The combined cardiac effect of the anabolic steroid, nandrolone and cocaine in the rat.

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Originality

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Duplication

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25/10/2005

Benjamin D. Phillis
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Benjamin D. Phillis

October, 2005

ADELAIDE
Table of Contents

DECLARATION ________________________________________ I

ACKNOWLEDGEMENTS ___________________________________ II

TABLE OF CONTENTS ___________________________________ III

ABBREVIATIONS ________________________________________ X

ABSTRACT ___________________________________________ XII

CHAPTER 1 ........................................................................ 1-1

Introduction __________________________________________ 1-1

1.1 Background ___________________________________________ 1-1

1.2 What are anabolic steroids? ____________________________ 1-1

1.3 General pharmacology of Anabolic steroids _____________ 1-2

1.3.1 Genomic effects of anabolic steroids ________________ 1-2

1.3.2 Non-genomic effects of anabolic steroids ______________ 1-3

1.4 Clinical use of AS ________________________________ 1-4

1.5 Patterns of AS abuse ________________________________ 1-4

1.5.1 Steroid abuse by athletes ___________________________ 1-4

1.5.2 Steroid abuse by sedentary teenagers _______________ 1-6

1.5.3 Prevalence of abuse ______________________________ 1-6

1.5.4 Abuse prevalence in Australia _____________________ 1-9

1.6 Cardiotoxicity of anabolic steroids ______________________ 1-9

1.6.1 Reduced coronary flow ____________________________ 1-11

1.6.2 Direct myocardial effects __________________________ 1-15

1.6.3 Hypertension _____________________________________ 1-21

1.7 Difficulties associated with anabolic steroid research _____________________________ 1-24

1.8 The polydrug abuse phenomenon ______________________ 1-25

1.9 The pharmacology of cocaine _________________________ 1-26

1.10 Preparations ______________________________________ 1-28

1.11 Metabolism ________________________________________ 1-29
1.12 Pharmacokinetics 1-30
1.13 Cocaine Cardiotoxicity 1-31
  1.13.1 Reduced coronary flow 1-34
  1.13.2 Cardiac arrhythmias 1-40
  1.13.3 Direct cardiac effects 1-43
1.14 Conclusion 1-47
1.15 Aims 1-48
1.16 Hypotheses 1-49
1.17 References 1-50

CHAPTER 2 2-1
General Methods 2-1
  2.1 Animals 2-1
  2.2 Radiotelemetry recording 2-1
  2.3 General anaesthesia 2-2
  2.4 Surgical implantation of radiotelemetry devices 2-2
    2.4.1 ECG implants 2-2
    2.4.2 Blood Pressure Implants 2-3
    2.4.3 Post-operative Management 2-4
  2.5 Analysis of radiotelemetry data 2-4
  2.6 Surgical procedure for ischaemia-reperfusion studies 2-4
  2.7 Continuous recording of cardiac parameters during ischaemia-reperfusion 2-6
  2.8 Induction of ischaemia and reperfusion 2-6
  2.9 Analysis of ischaemia-reperfusion data 2-7
  2.10 Determination of the extra-neuronal uptake of noradrenaline in isolated perfused hearts 2-11
    2.10.1 Pre-treatment 2-11
    2.10.2 Protocol 2-11
    2.10.3 Calculation of results 2-12
  2.11 Drugs 2-13
  2.12 References 2-15

CHAPTER 3 3-1
The effect of rat strain on the cardiovascular response to cocaine 3-1
  3.1 Introduction 3-1
    3.1.1 Is the cardiovascular response to intraperitoneal cocaine strain dependent? 3-1
  3.2 Aim 3-4
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3. Hypothesis</td>
<td>3-4</td>
</tr>
<tr>
<td>3.4. Methods</td>
<td>3-5</td>
</tr>
<tr>
<td>3.4.1 Animals</td>
<td>3-5</td>
</tr>
<tr>
<td>3.4.2 Protocol</td>
<td>3-5</td>
</tr>
<tr>
<td>3.4.3 Data Analysis</td>
<td>3-5</td>
</tr>
<tr>
<td>3.5. Results</td>
<td>3-6</td>
</tr>
<tr>
<td>3.5.1 Time course of the cocaine response</td>
<td>3-6</td>
</tr>
<tr>
<td>3.5.2 Between strain changes in the HR, SLA, DP and SP response to cocaine</td>
<td>3-6</td>
</tr>
<tr>
<td>3.6. Discussion</td>
<td>3-9</td>
</tr>
<tr>
<td>3.7. Conclusion</td>
<td>3-11</td>
</tr>
<tr>
<td>3.8 References</td>
<td>3-12</td>
</tr>
</tbody>
</table>

CHAPTER 4 | 4-1
The acute effect of nandrolone and cocaine on heart rate | 4-1

4.1 Introduction | 4-1
4.2 Aim | 4-2
4.3 Hypothesis | 4-2
4.4 Methodology | 4-3
4.4.1 Animals | 4-3
4.4.2 Protocol | 4-3
4.4.3 Drugs | 4-3
4.4.4 Statistical Analysis | 4-4
4.5 Results | 4-5
4.5.1 Plasma levels of nandrolone | 4-5
4.5.2 Pre-treatment response | 4-5
4.5.3 Cocaine response | 4-5
4.5.4 Effect of nandrolone pre-treatment on the response to cocaine | 4-5
4.6 Discussion | 4-8
4.7 Conclusion | 4-9
4.8 References | 4-10

CHAPTER 5 | 5-1
The effect of intravenous nandrolone in rats subjected to cardiac ischaemia and reperfusion | 5-1

5.1 Introduction | 5-1
5.2 Aim | 5-3
6.6.1 Blood pressure effects of cocaine 6-18
6.6.2 Effect of cocaine and ischaemia/reperfusion 6-18
6.6.3 Effect of the nandrolone-cocaine combination on arrhythmia and survival 6-20
6.6.4 Effect of pre-treatment on reperfusion arrhythmias 6-22
6.7 Conclusion 6-23
6.8 References 6-24

CHAPTER 7 7-1
Effects of chronic nandrolone administration 7-1
7.1 Introduction 7-1
7.2 Aims 7-2
7.3 Hypothesis 7-2
7.4 Methodology 7-3
  7.4.1 Animals 7-3
  7.4.2 Protocol 7-3
  7.4.3 Data Analysis 7-4
7.5 Results 7-7
  7.5.1 Change in body weight 7-7
  7.5.2 Change in organ weights 7-7
  7.5.3 Histopathology 7-8
  7.5.4 Cholesterol, triglycerides, LDL and HDL profiles 7-8
  7.5.5 Serology 7-8
  7.5.6 Plasma level of nandrolone 7-8
  7.5.7 Cardiovascular response to chronic nandrolone treatment 7-9
  7.5.8 Cardiovascular response to cocaine administration 7-9
  7.5.9 Cardiovascular response to ischaemia and reperfusion 7-10
  7.5.10 Zone at risk of infarction 7-10
  7.5.11 Survival Time 7-11
  7.5.12 Arrhythmia 7-11
7.6 Discussion 7-26
  7.6.1 Body weight 7-26
  7.6.2 Changes in organ weight 7-28
  7.6.3 Changes in cholesterol 7-30
  7.6.4 Serological Changes 7-32
  7.6.5 Plasma nandrolone level 7-32
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAT</td>
<td>Cholesterol Acetyltransferase</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Disease</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
</tr>
<tr>
<td>ANF</td>
<td>Atrial Natriuretic Factor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AS</td>
<td>Anabolic-Androgenic Steroid(s)</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>Antisense Oligodeoxynucleotide</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>AW</td>
<td>Albino Wistar</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BP</td>
<td>Blood Pressure</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
</tr>
<tr>
<td>CBF</td>
<td>Coronary Blood Flow</td>
</tr>
<tr>
<td>CFR</td>
<td>Coronary Flow Rate</td>
</tr>
<tr>
<td>chE1</td>
<td>Carboxysterase-1</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CK</td>
<td>Creatinine Kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac Output</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-Methyltransferase</td>
</tr>
<tr>
<td>CPK</td>
<td>Creatine Phosphokinase</td>
</tr>
<tr>
<td>CT</td>
<td>Core Temperature</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DECA</td>
<td>Decanoate</td>
</tr>
<tr>
<td>DOPEG</td>
<td>Dihydroxyphenylglycol</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>EMT</td>
<td>Extraneuronal Transporter for Monoamine Transmitters</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography - Mass Spectrometry</td>
</tr>
<tr>
<td>GGT</td>
<td>glutamyltransferase</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>hGH</td>
<td>Human Growth Hormone</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic Pituitary Adrenal (axis)</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HR</td>
<td>Heart Rate</td>
</tr>
<tr>
<td>5-HT</td>
<td>5, Hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>HTGL</td>
<td>Hepatic Triglyceride Lipase</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic Acid</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory Post-synaptic Potential</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LAD</td>
<td>Left Anterior Descending (artery)</td>
</tr>
<tr>
<td>LD_{10}</td>
<td>10% Lethal Dose</td>
</tr>
<tr>
<td>LD_{50}</td>
<td>50% Lethal Dose</td>
</tr>
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</table>
LDH  Lactate Dehydrogenase
LDL  Low Density Lipoprotein
LV   Left Ventricular
LVH  Left Ventricular Hypertrophy
MAO  Monoamine Oxidase
MBP  Mean Blood Pressure
MEAP Met-Enkephalin-Arg-Phe
MOPEG methoxymethoxyphenylglycol
mPOA Medial Preoptic Area
NA   Noradrenaline
NDMA N-nitrosodimethylamine
NIDA National Institute of Drug Abuse (USA)
NIH  National Institute of Health
NK-1 Neurokinin-1
NMDA N-methyl-D-aspartic acid
OCT2 Organic Cation Transporter 2
PAG  Periaqueductal Gray
PAI-1 Plasminogen Activator Inhibitor-1
PDGF Platelet Derived Growth Factor
PC   Phosphatidylcholine
PE   Phenylephrine
PEG  Polyethylene glycol
PGL2 Prostacyclin
PRP  Platelet Rich Plasma
PS   Phosphatidylserine
PVC  Premature Ventricular Contractions
RDI  Recommended Daily Intake
SA   Sodium Arachidonate
s.c. subcutaneous
SD   Sprague Dawley (rat)
SHR  Spontaneously Hypertensive Rat
SLA  Spontaneous Locomotor Activity
SNP  Sodium Nitroprusside
SP   Substance P
TC   Total Cholesterol
TNF  Tumour Necrosis Factor
TRIG Triglyceride
TXB2 Thromboxane
VF   Ventricular Fibrillation
VMN  Ventromedial Nucleus
VT   Ventricular Tachycardia
VTA  Ventral Tegmental Area
WKY  Wistar Kyoto
Abstract

Recent pharmacoepidemiological research shows a significant increase in the use of anabolic-steroids (AS) amongst teenagers and young adults and especially amongst sedentary, young males administering AS for cosmetic reasons. AS are often used in conjunction with psychostimulants. Survey findings suggest significant co-abuse of AS and cocaine. Unfortunately, despite documented evidence that both drugs alone can induce significant cardiovascular effects only very limited basic science research has been conducted into the potential for cardiotoxicity with this drug combination.

Nandrolone has been shown to be the AS of choice amongst many recreational users. Previous work has found heart rate (HR) increased significantly following cocaine hydrochloride (45mg/kg, i.p.) administration in albino Wistar rats (AW) chronically treated with nandrolone compared to the cocaine effect in vehicle treated controls (Phillis et al, 2000). Subsequent studies in this thesis established that this HR effect could not be attributed to an acute effect of the last nandrolone dose prior to cocaine administration. Dose-response relationships to cocaine (0,15,45mg/kg, i.p.) for cardiovascular variables using radiotelemetry were conducted in freely moving, conscious AW and Sprague-Dawley (SD) rats. These studies indicated that the cardiovascular response to cocaine was not strain dependent. This allowed direct comparisons to be made to literature values for cocaine effects in both strains.

In view of the rather moderate effects of nandrolone observed, the effect of AS pre-treatment on the response to cardiac ischaemia was assessed in order to simulate pre-existing cardiac disease. Nandrolone was administered i.v. at doses reflective of the plasma concentrations possibly achieved by chronic AS users (10-160µg/kg/min). SD rats were subsequently subjected to 15 minutes occlusion of the left anterior descending (LAD) coronary artery and 10 minutes reperfusion. A significant decrease in survival time during ischaemia was noted at the highest nandrolone dose (p<0.001) and significant decreases in the fraction of rats surviving ischaemia (40 & 160µg/kg/min, both p<0.05) compared to control. A significant increase in the Lambeth arrhythmia score was seen at the 3 highest nandrolone doses (all, p<0.05). A significant increase in the duration of VF during ischaemia was noted for the highest nandrolone dose (160µg/kg/min) compared to the lowest dose (10µg/kg/min). This pro-arrhythmic nandrolone effect could not be attributed to increases in myocardial noradrenaline (NA) by the blockade of extraneuronal reuptake, since nandrolone was shown to have too low an inhibiting potency on extraneuronal noradrenaline uptake in isolated perfused rat hearts. The severity of arrhythmia in rats receiving i.v. nandrolone was not increased by cocaine hydrochloride administration (0.5mg/kg/min, i.v) but rather was found to protect against the fatal VF induced by nandrolone alone (40µg/kg/min). In contrast to the acute effect of nandrolone, chronic nandrolone treatment of SD rats for 3, 6 or 9 weeks had no effect on the response to cardiac ischaemia or reperfusion. The basis of the difference between the effects of chronic and acute effects of nandrolone on ischaemia-induced dysrhythmia was not identified. It may relate to the much lower plasma nandrolone concentration achieved with
chronic nandrolone treatment. Alternatively, chronic treatment may result in down-regulation of the mechanism underlying enhanced arrhythmia after acute dosing.

This is the first study to show a significant and dose dependent increase in the Lambeth arrhythmia score during cardiac ischaemia in rats administered i.v. nandrolone. The mechanism of this effect remains unknown. Potential mechanisms include a CNS effect of nandrolone, an uncoupling of the protective effect of adenosine during early ischaemia by nandrolone or an increase in pro-arrhythmic endothelin-1 (ET-1). This study suggests that nandrolone abuse in patients with a pre-existing cardiac condition may precipitate life threatening cardiac arrhythmia.
Chapter 1

Introduction

1.1 Background
There is a growing body of epidemiological evidence indicating an increasing prevalence of the abuse of anabolic steroids in both image conscious, sedentary, teenage males and power athletes. In addition, anabolic steroid abuse is highly correlated with stimulant use. This thesis sets out to firstly review the reported cardiac consequences of abuse of anabolic steroids and the popular stimulant, cocaine, in the literature, and then examine the cardiovascular consequences of the co-administration of nandrolone (a commonly used steroid) and cocaine in an animal model. These experiments were performed in the absence of other confounding risk factors such as poor diet and heavy exercise.

1.2 What are anabolic steroids?
Anabolic steroids are synthetic derivatives of testosterone. All anabolic steroids have some androgenic action, as both the anabolic and androgenic effects are mediated through the same androgen receptor (Rockhold, 1993). Strictly speaking, they should be referred to as anabolic-androgenic steroids. For the sake of simplicity throughout this thesis they will be referred to as anabolic steroids (AS). One of the most popular AS is nandrolone, which has a high anabolic:androgenic ratio of 8:1 resulting in a lower chance of affecting primary and secondary sex characteristics when it is administered to increase muscle mass (Yu-Yahiro et al., 1989).

All endogenous androgens are based upon the ringed structure of cholesterol (Colby et al., 1988). As testosterone is highly subject to hepatic degradation, a number of modifications have been made to the structure of synthetic steroids to minimise this effect...

1. 17-β-esterification (eg. testosterone undecanoate)
2. 17-α-substitution (eg. methyl testosterone)
3. Modifications and additions to the steroid ring (eg. stanozolol and oxandrolone)
1.3 General pharmacology of Anabolic steroids

1.3.1 Genomic effects of anabolic steroids

The high lipophilicity of most AS allows ready passage across the lipid bi-layer of the target cell (Lukas, 1993) (Fig. 1.1). Binding to cytoplasmic steroid receptors and subsequent homodimer formation, elicits a conformational change in the steroid-receptor homodimer-complex, which exposes DNA binding regions (reviewed by Colby et al. 1988). Upon translocation to the nucleus, these “zinc finger” sequences allow binding to “hormone response elements” on DNA, leading to a subsequent increase in RNA transcription and protein production (Wright, 1980; Bahrke et al., 1998).

Almost every cell in the body contains androgen receptors. Therefore the anabolic or androgenic response is determined more by the location and type of cell than the nature of the AS (Bahrke et al., 1998).

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Fig. 1.1 Intercellular events following binding of AS to intercellular receptors. Lipophilic steroid passes readily into the cytoplasm, where it combines with a cytoplasmic steroid receptor. Identical receptor-steroid complexes dimerise, inducing a conformational change in the homodimer, which exposes DNA binding regions (“zinc fingers”). Translocation to the nucleus is induced by increased affinity for nuclear chromatin. Within the nucleus the “zinc fingers” bind DNA regions (“hormone response elements”) resulting in an increase in the number of RNA transcripts generated, and a subsequent increase in protein production.
1.3.2 Non-genomic effects of anabolic steroids

Genomic expression cannot explain all the observed effects of AS. A limited number of studies have examined the direct effect of androgens on coronary vasculature and found that the pharmacological effects occurred quickly, and were not consistent with the slow response normally attributed to changes in gene expression. It has been demonstrated that testosterone can block the vasodilator effect of adenosine in isolated perfused rat hearts (Ceballos et al., 1999). This effect was conserved even when testosterone was coupled to a large macromolecular complex, which prevented transport into the cytosol. Testosterone was still able to exert its inhibitory effect on vasodilation in the presence of cyclohexamidine, demonstrating that its acute effects were not dependent on RNA translation (protein synthesis). This vasoconstricting effect of testosterone appears to be mediated in part by the prostaglandin metabolic pathway. Inhibition of cyclooxygenase-1 (cox-1) by indomethacin was found to significantly reduce the vasoconstriction produced by testosterone. A reduction in vasoconstriction was also observed with a thromboxane A₂ (TXA₂) antagonist.

Most recently, Togna et al investigated the combined effect of testosterone and cocaine on platelet and endothelial function (Togna et al., 2003). Co-incubation of platelet rich plasma with testosterone and cocaine induced a significant increase in platelet aggregability in response to a subthreshold concentration of sodium arachidonate. Likewise, co-incubation of endothelium-intact, phenylephrine pre-contracted, rabbit aortic rings, with a subthreshold concentration of testosterone and cocaine caused an inhibition of acetylcholine mediated vascular relaxation. Incubation of platelets or aortic rings with these concentrations of either cocaine alone or testosterone alone had no effect on platelet aggregability or acetylcholine mediated relaxation. In addition, subthreshold concentrations of testosterone potentiated the inhibitory effect of higher concentrations of cocaine. The testosterone receptor antagonist, flutamide, did not prevent the effect observed with a concomitant incubation with both testosterone and cocaine, indicative of an androgen receptor independent mechanism in both cases. The lack of androgen receptor involvement suggests that testosterone can act on platelets and vessels through a non-genomic mechanism. The effects on vessels are possibly elicited through changes in membrane fluidity at the vascular endothelial cell level. Togna et al propose that the increase in aggregation seen with co-incubation of testosterone and cocaine may be due to modification of platelet membrane surface proteins and permeability changes.
1.4 Clinical use of AS

Steroids are used clinically to treat a broad spectrum of indications. Testosterone enanthate is used to treat the muscle wasting and hypogonadism associated with HIV (Forbes et al., 1992; Grinspoon et al., 1998). Androgens have also been used in the treatment of patients with excessive protein catabolism or insufficient protein synthesis, such as patients suffering from significant burns, malignancies and trauma (Chlebowski et al., 1983; Kopera, 1985). AS have been found to increase erythropoiesis and are therefore used to treat a variety of anaemias (Kibble et al., 1987; Marshall, 1988). Likewise, because of a leukopoietic effect via stimulation of proliferation of granulocytic colony forming cells, AS have also been found to provide a beneficial effect in the treatment of leukaemia (Kopera, 1985). Testosterone esters are also routinely used to treat short stature children in conjunction with human growth hormone (hGH) therapy (Hoberman et al., 1995). A variety of AS are used to promote bone growth in patients suffering osteoporosis (Kopera, 1985).

1.5 Patterns of AS abuse

1.5.1 Steroid abuse by athletes

Illicit use by athletes often involves the use of doses 10-1000 times the recommended dose for clinical use (Sullivan et al., 1999). In addition, users will often use 2 or more steroids simultaneously (Yates et al., 1992; Masonis et al., 1995), in a process known as “stacking” (Long et al., 2000) (Table 1.1). Many athletes use AS in “cycles” of 4-18 weeks (Kibble et al. 1987), decreasing the dose as competition dates approach (known as “tapering”). Successive cycles may be between 1 & 12 months apart (Kashkin et al., 1989). Many athletes will slowly increase the dose during a cycle until a peak dose is reached and then decrease the dose again towards the end of a cycle (Kashkin et al., 1989). This is known as “pyramiding”, and is said to reduce behavioural side effects.
<table>
<thead>
<tr>
<th>Product Name</th>
<th>Generic Name</th>
<th>Route</th>
<th>Reported Dose &amp; frequency (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anavar</td>
<td>Oxandrolone</td>
<td>oral</td>
<td>50mg/day x 16 weeks (Ferenchick et al., 1992), 7.5mg/day x 18 months (Winwood et al., 1990)</td>
</tr>
<tr>
<td>Anadrol-50</td>
<td>Oxymetholone</td>
<td>oral</td>
<td>46mg/day (Yates et al. 1992)</td>
</tr>
<tr>
<td>Deca-</td>
<td>Nandrolone decanoate (19, nortestosterone)</td>
<td>i.m.</td>
<td>200mg/week (Fineschi et al. 2001), 200mg/week (Cabasso, 1994), 200mg/week x 16 weeks (Ferenchick et al., 1992); 100mg/week x 8 weeks (McKillop et al. 1986)</td>
</tr>
<tr>
<td>Durabolin</td>
<td>Testosterone-cypionate</td>
<td>i.m.</td>
<td>400mg/week x 5 weeks (Sullivan et al., 1999), 83mg/day (Yates et al., 1992)</td>
</tr>
<tr>
<td>Depo-</td>
<td>Testosterone-cypionate</td>
<td>i.m.</td>
<td>700mg/week (Fineschi et al. 2001)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Methandrostenolone</td>
<td>oral &amp; i.m.</td>
<td>100mg/week (Palatini et al., 1996)</td>
</tr>
<tr>
<td>Dianabol</td>
<td>Boldenone-undecylenate</td>
<td>i.m.</td>
<td>100mg/week (Palatini et al., 1996)</td>
</tr>
<tr>
<td>Dianabol, i.m.</td>
<td>fluoxymesterone</td>
<td>oral</td>
<td>100mg/week (Palatini et al., 1996)</td>
</tr>
<tr>
<td>Equipoise</td>
<td>Methyltestosterone</td>
<td>oral</td>
<td>100mg/week (Palatini et al., 1996)</td>
</tr>
<tr>
<td>Metandren</td>
<td>Testosterone-propionate</td>
<td>i.m.</td>
<td>700mg/week (Fineschi et al. 2001)</td>
</tr>
<tr>
<td>Methyl</td>
<td>Testosterone-propionate</td>
<td>i.m.</td>
<td>700mg/week (Fineschi et al. 2001)</td>
</tr>
<tr>
<td>Testex Leo</td>
<td>Methenolone-enanthate</td>
<td>i.m., oral</td>
<td>100mg/week (Palatini et al., 1996)</td>
</tr>
<tr>
<td>Depot</td>
<td>Mesterolone</td>
<td>oral</td>
<td>100mg/week (Palatini et al., 1996)</td>
</tr>
<tr>
<td>Testex Leo</td>
<td>Testosterone-cyclopentilpropionate</td>
<td>i.m.</td>
<td>(Hausmann et al., 1998)</td>
</tr>
<tr>
<td>Testex Leo</td>
<td>Stanozolol</td>
<td>i.m., (veterinary)</td>
<td>70mg/week (Fineschi et al. 2001), 240mg/week x 5 weeks (Sullivan et al., 1999), 280mg/week x 2 years (Mewis et al. 1996), 40mg/day x 6 weeks (Kennedy, 1993), 15mg/day x 18 months (Winwood et al. 1990), 30mg/day (McKillop et al., 1986)</td>
</tr>
</tbody>
</table>

Table 1. Type, route, dose and dosing frequency of AS reportedly abused by athletes
1.5.2 Steroid abuse by sedentary teenagers

One of the most worrying trends in the abuse of AS has been recent reports of illicit use in sedentary teenagers. The main reasons for abuse have been variously reported as being to obtain a better body or larger muscles (Tanner et al., 1995; Nilsson et al., 2001), to “become brave” (Kindlundh et al., 1998; Kindlundh et al., 1999), to become intoxicated (Kindlundh et al., 1998), and to enhance sports performance (Bahrke et al. 1998).

A recent report also suggests that many HIV sufferers self-administer AS at supra-clinical doses in an attempt to minimise the muscle wasting associated with AIDS (Varriale et al. 1999).

Illicit AS can be easily purchased on the black market. Many of the steroids sold in this manner are from veterinary applications or diverted prescriptions. An extensive variety of steroids manufactured in foreign laboratories can be purchased on the street (Marshall, 1988).

1.5.3 Prevalence of abuse

An accurate indication of the prevalence of steroid abuse in the general population and in adolescents is hard to determine and is complicated by self-report data. Thompson et al report that of the drugs reportedly used by survey respondents, only 66% were found in urine (Thompson et al., 1992; Thomson et al., 1993). This is likely to be due to a combination of errors. Exaggeration of drug use is not uncommon in illicit drug users. Many respondents self-administering black-market AS may not have been supplied with the requested AS (DuRant et al., 1993a). As there is no obvious pharmacodynamic effect of AS, it is difficult for users to identify genuine drug. In addition, there is the potential in self-report surveys to confuse AS use with corticosteroids and glucocorticoids, prescribed for legitimate medical conditions (Handelsman et al. 1997).

Prevalence in gymnasia

An investigation of the prevalence of illicit steroid use in gymnasia across England, Wales and Scotland, reported a lifetime steroid use amongst 9.1% of men and 2.3% of women, where 6% of men and 1.4% of women were current users (Korkia, 1996; Korkia et al., 1997). Polydrug use was found to be common amongst the 1667 respondents, with 3.7% reporting co-administration of cocaine.
Prevalence in High School age students

The “Monitoring the Future” survey sponsored by the NIH (USA) estimated that in 2000 lifetime (“ever”) prevalence of AS use was 3.0, 3.5 and 2.5% amongst 8th, 10th and 12th grade students respectively (see also Table 1.2). AS and MDMA (“ecstasy”) were the only 2 drugs for which strong increases over the 1999 data were reported.

Steroid abuse in teenagers is not necessarily associated with participation in school sport. In a recent study, 25% of male users reporting lifetime steroid use did not participate in sport (Tanner et al., 1995).
<table>
<thead>
<tr>
<th>Location (Author, year)</th>
<th>Sample size</th>
<th>Age</th>
<th>Male (%)</th>
<th>Female (%)</th>
<th>Total (%)</th>
<th>Time-span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Richmond County, USA (DuRant et al., 1993b)</td>
<td>1,881</td>
<td>Overall 9th grade</td>
<td>5.4</td>
<td>1.5</td>
<td>4.2</td>
<td>Lifetime (“ever”)</td>
</tr>
<tr>
<td>Modesto, California, USA (Radakovich et al., 1993)</td>
<td>742</td>
<td>7th grade</td>
<td>4.7</td>
<td>3.2</td>
<td>3.8</td>
<td>Lifetime</td>
</tr>
<tr>
<td>Richmond County, USA (DuRant et al., 1994)</td>
<td>1,422</td>
<td>9th grade</td>
<td>4.8</td>
<td>2.9</td>
<td>3.8</td>
<td>Lifetime</td>
</tr>
<tr>
<td>USA (DuRant et al., 1995)</td>
<td>12,272</td>
<td>Overall</td>
<td>4.08</td>
<td>1.20</td>
<td>Lifetime</td>
<td></td>
</tr>
<tr>
<td>Rocky Mountain region, USA (Tanner et al., 1995)</td>
<td>6,930</td>
<td>Overall</td>
<td>4.0</td>
<td>1.3</td>
<td>2.7</td>
<td>Lifetime</td>
</tr>
<tr>
<td>Falkenberg, Sweden (Nilsson, 1995)</td>
<td>1,383</td>
<td>14-19 yrs</td>
<td>5.8</td>
<td>1.0</td>
<td>2.5</td>
<td>Current use</td>
</tr>
<tr>
<td>Nebraska, USA (Scott et al., 1996)</td>
<td>4,722</td>
<td>Overall 7th grade</td>
<td>4.5</td>
<td>0.8</td>
<td>2.5</td>
<td>Current use</td>
</tr>
<tr>
<td>Eastern States, Australia (Handelsman et al., 1997)</td>
<td>13,355</td>
<td>Overall</td>
<td>3.2</td>
<td>1.2</td>
<td>Lifetime &amp; current use</td>
<td></td>
</tr>
<tr>
<td>Uppsala, Sweden (Kindlundh et al., 1998)</td>
<td>2,742</td>
<td>Overall 16-17 yrs</td>
<td>1.7</td>
<td>0.1</td>
<td>Lifetime</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18-19 yrs</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. 2 Reports of prevalence of AS use appearing in the English literature since 1993 in High School age students.
1.5.4 Abuse prevalence in Australia

Very few studies have assessed the use of AS by Australian teenagers. In a recent study of drug use in 203 schools (grades 7-11) in Victoria and New South Wales, lifetime (“ever”) use of AS was reported to be 3.2% amongst males and 1.2% amongst girls, with 1.7% of boys and 0.4% of girls reporting use within the last month. The 1995 Australian national drug strategy household survey (Commonwealth Department of Health and Family Services, 1996) indicated a 0.2% prevalence of illicit AS use in the general population, with 28,800 people having used AS in the previous 12 months. The 1998 survey reported a lifetime use of steroids by 1% of the male population.

1.6 Cardiotoxicity of anabolic steroids

With the high doses of AS associated with illicit use a vast plethora of toxicological effects have been reported (Table 1.3). Despite these reports, very few studies have been conducted to determine the mortality rate amongst illicit users. Parssinen et al investigated the mortality rate amongst male power lifters placed 1st-5th in the weight series 82.5-124kg in Finnish championships during 1977-1982 (n=62) compared to population controls (Parssinen et al. 2000). The mortality rate amongst powerlifters was 12.9% during the 12-year follow-up, with a 4.6 fold higher risk of death than the population controls (95% CI 2.04-10.45; p=0.0002). The average age of death amongst powerlifters was 43 years (36-53 years). It must be cautioned however that AS use was never verified in these powerlifters, and although 2 deaths were from suicide – there is no strong evidence directly linking illicit AS use to an increased risk of deliberate death. At best this study can only be considered as finding a much higher mortality rate amongst power lifters at high risk of AS use. Future study should concentrate on finding evidence of premature death amongst known former and current AS users.
<table>
<thead>
<tr>
<th>Physiological System</th>
<th>Adverse Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic</td>
<td>Peliosis hepatitis</td>
<td>(Cabasso, 1994; Hausmann et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Hepatoma</td>
<td>(Creagh et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>Regenerative nodular hyperplasia</td>
<td>(Winwood et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Elevated serum aspartate amino transaminase (AST)</td>
<td>(Winwood et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Elevated liver function test values</td>
<td>(Winwood et al., 1990)</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Bleeding oesophageal varices</td>
<td>(Winwood et al., 1990)</td>
</tr>
<tr>
<td>Endocrine</td>
<td>Decreased testosterone</td>
<td>(Bijlsma et al., 1982)</td>
</tr>
<tr>
<td></td>
<td>Gynecomastia</td>
<td>(Neil, 1995)</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Reduced high density lipoprotein</td>
<td>(Kleiner et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Increased low density lipoprotein</td>
<td>(Glazer, 1991)</td>
</tr>
<tr>
<td></td>
<td>Coronary plaque formation</td>
<td>(Mewis et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Hypertension</td>
<td>(Kuipers et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>Ventricular tachycardia</td>
<td>(Appleby et al., 1994; Mewis et al., 1996; Niemenen et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Atrial fibrillation</td>
<td>(Sullivan et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Ventricular fibrillation</td>
<td>(Cammell et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>Left ventricular hypertrophy</td>
<td>(McKillop et al., 1986; Luke, 1990; Dickerman et al., 1995; Niemenen et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Right ventricular hypertrophy</td>
<td>(Luke, 1990; Kamell et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>Cardiomyopathy</td>
<td>(Mochizuki et al., 1988; McCarthy et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Myocardial infarction</td>
<td>(McNutt et al., 1988; Ferenechick et al., 1992; Kennedy, 1993; Kennedy et al., 1993a; Appleby et al., 1994; Fisher et al., 1996; Varrile et al., 1999; Fineschi et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Myocardial fibrosis</td>
<td>(Luke, 1990; Kamell et al., 1993; Niemenen et al., 1996; Hausmann et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Myocardial contraction band formation</td>
<td>(Luke, 1990; Fineschi et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Congestive heart failure</td>
<td>(Niaimen et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Platelet hyperaggregability</td>
<td>(McNutt et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>Thrombosis</td>
<td>(Montine et al., 1992; Toyama et al., 1994; Fisher et al., 1996; Niemenen et al., 1996; Varrile et al., 1999; McCarthy et al., 2000)</td>
</tr>
<tr>
<td>Reproductive</td>
<td>Suppression of spermatogenesis</td>
<td>(Steinberger et al., 1977; Bagatell et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Testicular atrophy</td>
<td>(Korkia et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Decreased testosterone</td>
<td>(Bijlsma et al., 1982; Sader et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Masculinizing in women</td>
<td>(Steinberger et al., 1977; Korkia et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>• Smaller breasts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Menstrual irregularities</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Clitoral enlargement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Fertility problems</td>
<td></td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Stroke</td>
<td>(Frankle et al., 1988; Mochizuki et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>Cerebral haemorrhage</td>
<td>(Kennedy et al., 1993a)</td>
</tr>
<tr>
<td></td>
<td>Schizophrenia</td>
<td>(Annitto et al., 1980)</td>
</tr>
<tr>
<td></td>
<td>Aggression</td>
<td>(Yates et al., 1992; Sa et al., 1993; Choi et al., 1994; Thiblin et al., 1997)</td>
</tr>
<tr>
<td>Other</td>
<td>Decreased serum calcium</td>
<td>(Bagatell et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Hypokalaemia</td>
<td>(Appleby et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Acne</td>
<td>(Bagatell et al., 1994)</td>
</tr>
</tbody>
</table>

Table 1.3 Reported side effects of AS abuse
AS use has been associated with many cardiovascular disturbances, including hypertension, myocardial infarction, ventricular and atrial fibrillation, left ventricular hypertrophy, cardiomyopathy, myocardial fibrosis, thrombosis and congestive heart failure (Table 1.3 & Appendix A). There are 3 main models that can help explain how AS may cause cardiotoxicity. It is probable that toxicity cannot be attributed solely to any one mechanism and is likely to result from a combination of effects...

1. Reduced coronary flow
   a. Vasospasm
   b. Thrombosis
   c. Arteriosclerosis

2. Direct myocardial effects
   a. Cardiac remodelling
   b. Myocardial cell injury

3. Hypertension

The following will consider each of these models in more detail and discuss additional factors, which may increase the risk of sudden cardiac death resulting from illicit AS use.

1.6.1 Reduced coronary flow

The observation of myocardial infarction associated with AS abuse (Table 1.3) suggests that AS can reduce coronary flow. Evidence for a number of potential mechanisms are outlined below.

Vasospasm

A number of athletes, known to be current AS users, dying from sudden cardiac death, have been found to have coronary arteries free from thrombus or atheroma (Luke, 1990; Kennedy et al., 1993b). Likewise, a number of AS abusing athletes, admitted for infarction have displayed angiographically normal arteries (Ferenchick et al., 1992) (Appendix A).

A depressed vasodilatory response in the brachial artery to sodium nitroprusside and methacholine has been reported in a patient self-administering nandrolone (Green et al., 1993), which returned to normal following 8 weeks abstinence. Chronic nandrolone treatment of rabbits has been found to reduce both endothelium dependent and independent relaxation of ex-vivo thoracic aorta by decreasing guanylyl cyclase (GC) activity (Ferrer et al., 1994a; Ferrer et al., 1994b).
Thrombosis

Several case reports have detailed thrombosis or platelet hyperaggregability associated with chronic high dose AS use (McNutt et al., 1988; Huie, 1994; Fisher et al., 1996; McCarthy et al., 2000).

Aggregation of male and female human platelets in response to a pro-aggregation stimulus is significantly greater in the presence of androgen (Johnson et al., 1975). Male rats and guinea pigs have been found to be 10 times more sensitive to aggregating stimulus than age matched females (Johnson et al., 1977). Male castration significantly reduced this sensitivity, suggesting a role for testosterone in aggregation promotion.

Androgens have been shown to affect the synthesis of a number of eicosanoids thereby increasing blood coagulation and enhancing the risk of thrombus formation. Testosterone has been demonstrated to increase the production of thromboxane A2 (platelet aggregator), while at the same time decreasing the production of prostacyclins (eg. PGI2), which normally inhibit platelet aggregation (Ferenchick, 1991; Weyrich et al., 1992; Melchert et al., 1995).

Several 17 α-alkylated androgens have been found to influence the coagulation/fibrinolytic system. Danazol, a steroid favoured by many weight lifters, significantly improves coagulation in type A and B haemophilia by increasing levels of factor VIII and IX (Laurell et al., 1979; Gralnick et al., 1983).

Disseminated intravascular coagulation often occurs in patients with prostatic carcinoma, presumably, as a result of thromboplastic substances released from tumour cells or activation of the intravascular coagulation system. A range of antiandrogens such as ketoconazole, flutamide and buserilline acetate have been found to be protective against coagulation in these patients (Litt et al., 1987; Martinez et al., 1988).

Whatever the role of AS in the formation of thrombi, it is unlikely that this represents a unifying aetiology for sudden death in steroid users. Many illicit users suffering myocardial infarction were found to have angiographically normal coronary arteries – free from atheroma or thrombus. It is more probable that the pro-coagulant effects of many androgens increase the risk of an adverse cardiac event occurring. This risk is likely to be increased dramatically in patients who also display significant atherosclerosis. Increased platelet aggregation induced by AS may also increase levels of platelet derived growth factor (PDGF), which increases cell proliferation and may contribute to atherosclerotic lesion (Melchert et al., 1995).
Atteriosclerosis

It has not proven possible to show a definite link between AS abuse and atherosclerosis resulting in turn in an adverse cardiovascular event. However, a number of studies have shown that AS change the normal blood lipid balance so that it resembles the pattern observed in patients with significant atherosclerosis (Glazer, 1991). A decreased high density lipoprotein (HDL) and elevated low density lipoprotein (LDL) profile has been reported in a number of athletes self-administering AS (Bowman, 1990).

An association exists between AS and increases in hepatic triglyceride lipase (HTGL) which increases high-density lipoprotein (HDL) catabolism. Reductions in HDL and increases in low-density lipoprotein (LDL) have been demonstrated in humans (Webb et al., 1984; Lenders et al., 1988; Thompson et al., 1989; Baldo-Enzi et al., 1990; Friedl et al., 1990; Glazer, 1991), cynomolgus monkeys (Weyrich et al., 1992), and rats (Leeds et al., 1986; Lewanowitsch et al., 2001) subjected to chronic AS treatment. Plasma HDL concentrations will often decrease in men, engaged in regular, intensive, vigorous exercise (Kibble et al. 1987). Increases in LDL may result in fatty streaks, endothelial injury, platelet aggregation, and increased cell proliferation resulting in advanced lesion. It appears that aromatisable AS such as testosterone do not induce HTGL as strongly as non-aromatisable steroids such as stanozolol (Thompson et al. 1989; Baldo-Enzi et al. 1990).

A further risk of altered plasma lipoprotein level is changed myocardial membrane phospholipid composition and an increased risk of cardiac arrhythmia. Myocardial tissue is composed of a phospholipid bilayer consisting of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphotidylserine (PS), sphinomyelin and phosphatidylinositol (Laarse, 1987). Besides de novo synthesis of cholesterol from acetyl-CoA, membranous cholesterol is also sourced from receptor mediated uptake of LDL (Laarse, 1987) (Fig. 1.2).
Fig. 1. 2 Pathways involved in cellular cholesterol supply, metabolism and removal. Source: Laarse et al., 1987. Low density lipoprotein is taken up into the cell by a receptor mediated mechanism, and is subjected to lysosomal breakdown resulting in an increase in intracellular free cholesterol. An increase in free cholesterol exerts an inhibitory effect on HMG-CoA reductase slowing the de novo cholesterol synthesis. Free cholesterol also prevents the synthesis of membrane bound LDL receptors, and activates cholesterol acyltransferase (ACAT) which stimulates the conversion of cholesterol to cholesterol esters.

The extent of cholesterol incorporation can change the physical properties of cellular lipid bilayers (Laarse, 1987). For example, Tulenko et al found that arterial smooth muscle cell plasma membranes isolated from 2% cholesterol fed rabbits showed a progressive elevation in the ratio of free cholesterol to phospholipid (Tulenko et al., 1998). Chen et al fed rabbits cholesterol (2% w/w) for 8 weeks and demonstrated that plasma membrane from arterial smooth muscle cells showed evidence of changes in membrane bilayer structural parameters and phospholipid composition (Chen et al., 1995). Treatment was found to increase the unesterified (free) cholesterol content by 67% and the bilayer width from 56-62Å. Interestingly, this increase in bilayer width was highly correlated (r=0.992) with membrane free cholesterol. No overall change in phospholipid content of the membrane was observed, but the contribution various phospholipid classes made to the makeup of the membrane changed significantly with cholesterol treatment. Membranes obtained from control rabbits exposed to free cholesterol in culture displayed similar changes in
phospholipid content of the membrane. Cholesterol enrichment of the plasma membrane was found to significantly decrease the activity of the Na+/K+ ATPase pump (transmembrane protein).

Because AS have been shown in both animals and humans to induce atherosclerotic changes in serum lipoprotein profiles it would be expected that nandrolone and similar AS may alter the composition and/or fatty acid content of the phospholipid membrane due to an increased availability of cholesterol for cellular uptake. However, Liang et al. used $^{31}$P NMR spectral analysis of whole heart phospholipids from male SD rats administered nandrolone decanoate (3.0mg/kg every 7-9 days for 9 injections following an initial loading dose) to find no significant difference in myocardial phospholipid profiles from drug treated rats and controls (Liang et al., 1992). Changes in phospholipid composition of the myocardial membrane were only found in rats subjected to exhaustive exercise. No differences were found between rats subjected to exercise and simultaneously administered nandrolone when compared to rats, which were exercised, but drug free. Unfortunately, Liang et al. made no attempt to quantify changes in LDL and HDL with nandrolone treatment so it is not known whether their negative results simply reflect a treatment strategy which does not have a significant effect on plasma lipoprotein.

1.6.2 Direct myocardial effects
Myocardial cells contain androgen receptors and it therefore seems reasonable that AS should have a direct effect on the cell. A number of autoradiographic studies have confirmed the presence of androgen receptors in male and female baboon hearts (McGill et al., 1980; McGill et al., 1981). Other studies have demonstrated the presence of myocardial androgen receptors in rat (Krieger et al., 1978), dog and human hearts (Marsh et al., 1998).

Cardiac remodelling
It is not unusual for significant increases in left ventricular mass to occur in athletes undertaking intensive aerobic exercise. An increase in heart tissue is matched by an increase in collateral vessel formation thereby increasing the oxygen supply to the new tissue. A number of autopsy studies following sudden cardiac death have noted significant left ventricular hypertrophy (LVH) in athletes abusing AS (Appendix A). Work conducted using mouse hearts suggest that this increase in heart muscle is not concomitant with new collateral vessel formation in AS treated rodents (Tagarakis et al., 2000). An echocardiographic study in hypertensive men conducted over 5 years found that elevated left ventricular mass index was the best indicator of future morbidity (Casale et al., 1986), although LVH has been found to correlate poorly with high blood pressure (BP) (Papademetriou et al., 1985).
Although cardiac hypertrophy has been associated with AS abuse in a number of autopsy studies (Luke, 1990; Cambell et al., 1993; Kennedy et al., 1993b; Dickerman et al., 1995) and detected with ECG and echocardiography in isolated cases (McKillop et al., 1986; Sullivan et al., 1999) these observations have proved difficult to reproduce with larger numbers of patients.

Salke et al report that weight training alone increases left ventricular (LV) posterior wall and ventricular septal thickness. No significant differences were found in athletes self administering large doses of AS (Salke et al., 1985). Likewise, Palatini et al noted no difference in functional and dimensional echocardiographic indexes between steroid using and non-using weight trainers (Palatini et al., 1996).

While a number of studies have noted overall increased LV mass or wall thickness in AS using subjects, adjustments for body surface area (BMI) or body mass often eliminate these significant differences. Most recently, Sader et al assessed changes in cardiac morphology and function using echocardiography in a group of 20 male, Australian bodybuilders, who either self administered AS or were drug free (Sader et al., 2001). While significant increases in LV mass were noted in the drug-using group (245±16 vs. 199±12g, p=0.04), this difference was eliminated when adjusted with the body mass index (BMI). After further adjustment for maximum strength capacity, the adjusted LV mass was quite similar between groups. Dickerman et al tested cardiac dimensions with echocardiography in a set of fraternal twins, who had both been weightlifting for 20 years (Dickerman et al., 1997a). One twin was drug free, but the other had a 15-year history of AS use. No difference could be found in the thickness of the LV posterior wall or the ventricular septum, despite an abnormal early diastolic filling velocity in the drug-using twin. In a further study Dickerman et al studied 16 competitive bodybuilders recruited for an echocardiographic study (Dickerman et al., 1997b). Eight of these heavyweight bodybuilders were drug free and the remainder self administered AS. Although a significant increase in LV posterior wall thickness and LV septal thickness was noted in the drug using group compared to drug free subjects, when adjusted for BMI no significant difference could be observed, although a significant decrease in the BMI adjusted left ventricular end diastolic dimension was noted in the drug using group. It was concluded that AS may potentiate concentric left ventricular hypertrophy with decreasing ventricular compliance without affecting cardiac function. Urhausen et al examined the echocardiographic characteristics of male, top-ranking bodybuilders who had a history of AS use (n=14) and those without such a history (n=7) (Urhausen et al., 1989). A significant increase in the LV posterior wall thickness was noted in the steroid-abusing group in comparison to control in the
absence of a significant increase in BP. These differences became non-significant when adjusted for body weight.

Sachtleben et al examined body composition and myocardial structure in male weight trainers reportedly using or not using AS (Sachtleben et al., 1993). Amongst the steroid users a significant increase was found in LV mass (182.8±26.9g vs. 210.6±42g, p<0.05) and interventricular septum thickness during the peak of a drug taking cycle, compared to the end of an “off cycle” of at least 8 weeks duration. In addition, LV diameter and posterior wall thickness was significantly greater in steroid administering weight trainers during an “on period” compared to non-using controls.

Stolt et al. found that physiologically adaptive LVH does not increase QT dispersion in endurance athletes, although the QT interval is prolonged due to increased vagal tone (Stolt et al., 1999). In subjects undergoing weight training and administering high doses of AS, an observation of increased QT dispersion despite short QT intervals was made. This may indicate altered myocardial structure in hypertrophied heart and may increase the risk of malignant arrhythmia.

The apparent contradiction between various echocardiographic studies of AS users may be related to the type of exercise that the subjects are practising. Isometric exercise results in increases in left ventricular internal dimension and wall thickness, whereas isotonic exercise has not been associated with this type of change (Kennedy, 1992). Athletes will often mix both forms of exercise. Many studies did not differentiate between users who were currently on a “cycle” and users who were undertaking a drug free period. Sachtleben et al. demonstrated that changes in cardiac morphology are evident between cycles (Sachtleben et al., 1993). In addition, the fact that 1-D echocardiographic measurements were made in some studies, without the use of 2-D controls increases the chances of erroneous dimensions (Urhausen et al., 1989). A number of studies utilising echocardiography to measure changes in cardiac dimensions have omitted to confirm the reported drug use of their subjects using urine-analysis (Salke et al., 1985; De Piccoli et al., 1991). This omission is most significant in studies comparing wall thickness changes between athletes on and off their drug taking cycle (Urhausen et al., 1989).

