Cardiovascular Effects of Aerobic Exercise Training in Hypoxic-induced Intrauterine Growth Restriction

by

Laura Marcela Reyes Martinez

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

University of Alberta

Abstract

Fetal hypoxia is one of the most common consequences of complicated pregnancies worldwide. It has been demonstrated that prenatal hypoxia leads to intrauterine growth restriction (IUGR). Being born growth restricted is associated with a decrease in cardiomyocyte proliferation, an increased susceptibility to cardiac ischemia/reperfusion (I/R) injury and impaired endothelial-dependent vascular function later in life, demonstrating that fetal environment during early development is important for cardiovascular health. Both I/R injury and endothelial dysfunction in hypoxic-induced IUGR offspring have been associated with an increase in the production of reactive oxygen species. Moreover, TNF-related weak inducer of apoptosis (TWEAK) induces cardiomyocyte proliferation through activation of the fibroblast growth factor-inducible molecule 14 (Fn-14) receptor. The TWEAK/Fn-14 pathway has not being studied in hypoxia-induced IUGR offspring. Early interventions are needed to ultimately reduce later life risk for cardiovascular disease. We tested whether aerobic exercise prevents the development of cardiovascular diseases in hypoxic-induced IUGR offspring. In addition, we tested whether the TWEAK/Fn-14 pathway play a role in cardiomyocyte proliferation, and this is associated with an increase susceptibility to cardiac I/R injury. Pregnant Sprague Dawley rats were exposed to control (21% oxygen) or hypoxia (11% oxygen) conditions from gestational day 15 to 21. Male and female offspring from normoxic (control) and hypoxic (IUGR) pregnancies were randomized at 10 weeks of age to either an exercise-trained or sedentary group. After acclimatization, rats ran on a treadmill for 6 weeks; 5 days/week, 30 min/day at 20 m/min. Twenty-four hours after the last bout of exercise, animals were euthanized and concentration response curves to phenylephrine and methylcholine were performed in second order mesenteric and

gastrocnemius muscle arteries, in the presence or absence of L-NAME (100 μM), MnTBAP (10 μM), apamin (0.1 μM) and TRAM-34 (10 μM), or indomethacin (5 μM). On the same experimental day, *ex vivo* cardiac function was determined using a working heart preparation. Hearts were perfused for 10 min in retrograde Langendorff mode, and then switched to working heart mode. Global, normothermic flow ischemia was induced for 10 min. Following ischemia, hearts were reperfused for 40 min. Superoxide production in cardiac tissue was assessed. In a second set of experiments, ventricular cardiomyocytes were isolated at postnatal day one. Proliferation and protein expression of Fn-14 were determined. Cardiomyocyte proliferation was also assessed in the presence or absence of TWEAK. Aerobic exercise training improved endothelium-derived hyperpolarization-mediated vasodilation only in IUGR male offspring. Moreover, aerobic exercise training improved baseline cardiac performance and decreased superoxide generation in male control offspring while in hypoxic-induced IUGR offspring the opposite effect was observed. There was no effect of IUGR or exercise on cardiac or vascular function in female offspring. Being born growth restricted was not associated with differences in the Fn-14 protein expression or cardiomyocyte proliferation. After being in culture for 72-hours, cardiomyocytes from IUGR male offspring had a decreased proliferation compared to controls. Our findings demonstrated that in IUGR populations, a common preventive strategy such as aerobic exercise may represent a secondary stressor to the cardiovascular physiology. The results from the present study also highlight that when examining the mechanisms by which exercise impacts the cardiovascular system in a susceptible population, sexual dimorphism must be considered.

Preface

This thesis is an original work by Laura Marcela Reyes Martinez. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "PREGNANCY COMPLICATIONS", No. AUP00000242, 3 May 2016.

Portions of Chapters 2 and Chapter 3 of this thesis have been published as "Reyes LM, Morton JS, Kirschenman R, DeLorey DS, Davidge ST. Vascular effects of aerobic exercise training in rat adult offspring exposed to hypoxia-induced intrauterine growth restriction. Journal of Physiology. 2015 Apr 15;593(8):1913-29". Reyes LM was responsible for the data collection and analysis as well as the manuscript composition. Morton JS assisted with the data collection and contributed to manuscript edits. Kirschenman R assisted with the data collection. DeLorey DS assisted with the exercise protocol. Davidge ST was the supervisory author and was involved with concept formation and manuscript composition.

Chapter 4 of this thesis has been published as "Reyes LM, Kirschenman R, Quon A, Morton JS, Shah A, Davidge ST. Aerobic exercise training reduces cardiac function in adult male offspring exposed to prenatal hypoxia. American Journal of Physiology Regulatory, Integrative and Comparative Physiology. 2015; 309: R489-98". Reyes LM was responsible for the data collection and analysis as well as the manuscript composition. Morton JS assisted with the data collection and contributed to manuscript edits. Kirschenman R, Quon A and Shah A assisted with the data collection. Davidge ST was the supervisory author and was involved with concept formation and manuscript composition.

Dedication

To David and Mateo

Acknowledgments

I consider myself a very lucky person. I had the opportunity of working under the supervision of Dr. Patricio López-Jaramillo and Dr. Ronald Garcia in Colombia, with whom I learned epidemiology and discovered my love for research and science. Working with Dr. López-Jaramillo also gave me the chance to meet one of his multiple collaborators, Dr. Sandra Davidge. Sandy, since I joined your lab, you have been a great role model and a mentor to me. I am very grateful for all you have done for me. During these years, I have not only learned about basic science in one of the most prestigious labs in the world, but I have also learned that hard work and passion is what drives an entire career. It has been a privilege to be part of the Davidge lab. Here I have met my dearest colleagues and friends. I have laughed and cried, I have pushed myself to my own limits; sometimes I was successful, and many times I was not, but I always have people that back me up, and I am very honoured to have worked with Jude, Jo, Meghan, Irene, Yan Yang, Subhadeep, Stephane, Rajan, Linn, Christian, Raven, Anita, Alison, Amin, Floor, Cindy, Lesley and Donna. I am also very thankful to my supervisory committee members, Dr. Zam Kassiri and Dr. Darren DeLorey for their input towards my research and for the invaluable feedback. I would also like to acknowledge my candidacy committee members Dr. Margie Davenport and Dr. Peter Mitchell for their mentorship. I would like to acknowledge the Faculty of Medicine and Dentistry, the Mazankowski Heart Institute and the Alberta Innovates Health Solutions, because none of my research would have been possible without the funding support I received from them through the scholarships I have won over the course of my PhD. The Department of Physiology also deserves a special mention for the opportunities given to me to keep my education up to the best quality standards. Finally, I would like to acknowledge my

friends and family for all their support during these years. They understood when I was not there for their celebrations or when they reached their own achievements, they supported me no matter what I was doing, and they were always there to listen to me. Last, but not least, I would like to recognize the role of my husband during my PhD. David you are the strength I need to wake up every day. You have always believed in me, you changed your life completely just to help me achieve what I believe is one of the biggest accomplishments of my life. You have always been there for me, you have heard my talks, and have learned what my research is all about, and now you know the importance of a healthy pregnancy, and reminded me of that every moment of our own pregnancy. You have taught me work ethics, and how to stand up for myself even when I was feeling vulnerable. You teach me every day how to overcome my fears, and have been more than patient with me, for that I will always be grateful. My PhD is certainly a combined effort. I would like to finish with something that Sandy always says, “it takes a village to raise a child”. I am very lucky to have been raised by so many good people. I wish you all the best, and I will be forever thankful.

Table of Contents

List of tables

List of figures

List of abbreviations

The following abbreviations have been used throughout this thesis.

Abbreviation	Meaning
ABG	Abdominal girth
ACh	Acetylcholine
AET	Aerobic exercise training
AHA	American Heart Association
Ang II	Angiotensin II
AT_1	Angiotensin II receptor 1
AT_{1a}	Angiotensin II receptor 1a
AT_2	Angiotensin II receptor 2
ATP	Adenosine triphosphate
AUC	Area under the curve
AV	Atrioventricular
BK_{Ca}	Large conductance calcium-activated potassium channel
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CCRC	Cumulative concentration response curve
CDK-2	Cyclin-dependent kinase 2
CVD	Cardiovascular diseases
cGMP	Cyclic guanosine monophosphate
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRL	Crown-rump length
DAG	Diacylglycerol
DHE	Dihydroethidium

EC_{80}	Concentration producing 80% of the maximum response
ECE	Endothelin converting enzyme
ECM	Extracellular matrix
EDH	Endothelium-dependent hyperpolarization
E_{max}	Maximum response
eNOS	Endothelial nitric oxide synthase
ETTs	Epoxyeicosatrienoic acids
ET-1	Endothelin-1
ET_A	Endothelin-1 receptor A
ET_B	Endothelin-1 receptor B
Egr-1	Early growth response factor-1
ERK 1	Extracellular signal-regulated kinase 1
ERK 2	Extracellular signal-regulated kinase 2
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
Fn-14	Fibroblast growth factor-inducible 14
GD	Gestational day
GHS	Reduced glutathione
GPx	Glutathione peroxidase
GR	Glucocorticoid receptor
GRx	Glutathione reductase
GSSG	Oxidized glutathione
GSK-3β	Glycogen synthase kinase-3β
H_2O_2	Hydrogen peroxide
HBSS	Hanks' balanced salt solution
HRV	Heart rate variability
HSP-70	Heat shock protein-70

HUVECs	Human umbilical endothelial cells
IC_{100}	Internal circumference equivalent to a transmural pressure of 100 mmHg
IGF-1	Insulin-like growth factor-1
IK_{Ca}	Intermediate conductance calcium-activated potassium channels
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
IP_3R	Inositol 1,4,5-triphosphate receptor
I/R	Ischemia/reperfusion
IUGR	Intrauterine growth restriction
IVCT	Isovolumetric contraction time
IVRT	Isovolumetric relaxation time
IVS	Inter ventricular septum
JNK	c-Jun N-terminal kinase
K^+	Potassium
KCl	Potassium chloride
K_{ATP}	ATP-sensitive potassium channel
K_{IR}	Inward-rectifying potassium channels
K_V	Voltage-gated potassium channels
LDH	Lactate dehydrogenase
L-NAME	N_ω-Nitro-L-arginine methyl ester hydrochloride
LTCC	L-type calcium channel
LV	Left ventricle
LVAW	Left ventricular anterior wall
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular-end diastolic pressure
$LVID_{dias}$	Left ventricular internal diameter in diastole

$LVID_{sys}$	Left ventricular internal diameter in systole
LVPW	Left ventricular posterior wall
LVV_{dias}	Left ventricular volume in diastole
LVV_{sys}	Left ventricular volume in systole
MaxiK	Large conductance calcium-activated potassium channels
MCh	Methylcholine
MDA	Malondialdehyde
MEGJ	Myoendothelial gap junctions
MLCK	Myosin light-chain kinase
MLCP	Myosin light-chain phosphatase
MMPs	Matrix metalloproteinases
MnTBAP	Mn(III)tetrakis(4-benzoic acid)porphyrin chloride
Na^+	Sodium
NAC	N-acetylcysteine
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrate
NADPH	Nicotinamide adenine dinucleotide phosphate
NCX	Sodium/calcium exchangers
NE	Norepinephrine
NMN	Nicotinamide mononucleotide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
$O_2^{\cdot -}$	Superoxide
$^{\cdot}OH$	Hydroxyl radical
$^1O_2$	Singlet oxygen

OCT	Optimal cutting medium
$ONOO^-$	Peroxynitrite
PAT	Pulmonary acceleration time
PE	Phenylephrine
pEC_{50}	Negative log of the effective concentration producing 50% of the maximum response
PET	Pulmonary ejection time
PG	Prostaglandins
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PGD	Prostaglandin synthase D
PGD_2	Prostaglandin D_2
PGF	Prostaglandin synthase F
PGF_2	Prostaglandin F_2
PGG	Prostaglandin synthase G
PGG_2	Prostaglandin G_2
PGHS	Prostaglandin H synthase
PGH_2	Prostaglandin H_2
PGI	Prostaglandin synthase I
PGI_2	Prostaglandin I_2, prostacyclin
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PKCε	Protein kinase C epsilon
PLB	Phospholamban
PLC	Phospholipase C
PMCA	Plasma membrane calcium pump
PND-1	Postnatal day one
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
r-TWEAK	Recombinant TNF-related weak inducer of apoptosis
RyR	Ryanodine receptor
SA	Sinus node
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum calcium-ATPase
SK_{Ca}	Small conductance calcium-activated potassium channel
SNP	Sodium nitroprusside
SOD	Superoxide dismutase enzymes
SOD-1	Copper-Zinc superoxide dismutase
SOD-2	Manganese superoxide dismutase
SOD-3	Superoxide dismutase-3
s- TWEAK	Soluble TWEAK
TIMPs	Tissue inhibitor of matrix metalloproteinases
TWEAK	TNF-related weak inducer of apoptosis
TXA2	Thromboxane A2
VIP	Vasoactive intestinal peptide
VSMC	Vascular smooth muscle cells

General introduction

Every year, nearly eight million women have a pregnancy-related complication[1] that leads to suboptimal conditions *in utero*, affecting the growth of the fetus.[2] A fetus who has not achieved its genetic growth potential for its gestational age is considered growth restricted.[3] According to the Society of Obstetricians and Gynecologists of Canada, "a fetus with an estimated fetal weight < 10th percentile on ultrasound that, because of a pathologic process, has not attained its biologically determined growth potential is considered growth restricted."[4] It has been estimated that intrauterine growth restriction (IUGR) affects approximately five million newborns per year in developed countries, while this rate is six times higher in developing countries.[5] It is also known that at least two percent of neonatal deaths can be attributed to IUGR.[6] Although IUGR has a vast impact early in life; an increasing body of evidence published within the last three decades has demonstrated that IUGR can have long-term consequences on the cardiovascular system.[7-9] More importantly, since cardiovascular diseases (CVD), are considered to be the leading cause of mortality worldwide,[10] a therapeutic approach to reduce the healthcare burden of being born growth restricted is needed. In order to understand the mechanisms underlying the pathophysiology of this phenomenon, a review of the cardiovascular system and how it is affected by IUGR is presented here.

1.1 The developmental origins of adult health and disease and the cardiovascular disease pandemic

Among non-communicable diseases, CVD are the leading cause of mortality.[10] Although populations from low and middle-income countries, especially those coming

from rural areas, have a lower risk factor for CVD compared to high-income countries,[11] mortality following a cardiovascular event remains higher in low and middle-income countries.[11] In Canada, the economic impact from these diseases has been estimated to cost around $21 billion in 2000.[12]

Ischemic heart events (angina and myocardial infarction) are major components of CVDs. In developed countries such as the United States, it has been estimated that every 40 seconds a person will have a myocardial infarction.[13] According to the INTERHEART study, ischemic heart events are associated with nine risk factors including smoking, abnormal lipid profile, hypertension, diabetes, abdominal obesity, psychosocial factors, regular alcohol consumption, low consumption of vegetables and fruits and a lack of regular physical activity.[14] The World Health Organization has recognized that up to 25% of the global burden of disease is due to an erroneous lifestyle and behaviour.[15]

Although CVDs are attributed to the risk accumulation throughout an individual lifetime,[16] a strong association between a low birth weight and the development of CVD such as ischemic heart disease,[7] insulin resistance,[17] and hypertension[8] has led to the theory that adverse influences early in development can result in changes in the structure and physiology of key organs, which result in an increased risk of chronic disease in adulthood. Since the first associations between prenatal growth and the later risk of developing CVD were made by Dr. Barker;[7,9] multiple epidemiological studies have been conducted to determine the role of fetal programming in the development of other non-communicable diseases.[18] Due to the reproducibility of these data, the validity of these findings is generally accepted. Together these findings have raised important

considerations for addressing IUGR as a risk factor in the development of CVD later in life; especially in countries where pregnancy-related complications are common[16] and in countries where modification of lifestyle to a Westernized lifestyle is also rapidly increasing.[15]

1.2 Etiology of intrauterine growth restriction

The etiology of IUGR is multifactorial, exemplified by the fact that many of the potential risk factors likely interact with each other because the growth of the fetus *in utero* reflects a balance between the fetus, the mother and the placenta,[19] alterations in any of which could lead to IUGR.

1.2.1 Fetal causes of intrauterine growth restriction

Fetal causes of IUGR account for ten to twenty percent of the total number of IUGR babies.[20] The fetal genome is a crucial determinant of growth in early life.[21] Fetal disorders associated with IUGR include chromosomal abnormalities (i.e. trisomy 13, 18, 21, and 22);[21-24] perinatal infections from viral or protozoan origin (i.e. cytomegalovirus, rubella, parvovirus, herpes virus, toxoplasmosis, and malaria);[25,26] multiple gestations;[27] and fetal malformation (congenital heart disease, congenital diaphragmatic hernia, abdominal wall defects and anencephaly).[28]

The mechanisms associated with the fetal causes of IUGR are varied and often interrelated. It is likely that a compromised karyotype impairs normal cell division leading to a reduction in cell number and fetal growth.[21] Moreover, IUGR can be both the result and/or a reaction to the presence of malformations.[28] Whereas, in the presence of an infection, the mechanisms associated with the development of IUGR

include not only apoptosis and delayed cell division in the fetus,[19] but also placental alterations such as necrotic avascular villi, edema and leukocyte infiltration, and trophoblast necrosis.[29,30]

1.2.2 Maternal and placental causes of intrauterine growth restriction

During pregnancy, maternal cardiovascular adaptations, such as an increase in cardiac output and arterial compliance, alongside a decrease in total vascular resistance,[31] must occur concurrently with the migration of extravillous trophoblast cells from the villi to the uterine stroma, where they invade and transform the spiral arterioles[32] in order to provide an adequate uterine perfusion that meets the needs of the growing fetus. Maternal and placental causes of IUGR are associated with a reduced uteroplacental blood flow or a diffusion limitation (oxygen, nutrients);[33] representing an environmental challenge to the growing fetus.

Placental causes of IUGR include abnormal trophoblast invasion,[34] placental infarcts,[34] placenta previa[35] and umbilical-placental vascular anomalies (i.e. an isolated single umbilical artery).[36] On the other hand, the maternal causes of IUGR include nutritional causes (poor pregnancy weight gain; maternal nutritional deprivation);[37] hypoxic causes (moderate-severe asthma,[38] cyanotic heart disease,[39] sickle cell disease[40]); vascular causes (chronic hypertension,[41] preeclampsia,[42] type I diabetes[43]); renal causes (chronic glomerulonephritis or tubulointerstitial disease);[44] environmental causes (high altitude,[45] smoking,[46] substance abuse [alcohol,[47] heroin,[48] cocaine[49]]); and poor obstetric history (previous stillbirth,[50] recurrent miscarriage[51] and IUGR history [52]).

1.3 Animal models of intrauterine growth restriction

Large epidemiological studies have demonstrated that there is an association between being born growth restricted and the development of CVD later in life.[7-9,17,18] A variety of animal models have been developed to manipulate the environment of the dam in order to assess different outcomes in a feasible timeline. Thus, these animal models are useful tools to elucidate the mechanisms by which the fetal environment is linked to the development of diseases later in life.[53,54]

Different strategies have been used in order to reproduce an IUGR phenotype, including the following. 1) Nutritional interventions: these models are based on the fact that maternal nutrition is an important determinant of fetal outcome. Pregnant dams are subjected to either a restriction in the intake of the total amount of calories,[55] proteins[56] or iron;[57] or subjected to over nutrition.[58] 2) Fetal overexposure to glucocorticoids: maternal stress or exogenous administration of glucocorticoids will initiate tissue differentiation prematurely, thus, *in utero* alterations of fetal homeostasis will occur. These *in utero* adaptations are no longer needed after birth, thus a mismatch between the fetal adaptations and their postnatal environment will predispose these offspring to CVD later in life.[59] 3) Fetal infections: inducing growth restriction by infecting the placenta with viruses such as cytomegalovirus.[60] 4) Hypoxic intervention: Since the fetus has limited reserves to compensate for a reduced oxygen supply, hypoxia is one of the most common insults in pregnancy complications.[61] Moreover, hypoxia is a common denominator in many conditions that challenge fetal development. For instance, placental infarcts,[34] sickle cell disease,[40] chronic hypertension,[41] and

moderate-severe asthma,[38] all involve a reduced oxygen supply to the fetus. Hence, this thesis will focus on the impact of prenatal hypoxia on later-life CVD in offspring.

1.4 Models of hypoxic-induced intrauterine growth restriction

Inducing hypoxia as a method to develop adverse uterine conditions can be achieved through a variety of approaches including the use of a hypoxic chamber to reduce the fraction of maternal inspired oxygen, or by surgically altering utero-placental perfusion. The latter, however, also induces a reduction in the nutrient delivery to the fetus.[62] In the following sections, a brief overview of each of the hypoxia-induced IUGR animal models will be discussed.

1.4.1 Mechanical reduction of blood flow

1.4.1.1 Bilateral uterine artery ligation

Bilateral uterine artery ligation is the most commonly used surgical model in rabbits, rats or mice.[63] This model causes an ~20% decrease in fetal weight compared to controls, while litter size remains the same in both groups.[54] However, a maternal or fetal demise in addition to variability in the fetal weight within the same litter (fetuses closest to the ligation are lighter compared to others in the same horn) is also observed. These complications raise concerns regarding the variability of the data that could be obtained within the same litter.[64]

1.4.1.2 Single umbilical artery ligation

A method used in both sheep[65] and guinea pigs[66] is ligation of one of the two umbilical arteries. This is a model for IUGR and also preterm labor. The method

comprises of the ligation of one umbilical artery close to the fetal abdomen; following which, a partial infarction occurs in the placenta and subsequently its function is compromised.

1.4.1.3 Reduced uterine perfusion pressure

This method is used as a model of preeclampsia in rabbits,[67] sheep,[68] guinea pigs,[69] nonhuman primates[70] and rats.[71,72] The development of IUGR, however, has also been associated with this animal model.[72] The reduction in uterine perfusions is achieved by clipping the abdominal aorta and for some animals (e.g. rat) due to the compensatory flow to the placenta by an increase in ovarian flow, it also involves clipping of both left and right ovarian arteries.[73]

1.4.2 Uteroplacental embolization

The pregnant sheep has been used extensively as an animal model of pregnancy complications because of its many similarities to human pregnancies (e.g. organogenesis occurs during early gestation, similarity of the vascular tree in the placenta, non litter bearing) and its accessibility to surgical instrumentation.[53] In sheep, a method to reduce the placental surface area of nutrient and oxygen exchange was developed. It mimics late gestation onset IUGR, by injecting microspheres (15-30 μm) into the maternal placental bed via the descending aorta or the fetal placental bed via the fetal umbilical vein.[53,54]

1.4.3 Caruncle ctomy

Sheep have a cotyledonary placenta. Thus, multiple areas of placental attachment are formed. The fetal portions are known as cotyledons whereas the maternal contact

sites are known as caruncles, and are visible in the non-pregnant uterus. Carunclectomy is a surgical procedure in sheep that involves the excision of the caruncles prior to mating; reducing the growth of the placenta. With the fetuses becoming hypoxic and hypoglycemic.[74]

1.4.4 Hypoxic chamber

Pregnant animals (sheep,[75] rodents,[76-109] or rabbits[110]) are placed in chambers in which the partial fraction of oxygen inspired by the dam is controlled at a value between 6.5-14%. The advantages of using this technique include that the level of oxygen, duration and timing of the insult can be manipulated. Moreover, it can be used to cause both acute and chronic hypoxic insults. One of the major limitations of this model is that the dam also becomes hypoxic, which represent another stressor to the pregnancy itself. In response to the low oxygen levels, some dams demonstrate a reduced food intake under hypoxic conditions.[90,103] Since activity levels are also reduced in hypoxic conditions, however, it is likely that nutrient supply is appropriately matched to metabolic demand.

1.5 Cardiovascular consequences of being born growth restricted following a hypoxic insult

In response to hypoxia, a series of fetal cardiovascular adaptations occur to maintain perfusion to major organs (brain, heart, and adrenal glands) that includes: redistribution of cardiac output, bradycardia, and increased arterial blood pressure.[111] Increased blood supply to the brain, heart and adrenal glands occurs at the expense of supply to other organs (e.g. the gastrointestinal track, kidneys, and muscle); as

demonstrated by a decreased blood flow velocity and an increased flow resistance observed in the descending aorta.[112] Moreover, other responses such as hypoglycemia,[113] increased cortisol[114] and noradrenaline[53] also occur. These adaptations and responses to an abnormal fetal environment, however, may induce permanent programming and, when they are no longer needed in the neonatal period, represent a mismatched condition in neonatal life thereby increasing the risk of later morbidity.[115] For the purpose of this thesis, I will focus on the effects of hypoxia on the cardiovascular system of the offspring.

1.5.1 Heart development

The development of the heart is a complex morphological process that encompasses the formation of a 4-chamber organ from a singular tubular structure. In rats, this process starts at gestational day (GD) 9[116] and ends at postnatal day (PND) 21.[117] During fetal life, the heart enlarges through a process of hyperplasia, whereas during postnatal life myocardial cell growth and maturation arises in three phases: a) PND-1 to PND-4 myocardial growth is due to hyperplasia and most of the cells are mononucleated; b) PND-6 to PND-14 there is a transitional phase where both hyperplasia and hypertrophy are observed and a rapid transition from mono to binucleated cells (terminally differentiated cells) occurs; and finally c) PND-14 and older where myocardial growth is secondary to hypertrophy.[117] Cardiomyocyte proliferation during fetal life can be regulated by hemodynamic forces[118] and/or circulating factors such as angiotensin II (Ang II),[119] cortisol[120] and insulin-like growth factor-1 (IGF-1).[121] Interestingly, the above-mentioned factors are also altered in IUGR offspring.

1.5.1.1 *Hemodynamic forces altering cardiomyocyte proliferation and maturation in intrauterine growth restricted offspring*

The fetal circulation is designed to deliver most of its oxygenated blood to the myocardium and the brain. This is achieved by the presence of intra- and extra-cardiac shunts which makes the fetal circulation parallel.[122] During hypoxia, the fetal circulation adapts to increased blood flow resistance in the maternal compartment.[123] An increase in the right ventricle afterload with a decrease in the left ventricle afterload guarantees redistribution of the cardiac output.[123] Alterations in fetal hemodynamics following IUGR are associated with cardiac remodeling.[124] It has been shown that increased wall stress induces cardiomyocyte proliferation in the developing heart.[125] The increase in blood volume, and therefore in shear stress, in the developing heart caused by hypoxia has been associated with an increase in heart weight;[126] suggesting an increase either in cardiomyocyte proliferation or size. Bae *et al.* showed that fetal cardiomyocytes (GD 21) from Sprague Dawley dams exposed to hypoxia (10.5% O_2) during the last third of pregnancy had an increased proportion of binucleated cardiomyocytes (indicative of terminally differentiated cells), and an increase in the size of the binucleated cells (an early marker of hypertrophy).[91]

1.5.1.2 *The role of circulating factors on cardiomyocyte proliferation and maturation in intrauterine growth restricted offspring*

Multiple locally generated circulating factors, acting in an autocrine or a paracrine fashion, can play a role in cardiomyocyte proliferation and maturation.[127] The role of these factors, however, in hypoxic-induced IUGR offspring is less understood. For instance, Ang II is known to promote cell proliferation of cardiac fibroblasts,[128]

vascular smooth muscle cells (VSMC),[129] and mononucleated cardiomyocytes from sheep;[119] whereas in rodents it promotes cardiomyocyte hypertrophy through the activation of the Ang II receptor 1 (AT_1).[128] The renin-angiotensin system (RAS) exists in various tissues and organs that function independently. There is a wide range of alterations of the RAS in rodent hypoxic IUGR offspring, including: *a)* an increase in renal renin and angiotensinogen mRNA;[130] *b)* an increase in renal AT_{1a} mRNA in male offspring;[131] and *c)* an increase cardiac protein expression of the AT_1 receptor in female offspring and an increase in the cardiac protein expression of the AT_2 receptor in male offspring.[92] These findings suggest that being born following a hypoxic insult will have a dichotomous effect on cardiomyocyte proliferation in male vs. female offspring.

Another factor associated with cardiomyocyte proliferation is activation of the IGF-1 receptor.[132] Previous findings from a maternal fasting animal model[133] and after uterine artery ligation[134] suggest that being born growth restricted is associated with a decrease in serum levels of IGF-1. Interestingly, in hypoxic-induced IUGR offspring, there is no difference in IGF-1 serum levels.[93]

Finally, an alternative factor that has been implicated in cardiomyocyte proliferation is endothelin-1 (ET-1). Paradis *et al.* have shown that *in vivo* newborn anoxia decreased cardiomyocyte proliferation and increased ET-1 production in the heart.[135] The blockade of ET-1 receptor A (ET_A) restored cardiomyocyte proliferation.[135] Moreover, in hypoxic IUGR offspring, ET-1 was shown to stimulate binucleation and inhibit proliferation of fetal cardiomyocytes; which denotes a premature transition of mononucleated cells to binucleated cells (terminally differentiated cells).[94] These events were reverted by using a non-selective ET-1 receptor antagonist,[94] suggesting that the

mechanisms by which hypoxic IUGR offspring impacted cardiomyocyte proliferation and binucleation are ET_A-mediated.

1.5.1.3 TNF-related weak inducer of apoptosis (TWEAK) and its potential role in cardiomyocyte proliferation in intrauterine growth restricted offspring

As previously described, regulation of cardiomyocyte proliferation is a process that can be altered by numerous factors that are interrelated. In response to mitotic stimuli (e.g. ET-1, Ang II, IGF-1), Cyclin D and cyclin-dependent kinase 2 (CDK-2) accumulate in the nuclei, enabling cell cycle progression from the first G_1 phase to the S phase.[136] The progression of the cell cycle from S to G_2 phase requires the sequestration of p21 and p27 (inhibitors of CDK) by Cyclin D to allow activity of Cyclin A and E.[136]

Conflicting evidence suggests that there might be other pathways involved in the development of cardiac tissues in IUGR offspring. Novoyatleva *et al.* found that the TNF-related weak inducer of apoptosis (TWEAK) was also associated with neonatal cardiomyocyte proliferation.[137] TWEAK, a member of the TNF factor family, is a type II transmembrane protein that can be further processed into a soluble cytokine (s-TWEAK) by of furin.[138] TWEAK is considered to be a multifunctional cytokine and has been extensively associated with cell migration, survival, differentiation, in addition to death and proliferation of different cell types such as smooth muscle cells, epithelial cells, myoblasts and astrocytes.[139] In neonatal cardiomyocytes isolated from Sprague Dawley rats, binding of either TWEAK or s-TWEAK with its receptor (protein fibroblast growth factor-inducible 14 [Fn-14]), has been shown to activate extracellular signal-regulated kinase (ERK 1/2) and phosphatidylinositol 3-kinase (PI3K), as well as phosphorylate glycogen synthase kinase-3β (GSK-3β), causing cardiomyocyte

proliferation (Figure 1.1). This phenomenon was only found to be active during the early stages in life since the TWEAK/Fn-14 pathway becomes quiescent after PND-10 following a down-regulation of Fn-14 expression in cardiomyocytes with age.[137]

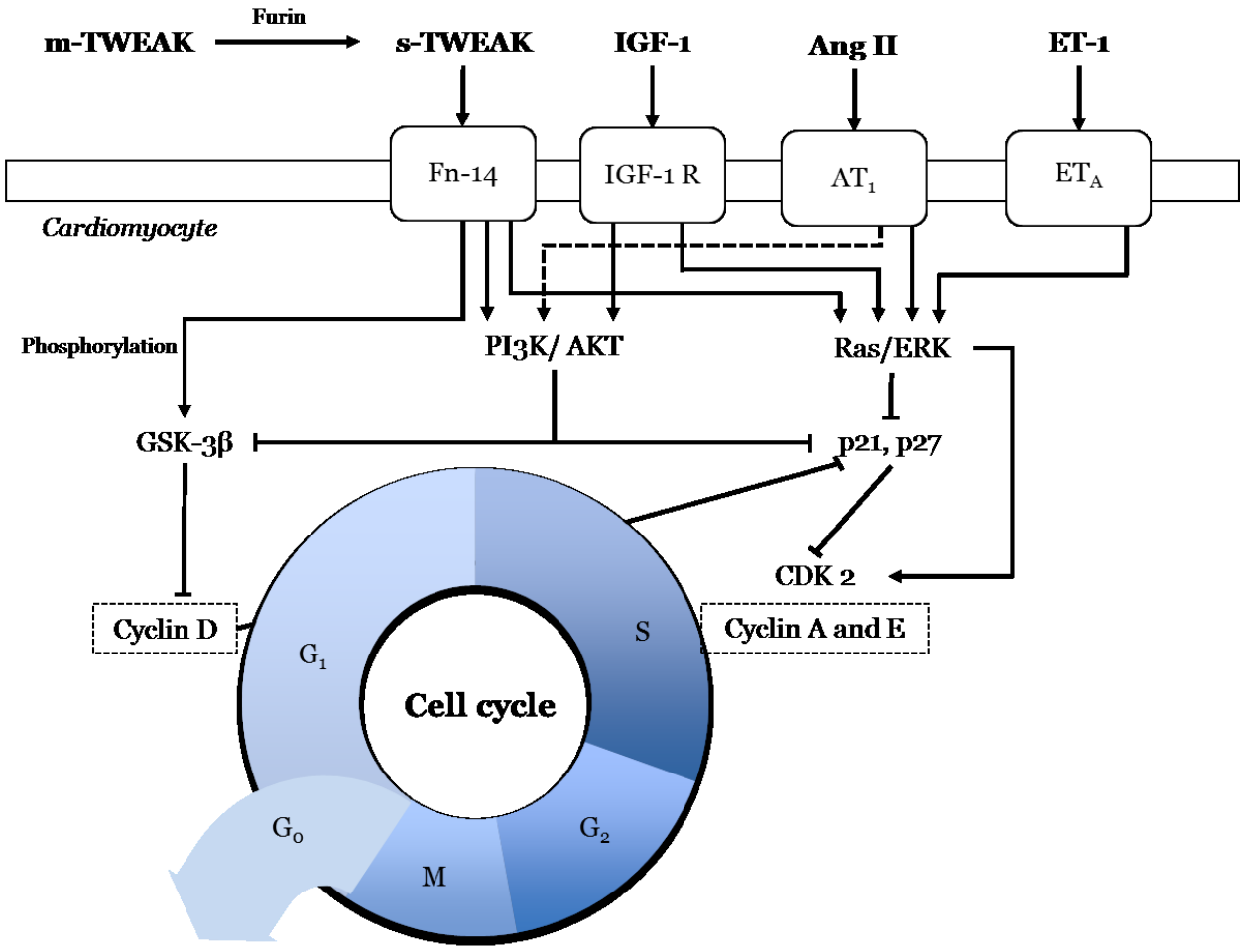

Figure 1.1 Signaling pathways involved in cardiomyocyte proliferation.
Ang II: Angiotensin; AT1: Ang II receptor 1; CDK 2: Cyclin-dependent kinase 2; ERK: extracellular signal-regulated kinase; ET-1: endothelin-1; ET_A: ET-1 receptor A; Fn-14: protein fibroblast growth factor-inducible 14; GSK-3β: glycogen synthase kinase-3β; IGF-1: insulin growth factor-1; IGF-1 R: insulin growth factor-1 receptor; m-TWEAK: membrane bound TNF-related weak inducer of apoptosis; PI3K: phosphatidylinositol 3-kinase; s-TWEAK: soluble TNF-related weak inducer of apoptosis. Modified from Botting *et al.* [136].

1.5.2 Heart structure

The heart consists of several cell types (cardiomyocytes, fibroblasts, endothelial cells, epicardial cells, smooth muscle cells, and pacemaker cells); which confer its structural, biochemical, mechanical and electrical properties. These cells are arranged in an cardiac extracellular matrix (ECM) which is a convoluted network of matrix proteins, signalling molecules, proteases and other cell types involved in tissue remodelling.[140] The ECM not only provides cardiac structural support, but also facilitates mechanical, electrical and chemical signalling during physiological or pathological stimuli.[140] An equilibrium between activity of the matrix metalloproteinases (MMPs; degrades collagen, elastin) and activity of their inhibitors (tissue inhibitor of MMPs (TIMPs)) is essential in cardiac remodelling,[141] and may be altered by prenatal hypoxia.

1.5.2.1 Cardiac remodelling in hypoxic-induced intrauterine growth restricted offspring

Hypoxic-induced IUGR offspring display an increase in both absolute heart weight and heart-to-body weight ratio. [88,90,91,95,103] This phenomenon could reflect either increased cardiomyocyte hypertrophy or fibrosis resulting from alterations in the expression of different components of ECM. Tong *et al.* found that neonatal hearts from hypoxic-induced IUGR offspring had an increased collagen I content with an increase in MMP-1 and -13 expression and an increase in the activity of TIMP-3 and -4.[96] These changes demonstrate a state where collagen I deposition exceeds its degradation. Soon after birth, the heart needs to adapt to changes in pressure and volume,[122] thus a continuous process of cardiac remodelling during postnatal development is necessary.

In alignment with the findings of Tong *et al.*, Xu *et al.* found that hypoxic IUGR adult offspring have an increase in cardiac collagen I and III deposition while MMP-2 expression was decreased.[89] Moreover, the ratio of β/α myosin heavy chain was increased in hypoxic IUGR adult offspring indicating ventricular remodelling and/or heart dysfunction.[89]

1.5.3 Physiology of postnatal cardiac function

It is necessary to understand cardiac physiology to comprehend the pathological implications of IUGR. The heart functions as a pump in the circulatory system. The right heart ejects blood through the pulmonary circulation to the lungs in order to oxygenate blood. The left heart ejects blood through the aorta and then the peripheral circulation throughout the body to supply organs and tissues with oxygen and nutrients. To do this, the heart combines mechanical and electrical stimuli to generate a cardiac cycle. Each cycle is initiated by the generation of an action potential in the sino-atrial (SA) node that travels through both atria and then through the atrioventricular (AV) bundle into the ventricles; allowing the atria to contract ahead of ventricular contraction.[142]

Changes in the polarization of the resting membrane of cardiomyocytes leads to cardiac contraction. In cardiac muscle, opening of fast sodium (Na^+) channels and voltage-activated calcium (Ca^{2+})-Na^+ channels initiates an action potential. Following this event, voltage-gated potassium (K^+) channels slowly open, thus flow of Ca^{2+} into the cell in combination with outflow of K^+ maintains a prolonged period of depolarization. A rapid loss of K^+ and closing of voltage-activated Ca^{2+}-Na^+ channels restores the membrane potential (repolarization). Subsequently, Ca^{2+} is released from the

sarcoplasmic reticulum. Intracellular Ca^{2+} then interacts with troponin to initiate cross-bridge formation and contraction.[142]

The cardiac cycle consists of a period of relaxation (diastole) followed by a period of contraction (systole). The cardiac cycle can be divided into 4 phases. Phase I (period of filling): there are three periods of filling during diastole. The first period consists of a rapid filling of the ventricles, blood flows from the veins to the atria, and about 80% of blood flows directly from the atria into the ventricles. In the second period, only small amount of blood flows into the ventricles. In the third period, the remaining 20% of blood is directed into the ventricles by contraction of the atria. Thus, in humans, the ventricular volume increases from 50 mL (blood that remains in the ventricle from the previous heartbeat, called end-systolic volume) to 120 mL (end-diastolic volume). Phase II (period of isovolumetric contraction): in this period contraction occurs in the ventricles but with no emptying, instead the pressure inside the ventricles increases (from 7 mmHg to 80 mmHg in the left ventricle and above 8 mmHg in the right ventricle) to close the atrioventricular valves and open the semilunar valves (aortic and pulmonary). Phase III (period of ejection): there are also three periods of systole. The first period is fast and about 70% of blood volume is ejected whereas in the last two periods the remaining 30% of the volume is ejected slowly. Phase IV (period of isovolumetric relaxation): at the end of systole, ventricular relaxation begins and allows the intraventricular pressure to decrease. The ventricular muscle relaxes with no changes in volume (period of isometric relaxation). The ventricles return to its starting point.[142]

The volume pump by the heart is regulated by the autonomic nervous system through chronotropism and inotropism. Parasympathetic nervous control of the heart comes from the Vagus nerve. Preganglionic efferent nerves fibers extend to the heart and synapse with ganglia located near the SA and AV nodes in the heart. Acetylcholine (ACh) is released and binds to nicotinic receptors and activates postganglionic efferent nerves. These fiber synapses with muscarinic receptors in the SA and AV nodes.[143] Sympathetic nervous control of the heart arises from the upper thoracic region of the spinal cord. Preganglionic efferent nerve fibers enter the paravertebral chains of ganglia (located on either side of the spinal column), and synapse with postganglionic sympathetic fibers and release ACh, which binds to nicotinic receptors. Sympathetic efferent fibers extend to the SA and AV nodes in the heart, releasing norepinephrine (NE) at synapses with β- adrenergic receptors.

1.5.3.1 Cardiac function in hypoxic-induced intrauterine growth restricted offspring

The functional implication of cardiac remodelling following a hypoxic pregnancy has also been assessed in the offspring of these pregnancies. Rueda-Clausen *et al.* used echocardiography to assess cardiac function *in vivo* and found that hypoxic IUGR male and female offspring had normal cardiac function in early adulthood. Once these animals aged, however, they found that both male and female offspring displayed signs of left ventricular diastolic dysfunction including increased isovolumetric relaxation time as well as an increased Tei index,[88] with no alterations in systolic function; suggesting that cardiac remodeling and stiffening impaired cardiac relaxation during diastole. Further, analyses of heart rate variability (HRV) in male hypoxic-induced IUGR offspring showed that there was an autonomic imbalance in these offspring where

there was a predominance of sympathetic over parasympathetic tone.[97] In addition, an increase in baroreflex gain was observed without any changes in basal heart rate or blood pressure.[97]

Cardiac function has also been assessed *ex vivo*. Using a modified Langendorff technique,[92,98-102,144,145] an aortic cannula is inserted into the ascending aorta and a perfusion solution is delivered to the heart in a retrograde manner via this cannula with subsequent perfusion of the coronary arteries.[146] Alternately, an isolated working heart preparation[88,89,107] has been used where the left atrium is cannulated and the perfusion solution passes from the atrium to the left ventricle where it is ejected via the aortic cannula. The coronary arteries are perfused (anterograde manner) in the course of ventricular ejection.[147] Discriminating by age, with these techniques, it has been determined that *a)* chick hypoxic IUGR embryos have a decreased left ventricular developed pressure (LVDP), a decreased myocardial contractility, decreased myocardial distensibility, and an increased left ventricular-end diastolic pressure (LVEDP).[144] Likewise, compared to controls, hearts from fetal IUGR sheep had an increased maximum rate of contraction and relaxation.[145] *b)* During early adulthood, there was either an increased myocardial contractility,[98] decreased LVDP and increased LVEDP in male hypoxic-induced IUGR offspring[89] or normal cardiac function.[92,99-102] Finally, *c)* with ageing, both male and female hypoxic-induced IUGR offspring had an increased LVEDP.[88] These findings suggest that cardiac function is impaired in hypoxic-induced IUGR offspring (alterations in cardiac contraction/relaxation, and presumably increased systemic vascular resistance with a reduction in stroke volume).

1.5.4 Physiology of vascular function

In order to understand the pathophysiological implications of IUGR in the vasculature, it is important to firstly demarcate the normal physiology of blood vessels. An artery is an elastic tube consisting of three layers: an intimal endothelial cell layer, a medial smooth muscle cell layer and an adventitial layer,[148] its main function is to deliver blood to different organs. Adequate tissue perfusion is attained by regulation of arterial tone; thus, arteries will vasodilate or vasoconstrict depending on tissue oxygen and nutrient demands. The regulation of these vascular responses comprises nervous mechanisms, together with endothelium-dependent, myogenic and humoral mechanisms, which vary among vascular beds.[149]

It has been determined that both sympathetic and parasympathetic neurons play a role in the regulation of vascular tone.[148-152] Upon sympathetic nerve stimulation, NE is released activating the α-adrenoceptors 1 and 2 in the VSMC and causing arterial vasoconstriction.[150] Other co-transmitters such as neuropeptide Y (NPY) and adenosine triphosphate (ATP) are vasoconstrictor molecules that are released together with NE. ATP activates P2 purinergic receptors, whereas NPY activates Y1 receptors in the VSMC.[150]

Since parasympathetic innervation of most peripheral resistance arteries is limited, the contribution of the parasympathetic nervous system to the regulation of vascular tone and hemodynamics is small.[148] It is important to note, however, that after a parasympathetic stimulus, ACh (one of the parasympathetic neurotransmitters)

triggers muscarinic receptors on either the VSMC (generating a vasoconstrictor response) or on endothelial cells (generating a vasodilator response).[152]

1.5.4.1 Endothelium-dependent vascular tone regulation

A delicate balance between vasodilator and vasoconstrictor mechanisms is needed in order to control vascular tone. Both vasodilation and vasoconstriction begin by the activation of one or multiple receptor-mediated pathways within the cell or by changes in intracellular Ca^{2+} concentration in the endothelial cells (vasodilation[153]), or in the VSMC (vasoconstriction[154]).

