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The Vascular Link Between Intrauterine Hypoxia and Postnatal Cardiovascular Pathology

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology

THE VASCULAR LINK BETWEEN INTRAUTERINE HYPOXIA AND POSTNATAL CARDIOVASCULAR PATHOLOGY

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ABSTRACT

The effect of intrauterine hypoxia on arterial development was evaluated with use of large and small animal models. Analyses included expression and deposition of extracellular matrix (ECM) proteins, differentiation and proliferation of vascular smooth muscle cells (VSMCs), intima formation and wall thickening. A comprehensive investigation of possible molecular, mechanical and hormonal mediators of altered arterial development was afforded by a sheep model with both acute and chronic hypoxemia studies, whereas a guinea pig model allowed for long-term study. Our findings show that chronically hypoxic fetal sheep and intrauterine growth restricted (IUGR) guinea pigs exhibit a reduction in elastic fibre content of the aorta. In adulthood, the deficiency in aortic elastic fibre content in growth restricted guinea pig offspring was amplified compared to the subtle changes observed in late fetal life. In severely hypoxic fetal sheep, more marked reduction in elastin content occurred with increases in wall thickness and VSMC content. Increased collagen paralleled elevated mRNA levels of procollagen I and transforming growth factor beta (TGF-β_1). Matrix metalloproteinase-2 (MMP-2) mRNA levels were inversely correlated with fetal arterial oxygen saturation and expression of its activator, membrane-type MMP (MTI-MMP), was elevated in severely hypoxic sheep. Marked neointima formation was also apparent in severely hypoxic fetuses concomitant with increased mRNA levels of E-selectin, indicating endothelial inflammation. These structural and molecular changes of the aorta in chronically hypoxic ovine fetuses occurred without changes in pressure or circulating cortisol levels. Further, while the hypoxic sheep showed no change in VSMC maturation, aortae of IUGR guinea pig fetuses and offspring had increased content of myosin heavy chain B (MHC-B), a marker

of undifferentiated VSMCs. Aortae of growth impaired guinea pig offspring exhibited a left shift in the length-tension curve as measured *ex vivo*. Thus altered aortic development in association with chronic hypoxia or IUGR leads to persistent structural abnormalities and reduced compliance in later life. In contrast, acute hypoxic study in fetal sheep demonstrated increased elastin content of the carotid artery in association with intermittent hemodynamic changes and elevated cortisol and thus highlight that beneficial adaptations are possible under certain intrauterine insults.

Key words: Hypoxia, Extracellular Matrix, Arterial Compliance

DEDICATION

To my daughters Reily and Zara

CO-AUTHORSHIP

The contribution of co-authors to chapters 2-4 of the thesis are acknowledged and outlined below.

Dr. Timothy Regnault Ph.D, Dr. Bryan Richardson M.D. and Dr. Robert Gagnon M.D., provided intellectual input and scientific expertise in the design and experimental details of the sheep studies outlined in Chapters 2 and 4. Dr. Regnault and Dr. Richardson conceived the experimental paradigm and provided intellectual input for Chapter 3 and reviewed and edited the manuscripts for Chapters 2-4. Dr. Robert Gros Ph.D provided technical assistance for the vessel function studies and reviewed the manuscript in Chapter 3. Graduate student, Karolina Piorkowska B.Sc weighed the guinea pig offspring weekly and assisted with guinea pig surgeries and post-mortems. Under my supervision graduate student, Sarah Folliot B.Sc performed a preliminary study for Chapter 4.

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AFP, α-fetoprotein

AGA, appropriate-for-gestational age

bFGF, basic fibroblast growth factor

CAD, coronary artery disease

CAM, cell adhesion molecule

CCO, combined cardiac output

CIHR, Canadian Institutes of Health Research

CVD, cardiovascular disease

DA, ductus arteriosus

DV, ductus venosus

ECM, extracellular matrix

EDFV, end diastolic flow velocity

EFW, estimated fetal weight

FHR, fetal heart rate

IGF, insulin-like growth factor

IVC, inferior vena cava

LGA, large-for-gestational age

MAP, mean arterial blood pressure

MHC-B, non-muscle myosin heavy chain B

MMP, matrix metalloproteinase

MTI-MMP, membrane-type metalloproteinase

NO, nitric oxide

NST, non-stress test

PAPP-A, pregnancy-associated plasma protein A

PCNA, proliferating cell nuclear antigen

PI, pulsatility index

PPAR-α; perioxisome proliferator-activating receptor

PWV, pulse wave velocity

RI, resistance index

SD, systolic-diastolic

SGA, small-for-gestational age

SMA, superior mesenteric artery

TGF-β, transforming growth factor beta

SNP, sodium nitropusside

TNF, tumor necrotic factor

UA, umbilical artery

UAL, uterine artery ligation

UCO, umbilical cord occlusion

VSMC, vascular smooth muscle cell

WHO, world health organization.

CHAPTER 1

THESIS TOPIC REVIEW

1.1 GENERAL INTRODUCTION

The group of pathological conditions affecting the heart and blood vessels known as cardiovascular disease (CVD) is the most likely cause of death in Canada and a burgeoning health problem worldwide. Traditionally, etiology and disease prevention have focused on genetics and environmental influences imposed during adult life such as smoking, diet and activity. However, it is now recognized that CVD can be traced to a vulnerability established *in utero*, independent of the classical risk factors. Early evidence of an impact of the fetal environment on later cardiovascular health emerged from the observation that low birth weight was predictive of hypertension, coronary artery disease, stroke, and other forms of CVD in adulthood (Barker, 2001). Numerous subsequent studies substantiated the association between impaired fetal growth and the development of adult-onset CVD in a variety of populations around the world including both developing and developed countries. Although developmental origins of CVD are irrefutable, little is known about the physiological mechanisms involved.

Structural abnormalities of the vasculature and heart, disturbed hemodynamics and progressive mechanical malfunction, are the known precursors of CVD in adults. Often overlooked, is the fact that susceptibility to such pathological changes is largely determined before birth. For instance, extracellular matrix composition, cellular phenotype and thus functional capacity of the cardiovascular system, are largely established during fetal and early neonatal life. Once this brief developmental window comes to a close, the heart and vessels no longer enjoy the plasticity they were afforded previously. Hence, an interference in remodelling of the cardiovascular system *in utero* will likely have long-term functional consequences and thereby initiate and accelerate progression towards CVD in individuals growth restricted in the womb.

1.2 FETAL GROWTH

Human development is orchestrated by a spatial and temporal regulation of proliferation, differentiation, organization and accretion of the cells and tissues. During the embryonic period

cells are arranged into the major organ systems, after which functional maturation along with an increase in mass of tissues, occur throughout the remaining fetal period. This time course of development occurs in parallel with changing hormonal levels in the fetal circulation which regulate uptake and metabolism of the maternally-supplied substrates utilized for growth. Substrate requirements are low during organogenesis and then rise exponentially at the end of the first trimester concurrent with rapid hyperplasia and hypertrophy. The rate of human fetal growth increases from 5g/day between 14 and 15 weeks of gestation to 10g/day at 20 weeks of gestation and then peaks at 32-35 weeks with a rate of 30-35g/day (Harding and Bocking, 2001). There is a plateau in growth towards term at approximately 41-42 weeks. Thus, the course of intrauterine growth follows a non-linear, sigmoid pattern. Normative growth curves have been constructed from sequential Estimates of Fetal Weight (EFW), calculated from a number of sonographic measurements such as head circumference, abdominal circumference and femur length. Together with gestational dating, these references are used for clinical assessment of fetal growth during pregnancy and at birth. This surveillance of fetal growth provides important information regarding the well-being of the fetus and the state of its supporting environment.

A body mass that is appropriate for gestational age (AGA) according to a normative growth curve suggests a healthy fetus or neonate and an optimal intrauterine milieu. Infants born large for gestational age (LGA) are those whose birth weights lie above the 90th percentile for a given gestational age. At the other extreme, small for gestational age (SGA) is most commonly defined as a fetal weight or birth weight below the 10th percentile at a given point in pregnancy and considered a proxy for suboptimal intrauterine growth (Harding and Bocking, 2001). Additional cut off values used for inference of poor fetal development include a birth weight under 2500g, as put forward by the World Health Organization (WHO) (UNICEF/WHO, 2004) and an EFW or birth weight that falls two standard deviations below the mean for gestational age (Harding and Bocking, 2001). A shortcoming in the use of normative growth curves for clinical evaluation and epidemiological study stems from the wide variation in individual fetal growth trajectories, that limits the value of weight as an indicator of growth impairment. A low birth weight infant may be constitutionally small having attained its growth potential *in utero*. On the other hand, a newborn of normal weight may have failed to reach its genetically pre-determined size. Genuine intrauterine growth restriction (IUGR) is defined as a deviation in the intrauterine

growth trajectory due to an intrinsic or environmental insult that results in small size at birth (Ergaz *et al*., 2005). When the developmental disturbance occurs in the early part of gestation in concert with rapid cell division and organ formation, there is a proportional diminution of body weight, length and head size that is termed symmetrical growth restriction (Cox *et al*., 2009). In contrast, asymmetrical IUGR characterized by disproportionate body morphology, arises when the insult coincides with rapid fetal growth in the second half of gestation and leads to relative sparing of critical organs such as the brain and heart at the expense of the liver, skeletal muscle and other non-critical organs (Cox *et al*., 2009). Measurement of relative body dimensions such as the ratio of mid-arm-to-head circumference and abdominal-to-head circumference facilitate accurate diagnosis of the more frequently occurring asymmetric pattern of IUGR (William, 2006). Identification of pathologic growth attenuation is less uncertain at post-mortem since the brain:liver ratio can be measured, which is the most reliable indicator of IUGR (William, 2006). For the obstetrician, recent technological advances allow for 3 dimensional ultrasound to calculate fetal brain:liver ratios, although the utility of this new method is yet undecided (William, 2006). Reliable diagnosis of IUGR in the perinatal period is an important goal given the short and long-term risks associated with this condition. Since demarcation of SGA and IUGR is frequently unclear in the clinical setting, the two terms are often used interchangeably in the context of human studies.

Currently, low birth weight is a high priority public health concern declared by international bodies such as the United Nations, since its mitigation would improve prenatal, neonatal and postnatal outcomes. According to the WHO, 15 percent of newborns worldwide are born with a birth weight under 2500g (UNICEF/WHO, 2004). Abnormal intrauterine growth is a considerable problem in underdeveloped nations where the rate of low birth rate is 6 times higher than that of developed countries, with rates above 30 percent in areas such as South Asia (de Onis *et al*., 1998). Among developed countries the incidence of low birth weight ranges from 3.9 percent to 11.3 percent, Canada ranking 9th with a rate of 6 percent and the United States ranking 25th with a rate of 8 percent (Raphael, 2010). In-hospital data compiled by the Canadian Institutes of Health Research (CIHR) and data including all births reported by Statistics Canada show that the incidence of SGA babies in Canada has increased from 1979 to 2005 (Raphael, 2010). In agreement, the March of Dimes proclaims that over the past 10 years the number of

SGA babies born in the United States has increased by 10 percent (Martin, 2007). Thus, suboptimal fetal growth is a long-standing health issue that over the past two decades has been exacerbated on a global scale with deepening poverty and in developed countries has failed to improve despite advances in perinatal care.

Fetuses that exhibit signs of IUGR are at increased risk for perinatal death, complications after birth and morbidity in the first year of life. Hence, such pregnancies are classified as high risk with obstetric management directed towards surveillance for optimal timing of delivery in consideration of fetal risk. The immediate concern posed by the IUGR pregnancy is perinatal demise. Infants classified as SGA have a death rate 20 fold higher than those born of normal weight and 43 percent of stillbirths have a body weight below the 10th percentile (Gardosi, 2005). The most frequent cause of perinatal death in cases of IUGR is asphyxia and congenital anomalies (McIntire *et al*., 1999). With perinatal asphyxia the newborn can suffer from complications that include multi-organ dysfunction (Wang *et al*., 2009), neonatal encaphalopathy (Bukowski *et al*., 2001), and metabolic acidemia (McIntire *et al*., 1999). Other dangers present after birth of an IUGR baby are meconium aspiration with persistent pulmonary hypertension and consequent respiratory complications (Fleischer *et al*., 1992; McIntire *et al*., 1999) as well as hypoglycemia and hypothermia (Doctor *et al*., 2001). In infants that survive poor intrauterine conditions and the subsequent hazards imposed by extrauterine surroundings, cerebral palsy (Jarvis *et al*., 2003) and developmental delay (Torrance *et al*., 2010) are reported more frequently. Overall the numerous adverse outcomes associated with impaired fetal growth, such as those discussed above, present in wide variance given the heterogeneous nature of IUGR.

The diverse etiologies of abnormal fetal growth emanate from the complexity in growth regulation. Factors affecting growth *in utero* can be divided into fetal, maternal and placental influences as shown in Table 1.1. The fundamental determinant of fetal size is established at fertilization in the newly constituted genetic profile of the conceptus that comprises an array of inherited genes, some of which set the intrauterine growth trajectory. Genetic influence accounts for 40 percent of the variation in birth weight (Magnus *et al*., 1984), with contributions from both the maternal and paternal genome. When inherited or acquired abnormalities in the genetic makeup arise, diminution in fetal growth frequently results, with 5-20 percent of SGA infants

Table 1.1: Factors Affecting Intrauterine Growth

Genetic (Estimated contribution 5-20 %)
Fetal Genome
Inherited/Acquired Genetic Abnormalties

Environmental (Estimated contribution 80-95%)
Maternal Size
Maternal Infection
Environmental Toxins
Multiple Gestation
Maternal Nutrient Intake
Maternal Vascular Disease
Maternal Hormones
Placental Factors (Estimated contribution 60%) *

* Genetic abnormalities, toxins, maternal infection nutrient intake and vascular disease can affect placental growth and development.

showing genetic disorders (Brodsky and Christou, 2004). However, whilst the genome sets the ultimate limits on fetal growth, size at birth is predominantly a function of the environment.

Disturbances in the intrauterine milieu that affect growth early in gestation include chronic congenital infection and exposure to environmental toxins. Infections documented to have consistent negative effects on birth weight when transmitted to the developing fetus, include rubella, syphilis, toxoplasmosis and cytomegalovirus (Khan and Kazzi, 2000). In terms of environmental toxins, effects on fetal growth have been reported with maternal intake of prescription and recreational drugs (Fulroth *et al*., 1989), alcohol and tobacco (Krol *et al*., 2009). Early exposure to infection and toxins together with fetal genetic disorders are considered to be the causes of symmetric IUGR, due to their effects on cellular proliferation (Cox *et al*., 2009). Yet, true symmetrical IUGR is rare, and the vast majority of live and stillborn IUGR births show an asymmetric pattern of growth. The latter pattern typically arises in the second half of gestation when the fetus is deprived of the substrates that fuel its rapid growth and maturation.

With advancement of pregnancy, the increasing reliance of the fetus on maternal provision of sustenance makes submissive the genetically predetermined growth trajectory to any challenges in this environment. The critical substrates utilized by the fetus for its growth and development are oxygen, glucose, amino acids and fatty acids. Thus, healthy intrauterine conditions depend on adequate concentrations of these substrates in the maternal circulation and their delivery across the maternal-fetal interface, which in turn, is a function of uterine and umbilical hemodynamics and placental transport mechanisms. Whereas fetal compromise in poor nations primarily arises from malnutrition, in affluent societies, IUGR is predominantly placental in nature. The placenta forms an active interface between the maternal and fetal circulations that mediates exchange of oxygen and nutrients. Therefore, when the normal process of placental development is prevented, the result is placental insufficiency and failure of the fetus to attain its growth potential.

1.2.1 Placental Influence on Fetal Growth

Human placentation begins within the first week of implantation as the trophoectoderm of the blastocyst differentiates into syncytiotrophoblast cells which proliferate and invade the uterine endometrium (Kingdom *et al*., 2000). Primary placental villi form as outward projections

of this primitive syncytia, while retaining an inner layer of proliferating cytotrophoblast cells from which multiple cell lineages emerge as facilitators of placental maturation (Kingdom *et al*., 2000). In the first trimester placenta, two types of chorionic villi are apparent: anchoring villi and floating villi. Anchoring villi adhere to the uterine wall and produce migratory daughter cells of the cytotrophoblast stem cells that continue invasion of the decidua and myometrium, thereby drawing maternal blood into the intervillous space (Chakraborty *et al*., 2002). Extending into the intervillous space are the floating villi within which mesenchyme derived from the embryoblast coalesce to form fetal blood vessels by the process of vasculogenesis (Demir *et al*., 1989). Thereafter, progressive branching of the villi along with angiogenesis establishes the fetal-umbilical circulation. Fetal blood flows from the paired umbilical arteries to the arteries and arterioles of the centrally located stem villi, which then lead to the capillaries of the tertiary and terminal villi. The terminal villi form bulging protusions that make contact with maternal blood and comprise 50 percent of the placental surface area after their rapid expansion in the third trimester (Kaufmann and Kingdom, 2000). For this reason, the heavily coiled capillaries within the terminal villi are the most important in materno-fetal exchange. Elaboration of the stem and terminal villi together with increased capillarization over the second half of gestation create a low impedance vascular bed that receives 30 - 45 percent of combined fetal cardiac output (CCO) (Kiserud and Acharya, 2004). Fetal-placental vascular resistance is reflected in the end diastolic flow velocity (EDFV) in the umbilical artery (UA) as measured by Doppler ultrasound, which becomes detectable with establishment of the fetal-umbilical circulation at the end of the first trimester and progressively increases thereafter.

Perfusion of the intervillous space by maternal blood is maximized by modification of the uterus-embedded spiral arteries located downstream from the ovarian and uterine arteries. A subset of extravillous trophoblast cells that emerge from stem cells of the anchoring villi, infiltrate the wall and lumen of the spiral arteries and subsequently degrade and replace the endothelial, elastic and smooth muscle layers (Lyall, 2006). In so doing, these formerly muscular arteries are transformed into dilated, non-contractile conduits. Consequently, maternal cardiac output, which is augmented early in pregnancy by placenta-derived hormones, is directed, unimpeded, towards the low resistance utero-placental vascular bed.

While placental blood flow is the primary determinant of oxygen and carbon dioxide exchange, nutrients such as glucose, fatty acids and amino acids require facilitated or active transport by membrane proteins. The terminally differentiated syncytiotrophoblast layer that borders the villi is actively involved in transplacental transport of these substrates through the various transporters, receptors and enzymes present on both its maternal-facing plasma membrane and basal membrane. Thus, optimal placental performance is not only dependent on vascular hemodynamics but on the structural integrity of the placental villi. Attainment of full placental development and function is temporally regulated by a multitude of growth factors (Canigga *et al*., 1999), their binding proteins (Hamilton *et al*., 1998), ECM components (Vettraino *et al*., 1996) and cell adhesion molecules (Burrows *et al*., 1994). When this intricate process fails, abnormalities in placental hemodynamics and transport properties threaten efficient delivery of critical substrates and hence the dependent fetus.

Impaired materno-fetal exchange accounts for 60 percent of IUGR fetuses without genetic abnormalities in Western society (Ghidini, 1996). The term placental insufficiency is used to describe placental malfunction and consequent reduction in substrate delivery that fails to meet increasing demands of the fetus and thereby results in asymmetric IUGR. This condition is consequent to aberrant placental formation. Such a disturbance in placental development and function can arise secondary to maternal vascular disorders such as thrombophilia and hypertension (Redline, 2006), congenital infection (Popek, 1992) and fetal chromosomal abnormalities (Mittal *et al*., 1998), thus explaining the rare occurrence of pure symmetrical IUGR. Alternatively, primary defects in placental development occur in the absence of genetic disorders and maternal disease, leading to either altered maternal or fetal vascular supply. In sum, placental insufficiency is an idiopathic or secondary condition and the most common underlying factor in asymmetric IUGR in developed countries of the world.

1.2.2 Altered Maternal Vascular Supply

Hemodynamics of the utero-placental circulation are clinically evaluated by Doppler velocimetry and described by the pulsatility index (PI), resistance index (RI) and systolic-diastolic (SD) ratio, in addition to the presence or absence of an early diastolic notch in the uterine artery. A PI two standard deviations above the mean and the absence of an early diastolic

notch reflect increased resistance of the uterine artery and indicate risk for IUGR and need for fetal surveillance (Harrington *et al*., 1996). This clinical presentation is associated with a placental injury characterized by underlying defective trophoblast invasion and inadequate spiral artery remodeling (Aviram *et al*., 2010). Since the metabolically active placenta consumes a significant fraction of oxygen delivered by the uterine artery, it is the first to respond to a reduction in maternal blood supply with an acceleration in maturation and increase in capillarization (Burton *et al*., 1995). Accordingly, it has been suggested that placental hypoxia is an antecedent to IUGR in cases of abnormal Doppler waveforms of the uterine artery (Kingdom *et al*., 2000). Progression from absence of an early diastolic notch to a negative notch is associated with a higher risk of mortality and poor outcome, however, this is rare and the majority of abnormalities in the maternal compartment are associated with moderate IUGR (Marsal, 2009).

1.2.3 Altered Fetal Vascular Supply

Severe IUGR arises when elaboration of the fetal villous tree is abnormal and inadequate. This placental phenotype is characterized by reduced villous branching, avascular villi, thickened exchange barrier and excessive syncytial knotting suggestive of increased apoptosis (Macara *et al*., 1995; Krebs *et al*., 1996). The result is increased resistance to blood flow in the fetal compartment, compromised integrity of the syncytiotrophoblast and hindered exchange of vital oxygen and nutrients. This severe type of IUGR owing to increased impedance of fetal-umbilical blood flow is diagnosed by an elevated PI, RI or SD and absent or reversed end diastolic flow velocity (EDFV) in the umbilical artery (UA) (Marsal, 2009). Fetal outcome relates to the extent of change in the UA. Compared to AGA fetuses, risk of perinatal mortality is 4 fold higher when an absent EDFV in the UA is observed and 10 fold higher when EDFV is reversed (Karsdorp *et al*., 1994). Recent evidence suggests that maternal serum markers of placental damage and dysfunction may be useful in defining this subset of severe placental insufficiency. The pregnancy-associated plasma protein A (PAPP-A) is synthesized by differentiating cytotrophoblasts in association with placental growth. In the absence of fetal genetic abnormalities, low levels of this protein in maternal plasma are predictive of severe IUGR and the commonly co-existing antenatal condition of preeclampsia (Costa *et al*., 2008). A study

conducted by the Kingdom group showed reduced plasma levels of PAPP-A in the first trimester together with a small placental size and elevated maternal concentrations of α-fetoprotein (AFP) in the second trimester, to have a positive predictive value of 100 percent and a false-positive rate of 0 percent for severe IUGR (Proctor *et al*., 2009). Elevated levels of AFP in the maternal circulation reflects compromised placental permeability due to breaks in the syncytium where apoptosis is most striking and fibrinoid deposits are found (Scifres and Nelson, 2009).

The form of placental failure characterized by injury to the fetal compartment and severe IUGR, occurs less frequently than those affecting the maternal compartment. This was shown by a study of 26 cases of IUGR excluding those associated with genetic or structural anomalies, twins and pre-existing maternal conditions, wherein 66 percent were associated with maternal underperfusion, 17 percent with injury to the fetal compartment and 17 percent to villitis of unknown etiology (Aviram *et al*., 2010). Similar distributions have been reported by other groups (Mayhew *et al*., 2007). The distinction between altered maternal vascular supply and fetal vascular supply as causes of IUGR is highlighted in Table 1.2. Collectively, placental insufficiency is the most prominent cause of IUGR in developed nations.

1.3 PLACENTAL INSUFFICIENCY AND CHRONIC FETAL HYPOXEMIA

Placental insufficiency hinders the delivery of all maternally-supplied substrates, yet oxygen deprivation has the most profound effect on fetal condition. In addition to Doppler velocimetry, a battery of tests is available for diagnosis and monitoring of fetal condition when IUGR is evident or for pregnancies at risk for IUGR, such as those complicated with pre-existing maternal vascular disease, multiple gestation and preeclampsia. Antenatal surveillance with use of such tools has supported the presence of fetal hypoxemia in pregnancies with small EFW and abnormal Doppler parameters. Altered fetal heart rate patterns observed by cardiotocography during non-stress tests (NST) are suggestive of fetal hypoxemia (Liang *et al*., 2002), occur frequently in IUGR pregnancies (Behrens *et al*., 1996) and are predictive of intrapartum fetal distress (Begum and Buckshee, 1998). Direct measurement of fetal blood oxygenation and other biochemical parameters are now possible with the use of cordocentesis, whereby fetal blood is

Table 1.2: Categories of IUGR

	Altered Maternal Vascular Supply	Altered Fetal Vascular Supply
Doppler Diagnostic criteria	Increased PI/RI/SD and absent or early diastolic notch in uterine artery	Increased PI/RI/SD and absent or reversed end diastolic flow velocity in umbilical artery
Placental Characteristics	Increased development of the peripheral villous tree and increased capillarization	Decreased villous branching, avascular villi, thickened exchange barrier, syncytial knotting, breached integrity of syncytiotrophoblast layer
Underlying Defect	Inadequate trophoblast invasion and remodeling of spiral arteries	Inadequate development of fetal compartment
Severity	Moderate IUGR	Severe IUGR
Incidence	Common (~66%)	Uncommon (~17%)

sampled *in utero* by ultrasound-guided insertion of a thin needle into the umbilial vein. Cordocentesis has revealed variable degrees of hypoxemia, hypercapnia, hyperlactatemia, hypoglycemia and acidemia in human IUGR fetuses. In 27 cases of severe IUGR without genetic abnormalities, as determined by ultrasonic measurement of fetal weight and dimensions, 65 percent were acidemic and hypoxemic; 60 percent were hypercapnic, 35 percent were hypoglycemic and 40 percent showed increased lactic acid concentration (Bon *et al*., 2007). Likewise, another study reported that of the 11 fetuses exhibiting abnormal FHR and absent EDFV in the UA, 64 percent were hypoxic and acidotic while 17 percent of the 21 fetuses with normal FHR and abnormal Doppler waveforms were acidotic and none of the 21 fetuses with normal FHR and EDFV were hypoxic (Pardi *et al*., 1993). Similar results were revealed and the predictive value of NST and Doppler evaluation demonstrated in a larger population of 2,873 pregnancies not complicated with infection, maternal disease or fetal genetic disorders (Liang *et al*., 2002). A large study conducted at St. Joseph's Health Care in London, Ontario, examined 27,043 singleton pregnancies and found a linear relationship between fetal oxygen saturation and birth weight across the entire range of birth weights (Lackman *et al*., 2001). Taken together, the abovementioned reports highlight the frequency of fetal hypoxia consequent to placental insufficiency and support oxygen as a primary determinant of fetal growth.

Remarkably, the fetus holds the ability to adapt and survive under hostile conditions such as oxygen deprivation. When utero-placental or umbilico-placental blood flow is reduced leading to insufficient oxygen delivery, the fetus is capable of maintaining oxygen consumption by increasing oxygen extraction. The placenta is involved in this response via increases in capillarization if the problem lies in the maternal compartment, whereas increased oxygen extraction occurs only across the umbilico-placental vascular bed and by the individual organs in cases of injury to the fetal villous and vasculature tree. These acute respiratory adjustments are effective in maintaining rate of oxidative metabolism over a wide range of placental flow abnormalities. However, when an acute reduction in oxygen delivery exceeds 50 percent, tissue supply becomes inadequate and fetal oxygen uptake is reduced (Rurak *et al*., 1990; Wilkening and Meschia, 1983). At this point, energy yield from cellular respiration cannot sustain normal growth and thus fetal strategy switches to one of survival that involves a departure from its prescribed developmental course. With more gradual and chronic hypoxia, slowing of growth

will occur before the reduction in oxygen delivery reaches 50 percent, so that oxygen uptake will be maintained and, in this case, the degree of growth restriction is related in a graded fashion with the severity of hypoxia (Richardson and Bocking, 1998). An induction of erythropoiesis with reduced oxygen delivery is a delayed response that enhances blood oxygen carrying capacity and correlates with the degree of hypoxia (Ostlund *et al*., 2000).

In the attempt to survive increasingly perilous conditions, the fetus employs a sequence of compensatory mechanisms that become accessible at specific gestational ages. The mature fetal cardiovascular system is effective in responding to hypoxia through redistribution of fetal cardiac output with preference to the brain, heart and adrenal glands at the cost of the other visceral organs, the carcass including skeletal muscle and the pulmonary bed (Iskovitz *et al*., 1987). In conjunction with systemic redistribution, the hemodynamic strategy involves maximizing the shunting and preferential streaming of blood that marks the fetal circulation. Increased shunting through the ductus venosus (DV) which connects the umbilical vein to the inferior vena cava (IVC), allows more of this highly saturated blood returning from the placenta to bypass the liver and accelerate toward the right atrium (Kiserud *et al*., 2000). Upon flowing through the foramen ovale located in the intra-atrium septum, this well-oxygenated stream of blood flows directly into the left atrium and then from the ascending aorta it is drawn into the dilated cerebral and myodcardial vascular beds. The deoxygenated streams returning from the upper and lower body are directed toward the tricuspid valve of the right atrium and pass through the ductus arteriosus (DA) to merge with the placenta-bound blood flowing through the descending aorta. Thus, hemodynamic adaptation to hypoxia also involves a shift of blood from the right to left ventricle by means of increased streaming through vascular shunts (Reed *et al*., 1987; Severi *et al*., 2000).

Increased afterload due to peripheral vasoconstriction of the fetal vessels and increased placental resistance consequent to the underlying placental defect, predominately affect the right ventricle. This is because the right ventricle ejects 65 percent of combined cardiac output (CCO), 40 percent of which flows directly into the descending aorta from the pulmonary trunk via the DA (Kiserud and Acharya, 2004). In contrast to the augmented afterload imposed on the right ventricle during chronic hypoxia, vasodilation of the coronary and cerebral circulations mitigates the afterload effect on the left ventricular ejection (Baschat *et al*., 2000).

Hemodynamic responses to placental injury and resultant hypoxia serve to protect those organs critical for survival, yet manifest as disproportionate body growth. Since the degree of circulatory adjustment varies with the severity of hypoxemia, its Doppler evaluation in conjunction with EDFV of the UA provides the practitioner with further information regarding the fetus suspected of IUGR. An increase in peak systolic velocity of the cerebral circulation together with a decrease in flow through the descending aorta, reveal preferential blood flow and suggest fetal hypoxemia (Arduini *et al*., 1992). These changes occur early in the response to hypoxia (Ferrazzi *et al*., 2002) and their magnitude reflects the severity of insult (Oros *et al*., 2011). With escalating fetal hypoxemia over hours or days, cardiac output redistribution reaches a maximum and eventually the depleted myocardium fails to cope with the high input impedance. At this point, ventricular output falls, velocity across the atrioventricular valves declines and bradycardia develops (Hecher *et al*., 1995). Augmented end diastolic ventricular pressure and resultant reversal of flow from the right atrium to the IVC during atrial contraction, are reflected in an increase in central venous pressure and pulsatility in the IVC and DV (Rizzo *et al*., 1995). The presence of such hemodynamic abnormalities suggest imminent intrauterine death and warrant premature delivery. Thus, circulatory adjustment permits acute and chronic adaptation to intrauterine hypoxia, correlates in magnitude to the degree of hypoxia and fails in the non-surviving fetus.

In addition to hemodynamic changes, the effort to overcome prolonged hypoxia *in utero* involves reducing oxygen expenditure through modifications in fetal metabolism. Studies have shown a 10 percent reduction in oxygen consumption within several hours of hypoxia (Milley, 1987) and a 20 percent reduction with prolongation of hypoxia over several days (Owens *et al*., 1987). To this end, energy consuming anabolic processes such as DNA and protein synthesis are curtailed while uptake of glucose and amino acids is reduced accordingly. The result is a slowing of overall fetal growth. This metabolic response leading to growth inhibition is mediated by a shift in the endocrine status of the fetus, as hormones responsible for global nutrient uptake and metabolic rate balance cellular respiration with the reduced substrate availability. For instance, the hypoxic IUGR fetus shows a reduction in circulating levels of insulin and insulin-like growth factor 1 (IGF-1), which promote cellular proliferation and uptake of glucose and amino acids. Further, with prolongation of oxygen deprivation the fetus resorts to decreases in

breathing and body movements in order to minimize energy expenditure and metabolic rate. When reduced fetal activity and muscle tone are observed with ultrasound, the obstetrician suspects severe fetal compromise.

1.3.1 Animal Models of Placental Insufficiency

Several techniques in a variety of species are currently used to induce chronic intrauterine hypoxia for the study of IUGR. The pregnant sheep is a long-standing model that has broadened our understanding of fetal and placental responses to oxygen deprivation as well as intrauterine growth and development in general (Bendeck *et al*., 1991; Gagnon *et al*., 1996). The utility in the sheep as a model of pregnancy is owing to its credibility in extrapolation to the human situation and its capacity to tolerate chronic placement of catheters in both fetal and maternal vessels, thus allowing for repeated blood sampling under steady-state conditions. With respect to the former, the sheep bears resemblance to the human in the number of fetuses and size at birth. As well, the structure of the villous tree is similar between the sheep and human (Leiser *et al*., 1997), although differences exist with respect to localization of the placental organ and extent of uterine invasion. The human placenta forms a single structure that destroys the basement membrane and the maternal endothelial layer so that maternal blood is in direct contact with the floating villi (Meekins *et al*., 1994). In contrast, fetal villi of the ovine fetus are contained in numerous placental elements termed cotyledons which contact the un-invaded maternal endothelium at specialized regions called caruncles (Stegeman, 1974). Despite these differences, the efficiencies of the placental transfer are similar between the sheep and human (Battaglia and Meschia, 1986).

Also comparable between the two species, are physiology under normal and pathological conditions (Ikeda *et al*., 2001) and the developmental time course of the major organ systems (Bendeck *et al*., 1991; Berry *et al*., 1972). For this reason, much of what we know in regards to fetal growth and development derives from sheep experiments. One example is the extensive characterization of cardiovascular development in the ovine fetus that parallels that of the human in temporal aspects of blood pressure elevation and maturational events such as cellular differentiation (Adler and Costabel, 1980; Burrell *et al*., 2003). Furthermore, advancement in our knowledge with respect to placental oxygen uptake and nutrient transport and their relation to

fetal growth was largely facilitated by maternal and fetal blood sampling in the chronically catheterized pregnant sheep.

