Cortisol Perturbation in the Pathophysiology of Septicaemia, Complicated Pregnancy and Weight Loss/Obesity

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Abstract

Cortisol, the principal glucocorticoid secreted from the adrenal glands, is essential for life. Healthy cortisol levels are maintained through negative feedback on the central nervous system (CNS) – pituitary stimulatory apparatus which regulates production of adrenocorticotropin (ACTH) and contains a light–entrained intrinsic CNS driven diurnal rhythm. Cortisol participates in a regulatory mechanism where inflammatory cytokines stimulate cortisol release and cortisol in turn suppresses cytokine release. The effects of cortisol in inflammatory states include elevating blood pressure and metabolic regulation. This thesis contains three exploratory studies examining circulating cortisolaemia using the best available methodologies (total and free cortisol and corticosteroid-binding globulin (CBG)) in clinical states characterized by immune activation/ inflammation and altered blood pressure. These clinical states include: (1) septic shock, (2) hypertensive disorders of pregnancy and (3) obesity-induced hypertension. Prior to the studies described here, little was know about cortisolaemia in these common pathological states.

Septic shock is a life threatening condition that complicates severe infection and is characterized by systemic inflammation and refractory hypotension. High plasma total cortisol levels and attenuated responses to synthetic ACTH stimulation are associated with increased mortality. The use of corticosteroids in septic shock has been highly controversial for decades, however recent trials have reported haemodynamic and survival benefits associated with the use of physiologic steroid replacement in patients with relative adrenal insufficiency (RAI) – currently defined as a total cortisol increment of 248 nmol/L or less following ACTH (250 μg) stimulation. However, CBG and albumin levels fall by around 50% with an increase in plasma free cortisol in critical illness. Hence, total cortisol may not reflect

the biologically active free (unbound) cortisol, suggesting that standard assays for plasma cortisol (which measure total plasma cortisol) underestimate HPA axis activity.

In this study, we have showed that plasma free cortisol is a better guide to circulating glucocorticoid activity in systemic infection than total cortisol. We have also validated the use of Coolens' method in estimating free cortisol in systemic infection, using plasma total cortisol and CBG measurements as plasma free cortisol is not performed in clinical laboratories. Free cortisol measurement allows better categorization of RAI and non-RAI groups with a free cortisol increment of 110 nmol/L as cut-off. Moreover, we have shown that survivors of RAI have normal adrenocortical function on follow-up testing suggesting a lack of functional adrenal reserve rather than adrenal damage during critical illness. Larger randomized controlled trials will be required to redefine RAI using free cortisol measurements and relate that to clinical outcomes and responses to corticosteroid therapy.

Nitric oxide (NO) is normally produced in the endothelium by the constitutive form of the NO synthase and this physiologic production is important for blood pressure regulation and blood flow distribution. Studies have shown that an overproduction of NO by the inducible form of NO synthase (iNOS) may contribute to the hypotension, cardiodepression and vascular hyporeactivity in septic shock. Clinical studies of non-selective inhibitors of the L-arginine nitric oxide pathway showed increased mortality from cardiovascular complications. However, glucocorticoids, which improve vasopressor sensitivity, may act by partially suppressing NO synthesis through selective direct inhibition of iNOS, and suppression of inflammatory cytokine synthesis. Hence, plasma nitrate/ nitrite (NOx) levels may provide a titratable end point to individualize glucocorticoid therapy in sepsis.

The NOx study in this thesis showed that cortisol (total and free), CBG and NOx correlated to illness severity. Free cortisol, and to a lesser extent total cortisol, but not NOx levels, predicted septic shock. NOx levels were characteristically stable within individuals but inter-individual differences were only partly accounted for by illness severity or renal dysfunction. NOx levels correlated weakly with cortisol, did not relate to the need for vasopressors and were not suppressed by hydrocortisone treatment. Thus, NOx is not a suitable target for glucocorticoid therapy in septic shock.

Pregnancy is the only sustained physiologic state of hypercortisolism in humans. A large body of data suggests that excessive foetal and prenatal glucocorticoid exposure leads to reduced birth weight and adverse health in offspring such as elevated blood pressure and insulin resistance. Pre-eclampsia and gamete donor pregnancies are associated with immune activation, elevated inflammatory cytokines as well as elevated blood pressure. Prior to the study described in this thesis however, there was no prospective data on maternal cortisolaemia in these complicated pregnancies.

My study has demonstrated for the first time that there was a substantial fall in plasma CBG levels in the last few weeks of gestation with a corresponding rise in free cortisol in normal pregnancy, a finding obscured for methodological reasons in past studies. This free cortisol elevation in late pregnancy may facilitate organ maturation in the foetus and perhaps prepare the mother for the metabolic demands of labour. In pre-eclampsia and gestational hypertension, plasma CBG, total and free cortisol levels were lower in late third trimester; and in IUGR, plasma CBG levels were suppressed from 28 weeks gestation until delivery but with no significant difference in plasma total and free cortisol. Women with assisted reproduction using donor gametes/ embryos had significantly lower plasma CBG, total and free cortisol levels even in those with normal pregnancy outcomes. Low CBG may be due to reduced

synthesis or enhanced inflammation-driven degradation. Low maternal cortisol may be due to a lack of placental corticotropin-releasing hormone, or reduced maternal ACTH, driving cortisol production. This unanticipated maternal hypocortisolism in complicated pregnancies may trigger precocious activation of the foetal HPA axis and could have implications for postnatal and adult health. Speculatively, since excess prenatal GCs increase HPA axis activity, we proposed that maternal hypocortisolism may predispose to the hypocortisolaemic state characterized by fatigue, pain and stress sensitivity, in offspring.

The third state of immune/ inflammatory activation associated with blood pressure dysregulation studied in this thesis is obesity. The epidemiologic relationship between obesity and hypertension is widely recognised. Central obesity in particular has been associated with exaggerated HPA responses to stimuli. Studies of severe dieting and starvation resulted in hypercortisolism and a significant decrease in CBG. The HPA axis and the renin-angiotensin-aldosterone system (RAAS) have been implicated in the pathophysiology of obesity-induced hypertension. However, there is little data on the effect of moderate weight loss (30% caloric restriction) on adrenocortical function, and the relation of adrenal hormones to altered blood pressure with weight loss.

In this study, measures of HPA axis and RAAS and blood pressure monitoring were performed in twenty-five obese subjects before and after a 12-week diet program (6000kJ/day). Short-term, moderate weight loss (mean 8.5 kg) was associated with a small reduction in blood pressure (mean arterial pressure 6 mmHg) and significantly reduced levels of aldosterone and renin but not cortisol levels. These findings suggest that aldosterone may have an important role in the blood pressure reduction with weight loss via a renin mediated mechanism, perhaps involving renal sympathetic tone. In contrast to severe caloric restriction, HPA axis activation does not occur with moderate weight loss. This suggests a threshold

effect of weight loss on the HPA axis where greater caloric restriction is required for HPA stimulation, or a counterbalancing of central and direct adrenal effects on HPA axis function.

Overall, these three exploratory studies have provided novel data on HPA axis function in systemic infection, pregnancy and in diet-induced weight loss. Each study offers a basis for further studies of HPA axis function in these disorders.

Statement of originality

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

I give consent to this copy of my thesis being made available in the University Library.

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Jui Ting Ho

April 2007

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Publications

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Ho JT, Lewis JG, O'Loughlin P, Bagley CJ, Romero R, Dekker GA, Torpy DJ. Reduced Maternal Corticosteroid-binding Globulin and Cortisol Levels in Pre-eclampsia and Gamete Recipient Pregnancies. *Clinical Endocrinology (Oxf)* 2007 Jun; 66(6):869-77

Ho JT, Keogh JB, Lewis JG, Clifton PM, Torpy DJ. Moderate weight loss reduces renin and aldosterone but does not influence basal or stimulated pituitary-adrenal axis function. *Hormone and Metabolic Research* 2007 (In Press)

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Dorin RI, Qualls CR, Pai HK, **Ho JT**, Lewis JG, Torpy DJ, Urban FK. Comparison of equilibrium estimates of free cortisol: Reversible changes in cortisol affinity for albumin in septic shock and relative adrenal insufficiency.

List of tables and figures

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Abbreviations

ACTH	Adrenocorticotropic hormone
AngII	Angiotensin II
APACHE II	Acute Physiology and Chronic Health Evaluation
AVP	Arginine Vasopressin
BMI	Body mass index
BW	Body Weight
CBG	Corticosteroid-binding globulin
CNS	Central Nervous System
CRH	Corticotropin-releasing hormone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
GC	Glucocorticoids
GH	Gestational hypertension
GR	Glucocorticoid receptor
GRE	Glucocorticoid-response element
HC	Healthy control
hGRα/ β	human Glucocorticoid receptor alpha/ bets
HPA	Hypothalamic-pituitary-Adrenal
HPLC	High performance liquid chromatography
ICU	Intensive care unit
ICV	Intracerebroventricular
IGF	Insulin like growth factor
IL	Interleukin
iNOS	inducible nitric oxide synthase
IUGR	Intrauterine growth restriction
MAP	Mean arterial pressure
MODS	Multiple organ dysfunction syndrome
MR	Mineralocorticoid receptor
MW	Molecular weight
NO	Nitric oxide

NOx	Nitrate/ nitrite
NOS	Nitric oxide synthase
POMC	Pro-opiomelanocortin
PRA	Plasma renin activity
RAAS	Renin-angiotensin-aldosterone system
RAI	Relative adrenal insufficiency
RM-ANOVA	Repeated measures analysis of variance
SEM	Standard error of the meas
SIRS	Systemic inflammatory response syndrome
S	Sepsis
SGA	Small for gestational age
SNS	Sympathetic nervous system
SS	Septic shock
Th1/Th2	T helper cell 1 and 2
TNF-α	Tumour necrosis factor–alpha
11β-HSD_1 & $_2$	11 beta hydroxysteroid dehydrogenase type 1 and 2

Chapter 1

Introduction

1.1 Historical developments

The anatomy of the adrenal glands was first described in 1563 by Bartholomeo Eustachius in his *Tabulae Anatomicae* (Eustachius, 1774). He offered, however, no hypothesis regarding their function. The existence of the suprarenal glands was also mentioned by Archangelus Piccolomineus in 1586 (Piccolomineus, 1586). In 1672, Adrianus Spigelius talked of the "capsulae renales" as merely there to occupy the space between the kidneys and the diaphragm (Baulin, 1588). Later, Nathaniel Highmore added that they might serve to absorb exudates from the large vessels nearby (Highmore, 1651). Progress toward discovering the real function of the adrenals was slight during the 18th and early 19th century. The year 1855 could be regarded as a watershed in the history of endocrinology. Thomas Addison's discovery that the adrenal cortex was essential to survival (Addison, 1855) preceded by nearly a century the demonstration with pure steroids that this gland produced at least two distinct hormones – eventually called glucocorticoids and mineralocorticoids – that are necessary for normal life (Gaunt *et al.*, 1975). During the intervening years many of the actions on glucose metabolism that would characterize glucocorticoids, and on salt and water balance that would characterize mineralocorticoids, were foreshadowed in the symptoms of addisonian patients (Addison, 1855) and adrenalectomized animals (Brown-Sequard, 1856), and in the effects of lipid extracts of adrenal cortex (Hartmann and Brownell, 1930). First ascribed to a single hormone, those actions stirred debate as to which were the most critical for survival. Abnormalities of electrolyte and water balance in addisonian patients and adrenalectomized animals, and the reversal of the abnormalities by administration of salt and adrenal extracts, favoured primacy of control of electrolytes (Gaunt *et al.*, 1975). The accompanying abnormalities of carbohydrate metabolism and their reversal by adrenal extracts led to the

proposal in 1932 that the "prepotent function" of the adrenal gland cortex was control of carbohydrate metabolism, accounting for all actions, including maintenance of life (Britton and Silvette, 1932). This sweeping view soon became untenable, and by 1940 the controversies had been settled by studies with pure glucocorticoids and mineralocorticoids.

Hypothalamic-pituitary relationships became a focus of endocrine research after the postulate of Geoffrey Harris (1948) that hypothalamic factors regulate pituitary hormone secretion. The hypothalamic-pituitary portal circulation was described in great detail by Popa and Fielding (1930), although they believed the direction of blood flow to be from the pituitary to the brain. Houssay et al observed a downward direction of portal blood flow in the living toad (McCann and Nemeroff, 1992).

Adrenocorticotropin (ACTH) was isolated in impure form in 1933 (Collip *et al.*, 1933), and the pure hormone was isolated a decade later (Li *et al.*, 1943; Sayers *et al.*, 1943). In 1955, it was shown that the addition of a hypothalamic fragment or extract to isolated anterior pituitary tissue stimulated ACTH release (Saffran and Schally, 1955). The identification of several hypothalamic releasing factors followed, including thyrotropin-releasing hormone (TRH) (1969), gonadotropin-releasing hormone (GnRH) (1971) and somatostatin (1973) by Guilleman and Schally, using tonnes of fresh animal hypothalamic materials (Medvei, 1993). However, it was not until 1981 that Vale and colleagues, using similar techniques and nearly half a million ovine hypothalami, isolated and determined the structure of ovine corticotropin-releasing hormone (oCRH) (Vale *et al.*, 1983). The amino acid sequence of human CRH (hCRH) was derived from the base sequence of the CRH gene (Shibahara *et al.*, 1983).

Cortisone, the ketone metabolite of the active principle of the adrenal glands, was isolated by Kendall and Reichstein in the late 1940s and shown to suppress immune functions (Raju,

1999). These scientists, along with Hench, received the Nobel Prize in Medicine or Physiology after Hench showed that cortisone produced a spectacular amelioration of rheumatoid arthritis (Hench *et al.*, 1949). The synthesis of cortisone acetate led to its successful use in Addison's disease in that same year. Subsequently, steroids with varying structures were developed for clinical use, and these exhibit a range of glucocorticoid-mineralocorticoid activity.

The concept of stress was introduced by Hans Selye (1907 – 1982) in the 1930's. Bernard (Bernard and Henry, 1949) and Cannon (Cannon, 1932), coined the terms "internal milieu" and "homeostasis" respectively, to introduce the concept of a constant internal environment in living organisms. This internal environment is assiduously maintained despite a multitude of threats, originating internally or externally. Selye observed the pathological effects of chronic stress including peptic ulcer, adrenal hypertrophy and immune impairment. The "general adaptation syndrome" described by Selye was proposed to comprise three stages: (1) the alarm reaction characterized biochemically by acute catecholamine and glucocorticoid hypersecretion; (2) the stage of resistance, with reduced biochemical and behavioural stress response; and (3) the stage of exhaustion, with the emergence of stress-induced disease (Selye, 1947). This has never been demonstrated (Sapolsky, 1992). Recent interest has focused on the responses to qualitatively different stressors, for example different stressors may activate different profiles of stress hormone changes (Rose *et al.*, 1985) and activate different ratios of the hypothalamic ACTH secretagogs - CRH and arginine-vasopressin (AVP) (Plotsky, 1991). Finally, the notion that the hypothalamic-pituitary-adrenal (HPA) axis and the immune system comprise a continuous feedback loop has arisen over the last 20 years; endocrine-immune relations are now a subject of intense investigation.

1.2 Physiology of the Hypothalamic-Pituitary-Adrenal Axis and the Stress System

Glucocorticoids (GCs), the end hormonal products and the final effectors of the HPA axis, regulate a variety of growth, metabolic, developmental, and immune functions and play a pivotal role in preserving basal and stress-related homeostasis (Simpson *et al.*, 1995; Lazar *et al.*, 2003). The integrity of the HPA axis and the precise regulation of its function are prerequisites for the successful response to any stressors. At the level of the hypothalamic-pituitary unit, CRH is released into the hypophyseal portal system and acts as the principal regulator of the anterior pituitary ACTH secretion, which in turn stimulates cortisol secretion from the adrenal cortex (Figure 1.1) (Tsigos and Chrousos, 1994). The binding of CRH on the CRH-R1 receptors of the corticotrophs is permissive for the secretion of ACTH, whereas AVP acts as a potent synergistic factor of CRH with little ACTH secretagogue activity by itself (Abou-Samra *et al.*, 1987). In non-stressful situations, both CRH and AVP are secreted in the portal system in a circadian and highly concordant pulsatile fashion (Chrousos, 1995). The amplitude of the CRH and AVP pulses increases in the early morning hours, resulting consequently in increases of both the amplitude and frequency of ACTH and cortisol secretory bursts in the circulation. Cortisol exerts a negative feedback effect on the secretion of CRH and AVP by the hypothalamus, on the secretion of ACTH by the anterior pituitary, and on suprahypothalamic centres that control the activity of the HPA axis. The inhibition of CRH/AVP and ACTH secretion serve to limit total tissue exposure to cortisol, thus minimizing the catabolic, lipogenic, anti-reproductive, and immunosuppressive effects of these hormones. Therefore, cortisol plays a key regulatory role in maintaining basal and stress-related homeostasis and preserving normal physiology (Simpson *et al.*, 1995; Chrousos *et al.*, 2001).

NOTE: This figure is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1 The hypothalamic-pituitary-adrenal axis, illustrating the major influences on cortisol secretion i.e. circadian factors, stress and negative feedback by cortisol at the hypothalamic and pituitary levels. (Kirk 2000)

The HPA axis and the sympathetic nervous system (SNS) are peripheral limbs of the stress system where CRH is the central integrator of the stress response. Paraventricular nucleus (PVN)-CRH neurons innervate cell bodies of the central control nuclei of the SNS in the brainstem (Saper *et al.*, 1976). CRH stimulates noradrenergic neurons of the locus cerulus (LC-NA system), one of the central control nuclei that regulate arousal (Figure 1.2). (Valentino and Wehby, 1988)

Functionally, the CRH and LC-NA systems seem to participate in a positive, reverberatory feedback loop so that activation of one system tends to activate the other as well (Chrousos,

1995). This includes projections of CRH-secreting neurons from the lateral PVN to the central sympathetic systems in the brainstem, and conversely, projections of catecholaminergic fibers from the LC-NA system, via the ascending noradrenergic bundle, to the PVN in the hypothalamus (Figure 1.2). Thus, CRH stimulates noradrenaline secretion through its specific receptors, while noradrenaline stimulates CRH secretion primarily through α_1-noradrenergic receptors. Autoregulatory, ultrashort negative feedback loops are also present in these neurons, with CRH and norepinephrine collateral fibers acting in an inhibitory fashion on presynaptic CRH and α2-noradrenergic receptors, respectively. The CRH, AVP, and noradrenergic neurons receive stimulatory input from the serotonergic, cholinergic, and histaminergic systems and inhibitory input from the γ-aminobutyric acid (GABA)/benzodiazepine and opioid peptide neuronal systems of the brain (Figure1.2) (Chrousos and Gold, 1992).

NOTE: This figure is included on page 7 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 Major components of the central and peripheral stress system. The paraventricular nucleus and the locus ceruleus/noradrenergic system are shown along with their peripheral limbs, the pituitary-adrenal axis, and the adrenomedullary and systemic sympathetic systems. CRH and central noradrenergic neurons mutually innervate and activate each other, while they exert presynaptic autoinhibition through collateral fibers. AVP from the paraventricular nucleus synergizes with CRH on stimulating ACTH secretion. The cholinergic and serotonergic neurotransmitter systems stimulate both components of the central stress system, while the γ-aminobutyric acid/benzodiazepine (GABA/BZD) and arcuate nucleus proopiomelanocortin (POMC) peptide systems inhibit it. (Chrousos, 1995)

Activation of the stress system leads to adaptive behavioral and physical changes. Centrally, the behavioral changes include enhanced arousal and accelerated motor reflexes, better attention span and cognitive function, decreased feeding and sexual behavior, and increased ability to withstand pain. Peripherally, the activation of the stress system results in increased sympathetic output, that is, increased release of noradrenaline from the sympathetic nerve terminals and adrenaline/ noradrenaline from the adrenal medulla and an increased secretion of glucocorticoids by the adrenal cortex. These changes are related to the physical adaptation that includes changes in cardiovascular function, intermediary metabolism, and modulation of the immune and inflammatory reaction.

1.3 Glucocorticoids

1.3.1 Biosynthesis and molecular actions

Cortisol, the principal glucocorticoid, is synthesized primarily in the zona fasciculata with a small contribution from the zona reticularis of the adrenal cortex. The hormone has a four-ring structure, similar to other steroid hormones, and contains 21-cardon atoms. The inner zones of the adrenal cortex possess the 17 α-hydroxylase enzyme necessary for cortisol synthesis.

Glucocorticoids exert their effects through the glucocorticoid receptor (GR) or mineralocorticoid receptor (MR), which belong to the superfamily of nuclear receptors that function as ligand-dependent transcription factors (Figure 1.3) (Carson-Jurica *et al.*, 1990; Encio and Detera-Wadleigh, 1991). GRs, originally identified in thymocytes (Munck and Brinck-Johnsen, 1968), are found in almost all nucleated cells. Alternative splicing of the human (h) GR in exon 9 generates two highly homologous receptor isoforms, hGRα and

hGRβ, which are identical through amino acid 727, but differ at their carboxyl-termini. hGRα is ubiquitously expressed in almost all human tissues and cells and represents the classic GR that functions as a ligand-dependent transcription factor, whereas hGRβ does not bind GCs and inhibits the transcriptional activity of hGRα in a dose-dependent manner (Hollenberg and Evans, 1988).

In the absence of ligand, hGRα resides mostly in the cytoplasm of cells as part of a large multiprotein complex, which consists of the receptor polypeptide, two molecules of heat-shock protein (hsp)-90, and several other proteins. Upon ligand binding, hGRα dissociates from the hsps and translocates into the nucleus of cells, where it modulates the transcriptional activity of glucocorticoid-responsive genes in either of two ways: by binding to specific sequences in the promoter region of target genes, the glucocorticoid-response elements (GREs), or through protein-protein interactions with other transcription factors, such as nuclear factor (NF)-κB, activator protein-1 (AP-1), and several signal transducers and activators of transcription (STATs) (Jonat *et al.*, 1990; Scheinman *et al.*, 1995). Newly characterized nuclear receptor co-regulators (co-activators or co-repressors) may also enhance or attenuate glucocorticoid signal transduction through different pathways, further accounting for the gene-, cell- and tissue-specific actions of glucocorticoids (McKenna *et al.*, 1999; McKenna and O'Malley, 2002).

The magnitude of a cell's response to GCs depends on the hormone level it is exposed to and its GC sensitivity, i.e. the efficiency of the GR-mediated signal transduction. Changes of tissue sensitivity to GCs may be associated with and influence the course of many pathologic states (Kino and Chrousos, 2001b). Such changes may present as glucocorticoid resistance or hypersensitivity, and may be a generalized or tissue-specific. Primary generalized glucocorticoid resistance has been described as a rare familial or sporadic syndrome mostly

due to inactivating mutations of the GR gene, while several autoimmune/inflammatory states, such as rheumatoid arthritis, osteoarthritis, Crohn's disease, ulcerative colitis and asthma, are often associated with resistance of the inflamed tissues to glucocorticoids (Bamberger *et al.*, 1996; McKenna *et al.*, 1999). In addition, septic shock and respiratory distress syndrome have been associated with systemic glucocorticoid resistance (Meduri *et al.*, 2002). On the other hand, glucocorticoid hypersensitivity has been suggested in visceral obesity-related insulin resistance, and in the acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus Type-1 (HIV-1) infection (Chrousos, 2000c; Kino and Chrousos, 2001a). Cellular sensitivity to GCs can be conferred by the expression of an inactive splice variant, GRβ (DeRijk *et al.*, 2002). Over expression of GRβ is proposed to contribute to GC resistance (Oakley *et al.*, 1999). Further, glucocorticoid receptor variants e.g. N363S and BclI restriction fragment polymorphism, may affect glucocorticoid sensitivity in a tissue-specific manner through altered GR function (DeRijk *et al.*, 2002). Other mechanisms, which potentially cause change of tissue sensitivity to glucocorticoids, may be considered in each step of the GR signalling cascade described above (Figure 1.3).

NOTE: This figure is included on page 10 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3 Shuttling of GRα between the cytoplasm and the nucleus and its transactivating or transrepressive activities. Possible sites of intervention, which may change the activity of GRα are indicated by numbers. GR: glucocorticoid receptor; GREs: glucocorticoid-responsive elements; TFREs: transcription factor responsive elements; HSP: heat shock proteins; TF: transcription factor. (Tomoshige 2005)

Since the discovery that the natural GCs, cortisol and cortisone, have much higher affinity for MRs than GRs, much evidence has indicated that under physiologic conditions some glucocorticoid effects, notably in the hippocampus, are mediated through MRs (Beaumont and Fanestil, 1983; Krozowski and Funder, 1983). Whereas MRs in mineralocorticoid target tissues are "protected" by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD_2), which inactivates cortisol to cortisone, in the hippocampus the enzyme is absent (De Kloet *et al.*, 1998). In uterine and placental cells, 11β-HSD_2 protects GRs from excessive glucocorticoid levels, thus protects the foetus from maternal hypercortisolism in mid-late pregnancy (Burton *et al.*, 1998; Waddell *et al.*, 1998).

GCs have higher affinity for MRs than GRs and therefore at low basal levels GCs occupy mainly unprotected MRs. As levels increase during the circadian cycle, MRs approach saturation and a substantial fraction of GRs become occupied. After stress, glucocorticoid levels may rise sufficiently to nearly saturate the GRs (De Kloet *et al.*, 1998). Hence it has been proposed that MRs mediate glucocorticoid feedback under basal diurnal conditions, but GRs mediate glucocorticoid feedback under stress conditions.

1.3.2 Regulation of cortisol secretion

The secretion of cortisol is regulated predominantly by ACTH from the anterior pituitary under the regulatory influence of hypothalamic CRH. Regulation of ACTH release is subject to three factors: stress, the circadian rhythm, and glucocorticoid negative feedback. Stress responses originate in the central nervous system (CNS) and increase hypothalamic CRH and thus pituitary ACTH secretion. In response to stress, ACTH release is greatly increased; consequently, the secretion of cortisol increases up to five-fold. These responses may attenuate circadian periodicity if the stress is prolonged.

The circadian rhythm is superimposed on episodic secretion; it is the result of CNS events that regulate both the number and magnitude of CRH and ACTH secretory episodes. The circadian rhythm exhibits diurnal rhythmicity and is light-entrained with peak cortisol blood levels attained shortly after waking, typically at ~ 6:00 a.m. to 8:00 a.m. and the lowest concentrations at approximately midnight (Figure 1.4) (Krieger, 1975; Sack *et al.*, 1992; Weibel *et al.*, 1995). Although this general pattern is consistent, there is considerable intra- and inter-individual variability, and the circadian rhythm may be altered by changes in sleep pattern, light-dark exposure, and meal times (Banky *et al.*, 1988). A food-entrained midday surge in cortisol levels occurs in humans. This surge is markedly attenuated by food deprivation (Quigley and Yen, 1979). The genesis of this food entrainment may attribute to neurotransmitter substrates in food, as high protein meals cause a greater surge in cortisol, and gastrointestinal hormones may be involved (Ishizuka *et al.*, 1983).

Figure 1.4 A graphic depiction of circadian rhythm of cortisol over a 24-hour period. (Weitzman 1971)

Regulation of the HPA axis is also linked to the immune system. Physiologic roles for cytokines in control of the HPA axis were first proposed by Besedovsky and Sorkin to account for the observation that an antigen challenge is followed after several days by a rise in cortisol levels coincident with immune activation (Besedovsky and Sorkin, 1977). Immune and nervous systems are envisioned as a link in a negative feedback loop, in which activated immune cells produce cytokines that signal increased immune activity to the brain, thereby stimulating the HPA axis which, through cortisol, suppresses the immune reactions (Besedovsky and Sorkin, 1977). Interleukin-1 (IL-1), IL-6 and tumour necrosis factor–α (TNF-α) have been shown to stimulate ACTH secretion and cortisol inhibits IL-2 synthesis (Rivier *et al.*, 1989; Koenig, 1991).

1.3.3 Cortisol in circulation and the free hormone hypothesis

In plasma, cortisol binds to corticosteroid-binding globulin (CBG) (approximately 70 – 80%) and albumin (10 – 20%). The plasma half-life of cortisol (60-90 minutes) is determined by the extent of plasma binding and the rate of metabolic inactivation. In basal conditions, about 5 - 6% of the circulating cortisol is free, which is generally considered physiologically active. Only the unbound cortisol and its metabolites are filterable at the glomerulus. Beyond cortisol levels which saturate CBG (approximately 400 – 500 nmol/L), plasma free cortisol increases disproportionately, as does cortisol urinary excretion.

The free hormone hypothesis holds that only the unbound, or “free” hormone, is able to interact with the receptor and is thus biologically active (Mendel, 1989). In its simplest form, this hypothesis ascribes to CBG the minor role of carrier protein and reservoir of cortisol. However, there is evidence that CBG has additional functions which will be discussed in the next section.

Two lines of evidence support the hypothesis that free cortisol is the biologically active fraction that enters tissues. First, metabolic clearance rates are inversely correlated with percent bound hormone, i.e. the greater the fraction of bound hormone, the slower the metabolic clearance (Siiteri *et al.*, 1982). This relationship was tested in a human trial; clearance rates of radioactively labelled cortisol were lower in subjects with higher CBG capacity (Bright, 1995). This indicates that cortisol available for metabolism is from the free fraction. If CBG were delivering a significant portion of bound cortisol to tissues, then the degradation rate would be unrelated to CBG capacity, as both free cortisol reaching the liver, and bound cortisol reaching tissues, would be degraded. Perhaps more compelling is the tight regulation of free cortisol levels in humans with abnormal CBG. Three separate mutations in the CBG gene have been shown to have decrease affinity for cortisol or reduced CBG levels (Van Baelen *et al.*, 1982; Emptoz-Bonneton *et al.*, 2000; Torpy *et al.*, 2001). In patients carrying these mutations, total cortisol levels are lower than in control individuals. The free cortisol levels are the same between groups. Therefore, total cortisol secretion is regulated to maintain a given free cortisol level; these data indicate that, in the regulation of total cortisol levels, free cortisol is the physiologically relevant fraction under normal conditions, but they do not rule out a role for a fraction of the large pool of circulating CBG-bound cortisol.

1.3.4 Factors affecting intracellular accumulation of free hormone

The stress response results in an increase in steady-state free cortisol levels via at least three different mechanisms: (1) enhanced synthesis, (2) decreased protein binding secondary to reduced expression of binding proteins, and (3) reduced elimination.

Hormone production. As mentioned earlier, cortisol production increases in response to stress. However, there is a poor correlation between cortisol production rates and total circulating cortisol levels. Interestingly, the correlation is greatly improved when total cortisol

levels are corrected for plasma CBG concentration (Bright, 1995). Approximately 70 - 80% of circulating cortisol is nonlinearly bound to CBG. Consequently, the fraction of free cortisol increases as total plasma cortisol levels increase.

Hormone elimination. The metabolic clearance of cortisol is characterized by two critical steps, the conversion to cortisone catalysed by the action of 11β-HSD_2 primarily in the kidneys, and the enzymatic reduction that takes place in the liver of both cortisol and cortisone to tetrahydrocortisols and tetrahydrocortisone, respectively, which are the end metabolites of glucocorticoids secreted in the urine. An interesting aspect of the metabolism of glucocorticoids is the recent data about the significance of the type 1 11β-HSD_1, an isoform expressed primarily in the liver, adipose tissue, kidneys, and brain. 11β-HSD_1 *in vivo* promotes the exact opposite chemical reaction compared to 11β-HSD_2 and acts as a reductase, catalyzing the conversion of cortisone to cortisol in humans and thus locally augments the concentration of the bioactive hormone. This tissue-specific amplification of the cortisol action, mediated by 11β-HSD_1, is considered an important factor that links tissue sensitivity to glucocorticoids to the pathophysiology of the HPA axis hyperactivation, especially under conditions that have been proposed to potentiate 11β-HSD_1 activity, such as visceral obesity (Seckl *et al.*, 2004). Although transgenic mice overexpressing 11β-HSD_1 develop a visceral obesity/ metabolic phenotype, the relevance of this to human obesity is controversial (Masuzaki *et al.*, 2001; Tomlinson *et al.*, 2004b).

P-Glycoprotein as a determinant of intracellular cortisol accumulation. It was thought previously that glucocorticoids move freely into and out of the cell by simple diffusion only. However, recent data demonstrated that many immune response effector cells (e.g., $CD4^+$ T cells, $CD8^+$ T cells, and natural killer [NK] cells) express a member of the large ATP-binding cassette superfamily of transport proteins also called traffic ATPases, P-glycoprotein (P-gp),

in the cell membrane that impedes cellular penetration of various small molecules (Chaudhary *et al.*, 1992; Drach *et al.*, 1992). Further, it has been shown that P-gp expression is capable of reducing intracellular GC penetration resulting in reduced GC binding and transactivating potency (Yates *et al.*, 2003).

While there is continued support for the free hormone hypothesis, there is evidence for more complex actions of CBG at the systemic and cellular levels. Many of these more complex actions allow for CBG to contribute to tissue distribution of cortisol, instead of solely regulating the amount of free cortisol that is available to all tissues.

1.4 Corticosteroid-binding globulin

Corticosteroid-binding globulin (CBG) is a 50-60 kDa glycoprotein with high cortisol-binding affinity (Kd ~ 10^{-8}). Human CBG gene, a 19 kb gene on chromosome 14 region q31-q32.1, encodes a 405 amino acid molecule that has a 22 amino acid signal peptide cleaved before secretion, resulting in a 383 amino acid glycoprotein (Hammond, 1990). The gene is also expressed in kidney, placenta and pancreas (Seralini *et al.*, 1990; Strel'chyonok and Avvakumov, 1990). CBG is a member of the serpin 260 superfamily but, rather than being a protease inhibitor, it is a substrate of elastase (Avvakumov and Strel'chyonok, 1988). One characteristic of these proteins is that they are cleaved by the serine proteases that they inhibit. Activated neutrophils at sites of inflammation carry and secrete serine proteases. Upon cleavage, CBG is thought to undergo a dramatic conformational change leading to release of bound cortisol.

1.4.1 Physiology of CBG

CBG is traditionally considered to have a transport role, distributing water-insoluble cortisol throughout the circulation, a buffer role perhaps blunting elevations of free cortisol during a secretory surge, or a reservoir function acting as a pool of cortisol during times of reduced cortisol secretion. Two lines of evidence have suggested a specific-tissue cortisol delivery role for CBG. These include the finding of a specific CBG interaction with human leukocyte elastase (HLE) and evidence for the presence of CBG receptors. HLE specifically cleaves CBG at the 344-345 residue leading to loss of a 39 amino acid C-terminal fragment and almost complete loss of CBG:cortisol binding affinity (Pemberton *et al.*, 1988). Hence, CBG may play a role in delivering cortisol specifically to inflammatory sites (Hammond, 1990). One report of specific, saturable, high-affinity CBG binding sites in prostate tissue suggested the presence of a cell surface CBG receptor, but no CBG receptor has been cloned (Hryb *et al.*, 1986). Recently, endocytic uptake of sex hormone binding globulin (SHBG)-sex steroid complexes by megalin, an LDL receptor analogue has been described (Hammes *et al.*, 2005). It is not known if similar mechanism applies to CBG-cortisol. If present, such a receptor may play a role in tissue specific CBG-cortisol delivery. Immunocytochemical detection of CBG has been reported in pituicytes and co-staining with ACTH suggests CBG is present selectively in corticotrophs (Perrot-Applanat *et al.*, 1984). Reports of intracellular localization of CBG and differential expression of CBG mRNA according to gestational stage in various tissues have led to the proposition that CBG may play a role in modulating access of cortisol to the glucocorticoid receptor, thereby altering tissue glucocorticoid sensitivity (Perrot-Applanat *et al.*, 1984; Challis *et al.*, 1995).

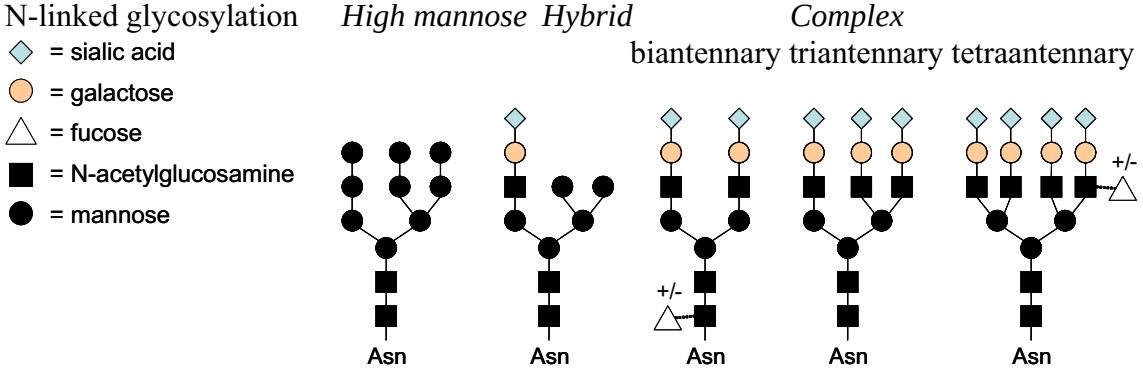
Levels of CBG are increased by oestrogen and pregnancy and decreased by insulin, or by glucocorticoids, such as prednisolone or in Cushing's syndrome (Hammond *et al.*, 1987; Schlechte and Hamilton, 1987; Hammond *et al.*, 1990). The effects of glucocorticoids on

CBG synthesis are glucocorticoid receptor dependent and those of oestradiol (and mitotane) are oestrogen receptor-α dependent (Cole *et al.*, 1999; Fernandez-Real *et al.*, 2001). High affinity CBG-cortisol binding is saturated beyond cortisol levels of 400 – 500 nmol/L hence the free cortisol levels rise exponentially at high cortisol concentrations (Ballard, 1979).