Animal Studies

Animal studies regarding increased cardiac mass with AS administration are also confusing and contradictory. The reported morphological and histological effects of AS are shown in Table 1. 4. Unfortunately, a number of investigations have failed to properly control for the age of the animals. It is probable that the rodent and canine myocardium is more sensitive to steroid administration during the immature phase of its lifecycle (Kinson et al., 1991). Kinson and associates found that male rats castrated 10 days after birth, incorporated significantly less (Azevedo et al.)-leucine into
cardiac protein than sham operated controls. This effect was significantly reversed by testosterone administration. At day 60, exogenous testosterone administration had no significant effect upon amino acid incorporation. This suggests that the sensitivity of the rodent heart to testosterone is age dependent. Previously, it has been demonstrated that exogenous testosterone administered to adult female mice induces a significant increase in ventricular wet weight, without a corresponding increase in DNA (Koenig et al., 1982) implying that cardiac growth in these animals was due to hypertrophy of existing cells. It is probable that increases in cardiac mass with steroid administration during the immature phase of life are due to hyperplasia, and increases in adults are due to hypertrophy (Kinison et al., 1991).

Tagarakis et al. investigated the combined influence of various AS and physical exercise (Tagarakis et al., 2000). It was found that treadmill exercised, female, SPF-NMRI mice administered Dianabol or Oral-Turinabol showed mild myocyte hypertrophy. In drug-free rats, exercise induced a significant increase in capillary density and shortened the intercapillary distance. These microvascular changes were not present in exercised, AS treated rats. These findings support the hypothesis that AS increase heart muscle mass and therefore myocardial oxygen demand, without an increase in the number of collateral vessels.

Marsh and colleagues demonstrated that mRNA encoding androgen receptors could be detected in myocytes of male and female adult rats, neonatal rat myocytes, rat heart, dog heart, and infant and adult human heart (Marsh et al., 1998). In addition, cultured cardiac cells from neonatal rat hearts have been shown to increase the rate of phenylalanine incorporation, and extent of ANP secretion, in response to testosterone and dihydrotestosterone (Azevedo et al., 1983; Marsh et al., 1998). Phenylalanine incorporation and ANP secretion have both been shown to be indicators of cardiac hypertrophy. Unfortunately, Marsh et al. only examined ANP secretion and amino acid incorporation in neonatal cardiac myocytes, which are still in a significant growth phase (Kolbeck-Ruhmkorff et al., 1993).

Animal studies suggest that while hypertrophy can be observed in immature, developing rat hearts it is uncertain whether this same effect can be observed in adult hearts. The work of Tagarakis et al. suggests that AS may inhibit the normal collateral formation associated with exercising rats (Tagarakis et al., 2000).
Myocardial Cell Injury

Some of the first investigations into the pathology of chronic AS use were conducted in the late 1970s. Behrendt and Boffin treated male rats with once weekly injections of methandrostenolone (1.65mg/kg, i.m.) (Behrendt et al., 1977). At the conclusion of the 13-week treatment period the heart muscle cells were examined with electron microscopy. Mitochondria and myofibrils were found to display changes similar to those associated with early heart failure. The mitochondria were found to be elongated and swollen, with sparse matrix and cristae. The myofibrils either disintegrated as a result of treatment, showed widened and twisted Z-bands, or a complete dissolution of the sacromeric units. Later studies found an increase in the intermediate sized, nonmyofibrillar filaments in muscle cells of the left ventricle (Behrendt, 1977).

Testosterone ($10^4$M) has been found to cause a significant release of cytoplasmic lactate dehydrogenase (LDH) from rat primary myocardial cell cultures (Melchert et al., 1992b). Transmission electron microscopy revealed a significant decrease in mitochondria number as well as disruption of the mitochondrial and plasma membranes (Melchert et al., 1992a).

Tseng et al administered nandrolone decanoate (20mg/kg, s.c./day/6 wks) to developing, spontaneously hypertensive rats and found evidence of small focal areas of myocardial cellular damage with inflammatory cellular or fibrotic infiltration (Tseng et al., 1994).

Direct myocardial damage would be expected to result in a loss of aerobic energy production, cell death and eventually result in fibrosis. Areas of extensive myocardial fibrosis have been demonstrated to significantly increase the risk of fatal ventricular arrhythmia.
<table>
<thead>
<tr>
<th>Author, date</th>
<th>Species, strain, age</th>
<th>AS, dose, frequency</th>
<th>Exercise status</th>
<th>Morphological changes</th>
<th>Histological changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Phillis et al., 2000)</td>
<td>Rat, AW, 12 weeks</td>
<td>Nandrolone decanoate, 20mg/kg, s.c./3xs weekly/15 days</td>
<td>sedentary</td>
<td>no change in heart weight/body weight ratio</td>
<td>None reported</td>
</tr>
<tr>
<td>(Woodiwiss et al., 2000)</td>
<td>Rat, SD, 75-90g</td>
<td>Nandrolone decanoate, 5mg/kg, i.m./2xs weekly/12 weeks</td>
<td>Voluntarily exercised on training wheels</td>
<td>Absolute heart weight was found to be significantly reduced compared to exercised-drug-free controls and non-exercised, non-treated rats.</td>
<td>None reported</td>
</tr>
<tr>
<td>(Tseng et al., 1994)</td>
<td>Rat, SHR, 6 weeks</td>
<td>Nandrolone decanoate, 20mg/kg, s.c./daily x 6 weeks</td>
<td>sedentary</td>
<td>No change in absolute ventricular weight. Significant increase in ventricular/100g body weight (cp. control). Significant increase in vertical ventricular diameters, and vertical ventricular circumferences. Heart dry weight and heart weight to final body weight was not significantly different in treated rats compared to control.</td>
<td>Evidence of small focal areas of myocardial cellular damage with inflammatory cellular or fibrotic infiltration.</td>
</tr>
<tr>
<td>(Liang et al., 1992)</td>
<td>Rat, SD, 4 weeks</td>
<td>Nandrolone decanoate, 3.0mg/kg, i.m./7-9 days/9 injections</td>
<td>Exercised on a rodent treadmill wearing a weight collar 5 days/week for 10 weeks</td>
<td>Heart weight was not significantly changed in the nandrolone treated group compared to control at the conclusion of 10 wks treatment. Likewise, the ratio for heart weight to final body weight was not significantly different between steroid treated rats and controls, regardless of exercise status.</td>
<td>None reported</td>
</tr>
<tr>
<td>(Liang et al., 1993)</td>
<td>Rat, SD, 4 weeks</td>
<td>Nandrolone decanoate, 3.0mg/kg, i.m./7-9 days/9 injections</td>
<td>Exercised on a rodent treadmill wearing a weight collar 5 days/week for 10 weeks</td>
<td>Heart weight was not significantly changed in the nandrolone treated group compared to control at the conclusion of 10 wks treatment. Likewise, the ratio for heart weight to final body weight was not significantly different between steroid treated rats and controls, regardless of exercise status.</td>
<td>None reported</td>
</tr>
<tr>
<td>(Bauman et al., 1988)</td>
<td>Rat, SD, 6 weeks</td>
<td>Stanozolol, 5mg, i.m./2xs weekly x 5 weeks</td>
<td>Swimming exercise, 3xs a week.</td>
<td>Exercising rats receiving nandrolone, with or without dietary protein supplementation demonstrated a significant increase in relative heart weight compared to non-exercised, non-drug-treated controls.</td>
<td>No pathologic condition was reported for any organ.</td>
</tr>
</tbody>
</table>

.../continued over
Table 1. 4 Morphological and histological myocardial changes reported in laboratory animals subjected to chronic AS treatment. For more details on histological changes see Appendix B.

1.6.3 Hypertension

The ability of AS to produce hypertension remains controversial and confusing. The observation that Conn’s syndrome, which produces increased aldosterone and subsequently results in hypertension, led to the suggestion that anabolic-androgenic steroids may also produce elevated BP (Skelton et al., 1969). Two early studies appeared to confirm this hypothesis. Methyltestosterone and testosterone (10mg, 5xs/wk) were found to produce hypertension within 6 weeks of administration, to rats drinking 1% saline. Fischer and Swain injected castrated male rats with testosterone cypionate (1mg/wk, i.m.) and found that after 3 weeks of treatment, BP had increased significantly in steroid pre-treated rats in comparison to vehicle treated controls (Fischer et al., 1977). More recently Tseng et al found nandrolone decanoate (20mg/kg, s.c., daily) accelerated the development of hypertension in young, spontaneously hypertensive rats in comparison to vehicle treated controls (Tseng et al., 1994). An approximately 20mmHg difference was found between nandrolone treated and vehicle treated rats at the conclusion of the treatment period. It is not known whether normotensive adult male rats would respond in the same manner to nandrolone treatment.

Despite these studies showing an increase in BP with AS treatment, a number of authors have found no evidence of hypertension with AS administration. Wolinsky found BP was unchanged following 4 weeks treatment of male rats with testosterone cypionate (Wolinsky, 1972). Brown and
Pilch found that exercising male rats administered methyltestosterone showed no significant change in heart rate (HR) or BP following 6 weeks of treatment (Brown et al., 1972). Likewise, Greenberg and associates found that the BP of both male and female dogs was unchanged 24 hrs after the injection of testosterone (10mg/kg, i.m.) (Greenberg et al., 1973).

Many human studies have also failed to find any indication of hypertension with AS use. Olsson et al report that 7.5mg/day of oxandrolone administered to 25 men and women with hyperlipoproteinaemia significantly reduced plasma triglycerides but had no effect on resting BP (Olsson et al., 1974). McKillop found significant LVH in a male body builder with an eight-year history of steroid abuse with no associated hypertension (130/80 mmHg) (McKillop et al., 1986). Friedl et al found no change in BP following 12 weeks chronic treatment of non-athletes with methyltestosterone (40mg/day, p.o.), testosterone enanthate (280mg/kg/week, i.m.), testolactone (280mg/kg, i.m.) or testosterone enanthate and testolactone (Friedl et al., 1990). Thompson et al and Urhausen et al found no change in BP in male power athletes administering a competitive regimen of steroids compared to their steroid free associates (Urhauen et al., 1989; Thompson et al., 1992). Sader et al found similar resting BP in AS using bodybuilders compared to their drug free colleagues (Sader et al., 2001).

Riebe et al measured BP at rest and during a standardised exercise test in AS abusing weight lifters, in their drug free associates, and in sedentary controls (Riebe et al., 1992). They found that the AS abusing group had significantly higher systolic BP at rest and during exercise. Adjusting rest and exercise BP for body weight or biceps size eliminated statistically significant differences between groups. This suggests that the higher rest and exercise BP values noted in AS users may simply reflect their larger body mass or be related to the larger arm circumference of these subjects. Blood pressure measurements were made using sphygmanomanometry, which is influenced by biceps size (Riebe et al., 1992). Variation of up to 13 cm was found between subject arm circumferences. The use of a standard sphygmanomanometry cuff in situations of abnormally large arm circumference can result in an over-estimate of BP (Urhauen et al., 1989). Further studies should include intraarterial BP measurement to avoid difficulties associated with BP measurement via indirect methods.

It is important to note that heavy resistance exercise itself can cause massive elevations in BP. MacDougall et al measured BP in the brachial artery in five experienced body builders performing a variety of resistance exercises until failure (MacDougall et al., 1985). The greatest peak pressures were recorded during the double leg press where the mean for the group was 320/250mmHg, with pressures in one subject exceeding 450/350mmHg. It was concluded that in young healthy subjects performing weight-lifting exercises, the mechanical compression of the blood vessels combines with a potent pressor and Valsalva response to produce extreme elevations in BP. While AS abuse
itself may not cause hypertension, other factors such as vasospasm and arteriosclerosis may alter the pressor response to resistance exercise.

It is possible that AS may cause increases in BP through stimulation of the hypothalamic-pituitary-adrenal (HPA) axis. In a recent study Schlussman and associates found that daily treatment of male SD rats with nandrolone decanoate (15mg/kg, i.m.) resulted in a significant increase in circulating adrenocorticotropin hormone (ACTH) and corticosterone 1 hour after the third steroid injection compared to vehicle treated controls (Schlussman et al., 2000; Schlussman et al., 2002). Adrenocorticotropin hormone levels did not remain elevated at 24 hours following nandrolone administration. Unfortunately, this study did not monitor BP changes over the nandrolone treatment period. Conversely, Alen et al measured ACTH levels in athletes self-administering AS for 26 weeks and then during withdrawal over the following 12 weeks and compared these results to a group exercising with the same physical intensity, but who were drug free for the entire period (Alen et al., 1985). After 8 weeks of AS treatment ACTH levels were found to decrease significantly in comparison to the control group and remained lower than control for the entire drug treatment period. Adrenocorticotropin hormone levels began to increase 6 weeks after cessation of AS administration. Unfortunately this study made no attempt to correlate serum hormone levels and BP. Schyvens et al recently studied the effect of exogenous ACTH (500µg/kg) on BP in telemetered mice (Schyvens et al., 2001). Blood pressure was clearly elevated after 2 days of treatment and by day 10 had risen to 136±4mmHg, which was significantly higher than vehicle treated controls (p<0.01). Unfortunately, plasma levels of ACTH were not progressively measured over the treatment period and therefore it was not possible to equate changes in BP with the concentration of circulating ACTH. Turner et al used the tail cuff method to record BP in male SD rats and found that systolic pressure increased significantly over 10 days of treatment with an ACTH dose of only 100µg/kg/day from 117±1 to 141±2 mmHg (Turner et al., 2001). Serum corticosterone was increased to 825±70 ng/mL in ACTH treated rats. Further studies by Lou et al found that both ACTH (500µg/kg/day) and corticosterone (120µg/kg/day) significantly increased BP compared to control (165±6 vs 127mmHg, p<0.001 for ACTH; 162±11 vs 111±6 for corticosterone, p<0.001) (Lou et al., 2001). It remains unclear whether high dose chronic treatment with AS increases BP to hypertensive levels through increased plasma concentrations of ACTH and corticosterone.

Both animal and human studies indicate that hypertension does not always occur after AS administration. Human data indicates that hypertension may occur in selected male patients or athletes. There is a lack of both human and animals studies designed to address the occurrence and potential mechanisms of steroid induced hypertension.
1.7 Difficulties associated with anabolic steroid research

It has proven extremely difficult to gather well-controlled clinical information from patients abusing AS. Competitive athletes are often unwilling to admit AS use (Nieminen et al., 1996). Patients presenting with serious cardiac conditions linked with AS abuse are commonly uncooperative with ongoing research and are usually lost to follow-up (Sullivan et al., 1999).

The dose of AS associated with a fatal abusive incident has never been reported (Melchert et al., 1992b). This implies that clinically relevant doses for investigations of toxicity are difficult to extrapolate. However, the plasma concentration following a therapeutic dose of testosterone enanthate in 19-40 year old males has been shown to reach an average of $4.37 \times 10^{-8}$M. As it is known that steroid users administer AS at doses 10-1000s the recommended therapeutic dose it is reasonable to suggest that plasma concentrations in the $10^{-7}$ to $10^{-4}$M range would not be unexpected (Melchert et al., 1992b; Welder, 1992).

Because of legal aspects of the non-therapeutic use of AS it is difficult to implement well controlled clinical trials. The unique administration pattern of many steroid users involving exceptionally high doses of various types and formulations of AS, administered by a variety of routes makes a human trial mimicking an abuse situation unethical (Parssinen et al., 2000). In addition the “drug free holidays” taken by some athletes immediately prior to competitions adds a further layer of complexity. Studies concerned with the cardiotoxic potential of AS are hampered by strenuous exercise in athletes and the highly modified diet of many weightlifters and body builders.

Many athletes will participate in a strict “pre-competition” diet in the 2-4 months prior to competing (Kleiner et al., 1990). Food diaries from body-builders preparing for competition have revealed diets typically high in protein (>3 times the recommended daily intake, RDI) (Kleiner et al., 1990), and cholesterol (>2.5 RDI) (Keith et al., 1996). Reports of male bodybuilders consuming up to a dozen eggs a day are not uncommon (Faber et al., 1986). In addition many competitors have increased gut motility due to laxative use. In an attempt to improve muscle definition in the hours prior to competition, many bodybuilders will reduce water intake, administer diuretics and undertake dehydrating exercises such as intensive exercise in a sauna (Kleiner et al., 1990). Heavy laxative and diuretic use can cause electrolyte disturbances increasing the risk of cardiac abnormalities (Hoes et al., 1994; Grobbe et al., 1995). In addition, severely restricted calcium (36% RDI) and reduced zinc (75% RDI) intake has been reported in women body builders (Kleiner et al., 1990). Many competitors believe that by “sodium-loading” they will be able to maximise their fluid loss. This consists of following a severely salt-restricted diet until the week of the competition.
Large amounts of salty foods are then consumed for the next 2-3 days before recommencing a sodium fast (Kleiner et al., 1990).

In addition AS are often not only administered in combination with stimulants, but also drugs supposedly helpful in assisting muscle building or definition, or to lessen the perceived side effects of AS, such as spironolactone (prevents K+ loss), Clomifén (increase gonadotropin levels) and contraspasmin (has β₁ & β₂ adrenergic effects) (Hausmann et al., 1998).

1.8 The polydrug abuse phenomenon

Users of illicit drugs normally do not restrict their use to one drug. Poly-drug abuse is common. A number of reports have indicated severe cardiac consequences from an AS and sympathomimetic combination. Reports include combinations of AS and amphetamine (Capezzuto et al., 1989; Appleby et al., 1994) as well as AS and cocaine (Kennedy et al., 1993b).

Because many AS users are athletes and are greatly concerned with their body image and health it could be presumed that they may be unique among drug users, and be unlikely to engage in polypharmacy (DuRant et al., 1993b). Meilman et al surveyed approximately 60,000 college students for use of various legal and illicit drugs. Amongst a subgroup of 175 AS users it was found that they were significantly more likely to use tobacco, marijuana, cocaine, amphetamines, sedatives, hallucinogens, opiates, inhalants and designer drugs than their steroid free colleagues (Meilman et al., 1995). In addition, DuRant et al found that cocaine was preferentially co-abused in AS users attending U.S. High Schools (DuRant et al., 1993b; DuRant et al., 1995). Cocaine was found to be the strongest co-varying factor with the use of AS (DuRant et al., 1993b; DuRant et al., 1995). The stability of this relationship over a 4-month period was also demonstrated (DuRant et al., 1994).

The number of 12th grade U.S. High School students reporting cocaine use in the past 12 months peaked in 1999 at 6.2%, and in 2001 was estimated at 4.8% (Johnston et al., 2002). The current "lifetime prevalence" of cocaine use in these students is estimated at 8.2% (Johnston et al., 2002). Therefore, although it appears that cocaine use has now stabilised, the prevalence of use remains high. The 2000 National Household Survey on Drug Abuse (NHSDA) estimated that there are 14 million illicit drug users in the US, of which 1.2 million are current cocaine users. This represents 0.5% of the population older than 12 years. Amongst youths aged between 16-17 prevalence of use in the past month is estimated at 1.1%. In combination with a significant prevalence of abuse of cocaine the reported lifetime prevalence of AS use has increased 1.2% to 3.7% in 12th grade students between 2000 and 2001 (Johnston et al., 2002). The percentage of students reporting use in
...past 12 months also increased during 2001 to approximately 2.4%. Thus considerable potential for co-abuse of AS and cocaine exists in the adolescent population.

Based on evidence of a significant potential for co-abuse of stimulants with AS, in addition to reports suggesting a high likelihood of abuse of this drug combination in adolescents, it was decided to further investigate the potential cardiac toxicity resulting from this drug combination. Despite the diverse pharmacology of AS and cocaine it has been observed that these two drugs share a very similar spectrum of cardiotoxic events, associated with both chronic and acute use. A possible synergistic or additive cardiotoxic interaction between these two drugs requires further investigation.

1.9 The pharmacology of cocaine

Cocaine (benzylmethylecgonine C17H21NO4) is an alkaloid extracted from the South American shrub Erythroxylon coca. Cocaine has both a sympathomimetic and a local anaesthetic effect, and is thought to have both central and peripheral actions. Cocaine can act on the CNS to prevent the neuronal reuptake of noradrenaline and to trigger the release of catecholamines from the adrenal medulla (Schindler et al., 1992). Cocaine also has a peripheral sympathetic effect through blockade of noradrenaline reuptake (Iversen, 1963). In addition Cocaine has been shown to cause noradrenaline efflux in isolated rat brain tissue (Azzaro et al., 1974).

A number of studies have provided evidence for the importance of the CNS in the cardiovascular response to cocaine. Wilkerson et al demonstrated that the BP response to intravenous cocaine in conscious dogs was decreased by more than 50% following pre-treatment with the ganglionic blocker, hexamethonium (Wilkerson, 1988). Similarly, anaesthesia with pentobarbital almost eliminated the pressor response to cocaine in comparison to the same rats given an identical dose of cocaine on a different day. Chiueh & Kopin demonstrated that cutting the splanchnic nerve reduced the cocaine-induced release of adrenaline and noradrenaline by 75 and 50%, respectively, in rats in which the noradrenaline metabolising enzyme, catechol O-methyltransferase (COMT) had been inhibited (Chiueh et al., 1978). Cocaine methiodide, which does not cross the blood-brain-barrier, infused intravenously into conscious rats until death has been found to be 6 times less potent than cocaine on a molar basis (Tella et al., 1992).

Many of the cardiac and behavioural effects of cocaine can be attributed to its ability to block the synaptic monoamine reuptake pump – causing the persistence of noradrenaline, dopamine and serotonin in the cleft (Fig. 1.3). Under normal physiological conditions when a sympathetic nerve is stimulated, the noradrenaline not bound to a receptor on a receiving nerve terminal, is taken back
up into the parent nerve terminal by the monoamine reuptake pump (Van Dyke et al., 1982). This prevents the accumulation of noradrenaline in the synaptic cleft and prolongation of a sympathetic response. Cocaine binds to the monoamine reuptake pump and prevents the removal of noradrenaline from the synaptic cleft. This results in a sympathomimetic response to the presence of cocaine. Under conditions of low sympathetic tone, synaptic noradrenaline effectively controls the rate of its own release by activating presynaptic α₂-adrenergic receptors (Yamaguchi et al., 1977). In the presence of cocaine, sympathetic tone is elevated and this negative feedback is insufficient to prevent accumulation of noradrenaline in the synaptic cleft (Cousineau et al., 1986).

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**Fig. 1. 3 Sympathomimetic action of cocaine.** Adapted from Van Dyke, 1982. Top: as an action potential is received at the synapse, vesicles of noradrenaline are released across the synaptic cleft to bind at receptors on the surface of the receiving nerve terminal. To prevent the persistence of this signal all unbound noradrenaline in the synaptic cleft is taken up by the parent nerve terminal via the monoamine reuptake pump. Bottom: cocaine binds to the monoamine reuptake pump and prevents the reuptake of noradrenaline within the synaptic cleft. This allows noradrenaline to persist and continuously activate noradrenaline receptors on the receiving nerve terminal resulting in a sympathomimetic response characterised by an increase in HR and BP.
Fig. 1. 4 The local anaesthetic action of cocaine. Adapted from Van Dyke, 1982. Top: Cocaine can block the transmission of sensory nerve impulses by changing the permeability of the axon membrane to sodium ions. In the absence of cocaine a sensory impulse is passed along the axon by a change in permeability to sodium ions, which reverse the charge difference between the axoplasm (negatively charged) and the axon membrane (positively charged). Bottom: In the presence of cocaine the nerve terminal cannot be stimulated because cocaine blocks the sodium channel and prevents ion flux.

Cocaine can also block the transmission of sensory nerve impulses and cause cardiac rhythm disturbances by changing the permeability of the axon membrane to Na⁺ and K⁺ (Fig. 1. 4).

1.10 Preparations
Cocaine is available in a number of different forms. Cocaine hydrochloride salt, colloquially termed “snow” or “Coke”, is often intranasally administered (“snorted”), it can also be injected (i.v. or i.p.) or taken orally (Manschreck et al., 1987; Lathers et al., 1988; Om, 1992; Das, 1993). There are two methods for the preparation of alkaloid cocaine. Cocaine hydrochloride salt can be mixed with buffered ammonia. The alkaloidal cocaine is extracted from solution using ether. The ether is then evaporated to produce a hard, crystalline substance known as “freebase” (Strang et al., 1993; Warner, 1993). Freebase cocaine does not decompose on heating and can be smoked (Om, 1992) which produces an extremely rapid and intense “high”, similar in onset of effect to intravenous cocaine (Pierce et al., 1986; Manschreck et al., 1987; Lathers et al., 1988). Alternatively, cocaine hydrochloride can be combined with “baking soda” (sodium bicarbonate) and heated until a solid forms (Cornish et al., 1996), known colloquially as “crack”. Crack produces a distinct “popping” sound when heated. Both crack and freebase cocaine can be smoked in pipes or mixed with marijuana or tobacco and smoked in a cigarette (Warner, 1993).
“Cutting agents” added on the street to increase cocaine bulk complicate understanding the toxicology of cocaine. These include procaine, lidocaine, tetracaine, caffeine, benzocaine, phencyclidine (“angel dust”), amphetamines, quinine, talc and strychnine (Garber et al., 1987; Lathers et al., 1988).

1.11 Metabolism
Cocaine is detoxified by plasma and hepatic cholinesterases to water-soluble metabolites, which can then be excreted in urine (Kamendulis et al., 1996). The major route of cocaine metabolism in humans involves carboxyesterase-1 (chE1) catalysed de-esterification to produce benzoylecgonine (BE) (Stewart et al., 1977; Brzezinski et al., 1997). Very little cocaine appears unchanged in the urine and most is excreted as the BE metabolite.

Carboxyesterase-2 (chE2) can also act on cocaine, producing ecgonine. Ecgonine is highly polar and almost completely metabolically inert (Pindel et al., 1997). Ecgonine accounts for between 32% and 49% of urinary metabolites.

![Diagram of metabolic pathways for cocaine mediated by carboxyesterases](image)

**Fig. 1.** Major metabolic pathways for cocaine mediated by carboxyesterases. Source: (Vitti et al., 1985)
Small amounts of cocaine are N-demethylated by cytochrome P450 to produce norcocaine. N-demethylation accounts for only about 2-6% of cocaine metabolism (Stewart et al., 1978; Inaba, 1989).

![Chemical structures of cocaine and norcocaine](image)

Fig. 1. 6 P-450 mediated n-demethylation of cocaine to produce nor-cocaine. Source: (Vitti et al., 1985).

It is important to note that when ethanol is consumed with cocaine a new toxic metabolite is formed, cocaethylene. In the presence of ethanol some cocaine undergoes transesterification with ethanol instead of hydrolysis with water forming an active metabolite (Brzezinski et al., 1997; Laizure et al., 2003). Co-ingestion of cocaine and alcohol has been found to reduce the fraction of cocaine dose converted to benzoylecgonine, and increase the formation of norcocaine (Pan et al., 1999a). The same carboxyesterases involved in cocaine metabolism are also thought to be implicit in cocaethylene metabolism (Brzezinski et al., 1997; Laizure et al., 2003).

1.12 Pharmacokinetics

Presumably, due to the difficulty of obtaining ethical approval for the use of cocaine in human subjects, very few studies have investigated pharmacokinetics in man. However, Chow and associates report average bioavailability of 132L, t½ of 48±13 minutes and 2.10L/min elimination clearance in five subjects given a 32mg intravenous dose of cocaine (Chow et al., 1985). This same dose was administered by Javaid and colleagues whose reported values are similar to those obtained by Chow et al (i.e. F=161L, t½=41.4±8.2 min., Cl<sub>elim.</sub>=2.45L/min) (Javaid et al., 1978; Javaid et al., 1993). It must be noted that both of these studies used subjects with previous histories of cocaine abuse. Javaid et al also quantified the differences in the kinetic profile of cocaine administered by the i.v. route compared to intranasal administration (Javaid et al., 1978). It was found that intranasal administration produced peak plasma cocaine levels in less than 30 minutes, and that plasma concentrations remained elevated past 60 minutes post inhalation. This resulted in a prolonged chronotropic effect of cocaine, which only declined after approximately 40 minutes. Importantly,
kinetic studies using rats have found that i.p. administration of cocaine closely parallels the kinetics of intranasal administration in humans (Wilkinson \textit{et al}., 1980; Lathers \textit{et al}., 1988).

Because cocaine is highly lipid soluble, peak levels are evident very rapidly in brain tissue. Experiments performed in rats demonstrate that cocaine rapidly crosses the blood brain barrier and distributes unequally in different brain regions (Javaid \textit{et al}., 1993). This unequal disposition pattern may account for differential biochemical effects in different brain regions. Low levels of cocaine were found in the nucleus accumbens and frontal cortex, whereas very high cocaine concentrations were discovered in the amygdala, caudate putamen and septum.

Oral cocaine administration to humans results in a predictably long lag period until peak plasma levels are reached. In 7 human volunteers who had all previously used cocaine for recreational purposes cocaine was not detected in the plasma until 30 minutes after the dose was administered (Wilkinson \textit{et al}., 1980). Peak plasma concentrations were recorded at 64±6.7 minutes.

Co-ingestion of alcohol and cocaine has been found to modify the pharmacokinetics of cocaine. Laizure \textit{et al} found that both cocaine and cocaethylene clearances are decreased by about 20% when given with alcohol (Laizure \textit{et al}., 2003). Co-administration of ethanol and cocaine in rats has been found to increase systemic bioavailability of cocaine by 37% when administered intraperitoneally (Pan \textit{et al}., 1999b).

1.13 Cocaine Cardiotoxicity

A usual recreational dose of cocaine by the intranasal route is about 100mg (Resnick \textit{et al}., 1977; Gradman, 1988). The doses of cocaine reported in the literature and associated with fatality vary widely. Plasma concentrations as low as 0.1mg/ml and as high as 24 mg/ml have been associated with sudden death (Wetti \textit{et al}., 1985; Vitullo \textit{et al}., 1989; Simkhovich \textit{et al}., 1994). Plasma concentration in post-mortem blood samples has been found to be unreliable in determining a toxic dose. This is because the rapid hydrolysis of cocaine in unpreserved blood and the site-dependent post-mortem release of cocaine from tissue stores results in large variation in concentration (Hearn \textit{et al}., 1991). Perimortem blood samples have proven difficult to collect due to the rapid onset of death following overdose, further limiting access to reliable data on blood concentrations of cocaine.
Chronic and acute administration of cocaine has been associated with a wide spectrum of adverse cardiac events including sudden cardiac death, cardiomyopathy, hypertension, myocardial infarction, myocardial fibrosis, myocarditis, ventricular tachycardia and fibrillation, cardiac hypertrophy, atherosclerosis, thrombosis and aortic rupture (Table 1.5)

The causes of cocaine-induced sudden death are likely to be multifactorial. Clinical and experimental evidence exists for at least three mechanisms of cocaine cardiotoxicity in humans.

1. **Reduced coronary flow**
   a. Vasospasm
   b. Thrombosis
   c. Arteriosclerosis

2. **Cardiac arrhythmias**
   a. Intracellular calcium flooding;
   b. Regional differences in the rate of repolarisation.

3. **Direct cardiac effect**: such as cardiac hypertrophy and pathological effects.
<table>
<thead>
<tr>
<th>Physiological System</th>
<th>Adverse Effect</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>Sudden cardiac death</td>
<td>(Bauman et al., 1994; Shen et al., 1995)</td>
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<tr>
<td></td>
<td>Cardiomyopathy</td>
<td>(Besse et al., 1997)</td>
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<td></td>
<td>Hypertension</td>
<td>(Hollander et al., 1997)</td>
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<tr>
<td></td>
<td>Myocardial infarction</td>
<td>(Isner et al., 1986; Simpson et al., 1986; Mittleman et al., 1987; Virmani et al., 1988)</td>
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<tr>
<td></td>
<td>Myocardial contraction band necrosis</td>
<td>(Simpson et al., 1986; Osawa et al., 1994)</td>
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<tr>
<td></td>
<td>Myocardial fibrosis</td>
<td>(Isner et al., 1986; Dressler et al., 1990)</td>
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<td></td>
<td>Myocarditis</td>
<td>(Virmani et al., 1988)</td>
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<td>Ventricular tachycardia</td>
<td>(Isner et al., 1986)</td>
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<td>Ventricular fibrillation</td>
<td>(Nanji et al., 1984)</td>
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<td>Cardiac hypertrophy</td>
<td>(Brickner et al., 1991; Om et al., 1993; Karch et al., 1995)</td>
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<td>Coronary vasoconstriction</td>
<td>(Lange et al., 1989)</td>
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<td></td>
<td>Atherosclerosis</td>
<td>(Dressler et al., 1990; Om et al., 1992)</td>
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<td>Thrombosis</td>
<td>(Isner et al., 1986)</td>
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<td></td>
<td>Aortic rupture</td>
<td>(Adkins et al., 1993; Baumgartner et al., 1997)</td>
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<td>Respiratory arrest</td>
<td>(Wetli et al., 1985)</td>
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<td>Cerebrovascular</td>
<td>Cerebral aneurysm</td>
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<td>Cerebral infarction</td>
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<td>Central nervous system</td>
<td>Seizures</td>
<td>(Jonsson et al., 1983; Campbell, 1988)</td>
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<td>Cerebral Vasculitis</td>
<td>(Ferenchick, 1991)</td>
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<td>Cerebral oedema</td>
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<td>Psychosis</td>
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<td>hyperthermia</td>
<td>(Wetli et al., 1985; Campbell, 1988)</td>
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<tr>
<td>Other</td>
<td>Metabolic acidosis</td>
<td>(Jonsson et al., 1983; Wetli et al., 1985)</td>
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<tr>
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<td>Severe weight loss</td>
<td>(Karch et al., 1998)</td>
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Table 1. 5 Spectrum of toxic events attributed to cocaine abuse.
One of the most documented outcomes of cocaine abuse is myocardial infarction (Coleman et al., 1982; Howard et al., 1985; Pasternack et al., 1985; Simpson et al., 1986; Weiss, 1986; Ascher et al., 1988; Hollander et al., 1997; Wilson, 1998). A number of models are available to explain reduced coronary flow potentially resulting in myocardial cell death.

1.13.1 Reduced coronary flow

Vasosconstriction

Cocaine can cause both direct and α-adrenoceptor-mediated vasoconstriction. The sympathomimetic action of cocaine results in a dramatic increase in myocardial oxygen demand. Under conditions of coronary artery narrowing, oxygen demand and supply are unmatched. The resulting trans-cardiac oxygen difference increases the risk of cardiac arrhythmia and subsequent infarction (Billman, 1990). The sympathomimetic action of cocaine can involve a significant pressor affect. The literature supports several cases of aortic rupture associated with cocaine abuse (Adkins et al., 1993; Baumgartner et al., 1997) Numerous case reports have noted the ability of cocaine to induce ischaemia in the absence of significant atheroma or thrombus (Osawa et al., 1994).

Reports of cerebral ischaemia and vasculitis attributable to chronic cocaine use suggest that severely reduced blood flow is not exclusive to the heart (Fredericks et al., 1991). Reduced regional cerebral blood flow has been demonstrated in the rat brain (Stein et al., 1993).

Direct mechanism

Cocaine has been shown to cause vasoconstriction in freshly explanted human coronary artery rings and sympathetic innervation independent constriction, in human umbilical cord (Chokshi et al., 1989). Isolated perfused rat hearts demonstrate a phentolamine resistant decrease in myocardial flow in response to cocaine. Glutaraldehyde fixing of cross-sections of this cardiac tissue has allowed a direct correlation between reduced coronary flow and coronary diameter to be made (Vitulo et al., 1989). Decreased coronary flow rate (CFR) in response to concentrations of cocaine thought to closely resemble plasma concentrations achieved with recreational dosing, has been evidenced in isolated, perfused rabbit hearts (Simkhovich et al., 1994).

α-mediated mechanism

Human studies

The α-adrenoceptor mediated mechanism of coronary artery narrowing by cocaine has been illustrated by a number of in vivo studies. Despite this, it has proven difficult to substantiate a conclusive link between arterial narrowing and myocardial infarction. Eisenberg et al investigated ventricular function immediately after intravenous cocaine using 2-dimensional echocardiography
and ECG. No evidence for acute myocardial ischaemia or left ventricular dysfunction was found in these subjects when administered intravenous doses of cocaine similar to those abused recreationally. (Eisenberg et al., 1993). However, it must be noted that this study was performed in current cocaine users who may exhibit tolerance to the cardiovascular effects of cocaine. As echocardiograms were only recorded at 2 and 7 minutes after infusion it is possible that cardiac complications occurred in the subacute period (greater than 7 minutes).

However, a number of clinical studies have shown a clear ability of cocaine to produce reductions in coronary diameter. A small dose of intranasal cocaine administered to patients undergoing cardiac catheterisation for the evaluation of chest pain was found to induce a pressor and chronotropic response, concomitant with an 8-12% reduction in coronary artery diameter which significantly reduced coronary sinus flow (Lange et al., 1989). Phentolamine administration returned BP, HR and coronary diameter to baseline.

Moliterno et al studied the effects of intranasal cocaine (2mg/kg) in coronary artery disease patients undergoing angiography, and found an average decrease in diameter of 7±1% in non-diseased coronary artery segments, and an extra 2% in diseased segments (Moliterno et al., 1994b). Flores et al performed angiography on 18 patients undergoing evaluation for chest pain (Flores et al., 1990). The decrease in luminal diameter following administration of an intranasal cocaine solution was found to be significantly greater in diseased segments of the left coronary artery than non-diseased (mean±SD, 29±23% vs 13±8%, p<0.05).

Brogan et al demonstrated that the vasoconstriction can also be attributed to the metabolites of cocaine (Brogan et al., 1992). Patients undergoing angiography for the evaluation of chest pain were administered either intranasal saline or cocaine HCl (2mg/kg). Significant vasoconstriction was noted at 30 minutes post cocaine administration, which returned to baseline by 60 minutes. At 90 minutes, plasma levels of benzoylecgonine and ethyl methyl ekgonine peaked, which corresponded to a further decrease in epicardial coronary artery diameter. Presumably, the vasoconstrictive effect of cocaine metabolites can explain the ischaemic effects of cocaine observed angiographically several hours after reported cocaine ingestion (Ascher et al., 1988). Interestingly, Nademanee et al undertook 24-hour Holter-monitor recordings from patients undergoing cocaine withdrawal to find significant ST elevation during the first 2 weeks of the drug-free period (Nademanee et al., 1989). All episodes of ST elevation were asymptomatic, suggesting that cocaine is capable of causing 'silent ischaemia' long after removal of the drug.
Animal studies

Hale and colleagues measured myocardial blood flow and circumflex artery diameter, before, and after, a high dose of cocaine (7 or 10 mg/kg, i.v. bolus) or saline in pentobarbital anaesthetised dogs (Hale et al., 1989). Artery diameter was found to decrease by 15±4% in response to cocaine, concomitant with a subepicardial regional myocardial blood flow reduction of 22%. A separate group of animals, also administered cocaine, showed evidence of decreased left ventricular function (reduction in HR and left ventricular dP/dt, and an increase in left ventricular end-diastolic pressure). Hayes et al attempted to find a dose-response relationship between cocaine dose and cross-sectional area of the coronary arteries. Constriction was found to be maximal at 60 minutes after a bolus injection of 0, 1, 3, 6, 9 mg/kg cocaine, corresponding with a 2±10, 17±8, 45±9, 33±8 and 46±10%, reduction in coronary diameter (Hayes et al., 1991). No attempt was made in this study to quantify the progression of myocardial ischaemia resulting from vasoconstriction. Investigation of the coronary sinus oxygen content following a low dose of cocaine (1 mg/kg, i.v.) in conscious dogs determined that while coronary vascular resistance increased by 24±3%, and myocardial oxygen consumption increased by 41±9% this was offset by an increase in the arterial oxygen content (by 2.8±0.3 vol%) and coronary blood flow (13±3%) (Shannon et al., 1993). This suggests that while oxygen demand is increased with cocaine, demand is matched by supply through an increase in arterial oxygen. A significant rise in haemoglobin from 12 to 14 g/dL which persisted for 60 minutes after cocaine injection was thought to contribute to the observed effect on arterial oxygen. The mechanism of this haemoglobin effect was not elucidated. It is uncertain whether the observed increase in arterial oxygen in this study is conserved at higher doses of cocaine.

It is probable that the vasoconstrictive effects of cocaine are time dependent. A biphasic effect of intravenous cocaine has been noted in pigs (Zimring et al., 1994). An initial, brief, but significant increase in coronary blood flow (CBF) was noted, followed by a more sustained decrease (17.0±3.3%). From 4 minutes following infusion, coronary artery diameters were found to decrease. This suggests that the vasoconstrictive response to cocaine only occurs after an initial vasodilation. It is possible that the dual-effect of cocaine on coronary vasculature can be attributed to its diverse pharmacology. It is probable that the vasodilation is caused by the local anaesthetic effect of cocaine and the vasoconstriction by the α-adrenergic response of the coronary arteries (Friedrichs et al., 1990; Benzaquen et al., 2001). Friedrichs et al found that both lidocaine and cocaine produced a significant decrease in coronary perfusion pressure, under conditions of constant coronary blood flow, suggesting that this transient vasodilation was due to a local anaesthetic effect (Friedrichs et al., 1990).
Although most studies investigating vasospasm have indeed observed small amounts of vasoconstriction it is uncertain whether the extent of constriction is sufficient to induce myocardial ischaemia (Benzaquen et al., 2001). It is probable that the degree of constriction induced by cocaine is much greater in diseased subjects. Miniature pigs subjected to regional endothelial denudation of the left anterior descending coronary artery, and fed a high cholesterol diet displayed myointimal thickening at the denuded site, which increased the constrictive effect of cocaine. Denuded coronary arteries displayed a significantly greater constriction (59±5%) than native ones (48±5%, P<0.05) (Egashira et al., 1991).

**Thrombosis**

A number of case reports on cocaine intoxication have demonstrated thrombus formation associated with cocaine use (Simpson et al., 1986; Virmani et al., 1988; Stenberg et al., 1989). It is not unusual for sudden cardiac death to be attributed to cocaine abuse, yet for the coronary arteries of the victim to be free of thrombus. In some of these cases it is possible that the fatal event may have been caused by a clot, which subsequently underwent thrombolysis (Rezkalla et al., 1990).

Rezkalla investigated cocaine induced platelet aggregation in platelet-rich plasma from cocaine-naïve patients (Rezkalla et al., 1993). Spontaneous, adrenaline and collagen-induced platelet aggregation tests produced no difference between control and cocaine treated samples. In response to adenosine diphosphate induced platelet aggregation, there was an increase in aggregation in the platelets incubated with cocaine in the presence of a 1µg dose of adenosine diphosphate (ADP). This suggests that cocaine may increase the sensitivity of platelets to certain aggregatory stimuli. However, *in vitro* platelet studies do not always accurately correlate with clinical events.

The incubation of platelet-rich plasma does not maintain the interaction of platelets with other blood cells such as leucocytes or erythrocytes (Heesch et al., 2000). Therefore Kugelmass et al investigated human platelet activation by examining P-selectin expression as a marker of platelet activation in whole blood (Kugelmass et al., 1993). Incubation of whole blood with cocaine (10µM to 13mM), induced significant increases in platelet associated fibrinogen (range of increase, 45±12% to 125±40%) and P-selectin expression (36±15% to 112±24%). However, it must be noted that only a minor provocation causes a release of stimulants from platelet granules, and the most potent platelet stimulators are other platelet products (Benzaquen et al., 2001). Virmani et al demonstrated serum cocaine concentrations of up to 80µM in autopsy patients with cocaine associated deaths (cited Kugelmass et al., 1993). It is therefore probable that while the higher mM cocaine doses used in this study have little clinical relevance Kugelmass *et al* still provide compelling
evidence of an increase in platelet associated fibrinogen and P-selectin expression at µM concentrations of cocaine.

A study performed in New Zealand White rabbits given daily intravenous infusions of cocaine (2mg/kg) or saline, examined segments of aorta for histological changes following 6 or 12 weeks of treatment (Eichhorn et al., 1992). The endothelium of aortic segments from cocaine treated rabbits produced high levels of the potent vasoconstrictor, thromboxane A2, generating a favourable milieu for thrombosis.

*In vivo* investigations of the effect of cocaine on platelets are limited. Using flow cytometry, Rinder et al observed the percent of activated platelets (those expressing P-selectin) in whole blood from chronic cocaine users compared to drug free controls (Rinder et al., 1994). It was found that cocaine users had a significantly greater (p=0.01) percent of activated platelets compared to controls due to a subset of cocaine users who showed an unusually high level of P-selectin expression. Plasma from these same subjects showed no effect upon *in vitro* platelet activation or aggregation, either directly or in concert with platelet agonists. However, in experiments where subjects received a blinded infusion of cocaine or placebo – the mean percent of activated platelets was found to rise sharply for both treatments. This suggests that *in vivo* α-granule release must occur by an indirect mechanism, and highlights the differences that may be observed between *in vivo* and *in vitro* investigations of aggregability. Heesch et al investigated the platelet affect of cocaine in 14 healthy, cocaine-naïve subjects in a randomised, double blind crossover study. Quantification of the platelet specific protein in blood following either cocaine (2mg/kg, intranasally) or placebo, revealed significant cocaine induced increases in platelet factor 4 and β thromboglobulin at 120 minutes following cocaine administration (Heesch et al., 2000). Microaggregate formation increased significantly at 40 and 80 minutes following cocaine administration. Bleeding time decreased following cocaine, but the change was not observed to be statistically significant. This suggests that exposure to a low dose of cocaine (2mg/kg) in naïve subjects can cause platelet activation, α-granule release and microaggregate formation. It is interesting that Rinder et al observed that activated platelets persisted for almost 6 hours after cocaine administration in chronic cocaine users (Rinder et al., 1994). Heesch et al observed a rapid clearing of microaggregates in naïve subjects. This suggests that chronic cocaine use may inhibit the reticuloendothelial system usually involved in removing microaggregates (Heesch et al., 2000).

The affect of cocaine on the plasma constituents involved in endogenous thrombosis and thrombolysis has not been well characterised. Moliterno et al found that cocaine (2mg/kg) administered intranasally to patients undergoing cardiac catheterisation produced a significant
ation in plasminogen activator inhibitor (PAI-1) activity compared to saline controls (Moliterno et al., 1994a). It is important to observe that PAI-1 is not specific to platelets, and is also produced by hepatocytes, mesothelial cells, monocytes and smooth muscle cells (Heesch et al., 2000). It must also be noted that this study enrolled patients with suspected coronary disease.

Kolodgie et al investigated the arteries from patients dying from cocaine-associated sudden death and who displayed acute coronary thrombosis (Kolodgie et al., 1991). A strong correlation (r=0.68) was found between the extent of luminal narrowing to the number of mast cells in coronary artery sections with thrombosis. This relationship was significantly stronger than the same correlation in arteries from patients where the cause of sudden death was attributed to significant coronary thrombosis and who had no history of cocaine abuse and a negative toxicology screen. These results suggest that cocaine may cause mast cell proliferation, which results in the release of pro-thrombotic mediators.

Both in vivo and in vitro studies appear to support the pro-thrombic effect of cocaine, either through a direct action on platelets or through an indirect mediator. Care must be taken in comparing in vivo data from cocaine-using and cocaine-naïve subjects, as differences in clearance of microaggregates may alter the number of cells remaining in the circulation displaying P-selectin expression. In aggregometer based studies it is expected that platelet-rich plasma in the absence of interactions with leucocytes and erythrocytes will display a different degree of coagulability compared to whole blood.

Arteriosclerosis

A limited number of studies have reported the prevalence of arteriosclerosis amongst cocaine users. It is presumed that coronary plaque formation results from either a direct effect of cocaine, or from a cocaine related stress that accelerates coronary artery disease from other causes (Dressler et al., 1990). For example it is probable that growth factors released from α-granules could indirectly accelerate the development of atherosclerosis.

Many investigations of the coronary effects of cocaine are concerned with users that have presented to hospitals for serious medical conditions or who have died. As a result they probably over-estimate the prevalence of coronary plaque formation amongst cocaine abusers (Dressler et al., 1990).
Stenberg et al. describe a fatal myocardial infarction in a 38-year-old patient sustaining simultaneous acute thrombosis of two major epicardial coronary arteries shortly after intravenous cocaine use (Stenberg et al., 1989). Likewise, Simpson et al. report autopsy findings of severe coronary obstructive lesions, as a result of chronic intimal proliferation and acute platelet thrombosis in a 21-year-old man, one hour following injection (Simpson et al., 1986). Isner et al. describe a fatal cardiac event in a 37-year-old man, with a history of drug abuse (Isner et al., 1986). Autopsy revealed thrombotic occlusion of the LAD coronary at a point where it had narrowed by 90% through plaque formation. The right coronary artery was reduced in diameter by 50%.

Dressler and associates examined hearts from 22 cocaine addicts at necropsy. It was found that 36% of fatalities demonstrated plaque formation greater than 75% in one of the 4 major epicardial coronary arteries, a mean of 1.8 narrowed arteries per subject (Dressler et al., 1990). Om and associates conducted an investigation into patients in whom the clinical presentation prompted referral for coronary angiography by the attending physician (Om et al., 1992). Urine toxicology screen was positive for cocaine in all 33 subjects analysed. Of these patients 60% had coronary artery disease (CAD). It was found that 40% of the patients studied had significant CAD, with ≥70% diameter stenosis. In addition there was enzymatic evidence of myocardial infarction in 12 of 33 patients (36%). In a more recent study of 70 patients presenting at US hospitals with cocaine associated myocardial infarction, it was found that 67% of these subjects met the criteria for CAD. Of these patients 95% had at least one stenosis of greater than or equal to 70%. Mittleman and Welti report 24 fatalities where there was toxicological evidence of cocaine use (Mittleman et al., 1987). Sixty-three percent of cases displayed significant arteriosclerosis. Forty-two percent of cases displayed stenosis estimated at 70 to 80%, 21% displayed 90% or more occlusion.