Vasodilator mechanisms

Multiple stimuli are known to initiate a vasodilator response including: shear stress, ACh, histamine, thrombin, serotonin, substance P, and bradykinin.[153] Endothelium-dependent vasodilation is primarily mediated via NO,[155] prostaglandin I2 (prostacyclin, PGI_2),[156] and endothelium-dependent hyperpolarization (EDH).[157] The relative contribution of each vasoactive agent depends on the species and strain of the experimental animal, its age and sex, its health status and the vascular bed examined (Figure 1.2).[149]

NO is a soluble gas synthesized from L-arginine and molecular oxygen by NO synthase (NOS) enzymes. The catalysis of L-arginine and O_2 requires a number of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin, and calmodulin.[158] There are three NOS isoforms (endothelial [eNOS], neuronal [nNOS] and inducible [iNOS]).[159] Both shear stress and/or the activation of receptors by any of the

substances mentioned above (e.g. ACh, bradykinin) will increase intracellular Ca^{2+} augmenting calmodulin binding to eNOS and increasing eNOS activity.[160] Following its production, NO is released by the endothelial cells and diffuses to the VSMC where it stimulates soluble guanylyl cyclase, producing cyclic guanosine monophosphate (cGMP). cGMP subsequently activates protein kinase G and leads to a decrease in the VSMC intracellular Ca^{2+} by phosphorylating phospholamban (PLB), activating large conductance Ca^{+2}-activated K^+ channels (BK_{Ca}) to release K^+ out of the cell, decreasing the inflow of Ca^{2+} through L-type Ca^{2+} channels (LTCC), and activating myosin light-chain phosphatase (MLCP).[161]

An increase in endothelial intracellular Ca^{2+} following a mechanical stress on the endothelial layer, such as shear stress, could also activate EDH-mediated vasodilation. EDH can be induced by multiple mechanisms including: molecules (epoxyeicosatrienoic acids (ETTs), hydrogen peroxide, C-type natriuretic peptide); activation of endothelial channels (small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}), intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca})); or activation of VSMC channels (Na^+/K^+ ATPase, inward-rectifying K^+ channels (K_{IR}), voltage-gated K^+ channels (K_V), BK_{Ca}).[162]

Activation of SK_{Ca} and IK_{Ca} channels occurs secondary to an increased endothelial intracellular Ca^{2+}, initiating a K^+ efflux to the extracellular space and resulting in hyperpolarization of the endothelial cell. Hyperpolarization of the VSMC is facilitated by myoendothelial gap junctions (MEGJ).[163] Certain EDH factors (ETTs, hydrogen peroxide and C-type natriuretic peptide) can also activate VSMC K^+ channels (BK_{Ca}, Na^+/K^+ ATPase, K_{IR}, and K_{ATP}) without endothelial hyperpolarization.[163]

Prostaglandins (PG) have a dual effect on vascular function, producing either vasodilation or vasoconstriction; thus, a special mention of the role of PGs needs to be made. The metabolism of arachidonic acid by the action of prostaglandin H synthase 1- and 2 (also referred to as cyclooxygenase (COX-1 and COX-2), which have both COX and peroxidase activity), produces multiple prostanoids including PGs and thromboxane A2 (TXA_2).[164] COX-1 and COX-2 first produce PGG_2, (an unstable intermediate) and then enable further reduction to PGH_2.[165] PGH_2 is a precursor for other PGs (PGD_2, F_2, I_2) and TXA_2, via the action of different synthases (PGD, PGF, PGI, and thromboxane synthase). The interaction of PGs with their specific receptors will generate different actions on the target cell.[166] Stimulation of the TP receptor promotes VSMC contraction. It is important to note that while TXA_2 is the preferential ligand for this receptor, other PGs can also activate the TP receptor.[167] Remarkably, PGI_2 can activate both the TP and IP receptor, with a greater affinity for the latter.[168]

PGI_2 is the principle metabolite of arachidonic acid.[169] PGI_2 is released by endothelial cells and activates the IP receptor in the VSMC, which is coupled to a Gs-protein.[170] This interaction activates cyclic adenosine monophosphate (cAMP) and protein kinase A, resulting in activation of plasmalemmal and sarcoplasmic reticulum Ca^{2+}-ATPases (SERCA). Moreover, PGI_2 could cause inhibition of Rho kinase[171] and could also activate K^+ channels (large conductance Ca^{2+}-dependent K^+ channel [BK_{ca}, MaxiK]; SK_{Ca}, ATP-sensitive K^+ channel [K_{ATP}], K_V[172-174]) leading to VSMC hyperpolarization. All of these events will subsequently result in decreased VSMC intracellular Ca^{2+} causing vasodilation (Figure 1.2).

Endothelial-dependent vasoconstrictor mechanisms

The most potent endothelial vasoconstrictor is ET-1.[175] The production of ET-1 involves enzymatic cleavage of its precursor prepro ET-1 to pro ET-1 and then big ET-1.[176] Big ET-1 is then cleaved to ET-1 by the action of endothelin converting enzyme (ECE)[177], chymase[178] and MMP-2.[179] ET-1 exerts its actions via activation of its ET_A and ET_B receptors. Stimulation of ET_A receptor coupled to $G_{12/13}$ in the VSMC induces phosphorylation of myosin light chain activating myosin light-chain kinase (MLCK).[176] Whereas stimulation of ET_A receptor coupled to Gq in the VSMC induces the activation of phospholipase C (PLC) to form inositol 1,4,5-triphosphate (IP_3) which binds to its receptor (IP_3R) in the sarcoplasmic reticulum to release Ca^{2+}. Moreover, stimulation of ET_A receptor coupled to Gq in the VSMC increases Ca^{2+} influx via activation of store-operated and receptor-operated Ca^{2+} channels[176] as well as LTCC.[175] Ca^{2+} binds to calmodulin thereby activating MLCK. This complex phosphorylates myosin at the 20-kDa myosin regulatory light chain leading to myosin-actin interactions and, consequently, vasoconstriction.[154] On the other hand, activation of ET_B receptor in the endothelial cells leads to an increase in intracellular Ca^{2+} inducing the activation of eNOS[180] and Ca^{2+}-dependent induction of COX-2.[181] Moreover, the release of the $G_{\beta\gamma}$ subunit is associated with activation of PI3K/AKT resulting in the production of NO (Figure 1.2).[176]

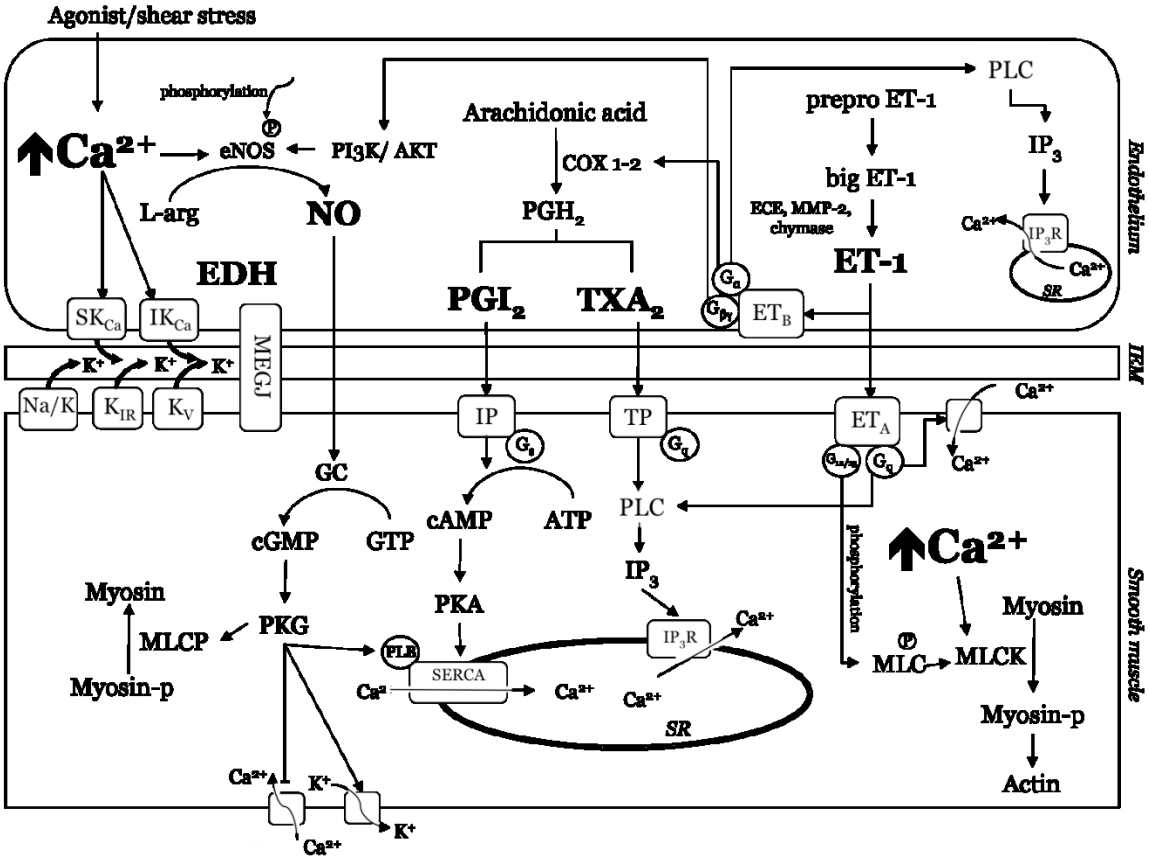

Figure 1.2. Vasodilator and vasoconstrictor pathways.
Summary of the vasodilator and vasoconstriction pathways. ATP: adenosine triphosphate; big ET-1: big endothelin-1; Ca^{2+}: calcium; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX 1-2: cyclooxygenase-1, and -2; EDH: endothelium-dependent hyperpolarization; ECE: ET-1 converting enzyme; eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; ET_{A-B}: ET-1 receptor A,B; GC: guanylyl cyclase; GTP: guanosine triphosphate; $IK_{Ca:}$ intermediate conductance Ca^{2+}-activated potassium (K^+) channels; IEL: internal elastic lamina; IP_3: inositol 1,4,5-triphosphate; IP_3R: IP_3 receptor; $K_{IR:}$ inward-rectifying K^+ channels; K_V: voltage-gated K^+ channels; L-arg: L-arginine; MEGJ: myoendothelial gap junction; MMP-2: matrix metalloproteinase-2; MLC: myosin light chain; MLCK: myosin light-chain kinase; MLCP: myosin light-chain phosphatase; Na/K: Na^+/K^+ ATPase; NO: nitric oxide; PGH_2: prostaglandin H_2; PGI_2: prostaglandin I_2 (prostacyclin); PKA: protein kinase A, PKG: protein kinase G, PLB: phospholamban; PLC: phospholipase C; SERCA: sarcoendoplasmic reticulum Ca^{2+}-ATPase; SK_{Ca}: small conductance Ca^{2+}-activated K^+ channels; SR: sarcoplasmic reticulum; TXA_2: thromboxane A_2.

1.5.4.2 Myogenic regulation of vascular tone

A myogenic response is defined as the ability of the VSMC to constrict or dilate in response to an increase or decrease in intravascular pressure respectively.[182] The development of a myogenic response starts with depolarization of VSMCs by an influx of Na^+, K^+, and Ca^{2+} through stretch-activated channels. Subsequently, an influx of extracellular Ca^{2+} to the VSMC through voltage-dependent Ca^{2+} channels will initiate Ca^{2+}-dependent activation of myosin light chain and further constriction.[183] In addition, it has been shown that another mechanism initiated by stretch is the activation of PLC leading to the formation of IP_3 and diacylglycerol (DAG). Activation of this pathway will increase intracellular Ca^{2+} concentration via release of Ca^{2+} from the sarcoplasmic reticulum.[183]

1.5.4.3 Vascular dysfunction in hypoxic-induced intrauterine growth restricted offspring

Being born growth restricted has been associated with vascular dysfunction in a variety of vascular beds, affecting both male and female offspring. Vascular dysfunction is a broad term used to outline a group of pathological events (vascular remodelling, smooth muscle or endothelial dysfunction) that will lead to impaired tissue perfusion. Endothelial dysfunction *per se* is an early predictor of CVD.[184]

Findings during fetal life have shown that male hypoxic IUGR fetuses have an increased thoracic aortic wall thickness with an increase in VSMC nuclei, suggesting hyperplasia of the VSMC.[103] Interestingly Giussani *et al.* also found that hypoxic IUGR

fetuses have an increased thoracic aortic wall thickness, while in adulthood the aortic wall thickness was not different when compared to control animals.[98]

Functional experiments in aortic rings showed that hypoxic-induced IUGR fetuses demonstrated increased Ang II-mediated vasoconstriction that was mediated via the AT_1 receptor.[109] Additionally, in the same vascular bed Camm *et al.* found an increased sensitivity to potassium chloride (KCl), with no changes in the sensitivity or maximal response to phenylephrine (PE) or vasodilator agents (methylcholine [MCh] or sodium nitroprusside [SNP]).[103] Conversely, Kim *et al.* found that prenatal exposure to hypoxia was associated with an increased maximal response to PE and increased basal production of NO in femoral arteries from sheep fetuses.[185] Since the authors did not explore agonist-mediated mechanisms of vasodilation in these arteries, conclusions regarding NO contribution to vasodilation cannot be drawn.

In addition, it has been shown that at PND-1 in hypoxic-induced IUGR offspring there was an enhanced femoral vasoconstrictor response to PE while this response in carotid arteries was decreased; demonstrating that neonatal vascular adaptations following a hypoxic insult are vascular bed specific.[90]

In adulthood, femoral arteries from male hypoxic-induced IUGR offspring had a decreased maximal vasodilation to MCh in one study;[84] and a decreased maximal vasodilation to both MCh and SNP in another study with a decreased contribution of NO compared to controls.[98] In the renal vascular bed, Tang *et al.* found that renal interlobar arteries from young adult male hypoxic-induced IUGR rat offspring have decreased ACh-mediated vasodilation in association with an increased PE-mediated

vasoconstriction. VSMC intracellular Ca^{2+} was increased, in association with an increase in the activity and expression of LTCC whereas K^+ currents, the activity of BK_{Ca} and the expression of MaxiK were all decreased.[108] In both male and female hypoxic-induced IUGR rat offspring, the contribution of NO to mesenteric artery vasodilation was reduced.[86,87,104] Interestingly, young and aged female hypoxic-induced IUGR rat offspring demonstrated an enhanced MEGJ/EDH-mediated vasodilation in mesenteric arteries as a possible compensatory mechanism to maintain vascular function.[86] Furthermore, Morton *et al.* found that the contribution of NO to flow-mediated mesenteric artery vasodilation was reduced in male and female hypoxic-induced IUGR offspring at a young age and this was sustained with ageing, while the EDH-mediated vasodilation was maintained in both groups but to a lesser extent in the aged female hypoxic IUGR offspring.[87]

Myogenic response of mesenteric arteries in male and female hypoxic-induced IUGR was not different from controls at 4-months of age.[105] Myogenic responses, however, were increased in male, but not female, hypoxic-induced IUGR offspring at 7 months.[105] These differences could be explained by a decreased modulation of the myogenic response by NO and prostaglandins in males and an increased modulation of myogenic responses by NO and prostaglandins in females.[105]

Bourque *et al.* found that although the vasoconstriction response of mesenteric arteries to ET-1 was not different in aged male and female hypoxic-induced IUGR offspring compared to controls.[106] Male, but not female hypoxic-induced IUGR offspring, however, had an increased conversion of big-ET-1 (precursor) to ET-1.[106] (Table 1.1).

1.5.5 Cardiovascular susceptibility to secondary stressors

The cardiovascular alterations that occur in hypoxic-induced IUGR offspring predispose them to an increased susceptibility to secondary stressors. There is a wide range of stressors that have been used to assess the impact of IUGR on the cardiovascular system, one of which is the *in vivo* pharmacological stimulation of the heart and/or vasculature with isoprenaline and carbachol (β adrenoreceptor and cholinergic agonists, respectively);[98,144] PE and SNP (α_1-adrenergic receptor agonist and NO-releasing drug, respectively).[97] In addition, it has been demonstrated that IUGR was associated with an early ageing vascular phenotype.[186] Moreover, the combination of IUGR and ageing has been shown to worsen cardiac and vascular function outcomes.[86-88] Lastly, exposing hypoxic-induced IUGR offspring to diets that mimic the Western diet have shown that male hypoxic IUGR offspring have an increased susceptibility of developing metabolic syndrome and both male and female hypoxic-induced IUGR offspring have an increased susceptibility to cardiac ischemia/reperfusion (I/R) injury.[187,188]

The high prevalence of myocardial infarction in the population (approximately one person will have a myocardial infarction every 42 seconds in the United States)[13] demonstrates the far-reaching impact of IUGR on cardiovascular health. In the following section, we are going to focus on the susceptibility to cardiac I/R injury in hypoxic-induced IUGR offspring.

1.5.5.1 Cardiac ischemia/reperfusion injury

Cardiac I/R injury refers to the damage that occurs to the myocardial tissue after the restoration of blood flow following a myocardial infarction. The pathophysiology of I/R injury encompasses a complex variety of mechanisms that are interrelated. It has been suggested that an exaggerated production of reactive oxygen species (ROS)[189] and intracellular Ca^{2+} overload[190] are key factors associated with I/R injury. These events will promote cellular injury and subsequent cardiomyocyte death.

One of the most remarkable features of the hypoxia-induced IUGR phenotype is an increased susceptibility of these animals to cardiac I/R injury.[89,92,99-102,107] Using the formerly mentioned *ex vivo* preparations (Langendorff system and the isolated working heart system), the heart can be subjected to a global ischemia followed by reperfusion period (30-180 min). Although the periods of ischemia vary among groups investigating the phenomenon (10-25 min), it has been shown that young adult hypoxic IUGR offspring had poorer cardiac function compared to their controls.

Li *et al.* and Xue *et al.* have shown that during reperfusion, hearts from male hypoxic-induced IUGR offspring had a decreased heart rate, LVDP and contractility with an increased LVEDP compared to controls.[92,100,101] Moreover, they found that hypoxic-induced IUGR offspring had an increased infarct size and an increase in the release of lactate dehydrogenase (LDH) in coronary effluent during the reperfusion period. In contrast, Xue *et al.* found that female hypoxic-induced IUGR offspring were not different from their counterparts in any *ex vivo* parameters measured.[101] This study also demonstrated that male offspring exposed to prenatal hypoxia had a decrease in cardiac levels of protein kinase C epsilon (PKCε) that was associated with the increased

the susceptibility of male offspring to myocardial damage while female offspring appear to be cardioprotected.[101]

Using a working heart system, Xu *et al.* found that during reperfusion, hearts from male hypoxic-induced IUGR offspring developed a reduced peak systolic pressure, cardiac output, and coronary flow compared to controls.[89] Moreover, the percent recovery of cardiac work developed during the reperfusion time was decreased in male hypoxic-induced IUGR offspring compared to controls.[89] Rueda-Clausen *et al.* confirmed that male hypoxic-induced IUGR offspring had a worse outcome after an I/R injury compared to controls; demonstrated by a decreased cardiac power developed during the reperfusion period.[107] However, in contrast to the study of Xue *et al.,*[101] female hypoxic-induced IUGR offspring also had a worse outcome after an I/R injury compared to controls. In addition, during reperfusion, hearts from both male and female hypoxic-exposed offspring had impairment in their glucose metabolism during reperfusion with a subsequent increase in proton (H^+) production reflecting a decreased myocardial energy efficiency compared to controls.[107] (Table 1.2).

To date there is no data regarding the effects of I/R injury in an *in vivo* model of hypoxic-induced IUGR offspring. Thus, the repercussions of an ischemic event on cardiac function and later life morbidity and mortality are still unknown in this animal model.

Table 1.1. Vascular dysfunction in hypoxic-induced intrauterine growth restricted offspring.

Author	Age	Sex		Findings	Vascular bed	Species
Camm *et al.*[103]	Fetuses	Male	↑	Wall thickness	Thoracic aorta	Wistar rat
			↑	VSMC nuclei		
			↑	Sensitivity to KCl		
Giussani *et al.*[98]	Fetuses	Male	↑	Wall thickness	Thoracic aorta	Wistar rat
			↑	Expression of nitrotyrosine		
Zhu *et al.*[109]	Fetuses	NS	↑	Ang II-mediated vasoconstriction, mediated via the AT_1 receptor	Thoracic aorta	Sprague Dawley rat
Kim *et al.*[185]	Fetuses	NS	↑	Maximal response to PE	Femoral arteries	Sheep
			↑	Basal production of NO		
Williams *et al.*[90]	Neonates	NS	↑	Vasoconstrictor response to PE	Femoral arteries	Sprague Dawley rat
			↓	Vasoconstrictor response to PE	Carotid arteries	
Allison *et al.*[84]	Adult	Male	↓	Maximal vasodilation to MCh	Femoral arteries	Sprague Dawley rat
Giussani *et al.*[98]	Adult	Male	↔	Wall thickness was not different when compared to control animals	Thoracic aorta	Wistar rat

			↓	Maximal vasodilation to MCh and SNP	Femoral arteries	
			↓	Contribution of NO to vasodilation compared to controls		
Tang *et al.*[108]	Adult	Male	↓	ACh-mediated vasodilation	Renal interlobar arteries	Sprague Dawley rat
			↑	PE-mediated vasoconstriction		
			↑	VSMC intracellular Ca^{2+}		
			↑	Activity and expression of LTCC		
			↓	Activity and expression of BKCa and MaxiK		
Williams *et al.*[104]	Adult (Young)	Male	↓	Contribution of NO to vasodilation	Mesenteric arteries	Sprague Dawley rat
	Adult (Aged)		↑	SOD modulation of MCh-mediated vasodilation at lower doses of MCh		
			↓	Contribution of NO to vasodilation		
Morton *et al.*[86]	Adult (Young)	Female	↓	Contribution of NO to vasodilation	Mesenteric arteries	Sprague Dawley rat
			↑	MEGJ/EDH-mediated vasodilation		
	Adult (Aged)	Female	↓	Contribution of NO to vasodilation		
			↑	MEGJ/EDH-mediated vasodilation		
	Adult (Aged)	Male	↓	Contribution of NO to vasodilation		

Morton *et al.*[87]	Adult (Young)	Female	↓	Contribution of NO to flow-mediated vasodilation	Mesenteric arteries	Sprague Dawley rat
	Adult (Aged)		↓	EDH-mediated flow vasodilation		
	Adult (Aged)	Male	↓	Contribution of NO to flow-mediated vasodilation		
	Adult (Young)					
Hemmings *et al.*[105]	Adult (Young)	Female	↔	Myogenic response was not different from controls	Mesenteric arteries	Sprague Dawley rat
	Adult (Young)	Male	↔			
	Adult (Aged)	Female	↑	Modulation of myogenic responses by NO and prostaglandins		
	Adult (Aged)	Male	↑	Myogenic response due to a decreased modulation of the myogenic response by NO and prostaglandins		
Bourque *et al.*[106]	Adult (Aged)	Male	↑	Conversion of big-ET-1 to ET-1	Mesenteric arteries	Sprague Dawley rat
	Adult (Aged)	Female	↔	Conversion of big-ET-1 to ET-1 was not different compared to controls		

NS: not specified. ↑: increased, ↓: decreased; ↔: no changes.

Table 1.2. Cardiac dysfunction after an ischemic event in hypoxic-induced intrauterine growth restricted offspring.

Author	Sex	Age	Time of ischemia	Findings	
Li *et al.*[100]	Male	6 month-old	25 min	↓	Heart rate, LVDP and contractility compared to controls
				↑	LVEDP compared to controls
				↑	Infarct size
Xu *et al.*[89]	Male	4 month-old and 7 month-old	20 min	↓	Peak systolic pressure compared to controls
				↓	Cardiac output compared to controls
				↓	Coronary flow compared to controls
				↑	LDH release during reperfusion
				↓	Percent recovery of cardiac work developed during reperfusion
Xue *et al.*[101]	Male	3 month-old	20 min	↓	LVDP and contractility compared to controls
				↑	LVEDP compared to controls
				↑	Infarct size
				↑	LDH release during reperfusion
	Female			↔	Not different from their counterparts
Xue *et al.*[92]	Male	3 month-old	20 min	↓	LVDP compared to controls
				↑	LVEDP compared to controls

				↑	Infarct size
				↑	LDH release during reperfusion
Rueda-Clausen *et al.*[107]	Male	4 month-old and 12 month-old	10 min	↓	Percent recovery of cardiac work developed during reperfusion
	Female			↓	Percent recovery of cardiac work developed during reperfusion
Patterson *et al.*[191]	Male	3 month-old	20 min	↓	LVDP and contractility compared to controls
				↑	LVEDP compared to controls
				↑	LDH release during reperfusion
Patterson *et al.*[102]	Male	3 month-old	20 min	↓	LVDP and contractility compared to controls
				↑	LVEDP compared to controls
				↑	LDH release during reperfusion

↑: increased, ↓: decreased; ←→: no changes

1.6 Mechanisms by which intrauterine growth restriction exerts its effects on the cardiovascular system

There are multiple mechanisms that can contribute to the etiology of fetal programming of CVD.[192] We will, however, focus on ones that might be associated with the development of CVD in later life after a hypoxic insult.

1.6.1 Oxidative stress and cardiovascular function

Reactive nitrogen species (RNS) and ROS are free radicals involved in cellular homeostasis (Table 1.3). Under pathological states, however, there is an increased production of ROS and RNS that exceeds the cell's capacity to metabolise/degrade these reactive species. In turn, increased production of ROS and RNS can disrupt cell function by causing lipid peroxidation and oxidation of proteins and DNA.[193] This is known as oxidative stress. An increasing body of evidence suggests that there is a link between oxidative stress and fetal programming of CVD later in life.

Table 1.3. Reactive oxygen and nitrogen species.

Reactive species	Symbol
Reactive oxygen species	
Superoxide	$O_2^{\cdot -}$
Hydroxyl radical	$^{\cdot}OH$
Hydrogen peroxide	H_2O_2
Singlet oxygen	$^1O_2$
Reactive nitrogen species	
Nitric oxide	NO
Peroxynitrite	$ONOO^-$

The $O_2^{\cdot -}$ radical is a product of oxygen reduction by plasmalemmal and mitochondrial-associated NADPH oxidases, xanthine oxidase, and mitochondrial respiration;[193] and it is converted to H_2O_2 by the actions of superoxide dismutase enzymes (SOD).[194] The most important RNS are NO and $ONOO^-$. The toxicity of NO is secondary to its combination with $O_2^{\cdot -}$ to produce the highly reactive product, $ONOO^-$.[195] Remarkably, the reaction rate for the production of $ONOO^-$ from $O_2^{\cdot -}$ is six times faster than the reaction rate of scavenging of $O_2^{\cdot -}$ by SOD.[195] The stability of $ONOO^-$ permits a greater opportunity for it to diffuse, conferring it's high toxicity properties to the cell.[195]

There are three SOD isoforms, two of which are located within the cell; SOD-1 (copper-zinc SOD [Cu-ZnSOD], located in the cytosol and mitochondrial

intermembrane space) and SOD-2 (manganese SOD [MnSOD], located in the mitochondrial matrix). SOD-3 is located in the extracellular space.[194] H_2O_2 can either react with ferrous salts to produce ˙OH (Fenton reaction);[196] or H_2O_2can be reduced to water by the enzymes catalase (CAT) or glutathione peroxidase (GPx).[197] GPx reduces H_2O_2 and in the process reduced glutathione (GSH) is oxidized to oxidized glutathione (GSSG). While glutathione reductase (GRx) reverses the process and regenerates GSH from GSSG.[198] Although GPx and CAT share common substrates, CAT has a lower affinity for H_2O_2.[199] There is also a second line of antioxidants, the non-enzymatic antioxidants which are either produced metabolically and are thus endogenous (*i.e.* glutathione, melatonin) or are derived from exogenous sources (*i.e.* vitamin C, E).[198]

Accumulated evidence suggests that oxidative stress is involved in the pathogenesis of cardiac dysfunction. For instance, ROS and RNS are both associated with cardiac hypertrophy.[200] Hypertrophic cardiomyocyte growth is characterized by the re-expression of fetal genes (β-myosin heavy chain, α-skeletal muscle, α-smooth muscle actin and atrial natriuretic factor) following a period of senescence after birth. In addition, cardiomyocyte growth is associated with the induction of genes such as c-Fos, c-Jun, and Erg-1; with a subsequent increase in mRNA, rRNA, and protein synthesis.[201] Ang II, NE, ET-1, interleukin 1β and 6, TNF, and fibroblast growth factor can elicit cardiomyocyte hypertrophy by activating multiple signal transduction pathways including tyrosine kinases (src, focal adhesion kinase), ERK1,2,5, p38, c-Jun N-terminal kinase (JNK)), protein kinase C (PKC), calcineurin, and PI3K/Akt.[202] The interactions of ROS

and RNS with these pathways (e.g. activation, disruption of protein-protein interactions) in combination with direct interaction with Ang II, NE, and ET-1, subsequently culminate in cardiomyocyte hypertrophy.

ROS and RNS also play a role in the pathophysiology of cardiac arrhythmia.[203] Cardiac arrhythmias are characterized by alterations in the cardiac action potential. Prolongation of the action potential (due to increased depolarization of the membrane via inflow of Na^+ and decreased repolarization of the membrane via K^+ outflow, leading to the activation of L-type Ca^{2+} channels and increased intracellular Ca^{2+}) as well as abnormal Ca^{2+} handling can trigger an arrhythmia.[204] ROS are known to have effects on both decreasing K^+ outflow[205] and increasing Na^+ inflow.[206] Moreover, ROS inhibit SERCA (decreasing Ca^{2+} uptake into the sarcoplasmic reticulum), and stimulate RyRs (activating Ca^{2+} release from the sarcoplasmic reticulum[207]), thus increasing intracellular Ca^{2+} and causing repolarization abnormalities that may lead to cardiac arrhythmias.

Finally, ROS and RNS have been shown to be involved in the pathophysiology of cardiac I/R injury.[208] During cardiac I/R injury, a disruption of the blood flow from the coronary arteries to the myocardium reduces oxygen supply to a level that is inadequate to maintain steady state metabolism.[209] Depending on the intensity of ischemia, a wide range of cellular changes occur following a reduction in oxygen availability. These cellular alterations include switching to anaerobic metabolism, a reduction of mitochondrial activity, accumulation of protons (decreasing pH), increased intracellular osmolality (increased Na^+ inflow [Na^+/H^+ exchanger]), Ca^{2+} overload (reduction of active

Ca^{2+} outflow, limitation of Ca^{2+} reuptake by the sarcoplasmic reticulum), increased mitochondrial $O_2^{\cdot -}$ formation, cell death, and tissue necrosis.[209-211]

Conversely, blood flow restoration is associated with normalization of the osmolality, normalization of the tissue pH (intracellular excess of H^+ is removed by activation of the Na^+/H^+ exchanger, the bicarbonate/Na^+ symporter and the Na^+ pump);[212] reestablishment of aerobic metabolism,[208] and generation of ROS and RNS ($O_2^{\cdot -}$, $^{\cdot}OH$, NO and $ONOO^-$[208,213]). The main sources of ROS and RNS during reperfusion are the mitochondria, xanthine oxidase, NADPH oxidase and uncoupled eNOS.[214] Moreover, during reperfusion, reestablishment of aerobic metabolism increases $O_2^{\cdot -}$ to a point which exceeds the cardiomyocyte antioxidant capacity.[214] The increased concentration of ROS and RNS cause lipid peroxidation of the mitochondrial and sarcolemmal membranes resulting in mitochondrial matrix swelling and apoptosis.[215] ROS and RNS activate inflammatory cascades and adhesive molecule generation thus interfering with capillary flow.[214] In addition, ROS and RNS can modify Ca^{2+} transporters leading to an increase in intracellular Ca^{2+}, which will further promote ROS and RNS generation.[216] ROS regulate LTCC, Na^+/Ca^{2+} exchangers (NCX), plasma membrane Ca^{2+} pumps (PMCA) in the sarcolemma and transverse tubules, and RyR and SERCA in the sarcoplasmic reticulum.[217] It is known that $O_2^{\cdot -}$, $^{\cdot}OH$ and H_2O_2 inactivate PMCA.[218] Moreover, $O_2^{\cdot -}$ and H_2O_2 enhance the activity of NCX.[219] Moreover, it has been shown that exogenous H_2O_2 increases the mitochondrial production of $O_2^{\cdot -}$ via an increase in Ca^{2+} inflow to the cell from the LTCC channels.[220] (Figure 1.3).

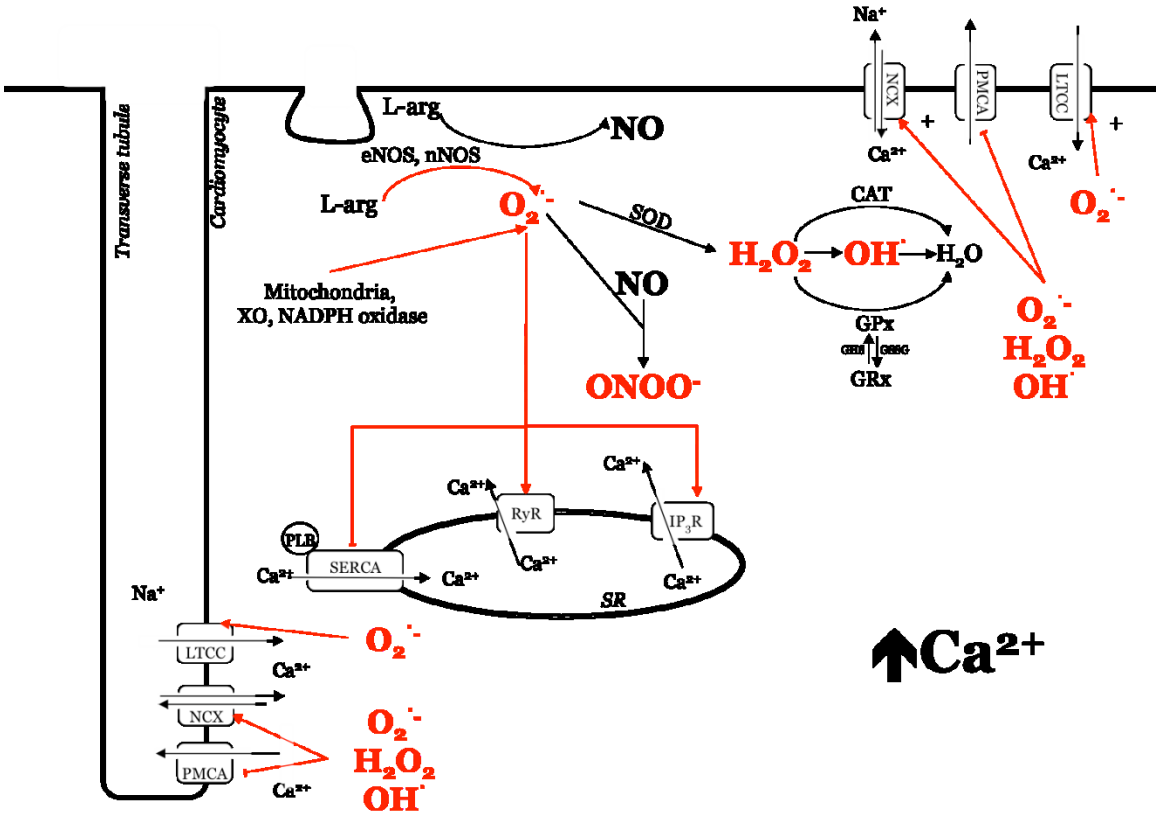

Figure 1.3. Crosstalk between ROS and RNS with calcium signalling during cardiac reperfusion.
Ca^{2+}: calcium; CAT: catalase; eNOS: endothelial nitric oxide synthase; GHS: reduced glutathione; GPx: glutathione peroxidase; GRx: glutathione reductase; GSSG: oxidized glutathione; H_2O: water; H_2O_2: hydrogen peroxide; IP_3R: inositol 1,4,5-triphosphate receptor; L-arg: L-arginine; LTCC: L-type Ca^{2+} channel; MLCK: myosin light-chain kinase; MLCP: myosin light-chain phosphatase; NCX: Na^+/Ca^{2+} exchanger; nNOS: neuronal nitric oxide synthase; NADPH oxidase: nicotinamide adenine dinucleotide phosphate-oxidase; NO: nitric oxide; $O_2^{\cdot-}$: superoxide; $ONOO^-$: peroxynitrite; $^{\cdot}OH$: hydroxyl radical; PLB: phospholamban; PMCA: plasma membrane Ca^{2+} pump; RyR: ryanodine receptor; SERCA: sarcoendoplasmic reticulum Ca^{2+}-ATPase; SOD: superoxide dismutase; SR: sarcoplasmic reticulum; XO: xanthine oxidase. Modified from Zhang *et al.*,[217].

In addition, oxidative stress participates in the disruption of vascular function by multiple mechanisms including the following. *1)* decreased NO bioavailability: increased $O_2^{\cdot -}$ production will lead to an increase in $ONOO^-$ formation which is known to inhibit PGI synthase,[221] inhibit guanylyl cyclase[222] and cause eNOS uncoupling which leads to further production of $O_2^{\cdot -}$ and reduced NO bioavailability.[223] *2)* Increase and/or potentiation of vasoconstrictor pathways: $O_2^{\cdot -}$ and H_2O_2 elevate pre-ET-1 mRNA and big-ET-1 synthesis, as well as ET-1 promoter activity.[224] In addition, ET- 1 activates NADPH oxidase in human endothelial cells.[225] Moreover, $O_2^{\cdot -}$ stimulates the release of Ca^{2+} from the sarcoplasmic reticulum[226] by inhibiting SERCA, or by activating IP_3R and ryanodine receptors (RyR).[227] Exogenous H_2O_2 activates COX[228] thereby increasing the production of vasoconstrictor prostanoids. This is particularly relevant in the context of ageing, where there is an increase in oxidative stress along with an increased COX expression and enhanced COX-dependent contractions.[229] In addition, increased PG-mediated vasoconstriction has been associated with conditions of vascular pathologies such as hypertension[230] and diabetes.[231] Further, ROS and RNS are known to activate and stabilize the TP receptor.[232] (Figure 1.4).

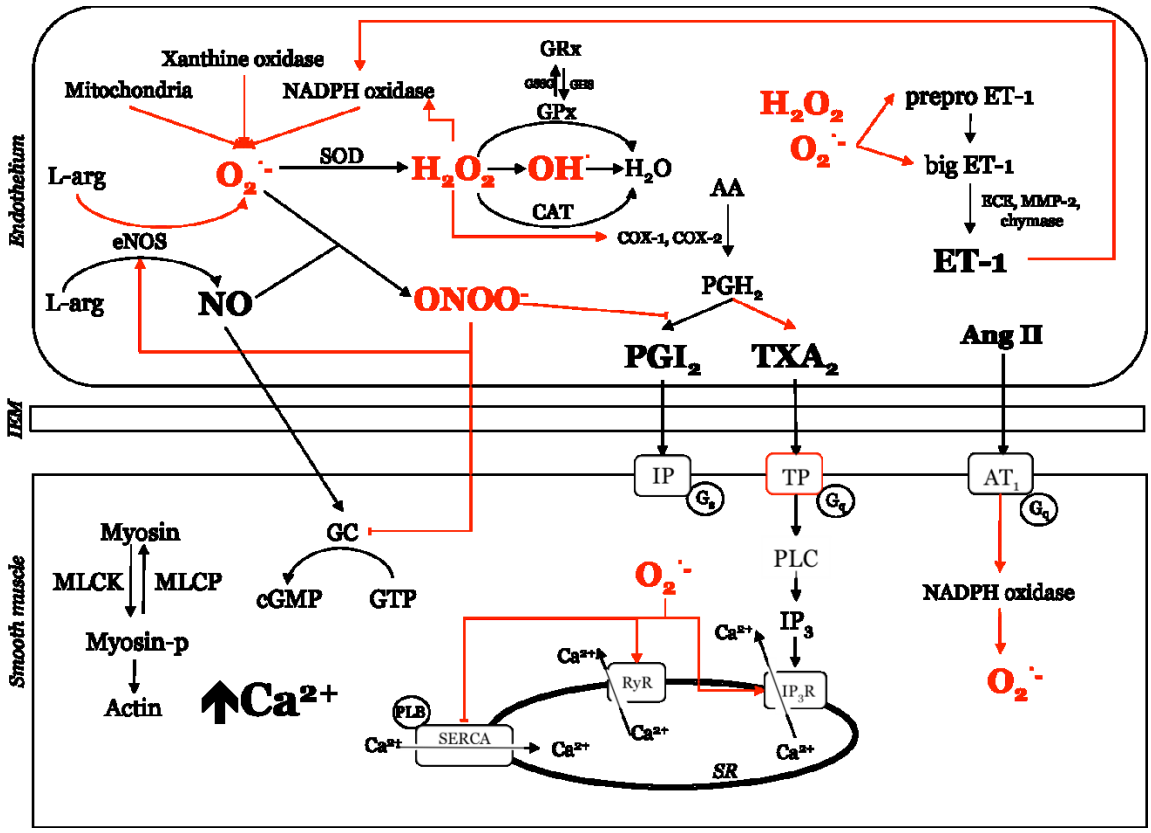

Figure 1.4. Effect of reactive oxygen and reactive nitrogen species on vasodilator and vasoconstrictor pathways.
AA: arachidonic acid; Ang II: Angiotensin II; ATP: adenosine triphosphate; AT1: Ang II receptor 1; big ET-1: big endothelin-1; Ca^{2+}: calcium; CAT: catalase; cGMP: cyclic guanosine monophosphate; COX-1,-2: cyclooxygenase-1,2; ECE: endothelin converting enzyme; eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; ET_{A-B}: ET-1 receptor A,B; GC: guanylyl cyclase; GHS: reduced glutathione; GPx: glutathione peroxidase; GRx: glutathione reductase; GSSG: oxidized glutathione; GTP: guanosine triphosphate; H_2O: water; H_2O_2: hydrogen peroxide; IEL: internal elastic lamina; IP_3: inositol 1,4,5-triphosphate; IP_3R: IP_3 receptor; L-arg: L-arginine; MMP-2: matrix metalloproteinase-2; MLCK: myosin light-chain kinase; MLCP: myosin light-chain phosphatase; NADPH oxidase: nicotinamide adenine dinucleotide phosphate-oxidase; NO: nitric oxide; $O_2^{\cdot-}$: superoxide; $ONOO^-$: peroxynitrite; $^{\cdot}OH$: hydroxyl radical; PGH_2: prostaglandin H_2; PGI_2: prostaglandin I_2 (prostacyclin); PLB: phospholamban; PLC: phospholipase C; RyR: ryanodine receptor; SERCA: sarcoendoplasmic reticulum Ca^{2+}-ATPase; SR: sarcoplasmic reticulum; TXA_2: thromboxane A_2.