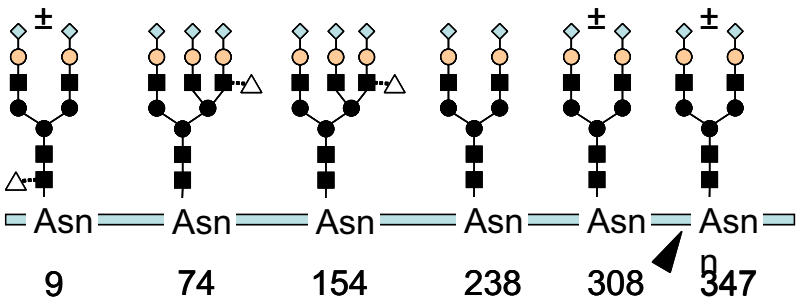
1.4.2 CBG glycosylation

Protein glycosylation, which involves a complex series of reactions catalysed by membrane bound glycosyltranferases and glycosidases in the endoplasmic reticulum and Golgi apparatus, is important for normal function of many proteins (Avvakumov and Hammond, 1994). CBG has six sequons that direct attachment of N-linked oligosaccharides. On average five of these sites are occupied with a mixture of bi- and tri-antennary oligosaccharides that are normally present in a 3:2 ratio (Figure 1.5). Glycosylation of Asn238 (site 4) is known to be important for acquisition of affinity for cortisol, either by constituting part of the binding site or by promoting correct folding of the protein (Avvakumov *et al.*, 1993). The role of sites of glycosylation other than Asn238 is poorly understood but it is known that glycosylation changes dramatically in the maternal blood during pregnancy (Strel'chyonok *et al.*, 1984; Benassayag *et al.*, 2001).

Figure 1.5
A) Protein N-Linked Glycosylation



B) Cartoon of glycosylation of CHO-cell-derived CBG adapted from Avvakumov *et al.* Site of cleavage by elastase is arrowed and partially occupied sites are indicated (±). Fucose is present at sub-stoichiometric levels.



1.4.3 CBG in Pregnancy

During pregnancy, increased placental production of oestrogen stimulates hepatic production of CBG, contributing to a 2-3 fold rise at term. CBG glycosylation shifts to a predominantly triantennary pattern in pregnancy with increased sialic acid groups, slowing clearance via the liver sialo-glycoprotein receptor (Strel'chyonok and Avvakumov, 1990). It has been proposed that non-lectin binding pregnancy-specific CBG (pregCBG) glycoforms may facilitate transplacental cortisol transport based on glycoform-specific enhanced syncytiotrophoblast membrane binding (Mitchell *et al.*, 2004). As pregCBG was still readily detectable in maternal serum up to 40 days post-partum (Figure 1.6), it has been suggested that pregCBG is more likely to be maternally derived rather than placental, or the half-life of the variant in the circulation was significantly longer than that of normal CBG (5 days).

NOTE: This figure is included on page 20 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.6 Demonstrating the change in pregCBG during gestation, and CBG concentrations in the postpartum period.(Strel'chyonok and Avvakumov, 1990)

1.4.4 CBG in stress and obesity/ insulin resistance

CBG participates actively in the stress response. Immune activation liberates IL-6 which stimulates cortisol secretion via activation of the hypothalamic CRH neuron (Papanicolaou *et al.*, 1998), and directly inhibits CBG gene transcription thereby increasing the free cortisol fraction and circulating glucocorticoid activity (Emptoz-Bonneton *et al.*, 1997; Tsigos *et al.*, 1998). *In vivo*, exogenous IL-6 reduces CBG concentrations by 50% in humans, and specific illnesses such as burns, sepsis and cardiac surgery are associated with similar falls in CBG levels, which correlate with the extent of IL-6 elevation (Zouaghi *et al.*, 1983; Emptoz-Bonneton *et al.*, 1997; Bernier *et al.*, 1998; Ho *et al.*, 2006).

A recent CBG knockout mouse model demonstrated a phenotype of fatigue and reduced activity levels, and interestingly, reduced survival following bacterial lipopolysaccharide

injection, perhaps related to reduced activity of hepatic glucocorticoid target genes and increased inflammatory mediators (Petersen *et al.*, 2006). Although there is no observed increased susceptibility of infection in human studies, these results suggest an effect of CBG or cortisol/CBG at specific tissue interfaces.

Obesity is associated with increased HPA function with elevations of cortisol secretion rates (Douyon and Schteingart, 2002), and reduced plasma CBG, perhaps due to a direct effect of insulin and pro-inflammatory cytokines on CBG gene transcription (Fernandez-Real *et al.*, 2002). Severe acute caloric restriction reduces CBG levels (Yanovski *et al.*, 1997) suggesting that CBG levels may fall with weight loss, thereby increasing circulatory free cortisol and predisposing to weight regain. Although epidemiological studies reveal a negative correlation between obesity and insulin levels and plasma CBG (Fernandez-Real *et al.*, 2002), little is known of the individual response of plasma CBG or cortisol levels to moderate diet-induced weight loss.

1.5 Physiologic actions of cortisol

Cortisol plays an important role in the metabolism of carbohydrates, lipids and protein, and has essential regulatory effects on immune and circulatory function.

1.5.1 Metabolic effects

The primary action on carbohydrate metabolism is to increase glucose production via effects on gluconeogenesis, and inhibit cellular uptake of glucose by inducing peripheral insulin resistance. The rate of gluconeogenesis is controlled principally by the activities of certain enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose-6-phosphatase. The genes encoding these proteins are controlled at the

transcriptional level by key hormones, particularly insulin, glucagon and glucocorticoids (Pilkis and Granner, 1992). Recently, a transcriptional coactivator, PGC-1, has been shown to be a key modulator of PEPCK promoter and may be a central therapeutic target of the insulin-cAMP axis in liver (Yoon *et al.*, 2001).

Cortisol also influences fat metabolism, activating lipolysis and inhibiting glucose uptake by the adipocytes. Excess cortisol will inhibit protein synthesis and activate proteolysis in muscles, liberating amino acids that can serve as substrates for gluconeogenesis (Pilkis and Granner, 1992). Cardiac muscle and the diaphragm are spared from this catabolic action. Cortisol is also involved in bone and mineral metabolism, activating osteoclasts, inhibiting osteoblasts, decreasing intestinal calcium uptake and increasing calcium urinary secretion by decreasing its renal reabsorption (Orth *et al.*, 1998).

1.5.2 Immunological and anti-inflammatory effects

Cortisol inhibits most immunologic and inflammatory responses. Excess cortisol secretion is followed by a fall in circulating lymphocytes, which results from the passage of lymphocytes from the circulation toward lymphoid organs (e.g. spleen, adenopathies, thoracic canal). The opposite effect is observed with granulocytes, which accumulate in blood circulation, whereas neutrophil migration toward inflammatory sites is inhibited (resulting from decreased secretion of chemokines), which contributes to a decreased local inflammatory reaction (Chrousos, 2000b). Macrophage secretion is inhibited by the production of migration inhibitory factor (Beishuizen and Thijs, 2001). Furthermore, cortisol stimulates eosinophil apoptosis (Beishuizen and Vermes, 1999).

Cortisol also inhibits the production of IL-12 by macrophages and monocytes, therefore influencing lymphocyte differentiation by acting on the Th1/Th2 balance (Chrousos, 2000b).

Cortisol also modulates the cytokine response observed during inflammation. On the cellular level, this action is mediated by the inhibition of the production and of the activity of proinflammatory cytokines (IL-1, IL-2, IL-3, IL-6, interferon-γ, TNF-α), chemokines, eicosanoids, bradykinin and migration inhibitory factor (Soni *et al.*, 1995; Beishuizen and Thijs, 2001, 2003). This inhibition results both from the direct interaction between the glucocorticoid-receptor complex and the GC responsive elements located on DNA, and from the inhibition of transcription factors such as nuclear factor-κB and activator protein-1 (Figure 1.7) (Almawi and Melemedjian, 2002). Simultaneously, cortisol stimulates the production of anti-inflammatory factors such as IL-1 receptor agonist, the soluble TNF receptor, IL-10 and transforming growth factor-β (Franchimont *et al.*, 1999). This anti-inflammatory activity is completed by inhibition of the production of cyclo-oxygenase-2 and of the inducible nitric oxide (NO) synthase, which are key enzymes in inflammation (Chrousos, 1995).

NOTE: This figure is included on page 24 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.7 Interaction between NF-κB and the activated GR. Cortisol or exogenous GCs freely cross into the cytoplasm and bind to specific GRs (GRα) to form the activated receptor (GC-GRα). GC-GRα complexes may influence NF-κB activity in five major ways: (1) physically interacting with the p65 subunit with formation of an inactive (GC-GRα/NF-κB) complex, (2) inducing the synthesis of the inhibitory protein IκBα via interaction with GC-responsive element DNA in the promoter of the IκB gene, (3) blocking degradation of IκBα via enhanced synthesis of IL-10, (4) impairing TNF-α-induced degradation of IκBα, and (5) competing for limited amounts of GR coactivators such as CREB-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1). GC-GRα may also decrease the stability of mRNA of several proinflammatory cytokines and other molecules. (Meduri *et al.*, 2002)

1.6 Cortisol and blood pressure Cortisol is critically involved in blood pressure regulation. This is exemplified in pathological conditions of cortisol deficiency and excess. Inadequate secretion of corticosteroids in Addison's disease causes life-threatening hypotension, whereas cortisol excess in Cushing's syndrome invariably elevates blood pressure.

Mechanisms of cortisol action on blood pressure

1.6.1 Salt and water homeostasis

This mechanism of GC-induced hypertension has been elucidated over the past two decades with the discovery of the 11β-HSD_2 enzyme (Ferrari and Krozowski, 2000). The non-selective mineralocorticoid receptor (MR) has the same *in vitro* affinity for its physiological substrate aldosterone as for the GC cortisol. However, circulating levels of cortisol are 100–1000-fold higher than those of aldosterone. The MR is protected from GC occupation by the 11βHSD_2, a gate-keeping enzyme, which converts cortisol to receptor-inactive cortisone. Kinetic properties of 11βHSD_2 suggest that the enzyme is saturated at high-normal physiological plasma cortisol levels (Ferrari, 2003). As a result, stimulation of the MR by cortisol leads to renal sodium retention, volume expansion and consequently an elevated blood pressure.

Significant sodium retention accompanies the rise in blood pressure when cortisol was given to normal male subjects. In keeping with this positive sodium balance and volume expansion, plasma renin, angiotensin II (AngII) and aldosterone concentrations were reduced and atrial natriuretic peptide is increased (Connell *et al.*, 1987; Williamson *et al.*, 1996). Despite these changes, the blood pressure elevating effect of cortisol is not primarily mediated by sodium retention, as demonstrated by co-administration of the type 1 (mineralocorticoid) receptor antagonist, spironolactone at 400 mg/day, a dose that antagonizes the sodium retaining effects of the synthetic mineralocorticoid 9'-fludrocortisone at 3 mg/day. Blood pressure rose with cortisol and spironolactone despite adequate type 1 receptor blockade (Williamson *et al.*, 1996). Further, the blood pressure rise produced by the synthetic steroids prednisolone, methylprednisolone, triamcinolone, and dexamethasone was not accompanied by urinary sodium retention or increased plasma volume (Whitworth *et al.*, 1989). Thus, while sodium

retention amplifies the hypertensive effects of cortisol, it is not the primary mechanism by which hypertension occurs.

1.6.2 Vascular tone

In addition to the effects on fluid and salt homeostasis in the kidney, cortisol increases vascular tone, thereby increasing peripheral resistance, an important determinant of blood pressure. This is exemplified in Addisonian crisis, where a complete lack of adrenal steroids results in vascular collapse aggravating the fluid depleted state and thereby contributing to life-threatening hypotension. In animal models, intravenous administration of hydrocortisone was shown to enhance vascular resistance in response to adrenaline, suggesting a permissive role of glucocorticoids for catecholamine action (Kadowitz and Yard, 1971; Yard and Kadowitz, 1972). In keeping with these findings, hydrocortisone administration in septic shock, an inflammatory state where relative adrenal insufficiency might be a contributing factor, reduced the time to cessation of vasopressor therapy in humans (Annane *et al.*, 1998; Briegel *et al.*, 1999).

Glucocorticoid receptors are widely distributed in the vasculature and, in the rat, 11βHSD is distributed in resistance vessels where it is appropriately sited to modulate access of glucocorticoids to vascular receptors (Walker and Edwards, 1991). Vascular tone is dynamically modulated by vascular smooth muscle cells (VSMCs) according to their state of contraction. *In vitro*, glucocorticoids have been shown to augment aortic contraction in response to catecholamines and angiotensin II by the upregulation of α1B-adrenergic and type I angiotensin II (AT_1) receptors in VSMCs (Sakaue and Hoffman, 1991; Ullian *et al.*, 1996). Both GRs and MRs have been identified by means of ligand binding and expression studies in VSMCs (Scott *et al.*, 1987). However, the role of the GR and MR in mediating these effects is unknown.

More recently, vascular endothelial cells (Ecs) have been recognised as another glucocorticoid target for modulating vascular tone. Ecs release nitric oxide (NO), which is a potent vasodilator relaxing juxtaposed VSMCs. In cortisol-treated humans, there was significant reductions in plasma nitrate/ nitrite (Nox) concentrations (Kelly *et al.*, 1998b). Physiological study of the NO system demonstrated a reduction on acetylcholine-induced vasodilatation in the forearm vascular bed following infusion of the NO synthase antagonist N^G-monomethyl-L-arginine (Mangos *et al.*, 2000). Glucocorticoids have complex effects on the NO system *in vitro*, including inhibition of inducible nitric oxide synthase (iNOs) and endothelial eNOS isoforms, inhibition of transmembrane arginine transport and synthesis of NO synthase cofactor tetrahydrobiopterin (Radomski *et al.*, 1990; Simmons *et al.*, 1996). While the iNOS has not been implicated in the basal regulation of vascular tone, inhibition of transmembrane arginine transport or *de novo* intracellular arginine synthesis are possible mechanisms by which vascular NO system activity and thus, elevate blood pressure (Whitworth *et al.*, 2002).

1.6.3 Heart

The heart is a corticosteroid target organ expressing GR, MR and 11β-HSD_1 (Kayes-Wandover and White, 2000). Cortisol treatment increases cardiac output, measured using Flick technique or Doppler echocardiography. Heart rate is unchanged and the increase in cardiac output is primarily due to an increase in stroke volume accompanying the expansion of plasma volume. However, pre-treatment with the β-adrenoreceptor antagonist atenolol prevents the increase in cardiac output, but does not prevent the rise in blood pressure, suggesting that cortisol-induced hypertension is not dependent on increased cardiac output (Pirpiris *et al.*, 1993). While no cardiac-specific glucocorticoid-mediated effects have been demonstrated with regard to blood pressure regulation, corticosteroids play a major role in

cardiac remodelling. Excessive MR activation by aldosterone causes cardiac hypertrophy and fibrosis. In keeping with these deleterious effects, conversely, MR blockade in patients with acute or chronic left ventricular failure drastically reduces hospitalisation and mortality (Pitt *et al.*, 2003). The physiological role of 11β-HSD_1 in the heart remains unclear.

1.6.4 Brain

The central nervous system is another potential site of blood pressure regulation by corticosteroids. GR, MR and 11β-HSD_1 are widely expressed in the brain, whereas 11β-HSD_2 expression is much more restricted (Seckl, 1997). Thus the majority of MRs in the brain seem to be permanently occupied by glucocorticoids and only spatially protected from glucocorticoids by 11β-HSD_2 (De Kloet *et al.*, 2000). Selective activation of MRs in the brain by intracerebroventricular (ICV) infusions of aldosterone increases salt appetite and central sympathetic nervous system drive to the periphery, thereby producing hypertension through multiple mechanisms (Gomez-Sanchez, 2004). By contrast, ICV infusion of corticosterone alone does not alter blood pressure but reverses aldosterone-induced hypertension, suggesting an antagonistic effect of glucocorticoids (Gomez-Sanchez *et al.*, 1990). These studies show that the brain regulates corticosteroid-mediated blood pressure through regulation of salt appetite and sympathetic nervous activity.

1.7 Stress and the adaptive response

Stress is a state of threatened homeostasis caused by intrinsic or extrinsic adverse forces and is counteracted by an intricate repertoire of physiologic and behavioural responses that aim to re-establish the challenged body equilibrium (Selye, 1947). Crucial functions of the stress system response are mediated by the HPA axis and the central and peripheral components of the autonomic nervous system. The adaptive response to stress is determined for every

individual by a multiplicity of genetic, environmental, and developmental factors. Alterations of the ability to respond to stressors, as for example, inadequate, excessive, and/or prolonged reactions, may lead to disease. Moreover, excessive and/or chronically imposed stressors may have an adverse impact on a variety of physiologic functions, such as growth, reproduction, metabolism, and immunocompetence, as well as on personality development and behaviour (Chrousos, 1998; Phillips, 2001). Thus, chronic stress can be defined as a pathologic state of prolonged threat to homeostasis by persistent or frequently repeated stressors and is considered a significant contributing factor in the pathophysiology of manifestations that characterize a wide range of diseases and syndromes. In addition to duration, the timing of major stressful events is also a decisive parameter and may contribute to pathology independently, especially if placed early in the life course. Prenatal life, infancy, childhood, and adolescence are periods of increased vulnerability to stressors and increased plasticity for the stress system, and therefore critical in the evolving process of forming the individual's general matrix of adaptive stress response (Phillips, 2001).

Failure of the stress system to establish both an adequate and time-limited response to severe or chronic stress, if not lethal, leads progressively to a shift of the internal milieu outside the genetically and environmentally programmed homeostatic range, toward a new state of equilibrium dictated by the imposed stressors and described as an *allostatic state*. The process leading to this re-established state, *allostasis*, aims to achieve viability under the altered conditions, but in the long term the individual that remains in an allostatic state suffers cumulative deleterious consequences (*allostatic load*) caused by both the persistent stressor and the prolonged duration of the adaptive response (Fig. 1.8) (McEwen, 1998; Chrousos, 2000a; Charmandari *et al.*, 2003). For example, the metabolic syndrome which is characterized by central obesity, insulin resistance, dyslipidaemia, and hypertension can be described as a state of deranged metabolic homeostasis. Obesity, a systemic low grade

inflammatory condition, seems to constitute a chronic stressful state that may cause HPA axis hyperactivation contributing to the derangement of the metabolic equilibrium.

NOTE: This figure is included on page 30 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.8 Homeostatic regulation curves. A homeostatic system, such as that between glucocorticoids and a glucocorticoid response, has an inverse U-shaped dose-response curve relation, in which there is an optimum effect range in the middle and suboptimal actions in the two sides. A homeostatic system is in *eustasis* when it operates optimally and in *allostasis* when it operates suboptimally. Allostasis signifies failure of attaining homeostasis and an adaptive alteration in a system e.g. elevated blood pressure, which may form part of an allostatic load leading to morbidity and mortality. (Charmandari 2003)

The HPA axis is one of the most important allostatic (or adaptive) systems; when active, it allows the organism to respond to a challenge or stressful condition, but its inactivation is equally important, since the allostatic load can over exposed the organism to mediators of

neural, endocrine and immune stress, with different pathophysiological consequences. The effect of aging on adrenal steroidogenesis is well recognised, this mainly affects adrenal androgens dehydroepiandrosterone (DHEA) and its sulphate (DHEAS) (Parker *et al.*, 2000). Much evidence suggests that the primary defect in allostatic systems in aging might be a prolonged response to stressful conditions, due to the inability to shut off the allostatic response after the end of the stress. Consequently, the HPA axis would become less resilient with age in responding to stimuli (Ferrari *et al.*, 2001). However, in spite of the subtle age-related changes of HPA function, the circulating glucocorticoid levels are relatively constant or even show a trend toward an increase with age. Both plasma cortisol and ACTH usually fall within normal ranges in physiological aging, but a trend toward higher nocturnal levels is often reported. Further, it has been shown that the cortisol response to ACTH stimulation is similar in older and younger subjects (Parker *et al.*, 2000).

1.7.1 Cortisol and stress

Glucocorticoids are involved in almost every physiologic, cellular, and molecular process. Hence, dysregulation of HPA axis activity and/or altered tissue sensitivity to glucocorticoids may lead to disease (Chrousos *et al.*, 1998; Charmandari *et al.*, 2003).

During stress, adrenal hormone biosynthesis is characterized by a shift away from aldosterone and androgens towards cortisol production. Hence, there is a relative fall in aldosterone production while renin levels rise. However, the clinical significance and consequences of this relative fall in aldosterone secretion is unknown, and mineralocorticoid treatment in sepsis remains controversial. These events are associated with an attenuation of the circadian rhythm of cortisol secretion secondary to an increase in CRH and ACTH production, stimulated by inflammatory cytokines, vagal stimulation and reduced cortisol negative feedback (Chrousos, 2000b).

The pleiotropic effects of cortisol, described earlier, are directed to the maintainence of homeostasis during acute stress. Metabolic effects, such as hyperglycaemia, increase energy substrates during a period that requires increased metabolism and transfer of available glucose to insulin-independent cells (e.g. central nervous system, inflammatory cells). Cardiovascular effects increase and re-direct blood flow. Cortisol counteracts almost every step of the inflammatory cascade, modulating the immune response such as shifting from a Th1 to Th2 helper T cell response. These different mechanisms are integrated in the adaptive response to stress. Inflammatory cytokines (e.g. TNF-α, IL-6, IL-1) acutely activate the hypothalamic–pituitary axis, which reduces the inflammatory response; cortisol, in turn, exerts a negative feedback on the hypothalamic–pituitary axis, eventually terminating the immunosuppressive and catabolic effects of cortisol (Tsigos and Chrousos, 2002). However, over time, prolonged exposure to cytokines might lead to an altered response of the HPA axis. In these states, dissociation between central activation of the HPA axis and the adrenal cortex may occur (Bornstein and Chrousos, 1999). Thus, frequently, ACTH levels are low relative to elevated concentrations of cortisol, perhaps reflecting hypertrophy/ hyperplasia of the adrenal gland. This dissociation suggests a long-term reset of the HPA axis maintained by reduced cortisol negative feedback at the brain/ pituitary and extra-pituitary mechanisms of adrenal regulation that can be both neural and immune (Ehrhart-Bornstein *et al.*, 1998; Bornstein and Chrousos, 1999).

The concentration of circulating cortisol that is 'normal' for the response to stress depends on the nature and intensity of the stressor as well as tissue glucocorticoid sensitivity which may be altered by environmental factors, such as cytokines, or genetic factors such as those known to influence glucocorticoid receptor function.

Dysregulation or altered regulation of the HPA axis has been implicated in the pathophysiology of many of the major human disorders, including psychiatric, metabolic, inflammatory and autoimmune states. This thesis will focus on three states of altered cortisol secretion in association with immune activation and blood pressure dysregulation. These include (1) septic shock, characterized by hypotension and cortisol hypersecretion; (2) pre-eclampsia and gestational hypertension, characterized by hypertension in the setting of pregnancy-induced cortisol hypersecretion and (3) the weight loss induced blood pressure fall, where cortisol and/ or aldosterone may have a role (Figure 1.9). These three conditions are associated with immune activation which can affect the HPA and RAAS axes, circulating free hormone concentrations and tissue sensitivity to glucocorticoids. Therefore, the overall aim of this thesis is to study the effects of these inflammatory conditions on the HPA axis in the setting of abnormal blood pressure regulation.

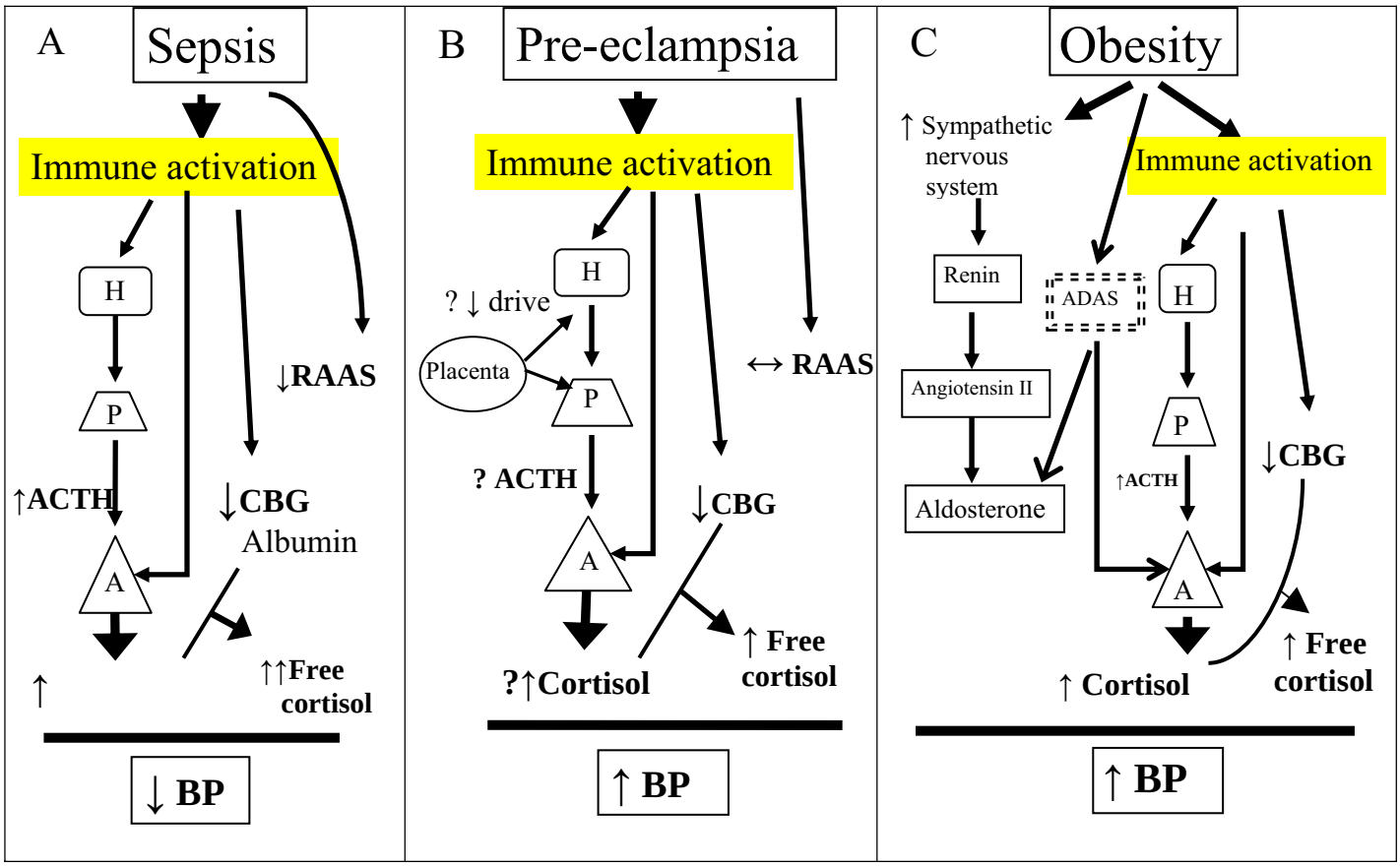


Figure 1.9 Schematic diagrams showing three states of altered cortisol secretion associated with immune activation and blood pressure dysregulation. Study of the HPA axis under these conditions forms the basis of this thesis.

(Abbreviations: H-Hypothalamus, P–Pituitary, A-Adrenal, RAAS–renin-angiotensin-aldosterone system, CBG–corticosteroid-binding globulin, ADAS–adipocyte derived adrenostimulatory factor, BP – blood pressure)

1.7.2 Cortisol and sepsis

My studies in this area will be discussed in Chapters 3 and 4. Septic shock is a clinical syndrome that complicates severe infection and has a mortality of 50%. The initial phase of sepsis is associated with intense inflammatory activity, secondary to the identification of infectious components by the immune system. The anti-inflammatory effects of glucocorticoids led to their application in sepsis. The first trials of corticosteroid therapy in severe sepsis were done with short-term (< 24 hours), extremely high doses glucocorticoid dosing e.g. 40,000 mg hydrocortisone equivalent in a single dose. Those trials did not show any benefit on duration of shock or survival (Bone *et al.*, 1987; No authors, 1987). A meta-analysis concluded that glucocorticoids have no favourable effects on morbidity and mortality in severe sepsis, and even suggested an increased risk for superinfection-related death (Cronin *et al.*, 1995).

However, ongoing positive anecdotal experience and a new concept of relative adrenal insufficiency (RAI) in septic shock led to reconsideration of the use of glucocorticoids. Absolute adrenal insufficiency is rare among critically ill patients. Suboptimal cortisol production during septic shock has been termed “functional” or “relative” adrenal insufficiency (Zaloga, 2001). Conceptually RAI is defined as an inadequate cortisol response to severe illness associated with rapid clinical and haemodynamic improvement following stress-dose (200 - 300 mg hydrocortisone per day) glucocorticoid therapy (Rothwell *et al.*, 1991; Annane *et al.*, 2000; Beishuizen and Thijs, 2001; Zaloga, 2001; Ligtenberg and Zijlstra, 2004). The presence of “relative” adrenal failure was associated with a significant increase in mortality (Briegel *et al.*, 1996). A multicentre, randomised, prospective study in septic shock patients showed that the use of stress-dose corticosteroids significantly improved 28 day survival and facilitated withdrawal of vasopressor support in patients with RAI (defined here

by a cortisol increment of 248 nmol/L or less, i.e. $\delta \leq 248$) but not in patients without RAI (Annane *et al.*, 2002).

In addition to reduced cortisol levels in sepsis, tissue glucocorticoid resistance may compound the functional glucocorticoid deficiency. Several factors may be involved and these probably interact: decreased access of cortisol to the inflammatory site secondary to the reduction in circulating CBG; modulation of local cortisol level by a reduction in the cleavage of CBG–cortisol complex (anti-elastase activity); reduction in the number and affinity of glucocorticoid receptors, shown on lymphocytes treated with different cytokines; and a rise in the conversion of cortisol in inactive cortisone by increased activity of the 11 β-HSD stimulated by IL-2, IL-4 and IL-13; and direct inhibition of the genomic and nongenomic effects of glucocorticoids (Figure 1.10) These different mechanisms can account for decreased activity of glucocorticoids while plasma cortisol level is apparently appropriate.

Currently, there is no consensus about diagnostic criteria for RAI or the indications for glucocorticoid treatment in septic shock. Considerable disagreement exists over what cortisol level is "normal" or "appropriate" in septic shock, what constitutes an adequate response to ACTH, and what dose of synthetic ACTH should be used for stimulation testing. It has been proposed that free cortisol more accurately reflects HPA axis activation (Hamrahian *et al.*, 2004). However, this study had a heterogeneous patient population and without septic shock patients. My study described in this thesis compares total and free cortisol measurements, following ACTH stimulation testing, in septic shock and sepsis during illness and recovery. We also compare measured free cortisol using an equilibrium-dialysis validated ultrafiltration assay with a calculation technique - Coolens' method. If the use of this method is reliable in septic shock, it would obviate the need for complex non-automated methods of free cortisol measurement. I anticipated that free cortisol (measured or calculated) may contribute

significantly to future studies that attempt to use plasma cortisol to predict responses to hydrocortisone therapy and clinical outcomes.

NOTE: This figure is included on page 37 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.10 Regulated versus dysregulated response. Once activated, NF-κB and GRα can mutually repress each other through a protein-protein interaction that prevents their DNA binding and subsequent transcriptional activity. Activation of one transcription factor in excess of the binding (inhibitory) capacity of the other shifts cellular responses toward increased (dysregulated) or decreased (regulated) transcription of inflammatory mediators over time. (A) GC-GRα activation sufficient to maintain NF-κB levels in homeostasis (GC-GRα-regulated response) and to achieve a reduction in inflammation over time is shown. (B) An excess of NF-κB activation is shown, leading to protracted inflammation (NF-κB-driven dysregulated response) over time. (Meduri 2004)

1.7.3 Cortisol in pre-eclampsia and intrauterine growth restriction

Pregnancy is the only physiologic state of hypercortisolism that is sustained over many months in humans. Typically blood and urine cortisol levels rise progressively through gestation reaching a two to three-fold increase by term (Carr *et al.*, 1981). The hypercortisolism of pregnancy may be partly explained by an oestrogen-stimulated increase in CBG (Nolten and Rueckert, 1981; Lindsay and Nieman, 2005). In addition, placental production of CRH stimulates maternal pituitary ACTH release which in turn, increases cortisol secretion. Although the precursor peptide, pro-opiomelanocortin (POMC) is produced in placenta, ACTH is not thought to be secreted (Goland *et al.*, 1988). Normal maternal cortisol responses to stress indicate that the maternal hypothalamus retains an influence over the HPA axis and there is diminished sensitivity of the axis to cortisol feedback during pregnancy (Lindsay and Nieman, 2005). In early pregnancy, maternal cortisol suppresses foetal ACTH production. The foetal adrenal does not produce cortisol until maturation of the adrenal enzyme 3β-HSD at 28 - 30 weeks. In the third trimester, the foetus is 80 - 90 % protected against maternal hypercortisolism by placental 11β-HSD_2 which inactivates cortisol to cortisone, hence foetal ACTH and cortisol rise. At term, however, 25% of foetal cortisol is of maternal origin since an anatomical bypass at the syncytiotrophoblast cell avoids cortisol inactivation (Beitins *et al.*, 1973). These relative effects of maternal and foetal cortisol are important since appropriately timed exposure of foetal tissues, especially the brain, lung, kidneys and pancreas are required for normal development.

Hypertensive disorders (pre-eclampsia and gestational hypertension) complicate 12 to 22 percent of pregnancies, and are major causes of maternal and perinatal mortality and morbidity. The foetal consequences of chronic placental hypoperfusion are foetal growth restriction and oligohydramnios. Pathogenesis of pre-eclampsia is complex, and although research addressing this disorder has been intensive, it has not resulted in substantial

improvement in prediction or prevention of the disorder. Cortisol can elevate blood pressure through several pathways as described in previous section, and therefore may have a role in the pathogenesis of pre-eclampsia (Kelly *et al.*, 1998a). The inflammatory state of pre-eclampsia may stimulate cortisol secretion and may account for previously reported lowered CBG concentrations (Potter *et al.*, 1987). Conversely, placental dysfunction may diminish the hypercortisolism of pregnancy through lowered placental drive to the maternal HPA axis (Figure 1.9B).

Epidemiological studies showed blood pressure levels in adult life correlated inversely with birthweight at term (Barker *et al.*, 1989). Low birthweight humans are also at increased risk of type 2 diabetes, obesity, cardiac mortality as well as psychological/ psychiatric disorders (Table 1.1) (Seckl and Meaney, 2004). Low birthweight babies and adults have increased basal and stimulated cortisol levels. An influence of prenatal GCs is suggested by a correlation between low birthweight and antenatal exogenous GCs, or with measured placental 11β-HSD_2 activity (Seckl and Meaney, 2004). Animal studies suggest that either maternal under-nutrition (which reduces placental 11β-HSD_2, hence increasing prenatal GC exposure) or direct prenatal GC over-exposure lead to permanent alterations or 'programming' of the developing organism which, later in life, increases the risk of developing high blood pressure (Alexander, 2006). A further mechanism of foetal 'programming' of high blood pressure may include altered expression of specific transcription factors such as the glucocorticoid receptors (Reynolds *et al.*, 2002; Whorwood *et al.*, 2002).

Table 1.1 Human and animal data that link prenatal glucocorticoid excess and stress to postnatal development of metabolic syndrome and psychiatric disorders.

<table>
<tr><td colspan="2">Animal Data</td></tr>
<tr><td>Experiments
Maternal administration of dexamethasone
Carbenoxolone</td><td rowspan="2">Implications on offspring
Reduced birthweight
Reduced nephron number
Reduced beta cell mass
Alter cardiac norepinephric responses
Increased cortisol secretion
Increased anxiety
Increased blood pressure
Increased adult weight</td></tr>
<tr><td>Mechanisms
Maternal under-nutrition reduces placental 11β-HSD$_2$
Cortisol excess acts to alter GR exon methylation, epigenetic effect on maternal-cortisol, offspring-cortisol responses</td></tr>
<tr><td colspan="2">Human data</td></tr>
<tr><td colspan="2">Epidemiology: low birthweight is associated with hypertension, type II diabetes mellitus, insulin resistance and increased cardiovascular mortality in adult life
Low birth weight babies have higher cortisol levels as adults
Exogenous glucocorticoids lower birthweight
Reduced 11β-HSD$_2$ (mutations/less activity) correlated with birthweight
Dexamethasone given to pregnant women with congenital adrenal hyperplasia → increased emotional/ behavioural problems in offspring
Prenatal stress may increase vulnerability to schizophrenia, probable effect on cortisol – dopaminergic system</td></tr>
</table>

Hence, the available evidence would suggest that maternal cortisol levels may be altered in complicated pregnancy such as pre-eclampsia, and that such alterations may have health implications for offspring. However, there is surprisingly very little knowledge regarding maternal HPA function in high risk pregnancies such as pre-eclampsia and intrauterine growth retardation, and in gamete recipient pregnancy. My study described in this thesis has produced novel data showing that certain high risk pregnancies are associated with low maternal cortisol, an area for which there are no animal data (Chapter 5).

1.7.4 Cortisol and obesity

The prevalence of obesity has doubled in the last 15 years (Cameron *et al.*, 2003). The major concerns are the co-morbidities such as type 2 diabetes, cardiovascular disease, stroke, and certain types of cancers (Bjorntorp, 1998). The metabolic syndrome is a cluster of abnormalities including truncal obesity, insulin resistance, high blood pressure, dyslipidaemia, and disturbances in glucose homeostasis (Reaven, 1993). There is now strong evidence for an established difference between central (visceral) and peripheral (gluteo-femoral) fat accumulation in the association with these diseases. Central obesity is strongly associated with high cardiovascular morbidity and mortality (Bjorntorp, 1998; Wajchenberg, 2000).