It is notable that cocaine can cause a reduction in coronary flow by the same mechanisms attributable to AS. Both AS and cocaine have been found to induce vasospasm. Chronic use of both drugs has been associated with thrombosis and arteriosclerosis.

1.13.2 Cardiac arrhythmias

Cardiac arrhythmia is commonly associated with cocaine administration (Lathers et al., 1988; Osawa et al., 1994; Shen et al., 1995; Gamouras et al., 2000; Littmann et al., 2000). A number of hypotheses have been forwarded to explain cocaine-mediated arrhythmogenesis.
Intracellular calcium flooding hypothesis

Cocaine has been shown to cause significant increases in intracellular calcium levels in ferret papillary muscle (Perreault et al., 1990). Significant release of calcium from the sarcoplasmic reticulum has been shown to result from cocaine administration to rabbits (Tomita et al., 1993). It is probable that cocaine elicits this “calcium flooding” through strong stimulation of α and β adrenoceptors (Billman, 1995). A number of studies have shown that the inotropic and chronotropic effects of cocaine can be attenuated using α and β-antagonists. Branch and Knuepfer examined the effect of a range of α and β adrenoceptor antagonists on the response to cocaine (5mg/kg, i.v.) (Branch et al., 1992). Cocaine was found to elicit an immediate (peak) and sustained pressor response with a simultaneous decrease in HR. This effect was virtually eliminated by alpha-1 adrenoceptor antagonists. Evidence was found for a contribution of alpha-2 adrenoceptors to the vasoconstrictor effect of cocaine. Beta-1 adrenoceptor blockade was found to attenuate the bradycardic effect of cocaine. Similar results have been reported in squirrel monkeys (Schindler et al., 1992).

Large increases in intracellular calcium can produce oscillations of membrane voltage during diastole (during repolarisation of the action potential). If membrane potential reaches threshold during these oscillations, a repetitively sustained action potential results (oscillatory afterdepolarisation) (Billman et al., 1988). This has been found to result in coupled extrasystoles and paroxysmal tachycardia (Bozler, 1943). It is probable that calcium overload causes the initiation and maintenance of ventricular fibrillation.

Because of the apparent importance of intracellular calcium release in arrhythmia formation it would be expected that calcium channel blockers would decrease the risk of arrhythmia formation. Billman and Hoskins demonstrated that verapamil can prevent ventricular fibrillation in dogs subjected to an exercise plus ischaemia plus cocaine test (Billman et al., 1988). No arrhythmia was observed in the absence of cocaine. Work conducted in isolated feline cardiac myocytes demonstrates that cocaine can prolong action potential duration resulting in extrasystoles (Kimura et al., 1992). The frequency of arrhythmia formation was found to be enhanced by catecholamines and attenuated by verapamil. Nimodipine has been shown to prevent cocaine induced arrhythmia in squirrel monkeys (Manger et al., 1989).

Myocardial contraction band necrosis is attributable to calcium overload, and has been noted in a number of clinical case reports. Contraction bands are formed by hypercontraction of some sarcomeres, interspersed with some sarcomeres that are torn apart (Virmani et al., 1988). Tazalaar et
al investigated 30 cases of cocaine associated fatalities and found myocardial contraction band necrosis in 93% of subjects, a significantly greater frequency of incidence than controls (fatalities due to hypnotic-sedative overdose) (Tazelaar et al., 1987). The diffuseness of the contraction bands correlated directly with the amount of cocaine found in the blood at autopsy. The presence and number of contraction bands was independent of other drugs found in the urine and blood, the number of sections of myocardium examined and a history of attempted resuscitation. Virmani et al found evidence of myocardial contraction bands in 23% of patients dying from natural causes whose toxicology screen was positive for cocaine or its metabolites (Virmani et al., 1988). The same pathology was noted in 3 out of 9 homicide victims with plasma testing positive for cocaine.

**Catelohamine induced arrhythmia hypothesis**

The ability of cocaine to inhibit the neuronal re-uptake of noradrenaline, increase the neural efflux of noradrenaline and release catecholamines from the adrenal medulla results in a significant increase in circulating catecholamines. Trouve et al demonstrated in the squirrel monkey that increases in HR and BP with cocaine administration were the direct result of increased plasma concentrations of dopamine, noradrenaline and adrenaline (Trouve et al., 1990). These changes were accompanied by transient episodes of arrhythmia. In later experiments Trouve et al demonstrated a similar elevation of catecholamines following cocaine administration to SD rats (60mg/kg, i.p.), which induced increases in BP and cardiac arrhythmia (Trouve et al., 1991). Administration of nitrendipine (calcium channel blocker) alone or a combination of enalaprilat (angiotensin converting enzyme) and diazepam decreased BP and returned cardiac rhythm to normal. A similar effect on cardiac rhythm and cardiovascular variables with nimodipine was observed in cocaine treated squirrel monkeys (Manger et al., 1989). Nitrendipine administration in conjunction with cocaine infusion was found to significantly increase survival time and increase the lethal dose of cocaine in SD rats (Trouve et al., 1986).

**Local anaesthesia hypothesis**

Cocaine can block both sodium (Shakalis et al., 1967; Pitts et al., 1989) and potassium channels to delay repolarisation. The interaction of cocaine with cardiac sodium channels has been found to be pH dependent (Crumb et al., 1995).

The QT interval on the ECG has been used as an indicator of the ability of cocaine to inhibit repolarisation. Regional cardiac differences in the rate of repolarisation could allow for excitability to recover in one area of the heart before others have fully repolarised (Kabas et al., 1990). Tissue that has remained in a depolarised state longer than the surrounding tissue may re-excite the fully repolarised regions such that a premature impulse is generated.
Studies on the heterogeneity of repolarisation caused by cocaine remain contradictory. While a small number of studies have shown QT interval lengthening in a canine model of cocaine toxicity, human studies have failed to show this same effect. This is most probably related to the small dose of cocaine that clinical studies are ethically restricted to using.

Billman and Latti found that 1mg/kg, i.v. of cocaine significantly increased the canine QT interval (Billman et al., 1993). This outcome was confirmed by 2 studies using doses of 3 and 4 mg/kg, i.v. respectively in dogs (Kabas et al., 1990; Temesy-Armos et al., 1992). Schwartz et al observed elevations in the PR, QRS and QT interval of dogs administered a bolus dose of cocaine (Schwartz et al., 1989).

Despite the observation of cocaine induced arrhythmia in dogs, human data remains equivocal. Daniel et al found that although 2mg/kg of intransally administered cocaine caused an increase in HR in human subjects, no change in ECG parameters were observed (Daniel et al., 1995). Lange and associates also noted no significant change in ECG with administration of the same cocaine dose (Lange et al., 1989). A study of 45 patients admitted to hospital with a history of cocaine use in the last 24 hours found that both subjects presenting with chest pain and those without anginal pain had prolonged QT, QTc and QTc dispersion (Gamouras et al., 2000). The frequency of repolarisation abnormalities was significantly greater in the chest pain group than the pain free subjects. Additionally, three examples of lethal ventricular fibrillation were observed in patients with chest pain.

1.13.3 Direct cardiac effects

Histological observations

Dressler examined 22 fatalities directly attributed to cocaine use, and found that 32% of these subjects had 1 or more foci of ventricular wall necrosis (Dressler et al., 1990). Welder found that adult primary myocardial cell cultures exposed to cocaine (10^{-3}M and 10^{-5}M) displayed vacuolisation, granulation and pseudopodia formation as early as an hour after exposure, as well as depressed contractility and significant lactate dehydrogenase (LDH) release, but did not significantly reduce cell viability (Welder, 1992; Welder et al., 1992).

Morphological changes

Human studies

The dangers of cardiac hypertrophy have already been previously considered. Left ventricular hypertrophy in cocaine users has been extensively reported. Om et al undertook an echocardiographic study of 58 normotensive cocaine users (Om et al., 1993). Exclusion criteria included valvular abnormalities, HIV or excessive physical work. Mean values for both men and
women were greater than normotensive controls (men 112±41 vs 83±23 gm/m², p=0.001; women 110±53 vs 82±14 gm/m², p=0.037). No correlation was found between the route of administration and the presence of hypertrophy. Karch et al confirmed these findings by performing an autopsy study comparing the heart weights in male trauma fatalities testing positive for cocaine with fatalities testing negative for cocaine and historical controls (Karch et al., 1995). Cocaine positive fatalities (375±82 g) were found to have a significantly (p<0.01) higher heart mass than cocaine negative (337±54 g, p<0.01) controls. The same changes were not evident in women. No attempt was made to control for the magnitude of cocaine doses abused, nor for the individual patterns of abuse. In a further retrospective autopsy study, cocaine, and its metabolite concentrations and organ weights were compared in cocaine users dying of lethal trauma (eg gun shot wound, burns, suicide etc) with subjects dying a cocaine-related death (Karch et al., 1998). Body mass index was significantly lower and heart mass significantly greater in subjects in which the cause of death was related to their cocaine abuse, than in recreational cocaine users suffering lethal trauma. Brickner et al subjected 30 male, normotensive, chronic cocaine abusers to 2 dimensional echocardiography and compared results to race and aged matched controls (Brickner et al., 1991). A significant elevation in left ventricular mass index was noted in the cocaine group (103±24 vs 77±14 g/m², p=0.0001).

**Animal Studies**

Sutliff et al report that rabbits administered cocaine (4mg/kg, i.v., twice daily) for 3 weeks demonstrated a significant increase in cardiac and aortic wet weight without a corresponding increase in body weight (Sutliff et al., 1996). They also demonstrated that expression of the c-fos proto-oncogene in the aorta increased significantly 60 minutes following a single i.v. dose of cocaine (4mg/kg). Besse et al measured cocaine induced increases in wet weight of the rat heart, and changes in expression of genes encoding cardiac hormones (Besse et al., 1997). Atrial natriuretic factor (ANF) has been shown to have increased secretion under conditions of cardiac hypertrophy (Mercadier et al., 1989; Marsh et al., 1998). Twenty-eight days treatment with cocaine (40mg/kg, i.p./day) significantly increased left ventricular weight, significantly elevated ANF secretion and caused an increase in expression of the β-myosin heavy chain compared to saline controls. Although, increases in ANF can occur in response to elevated BP as well as hypertrophy (Foltin et al., 1990; Lange et al., 1990; Pitts et al., 1998), the ANF increases observed by Besse et al were found to occur in the absence of an appreciable BP effect of cocaine. Johansson et al found that BP returns to normal, 5 days after beginning of chronic infusion, as a result of functional and dispositional tolerance (Johansson et al., 1992). Chronic cocaine users will often have normal systolic pressure (Brickner et al., 1991).
There are a number of hypotheses linking cocaine abuse and cardiac hypertrophy...

1) **The α-adrenergic stimulation hypothesis:** Elevated catecholamine levels have been linked to LVH. Simpson demonstrated that NA administration to neonatal rat myocardial cell culture resulted in a significantly greater accumulation of cell protein compared to untreated cells (Simpson, 1985). This hypertrophic effect was significantly reduced by α-adrenoceptor blocking agents (terazosin and prazosin). Meidell et al demonstrated in the same culture system that NA increases the production of myofibrillar protein without significantly altering degradation rates (Meidell et al., 1986). Unfortunately, because these studies were conducted using neonatal cells, it is not known whether adult cells will show the same anabolic effect.

Chronic, subhypertensive levels of NA have been shown to cause LVH in dogs (Laks et al., 1973). The effect of α-adrenoceptor blockade on cardiac hypertrophy induced by aortic banding has been studied in guinea pigs (Tamai et al., 1989). Bunazosin was found to protect against LVH, whereas a β-blocker had no effect. It is not understood whether this protective effect results from a lowering of afterload or a reduction in myocardial protein synthesis.

It is uncertain what role α-adrenergic stimulation plays in human cardiac hypertrophy as the density of α receptors on human myocardial cells is very low (Turlapaty et al., 1985). Shub et al observed LVH in only 20% of patients presenting with adrenal medullary tumour (pheochromocytoma), which produced abnormally high catecholamine levels (Shub et al., 1986).

2) **The intermittent BP elevation model:** Even small doses of intravenous cocaine can cause a significant, transient increase in human BP (Foltin et al., 1990; Lange et al., 1990; Pitts et al., 1998). Such intermittent increases in BP may serve as a physiological basis for the production of cardiac hypertrophy.

Devereaux et al showed that LVH as detected by echocardiography can be found in a significant number of patients with mild hypertension (Devereaux, 1990). Normotensive subjects who have an unusually large systolic response to exercise have been found to have a greater probability of LVH (Gottdiener et al., 1990). Out
of 22 men with an abnormal pressure response to exercise, 64% of these were found to have LVH. This suggests that transient increases in BP (such as those produced by cocaine) may be sufficient to cause cardiac hypertrophy, and that resting hypertension is not a prerequisite for LVH. Brickner \textit{et al} found LVH in normotensive cocaine users (Brickner \textit{et al.}, 1991).

3) \textbf{Increased proto-oncogene expression:} It is possible that cocaine induced increases in circulating noradrenaline can directly induce the proto-oncogenes \textit{c-myc} and \textit{c-fos} which are responsible for cardiac growth. Kolbeck-Ruhmkorff \textit{et al} observed that mRNA from \textit{c-myc} and \textit{c-fos} was absent in freshly excised rat hearts, and baseline activity was only observed after hearts had been perfused in the working mode (Kolbeck-Ruhmkorff \textit{et al.}, 1993). Hearts, which were perfused with noradrenaline, had a 3.8-fold increase in the expression of \textit{c-myc} and \textit{c-fos} after 90 minutes over baseline levels. Chronic cocaine administration to rabbits has been found to cause a time-dependent increase in \textit{c-fos} in the aorta using Western blotting (Sutliff \textit{et al.}, 1996). Zimmer found that administration of noradrenaline to isolated, perfused, working rat hearts, combined with an increase in pre-and afterload caused the \textit{c-fos} and \textit{c-myc} mRNA signal to appear earlier, to be more pronounced, and to persist for a longer period of time (Zimmer, 1997). This suggests that the combination of two hypertrophy-inducing stimuli may induce a greater degree of cardiac hypertrophy than either alone. This may be especially important in athletes abusing cocaine, where intensive exercise is already causing dramatic increases in pre and afterload.
1.14 Conclusion

Despite the similarity in the spectrum of cardiotoxic events attributed to cocaine and AS, very few studies have investigated the cardiac consequences of co-abuse of these drugs. Far more experimental evidence is available for potential mechanisms of cocaine cardiotoxicity than for AS. Clinical investigation of the cocaine-AS combination is hampered by confounding factors associated with AS use, such as poor diet, strenuous exercise, multiple formulations by multiple routes, "drug free holidays", street contaminants, and uncertainty over the dose magnitude associated with fatalities. Accordingly, experimental studies in animals should provide the mechanism for investigating the cardiovascular consequences of this drug combination free of the confounding factors in human subjects. However, there have been only three experimental studies which have investigated a potential additive cardiotoxic effect of these two drugs (Tseng et al., 1994; Phillis et al., 2000; Togna et al., 2003). For this reason the studies in this thesis were designed to investigate in detail the cardiovascular affects of the AS, nandrolone, and its affects in combination with cocaine treatment, in normal rats and in rats with compromised cardiac perfusion. The general aim of this thesis was to address some of the issues raised in this chapter such as polypharmacy and pre-existing cardiac pathology. As AS have been shown to display both genomic and non-genomic effects it was necessary to administer nandrolone in both an acute and chronic manner.
1.15 Aims

1. To determine whether our previously reported cardiovascular effects of cocaine in the Wistar strain of rat extend to the Sprague-Dawley strain of rat.

2. To determine if the previously reported enhancement of the cardiovascular effects of cocaine by chronic nandrolone pre-treatment in the conscious rat occurs following acute nandrolone treatment.

3. a. To determine whether acute or chronic nandrolone treatment exacerbates the effects of myocardial ischaemia/reperfusion in the anaesthetised rat.

   b. To determine the effects of acute or chronic nandrolone treatment combined with acute cocaine administration in the ischaemic or reperfused myocardium in the anaesthetised rat.

4. To determine, in an isolated rat heart model, the effects of nandrolone on the extraneuronal uptake of noradrenaline.
1.16 Hypotheses

1. Cocaine has identical dose-related cardiovascular effects in conscious Albino Wistar and Sprague-Dawley rats.

2. Acute nandrolone treatment potentiates the cardiovascular effects of cocaine in conscious rats.

3. a. Acute and chronic nandrolone treatment exacerbates the effects of myocardial ischaemia/reperfusion in the anaesthetised rat.

   b. Acute and chronic nandrolone treatment exacerbates the effects of cocaine in the ischaemic or reperfused myocardium.

4. Nandrolone inhibits the extraneuronal uptake of noradrenaline in the isolated rat heart in a concentration dependent manner.
1.17 References


CHAPTER 1 Introduction


CHAPTER 1 Introduction


CHAPTER 1 Introduction


2.1 Animals

All rats were purchased from Central Animal Supplies (Waite Campus, Adelaide University). Rats were provided with standard rat chow and water *ad libitum*. Animals were maintained in a standard 12 hour light-dark cycle, beginning at 7 AM, and subjected to a constant room temperature of 22±2°C. The experiments were conducted in strict accordance with the guidelines of the “Principles of laboratory care” (NIH publication No. 85-23, revised 1985), the Australian Code of Practice for the care of animals for scientific purposes of the National Health and Medical Research Council and the Adelaide University Ethics Committee.

2.2 Radiotelemetry recording

Radiotelemetry is designed to broadcast physiological data from transmitters implanted in the abdominal cavity of laboratory animals to a remote receiver. This allows the recording of cardiovascular variables in freely moving, non-anaesthetised, laboratory animals, reducing stress responses to a minimum.

The experiments presented in this thesis made use of 2 types of Data Sciences radiotelemetry implants (Data Sciences, St. Paul, MN) – the ECG implant (TA11CTA-F40) and the BP implant (TA11PA-C40).

Radio signals from the implants were collected by a receiver (RA1020 Receiver, Data Sciences, St Paul, MN), which was connected to a computer running LabPro software (Data Sciences, St Paul, MN) (Fig. 2.1). Each implant was calibrated for temperature to conform to the manufacturer’s configuration settings. The waveform-sampling rate was set at 1000Hz with a 250Hz filter.
2.3 General anaesthesia

When implanting radiotelemetry implants, rats were anaesthetised with a mixture of methohexidine sodium [Brictal™] (10mg/ml) and pentobarbital sodium [Nembutal™] (60mg/ml), administered intraperitoneally (i.p.) in the ratio 10:1 at 5ml/kg. In studies involving preparation for ischaemia-reperfusion, anaesthesia was induced by pentobarbital sodium (60mg/kg). Prior to commencement of surgery, the rats were tested via the pedal reflex and continuously monitored throughout the experiment to ensure an adequate level of anaesthesia was maintained.

2.4 Surgical implantation of radiotelemetry devices

2.4.1 ECG implants

ECG radiotelemetry implants (Physiotel implant, model TA11CTA-F40, Data Sciences, St Paul, MN) allowed the measurement of electrocardiogram, (ECG), heart rate (HR), core temperature (CT) and spontaneous locomotor activity (SLA) in freely moving, fully conscious rats. Each implant (approx. 30mm x 17mm) had two electrodes covered by silastic tubing, with approximately 10mm of exposed wire near the end of the electrodes. The tips of each electrode were also covered with silastic tubing to prevent local irritation developing (Fig. 2.2).
ECG implants were placed in the peritoneal cavity and stitched to the abdominal wall with non-absorbable suture. The positive electrode was secured subcutaneously just left of the xyphoid process. The negative electrode was tunneled under the skin to the area of the right scapula and stitched in place. Both electrodes were immobilised with non-absorbable suture to prevent migration and to reduce movement interference.

![ECG Radiotelemetry Implant](image)

**Fig. 2.2 Data Sciences ECG radiotelemetry implant.** Implants consist of a pair of electrodes made from helically wound stainless steel extending from an epoxy encapsulated electronics module. Source: [Brockway et al., 1993].

2.4.2 **Blood Pressure Implants**

Radiotelemetry implants (Physiotel implant, model TA11PA-C40, Data Sciences, St Paul, MN) allowed the measurement of HR, diastolic pressure (DP), systolic pressure (SP) and SLA. The implants consist of a small, cylindrical, biocompatible plastic housing about 2cm long, which contains the pressure recording electronics. From this protrudes a fluid filled catheter (0.7mm diameter) with an anti-thrombogenic tip (Fig. 2.3). Each implant was calibrated for temperature and blood pressure (BP) to conform to the manufacturer’s configuration settings. The waveform-sampling rate was set at 1000Hz with a 250Hz filter.

Blood pressure implants were placed in the peritoneal cavity and stitched to the abdominal wall with non-absorbable suture. The sensing catheter was placed in the descending aorta below the renal arteries, pointing “up-stream”. The catheter was lightly glued in place using tissue adhesive. Full details of the implants and surgical procedure is described elsewhere (Brockway et al., 1991; Guiol et al., 1992).
2.4.3 Post-operative Management

Wound sites were irrigated with bupivacaine 0.5% to reduce postoperative pain. Injectable antibiotic (Tribissen™) and topical antibiotic powder were administered post operatively. Rats were allowed to recover for a minimum of 10 days before experimentation was begun.

2.5 Analysis of radiotelemetry data

Drug responses were averaged (mean±S.E.) and plotted as a function of time. To maintain consistency with previously published radiotelemetry data (Phillis et al., 2000; Phillis et al., 2001) the area under the curve (AUC) was also determined for each parameter using the trapezoidal method. AUC was calculated from the raw data using Graphpad PRISM 4.02 (Graphpad Software, San Diego, California, USA). Between treatment group differences were analysed using either a single-factor ANOVA or a Student’s t-test.

2.6 Surgical procedure for ischaemia-reperfusion studies

Induction of ischaemia and reperfusion was conducted using the methodology established by McLennan (McLennan et al., 1985; 1988). Following induction of anaesthesia in Sprague-Dawley rats, the trachea was cannulated in preparation for artificial ventilation. Animals were kept warm on a heating table maintained at 37°C. The left femoral artery and vein were exposed and catheters inserted to allow for BP measurement and intravenous drug administration, respectively. The femoral artery catheter was attached to a BP transducer (Narco Bio-Systems P-1000B). The femoral
vein catheter was attached to a syringe pump. All lines were filled with heparinised saline to maintain patency (Fig. 2.4 right, top).

The chest was opened at the left fifth intercostal space. The fourth and fifth ribs were sectioned approximately 2 and 7mm from the sternum and artificial ventilation was commenced immediately. Animals were ventilated with room air with a stroke volume of 1.8ml/100g and a rate of 62 strokes/min. The pericardium was then opened and the heart exteriorised by the application of gentle pressure on the right chest wall. A 6/0 braided silk ligature attached to a 12mm atraumatic taper needle (Vascular 1153-11, Davis and Geck, Australia) was threaded around the left anterior descending coronary artery about 3mm from its origin, and the heart replaced in the chest cavity. A short section (~5mm) of polyethylene tubing was placed over both free ends of the ligature.

Pre-oxidised Needles (21Gx1) were used as ECG electrodes (lead II ECG) and were inserted subcutaneously on opposite sides of the chest (Fig. 2.4 left, top).
2.7 Continuous recording of cardiac parameters during ischaemia-reperfusion.

Cardiac parameters (HR, BP and ECG) were continuously recorded using Acq-Knowledge 3.5.6 software (BioPac), which allowed waveforms to be stored in graphical format for off-line analysis (Fig. 2.4 right, bottom).

2.8 Induction of ischaemia and reperfusion

Tightening the ligature around the LAD coronary artery and pulling it down a retaining slit in the side of the polyethylene tubing for a period of 15 minutes induced ischaemia. The ligature was then released and reperfusion initiated for a further 10 minutes.
Following completion of the procedure, the heart was excised into ice cold saline and cannulated onto a 10 ml syringe. The ligature was re-tightened and the heart was perfused with Evan's blue dye to determine the "area at risk of ischaemia" (%z/r). The underperfused region (pink) was cut out of the heart, weighed, and expressed as a ratio of total weight.

2.9 Analysis of ischaemia-reperfusion data

Ventricular tachycardia (VT) was defined as 4 or more consecutive ventricular premature beats (Fig. 2.5 top). Ventricular fibrillation (VF) was defined as a signal that changed from beat to beat in morphology and rate (Fig. 2.5 bottom).

Constructing a contingency table and applying the Chi-squared test evaluated differences in the total number of surviving rats responding with VT or VF during ischaemia or reperfusion. Fischer's Exact test was applied to 2x2 contingency tables as a post-hoc test. The fraction rats responding with VT, VF or VT+VF was also calculated for each treatment during ischaemia and reperfusion and analysed using the same contingency table method described above. Survival time during ischaemia and reperfusion was presented as mean±SE and analysed using a single-factor ANOVA with Dunnett's post-hoc test (in comparison to control). For rats which died of VF the time of death was taken to be the beginning of the period of fatal VF.

Average duration (s) of VT or VF or total dysrhythmia (VT+VF) during ischaemia and reperfusion was calculated for each treatment, and presented as mean±SE. For fatal VF a maximum of 120s was recorded for a single episode. Personal communication with Abeywardena et al revealed that 120s is the maximum period of VF for which a rat has survived under this procedure. Differences between treatment groups in the duration of VT, VF and VT+VF were analysed with a Kruskall-Wallis test with Dunn's multiple comparisons test post-hoc.

The severity of dysrhythmia observed during occlusion and reperfusion was scored using the Lambeth convention (Walker et al., 1988) and presented as mean±SE. The small number of rats that died without displaying arrhythmia were excluded from the score (number of rats excluded is noted in the legend figure). The scoring method utilised was as follows:
Occlusion

0 = 0-49 premature ventricular contractions (PVC)
1 = 50-499 PVCs
2 = more than 499 PVCs and/or 1 episode of spontaneously reverting VT or VF
3 = more than 1 episode of VT and/or VF, less than 60s total combined duration
4 = VT and/or VF, 60-119s total combined duration
5 = VT and/or VF, more than 119s total combined duration
6 = Fatal VF starting at more than 15 min after occlusion
7 = Fatal VF starting at between 4 min and 14 min 59s after occlusion
8 = Fatal VF starting at between 1 min and 3 min 59s after occlusion
9 = Fatal VF starting before 1 min after occlusion

Reperfusion

0 = 0-49 ventricular extra beats (VEB)
1 = 50-249 VEBs
2 = >249 VEBs and/or 1 episode of VT or VF
3 = more than one episode of VT or VF <20s combined duration
4 = VT and/or VF, 20-60s combined duration
5 = VT and/or VF, more than 60s combined duration
6 = Fatal VF starting more than 5 min after reperfusion
7 = Fatal VF starting between 2 and 5 min after reperfusion
8 = Fatal VF starting between 20s and 2 min after reperfusion
9 = Fatal VF starting within 20s of reperfusion.

Differences in Lambeth arrhythmia score or duration of arrhythmia was determined using a Kruskal-Wallis non-parametric test, with Dunn’s post hoc test. Differences in “area at risk of ischaemia” and total survival were calculated using a one-way ANOVA with Dunnett’s post-hoc test. In all statistical tests the level for statistical significance was p<0.05.

Blood pressure and HR was recorded during ischaemia and reperfusion. Cardiovascular variables were recorded immediately prior to ischaemia and at 300, 600 and 900s. The same variables were recorded prior to reperfusion and at 600s. If VT or VF was present at the designated time, a BP or HR reading was taken within 60s before or after the designated time. If no period of normal beat was evident for 60s before or after the designated time, a BP or HR reading was not recorded. All data was expressed relative to the value at 0s of ischaemia or reperfusion. All cardiovascular variables were presented as mean±SE. Because repeated measures ANOVA is not appropriate for

CHAPTER 2 General Methods
use when data sets contain missing values, between and within group differences were assessed
during ischaemia and reperfusion using two-way ANOVA. Differences in BP and HR during
ischaemia between groups were calculated using Bonferroni’s multiple comparisons post-hoc test.
Post-hoc tests were applied only to ‘treatment’ (or ‘dose’) and ‘interaction’. Post-hoc tests were
calculated using Microsoft Excel from the following formula (Neter et al., 1990):

For each comparison:

\[
t = \frac{\text{mean}_1 - \text{mean}_2}{\sqrt{\frac{\text{MS}_{\text{residual}}}{N_1 + \frac{1}{N_2}}}}
\]

mean\(_1\) & mean\(_2\): mean values for the comparison

N\(_1\) & N\(_2\): number of values contributing to the mean

MS\(_{\text{residual}}\): The mean square for residuals (taken from the ANOVA results Graphpad PRISM 4.02)

To determine the significance level the computed value for the t ratio (above) was compared
against the standard values (denoted t\(^*\)). If t > t\(^*\) then the comparison was significant at the
nominated significance level.

t\(^*\) = TINV(probability, deg._freedom)

TINV: MS Excel formula for the inverse of the Student’s t-distribution

Probability: significance level (\(\alpha\))

Deg._freedom: Degrees of freedom for residuals (taken from ANOVA results Graphpad PRISM
4.02).
Fig. 2.5 **TOP**: ECG trace showing a period of VT during cardiac ischaemia. **BOTTOM**: a period of VF during ischaemia.
2.10 Determination of the extra-neuronal uptake of noradrenaline in isolated perfused hearts.

2.10.1 Pre-treatment
Approximately 24 hours prior to sacrifice rats were treated with reserpine (2.3mg/kg, i.p.) to deplete vesicular noradrenaline and 18 hours before with pargyline HCl (75mg/kg, i.p.) to inhibit monoamine oxidase (Bryan et al., 1986). A further dose of pargyline was administered 2-3 hours prior to sacrifice.

2.10.2 Protocol
The protocol for the determination of extraneuronal uptake of noradrenaline was adapted from Bryan et al. (1986). Rats were anaesthetised with halothane, decapitated and the heart excised into chilled, calcium-free-Krebs. This prevented the hearts from beating until they were attached to the aortic cannula, and perfusion with Krebs had begun. Hearts were cannulated onto a Langendorff apparatus via the aorta and retrogradely perfused at a constant flow of 10ml/min with Krebs bicarbonate solution at 37°C and gassed with carbogen (95% O₂/5% CO₂). Perfusion pressure was monitored using a transducer (Statham P23XL) attached to a side arm of the perfusion line before the Krebs entered the aortic cannula. Pressure data were monitored using an eight-channel Maclab™ unit (MacLab/8c, AD instruments, Castle Hill, NSW). An equilibration phase of 5 minutes allowed the perfusion pressure to stabilise below 60mmHg. The flow of Krebs from the apex of the heart was monitored to ensure that a flow of 10ml/min had been maintained during the perfusion.

Hearts were then pre-perfused for 20 minutes with Krebs containing cocaine (27μM) to eliminate neuronal NA reuptake and U-0521 (50μM) to block COMT mediated NA metabolism. At this time either corticosterone, nandrolone (various concentrations) or vehicle control was also added to the Krebs. Hearts were then perfused for 2 minutes with ascorbic-Krebs (0.58mM), cocaine, U-0521, the inhibitor of interest and ³H-NA (0.85μM). Prior to commencement of the ³H-NA perfusion a ~1ml sample of perfusion medium was collected. At the conclusion of the ³H-NA perfusion, hearts were blotted dry. Care was taken to ensure that the intraventricular fluid was gently squeezed out and blotted. Blotted hearts were then cut open and stored overnight in 7.5mls of 0.4M perchloric acid at 4-6°C.
The next day hearts were cut into approximately 1mm slices, homogenised in the perchloric acid and then centrifuged at 10 000 x g. One ml of the resulting supernatant was added to 5ml of scintillation fluid (Optiphase) and subjected to beta scintillation counting (Beckman, USA).

Separate experiments were conducted to determine the amount of tissue radioactivity associated with the extracellular space. These experiments were identical to those just described except 14C-sorbitol was substituted for 3H-NA. The data from these experiments was used to calculate the intracellular concentration of 3H-NA (Equation 2.1).

2.10.3 Calculation of results

The extent of extraneuronal uptake was calculated as the amine content of the heart tissue, corrected for the amine in the extracellular space (calculated from the 14C-sorbitol content of the heart) divided by the total perfusion time (2 min) (Equation 2.1). Experiments performed in 3 hearts found that the extracellular space contained the equivalent of 293pmol/g.

$$uptake - 2 = \left[ \left( \frac{HH - B}{SA} \times \frac{1}{Wt} \times 1000 \right) - SS \right] \times \frac{1}{time} \text{ pmol/g/min}$$

$HH =$ radioactivity associated with 1ml of supernatant from heart homogenate (DPM)
$SA =$ specific activity (DPM/nmol)
$B =$ blank (DPM)
$Wt =$ weight (g)
$SS =$ average amount of 3H-NA uptake into the extracellular space (pmol/g)
$Time =$ perfusion time (min)

Equation 2.1 Extraneuronal reuptake of noradrenaline from isolated perfused rat heart (pmol/g/min). The amount of radioactivity (DPM) associated with 1ml of perchloric acid was multiplied by the total volume of supernatant (7.5ml). Uptake was adjusted for weight and the total perfusion time (2 minutes).

Uptake of NA (mean±SE) was expressed as a log₁₀ function of the inhibitor concentration. Graphpad™ Prism 4.0 for Windows (GraphPad Software, San Diego, California, USA) was used to fit the data to a sigmoidal dose response curve, from which the IC₅₀ was determined for nandrolone and corticosterone.

CHAPTER 2 General Methods
2.11 Drugs

The anaesthetic methohexitone sodium [Brietal™] was obtained from Eli-Lilly (Sydney, Australia) and pentobarbital sodium [Nembutal™] from Rhone Merieux Pty Ltd (Pinkenba, Australia). Noradrenaline bitartrate and activated alumina were obtained from Sigma Pharmaceuticals (St. Louis, MO, USA).

Antibiotics used during surgery included Tribriessen™ injectable antibiotic (Trimethoprim 80mg/ml & sulfadiazine 400mg/ml) from Jurox (Silverwater, NSW, Australia) and topical antibiotic powder (neomycin sulphate 2.5mg/g, sulphacetamide sodium 100mg/g, nitrofurazone 2mg/g, benzocaine 5mg/g) from Apex (St. Mary’s, NSW, Australia). Bupivacaine 0.5% (Marcain™) used to manage post-operative pain was obtained from Astra Pharmaceuticals (North Ryde, NSW, Australia).

[ring-2,5,6-3H] levo-Noradrenaline (1.48-2.96TBq/mmol) was purchased from New England Nuclear (Boston, MA, USA). All stocks of 3H-NA were purified before use by a batch alumina process previously reported (de la Lande et al, 1967). An aliquot of the manufacturer’s stock solution was added to 30 ml tubes containing 10ml of HCl (1M), 500mg of acid-activated alumina, ascorbic acid (57mM), and EDTA (27mM). The solution in the tube was continuously bubbled with N2 and adjusted to pH 8.4 using sodium carbonate solutions (1M and 0.1M). The pH was maintained at 8.4 for 4 minutes, after which the solution was allowed to settle and the effluent decanted. The alumina was then washed twice with 10ml of distilled water, before eluting the catecholamine with acetic acid. Five ml of acetic acid (300mM) was added and left for 5 minutes, after which time the supernatant was decanted. A further 2mls of acetic acid was added and both fractions of decanted acetic acid were pooled. The acetic acid stock was then frozen in a 100ml conical flask by suspension in a slurry of dry ice and alcohol, and subjected to freeze drying overnight. Prior to use, the 3H-NA was reconstituted into ascorbic, carbogen bubbled Krebs.

The final concentration of labelled noradrenaline used was 1.06x10⁻⁷M (specific activity = 51.8μCi/nmol).

D-[¹⁴C]-sorbitol (7.40-13.0GBq/mmol) was also obtained from New England Nuclear. The final concentration of labelled sorbitol used was 63x10⁻⁴M (specific activity = 256μCi/nmol).

125mg Reserpine (Sigma) was mixed with 125mg of citric acid, 5mls of benzyl alcohol and 45mls of saline (0.9%). Sonication and gentle heating over 45 minutes was required to dissolve all reserpine, which was subsequently distributed into 2ml aliquots.

CHAPTER 2 General Methods
Nandrolone, corticosterone acetate and pargyline were purchased from Sigma (St. Louis, MO, USA). Nandrolone and corticosterone were dissolved in 100% ethanol when used in isolated perfused heart experiments. When nandrolone was prepared for infusion small milligram quantities were stored in a 100% ethanol solution, from which it was diluted with saline to a stock solution containing 8.7% ethanol. The final ethanol concentration in nandrolone infusions ranged from 4.0 to 6.3% (4.0 to 6.3 μl/min).

Ascorbic Kreb’s was made with the following composition (in mM): NaCl 118, KCl 4.7, NaHCO₃ 25, D-glucose 5.6, KH₂PO₄ 1.2, CaCl₂ 2.5, MgCl₂ 0.74, EDTA 0.012, ascorbic acid 0.29. Krebs solution was filtered through a 1.2-μm Millipore filter before use. All Krebs components were analytical grade purity and were obtained from BDH (Kilsyth, Victoria, Australia).

Cocaine Hydrochloride was purchased from Fauldings Pty Ltd (Salisbury, South Australia). U-0521 (Upjohn). All doses of cocaine were administered as the hydrochloride salt.

The vehicle for nandrolone decanoate (DECA-50, Nature Vet Pty Ltd, Agnes Banks, NSW, Australia) was arachis (peanut) oil supplemented with 10% benzyl alcohol. Benzyl alcohol was purchased from Sigma Chemicals (St. Louis, MO, US)
2.12 References


Chapter 3

The effect of rat strain on the cardiovascular response to cocaine

3.1. Introduction

3.1.1 Is the cardiovascular response to intraperitoneal cocaine strain dependent?

The clinical observation that the dose of cocaine associated with adverse cardiac effects (Isner et al., 1986) and with lethality (Nanji et al., 1984; Smart et al., 1987) varied considerably from patient to patient without being necessarily related to the route of administration, led to the hypothesis that these differences were of genetic origin (Heavner et al., 1998). Despite these putative pharmacogenetic differences in the cardiovascular effects of cocaine in human subjects no published data is available on the effect of race on the cardiac cocaine response. Similarly, very few researchers have examined the potential strain dependency of the cardiovascular response to cocaine in rats. It remains unknown whether a difference in the cardiovascular response to cocaine exists between the 2 most commonly used laboratory rat strains (Sprague Dawley, SD and Albino Wistar, AW). As much of the published work on the cardiovascular effects of cocaine has used SD animals we were obliged to confirm our early data obtained with AW rats in SDs. Many of the pilot studies for this thesis were conducted using AW rats. Because ischaemia-reperfusion in SD rats was used in later studies (Chapters 5-7) to further investigate the potential cardiotoxicity of nandrolone and cocaine it was necessary to determine whether the cardiovascular response to cocaine was similar between the 2 strains.

Ishizuka et al found that restrained Wistar Kyoto (WKY) rats showed a significantly lesser mean blood pressure (MBP) and HR response to cocaine than when they were freely moving (Ishizuka et al., 1989). This same effect was not noted for spontaneously hypertensive rats (SHR). However, restrained SHRs were found to have a significantly greater change in the initial MBP and HR response to cocaine than WKY rats. This effect could not be observed in freely moving rats. This not only suggests that the cardiovascular response to cocaine was strain dependent in these rats, but that it was also affected by the extent of restraint. Later studies by Jin et al found that the $K_d$ and $B_{max}$ of the high affinity site on the cocaine receptor were lower in SHRs than in WKY rats in the striatum (Jin et al., 1991). In the hippocampus the $K_d$ of the high-affinity site was found to be lower.
in SHRs. This suggests that the differences in cocaine binding in WKY and SHRs may provide a neurochemical basis for the different responses of the two strains to cocaine administration.

Shi et al compared the cardiac effect of cocaine in rats identified as being genetically slow amygdala kindling rats (slow) with the effect in fast amygdala kindling rats (fast) (Shi et al., 1999). These 2 rat strains were originally developed for use in epilepsy research (F, cross between Wistar and Long Evans rats) and were found to have a differential sensitivity to the cardiotoxicity of cocaine. Animals were halothane anaesthetised, mechanically ventilated and were administered cocaine (3 or 4 mg/kg, i.v.) until they died. Arrhythmias developed at much lower cumulative cocaine doses in Slow-kindling rats than in Fast-kindling rats (15±1 versus 42±3 mg/kg, p<0.01). The lethal cocaine dose was significantly lower in Slow than in Fast strains. It was found that the difference in sensitivity to arrhythmia between the two strains was abolished by the non-selective α-adrenoceptor blocker, phentolamine, suggesting that genetic differences between Fast and Slow rats may be related to the α-adrenergic receptor or factors acting via the α-adrenergic receptor. Additional studies showed that isolated, perfused hearts from slow kindling rats required lower cocaine doses to develop cardiac arrhythmias and arrest compared to hearts from genetically Fast amygdala kindling rats (Heavner et al., 1998).

Branch and Knuepfer have conducted a comprehensive investigation of within strain effects of cocaine in SD rats (Branch et al., 1994b; a). They found that male SD rats showed a differential response in cardiac output (CO) in response to cocaine while arterial pressure and HR responses were consistent. Based on the CO response to cocaine (5mg/kg, i.v.) it was found that a population could be split into responders (a mean decrease in CO of 15% or more) and non-responders (smaller negative or positive increase in CO). The decrease in CO was found to be related to the rate of rise of plasma cocaine levels and was not necessarily dependent on the absolute dose (Branch et al., 1992). It was found that the differential cardiovascular responsiveness in these rats was mediated by central sympathetic excitation, which is dependent on α₁-adrenergic receptor activation, reduced by β adrenergic receptor activation and is not mediated by cyclo-oxygenase metabolites. These changes could not be attributed to differences between responders and non-responders in cocaine metabolism or in direct cardiac responsiveness to cocaine (Branch et al., 1994b).
Ruth et al found that the HR response to cocaine (0-15mg/kg, i.p.) in four inbred mouse strains varied significantly (Ruth et al., 1988). Because HR was measured in mice held in restraining tubes by subcutaneous needle electrodes, it is not known whether the different HR responses simply reflect a strain dependent stress response. No strain differences were found in the concentration of [3H]-cocaine in the brain following injection of 5mg/kg, suggesting no pharmacokinetic explanation for the strain differences.

A number of investigations have suggested that different behavioural responses to cocaine may be due to strain. These strain differences could result from pharmacokinetic or pharmacodynamic factors (Ruth et al., 1988). Only a small number of studies have examined whether the cardiovascular response to cocaine is strain dependent.

George et al found that the SLA response to various cocaine doses (0-60mg/kg, i.p.) was significantly different among 4 different inbred rat strains (ACI [ACI/Nih], LEW [LEW/CRLBR], NBR [NBR/Nih] and F344 [F-344/CRLBR] (George et al., 1991). NBR rats showed a high degree of locomotor sensitivity to cocaine, compared to the other 3 strains. A full dose-response curve could not be completed for ACI rats as a significant number of seizures and deaths were observed at the 40mg/kg dose. No differences between these strains were found in ligand binding to striatal dopaminergic transporters or receptor sites. Interestingly, further studies found that the NBR strain showed the lowest LD_{50} with cocaine (George, 1991). However, over all 4 strains, no significant correlation was found between the SLA effects of cocaine and lethality. Sensitivity to cocaine-induced lethality was found to be highly correlated with baseline levels of ambulatory activity (r=0.99, p<0.01). Similarly, Cailhol and Mormede found that in WKY, WKHA and SHRs administered cocaine (0-20mg/kg, i.p.) female SHRs showed significantly greater locomotor stimulation compared to WKY (p<0.001) and WKHA (p<0.001) females (Cailhol et al., 1999). This same strain effect was not observed for males.

Despite observations of differential behavioural responses or HR in inbred rat or mouse strains in the response to cocaine, a differential CO response between SD rats, and BP and HR differences in normotensive versus hypertensive rats, none of these responses to cocaine have been assessed for strain dependency in commonly used laboratory normotensive rat strains. This study aimed to characterise the cardiovascular response to i.p. cocaine in fully conscious, freely moving AW and SD rats. To avoid changes in HR (Ruth et al., 1988) and BP between strains due to a strain-dependent stress response; conscious, freely moving rats were used for this study.
3.2. Aim

- To determine whether a strain related effect in response to cocaine in terms of HR, BP and SLA can be observed between SD and AW rats.

3.3. Hypothesis

The HR, SLA, DP and SP response to cocaine will not be statistically significantly different between strains.
3.4. Methods

3.4.1 Animals
Albino Wistar or SD rats were obtained at 12 weeks and housed as per 2.1 General Methods. Animals were implanted with radiotelemetry devices for recording HR, SLA, DP and SP as per General Methods 2.4.

3.4.2 Protocol
On each test day the animals were administered cocaine HCl i.p. and then returned to their home cages. Simultaneous recordings of HR, SLA, DP and SP were carried out for 150 mins. Baseline values were determined at 290-300 minutes following drug administration. To avoid recording the immediate stress response following handling (Irvine et al., 1997) and drug injection, all variables were recorded from 15 minutes onwards. Test days were separated by a minimum of 48 hours. Dose response curves were generated for cocaine (0, 15, 45 mg/kg). Each dose was administered on 3 occasions.

3.4.3 Data Analysis
Results for HR, SLA, DP and SP for each group of triplicates were averaged and plotted as a function of time. In order to maintain consistency with previously published work (Phillis et al., 2000; Phillis et al., 2001) which examined cardiovascular responses to sympathomimetic drugs, between strain comparisons were made by calculating area under the curve (AUC). AUC was determined by the trapezoidal method using Graphpad PRISM 4.02. The baseline for HR and BP was the average of the values at 290, 295 and 300 minutes following drug administration. The baseline for SLA was zero. Between strain differences in the AUC for the response to saline or cocaine were determined using an unpaired t-test at each dose.
3.5. Results

3.5.1 Time course of the cocaine response

Both strains showed an increase in SLA and BP in response to cocaine. A small initial decrease in HR was observed in comparison to saline at the highest cocaine dose. In both AW and SD rats this effect lasted until 45 minutes post injection (Fig 3.1 C). Heart rate decreased by 10.9±3.5% (48.3±15.6 bpm) in AW rats and 10.7±3.4% (42.1±13.2 bpm) in SD rats in response to cocaine (45mg/kg) in comparison to the effect of saline at 15 minutes post injection. No significant difference was found in the size of this decrease between the two strains (unpaired t-test). An initial decrease in response to cocaine in comparison to the response to saline was not observed for SLA (Fig 3.1 D-F), DP (Fig 3.2 A-C) or SP (Fig 3.2 D-F).

3.5.2 Between strain differences in the HR, SLA, DP and SP response to cocaine.

Cardiovascular effects

AW rats were found to have a significantly greater HR response to saline \([t(11)=3.816, \ p<0.01]\) compared to the SD strain (Fig 3.1 A). This difference was not evident for either concentration of cocaine (15 or 45mg/kg) (Fig 3.1 B & C). No significant difference between strains was found for the magnitude of the AUC for the DP or SP response to saline or cocaine (Fig 3.2 A-C Fig 3.2 D-F).

SLA effects

AW rats were found to have a greater AUC for the SLA response to saline compared to SD rats \([t(11)=2.714, \ p<0.05]\) (Fig 3.1 D). Conversely, the area under the SLA versus time graph was found to be greater in SD rats \([t(11)=2.446, \ p<0.05]\) compared to AW for the response to cocaine (15mg/kg) (Fig 3.1 E). No significant difference between strains was observed at 45mg/kg (Fig 3.1 F).
Fig 3. 1 **Left panel:** The heart rate (HR) response in AW (▲) and SD (▼) rats administered cocaine. (A) 0mg/kg [n=6-7], Inset: AUC, *p<0.01, unpaired t-test. (B) 15mg/kg [n=6-7], Inset: AUC. (C) 45mg/kg [n=3-5], Inset: AUC. **Right panel:** The spontaneous locomotor activity response (SLA) in AW (▲) and SD (▼) rats administered cocaine. (D) 0mg/kg [n=6-7], Inset: AUC, *p<0.05 (E) 15mg/kg [n=6-7], Inset: AUC, *p<0.05. (F) 45mg/kg [n=3-5], Inset: AUC. On all time-course graphs the value at 300 minutes is the average of the values at 290, 295 and 300 minutes following drug administration.
Fig 3. 2 *Left panel:* diastolic pressure (DP) response in SD (■) and AW (▲) rats administered cocaine. (A) 0mg/kg [n=6-7], *Inset:* AUC (B) 15mg/kg [n=6-7], *Inset:* AUC (C) 45mg/kg [n=3-5], *Inset:* AUC. *Right panel:* systolic pressure response (SP) in SD (■) and AW (▲) rats administered cocaine. (D) 0mg/kg [n=6-7], *Inset:* AUC (E) 15mg/kg [n=6-7], *Inset:* AUC (F) 45mg/kg [n=3-5], *Inset:* AUC. Mean±S.E. On all time-course graphs the value at 300 minutes is the average of the values at 290, 295 and 300 minutes following drug administration.
3.6. Discussion

Although a significantly greater HR response to saline was observed in AW rats compared to SD, no such difference was observed for cocaine. Likewise, no significant difference between strains was found for the DP and SP response to saline or cocaine. This suggests that direct comparisons can be made between the cardiovascular responses to cocaine in the 2 most commonly used rat strains in the published literature. This preliminary study also supports the use of SD rats in later chapters of this thesis to investigate the cardiac effects of cocaine during ischaemia-reperfusion and allows a comparison to be made to previously published work (Phillis et al., 2000). A pronounced and significantly greater SLA response to cocaine (15mg/kg) was found in SD rats compared to AW rats. This suggests that while the behavioural effects of cocaine may be strain dependent at some doses, the cardiovascular effects are independent of strain.