The methods employed for induction of chronic hypoxemia in fetal sheep include preconceptual carunclectomy (Trahair *et al*., 1997), maternal hyperthermia (Regnault *et al*., 2003), uterine artery (Clapp *et al*., 1980) and umbilical-placental embolization (Murotsuki *et al*., 1996). Of these models, maternal hyperthermia and umbilical-placental embolization simulate asymmetric IUGR. In the latter model, chronic fetal hypoxemia is produced by injection of non-radioactive microspheres into the umbilical circulation via the maternal abdominal aorta of the chronically instrumented ewe. The infused microspheres accumulate in the fetal villi and maternal epithelium at the maternal-fetal interface, thereby disrupting contact between the two compartments (Cheung *et al*., 2004). This breach in the placental exchange surface compromises placental blood flow and thereby leads to chronic fetal hypoxemia, abnormal EDFV of the UA and asymmetric IUGR (Gagnon *et al*., 1994). Thus, umbilical-placental embolization closely approximates human placental insufficiency characterized by reduced fetal supply. Further, catheterization of the fetal vessels allows for monitoring of fetal hypoxemia, the level of which is controlled through the number of microspheres injected.

Several groups worldwide have adopted the umbilical-placental embolization model. As in human IUGR pregnancies, the extent of fetal compromise inflicted by umbilical-placental embolization is reflected in the degree of circulatory changes. An increase in UA resistance occurs with a 30 percent reduction in fetal arterial oxygen content over 10 or 21 days of embolization (Murotsuki *et al*., 1997). Abnormal EDFVs are accompanied by increases in cerebral and carotid blood flow by 130 and 40 percent, respectively, in progressively acidotic fetuses subjected to severe embolization for 6 hours (Gagnon *et al*., 1997). Moreover, FHR variability, which is a clinical indication for cardiac deterioration and impending fetal death, is only altered in the ovine fetus with metabolic and respiratory acidosis (Gagnon *et al*., 1996).

Not only does the embolized ovine fetus parallel the severe IUGR human fetus in terms of circulatory adaptations, but the endocrine changes driving these fetal responses and the general intrauterine milieu are comparable. Catecholamines which play a role in the hemodynamic response to intrauterine hypoxemia are elevated in the amniotic fluid (Divers *et al*., 1981) and umbilical vein (Okamura *et al*., 1990) of underdeveloped human fetuses and in the

circulation of chronically hypoxic ovine fetuses (Gagnon *et al*., 1994). Whereas norephinephrine increases progressively during prolonged hypoxemia, the increase in cortisol, another regulator of fetal vasoactivity and metabolism, is transient (Gagnon *et al*., 1994). In agreement, cordocentesis has revealed elevated levels of cortisol in umbilical venous blood of IUGR compared to AGA pregnancies (Cortelazzi *et al*., 2003). While, endocrine factors known to inhibit growth are elevated in the sheep and human fetus subjected to placental insufficiency, growth-promoting hormones are reduced. Most important among the latter are insulin and insulin-like-growth factor-1 (IGF-1), which are both decreased in the plasma of embolized sheep fetuses (Thorn *et al*., 2009) and in the umbilical circulation of human IUGR fetuses (Economides *et al*., 1991). Amino acids and glucose are major stimulators of the anabolic actions of the insulin-IGF-1 axis. Fittingly, a reduced supply of essential amino acids (Bloomfield *et al*., 2002) along with hypoglycemia (Joyce *et al*., 2001) are characteristic of umbilical-placental embolization in sheep and failed development of the human villous tree.

Placental insufficiency is a compound insult that incites an array of fetal and placental responses. Although no animal model truly mimicks normal or disordered human pregnancy, umbilical-placental embolization of the pregnant sheep captures many aspects of the intrauterine challenges faced by the severe IUGR fetus and is a valuable tool for the study of fetal development. Unfortunately, like other large animal models, the expense and logistic difficulties using sheep are significant deterrents and therefore its use in prenatal study is currently in decline. Alternatively, many research groups take advantage of rodent models which offer convenience in terms of low cost, large litter sizes, shorter gestations and life spans. Although there exists fundamental differences in the timing and nature of organ development in most rodents compared to humans, such models are useful in long-term study of offspring after *in utero* insults.

Unlike other rodents, and similar to the human and sheep, the guinea pig develops predominately in the prenatal period and is relatively mature at birth. Another analogy lies in placental structure. The placenta of both the guinea pig and human belong to the haemochorial-discoid type, which is defined by its formation as a singular organ and its invasive destruction of the maternal epithelium by which direct contact between the fetal trophoblast and maternal blood is achieved (Mess, 2007). The guinea pig placenta allows for counter-current exchange and

maximal exchange efficiency so that there is little room for improvement in response to a reduction in maternal blood flow (Harding and Bocking, 2001).

An established method for IUGR that is suitable for guinea pigs is uterine artery ligation (UAL). The ligation model was originally developed in the rat, and has more recently been applied to the guinea pig which offers more in terms of comparative physiology. Whereas the umbilical-placental embolization model resembles human IUGR with abnormal EDFVs of the UA, the UAL technique is similar to moderate IUGR caused by poor placental perfusion because it leads to reduction in maternal placental blood flow. The degree of placental blood flow restriction is proportional to the associated decreases in fetal and placenta weight (Jansson *et al*., 1986). In fact, the relationship between placental blood flow and fetal weight holds true for spontaneous IUGR within a litter of an un-operated uterus (Myers *et al*., 1982). The high fetal mass of the guinea pig renders it highly reliant on maternal supply late in gestation and thus susceptible to UAL. Growth restricted fetal guinea pigs exhibit brain sparing as reflected by relative maintenance of brain and adrenal weights together with reductions in weight of the liver and spleen (Lafeber *et al*., 1984). Evidence shows that asymmetric IUGR in the UAL guinea pig is associated with intrauterine hypoxia (Demter *et al*., 1991), hypoglycaemia and reduced availability of essential amino acids (Jansson and Persson, 1990). It is important to note, however, that compared to the embolization model the insult imposed by UAL is of longer duration as the procedure is typically performed at mid-gestation.

1.4 UMBILICAL CORD OCCLUSION AND ACUTE FETAL HYPOXIA

Intrauterine hypoxemia and consequent impairment in fetal growth can arise from reductions in umbilical blood flow. Enclosed in the umbilical cord are the two umbilical arteries which carry deoxygenated blood from the fetal circulation to the placenta and the umbilical vein which returns oxygenated blood from the placenta to the fetal tissues. Within the cord, the umbilical vessels are embedded in a loose proteoglycan-rich matrix called Wharton's jelly. Cushioning by Wharton's jelly along with coiling of the cord protect the umbilical vessels from forces of torsion and compression such as occur during fetal movement or uterine contraction.

Thus, complications of the umbilical cord including hypercoiling, hypocoiling (Predanic., 2005), true knots (Clerici *et al*., 2007) and loops of the umbilical cord (Clapp *et al*., 2003), render the fetus susceptible to fetal blood flow reductions, hypoxic stress, asphyxia and IUGR. Such abnormalities can result in chronic or intermittent disruption of the umbilical circulation and are associated with an increased risk of low birth weight, non-reassuring FHR patterns, delivery complications and adverse neonatal outcomes (Baergen., 2007; Osak *et al*., 1997). In fact, compromise of the umbilical circulation is implicated in 20 percent of still births (Baergen, 2007).

A nuchal cord, whereby the umbilical cord encircles the fetal neck, is commonly detected in human pregnancy during prenatal care or at delivery. The incidence of cord entanglement in normal pregnancies has been reported as 7 percent in the first trimester (Plasencia *et al*., 2010), 8 percent (Lal *et al*., 2008) and 12 percent (Clapp *et al*., 2003) in the second trimester, and at the time of delivery the occurrence has been reported as high as 37 (Clapp *et al*., 2003) and 27 percent (Lal *et al*., 2008). These studies also highlight that the presence of a nuchal cord may be sporadic, resolving and then later reappearing (Lal *et al*., 2008). The majority of nuchal cords are benign, however when symptomatic, variable-type FHR decelerations suggestive of fetal hypoxemia arise and vary in magnitude according to the severity and duration of umbilical cord compression (Agrawal *et al*., 2003; Clapp *et al*., 2003). Recurrent fetal hypoxemia due to intermittent cord occlusion and the associated adverse outcomes including low Apgar scores, operative delivery, spastic cerebral palsy and perinatal demise, are more likely when the nuchal cord persists or concurs with multiple loops, high tension or crossing of the cord (Clapp *et al*., 1999). Fetuses entangled in the umbilical cord are particularly at risk for distress during labour and delivery. In fact, nuchal cord is the most common cause of non-reassuring FHR tracings at the time of delivery, which coincide with reductions in pH and base excess in the umbilical vein (Agrawal *et al*., 2003). In all, nuchal cords are a very frequent obstetric concern that may lead to repeated compressions of the umbilical cord and associated recurrent episodes of acute fetal hypoxemia of various degrees.

1.4.1 Animal Model of Umbilical Cord Occlusion

Experimental study of umbilical cord compression in the ovine fetus has been undertaken by several groups. The model involves surgical preparation of the pregnant sheep later in gestation, wherein an inflatable occluder cuff is tied around the umbilical cord of the exteriorized fetus (Green *et al*., 1999; Wassink *et al*., 2007). Partial or complete umbilical cord occlusion (UCO) of varied duration is produced by infusion of saline into the inflatable cuff at the time of experimental study. This technique has allowed a thorough characterization of fetal responses to acute hypoxia. Upon umbilical cord compression, the abrupt reduction in fetal oxygenation immediately activates the chemoreflex primarily through transduction by the carotid bodies (Giussani *et al*., 1993). Efferent signals are conducted along the muscarinic pathway to induce transient bradycardia while peripheral vasoconstriction results from sympathetic stimulation of α- adrenergic receptors (Giussani *et al*., 1993). This is followed by a progressive increase in fetal mean arterial blood pressure (MAP) due to both increased sympathetic output and umbilical resistance, in concert with a redistribution of cardiac output in favour of the brain, adrenals and heart (Wassink *et al*., 2007; Yan *et al*., 2009). With prolongation of umbilical cord occlusion over one minute, release of catecholamines and other vasoactive agents into the fetal circulation return FHR to baseline and sustain the blood flow redistribution (Wassink *et al*., 2007). When hypoxia is unremitting, fetal cardiovascular defences are overwhelmed, at which time further FHR reduction and hypotension will develop alongside cardiac failure (Hernandez-Andrade *et al*., 2005; Yan *et al*., 2009). Deterioration in ovine fetuses subjected to UCO of long duration (5-10 min) concurs with bradycardia, analogous to that seen in clinical cord compression.

Another aspect of perinatal physiology elucidated by the umbilical cord occlusion technique is the nature and maturation of cardiovascular regulatory mechanisms and defence responses of the fetus. Sympathetic nervous activity (Fletcher *et al*., 2003) and chemoreflex function (Kiserud *et al*., 2001) are present very early in gestation. However, relative to term fetuses the response to hypoxia is blunted in immature fetuses between 60 and 70 percent of gestation, with a progressive increase in magnitude thereafter (Fletcher *et al*., 2006). Although early in gestation the fetus is able to survive prolonged hypoxia, it is particularly vulnerable to progressive hypotension and hypoperfusion of the tissues given the incapacity of peripheral vasoconstriction and redistribution of cardiac output (Bennet *et al*., 1999). Furthermore, sheep

studies have revealed modified fetal responses when severe intermittent hypoxia is prolonged (Green *et al*., 1999; Unno *et al*., 1997) and when acute hypoxia is superimposed on pre-existing hypoxia (Imamura *et al*., 2004) or occurs after an epidsode of severe hypoxia (Thakor and Giussani, 2009). Interestingly, the increase in plasma cortisol in response to acute hypoxia is enhanced after moderate long-term hypoxia (Imamura *et al*., 2004), yet attenuated after repeated severe hypoxia over several hours (Unno *et al*., 1997). Thus, fetal defenses are remarkably effective in adapting to multiple or superimposed intrauterine insults and increasingly so with advancement of gestation, yet when the threshold of tolerance is reached failure in these systems quickly results in fetal demise.

1.5 FETAL PROGRAMMING

Fetal outcome under various intrauterine insults is a product of their associated perturbations in the intrauterine milieu that supports ongoing development. The conceptus is responsive to such changes allowing continuance of organ formation, maturation and growth despite deficient sustenance of even an extreme degree. This plastic nature of development has long been recognized in the observation that neonatal outcome relates to maternal state and for this reason promotion and maintenance of maternal health has traditionally been a primary goal in obstetric management. Yet, more recently it has been discovered that the influence of the intrauterine environment extends well beyond the immediate postnatal period. Indeed, fetal experience is now recognized as an important determinant of long-term health and risk for adult-onset degenerative diseases. This phenomenon was first brought to light by seminal epidemiological data collected in the 1980s by David Barker and colleagues that showed low birth weight to be predictive of death from ischemic heart disease in adult life (Barker and Osmond, 1986). Soon after, a number of other chronic conditions were linked to birth measures indicative of impaired intrauterine growth; relationships that have now been substantiated by numerous studies in various populations around the world. The mounting list of degenerative diseases with prenatal origins includes coronary artery disease, Type-2 diabetes, kidney failure, osteoporosis, polycystic ovarian syndrome and cancer (Gluckman *et al*., 2008; Ozanne and Constancia 2007). Many of

these diseases are strongly interrelated, and human studies have further demonstrated the presence of common precursors in those determined to be growth impaired *in utero*. For instance, central obesity (Harding 2001), hypertension (Barker *et al*., 1990) and insulin resistance (Jaquet *et al*., 2000), which together constitute the metabolic syndrome, tend to manifest in children and adults born small.

This abnormal physiology is not merely a manifestation of the severely growth restricted, rather phenotypic changes quantifiable in postnatal life can occur with modest intrauterine insults that impact minimally upon fetal size. In fact, the incidence of the interrelated metabolic and cardiovascular disorders mentioned above, are inversely correlated with birth weight along the entire continuum of birth weight (Barker, 2001). Hence, one may deduce that small changes in oxygen and nutrient supply to the fetus in the absence of overt maternal disease or deficiency, conveys a relative disadvantage to the offspring. Evidence of this is provided by a recent study showing maternal thinness and an unbalanced diet during pregnancy to have a subtle effect on size of the newborn, yet later in life the offspring show a propensity toward high blood pressure, insulin resistance and altered stress responses (Wabitsch, 2000). On the other hand, maternal obesity and excessive weight gain during pregnancy increase the risk for macrosomic babies that tend to become obese and develop cardiovascular disease and type-2 diabetes later in life (Desai *et al*., 2010). Thus, at least for the metabolic syndrome, the correlation between disease and birth weight appears to be U-shaped, such that the highest risk occurs in the smallest and largest babies. Taken together, the above highlight that long-term outcome of the fetus warrants consideration not only in cases of maternal disease and antenatal disorders, but also with respect to nutritional inadequacies related to common Western dietary habits.

1.5.1 Mechanisms of Programming

The current impetus within the newly emerged area of fetal programming is in the understanding of underlying mechanisms. In this respect, epidemiological analyses are lacking in the insight they yield. First, population studies rely on birth measures as crude proxies of intrauterine growth and thus are imprecise in their identification of infants who have experienced genuine growth restriction. As well, postnatal risk factors and variability in the timing, nature and duration of the intrauterine insult causing growth restriction are confounding variables

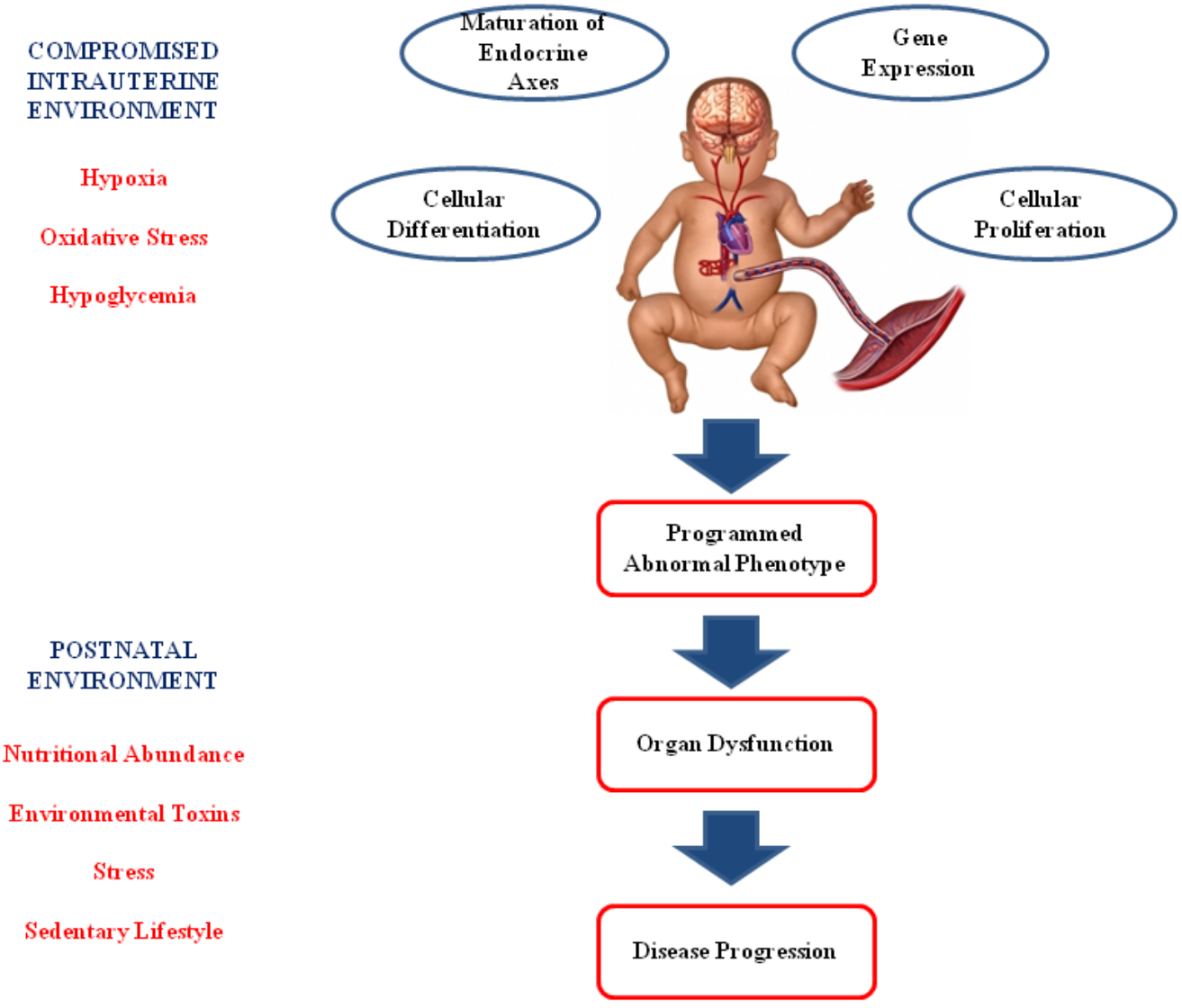
inherent in the study of human IUGR. Thus, for elucidation of the underlying mechanisms responsible for developmentally-related disease risk, animal models emulating specific prenatal insults have been applied. Various species subjected to a range of prenatal challenges including maternal anemia, malnutrition, glucocorticoid administration, stress and placental insufficiency as described previously, have reproduced the human phenotypes, thus confirming that suboptimal intrauterine conditions lead to pathological conditions. Further, investigation with animal models has established the broad tenets of fetal programming (Figure 1.1) which currently guide ongoing research in the field.

The accepted concept of fetal programming holds that adult-onset disease is the culmination of persistent changes in the structure, metabolism, gene expression and homeostatic regulation of organ systems that occur *in utero*. These programmed changes result directly from the intrauterine insult or secondary to the associated fetal response and relate to the modality, duration and timing of the stimulus. Organs and tissues are particularly vulnerable during times of rapid proliferation or differentiation and lose much of their plasticity once these developmental windows come to a close. In the human, critical periods of the various organs range from preconception to neonatal life. For instance, nephrogenesis in the kidney begins in the 5th week of pregnancy and accelerates until 35 weeks, after which the number of nephrons is set for life (Schreuder *et al*., 2007). The kidney is among the organs sacrificed in the fetal attempt to survive substrate deficiency and altered kidney development leading to a permanent reduction in nephron number and kidney mass is an established contributor to programmed hypertension and renal failure in both IUGR humans and rodents (Schreuder *et al*., 2007; Wlodek *et al*., 2008).

In addition to enduring structural changes, programmed organ dysfunction can result from persistent changes in gene expression patterns thought to arise from epigenetic modifications (Ozanne and Constancia, 2007). Since the epigenome is heritable, this mechanism may account for transmission of *in utero*- derived abnormalities across generations (Ozanne and Constancia, 2007). Evidence of epigenetic modification has been demonstrated in rat offspring growth restricted by maternal protein restriction displaying changes in DNA methylation of genes involved in the regulation of muscle metabolism, such as peroxisome proliferator-activated receptor PPAR-α (Lillycrop *et al*., 2005). Moreover, deviation in function at the organ and

Figure 1.1: Mechanisms of Programming

When the conceptus is subjected to an intrauterine insult, the plasticity of its growth and development allows it to compensate through changes in gene expression, cellular proliferation and differentiation and maturation of regulatory systems. The resulting phenotype is determined by the specific nature of the insult, as well as its timing and duration. After birth many organs such as the heart and blood vessels have limited ability to recover any deficiencies in organ structure established during intrauterine development and thus programmed phenotypic abnormalities may result in organ dysfunction that later leads to disease progression. The rate at which disease progression occurs depends on an interaction between the abnormal phenotype and the postnatal environment.


COMPROMISED INTRAUTERINE ENVIRONMENT
Hypoxia
Oxidative Stress
Hypoglycemia
Maturation of Endocrine Axes
Gene Expression
Cellular Differentiation
Cellular Proliferation
Programmed Abnormal Phenotype
POSTNATAL ENVIRONMENT
Nutritional Abundance
Environmental Toxins
Stress
Sedentary Lifestyle
Organ Dysfunction
Disease Progression

cellular levels, as exemplified above, propagate irregularities at the systemic level. For instance, in the growth restricted animal and human, wasted muscle mass and altered distribution of fibre types together with dysregulation of molecular pathways involved in muscle metabolism, promote a state of insulin resistance that is enduring and progressive (Jaquet *et al*., 2000). Therefore, a specific intrauterine challenge likely induces multiple mechanisms of programming thereby producing a synergistic effect on pathogenesis and this explains the interrelated spectrum of disorders that have been linked to low birth weight.

Once the surviving fetus exits the deprived intrauterine environment, it must cope in extrauterine life with all of the sacrifices it made to arrive. Shortly after birth the organ systems no longer enjoy the plasticity that was afforded to them *in utero*, and thus any debility in their structure, morphology, cell number or gene expression may be largely fixed going forward in postnatal life. This premature disadvantage leads to progressive dysfunction that is accelerated with the burden of aging and superimposition of postnatal lifestyle factors. Further, the newborn which has adapted to a state of starvation *in utero*, may be met with an incongruent environment of relative overabundance. Trained to conserve energy, the IUGR baby exploits this opportunity of nutrient overabundance albeit with negative long-term consequences. Those growth restricted *in utero* exhibiting rapid catch-up growth during infancy are at higher risk for obesity, cardiovascular disease and insulin resistance, compared to their steadily growing counterparts (Barker, 2005). Thus, the interaction of the programmed phenotype with the postnatal environment is a significant factor in the quality, severity and pace of pathogenesis. In this light, compared to nutritionally deprived fetuses born into an impoverished society, fetal programming of fetuses subjected to placental insufficiency and born into a well-nourished postnatal environment may be more detrimental.

1.5.2 Programming of Cardiovascular Disease

Cardiovascular disease (CVD) which outweighs all other common degenerative conditions in terms of the burden it imposes, is the most commonly examined in the epidemiological study of programming. This wide-spread disease encompasses a group of interrelated and progressive conditions affecting the heart and blood vessels, including hypertension, atherosclerosis and coronary artery disease. In Canada, CVD accounts for 34 and

32 percent of female and male deaths, respectively, and is associated with morbidity costs of 18 billion dollars per year (Statistics Canada, 2003). Despite advancement in pharmacological treatments over the past 4 decades and an overall reduction in smoking, CVD remains, by far, the number one killer in North America (Bitton and Gaziano, 2010). On an international scale, the gravity of CVD is mounting with increasing prevalence in developing nations (Gersh *et al*., 2010), rising rates of obesity and type 2 diabetes and the recent emergence of childhood hypertension in affluent regions (Feber and Ahmed, 2010).

The original studies which revealed prenatal origins of CVD were conducted by Barker and Osmond, who related death rates from ischemic heart disease to birth weights of men which were recorded between 1911 and 1930 in Hertfordshire, England. They discovered men who weighed 5.5 pounds or less at birth to have the highest risk of deathfrom heart disease (Barker and Osmond, 1986; Barker *et* al., 1989). In subsequent studies, Barker and colleagues revealed blood pressure in adult life to be inversely related to birth weight, length and head circumference at birth, in both men and women of the UK (Barker *et* al., 1992; Barker *et* al., 1990). The relation to other forms of CVD followed: in 1996 they reported mortality from stroke and coronary heart disease in 13,249 men from the UK to be strongly associated with small head circumference, thinness, shortness and weight at birth (Martyn *et al*., 1996). A couple of years later, the same group found the degree of atherosclerotic narrowing in the carotid arteries to be greatest in people with low birth weight, independent of gestational age at birth (Martyn *et al*., 1998).

Since the time of these first discoveries, numerous cohort and longitudinal studies in populations worldwide have confirmed the existence of a relationship between birth weight as a proxy for fetal growth *in utero* and cardiovascular health in adults, adolescents and children. For example, in a large cohort of US men aged 40-75, Curhan *et al*. (1996b), reported birth weight to be inversely correlated to the incidence of hypertension. Later, this group replicated the findings in female subjects (Curhan *et* al., 1996a). Similar studies in Scotland, Sweden and the Netherlands have demonstrated a trend of increasing adult blood pressure with lower birth weights, in both men and women (Law *et* al., 1993; Leon *et* al., 1996; Macintyre *et al*., 1991). The majority of studies with adolescent subjects have found birth weight to be negatively correlated with blood pressure in this age group, despite the confounding influence of puberty. For instance, work by Taittonen *et al*. (1996) revealed low birth weight to be predictive of high

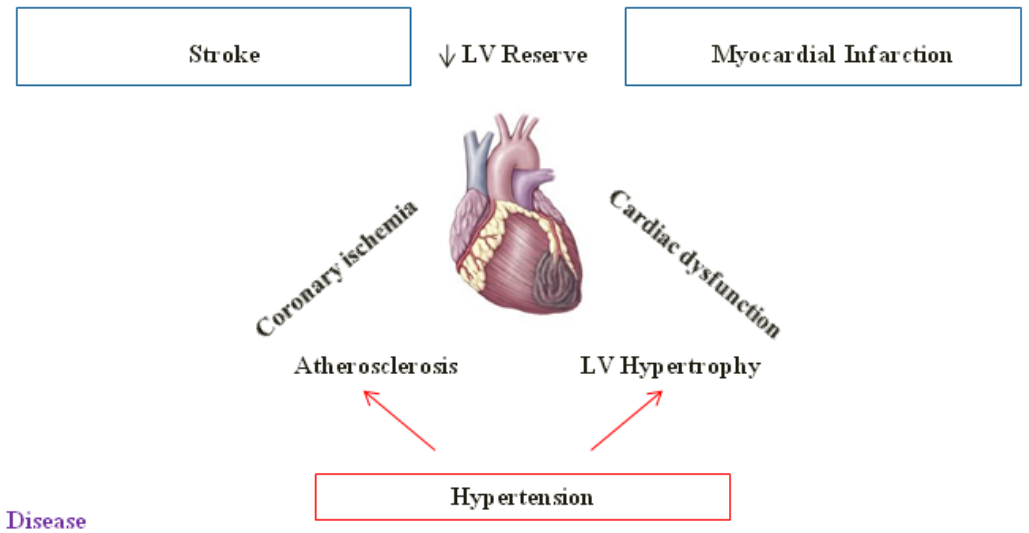
systolic blood pressure in a group of young adults from Finnland. In another study, systolic blood pressure increased in relation to decreasing birth weight, in a large population of Swedish men aged 17-21 years born in 1973 (Nilsson *et al*., 1997). Likewise, a study carried out in Jamaica revealed similar results in a group of children 6-16 years of age, born after 28 weeks gestation (Forrester *et* al., 1996). The same inverse relationship between blood pressure and birth weight has been demonstrated in a group of UK children aged 5-7, born between 1983 and 1985 (Whincup *et al*., 1989). Thus, it appears that the effect of prenatal environmental triggers on cardiovascular health becomes quantifiable in childhood, a time when the influences of ageing and lifestyle are minimal. These early epidemiological studies have identified the phenomenon of intrauterine programming and firmly established developmental links to CVD. The next step is elucidating the means by which developmental disturbances translate to long-term disease vulnerability. Clues to the answer of the mechanistic questions that remain unresolved lie in the disease process and its developmental correlates.

1.6 CARDIOVASCULAR DISEASE PROGRESSION IN ADULT LIFE

The general process of CVD progression is depicted in Figure 1.2. When CVD is not congenital, its evolution occurs over decades and is set in motion by an injury to the arterial wall in the form of mechanical overload (Li *et al*., 1998), oxidative stress (Chrissobolis *et al*., 2011), inflammation (Barbato *et al*., 2005) or a combination of these stimuli. This initial trigger may be brought on by any of the postnatal cardiovascular risk factors such as smoking, diet and obesity or more insidiously by the normal process of ageing (Kannel *et al*., 1997). Consequent modification in the responsiveness and mechanical behaviour of the vascular wall leads to a vicious cycle of arterial dysfunction and increasing blood pressure that may fester for years before the onset of symptomatic CVD (Campuzano *et al*., 2006; McCall *et al*., 2010). Eventually, cardiac dysfunction ensues once compensatory mechanisms fail to manage the increasing load placed on the heart by this progressive disturbance in the hemodynamic

Figure 1.2 Progression of Cardiovascular Disease

The gradual progression of cardiovascular disease occurs over decades and is instigated by an initial insult to the vascular wall. The compensatory response of the vascular wall leads to arterial dysfunction and thereby a disturbance in hemodynamics. The resultant mechanical stress placed on the vascular wall leads to further arterial damage and dysfunction and so a vicious cycle continues. Altered hemodynamic forces also place excessive strain on the heart, inducing compensatory remodeling of the left ventricle (LV) that eventually becomes maladaptive. Eventually, the over-stressed and under-perfused heart may be unable to cope.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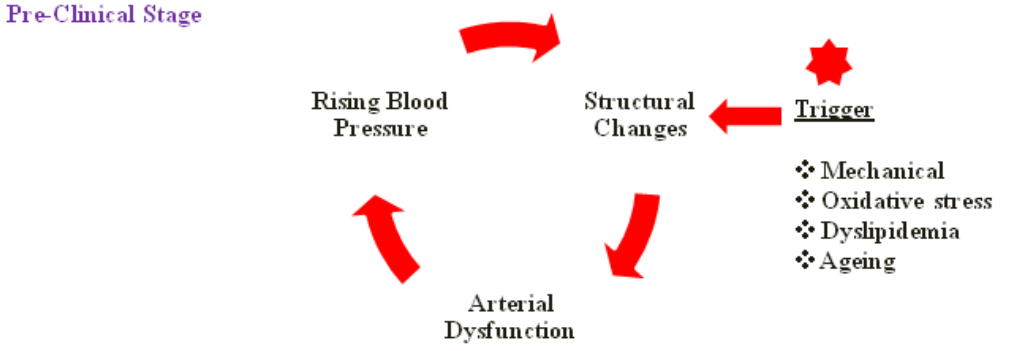
Coronary ischemia
Cardiac dysfunction
Atherosclerosis
LV Hypertrophy
Hypertension
Disease


Pre-Clinical Stage
Rising Blood Pressure
Structural Changes
Trigger
❖ Mechanical
❖ Oxidative stress
❖ Dyslipidemia
❖ Ageing
Arterial Dysfunction

environment (Graham and Trafford, 2007). Congestive heart failure, myocardial infarction and stroke are the ultimate and fatal outcomes of this pathological course. In sum, the gradual disruption in hemodynamic forces that parallels arterial and cardiac decline is instigated by a vascular reaction to adverse stimuli. In this light CVD is primarily a disorder of the arterial wall.