A hypothetical role for glucocorticoids in human obesity has been suggested, since the abdominal obesity phenotype and syndromes of endogenous or exogenous hypercortisolism (Cushing's syndrome) share several similarities, with particular emphasis on all features of the metabolic syndrome. An emerging body of evidence indicates that this may be related, at least in part, to a condition of functional hypercortisolism, due to subtle alterations of the HPA axis combined with alterations of cortisol metabolism in extra-adrenal tissues (Smith, 1996; Pasquali and Vicennati, 2000a). These effects, however, may require the concerted action of many other hormones, including increased insulin levels, altered catecholamine regulation, growth hormone (GH) reduction, and decreased (in males) or increased (in females) free androgen availability (Bjorntorp, 1996). Dysregulation of the HPA axis in abdominal obesity has been convincingly demonstrated by many dynamic studies, showing increased cortisol secretion after physiologic or psychic stress after stimulation with various neuropeptides and secretagogues (Pasquali and Vicennati, 2000b). These studies suggest central neuroendocrine dysregulation in obesity, particularly of abdominal type, leading to slightly abnormal net cortisol production, either continuous or episodic.

Obesity is also characterized by an increased cortisol metabolic clearance rate. Higher numbers of glucocorticoid receptors have been demonstrated in abdominal than in subcutaneous adipocytes, which favours an increase in intracellular cortisol action and metabolism in the abdominal fat (Rebuffe-Scrive *et al.*, 1985). In addition, alterations of the activity of two enzyme systems, such as impaired activity of 11β-HSD_1, which reactivates cortisol from inactive cortisone in the liver and the adipose tissue, and enhanced activity of the 5-α-reductase, which metabolizes cortisol to its tetrahydro-derivates, have been described in patients with increased central adiposity (Sandeep and Walker, 2001; Tomlinson *et al.*, 2004a). The importance of the role of excess glucocorticoids produced within the adipose tissue in determining abdominal obesity has been emphasized by studies demonstrating that transgenic mice overexpressing 11β-HSD_1 selectively in the adipose tissue have increased levels of corticosterone and are characterized by the development of visceral obesity and all features of the metabolic syndrome, particularly insulin-resistant diabetes, hypertension and hyperlipidaemia (Masuzaki *et al.*, 2001). The recent identification of adipocyte-derived adrenostimulatory factor(s) which stimulate aldosterone and cortisol release *in vitro* may provide an additional link between obesity and hypertension (Ehrhart-Bornstein *et al.*, 2003). Therefore, both neuroendocrine dysregulation of the HPA axis, as well as peripheral alterations of cortisol metabolism, may play a role in the pathophysiology of the human abdominal phenotype of obesity (Figure 1.7C).

The treatment of obesity is difficult. Although caloric restriction reliably leads to loss of fat (and lean) mass, weight regain occurs in 80% of subjects within 2 years. It is known that starvation leads to hypercortisolism due to central activation of the HPA axis, presumably to facilitate a catabolic state (Douyon and Schteingart, 2002). However, it is not known if mild to moderate caloric restriction, of the order of 30%, leads to HPA axis activation. This is important as caloric restriction of this degree is consistent with commercial and medically

prescribed dietary weight loss strategies. Moreover, activation of the HPA axis may contribute to the weight regain phenomenon. Our study on the effect of moderate diet-induced weight loss on the HPA function and blood pressure regulation will be discussed in Chapter 6.

1.8 Summary

The stress response with the resultant activation of the HPA axis and central nervous system can lead to peripheral endocrine-metabolic and haemodynamic consequences. Chronic HPA axis activation may lead to pathological states. The sensitivity to environmental stressors is individually diverse and the adaptational ability is highly variable. Dissociation between feedback regulatory mechanisms and peripheral sensitivity may lead to dysregulation of the HPA axis and disease. Our studies in septic shock, hypertensive disorders of pregnancy and the blood pressure effect of moderate weight loss have allowed us to examine the role of HPA axis in three conditions associated with immune activation, altered cortisol levels and pathophysiologically important effects on blood pressure.

Chapter 2

Methods

2.1 Introduction

This chapter will discuss the methods that are common to the studies presented in this thesis. These procedures been used by our group in other studies and by other investigators, and are accepted methods of assessing adrenocortical function in human disorders. When a new method has been established (i.e. the validation of Coolens' method in septicaemia), this will be described in detail in the relevant chapters.

2.2 Methods

2.2.1 Blood pressure measurements

Resting blood pressure

Resting blood pressure (mean of 3 measurements) was measured by mercury sphygmomanometers or automated oscillometric blood pressure devices (Dinamap™, 845XT/XT-IEC, Tampa, Florida), with subjects in a seated position.

24 hour ambulatory blood pressure

Ambulatory blood pressures were measured with Cardiometrics ambulatory BP devices (Cardiometrics Inc). The measurement interval was set at 30 minutes whilst the subject was awake and 60 minutes whilst the subject was asleep. These blood pressures were recorded on the device, and the average day (diurnal) or night (nocturnal) blood pressures were derived from data downloaded to a computer with specific Cardiometrics software.

Invasive arterial blood pressure measurement

This technique involved direct measurement of arterial pressure by placing a cannula in an artery (usually radial, femoral, dorsalis pedis or brachial) and connected to an electronic pressure transducer. This technique was only performed in the intensive care unit with close monitoring.

2.2.2 ACTH stimulation tests

Several protocols have been used to assess the response to exogenous ACTH. The agent used was synthetic ACTH (1-24) (Synacthen, Novartis, North Ryde, NSW, Australia), which had the full biologic potency of native ACTH (1-39). Short (one hour or less) tests involved administration of a single "low" (1 mcg) or "high" (250 mcg) Synacthen dose. These doses result in supraphysiological plasma ACTH concentrations; mean levels approximate 1320 pmol/L after the high-dose ACTH test and about 418 pmol/L after the low-dose test. There were no untoward side effects.

Low-dose ACTH stimulation test

A test involving more physiological plasma concentrations of ACTH theoretically provides a more sensitive index of adrenocortical responsiveness. The low-dose ACTH test was performed by measuring serum cortisol immediately before and 30 minutes after intravenous injection of 1.0 μg Synacthen.

High-dose ACTH stimulation test

This test consisted of measuring serum cortisol immediately before and 30 and 60 minutes after intravenous injection of 250 μg of Synacthen.

2.2.3 Hormone assays

Plasma total cortisol concentration

Plasma total cortisol was measured by enzyme-linked fluorescent assay (AxSYM Cortisol assay kit, Abbott Laboratories). This assay utilizes Fluorescence Polarization immunoassay technology for the quantitative measurement of cortisol in serum. The intra and inter-assay coefficients of variation were 5.0 and 6.5% respectively.

Plasma free cortisol concentration

Plasma free cortisol was measured by an in-house ultrafiltration/ligand binding method. ^{3}H cortisol (0.1 μCi, Amersham TRK 407) in ethanol was evaporated to dryness in a glass tube. Plasma (0.5 ml) was then added for a 30-min incubation at 37^{o}C, after which 50 μl was retained for total radioactivity determination and the remainder added to a preconditioned ultrafiltration device (Millipore UFC3LGC00) and centrifuged for 30 min at 14,000 rpm using a microfuge. Comparison of the ultrafiltrate radioactive counts (50μl) with the equilibrated non-centrifuged plasma radioactivity (50μl) determined the % free cortisol. Radioactivity was counted in 2 ml of xylene based scintillant (PCS II, Amersham). The recovery of radioactivity is 100%. There is no adsorption of ^{3}H cortisol to either the filter or the polypropylene used in the filter device and carrier tube.

Validation of free cortisol assay

The ultrafiltration method of measuring free cortisol was compared to the gold standard of equilibrium dialysis using 19 samples from septic shock patients and 10 samples from normal individuals. For each sample, 1ml of plasma was dialysed against 250ul phosphate buffer solution using an Eppendorf tube device with a presoaked dialysis membrane (6-8000 MW cut off) for 18 hours at 37^{o}C. The total and post dialysis samples were analysed by plasma

direct ELISA. The methods yield near identical cortisol values and the correlation coefficient and R^2 for regression are R=0.99 and R^2=0.99 respectively (Figure. 2.1).

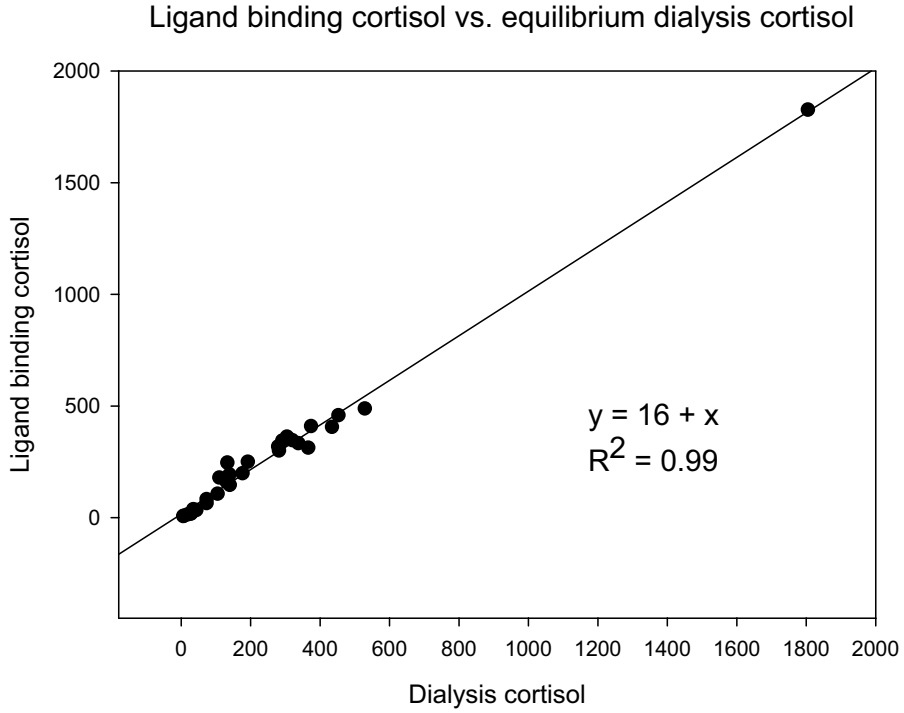


Figure 2.1 Validation of free cortisol assay (ligand binding/ultrafiltration method) against equilibrium dialysis using 19 septic shock samples and 10 control samples.

Plasma corticosteroid-binding globulin concentration

Plasma CBG was measured using an in-house method. This was a two site non-competitive direct enzyme-linked immunosorbent (ELISA) assay for CBG using monoclonal and polyclonal antibodies (Lewis *et al.*, 2003). ELISA plates (Falcon 3912 microtest III; Beckton Dickinson, Oxnard, CA, USA) were coated for 5 – 7 days at 4 ^{o}C with 100 μg/ well of sheep anti-CBG serum, diluted in phosphate-buffered saline (PBS), 30 μl of antibody in 10 ml PBS. Following coating the plates were washed and blocked with assay buffer (150 μl/ well) for 1 hour at 20 ^{o}C. The plates were emptied by inversion and either 100 μl of standard or patient plasma (1:1000 dilution in assay buffer) added to each well. Following overnight incubation

at 4 ^{o}C, the plates were washed and supernatant 12G2 (1:20 dilution) was added for 2 hours at 20 ^{o}C. The plates were then washed and 100 µl of antimouse IgG Fc-peroxidase (1:1000 dilution) added for 1 hour, after which the plates were finally washed and substrate added, colour developed and terminated prior to spectrophotometry at 450 nm. The value assigned to the standard plasma was determined by calibration with purified CBG and an assumed molecular weight of 52,000. The use of antimouse IgG Fc-peroxidase for detecting monoclonal antibody 12G2 in the ELISA rather than antimouse IgG-peroxidase that displaces some of the monoclonal antibody bound to CBG via the immobilised polyclonal antibody suggests that the monoclonal antibody binding site for CBG and the recognition sites on the monoclonal antibody by antimouse IgG-peroxidase are in close proximity. The use of of antimouse IgG Fc-peroxidase affords greater spatial separation, thus negating steric hindrance.

Urinary free cortisol/ cortisone

Urine free cortisol and cortisone were measured using a commercial high performance liquid chromatography (HPLC) system. The solvent-delivery system was controlled by Millennium 2010 Chromatography Manager (Waters, Milford MA). The intra- and inter-assay precisions were 3 and 10.5% respectively.

Plasma renin concentration

Plasma renin concentration was measured by Nichols Advantage Direct Renin assay (Nichols Institute Diagnostics, San Clemente, CA). The assay used two specific monoclonal antibodies. The first monoclonal antibody is biotin-labeled and was used to capture renin by recognizing both active renin and prorenin. The second monoclonal antibody is labeled with acridinium ester, and this labeled antibody detected active renin and activated prorenin. The chemiluminescence reaction was initiated by the addition of alkaline peroxide. The amount of

bound labeled antibody, as determined by relative light units, was directly proportional to the concentration of active renin in the sample. The limit of detection for this assay was 0.8 μU/mL. The intra- and inter-assay precision were 3.8 and 4% respectively.

Plasma aldosterone concentration

Plasma aldosterone was measured by Nichols Advantage assay. This assay was based upon competitive binding between aldosterone in the patient sample and acridinium ester labelled aldosterone for a specific mouse monoclonal antibody to human aldosterone. The sensitivity for this assay was estimated to be ≤ 1.2 ng/dL. The intra- and inter-assay precision were 4.0 and 6.0% respectively.

Plasma nitrate/ nitrite (NOx) concentration

Plasma NOx concentration was measured after nitrate reduction to nitrite by the addition of nitrate reductase. Briefly, plasma samples were ultrafiltered through a 10 kDa molecular cut-off filter device (UFC3LGC00, Millipore Corporation, Billerica, MA). The ultrafiltrates were incubated at room temperature for 3 hours in the presence of nitrate reductase (Nitrite/ nitrate colorimetric assay kit, catalog no, 780001, Cayman Chemical Company, Ann Arbor, MI). Total nitrite in the supernatant was subjected to the Griess reaction and assayed spectrophotometrically at 540 nm. The detection limit is approximately 2.0 μM.

2.2.4 Coolens' method

In human plasma the major fraction (about 70%) of cortisol is bound to CBG, approximately 20% is bound to albumin and 10% is unbound (free). Since free cortisol constitutes the biologically active form of the hormone, its measurement is theoretically advantageous. The available methods, ultracentrifugation or equilibrium dialysis, are technically demanding. However, the concentrations of total cortisol and CBG can be measured reliably by standard techniques. Free cortisol can then be easily calculated from these data using Coolens' method based on binding equilibria (Coolens *et al.*, 1987). Two simultaneous binding equilibria determine the binding of cortisol in human plasma: the saturable binding of cortisol to CBG and the non-saturable binding to albumin. From these equilibria the following quadratic equation is derived:

$$U^2.\ K\ (1 + N) + U[1 + N + K(G - T)] - T = 0$$

In this equation U, T and G correspond, respectively, to the molar concentrations of unbound cortisol, total cortisol and CBG. K represents the affinity of CBG for cortisol at 37^{o}C and N the ratio of albumin-bound to unbound cortisol. A value of 3×10^7 M^{-1} is used for K and a constant value of 1.74 for N. When all concentrations are expressed as μM, this equation can be solved for U in the following was:

$$U = \sqrt{Z^2 + 0.0122T} - Z$$

Wherein $Z = 0.0167 + 0.182\ (G - T)$.

The calculated free cortisol (y) correlated well with those (x) obtained by centrifugal ultrafiltration at 37^{o}C (Figure 2.2).

NOTE: This figure is included on page 51 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.2 Unbound (free) cortisol, calculated from total cortisol and CBG, as a function of unbound cortisol, measured by ultrafiltration in plasma samples of normal subjects. 100 ng/ml of unbound cortisol corresponds to 0.276 μM. (Coolens 1987)

2.3 Statistical analyses

For all studies results were expressed as mean ± SEM and a P value < 0.05 was used to indicate statistical significance. The statistical analyses for each study are described in the relevant chapter. All descriptive and inferential statistical calculations were performed using the Statistica (Statsoft, Tulsa. OK) computer package.

Chapter 3

The hypothalamic-pituitary-adrenal axis in critical illness

3.1 Summary

Adequate adrenocortical function is essential to survive in critical illness. Severe illness and stress activate the HPA axis with sustained secretion of cortisol. The failure of an appropriate neuroendocrine response can lead to vasopressor dependent refractory hypotension (shock). This state of relative or functional adrenal insufficiency (RAI) is characterized by an inadequate production of cortisol in relation to an increased demand during period of severe stress, particularly prolonged critical illness. Recent studies with stress doses of corticosteroid in septic shock have shown marked haemodynamic improvement in patients with relative adrenal insufficiency. However, plasma total cortisol may not reflect the biologically active free cortisol as cortisol binding proteins – CBG and albumin levels decrease markedly in critical illness.

The hypothesis of the study was that free plasma cortisol may correspond more closely to illness severity than total cortisol in severe sepsis. The aims were to compare total with free cortisol, measured directly and estimated by Coolens' method, CBG and albumin in patients with septic shock (with and without RAI) and sepsis during acute illness, recovery and convalescence.

The study showed that free cortisol levels reflected illness severity more closely than total cortisol, comparing septic shock (SS), sepsis (S) and control (HC) groups (basal free cortisol: SS 186 vs. S 29 vs. HC 13 nmol/L, $P<0.001$ compared to basal total cortisol: SS 880 vs. S 417 vs. HC 352 nmol/L, $P<0.001$). Stimulated free cortisol increments varied greatly with illness category; SS 192 vs. S 115 vs. HC 59 nmol/L, $P=0.004$, whereas total cortisol

increments did not; SS 474 vs. S 576 vs. HC 524 nmol/L, *P*=0.013. The lack of increase in total cortisol with illness severity was due to lower CBG and albumin. One-third of patients with septic shock (15/45), but no sepsis patients met a recently described criterion for RAI (total cortisol increment after tetracosactrin ≤248 nmol/L). RAI patients had higher basal total cortisol (1157 vs. 756 nmol/L, *P*=0.028) and basal free cortisol (287 vs. 140 nmol/L, *P*=0.017) than non-RAI patients. Mean cortisol increments in RAI were lower (total: 99 vs. 648 nmol/L, *P*<0.001, free: 59 vs. 252 nmol/L, *P*<0.001). These differences were not due to altered CBG or albumin levels. Free cortisol levels normalized more promptly than total cortisol in convalescence. Calculated free cortisol by Coolens' method compared closely to measured free cortisol.

In conclusion, free cortisol is likely to be a better guide to cortisolaemia in systemic infection as it corresponds more closely to illness severity. The attenuated cortisol increment after tetracosactrin in RAI is not due to low cortisol-binding proteins. Free cortisol levels can be determined reliably using total cortisol and CBG levels.

3.2 Introduction

The maintenance of normal, coordinated physiologic functioning of various organ systems of the body is the primary role of the endocrine system (Rolih and Ober, 1995). Stress is the state of threatened homeostasis. The physiologic response to stress involves activation of stress regulatory centres in the central nervous system (CNS), with consequent stimulation of the HPA axis and the autonomic nervous system. The stress system influences other endocrine systems (i.e., those controlling gonadal, thyroidal, and growth functions) and exerts complex effects on the immune/inflammatory reaction.

Critical illness is an acute medical condition that is immediately or imminently life-threatening. One or more organ systems deteriorate to such an extent that they can no longer support the independent functioning of the patient (e.g., cardiac failure, renal failure, respiratory failure). Common to many forms of critical illness are activation of systemic inflammatory cascades and decreased perfusion of one or more organ systems. Cytokines are soluble proteins that activate and regulate T and B lymphocytes and mediate many of the manifestations of the systemic inflammatory response. Cytokines affect endocrine glands at many different levels. In particular, they affect the release of hormones from the hypothalamus and the pituitary. They also act on the pituitary as paracrine and/or autocrine regulators, modulating hormone secretion and cell growth. The major cytokines that affect the hypothalamic-pituitary axis are IL-1, IL-2, IL-6, TNF, and IFN. The predominant effects of these cytokines are stimulation of the HPA axis, suppression of the hypothalamic–pituitary–gonadal (HPG) axis and hypothalamic–pituitary–thyroid (HPT) axis, and release of growth hormone.

3.2.1 The HPA axis in stress and critical illness

The principal CNS centres of the stress system are CRH/ AVP and locus ceruleus–norepinephrine neurons of the hypothalamus and brainstem, respectively, which regulate the HPA axis and the sympathetic nervous system. The end hormones of these systems, cortisol and the catecholamines, act to maintain behavioural, cardiovascular, metabolic, and immune homeostasis during stress (Chrousos, 1998).

Cortisol has long been recognized as essential for survival in critical illness. The normal daily production of cortisol is approximately 12 – 15 mg/m^2 of body surface area per day; this production can increase up to fivefold in critical illness (Chernow *et al.*, 1987; Schein *et al.*,

1990). The type and severity of stress influence the degree of HPA axis activation. An adequate increase in cortisol production is crucial for survival as it provides metabolic substrates to vital organs, such as the brain, elevates blood pressure and postpones catabolism. Conversely, elevation of plasma cortisol contributes to the hyperglycaemia, leucocytosis, and hypercatabolism seen in most forms of critical illness. The increased circulating cortisol restrains the inflammatory response from overreacting, but it may also be responsible for the unrestrained progression of an infection.

The activity of the HPA axis displays a biphasic pattern during the course of critical illness (Vermes *et al.*, 1995). The high plasma cortisol concentrations present during the initial phase after surgery, trauma, or sepsis are associated with augmented ACTH release, which in turn, is driven by CRH, cytokines, and the noradrenergic system (Rivier and Vale, 1983; Elenkov *et al.*, 1999). In prolonged critical illness, plasma ACTH is low whereas cortisol concentrations usually remain elevated; this indicates that cortisol release in this phase may be driven by an alternative pathway, possibly involving endothelin. An altered pituitary cortisol feedback and a hypersecretion of peptides with CRH- or ACTH-like activity may also be involved in the regulation of the HPA axis (Vermes *et al.*, 1995). Furthermore, IL-6 has been demonstrated to stimulate adrenal cortisol secretion directly (Path *et al.*, 1996; Path *et al.*, 1997). Another adaptive response to prolonged critical illness is the shift of steroid production in the adrenals from mineralocorticoid and androgens to cortisol (Parker *et al.*, 2000). The mechanism for this is unknown, though cytokines have been suggested to play a role. These mechanisms may ultimately fail, as indicated by a substantially higher incidence of adrenal insufficiency in prolonged critical illness (Barquist and Kirton, 1997). This prolonged imbalance between immunosuppressive and immunostimulatory hormones of adrenocortical origin may have devastating consequences.

3.2.2 Septic shock

Sepsis is a clinical syndrome that complicates severe infection and is characterized by systemic inflammation and widespread tissue injury. Septic shock is sepsis with hypotension despite adequate fluid resuscitation combined with perfusion abnormalities that may include lactic acidosis, oliguria, or an acute alteration in mental status (No authors, 1992). Septic shock is a form of vasodilatory shock resulted from a marked reduction in systemic vascular resistance, often associated with an increase in cardiac output. Septic shock is among the leading causes of death in intensive care units with a 60% mortality rate. Factors that predispose patients to septic shock include age greater than 65years, chronic organ failure, co-morbidities that impair immune function and genetic factors such as male gender and non-Caucasian ethnic group (Alberti *et al.*, 2002; Martin *et al.*, 2003; Martin *et al.*, 2006).

Sepsis is a complex process involving activation of circulating phagocytic cells and generation of pro-inflammatory and anti-inflammatory mediators. The interaction between pro-inflammatory and anti-inflammatory mediators can be viewed as a struggle between opposing influences. When this balance in the inflammatory process is lost, these mediators can lead to damage to host tissue and changes in the neuroendocrine-immune status.

The time-window for intervention is short, and treatment must promptly control the source of infection and restore haemodynamic homeostasis. Adequate fluid resuscitation, appropriate antimicrobial therapy and protective ventilation form the basis of sepsis management. Adjuvant therapies have been the subject of ongoing investigation, mostly involving modulation of the inflammatory response in sepsis, however they have largely failed to yield positive results in improving outcomes (Dellinger *et al.*, 2006).

The use of corticosteroid as adjunctive therapy in severe sepsis and septic shock has been a source of controversy for the past 35 years. Despite a wealth of preclinical data supporting both survival and physiologic benefit for early steroid use, the data in human sepsis have been much less convincing (Hinshaw *et al.*, 1981; Ottosson *et al.*, 1982). There have been reports suggesting the potential for harm associated with the administration of high-dose corticosteroid in patients with severe sepsis (Minneci *et al.*, 2004). Recent trials have reported haemodynamic and survival benefit associated with the use of more physiologic steroid replacement in patients with vasopressor-dependent septic shock (Annane *et al.*, 2002). These results coupled with the observation of "relative adrenal insufficiency" may redefine a role for corticosteroid therapy in the management of septic shock.

3.2.3 Relative adrenal insufficiency (RAI)

Secretory failure of the adrenal glands was first implicated as a factor in the pathogenesis of circulatory collapse associated with infection after original reports of Waterhouse (Waterhouse, 1911) and Friderichsen (Friderichsen, 1918). More recent evidence for the critical role of intact adrenal activity in critical illness came from the observation that the use of sedative agent etomidate, which inhibits synthesis of cortisol, was associated with increased mortality (Ledingham and Watt, 1983). These observations emphasize that the functional integrity of the HPA axis is essential to survival in critical illness.

Relative adrenal insufficiency often develops in septic patients and resolves when the patient recovers. This suggests that mediators such as TNFα, IL-6 and corticostatin released during septic shock may impair the functioning of the HPA axis. Corticostatin, a defensin peptide produced by immune cells, has been shown to impair adrenocortical function by competing with ACTH through binding to its receptor (Zhu and Solomon, 1992). Other possible

mechanism underlying the diminished cortisol level might be a decreased ACTH secretion as discussed above.

Whilst absolute adrenal insufficiency is uncommon among critically ill patients, with an incidence estimated to be ≤ 3 % (Burry and Wax, 2004), occult and transient relative adrenal insufficiency is under recognised (Baldwin and Allo, 1993). The prevalence of relative adrenal insufficiency is unlikely to have altered over the past decade; the recognition of this pathological phenomenon has certainly increased. RAI is conceptually defined by some investigators as an inadequate cortisol response to severe illness associated with rapid clinical and haemodynamic improvement following stress-dose glucocorticoid therapy (Rothwell *et al.*, 1991; Annane *et al.*, 2000b; Beishuizen and Thijs, 2001). However, there is no consensus about diagnostic criteria or indications for treatment, and considerable disagreement exists over what constitutes an "appropriate" cortisol level in septic shock or an adequate response to ACTH stimulation.

Cortisol levels vary widely in patients with septic shock (Schein *et al.*, 1990). Studies found increased mortality associated with both low and high plasma cortisol levels (Moran *et al.*, 1994; Bouachour *et al.*, 1995). In 2000, Annane et al observed that the mortality from septic shock was greatest in patients whose baseline cortisol levels exceed 938 nmol/L and an increment of 248 nmol/L or less after high dose (250 μg) ACTH stimulation (Annane *et al.*, 2000b). Subsequently, they conducted the largest multicentre, double-blind, placebo-controlled trial of 300 septic shock patients and showed that the use of stress-dose corticosteroids (hydrocortisone 200 mg/day) significantly improved 28 day survival and facilitated withdrawal of vasopressor support in patients with RAI (defined by a cortisol increment of 248 nmol/L or less, i.e. $\delta \leq 248$) but not in patients without RAI (Annane *et al.*, 2002).

In critical illness, mean CBG and albumin levels fall by around 50% with an increase in plasma free cortisol. Hence, total cortisol may not reflect the biologically active free (unbound) cortisol, suggesting that standard assays for plasma cortisol (which measure total plasma cortisol) underestimate HPA axis activity. It has been proposed that free cortisol more accurately reflects HPA axis activation (Hamrahian *et al.*, 2004). Indeed, a recent study found that baseline and ACTH-stimulated cortisol levels in a heterogeneous group of critically ill patients (n=66) were lower in the subgroup with hypoalbuminaemia (albumin< 2.5g/dL, n=36). However, free cortisol levels were similar in the hypoalbuminaemic and normoalbuminaemic groups (Hamrahian *et al.*, 2004). The study has two major limitations. First, the critically ill patients did not include any patients with septic shock. Second, the degree of haemodynamic instability at the time of plasma cortisol sampling was not reported; thus it is impossible to correlate the cortisol level with severity of illness or physiologic status. In addition, the applicability of the study is uncertain because the free cortisol assay is not available at most clinical centres.

3.3 Hypothesis and aims

The principal hypothesis of this study was that the free cortisol would be a better measure of circulating glucocorticoid activity than total cortisol in systemic infection.

The aims were:

1. To compare total and free plasma cortisol levels under basal and tetracosactrin-stimulated conditions, in patients with septic shock (with and without currently defined RAI), sepsis and in healthy controls
2. To determine the rates of recovery of RAI in survivors,

The Coolens' method may be practically useful in critical illness as it estimates free cortisol levels from total cortisol and CBG levels (Coolens *et al.*, 1987). If reliable in septic shock, use of Coolens' method would obviate the need for complex non-automated methods of free cortisol measurement such as ultracentrifugation or equilibrium dialysis. However, the method has not been validated in septic shock.

The third aim of the study was to evaluate the use of the Coolens' method in estimating free cortisol in septic shock.

3.4 Research design and methods

This prospective study was conducted between April 2004 and January 2005 in the Intensive Care Unit and Endocrine Test Unit of the Royal Adelaide Hospital. The study protocol was approved by the Royal Adelaide Hospital Ethics Committee and informed consent was obtained from each patient or their next-of-kin for follow-up testing and/or analysis of initial basal/stimulated cortisol levels. The study did not alter therapy and each patient's clinical care was determined by their own physician.

Patients and volunteers

Definitions of septic shock and sepsis followed the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee criteria (No authors, 1992). Septic shock was defined as the presence of systemic inflammatory response syndrome (SIRS), documented infection or positive blood culture and organ dysfunction, hypoperfusion abnormality or sepsis-induced hypotension refractory to adequate fluid resuscitation or the use of inotropic or vasopressor support. SIRS is manifested by two or more of the following: fever (temperature > 38^{o}C) or hypothermia (temperature < 35.5^{o}C); tachycardia (> 90 beats/min); tachypnea (> 20 breaths/min); leucocytosis or leucopenia (white blood cell count > 12000 or < 4000 cells/μL) or immature neutrophils (bands > 10%). The criteria for organ dysfunction or hypoperfusion abnormality included one of the following: altered mental status; hypoxemia (PaO_2 < 75 mmHg); oliguria (urine output < 30ml/h); elevated plasma lactate level (> 2 mmol/L). The definition of hypotension was: systolic blood pressure < 90 mmHg or a reduction in systolic blood pressure of 40 mmHg or more from baseline.

Septic shock patients who met the entry criteria were recruited from ICU. Sepsis patients were recruited from general wards after referral from their physician. Tetracosactrin tests were performed while patients still met the diagnostic criteria for sepsis, within 24 hours of diagnosis. Healthy controls were recruited by advertisement for the tetracosactrin stimulation test.

Exclusion criteria included: age under 18 years, pregnancy, irreversible underlying disease such as advanced malignancy or AIDS, or conditions known to disrupt the hypothalamic-pituitary-adrenal axis such as the use of glucocorticoids and pre-existing hypocortisolism. Patients with cirrhosis, pulmonary embolism and acute myocardial infarction were excluded.

Tetracosactrin (ACTH 1-24) stimulation tests

Blood samples were collected immediately before intravenous tetracosactrin (250 μg ACTH 1-24, Synacthen, Novartis, North Ryde, NSW, Australia) and at 30 and 60 minutes post-injection. Surviving patients were offered a second ACTH stimulation test performed within 48 hours of hospital discharge and a third ACTH stimulation test 6-12 weeks after the second test. Follow up ACTH stimulation tests were not performed in the control subjects.

Clinical Evaluation

Information relating to: [1] age and gender; [2] infection site/s and organisms [3] severity of illness at baseline using the Acute Physiology and Chronic Health Evaluation (APACHE) II scoring system; [4] disease outcome including mortality; [5] use of hydrocortisone; and [6] time-to-discontinuation of vasopressor support was collected.

Laboratory measurements

Blood samples were collected for plasma total and free cortisol, CBG and albumin. Hormone assays were described in Chapter 2.

Validation of ultrafiltration/ ligand binding method for free cortisol measurement

Validation of the ligand binding assay against equilibrium dialysis method was performed using samples from 19 septic shock patients and 10 samples from normal individuals. For each sample, 1ml of plasma was dialysed against 250ul phosphate buffer solution using an eppendorf tube device with a presoaked dialysis membrane (6-8000 MW cut off) for 18 hours at 37^{o}C. The total and post dialysis samples were analysed by plasma direct ELISA.

Calculated free cortisol was derived using the Coolens' equation, which was described in detail in Chapter 2.

$$U^2.K\ (1 + N) + U[1 + N + K(G - T)] – T = 0$$

cortisol (T), CBG (G), U unbound cortisol, K affinity of CBG for cortisol at 37^o (Coolens et al., 1987a). N is the ratio of albumin bound to free cortisol and 1.74 is the value conventionally used. The value of N would be expected to change with altered concentrations of plasma albumin, as observed in septic shock. We addressed this by investigating the distribution of cortisol (600 nmol/L) in varying concentrations of purified human serum albumin solutions (Sigma A-9511) using equilibrium dialysis. We used these experimentally derived values of N to further calculate free cortisol thus compensating for variations in plasma albumin.

Statistical Analysis

The plasma cortisol increment was defined as the difference between the basal cortisol and the highest concentration after tetracosactrin. Descriptive statistics were reported as mean ± standard error (SE). The data from the three groups (septic shock, sepsis and control) were analyzed by Kruskal-Wallis one way analysis of variance on ranks to investigate group effects, time effects, and group by time interaction. Relative adrenal insufficiency was empirically defined using the currently most validated criterion, that of Annane et al (11). The RAI ($\delta \leq 248$) and non-RAI ($\delta > 248$) groups were compared using nonparametric (Mann-Whitney rank sum test) analysis. Statistical significance was set at $P< 0.05$. Statistical analyses were performed using the Statistica software package (Statsoft, Tulsa, OK).

3.5 Results

Eighty-four patients were screened and 64 were enrolled. Principal exclusions were the current use of glucocorticoid therapy, cirrhosis and malignancy. There were 45 septic shock (SS) patients, 19 with sepsis (S) and 10 healthy controls (HC). The clinical characteristics of

the patients are summarized in Table 3.1. Control subjects were younger than patients and more males than females were tested in the sepsis group. Follow up tetracosactrin stimulation tests were performed in 20 SS and 14 S patients.

Table 3.1 Clinixal characteristics of patients with septic shock, sepsis and the healthy controls.

Characteristic	Septic shock (n = 45)	Sepsis (n = 19)	Healthy control (n = 10)
Age (yr)	64 ± 2	55 ± 4	38 ± 4
Gender (%)			
Men	53 (24)	79 (15)	50 (5)
Women	47 (21)	21 (4)	50 (5)
Site of infection (%)			
Chest	38 (17)	26 (5)	N/A
Abdomen	27 (12)	21 (4)	
Urinary tract	4 (2)	21 (4)	
Bone	2 (1)	6 (1)	
Skin	7 (3)	26 (5)	
Vascular	4 (2)	0	
Multiple	7 (3)	0	
unknown	11 (5)	0	
Type of organism (%)			
Gram positive	38 (17)	42 (8)	N/A
Gram negative	24 (11)	11 (2)	
Fungus	2 (1)	0	
Multiple	20 (9)	5 (1)	
Unknown	16 (7)	42 (8)	
Severity of illness score (APACHE II)	25 ± 8	N/A	N/A
Duration of vasopressor support (days)	6.3 ± 1.2	N/A	N/A
Duration of hydrocortisone therapy (days)	6.2 ± 1.4	N/A	N/A
Mortality (%)	37	0	N/A

N/A denotes not applicable

Comparison of total and free cortisol, CBG and albumin levels: SS, S and controls

Free cortisol levels corresponded more closely to illness severity than total cortisol (Fig. 3.1). Basal free cortisol levels were SS 186 ± 24 vs. S 29 ± 5 vs. HC 13 ± 2 nmol/L; i.e., a 6.4 fold difference SS vs. S ($P < 0.001$), and basal total cortisol levels were: SS 880 ± 79 vs. S 417 ± 45 vs. HC 352 ± 34 nmol/L; a 2.1 fold difference SS vs. S ($P < 0.001$). Following tetracosactrin stimulation, free cortisol levels were: SS 192 ± 36 vs. S 115 ± 12 vs. HC 59 ± 7 nmol/L, ($P = 0.004$) and total cortisol increments were: SS 474 ± 79 vs. S 576 ± 36 vs. HC 524 ± 54 nmol/L ($P = 0.013$). Higher free cortisol with illness severity may be due to lower levels of cortisol binding proteins (Fig. 3.1). Sepsis patients had 35% lower CBG and 29% lower albumin levels than controls and septic shock was associated with a further 19% fall in CBG and a 29% fall in albumin levels compared to the sepsis group (Fig. 1). CBG levels were: SS 335 ± 17 vs. S 412 ± 40 vs. HC 633 ± 43 nmol/L ($P < 0.001$); albumin levels were: SS 19 ± 1, S 27 ± 1, HC 38 ± 1 g/L ($P < 0.001$).