It is perhaps not surprising that there are no significant cardiovascular differences between these 2 strains at the 45mg/kg dose considering that SD rats are not unrelated to Wistars. Sprague Dawley rats are believed to have originated from Wistar females crossed to a ‘hybrid’ male of unknown origin (Lindsey, 1979; Pass et al., 1993).

Tolerance to the cardiovascular effects of cocaine is unlikely to explain the lack of a strain effect. Ansah et al reported no tolerance to the HR, SLA and core temperature (CT) response to cocaine in Wistar rats (Ansah et al., 1996). Rats were treated with a once daily dose of cocaine (20mg/kg, i.p.) and HR, SLA and CT measured using radiotelemetry implants. The tachycardic response to cocaine did not change between days 1 and 30 of administration, indicating that the HR response did not undergo sensitisation or display tolerance. The SLA response was found to show a marginally significant (p=0.0514) increase in cocaine administered animals across the 30 day treatment period, suggesting that cocaine may induce sensitisation to behavioural effects. However, changes in SLA over 30 days were cyclical in nature and it was acknowledged that the “pattern of response may be due to variability in animals, rather than a physiological effect”. The present study used an interdosing interval of 48 hours. Previous studies in non-telemetered animals have found sensitisation to the behavioural effects of intermittent cocaine, and tolerance to continuously administered cocaine, cardiovascular effects were not reported (Reith et al., 1987).

The increases in BP with cocaine evident in this study are consistent with work by Pan and Hedaya who examined the cardiac effects of i.p. cocaine in the male rat and demonstrated a very mild but sustained cardiovascular effect (Pan et al., 1998). Pan and Hedaya designed a novel in vivo model of drug disposition, which allowed investigation of simultaneous pharmacokinetic and dynamic effects of cocaine in the same animal (male Wistar rat) by multiple routes. Gastric, abdominal and femoral

CHAPTER 3 The effect of rat strain on the cardiovascular response to cocaine
vein catheters allowed administration of cocaine by the p.o., i.p. or i.v. route without handling the animal. Mean arterial pressure following cocaine (30mg/kg, i.p.) was found to increase by approximately 13% above baseline at its peak, and sustained a higher level of pressure over the 250 minutes of the study compared to i.v. administration. Likewise, both i.p. and i.v. cocaine administration produced a decrease in HR of about 25% at its lowest point. With i.p. administration the bradycardic effect of cocaine was prolonged for more than 60 minutes longer than i.v. cocaine. Although an initial decrease in HR was observed in the present study this was not of the magnitude reported by Pan and Hedaya in awake, freely moving rats. Later studies in this thesis found that i.v. cocaine administration produced more significant bradycardia (a reduction of approximately 70bpm from baseline) (Fig. 6.4). However, studies in chapter 6 were conducted in anaesthetised animals. Pan and Hedaya showed the long lasting nature of the cardiac response to i.p. cocaine in the rat by demonstrating that the QRS interval in rats administered i.p. cocaine was still prolonged at 250 minutes following injection. In comparison the QRS interval of rats administered i.v. cocaine had returned to near baseline levels by approximately 50 minutes.

Most available literature on the cardiovascular effects of cocaine in rats concerns i.v. administration. However, the cardiovascular effects of i.p. cocaine remain important. It has been demonstrated that the distribution kinetics of cocaine following i.p. injection in rats is similar to the absorption profile obtained by intranasal administration in humans (Javaid et al., 1993). Intranasal insufflation ("snorting") and smoking have increased in popularity over the past 15 years (Hatsukami et al., 1996). Current estimations of the proportion of current recreational cocaine users administering cocaine by nasal insufflation range from 30-40% (Gossop et al., 1994; Ferri et al., 1999; Gossop et al., 2000). Javaid et al found that the plasma concentration of cocaine in rats administered i.p. cocaine reached maximum at about 10 minutes post injection and was maintained at this level until approximately 60 minutes (Javaid et al., 1993). Javaid noted a similar profile in patients administered either a 32 or 96mg cocaine dose intranasally (Javaid et al., 1978; Javaid et al., 1993). The maintenance of a constant plasma cocaine concentration for the first hour following administration is consistent with the results presented here. In both strains BP remained elevated for the first 60 minutes following cocaine administration. Although HR initially declined in response to cocaine, peak HR wasn't obtained until 120-140 minutes post injection. Wilkerson and colleagues observed a "sustained peak" plasma cocaine concentration in male subjects snorting doses of cocaine between 0.19 and 0.75 mg/kg (Wilkinson et al., 1980). A peak response was achieved by about 15 minutes post administration which was maintained until approximately 70 minutes post administration.
3.7. Conclusion

The HR and BP response to intraperitoneal cocaine is not strain dependent. Spontaneous locomotor activity was found to show a strain dependent response to cocaine at 15mg/kg which was significantly greater in SD rats and abolished at high concentration (45mg/kg, i.p.). Therefore behavioural effects at low concentrations of cocaine may be strain dependent, but this does not affect the cardiovascular response.
3.8. References


Chapter 4

The effect of acute nandrolone and cocaine on heart rate

4.1 Introduction

Recent reports suggest that testosterone can have an acute effect on vessels and platelets in vitro, independent of the slow responses usually associated with changes in gene expression. Co-incubation of human platelet rich plasma with testosterone and cocaine has been found to induce a significant increase in platelet aggregability in response to a sub-threshold concentration of sodium arachidonate even in the presence of the androgen antagonist, flutamide (Togna et al., 2003). Togna et al. also showed that co-incubation of endothelium-intact, phenylephrine pre-contracted, rabbit aortic rings with testosterone, potentiated the cocaine induced inhibition of acetylcholine mediated vascular relaxation by an androgen-independent mechanism. Similarly, Ceballos et al. showed that testosterone can block the vasodilator effect of adenosine in isolated perfused rat hearts (Ceballos et al., 1999). This effect occurred even when testosterone transport into the cytosol was prevented or protein synthesis inhibited.

In light of recent reports of the acute effects of testosterone (Ceballos et al., 1999; Togna et al., 2003), the previously reported (Phillis et al., 2000) interaction between nandrolone and cocaine in terms of HR was re-investigated. The aim was to determine whether this interaction required chronic exposure to nandrolone or whether it might have resulted from an effect of the last dose of nandrolone prior to acute cocaine injection. In order to determine whether the enhanced chronotropy was simply due to a direct, acute effect of nandrolone it was necessary to observe whether prior exposure to a single nandrolone dose (20mg/kg, s.c.) changed the HR response to cocaine, as per the previously reported chronic study (Phillis et al., 2000).
4.2 Aim

- To evaluate whether a single subcutaneous injection of nandrolone (20mg/kg, s.c.) can significantly increase the HR, core temperature (CT) and SLA response to cocaine (45mg/kg, i.p.) compared to vehicle treated controls.

- To determine the plasma concentration of nandrolone 250 minutes after injection of a single subcutaneous dose (20mg/kg, s.c.).

4.3 Hypothesis

Potentiation of the tachycardiac effect of intraperitoneal cocaine by nandrolone occurs after chronic administration of nandrolone but not after acute administration.
4.4 Methodology

4.4.1 Animals
Eighteen male albino Wistar rats were purchased at 12 weeks and were housed, and implanted with ECG radiotelemetry implants (Physiotel implant, model TA11CTA-F40, Data Sciences, St. Paul, Minn., USA) as described in general methods, sections 2.1, 2.3 and 2.4.1, respectively.

4.4.2 Protocol
Rats were divided into 3 equal groups, and were assigned to receive either a single dose of nandrolone (20mg/kg, s.c.), saline or vehicle. Rats were allowed an acclimatisation period of 30 minutes following activation of the radiotelemetry implants. Nandrolone decanoate (20mg/kg), vehicle or saline were then administered subcutaneously. In accordance with the protocol from the previously reported chronic study (Phillis et al., 2000), telemetry monitoring was then continued for 210 minutes before the administration of cocaine (45mg/kg, i.p.). The effect of cocaine was recorded for a further 180 minutes.

Locomotor activity was used as a pharmacodynamic measure of cocaine effect and was found to peak at 40 minutes (250 minutes after pre-treatment with nandrolone, saline or vehicle). In order to determine whether there were appreciable levels of nandrolone present at the onset of the peak cocaine effect, a separate group of animals were administered either nandrolone (20mg/kg, s.c., n=3) or vehicle (n=3). Animals were anaesthetised with pentobarbital (45mg/kg, i.p.) 15 minutes before blood collection. Blood was collected from the abdominal aorta after 250 minutes. Blood was centrifuged for 10 minutes at 7 000 x g and the resulting plasma frozen in 5ml aliquots. Plasma was sent on ice to the Racing Forensic Laboratories (Randwick, NSW) where it was assayed for nandrolone using GC/MS. The limit of detection was 2ng/ml.

4.4.3 Drugs
Vehicle was prepared from 10% benzyl alcohol and peanut oil.
4.4.4 **Statistical Analysis**

*Area under the curve calculations*

The mean and S.E. were calculated for each group of rats at each sampling interval for each variable recorded. To maintain consistency with previously published work (Phillis *et al.*, 2000; Phillis *et al.*, 2001) that examined HR, CT and SLA responses to sympathomimetic drugs, area under the curve (AUC) was used to compare drug responses between treatment groups. Calculation of AUC was by the trapezoidal method. When analysing both the response to treatment and the response to cocaine the same baseline was used, which was determined to be the average of the last 3 values for the HR, or the CT response to treatment with nandrolone, saline or vehicle (i.e. values at 200, 205 and 210 minutes). A baseline of zero was used for SLA.

The HR and SLA response to treatment with nandrolone, vehicle or saline was compared to the treatment plus cocaine response by calculating the AUC for the first 180 minutes of the treatment response and comparing this to the 180 minutes over which the cocaine response was recorded. The CT response to cocaine was below baseline at a number of time points and the response to pre-treatment with nandrolone, vehicle and saline was largely above baseline (see Fig. 4.2). As the CT response to cocaine was found to involve 2 phases, an initial decrease (0-40s) and a subsequent increase (45-180s) the AUC was analysed separately for each of these phases, and the net area determined.

*Method for determining whether pre-treatment or pre-treatment plus cocaine significantly altered AUC*

Differences between groups in the HR or CT response to treatment alone or treatment plus cocaine were determined using a one-way ANOVA.

*Method for determining whether cocaine significantly increased the AUC response compared to treatment alone*

To determine whether cocaine had an effect on HR or CT an unpaired t-test was used to examine differences in the AUC response in the presence and absence of cocaine.
4.5 Results

4.5.1 Plasma levels of nandrolone
Nandrolone was not detected in plasma from vehicle treated rats. A single dose of nandrolone (20mg/kg, s.c.) produced a plasma nandrolone level of 12.1±3.2nM (3±1 ng/ml) at 250 minutes after injection.

4.5.2 Pre-treatment response
No significant difference was observed in the HR (Fig. 4. 1 C.), SLA (Fig. 4. 1 D.) or CT (Fig. 4. B., C.) response to treatment with nandrolone, saline or vehicle (single-factor ANOVA).

4.5.3 Cocaine response
In all treatment groups cocaine was found to significantly increase the area under the HR versus time curve in comparison to treatment with nandrolone \[t(10)=7.137, p<0.001\], saline \[t(8)=2.916, p<0.05\] or vehicle \[t(9)=5.665, p<0.001\] alone (Fig. 4. 1 C.). Likewise, cocaine significantly increased locomotor activity compared to nandrolone alone \[t(10)=3.820, p<0.01\], saline alone \[t(10)=3.711, p<0.01\] and vehicle alone \[t(10)=3.931, p<0.01\] (Fig. 4. 1 D.).

The CT response to cocaine appeared to be biphasic (Fig. 4. 2). An initial and immediate decrease in CT was observed which lasted for the first 10-20 minutes following cocaine injection. This decrease was not evident in the absence of cocaine. The initial decrease was followed by an increase which reached maximum at approximately 80 minutes post cocaine injection in all pre-treatment groups. The 2 main parts of the biphasic CT response were analysed separately (ie. 0-40 minutes \(\text{AUC}_{0-40 \text{ minutes}}\) and 45-180 minutes \(\text{AUC}_{45-180 \text{ minutes}}\)). Cocaine caused a significant decrease in \(\text{AUC}_{0-40 \text{ minutes}}\) compared to treatment with nandrolone \[t(9)=2.897, p<0.05\], saline \[t(10)=6.339, p<0.001\] or vehicle \[t(8)=4.268, p<0.01\] alone (Fig. 4. 2 B.). A significant increase in the \(\text{AUC}_{45-180 \text{ minutes}}\) was demonstrated in response to cocaine in all pre-treatment groups i.e. nandrolone \[t(10)=3.132, p<0.05\], saline \[t(10)=3.053, p<0.05\] and vehicle \[t(10)=3.557, p<0.01\] pre-treatment (Fig. 4. 2 C.).

4.5.4 Effect of nandrolone pre-treatment on response to cocaine
There was no significant difference in the HR (Fig. 4. 1 C.) or SLA (Fig. 4. 1 D.) response to cocaine in rats treated with nandrolone, compared to those treated with vehicle. Similarly, no difference was found in the response to cocaine in the 3 different pre-treatment groups for 0-40s and 45-180s of the CT response (Fig. 4. 2 B., C.).
Fig. 4. 1 Time course of the HR (A.) and SLA (B.) response to treatment with nandrolone (●), saline (○) or vehicle (▼) alone (solid lines) compared with the subsequent treatment with cocaine (dotted lines) in rats pre-treated with nandrolone (●), saline (○) or vehicle (▼). Dotted vertical lines represent the last x value used for calculating AUC. Area under the curve (AUCₙₐ₅₀) for the HR (C.) and SLA (D.) versus time response to treatment alone (N = nandrolone, S = saline, V = vehicle) and pre-treatment plus cocaine (N+C = nandrolone+cocaine, S+C = saline + cocaine, V+C = vehicle + cocaine). Mean±SE, n=5-6. *p<0.05, **p<0.01, ***p<0.001 significantly different from treatment alone, unpaired t-test.
**Fig. 4.** 2 (A) Time course of the core temperature (CT) response to treatment with nandrolone (☐), saline (○) or vehicle (△) alone (solid line) compared with the subsequent treatment with cocaine (dotted line) in rats pre-treated with nandrolone (●), saline (○) or vehicle (△). The decrease in CT in response to cocaine (denoted “area 1”) was analysed separately from the increase (“area 2”). **Area under the curve for area 1 (AUC_{0-40 minutes}) (B.) and area 2 (AUC_{45-180 minutes}) (C.) for treatment alone (N = nandrolone, S = saline, V = vehicle) and treatment + cocaine (N+C = nandrolone + cocaine, S+C = saline + cocaine, V+C = vehicle + cocaine).** Mean±SE, n=4-6. *p<0.05, **p<0.01, ***p<0.001 significantly different from treatment alone, unpaired t-test.
4.6 Discussion

These results indicate that a single dose of nandrolone (20mg/kg, s.c.) to naïve rats, results in a quantifiable plasma concentration, at 250 minutes following injection.

Although intraperitoneally administered cocaine was found to significantly increase the AUC_{0-18} \text{ minutes} for HR and SLA and the AUC_{45-180} \text{ minutes} for CT in comparison to the response to treatment alone, the magnitude of this difference was not significantly different between treatment groups. This suggests that while a significant cocaine effect can be observed with a dose of 45mg/kg, pre-treatment with a single nandrolone dose (20mg/kg, s.c.) does not alter the HR, SLA or CT response to cocaine. While a single dose resulted in an appreciable plasma nandrolone level, this did not potentiate the HR effect of cocaine as was observed previously with chronic nandrolone treatment (Phillis et al., 2000).

This study suggests that the enhanced chronotropic response to cocaine (45mg/kg, i.p.) resulting from chronic nandrolone treatment reported previously, was unlikely to be due to an acute effect of the previous nandrolone injection. However, it cannot be excluded that a direct, acute effect of nandrolone might be observed with a higher single dose.
4.7 Conclusion

A single subcutaneous dose of nandrolone (20mg/kg) distributes from the site of injection to produce detectable levels of nandrolone at 4 hours or more following injection. A single subcutaneous injection of nandrolone does not change the HR response to cocaine (45mg/kg, i.p.) in comparison to other pre-treatment groups. The SLA and CT response to cocaine are also unaffected by pre-treatment. Thus the effect of nandrolone upon the HR response to subsequent cocaine administration (observed previously) is a result of the chronic effects of nandrolone treatment rather than a direct drug-drug interaction.
4.8 References


Chapter 5

The effect of intravenous nandrolone in rats subjected to cardiac ischaemia and reperfusion

5.1 Introduction

The cardiovascular consequences of AS administration to rats reported to date have been moderate. Chronic nandrolone administration to rats has been associated with a significantly heightened HR response to cocaine (Phillis et al., 2000), with accelerated development of hypertension in developing, spontaneously hypertensive rats (Tseng et al., 1994) and with left ventricular hypertrophy (Tseng et al., 1994; Trifunovic et al., 1995) in sedentary rats.

As discussed at length in chapter 1 (1.6.1), a number of human case reports have linked AS to atherosclerosis (Mewis et al., 1996) and thrombosis, (Fisher et al., 1996; Nieminen et al., 1996) both of which significantly increase the risk of cardiac ischaemia. Consequently, it was decided to investigate the effect of nandrolone administration in the presence of reduced perfusion. It is probable that many of the cardiotoxic effects attributed to AS, which have been discussed in chapter 1, including direct myocardial toxicity (1.6.2), left ventricular hypertrophy (1.6.2), myocardial infarction (1.6.1 & table 1.3), hypertension (1.6.3) and hyperlipidaemia (1.6.1) can significantly increase the risk of ventricular arrhythmia during both ischaemia and reperfusion.

There are more than 50 years experience with the rat ischaemia-reperfusion model. (Heimberger, 1946). The model involves occluding a coronary artery, usually the left anterior descending (LAD) artery because of its easy accessibility, and observing the frequency and duration of resulting premature ventricular contractions (PVC), ventricular tachycardia (VT) and ventricular fibrillation (VF). The occlusion is often then removed, so that an examination of reperfusion arrhythmia can be undertaken.

It is important to note that whereas return to normal sinus rhythm following VF is very rare in humans, it is common in rats, (Johnston et al., 1983b) and is most probably related to heart size. Abeywardena et al (personal communication, 2001) reported a period of VF in a rat lasting more than 100s, which reverted to normal rhythm. While VF is usually fatal in humans,
electrocardiographic findings have been reported from a patient who displayed two transient bouts of VF, which spontaneously reverted to sinus rhythm at the end of an asymptomatic ischaemic episode (Maseri et al., 1982).

Cardiac ischaemia produced by ligation of the LAD coronary artery in the anaesthetised rat induces 3 distinct phases of arrhythmia.

1. An early phase 4-10 minutes following occlusion of the LAD coronary artery (Botting et al., 1983; Manning et al., 1984).
2. An intermediate phase starts between 1.5 and 4 hours following ischaemia. (Botting et al., 1983; Johnston et al., 1983a; b; Clements-Jewery et al., 2002)
3. A late phase is evident in most rats surviving for greater than 24 hours post permanent-occlusion, manifest by multifocal premature ventricular contractions (PVC) (Curtis et al., 1987).

The degree of arrhythmia is dependent upon the size of the “area at risk of infarction” or the “occluded zone”. At the conclusion of the protocol the under perfused area of the heart can be determined by retrogradely perfusing the heart via the aorta with Evan’s blue dye. The lack of effective coronary collaterals in the rat heart results in reproducible results for the occluded zone. (Evans et al., 1985)

Ischaemia-reperfusion experiments can be performed in isolated perfused hearts. However, it has been observed that the duration and frequency of arrhythmia in isolated perfused hearts is significantly less than that elicited in vivo (Daugherty et al., 1981). The present study was concerned with an in vivo model of the effect of nandrolone infusion.

The selection of anaesthetic is important in in vivo studies of ischaemia-reperfusion. Both halothane and chloroform have been found to have varying degrees of antiarrhythmic action. Halothane has been shown to be cardioprotective in terms of ischaemia induced arrhythmia in the rat, compared to conscious controls, without changing the size of the occluded zone. (MacLeod et al., 1983) Halothane anaesthetised rats have a greater tendency to die during ischaemia from non-arrhythmic cardiac output failure. (Au et al., 1983). Pentobarbitone has not been shown to have the same antiarrhythmic action. (Au et al., 1983)

Previous studies with chronic, (and now acute) nandrolone treatment have not been found to significantly modify HR or BP in the absence of cocaine in normal adult rats. It was therefore decided to investigate if nandrolone pre-treatment will produce a significant cardiovascular outcome in rats subsequently exposed to cardiac ischaemia. The absorption, distribution and
elimination of nandrolone as administered previously is complicated by the use of oil-based preparations. In order to ensure maximum bioavailability and simplify interpretation of the pharmacodynamic data from acute nandrolone administration it was decided to intravenously administer nandrolone.

5.2 Aim

To assess whether acute nandrolone administration increases the frequency and duration of arrhythmia in rats subjected to cardiac ischaemia and reperfusion.

5.3 Hypothesis

Intravenous nandrolone will cause a dose related increase in arrhythmia, and a corresponding decrease in survival time during LAD coronary artery occlusion and reperfusion in the anaesthetised rat.
5.4 Methodology

5.4.1 Animals

Male Sprague Dawley rats weighing 358±4g on the day of experiment were fasted overnight before surgery for ischaemia, and were randomly assigned to either of 5 treatment groups.

0N: nandrolone vehicle + saline;
10N: 10µg/kg/min nandrolone + saline;
40N: 40µg/kg/min nandrolone + saline;
80N: 80µg/kg/min nandrolone + saline;
160N: 160µg/kg/min nandrolone + saline.

5.4.2 Protocol

Rats were anaesthetised with pentobarbital sodium (60mg/kg, i.p.) and a ligature placed around the LAD coronary artery as per General Methods 2.6. The left femoral artery and vein were catheterised for BP measurement and i.v. drug administration respectively. A five-minute equilibration period was allowed following the completion of surgery. Cardiac parameters were recorded continuously from the conclusion of surgery (General Methods 2.7) and data was analysed according to General Methods 2.9. Nandrolone or the nandrolone vehicle was then administered at 70µl/min for 10 minutes in all pre-treatment groups. As cocaine was to be infused prior to ischaemia in subsequent experiments a control infusion of saline was carried out at 100µl/min for a further 10 minutes (Fig. 5.1).

In a separate group of 6 animals blood samples were collected to determine plasma nandrolone levels. Rats were anaesthetised with pentobarbital (60mg/kg, i.p.) and a catheter inserted into the femoral vein. A 10-minute infusion at 70µl/min was conducted with nandrolone (40 or 160µg/kg). A further 10 minutes later, approximately 7ml of blood was collected from the abdominal descending aorta into heparin lithium gel tubes, centrifuged and the resulting plasma aliquotted into 5ml tubes and frozen. Samples were sent on ice to the Australian Racing Forensic Laboratory for analysis of plasma nandrolone levels (GC-MS gas chromatography, mass spectrometry).
5.4.3 Data analysis
Blood pressure and HR during drug infusion was recorded and expressed relative to the value at the beginning of infusion (t=0s). Significance of differences between groups in the BP or HR response to nandrolone or vehicle infusion with respect to time was determined using a two-factor-repeated-measures-ANOVA.

All cardiovascular responses during ischaemia and reperfusion were expressed relative to the value at 0s of ischaemia or reperfusion. This normalised data was analysed using a two-factor ANOVA. As a number of rats in the nandrolone treated groups died from VF during ischaemia it was not possible to analyse between group differences using repeated measures as reliability of the test decreases significantly with missing values.

Arrhythmia data was analysed according to General methods 2.9.

5.4.4 Drugs
Nandrolone was dissolved in a 100% ethanol solution from which it was diluted to a stock solution with an ethanol concentration of 8.7%. The final concentration of ethanol in the nandrolone infusion was 1.8-4.6% (average, 3.0±0.1%). The average amount of ethanol contained in the 700μl nandrolone or vehicle infusions was 21.0±0.4μl.
1. Open chest and place ligature loosely around LAD coronary artery.
2. Nandrolone or vehicle infusion (0, 10, 40, 80, 160μg/kg/min, i.v.).
4. Ischaemia.
5. Reperfusion.
6. Collect blood & excise heart.

**Fig. 5. 1 Ischaemia-reperfusion protocol.** Rats were allowed a 5-minute equilibration period from opening of the chest until the commencement of a 10-minute nandrolone or vehicle infusion. This was followed immediately by a 10-minute saline infusion before the induction of ischaemia. Ischaemia was maintained for 15 minutes and reperfusion for 10 minutes. At the conclusion of the procedure the heart was excised in order to determine the “area at risk of infarction”.

CHARTER 5 The effect of intravenous nandrolone in rats subjected to cardiac ischaemia and reperfusion 5-6
5.5 Results

5.5.1 Plasma level of nandrolone
The plasma level of nandrolone was measured 10 minutes after the conclusion of infusion in a separate group of 6 rats. In rats administered 160μg/kg/min for 10 minutes (n=3), the resultant plasma concentration was significantly greater [t(4)=4.686, p<0.01] than rats infused with 40μg/kg/min (n=3) (915±135 vs 281±15nM, respectively).

5.5.2 Area at risk of Ischaemia
No significant difference was found in the ‘area at risk of ischaemia’ between the various pre-treatment groups (Fig. 5. 2)

5.5.3 Cardiovascular response to pre-ischaemia nandrolone infusion
The dose of nandrolone infused was not found to significantly change the ΔSP (Fig. 5. 3 A.), ΔDP (Fig. 5. 3 B.) or ΔHR (Fig. 5. 3 C.) from 0s of infusion. However, ‘time’ was found to cause a significant decrease in ΔHR (p<0.0001). No overall interaction between nandrolone ‘dose’ and ‘time’ was evident in ΔSP, ΔDP or ΔHR.

5.5.4 Cardiovascular response to ischaemia and reperfusion

Ischaemia
Nandrolone dose was not found to significantly effect the change in SP (Fig. 5. 4), DP (Fig. 5. 4) or HR (Fig. 5. 4) from 0s of ischaemia. However, ‘time’ was found to significantly effect ΔSP and ΔDP (both, p<0.01). No significant, overall ‘interaction’, was found between ‘time’ and ‘dose’ throughout ischaemia for any of the variables measured.

Reperfusion
The ΔSP (Fig. 5. 4 D.), ΔDP (Fig. 5. 4 E.) and ΔHR (Fig. 5. 4 F.) between the beginning and end of reperfusion was not found to vary significantly.
Ischaemia
Survival time decreased significantly (p<0.01) during ischaemia in rats administered the highest nandrolone dose (160µg/kg/min) compared to vehicle treated rats (p<0.001) (Fig. 5.5). While no other significant decreases in survival time during ischaemia were evident between treatment groups, the fraction of rats surviving ischaemia was found to be significantly lower ($\chi^2=12.29$, p<0.05) in the 40N (p<0.05) and 160N (p<0.05) treatment groups in comparison to vehicle treated controls (Table 5.1). Five deaths during ischaemia which were not due to VF and therefore could not be scored using the Lambeth convention were excluded from all survival calculations. These exclusions are noted in Fig. 5.5.

Reperfusion
No significant difference between groups was observed in the number of rats surviving reperfusion (Table 5.1). No statistically relevant decreases in survival time were observed in any group in comparison to control. (Fig. 5.5). One death during reperfusion which was not due to VF and therefore could not be scored using the Lambeth convention was excluded from survival calculations (Fig. 5.5)
5.5.6 Arrhythmia

Lambeth scores showed a significant increase ($\chi^2=13.69, p<0.01$) for the 3 highest doses of nandrolone, compared to vehicle treated rats (all $p<0.05$) during ischaemia (Table 5.2). Conversely, there were no differences found in Lambeth scores between nandrolone treatments and vehicle during reperfusion (Table 5.2).

Ischaemia

A significant difference was found between the fraction of rats responding to ischaemia with VF and those without any VF ($\chi^2=13.66, p<0.01$) (Table 5.3). A significantly greater fraction of rats treated with the highest dose of nandrolone (160µg/kg/min) responded with VF compared to the vehicle control (0µg/kg/min) ($p<0.05$). Likewise, significant increases in the frequency of VF were found in all doses of nandrolone in comparison to the lowest nandrolone dose of 10µg/kg/min (40µg/kg/min, $p<0.05$; 80µg/kg/min, $p<0.05$; 160µg/kg/min, $p<0.01$). No other significant changes in the fraction of rats responding with VT, or VT+VF were found during ischaemia (Table 5.3).

Rats displayed arrhythmia of longer duration during cardiac ischaemia than in reperfusion. The mean duration of VF during cardiac ischaemia was found to increase with nandrolone treatment in an approximately dose related manner (Fig. 5.6.B). A statistically significant increase in mean VF duration ($\chi^2=15.19, p<0.01$) was noted in rats treated with the highest dose of nandrolone (160µg/kg/min) compared to the lowest nandrolone dose (10µg/kg/min) ($p<0.05$). No obvious dose related effect was evident for mean VT duration, neither were any between group differences evident (Fig. 5.6.A). While increases in mean VT+VF duration displayed a dose related effect, none of these increases were significantly different from control (Fig. 5.7).

Increased mortality in nandrolone treated rats did not skew calculations of arrhythmia duration. Although the survival time and the fraction of rats surviving cardiac ischaemia was significantly different between groups, no significant difference in the duration of VT, VF and VT+VF was noted in rats surviving ischaemia (data not shown).
Reperfusion

No significant differences were found in the fraction of surviving rats responding with VT, VF or VT+VF during reperfusion (Table 5.3).

There was very little VT (Fig. 5.6.C) or VF (Fig. 5.6.D) during reperfusion in these rats, with no significant differences in the response between treatment groups. Much smaller mean durations of VT+VF were noted in cardiac reperfusion compared to cardiac ischaemia, with no significant differences between the responses of the various treatment groups (Fig. 5.7).
Fig. 5. 2 Zone at risk of infarction (\%z/r) in rats administered nandrolone (0, 10, 40, 80, 160 μg/kg/min). \( z \) = Mass of the underperfused heart region (area at risk of infarction), \( r \) = total heart mass (refer General Methods 2.7). **Legend:** 0N=0μg/kg/min nandrolone + saline (0.1ml/min/10 minutes), 10N=10μg/kg/min nandrolone + saline, 40N=40μg/kg/min nandrolone + saline, 80N=80μg/kg/min nandrolone + saline, 160N=160μg/kg/min + saline. Single-factor ANOVA, no significant differences. Mean±S.E., n=10-14.
Fig. 5. 3 Change in A. systolic pressure (SP), B. diastolic pressure (DP) and C. heart rate (HR) in rats infused for 10 minutes with nandrolone 0, 10, 40, 80, 160 µg/kg/min. Legend: ● 0 µg/kg/min nandrolone, △ 10 µg/kg/min nandrolone, * 40 µg/kg/min nandrolone, ◦ 80 µg/kg/min nandrolone, ○ 160 µg/kg/min nandrolone. Repeated-measures-two-factor-ANOVA. SP: DOSE [F(4,56)=0.938, p>0.05], TIME [F(1,56)=0.108, p>0.05], INTERACTION [F(4,56)=0.856, p>0.05]. DP: DOSE [F(4,56)=0.982, p>0.05], TIME [F(1,56)=0.428, p>0.05], INTERACTION [F(4,56)=1.106; p>0.5]. HR: DOSE [F(4,54)=0.655, p>0.05], TIME [F(1,54)=87.28, p<0.0001], INTERACTION [F(4,54)=2.247; p>0.5], Mean±S.E., n=10-14.
Fig. 5. 4 Change in A. Systolic (SP), B. diastolic pressure (DP) C. heart rate (HR) during cardiac ischaemia in rats treated with nandrolone (0, 10, 40, 80,160 µg/kg/min). Legend: ● 0µg/kg/min nandrolone, ▲ 10µg/kg/min nandrolone, * 40µg/kg/min nandrolone, ○ 80µg/kg/min nandrolone, ● 160µg/kg/min nandrolone. Two-factor ANOVA SP: DOSE $F(4,134)=0.932$, p>0.05, TIME $F(2,134)=6.291$, p<0.01, INTERACTION $F(8,134)=0.384$, p>0.05, DP: DOSE $F(4,134)=0.679$, p>0.05, TIME $F(2,134)=5.225$, p<0.01, INTERACTION: $F(8,134)=0.486$, p>0.05. HR: DOSE $F(4,125)=1.259$, p>0.05, TIME $F(2,125)=0.265$, p>0.05, INTERACTION: [F(8,125)=0.198, p>0.05]. Mean±S.E. , n=5-14 (BP), n=4-14 (HR). Change in D. Systolic (SP) and E. diastolic pressure (DP) and F. heart rate (HR) at the conclusion of reperfusion in rats treated with nandrolone (0, 10, 40, 80, 160 µg/kg/min). Two-factor ANOVA SP: no significant difference, DP: no significant difference, HR: no significant difference. Mean±S.E., n=5-13.
Fig. 5. Survival time during cardiac ischaemia (I) and reperfusion (R) in rats treated with nandrolone (0, 10, 40, 80, 160µg/kg/min) and saline immediately prior to commencement of ischaemia. Parentheses: indicate number of rats in each treatment group excluded because of death unrelated to VF. Legend: 0N=0µg/kg/min nandrolone + saline (0.1ml/min/10 minutes), 10N=10µg/kg/min nandrolone + saline, 40N=40µg/kg/min nandrolone + saline, 80N=80µg/kg/min nandrolone + saline, 160N=160µg/kg/min + saline. Single-factor ANOVA, Dunnett’s post-hoc test. (I) $F(4,51)=3.803, p<0.01$. "p<0.001 significantly different from 0µg/kg/min. (R) no significant difference. “n” value excludes the 6 rats in parentheses indicated in the figure excluded because of death unrelated to VF. Mean±S.E. n=10-13 (ischaemia), n=6-13 (reperfusion)
<table>
<thead>
<tr>
<th>Dose</th>
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<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>0N</td>
<td>13/13 (100%)</td>
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<tr>
<td>10N</td>
<td>13/13 (100%)</td>
</tr>
<tr>
<td>40N</td>
<td>6/10* (60%)</td>
</tr>
<tr>
<td>80N</td>
<td>7/10 (70%)</td>
</tr>
<tr>
<td>160N</td>
<td>6/10* (60%)</td>
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</tbody>
</table>

**Table 5.1 Number of rats surviving cardiac ischaemia (I) or reperfusion (R).** Includes only those rats which obeyed the Lambeth scoring system (ie. excludes death unrelated to VF. See caption for Fig. 5.5). Parentheses: percentage of rats surviving ischaemia and reperfusion. **Legend:** 0N=0μg/kg/min nandrolone + saline, 10N=10μg/kg/min nandrolone + saline, 40N=40μg/kg/min nandrolone + saline, 80N=80μg/kg/min nandrolone + saline, 160N=160μg/kg/min + saline. Chi-squared test with Fischer’s exact test post-hoc in comparison to 0N. *p<0.05 significantly different from 0N (0μg/kg/min).

<table>
<thead>
<tr>
<th>Treatment Type</th>
<th>I</th>
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<tr>
<td>0N</td>
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<td>3±1</td>
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<tr>
<td>160N</td>
<td>5±1*</td>
<td>1±1</td>
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**Table 5.2 Lambeth arrhythmia score during cardiac ischaemia (I) or reperfusion (R) in rats treated with nandrolone (0-160μg/kg/min).** **Legend:** 0N=0μg/kg/min nandrolone + saline, 10N=10μg/kg/min nandrolone + saline, 40N=40μg/kg/min nandrolone + saline, 80N=80μg/kg/min nandrolone + saline, 160N=160μg/kg/min + saline. Kruskal-Wallis, Dunn’s post-hoc comparisons made to 0N. *p<0.05 significantly different from 0μg/kg/min. Mean±S.E. n=10-13 (ischaemia), n=6-13 (reperfusion).
### Table 5.3 Fraction of nandrolone (0, 10, 40, 80, 160μg/kg/min) treated rats responding with VT, VF or VT+VF during a 15-minute period of cardiac ischaemia (I) and during 10 minutes of cardiac reperfusion (R). Parentheses: percent rats responding with VT, VF or VT+VF. Legend:

- **0N**=0μg/kg/min nandrolone + saline (0.1ml/min/10 minutes),  **10N**=10μg/kg/min nandrolone + saline,  **40N**=40μg/kg/min nandrolone + saline,  **80N**=80μg/kg/min nandrolone + saline,  **160N**=160μg/kg/min + saline. Chi-squared test, Fischer’s Exact Test post-hoc. *p<0.05 significantly different from 0N; **p<0.05, ***p<0.01 significantly different from 10N. Mean±S.E.
Fig. 5.6 Average duration of VT in cardiac ischaemia (A.) and cardiac reperfusion (C.) and duration of VF in ischaemia (B.) and reperfusion (D.) in rats infused with nandrolone (0,10,40,80,160μg/kg/min). Legend: 0N=0μg/kg/min nandrolone + saline (0.1ml/min/10 minutes), 10N=10μg/kg/min nandrolone + saline, 40N=40μg/kg/min nandrolone + saline, 80N=80μg/kg/min nandrolone + saline, 160N=160μg/kg/min + saline. Kruskal-Wallis test with Dunn’s multiple comparisons test. *p<0.05 significantly different from 10μg/kg/min. Mean±S.E. (n=10-14 ischaemia, n=6-13 reperfusion).
Fig. 5. 7 Average duration of VT+VF during cardiac ischaemia (I) and cardiac reperfusion (R) in rats treated with nandrolone (0, 10, 40, 80, 160µg/kg/min) and saline immediately prior to commencement of ischaemia. Legend: 0N=0µg/kg/min nandrolone + saline (0.1ml/min/10 minutes), 10N=10µg/kg/min nandrolone + saline, 40N=40µg/kg/min nandrolone + saline, 80N=80µg/kg/min nandrolone + saline, 160N=160µg/kg/min + saline. Kruskal Wallis test. Mean±S.E. n=10-14 (ischaemia), n=6-13 (reperfusion).
5.6 Discussion

This is the first study to investigate changes in haemodynamics following nandrolone infusion in male rats, although BP and ECG assessment in human volunteers administered i.v. testosterone at physiological and super-physiological doses have been reported (White et al., 1999). White et al. reported no ECG or BP changes. However, a maximum testosterone dose of only six times the endogenous level was used. It is probable that plasma concentrations reached with illicit use are several orders of magnitude higher than this.

The absorption, distribution and elimination of nandrolone as administered previously is complicated by the use of oil-based preparations. In order to ensure maximum bioavailability and simplify interpretation of the pharmacodynamic data from acute nandrolone administration it was decided to intravenously administer nandrolone. As mentioned previously, there are no reports in the literature equating the cardiac effect of nandrolone with plasma nandrolone concentrations. This makes selection of doses for i.v. administration exceptionally difficult. As plasma nandrolone concentrations following an abusive cycle have not been reported it is very difficult to determine a pharmacologically relevant dose. Reports of endogenous testosterone levels in adult males range from 1.21 to 3.47 X 10^{-8}M (Strauss et al., 1985; Rowland et al., 1987; Melchert et al., 1992). A therapeutic dose of testosterone enanthate administered to 19-40 year old males has been associated with a mean plasma concentration of 4.37 X 10^{-8}M. As illicit use of AS is associated with a much higher dose than would be clinically prescribed (10-1000 times the recommended therapeutic dose) it was decided that infusion doses of nandrolone of 10-160μg/kg/min would be used and plasma nandrolone concentrations measured following infusion. The plasma concentration achieved with an infusion of 40μg/kg/min of nandrolone was 281±15nM (28.1x10^{-9}M) and with the highest dose (160μg/kg/min) was 915±135nM (91.5±13.5x10^{-9}M). This suggests that the plasma nandrolone concentration achieved with infusion of 160μg/kg/min was approximately 25-75 fold greater than the reported endogenous male testosterone level (Strauss et al., 1985; Rowland et al., 1987; Melchert et al., 1992).

We found no evidence of an haemodynamic effect of acutely administered nandrolone. No significant difference was found in the change in BP or change in HR from baseline between treatment groups. Chronic treatment with nandrolone may be necessary to observe the cardiovascular effects commonly attributed to AS abuse. However, 'time' alone was found to cause a significant decrease in HR during the 10-minute nandrolone infusion. This most probably reflects bradycardia produced in response to the open chest. To prevent this effect obscuring a possible...
haemodynamic action of nandrolone, recordings of BP and HR were made in chapter 7 before the chest was opened for induction of ischaemia (Fig. 7.1).

It was observed that the BP and HR response to ischaemia was unaffected by the ‘dose’ of nandrolone used. ‘Time’ alone was found to have a significant affect on SP and DP during ischaemia. The time-dependent affect is most probably due to the adaptation to the ischaemic insult. A significant decrease in SP and DP in all treatment groups can be noted evident during the first 5 minutes of ischaemia. Heart rate remained approximately the same or decreased. Blood pressure was found to increase again following the 5th minute of ischaemia in all treatment groups.

A decrease in BP and HR immediately following induction of ischaemia has been noted by several researchers in both conscious (Botting et al., 1983; Curtis et al., 1987) and pentobarbitone sodium anaesthetised rats (Au et al., 1983; McLennan et al., 1985). While most studies performed in anaesthetised rats have noted a decrease in HR immediately following occlusion, at least one investigator has noted a small increase in conscious animals (Johnston et al., 1983a; b). After the initial decrease the slow increase in pressure and rate during ischaemia most probably reflects an adaptation to the cardiac insult. The ‘time’ effect may also be contributed to by a decrease in survival as ischaemia progressed and by arrhythmia which made it difficult to record accurate BP and HR values. No BP or HR values could be reported while a rat was experiencing VT or VF. If the duration of arrhythmia was greater than 60s before or 60s after 0s, 300s, 600s or 900s of ischaemia no cardiovascular values could be reported (see General Methods 2.9). The combination of increased fatality and an increase in the number of rats experiencing arrhythmia as ischaemia progressed resulted in a decrease in ‘n’ between the beginning of ischaemia and the end of reperfusion. This may have contributed to the effect of time on BP observed during ischaemia.

Reperfusion induced arrhythmias are best observed following an ischaemic period of 5-10 minutes (Manning et al., 1984). As we were interested in observing arrhythmia resulting from ischaemia we chose a 15 minute LAD coronary artery occlusion time. Previous studies have indicated that this results in a much greater degree of arrhythmia during ischaemia than in reperfusion (McLennan et al., 1988). This effect was confirmed in the present study, where very few arrhythmic events were noted during reperfusion.

The main finding in this study was an increased number of fatalities during cardiac ischaemia in rats receiving nandrolone at the three highest doses, reflected in a decreased survival time during ischaemia. This increased mortality corresponded with an increase in the duration of VF and a significantly increased Lambeth score for all three doses of nandrolone compared to control. These effects could not be attributed to variation in the area at risk of infarction. No arrhythmia was
evident in the absence of cardiac insult. Nandrolone had no significant effect on cardiac rhythm or mortality during reperfusion.

5.6.1 Potential mechanism for an acute nandrolone effect during cardiac ischaemia
Very few researchers have attempted to investigate the acute effects of AS. Our results indicate that nandrolone alone did not significantly change BP before or after ischaemia, and that dose related increases in Lambeth score and VF were only observed during ischaemia. Many mechanisms could explain these results. The increase in arrhythmia and decrease in survival during cardiac ischaemia is potentially related to increases in catecholamine release during LAD coronary artery occlusion.

Myocardial ischaemia has been demonstrated to give rise to an accumulation of catecholamines in the myocardium by 3 main mechanisms…

1. An increase in plasma catecholamines via stimulation of the adrenal medulla (Daugherty et al., 1986);
2. reflex increase in cardiac sympathetic nerve activity, accompanied by a local release of catecholamines from the sympathetic nerve endings of the myocardium via a reversal of uptake-1 (Schomig, 1990; Dart et al., 1993; Du et al., 1993; Du et al., 1998);
3. local metabolic release mechanisms, independent of central nervous system involvement (Schomig, 1990).

Sex steroids such as testosterone have been shown to inhibit acutely the re-uptake of noradrenaline by smooth muscle in isolated, perfused rat hearts (Iversen et al., 1970; Salt, 1972). Histochemical/fluorescence studies have associated this uptake with cardiac muscle cells (Clarke et al., 1969). Uptake-2 has also been demonstrated in rat cardiac myocytes (Obst et al., 1996). It is possible that a synergy exists between ischaemia-induced release of catecholamines and inhibition of smooth muscle re-uptake by nandrolone, resulting in increased VF during an ischaemic period. A number of authors have postulated that because fixed coronary plaques can prevent the extraneuronal uptake (uptake-2) of noradrenaline, the risk of ischaemia is greatly increased in these patients (Lathers et al., 1988; Kabas et al., 1990). Although both testosterone and androsterone have been shown to block uptake-2, it is unknown whether nandrolone and similar AS can also block this mechanism. This question was addressed in chapter 8.
5.7 Conclusion

- Intravenous nandrolone (10 to 160μg/kg/min) in the absence of ischaemia did not cause a significant change in BP or HR.
- No arrhythmia was observed in the absence of cardiac ischaemia.
- Acute administration of i.v. nandrolone caused a dose related increase in ischaemia induced VF resulting in a significantly increased Lambeth arrhythmia score at the 3 highest doses, and consequently a decrease in survival.
5.8 References


CHAPTER 5 The effect of intravenous nandrolone in rats subjected to cardiac ischaemia and reperfusion 5-23


CHAPTER 5 The effect of intravenous nandrolone in rats subjected to cardiac ischaemia and reperfusion


The effect of intravenous cocaine in rats subjected to cardiac ischaemia and reperfusion

6.1 Introduction
Cocaine has been shown to induce myocardial ischaemia leading to increased risk of severe cardiac rhythm disturbances in human subjects, including fatal (Nanji et al., 1984; Osawa et al., 1994; Gamouras et al., 2000) and non-fatal arrhythmia (Ascher et al., 1988). Prenatal cocaine exposure has been demonstrated to increase the incidence of atrial and ventricular arrhythmia in the neonatal period (Frassica et al., 1994).

The cardiac effects of cocaine are long lasting. Ambulatory 24 hour monitoring (Holter recording) has revealed evidence of ischaemia in cocaine users up until 2 weeks following withdrawal (Nademanee et al., 1989).

A number of putative mechanisms for cocaine-induced arrhythmia in animal models have been proposed, including coronary artery vasoconstriction (refer 1.13.1), changes to cardiac conduction (refer 1.13.2) and calcium mobilisation (refer 1.13.2). It is unlikely that a single unifying explanation can be forwarded to explain the arrhythmic effects of cocaine, and is probable that arrhythmia is due to a number of mechanisms acting in concert.

In view of the suspected interaction between cocaine and nandrolone, discussed in chapter 1, and in view of the potentiating effects of acute nandrolone during ischaemia, (described in chapter 5) the effect of cocaine in the presence and absence of nandrolone was investigated in this model of ischaemia-reperfusion.
6.2 Aims

1. To determine whether i.v. cocaine increases ischaemia-induced cardiac arrhythmia.

2. To determine whether i.v. cocaine modifies the potentiating effect of acute nandrolone on ischaemia-induced cardiac arrhythmia.

6.3 Hypotheses

1. Cocaine will potentiate arrhythmia in ischaemic hearts.

2. Cocaine will potentiate the effects of nandrolone on occurrence and severity of arrhythmia in ischaemic hearts.
6.4 Methodology

6.4.1 Animals
Ten week old, male Sprague Dawley rats were fasted overnight and randomly assigned to either of 5 treatment groups, ...

1. 40N+C: nandrolone (40µg/kg/min) + cocaine (0.5mg/kg/min);
2. 40N+S: nandrolone (40µg/kg/min) + saline
3. S+C: saline + cocaine (0.5mg/kg/min)
4. 0N+C: vehicle + cocaine (0.5mg/kg/min)
5. 0N+S: vehicle + saline

Results from chapter 5 were used for treatment groups 2 and 5.