Upon exposure to an injurious stimulus, the endothelium becomes activated and initiates the homeostatic response of the artery. A host of inflammatory genes are induced, including cytokines, cell adhesion molecules (CAMs) and regulatory enzymes that collaborate in the mediation of structural and functional changes of the arterial wall (Anggrahini *et al*., 2009; Barron *et al*., 1997; Rectenwald *et al*., 2000). Pivotal among these proteins are the matrix metalloproteinases (MMPs), particularly the gelatinases MMP-2 and MMP-9, which drive compensatory remodeling (Yasmin *et al*., 2005). MMPs cleave ECM components so as to restructure the arterial media and when chronically stimulated, promote an ongoing cycle of degradation and synthesis. The primary substrates of MMP-2 and 9 are the basement membrane proteins, collagen Type IV, fibronectin and laminin (Knauper and Murphy, 1998). Through degradation of these proteins, the gelatinases disrupt the subendothelium thereby altering endothelial permeability (Rosenburg *et al*., 1998). Under normal conditions vascular smooth muscle cells (VSMCs) are in a quiescent contractile state. However, upon activation which requires degradation of the basement membrane by MMP-2, contractile VSMCs revert to a proliferative, migratory and synthetic state (Bendeck and Tempo, 1994; Mochizuki *et al*., 2001; VanSaun and Matrisian, 2006). The phenotypically modulated VSMCs migrate to the intima and secrete collagen and proteoglycans under the stimulation of pro-inflammatory cytokines, such as transforming growth factor (TGF-β) (Wolf *et al*., 1994). The result is altered composition and hypertrophy of the media, in addition to thickening of the pre-existing fibrocellular intima that is the precursor to atherosclerotic lesions in specific arterial segments (Stary *et al*., 1992). The proteoglycans within the thickened intima bind circulating lipoproteins, establishing Type I arterial lesions (Nakashima *et al*., 2008). These retained lipoproteins undergo oxidation, forming cytotoxic aggregates that recruit monocytes, which in turn, bind to the CAMs expressed on the activated endothelium (Nakashima *et al*., 2008; Glass and Witztum, 2001). The CAMs together with chemokines allow transmigration of the monoctyes and modifed lipoproteins into the subendothelial space through the denuded basement membrane (Barron *et al*., 1997). This is

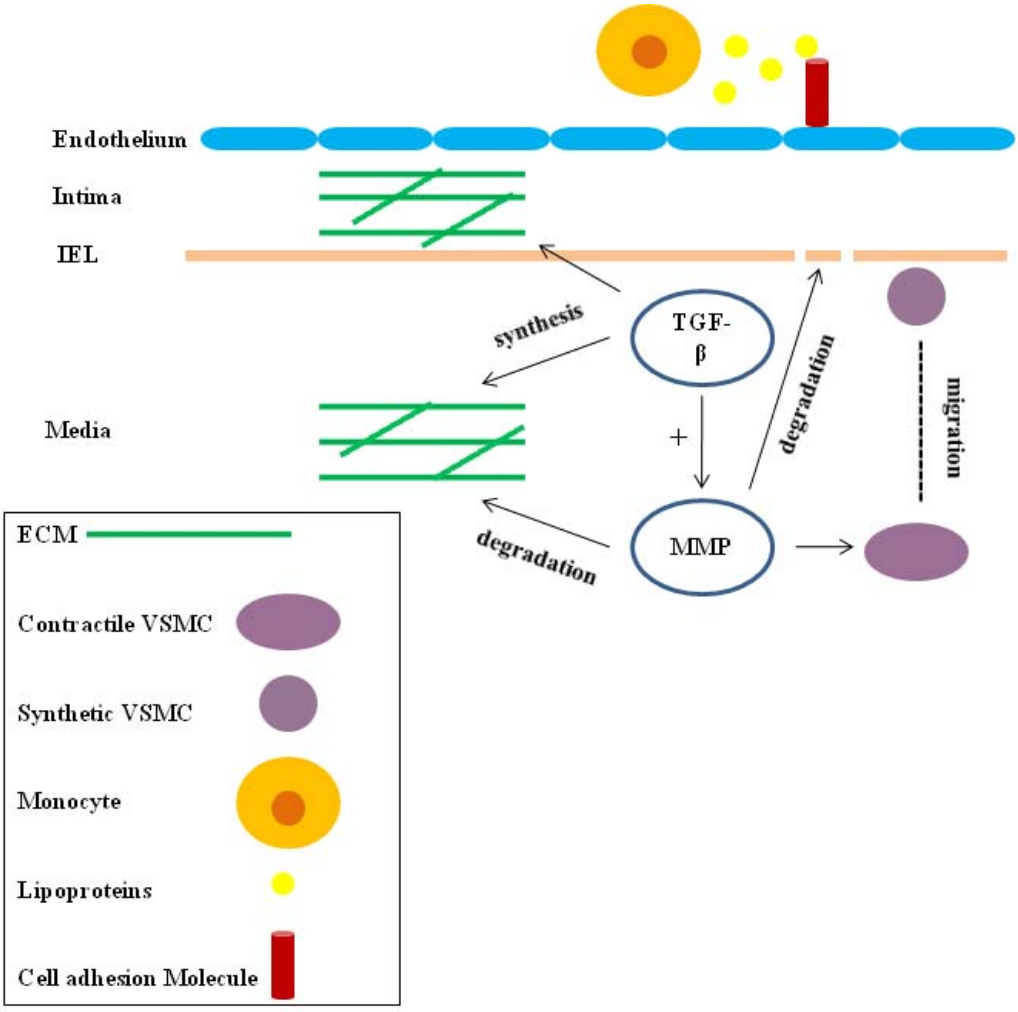
followed by differentiation of the monocytes into macrophages which internalize the oxidized lipoproteins, forming high oxygen consuming foam cells (Glass and Witztum, 2001). Such cholesterol-rich foam cells characterize complex lesions that protrude from the arterial wall causing narrowing of the lumen and symptoms of ischemia (Nakashima *et al*., 2008). The arterial response to an adverse trigger is portrayed in Figure 1.3.

Site-dependent modifications in arterial structure and function arising from the cascade of molecular events described above, have synergistic effects on homeostasis of the system as a whole. Injury and dysfunction of the endothelium characterized by reduced nitric oxide (NO) and impaired vasodilatory responses are fundamental in the early process of atherosclerosis and hypertension (Campuzano *et al*., 2006). In patients destined to develop coronary artery disease (CAD), flow-mediated vasodilation may be abolished or even reversed to a constrictor response in damaged arteries such as the coronary arteries, while vasodilatory capacity is preserved in small atherosclerotic-resistant arteries (Campuzano *et al*., 2006). Since the endothelium mediates the contractile state of underlying smooth muscle through tonic and responsive release of endothelial-derived relaxing factors, its dysfunction increases vascular tone and blood pressure and impedes organ perfusion. Whereas mean arterial blood pressure (MAP) or in other words the steady component of blood pressure, is increased by endothelial dysfunction of the peripheral circulation, the pulsatile component of intravascular pressure is amplified through increased stiffness of central arteries (London *et al*., 1999). The disturbance in hemodynamics consequent to endothelial dysfunction, atherosclerosis and central stiffening, further promotes arterial damage and so a cycle of progressive arterial dysfunction and increasing blood pressure continues.

The left ventricle responds to a mounting afterload by remodeling of its extracellular and cellular components. This reactive process in the heart is also mediated by the MMPs and profibrotic cytokines (Khan and Sheppard, 2006; Spinale, 2007). Activation of MMP-2 and MMP-9 results in increased turnover of matrix proteins, which form a network that maintains orientation of adjacent cardiomyocytes and alignment of the myofibrils within them (Graham *et al*., 2008). As the cycle of degradation and synthesis of fibrillar proteins continues, the former intricately structured matrix becomes disordered. In addition, persistent expression of profibrotic cytokines, particularly TGF-β, causes excessive accumulation of interstitual collagen (Khan and

Figure 1.3 Arterial Response to Injurious Stimuli

In response to an adverse stimulus, the endothelium induces an inflammatory response. This involves expression of the matrix metalloproteinases (MMPs) which degrade extracellular matrix (ECM) proteins within the media. MMP-2 degrades basement membrane proteins including collagen IV and thereby disrupts the internal elastic lamina (IEL) and facilitates phenotypic switch of contractile vascular smooth muscle cells (VSMCs) to synthetic VSMCs which migrate to the intima and proliferate. The expression and activity of MMP-2 is induced by the pro-inflammatory cytokine, transforming growth factor (TGF-β). This cytokine stimulates ECM synthesis and contributes to enlargement of the media and intima. The cell adhesion molecules (CAMs) are expressed on the endothelial surface and allow circulating monocytes and lipoproteins to adhere to and migrate across the denuded endothelium.


Endothelium
Intima
IEL
synthesis
TGF-β
degradation
migration
Media
+
degradation
MMP
ECM
Contractile VSMC
Synthetic VSMC
Monocyte
Lipoproteins
Cell adhesion Molecule

Sheppard, 2006). Given that collagen is the primary determinant of ventricular stiffness, fibrosis of the heart leads to reduced ventricular filling during diastole (Graham and Trafford, 2007). Adding to the problem are the cellular changes involved in reactive remodeling of the heart such as cardiomyocyte hypertrophy, phenotypic modulation and apoptosis, which together alter contractile properties of the ventricular wall (Chien *et al*., 1991). These cellular and extracellular adaptations of the chronically overloaded heart are induced in the attempt to normalize wall stress, but become maladaptive over time leading to systolic and diastolic dysfunction.

Thus, with advanced CVD, not only is the heart at an increasing mechanical disadvantage in dealing with the excessive hemodynamic load, but atherosclerotic lesions in the coronary circulation impede the delivery of oxygen and nutrients that are in high demand. Cardiac ischemia and associated injury due to coronary blockage further promote interstitial fibrosis and myocyte apoptosis through a reparative process (Sun, 2009). Thus, if left unchecked, arterial dysfunction, hemodynamic disturbance and associated cardiac responses, culminate in congestive heart failure and myocardial infarction.

1.6.1 The Role of Central Arterial Stiffening in the Progression of CVD

Augmented pulsatile load due to central arterial stiffening is a hallmark of advancing CVD that plays a major role in cardiac stress and is both a consequence and contributor to arterial damage. This pulsatile component of hemodyanmics is a product of the pressure wave propagated by intermittent left ventricular ejection. At any point along the arterial tree, the amplitude and profile of this waveform is determined by the summation of the forward wave traveling from the heart and a reflected wave returning from the periphery (Mitchell *et al*. 2004; O'Rourke *et al*., 2002). Synchronization of forward and reflected waves is largely a function of the viscoelastic properties of conduit arteries, particularly the thoracic aorta (Cohn, 2001; London and Guerin, 1999). Upon receiving left ventricular ejection, the aorta and other large arteries distend to store a proportion of the blood mass, while the remaining flows forward at a finite speed (Cohn, 2001). This transmission velocity determines time of arrival of the pulse wave at the distal vasculature, where high resistance causes reflection of the wave back towards the heart (London and Guerin, 1999; Safar *et al*., 2003). Thus, when the central arteries are highly distensible as in the young and healthy, over half of cardiac output is buffered, pulse wave

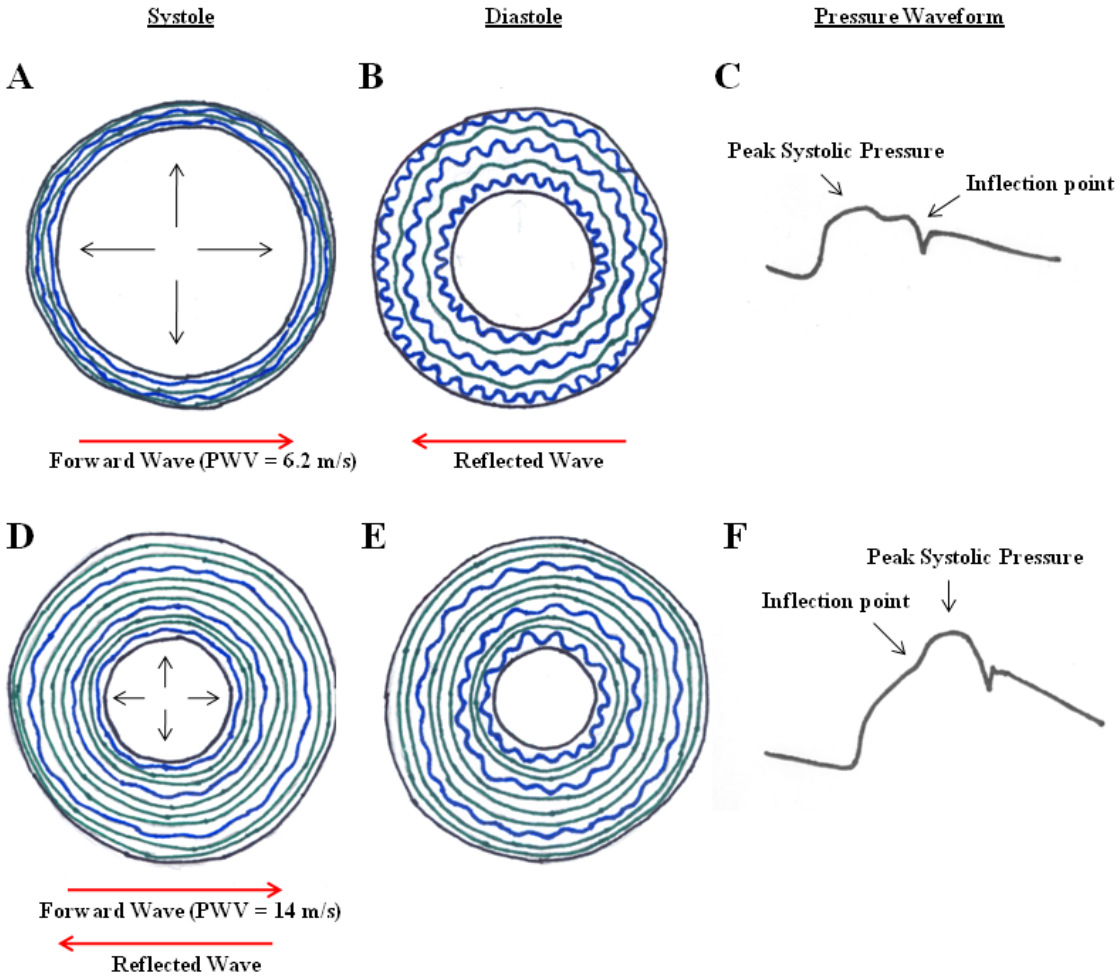
velocity (PWV) is low, and the reflected wave returns after the aortic valves have closed (London and Guerin, 1999). In this case diastolic flow is enhanced, as the reflected wave merges with the reservoir that is propagated forward by elastic recoil of conduit arteries. In sum, compliant arteries dampen pressure oscillations generated by cardiac contraction and ensure adequate coronary perfusion and continuous flow into the microcirculation during diastole. By contrast, stiff large arteries such as those in the hypertensive and pre-hypertensive state, propel a greater proportion of ventricular output, at a faster rate toward the periphery (Mitchell *et al*., 2000). An early return of the reflected wave further augments central systolic pressure and produces a large and rapid drop in pressure during diastole (Mitchell *et al*., 2004). This high systolic load that places excessive strain on the heart, together with diminished coronary perfusion exacerbate pre-existing atherosclerosis and promotes cardiac ischemia and failure. Disturbance in pulsatile dynamics as described is an important antecedent to CVD and arises from abnormalities in the viscoelastic behaviour of the arterial wall. The relation between viscoelastic properties of the aortic wall and pulsatile load is depicted in Figure 1.4.

1.6.2 Compliance and the Extracellular Matrix

Abnormalities in the viscoelastic properties of central arteries present in those destined to develop CVD are preceded by structural changes within the vascular wall. The viscoelastic properties of the arterial wall are primarily determined by the relative proportions and orientations of ECM constituents which bear most of the intravascular load during passive distension. Most important are the proteins, elastin and collagen. Elastic macromolecules endow the vessel with the ability to expand and recoil in response to changes in distending pressure, while collagen limits elasticity of the arterial wall with opposing tensile strength (Fonck *et al*., 2007; Shadwick *et al*., 1999). The parallel arrangement of these extracellular fibers underlies the non-linear relationship between arterial compliance and blood pressure (Shadwick *et al*., 1999). At low distending pressures, the collagenous units are minimally recruited, while the elastic component predominately accommodates the volume load. With intensifying pressure, the change in dimension of an artery is increasingly dependent on inextensible collagen elements as the elastic fibers approach maximal tension and thus distensibility decreases (Roach and Burton,

Figure 1.4 Viscoelastic Properties of Central Arteries and Pulsatile Load

A, a highly compliant aorta. Upon receiving left ventricular ejection, elastic fibres (blue) within the media extend, allowing an increase in lumen diameter. A highly compliant aorta slows the velocity with which the ejected blood mass flows forward as the forward wave. This velocity is measured as pulse wave velocity (PWV). The average PWV for a healthy, individual under the age of 30 is 6.2 m/s (The reference values for arterial stiffness collaboration, 2010). B, During diastole elastic recoil of the elastic fibres reduces the lumen diameter and the reflected wave returns. C, The return of the reflected wave is visible on a pressure waveform as an inflection point. In a young, healthy individual the inflection point occurs after peak systolic pressure. Thus, highly compliant vessels mitigate the amplitude of the pressure wave. D, A stiff aorta. The forward wave is propagated at a higher speed when the aorta is stiff. The average PWV for a hypertensive patient over the age of 70 is 14 m/s (The reference values for arterial stiffness collaboration, 2010). In this case, the reflected wave returns during systole. E, in diastole there is a dramatic drop in blood pressure. F, The inflection point is visible before peak systolic pressure. Thus peak systolic pressure is augmented and the amplitude of the pressure waveform is greater. The pressure waveforms were adapted from Murgo *et al*., (1980).


Systole
Diastole
Pressure Waveform
A
B
C
Peak Systolic Pressure
Inflection point
Forward Wave (PWV = 6.2 m/s)
Reflected Wave
D
E
F
Peak Systolic Pressure
Inflection point
Forward Wave (PWV = 14 m/s)
Reflected Wave

1957; Shadwick *et al*., 1999). Whereas PWV and other non-invasive indices are used to measure pulsatile load and central arterial compliance *in vivo*, generation of the length-tension relationship *ex vivo* allows for direct measurement of passive wall mechanics in arterial rings (Kingwell *et al*., 1997). In arteries such as the aorta that function in buffering of left ventricular ejection, high elastin content translates to high compliance because these deformable proteins shoulder most of the load throughout the physiological pressure range. Reduced elastin content and excessive collagen accumulation owing to reactive vascular remodeling or to the repeated cyclic stress that occurs over time (Et-Taouil *et al*., 2003), predominantly occur in the large elastic arteries. Consequent central arterial stiffening and its associated disturbance in the pulsatile hemodynamic load are strong and independent predictors of hypertension and CVD (Abhayaratna *et al*., 2008). Thus, alteration in ECM composition of central arteries is a structural correlate to the hemodynamic abnormalities that precede overt CVD.

Possibly germane to programming of CVD, is the fact that vascular ECM proteins are deposited during a brief developmental window, after which the relative proportions of these components within the vascular wall are largely fixed. Thus, it is plausible that the propensity toward CVD in adults who were growth restricted in the womb arises from interference in the deposition of these proteins *in utero* and thereaby acceleration in the progression of central arterial stiffening and CVD in postnatal life.

1.7 REMODELING OF THE EXTRACELLULAR MATRIX DURING DEVELOPMENT

Organization of the arterial system during development is accomplished in a precise time and site-specific pattern by a complex interaction between hormones, growth factors, cytokines and hemodynamic forces (Bendeck *et al*., 1994; Hutana *et al*., 2007; Swee *et al*., 1995). These modulators delineate anatomically-defined differential gene expression that gives rise to the longitudinal variation in composition and geometry that characterizes the mature vascular tree. At the proximal end of the vascular tree, elastic fibres are present in abundance within the extracellular matrix (ECM) and the ratio of elastic fibres-to-collagenous fibres decreases toward

the periphery which is populated by small muscular arteries. Elastic fibres are arranged in thick, fenestrated concentric layers (Rosenbloom *et al*., 1993). Construction of a functional elastic fiber begins with the synthesis and deposition of the precursor molecule, tropoelastin, into the extracellular space by embryonic-type vascular smooth muscle cells (VSMCs) possessing synthetic properties. Once in the matrix, the rod-like structures are aligned within a microfibrillar scaffold and covalently cross-linked to form the highly insoluble elastin protein (Debelle and Tamburro, 1999). The regulation of the single elastin gene is primarily exerted at the transcriptional level (Perrin *et al*., 1997; Swee *et al*., 1995). Insulin-like growth factor-1 (IGF-1) and interleukin – 1β are known enhancers of tropoelastin mRNA expression, whereas tumor necrosis factor -α (TNF-α) and transforming necrotic factor -α (TNF-α) downregulate gene transcription (Rich *et al*., 1992; Swee *et al*., 1995). Cortisol is a prominent example of a hormonal regulator acting upstream, which promotes elastin accumulation as well as collagen deposition (Rich *et al*., 1992; Bendeck *et al*., 1991). In addition to these hormones and growth factors, *in vitro* studies have shown tropoelastin mRNA expression and soluble elastin protein levels to be downregulated under conditions of hypoxia in cultured VSMCs (Durmowicz *et al*., 1991), whereas procollagen gene expression is upregulated in hypoxic VSMCs and myofibroblasts (van Vlimmeren *et al*., 2010). Further, reactive oxygen species prevent proper elastic fibre assembly *in vitro* through reduced cross-linked and interference in protein binding (Akhtar *et al*., 2010).

Collagenous fibers have high tensile strength and thus provide structural integrity to the tissues. Their presence in the arterial wall increases progressively toward the periphery where they play a part in the reflection properties of small arteries. In the heart, collagen proteins are the predominant components of the fibrillar network that supports individual cardiomyocytes and aligns the myofibrils within the myocyte (Graham and Trafford, 2008). In this way, collagen fibres contribute to systolic contraction through coordination of sarcomere shortening and are the primary determinants of compliance during diastole (Khan and Sheppard, 2006). Thus, proper functioning and health of the heart are largely dependent on the content and organization of collagen fibrils.

Whereas elastin derives from a single gene, several types of collagen proteins exist. The predominant collagens of the heart and vasculature are collagens Type I and III: the tensile

strength of the more abundant collagen Type I is substantially greater than that of collagen III (Qui *et al*., 2007). Once secreted into the extracellular matrix by synthetic VSMCs, the procollagen precursor is assembled into a triple helix and subsequently stabilized by posttranslational processing into a fibrillar unit (Van Der Rest *et al*., 1991). TGF-β1 and IGF-1 are known to stimulate collagen gene transcription, whereas interferon-γ, basic fibroblast growth factor (bFGF) and nitric oxide have been found to inhibit collagen synthesis (Ford *et al*., 1999; Kypreos and Sonenshein, 1998; Lawrence *et al*., 1994; Reiser *et al*., 1996).

Given that noncellular proteins are synthesized and secreted into the extracellular space by synthetic VSMCs, the deposition rate of collagen and elastin depend on the content of this cellular phenotype within the media (Durmowicz *et al*., 1996). VSMCs exhibiting high rates of proliferation and the ability to produce ECM molecules predominate in early gestation and gradually switch to the mature contractile cells whose principal function is regulation of vasomotor tone, blood pressure and blood flow distributions (Chern *et al*., 1995; Hutana *et al*., 2007). In the ovine fetus and human, this phenotypic transition largely occurs in the last third of pregnancy (Hutana *et al*., 2007; Owens *et al*., 2004). Therefore, ECM protein accumulation rises sharply with VSMC proliferation rates early in gestation, is curbed by cellular phenotypic maturation in late gestation and varies across vascular beds in relation to the synthetic to contractile phenotype ratio.

Modifications in the composition and geometry of the fetal vascular tree over the course of gestation also parallel hemodynamic changes, in order to redefine arterial mechanics in harmony with the new loading conditions. A general thickening of the arterial wall follows the developmental rise in MAP, increasing blood flow rates stimulate diameter enlargement and angiogenesis of the microvasculature respond to the growing perfusion demands of peripheral tissues (Bendeck *et al*., 1991; Cho *et al*., 1992). In late gestation, cyclic stretch induced by pulsatile flow becomes a potent stimulus of elastogenesis, whereas collagen synthesis appears unrelated to blood flow at this time. (Bendeck *et al*., 1994; Wells *et al*., 1999). Thus, elastin accumulates at a high rate in the aorta and other proximal arteries where its deformability is required for accommodation of pulsatile ventricular ejection.

During ovine and human pregnancies, collagen synthesis reaches a maximum early in gestation with a subsequent plateau, whereas elastin accumulates at a slower but steady rate from

early to late gestation (Bendeck *et al*., 1994; Berry *et al*., 1972). In late gestation, elastin synthesis begins to accelerate, reaching a peak in the immediate postnatal period. This accelerated elastin deposition is a key event in the critical phase of arterial remodeling that is initiated by a near-term rise in cortisol and subsequently follows the maturational changes in the hemodynamic environment. After birth, a brief and dramatic period of continued geometric remodeling and deposition of ECM components adapt the vasculature to the profound pressure and flow changes generated by the loss of the placenta, closing of fetal shunts and redistribution of cardiac output that accompany parturition and birth (Bendeck *et al*., 1994; Leung *et al*., 1977). Upon conclusion of this developmental remodeling, arterial structure and mechanics are suited to promote cardiovascular homeostasis in extrauterine life. Thereafter, rates of ECM protein synthesis decline rapidly. In fact, the half life of highly resilient mature elastin is 40 years (Shapiro *et al*. 1991) and its post-development biosynthesis is negligible under normal conditions (Mariencheck *et al*. 1995).

1.8 SCOPE OF THESIS

An interference in the maturation and remodeling of the heart and blood vessels *in utero* may have long-term consequences for cardiovascular health. Hypoxia due to placental insufficiency or a nuchal cord manifests in the second half of gestation, concurrent with a critical period of arterial development (Bendeck *et al*., 1994). The fetal response to hypoxia involves many of the known regulators of arterial remodeling, including hemodynamic conditions (Bendeck *et al*., 1994), the hormonal milieu (Bendeck *et al*., 1991) and local expression of growth factors (Ford *et al*., 1999; Kypreos and Sonenshein, 1998; Lawrence *et al*., 1994; Reiser *et al*., 1996). The two important developmental events that occur during this critical period are the phenotypic switch of VSMCs (Hutana *et al*., 2007) and the rapid deposition of ECM proteins (Bendeck *et al*., 1994). The former establishes the contractile properties of the vascular cells that are important for the peripheral vasculature. The latter sets the ECM composition that determines the buffering capacity of the proximal circulation. The buffering capacity or compliance, of central vessels is strongly and independently linked to the development of CVD in adulthood

(Abhayaratna *et al*., 2008). However, reduced arterial compliance due to altered ECM deposition during adverse fetal development with hypoxia and growth restriction is largely unexplored as a mechanism for programming of CVD.

The overall goal of the thesis is to determine whether disturbances in arterial development occurs in response to fetal hypoxemia and whether such changes translate to long-term compromise in arterial function. Chapters 2-4 address the following hypotheses:

1. Placental insufficiency with chronic hypoxemia in the late gestation ovine fetus leads to structural abnormalities of the aorta in terms of ECM and VSMC content. Such changes are related to altered expression of MMP-2 and TGF-β_1 and a delay in the differentiation of VSMCs. These changes are directly related to the extent of fetal oxygen deprivation. The aortic response is also reflected in the umbilical artery and differs from that of the superior mesenteric artery.

2. Placental insufficiency with fetal growth restriction in the ligated pregnant guinea pig results in structural abnormalities of the aorta that persist into adulthood and manifest as a reduction in compliance. Altered aortic structure is associated with a delay in the maturation of VSMCs.

3. Intermittent umbilical cord occlusion with acute but limited hypoxemia in the late gestation ovine fetus leads to an increased elastin content of the carotid artery and a reduced elastin content of the superior mesenteric artery, in association with hemodynamic and cortisol responses.

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CHAPTER 2

Chronic Intrauterine Hypoxia Interferes With Aortic Development in the Late Gestation Ovine Fetus

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2.1 INTRODUCTION

Cardiovascular disease (CVD) imposes a substantial burden on Western society, with an annual death toll of 1 million Americans constituting 34.3% of deaths and morbidity costs of $503.2 billion per year (AHA, 2010). Currently, aetiology and disease prevention centre on genetics and environmental or lifestyle conditions in postnatal life. However, it is now recognized that in addition to and independent of these traditional risk factors, CVD can be traced to a vulnerability established *in utero*. This influence of the fetal environment on later health has been substantiated over the past decade by consistent observation that low birth weight and other indicators of impaired fetal growth are predictive of various degenerative adult diseases including CVD (Barker *et al*. 1989; Krochik *et al*. 2010). Although developmental origins of CVD are now widely acknowledged, the underlying mechanisms have not been elucidated.

In the developed world placental insufficiency accounts for 60% of infants who are identified as being intrauterine growth restricted (IUGR) (Ghidini, 1996). This antenatal condition arises when abnormal formation of the placental exchange surface leads to progressive disturbance of oxygen and nutrient exchange between mother and fetus (Krebs *et al*. 1996; Regnault *et al*. 2007). The resultant fetal hypoxemia manifests in the second half of gestation, concurrent with a critical period of arterial development. During this time, deposition of extracellular matrix (ECM) proteins accelerates in a time and site-dependent manner, and the vascular smooth muscle cells (VSMCs) that synthesize non-cellular proteins undergo differentiation (Berry *et al*. 1972; Bendeck & Langille, 1991; Wells *et al*. 1999). Given that oxygen tension (van Vlimmeren *et al*. 2010) and factors involved in the fetal response to hypoxemia (Durmowicz *et al*. 1994; Yee *et al*. 1996) are known mediators of ECM remodeling

and VSMC differentiation, enduring arterial defects established *in utero* may underlie susceptibility to CVD in offspring growth restricted by placental insufficiency.

One important outcome of development is the establishment of a relative abundance in elastin proteins in the aorta and its major branches that endows these vessels with a high degree of elasticity. The viscoelastic properties of large arteries govern pulse pressure dynamics and are thus major determinants of cardiac workload. In fact, central arterial stiffening due to altered composition of the ECM is a strong and independent predictor of CVD, as it promotes hypertension and cardiac hypertrophy (Abhayaratna *et al*. 2008). We therefore propose that interference in fetal arterial development as a result of chronic hypoxemia leads to structural abnormalities characteristic of arterial stiffening. To test our hypothesis, we used an established ovine model of placental insufficiency (Gagnon *et al*. 1994) whereby maternal-fetal blood gas exchange is restricted by embolization of the placental circulation and induced fetal hypoxemia is monitored and controlled. Accordingly, we have examined the effect of varying degrees of hypoxemia on aortic composition of elastic and collagenous fibres, VSMC content and phenotype. Known regulators of pathological arterial remodeling in adults, including transforming growth factor beta (TGF-β) and matrix metalloproteinase-2 (MMP-2) were also examined as possible mechanistic links. We demonstrate that upregulation of these molecular mediators in response to chronic hypoxia accompanies altered transcription and accumulation of extracellular and intracellular proteins leading to aberrant arterial morphology.

2.2 METHODS

2.2.1 Surgical Procedures

Embolization of the placenta in pregnant sheep is an established model of placental insufficiency. Surgical preparation and experimental manipulations were performed as previously described (Gagnon *et al*. 1994). All surgical and experimental procedures were approved by the Canadian Council on Animal Care Regulations and The University of Western Ontario Animal Ethics board.

Pregnant Western ewes between 112 and 114 days of gestation (term = 147 days) were chronically instrumented using sterile technique under general anesthesia (1g thiopental sodium in solution, intravenously (IV) for induction, Abbott Laboratories Ltd., Montreal, QC; followed by 1% to 1.5% isoflurane in O_2 for maintenance). Prior to surgery, an analgesic was given intramuscularly to the ewe (0.2 g ketoprofen, Merial Canada Inc., Baie B'urse, QC). A midline incision was made in the lower abdominal wall and the uterus was palpated to determine the fetal number and position. The lower body of the fetus was exteriorized through an incision in the uterine wall, and polyvinyl catheters (Scientific Commodities, Lake Havasu city, AZ) were placed in the right and left fetal femoral arteries, the fetal hind limb vein, and the right maternal femoral vein. Once the fetus was returned to the uterus, a catheter was placed in the amniotic cavity by attachment to the fetal hind skin. Antibiotics were administered intra-operatively to the mother (IV) (0.2 g trimethoprim and 1.2 g sulfadorine, Schering Canada Inc., Pointe-Claire, QC), fetus (IV) and amniotic cavity (1 million IU penicillin G sodium, Pharmaceutical Partners of Canada, Richmond Hill, ON). The uterus and abdominal wall incisions were sutured in layers and catheters were exteriorized through the maternal flank and secured to the back of the ewe in a plastic pouch.

During the postoperative period (3-4 days) the antibiotic regime was continued daily [mother (IV) 0.2 g trimethoprim; fetus (IV) and amniotic cavity 1 million IU penicillin G sodium]. Arterial blood was sampled daily for evaluation of maternal and fetal condition and catheters were flushed with heparinized saline to maintain patency. Animals were housed in individual metabolic cages with food and water available ad libitum. The housing facility was temperature (16°C) and humidity (50%) controlled, with a 12:12 hour light-dark cycle.

2.2.2 Experimental Design

Nineteen sheep were studied. Experiments were initiated 3 or 4 days after surgery at a gestational age of 116-118 days. Placental embolization was performed in experimental animals by bolus injections of latex microspheres (15 μm or 30 μm; Interactive Medical Technology Laboratories, Los Angeles, CA) into the fetal abdominal aorta distal to the renal artery via a fetal femoral arterial catheter. Injections were initiated at 1000 hrs on day 1, following a 2-hour baseline period, and repeated at 10-minute intervals until a stable reduction of arterial oxygen

saturation at the desired level of fetal hypoxia was maintained for at least an hour. The target level of arterial oxygen saturation was 40-50% for the moderately hypoxic group and 30-40% for the severely hypoxic group. On subsequent days embolization was performed after the 2 hour baseline period if fetal arterial oxygenation rose above targeted values. Control fetuses were injected with saline only.

2.2.3 *In Vivo* Physiological Parameters

On selected days (experimental day 1,5,8,12 and 15) the embolized and Control groups were subjected to a blood sampling and cardiovascular monitoring regime. Fetal arterial and maternal venous blood samples taken at 0900hrs (baseline), 1300hrs and 1600hrs were analyzed for blood gases, lactate, glucose and pH using a blood gas analyzer (ABL-725, Radiometer, Copenhagen, Denmark) and corrected for fetal temperature (T = 39.2°C). Plasma aliquots from samples taken at 0900 hrs were stored at – 80 °C for later cortisol analysis. Fetal arterial blood pressure (MAP), adjusted for amniotic fluid pressure, was continuously monitored between 0800 and 1600 hrs with pressure transducers (Cobe, Arvada, CO) and recorded on a data acquisition system (Powerlab model ML 795, ADI Instruments, Colorado Springs, CO). Fetal heart rate (FHR) was derived from the arterial blood pressure waveform. During baseline periods, starting at 0900 hrs, 20 min averages of fetal mean arterial blood pressure (MAP) and heart rate (FHR) were calculated for each fetus, using Powerlab software (Powerlab, ADI Instruments, Colorado Springs, CO). Average fetal arterial oxygen saturation values for each fetus were calculated using blood gas measurements taken at 0900 hrs, 1300 hrs and 1600 hrs on each experimental day, excluding baseline of day 1.

2.2.4 Post-Mortem and Tissue Preparation

On day 15, after the final blood sample at 1600 hrs, ewes and fetuses were sacrificed with an overdose of barbiturate (30 mg pentobarbital sodium, Fatal-Plus; Vortech Pharmaceuticals, Dearborn, MI). The fetus was weighed, sexed and dissected to obtain brain and liver weight. The descending aorta proximal to the diaphragm was perfused with saline and then fixed with 4% paraformaldehyde at physiological pressure (120 mmHg). The thoracic aorta immediately distal

to the aortic arch and the superior mesenteric artery (SMA), were excised, cleared of fat and connective tissue, fast frozen in liquid nitrogen and stored at - 80°C for later analyses. Fixed aortae were embedded in paraffin and cut in 5 μm cross-sections that were baked onto positively charged glass plates by heating in a 50 ºC oven for 2 days.

2.2.5 Plasma Cortisol Concentration

Baseline plasma samples were analyzed for cortisol concentration using an enzyme-linked immunosorbent (ELISA) assay according to the manufacturer's instructions (ALPCO Diagnostics, Salem, NH). The absorbance of sample triplicates was measured on a microtiter plate reader at 450 nm and the mean optical density calculated from a 4-parameter standard curve. The intra-assay and inter-assay coefficient of variation for the cortisol ELISA was 5.6% and 7.1%, respectively.

2.2.6 RNA Extraction and Quantitative qPCR in the Aorta and SMA

Total RNA was extracted from frozen thoracic aortae and SMA using the Trizol method (Invitrogen Life Technologies Co., Burlington, ON). RNA integrity of each sample was assessed using 1.2 % agarose electrophoresis with ethidium bromide staining. Complementary DNA was synthesized from 2 μg of purified RNA using oligo(dT) primers and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies Co., Burlington, ON). Standard curves for each primer set (Appendix) were generated in order to determine optimal concentrations of input cDNA and PCR efficiency. PCR efficiencies for each primer set were 90% -100%. cDNA products were used as templates for quantitative real-time polymerase chain reaction (qRT-PCR) for measurement of gene expression levels using the SYBR green system (Bio-Rad Laboratories Mississauga, ON) on a Bio-Rad CFX384 real time PCR detector. Amplification was performed in triplicate at 95˚C for 3 min, followed by 39 cycles at 95˚C for 15s, 59˚C for 15s and 72˚C for 15s. Melting curve analyses after each run and the presence of a single band of appropriate size in 1.8% agarose gel confirmed amplification of a single product.

