IMPACT OF CHRONIC PRENATAL HYPOXIA ON VASCULAR FUNCTION DURING FETAL, NEONATAL AND ADULT LIFE

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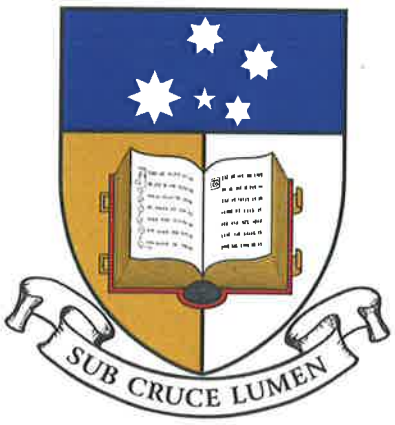

This thesis is dedicated to (Auntie) Chriss Kirk, for everything you taught me about life and nature as a child, and to Jamie, because I love you.

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ABSTRACT

Changes in vascular function may contribute to the increased risk of developing adult hypertension and cardiovascular disease that is associated with impaired fetal growth. Impaired fetal growth may be a consequence of placental insufficiency with an associated restriction of both fetal oxygen and nutrient supply; however the impact of prenatal hypoxia on vascular function is unclear. Therefore, the aim of this thesis was to determine the effects of chronic hypoxia before birth on vascular function during fetal, neonatal and adult life.

The effects of chronic hypoxia during the last week of pregnancy in rats on neonatal and adult offspring vascular function were determined. Hypoxia also reduced maternal food intake, therefore the vascular consequences of equivalent undernutrition were also assessed. Femoral artery vasoconstriction to phenylephrine (α_1 adrenergic receptor agonist) was increased only by maternal hypoxia, however, indicating a specific impact of prenatal hypoxia, or the interaction of hypoxia and undernutrition, on neonatal peripheral vascular function.

Maternal hypoxia reduced sensitivity to endothelium-dependent vasodilation in small mesenteric arteries from 4 month offspring. Whereas endothelial function decreased with age in control offspring, no age-effect was observed in hypoxia or undernutrition offspring, and at 7 months sensitivity did not differ among groups. However, at this age the endothelial pathways which mediated relaxation differed in offspring from hypoxia dams; specifically, there was a loss of nitric oxide-mediated endothelium-dependent relaxation,

attributable in part to increased vascular oxidative stress. Sensitivity to phenylephrine was also increased in mesenteric arteries from 7 month offspring of hypoxia dams.

In the second experimental series, the effects of chronic hypoxia on fetal vascular function were studied in a large animal model, the sheep. Interestingly, the sensitivity of smaller mesenteric artery branches to noradrenaline increased in direct relation to the degree of placental insufficiency, as indicated by both the gestational fetal arterial PO_2 and PCO_2.

Prenatal hypoxia therefore impacts vascular function throughout fetal, neonatal and adult life. The persistence of adaptive changes in peripheral vascular function following chronic hypoxia may impair later regulation of peripheral vascular resistance, and increase the risk of developing hypertension in individuals whose prenatal growth was impaired.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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PUBLICATIONS ARISING FROM THIS THESIS

- **Williams, SJ**, Campbell, ME, McMillen, IC and Davidge, ST. (2005) Differential effects of maternal hypoxia or nutrient restriction on carotid and femoral vascular function in neonatal rats. ***American Journal of Physiology, Regulatory, Integrative & Comparative Physiology*** 288(2):R360-7

- **Williams, SJ**, Hemmings, DG, Mitchell, JM, McMillen, IC and Davidge, ST. (2005) Effects of maternal hypoxia or nutrient restriction during pregnancy on endothelial function in adult male rat offspring. ***Journal of Physiology*** 565(1):125-35. *Included in a special issue of the Journal focussed on developmental programming of adult disease.*

IN PREPARATION

- Williams, **SJ,** Davidge, ST, Morrison, JM and McMillen, IC. Chronic hypoxia increases sensitivity to noradrenaline in small mesenteric arteries from fetal sheep in late gestation.

RELATED PUBLICATIONS

- Veerareddy, S, Campbell, ME, **Williams, SJ**, Baker, PN, and Davidge, ST. (2004) Myogenic reactivity is enhanced in rat radial uterine arteries in a model of maternal undernutrition. ***American Journal of Obstetrics & Gynecology*** 191(1):334-9

- Hemmings, DG, **Williams, SJ** and Davidge, ST. (2005) Increased myogenic tone in 7-month-old adult male but not female offspring from rat dams exposed to hypoxia during pregnancy. ***American Journal of Physiology, Heart & Circulatory Physiology*** Aug 289(2) H674-82

- Campbell, ME, **Williams, SJ,** Veerareddy, S and Davidge, ST. (2005) Maternal nutrient restriction reduces carotid artery constriction without increasing nitric oxide synthesis in the late gestation rat fetus. ***Pediatric Research*** 58(5) 840-4

- Xu, Y*, **Williams, SJ*,** O'Brien, D, and Davidge, ST. (2006) Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. ***FASEB Journal*** (Epub April 2006). **These authors contributed equally to this work.*

ABBREVIATIONS

5-HT	5- Hydroxytryptamine
A II	Angiotensin II
AA	Arachidonic acid
AC	Adenylate cyclase
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AT_1	Angiotensin type 1 receptor
AT_2	Angiotensin type 2 receptor
BH_4	Tetrahydrobiopterin
BK	Bradykinin
C	Control group
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CM	Calmodulin
CX	Carunclectomy
d	Days
dGA	Days gestational age
DMEM	Dulbecco's modified eagle medium
EC_{50}	Effective concentration (50% response)
EDHF	Endothelium-derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET_A	Endothelin type A receptor
ET_B	Endothelin type B receptor

GC	Guanylate cyclase
g/dL	Grams per decilitre
GMP	Guanosine monophosphate
h	Hours
H	Hypoxia group
Hb	Hemoglobin
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HIF-1	Hypoxia inducible factor-1
H_2O_2	Peroxide
iNOS	Inducible nitric oxide synthase
IP	Prostacyclin receptor
IP_3	Inositol trisphosphate
IUGR	Intrauterine growth restriction
KPSS	High potassium physiological saline solution
LNA	LN-nitro arginine
L-NAME	N^{ω}-nitro-$_L$-arginine methyl ester
Meclofenamate	Meclofenamic acid
Methacholine	Acetyl-β-methylcholine chloride
mg	Milligrams
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mmHg	Millimetres of mercury
mmol/L	Millimoles per litre
μmol/L	Micromoles per litre
μm	Micrometres
$mNmm^{-1}$	Milli newtons per millimetre square
MnTMPyP	Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin

mo	Months
N	Normoxic group
NA	Noradrenaline
$NADPH_{ox}$	NAD(P)H oxidase
nmol/L	nanomoles per litre
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Nitrates
NPY	Neuropeptide Y
NR	Nutrient restriction group
O_2^{*-}	Superoxide anion
ODQ	1H-(1,2,4)Oxadiazole(4,3-a)quinoxalin-1-one
$ONOO^{*-}$	Peroxynitrite
P-450	Cytochrome P-450
PCO_2	Partial pressure of carbon dioxide
PE	Phenylephrine
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGHS	Prostaglandin G/H synthase
PGI_2	Prostacyclin
PGIS	Prostacyclin synthase
PKA	Protein kinase A
PLA_2	Phospholipase A_2
PO_2	Partial pressure of oxygen
PSS	Physiological saline solution
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rat
SNP	Sodium nitroprusside

SNS	Sympathetic nervous system
SOA	Superoxide anion
SOD	Superoxide dismutase
SP	Substance P
TF	Thin filaments
TxA_2	Thromboxane
TxAS	Thromboxane synthase
U46619	9,11-dideoxy-11α,9α -epoxymethano-prostaglandin $F_{2\alpha}$

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CHAPTER 1

LITERATURE REVIEW

1.1 OVERVIEW

In human populations across the world, epidemiological studies have demonstrated that being of low birth weight is associated with an increased risk of developing cardiovascular disease during adult life. Numerous experimental studies have investigated the biological basis of this association. From these studies, it is apparent that both structural and functional elements of key systems involved in the regulation of cardiovascular physiology may be programmed by the *in utero* environment, increasing the individual's risk of developing later disease(McMillen & Robinson, 2005). An interesting series of studies have now also demonstrated that the function of arteries within peripheral circulations is impaired from infancy through to early adult life in persons who were small for their gestational age at birth. Impaired prenatal growth may therefore result in changes in vascular function which contribute to the increased the risk of developing hypertension and cardiovascular disease during adult life.

This review will firstly consider the evidence that impaired growth *in utero* impacts later cardiovascular health and vascular function within human populations. To understand the origins of changes in vascular function following intrauterine growth restriction (IUGR), several major clinical etiologies of IUGR will be considered. Important aspects of the regulation of vascular function will be briefly reviewed, to facilitate the detailed investigation of the time-course of changes in vascular function following reduced fetal oxygen or nutrient supply. Specifically, the integrated neural, endocrine, paracrine and autocrine fetal responses to acute stress which modify regional vascular function and the impact of prior fetal compromise on these responses will be discussed. The currently available data regarding the long-term functional consequences of specific perturbations during prenatal development will then be considered in relation to both endothelial and

vascular smooth muscle function. The relevance of currently available data to the development of the experimental hypotheses will be highlighted throughout.

1.2 EFFECTS OF PRENATAL GROWTH ON POSTNATAL HEALTH

1.2.1 Intrauterine growth restriction and the incidence of cardiovascular disease

Interest in the impact of impaired fetal growth on health after birth has grown considerably following the early observations of Barker and colleagues in 1986, who noted that there was an association between high infant mortality in specific geographic regions within the UK and high cardiovascular mortality rates approximately 50 years later (Barker & Osmond, 1986). Further investigations demonstrated that in populations within England where detailed birth records were available, infants born small for gestational age were at an increased risk of developing cardiovascular disease in adult life (Barker *et al.*, 1989b; Martyn & Barker, 1994). Subsequent investigations have confirmed the inverse association between birth weight and risk of developing cardiovascular disease in diverse populations, including individuals from Croatia (Kolacek *et al.*, 1993), India (Stein *et al.*, 1996), the United States (Curhan *et al.*, 1996a; Rich-Edwards *et al.*, 1997), Sweden (Leon *et al.*, 1996; Leon *et al.*, 1998) and Finland (Eriksson *et al.*, 1999).

Evidence that the underlying cause of reduced growth *in utero* was significant in determining later risk of cardiovascular disease was provided by data which identified associations between adult cardiovascular disease and indices of relative size at birth including ponderal index (Eriksson *et al.*, 1999; Forsen *et al.*, 1999; Eriksson *et al.*, 2001), head circumference (Barker *et al.*, 1993; Stein *et al.*, 1996) and abdominal circumference (Barker *et al.*, 1995). These studies implied that both reduced and disproportionate fetal growth, which may result from IUGR, were associated with an increased risk of developing

cardiovascular disease. The incidence of cardiovascular disease in adulthood was further related to patterns of growth during infancy and childhood (Eriksson *et al.*, 1999). In several studies it was demonstrated that male infants who were small at birth and at 1 year of age were at greatest risk of developing cardiovascular disease (Barker *et al.*, 1989b; Osmond *et al.*, 1993; Eriksson *et al.*, 2001). Rapid weight gain between ages 2-7 years in male children also carried an increased risk of developing cardiovascular disease (Eriksson *et al.*, 2001), however, and rapid catch up growth was also associated with increased cardiovascular mortality in women (Osmond *et al.*, 1993; Forsen *et al.*, 1999).

On the basis of the body of data linking prenatal growth to postnatal health, a hypothesis was suggested: fetal adaptations to a sub-optimal *in utero* environment result in permanent structural and functional differences in key homeostatic systems, predisposing the individual to the later development of cardiovascular and metabolic disease (Barker, 1995). Evidence to support this hypothesis has since been generated through numerous epidemiologic, clinical and basic studies.

1.2.2 Intrauterine growth restriction and adult blood pressure

Hypertension has long been recognized as an important risk factor for cardiovascular morbidity and mortality. While the relation between cardiovascular risk and blood pressure is J-shaped in some studies (Cruickshank *et al.*, 1987; Staessen *et al.*, 1989), it is well established that the risk of cardiovascular disease is positively related to blood pressure across the normotensive range, such that even modest increases in blood pressure increase the risk of cardiovascular disease (Hansson, 2000; Greenberg, 2003). Meta-analysis comparisons of the cardiovascular risk reduction produced by different antihypertensive agents have also demonstrated that risk reduction is primarily related to the degree of the

decrease in blood pressure, and not to specific treatment regimes (Turnbull, 2003; Lawes *et al.*, 2004).

In a large number of studies, an inverse association has been identified between weight at birth and adult systolic blood pressure (Barker *et al.*, 1989a; Barker *et al.*, 1990; Barker *et al.*, 1992; Law *et al.*, 1993; Huxley *et al.*, 2000) and this association has been observed across the full birth weight range. A systematic review conducted in 2000 that used meta-analysis to compare results from 80 studies and represented data from approximately 440,000 individuals of varying ages confirmed the inverse relation between birth weight and blood pressure, which was observed from childhood through to adult life (Huxley *et al.*, 2000). This meta-analysis further identified an inverse association between head circumference and systolic blood pressure, although other indicators of relative fetal growth did not show significant association with systolic blood pressure. These data suggest that elevated blood pressure may be an important contributor to the increased cardiovascular mortality in individuals who were growth-restricted *in utero*.

The degree to which adult blood pressure is attributable to birth weight is controversial (Huxley *et al.*, 2002), and there has also been suggestion that publication bias within the literature may have contributed to an overstatement of the impact of birth weight on later blood pressure (Huxley *et al.*, 2002; Schluchter, 2003). It must be considered, however, that birth weight is only a proxy measure of fetal growth, which summarizes a complex process. Further, larger studies in particular may require use of questionnaire-based, self-reported data of varying accuracy, and the inclusion of data from individuals receiving treatment for hypertension (Curhan *et al.*, 1996a; Curhan *et al.*, 1996b). These factors may reasonably dilute the measurable effects of size at birth on blood pressure. The coincident increase in

cardiovascular mortality suggests, however, that differences in blood pressure regulation, irrespective of the absolute increase in blood pressure, are of relevance to understanding susceptibility to cardiovascular disease in individuals of low birth weight.

1.2.2.1 Programming of blood pressure in animal studies

The epidemiologic associations between birth weight and blood pressure are further supported by numerous observations of elevated blood pressure in adult offspring following the experimental reduction of fetal growth in animals. Increases in blood pressure in adult offspring have now been reported in guinea pigs (Persson & Jansson, 1992) and rats (Payne *et al.*, 2003; Payne *et al.*, 2004) where fetal growth restriction was induced by uteroplacental limitation, and in male and female rat litter-runts compared to normally grown rat pups (Woods & Weeks, 2004). A number of investigations have also reported elevated blood pressure in offspring of dams malnourished using various protocols during pregnancy (Langley & Jackson, 1994; Langley-Evans *et al.*, 1999; Vickers *et al.*, 2000; Ozaki *et al.*, 2001; Battista *et al.*, 2002; Lamireau *et al.*, 2002; Brawley *et al.*, 2003; Khan *et al.*, 2003; Khan *et al.*, 2004). It must be noted, however, that other studies have reported no change in blood pressure following either uterine artery ligation (Jansson & Lambert, 1999) or undernutrition (Holemans *et al.*, 1999). Differences in the undernutrition protocol employed may contribute to these observations. There is also evidence that the method of measurement may have a significant impact on the observed effects of impaired fetal growth on adult blood pressure. Restraint procedures commonly used during the determination of blood pressure by the tail-cuff method in rats can increase blood pressure as measured by telemetry (Irvine *et al.*, 1997). The cardiovascular response to stress may also be augmented by prenatal exposure to an adverse *in utero* environment. No change in basal blood pressure was observed in adult male pups from protein restricted dams when

blood pressures were measured by telemetry. The stress-induced increases in systolic and diastolic blood pressure were greater, however, in pups from protein-restricted dams than from control dams (Tonkiss *et al.*, 1998). Prenatal hypoxia also increased the stress-induced elevation in blood pressure in male adult rat offspring without influencing basal blood pressure (Peyronnet *et al.*, 2002). While the body of experimental data supports the hypothesis that impaired fetal growth increases postnatal blood pressure, the potential contribution of differences in the cardiovascular sensitivity to stress must also be considered.

1.2.3 Current hypotheses and proposed mechanisms

It has recently been suggested that the programming of adult health during early development may be considered as arising through (a) a loss of functional capacity related to limitations to the total growth, or maturation trajectory for specific organs (b) an adaptive response of the fetus which conveys immediate or short-term survival advantage or (c) a predictive adaptive response of the fetus which may convey little or no short-term survival advantage, but may facilitate postnatal survival in the predicted environment (eg. famine) (Gluckman & Hanson, 2004; McMillen & Robinson, 2005). It is further proposed that when the actual environment differs substantially to the predicted environment, predictive adaptations are rendered maladaptive and therefore contribute to the predisposition to develop adult onset cardiovascular disease (Gluckman & Hanson, 2004). While evidence supports the biological operation of all 3 proposed mechanisms, the relative contribution of each pathway to the determination of individual adult health is likely to depend considerably on the specific perturbations experienced during development.

This review will primarily consider the impact of impaired prenatal development on vascular function as a proposed pathway linking fetal adaptation to a sub-optimal *in utero* environment to cardiovascular health during adult life. It is not yet clear how predictive adaptation may contribute to the programming of vascular function, whereas immediate adaptive changes in fetal vascular function are known to occur in response to acute hypoxia (Cohn *et al.*, 1974; Giussani *et al.*, 1993) and chronic maternal undernutrition (Ozaki *et al.*, 2000; Nishina *et al.*, 2003). The persistence of changes in vascular function originating in response to changes in the *in utero* environment, but of no subsequent adaptive value may therefore best describe the current paradigm of vascular programming. However, one study has reported that while small artery vasodilator function was impaired in the offspring of rat dams fed a high fat diet during pregnancy, this effect was reversed in offspring fed a postnatal diet high in fat (Khan *et al.*, 2004). Predictive adaptive changes in vascular function may therefore prove an interesting future direction within the field.

1.2.4 Evidence for the in utero programming of postnatal vascular function

Peripheral vascular resistance is dependent on the tone of resistance arteries, and alterations in vascular tone contribute substantially to blood pressure regulation. The vascular tone of resistance arteries is dynamically regulated by neuroendocrine and local mechanisms, which collectively determine the balance between vasoconstrictor and vasodilator signals to modify peripheral resistance. Evidence to support the programming of endothelial and vascular smooth muscle function, arterial structure and sympathetic activity in humans will be considered below.

1.2.4.1 Endothelium-dependent and -independent vasodilation

Endothelial function is a key modulator of vascular homeostasis and endothelium-dependent relaxation is considered an indicator of vascular health. Martin *et al.* assessed differences in vasodilation of cutaneous blood vessels in neonatal infants of low or appropriate birth weight. Endothelium-dependent relaxation to acetylcholine was significantly impaired in 3 day-old, low birth weight infants, while no differences in endothelium-independent relaxation were observed (Martin *et al.*, 2000a). Interestingly, further investigations by the same group revealed that while low birth weight was associated with endothelial impairment in term infants, endothelial function was unaffected in preterm, IUGR infants (Norman & Martin, 2003). These data highlight that being small for gestational age rather than prematurity is of relevance to vascular function after birth, and also suggest that changes in the *in utero* environment during late gestation may have a greater impact than changes earlier in gestation. In contrast, 3 month old infants who had been of low birth weight demonstrated no change in endothelium-dependent relaxation to acetylcholine in cutaneous vessels, while endothelium-independent relaxation was impaired (Goh *et al.*, 2001). Cutaneous vascular endothelial responses were impaired in 9 year old children who were of low birth weight (Martin *et al.*, 2000b), with no change in endothelium-independent relaxation to a nitric oxide donor. These data suggest that vasodilator pathways are perturbed in infants and children who were of low birth weight, and that changes in vasodilator responses precede the influence of adult lifestyle factors on vascular function.

Vascular function has also been examined in young adults who were of low birth weight. Flow-mediated endothelium-dependent vasodilation of the brachial artery was impaired in 19-20 year old low birth weight individuals relative to appropriate for gestational age

individuals, while relaxation to a nitric oxide donor was similar (Goodfellow *et al.*, 1998). These findings were confirmed in a larger cohort of adults aged 20-28 years, where brachial artery endothelium-dependent, but not -independent relaxation was impaired in individuals of low birth weight (Leeson *et al.*, 2001). When the influence of other cardiovascular risk factors was considered, it was found that the effects of birth weight were most significant in individuals who were otherwise at low cardiovascular risk (Leeson *et al.*, 2001). In contrast, endothelial function did not differ in the forearm vascular bed in adults aged 28 years, despite increased plasma von Willebrand factor levels (McAllister *et al.*, 1999), which is considered to be a strong marker of endothelial dysfunction, (Lip & Blann, 1997) and a risk factor for coronary heart disease (Whincup *et al.*, 2002). Combined analysis of male and female vascular responses in this study may have limited the statistical power, as the endothelial function of men versus women differs substantially (Celermajer *et al.*, 1994a; Celermajer *et al.*, 1994b; Sarabi *et al.*, 1999), whereas the small subject numbers may not have supported gender-specific analysis. Both human (Murray *et al.*, 2001) and animal studies (Ozaki *et al.*, 2001; Franco *et al.*, 2002a) indicate significant gender-dependency of the effects of perturbations of the *in utero* environment on the adult vasculature.

1.2.4.2 Arterial Compliance

Studies focused on the effects of low birth weight on compliance in conduit arteries have yielded conflicting results. While compliance was reported to be reduced in low birth weight children (Martin *et al.*, 2000b) and young adults (Murray *et al.*, 2001; te Velde *et al.*, 2004) other studies reported no significant effect (Kumaran *et al.*, 2000; Montgomery *et al.*, 2000) Recently, although neither arterial stiffness or endothelial function differed in a

cohort of teenagers that were classified as IUGR at birth, the end-diastolic diameters of both the abdominal aorta and popliteal artery were significantly smaller (Brodszki *et al.*, 2005). The effects of impaired prenatal growth on conduit artery structural development and compliance therefore currently require further clarification.

1.2.4.3 Function of the sympathetic nervous system

The sympathetic nervous system contributes substantially to the regulation of peripheral vascular tone, and there is some evidence to suggest that sympathetic tone may be increased in individuals of low birth weight. It has previously been reported that pulse rate was elevated in individuals of low birth weight (Phillips & Barker, 1997). In a cohort of monozygotic and dizygotic twins who were studied as teenagers, lower birth weight for gestational age was also associated with higher sympathetic activity, as indicated by a short cardiac pre-injection period at rest and increased sympathetic reactivity to mental stress, without changes in parasympathetic activity (IJzerman *et al.*, 2003). When the effects of birth weight were compared within pairs however, the effect was observed only in dizygotic twins. The authors suggested that this implied that genetic factors mediated the association of low birth weight, increased sympathetic activity, and increased blood pressure (IJzerman *et al.*, 2003). However, it should be noted that while the mean birth weight of monozygotic and dizygotic twins were similar, mean within-pair differences in birth weight were not reported. It is therefore unclear that differences in birth weight discordance in monozygotic versus dizygotic twins in this study did not contribute to the lack of effect in monozygotic twins when analysed using within-pair statistics.

There is therefore a body of evidence demonstrating that fetal growth influences vascular endothelial function during neonatal, childhood and early adult life, and may also decrease arterial wall compliance or lumen diameter in conduit arteries and enhance sympathetic activity. The mechanisms underlying changes in vascular function in individuals born small for gestational age may be related to the specific nature and timing of deficits in fetal substrate supply. Consideration must therefore be given to the major clinical etiologies of impaired fetal growth, and the ensuing fetal cardiovascular adaptations.

1.3 THE REGULATION OF FETAL GROWTH

1.3.1 Fetal growth and intrauterine growth restriction

Fetal growth is determined both by the genetic potential for growth, and constraint of this growth imposed by factors affecting substrate delivery to the fetus, including fetal number (Imaizumi, 2001; Garite *et al.*, 2004b), the size and functional capacity of the placenta (Molteni *et al.*, 1978; Woods *et al.*, 1980), maternal size and parity (Voorhorst *et al.*, 1993; Zhang & Bowes, 1995), maternal nutrition (Mitchell *et al.*, 2004), and the reduced inspired oxygen/impaired uteroplacental blood flow which occurs during pregnancy at high-altitude (McCullough & Reeves, 1977; Moore *et al.*, 1982b). Fetal growth may be further constrained by maternal health, (Hameed *et al.*, 2001), environmental exposures (Levario-Carrillo *et al.*, 2004) and in humans particularly by behavior during pregnancy, including smoking, alcohol consumption or substance abuse (Lundsberg *et al.*, 1997; Bateman & Chiriboga, 2000; England *et al.*, 2001). Clinically, a fetus or infant is considered to have experienced IUGR when the body weight for gestational age falls within the 10^{th} percentile of the reference population (Battaglia & Lubchenco, 1967). This definition does not differentiate between infants who are constitutionally small, and those small due to

pathological constraint of prenatal growth (Gardosi *et al.*, 1992). However, chronic reduction in fetal substrate supply frequently results in asymmetric fetal growth, and comparison of growth indices including length, head and abdominal circumference may provide a more informative measure of prenatal development (Galan *et al.*, 2002). The incidence of IUGR varies among populations, occurring in 4-7% of pregnancies in developed countries and affecting a substantially larger proportion of infants born in developing countries (Villar *et al.*, 1986; de Onis *et al.*, 1998). The clinical causes of IUGR are diverse, and the predominating etiology also varies significantly among populations. Several major causes of IUGR will be considered below.

1.3.2 Maternal malnutrition

Birth weight was reduced by severe maternal undernutrition during late gestation in women who were exposed to a defined period of famine during the Dutch Hunger Winter (Stein & Susser, 1975a, b; Stein *et al.*, 2004). In developing countries, the chronic undernutrition of women throughout life, including during pregnancy, leads to reduced offspring birth weight both by reducing maternal size and pre-pregnancy weight, and limiting fetal nutrient supply (Barker, 2001). The total nutritional intake and the composition of maternal nutrition during pregnancy may each be important determinants of fetal growth. Across the normal birth weight range, the proportion of energy derived from protein during early pregnancy (as assessed by food-frequency questionnaire) was positively associated with birth weight in a cohort of South Australian women (Moore *et al.*, 2004b). It has also been demonstrated that balanced protein-energy supplementation for the last 20 weeks of pregnancy improved fetal growth, and reduced the risk for the birth of a small for gestational age infant in rural Gambia (Ceesay *et al.*, 1997). A positive effect of protein supplementation on infant birth weight has now been described in several developing countries (Merialdi *et al.*, 2003).

Interestingly, however, a high-protein supplement given to pregnant American women who were unlikely to have a limited protein intake before supplementation was associated with a small, but significant increase in the incidence of IUGR (Rush *et al.*, 1980). It is also of note that adult systolic blood pressure was positively related to red meat consumption during late pregnancy in the offspring of Scottish women advised to eat a low carbohydrate/high protein diet while pregnant (Shiell *et al.*, 2001). Adult systolic blood pressure was also inversely related to birth weight, while lower birth weight was associated with lower carbohydrate intake during pregnancy. However, in this study there was little impact of controlling for birth weight on the association between dietary composition during late pregnancy and adult offspring blood pressure, which may suggest a direct effect of maternal dietary composition on cardiovascular function in adult offspring.

Although an extensive range of macro- and micronutrient supplement interventions have been trialed during pregnancy, the majority of supplementation studies have demonstrated little effect on the incidence of low birth weight or IUGR (Merialdi *et al.*, 2003). Similarly, several studies have reported no substantial impact of maternal eating disorders on birth weight within Western populations (Conti *et al.*, 1998; Franko *et al.*, 2001). It therefore seems likely that maternal nutrition is a major limiting factor for total fetal growth, and hence a major etiology of IUGR, only within specific populations. It must be noted, however, that low weight gain during pregnancy remains a significant risk factor for IUGR (Ramakrishnan, 2004). Experimentally, undernutrition protocols in pregnant rats have been widely used to investigate the cardiovascular effects of IUGR. Considerable differences in the timing of organogenesis in rat versus human pregnancies may contribute to the significant effects of undernutrition on birth weight in this species. Interestingly, experimental studies also suggest that undernutrition in pregnant rats may impair

uteroplacental vascular function, which could further limit fetal oxygen and nutrient supply (Itoh *et al.*, 2002; Veerareddy *et al.*, 2004). There is substantial evidence that both maternal macro and micronutrient status throughout pregnancy impacts fetal development, and may increase the risk of cardiovascular disease in adult life while exerting only a subtle influence on infant birth weight (Barker, 2001; Shiell *et al.*, 2001).

1.3.3 Maternal smoking

The number of women who smoke before and during pregnancy remains high in many populations, and smoking represents a common cause of IUGR. In a large cohort of American women, 18% of white and 14% of black women self-reported smoking during pregnancy between 1990-1994 (Ananth *et al.*, 2005). The reported prevalence of smoking during pregnancy in both white and black women was lower in 1995-1999, at 14.2% and 10% respectively (Ananth *et al.*, 2005). While this study does suggest that the prevalence of smoking during pregnancy in the US is decreasing, the number of infants exposed to components of cigarette smoke before birth remains high, and is also likely to be under-reported. In the UK in 1999, analysis of saliva concentrations of the nicotine metabolite cotinine indicated that the prevalence of smoking during pregnancy was 30% within a small representative sample of pregnant women in England (Owen & McNeill, 2001). From 1991-1998 the prevalence of smoking during pregnancy in Denmark was similar, at 29.6% (Mortensen *et al.*, 2001), while the self-reported prevalence of smoking in a representative cohort of pregnant South Australian women between 1998-2000 was 19.1% (Moore *et al.*, 2004b).

Maternal smoking exposes the fetus to a range of toxic substances and carries numerous reproductive risks. Among these, smoking during pregnancy has been clearly associated

with decreased birth weight in term infants (England *et al.*, 2001; Steward & Moser, 2004; Gomez *et al.*, 2005). Fetal growth may be limited by maternal smoking through several mechanisms. Carbon monoxide (CO) inspired in cigarette smoke binds tightly to both maternal and fetal hemoglobin, reducing oxygen carrying capacity in both circulations and thus oxygen delivery to the fetus. The level of maternal expired CO at delivery, used as a measure of maternal smoking during pregnancy, was associated with a dose-dependent decrease in the birth weight of term infants born to a cohort of French women (Gomez *et al.*, 2005). Smoking also impairs vascular function in both the maternal-placental and fetal-placental circulation, and may therefore limit placental perfusion from both the fetal and maternal interface. The expression and activity of endothelial nitric oxide synthase (eNOS), which produces the vasodilator nitric oxide, was significantly reduced in umbilical vein endothelial cells collected from the umbilical cord of infants whose mothers smoked (Andersen *et al.*, 2004). Stereologic examination of placentas from women who smoked indicated that the length, volume and total surface area of fetal placental capillaries were reduced by smoking (Larsen *et al.*, 2002). Smoking during pregnancy therefore remains a significant modifiable risk factor for IUGR.

1.3.4 Pregnancy at high-altitude

Approximately 140 million people across the world live at an altitude greater than 2500m above sea level and are therefore exposed to chronic hypobaric hypoxia throughout life (Moore *et al.*, 2004a). A series of investigations across regions of high and low-altitude in the US (McCullough & Reeves, 1977; McCullough *et al.*, 1977; Yip, 1987; Unger *et al.*, 1988; Jensen & Moore, 1997), India (Wiley, 1994), Peru (Mortola *et al.*, 2000), Bolivia (Giussani *et al.*, 2001; Keyes *et al.*, 2003), and in the Tibet Autonomous Region of China (Moore *et al.*, 2001) have demonstrated that birth weight is reduced and infant mortality

increased in populations at high-altitude. Birth weights in preterm deliveries have indicated that lower birth weight at high-altitude primarily results from slower growth during late gestation (Unger *et al.*, 1988). Interestingly, when the effects of altitude on birth weight were compared in people of differing ancestry, the altitude-related decrease in birth weight was smallest in Tibetan, slightly increased in Andean, intermediate in European and greatest in Chinese populations (Moore *et al.*, 2004a). As birth weight was relatively protected in Tibetan and Andean populations, who have who have lived at high-altitude for longest, these data imply that genetic adaptation to pregnancy at high-altitude (Moore *et al.*, 2004a) optimizes fetal growth.

The contribution of poor maternal nutrition resulting from the relatively low socioeconomic status of populations at high-altitude to the effect of altitude on birth weight was assessed by Giussani et al. in 2001, who compared birth weights in high- and low-income populations residing at high (La Paz) and low (Santa Cruz) altitudes within Bolivia (Giussani *et al.*, 2001). Birth weight was lower at high- than at low-altitude. However, within the high-altitude population, birth weight was lower in high-income families than in low-income families, whereas at low-altitude this effect was reversed (Giussani *et al.*, 2001). These data imply that hypoxia, rather than impaired nutrition decreases birth weight at high-altitude. As the low-income families at high-altitude were predominantly of Amerindian heritage, while the high-income/high-altitude group were predominantly of European ancestry, these data may also further support the hypothesis that genetic adaptation to pregnancy at high-altitude reduces the effect of hypoxia throughout gestation on birth weight (Giussani *et al.*, 2001).

Birth weight was directly related to maternal arterial oxygen concentration during the third trimester in a population of pregnant American women residing at 3100m, implying that reduced fetal growth at high-altitude reflects decreased fetal oxygen supply (Moore *et al.*, 1982b). However, a study of maternal arterial blood gas status throughout pregnancy in women living at low- and high-altitude in Peru demonstrated that although both PO_2 and O_2 saturation were lower at high- than low-altitude, arterial oxygen content was maintained by increased hematocrit (McAuliffe *et al.*, 2001). In women from families that had lived at high-altitude for less than 3 generations, hemoglobin, oxygen saturation and oxygen content declined during late gestation while remaining constant in women of longer high-altitude ancestry (McAuliffe *et al.*, 2001). It is therefore apparent that for a large proportion of women living at high-altitude, decreased maternal arterial oxygen content alone does not account for reduced birth weight.

Fetal oxygen supply is dependent on both maternal arterial oxygen content and utero-placental blood flow. When pelvic blood flow changes during pregnancy were assessed in American women living at low- and high-altitude, both volumetric uterine artery blood flow and the proportion of iliac artery flow shunted towards the uterine artery were lower in women living at high-altitude (Zamudio *et al.*, 1995). The circulating blood volume of non-pregnant women living at high-altitude was also lower than women living at moderate-altitude, such that although a similar volume increase occurred during pregnancy, total blood volume remained lower in pregnant women at high-altitude (Zamudio *et al.*, 1993). Living at high-altitude increases the risk of developing preeclampsia (Moore *et al.*, 1982a; Zamudio *et al.*, 1993; Jensen & Moore, 1997; Palmer *et al.*, 1999; Keyes *et al.*, 2003), and even among normotensive pregnant women at high-altitude the normal decline in arterial blood pressure during pregnancy was impaired (Palmer *et al.*, 1999). Therefore, in addition

to reductions in total maternal arterial O_2 content in some instances, fetal substrate supply may be further reduced by decreases in utero-placental blood flow secondary to impaired maternal cardiovascular adaptation to pregnancy in many women living at high-altitude.

1.3.5 Uteroplacental insufficiency

It has been suggested that in developed countries, uteroplacental insufficiency accounts for the majority of clinical IUGR cases (Henriksen & Clausen, 2002). Uteroplacental insufficiency, arising from impaired development or function of the placenta, or impaired utero-placental or feto-placental blood flow may limit the supply of both oxygen and nutrients to the fetus. A series of studies which analysed umbilical vein and artery blood samples in a cohort of appropriate and small for gestational age human fetuses in London during the 80's indicated that small for gestational age fetuses were hypoglycemic (Economides & Nicolaides, 1989), hypoxemic, hypercapnic, hyperlacticemic and acidotic during mid-late gestation (Nicolaides *et al.*, 1989). In IUGR pregnancies, maternal venous plasma concentrations of essential amino acids were higher than in healthy pregnancies, while fetal plasma levels were lower, and correlated with the degree of fetal hypoxia (Economides *et al.*, 1989), implying that amino acid supply to the fetus was limited by placental function as opposed to maternal nutrition. These data therefore support the suggestion that uteroplacental insufficiency represents the major clinical etiology of IUGR within developed populations.