6.4.2 Protocol
A five-minute equilibration period was allowed following the completion of surgery. Nandrolone (40µg/kg/min), saline or the nandrolone vehicle was then administered at 70µl/min for 10 minutes. Cocaine (0, 0.5mg/kg/min, i.v.) was then infused at 100µl/min for a further 10 minutes. The total dose of cocaine received was 5.0mg/kg.

Ischaemia and reperfusion was conducted as per chapter 5. The protocol used is summarised in (Fig. 6.1).

6.4.3 Data analysis
The effect of pre-treatment with nandrolone, saline or vehicle on BP and HR was determined using a repeated-measures-two-factor-ANOVA. The effect of cocaine or saline on BP and HR in pretreated rats was determined using a two-factor-ANOVA. Differences between groups (post-hoc) were determined for ‘treatment’ and ‘interaction’ using Bonferroni’s test (with correction for multiple comparisons – see General Methods 2.9). Post-hoc tests for ‘treatment’ are shown in Fig. 6.3 and Fig. 6.4. The post-hoc tests for ‘interaction’ and shown in Appendix G. Changes in BP and HR during ischaemia and reperfusion and the duration and frequency of arrhythmia etc were determined as per General Methods 2.9. Because cocaine depressed both BP and HR and this effect had not returned to baseline by the beginning of ischaemia the effect of ischaemia on cardiovascular variables was determined separately in cocaine and saline treated rats.
1. Open chest and place ligature loosely around LAD
2. Nandrolone (40µg/kg/min, i.v.), vehicle or saline infusion
3. Cocaine (0.5mg/kg/min) or saline
4. Ischaemia
5. Reperfusion
6. Excise heart

**Fig. 6.1 Ischaemia-reperfusion protocol.** Rats were allowed a 5-minute equilibration period from opening of the chest until the commencement of a 10-minute nandrolone, saline or vehicle infusion. This was followed immediately by a 10-minute cocaine infusion before the induction of ischaemia. Ischaemia was maintained for 15 minutes and reperfusion for 10 minutes. At the conclusion of the procedure the blood was collected and the heart excised.
6.5 Results

To aid interpretation of the degree of any additional potential arrhythmia caused by cocaine in conjunction with nandrolone, the nandrolone alone (40μg/kg/min) results from the previous chapter (40N+S), have been included here and in all statistical analyses. In order to demonstrate the arrhythmic effect of cocaine alone, the vehicle plus saline results (0N+S) from chapter 5 have also been included. Tabular results have these groups shaded in grey.

6.5.1 Area at risk of ischaemia

The area at risk of ischaemia was not significantly different between pre-treatment groups *data not shown*.

6.5.2 Cardiovascular response to pre-treatment

Response to nandrolone, vehicle or saline pre-treatment

The change (Δ) in BP and HR from 0s of infusion was noted at 300s and 600s of infusion. Treatment with vehicle, saline or nandrolone was not found to significantly effect Δ SP (Fig. 6.2 A.), Δ DP (Fig. 6.2 B.) or Δ HR (Fig. 6.2 C.). Time was found to have a significant effect upon Δ DP (p<0.05) and Δ HR (p<0.001). No interaction between ‘time’ and ‘treatment’ was observed for any of the variables measured.

Response to cocaine or saline

The SP, DP and HR response to cocaine or saline was expressed as change (Δ) from baseline, where the baseline was the value 40s prior to the start of drug infusion.

All rats treated with cocaine demonstrated a strong initial, but transient, pressor response to cocaine (Fig. 6.3). Both the ΔSP and the ΔDP from baseline in all rats administered cocaine peaked at 40s post administration. At all time points subsequent to 100s a sharp depressor effect was observed. No significant fluctuation in either ΔSP or ΔDP was recorded in nandrolone or vehicle infused rats administered saline.

Due to the bi-phasic nature of the BP response to cocaine both phases were analysed separately by repeated-measures-ANOVA. The ΔSP (Fig. 6.3 A.) and ΔDP (Fig. 6.3 B.) response to cocaine was divided into pressor (0-100s) and depressor phases (100-600s). Heart rate (Fig. 6.3 C.) did not display a biphasic response to cocaine and the whole 600s of infusion was analysed simultaneously.
The Pressor Phase
'Treatment' alone was found to have a significant effect on $\Delta$SP (p<0.01) and $\Delta$DP (p<0.001) during the first 100s of the 10 minute drug infusion period. Most of the significant difference between cocaine and saline treated groups was found at 40s of cocaine infusion corresponding to the peak of the cocaine effect (see Fig. 6.3 Inset A & B. for details of post-hoc tests). Pre-treatment with nandrolone was not found to alter the BP response to cocaine. 'Time' alone also had a significant effect on $\Delta$SP and $\Delta$DP (both, p<0.0001). A significant interaction was observed between 'treatment' and 'time' for both $\Delta$SP and $\Delta$DP (both, p<0.0001). To aid the clarity of Fig. 6.3 these significant differences are noted separately in Appendix G.

The Depressor Phase
Treatment alone was found to have a significant effect on $\Delta$SP (p<0.01) and $\Delta$DP (p<0.0001) during 100-600s of infusion. Post-hoc tests (Bonferroni's) did not detect any significant differences between groups (Fig. 6.3). Pre-treatment with nandrolone was not found to alter the BP response to cocaine. Time was also found to significantly affect $\Delta$SP and $\Delta$DP (both, p<0.0001). A significant interaction was observed between 'treatment' and 'time' for $\Delta$SP (p<0.0001) and $\Delta$DP (p<0.0001) (see Appendix G for post-hoc tests).

HR Changes during 10 minutes of drug infusion (cocaine or saline)
Cocaine was found to cause a profound decrease in HR producing at the lowest point an average HR of 309±21, 310±14 and 323±11 bpm in rats pre-treated with infused saline, vehicle or nandrolone (40μg/kg/min), respectively, compared with pre-cocaine values of 377±10, 399±8 and 388±7, respectively. Nandrolone pre-treatment was not found to significantly alter the effect of cocaine on HR compared with saline and vehicle pre-treatment. 'Treatment' alone was found to have a significant effect on $\Delta$HR (p<0.05). This effect was most pronounced at 300s of infusion where the $\Delta$HR was found to be significantly greater in the 40N+S and 0N+S groups compared to 40N+C (p<0.01 and p<0.001, respectively) (Fig. 6.4). 'Time' alone was also found to vary significantly (p<0.0001). A significant interaction (p<0.0001) between 'treatment' and 'time' was observed for $\Delta$HR (see Appendix G for post-hoc tests).

6.5.3 Cardiovascular responses to ischaemia and reperfusion
Cocaine was found to produce a significant cardio-depressant effect in the absence of ischaemia in terms of BP and HR. Therefore, only the effect of coronary occlusion and reperfusion on cardiovascular responses in cocaine treated groups (S+C, 0N+C, 40N+C) are presented below. As reported in the previous chapter, there was no significant 'treatment' or 'time' effect for non-cocaine treated groups (0N+S, 40N+S), neither was an interaction observed for the SP, DP or HR.
response (two-factor-ANOVA) during ischaemia. No significant difference between these 2 groups was evident during reperfusion for any of the cardiovascular variables measured (unpaired t-test).

Ischaemia
No effect of ‘treatment’ or ‘time’ was observed for both ΔSP and ΔDP during ischaemia (Fig. 6.5 A, B). Likewise, no interaction was found between ‘treatment’ and ‘time’ for BP changes.

‘Treatment’ alone caused a significant difference in the ΔHR response during ischaemia (Fig. 6.5 C). However, no significant difference was found between treatment groups at each time point (Bonferroni’s). ‘Time’ alone did not significantly change the ΔHR response during ischaemia. Likewise, no interaction was observed.

Reperfusion
No significant difference between groups was found for the ΔSP (Fig. 6.5 D), ΔDP (Fig. 6.5 E) or ΔHR (Fig. 6.5 F) response during reperfusion.

6.5.4 Survival
Nine out of a total group of 63 rats (14%) died from causes not related to VF. Six of these rats were from treatment groups receiving cocaine (4 in ischaemia and 2 in reperfusion). An additional 6 deaths could be attributed to VF. As the former rats do not obey the Lambeth convention they could not be scored for arrhythmia severity. All exclusions have been noted in Fig. 6.6. Survival data has been separated into rats which complied with the Lambeth convention and those that didn’t (Table 6.1).

Ischaemia
In rats which complied with the Lambeth convention (Fig. 6.6 top) there was a significant decrease in survival during ischaemia (p=0.0454). As the ‘p’ value was so close to 0.05, post-hoc tests (Tukey’s) were not able to detect where this significance lay. As survival was 100% in rats obeying the Lambeth convention during reperfusion, statistical tests of survival were not conducted. When all rats were included in survival calculations regardless of whether they obeyed the Lambeth convention or not there was no difference in the length of survival during ischaemia and reperfusion (Fig. 6.6, bottom). However, when examining the fraction of rats surviving ischaemia compared to the number dying of VF it was found that survival was significantly greater ($\chi^2=13.49$, p<0.01) in the 0N+S (p<0.05) and 40N+C (p<0.05) group in comparison to 40N+S (Table 6.1).
Reperfusion
None of the 53 rats complying with the Lambeth convention died during reperfusion. Survival time and the fraction of surviving rats did not differ between treatment groups when all-cause death was considered (Fig. 6.6).

6.5.5 Arrhythmia
The infusion of cocaine alone produced no VT or VF.

Ischaemia
During ischaemia, cocaine did not significantly change the number of rats responding with VT, VF or VT+VF (Table 6.3) neither was the average duration of VT (Fig. 6.7.A), VF (Fig. 6.7.B) or VT+VF (Fig. 6.9) significantly different between groups. No significant difference in the Lambeth score was observed between groups (Table 6.2). Despite no significant difference in the number of rats experiencing an arrhythmic episode (Table 6.3), the duration of any episodes observed (Fig. 6.7) or the Lambeth score (Table 6.2), a significantly greater number of rats were observed to die of VF during ischaemia in nandrolone pre-treated rats administered saline (40N+S) compared to the 0N+C (p<0.05) and 40N+C (p<0.05) treatment groups (Table 6.1). When rats dying from ischaemia were excluded from statistical analysis there was only a minor change in the mean duration of VF during ischaemia for all groups (Fig. 6.8), although the duration of VF was now found to be significantly greater (p<0.05) in the 40N+S group compared to 0N+S (p<0.05). No significant differences were observed between groups for VT or VT+VF (data not shown).

Reperfusion
The number of rats responding with VT, VF or VT+VF was not significantly different between treatment groups during reperfusion (Table 6.3) neither was the duration of VT (Fig. 6.7.C), VF (Fig. 6.7.D) or VT+VF (Fig. 6.9) significantly different between groups. Similarly, the Lambeth score was also unaffected by any pre-treatment (Table 6.2).
Fig. 6. 2 Change in A. SP, B. DP, C. HR in rats pre-treated with vehicle (70μl/min), saline (70μl/min) or nandrolone (40μg/kg/min). Legend: X saline (100μl/min) + cocaine (0.5mg/kg/min) [S+C], □ nandrolone vehicle (70μl/min) + cocaine [0N+C], ○ nandrolone (40μg/kg/min) + cocaine [40N+C], ■ nandrolone vehicle (70μl/min) + saline (100μl/min) [0N+S], *nandrolone (40μg/kg/min) + saline [40N+S]. Repeated-measures-ANOVA, Bonferroni’s post-hoc test. SP: TREATMENT [F(4,59)=0.978, p>0.05]; TIME [F(1,59)=3.567, p>0.05]; INTERACTION [F(4,59)=1.315, p>0.05]. DP: TREATMENT [F(4,59)=0.965, p>0.05]; TIME [F(1,59)=5.56, p<0.05]; INTERACTION [F(4,59)=1.502, p>0.05]. HR: TREATMENT [F(4,59)=0.302, p>0.05]; TIME [F(1,59)=136.5, p<0.001]; INTERACTION [F(4,59)=0.546, p>0.05]; Mean±S.E., n=12-14.
Fig. 6. 3 Change in A. systolic pressure (SP), B. diastolic pressure (DP) relative to the value at 40s prior to the start of infusion of cocaine (0.5mg/kg/min). Legend: X saline (100μl/min) + cocaine (0.5mg/kg/min) [S+C], ▼ nandrolone vehicle (70μl/min) + cocaine [ON+C], ○ nandrolone (40μg/kg/min) + cocaine [40N+C]. ■ nandrolone vehicle (70μl/min) + saline (100μl/min) [ON+S], ★ nandrolone (40μg/kg/min) + saline [40N+S].

Repeated-measures-ANOVA, Bonferroni’s post-hoc test. The line at x=100s represents the end of the pressor response to cocaine. SP (0-100s) see Inset A: TREATMENT [F(4,295)=3.790, p<0.01] *** p<0.001 significantly different from S+C, *p<0.05, ###p<0.001 significantly different from ON+C, **p<0.01 significantly different from 40N+C; TIME [F(5,295)=24.53, p<0.0001]; INTERACTION [F(20,295)=5.866, p<0.0001] see Appendix G for ‘interaction’ post-hoc tests. SP (100-600s): TREATMENT [F(4,354)=4.928, p<0.01]; TIME [F(6,354)=19.83, p<0.0001]; INTERACTION [F(24,354)=6.35, p<0.0001] see Appendix G for ‘interaction’ post-hoc tests. DP (0-100s) see Inset B: TREATMENT [F(4,295)=5.578, p<0.001] **p<0.01, ###p<0.001 significantly different from S+C, #p<0.05, ###p<0.001 significantly different from ON+C, +p<0.05, +++p<0.001 significantly different from 40N+C; TIME [F(5,295)=25.95, p<0.0001]; INTERACTION [F(20,295)=6.749, p<0.0001] see Appendix G for ‘interaction’ post-hoc tests. DP (100-600s): TREATMENT [F(4,354)=7.99, p<0.0001]; TIME [F(6,354)=30.11, p<0.0001]; INTERACTION [F(24,354)=7.067, p<0.0001] see Appendix G for ‘interaction’ post-hoc tests. Mean±S.E. n=11-14.
Fig. 6.4 Change in heart rate (HR), relative to the value at 40s prior to the start of infusion of cocaine (0.5mg/kg/min). Legend: X saline (100μl/min) + cocaine (0.5mg/kg/min) [S+C], ▼ nandrolone vehicle (70μl/min) + cocaine [0N+C], ○ nandrolone (40μg/kg/min) + cocaine [40N+C], ■ nandrolone vehicle (70μl/min) + saline (100μl/min) [0N+S], ▲ nandrolone (40μg/kg/min) + saline [40N+S]. Repeated-measures-ANOVA, Bonferroni's post-hoc test. HR (0-600s): TREATMENT [F(4,638)=3.227, p<0.05] ++p<0.01, +++p<0.001; TIME [F(11,638)=37.54, p<0.0001]; INTERACTION [F(44,638)=5.762, p<0.0001] see Appendix G for interaction post-hoc tests. Mean±S.E. n=11-14.
Fig. 6. 5 Left: Change in A. Systolic (SP), B. diastolic pressure (DP) and C. HR during cardiac ischaemia. Legend: ▼ nandrolone vehicle (70 μl/min) + cocaine, ○ nandrolone (40 μg/kg/min) + cocaine, X saline (100 μl/min) + cocaine (0.5 mg/kg/min). Two-factor-ANOVA, Bonferroni’s post-hoc test. SP: TREATMENT [F(2,81)=2.561, p>0.05]; TIME [F(2,81)=0.755, p>0.05]; INTERACTION [F(4,81)=1.412, p>0.05]. DP: TREATMENT [F(2,81)=2.932, p>0.05]; TIME [F(2,81)=0.793, p>0.05]; INTERACTION [F(4,81)=0.883, p>0.05]. HR: TREATMENT [F(2,82)=3.754, p<0.05]; TIME [F(2,82)=0.021, p>0.05]; INTERACTION [F(4,82)=1.4, p>0.05]. Mean±S.E., n=10-12. Right: Change in D. SP, E. DP and F. HR at the conclusion of reperfusion. Legend: 0N+C = nandrolone vehicle (70 μl/min) + cocaine, 40N+C = nandrolone (40 μg/kg/min) + cocaine, S+C = saline (100 μl/min) + cocaine (0.5 mg/kg/min). Single-factor-ANOVA. SP: [F(2,22)=0.7, p>0.05]. DP: [F(2,22)=0.693, p>0.05], HR: [F(2,23)=0.66, p>0.05]. Mean±S.E., n=7-10.
Fig. 6.6 Survival time in ischaemia (I) and reperfusion (R). Parentheses indicate number of rats in each pre-treatment group dying from causes unrelated to VF. **Legend:** S+C = saline (100μl/min) + cocaine (0.5mg/kg/min), 0N+C = vehicle (70μl/min) + cocaine, 0N+S = vehicle + saline (100μl/min), 40N+C = nandrolone (40μg/kg/min) + cocaine, 40N+S = nandrolone + saline. **TOP:** death defined according to Lambeth convention.

**Between group differences:** Single-factor ANOVA. (I) [F(4,48)=2.634, p<0.05] (R) Invalid to perform ANOVA when survival = 100%. Mean±S.E. n=10-13 (ischaemia), n=6-13 (reperfusion). **BOTTOM:** death from all causes.

**Between group differences:** Single-factor ANOVA. (I) no significant difference (R) no significant difference. Mean±S.E., n=12-14 (ischaemia), n=7-13 (reperfusion).
Table 6.1 Number of rats surviving as a fraction of rats alive at the beginning of ischaemia. Left hand column: fraction of rats surviving which obey the Lambeth convention. Right hand column: fraction of all rats surviving. Parentheses: percentage of rats surviving ischaemia and reperfusion. Legend: S+C = saline (100µl/min) + cocaine (0.5mg/kg/min), 0N+C = vehicle (70µl/min) + cocaine, 0N+S = vehicle + saline (100µl/min), 40N+C = nandrolone (40µg/kg/min) + cocaine, 40N+S = nandrolone + saline. Chi-squared test with Fischer’s Exact test post-hoc. *p<0.05 significantly different from 40N+S.

<table>
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<th>R</th>
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<td>(100%)</td>
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<td>(100%)</td>
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<td>(100%)</td>
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<td>(78%)</td>
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<td>13/14</td>
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<td>(100%)</td>
<td>(93%)</td>
<td>(100%)</td>
</tr>
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<td>11/12</td>
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<td>(100%)</td>
<td>(100%)</td>
<td>(92%)</td>
<td>(100%)</td>
</tr>
<tr>
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<td>7/12</td>
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<td>(60%)</td>
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Table 6.2 Lambeth convention score. Legend: S+C = saline (100µl/min) + cocaine (0.5mg/kg/min), 0N+C = nandrolone vehicle (70µl/min) + cocaine, 0N+S = nandrolone vehicle (70µl/min) + saline (100µl/min), 40N+C = nandrolone (40µg/kg/min) + cocaine, 40N+S = nandrolone (40µg/kg/min) + saline. Kruskal-Wallis, no significant differences. Mean±SE, n=10-13 (ischaemia), n=6-13 (reperfusion).
<table>
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<th>Fraction responding with VF</th>
<th>Fraction responding with VT or VF</th>
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</tr>
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<td>(20.0)</td>
<td>(33.3)</td>
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<tr>
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<td>7/13</td>
<td>1/9</td>
<td>5/13</td>
</tr>
<tr>
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<td>(11.1)</td>
<td>(38.5)</td>
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<td>(41.7)</td>
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</tr>
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<td>(28.6)</td>
<td>(58.3)</td>
</tr>
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</table>

Table 6.3 Fraction of surviving rats responding with VT, VF or VT+VF during 15 minutes of ischaemia and during 10 minutes reperfusion. Parentheses: percent of surviving rats responding with VT, VF or VT+VF during 10 minutes of reperfusion. Legend: S+C = saline (100μl/min) + cocaine (0.5mg/kg/min), 0N+C = nandrolone vehicle (70μl/min) + cocaine, 0N+S = nandrolone vehicle (70μl/min) + saline (100μl/min), 40N+C = nandrolone (40μg/kg/min) + cocaine, 40N+S = nandrolone (40μg/kg/min) + saline. Chi-squared test, no significant differences.
Fig. 6.7 Average duration of VT in ischaemia (A.) and reperfusion (C.) and duration of VF in ischaemia (B.) and reperfusion (D.). Legend: S+C = saline (100μl/min) + cocaine (0.5mg/kg/min), 0N+C = nandrolone vehicle (70μl/min) + cocaine, 0N+S = nandrolone vehicle (70μl/min) + saline (100μl/min), 40N+C = nandrolone (40μg/kg/min) + cocaine, 40N+S = nandrolone (40μg/kg/min) + saline. Kruskal-Wallis test, no significant differences. Mean±S.E., n=12-14 (ischaemia), n=7-13 (reperfusion).
**Fig. 6. 8** Average duration of VF in ischaemia in rats surviving ischaemia. Legend: S+C = saline (100µl/min) + cocaine (0.5mg/kg/min), 0N+C = nandrolone vehicle (70µl/min) + cocaine, 0N+S = nandrolone vehicle (70µl/min) + saline (100µl/min), 40N+C = nandrolone (40µg/kg/min) + cocaine, 40N+S = nandrolone (40µg/kg/min) + saline.

Mean±S.E., n=10 (S+C), n=10 (0N+C), n=13 (0N+S), n=11 (40N+C), n=10 (40N+S). kw=11.35, p<0.05. *p<0.05

Significantly different from 0N+S, Kruskal-Wallis test with Dunn’s multiple comparisons test.

**Fig. 6. 9** Average duration of VT+VF in ischaemia (I) and reperfusion (R). Legend: S+C = saline (100µl/min) + cocaine (0.5mg/kg/min), 0N+C = nandrolone vehicle (70µl/min) + cocaine, 0N+S = nandrolone vehicle (70µl/min) + saline (100µl/min), 40N+C = nandrolone (40µg/kg/min) + cocaine, 40N+S = nandrolone (40µg/kg/min) + saline.

Mean±S.E., n=12-14 (ischaemia), n=7-13 (reperfusion). (I) no significant difference, (R) no significant difference. Kruskal-Wallis test, no significant differences.
6.6 Discussion

6.6.1 Blood pressure effects of cocaine

While cocaine displayed an obvious initial pressor effect, there was a rapid decline in systolic and diastolic pressures at all time points past 40s. Heart rate showed little or no initial increase, but steadily decreased from baseline, reaching a maximum depression from baseline of between 60 and 70 bpm. The cardiovascular response to infused cocaine was unaffected by pre-treatment with nandrolone. No arrhythmia was observed during cocaine infusion.

The initial pressor response to intravenous cocaine, followed by an immediate depressor effect has been previously reported in rats. Cocaine (2mg/kg/min, i.v.) in rats under pentobarbital anaesthesia (30mg/kg, i.v.) was found to produce a transient pressor response, followed by a gradual depressor phase (Tella et al., 1992). The magnitude of the pressor response and the extent of the depression induced in BP was found to be dependent on the extent of anaesthesia. Similarly, the response to both cocaine (0.16-5mg/kg) and procaine (0.31 & 1.25 mg/kg) in pentobarbital anaesthetised (65mg/kg, i.p.) rats has been shown to involve a dose related decrease in arterial pressure immediately following a short pressor phase in the first minute (Pitts et al., 1987). A dampened cardiovascular response to cocaine has been previously reported in anaesthetised canines. A cocaine dose of 1mg/kg, when administered to conscious dogs, increased mean BP 44±5.2 mmHg. This dose, administered 30 min after dogs were anaesthetised with pentobarbital (32mg/kg, i.v.), increased BP only 9.5±3.9 mmHg. Pentobarbital anaesthetised (25mg/kg, i.v.) dogs subjected to a cocaine infusion (4mg/kg) displayed no significant change in HR or BP (Fraker et al., 1990). This same dose had elevated BP and HR by 58% and 43%, respectively in conscious animals. A similar diminished pressure response to cocaine after pentobarbital has also been demonstrated in mice (Wang et al., 1999).

6.6.2 Effect of cocaine and ischaemia/reperfusion

Nandrolone pre-treatment was not found to significantly effect the ΔSP and ΔDP response during ischaemia or reperfusion. The significant effect of ‘treatment’ observed for HR during ischaemia may be due to the high variability of the S+C group (Fig. 6.5). Post-hoc tests were negative making the biological relevance of the significant treatment effect difficult to interpret.

Although it was evident that the dose of cocaine used (total dose=5mg/kg) was sufficient to cause a significant initial pressor response, it may have been too low to produce arrhythmia. Alternatively, a lack of a pro-arrhythmic action may reflect different effects associated with slow cocaine infusion and bolus administration. Bolus doses of cocaine in excess of 1mg/kg have been associated with
VF when combined with coronary occlusion and treadmill exercise in the dog (Billman, 1993). Bolus doses greater than 3mg/kg, i.v. have been associated with ventricular conduction slowing in the dog (Kabas et al., 1990). Bolus iv doses of 2mg/kg produced moderately severe arrhythmia in anaesthetised dogs (Mehta et al., 2003). However, a slow cocaine infusion of 0.11mg/kg/min (total dose, 20 mg/kg) in anaesthetised dogs with normal hearts had no pro-arrhythmic effects but was found to raise the threshold for VF (ie. reduced ventricular vulnerability to fibrillation) (Tisdale et al., 1996). Accordingly, it is possible that cocaine may display class I anti-arrhythmic properties (Shakalis et al., 1967; Pitts et al., 1989; Crumb et al., 1995) under some circumstances (refer 1.13.2 for review).

Because cocaine administration simultaneous with alcohol ingestion has been shown to produce a highly toxic, hepatic metabolite called cocaethylene (Wilson et al., 1995; McCance-Katz et al., 1998; Pan et al., 1999a; b; Ponsoda et al., 1999; Wilson et al., 2001; Wilson et al., 2002; Laizure et al., 2003), the S+C control group was included in this study to control for possible formation of this metabolite. This was necessary because the vehicle for the nandrolone used in this study was dilute ethanol. Cocaethylene (11.25 & 7.5 mg/kg) has been found to significantly depress the myocardium, decreasing stroke volume, mean arterial pressure and dP/dt in dogs (Wilson et al., 1995).

The combination of cocaine and ethanol itself has been found to considerably increase toxicity. Alcohol drinking in rats has been found to alter the pharmacokinetics of cocaine, increasing bioavailability, elimination half-life and increasing the formation of norcocaine (McCance-Katz et al., 1998; Pan et al., 1999a; b). Human hepatocytes treated with ethanol have been found to be depleted in reduced glutathione (GSH), making them more susceptible to cocaine-induced oxidative damage (Ponsoda et al., 1999). Bolus administration of cocaine and ethanol to mongrel dogs has been found to result in prolonged cardiotoxicity and ventricular arrhythmias (Wilson et al., 2001). Cocaine and ethanol in combination were found to be more toxic than either substance alone.

The pre-ischaemic HR and BP response to cocaine in rats pre-treated with ethanol containing vehicle was not significantly different from the response in rats pre-treated with saline or nandrolone. Ischaemia did not promote any significant difference between the HR or BP response of vehicle treated rats administered cocaine and rats pre-treated with saline or nandrolone and subsequently infused with cocaine. The extent, duration and frequency of arrhythmia was not significantly different in the S+C group in comparison to 0N+C. It is probable that the very low doses of ethanol used in this study did not result in appreciable cocaethylene formation.

CHAPTER 6 The effect of intravenous cocaine in rats subjected to ischaemia and reperfusion 6-19
6.6.3 *Effect of the nandrolone-cocaine combination on arrhythmia and survival*

No significant increase was noted in Lambeth score during ischaemia for any pre-treatment, neither were any significant differences between pre-treatment groups noted for occurrence and duration of VT, VF or VT+VF. Survival time during ischaemia was unaffected by pre-treatment, but the fraction of rats which obeyed the Lambeth convention and survived ischaemia was significantly less in the 40N+S group compared to the 0N+S and 40N+C groups (Table 6.1 Left column). This implies that rats in the 40N+S treatment group died towards the end of the ischaemic period and therefore did not significantly affect average group survival time. Importantly, the enhanced mortality from *fatal* VF in ischaemia due to nandrolone pre-treatment was not present in those rats also pretreated with cocaine. However, although cocaine significantly protected against *fatal* VF in ischaemia this was not reflected in statistically significant reductions in Lambeth score or in the occurrence or duration of VT or VF by the combined treatment. To determine whether rats dying during ischaemia skewed the arrhythmia duration results, the duration of arrhythmia was determined only in rats surviving ischaemia (Fig. 6.5). However, the only significant difference observed was a significant decrease (p<0.05) in the duration of VF in the 0N+S group compared to the 40N+S group. The 40N+C combination was not found to confer a statistically significant decrease in VF duration compared to nandrolone alone. An intermediate dose of nandrolone was selected in these experiments in order to test for potentiation of arrhythmia with combined treatment. Since cocaine appeared instead to be protective against *fatal* VF, the choice of a higher dose of nandrolone such as 160μg/kg/min, which produced more significant increases in the occurrence and duration of VF in ischaemia (chapter 5), may have provided a more sensitive test for the combined effect of cocaine on arrhythmia scores.

Cocaine’s apparent protective effect against *fatal* VF in nandrolone treated rats may possibly be explained by two previously described mechanisms:

1. **Anti-arrhythmic effect.** Although cocaine’s inhibitory effect on cardiac Na+ and K+ channels contributes to its pro-arrhythmic effects (Billman, 1990; Kimura et al., 1992), it is possible that anti-arrhythmic effects may occur under some circumstances, as discussed above (6.6.2). Like Tisdale et al., the current experiments used iv infusion as opposed to iv bolus (Tisdale et al., 1996). However, if a membrane stabilising effect underlies the protective effect of cocaine in these experiments, it was only apparent in the presence of nandrolone. It was difficult to investigate antiarrhythmic activity in the current study as the basic levels of arrhythmia were low. Future experiments should use a higher concentration of nandrolone in order to provide a higher basal level of arrhythmia.
2. Blockade of NA release by the neuronal monoamine pump. This important pump normally transports NA into the nerve ending and is potently inhibited by cocaine (Iversen, 1963; Iversen et al., 1969). Although still controversial (Daugherty et al., 1986) there is evidence that elevated intracardiac catecholamines contribute to arrhythmogenesis in ischaemia. Intracardiac catecholamine concentrations are known to be elevated in ischaemia (Schomig et al., 1984; Schomig et al., 1987; Schomig, 1990). Initially in vivo this occurs by enhanced exocytotic release due to increased sympathetic nervous system activation (Dart et al., 1983; Dart et al., 1987). However as ischaemia progresses, due to accumulation of intracellular Na+ there is reverse transport of NA by the neuronal catecholamine uptake transporter, which is dependent on the transmembrane Na+ gradient (Schomig, 1990; Dart et al., 1993; Du et al., 1998). This carrier mediated efflux has been shown to be blocked in rats with LAD occlusion (Du et al., 1998) and in isolated perfused hearts (Schomig et al., 1984) by inhibitors of the neuronal uptake transporter such as cocaine, desipramine, nisoxetine and (+)-oxaprotiline (Schomig et al., 1984; Schomig et al., 1988; Du et al., 1998). In this setting uptake-1 inhibitors such as cocaine are protective against arrhythmia formation. When the neuronal uptake pump results in reverse transport during ischaemia, the only mechanism available for local clearance of NA in the heart is extraneuronal uptake and metabolism (Schomig, 1990).

Inhibition of extraneuronal uptake is one non-genomic property of a number of steroid hormones including testosterone (Iversen et al., 1970). If nandrolone were able to inhibit extraneuronal uptake, this may not be important in the normoxic heart but could result in enhanced extracellular concentrations during ischaemia. Cocaine would be expected to reduce these extracellular concentrations. In the present experiments the protective effect of cocaine alone on ischaemia-induced arrhythmia as described by Du et al. was not reproduced in vivo. However, the extent of this protection may depend on a number of factors including cocaine concentration and duration of exposure. Furthermore, one would expect this particular effect of cocaine to be more pronounced where non-neuronal metabolism of NA is blocked.

The effects of combined nandrolone and cocaine, in the setting of ischaemia, contrast with the combined effects previously demonstrated in the absence of cardiac ischaemia, in which nandrolone pre-treatment enhanced the positive chronotropic effect of cocaine. In the latter case, enhancement of HR response only occurred after chronic treatment with nandrolone. A single subcutaneous dose of nandrolone had no effect; although it could not be excluded that acute nandrolone at higher doses might have influenced the positive chronotropic effect of cocaine since a single subcutaneous dose of cocaine did not achieve the same plasma levels as those achieved after chronic dosing or after iv infusion. In the present experiments, although high plasma levels of nandrolone were achieved, the presence of anaesthesia limited the assessment to an interaction with the decreased HR response to cocaine.
In light of the more prominent effects of chronic nandrolone in the absence of cardiac ischaemia, the effects of chronic treatment with nandrolone in the ischaemia/reperfusion model, both in the presence and absence of cocaine, were investigated and are described in the next chapter.

6.6.4 Effect of pre-treatment on reperfusion arrhythmias

In rats obeying the Lambeth convention, reperfusion produced no significant dysrhythmia. Consequently, survival was 100% for all rats. This is similar to results from chapter 5 where arrhythmia activity was low during reperfusion, presumably due to the longer period of ischaemia (McLennan et al., 1988).
6.7 Conclusion

- In the anaesthetised rat, iv cocaine infusion at 0.5mg/kg/min (5mg/kg total dose) caused a sustained fall in HR and a biphasic change in BP, consisting of an initial transient increase, followed by a sustained decrease in BP. These haemodynamic effects of cocaine were unaffected by nandrolone pre-treatment.

- Intravenous cocaine infusion at 0.5mg/kg/min was not pro-arrhythmia in rats with normal coronary flow, nor did it enhance the incidence of arrhythmia in rats subjected to cardiac ischaemia.

- Intravenous cocaine infusion significantly reversed the increased mortality from fatal VF during cardiac ischaemia which was induced by iv nandrolone pre-treatment. However, this protective effect of cocaine was not reflected in a statistically significant reduction in arrhythmia score nor the occurrence and duration of VF or VT during ischaemia.

- No effects of pre-treatment were detected for mortality or arrhythmia in reperfusion.
6.8 References


CHAPTER 6 The effect of intravenous cocaine in rats subjected to ischaemia and reperfusion 6-25


CHAPTER 6 The effect of intravenous cocaine in rats subjected to ischaemia and reperfusion

6-26
Chapter 7

Effects of chronic nandrolone administration

7.1 Introduction

Although an increase in arrhythmia has been observed during ischaemia with infused nandrolone (chapter 5), this is not the usual form of drug delivery. Recreational AS users most often administer steroids chronically by the intramuscular or subcutaneous route. In this chapter the effects of chronic treatment were examined. Plasma levels of nandrolone following chronic treatment were measured so that a comparison could be made to the level obtained with intravenous treatment.

The same chronic dosing regimen (nandrolone decanoate 20mg/kg, s.c./3xs weekly) which was previously used over 3 weeks of treatment (Phillis et al., 2000) was used again for up to 9 weeks. This dose of nandrolone has been shown to increase the rate of elevation in BP of developing, spontaneously hypertensive rats and to produce myocardial inflammatory and fibrotic changes (Tseng et al., 1994). A nandrolone dose of 20mg/kg is approximately 60 times the therapeutic dose (Johansson et al., 1997), and within the range of doses used illicitly by athletes (Yu-Yahiro et al., 1989).

A number of other potentially toxic effects of chronic steroid treatment were also investigated. In addition to arrhythmia, chronic steroid treatment has been associated with hypertension (for review see 1.6.3) (Hall et al., 1982; Tseng et al., 1994), cardiac hypertrophy (see 1.6.2 ‘cardiac remodelling’) (Kennedy, 1993; Huie, 1994), hyperlipidemia (see 1.6.1 ‘arteriosclerosis’) (Leeds et al., 1986; Glazer, 1991) and cellular damage to the liver (Ishak et al., 1987; Gragerta et al., 1993; Boada et al., 1999) and myocardium (see 1.6.2 ‘myocardial cell injury’) (Tseng et al., 1994; Trifunovic et al., 1995; Norton et al., 2000).
7.2 Aims

1. To determine the cardiovascular effects of chronic nandrolone treatment in pentobarbital anaesthetised, close-chested rats, particularly during cardiac ischaemia and reperfusion.

2. To determine if this chronic regimen of nandrolone treatment results in changes in cholesterol, blood chemistry and pathology.

7.3 Hypothesis

Chronic nandrolone treatment will cause an increase in the degree of arrhythmia during ischaemia, which will increase in severity according to the length of nandrolone pre-treatment.
7.4 Methodology

7.4.1 Animals

Male Sprague Dawley rats were purchased at 9-10 weeks old, 2 weeks before the commencement of treatment. Rats were housed in groups of 4-6 in wire mesh cages. One hundred and eight animals were randomly assigned to one of 4 treatment groups.

**Group 1, 3N & 3V:** Treated with nandrolone (20mg/kg, s.c., n=14) or vehicle (n=14) for 3 weeks.
**Group 2, 6N & 6V:** Treated with nandrolone (20mg/kg, s.c., n=14) or vehicle (n=14) for 6 weeks.
**Group 3, 9N & 9V:** Treated with nandrolone (20mg/kg, s.c., n=14) or vehicle (n=14) for 9 weeks.
**Group 3, 9N+C, 9V+C:** Treated with nandrolone (20mg/kg, s.c., n=12) or vehicle (n=12) for 9 weeks and subjected to a 10 minute cocaine infusion (0.5mg/kg/min) immediately prior to induction of ischaemia.

Although only 12 rats were required in each treatment group, 14 were treated in order to account for animals, which may be lost due to surgical preparation for ischaemia. Rats were dosed according to a staggered treatment schedule, such that a maximum of 8 rats were ready for ischaemia-reperfusion on any one-day.

A separate group of 16 rats were treated in order to provide data on plasma nandrolone levels at 3 and 9 weeks of treatment. These rats were randomly assigned to either of 4 groups.

**Group A.** Treated with nandrolone (20mg/kg, s.c., n=4) for 3 weeks.
**Group B.** Treated with vehicle for 3 weeks (n=4).
**Group C.** Treated with nandrolone (20mg/kg, s.c., n=4) for 9 weeks.
**Group D.** Treated with vehicle for 9 weeks (n=4).

7.4.2 Protocol

Rats were fasted for 12 hours prior to use. Anaesthesia and cannulation were conducted as per “general methods”. Blood pressure was recorded for 5 minutes prior to the chest being opened, and for 5 minutes following completion of preparative surgery and the equilibration period.

Rats in groups 1-3 were maintained under ischaemic conditions for 15 minutes after which reperfusion was initiated for a further 10 minutes. Rats in group 4 were challenged with a 10-minute cocaine infusion (0.5mg/kg/min) prior to initiation of ischaemia (Fig. 7.1).
At the conclusion of the protocol blood was collected into heparinized eppendorff tubes for Na⁺ and K⁺ assay, to determine whether potential changes in cardiac rhythm were due to alterations in plasma electrolytes. Approximately 1ml of blood was collected into EDTA tubes for analysis of cholesterol. The heart was excised into ice-cold saline, re-occluded and perfused with Evan's blue dye (refer 2.8). The liver, kidneys, adrenal gland, spleens and testes were removed and weighed to determine the potential toxicological effect of chronic nandrolone pre-treatment. The heart and kidneys from 4 rats treated with nandrolone for determination of steroid plasma levels and hearts from four controls were sent to Veterinary Pathology at the Institute of Medical and Veterinary Science (IMVS), Royal Adelaide Hospital, South Australia for preparation of sections for haematoxin and cosin staining and light microscopy. The sections were assessed by an independent veterinary pathologist, blinded to the treatments.

Blood samples were stored at -20° until required. Serum lipoprotein was determined using a COBAS-Bio Centrifugal Analyser (Roche Diagnostics, USA). Total plasma cholesterol (TC) and triglyceride (TRIG) concentration were determined using commercial kits (Roche Products (UNIMATE 7 (07 3664 3) and UNIMATE 5 (07 3679 1)) while high density lipoprotein (HDL) concentrations were determined using the polyethylene glycol (PEG) method. Low-density lipoprotein (LDL) levels were calculated using the Friedewald equation \( \left( \text{Equation 7.1} \right) \)

\[ \text{LDL} = \text{TC} - \left( \text{HDL} + (\text{TRIG} \times 0.45) \right) \]

\( \text{Equation 7.1 Friedewald equation for determining low density lipoprotein.} \) LDL=low density lipoprotein, TC=total plasma cholesterol, HDL=high density lipoprotein, TRIG=triglyceride.

Assays for TC, TRIG and HDL were performed in duplicate for the plasma from each rat. Results were discarded if the duplicate values varied by more than 10%.

Plasma from rats treated for 3 & 9 weeks \((n=3)\) and their respective vehicle controls \((n=3)\) were analysed for blood chemistry, total bilirubin and protein, liver function and lactate dehydrogenase (LD) at the Institute of Medical and Veterinary Science (Queen Elizabeth Hospital, Woodville South, SA).

7.4.3 Data Analysis
Changes in body weight over the days of treatment were graphed as a function of time \(\text{(mean±SE)}\) and analysed using repeated-measures-two-factor-ANOVA with Bonferroni's post hoc test. Terminal tissue weights, serum lipoprotein levels and plasma nandrolone concentrations were analysed using single-factor-ANOVA and Bonferroni's post-hoc test between selected pairs. To
determine the cardiovascular effect of chronic nandrolone treatment, the SP, DP and HR was
determined at 2 points (200s apart) during the 5 minute recording period. An average of these
values was determined and a group average calculated for each treatment. These values were then
analysed using single-factor-ANOVA and Bonferroni’s post-hoc test between selected pairs. The
effect of cocaine infusion in nandrolone and vehicle treated rats on cardiovascular parameters was
expressed as change (Δ) in SP, DP and HR from 40s prior to the start of infusion. Differences
between groups were then analysed using a repeat-measures-2-factor-ANOVA. In previous
chapters cardiovascular variables were reported as AUC to maintain consistency with the author’s
previously published work (refer 2.5 ‘Analysis of Radiotelemetry Data’). However, ANOVA is
more widely used as a statistical analysis method for this type of data. Results obtained with
ischaemia and reperfusion were presented as per General Methods 2.9.
a. Groups 1, 2, 3

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**Ischaemia**  
**Reperfusion**

1. BP recording in closed chested animal.
2. Open chest and place ligature around LAD coronary artery ready for induction of ischaemia.
3. Equilibration period.
4. Ischaemia.
5. Reperfusion.
6. Collect blood & excise heart.

b. Group 4 only

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**Cocaine infusion**  
**Ischaemia**  
**Reperfusion**

1. Close-chest BP & HR recording
2. Cocaine infusion (0.5mg/kg/min, i.v.)
3. Open chest and place ligature around LAD coronary artery ready for induction of ischaemia
4. Equilibration Period
5. Ischaemia
6. Reperfusion
7. Collect blood & excise heart

**Fig. 7.1 Ischaemia-reperfusion protocol.** Group 4 is subjected to a cocaine infusion (0.5mg/kg/min) for 10 minutes prior to commencement of ischaemia.

**CHAPTER 7 Effects of chronic nandrolone administration** 7-6
7.5 Results

7.5.1 Change in body weight

Body weights were recorded for the days prior to sacrifice (Fig. 7.2). The final day of treatment was not included in this data because rats were fasted prior to sacrifice. Treatment with nandrolone for 12 days was not found to significantly vary body weight. However, treatment for 33 days was found to significantly vary body weight (p<0.05). Vehicle treated rats were found to have a significantly greater body weight at days 22-26 (all, p<0.05), days 29-31 (all, p<0.01) and day 33 (p<0.001) compared to nandrolone treated animals (Fig. 7.2 B). Likewise, vehicle treated rats were found to have a significantly greater (p<0.001) body weight in comparison to nandrolone at day 24 (p<0.05), days 26-29 (p<0.01) and at all other time points thereafter (p<0.001) in rats treated for 54 days (Fig. 7.2 C). ‘Time’ was found to have a significant effect on body weight in rats treated for 12 days (p<0.0001), 33 days (p<0.0001) and 54 days (p<0.0001). An interaction between ‘time’ and ‘treatment’ was noted in rats treated for 12 days (p<0.0001), 33 (p<0.0001) and 54 days (p<0.0001). In rats treated for 33 days this interaction became significant at day 22 (p<0.05) and was evident at days 24-26 (p<0.05), days 29-31 (all, p<0.01) and day 33 (p<0.001). Similarly, in rats treated for 54 days the interaction became statistically significant at day 24 (p<0.05) and continued to be significant at all points thereafter (days 26-29, p<0.01; days 31-54, p<0.001).

7.5.2 Change in organ weights

Because of a large difference between body weights of nandrolone and vehicle treated rats, organ weights are presented as ‘average terminal tissue weight/100g terminal body weight’ (Table 7.1). Relative kidney weights were found to change significantly (p<0.0001) over the treatment period such that kidneys from nandrolone treated rats at 3, 6 or 9 weeks of nandrolone administration were found to be significantly larger than the respective control (all, p<0.001). Significant changes were also observed in testicular weight over the duration of the treatment period (p<0.0001). The relative weight of the testes was found to be significantly greater in nandrolone treated rats at 9 weeks in comparison to the corresponding vehicle control (p<0.01). Significant changes were also observed in the relative weight of the adrenals (p<0.0001). Significantly larger relative adrenal weights were noted for rats administered nandrolone for 6 or 9 weeks compared to control (both, p<0.001). Nandrolone treatment was not found to change spleen weight, but relative liver weights were significantly changed (p<0.01). The relative weight of the liver was significantly less (p<0.05) in rats treated for 3 weeks with nandrolone compared to control. Heart weight normalised for body weight was found to be significantly changed by nandrolone treatment (p<0.0001), such that the
relative heart weight was significantly greater in nandrolone treated rats at 6 and 9 weeks (both, p<0.0001).

7.5.3 Histopathology
H & E staining and light microscopy revealed no significant difference in histology of hearts and kidneys from rats treated for 9 weeks with nandrolone and corresponding vehicle controls.

7.5.4 Cholesterol, triglycerides, LDL and HDL profiles.
Total cholesterol (TC) was found to be lower in all nandrolone groups in comparison to the vehicle control (Table 7.2) but was only found to be statistically significantly different from vehicle at 3 weeks of treatment (p<0.01). Total cholesterol in nandrolone treated rats was found to increase significantly at week 9 compared to week 3 of nandrolone treatment (p<0.001). Although triglyceride levels were consistently lower in nandrolone treated rats compared to control, statistically relevant differences were not detected. High density lipoprotein (HDL) was only decreased in rats treated for 3 weeks with nandrolone in comparison to vehicle (p<0.05). High density lipoprotein was found to be significantly elevated at 9 weeks of nandrolone treatment in comparison to 3 weeks steroid treated rats (p<0.001). No statistically significant differences were detected between treatment groups in LDL or the HDL: LDL ratio, neither were any differences within groups observed as treatment progressed.

7.5.5 Serology
It was found that glucose [t(6)=6.070, p<0.001] and bicarbonate [t(6)=2.449, p<0.05] levels were elevated at 3 and 9 weeks of nandrolone treatment, respectively compared to the corresponding vehicle treated controls (Table 7.3). Sodium [t(6)=3.576, p<0.05], phosphate [t(6)=4.642, p<0.01], alkaline phosphatase (ALP) [t(6)=2.666, p<0.05], aspartate aminotransferase (AST) [t(6)=2.741, p<0.05] and lactate dehydrogenase (LD) [t(6)=2.588, p<0.05] were all found to have significantly lower values in rats treated for 3 weeks with nandrolone in comparison to animals administered vehicle for the same period of time. Significantly lower values at 3 and 9 weeks of treatment were observed for creatinine [t(6)=2.976, p<0.05 and t(6)=2.778, p<0.05, respectively], total protein [t(6)=6.128, p<0.001 and t(6)=3.595, p<0.05, respectively], and albumin [t(6)=2.782, p<0.05 and t(6)=3.800, p<0.01, respectively] compared to control. No statistically significant change between groups was observed for potassium, chloride, urea, calcium, total bilirubin, glutamyltransferase (GGT) and alanine aminotransferase (ALT).

7.5.6 Plasma level of nandrolone
Nandrolone was not detectable in controls.
Plasma nandrolone concentration was found to increase at 3 weeks (p<0.01) and 9 weeks (p<0.001) compared to vehicle control (Table 7.4).

7.5.7 Cardiovascular response to chronic nandrolone treatment

Effect of nandrolone on DP and SP
In rats under pentobarbital anaesthesia nandrolone, pre-treatment did not significantly affect either DP or SP (Fig. 7.3).

Effect of nandrolone on HR
Heart rate in nandrolone treated rats was found to vary significantly (p<0.01) in response to chronic nandrolone treatment. However, no significant differences were observed when rats treated for 3, 6 or 9 weeks were compared to their respective controls. When comparing within treatment groups, a significantly lower HR was noted in nandrolone treated rats at 9 weeks of treatment compared to 3 and 6 weeks (p<0.001 and p<0.05 respectively) (Fig. 7.4).