Fold change values calculated relative to the average mRNA for a given gene were normalized to the reference gene (S15).

2.2.7 Collagen and Elastin Staining in the Aorta

After deparaffinization in xylene, slides were rehydrated by passage through a decreasing ethanol series. Collagen content was measured in cross-sections stained with 1% Sirius red F3BA (Sigma-Aldrich Canada Ltd., Oakville ON) in a saturated aqueous solution of picric acid, for 1 hour. Additional aortic sections were stained 30 minutes in 0.2 % Orcein (Sigma-Aldrich Canada Ltd., Oakville ON) for identification of elastic fibres. Stained cross-sections were captured on a microscope (Leica DM RB) at 10x magnification. Duplicates of 3 cross-sections per animal and 4-5 areas per cross-section were used for analyses. Wall thickness was measured as the distance between the internal and external elastic laminae. For collagen quantification the tunica media was selected, whereas elastin content measurement included both the tunica media and the internal elastic lamina. The area positive for protein (elastin or collagen) was identified by color thresholding using image analysis software (Image Pro 6.0, MediaCybernetics, Bethesda, MD) and expressed relative to the sum of area non-stained. Total protein content (elastin or collagen) was calculated by multiplying the average wall thickness for each vessel by the percent area stained, as previously reported (Kobs *et al*. 2005). Orcein slides were captured at 40x magnification for measurement of intima thickness. If an intima was present its thickness was measured perpendicular to the medial border. A thickness score of 0 was given if no intima was present. The perpendicular distance between the internal elastic lamina and the first elastic layer within the media was measured.

2.2.8 Immunofluorescent Staining for α Actin, MHC-B and PCNA in the Aorta

Deparaffinized cross-sections of the aorta were incubated at room temperature for 10 min in Background Sniper (Biocare Medical LLC, Brampton, ON) for blockage of nonspecific binding, followed by incubation with primary antibodies diluted in Universal Antibody Diluting Solution (Dako Canada Inc., Burlington ON) in a humidified chamber at 4ºC overnight: 1:4000 dilutions of monoclonal mouse α-actin antibody (Boehringer Ingelheim Ltd., Mannheim Germany) along with 1:3000 dilutions of polyclonal rabbit nonmuscle myosin heavy chain II-B

(MHC-B) (Covance Inc., Emeryville, CA). After washing in phosphate-buffered saline, slides were incubated in 1:400 dilutions of Molecular Probes secondary antibodies Alexa Fluor® 568 and 405 (Invitrogen Life Technologies Co., Burlington, ON) at room temp for 30 min in a black covered humidity chamber. After washing, slides were counterstained for 15 min using Sytox Green (Invitrogen Life Technologies Co., Burlington ON). Additional slides were incubated in 1:400 dilutions of rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology INC., CA) and counterstained with Hoechst 3342 (Invitrogen Life Technologies Co., Burlington,ON) for 10 min. Replacement of the primary antibody with PBS or IgG were used as negative controls. Fluorescence VectaShield mounting medium (Vector Laboratories Burlington, ON) was used for mounting. Slides were stained in duplicate and simultaneously to minimize variation in staining intensity. All antibodies were tested for specificity by Western Blot. Sections were imaged on a microscope (Zeiss) and captured at 20x magnification using a camera and software for image capture and analysis (Axiovision 4.0, Carl Zeiss Microimaging LLC, Thornwood, NY). The area positive for staining was identified by color thresholding using image analysis software (Image Pro 6.0, MediaCybernetics, Bethesda, MD). The sum of area stained and the number of stained objects were expressed as a percentage of an area of constant dimension within the media.

2.2.9 Statistical Procedures

All statistical procedures were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Between group differences were assessed using one-way ANOVA with Bonferroni *post hoc* tests, or Kruskal-Wallis with Dunns *post hoc* test when data were determined to be non-parametric. Within group comparisons of baseline values of blood pressure and heart rate, across the 15 experimental days, were made by one-way ANOVA for repeated measures. Two way ANOVA was performed when missing values did not allow for repeated measures analysis. Relationships between arterial measurements and average fetal arterial oxygen saturation values over the course of the embolization period were expressed as Spearman's correlation coefficient and included animals from all 3 study groups. Significance was set at $p < .05$ and all data are presented as mean ± SEM.

2.3 RESULTS

2.3.1 Fetal Growth Restriction and Physiological Parameters

Our goal was to produce a level of chronic hypoxia typical of clinical IUGR and likely to result in fetal survival. In order to determine the dose-response effect, graded severity of hypoxia between Moderate and Severe groups was produced by embolization. Excluded from the study were fetuses from 2 ewes that developed mastitis during the experiment and 1 fetus that was severely hypoxic during and following surgery. Included in data analyses were 5 animals in each of the Moderate and Severe groups and 6 animals in the Control group. The ratio of female to male fetuses in Control, Moderate, and Severe groups was 2:3, 3:2, 3:3 respectively. Average blood gas values, pH, haemoglobin, glucose and lactate concentrations over the course of the embolization period are presented in Table 2.1. Hypoxia of each degree was not accompanied by cumulative metabolic acidosis or lactic acid accumulation. Fetuses subjected to Severe hypoxia displayed asymmetric fetal growth restriction, as reflected by reduced fetal body weight ($p < .05$) and increased brain-to-liver ratio ($p < .05$), relative to Control. Average fetal weight was 3.6 ± 0.3 kg for the Control group, 3.3 ± 0.4 kg for the Moderate group and 2.7 ± 0.2 kg for the Severe group. The brain-to-liver ratio was 0.38 ± 0.06, 0.47 ± 0.04 and 0.59 ± 0.06 for the Control, Moderate and Severe groups, respectively.

Baseline values of MAP and FHR were not different between groups or within groups over the 15 day study period (included in analysis are days 1, 5, 8, 12, 15). Baseline MAP on day 1 was 40 ± 4 mmHg for the Control group; 40 ± 2 mmHg for the Moderate group and 40 ± 2 mmHg for the Severe group. Baseline FHR on day 1 was 171 ± 14 bpm for the Control group; 179 ± 3 bpm for the moderate group; and 171 ± 12 bpm for the Severe group. Plasma cortisol concentration was not different between groups at baseline on day 1 of the study and was also unchanged within groups across the 15 day embolization period (included in analysis are days 1, 5, 8, 12, 15). Cortisol concentration on baseline of day 1 was 3.2 ± 0.4 μg/dL for the Control group; 5.1 ± 3.0 μg/dL for the Moderate group; and 3.8 ± 0.4 μg/dL for the Severe group. On day 15 of the study, circulating cortisol concentration was 4.5 ± 0.8 μg/dL for the Control group; 4.8 ± 2.5 μg/dL for the Moderate group; and 2.3 ± 0.1 μg/dL for the Severe group.

2.3.2 Aberrant ECM Remodeling Leads to Compositional Changes in the Aorta

Collagen I and III are the predominant fibrillar collagens present in blood vessels. In all 3 study groups, mRNA levels of procollagen Iα were higher than those of procollagen III. Real-time PCR revealed procollagen Iα mRNA levels of the thoracic aorta to be elevated in severely hypoxic animals compared to control ($p < .05$) and a similar trend observed with respect to Collagen III (Figure 2.1). Procollagen Iα and III mRNA levels were inversely related with

Table 2.1: Fetal blood gases, pH, Haemoglobin (Hb), glucose and lactate

GROUP	pH	PaCO2 (mmHg)	PaO2 (mmHg)	SaO2 (%)	Hb (mmol/L)	Glucose (mmol/L)	Lactate (mmol/L)
CONTROL	7.32 ± 0.01	52.1 ± 0.6	20.7 ± 0.7	51.3 ± 0.8	9.9 ± 0.8	1.1 ± 0.0	1.2 ± 0.1
MODERATE	7.31 ± 0.01	55.5 ± 1.2	18.1 ± 0.6	44.1 ± 1.1	10.1 ± 0.5	1.0 ± 0.1	1.7 ± 0.4
SEVERE	7.32 ± 0.01	53.8 ± 0.5	15.5 ± 0.5 **	35.6 ± 1.3 ***	9.3 ± 1.2	1.2 ± 1.1	1.7 ± 0.1

Blood sampled from the fetal femoral artery at selected time points was analyzed using a blood gas analyzer. Average values over the course of the study were calculated for each group and presented as ± SEM. Group differences were analyzed using the Kruskal-Wallis with Dunns test for *post hoc* comparisons. Fetal arterial partial pressure of oxygen (PaO_2) and oxygen saturation (SaO_2) were reduced in Moderate and Severe groups compared to control, without changes in haemoglobin (Hb), lactate concentration or pH.

* $P < .05$ hypoxic groups vs. control

average fetal arterial oxygen saturation, Spearman's coefficient was $R = - 0.69$ ($p < .05$) and $R =$

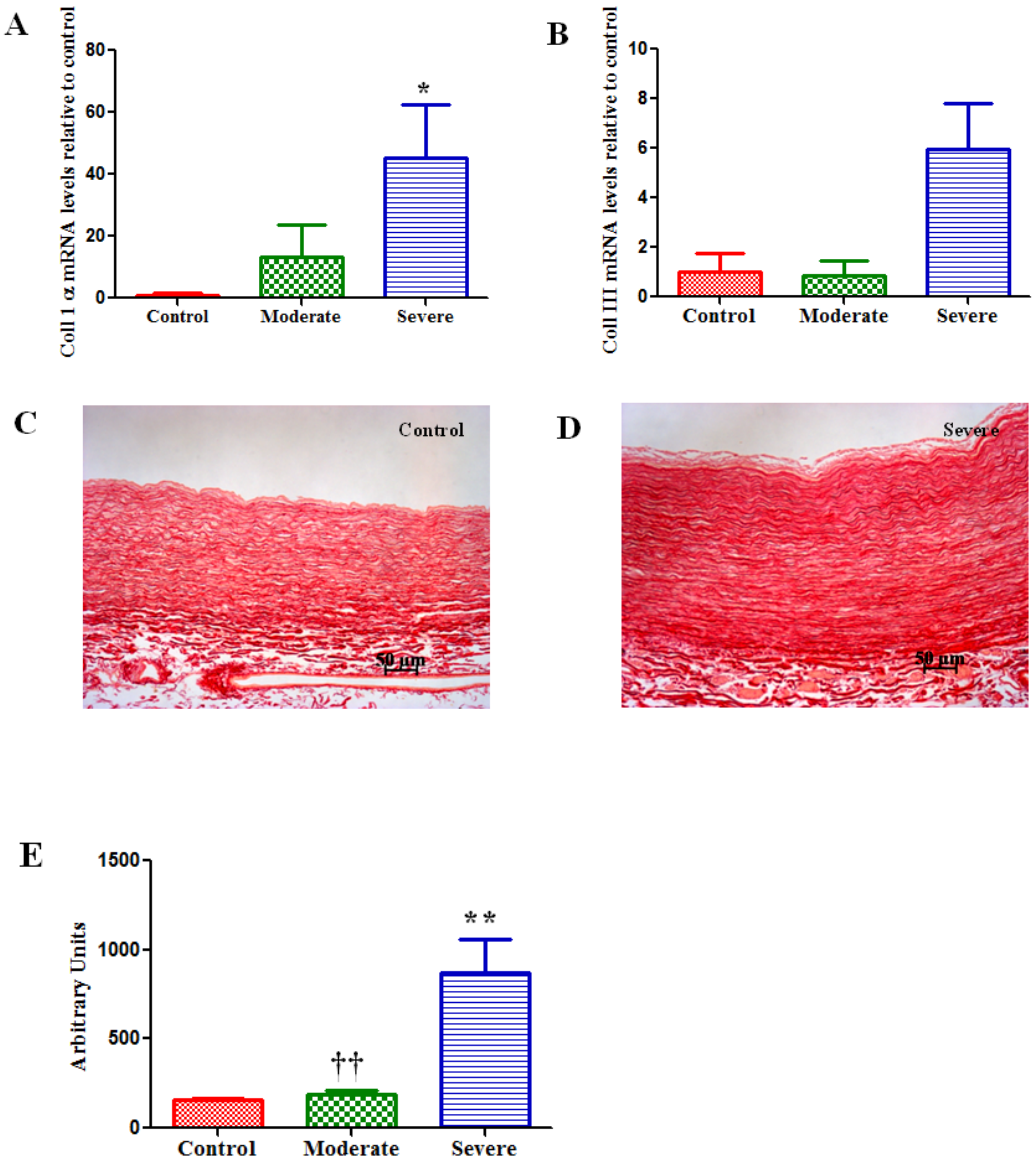
.

Figure 2.1. Procollagen Iα and III mRNA levels and total collagen content of the aorta are increased in severely hypoxic fetuses

A, Real-time PCR showed procollagen Iα mRNA levels to be increased in the thoracic aorta of severely hypoxic group versus control. B, A comparable pattern was found with respect to Procollagen III, although changes were not significant. Collagen content of the media was measured in cross-sections of the descending aorta stained with a Sirius-red dye: shown are cross-sections from a Control (C) and Severe (D) animal. E, A 5x increase in total collagen content compared to Control was measured in the Severe group. Comparisons between groups were made using Kruskal-Wallis statistic with Dunns *post hoc* test. Data are presented as ± SEM.

* $p < .05$ hypoxic groups vs. Control

† $p < .05$ Moderate vs. Severe group


A
Coll 1 α mRNA levels relative to control
80
60
40
20
0
*
Control
Moderate
Severe
B
Coll III mRNA levels relative to control
10
8
6
4
2
0
Control
Moderate
Severe
C
Control
50 µm
D
Severe
50 µm
E
Arbitrary Units
1500
1000
500
0
**
††
Control
Moderate
Severe

- 0.72 ($p < .05$), respectively. mRNA levels of tropoelastin were not different between groups, levels relative to control were 1.00 ± 0.5 for the Control group; 1.38 ± 1.37 for the Moderate group; and 2.17 ± 1.21 for the Severe group. Reflecting patterns of procollagen mRNA expression, total collagen content within the media of the thoracic aorta measured using the Sirius-red dye, was also increased in the severely hypoxic animals compared to normoxic animals ($P < .01$). However, the relative collagen content in the aortic media did not differ among groups: 3.57 ± 0.50 for the control group; 2.79 ± 2.24 for the Moderate group and 2.66 ± 0.57 for the Severe group. Compared to the Control group, relative elastic fiber content was reduced by 12% in the Moderate group and by 39% ($p <.05$) in the Severe group (Figure 2.2). Spearman's correlation analysis showed a positive relationship between relative elastic fiber content with fetal average arterial oxygen saturation ($R = 0.55$, $p < .05$). Total elastic fiber content was constant across groups: 99.09 ± 5.43 for the Control group, 142.13 ± 9.98 for the Moderate group and 120.10 ± 8.65 for the Severe group.

2.3.3 Regulators of ECM Protein Synthesis and Degradation

TGF-β_1 mRNA levels as measured by real-time PCR were increased in the Severe hypoxic group versus Control ($p < .05$), but similar between Moderate and Control groups (Figure 2.3). TGF-β_1 mRNA levels in the aorta exhibited an inverse relationship with fetal arterial oxygen saturation ($R = -0.7$, $p < .05$) (Figure 2.3). mRNA levels of MMP-2 were also inversely correlated with fetal arterial oxygen saturation ($R = -0.6$, $p < .05$) and showed a similar pattern of change among groups, although significance was not reached (Figure 2.4). MTI-MMP mRNA levels were increased in the severely hypoxic animals compared to Control ($p < .05$) and

again showed an inverse relationship with arterial oxygen saturation ($R = -0.66$, $p < .01$) (Figure 2.4).

2.3.4 Aortic VSMC Content and Wall Thickness

Differentiated VSMC content in the aortic media as, reflected by the fluorescent staining of the α-actin protein, was increased in a graded fashion in response to Moderate and Severe hypoxia (Figure 2.5). Compared to Control, total α actin content was 45 % higher in the Moderate group and 65% ($p < .05$) higher in the Severe group; and the percentage of α actin

Figure 2.2. Relative elastic fiber content within the aortic media is reduced in hypoxic fetuses Relative elastic fiber content in the media measured in cross-sections of the thoracic aorta in Control (A), Moderate (B) and Severe (C) groups, is shown. This decrease in relative elastic fiber content was graded across Moderate and Severe group, with a 12% and 39% decrease respectively. Kruskal-Wallis with Dunns *post hoc* test were used for multiple comparisons.

* $p < .05$ hypoxic groups vs. Control

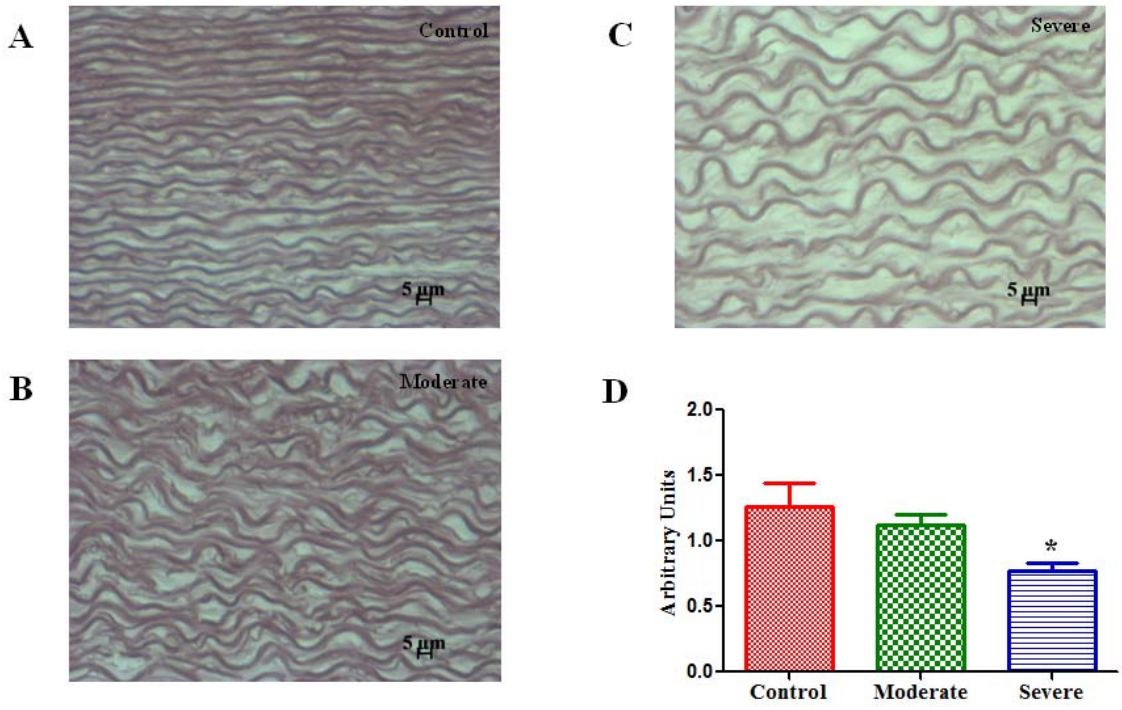

A
Control
5 µm
C
Severe
5 µm
B
Moderate
5 µm
D
Arbitrary Units
2.0
1.5
1.0
0.5
0.0
*
Control
Moderate
Severe

Figure 2.3. Severe hypoxia increases expression of TGF-β_1 in the thoracic aorta

A, mRNA levels of TGF-β_1 measured by real-time PCR were increased in severely hypoxic fetuses. Kruskal-Wallis with Dunns *post hoc* test were used for multiple comparisons. B, Spearman's correlation revealed an inverse relationship TGF-β_1 mRNA levels and fetal arterial oxygen saturation was determined using Spearman's correlation ($R = -0.70$, $p < .05$).

** $p < .01$ hypoxic groups vs. Control

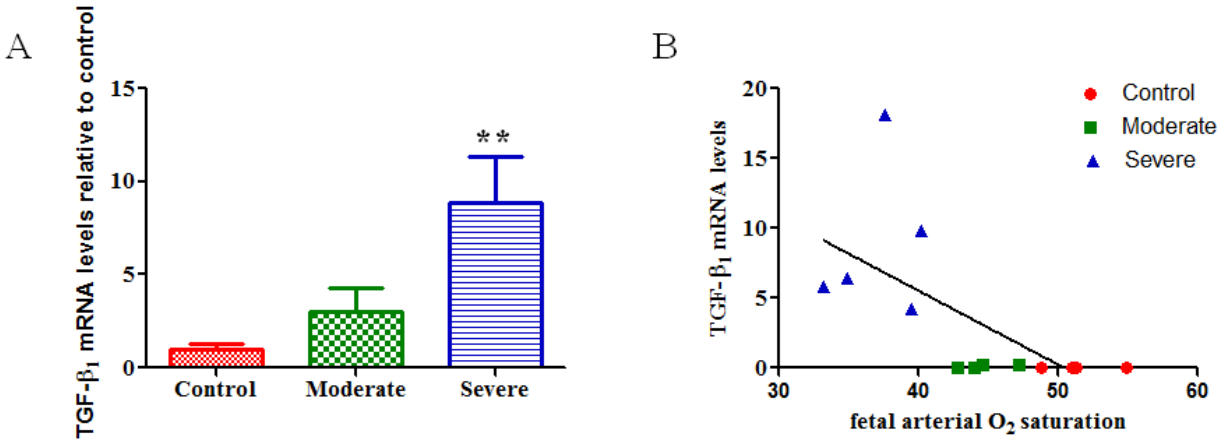

A
TGF-β1 mRNA levels relative to control
15
10
5
0
**
Control
Moderate
Severe
B
TGF-β1 mRNA levels
20
15
10
5
0
30
40
50
60
fetal arterial O2 saturation
Control
Moderate
Severe

Figure 2.4. mRNA levels of MMP-2 and co-activator MTI-MMP are increased in the thoracic aorta of severely hypoxic fetuses

A, matrix metalloproteinase-2 (MMP-2) mRNA levels in the thoracic aorta measured by real-time PCR are increased in severely hypoxic fetuses compared to Control ($p = 0.059$). B, An inverse correlation between MMP-2 mRNA levels in the thoracic aorta and fetal arterial oxygen saturation was found ($R = - 0.60$, $p < .05$). C, Severely hypoxic fetuses exhibited increased mRNA levels of membrane-type matrix metalloproteinase (MTI-MMP) which is required to activate MMP-2. D, MTI-MMP exhibited an inverse relationship with fetal arterial oxygen saturation ($R = -0.66$, $p < .01$). Kruskal-Wallis test with Dunns *post hoc* were used for multiple comparisons and Spearman's was used for correlational analyses. Data are shown as ± SEM.

* $p < .05$ Hypoxic groups vs. Control

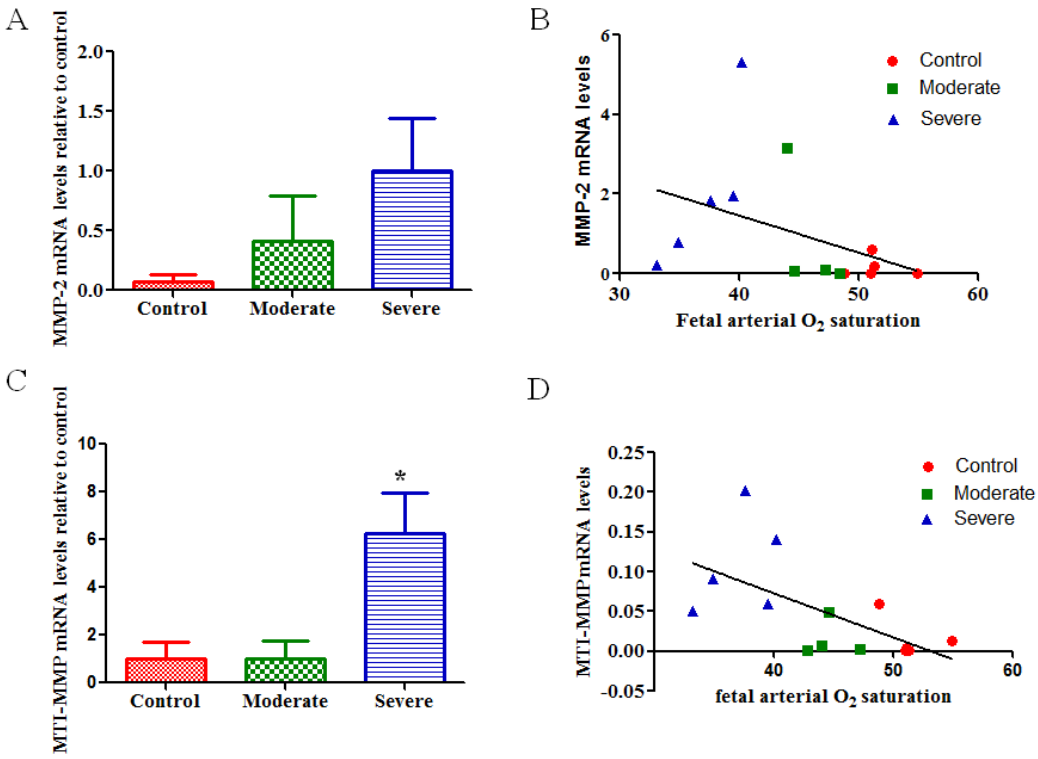
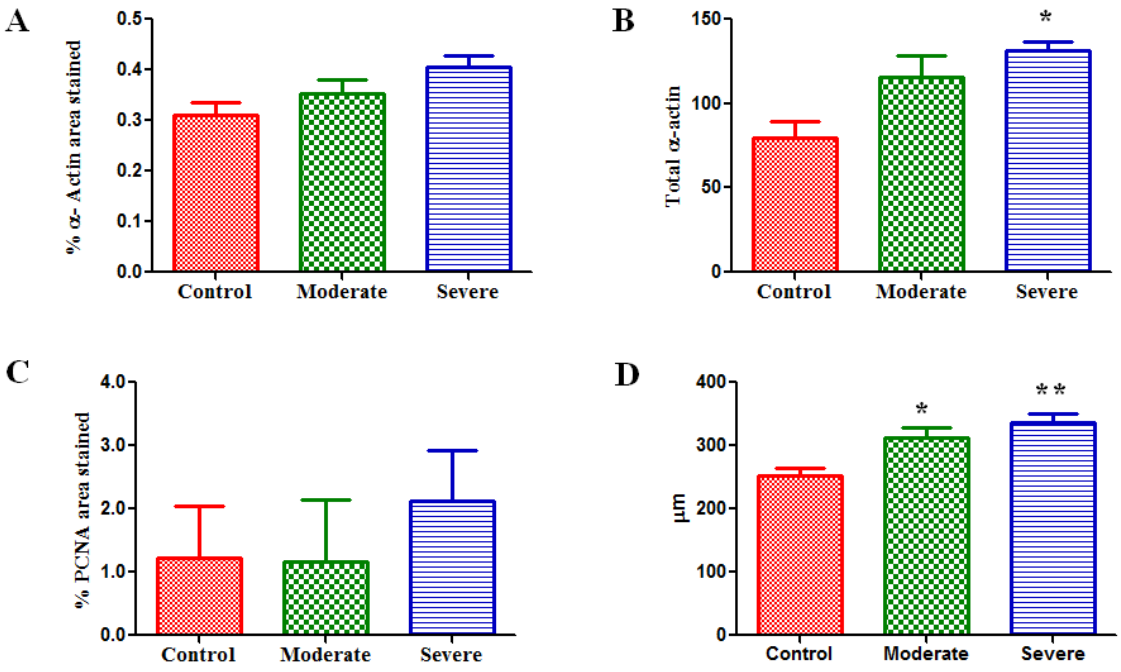

A
MMP-2 mRNA levels relative to control
2.0
1.5
1.0
0.5
0.0
Control
Moderate
Severe
B
MMP-2 mRNA levels
6
4
2
0
30
40
50
60
Fetal arterial O2 saturation
Control
Moderate
Severe
C
MTI-MMP mRNA levels relative to control
10
8
6
4
2
0
*
Control
Moderate
Severe
D
MTI-MMP mRNA levels
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
40
50
60
fetal arterial O2 saturation
Control
Moderate
Severe

Figure 2.5. Hypertrophy of the aortic media in response to chronic intrauterine hypoxia is due in part to an increase in VSMC content

A, Relative to Control, the % area stained for α-actin was increased by 13% in the Moderate group and by 30% in the Severe group. B, The total α-actin content showed a similar pattern, with statistical significance between Control and Severe groups. Total α actin content was increased by 45% and 65% in Moderate and Severe groups respectively, compared to Control. C, No significant difference between groups was found with respect to the % area stained for Proliferating Cell Nuclear Antigen (PCNA), a marker for cell proliferation. D, Media thickness was increased by 23% and 33% by Moderate and Severe hypoxia respectively. Group differences were assessed with the Kruskal-Wallis statistic and Dunns *post hoc* test. Data are shown as ± SEM.

* $p < .05$ hypoxic groups vs. Control


A
% α- Actin area stained
0.5
0.4
0.3
0.2
0.1
0.0
Control
Moderate
Severe
B
Total α-actin
150
100
50
0
*
Control
Moderate
Severe
C
% PCNA area stained
4.0
3.0
2.0
1.0
0.0
Control
Moderate
Severe
D
µm
400
300
200
100
0
*
**
Control
Moderate
Severe

within the media was increased relative to Control, by 13% in the Moderate group and 30% in the Severe group. The % α-actin within the media was inversely correlated with fetal arterial oxygen saturation ($R = -0.61$, $p < .05$). The presence of MHC-B, a marker of undifferentiated synthetic-type VSMCs in the media, was not altered by hypoxia. As well, no differences between groups were found for the percentage of cells in the media or staining of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation. Aortic media thickness was increased by 23% in the Moderate and 33% ($p < .01$) in the Severe group, relative to Control animals (Figure 2.5). Wall thickness directly related with average fetal arterial oxygen saturation (Spearman's coefficient $R = - 0.67$, $p < .05$).

2.3.5 Intima Hyperplasia in Hypoxic Fetuses Associated with Endothelial Activation

The presence of a fibrous neointima was predominantly found in severely hypoxic fetuses and the thickness of the intima was markedly increased in this group compared to Control and Moderate animals, suggesting intima hyperplasia to occur in response to Severe fetal hypoxia (Figure 2.6). The Severe group exhibited a 14-fold increase in intima thickness and an 11-fold increase in the intima:media thickness, compared to Control ($p < .05$). The thickness of the area between the internal elastic lamina and the first elastic fiber within the media was also markedly increased in the Severe group relative to Control ($p < 0.05$) (Figure 2.6). E-selectin was increased in severely hypoxic fetuses ($p < .05$) and mRNA levels of E-selectin showed an inverse relationship with average fetal arterial oxygen saturation (Spearman's coefficient $R = - 0.67$, $p < .05$). (Figure 2.7)

2.3.6 Fetal Hypoxia has a Differential Effect on the Superior Mesenteric Artery (SMA)

No differences in mRNA levels of procollagen I α and III were observed in the SMA. mRNA levels of procollagen Iα relative to Control were 1.00 ± 0.46 for the Control group; 0.65 ± 0.22 for the Moderate group and 1.57 ± 0.73 for the Severe group. mRNA levels of procollagen III relative to Control were 1.00 ± 0.33 for the Control group; 0.93 ± 0.47 for the Moderate group; and 0.85 ± 0.27 for the Severe group. TGF-β_1, MMP-2 and E-selectin mRNA levels also showed no changes among groups.

Figure 2.6: Intima hyperplasia of the aorta in severely hypoxic fetuses

A, The presence of an intima was largely lacking in descending aortae from Control and moderately hypoxic fetuses. B, An intimal layer was observed in Severely hypoxic fetuses; its size variable and often strikingly thick. C, Relative to Control, a 14 fold increase in the thickness of the intima on the luminal (L) side of the internal elastic lamina and a 2 fold increase in the thickness of the medial intima (the distance between the internal elastic lamina and the first elastic fiber within the media, as indicated by the arrow in B) was measured in Severe aortae. D, The ratio of intima-to-media thickness was also notably increased in Severe animals compared to Control and Moderate. The Kruskal-Wallis with Dunns *post hoc* were used to assess group differences. Data are presented as ± SEM.

* $p < .05$ Hypoxic groups vs. Control

† $p < .05$ Moderate vs. Severe groups

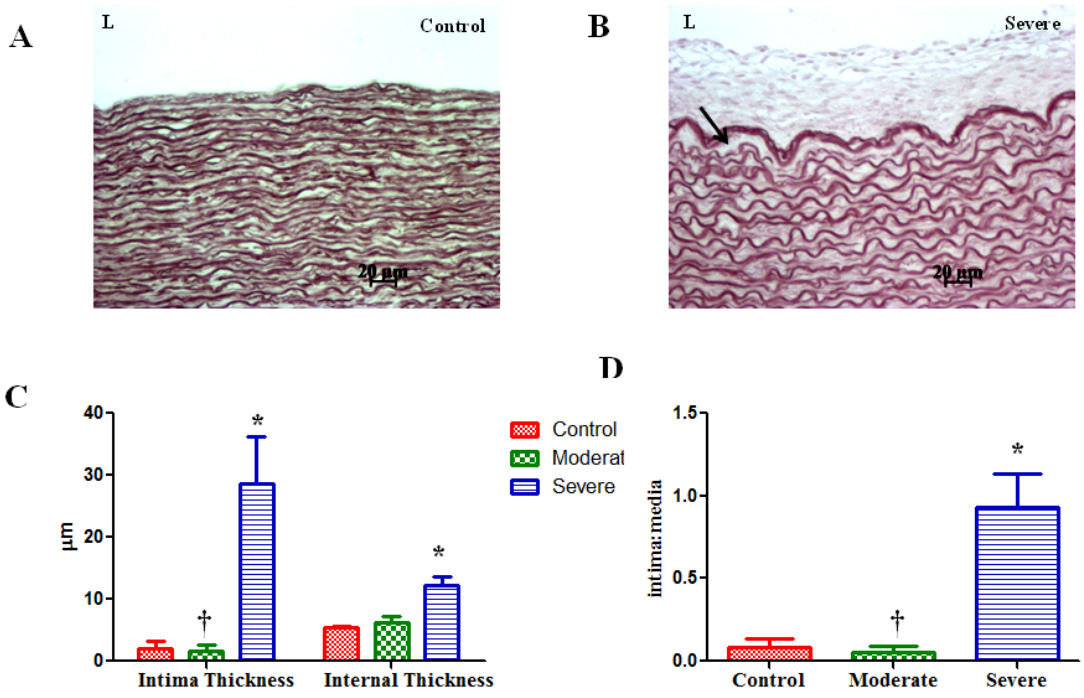
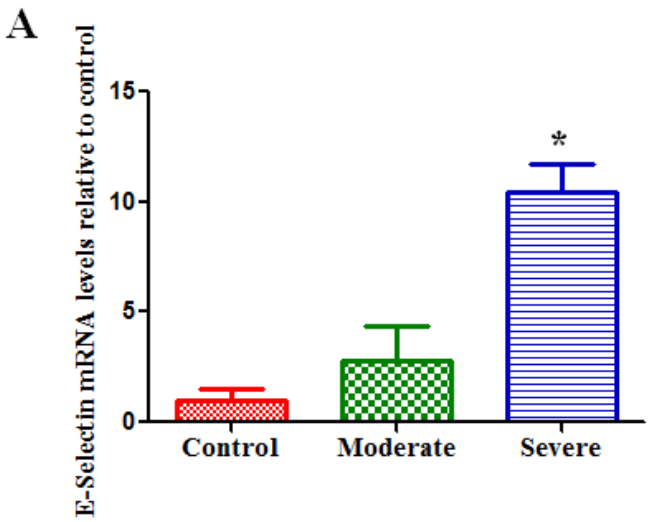

A
L
Control
20 µm
B
L
Severe
20 µm
C
µm
40
30
20
10
0
*
†
*
Intima Thickness
Internal Thickness
Control
Moderat
Severe
D
intima:media
1.5
1.0
0.5
0.0
*
†
Control
Moderate
Severe

Figure 2.7. Intima hyperplasia of the aorta in response to Severe hypoxia was accompanied by increased mRNA levels of E-selectin

A, E-selectin is a cell-adhesion molecule that increases in response to endothelial activation. mRNA levels of E-selectin as measured by real-time PCR were increased in thoracic aortae of severely hypoxic fetuses.