Several authors have identified significant increases in the incidence of abnormalities that may impair function in the placentas of IUGR infants, in particular changes in placental vascularisation (Giles *et al.*, 1985; Lee & Yeh, 1986; Krebs *et al.*, 1996; Salafia *et al.*, 1997) and in the structure of placental terminal villi (Macara *et al.*, 1996). There is also

evidence that the rate of apoptosis within IUGR placentas may be increased (Smith *et al.*, 1997). The activity of an amino acid transporter (system A) in isolated syncytiotrophoblast membrane vesicles was lower in samples obtained from the placentas of small for gestational infants than from appropriately grown infants (Mahendran *et al.*, 1993). By limiting the transfer of factors between maternal and fetal circulations, these abnormalities clearly may reduce fetal growth; however the underlying cause of such placental insufficiency is generally idiopathic. Gestational hypertension also impairs transfer of oxygen and nutrients to the developing fetus through decreased placental perfusion, and gross placental damage, and represents a significant clinical etiology of IUGR (Xiong *et al.*, 1999; Lackman *et al.*, 2001; Buchbinder *et al.*, 2002).

It is clear that when IUGR results from maternal smoking, residence at high altitude or placental insufficiency, which accounts for a large proportion of IUGR cases within developed countries, the supply of both oxygen and nutrients to the fetus is limited. The studies described within this thesis therefore focused on understanding the short- and long-term impact of decreased fetal oxygen in addition to nutrient supply on vascular function.

1.3.6 Prenatal diagnosis of intrauterine growth restriction

A fetus is considered to be small for gestational age when the estimated fetal weight for gestation, based on menstrual dating and fetal ultrasound biometric measures, falls more than two standard deviations below the population mean (Ergaz *et al.*, 2005). The cohort of fetuses identified as small for gestational age includes those that may appear small due to inaccurate gestational age determination, or be small as a result of constitutional factors in addition to fetuses that are IUGR due to the pathological limitation of fetal growth. The

biometric and hemodynamic characteristics of IUGR fetuses therefore provide useful clinical measures to assess whether fetal growth has been pathologically limited *in utero*.

1.3.6.1 Biometric measures in intrauterine growth restricted infants

While the primary factor considered in the diagnosis of IUGR is the estimated fetal weight, numerous studies have indicated that when fetal substrate supply is limited the growth of some organs, most notably the brain, is preferentially maintained while the growth of the liver in particular is disproportionately reduced (Campbell & Thoms, 1977; Murao *et al.*, 1990; Baker *et al.*, 1995; McMillen *et al.*, 2001). The ratio of head to abdominal circumference can be determined by ultrasound and has been used as an indicator of growth asymmetry in human fetuses (Crane & Kopta, 1979). Fetal thinness can also be assessed using ponderal index, which is calculated as weight/length^3 and has also been widely used clinically as an indicator of IUGR.

1.3.6.2 Doppler ultrasound assessment of hemodynamics in the growth restricted fetus

Doppler ultrasonography has become an important tool in the prenatal diagnosis of IUGR. By assessing the nature of blood flow through key fetal arteries and veins, regional vascular resistance can be inferred which provides a measure of the efficiency of placental perfusion and the distribution of fetal cardiac output. The Doppler signal is most often qualitatively analysed to determine the pattern of blood flow within the blood vessel investigated. An increase in the pulsatility index, the absence or the reversal of blood flow during diastole in the umbilical artery indicates increased placental vascular resistance characteristic of placental insufficiency, and is strongly associated with IUGR (Karsdorp *et al.*, 1994;

Arbeille, 1997). The mechanisms of brain sparing in IUGR fetuses involve a significant reduction in cerebral vascular resistance, which is reflected in decreased pulsatility of the middle cerebral or carotid artery (Wladimiroff *et al.*, 1987). A change in the ratio of cerebral: umbilical or cerebral: aortic pulsatility indices that indicate decreased cerebral vascular resistance concomitant with increased placental vascular resistance are considered to be reliable indicators of IUGR (Arbeille, 1997). The ductus venosus is a shunt which allows a proportion of well-oxygenated umbilical venous blood returning from the placenta to bypass the liver circulation, and instead flow directly into the inferior vena cava to return to the heart. Doppler ultrasonography has demonstrated that the proportion of blood flow shunted through the ductus venosus was significantly increased, while blood flow through the intrahepatic vein was decreased in a cohort of IUGR fetuses when compared to control fetuses (Bellotti *et al.*, 2004).

1.3.7 Intrauterine growth restriction - perinatal morbidity and mortality

In both developing and developed countries perinatal morbidity and mortality is higher in IUGR infants than in those appropriately grown at birth (McIntire *et al.*, 1999). IUGR infants represent a greater proportion of preterm births than term births (Lackman *et al.*, 2001), related in part to induced delivery in cases where it is considered that the risks of intrauterine death outweigh the risks of neonatal death (Yu & Upadhyay, 2004). While there is little debate that perinatal mortality is higher in IUGR infants (Kramer *et al.*, 1990; Lackman *et al.*, 2001; Zaw *et al.*, 2003), a continuous inverse relation between neonatal death and birth weight percentile for gestational age has been described in several studies (Kramer *et al.*, 1990; McIntire *et al.*, 1999).

IUGR infants are at greater risk for a range of serious perinatal morbidities, including hypoxic-ischemic encephalopathy (McIntire *et al.*, 1999; Bukowski *et al.*, 2003; Gilbert & Danielsen, 2003), intravetricular hemorrhage (Gilbert & Danielsen, 2003), necrotizing enterocolitis (Gilbert & Danielsen, 2003; Garite *et al.*, 2004a), and respiratory distress syndrome (McIntire *et al.*, 1999; Gilbert & Danielsen, 2003; Zaw *et al.*, 2003). Premature IUGR infants may also be at greater risk of retinopathy of prematurity (Zaw *et al.*, 2003; Garite *et al.*, 2004a). IUGR infants are also more likely to be hypothermic, hypoglycaemic, hypocalcaemic and polycythaemic (Kramer *et al.*, 1990), and beyond delivery at 29 weeks gestation are associated with a significantly longer length of hospital stay and higher cost of care than appropriate for gestational age infants (Gilbert & Danielsen, 2003).

Changes in regional vascular resistance are therefore a consistent clinical characteristic of IUGR fetuses, which highlights the importance of understanding both the short-term and long-term effects of reduced fetal substrate supply on regional vascular function. Such changes may be of relevance to both the short and long-term adverse consequences of IUGR. To further understand the impact of IUGR on vascular function, consideration must be given to the key pathways that regulate vascular tone and may also be implicated in cardiovascular disease.

1.4 OVERVIEW OF VASCULAR FUNCTION

1.4.1 Endothelial function and vascular homeostasis

The lumen of each blood vessel throughout the body is lined by endothelial cells, which contribute substantially to the regulation of local vascular tone. The importance of endothelial function in modulating vascular responses was not recognized, however, until the relatively recent observations of Furchgott and Zawadzki, who first reported that

vasodilation to acetylcholine in isolated artery preparations from rabbits was dependent on the presence of an intact endothelial layer (Furchgott & Zawadzki, 1980). Since this time, a plethora of investigations have demonstrated that endothelial production of vasodilator substances in response to numerous endocrine/paracrine, metabolic and mechanical stimuli mediate vasodilation. Figure 1 summarizes the key pathways involved in the release of endothelium-derived vasodilator factors.

The first endothelium-derived vasodilator identified was nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Palmer *et al.*, 1988; Palmer & Moncada, 1989). Inhibition of NO synthesis in humans resulted in an immediate decrease in forearm blood flow (Vallance *et al.*, 1989; Baan *et al.*, 1997) and increase in blood pressure (Haynes *et al.*, 1993), highlighting the contribution of NO to hemodynamic regulation *in vivo*. NO is produced via the actions of 3 enzyme isoforms, endothelial NO synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Each NOS isoform produces NO through the conversion of L-arginine and oxygen to L-citrulline and NO. Expression of all 3 isoforms has been observed throughout the cardiovascular system (Papapetropoulos *et al.*, 1999), however in endothelial cells eNOS expression predominates, while high iNOS expression may be induced under certain circumstances. NO derived from nNOS within perivascular nerves may also contribute to the regulation of cerebral artery tone (Gonzalez *et al.*, 1997).

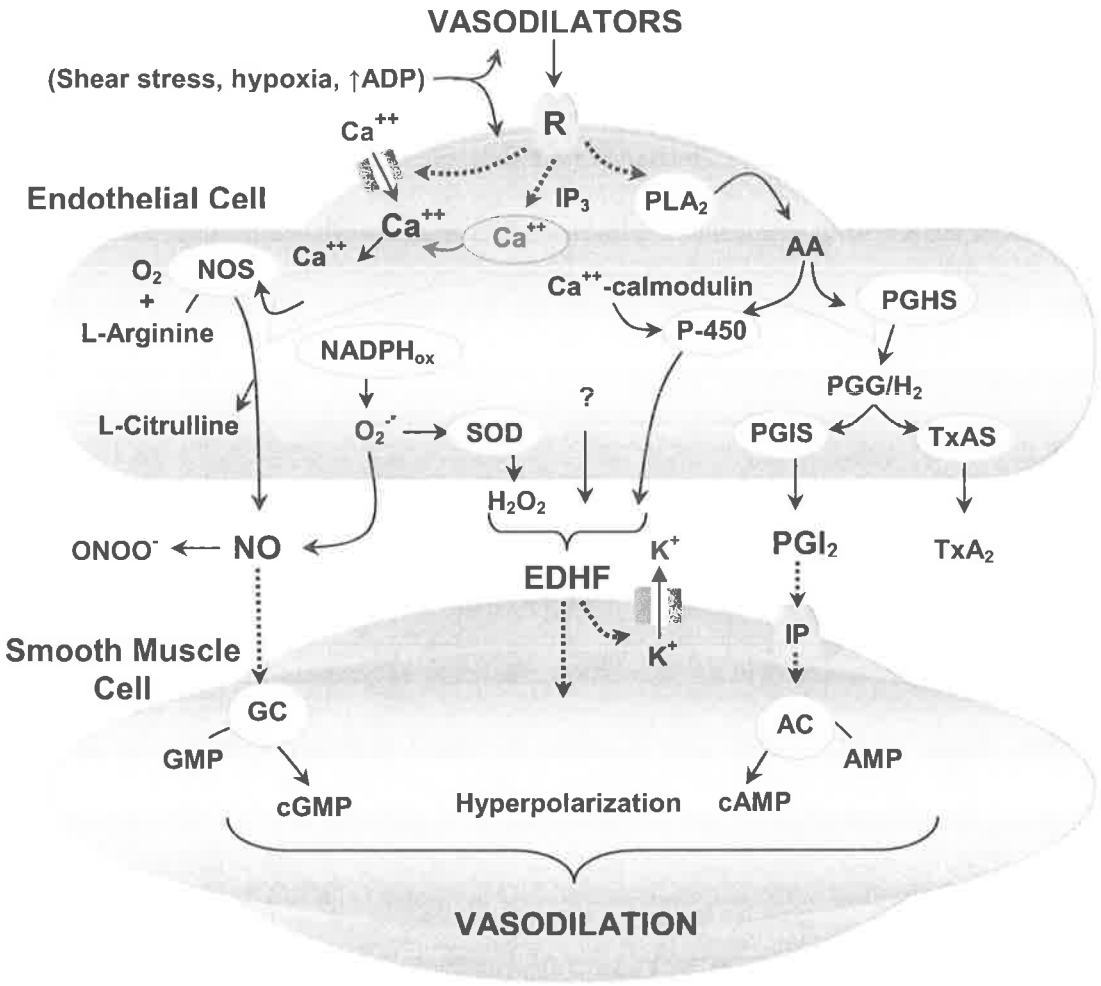


Figure 1.1: Endothelium-dependent vasodilation

Activation of endothelial cell receptors results in an influx of extracellular Ca^{++} combined with inositol trisphosphate (IP_3)-mediated release of Ca^{++} from the sarcoplasmic reticulum. The elevated intracellular Ca^{++} level activates calmodulin, which then activates nitric oxide synthase (NOS) to form the vasodilator nitric oxide (NO) through the conversion of L-arginine to L-citrulline. The diffusion of NO into vascular smooth muscle cells activates guanylate cyclase (GC) to form cyclic GMP (cGMP). In the presence of superoxide anion (O_2^{-*}), which can be produced by numerous processes within endothelial cells including NADPH oxidase ($NADPH_{ox}$) activity, NO is rapidly scavenged to form peroxynitrite ($ONOO^-$). Under physiological conditions O_2^{-*} is scavenged by superoxide dismutase (SOD), however, leading to the formation of H_2O_2 which may contribute to EDHF-mediated vasodilation. Endothelial G-protein coupled receptor activation promotes release of arachidonic acid from the plasma membrane by phospholipase A_2 (PLA_2), which is metabolized by prostaglandin G/H synthase to form the intermediate prostaglandin product PGG/H_2. This intermediate is further converted to form the vasodilator prostacyclin (PGI_2) or vasoconstrictor thromboxane (TxA_2) via the actions of specific isomerases (PGIS or TxAS). PGI_2 binds to IP receptors on vascular smooth muscle cells, activating adenylate cyclase (AC) to produce cyclic AMP (cAMP) and promote vasodilation. Endothelium-derived hyperpolarizing factor (EDHF) may include components from several different pathways; however one established component is arachidonic acid metabolites produced via the actions of cytochrome P-450 (P-450). Ultimately, EDHF results in K+ channel activation and smooth muscle cell hyperpolarization (modified from (Vanhoutte, 2004)).

In endothelial cells, sequential myristoylation and palmitoylation events facilitate the trafficking of eNOS to caveolae where the enzyme is predominantly located (Garcia-Cardena *et al.*, 1996b; Shaul *et al.*, 1996). Within caveolae eNOS activity is negatively regulated by interaction with caveolin-1 (Michel *et al.*, 1997). The binding of Ca^{2+}-calmodulin following an agonist-induced increase in intracellular [Ca^{2+}] facilitates dissociation of eNOS and caveolin-1 (Michel *et al.*, 1997), while agonist-induced de-palmityolation also promotes the translocation of eNOS to the cytosol (Robinson *et al.*, 1995). There is also evidence that binding of heat shock protein-90 promotes eNOS activity (Garcia-Cardena *et al.*, 1998). Both tyrosine (Garcia-Cardena *et al.*, 1996a) and serine (Fulton *et al.*, 1999; Gallis *et al.*, 1999; Michell *et al.*, 1999) phosphorylation events modify eNOS sensitivity to Ca^{2+}-calmodulin to further regulate eNOS activity (Papapetropoulos *et al.*, 1999; Shaul, 2002). The availability of the obligatory co-factors tetrahydrobiopterin, FAD, FMN and NADPH further determines eNOS activity (Papapetropoulos *et al.*, 1999; Shaul, 2002). In the absence of tetrahydrobiopterin eNOS uncouples, resulting in the production of superoxide anion rather than NO (Wever *et al.*, 1997; Vasquez-Vivar *et al.*, 1998). Although only eNOS is regulated by interaction with caveolin-1 (Michel *et al.*, 1997), activity of both nNOS and eNOS is Ca^{2+}-calmodulin dependent, and therefore regulated by intracellular [Ca^{2+}]. iNOS activity is relatively Ca^{2+}-independent, however, as calmodulin is able to bind this isoform without prior activation by Ca^{2+} (Andrew & Mayer, 1999). Significant endothelial expression of iNOS occurs in response to cytokines or other inflammatory factors, and results in rapid increase in local NO production (Andrew & Mayer, 1999). Endothelial cell NOS activity is therefore highly subject to post-translational modulation of enzyme activity through several positive and negative-regulatory pathways.

One of the early observations relating to endothelial function was that when cultured, endothelial cells release both vasodilator prostacyclin and vasoconstrictor thromboxane prostaglandins (Ingerman-Wojenski *et al.*, 1981). The rate-limiting step in prostaglandin synthesis is the formation of PGG/H_2, which is synthesized from arachidonic acid by two enzyme isoforms, prostaglandin G/H synthase (PGHS)-1 and PGHS-2. Expression of PGHS-1 is constitutive, while PGHS-2 expression is inducible in response to several factors, including reactive oxygen species (Cosentino *et al.*, 2003) and cytokines (Mark *et al.*, 2001). The intermediate prostaglandin PGH_2 may exert a direct vasoconstrictor influence on the vascular smooth muscle via the thromboxane receptor (Davidge, 2001), however PGH_2 is normally converted to prostacyclin or thromboxane via the activity of specific isomerases. The balance of vasodilator to vasoconstrictor prostaglandin production therefore determines the overall endothelium-derived prostanoid influence on smooth muscle tone. Inhibition of prostaglandin production *in vivo* reduced both basal (Duffy *et al.*, 1998) and stimulated (Duffy *et al.*, 1999; Schrage *et al.*, 2004) forearm blood flow in healthy young adults, although to a lesser extent than NOS inhibition (Duffy *et al.*, 1999; Schrage *et al.*, 2004). It should be noted, however, that in some studies PGHS inhibition did not modify forearm blood flow (Baan *et al.*, 1997; Schrage *et al.*, 2005). While the contribution of prostaglandins to vascular smooth muscle tone may be small under resting conditions in healthy young adults, a switch from predominantly vasodilator to vasoconstrictor prostaglandin production occurs during aging (Stewart *et al.*, 2000). Changes in the prostaglandin pathway may therefore contribute to the age-related decline in endothelial function.

It was also recognized that a third factor(s) mediated endothelium-dependent responses by producing hyperpolarization of smooth muscle cells (Chen *et al.*, 1988; Feletou &

Vanhoutte, 1988). Endothelial-derived hyperpolarizing factor (EDHF) comprises several pathways, including cytochrome P-450 metabolites of arachidonic acid (Hecker *et al.*, 1994), hydrogen peroxide (Matoba & Shimokawa, 2003), and transfer across endothelium-smooth muscle gap junctions (Edwards *et al.*, 1999a). EDHF effects hyperpolarization by modulating smooth muscle potassium channel activity (Corriu *et al.*, 1996; Zygmunt & Hogestatt, 1996).

1.4.2 Endothelial function in cardiovascular disease

Endothelial function, in particular the production of NO, plays a key role in both short- and long-term modulation of vascular function. The reported impairment in endothelial function in patients with hypertension (Linder *et al.*, 1990; Panza *et al.*, 1990) may therefore also contribute to promoting smooth muscle proliferation, hypertrophy and vascular remodeling in hypertension. Vascular smooth muscle cell proliferation is opposed *in vitro* by NO (Garg & Hassid, 1989), and chronic L-arginine supplementation to spontaneously hypertensive rats (SHR) resulted in partial reversal of the VSMC hypertrophy which is characteristic of cultured smooth muscle cells from adult SHR rats (Somoza *et al.*, 2004). In large arteries, evidence suggests that local NO production also increases arterial distensibility (Kinlay *et al.*, 2001; Wilkinson *et al.*, 2002). Production of cGMP following NO diffusion into vascular smooth muscle regulates the expression of an array of genes (Pilz & Casteel, 2003). Cross-talk between NO and the endothelin-1 (Vanhoutte, 2000) and angiotensin II (de Gasparo, 2002) pathways also provides tonic regulation of their vasoconstrictor and hypertrophic effects on smooth muscle function.

Even before the identity of endothelium-derived relaxing factor was known, it was inferred that superoxide anion inactivated factors released from the endothelium, as superfusion of

isolated artery preparations with superoxide dismutase potentiated the effects of acetylcholine (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986). Evidence has now also demonstrated that the scavenging of NO by superoxide anion to form the highly reactive nitrogen radical peroxynitrite ($ONOO^{-*}$) contributes substantially to endothelial dysfunction in cardiovascular disease states (Cai & Harrison, 2000). Superoxide anion is produced via several pathways within endothelial cells, including the enzymatic activity of xanthine oxidase, NADPH oxidase, and uncoupled eNOS and leakage from mitochondria (Li & Shah, 2004). When produced in low, physiological quantities, superoxide anion is converted to H_2O_2 by superoxide dismutase. The cross-reaction between superoxide anion and NO occurs at a substantially faster rate, however, leading to peroxynitrite formation when superoxide and NO formation are excessive. By reducing the bio-availability of NO, excessive production of superoxide anion impairs endothelium-dependent relaxation. Peroxynitrite formation also has direct, cytotoxic effects, resulting in protein nitrosylation (Ashraf *et al.*, 2002; Mishra *et al.*, 2002; Zanelli *et al.*, 2002) and lipid peroxidation (Numagami *et al.*, 1997). Interestingly, increased production of peroxynitrite has also been demonstrated to reduce the expression of prostacyclin synthase within vascular cells, which may ultimately favour production of vasoconstrictor prostaglandins (Zou *et al.*, 1997; Cooke & Davidge, 2002).

1.4.3 Vascular smooth muscle function

The tone of vascular smooth muscle within resistance arteries determines the diameter, and hence resistance to flow that is provided by blood vessels throughout the body. Regulation of vascular smooth muscle tone is mediated by an array of endothelial-derived paracrine and circulating endocrine factors in addition to innervation by sympathetic nerve terminals (in most vascular beds unopposed by parasympathetic nerve terminals). Vascular tone is

also modulated in response to direct metabolic and mechanical stimuli. The key features of pharmacomechanical coupling in vascular smooth muscle are reviewed in figure 2. Vascular smooth muscle tone can also be modified by changes in membrane potential (electromechanical coupling) elicited by changes in the activity of ion channels, which respond to metabolic or mechanical stimuli, including intracellular [ATP]/[ADP], [H^+] and stretch.

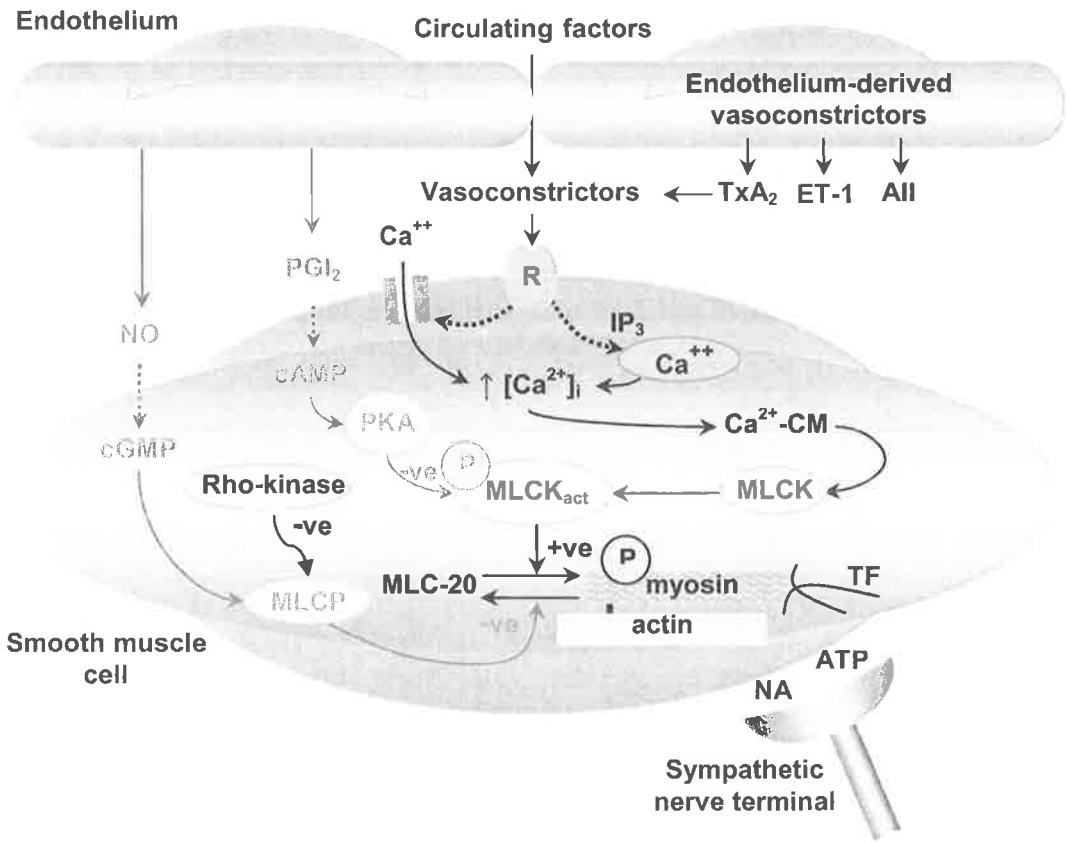


Figure 1.2: Pharmacomechanical coupling in vascular smooth muscle

Vascular smooth muscle constriction occurs in response to numerous circulating, endothelium-derived (including thromboxane, (TxA_2) endothelin-1 (ET-1) and angiotensin II (AII)) and sympathetic nerve terminal-derived vasoconstrictor factors (noradrenaline (NA) and ATP). Smooth muscle cell receptor activation results in an elevation of intracellular Ca^{2+} levels that is mediated by the opening of plasma membrane Ca^{2+} channels, and release of Ca^{2+} from the sarcoplasmic reticulum via both Ca^{2+}-induced-Ca^{2+}-release and binding of inositol trisphosphate (IP_3) produced via the phospholipase C/phosphatidyl-inositol bisphosphate signaling pathway to sarcoplasmic reticulum IP_3 receptors. Elevated intracellular Ca^{2+} binds to calmodulin (CM), producing a conformational change which allows it to activate myosin light chain kinase (MLCK). Activated MLCK ($MLCK_{act}$) phosphorylates the regulatory myosin light chain-20 (MLC-20), which facilitates the interaction of actin and the myosin hexamer (composed of 2 myosin heavy chains, 2 MLC-20 chains and 2 MLC-17 chains) by enabling activation of the myosin Mg^{2+}-ATPase by actin. Cross-bridge cycling between actin, and myosin, which is anchored at the tail end by thick filaments (TF), produces contraction of the smooth muscle cell. Production of cyclic AMP (cAMP) inhibits smooth muscle constriction by phosphorylating $MLCK_{act}$, which reduces the affinity of $MLCK_{act}$ for Ca^{2+}-CM. Production of cGMP leads to decreases in intracellular Ca^{2+} concentrations and the activation of myosin light chain phosphatase (MLCP), which dephosphorylates myosin. The activity of MLCP is also regulated by phosphorylation through the Rho A-GTP-activated Rho-kinase pathway which can be activated by G-protein activity in response to ligand binding to G-protein-coupled-receptors. Phosphorylation of MLCP by Rho-kinase reduces the enzyme's affinity for myosin light chain and, by decreasing the activity of this regulatory enzyme, results in Ca^{2+} sensitization of the smooth muscle contractile apparatus (modified from (Ogut & Brozovich, 2003)).

1.4.4 Vascular smooth muscle in cardiovascular disease

Hypertension is associated with changes in resistance artery structure, and vascular smooth muscle function which differ according to the size and function of the artery, and etiology of hypertension. Resistance artery lumen diameter may be reduced, and media/lumen ratio increased as a consequence of either eutrophic (Korsgaard *et al.*, 1993) or hypertrophic (Sihm *et al.*, 1995; Rizzoni *et al.*, 2000) remodeling processes or a combination of both (Intengan *et al.*, 1999a 584). In eutrophic remodeling both internal and external artery diameters are reduced in the absence of changes in medial thickness, and this form of remodeling has been associated with essential hypertension in humans (Korsgaard *et al.*, 1993) and genetic models of hypertension in animals (Baumbach & Heistad, 1989; Intengan *et al.*, 1999b). Hypertrophic resistance artery remodeling, which involves hypertrophy/proliferation of smooth muscle cells leading to medial thickening, has also been described in essential hypertension (Sihm *et al.*, 1995), and in experimental studies where hypertension was induced in rats (Deng & Schiffrin, 1992). Evidence suggests that resistance artery remodeling may be promoted by vasoconstrictor signaling pathways, in particular endothelin-1 (d'Uscio *et al.*, 1997; Amiri *et al.*, 2004) and angiotensin II (Schiffrin & Touyz, 2004), and loss of NO-regulation of vascular smooth muscle cell phenotype (Garg & Hassid, 1989), effects which may be partially mediated by increased local reactive oxygen species generation (Touyz *et al.*, 2003).

Vascular smooth muscle function may also be modified in hypertension. Enhanced sensitivity to noradrenaline has been described in the isolated perfused mesenteric arcade during early stages of hypertension in Dahl salt-sensitive rats (Kong *et al.*, 1991b) and in young, pre-hypertensive spontaneously hypertensive rats (Kong *et al.*, 1991a). However, while maximal tension generation to a range of vasoconstrictor factors, including NA and

AII was enhanced, no differences were observed in vasoconstrictor sensitivity either in isolated human artery preparations (Aalkjaer *et al.*, 1987; Egan *et al.*, 1987) or *in vivo* (Egan *et al.*, 1987). It was suggested that enhanced maximal constriction may result from the potentiation of contraction by structural remodeling (Aalkjaer *et al.*, 1987; Egan *et al.*, 1987). It has also been demonstrated *in vitro*, however, that 3 days' chronic vasoconstriction to endothelin-1 resulted in inward remodeling of rat resistance arteries (Bakker *et al.*, 2002). Differential vasoconstrictor function may therefore contribute to initiating the resistance artery structural remodeling observed in hypertension.

Vascular tone is therefore dependent on the complex interactions between neural, endocrine, paracrine/autocrine, metabolic and mechanical stimuli, which influence endothelial and vascular smooth muscle function. In cardiovascular disease states imbalance between vasoconstrictor and vasodilator influences on vascular smooth muscle tone may promote remodeling and functional changes within small arteries that lead to increased peripheral vascular resistance. To investigate the potential pathways which may mediate the association between fetal growth restriction and pathological changes in vascular function, it is important to consider the impact of perturbations in the *in utero* environment on fetal vascular function.

1.5 FETAL VASCULAR ADAPTATIONS TO REDUCED SUBSTRATE SUPPLY

In the fetal sheep, cardiovascular adaptation is evident in response to acute hypoxia very early in gestation (Iwamoto *et al.*, 1989; Kiserud *et al.*, 2001). There is also evidence that the increase in femoral vascular resistance in response to acute hypoxia is greater in fetuses that were previously chronically hypoxic, hypoglycemic or acidotic (Gardner *et al.*, 2002a).

Chronic fetal exposure to a compromised *in utero* environment may therefore increase the capacity for peripheral vasoconstriction in the fetus. The persistence of such fetal adaptation may indeed contribute to changes in postnatal vascular function. This section will therefore review the fetal cardiovascular responses to either acute or chronic perturbations of the *in utero* environment, with particular focus on changes in vascular function.

1.5.1 Fetal circulation and the distribution of cardiac output during normoxia

The presence of 3 vascular shunts within the fetal circulatory system enables blood flow from the placenta to be optimally distributed throughout fetal tissue, so that well-oxygenated blood is supplied to oxygen-sensitive organs, in particular the brain and heart. In a normoxic fetal sheep, approximately two thirds of umbilical vein blood flow enters the portal vein and flows through the liver to the inferior vena cava to be returned to the heart (Jensen *et al.*, 1999). The remainder is shunted via the ductus venosus directly to the inferior vena cava. Both superior and inferior vena cava blood flow enters the right atrium, where the presence of the foramen ovale permits a substantial proportion of well-oxygenated blood to cross through the left atrium into the left ventricle. This allows perfusion of the brain and heart with well oxygenated blood. The third shunt allows vena cava blood pumped through the right ventricle into the pulmonary artery to be returned to the descending aorta via the ductus arteriosus, such that only a small proportion of combined ventricular output perfuses the lungs, whereas right-ventricular output primarily supplies the lower fetal body.

In fetal sheep, under normoxic conditions approximately 45% of fetal combined ventricular output is distributed to the placenta via the umbilical arteries, while the remaining 55% is

distributed throughout the fetal body. Of this, ~ 30% of cardiac output flows to the fetal carcass (skeletal muscle, skin, connective tissue and bone), and 11% to the lungs, while small fractions of cardiac output are directed toward the heart (2.6%), brain (3%), kidneys (2.3%), small intestines (2.6%) and adrenals (0.006%) (Jensen *et al.*, 1999).

1.5.2 Fetal circulatory response to acute hypoxia

In response to acute hypoxia, fetal heart rate transiently decreases, while arterial blood pressure progressively increases and cardiac output is redistributed by well-characterized changes in regional blood flow (Cohn *et al.*, 1974; Peeters *et al.*, 1979; Iwamoto *et al.*, 1983; Itskovitz *et al.*, 1987; Bocking *et al.*, 1988). Blood flow to the brain, heart and adrenals is conserved during fetal hypoxia whether it results from maternal hypoxia (Cohn *et al.*, 1974; Peeters *et al.*, 1979), reduced umbilical blood flow (Itskovitz *et al.*, 1987) or reduced uterine blood flow (Jensen *et al.*, 1991; Jensen *et al.*, 1999). At the same time, blood flow and/or oxygen delivery to peripheral tissues is reduced by hypoxia; however the nature of reductions in blood flow to the peripheral circulations varies according to the underlying cause of hypoxia. Maternal hypoxia reduces blood flow to the fetal carcass, lungs, kidneys and intestines (Cohn *et al.*, 1974), whereas reduced umbilical blood flow has been reported to reduce flow to the lungs and increase blood flow to the carcass, kidneys and intestines (Itskovitz *et al.*, 1987). Due to increased ductus venosus blood flow and preferential shunting of ductus venosus blood across the foramen ovale following umbilical cord compression, oxygen delivery to brain and heart tissue was increased, however, while all other tissues received less oxygen during umbilical cord occlusion (Itskovitz *et al.*, 1987). The effects of reduced uterine blood flow on peripheral circulations are dependent on the timing and degree of the reduction in uterine blood flow (Jensen *et al.*, 1999).

1.5.2.1. Impact of fetal growth restriction on the response to acute hypoxia

Several studies have also examined the impact of fetal growth restriction on the redistribution of cardiac output in response to acute hypoxia in fetal sheep. When fetal growth restriction was achieved by repeated microsphere embolization of the uteroplacental bed, a greater proportion of cardiac output was distributed to brain, heart and adrenal tissue and a smaller proportion to lung tissue at baseline (Block *et al.*, 1984). Superimposed acute hypoxia produced a greater increase in the proportional blood flow to the brain, heart and adrenals in embolized than control fetuses, and decreased lung blood flow only in embolized fetuses (Block *et al.*, 1984). The effects of long-term high-altitude hypoxia on regional fetal blood flow and the cardiovascular response to acute hypoxia in fetal sheep were assessed by comparison of data from fetal sheep at high-altitude with previously reported data from fetal sheep at low-altitude. At baseline, carcass blood flow was lower in fetuses at high-altitude, while acute hypoxia produced a similar increase in brain, heart and adrenal blood flow in fetuses at high- and low-altitude (Kamitomo *et al.*, 1993). A prior history of hypoxemia, acidemia or hypoglycemia for at least 6 days in fetal sheep during late gestation also augmented the increase in femoral vascular resistance in response to acute hypoxia (Gardner *et al.*, 2002a), although changes in other vascular beds were not assessed.

These studies provide evidence that there is a basal redistribution of cardiac output in growth-restricted fetal sheep, similar to clinical Doppler ultrasonography observations of IUGR fetuses. Further, the vascular response to a further period of acute hypoxia may be potentiated by prior fetal compromise.

1.5.3 Neural/neuroendocrine component of the fetal response to hypoxia

1.5.3.1 Contribution during acute hypoxia in fetal sheep

The initiation of the fetal cardiovascular response to acute hypoxia is reflex, and is dependent on carotid, but not aortic chemoreceptor activation, which results in a rapid-onset vagally-mediated bradycardia and a sympathetically-mediated increase in peripheral vascular resistance (Bartelds *et al.*, 1993; Giussani *et al.*, 1993). The involvement of α-adrenergic sympathetic efferent pathways has been further confirmed by blockade of α-adrenergic receptors with phentolamine, which prevented the rapid femoral vasoconstriction in response to acute hypoxia in intact, normoxic fetal sheep (Giussani *et al.*, 1993). Fetal sheep that had undergone chemical sympathectomy (with 6-hydroxydopamine) also did not demonstrate vasoconstriction in the carcass, mesenteric, splanchnic or splenic vascular beds in response to acute hypoxia, as assessed by microsphere analysis of regional blood flow (Iwamoto *et al.*, 1983).

Acute hypoxia results in a rapid increase in fetal plasma catecholamine concentrations, which is strongly related to the degree of hypoxemia (Cohen *et al.*, 1982; Lewis *et al.*, 1982) and primarily derived from the fetal adrenal (Cohen *et al.*, 1984; Jones *et al.*, 1988). Adrenal medulla catecholamine release may occur through either direct effects of hypoxia on the adrenal (Adams *et al.*, 1996) or through stimulation via splanchnic nerves, however in mid-late gestation fetal sheep catecholamine release is primarily neurally mediated (Cheung, 1990).