7.5.8 Cardiovascular response to cocaine administration
In 2 separate groups of 12 rats, the effect of cocaine following 9 weeks treatment with nandrolone or vehicle was investigated. The cardiovascular effect was measured as change (Δ) from baseline (40s prior to the start of cocaine infusion). Cocaine (0.5mg/kg/min) administered as a 10-minute infusion to rats prior to arterial occlusion was found to induce an immediate, sharp elevation in SP and DP in rats treated for 9 weeks with nandrolone or vehicle. Change in SP (ΔSP) and DP (ΔDP) is shown in Fig. 7.5, A. B.. Maximal ΔSP in response to cocaine was found to occur at 40s following the start of infusion (Fig. 7.5, A.). Maximal ΔDP occurred at 60s (Fig. 7.5, B.). The ΔSP and ΔDP response to cocaine was not significantly modified by pre-treatment. While both SP and DP displayed an early increase, this was followed by a slow decrease over time. At 600s, following the start of infusion, both DP and SP displayed a decrease in pressure below baseline. Consequently, ‘Time’ alone was found to significantly effect ΔSP and ΔDP (p<0.0001). The time at which maximum SP and DP was obtained in response to cocaine was unaffected by pretreatment. No significant interaction (between ‘treatment’ and ‘time’) was observed for ΔSP and ΔDP (Fig. 7.5, A, B).

Heart rate in nandrolone treated rats administered cocaine increased slowly until 160s, whereas HR in vehicle treated rats reached maximum by 40s and then declined rapidly between 180s and 300s. The change in HR (ΔHR) is shown in Fig. 7.5, C. No effect of treatment on ΔHR was found. However, ‘time’ alone was found to have a significant effect on ΔHR (p<0.0001). A significant interaction (p<0.01) in terms of ΔHR was found between ‘time’ and ‘treatment’, such that the ΔHR
response to cocaine was significantly greater (p<0.01) in nandrolone treated rats compared to vehicle at the final time point of infusion (600s) (Fig. 7. 5, C).

7.5.9 Cardiovascular response to ischaemia and reperfusion

Because SP, DP and HR was still depressed at the end of the cocaine infusion period (eg, vehicle treated rats receiving cocaine ΔSP: -9.5±8.69mmHg, ΔDP: -7.0±7.39mmHg, ΔHR: -33±14.23mmHg) the cardiovascular response to ischaemia and reperfusion in rats receiving cocaine was analysed separately from rats receiving nandrolone or vehicle only.

Ischaemia

Nandrolone or vehicle treatment only

The change (Δ) in SP, DP and HR was calculated from time zero of ischaemia at 300s, 600s and 900s of occlusion (Fig. 7. 6). A significant effect of treatment was only found for ΔHR (p<0.05) (Fig. 7. 6 C). Post-hoc tests (Bonferroni’s) failed to detect any significant differences in HR at any time point during ischaemia. No effect of ‘time’ or any interaction between ‘treatment’ and ‘time’ was found for any of the variables measured.

9 weeks nandrolone or vehicle treatment, plus cocaine

Treatment was found to significantly affect ΔSP (Fig. 7. 7 A.) and ΔDP (Fig. 7. 7 B.) (both, p<0.01). Post-hoc tests did not reveal where this significance lay. Treatment did not significantly affect ΔHR (Fig. 7. 7 C.). Likewise ‘time’ alone was not found to significantly change either the change in BP or the change in HR. No significant interaction was found between ‘treatment’ and ‘time’ for the BP or HR changes.

Reperfusion

Nandrolone or vehicle treatment only

The change in SP, DP and HR was calculated from time zero of reperfusion at the conclusion of the 10 minute reperfusion period (600s). No significant difference was found between treatment groups for the ΔSP (Fig. 7. 6 D.), ΔDP (Fig. 7. 6 E.) or ΔHR (Fig. 7. 6 F.) responses.

9 weeks nandrolone or vehicle treatment, plus cocaine

No significant difference was found between treatment groups for the SP (Fig. 7. 7 D.), DP (Fig. 7. 7 E.) or HR (Fig. 7. 7 F.) response during reperfusion.

7.5.10 Zone at risk of infarction

The area at risk of infarction was consistent between treatment groups (data not shown). No significant differences were found between or within treatment groups for the duration of the study (single-factor ANOVA).
7.5.11 Survival Time

Five out of a total of 98 rats (~5%) died from complications unrelated to VF (8 died from VF). Severity of arrhythmia was not scored in these rats neither were they included in survival calculations. The number of exclusions from each treatment group is noted in parentheses above Fig. 7.8.

No significant difference was found between the survival times of the various treatment groups in ischaemia and reperfusion (Fig. 7.8), neither were significant differences found in the number of rats surviving ischaemia and reperfusion (Table 7.5).

7.5.12 Arrhythmia

Ischaemia

No significant difference was found between the fraction of rats responding with VT, VF or VT+VF during ischaemia (Table 7.7). Likewise, no significant differences were found between the duration of VT (Fig. 7.9.A), VF (Fig. 7.9.B) or VT+VF (Fig. 7.10) during ischaemia, neither were any differences in Lambeth arrhythmia score detected between groups (Table 7.6). To ensure that fatalities during ischaemia did not skew the results, arrhythmia duration was also calculated in rats which survived ischaemia. The duration of VT, VF or VT+VF during ischaemia was not significantly different in rats which survived occlusion (data not shown).

Reperfusion

No significant difference between groups for the fraction of rats responding with arrhythmia was noted during reperfusion (Table 7.7). At no time was the frequency of VT (Fig. 7.9.C), VF (Fig. 7.9.D) or VT+VF (Fig. 7.10), or the arrhythmia score (Table 7.6) statistically different between groups.
Fig. 7.2 Body weight in rats treated with nandrolone (20mg/kg, s.c./3xs weekly) over A. 12 days, B. 33 days or C. 54 days prior to sacrifice. ■ Nandrolone, ○ vehicle. Repeated Measures, 2-factor ANOVA, Bonferroni’s post-hoc test. A. TREATMENT [F(1,130)=0.359, p>0.05], TIME [F(5,130)=65.57, p<0.0001], INTERACTION [F(5,130)=9.947, p<0.0001] (see text for post-hoc results). B. TREATMENT [F(1,364)=6.451, p<0.05] *p<0.05, **p<0.01, ***p<0.001 significantly different from vehicle treated rats, TIME [F(14,364)=58.89, p<0.0001], INTERACTION [F(14,364)=84.38, p<0.0001] (see text for post-hoc results). C. TREATMENT [F(1,598)=14.10, p<0.0001] *p<0.05, **p<0.01, ***p<0.001 significantly different from vehicle treated rats, TIME [F(23,598)=50.8, p<0.0001], INTERACTION [F(23,598)=107.2, p<0.0001] (see text for post-hoc results). n=13-14, Mean±S.E.
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<td>(0.32±0.00)</td>
<td>(0.53±0.01) ***</td>
<td>(0.31±0.01)</td>
</tr>
<tr>
<td><strong>Testes</strong></td>
<td>1.58±0.02*</td>
<td>1.40±0.08</td>
<td>1.46±0.02*</td>
<td>1.64±0.02***</td>
<td>1.48±0.02</td>
<td>1.64±0.03***</td>
</tr>
<tr>
<td></td>
<td>(0.39±0.01)</td>
<td>(0.35±0.02)</td>
<td>(0.39±0.01)</td>
<td>(0.36±0.00)</td>
<td>(0.39±0.01) **</td>
<td>(0.33±0.01)</td>
</tr>
<tr>
<td><strong>Adrenals</strong></td>
<td>0.033±0.001</td>
<td>0.028±0.001</td>
<td>0.031±0.001</td>
<td>0.027±0.001</td>
<td>0.035±0.002</td>
<td>0.030±0.002</td>
</tr>
<tr>
<td></td>
<td>(0.008±0.0003)</td>
<td>(0.007±0.0002)</td>
<td>(0.008±0.0003)***</td>
<td>(0.006±0.0002)</td>
<td>(0.009±0.0004)***</td>
<td>(0.006±0.0004)***</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>0.53±0.01</td>
<td>0.52±0.04</td>
<td>0.47±0.01</td>
<td>0.55±0.02</td>
<td>0.50±0.02</td>
<td>0.55±0.02</td>
</tr>
<tr>
<td></td>
<td>(0.13±0.003)</td>
<td>(0.128±0.010)</td>
<td>(0.125±0.004)</td>
<td>(0.121±0.004)</td>
<td>(0.13±0.01)</td>
<td>(0.11±0.00)</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>10.50±0.52</td>
<td>12.34±0.77</td>
<td>9.66±0.34***</td>
<td>13.34±0.46</td>
<td>9.65±0.49***</td>
<td>12.96±0.66</td>
</tr>
<tr>
<td></td>
<td>(2.60±0.10) *</td>
<td>(3.09±0.18)</td>
<td>(2.60±0.11)</td>
<td>(2.93±0.10)</td>
<td>(2.52±0.09)</td>
<td>(2.60±0.1)</td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td>1.69±0.05</td>
<td>1.61±0.066</td>
<td>1.65±0.04</td>
<td>1.71±0.034</td>
<td>1.70±0.05</td>
<td>1.79±0.07</td>
</tr>
<tr>
<td></td>
<td>(0.43±0.01)</td>
<td>(0.40±0.01)</td>
<td>(0.44±0.01) ***</td>
<td>(0.37±0.01)</td>
<td>(0.45±0.01) ***</td>
<td>(0.36±0.01)</td>
</tr>
<tr>
<td><strong>Body weight</strong></td>
<td>40±3.9</td>
<td>399±10</td>
<td>373±0.000000000000 ***</td>
<td>456±0.000000000000000000 XXX</td>
<td>381±0.000000000000000000 XXX</td>
<td>498±0.000000000000000000 XXX</td>
</tr>
</tbody>
</table>

**Table 7.1 Terminal tissue weight in grams.** *Paretheses:* terminal tissue weight/100g body weight (normalised). **Note:** body weight refers to the body weight on the day of sacrifice following overnight fasting. Single-factor ANOVA, Bonferroni's post-hoc test (between selected pairs). **Legend:** 3N = 3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. **Kidney (absolute wt.):** [F(5,146)=49.47, p<0.0001], **kidney (normalised):** [F(5,146)=143.8, p<0.0001], **testes (absolute wt.):** [F(5,46)=6.082, p<0.0001], **testes (normalised):** [F(5,46)=6.169, p<0.0001], **adrenals (absolute wt.):** [F(5,139)=3.709, p<0.01], **adrenals (normalised):** [F(5,139)=15.47, p<0.0001], **spleen (absolute wt.):** no significant difference, **spleen (normalised):** no significant difference, **liver (absolute):** [F(5,70)=8.717, p<0.0001], **liver (normalised):** [F(5,70)=3.776, p<0.01], **heart (absolute):** no significant difference, **heart (normalised):** [F(5,67)=11.65, p<0.0001]. *p<0.05, **p<0.01, ***p<0.001 significantly different from respective vehicle control, ****p<0.001 significantly different from 3N, ***p<0.001 significantly different from 6N, XXp<0.05, XXXp<0.01, XXXXp<0.001. Significantly different from 3V Mean±S.E., n=12-14.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total cholesterol (TC) mmol/l</th>
<th>Triglyceride (TRIG) mmol/l</th>
<th>High density lipoprotein (HDL) mmol/l</th>
<th>Low density lipoprotein (LDL) mmol/l</th>
<th>HDL/LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3N*</td>
<td>1.22±0.07 (n=8) **</td>
<td>0.30±0.04 (n=9)</td>
<td>0.75±0.05 (n=8) **</td>
<td>0.34±0.04 (n=8)</td>
<td>2.34±0.25 (n=8)</td>
</tr>
<tr>
<td>3V</td>
<td>1.76±0.12 (n=10)</td>
<td>0.43±0.14 (n=6)</td>
<td>1.09±0.1 (n=6)</td>
<td>0.59±0.08 (n=4)</td>
<td>2.07±0.29 (n=4)</td>
</tr>
<tr>
<td>6N</td>
<td>1.4±0.09 (n=7)</td>
<td>0.31±0.07 (n=4)</td>
<td>1.01±0.07 (n=4)</td>
<td>0.36±0.04 (n=3)</td>
<td>2.83±0.09 (n=3)</td>
</tr>
<tr>
<td>6V</td>
<td>1.77±0.15 (n=6)</td>
<td>0.46±0.12 (n=5)</td>
<td>1.07±0.05 (n=7)</td>
<td>0.42±0.14 (n=4)</td>
<td>3.56±0.98 (n=4)</td>
</tr>
<tr>
<td>9N</td>
<td>1.71±0.06 (n=10) **</td>
<td>0.34±0.05 (n=6)</td>
<td>1.19±0.06 (n=9) **</td>
<td>0.48±0.06 (n=4)</td>
<td>2.59±0.32 (n=4)</td>
</tr>
<tr>
<td>9V</td>
<td>1.80±0.11 (n=9)</td>
<td>0.52±0.14 (n=9)</td>
<td>1.12±0.09 (n=9)</td>
<td>0.45±0.11 (n=6)</td>
<td>3.14±0.52 (n=6)</td>
</tr>
</tbody>
</table>

Table 7.2 Total cholesterol (TC), triglyceride (TRIG), high density lipoprotein (HDL), low density lipoprotein (LDL) in rats treated with nandrolone for 3, 6 or 9 weeks compared to respective vehicle controls. Legend: 3N=3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Single-factor-ANOVA, Bonferroni's post-hoc test between selected pairs. TC: [F(5,44)=5.179, p<0.001], TRIG: no significant difference, HDL: [F(5,37)=5.24, p<0.001], LDL: no significant differences, HDL/LDL: no significant differences. *p<0.05, **p<0.01 significantly different from 3V. ***p<0.01, ****p<0.001 significantly different from 3N. Mean±S.E.
| Glucose (mmol/L) | Sodium (mmol/L) | Potassium (mmol/L) | Bicarbonate (mmol/L) | Chloride (mmol/L) | Creatinine (mmol/L) | Urea (mmol/L) | Calcium (mmol/L) | Phosphate (mmol/L) | Total protein (g/L) | Albumin (g/L) | Total bilirubin (µmol/L) | ALP (U/L) | AST (U/L) | GGT (U/L) | ALT (U/L) | LD (U/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7.8±0.1 | 139±0 | 4.3±0.2 | 17±1 | 106±2 | 0.07±0.002 | 6.9±0.5 | 2.4±0.05 | 2.4±0.26 | *** | 29±1 | 1.0± | 98±8 | 137±13 | 140 | 25±4 | 35±5 |
| 4.6±0.5 | 141±0 | 4.2±0.2 | 14±2 | 105±2 | 0.091±0.005 | 7.6±0.7 | 2.5±0.07 | 3.6±0.04 | 50±2 | 32±1 | 24±0 | 128±7 | 190±15 | 140 | 22±4 | 69±5 |
| 8.2±0.3 | 139±1 | 4.2±0.1 | 18±1 | 105±2 | 0.075±0.002 | 7.5±0.3 | 2.5±0.09 | 2.0±0.20 | 53±1 | 27±1 | 12±0 | 91±16 | 25±13 | 140 | 40±6 | 701±17 |
| 9.2±0.3 | 139±1 | 4.1±0.2 | 14±1 | 106±1 | 0.085±0.003 | 6.3±0.5 | 2.6±0.05 | 1.89±0.27 | 58±1 | 32±1 | 24±0 | 137±21 | 216±19 | 130 | 488±5 | 595±39 |

Reference Values (mean±S.D.):
- Glucose (mmol/L): 7.8±0.1
- Sodium (mmol/L): 139±0
- Potassium (mmol/L): 4.3±0.2
- Bicarbonate (mmol/L): 17±1
- Chloride (mmol/L): 106±2
- Creatinine (mmol/L): 0.07±0.002
- Urea (mmol/L): 6.9±0.5
- Calcium (mmol/L): 2.4±0.05
- Phosphate (mmol/L): 2.4±0.26
- Total protein (g/L): 50±2
- Albumin (g/L): 29±1
- Total bilirubin (µmol/L): 1.0±
- ALP (U/L): 98±8
- AST (U/L): 137±13
- GGT (U/L): 140
- ALT (U/L): 25±4
- LD (U/L): 35±5

**Table 7.3 Serology for rats treated with 3 or 9 weeks with nandrolone or vehicle, and literature values.**

**Legend:** 3N=3 weeks nandrolone treatment, 3V=3 weeks vehicle treatment, 6N=6 weeks nandrolone treatment, 6V=6 weeks vehicle treatment, 9N=9 weeks nandrolone treatment, 9V=9 weeks vehicle treatment, 2N+C=9 weeks nandrolone treatment + cocaine, 9V+C=9 weeks vehicle treatment + cocaine. Mean±SE, n=4. ALP: alkaline phosphatase, AST: aspartate aminotransferase, GGT: gamma glutamyltransferase, ALT: alanine aminotransferase, LD: lactate dehydrogenase.

**Effect of nandrolone treatment on serology:** unpaired t-test *p<0.05, **p<0.01, ***p<0.001 significantly different from 3 wks vehicle. †p<0.05, ‡p<0.01 significantly different from 9 wks vehicle. **Effect of treatment duration on serology:** unpaired t-test *p<0.05, ††p<0.01, ‡‡p<0.001 significantly different from 3 wks vehicle. **p<0.01, ***p<0.001 significantly different from 3 wks nandrolone. Note: The age of rats has been noted in parentheses for literature serology values (only where the age of rats has been published).
<table>
<thead>
<tr>
<th>Nandrolone Treatment</th>
<th>n</th>
<th>Plasma nandrolone concentration (nM)</th>
<th>Plasma nandrolone concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3N</td>
<td>3</td>
<td>90±18**</td>
<td>25±5**</td>
</tr>
<tr>
<td>3V</td>
<td>3</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>9N</td>
<td>3</td>
<td>172±23*** #</td>
<td>47±6*** #</td>
</tr>
<tr>
<td>9V</td>
<td>3</td>
<td>0±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

Table 7.4 Plasma nandrolone concentrations. Gas chromatography - mass spectrometry method.

Legend: 3N = 3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. One-way-ANOVA, Bonferroni’s post-hoc test between selected pairs [F(3,8)=33.82, p<0.0001], **p<0.01, ***p<0.001 significantly different from respective vehicle control. ##p<0.05, significantly different from 3N, Mean±S.E., n=3.

Fig. 7.3 SP (open bars) and DP (filled bars) in pentobarbital anaesthetised rats at 3, 6 & 9 weeks of nandrolone (20mg/kg, s.c.) or vehicle administration. Legend: 3N = 3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Single-factor ANOVA, no significant differences. Mean±S.E., n=12-14.
Fig. 7. HR in pentobarbital anaesthetised rats at 3, 6 & 9 weeks of nandrolone (20mg/kg, s.c.) or vehicle administration. Legend: 3N = 3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Single-factor ANOVA, Bonferroni’s post hoc test between selected pairs. [F(5,128)=4.022, p<0.01], *p<0.05, ***p<0.001 significantly different from 9 weeks nandrolone, Mean±SE, n=12-14.
Fig. 7 5 A. SP, B. DP and C. HR response to a 10-minute cocaine (0.5mg/kg/min) infusion in rats treated for 9 weeks with nandrolone (■) (20mg/kg, s.c./3 X weekly) or vehicle (▲).

**Between group differences:** Repeat-measures-2-factor-ANOVA, A. SP: TREATMENT [F(1,242)=1.767, p>0.05], TIME [F(11,242)=38.92, p<0.0001], INTERACTION [F(11,242)=1.819, p>0.05], B. DP: TREATMENT [F(1,242)=1.633, p>0.05], TIME [F(11,242)=41.40, p<0.0001], INTERACTION [F(11,242)=0.685, p>0.05], C. HR: TREATMENT [F(1,132)=3.178, p>0.05], TIME [F(11,132)=10.85, p<0.0001], INTERACTION [F(11,132)=2.426, p<0.01] (see text for post-hoc results). Mean±SE, n=11-12.
Fig. 7.6 Left: Change in A. SP, B. DP and C. HR during cardiac ischaemia in rats treated for 3, 6 or 9 weeks with nandrolone or vehicle. Legends: 3N (●) = 3 weeks nandrolone treatment, 6N (★) = 6 weeks nandrolone treatment, 9N (△) = 9 weeks nandrolone treatment, 3V (□) = 3 weeks vehicle treatment, 6V (□) = 6 weeks vehicle treatment, 9V (△) = 9 weeks vehicle treatment. Two-factor-ANOVA, Bonferroni’s post-hoc test. SP: TREATMENT [F(5,181)=2.086, p>0.05], TIME [F(2,181)=0.647, p>0.05], INTERACTION [F(10,181)=0.09, p>0.05]. DP: TREATMENT [F(5,181)=2.211, p>0.05], TIME [F(2,181)=0.535, p>0.05], INTERACTION [F(10,181)=0.108, p>0.05]. HR: TREATMENT [F(5,160)=2.626, p<0.05], TIME [F(2,160)=0.115, p>0.05], INTERACTION [F(10,160)=0.373, p>0.05]. Mean±S.E., n=9-12. Right: Change in D. SP, E. DP, F. HR from the beginning of reperfusion in rats treated for 3, 6 or 9 weeks with nandrolone. Single-factor-ANOVA. SP: no significant difference, DP: no significant difference, HR: no significant difference. Mean±S.E., n=9-12.
Fig. 7.7 Left: Change in A. SP, B. DP & C. HR during cardiac ischaemia in rats treated for 9 weeks with nandrolone or vehicle plus a 10 minute cocaine infusion (0.5mg/kg/min) (ie. 9N+C & 9N+V). Two-factor-ANOVA, Bonferroni’s post-hoc test SP: TREATMENT [F(1,63)=9.892, p<0.01], TIME [F(2,63)=0.313, p>0.05], INTERACTION [F(2,63)=0.183, p>0.05]. DP: TREATMENT [F(1,63)=9.296, p<0.01]. TIME [F(2,63)=0.515, p>0.05], INTERACTION [F(2,63)=0.237, p>0.05] HR: TREATMENT [F(1,39)=3.252, p>0.05], TIME [F(2,39)=0.2709, p>0.05], INTERACTION [F(2,39)=0.4223, p>0.05]. Mean±S.E., n=10-12. Right: Change in D. SP, E. DP, F. HR from the beginning of reperfusion in rats treated for 9 weeks with nandrolone or vehicle plus a 10 minute cocaine infusion (0.5mg/kg/min). Single-factor-ANOVA. SP: no significant difference, DP: no significant difference, HR: no significant difference. Mean±SE, n=9-12.
Fig. 7. Survival time during ischaemia (I) and reperfusion (R) in rats treated with nandrolone (20mg/kg, s.c./3xs weekly) or vehicle and in rats treated with cocaine (0.5mg/kg/min). Parentheses: total number of rats in each treatment group with death not complying with the Lambeth convention. Legend: 3N=3 week nandrolone treatment, 3V = 3 week vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Single-factor ANOVA, no significant differences. Mean±SEM, n=9-13.
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Ischaemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>3N</td>
<td>11/13</td>
<td>11/11</td>
</tr>
<tr>
<td>3V</td>
<td>9/10</td>
<td>9/9</td>
</tr>
<tr>
<td>6N</td>
<td>10/12</td>
<td>10/10</td>
</tr>
<tr>
<td>6V</td>
<td>10/11</td>
<td>10/10</td>
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<tr>
<td>9N</td>
<td>11/12</td>
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<tr>
<td>9V</td>
<td>12/12</td>
<td>12/12</td>
</tr>
<tr>
<td>9N+C</td>
<td>10/11</td>
<td>10/10</td>
</tr>
<tr>
<td>9V+C</td>
<td>12/12</td>
<td>12/12</td>
</tr>
</tbody>
</table>

Table 7.5 Number of rats surviving as a fraction of rats alive at the beginning of ischaemia.

Legend: 3N = 3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Chi-squared test, no significant differences. Mean±SEM, n=9-13.

<table>
<thead>
<tr>
<th>I</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3N</td>
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</tr>
<tr>
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<td>2±1</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>9V+C</td>
<td>2±0</td>
</tr>
</tbody>
</table>

Table 7.6 Lambeth convention arrhythmia score in rats treated with nandrolone (20mg/kg, s.c. /3xs weekly) or vehicle and in rats treated with cocaine (0.5mg/kg/min) prior to ischaemia (I) and reperfusion (R). Legend: 3N = 3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Mean±SEM, n=9-14. Kruskal-Wallis test, no significant differences.
<table>
<thead>
<tr>
<th>Time</th>
<th>N</th>
<th>V</th>
<th>N</th>
<th>V</th>
<th>N</th>
<th>V</th>
<th>N</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 wks</td>
<td>10/14</td>
<td>11/12</td>
<td>4/11</td>
<td>5/9</td>
<td>7/14</td>
<td>7/12</td>
<td>0/11</td>
<td>3/9</td>
</tr>
<tr>
<td></td>
<td>(71.4)</td>
<td>(91.7)</td>
<td>(36.4)</td>
<td>(55.5)</td>
<td>(50.0)</td>
<td>(58.3)</td>
<td>(0.0)</td>
<td>(33.3)</td>
</tr>
<tr>
<td>6 wks</td>
<td>10/12</td>
<td>7/12</td>
<td>4/10</td>
<td>4/10</td>
<td>5/12</td>
<td>7/12</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>(83.3)</td>
<td>(50.0)</td>
<td>(40.0)</td>
<td>(40.0)</td>
<td>(41.7)</td>
<td>(58.3)</td>
<td>(20.0)</td>
<td>(0.0)</td>
</tr>
<tr>
<td>9 wks</td>
<td>6/12</td>
<td>7/12</td>
<td>4/11</td>
<td>7/12</td>
<td>4/12</td>
<td>4/12</td>
<td>1/11</td>
<td>2/12</td>
</tr>
<tr>
<td></td>
<td>(50.0)</td>
<td>(58.3)</td>
<td>(36.4)</td>
<td>(58.3)</td>
<td>(33.3)</td>
<td>(33.3)</td>
<td>(9.1)</td>
<td>(16.7)</td>
</tr>
<tr>
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<td>6/12</td>
<td>6/12</td>
<td>3/10</td>
<td>4/12</td>
<td>3/12</td>
<td>3/12</td>
<td>0/10</td>
<td>1/12</td>
</tr>
<tr>
<td>cocaine</td>
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<td>(33.3)</td>
<td>(25.0)</td>
<td>(25.0)</td>
<td>(0.0)</td>
<td>(8.3)</td>
</tr>
<tr>
<td></td>
<td>(50.0)</td>
<td>(50.0)</td>
<td>(33.3)</td>
<td>(33.3)</td>
<td>(25.0)</td>
<td>(25.0)</td>
<td>(0.0)</td>
<td>(8.3)</td>
</tr>
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Table 7.7 Fraction of rats responding with VT or VF or VT+VF during 15 minutes ischaemia (I) and 10 minutes reperfusion (R) after 3,6 or 9 weeks of nandrolone treatment or 9 weeks of steroid plus a 10 minute cocaine infusion (0.5mg/kg/min). Parentheses: Percent of rats responding with VT, VF or VT+VF during ischaemia and reperfusion. Legend: N=nandrolone, V=vehicle. Chi-squared test, no significant differences.

CHAPTER 7 Effects of chronic nandrolone administration 7-23
Fig. 7. Average duration of VT in ischaemia (A.) and reperfusion (C.) and duration of VF in ischaemia (B.) and reperfusion (D.) in rats treated with nandrolone (20mg/kg, s.c. 3xs weekly for 3, 6 or 9 weeks), respective control or nandrolone (9 weeks treatment) and cocaine (0.5mg/kg, i.v.). Legend: 3N = 3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Kruskal-Wallis test, no significant differences. Mean±S.E., n=9-13.
Fig. 7.10 Average duration of VT+VF during ischaemia (I) or reperfusion (R) in rats pre-treated with nandrolone (20mg/kg, s.c. /3xs weekly) or vehicle and in rats treated with cocaine (0.5mg/kg/min). Legend: 3N=3 weeks nandrolone treatment, 3V = 3 weeks vehicle treatment, 6N = 6 weeks nandrolone treatment, 6V = 6 weeks vehicle treatment, 9N = 9 weeks nandrolone treatment, 9V = 9 weeks vehicle treatment, 9N+C = 9 weeks nandrolone treatment + cocaine, 9V+C = 9 weeks vehicle treatment + cocaine. Mean±SEM, n=9-14, Kruskal-Wallis test, no significant differences.
7.6 Discussion

7.6.1 Body weight

Nandrolone significantly retarded the increase in body weight observed in vehicle treated controls (Fig. 7.2) when treatment was continued for 6 or 9 weeks. Johansson observed the same effect on body weight in several studies in which a daily nandrolone dose (15mg/kg, s.c.) was found to induce a significant decrease in body weight compared to control after 14 days of treatment (Hallberg et al., 2000; Johansson et al., 2000a; Johansson et al., 2000b). Doses of 2mg/daily and 20mg/bi-weekly have both been found to significantly reduce body weight compared to control (Long et al., 1996; Long et al., 2000).

It appears that the anabolic effect of nandrolone on body weight in rats is related to 3 main factors: gender, the dose and the extent of exercise.

1. **Gender:** Yu-Yahiro et al (1989) administered nandrolone decanoate (~100mg/kg) or 0.5cc of physiological saline to 6-week-old male or female Wistar rats, once weekly for six treatments (Yu-Yahiro et al., 1989). While nandrolone treated female rats gained weight at a similar rate to their saline treated cage-mates, male control rats gained significantly more weight than treated rats (control, 234.4±5.4g versus treated, 144.5±4.0g). Bisschop et al, 1997 delivered a low (1.5mg/kg, i.m., weekly) and a high (7.5mg/kg, i.m., weekly) dose of nandrolone to male and female rats over 5 weeks (Bisschop et al., 1997). While both doses of nandrolone were found to increase body weight from week 1 of treatment in female rats, a marked decrease in weight was noted for male rats receiving the highest dose in comparison to saline controls after 5 weeks of treatment. A number of studies have demonstrated a retarded somatic body weight gain over several weeks of nandrolone treatment compared to control. Trifunovic et al (1995) found a significant suppression of growth rate in male rats treated with nandrolone (5mg/kg, i.m., biweekly) for 8 weeks compared to arachis oil treated controls (Trifunovic et al., 1995). Interestingly, this significantly lower weight gain than control could not be attributed to differences in food intake, gastrointestinal energy absorption or an altered ratio of fat vs. protein metabolism. Tseng et al (1994) also found that nandrolone (20mg/kg, s.c.) decreased average terminal body weight in comparison to vehicle treated controls (Tseng et al., 1994).

Conversely, a small number of publications have reported no significant change in body weight in male nandrolone treated rats. Liang et al (1992) found no change in the body weight of rats administered nandrolone at 3.0mg/kg, i.m., ~7-9 days following a loading
dose of 3.0mg/kg on the first day of treatment (Liang et al., 1992). This is probably related to the use of juvenile rats (4 weeks old) and a lower dose of nandrolone than used in other studies.

2. Dose: A study by Kochakian and colleagues (1959) found that body weight gain was stimulated in male rats by low doses of testosterone and inhibited by large doses (Kochakian et al., 1959). A number of studies using low AS concentrations in non-exercised rats have not observed the same slowing of weight gain that has been observed with larger nandrolone doses. Lewanowitsch and Irvine (2000) failed to observe an appreciable difference in the body weight of male rats administered a daily injection of testosterone (1mg/kg) for 2 weeks (Lewanowitsch et al., 2001). Likewise, Bisschop and co-workers found no change in the body weight of male rats treated weekly for 5 weeks with low dose nandrolone (1.5mg/kg, i.m.), but found a significant decrease in weight, compared to control with high dose treatment (7.5mg/kg, i.m.) (Bisschop et al., 1997). More recently, Johansson and colleagues (2000) found that daily doses of nandrolone decanoate (15mg/kg, s.c) resulted in nandrolone rats being significantly smaller than vehicle treated controls (p<0.0001) after 2 weeks of treatment (Johansson et al., 2000a). Further studies confirmed this observation (Johansson et al., 2000b).

3. Exercise: Woodiwiss et al (2000), found that body weight significantly decreased in male Sprague-Dawley rats administered a biweekly injection of nandrolone decanoate (5mg/kg, i.m.) compared to similar sedentary control treated rats (Woodiwiss et al., 2000). Rats exercised according to a voluntary running schedule, but similarly treated with nandrolone were found to increase in body weight but this increase was not significantly different from drug-free exercised controls.

The present investigation supports those previous studies which have found a decrease in body weight in male rats treated with high concentrations of AS (Long et al., 1996; Bisschop et al., 1997; Johansson et al., 2000a; Johansson et al., 2000b; Long et al., 2000). Trifunovic et al (1995) proposed a number of explanations for the observation of lower body weight observed for male rats with high dose, chronic nandrolone treatment compared to vehicle controls seen in a number of studies (Trifunovic et al., 1995). These hypotheses apply equally to our own observations. As Trifunovic and co-workers found that plasma testosterone concentrations were markedly decreased as a result of exogenous steroid administration it was postulated that suppression of endogenous sex steroids or other growth factors may explain some of the observed decreases in body weight. Changes in
androgen receptor expression or an altered receptor response to exogenous, compared with endogenous androgens, could also possibly explain the observation of decreased somatic growth.

7.6.2 Changes in organ weight

Although many studies chronically administering AS to rats have observed changes in organ weight it is difficult to determine whether these changes are simply related to reduced growth with high dose AS.

1. Changes to heart weight: In the present study it was found that treatment for 6 or more weeks significantly increased the relative wet weight of the heart compared to vehicle treated rats. This is consistent with the cardiac hypertrophy noted in sudden cardiac death attributed to AS abuse (Luke, 1990; Cambell et al., 1993; Kennedy, 1993; Dickerman et al., 1995; Hausmann et al., 1998). Conversely, Trifunovic et al (1995) noted a significant decrease in absolute, total wet heart weight, and absolute left ventricular, wet and dry weight in nandrolone treated rats (5mg/kg, i.m./bi-weekly/8wks) compared to controls (Trifunovic et al., 1995). It was acknowledged that the observed decrease in the absolute weight of the wet and dry left ventricle may be attributable to the significant decrease in body weight induced by high doses of nandrolone. Following an identical treatment regimen to the above study, Norton et al (2000) found a significant decrease in absolute right ventricular mass, but not absolute left ventricular mass (Norton et al., 2000) after 3 months of treatment. Absolute left ventricular mass was unchanged by treatment but was increased significantly (p<0.01) in comparison to control when adjusted for body weight. A significant increase in relative ventricular weight (ventricular/100g body weight) and vertical ventricular diameter was found in spontaneously hypertensive rats treated for 6 weeks with nandrolone (20mg/kg, s.c. daily) compared to control (Tseng et al., 1994). Minkin et al (1993) found no effect of chronic nandrolone treatment for eight weeks on absolute wet heart weight (Minkin et al., 1993). However, they noted that hearts from nandrolone treated rats (especially at the highest dose of 50mg/wk) appeared harder and smaller than those from control animals. The histology results presented in this thesis do not support a direct toxic effect of nandrolone on the heart. This is consistent with work by Bauman who failed to find any indication of a cardiotoxic effect of stanozolol using light microscopy (Bauman et al., 1988).

A more detailed study is required to determine the hypertrophic potential of nandrolone. It is unclear from the results presented in this thesis whether the observed increase in relative heart weight was simply due to an overall decrease in body weight. Absolute heart weight
was not significantly different between pre-treatment groups. Small animal echocardiography may provide a means of determining changes in wall thickness and cardiac mass as a result of nandrolone treatment.

2. **Liver:** Although a significant decrease was observed in the present study in the relative weight of the liver with nandrolone at weeks 3 of treatment – no such difference occurred at 9 weeks. Chronic stanozolol treatment has been shown to significantly reduce relative (Bauman et al., 1988), and absolute (Molano et al., 1999) liver weight in exercised rats. Although we have reported minimal effect of nandrolone on liver weight, Yu-Yahiro and colleagues (1989) found that decreases in liver weight due to nandrolone treatment could be attributed to lower liver lipid content (Yu-Yahiro et al., 1989).

3. **Kidneys:** This thesis reports significant increases in both absolute and relative kidney weights at 3, 6 and 9 weeks of nandrolone treatment in comparison to the respective vehicle controls. Increases in kidney weight have been consistently associated with chronic, high dose nandrolone administration to rats. Tseng et al reported chronic nandrolone treatment, induced increases in absolute and relative kidney weight in sedentary rats (Tseng et al., 1994). Similarly, Yu-Yahiro et al report significant increases in absolute kidney size in rats treated for 6 weeks with nandrolone decanoate (Yu-Yahiro et al., 1989). Minkin et al reported a significant and dose dependant increase in the kidney weight of nandrolone decanoate treated rats (Minkin et al., 1993), although no mechanism to explain this effect was provided. Blantz et al treated ovariectomised female rats with nandrolone decanoate and found a significant increase in absolute kidney weight after 6 and 16 weeks weeks of treatment compared to vehicle treated controls (Blantz et al., 1988). This increase in kidney size at 16 weeks was found to be due to tubular hypertrophy and modest increases in glomerular size. Interestingly, these authors also found that although kidney size was significantly increased with nandrolone treatment no kidney abnormalities were observed with light microscopy. The present study is consistent with this finding. Shukla et al found that testosterone injection into both male and female mice produced elevated levels of glucosyleramid synthase, decreased levels of the hydrolase and increased kidney growth (Shukla et al., 1992). Injection of 17 beta-oestradiol (which has been found to produce decreases in mouse kidney size) had the opposite effect on enzymes controlling the level of tissue glucosyleramide. These authors conclude that testosterone may cause increases in kidney size through the production of kidney glycosphingolipids. It is also possible that the increase in heart and kidney weights observed in nandrolone treated rats are due to an increase in protein turnover, such that decreases in body weight reflect a shift of carcass.
protein to the synthesis of new tissue at organ sites (Tseng et al., 1994). It is likely that the increase in kidney size with nandrolone treatment reported in this thesis cannot be attributed to a single mechanism.

4. **Other organs** The effect of nandrolone on testicular weight in the present study was difficult to evaluate. While absolute weight was significantly increased at week 3 and decreased at week 6 of nandrolone treatment, relative weights were significantly increased in comparison to control at week 9. Nandrolone was found to cause a consistent increase in the relative weight of adrenals which was significant 6 and 9 weeks of treatment in comparison to vehicle control. The toxicological significance of this increase in adrenal weight is also difficult to determine. Schlussman et al (2002) found administration of nandrolone decanoate (15mg/kg X 3 each day) to rats produced significant elevations in circulating levels of adrenal corticotropin hormone (ACTH) and corticosterone after the third day of treatment (Schlussman et al., 2002) resulting from significantly increased POMC synthesis in the anterior pituitary. It is possible that the increase in adrenal size in the present study reflects increased stimulation of the adrenal cortex by ACTH.

Both Tseng et al and Minkin et al observed that chronic nandrolone treatment had no effect on testicular weight (Minkin et al., 1993; Tseng et al., 1994). While Yu-Yahiro report a decrease in testicular weight in treated rats they did not report organ weights relative to body weight (Yu-Yahiro et al., 1989). Consistent with our own results Tseng et al found a significant increase in relative adrenal weight with nandrolone administration, but not in absolute weight.

7.6.3 Changes in cholesterol
TC, TRIG, HDL and LDL all displayed a tendency to be lower in nandrolone treated rats at 3 and 6 weeks of treatment compared to the vehicle control for 3 and 6 weeks of administration. However, a statistically significant decrease in nandrolone treated rats was only observed for TC and HDL at 3 weeks in comparison to control. No significant cholesterol, triglyceride or lipoprotein changes were observed at 6 or 9 weeks in comparison to control.

Overall, it appears that a large dose of nandrolone administered over a significant period of time failed to induce a significant change in serum lipoprotein concentrations in comparison to control values. There is evidence to suggest that the lack of a profound change in the lipoprotein profile of these rats over the treatment period is related to two main factors...
1. **Route of administration.** Oral use of AS produces the classical decline in HDL and elevation in LDL, increasing the risk for cardiovascular disease more than any other known agent (Frisch *et al.*, 1999). Use of injectable AS does not appear to produce such a detrimental outcome. Unfortunately because no AS is available for both oral and parenteral human use it has not been possible to test this hypothesis using the same AS. However, a number of studies have been performed examining the effect of oral and parenteral preparations of different AS.

Thompson *et al* (1989) performed a crossover design study of the effect of oral stanozolol and intramuscular testosterone in 11 weight lifters (Thompson *et al*., 1989). They report that while stanozolol significantly reduced HDL (-33%), increased LDL (+29%) and increased hepatic triglyceride lipase (HTGL) activity by 123%, intramuscular testosterone produced only a modest 9% decrease in HDL, a 16% decline in LDL and a 25% increase in HTGL activity. This effect cannot be attributed to a greater androgenic potential of stanozolol as testosterone was found to more effectively suppress FSH and LH secretion.

2. **Aromatisation of nandrolone to estradiol.** The available data suggests that those AS which are not readily converted to oestrogenic metabolites may significantly depress HDL by increasing the activity of HTGL, compared to compounds such as nandrolone which are readily metabolised to estradiol (Hannan *et al*., 1991; Obasanjo *et al*., 1996). Obasanjo and associates treated Cynomolgus macaques with nandrolone over 2 years and found a surprising elevation in plasma estradiol – thought to be due to conversion of androgen to 17β-estradiol.

Friedl *et al* (1990) compared the ability of testosterone enanthate (oestrogenic activity), 17α-methyltestosterone (little or no oestrogenic activity) and testolactone (aromatase inhibitor) to induce changes in plasma HDL in non-athletic, male subjects (Friedl *et al*., 1990). Testosterone enanthate (280mg/wk, i.m.) produced significant increases in plasma estradiol concentrations, which reached a maximum at 8 weeks of treatment. No significant change in HDL was observed over 12 weeks of testosterone enanthate treatment compared to the other testosterone preparations. Conversely, methyltestosterone and testolactone produced no increase in estradiol over time, but both displayed significantly depressed HDL compared to testosterone enanthate, presumably due to an observed significant increase in HTGL activity over time. Methyltestosterone also significantly elevated plasma LDL. These results suggest that a decrease in plasma HDL is related to the inability of the administered...
AS to be aromatised to estradiol. AS with 17α-substitutions are likely to decrease HDL through increases in HTGL activity.

Bauman et al (1988) reported changes in triglycerides, cholesterol, LDL and HDL in pooled plasma from male rats treated for 5 weeks with stanozolol (5mg, equivalent to ~15mg/kg twice weekly) (Bauman et al., 1988). In steroid treated, exercised rats, receiving a 16.6% w/w protein dietary supplement, levels of TRIG, TC, HDL and the TC/HDL ratio were all found to decrease in comparison to sedentary, untreated, non-dietary supplemented rats. These decreases were conserved in similarly treated rats, which received no dietary supplement. Our own laboratory (Lewanowitsch et al., 2001) found that 2 weeks treatment with testosterone (1mg/kg, s.c) produced a significant decrease in HDL in female rats.

7.6.4 Serological Changes
Comparisons of sodium, potassium and chloride ion levels to literature values for veterinary pathology reported by Bauman et al revealed values which were within the normal range (Bauman et al., 1988).

The significant decrease in total plasma protein demonstrated at 3 and 9 weeks of treatment in comparison to control treated rats is consistent with the observations of Hall & Hungerford (1982). Mononephrectomised, female, Sprague-Dawley rats were administered nandrolone (10mg/kg/day) which was found to significantly decrease plasma protein but leave plasma sodium unchanged after 5 weeks of treatment.

7.6.5 Plasma nandrolone level
To the best of the author's knowledge this is the first study to measure plasma concentrations of nandrolone following chronic subcutaneous treatment in the rat. These results indicate that the nandrolone plasma concentration increased to a maximum of 172±23 nM with a dose of 20mg/kg, s.c. /3 days week to week 9, and that levels of endogenous 19-nortestosterone in vehicle treated rats were below the limits of detection. Nandrolone plasma levels at 9 weeks of treatment were significantly increased compared to 3 weeks, suggesting that 'cycle length' in recreational AS users is a key determinant of plasma AS concentration. The plasma nandrolone concentration following acute intravenous administration of 160μg/kg/min nandrolone (chapter 5) was more than 5 times greater than the highest concentration achieved with chronic administration. As previously discussed there is a dearth of information regarding plasma levels of AS following chronic treatment, and consequently it is difficult to correlate cardiac consequences to plasma concentrations of AS. Abu-Shakra et al only demonstrated apoptic damage of murine-C2-skeletal-
muscle cells at concentrations of >10μM (Abu-Shakra et al., 1997). This may explain the lack of cardiac pathology in the present study where plasma nandrolone concentrations were <0.2μM after chronic treatment.

7.6.6 Effect of treatment on BP and HR

No significant difference was noted in SP, DP or HR in nandrolone treated rats at the end 3, 6 or 9 weeks treatment in comparison to controls in close-chested rats which were pentobarbital-anaesthetised in preparation for ischaemia. Although BP in this study was tested in anaesthetised animals these results are still consistent with earlier pilot studies performed in freely moving, conscious rats chronically instrumented with radiotelemetry devices. In this pilot study, each of 5 rats acted as their own control during administration of nandrolone for 9 weeks using the same treatment protocol as presented in the current study. No effect of nandrolone decanoate was found during or between the start and finish of nandrolone treatment (Appendix C). A lack of effect on pressure was also noted by Trifunovic and colleagues (1995) using tail-cuff plethysmography on rats chronically treated for 3 months with a low dose of nandrolone (5mg/kg, i.m./bi-weekly) (Trifunovic et al., 1995). Likewise, chronic treatment of rabbits for 4, 8 or 12 weeks with nandrolone decanoate (10mg/kg/week, i.m.) failed to increase BP measured directly in the car artery in comparison to control (Ferrer et al., 1994). However, Tseng and associates (1994) found that nandrolone accelerated the development of hypertension in spontaneously hypertensive rats.

These results are also consistent with those of Hall and Hungerford (1982) who found that nandrolone administered according to a daily chronic schedule in young male rats drinking 1% saline did not induce hypertension. Rats similarly treated with testosterone were found to be hypertensive after 5 weeks of administration. As a result it is hypothesised that demethylation of testosterone at C10 (such as in nandrolone) completely destroys any effect on BP. The effect of testosterone in the absence of saline drinking water and in adult male rats has not been investigated.

Consistent with the lack of an effect of nandrolone on BP, no significant difference was found in the SP and DP response to ischaemia and reperfusion in the various treatment groups (Fig. 7.6). The high variability associated with 900s of ischaemia may explain an apparent treatment effect for HR at this point (Bonferroni’s test, negative).

7.6.7 Cardiovascular effect of cocaine infusion

The pressor effect of cocaine was found to be much more pronounced in this chronic study than in chapter 6 and the extent of bradycardia far less severe. This difference is most probably related to
variation in methodology. In chapter 6, BP and HR were measured in open-chested rats. In an attempt to obtain a more accurate measure of these parameters the chest was not opened in chronically treated rats until after the 10 minute cocaine infusion was concluded. The difference in the BP and HR between the chronic and acute pre-treatments is most probably related to whether these parameters were measured in open or close-chested rats. Interestingly, nandrolone pre-treatment was not found to significantly affect the HR response to cocaine when compared to vehicle treated control (Fig. 7.5). This contrasts to the significantly increased HR in conscious, freely moving rats administered nandrolone (20mg/kg, s.c.) for 3 weeks and challenged with i.p. cocaine (45mg/kg) (Phillis et al., 2000). This may be related to the use of anaethetised rats in this present study and the profile of the cocaine response. Whereas i.p. cocaine causes long lasting cardiovascular responses the effect of i.v. cocaine is only of short duration (i.e. in this study BP returned to baseline between 3 and 6 minutes of infusion). Future studies should investigate the effect of i.v. cocaine in conscious animals.

The biological relevance of the combination effect of nandrolone and cocaine on SP and DP during ischaemia is difficult to interpret (Fig. 7.7). Although a ‘treatment’ effect was noted for both SP and DP during ischaemia, the post-hoc tests were all non-significant. Nandrolone pre-treatment appeared to reduce the magnitude of the decreases in SP and DP observed during ischaemia, but not reperfusion. Because chronic nandrolone treatment alone did not cause a significant increase in arrhythmia duration or frequency it does not appear that the ‘protective’ effect of the nandrolone and cocaine combination on BP decreases confers any protection against arrhythmia.

7.6.8 Effect of pre-treatment on the frequency and duration of arrhythmia and on survival

Although treatment with nandrolone had profound effects on body and organ weights – no difference was found in the frequency and duration of arrhythmia. In addition there were no significant effects of nandrolone pre-treatment on the cardiovascular parameters measured consistent with previous results obtained with 3 weeks pre-treatment (Phillis et al., 2000). Furthermore, rats treated with nandrolone + cocaine showed no significant difference from control during ischaemia and reperfusion in terms of the survival measures used and the extent of arrhythmia. These results suggest that nandrolone administered chronically at a dose relevant to an abusive regimen does not significantly alter the degree of arrhythmia produced by ischaemia or reperfusion but that acute intravenous administration at a dose of 40µg/kg/min and above does. We can speculate on 2 possible explanations for the observed effects…
1. **Down regulation of the mechanism responsible for arrhythmia formation.** It is possible that chronic nandrolone treatment caused downregulation of an intermediate factor or receptor involved in arrhythmia formation. Although there is no example in the literature of tolerance to nandrolone or other AS, it would be expected that changes in receptor expression would be induced by nandrolone as evidenced by almost all agonists and antagonists in many different organ systems.