1.6.1.1 Role of oxidative stress in the cardiovascular function of hypoxic-induced intrauterine growth restricted offspring

It has been established that in hypoxic-induced IUGR offspring there is an upregulated expression of genes related with oxidative stress and metabolism in the heart.[61] Moreover, using a metabolomics approach, it has been shown that carotid arteries from newborn sheep born from hypoxic ewes have increased levels of malate, fumarate, GSH, GSSG, nicotinamide, nicotinamide mononucleotide (NMN), nicotinamide adenine dinucleotide (NAD^+), and nicotinamide adenine dinucleotide hydrate (NADH); suggesting not only an increased oxidative stress with hypoxia but also a compensatory upregulation of key pathways to overcome the hypoxic insult.[233] Moreover, the ratio of GSH to GSSG in the myocardium as well as levels of cardiac MDA were higher in aged, male hypoxic-induced IUGR offspring compared to controls.[234] In addition, there is enhanced cardiac expression of heat shock protein-70 (HSP-70, an oxidative stress marker) as well as an increased aortic expression of nitrotyrosine (a footprint of $ONOO^-$ generation).[98]

Thoracic aortas from hypoxic-induced IUGR fetuses have an increase in $O_2^{\bullet -}$ production concomitant with a decrease in SOD-1 mRNA and protein expression, and elevation of the NADPH oxidase isoform 4. Further, incubating thoracic aortas with apocynin (an NADPH oxidase inhibitor) and tempol (an SOD mimetic) inhibited the maximal vasoconstrictor response to Ang II only in hypoxic-induced IUGR fetuses.[109] Williams *et al.* found that incubating mesenteric arteries from aged, male hypoxic-induced IUGR offspring with

exogenous SOD enhanced MCh-mediated vasodilation at lower doses of MCh, suggesting that local $O_2^{\cdot -}$ could influence endothelial dependent vasodilation.[104]

1.6.1.2 Effect of antioxidant treatment on the cardiovascular system of hypoxic-induced intrauterine growth restricted offspring

Based on the fact that oxidative stress has a potential role in the pathophysiology of fetal programming of the heart and vascular function, a growing body of evidence has demonstrated the effects of antioxidant treatment in the cardiovascular system of hypoxic-induced IUGR offspring. Itani *et al.* showed in the chick embryo that melatonin increased CAT and GPX cardiac expression, restored cardiac dysfunction (increased LVDP and restored myocardial contractility and distensibility) and repaired femoral vasodilation to ACh by enhancing NO-dependent and -independent mechanisms.[144] Moreover, Giussani *et al.* found that in Wistar rats, maternal treatment with vitamin C decreased the cardiac protein expression of HSP-70 and the aortic expression of nitrotyrosine from hypoxic-induced IUGR fetuses.[98]

Interestingly, treatment of the dam also had consequences in young and aged offspring. Vitamin C treatment improved cardiac contractility as well as enhanced vasodilation of femoral arteries to MCh through increasing NO-independent mechanisms of vasodilation.[98] Kane *et al.* found that vitamin C treatment recovered the predominance of sympathetic tone and decreased the baroreflex gain in these animals to control levels.[97] In addition, treating dams exposed to hypoxia during pregnancy with NAC decreased susceptibility to I/R

injury (during reperfusion: improved LVDP, decreased LVDEP, and decreased myocardial LDH release) of male hypoxic-induced IUGR offspring.[102] Moreover, aged hypoxic-induced IUGR offspring born from dams treated with allopurinol had better femoral artery vasodilation to MCh compared to non-treated hypoxic IUGR offspring.[84]

1.7 Prevention of the development of cardiovascular diseases in populations affected by fetal programming

An increasing body of evidence has shown that oxidative stress is involved in the pathophysiology of the cardiovascular phenotype of hypoxic-induced IUGR offspring. Thus, until now research has focused on providing antioxidant treatments to the dam in order to prevent the development of CVD in the offspring. Although this strategy has shown improvements in offspring vascular and cardiac function, replicating these results in humans has proven difficult. For example, antioxidant treatment with vitamin C and E during pregnancy did not show a reduction in the risk of preeclampsia and other serious complications in pregnancy (preterm birth, IUGR).[235] It is important to note that the equivalent doses of antioxidants needed to replicate the data in humans are very high and may be deleterious for pregnant women.[236] Moreover, interventions during pregnancy present major ethical challenges.[237] Hence, it is necessary to consider treating the offspring instead. As previously stated, CVDs are a result of risk accumulation throughout an individual's lifetime[16] and timely interventions in early life can improve health outcomes later in life.[238] Thus, preventive strategy approaches during youth are needed. Multiple approaches could be taken to

develop a preventive intervention, however, as with pregnant women, research in children also presents major ethical challenges.[239] Therefore, interventions that do not involve the intake of medications or biological products are more feasible. Taking into consideration that a lack of regular physical activity in the general population is a risk factor to develop CVD,[14] and that worldwide polices which include physical activity have been implemented to decrease the burden of CVD at all ages,[240] we decided to test whether aerobic exercise training could be used as a therapeutic strategy to prevent the development of CVD in hypoxic-induced IUGR offspring later in life .

1.7.1 Aerobic exercise training as a potential treatment of cardiovascular diseases

Physical activity is the movement of muscles that results in energy expenditure. When this activity is planned and includes repetition of movements it is considered exercise.[241] Exercise can be defined as either aerobic or resistance depending on the number of muscles involved in the activity, the increase in heart rate, energy expenditure and the resultant increase in muscular strength.[242] Aerobic exercise training (AET) is an activity that involves the dynamic movement of large muscle groups resulting in substantial increases in heart rate and energy expenditure.[242]

Although many strategies have been proposed to prevent CVD, AET is one of the most practical and effective preventive treatments that has been found to date.[243] The American College of Sport Medicine guidelines for exercise testing

and prescription recommends 150 min•week^{-1} of moderate intensity aerobic activity to delay premature mortality and reduce the risk of chronic diseases.[244] AET has been associated with the improvement of dyslipidemia, high blood pressure and glucose impairment (risk factors to develop CVD).[245,246] Therefore, AET has been used in both primary and secondary prevention to reduce premature death from CVD.[247] The molecular mechanisms associated with the improvement of cardiac and vascular function are discussed in the following sections.

1.7.1.1 Cardiac structural and functional adaptations following aerobic exercise training

During acute bouts of exercise, the cardiovascular system needs to increase muscle blood flow to match the oxygen demands. This is achieved by increasing the heart rate, stroke volume, systolic blood pressure and myocardial contractility while decreasing the systemic vascular resistance.[248] To guarantee maximal levels of aerobic performance, the cardiovascular system experiences a series of adaptations following repeated bouts of exercise. The effects of AET upon the cardiac structure and function are dependent on the exercise-training paradigm (frequency, intensity and duration).[249] The cardiac adaptations following AET include: *1)* a reduction in resting and submaximal heart rate;[250,251] *2)* an increase in left and right ventricle mass in association with an increase in end-diastolic volume with minimal changes in wall thickness;[252,253,254] *3)* an increase stroke volume;[253,255] *4)* an increase in cardiac output;[255,256] and *5)* enhance myocardial lipid and glucose oxidation.[257,258] The mechanisms

implicated in the above-mentioned changes have been extensively studied. An increase in hemodynamic load and mechanical stress, as well as an increase in sympathetic activity and the release of hormones and growth factors (i.e. growth hormone, thyroid hormone, IGF-1, vascular endothelial growth factor)[259] lead to the activation of different signalling pathways involved in cardiomyocyte hypertrophy,[260,261] angiogenesis,[262] mitochondrial biogenesis[263-265] and cardiomyocyte contractility.[266-268] These adaptations will either maintain or improve cardiac function. [259]

1.7.1.2 Role of aerobic exercise training in ischemia/reperfusion injury

There are several mechanisms by which AET could potentially confer cardioprotection after I/R injury, such as: *1)* increased levels of heat shock proteins which will increase the protein levels and activity of SOD-2 in the heart;[269] *2)* improved NO signalling by altering phosphorylation of eNOS with a subsequent increase in NO bioavailability;[270] *3)* enhanced calcium handling;[271-273] and *4)* reduction of oxidative stress.[274] Indeed, a reduction in oxidative stress is one of the main mechanisms by which AET may confer cardioprotection. Several studies have shown that SOD-1, SOD-2, catalase and GPx protein expression and/or activity are all upregulated following aerobic exercise training.[274] Moreover, AET has been associated with an improvement of oxidative stress in cardiovascular diseases such as heart failure, hypertension and myocardial infarction.[275] In addition, AET has been associated with an increase in the protein expression of SERCA-2a and unaltered PLB protein expression, suggesting an increase in SERCA-2a activity.[271,272] Furthermore, AET is associated with the

prevention of calpain activation;[273] which is a calcium-dependent, cytosolic cysteine protease[276] that degrades both SERCA-2a and PLB. Taken together, these data suggest that AET may confer cardioprotection by improving cardiomyocyte calcium handling.

1.7.1.3 Role of aerobic exercise training in endothelial vascular function

One of the major benefits related to AET is an increase in vascular NO concentration.[277] This increase can be explained by: *1)* an increase in shear stress secondary to an increase in pulse pressure and pulsatility accompanied by an increase in heart rate;[278] *2)* an increase in the expression of eNOS in the vasculature;[279] and *3)* an increase in tetrahydrobiopterin concentration, which is associated with an improvement of eNOS coupling.[280] Moreover, AET has been associated with an increase in NO-mediated vasodilation[281] and with a modulation of ROS by either increasing the expression of antioxidant enzymes such as SOD-1 in the vasculature,[282] or by reducing the expression and activity of their sources, such as NADPH oxidase.[283] AET has also been associated with an improvement of vascular function by increasing EDH-mediated vasodilation,[284] reducing plasma ET-1,[285] and decreasing expression of the AT_1 receptor.[283]

1.7.2 Aerobic exercise training in compromised populations

Conflictingly, however, there also exists considerable evidence contradicting the effects of AET in improving both vascular function and cardioprotection. These differences in the results demonstrate the large variety of training paradigms, controls, animal models, and diseased-animal models that

have been used; complicating the comparison of the studies. Thus, instead of adopting one of the possible mechanisms as a dogma, and thereby negating the others, we should assume that interactions between all of the factors involved can exist and that more research needs to be carried out to pinpoint specific details of the role of AET in cardiovascular function. This issue is particularly relevant in compromised populations. For instance, in the case of oxidative stress, compromised populations (e.g. with heart failure, hypertension) may have an excessive formation of ROS in response to exercise, which would lower the availability of NO and potentially cause cellular damage following AET.[286] Interestingly, in streptozocin-diabetic rats intensive exercise did not improve endothelium-dependent vasodilation in the thoracic aorta.[287] In addition, exercise did not improve vascular function as assessed by brachial artery flow-mediated dilation in subjects with type 2 diabetes and peripheral artery disease.[288] Likewise, in humans exercise has been shown to cause transient myocardial damage[289] and atrial fibrillation.[290] Moreover, Laher *et al.* found that in 8 month old obesogenic/diabetic *db/db* mice, AET increased myocardial oxidative stress, did not upregulate any SOD isoforms or catalase, and negatively altered glutathione homeostasis.[291] Thus, previous findings suggest that improving vascular function or achieving cardioprotection through exercise in diseased populations is uncertain and further investigation is required to determine the effects of AET in a susceptible population such as IUGR offspring.

1.7.2.1 Aerobic exercise training in intrauterine growth restricted populations

The effect of AET in IUGR populations is impacted by the severity of growth restriction and it depends on additional factors such as the propensity to exercise, exercise ability and physiological responses to exercise.[292] It has been established that extremely low birth weight is associated with a decreased exercise capacity[293] and reduced participation in physical activity.[294] Recent reports have shown that young males born with low birth weight can develop exercise-induced hypertension[295] and exercise-induced cardiac fatigue.[296] Both conditions could be associated with an increase in sympathetic tone in IUGR, and demonstrate that the impact of AET in IUGR offspring health should be further determined. In growth-restricted animal models (such as models of protein restriction or placental insufficiency following uterine artery ligation), evidence has shown that AET reduces body weight and body fat mass,[297-299] improves metabolic function by increasing insulin sensitivity,[300] and results in increased relative islet surface area and β-cell mass.[299] Moreover, AET increases muscle mitochondrial biogenesis and mitochondrial function.[298] The impact of AET on cardiovascular function, however, has been less studied and is, therefore, less understood. Regarding vascular function, using an animal model of protein restriction Oliveira *et al.* found that AET in male offspring decreased Ang II-dependent vasoconstriction in aortic rings by increasing SOD-2 protein expression, upregulating AT_2 protein expression and decreasing protein expression of a NADPH oxidase subunit.[301] Conversely, AET was associated with an increase in heart mass (total heart weight) that was likely due to an increased

AKT protein expression and phosphorylation.[302] Cardiac function following AET, however, has not yet been assessed. Moreover, whether AET could be used as a preventive therapeutic approach in hypoxic-induced IUGR offspring has not been determined.

In summary, being born growth restricted after a hypoxic insult is associated with early onset CVD characterized by: *1)* cardiac hypertrophy, *2)* endothelial dysfunction and *3)* increased susceptibility to cardiac I/R injury. One of the possible pathophysiological mechanisms associated with the development of CVD in hypoxic-induced IUGR offspring is an increase in ROS and RNS in the endothelium and the myocardium. To date, no therapeutic approaches during early life to prevent the early onset of CVD in hypoxic-induced IUGR offspring have been assessed. AET has been associated with an improvement of endothelial dysfunction and cardioprotection by improving oxidative stress and calcium handling. The effects of AET on the cardiovascular health of hypoxic-induced IUGR offspring, however, have not been defined. Moreover, it is unknown whether AET would be beneficial or would rather be a secondary stressor in an already susceptible population. Therefore, we will test whether AET could be used as a preventive therapy to improve vascular endothelial function and to decrease susceptibility to cardiac I/R injury using a hypoxic-induced IUGR rat model.

1.8 Hypotheses

Cardiac development and function

- Hypoxic-induced IUGR offspring have a decrease in cardiomyocyte proliferation and an increase in cardiomyocyte binucleation at PND-1 secondary to a decreased expression of Fn-14.
- Alterations in the TWEAK/Fn-14 pathway and, therefore, cardiac development could be associated with an increased susceptibility to cardiac I/R injury in hypoxic-induced IUGR offspring.
- Adult hypoxic-induced IUGR offspring are susceptible to cardiac I/R injury because of an increase in cardiac oxidative stress.
- AET will confer cardioprotection to hypoxic-induced IUGR offspring by improving cardiac oxidative stress and calcium handling.

Endothelial vascular function

- Adult hypoxic-induced IUGR offspring have endothelial dysfunction because of an increase in vascular oxidative stress.
- AET will improve vascular endothelial function in hypoxic-induced IUGR offspring by decreasing ROS and RNS and, therefore, increasing NO bioavailability.

1.9 Objectives

- To determine whether hypoxic-induced IUGR offspring have a decreased cardiomyocyte proliferation and an increased cardiomyocyte binucleation at PND-1 secondary to a decreased expression of Fn-14.
- To assess whether the serum concentration of s-TWEAK and the cardiac protein expression of Fn-14 is altered in young hypoxic-induced IUGR offspring.
- To test whether AET could improve NO-induced vasodilation in IUGR offspring by modulating the ROS contribution to vasodilation in mesenteric arteries from hypoxic-induced IUGR offspring.
- To evaluate whether AET could improve cardiac performance after I/R injury in hypoxic-induced IUGR offspring by increasing the cardiac protein expression of antioxidant enzymes such as SOD-1, SOD-2, CAT and GPx.

2 Aerobic exercise training protocol development and phenotypic characteristics in the hypoxic-induced intrauterine growth restricted animal model[1]

2.1 Introduction

As previously mentioned in Chapter 1, during a compromised pregnancy the *in utero* environment can affect fetal growth and development resulting in IUGR; which has been associated with an increased risk of chronic disease in adulthood, such us ischemic heart disease, insulin resistance, diabetes and stroke.[7,17,303] Prenatal hypoxia is a critical insult causing IUGR in many pregnancy complications.[32] We have previously shown in a rat model that a prenatal hypoxic insult leads to decreased cardiac performance after ischemia,[88,89] endothelial dysfunction[86,87,104,106] and increased susceptibility to secondary stressors (e.g. aging or a high fat diet) in adult offspring.[88,187] Thus, offspring who are at an increased risk of cardiovascular disease due to their prenatal environment are more likely to require prevention or treatment options.

The American Heart Association (AHA) has developed the concept of an ideal cardiovascular health; which encompasses the simultaneous presence of

[1] A portion of this Chapter was published in a peer reviewed journal:
Reyes LM, Morton JS, Kirschenman R, DeLorey DS, Davidge ST. Vascular effects of aerobic exercise training in rat adult offspring exposed to hypoxia-induced intrauterine growth restriction. J Physiol. 2015 Apr 15;593(8):1913-29.
Reyes LM: Contribution: Data collection: 80%, data analyses: 100%, manuscript writer: 100%, paper submission: 100%.
Kirschenman R Contribution: Data collection.
DeLorey DS Contribution: Input on experimental design, critical review of the manuscript.
Morton JS and Davidge ST Contribution: Critical review of the manuscript.

healthy behaviors such as abstinence of smoking, normal body mass index, physical activity and having a healthy diet in addition to the absence of dyslipidemia, high blood pressure and glucose impairment.[304] Aerobic exercise training (AET) has been associated with improvement of the aforementioned health conditions.[245,246] Moreover, although many strategies have been proposed to prevent cardiovascular diseases, AET is one of the most practical and effective preventive treatments that has been found to date.[243]

It is important to note, however, that extremely low birth weight has been associated with a decreased exercise capacity[293] and reduced participation in physical activity.[294] Moreover, an increasing body of evidence suggests that the cardiovascular benefits following AET in compromised populations are debatable;[287,288,291] thus, further investigation is required to determine the effects of AET in a susceptible population such as IUGR offspring.

Evidence has shown that AET in growth-restricted animal models (such as models of protein restriction or placental insufficiency following uterine artery ligation) reduces body weight and body fat mass[297-299] and improves metabolic function.[299,300] AET, however, has also been suggested to chronically activate stress responses in rodents.[305,306] Moreover, being born growth restricted has been associated with increased mineralocorticoid mRNA expression in the hippocampus and increased free corticosterone in plasma after a 30-min restraint stress test.[307] Thus, whether AET as an intervention could be a secondary stressor in an already susceptible IUGR population is unknown.

2.2 Objectives

This study is divided into two parts. In the first part, our objective was to determine whether aerobic exercise capacity was different in control and IUGR offspring and to then choose an exercise training paradigm aligned with the American College of Sport Medicine guidelines for exercise testing and prescription to prevent cardiovascular diseases. In the second part of the study, our objectives were to evaluate the impact of AET on food consumption, body weight and body composition in IUGR offspring. Furthermore, we aimed to establish whether AET was associated with any modifications in serum corticosterone levels (e.g. an indicator of a stress response) in IUGR offspring.

2.3 Methods

2.3.1 Animal model used for Chapters 2, 3 and 4

2.3.1.1 Dams

Three month-old Sprague Dawley rats (Charles River, Wilmington, MA), were housed in the University of Alberta animal facility where room conditions were as follows: 35% humidity, 10:14 hour light:dark cycle, and fed *ad libitum* with standard rodent chow. After an acclimatization period of one week, rats were mated overnight. Upon confirmation of pregnancy (presence of sperm in a vaginal smear designated as gestational day (GD) 0), female rats were single housed and then exposed to control (room air) or hypoxic (11% oxygen) conditions from GD 15 to 21. We chose this hypoxic treatment, since the last third of pregnancy is characterized by the largest increase in weight gain by the offspring; moreover, data from our lab have previously shown that the use of

hypoxia during pregnancy stopped the normal offspring growth trajectory with subsequent brain sparing;[88,90] thus, throughout my thesis, offspring born from dams exposed to hypoxia are referred to as IUGR offspring and offspring from dams exposed to normoxia are referred to as control offspring.

2.3.1.2 Offspring

At the time of birth (GD 22), anthropometric parameters such as body weight, crown to rump length and abdominal girth were measured. Litters were randomly reduced to eight pups (four males and four females) to control access to maternal nutrition. Offspring were weaned at three weeks and at ten weeks of age two males and two females from each litter where randomly allocated to the exercise training group while two males and two females where assigned to a sedentary group. All procedures in this study were approved by the University of Alberta Animal Welfare Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.3.1.3 Animal model used for Chapter 5

For Chapter 5, a different subset of animals was generated using the same hypoxia-induced animal model described above. A total of 22 dams (12 in the control group, and 10 in the IUGR group) were used. Anthropometric parameters of the offspring, such as body weight, crown to rump length, abdominal girth and heart weight, were measured at PND- 1. Four to six female and four to six male pups from each litter were used for cardiomyocyte isolation. The details of the cardiomyocyte isolation protocol will be presented in Chapter 5.

2.3.2 Exercise tolerance test

At ten weeks of age, a subset of offspring (three/group: control and IUGR; male and female) were familiarized with running on a motor-driven treadmill (Animal treadmill: Exer 3/6 Columbus Instruments, OH, USA) for 10 min at 25 m min^{-1}; 10° grade; on four consecutive days. After familiarization, each rat performed a progressive, incremental exercise test to fatigue. Testing began at a speed of 25 m min^{-1} and 10° grade for three min, treadmill speed was then increased to 40 m min^{-1} with the grade held constant for three min. Subsequently, treadmill speed was increased progressively by five m min^{-1} every minute until rats were not able to continue running. Total exercise time, maximal treadmill speed and total distance run were recorded for each rat to determine maximal exercise capacity.

2.3.3 Exercise intervention

At ten weeks of age, offspring were progressively habituated to motor-driven treadmill running during five consecutive days and then exercised for 30 min at 20 m min^{-1}; five° grade; five days $week^{-1}$; for six weeks. This exercise protocol was adapted from Jendzjowsky *et al.*[308] Rats were encouraged to run with a jet of air applied to the hindquarters. Offspring in the sedentary group were exposed to the same room environment for the same period of time as the training group.

2.3.4 Food intake, body weight, body composition analysis

Food intake and body weight were calculated weekly from ten to 16 weeks of age. Food consumption was determined by the provision of a weighed (approx.

400 grams) portion of chow and subsequent weighing of the food remnants. At 15 weeks of age, whole body composition was measured in conscious offspring using echoMRI™ (Houston, TX, USA) that is housed in the Cardiovascular Research Centre core facility at the University of Alberta. The percentage of fat and lean tissue relative to body weight was calculated for each animal.

2.3.5 Corticosterone assay

At 16 weeks of age, and 24 hours after the last bout of exercise, offspring were anesthetized with a single dose (1.5 mL in a four litre chamber) of inhaled isoflurane, a blood sample was collected by venipuncture of the inferior vena cava and then the animals were euthanized by exsanguination. Serum was separated by centrifugation at 3000 rpm for 15 min and then frozen at -80 °C and stored until analysis. Serum corticosterone levels were determined according to the manufacturer's instructions using a commercially available, colorimetric competitive enzyme immunoassay (Enzo Life Sciences, Inc., Farmingdale, NY, USA). The concentration of corticosterone was determined by interpolation from the standard curve. The inter- and intra-assay coefficients of variation obtained were 5.8 % and 4.3 %, respectively.

2.3.6 Statistical analyses

Data were presented as mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to assess normality of continuous data. Body weights, crown-rump length, abdominal girth, total exercise time, maximal treadmill speed and total distance run were analyzed using two-sample *t*-tests or a Mann-Whitney test when data were not normally distributed. Male and female offspring

were analyzed separately due to their phenotypic differences. This study had a two-way ANOVA design where the effect of being born growth restricted and the effect of AET were determined for each sex. Therefore, food intake, body weight, body composition and corticosterone levels were tested using a two-way ANOVA followed by a Bonferroni post-hoc test. All data were analyzed using GraphPad Prism 6 statistical software (GraphPad Software, USA). A $p < 0.05$ value was considered statistically significant.

2.4 Results

2.4.1 Animal model

At the time of birth, both male and female offspring from dams exposed to hypoxia had a lower body weight and reduced abdominal girth than offspring from dams in normoxic conditions. In addition, the crown-rump length to abdominal girth ratio was increased in male IUGR offspring suggesting an asymmetric growth restriction phenotype (Table 2.1).

Table 2.1. Morphological characteristics at birth of male and female offspring born from normoxic dams (control) or dams exposed to hypoxia (IUGR).

Parameter	Control	IUGR
Male offspring		
Body weight (g)	7.2 ± 0.1	6.2 ± 0.2****
Crown-rump length (mm)	45.2 ± 0.5	44.1 ± 0.5
Abdominal girth (mm)	47.7 ± 0.5	44.8 ±0.7 **
CRL ABG^{-1} ratio	0.94 ± 0.006	0.97 ±0.007**
Female offspring		
Body weight (g)	6.7 ± 0.1	5.9 ± 0.1****
Crown-rump length (mm)	44.1 ± 0.6	43.1 ± 0.5
Abdominal girth (mm)	46.4 ± 0.6	44.2 ± 0.8*
CRL ABG^{-1} ratio	0.94 ± 0.006	0.95 ± 0.01

Data presented as mean ± SEM. CRL: crown-rump length; ABG: abdominal girth. * $p<0.05$, ** $p<0.01$ and ****$p<0.0001$ compared to control offspring.

2.4.2 Exercise tolerance test

There were no differences regarding time, speed reached at the end of the exercise tolerance test or distance run between control and IUGR offspring in either male (Figure 2.1 A-C) or female (Figure 2.1 D-F) offspring. Given the similar exercise capacity in all groups, the experimental exercise training intervention performed represented the same absolute work rate and relative exercise intensity in both control and IUGR groups.

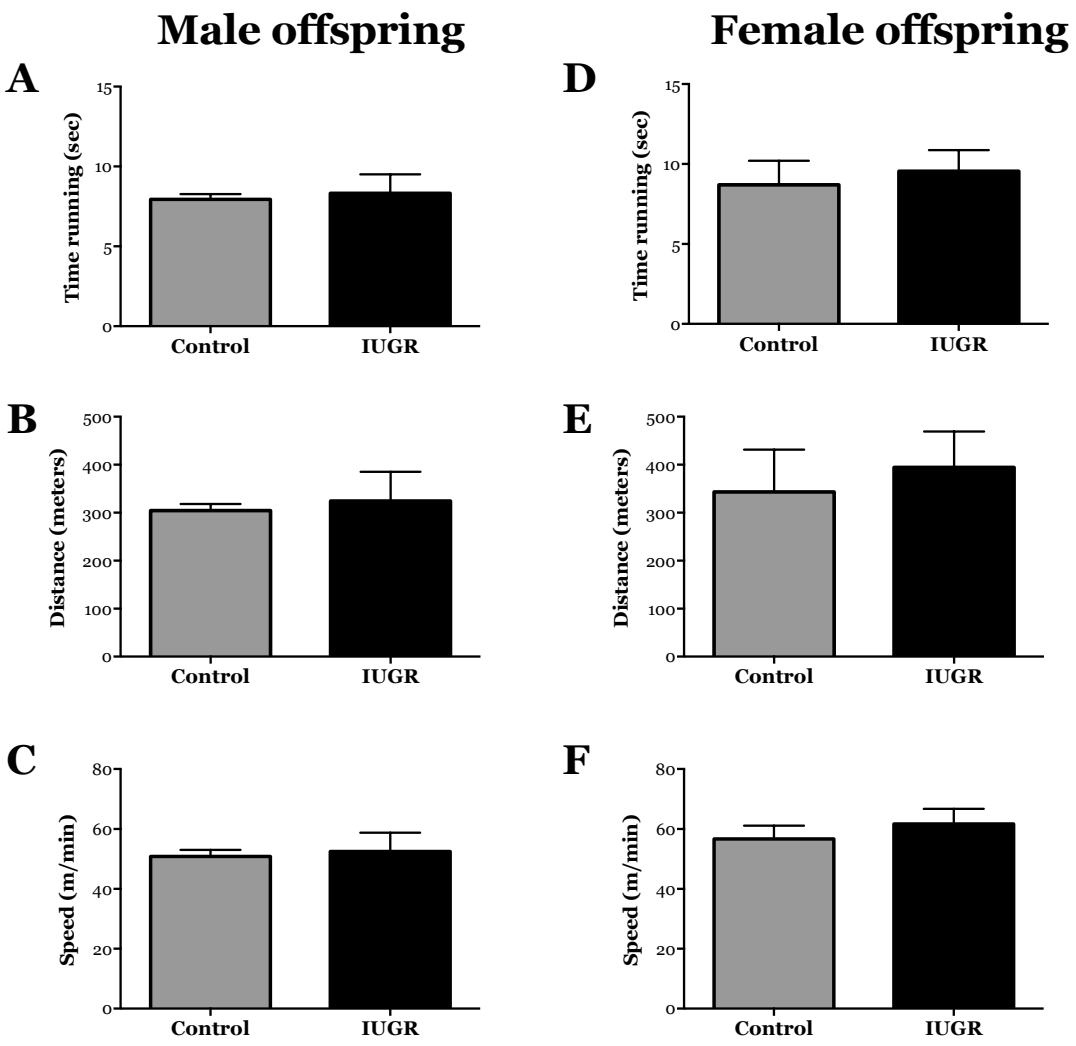

Figure 2.1. Male and female, control and IUGR offspring's time, speed reached and distance run during an exercise tolerance test.
Panel A- Time running before exhaustion during an exercise tolerance test, summary data from male control (n=3) and IUGR (n=3) offspring. Panel B- Distance run during an exercise tolerance test, summary data from male control (n=3) and IUGR (n=3) offspring. Panel C- Speed reach during an exercise tolerance test, summary data from male control (n=3) and IUGR (n=3) offspring. Panel D- Time running before exhaustion during an exercise tolerance test, summary data from female control (n=3) and IUGR (n=3) offspring. Panel E- Distance run during an exercise tolerance test, summary data from female control (n=3) and IUGR (n=3) offspring. Panel F- Speed reach during an exercise tolerance test, summary data from female control (n=3) and IUGR (n=3) offspring. Data are summarized and presented as mean ± SEM.

2.4.3 Food intake, body weight and body composition

During the entire six weeks of the experimental protocol, control male offspring had a lower food consumption compared to IUGR male offspring ($p<0.0001$). Aerobic exercise, however, did not have an effect on food consumption in either male control or IUGR offspring (Figure 2.2 A). During the six weeks of AET, body weight was not different between male control and IUGR, sedentary or exercised offspring (Figure 2.2 B). Interestingly, while AET decreased the percentage of fat tissue in only the male control offspring (Figure 2.2 C, $p=0.01$); it did not change the percentage of lean tissue in any of the offspring (Figure 2.2 D).

In female offspring, there were no differences regarding food consumption or body weight gain during the experimental period (Figure 2.3 A, 2.3 B). Being born growth restricted, however, was associated with an increased percentage of fat tissue (Figure 2.3 C, $p=0.02$) and AET increased the percentage of lean tissue in both control and IUGR offspring (Figure 2.3 D, $p=0.03$).

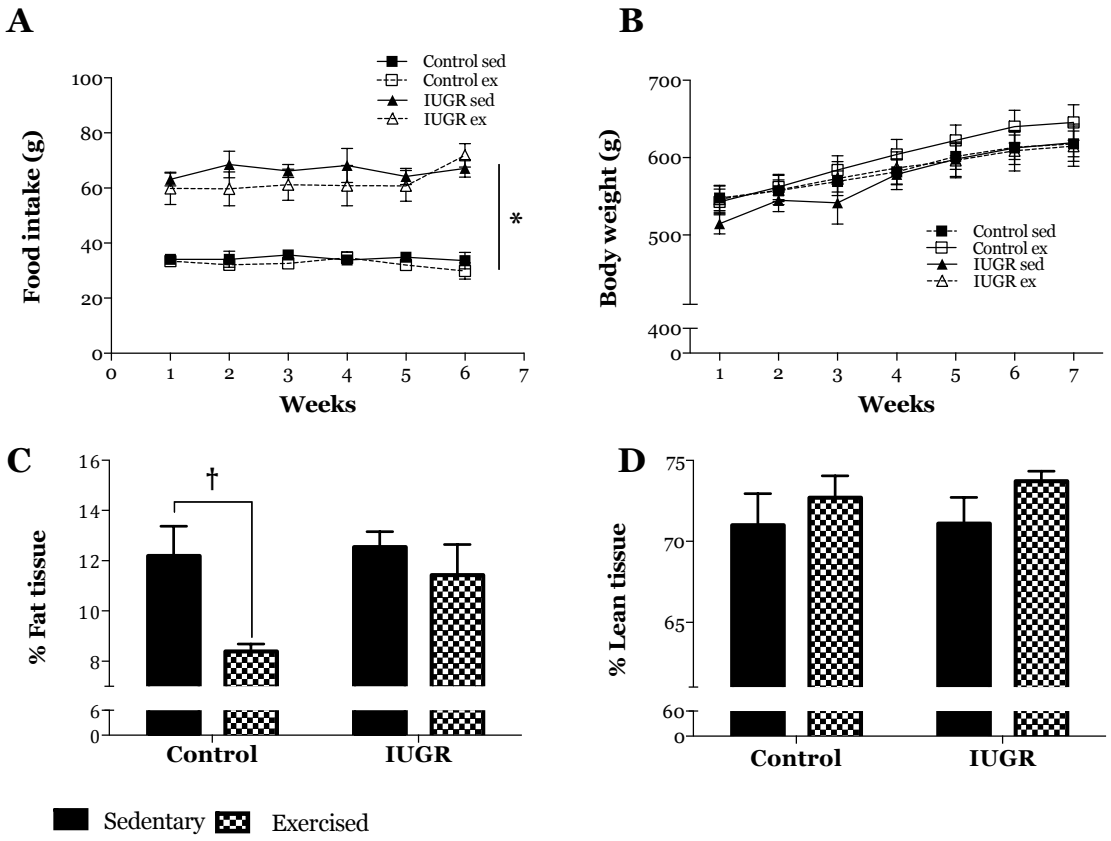

Figure 2.2. Food intake, body weight, and percentage of fat and lean tissue from male control and IUGR, sedentary and exercised offspring.
Panel A- Summary data of weekly food consumption. Groups include: control sedentary offspring (n=6); control exercised offspring (n=6); IUGR sedentary offspring (n=6) and IUGR exercised offspring (n=6). * $p<0.0001$ group effect control vs. IUGR offspring. Panel B- Summary data of body weight during the 6 weeks of exercise training. Groups include: control sedentary offspring (n=6); control exercised offspring (n=6); IUGR sedentary offspring (n=6) and IUGR exercised offspring (n=6). Panel C- Summary data of percentage of fat tissue after 6 weeks of exercise training; n=6-8. Data are summarized and presented as mean ± SEM. † $p<0.05$ after a Bonferroni *post hoc* test control sedentary vs. control exercised. Panel D- Summary data of percentage of lean tissue after 6 weeks of exercise training; n=6-8. Data are summarized and presented as mean ± SEM.

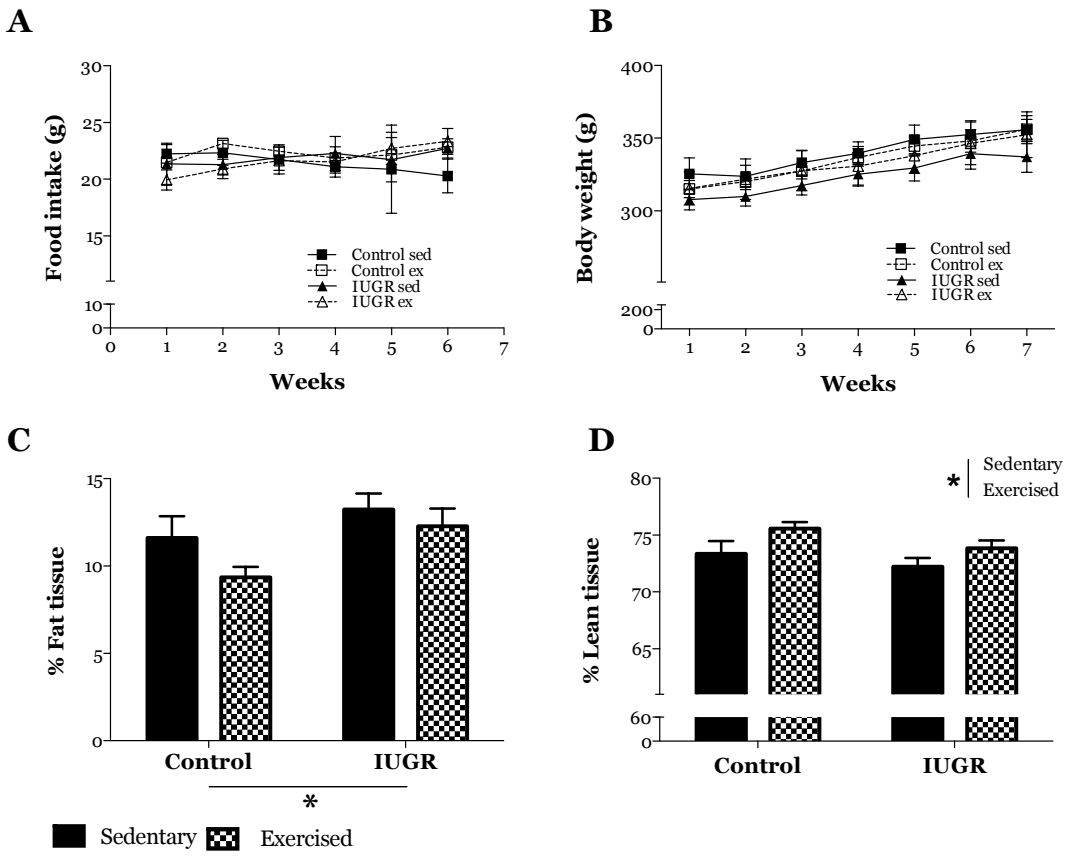

Figure 2.3. Food intake, body weight, and percentage of fat and lean tissue from female control and IUGR, sedentary and exercised offspring.
Panel A- Summary data of weekly food consumption. Groups include: control sedentary offspring (n=7); control exercised offspring (n=7); IUGR sedentary offspring (n=7) and IUGR exercised offspring (n=7). Panel B- Summary data of body weight during the 6 weeks of exercise training. Groups include: control sedentary offspring (n=7); control exercised offspring (n=7); IUGR sedentary offspring (n=7) and IUGR exercised offspring (n=7). Panel C- Summary data of percentage of fat tissue after 6 weeks of exercise training; n=8-10. Data are summarized and presented as mean ± SEM. * $p < 0.05$ group effect control vs. IUGR offspring. Panel D- Summary data of percentage of lean tissue after 6 weeks of exercise training; n=6-8. Data are summarized and presented as mean ± SEM. * $p < 0.05$ group effect sedentary vs. exercised offspring.

2.4.4 Serum corticosterone concentration

A two-way ANOVA analysis showed that being born growth restricted did not alter serum corticosterone levels in male offspring (61.6 ± 6.8 ng mL^{-1} sedentary control vs. 58.4 ± 7.6 ng mL^{-1} sedentary IUGR; p=0.67). In addition, there was a group effect of AET, with decreased serum corticosterone levels in both control and IUGR male offspring (45.4 ± 8.2 ng mL^{-1} exercised control and 41.9 ± 8.8 ng mL^{-1} exercised IUGR; p=0.05).

In female offspring, neither being born growth restricted nor performing AET had an effect on serum corticosterone levels (60.7 ± 7.9 ng mL^{-1} sedentary control; 55.7 ± 6.3 ng mL^{-1} exercised control; 58.0 ± 7.7 ng mL^{-1} sedentary IUGR; 59.7 ± 4.7 ng mL^{-1} exercised IUGR).

2.5 Discussion

AET is a universally accepted strategy in the prevention of cardiovascular diseases. This strategy, however, has not been assessed in a model of growth restriction such as hypoxic-induced IUGR offspring. Moreover, since being born growth restricted has been associated with a decreased exercise capacity and an increased susceptibility to secondary insults, it was necessary to develop a reliable aerobic exercise protocol that would ensure that if differences were found among the groups in any of the parameters assessed, these differences were not associated with the protocol *per se*.

A variety of training paradigms (voluntary vs. forced exercise; frequency; intensity and time) have been utilized to investigate physiological adaptations to exercise. Our protocol was based on findings from Jendzjowsky *et al.*, where the authors

calculated that the given exercise protocol corresponded to approximately 50% of maximal exercise tolerance in rodents.[308] Moreover, the chosen training paradigm was in alignment with the American College of Sport Medicine guidelines for exercise testing and prescription[244] which recommend 150 min•week^{-1} of moderate intensity aerobic activity to delay premature mortality and reduce the risk of chronic diseases (including cardiovascular diseases).

Since there was a possibility that exercise capacity could be altered by IUGR in our model, we carried out a pilot study to assess exercise capacity in both IUGR and control offspring prior to setting the experimental exercise protocol. In the present study, exercise tolerance was not different between control and IUGR offspring and, therefore, exercise training was completed at the same absolute work rate and relative intensity in all groups. Thus, group and sex differences in any of the parameters that we assessed throughout my thesis were not attributable to differences in the training stimulus.

Being born growth restricted has been associated with either an increase in basal levels of serum corticosterone[309] or an increase in serum corticosterone levels following a restraint stress test.[307,310] In the present study we found that neither exposure to hypoxia *in utero* nor AET was associated with changes in corticosterone levels in female offspring, while AET decreased corticosterone levels in male control and IUGR offspring. These findings are in accordance with Boaventura *et al.*, who found that after 5 months of running in a motorized wheel, male IUGR offspring had lower corticosterone levels compared to sedentary IUGR offspring.[311] Moreover, these results suggest that AET does

not evoke a stress response when used as an intervention in a hypoxia-induced IUGR animal model.

Our findings have demonstrated that hypoxia during the last third of pregnancy reduces offspring birth weight and abdominal girth. It is possible that the reduced pup size was due to preterm birth, however, we noticed that the dams exposed to hypoxia delivered their litters later than the control dams, suggesting that exposing the dams to hypoxic conditions is not associated with preterm delivery. Since this is only an observation, further investigation regarding the effects of hypoxia in the initiation of parturition should be considered. In addition, IUGR offspring underwent a rapid growth phase postnatally and their body weights were not different compared to control offspring by the beginning of the aerobic exercise intervention at ten weeks of age. Interestingly, our findings regarding body composition and body weight suggest that, in contrast to control offspring, AET has no effect on the percentage of fat mass in either male or female IUGR offspring. Thus, AET in an IUGR population may not be effective in decreasing fat accumulation or in the prevention of obesity. In accordance with previous studies,[312,313] our findings show that male IUGR offspring had increased food consumption. Moreover, we found a disparity between increased food consumption and weight gain in male control and IUGR offspring. As noted, fat tissue accumulation was not different among the groups; suggesting that in male IUGR offspring there could be metabolic alterations associated with this phenomenon. It has already been established that IUGR is associated with a reduction in proteins associated with nutrient absorption and transport (albumin and transferrin) as well as energy metabolism (ubiquinol-cytochrome c reductase, mitochondrial succinate dehydrogenase complex subunit A and

phosphoenolpyruvate carboxykinase 2) in the fetal gut, predisposing the gut to metabolic defects during gestation and neonatal periods.[314] These alterations might make them prone to obesity later in life. Although in our model of IUGR offspring, total body weight was not increased compared to control offspring, we have previously described that IUGR offspring have an increased susceptibility to develop intra-abdominal fat deposition, insulin resistance, impaired glucose tolerance and dyslipidemia with a high fat diet compared to control offspring.[187] Since female IUGR offspring had a higher percentage of fat tissue compared to female control offspring we suggest that the catch-up growth observed in female offspring was due to fat tissue accumulation; suggesting a greater metabolic efficiency.

In conclusion, AET capacity was similar in control and IUGR offspring for both sexes. Using previously established protocols for exercise training, we did not observe increased corticosterone levels suggesting that this intervention did not evoke a stress response. Thus we used this approach to test the hypothesis that exercise training could be a preventive strategy to decrease the cardiovascular burden of being born growth restricted.

3 Vascular effects of aerobic exercise training in hypoxic-induced intrauterine growth restricted adult offspring[2]

3.1 Introduction

After we had established that being born growth restricted did not compromise aerobic exercise capacity and, therefore, that group and sex differences in any of the responses assessed were not attributable to the training stimulus (Chapter 2), we next evaluated the impact of AET on vascular function in IUGR offspring.

Being born growth restricted has been associated with vascular dysfunction in a variety of vascular beds, affecting both male and female offspring and characterized by: increased arterial wall stiffness, reduced arterial diameter, altered collagen deposition, increased vasoconstrictor capacity, decreased EDH- and NO-mediated vasodilation.[86,87,106,315,316]

As previously discussed in Chapter 1, oxidative stress has been proposed as a mechanism for vascular dysfunction in IUGR.[62] Being born from a hypoxic pregnancy, independent of IUGR, has been associated with increased aortic nitrotyrosine levels;[98]

[2] A version of this Chapter was published in a peer reviewed journal:
Reyes LM, Morton JS, Kirschenman R, DeLorey DS, Davidge ST. Vascular effects of aerobic exercise training in rat adult offspring exposed to hypoxia-induced intrauterine growth restriction. J Physiol. 2015 Apr 15;593(8):1913-29.
Reyes LM: Contribution: Data collection: 80%, data analyses: 100%, manuscript writer: 100%, paper submission: 100%.
Morton JS Contribution: Data collection (mounted all the vessels in the wire myography) and critical review of the manuscript.
Kirschenman R Contribution: Handle the animals in the exercise protocol.
DeLorey DS Contribution: Input on experimental design, critical review of the manuscript.
Davidge ST Contribution: Critical review of the manuscript.

which is a footprint of $ONOO^-$, a powerful oxidant produced by the reaction of NO with $O_2^{\cdot -}$.[195] Further, maternal treatment with antioxidants during a hypoxic pregnancy was shown to rescue endothelial dysfunction in the offspring at adulthood.[98] Several other studies have shown that a vascular oxidant tone affecting NO bioavailability is functional in fetal life and that it can be modified by hypoxic conditions and by exposure to antioxidants or to agents that increase NO bioavailability in the maternal and fetal circulation.[317-319]

AET has been shown to improve vascular function, and thus has been proposed as an intervention to prevent cardiovascular diseases. Exercise has been associated with an improvement of vascular function by increasing EDH-mediated vasodilation in gastrocnemius muscle arteries from spontaneously hypertensive rats;[284] increasing the expression of eNOS in the aorta;[279] increasing NO-mediated vasodilation in epicardial coronary arteries;[281] reducing plasma ET-1,[285] and decreasing ROS generation in aortic endothelial cells.[282] Interestingly, however, it also has been shown that exercise did not improve vascular function in Streptozotocin-diabetic rats[287] or in subjects with type-2 diabetes and peripheral artery disease.[288] Thus, exercise training may not be beneficial in conditions with a susceptible vascular pathology.

Although there has been an increase in the amount of research focused on the physiologic effects of AET in IUGR populations, this research has mainly encompassed the metabolic effects of exercise training.[297-300] Using a protein restriction animal model of IUGR, Oliviera *et al.*, found that in male offspring, AET was associated with a decrease in Ang II-dependent vasoconstriction in the thoracic aorta, and was associated with an increased expression of SOD-2 and a decreased expression of the p47 phox

subunit of the NADPH oxidase.[301] These findings suggest that AET can improve vascular function in IUGR populations. Thus, the aim of our study was to determine whether AET could be used as an early intervention strategy to improve vascular function by improving the mechanisms of vasodilation in hypoxic-induced IUGR adult offspring.