* $p < .05$ hypoxic groups vs. Control


A
E-Selectin mRNA levels relative to control
15
10
5
0
*
Control
Moderate
Severe

2.4 DISCUSSION

This study is the first to investigate aortic development in the late gestation ovine fetus under varying degrees of oxygen deprivation *in utero*. Two degrees of fetal hypoxemia without metabolic acidosis were produced by umbilical-placental embolization, an established model of placental insufficiency. This antenatal disorder is the predominant cause of IUGR in Western society (Ghidini, 1996) and occurs when abnormal placental villous and arterial structure lead to inadequate transplacental transport of oxygen and nutrients that fails to meet increasing demands of the growing fetus (Regnault *et al*. 2007; Todros *et al*. 1999). The resultant fetal hypoxemia is the major stimulus driving fetal adaptations including slowing of growth and altered organ development (Lackman, 2001; Giussani, 2007). This model has previously been shown to produce abnormal umbilical artery Doppler waveforms (Gagnon, 1994) which reflect increased umbilical-placental vascular resistance and are used clinically to diagnose placental insufficiency and assess its severity. Embolization leading to severe hypoxia in the current study increased the ratio of brain-to-liver weight, an indication of the asymmetric pattern of growth restriction that occurs in human placental insufficiency (Dashe, 2000) whereby brain growth is preserved relative to non-critical organs.

2.4.1 Mechanical Consequences of Altered Aortic ECM Composition

The structural phenotype of the aorta produced by Severe hypoxia was characterized by reduced relative elastin content, together with media thickening due to both increased collagen accumulation and VSMC hypertrophy. Relative proportions and orientation of the arterial wall components are principal determinants of the viscoelastic behaviour of the aortic wall and thus pivotal in systemic hemodynamics. Viscosity is largely influenced by the VSMCs (Wells, 1998) whereas elasticity is a function of the ECM proteins, elastin and collagen (Roach & Burton, 1957). Deformable elastic fibres bear circumferential tension at low distending pressures affording generous responses in dimension. Stiffening of the arterial wall with rising pressure is ascribed to engagement of the inextensible collagen fibres as elastic fibres become taut. Given that this transfer of intravascular load from elastin to collagen occurs over the physiological pressure range, the relative reduction in elastic fibre content evident in fetuses subjected to

hypoxia in the present study may result in collagen recruitment at lower distending pressure. The stiffening effect of an increased collagen-to-elastin ratio is palpable in the pathological deficits in elastogenesis characterized by multiple genetic diseases (Salaymeh & Banerjee, 2001; Nemes *et al*. 2008), and has been demonstrated experimentally by ex-vivo protein digestion (Fonck *et al*. 2007; Roach & Burton, 1957).

2.4.2 Developmental Timing of Hypoxic Insult

The composition and architecture of arterial wall components requisite for proper functioning of the mature vascular tree evolve from precise time and site dependent gene expression and cellular differentiation during fetal and neonatal life. Since the time course and regulation of cardiovascular development in the sheep are analogous to that in the human (Berry *et al*. 1972; Wells *et al*. 1998), our findings hold significance in the context of clinical fetal growth restriction. In both species, VSMCs exhibiting high rates of proliferation and the ability to synthesize ECM molecules predominate in early gestation and gradually switch to mature contractile cells over the last third of pregnancy (Hutana *et al*. 2007). The current study failed to find an effect of intrauterine hypoxia on media cellularity or on VSMC proliferation and maturation, possibly because the majority of phenotypic modulation of the synthetic type cell had occurred by this later stage in gestation. This suggests that the relative increase in α actin, which is the most abundant protein in differentiated contractile cells (Fatigati & Murphy, 1984), was associated with VSMC hypertrophy. In this case, increased α actin and collagen synthesis may be due to an upregulation in the activity of the residual population of synthetic-type VSMCs rather than to proliferation of these cells. It is possible that VSMC proliferation leading to an increase in total cell content concomitant with media hypertrophy occurred earlier in the insult, and was not detected by PCNA staining at day 15 of hypoxia. In both human and sheep, collagen synthesis by VSMCs peaks early in gestation and plateaus near term, whereas elastin synthesis starts to accelerate in late gestation and reaches a maximum shortly after birth (Bendeck & Langille, 1991; Berry *et al*. 1972). Our fetal sheep were hypoxic between 116 and 132 days of gestation, coinciding with this period of rapid elastin accumulation, wherein a considerable increase in the elastin-to-collagen ratio takes place. There is a brief continuation of arterial remodeling after birth during which occurs rapid deposition of ECM macromolecules, and their

cross-linking as the final step in fibre formation (Bendeck *et al*. 1991; Wells *et al*. 1999). Thereafter, rates of ECM protein turnover decline substantially. In fact, the half life of highly resilient mature elastin is 40 years (Shapiro *et al*. 1991) and its post-development biosynthesis is negligible under normal conditions (Mariencheck *et al*. 1995). Thus if the reduction in relative elastic fibre content measured in the present study is due to upregulated protein degradation, the functional consequences may be permanent.

2.4.3 Mediators of Hypoxic-Induced Changes within the Aortic Media

Formation of the vasculature over the course of gestation is orchestrated by the varying impact of endocrine, autocrine, paracrine and mechanical stimuli. Cortisol is a potent regulator of elastin (Yee *et* al. 1996) and collagen synthesis, (Leitman *et al*. 1984) that mediates an acceleration in ECM accumulation from 120 days of gestation to term in the ovine fetus (Bendeck *et al*. 1991). The current study failed to find an effect of chronic hypoxia on baseline circulating cortisol levels over the 15-day embolization period. An increase in cortisol is a known response to acute hypoxia (Green *et al*. 2000, Thompson *et al*., 2011), however, during chronic hypoxia this response has been previously shown to be transient (Gagnon *et al*. 1994) or absent (Kerr *et al*. 1992). Hemodynamic conditions within the growing vascular tree parallel modifications in composition and geometry of the arterial wall and become highly influential in the rapid deposition of elastin that occurs in the late gestation and early postnatal periods (Bendeck *et al*. 1994). Embolization in the present study did not result in fetal hypertension in either Moderate or Severe groups, as reported by previous studies (Louey *et al*. 2007). Yet, human fetuses growth restricted by placental insufficiency have been shown to exhibit abnormal hemodynamics characterized by reduced blood flow velocity in the descending aorta and increased blood flow velocity in the carotid artery, as a result of increased placental vascular resistance and redistribution of cardiac output (Mori *et al*. 1993). Since blood flow stimulates the site-dependent upregulation of elastin accumulation during the critical perinatal induction period of elastogenesis (Bendeck *et al*. 1994), diminished blood flow through the aorta must be considered a potential cause of the hypoxia-related reduction in relative elastic fibre content found in the present study. Since tropoelastin mRNA levels were not affected by chronic

hypoxia, post-transcriptional or post-translational mechanisms involved in the construction of a functional elastin fibre may be altered.

Progressive organization of the ECM scaffold that permits enduring changes in vessel structure and size during fetal development, is mediated by a family of proteolytic enzymes, matrix metalloproteinases (MMPs). The elastinolytic and collagenolytic gelatinase, MMP-2, has been identified as a key regulator of postnatal pathological remodeling of the heart and blood vessels leading to fibrosis and cardiovascular dysfunction (Polyakova *et al*. 2004). The activities of MMP-2 include degradation of elastin, fibronectin, and non-fibrillar collagen particularly the basement membrane collagen IV. Activation of MMP-2 is accomplished by proteolysis by the stromelysin, membrane-type MMP (MTI-MMP) which is inserted into the cell membrane as a fully activated endopeptide (Knauper & Murphy, 1998). Previously, the activity of MMP-2 has been correlated with the expression of MTI-MMP (Zahradka et al. 2004). Our data show elevated MTI-MMP mRNA levels in severely hypoxic fetuses and a similar trend with respect to MMP-2, as well as a significant correlation between MMP-2 mRNA levels and fetal arterial oxygen saturation, suggesting that MMP-2 played a role in the aberrant aortic remodeling observed in these animals. Our data parallels a study by He *et* al. 2007, that reports increases in mRNA levels of MMP-2 and MTI-MMP along with increases in pro-MMP-2 protein levels and activity in response to chronic hypoxia in adult rats. Also pertinent to our findings, increased circulating concentrations of MMP-2 and MMP-9 as well as reduced relative plasma levels of the tissue inhibitiors of MMPs (TIMPs), have been reported in growth restricted infants (Sesso & Franco, 2010).

In addition to the regulatory effects of hypoxia and oxidative stress on the transcription and activity of MMP-2, induction occurs in response to growth factors such as TGF-β. TGF-β_1 regulates multiple cellular activities and has been implicated in control of growth and differentiation of various organs during development, including blood vessels (Saltis & Bobik A, 1995; Yee *et al*. 1996). In the adult, TGF-β_1 is recognized as an important mediator of compensatory structural remodeling of the myocardium and arteries in response to mechanical overload, (Li *et* al. 1998) hypoxia (Chen *et al*. 2006) and oxidative stress (Zhao *et al*. 2008). We report increased mRNA levels of TGF-β_1 in severely hypoxic fetuses. Relevant to our morphological and PCR data, the effects of TGF-β_1 include direct stimulation of collagen I and

III transcription (Ross & Tranquillo, 2003), upregulation of α actin expression (Owens *et al*. 1988), and induction of MMP-2 transcription and activity (Ross & Tranquillo, 2003).

2.4.4 Mediators of Hypoxic-Induced Aortic Intima Hyperplasia

Both MMP-2 and TGF-β_1 are known to be involved in the formation of a fibrous intima that is the initiating event in the atherogenic process (Stary *et al*. 1992), and was observed in our severely hypoxic fetuses. Elastin derived peptides released upon proteolytic activity of MMP-2 directly contribute to switching of VSMCs into the migratory, proliferative and secretory phenotype (Mochizuki *et al*. 2001). Degradation of the subendothelial basement membrane by MMP-2 compromises endothelial barrier function (Rosenberg *et al*. 1998) and allows VSMC migration to the intima (Bendeck & *Zempo et al.* 1994) where they proliferate and secrete ECM proteins under stimulation by growth factors including TGF-β, thereby leading to intima thickening. The role of TGF-β_1 in neointima formation has been demonstrated by reduction in the size of intimal lesions after balloon injury with inhibition of TGF-β_1 (Wolf *et al*. 1994) . Activated endothelial cells which produce growth-promoting TGF-β_1, promote expression of cell adhesion molecules (CAMs) that facilitate the interaction of platelets and leukocytes with the vascular endothelium. E-selectin, which we reported to be increased in severely hypoxic aortae, is among the CAMs expressed during the initial phase of intima thickening in response to vascular injury (Barron *et al*. 1997). Changes in mRNA levels of procollagen, TGF-β_1, MMP-2 and MTI-MMP were absent from the superior mesenteric artery, implying a differential response to hypoxia depending on the type and location of the arterial segment. The SMA is an atherosclerotic-resistant artery, not prone to intima hyperplasia as is the aorta, and hence it's cellular response to hypoxia or injury may not include endothelial activation by MMP-2.

2.5 CONCLUSIONS

Fetuses subjected to hypoxia exhibited structural abnormalities resembling those present in adults destined to develop CVD, that were at least in part, attributable to oxygen-regulated growth factors and enzymes. It should be noted, that the duration of embolization equilibrates to 10% of ovine gestation yet hypoxia is endured throughout the second half of gestation during

placental insufficiency, thus arterial responses measured in the present study may be mild or the phenotypic outcome may differ in comparison to the clinical situation. A defect in the original architecture of the aortic wall in growth restricted infants may alter its biological responses and mechanical behaviour, thereby accelerating the progression to hypertension and CVD in postnatal life.

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CHAPTER 3

Central Stiffening in Adulthood Linked to Aberrant Aortic Remodeling Under Suboptimal Intrauterine Conditions

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3.1 INTRODUCTION

Seminal epidemiological studies over the past two decades have established a link between the fetal experience and long term health. Suboptimal conditions in the womb commonly arise from an interference in substrate delivery and availability that prevents the fetus from sustaining its growth trajectory. Such is the case of placental insufficiency, whereby abnormalities in the placental exchange surface impede maternal-fetal blood flow and frequently lead to chronic fetal hypoxemia (Krebs *et al*., 1996; Mayhew *et al*., 2004). This antenatal condition accounts for 60% of neonates who are identified as intrauterine growth restricted (IUGR) in the developed world (Ghidini, 1996). Morphometric indices of growth impairment are predictive of a number of chronic diseases in adulthood, among them Cardiovascular disease (CVD) (Barker *et* al., 1989; Curhan *et al*., 1996; Krochik *et al*., 2010). Despite widespread knowledge of this pre-birth risk factor, the developmental disturbance that renders the low birth weight fetus vulnerable to later CVD is currently ill-defined.

In pregnancies complicated by placental insufficiency, fetal compromise apparent as slowed and disproportionate growth manifests in the second half of gestation, when rapid growth and maturation of organs are heavily dependent on transported substrates. This coincides with an important phase of arterial remodeling, during which occurs time and site-dependent deposition of extracellular matrix (ECM) proteins and transition of vascular smooth muscle cells (VSMC) from synthetic to contractile-type cells. These processes establish the mechanical and functional properties required for hemodynamic homeostasis in extra-uterine life (Berry *et al*., 1972; Wells *et al*., 1999). Interesting, the accumulation of elastin which predominates in large arteries, is limited to a brief window that spans late gestation and the early neonatal period (Bendeck *et al*., 1994; Berry *et al* ., 1972). The resultant high elastin content of the proximal circulation imparts these vessels with a high degree of compliance that is critical for long-term cardiovascular health, as it dampens and synchronizes the pressure waves generated by cardiac ejection. In fact, central arterial stiffening due to altered composition of the ECM is a strong and independent predictor of CVD (Abhayaratna *et al*., 2008; Blacher and Safar, 2005). Given that elastin content is fixed upon conclusion of developmental remodeling (Mariencheck *et al*., 1995; Shapiro *et al*., 1991) and that fetal hypoxemia alters known regulators of ECM deposition (Durmowicz *et al*.,

1994; van Vlimmeren *et al*., 2010), permanent central arterial stiffening may arise from aberrant arterial formation *in utero*, in fetuses growing under placental insufficiency.

Perturbed arterial development and subsequent wall stiffening as a mechanism for intrauterine programming of cardiovascular sequellae, was explored in the present study. Growth of the fetal guinea pig was impaired by means of an established model of placental insufficiency, whereby placental blood flow is reduced throughout the second half of gestation by uterine artery ligation (UAL) (Jansson *et al*., 1986; Jones and Parer, 1983). The effects of growth restriction on aortic development in the near term fetus was assessed by measurement of wall dimensions, ECM protein composition, VSMC content and phenotype. Adult growth restricted offspring were studied in order to determine the permanence of any structural abnormalities of the aorta established *in utero* and its elastic properties via measurement of the length-tension relationship *ex vivo.*

3.2 METHODS

3.2.1 Surgical Procedures and Experimental Design

All surgical and experimental protocols were approved by The University of Western Ontario Animal Use Subcommittee. Chronic placental insufficiency was induced in time-mated guinea pigs by UAL. This technique is commonly used to impair intrauterine growth in rodents (Alexander, 2003; Briscoe *et al*., 2004), as it depletes uterine capacity leading to discordant fetal growth within litters and variable fetal growth restriction (Detmer and Carter, 1992; Jansson *et al*., 1986). Pregnant guinea pigs at 28-30 days of gestation (term ~ 67 days) were induced in an anesthetic chamber (4-5% Isoflurane with 2L/min O_2; followed by 2.5-3% Isoflurane with 1L/min O_2 for maintenance). The volume of the anesthetic chamber was 3 L. Immediately after induction, a subcutaneous injection of Robinul (Glycopyrrolate, 0.01 mg/kg, Sandoz Can Inc., Montreal QC) was administered. A midline incision was made below the umbilicus in order to retrieve the mesometrium associated with one horn of the uterus and subsequently UAL was performed at the base of the arterial arcade. In order to maximize fetal survival, the uterine horn with the smallest number of embryos was ligated. The ligature remained in place for the duration of the experiment and fetuses from the un-operated horn served as control. A subcutaneous

injection of Temgesic (Buprenorphine, 0.025mg/kg, Schering-Plough Co., Kenilworth NJ) was administered immediately following surgery.

At 63-66 days UAL pregnant guinea pigs were sedated with an intramuscular injection of Versed (Midazolam, 5mg/kg, Sandoz Canada Inc., Boucherville QC) and after 10 minutes an intramuscular injection of Vetalar (Ketamine, 50mg/kg, Bioniche Animal Health Canada Inc., Belleville ON) together with Rompun (Zylazine, 3mg/kg, Bayer Inc., Toronto ON) were administered for anaesthesia. Subsequently, Robinul was injected subcutaneously (0.025mg/kg) and Xylocaine 2% (Lidocaine, AstraZeneca Can Inc., Mississauga ON) was injected along the incision line previously made during surgery. An adjacent incision was then made below the umbilicus, the fetuses removed and treated with Vetalar as above. After caesarean section, the mother was sacrificed with Euthanyl Forte (Pentobarbital Sodium, Bimeda-MTZ Animal Health Inc., Cambridge ON) by intracardiac injection. The placement of the ligature was confirmed at autopsy. Body weight and the brain:liver ratio of each fetus were measured.

An additional 3 ligated pregnant guinea pigs were allowed to deliver spontaneously at term, at which time the pups were weighed and then returned to their mothers. At 20 days, guinea pig offspring were weaned, separated by sex, placed on standard chow and housed in group cages in a temperature (18°C) and humidity (30%) controlled environment, with a 12:12 hour light-dark cycle. From the time of birth, guinea pig offspring were weighed weekly. At 13-15 months of age which corresponds to mid-adulthood (Kind *et al*., 2003), offspring were sacrificed by intracardiac injection of Euthanyl Forte (Pentobarbital Sodium, Bimeda-MTZ Animal Health Inc., Cambridge ON).

3.2.2 Fetal and Adult Groupings

In order to preserve the integrity of fetal tissue by rapid organ collection during caesarean section, only the most medial and lateral fetus were studied with horns of more than 3 fetuses. Groupings of late gestation fetuses were based on morphometric indices of fetal growth. Fetuses were defined as appropriate for gestational age (AGA) if their body weights were within the 25th and 75th percentile of all fetuses, small for gestational age (SGA) if their body weights were below the 25th percentile and brain:liver ratios were below the group median, and intrauterine growth restricted (IUGR) if their body weights were below the 25th percentile and brain:liver

ratios were above the group median. The group median included all fetuses of each litter. The range of fetal weights used in the present study for classification into appropriate for AGA, SGA and IUGR, are comparable to those used by other studies (Briscoe *et al*., 2004; Lafeber *et al*., 1984). The group median of the brain:liver ratio including all fetuses was 0.85, a ratio above this value has been previously defined as IUGR in guinea pigs (Carter, 1993). For groupings of offspring, the smallest pups in the ligated uterine horn were considered growth impaired and the remaining pups were considered AGA.

3.2.3 Staining for Collagen and Elastin

Aortae of fetal guinea pigs were perfusion fixed *in situ* with 4% paraformaldehyde at physiological pressure via the left ventricle and subsequently a segment of approximately 1.5 cm was excised immediately distal to the aortic arch for histological analyses. Aortae of adult offspring collected from the same anatomical location were placed in 4% paraformaldehyde for fixation. Fixed aortae from fetal and adult guinea pigs were cut into cross-sections, embedded in paraffin and cut in 5 μm cross-sections that were baked onto positively charged glass plates by heating in a 50 ºC oven for 2 days. After deparaffinization, aortic cross-sections were rehydrated by passage through a decreasing ethanol series. Collagen content was measured in cross-sections stained with 1% Sirius Red F3BA (Sigma-Aldrich Canada Ltd., Oakville ON) in a saturated aqueous solution of picric acid, for 1 hour. Additional aortic sections were stained 30 minutes in 0.2 % Orcein (Sigma-Aldrich Canada Ltd., Oakville ON) for identification of elastic fibres. Stained cross-sections were captured on a microscope (Leica DM RB) at 423x magnification. Duplicates of 2-3 cross-sections per animal and 5-6 areas per cross-section were used for analysis. Animal identity corresponding to each slide was blinded to the operator for analyses. Wall thickness was measured as the distance between the internal and external elastic laminae. For collagen quantification the tunica media was selected, whereas elastin content measurement included both the tunica media and the internal elastic lamina. The area positive for protein (elastin or collagen) was identified by color thresholding using image analysis software (Image Pro 6.0, MediaCybernetics, Bethesda, MD) and expressed relative to the sum of area non-stained. Total protein content (elastin or collagen) was calculated by multiplying the average wall thickness for each vessel by the percent area stained, as performed by previous studies

(Kobs *et al*., 2005). In orcein stained cross-sections the individual elastic laminae from internal to external elastic lamina were counted.

3.2.4 Immunofluorescent Staining for α-actin and MHC-B

Additional deparaffinized and rehydrated aortic cross-sections were incubated at room temperature for 10 min in Background Sniper (Biocare Medical LLC, Brampton, ON) for blockage of nonspecific binding, followed by incubation with primary antibodies diluted in Universal Antibody Diluting Solution (Dako Canada Inc., Burlington ON) in a humidified chamber at 4ºC overnight: 1:4000 dilutions of monoclonal mouse immunoglobulin G α-actin antibody (Boehringer Ingelheim Ltd., Mannheim Germany) along with 1:2000 dilutions of polyclonal rabbit nonmuscle myosin heavy chain II-B (MHC-B) (Covance Inc., Emeryville, CA). After washing in phosphate-buffered saline, slides were incubated in 1:400 dilutions of Molecular Probes secondary antibodies Alexa Fluor® 568 and 405 (Invitrogen Life Technologies Co., Burlington, ON) at room temp for 30 min in a black covered humidity chamber. After washing, slides were counterstained for 15 min using Sytox Green (Invitrogen Life Technologies Co., Burlington ON). Replacement of the primary antibody with PBS or IgG was used as a negative control. Fluorescence VectaShield mounting medium (Vector Laboratories Burlington, ON) was used for mounting. Slides were stained in duplicate and simultaneously to minimize variation in staining intensity and all analyses were performed blinded to the operator. All antibodies were tested for specificity by Western Blot. Sections were imaged on a microscope (Zeiss) and captured at 40x objective using a camera and software for image capture and analysis (Axiovision 4.0, Carl Zeiss Microimaging LLC, Thornwood, NY). For each cross-section, 10-12 fields were analyzed. The area positive for staining was identified by color thresholding using image analysis software (Image Pro 6.0, MediaCybernetics, Bethesda, MD). The sum of area stained for α-actin and MHC-B and the number of cells stained with Sytox Green were expressed as a percentage of an area of constant dimension within the media.

3.2.5 Length-Tension Relationship in Adult Aortae

Immediately after sacrifice, an aortic segment excised just distal to the segment used for histology was immediately placed in ice-cold Krebs solution (118 mM NaCl; 25 mM $NaHCO_3$; 11.1 mM D-glucose; 4.71 mM KCl; 2.56 mM $CaCl_2 \cdot 2H_2O$; 1.13 mM $NaH_2PO_4 \cdot 2H_2O$; 1.12 mM $MgCl_2 \cdot 6H_2O$; 0.114 mM Ascorbic acid; 0.0297 mM disodium EDTA) for *in vitro* measurement of compliance, as previously described for rats (Kingwell *et al*., 1997). Three aortic rings from each animal were mounted isometrically onto 2 parallel stainless steel wires, one connected to a micrometer for fine distance adjustments and the other connected to a force transducer (FT03; Grass Instruments) attached to a digital display (P11T; Grass Instruments). An initial pre-stretch of each vessel was performed by stretching the rings from the zero tension position to a maximal stretch of 3mm in 500 μm increments, at 2 min intervals. Following the pre-conditioning, the aortic rings were allowed to equilibrate at zero tension for 15 min. A length-tension curve was then generated by increasing the distance by 500 μm at 2 min intervals from the zero tension position until the vessel snapped or no further response was observed. The length-tension relationship was then fitted by a linear equation, the slope of which relates directly to stiffness.

3.2.6 Statistical Procedures

All statistical procedures were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Between group differences were assessed using one-way ANOVA with Bonferroni *post hoc* tests, or unpaired t-test. Differences between length-tension curves were assessed using a two-way ANOVA. All data are presented as mean ± SEM and significance was set at $p < .05$.

3.3 RESULTS

3.3.1 UAL Leads to Fetal Growth Impairment and Postnatal Catch-Up Growth

Twenty six late gestation fetuses were studied (AGA: n = 12; SGA: n = 8; IUGR: n = 6). Body weights and brain:liver ratios of fetuses are shown in Figure 3.1. All fetuses but one in the

IUGR group were derived from the ligated horn. Twelve guinea pig offspring born from 3 surgically prepared pregnant sows were studied. The birth weights of guinea pig offspring are shown in Figure 3.2. The smallest pups had birth weights approximately 20 % less than the AGA pups and comparable to the late gestation SGA fetuses and were thus defined as SGA (n = 5). The remaining pups of the litter were AGA (n = 7). During the postnatal period, SGA offspring exhibited catch-up growth that occurred by ~ 140 days (Figure 3.2). In later adulthood, at the time of sacrifice (14 months), the body weights of AGA offspring were not significantly different from that of SGA offspring (1.0 ± 0.1 vs. 1.0 ± 0.1 kg).

3.3.2 Abnormal Aortic Structure Established *In Utero* is Magnified in Adulthood.

The relative area positive for elastic fibre staining and the number of elastic lamellae adjusted for media thickness in aortae of late gestation fetuses and adult offspring are shown in Figure 3.3. In late gestation, relative elastic fibre staining in the aorta was reduced by 10 % and 14 % in SGA and IUGR fetuses, respectively, compared to AGA fetuses ($p = 0.14$). The total number of elastic laminae in late gestation fetuses was 14.6 ± 0.8 for the AGA group; 13.4 ± 0.4 for the SGA group and 13.2 ± 0.5 for the IUGR group. The total number of elastic laminae relative to media thickness was similar between fetal groups. Media thickness was 69.0 ± 0.1 μm in AGA fetuses; 58.5 ± 3.7 μm in SGA fetuses and 53.9 ± 3.3μm in IUGR fetuses. In adulthood, relative elastic fibre staining of the aorta in SGA offspring was 51 % lower than that of AGA offspring ($p < .01$). Relative to AGA, SGA adults had a smaller total number of elastic lamellae (14.5 ± 0.9 in AGA vs. 12.4 ± 0.7 in SGA). The ratio of elastic laminae-to-media thickness was 25 % lower in SGA compared to AGA adult offspring ($p < .01$), whereas media thickness and the ratio of media thickness-to-body weight were similar between groups. Permanence in the total number of elastic lamellae between late gestation and adulthood was demonstrated in that the total number of elastic lamellae was similar in normally grown fetuses and adults: 14.6 ± 0.8 in AGA late gestation fetuses and 14.5 ± 0.9 in AGA adult offspring. In both SGA and IUGR late gestation fetuses, the relative area positive for collagen staining was increased by 100 % relative to that of AGA fetuses (AGA: 0.69 ± 0.14; SGA: 1.46 ± 0.40; IUGR: 1.38 ± 0.29, $p = .06$). Compared to AGA, total collagen content was increased by 29% and 18% in SGA and IUGR fetuses respectively (AGA: 18.10 ± 2.49; SGA: 23.42 ± 4.65; IUGR: 21.28 ± 2.49).

Figure 3.1 Body Weight and Brain:Liver Ratio of Late Gestation Fetuses

Late gestation fetuses were grouped according to body weight percentiles and the brain:liver ratio which is an accurate indicator of asymmetric intrauterine growth restriction (IUGR). Average body weight of small-for-gestational-age (SGA) fetuses (n = 8) was 25 % lower than that for appropriate-for-gestational-age (AGA) fetuses (n = 12) (A). IUGR fetuses (n = 6) had body weights 40 % lower than AGA fetuses and 20 % lower than SGA fetuses (A). The brain:liver ratio was similar between AGA and SGA fetuses, and 38 % lower in AGA vs. IUGR fetuses.

* $p < .05$ AGA vs. SGA and IUGR

† $P < .05$ SGA vs. IUGR

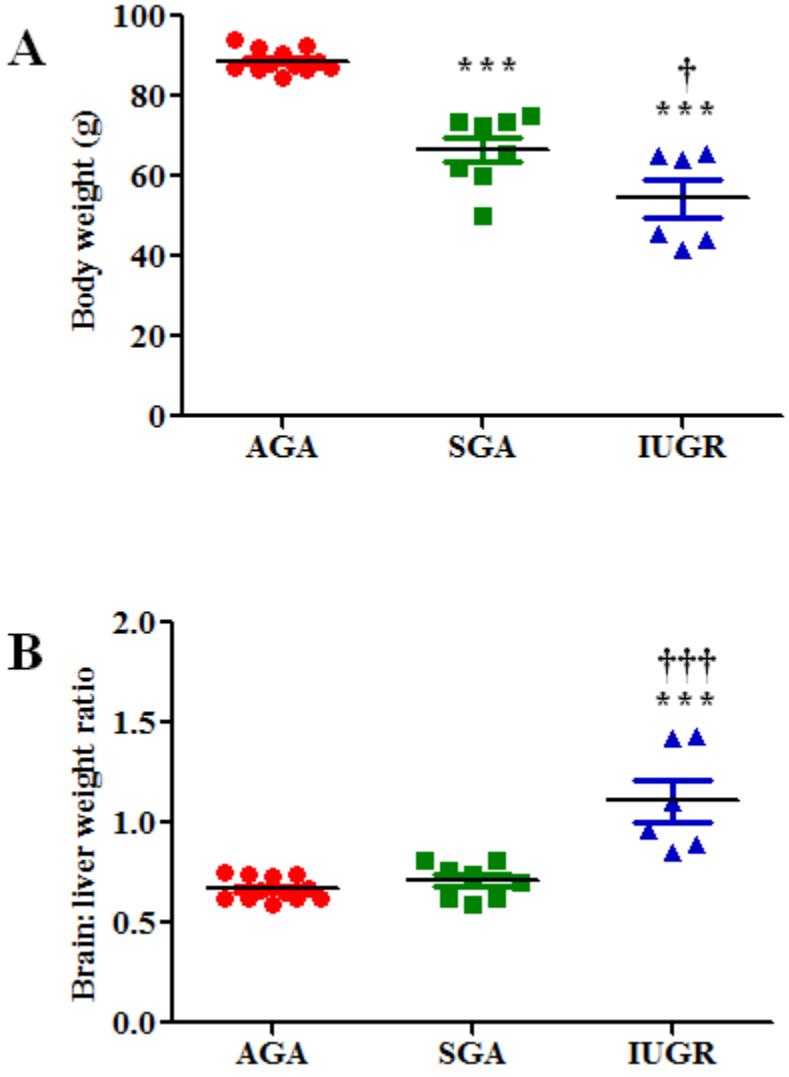
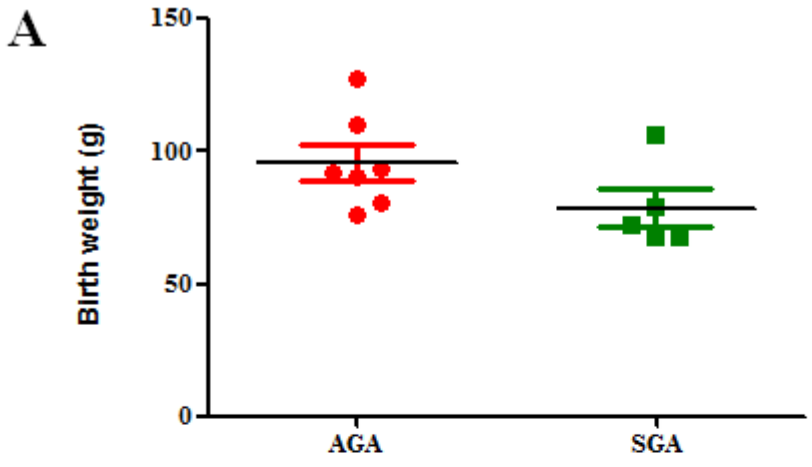

A
Body weight (g)
100
80
60
40
20
0
AGA
SGA
IUGR
***
†
***
B
Brain: liver weight ratio
2.0
1.5
1.0
0.5
0.0
AGA
SGA
IUGR
†††
***

Figure 3.2 Birth Weight and Catch-up Growth in Offspring

Guinea pig offspring were grouped at the time of birth into an appropriate-for-gestational-age (AGA) (n = 7) and a small-for-gestational-age (SGA) (n = 5) group, on the basis of birth weight. Compared to AGA adults, average birth weight of SGA adults was 18 % lower (A). In postnatal life SGA offspring exhibited catch up growth that occurred by ~ 140 days (B).