2. **Plasma nandrolone concentrations with chronic treatment were too low.** Significant potentiation of ischaemia-induced arrhythmia was noted in acutely treated rats with intravenous administration of nandrolone (40µg/kg/min) which produced a plasma concentration of 281nM, a lower dose had no effect. Nandrolone treatment for 9 weeks in the present study produced a plasma concentration of 172nM. It is possible that chronic nandrolone treatment did not reach the threshold concentration required for arrhythmia formation.
7.7 Conclusion

Treatment with nandrolone for 3 or 9 weeks produced a significant elevation in the plasma concentration of 19-nor-testosterone. A significant catabolic effect of nandrolone was observed at 6 and 9 weeks of treatment but no BP or arrhythmia effect was observed in the absence of ischaemia. Furthermore, no evidence of significant effects of nandrolone were noted for the number of rats surviving, the survival time, the Lambeth arrhythmia score, or the duration of VT, VF and VT+VF in either ischaemia or reperfusion. The dose related increase in VF, Lambeth score and decrease in survival with intravenous nandrolone treatment described in chapter 5 could not be replicated with chronic subcutaneous administration. This is possibly due to the lower plasma steroid level achieved with chronic treatment or a progressive down-regulation of a receptor or intermediate factor involved in arrhythmia formation with acute treatment.
7.8 References


CHAPTER 7 Effects of chronic nandrolone administration 7-38


CHAPTER 7 Effects of chronic nandrolone administration 7-39

Chapter 8

The effect of nandrolone on extraneuronal [³H]-noradrenaline reuptake in rat heart

8.1 Introduction

8.1.1 Summary

Results to date have demonstrated that infused nandrolone (40-160µg/kg/min) can increase the incidence and duration of arrhythmia in rats subjected to ischaemia (Ch. 5). As there was a maximum of 30 minutes between the beginning of nandrolone infusion and the end of ischaemia, the mechanism of this effect is most likely to be non-genomic. Both a single s.c. injection and i.v. administration of nandrolone were found to have no significant cardiovascular effect, suggesting that ischaemia is required in order to observe an adverse cardiovascular effect. In this setting one of the ways by which nandrolone may act non-genomically is through blockade of the extraneuronal reuptake (uptake-2) of noradrenaline. The resulting decrease in extracellular NA clearance combined with increased release of NA from cardiac nerves due to ligation of the LAD coronary artery may possibly explain the dose dependent increase in arrhythmia. Cocaine can inhibit neuronal reuptake (uptake-1) of noradrenaline (refer 1.9), whereas endogenous androgens such as testosterone have been demonstrated to prevent the extraneuronal reuptake of catecholamine (Iversen et al., 1970; Salt, 1972). However, it is not known whether nandrolone significantly inhibits the reuptake of noradrenaline from cardiac muscle in the same manner as other steroids such as corticosterone.

8.1.2 Extraneuronal uptake

Iversen first reported the extraneuronal reuptake mechanism for noradrenaline in 1965 (Iversen, 1965). He found that when isolated rat hearts were perfused with a high concentration of ³H-noradrenaline or ³H-adrenaline (1-40µg/ml) there was a rapid increase in ³H-catecholamine uptake at perfusion concentrations above 0.5µg/ml which was obviously not related to uptake-1 alone (Fig. 8.1). The large increase in the concentration of catecholamine in the heart tissue couldn’t be explained in terms of simple diffusion as the intracellular concentration of NA was several orders of magnitude higher than the perfusing medium.
Iversen named this new uptake mechanism "uptake-2". It was found to differ from neuronal uptake in 2 main aspects...

1. The process was reversible. Noradrenaline disappeared from the tissue if hearts were perfused with catecholamine free solution. This suggested that uptake-2 could act in both directions.

2. The uptake-2 process appeared to be completely inoperative at low concentrations.

Initially it was thought that uptake-1 operated only at low catecholamine concentrations and that extraneuronal uptake only occurred at high catecholamine concentration (Simmonds et al., 1968). However, later experiments using tritiated noradrenaline showed active uptake at all concentrations (Lightman et al., 1969). It was demonstrated that at low noradrenaline concentrations the intracellular metabolising enzymes for noradrenaline – catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) are not fully saturated. The metabolism of noradrenaline produces methoxyhydroxyphenylglycol (MOPEG) and dihydroxyphenylglycol (DOPEG), which diffuse rapidly out of the myocardial cells (Fiebig et al., 1978). Thus, uptake of ³H-NA acts like a 'leaky-pump' whereby ³H-NA crosses the myocardial membrane at the same time that radiolabelled metabolites are being lost. This implies that in initial experiments where MAO and COMT were not inhibited, radiolabelled metabolites were able to diffuse out of the myocardial cells into the perfusate. As only the radioactivity in the tissue was measured, this resulted in an under-estimate of the rate of uptake of noradrenaline by cardiac myocytes.

In 1969 Kalsner demonstrated that 17β-oestradiol, progesterone and deoxycorticosterone potentiated the contraction response of sympathomimetic amines in rabbit aortic strips (Kalsner, 1969b). This effect was also demonstrated using hydrocortisone (Kalsner, 1969a). Based on this observation Iversen examined whether this effect could be explained by extraneuronal blockade of noradrenaline reuptake (Iversen et al., 1970). He tested a range of steroid hormones (not including nandrolone) and found that oestrogen and corticosterone displayed potent inhibition. Further studies tested the ability of a range of steroid hormones including male hormones to block the reuptake of ³H NA in perfused hearts (Salt, 1972). He found that androstenedione and testosterone both at a perfusion concentration of 10μg/ml caused an 88.7±4.0% and 82.9±2.2% inhibition of uptake-2 respectively. It is probable that this degree of inhibition was underestimated in these experiments due to a lack of COMT and MAO inhibition resulting in an apparently lower rate of NA reuptake than would otherwise be expected.
Iversen and colleagues also demonstrated that a range of haloalkylamines related to phenoxybenzamine also cause inhibition of uptake-2 (Iversen et al., 1972).

Subsequent to Iversen's pioneering work a number of other characteristics of extraneuronal noradrenaline reuptake have become obvious. These include

1. Uptake-2 has a broad substrate specificity which is not restricted solely to $^3$H-catecholamines or $^3$H-phenethylamines, but extends to tritiated resorcinols and imidazoline derivatives and also tritiated histamine and serotonin (Grohmann et al., 1984).

2. Resting membrane potential modulates uptake-2. Vascular smooth muscle can be depolarised by $\alpha_1$-adrenoceptor agonists (eg. noradrenaline and methoxamine) and hyperpolarized by nicorandil (a vasodilator). Trendelenburg utilised these properties in segments of rabbit main pulmonary artery to show that nicorandil increased the extraneuronal accumulation of $[^3]$H-isoprenaline (Trendelenburg, 1987).

Evidence suggests that the uptake that Iversen observed in isolated, perfused hearts was due to uptake by myocardial cells and cannot be attributed solely to uptake by the venules, arterioles and other vasculature. Early support for this was provided by a histochemical/fluorescence study by Clarke et al in 1969 which showed that noradrenaline appeared to be predominantly associated with the cardiac muscle cells in rat hearts perfused with high concentrations of noradrenaline (Clarke et al).
More recently, catecholamine uptake has been characterised in rat cardiac myocytes (Obst et al., 1996). It was found that uptake was largely inhibited by corticosterone and unaffected by cocaine and desipramine. Babin-Ebell & Gliese demonstrated that O-methylisoprenaline, inhibited extraneuronal reuptake of $^3$H-NA by 61.2±3.8% in rat atrium and 16.0±3.5% in rat ventricle (Babin-Ebell et al., 1995).

The transporter for uptake-2 has been recently cloned (Grundemann et al., 1998). Overlap in the sensitivity to inhibitors of catecholamine uptake and antagonists of the renal transport of organic cations by the transporter protein organic cation transporter 2 (OCT2) suggested that the extraneuronal transporter may belong to the family of amphiphilic solute facilitators. Successful cloning of the transporter from a human kidney carcinoma cell line (Caki-1) previously shown to demonstrate uptake-2 in vitro, resulted in the naming of the uptake-2 transporter, EMT (extraneuronal transporter for monoamine transmitters) (Grundemann et al., 1998). In accordance with data from perfused rat hearts it was demonstrated that human embryonic kidney cells (293) stably transfected with the EMT cDNA take up adrenaline three times more efficiently than noradrenaline (Grundemann et al., 1998). The rat EMT demonstrates 93 and 91% homology to its human and bovine counterparts (Paczkowski et al., 1999). The lack of any major pharmacological difference in the pharmacological properties of rat, human and bovine EMTs suggest that data obtained previously in studies on rat tissues can be extrapolated to human EMT (Paczkowski et al., 1999).
8.2 Aim

- To determine whether nandrolone can inhibit extraneuronal noradrenaline reuptake in rat heart.
- Based on the plasma concentration of nandrolone achieved with acute dosing (Ch. 5) to determine whether nandrolone mediated inhibition of noradrenaline uptake is likely to explain the observed cardiac effects of nandrolone.

8.3 Hypothesis

Nandrolone will significantly inhibit the reuptake of noradrenaline in vitro at concentrations consistent with those at which it potentiated ischaemia-induced arrhythmia.
8.4 Method

Albino Wistar rats were obtained at 10-12 weeks of age and housed according to General Methods 2.1. The protocols for the investigation of uptake inhibition and the methodology for analysis of results are contained in General Methods 2.10.

In order to provide a rapid means of determining whether nandrolone could inhibit the reuptake of noradrenaline in rat cardiac tissue, a series of pilot experiments were conducted using atrial and ventricular slices. It was hoped that a simple heart slice preparation could provide a means for the simultaneous study of several tissue samples obtained from the same heart. The results of this study and a brief discussion are contained in Appendix D. Although good uptake of ³H-NA could be demonstrated in tissue slices, this could not be significantly inhibited with well established inhibitors of extraneuronal reuptake (i.e. corticosterone or O-methyl-isoprenaline).
8.5 Results

The IC_{50} value for nandrolone to inhibit extraneuronal uptake of [¹H]-NA (0.85µM) was more than 2,000 times greater than that of corticosterone. The IC_{50} of nandrolone was found to be 3.95x10^{-11}M compared to an IC_{50} for corticosterone of 1.78x10^{-4}M (Fig. 8.2)

Fig. 8.2 Uptake of [³H]-NA into isolated, perfused rat hearts. Mean±SE, n=10 (vehicle), n=3-6 (all other concentrations).
8.6 Discussion

The IC₉₀ value obtained for corticosterone (1.78x10⁻⁶M) compares favourably with the reported literature values. Iversen and Salt report an IC₉₀ value for corticosterone of 7.87x10⁻⁶M (Iversen et al., 1970). In a later study Salt reports the IC₉₀ value as 2.7x10⁻⁶M±1.18x10⁻⁶M (Salt, 1972). Neither of these studies inhibited metabolism of noradrenaline.

It is unlikely that blockade of the extraneuronal reuptake of noradrenaline can explain the cardiac effects of intravenous nandrolone on cardiac events during LAD coronary artery ischaemia or on the positive chronotropic effect of cocaine in rats pretreated with nandrolone for 3 weeks (Phillis et al., 2000). The mean plasma level of nandrolone obtained in Ch. 7 following 9 weeks of treatment with nandrolone was 172±22.5nM (1.72x10⁻⁷±2.25x10⁻⁸M). Even when nandrolone was infused at high concentration (160μg/kg/min) the mean plasma concentration only reached 915±135nM (9.15x10⁻⁷±1.35x10⁻⁷M). The IC₉₀ for nandrolone was found to be more than 4x10³ times greater than this plasma level of nandrolone. It is therefore probable that inhibition of the extraneuronal reuptake of noradrenaline cannot fully explain its cardiac effects.
8.7 Conclusion

Nandrolone inhibits extraneuronal reuptake of noradrenaline with low potency compared to corticosterone in isolated perfused rat heart. The plasma levels of steroid produced by chronic treatment with a high dose of nandrolone and the plasma levels resulting from intravenous infusion do not approach the concentration associated with the IC$_{50}$ of nandrolone for extraneuronal noradrenaline reuptake. Blockade of the extraneuronal reuptake of noradrenaline is unlikely to provide a mechanism for the *in vivo* potentiating effect of nandrolone on ischaemia induced cardiac arrhythmia.
8.8 References


Chapter 9

Discussion

The published literature concerning the cardiovascular effects of AS abuse in humans is complicated by a large number of confounding variables including abuse of multiple steroid types, administration route, dose, age, gender, diet and exercise. Similar problems are associated with animal studies investigating the cardiovascular consequences of AS administration. The present study has sought to minimise these variables and investigate the cardiac effects of nandrolone in normotensive, non-exercised, adult male rats.

9.1 Rat strain

Comparison of the effect of cocaine on the two most common laboratory rat strains used in this thesis indicated that the cardiovascular response to cocaine in the two strains was comparable. These results reassure us that the use of the SD rat for the experiments presented here was appropriate and would allow comparison with the majority of previous studies.

9.2 Effects of nandrolone alone

9.2.1 Effects of nandrolone in non-ischaemic rats

Chronic nandrolone treatment of the rats did not increase body weight. This effect has been previously reported by a number of investigators in adult male rats receiving s.c. nandrolone decanoate using a range of doses (Long et al., 1996; Hallberg et al., 2000; Johansson et al., 2000a; Johansson et al., 2000b; Long et al., 2000) and contrasts with the increased body weight gain seen in females or immature males (Lewanowitsch et al., 2001).

Histological examination of the hearts from nandrolone treated animals showed no evidence of cellular toxicity in comparison to hearts from vehicle treated controls. This is in accordance with previous studies where light microscopy has failed to provide any indication of a cardiotoxic effect of AS in hearts from stanozolol treated rats (Bauman et al., 1988), in liver from oxandrolone or testosterone treated rats (Horvath et al., 1971), and in testes and muscle from nandrolone treated rats (Yu-Yahiro et al., 1989).
Neither acute nor chronic treatment of conscious rats with s.c. nandrolone resulted in significant changes in BP or HR. There was also no effect of nandrolone on these cardiovascular variables in anaesthetised rats when the drug was administered i.v.. This is consistent with previous findings in the rat (Hall et al., 1982; Trifunovic et al., 1995) and rabbit (Ferrer et al., 1994). This suggests that tachycardia and hypertension reported with the use of AS in humans is either species specific (occurring in humans but not rats) or additional elements are required before it is evident.

9.2.2 Cardiovascular effects of nandrolone in rats subjected to cardiac ischaemia and reperfusion
In the anaesthetised animals subjected to cardiac ischaemia and reperfusion, arrhythmia and mortality during ischaemia were increased by acute nandrolone pre-treatment. This suggests that perhaps in some of the reported cases of sudden cardiac death associated with steroid abuse, the steroids may have exacerbated an ischaemic episode.

The mechanism of the observed increases in arrhythmia during ischaemia following nandrolone infusion is uncertain. The possibility of an interaction at the level of noradrenaline transporters was examined in chapter 8. The likelihood of nandrolone inhibiting non-neuronal transport of NA was excluded due to its low potency relative to the plasma levels achieved in these in vivo experiments. The IC\textsubscript{50} for nandrolone inhibition of extraneuronal reuptake was more than 2x10\textsuperscript{5} times greater than the IC\textsubscript{50} for corticosterone, and more than 4x10\textsuperscript{3} times greater than the plasma concentration of nandrolone achieved with i.v. infusion (160µg/kg/min). This suggests that the contribution that blockade of extraneuronal reuptake by nandrolone makes to myocardial noradrenaline concentration during an ischaemic period is very low. There are a number of potential mechanisms for the pro-arrhythmic effect of nandrolone during ischaemia that are worthy of further investigation...

1. Uncoupling of the protective effect of adenosine on NA efflux during ischaemia
It is possible that the increased arrhythmia observed is in part due to a loss of a protective effect of adenosine. During early ischaemia a reflex increase in cardiac sympathetic nerve activity is accompanied by local exocytotic NA release (Schomig, 1990). A concomitant rise in adenosine causes pre-synaptic inhibition of NA release (Richardt et al., 1987; Richardt et al., 1994; Schreieck et al., 1999). Schreieck and Richardt demonstrated in isolated perfused rat hearts that a range of variously selective adenosine receptor antagonists promoted arrhythmia in hearts subjected to regional ischaemia (Schreieck et al., 1999). A similar arrhythmogenic effect was observed when endogenous adenosine was inhibited. Conversely, when adenosine breakdown was blocked or adenosine production promoted, the occurrence of arrhythmia was significantly decreased.
Testosterone has been shown to block the vasodilatator effect of adenosine in isolated perfused rat hearts and was unaffected by cyclohexamidé, or coupling to a large dextran-lysine complex. The effect was found to be of rapid onset, disappearing quickly after removal of the steroid (Ceballos et al., 1999). These findings indicate that the effects are likely to be mediated by a mechanism which is independent of nuclear binding or protein synthesis, and occurring at the cell surface. If nandrolone acts in a similar way as testosterone and is active against adenosine released during myocardial ischaemia it is possible that the pro-arrhythmic effect of nandrolone observed in the present study reflects an effect against physiological mechanisms activated in early ischaemia to prevent NA overflow. It is unknown whether nandrolone acts in the same manner as testosterone to block the vasodilator effects of adenosine. It is also not known whether the blockade of the vasodilator effect of adenosine by testosterone noted by Ceballos et al is due to an interaction at the receptor level, at the receptor lipid interface, or in a pathway triggered by adenosine receptor activation. Furthermore, it is unclear how adenosine provides its protective effect as Schreieck and Richardt found that the antiarrhythmic effect of adenosine could not be correlated with a decrease in ischaemic injury (Schreieck et al., 1999).

2. CNS action of nandrolone

It is possible that the increases in arrhythmia observed during ischaemia are related to a CNS effect of nandrolone. In adult male rats, electrophoretic application of testosterone has been found to increase the firing rate of individual neurons in the anterior hypothalamus within 2-30s (Yamada, 1979). A similar effect has been observed in the lateral hypothalamus (Orsini et al., 1985). Recent work has focussed on examining changes in the expression of receptors for various brain neurotransmitters in response to treatment with nandrolone and other AS. Chronic nandrolone treatment has been shown to affect the expression of dopamine receptors (Kindlundh et al., 2001), serotonin receptors in the hippocampal CA1, medial globus pallidus and nucleus accumbens shell (Kindlundh et al., 2003), to change expression of subunits of the NMDA receptor in the hippocampus and hypothalamus (Le Greves et al., 1997) and significantly enhance SP immunoreactivity in the amygdala, hypothalamus, striatum and periaqueductal grey (PAG) (Hallberg et al., 2000). The current literature on these CNS changes and others are summarised in Appendix E. It is not known if nandrolone and similar AS act upon brain regions important in maintaining normal cardiovascular homeostasis, particularly with respect to central responses activated by cardiac ischaemia.

3. Arrhythmia induction through endothelin-1

A number of recent studies have associated the potent vasoconstricting peptide, endothelin-1 (ET-1) with arrhythmogenesis during LAD coronary artery ischaemia. Szabo et al demonstrated that intracoronary ET-1 infusion (30 or 60pmol/min/30 min) has a direct arrhythmogenic effect in the
absence of coronary occlusion (Szabo et al., 2000). However, it is unlikely that the concentrations of ET-1 used by Szabo resemble the concentration that is achievable in a physiological situation. Lin and Yuan demonstrated that i.v. administration of pre-proET-1 mRNA antisense oligodeoxynucleotide (AS-ODN) 2 hrs before coronary occlusion in rats resulted in a dose dependent protection against VT and VF (Lin et al., 2002). Mortality was reduced from 16.7% following administration of saline to 0% with the highest dose of AS-ODN (90nmol/kg). Administration of sense oligodeoxynucleotide resulted in an incidence of VT and VF similar to control. A similar dose dependent effect was demonstrated for VT and mortality in the cat in the same study using intracoronary injection of the ET<sub>A</sub> receptor antagonist, BQ610. However, the role of ET-1 in the induction and maintenance of ischaemia is not without controversy. Iskit and Guc found that the ET<sub>A</sub>-ET<sub>B</sub> receptor blocker, bosentan was not protective against ischaemia and reperfusion induced arrhythmia in rats (Iskit et al., 1996).

Following the conclusion of the ischaemia/reperfusion protocol performed in chapter 5, approximately 5ml of blood was extracted from the descending aorta. A preliminary investigation of the ET-1 plasma concentration (pg/ml) in rats pre-treated with vehicle or nandrolone (80μg/kg/min) showed no significant difference between controls and nandrolone infused rats (Appendix F). Before it is possible rule out a nandrolone effect on ET-1, it is necessary to know the plasma ET-1 concentration immediately prior to ischaemia, rather than at the end of reperfusion. The experiments conducted in support of this thesis did not involve blood sampling throughout ischaemia/reperfusion. Future work should attempt to equate nandrolone and ET-1 plasma levels during cardiac ischaemia.

Recently evidence has begun to emerge which suggests that testosterone may increase the vasoconstrictive effect of ET-1 on porcine coronary vasculature (Teoh et al., 2000). At present there is no data on the affects of nandrolone on ET-1 in rat coronary arteries or any evidence that can provide a direct link between increased plasma ET-1 resulting in VF during ischaemia in nandrolone treated rats.

9.3 Effects of cocaine alone

Intraperitoneal cocaine administered to conscious animals resulted in an increase in BP, HR and SLA as would be expected. However, the responses are somewhat prolonged and smaller in peak effect when compared to literature values where the drug was administered i.v. For example, the HR increase in response to i.v. cocaine in vehicle pre-treated anaesthetised rats (close-chested), was found to peak at about 40s of infusion (0.5mg/kg/min, total dose = 5mg). In comparison, when cocaine (45mg/kg, i.p.) was administered i.p. to fully-conscious SD rats, HR was found to peak at
approximately 90 minutes. Despite these large differences in the cardiovascular effect of cocaine by the 2 administration routes, the i.p. route is appropriate to mimic the pharmacokinetic profile of cocaine after intranasal administration, which is one of the most popular routes of administration in polydrug users.

When cocaine was administered acutely by the i.v. route to anaesthetised rats pre-treated with either saline or ethanol vehicle, and subjected to ischaemia and reperfusion, there was no increase in arrhythmia in comparison to control. This may reflect a lower plasma cocaine concentration achieved with infusion as opposed to iv bolus administration (Mehta et al., 2003). Moreover, anaesthetised animals display blunted responses to the sympathomimetic effects of cocaine (Pitts et al., 1987; Fraker et al., 1990; Tella et al., 1992a; Wang et al., 1999).

9.4 Cocaine effects in combination with chronic nandrolone

Previous work found that chronic pre-treatment with nandrolone, significantly (p<0.05) potentiated the HR response to cocaine (45mg/kg, i.p.) in conscious rats (Phillis et al., 2000). In anaesthetised rats, chronic nandrolone pre-treatment did not significantly change the BP or HR response to i.v. cocaine (0.5mg/kg/min, total dose = 5mg). The interaction between ‘treatment’ and ‘time’ at 600s of infusion for the HR response to cocaine may be explained by the high variability associated with the vehicle response at this point (Fig. 7.B.C). It is possible that the failure to observe a significantly elevated HR response to i.v. cocaine in chronically nandrolone treated rats compared to vehicle controls may be related to the use of anaesthesia. Wilkerson found that pentobarbital anaesthesia significantly reduced the BP, HR and rate-pressure response to cocaine (1mg/kg, i.v.) in dogs (Wilkerson, 1988). This suggests that pentobarbital anaesthesia may have ‘dampened’ the cardiovascular response to i.v. cocaine in the SD rats used in chapter 7. It is difficult to compare the effect of i.p. cocaine in conscious, freely moving rats, with the effect of i.v. cocaine in anaesthetised rats. Therefore, further work examining the cardiovascular effects of i.v. cocaine in fully conscious, chronically nandrolone treated rats is warranted.

It is possible that the previously observed effect of nandrolone on the HR response to cocaine may reflect a pharmacokinetic interaction between nandrolone and cocaine (see 9.7) which was not present in rats treated acutely with nandrolone. The possibility of a pharmacokinetic interaction between nandrolone and cocaine requires further investigation (Long et al., 2000) (see 9.7 Future Studies).

A number of models have been proposed to explain a reported pro-arrhythmic effect of cocaine including increases in intracellular calcium, increased catecholamine concentration and the local
anaesthetic properties of cocaine. These are reviewed extensively in 1.13.2. Likewise, chronic nandrolone treatment has been associated with a number of cardiac conditions which could be expected to increase the risk of ischaemic events, including, left ventricular hypertrophy (1.6.2), hypertension (1.6.3), arteriosclerosis (1.6.1), direct toxicity (1.6.2) and acute myocardial infarction (1.6.1 & table 1.3). However, in the present study cocaine (i.v.) in combination with chronic nandrolone had no effect upon the frequency or duration of arrhythmia. This may have been due to a low basal arrhythmic activity following chronic nandrolone treatment.

9.5 Cocaine effects in combination with acute nandrolone

Acute nandrolone (40μg/kg/min) administration followed by i.v. cocaine (0.5mg/kg, s.c.) did not produce a significantly different BP or HR response profile compared to saline or vehicle pre-treated animals. However, cocaine appeared to protect against fatal VF during ischaemia which occurred in the presence of nandrolone.

It is possible that the protective action of cocaine is a centrally mediated effect. Bonci and Williams demonstrated that cocaine (10mg/kg, i.p.) can change the normal synaptic regulation of dopamine cells in the VTA (Bonci et al., 1996). In the absence of cocaine pre-treatment, D1 receptor activation increased the amplitude of GABA\(_B\) mediated inhibitory post synaptic potentials (IPSP). Rats which received cocaine for 2 weeks showed inhibition of the GABA\(_B\) IPSP. This inhibition was blocked by adenosine A1 receptor antagonists and compounds disrupting cAMP metabolism. In a similar study Baldo et al used a range of adenosine agonists and antagonists to show that cocaine withdrawal induces changes in the expression of adenosine receptors in the nucleus accumbens (Baldo et al., 1999). Although both of these studies were concerned with mechanisms of brain reward, it is possible that similar changes in synaptic transmission may mediate the cardiovascular response to nandrolone. Testosterone has been shown to have an effect at adenosine receptors (Ceballos et al., 1999). Therefore, it is possible that central adenosine receptors may provide a site of interaction of AS and cocaine.

9.6 Experimental Limitations

As with all studies, a limited number of factors could be examined. A number of variables are worthy of further study and should be considered in the interpretation of the results presented in this report. These variables include species, strain, gender, exercise, stress, drug doses and routes of administration.
9.6.1 Species

It is clear that the rat model has limitations and that considerable care must be exercised in extrapolating results from this model to humans. However, although there are species differences in the response to AS and cocaine there are also many similarities. Similar plasma profiles are produced in both rats and humans after i.v. cocaine administration (Javaid et al., 1978; Jeffcoat et al., 1989; Pan et al., 1998). Both rats and humans show similar pharmacodynamic responses to cocaine characterised by dose related increases in SLA, CT, HR and BP (Folien et al., 1988; Lange et al., 1989; Lomax et al., 1990; Tella et al., 1992a; Ansah et al., 1996; Evans et al., 1996; Horowitz et al., 1997) and similar ECG changes (Opitz et al., 1995; Littmann et al., 2000; Phillips et al., 2000). Isolated vessels from both humans and rats show the same vasoconstrictive response to exogenously applied cocaine (Isner et al., 1989; Vitullo et al., 1989; Chokshi et al., 1990; Benzaquen et al., 2001). Likewise, similarities exist between rats and humans in the behavioural effects of AS (Anitto et al., 1980; Conacher et al., 1989; Choi et al., 1994; Long et al., 1996) and the induction of changes in plasma lipid profiles (Webb et al., 1984; Yu-Yahiro et al., 1989; Zuliani et al., 1989; Glazer, 1991; Lewanowitsch et al., 2001).

9.6.2 Strain

While a significant number of investigators have examined the strain related behavioural effects of cocaine, research on cardiovascular outcomes is limited. The possible effect of strain on the cardiovascular response to cocaine is discussed at length in chapter 3. Evidence provided in chapter 3 indicates that the BP and HR response to i.p. cocaine in the 2 strains (AW and SD) used in the present study, was not significantly different. However, wistar rats showed a significantly greater SLA response to saline. The opposite effect was observed with the administration of cocaine, whereby the 15mg/kg dose caused a significantly greater SLA response in SD rats. This implies that while there is evidence that suggests a strain related behavioural response to cocaine (George et al., 1991; Callhol et al., 1999) a similar differential cardiovascular response is not observed.

9.6.3 Gender

Although the studies in this thesis were confined to male animals, gender may be an important factor in the response to cocaine and nandrolone. Morishima et al infused cocaine (2mg/kg/min) until circulatory collapse in male and female rats. It was found that the dose required to produce cardiovascular toxic manifestations was significantly lower in male rats than females (Morishima et al., 1993). Lukas et al studied the gender dependency of positive and negative psychological effects of cocaine and correlated these changes to plasma concentration and HR (Lukas et al., 1996). It was found that intranasal cocaine HCl caused a significantly higher peak plasma concentration in males (144.4±17.5ng/ml) compared to females in the follicular stage of the menstrual cycle.
(73.2±9.9 ng/ml). When the same group of women were administered cocaine during their luteal phase the plasma concentration was lower (54.7±8.7 ng/ml). Despite a significant difference between plasma cocaine levels the HR response to cocaine was not significantly different between groups. This suggests that women may be more sensitive than males to the HR effects of cocaine, but that this effect is not altered by the hormonal fluctuation seen in women. Future studies should consider the importance of gender.

9.6.4 Exercise

The addition of anaerobic exercise would have been an interesting series of experiments to add to this project but this is quite difficult to undertake using rats as the experimental animal. Most reports using rats have used an aerobic exercise regime such as treadmill running which is not appropriate to simulate AS users’ regimes but may be acceptable as a form of stress stimulating the HPA axis. Conlee and colleagues demonstrated that treadmill running caused elevations in plasma catecholamines and corticosterone which were further significantly increased by cocaine (12.5 mg/kg, i.p.) (Conlee et al., 1991). Noradrenaline in treadmill exercised rats increased by greater than 2.3 times in cocaine treated rats compared to exercised, saline controls. Comparable increases in plasma catecholamines were also observed by Ojuka et al and Han et al using treadmill exercise and cocaine (20 mg/kg, i.p. and 5 mg/kg, i.v., respectively) (Han et al., 1996; Ojuka et al., 1996). Because adrenaline has been found to be synergistic with cocaine in reducing coronary flow rate (CFR) in isolated, perfused hearts (Avakian et al., 1990) the additive effect of exercise on the catecholamine increase in response to cocaine may be especially important in potentiating the adverse effect of cocaine on the heart.

Han et al also found a sudden increase in plasma lactate within 2 minutes of commencing treadmill exercise in cocaine treated rats, compared to vehicle controls (Han et al., 1996). This difference remained statistically significant (p<0.05) even following a 20 minute recovery period. It was not determined whether this increase in lactate was due to a vasoconstriction mediated reduction of blood flow resulting in hypoxia and lactate production. Similar lactate increases in exercised rats have been observed in animals subjected to chronic cocaine treatment (Kelly et al., 1995).

9.6.5 Stress

Stress is an important variable in determining the cardiovascular response to cocaine in the rat. It has been demonstrated that cocaine (5 mg/kg, i.v.) administration and air-jet stress both cause a bimodal cardiac output (CO) response in rats thus allowing the classification of ‘responders’ (decreased CO) and ‘non-responders’ (increased CO) (Knuepfer et al., 1993). Heart rate and arterial pressure did not display the same bi-modal distribution. This suggests that in a sub-population of
rats ('responders') the cardiac output in response to cocaine is strongly influenced by stress. Later work has found that a similar response is elicited using cold water stress, and that the differences in CO within a population of rats in response to cold water or cocaine could be eliminated by administering nicardipine (calcium channel blocker, 25μg/kg, i.v.) or atropine bromide (peripheral anti-muscarinic, 0.5mg/kg, i.v.). This suggests that the haemodynamic response to both cocaine and stress is influenced by calcium channel dependent vascular smooth muscle contraction and muscarinic inhibition of catecholamine release.

In the present study in order to minimise the stress component of the response to cocaine, cardiovascular variables in chapters 3 and 4 were collected using radiotelemetry. Radiotelemetry eliminates the extreme stress associated with restraint during tail-cuff measurements of BP (Clement et al., 1989; Irvine et al., 1997; McDougall et al., 2000). Handling of the animal is limited to the time of drug injection.

9.6.6 Route of drug administration

A small number of human studies have investigated the pharmacokinetics of cocaine via different routes, although resulting plasma concentrations have been rarely equated to cardiovascular effects. Jeffcoat et al observed the disposition of radiolabelled cocaine after 3 different administration routes: i.v. injection, snorting and smoke inhalation (Jeffcoat et al., 1989). The plasma concentration-time profile for cocaine and the major metabolite (benzoyl ecgonine) for i.v. administration and smoke inhalation were almost identical. Whereas i.v. injection and smoking produced large and rapid increases in parent drug and metabolite concentration, administration via nasal insufflation (snorting) displayed a long lag time to peak cocaine concentration of 44.7±17.0 min. Cocaine plasma concentration remained appreciably higher than that via the other routes until about 250 minutes post insufflation. Benzoyl ecgonine concentrations reached a maximum within approximately 200 minutes and remained elevated until the conclusion of the experiment at 500 minutes post insufflation. Branch and Knuepfer provide evidence to suggest that benzoyl ecgonine can cause a profound simultaneous pressor and bradycardic effect upon infusion (1mg/kg) in rats (Branch et al., 1994). The maintenance of a high cocaine and benzoyl ecgonine plasma concentration following nasal insufflation explains the observation of a consistently high HR (>180% baseline) until greater than 60 minutes post administration (Javaid et al., 1978). Obvious methodological problems prevent administration of cocaine to rats by nasal insufflation. Fortunately, Javaid and Davis demonstrated that the disposition pattern of cocaine after i.p. administration in the rat is similar to that observed in humans after intranasal administration (Javaid et al., 1993). Most animal studies concerned with cocaine cardiotoxicity have used i.v. cocaine.
administration. However, fatal cardiotoxic incidents have been reported for subjects using intranasal administration and smoking (Wetli et al., 1985; Mittleman et al., 1987).

9.6.7 Cardiac ischaemia

A number of human case reports have linked AS abuse to conditions associated with lowered perfusion and increased risk of cardiac ischaemia. Steroid abuse has been linked to athlerosclerosis (Mewis et al., 1996) and thrombosis, (Fisher et al., 1996; Nieminen et al., 1996) both of which significantly increase the risk of cardiac ischaemia. Likewise cocaine has been associated with reduced perfusion (Lange et al., 1989; Lange et al., 1990) through a direct vasoconstrictive action (Vitullo et al., 1989; Chokshi et al., 1990; Simkhovich et al., 1994). In the present study, chronic pretreatment with nandrolone did not alter the cardiovascular response to ischaemia or the magnitude and duration of arrhythmia elicited by a 15-minute ligation of the LAD coronary artery.

In order to maximise bioavailability acutely, nandrolone was infused i.v. to a group of rats, and found to elicit a dose dependent increase in VF during ischaemia, and a significant increase in the Lambeth arrhythmia score compared to vehicle infused controls. This discrepancy in the arrhythmic response between acute and chronic dosing may be related to the much higher plasma nandrolone concentration achieved with i.v. infusion. Alternatively, it is also possible that chronic treatment caused downregulation of receptor(s) or a transduction cascade intermediate, or activated a negative feedback mechanism critical for increasing arrhythmia with i.v. infusion. For example, nandrolone has been demonstrated to have a biphasic and treatment duration dependent effect on HPA activity. Chronic nandrolone treatment for 14 days at both 15 and 45mg/kg significantly decreased circulating ACTH, 24 hrs after the last injection, while corticosterone only displayed a significant reduction at the highest dose at the same time point (Zhou et al., 1998). Short term treatment with 15mg/kg, i.m. in a different study caused increases in circulating levels of both ACTH and corticosterone after 3 days treatment, an effect which was lost 24 hours after drug withdrawal (Schlussman et al., 2000). Schlussman and associates suggest that short term nandrolone treatment may transiently activate the HPA axis whereas chronic administration results in inhibition. A similar bimodal effect on corticosterone and ACTH has been noted with acute and chronic cocaine administration (Zhou et al., 1996). While changes in circulating ACTH are an example of a mediator which is variably affected by acute or chronic nandrolone treatment it may not explain the resulting differences in the arrhythmic propensity of acute or chronic nandrolone treatment in the present study. In fact, infusion of the melanocortin peptide ACTH-(1-24) (adrenocorticotropin) during a 5 minute occlusion of the rat LAD coronary artery has been shown to afford a dose dependent protection against reperfusion arrhythmias (Bazzani et al., 2001; Bazzani et al., 2002). Conversely, the reverse effect was observed with the melanocortin MC3/MC4 receptor.
antagonist SHU 9119 (Mioni et al., 2004). If it is assumed from the work of Schlussman that our i.v. infusion of nandrolone caused an increase in ACTH in a similar manner to acute i.m. administration we would predict an anti-arrhythmic effect. This contrasts to the increase in VF frequency and duration that was actually observed. More work is required to determine exact ACTH levels following i.v. administration to determine whether changes in circulating ACTH evoked by acute or chronic nandrolone administration can explain the difference in the occurrence of VF during ischaemia following these different treatment regimes.

Pre-ischaemic, i.v. cocaine, administered to anaesthetised rats regardless of acute or chronic pre-treatment with nandrolone failed to significantly affect the frequency and duration of arrhythmia during ischaemia. It is probable that this effect is related to the bradycardia seen with cocaine administration in pentobarbital treated rats which itself makes observation of a true effect of cocaine on cardiac rhythm difficult to observe. Pentobarbital was utilised because it has been shown to display limited effect on cardiac rhythm, whereas urethane, halothane and chloroform have been shown to have an anti-arrhythmic effect (Curtis et al., 1987). Unfortunately, pentobarbital has been demonstrated to have a cardio-depressant effect when co-administered with cocaine (Pitts et al., 1987; Tella et al., 1992b) and causes a significant decrease in the peak pressor response to cocaine in dogs (Fraker et al., 1990) and mice (Wang et al., 1999).

It was noted that in rats administered cocaine, there was an increasing tendency for death to occur which is unrelated to VF, and prevents scoring according to the Lambeth convention. Without the observation of fatal VF it is also difficult to assign an accurate survival time. As measurements of cardiac output were not taken, it was not possible to determine the exact cause of these deaths. In most rats the Lambeth scoring method is necessary to obtain meaningful results from data which show a large standard deviation.

Another of the disadvantages of the model used in this study is that artificial ventilation in open-chested animals allows animals to survive periods of arrhythmia that would otherwise prove fatal. Therefore, it is concluded that the anaesthetised ischaemia-reperfusion rat model is not ideal for the evaluation of the cardiovascular effect of drugs with a significant sympathetic component.

9.7 Future studies

Future studies must concentrate on the mechanism of the observed increase in arrhythmia with infused nandrolone. In the present study it is uncertain whether the pro-arrhythmic effect of nandrolone during ischaemia is steroid-receptor mediated or is due to a non-specific toxic effect. Pre-ischaemic infusion of a steroid receptor antagonist such as flutamide to determine whether the
pro-arrhythmic effects of nandrolone during ischaemia can be reduced or eliminated will help determine whether toxicity is receptor mediated.

Nandrolone readily crosses the blood brain barrier due to its high lipophilicity. An assessment of the nandrolone concentration in various brain regions following the drug infusion protocol used in this thesis will help elucidate brain regions potentially affected by nandrolone infusion. Identifying possible brain regions involved in cardiovascular homeostasis and the effect of nandrolone on neurotransmitter release and receptor expression would greatly contribute to an understanding of the cardiotoxicity of nandrolone. The effect of nandrolone on neurotransmission in the CNS and periphery is unknown. Due to the low potency of nandrolone for extracranial NA reuptake, it is unlikely that increased myocardial NA can explain the observed arrhythmia during ischaemia. No studies have been conducted examining the effect of centrally applied anabolic steroid on cardiotoxicity. However, a CNS dependent mechanism of cardiotoxicity is not without precedent. Tyrosine, the amino-acid precursor of the catecholamine neurotransmitters is an example of a drug which can act centrally to increase the incidence of arrhythmia during cardiac ischaemia. It has been found that tyrosine injected into the lateral cerebral ventricle of pigs induces ventricular fibrillation in 100% of pigs compared to 52% in drug free, conscious controls (Kirby et al., 1992). This effect was attributed to an increase in CNS catecholamine synthesis. It is possible that nandrolone may also act centrally through an as yet unknown pathway to cause an increase in cardiac arrhythmia.

The direct effects of nandrolone on the heart remain uncertain. Ceballos et al. demonstrated that testosterone when confined to the coronary vascular lumen can block the adenosine vasodilator effect in isolated perfused rat heart (Ceballos et al., 1999). Future experiments should investigate whether nandrolone can also show this effect. The likelihood of the importance of an adenosine-mediated pro-arrrhythmic effect of nandrolone is indicated by the effect of adenosine antagonists during ischaemia. Both, adenosine antagonism with theophylline, 8-phenyltheophylline or xanthine amine congener and inhibition of endogenous adenosine release ((S-(4-nitrobenzyl)-6-thioinosine) have been shown to cause an increase in cardiac arrhythmia in isolated perfused rat hearts (Gorge et al., 1998; Schrieck et al., 1999). It is possible that the pro-arrrhythmic effects of nandrolone may be related to an inhibition of endogenous adenosine release or an antagonism of cardiac adenosine receptors.

To date investigations with nandrolone have provided no evidence of a hypertensive effect with acute or chronic treatment. The reported increase in BP by chronic 19, methyl testosterone (Molteni et al., 1969) or testosterone (Fischer et al., 1977) treatment should be confirmed using radiotelemetry. As animal handling can induce stress related increases in BP and HR, it is imperative to ensure that previous studies using tail cuff measurements of BP in response to

CHAPTER 9 Discussion
testosterone (Hall et al., 1982) have not unintentionally reported a significant stress component. It is also important to determine whether the accelerated hypertension in nandrolone treated developing SHRs observed by Tseng et al can be replicated using radiotelemetry (Tseng et al., 1994). Only a small number of conflicting studies have addressed the putative hypertensive effect of AS in rats and consequently the ability of AS to induce hypertension remains controversial and confusing (Brown et al., 1972; Wolinsky, 1972; Greenberg et al., 1973; Fischer et al., 1977; Hall et al., 1982). These studies have used a range of AS, different treatment periods and species, and examined both exercising or non-exercising animals all of which alone may have a significant effect upon the cardiovascular response to AS.

Despite the significant increase in relative heart size observed in this study in response to 6 and 9 weeks nandrolone treatment, no pathology was detected in hearts compared to vehicle treated rats. Future studies involving chronic nandrolone treatment should use higher resolution microscopy. A study by Abu-Shakra and associates demonstrated apoptotic cell death in murine skeletal muscle cells treated with stanozolol (Abu-Shakra et al., 1997). However, the doses of stanozolol that cells were exposed to was exceptionally high (>10μM). A steroid antagonist, failed to reverse cell death suggesting a direct toxic effect of stanozolol rather than a receptor mediated effect. It is not known whether AS concentrations of greater than 10μM are achievable in vivo. In the present study, plasma concentrations of nandrolone associated with arrhythmia during ischaemia only reached approximately 0.92μM. Although the nandrolone doses used in the present study are reflective of doses of AS used by recreational drug users (Yu-Yahiro et al., 1989; Johansson et al., 1997) it is possible that this concentration was too low in order to observe myocardial tissue damage. It is possible that a direct effect of nandrolone was missed in this study because of the use of low resolution light microscopy. Horvath et al failed to find a toxic effect of oxandrolone and testosterone on rat hepatocytes using light microscopy and only observed proliferation of the smooth-surfaced endoplasmic reticulum when using electron microscopy (Horvath et al., 1971).

It is possible that the significant increase in chronotropy by i.p. cocaine, reported previously in fully-conscious chronically nandrolone treated rats in comparison to vehicle controls, is due to a pharmacokinetic interaction between nandrolone and cocaine. Cytochrome P-450 is integral to the metabolism of testosterone and nandrolone, being responsible for hydroxylation at several positions (Juchau et al., 1976). Testosterone has also been found to be capable of inducing cytochrome P450 in female mice (Hong et al., 1989). Hong et al investigated the P4502E1-dependent renal metabolism of the carcinogen N-nitrosodimethylamine (NDMA) after 2 days treatment of female mice with testosterone (500mg/kg, s.c.). Renal NDMA demethylase activity was found to be elevated 17-fold, corresponding with a significant elevation in P4502E1. A similar effect has been observed for hepatic cytochrome P450 in female rats administered oxandrolone but
not in male rats (Waskiewicz et al., 1995). Cocaine is N-demethylated by cytochrome P-450 (Misra et al., 1974). Chronic treatment with cocaine (30mg/kg/day, i.p.) for 2 weeks has been shown to significantly increase tumour necrosis factor-alpha (TNF-α) and creatine phosphokinase (CPK) in mice hearts and sera. Pre-treatment with the P450 inhibitors cimetidine or metyrapone was found to attenuate or abolish the effect of cocaine on TNF-α and CPK. Similarly, isolated hepatocytes have been shown to have an increased susceptibility to cocaine-induced cellular damage when liver cells were isolated from rats in which P450 isoforms had been induced via phenobarbital or dexamethasone (Jovey et al., 1993; Poet et al., 1996). In addition it has been shown that chronic cocaine administration can induce its own metabolism in mice (Powers et al., 1999). The fact that both nandrolone and cocaine are metabolised to some extent by cytochrome P450 suggests that chronic treatment with either drug could alter the cardiac response elicited by the other, and requires further attention.
9.8 Conclusion

High doses of nandrolone, administered by either chronic s.c. or acute s.c. or i.v. route, had no significant cardiovascular effect in the sedentary rat. In conscious animals, chronic, but not single dose s.c. nandrolone potentiated the tachycardia resulting from i.p. cocaine administration. In anaesthetised rats in which the pressor and chronotropic response to cocaine was blunted and transient, there was no effect of acute nandrolone on cardiovascular responses to i.v. cocaine.

In anaesthetised rats pretreated with i.v. nandrolone and subjected to cardiac ischaemia there was an increase in the duration and frequency of VF and significantly increased mortality. No effect of nandrolone pretreatment was evident during cardiac reperfusion. The effect of nandrolone on ischaemia-induced arrhythmia was not observed after chronic nandrolone dosing for up to 9 weeks. The mechanism of the potentiating effect of acute i.v. nandrolone on arrhythmia was not identified but is unlikely to be due to decreased clearance of noradrenaline by cardiac cells since nandrolone was shown to be too weak an inhibitor of extraneuronal noradrenaline uptake in the isolated perfused rat heart, displaying an IC\textsubscript{50} several orders of magnitude greater than the plasma levels associated with potentiation of arrhythmia \textit{in vivo}. The failure of chronic s.c. nandrolone to potentiate ischaemia induced arrhythmia and mortality may be due to failure of the chronic dosing regime to achieve sufficiently high plasma levels of nandrolone, or alternatively it may be due to down regulation of the effect with chronic exposure to the drug.

In anaesthetised animals iv cocaine infusion had no significant effect on ischaemia-induced arrhythmia when administered alone, and did not exacerbate the effects of infused nandrolone. On the contrary, co-administration of cocaine appeared to reverse the increased mortality and arrhythmia during ischaemia due to nandrolone.

The mechanism of the potentiating effect of acute nandrolone on ischaemia-induced arrhythmia requires further study. However these data suggest that AS administration might precipitate adverse cardiac events in individuals predisposed to cardiac ischaemia. Similarly, the mechanism of the apparently protective effect of cocaine is unclear. However, further studies are necessary to determine whether a similar effect occurs in conscious animals in which the pressor and increased HR responses to cocaine are more pronounced. Moreover, the administration of iv cocaine by infusion may have limited its pro-arrhythmic effects. It is possible that a different interaction with nandrolone or ischaemia may have been observed if bolus doses of cocaine, or higher doses of cocaine, had been administered.
In conclusion, if the results presented here extend to humans, the use of these drugs may be tolerated in healthy individuals and not lead to cardiac events. However, in individuals with compromised coronary perfusion they are likely to precipitate fatal consequences.
9.9 References


CHAPTER 9 Discussion


Appendix A.