3.2 Objectives

Our primary question in any vascular bed, when considering endothelial dysfunction as an outcome, is to address the main mechanisms of vasodilation. We have previously shown that mesenteric arteries from IUGR offspring have a reduced NO component of vasodilation while maintaining EDH, with no contribution of prostaglandins to vasodilation. Therefore, our first objective was to test whether AET could improve NO-induced vasodilation in IUGR offspring. Further, we investigated whether AET could modulate the ROS contribution to vasodilation in mesenteric arteries from IUGR offspring. The second objective of our study was to determine the role of AET in the mechanisms of vasodilation in the gastrocnemius muscle artery (a vascular segment from a muscle group recruited during treadmill exercise training) from hypoxic-induced IUGR offspring.

3.3 Methods

3.3.1 Vascular Function

At 16 weeks of age, and 24 hours after the last bout of exercise, offspring were anesthetized with a single dose (1.5 mL in a 4 litre chamber) of inhaled isoflurane and euthanized by exsanguination. Vascular function was assessed using wire myography from sedentary and exercised, control and IUGR, male and female offspring. Second

order mesenteric arteries and first or second order arteries from the medial head of the gastrocnemius muscle (defined as the first or second branches of the feed artery that traverses the superficial portion of the muscle) were isolated and dissected in ice-cold physiological saline solution (in mM: 10 HEPES, 5.5 glucose, 1.56 $CaCl_2$, 4.7 KCl, 142 NaCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, pH 7.4).

Arteries were mounted on two 40 μm wires attached to a wire myograph (DMT, Copenhagen, Denmark) to allow isometric tension recordings. Vessels were normalized to their optimal resting tension (set to 0.8 x IC_{100} [the internal circumference equivalent to a transmural pressure of 100 mmHg]) by increasing their diameter in a stepwise manner. Following a 30 min equilibration period, functional endothelial and smooth muscle integrity were checked by exposing the vessels twice to a single dose of PE (10μM) and then a single dose of MCh (3 μM). A cumulative concentration response curve (CCRC) to PE (0.001 to 100 μM for mesenteric arteries and 0.01 nM to 100μM for gastrocnemius muscle arteries) was performed to determine the EC_{80} dose [the concentration producing 80% of the maximum response (E_{max}) for the vasoconstrictor]. Responses were normalized to artery length. For both vascular beds assessed, the maximum contractile response of smooth muscle cells was tested using a high potassium solution at the end of the experimental protocol (in mM: 10 HEPES, 5.5 glucose, 4.9 $CaCl_2$, 124 KCl, 24 NaCl, 2.4 $MgSO_4$, 1.18 KH_2PO_4, pH 7.4).

To investigate vascular responses to the endothelium-dependent vasodilator, MCh (0.01 nM to 3 μM), a CCRC was performed following preconstriction with the EC_{80} concentration of PE. CCRCs to MCh and PE were performed in mesenteric arteries with separate baths used to incubate the arteries with the following inhibitors: N_ω-Nitro-L-

arginine methyl ester hydrochloride (L-NAME; 100 μM) to inhibit NOS activity, or Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP; 10 μM) to scavenge $ONOO^{-}$.[320]

Moreover, CCRCs to MCh and PE were performed in gastrocnemius muscle arteries with separate baths used to incubate the arteries with the following inhibitors: L-NAME (100 μM), a combination of Apamin (0.1 μM) and TRAM-34 (10 μM) to block SK_{Ca} and IK_{Ca} channels, or indomethacin, a cyclooxygenase inhibitor (5 μM).

3.3.2 Statistical analyses

Data were presented as mean ± SEM. The Shapiro-Wilk test was used to assess normality of continuous data. This study had a two-way ANOVA design where the effect of being born growth restricted and the effect of AET were determined. The effect of L-NAME, MnTBAP, Apamin + TRAM-34 or indomethacin on vasoconstriction and vasodilation was compared within each study group using a one-way ANOVA followed by a Dunnett's post-hoc test analysis of pEC_{50} (the negative log of the effective concentration producing 50% of the maximum response) or E_{max} data.

The contribution of NO, ROS, EDH or prostaglandins to vasodilation was further compared between study groups by assessment of the delta of the vasodilation area under the curve (AUC) with or without inhibitors. The effect of being born growth restricted and the effect of AET were tested using a two-way ANOVA followed by a Bonferroni post-hoc test.

Female and male offspring data were analyzed separately due to phenotypical differences. Statistical significance was defined as $p<0.05$. All data were analyzed using GraphPad Prism 6 statistical software (GraphPad Software, USA).

3.4 Results

3.4.1 Mesenteric arteries vascular function

3.4.1.1 Phenylephrine-induced vasoconstriction

Compared to controls, being born growth restricted did not modify PE-induced vasoconstriction in either male ($p=0.07$) or female ($p=0.6$) offspring. In addition, compared to sedentary animals, AET had no effect on maximal vasoconstriction to PE: male control offspring (E_{max}: 9.9 ± 0.4 mN mm^{-1} sedentary vs. 10.8 ± 0.7 mN mm^{-1} exercised), male IUGR offspring (E_{max}: 9.1 ± 0.7 mN mm^{-1} sedentary vs. 9.4 ± 0.7 mN mm^{-1} exercised), female control offspring (E_{max}: 7.4 ± 0.4 mN mm^{-1} sedentary vs. 8.1 ± 0.6 mN mm^{-1} exercised) or female IUGR offspring (E_{max}: 7.5 ± 0.3 mN mm^{-1} sedentary vs. 8.4 ± 0.5 mN mm^{-1} exercised).

In males, there were no changes regarding maximal vasoconstriction to PE in the presence or absence of L-NAME or MnTBAP in any of the groups (Table 3-1). In females, the presence of L-NAME increased maximal PE-induced vasoconstriction only in control sedentary offspring (Table 3.1). Vascular sensitivity to PE, however, was unchanged following the addition of L-NAME or MnTBAP to mesenteric arteries from all groups (Figure 3.1).

Table 3.1. Mesenteric artery, phenylephrine-induced vasoconstriction in the presence or absence of L-NAME or MnTBAP from male and female, control and IUGR, sedentary and exercised offspring.

	No inhibitors	L-NAME	MnTBAP
Male offspring (E_{max}; mN mm^{-1})			
Control Sedentary offspring	9.87 ± 0.37	9.91 ± 0.4	10.1 ± 0.89
Control Exercised offspring	10.76 ± 0.68	11.63 ± 0.86	10.41±0.81
IUGR Sedentary offspring	8.41 ± 0.6	9.27 ± 0.54	8.34±0.6
IUGR Exercised offspring	9.35 ± 0.72	10.13 ± 0.97	10.7±0.46
Female offspring (E_{max}; mN mm^{-1})			
Control Sedentary offspring	7.37 ± 0.42	9.33±0.38**	8.08±0.47
Control Exercised offspring	8.1 ± 0.6	9.38±0.55	8.45±0.43
IUGR Sedentary offspring	7.55 ± 0.33	8.15±0.45	6.81±0.39
IUGR Exercised offspring	8.37 ± 0.46	9.43±0.68	8.07±0.49

Data presented as mean ± SEM.** $p<0.01$ compared to no inhibitors and MnTBAP after a one-way ANOVA followed by a Dunnett's post-hoc comparison.

3.4.1.2 Vasoconstriction to high potassium solution

In both male and female offspring, neither IUGR nor AET affected the maximum vasoconstriction to high potassium solution; male control offspring (9.4 ± 0.3 mN mm^{-1} sedentary vs. 9.6 ± 0.9 mN mm^{-1} exercised), male IUGR offspring (8.9 ± 0.5 mN mm^{-1}

sedentary vs. 9 ± 0.4 mN mm^{-1} exercised); female control offspring (8.2 ± 0.4 mN mm^{-1} sedentary vs. 9.2 ± 0.6 mN mm^{-1} exercised) or female IUGR offspring (7.7 ± 0.2 mN mm^{-1} sedentary vs. 8.5 ± 0.4 mN mm^{-1} exercised).

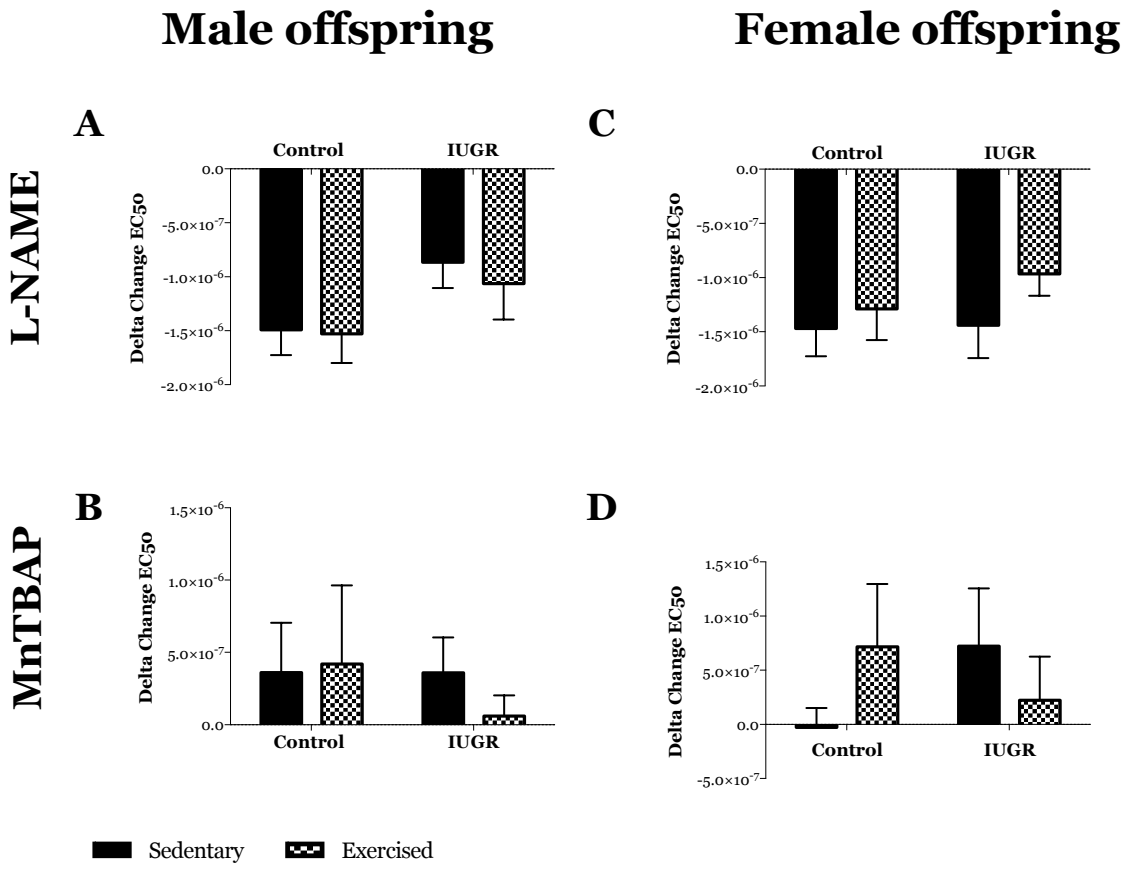

Figure 3.1. Mesenteric artery sensitivity to phenylephrine-induced vasoconstriction in the presence or absence of L-NAME or MnTBAP from male and female, control and IUGR, sedentary and exercised offspring.
Panel A- Delta change in the phenylephrine pEC_{50} following addition of L-NAME in males (n=10-13); Panel B- Delta change in the phenylephrine pEC_{50} following addition of MnTBAP in males (n=7-9). Panel C- Delta change in the phenylephrine pEC_{50} following addition of L-NAME in females (n=11-15); Panel D- Delta change in the phenylephrine pEC_{50} following addition of MnTBAP in females (n=7-10). Groups include: sedentary offspring (solid) and exercised offspring (hatched). Data are presented as mean ± SEM.

3.4.1.3 Methylcholine-induced vasodilation in male offspring

In male offspring, there was an interaction effect ($p<0.04$) between prenatal environment and activity status on MCh-induced vasodilation, whereby exercise increased vasodilation in control but decreased responses in IUGR offspring (Figure 3.2 A).

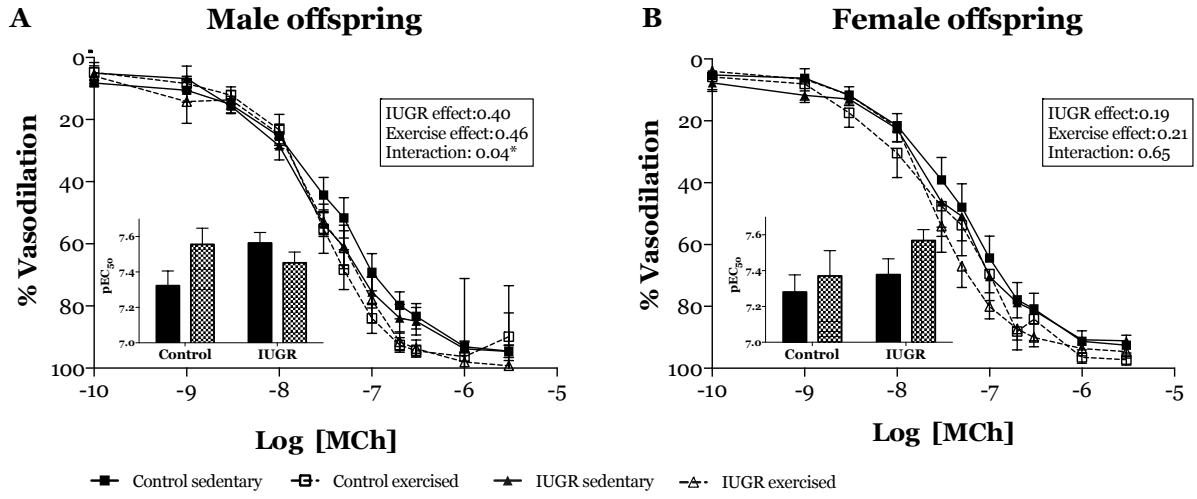

Figure 3.2. Cumulative concentration response curves to methylcholine in mesenteric arteries from male and female, control and IUGR, sedentary and exercised offspring.
Percent vasodilation to methylcholine (MCh) in mesenteric arteries from Panel A- males (n=8-13) and Panel B- females (n=8-13). Groups include: control sedentary offspring (solid lines, closed squares), control exercised offspring (dashed lines, open squares), IUGR sedentary offspring (solid lines, closed triangles) and IUGR exercised offspring (dashed lines, open triangles). Data are presented as mean ± SEM and summarized as pEC_{50} in the inset bar graph figures: sedentary offspring (solid) and exercised offspring (hatched). * $p < 0.05$ for a statistically significant interaction between aerobic exercise training and IUGR in male offspring.

L-NAME decreased mesenteric artery sensitivity to MCh (Figure 3.3 A; Table 3.2); demonstrating that there was an NO contribution to vasodilation in all male groups. Maximal vasodilation was also decreased in the presence of L-NAME in control sedentary and IUGR exercised offspring (Figure 3.3 A; Table 3.2). Based on analysis of

the delta AUC for all male groups, neither being born IUGR nor exercise affected the NO contribution to vasodilation in male offspring (Figure 3.3 B).

The addition of MnTBAP did not affect sensitivity or maximal vasodilation of mesenteric arteries to MCh in any of the groups (Figure 3.3 A; Table 3.2). Moreover, analysis of delta AUC did not show a significant group effect of either phenotype or exercise on MnTBAP treatment (Figure 3.3 B).

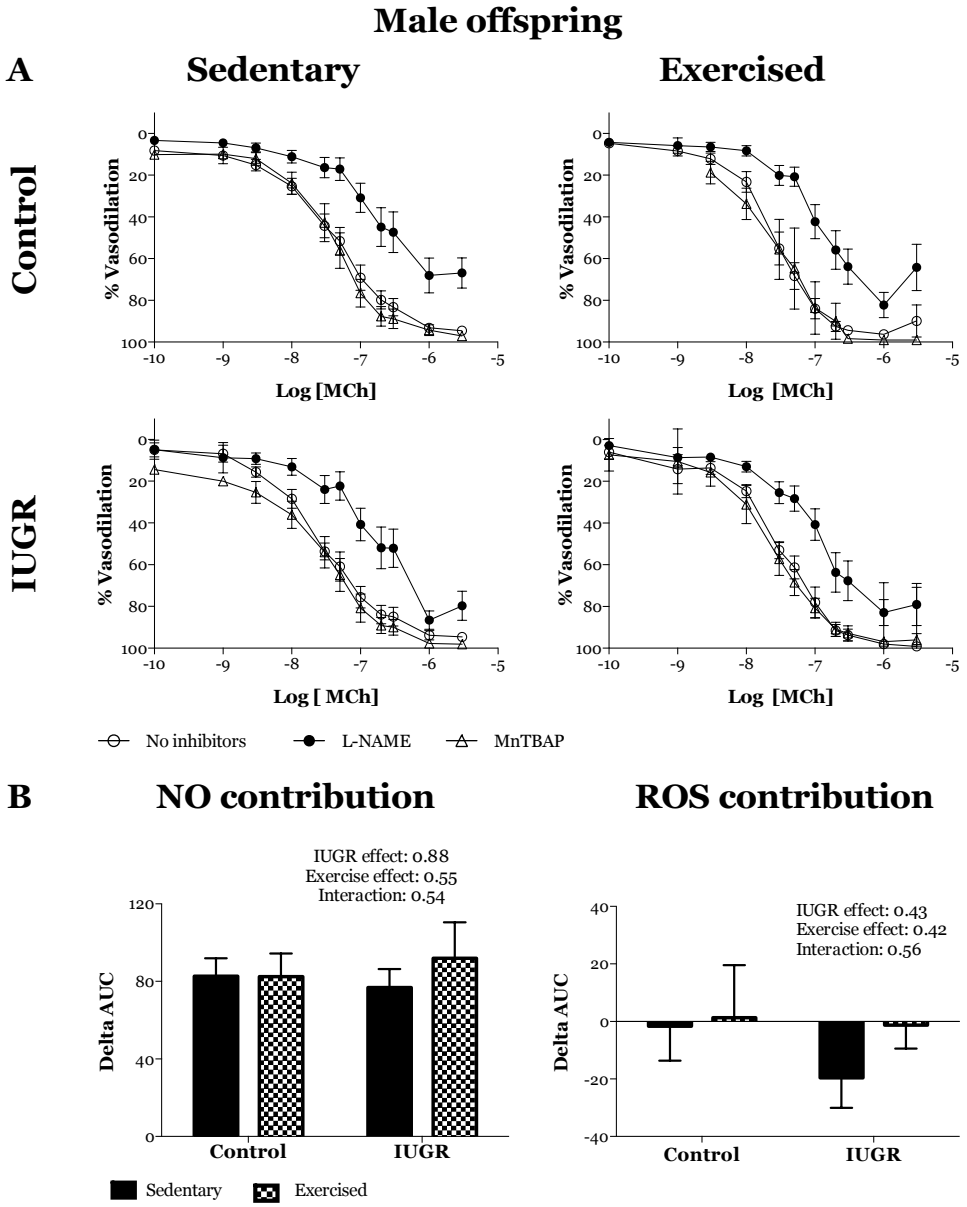

Figure 3.3. Cumulative concentration response curves to methylcholine in mesenteric arteries from male control and IUGR, sedentary and exercised offspring in the presence or absence of L-NAME or MnTBAP.

Panel A- Percent vasodilation to MCh in mesenteric arteries from males (n=7-13). Responses to methylcholine in control and IUGR animals in sedentary or exercised groups are shown in the absence of inhibitors (open circles); in the presence of L-NAME (closed circles); or in the presence of MnTBAP (open triangles). Panel B- Summary data of the delta AUC in vessels incubated with no inhibitor or L-NAME, equivalent to the contribution of NO to the vasodilator response to MCh; and in vessels incubated with no inhibitor or MnTBAP equivalent to the contribution of ROS to the vasodilator response to MCh. Data are presented as mean ± SEM.

Table 3.2. Mesenteric artery summary data (pEC_{50} and E_{max}) of vasodilation to methylcholine from male control and IUGR, sedentary and exercised offspring.

	No inhibitors	L-NAME	MnTBAP
		pEC_{50}	
Control sedentary offspring	7.32±0.08	6.8±0.14**	7.36±0.08
Control exercised offspring	7.56±0.06	7.01±0.1**	7.47±0.2
IUGR sedentary offspring	7.56±0.09	6.76±0.15***	7.43±0.11
IUGR exercised offspring	7.45±0.06	6.98±0.11**	7.59±0.09
		E_{max} (%)	
Control sedentary offspring	93.16±1.76	68.04±8.28**	94.24±2.40
Control exercised offspring	96.26±1.39	82.27±5.96	99.03±0.44
IUGR sedentary offspring	93.76±2.08	86.54±4.25	97.73±0.81
IUGR exercised offspring	98.01±2.56	82.87±6.15*	96.81±1.58

Data presented as mean ± SEM.*, **, *** $p<0.05$, $p<0.01$, $p<0.001$ vs. artery with no inhibitor after Dunnett's post-hoc test.

3.4.1.4 Methylcholine-induced vasodilation in female offspring

Neither phenotype nor AET had an effect on vasodilator responses to MCh in female offspring (Figure 3.2 B).

In female offspring, a significant reduction in sensitivity to MCh following the addition of L-NAME occurred in control sedentary and exercised offspring as well as IUGR exercised offspring (Figure 3.4 A, Table 3.3). Maximal vasodilation, however, was not reduced in the presence of L-NAME in any female group (Table 3.3). Analysis of the delta AUC demonstrated that there was a significant interaction of phenotype and

exercise suggesting that AET only improved NO-mediated vasodilation in control offspring ($p=0.03$; Figure 3.4 B).

MnTBAP did not affect vascular sensitivity to MCh or maximal vasodilation in either sedentary or exercised, control or IUGR female offspring (Figure 3.4 B; Table 3.3). Delta AUC analysis demonstrated that neither AET nor being born IUGR had an effect on the contribution of ROS to vasodilation (Figure 3.4 B).

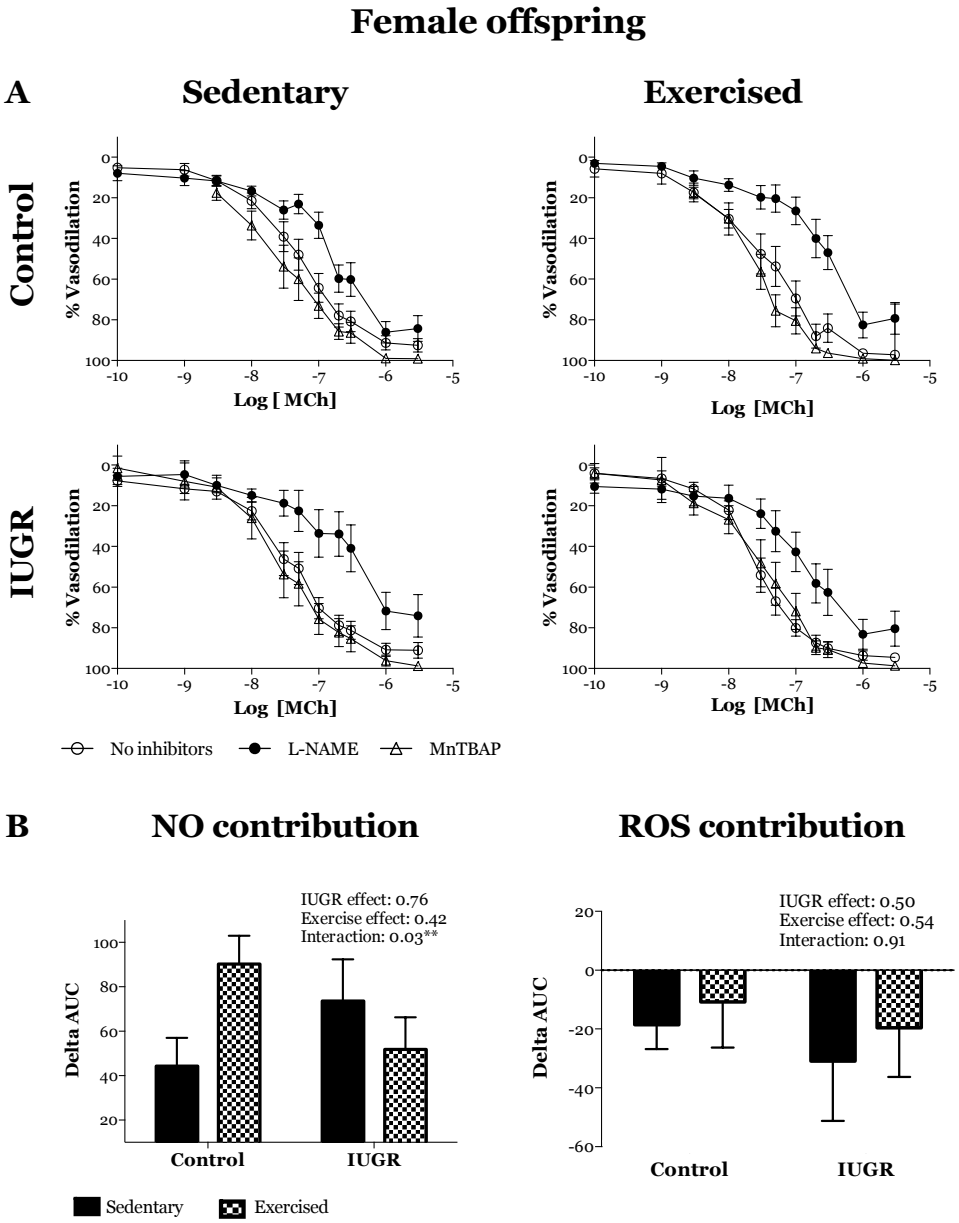

Figure 3.4. Cumulative concentration response curves to methylcholine in mesenteric arteries from female control and IUGR, sedentary and exercised offspring in the presence or absence of L-NAME or MnTBAP.
Panel A- Percent vasodilation to MCh in mesenteric arteries from females (n=7-13). Responses to methylcholine in control and IUGR animals in sedentary or exercised groups are shown in the absence of inhibitors (open circles); in the presence of L-NAME (closed circles); or in the presence of MnTBAP (open triangles). Panel B- Summary data of the delta AUC in vessels incubated with no inhibitor or L-NAME, equivalent to the contribution of NO to the vasodilator response to MCh; and in vessels incubated with no inhibitor or MnTBAP equivalent to the contribution of ROS to the vasodilator response to MCh. Data are presented as mean ± SEM. ** $p < 0.01$ for a statistically significant interaction between aerobic exercise training and IUGR in the presence of L-NAME.

Table 3.3. Mesenteric artery summary data (pEC_{50} and E_{max}) of vasodilation to methylcholine from female, control and IUGR, sedentary and exercised offspring.

	No inhibitors	L-NAME	MnTBAP
		pEC_{50}	
Control sedentary offspring	7.28±0.09	6.76±0.06*	7.49±0.26
Control exercised offspring	7.37±0.14	6.55±0.12***	7.55±0.09
IUGR sedentary offspring	7.37±0.08	6.39±0.51	7.49±0.14
IUGR exercised offspring	7.57±0.06	6.88±0.16**	7.38±0.12
		E_{max} (%)	
Control sedentary offspring	91.29±3.31	86.09±5.07	98.92±0.83
Control exercised offspring	96.47±1.81	82.57±6.24	99.11±0.39
IUGR sedentary offspring	90.75±3.13	71.75±9.07	96.06±2.51
IUGR exercised offspring	93.64±2.26	83.19±7.21	97.26±1.28

Data presented as mean ± SEM.*, **, *** $p<0.05$, $p<0.01$, $p<0.001$ vs. artery with no inhibitor after Dunnett's post-hoc test.

3.4.2 Gastrocnemius muscle arteries vascular function

3.4.2.1 Phenylephrine-induced vasoconstriction

There were no changes regarding sensitivity to PE or maximal PE-induced vasoconstriction in the presence or absence of L-NAME, indomethacin or Apamin + TRAM-34 in any of the groups (male and female, control and IUGR, sedentary and exercised; Table 3.4).

3.4.2.2 Vasoconstriction to high potassium solution

As was observed in mesenteric arteries, in both male and female offspring neither IUGR nor AET affected maximum vasoconstriction to high potassium solution; male

control offspring (10.6 ± 0.9 mN mm^{-1} sedentary vs. 9.7 ± 1.2 mN mm^{-1} exercised), male IUGR offspring (12.1 ± 0.8 mN mm^{-1} sedentary vs. 11.6 ± 0.8 mN mm^{-1} exercised); female control offspring (10.3 ± 0.7 mN mm^{-1} sedentary vs. 8.9 ± 0.5 mN mm^{-1} exercised) or female IUGR offspring (8.9 ± 0.5 mN mm^{-1} sedentary vs. 9.6 ± 0.7 mN mm^{-1} exercised).

Table 3.4. Gastrocnemius muscle arteries phenylephrine-induced vasoconstriction in the presence or absence of L-NAME, Apamin + TRAM-34 and indomethacin, from male and female, control and IUGR, sedentary and exercised offspring.

	No inhibitors	L-NAME	Apamin + TRAM-34	Indomethacin
Male offspring (E_{max}; mN mm^{-1})				
Control Sedentary offspring	12.72 ± 0.47	11.33 ± 1.28	9.57 ± 1.09	12.22 ± 0.79
Control Exercised offspring	12.47 ± 0.80	9.97 ± 1.24	11.33 ± 1.32	10.69 ± 0.38
IUGR Sedentary offspring	12.29 ± 1	11.68 ± 2.2	10.29 ± 0.64	11.47 ± 1.68
IUGR Exercised offspring	11.4 ± 1.5	8.82 ± 0.99	11.03 ± 1.08	11.63 ± 1.27
Female offspring (E_{max}; mN mm^{-1})				
Control Sedentary offspring	11.9 ± 0.64	12.49 ± 1.17	9.76 ± 0.53	10.99 ± 0.76
Control Exercised offspring	10.94 ± 0.64	10.94 ± 1.8	9.95 ± 0.72	9.23 ± 0.69
IUGR Sedentary offspring	11.9 ± 0.58	8.39 ± 1.63	8.66 ± 0.37	10.2 ± 0.68
IUGR Exercised offspring	10.25 ± 1.14	9.28 ± 1.87	8.42 ± 0.62	10 ± 0.95

Data presented as mean ± SEM.

3.4.2.3 Methylcholine-induced vasodilation in male offspring

In male offspring, there was an interaction between being born IUGR and AET ($p=0.04$) whereby maximal vasodilation to MCh tended to be increased following exercise only in IUGR offspring (E_{max}; 76 ± 4.9% sedentary control vs. 61.9 ± 9.2% exercised control; 71.22 ± 8.1% sedentary IUGR vs. 89.9 ± 6.8% exercised IUGR).

Following the addition of L-NAME, a decrease in the MCh E_{max} demonstrated that NO contributed to vasodilation in gastrocnemius muscle arteries from control sedentary offspring, but not IUGR sedentary offspring. The variability in the IUGR offspring data, however, was greater and thus careful interpretation of the data is required (Figure 3.5, Table 3.5). Interestingly, NO significantly contributed to vasodilation in both control and IUGR groups following exercise ($p<0.01$ and $p<0.001$; Table 3.5). In addition, we found that EDH contributed to vasodilation in gastrocnemius muscle arteries from control sedentary offspring ($p<0.05$) as well as IUGR exercised offspring ($p<0.001$, Table 3.5). There was no significant contribution of prostaglandins to vasodilation in any male group. Further, sensitivity to MCh was unaltered by any inhibitor in control or IUGR, sedentary or exercised offspring (Table 3.5).

Analysis of the delta AUC demonstrated that neither phenotype nor AET had an effect on either NO-mediated vasodilation (Figure 3.6 A) or prostaglandin-mediated vascular responses (Figure 3.6 C). Further, EDH-mediated vasodilation was increased only in IUGR exercised male offspring (Figure 3.6 B).

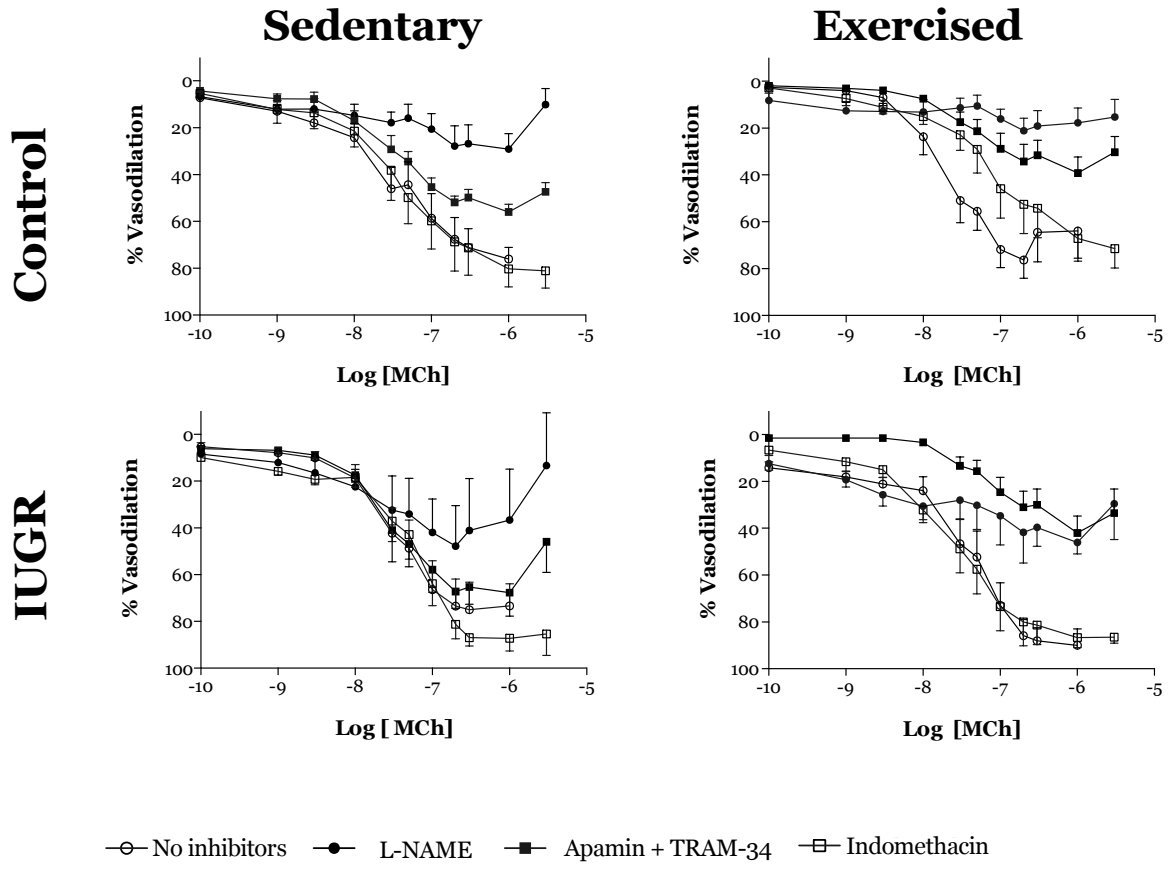

Figure 3.5. Cumulative concentration response curves to methylcholine of gastrocnemius muscle arteries from male, control and IUGR, sedentary and exercised offspring in the presence or absence of L-NAME, Apamin+TRAM-34 or indomethacin.

Percent vasodilation to MCh in gastrocnemius muscle arteries from male offspring (n=4-8). Responses to methylcholine in control and IUGR animals in sedentary or exercised groups are shown in the absence of inhibitors (open circles); in the presence of L-NAME (closed circles); in the presence of Apamin+TRAM-34 (closed squares); or in the presence of indomethacin (open squares). Data are presented as mean ± SEM.

Table 3.5. Gastrocnemius muscle artery summary data (pEC_{50} and E_{max}) of vasodilation to methylcholine from male control and IUGR, sedentary and exercised offspring.

	No inhibitors	L-NAME	Apamin + TRAM-34	Indomethacin
	pEC_{50}			
Control Sedentary offspring	7.51±0.16	7.73±0.63	7.52±0.09	7.37±0.18
Control Exercised offspring	7.73±0.26	7.73±0.6	7.44±0.15	7.05±0.23
IUGR Sedentary offspring	7.60±0.14	8.04±0.61	7.63±0.14	7.21±0.08
IUGR Exercised offspring	7.37±0.09	.	7.19±0.17	7.57±0.13
	E_{max} (%)			
Control Sedentary offspring	76.03±4.89	29.09±6.48***	55.96±3.19*	80.25±7.66
Control Exercised offspring	58.88±10.68	17.72±6.25**	39.16±6.72	67.17±9.63
IUGR Sedentary offspring	71.22±8.12	36.6±21.65	67.65±0.81	87.17±5.28
IUGR Exercised offspring	89.92±6.82	46.06±4.87***	42.03±7.13***	86.63±4.17

Data presented as mean ± SEM.*, **, *** $p<0.05$, $p<0.01$, $p<0.001$ vs. artery with no inhibitor after Dunnett's post-hoc test.

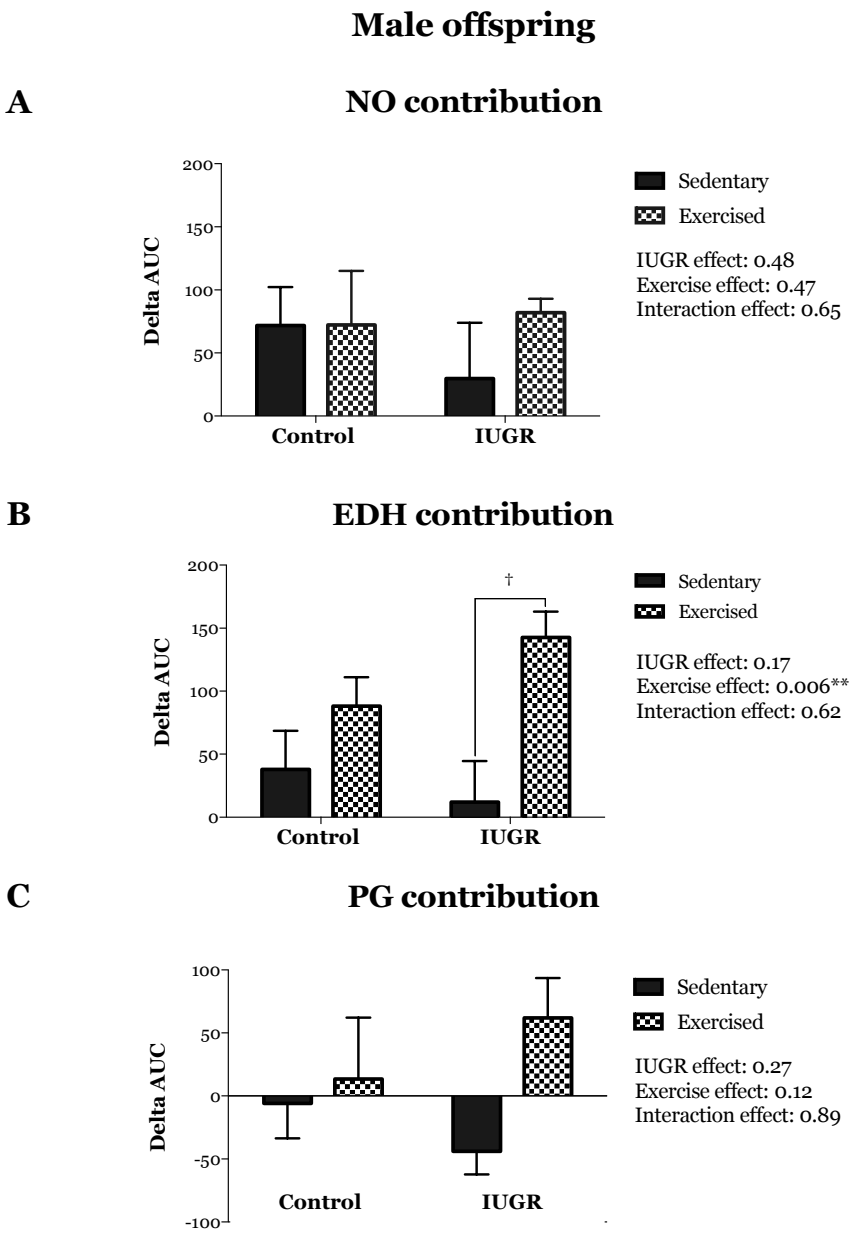

Figure 3.6. Nitric oxide, EDH and prostaglandin contribution to gastrocnemius muscle artery vasodilation in male control and IUGR, sedentary and exercised offspring.

Summary data of vasodilator responses to MCh in gastrocnemius muscle arteries (n=4-8). Panel A- Summary data of the delta AUC in vessels incubated with no inhibitor or with L-NAME; equivalent to the contribution of NO to vasodilator responses to MCh. Panel B- Summary data of the delta AUC in vessels incubated with no inhibitor or with Apamin+TRAM-34; equivalent to the contribution of EDH to vasodilation. Panel C- Summary data of the delta AUC in vessels incubated with no inhibitor or with indomethacin; equivalent to the contribution of prostaglandins (PG) to vasodilation. Data are presented as mean ± SEM; sedentary offspring (closed bars); exercised offspring (hatched bars). ** $p < 0.01$ for a statistically significant exercise effect. † $p < 0.05$ vs. sedentary IUGR offspring after a Bonferroni post-hoc test.

3.4.2.4 Methylcholine -induced vasodilation in female offspring

Being born growth restricted impaired maximal vasodilation to MCh in female offspring (E_{max}; 93.1 ± 0.3% control vs. 84.3 ± 4.2% IUGR, p=0.02). Performing aerobic exercise did not improve vasodilation in either control (E_{max}; 93.1 ± 0.3% sedentary vs. 92.8 ± 5.5% exercised) or IUGR female offspring (E_{max}; 84.3 ± 4.2% sedentary vs. 70. ± 10.9% exercised).

In control sedentary females, NO contributed to vasodilation (Figure 3.7, ΔpEC_{50} p<0.05, ΔE_{max} p<0.001; Table 3.6). Following exercise, a contribution of NO to vasodilation was still observed in control offspring (ΔE_{max} p<0.001; Table 3.6). While neither EDH nor prostaglandins were found to significantly contribute to vasodilation in either sedentary or exercised control offspring, the variability of the data may have precluded the detection of more minor contributions of these vasodilator pathways. In IUGR sedentary or exercised female offspring, there were no significant changes in sensitivity to MCh or maximal vasodilation after the addition of any inhibitor (Figure 3.7; Table 3.6). As observed in arteries from male IUGR offspring, however, the variability of the data was greater than in control groups, complicating the interpretation.

Analysis of the delta AUC demonstrated that being born growth restricted was associated with a reduced NO vasodilator component (p=0.007, Figure 3.8 A), an increased involvement of prostaglandin-mediated vasoconstriction (p=0.002, Figure 3.8 C) and had no effect on EDH-mediated vasodilation (Figure 3.8 B).

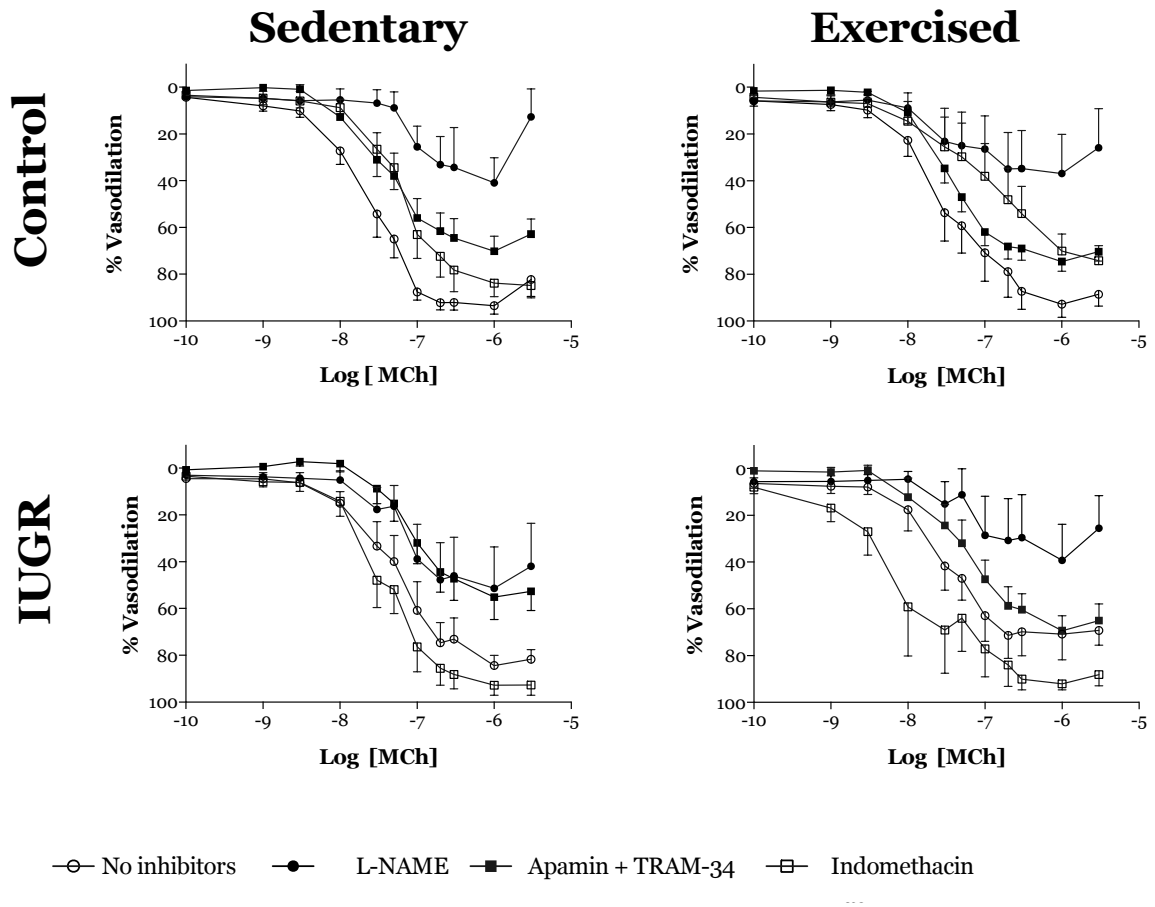

Figure 3.7. Cumulative concentration response curves to methylcholine of gastrocnemius muscle arteries from female, control and IUGR, sedentary and exercised offspring in the presence or absence of L-NAME, Apamin+TRAM-34 or indomethacin.