During acute hypoxia the fetal vasculature is exposed to circulating catecholamines derived from the adrenal, and NA released from sympathetic nerve terminals. Interestingly, when

the sensitivity to catecholamines was compared in isolated vascular rings of intrahepatic vein, and ductus venosus from late gestation fetal sheep, both sensitivity and maximal constriction to NA were greater in sections of intrahepatic vein than in ductus venosus sections (Tchirikov *et al.*, 2003). Differences in regional catecholamine responsiveness within the fetal vasculature may therefore contribute to the redistribution of cardiac output during acute hypoxia. When the effects of acute hypoxia *in vitro* on constriction to NA or electrical field stimulation of periarterial nerves were assessed in femoral arteries from chicken embryos, however, maximal constriction to both stimuli was reduced under hypoxic conditions (Ruijtenbeek *et al.*, 2002). The decrease in constriction induced by smooth muscle cell depolarizaton during acute *in vitro* hypoxia was smaller than the decrease in NA-induced constriction, while the effects of hypoxia did not appear to be dependent on the presence of endothelium (Ruijtenbeek *et al.*, 2002). Local hypoxia may have a specific inhibitory effect on α1-adrenergic receptor pharmacomechanical coupling, as opposed to decreased constriction capacity due to energy depletion during hypoxia (Ruijtenbeek *et al.*, 2002). Vasoconstriction of small mesenteric arteries in response to acute hypoxia in the chick embryo *in vivo* at 0.8 of incubation was abolished by phentolamine infusion, however, implying that decreased mesenteric blood flow during hypoxia is dependent on α-adrenergic activation (Rouwet *et al.*, 2000). Therefore, α-adrenergic receptors mediate peripheral vasoconstriction during hypoxia in late-incubation chick embryos despite the direct effects of local hypoxia on pharmacomechanical coupling.

1.5.3.2 Contribution during acute hypoxia in growth restricted fetuses

Circulating NA concentrations are higher in compromised fetal sheep (Jones & Robinson, 1983; Hooper *et al.*, 1990; Gagnon *et al.*, 1994; Simonetta *et al.*, 1997; Smolich & Esler,

1999; Gardner *et al.*, 2002a). Circulating NA levels were also higher, and the decrease in arterial blood pressure in response to α1-adrenergic receptor blockade with phentolamine greater in hypoxic, placentally restricted fetal sheep than in control fetuses (Danielson *et al.*, 2005). Growth-restricted, hypoxic fetuses may therefore be more dependent on α-adrenergic signaling for the maintenance of blood pressure than control fetuses.

A series of investigations in chicken embryos have determined the impact of chronic hypoxia during incubation on the innervation and sensitivity to catecholamines in peripheral arteries. Chronic hypoxia (15% O_2) from either d6-19 or throughout incubation increased the density of sympathetic nerve terminals in femoral arteries of 19d chick embryos (where hatching occurs at 21d) (Ruijtenbeek *et al.*, 2000; Rouwet *et al.*, 2002). Adult chickens that were exposed to hypoxia *in ovo* did not demonstrate sympathetic hyperinnervation in branches of the femoral artery (Villamor *et al.*, 2004), however hyperinnervation of peripheral arteries may be an important adaptation in the chick embryo during hypoxia.

Chronic hypoxia may also modify the sensitivity of peripheral blood vessels to catecholamine-induced vasoconstriction. In chicken embryos, incubation under chronic hypoxia from d6-19 reduced the sensitivity of femoral arteries to NA by increasing periarterial neuronal NA reuptake (Ruijtenbeek *et al.*, 2000). There was no change in NA sensitivity in small mesenteric arteries of 19d old chick embryos that were hypoxic throughout incubation, however, while the baseline adrenergic tone in these arteries was elevated (Rouwet *et al.*, 2002). In sheep, when maternal hypoxia was induced for 5 days during late gestation by infusion of nitrogen gas into a maternal tracheal catheter, the maximal tension generated in response to α1-adrenergic receptor activation with

phenylephrine (PE) was increased in isolated femoral arteries (Kim *et al.*, 2005). Interestingly, when NO synthesis was inhibited using L-NAME, both sensitivity and maximal constriction to PE were greater in femoral arteries from fetal sheep exposed to maternal hypoxia compared to normoxic controls (Kim *et al.*, 2005). In this study, maternal hypoxia did not modify vascular responses to PE in renal arteries, suggesting that the effects of hypoxia on adrenergic responsiveness are vascular bed-specific, and may overcome increased local vasodilator NO production.

1.5.4 Endocrine/Paracrine component of the fetal response to hypoxia

1.5.4.1 Vasopressin

Fetal sheep plasma concentrations of the vasoconstrictor vasopressin, which is synthesized and released from the pituitary, are substantially elevated in response to acute hypoxia (Rurak, 1978; Daniel *et al.*, 1983; Stegner *et al.*, 1984). Antagonism of the V_1 vasopressin receptor in fetal sheep during hypoxia also partially reversed the fetal hypertension and bradycardia, decreased vascular resistance in the placenta, gut and liver, increased vascular resistance in the brain and decreased blood flow to both the brain and heart (Perez *et al.*, 1989). These data support the conclusion that the increase in vasopressin concentrations contribute functionally to the fetal cardiovascular response to hypoxia. In carotid denervated fetuses, however, femoral vascular resistance was not increased by hypoxia despite an increase in plasma vasopressin concentrations to levels that were similar to intact fetuses (Giussani *et al.*, 1994b).

1.5.4.2 Angiotensin II

Angiotensin II (AII) is produced through the enzymatic activation of angiotensinogen, which is first cleaved to angiotensin I by renin, and subsequently converted to the active AII form by angiotensin converting enzyme (ACE). Two distinct receptors bind AII; AT_1 and AT_2, both of which are expressed throughout the vasculature. Infusion of the angiotensin converting enzyme inhibitor captopril did not modify the cardiovascular response to hypoxia in intact late gestation fetal sheep; however in fetuses that had undergone carotid sinus denervation, captopril infusion blocked the blood pressure elevation and reduced the increase in femoral vascular resistance which occurred in vehicle infused denervated fetal sheep (Green *et al.*, 1998a). Interestingly, the contribution of angiotensin II to the maintenance of fetal arterial blood pressure was greater during late-gestation in fetal sheep that were chronically hypoxemic and growth restricted as a consequence of placental restriction (Edwards *et al.*, 1999b). Plasma renin activity (Konje *et al.*, 1996) and circulating AII (Kingdom *et al.*, 1993) were also higher in growth restricted human infants than in normally grown infants.

1.5.4.3 Cortisol

The fetal endocrine response to acute hypoxia includes an elevation of fetal plasma adrenocorticotrophic hormone (ACTH) and cortisol concentrations (Jones *et al.*, 1977; Towell *et al.*, 1987; Jackson *et al.*, 1989; Giussani *et al.*, 1994a; Gardner *et al.*, 2002b). Administration of synthetic glucocorticoids increases fetal arterial blood pressure and both peripheral and central vascular resistance, suggesting an effect of glucocorticoids on vascular function (Derks *et al.*, 1997; Anwar *et al.*, 1999; Schwab *et al.*, 2000; Fletcher *et al.*, 2002; Quaedackers *et al.*, 2005). It is also possible that glucocorticoids mediate rapid changes in peripheral resistance by increasing secretion of other endocrine vasoconstrictor

factors, including adrenaline and NPY (Fletcher *et al.*, 2002). Infusion of cortisol for 5 days in fetal sheep at 129d gestation increased both circulating AII and the hypotensive response to inhibition of the angiotensin type 1 receptor, implying that the renin angiotensin system may mediate the increase in fetal arterial pressure during glucocorticoid exposure (Forhead *et al.*, 2000). Further, fetal arterial blood pressure did not increase when cortisol infusion for 5 days was combined with infusion of the AT1 receptor antagonist GR138950 (Forhead & Fowden, 2004). These studies implicate an increase in renin angiotensin system activity in the fetus following elevated glucocorticoid exposure, which may contribute substantially to the observed pressor effects.

A number of studies have demonstrated that fetal plasma cortisol concentrations are elevated in fetuses by chronic compromise, including uteroplacental insufficiency (Phillips *et al.*, 1996), high-altitude hypoxia (Imamura *et al.*, 2004) and chronic umbilical cord occlusion (Gardner *et al.*, 2001a). There is some evidence that glucocorticoid exposure modifies peripheral vascular function, which may contribute to the maintenance of cardiac output redistribution in growth-restricted fetuses. Infusion with the synthetic glucocorticoid betamethasone for 48h in fetal sheep at 128d gestation increased both the sensitivity and maximal constriction to vascular smooth muscle depolarization in isolated femoral artery branches at 130d gestation. There were no differences, however, in the vasoconstriction to NA or the thromboxane mimetic U46619 (Anwar *et al.*, 1999). Betamethasone infusion also decreased sensitivity to endothelium-dependent relaxation by bradykinin, but not acetylcholine, and decreased sensitivity to forskolin, which activates adenylate cyclase to produce cAMP (Anwar *et al.*, 1999). Similarly, dexamethasone infusion in fetal sheep for 48h at 110-111dGA increased both sensitivity and maximal vasoconstriction to endothelin-1 in isolated femoral arteries at 112-113dGA, while isolated middle cerebral arteries

exhibited tachyphylaxis in response to endothelin-1 after infusion (Docherty *et al.*, 2001a). Following a course of maternally administered dexamethasone injections given between 103-117dGA, both the sensitivity and maximal constriction to endothelin-1 were also increased in isolated femoral artery branches from fetuses at 119dGA (Molnar *et al.*, 2002). In this study, dexamethasone increased sensitivity to endothelium-dependent vasodilation to bradykinin, with no change in dilation to acetylcholine (Molnar *et al.*, 2002). Inhibition of NO synthesis with L-NAME did not inhibit relaxation to acetylcholine in arteries from dexamethasone-exposed fetal sheep, while it decreased sensitivity in arteries from control fetuses (Molnar *et al.*, 2002).

1.5.4.4 Endothelin-1

Big endothelin-1 is produced and converted to endothelin-1 by endothelin converting enzyme within endothelial cells, and is primarily considered to act as a paracrine/autocrine factor, via either ET_A or ET_B receptors on smooth muscle cells to elicit vasoconstriction. Activation of ET_B receptors on endothelial cells results in endothelium-dependent vasodilation however, and the prevailing effect of endothelin-1 is therefore dependent on the balance of endothelial and smooth muscle effects. There was no change in plasma endothelin-1 levels in response to a 1h period of acute hypoxia in fetal sheep at ~126dGA, nor was the increase in femoral vascular resistance during hypoxia modified by inhibition of the ET_A receptor (Green *et al.*, 1998b). Interestingly, however, inhibition of the ET_A receptor did increase carotid blood flow and reduce carotid vascular resistance during both normoxia and hypoxia (Green *et al.*, 1998b). These data imply that endothelin-1 may exert a tonic influence on carotid vascular function, but is not essential to the cardiovascular response to acute hypoxia in the fetal sheep. While there was no apparent role for

endothelin-1 in the fetal response to acute hypoxia, there is more evidence to suggest that endothelin-1 may contribute to the fetal adaptation to chronic hypoxia *in utero*. PreproET, the precursor to ET-1, is expressed in endothelial cells in response to several stimuli, including hypoxia, (Rakugi *et al.*, 1990), and also the hormonal factors AII (Vanhoutte, 2000), cortisol (Vanhoutte, 2000), and erythropoietin (Vogel *et al.*, 1997), all of which have been demonstrated to be elevated in the fetal circulation when fetal growth is impaired (Kingdom *et al.*, 1993; Snijders *et al.*, 1993; Phillips *et al.*, 1996). Expression of endothelin-1 is regulated in part by hypoxia inducible factor 1α (Yamashita *et al.*, 2001), and elevations in plasma endothelin-1 levels have also been reported in human IUGR infants (Erdem *et al.*, 2003; Arslan *et al.*, 2004), suggesting a role for endothelin-1 in the vascular adaptation to an adverse *in utero* environment. It has also been demonstrated that femoral, middle cerebral, adrenal and renal arteries isolated from fetal sheep aged 110 – 145d gestation all constrict in response to endothelin-1 *in vitro* (Docherty *et al.*, 2001b). Increased endothelin-1 concentrations would therefore be expected to influence regional blood flow in the fetus. Interestingly, following 48h exposure to dexamethasone at 110dGA both sensitivity and maximal constriction to endothelin-1 were increased in femoral, but not middle cerebral arteries from fetal sheep (Docherty *et al.*, 2001a). As elevated plasma cortisol levels are a characteristic endocrine feature of growth restricted fetuses, it is possible that chronic hypoxia and elevated glucocorticoid concentrations result in increased synthesis and increased sensitivity to endothelin-1 respectively in growth restricted fetuses.

1.5.5 Local vasodilator contribution to the fetal response to hypoxia

1.5.5.1 Nitric oxide, evidence from fetal sheep in vivo

Studies that have inhibited synthesis of the vasodilator NO in fetal sheep have clearly demonstrated that it is an important modulator of basal fetal vascular tone and cardiovascular homeostasis. When NO synthesis is inhibited, fetal arterial blood pressure increases quickly (Chang *et al.*, 1992; Reller *et al.*, 1995; Fan *et al.*, 1996; Green *et al.*, 1996; Chlorakos *et al.*, 1998; Fan *et al.*, 1998; Yu *et al.*, 2002; Coumans *et al.*, 2003). The pressor effects of NOS inhibition can be attributed to widespread vasoconstriction resulting from the loss of NO-modulation of basal vascular tone. Increased vascular resistance following NOS inhibition has been demonstrated in the femoral (Green *et al.*, 1996) mesenteric (Fan *et al.*, 1996; Fan *et al.*, 1998) cerebral (McCrabb & Harding, 1996; Coumans *et al.*, 2003), myocardial (Reller *et al.*, 1995) and umbilico-placental (Chang *et al.*, 1992; Chlorakos *et al.*, 1998; Coumans *et al.*, 2003) circulations in fetal sheep.

The substantial effects of NOS inhibition on basal fetal cardiovascular function complicate the interpretation of studies that have attempted to determine the role of NO in mediating specific regional vascular changes in the fetus in response to hypoxia. A series of studies conducted by Gardner and colleagues have addressed this issue by utilizing a NO-clamp where simultaneous infusion of the NOS inhibitor L-NAME and the NO donor sodium nitroprusside (SNP) blocks *de novo* synthesis of NO while compensating for the loss of basal NO production (Gardner *et al.*, 2001b). Using this method, it has been demonstrated that basal umbilical blood flow is substantially dependent on NO production, while NO does not appear to contribute to the enhanced umbilical blood flow in response to acute hypoxia (Gardner *et al.*, 2001b). Interestingly, chronic prior umbilical cord compression in

fetal sheep during late gestation reduced the pressor response, and increased umbilical vascular conductance in response to an acute period of hypoxia (Gardner & Giussani, 2003). When NO synthesis was blocked using the NO-clamp technique these changes were reversed. These data may imply that a period of chronic fetal compromise leads to a switch from a high-pressure, vasoconstrictor-mediated blood flow redistribution to a lower-pressure, vasodilator-mediated blood flow redistribution in response to acute hypoxia (Gardner & Giussani, 2003).

The NO-clamp was also used to investigate the role of NO in the peripheral vascular responses to acute hypoxia. The increase in femoral vascular resistance in response to acute hypoxia was augmented by NO-clamp in fetal sheep at ~130 d gestation, suggesting that local NO production offsets the peripheral vasoconstriction that occurs in response to acute hypoxia (Morrison *et al.*, 2003). Interestingly, it was also demonstrated that a period of chronic umbilical cord occlusion up-regulated NO synthesis in the femoral vascular bed, which diminished the increase in femoral vascular resistance that occurred in response to acute hypoxia in 130dGA fetal sheep (Gardner *et al.*, 2002b). Chronic high-altitude hypoxia increased both maternal and fetal nitrate concentrations substantially (Zhang *et al.*, 1998), further implying that chronic hypoxia increases NO production within the fetal vasculature.

1.5.5.2 Nitric oxide and vascular relaxation - in vitro studies

Acute hypoxia *in vitro,* which was achieved by bubbling the buffer with 95% N_2 and 5% CO_2 decreased maximal relaxation to acetylcholine in isolated carotid arteries from fetal guinea pigs in late gestation suggesting that hypoxia may have a direct inhibitory effect on

endothelial function (Thompson & Weiner, 1999). Similar data were obtained from femoral arteries isolated from chick embryos during late incubation where acute hypoxia *in vitro* abolished endothelium-dependent relaxation to acetylcholine (Ruijtenbeek *et al.*, 2002). As acute hypoxia increased sensitivity to the NO donor SNP and inhibition of NOS activity with L-NAME did not modify contractile responses during hypoxia, there is some evidence that the direct effects of hypoxia may involve decreased NO bio-availability (Ruijtenbeek *et al.*, 2002).

Interestingly, there is also some evidence that prolonged hypoxia in the fetal guinea pig up-regulates NO production in the carotid artery. While 4 days' chronic maternal hypoxia impaired both sensitivity and maximal relaxation to acetylcholine in fetal guinea pig carotid arteries, endothelium-dependent relaxation did not differ in carotid arteries following 7 days maternal hypoxia (Thompson & Weiner, 1999). When NO synthesis was inhibited by LNA there were no differences in relaxation to acetylcholine among groups (Thompson & Weiner, 1999), implying that differences in NO synthesis may account for the impaired endothelial function following 4 days maternal hypoxia, and further, that NO production increases after 7 days' hypoxia which restores endothelial function. This was further established in a subsequent study, where 14 days maternal hypoxia (12% O_2) decreased contraction to the vasoconstrictors PGF_{2a} and U46619 in fetal carotid arteries (Thompson *et al.*, 2004). Constriction to each agonist was enhanced by the inhibition of prostaglandin or NO synthesis, however in each instance the enhancement in constriction was greatest in arteries from hypoxia-exposed fetuses (Thompson *et al.*, 2004).

In contrast, in the chicken embryo, sensitivity to acetylcholine was reduced in isolated femoral arteries following chronic hypoxia during incubation (15% O_2) (Ruijtenbeek *et al.*,

2003b). Inhibition of NO synthesis with L-NAME did not modify sensitivity to acetylcholine in femoral arteries from hypoxia-incubated chick embryos, while sensitivity was reduced by L-NAME in arteries from control embryos, implying that hypoxia decreased the contribution of NO to endothelial function in femoral arteries (Ruijtenbeek *et al.*, 2003b). These findings were confirmed in a further study where hypoxia (15%) from d6-d19 of incubation decreased sensitivity to acetylcholine in femoral arteries relative to arteries isolated from control embryos, while differences between the groups were abolished in the presence of L-NAME (Villamor *et al.*, 2004). In the same study, no changes were observed in relaxation to acetylcholine in isolated intrapulmonary arteries (Villamor *et al.*, 2004). Differences in the effects of chronic hypoxia on the function of isolated guinea pig carotid arteries and chick embryo femoral arteries may reflect species differences, or may imply that the vascular effects of chronic hypoxia on NO production differ between regional circulations.

There is therefore substantial redundancy within the fetal cardiovascular response to hypoxia. The relative roles of each regulatory pathway may depend on gestational age and the functional capacity of other key systems. A central role for adrenergic vasoconstriction in peripheral vascular beds has been demonstrated during acute hypoxia, however, and vascular adrenergic activity may be of increased relevance in chronic hypoxia. Chronic hypoxia may also produce specific effects on endothelial function, in particular NO production. In light of the complex interactions between different pathways which regulate vascular function, *in vitro* preparations which allow the investigation of specific pathways are particularly valuable in determining the impact of fetal growth restriction on vascular function.

1.5.6 Fetal circulatory effects of maternal undernutrition

There is currently less information regarding the fetal vascular effects of maternal undernutrition. In an interesting recent report, however, the degree of ductus venosus shunting of blood flow in human fetuses was directly related to maternal slimness or skin fold thickness such that there was relative sparing of liver blood flow in the fetuses of thin mothers (Haugen *et al.*, 2005). When maternal diet was assessed by a food-frequency questionnaire in the same study, an unbalanced or imprudent diet was also associated with increased liver blood flow and reduced ductus venosus flow (Haugen *et al.*, 2005), suggesting that in human fetuses the distribution of fetal cardiac output is influenced by maternal nutrition but that the effects of undernutrition on fetal cardiac output distribution differ from hypoxia.

In fetal sheep, maternal undernutrition during the first 70 days of gestation increased basal femoral vascular resistance, and also augmented the increase in femoral vascular resistance in response to acute maternal hypoxia (Hawkins *et al.*, 2000). Similarly, late gestation fetuses that were spontaneously hypoglycemic for at least 6 days after surgery had higher baseline femoral vascular resistance and a greater increase in vascular resistance in response to acute hypoxia than control fetuses (Gardner *et al.*, 2002a). *In vitro* studies may suggest that the increased basal femoral vascular resistance in fetal sheep that were undernourished *in utero* may relate to changes in endothelial function or smooth muscle sensitivity to NO. Either global or protein maternal undernutrition during the first 70 days of gestation impaired both sensitivity and maximal relaxation to acetylcholine in isolated femoral arteries from mid-gestation fetuses (Nishina *et al.*, 2003). Following protein restriction, but not global undernutrition, sensitivity and maximal relaxation to the NO donor SNP was also reduced in femoral arteries from mid-gestation fetal sheep (Nishina *et*

al., 2003). The mechanisms whereby endothelial function is impaired may therefore differ depending on the specific nature of the maternal undernutrition. Sensitivity to both acetylcholine and SNP were also reduced in femoral arteries from late gestation fetuses following 50% maternal undernutrition from d0-d70 of gestation, however in this study there were no vascular effects of a 15% reduction in maternal nutrition during this gestational period (Ozaki *et al.*, 2000).

1.6 PROGRAMMING OF ADULT VASCULAR FUNCTION IN ANIMALS

A range of animal studies have now investigated the sensitivity of several vascular pathways to perturbations in prenatal development, and the potential for the adult function of these pathways to be modified by deficits in fetal substrate supply. These studies have not only confirmed human data indicating that impaired fetal growth may program later vascular function, but have highlighted key candidate mechanisms. Primarily, investigations have focused on differences in endothelial function, and sensitivity to vasoconstrictor factors within small, resistance-sized arteries from peripheral vascular beds. The key vascular effects of different perturbations before birth on adult vascular function are summarized in table 1.

1.6.1 Impact of prenatal malnutrition on postnatal endothelial function

1.6.1.1 Global undernutrition during pregnancy

Nutritional restriction by 50% during the second half of pregnancy in Wistar rats decreased maximal endothelium-dependent relaxation to both acetylcholine and bradykinin in small mesenteric arteries from female offspring aged 3-4 months (Holemans *et al.*, 1999).

Table 1.1: Programming of adult vascular function in animal studies

Experimental protocol	Effect on fetal growth	Age at follow-up	Artery type	Vasodilator responses	Vasoconstrictor responses	Reference
Global undernutrition						
50% restriction d11-d23 pregnancy.	↔ Litter size	100-120d (♀)	Small mesenteric arteries	↓ ACh & BK (max) ↑ SNP (max)	↓ KPSS (max) ↔ NA	(Holemans *et al.*, 1999)
30% restriction during pregnancy in Wistar rats.	↓ Body weight (day1) ↔ Litter size ↑ % Liver weight ↓ % Lung wt	20, 100 and 200 days (♂/ ♀)	Femoral artery (d20) 2° femoral artery branches (d100/d200)	*d100* ↓ SNP (max) (♂/ ♀) *d200* no significant effects	*d20* ↓ PE and NA ↑ U46619 (♂) *d100* ↑ U46619 (max) (♂) *d200* ↑ U46619 (max) (♂) ↑ KPSS (sensitivity) (♂)	(Ozaki *et al.*, 2001)
50% restriction during pregnancy in Wistar rats.	↓ Birth weight ↔ Litter size	14 weeks (♂/ ♀)	Aorta, +/- endothelium	↓ ACh (sensitivity & max) (♂/ ♀) ↔ SNP	↔ NA	(Franco *et al.*, 2002a)
	↓ Birth weight ↔ Litter size	14 weeks (♂)	Mesenteric microvessels	↓ ACh and BK (max) SOD & MnTMPyP improved dilation in restricted offspring ↔ Dilation to SNP	- *not determined* -	(Franco *et al.*, 2002b)
	↓ Birth weight	16 weeks (♂)	Mesenteric microvessels	↑ SOA production due to NADPH oxidase, reduced by losartan ↓ ACh and BK (max) improved by apocynin	- *not determined* -	(Franco *et al.*, 2003)
	↓ Birth weight ↔ Litter size	16 weeks (♂)	Mesenteric microvessels	↓ ACh and BK (max) Vitamin C and E improved dilation ↔ Dilation to SNP	- *not determined* -	(Franco Mdo *et al.*, 2003)
	↓ Birth weight ↔ Litter size		Mesenteric microvessels	↓ ACh and BK (max) improved by BH4		(Franco *et al.*, 2004)
Protein restriction						
24 or 8% casein from d14 pregnancy until postnatal d50	↓ Adult body weight	140-180d (♂)	Iliac artery	- *not measured* -	↓ NA (max & sensitivity) ↓ methoxamine (max & sensitivity)	(Del Basso *et al.*, 1983)
18 or 9 % casein during pregnancy in Wistar rats.	↔ Litter size ↓ Birth weight in 6% pups only	12 weeks	Pial microvessels	↓ Max dilation to ACh & SP ↓ max dilation to SNP (lower expression of sGC)	↔ U46619	(Lamireau *et al.*, 2002)
9% casein protein restriction during pregnancy.	↔ Litter size ↔ Birth weight ↔ Pregnant offspring weight gain ↔ F_1 fetal/placental weights	~5 months pregnant (♀)	Thoracic aorta 3° Mesenteric arcade	*Thoracic aorta* ↔ ACh & isoprenaline *Mesenteric arcade* ↓ ACh (sensitivity) ↓ isoprenaline (max) ↔ Dilation to isoprenaline in presence of L-NAME	*Thoracic aorta* ↔ PE and KPSS *Mesenteric arcade* ↔ PE & KPSS	(Torrens *et al.*, 2002)
9% casein protein restriction during pregnancy in Wistar rats.	↔ birth weight ↓ body weight at 65d	87d or 164d (♂)	Small mesenteric arteries	*87d* ↓ BK & SNP (max) ↓ ACh & SNP (sensitivity) *164d* ↓ ACh & BK (max) Effect of age in C but not PR response to SNP	*87d* ↓ U46619 (max & sensitivity) *164d* ↔ U46619 at both ages: ↔ KPSS or PE	(Brawley *et al.*, 2003)
High fat						
30% or 20% lard 10d before mating until 16d postpartum in Sprague Dawley rats	↔ neonatal weight	15d 60d	Femoral artery (15d)	*15d* ↓ ACh dilation ↓ NO donor (max) *60d* ↓ ACh (max) ↔ NO donor	*15d* ↔ NA *60d* ↑ NA (sensitivity) ↔ U46619	(Koukkou *et al.*, 1998)
20% animal lard 10d before mating until 21d post partum		160d (♀)	3° branches of femoral arteries	↓ ACh (max) ↔ NO donor	↔ NA, KPSS and U46619	(Ghosh *et al.*, 2001)
20% animal lard 10d before mating and during pregnancy in Sprague Dawley rats	↔ neonatal weight	180d (♂/ ♀)	Femoral arteries and mesenteric artery 3° branches	*Mesenteric* ↓ ACh (max) ↓ EDHF-mediated dilation (♂/ ♀) *Femoral* ↑ ACh (max) (♂) ↑ NO-mediated dilation (♂)	*Femoral* ↓ AII (max) (♂/ ♀) *Mesenteric and femoral* ↔ NA and PE (♂/ ♀)	(Taylor *et al.*, 2004)
20% animal lard added to diet, 10 days before mating until 21d post partum in Sprague Dawley rats			3° mesenteric artery branches	↓ ACh (max) effect reversed if fed high fat diet after weaning (♂/ ♀) ↔ NO donor (♂/ ♀)	- *not reported* -	(Khan *et al.*, 2004)
	↔ birth weight and adult body weight.	180d	Thoracic aorta	↓ ACh (max) (♂/ ♀) ↔ NO donor	↔ PE	(Armitage *et al.*, 2005)

Table 1 continued

Experimental protocol	Effect on fetal growth	Age at follow-up	Artery type	Vasodilator responses	Vasoconstrictor responses	Reference
Uteroplacental insufficiency						
Clipped abdominal aorta and ovarian arteries on d14 of pregnancy in Wistar rats.	↓ Birth weight and ↓adult weight	4, 8 and 12 weeks (♂)	Thoracic aortic strip	*4, 8 and 12 weeks* ↓ ACH (max) with no effect of L-NAME or ODQ in IUGR rats ↔ SNP	*4, 8 and 12 weeks* ↑ PE (max) in IUGR no effect of L-NAME endothelial removal, or ODQ *12 weeks* ↑ PE (sensitivity)	(Payne *et al.*, 2003)
(As above, with postnatal high salt diet)	↓ Birth weight and ↓adult weight	12 weeks (♂)	Thoracic aortic strip	↓ ACh (max) NOx production lower in IUGR rats	↑ PE (max & sensitivity) no effect of endothelium removal or L-NAME	(Payne *et al.*, 2004)
Bilateral uterine artery ligation on d13 of pregnancy in Wistar rats.	↓ Litter size ↑ Birth weight	3 weeks (♂)	1° mesenteric, renal, femoral and saphenous arteries	*Renal artery* ↑ Isoproterenol (max) ↔ Forskolin & diterpene *other arteries unaffected*	*Renal artery* ↑ PE and 5-HT (max) ↓ NA (max) ↓ PE (sensitivity) *other arteries unaffected*	(Sanders *et al.*, 2004)
Heterozygous offspring from eNOS -/- dams vs eNOS +/+ dams	↓ Litter size ↔ Birth weight	7-8 weeks	Carotid and mesenteric arteries	*Carotid* ↓ ACh and Isoproterenol (max) *Mesenteric* ↓ ACh and Isoproterenol (max & sensitivity)	*Carotid and mesenteric* ↑ 60mM KPSS ↑ PE	(Longo *et al.*, 2005)
Chronic hypoxia						
15% O2 from d6-19 of incubation in chick embryo	↔ Hatch weight ↔ Mortality	14-15 weeks (♂/ ♀)	Femoral artery and side-branch	*Femoral artery* ↔ ACh, SNP Side branch ↔ ACh, SNP ↓ L-NAME effect on ACh (♂/ ♀)	*Femoral artery* ↔ KPSS, NA ↑ EFS (sensitivity) (♂/ ♀) *Side branch* ↔ KPSS, NA, EFS	(Ruijtenbeek *et al.*, 2003a)
	↓ adult body weight	3-4 weeks	Femoral artery side branches	↔ ACh or effect of L-NAME on ACh relaxation	↔ KPSS, but L-NAME did not ↑ response in hypoxia chickens	(Ruijtenbeek *et al.*, 2003b)
Anemia (20 days in late gestation) in fetal sheep	↔ birth weight ↓ adult body weight	7 months	Small mesenteric arteries	↔ ACh, Adenosine or SNP	↔ KPSS, NA, tended to ↑ AII (max)	(Davis *et al.*, 2002)

Endothelium-dependent vasodilation to acetylcholine was also reduced in aorta from 3.5-4 month male and female offspring following 50% nutritional restriction throughout pregnancy in Wistar rats (Franco *et al.*, 2002a), and these observations were confirmed in mesenteric microvessels from male offspring of the same nutritional protocol in several subsequent studies (Franco *et al.*, 2002b; Franco Mdo *et al.*, 2003; Franco *et al.*, 2004).

Holemans *et al.* also reported that sensitivity to the exogenous NO donor SNP was enhanced following maternal undernutrition during pregnancy. This suggested that production of, rather than sensitivity to NO may be impaired following nutritional restriction (Holemans *et al.*, 1999). However, 30% nutrient restriction throughout pregnancy decreased sensitivity to SNP in femoral artery branches from both male and female offspring aged 3.5 months (Ozaki *et al.*, 2001), while 50% nutrient restriction throughout pregnancy did not alter sensitivity to SNP in mesenteric microvessels of 3.5-4 month male offspring (Franco *et al.*, 2002a; Franco *et al.*, 2002b; Franco Mdo *et al.*, 2003). The impact of prenatal undernutrition on vascular smooth muscle sensitivity to NO may therefore be dependent on the timing of the nutritional insult, vascular bed or arterial size, and may contribute to impairment of the NO pathway in some, but not all nutritionally programmed vascular dysfunction.

A series of studies have examined the role of enhanced vascular oxidative stress in impairing endothelium-dependent vasodilation, through the scavenging of NO by superoxide anion following maternal undernutrition during pregnancy. The impaired response to both acetylcholine and bradykinin in mesenteric microvessels of male offspring from 50% nutrient restricted dams was improved by exogenous addition of superoxide dismutase, or the superoxide dismutase mimetic manganese (III) tetrakis (1-methyl-4-

pyridyl) porphyrin (Franco *et al.*, 2002b). It was also demonstrated that increased NADPH-oxidase activity contributed to elevated superoxide anion production in undernourished offspring (Franco *et al.*, 2003). This was reduced by angiotensin type 1 (AT_1) receptor blockade with losartan (Franco *et al.*, 2003), suggesting that angiotensin II may play a role in the elevated vascular oxidative stress in male offspring following 50% undernutrition. Administration of antioxidant vitamin C or vitamin E for 15 days restored endothelium-dependent vasodilation in undernourished male offspring (Franco Mdo *et al.*, 2003), providing further evidence that oxidative stress mediated the observed endothelial dysfunction.

The activity of NO synthase is dependent on the presence of several co-factors, including tetrahydrobiopterin. In the absence of sufficient tetrahydrobiopterin, the enzyme may uncouple and produce superoxide anion (Wever *et al.*, 1997; Vasquez-Vivar *et al.*, 1998). Superfusion of 100nM tetrahydrobiopterin improved endothelium-dependent vasodilation in mesenteric microvessels (Franco *et al.*, 2004). When NOS activity was assayed in mesenteric arterioles, however, activity was significantly lower in offspring from undernourished dams despite the presence of 10μM tetrahydrobiopterin within the assay buffer (Franco *et al.*, 2004). It is therefore not yet clear what role tetrahydrobiopterin deficiency may play in the reduced bio-availability of NO within small peripheral arteries of undernourished offspring.

1.6.1.2 Protein undernutrition

Through a number of publications, it has been demonstrated that reduced protein intake in pregnant rats programs hypertension in adult offspring (Langley & Jackson, 1994; Langley-

Evans *et al.*, 1999; Lamireau *et al.*, 2002; Brawley *et al.*, 2003). When the effects on vascular function were assessed, endothelium-dependent vasodilation to acetylcholine and substance P was impaired in pial microvessels from offspring of pregnant rats fed a 50% protein restriction (9% casein) diet throughout pregnancy (Lamireau *et al.*, 2002). Reduced vasodilation may have been effected in this study through reduced vascular smooth muscle NO sensitivity, as dilation to SNP was also reduced, and expression of soluble guanylate cyclase lower in pial arteries (Lamireau *et al.*, 2002). When the function of conduit and resistance arteries was examined in the pregnant female offspring of rat dams that were fed 9% casein throughout pregnancy, while aortic endothelial function was unaffected, small mesenteric artery endothelium-dependent vasodilation to acetylcholine and isoprenaline was impaired (Torrens *et al.*, 2002). When vasodilation to isoprenaline was compared in the presence of the NOS inhibitor L-NAME relaxation responses no longer differed between the pregnant female offspring of control and protein-restricted dams (Torrens *et al.*, 2002), suggesting that bio-available NO was also reduced in the peripheral vasculature following protein restriction *in utero*. Male offspring from dams fed a 9% casein diet throughout pregnancy also demonstrated impaired endothelium-dependent responses to acetylcholine and bradykinin at both 3 and 5.5 months of age (Brawley *et al.*, 2003). At 3 months, this was accompanied by reduced sensitivity and maximal dilation to SNP. In control offspring, but not protein restricted offspring, sensitivity to exogenous NO decreased with age, however, and at 5.5 months vasodilation to SNP was similar between groups (Brawley *et al.*, 2003).

These studies clearly demonstrate that both global, and protein-undernutrition during pregnancy impair endothelial function, and diminish the contribution of NO to endothelium-dependent vasodilation, in adult male and female offspring either with,

or without changes in vascular smooth muscle sensitivity to exogenous NO. Further, NO bio-availability may be decreased via enhanced vascular oxidative stress in offspring from undernourished dams, while there is some data to suggest that NO synthesis may be reduced by limited co-factor availability.

1.6.1.3 High fat diet during pregnancy

Fetal malnutrition throughout pregnancy resulting from an imbalanced maternal diet rich in saturated animal fat may be of increasing relevance in both developed and developing populations. As previously observed in offspring from dams undernourished, or fed a low protein diet during pregnancy, offspring of dams fed a pregnancy diet high in fat demonstrate elevated postnatal blood pressure (Khan *et al.*, 2003; Khan *et al.*, 2004).