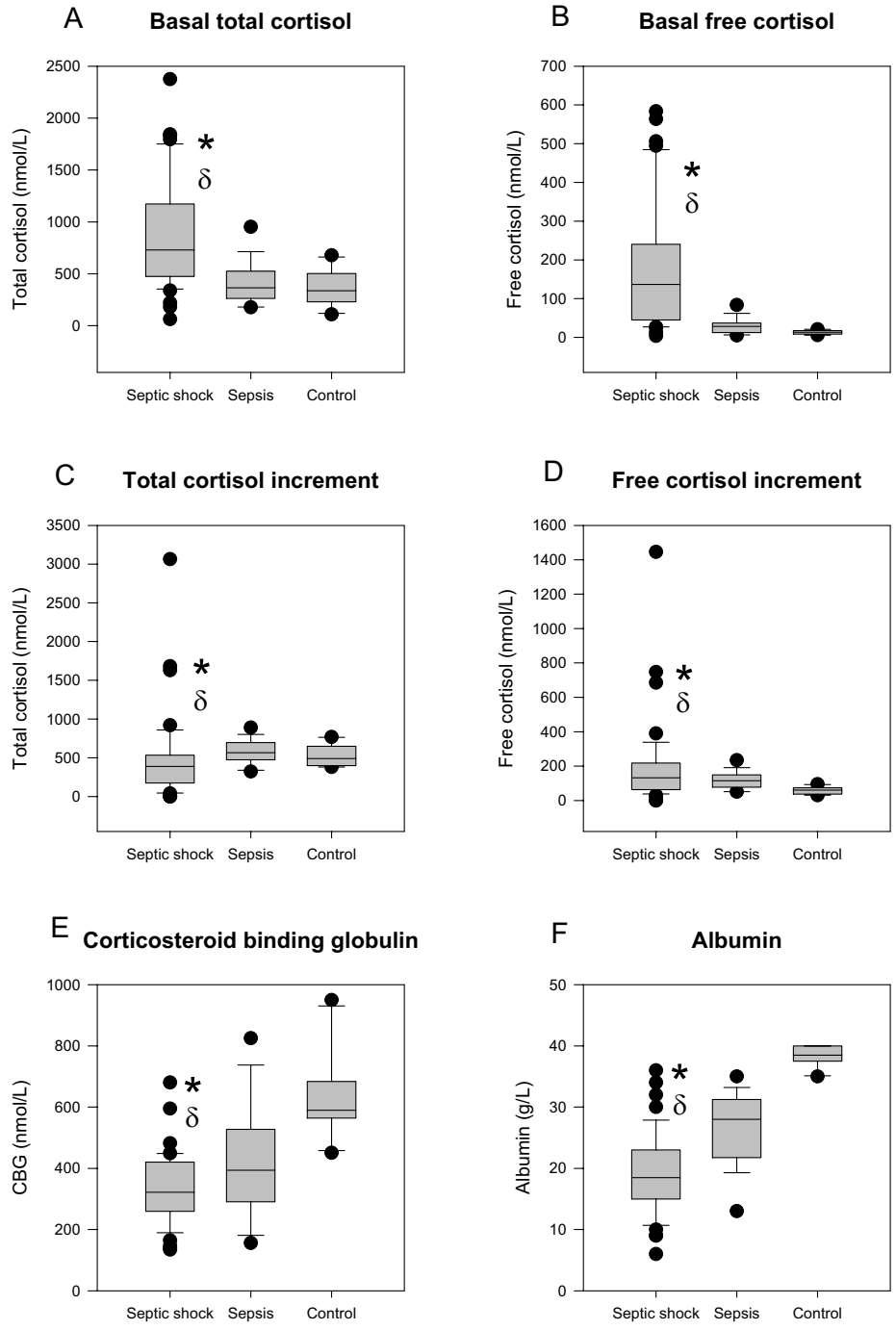


Figure 3.1 Basal total and free plasma cortisol, and cortisol increments after tetracosactrin (ACTH 1-24), CBG and albumin levels in patients with patients with septic shock (n=45), sepsis (n=19) and in healthy controls (n=10). The plots represent median, 25th and 75th percentiles as vertical boxes with error bars (10th and 90th percentiles), * denotes $P < 0.05$ for septic shock vs. sepsis and δ for septic shock vs. control. The basal total (Panel A) and free cortisol concentrations (Panel B) were significantly higher in the septic shock group than in the sepsis and control groups. Free cortisol increments (Panel D) corresponded to sepsis and its severity whereas total cortisol increments did not (Panel C). Corticosteroid-binding globulin (CBG) and plasma albumin levels (Panel E and F) fell significantly with illness severity.

Relative adrenal insufficiency: total and free cortisol levels

One-third (15/45, 33%) of septic shock patients met the current empiric definition of relative adrenal insufficiency (RAI, $\delta \leq 248$). None of the sepsis patients had RAI. Nine out of the fifteen patients with $\delta \leq 248$ (60%) and fifteen out of thirty (50%) of patients with non-RAI ($\delta > 248$) received stress-dose hydrocortisone therapy. There was no difference in the duration of vasopressor support between the two groups (RAI, 8.3 ± 2.4 vs. non-RAI, 4.4 ± 1.0 days, $P > 0.05$). There was also no difference in the duration of hydrocortisone therapy between the two groups (RAI, 7.0 ± 2.2 vs. non-RAI, 3.0 ± 1.0 days, $P > 0.05$). Among patients with $\delta \leq 248$, 33% (3/9) of hydrocortisone treated compared to 50% (3/6) of non-hydrocortisone treated patients died by day 28. In contrast, 50% (15/30) of patients with $\delta > 248$ who received hydrocortisone and 20% (3/15) of non-hydrocortisone treated patients died by day 28. Overall, there was no significant difference in the 28 day mortality of septic shock patients with and without RAI (40% vs. 35%, respectively, $P > 0.05$).

Total and free cortisol, CBG and albumin levels in septic shock patients are shown in Fig. 3.2. Patients with $\delta \leq 248$ had higher basal total cortisol (1154 ± 145 vs. 756 ± 87 nmol/L, $P = 0.028$) and basal free cortisol levels (287 ± 51 vs. 140 ± 22 nmol/L, $P = 0.017$). As expected by definition, total cortisol increments were less in the RAI group than the non-RAI group (99 ± 20 vs. 648 ± 101, $P < 0.001$). Free cortisol increments were also significantly less (59 ± 11 vs. 252 ± 48, $P < 0.001$). Lower cortisol increments in RAI were not related to CBG or albumin concentrations as these were similar: CBG: RAI 350 ± 38 vs. non-RAI 328 ± 18, $P > 0.05$; albumin: RAI 19 ± 2 vs. non-RAI 19 ± 1, $P > 0.05$.

Thirteen of fifteen (87%) patients with $\delta \leq 248$ had a free cortisol increment after tetracosactrin of <110nmol/L and 27/31 (87%) of patients with $\delta > 248$ exceeded this

threshold. Hence, a free cortisol increment of 110 nmol/L categorizes RAI and non-RAI patients similarly to a total cortisol response of 248 nmol/L.

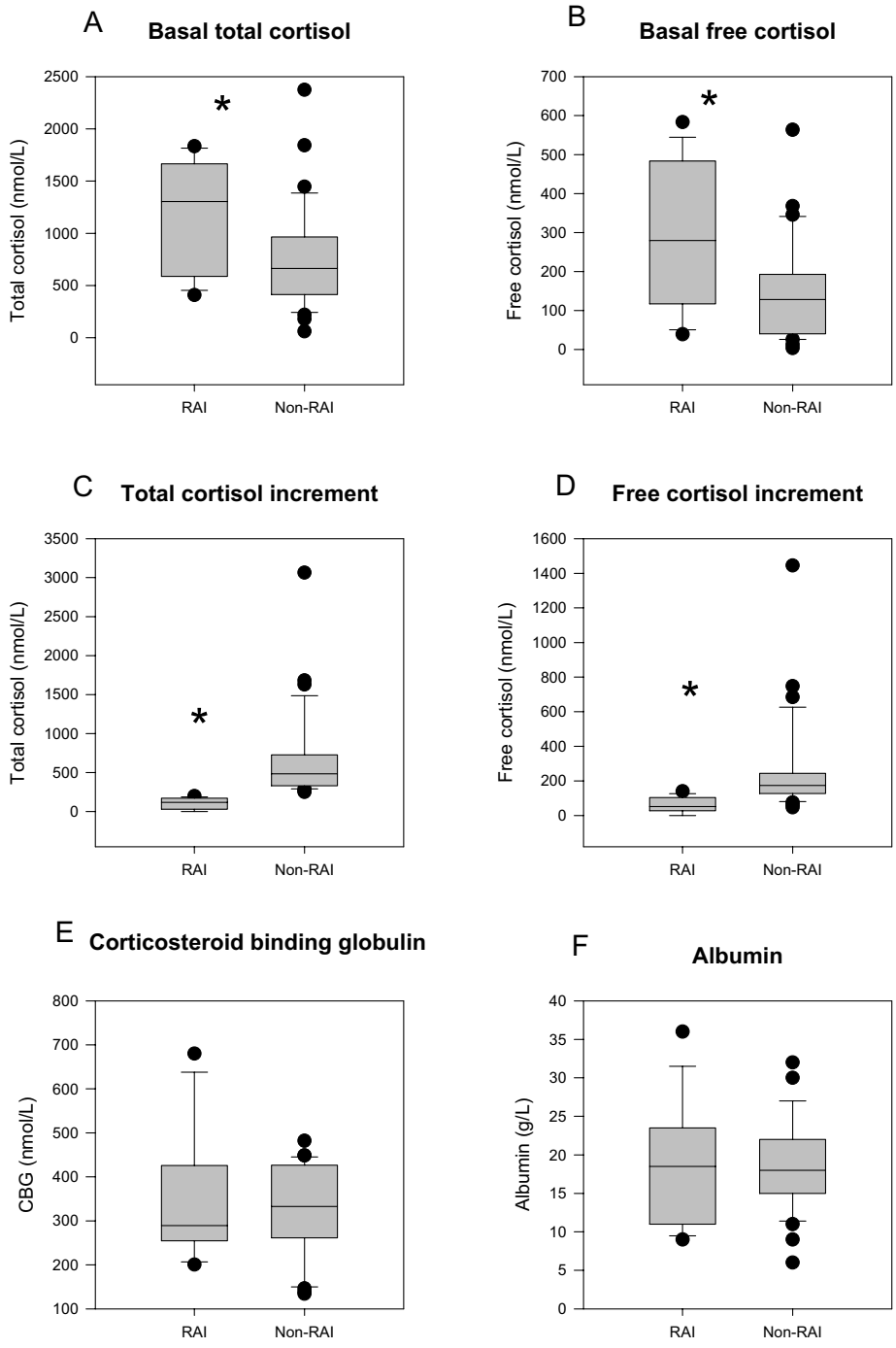
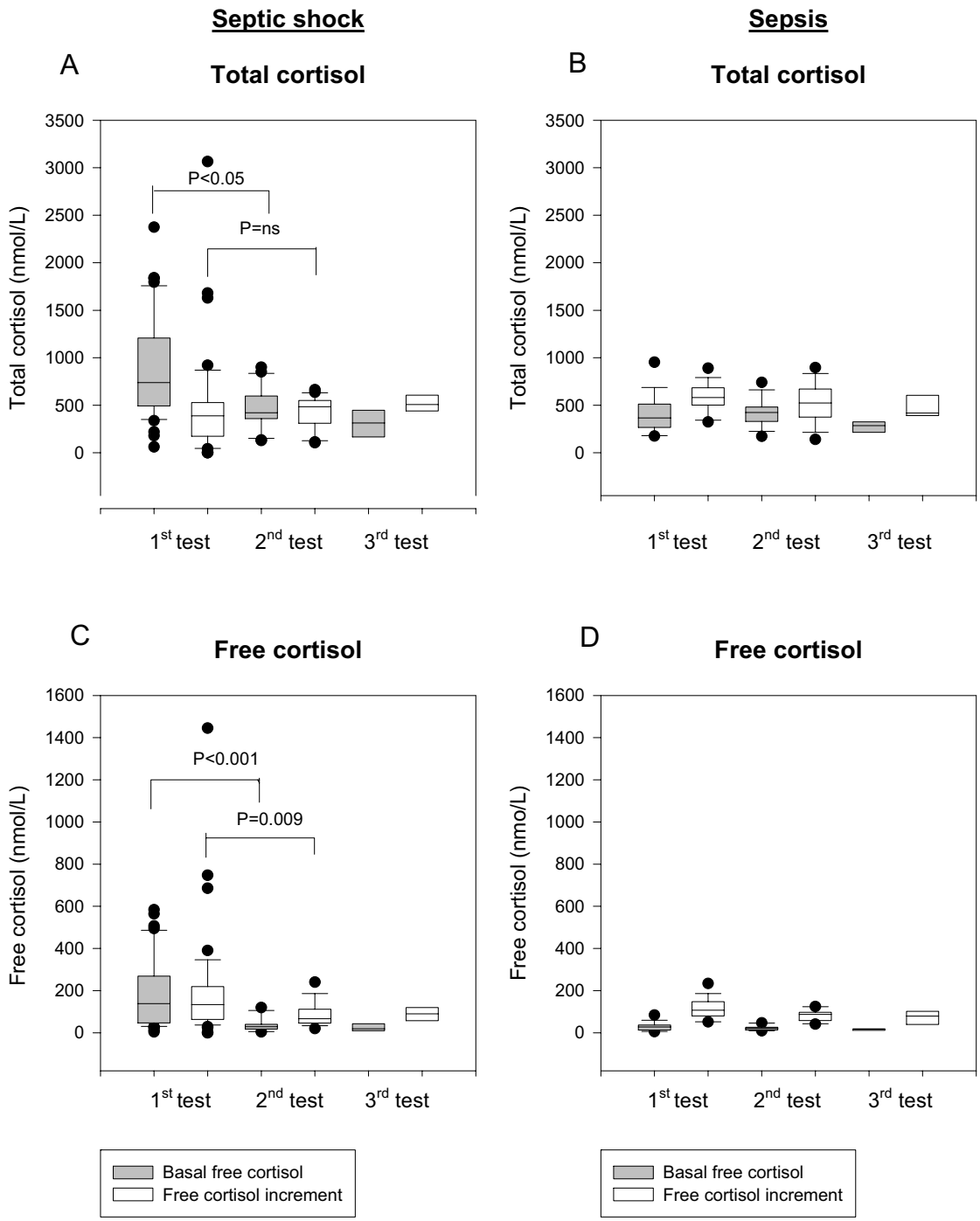


Figure 3.2 Basal and tetracosactrin-stimulated total and free cortisol levels in septic shock patients with RAI (delta cortisol ≤ 248 nmol/L, n=15) and without RAI (delta cortisol > 248 nmol/L, n=30). The plots represent median, 25th and 75th percentiles as vertical boxes with error bars (10th and 90th percentiles), * denotes $P<0.05$ for RAI vs. non-RAI. Patients with RAI had significantly higher basal total cortisol (Panel A) and free cortisol levels (Panel B), and lower total and free cortisol increment post tetracosactrin stimulation (Panel C and D). There was no difference in corticosteroid-binding globulin (CBG) and plasma albumin levels between the groups.

Results of repeated tetracosactrin tests after recovery from septic shock and sepsis

By the time of hospital discharge, follow-up tests showed free cortisol levels had fallen to baseline but total cortisol remained elevated (Fig 3.3). Among septic shock survivors, mean basal total cortisol levels remained high at the second test (1^{st} test 880 ± 79 vs. 2^{nd} test 464 ± 45 nmol/L, $P < 0.05$) but mean basal free cortisol had significantly declined (1^{st} test 186 ±24 vs. 2^{nd} test 38 ± 8 nmol/L, $P < 0.001$). Basal total cortisol did, however, fall by the third tetracosactrin stimulation test. In septic shock patients, total cortisol increments after tetracosactrin did not change significantly on re-testing (1^{st} test 477 ± 79 vs. 2^{nd} test 441 ± 36 nmol/L, P = ns) but there was a fall in free cortisol by the second test (1^{st} test 192 ± 36 vs. 2^{nd} test 84 ± 14 nmol/L, $P = 0.009$). Persistently elevated total cortisol in the SS group, despite a fall in free cortisol reflects the effect of rising CBG (1^{st} test 335 ± 17 vs. 2^{nd} test 502 ± 37, $P < 0.001$; 2^{nd} test vs. 3^{rd} test 569 ± 24 nmol/L, $P > 0.05$) and rising albumin **(**1^{st} test 19 ± 1 vs. 2^{nd} test 24 ± 2, $P = 0.004$; 2^{nd} vs. 3^{rd} test 35 ± 2 nmol/L, $P < 0.001$). Of the six patients (with $\delta \leq 248$) re-tested with tetracosactrin, all had delta cortisol > 248 nmol/L at the time of the second test. Sepsis patients exhibited no significant change in basal or stimulated total and free cortisol on re-testing. Among sepsis patients, mean CBG levels rose slightly but non-significantly on re-testing (1^{st} test 412 ± 40 vs. 2^{nd} test 526 ± 45 vs. 3^{rd} test 576 ± 71 nmol/L, $P > 0.05$). Albumin levels rose significantly **(**1^{st} test 19 ± 1 vs. 2^{nd} test 30 ± 2 vs. 3^{rd} test 36 ± 0.4 nmol/L, $P = 0.05$).


Septic shock
Sepsis
A
Total cortisol
B
Total cortisol
Total cortisol (nmol/L)
3500
3000
2500
2000
1500
1000
500
0
P<0.05
P=ns
1st test
2nd test
3rd test
C
Free cortisol
D
Free cortisol
Free cortisol (nmol/L)
1600
1400
1200
1000
800
600
400
200
0
P<0.001
P=0.009
Basal free cortisol
Free cortisol increment

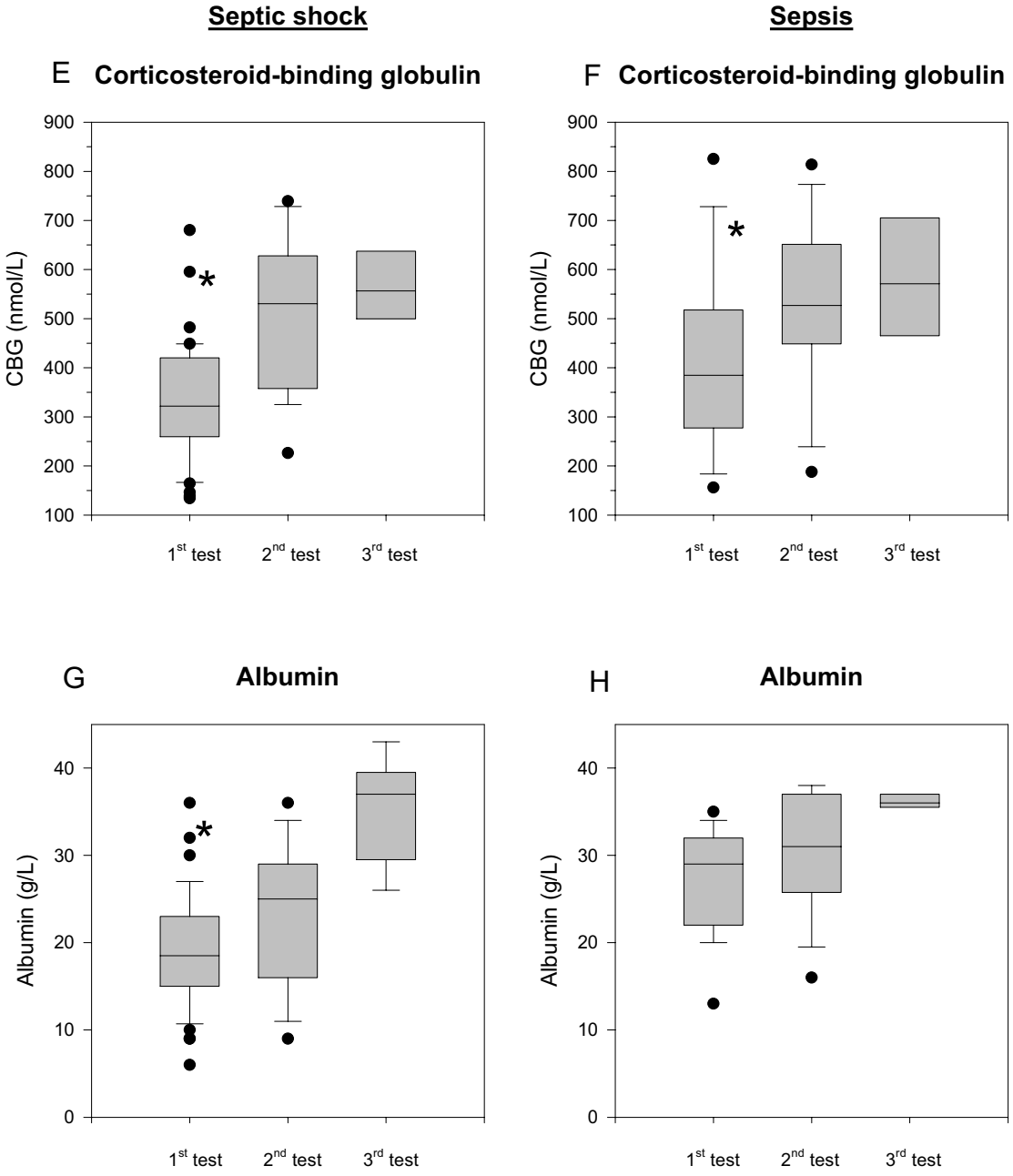


Figure 3.3 Cortisol (total and free), CBG and albumin levels from serial tetracosactrin stimulation tests in septic shock patients. Results are shown as median, 25th and 75th percentiles with standard errors, * denotes $P < 0.05$ for SS vs S vs HC. Tests were performed at the following times: 1st test: at the time of illness, 2nd test: immediately prior to hospital discharge and 3rd test: 6-12 weeks after discharge. 20 SS and 10 S patients had the second test and 14 SS and 8 S patients had the third test. In the septic shock group basal total cortisol fell on the second test but free cortisol fell to baseline. Also, total cortisol increments after tetracosactrin did not change by the second test but the free cortisol had normalised. Differences in total and free cortisol in the sepsis group were smaller.

Calculated and measured free cortisol

Calculated free cortisol (Coolens' method) and measured free cortisol (ultracentrifugation) were in close agreement (r = 0.9, $P < 0.001$, Fig. 3.4a). We also compared free cortisol by ultrafiltration with free cortisol calculated to take account of albumin concentration (Fig 3.4b) using the experimentally derived values of N (Table 3.2). Furthermore, we compare the correlation between calculated and measured free cortisol in patients with hypoalbuminemia (serum albumin ≤ 25 g/L) to those with normal albumin concentrations. As shown in Figure 4C, the correlation coefficient (r) of 0.911, $P < 0.001$, is obtained for calculated versus measured free cortisol in patients with hypoalbuminemia as compared to r = 0.936 for the normoproteinemic group. Overall, adjustment of the Coolens' equation constant N to take account of varying albumin concentrations had little effect on estimated free cortisol.

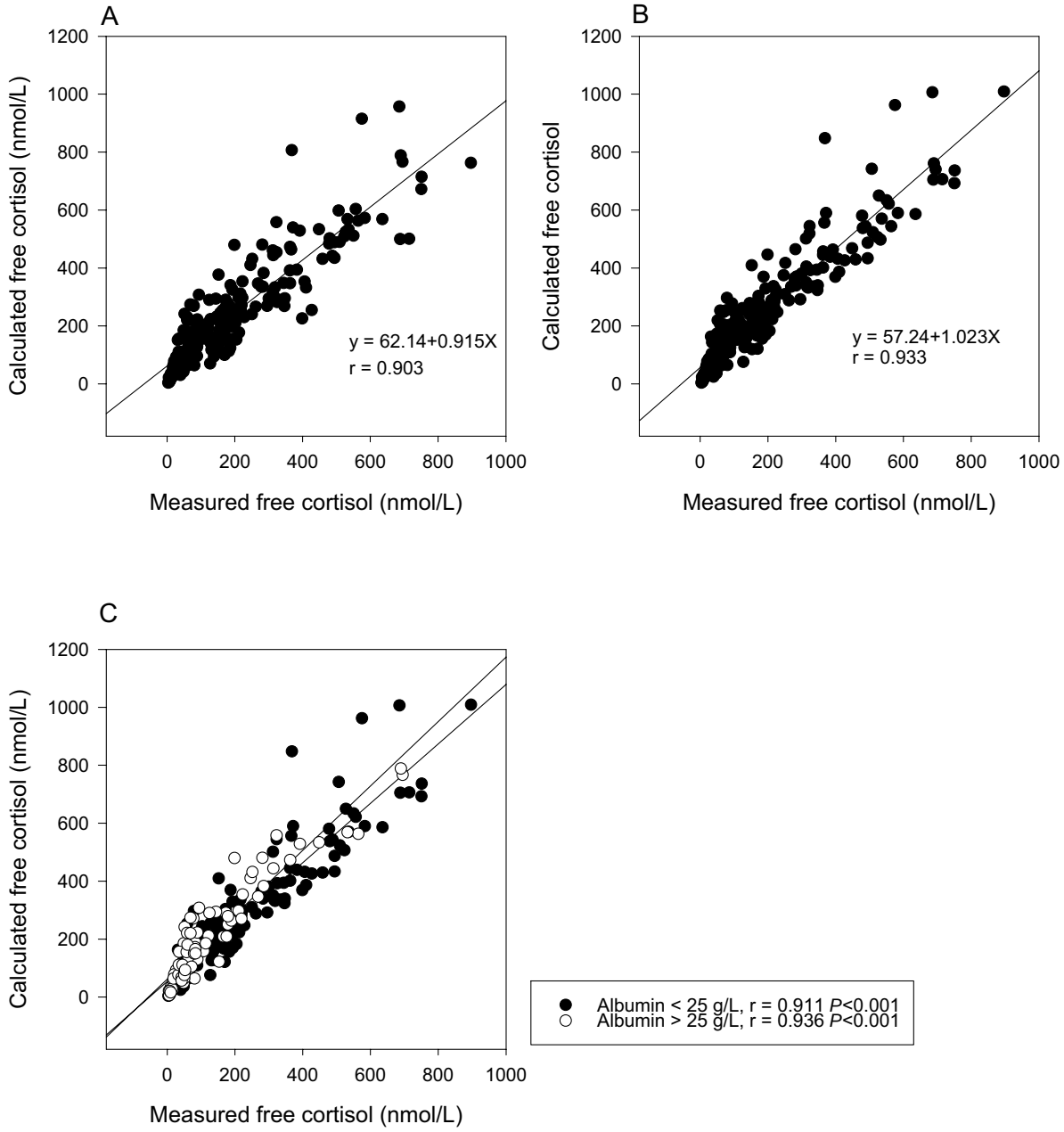


Figure 3.4 Comparisons between measured free cortisol by ultrafiltration method and (A) calculated free cortisol by Coolens' equation (the substitute for N is 1.74) and (B) calculated free cortisol using experimentally derived N to take account of varying plasma albumin concentrations, as shown in Table 3.2. Graph (C) compares the correlation of calculated vs. measured free cortisol in patients with hypoalbuminemia to those with normal albumin concentrations. All samples from the septic shock, sepsis and control groups were used in these analyses. The data suggest that adjustment of the Coolens' equation for varying plasma albumin concentrations is probably not warranted.

Table 3.2 The effect of varying concentrations of purified human serum albumin in a solution containing 600nmol/L cortisol on free cortisol fraction obtained by equilibrium dialysis, and on N the ratio of albumin bound: free cortisol. A constant N (1.74) is conventionally used in the Coolens' equation to calculate free cortisol. We used these experimentally derived values of N to re-calculate free cortisol thus compensating for variations in plasma albumin, as shown in Figure 3.4.

Albumin (g/L)	% free cortisol	N (ratio albumin bound: free)
40	29	2.45
20	37	1.69
10	50	1.0
5	68	0.47
1	76	0.32

3.6 Discussion

This study suggests that free plasma cortisol is likely to provide a more accurate reflection of circulating glucocorticoid activity than total plasma cortisol. Our findings in support of this proposition are [1] ACTH-stimulated free cortisol increments varied markedly in septic shock, sepsis and healthy controls, whereas total cortisol increments were nearly identical across all groups, [2] basal free cortisol levels were several-fold more elevated than total cortisol in septic shock, compared to sepsis and healthy controls, [3] after resolution of septic shock, basal free cortisol levels fell promptly but total cortisol levels remained elevated, [4] there is less overlap between RAI ($\delta \leq 248$) and non-RAI ($\delta > 248$) patients when basal free cortisol levels were used rather than basal total cortisol levels. In addition, we found that

Coolens' method predicts free cortisol, as measured by ultracentrifugation in a method validated by equilibrium dialysis, in septic shock.

Relative adrenal insufficiency, by some of the investigators, is based on mortality and treatment responses to hydrocortisone. Our study can not support or refute the concept of RAI, but suggests that free cortisol may be a better guide to cortisolaemia.

Cortisol circulates in plasma in three fractions; 80-90% is bound to the 52-56 kDa glycoprotein, corticosteroid-binding globulin (CBG), 5-10% is loosely bound to albumin and up to 5% circulates as free or unbound cortisol, under unstressed conditions (Hammond *et al.*, 1990). High-affinity cortisol-CBG binding (Ka 3 x 10^7 L/mol) is saturated at 500-600 nmol/L of cortisol, as each molecule of CBG binds only a single molecule of cortisol (Ballard, 1979; Hammond *et al.*, 1991). In our group of critically ill patients, mean CBG levels were reduced by 48% in septic shock and by 35% in sepsis. Correspondingly, the basal free cortisol fraction in plasma was 23% in septic shock, 7% in sepsis and 3.6% in controls. Despite increased free cortisol in septic shock, total cortisol levels may remain stable due to reductions in bound cortisol - our data show this as there was little difference in basal and stimulated total cortisol in sepsis and septic shock, but marked differences in free cortisol levels.

Free cortisol measurement, using either ultracentrifugation or equilibrium dialysis to separate free from bound cortisol, is not offered in routine clinical laboratories. Free cortisol measurement is time consuming and non-automated. There are few clinical studies comparing free cortisol to total cortisol. However, we found that the Coolens' calculation method, which relies on a number of assumptions, along with routine immunoassay of both total cortisol and CBG, applies in septic shock. The validity of this equation has been

questioned particularly in critical illness where marked reduction of cortisol-binding proteins is observed (Vogeser *et al.*, 1999). However, our data reveal that adjustment of the Coolens' equation constant N to take account of varying albumin concentrations had little effect on estimated free cortisol (Fig. 3.4). These data accords with *in vitro* data using albumin solutions and ^{3}H cortisol distribution analysed by ultrafiltration (Lewis *et al.*, 2005). Hence, the Coolens' method, without adjustment of the N value could be used in septic shock and may be more broadly applicable if free cortisol were shown to be superior to total cortisol in assisting the decision to use hydrocortisone therapy. We recognise that a constant value (K) used for the affinity of CBG for cortisol could constitute a limitation, if CBG binding affinity changes in septic shock or in hereditary CBG variants with reduced affinity. The calculated free cortisol would be markedly underestimated if these abnormal forms of CBG cannot be detected immunologically. Since the frequency of these inherited CBG abnormalities is very low and the Coolens' equation was reliable, these factors do not affect the clinical usefulness of the proposed method.

A recent report involving 66 critically ill patients, of whom 18 had sepsis but none was reported to have septic shock, suggested that low cortisol binding proteins, rather than low free cortisol levels, may underlie reports of reduced total cortisol in critical illness (Hamrahian *et al.*, 2004). Our study supports this contention and suggests that free cortisol should be evaluated in treatment trials in septic shock. We also examined the possibility that diminished cortisol increments after ACTH may relate to low cortisol binding proteins. However, total cortisol increments of ≤ 248 nmol/L were not associated with lower cortisol-binding proteins in our study.

It has been proposed that low cortisol levels in critical illness may be due to adrenal haemorrhage or necrosis, pituitary ACTH deficits, and/or genetic polymorphisms that limit

maximal adrenocortical hormone secretion (Beishuizen *et al.*, 2001). Our follow-up data reveal prompt recovery from RAI in septic shock survivors, although this observation involved only six patients. Moreover, follow-up testing of septic shock patients showed an overall return of free cortisol levels to normal values post-illness which is in keeping with the concept of a lack of functional adrenal reserve rather than adrenal damage during critical illness.

A design limitation of our study is that we can not analyze free or total cortisol levels with respect to patient outcomes, as therapy was not randomized and our numbers are not sufficient to determine treatment effects. For example, we did not show a significant difference in mortality between RAI and non-RAI patients in septic shock. Although we found that a free cortisol increment of 110 nmol/L categorized patients similarly to a total cortisol increment after tetracosactrin of 248 nmol/L, a different free cortisol cut-off may correspond to the effects of hydrocortisone treatment, but to demonstrate such an effect would require a therapeutic trial. There were more males than females in the sepsis group and the controls were younger. However, due to the relatively minor effects of age and gender on the HPA axis function (Waltman *et al.*, 1991; Parker *et al.*, 2000; Fernandez-Real *et al.*, 2002), we do not expect that this would have significantly altered the results.

The concept of RAI is controversial, since it is not immediately apparent why reduced adrenal reserve, as evidenced by reduced cortisol response to ACTH despite high basal total cortisol, is predictive of responsiveness to parenteral hydrocortisone in septic shock. Traditionally, adrenal function would be considered normal with ACTH stimulation testing if basal cortisol levels are elevated irrespective of the magnitude of the increment in response to ACTH. On the other hand, the identification of patients who can not increase cortisol secretion after exogenous ACTH may be revealing a subgroup that require more cortisol despite high basal

levels, perhaps due to acquired tissue glucocorticoid resistance. It is relevant here that one stress-dose hydrocortisone regimen (10mg/hr) produces total cortisol levels of approximately 3,100 nmol/L, well above the levels seen in our septic shock patients (Keh *et al.*, 2003). Nevertheless, the concept has empirical support in prognostic (Rothwell *et al.*, 1991; Annane *et al.*, 2000) and hydrocortisone therapy (Annane *et al.*, 2002) studies. While our data do not support or refute the concept of RAI it appears relevant that free cortisol increments after tetracosactrin varied markedly with sepsis severity, whereas total cortisol increments, on which the most validated empiric definitions of RAI rely, did not vary with sepsis severity. Hence, we think that free cortisol measures may contribute usefully to studies that attempt to use plasma cortisol to predict responses to hydrocortisone.

In comparison with a large therapeutic trial (n=299) in septic shock, the prevalence of RAI (33% vs. 77%) and mortality rate (37 % vs. 55%) were lower, despite comparable patient selection criteria (Annane *et al.*, 2002). Furthermore, in our patients with RAI, mean basal total cortisol levels were higher in RAI than in non-RAI, whereas in the study of Annane et al (Annane *et al.*, 2002), basal total cortisol was lower in RAI. The illness severity of the patients in the two studies is difficult to compare as different severity indices were used, although our mean APACHE II scores were similar to other studies of septic shock (Ligtenberg and Zijlstra, 2004). It should be recognised that etomidate, an inhibitor of 11-β hydroxylase, was administered to some patients in the study by Annane et al, and this could have contributed to the observed higher frequency of RAI. In the current study no patient received etomidate.

3.7 Conclusion

Free cortisol more closely reflects sepsis severity, and falls to normal more quickly after recovery than total cortisol. Lower total cortisol increments in RAI, using one current definition, were due to reduced free cortisol increments rather than lower cortisol binding proteins. Our data indicate that the Coolens' calculation is a reliable predictor of measured free cortisol and would be more practical than direct measurement. In the development of the notion that cortisol increments after tetracosactrin, and basal cortisol measures, may predict responsiveness to hydrocortisone treatment, our data suggest that free cortisol may be a better index of cortisolaemia than the total cortisol measures hitherto advocated.

3.8 Development of a cubic equilibrium equation in estimating free cortisol

Validation of the use of Coolens' equation in severe sepsis has led to the development of a cubic equilibrium equation in estimating free cortisol by our collaborators, Dr. Richard Dorin and his team from New Mexico, USA. Using our measured and calculated free cortisol data, they have validated the equilibrium solution and related model of free cortisol in equilibrium with CBG and albumin by comparing estimates of free cortisol, derived using published or derived dissociation constants (K_d) for CBG (K_C) and albumin (K_A).

$$Eq.13) \qquad F = \frac{-((1+N)K_C + TotC - TotF) + \sqrt{((1+N)K_C + TotC - TotF)^2 + 4(1+N)TotF * K_C}}{2(1+N)}.$$

F represents free cortisol, *C* = unbound CBG, *FC* = CBG-bound cortisol and *TotC* = total CBG. N is equal to 1.74

Group-specific K_A estimates were derived using least squares solution for non-linear regression, and individual K_A estimates were derived using measured free cortisol. In a dynamic model, equilibrium is achieved rapidly (<0.3 second). Septic shock patients had a higher K_A (141 μM , 127-156 95% CI) compared to septic and control subjects (K_A = 105 μM) (P=0.004). In a paired analysis, K_A decreased significantly following recovery from sepsis (*P*=0.04). Among septic shock subjects, those with relative adrenal insufficiency (RAI), had significantly higher K_A than non-RAI (*P*=0.03). Comparing Coolens' equation (A) and cubic equilibrium equation (B) in Figure 3.5, the cubic equilibrium equation predicts measured free cortisol accurately under conditions of variable CBG and albumin concentration. As septic shock and RAI are associated with a reversible increase in K_A, and the decreased affinity of cortisol for albumin contributes substantially to the relative increase in free cortisol observed, performance of the cubic equilibrium is superior to Coolens' equation. The cubic equation provides a reasonably accurate estimation of free cortisol and new insights related to reversible changes in cortisol binding affinity for albumin in septic shock and RAI, and has additional applications useful to modelling and hypothesis development.