Percent vasodilation to MCh in gastrocnemius muscle arteries from female offspring (n=4-7). Responses to methylcholine in control and IUGR animals in sedentary or exercised groups are shown in the absence of inhibitors (open circles); in the presence of L-NAME (closed circles); in the presence of Apamin+TRAM-34 (closed squares); or in the presence of indomethacin (open squares). Data are presented as mean ± SEM.

Table 3.6. Gastrocnemius muscle artery summary data (pEC_{50} and E_{max}) of vasodilation to methylcholine from female, control and IUGR, sedentary and exercised offspring.

	No inhibitors	L-NAME	Apamin + TRAM-34	Indomethacin
	pEC_{50}			
Control Sedentary offspring	7.61±0.07	7.11±0.18*	7.45±0.08	7.23±0.08
Control Exercised offspring	7.53±0.12	7.59±0.42	7.29±0.13	6.87±0.34
IUGR Sedentary offspring	7.29±0.11	7.22±0.19	7.1±0.1	7.43±0.08
IUGR Exercised offspring	7.56±0.13	7.21±0.30	7.28±0.11	8.1±0.22
	E_{max} (%)			
Control Sedentary offspring	93.4±3.47	32.61±12.25***	70.13±6.33	83.84±5.67
Control Exercised offspring	92.8±5.49	36.85±16.61**	72.10±3.97	70.04±7.17
IUGR Sedentary offspring	84.34±4.15	51.39±17.62	55.16±9.54	92.81±4.24
IUGR Exercised offspring	70.79±10.87	39.26±15.26	69.3±6.24	92.11±2.48

Data presented as mean ± SEM.*, **, *** $p<0.05$, $p<0.01$, $p<0.001$ vs. artery with no inhibitor after Dunnett'spost-hoc test.

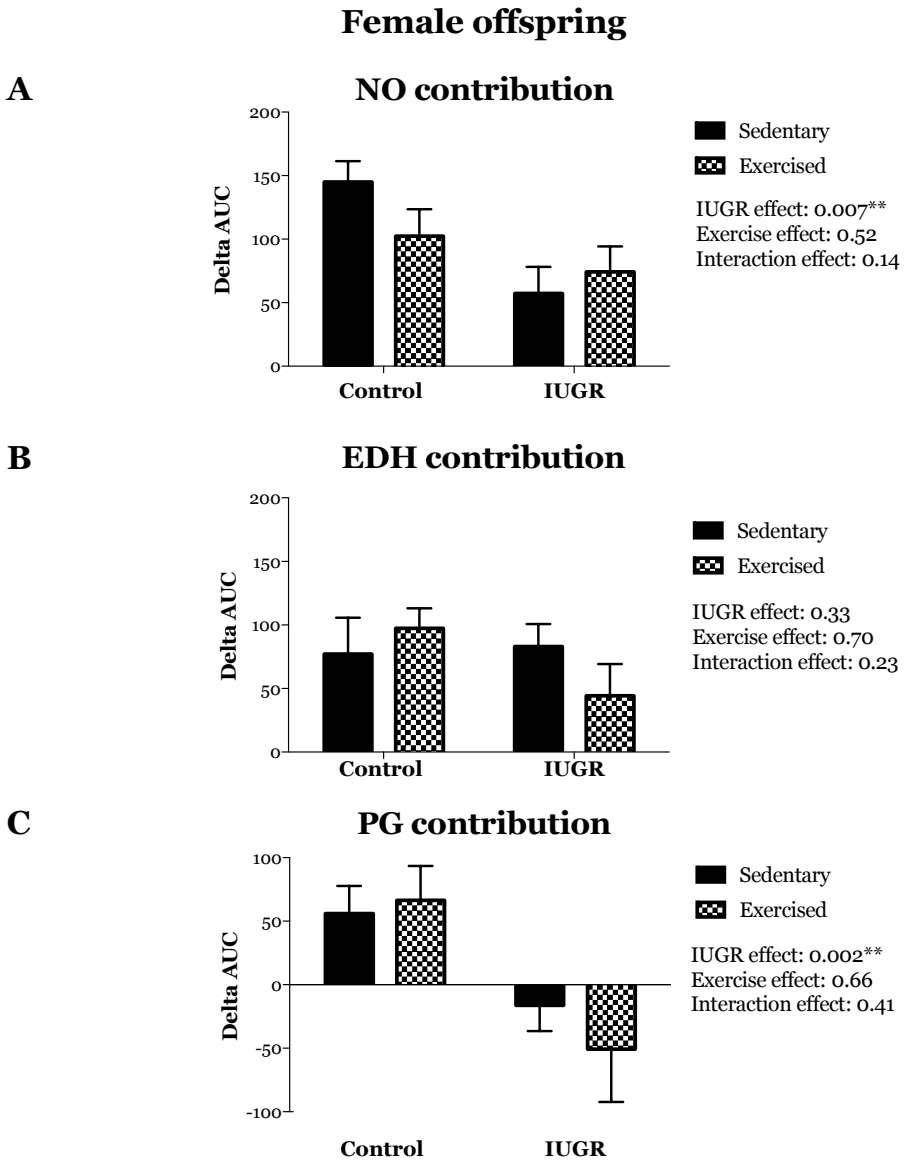

Figure 3.8. Nitric oxide, EDH and prostaglandin contribution to gastrocnemius muscle artery vasodilation in female, control and IUGR, sedentary and exercised offspring.

Summary data of vasodilator responses to MCh in gastrocnemius muscle arteries (n=4-7). Panel A- Summary data of the delta AUC in vessels incubated with no inhibitor or with L-NAME; equivalent to the contribution of NO to the vasodilator responses to MCh. Panel B- Summary data of the delta AUC in vessels incubated with no inhibitor or with Apamin+TRAM-34; equivalent to the contribution of EDH to vasodilation. Panel C- Summary data of the delta AUC in vessels incubated with no inhibitor or with indomethacin; equivalent to the contribution of prostaglandins (PG) to vasodilation. Data are presented as mean ± SEM; sedentary offspring (closed bars); exercised offspring (hatched bars). Being born growth restricted was associated with a reduced NO vasodilator component, an increased involvement of prostaglandin-mediated vasoconstriction, and had no effect on EDH-mediated vasodilation. ** $p < 0.01$ for a statistically significant IUGR effect.

3.5 Discussion

Using a hypoxia-induced IUGR model, we found that female IUGR offspring had reduced NO-mediated vasodilation in both mesenteric and gastrocnemius muscle arteries; while in male IUGR offspring NO-mediated vasodilation was reduced only in the gastrocnemius muscle arteries. Furthermore, we demonstrated that female IUGR offspring had a decreased vasodilator response in gastrocnemius muscle arteries and there was an increase in prostaglandin-mediated vasoconstriction. Interestingly, while in control offspring AET increased vasodilator responses in mesenteric arteries from males and enhanced NO-modulation in mesenteric arteries from females in IUGR offspring, AET only improved EDH-mediated vasodilation in gastrocnemius muscle arteries from males.

Previous studies have also associated a hypoxic insult during the last third of pregnancy with vascular dysfunction in the offspring. Prenatal hypoxia in rats has been associated with increased fetal aortic thickness,[98,103] and with increased vascular $ONOO^-$ generation,[98] which can lead to endothelial dysfunction. In addition, it has been shown in neonatal rats that there was an enhanced femoral vasoconstrictor response to PE while this response in carotid arteries was decreased; demonstrating that neonatal vascular adaptations following a hypoxic insult are vascular bed specific and may be pivotal in increasing blood flow to vital organs such as the brain while reducing blood flow to other organs.[90] In both male and female IUGR offspring as adults, the contribution of NO to mesenteric artery vasodilation was reduced.[86,87,98,104] In one previous study, only female IUGR offspring demonstrated an enhanced MEGJ/EDH-mediated vasodilation in mesenteric arteries as a possible compensatory

mechanism to maintain vascular function.[86] In the current study, however, EDH-mediated vasodilation was not significantly enhanced in gastrocnemius muscle arteries from either male or female IUGR offspring.

The present study also demonstrated that exposure to hypoxia *in utero* was associated with an enhanced PG-mediated vasoconstriction in the gastrocnemius muscle arteries in IUGR female offspring. Mechanisms that have been associated with increased prostaglandin-mediated vasoconstriction include: upregulation of COX-1 and 2; activation of COX-1 and 2 by ROS and enhanced activation of thromboxane receptors. Interestingly, increased PG-mediated vasoconstriction has been associated with conditions of vascular pathologies such as hypertension[230] and diabetes[231] as well as aging.[321] The increased PGHS-dependent constriction observed in our study of young female adults exposed to prenatal hypoxia suggests the development of vascular pathology prior to overt disease.

Our data suggest that the mechanisms which lead to endothelial dysfunction in IUGR offspring differ according to sex; with a greater negative impact on female IUGR offspring compared to male IUGR offspring. Contrary to our findings, Ozaki *et al.* found that both hypertension and vascular dysfunction in the offspring of protein-restricted dams was predominant among male offspring.[322] In a model of uteroplacental insufficiency fetal programming, however, female offspring exhibited increased arterial wall stiffness in uterine and renal arteries in the absence of altered vascular reactivity in either mesenteric or femoral vascular beds.[323] Further, previous findings from our laboratory have determined that in aged female IUGR offspring NO-mediated, flow-induced vasodilation was reduced and there was a predominant

EDH-mediated vasodilator component compared to male IUGR offspring.[87] In addition, with aging the mechanisms involved in preserving EDH-mediated vasodilation were different between sexes; a decrease in the contribution of MEGJ to vasodilation has been found in aged male IUGR offspring compared to aged female IUGR offspring.[86] It has also been reported that a hypoxic insult during pregnancy increased myogenic tone in mesenteric arteries in only male IUGR offspring.[105] We can conclude that the type and severity of the insult that create a phenotype, the vascular bed assessed, as well as the sex of the offspring all play a role in the presentation of vascular dysfunction later in life following a compromised pregnancy.

AET has been shown to improve vascular function by increasing NO bioavailability[278] and by reducing ROS.[275] Our findings in control female offspring showed that AET enhanced NO-mediated vasodilation without affecting total vasodilation responses in mesenteric arteries. This could be due to a reduction in other vasodilatory pathways such as EDH or PG; however, it is also possible that there is the potential for redundancy of vasodilator mechanisms.

Our results regarding ROS did not demonstrate improved vasodilation with a $ONOO^-$ scavenger. The impairment of NO-mediated vasodilation observed in female IUGR offspring, therefore, was not likely to be secondary to a reduction in NO bioavailability via scavenging by $O_2^{\cdot-}$ and might instead be due to a decrease in NO production. Further experiments are needed in order to assess this observation.

In accordance with our findings, it has previously been demonstrated that exercise training was associated with an increase in EDH-mediated vasodilation in

animal models of disease such as hypertension[284,324] and diabetes.[325] The mechanism by which this improvement may occur is not completely understood and remains under investigation. Exercise training, however, has been shown to increase whole cell K^+ current activation and improve functional gating of K_{ca} channels without affecting protein expression in coronary arteries from diabetic dislipidemic pigs.[326] In addition, Milkau *et al.* determined that EDH-mediated vasodilation was crucial for active hyperemia, and was mediated through the activation of endothelial SK_{ca} and spread along the vascular wall via connexin 40 endo-endo and myo-endo gap junctions.[327] Upregulation of SK_{ca} and connexin 40 or improvement of their function could, therefore, be involved in the beneficial effect of AET in the vasculature observed in male IUGR offspring.

In conclusion, exposure to hypoxia *in utero* was associated with decreased NO-mediated vasodilation in female offspring in both mesenteric and gastrocnemius muscle arteries. An increase in PG-mediated vasoconstriction in gastrocnemius muscle arteries was also observed in female IUGR offspring. Our data suggest that exercise enhanced NO-mediated vasodilation in female control offspring but not IUGR offspring. Furthermore, exercise improved EDH-mediated vasodilation only in male IUGR offspring. Results from the present study highlight that understanding the mechanisms by which exercise impacts specific vascular beds in a susceptible population is essential. Exercise may not prove to be a beneficial instrument for specific vascular pathways affected by prenatal hypoxia, particularly in female offspring.

4 Aerobic exercise training reduces cardiac function in adult male offspring exposed to prenatal hypoxia[3]

4.1 Introduction

Intrauterine growth restriction following a hypoxic insult has been associated with specific cardiac structural changes from early stages in development. It has been established that chronic exposure to hypoxia during pregnancy is associated with a decrease in fetal and neonatal cardiomyocyte proliferation and an increase in cardiomyocyte apoptosis.[91,96] Furthermore, an increase in collagen deposition soon after birth[96] and later in life[89] has been found in offspring exposed to hypoxia *in utero*. Moreover, cardiovascular functional changes in offspring born from hypoxic pregnancies have also been reported. Kane *et al.*[97] found that chronic exposure to hypoxia during pregnancy leads to an increased sympathetic output and increase in baroreflex gain in adult offspring. In addition, hypoxia-induced IUGR has been associated with an increased oxidized/reduced glutathione ratio in the myocardium; demonstrating an increase in oxidative stress.[234] Moreover, adult offspring born from hypoxic pregnancies have an increased susceptibility to cardiac I/R injury.[89,100,101,107]

[3] A version of this Chapter was published in a peer reviewed journal:
Reyes LM, Kirschenman R, Quon A, Morton JS, Shah A, Davidge ST. Aerobic exercise training reduces cardiac function in adult male offspring exposed to prenatal hypoxia. Am J Physiol Regul Integr Comp Physiol. 2015; 309: R489-98.
Reyes LM: Contribution: Data collection: 80%, data analyses: 100%, manuscript writer: 100%,, paper submission: 100%..
Kirschenman R Contribution: Handle the animals in the exercise protocol.
Quon A Contribution: Prepared the samples and performed the western blots.
Shah A Contribution: Data collection.
Morton JS and Davidge ST Contribution: Critical review of the manuscript.

As previously addressed in Chapter 1, there are several mechanisms by which AET could potentially improve cardiac outcomes; however, a reduction in ROS is one of the main mechanisms by which AET may confer cardioprotection. AET has been shown to improve the antioxidant capacity of cardiomyocytes; several studies have shown that SOD-1, SOD-2, CAT and GPx protein expression and/or activity are upregulated following AET.[274] Exercise, nonetheless, has also been shown to cause transient myocardial damage [289] and atrial fibrillation.[290] Likewise, AET was shown to negatively impact ROS modulation in an animal model of obesity and diabetes.[291] Thus, whether cardioprotection can be achieved through exercise in compromised populations is debatable and further investigation is required to determine the effects of AET in a susceptible population such as IUGR offspring. We have shown (Chapter 3, [328]) that AET had a beneficial vascular effect in only male IUGR offspring; where an enhanced EDH-mediated vasodilation in gastrocnemius muscle arteries was found. The impact of AET on cardiac performance in IUGR offspring, however, remains unknown.

4.2 Objectives

The present study encompasses three objectives. The first aim was to evaluate whether AET could alter cardiac structure. The second aim was to determine whether AET could improve cardiac performance after I/R injury in IUGR offspring. Finally, we wanted to assess the role of ROS in the pathophysiology of I/R injury in IUGR offspring. Thus, the third aim was to investigate whether AET improved cardiac protein expression of antioxidant enzymes such as SOD-1, SOD-2 and CAT, and reduced cardiac $O_2^{\cdot-}$ production.

4.3 Methods

4.3.1 In vivo Echocardiography

At 15 weeks of age, rats (control and IUGR, sedentary and exercised, male and female) were anesthetized with inhaled isoflurane (2% mixed with 1.5 L/min 19.5% O_2. for the initial induction, and thereafter 1.5% after the loss of consciousness). Rats were immobilized on a heating platform in a supine position and their extremities were fixed to electrodes on the heating platform surface. Electrocardiogram electrodes continuously monitored heart rate and respiratory rate. Body temperature was monitored using a rectal probe. After depilating the chest area, a transthoracic echocardiography was performed using the Vevo 2100 digital imaging platform (VisualSonics, Canada) with a 13-23 MHz transducer. M-mode images from the parasternal short and long axis views, as well as pulse-wave Doppler images were taken. Animals from the exercised groups did not perform the exercise protocol on the day of echocardiographic assessment. The following formulas were used to calculate echocardiogram parameters; left ventricular internal diameter in diastole ($LVID_{dias}$); left ventricular internal diameter in systole ($LVID_{sys}$), left ventricular volume in diastole (LVV_{dias}) and left ventricular volume in systole (LVV_{sys}).

Left ventricular volume; diastole = $(7.0/(2.4 + LVID_{dias})) * LVID_{dias}^3$

Left ventricular volume; systole = $(7.0/(2.4 + LVID_{sys})) * LVID_{sys}^3$

% Ejection fraction = $100 * ((LVV_{dias} - LVV_{sys}) / LVV_{dias})$

% Fractional shortening = $100 * ((LVID_{dias} - LVID_{sys}) / LVID_{dias})$

Left ventricular mass = 1.053 * (($LVID_{dias}$ + $LVPW_{dias}$ + IVS_{dias})^3 – $LVID_{dias}^3$)

4.3.2 Ex vivo Ischemia/Reperfusion protocol

At 16 weeks of age, and 24 hours after the last bout of exercise, offspring were anesthetized with a single dose (1.5 mL in a 4 litre chamber) of inhaled isoflurane. When the pedal reflex was absent, hearts were rapidly excised and the aortas were fixed to a cannula and perfused in a retrograde Langendorff mode with Krebs–Henseleit solution [in mmol/L: 120 NaCl, 25 $NaHCO_3$, 5.5 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$ and 2.5 $CaCl_2$ (pH 7.4 gassed with 95% O_2 and 5% CO_2)]. The left atrium was cannulated and hearts were subsequently perfused in an anterograde working heart mode. Hearts were paced at 300 b.p.m. The working heart protocol included 30 min of baseline (pre-ischemia), followed by 10 min of global, no-flow ischemia and 40 min of reperfusion. This protocol was based on previous data where isolated heart experiments were carried out in young and aged offspring.[107,187] Measurements of cardiac function were obtained every 10 min. Signals from all sensors (flow, pressure, temperature, ECG) were acquired using an interface and recorded using the Isoheart Software (Harvard Apparatus, USA). Cardiac performance during the I/R protocol was determined as previously described[107,329] by calculating cardiac power [peak systolic pressure (mmHg) – maximal preload (mmHg) * cardiac output (mL/ min) * 0.13] / heart dry weight (g).

4.3.3 Western blot analyses in non-perfused hearts

Non-perfused heart tissue was homogenized in a lysis buffer containing [in mmol/L: 20 Tris (pH 7.4), 5 EDTA, 10 sodium pyrophosphate tetrabasic, 100 sodium fluoride, and 1% NP-40, and Protease Inhibitor Cocktail (1X Halt™ protease inhibitor, Thermo scientific, USA)]. Protein concentrations were determined by bicinchoninic acid

assay (Pierce). A total of 100 μg of protein was loaded and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed. Membranes were blocked with blocking buffer for fluorescent western blotting (Rockland Immunochemicals Inc., USA) and they were incubated with anti-SOD-1 C-17 (1:1,000; Santa Cruz Biotechnology, USA), anti-SOD-2 FL (1:2,000; Santa Cruz Biotechnology, USA), anti-CAT (1:1,000; Abcam, UK), or anti-GPx (1:5,000; Abcam, UK) and re-probed with anti-α-tubulin (1:2,500; Abcam, UK) for protein loading control. Finally, membranes were probed with the following secondary antibodies (1:10,000; IRDye 800 donkey and 1:10,000; IRDye 680 donkey; USA). Imaging was performed on a Li-Cor Odyssey system. Protein bands were quantified with corresponding software (Li-Cor Biosciences, Canada). Results were normalized to α-tubulin.

4.3.4 Superoxide detection in cardiac tissue

A piece of non-perfused left ventricle was embedded in optimal cutting medium (OCT) and snap-frozen in liquid nitrogen and stored at -80 °C until use. Sections were cut at 20 μm using a cryostat. $O_2^{\cdot -}$ generation was measured by staining the tissues with dihydroethidium (DHE). DHE is a cell permeable compound which reacts with intracellular and extracellular $O_2^{\cdot -}$ to produce ethidium. Fluorescence is generated when ethidium then binds to nuclear DNA. Four slides with four sections (one from each group: control sedentary, control exercised, IUGR sedentary, and IUGR exercised) were prepared for male and female offspring. This allowed measurements for each tissue to be replicated four times. Slides were thawed, washed three times with Hanks' balanced salt solution (HBSS), and incubated for 10 min at 37 °C. After removal of HBSS, fresh DHE

(200 µmol/L) was added and the slides were incubated for 30 min at 37 °C. After removal of DHE and washes with HBSS, slides were immediately viewed using a fluorescence microscope (IX81 Olympus, Japan) and images were obtained with cellSense (Olympus, Japan). All samples were analyzed within 1 hour of staining. Images are presented at 20X magnification.

4.3.5 Statistical analyses

The data are presented as mean ± SEM. This study had a two-way ANOVA design where the effect of being born growth restricted and the effect of AET were determined. Female and male offspring data were analyzed separately due to differences in their phenotype. All the variables were tested using a two-way ANOVA followed by a Bonferroni *post hoc* test. To determine cardiac performance after ischemia, a mean of the baseline cardiac power in each group was estimated and then the percentage recovery of cardiac power was calculated. For western blot analyses, comparisons between the groups were performed by normalizing to the sedentary control group and then assessing the percent change in the other groups (control exercised, IUGR sedentary and IUGR exercised). For DHE analyses, the mean intensity of the staining was normalized to the number of nuclei/picture using ImageJ software (National Institutes of Health, USA), and an average of the four pictures was calculated. Comparisons between the groups were performed by normalizing to the sedentary control group and then assessing the percent change in the other groups (control exercised, IUGR sedentary and IUGR exercised). Statistical significance was defined as $p \leq 0.05$. All data were analyzed using GraphPad Prism 6 statistical software (GraphPad Software, USA).

4.4 Results

4.4.1 Echocardiography

In male offspring, neither being born from a hypoxic environment nor AET affected left ventricular wall dimensions or heart function variables such as ejection fraction, fractional shortening or stroke volume (Table 4.1). No changes in the aortic or pulmonary valve flow were found and mitral valve function was preserved in all groups (Table 4.2).

In female offspring, there were no differences among the groups regarding left ventricular wall dimensions and systolic function (ejection fraction, fractional shortening and stroke volume, Table 4.3). Moreover, we found that compared to control sedentary female offspring, pulmonary valve peak velocity was increased in IUGR sedentary female offspring (control 910.3±49.5 ms vs. IUGR 1028.3±74.8 ms; $p<0.05$, Table 4.4). There were no differences among the groups regarding diastolic function (mitral valve function, Table 4.4).

Table 4.1. Echocardiographic data obtained from male control and IUGR, sedentary and exercised offspring.

	Control Sedentary	Control Exercised	IUGR Sedentary	IUGR Exercised
Morphologic parameters				
IVS; diastole (mm)	1.21±0.08	1.02±0.03	1.18±0.06	1.22±0.08
IVS; systole (mm)	1.69±0.12	1.50±0.10	1.68±0.05	1.76±0.13
LVAW; diastole (mm)	2.16±0.07	2.29±0.15	2.31±0.16	1.97±0.10
LVAW; systole (mm)	3.55±0.10	3.79±0.22	3.70±0.19	3.46±0.12
LVID; diastole (mm)	8.34±0.28	7.99±0.28	8.05±0.24	8.34±0.09
LVID; systole (mm)	4.49±0.36	4.45±0.16	4.44±0.25	4.57±0.16
LVPW; diastole (mm)	2.44±0.14	2.46±0.18	2.24±0.06	2.20±0.08
LVPW; systole (mm)	3.70±0.21	3.44±0.20	3.52±0.11	3.39±0.15
LV mass (mg)	1211.30±61.74	996.81±82.03	1053.42±43.95	1101.02±50.63
LV mass/body weight (mg/g)	1.89±0.12	1.70±0.10	1.71±0.06	1.86±0.07
Volume parameters				
LV volume; diastole (uL)	383.38±27.46	346.95±27.62	352.21±23.41	378.70±9.10
LV volume; systole (uL)	100.29±16.85	91.08±7.81	93.11±11.67	96.72±7.63
Systolic function				
Stroke volume (uL)	296.43±18.98	289.74±21.94	286.18±13.33	312.20±16.19
Ejection fraction (%)	82.74±1.81	82.52±1.51	80.04±1.43	80.29±1.61
Fractional shortening (%)	53.96±1.98	53.29±1.76	50.66±1.39	50.95±1.69
Cardiac output (mL/min)	91.16±6.96	102.73±11.09	88.30±6.26	111.96±10.37

Data are presented as mean ± SEM. IVS: inter ventricular septum; LVAW: left ventricular anterior wall; LVID: left ventricular internal diameter; LVPW: left ventricular posterior wall; LV: left ventricle.

Table 4.2. Pulse-wave Doppler data obtained from male control and IUGR, sedentary and exercised offspring.

	Control Sedentary	Control Exercised	IUGR Sedentary	IUGR Exercised
Aortic valve flow				
Ejection time (ms)	89.54±9.57	75.45±4.21	76.18±3.39	74.69±3.04
Outflow maximum ejection velocity (mm/s)	1024.37±40.64	1139.50±114.36	985.09±47.14	984.36±90.97
Peak pressure gradient (mmHg)	4.26±0.33	5.40±1.00	4.09±0.42	4.07±0.80
Pulmonary valve flow				
Acceleration time [PAT] (ms)	36.89±1.35	34.64±4.36	35.26±2.02	35.07±3.00
Ejection time [PET] (ms)	77.78±2.34	74.08±2.48	78.57±2.96	77.38±1.98
Valve peak velocity (ms)	874.85±60.73	921.75±22.41	952.33±42.96	933.88±78.85
PAT/PET	0.48±0.02	0.47±0.06	0.45±0.02	0.45±0.03
Peak pressure gradient (mmHg)	3.21±0.50	3.41±0.16	3.69±0.34	3.64±0.63
Mitral valve				
Ejection time (ms)	55.38±3.90	54.47±3.29	54.05±3.05	55.38±3.76
IVCT (ms)	26.55±1.96	22.71±3.02	26.49±3.05	21.97±2.57
IVRT (ms)	25.68±1.48	25.99±1.89	26.52±1.10	29.22±2.82
E velocity (mm/s)	795.12±41.65	763.14±51.55	706.50±38.09	754.74±54.62
A velocity (mm/s)	599.07±58.06	533.14±31.68	539.39±44.99	557.08±49.02
E/A ratio	1.40±0.12	1.44±0.06	1.34±0.06	1.37±0.07
Tei index	0.99±0.09	0.93±0.13	1.02±0.11	0.96±0.11

Data are presented as mean ± SEM. IVCT: isovolumetric contraction time; IVRT: isovolumetric relaxation time.

Table 4.3. Echocardiographic data obtained from female control and IUGR, sedentary and exercised offspring.

	Control Sedentary	Control Exercised	IUGR Sedentary	IUGR Exercised
Morphologic parameters				
IVS; diastole (mm)	0.95±0.08	0.97±0.05	1.10±0.12	1.08±0.10
IVS; systole (mm)	1.58±0.05	1.43±0.12	1.88±0.33	1.74±0.15
Diastolic diameter (mm)	7.13±0.19	6.95±0.36	6.75±0.28	7.22±0.15
Systolic diameter (mm)	3.26±0.17	3.43±0.33	2.75±0.32	3.23±0.19
LVAW; diastole (mm)	1.68±0.08	1.78±0.11	1.70±0.14	2.03±0.13
LVAW; systole (mm)	3.19±0.08	2.95±0.13	3.12±0.30	3.47±0.14
LVID; diastole (mm)	7.20±0.22	7.09±0.39	6.90±0.26	7.18±0.14
LVID; systole (mm)	3.63±0.21	3.77±0.33	3.05±0.36	3.59±0.21
LVPW; diastole (mm)	1.88±0.08	1.86±0.19	1.85±0.10	1.83±0.08
LVPW; systole (mm)	3.01±0.10	3.09±0.22	3.23±0.15	3.04±0.06
LV mass (mg)	703.04±29.53	656.44±68.82	673.80±63.43	696.42±53.64
LV mass/ body weight (mg/g)	2.04±0.14	1.99±0.27	2.09±0.14	1.94±0.14
Volume parameters				
LV volume; diastole (uL)	275.14±18.21	267.77±33.27	249.96±20.27	270.90±12.02
LV volume; systole (uL)	57.92±6.82	64.43±13.42	41.48±10.61	55.70±7.81
Systolic function				
Stroke volume (uL)	224.40±13.14	204.12±20.28	206.45±14.41	231.42±9.95
Ejection fraction (%)	83.56±1.67	80.73±2.40	87.54±2.37	84.40±1.71
Fractional shortening (%)	54.42±2.17	51.13±2.48	59.89±3.38	55.32±2.15
Cardiac output (mL/min)	76.91±4.24	69.31±7.71	68.09±6.01	72.92±6.13

Data are presented as mean ± SEM. IVS: inter ventricular septum; LVAW: left ventricular anterior wall; LVID: left ventricular internal diameter; LVPW: left ventricular posterior wall; LV: left ventricle.

Table 4.4. Pulse-wave Doppler data obtained from female control and IUGR, sedentary and exercised offspring.

	Control Sedentary	Control Exercised	IUGR Sedentary	IUGR Exercised
Aortic valve flow				
Ejection time (ms)	69.85±2.74	74.32±3.70	70.80±4.52	69.98±3.91
Outflow maximum ejection velocity (mm/s)	972.32±49.29	795.91±38	908.58±44.41	930.93±61.80
Peak pressure gradient (mmHg)	3.85±0.38	2.56±0.24	3.35±0.33	3.56±0.48
Pulmonary valve flow				
Acceleration time [PAT] (ms)	31.93±2.49	36.41±4.41	32.29±2.57	27.75±3.30
Ejection time [PET] (ms)	80.16±2.51	77.32±6.58	82.92±2.82	77.20±5.59
Valve peak velocity (ms)	910.26±49.47	809.86±42.15	1028.34±74.82	904.50±87.32
PAT/PET	0.40±0.03	0.47±0.03	0.39±0.03	0.37±0.04
Peak pressure gradient (mmHg)	3.39±0.40	2.66±0.29	4.36±0.63	3.46±0.69
Mitral valve				
Ejection time (ms)	51.26±3.08	54.15±5.36	56.01±3.76	55.97±3.76
IVCT (ms)	25.78±2.30	27.24±2.88	21.97±2.92	23.75±4.49
IVRT (ms)	24.57±1.90	26.01±2.51	23.47±0.44	24.40±1.36
E velocity (mm/s)	798.09±29.84	719.75±53.43	744.31±47.03	771.69±48.54
A velocity (mm/s)	533.35±27.13	542.25±27.43	523.06±47.29	602.02±79.17
E/A ratio	1.52±0.09	1.33±0.10	1.46±0.12	1.35±0.12
Tei index	1.03±0.10	1.03±0.11	0.86±0.14	0.92±0.17

Data are presented as mean ± SEM. IVCT: isovolumetric contraction time; IVRT: isovolumetric relaxation time. Underline indicates a two-way ANOVA IUGR effect.

4.4.2 Ischemia/Reperfusion protocol

In male offspring, cardiac performance was similar in control and IUGR sedentary groups during the pre-ischemia period ($p>0.05$; Figure 4.1 A, 4.1 B). A Bonferroni *post hoc* reveled that AET increased baseline cardiac performance in control offspring, while cardiac performance in male IUGR offspring was reduced (Figure 4.1 A, 4.1 B). During reperfusion, being born growth restricted was associated with a decrease in cardiac power ($p=0.03$; Figure 4.1 C). Moreover, being born growth restricted was associated with a decrease in the percent recovery following the ischemic insult ($p=0.04$; Figure 4.1 D).

In female offspring, there were no differences in cardiac performance between control and IUGR sedentary offspring during the pre-ischemia period (Figure 4.2 A, 4.2 B). Interestingly, and contrary to our observations in male offspring, AET did not affect pre- or post-ischemic cardiac performance in either control or IUGR female offspring (Figure 4.2 A, 4.2 B, 4.2 C). Consequently, the percent recovery of cardiac power following the ischemic event was also similar in all groups (Figure 4.2 D).

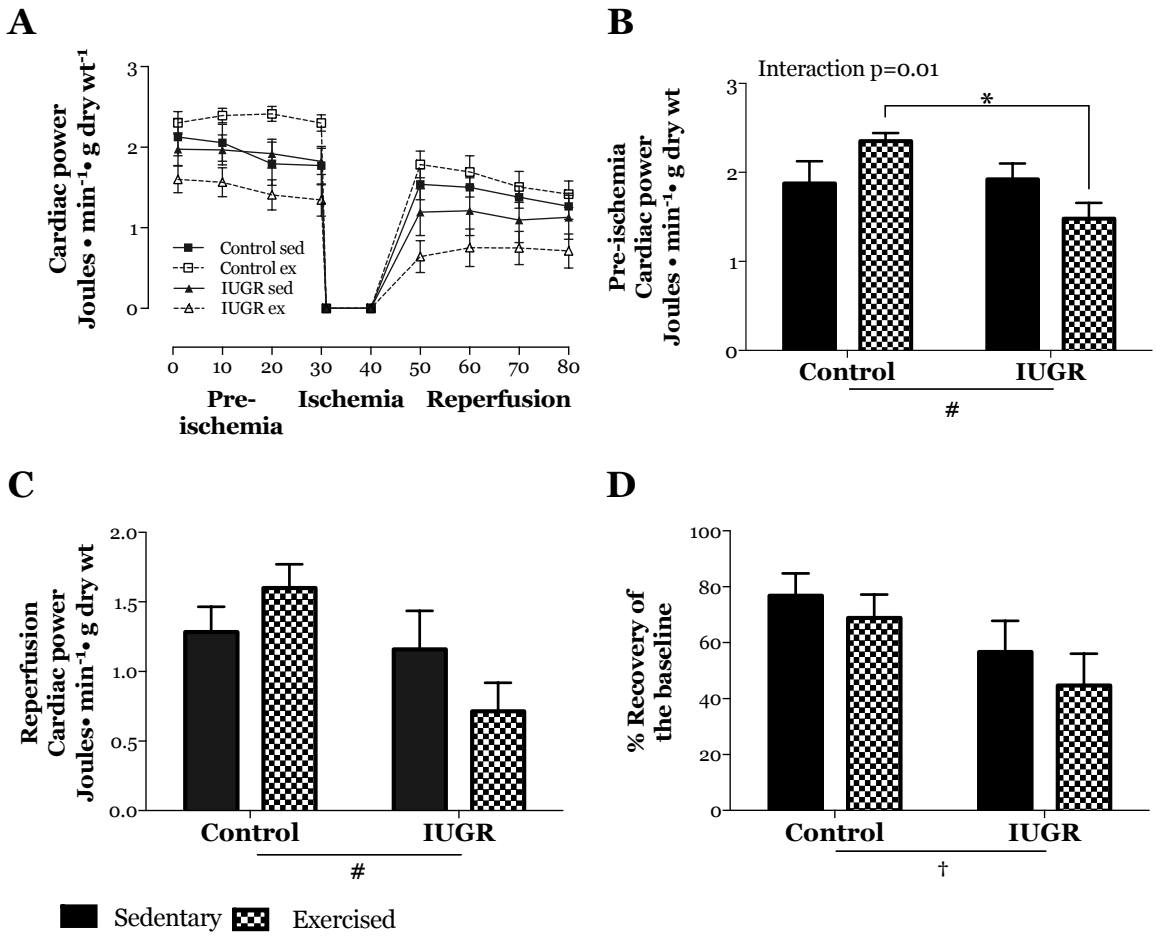

Figure 4.1. Male cardiac performance during ischemia/reperfusion protocol.
Panel A- A baseline cardiac performance of 30 min was obtained, followed by 10 min of global, no-flow ischemia and 40 min of reperfusion. Groups include: control sedentary offspring (solid line, closed squares, n=7); control exercised offspring (dashed line, open squares, n=6); IUGR sedentary offspring (solid line, closed triangles, n=9) and IUGR exercised offspring (dashed line, open triangles, n=8). Panel B- Summary data of cardiac performance during pre-ischemia. Panel C- Summary data of cardiac power during reperfusion. Panel D - Summary data of percent recovery of cardiac power after the ischemic event. Data are summarized and presented as mean ± SEM. Sedentary male offspring (closed bars); exercised male offspring (hatched bars). Data were analyzed by 2-way ANOVA; # p=0.03, † p=0.04: group effect control vs. IUGR; * p<0.05 Bonferroni *post hoc* test control exercised vs. IUGR exercised.

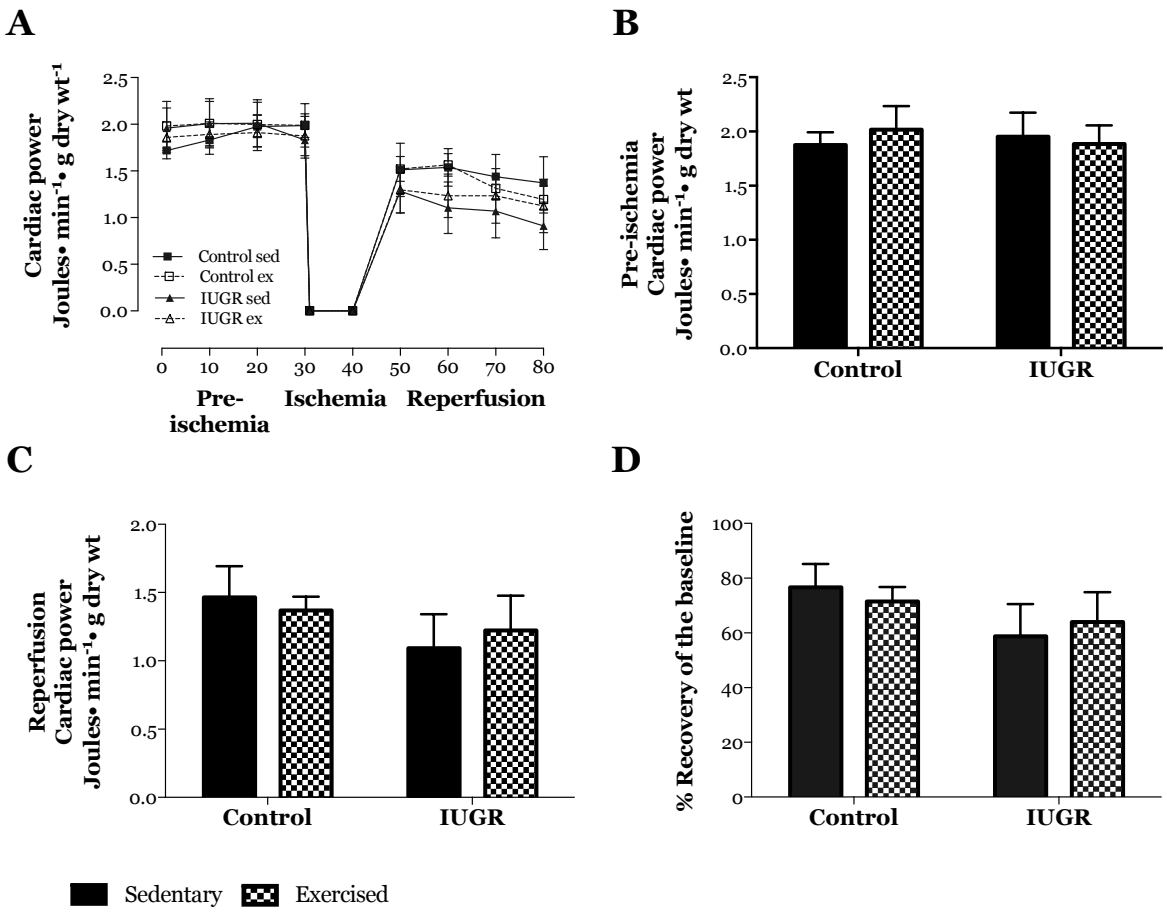

Figure 4.2. Female cardiac performance during ischemia/reperfusion protocol.
Panel A- A baseline cardiac performance of 30 min was obtained, followed by 10 min of global, no-flow ischemia and 40 min of reperfusion. Groups include: control sedentary offspring (solid line, closed squares, n=6); control exercised offspring (dashed line, open squares, n=8); IUGR sedentary offspring (solid line, closed triangles, n=7) and IUGR exercised offspring (dashed line, open triangles, n=8). Panel B - Summary data of cardiac performance during pre-ischemia. Panel C- Summary data of cardiac power during reperfusion. Panel D - Summary data of percent recovery of cardiac power after the ischemic event. Data are summarized and presented as mean ± SEM and were analyzed by 2-way ANOVA. Sedentary female offspring (closed bars); exercised female offspring (hatched bars).

4.4.3 Protein expression of antioxidant enzymes in non-perfused hearts

In male offspring, cardiac protein expression of SOD-1 (Figure 4.3 A), SOD-2 (Figure 4.3 B), catalase (Figure 4.3 C) and glutathione peroxidase (control sedentary 100±24.0% vs. exercised 112.4±15.3%; and IUGR sedentary 131.4±38.9% vs. exercised 103.6±25.5%) was not different among the groups.

Conversely in female offspring, AET increased cardiac protein expression of SOD-1 in both control offspring (sedentary 100±15.3% vs. exercised 272.7±86.4%); and IUGR offspring (sedentary 163.8±33.6% vs. exercised 231.2±65.0%; $p<0.05$; Figure 4.4 A). Cardiac protein expression of SOD-2 (Figure 4.4 B), catalase (Figure 4.4 C) and glutathione peroxidase (control sedentary 100±13.9% vs. exercised 82.2±9%; and IUGR sedentary offspring 129.9±24.2% vs. exercised 109.6±22.4%) were not different among the groups.

4.4.4 Superoxide production in non-perfused hearts

In male offspring, AET decreased $O_2^{\cdot -}$-generation in control offspring while in IUGR offspring; AET had the opposite effect (control sedentary 100±15.9% vs. exercised 62.9±6.9%; and IUGR sedentary 74.3±16.5% vs. exercised 93.1±10.5%; interaction effect $p=0.05$; Figure 4.5 A-4.5 F).

In female offspring, neither IUGR nor AET had an effect on $O_2^{\cdot -}$ production (Figure 4.6 A- 4.6 F).