Both endothelium-dependent relaxation and relaxation to SNP were impaired in femoral arteries from 15d offspring of pregnant rats fed 30% lard from 10 days prior to mating until d16 post partum (Koukkou *et al.*, 1998). Endothelium-dependent dilation to acetylcholine was also reduced, although dilation to SNP did not differ, in 60d offspring following consumption of a maternal diet including 20% lard across the same time (Koukkou *et al.*, 1998). In rats, a maternal diet high in animal fat during pregnancy has now been demonstrated to impair endothelium-dependent relaxation to acetylcholine in small femoral artery branches from female offspring (Ghosh *et al.*, 2001), and in both mesenteric artery branches (Khan *et al.*, 2003; Taylor *et al.*, 2004) and thoracic aorta (Armitage *et al.*, 2005) from male and female offspring.

Impaired dilation to acetylcholine in mesenteric artery branches in both sexes was attributed primarily to impairment in endothelium-derived hyperpolarizing factor-mediated relaxation (Taylor *et al.*, 2004). However, in the same study enhanced dilation to acetylcholine was observed in femoral arteries from male but not female offspring, which was accounted for by enhanced NO-mediated endothelium-dependent relaxation in this artery (Taylor *et al.*, 2004). Perhaps the most intriguing data generated in this series of studies demonstrated that the endothelial dysfunction programmed in offspring by a high fat diet during pregnancy was reversed in offspring that were maintained on a high fat diet after weaning, although these offspring still developed high blood pressure (Khan *et al.*, 2004). These data provide evidence that the vasculature may undergo 'predictive adaptive' changes (Gluckman & Hanson, 2004) related to the specific *in utero* environment, however further work is required to determine how this may factor into vascular dysfunction programmed via other experimental protocols.

1.6.2 Uteroplacental insufficiency and postnatal endothelial function

Endothelial function was assessed in growth-restricted male rat offspring from dams where the maternal abdominal aorta and ovarian arteries were clipped on d14 of gestation, which reduced uteroplacental blood flow and offspring birth weight (Payne *et al.*, 2003). At 4, 8 and 12 weeks postnatal age, maximal endothelium-dependent dilation to acetylcholine was reduced in strips of thoracic aorta, while inhibition of NO synthesis, or guanylate cyclase activity did not further reduce relaxation to acetylcholine in growth-restricted offspring (Payne *et al.*, 2003). At 4 weeks, both basal and stimulated NO production was also lower in growth-restricted offspring (Payne *et al.*, 2003). These data imply that the observed impairment in endothelial function may have resulted from decreased activity of the NO pathway.

In a second study from the same group, the previous observations of impaired endothelium-dependent dilation to acetylcholine, and lower NO production were confirmed in aortic strips from growth-restricted male offspring, and the additional effects of a postnatal diet high in salt examined (Payne *et al.*, 2004). While statistical comparisons between growth-restricted offspring fed normal and high salt diet were not reported, maximal dilation to acetylcholine appeared to be further impaired by postnatal high salt diet in growth-restricted offspring, whereas the effects of high salt diet on arteries from control offspring were less pronounced. In addition, no effect of high-salt diet on adult mean arterial blood pressure was evident in controls (131±3 vs 129±2mmHg), whereas high salt appeared to further elevate mean arterial blood pressure in growth-restricted offspring (144±4 versus 171±12mmHg) (Payne *et al.*, 2004). These data provide some evidence that the prenatal environment may program relative sensitivity to the effects of lifestyle factors, which then augment the detrimental cardiovascular effects of impaired prenatal growth.

While the previous studies used surgical methods to reduce uteroplacental blood flow, the vascular consequences in offspring from a genetic model of uteroplacental insufficiency have recently been described. The development of an endothelial NO synthase (eNOS) knockout mouse has enabled assessment of vascular function in heterozygous offspring mothered by either eNOS +/+, or eNOS -/- dams. Vascular adaptation to pregnancy in eNOS -/- dams is impaired, resulting in smaller litter sizes, with similar sized pups (Longo *et al.*, 2005). Interestingly, relaxation to acetylcholine in carotid and mesenteric arteries from 7-8 week old heterozygous offspring of eNOS +/+ dams did not differ to wild-type offspring (Longo *et al.*, 2005). However, endothelial function in both carotid and mesenteric arteries from heterozygous offspring of eNOS -/- dams was impaired, and was

instead similar to endothelial function in eNOS -/- offspring (Longo *et al.*, 2005). This study therefore elegantly demonstrates that phenotypic postnatal vascular function is dependent on development *in utero*, independent of genetic inheritance.

1.6.3 Prenatal hypoxia and postnatal endothelial function

Despite the relevance of oxygen limitation before birth in clinical IUGR, the effects of prenatal hypoxia on postnatal vascular function have only been examined in a few studies. While an ambient oxygen concentration of 15% from day 6 – 19 of incubation in chick embryos did not alter dilation to acetylcholine in the femoral artery, or femoral artery branches from 14-15 week old chickens, NO synthase inhibition by L-NAME had less effect on dilation to acetylcholine in chickens exposed to hypoxia *in ovo* (Ruijtenbeek *et al.*, 2003a). These results suggest that a period of hypoxia during development also impairs functioning of the NO pathway, however at 14-15 weeks, compensatory changes in the function of other endothelial vasodilator pathways maintain endothelial function. The impairment of NO-mediated endothelial function may also interact with postnatal age, as younger chickens (aged 3-4 weeks) that were exposed to the same hypoxia protocol before hatching did not demonstrate changes in dilation to acetylcholine, nor in the effect of L-NAME on dilation to acetylcholine (Ruijtenbeek *et al.*, 2003b). In this study L-NAME did not increase constriction to a depolarizing potassium solution in femoral branches from hypoxia exposed chickens, however constriction was increased by L-NAME in arteries from controls. Impairment in basal NO synthesis may therefore precede changes in stimulated NO synthesis following hypoxia during development.

The effects of anemia during late gestation on the subsequent function of mesenteric arteries in 7 month old sheep was assessed in one study, which reported no significant

changes in dilation to acetylcholine, adenosine or SNP (Davis *et al.*, 2002). The role of NO, or other specific endothelium-derived vasodilators in mediating dilation to acetylcholine were not examined in this study. Several potentially confounding factors should also be considered; fetal anemia comprises a reduced hematocrit, unlike fetal hypoxemia due to placental insufficiency where hematocrit is generally increased. Any shear stress component contributing to the long-term impact of prenatal hypoxia may therefore differ when hypoxia results from anemia. It should also be noted that arteries were collected from sheep under halothane and (1:1) nitrous oxide: oxygen anesthesia. Nitrous oxide inhalational anesthesia has previously been demonstrated to modify mesenteric arcade vascular function in dogs (Tverskoy *et al.*, 1985), however it is unclear whether this mode of anesthesia results in persistent changes in isolated mesenteric artery function.

1.6.4 Prenatal malnutrition and postnatal arterial vasoconstrictor function

1.6.4.1 Global undernutrition

The impact of maternal undernutrition during pregnancy on vasoconstrictor responses of peripheral arteries in adult life has also been examined. Vasoconstriction to depolarizing potassium was reduced, while constriction to NA was unaltered in small mesenteric arteries from the 100-120d old female rat offspring of dams who were undernourished by 50% for the second half of pregnancy (Holemans *et al.*, 1999). In contrast, femoral arteries from offspring of dams undernourished by 30% were less sensitive to PE or NA at 20d, while the overall constriction to the thromboxane mimetic U46619 was enhanced (Ozaki *et al.*, 2001). As these pups aged no persistent difference in NA or PE sensitivity was noted, however femoral artery branches from male pups 100 and 200d old still demonstrated enhanced maximal constriction to U46619, while by 200d sensitivity to depolarizing

potassium-induced constriction was also enhanced (Ozaki *et al.*, 2001). No differences were noted in vasoconstrictor responses of adult female rat offspring in this study.

1.6.4.2 Protein restriction

The majority of reports on the effects of protein restriction during pregnancy on adult vascular function have not identified alterations in vasoconstrictor responses. An early study reported that protein undernutrition (8% casein with supplemental methionine) from d14 of pregnancy until 50 days postnatal age decreased iliac artery sensitivity and maximal vasoconstriction to both NA and methoxamine (α1 adrenergic receptor agonist) in 140-180d male offspring (Del Basso *et al.*, 1983). No differences were observed in the vasoconstrictor responses of pial microvessels of 12 week offspring (Lamireau *et al.*, 2002), or in thoracic aorta or small mesenteric arteries of 5 month pregnant offspring (Torrens *et al.*, 2002) following maternal protein restriction during pregnancy. A transient decrease in sensitivity, and maximum constriction to U46619 was observed in 87d male offspring from dams that were 50% protein restricted throughout pregnancy, but this effect was no longer observed by 164d, and was not associated with changes in other vasoconstrictor pathways (Brawley *et al.*, 2003).

1.6.4.3 High fat diet

Currently, there is little evidence to suggest that a maternal diet high in fat throughout pregnancy and lactation influences the vasoconstrictor responses of arteries from adult offspring. Sensitivity to NA was increased in femoral arteries from 60d offspring of dams fed 20% lard throughout pregnancy and lactation (Koukkou *et al.*, 1998). Maximal constriction to AII was reduced in femoral arteries from 180d old male and female

offspring from lard-fed dams (Taylor *et al.*, 2004). However, several other studies have reported no differences in the vasoconstrictor responses of arteries from either male or female offspring of lard-fed dams (Ghosh *et al.*, 2001; Khan *et al.*, 2003; Khan *et al.*, 2004; Armitage *et al.*, 2005).

In rats it therefore appears that global undernutrition throughout pregnancy may enhance sensitivity to thromboxane in mesenteric arteries of male offspring throughout postnatal life. However, there is currently little evidence to suggest that malnutrition resulting from decreased protein intake, or increased saturated fat intake throughout pregnancy produces clear or consistent effects on vasoconstrictor function of the peripheral vasculature of adult offspring.

1.6.5 Placental insufficiency and postnatal arterial vasoconstrictor function

In contrast to the limited effects of various maternal undernutrition protocols on vasoconstrictor function in arteries of adult offspring, several studies have reported enhanced vasoconstrictor responses in offspring where placental insufficiency was induced during pregnancy. Payne et al. reported that reduced uteroplacental perfusion in pregnant rats produced growth-restricted pups that had increased maximum constriction to the α1 adrenergic agonist PE in thoracic aorta at 4, 8 and 12 weeks postnatal age in males (Payne *et al.*, 2003). By 12 weeks growth-restricted male offspring also had enhanced sensitivity to PE in this study (Payne *et al.*, 2003). A second report from this group confirmed enhanced sensitivity, and maximal constriction to PE in 12 week male offspring that were growth-restricted *in utero* as a consequence of reduced uteroplacental perfusion (Payne *et al.*, 2004). Bilateral uterine artery ligation, resulting in reduced litter size, but increased pup birth weight also increased maximal vasoconstriction to PE and 5-hydroxytryptamine (5-

HT) in renal arteries from offspring aged 3 weeks. While α-adrenergic mediated constriction was increased, maximal constriction to NA decreased in renal arteries, consistent with an enhanced β-adrenoceptor mediated vasodilatory effect which was confirmed through assessment of renal artery dilation to isoproterenol (Sanders *et al.*, 2004). Mesenteric, femoral and saphenous arteries were also examined in this study; however no other arteries demonstrated functional changes at this age.

Vasoconstrictor responses were also compared in the heterozygous mouse offspring of eNOS +/+ and eNOS -/- mice, where eNOS -/- dams, through impaired cardiovascular adaptation to pregnancy may be considered to present a model of uteroplacental insufficiency. Interestingly, consistent with the effects of surgically induced uteroplacental insufficiency, 7-8 week old heterozygous offspring from eNOS -/- dams demonstrated increased potassium-induced contraction, and enhanced maximal constriction and sensitivity to PE in both carotid and mesenteric arteries (Longo *et al.*, 2005). Unlike the effects of intrauterine stress on the renal artery reported by Sanders et al., dilation to the β adrenergic agonist isoproterenol was decreased in offspring from eNOS -/- dams, suggesting that vasoconstriction to NA would also be enhanced in arteries from these offspring (Longo *et al.*, 2005). Heterozygous offspring who were mothered by an eNOS +/+ dam did not demonstrate changes in vasoconstrictor responses.

1.6.6 Prenatal hypoxia and postnatal arterial vasoconstrictor function

From the results discussed so far there is evidence to suggest that uteroplacental insufficiency, which reduces both fetal oxygen and nutrient supply, increases vasoconstrictor responses in arteries from adult offspring, while there is limited evidence to support enhanced vasoconstrictor responses in offspring exposed to prenatal undernutrition

alone. This may suggest that effects on vasoconstrictor sensitivity in adult life relate more to deficits in fetal oxygen than nutrient supply. The long-term effects of chronic hypoxia *in ovo* on the vasoconstrictor responses of the femoral artery and femoral artery side branches have been determined in male and female chickens aged 14-15 weeks. At this age, femoral arteries were more sensitive to electrical field stimulation, but did not demonstrate changes in NA or potassium sensitivity (Ruijtenbeek *et al.*, 2003a). Side branches of femoral arteries did not demonstrate changes in vasoconstriction to NA, potassium or electrical field stimulation. Consistent with the results of this study, no changes in vascular contraction induced by 63 mM potassium were observed in the side-branches of the femoral arteries from 3-4 week old chickens (Ruijtenbeek *et al.*, 2003b). It should also be noted that chronic hypoxia during development did not increase vascular adrenergic sensitivity in chick embryos, while maximal constriction to PE was increased in femoral arteries from fetal sheep following maternal hypoxia, and there may be species differences in the impact of hypoxia on vascular function. The timing of development of the sympathetic nervous system, including peripheral vascular innervation differs considerably among species. Such differences may result in differential sensitivity of components of the sympathetic nervous system to the impact of reduced oxygen supply during development. Experimental fetal anemia during late gestation, which reduced fetal blood oxygen content significantly, tended to increase maximum constriction angiotensin II in mesenteric arteries from 7 month old sheep without modifying constriction to potassium or NA (Davis *et al.*, 2002).

1.7 SUMMARY

Studies in both humans and animals have identified changes in vascular function following IUGR which may contribute to the increased risk of developing adult hypertension and cardiovascular disease. Where fetal growth restriction occurs as a consequence of impaired

utero-placental blood flow or placental dysfunction the fetus experiences limitation of both oxygen and nutrient supply. Currently, although studies have described the effects of maternal undernutrition on adult vascular function, the long-term effects of prenatal hypoxia on vascular function in mammals are unclear. The available evidence may suggest, however, that there are differential effects of hypoxia and undernutrition on the balance of endothelial function and smooth muscle vasoconstrictor sensitivity within the peripheral circulation. The fetal circulatory response to hypoxia is well characterized, and prenatal adaptation to hypoxia represents a strong candidate mechanism that may link the fetal vascular adaptation to a sub-optimal intrauterine environment to later vascular phenotype, and cardiovascular disease risk. However, the specific effects of hypoxia on vascular function before birth, and persistence of changes in vascular function into postnatal life remain to be determined.

1.8 GENERAL AIM

The general aim of the research described within this thesis was therefore to determine the effects of chronic hypoxia before birth on vascular function. This general aim translated into the following experimental aims, which focused investigations on the effects of prenatal hypoxia on vascular function during prenatal, early postnatal and adult life.

1.9 SPECIFIC AIMS AND EXPERIMENTAL APPROACH

Chapter 2

To determine the effects of chronic hypoxia in late gestation on regional vascular responses in newborn rat pups to vasoconstrictor factors which are known to be implicated in the redistribution of fetal cardiac output.

- Pregnant rats were housed in room air or in chambers maintained at 12% O_2 from d15 to d21 of gestation (term = 22 days). Chronic hypoxia during pregnancy has previously been demonstrated to reduce maternal food intake (Van Geijn *et al.*, 1980; de Grauw *et al.*, 1986). Therefore, the effects of equivalent nutritional restriction alone during the last week of pregnancy were determined in a third group of pregnant rats.
- Within 4-12h of birth vascular function was assessed in carotid and femoral arteries isolated from neonatal offspring of all 3 animal groups. Specifically, vasoconstriction to the a1 adrenergic receptor agonist, phenylephrine, and to endothelin-1 were assessed, as both adrenergic and endothelin-1 mediated vasoconstriction have been implicated in the fetal vascular adaptation to hypoxia.
- This study also characterized the effects of maternal hypoxia on weight gain during pregnancy, neonatal size and relative organ proportions at birth.

Chapter 3

1. **To determine the impact of chronic hypoxia during late gestation on endothelium-dependent vasodilation in resistance arteries from adult rat offspring.**
2. **To determine whether chronic hypoxia during late gestation modifies the contribution of specific endothelial-derived vasodilators to endothelium-dependent relaxation in resistance arteries from adult rat offspring.**

- Rodent studies are well suited to assessing the long-term effects of impaired prenatal development, and were therefore used for this series of studies. Vascular function of small mesenteric arteries was assessed in male offspring from control, hypoxia and nutrient restriction dams at 4 or 7 months of age. Specifically,

vasoconstriction to phenylephrine was assessed, and endothelium-dependent vasodilation to the acetylcholine analogue, methacholine determined. The contribution of NO and prostaglandin synthesis to endothelium-dependent responses was also assessed using specific enzyme inhibitors, while the significance of NO scavenging by superoxide anion was determined by incubation with exogenous superoxide dismutase.

Chapter 4

To determine the impact of placental insufficiency, and associated chronic hypoxia on vasoconstrictor responses of resistance arteries in the fetal sheep to NA, a key factor involved in the fetal circulatory response to acute hypoxia, and implicated in the cardiovascular adaptation to chronic hypoxia.

- Small arteries within the fetal sheep mesenteric arcade are of suitable size and viability to study using wire myography. This investigation was therefore able to determine the impact of fetal compromise on the function of small peripheral arteries during fetal life. Surgery was performed in non-pregnant ewes to remove the majority of endometrial caruncles, in order to reduce subsequent placental size, and thus limit fetal growth. Between 110-118d gestation, vascular catheters were implanted in control and placentally restricted fetal sheep for assessment of fetal arterial blood gas status. In late gestation (136-141d gestation where term is ~150d) post mortem was performed and a section of mesenteric arcade collected. Vasoconstrictor responses to noradrenaline were determined in both 3rd and 4th order small mesenteric arteries.

CHAPTER 2

DIFFERENTIAL EFFECTS OF MATERNAL HYPOXIA OR NUTRIENT RESTRICTION ON CAROTID AND FEMORAL VASCULAR FUNCTION IN NEONATAL RATS

2.1 SUMMARY

In response to reduced oxygen or nutrient supply, the fetus may redistribute cardiac output to conserve brain and heart growth, at the expense of the peripheral tissues. It is not known, however, whether alterations in vascular function are maintained after birth, or whether reduced fetal oxygen, versus nutrient supply produce distinct effects. Using a pressure myograph, we examined isolated carotid and femoral artery responses to phenylephrine and endothelin-1 in neonatal rats, following either reduced maternal oxygen or global nutrient restriction during late gestation. Timed-pregnant Sprague Dawley rats were randomly assigned to control, (n=10), hypoxia, (12% O_2, n=9) or nutrient restriction (40% of control diet, n=7) protocol and treated from d15-21 of pregnancy. Pups were collected 3-12h after birth. Neonatal weights ($P < 0.001$), and relative liver weights ($P < 0.001$) were lower in hypoxia and nutrient restriction offspring compared to control, while relative heart weights were greater in hypoxia than in control or nutrient restriction offspring ($P < 0.01$). Constriction to phenylephrine was reduced in carotid arteries from hypoxia and nutrient restriction neonates compared to control ($P<0.001$), while the femoral artery response was greater in hypoxia neonates compared to control or nutrient restriction pups ($P<0.01$). Only hypoxia reduced carotid responses to endothelin-1, while no differences were observed in the endothelin-1 responses in femoral arteries. Maternal hypoxia and nutrient restriction produced distinct effects on heart growth and neonatal vascular function, suggesting that regional changes in cardiovascular function following poor fetal growth are dependent on the nature of the insult *in utero*.

2.2 INTRODUCTION

Intrauterine growth restriction, (IUGR) is associated with increased morbidity and mortality in the perinatal period (Ghidini, 1996). A series of epidemiological studies has also demonstrated that poor growth *in utero* is associated with increased risk of developing cardiovascular disease and hypertension in later life (Barker, 1995, 1998; Huxley *et al.*, 2000). It has been suggested that the mechanisms by which the fetus adapts to a sub-optimal intrauterine environment may allow its continued growth *in utero*, but also increase the subsequent risk of developing cardiovascular disease (Barker, 1995; McMillen *et al.*, 2001). It has now been demonstrated that infants (Martin *et al.*, 2000a; Goh *et al.*, 2001; Norman & Martin, 2003), children (Martin *et al.*, 2000b) and young adults (Goodfellow *et al.*, 1998; Leeson *et al.*, 2001) who were of low birth weight have impaired peripheral vascular function compared with contemporaries of appropriate birth weight. This may suggest that when growth *in utero* is restricted, early alterations in local vascular function result in a persistent impairment in peripheral vascular function after birth. While such changes may contribute to the increased risk of developing hypertension and cardiovascular disease in adult life, it is not yet clear how specific deficits in the *in utero* environment may alter vascular function after birth.

Placental insufficiency, which limits the supply of both oxygen and nutrients to the fetus, is a major clinical cause of IUGR, (Henriksen & Clausen, 2002; Gagnon, 2003). There is now substantial evidence that in animal models, poor maternal nutrition during pregnancy can impair fetal (Ozaki *et al.*, 2000; Nishina *et al.*, 2003) and adult vascular function, (Holemans *et al.*, 1999; Franco *et al.*, 2002a; Franco *et al.*, 2002b; Brawley *et al.*, 2003). Similarly, acute hypoxia *in vitro*, and chronic maternal hypoxia for four days has been demonstrated to impair endothelial function in the fetal guinea pig carotid artery, however

this impairment was not observed following seven days chronic hypoxia (Thompson & Weiner, 1999). Although it has been clearly demonstrated that one of the important fetal adaptations to either acute or sustained hypoxia is a redistribution of fetal cardiac output, to ensure a preferential supply of nutrients to, and thus maintained growth of the brain and heart at the expense of the periphery (Cohn *et al.*, 1974; Peeters *et al.*, 1979), it is not yet known whether changes in regional vascular function following fetal hypoxia may persist after birth.

The redistribution of fetal cardiac output during hypoxia may be partially dependent on the reported increased circulating levels of several vasoactive factors, including noradrenaline (Giussani *et al.*, 1993; Simonetta *et al.*, 1997) and endothelin-1 (Hashiguchi *et al.*, 1991; Arslan *et al.*, 2004), but may also involve changes in the local vascular responses to these circulating factors. The effects of chronic hypoxia *in utero* on the regional vascular responses to these vasoconstrictors after birth are not yet known.

In order to determine whether reduced oxygen supply *in utero* influences regional vasoconstrictor responses at birth, prior to the additional influences of various determinants of postnatal growth, we have investigated the effects of chronic maternal hypoxia on the vascular responses of two distinct arteries from neonatal offspring: the carotid artery, which supplies blood flow to the brain, and the femoral artery, which supplies the periphery. We have tested the hypothesis that chronic maternal hypoxia in rats reduces neonatal carotid artery responses to the vasoconstrictors phenylephrine and endothelin-1, while increasing the responses of neonatal femoral arteries to these factors. As it has previously been demonstrated that chronic hypoxia reduces food intake in pregnant rats (Van Geijn *et al.*, 1980; de Grauw *et al.*, 1986; Gleed & Mortola, 1991), we also determined the effects of

maternal nutritional restriction during late gestation on the vascular responses of arteries from the neonatal offspring.

2.3 MATERIALS AND METHODS

2.3.1 Animals

All procedures in this study were approved by both the University of Adelaide Animal Ethics Committee and the University of Alberta Animal Welfare Committee. Female Sprague Dawley rats were obtained at 3 months of age, (Charles River, Quebec, Canada) and were mated within the animal facility after a minimum 1-week acclimatisation. Pregnancy was confirmed by the presence of sperm in a vaginal smear examined microscopically the following morning, and this was considered day 0 of pregnancy. All rats received food (standard lab rat chow) *ad libitum* from day 0 – 15 of pregnancy. On day 15, rats were randomized to control, maternal hypoxia or maternal nutrient restriction protocols. Throughout pregnancy, rats were housed individually in standard rat cages which were maintained in a clean conventional facility, with 60% humidity, a 12h light: 12 h darkness light cycle, and *ad libitum* access to water. Control group rats (n=10) were housed in room air on a rack in the same room as the chambers that hypoxia and nutrient restricted dams were housed in, and were fed *ad libitum* throughout pregnancy. Food intake, and weight gain were measured daily in all pregnant rats.

2.3.2 Maternal Hypoxia

In order to reduce maternal oxygen supply during late gestation, 9 pregnant rats were placed inside a plexiglass chamber (volume: 140L) on day 15 of pregnancy, which was maintained at 12% oxygen by continuous infusion of a mixture of nitrogen and compressed air, without additional infusion of carbon dioxide. Previous investigations have

demonstrated that maternal hypoxia, ranging between 9.0-14.0% oxygen for varying lengths of time reduced fetal growth, and altered organ proportions, validating the use of this technique to induce asymmetric fetal growth restriction (Van Geijn *et al.*, 1980; de Grauw *et al.*, 1986). The oxygen concentration of the chamber was monitored throughout treatment using a portable oxygen analyser, which was calibrated daily (Hudson RCI, Temecula, CA, USA). The chamber, which housed a maximum of three pregnant rats maintained individually in separate standard rat cages at any time, was opened briefly once/day to weigh rats and food, and to clean cages. Rats were removed from the chamber on the morning of day 22 of pregnancy, and housed in room air until delivery.

2.3.3 Maternal Nutrient Restriction

A group of pregnant rats (n=7) randomized to a nutrient restriction protocol were placed inside a second, identical plexiglass chamber on day 15 of pregnancy, which was continuously infused with compressed air. Oxygen concentration was checked periodically to ensure rats were exposed to 21% oxygen. The chamber was opened briefly once/day to allow rats to be weighed and cages cleaned. From day 15 rats were administered 11.5 $\pm$ 1g standard rat chow/day, which was equivalent to the lowest food intake recorded in rats exposed to maternal hypoxia, and represented 40 % of control food intake during this time. The number of dams used for the hypoxia and nutrient restriction groups varied due to problems encountered with equipment function and availability. While rats were intended to remain in the chamber until day 22 of pregnancy, in 6 of the 7 rats, delivery occurred on day 21, and thus rats were removed from the chamber at this time. All rats were returned to normal diet following delivery.

2.3.4 Tissue collection

Within 3-12h after birth, all pups were weighed, and litter size was reduced to 8 pups, which were returned to the dam for use in other studies. The remaining neonatal rats were collected at this time, and were euthanised by decapitation. Brain, heart, lung, liver and kidneys were dissected from 1-4 pups per litter for the determination of relative organ proportions. Vascular function experiments were performed in arteries isolated from one neonate/litter from a total of 7 control, 7 maternal hypoxia and 5 maternal nutrient restriction litters; in the remainder of animals the timing of birth prevented vascular experiments being performed, however maternal and neonatal weight/organ weight data pertinent to the animal model has been included. Carotid and femoral arteries were gently dissected free of connective tissue in ice-cold Dulbeccos Modified Eagle Medium buffer (Loutzenhiser *et al.*, 2002) (DMEM, mmol/L; 1 sodium pyruvate, 25 sodium bicarbonate, 5 HEPES, 5 D-Glucose, containing 1.8 calcium, 5.4 potassium, and 137.2 sodium; pH 7.4, Sigma, Missouri, USA) for the assessment of vascular function. For each vessel, the dissection was extended to provide the longest segment of artery that is practical to dissect from a rat at this developmental stage, (2-3mm), and the full segment was mounted for study, thus avoiding any regional differences in vessel reactivity.

2.3.5 Vessel preparation and equipment

Following dissection, 2-3 mm sections of carotid and femoral arteries were mounted on a pressurized myograph system (Living Systems, Burlington, VT, USA) in a 2.5 ml organ bath filled with DMEM. This technique was chosen as it applies minimal mechanical stress to the artery being studied, making it ideal for the study of delicate neonatal arteries. It should be noted, however, that endothelial function was not directly assessed in this protocol. Briefly, one end of the artery was gently opened and slipped over the tip of a glass

cannula (60-80 μm diameter), which was then secured with a short section of synthetic thread. Blood was then flushed from the preparation by gently flowing medium through the lumen of the artery. A second glass cannula was positioned inside the open end of the artery, and secured by thread. Flow through one cannula was blocked by means of a stop-cock, while flow through the second cannula enabled the artery preparation to be pressurized at 12 mmHg using a pressure-servo regulated peristaltic pump (Living Systems, Burlington, VT, USA). The preparation was allowed to stabilize for 30 min at 37°C. This pressure was chosen on the basis of preliminary studies, which indicated that 12 mmHg was sufficient to maintain viable carotid and femoral arteries, with adequate lumen visibility to allow accurate measurement. Vessel viability was reduced at higher pressures. Following stabilization, concentration response curves were performed to determine the vascular response to increasing concentrations of the vasoconstrictor phenylephrine (1 nmol/L – 50 μmol/L, Sigma, St Louis, MO, USA). After 4 min exposure to each concentration, artery lumen diameter and wall thickness was measured in two places using an inverted microscope (Nikon, TS100-F) and video camera (Sony, CCD monochrome XC-ST30) in concert with a video dimension analyser (Living Systems, Burlington, VT, USA, measurement precision 1-2 μm), and subsequently averaged. In a subset of arteries, after completion of the phenylephrine concentration response curve, vessels were washed in DMEM for 1 h, before the vascular responses to endothelin-1 were determined by the same method (0.1 nmol/L – 0.1 μmol/L, Sigma, St Louis, MO, USA). Fresh medium was supplied at 10 min intervals throughout the experiment.

2.3.6 Data Analysis

All data are presented as mean ± standard error of the mean. Maternal characteristics, neonatal weight, organ weights and relative organ proportions were compared using One-

Way ANOVA and, where relevant, Tukey post-hoc analysis. Maternal weight gain and food intake before, and after the start of treatment were compared using Two-Way ANOVA for repeated measures, followed by Tukey post hoc test. Artery lumen diameter, and wall thickness at baseline were compared among groups using One-Way ANOVA and Tukey post hoc test. Concentration response curves are presented graphically as the mean percent constriction ± standard error of the mean at each point. Percent constriction was determined as the artery lumen diameter following drug administration expressed as a fraction of baseline lumen diameter. Vascular responses in this study were compared among groups using Two-Way ANOVA for repeated measures, as the nature of the concentration response curve generated by femoral arteries to phenylephrine made calculation of EC_{50} values inappropriate. Where possible, comparisons of EC_{50} values were also performed however, and for this purpose EC_{50}s were calculated using Sigma Plot 8.0 Pharmacology Standard Curves Analysis. Statistical significance was defined as $P<0.05$, and is indicated within figures by the use of different letters.

2.4 RESULTS

2.4.1 Maternal Characteristics

There was no difference in maternal age or weight at the start of pregnancy, or in the observed litter size among groups (Table 1). There were also no significant differences in the number of stillborn pups among treatment groups (Table 1). Food intake prior to treatment was not different among groups. Maternal hypoxia significantly reduced food intake during the treatment period, compared to control, ($P<0.001$) while food intake in the nutrient restriction group was also lower ($P<0.001$) than control, but not significantly different to the hypoxia group (Figure 1A). Similarly, weight gain prior to pregnancy was not different among groups, but weight gain during treatment was significantly reduced in

both maternal hypoxia and maternal nutrient restriction groups (P<0.001). Maximal weight gain on day 21 of pregnancy was greater in control (C) than in maternal hypoxia (H) or nutrient restriction (NR), (C: 149.8 ± 2.5g Vs H: 132.8 ± 2.2g or NR: 126.0 ± 1.7g, P<0.001). When the day of delivery was compared between groups, delivery occurred earlier in nutrient restriction dams than in hypoxia dams (H: 22.4 ± 0.2d vs NR: 21.2 ± 0.1d, P<0.05), however neither group was significantly different from control (C: 21.8 ± 0.1d).

Table 2.1: ***Maternal and litter characteristics***

Group	Age (weeks)	Pre-pregnant Weight (g)	Litter size (#)	Total number Stillborn/live pups
Control (n=10)	15.3 + 0.6	307 + 7	15.4 + 0.7	3/151
Hypoxia (n=9)	16.0 + 0.7	312 + 8	13.6+ 0.6	8/114
Nutrient Restriction (n=7)	15.3 + 0.4	318 + 5	15.4 + 0.7	1/108

There were no significant differences among the groups

Figure 2.1A

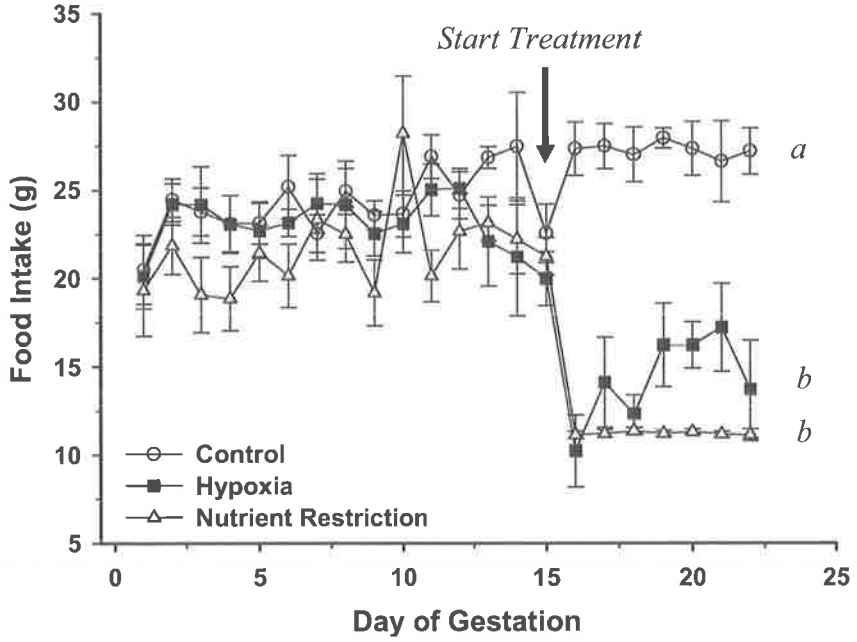


Figure 2.1B

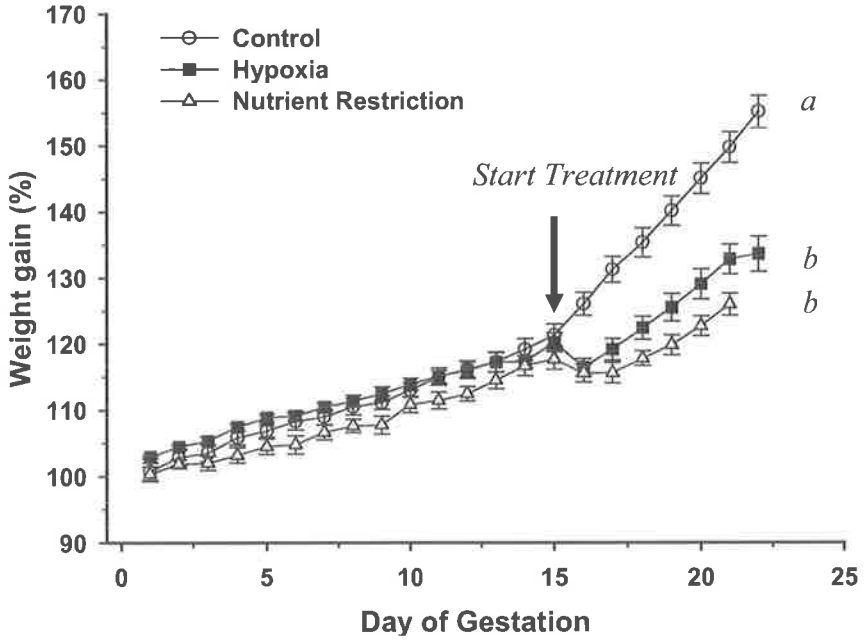


Figure 2.1: Maternal food intake and weight gain during pregnancy

Food intake **(A)** and weight gain **(B)** during pregnancy in the maternal hypoxia (black squares, n=9), nutrient restriction (grey triangles, n=7) and control (open circles, n=10) groups. Different letters denote significant differences ($P<0.05$) among groups.