Reported fatal and non-fatal cardiovascular outcomes resulting from anabolic steroid use
<table>
<thead>
<tr>
<th>Authors</th>
<th>Gender &amp; age</th>
<th>Exercise Status</th>
<th>History of steroid use</th>
<th>Metabolites Detected</th>
<th>Fatal/Non-Fatal</th>
<th>Clinical details</th>
<th>Diagnosis &amp; details of follow-up (Explanations)</th>
</tr>
</thead>
</table>
| (Appleby et al., 1994) | Male, 31 y.o. | Bodybuilder     | Had taken anabolic steroid and amphetamine courses over 5-10 years (type of steroids not reported). Used 2 Frumul (80mg furosemide and 19mg amisulod) tablets per day to reduce body fluid and increase muscle definition. Consumed 5g of potassium per day and additional magnesium as a health tonic. | Drug screen not performed | Non fatal       | Collapsed on stage during a body building competition with chest pain. Blood pressure was 150/70, pulse 100 regular, jugular venous pressure not raised, heart sound normal and chest clear. On ECG there was significant ST elevation in leads II, III and aVF, with tented T-waves and ST depression in I, aVL and leads V5-V6. Chest x-ray was normal. The peak creatine kinase was significantly elevated (163 U/L). Post thrombolysis ECG showed resolution of the ST changes without development of significant Q-waves, however there was inferolateral T-wave inversion in leads II, III, V5 and V6. | Myocardial infarction  
Patient refused to accept that he had suffered a myocardial infarction, discharged himself and was lost to follow-up. |
| (Bowman, 1990)       | Male, 23 y.o. | Bodybuilder     | An abuse history spanning 5 years and most recently 5 weeks previously.                  |                      | Non fatal       | 1.5-hour history of severe tight retrosternal chest pain. A non-smoker with no family history of heart disease. Examination of the ECG recorded on admission showed evidence of an acute lateral infarction. Treatment with streptokinase did not prevent formation of a full thickness infarct with a rise in cardiac enzymes. | Myocardial infarction  
Cardiac catheterisation found evidence of septal ischaemia and significant athero-occlusive plaque formation in the left anterior descending artery and large oblique marginal branch. |
<table>
<thead>
<tr>
<th>Authors</th>
<th>Gender &amp; age</th>
<th>Exercise Status</th>
<th>History of steroid use</th>
<th>Metabolites Detected</th>
<th>Fatal/Non-Fatal</th>
<th>Clinical details</th>
<th>Diagnosis &amp; details of follow-up (recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Dickerman et al, 1995)</td>
<td>Male, 20 y.o.</td>
<td>Amateur bodybuilder</td>
<td>Reportedly abused a range of steroids including Primobolen-Depot (methenolone depot), Testosterone (veterinarian testosterone enanthate) and Launabolin (veterinarian nandrolone lactate). At the time of death he had just completed a 3-month cycle of steroids. At peak cycle he was administering approximately 700mg/week of anabolic steroids.</td>
<td>Drug screen not performed.</td>
<td>Fatal</td>
<td>Suffered an instantaneous death with idiopathic bilateral pulmonary haemorrhage. There was no past or family history of cardiac disease. Heart displayed evidence of significant concentric left ventricular hypertrophy and weighed 550g. Evidence of mild atherosclerosis.</td>
<td>Ventricular hypertrophy and pulmonary haemorrhage.</td>
</tr>
<tr>
<td>(Feencheck et al, 1992)</td>
<td>Male, 37 y.o.</td>
<td>competitive weightlifter</td>
<td>A history of intermittent steroid abuse spanning 7 years. At the time of admission he was completing a 16 weeks cycle of steroid use. He admitted to the abuse of several intramuscularly administered steroids including nandrolone decanoate, boldenone, testosterone cypionate and stanozolol. He also used oral oxandrolone.</td>
<td>Drug screen not performed.</td>
<td>Non-fatal</td>
<td>45-minute history of chest pain radiating to the left arm and associated with nausea, diaphoresis and shortness of breath. Admission blood pressure was normal (80/120mmHg). No risk factors for arterial disease (including no history or family history of sustained hypertension, diabetes mellitus, smoking or known hyperlipidaemia) ECG demonstrated ST segment elevation and appearance of new Q waves 0.04 mv's in duration indicative of myocardial infarction. An increase in cardiac enzyme activity was reported (creatin kinase and lactate dehydrogenase). Treatment was begun with intravenous tissue plasminogen activator and heparin. Recovery was uneventful. Cardiac catheterisation performed 3 days after admission showed angiographically normal arteries and normal left ventricular function.</td>
<td>Myocardial infarction. Exercise stress testing 4 months after discharge showed no ischaemic changes.</td>
</tr>
<tr>
<td>Authors</td>
<td>Gender &amp; age</td>
<td>Exercise Status</td>
<td>History of steroid use</td>
<td>Metabolites Detected</td>
<td>Fatal/Non-Fatal</td>
<td>Clinical details</td>
<td>Diagnosis &amp; details of follow-up &amp; pathology</td>
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<td>(Fishbe et al., 1996)</td>
<td>Male, 43 y.o.</td>
<td>Amateur bodybuilder</td>
<td>Duration of abuse is unknown. At the time of admission he was administering methandienone (orally), nandrolone decanoate (i.m.) and testosterone (i.m.)</td>
<td>Admitted to hospital complaining of severe central back pain associated with sweating, nausea and vomiting.</td>
<td>Non fatal</td>
<td>No past medical history of note. No current prescription medicine. Blood pressure was normal (110/70). Blood count, urea, electrolytes, blood glucose and chest x-ray were all normal. ECG revealed an acute inferior infarct with ST elevation in leads II &amp; III. Echocardiography revealed dilation of all the cardiac chambers and the left ventricular ejection fraction was estimated to be 30%.</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>(Haasmann et al., 1998)</td>
<td>Male, 23 y.o.</td>
<td>Amateur bodybuilder</td>
<td>Abused a wide range of performance enhancing drugs. The following were found in his apartment following death... Testex Leo 250 prolongam (testosterone cyclopentylpropionate) Primobolan Depot 100mg (methenolone enanthate) Proviron 25mg (mesterolone) Thybon 100μg (isothyroxine hydrochloride) Adalactone 100mg (spironolactone) Clomifene 25mg Contraspemin 0.02mg (clenbuterol hydrochloride)</td>
<td>Using enzyme immunosassay (ELA) and gas chromatography-mass spectrometry (GC-MS) metabolites of mesterolone, methandienone, testosterone, nandrolone and clenbuterol were detected in the urine.</td>
<td>Fatal</td>
<td>The autopsy revealed cardiac hypertrophy with dilation of the right ventricle and focal induration of the endocardium. Histology revealed enlargement and nuclear polymorphism of the left ventricular muscle fibres. Intersitial myocardial fibrosis and disseminated focal necrosis.</td>
<td>Cardiac hypertrophy</td>
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</table>

Exercise Status - Amateur bodybuilder

Gender & age - Male, 43 y.o.

Gender & age - Male, 23 y.o.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Gender &amp; age</th>
<th>Exercise Status</th>
<th>History of steroid use</th>
<th>Metabolites Detected</th>
<th>Fatal/Non-Fatal</th>
<th>Clinical details</th>
<th>Diagnosis &amp; details of follow-up</th>
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<tr>
<td>(Hase, 1994)</td>
<td>Male, 25 y.o.</td>
<td>Amateur weightlifter</td>
<td>16 weeks prior to admission he had been administering 100mg of nandrolone decanoate weekly (i.m.). He continued this practice for 6 weeks. Following a 4-week break he continued nandrolone administration at 200mg for 6 weeks.</td>
<td>A urine toxicology screen was negative for amphetaamines, barbiturates, benzodiazepines, cocaine and phencyclidine. The urine was not tested for anabolic steroids.</td>
<td>Non-fatal</td>
<td>Nod to severe fatigue after a heavy weight training session. Began experiencing substernal chest discomfort, which progressed to crushing pain. No significant prior medical history. Family history of infarction i.e. grandfather died at 40 from myocardial infarction and an uncle survived an AMI at the same age. Increases in cardiac enzymes observed. Cholesterol was normal. Echocardiography revealed mild hypokinesia. Coronary angiography revealed a large proximal left anterior descending artery lesion consistent with a thrombus.</td>
<td>Myocardial infarction 8 months post infarction; the patient denied experiencing any further cardiac symptoms with exercise or any additional steroid use. Cholesterol levels were measured again and found to be at acceptable levels.</td>
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<td>(Kennedy, 1993)</td>
<td>Male, 24 y.o.</td>
<td>Competitive bodybuilder</td>
<td>2-year history of anabolic steroid abuse. Over the 6 weeks prior to admission he had been taking oral stanozolol (40 mgs daily), nandrolone (200 mgs, i.m., twice weekly) and Sustanon 250 (testosterone esters = 1ml, i.m. weekly).</td>
<td>Drug screen not performed</td>
<td>Non-fatal</td>
<td>Presented 3 days after the development of recurrent, dull central chest pain radiating to his left arm. No family history of heart disease or lipid disorders. Smoked 30 cigarettes daily, a 10-year habit. Serial electrocardiograms and cardiac enzyme measurements confirmed lateral myocardial infarction. Echocardiography revealed a small area of apical infarction. His blood pressure remained intermittently elevated (maximum 195/110mmHg).</td>
<td>Myocardial infarction Awaiting coronary angiography.</td>
</tr>
<tr>
<td>(Kennedy et al, 1993a)</td>
<td>Male, 27 y.o.</td>
<td>Body builder</td>
<td>6-year history of anabolic steroid abuse.</td>
<td>Gas chromatographic and mass spectroscopic analysis of urine upon autopsy detected stanozolol and nandrolone.</td>
<td>Fatal</td>
<td>Following a 6-year history of steroid abuse the patient had suffered an uncomplicated inferolateral myocardial infarction 10 months previously. 3 months following this he resumed weight lifting and 8 months later resumed steroid use. At autopsy the coronary arteries were found to arise normally, there was no significant atherosclerosis in the coronary, carotid or cerebral circulations. A right cerebellar haemorrhage was present which had spread into the pons and vestibular systems. There was no evidence of vasculitis in the cerebral or coronary circulation.</td>
<td>Cerebral haemorrhage</td>
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<tr>
<td>Authors</td>
<td>Gender &amp; age</td>
<td>Exercise Status</td>
<td>History of steroid use</td>
<td>Metabolites Detected</td>
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<tr>
<td>Kennedy et al., 1993b</td>
<td>Male, 18 y.o.</td>
<td>Professional footballer</td>
<td>Not available</td>
<td>Oxymesterone glucuronide (metabolite of oxymesterone)</td>
<td>Fatal</td>
<td>Petechial surface haemorrhage present on the epicardial surface of the heart. Coronary arteries were normal, equidominant and free of atheroma or thrombus. Histological analysis revealed myocyte disarray with variation in fibre size and thickening of the walls of the intramural arteries consistent with hypertrophic cardiomyopathy.</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>Kennedy et al., 1993b</td>
<td>Male, 24 y.o.</td>
<td>Professional footballer</td>
<td>Not available</td>
<td>Oxymesterone glucuronide</td>
<td>Fatal</td>
<td>Petechial haemorrhages present on the posterior surface of the heart, and 2 haemorrhages on the anterior surface consistent with cardiac puncture. The coronary arteries were normal and were equidominant. Likewise, there was no evidence of thrombus, no valvular disorder and only minimal atherosclerosis. An extensive area of scarring was found in the interventricular septum. There were multiple foci of fibrosis within the septum with adjacent hypertrophic myocytes and in places a mononuclear and lymphocytic infiltrate consistent with myocarditis.</td>
<td>Myocarditis</td>
</tr>
<tr>
<td>Luke, 1990, Cambell et al., 1993</td>
<td>Male, 21 y.o.</td>
<td>At least 7-year history of high intensity sports. Four years in varsity football. Subsequently involved in bodybuilding.</td>
<td>History of parenteral steroid abuse for several months before decease. Police reports indicate possession of testosterone cypionate (200mg/ml), nandrolone decanoate (100mg/ml &amp; 200mg/ml), Drugs reported to have been administered twice weekly. The last injection occurred approx. 1 week before decease.</td>
<td>Urine: 19-norandrosterone (250ng/ml), 19-nor-testolchodiol (52 ng/ml), and 19-nor-epiandrosterone (52 ng/ml) (nandrolone decanoate metabolites) Blood: found to be unsuitable for analysis.</td>
<td>Fatal</td>
<td>Collapsed while using a bench press at a local gym. Paramedics reporting to the gym found him in ventricular fibrillation. Autopsy found marked left-sided cardiac hypertrophy. The coronary arteries showed no evidence of atherosclerosis. The cardiac valves were unremarkable. Sectioned myocardium revealed extensive regional fibrosis with principal involvement of the subepicardial, and the central aspects of the left ventricle and the interventricular septum and of the right ventricle and atria. Sectioned myocardium revealed evidence of extensive left and right ventricular hypertrophy and dilatation. Microscopic examination also found evidence of contraction band formation.</td>
<td>Cardiac hypertrophy and regional myocardial fibrosis.</td>
</tr>
<tr>
<td>McNutt et al., 1988</td>
<td>Male, 23 y.o.</td>
<td>World class weight lifter</td>
<td>Intramuscular and oral AS for 6 weeks prior to admission. Long-term abuse not reported.</td>
<td>Drug screen not performed</td>
<td>Non fatal</td>
<td>No past or family history of cardiac disease. Laboratory studies revealed an elevated creatine kinase. The ECG disclosed both elevated ST segment and the development of new Q waves. Cardiac catheterisation performed 10 days after infarction demonstrated normal coronary arteries and apical dyskinesis. The subject was found to have elevated serum cholesterol at admission in conjunction with platelet hyperaggregability. Twenty-four days post-infarction after discontinuation of steroids the total serum cholesterol was found to have reduced dramatically.</td>
<td>Acute myocardial infarction. No follow-up reported</td>
</tr>
<tr>
<td>Authors</td>
<td>Gender &amp; age</td>
<td>Exercise Status</td>
<td>History of anabolic use</td>
<td>Metabolites Detected</td>
<td>Fatal/Non-Fatal</td>
<td>Clinical details</td>
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<td>(Mewes et al., 1996)</td>
<td>Male, 28 y.o.</td>
<td>Bodybuilder</td>
<td>2 year history of anabolic steroid administration (280mg/week). Six months prior to admission drug administration had been discontinued.</td>
<td>Drug screen not performed</td>
<td>Non fatal</td>
<td>Presented with ventricular tachycardia. The ECG recorded at sinus rhythm disclosed a QRS pattern, R reduction in V1-V4, and T-wave inversions in V1-V6 and to II, III, and aVF. Echocardiography revealed a dilated left ventricle with diffuse hypokinesia and fractional shortening of 29%. Coronary angiography revealed severe CHD with stenosis lesions in the right coronary artery and evidence of revascularised thrombus in the left anterior descending artery. Lack of perfusion in the apex was discovered using thallium myocardial scintigraphy at rest. Patient was discharged with 200mg midodrine in addition to antianginal therapy including aspirin. No further follow-up was reported.</td>
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<tr>
<td>(Mochizuki et al., 1988)</td>
<td>Male, 32 y.o.</td>
<td>Competitive weightlifter</td>
<td>Had used anabolic steroids intermittently since 16 y.o. Had used a wide variety of steroids using multiple combinations in high doses concomitantly. Oral compounds used included methandrostenedione (Dianabol), oxandrolone (Anavar), and stanozolol (Winstrol). Intramuscularly injected compounds included nandrolone decanoate (Deca-Durabolin) and nandrolone phenylpropionate (Durabolin). In addition he reported use of human chorionic gonadotropin and tamoxifen.</td>
<td>Drug screen not performed</td>
<td>Non fatal</td>
<td>Complaints of blurred vision and slurred speech, as well as right-sided numbness, paresthesia, and ataxia. A CT scan and an EEG were found to be normal. Discharged at 7 days with right hemiparesis and dysarthria. Four months after discharge the patient presented again because of the sudden onset of left-sided facial drooping and hemiparesis. A CT scan displayed a large ischaemic hypodensity in the area of the right middle cerebral artery, which indicated an ischaemic cerebrovascular accident (CVA). An echocardiogram demonstrated severely impaired left ventricular performance consistent with cardiomyopathy without thrombus. 24 hour Holter recording showed frequent premature atrial depolarization and rare premature ventricular contractions. After stabilisation he was referred for rehabilitation – on admission he was found to have flaccid left sided hemiparesis and sensory loss, blood pressure of 124/80, heart rate 86 bpm and regular. There was no evidence of carotid bruits or cardiac murmurs, gallops or rubs. An ECG showed sinus rhythm with non-specific ST-T wave abnormalities in the inferolateral leads. Chest x-ray showed cardiomegaly. An echocardiogram provided evidence of a severely reduced left ventricular ejection fraction with akinesia of the anterior wall and septum, modestly severe hypokinesia of the inferior wall and mild hypokinesia of the lateral wall. Doppler studies of the carotid arteries showed minimal intimal thickening but no stenosis or plaques.</td>
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Diagnosis & details of follow-up (if applicable)

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<thead>
<tr>
<th>Authors</th>
<th>Gender &amp; age</th>
<th>Exercise Status</th>
<th>History of steroid use</th>
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</tr>
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<tbody>
<tr>
<td>Toyama et al., 1994</td>
<td>Male, 61 y.o.</td>
<td>Not reported but presumably sedentary</td>
<td>Presented initially with gingival bleeding at which time pancytopenia was noted. Detailed examinations revealed aplastic anemia for which 45mg of methenolone enanthate was prescribed (presumably on a weekly basis). No coronary risk factors, including diabetes or hypertension. Presented 3 months later with severe epigastric pain.</td>
<td>Not relevant (non-abuse situation)</td>
<td>Non fatal</td>
<td>ST elevation was observed in leads II, III and aVF on ECG examination. Acute myocardial infarction was diagnosed from the ECG and echocardiographic findings. Peak value of creatinine phosphokinase was 4,794 IU/l on day 2 of hospitalisation. No atherosclerotic lesions were observed, but multiple filling defects were observed in segments 1-3 of the right coronary artery, and the distal ends of these regions displayed delayed flow. These filling defects strongly suggested the presence of thrombi.</td>
<td>Cardiac catheterisation was performed on day 48 following hospitalisation. No follow-up reported</td>
</tr>
<tr>
<td>Toyama et al., 1994</td>
<td>Female, 59 y.o.</td>
<td>Not reported, but presumably sedentary</td>
<td>Visited the outpatient clinic in August 1977 complaining of easy fatigability and palpitations. Severe anaemia was observed and the patient was admitted. She was treated with methenolone enanthate (100mg). Treatment was continued in 1982 with oxymetholone (30mg). No coronary risk factors except for steroid induced glucose intolerance.</td>
<td>Not relevant (non-abuse situation)</td>
<td>Non fatal</td>
<td>Infarction occurred in November 1990, and again in February 1991 First infarction At the time of the first infarction ST elevations were observed in leads II, III and aVF on ECG analysis. Acute myocardial infarction of the inferior wall was diagnosed. The peak value of serum creatinine phosphokinase was 1,802 IU/l. Second infarction Relapse of the inferior wall was diagnosed based on ECG findings. The peak value of serum creatinine phosphokinase was 707 IU/l. Left ventriculography revealed akinetic motion of the posterior basal wall. Coronary arteriography showed no sclerotic lesions in the left coronary artery. Thrombotic filling defects were observed at segment 2 of the right coronary artery, and a complete occlusion was seen beyond these lesions.</td>
<td>Cardiac catheterisation on the 24th day of second hospital admission. No follow-up reported</td>
</tr>
<tr>
<td>Authors</td>
<td>Gender &amp; age</td>
<td>Exercise Status</td>
<td>History of steroid use</td>
<td>Metabolites Detected</td>
<td>Fatal/Non-Fatal</td>
<td>Clinical details</td>
<td>Diagnosis &amp; details of follow-up</td>
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<tr>
<td>(Vezzali et al., 1999)</td>
<td>Male, 39 y.o. (HIV+)</td>
<td>Not reported but presumably sedentary</td>
<td>8 month history of testosterone enanthate (500mg, IM) administration every 2 weeks to prevent muscle wasting (non-physician approved)</td>
<td>Admitted with severe squeezing chest pain localized to the midsternum of 4 hours duration. Concomitant symptoms included mild shortness of breath, nausea and diaphoresis.</td>
<td>Non-fatal</td>
<td>Patient reported being HIV+ for 7 years with a CD4 count of 400/ml and a viral load of zero. Physical findings were normal besides a mild increase in heart rate (100 bpm) and an audible S4 gallop at the apex. The initial ECG showed ST segment depression and T-wave inversion of the mid and left precordial leads consistent with non-Q-wave infarction. Serial creatinine kinase (CK) and CK-MB enzymes were high, with peaks of 236 U/L and 35 mg/dl, respectively at 24 hours. Two-dimensional echocardiography showed left ventricular anterior wall hypokinesia. A fasting lipid profile on day-2 post admission revealed serum total cholesterol of 272 mg/dl, low-density lipoprotein 167 mg/dl, high-density lipoprotein 44 mg/dl, and triglycerides 306 mg/dl. A submaximal exercise stress thallium scintigram revealed a conspicuous perfusion defect of the left ventricular anterior wall. Coronary angiograms showed a ruptured plaque and a large, non-occlusive, superimposed thrombus of the mid-left anterior descending artery.</td>
<td>Acute myocardial infarction</td>
</tr>
</tbody>
</table>
References


Reported histological changes in laboratory animals administered anabolic steroids
<table>
<thead>
<tr>
<th>Author/date</th>
<th>Treatment Regime</th>
<th>Species/strain/sex</th>
<th>Observations</th>
</tr>
</thead>
</table>
| (Baeren et al., 1988) | Control: no exercise, protein supplementation, or steroid.                         | Rat, SD, male     | - Liver and adrenal weight significantly decreased in all exercised groups compared to controls.  
- Testicular weight was significantly less in rats administered steroid.  
- Heart weight was significantly greater in steroid treated rats compared to control.  
- Hematoxylin and eosin-staining of liver, kidneys, adrenals, heart, and testes from treated rats revealed no significant pathology. |
| (Behrendt, 1977)   | Methandienone (Dianabol®) (1.65mg/kg/week/13 weeks); Control: sesame seed oil    | Rat, Wistar, female | - Electron microscopy revealed an increase in intermediate sized, non-myofibrillar filaments in muscle cells of the left cardiac ventricle in steroid treated rats compared to control. |
| (Behrendt et al., 1977) | Methandienone (Dianabol®) (1.65mg/kg/week/3 weeks); Control: sesame seed oil    | Rat, Wistar, female | - Electron microscopy found that the mitochondria and myofibrils from heart muscle cells in steroid treated rats showed swelling and elongation similar to the morphology changes observed in early heart failure.  
- The matrix was sparse with significantly reduced cristae. The myofibrils showed either disintegration and widened and twisted Z-bands or a complete dissolution of the sarcormeric units. |
| (Buschop et al., 1997) | Nandrolone decanate (1.5 or 7.5mg/kg, i.m./week/5 weeks); Control: saline        | Rat, Wistar, male and female | - Hematoxylin and eosin-stained slides of the diaphragm and gastrocnemius from both male and female rats demonstrated that nandrolone caused a significant increase in type IIa/b dimensions.  
- Nandrolone caused an increase in type I fibre dimensions in the gastrocnemius in both sexes. |
| (Besada et al., 1999) | Stanazolol (5mg/kg, i.p. /day) for 12 hours, or 1, 2, 3, 4, 6, 8, 10, 20 days.   | Rat, SD, male     | - Hepatic cytochrome P450 and B5 content increased significantly for acutely treated rats (0.5-4 days) but significantly decreased at days 60 and 90.  
- No effect was found on serum transaminase enzymes in any of the groups, either in acute or chronic studies.  
- Light microscopic evaluation of H/E stained liver sections from animals at day 3 and 4 of treatment revealed slight to moderate multifocal lobular inflammation with siderophilic degeneration and evident Kupffer cells reactivity of the liver tissue compared to control groups. These changes were not evident in rats treated for shorter periods of time.  
- Cytoplasmic vacuolization and lipidic degeneration was evident in the majority of the chronically treated rats. Increased mitosis and binucleation and variability in the size of cell nuclei was seen in 90 days treated rats but only in 3 livers from 60 day treated rats.  
- Eleven livers from short-term and long-term treated animals showed a subcapsular inflammatory lesion (sarkogranuloma) and an evident enlargement and hyalinosis of centrilobular vein wall (eight livers). |
| (Horvath et al., 1973) | Oxandrolone or testosterone administered at 1mg in 1ml of water by stomach tube,  
for 3 days. Rats were killed 16 hours after the last dose on day 4.              | Rat, SD, female    | - Light microscopy detected no major alterations in the liver.  
- Electron microscopy detected significant endoplasmic reticulum (ER) alterations in oxandrolone treated rats. In some hepatocytes, normal rough-surfaced-ER could hardly be seen.  
- The cisternae in the hepatocytes were extremely dilated and had only a few ribosomes attached to them.  
- Oxandrolone caused wide variations in the size and form of the mitochondria.  
- Testosterone produced only mild changes in hepatocytes. The rough-surfaced-ER was conspicuous but had a normal ultrastructure. The compartment of the cytoplasm occupied by the smooth-surfaced-ER was larger than in normal midzonal hepatocytes.  

<table>
<thead>
<tr>
<th>Author/date</th>
<th>Treatment Regime</th>
<th>Species/strain/sex</th>
<th>Observations</th>
</tr>
</thead>
</table>
| (Tseng et al., 1994)| Nandrolone (20mg/kg, s.c./day/1-6 weeks) or corn oil control. | Rat, SHK, Male     | • Ventricle tissue was stained with Hematoxylin and eosin and examined for histopathological damage by light microscopy. A semi-quantitative grading scale was used by a blinded observer. One this scale 0=no obvious pathology, 1=increased diffuse cellularity around great vessels or heart valves, 2=1-5 small focal areas of cellular damage with inflammatory cellular or fibrotic infiltration, 3=more than 5 such areas, 4=presence of at least one large lesion (greater than one-third of the thickness of the chamber wall at that point), and 5=more than one large lesion.  
• Nandrolone treated rats were found to have greater myocardial damage than control treated rats (3.83±0.4 vs. 2.83±0.17, P<0.05). |
| (Yu-Yahiro et al., 1989) | Either 0.5cc of saline or nandrolone decanoate (50mg/ml of approximately 100mg/kg). | Rat, Wistar, Male and female | • Treated livers in both males and females showed no obvious peliosis or hepatic dysplasia.  
• Sudan Black B and Oil Red O staining for lipids showed that male but not female treated livers had much lower lipid content than those of control.  
• All females had abnormal vacuolization, stromal edema, and peliosis of the uteri.  
• No pathologic changes were observed in testes, spine, ovaries or muscle.  
• There were no differences in DNA or RNA content in any tissues, as measured by methyl green/pyronin staining.  
• In a separate group of all rats, half were treated with nandrolone and half with control, testes and liver were found to be significantly heavier in the control versus the treated group.  
• Kidneys were heavier in the treated rats versus the control. |
References


Appendix C

Pilot study: chronic effect of nandrolone in telemetered rats
C.1 Introduction
The ability of AS to produce hypertension remains controversial and confusing. However, Tseng et al (1994) found that nandrolone decanoate (20mg/kg, s.c., daily) accelerated the development of hypertension in young, spontaneously hypertensive rats in comparison to vehicle treated controls (Tseng et al., 1994). Fischer and Swain injected castrated male rats with testosterone cypionate (1mg/wk, i.m.) and found that after 3 weeks of treatment, BP had increased significantly in steroid pre-treated rats in comparison to vehicle treated controls (Fischer et al., 1977). In human subjects, Riebe and associates measured BP at rest and during a standardised exercise test in AS abusing weight lifters, in their drug free associates and in sedentary controls (Riebe et al., 1992). They found that the AS abusing group had significantly higher SP at rest and during exercise. Despite these studies suggesting a causal link between hypertension and AS a significant number of investigations in both rats (Brown et al., 1972; Wolinsky, 1972) and human subjects (Olsson et al., 1974; McKillop et al., 1986; Urhausen et al., 1989; Friedl et al., 1990; Thompson et al., 1992) have failed to find any evidence of AS related BP changes.

It was decided to use radiotelemetry to investigate whether chronically administered nandrolone could induce hypertension in AW rats. Because of the small number of telemetry implants available for monitoring BP each rat served as its own control.

C.2 Aim
To determine whether non-anaesthetised, freely moving AW rats display a significant change in HR, BP and SLA over 9 weeks of treatment with nandrolone (20mg/kg, s.c.).

C.3 Hypothesis
Chronic treatment with nandrolone decanoate will result in a significant increase in BP at the conclusion of 9 weeks of treatment compared to the pre-treatment period. Heart rate and SLA will be unchanged by nandrolone treatment.
C.4 Method

C.4.1 Animals
Albino-Wistar rats were purchased at 12 weeks and were housed according to General Methods 2.1. Rats were anaesthetised (2.3) and surgically implanted with Data-Sciences blood pressure implants (2.2) as per 2.4.2. Post-operative care was carried out following the details outlined in 2.5.

C.4.2 Treatment
Rats were treated for 9 weeks with nandrolone decanoate (20mg/kg, s.c.) 3 days a week (Monday, Wednesday, Friday).

C.4.3 Protocol
Rats were injected subcutaneously with nandrolone (20mg/kg, s.c.) and returned to their home cage for 60 minutes. At the conclusion of this acclimatisation period, telemetry recordings of HR, SLA and BP were made for 60 minutes. This protocol was conducted 3 times weekly (Monday, Wednesday and Friday) and continued for 9 weeks (61 days).

C.4.4 Data Analysis
All parameters were graphed as functions of time (days). The HR, BP and SLA before initiation of treatment was compared to the value of these variables on the final day of nandrolone administration using a Student’s t-test.
C.5 Results

C.5.1 Changes in body weight

Rats weighed 571.6±22g at the beginning of the treatment period. By the 9th week of treatment weight had decreased by 74±13g (Fig. C.1). Body weight decreased significantly at day 61 compared to day 1 [t(8)=2.595, p<0.05] (Fig. C.1 inset).

C.5.2 Changes in cardiovascular parameters

Although SP and DP appeared to decrease slightly between day 38 and day 61, no statistically significant changes were evident between day 1 and 61 of treatment (Fig. C.2).

C.5.3 Changes in cardiovascular parameters

Likewise, no significant changes in SLA were evident between the start and conclusion of nandrolone treatment. Spontaneous locomotor activity peaked between day 20 and 30 of treatment (Fig. C.3). This corresponded to a peak in HR, which was also evident between 20 and 30 days (Fig. C.2). No significant changes were evident in HR between the beginning and end of nandrolone treatment.
Fig. C. 1 Body weight following a 3xs weekly administration of nandrolone (20mg/kg, s.c.).
Inset: Weight at start and conclusion of treatment. *p<0.05 significantly different from day 61, unpaired t-test. Mean±S.E., n=5

Fig. C. 2 Diastolic (▲) and systolic pressure (■), and heart rate (▼) in rats treated with nandrolone (20mg/kg, s.c.) for 9 weeks. Mean±S.E., n=5
Fig. C. 3 Spontaneous locomotor activity (SLA) response to nandrolone treatment (20mg/kg, s.c.) over 61 days. Mean±S.E., n=5
C.6 Key Findings

- No significant hypertensive effect of nandrolone was observed during 9 weeks of high dose treatment.
- The peak in SLA and HR that developed between day 20 and 30 of nandrolone treatment requires further investigation. This change was not evident in SP and DP.
- Nandrolone significantly reduced total body weight. It is unknown whether this reflects changes in water composition, protein or fat disposition.

C.7 Future work

- In order to properly characterise the peak in SLA and HR between days 20 and 30 of treatment this study needs to be repeated with the appropriate vehicle control animals.
C.8 References


Appendix D

Pilot study: extraneuronal noradrenaline uptake in rat heart slices
D.1 Introduction

Bönisch *et al.* demonstrated good uptake of isoprenaline from isolated sections of rat aorta and ventricle (Bönisch *et al.*, 1974). Babin-Ebell and Gliese demonstrated that isolated 20mg strips of human atrium displayed strong uptake-2, which was sensitive to inhibition by O-methylisoprenaline (OMI) (Babin-Ebell *et al.*, 1995). The investigation of Babin-Ebell *et al.* was the first study to show that uptake-2 occurs in human heart tissue.

In search of a simple method for investigating the ability of nandrolone to inhibit extraneuronal noradrenaline reuptake it was decided to use slices of isolated rat ventricle and atria. It was hoped that large numbers of slices could be generated from each heart, minimising the number of animals used and allowing an efficient generation of data.

D.2 Aim

- To establish a simple method using isolated rat ventricular / atrial slices and known inhibitors of extraneuronal noradrenaline (corticosterone and O-methylisoprenaline{OMI}) to inhibit the uptake of $[^3]$H]-NA.

- To compare the uptake inhibition produced with nandrolone to that induced by corticosterone using this method.

D.3 Hypothesis

Nandrolone will inhibit the extraneuronal reuptake of $[^3]$H]-NA in rat ventricular slices with a similar potency to corticosterone.
D.4 Method

Rats were anaesthetised with halothane, decapitated, the heart exteriorised and excised into ice-cold, pre-carbogen bubbled, calcium-free-Krebs solution. The tissue of interest was then isolated. Left ventricles were cut with a scalpel into slices of 20±2mg. Atria were cut in halves longitudinally. Tissues were then weighed on paraffin film and pre-incubated in 2mls of Krebs containing nialamide (350µM) for 1 hour to inhibit monoamine oxidase (MAO). Tissues were then further incubated in nialamide-free solution, in the presence of cocaine (27µM) and U-0521 (50µM) to inhibit uptake-1 and COMT respectively and in the presence or absence of an uptake-2 inhibitor (corticosterone or O-methyl-isoprenaline) at various concentrations. Finally tissues were incubated for 30 minutes in ³H-NA (see results for concentration) still in the presence of inhibitors of NA re-uptake and metabolism.

At the conclusion of the incubation with ³H-NA, tissues were rinsed for 5s in ice-cold-Krebs to remove extracellular ³H-NA before being transferred to 5ml tubes containing 2ml of HCl (0.1M). Radioactivity was extracted overnight at 4-6°C. One ml of supernatant was then transferred to 5ml of HI-SAFE scintillation fluid for scintillation counting. Uptake was calculated according to the following formula...

\[
uptake - 2 = \left[ \frac{(ST - B) \times 2}{SA} \right] \times \frac{1}{Wt.} \text{ pmol/mg}
\]

ST = radioactivity associated with 1ml of supernatant (DPM - blank)

SA = specific activity (\(SA = \frac{DPM - \text{blank}}{[NA]\text{nmol}}\))

Wt = weight (mg)
D.5 Results

To ensure that the protocol was sufficiently robust to detect noradrenaline uptake it was decided to examine the blockade of uptake-1 by cocaine in rat ventricular slices in the absence of uptake-2 inhibition. Reuptake of $^3$H-NA (1.8μM) was found to be inhibited by 68% in ventricular slices incubated with cocaine (27μM) in comparison to vehicle (saline) treated controls (Fig. D. 1). Uptake associated with left ventricle incubated with corticosterone (25μM) and $^3$H-NA (27μM) was not found to be significantly different from control. Similarly, there was no difference in the uptake of $^3$H-NA from atria and ventricle incubated with corticosterone (85μM) or vehicle when the incubating concentration of $^3$H-NA was 1.8μM. No effect of corticosterone was found on ventricular slices, even with a high ratio of inhibitor : NA (eg. 85μM corticosterone : 8.9mM NA). However, corticosterone (85 μM) was found to significantly reduce $[^3]$H-NA uptake in rat atrial tissue [t(6)=2.669, p<0.05]. The magnitude of the reduction was 22%. Similarly OMI (100μM) reduced $[^3]$H-NA uptake in atrial tissue by 32% [t(6)=3.187, p<0.05].
<table>
<thead>
<tr>
<th>#</th>
<th>Tissue</th>
<th>Noradrenaline (nmol/ml)</th>
<th>n</th>
<th>Inhibitor (nmol/ml)</th>
<th>cocaine</th>
<th>Uptake (pmol/mg) (% inhibition)</th>
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<tr>
<td>1.</td>
<td>Ventricle</td>
<td>1.80</td>
<td>4</td>
<td>-</td>
<td>(+)</td>
<td>1.10±0.12**</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>-</td>
<td>(-)</td>
<td>3.43±0.26</td>
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<td>Ventricle</td>
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<td>12.55±0.81</td>
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<td>4</td>
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<td>6.99x10⁻³ ± 3.4x10⁻⁴#</td>
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<td>8.03x10⁻³ ± 6.7x10⁻⁴</td>
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</table>

Fig. D. 1³H-NA uptake in various isolated rat tissues. Legend: CORT = corticosterone, OMI = O-methyl-isoprenaline. **p<0.001 significantly different from control (no cocaine), #p<0.05 significantly different from vehicle, unpaired t-test.
D.6 Key findings

- Inhibition of extraneuronal uptake (uptake-2) of $[^3]$H-NA was only demonstrated in atria with a high inhibitor concentration and a low noradrenaline concentration. The magnitude of this inhibition is significantly less than that reported in isolated perfused rat hearts. Salt et al. report extraneuronal inhibition of $[^3]$H-NA of greater than 85% with similar inhibitor concentrations in isolated perfused hearts (Salt, 1972).

- Although extraneuronal reuptake of $[^3]$H-NA could be detected using this method it could not be appreciably inhibited in ventricular slices.

D.7 LIMITATIONS

- The difficulty of inhibiting $[^3]$H-NA reuptake with common uptake-2 inhibitors makes this an unsuitable model in which to investigate the potential ability of nandrolone to inhibit extraneuronal reuptake. Personal communication with H. Bonisch (2001) confirmed that he had also been able to show good uptake-2 in ventricular slices, but was unable to inhibit this uptake with common uptake-2 inhibitors. These results were never published.
D.1 References


Appendix E

Effect of anabolic steroids in the CNS
Evidence for a CNS effect

GABA
Micromolar concentrations of stanozolol and 17α-methyltestosterone (17,α-MT) have been shown to significantly inhibit binding of [3H]flunitrazepam (a GABA<sub>A</sub> receptor ligand) to the rat brain cerebrocortical membranes (Masonis et al., 1995).

17, α-MT, stanozolol and nandrolone have been found to induce rapid and reversible modulation of GABAergic currents in neurons of the ventromedial nucleus of the hypothalamus (VMN) and the medial pre-optic area (mPOA) (Jorge-Rivera et al., 2000).

Dopamine
Chronic nandrolone (1,5,15mg/kg/day) treatment of male SD rats has been found to cause significant down-regulation of 5-HT<sub>1B</sub> receptor density in the hippocampal CA1 and in the medial globus pallidus and a significant up-regulation of 5HT<sub>2</sub> receptor density in the nucleus accumbens shell at all nandrolone doses (Kindlundh et al., 2003).

Rats administered nandrolone (3mg/kg, i.m./weekly) for 6 weeks were found to display increased DA and serotonin (5-HT) metabolism in brain regions regulating affective, emotional and motivational behaviour (Thiblin et al., 1999). These changes are likely to reflect an increase in neuronal activity as no effect on synthesis rate or monoamine-oxidase (MAO) enzymatic activity could be detected.

Opioid
Chronic nandrolone treatment of male rats (15mg/kg, i.m./daily/2 weeks) modulates the density of DA receptors in brain regions associated with reward and behaviour (Kindlundh et al., 2001), presumably reflecting an altered dopaminergic activity. Autoradiography revealed specific binding of D<sub>1</sub>-like receptors was significantly down regulated in the caudate putamen and the nucleus accumbens core and shell. D<sub>2</sub>-like receptor densities were downregulated in the nucleus accumbens shell, but upregulated in the caudate putamen, the nucleus accumbens core and the ventral tegmental area.

14 days treatment with an AS cocktail (nandrolone, testosterone and boldenone undecylenate) significantly decreased β-endorphin immunoreactivity in the rostral area of the arcuate nucleus in the rat brain (Menard et al., 1995).

Johansson et al. found that 14 days treatment of male SD rats with nandrolone decanoate (5 or 15mg/kg, i.m./daily) induced a 20-fold increase in β-endorphin in the ventral tegmental area (VTA) at the highest dose of nandrolone (Johansson et al., 1997).

NMDA
Treatment of rats with chronic nandrolone (15mg/kg, i.m./daily) has been demonstrated to increase the immunoreactivity of dynorphin-B and Met-Enkephalin-Arg-Phe (MEAP) in hypothalamus, striatum and periaqueductal grey matter compared to controls (Johansson et al., 2000). Nandrolone was found to cause an imbalance between the dynorphin and enkephalin opioid system in the nucleus accumbens, hypothalamus and periaqueductal grey (PAG). This imbalance persisted after a 3 week withdrawal period.

Le Grevès et al treated SD rats with nandrolone decanoate (5 and 15mg/kg, i.m., daily) for 14 days and investigated the change in expression of the NR1, NR2A and NR2B NMDA receptor subunits (Le Grevès et al., 1997). Using northern blotting it was found that both doses of nandrolone significantly reduced the mRNA of the NR2A subunit in the hippocampus. Conversely, in the hypothalamus the NR2A subunit mRNAs exhibited a significant decrease decrease at both doses. The mRNA level of the NR2B subunit significantly decreased only at the lower nandrolone dose.

Glucocorticoid
Aihima et al observed that in adrenalectomised rats administered a cocktail of anabolic steroids for one week (testosterone cypionate, 2mg/kg, nandrolone 2mg/kg and boldenone, 1 mg/kg, i.m.) that the density of glucocorticoid-receptor immunoreactive cells in the CA1 and granular layer of the dentate gyrus increased significantly in comparison to untreated rats (Aihima et al., 1992). This suggests that AS activated glucocorticoid receptors may regulate the expression of genes whose products play an important role in the cognitive disorders associated with AS abuse.

Substance P
Johansson and colleagues administered nandrolone (15mg/kg/day) to male Sprague Dawley rats for 14 days and found significantly enhanced SP immunoreactivity in the amygdala, hypothalamus, striatum and PAG (Hallberg et al., 2000). Drug withdrawal did not reverse these changes in the PAG.
References


Appendix F

Effect of nandrolone on plasma ET-1
F.1 Introduction

*In vivo* rat models have demonstrated that intracoronary Endothelin-1 (ET-1) can cause cardiac arrhythmia in the absence of cardiac ischaemia (Szabo et al., 2000). Conversely, pre-proET-1 mRNA antisense oligodeoxynucleotide (i.v.) has been shown to be protective against VT and VF during cardiac arrhythmia induced by LAD occlusion resulting in a significant increase in survival (Lin et al., 2002). Recently evidence has begun to emerge which suggests that testosterone may increase the vasoconstrictive effect of ET-1 on porcine coronary vasculature (Teoh et al., 2000). These effects were found to be endothelium and androgen receptor independent. If nandrolone can also increase the vasoconstrictive effect of ET-1 this may explain the significant increase in Lambeth arrhythmia score during ischaemia in rats treated with nandrolone compared to vehicle treated animals.

F.2 Aim

To determine the plasma concentration of ET-1 in post-reperfusion animals.

F.3 Hypothesis

Plasma ET-1 concentration will be significantly increased in nandrolone treated rats compared to vehicle treated animals.

F.4 Method

Following the ischaemia-reperfusion procedure in chapter 5, 3-5ml of blood was collected from the abdominal aorta into heparinised eppendorf tubes from rats which survived cardiac reperfusion and had been treated with either vehicle or nandrolone (80μg/kg/min). Blood was centrifuged and the plasma fractionated off into 0.5ml aliquots and frozen at -20°C until assay. Plasma was assayed for ET-1 using a radioimmunoassay method as detailed elsewhere (Zhang et al., 1998).

The significance of differences between the plasma concentration of ET-1 in nandrolone and vehicle treated animals was determined using a Student’s unpaired t-test.

F.5 Results

No significant difference was found between the plasma concentration of ET-1 in nandrolone and vehicle treated animals (*Fig. F. 1*).
**Fig. F. 1** Plasma ET-1 in rats administered vehicle or nandrolone (80μg/kg/min) following the conclusion of ischaemia/reperfusion. Mean±S.E., n=5-6. *Between group differences:* unpaired Student's t-test: no significant difference.

**F.6 Conclusions & Future Directions**

Although no significant change in plasma ET-1 concentration was noted in the present study, it is possible that any increases in ET-1 which occurred during ischaemia had returned to normal by the time that blood was collected. Collection of blood at time points throughout ischaemia may help determine whether nandrolone can act to increase ET-1 concentration and thus cause an increase in arrhythmia. Further studies are required in order to test whether nandrolone can act to enhance the vasoconstrictive effect of ET-1 in isolated rat coronary vessels, in a manner similar to that of testosterone in porcine coronary artery rings (Teoh et al., 2000).
F.7 References


Fig G.1 Change in A. systolic pressure (SP) relative to the value at 40s prior to the start of infusion of cocaine (0.5mg/kg/min). B. The pressor response to cocaine (first 100s of cocaine infusion). C. The depressor response to cocaine (100-600s of cocaine infusion).

Legend: X saline (100μl/min) + cocaine (0.5mg/kg/min) [S+C], ▽ nandrolone vehicle (70μl/min) + cocaine [ON+C], ○ nandrolone (40μg/kg/min) + cocaine [40N+C], ▼ nandrolone vehicle (70μl/min) + saline (100μl/min) [ON+S], * nandrolone (40μg/kg/min) + saline [40N+S]. The error bars have been removed from figures B. & C. to retain clarity for symbols showing significance levels. Repeated-measures ANOVA, Bonferroni’s post-hoc test. The line at x=100s represents the end of the pressor response to cocaine. SP (0-100s) see Inset A: TREATMENT [F(4,295)=3.790, p<0.01]; TIME [F(5,295)=24.53, p<0.0001]; INTERACTION [F(20,295)=5.866, p<0.0001] *p<0.05, **p<0.01, ***p<0.001 significantly different from S+C; #p<0.05, ##p<0.01, ###p<0.001 significantly different from ON+C; +p<0.05, ++p<0.01, +++p<0.001 significantly different from 40N+C. C. SP (100-600s): TREATMENT [F(4,354)=4.928, p<0.01]; TIME [F(6,354)=19.83, p<0.0001]; INTERACTION [F(24,354)=6.35, p<0.0001] *p<0.05, **p<0.01, ***p<0.001 significantly different from S+C; #p<0.05, ##p<0.01, ###p<0.001 significantly different from ON+C; +p<0.05, ++p<0.01, +++p<0.001 significantly different from 40N+C.
Fig 2. Change in A. diastolic pressure relative to the value at 40s prior to the start of infusion of cocaine (0.5mg/kg/min). B. The pressor response to cocaine (first 100s of cocaine infusion). C. The depressor response to cocaine (100–600s of cocaine infusion).

Legend: X saline (100μl/min) + cocaine (0.5mg/kg/min) [S+C], ▼ nandrolone vehicle (70μl/min) + cocaine [0N+C], ○ nandrolone (40μg/kg/min) + cocaine [40N+C], ■ nandrolone vehicle (70μl/min) + saline (100μl/min) [0N+S], *nandrolone (40μg/kg/min) + saline [40N+S]. The error bars have been removed from figures B. & C. to retain clarity for symbols showing significance levels. Repeated-measures-ANOVA, Bonferroni’s post-hoc test. The line at x=100s represents the end of the pressor response to cocaine. DP (0-100s) see Inset B: TREATMENT [F(4,295)=5.578, p<0.001]; TIME [F(5,295)=25.95, p<0.0001]; INTERACTION [F(20,295)=6.749, p<0.0001]. **p<0.01, ***p<0.001 significantly different from S+C; #p<0.05, ###p<0.001 significantly different from 0N+C; +p<0.05, +++p<0.001 significantly different from 40N+C. DP (100-600s): TREATMENT [F(4,354)=7.99, p<0.0001]; TIME [F(6,354)=30.11, p<0.0001]; INTERACTION [F(24,354)=7.067, p<0.0001] **p<0.01, ***p<0.001 significantly different from S+C; #p<0.05, ##p<0.01, ###p<0.001 significantly different from 0N+C; +p<0.05, ++p<0.01 +++p<0.001 significantly different from 40N+C. Mean±S.E. n=11-14.
HR response to cocaine infusion

Fig G.3 Change in heart rate (HR), relative to the value at 40s prior to the start of infusion of cocaine (0.5mg/kg/min). The error bars have been removed to retain clarity for symbols showing significance levels. Legend: X saline (100μl/min) + cocaine (0.5mg/kg/min) [S+C], ▼ nandrolone vehicle (70μl/min) + cocaine [ON+C], o nandrolone (40μg/kg/min) + cocaine [40N+C], ■ nandrolone vehicle (70μl/min) + saline (100μl/min) [ON+S], *nandrolone (40μg/kg/min) + saline [40N+S]. Repeated-measures-ANOVA, Bonferroni's post-hoc test. HR (0-600s): TREATMENT [F(4,638)=3.227, p<0.05]; TIME [F(11,638)=37.54, p<0.001]; INTERACTION [F(44,638)=5.762, p<0.0001] **p<0.01, ***p<0.001 significantly different from S+C; #p<0.05, ##p<0.01, ###p<0.001 significantly different from ON+C, +p<0.05, ++p<0.01 +++p<0.001 significantly different from 40N+C. Mean±S.E. n=11-14.