Male offspring

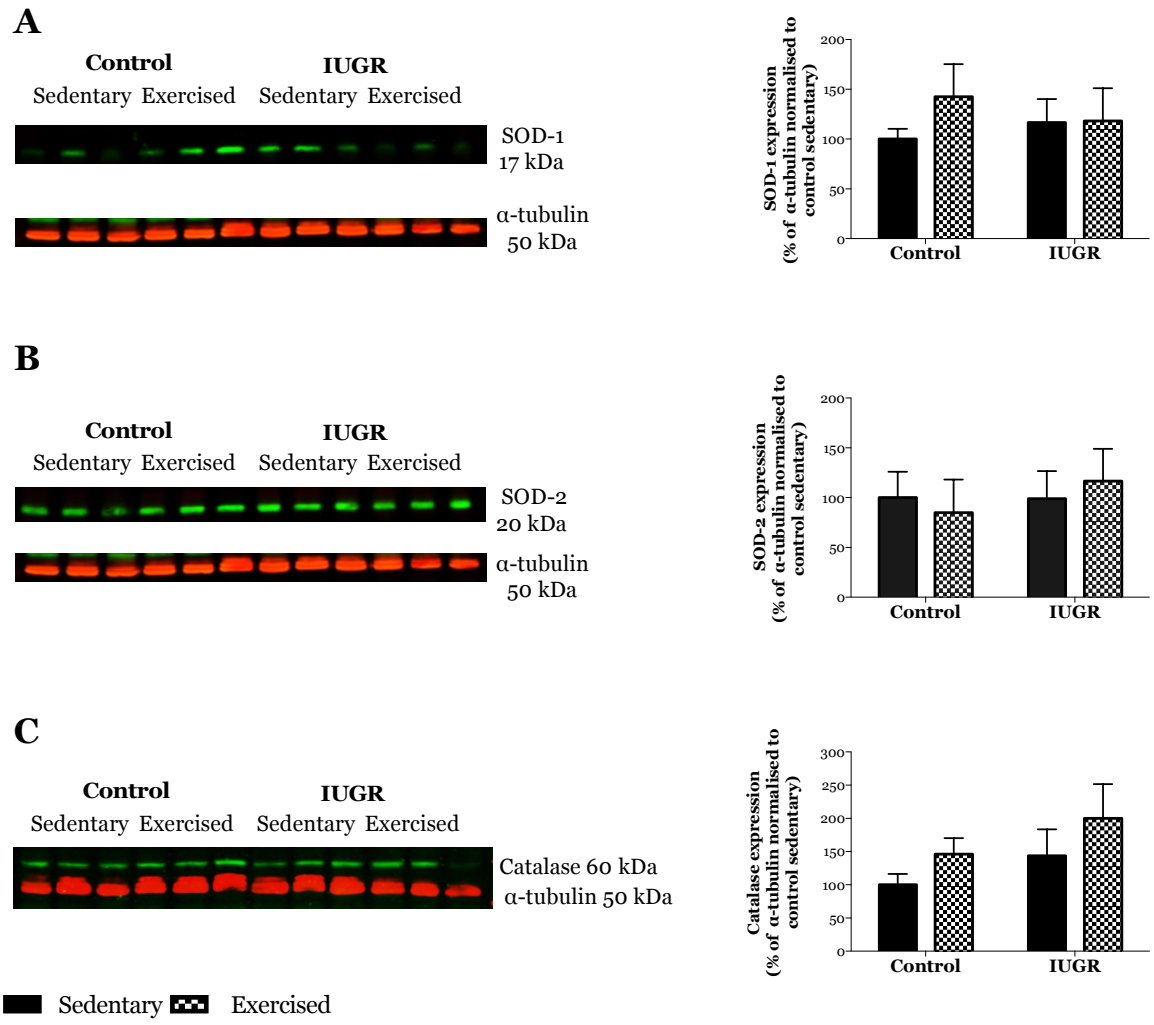

Figure 4.3. Cardiac protein expression of antioxidant enzymes in male control and IUGR, sedentary and exercised offspring.
Protein expression in non-perfused cardiac tissue of antioxidant enzymes in male sedentary offspring (closed bars, n=9) and male exercised offspring (hatched bars, n=7). All data are presented as a ratio of the protein of interest to α-tubulin. The sedentary control group was normalized to 100% and then the percent change of the other groups (control exercised, IUGR sedentary and IUGR exercised) was assessed. Panel A - Representative image of a western blot membrane probed for SOD-1, α-tubulin and summary data of SOD-1 protein expression. Panel B - Representative image of a western blot membrane probed for SOD-2, -tubulin and summary data of SOD-2 protein expression. Panel C - Representative image of a western blot membrane probed for catalase, α-tubulin and summary data of catalase protein expression. Data are summarized and presented as mean ± SEM and were analyzed by 2-way ANOVA.

Female offspring

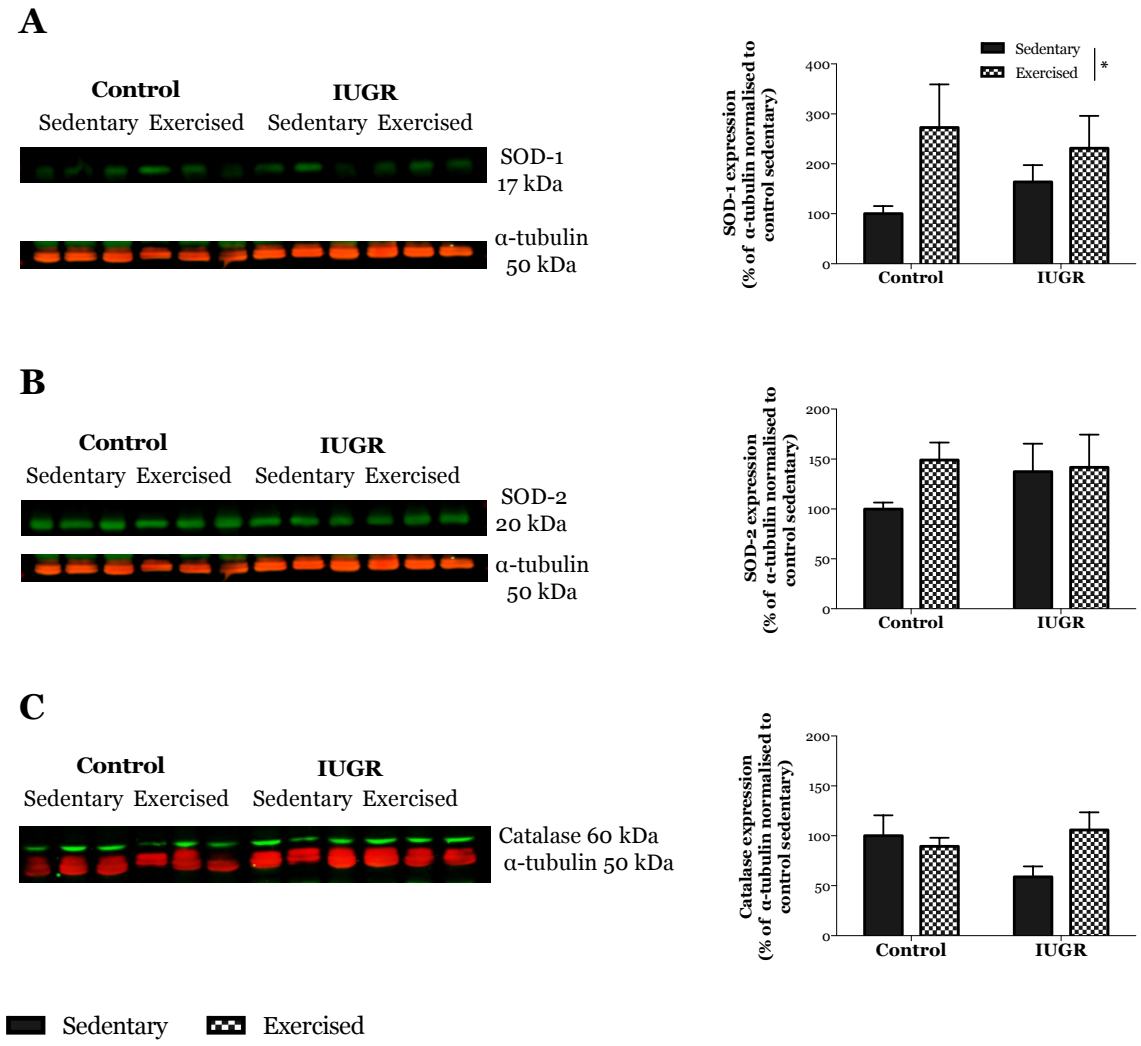

Figure 4.4. Cardiac protein expression of antioxidant enzymes in female control and IUGR, sedentary and exercised offspring.
Protein expression in non-perfused heart tissues of antioxidant enzymes in female sedentary offspring (closed bars, n=9) and female exercised offspring (hatched bars, n=7-9). All data are presented as a ratio of the protein of interest to α-tubulin. The sedentary control group was normalized to 100% and then the percent change of the other groups (control exercise, IUGR sedentary and IUGR exercise) was assessed. Panel A - Representative image of a western blot membrane probed for SOD-1, α-tubulin and summary data of SOD-1 protein expression. Panel B - Representative image of a western blot membrane probed for SOD-2 , α-tubulin and summary data of SOD-2 protein expression. Panel C - Representative image of a western blot membrane probed for catalase, α-tubulin and summary data of catalase protein expression. Data are summarized and presented as mean ± SEM and were analyzed by 2-way ANOVA; * $p<0.05$: group effect sedentary vs. exercised.

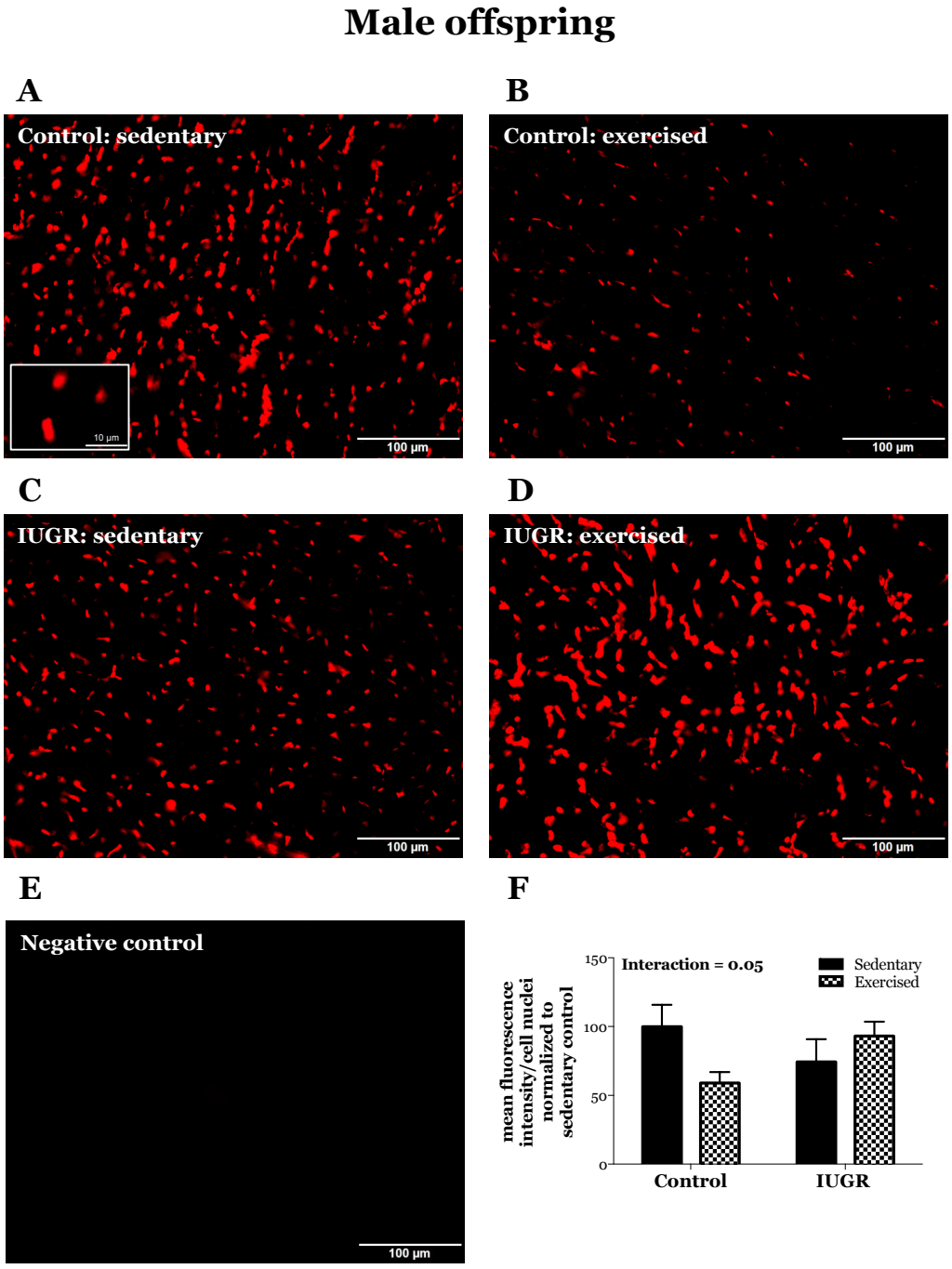

Figure 4.5. DHE staining in non-perfused hearts from male control and IUGR, sedentary and exercised offspring.

$O_2^{\cdot -}$ production as assessed by DHE staining in non-perfused hearts. The sedentary control group was normalized to 100% and then the percent change of the other groups (control exercised, IUGR sedentary and IUGR exercised) was assessed. Representative images of Panel A - male control sedentary Panel B - male control exercised, Panel C - male IUGR sedentary, Panel D - male IUGR exercised and Panel E - negative control for DHE staining. Panel F- Summary data of male sedentary offspring (closed bars, n=4-5) and male exercised offspring (hatched bars, n=4-5). Data are summarized and presented as mean ± SEM and were analyzed by 2-way ANOVA.

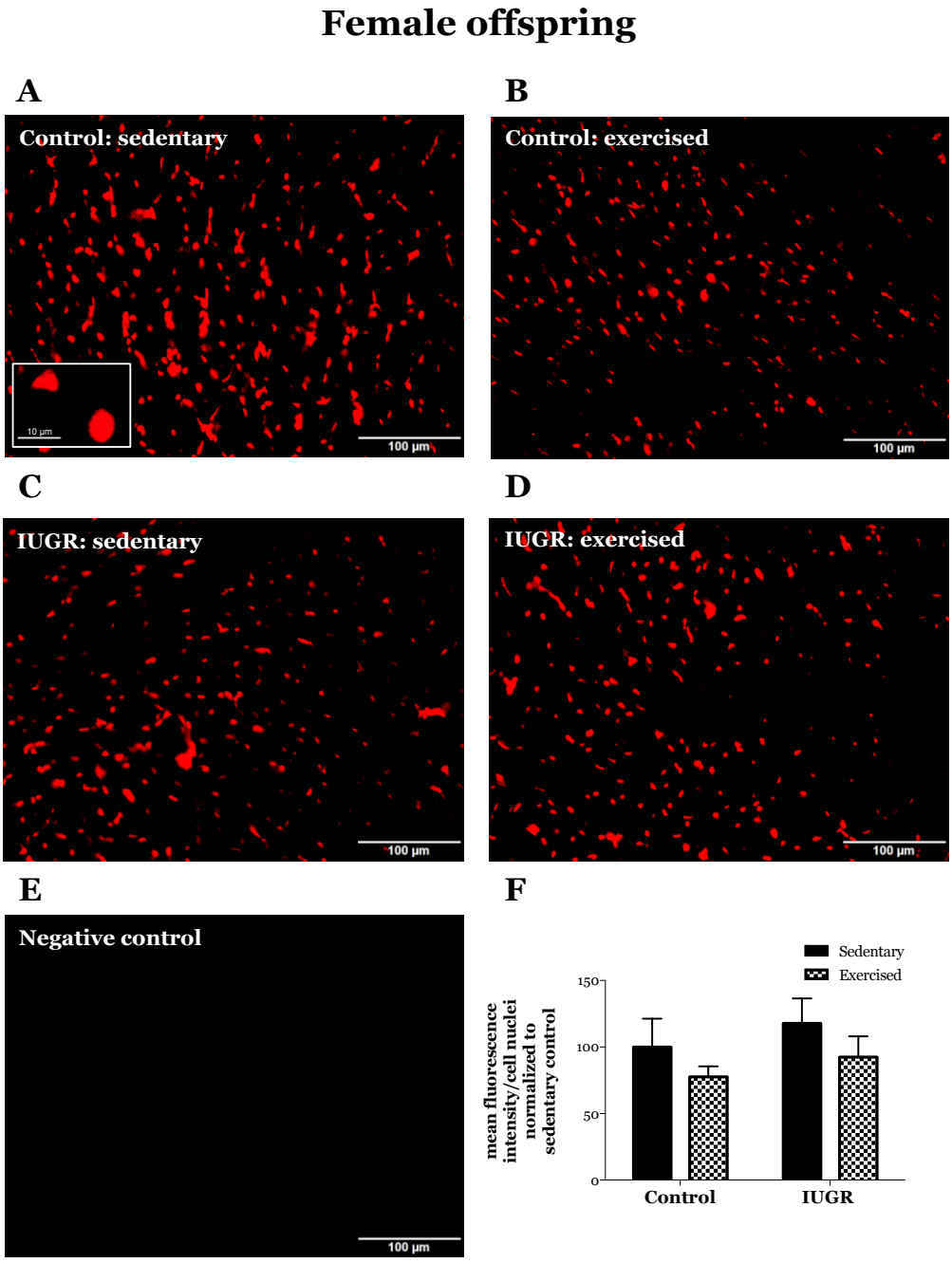

Figure 4.6. DHE staining in non-perfused hearts from female control and IUGR, sedentary and exercised offspring.
$O_2^{\cdot -}$ production as assessed by DHE staining in non-perfused hearts. The sedentary control group was normalized to 100% and then the percent change of the other groups (control exercised, IUGR sedentary and IUGR exercised) was assessed. Representative images of Panel A - female control sedentary Panel B - female control exercised, Panel C - female IUGR sedentary, Panel D - female IUGR exercised and Panel E - negative control for DHE staining. Panel F - Summary data of female sedentary offspring (closed bars, n=3-6) and female exercised offspring (hatched bars, n=5). Data are summarized and presented as mean ± SEM and were analyzed by 2-way ANOVA.

4.5 Discussion

In this study we tested aerobic exercise as an intervention to prevent susceptibility to cardiac I/R injury in hypoxic-induced IUGR adult offspring. To the best of our knowledge, this is the first study designed to determine the impact of AET on cardiac function in offspring born from hypoxic pregnancies. As expected, in male control offspring AET improved basal cardiac performance and decreased cardiac $O_2^{\cdot -}$ generation. In male IUGR offspring, however, the opposite was observed whereby AET reduced cardiac performance and increased cardiac $O_2^{\cdot -}$ generation. Recovery of cardiac performance during the reperfusion period was not affected by AET in either control or IUGR offspring. The interaction of growth restriction and exercise, therefore, had a detrimental effect on cardiac function in male offspring. In females, there was no effect of IUGR or AET on basal cardiac performance nor did AET have an effect on the recovery of cardiac performance during the reperfusion period. We further observed that AET increased cardiac SOD-1 expression in both control and IUGR female offspring that was not observed in male offspring. The female sex, therefore, appeared to be protected against the detrimental effects of a compromised *in utero* environment on cardiac function and this may have been due to an improved antioxidant status. In males, exercise was found to have a differential effect on the levels of oxidative stress, as assessed by $O_2^{\cdot -}$ levels, dependent on the *in utero* condition experienced by the offspring. Since the expression levels of antioxidants measured in this study were found to be unaltered, this might be due to differential expression of pro-oxidants or differential activity levels of either pro- or antioxidants.

Our findings in male IUGR exercised offspring imply a basal cardiac maladaptation to AET in this group. Altered cardiac function in male IUGR exercised offspring could be secondary to an increase in sympathetic tone following exercise. Being born small for a given gestational age at term has been associated with reduced HRV[330] and an increase in plasma NE;[331] which suggests that IUGR is associated with an imbalance in autonomic tone. In a swine model of myocardial infarction, Duncker *et al.,*[332] found that during exercise swine had an exaggerated withdrawal of cardiac parasympathetic tone, increased plasma levels of NE and epinephrine, and a blunted β-adrenergic cardiac response while β-adrenergic vasodilation in the coronary vasculature was maintained; suggesting cardiac specific β-adrenergic desensitization. Thus, in our animal model, a preexisting autonomic imbalance could lead to a state of β-adrenergic desensitization, which would be detrimental only under stress conditions such as aerobic exercise and would impair the normal cardiac adaptations to AET. Moreover, we also found that AET in male offspring decreased cardiac $O_2^{\cdot -}$ generation in controls while the opposite effect occurred in IUGR offspring; with no changes in the protein expression of any of the antioxidant enzymes studied. Since it has been previously shown that vagal nerve stimulation modulates cardiac redox status and adrenergic drive in mice with chronic heart failure,[333] an association between an increased sympathetic tone, and the increase in ROS in IUGR exercised offspring could be made.

In female offspring, despite preserved *ex vivo* cardiac function, we demonstrated that IUGR offspring had an increase in pulmonary valve peak velocity *in vivo*. It has been previously described that female IUGR offspring exhibited signs of pulmonary hypertension only with aging.[88] Changes in the pulmonary valve peak velocity in our

young female offspring suggest that cardiac remodeling could be occurring in the absence of overt signs of cardiac dysfunction either *ex vivo* or *in vivo*; AET did not alter any other echocardiogram functional or morphological parameters in either control or IUGR offspring. Morphological adaptations of the heart, however, may be modest unless comparing well trained and untrained populations,[334] or severe pathological states.[335] Regarding the antioxidant balance in female offspring, AET increased SOD-1 while it had no effect on either the protein expression of the other antioxidant enzymes measured or $O_2^{\cdot -}$ production. These results are in accordance with McDonald *et al.*[336] who found that in Streptozotocin-diabetic rats SOD-1, but not SOD-2, was upregulated after 6 weeks of AET on a treadmill. An increased antioxidant capacity might go some way to explaining the preserved cardiac function observed in this sex.

In conclusion, a decrease in cardiac performance before ischemia in association with an increase in ROS, demonstrated that the impact of AET on cardiac function in male IUGR offspring was greater than in the female IUGR offspring. Interestingly, our previous findings in littermates demonstrated a more pronounced vascular dysfunction associated with AET in female compared to male offspring.[86,328] Since it has been previously shown that being born IUGR secondary to a hypoxic pregnancy was associated with an increased cardiovascular and metabolic susceptibility to secondary stressors such as aging and a high-fat diet;[86,88,106,107,187] we could interpret that AET may represent a secondary stressor to cardiac function that is not well tolerated in male hypoxic-induced IUGR offspring. Our data support the fact that the impact of any insult during fetal development is crucial to cardiovascular development. Moreover, well-

established preventive strategies to decrease the risk of cardiovascular diseases later in life may be detrimental in this susceptible population by further increasing risk.

5 The role of TWEAK in IUGR offspring cardiomyocyte proliferation

5.1 Introduction

As previously discussed in Chapter 1, cardiac development in in early fetal life is associated with cardiomyocyte hyperplasia, whereas in late fetal and early postnatal life it is associated with cardiomyocyte hypertrophy.[337] Bae S et al. showed that hypoxic-induced IUGR fetal cardiomyocytes at GD 21 in rats had an increased proportion of binucleated cardiomyocytes (indicative of terminally differentiated cells) with an increase in the size of binucleated cells (an early marker of hypertrophy).[91] Moreover, compared to cardiomyocytes from normoxic dams, cardiomyocytes from hypoxic-induced IUGR offspring had an increased rate of apoptosis.[91] In addition, it has been shown that compared to cardiomyocytes in offspring born from normoxic dams, cardiomyocytes from hypoxic-induced IUGR offspring proliferate less.[94] Taken together, these data suggest that cardiomyocytes from hypoxic-induced IUGR offspring have decreased cardiomyocyte proliferation and an increase in cardiac hypertrophy and fibrosis.

Heart development and cardiomyocyte proliferation are complex processes regulated by neurohormonal stimuli (i.e. Ang II, IGF-1, ET-1) as well as hemodynamic stimulus, finishing soon after birth.[125] Cardiomyocyte proliferation in adulthood, however, is a limited.[338] Novoyatleva et al.,[137] conversely, found that TWEAK was associated with both neonatal and adult cardiomyocyte proliferation. In neonatal cardiomyocytes isolated from Sprague Dawley rats,

binding of TWEAK with its receptor (Fn-14) causes cardiomyocyte proliferation. This phenomenon was physiologically relevant during the early stages in life since the TWEAK/Fn-14 pathway becomes quiescent after the completion of cardiac development at approximately PND-10 due to a down-regulation of Fn-14 expression in cardiomyocytes with age.[137] Interestingly, overexpression of Fn-14 resulted in adult cardiomyocyte proliferation.[137]

The role of TWEAK/Fn-14 pathway in cardiomyocyte proliferation has not been studied in offspring born growth restricted after a hypoxic insult (IUGR offspring). Moreover, it has been demonstrated that after a myocardial infarction, Fn-14 protein expression is upregulated.[339,340] Suggesting a potential link between the increased susceptibility to I/R injury in IUGR offspring and TWEAK/Fn-14 alterations in 4-month old offspring.

5.2 Objectives

The aims of this project in early postnatal life (PND-1) were: a) to determine whether being born growth restricted is associated with a decrease in cardiomyocyte proliferation and an increase in cardiomyocyte binucleation; b) to assess whether a decreased expression of Fn-14 in neonatal cardiomyocytes is associated with a decrease in cardiomyocyte proliferation in IUGR offspring; and c) to evaluate whether cardiomyocyte proliferation in response to recombinant TWEAK is different between control and IUGR offspring. Finally, the aims of this project in adult offspring (4-month old) were: a) to assess whether the serum

concentration of s-TWEAK and b) the cardiac protein expression of Fn-14 in 4-month old offspring is altered in IUGR offspring.

5.3 Methods

5.3.1 Cardiomyocyte isolation

As stated in section 2.3.2 from Chapter 2, a subset of dams was used for this set of experiments. On PND-1, four to six female and four to six male pups from each litter were used for cardiomyocyte isolation. After euthanasia, ventricles were dissected in a dissociation buffer (in mM: 116 NaCl, 20 HEPES, 0.8 Na2HPO4, 5.6 glucose, 5.4 KCl, 0.8 MgSO4, pH 7.35). Multiple enzymatic digestions with a dissociation buffer containing collagenase type II (0.4 mg/mL) and pancreatin (0.6 mg/mL) were performed at 37°C. Following this procedure, the digested solution was filtered through a 70 μm filter and spun at 1300 g. Cardiomyocytes were counted using the Trypan Blue exclusion test of cell viability (Sigma, USA). Afterwards, cardiomyocytes were separated and resuspended in different culture medias according to the experimental protocol being performed (determination of proliferation and binucleation or stimulation with recombinant TWEAK). Cardiomyocytes were cultured in 48-well plates and grown in a humidified atmosphere at 37°C with 5% CO2/ 95% air.

5.3.2 Determination of proliferation and binucleation

Approximately 30% of isolated cardiomyocytes were resuspended in medium-199 (Gibco, Life Technologies, USA) supplemented with 2% albumin

(Sigma, USA), 2 mM L-carnitine (Sigma, USA), 5 mM creatine (Sigma, USA), 5 mM taurine (Sigma, USA) and penicillin-streptomycin (Life Technologies, USA).

Twenty-four hours after isolation, cardiomyocytes were fixed in methanol. Cell proliferation and binucleation were determined by immunofluorescence using an overnight incubation with anti-Ki-67 antibody (ab16667; 1:50; Abcam, Canada) and anti-heavy chain cardiac myosin antibody (ab50967; 1:150, Abcam, Canada). Cardiomyocytes were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 546-conjugated anti-mouse secondary antibodies (1:200; Abcam, Canada) for 40 min in the dark. Nuclei were subsequently stained with DAPI (300 nM; Thermo scientific, USA). To determine cardiomyocyte proliferation and binucleation, three replicate wells were stained and pictures of four random fields per well were taken for each of the control and IUGR, male and female offspring groups. Ratios between Ki-67 positive nuclei/total nuclei number and number of binucleated cells/total number of cells per picture were calculated.

5.3.3 Cardiomyocyte stimulation with TWEAK

The remaining 70% of cardiomyocytes were initially incubated for 48 hours in medium-199 (Gibco, Life Technologies, USA) supplemented with 5% horse serum, 20 mM cytosine b-D-arabinofuranoside (araC; Sigma, USA), and penicillin-streptomycin (Life Technologies, USA) to prevent the proliferation of non-myocyte cells. Subsequently, cardiomyocytes were washed and culture in medium-199 supplemented with 2% albumin with or without TWEAK

recombinant protein (r-TWEAK, 100 ng/mL; Biolegend, USA). A combination of r-TWEAK and a Fn-14 receptor antibody (100 μg/mL; Abcam, Canada); a Fn-14 receptor antibody alone and finally, medium-199 supplemented without albumin. After the cardiomyocytes were stimulated for 72 hours with r-TWEAK, cells were fixed in methanol and proliferation was determined as previously described. Cardiomyocytes were visualized under an Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group, Canada), using Cellsense imaging software (Olympus, Japan). All images presented are in the original (10X) magnification.

5.3.4 TWEAK receptor protein expression in PND-1 and 4-month old offspring hearts

Heart tissue from the remaining pups collected at PND-1 and from 4-month old non-perfused, sedentary animals previously generated for Chapters 2, 3 and 4 was used to determine the protein expression of Fn-14. Tissue preparation was performed as described previously in section 4.3.3 from Chapter 4, with an additional step in which the membranes were incubated with anti-TWEAK receptor (1:100; Abcam, Canada) and re-probed with anti-α-tubulin (1: 50 000; Abcam, Canada) as a protein loading control. Finally, membranes were probed with the following secondary antibodies: IRDye 800CW donkey anti-rabbit IgG and IRDye 680RD donkey anti-mouse IgG secondary antibodies (1:10000; Li-Cor Biosciences, Lincoln NE USA). The protein bands were imaged using the Li-Cor Odyssey Imaging Systems v3.0. Densitometry analysis was performed using ImageStudio v.5.2.5. (LI-COR Biosciences, Lincoln NE USA). Data were normalized to the α-tubulin loading control.

5.3.5 Soluble TWEAK in serum

Serum from 4-month old male and female control and IUGR offspring previously generated for Chapters 2, 3 and 4 were used to determine s-TWEAK levels using a commercially available, standard sandwich enzyme-linked immunosorbent assay (LifeSpan Biosciences, Inc., USA) according to the manufacturer's instructions. The concentration of s-TWEAK was calculated by interpolation of the results from the standard curve. The inter- and intra-assay coefficients of variation obtained were 6.45% and less than 11% respectively.

5.3.6 Statistical analyses

Data were presented as mean ± SEM. The Shapiro-Wilk test was used to assess normality of continuous data. Data were analyzed using two-sample t-tests or a Mann-Whitney test when data were not normally distributed. The effect of the addition of r-TWEAK on cardiomyocyte proliferation was analyzed using a two-way ANOVA followed by a Bonferroni post-hoc test. Comparisons between the groups were performed by normalizing the control offspring to 100% and then assessing the percent change of the IUGR offspring. Female and male offspring data were analyzed separately due to differences in their phenotype. Statistical significance was defined as $p<0.05$. All data were analyzed using GraphPad Prism 7 statistical software (GraphPad Software, USA).

5.4 Results

5.4.1 Animal model

Morphological characteristics of male and female offspring from normoxic and hypoxic pregnancies are provided in Table 5.1. At PND-1; both male and female offspring from dams exposed to hypoxia had a reduced crown-rump length. Compared to male offspring from dams in normoxic conditions, male offspring exposed to hypoxia also had a reduced abdominal girth. The percentage of stillbirths was higher in dams exposed to hypoxia compared to control dams (11.7% vs. 1.9%; $p<0.0001$). The litter size, however, was not different among the groups (14 vs. 15; $p=0.14$).

Table 5.1. Morphological characteristics at PND-1 of male and female offspring born from normoxic dams (Control) or dams exposed to hypoxia (IUGR).

Parameter	Control (n=9)	IUGR (n=8)
Male offspring		
Body weight (g)	7.8 ± 0.2	7.4 ± 0.2
Crown-rump length (mm)	45.3 ± 0.4	43.2 ± 0.5**
Abdominal girth (mm)	49.4 ± 0.7	46.9 ±0.8 *
CRL.ABG-1 ratio	0.92 ± 0.01	0.92 ±0.01
Heart weight (mg)	41.6 ± 1.5	39.8 ± 2.2
Heart weight/body weight -1 ratio	5.1 ± 0.2	5.3 ± 0.2
Female offspring		
Body weight (g)	7.3 ± 0.2	6.9 ± 0.2
Crown-rump length (mm)	44.3 ± 0.5	42 ± 0.5**
Abdominal girth (mm)	47 ± 0.7	45.6 ± 0.8
CRL.ABG-1 ratio	0.94 ± 0.02	0.92 ± 0.01
Heart weight (mg)	40.3 ± 1.2	38.2 ± 2.2
Heart weight/body weight -1 ratio	5.2 ± 0.1	5.6 ± 0.3

Data presented as mean ± SEM. CRL: crown-rump length; ABG: abdominal girth. * $p \leq 0.05$, ** $p < 0.01$ compared to control offspring.

5.4.2 Fn-14 protein expression in postnatal day 1 offspring

No differences among the groups were found regarding cardiomyocyte Fn-14 protein expression in either male (Figure 5.1 A and B) or female offspring at PND-1 (Figure 5.1 A and C).

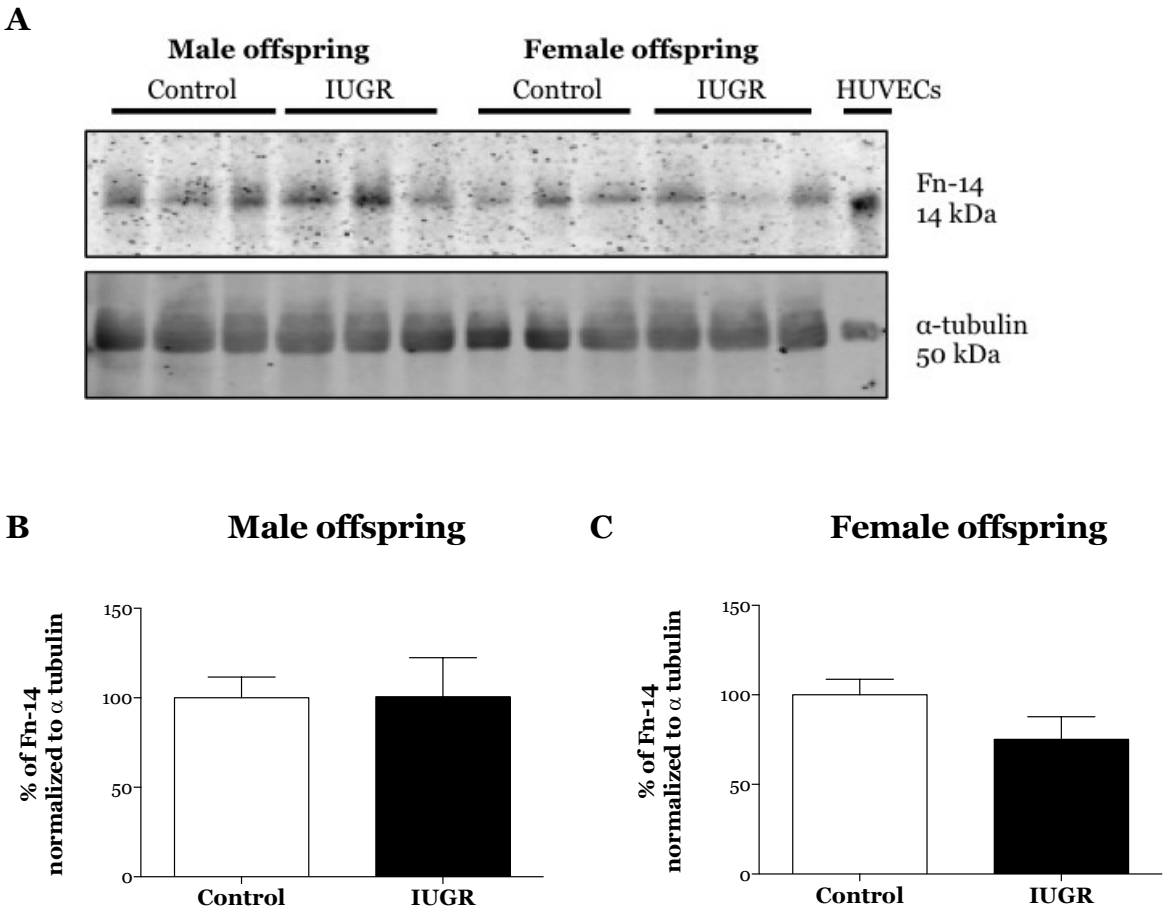

Figure 5.1. PND-1 cardiac tissue Fn-14 protein expression in control and IUGR, male and female offspring.
All data are presented as a ratio of Fn-14 to α-tubulin. The control group was normalized to 100% and then the percent change of the IUGR group was assessed. Panel A- Representative image of a western blot membrane probed for Fn-14 and α-tubulin in male and female control and IUGR offspring. Panel B- Summary data of Fn-14 protein expression in male control offspring (open bars, n=5) and IUGR offspring (closed bars, n=6). Panel C- Summary data of Fn-14 protein expression in female control offspring (open bars, n=6) and IUGR offspring (closed bars, n=6). Data are summarized and presented as mean ± SEM.

5.4.3 Proliferation and binucleation of cardiomyocytes at PND-1

There were no differences among the groups regarding the proliferation or binucleation of cardiomyocytes at PND-1 (Figure 5.2).

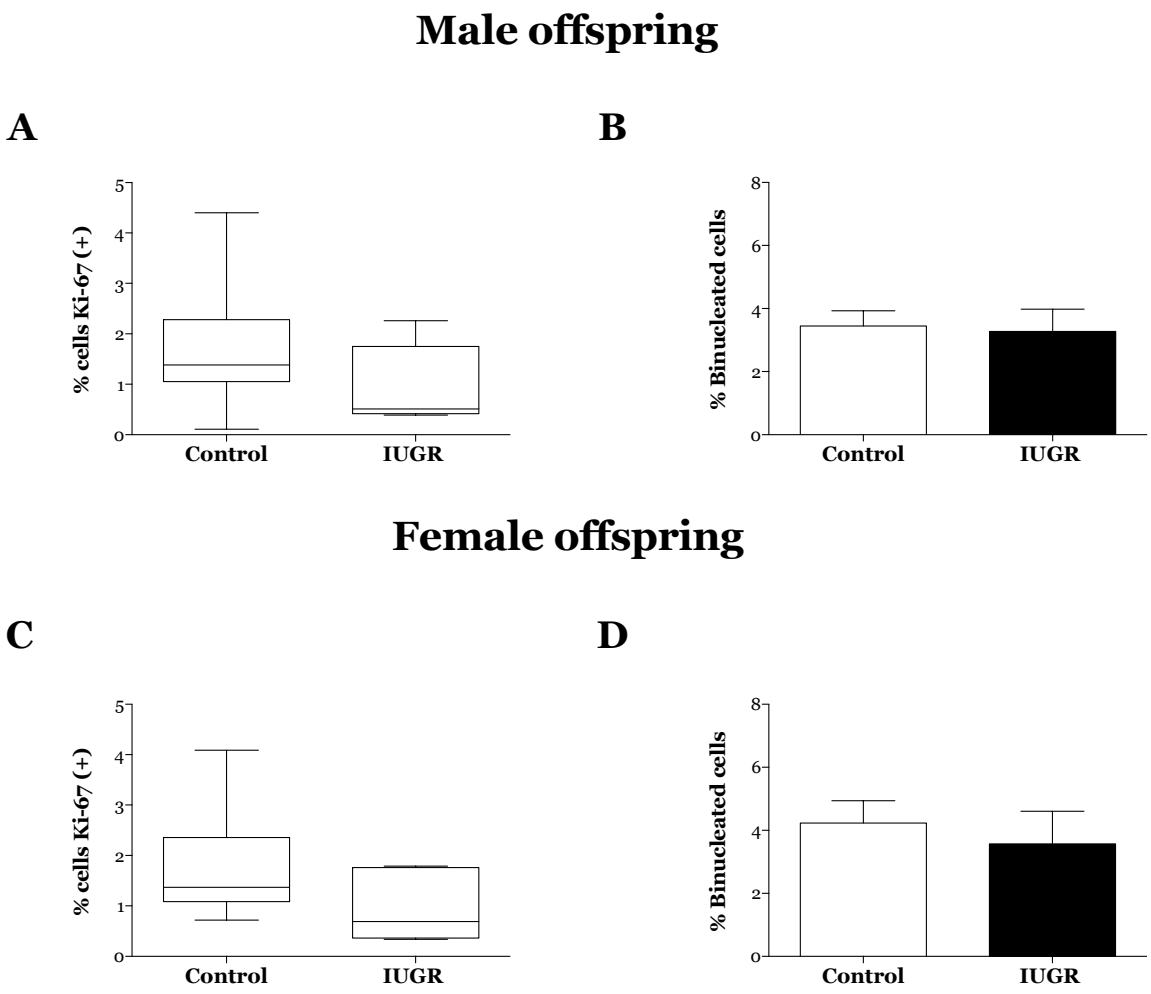

Figure 5.2. PND-1 cardiomyocyte proliferation and binucleation in control and IUGR, male and female offspring.
Panel A- Cardiomyocyte proliferation was determined by the percentage of ki-67 positive nuclei in control (n=8) or IUGR (n=7) male offspring. Panel B- The ratio between the total number of binucleated to mononucleated cardiomyocytes was determined in control (n=8) or IUGR (n=7) male offspring. Panel C- Cardiomyocyte proliferation was determined by the percentage of ki-67 positive nuclei in control (n=9) or IUGR (n=6) female offspring. Panel D- The ratio between the total number of binucleated to mononucleated cardiomyocytes was determined in control (n=9) or IUGR (n=6) female offspring. Non-normally distributed data are presented as median and 5-95% confidence interval. If data are normally distributed; they are summarized and presented as mean ± SEM.

5.4.4 Cardiomyocyte stimulation with recombinant TWEAK

In male offspring, being born growth restricted was associated with a decreased cardiomyocyte proliferation after being in culture for 72 hours. The addition of r-TWEAK increased cardiomyocyte proliferation to the same extent in control and IUGR male offspring (Figure 5.3 A, B). The addition of r-TWEAK in the presence of the Fn-14 receptor blocker decreased cardiomyocyte proliferation in both control male offspring [2.1±0.3 (r-TWEAK) vs. 0.6±0.2 (r-TWEAK+ Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.005], and IUGR male offspring [1.7±0.3 (r-TWEAK) vs. 0.4±0.1 (r-TWEAK+ Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.0003]. The Fn-14 receptor antibody alone had no effect on cardiomyocyte proliferation in control male offspring [1.1±0.3 (medium-199 supplemented with 2% albumin) vs. 0.7±0.5 (Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.57], or IUGR male offspring [0.8±0.1 (medium-199 supplemented with 2% albumin) vs. 0.7±0.2 (Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.71]. Cardiomyocyte proliferation was lower in the presence of albumin free medium compared to medium-199 supplemented with 2% albumin in control male offspring [1.1±0.3 (medium-199 supplemented with 2% albumin) vs. 0.03±0.0 (medium-199 without albumin) % Ki-67 (+) cardiomyocytes; p=0.04], but not IUGR male offspring [0.8±0.1 (medium-199 supplemented with 2% albumin) vs. 0.4±0.1 (medium-199 without albumin) % Ki-67 (+) cardiomyocytes; p=0.07].

In contrast, in female offspring there were no differences in cardiomyocyte proliferation between control and IUGR offspring; and the addition of r-TWEAK

increased proliferation in both control and IUGR offspring (Figure 5-3 C, D). The addition of r-TWEAK in the presence of the Fn-14 receptor blocker decreased cardiomyocyte proliferation in both control female offspring [2.4±0.3 (r-TWEAK) vs. 0.9±0.2 (r-TWEAK+ Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.02], and IUGR female offspring [2.8±0.7 (r-TWEAK) vs. 0.4±0.1 (r-TWEAK+ Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.009]. The Fn-14 receptor antibody alone had no effect on cardiomyocyte proliferation in control female offspring [1.1±0.3 (medium-199 supplemented with 2% albumin) vs. 0.8±0.7 (Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.67], or IUGR female offspring [0.8±0.2 (medium-199 supplemented with 2% albumin) vs. 0.2±0.1 (Fn-14 receptor blocker) % Ki-67 (+) cardiomyocytes; p=0.17]. Cardiomyocyte proliferation was lower in the presence of albumin free medium compared to medium-199 supplemented with 2% albumin in control female offspring [1.1±0.3 (medium-199 supplemented with 2% albumin) vs. 0.3±0.3 (medium-199 without albumin) % Ki-67 (+) cardiomyocytes; p=0.04], and IUGR male offspring [0.8±0.2 (medium-199 supplemented with 2% albumin) vs. 0.1±0.01 (medium-199 without albumin) % Ki-67 (+) cardiomyocytes; p=0.04].