2.4.2 Neonatal and Organ Weights

Neonatal body weight was reduced by both hypoxia and nutrient restriction protocols, compared to control (C: 6.5 ± 0.1g Vs H: 5.9 ± 0.1g or NR: 5.6 ± 0.1g, $P<0.001$, Figure 2A). Neonatal heart and brain weights were significantly higher in offspring from hypoxia than nutrient restriction dams, while liver weights were lower in both groups compared to control ($P<0.05$, Table 2). Lung weights were not different between groups; however combined kidney weights were significantly lower in pups from nutrient restriction dams than from either control or hypoxia dams (Table 2). When organ weights were expressed as a proportion of body weight, it was observed that heart weights constituted a greater proportion of body weight in pups from hypoxia than from either control or nutrient restriction dams. (Figure 2B, $P<0.05$) Proportional brain weights were also greater in offspring from hypoxia than control dams, (Figure 2C, $P<0.05$). The relative brain weight in pups from nutrient restriction dams tended to be greater than control, (Figure 2C, $P=0.076$), but was not different from hypoxia. Proportional liver weight was reduced in offspring from both hypoxia and nutrient restriction dams compared to control (Figure 2D, $P<0.01$), while there was no significant difference in the proportional weight of either lung or kidney between groups (data not shown).

Table 2.2: Neonatal organ weights

	Control (n=17 pups/10 litters)	Maternal Hypoxia (n=17 pups/9 litters)	Maternal Nutrient Restriction (n=14 pups/7 litters
Brain (mg)	$276.6 \pm 6.5^{a,b}$	291.1 ± 5.1^{a}	261.2 ± 5.3^{b}
Heart (mg)	$42.3 \pm 1.5^{a,b}$	46.6 ± 2.6^{a}	35.1 ± 1.7^{b}
Liver (mg)	338.2 ± 16.4^{a}	270.2 ± 17.5^{b}	237.2 ± 7.1^{b}
Kidney (mg)	62.9 ± 2.0^{a}	58.0 ± 2.8^{a}	48.8 ± 2.6^{b}
Lung (mg)	130.7 ± 6.7^{a}	106.7 ± 7.5^{a}	121.1 ± 6.2^{a}

Different letters denote significant differences among groups ($P<0.05$)

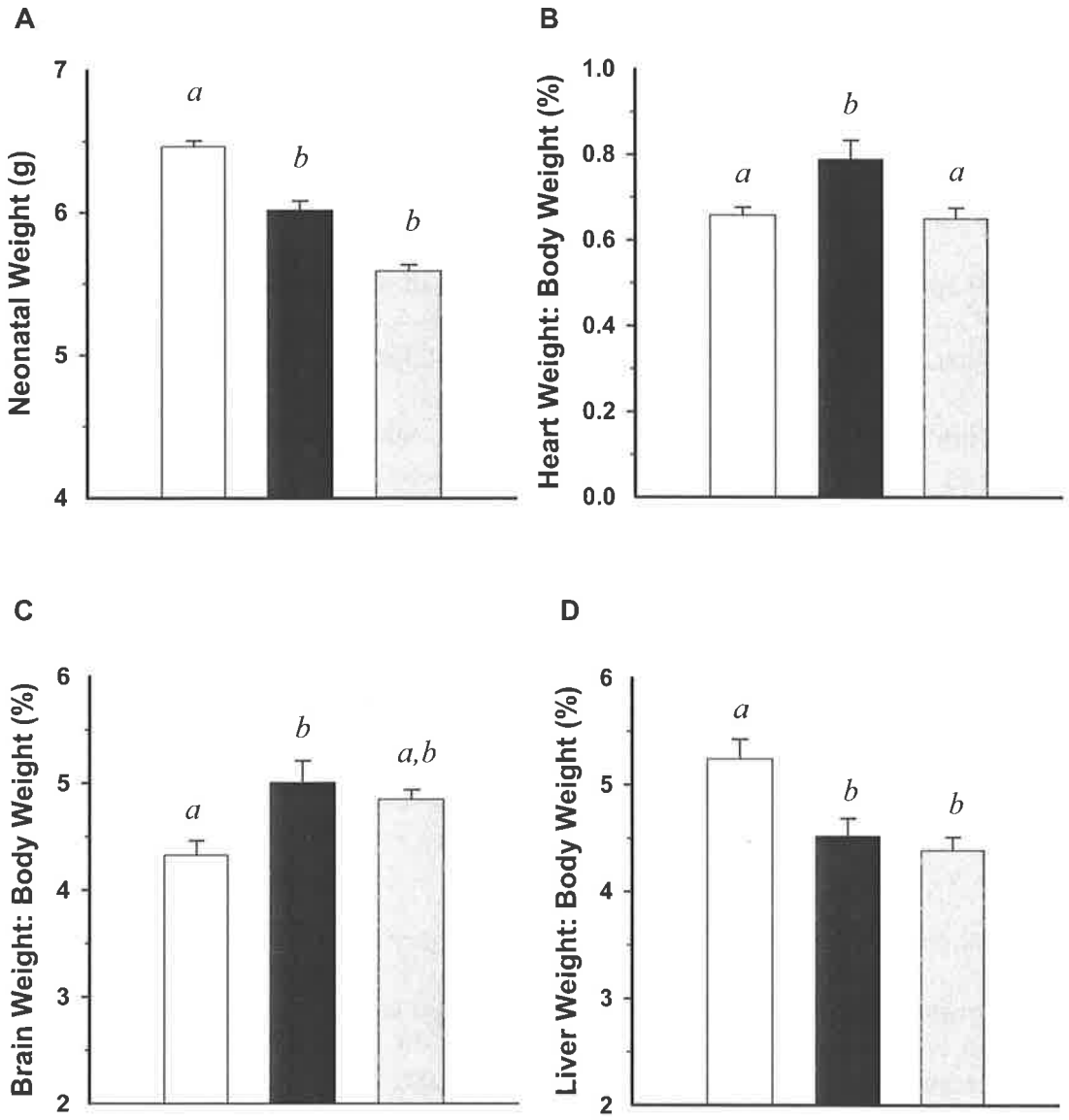


Figure 2.2: Neonatal weights and relative organ proportions

(A) Neonatal weight in offspring from hypoxia (black bar, n=9 litters), nutrient restriction (grey bar, n=7 litters) and control (open bar, n=9 litters) dams. **(B)** Pups from hypoxia dams had greater relative heart weights ($P<0.05$) than pups from either control or nutrient restriction. **(C)** Relative brain weights were greater in pups from hypoxia than control dams, ($P<0.05$) while pups from nutrient restricted dams were not different from either. **(D)** Relative liver weights were lower in pups from both hypoxia and nutrient restriction dams than control ($P<0.001$).

2.4.3 Carotid and Femoral Vascular Responses

After equilibration, and prior to the beginning of the experiment, the lumen diameters of carotid and femoral arteries were not significantly different among groups (Carotid: C: 146.6 ± 17.9μm, H: 137.4 ± 13.2μm, NR: 144.4 ± 18.0μm, Femoral: C: 167.8 ± 19.1μm, H: 186.5 ± 19.1μm, NR: 159.1 ± 26.1μm). Arterial wall thickness was also not different among groups, (Carotid: C: 41.2 ± 7.6μm, H: 30.7 ± 4.3μm, N: 43.0 ± 9.6μm, Femoral: C: 24.5 ± 3.1μm, H: 24.0 ± 3.6μm, N: 28.5 ± 6.6μm. Similarly, the wall: lumen ratio of carotid, and femoral arteries at baseline were not different among groups (Carotid: C: 0.30 ± 0.06, H: 0.22 ± 0.02, NR: 0.32 ± 0.09, Femoral: C: 0.16 ± 0.02, H 0.13 ± 0.02, N: 0.16 ± 0.04). While carotid artery wall thickness tended to be greater (P=0.09) than femoral artery wall thickness in the control group, there was no significant difference in wall thickness between the two arteries in maternal hypoxia or maternal nutrient restriction groups.

Both arterial constriction in response to increasing concentrations of phenylephrine, and the maximal constriction attained were lower in the carotid artery of pups from either hypoxia or nutrient restriction dams compared to control (P=0.002, Figure 3A), while the sensitivity to phenylephrine was not different among groups (EC_{50}: C: 45.1 ± 26 nmol/L, H: 138.0 ± 59.8nmol/L, N: 89.5 ± 25nmol/L). In contrast, femoral artery constriction in response to increasing concentrations of phenylephrine was significantly enhanced in arteries of pups from hypoxia dams as compared to those from either control or nutrient restriction dams (P=0.003, Figure 3B). Femoral arteries did not sustain constriction at the highest doses of phenylephrine, and thus the maximal constriction was compared at a concentration of 1μmol/L. Maximal constriction was greatest in femoral arteries from pups of hypoxia dams, and was significantly higher than those from control or nutrient restriction dams (H: 55.2 ± 9.2% vs C: 27.8 ± 5.4% or NR: 33.0 ± 7.2%, P<0.01).

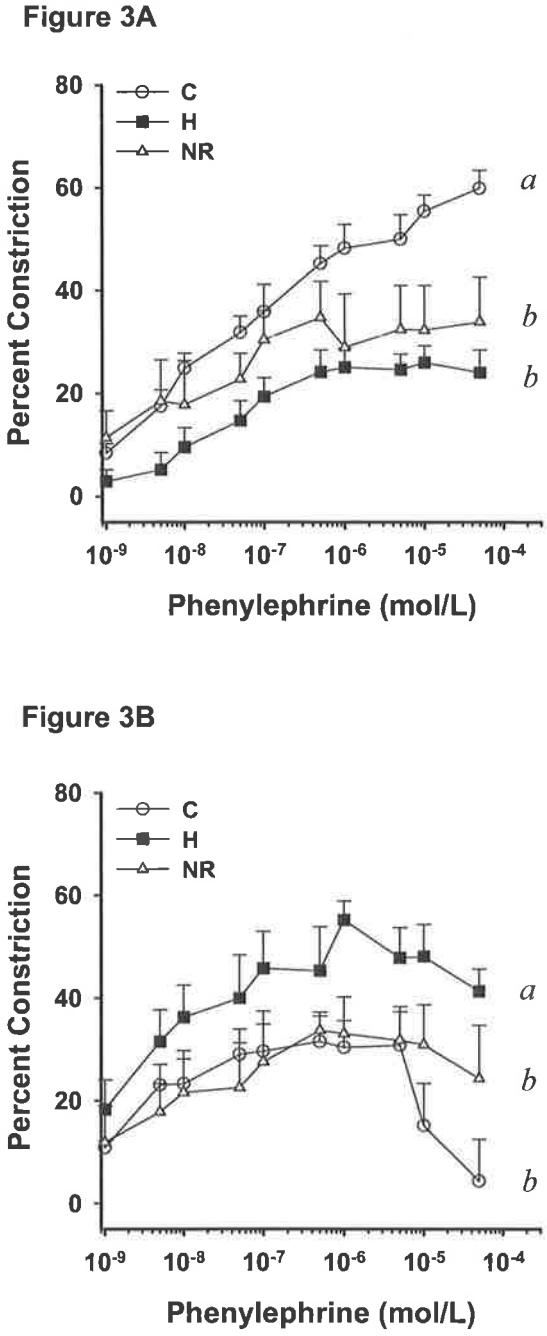
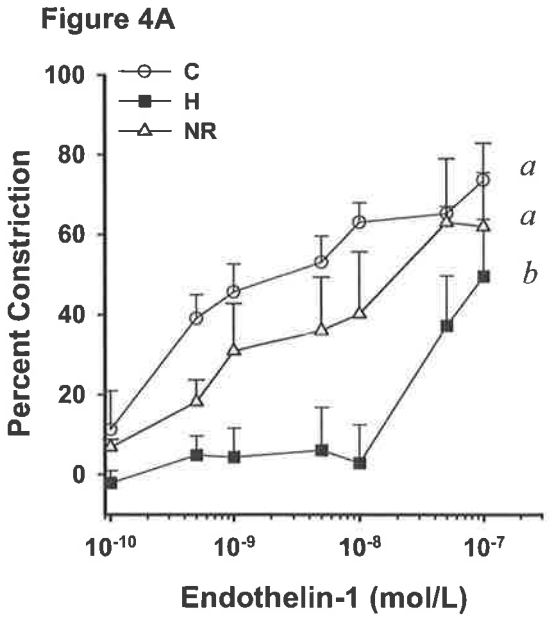


Figure 2.3: Neonatal artery vasoconstriction to phenylephrine

(A) Constriction to phenylephrine in carotid arteries from offspring of hypoxia (H, black squares, n=7) nutrient restriction (NR, grey triangles, n=5) and control (C, open circles, n=6) dams. **(B)** Constriction to phenylephrine in femoral arteries from offspring of hypoxia (n=6) nutrient restriction (n=4) or control (n=7) dams. Different letters denote significant differences ($P<0.01$) among groups.

In a subset of experiments, the vascular response to endothelin-1 was also determined. The cumulative response to increasing endothelin-1 concentration was less in carotid arteries of neonates from hypoxia, but not nutrient restriction dams, compared to control (Figure 4A, $P<0.05$). There were no differences, however, in the maximal carotid artery constriction to endothelin-1, or in the sensitivity to endothelin-1 among groups (EC_{50}: C: 7.11 ± 2.78nmol/L, H: 30.3 ± 15.0nmol/L, N: 11.2 ± 10.5nmol/L). No differences were apparent in the femoral artery response to increasing endothelin-1 concentrations, sensitivity, (EC_{50}: C: 26.0 ± 13.1nmol/L, H: 27.1 ± 6.84nmol/L, N: 56.1 ± 18.4nmol/L. or in the maximal constriction at 0.1 μmol/L endothelin-1 among groups (Figure 4B).



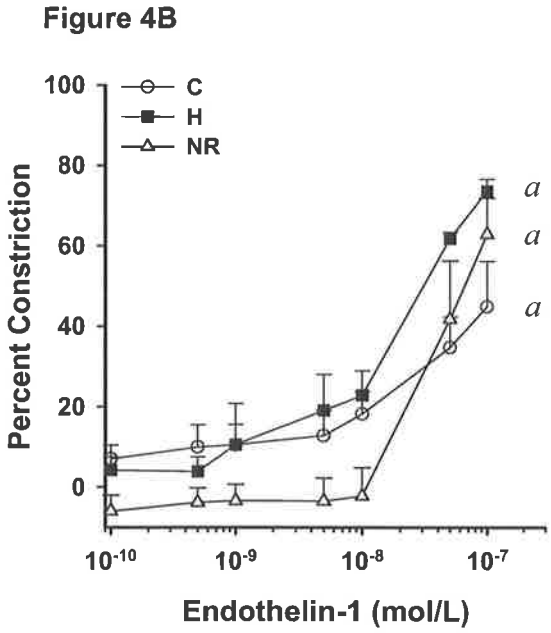


Figure 2.4: Neonatal artery vasoconstriction to endothelin-1

Vasoconstriction to endothelin-1 in **(A)** carotid arteries from offspring of hypoxia (H, black squares, n=3), nutrient restriction (NR, grey triangles, n=3) or control (C, open circles, n=3) dams. **(B)** Femoral artery constriction to endothelin-1 (control, n=5, hypoxia, n=2, nutrient restriction, n=4). Different letters denote significant ($P<0.05$) differences among groups.

2.5 DISCUSSION

Reduction in either maternal oxygen or nutrient supply during the last week of gestation resulted in reduced neonatal size, and perturbations of neonatal organ weight and proportion. These effects are consistent with previous reports of the impact of reduced oxygen or nutrient supply during pregnancy on fetal growth (Van Geijn *et al.*, 1980; Xiao *et al.*, 2000). Conservation of brain and heart growth in offspring from maternal hypoxia dams, and reduced liver growth in offspring from both hypoxia, and nutrient restriction dams suggests that that the two treatments caused changes in peripheral resistance that redistributed cardiac output. Chronic maternal hypoxia, however, specifically increased relative neonatal heart weight, suggesting that fetal growth was differentially affected by the two maternal treatments. This study, to our knowledge, is the first to determine the impact of reduced oxygen and/or nutrient supply *in utero* on regional vasoconstrictor responses in the neonatal rat. While the changes in vascular function observed in offspring from maternal hypoxia supported our hypothesis, interestingly, maternal nutrient restriction produced distinct effects on vascular function, highlighting that the neonatal vascular consequences of fetal growth restriction are dependent on the specific nature of the *in utero* environment.

Several authors have used chronic maternal hypoxia to restrict fetal growth in rats previously, and have demonstrated that across a range of oxygen reductions imposed at different times throughout gestation, this treatment results in reduced fetal, or neonatal body weight, and altered fetal organ growth (Van Geijn *et al.*, 1980; de Grauw *et al.*, 1986; Gleed & Mortola, 1991; Peyronnet *et al.*, 2000; Xiao *et al.*, 2000; Mamet *et al.*, 2002; Ostadalova *et al.*, 2002; Peyronnet *et al.*, 2002; Li *et al.*, 2003a). Decreased maternal food intake as a consequence of maternal hypoxia has also been reported previously (Van Geijn

et al., 1980; de Grauw *et al.*, 1986; Gleed & Mortola, 1991). It is interesting to postulate that this may have effectively resulted in a greater cumulative substrate deficit, and thus constituted a dual insult for the offspring exposed to maternal hypoxia. Although there was a decrease in food intake in the dams exposed to chronic hypoxia, it is likely that the available oxygen supply also limited metabolism, such that nutrient supply was appropriate to metabolic demand. The partial recovery of maternal appetite as hypoxia treatment continued indicates that the pregnant dam may be able to adapt to reduced oxygen. In contrast, the decreased available food in the nutrient restriction group may have presented a greater fetal nutrient deficit. The similar effects of the two treatments on birth weight, and organ growth do not suggest that the restriction of fetal growth was more severe in dams exposed to hypoxia.

The increase in heart weight and proportion observed in offspring from maternal hypoxia treated dams in this study is consistent with previous reports in fetal rats following chronic maternal hypoxia (10.5%) from d19 to d21 of gestation (Xiao *et al.*, 2000). Intermittent hypobaric hypoxia either pre or postnatally in rats also increased heart weight and proportion of body weight, (Ostadalova *et al.*, 2002). It is interesting that several recent studies have demonstrated perturbations in heart development and function following chronic maternal hypoxia (Xiao *et al.*, 2000; Li *et al.*, 2003a).

Our results demonstrated that reductions in maternal oxygen, or nutrient supply produced effects on vascular function, in the absence of significant structural differences, that were consistent with the redistribution of cardiac output, and remained evident in the neonate at birth. While both maternal hypoxia and maternal nutrient restriction reduced the carotid artery response to phenylephrine, only hypoxia increased the femoral artery response to this

vasoconstrictor. Thus changes in vascular function, which serve to maintain brain blood flow *in utero* in fetuses exposed to either hypoxia or nutrient restriction, are still evident some 12h after birth. Only exposure of the fetus to maternal hypoxia, however, resulted in an enhanced femoral vasoconstrictor response to phenylephrine, which highlights that there is a differential effect of restriction of oxygen or nutrient supply on peripheral vascular function in the neonate. Furthermore, while there was a marked reduction in the carotid artery response to endothelin-1 after exposure to maternal hypoxia, there was no effect of maternal nutrient restriction on the carotid artery vasoconstrictor response to this agonist. There were also no differences observed in the femoral artery response to endothelin-1 between neonates exposed to maternal hypoxia or nutrient restriction.

In this study, DMEM was not bubbled with 5% CO_2 since we were using a HEPES buffered system, rather than a bicarbonate buffered system, and the absence of bubbles within the organ bath facilitated measurement of vascular responses with the video dimension analyser. We have been unable to find evidence to demonstrate that HEPES is not able to maintain intracellular pH in vascular tissue, however it is possible that intracellular pH was affected by the absence of CO_2. Overall, cells maintain intracellular pH through a number of intrinsic mechanisms, including Na^+H^+ exchange and phosphates. Indeed, it has been shown that the HEPES "closed" system as well as the bicarbonate "open" system are both well poised to defend the cell against pH changes (Goldsmith and Hilton, Kidney Int. 1992; 41: 43-49), and we therefore consider that this is unlikely to account for the differences in vascular function observed among groups.

The regional changes in vascular function which were observed following each maternal insult may reflect specific fetal vascular adaptations to either reduced oxygen or nutrient

supply. While fetal plasma noradrenaline levels have consistently been reported as increased in response to hypoxia (12, 38, (Gardner *et al.*, 2002a), maternal fasting also results in increased fetal plasma noradrenaline concentrations (Fowden *et al.*, 1998). Spontaneously hypoglycemic fetal sheep do not have increased basal noradrenaline concentrations, but there is a greater increase in plasma noradrenaline in these fetuses in response to acute hypoxia (Gardner *et al.*, 2002a). These findings may suggest that catecholamines are involved in the fetal adaptation to both hypoxia and undernutrition. In vascular tissue, expression of the precursor protein for endothelin-1 is increased by hypoxia-inducible factor-1 (HIF-1) signalling (Hu *et al.*, 1998), and the reported increase in fetal plasma endothelin-1 levels in response to hypoxia (1, 16) may represent a specific adaptation to a reduced oxygen supply.

While this study has demonstrated that either reduced oxygen or nutrient supply during gestation may alter neonatal vascular responses, the mechanisms involved in these alterations are not yet clear. Neonatal carotid artery constriction in response to increasing concentrations of both phenylephrine and endothelin-1 was lower following chronic maternal hypoxia, while sensitivity to each agonist was unchanged. However, the maximal constriction to endothelin-1 was not significantly different among groups, which may suggest that differences in contractile capacity of carotid arteries did not account for the observed responses. The effect of hypoxia on fetal vascular smooth muscle development is currently unclear. Tension generated to depolarising KCl was lower in coronary arteries from fetal sheep exposed to long term, high altitude hypoxia (Garcia *et al.*, 2000). Consistent with the results of the current study however, where no significant effect on carotid wall or lumen size was observed, this treatment appears to have little effect on cerebral artery size, or structure in the fetal sheep (Longo *et al.*, 1993). Acute hypoxia *in*

vitro also slightly decreased the response to depolarising KCl in chick embryo femoral arteries (Ruijtenbeek *et al.*, 2002), however it has previously been demonstrated in chick embryos that chronic hypoxia increased the aortic wall: lumen ratio as a consequence of medial hypertrophic growth (Rouwet *et al.*, 2002), which would suggest that conduit artery smooth muscle mass may be increased, at least in some arteries, by chronic hypoxia. However, in our study there was no difference in the wall thickness or wall to lumen ratio in either the carotid or femoral arteries among the groups suggesting that specific structural changes of the arteries do not account for the observed differences in vasoconstriction.

Maternal nutrient restriction did not reduce carotid artery responses to endothelin-1 in this study, which may indicate that the decreased response was specific to phenylephrine, rather than related to changes in endothelial or vascular smooth muscle function. While previous authors have investigated the effects of maternal undernutrition on isolated vascular function in resistance-sized arteries from peripheral vascular beds in fetal sheep (Ozaki *et al.*, 2000; Nishina *et al.*, 2003), and adult rat (Holemans *et al.*, 1999; Franco *et al.*, 2002a; Brawley *et al.*, 2003), to our knowledge, the effects on carotid artery function have not previously been examined. The increase in femoral artery vasoconstriction following maternal hypoxia treatment was specific to phenylephrine, although the limited data available for endothelin-1 in this vascular bed should be interpreted cautiously.

The sympathetic nervous system has been clearly demonstrated to be involved in the redistribution of cardiac output in response to acute hypoxia in the fetus (Giussani *et al.*, 1993), and it has also been demonstrated that circulating noradrenaline levels are higher in low, than in high birth weight piglets at 3 months of age (Poore *et al.*, 2002). Two recent studies have investigated the effects of chronic hypoxia during incubation on the

functioning of peripheral arteries in the chick embryo, and have demonstrated increased femoral periarterial innervation (Ruijtenbeek *et al.*, 2000) and increased basal sympathetic tone in mesenteric arteries (Rouwet *et al.*, 2002) respectively. Our results would suggest an increased responsiveness of peripheral arteries to α1 adrenergic receptor activation following chronic hypoxia in the fetal rat, which is still evident at birth.

While there were no differences in the responses of femoral arteries from offspring of nutrient restricted dams relative to control in this study, it has previously been demonstrated that nutrient restriction impaired endothelial dependent, and independent relaxation of resistance sized arteries from the femoral vascular bed in fetal sheep (Ozaki *et al.*, 2000; Nishina *et al.*, 2003). It is possible that any reductions in femoral blood flow following maternal nutritional restriction affect the functioning of resistance sized arterioles, but not conduit arteries. Interestingly, it has previously been demonstrated that reduced maternal protein intake produces alterations in the function of small mesenteric arteries, without affecting responses in the thoracic aorta of adult male offspring (Torrens *et al.*, 2002). The differences we observed between the responses of femoral arteries from the offspring of hypoxia and nutrient restricted dams may reflect underlying differences in the mechanisms mediating redistribution of cardiac output in response to these two treatments. Several possible mechanisms may contribute to the altered regional vascular responses to phenylephrine and endothelin-1 observed in this study, including regional changes in receptor expression, calcium sensitivity, or vascular smooth muscle maturation.

In conclusion, we have demonstrated that both reduced maternal oxygen and nutrient supply in late gestation restricted fetal growth, and led to perturbations of neonatal vascular responses to vasoconstrictor agents in the absence of changes in artery wall thickness, or

wall: lumen diameter ratio. As regional vascular responses were differentially altered by reduced oxygen versus nutrient supply, this study further demonstrates that changes in vascular function at birth are dependent on the specific nature of the insult during development. The regional changes in vascular function observed in this study may underlie the redistribution of cardiac output during fetal life, and thus may have contributed to conserving brain growth during development. Persistent alterations in vascular function in the IUGR neonate may impact cardiovascular regulation in these infants, however, and thus contribute to the development of adverse postnatal outcomes associated with impaired fetal growth. These data also highlight the heterogeneity of vascular effects following impaired fetal growth that may be expected within human populations, where the underlying causes of intrauterine growth restriction are diverse.

CHAPTER 3

EFFECTS OF MATERNAL HYPOXIA OR NUTRIENT RESTRICTION DURING PREGNANCY ON ENDOTHELIAL FUNCTION IN ADULT MALE RAT OFFSPRING

3.1 SUMMARY

Compromised fetal growth impairs vascular function, however it is unclear whether chronic hypoxia *in utero* affects adult endothelial function. We hypothesized that maternal hypoxia (H, 12% O_2, n=9) or nutrient restriction (NR, 40% of control, n=7) imposed from d15-21 pregnancy in rats would impair endothelial function in adult male offspring (relative to control, C, n=10). Using a wire myograph, endothelium-dependent relaxation to methacholine was assessed in small mesenteric arteries from 4 and 7 month (mo) male offspring. Nitric oxide (NO) mediation of endothelium-dependent relaxation was evaluated using L-NAME (NO-synthase inhibitor). Observed differences in the NO pathway at 7mo were investigated using exogenous superoxide dismutase (SOD) to reduce NO scavenging, and sodium nitroprusside (SNP, NO-donor) to assess smooth muscle sensitivity to NO. Sensitivity to methacholine-induced endothelium-dependent relaxation was reduced in H offspring at 4mo ($P<0.05$), but was not different among groups at 7mo. L-NAME reduced methacholine sensitivity in C ($P<0.01$), H ($P<0.01$) and NR ($P<0.05$) offspring at 4mo, but at 7mo L-NAME reduced sensitivity in C ($P<0.05$), tended to in NR ($P=0.055$) but had no effect in H offspring. SOD did not alter sensitivity to methacholine in C, but increased sensitivity in H offspring ($P<0.01$). SNP responses did not differ among groups. In summary, prenatal hypoxia, but not nutrient restriction impaired endothelium-dependent relaxation at 4mo, and reduced NO-mediation of endothelial function at 7mo, in part through reduced NO bio-availability. Distinct effects following reduced maternal oxygen versus nutrition suggest that decreased oxygen supply during fetal life may specifically impact adult vascular function.

3.2 INTRODUCTION

A series of epidemiological studies have demonstrated that poor growth *in utero* is associated with an increased risk of developing cardiovascular disease, including hypertension, in later life (Barker, 1995, 1998; Huxley *et al.*, 2000). Alterations in peripheral vascular endothelial function following restriction of fetal growth may contribute to the development of cardiovascular disease in adult life. Impaired endothelium-dependent vascular relaxation has recently been demonstrated in the peripheral vascular beds of low birth weight infants (Martin *et al.*, 2000a; Norman & Martin, 2003), children (Martin *et al.*, 2000b) and young adults (Goodfellow *et al.*, 1998; Leeson *et al.*, 2001). The mechanisms underlying impaired endothelial function in individuals of low birth weight, however, are currently unclear.

Placental insufficiency, a major clinical cause of fetal growth restriction (Henriksen & Clausen, 2002), reduces the supply of both oxygen, and nutrients to the fetus (Owens *et al.*, 1989). There is evidence that either undernutrition (Ozaki *et al.*, 2000; Nishina *et al.*, 2003) or hypoxia (Thompson & Weiner, 1999) *in utero* may influence fetal endothelial function. Endothelial function is also impaired in adult rat offspring following global undernutrition (Franco *et al.*, 2002a; Franco *et al.*, 2002b), or protein restriction during pregnancy (Torrens *et al.*, 2002; Brawley *et al.*, 2003). Reduced production of (Franco *et al.*, 2002a) and/or sensitivity to endothelial-derived NO (Ozaki *et al.*, 2001; Brawley *et al.*, 2003) may contribute to these effects on adult endothelial function. Although placental insufficiency reduces both oxygen and nutrient supply to the fetus, the effects of reduced fetal oxygen supply on adult endothelial function are currently less clear. In chickens, chronic hypoxia *in ovo* increased adult vascular contractile responses, but did not affect endothelium-dependent relaxation to acetylcholine in femoral artery branches (Ruijtenbeek *et al.*,

2003a). However, NO modulation of this vascular relaxation to acetylcholine was reduced in chickens exposed to hypoxia before hatching (Ruijtenbeek *et al.*, 2003a). Thus, although there is evidence to suggest that chronic hypoxia during development may impact the adult vascular NO pathway, the consequences for endothelial function in mammals remain unclear.

To clarify the effects of prenatal hypoxia on postnatal endothelial function, we investigated the effects of chronic maternal hypoxia on endothelium-dependent relaxation in isolated mesenteric arteries from adult male offspring. Since food intake is reduced in pregnant rats exposed to hypoxia, (Van Geijn *et al.*, 1980; de Grauw *et al.*, 1986; Gleed & Mortola, 1991; Williams *et al.*, 2005) we also examined the effects of reduced maternal nutrition across the same period of gestation. We hypothesized that either reduced maternal oxygen, or nutrient supply during the third week of pregnancy would reduce endothelium-dependent relaxation in adult male offspring. Since the endothelial-derived vasodilators NO and prostacyclin are important regulators of resistance artery tone, we also specifically assessed the role of NO and prostaglandins in mediating endothelium-dependent relaxation.

3.3 MATERIALS AND METHODS

3.3.1 Animals

All procedures in this study were approved by both the University of Adelaide Animal Ethics Committee and the University of Alberta Animal Welfare Committee. Female Sprague Dawley rats were obtained at 3 months of age, (Charles River, Quebec, Canada) and were mated within the animal facility after a minimum 1-week acclimatisation. Pregnancy was confirmed by the presence of sperm in a vaginal smear examined microscopically the following morning, and this was considered day 0 of pregnancy (term =

22 days). All rats received food (standard lab rat chow) *ad libitum* from day 0 – 15 of pregnancy. On day 15, rats were randomized to control (C, n=10), maternal hypoxia (H, n=9) or maternal nutrient restriction (NR, n=7) protocols. Throughout pregnancy, rats were housed individually in standard rat cages which were maintained in a clean conventional facility, with 60% humidity, a 12h light: 12 h darkness light cycle, and *ad libitum* access to water. Control group rats were housed in room air, and fed *ad libitum* throughout pregnancy. Food intake, and weight gain were measured daily in all pregnant rats.

3.3.2 Maternal Hypoxia or Nutrient Restriction Protocols

As has previously been described, (Williams *et al.*, 2005) maternal oxygen supply was reduced during late gestation by placing rats housed in standard individual cages (Volume: 20.7 L) inside a plexiglass chamber (Volume: 140 L) on day 15 of pregnancy, which was maintained at 12% oxygen by continuous infusion of a mixture of nitrogen and compressed air, without additional infusion of carbon dioxide. The chamber was opened briefly once/day to weigh rats and food, and to clean cages. After re-closing the chamber, the oxygen concentration was decreased from 21% to 12% oxygen over 30-35 minutes. Oxygen concentration of the chamber was monitored throughout treatment using a portable oxygen analyser, which was calibrated daily (Hudson RCI, Temecula, CA, USA). For comparative purposes, pregnant rats randomised to NR protocol were placed inside a second, identical plexiglass chamber on day 15 of pregnancy, which was continuously infused with compressed air. Oxygen concentration was checked periodically to ensure rats were exposed to 21% oxygen. Rats were fed 11.5±1g standard rat chow/day, which was equivalent to the lowest food intake recorded in rats exposed to maternal hypoxia, and represented 40% of control food intake during this time. We have previously described in

detail the effects of these treatments on maternal food intake and weight gain throughout pregnancy (Williams *et al.*, 2005).

3.3.3 Postnatal Animal Care and Tissue Collection

Within 3-12h of birth in all groups, all pups were weighed, and litters were reduced to 8 pups/dam in order to standardise postnatal nutrition among litters. All dams were housed in room air, and fed *ad libitum* while nursing pups. Offspring were weaned at 3 weeks of age, and aged within the animal facility, with *ad libitum* access to food, for use at 4 or 7 months of age. At each age, 1-2 male offspring per litter were randomly selected for vascular experiments. Female offspring generated in this study were used in a separate series of experiments. On the day of experiment, male offspring were anaesthetised by intraperitoneal injection of 42.25 mg/kg sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Ontario, Canada) and euthanized by exsanguination. At this time, a section of the mesenteric arcade 5-10 cm distal from the pylorus was removed, and placed in ice-cold HEPES buffered Physiological Saline Solution (HEPES-PSS, mmol/L: NaCl 142.0, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.56, Glucose 5.5, HEPES 10.0, pH 7.4) for subsequent artery dissection.

3.3.4 Wire Myography for Assessment of Vascular Function

Second-order mesenteric arteries of 250-350 μm internal diameter were dissected free of fat and connective tissue, and cut into approximately 2 mm long sections. Two 20 μm diameter wires were threaded through the lumen of each artery section, which was then mounted onto an isometric wire myograph system (Kent Scientific, Litchfield, CT) in a 5 ml organ bath containing warmed (37°C) HEPES-PSS. The length of each artery section was measured using a micrometer, and following a 30-minute equilibration period a passive

circumference-tension curve was performed for each segment in order to set optimum resting tension. Arteries were then allowed a further 30-minute equilibration period. Cumulative concentration-response curves to the α1 adrenergic receptor agonist phenylephrine, (100 nmol/L – 50 μmol/L) were performed in order to pre-constrict arteries to the same extent before measuring vasodilator responses. The concentration of phenylephrine required to produce 50% of the maximal vasoconstrictor response to this agonist was calculated for each artery, and this concentration was administered prior to performing vasodilation curves. Following a 30-minute wash period arteries were pre-constricted and cumulative concentration-response curves were then performed using the stable acetylcholine analogue acetyl-β-methylcholine chloride (methacholine, 1 nmol/L–1 μmol/L, Sigma), which produces an endothelium-dependent arterial relaxation. Methacholine concentration response curves were then repeated following a 15 minute incubation with N^{ω}-nitro-$_L$-arginine methyl ester (L-NAME; NO synthase inhibitor, 100 μmol/L Calbiochem) or meclofenamic acid (Meclofenamate, Prostaglandin H Synthase inhibitor, 1 μmol/L, Sigma).

In 7 month offspring, additional experiments were performed to further investigate the differences observed in NO-mediation of endothelium-dependent relaxation. Specifically, following a 30-minute wash period arteries were incubated for 1 hour with polyethylene glycol-superoxide dismutase (SOD, 50 units/ml, Sigma). Arteries were pre-constricted with phenylephrine as above, prior to repeating methacholine concentration response curves. We have previously validated this SOD protocol for use in wire myography preparations using both rat (Davidge *et al.*, 1998; O'Brien *et al.*, 2001) and mouse arteries (Cooke & Davidge, 2003). Vascular relaxation to the exogenous NO donor sodium nitroprusside, (SNP, 1

nmol/L – 1 μmol/L) was also determined in arteries from 7 month offspring in order to assess vascular smooth muscle sensitivity to NO. The number of animals used to investigate differences in the nitric oxide and prostaglandin pathways differed between groups due to variation in the number of viable artery segments mounted within individual experiments. In some instances less than 4 male offspring were available from each litter, which also influenced the number of animals used for each experiment.