A
150
100
50
0
Birth weight (g)
AGA
SGA

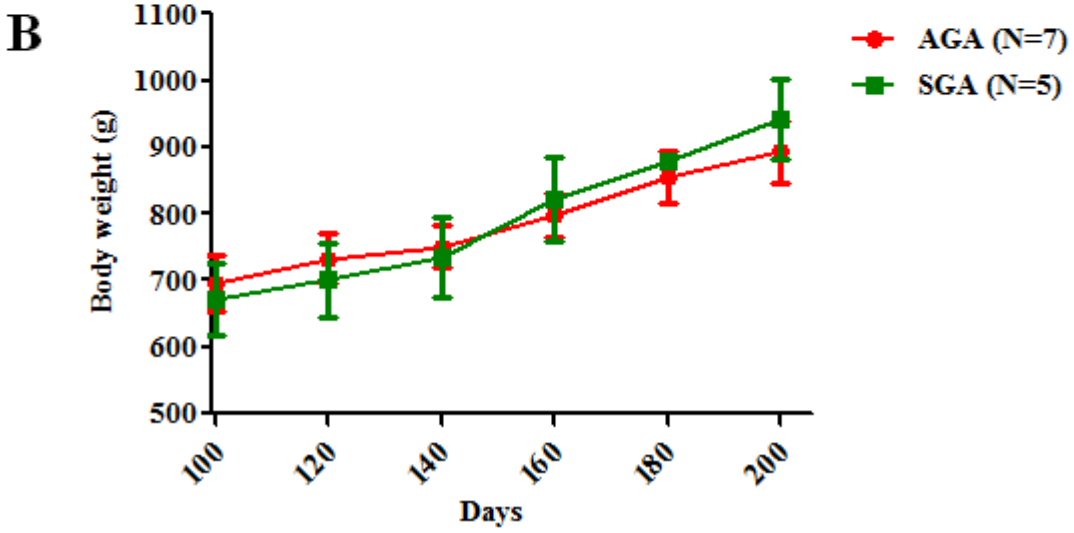
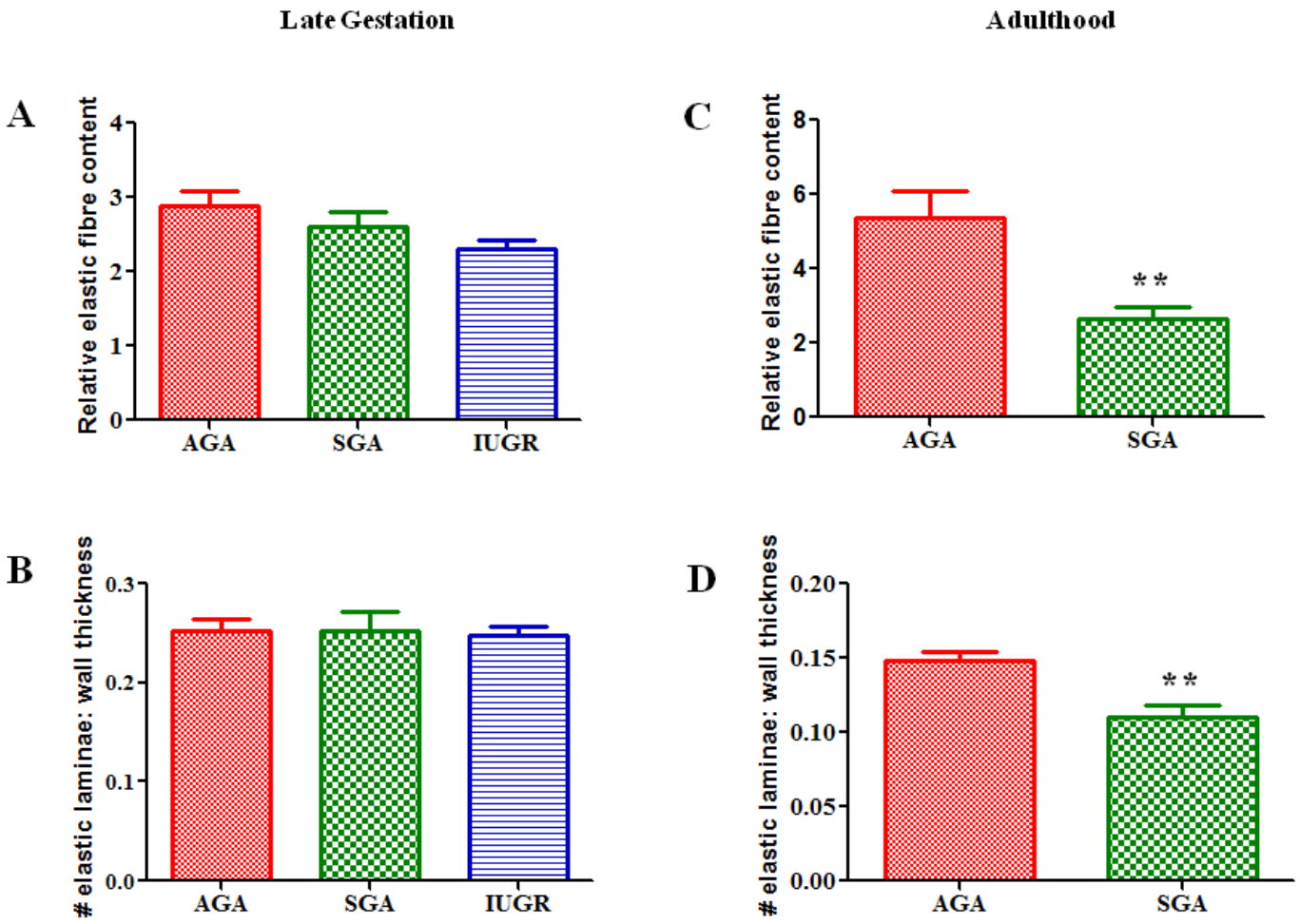

B
1100
1000
900
800
700
600
500
Body weight (g)
100
120
140
160
180
200
Days
AGA (N=7)
SGA (N=5)

Figure 3.3 Elastic Fibre Content in Fetuses and Offspring

A trend towards a stepwise reduction in the relative area stained positive for elastic fibres within the aortic media, was observed across appropriate-for-gestational-age (AGA), small-for-gestational-age (SGA) and intrauterine growth restricted (IUGR) late gestation fetuses (A). The number of individual circumferential elastic laminae within the aortic media remained normalized to wall thickness in SGA and IUGR late gestation fetuses (B). In adulthood, the relative area stained positive for elastic fibres was markedly lower in SGA compared to AGA offspring (C). As well, the ratio of the number of elastic laminae-to-media thickness was reduced in SGA relative to AGA offspring (D).

** $p < .01$ AGA vs. SGA


Late Gestation
Adulthood
A
B
C
D
Relative elastic fibre content
elastic laminae: wall thickness
AGA
SGA
IUGR
**
**

In adulthood, no differences were observed for relative collagen content, whereas total collagen content was increased by 41% in SGA relative to AGA offspring ($p = 0.1$).

There were no differences in the percent area stained for α-actin in the aortaes of late gestation fetuses (AGA: 46.3 ± 4.2; SGA: 46.2 ± 6.9; IUGR: 48.9 ± 4.1). The percent area stained for MHC-B was increased 6-fold in the SGA group compared to AGA fetuses, but no significant differences were found between AGA and IUGR fetuses (Figure 3.4). The percent area stained for α-actin in cross-sections of aortae from adult offspring, are shown in Figure 3.5. In aortae of adult SGA offspring, the % area stained for α-actin was 33% higher and the total α-actin content was 56% higher compared to the AGA group ($p < .05$). The number of cells per area within the media was 2.0 ± 0.0 for the AGA adults and 1.6 ± 0.0 for the SGA group. Figure 3.4 shows a 3 fold increase in the % area stained for MHC-B was observed in SGA relative to AGA offspring ($p < .05$).

3.3.4 Reduced Aortic Compliance in SGA Offspring

The length-tension curve was shifted to the left in SGA adult offspring compared to control adult offspring (Figure 3.6).

3.5 DISCUSSION

This study is the first to demonstrate experimentally, a link between aberrant arterial development in the fetal guinea pig under substrate deprivation and central arterial stiffening in adulthood. We showed adult guinea pigs born of low birth weight to develop increased aortic stiffness that is a consequence of altered media composition likely originating *in utero*. The marked reduction in relative elastic fibre content observed in SGA adult offspring was present to a lesser degree in SGA and IUGR near term fetuses. This apparent postnatal magnification of the subtle offset in balance between the elastic and stiff wall components was due to media hypertrophy without concomitant deposition of elastin proteins. The high expression of α-actin in adult SGA offspring suggests that VSMC proliferation or hypertrophy occurred within the aortic media. Low birth weight offspring also displayed increased collagen content along with an abundance of embryonic-type VSMCs that are capable of synthesizing ECM proteins. The

Figure 3.4 MHC-B in SGA Fetuses and Offspring

The % area stained for MHC-B, a marker for synthetic-type VSMCs, was increased 6-fold in the small-for-gestational-age (SGA) group relative to appropriate-for-gestational-age (AGA) fetuses (A). Aortae from adult SGA offspring also exhibited an increase in the % area stained for MHC-B (B). Shown are fluorescent staining of MHC-B proteins (red) and nuclei (green) within the aortic media of AGA (C) and SGA (D) adult offspring.

* $p < .05$ AGA vs. SGA

*** $p < .001$ AGA vs. SGA

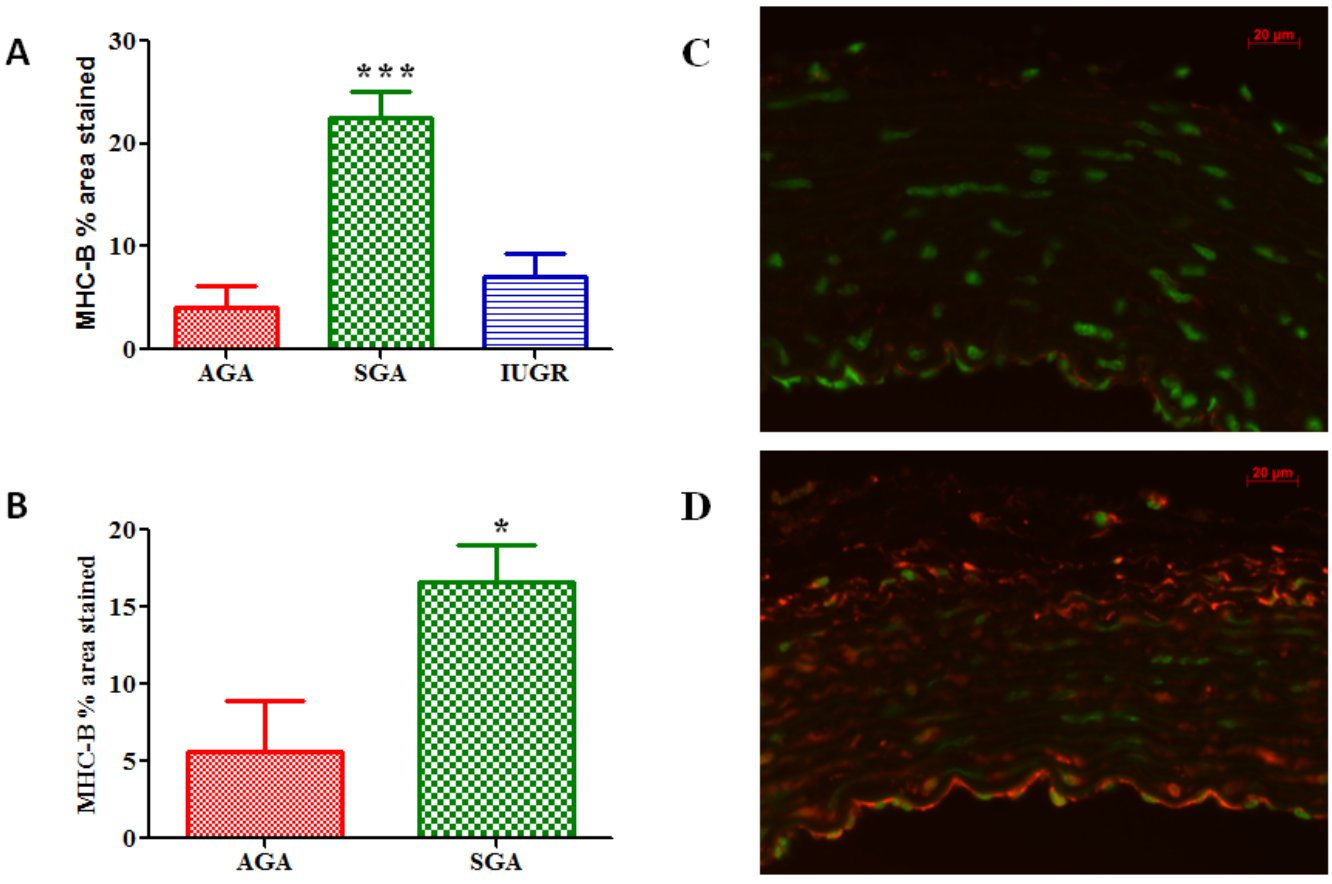

A
MHC-B % area stained
30
20
10
0
***
AGA
SGA
IUGR
B
MHC-B % area stained
20
15
10
5
0
*
AGA
SGA
C
20 µm
D
20 µm

Figure 3.5 α-actin in SGA offspring

The % area stained for the α-actin protein within the aortic media was increased in small-for-gestational-age (SGA) compared to appropriate-for-gestational-age (AGA) adult offspring.

* $p < .05$ AGA vs. SGA

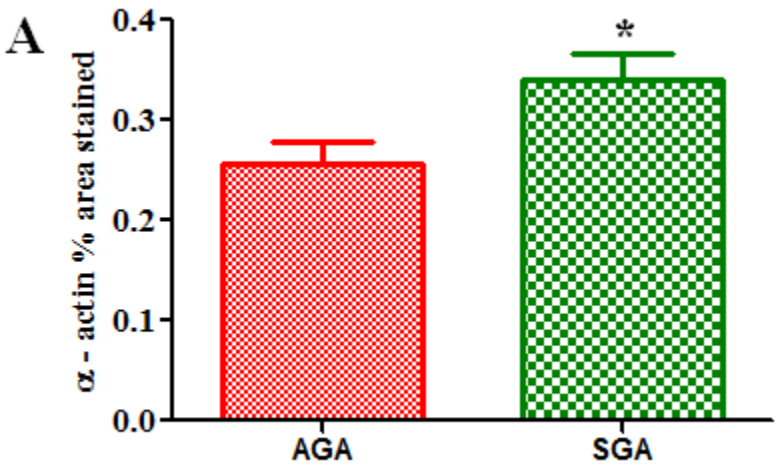
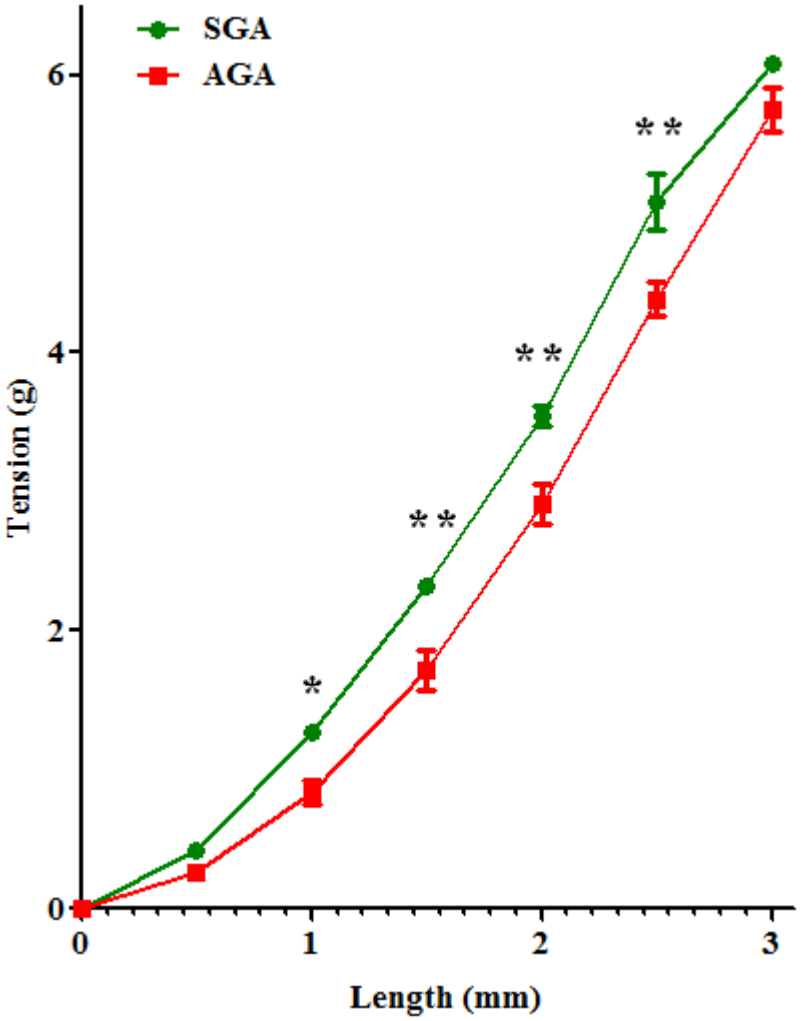

A
0.4
0.3
0.2
0.1
0.0
α - actin % area stained
*
AGA
SGA

Figure 3.6 Length-Tension Curve in SGA Offspring

The length-tension curve was shifted to the left in aortae from SGA compared to AGA adult offspring.

* $p < .05$ AGA vs. SGA

*** $p < .001$ AGA vs. SGA


SGA
AGA
Tension (g)
Length (mm)
0
2
4
6
0
1
2
3
*
**
**
**

striking increase in staining for the marker of these synthetic VSMCs was also present in SGA fetuses, suggesting that a delayed VSMC maturation *in utero* leads to permanent phenotypic characteristics in the offspring. Our measurement of reduced aortic compliance in low birth weight offspring by *ex-vivo* generation of length tension curves, substantiates the positive correlations between birth weight and arterial compliance previously reported in human children, adolescents and adults (Bradley *et al*., 2010; Cheung*et al*., 2004; Mzayek *et al*., 2009; te Velde *et al*., 2004). Further, our data suggest that aortic dysfunction in low birth weight offspring is linked to structural and cellular defects programmed by intrauterine deficiency that persist and are magnified postnatally. Thus, we provide evidence for a mechanism underlying the high risk of hypertension and CVD repeatedly reported in low birth weight human adults (Law *et al*., 1993; Martyn *et al*., 1998; Nilsson *et al*., 1997; Osmond *et al*., 1993).

3.5.1 Enduring Changes in ECM Composition Established *In Utero*

Elastin precursors are synthesized during a brief developmental window, predominantly in proximal arteries, and once deposited as insoluble proteins will endure the lifetime of an individual (Mariencheck *et al*., 1995; Shapiro *et al*., 1991). These proteins comprise 90% of elastic fibres which bear circumferential tension at low distending pressures, affording properties of distension and recoil of the vascular wall (Roach and Burton, 1957). Given that transfer of intravascular load from elastin to collagen occurs over the physiological pressure range, the relative reduction in elastic fibre content evident in low birth weight offspring results in collagen recruitment at lower distending pressure and wall stiffening. The reduced elastic fibre content in adult life may be remnant of changes originating *in utero*. We observed a subtle decrease in the total number of elastic laminae and relative content of elastin fibres in late gestation fetuses that were graded in relation to severity of growth impairment, the latter associated with a reduced thickness of the elastic laminae as the number of elastic laminae remained normalized to wall thickness. A disturbance in elastin deposition is permanent since these proteins are not appreciably synthesized in postnatal life once developmental remodeling is complete (Mariencheck *et al*., 2005). The stability of elastic components was demonstrated in the present study, as the total number of elastic laminae was comparable between AGA fetuses and AGA adults. A fixed ECM ratio established before birth that is slightly deficient in elastic components,

together with accumulation of other wall constituents during normal postnatal arterial growth, likely account for abnormal ECM composition and associated aortic stiffening in later life. The present study did not allow for examination of an adult IUGR group, yet a greater degree of structural changes translating to a further stiffening of the aorta is likely in such offspring. Nevertheless, evident stiffening in the moderately growth impaired adult offspring, speaks to the potency of placental intrauterine insults as a trigger for progression of cardiovascular pathology.

3.5.2 Functional Consequence of Programmed Aortic Structure

Dynamic elasticity of the aorta and its major branches provided by passive mechanical properties of ECM proteins, dampen pressure oscillations generated by ventricular ejection thereby minimizing energetic demands placed on the heart (Abhayaratna *et al*., 2008). A compromise in this buffering function of the proximal circulation leads to progressive hemodynamic disturbance and cardiac malfunction. In fact, noninvasive indices of aortic stiffness are independent and powerful predictors of hypertension and CVD (Abhayaratna *et al*., 2008). Therefore, the present study provides evidence that aortic stiffening underlies vulnerability to CVD in individuals who failed to reach their intrauterine growth trajectories. Aortic stiffening in SGA guinea pig offspring concurred with a phenotype resembling hypertensive remodeling and present in human adults destined to develop CVD, that is VSMC phenotype modulation and hypertophy, increased collagen accumulation and reduced elastin content due to protein degradation and fibre fragmentation (Et-Taouil *et al*., 2003) Our data suggest that premature initiation and acceleration of this adverse remodeling and associated wall stiffening arise from aberrant arterial development under placental insufficiency-induced substrate deprivation.

3.5.3 Enduring Changes in VSMC Phenotype Established *In Utero*

Cellular phenotype was identified in the current study by the presence of MHC-B, a commonly used marker for embryonic VSMCs that are highly proliferative and migratory and capable of synthesizing large amounts of ECM proteins. Over the second half of gestation, these synthetic-type cells undergo a phenotypic switch to mature cells that express a host of proteins required for their contractile function, such as α-actin (Hutanu *et al*., 2007). Differentiation is

reversible, as phenotypic modulation of contractile VSMCs to their synthetic precursors in response to injury and local environmental cues contributes importantly to pathological remodeling in postnatal life (Chen *et al*., 2010; Raines and Ross, 1993). Our data reveal a marked increase in relative MHC-B content in SGA fetuses and adult offspring that was absent from IUGR fetuses. It is possible that interference in phenotypic switching *in utero* resulting in permanent changes in gene expression underlies the high MHC-B content associated with moderate growth restriction observed in both fetal and adult life. Enduring changes in gene expression patterns arising from transitory alterations in the intrauterine milieu are thought to be central in fetal programming of chronic disease (Ozanne and Constancia, 2007). A proposed mechanism for persistence of adaptations in cellular identity over time in an individual and across generations is a change in the epigenotype. Evidence suggests VSMC phenotype to be regulated at the level of chromatin and thus susceptible to epigenetic modifications (McDonald *et al*., 2006; Qiu and Li, 2006). With respect to the discrepancy in cellular response in moderately versus severely growth restricted fetuses observed in the present study, we have previously shown changes in gene expression and protein deposition in the aorta to be dependent on the severity of intrauterine hypoxia, and this dose-response relationship varied with the outcome measured (Thompson *et al*., 2011a). Thus, intracellular signals regulating VSMC differentiation may be differentially affected by the level of hypoxia or oxidative stress reached in the IUGR fetuses and this may account for the maintenance of VSMC maturation despite slowed overall growth in this group. Currently, comparisons between symmetrically and asymmetrically growth restricted fetuses in terms of developmental outcomes have not been investigated.

3.6 CONCLUSIONS

Placental insufficiency in the pregnant guinea pig results in a reduction in relative elastic fibre content of the fetal aorta that is related to the severity of growth restriction; a finding that agrees with recent studies in the hypoxic sheep fetus (Thompson *et al*., 2011b). The current study is novel in that it examines both the immediate and long-term effects of fetal growth impairment on aortic structure and function. In so doing, the subtle reduction in elastic fibre

content exhibited by the growth restricted fetus was shown to be amplified later in adulthood and this was associated with a decreased compliance of the aorta. Aortae of growth impaired offspring also deviated from those of normal birth weight offspring with respect to the content of collagen and VSMCs as well as VSMC phenotype. Our data suggest that altered VSMC phenotype in the SGA offspring may derive directly from an interference in VSMC maturation *in utero*.

This study provides evidence that fetal growth impairment is associated with changes in media composition and cellular properties of the aorta that persist postnatally and lead to reduced compliance in adulthood. Thus, we have identified a developmental disturbance that may be key in the programming of cardiovascular pathology that is known to occur in low birth weight human offspring. Clinical inferences drawn from this model with respect to phenotypic outcomes are validated in that the timing of developmental processes are likely similar between the guinea pig and human, since both are precocious developers. Identification of phenotypes consequent to particular prenatal insults is the first step in testing prenatal and postnatal therapeutic targets for the SGA and IUGR infant. The potential of early and aggressive intervention in offspring of complicated pregnancy has not been realized, since this population has essentially been omitted in current CVD risk estimation, treatment and preventative strategies. Hence, further investigation with use of animal models in the characterization of cardiovascular profiles, is required to address these individuals who are born susceptible.

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CHAPTER 4

The Effect of Intermittent Umbilical Cord Occlusion on Elastin Composition in the Ovine Fetus

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4.1 INTRODUCTION

A nuchal cord occurs when the umbilical cord becomes wrapped around the fetal neck and is detected in 25 percent of human pregnancies at the time of birth (Collins *et al*., 1995; Tantbirojn *et al*., 2009). In late gestation, episodes of acute fetal hypoxemia apparent by abnormal heart rate patterns have been linked to the presence of a nuchal cord and are thought to arise from intermittent umbilical cord compression and consequent reductions in umbilical blood flow (Tantbirojn *et al*., 2009). These recurrent intrauterine insults have the potential to alter fetal development in relation to the frequency and severity of insult. The cardiovascular system may be particularly susceptible to programming effects since the circulatory response to acute hypoxemia involves mediators of its development, yet vascular changes in response to umbilical cord occlusion remain unexplored.

Essential to growth and development of the arterial system is a temporal and spatial adaptation of the vasculature to maturational changes in the physical forces it sustains (Bendeck *et al*., 1994; Bendeck *et al*., 1991). This process involves geometric and compositional remodeling of the arterial wall, achieved by synthesis and deposition of structural proteins into the extracellular matrix by vascular smooth muscle cells (Bendeck *et al*., 1994; Wells *et al*., 1999). The elastin protein is a primary constituent of the matrix which endows the vascular wall with the ability to expand and recoil and is therefore a major determinant of its viscoelastic property (Kelleher *et al*., 2004; Shadwick, 1999). Unlike other arterial wall constituents, elastin is not appreciably synthesized in postnatal life once developmental remodeling is complete (Davis, 1993; Keeley and Alatawi, 1991; Mariencheck *et al*., 1995).

In mammals the majority of elastin deposition occurs in late gestation wherein hemodynamic forces become highly influential, and it is during this time when symptomatic nuchal cord is likely to manifest (Bendeck *et al*., 1994). The fetal circulatory response to umbilical cord occlusion involves a transient rise in arterial blood pressure accompanied by a redistribution of cardiac output in favor of vital organs such as the brain (Green *et al*., 1991; Green *et al*., 2001; Kaneko *et al*., 2003). Since elastin deposition is stimulated by blood flow in late gestation, the hemodynamic response to intermittent hypoxia may give rise to an increase in elastin content in the carotid artery which is the major supplier of blood to the brain (keeley and Alatawi, 1991; Driss *et al*., 1997). Other possible mediators of perturbations in vascular

development are hormones and growth factors involved in the response to acute hypoxia, including angiotensin II, TGF-β, and cortisol which is a potent stimulus for matrix protein deposition in late gestation (Bendeck and Langille, 1991; Sundgren *et* al., 2003; Zhang *et* al., 2003).

The present study used the chronically catheterized ovine fetus to determine elastin content of the carotid artery and superior mesenteric artery in relation to severity of intermittent umbilical cord occlusion as well as characterize the corresponding fetal response in terms of arterial blood pressure and circulating cortisol concentration. While the duration and severity of UCO varies widely in the human situation, we studied varying degrees of acute hypoxia likely to result in fetal survival and previously shown to produce changes in brain development (Falkowski *et* al., 2002; Rocha *et* al., 2004). With regard to arterial development, we chose to focus on the extracellular matrix protein elastin given that once deposited during the brief window of early development, highly resilient mature elastin proteins do not undergo turnover and thus will endure the lifetime of an individual (Davis, 1993; Mariencheck *et al*., 1995). Furthermore, the abundance of elastin in large conduit arteries imparts characteristics of distensibility and elastic recoil that promote cardiovascular homeostasis by dampening the pressure oscillations produced by cardiac ejection (Shadwick, 1999). Thus an alteration in deposition of elastin during development may have long-term consequences for cardiovascular health.

4.2 METHODS

All surgical and experimental procedures followed the guide to the care and use of experimental animals approved by the Canadian Council on Animal Care Regulations and The University of Western Ontario Animal Ethics board. UCO is an established model of acute hypoxia in the ovine fetus (Green *et al*., 2001; Gardner and Giussani, 2003; Wassink *et al*., 2007). Surgical preparation and experimental manipulations were performed as previously described (Green *et al*., 2001).

4.2.1 Surgical Procedures

Pregnant mixed Western ewes between 113 and 117 days of gestation (term = 147 days) were chronically instrumented using sterile technique under general anesthesia (1g thiopental sodium in solution, intravenously (IV) for induction; Abbott Laboratories Ltd, Montreal, Canada; followed by 1% to 1.5% halothane in O_2 for maintenance). Prior to surgery, an analgesic was given intramuscularly to the ewe (0.2 g ketoprofen, Merial Canada Inc, Quebec, Canada). A midline incision was made in the lower abdominal wall, and the uterus was palpated to determine the fetal number and position. The upper body of the fetus and the proximal portion of the umbilical cord were exteriorized through an incision in the uterine wall. Polyvinyl catheters (Scientific Commodities, Lake Havasu city, AZ) were placed in the right and left fetal brachiocephalic arteries (0.72 : 1.22 mm) for measurement of blood pressure and sampling, the right fetal brachiocephalic vein (0.72 : 1.22 mm) for administration of antibiotics and transfusion of maternal blood, and the right maternal femoral vein (1.68 : 2.39 mm) for administration of antibiotics, sampling and euthanasia. In experimental animals an inflatable silicone occluder cuff (OCHD16; In Vivo Metric, Healdsburg, CA, USA), was positioned around the umbilical cord and secured to the abdominal skin, and the volume required for complete inflation was determined (4 – 6 cc). Once the fetus was returned to the uterus, a catheter was placed in the amniotic fluid cavity for measurement of amniotic pressure. Antibiotics were administered intra-operatively to the mother, (0.2 g trimethoprim and 1.2 g sulfadorine (IV), Schering Canada Inc, Pointe-Claire, Quebec, Canada) fetus (IV) and amniotic cavity (1 million IU penicillin G sodium, Pharmaceutical Partners of Canada, Richmond Hill, Ontario, Canada). The uterus and abdominal wall incisions were sutured in layers and catheters exteriorized through the maternal flank and secured to the back of the ewe in a plastic pouch.

Ewes were allowed a 3-4 day postoperative period prior to experimentation, during which the antibiotic regime was administered. Arterial blood was sampled for evaluation of maternal and fetal condition and catheters were flushed with heparinized saline to maintain patency.

4.2.2 Experimental Design

Umbilical cord occlusion was achieved by completely inflating the cuff with sterile saline using the predetermined volume. Over a five-hour period, the mild group received a single one-

minute occlusion every hour (n = 6), the moderate group a single two-minute occlusion every hour (n = 4), the severe group a single three-minute occlusion every hour (n = 6) and the control group received no occlusion (n = 7). For each experimental day, the five-hour occlusion series was preceded by a two-hour baseline and followed by a two-hour recovery period. On days one and four, fetal blood samples were taken during baseline and recovery for measurement of cortisol, blood gases, lactate and pH. Additionally, fetal blood gases, lactate and pH were measured five minutes before the occlusion, at the end of the occlusion, and five minutes after the occlusion, for the first and last occlusion of each day.

4.2.3 *In Vivo* Physiological Parameters

Blood was analyzed for blood gases, oxygen saturation, lactate and pH using a blood gas analyzer (ABL-725, Radiometer, Copenhagen, Denmark) and corrected for fetal temperature (T = 39.5°C). Plasma aliquots from samples drawn at baseline, post-UCO (first and last UCO) and recovery were stored at – 80 °C for later cortisol analysis. Fetal arterial blood pressure, adjusted for amniotic fluid pressure, was continuously monitored with pressure transducers (Cobe, Arvada, CO) and recorded on a data acquisition system (Powerlab model ML 795, ADI Instruments, Colorado Springs, CO). Fetal heart rate (FHR) was derived from the arterial blood pressure waveform. The control group was subjected to the same blood sampling and cardiovascular monitoring regime as the experimental group.

On the fourth day of study ewes and fetuses were sacrificed with an overdose of barbiturate after the 2-hr recovery period (30mg pentobarbital sodium, Fatal-Plus; Vortech Pharmaceuticals, Dearborn, MI); the fetus was delivered immediately by cesarean section and weighed. Approximately two cm of the carotid artery was taken above the aortic arch and the superior mesenteric was excised between its origin and the first pancreatic branch. Vessels were stripped of connective tissue, fast frozen in liquid nitrogen and stored at -80° C for later analysis.

4.2.4 Biochemical Measurement of Elastin Composition

Thawed samples were diced, and weighed. Extraction and quantification of elastin was performed as previously described (Burkhardt *et al*., 2008; Cheng *et al*., 2008; Foronjy *et al*., 2008). After tissues were weighed they were treated with 0.25M oxalic acid and then placed into

a boiling water bath for 60 minutes for extraction of insoluble elastin. After centrifugation (3000 xg for 10 minutes), the liquid was retained and the extraction procedure repeated for the remaining residue. It was confirmed that two heat extractions resulted in complete solubilization of elastin. Elastin was recovered from the liquid extract and precipitated using fastin dye reagent: 5,10,15,20-tetraphenyl-21,23-pophrine sulfonate, and quantified according to the manufacturer's instructions for the Fastin elastin assay (Biocolor, Belfast, Ireland). Absorbance of standards (0, 12.5, 25, 50 and 70 μg/μL) and samples were read on a microplate reader (Multiskan Ascent, Thermo labsystems, Fischer Scientific, Ottawa, Canada) using a 509 nm blue green filter. The amount of elastin present was determined from the standard curve and expressed as μg per mg tissue. The intra-assay and inter-assay coefficient of variation for the Fastin elastin assay was 5.9% and 9.9% respectively.

4.2.5 Plasma Cortisol Concentration

Cortisol plasma concentration was measured using an enzyme-linked immunosorbent (ELISA) assay (ALPCO Diagnostics, Salem, NH); the intra-assay and inter-assay coefficient of variation for the cortisol ELISA was 5.6% and 7.1%, respectively.