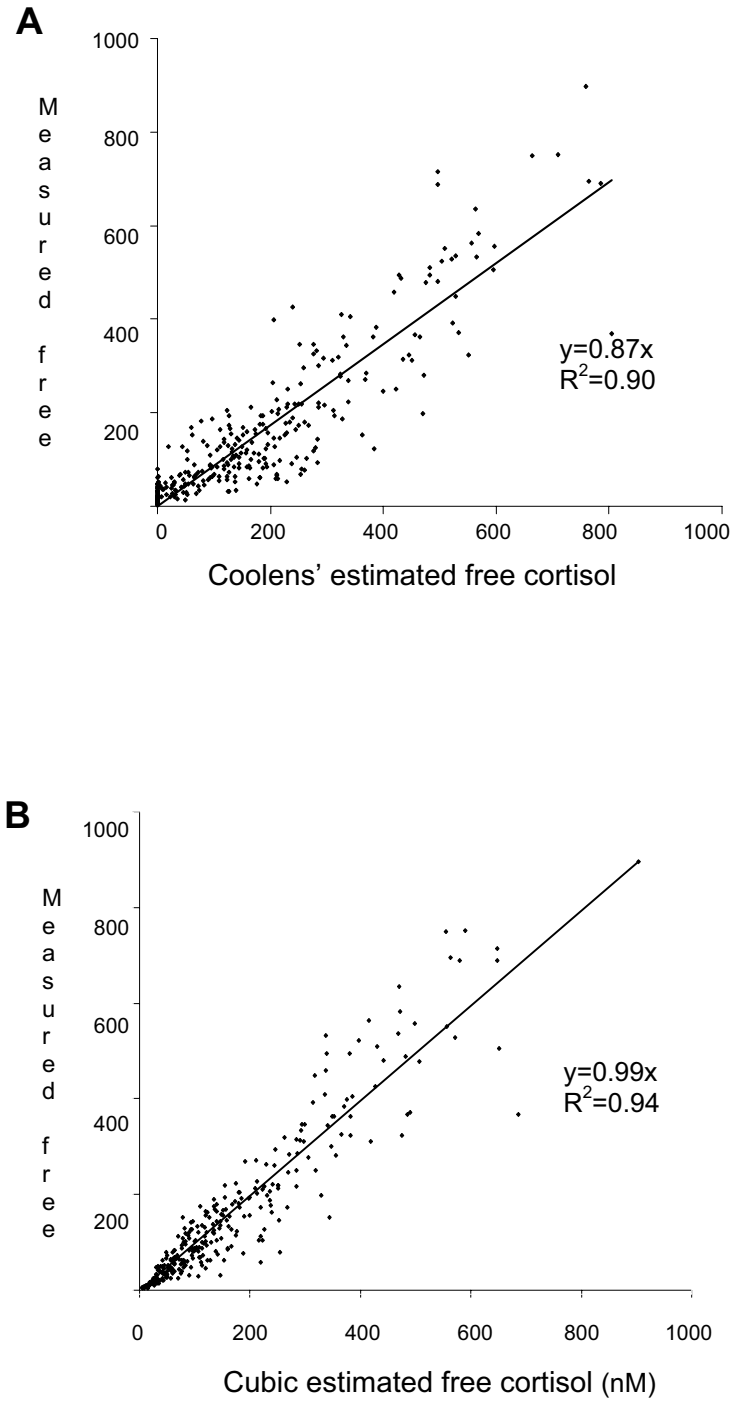


Figure 3.5 Correlation between measured free cortisol (ultrafiltration/ligand binding method) and free cortisol estimated using either (A) Coolens equation or (B) cubic equilibrium equation. Free cortisol estimates by the cubic equation are obtained using group-specific K_A values derived using the non-linear least squares solution.

Chapter 4

Nitric oxide as a potential physiologically relevant measure of cortisol action in tissues

4.1 Summary

Nitric oxide (NO) is normally produced in the endothelium by the constitutive form of NO synthase. This physiological production of NO is important for blood pressure regulation and blood flow distribution. Extensive research has suggested that overproduction of NO by the inducible form of NO synthase (iNOS) may contribute to the hypotension, cardiodepression and vascular hyporeactivity in septic shock. Lipopolysaccarides and cytokines, such as TNF-α, IL-1 and interferon-γ, have been shown to induce iNOS in the endothelium, vascular smooth muscle cells macrophages and various parenchymal cells. Measurement of NO *in vivo* is difficult due to its short half life, but plasma nitrate/ nitrite (NOx), the stable products of NO oxidation, are indicators of NO activity (Wong *et al.*, 1995; Krafte-Jacobs *et al.*, 1997; Spack *et al.*, 1997). Plasma NOx level correlates with sepsis severity and may predict a greater risk of developing more severe organ dysfunction and mortality.

Clinical studies of non-selective inhibitors of the L-arginine nitric oxide pathway showed an increase in cardiovascular death from pulmonary hypertension, myocardial ischaemia and reduced cardiac output. However, glucocorticoids, which improve vasopressor sensitivity, may act by partially suppressing NO synthesis through selective direct inhibition of iNOS, and suppression of inflammatory cytokine synthesis.

It has been shown that exogenous hydrocortisone administration reduces plasma NOx and this response correlates with a reduced requirement for vasopressor treatment in septic shock.

Hence, NOx levels may provide a titratable end point to individualize glucocorticoid therapy in sepsis. As NOx levels may reflect the balance of cellular glucocorticoid receptor-α activity, NOx may be a tissue marker of glucocorticoid activity, as well as being directly relevant to haemodynamic status in sepsis. The use of a tissue marker of glucucocorticoid sufficiency in sepsis may be a better guide to the use of exogenous hydrocortisone in sepsis since since cortisol levels have limited value in this regard despite the potential value of free cortisol over total cortisol described elsewhere in this thesis.

The hypotheses of the study are that the severity of sepsis correlates with plasma NOx, that NOx levels correlate with cortisolaemia, and that NOx levels may relate to vasopressor requirement. The aims were to (1) relate plasma NOx to illness severity, (2) determine the relation between NOx and cortisol levels, in severe sepsis, and (3) determine if NOx levels relate to the need for vasopressors.

Plasma NOx, total and free cortisol, and CBG concentrations were measured for 7 days following admission of sepsis/septic shock patients to the intensive care unit. The study showed that patients who developed septic shock (n=35) had higher plasma NOx, total and free cortisol, and lower CBG concentrations than the sepsis alone group (n=27). Cortisol, CBG and NOx levels correlated with illness severity. Free cortisol, and to a lesser extent total cortisol, but not NOx levels, predict septic shock. NOx levels were characteristically stable within individuals but inter-individual differences were only partly accounted for by illness severity or renal dysfunction. NOx levels did correlate weakly with cortisol. However, NOx levels did not relate to the need for vasopressors. NOx levels were not suppressed by hydrocortisone treatment, although hydrocortisone therapy was given to deteriorating patients whose NOx levels may have otherwise been expected to rise.

In conclusion, nitric oxide production is correlated with illness severity and cortisolaemia but did not relate to vasopressor requirement, suggesting it will not comprise a suitable target for individualized hydrocortisone therapy.

4.2 Introduction

Nitric oxide, a ubiquitous biological molecule produced by numerous cell types, is implicated in a wide range of disease processes, exerting both detrimental and beneficial effects at the cellular and vascular levels. NO is believed to play a key role in the pathogenesis of septic shock, as clinical data provided evidence of increased NO production in sepsis, contributing to hypotension and myocardial dysfunction. Treatment with non-selective inhibitors of NO synthesis has been shown to improve haemodynamic parameters and survival in several animal models of septic shock. In human septic shock, inhibition of NO synthesis has been shown to have haemodynamic improvement in the short-term, but its long-term beneficial effect is uncertain. The aim of this chapter is to discuss our observations of NOx in sepsis, which were directed towards the potential use of NOx as a hydrocortisone treatment target in the management of sepsis/septic shock.

4.2.1 Nitric oxide synthesis and metabolism

NO is a gaseous, electrically neutral free radical that is highly water-soluble. It is an ideal local physiological messenger because of its small size, lipophilic nature and brief duration of action, having a half-life of only 0.1 to 10 seconds (Ignarro, 1991). This highly reactive oxidation product of nitrogen is produced normally by many cell types, including endothelial cells, resulting in the potential for widespread effects in the cardiovascular, central nervous and immune systems (Nathan, 1992).

NO is synthesized enzymatically from the amino acid L-arginine by different isoenzymes of the nitric oxide synthase (NOS). Two of these isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), are calcium-dependent and constitutively present. These enzymes function under resting conditions to generate small transient surges in NO in response to elevated intracellular ionized calcium levels. The third isoform, inducible NOS (iNOS), is calcium-independent and may be present in a wide range of cell types. iNOS is not expressed under basal conditions except in intestinal, bronchial, and renal tubular epithelial cells (Geller and Billiar, 1998). Rather, iNOS expression occurs in the face of cellular stress and inflammation, including hypoxia and sepsis (Zamora *et al.*, 2000). The regulation of iNOS expression is thought to occur primarily at the level of gene expression. In contrast to constitutive isoforms of NOS, iNOS expression results in sustained production of larger quantities of NO for extended periods of time. Thus, iNOS-generated NO can lead to sustained vasodilatation or cell toxicity and subsequently can contribute to the pathophysiology of inflammation, sepsis and shock.

Most of the effects of NO are mediated through a guanosine 3', 5'-cyclic monophosphate (cGMP)-dependent mechanism. eNOS derived NO helps maintain perfusion of the microcirculation via vasodilatation and opposes sympathetic and chemically mediated vasoconstriction that might otherwise adversely affect tissue perfusion. At basal concentrations, NO protects the integrity of the endothelium and functions as a radical scavenger and exerts cytoprotective effects. The net result is a decrease in endothelial damage, vascular inflammation and oxidant injury. Conversely, exaggerated NO production can result in tissue damage (Figure 4.1) (Nathan and Xie, 1994; Kirkeboen and Strand, 1999).

NOTE: This figure is included on page 86 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.1 Dichotomous effects of endothelial nitric oxide synthase (eNOS)-derived and inflammatory nitric oxide synthase (iNOS)-derived nitric oxide (NO). (Kirkeboen 1999)

4.2.2 The role of nitric oxide in sepsis

The finding of elevated circulating plasma nitrite/nitrate (NOx), the stable by-products of NO, in septic patients, together with the reduction in vascular tone seen after endotoxin or proinflammatory cytokine administration, leads to the suggestion that NO is involved in the cardiovascular alterations and direct cellular toxicity of septic shock. Proinflammatory cytokines associated with sepsis (IL-1, IL-6, TNF, interferon-γ, IL-1β) and endotoxin (lipopolysaccharide) increase both iNOS expression and NO production (Liu *et al.*, 1993; Nathan and Xie, 1994b). Additional insight is derived from studies in iNOS-deficient mice demonstrating a resistance to endotoxin-induced hypotension and mortality (MacMicking *et*

al., 1995; Wei *et al.*, 1995; Gunnett *et al.*, 1998). These data confirm that pathologic levels of iNOS expression and NO synthesis contribute to the excessive vasodilatation, loss of systemic vascular resistance and vascular leak that are characteristic of septic shock.

In addition to its adverse haemodynamic effects in sepsis, NO overproduction has other physiologic and cellular consequences. In high levels, NO acts as a negative inotrope on the myocardium (Vincent, 1998; Flesch *et al.*, 1999). Physiologic concentrations of NO preserve hepatic, splanchnic and renal microcirculatory flow, minimizing tissue hypoperfusion during low-flow states. In contrast, pathologic levels of NO in septic shock can induce hepatocyte damage and increase gut epithelial permeability (Salzman *et al.*, 1995). Finally, many investigators have advocated that high levels of NO in sepsis exert cytotoxic effects through the formation of $ONOO^-$, resulting in damage to DNA and membrane phospholipids and impairment of mitochondrial respiration (Szabo and Ohshima, 1997; Vincent *et al.*, 2000).

While measurement of NO in vivo is difficult because of its short half-life, plasma NOx has been used as an indicator of NO activity in clinical studies. Plasma NOx has been shown to be increased in neonates, children and adults with sepsis (Ochoa *et al.*, 1991; Wong *et al.*, 1995). Further, high NOx levels have been reported to correlate with the degree of vascular failure and hypotension, and in part, to the degree of inflammation in paediatric sepsis. Children with higher circulating IL-6, consistent with markers of inflammation and disease severity, have higher plasma NOx concentrations (Doughty *et al.*, 1998). Whether NO contributes to the development of multiple organ dysfunction syndrome (MODS) in patients with sepsis is still a matter of debate (Lorente *et al.*, 1993).

4.2.3 Nitric oxide and cortisol

Given the roles of NO as potent vasodilator, immunomodulator, and potential cytotoxin, it represents a potential therapeutic target. Experimental studies in animals and humans on the effects of NO blockade with NOS inhibitors have yielded inconclusive results. Most of these studies have used L-arginine analogs that non-selectively and competitively inhibit NOS. The discrepancies published are likely related to model variability, specificity, and differences in the inhibitor type and dosing regimen. However, some generalized conclusions have emerged regarding the physiology of NOS inhibition in septic shock: NO blockade can increase mean arterial pressure and reverse hypotension, vascular leak, and vasopressor hyporeactivity (Kilbourn *et al.*, 1990; Hollenberg *et al.*, 1993; Lorente *et al.*, 1993; Avontuur *et al.*, 1999; Grover *et al.*, 1999); blocking NO with NOS inhibitors tends to decrease cardiac contractility and cardiac output while increasing pulmonary vascular resistance (Lorente *et al.*, 1993; Petros *et al.*, 1994; Cobb, 2001); and non-selective NOS blockade can abolish the protective effects of eNOS on the endothelium and microcirculatory flow, potentially worsening organ injury in the liver, gut, kidney, and heart (Harbrecht *et al.*, 1994; Cobb and Danner, 1996). The potential for negative effects of non-specific NOS inhibitors was highlighted in 1999 when a phase III prospective, randomized trial was terminated prematurely owing to increased mortality in the treatment group (Cobb, 1999; Grover *et al.*, 1999).

Glucocorticoids, on the other hand, can inhibit the induction of iNOS selectively, and the production of inflammatory cytokines. As discussed earlier, NOS activity is stimulated by inflammatory cytokines such as IL-6 and IL-8, IL-1β and TNF-α, and the output of these inflammatory cytokines reflects the net balance of glucocorticoid receptor-α and NF-κB activity at the cellular level (Franchimont *et al.*, 2002; Meduri *et al.*, 2002). Cortisol can also augment the blood pressure elevating of noradrenaline, the chief vasopressor used in septic shock. Several studies have shown that initiation of stress-dose hydrocortisone therapy in

septic shock is associated with improved survival and earlier vasopressor therapy withdrawal (Briegel *et al.*, 1999; Bollaert, 2000; Annane *et al.*, 2002). (See Chapter 3) Keh et al. found that hydrocortisone infusion 10mg/hr for 3 days, followed by placebo in a cross-over design in 20 septic shock patients, was associated with a mean 32% fall in plasma NOx levels compared to placebo. The reduction of plasma NOx correlated with reduced vasopressor support in septic shock patients treated with hydrocortisone infusion (Figure 4.2) (Keh *et al.*, 2003).

Plasma free cortisol is a better guide to circulating glucocorticoid activity than total cortisol, and it corresponds more closely to illness severity in severe sepsis (Chapter 3). However, there is no data on the relationship between adrenocortical function and NO in sepsis.

NOTE: This figure is included on page 89 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.2 NOx plasma concentration. HC administration, but not placebo, reduced the NOx plasma concentration, indicating inhibition of nitric oxide formation. Note that NOx remained unchanged after cessation of HC infusion. On Day 3, NOx was significantly different between HC-1 and HC-2 ($p < 0.02$, Mann-Whitney-U test). (*Line chart*) p values of different treatment periods represent results of variance analysis (Friedman test). (*Bar chart*) Compared with placebo, a 3-day exposure to hydrocortisone reduced NOx to approximately 32% ($p = 0.009$). *Triangles* = baseline values; *closed circles* = hydrocortisone; *open circles* = placebo. (Keh 2003)

4.3 Hypotheses and Aims

This study aims to define the relationship between adrenal function and NOS activity and relate NOx levels to vasopressor initiation and discontinuation in severe sepsis. This may help refine patient selection criteria for hydrocortisone therapy and optimise treatment regimens.

The hypotheses of the study are:

1. That sepsis severity correlates with plasma NOx level
2. That plasma NOx and cortisolaemia are related
3. That plasma NOx, predicts the requirement for vasopressor support.

The aims of the study are:

1. To observe the relation between plasma NOx levels and the severity of sepsis, as defined by APACHE II scoring system
2. The relation between NOx and endogenous glucocorticoid in severe sepsis
3. To determine if NOx levels relate to the ability to withdraw vaopressors.

4.4 Research design and methods

Subjects

This study was approved by the Royal Adelaide Hospital Ethics Committee and informed consent was obtained from the patients or their next-of-kin. Patients were recruited within 24 hours of admission to the intensive care unit (ICU) with a diagnosis of the sepsis syndrome. The criteria for sepsis syndrome were based on the recommendations of Bone et al (Bone, 1991), as discussed in Chapter 3.

Treatment decisions were not influenced by the study protocol and corticosteroid treatment was the decision of treating physicians. Vasopressor regimen was standardized and titrated against a mean arterial pressure (MAP) of at least 70 mmHg. Briefly, noradrenaline/ adrenaline infusion (6 mg in 100 ml 0.9 % saline) was commenced at 2 – 4 μg/min, and then titrated to a target MAP determined by the treating doctor. Once the target MAP was stable for at least 4 hours, the infusion rate was reduced by 5 μg/min half hourly if infusion rate > 10 μg/min or 2 μg/min half hourly if infusion rate < 10 μg/min. If MAP > 20 mmHg above goal, the infusion rate was reduced by 50% and if MAP fell below target for more than 5 minutes, the infusion rate was increased to meet target MAP.

Clinical Evaluation

Information relating to: [1] age and gender; [2] infection site/s and organisms [3] severity of illness on admission using the Acute Physiology and Chronic Health Evaluation (APACHE) II scoring system; [4] use of hydrocortisone; [5] outcome measures including mortality; and [6] vasopressor support was collected.

Blood samples

On admission and each morning for up to seven consecutive days, blood was collected for routine laboratory tests, plasma total and free cortisol, corticosteroid-binding globulin and nitrite/ nitrate (NOx) concentrations. Samples were immediately centrifuged at 2000 g at 4 ^{o}C for 15 min, and plasma was stored at -80 ^{o}C. All samples from an individual subject were measured in the same assay to eliminate inter-assay variation. Hormone assays are discussed in Chapter 2.

Statistical analysis

Summary statistics were reported as mean ± standard error, together with the number of observations (n). Differences between two means according to groups were analyzed by Student's unpaired t test. Tables of categorical data were analyzed by Fisher's exact test (2 × 2 tables) or an exact test on the relative risk. The exact Fisher-Freeman-Halton test was used to analyze $r \times c$ tables. Ordinary least-products regression analysis was used for the prediction of one continuous variable by another, resulting in P values for slope = 0, and correlation coefficients (r). Stepwise binomial logistic regression analysis was used to identify predictors of shock or death. Repeated measures analysis of variance (RM-ANOVA) with the Greenhouse-Geisser adjustment for serial autocorrelation was used to test for differences between groups over time, though this technique was handicapped by missing values. When multiple comparisons were made (e.g. Tables 4.1 and 4.2), the Ryan-Holm stepdown Bonferroni procedure was used to control the familywise Type I error-rate, by adjusting P to P'. Two-sided $P \leq 0.05$ (or $P' < 0.05$) was regarded as statistically significant. Continuous variables were analyzed using SYSTAT 10 (SPSS Inc., Chicago IL). Categorical variables were analyzed using StatXact 7 (Cytel Software Corporation, Cambridge MA).

4.5 Results

Baseline characteristics

Sixty two patients enrolled into the study were retrospectively divided into two groups; those with sepsis (n = 27) and those with septic shock (n = 35). Clinical characteristics are outlined in Table 4.1 Patients who developed septic shock were older and had greater illness severity than the sepsis group at study entry. 51% (18/35) of the septic shock group received corticosteroid supplementation (hydrocortisone equivalent dose of 200 – 300 mg per day). Steroid recipients had greater admission illness severity with a mean APACHE II score of 25

compared to 18 in the non-hydrocortisone treated group. No significant differences between hydrocortisone and non-hydrocortisone treated septic shock groups were evident for age, sex, duration of vasopressor requirement or mortality.

Seven sepsis patients received corticosteroids, four for exacerbation of chronic obstructive pulmonary disease, two for immunosuppression following bone marrow transplantation and one for severe epiglottitis. The two sepsis patient deaths resulted from myocardial infarction and massive stroke.

Table 4.1 Clinical chaacteristics of patients with sepsis and septic shock

	Septic shock (n = 35)	Sepsis (n = 27)	Two-sided P	Two-sided P'
Age (years)	60 ± 2.6	50 ± 3.7	0.03*	0.21
Gender (M:F)	22 : 13	19 : 8	0.60#	>0.99
APACHE II	22 ± 1.1	17 ± 1.4	0.01*	0.08
Dead at <28 days	9	2	0.094#	0.47
Main source of infection				
Pulmonary	20	18		
Gastrointestinal	5	2	0.86##	>0.99
Genitourinary	2	1		
Others	8	6		
Microbiology				
Gram positive	14	12		
Gram negative	9	4		
Fungus	2	0	0.49##	>0.99
Multiple	8	7		
Unknown	2	4		
Corticosteroid use				
No. of patients	18	7	0.03#	0.21
Duration of use (days)	4 ± 0.6	4 ± 1.1	0.81*	>0.99
Vasopressor support				
No. of patients	35	0	<0.0001#	<0.001
Duration of use (days)	3 ± 0.3	0	<0.0001*	<0.001

P', raw P value corrected for 10 simultaneous hypotheses by the Ryan-Holm stepdown Bonferroni technique. * t test. # Fisher exact test. ## Fisher-Freeman-Halton exact test.

NOx, illness severity, shock and survival

Indices of stress hormone release (plasma total and free cortisol, CBG concentrations), nitric oxide synthesis (plasma NOx) and serum albumin and creatinine concentrations on admission are presented in Table 4.2. Illness severity represented by APACHE II scores correlated positively with plasma total and free cortisol and NOx concentrations on admission (Figure 4.3). Using stepwise logistic regression analysis, free cortisol on admission was a better predictor of septic shock ($P = 0.017$) than total cortisol ($P = 0.03$). Serial NOx concentrations of the non-survivors (n=9) with septic shock who died were higher than that of the survivors ($P = 0.005$). However, plasma NOx was not a significant predictor of shock. The relative risk of septic shock was 1.175 greater only when plasma NOx concentration was more than 50 μmol/L (exact Cochran-Armitage test). Age of the patients was a significant predictor of mortality ($P = 0.0181$).

Although total and free cortisol and CBG concentrations at admission were similar, there was a 4- to 5-fold difference in total and free cortisol concentrations between survivors and non-survivors on days preceding death (survivors vs. non-survivors: total cortisol - 905 vs. 3688 nmol/L, $P = 0.015$ on day 3; 719 vs. 3099 nmol/L, $P = 0.005$ on day 6; and free cortisol – 283 vs. 1006 nmol/L, $P = 0.05$ on day 3, 176 vs. 1036 nmol/L, $P = 0.006$ on day 6). Plasma CBG concentrations were also significantly lower in the non-survivors, 162 vs. 317 nmol/L survivors, $P < 0.001$.

Table 4.2 Plasma concentrations of cortisol, NOx, albumin, and creatinine (CBG) on admission in septic shock and sepsis groups.

	Septic shock (n = 35)	Sepsis (n = 27)	Two-sided P	Two-sided P'
Total cortisol (nmol/L)	1667± 281	830 ± 138	0.02	0.06
Free cortisol (nmol/L)	524 ± 101	205 ± 48	0.01	**0.04**
CBG (nmol/L)	282 ± 20	380 ± 22	0.002	**0.01**
NOx (µmol/L)	72 ± 7.2	46 ± 9	0.03	0.06
Albumin (g/L)	19 ± 1	26 ± 2	0.001	**0.006**
Creatinine (µmol/L)	179 ± 24	112 ± 21	0.05	0.06

P', raw P value corrected for 6 simultaneous hypotheses by the Ryan-Holm stepdown Bonferroni technique. All analyses by unpaired t test.

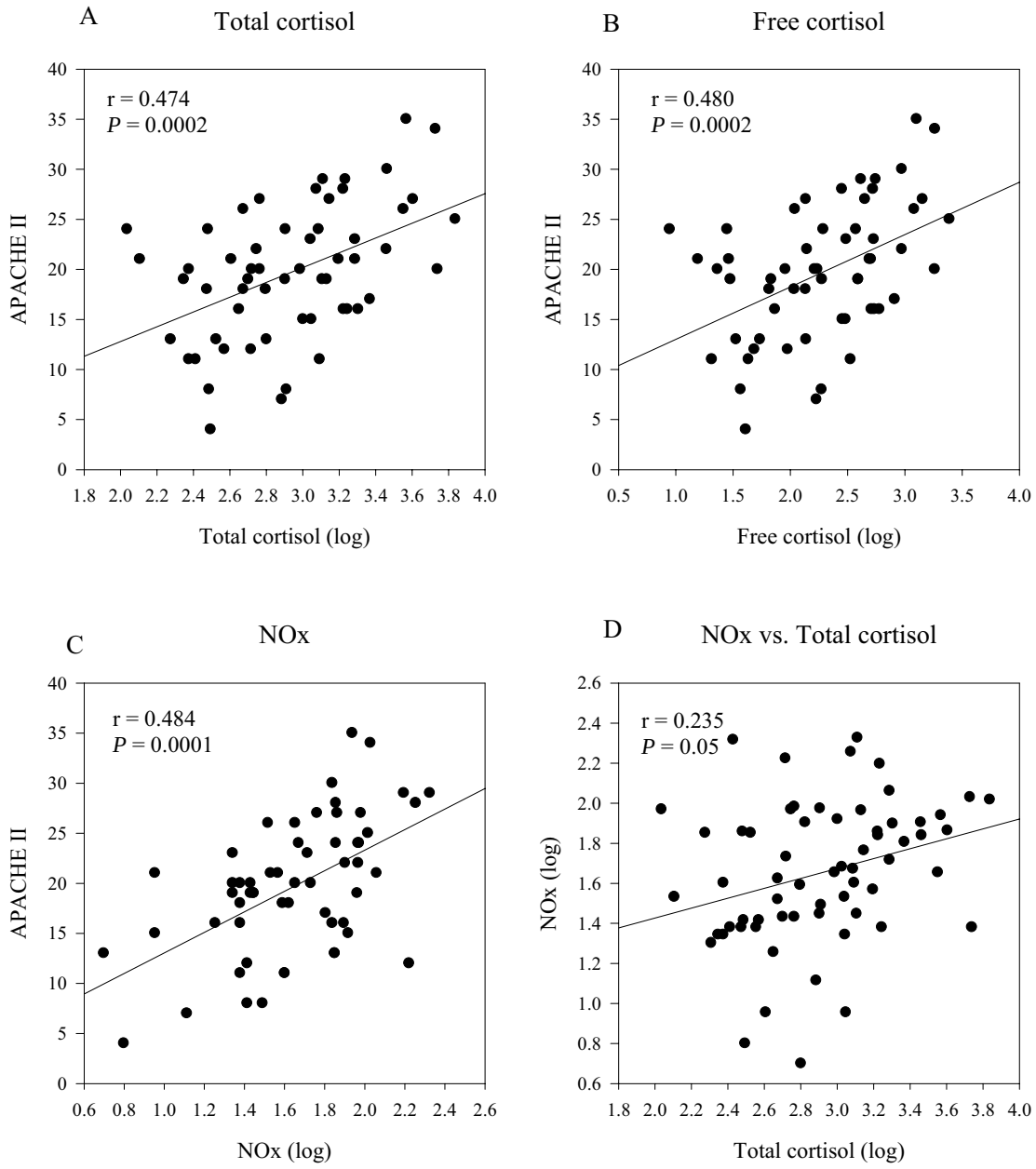


Figure 4.3 Illness severity represented by APACHE II scores correlated positively with plasma total and free cortisol and NOx on admission (A, B and C). The correlation between Nox and total cortisol is only moderate (D).

Plasma NOx and cortisol concentrations

There was a very high inter-individual variability in plasma NOx concentrations (range 22 – 180 μmol/L). However within individuals, there was little diurnal variation in NOx concentrations and only a non-significant decrease from baseline to the end of study (P = 0.61) (Fig. 4.4C).

Plasma total and free cortisol concentrations were significantly elevated in patients with septic shock (Table 4.2). However, there is a wide variation between individuals (range: total cortisol 128 – 2018 nmol/L and free cortisol 40 – 600 nmol/L). The correlation between plasma NOx and cortisol measurements was only moderately positive (P = 0.05) (Figure 4.3D).

Plasma NOx and renal function

There was a positive correlation between plasma NOx and serum creatinine (Table 4.3), since plasma NOx concentrations were higher in patients with serum creatinine concentrations greater than 200 μmol/L (n = 10). However, when the confounding effect of creatinine was excluded, plasma NOx still correlated positively with illness severity (r = 0.39, P = 0.007). There were no differences in NOx concentrations between survivors and non-survivors, when creatinine was considered as a covariate (P = 0.11).

Plasma NOx and vasopressor support

By Day 3, ten of the twenty four survivors continued to receive vasopressor support in excess of 5 μg/kg/min of noradrenaline. Mean length of vasopressor support for the duration of the study was 4 days (range 2 - 8 days). 44% of the deaths occurred within 72 hours while receiving vasopressors. There was no relationship, however, between the requirement for vasopressors and plasma NOx concentrations, either at commencement of vasopressors (P = 0.62), highest dose required (P = 0.92) or discontinuation (P = 0.72).

Hydrocortisone treatment and NOx

Hydrocortisone therapy increased plasma cortisol concentrations 2- to 5-fold with peak levels within 24 hours (Figure 4.4A and B). However, serial NOx concentrations were similar in hydrocortisone treated and untreated patients (Figure 4.4C). Initiation of hydrocortisone was not associated with any significant fall in NOx levels; however this was an uncontrolled observation i.e. no subjects were commenced on hydrocortisone for reasons other than clinical deterioration, which may elevate NOx.

Table 4.3 Ordinary least-products regression analysis between plasma NOx and cortisol measurements (total and free cortisol, CBG) and renal function

Dependent variable		Independent variables			
NOx		Total cortisol	Free cortisol	CBG	Creatinine
	n	62	62	62	62
	r	0.04	0.03	0.18	0.5
	P	0.90	0.90	0.54	< 0.001
	P'	> 0.999	> 0.999	> 0.999	< 0.004

P′, raw P value corrected for 4 simultaneous hypotheses by the Ryan-Holm stepdown Bonferroni technique.

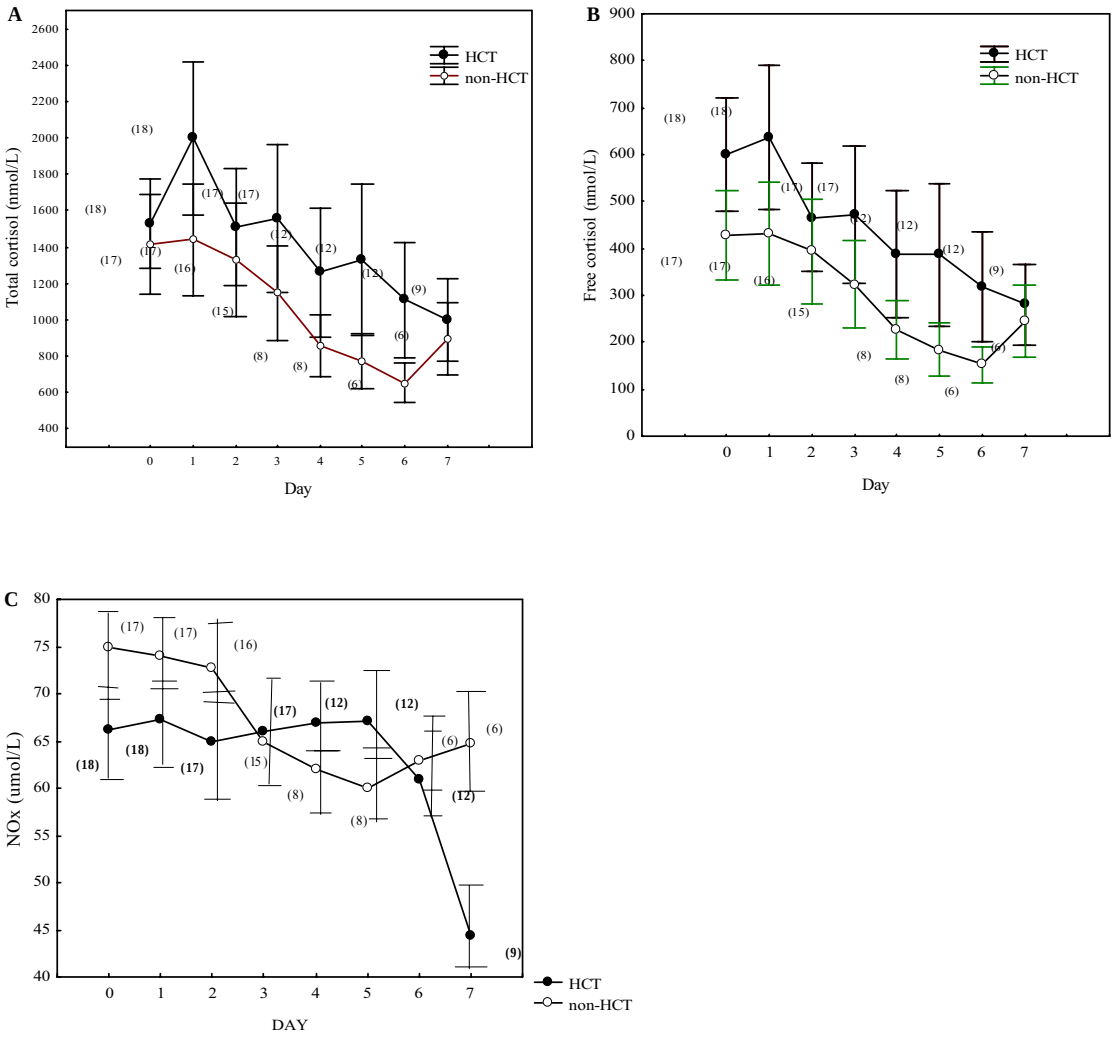


Figure 4.4 Serial plasma total and free cortisol, and NOx concentrations in hydrocortisone treated (n = 18) and untreated (n = 17) septic shock patients. Intravenous hydrocortisone increased individual's plasma cortisol concentrations by 2- to 5-fold within 24 hours, mean levels increased up to 2-fold (A and B). Both total and free cortisol concentrations remained elevated during the study period. Plasma NOx was not readily suppressed by hydrocortisone; the fall in NOx concentrations was only evident after day 5 of treatment (C).
(number) represents the number of observations at that particular time point

4.6 Discussion

Plasma NOx, total and free cortisol concentrations were elevated in relation to illness severity, as measured by APACHE II score or sepsis category (sepsis/septic shock). However, plasma NOx did not predict shock, nor did NOx relate to vasopressor discontinuation. Hence, NOx would not appear to be a promising marker of tissue glucocorticoid sufficiency to guide individualized hydrocortisone therapy in severe sepsis.

NOx levels were very high in sepsis and displayed a high inter-individual variability that could only be partly accounted for by illness severity even when the confounding effect of renal function was eliminated. Conversely, NOx levels were remarkably stable within individuals suggesting individual constitutional factors may play a major role in the NOx response to illness. These characteristics undermine the value of NOx as a marker of iNOS mediated vascular instability and a target for therapy using inhibitors of iNOS such as exogenous hydrocortisone.

Our finding of higher NOx levels in patients with septic shock than sepsis is in agreement with other investigators (Spack *et al.*, 1997; Doughty *et al.*, 1998; MacKenzie *et al.*, 2001). However, the correlation with illness severity was only moderate and there was no association with mortality. In contrast to the study by Keh et al, we found no relationship between vasopressor support and plasma NOx levels.

A limitation of this study was that the time of hydrocortisone administration was not standardized as it was the decision of the treating intensivist. The subjects in the Keh et al. study received hydrocortisone infusion (10mg/h) at the time of septic shock diagnosis, whereas in this study hydrocortisone was usually administered when patients deteriorated despite volume replacement and on moderate to high dose inotropic support. Hence, there was

a 24 to 48 hours lag time among subjects who received hydrocortisone therapy and a high inter-individual variability in NOx levels at the time of treatment.

Abnormalities of the NO pathway have been identified in specific cells from patients with chronic diseases (Sakurai *et al.*, 1995; Godkin *et al.*, 1996) but the cells responsible for hyperproduction of NO in sepsis are yet to be determined. Studies have demonstrated tissue heterogeneity in NO activity as well as differences in the response to different inflammatory stimuli (Salter *et al.*, 1991; Cunha *et al.*, 1994). It has also been proposed that iNOS activity is compartmentalized and restricted to the site of inflammation (Schoedon *et al.*, 1995; Annane *et al.*, 2000a). This may account for the great variability in NOx concentrations between individuals with sepsis. It could also explain the persistent elevation of NOx on days after the onset of sepsis despite glucocorticoids, and the lack of correlation with vasopressor requirement.

Plasma NOx measurement represents an indirect but acceptable surrogate for NO production in most studies. However, NOx levels can be affected by diet, renal function and hydration, all of which may be altered in severe illness. This could make interpretation of data difficult and yield conflicting results. In addition, systemic concentration of NOx may not reflect local production of NO. Measurements of other intermediates such as cyclic guanosine monophosphate (cGMP) and nitrosothiol, or immunohistochemical detection of iNOS gene expression may prove more useful.

4.7 Conclusion

In conclusion, both total and free cortisol and NOx levels correlated with sepsis severity. However, perhaps due to individual constitutive factors influencing NO production, we did not find that NOx levels predicted the development of shock or the capacity to discontinue vasopressor support in shock patients. Overall, these observations do not suggest that NOx may be a useful target for hydrocortisone therapy, or a marker of tissue glucocorticoid sufficiency in sepsis.

Chapter 5

The maternal hypothalamic-pituitary-adrenal axis in pregnancy

5.1 Summary

Pregnancy is a transient, physiologic period of increased hypothalamic-pituitary-adrenal function with progressive elevation of circulating cortisol and ACTH levels through gestation. The causes of increased ACTH may include placental synthesis of biologically active CRH, CNS/ hypothalamic-pituitary desensitization to cortisol feedback, or enhanced pituitary responses to corticotropin-releasing factors. The hypercortisolism of pregnancy may act to redirect immune responses and alter maternal metabolism to increase substrate availability and fat stores, to ensure a healthy pregnancy. Foetal over-exposure to increased concentrations of glucocorticoids (GCs), either endogenous or exogenous, may influence development and have profound effects on the incidence of postnatal and adult disease. Animal and human studies indicate that GC-induced disruption of the HPA axis can occur at all levels including altered ACTH expression, plasma GC concentrations, GC metabolism and tissue GR density (Bertram and Hanson, 2002). These perturbations may lead to adult disorders such as type 2 diabetes, metabolic syndrome and cardiovascular disease, which in turn, have been linked by epidemiological studies with low birth weight (Barker, 2001).