3.3.5. Detection of Endothelial Nitric Oxide Synthase (eNOS) Protein by Immunofluorescence

The primary changes in vascular function observed in this study implicated changes in the endothelial NO pathway, therefore we determined relative staining for eNOS within mesenteric arteries using immunofluorescence. A small section of the mesenteric arcade was embedded in Optimal Cutting Temperature (OCT) Compound (Tissue-Tek) before being snap-frozen in liquid nitrogen and stored at –80°C. Frozen embedded tissue was sectioned (10 μm) using a cryostat (-20°C) and sections mounted on superfrost slides, which were wrapped in foil and stored at –80°C until used in immunofluorescence experiments. Slides were allowed to thaw to room temperature for 1h prior to use. Non-specific antibody binding was blocked by incubating sections in 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1h. Experimental sections were then incubated with eNOS primary antibodies (1:100, Affinity Bioreagents, PA3-031) overnight at 4°C, while control sections on each slide were incubated with 2% BSA alone during this time. After slides were washed in PBS, they were incubated with a Rhodamine-conjugated secondary antibody (Molecular Probes, A-11010). Slides were washed well, before being mounted with a DAPI-containing 2:1 Vectashield H-1200 mounting solution (Vector Laboratories Inc, Burlington, Ontario, Canada). Slides were then stored in the dark at 4°C

before analysis (within 2 days) using an Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group, Ontario, Canada). Mesenteric arteries were identified morphologically within sections, and images were captured using Slidebook 2D, 3D Timelapse Imaging Software (Intelligent Imaging Innovations Inc, Colorado, USA). Sections from all groups were stained simultaneously, and images were renormalised after capture to display the same range of fluorescence intensity. Adobe Photoshop was used to perform densitometry analysis of eNOS staining. From each animal the relative intensity of staining within 2-7 arteries was determined, and subsequently averaged. Prior to performing these experiments the specificity of the eNOS antibody was confirmed within our laboratory using blocking peptides, and was subsequently confirmed by the endothelial localisation within images.

3.3.6 Data Analysis

Data are presented as mean ± SEM. Neonatal characteristics were compared using One- or Two-Way ANOVA as appropriate and, where relevant, Tukey post-hoc analysis. Cumulative concentration response curves were summarised for statistical purposes by calculation of EC_{50} values using the Hill Slope Equation (Sigma Plot 8.0 Pharmacology Standard Curves Analysis). The effect of treatment group on vascular sensitivity, as determined by comparison of the EC_{50} values, was assessed using One- or Two-Way ANOVA with Tukey post hoc test. The effect of incubating arteries with inhibitors of NO or prostaglandin synthesis, or with SOD on vascular responses to methacholine within groups was determined by use of a paired t-test. Relative immunofluorescence staining levels are presented as median values, and were compared among groups using a Kruskal-Wallace ANOVA on ranks. Statistical significance was defined as $P<0.05$.

3.4 RESULTS

3.4.1 Neonatal Outcomes and Postnatal Weight

The effects of the maternal hypoxia or nutrient restriction protocols used in this study on maternal food intake and weight gain during pregnancy, and on neonatal body weight and growth parameters have been described in detail previously (Williams *et al.*, 2005). Prior to reduction of each litter to 8 pups, litter size was not different among groups, however birth weight was reduced by both maternal hypoxia (H) and nutrient restriction (NR), compared to control (C) (Chapter 2, $P<0.001$). At both 4 and 7 months of age, male H offspring were significantly smaller than those from either C or NR dams (Table 1, $P<0.01$).

Table 3.1: Offspring Characteristics

	Control	Hypoxia	Nutrient Restriction
Weight at 4 months (g)	612.2 ± 13.3^{a} *(12)*	537.9 ± 15.6^{b} *(10)*	609.0 ± 12.5^{a} *(8)*
Weight at 7 months (g)	685.4 ± 20.8^{a} *(17)*	602.0 ± 18.0^{b} *(13)*	667.2 ± 17.2^{a} *(7)*

Different letters denote significant differences among groups. For each group, n values are indicated within parentheses.

3.4.2 Mesenteric Artery Size and Constriction to Phenylephrine

Mesenteric artery diameter was not different among groups at either 4 or 7 months of age (4 months: C, 296 ± 14μm; H, 269 ± 16μm; NR, 263 ± 19μm; 7 months: C, 290 ± 12μm; H, 270 ± 14μm; NR, 305 ± 18μm). Sensitivity to phenylephrine-induced vasoconstriction did not differ among groups at 4 months (Figure 1A). Mesenteric artery sensitivity to phenylephrine increased with age in H offspring, such that arteries were significantly more sensitive to phenylephrine than control by 7 months (Figure 1B, $P<0.05$). There was no

significant effect of age on the sensitivity of mesenteric arteries from NR offspring to phenylephrine, however at 7 months EC_{50} values did not differ statistically between H and NR offspring (Figure 1C). Maximum tension generated in response to phenylephrine did not differ among groups at either 4 or 7 months, (4 months: C: 3.58±0.22 mN/mm^2, H: 3.36±0.26 mN/mm^2, NR: 3.18±0.26 mN/mm^2, 7 months: C: 3.75±0.20 mN/mm^2, H: 3.58±0.22 mN/mm^2, NR: 3.91±0.27 mN/mm^2). There was a trend ($P=0.06$) for maximum phenylephrine-induced tension to increase from 4 to 7 months in all groups.

3.4.3 Endothelium-dependent Relaxation

At 4 months, sensitivity to the endothelium-dependent vasodilator methacholine was reduced in H offspring compared to either C or NR offspring (Figure 2A, $P<0.05$). At 7 months, however, the response to methacholine was not different among groups (Figure 2B, $P>0.05$). In C offspring, the vascular sensitivity to methacholine decreased between 4 and 7 months, as evidenced by the increased EC_{50} value (Figure 2C, $P<0.05$). There was no effect of increasing age on the endothelium-dependent relaxation of mesenteric arteries from H or NR offspring.

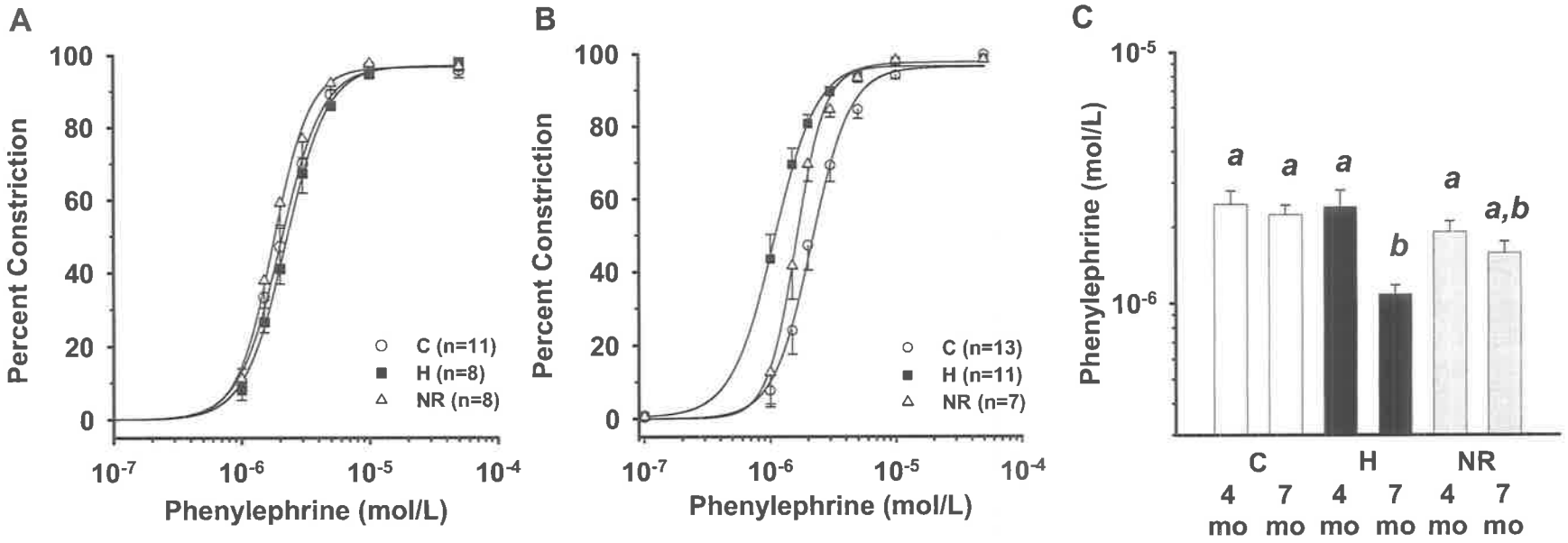


Figure 3.1: ***Mesenteric artery constriction to phenylephrine at 4 and 7 months.***

(A) Vasoconstriction to phenylephrine in mesenteric arteries from 4 month old offspring of control (C, open circles, n=11), hypoxia (H, black squares, n=8) or nutrient restriction dams (NR, grey triangles, n=8). (B) Vasoconstriction to phenylephrine in mesenteric arteries from 7 month old offspring of control (C, open circles, n=13), hypoxia (H, black squares, n=11) or nutrient restriction (NR, grey triangles, n=7) dams. (C) Sensitivity (EC_{50} values) to phenylephrine in mesenteric arteries from offspring of control (C, open bars), hypoxia (H, black bars) or nutrient restricted dams (NR, grey bars) at 4 or 7 months of age. Different letters denote significant differences ($P<0.05$) among groups.

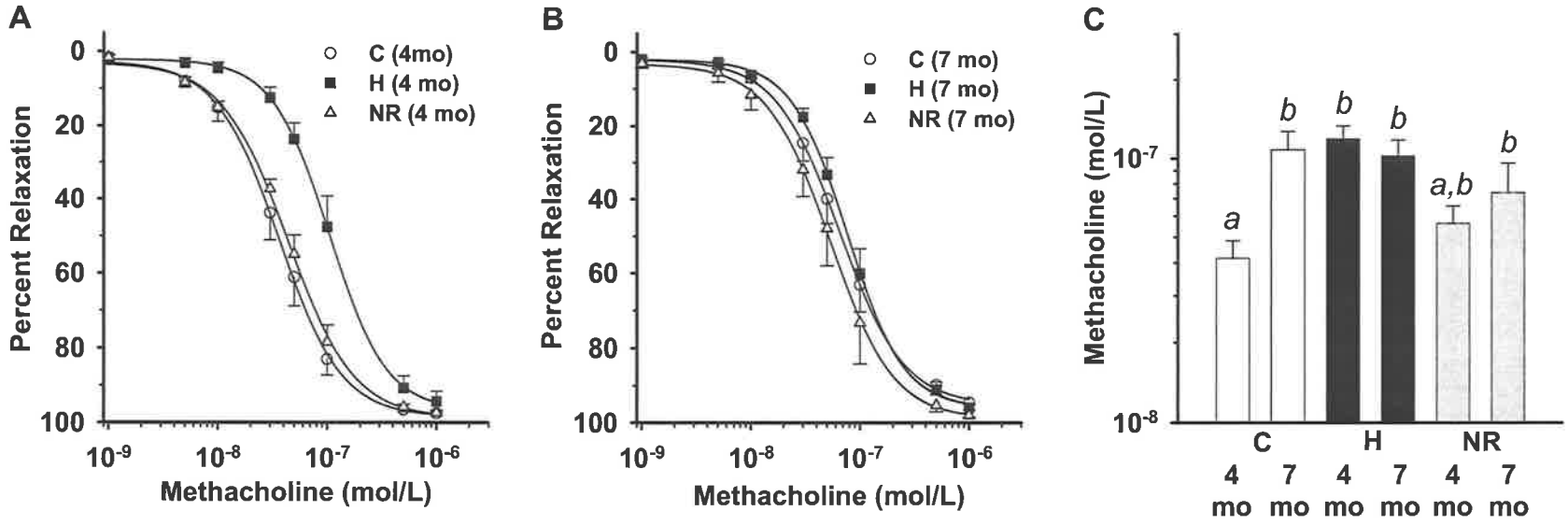


Figure 3.2: Mesenteric artery relaxation to methacholine at 4 and 7 months.

(A) Endothelium-dependent relaxation to methacholine in mesenteric arteries from 4 month old offspring of control (C, open circles, n=10), hypoxia (H, black squares, n=7) or nutrient restriction dams (NR, grey triangles, n=8). **(B)** Methacholine-induced endothelium-dependent relaxation in mesenteric arteries from 7 month offspring of control (C, n=14), hypoxia (H, n=12) or nutrient restricted (NR, n=6) dams. **(C)** Endothelial sensitivity to methacholine (EC_{50} values) in mesenteric arteries from offspring of control (C, open bars), hypoxia (H, black bars) or nutrient restricted dams (NR, grey bars) at 4 or 7 months. Different letters denote significant differences ($P<0.05$) among groups

3.4.4. Prostaglandin Mediation of Endothelium-Dependent Responses

Meclofenamate (prostaglandin H synthase inhibitor) reduced the vascular sensitivity to methacholine in 4 month old NR offspring (EC_{50}: 54.0±8.4 nmol/L vs 85.8±21.7 nmol/L, $P<0.05$), but did not affect vascular relaxation in the other groups. Interestingly, at 7 months meclofenamate tended to increase sensitivity to methacholine-induced vasodilation in both H (EC_{50}: 103.1±30.1 nmol/L vs 78.6±28.5 nmol/L, $P=0.06$), and NR offspring (EC_{50}: 61.1±18.2 nmol/L vs 36.0±7.1 nmol/L, $P=0.07$), suggesting the inhibition of a vasoconstrictor as opposed to vasodilator prostaglandin.

3.4.5 Nitric Oxide Mediation of Endothelium-Dependent Responses

At 4 months, L-NAME (NO synthase inhibitor) reduced the vascular sensitivity to methacholine in both C (Figure 3A, EC_{50}: 45.5±12.1 nmol/L vs 104.2±25.9 nmol/L, $P<0.01$) and H offspring (Figure 3B, EC_{50}: 112.3±17.7 nmol/L vs 190.6±21.9 nmol/L, $P<0.01$), but did not significantly change the EC_{50} value in NR offspring (Figure 3C, EC_{50}: 72.6±30.2 nmol/L vs 105.7±25.5 nmol/L, $P=0.08$). The methacholine concentration-response curve was significantly shifted across the lower concentration range in NR offspring however, such that L-NAME significantly increased the EC_{20} value (Figure 3C, EC_{20}: 11.1±3.3 nmol/L vs 24.7±7.6 nmol/L, $P<0.05$).

At 7 months, L-NAME reduced sensitivity to endothelium-dependent vasodilation by methacholine in C offspring (Figure 4A, EC_{50}: 82.2±17.2 nmol/L vs 187.1±39.7 nmol/L, $P<0.01$) and also tended to reduce sensitivity to methacholine at EC_{50} in NR offspring (Figure 4C, EC_{50}: 69.5±24.5 nmol/L vs 114.4±14.0 nmol/L $P=0.055$). The relaxation to methacholine was more variable in NR offspring, and for those animals that showed higher sensitivity to methacholine (n=4 out of 6), there was a significant increase in the EC_{50} value

in the presence of L-NAME (36.5±7.0 nmol/L vs 105.0±11.7 nmol/L, $P<0.05$). In contrast, there was no effect of L-NAME on vascular sensitivity to methacholine-induced relaxation in 7 month old H offspring, as there was no change in either EC_{50} or EC_{20} values (Figure 4B) in the presence of L-NAME.

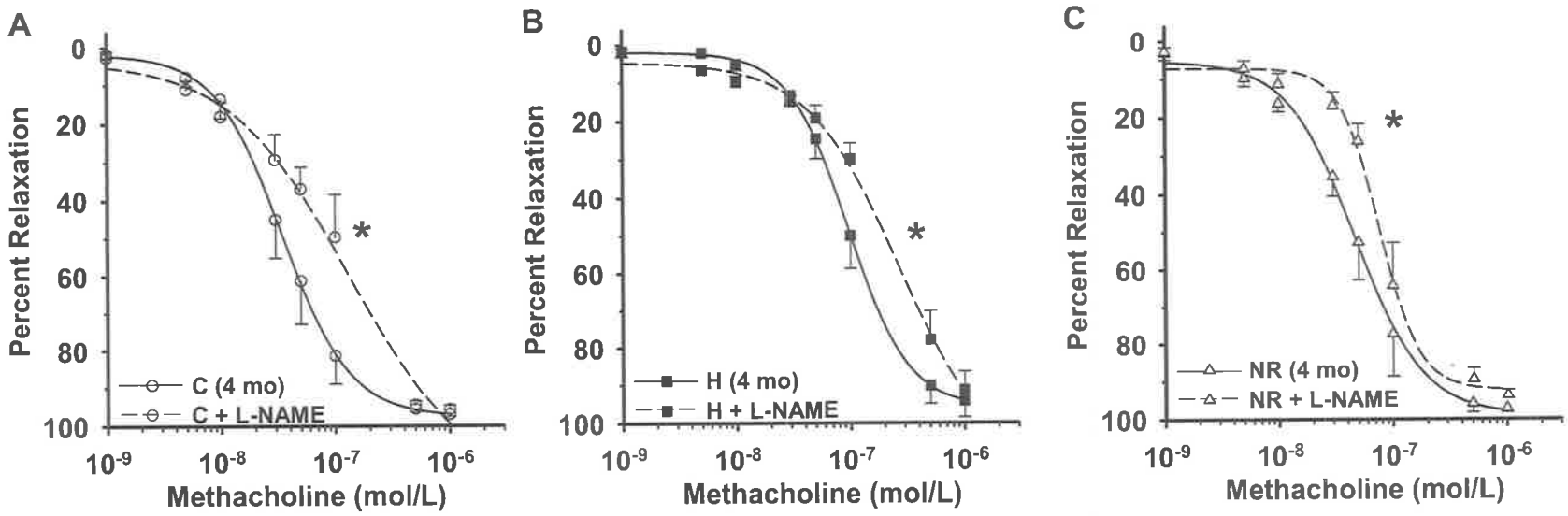


Figure 3.3: Effect of L-NAME on relaxation to methacholine at 4 months.

Endothelium-dependent relaxation to methacholine in the absence (solid line) or presence of L-NAME (NO synthase inhibitor, dashed line) in mesenteric arteries of offspring from **(A)** control (C, open circles, n=6, EC_{50} shift, $P<0.01$), **(B)** hypoxia (H, black squares, n=6, EC_{50} shift, $P<0.01$) and **(C)** nutrient restricted dams (NR, grey triangles, n=5, EC_{50} shift, $P=0.08$, EC_{20} shift, $P<0.05$) at 4 months. *Indicates a significant increase in methacholine EC_{50} or EC_{20} in the presence of L-NAME.

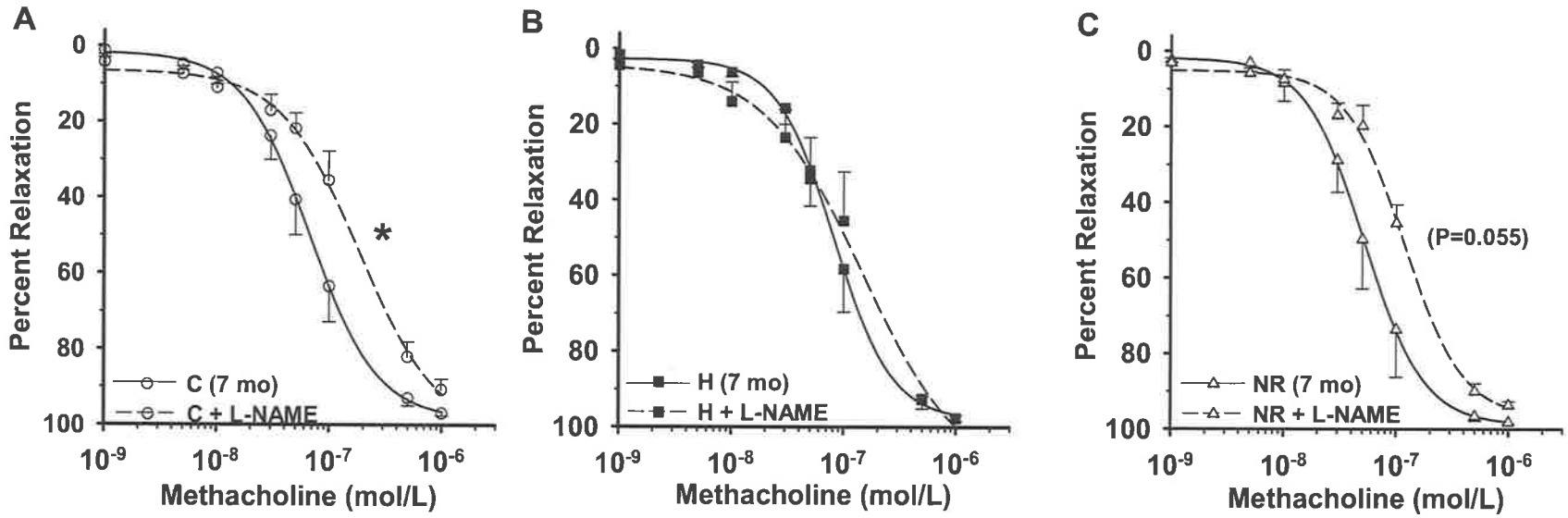


Figure 3.4: ***Effect of L-NAME on relaxation to methacholine at 7 months.***

Endothelium-dependent relaxation to methacholine in the absence (solid line) or presence (dashed line) of L-NAME in mesenteric arteries of offspring from **(A)** control (C, open circles, n=7, EC_{50} shift, $P<0.05$), **(B)** hypoxia (H, black squares, n=6 EC_{50} and EC_{20} shift NS), and **(C)** nutrient restricted dams, (NR, grey triangles, n=6, EC_{50} shift, $P=0.055$, EC_{20} shift NS) at 7 months. *Indicates a significant increase in methacholine EC_{50} in the presence of L-NAME.

In order to further investigate the reduced NO-mediation of endothelium-dependent relaxation that was observed only in 7 month H offspring, the effect of exogenous superoxide dismutase (to prevent scavenging of NO by superoxide anion) on relaxation to methacholine was determined. Superoxide dismutase incubation did not significantly alter vasodilation to methacholine in C offspring (Figure 5A), and no significant effect was observed on EC_{50} values from H offspring (Figure 5B). However, superoxide dismutase did significantly enhance the relaxation to low concentrations of methacholine such that the EC_{20} value was significantly reduced in H offspring (Figure 5B, EC_{20}: 30.1±4.4 nmol/L vs 10.1±1.6 nmol/L, $P<0.001$).

To determine the vascular smooth muscle sensitivity to exogenous NO, endothelium-independent relaxation to the NO donor sodium nitroprusside was assessed in 7 month offspring. There was no difference in the sensitivity, or maximal relaxation to sodium nitroprusside among groups (Figure 6).

Figure 5

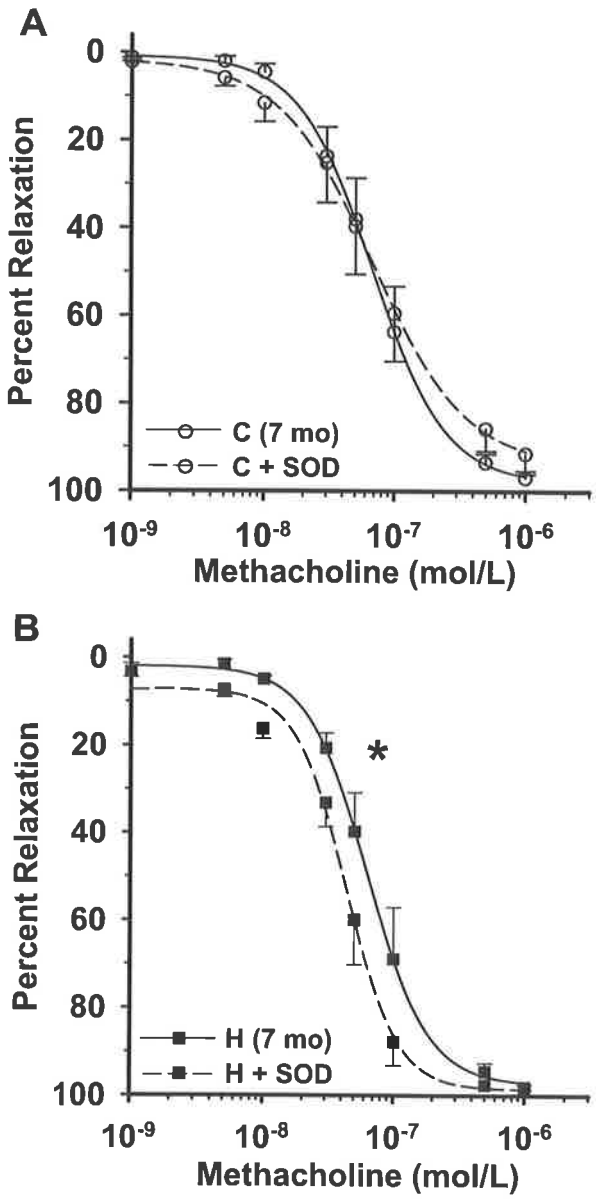


Figure 3.5: Effect of superoxide dismutase on relaxation to methacholine at 7 months.

(A) Endothelium-dependent relaxation to methacholine in the absence (solid line) or presence of exogenous superoxide dismutase (SOD, dashed line) in offspring from control (C, open circles, n=7) and **(B)** hypoxia dams (H, black squares, n=5). *Indicates a significant decrease in EC_{20} in the presence of superoxide dismutase.

Figure 6

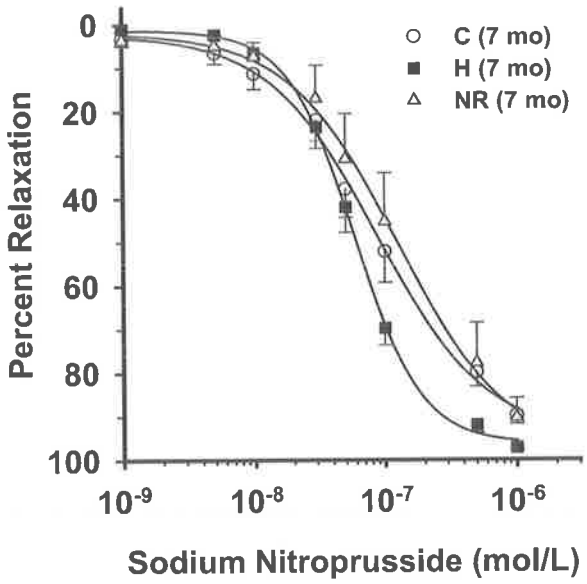


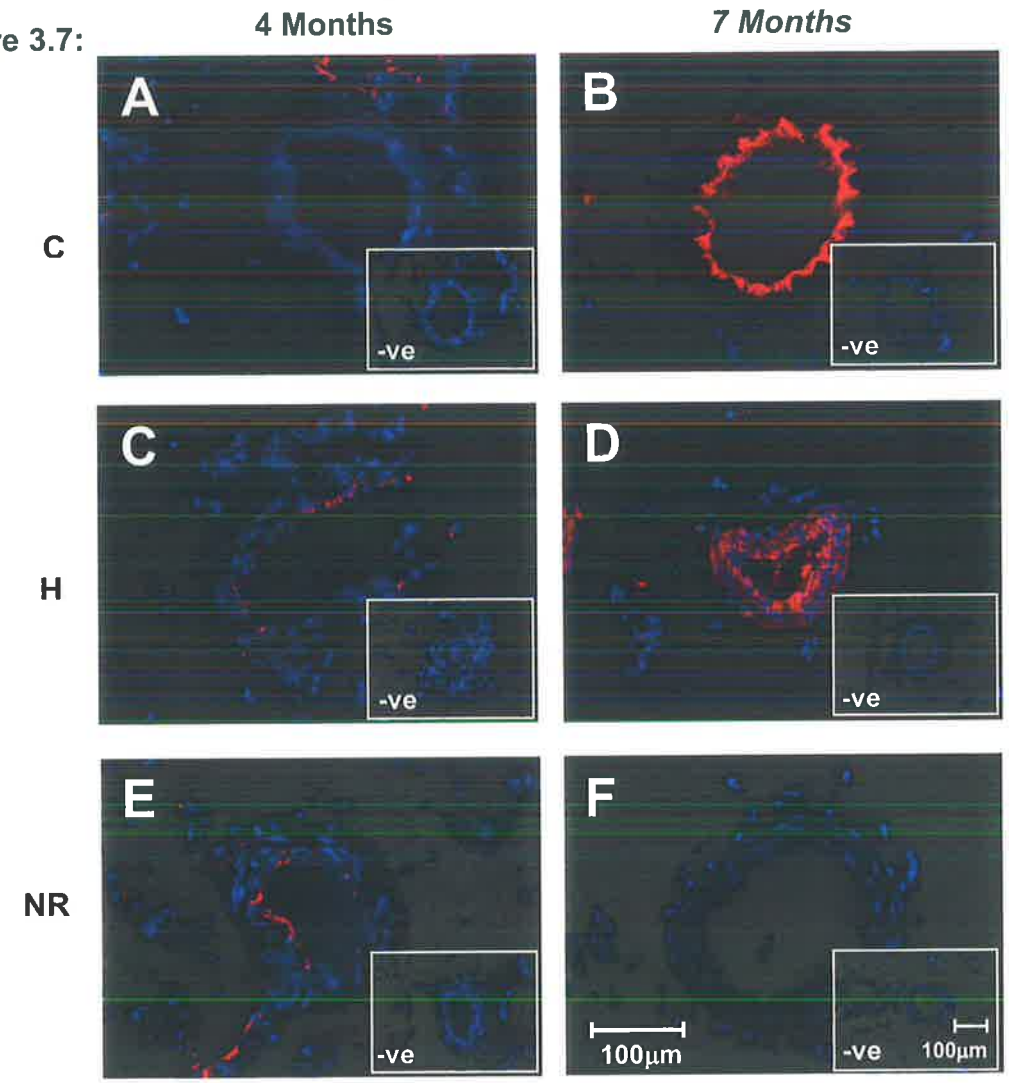
Figure 3.6: Mesenteric artery relaxation to sodium nitroprusside at 7 months.

Endothelium-independent relaxation of mesenteric arteries to sodium nitroprusside in 7 month old offspring of control (C, open circles, n=8), hypoxia (H, black squares, n=4) or nutrient restriction dams (NR, grey triangles, n=4).

Immunostaining for Endothelial Nitric Oxide Synthase (eNOS) in Mesenteric Arteries

Figure 7 shows representative images of vascular staining for eNOS in sections of mesenteric arteries from C, H and NR offspring at 4 and 7 months. The relative intensity of immunofluorescent staining for eNOS increased with increasing age in C offspring ($P<0.01$, Figure 7G), but not in either H or NR offspring. The relative staining intensity for eNOS was lower in arteries from NR offspring than from either C or H offspring at 7 months ($P<0.05$, Figure 7G).

Figure 3.7:


4 Months
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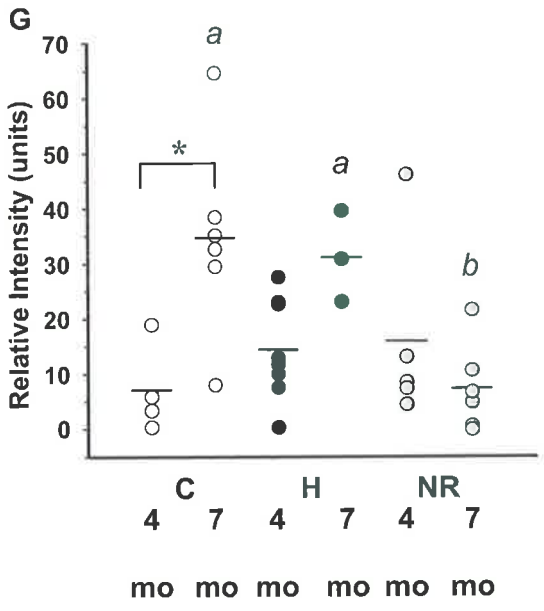
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Relative Intensity (units)
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Figure 3.7: Localisation of staining for eNOS within mesenteric arteries at 4 and 7 months.

Immunofluorescent staining identified expression of eNOS (red) in mesenteric arteries from all groups at both 4 and 7 months, which were visualised by aid of DAPI nuclear stain (blue). Representative images show arteries from control offspring at **(A)** 4 and **(B)** 7 months, hypoxia offspring at **(C)** 4 and **(D)** 7 months and nutrient restriction offspring at **(E)** 4 and **(F)** 7 months along with the appropriate negative control for each (inset). **(G)** Relative fluorescence intensity was normalised to the negative control in each instance, and the median intensity compared among groups (C [4mo], n=4, C [7mo], n=6, H [4mo], n=7, H [7mo], n=3, NR [4mo], n=5, NR [7mo], n=6) Individual data points represent the mean relative intensity for each animal, calculated as the mean intensity from 2-7 arteries per animal. * Indicates a significant difference between 4 and 7 month control offspring while different letters denote significant differences among groups at 7 months. No differences were observed among groups at 4 months.

3.5 DISCUSSION

In this study we examined the effects of maternal hypoxia during late gestation on vascular endothelial function in adult male rat offspring. To control for the reduction in maternal appetite that occurs in association with maternal hypoxia (Van Geijn *et al.*, 1980; de Grauw *et al.*, 1986; Gleed & Mortola, 1991), we also examined vascular responses in offspring from dams that were nutrient restricted in late gestation. We have previously shown that both maternal hypoxia and nutrient restriction during late gestation reduce birth weight and alter proportional organ weights, suggesting that each results in asymmetric fetal growth restriction (Williams *et al.*, 2005). We have also shown that maternal hypoxia and nutrient restriction differentially affect carotid and femoral artery vasoconstrictor responses in the neonate (Williams *et al.*, 2005). Interestingly, in the present study sensitivity to phenylephrine was also increased specifically by maternal hypoxia in mesenteric arteries, although this was observed only at 7 months. The current study extends our investigations into the role of prenatal hypoxia in impairing postnatal vascular function by examining endothelium-dependent responses from adult H and NR offspring. To avoid variation in endothelial function related to changing estrogen levels in rats throughout the estrous cycle and with advancing age (Davidge & Zhang, 1998; Wight *et al.*, 2000) these experiments focused on male offspring. To our knowledge, these are the first data to demonstrate perturbations of small artery endothelial function in adult rat offspring following chronic maternal hypoxia.

The sensitivity of mesenteric arteries from 4 month H offspring to endothelium-dependent relaxation by methacholine was significantly reduced when compared to either C or NR offspring. At this age, L-NAME reduced sensitivity to methacholine in all offspring, while meclofenamate did not affect methacholine sensitivity in either H or C offspring. While the

degree of NO or prostaglandin-mediation of methacholine-induced relaxation may vary among groups, our results do not suggest substantial differences in the contributions of these pathways in mesenteric arteries from 4 month H offspring. Our data may therefore imply that other mechanisms mediate the observed impairment in endothelial function. Perhaps the most likely mechanism implicated in H offspring is a deficit in endothelium-derivered hyperpolarizing factor (EDHF) mediated relaxation. EDHF-mediated relaxation was impaired in offspring of dams fed a high fat diet during pregnancy (Taylor *et al.*, 2004), however the effects of chronic prenatal hypoxia on this pathway have not previously been described.

In C offspring, mesenteric artery sensitivity to methacholine-induced endothelium-dependent relaxation decreased with increasing age. However, in arteries from H or NR offspring, methacholine EC_{50} values did not further shift with age, demonstrating no age-dependent change in endothelial sensitivity to methacholine. These data suggest a possible premature aging of the vasculature in H and NR offspring. Previously, when the effects of moderate maternal undernutrition throughout pregnancy on endothelial function in femoral artery branches were examined in 3.5 and 7 month rat offspring, increasing age reduced maximal relaxation similarly in control, and restricted offspring (Ozaki *et al.*, 2001). The lost interaction of age with endothelial function in NR offspring from the current study may reflect differences in the femoral versus mesenteric vascular beds, or the timing or severity of the undernutrition used in these studies.