4.2.6 Data Analyses and Statistical Procedures

Analyses of the raw blood pressure signal and heart rate data were performed using powerlab software (Powerlab, ADI Instruments, Colorado Springs, CO). During the baseline and recovery periods on days one and four, 20 min averages of fetal mean arterial blood pressure (MAP), systolic pressure (SysP), pulse pressure (PP) and heart rate (FHR) were calculated for each fetus. Within group comparisons of baseline and recovery values across the four experimental days were made by a one-way ANOVA for repeated measures. For each UCO on days one and four, 30 second averages of MAP and PP data and 10 second averages of FHR data were calculated over two minutes prior to the onset of occlusion and over the course of the response, for determination of the maximum change (Δ) in MAP and the duration of the rise in MAP. Also, 10-minute averages of MAP, PP and SysP, from the onset of UCO were measured and used to calculate the mean pre to post-UCO change in these variables. Differences between groups in elastin content of the carotid and superior mesenteric arteries were assessed using a

one-way ANOVA. Cortisol concentrations were analyzed using repeated measures, and group means were calculated. A post hoc Bonferroni test was performed with findings of significant difference. Significance was set at $p < .05$ and results are presented as mean ± SEM.

4.3 RESULTS

Gestational age and birth weight were similar across groups. Experimental groups comprised 7 control, 6 mild, 4 moderate and 6 severe animals. There were no significant differences in fetal oxygenation and MAP at baseline on day 1 between the four groups [(control PaO_2: 23.6 ± 0.9 mmHg; MAP: 37.6 ± 4.5 mmHg), (mild PaO_2: 23.3 ± 0.9 mmHg; MAP: 39.7 ± 3.7 mmHg), (moderate PaO_2: 22.1 ± 0.4 mmHg; MAP: 42.5 ± 2.5 mmHg), (severe PaO_2: 22.0 ± 1.2 mmHg; MAP: 40.0 ± 3.4 mmHg)]. There was no change in baseline MAP over the four day experiment. As well, baseline cortisol levels were similar among groups on day 1 and within groups across the study [(day 1: control: 2.4 ± 0.3 μg/dL; mild: 3.4 ± 0.1 μg/dL; moderate: 3.1 ± 0.9 μg/dL; severe: 2.5 ± 0.6 μg/dL), (day 4: control: 5.4 ± 2.6 μg/dL; mild: 6.8 ± 4.0 μg/dL; moderate: 7.0 ± 3.3 μg/dL; severe: 3.2 ± 0.6 μg/dL).

Fetal arterial oxygen pressure (PaO_2), oxygen saturation (O_2 sat), and carbon dioxide pressure ($PaCO_2$) changed in a graded fashion across mild, moderate and severe groups (Table 4.1). Fetal arterial oxygenation and pH returned to pre-occlusion levels by 5 min post-occlusion in all experimental groups. An immediate deceleration in fetal heart rate accompanied umbilical cord occlusion of each degree, with a return to baseline within three minutes (Figure 4.1). Mild, moderate and severe occlusion produced a transient rise in MAP, Sys P and PP. The max Δ in MAP, and the mean Δ in MAP associated with cord occlusion in each group are shown in Table 4.2. The mean level of MAP over the 10 minute period following the onset of UCO, as well as the duration of the rise in MAP post-UCO are also shown in Table 4.2. The mean Δ in SysP stimulated by UCO increased in magnitude across groups (mild: 12.3 ± 1.0 mmHg; moderate: 17.6 ± 1.1 mmHg; severe: 20.0 ± 1.5 mmHg, $p < .01$). Although PP increased in response to UCO, the mean Δ in PP did not differ between groups (mild: 5.2 ± 0.3 mmHg; moderate 4.9 ± 0.7 mmHg; severe: 6.2 ± 0.8 mmHg).

Table 4.1.

Blood gases, lactate, and pH values pre and end UCO

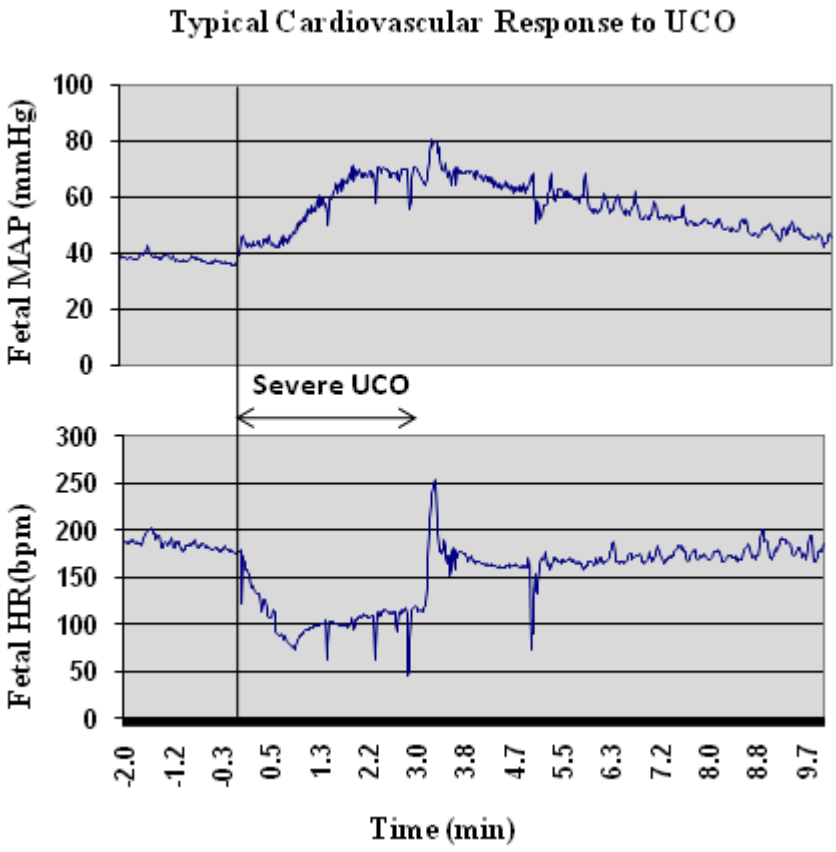
	PaO2 (mmHg)		O2 sat (%)		CaO2 (mmHg)		Lact (mmol/L)		pH	
	pre-UCO	**end-UCO**	**pre-UCO**	**end-UCO**	**pre-UCO**	**end-UCO**	**pre-UCO**	**end-UCO**	**pre-UCO**	**end-UCO**
Mild (n = 6)	23.6 ± 0.5	9.8 ± 0.9 ** ††	63.4 ± 1.8	12.8 ± 3.3 ** ††	48 ± 0.6	53.7 ± 1.1 ** ††	1.1 ± 0.1	1.0 ± 0.1 ††	7.36 ± 0.2	7.32 ± 0.0 ** ††
Moderate (n = 4)	22.2 ± 0.7	7.3 ± 0.8 ** ††	57.6 ± 1.9	7.4 ± 1.6 ** ††	49 ± 0.7	62.1 ± 1.4 ** ††	1.4 ± 0.2	1.9 ± 0.3 ** ††	7.35 ± 0.1	7.26 ± 0.1 ** ††
Severe (n = 7)	23.0 ± 0.7	4.7 ± 1.1 ** ††	66.5 ± 1.1	2.8 ± 2.0 ** ††	47.9 ± 0.5	68.0 ± 2.3 ** ††	1.1 ± 0.2	2.2 ± 0.1 ** ††	7.37 ± 0.0	7.23 ± 0.0 ** ††

Fetal arterial blood samples were drawn and analyzed for blood gases, lactate (lact) and pH; 5 min before (pre) and at the end of (end) umbilical cord occlusion (UCO). Hypoxia in response to UCO occurred in a graded fashion across mild, moderate and severe UCO groups.

** Pre vs. end UCO differences are all significant at $p < .01$ for all groups, except for lactate values for the mild group

†† End UCO values between the groups are all significant at $p < .01$.

Figure 4.1: Typical Cardiovascular Response to UCO The typical response to umbilical cord occlusion (UCO) of each degree was characterized by a transient fall in fetal heart rate and rise in fetal mean arterial blood pressure. Shown is the blood pressure and heart rate response to severe UCO. The 3-min UCO was initiated at time 0 min (as indicated by the horizontal line). Blood pressure was measured from the brachiocephalic artery and heart rate was derived from the arterial blood pressure waveform.


Typical Cardiovascular Response to UCO
Fetal MAP (mmHg)
100
80
60
40
20
0
Severe UCO
Fetal HR(bpm)
300
250
200
150
100
50
0
-2.0
-1.2
-0.3
0.5
1.3
2.2
3.0
3.8
4.7
5.5
6.3
7.2
8.0
8.8
9.7
Time (min)

A trend towards increasing elastin content of the carotid artery with increasing severity of fetal hypoxemia was apparent, the difference was only significant for the severe group ($p < .05$) (Table 4.2, Figure 4.2). In contrast, elastin content of the superior mesenteric artery did not differ between the three UCO groups and the control group (Figure 4.2).

There was no consistent cortisol response to mild UCO on day one or day four. However, repeated measures analysis revealed post-UCO plasma cortisol concentration to be consistently elevated in all fetuses made moderately or severely hypoxic on days one and four ($p < 0.05$). The percent change in cortisol concentration associated with UCO on day one for the moderate group was 69.4 ± 8.9% and for the severe group 95.2 ± 17.6 %; and day four for the moderate group was 76.8 ± 38.6 % and for the severe group 112.1 ± 32.7%.

4.4 DISCUSSION

The present study is the first to examine the effect of acute intermittent hypoxia on fetal arterial remodeling. Three degrees of acute, reversible hypoxemia without cumulative acidosis due to varied duration of UCO were produced repeatedly over 4 days in the late gestation ovine fetus. Fetuses exposed to intermittent hypoxia exhibited increased elastin content of the carotid artery in relation to the control group, with the most pronounced change observed in the severe UCO group. In contrast, no change in elastin content of the superior mesenteric artery was found. A transient rise in fetal blood pressure accompanied hypoxemia of each degree, and was increased in magnitude across mild, moderate and severe groups. We surmise that this circulatory adjustment to UCO accounts for the differential response in protein accumulation between the carotid and the superior mesenteric artery.

Compliance of central conduit arteries which is largely a function of the abundant elastin protein is an important and independent determinant of cardiovascular health because it determines pulsatile load of the system (Shadwick, 1999). Since under normal conditions there is no appreciable synthesis of elastin after development, the content of elastin is largely determined in fetal life. Degradation and fragmentation of elastin over age in postnatal life due to repeated bouts of cyclic stretch have been implicated in the progression of hypertension and cardiovascular disease (Greenwald, 2007; Silvia *et* al., 2006).

Table 4.2

Elastin content of the carotid artery and fetal circulatory response to UCO

Group	Elastin	Mean Δ	Max Δ	Post mean	Duration
Control (n = 7)	5.7 ± 0.4	2.1 ± 0.5	4.0 ± 1.7	41.51 ± 2.8	-
Mild (n = 6)	7.0 ± 0.7	10.0 ± 0.9**	21.1 ± 2.5**	47.6 ± 2.5	10.4 ± 2.5
Moderate (n = 4)	7.4 ± 0.7	14.8 ± 0.9**†	32.4 ± 1.6**†	52.0 ± 1.0	14.3 ± 0.6†
Severe (n = 6)	9.5 ± 1.0 *	17.0 ± 0.7**††	34.5 ± 0.6**††	54.0 ± 2.4*	17.4 ± 1.2††

Elastin content (μg/mg tissue); mean Δ in fetal MAP (mmHg) was calculated from 2 min averages pre-UCO and 10-min averages from the onset of UCO; max Δ in fetal MAP was calculated from pre-UCO averages and the highest value achieved post-UCO. Control values were derived by the same calculations at time points matching UCO data. Duration of the rise in MAP was measured as the time (min) from the onset of UCO to the return of baseline values. For differences between group a one-way ANOVA was used

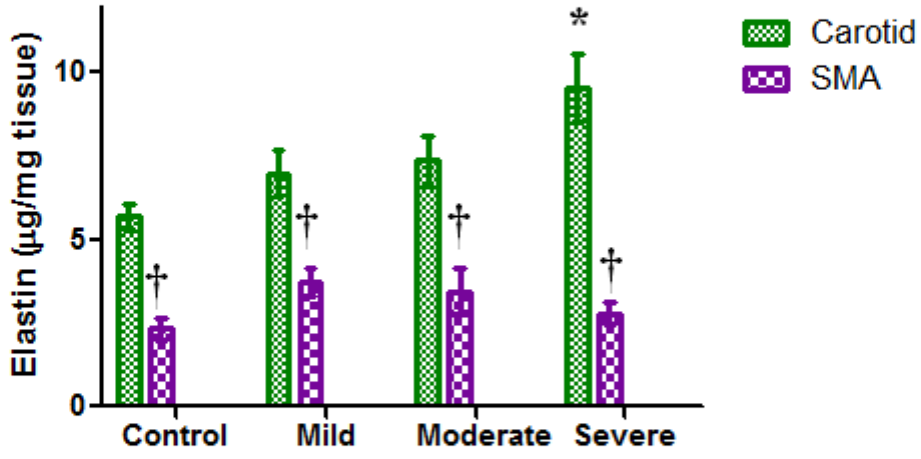
* $p < 0.05$ vs. control ; ** $p < 0.01$ vs. control

† $p < 0.05$ vs. mild; †† $p < 0.01$ vs. moderate

Figure 4.2: Elastin Content of Carotid vs. Superior Mesenteric Artery Elastin composition in the carotid vs. superior mesenteric artery (SMA). The control group (n = 7) recieved no umbilical cord occlusions (UCO); the mild hypoxic groups (n = 6) received one min UCO/hr; the moderate hypoxic group (n = 4) two min UCO/hr and the severe hypoxic group (n = 6) received 3 min UCO/hr. Elastin composition was higher in the severe group compared to control ($p < .05$). Values are expressed as μg/mg tissue and ± SEM.

* Hypoxic groups vs. control $p < .05$

†† SMA vs. carotid artery $p < .01$


Elastin (µg/mg tissue)
10
5
0
Control
Mild
Moderate
Severe
*
†
Carotid
SMA

Therefore, the increase in elastin concentration of the fetal carotid artery in response to acute intermittent hypoxia may protect against the development of cardiovascular disease in postnatal life; thus the present study provides evidence for potentially beneficial programming in response to an acute prenatal insult.

4.4.1 Acute Hypoxia, Hemodynamic Stimuli and Deposition of Elastin

Alterations in vascular development produced by repeated UCO may be mediated by the changes in hemodynamic conditions and circulating hormones that occur in response to acute hypoxia or by direct effects of oxygen tension. Fetuses exposed to intermittent hypoxia in this study exhibited increased elastin content of the carotid artery in relation to control, with the most pronounced change in the severe group. We propose that the increase in elastin content of the carotid artery resulted primarily from hypoxic-induced changes in hemodynamic regulators of protein deposition. A transient rise in fetal blood pressure accompanied hypoxemia of each degree, and was increased in magnitude across mild, moderate and severe groups. It is known that redistribution of cardiac output with preference to the brain accompanies elevated blood pressure during acute hypoxia (Green *et al*., 1999; Kaneko *et* al., 2003). Although blood flow was not measured in the present study, an increase in blood flow constituting both pressure and resistance elements and proportional to the observed increase in blood pressure is expected to occur in the carotid artery. Animals were examined in late gestation which corresponds to a time when elastin synthesis accelerates and becomes highly related to blood flow, in both human and sheep (Bendeck *et al*., 1994). The regulatory role of blood flow in elastin deposition during development has been demonstrated in fetal sheep by correlational analysis in various vessels and by experimental manipulations in neonatal rabbits whereby increases in local blood flow stimulate increases in arterial elastin content and decreases in elastin accumulation result from reductions in blood flow, with no effect on collagen (Langille *et al*., 1989; Leung *et al*., 1977). Only severe hypoxemia produced an average level of fetal MAP over the 10 minute period following the onset of UCO that was significantly greater than control values; and this level approaches that previously reported in newborn lambs at the time of peak elastin synthesis (Bendeck *et al*., 1994). Therefore, a developmentally determined threshold level of blood flow stimulating an up-regulation in elastin synthesis may have been achieved during severe UCO,

resulting in the marked response of elastin accumulation in the carotid artery observed in this group.

Interestingly, elastin content of the superior mesenteric artery was found to be unchanged by UCO. Blood flow through the carotid artery versus the superior mesenteric artery would differ dramatically during UCO due to local changes in vascular resistance that function in redistributing cardiac output with preference to the brain, whereas blood pressure would be similar between these two vessels. Previous studies have reported blood flow to the digestive tract to be maintained during complete occlusion similar in degree to that used in the present study and to only decrease in more severe hypoxemia with developing acidosis (Itskotvitz *et al*., 1987; Peeters *et al*., 1989). It is important to note that the relation between hemodynamic forces and elastin accumulation has been studied primarily in large arteries, thus it is possible that a different regulatory function of mechanical stimuli pertains to remodeling of small muscular arteries such as the superior mesenteric artery.

4.4.2 Other Possible Mediators of Augmented Elastin Deposition

In addition to locally mediated arterial remodeling, changes in circulating hormones induced by acute hypoxemia may contribute to the high elastin content observed with severe UCO. In agreement with previous work, we found circulating cortisol to rise in response to UCO (Green *et al*., 2000; Roelfsema *et al*., 2005; Unno *et al*., 1997). Cortisol stimulates both elastin and collagen synthesis and is thought to mediate the precipitous rise in protein accumulation that occurs in late gestation (Bendeck *et al*., 1994). However, plasma cortisol elevations which would presumably have a systemic effect, do not explain the differential response of the carotid versus the superior mesenteric artery to acute hypoxia. Alternatively, muscular arteries may respond differently to cortisol. Nevertheless, there was no cortisol response to mild UCO, whereas elastin content was increased in mild, moderate and severe compared to control.

Changes in oxygen tension are known to cause modifications in arterial structure and function. The oxygen-regulated response to hypoxia in vessels undergoing growth and development has been studied extensively in neonates exposed to chronic hypobaric hypoxia. The accelerated elastin accumulation which contributes substantially to morphometric changes in this model is attributed primarily to hypoxic-stimulated changes in mechanical load rather than

to changes in oxygen tension (Durmowicz *et al*., 1991; Stenmark *et al*., 2006; Stenmark *et* al., 1994). In fact, elastin production is blunted in cultured smooth muscle cells exposed to hypoxia (Rabinovitch *et al*., 1983). Additional effects of hypoxia including changes in matrix protein turnover and vasoconstriction have been shown to occur only with prolongation of hypoxia therefore transient reductions in oxygenation produced by UCO in the present study were likely inconsequential in comparison to hemodynamic influences (Ambalavanan *et* al., 2007; Zaidi *et* al., 2002).

Quantitative analysis of elastin composition was undertaken as opposed to measurement of mRNA expression since absolute and relative content of elastin determines arterial stiffness. However the increase in elastin relative to tissue weight suggests that either elastin alone is increased or the other wall components have decreased. It is probable that the former has occurred since collagen deposition and smooth muscle cell proliferation are known to increase in response to hypoxia, cortisol and mechanical load (Bendeck *et al*., 1991; Kelleher *et al*., 2004). Structural constituents of the arterial wall control passive mechanical properties: elastin is the primary determinant of elastic modulus at low distending pressure, while extensibility at high pressure is a function of collagen (Shadwick, 1999). Absolute and relative increases in elastin quantity enhance arterial distensibility at physiological pressures over which mechanical load is transferred from elastin to collagen. The organization and cross-linking of ECM proteins which may have been altered by acute hypoxia, also play a role in arterial mechanics (Cheng *et* al., 2008).

4.5 CONCLUSIONS

In summary, acute, reversible fetal hypoxemia due to intermittent UCO appears to accelerate developmental deposition of elastin in the carotid artery in relation to the severity of insult, whereas matrix elastin content of the superior mesenteric artery is not affected. Although there are several possible mediators of this synthetic response, the current data suggest hemodynamic stimuli to play the primary role. As this study is the first to reveal perturbations in fetal arterial remodeling in response to intermittent hypoxemia, it implores a number of potential

routes for future inquiries including the effect on additional structural factors such as collagen content and cross-linking, resultant modifications in vascular morphology and long-term consequences for postnatal arterial function.

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CHAPTER 5

DISCUSSION

5.1 SUMMARY

Exploration of potential mechanisms involved in the developmental origins of CVD was the overall goal of this thesis. The main hypothesis tested was that a perturbation in arterial development during intrauterine life leads to permanent structural abnormalities and thereby arterial dysfunction in postnatal life. As the fundamental developmental disturbance, attention was directed towards the deposition of ECM proteins, elastin and collagen. The rationale being that the absolute and relative content of elastin and collagen within the aorta and other proximal arteries are the primary determinants of arterial compliance (Fonck *et al*., 2007; Shadwick, 1999). In turn, reduced arterial compliance, or in other words wall stiffening, of the central vasculature is an important hallmark of CVD (Abhayaratna *et al*., 2008). Further, ECM composition of the arterial wall is to a large extent fixed upon conclusion of developmental remodeling, given that under normal conditions the elastin protein is not appreciably synthesized postnatally after approximately one month of age in the sheep and human (Bendeck *et al*., 1994; Mariencheck *et al*., 1995). Thus, a deficiency in elastin content established *in utero* will likely have long-term cardiovascular consequences. Such a deficiency may be amplified in postnatal life when stimuli such as augmented mechanical load or injury lead to hypertrophic remodeling. This adverse remodeling which precedes hypertension and CVD involves phenotypic switching of VSMCs to their embryonic precursors that proliferate and deposit collagen and other proteins into the ECM (Owens *et al.,* 2004). Since VSMC differentiation from embryonic VSMCs to mature contractile cells occurs over the second half of gestation (Owens *et al*., 2004), this developmental milestone was also examined in the context of intrauterine compromise. The key finding of the thesis is that aberrant aortic remodeling during the second half of gestation characterized by altered deposition of the ECM constituents, in the growth restricted fetus, leads to persistent structural abnormalities and increased wall stiffness in adulthood. Prior to these studies, interference in ECM protein deposition and consequent central arterial stiffening as a link between suboptimal intrauterine conditions and later CVD remained largely unexamined.

Our studies present a novel perspective on programming of CVD as they show mechanical disadvantage and structural abnormalities of the proximal circulation linked to alteration in the expression and activity of key regulatory molecules involved in arterial

remodeling during fetal development. To date, investigation of programming with respect to cardiovascular pathology has primarily focused on kidney and heart development in addition to small artery function. Evidence suggests that substrate deficiency *in utero* results in delayed maturation and reduced proliferation of cardiomyocytes (Louey *et al*., 2007) and thereby conveys a potentially permanent disadvantage to the heart. Yet, this fails to explain the increased blood pressure consistently demonstrated in the growth restricted rodent (Alexander, 2003; Ceravolo *et al*., 2007) and human (Barker *et al*., 1990; Curhan *et al*., 1996), that presents as early as childhood (Arends *et al*., 2005). A potential mechanism of this programmed hypertension that has been extensively studied is altered kidney development and consequent disturbance in blood pressure regulation (Thompson and Regnault, 2011). Human studies report reduced kidney size and volume (Konje *et al*., 1997; Spencer *et al*., 2001) and decreased nephron number (Hinchliffe *et al*., 1992) in adults born small. In support of this, diminished glomeruli number has been found in postnatal rats exposed to placental insufficiency *in utero* (Moritz *et al*., 2009; Wlodek *et al*., 2008) In addition to kidney structure, vascular responsiveness as a correlate to hypertension in growth restricted offspring has been explored by several groups. Although several studies have reported endothelial dysfunction in human adults who were born of low birth weight (Brodszki *et al*., 2005; Goodfellow *et al*., 1998), rodent studies have yielded inconsistent results that are variable between the arterial segment under study (Mazzuca *et al*., 2010 Morton *et al*., 2010; Payne *et al*., 2003). Our preliminary studies revealed no change in responses of the small mesenteric artery in growth restricted adult guinea pig to a number of vasoconstrictive and vasodilatory agents. However, we did find premature intima thickening of the aorta in concert with elevated levels of E-selectin, a marker of endothelial damage, in growth restricted ovine fetuses. The atherosclerotic process is closely tied to endothelial damage and hence, it is possible that later development of endothelial dysfunction in growth restricted offspring derives directly from this *in utero* intimal response. Taken together, human studies and some animals studies show that endothelial dysfunction is associated with IUGR, yet it is still unclear as to whether this derives directly from abnormalities in vascular properties and/or gene expression established before birth or is a secondary effect arising in postnatal life. Our data however, provide evidence that the increased risk for hypertension and CVD in growth restricted offspring can be traced to a direct effect on aortic development that results in a premature initiation of arterial stiffening and

intima thickening. These findings provide important insight into the developmental origins of CVD.

5.1.2 Hypoxia as a Programming Agent

As a cause of intrauterine growth restriction, we simulated the human condition of placental insufficiency using both a large and small animal model. These models bear clinical relevance since both species are prenatal developers as is the human and placental insufficiency is the major cause of IUGR in developed countries (Kingdom *et al*., 2000). Compared to the commonly used nutritional models, placental insufficiency is studied less in the context of fetal programming. The insult imposed by placental insufficiency is a reduction in nutrient supply of which oxygen deprivation is a primary cause of blunted fetal growth and perturbed organ development (Giussani *et al*., 2007; Lackman *et al*., 2001). The duration of fetal hypoxia in the ovine model equates to 10 percent of gestation and was applied in late pregnancy, whereas hypoxia was endured over the second half of gestation in the ligated guinea pig. Another difference between the two models lies in the nature of fetal hypoxia. The ovine model resembles human IUGR arising from a failure in elaboration of the fetal villous tree and associated with abnormal UA EDFV (Macara *et al*., 1995; Krebs *et al*., 1996), while the guinea pig model approximates human IUGR due to inadequate spiral remodeling and associated with reduced uterine blood flow (Aviram *et al*., 2010). One advantage of the sheep model was the ability to measure fetal arterial oxygen content *in vivo* and thus correlate the various measured outcomes to the level of oxygenation. This study confirmed that oxygen deprivation is the key insult of placental insufficiency and is instrumental in programming of cardiovascular dysfunction. The two models of chronic hypoxia produced a similar aortic phenotype with respect to ECM composition, however, there were differences in terms of wall growth likely due to the differential durations of the insult. This speaks to the relation between phenotypic outcome and timing of the insult. A third model of acute, intermittent hypoxia in the ovine fetus was studied, allowing for comparison of the vascular response to acute versus chronic hypoxia. A novel dimension of all three models was the assessment of the dose-response effect. Three degrees of acute hypoxia were produced in the acute study, two degrees of chronic hypoxia in the embolization study and a SGA and IUGR group were delineated in the guinea pig model. Taken

together, the data suggest that the vascular response and resulting phenotype is indeed dependent on the level of hypoxia and in turn, this relationship dependent on the outcome measured.

5.1.3 Severity of Insult

There is wide variation in the degree of human fetal growth impairment. Hence, it is important to note that relative to the spectrum of clinical placental insufficiency, our ovine model represents a mild insult given that it was applied over 10 percent of gestation, whereas human fetuses endure chronic hypoxia throughout the second half of gestation. As well, neither moderate nor severe hypoxic ovine fetuses developed metabolic acidosis or hypercapnia. Results of the ovine study suggest that elastin accumulation is particularly sensitive to arterial oxygenation, whereas collagen mRNA expression and accumulation is increased only with arterial oxygen saturation values below 40 percent. Interestingly, similar to the aorta, the umbilical artery of chronically hypoxic ovine fetuses showed a marked reduction in the size of the internal elastic lamina that was graded across moderate and severe groups (Figure 5.1). Elastin deficiency in growth impaired fetal guinea pigs was more subtle not reaching statistical significance, yet later in adult life this deficiency was amplified in concert with increased collagen and VSMC content. The SGA offspring studied had birth weights ~ 20 percent lower than the AGA offspring, a difference similar to those produced by nutritional models in rodents (Delahye *et al*., 2010). The fact that such a slight developmental disturbance in mildly growth restricted offspring is apparent functionally in adulthood is intriguing and suggests intrauterine compromise or birth weight to be a strong predictor of CVD. In agreement, human epidemiological studies report blood pressure to be inversely related to human birth weight, along the normal range of birth weights (Barker, 2005).

5.1.4 Reduced Aortic Compliance Linked to Programming of CVD

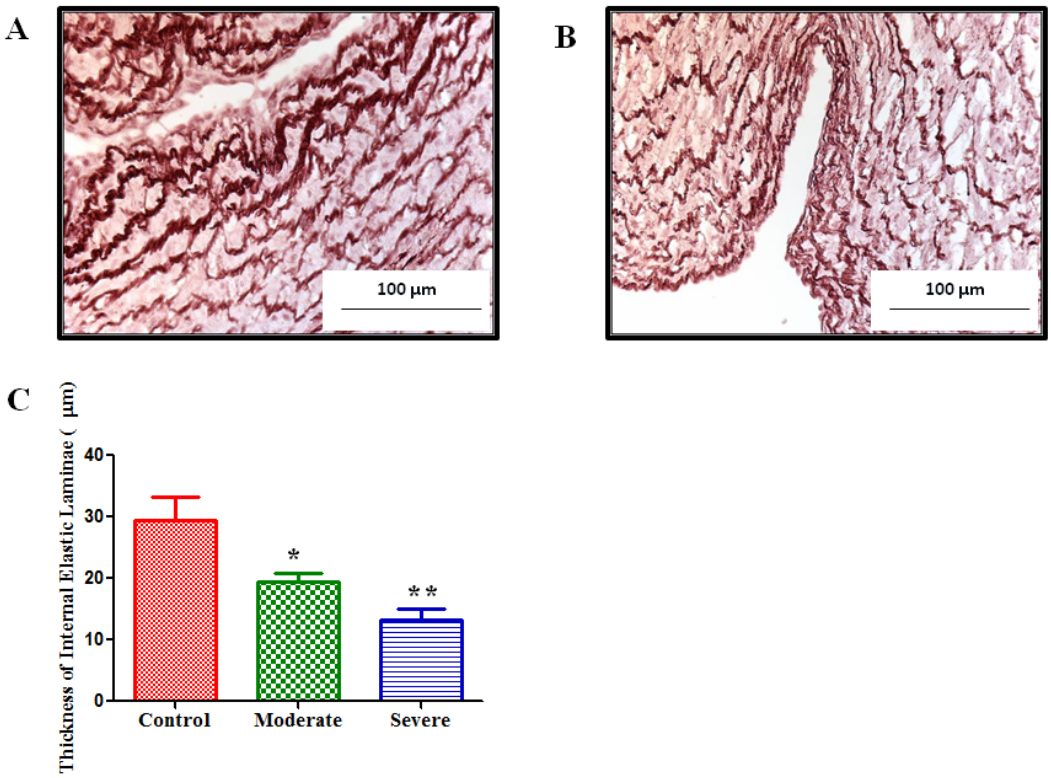
The main hypothesis that perturbed aortic remodeling in the substrate deprived fetus leads to persistent structural abnormalities and reduced compliance in postnatal life was confirmed by the two models of placental insufficiency. These studies were the first to demonstrate that altered ECM composition of the aorta occurs in the chronically hypoxic fetus, that this deficiency is indeed persistent and that the later consequence is reduced aortic

Figure 5.1 Thickness of Elastic Lamina in Hypoxic fetal sheep

The concentric layers of internal elastic laminae of an umbilical artery from a control ovine fetus (A) and severely hypoxic fetus (B) are shown. The thickness of the internal elastic laminae was reduced in moderate and severe fetuses, compared to control.

* $p < .05$ hypoxic animals vs. control

** $p < .01$ hypoxic animals vs. control


A
100 µm
B
100 µm
C
Thickness of Internal Elastic Laminae (µm)
40
30
20
10
0
*
**
Control
Moderate
Severe

compliance. The buffering capacity of the arterial system is a function of central arterial compliance and a compromise in this capacity leads to progressive hemodynamic disturbance and cardiac malfunction (Mitchell *et al*., 2000). In fact, noninvasive indices of aortic stiffness are independent and powerful predictors of hypertension and CVD (Abhayaratna *et al*. 2008). Further, aortic stiffening in adults is associated with increased wall thickness, excessive collagen accumulation, elastin degradation and fragmentation, VSMC proliferation and phenotypic switching (Et-Taouil *et al*. 2003), all of which bear resemblance to the phenotypes observed in our growth impaired animals. Therefore, our data suggest that the high risk for development of hypertension and CVD in those growth restricted *in utero* is tied to an interference in aortic ECM remodeling that manifests as blunted viscoelastic capacity.

Although central arterial compliance has been largely unexplored with animal models of IUGR, several studies have non-invasively measured this CVD correlate in IUGR human offspring. The majority of human studies reveal an association betwen birth weight and arterial compliance. For instance, Martyn *et al*. (1995) examined arterial compliance in a group of middle-aged adults born between 1939 and 1940 in the UK. In this group, pulse wave velocity (PWV) as a measure of arterial stiffness in the central and peripheral arteries was higher in those whose measurements of weight, length and abdominal circumference were low at birth. The inverse relationship between birth measurements and PWV in this study was stronger in the central segment compared with that of the peripheral segment (Martin *et al*., 1995). A relation between central PWV and birth weight was also reported by Oren and colleagues, in a cohort of young adults living in the Netherlands born between 1970 and 1973 (Oren *et al*., 2003) (see Figure 1.4). On the other hand, two studies have failed to find a significant correlation between birth weight and indices of arterial compliance in young adults. The first measured PWV of peripheral arterial segments in men and women aged 25 years; and the other calculated the carotid artery distensibility coefficient and central PWV in a group of normotensive subjects aged 19-24 (Broyd *et* al, 2005; Montgomery *et al*., 2000).

An affect of fetal growth on arterial compliance has also been reported in children. Peripheral conduit artery stiffness in 8 year olds measured by PWV was found to be higher in those born preterm and SGA compared to those who were preterm and AGA (Cheung *et* al., 2004). Likewise, work in Spain found augmentation index, a surrogate measure of central arterial

stiffness, to be highest in the lowest birth weight category of children 7-18 years in age (Lurbe *et* al., 2003). Conversely, stiffness index of the aorta in a group of 9 year old children was found to be similar between those whose birth weights were AGA and those whose birth weights were 3 standard deviations below the age related mean (Ley *et* al., 1997).

A shortcoming of the aforementioned studies in yielding inconsistent results regarding the effect of fetal growth restriction on later arterial function may lie in the use of birth weight as a proxy for intrauterine growth rate and the etiological variation in IUGR. This limitation was eradicated in two prospective studies by Mori *et al*., which performed non-invasive measurements of arterial distensibility in SGA newborns identified as having placental insufficiency *in utero* by abnormal UA EDFV and AGA infants with normal flow waveforms. Results of the first study revealed stiffness index of the aorta and carotid artery to be greatest in the lowest birth weight subgroup of the infants who suffered from placental insufficiency, and were significantly different from that of the AGA infants (Mori and Yoshiyuki, 2006). The second study found large artery stiffness to be increased in infants identified as having placental insufficiency during pregnancy, with the largest changes in the most severely compromised fetuses (Mori *et al*., 2006). Our data expound these human findings in that they confirm the developmental origins of arterial stiffening and demonstrate that this dysfunction is a direct result of hypoxic intrauterine insults rather than a secondary effect of postnatal catch-up growth or abnormalities in other organ systems.