The aim of this study was to measure and contrast maternal cortisol and CBG levels in pregnancies with normal outcomes, pre-eclampsia, intra-uterine growth restriction and in gamete recipients. This was a prospective study of 93 women at high risk of pre-eclampsia, including gamete recipients (n=22) and 33 controls. Plasma total and free cortisol and CBG were measured every 2 weeks from 16 weeks gestation until delivery.

In this study, 42% of the high risk group had complications, including pre-eclampsia (n=11), gestational hypertension (n=16) and small for gestational age (SGA) neonates (n=12). There were no complications in the controls. In all groups, plasma CBG concentrations increased progressively across gestation ($P<0.05$), in parallel to total cortisol, but fell significantly from 36 weeks gestation onwards with a corresponding rise in free cortisol concentrations. In pre-eclampsia and gestational hypertension, plasma CBG, total and free cortisol concentrations were lower from 36 weeks onwards ($P<0.05$). In IUGR, plasma CBG concentrations were suppressed from 28 weeks gestation until delivery ($P<0.05$), but with no significant difference in plasma total and free cortisol. Gamete recipients had significantly lower plasma CBG from 20 weeks gestation onwards, and plasma total and free cortisol were reduced at 24 and 32 weeks onwards, respectively.

In conclusion, maternal plasma CBG, total and free cortisol concentrations are reduced in pre-eclampsia/ gestational hypertension, and markedly reduced in gamete recipients. Low CBG may be due to reduced synthesis or enhanced inflammation-driven degradation. Low maternal cortisol may be due to a lack of placental corticotropin-releasing hormone, or reduced maternal ACTH, driving cortisol production. This unanticipated maternal hypocortisolism in complicated pregnancies may trigger precocious activation of the foetal HPA axis and could have implications for postnatal and adult health.

5.2 Introduction

5.2.1 Maternal hypothalamic-pituitary-adrenal axis

Pregnancy results in profound anatomical and physiological changes in almost every organ system. These adaptations to the pregnant state occur just after conception and evolve through to parturition. The purpose of these adaptations is to accommodate the needs of the maternal – foetal unit to ensure a healthy pregnancy.

During gestation, the placenta functions as a major endocrine organ where the syncytiotrophoblasts produce steroid and protein hormones and cytotrophoblasts manufacture a variety of neuropeptides and growth factors. Pregnancy is the only condition in humans where CRH can be readily measured in peripheral plasma. Normally, dilution of hypothalamic CRH, after its secretion into the hypophyseal-portal circulation, precludes measurement of CRH in the periphery. During pregnancy, circulating immunoreactive CRH in plasma increases exponentially up to 1000 times its non-pregnant level, from 8-10 weeks of gestation onward (Figure 5.1) (Goland *et al.*, 1988). This is the result of CRH production mainly by the placenta, decidua and foetal membrane rather than of hypothalamic origin (Sasaki *et al.*, 1984; Jones *et al.*, 1989). Systemic maternal effects of elevated CRH in pregnancy are thought to be limited due to binding of free bioactive CRH to CRH-binding protein (CRH-BP), a 322 amino acid glycoprotein (Potter *et al.*, 1991). Circulating CRH-BP levels in early and midgestation are similar to non-pregnant levels, suggesting that CRH-BP is not stimulated by elevated oestrogen levels in pregnancy, in contrast to CBG (Linton *et al.*, 1993). CRH-BP fall by around 60% in late third trimester, leading to elevations in free CRH (Linton *et al.*, 1993) (Figure 5.1). The control of placental CRH synthesis and secretion is quite different from that in the hypothalamus. In contrast to the negative feedback effect of cortisol on hypothalamic CRH release, placental CRH secretion is promoted by cortisol,

establishing a potent positive feedback loop (Fig. 5.2). There is no apparent circadian rhythm in plasma CRH despite preservation on circadian patterns of ACTH and cortisol, indicating that the maternal hypothalamus retains an influence over the HPA axis during pregnancy. Maternal CRH has been conceptualised as a biological clock that determines the length of gestation (McLean *et al.*, 1995; Smith, 1998). Further CRH can induce vasodilatation in the uterine arteries, and may regulate placental blood flow (Clifton *et al.*, 1994).

NOTE: This figure is included on page 106 of the print copy of the thesis held in the University of Adelaide Library.

Figure 5.1 Mean plasma CRH concentrations in seven women throughout pregnancy. Sequential samples were obtained at 1- to 2-week intervals beginning at 12 weeks gestation. (Goland 1988)

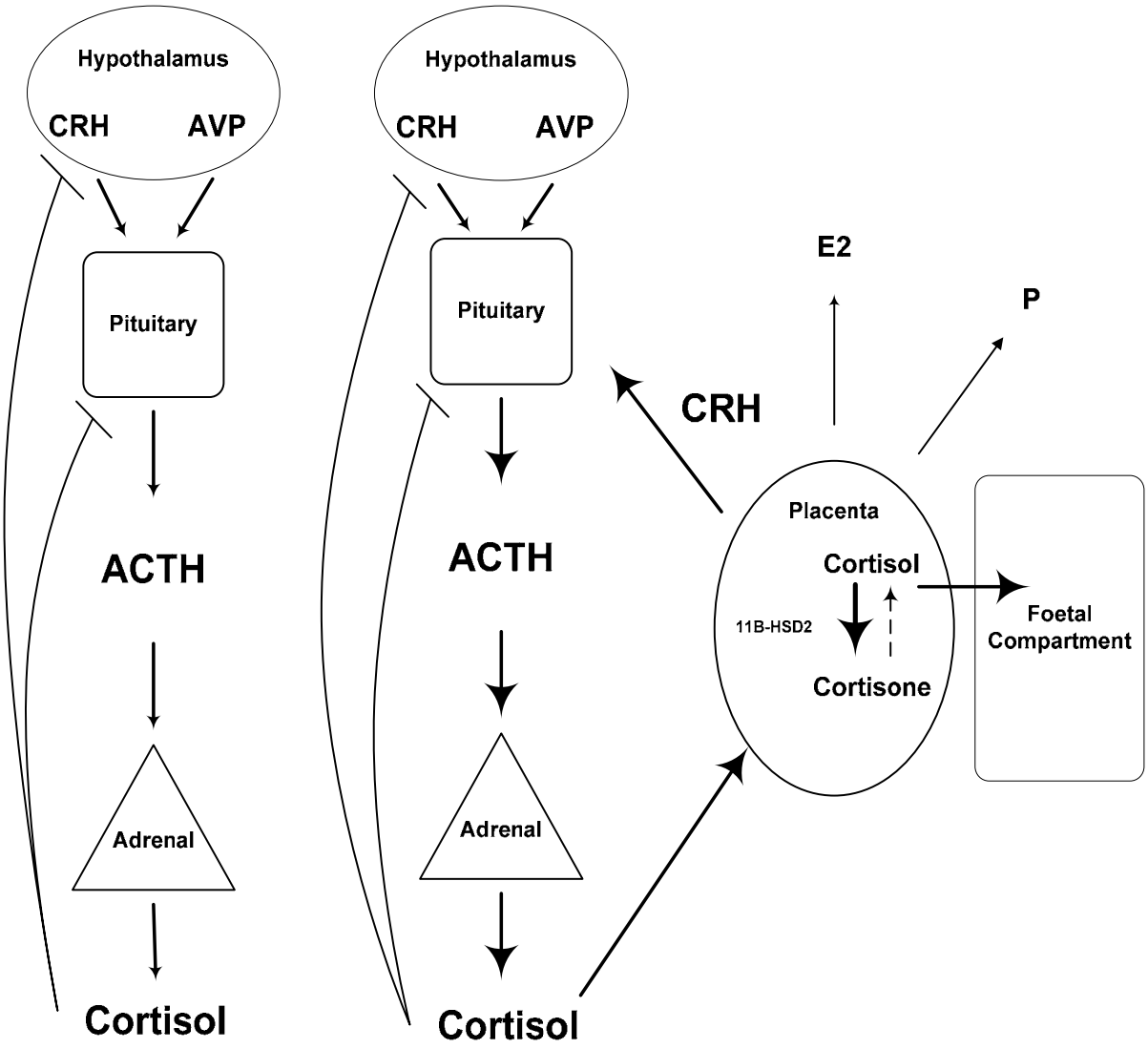


Figure 5.2 A schematic representation of the HPA axis in the (A) non-pregnant and (B) pregnant states. In the latter, cortisol participates in a positive feedback loop by stimulating placental CRH secretion, whereas inhibiting hypothalamic CRH release.

Pregnancy is the only physiologic state where increased HPA axis activity is sustained over a long period. Plasma total and free cortisol, and urine free cortisol levels increase progressively in pregnancy (Carr *et al.*, 1981). The enhanced cortisol production results from both an increase in maternal plasma ACTH concentrations (Fig. 5.3) and hyper-responsiveness of the adrenal cortex to ACTH stimulation during pregnancy (Nolten and

Rueckert, 1981). The circadian rhythm of maternal plasma ACTH levels is maintained throughout pregnancy, probably due to AVP secreted by the parvicellular paraventricular nuclei (Mastorakos and Ilias, 2003). There continues to be a diurnal rhythm of cortisol secretion with a trend toward a greater morning to evening difference as pregnancy progresses, and there is a normal cortisol response to stress and at the time of labour (Nolten *et al.*, 1980). Furthermore, oestrogen-induced increase in hepatic production of CBG results in decreased metabolic clearance of cortisol and a doubling of cortisol's half-life in plasma. Consequently, a steady rise in total and free cortisol is noted across gestation, peaking during the third trimester about two to three times non-pregnant values (Nolten *et al.*, 1980).

Figure 5.3 Mean (±SE) total plasma concentrations of cortisol and ACTH during normal pregnancy. Blood samples were obtained weekly at 0800 to 0900 hours, during labour and on the second postpartum day. The vertical bars correspond to the magnitude of the standard error of the mean. (Carr 1981)

This transient, physiologic state of hypercortisolism may act to adapt the maternal body to gestation and parturition, for example by liberating metabolic substrates such as glucose and amino acids and promoting fat storage for the demands of parturition and lactation. Maternal cortisol may also be involved in the functional maturation of foetal organ systems, particularly the lung, liver, brain and pancreas in late gestation. Cortisol is a potent immunomodulator that inhibits most immunologic and inflammatory responses. During pregnancy, cell-mediated immune function and T helper 1 cell (Th1) cytokine production (IL-12, IF-γ) are suppressed and humoral immunity and Th2 cytokine production (IL-4, IL-10) are enhanced. Convincing evidence exists to indicate that changes in the production of cortisol play major roles in modulating the balance between Th1 and Th2 cytokines in pregnancy (Chrousos, 1995). These cytokine patterns are reversed in the postpartum period which may explain the increased postpartum vulnerability to autoimmune diseases, analogous to the steroid withdrawal syndrome.

In the post partum period, maternal plasma cortisol levels show a decline toward normal levels, as the HPA axis gradually returns to its pre-pregnant dynamic state. Immediately after parturition, the maternal HPA axis is mildly suppressed, comparable to the post-cure situation of Cushing's syndrome. Dynamic testing of the HPA axis shows transiently suppressed hypothalamic CRH secretion at 3 and 6 weeks postpartum, and normal responses after 12 weeks (Magiakou *et al.*, 1996). Although suppressed ACTH responses are noted post partum, total cortisol levels are within the normal range, probably as a result of elevated CBG concentrations (Strel'chyonok *et al.*, 1984; Chrousos *et al.*, 1998).

5.2.2 Cortisol and foetal development

Glucocorticoids have potent effects upon foetal tissue development. It is the accelerated maturation of organs, notably the lung that underpins the widespread use of GCs in obstetric and neonatal practice in preterm births. Glucocorticoid receptors are expressed in most foetal tissues, including lung, brain, liver and gut from early embryonic stages (Winter, 1982; Pavlik and Buresova, 1984; Yang *et al.*, 1990). As term approaches, selected foetal tissues including liver and lung express 11-ketosteroid reductase activity that promotes local conversion of cortisone to cortisol (Winter, 1982). Thus, cortisol serves as an important stimulus to prepare the foetus for extrauterine survival.

The foetal HPA axis is characterized by maturation of the foetal pituitary first, with HPA activity beginning at midgestation (Gitau *et al.*, 2001). The foetal adrenal is composed of three functional zones: the foetal zone which is the principal site of DHEAS synthesis, the outer definitive zone which produces mineralocorticoids, and the transitional zone which expresses CYP17 (p450c17) and 3β-HSD enzymes for cortisol synthesis (Pepe and Albrecht, 1995; Mesiano and Jaffe, 1997). Until 28 -30 weeks, foetal ACTH is suppressed by maternal cortisol and the foetal adrenal does not produce cortisol, awaiting maturation of the 3β-HSD enzyme (Bolt *et al.*, 2002). For most of gestation, the foetus is protected from the effects of maternal hypercortisolism by placental 11β-HSD_2 which inactivates cortisol to cortisone (Seckl *et al.*, 2000; Fowden and Forhead, 2004). 11β-HSD_2 is, therefore, a key factor in limiting foetal and placental exposure to maternal glucocorticoids. Nevertheless, this barrier is incomplete as 10 – 20% of active maternal GCs cross intact to the foetus reflecting an anatomical bypass of the enzyme via the syncytiocytotrophoblast (Benediktsson *et al.*, 1997). Although about one-third of variations in foetal cortisol levels are attributable to maternal source, foetal stress responses are independent of maternal responses (Gitau *et al.*, 2001). Hence, increased foetal glucocorticoid exposure can occur due to increased maternal cortisol

levels, decreased placental 11β-HSD_2 activity or increased cortisol output by the foetal adrenal.

The degree to which maternal hypercortisolism in pregnancy is required, and how precisely it needs to be regulated is not well understood. It has been reported that, prior to the availability of synthetic glucocorticoids, pregnancy in untreated women with Addison's disease was invariably fatal. There are, however, no data on the outcomes of pregnancy in treated Addison's. Anecdotally, some women require an increase in glucocorticoid dosage to avoid incipient features of hypocortisolism, such as hypotension. Although rises in maternal cortisol are associated with successful pregnancy, excessive maternal cortisol such as in Cushing's syndrome, is associated with a high maternal as well as foetal morbidity and mortality. It has been proposed that foetal exposure to excessive GCs may restrict foetal growth, and programme permanent alterations in its cardiovascular, endocrine and metabolic systems (Barker, 1995; Bertram *et al.*, 2001).

Theoretically, excessive maternal hypercortisolism may play a role in pregnancy-induced hypertension, a condition which affects at least 5% of pregnancies. As described earlier (Chapter 1) cortisol's pleiotropic effects on the cardiovascular system may lead to blood pressure elevation.

5.2.3 Pre-eclampsia

Pre-eclampsia occurs in 3-5% of pregnancies and is a major cause (15-20%) of maternal mortality in developed countries and a leading cause of intra-uterine growth restriction and (iatrogenic) preterm birth (No authors, 2000). Pre-eclampsia is a syndrome defined by the new onset hypertension (BP > 140/90 on 2 occasions ≥ 6 hours apart) and proteinuria (>

300mg per 24 hours) after 20 weeks of gestation. Additional features include oedema, visual disturbances, headache, abdominal pain, thrombocytopenia and abnormal liver function. Many of these result from mild to severe microangiopathy of target organs, such as brain, liver, kidney and placenta.

The aetiology of pre-eclampsia is unknown. The diverse range of clinical features has led to controversy in both diagnosis and options for therapy (Sibai *et al.*, 2005). The pathophysiology of pre-eclampsia may be a complex process involving genetic, endocrine and immune factors (Table 5.1). Although most cases of pre-eclampsia are sporadic, genetic factors are thought to play a role in disease susceptibility. Studies have suggested that both maternal and paternal contributions to foetal genes may have a role in defective placentation and subsequent pre-eclampsia.

Table 5.1 Mechanisms and hypotheses implicated in the pathogenesis of pre-eclampsia

Possible causes of preeclampsia
• *Placental dysfunction* Defective trophoblast invasion Poor perfusion/ischaemia Release of factors into maternal circulation: Corticotropin releasing hormone * Neurokinin B
• *Endothelial dysfunction* Hyperdynamic disease model * Raised sensitivity to pressors * Increased platelet activation Release of nitric oxide and vascular endothelial growth factor (VEGF)*
• *Dyslipidaemia* Oxidative stress* Lipid peroxidation
• *Immune maladaptation* Switch to Th1 cell mediated immunity* Increased levels of tumour necrosis factor-alpha, interleukin-1, interleukin-6*
• *Genetic-conflict hypothesis* Primipaternity and paternal factors

* Mechanisms that may be influenced by cortisol

It is proposed that the initiating event in pre-eclampsia may be reduced uteroplacental perfusion as a result of abnormal cytotrophoblast invasion of spiral arterioles (Zhou *et al.*, 1997). Placental ischaemia is thought to lead to widespread activation/dysfunction of the maternal vascular endothelium that results in enhanced formation of endothelin and thromboxane, increased vascular sensitivity to angiotensin II, and decreased formation of vasodilators such as nitric oxide and prostacyclin. Increased placental expression and secretion of sFlt-1 (soluble fms-like tyrosine kinase 1), a naturally occurring circulating antagonist of vascular endothelial growth factor (VEGF) may play a central role in the pathogenesis of endothelial dysfunction in pre-eclampsia (Chambers *et al.*, 2001; Kino and Chrousos, 2001b; Maynard *et al.*, 2003). Elevated levels of VEGF correlate with the severity of the hypertension (Kupferminc *et al.*, 1997) and, possibly extravasation of plasma protein and the subsequent development of proteinuria (Sharkey *et al.*, 1996). Immune maladaptation may also exaggerate endovascular and endothelial cell dysfunction by increasing decidual release of Th1 cytokines (predominantly tumour necrosis factor–alpha, interleukins-1 and 6), proteolytic enzymes, and free radical species (Dekker and Sibai, 2001). Interleukins 1 and 6 are potent stimuli of hypothalamic CRH release, acting through eicosanoid cyclooxygenase pathway (Navarra *et al.*, 1991). Within this model, many of the interacting immune-cardiovascular interactions could be influenced by cortisol (e.g. cytokine release, the effects of VEGF) or could themselves influence cortisol secretion.

Few studies have investigated the role of cortisol and corticosteroid binding globulin (CBG) in pregnancy-induced hypertension and pre-eclampsia. McCalla et al. reported a reduction of 11β-HSD_2 activity in placentae of women who developed pre-eclampsia, and a significantly higher cortisol level in umbilical cord blood was associated with decreased foetal weight (McCalla *et al.*, 1998). Potter et al. reported that the total plasma CBG concentrations were significantly lower in those who developed hypertension in pregnancy than those who

remained normotensive. A fall in levels of CBG often immediately preceded the onset of hypertension. An earlier development of hypertension is associated with a greater diminution of CBG, and the CBG level was directly related to the birth weight of the infant at 34-36 weeks gestation (Potter *et al.*, 1987).

The renin-angiotensin-aldosterone (RAA) system is strongly stimulated in pregnancy with aldosterone levels rising up to four-fold by term, associated with a decline in angiotensin II vascular responsiveness (Wilson *et al.*, 1980; Brown *et al.*, 1992). Excessive RAAS activity was a candidate in the pathogenesis of pre-eclampsia. However, plasma renin activity (PRA), plasma angiotensin II (AngII) and aldosterone concentrations are reduced compared with normal pregnancy. Although plasma AII concentrations are reduced in pre-eclampsia, there is heightened pressor sensitivity to infused AII - the mechanism(s) for this are unknown (Brown *et al.*, 1997). Hence, the RAAS system is not thought to play a significant role in the development of pre-eclampsia.

5.2.4 Intrauterine growth restriction (IUGR)

IUGR, or small for gestational age, refers to newborns with a birth weight and/ or birth length below the 10^{th} percentile for their gestational age with pathologic restriction of foetal growth due to adverse genetic or environmental influences (Lepercq and Mahieu-Caputo, 1998; Wollmann, 1998). IUGR is often unexplained but may be due to identifiable foetal, placental, or maternal factors. There is significant overlap among these factors. Maternal medical disorders (e.g. nephropathy, collagen vascular disease) and obstetric complications (e.g. pre-eclampsia) are associated with vasculopathy and diminished uteroplacental perfusion.

Epidemiological data demonstrated an association between reduced birth weight and the later development of the metabolic syndrome, comprising arterial hypertension, obesity, insulin

resistance, type II diabetes mellitus and cardiac mortality in adults (Barker, 1993; Phillips *et al.*, 1994). This association may be the result of the adaptational changes of the foetal-endocrine-metabolic mechanisms to the impaired intrauterine milieu to assure survival in the short term. The persistence of these changes after birth can be detrimental in adult life. This hypothesis is also known as the Barker's hypothesis, after its founder David Barker. More recently, it has been proposed that premature activation of the offspring's HPA axis may also predispose to depression, anxiety, eating disorders and antisocial behaviour (Susser *et al.*, 1998).

A common mechanism may underlie foetal programming through maternal undernutrition and GC exposure. Dietary protein restriction during rat pregnancy selectively attenuates 11β-HSD_2 but not other placental enzymes (Langley-Evans *et al.*, 1996; Lesage *et al.*, 2001). Increasing the exposure of rat foetuses to maternal GCs by inhibiting 11β-HSD_2 with carbenoxolone also causes postnatal hyperglycaemia, elevated blood pressure and increased GR density (Lindsay *et al.*, 1996; Bertram *et al.*, 2001). In human, 11β-HSD_2 gene mutations cause low birth weight (Dave-Sharma *et al.*, 1998), and reduced placental 11β-HSD_2 activity is associated with IUGR (Shams *et al.*, 1998).

Prenatal excesses of glucocorticoids may predispose to the metabolic syndrome through alterations to kidney development including glomerular number, increased expression of catecholamine receptors, induction of insulin like growth factors (IGFs), increased glucocorticoid sensitivity and indirect effects on carbohydrate and fat homeostasis (Bertram and Hanson, 2002). Psychiatric effects of glucocorticoids may result from altered dopamine and serotonin sensitivity as well as hippocampal glucocorticoid receptor (GR) expression (Seckl *et al.*, 2000).

In the long term, prenatal glucocorticoid exposure may permanently reset the endocrine axes by changing the set point and sensitivity of the feedback mechanisms (Bertram and Hanson, 2002). This leads to permanent changes in basal hormone levels and in the endocrine responses to stimuli. Basal and stimulated glucocorticoid concentrations are known to be high in adult sheep, rats and guinea pigs over-exposed to glucocorticoids *in utero* (Langley-Evans *et al.*, 1996; Challis *et al.*, 2001). Similarly, in humans, basal hypercortisolaemia and greater adrenocortical responsiveness to ACTH are observed in adults who were small at birth (Phillips *et al.*, 1998; Reynolds *et al.*, 2001). Persistently enhanced HPA function in the adult may contribute to the pathogenesis of cardiovascular and metabolic diseases (Benediktsson *et al.*, 1993). Moreover, studies indicate that this HPA axis overactivation can have long-lasting effects on the individual's ability to face stress, with a higher incidence of antisocial behaviour or even schizoid personality (Hoek *et al.*, 1996; Neugebauer *et al.*, 1999). Therefore, it seems that intrauterine life exerts a lasting effect on both the somatic and psychosocial health of the individual.

5.2.5 Assisted reproduction

Advances in assisted reproductive technology including the use of gamete donation have revolutionised reproductive medicine. However, the use of donated gametes i.e. donor insemination, oocytes or donated embryos, may affect the maternal-foetal immune interaction and increase the risk of pregnancy complications such as placenta previa and abruption, gestational diabetes, pre-eclampsia and perinatal complications (Wang *et al.*, 2002; Van Voorhis, 2006). Perinatal mortality has also been reported to be increased due, at least in part, to these obstetrical complications, low birth weight, and multiple gestation (Jackson *et al.*, 2004). At present, there is no data on cortisol in gamete or embryo recipients.

5.3 Hypothesis and Aims

The principal hypothesis of this study was that disordered maternal hypercortisolism (as defined by novel measures of plasma free cortisol and CBG) may be associated with the development of pre-eclampsia and IUGR.

The first aim of this study was to prospectively measure maternal cortisolaemia in normal pregnancy and in pre-eclampsia. The second and third aims were to measure cortisol in women with growth restricted foetuses and in those who conceived using donated gametes respectively.

Free cortisol measurement is not offered in routine clinical laboratories. The Coolens' method may be practically useful in pregnancy as it estimates free cortisol levels from total cortisol and CBG levels (Coolens *et al.*, 1987). However, the method has not been validated in pregnancy, and such validation was one objective of this study.

5.4 Research design and methods

Subjects and study design

This prospective study was conducted between April 2004 and December 2005 at the Women's and Children's Hospital (Adelaide, Australia). The study protocol was approved by the Women's and Children's Hospital Ethics Committee and all women gave informed consent for their participation.

Women with one or more of the following clinical risk factors for pre-eclampsia and intrauterine growth restriction were recruited from the 'High Risk Pregnancy Clinic' during the first trimester of pregnancy by a senior obstetrician. Inclusion criteria were pre-eclampsia

in a previous pregnancy, family history of pre-eclampsia, chronic hypertension, antiphospholipid syndrome, previous delivery of growth restricted infants, previous preterm delivery or stillbirth and pregnancy after gamete or embryo donation. These risk factors are known to increase the risk of pre-eclampsia or IUGR (Sibai et al., 2005). Of the 150 women screened, 97 women were eligible for the study and were enrolled into the 'high risk' group.

Women were excluded if they were unable or unwilling to provide informed consent or had any of the following: known multiple pregnancy, factors that may affect the HPA axis such as glucocorticoid use, pre-existing adrenocortical insufficiency or foetal congenital and/ or chromosomal anomalies.

Thirty-three 'low risk' women (those with apparent normal pregnancy and none of the above risk factors) were recruited from the antenatal clinics during the first trimester to establish normal values for maternal plasma concentrations of total and free cortisol, and CBG.

Complicated pregnancy definitions

Pregnancy complications included: (i) the development of pre-eclampsia in the mother, (ii) gestational hypertension, and (iii) the birth of an infant who was small for gestational age (SGA). Pre-eclampsia was defined as new onset hypertension (blood pressure greater than 140 mmHg systolic or 90 mmHg diastolic) measured on 2 occasions at least 4 hours apart and proteinuria (≥ 300mg per 24 hours) after 20 weeks of gestation (Brown *et al.*, 2001). In the absence of proteinuria, the condition is suspected if the hypertension is accompanied by headache, visual disturbances, abdominal pain or abnormal laboratory tests (thrombocytopenia, hyperuricaemia, disordered liver function). Gestational hypertension was hypertension as defined above, without other signs of pre-eclampsia. The onset of pre-eclampsia was defined as the time when hypertension and proteinuria were first documented; the diagnosis was confirmed in each case by two senior clinical staff independently.

Small for gestational age (SGA) was defined as a birth weight below the customised 10th percentile for gestational age. The birth weight percentile was customised for each birth by the factors: gender, parity, maternal ethnicity, body mass index (BMI) and maternal height (Gardosi *et al.*, 1992; Wilcox *et al.*, 1993). The use of customised foetal growth standards can reduce the rates of false-positive and false-negative diagnosis of IUGR (Mongelli and Gardosi, 1996; De Jong *et al.*, 2000). There is increasing evidence that customising foetal growth curves improves the distinction between genetically small and growth-restricted foetuses in different populations (Sciscione *et al.*, 1996). Entry of these variables, along with birth weight and gestation, into a computer program (Gestation network centile calculator http://www.gestation.net/birthweight_centiles/birthweight_centiles.htm) generated a customised birth weight centile for each individual infant.

Laboratory measurements

Maternal blood samples were collected by venepuncture during the course of routine afternoon (1400 -1700 hour) antenatal visits. None of the samples was collected within 1 hour of a meal and patients were advised to abstain from caffeine-containing drinks on the day of collection. Samples were initially obtained at 12 - 20 weeks (visit 1); then every 4 weeks until 24 weeks, and every 2 weeks until 38 weeks. Clinical information including body weight, blood pressure and urinalysis for protein were collected at every visit. No samples were collected after antenatal steroid administration and samples that were collected at the time of labour were not included in the analysis as the cortisol levels would be elevated.

Plasma was separated within 2 hours of collection and was stored at -20^{o}C until assay for CBG, total and free cortisol levels. Samples from individual subjects were measured in the same assay to eliminate inter-assay variation. Hormone assays for plasma total and free cortisol and CBG were described in Chapter 2.

Statistical analysis

Descriptive statistics were reported as mean ± standard error (SEM) after a normality test showed that cortisol and CBG data were normally distributed. Patient groups included those with (a) gestational hypertension, (b) pre-eclampsia, (c) SGA, (d) assisted reproduction with gamete/ embryo donation and (e) controls. Chi-square analysis was used to test for any significant differences between the categorical variables that characterised the patients at entry (Table 5.2). Differences in mean hormone measurements with advancing gestational age in control subjects were sought by one-way repeated measures Analysis of Variance (ANOVA), followed by post hoc Student-Newman-Keuls test. Differences between two groups were examined using the Student's *t* test or two-way analysis of variance. Differences between more than two groups at different time points were examined by repeated measures ANOVA followed by Fisher's least significant difference test. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using the Statistica software package (Statsoft, Tulsa, OK).

5.5 Results

Characteristics of the women and infants

The characteristics of the volunteers are shown in Table 5.1. Of the 136 pregnant women enrolled, eight withdrew from the study and two miscarriages occurred before 15 weeks. There were 93 high-risk pregnancies, of whom 22 were gamete recipients, and 33 controls. Within the high risk group, 42% developed complications - 11 had pre-eclampsia, 16 had gestational hypertension and 12 pregnancies resulted in the birth of SGA infants. There were no pregnancy-related complications in the control group. There were no significant differences between groups with regard to maternal education (X^2=6.283, P=0.179), working

status (X^2=1.544, P=0.819), baby gender (X^2=8.695, P=0.069) and season of birth (X^2=11.036, P=0.526).

Women with pre-eclampsia had a greater body mass index, higher systolic and diastolic blood pressure, but were not significantly different in age to controls, at enrolment. Eight women (73%) were treated with antihypertensive agents and two required magnesium sulphate therapy. The mean gestational period was shorter than the controls (Table 5.2); one woman delivered before 37 weeks and three delivered before 34 weeks gestation. The mean infant birth weight was lower than the controls.

The age of women who developed gestational hypertension was similar to controls, but they had a higher BMI. Five women (31%) were treated with antihypertensive agents. There was no difference in the gestation period or birth weight (Table 5.2).

Two of the twelve SGA infants were born to gamete recipients, two to women with pre-eclampsia and four to women with a thrombophilic disorder - antiphospholipid antibody syndrome or hyperhomocystinemia. Five infants were delivered before 37 weeks and three delivered before 28 weeks gestation. Antenatal steroid was administered immediately prior to delivery in five women who delivered before 34 weeks gestation.

The mean age of the twenty-two gamete recipients (GR) was 42.7 years (range 38 -55). They had a similar mean BMI to controls and delivered at a mean of 38 weeks gestation. Infant birth weights were comparable to controls.

Table 5.2 Characteristics of pre-eclampsia (PE), gestational hypertension (GH), intrauterine growth restriction (IUGR), assisted reproduction by gamete donation (GR) and control groups.

Characteristic	PE (n=11)	GH (n=16)	IUGR (n=12)	GR (n=22)	Control (n=33)
Age (yr)	38.4 ± 1.9	31.4 ± 1.4	32.4 ± 2.1	42.7 ±1.3 *	32.3 ± 0.9
Ethnicity (%) Caucasian	100	94	100	100	90
Married/de facto(%)	100	100	91	100	100
Education (n) Tertiary: Secondary	5: 4	6: 10	3: 9	13: 9	20: 13
Working status (%)	49	42	38	60	57
Tobacco use (n)	0	1	1	1	2
Alcohol use (n)	0	1	1	1	1
Baby's gender(M:F)	4: 7	12: 4	8: 3	8: 14	19: 14
Season at birth (n) spr: sum: au: win	3: 2: 3: 4	4: 5: 4: 3	3: 1: 3: 5	2: 11: 4: 5	8: 13: 7: 5
BMI (kg/m^2)	31.3±2.3 *	34 ±2.8 *	27.1 ± 1.6	26 ± 1.8	26.6 ± 1.0
Gestational age at delivery (weeks)	36.1±1.6 *	37.3 ± 0.8	35.3 ± 2.2*	38 ± 0.5	39.2 ± 0.2
Birth weight (g)	2544±327*	3409± 144	2270±332*	3116 ± 117	3525 ± 89
BP at week 16 Systolic (mmHg) Diastolic (mmHg)	125 ± 5.1 75 ± 5.2	120 ± 2.8 74 ± 1.8	111 ± 2.4 67 ± 2.3	114 ± 2.2 71 ± 2.1	109 ± 2.2 65 ± 1.8
BP at delivery Systolic (mmHg) Diastolic (mmHg)	148 ±5.4 * 85 ± 4.1 *	128 ± 8.8 80 ± 2.5	118 ± 7.5 70 ± 7.2	126 ± 3.2 81 ± 2.1	118 ± 4.0 70 ± 1.8

Abbreviations: spr –spring, sum - summer, au - autumn, win - winter

Values are represented as means ± SEM

* denotes $P < 0.05$ when compared to control

Cortisol measurements in control subjects

Total cortisol concentrations increased 2.4-fold, rising from 336 ± 16 nmol/L (n = 33) at 16 weeks gestation to 810 ± 82 nmol/L (n = 31) at 38 weeks gestation ($P < 0.05$; Fig. 5.4). Free cortisol concentrations also rose progressively from 16 weeks, reaching a 1.8 fold increase by 36 weeks ($P <0.001$). At 16 weeks gestation, the CBG concentration (496 ± 22 nmol/L) was already above the non-pregnant range of 298 – 426 nmol/L. Plasma CBG levels increased progressively across gestation but declined at 36 weeks with a significant corresponding rise in free cortisol.

Cortisol measurements in pre-eclampsia and gestational hypertension

Plasma total and free cortisol and CBG concentrations of women who developed pre-eclampsia (PE) and gestational hypertension (GH) and normal controls (C) are shown in Fig. 5.5. Women with gestational hypertension and pre-eclampsia had lower plasma total and free cortisol concentrations than controls, reaching statistical significance in late third trimester ($P < 0.05$ at 36 -38 weeks). Similarly, plasma CBG concentrations tended to be lower in the PE and GH groups from early pregnancy, reaching statistical significance at 36 -38 weeks (Fig 5.5).

Cortisol measurements in small for gestational age pregnancies

Among the SGA group, plasma CBG concentrations were lower by up to 20 % ($P < 0.05$) from 28 weeks until 38 week, compared to the controls. However, there was no significant difference in plasma total and free cortisol concentrations (Fig. 5.6).

Cortisol measurements in gamete recipient pregnancy

Among the gamete recipient (GR) group, the CBG concentrations were suppressed by up to 23 % from 20 weeks gestation until delivery, compared to the control group (Fig. 5.7). Plasma

total and free cortisol concentrations were decreased significantly from 24 and 32 weeks onwards, respectively (Fig. 5.7).

Comparing measured free cortisol to calculated free cortisol concentrations

Calculated free cortisol was derived using the Coolens' equation: $U^2.K\ (1+N)+U[1+N+K(G-T)]-T=0$, (cortisol (T), CBG (G), U unbound cortisol, K affinity of CBG for cortisol at 37^o).(Coolens et al., 1987b) N is the ratio of albumin bound to free cortisol and 1.74 is the value conventionally used. The control samples taken between 16 to 36 weeks gestation were used in this analysis. A Bland-Altman plot was used to compare Coolens' equation with measured free cortisol concentrations (Bland and Altman, 1986) Figure 5.8 displayed a scatter diagram of the ratios of the two methods plotted against the geometric mean of the two measurements. The mean ratio was 2.3 and the limits of agreement were 0.3 and 4.3. The correlation coefficient ($r = 0.391$) and coefficient of determination ($r^2 = 0.153$, $P < 0.001$) indicate that Coolens' equation does not reliably estimate free cortisol in pregnancy.

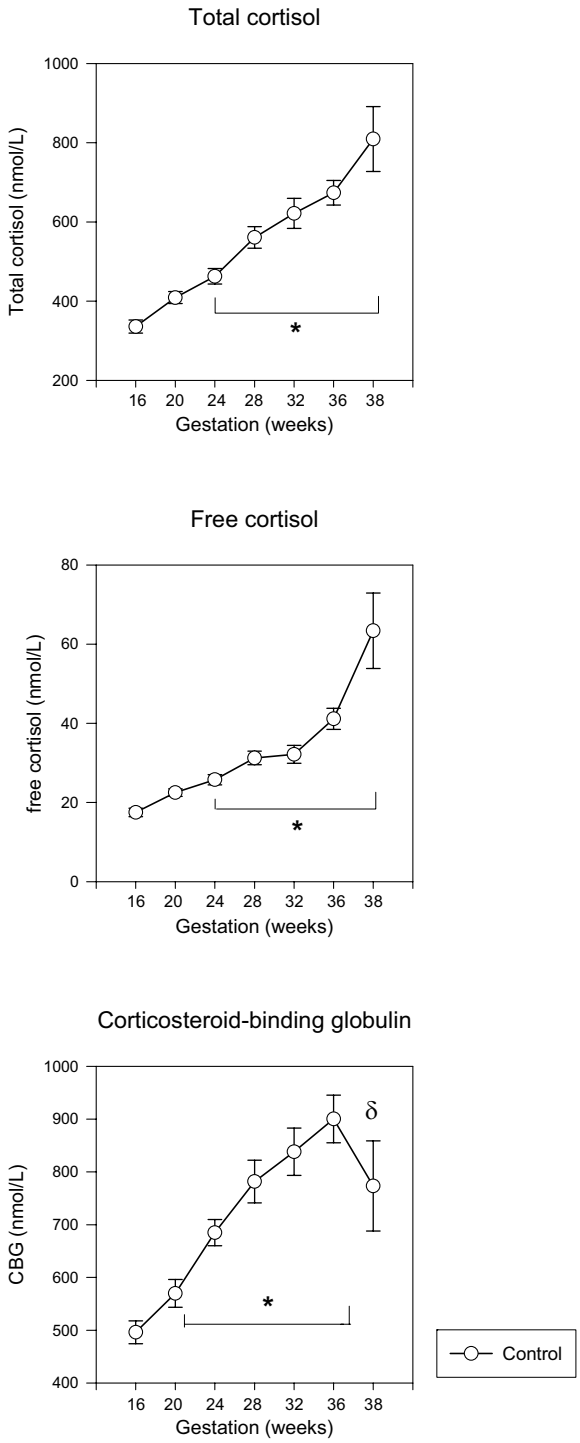


Figure 5.4 Plasma total and free cortisol, and corticosteroid-binding globulin (CBG) concentrations in control subjects (n=33). Total cortisol, free cortisol and CBG levels increased progressively throughout pregnancy with a 2.4 fold-rise by the third trimester (* denotes all levels increased relative to 16 weeks gestation). A marked increase in free cortisol levels is noted at 38 weeks (δ denotes $P < 0.05$ for 36 weeks vs. 38 weeks), due to both increased cortisol and reduced CBG.