Endothelium-dependent relaxation in C offspring was significantly mediated by NO at both 4 and 7 months, as L-NAME decreased arterial sensitivity to methacholine at both time-points. In NR offspring, L-NAME also tended to reduce sensitivity to methacholine at both

ages. While the effect of L-NAME on the relaxation to methacholine in NR offspring at 7 months was more variable than in C or H offspring, these data suggest that the NO pathway contributes to endothelium-dependent relaxation in this group. In contrast, by 7 months there was no apparent role for NO in mediating vasodilation to methacholine in H offspring. Interestingly, chronic hypoxia *in ovo* also reduced NO-mediation of endothelium-dependent relaxation in femoral artery branches from chickens aged 14-15 weeks without changing overall endothelial responses to acetylcholine (Ruijtenbeek *et al.*, 2003a). Endothelial NO production is important in regulating short-term vascular tone (Vallance *et al.*, 1989; Coffman, 1994), arterial distensibility (Kinlay *et al.*, 2001; Wilkinson *et al.*, 2002), and vascular smooth muscle proliferation (Cornwell *et al.*, 1994; Fukumoto *et al.*, 1999). Changes in this pathway may therefore affect long-term cardiovascular health, and have been implicated in clinical hypertension (Linder *et al.*, 1990; Panza *et al.*, 1990). Impairments in the NO pathway have now also been implicated in the vascular dysfunction programmed through a range of dietary models of fetal growth restriction, including models of maternal protein (Torrens *et al.*, 2002) and global (Franco *et al.*, 2002a; Franco *et al.*, 2004) undernutrition. Our data demonstrate that maternal hypoxia, associated with decreased appetite during late gestation also reduces NO-mediation of endothelial function in adult offspring. However, offspring from pregnancies where maternal nutrition was similarly reduced during this time did not demonstrate reduced NO-mediation of relaxation to methacholine. This suggests that hypoxia alone, or the interaction of hypoxia and undernutrition during the last week of pregnancy impaired NO-mediated endothelial function in adult male rats.

To further investigate the loss of NO-mediated relaxation in 7 month-old H offspring, we determined the effect of exogenous superoxide dismutase (SOD), which reduces the

scavenging of NO by superoxide anion (Rubanyi & Vanhoutte, 1986; Davidge *et al.*, 1998; Cooke & Davidge, 2003). In C offspring, the addition of SOD did not affect the response to methacholine, demonstrating that superoxide anion did not influence endothelial function in this group. In H offspring, the EC_{50} value was not significantly shifted, but the reduction in EC_{20} value by SOD suggests that local superoxide anion concentrations influence endothelium-dependent relaxation across the nanomolar agonist concentration range, which may be of physiological relevance. Enhanced vascular oxidative stress has previously been demonstrated to impair endothelial function in adult offspring from nutrient restricted dams (Franco *et al.*, 2002b), however this is the first assessment in adult offspring following chronic hypoxia during development.

There were no differences in the vascular responses to the NO donor, sodium nitroprusside among the three groups, suggesting that the loss of NO-mediated relaxation in H offspring is due to reduced bio-available NO rather than decreased vascular smooth muscle sensitivity to relaxation by NO. Undernutrition during pregnancy has previously been reported to increase (Holemans *et al.*, 1999), decrease (Ozaki *et al.*, 2001; Brawley *et al.*, 2003) or not alter (Franco *et al.*, 2002a; Franco *et al.*, 2002b) vascular sensitivity to sodium nitroprusside, however the causes of these heterogeneous effects are not yet clear.

In this study, the relative staining for eNOS increased with age only in C offspring. Previous studies have also demonstrated greater expression of eNOS protein in aorta from old compared to young male rats, while both endothelium-dependent dilation *in vivo* and eNOS activity in the aorta (Cernadas *et al.*, 1998; van der Loo *et al.*, 2000), and mesenteric arteries (Matz *et al.*, 2000) was reduced by aging. Interestingly there was no significant increase in staining for eNOS with increasing age in the mesenteric arteries of either H or

NR offspring, and at 7 months, staining for eNOS was lower in arteries from NR offspring than in either C or H offspring. Consistent with these findings, eNOS mRNA expression and enzyme activity were both reduced in aorta following undernutrition throughout pregnancy (Franco *et al.*, 2002a). The sustained endothelial eNOS expression in 7 month H offspring further supports data suggesting that the loss of NO-mediated endothelial function may result from decreased bio-available NO, rather than a reduced capacity to produce the vasodilator.

Finally, we also evaluated the role of prostaglandin production in mediating endothelium-dependent responses. Significant prostaglandin-mediated relaxation was only observed in the 4 month old NR offspring. The trend for sensitivity to methacholine to be increased by meclofenamate in both H and NR offspring at 7 months of age suggests that in these groups there may be inhibition of a vasoconstrictor rather than vasodilator prostaglandin. It has previously been demonstrated that aging increased production of vasoconstrictor, as opposed to vasodilator prostaglandins within the endothelium, (Stewart *et al.*, 2000) and it is possible that a similar change in the balance of prostanoid production may contribute to this observation. Endothelium-dependent vasodilation is also mediated by other factors, such as endothelial-derived hyperpolarising factor(s). Our results suggest changes in this pathway may occur following reduced oxygen supply *in utero*.

In summary, chronic maternal hypoxia during pregnancy impaired endothelium-dependent relaxation in mesenteric arteries from 4 month offspring, which was not attributable to overt changes in the NO or prostaglandin pathways. Maternal hypoxia also reduced NO-modulation of endothelium-dependent relaxation in 7 month offspring, when compared to C or NR offspring, and increased sensitivity to alpha-adrenoceptor mediated

vasoconstriction to phenylephrine compared to control. Thus, at both ages studied, our data suggest that the balance of vasoconstrictor to vasodilator responses in small mesenteric arteries was perturbed by prenatal hypoxia. These effects were not observed in NR offspring, where maternal nutrition was reduced during pregnancy to account for reduced appetite in H dams (Williams *et al.*, 2005). Therefore, these data provide evidence that reduced oxygen supply during late gestation, either independently or through interaction with reduced nutrition, impacts adult endothelial function in a mammalian model. The causes of intrauterine growth restriction within human populations are heterogeneous, and frequently involve reduction of both fetal oxygen and nutrient supply. It is therefore important to recognise that the programming of later cardiovascular function, including endothelial function, may reflect the specific nature of the *in utero* environment.

CHAPTER 4

CHRONIC HYPOXIA INCREASES SENSITIVITY TO NORADRENALINE IN SMALL MESENTERIC ARTERIES FROM FETAL SHEEP IN LATE GESTATION

4.1 SUMMARY

Changes in mesenteric resistance artery function may contribute to both the short-term and long-term adverse consequences of intrauterine growth restriction (IUGR). We hypothesized that placental restriction and associated chronic hypoxia would increase sensitivity to noradrenaline-induced vasoconstriction in small mesenteric artery branches from late gestation fetal sheep. Carunclectomy was performed in non-pregnant ewes to restrict subsequent placental and fetal growth (CX). Catheters were surgically inserted in the carotid artery and jugular vein in control and CX fetuses between 110-118 dGA. Using a wire myograph, vasoconstriction to noradrenaline was assessed in 3rd and 4th order mesenteric artery branches collected at post-mortem between 136-141 dGA (term ~150d). The mean gestational PO_2 was used to categorise fetuses as hypoxic (PO_2<17.0mmHg, n=5) or normoxic (n=7). All hypoxic fetuses were CX, whereas the normoxic group included both control and CX fetuses (PO_2: H, 13.5±0.9mmHg, versus N: 20.1±0.8mmHg, P<0.001). Hypoxic fetuses were also characterized by hypercapnia (P<0.001), hypoglycemia (P<0.01), lower body weight (P<0.001) and greater relative brain weight (P<0.01). Sensitivity to noradrenaline was increased in 4th order fetal mesenteric arteries in the H group (EC_{50}: N, 6.4±0.24μM versus H, 3.07±0.96μM, P<0.01). Further, noradrenaline EC_{50} values correlated with both mean gestational PO_2 (R^2=0.68, P<0.01) and PCO_2 (R^2=0.71, P=0.001) when H and N groups were combined. Interestingly, the sensitivity of 3rd order arteries to noradrenaline did not differ between the H and N groups and was not related to the prevailing fetal arterial blood gases. In summary, sensitivity to noradrenaline increased in 4th order, but not 3rd order mesenteric arteries in direct relation to the degree of placental insufficiency, indicated by fetal hypoxia and hypercapnia during late gestation. These data suggest a specific effect of chronic fetal compromise on smaller resistance arteries.

4.2 INTRODUCTION

Intrauterine growth restriction (IUGR) is associated with elevated perinatal morbidity and mortality (Kramer *et al.*, 1990; McIntire *et al.*, 1999; Lackman *et al.*, 2001), and has been further associated with an increased risk of developing hypertension during adult life (Barker *et al.*, 1989a; Barker *et al.*, 1990; Huxley *et al.*, 2000). One of the major clinical causes of IUGR is placental insufficiency (Henriksen & Clausen, 2002), where fetal growth is restricted by the limitation of both oxygen and nutrient supply. Fetal adaptations to reduced substrate supply may result in structural and functional differences within key homeostatic systems, which predispose individuals to the later development of disease (Barker, 1995). The fetal cardiovascular adaptation to acute hypoxia involves the redistribution of cardiac output to maintain blood flow and oxygen delivery to the brain, heart and adrenals while blood flow to peripheral circulations is reduced (Cohn *et al.*, 1974; Peeters *et al.*, 1979). Both clinical (Wladimiroff *et al.*, 1987; Arbeille, 1997) and experimental (Block *et al.*, 1984; Kamitomo *et al.*, 1993) evidence suggests that a similar redistribution of cardiac output is sustained in chronically hypoxic, growth-restricted fetuses to result in the sparing of brain and heart growth with increased vascular resistance within peripheral circulations, including the mesentery (Robel-Tillig *et al.*, 2002). Sympathetic nervous system (SNS) activation plays a key role in the fetal response to acute hypoxia, including the rapid increase in peripheral vascular resistance (Giussani *et al.*, 1993). Plasma noradrenaline concentrations are also elevated in chronically hypoxic, growth restricted fetuses (Gagnon *et al.*, 1994; Simonetta *et al.*, 1997; Smolich & Esler, 1999), suggesting a sustained increase in SNS activity that may contribute to maintaining elevated peripheral vascular resistance. Indeed, α-adrenergic receptor blockade produced a greater decrease in arterial blood pressure in chronically hypoxic, placentally restricted fetal sheep than in normoxic controls during late gestation (Danielson *et al.*, 2005). It is not

known, however, whether the increased contribution of α-adrenergic receptor signaling to the maintenance of blood pressure in the growth restricted fetus also involves an increase in the responsiveness of small peripheral arteries to noradrenaline-mediated vasoconstriction.

The mesenteric circulation is a peripheral vascular bed where changes in fetal vascular function related to IUGR may be of most relevance to later health. Necrotizing enterocolitis is among the serious neonatal complications associated with IUGR (Gilbert & Danielsen, 2003; Garite *et al.*, 2004a). This condition is initiated when persistent mesenteric hypo-perfusion following prolonged hypoxia or asphyxia results in ischemic injury to the gut (Hsueh *et al.*, 2003). Changes in mesenteric artery function in the IUGR fetus that promote vasoconstriction may contribute to the increased susceptibility of IUGR infants to necrotizing enterocolitis. The mesenteric arcade also plays a significant role in blood pressure regulation during adult life (Christensen & Mulvany, 1993). In several studies small mesenteric artery function was impaired in adult offspring following the restriction of fetal growth in rats (Holemans *et al.*, 1999; Franco *et al.*, 2002b; Torrens *et al.*, 2002). We have also demonstrated in Chapter 3 that prenatal hypoxia resulted in an age-dependent increase in small mesenteric artery sensitivity to the α_1 adrenergic receptor agonist phenylephrine. The mesenteric arcade therefore represents a vascular bed which may be particularly sensitive to changes in the *in utero* environment, however there is currently no information regarding the impact of fetal growth restriction on small mesenteric artery function in the fetus.

In this study we therefore aimed to determine the effects of placental insufficiency leading to chronic fetal hypoxia and growth restriction, on the responses of fetal small mesenteric arteries to noradrenaline. We tested the hypothesis that placental restriction and associated

chronic hypoxia would increase sensitivity to noradrenaline-induced vasoconstriction in 3rd and 4th order mesenteric artery branches from late gestation fetal sheep.

4.3 METHODS

4.3.1 Animals and Surgery

All procedures were approved by the University of Adelaide Animal Ethics Committee. Surgical procedures were performed using aseptic technique under general anesthesia, which was induced by intravenous injection of sodium thiopentone (1.25g Pentothal, Rhone Merieux, Pinkenba, Queensland, Australia), and maintained after intubation by ventilation with 2-4% halothane (Fluothane, ICI, Melbourne, Vic, Australia) in oxygen. In 6 non-pregnant merino ewes surgery was performed to remove the majority of uterine endometrial caruncles (CX) as previously described (Edwards *et al.*, 1999b) in order to restrict subsequent placental growth. After surgery, ewes were observed for 4-7 days, and were allowed a minimum 10 week recovery period before being mated.

Ultrasound was performed at approximately 60 days post-mating to confirm pregnancy. In all ewes (CX, n = 6, control n = 4), vascular catheters were implanted in a fetal carotid artery and jugular vein, maternal jugular vein and amniotic cavity between 110 and 118 days gestation. All catheters were filled with heparinized saline (50IU/ml heparin sodium, Pharmacia Australia, Rydalmere, NSW, Australia in sterile saline, Baxter Healthcare, Old Toongabbie, NSW, Australia) and the fetal catheters exteriorized through an incision made in the ewe's flank. In instances where ewes carried twin fetuses, both twins were catheterized where possible. Antibiotics (Norocillin; 150mg/ml procaine penicillin and 112.5mg/ml benzathine penicillin, Norbrook Laboratories, New Gisborne, Vic, Australia and Streptomycin, 125mg/ml Dihydrostreptomycin in sterile saline, Sigma, St Louis, MO,

USA) were administered intramuscularly to both ewe and fetus at the time of surgery. Following surgery ewes received the analgesic Xylazine ($0.02mg.kg^{-1}$) during the recovery period. Ewes received the same regime of antibiotics for 3 days post-surgery, while fetuses received intra-amniotic infusion of Ampicillin (100mg/ml, Ampicillin sodium, CSL Limited, Parkville, VIC, Australia) for 4 days post-surgery.

4.3.2 Blood Sampling

Vascular catheters were maintained patent by regular flushing with heparinized saline every 1-3 days. After a minimum of 4 days recovery, fetal (3ml) and maternal (5ml) blood samples were collected 3 times per week. An additional 0.6 ml fetal arterial blood sample was collected for measurement of fetal blood PO_2, PCO_2, pH, O_2 saturation, hemoglobin and hematocrit 3-5 times per week (ABL 520 analyser, Radiometer, Copenhagen, Denmark).

4.3.3 Post Mortem and Tissue Collection

A total of 6 CX and 4 control ewes and their fetuses were euthanased with an overdose of sodium pentobarbitone (Lethabarb, Virbac Pty Ltd, Peakhurst, NSW, Australia) between 136 and 141d of gestation. Fetuses were exteriorized by hysterectomy, weighed and decapitated. In 7 fetuses of CX ewes, and 5 fetuses of control ewes, a section of small intestine and associated mesenteric arcade was collected at 15-30 cm distal to the pylorus, and immediately placed in ice cold HEPES-buffered physiological saline solution (HEPES-PSS, mmol/L: NaCl 142.0, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.56, Glucose 5.5, HEPES 10.0, pH 7.4). Major organs were then dissected and weighed.

4.3.4 Wire Myography

The typical branching pattern observed in the collected section of fetal mesenteric arcade is demonstrated in Figure 1. Both 3rd and 4th order mesenteric artery branches (diameter ~200-300μm) were dissected clean of fat and connective tissue before being cut into approximately 2mm long sections. Two 25μm diameter wires were threaded through the lumen of each arterial section before mounting on an isometric wire myograph system (Kent Scientific, Litchfield, CT, USA) in separate 5ml organ baths for assessment of vasoconstrictor responses. A micrometer was used to measure the length of each artery segment, and after a 30 minute equilibration period the optimum resting tension for each artery was determined by performing a passive circumference-tension curve. In a preliminary series of experiments, the contractile characteristics of 4th order mesenteric arteries were assessed by incremental stretching of arteries maximally constricted in 125mM K^+ HEPES-PSS (mmol/L: KCl 123.7, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.17, $CaCl_2$ 5.0, HEPES 10.0, glucose 5.5, sucrose 45.0) and the same arteries maximally relaxed in Ca^{++} free HEPES-PSS (mmol/L: NaCl 142.0, KCl 4.7, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.17, HEPES 10.0, EGTA 2.0) to determine the optimum resting tension (Figure 2B). The contractile characteristics of 3rd order mesenteric arteries were subsequently determined using the same protocol (Figure 2A). Data were collected from 3rd order arteries following the completion of a pharmacologic protocol, however, to maximize data collection from the experimental animals. Thus active tension generation in 3rd order arteries was lower than that observed in 4th order arteries. For both 3rd and 4th order mesenteric arteries, active tension generation was optimal at approximately 80% of the passive tension which would be achieved at a transmural pressure of 45 mmHg. Beyond this degree of baseline stretch passive tension contributed substantially to the total developed tension.

Figure 4.1:

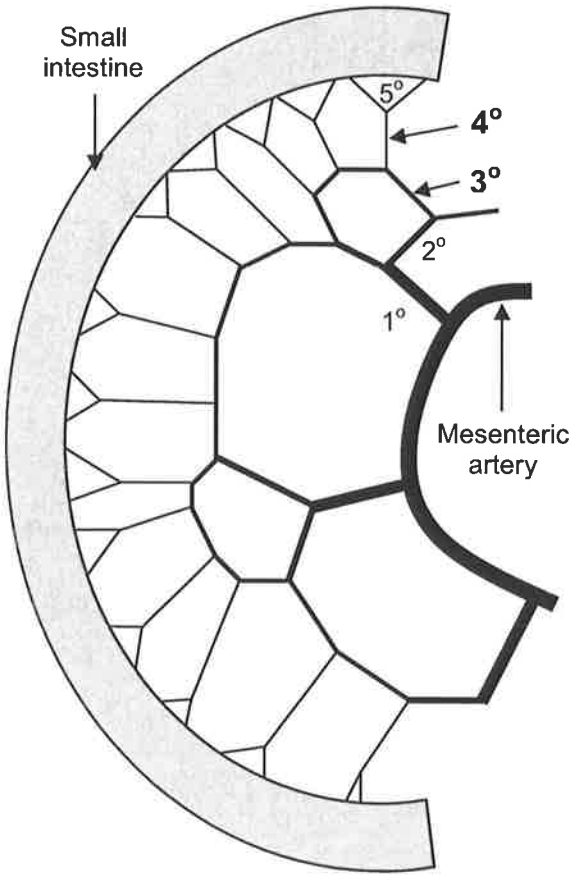

Figure 4.1: Fetal sheep mesenteric arcade branching pattern.

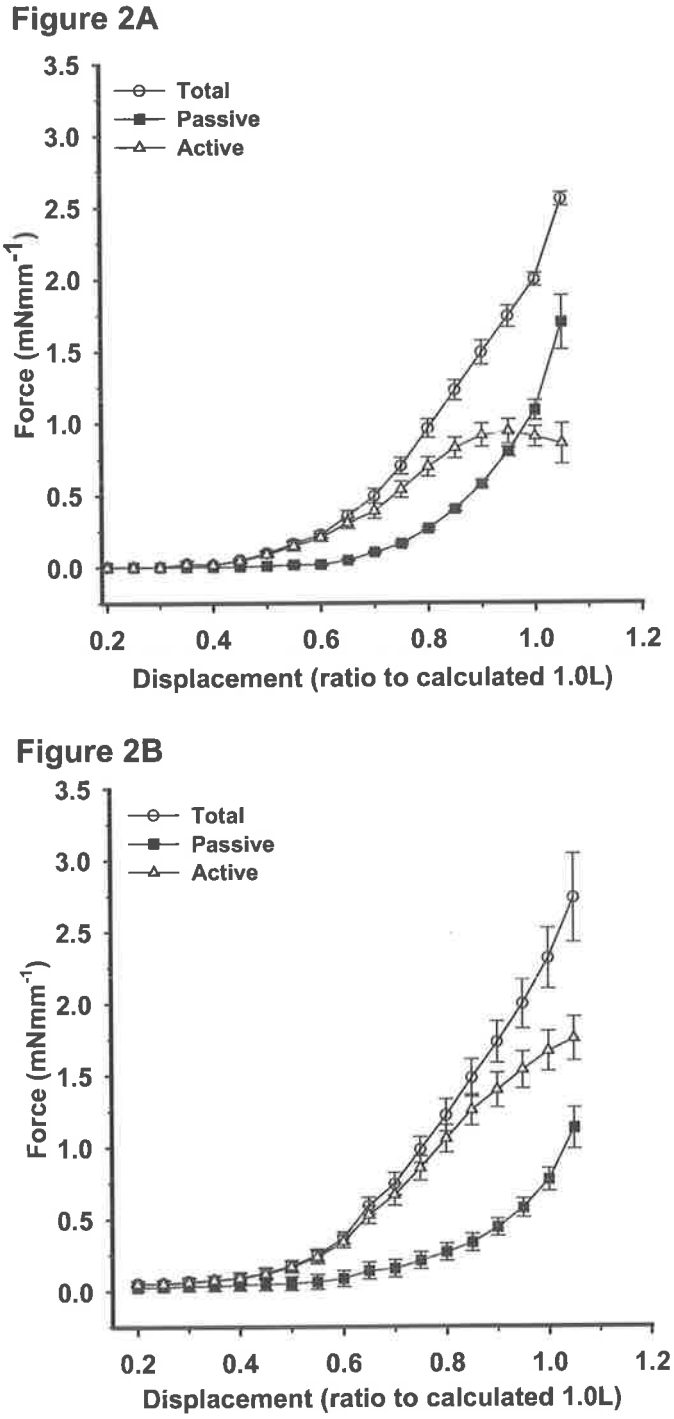

Figure 4.2: Contractile characteristics of mesenteric arteries

Relation of total, passive and calculated active tension generated during incremental stretch in **(A)** 3rd order and **(B)** 4th order mesenteric arteries from fetal sheep during late gestation. Each figure represents the mean ± SEM of data from 10 arterial segments collected from 5 fetal sheep.

Arteries were allowed a further 30 minutes equilibration to optimum resting tension before a cumulative concentration-response curve to noradrenaline (100nmol/L – 50μmol/L, Sigma) was performed. Preliminary experiments using the α_1 adrenergic receptor agonist phenylephrine indicated that arteries from both normoxic and hypoxic fetal sheep exhibited significant tachyphylaxis in response to this agonist. This interesting observation may relate to the ontogenic expression or activity of adrenergic receptors within the fetal mesenteric arcade, and warrants future investigation. It was not possible, however, to assess differences in vascular sensitivity between normoxic and hypoxic fetal sheep using this agonist and thus experiments were conducted with noradrenaline. Endothelial function was confirmed at the completion of each concentration response curve by determining the vascular response to administration of a bolus dose (50μmol/L) of the acetylcholine analogue acetyl-β-methylcholine chloride (Sigma).

4.3.5 Glucose assay

Fetal plasma glucose concentrations were assayed in 5 hypoxic and 5 normoxic fetuses at two time-points each (130-133dGA and 137-140dGA) by enzymatic analysis using the COBAS MIRA automated analysis (Roche Diagnostica, Basel Switzerland). This assay has previously been validated for sheep plasma samples. (Edwards & McMillen, 2001) The intra assay coefficient of variation was less than 10%.

4.3.6 Data analysis

The calculated mean arterial PO_2 throughout late gestation was used to categorize all fetuses as either hypoxic (PO_2 < 17.0 mmHg) or normoxic ($PO_2 \geq$ 17.0 mmHg) for analysis. The normoxic group included fetuses from 5 control (4 twin fetuses from 3 sets of twins and 1 singleton fetus) and 2 CX (1 twin fetus and 1 singleton fetus) pregnancies, of

which 4 fetuses were male and 3 female. All hypoxic fetuses were from 4 CX pregnancies and included 3 fetuses from 2 sets of twins and 2 singleton fetuses. The hypoxic group comprised 2 male and 3 female fetuses. The total number of animals used in these experiments was limited by unforseen delays in equipment availability and post-surgery animal losses related to maternal or fetal complications. Cumulative concentration response curves for noradrenaline were summarized by calculation of EC_{50} values using the Hill Slope Equation (Sigma Plot 8.0 Pharmacology Standard Curves Analysis). There was no significant effect of either fetal number or sex on mesenteric artery sensitivity to noradrenaline. When the vascular responses were compared using surgical treatment (PR or Control) and hypoxia (ie H or N) as the major factors in the analysis, there was no separate main effect of surgical treatment or interaction between surgical treatment and chronic hypoxia. Thus data were analysed on the basis of whether the fetus was hypoxic (H) or normoxic (N). Statistical analyses were performed using the $-\log_{10}$ of EC_{50} values; however for ease of presentation, data are presented graphically as the EC_{50} concentration. Data were analysed using t-test, two-way ANOVA with repeated measures or linear regression analysis as appropriate and are presented as mean ± SEM.

4.4 RESULTS

4.4.1 Fetal outcome and arterial blood gas status

Both the mean arterial PO_2 ($P<0.001$) and oxygen saturation ($P<0.001$) were lower in hypoxic than normoxic fetuses (Table 1). Hypoxic fetuses were also hypercapnic ($P<0.001$), and tended to have a lower arterial pH ($P=0.07$) across late gestation (Table 4.1).

Table 4.1: Fetal mean arterial blood gases

	PO_2 (mmHg)	O_2 Saturation (%)	PCO_2 (mmHg)	pH	Hb (g/dL)	Hematocrit (%)
Normoxic (n=7)	20.1±0.8	61.7±2.6	49.6±0.9	7.38±0.01	11.6±0.4	35.6±1.2
Hypoxic (n=5)	13.5±0.9[§]	32.6±2.8[§]	55.7±0.7[§]	7.36±0.01	13.3±1.3	40.9±3.9

Values are mean ± SEM. [§] Indicates a significant difference ($P<0.001$) between normoxic and hypoxic groups.

There was no difference in the gestational age of the normoxic (139.1 ± 0.4dGA) and hypoxic (139.8 ± 0.5dGA) groups at post mortem, but fetal body weight was significantly lower in the hypoxic compared with the normoxic group (Table 4.2, $P<0.001$). Relative brain weight was greater ($P<0.01$), and relative heart weight tended to be greater ($P=0.06$) in hypoxic fetuses. The relative total kidney weight was not different between groups, while relative liver weight was lower ($P<0.01$) in hypoxic fetuses (Table 4.2).

Table 4.2: ***Fetal body and organ weights***

	Body (kg)	Brain (g)	Heart (g)	Liver (g)	Kidney (g)
Normoxic (n=7)	4.77±0.24	56.8±1.2	30.9±1.7	115.1±8.4	26.2±2.6
Hypoxic (n=5)	2.59±0.40§	48.7±3.4*	20.7±1.1**	45.3±9.7§	16.4±2.4*
Relative organ weights (% BW)					
Normoxic (n=7)		1.21±0.05	0.65±0.02	2.41±1.20	0.55±0.04
Hypoxic (n=5)		2.00±0.16**	0.87±0.12	1.70±0.10**	0.64±0.02

Values are mean ± SEM. Significant differences between normoxic and hypoxic groups are indicated as *P<0.05, **P<0.01, § P<0.001.

4.4.2 Mesenteric artery diameter and constriction to noradrenaline

The diameter of 3rd order arteries was significantly greater than 4th order arteries (P<0.01) but there was no difference in the diameter of either 3rd or 4th order arteries between hypoxic and normoxic fetuses (3rd order: N, 304.3±24.5μm, H, 313.7±29.0μm, 4th order: N, 186.0±24.5μm, H, 203.9±35.5μm). The sensitivity of 3rd order mesenteric arteries to noradrenaline induced constriction was also not different between normoxic and hypoxic fetuses (Figure 3A, C). In contrast, 4th order arteries from hypoxic fetuses were significantly more sensitive to noradrenaline than 4th order arteries from normoxic fetuses (Figure 3B, C, P<0.01). The maximum tension developed in response to noradrenaline did not differ significantly between 3rd and 4th order arteries or between normoxic and hypoxic fetuses (3rd order: N, 1.9±0.2mNmm^{-1}, H, 1.9±0.2mNmm^{-1}, 4th order: N, 1.3±0.2 mNmm^{-1}, H, 1.8±0.2mNmm^{-1}). Maximum tension developed in response to noradrenaline was

directly related to diameter in 3rd order arteries (R^2=0.51, P<0.05, max tension = 0.445 + (0.0045 x diameter), but was not in 4th order arteries.

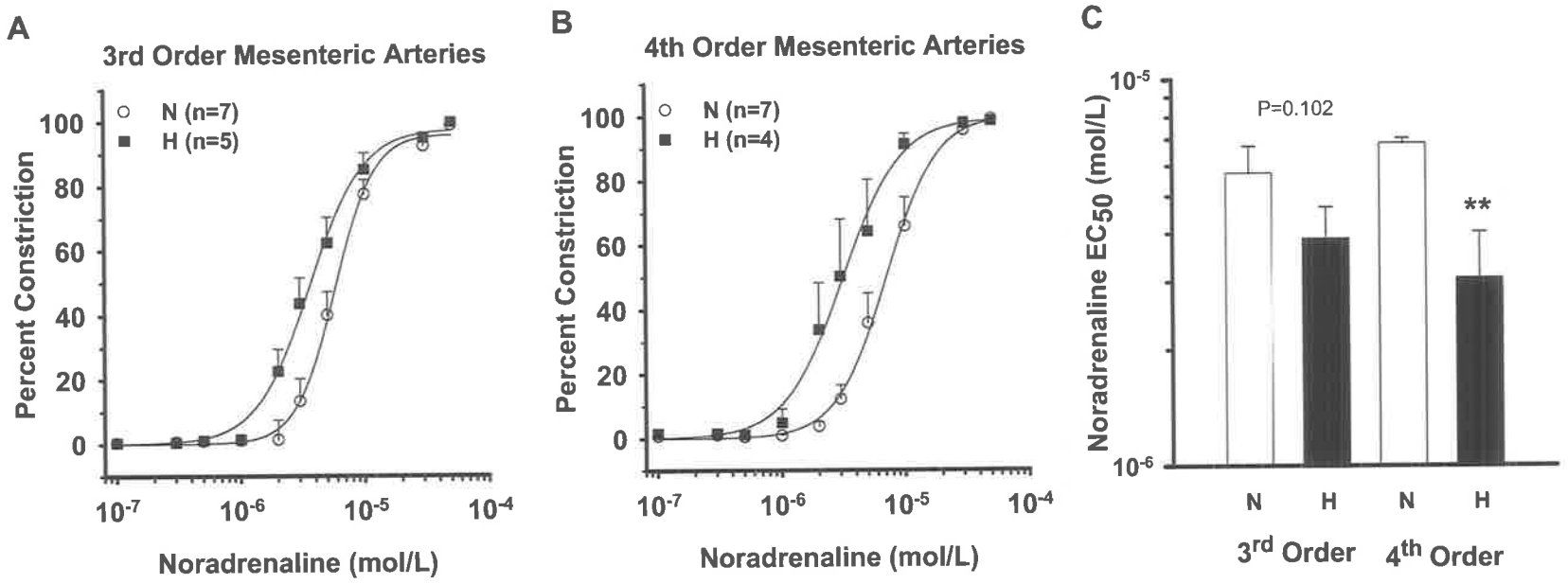

Figure 4.3: ***Fetal sheep mesenteric artery constriction to noradrenaline.***

Vasoconstriction to noradrenaline in **(A)** 3rd order and **(B)** 4th order mesenteric arteries from normoxic (N, open circles) and hypoxic (H, black squares) fetal sheep. **(C)** Sensitivity (EC_{50} values) to noradrenaline in 3rd and 4th order arteries from normoxic and hypoxic fetal sheep. **Indicates that within 4th order arteries the EC_{50} value was significantly lower ($P<0.01$) in arteries from hypoxic fetal sheep than from normoxic fetal sheep.

4.4.3 Relationship between vascular sensitivity to noradrenaline and fetal arterial blood gas status

There was no relationship between the sensitivity of the 3rd order mesenteric arteries to noradrenaline and mean gestational PO_2, PCO_2 or pH when data from the hypoxic and normoxic fetuses were combined (Figure 4A). The sensitivity of 4th order mesenteric arteries to noradrenaline was inversely related to the mean gestational arterial PO_2 such that the EC_{50} value increased as PO_2 increased (Figure 4B, $-\log_{10}EC_{50} = 6.344 - (0.0574 \times PO_2)$ $R^2=0.68$, $P<0.01$) and to mean PCO_2 (Figure 5B, $-\log_{10} EC_{50} = 2.077 + (0.0628 \times PCO_2)$ $R^2=0.71$, $P=0.001$). Partial correlational analysis indicated that the effects of PO_2 and PCO_2 on vascular sensitivity to noradrenaline were not independent. Although there was no significant relationship between the sensitivity of 3rd order mesenteric arteries to noradrenaline and any mean gestational blood gas measure, the sensitivity of 3rd order arteries was directly related to the sensitivity of 4th order arteries ($R^2 = 0.52$, $P<0.05$, [3rd order $-\log_{10} EC_{50}$] = $1.120 + (0.777 \times$ [4th order $-\log_{10} EC_{50}$]), implying that the large increase in 4th order mesenteric artery sensitivity to noradrenaline in hypoxic fetuses was associated with a smaller increase in 3rd order mesenteric artery sensitivity in hypoxic fetuses.

Figure 4.4

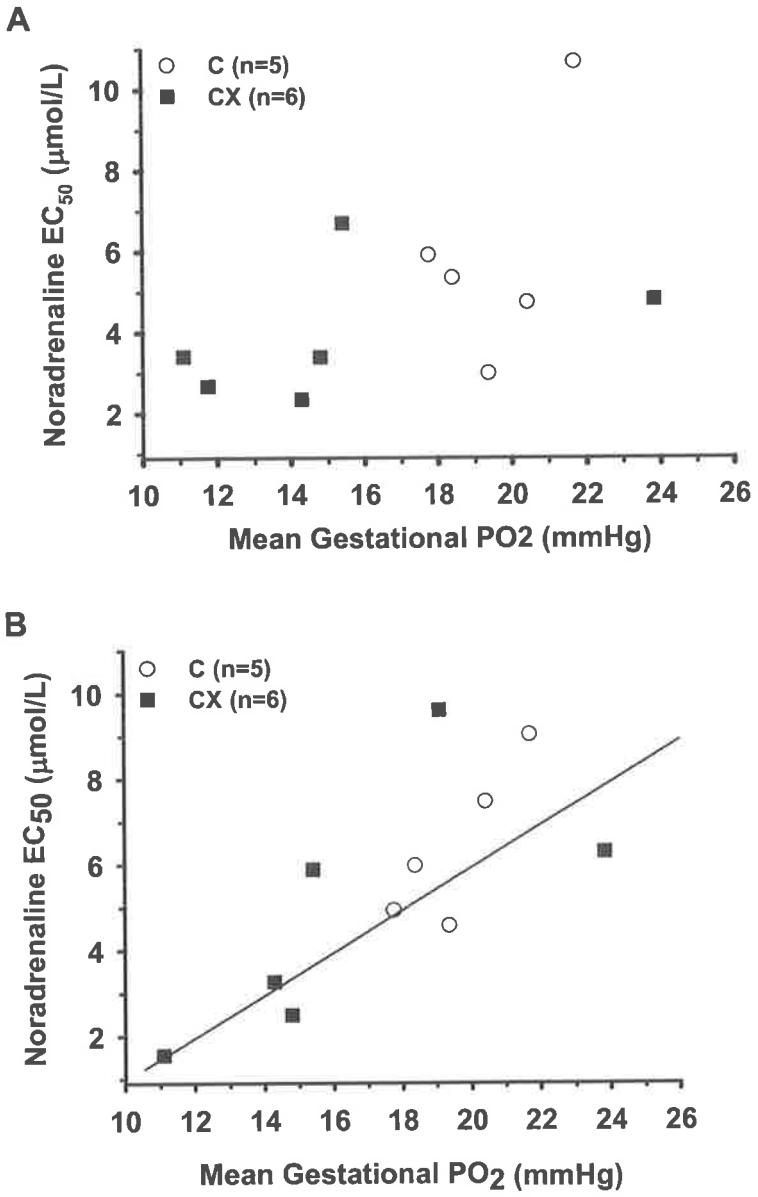

Figure 4.4: ***Mesenteric artery sensitivity to noradrenaline and mean arterial PO_2.***

The effect of prevailing PO_2 on sensitivity to noradrenaline was assessed in **(A)** 3rd and **(B)** 4th order mesenteric arteries collected from fetuses of control (C, open circles) or carunclectomy (CX, black squares) pregnancies. While no relation was present in 3rd order arteries, in 4th order arteries noradrenaline EC_{50} values were directly related to the mean gestational PO_2 ($R^2 = 0.68$, $P = 0.002$).