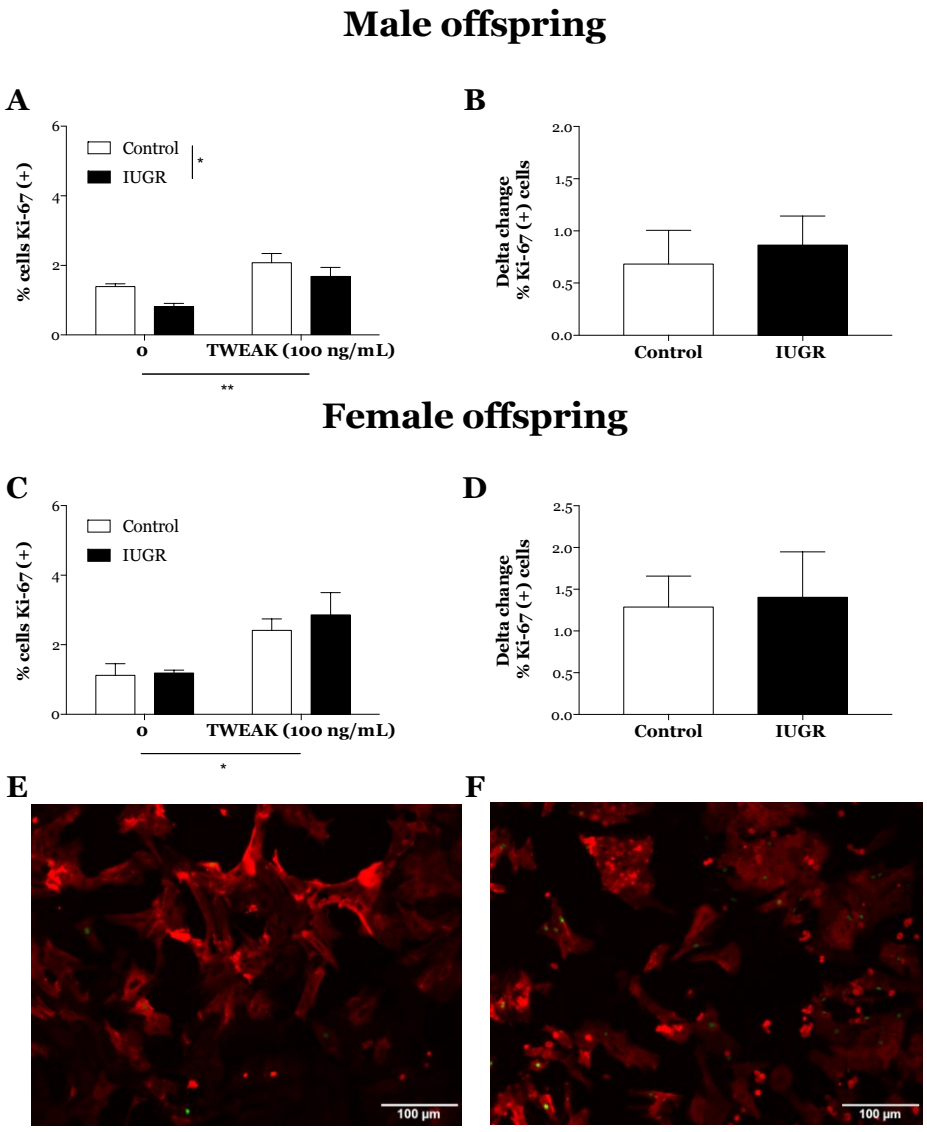

Figure 5.3. Proliferation of PND-1 cardiomyocytes exposed to recombinant TWEAK in control and IUGR, male and female offspring.
Cardiomyocyte proliferation was determined by the percentage of ki-67 positive nuclei in control and IUGR, male and female offspring. A two-way ANOVA analysis was performed to determine the effect of being born growth restricted and/or the addition of recombinant TWEAK on cardiomyocyte proliferation. Data was normalized to 100% of the control offspring and then, the percentage of change in IUGR offspring was assessed. Panel A- Summarized data of male control offspring (open bars; n=3) and male IUGR offspring (closed bars; n=3). Panel B- Delta change in proliferation (Ki-67 positive nuclei) in control and IUGR male offspring. Panel C- Summarized data of female control offspring (open bars; n=3) and female IUGR offspring (closed bars; n=3). Panel D- Delta change in proliferation (Ki-67 positive nuclei) in control and IUGR female offspring. Panel E- Representative immunofluorescence images of proliferating cardiomyocytes. (Red channel: anti-heavy chain cardiac myosin; Green channel: anti-Ki-67). Panel F- Representative immunofluorescence images of proliferating cardiomyocytes. (Red channel: anti-heavy chain cardiac myosin; Green channel: anti-Ki-67). Data are summarized and presented as mean ± SEM.

5.4.5 Serum levels of TWEAK

A series of serum dilutions (1:100; 1:10; 1:2 and neat serum) were tested to determine the serum concentration of s-TWEAK in the experimental groups at PND-1. Despite this, the values of s-TWEAK were lower than the kit's sensitivity range (10 pg/mL) and, therefore, remained non-detectable.

5.4.6 Fn-14 protein expression in 4-month old offspring

Protein expression of the Fn-14 receptor remained quiescent in cardiac tissues from control and IUGR, male and female 4 month old offspring (Figure 5.4).

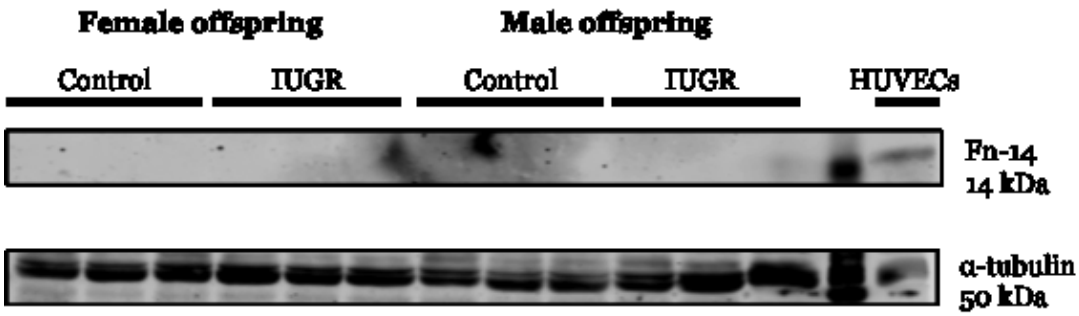

Figure 5.4. Cardiac tissue Fn-14 protein expression in 4-month old control and IUGR, male and female offspring.
Panel A- Representative image of a western blot membrane probed for Fn-14 and α-tubulin in male control (n=6) and IUGR offspring (n=6); and female control (n=6) and IUGR (n=6) offspring. Human umbilical vein endothelial cell (HUVECs) lysates were used as a positive control.

5.5 Discussion

TWEAK is a multifunctional cytokine that has recently gained attention because of its involvement in cardiomyopathy[339-342], and neonatal cardiomyocyte proliferation.[137] To the best of our knowledge, this is the first time that the role of the TWEAK/Fn-14 pathway in the susceptibility of IUGR offspring to cardiovascular diseases has been assessed. We found that being born growth restricted was not associated with differences in Fn-14 protein expression or cardiomyocyte proliferation at PND-1 in either male or female offspring. However, after being in culture for 72-hours, IUGR male offspring had a decreased cardiomyocyte proliferation compared to controls. The addition of r-TWEAK, however, increased proliferation in both groups to the same extent. In addition, in female offspring, cardiomyocyte proliferation in culture was not reduced in IUGR offspring and the addition of r-TWEAK increased proliferation to the same extent in both control and IUGR offspring. Cardiomyocyte proliferation among all groups was inhibited by the Fn-14 receptor blocking antibody.

In this subset of animals, hypoxia during pregnancy was associated with a decreased CRL but not ABG or body weight in IUGR female offspring; while male IUGR offspring had a decreased CRL and ABG. It is important to note, however, that for the purpose of this study the litter was handled, and measurements taken, 24-hours following birth. Thus, milk consumption during this initial 24-hour period may have affected the body weight of offspring by the time the

measurements were collected. Nonetheless, our findings suggest that hypoxia induced a stronger phenotype in male offspring.

Heart development is an active process which occurs both prenatally and during early postnatal life.[117] The fact that there were no differences in the percentage of binucleation of cardiomyocytes at PND-1 implies that there is still opportunity for growth and differentiation in these cells. Importantly, although we could not find any differences in cardiomyocyte proliferation in our hypoxic-induced IUGR offspring at PND-1; cardiomyocytes from male IUGR offspring proliferated less than controls when in culture for 72-hours. These findings suggest that cardiomyocyte proliferation capacity could be compromised, as shown previously by Tong et al.[96] at other time points (GD 21 and PND-7). Further, a large variation in the proliferation of cardiomyocytes was observed in the control group for both male and female offspring, which reduced the power of our study. However, ex vivo cardiomyocytes showed consistently less proliferation in IUGR offspring compared to controls; suggesting a potential susceptibility to impaired cardiac development.

We hypothesized that the differences in cardiomyocyte proliferation could be attributed to a decrease in cardiac protein expression of Fn-14. Since it has been shown that both TWEAK and its receptor are highly upregulated after a myocardial infarction,[339,340] we hypothesized that these differences would remain in adult life and would be associated with the increased susceptibility to CVD observed in our hypoxic-induced IUGR model. In addition, it has been demonstrated that genetic manipulation of TWEAK, its downstream targets or

the induction of its receptor all result in dilated cardiomyopathy, cardiomyocyte hypertrophy and ECM remodelling in adult rodents.[341,342] Furthermore, mice treated with adenovirus expressing soluble murine TWEAK had a decreased expression of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α, the master regulator of mitochondrial biogenesis.[343] Decreased PGC1-α expression has been associated with decreased mitochondrial biogenesis and cardiac I/R injury.[344] However, in the current study cardiac protein expression of Fn-14 at PND-1 was not different among the groups. Moreover, the Fn-14 cardiac protein receptor remained quiescent and s-TWEAK serum concentrations were undetectable; thus, it can only be surmised that other mechanisms may be involved in the development of cardiac diseases later in life in IUGR offspring.

In conclusion, activation of the TWEAK/Fn-14 pathway leads to increased proliferation but does not appear to contribute to the reduced cardiomyocyte proliferation capacity observed in the prenatal hypoxic-exposed male offspring. Interestingly, reduced cardiomyocyte proliferation capacity was compromised in male, but not female, IUGR offspring. Previous studies from our laboratory[88,107] as well as data shown in Chapter 4, have shown that male IUGR offspring appear to have a greater cardiac dysfunction phenotype compared to females and it could be speculated that this reduced proliferative capacity may have a role to play in the susceptibility of male offspring to cardiovascular disease. Finally, other mechanisms involved in cardiomyocyte proliferation likely have an important role in the long-term consequences of being born growth restricted, and, therefore, further studies are needed.

6 General discussion and future directions

It is well-established that being born growth restricted has long-term cardiovascular consequences.[7-9,17,18,345] Of the many *in utero* stressors that a fetus can be subjected to, hypoxia is one of the most clinically relevant since there are limited reserves to compensate for a reduced oxygen supply.[61] Therefore, I used hypoxia as a method to develop adverse uterine conditions and to create an IUGR animal model that would develop CVD later in life. As stated throughout my thesis, many strategies have been proposed to prevent CVD; however, and despite large volumes of research that have attempted to discover cardiovascular protective mechanisms, exercise is one of the most practical and effective preventive treatments that has been found to date.[243] This strategy, however, had not been assessed in a model of growth restriction such as hypoxic-induced IUGR offspring. Therefore, we tested the hypothesis that AET could be used as a preventive strategy to decrease the impact of IUGR on later life cardiovascular health. In addition, since an early influence on the development of CVD later in life in hypoxic-induced IUGR offspring could involve altered cardiac morphology, we also wanted to explore the potential role of the TWEAK/Fn-14 pathway in cardiomyocyte proliferation.

6.1 Summary of the most significant findings

6.1.1 Changes in the cardiovascular function of hypoxic-induced IUGR offspring

We found that exposing rat dams to hypoxia during the last third of their pregnancy impacted not only offspring growth, but also impacted their cardiovascular health later in life. In terms of the vascular pathophysiology of adult IUGR offspring, females had reduced NO-mediated vasodilation in both mesenteric and gastrocnemius muscle arteries, while in male IUGR offspring NO-mediated vasodilation was reduced in only the gastrocnemius muscle arteries. Furthermore, we demonstrated that female IUGR offspring had a decreased total vasodilator response in gastrocnemius muscle arteries and there was an increase in prostaglandin-mediated vasoconstriction.

Echocardiographic assessments showed that there were no changes in cardiac function *in vivo* in hypoxic-induced IUGR offspring. However, male IUGR offspring but not female IUGR offspring had an increased susceptibility to cardiac I/R injury. It has been previously established that both male and female hypoxic-induced IUGR offspring have an increased proton production during reperfusion, in addition to a decrease in the relative proportion of ATP derived from fatty acid oxidation and a relative increase in the proportion of ATP derived from both the catabolism of carbohydrates and glycolysis.[107] It has been previously determined that the presence of high levels of circulatory fatty acids during reperfusion is detrimental to cardiac recovery.[346] Moreover, Bartkevics *et*

al., found that hemodynamic recovery of hearts perfused using Krebs buffer supplemented with 1.2 mM palmitate was lower compared to hearts perfused without palmitate.[347] We used a palmitate-free Krebs buffer in our preparations. Thus, it would be expected that cardiac recovery from I/R injury of both controls and IUGR offspring would be greater compared to previous studies derived from our lab which used palmitate-supplemented buffer,[89,107] without compromising the hypoxic-induced IUGR phenotype. Interestingly, that was not the case for female IUGR offspring, suggesting that they may be cardioprotected.

Finally, since hypoxic-induced IUGR offspring display a cardiac remodelling phenotype,[89] we investigated the potential role of the TWEAK/Fn-14 pathway in cardiomyocyte proliferation in neonatal offspring. Cardiomyocytes from male IUGR offspring but not female IUGR offspring had less ability to proliferate compared to controls after being in culture for 72-hours; demonstrating again that hypoxia sequelae are worse in male offspring compared to female offspring. There were no differences in cardiac Fn-14 protein expression at PND-1 in either sex.

6.1.2 The role of aerobic exercise training in the prevention of cardiovascular diseases of hypoxic-induced IUGR offspring

To our knowledge this is the first time that AET has been used therapeutically in a hypoxic-induced IUGR animal model. Since being born growth restricted has been associated with a decreased capacity for exercise[293,294] and an increased susceptibility to secondary insults,[186-188] our first aim was to

develop a reliable exercise protocol that did not evoke a stress response in IUGR offspring so that we could be confident that the differences among the groups were not associated with the protocol *per se*. Consequently, assessment of vascular function in mesenteric and gastrocnemius muscle arteries in hypoxic-induced IUGR offspring showed that AET in IUGR offspring enhanced EDH contribution to vasodilation in gastrocnemius muscle arteries without any major improvements in overall vasodilation in males. In contrast, in male IUGR offspring AET reduced basal cardiac performance *ex vivo* and increased cardiac $O_2^{\cdot -}$ generation with no alteration of any antioxidant enzyme assessed. Conversely, in female IUGR offspring AET did not affect cardiac performance *ex vivo,* while there was an increased cardiac SOD-1 expression with no changes in $O_2^{\cdot -}$ generation. These findings suggest that AET improved EDH-mediated vasodilation in gastrocnemius muscle arteries in male IUGR offspring (perhaps as a compensatory mechanism), while it had a detrimental effect on cardiac function in male IUGR offspring and had no effect on female IUGR cardiovascular function.

6.2 Significance of my results

Contrary to my findings in the hypoxic-induced IUGR model, evidence has shown that AET in other animal models of IUGR improved the metabolic profile and reduced Ang II-dependent vasoconstriction of IUGR offspring.[297-300,302] AET can confer a cardiovascular protective phenotype by triggering a transient increase in the production of ROS.[348] This transient increase of ROS can upregulate the protein expression and function of multiple antioxidant enzymes,

thereby increasing the antioxidant capacity of the system.[349] It has been well-established that these processes depend on the mode, intensity and duration of exercise.[350] In 1992, The HERITAGE Family Study was funded to assess the individual differences in the response to regular exercise training in a sedentary population; and to determine the role of the genotype in the cardiovascular and metabolic responses to AET. Approximately, 750 individuals were recruited and were subjected to 20 weeks of supervised AET. One of the many publications derived from this multicenter project found that AET was associated with minimal changes in body composition, lipid profile, glucose tolerance and resting blood pressure. After stratification of the data by the response to the AET (measured by the improvement in their personal VO_{2max}), body weight and the percentage of fat mass loss was greater in the people who had a better response to the AET. The magnitude of these changes was relatively small. Therefore, the variation of the response of each individual to an exercise intervention must be considered.[351]

Evidence from compromised populations (e.g. diabetes, obesity, myocardial infarction), however, has shown that AET may be detrimental;[287-291] and one of the possible mechanisms associated with this phenomenon is increased oxidative stress.[291] Therefore, we could speculate that in hypoxic-induced IUGR offspring there is either a cellular state where there is an excessive production of ROS to begin with; or that the AET paradigm used in my studies exceeded the threshold for the hormetic effect of exercise in this particular population.

The plasticity of the cardiovascular system declines with age; moreover, with ageing there is an increased risk of developing inadequate responses to new environmental challenges.[352] We started the AET protocol when offspring were 2.5-months old, during adolescence.[353] It has to be considered, however, that IUGR is associated with an early ageing vascular phenotype.[186] Thus, it is plausible that because of the exaggerated ageing phenotype, a hypoxic-induced IUGR phenotype may have already been established and, therefore, AET was unable to prevent the ongoing progression of CVD.

Findings from my thesis showed that consideration of sexual dimorphism is essential. Female IUGR offspring had endothelial dysfunction with normal cardiac function whereas male IUGR offspring showed an increased susceptibility to cardiac I/R injury without overt signs of endothelial dysfunction. Since the cardiovascular system is integrated, it is possible that the establishment of dysfunction in one of the organs (i.e. endothelial dysfunction) will trigger compensatory mechanisms to protect the other organ (i.e. the heart) or vice versa. Previous studies have demonstrated a potential role for PKCε in mediating cardioprotective mechanisms in females. Male hypoxic-induced IUGR offspring were shown to have a decreased cardiac mRNA and protein expression of PKCε secondary to an increased methylation of the PKCε promoter region at the early growth response factor-1 (Egr-1) binding site.[354] Moreover, it has been demonstrated that there is an increase in the cardiac protein expression of estrogen receptors in female offspring,[191,354] and that estrogen receptors can bind

to the promoter regions of PKCε[191,354] conferring female hypoxic-induced IUGR offspring cardioprotection against I/R injury.

We assessed the TWEAK/Fn-14 pathway in cardiomyocyte proliferation because it is known that IUGR is associated with a decrease in cardiomyocyte proliferation.[94] The mechanisms that support this association, however, are not fully understood. Multiple locally generated factors acting in an autocrine or paracrine fashion are associated with cardiomyocyte proliferation and maturation (e.g. Ang II, cortisol, IGF-1, ET-1). These factors, or the receptors by which they exert their function, are altered in IUGR offspring; therefore, results derived from them would be difficult to interpret. Interestingly, the TWEAK/Fn-14 pathway, which has recently been shown to have a role in cardiomyocyte proliferation,[137] has never been studied in IUGR offspring. However, we did not find any differences in cardiac Fn-14 protein expression or in the response of cardiomyocytes from hypoxic-induced IUGR offspring to s-TWEAK. Likewise, we assessed the TWEAK/Fn-14 pathway in adult offspring since previous studies have suggested that the increased susceptibility to I/R injury in hypoxic-induced IUGR offspring is programmed during cardiac development[102] and genetic manipulation of the TWEAK/Fn-14 pathway has been shown to result in cardiomyopathy and ECM remodelling.[341,342] Furthermore, alterations in the TWEAK/Fn-14 pathway have been associated with a decreased expression of PGC1-α;[343] which is known to be decreased in cardiac I/R injury.[344] Therefore, the TWEAK/Fn-14 pathway was considered to be a good candidate as the link between prenatal hypoxia and increased susceptibility to CVD later in life.

Contrary to our hypothesis, Fn-14 cardiac protein expression was quiescent in adult IUGR offspring, and thus, likely not a primary pathway affecting cardiovascular development.

6.3 Future directions

The results from my research need to be interpreted carefully. Since our studies were designed as a proof-of-principle of the role of aerobic exercise in ameliorating CVD in hypoxic-induced IUGR offspring. Hence, we cannot suggest that young adults born growth restricted should not enroll in physical activity or AET. It is important to note, however, that our studies raised a concern that the benefits of AET may differ between populations. We suggest that the dogma that is currently applied to healthy populations should not be arbitrarily applied to other specific populations (e.g. hypoxic-induced IUGR offspring) without increasing the amount of research and spanning the current knowledge gap. For example, different training paradigms should be assessed in order to find a protocol that provides favourable vascular and cardiac outcomes. Considering that it has previously been shown that young males born with a low birth weight can develop exercise-induced hypertension[295] and exercise-induced cardiac fatigue,[296] a comparison of multiple exercise training paradigms is needed; such as reducing the intensity of exercise but changing the duration of the exercise to achieve cardiovascular improvement. Moreover, it is important to acknowledge that in the current study there may have been an existing, but asymptomatic, cardiovascular pathology when the AET protocol was applied at 2.5 months of age in the rat; thus compromising the ability of AET to effectively prevent the

progression of CVD in this model. In this case, starting aerobic exercise earlier in life (after weaning) or using AET during pregnancy could be a better approach to prevent the development of CVD in offspring; however, the translation of these approaches to human populations would not be straightforward.

Evidence has shown that performing AET during pregnancy decreases the risk of preeclampsia and gestational hypertension,[355] gestational diabetes,[356] and excessive weight gain,[357] without impacting offspring growth or development.[358] Remarkably, all of these pregnancy complications are associated with fetal programming of CVD later in life. Hence, AET has been assessed during complicated pregnancies to determine if it improves both maternal and offspring outcomes. Vega *et al.* showed that, in an animal model of maternal obesity, the maternal serum concentration of triglycerides, cholesterol, and insulin returned to normal levels after AET. Interestingly, female offspring of obese dams had no metabolic alterations, while male offspring had increased serum levels of triglycerides and leptin in addition to an increased fat deposition. Male offspring from obese exercised dams had a reduction to control levels of serum triglycerides, and a decrease in serum leptin levels compared to male offspring of non-exercised obese dams.[359] Volpato *et al.,* however, found that diabetic pregnant rats exposed to swimming during pregnancy had increased plasma levels of malondialdehyde, with decreased levels of glutathione peroxidase and elevated $O_2^{\cdot -}$ activity compared to non-diabetic pregnant dams.[360] Fetuses from these exercised diabetic rats had a lower body weight and placental weight compared to non-exercised diabetic rats.[360] These findings suggest that special

considerations have to be made in the case of treating pregnant dams exposed to hypoxia with AET.

Regarding vascular function, our studies focused on the contribution of ROS to endothelial dysfunction. To do so, we assessed mesenteric arteries in the presence or absence of an $ONOO^-$ scavenger (MnTBAP); however, $O_2^{\bullet -}$ production and SOD function and expression were not determined. Thus, based on our current studies we cannot rule out the role of ROS in endothelial dysfunction and/or the role of AET in the amelioration of oxidative stress in hypoxic-induced IUGR offspring. Our data suggest that there was no beneficial effect of AET on either mesenteric or GMA artery function in female hypoxic-induced IUGR offspring. The absence of this effect in these particular vascular beds does not exclude that other vascular adaptations to AET may have occurred. It is important to note that neither conduit artery function nor flow-mediated vasodilation have been assessed following AET in this animal model. Increased shear stress is one of the main mechanisms by which aerobic exercise improves vascular function.[278] Therefore, assessment of flow-mediated vasodilation *in vivo* or *ex vivo* will provide additional evidence on how AET impacts vascular function in hypoxic-induced IUGR offspring. Moreover, it has been established that AET reduces central sympathetic outflow in healthy[361] and diseased populations (e.g. heart failure).[362] AET also enhances arterial baroreflex sensitivity,[363] which plays a critical role in the acute regulation of blood pressure by changing the autonomic nervous activity in the vasculature or in the heart.[364] Findings from previous studies in our laboratory suggest that autonomic nervous system function may be

impaired in hypoxic-induced IUGR restricted offspring.[88] Thus, it would be helpful to first determine the baseline autonomic nervous system function in our animal model in order to later determine whether aerobic exercise can influence autonomic nervous system function in hypoxic-induced IUGR offspring.

In addition, in the cardiac studies we quantified the protein expression of SOD-1,-2 and catalase in combination with $O_2^{\cdot -}$ production. In male hypoxic-induced IUGR offspring, AET was associated with an increase in $O_2^{\cdot -}$ production, with no changes in the main cardiac antioxidant enzymes. It would have been useful to also know the activity of these enzymes in order to understand better how AET impacted the ROS production- scavenging balance. Our findings showed that AET deteriorated cardiac function *ex vivo* in male hypoxic-induced IUGR offspring without compromising cardiac function *in vivo*. The cardioprotective role of AET, however, has not been assessed using an *in vivo* I/R protocol. AET has been shown to reduce myocardial infarction area size,[365] and improves cardiac function during[366] and after[367] ischemia. Using an *in vivo* I/R protocol would allow determination of whether AET improves cardiac function after an ischemic event, or whether AET decreases the risk of developing heart failure after an infarction. Moreover, in an *in vivo* I/R model one can assess the input of the systemic circulation, and the autonomic nervous system during the ischemic and reperfusion phase.[368] This is important because following an ischemic event, there is an inflammatory reaction that is required for scar formation.[369] This process is modulated by the ECM,[370] and is also associated with angiogenesis.[371] Thus, an *in vivo* I/R model can provide a systemic approach

to understand the impact of AET in the recovery of myocardial injury. In addition, an *in vivo* cardiac I/R injury model would allow assessment of the role of the TWEAK/Fn-14 pathway in I/R injury or EMC remodelling after an ischemic event. It has been previously determined that Fn-14 is upregulated 28 days after the induction of myocardial infarction in CD-1 mice.[340] Thus, although TWEAK was shown to be quiescent in adult IUGR offspring, the increased susceptibility to CVD later in life places adult IUGR offspring at risk of overexpressing the TWEAK/Fn-14 pathway and, therefore, to have worsened cardiac function after a myocardial infarction than control offspring.

Regarding heart development, our findings suggest that cardiomyocyte proliferation is compromised in male IUGR offspring. Our study was designed to determine whether cardiomyocyte proliferation was impaired in IUGR offspring at a specific time point (PND-1). Heart development, however, is an active process that terminates early in postnatal life in rats (PND-21).[117] Hence, in order to fully understand how hypoxia impacts cardiac development, assessments of cardiomyocyte proliferation and binucleation at different time-points are needed. Moreover, determining the size of the cardiomyocytes, and the presence of alterations in the ECM composition or assessment of whether there is a functional change that can be measured by echocardiography, would complement these data. Our findings also indicate that the TWEAK/Fn-14 pathway was not associated with cardiomyocyte proliferation, denoting that alternative pathways involved in cardiomyocyte proliferation should be assessed in our animal model.

6.4 Study limitations

There are some study limitations that need to be considered. Regarding the AET protocol *per se*, one of the limitations of this study is that AET was forced and not voluntary. Forced exercise is associated with a loss of control over the animal activity pattern, especially when the exercise training occurs during the daylight cycle, causing both stress and disruption of the normal rodent circadian rhythm.[372] Moreover, previous evidence has shown that forced exercise increases serum corticosterone levels,[305,306,373] and increases anxiety-like behaviour in open field testes.[306] Our data, however, suggest that in our animal model AET did not evoke a stress response when used as an intervention in a hypoxia-induced IUGR animal model.

The results derived from these projects are partially translational since they were conducted in an animal model. We designed these experiments, however, to provide a proof of principle. Given the nature of this project, the execution of a prospective cohort including children born IUGR and with a subsequent treatment and follow up structure would be perhaps more clinically relevant; however, at the current time this type of study is not feasible because CVD have an extend subclinical phase, and therefore, is not cost-effective.

Finally, our findings regarding the role of TWEAK/Fn-14 pathway in cardiomyocyte proliferation need to be interpreted carefully. For this set of experiments, in some groups we have a low n number, which reflects the variability of the data. Moreover, we used ki-67 as a marker of proliferation. ki-67 is a nuclear protein expressed in all phases of the cell cycle, excluding resting cells

in G_0.[374] Thus, it could be arguable that using this marker, the proliferation effect could be either under or overestimated, as compare to other markers that target the cardiomyocytes in their S-phase (DNA synthesis), such as bromodeoxyuridine (BrdU),[375] or proliferating cell nuclear antigen (PCNA).[376] We used ki-67 as a proliferation marker, however, based on the fact that cells can enter a quiescent state even after DNA synthesis completion,[377] thus, a proliferation marker is actually an indicative of the proliferation potential of the cell rather than a predictor of proliferation.[378] In addition, it has been established that PCNA does not work well in very slow proliferative cells in S-phase.[376]

6.5 Conclusion

The impact of IUGR on cardiovascular health later in life is far reaching. Notably, IUGR is now considered as a risk factor to develop CVD later in life. Given the burden of CVD to public health, it is imperative that we create and evaluate strategies to prevent the development of CVD, especially in populations at increased risk. Our research demonstrated that aerobic exercise, which is a well-established strategy for primary and secondary prevention of CVD, had a detrimental effect on cardiac function from male hypoxic-induced IUGR offspring. An IUGR population can be considered to have a cardiovascular physiology that has altered to promote survival of the fetus in an adverse uterine environment, and once born these cardiovascular adaptations are maintained unnecessarily and may, in this respect, cause a mismatch in development vs. environment. Our findings demonstrated that in populations at risk, such as these IUGR populations, a common preventive strategy such as aerobic exercise

may represent a secondary stressor to the cardiovascular physiology. Hence, more research is needed in order to establish an exercise paradigm that does not impact cardiac function, but instead exerts cardiovascular benefits. Our results highlight the fact that cardiovascular adaptations to IUGR and AET are sex-dependent and, therefore, findings from male and female populations should not be extrapolated to the other sex. Finally, in the long term, individualization of health care in populations at risk may give better results than adopting health policies suggested for the general population.

References

1 Lewis, G. Reviewing maternal deaths to make pregnancy safer. *Best Pract Res Clin Obstet Gynaecol* **22**, 447-463, doi:10.1016/j.bpobgyn.2007.10.007 (2008).

2 Baschat, A. A. Pathophysiology of fetal growth restriction: implications for diagnosis and surveillance. *Obstet Gynecol Surv* **59**, 617-627 (2004).

3 Ross, M. G. M., R. Z. . Fetal Growth Restriction. (2008). <http://emedicine.medscape.com/article/261226-overview#showall>.

4 Lausman, A., Kingdom, J. & Maternal Fetal Medicine, C. Intrauterine growth restriction: screening, diagnosis, and management. *J Obstet Gynaecol Can* **35**, 741-748, doi:10.1016/S1701-2163(15)30865-3 (2013).

5 de Onis, M., Blossner, M. & Villar, J. Levels and patterns of intrauterine growth retardation in developing countries. *Eur J Clin Nutr* **52 Suppl 1**, S5-15 (1998).

6 Lawn, J. E., Cousens, S., Zupan, J. & Lancet Neonatal Survival Steering, T. 4 million neonatal deaths: when? Where? Why? *Lancet* **365**, 891-900, doi:10.1016/S0140-6736(05)71048-5 (2005).

7 Barker, D. J., Winter, P. D., Osmond, C., Margetts, B. & Simmonds, S. J. Weight in infancy and death from ischaemic heart disease. *Lancet* **2**, 577-580 (1989).

8 Barker, D. J., Bull, A. R., Osmond, C. & Simmonds, S. J. Fetal and placental size and risk of hypertension in adult life. *BMJ* **301**, 259-262 (1990).

9 Barker, D. J., Osmond, C., Simmonds, S. J. & Wield, G. A. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. *BMJ* **306**, 422-426 (1993).

10 Global Status Report on Noncommunicable Diseases 2010. *Global Status Report on Noncommunicable Diseases 2010*, 1-162 (2011).

11 Yusuf, S. *et al.* Cardiovascular risk and events in 17 low-, middle-, and high-income countries. *N Engl J Med* **371**, 818-827, doi:10.1056/NEJMoa1311890 (2014).

12 Canada, P. H. A. o. *Economic Burden of Illness in Canada 2000*, <http://www.phac-aspc.gc.ca/cd-mc/cvd-mcv/cvd_ebic-mcv_femc-eng.php> (2000).

13 Mozaffarian, D. *et al.* Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation* **133**, e38-e360, doi:10.1161/CIR.0000000000000350 (2016).

14 Yusuf, S. *et al.* Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* **364**, 937-952, doi:10.1016/S0140-6736(04)17018-9 (2004).

15 *World Health Organization. Health Transition.*, <http://www.who.int/trade/glossary/story050/en/> (2015).

16 Fuster, V., Kelly, B. B. & Vedanthan, R. Global cardiovascular health: urgent need for an intersectoral approach. *J Am Coll Cardiol* **58**, 1208-1210, doi:10.1016/j.jacc.2011.05.038 (2011).

17 Jaquet, D., Gaboriau, A., Czernichow, P. & Levy-Marchal, C. Insulin resistance early in adulthood in subjects born with intrauterine growth retardation. *J Clin Endocrinol Metab* **85**, 1401-1406, doi:10.1210/jcem.85.4.6544 (2000).

18 Barker, D. J., Osmond, C. & Law, C. M. The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. *J Epidemiol Community Health* **43**, 237-240 (1989).

19 Pollack, R. N. & Divon, M. Y. Intrauterine growth retardation: definition, classification, and etiology. *Clin Obstet Gynecol* **35**, 99-107 (1992).

20 Hendrix, N. & Berghella, V. Non-placental causes of intrauterine growth restriction. *Semin Perinatol* **32**, 161-165, doi:10.1053/j.semperi.2008.02.004 (2008).

21 Monk, D. & Moore, G. E. Intrauterine growth restriction--genetic causes and consequences. *Semin Fetal Neonatal Med* **9**, 371-378, doi:10.1016/j.siny.2004.03.002 (2004).

22 Cereda, A. & Carey, J. C. The trisomy 18 syndrome. *Orphanet J Rare Dis* **7**, 81, doi:10.1186/1750-1172-7-81 (2012).

23 Baty, B. J., Blackburn, B. L. & Carey, J. C. Natural history of trisomy 18 and trisomy 13: I. Growth, physical assessment, medical histories, survival, and recurrence risk. *Am J Med Genet* **49**, 175-188, doi:10.1002/ajmg.1320490204 (1994).

24 Tonni, G., Ventura, A., Pattacini, P., Bonasoni, M. & Ferrari, B. Complex cardiac defect, bowing of lower limbs and multiple anomalies in trisomy 22. Ultrasound, post-mortem CT findings with necropsy confirmation. *Fetal Pediatr Pathol* **31**, 439-447, doi:10.3109/15513815.2012.659409 (2012).

25 Adams Waldorf, K. M. & McAdams, R. M. Influence of infection during pregnancy on fetal development. *Reproduction* **146**, R151-162, doi:10.1530/REP-13-0232 (2013).

26 Longo, S., Borghesi, A., Tzialla, C. & Stronati, M. IUGR and infections. *Early Hum Dev* **90 Suppl 1**, S42-44, doi:10.1016/S0378-3782(14)70014-3 (2014).

27 Papageorghiou, A. T., Bakoulas, V., Sebire, N. J. & Nicolaides, K. H. Intrauterine growth in multiple pregnancies in relation to fetal number, chorionicity and gestational age. *Ultrasound Obstet Gynecol* **32**, 890-893, doi:10.1002/uog.6140 (2008).

28 Khoury, M. J., Erickson, J. D., Cordero, J. F. & McCarthy, B. J. Congenital malformations and intrauterine growth retardation: a population study. *Pediatrics* **82**, 83-90 (1988).

29 Pereira, L. *et al.* Intrauterine growth restriction caused by underlying congenital cytomegalovirus infection. *J Infect Dis* **209**, 1573-1584, doi:10.1093/infdis/jiu019 (2014).

30 Galbraith, R. M. *et al.* The human materno-foetal relationship in malaria. II. Histological, ultrastructural and immunopathological studies of the placenta. *Trans R Soc Trop Med Hyg* **74**, 61-72 (1980).

31 Melchiorre, K., Sharma, R. & Thilaganathan, B. Cardiac structure and function in normal pregnancy. *Curr Opin Obstet Gynecol* **24**, 413-421, doi:10.1097/GCO.0b013e328359826f (2012).

32 Kingdom, J. C. & Kaufmann, P. Oxygen and placental villous development: origins of fetal hypoxia. *Placenta* **18**, 613-621; discussion 623-616 (1997).

33 Jansson, T. & Powell, T. L. IFPA 2005 Award in Placentology Lecture. Human placental transport in altered fetal growth: does the placenta function as a nutrient sensor? -- a review. *Placenta* **27 Suppl A**, S91-97, doi:10.1016/j.placenta.2005.11.010 (2006).

34 Mifsud, W. & Sebire, N. J. Placental pathology in early-onset and late-onset fetal growth restriction. *Fetal Diagn Ther* **36**, 117-128, doi:10.1159/000359969 (2014).

35 Schneiderman, M. & Balayla, J. A comparative study of neonatal outcomes in placenta previa versus cesarean for other indication at term. *J Matern Fetal Neonatal Med* **26**, 1121-1127, doi:10.3109/14767058.2013.770465 (2013).

36 Mailath-Pokorny, M., Worda, K., Schmid, M., Polterauer, S. & Bettelheim, D. Isolated single umbilical artery: evaluating the risk of adverse pregnancy outcome. *Eur J Obstet Gynecol Reprod Biol* **184**, 80-83, doi:10.1016/j.ejogrb.2014.11.007 (2015).

37 Belkacemi, L., Nelson, D. M., Desai, M. & Ross, M. G. Maternal undernutrition influences placental-fetal development. *Biol Reprod* **83**, 325-331, doi:10.1095/biolreprod.110.084517 (2010).

38 Namazy, J. A. *et al.* Effects of asthma severity, exacerbations and oral corticosteroids on perinatal outcomes. *Eur Respir J* **41**, 1082-1090, doi:10.1183/09031936.00195111 (2013).

39 Hsieh, T. T., Chen, K. C. & Soong, J. H. Outcome of pregnancy in patients with organic heart disease in Taiwan. *Asia Oceania J Obstet Gynaecol* **19**, 21-27 (1993).

40 Chakravarty, E. F., Khanna, D. & Chung, L. Pregnancy outcomes in systemic sclerosis, primary pulmonary hypertension, and sickle cell disease. *Obstet Gynecol* **111**, 927-934, doi:10.1097/01.AOG.0000308710.86880.a6 (2008).

41 August, P., Jeyabalan, A. & Roberts, J. M. in *Chesley's Hypertensive disorders in pregnancy* (eds Robert N. Taylor, James M. Roberts, F. Gary Cunningham, & Marshall D. Lindheimer) Ch. 18, 397-417 pages (Elsevier/AP, Academic Press is an imprint of Elsevier, 2015).

42 Long, P. A., Abell, D. A. & Beischer, N. A. Fetal growth retardation and pre-eclampsia. *Br J Obstet Gynaecol* **87**, 13-18 (1980).

43 Howarth, C., Gazis, A. & James, D. Associations of Type 1 diabetes mellitus, maternal vascular disease and complications of pregnancy. *Diabet Med* **24**, 1229-1234, doi:10.1111/j.1464-5491.2007.02254.x (2007).

44 Jones, D. C. & Hayslett, J. P. Outcome of pregnancy in women with moderate or severe renal insufficiency. *N Engl J Med* **335**, 226-232, doi:10.1056/NEJM199607253350402 (1996).

45 Keyes, L. E. *et al.* Intrauterine growth restriction, preeclampsia, and intrauterine mortality at high altitude in Bolivia. *Pediatr Res* **54**, 20-25, doi:10.1203/01.PDR.0000069846.64389.DC (2003).

46 Blatt, K., Moore, E., Chen, A., Van Hook, J. & DeFranco, E. A. Association of reported trimester-specific smoking cessation with fetal growth restriction. *Obstet Gynecol* **125**, 1452-1459, doi:10.1097/AOG.0000000000000679 (2015).

47 Jones, K. L., Smith, D. W., Ulleland, C. N. & Streissguth, P. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* **1**, 1267-1271 (1973).

48 Thangappah, R. B. Maternal and perinatal outcome with drug abuse in pregnancy. *J Obstet Gynaecol* **20**, 597-600, doi:10.1080/01443610020001422 (2000).

49 Gouin, K., Murphy, K., Shah, P. S., Knowledge Synthesis group on Determinants of Low Birth, W. & Preterm, B. Effects of cocaine use during pregnancy on low birthweight and preterm birth: systematic review and metaanalyses. *Am J Obstet Gynecol* **204**, 340 e341-312, doi:10.1016/j.ajog.2010.11.013 (2011).

50 Visser, G. H., Huisman, A., Saathof, P. W. & Sinnige, H. A. Early fetal growth retardation: obstetric background and recurrence rate. *Obstet Gynecol* **67**, 40-43 (1986).

51 Gunnarsdottir, J., Stephansson, O., Cnattingius, S., Akerud, H. & Wikstrom, A. K. Risk of placental dysfunction disorders after prior miscarriages: a population-based study. *Am J Obstet Gynecol* **211**, 34 e31-38, doi:10.1016/j.ajog.2014.01.041 (2014).

52 Voskamp, B. J. *et al.* Recurrence of small-for-gestational-age pregnancy: analysis of first and subsequent singleton pregnancies in The Netherlands.

Am J Obstet Gynecol **208**, 374 e371-376, doi:10.1016/j.ajog.2013.01.045 (2013).

53 Morrison, J. L. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. *Clin Exp Pharmacol Physiol* **35**, 730-743, doi:10.1111/j.1440-1681.2008.04975.x (2008).

54 Vuguin, P. M. Animal models for small for gestational age and fetal programming of adult disease. *Horm Res* **68**, 113-123, doi:10.1159/000100545 (2007).

55 Garofano, A., Czernichow, P. & Breant, B. Beta-cell mass and proliferation following late fetal and early postnatal malnutrition in the rat. *Diabetologia* **41**, 1114-1120, doi:10.1007/s001250051038 (1998).

56 Zohdi, V., Lim, K., Pearson, J. T. & Black, M. J. Developmental programming of cardiovascular disease following intrauterine growth restriction: findings utilising a rat model of maternal protein restriction. *Nutrients* **7**, 119-152, doi:10.3390/nu7010119 (2014).

57 Gambling, L. *et al.* Iron deficiency during pregnancy affects postnatal blood pressure in the rat. *J Physiol* **552**, 603-610, doi:10.1113/jphysiol.2003.051383 (2003).

58 Picone, O. *et al.* Hyperlipidic hypercholesterolemic diet in prepubertal rabbits affects gene expression in the embryo, restricts fetal growth and

increases offspring susceptibility to obesity. *Theriogenology* **75**, 287-299, doi:10.1016/j.theriogenology.2010.08.015 (2011).

59 Fowden, A. L., Valenzuela, O. A., Vaughan, O. R., Jellyman, J. K. & Forhead, A. J. Glucocorticoid programming of intrauterine development. *Domest Anim Endocrinol* **56 Suppl**, S121-132, doi:10.1016/j.domaniend.2016.02.014 (2016).

60 Li, R. Y. & Tsutsui, Y. Growth retardation and microcephaly induced in mice by placental infection with murine cytomegalovirus. *Teratology* **62**, 79-85, doi:10.1002/1096-9926(200008)62:2<79::AID-TERA3>3.0.CO;2-S (2000).

61 Zhang, L. Prenatal hypoxia and cardiac programming. *J Soc Gynecol Investig* **12**, 2-13, doi:10.1016/j.jsgi.2004.09.004 (2005).

62 Giussani, D. A. & Davidge, S. T. Developmental programming of cardiovascular disease by prenatal hypoxia. *J Dev Orig Health Dis* **4**, 328-337, doi:10.1017/S204017441300010X (2013).

63 Janot, M., Cortes-Dubly, M. L., Rodriguez, S. & Huynh-Do, U. Bilateral uterine vessel ligation as a model of intrauterine growth restriction in mice. *Reprod Biol Endocrinol* **12**, 62, doi:10.1186/1477-7827-12-62 (2014).

64 Nusken, K. D., Warnecke, C., Hilgers, K. F. & Schneider, H. Intrauterine growth after uterine artery ligation in rats: dependence on the fetal

position in the uterine horn and need for prenatal marking of the animals. *J Hypertens* **25**, 247-248, doi:10.1097/01.hjh.0000254371.70823.d4 (2007).

65 Emmanouilides, G. C., Townsend, D. E. & Bauer, R. A. Effects of single umbilical artery ligation in the lamb fetus. *Pediatrics* **42**, 919-927 (1968).

66 Lafeber, H. N., Rolph, T. P. & Jones, C. T. Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. *J Dev Physiol* **6**, 441-459 (1984).

67 Losonczy, G., Brown, G. & Venuto, R. C. Increased peripheral resistance during reduced uterine perfusion pressure hypertension in pregnant rabbits. *Am J Med Sci* **303**, 233-240 (1992).

68 Clark, K. E., Durnwald, M. & Austin, J. E. A model for studying chronic reduction in uterine blood flow in pregnant sheep. *Am J Physiol* **242**, H297-301 (1982).

69 Golden, J. G., Hughes, H. C. & Lang, C. M. Experimental toxemia in the pregnant guinea pig (Cavia porcellus). *Lab Anim Sci* **30**, 174-179 (1980).

70 Abitbol, M. M., Ober, M. B., Gallo, G. R., Driscoll, S. G. & Pirani, C. L. Experimental toxemia of pregnancy in the monkey, with a preliminary report on renin and aldosterone. *Am J Pathol* **86**, 573-590 (1977).

71 Abitbol, M. M. Simplified technique to produce toxemia in the rat: considerations on cause of toxemia. *Clin Exp Hypertens B* **1**, 93-103 (1982).

72 Alexander, B. T. Placental insufficiency leads to development of hypertension in growth-restricted offspring. *Hypertension* **41**, 457-462, doi:10.1161/01.HYP.0000053448.95913.3D (2003).

73 Li, J., LaMarca, B. & Reckelhoff, J. F. A model of preeclampsia in rats: the reduced uterine perfusion pressure (RUPP) model. *Am J Physiol Heart Circ Physiol* **303**, H1-8, doi:10.1152/ajpheart.00117.2012 (2012).