Values are represented as means ± SEM

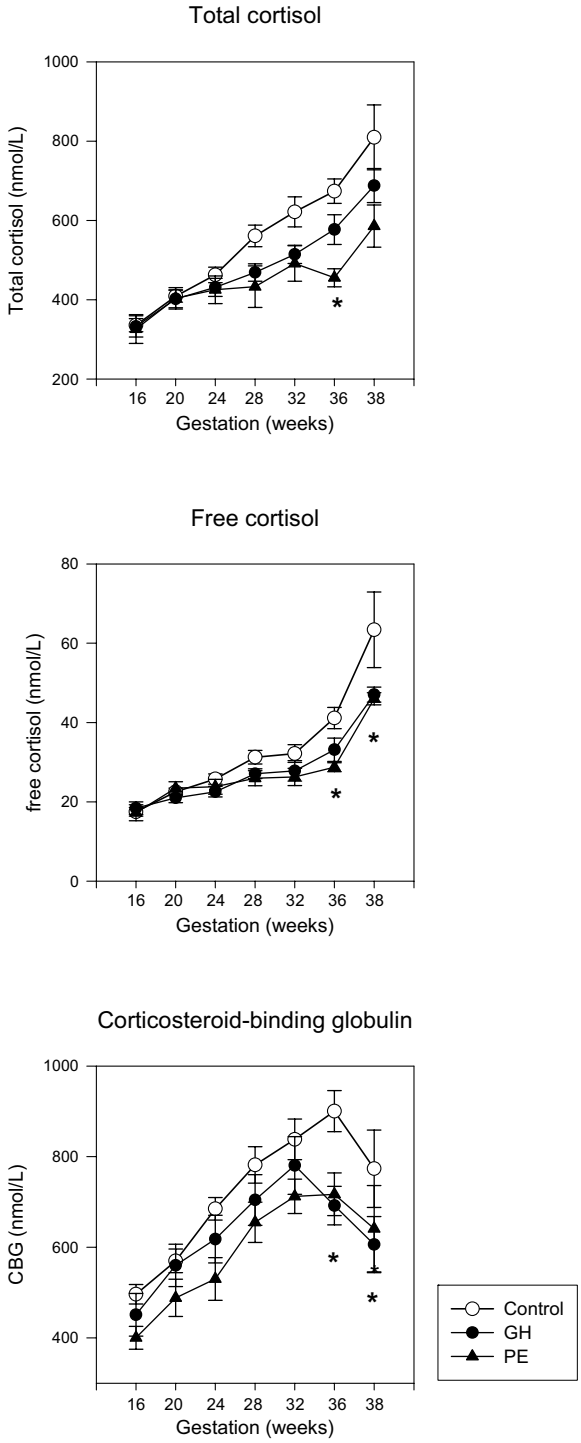


Figure 5.5 Comparing subjects with pre-eclampsia (PE, n=11), gestational hypertension (GH, n=16) and controls (n=33). Plasma total and free cortisol and CBG levels in PE and GH were significantly lower than controls (at the points marked *).

Values are represented as means ± SEM

* denotes $P < 0.05$ when compared to control

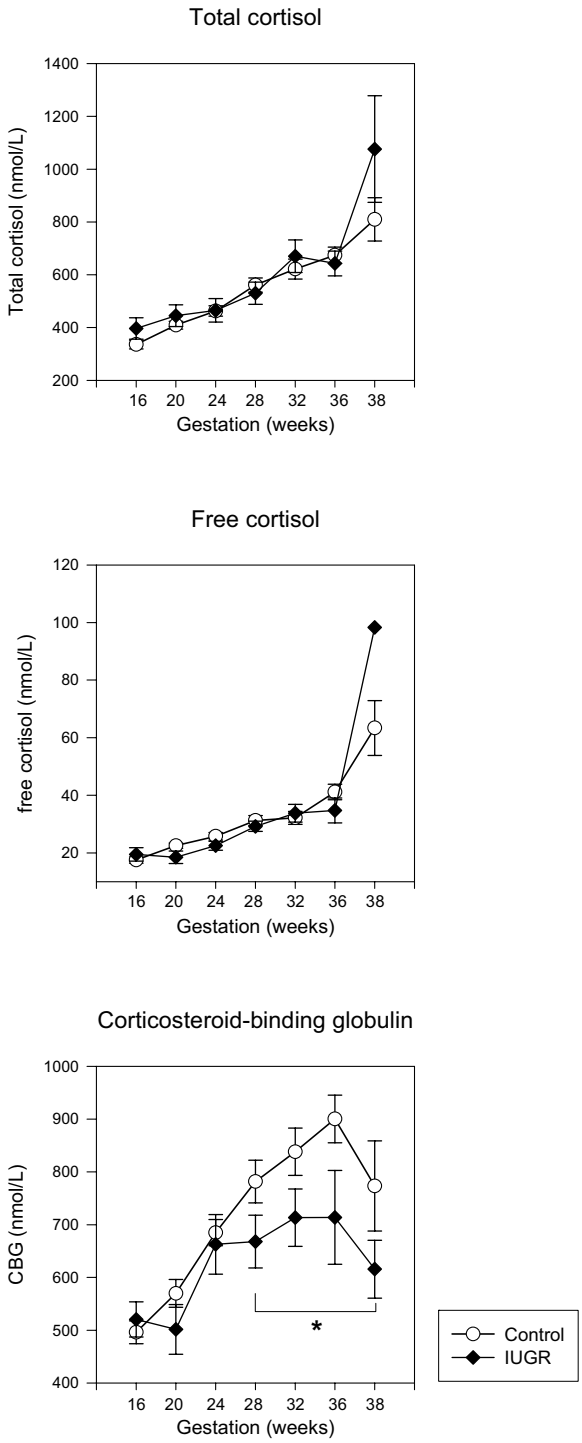


Figure 5.6 Plasma total and free cortisol and CBG concentrations in women who had intrauterine growth restricted neonates (n=12) and controls. Plasma CBG concentrations were suppressed from 28 weeks gestation until delivery ($P < 0.05$). However, there was no significant difference in plasma total and free cortisol between groups.

Values are represented as means ± SEM

* denotes $P < 0.05$ when compared to control

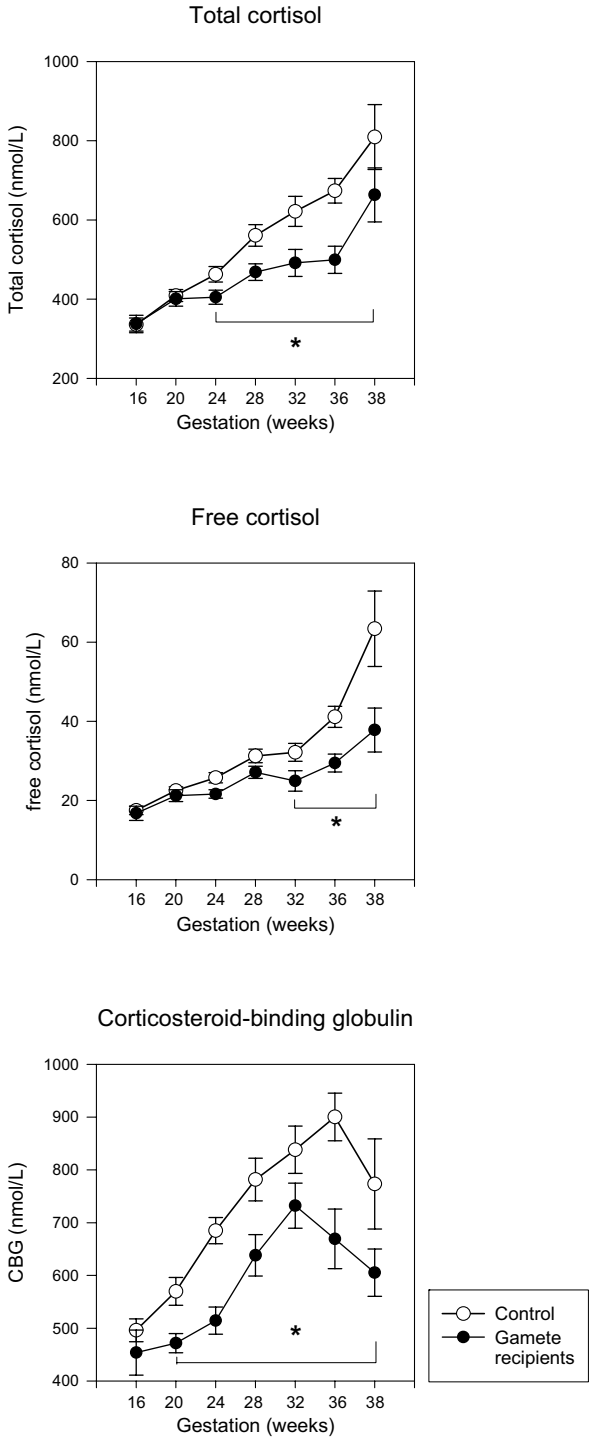


Figure 5.7 Plasma total and free cortisol and CBG concentrations in women with assisted fertilization using donor oocytes/ embryos (n=22). Plasma CBG concentrations were suppressed by up to 23 % from 20 weeks gestation until delivery, compared to the controls. Plasma total and free cortisol concentrations were reduced significantly at 24 and 32 weeks onwards, respectively.

Values are represented as means ± SEM

* denotes $P < 0.05$ when compared to control

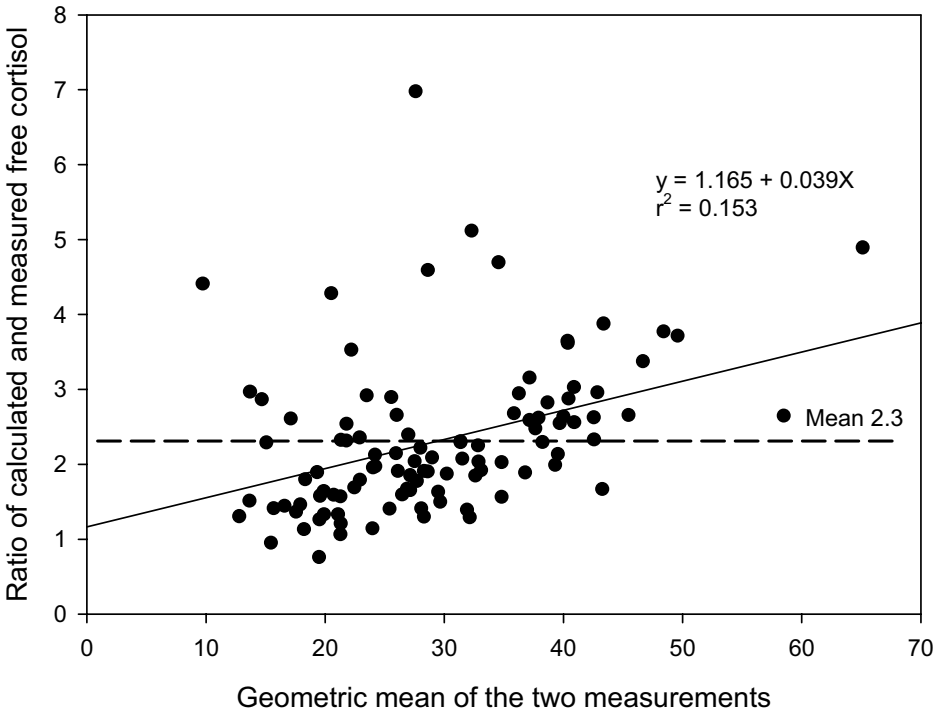


Figure 5.8 Comparing measured free cortisol by ultrafiltration/ ligand binding method to calculated free cortisol by Coolens' method using Bland-Altman plot. The display of the plots and the limits of agreement suggested that Coolens' method cannot estimate free cortisol reliably in pregnancy.

5.6 Discussion

We describe several novel observations regarding maternal cortisol in normal and complicated pregnancy. The major findings are: [1] in normal pregnancy, maternal plasma CBG concentrations rose progressively, in parallel with total cortisol across gestation but declined from 36 weeks onwards, with a corresponding rise in free cortisol; [2] in hypertensive disorders of pregnancy, plasma total and free cortisol and CBG concentrations were reduced from 36 weeks onwards; [3] women with growth restricted infants had

markedly reduced plasma CBG from 28 weeks onwards; [4] women with assisted reproduction by gamete donation had significantly lower plasma CBG throughout gestation, and reduced total and free cortisol concentrations at 24 and 32 weeks onwards, respectively and; [5] Coolens' method does not estimate free cortisol reliably in pregnancy.

Pregnancy is a physiologic state of sustained HPA axis hyperactivity. Progressive elevations of maternal cortisol throughout gestation have been attributed to increased secretion of placental CRH which stimulates maternal ACTH release (Carr *et al.*, 1981; Nolten and Rueckert, 1981; Sasaki *et al.*, 1984; Allolio *et al.*, 1990; Petraglia *et al.*, 1991; Petraglia *et al.*, 1993; Zoumakis *et al.*, 1997). In contrast to the negative feedback effects of cortisol on hypothalamic CRH, cortisol stimulates placental CRH release establishing a potent positive feedback loop (Jones *et al.*, 1989; Petraglia *et al.*, 1991). In addition, the continued influence of maternal ACTH on plasma cortisol levels implies a downregulation of the negative feedback effect of maternal cortisol at the hypothalamic-pituitary unit.

Cortisol has important effects on growth and organ differentiation, particularly the brain, lungs, kidneys and pancreas of the foetus. CBG is a plasma glycoprotein with a high binding affinity for cortisol (Seal and Doe, 1966). Maternal CBG concentrations increase progressively throughout gestation, reflecting increased hepatic biosynthesis stimulated by circulating oestrogen (Rosenthal *et al.*, 1969). Moreover, a non-lectin binding form of CBG comprising 10% of total CBG appears (Strel'chyonok *et al.*, 1984). This "pregnancy-specific" CBG is probably maternal in origin and may have an increased binding affinity to syncytiotrophoblast cell membranes compared with normal CBG (Strel'chyonok and Avvakumov, 1990; Benassayag *et al.*, 2001). Functionally, pregnancy-specific CBG may facilitate maternal-foetal cortisol transfer. This is of particular interest given the role of syncytiotrophoblasts as a shunt, bypassing 11β-HSD_2, for cortisol transfer to the foetus

(Brown *et al.*, 1996). Pregnancy CBG may also have a role in the regulation of placental CRH release (Mitchell *et al.*, 2004).

Maturation of the foetal HPA axis is characterised by diminishing suppression of foetal ACTH as 11β-HSD_2 activity rises and maturation of 3β-HSD in the third trimester allowing cortisol synthesis. At term, approximately 75% of cortisol in the foetal circulation is of foetal origin (Beitins *et al.*, 1973). Precocious activation of this axis has been reported in premature infants (Parker *et al.*, 1995), perhaps due to immune stimulus from chorioamnionitis which occur in 50% of preterm births (Watterberg, 2004). Recent studies suggest that subtle abnormalities of the HPA axis may be the cause of prenatal growth restriction and development of the metabolic syndrome in adult life (Barker *et al.*, 1993).

We found a progressive rise in plasma CBG, total and free cortisol concentrations with advancing gestation in normal pregnant women, consistent with the observations of others (Carr *et al.*, 1981; Nolten and Rueckert, 1981; Demey-Ponsart *et al.*, 1982). However, we have demonstrated for the first time that there is a substantial fall in plasma CBG levels in the last few weeks of gestation with a corresponding rise in free cortisol. A larger sample size as well as frequent sampling across gestation may account for this finding. Previous studies have often used pooled late pregnancy samples which would conceal the fall in CBG (Scott *et al.*, 1990). Furthermore, we directly quantified plasma CBG, whereas many past studies used indirect binding techniques. Physiologically, this free cortisol elevation in late pregnancy may facilitate organ maturation in the foetus and perhaps prepare the mother for the metabolic demands of labour.

The finding of reduced levels of total and free cortisol in pregnancy related disorders is surprising, especially in pre-eclampsia as these states are associated with immune activation

which generally stimulates HPA axis activity. Several studies reported no difference in plasma cortisol in normal and hypertensive pregnancies (Laatikainen *et al.*, 1991; Walker *et al.*, 1995), however, samples were collected at few time points in the third trimester after hypertension diagnosis. Low cortisol in pre-eclampsia may be due to reduced maternal ACTH secondary to enhanced negative feedback at the hypothalamic level or inability of the dysfunctional placenta to drive the maternal HPA axis. A recent study showed that elevated cortisol levels at 15 weeks gestation predicted preterm birth, however there were no data on pregnancy complications (Sandman *et al.*, 2006). Our finding of reduced CBG concentration in hypertensive pregnancies is in agreement with one other study (Potter *et al.*, 1987), however, we have further shown that plasma CBG failed to rise beyond 32 to 34 week gestation in these disorders and measured free and total cortisol. It is proposed that the initiating event in pre-eclampsia may be reduced uteroplacental perfusion as a result of abnormal cytotrophoblast invasion of spiral arterioles (Zhou *et al.*, 1997) and immune maladaptation (Dekker and Sibai, 1998). Low maternal oestriol levels were observed in hypertensive pregnancies (Warren *et al.*, 1995). Reduced CBG concentrations in pre-eclampsia and gestational hypertension may be a consequence of increased catabolism peripherally and decreased synthesis induced by the circulating cytokines or reduced plasma oestriol levels. Reduced CBG synthesis may affect the relatively specific triantennary pregnancy glycoforms but we have not examined these.

In gamete recipients, plasma CBG was markedly reduced from early gestation, even in those with a normal pregnancy outcome. Suppressed CBG levels in gamete recipients may result from increased levels of pro-inflammatory cytokines, as a consequence of the total allograft immune status of the foetus. Similar to the pre-eclampsia group, low total and free cortisol were noted, again suggesting unexpected HPA axis underactivity, perhaps due to placental dysfunction.

Free cortisol measurement, using either ultracentrifugation or equilibrium dialysis is not offered by routine clinical laboratories. Coolens' method allows calculation of free cortisol concentration from the concentrations of total cortisol and of CBG, based on binding equilibria (Coolens *et al.*, 1987). We have validated the use of Coolens' method in estimating free cortisol in severe sepsis (Ho *et al.*, 2006). However, the relatively lack of correlation between calculated and measured free cortisol in pregnancy suggests altered binding affinity of CBG for cortisol in pregnancy or interference of the equilibrium by other pregnancy-associated steroids e.g. progesterone.

Strengths of the study include its prospective design, frequent measurements of cortisol through gestation and sample size. However, cortisol levels drawn in the afternoon, a time when the levels are low and stable, is a limited measure of HPA activity and can not provide a full circadian pattern of cortisol secretion. Urine measures are confounded in pregnancy by the effects of altered renal tubular flow rates and cortisol metabolism. Hence, further studies need to exclude the possibility of altered circadian cortisol release, perhaps through saliva sampling throughout the day. In contrast, CBG levels do not exhibit circadian variation.

One of the limitations of this study is the lack of age matching between groups. This reflects the recruitment criteria where risk factors are commoner in older women (e.g. previous pre-eclampsia) or the use of reproductive technology, where gamete donation is mainly performed in older women. There are no data to suggest reduced maternal cortisolaemia with age, nor do cortisol and CBG decline appreciably with age in non-pregnant individuals (Parker *et al.*, 2000). Due to recruitment requirements, we were not able to match the age between groups and these differences can not be statistically corrected for due to lack of overlap between groups. Although an effect of age on maternal cortisol/ CBG would be of interest, we note

that age had no effect on cortisol within controls or patient groups; hence we think that maternal age is unlikely to account for our findings. This study is also not designed to assess the association of the patients' socioeconomic status or season of birth with the development of adverse outcomes.

5.7 Conclusion

Maternal plasma CBG, total and free cortisol concentrations are reduced in gestational hypertension, pre-eclampsia and in gamete recipient pregnancies. These findings may relate to placental dysfunction and could lead to compensatory activation of the foetal HPA axis. Such activation, if it leads to disordered HPA function in adult life could have implications for metabolic and psychological health.

5.8 Implications and future directions

Despite the wealth of animal data, little is known of the maternal HPA axis in complicated human pregnancies, nor its relation to the neuroendocrine programming of the foetus. The mechanism of neuroendocrine programming is clearly complex, but it may represent a common pathway by which a wide variety of external influences, such as maternal nutrition and illness, have long-term effect on the foetus.

Our finding of low maternal cortisol levels in complicated pregnancies has provided novel and important knowledge about the maternal HPA axis in pregnancy. Relative maternal hypocortisolism may trigger precocious activation of the foetal HPA axis. Further, neonatal vulnerability to stress may be enhanced by prematurity, maternal parity, multiple birth and low birth weight. Chronic hyperactivation state may lead to pathological syndromes. As CRH

coordinates behavioral, neuroendocrine, autonomic and immunologic adaptation during stress, increased and prolonged production of CRH may explain the pathogenesis and some manifestations of these syndromes. Examples of increased HPA axis dysfunction include melancholic depression, obsessive-compulsive disorder, chronic alcoholism and psychosocial dwarfism (Chrousos, 1998).

Studies to investigate postnatal HPA axis may provide further knowledge on the maternal physiology and foetal programming events. In order to avoid future economic and psychological burden of the metabolic syndrome and affective disorder, special attention should be paid to optimize maternal and intrauterine environments during pregnancy.

Chapter 6

Obesity, hypertension & the hypothalamic-pituitary-adrenal axis

6.1 Summary

The hypothalamic-pituitary-adrenal axis (HPA) and the renin-angiotensin-aldosterone system (RAAS) have been implicated in the pathophysiology of obesity-induced hypertension. However, there are little data on the effect of moderate weight loss on the blood pressure response to salt loading and adrenocortical function.

Twenty five obese subjects (age 39 – 63 years) followed a 12 week weight loss diet (6000kJ/day) before and after which they followed a high (250mmol/d) or low salt (30 mmol/day) diet for 2 weeks crossed-over and randomised. After each diet, 24-hr ambulatory blood pressure, plasma aldosterone, renin levels, 24-hour urinary free cortisol/cortisone, CBG, low dose (1 mcg IV) ACTH stimulation tests with measures of total and free cortisol were performed.

The results showed that mean arterial pressure fell by 6 mmHg after 8.5 kg weight loss. Salt loading elevated day time blood pressure by 6 mmHg (systolic) and 3 mmHg (diastolic) but the effect was not altered by weight loss. Plasma aldosterone and renin levels fell with weight loss (aldosterone: 853 ± 156 to 635 ± 73 pmol/L; renin: 35.4 ± 7 to 24 ± 3 mU/L $P < 0.05$). All cortisol measures were unaffected by weight reduction.

In conclusion, short-term, moderate weight loss was associated with a small reduction in blood pressure and reduced levels of aldosterone and renin. The blood pressure elevating effect of a salt load was not altered. These findings suggest that aldosterone may have an important role in the BP fall with weight loss via a renin mediated mechanism, perhaps

involving renal sympathetic tone. In contrast to severe caloric restriction, HPA axis activation does not occur with moderate weight loss. This suggests a threshold effect of weight loss on the HPA axis where greater caloric restriction is required for HPA stimulation, or a counterbalancing of central and direct adrenal effects on HPA axis function.

6.2 Introduction

Obesity is now developing to alarming proportions worldwide, with devastating economic and medical health burdens on humanity (Hall *et al.*, 2001; El-Atat *et al.*, 2003). There is strong evidence for the association between central (abdominal, visceral) obesity and the metabolic syndrome, which is characterised by hypertension, dyslipidaemia and insulin resistance (Bjorntorp and Rosmond, 2000).

The relationship between obesity and hypertension is now widely recognised, with population studies showing that excess weight gain is the one of the best predictors for development of hypertension (Kannel *et al.*, 1993; Hall *et al.*, 2002). Results from the Framingham Heart Study suggest that obesity accounts for 78% and 65% of essential hypertension in men and women, respectively, and quantitatively 1 kg of excess fat is associated with a 1 mmHg increase in systolic blood pressure (Kannel *et al.*, 1993). Animal experiments and human studies have confirmed this causation and have provided insight into the mechanisms involved (Kannel *et al.*, 1993; Hall *et al.*, 2001; Hall *et al.*, 2002; Rocchini, 2002). Among others, enhanced sympathetic and renin-aldosterone-angiotensin system activity, hyperactivity of the HPA axis, renal dysfunction, hyperinsulinaemia and hyperleptinaemia are major contributory mechanisms to obesity-induced hypertension (Figure 6.1).

NOTE: This figure is included on page 138 of the print copy of the thesis held in the University of Adelaide Library.

Figure 6.1 A schematic outline of factors that contribute to the pathogenesis of obesity-induced hypertension. (El-Atat 2003)

Adipose tissue as a source of inflammatory cytokines and hormones

Visceral adipose tissue is currently recognised as a rich milieu and source of inflammatory cytokines, such as TNF-α, IL-6, C- reactive protein and plasminogen activator inhibitor. As such, obesity has been suggested to be a low-grade inflammatory condition which may be important in the causation and progression of hypertension and atherosclerosis (Festa *et al.*, 2000; Hall *et al.*, 2002; Castro *et al.*, 2003). In addition, the inflammatory cytokines can, in turn, stimulate the HPA axis. There is also increasing evidence that adipose tissue possess a local RAAS which may synthesize all components of the RAAS. This chapter will focus on the role of the HPA axis, renin-aldosterone-angiotensin and sympathetic nervous systems in the pathogenesis of obesity-induced hypertension and the effects with moderate diet-induced weight loss.

6.2.1 Obesity and the HPA axis

Patients with Cushing's syndrome provide clear evidence that cortisol exerts powerful effects on energy balance, a major outcome of these actions being intra-abdominal fat deposition. Insulin resistance and hyperinsulinaemia have been well characterised in all types of obesity; however, viscerally obese subjects displayed more pronounced insulin resistance and a higher risk for type 2 diabetes mellitus (Bjorntorp, 1988). Cortisol and androgens have been implicated as mediating factors (Haffner, 2000). Cortisol may act through several mechanisms including an increase in hepatic glucose production by stimulation of gluconeogenesis, increase in peripheral insulin resistance, and stimulation of lipoprotein lipase in abdominal adipocytes, which in turn increases intra-abdominal fat deposition. Based on this and other observations, it has been suggested that visceral obesity is associated with relative functional hypercortisolism.

Evidence of altered HPA axis function has been described in animal models of obesity and in obese subjects. In rodents with spontaneously occurring obesity due to the presence of a double recessive obese gene (fa/fa) Zucker rat, a number of behavioural, metabolic, and endocrine disorders have been described (Guillaume-Gentil *et al.*, 1990). These animals have higher morning plasma corticosterone and exhibit greater cortisone response to stress than lean control. Further, they showed an early escape from dexamethasone suppression; features consistent with a hyperactive HPA axis. It seems doubtful, however, that the elevated level of corticosterone is solely responsible for the obesity because administration of this hormone does not cause severe obesity as in the rat models mentioned, suggesting that other factors are also involved.

Many clinical studies have looked at whether obese individuals display glucocorticoid-like abnormalities, but until relatively recently the data was confusing, partly due to cohort

variation (especially with regard to the degree and pattern of adiposity), and partly because of a focus on basal glucocorticoid levels. Studies that delineate the pattern of adiposity and assess dynamic as well as basal HPA axis function have now yielded a clearer picture of the role of cortisol in obesity. Firstly, viscerally obese individuals display abnormal glucocorticoid metabolism in the visceral adipose tissue, resulting in a localised increase in glucocorticoid availability (Bujalska *et al.*, 1997). Secondly, viscerally obese individuals usually have normal basal plasma cortisol levels, and normal inhibitory cortisol feedback, but consistently display exaggerated HPA axis responses to everyday environmental stimuli such as physical and mental stress (Marin *et al.*, 1992; Weaver *et al.*, 1993; Korbonits *et al.*, 1996). This is consistent with the common finding that viscerally obese subjects display increased 24-h total urinary cortisol metabolite excretion despite having normal basal cortisol levels. It has also been shown that the pulse frequency of ACTH release from pituitary is increased from obese subjects, suggestive of an altered CNS drive (Pasquali *et al.*, 1993). In addition to HPA axis activation, a parallel central activation of the sympathetic nervous system occurs in response to environmental stress (Ljung *et al.*, 2000). This dual activation may contribute to insulin resistance and to the pattern of body fat accumulation. An additional consequence may be blood pressure elevation as seen in this type of obesity. It has been suggested that the feedback regulation of the HPA axis plays important role in this chain of events via glucocorticoid receptors in the brain. Restriction fragment length polymorphism of the glucocorticoid receptor gene can lead to abdominal obesity, insulin resistance and hypertension (Rosmond *et al.*, 2000; Rocchini, 2002).

CBG

Obesity is associated with increased levels of inflammatory cytokines which are thought to play a role in atherogenic processes and their vascular sequelae (Yeh, 2004). The fat-derived pro-inflammatory cytokines, TNF_{α} and IL-6 stimulate plasma cortisol secretion and also

reduce CBG synthesis (Bernardini *et al.*, 1990; Emptoz-Bonneton *et al.*, 1997). Apart from cytokines, other factors which may alter circulating CBG concentrations include insulin, which reduces CBG and free fatty acids, and interferes with cortisol:CBG binding (Martin *et al.*, 1988; Hammond *et al.*, 1991) . An epidemiological study reveals a negative correlation between obesity, and insulin and CBG levels (Fernandez-Real *et al.*, 2002).

Effects of diet and weight loss on HPA axis function

It has been suggested that food intake can modulate the HPA axis response to stress and that glucocorticoids plays a role in the neuroendocrine control of food intake and energy expenditure (Leal and Moreira, 1997; Cavagnini *et al.*, 2000). Food consumption can be increased directly by glucocorticoids through stimulation of neuropeptide Y and inhibition of CRH and melanocortin release; however, the system that regulates energy homeostasis is complex. Although studies of food restriction in rats indicate that feeding is a major synchronizer of rhythms in HPA axis activity, the information in humans is less clear.

One study of severe dieting (800 calorie/day) in healthy obese women found that weight loss did not significantly alter the ACTH response to ovine CRH stimulation but the total plasma cortisol response decreased significantly with weight loss, and appeared to be related to a significant decrease in CBG (Yanovski *et al.*, 1997). However, little is known of the HPA axis function with moderate weight loss.

6.2.2 Renin-aldosterone-angiotensin system (RAAS)

Obesity may cause sodium retention and hypertension through alteration in the RAAS. The RAAS is an important determinant of efferent glomerular arteriolar tone and tubular sodium reabsorption. Three mechanisms appear to be especially important in mediating increased

sodium reabsorption associated with weight gain: (1) activation of the RAAS system, (2) increased renal sympathetic activity, and (3) altered intrarenal physical forces. Another mechanism, hyperinsulinaemia, has also been suggested to raise arterial pressure in obese subjects, although most of the available evidence suggests that elevated insulin levels do not elevate blood pressure in dogs or humans (Hall, 1993).

The RAAS seems to be activated in obesity despite a state of volume expansion and sodium retention. Elevated plasma aldosterone levels have been reported in obese individuals (Rocchini *et al.*, 1989; Egan *et al.*, 1994; Goodfriend *et al.*, 1998), and it is postulated that an adipocyte-derived factor may cause release of a hepatic factor that in turn steps up aldosterone synthesis (Egan *et al.*, 1994; Goodfriend *et al.*, 1999). The recent identification of adipocyte-derived factor/s which stimulate aldosterone and cortisol release in vitro may provide an additional link between obesity and hypertension (Ehrhart-Bornstein *et al.*, 2003). There are reports that obese subjects have increased plasma rennin activity (PRA), plasma angiotensinogen, angiotensin-converting enzyme (ACE) activity, and plasma angiotensin (Ang) II levels (Egan *et al.*, 1994; Cooper *et al.*, 1997; Engeli and Sharma, 2001). In addition to elevating blood pressure, activation of the RAAS may also contribute to glomerular injury and nephron loss associated with obesity because ANG II formation constricts the efferent arterioles and exacerbates the rise in glomerular hydrostatic pressure caused by systemic hypertension (Hall, 1986; Hall *et al.*, 1999).

It has been suggested that obese subjects have an enhanced sensitivity to dietary salt through failure to suppress renin and angiotensin and that weight loss completely obliterates this effect. Rocchini et al. showed a reduced sensitivity of blood pressure to salt intake, in association with a significant 27% fall in plasma aldosterone and no change in PRA after 20-weeks of weight loss in obese adolescents (Rocchini *et al.*, 1989; Wedler *et al.*, 1992). In

contrast, Tuck et al. demonstrated significant reductions in both PRA and aldosterone with weight loss in obese adults (Weinberger, 1996). However, the finding of this study could be confounded by existing hypertension and diabetes – conditions associated with low plasma renin (Moran *et al.*, 1994).

6.2.3 Sympathetic nervous system

Obesity may also cause sodium retention and hypertension through stimulation of the sympathetic nervous system. It have been suggested that the sympathetic nervous system in obese individuals is chronically activated in an attempt to prevent further weight gain and that obesity-induced hypertension is a by-product of the overactive sympathetic nervous system (Landsberg and Krieger, 1989). Hall et al. have shown that α- and β-adrenergic blockade reduced arterial pressure to a much greater extent in obese than normal dogs. These investigators also demonstrated that renal denervation prevents both the hypertension and sodium retention associated with obesity (Kassab *et al.*, 1995). Clonidine, which blocks central α2 receptors and thereby reduces the central sympathetic drive, has been shown to markedly blunt hypertension, sodium resistance and insulin resistance in dogs fed a high-calorie diet (Rocchini *et al.*, 1999).

In summary, the association between obesity and hypertension has long been recognised. However, the exact mechanism whereby obesity causes hypertension is still unknown. The HPA axis, RAAS and sympathetic nervous systems have been implicated in the pathogenesis of obesity hypertension, however little is known about the effects of weight loss on these systems.

6.3 Hypothesis and Aims

The hypothesis was that weight loss may lead to activation of HPA axis function through central mechanisms, and suppression of aldosterone may occur without significant change in renin, due to reduced putative adipocyte-derived adrenostimulatory (ADAS) factors.

The aims were to investigate the effect of commonly achievable moderate weight loss on blood pressure, salt sensitivity and simultaneously examine measures of

(1) The HPA axis, under basal conditions (urine free cortisol) and ACTH-stimulated conditions, and also urine free cortisol/ cortisone ratio as an index of 11β-HSD activity, and
(2) The renin-angiotensin-aldosterone axis under stimulated (salt-restricted) and suppressed (salt loaded) conditions.

6.4 Research design and methods

Subjects

One hundred and one subjects were recruited by newspaper advertisement. Inclusion criteria were age 20 - 65 years and BMI 27 – 40 kg/m^2. Exclusion criteria were diabetes mellitus or fasting glucose ≥ 7.0 mmol/L, resting blood pressure > 150/95 mm Hg, medications affecting study measurements, active liver or kidney disease or malignancy, current gastrointestinal disease, pregnancy or lactation and more than 50g alcohol/day. The study protocol was approved by the Human Ethics Committee of the Commonwealth Scientific Industrial Research Organisation (CSIRO) and subjects provided informed written consent. The trial was registered with the Australian Clinical Trials Registry (ACTR) N012605000614695.

Forty four subjects were selected and matched for age, gender and BMI, 14 withdrew for personal reasons, 2 were lost to follow-up, 2 were unable to comply with the protocol and 1 withdrew for medical reasons unrelated to the study. Twenty five subjects (17 female, 8 male) completed the study (Table 6.1).

Table 6.1 Baseline characteristics

	Total	**LC**	**HC**
AGE (yr)	48.7 ± 7.6	50.1 ± 7.0	46.9 ± 8.2
BMI (kg/m^2)	32.9 ± 4.3	32.6 ± 4.7	33.2 ± 4.0
Weight (kg)	94.2 ± 17.3	91.5 ± 20.6	97.6 ± 12.0
Glucose (mmol/L)	5.9 ± 0.7	5.9 ± 0.8	5.8 ± 0.4
Insulin (mU/L)	14.8 ± 11.2	16.9±13.9	12.1 ± 11.2
Total cholesterol (mmol/L)	5.5 ± 1.1	5.3 ± 0.9	5.7 ± 1.1
Triglycerides (mmol/L)	1.6 ± 0.8	1.7 ± 0.9	1.4 ± 0.6
HDL-cholesterol (mmol/L)	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3
LDL-cholesterol (mmol/L)	3.6 ± 1.2	3.5 ± 1.0	3.8 ± 1.5
SBP (mmHg)	123.0 ± 13.2	122.7 ± 14.4	123.2 ± 12.2
DBP (mmHg)	75.4 ± 7.0	74.6 ± 6.6	76.4 ± 19.3

Values are Mean ± SD
LC – low carbohydrate, HC – high carbohydrate

Study protocol

The study protocol is summarized in Figure 6.2. This was a 4 week weight stable period in a 2 week randomised crossover design, of either a low salt, 30 mmol Na, 100 mmol K or high salt, 250 mmol Na diet. Potassium was kept constant at 100 mmol/day. Eighteen salt tablets were consumed per day (600-mg per tab [10.5 mmol sodium], (Salt Tablets 3M Pharmaceuticals Pty Ltd, Thornleigh, NSW, Australia). Slow Sodium tablets (Novartis

Pharmaceuticals Australia Pty Ltd. North Ryde, NSW. Australia) were consumed if volunteers could not tolerate salt tablets.