Figure 4.5

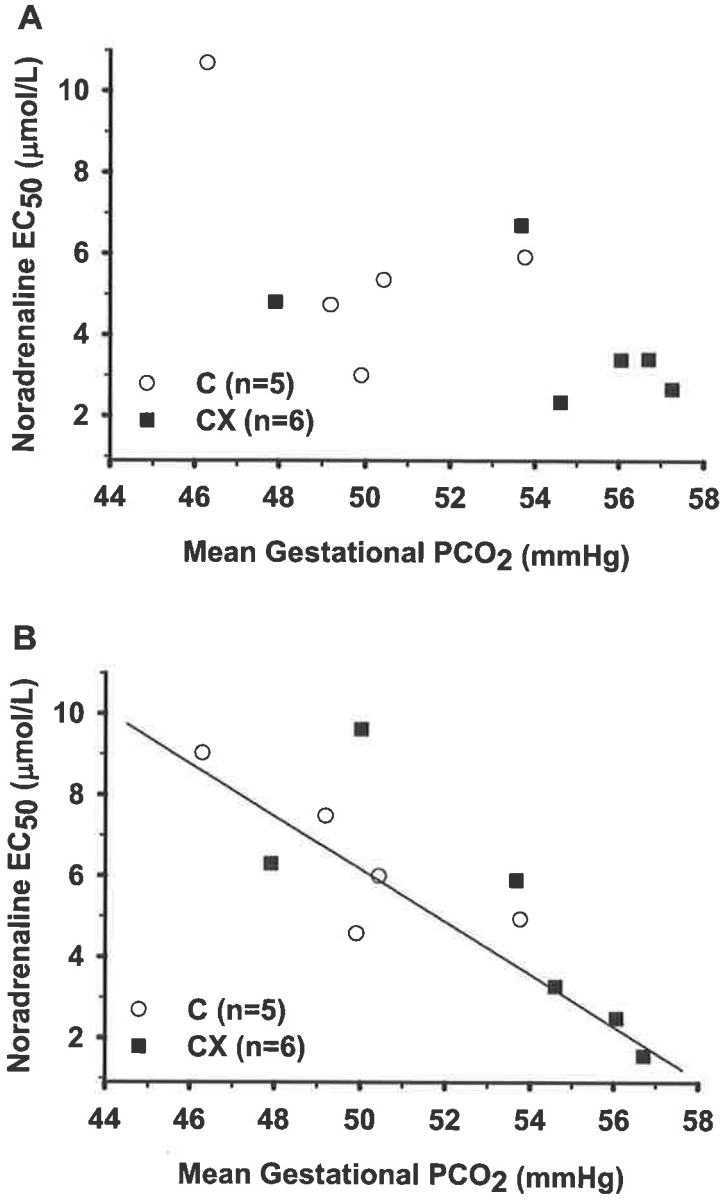

Figure 4.5: Mesenteric artery sensitivity to noradrenaline and mean arterial PCO_2.

The effect of prevailing PCO_2 on sensitivity to noradrenaline was assessed in **(A)** 3rd and **(B)** 4th order mesenteric arteries collected from fetuses of control (C, open circles) or carunclectomy (CX, black squares) pregnancies. While no relation was present in 3rd order arteries, in 4th order arteries the noradrenaline EC_{50} was directly related to PCO_2 ($R^2 = 0.71$, $P = 0.001$).

Fetal plasma glucose levels were lower in the hypoxic than normoxic group (N, 1.19±0.06, H, 0.68±0.11, $P<0.01$). There was no relationship, however, between plasma glucose concentration and the sensitivity of either the 3rd or 4th order mesenteric arteries to noradrenaline.

4.5 DISCUSSION

We have examined the impact of placental insufficiency, resulting in chronic fetal hypoxemia, hypercapnia and hypoglycemia on vasoconstriction to noradrenaline in small mesenteric arteries from fetal sheep during late gestation. The most significant finding of this study was that the sensitivity of 4th order mesenteric arteries to noradrenaline increased in direct relation to the degree of placental insufficiency, as indicated by the degree of both chronic fetal hypoxia and hypercapnia. As chronic fetal compromise is associated with elevated plasma catecholamine concentrations (Gagnon *et al.*, 1994; Simonetta *et al.*, 1997; Smolich & Esler, 1999), such changes in vascular sensitivity may play a significant role in sustaining a redistribution of cardiac output away from the fetal mesenteric circulation in IUGR fetuses. Interestingly, there was no significant effect of chronic fetal compromise on the sensitivity of 3rd order mesenteric arteries to noradrenaline. Noradrenaline EC_{50} values of 3rd and 4th order arteries were positively related, however, implying that a small increase in 3rd order artery sensitivity occurs in parallel with a larger increase in 4th order artery sensitivity within the mesenteric arcade of compromised fetuses. It is of particular interest that in this study the impact of placental insufficiency on vascular function was greatest in 4th order mesenteric arteries. These data may suggest that the smaller fetal resistance vessels, which are important to the regulation of blood pressure and regional blood flow, may also be more sensitive to perturbations in the *in utero* environment.

The experimental induction of restriction of placental growth in the sheep is a well characterized model which has been used to study the effects of chronic placental insufficiency on the fetal cardiovascular system (Robinson *et al.*, 1979; Edwards *et al.*, 1999b; Danielson *et al.*, 2005). In this study, as in others, the removal of the majority of endometrial caruncles from the sheep uterus prior to conception resulted in chronic fetal hypoxia in most, but not all of the fetuses in the CX group. There is a significant compensatory hypertrophic response in the remaining placentomes following mating which serves to limit the impact of the carunclectomy surgery on placental and hence fetal growth in some pregnancies (Robinson *et al.*, 1979; Robinson *et al.*, 1994; McMillen *et al.*, 2001). In the present study, there was only a significant effect of uterine carunclectomy on vascular sensitivity to noradrenaline when placental insufficiency ie chronic fetal hypoxaemia and hypoglycaemia was induced. These data therefore imply that the changes in fetal vascular function observed were a consequence of placental insufficiency. In human pregnancies, cordocentesis studies have demonstrated that IUGR fetuses are hypoxic, hypercapnic, (Nicolaides *et al.*, 1989) and hypoglycemic (Economides & Nicolaides, 1989), similar to the hypoxic group of fetal sheep within this study. While human IUGR fetuses were also acidotic (Nicolaides *et al.*, 1989), in our study the respiratory acidosis resulting from impaired placental clearance of CO_2 was generally compensated, such that there was no change in fetal arterial pH in the hypoxic group. The changes in body weight, and proportional organ weights in the hypoxic group were also consistent with asymmetric intrauterine growth restriction (Crane & Kopta, 1979; Robinson *et al.*, 1994; McMillen *et al.*, 2001), where brain growth was relatively conserved, while liver growth was disproportionately reduced.

While sensitivity to noradrenaline was specifically increased in 4th order arteries from hypoxic fetuses, in this study it was not possible to establish whether either chronic hypoxia or hypercapnia was the major factor responsible for determining mesenteric artery function. It is interesting, however, that although plasma glucose concentrations were lower in hypoxic fetuses there was no significant relation between plasma glucose concentration and the 3rd or 4th order mesenteric artery contractile response to noradrenaline. Hypoxia and hypercapnia represent strong biological stimuli. An acute reduction in oxygen supply elicits an adaptive fetal cardiovascular response observable by Doppler ultrasound from as early as 0.3-0.5 of gestation in fetal sheep (Kiserud *et al.*, 2001). By 0.7 of gestation in fetal sheep, the cardiovascular response to acute hypoxia includes an increase in peripheral vascular resistance (Iwamoto *et al.*, 1989). During late gestation, hypoxia sensed by fetal carotid chemoreceptors results in rapid, sympathetically-mediated peripheral vasoconstriction, which contributes to the centralization of cardiac output and a gradual increase in fetal arterial blood pressure (Giussani *et al.*, 1993). Vasoconstriction within the fetal sheep femoral vascular bed (Giussani *et al.*, 1993) and the chick embryo mesenteric arcade (Rouwet *et al.*, 2000) during acute hypoxia is significantly dependent on α-adrenergic receptor activation as each response was inhibited by phentolamine blockade of α-adrenergic receptors. Acute hypoxia therefore clearly modulates SNS activity and vasoconstriction within peripheral vascular beds, including the mesentery, via the activation of α-adrenergic receptors.

The fetal cardiovascular response to acute hypercapnia is less clear, and is difficult to separate from responses to parallel changes in arterial pH (Boekkooi *et al.*, 1992; Chen & Wood, 1993). Previously, 1 hour normoxic hypercapnia increased fetal heart rate after ~15-20 minutes in intact, but not sinoaortic (Raff *et al.*, 1991) or carotid sinus denervated and

vagotomized (Chen & Wood, 1993) fetal sheep, suggesting that fetal peripheral chemoreceptors are sensitive to CO_2. In these studies hypercapnia did not increase fetal mean arterial blood pressure, however, and the influence of hypercapnia on the peripheral circulation was not assessed (Raff *et al.*, 1991; Chen & Wood, 1993). A very brief (< 1 minute) increase in fetal arterial CO_2 produced fetal bradycardia which was significantly related to the change in arterial pH (Boekkooi *et al.*, 1992). Increasing basal fetal arterial PCO_2 to ~64mmHg by maternal CO_2 inhalation did not change the chemoreceptor response to acute uterine artery occlusion (Boekkooi *et al.*, 1992), however, suggesting that in the late gestation fetal sheep the chemoreflex responses to hypoxia and hypercapnia are not interdependent. In contrast, the increase in plasma vasopressin concentration was augmented during hypercapnic hypoxia comapred to normocapnic hypoxia, implying some synergistic effects (Raff *et al.*, 1991). More available data may support the impact of acute hypoxia than hypercapnia on peripheral vascular adrenergic tone, however it should be considered that both chronic fetal hypoxia and hypercapnia may be of relevance to clinical placental insufficiency (Nicolaides *et al.*, 1989).

As described in chapter 2, chronic maternal hypoxia, but not undernutrition in pregnant rats increased vasoconstriction to the α_1 adrenergic receptor agonist phenylephrine in femoral arteries isolated from neonatal offspring. Consistent with these data, maximal constriction to phenylephrine was also increased in isolated femoral arteries from fetal sheep after 5 days chronic hypoxia, induced by the continuous infusion of nitrogen into the maternal trachea (Kim *et al.*, 2005). In placentally restricted fetal sheep that were hypoxic and hypercapnic, *in vivo* blockade of α-adrenergic receptors with phentolamine during late gestation resulted in a greater decrease in mean arterial blood pressure than in control fetuses (Danielson *et al.*, 2005). These data may imply a functional role for enhanced

vascular sensitivity to adrenergic activation in the maintenance of peripheral resistance and hence fetal arterial blood pressure during chronic placental insufficiency. Danielson et al. observed no differences in pressor responses to phenylephrine between control and placentally restricted fetal sheep (Danielson *et al.*, 2005). However, the administration of a single bolus dose in this study may not have identified any differences in α_1-adrenergic sensitivity. Indeed, in the present study there were no differences in the maximal tension developed in response to noradrenaline in isolated arteries, while the enhanced vascular sensitivity, coupled with elevated plasma catecholamines may result in increased basal vasoconstrictor tone within 4th order mesenteric arteries.

Several mechanisms may mediate the enhanced sensitivity to noradrenaline observed in 4th order mesenteric arteries from the hypoxic group. In adult rats, the relative expression of α_1 adrenergic receptor subtypes differs along the arterial tree, with the α_{1D} adrenergic receptor subtype predominating in the superior mesenteric artery while α_{1B} subtype expression and functional contribution is greater in mesenteric resistance vessels (Piascik *et al.*, 1997). Hypoxia specifically up-regulated the expression and functional contribution of α_{1B} adrenergic receptors in rat aortic vascular smooth muscle cells during cell culture, tissue culture and in adult rats housed under normobaric hypoxia for 8h, without modifying expression of the α_{1D} receptor subtype (Eckhart *et al.*, 1996). Synthetic glucocorticoid exposure also increased α_{1B} adrenergic receptor expression in cultured smooth muscle cells (Sakaue & Hoffman, 1991). It is interesting to postulate that in the hypoxic group, either hypoxia or elevated glucocorticoids (Phillips *et al.*, 1996) may have increased α_{1B} subtype expression, which had a relatively greater impact in the distal mesenteric artery branches. Within an isolated-perfused mesentery preparation from normally grown fetal piglets,

inhibition of α_{1A} but not α_{1B} adrenergic receptor subtype significantly inhibited the pressor response to the α_1 agonist methoxamine (Hoang *et al.*, 1996), however, and the functional adrenergic receptor subtypes in the fetal sheep mesenteric arcade remain to be determined. In the mesenteric arcade from both fetal and neonatal piglets α_2 adrenergic receptors also contribute to vasoconstrictor responses (Ferrara *et al.*, 1996; Hoang *et al.*, 1996). Differences in α_2 adrenergic receptor expression may therefore also be of relevance.

Impairments in endothelial function may also have contributed to the enhanced sensitivity to noradrenaline observed in 4th order mesenteric arteries. Previously, maternal undernutrition decreased endothelium-dependent vasodilation in femoral artery branches from mid and late-gestation fetal sheep (Ozaki *et al.*, 2000; Nishina *et al.*, 2003). Similarly, chronic *in ovo* hypoxia impaired endothelium-dependent relaxation in femoral arteries isolated from chick embryos near to hatch (Ruijtenbeek *et al.*, 2003b; Villamor *et al.*, 2004). In fetal guinea pigs, chronic maternal hypoxia for 4 days transiently impaired fetal carotid artery endothelial function, which was fully compensated by up-regulation of NO production after 7 days hypoxia (Thompson & Weiner, 1999). While species differences should not be overlooked, comparison of these data may suggest that the impact of chronic hypoxia on endothelial function is regionally specific. In both *in vitro* (Li *et al.*, 2003b) and *in vivo* (Groenendijk *et al.*, 2005) preparations endothelial NO synthase expression, and/or NO production is increased by shear stress arising from increased flow velocity. Prolonged changes in regional blood flow may therefore provide a direct mechanism whereby endothelial function is differentially modified within vascular beds that receive more, or less blood flow during hypoxia. Consistent with this hypothesis, vascular NO production was directly related to flow rate in an *in vitro* perfused mesenteric arcade preparation from neonatal piglets (Reber *et al.*, 2001). Interestingly, reducing blood flow for 5 hours through

an *in vivo* mesenteric arcade loop preparation from neonatal piglets also increased the subsequent sensitivity of isolated mesenteric artery rings to noradrenaline, (Nowicki, 1999). Nitric oxide contributes substantially to the regulation of mesenteric blood flow in the normally grown mid (Fan *et al.*, 1998) and late-gestation fetal sheep (Fan *et al.*, 1996) and the impact of placental insufficiency on fetal mesenteric artery endothelial function warrants future investigation.

IUGR infants may be at increased risk of experiencing a severe hypoxic or asphyxic episode during late gestation. In normally grown fetal sheep at 0.7 of gestation, phentolamine infusion inhibited the biphasic mesenteric hypo-perfusion that followed an acute asphyxic episode, which peaked at approximately 1h, and between 4.5-6h after the insult in the vehicle-infused control fetuses (Quaedackers *et al.*, 2004). Increased sensitivity to noradrenaline, observed in smaller arteries in the present study may compound intestinal ischemia following a severe hypoxic or asphyxic insult, by augmenting the normal increase in mesenteric vascular resistance. Changes in mesenteric vascular function may therefore contribute to the increased risk of necrotizing enterocolitis in IUGR infants.

In conclusion, we have demonstrated that placental insufficiency results in a specific increase in the sensitivity of smaller fetal mesenteric arteries to noradrenaline, in direct relation to the degree of fetal hypoxia and hypercapnia observed during late gestation. Enhanced sensitivity of smaller artery branches to noradrenaline may play an important role in the maintenance of blood pressure in the chronically hypoxic, growth restricted fetus. However, the maintenance of enhanced adrenergic sensitivity within the mesenteric arcade may also increase the susceptibility of the IUGR infant to ischemic gut injury within the perinatal period. A persistent increase in the vascular sensitivity to adrenergic

vasoconstriction may also contribute to the peripheral vascular dysfunction during adult life which has been observed following the restriction of fetal growth in animal studies (McMillen & Robinson, 2005). Finally, these data may further support the evidence presented in chapters 2 and 3 which suggests that there is a specific impact of prenatal hypoxia on peripheral vascular sensitivity to adrenergic vasoconstriction.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 OVERVIEW

The general aim of the research presented within this thesis was to determine the impact of chronic prenatal hypoxia on vascular function. A significant body of work has demonstrated that maternal dietary perturbations during pregnancy influence the vascular function, in particular endothelial function of adult offspring. It was previously unclear, however, whether chronic prenatal hypoxia produced a differential impact on subsequent vascular function. This issue was important to clarify as in most instances IUGR is the end-result of a combined reduction in fetal oxygen and nutrient supply. The additional impact of chronic hypoxia on vascular function may therefore be of relevance to understanding the mechanisms that increase cardiovascular risk in persons whose prenatal growth was limited by several of the major causes of IUGR. Currently, several available pharmacologic strategies effectively lower blood pressure after the onset of hypertension (Turnbull, 2003; Lawes *et al.*, 2004); however the underlying etiology of essential hypertension remains unknown in the majority of cases. Understanding the mechanisms whereby IUGR produces long-term modifications in vascular function, which may impair the regulation of peripheral vascular resistance and thus increase cardiovascular risk during adult life, may therefore provide significant insight into one potential initiating mechanism of this serious condition.

Two key experimental techniques were used during these studies to assess vascular function *in vitro*; pressure and wire-based myography. Arteries collected from neonatal rat pups were of narrow diameter and highly fragile. Use of the pressure myograph overcame difficulties associated with these characteristics. To investigate the endothelial pathways that may contribute to changes in adult vascular function following IUGR, use of the wire myograph, where multiple vessels may be studied simultaneously, allowed efficient assessment of multiple pathways. Similarly, use of the wire myograph to study arteries

from fetal sheep enabled maximal use of tissues from these valuable animals. Results gained through the course of this research indicated that there was a specific impact of prenatal hypoxia, or the interaction of hypoxia and undernutrition, on postnatal vascular function. Figure 5.1 summarises key observations from this thesis, with comparison to previous reports regarding the impact of maternal malnutrition during pregnancy on vascular smooth muscle and endothelial function. These data thereby also highlight the complexity of vascular programming, where multiple mechanisms must eventually lead to the programming of vascular function. For instance, while inappropriate fetal glucocorticoid exposure has been implicated in the programming of adult cardiovascular phenotype (McMillen & Robinson, 2005), and also impacts fetal vascular function (Forhead *et al.*, 2000; Docherty *et al.*, 2001a; Molnar *et al.*, 2002) it would be expected that fetal glucocorticoid exposure would be elevated by both maternal hypoxia and undernutrition protocols. Additional mechanisms must therefore also be involved in determining the long-term vascular consequences of IUGR. The fetal cardiovascular adaptation to acute reductions in oxygen supply *in utero* has been well characterised, and is known to involve activation of a cascade of vascular signalling pathways. It is not yet clear how this initial response is modified during sustained fetal compromise. Furthermore, the activity of each pathway may depend on the functional capacity of the others (Green *et al.*, 1998a), which may differ in growth restricted fetuses (Edwards *et al.*, 1999b; Danielson *et al.*, 2005). The neural and hormonally mediated redistribution of fetal cardiac output also results in regional changes in both mechanical and metabolic factors that further regulate local vascular function. The regional specificity of the effects of prenatal hypoxia on neonatal vascular function (Chapter 2), and previously reported in chick embryos following chronic hypoxia *in ovo* (Villamor *et al.*, 2004) makes it tempting to postulate that changes

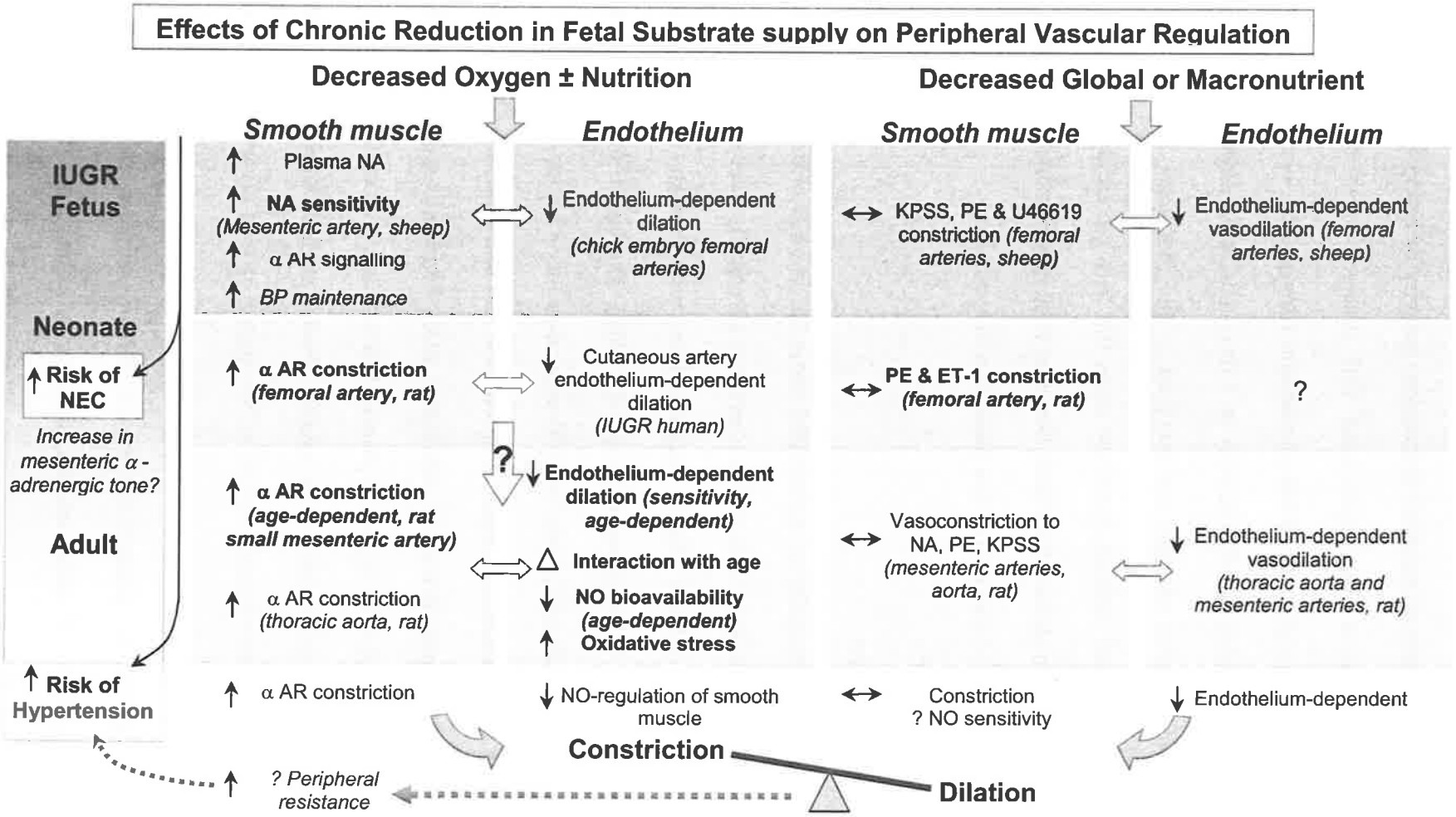

Figure 5.1: Overview of the impact of chronic fetal substrate reduction on vascular function

Data included within this thesis is indicated by bold text. For detailed discussion of the other studies, please refer to Chapter 1. AR; adrenergic receptor, ET-1; endothelin-1, IUGR; intrauterine growth restriction, NA; noradrenaline, NEC; necrotizing enterocolitis, NO; nitric oxide, PE; phenylephrine.

in regional blood flow may directly modulate local endothelial function, thus initiating the observed impairment in peripheral endothelial function. Activity of eNOS is significantly regulated by changes in shear stress associated with changes in local blood flow. Interestingly, in a perfused mesenteric arcade preparation from piglets, changes in blood flow had a much greater impact on NOS activity at 3 days than at 35 days postnatal age (Nowicki, 1999) Whether such changes are involved in the long-term impairment of peripheral vascular endothelial function is not yet clear, but does represent an interesting future direction. Much remains to be determined; however what may be concluded from the investigations that have formed the basis of this thesis is reviewed below.

5.1.1 Fetal vascular function

The surgical removal of the majority of endometrial caruncles in ewes prior to conception enabled the investigation of vascular function in a group of chronically hypoxic, placentally restricted fetal sheep that shared many characteristics with human IUGR fetuses (Economides & Nicolaides, 1989; Economides *et al.*, 1989). The larger size of the fetal sheep provided access to small artery branches within the mesenteric arcade, which may be of most relevance to the regulation of regional blood flow and blood pressure. This study provides the first direct evidence that small mesenteric artery function is modified by fetal growth restriction. Interestingly, the impact of placental insufficiency, as evidenced by fetal hypoxia and hypercapnia, was greatest in the smaller arteries studied, where the vascular sensitivity to noradrenaline was directly related to the degree of fetal compromise. It was also interesting that although fetuses within the hypoxic group were also hypoglycaemic, there was no significant relation between the degree of hypoglycaemia and sensitivity to noradrenaline in either 3rd or 4th order arteries, which may suggest that plasma glucose

levels do not exert a direct modulatory influence on fetal vascular catecholamine sensitivity within small mesenteric arteries.

5.1.2 Neonatal vascular function

In pregnant rats, exposure to chronic maternal hypoxia during the last week of gestation resulted in a substantial reduction in food intake and weight gain. When the effects of an equivalent level of undernutrition in the absence of hypoxia were determined, weight gain during pregnancy was reduced to a similar level. At birth, both protocols resulted in smaller, asymmetrically grown neonates without significant reduction in litter size. Neonatal proportional heart size was only increased by maternal hypoxia, however, suggesting that there was a specific effect of hypoxia on fetal growth. While maternal hypoxia did represent a dual insult during fetal development, and the possibility exists that this would therefore exert a more severe influence on the fetus overall, the generally similar effects on birth weight and growth asymmetry do not support this conclusion. It is therefore considered that the two maternal protocols differed in nature, but not necessarily in the degree of impact on fetal growth.

The changes in vascular function observed in neonates from either hypoxia or undernutrition dams were consistent with local vascular adaptations that may have maintained a centralization of cardiac output during fetal life. Following both protocols there was a decrease in the constrictor response of isolated carotid arteries to phenylephrine. Only maternal hypoxia reduced carotid artery constriction to endothelin-1, however, implying that the vascular adaptation to hypoxia and undernutrition versus undernutrition alone was specifically related to the prenatal environment. In the fetal sheep, inhibition of the endothelin type A receptor in vivo reduced basal carotid artery vascular

resistance, which may suggest a role for endothelin in regulating basal carotid artery tone (Green *et al.*, 1998b). Although endothelin-1 generally is produced and acts locally, increased plasma concentrations of this factor have also been reported in IUGR infants at birth (Arslan *et al.*, 2004). Expression of the endothelin-1 precursor preproendothelin-1 is also up-regulated by hypoxia (Rakugi *et al.*, 1990), and the possibility therefore exists that during maternal hypoxia the fetal carotid artery responsiveness to endothelin-1 was specifically reduced to compensate for a hypoxia-mediated increase in endothelin-1 production. Indeed, in umbilical arteries isolated from IUGR pregnancies, vascular responsiveness to endothelin-1 was also reduced (Bodelsson *et al.*, 1995), further suggesting that decreased vasoconstriction to this factor may be an important adaptation within circulations where blood flow is increased during chronic hypoxia. Bodelsson *et al.* reported that while vasoconstriction to endothelin-1 and noradrenaline were reduced, constriction to serotonin and the thromboxane mimetic U46619 did not differ in umbilical arteries from normal or IUGR pregnancies (Bodelsson *et al.*, 1995), which does further suggest that down regulation of certain vasoconstrictor pathways contributes to the vascular adaptation to IUGR.

As neonatal carotid artery vasoconstriction to both phenylephrine and endothelin-1 were reduced following maternal hypoxia, it is also possible, however, that enhanced basal endothelial function offset constrictor responses to each factor (Thompson *et al.*, 2004). Since vasoconstriction to endothelin-1 did not differ in carotid arteries from pups of control and nutrient restriction dams, however, increased basal endothelium-derived vasodilator production would not explain the suppression of phenylephrine-induced constriction observed in these neonates. The impact of hypoxia and/or nutrient restriction before birth

on neonatal endothelial function was not assessed through this project, but warrants future investigation.

Maternal hypoxia increased vasoconstriction to phenylephrine in femoral arteries isolated from neonatal pups without modifying sensitivity. This is in contrast to the effects of placental insufficiency on small mesenteric arteries from fetal sheep described in chapter 4, where chronic fetal compromise increased sensitivity but not maximal vasoconstriction to noradrenaline. A recent preliminary study has, however, reported a similar increase in maximal constriction to phenylephrine in femoral arteries isolated from fetal sheep after 5 days of maternal hypoxia (Kim *et al.*, 2005). Our observations in fetal mesenteric arteries also strongly suggest that the impact of fetal compromise on the vascular adrenergic pathway is region specific within the vascular tree. It is therefore possible that the adaptation to prenatal hypoxia involves an increase in maximal constriction in peripheral conduit arteries, while sensitivity is increased within small artery branches.

This study has clearly identified that reduced oxygen and/or nutrient supply during the last week of gestation in the rat produced asymmetric fetal growth restriction, and modified regional vascular function in the neonate in a manner consistent with the redistribution of cardiac output during fetal life. The differential impact of maternal hypoxia versus undernutrition further implied that changes in vascular function observed after birth may relate to the specific etiology of fetal growth restriction. By focussing this study on vascular function shortly after birth we were also able to demonstrate that, prior to any differences in postnatal growth among groups, differences in the regulation of regional vascular function were related to the specific nature of the intrauterine environment.

5.1.3 Adult vascular function

To determine the long-term vascular consequences of reduced oxygen and/or nutrient supply before birth, small mesenteric artery function was assessed in the adult offspring of maternal hypoxia and nutrient restriction dams. This study focussed on male offspring only, to facilitate a more detailed investigation of the vascular pathways influenced by the prenatal environment; however the effects of these protocols on vascular function in female offspring were assessed in a related investigation. Interestingly, maternal hypoxia or nutrient restriction also resulted in differential effects on vascular function during adult life. At 4 months, no differences were observed in constriction to phenylephrine among male offspring from control, hypoxia or nutrient restriction dams, while endothelium-dependent vasodilation was impaired in only hypoxia offspring. By 7 months, sensitivity to phenylephrine-mediated constriction was substantially increased in hypoxia offspring, with a smaller effect observed in nutrient restriction offspring. At this age, sensitivity to endothelium-dependent vasodilation did not differ among the groups. It is apparent, however, that both at 4 and 7 months, the balance of vasoconstrictor to vasodilator responses assessed in small mesenteric arteries was shifted toward vasoconstriction in offspring exposed to prenatal hypoxia. The age-dependent increase in the adrenergic sensitivity of small mesenteric arteries from offspring of hypoxia dams is also interesting given the enhanced contractile responses to noradrenaline and phenylephrine observed in fetal and neonatal arteries respectively following chronic prenatal hypoxia. Previously, feeding a globally restricted, protein deficient or high fat diet during pregnancy in rats did not modify sensitivity to adrenergic receptor-mediated vasoconstriction in aorta or mesenteric artery preparations, while endothelial function was impaired in these studies (reviewed in chapter 1). In contrast, uteroplacental insufficiency secondary to uterine artery ligation did increase sensitivity and maximal constriction in adult male rat offspring (Payne

et al., 2003; Payne *et al.*, 2004). Similarly, in eNOS heterozygous offspring from eNOS -/- mouse dams, which may also be considered a model of uteroplacental insufficiency, constriction to phenylephrine was enhanced in both isolated mesenteric and carotid arteries (Longo *et al.*, 2005). Taken together, these results provide a strong argument that reduced fetal oxygen and nutrient supply impacts vascular adrenergic receptor function, while reduced nutrition alone may not.

Comparison of the effects of age on endothelial function within the 3 experimental groups indicated that while arteries from control offspring demonstrated the expected decline in endothelial function with advancing age, this effect was absent in offspring from both hypoxia and undernutrition dams. The interaction between the prenatal environment and the complex process of vascular aging has not been extensively studied, but does warrant further consideration. Results obtained in this study may suggest that aspects of normal vascular aging may occur prematurely, with modification of the normal time-course of functional age-related changes following reduced oxygen and/or nutrient supply *in utero*. Such differences may have considerable relevance to the development of cardiovascular disease in individuals whose prenatal growth was impaired.

Experiments to determine the contribution of NO, and prostaglandin production to endothelium-dependent vasodilation indicated that the impairment in endothelial function observed in 4 month old offspring from hypoxia exposed dams did not result from a loss of function of either of these vasodilator pathways. These data may thereby imply that changes in the endothelium-derived hyperpolarizing factor pathway mediated the decrease in endothelial sensitivity to methacholine observed at this time-point. At 7 months, although sensitivity to endothelium-dependent vasodilation did not differ among groups,

NO no longer contributed to endothelial responses in offspring from hypoxia dams. No differences in vascular smooth muscle sensitivity to the NO donor sodium nitroprusside were observed among the three groups at this age. As exogenous superoxide dismutase improved endothelium-dependent dilation in only offspring from hypoxia dams, the loss of NO-mediated relaxation may be related, in part, to reduced NO bioavailability secondary to enhanced local scavenging by superoxide anion. This is further supported by data indicating that eNOS expression was not different in mesenteric arteries from offspring of control or hypoxia dams at 7 months, although the age-dependent increase in eNOS expression in offspring from control dams was not observed in arteries from hypoxia dams. Interestingly, consistent with previous reports, eNOS expression was lower in mesenteric arteries from offspring of undernourished dams (Franco *et al.*, 2002a).

As reviewed in chapter 1, numerous studies have now demonstrated that maternal dietary manipulations throughout gestation in rats impair endothelial function during adult life, and in many instances these studies implicated changes in the function of the NO pathway. The lack of effect of maternal undernutrition during the last week of pregnancy on endothelium-dependent vasodilation in this study may suggest that the critical window for vascular programming by nutritional perturbations occurs earlier in gestation in rats. The restriction of maternal nutrition for only the last week of pregnancy is also unlikely to represent a common biological occurrence, whereas fetal oxygen supply is more likely to become limited during late gestation (Robinson *et al.*, 1994).

It is interesting that the NO pathway appears to be particularly sensitive to the impact of an adverse *in utero* environment. The mechanisms that may result in long term reductions in vascular NO bio-availability following IUGR are not yet clear, and may involve differential

impact on eNOS expression (Franco *et al.*, 2002a), soluble guanylate cyclase expression (Lamireau *et al.*, 2002), cofactor availability (Franco *et al.*, 2004) or NO scavenging (Franco *et al.*, 2002b; Franco *et al.*, 2003; Franco Mdo *et al.*, 2003), which may be further dependent on the etiology of IUGR. Changes in the targeting of eNOS within endothelial cells or in important protein-protein regulatory mechanisms also remain to be investigated. The potential consequences of prolonged decreases in NO bioavailability are much clearer, however. As NO provides important modulation of short and long-term vascular smooth muscle function, impaired NO bio-availability may have a substantial impact on vascular smooth muscle phenotype. Interestingly, NO also de-activates noradrenaline under *in vitro* conditions (Kolo *et al.*, 2004), suggesting that the loss of NO may further enhance adrenergic signalling within small arteries. As the NO pathway appears to be sensitive to both hypoxia concomitant with undernutrition during late gestation, and maternal malnutrition throughout gestation alone, it may also represent a potential therapeutic target in individuals who were born small for gestational age regardless of the underlying cause of IUGR.

5.2 CONCLUSIONS

Changes in regional vascular function may be central to the fetal adaptation to a reduction in substrate supply *in utero*, and regional changes in fetal vascular resistance represent one of the most reliable clinical indicators of IUGR and chronic fetal hypoxia (Karsdorp *et al.*, 1994; Arbeille, 1997). The data presented within this thesis provide clear evidence that fetal compromise, including the reduction of oxygen and nutrient supply *in utero*, results in prolonged changes in the regulation of vascular function. Differences in the regulation of peripheral vascular tone following IUGR may contribute to the increased incidence of

certain complications within the perinatal period, and may also contribute to the elevated risk of developing hypertension during adult life.

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"Science is a wonderful thing if one does not have to earn one's living at it."

Albert Einstein