5.1.5 Chronic Hypoxia and VSMC Maturation

An interesting contrast in the effect of substrate deficiency on VSMC maturation between degrees of intrauterine compromise was demonstrated in sheep and guinea pigs subjected to conditions of placental insufficiency. Chronically hypoxic fetal sheep displayed no change in staining for a marker of synthetic-type VSMCs (MHC-B) and we suspected that this was due to the late timing of the insult as VSMC differentiation is largely complete by term (Owens *et al*., 2004) (see Chapter 2 Discussion 2.4.2). However, fetal guinea pigs subjected to chronic hypoxia throughout the second half of gestation with high brain:liver ratios suggestive of adaptation to oxygen deprivation, also did not show changes in VSMC maturation. Conversely, both SGA guinea pig fetuses and offspring exhibited a marked increase in MHC-B content, suggesting that

delayed VSMC maturation *in utero* translates to long-term changes in cellular identity (see Chapter 3 Discussion 3.5.3). A proposed mechanism for persistence of adaptations in cellular identity over time in an individual and across generations is a change in the epigenotype. Evidence suggests VSMC phenotype to be regulated at the level of chromatin and thus susceptible to epigenetic modifications (McDonald *et al*., 2006; Qiu and Li, 2006). With respect to the contradictory results between SGA and IUGR guinea pig fetuses, intracellular signals regulating VSMC differentiation may be differentially affected by the level of hypoxia (Owens *et al*., 2004) or oxidative stress reached in the IUGR fetuses and this may account for maintenance of VSMC maturation despite slowed overall growth in this group. A similar result was recently revealed in our laboratory with respect to several maturational indicators of brain development, such that compared to the AGA group, SGA guinea pig fetuses showed delayed brain maturation that was not apparent in the IUGR fetuses (Piorkowski *et al*., unpublished data).

5.1.6 Chronic Hypoxia and Intima Thickening

As revealed by the embolization model, the aortic defect associated with placental insufficiency was characterized by intima formation and thickening along with changes of the media. This phenotype was observed in only the severely hypoxic animals. Akin to the aortae of our normoxic ovine fetuses, the intima of normal human fetuses and infants is exclusively an endothelial layer which is closely adherent to the internal elastic lamina (Sasaguri *et al*. 1994). In atherosclerotic-prone arteries, such as the descending aorta, a subendothelial fibrous layer lined on the medial side by a narrow zone of proliferating VSMCs develops over the first two decades of life (Sasaguri *et al*, 1994). Over the 3rd and 4th decade of life, retention of lipoproteins by the ECM of the pre-existing intimal layer instigates the development of atherosclerotic lesions (Stary *et al*. 1992). Thus, our observation of premature formation of an intimal zone on the medial and luminal side of the internal elastic lamina in aortae of fetuses subjected to severe hypoxia implies that IUGR by intrauterine hypoxia increases the risk for atherosclerosis in adulthood. In support of this, increased aortic intima-media thickness has been found in human IUGR fetuses and children (Cosmi *et al*., 2009). The sheep fetus most closely approximates the human in terms of timing of developmental processes and physiology under normal and pathological conditions (Ikeda *et al*., 2001).

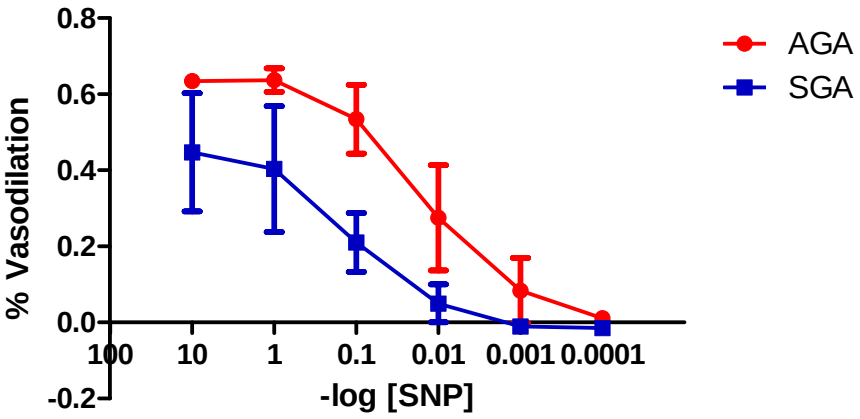
Endothelial damage results from the atherosclerotic process as well as increased intravascular load due to central arterial stiffening. Indeed, atherosclerosis, endothelial dysfunction and central arterial stiffening are strongly interrelated (Campuzano *et al*., 2006; McCall *et al*., 2010). Thus, intima formation and altered ECM composition initiated *in utero* may lead to endothelial dysfunction in conjunction with atherosclerosis and arterial stiffening. In fact, aortae exhibiting intima thickening also showed increased mRNA levels of E-selectin. E-selectin is involved in the inflammatory response, facilitates the atherogenic process and is used as an indicator of endothelial damage (Barron *et al*., 1997).

Human studies demonstrate blunted flow-mediated dilation in children (Martin *et al*., 2000), adolescents (Goodfellow *et al*., 1998) and adults (Leeson *et al*., 2001) who were born of small birth weight. Using the guinea pig study, we investigated the endothelial-dependent responses of the superior mesenteric artery to methalcholine using pressure myography and found no differences in these responses between AGA and SGA offspring. Further, a vessel-bath apparatus was used to assess responses of the guinea pig aorta. Interestingly, while the superior mesenteric artery appeared very sensitive to methacholine, the aorta failed to respond to this agonist, perhaps due to species differences in responsiveness or abundance of proximal aortic receptors. There appeared to be a blunted vasodilatory response of the guinea pig aorta to the endothelial-independent agonist, sodium nitropusside, however these preliminary results remain inconclusive (Figure 5.2). Extention of these preliminary studies is required to decipher the utility of the guinea pig for investigation of vessel function.

The association between IUGR and endothelial function has typically been investigated in rats. Morton and colleagues showed an inhibition of vasodilation of the superior mesenteric artery in response to methacholine using a wire myograph system, in young and aged rats which were growth restricted in a hypoxic chamber (Morton *et al*., 2010). On the other hand, another study reported uterine artery ligation of the pregnant rat to result in endothelial dysfunction of the uterine artery but no changes in the mesenteric, renal or femoral arteries in female offspring (Mazzuca *et al*., 2010). Thus, results of these rodent studies are inconsistent.

Figure 5.2. Preliminary Results: Response of Adult Aortae to SNP

Aortae of adult guinea pig offspring were immediately placed in ice-cold Krebs at post-mortem and placed in a vessel-bath apparatus for measurement of responsiveness. The response of SGA aortae (n = 4) to sodium nitropusside (SNP), a endothelium-independent vasodilatory agonist, appeared blunted compared to AGA aortae (n = 4).


0.8
0.6
0.4
0.2
0.0
-0.2
% Vasodilation
100
10
1
0.1
0.01
0.001
0.0001
-log [SNP]
AGA
SGA

5.1.7 Mediators of Hypoxic-induced Perturbations in Arterial Development

Another advantage of the sheep model was that it allowed comprehensive investigation into possible molecular, hormonal and mechanical links to the observed changes in arterial phenotype. The acute hypoxia model showed transient increases in circulating cortisol concentration in response to acute hypoxia that did not differ in degree in relation to the severity of insult. No lasting change in plasma coritsol levels were produced in chronic hypoxic ovine fetuses, as previously reported (Gagnon *et al*. 1994; Kerr *et al*. 1992). Acute hypoxia was associated with immediate and transient elevations in blood pressure that were increased in a graded fashion across mild, moderate and severe groups and this hemodynamic response is assumed to be the primary stimulus for increased carotid elastin content in fetuses of the latter group. In agreement with other studies (Louey *et al*., 2007), chronic hypoxia generated no change in baseline blood pressure across the 15 days of study. Still, it is possible that perturbed aortic remodeling in the chronically hypoxic fetus is related to altered blood flow due to redistribution of cardiac output.

Chronic hypoxia was associated with increased expression of molecular regulators that have been implicated in the vascular response to adverse stimuli as well as in developmental remodeling. The mRNA levels of the pro-fibrotic growth factor, TGF-β_1, showed a similar pattern to that of collagen I as well as the total collagen content, that is, an increase in severely hypoxic fetuses with similar levels between normoxic and moderately hypoxic groups. TGF-β_1 is a known inducer of the MMP-2 (Ross & Tranquillo, 2003) and both have been implicated as key players in the adverse arterial and cardiac remodeling that contributes importantly to the progression of CVD (Chen *et al*. 2006; Mochizuki *et al*. 2001; Zhao *et al*. 2008). As well, MMP-2 and TGF-β_1 are both involved in intima thickening, the former through facilitation of VSMC migration (Bendeck *& Zempo et al.* 1994) and degradation of the internal elastic lamina and the basement membrane of the endothelium (Rosenberg *et al*. 1998). The migrated VSMCs deposit ECM proteins into the intima under the stimulation of TGF-β_1 and other inflammatory cytokines (Bendeck & Zempo *et al.* 1994). Along with an increase in mRNA levels of TGF-β_1, aortae of severely hypoxic ovine fetuses exhibited increases in mRNA levels of MMP-2 and its protease activator, MTI-MMP. The activity of MMP-2 has been previously correlated with the mRNA expression of MTI-MMP, independent of protein levels of MMP-2 (Zahradka *et al*.

2004). As well, inverse correlations between fetal arterial oxygen saturation and mRNA levels of procollagen I and III, TGF-β_1, MMP-2 and MTI-MMP were observed in severely hypoxic ovine fetuses. These correlational analyses provide an alternative method of assessing the effect of hypoxia on various outcomes, given the limitation of sample size common to large animal models.

In accord with the aortic response, our preliminary data show increases in TGF-β_1, MMP-2, MTI-MMP and procollagen III mRNA levels in the right ventricle of severely hypoxic fetus, with no differences found in the left ventricle (Figures 5.3 & 5.4). The differential response of the right versus left ventricle may be owing to the redistribution of arterial and cardiac blood flow that accompanies hypoxia. It is known that there is a right-left shift in blood flow through the myocardium during chronic hypoxia and an increase in afterload imposed on the right ventricle due to peripheral vasoconstriction, whereas cerebral vasodilation relatively reduces impedance of the left ventricle (Baschat *et al*., 2000). The total and relative weights of the heart were not different between hypoxic and control fetuses. This may be attributable to the hypoxic-induced reduction in proliferation of cardiomyocytes in both the left and right ventricle previously reported (Louey *et al*., 2007). The postulation that the differential effect of hypoxia on the left versus the right ventricle is due to hemodynamic factors, is supported by our preliminary data which show moderate and severe acute hypoxia to be associated with increases in MMP-2 in only the right ventricle (Figure 5.5). The primary stimulus in this model is the transient, yet, dramatic hemodynamic response which involves a redistribution of cardiac output. This acute model also shows an increase in the weight of the right ventricle relative to total heart weight (Figure 5.6). Mechanical load is an inducer of collagen deposition and cardiomyocyte proliferation, thus perhaps acute fetal hypoxia differs from chronic hypoxia in that the former induces and the latter inhibits cardiomyocyte proliferation.

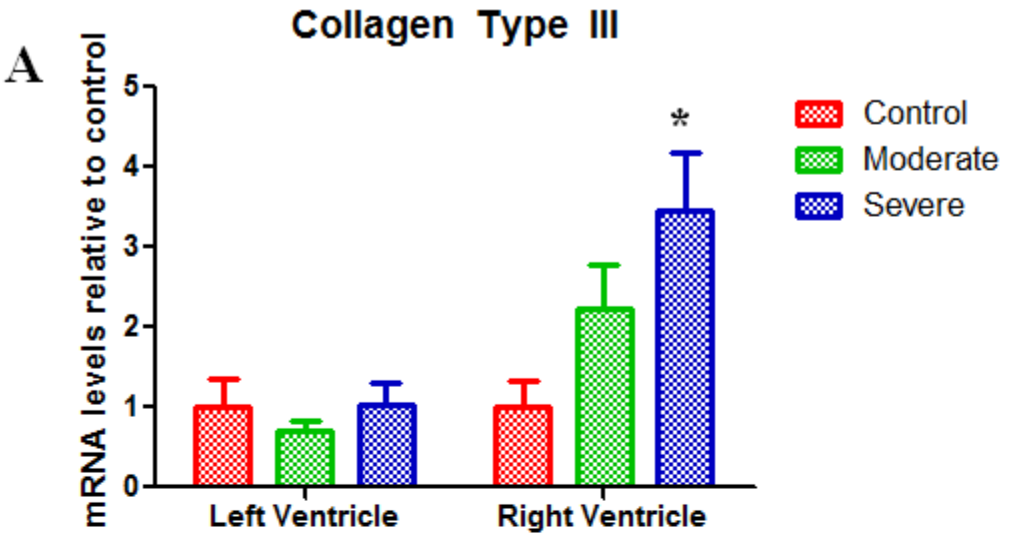
5.1.8 Site-Specific Responses to Hypoxia

The acute and chronic hypoxic ovine studies highlight that various insults stimulate differential responses among anatomically distinct vascular beds. In chronically hypoxic fetuses, mRNA levels of procollagen I and III, TGF-β_1, MMP-2 and MTI-MMP in the superior mesenteric artery were not different from normoxic fetuses. The fact that the superior mesenteric

Figure 5.3 Effect of Chronic Hypoxia on Collagen I and III mRNA levels in the Left and Right Ventricle

Collagen III mRNA levels in the right ventricle of severely hypoxic ovine fetuses, as measured by Real-time PCR, were increased relative to control fetuses, while levels between control and moderate fetuses were not different (A). No difference in mRNA levels of collagen I mRNA were found between the groups, although a trend toward an increase in hypoxic animals was observed (B).

* $p < .05$


A
Collagen Type III
mRNA levels relative to control
0
1
2
3
4
5
*
Left Ventricle
Right Ventricle
Control
Moderate
Severe

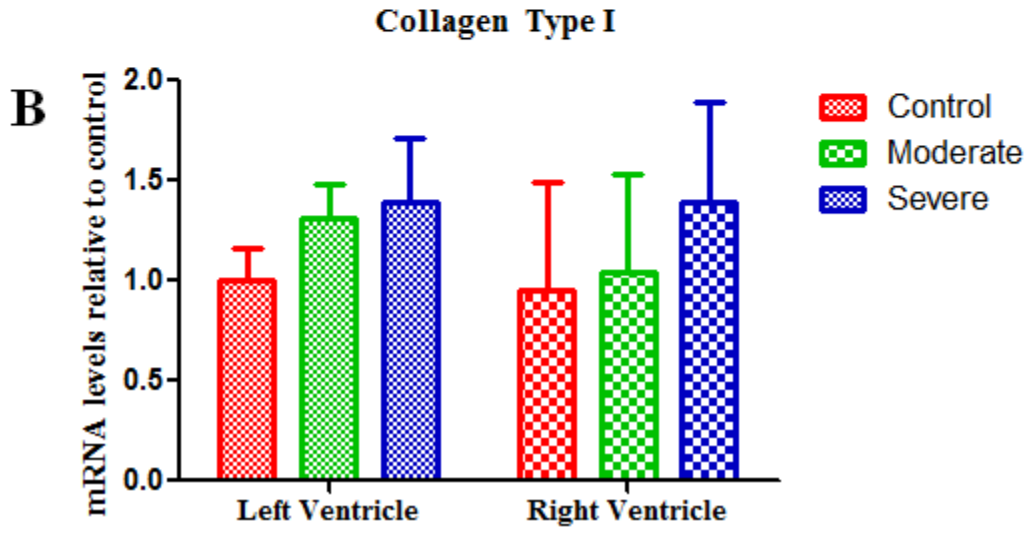
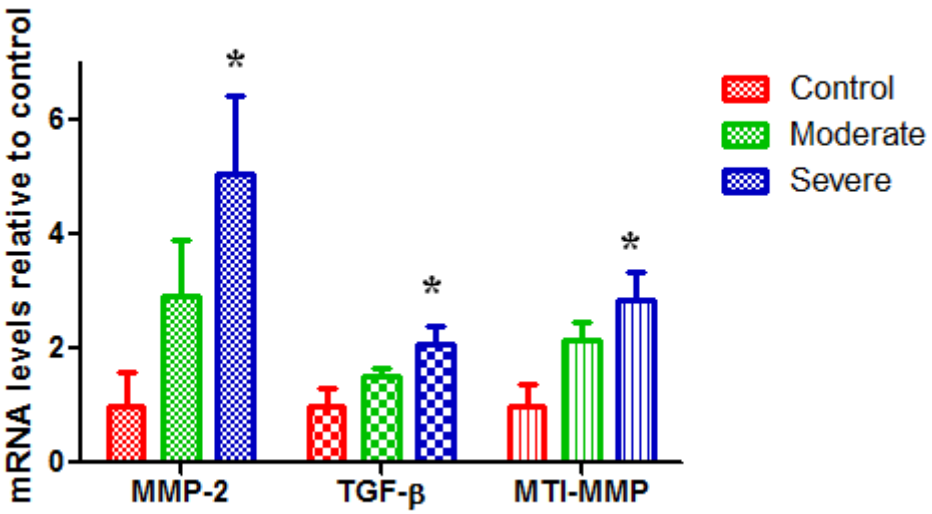

B
Collagen Type I
mRNA levels relative to control
0.0
0.5
1.0
1.5
2.0
Left Ventricle
Right Ventricle
Control
Moderate
Severe

Figure 5.4 Effect of Chronic Hypoxia on TGF-β_1, MMP-2 and MTI-MMP mRNA levels in Right Ventricle

In the right ventricle of severely hypoxic animals the mRNA levels of TGF-β_1, matrix metalloproteinase (MMP-2) and its activator, membrane-type MMP (MTI-MMP), are increased compared to control.

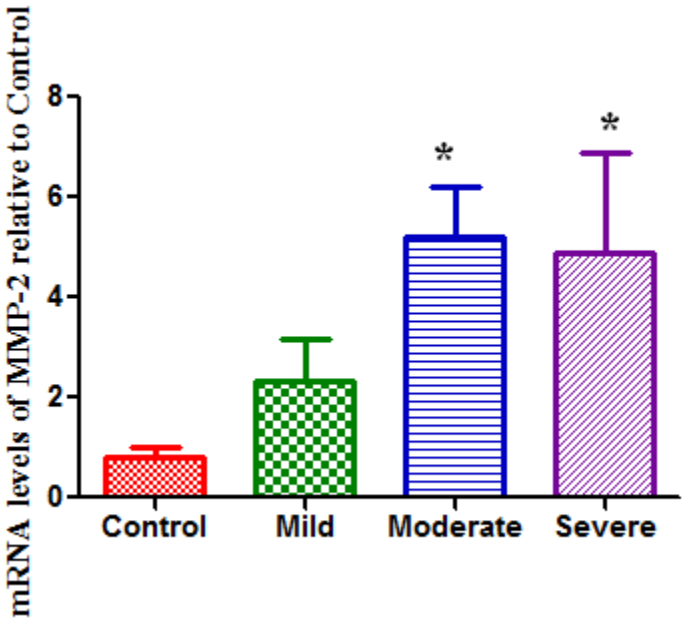
* $p < .05$


mRNA levels relative to control
0
2
4
6
*
*
*
MMP-2
TGF-β
MTI-MMP
Control
Moderate
Severe

Appendix 5.5 Effect of Acute Hyoxia on mRNA levels of MMP-2 in the Right Ventricle

In the right ventricle, mRNA levels of matrix metalloproteinase (MMP-2) are elevated in ovine fetal sheep subjected to moderate and severe umbilical cord occlusion (UCO), compared to control animals.

* $p < .05$


mRNA levels of MMP-2 relative to Control
8
6
4
2
0
*
*
Control
Mild
Moderate
Severe

artery is an atherosclerotic-resistant artery, not prone to intima hyperplasia as is the aorta (Stary *et al*., 1992), suggests that cellular responses including the process of endothelial activation by MMP-2 are not induced under conditions of hypoxia, injury or oxidative stress. Perhaps, cellular proliferation or hypertrophy occurred in the superior mesenteric artery in response to hypoxia, since it is a muscular artery. Furthermore, differential responses depending on the type and location of the arterial segment was also shown in the acute UCO study, wherein elastin deposition was induced in the carotid artery with no changes observed in the superior mesenteric artery.

5.2 CONCLUSIONS

We provide evidence that the propensity for hypertension and CVD in IUGR offspring may be directly linked to disturbances in aortic development characterized by altered ECM composition and intima thickening, at least in the case of placental insufficiency. These changes occur in concert with induction of TGF-β, MMP-2 and E-selectin, molecular mediators of the inflammatory vascular response and adverse remodeling. Further, the guinea pig study provides evidence that this aberrant aortic development has long-term structural and functional consequences in terms of aortic compliance. Thus, we reveal aortic stiffening to be an important link between CVD in adulthood and hypoxic intrauterine insults. We demonstrate that the cellular response of the superior mesenteric artery to chronic intrauterine hypoxia differs from that of the aorta, while the cellular response of the right ventricle mirrors that of the aorta. As well, our studies not only demonstrate differential responses to varying degrees of hypoxia, but also show differential responses to acute versus chronic hypoxia. Whereas the sheep and guinea pig chronic hypoxic models provide evidence for a mechanistic link to developmental origins of CVD, the acute hypoxic study suggests that an intrauterine insult can result in programming of a beneficial phenotype rather than a vulnerable phenotype.

5.3 FUTURE DIRECTIONS

It is now widely acknowledged that the intrauterine environment plays a significant role in long-term cardiovascular health. This field of study has put forward a novel aspect of CVD which warrants a redefinition of the long established paradigm that presumes etiology to stem from a combination of genetics and postnatal lifestyle factors. Indeed, the associations between birth weight and chronic adult disease are independent of these traditional risk factors. Yet, clinical risk profiling and guidelines for preventative strategies currently do not take into account environmental triggers imposed before birth. In fact, individuals born from compromised pregnancies are not identified as high risk targets in the American Heart Association's impact goals for 2020 (Lloyd-Jones, 2010). Pre-birth factors may account for the portion of CVD left unexplained by those large scale analyses such as the Framington Heart Study (Bitton and Gaziano, 2010), which have characterized risk distribution within populations. Hence, early and aggressive preventative measures in those born susceptible may alleviate the burden of CVD. We show that changes in elastic fibres of the umbilical artery are reflective of changes in the aorta, both related to the degree of hypoxia. Thus, the umbilical artery which can be harvested at birth in human pregnancies may be useful as a marker of vascular programming and thereby identify fetuses that may benefit from early preventative measures. Further investigation into potential markers of perturbed cardiovascular development that can be used clinically, such as circulating levels of TIMPs and MMPs in umbilical blood, is warranted.

Before we can begin to integrate fetal experience into current preventative policy and clinical practice, a deeper understanding of the phenotypic outcomes and underlying molecular mechanisms is paramount. Our data provide important insight into the vascular phenotype of hypoxic IUGR fetuses, the molecular mediators involved in this outcome and the long-term functional consequences. Further investigation is required to identify upstream regulators of the vascular and cardiac response to hypoxia during fetal life. Possible upstream regulators are hormones such as angiotensin II, reactive oxygen species due to oxidative stress, or direct effects of oxygen tension may play a role. Once a complete picture of the cellular response is uncovered, potential prenatal inventions such as maternal administration of antioxidants can be tested. Another finding of the thesis that implores further investigation is the premature intima

thickening in aortae of hypoxic ovine fetuses. It would be interesting to determine whether this process is indeed associated with endothelial damage and leads to acceleration of the atherosclerotic process in postnatal life.

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Appendix 1. Primer Table

Gene	Primer (5' - 3')	Reference no
Tropoelastin	FWD CCTTGGAGGTGTGTCTCCAG REV GAACGTTGATGAGGTCGTGA	M26188.1
Collagen Iα2	FWD GCTCTGCGACACAAGGAGTC REV AGGATTGGCATGTTGCTAGG	NM_000089
Collagen III	FWD CATTCTTTGAATCCTAGCCCAT REV CCAGGTTGAGGTAGGGTGAA	NM_000090
MMP-2	FWD CTGATGGCGCCCATTTATAC REV GATGAACCGGTCCTTGAAGA	NM_001166180.1
MTI-MMP	FWD CCATCATGGCACCCTTTTAC REV CAAACATCTCCCCTCGAAGC	NM_001166181.1
TGF-β	FWD GCACGTGGAGCTGTACCAGAA REV GACGTCAAAGGACAGCCACTC	NM_001009400
E-Selectin	FWD CTCCCCGTCCAAGAACTACA REV CGCCTCTACCTGTCCTTGAG	Nm_001145667
15S	FWD ATCATTCTGCCCGAGATGGTG REV TGCTTTACGGGCTTGTAGGTG	AY949774

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Appendix 3. Ethics protocol (Chapters 2 & 4)

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, _ London, Ontario _ CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 _ FL 519-661-2028 _ www.uwo.ca / animal
10.01.08
***This is the 3rd Renewal of this protocol**
*A Full Protocol submission will be required in **2009**
Dear Dr. **Richardson**
Your Animal Use Protocol form entitled:
Fetal Brain Development: The Impact of Acute and Chronic Hypoxia
has had its yearly renewal approved by the Animal Use Subcommittee.
This approval is valid from **10.01.08 to 09.30.09**
The protocol number for this project remains as **2005-061**
1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
c. . Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.
REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. **B Matushewski, W Lagerwerf**

Appendix 4. Ethics protocol (Chapter 3)

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, _ London, Ontario _ CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 _ FL 519-661-2028 _ www.uwo.ca / animal

06.03.2010

This is the Original Approval for this protocol

*A Full Protocol submission will be required in
06.30.2014*

Dear Dr. Regnault:

Your Animal Use Protocol form entitled:

In Utero Origins of Adult Insulin Resistance

Funding Agency CIHR - Grant #R3826A09

has been approved by the University Council on Animal Care. This approval is valid from **06.03.2010 to 06.30.2011.** The protocol number for this project is **2010-229**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.

If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species Strain Other Detail	**Pain Level**	**Animal # Total for 4 Years**
Guinea Pig Hartley Pregnant ~25 Days on Arrival C	556	

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components
(biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and
have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. **Approved - T. Regnault, W. Lagerwerf**

CURRICULUM VITAE

Jennifer Anne Thompson

EDUCATION

Doctorate (PhD) Physiology

- The University of Western Ontario, Canada, Expected to receive 08/2011. Supervisors: Drs Tim Regnault, Bryan Richardson. Thesis entitled: *The effect of fetal hypoxia on cardiovascular structure and function.*

Master's (MSc) Kinesiology

- The University of Western Ontario, Canada, received 08/2005. Supervisor: Dr. Michelle Mottola. Thesis entitled: *The Acute cardiovascular response to low intensity resistance training in the antenatal hospitalized patient.*

Bachelor's, Honours (BA) Kinesiology

- The University of Western Ontario, Canada, received 04/2004. Concentration: Exercise Physiology. Honours thesis entitled: *Effect of low body negative pressure on arterial compliance.*

ACADEMIC/EMPLOYMENT HISTORY

09/2007 – present **Tutorial Instructor**

- Physiology 130/1021, Department of Physiology and Pharmacology, The University of Western Ontario

09/2007 – 04/2008 **Assistant supervisor**

- Laboratory Experience 480, The University of Western Ontario

01/2006 – 04/2006 **Teacher's Assistant**

- Biochemistry and Exercise ,
  Faculty of Health Science, The University of Western Ontario

09/2005 – 12/2005 **Tutorial Instructor**

- Anatomy 221, Faculty of Health Sciences,
  The University of Western Ontario

05/2005-09/2005 **Research Assistant**

- Exercise and Pregnancy Laboratory, School of
  Kinesiology, The University of Western Ontario

09/2004-05/2005 **Teacher's Assistant**

- Anatomy, Faculty of Health Sciences
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05/2004-09/2004 **Research Assistant**

- Neurovascular Laboratory, School of
  Kinesiology, The University of Western Ontario

AWARDS, HONOURS, FELLOWSHIPS, SCHOLARSHIPS

Scholarships

Ontario Graduate Scholarship in Science and Technology, 2010/2011

Studentship; Lawson Health Research Institute, 2010/2011 (Declined)

Ontario Graduate Scholarship, 2009/2010

Schulich Scholarship for Medical Research, 2008-2010

Western Graduate Research Scholarship, 2006-2010

Graduate Student Enrichment Program, CHRI, 2006

Awards

Graduate Thesis Research Award, The University of Western Ontario, 2011

Travel Award, Perinatal Biology Conference, Aspen, Colorado, 2010

New Investigator's Award for Best Oral Presentation: Society for Gynecological Investigation 57th Annual Meeting, 2010 Orlando, Florida

Travel Award, Canada Institutes of Health Research (CIHR): Institute of Human Development, Child and Youth Health (IHDCY), 2009

Graduate Thesis Research Award, The University of Western Ontario, 2009

Peggy Collins Memorial Award, The University of Western Ontario, 2003

Honours

Dean's Honour List, The University of Western Ontario, 05/2005

COMMITTEES

Society of Graduate School Council, 2005

PUBLICATIONS AND REVIEWS

Peer Reviewed Original Articles in Press

Thompson JA, Richardson BS, Gagnon R, Regnault TRH. Chronic intrauterine hypoxia interferes with aortic development in the late gestation ovine fetus. *Journal of Physiology.* 589 (Pt 13); 3319-3332, 2011.

Thompson JA Folliot SA, Richardson BS, Gagnon R, Regnault TRH. The Effect of Intermittent Umbilical Cord Occlusion on Elastin Accumulation in the Ovine Fetus. *Reproductive Sciences*. Accepted Nov 2010

Thompson JA, Gros R, Piorkowski K, Richardson BS, Regnault TRH. Central arterial stiffening in adulthood linked to aberrant aortic remodeling under suboptimal intrauterine conditions. *American Journal of Physiology Integrative, Comparative and Regulatory Physiology*. Accepted Sept 2011

Invited Reviews in Press

Thompson JA and Regnault TRH. In utero origins of the metabolic syndrome. *Seminars in Reproductive Medicine*. 29 (3); 211-224, 2011.

Currently Submitted Manuscript and Reviews in Review

Peer Reviewed Abstracts

Thompson JA Fetal programming of vascular dysfunction: role of cytokines and endothelial activation. *Reproductive Sciences.*

Thompson JA, Richardson BS, Regnault TH. Hypoxic-related changes in collagen I, III and MMP-2 mRNA levels of the heart and aorta in the late gestation fetus. *Reproductive Sciences.* Vol 17(3) supp. 2010, 346.

Thompson JA, Richardson BS, Gagnon R, Regnault TH. Vascular structure in an ovine model of placental insufficiency. *Reproductive Sciences.* Vol 16(3) supp. 2009

Thompson JA, Richardson BS, Regnault TH, Gagnon R. Intermittent umbilical cord occlusions alter developmental deposition of elastin in the carotid artery. *Reproductive Sciences.* 15(1) supp. 2008, 127.

Thompson JA, Mitchell GF, Gratton R, Mottola MF. The acute affect of exercise intervention on arterial compliance in hospitalized antenatal women. *Journal of the American College of Sports Medicine.* 39(5) supp. 2007, S271.

Kilmer CA, **Thompson JA**, Shoemaker JK, Rice C, Marsh GD. Blood flow in the human anterior tibial artery following ischemic isometric exercise. *Medicine and Science in Sports and Exercise*. 37(5) supp. May 2005, p S222.

ORAL PRESENTATIONS

Invited Presentations

Thompson JA Cardiovascular development in the chronically hypoxic fetus. Oregon Health and Science University, Department of Cardiology. Portland, Oregon, Sept 2010

International Presentations

Thompson JA Fetal programming of vascular dysfunction: role of cytokines and endothelial activation. Society for Gynecological Investigation 58th Annual Meeting. Miami, Florida. March 2011

Thompson JA Hypoxic-related changes in collagen I, III and MMP-2 mRNA expression in the heart and aorta in the late gestation fetus. Society for Gynecological Investigation 57th Annual Meeting. Orlando Florida, March 2010 **** Best oral presentation**

Thompson JA Aortic protein content and wall thickness in the chronically hypoxic fetus. 56th Annual Meeting of the Society for Gynecological Investigation. Glasgow, Scotland, March 2009

Thompson JA Vascular structure in an ovine model of placental insufficiency. 35th Annual Meeting of the Fetal and Neonatal Physiological Society. Maastricht, the Netherlands, June 2008

National Presentations

Thompson JA Placental insufficiency leads to aberrant aortic remodeling. Perinatal Investigator's Meeting. Kingston, Ontario, Nov 2010

Thompson JA Perturbations in vascular remodeling induced by chronic hypoxia lead to arterial stiffening in adulthood. 21st Annual Spring Research Meeting of the Ontario Hypertensive Society. Nattawasaga, Ontario, May 2010

Thompson JA Perturbations in vascular remodeling induced by chronic hypoxia lead to arterial stiffening in adulthood. Obstetrics/Gynecology Department Paul Hardy Research Day. London, Ontario May 2010

Thompson JA Fetal programming of CVD: placental insufficiency. Department of Physiology and Pharmacology. London, Ontario, March 2010

Thompson JA Vascular development in the chronically hypoxic ovine fetus. 20th Annual Spring Research Meeting of the Ontario Hypertensive Society, Peterborough Ontario, May 2009

Thompson JA Vascular development in the chronically hypoxic ovine fetus. TOFS series. London, Ontario, April 2009

Thompson JA Intermittent umbilical cord occlusion alters developmental deposition of elastin in the carotid artery, TOFS series. London, Ontario, Feb 2008

Poster Presentations (National and International)

Thompson JA Aortic stiffening in growth restricted offspring linked to altered remodeling in the womb. Society for Gynecological Investigation 58th Annual Meeting. Miami, Florida, March 2011

Thompson JA Structural abnormalities induced in utero lead to vascular dysfunction in adulthood. Perinatal Biology Conference. Aspen, Colorado, Aug 2010

Thompson JA Aortic protein content and wall thickness in the chronically hypoxic fetus. 32nd Annual Perinatal Investigators Meeting, Kingston, Ontario, Nov 2008

Thompson JA Vascular structure in an ovine model of placental insufficiency. Developmental Origins of Health and Disease Symposium, Ann Arbor, Michigan, Oct 2008 **** winner for best poster**

Thompson JA Intermittent umbilical cord occlusion alters developmental deposition of elastin in the carotid artery. 55th Annual Meeting of the Society for Gynecological Investigation, San Diego, California, March 2008

Thompson JA The acute affect of an exercise intervention on arterial compliance in hospitalized antenatal women. 54th Annual Meeting of the American College of Sports Medicine, New Orleans, Louisiana, May, 2007

ADDITIONAL TRAINING

Member of toastmasters 2005 – 2007