The salt protocol was followed by 12 weeks of weight loss in which subjects were randomized to one of 2 dietary weight loss patterns, described below. After weight loss the salt loading protocol was repeated. Compliance with the diet was evaluated by serial measurement of 24-hour urinary sodium and potassium excretion as previously described (Vertes *et al.*, 1977).

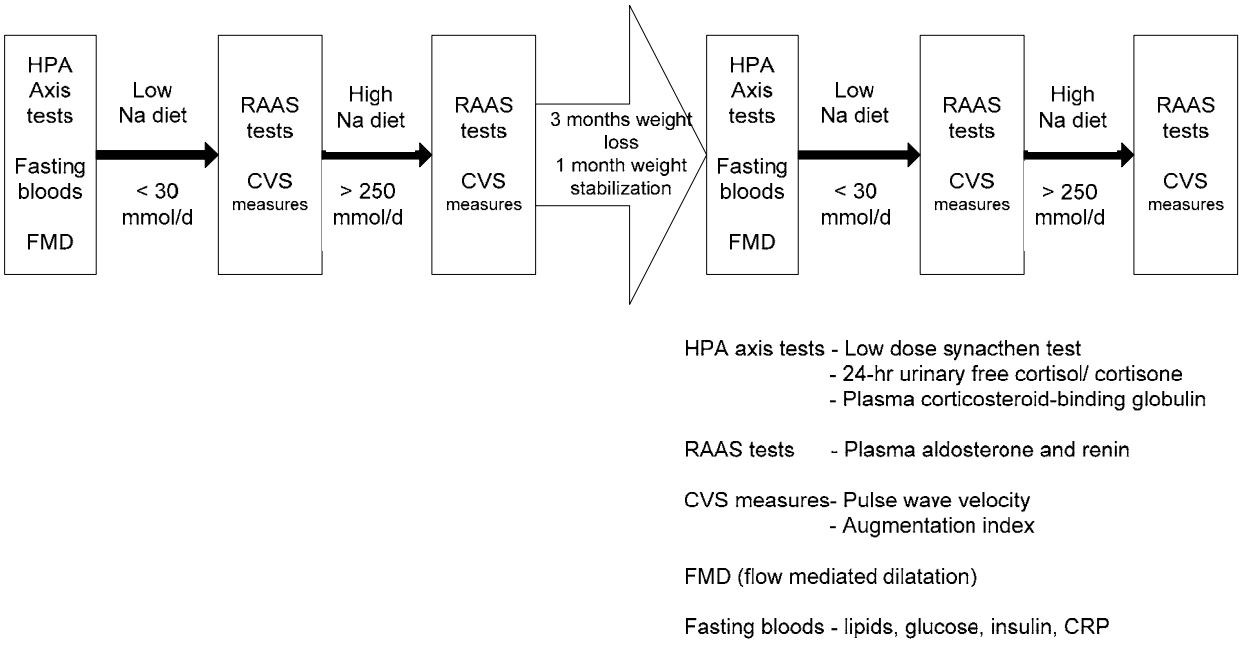


Figure 6.2 Study Protocol

Dietary methodology during weight loss

In an outpatient randomized parallel design subjects were prescribed weight loss diets that were designed to be approximately 6000kJ so that weight loss would approximate 0.5-1 kg per week. Daily food records were maintained to promote compliance and volunteers saw a dietitian individually every 2 weeks.

Body weight, height, waist circumference and body composition

Fasting body weight (Mercury digital scales, model AMZ14, Japan) was recorded in light clothing without shoes at baseline and every 2 weeks during weight loss.

Height was measured on a stadiometer (Seca, Germany) at baseline. Body mass index (BMI) was calculated by weight (kg)/height (m^2).

Waist circumference (cm) was recorded as the smallest measurement (mean of three) between the iliac crest and the lateral costal margin.

Fat mass, lean mass and percent body fat were measured by single frequency bioelectrical impedance analysis (BIA) (Bioimpedance meter IMP5, Impedimed Pty Ltd. Mansfield, Qld Australia).

Laboratory measurements

At baseline subjects had HPA axis testing including a low-dose synacthen stimulation test, a 24-hour urinary free cortisol/cortisone, a 24-hour ambulatory blood pressure and fasting bloods for the measurement of plasma total and free cortisol and CBG (methods described in Chapter 2). HPA axis tests were repeated after the weight loss program. At the end of each dietary salt phase, mid-morning sitting (10 minutes) plasma aldosterone and renin were collected in addition to a 24-hour urinary sodium, potassium, free cortisol and ambulatory blood pressure.

Statistical analyses

Descriptive statistics were reported as mean ± standard error (SEM). Analysis of variance (ANOVA) with repeated measures was used. All analyses were performed using SPSS for Windows 10.0 (Chicago, USA) and statistical significance was set at $P < 0.05$.

6.5 Results

Weight

Mean weight loss was 8.5 ± 0.8 kg (8.9 ± 0.7%). The range of weight loss was 1.2 - 14.7 kg and all subjects lost weight.

24hr Ambulatory blood pressure: Response to salt loading

The volunteers were relatively salt resistant (MAP changes with salt intake < 5 mmHg) before and after weight loss. Salt loading led to a rise in MAP of 4 mmHg (92.7 - 96.7 mmHg) before weight loss and 3 mmHg after weight loss. The effect of salt on BP was statistically significant but there was no significant difference in salt sensitivity after weight loss (Figure 6.3).

Daytime systolic blood pressure (SBP) rose after the salt load before (5.7 ± 1.5 mmHg, $P < 0.05$) and after weight loss (5.0 ± 1.4 mmHg, $P < 0.05$). Daytime diastolic blood pressure (DBP) also rose after the salt load before (2.5 ± 1.0 mmHg, $P < 0.05$) and after weight loss (1.8 ±1.0 mmHg, $P < 0.05$). Night time SBP rose in response to salt before (5.8 ± 1.9 mmHg, $P < 0.05$) and after weight loss (4.2 ± 1.7 mmHg, $P < 0.05$). Night time DBP rose by 2.5 ± 1.0 mmHg, $P < 0.01$ before and by 2.4 ± 1.3 mmHg, $P < 0.01$ after weight loss.

Resting (seated) blood pressure before and after weight loss

Both resting SBP and DBP were lower at the end of weight loss, SBP 123 ± 3 vs. 116 ± 3 ($P < 0.01$), DBP 75 ± 1 vs. 68 ± 1 mmHg ($P < 0.001$). SBP remained reduced after 4 weeks of weight maintenance, at week 24, 119 ± 3 mmHg ($P < 0.05$) and DBP was 73 ± 2 mmHg (P=0.066) with weight regain of 0.25 ± 0.2 kg.

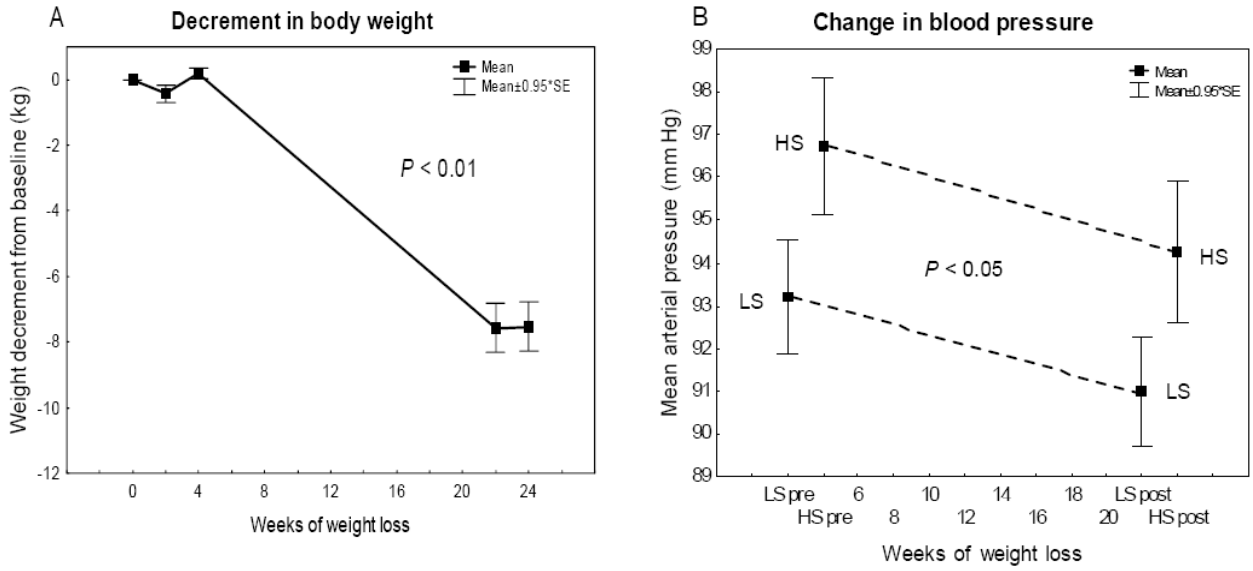


Figure 6.3 Mean arterial pressures (B) fell significantly after 7.7 kg weight loss (A). Blood pressure response to salt loading was not altered by weight loss.

(LS and HS denote low and high salt phases respectively)

Urinary sodium and potassium

Compliance with the protocol was established by sodium excretion during low salt phase of 32.2 ± 33.6 mmol/24hrs before and after 61.1 ± 47.8 mmol/24hrs weight loss and during the high salt phase 242 ± 99 and 268 ± 127 mmol/24hrs before and after weight loss respectively. The difference in sodium was approximately 200mmol/24hr on both occasions. Potassium excretion was 89 ± 26 and 81 ± 33 mmol/24hrs, low and high salt respectively before weight loss and 96 ± 60 and 93 ± 54 mmol/24hrs low and high salt respectively after weight loss.

Renin-Angiotensin-Aldosterone Axis

After weight loss, salt-restricted renin levels were 33 % lower than pre-weight loss values and correlated with reduction in body weight (r = 0.4, P = 0.012). Salt-suppressed renin levels were lower after weight loss (Table 6.2. Figure 6.4A). The decrease in mean arterial

pressure after weight loss was not correlated with the change in plasma renin. Aldosterone responded to weight loss as plasma renin did, but to a lesser extent (Figure 6.4B). There was no relationship between the fall in blood pressure with weight loss and the changes in renin or aldosterone with weight loss.

Cortisol and corticosteroid-binding globulin

There was no significant change in baseline or synacthen stimulated total and free cortisol levels following 12 weeks of diet-induced weight loss (Table 6.2). There was no change in the plasma total and free cortisol responses to cosyntropin. There was also no significant change in CBG levels with weight loss (Figure 6.5). There was a non-significant rise in urinary free cortisol with salt load in both pre- and post-weight loss phases; paralleled with the increase in urinary volume. This may represent an altered handling of salt and fluid by the kidneys in the event of salt loading (Mericq and Cutler, Jr., 1998). There was no change in the F/E ratios with moderate weight loss or salt loading.

Table 6.2 Hormones of the renin-angiotensin-aldosterone system and hypothalamic-pituitary-adrenal axis pre- and post-weight loss.

	Before weight loss		**After weight loss**	
	Low salt	**High salt**	**Low salt**	**High salt**
Renin (mU/L)	35.4 ± 7	8 ± 1.5	24 ± 3*	7 ± 1.1
Aldosterone (pmol/L)	853 ± 156	235 ± 72	635 ± 73*	208 ± 29
24 urine free cortisol (nmol/day)	52.2 ± 6.5	81.5 ± 8.5	56 ± 7.8	68.6 ± 7.3
11 βHSD1 activity F/E ratio	0.24 ± 0.03	0.3 ± 0.03	0.24 ± 0.02	0.28 ± 0.03
Total cortisol (nmol/L)				
0 min	387 ± 34		356 ±17	
30 min	820 ± 29		796 ± 34	
60 min	628 ± 28		664 ± 32	
Free cortisol (nmol/L)				
0 min	20 ± 3		16 ± 1	
30 min	61 ± 3		55 ± 4	
60 min	38 ± 3		48 ± 3	
CBG (nmol/L)	538 ± 55		555 ± 38	

* $P < 0.05$

Values are mean ± SEM

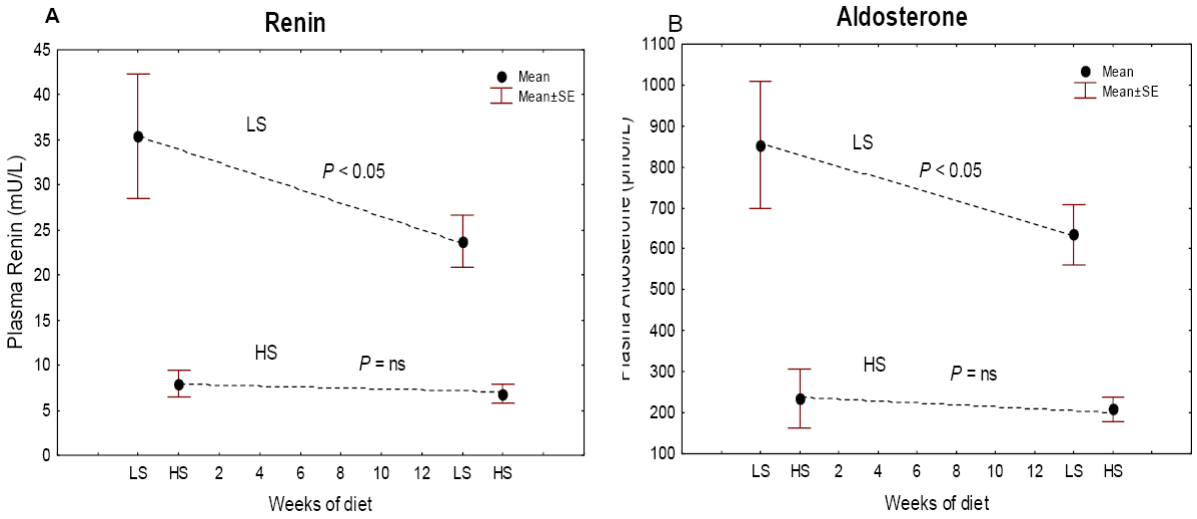


Figure 6.4 Plasma aldosterone and renin levels fell with weight loss. Salt-restricted renin levels (A) were 33 % lower than pre-weight loss values and correlated with reduction in body weight (r = 0.4, *P* = 0.012). Aldosterone (B) responded to weight loss as plasma renin did, but to a lesser extent.

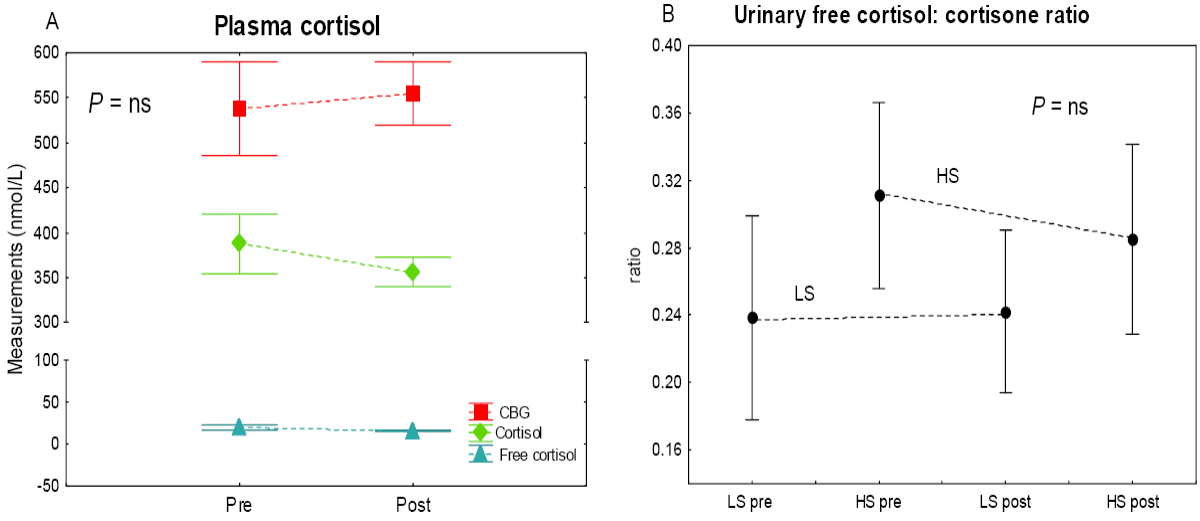


Figure 6.5 All cortisol measures including total and free cortisol responses to cosyntropin and CBG levels were unaffected by weight reduction. The 11 beta hydroxysteroid dehydrogenase 1 (11β-HSD1) activity, represented by the ratio of urinary free cortisol to cortisone was also unchanged.

6.6 Discussion

The main findings from this study were (1) stability of HPA axis function with weight loss, suggesting a lack of both starvation-like central activation of the HPA axis, and the putative peripheral direct adreno-suppressive effects of weight loss, or a balanced counteracting effect of these influences, (2) reduced renin and aldosterone levels compatible with a central effect of weight loss on the RAAS rather than a direct adrenocortical effect via putative adrenostimulatory factors., and (3) salt sensitivity of blood pressure was not altered by weight loss in this relatively salt-insensitive group.

The lack of effect of moderate dietary restriction on HPA axis function was the major novel finding from this study. The significance of this finding is that it is the first to suggest a threshold effect whereby moderate diet restriction does not perturb the HPA axis. Cortisol has been proposed to play a role in obesity, but it is unlikely that the HPA axis activation plays a role in weight regain after diet-induced weight loss.

Severe dietary restriction or starvation is well known to induce prompt hypercortisolism (Yanovski *et al.*, 1997; Douyon and Schteingart, 2002). In anorexia nervosa for example, ACTH levels are generally in the normal range and the ACTH response to CRH is reduced along with increased CRH levels in the cerebral spinal fluid suggestive of CRH neuron activation, along with reduced sensitivity to dexamethasone feedback (Douyon and Schteingart, 2002). Functionally, starvation-induced hypercortisolism probably acts to increase muscle catabolism providing substrate, particularly alanine, for gluconeogenesis. High cortisol levels also produce insulin resistance thereby reserving substrate such as glucose for glucose dependent tissues such as the brain. Severe caloric restriction (800 Kcal/d) also reduces CBG (by 37%) which would further elevate free cortisol levels in the setting of diminished cortisol feedback.

In contrast, we found no effect of moderate dietary restriction and weight loss on basal urine free cortisol measured by specific HPLC, the urine cortisol/cortisone ratio, a measure of 11β-HSD_1 activity, or any effect on basal or ACTH-stimulated total or free cortisol levels and plasma CBG. These findings suggest that moderate dieting/weight loss does not alter HPA axis function, at least to the extent measurable with these experimental methodologies.

The lack of changes in all the measured HPA axis parameters suggest that there is a threshold effect with only relatively severe dietary restriction producing stimulation of the HPA axis. A lack of effect of weight loss on urine cortisol and cortisone may be unexpected given the putative role of adipose tissue 11β-HSD enzyme activity in cortisol regeneration. However, recent studies suggest that this enzyme is inhibited in obesity, downplaying its role in HPA perturbations of obesity. Presumably, weight loss of this magnitude does not alter 11β-HSD activity (Tomlinson *et al.*, 2004a). Despite these negative HPA axis findings, our results do not rule out the possibility of a net balance of counteracting effects on the HPA axis, such as may result from increased central activation of the HPA axis and reduced effects from putative adipocyte-derived adrenostimulatory factors leading to net stability of HPA axis function.

In this regard, the effects of weight loss on the mineralocorticoid axis are instructive. It has been reported that adrenostimulatory factors have a greater effect on aldosterone than cortisol release (Ehrhart-Bornstein *et al.*, 2003). However, in our weight loss model, we noted a greater proportionate fall in plasma renin levels than in aldosterone suggesting that the effect of moderate weight loss on the RAAS was via the juxta-glomerular apparatus and renin secretion, perhaps via reduced renal sympathetic drive to the renal β-receptor at the juxta-glomeriular apparatus. The finding with respect to reduced renin with weight loss contradicts some, but not all, studies of weight loss (Rocchini *et al.*, 1989; Engeli and Sharma, 2001).

Concomitant suppression of renin and aldosterone with weight loss suggests an effect of weight loss on the juxta-glomerular secretion of renin, rather than a direct effect via putative adrenostimulatory factors on the zona glomerulosa. This effect may be mediated by the known effect of the sympathetic nervous system, reportedly overactive in obesity, on the β-adrenoceptors of the juxta-glomerular apparatus, the renal site of renin production (Landsberg and Krieger, 1989).

The study has several limitations. The findings arose from short-term (3-month) weight loss. However, it appears unlikely that long term follow up would reveal changes in HPA axis function that were not evident in this period. It is possible, however, that the RAAS changes may ameliorate over time. The numbers of volunteers studied were sufficient to observe hormonal changes that were likely to be clinically significant (> 20%). Our study was not directed towards the study of salt sensitivity to blood pressure. The population heterogeneity of BP response to salt in normotensives ranges from 1-34% (Weinberger, 1996). Various arbitrary definitions of salt sensitivity have been described, but using a change in mean arterial pressure of ≥ 10mmHg as sensitive and ≤ 5mmHg as insensitive (Sullivan, 1991; Wedler *et al.*, 1992; Weinberger, 1993), approximately 26% of normotensives and 51% of hypertensives are salt sensitive (Weinberger, 1996). There are conflicting data on the issue of increased salt sensitivity in obese subjects. The present study describes volunteers who were quite salt insensitive, with salt intake related MAP changes of 4mmHg before and 3mmHg after weight loss, hence it would be difficult to determine a meaningful fall in salt sensitivity. A group of hypertensive obese subjects, or a group of normotensives with the apparently familial/genetic tendency to salt sensitivity may display a reduced salt sensitivity to BP. This was shown in a study of obese adolescents who were salt sensitive (MAP change with salt intake of 12mmHg) and had their salt sensitivity reduced to MAP 1mmHg with weight loss

(Rocchini *et al.*, 1989). Salt sensitivity involves renal salt handling and a range of putative physiologic/genetic factors of uncertain relative importance. Salt sensitive individuals have lower renin and aldosterone levels suggesting that the RAAS is not a major determinant of the salt sensitivity phenotype. Hence it would not necessarily follow that the fall in aldosterone with weight loss noted would reduce the salt sensitivity of blood pressure.

Particular strengths of this study included the sensitivity of the measures chosen to study the RAAS and HPA axes, the use of potassium equivalent diets when altering salt intake and the use of measurements taken after the acute weight loss phase.

6.7 Conclusion

This study revealed the expected effects of short-term weight loss on blood pressure and aldosterone levels, with lowered renin suggesting a renal rather than direct adrenal effect of weight loss on the mineralocorticoid axis. We have shown for the first time that basal and ACTH-stimulated cortisol secretion are unaltered by moderate weight loss, in contrast to the effects of starvation and the known effects of obesity on central HPA axis function. The lack of effect of moderate weight loss on HPA axis function may represent a counterbalancing effect of influences on the HPA axis, but suggest that global HPA axis is likely to be unaltered by this widely employed diet/weight loss strategy.

Chapter 7

Discussion and future perspectives

This thesis has studied HPA axis function in sepsis and in complicated pregnancy as well as adrenocortical function in mild diet-induced weight loss. Each of these conditions, although disparate, is characterized by HPA/ adrenocortical perturbation and immune activation (Figure 7.1). In addition, there is insufficient data to date on total/ free cortisolaemia – a lack of knowledge that impedes our ability to determine if cortisol has a pathogenic role.

My findings from these studies have provided novel data on the pathophysiologic regulation of cortisol in disorders which have perturbation of immune and blood pressure regulation as their hallmark. Understanding the pathophysiology of cortisol will enable us to better prognosticate relative adrenal insufficiency in critical illness and understand the role of the adrenal cortex in pre-eclampsia and obesity-related hypertension.

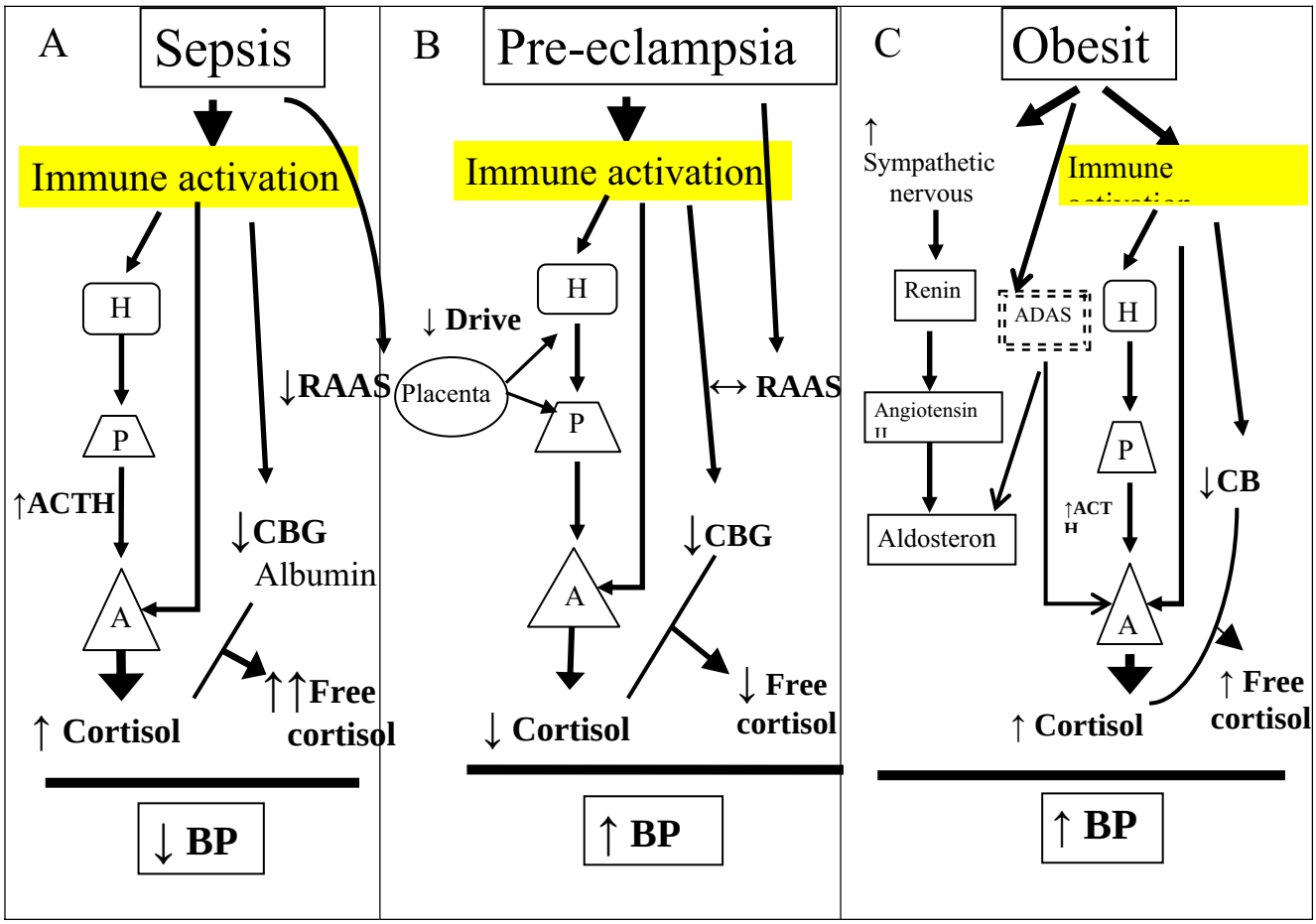


Figure 7.1 Heuristic diagram showing alterations of the hypohtlamic-pituitary-adrenal axis in three conditions: sepsis, complicated pregnancy and in obesity. Each of these conditions, although disparate are associated with immune system activation and pathological alterations of blood pressure regulation.

Studies described in this thesis were directed towards identifying cortisolaemia (total/ free cortisol) in each state so as to determine the potential role of altered HPA axis function in the pathophysiology of these conditions.

Note that the alterations in pregnancy are described relative to healthy pregnancy. RAAS: renin-angiotensin-aldosterone system

In the sepsis study, we have demonstrated that free cortisol is a better guide to circulating glucocorticoid bioactivity than total cortisol in systemic inflammation as it corresponds more closely to illness severity (Chapter 3). Most published studies measured plasma total cortisol

in the assessment of adrenal function in critical illness. However, activation of systemic inflammatory cascade leads to marked reduction in plasma CBG and increased free cortisol fraction (Figure 7.1A). Thus, total cortisol may not represent adrenal function reliably in critical illness. Our study is the first to compare total and free cortisol in systemic infection, and we show that free cortisol is superior to total cortisol in this clinical setting. We have also validated the Coolens' method in estimating free cortisol in systemic infection, by using total cortisol and CBG levels.

The concept of relative adrenal insufficiency in septic shock remains controversial. Cortisol levels vary widely in patients with septic shock. Studies found increased mortality associated with both low and high plasma cortisol levels (Moran *et al.*, 1994; Bouachour *et al.*, 1995). The current empirical definition of RAI is based on a total cortisol increment of 248 nmol/L or less (Annane *et al.*, 2000b). Our study suggests a free cortisol increment of 110 nmol/L or less may better categorize RAI. However, this would need to be elucidated with larger randomized controlled trials with respect to outcomes and clinical responses to corticosteroid therapy. It has been proposed that low cortisol levels in critical illness may be due to adrenal haemorrhage or necrosis, pituitary ACTH deficits, and/or genetic polymorphisms that limit maximal adrenocortical hormone secretion (Beishuizen and Thijs, 2001). Our follow-up data reveal prompt recovery from RAI in septic shock survivors, which is in keeping with the concept of a lack of functional adrenal reserve rather than adrenal damage during critical illness.

These novel findings point towards the use of free rather than total cortisol in studies aimed at evaluating illness severity and hydrocortisone therapy in functional or relative adrenal insufficiency. As tissue glucocorticoid resistance in infection limits the utility of plasma cortisol measures to predict responses to hydrocortisone, we studied an *in vivo* biological

product, NOx - a plasma marker of nitric oxide synthase activity, as a target for hydrocortisone therapy in systemic infection. The finding that NOx is not readily suppressed by glucocorticoid therapy in septic shock is contrary to another study (Keh *et al.*, 2003). Although NOx levels correlate to illness severity, it is not superior to total or free cortisol levels. Further, there is high inter-individual variability despite high stability within individuals. The reason for the inter-individual differences seems to extend beyond illness severity and may reflect genetic polymorphisms in enzymes of the iNOS system. These differences would preclude the use of actual NOx levels as targets for glucocorticoid therapy and the use of a percent decrement in NOx with GC therapy is obviously not possible if NOx does not respond reliably to exgogenous GCs.

The pregnancy study has yield exciting results of considerable clinical interest. We have demonstrated for the first time that plasma CBG, total and free cortisol rise progressively across gestation, but CBG falls substantially in the last few weeks of gestation with a corresponding rise in free cortisol. Speculatively, this free cortisol elevation in late pregnancy may facilitate organ maturation in the foetus and perhaps facilitate labour in part through stimulation of placental CRH release. The finding of maternal hypocortisolism and low CBG levels in late third trimester in high risk pregnancies including pre-eclampsia, intrauterine growth restriction and assisted reproduction is unexpected, as these states are associated with immune activation which generally stimulates HPA axis activity. Low cortisol in pre-eclampsia may be due to reduced maternal ACTH secondary to enhanced negative feedback at the hypothalamic level or inability of the dysfunctional placenta to drive the maternal HPA axis (Figure 7.1B). Reduced CBG concentrations in high risk pregnancies may be a consequence of increased catabolism peripherally and decreased synthesis induced by the circulating cytokines or reduced plasma oestriol levels.

Strikingly, plasma CBG was markedly reduced from early gestation in gamete recipients, even in those with a normal pregnancy outcome. Suppressed CBG levels in gamete recipients may result from increased levels of pro-inflammatory cytokines, as a consequence of the total allograft immune status of the foetus.

Finally, we have demonstrated that Coolens' method cannot estimate free cortisol reliably in pregnancy suggesting an altered binding affinity of CBG for cortisol in pregnancy or interference of the equilibrium by other pregnancy-associated steroids.

The long term effects of maternal hypocortisolism on the foetus/ neonate are unknown. Human and animal studies suggest that excessive prenatal exposure to glucocorticoids may permanently program physiology, leading to increased risks of cardiovascular, metabolic and neuroendocrine disorders in adulthood (Barker, 1993; Phillips, 2001; Bertram and Hanson, 2002). Studies indicate that maternal hypercortisolism, both endogenous and exogenous, is associated with lower birthweight, neonatal hypercortisolism and hyperactivity of the HPA axis.

Whether maternal hypocortisolism, as suggested by our studies in complicated pregnancy, lead to hypoactivity of HPA axis post-partum and/ or of the offspring has not been examined. We hypothesize that maternal hypocortisolism may lead to postnatal HPA axis hypofunctioning. Persistent hypoactivity of the neonatal HPA axis into adulthood may predispose to a spectrum of conditions known as hypocortisolaemic disorders such as chronic fatigue syndrome, fibromyalgia and atypical depression (Heim *et al.*, 2000; Wust *et al.*, 2004). This study has provided novel knowledge on the physiology of cortisol in human pregnancy and may pave the way for future studies on the association between a relatively underactive maternal HPA axis and a new pathway to a foetal origin of adult disease.

In contrast to the inflammatory conditions of sepsis and pre-eclampsia, obesity represents a chronic inflammatory state with functional hypercortisolism, due to subtle central alterations of the HPA axis. Obesity and blood pressure are strongly associated and the adrenocortical hormones aldosterone and cortisol are potentially important links in this association. The recently isolated but incompletely charaterized adipocyte-derived adrenostimulatory factor (ADAS) was shown to stimulate aldosterone, and to a lesser extent cortisol release *in vitro*. Our study revealed the expected effects of short-term weight loss on blood pressure and aldosterone levels, with lowered renin suggesting a renal sympathetic rather than direct adrenal effect of weight loss on the mineralocorticoid axis. We have shown for the first time that basal and ACTH-stimulated cortisol secretion are unaltered by moderate weight loss, in contrast to the effects of starvation and the various described effects of obesity on central HPA axis function. The lack of effect of moderate weight loss on HPA axis function may represent a counterbalancing effect of influences on the HPA axis, but suggest that global HPA axis function is likely to be unaltered by this widely employed diet/weight loss strategy. Further, the HPA axis may not contribute to the weight regain phenomenon.

Future studies

The studies in this thesis have yield novel results with potentially broad clinical implications. Further studies could address the generalizability of our findings and explore those implications.

Our septic shock study has shown that free cortisol is a better guide to glucocorticoid activity in systemic infection. However, we are unable to analyse free cortisol with respect to patient outcomes as therapy was not randomized and our numbers are not sufficient to determine

treatment effects. Future research in this field should involve large randomised controlled trials that aim to (i) define relative adrenal insufficiency by low dose vs. standard ACTH stimulation tests using free cortisol measurements; (ii) stratify illness severity, prognosis and outcomes based on free cortisol levels and (iii) identify reliable biomarkers of acute inflammatory response that may guide glucocorticoid therapy in critical illness.

In pregnancy, although our cortisol measures were prospective and taken frequently through gestation, plasma cortisol drawn once at each study time point is a limited measure of HPA activity and can not provide a full circadian pattern of cortisol secretion in pregnancy. Urine measures are confounded in pregnancy by the effects of altered renal tubular flow rates and cortisol metabolism. Hence, future studies should utilise salivary cortisol sampling throughout the day to assess the circadian pattern of cortisol secretion and to exclude the possibility of altered cortisol release in complicated pregnancies.

The novel finding of maternal hypocortisolism in complicated pregnancies in late gestation may have implications for offspring and mothers as discussed earlier. This study did not measure post-partum maternal cortisolaemia and CBG which could potentially provide useful information on the HPA function of those women with hypocortisolism during pregnancy. Moreover, there were no outcome data on these women and their babies. Future studies should focus on the effects of maternal HPA function on (i) the physical and psychological health of the mother postnatally, and (ii) outcomes of their babies. Dynamic assessment of the neonatal HPA axis may reveal low basal cortisol levels and a diminished cortisol response to stimulation. To study the long term effects of these changes, a prospective, longitudinal study using animal models may be necessary.

We still know remarkably little about the maternal HPA axis in pregnancy and how to optimize the maternal and intrauterine environment. Information from these studies may help clinicians to improve maternal and infant health, and at an individual level, the identification of high risk groups who may respond to early lifestyle (e.g. weight reduction) and/ or pharmacological interventions (e.g. antihypertensive therapy).

One of the limitations of the obesity study is the short-term (3 month) weight loss period. However, it is unlikely that long term follow up would reveal changes in HPA axis function that were not evident in this period. It is possible, however, that the RAAS changes may ameliorate over time. Future studies would aim to conduct a longer (≥ 6 months) weight loss program with an extended follow up period. We would also aim to measure the ADAS factors pre- and post weight loss when this becomes technically possible.

The last few decades have witnessed an upsurge in the understanding of the action of glucocorticoids and their involvement in human pathophysiology. Glucocorticoids are involved in almost every physiologic, cellular, and molecular network of the human organ systems. However, alterations in the activity of the HPA axis and/or tissue sensitivity to glucocorticoids may lead to disease if the adaptive response of the body to maintain homeostasis is compromised. Abnormal cortisol responses may imprint illnesses and may have long term consequences such as foetal origin of adult disease.

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