74 Robinson, J. S., Kingston, E. J., Jones, C. T. & Thorburn, G. D. Studies on experimental growth retardation in sheep. The effect of removal of a endometrial caruncles on fetal size and metabolism. *J Dev Physiol* **1**, 379-398 (1979).

75 Brain, K. L. *et al.* Induction of controlled hypoxic pregnancy in large mammalian species. *Physiol Rep* **3**, doi:10.14814/phy2.12614 (2015).

76 Pham, H. *et al.* Impact of inhaled nitric oxide on white matter damage in growth-restricted neonatal rats. *Pediatr Res* **77**, 563-569, doi:10.1038/pr.2015.4 (2015).

77 Iqbal, W. & Ciriello, J. Effect of maternal chronic intermittent hypoxia during gestation on offspring growth in the rat. *Am J Obstet Gynecol* **209**, 564 e561-569, doi:10.1016/j.ajog.2013.08.027 (2013).

78 Wang, Z., Huang, Z., Lu, G., Lin, L. & Ferrari, M. Hypoxia during pregnancy in rats leads to early morphological changes of atherosclerosis in adult offspring. *Am J Physiol Heart Circ Physiol* **296**, H1321-1328, doi:10.1152/ajpheart.00440.2008 (2009).

79 Ream, M., Ray, A. M., Chandra, R. & Chikaraishi, D. M. Early fetal hypoxia leads to growth restriction and myocardial thinning. *Am J Physiol Regul Integr Comp Physiol* **295**, R583-595, doi:10.1152/ajpregu.00771.2007 (2008).

80 Jakoubek, V., Bibova, J., Herget, J. & Hampl, V. Chronic hypoxia increases fetoplacental vascular resistance and vasoconstrictor reactivity in the rat. *Am J Physiol Heart Circ Physiol* **294**, H1638-1644, doi:10.1152/ajpheart.01120.2007 (2008).

81 Thaete, L. G., Dewey, E. R. & Neerhof, M. G. Endothelin and the regulation of uterine and placental perfusion in hypoxia-induced fetal growth restriction. *J Soc Gynecol Investig* **11**, 16-21 (2004).

82 Thaete, L. G., Neerhof, M. G. & Caplan, M. S. Endothelin receptor A antagonism prevents hypoxia-induced intrauterine growth restriction in the rat. *Am J Obstet Gynecol* **176**, 73-76 (1997).

83 Van Geijn, H. P., Kaylor, W. M., Jr., Nicola, K. R. & Zuspan, F. P. Induction of severe intrauterine growth retardation in the Sprague-Dawley rat. *Am J Obstet Gynecol* **137**, 43-47 (1980).

84 Allison, B. J. *et al.* Divergence of mechanistic pathways mediating cardiovascular aging and developmental programming of cardiovascular disease. *FASEB J* **30**, 1968-1975, doi:10.1096/fj.201500057 (2016).

85 Lee, A. M., Morrison, J. L., Botting, K. J., Shandala, T. & Xian, C. J. Effects of Maternal Hypoxia during Pregnancy on Bone Development in Offspring: A Guinea Pig Model. *Int J Endocrinol* **2014**, 916918, doi:10.1155/2014/916918 (2014).

86 Morton, J. S., Rueda-Clausen, C. F. & Davidge, S. T. Mechanisms of endothelium-dependent vasodilation in male and female, young and aged offspring born growth restricted. *Am J Physiol Regul Integr Comp Physiol* **298**, R930-938, doi:10.1152/ajpregu.00641.2009 (2010).

87 Morton, J. S., Rueda-Clausen, C. F. & Davidge, S. T. Flow-mediated vasodilation is impaired in adult rat offspring exposed to prenatal hypoxia. *J Appl Physiol (1985)* **110**, 1073-1082, doi:10.1152/japplphysiol.01174.2010 (2011).

88 Rueda-Clausen, C. F., Morton, J. S. & Davidge, S. T. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. *Cardiovasc Res* **81**, 713-722, doi:10.1093/cvr/cvn341 (2009).

89 Xu, Y., Williams, S. J., O'Brien, D. & Davidge, S. T. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac

remodeling and impairs postischemic recovery in adult male offspring. *FASEB J* **20**, 1251-1253, doi:10.1096/fj.05-4917fje (2006).

90 Williams, S. J., Campbell, M. E., McMillen, I. C. & Davidge, S. T. Differential effects of maternal hypoxia or nutrient restriction on carotid and femoral vascular function in neonatal rats. *Am J Physiol Regul Integr Comp Physiol* **288**, R360-367, doi:10.1152/ajpregu.00178.2004 (2005).

91 Bae, S., Xiao, Y., Li, G., Casiano, C. A. & Zhang, L. Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart. *Am J Physiol Heart Circ Physiol* **285**, H983-990, doi:10.1152/ajpheart.00005.2003 (2003).

92 Xue, Q., Dasgupta, C., Chen, M. & Zhang, L. Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. *Cardiovasc Res* **89**, 300-308, doi:10.1093/cvr/cvq303 (2011).

93 Tapanainen, P. J. *et al.* Maternal hypoxia as a model for intrauterine growth retardation: effects on insulin-like growth factors and their binding proteins. *Pediatr Res* **36**, 152-158, doi:10.1203/00006450-199408000-00004 (1994).

94 Paradis, A., Xiao, D., Zhou, J. & Zhang, L. Endothelin-1 promotes cardiomyocyte terminal differentiation in the developing heart via

heightened DNA methylation. *Int J Med Sci* **11**, 373-380, doi:10.7150/ijms.7802 (2014).

95 Xiao, D., Ducsay, C. A. & Zhang, L. Chronic hypoxia and developmental regulation of cytochrome c expression in rats. *J Soc Gynecol Investig* **7**, 279-283 (2000).

96 Tong, W., Xue, Q., Li, Y. & Zhang, L. Maternal hypoxia alters matrix metalloproteinase expression patterns and causes cardiac remodeling in fetal and neonatal rats. *Am J Physiol Heart Circ Physiol* **301**, H2113-2121, doi:10.1152/ajpheart.00356.2011 (2011).

97 Kane, A. D., Herrera, E. A., Camm, E. J. & Giussani, D. A. Vitamin C prevents intrauterine programming of in vivo cardiovascular dysfunction in the rat. *Circ J* **77**, 2604-2611 (2013).

98 Giussani, D. A. *et al.* Developmental programming of cardiovascular dysfunction by prenatal hypoxia and oxidative stress. *PLoS One* **7**, e31017, doi:10.1371/journal.pone.0031017 (2012).

99 Li, G., Bae, S. & Zhang, L. Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. *Am J Physiol Heart Circ Physiol* **286**, H1712-1719, doi:10.1152/ajpheart.00898.2003 (2004).

100 Li, G. *et al.* Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *J Soc Gynecol Investig* **10**, 265-274 (2003).

101 Xue, Q. & Zhang, L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: role of protein kinase C epsilon. *J Pharmacol Exp Ther* **330**, 624-632, doi:10.1124/jpet.109.153239 (2009).

102 Patterson, A. J., Xiao, D., Xiong, F., Dixon, B. & Zhang, L. Hypoxia-derived oxidative stress mediates epigenetic repression of PKCepsilon gene in foetal rat hearts. *Cardiovasc Res* **93**, 302-310, doi:10.1093/cvr/cvr322 (2012).

103 Camm, E. J. *et al.* Partial contributions of developmental hypoxia and undernutrition to prenatal alterations in somatic growth and cardiovascular structure and function. *Am J Obstet Gynecol* **203**, 495 e424-434, doi:10.1016/j.ajog.2010.06.046 (2010).

104 Williams, S. J., Hemmings, D. G., Mitchell, J. M., McMillen, I. C. & Davidge, S. T. Effects of maternal hypoxia or nutrient restriction during pregnancy on endothelial function in adult male rat offspring. *J Physiol* **565**, 125-135, doi:10.1113/jphysiol.2005.084889 (2005).

105 Hemmings, D. G., Williams, S. J. & Davidge, S. T. Increased myogenic tone in 7-month-old adult male but not female offspring from rat dams exposed to hypoxia during pregnancy. *Am J Physiol Heart Circ Physiol* **289**, H674-682, doi:10.1152/ajpheart.00191.2005 (2005).

106 Bourque, S. L. *et al.* Prenatal hypoxia causes long-term alterations in vascular endothelin-1 function in aged male, but not female, offspring. *Hypertension* **62**, 753-758, doi:10.1161/HYPERTENSIONAHA.113.01516 (2013).

107 Rueda-Clausen, C. F., Morton, J. S., Lopaschuk, G. D. & Davidge, S. T. Long-term effects of intrauterine growth restriction on cardiac metabolism and susceptibility to ischaemia/reperfusion. *Cardiovasc Res* **90**, 285-294, doi:10.1093/cvr/cvq363 (2011).

108 Tang, J. *et al.* Chronic hypoxia in pregnancy affected vascular tone of renal interlobar arteries in the offspring. *Sci Rep* **5**, 9723, doi:10.1038/srep09723 (2015).

109 Zhu, X. *et al.* Prenatal hypoxia enhanced angiotensin II-mediated vasoconstriction via increased oxidative signaling in fetal rats. *Reprod Toxicol* **60**, 21-28, doi:10.1016/j.reprotox.2016.01.001 (2016).

110 Chen, Y. F., Wang, Z. H., Chen, Z. K., Lv, G. R. & Ferrari, M. Intermittent maternal hypoxia has an influence on regional expression of endothelial nitric oxide synthase in fetal arteries of rabbits. *Pediatr Res* **73**, 706-712, doi:10.1038/pr.2013.39 (2013).

111 Thornburg, K. L. Fetal response to intrauterine stress. *Ciba Found Symp* **156**, 17-29; discussion 29-37 (1991).

112 Bilardo, C. M., Nicolaides, K. H. & Campbell, S. Doppler measurements of fetal and uteroplacental circulations: relationship with umbilical venous blood gases measured at cordocentesis. *Am J Obstet Gynecol* **162**, 115-120 (1990).

113 Economides, D. L., Nicolaides, K. H. & Campbell, S. Metabolic and endocrine findings in appropriate and small for gestational age fetuses. *J Perinat Med* **19**, 97-105 (1991).

114 Economides, D. L., Nicolaides, K. H., Linton, E. A., Perry, L. A. & Chard, T. Plasma cortisol and adrenocorticotropin in appropriate and small for gestational age fetuses. *Fetal Ther* **3**, 158-164 (1988).

115 Fowden, A. L., Giussani, D. A. & Forhead, A. J. Intrauterine programming of physiological systems: causes and consequences. *Physiology (Bethesda)* **21**, 29-37, doi:10.1152/physiol.00050.2005 (2006).

116 Marcela, S. G. *et al.* Chronological and morphological study of heart development in the rat. *Anat Rec (Hoboken)* **295**, 1267-1290, doi:10.1002/ar.22508 (2012).

117 Clubb, F. J., Jr. & Bishop, S. P. Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. *Lab Invest* **50**, 571-577 (1984).

118 Granados-Riveron, J. T. & Brook, J. D. The impact of mechanical forces in heart morphogenesis. *Circ Cardiovasc Genet* **5**, 132-142, doi:10.1161/CIRCGENETICS.111.961086 (2012).

119 Sundgren, N. C., Giraud, G. D., Stork, P. J., Maylie, J. G. & Thornburg, K. L. Angiotensin II stimulates hyperplasia but not hypertrophy in immature ovine cardiomyocytes. *J Physiol* **548**, 881-891, doi:10.1113/jphysiol.2003.038778 (2003).

120 Giraud, G. D., Louey, S., Jonker, S., Schultz, J. & Thornburg, K. L. Cortisol stimulates cell cycle activity in the cardiomyocyte of the sheep fetus. *Endocrinology* **147**, 3643-3649, doi:10.1210/en.2006-0061 (2006).

121 Sundgren, N. C. *et al.* Extracellular signal-regulated kinase and phosphoinositol-3 kinase mediate IGF-1 induced proliferation of fetal sheep cardiomyocytes. *Am J Physiol Regul Integr Comp Physiol* **285**, R1481-1489, doi:10.1152/ajpregu.00232.2003 (2003).

122 Murphy, P. J. The fetal circulation. *Continuing Education in Anaesthesia, Critical Care & Pain* **5**, 107-112, doi:10.1093/bjaceaccp/mki030 (2005).

123 Baschat, A. A. Fetal responses to placental insufficiency: an update. *BJOG* **111**, 1031-1041, doi:10.1111/j.1471-0528.2004.00273.x (2004).

124 Cohen, E., Wong, F. Y., Horne, R. S. & Yiallourou, S. R. Intrauterine growth restriction: impact on cardiovascular development and function

throughout infancy. *Pediatr Res* **79**, 821-830, doi:10.1038/pr.2016.24 (2016).

125 Thornburg, K. *et al.* Regulation of the cardiomyocyte population in the developing heart. *Prog Biophys Mol Biol* **106**, 289-299, doi:10.1016/j.pbiomolbio.2010.11.010 (2011).

126 Rouwet, E. V. *et al.* Hypoxia induces aortic hypertrophic growth, left ventricular dysfunction, and sympathetic hyperinnervation of peripheral arteries in the chick embryo. *Circulation* **105**, 2791-2796 (2002).

127 Engelmann, G. L., Boehm, K. D., Haskell, J. F., Khairallah, P. A. & Ilan, J. Insulin-like growth factors and neonatal cardiomyocyte development: ventricular gene expression and membrane receptor variations in normotensive and hypertensive rats. *Mol Cell Endocrinol* **63**, 1-14 (1989).

128 Sadoshima, J. & Izumo, S. Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ Res* **73**, 413-423 (1993).

129 Watanabe, T., Pakala, R., Katagiri, T. & Benedict, C. R. Serotonin potentiates angiotensin II--induced vascular smooth muscle cell proliferation. *Atherosclerosis* **159**, 269-279 (2001).

130 Grigore, D. *et al.* Placental insufficiency results in temporal alterations in the renin angiotensin system in male hypertensive growth restricted

offspring. *Am J Physiol Regul Integr Comp Physiol* **293**, R804-811, doi:10.1152/ajpregu.00725.2006 (2007).

131 Wlodek, M. E. *et al.* Normal lactational environment restores nephron endowment and prevents hypertension after placental restriction in the rat. *J Am Soc Nephrol* **18**, 1688-1696, doi:10.1681/ASN.2007010015 (2007).

132 Kajstura, J., Cheng, W., Reiss, K. & Anversa, P. The IGF-1-IGF-1 receptor system modulates myocyte proliferation but not myocyte cellular hypertrophy in vitro. *Exp Cell Res* **215**, 273-283, doi:10.1006/excr.1994.1343 (1994).

133 Straus, D. S., Ooi, G. T., Orlowski, C. C. & Rechler, M. M. Expression of the genes for insulin-like growth factor-I (IGF-I), IGF-II, and IGF-binding proteins-1 and -2 in fetal rat under conditions of intrauterine growth retardation caused by maternal fasting. *Endocrinology* **128**, 518-525, doi:10.1210/endo-128-1-518 (1991).

134 Vileisis, R. A. & D'Ercole, A. J. Tissue and serum concentrations of somatomedin-C/insulin-like growth factor I in fetal rats made growth retarded by uterine artery ligation. *Pediatr Res* **20**, 126-130, doi:10.1203/00006450-198602000-00006 (1986).

135 Paradis, A. N., Gay, M. S., Wilson, C. G. & Zhang, L. Newborn hypoxia/anoxia inhibits cardiomyocyte proliferation and decreases

cardiomyocyte endowment in the developing heart: role of endothelin-1. *PLoS One* **10**, e0116600, doi:10.1371/journal.pone.0116600 (2015).

136 Botting, K. J. *et al.* Early origins of heart disease: low birth weight and determinants of cardiomyocyte endowment. *Clin Exp Pharmacol Physiol* **39**, 814-823, doi:10.1111/j.1440-1681.2011.05649.x (2012).

137 Novoyatleva, T. *et al.* TWEAK is a positive regulator of cardiomyocyte proliferation. *Cardiovasc Res* **85**, 681-690, doi:10.1093/cvr/cvp360 (2010).

138 Chicheportiche, Y. *et al.* TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem* **272**, 32401-32410 (1997).

139 Winkles, J. A. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov* **7**, 411-425, doi:10.1038/nrd2488 (2008).

140 Bowers, S. L., Banerjee, I. & Baudino, T. A. The extracellular matrix: at the center of it all. *J Mol Cell Cardiol* **48**, 474-482, doi:10.1016/j.yjmcc.2009.08.024 (2010).

141 Moore, L., Fan, D., Basu, R., Kandalam, V. & Kassiri, Z. Tissue inhibitor of metalloproteinases (TIMPs) in heart failure. *Heart Fail Rev* **17**, 693-706, doi:10.1007/s10741-011-9266-y (2012).

142 Hall, J. E. & Guyton, A. C. in *Guyton and Hall textbook of medical physiology* 101-113 (Saunders/Elsevier, 2011).

143 Drew, R. C. S., L.I. in *Primer on the autonomic nervous system* Ch. 36, 177-180 (Academic Press, 2004).

144 Itani, N., Skeffington, K. L., Beck, C., Niu, Y. & Giussani, D. A. Melatonin rescues cardiovascular dysfunction during hypoxic development in the chick embryo. *J Pineal Res* **60**, 16-26, doi:10.1111/jpi.12283 (2016).

145 Tare, M. *et al.* Maternal melatonin administration mitigates coronary stiffness and endothelial dysfunction, and improves heart resilience to insult in growth restricted lambs. *J Physiol* **592**, 2695-2709, doi:10.1113/jphysiol.2014.270934 (2014).

146 Dhein, S. in *Practical Methods in Cardiovascular Research* (eds Stefan Dhein, Friedrich Wilhelm Mohr, & Mario Delmar) 155-172 (Springer Berlin Heidelberg, 2005).

147 de Leiris, J., Harding, D. P. & Pestre, S. The isolated perfused rat heart: a model for studying myocardial hypoxia or ischaemia. *Basic Res Cardiol* **79**, 313-321 (1984).

148 Storkebaum, E. & Carmeliet, P. Paracrine control of vascular innervation in health and disease. *Acta Physiol (Oxf)* **203**, 61-86, doi:10.1111/j.1748-1716.2011.02333.x (2011).

149 Hill, C. E., Phillips, J. K. & Sandow, S. L. Heterogeneous control of blood flow amongst different vascular beds. *Med Res Rev* **21**, 1-60 (2001).

150 Lombard, J. H. C., A.W. in *Primer on the autonomic nervous system* Ch. 38, xxvii, 459 p. (Academic Press, 2004).

151 Burnstock, G. Autonomic neurotransmission: 60 years since sir Henry Dale. *Annu Rev Pharmacol Toxicol* **49**, 1-30, doi:10.1146/annurev.pharmtox.052808.102215 (2009).

152 Furchgott, R. F. & Zawadzki, J. V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376 (1980).

153 Durand, M. J. & Gutterman, D. D. Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. *Microcirculation* **20**, 239-247, doi:10.1111/micc.12040 (2013).

154 Akata, T. Cellular and molecular mechanisms regulating vascular tone. Part 1: basic mechanisms controlling cytosolic Ca2+ concentration and the Ca2+-dependent regulation of vascular tone. *J Anesth* **21**, 220-231, doi:10.1007/s00540-006-0487-5 (2007).

155 Tousoulis, D., Kampoli, A. M., Tentolouris, C., Papageorgiou, N. & Stefanadis, C. The role of nitric oxide on endothelial function. *Curr Vasc Pharmacol* **10**, 4-18 (2012).

156 Moncada, S. & Vane, J. R. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. *Pharmacol Rev* **30**, 293-331 (1978).

157 Feletou, M. & Vanhoutte, P. M. Endothelium-derived hyperpolarizing factor: where are we now? *Arterioscler Thromb Vasc Biol* **26**, 1215-1225, doi:10.1161/01.ATV.0000217611.81085.c5 (2006).

158 Moncada, S. & Higgs, E. A. Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J* **9**, 1319-1330 (1995).

159 Forstermann, U. *et al.* Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* **23**, 1121-1131 (1994).

160 Forstermann, U., Pollock, J. S., Schmidt, H. H., Heller, M. & Murad, F. Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* **88**, 1788-1792 (1991).

161 Lincoln, T. M., Dey, N. & Sellak, H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol (1985)* **91**, 1421-1430 (2001).

162 Morton, J. S. & Davidge, S. T. Arterial endothelium-derived hyperpolarization: potential role in pregnancy adaptations and complications. *J Cardiovasc Pharmacol* **61**, 197-203, doi:10.1097/FJC.0b013e31827b6367 (2013).

163 Luksha, L., Agewall, S. & Kublickiene, K. Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis* **202**, 330-344, doi:10.1016/j.atherosclerosis.2008.06.008 (2009).

164 Smith, W. L., DeWitt, D. L. & Garavito, R. M. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* **69**, 145-182, doi:10.1146/annurev.biochem.69.1.145 (2000).

165 Miyamoto, T., Ogino, N., Yamamoto, S. & Hayaishi, O. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem* **251**, 2629-2636 (1976).

166 Tsuboi, K., Sugimoto, Y. & Ichikawa, A. Prostanoid receptor subtypes. *Prostaglandins Other Lipid Mediat* **68-69**, 535-556 (2002).

167 Gluais, P., Lonchampt, M., Morrow, J. D., Vanhoutte, P. M. & Feletou, M. Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: the Janus face of prostacyclin. *Br J Pharmacol* **146**, 834-845, doi:10.1038/sj.bjp.0706390 (2005).

168 Feletou, M., Huang, Y. & Vanhoutte, P. M. Vasoconstrictor prostanoids. *Pflugers Arch* **459**, 941-950, doi:10.1007/s00424-010-0812-6 (2010).

169 Parkington, H. C., Coleman, H. A. & Tare, M. Prostacyclin and endothelium-dependent hyperpolarization. *Pharmacol Res* **49**, 509-514, doi:10.1016/j.phrs.2003.11.012 (2004).

170 Majed, B. H. & Khalil, R. A. Molecular mechanisms regulating the vascular prostacyclin pathways and their adaptation during pregnancy and in the newborn. *Pharmacol Rev* **64**, 540-582, doi:10.1124/pr.111.004770 (2012).

171 Wikstrom, K., Kavanagh, D. J., Reid, H. M. & Kinsella, B. T. Differential regulation of RhoA-mediated signaling by the TPalpha and TPbeta isoforms of the human thromboxane A2 receptor: independent modulation of TPalpha signaling by prostacyclin and nitric oxide. *Cell Signal* **20**, 1497-1512, doi:10.1016/j.cellsig.2008.04.006 (2008).

172 Dong, H., Waldron, G. J., Cole, W. C. & Triggle, C. R. Roles of calcium-activated and voltage-gated delayed rectifier potassium channels in endothelium-dependent vasorelaxation of the rabbit middle cerebral artery. *Br J Pharmacol* **123**, 821-832, doi:10.1038/sj.bjp.0701680 (1998).

173 Dumas, M., Dumas, J. P., Rochette, L., Advenier, C. & Giudicelli, J. F. Role of potassium channels and nitric oxide in the effects of iloprost and prostaglandin E1 on hypoxic vasoconstriction in the isolated perfused lung

of the rat. *Br J Pharmacol* **120**, 405-410, doi:10.1038/sj.bjp.0700912 (1997).

174 Tanaka, Y., Yamaki, F., Koike, K. & Toro, L. New insights into the intracellular mechanisms by which PGI2 analogues elicit vascular relaxation: cyclic AMP-independent, Gs-protein mediated-activation of MaxiK channel. *Curr Med Chem Cardiovasc Hematol Agents* **2**, 257-265 (2004).

175 Van Renterghem, C. *et al.* Molecular mechanism of action of the vasoconstrictor peptide endothelin. *Biochem Biophys Res Commun* **157**, 977-985 (1988).

176 Horinouchi, T., Terada, K., Higashi, T. & Miwa, S. Endothelin receptor signaling: new insight into its regulatory mechanisms. *J Pharmacol Sci* **123**, 85-101 (2013).

177 Kashiwabara, T. *et al.* Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect in vivo. *FEBS Lett* **247**, 73-76 (1989).

178 Kishi, F. *et al.* Novel 31-amino-acid-length endothelins cause constriction of vascular smooth muscle. *Biochem Biophys Res Commun* **248**, 387-390, doi:10.1006/bbrc.1998.8980 (1998).

179 Fernandez-Patron, C., Radomski, M. W. & Davidge, S. T. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res* **85**, 906-911 (1999).

180 Hirata, Y. *et al.* Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Invest* **91**, 1367-1373, doi:10.1172/JCI116338 (1993).

181 Filep, J. G., Battistini, B., Cote, Y. P., Beaudoin, A. R. & Sirois, P. Endothelin-1 induces prostacyclin release from bovine aortic endothelial cells. *Biochem Biophys Res Commun* **177**, 171-176 (1991).

182 Davis, M. J. & Hill, M. A. Signaling mechanisms underlying the vascular myogenic response. *Physiol Rev* **79**, 387-423 (1999).

183 Meininger, G. A. & Davis, M. J. Cellular mechanisms involved in the vascular myogenic response. *Am J Physiol* **263**, H647-659 (1992).

184 Gokce, N. *et al.* Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol* **41**, 1769-1775 (2003).

185 Kim, Y. H. *et al.* Chronic hypoxia alters vasoconstrictive responses of femoral artery in the fetal sheep. *J Korean Med Sci* **20**, 13-19, doi:10.3346/jkms.2005.20.1.13 (2005).

186 Nilsson, P. M., Lurbe, E. & Laurent, S. The early life origins of vascular ageing and cardiovascular risk: the EVA syndrome. *J Hypertens* **26**, 1049-1057, doi:10.1097/HJH.0b013e3282f82c3e (2008).

187 Rueda-Clausen, C. F. *et al.* Hypoxia-induced intrauterine growth restriction increases the susceptibility of rats to high-fat diet-induced metabolic syndrome. *Diabetes* **60**, 507-516, doi:10.2337/db10-1239 (2011).

188 Shah, A. *et al.* Effect of resveratrol on metabolic and cardiovascular function in male and female adult offspring exposed to prenatal hypoxia and a high-fat diet. *J Physiol* **594**, 1465-1482, doi:10.1113/JP271133 (2016).

189 Myers, M. L., Bolli, R., Lekich, R. F., Hartley, C. J. & Roberts, R. Enhancement of recovery of myocardial function by oxygen free-radical scavengers after reversible regional ischemia. *Circulation* **72**, 915-921 (1985).

190 Brooks, W. W., Conrad, C. H. & Morgan, J. P. Reperfusion induced arrhythmias following ischaemia in intact rat heart: role of intracellular calcium. *Cardiovasc Res* **29**, 536-542 (1995).

191 Patterson, A. J., Chen, M., Xue, Q., Xiao, D. & Zhang, L. Chronic prenatal hypoxia induces epigenetic programming of PKC{epsilon} gene repression

in rat hearts. *Circ Res* **107**, 365-373, doi:10.1161/CIRCRESAHA.110.221259 (2010).

192 Alexander, B. T., Dasinger, J. H. & Intapad, S. Fetal programming and cardiovascular pathology. *Compr Physiol* **5**, 997-1025, doi:10.1002/cphy.c140036 (2015).

193 Droge, W. Free radicals in the physiological control of cell function. *Physiol Rev* **82**, 47-95, doi:10.1152/physrev.00018.2001 (2002).

194 Fridovich, I. The biology of oxygen radicals. *Science* **201**, 875-880 (1978).

195 Beckman, J. S. & Koppenol, W. H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* **271**, C1424-1437 (1996).

196 Winterbourn, C. C. Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett* **82-83**, 969-974 (1995).

197 Chance, B., Sies, H. & Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59**, 527-605 (1979).

198 Pham-Huy, L. A., He, H. & Pham-Huy, C. Free radicals, antioxidants in disease and health. *Int J Biomed Sci* **4**, 89-96 (2008).

199 Kirkman, H. N. & Gaetani, G. F. Mammalian catalase: a venerable enzyme with new mysteries. *Trends Biochem Sci* **32**, 44-50, doi:10.1016/j.tibs.2006.11.003 (2007).

200 Sabri, A., Hughie, H. H. & Lucchesi, P. A. Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. *Antioxid Redox Signal* **5**, 731-740, doi:10.1089/152308603770380034 (2003).

201 Schaub, M. C., Hefti, M. A., Harder, B. A. & Eppenberger, H. M. Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. *J Mol Med (Berl)* **75**, 901-920 (1997).

202 Sugden, P. H. Signalling pathways in cardiac myocyte hypertrophy. *Ann Med* **33**, 611-622 (2001).

203 Tse, G., Yan, B. P., Chan, Y. W., Tian, X. Y. & Huang, Y. Reactive Oxygen Species, Endoplasmic Reticulum Stress and Mitochondrial Dysfunction: The Link with Cardiac Arrhythmogenesis. *Front Physiol* **7**, 313, doi:10.3389/fphys.2016.00313 (2016).

204 Tse, G. Mechanisms of cardiac arrhythmias. *J Arrhythm* **32**, 75-81, doi:10.1016/j.joa.2015.11.003 (2016).

205 Lu, Z. *et al.* Reactive oxygen species-induced activation of p90 ribosomal S6 kinase prolongs cardiac repolarization through inhibiting outward K+ channel activity. *Circ Res* **103**, 269-278, doi:10.1161/CIRCRESAHA.107.166678 (2008).

206 Ma, J. H., Luo, A. T. & Zhang, P. H. Effect of hydrogen peroxide on persistent sodium current in guinea pig ventricular myocytes. *Acta*

Pharmacol Sin **26**, 828-834, doi:10.1111/j.1745-7254.2005.00154.x (2005).

207 Li, Q., Su, D., O'Rourke, B., Pogwizd, S. M. & Zhou, L. Mitochondria-derived ROS bursts disturb Ca(2)(+) cycling and induce abnormal automaticity in guinea pig cardiomyocytes: a theoretical study. *Am J Physiol Heart Circ Physiol* **308**, H623-636, doi:10.1152/ajpheart.00493.2014 (2015).

208 Venardos, K. M., Perkins, A., Headrick, J. & Kaye, D. M. Myocardial ischemia-reperfusion injury, antioxidant enzyme systems, and selenium: a review. *Curr Med Chem* **14**, 1539-1549 (2007).

209 Hearse, D. J. Ischemia at the crossroads? *Cardiovasc Drugs Ther* **2**, 9-15 (1988).

210 Nohl, H. & Jordan, W. The mitochondrial site of superoxide formation. *Biochem Biophys Res Commun* **138**, 533-539 (1986).

211 Kalogeris, T., Baines, C. P., Krenz, M. & Korthuis, R. J. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol* **298**, 229-317, doi:10.1016/B978-0-12-394309-5.00006-7 (2012).

212 Piper, H. M., Garcia-Dorado, D. & Ovize, M. A fresh look at reperfusion injury. *Cardiovasc Res* **38**, 291-300 (1998).

213 Moens, A. L., Claeys, M. J., Timmermans, J. P. & Vrints, C. J. Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process. *Int J Cardiol* **100**, 179-190, doi:10.1016/j.ijcard.2004.04.013 (2005).

214 Zhou, T., Chuang, C. C. & Zuo, L. Molecular Characterization of Reactive Oxygen Species in Myocardial Ischemia-Reperfusion Injury. *Biomed Res Int* **2015**, 864946, doi:10.1155/2015/864946 (2015).

215 Levraut, J., Iwase, H., Shao, Z. H., Vanden Hoek, T. L. & Schumacker, P. T. Cell death during ischemia: relationship to mitochondrial depolarization and ROS generation. *Am J Physiol Heart Circ Physiol* **284**, H549-558, doi:10.1152/ajpheart.00708.2002 (2003).

216 Hidalgo, C. & Donoso, P. Crosstalk between calcium and redox signaling: from molecular mechanisms to health implications. *Antioxid Redox Signal* **10**, 1275-1312, doi:10.1089/ars.2007.1886 (2008).

217 Zhang, H. *et al.* ROS regulation of microdomain Ca(2+) signalling at the dyads. *Cardiovasc Res* **98**, 248-258, doi:10.1093/cvr/cvt050 (2013).

218 Kaneko, M., Elimban, V. & Dhalla, N. S. Mechanism for depression of heart sarcolemmal Ca2+ pump by oxygen free radicals. *Am J Physiol* **257**, H804-811 (1989).

219 Goldhaber, J. I. Free radicals enhance Na+/Ca2+ exchange in ventricular myocytes. *Am J Physiol* **271**, H823-833 (1996).

220 Viola, H. M., Arthur, P. G. & Hool, L. C. Transient exposure to hydrogen peroxide causes an increase in mitochondria-derived superoxide as a result of sustained alteration in L-type Ca2+ channel function in the absence of apoptosis in ventricular myocytes. *Circ Res* **100**, 1036-1044, doi:10.1161/01.RES.0000263010.19273.48 (2007).

221 Zou, M. H. & Ullrich, V. Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett* **382**, 101-104 (1996).

222 Weber, M., Lauer, N., Mulsch, A. & Kojda, G. The effect of peroxynitrite on the catalytic activity of soluble guanylyl cyclase. *Free Radic Biol Med* **31**, 1360-1367 (2001).

223 Vasquez-Vivar, J. *et al.* Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* **95**, 9220-9225 (1998).

224 Kahler, J. *et al.* Oxidative stress increases synthesis of big endothelin-1 by activation of the endothelin-1 promoter. *J Mol Cell Cardiol* **32**, 1429-1437, doi:10.1006/jmcc.2000.1178 (2000).

225 Duerrschmidt, N., Wippich, N., Goettsch, W., Broemme, H. J. & Morawietz, H. Endothelin-1 induces NAD(P)H oxidase in human endothelial cells. *Biochem Biophys Res Commun* **269**, 713-717, doi:10.1006/bbrc.2000.2354 (2000).

226 Suzuki, Y. J. & Ford, G. D. Superoxide stimulates IP3-induced Ca2+ release from vascular smooth muscle sarcoplasmic reticulum. *Am J Physiol* **262**, H114-116 (1992).

227 Camello-Almaraz, C., Gomez-Pinilla, P. J., Pozo, M. J. & Camello, P. J. Mitochondrial reactive oxygen species and Ca2+ signaling. *Am J Physiol Cell Physiol* **291**, C1082-1088, doi:10.1152/ajpcell.00217.2006 (2006).

228 Harlan, J. M. & Callahan, K. S. Role of hydrogen peroxide in the neutrophil-mediated release of prostacyclin from cultured endothelial cells. *J Clin Invest* **74**, 442-448, doi:10.1172/JCI111440 (1984).

229 Matz, R. L., de Sotomayor, M. A., Schott, C., Stoclet, J. C. & Andriantsitohaina, R. Vascular bed heterogeneity in age-related endothelial dysfunction with respect to NO and eicosanoids. *Br J Pharmacol* **131**, 303-311, doi:10.1038/sj.bjp.0703568 (2000).

230 Vanhoutte, P. M., Feletou, M. & Taddei, S. Endothelium-dependent contractions in hypertension. *Br J Pharmacol* **144**, 449-458, doi:10.1038/sj.bjp.0706042 (2005).

231 Matsumoto, T., Kakami, M., Noguchi, E., Kobayashi, T. & Kamata, K. Imbalance between endothelium-derived relaxing and contracting factors in mesenteric arteries from aged OLETF rats, a model of Type 2 diabetes. *Am J Physiol Heart Circ Physiol* **293**, H1480-1490, doi:10.1152/ajpheart.00229.2007 (2007).

232 Wilson, S. J. *et al.* Activation-dependent stabilization of the human thromboxane receptor: role of reactive oxygen species. *J Lipid Res* **50**, 1047-1056, doi:10.1194/jlr.M800447-JLR200 (2009).

233 Goyal, R. & Longo, L. D. Metabolic Profiles in Ovine Carotid Arteries with Developmental Maturation and Long-Term Hypoxia. *PLoS One* **10**, e0130739, doi:10.1371/journal.pone.0130739 (2015).

234 Rueda-Clausen, C. F. *et al.* Effects of hypoxia-induced intrauterine growth restriction on cardiac siderosis and oxidative stress. *J Dev Orig Health Dis* **3**, 350-357, doi:10.1017/S2040174412000219 (2012).

235 Rumbold, A., Duley, L., Crowther, C. A. & Haslam, R. R. Antioxidants for preventing pre-eclampsia. *Cochrane Database Syst Rev*, CD004227, doi:10.1002/14651858.CD004227.pub3 (2008).

236 Talaulikar, V. & Manyonda, I. Vitamins C and E for the prevention of pre-eclampsia: time to give up the ghost*. *BJOG* **116**, 1416-1417, doi:10.1111/j.1471-0528.2009.02280.x (2009).

237 Chervenak, F. A. & McCullough, L. B. An ethically justified framework for clinical investigation to benefit pregnant and fetal patients. *Am J Bioeth* **11**, 39-49, doi:10.1080/15265161.2011.562595 (2011).

238 Godfrey, K. M., Gluckman, P. D. & Hanson, M. A. Developmental origins of metabolic disease: life course and intergenerational perspectives.

Trends Endocrinol Metab **21**, 199-205, doi:10.1016/j.tem.2009.12.008 (2010).

239 Roth-Cline, M., Gerson, J., Bright, P., Lee, C. S. & Nelson, R. M. Ethical considerations in conducting pediatric research. *Handb Exp Pharmacol* **205**, 219-244, doi:10.1007/978-3-642-20195-0_11 (2011).

240 in *Global Recommendations on Physical Activity for Health WHO Guidelines Approved by the Guidelines Review Committee* (2010).

241 Caspersen, C. J., Powell, K. E. & Christenson, G. M. Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research. *Public Health Rep* **100**, 126-131 (1985).

242 Howley, E. T. Type of activity: resistance, aerobic and leisure versus occupational physical activity. *Med Sci Sports Exerc* **33**, S364-369; discussion S419-320 (2001).

243 Powers, S. K., Quindry, J. C. & Kavazis, A. N. Exercise-induced cardioprotection against myocardial ischemia-reperfusion injury. *Free Radic Biol Med* **44**, 193-201, doi:10.1016/j.freeradbiomed.2007.02.006 (2008).

244 Medicine, A. C. o. S. *ACSM's guidelines for exercise testing and prescription*. (Lippincott Williams & Wilkins, 2013).

245 Gordon, B., Chen, S. & Durstine, J. L. The effects of exercise training on the traditional lipid profile and beyond. *Curr Sports Med Rep* **13**, 253-259, doi:10.1249/JSR.0000000000000073 (2014).

246 Lackland, D. T. & Voeks, J. H. Metabolic syndrome and hypertension: regular exercise as part of lifestyle management. *Curr Hypertens Rep* **16**, 492, doi:10.1007/s11906-014-0492-2 (2014).

247 Eijsvogels, T. M., Molossi, S., Lee, D. C., Emery, M. S. & Thompson, P. D. Exercise at the Extremes: The Amount of Exercise to Reduce Cardiovascular Events. *J Am Coll Cardiol* **67**, 316-329, doi:10.1016/j.jacc.2015.11.034 (2016).

248 Moore, R. L. & Korzick, D. H. Cellular adaptations of the myocardium to chronic exercise. *Prog Cardiovasc Dis* **37**, 371-396 (1995).

249 Wilson, M. G., Ellison, G. M. & Cable, N. T. Basic science behind the cardiovascular benefits of exercise. *Br J Sports Med* **50**, 93-99, doi:10.1136/bjsports-2014-306596rep (2016).

250 Bonaduce, D. *et al.* Intensive training and cardiac autonomic control in high level athletes. *Med Sci Sports Exerc* **30**, 691-696 (1998).

251 Smith, M. L., Hudson, D. L., Graitzer, H. M. & Raven, P. B. Exercise training bradycardia: the role of autonomic balance. *Med Sci Sports Exerc* **21**, 40-44 (1989).

252 Utomi, V. *et al.* Systematic review and meta-analysis of training mode, imaging modality and body size influences on the morphology and function of the male athlete's heart. *Heart* **99**, 1727-1733, doi:10.1136/heartjnl-2012-303465 (2013).

253 Petersen, S. E. *et al.* Sex-specific characteristics of cardiac function, geometry, and mass in young adult elite athletes. *J Magn Reson Imaging* **24**, 297-303, doi:10.1002/jmri.20633 (2006).

254 Levy, W. C., Cerqueira, M. D., Abrass, I. B., Schwartz, R. S. & Stratton, J. R. Endurance exercise training augments diastolic filling at rest and during exercise in healthy young and older men. *Circulation* **88**, 116-126 (1993).

255 Chen, L. *et al.* Effects of exercise training on myocardial fatty acid metabolism in rats with depressed cardiac function induced by transient ischemia. *Jpn Circ J* **65**, 550-555 (2001).

256 Jin, H. *et al.* Effects of exercise training on cardiac function, gene expression, and apoptosis in rats. *Am J Physiol Heart Circ Physiol* **279**, H2994-3002 (2000).

257 Dobrzyn, P. *et al.* Expression of lipogenic genes is upregulated in the heart with exercise training-induced but not pressure overload-induced left ventricular hypertrophy. *Am J Physiol Endocrinol Metab* **304**, E1348-1358, doi:10.1152/ajpendo.00603.2012 (2013).

258 Gertz, E. W., Wisneski, J. A., Stanley, W. C. & Neese, R. A. Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments. *J Clin Invest* **82**, 2017-2025, doi:10.1172/JCI113822 (1988).

259 Bernardo, B. C. & McMullen, J. R. Molecular Aspects of Exercise-induced Cardiac Remodeling. *Cardiol Clin* **34**, 515-530, doi:10.1016/j.ccl.2016.06.002 (2016).

260 Kemi, O. J., Loennechen, J. P., Wisloff, U. & Ellingsen, O. Intensity-controlled treadmill running in mice: cardiac and skeletal muscle hypertrophy. *J Appl Physiol (1985)* **93**, 1301-1309, doi:10.1152/japplphysiol.00231.2002 (2002).

261 Gibb, A. A. *et al.* FVB/NJ Mice Are a Useful Model for Examining Cardiac Adaptations to Treadmill Exercise. *Front Physiol* **7**, 636, doi:10.3389/fphys.2016.00636 (2016).

262 Brown, M. D. Exercise and coronary vascular remodelling in the healthy heart. *Exp Physiol* **88**, 645-658 (2003).

263 Arcos, J. C., Sohal, R. S., Sun, S. C., Argus, M. F. & Burch, G. E. Changes in ultrastructure and respiratory control in mitochondria of rat heart hypertrophied by exercise. *Exp Mol Pathol* **8**, 49-65 (1968).

264 Aldinger, E. E. & Sohal, R. S. Effects of digitoxin on the ultrastructural myocardial changes in the rat subjected to chronic exercise. *Am J Cardiol* **26**, 369-374 (1970).

265 Vettor, R. *et al.* Exercise training boosts eNOS-dependent mitochondrial biogenesis in mouse heart: role in adaptation of glucose metabolism. *Am J Physiol Endocrinol Metab* **306**, E519-528, doi:10.1152/ajpendo.00617.2013 (2014).

266 Crews, J. & Aldinger, E. E. Effect of chronic exercise on myocardial function. *Am Heart J* **74**, 536-542 (1967).

267 Tibbits, G. F., Barnard, R. J., Baldwin, K. M., Cugalj, N. & Roberts, N. K. Influence of exercise on excitation-contraction coupling in rat myocardium. *Am J Physiol* **240**, H472-480 (1981).

268 Tibbits, G., Koziol, B. J., Roberts, N. K., Baldwin, K. M. & Barnard, R. J. Adaptation of the rat myocardium to endurance training. *J Appl Physiol Respir Environ Exerc Physiol* **44**, 85-89 (1978).

269 Suzuki, K. *et al.* Heat shock protein 72 enhances manganese superoxide dismutase activity during myocardial ischemia-reperfusion injury, associated with mitochondrial protection and apoptosis reduction. *Circulation* **106**, I270-276 (2002).

270 Calvert, J. W. *et al.* Exercise protects against myocardial ischemia-reperfusion injury via stimulation of beta(3)-adrenergic receptors and

increased nitric oxide signaling: role of nitrite and nitrosothiols. *Circ Res* **108**, 1448-1458, doi:10.1161/CIRCRESAHA.111.241117 (2011).

271 Kemi, O. J., Ceci, M., Condorelli, G., Smith, G. L. & Wisloff, U. Myocardial sarcoplasmic reticulum Ca2+ ATPase function is increased by aerobic interval training. *Eur J Cardiovasc Prev Rehabil* **15**, 145-148, doi:10.1097/HJR.0b013e3282efd4e0 (2008).

272 Kemi, O. J. *et al.* Aerobic interval training enhances cardiomyocyte contractility and Ca2+ cycling by phosphorylation of CaMKII and Thr-17 of phospholamban. *J Mol Cell Cardiol* **43**, 354-361, doi:10.1016/j.yjmcc.2007.06.013 (2007).

273 French, J. P. *et al.* Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain. *FASEB J* **22**, 2862-2871, doi:10.1096/fj.07-102541 (2008).

274 Powers, S. K., Sollanek, K. J., Wiggs, M. P., Demirel, H. A. & Smuder, A. J. Exercise-induced improvements in myocardial antioxidant capacity: the antioxidant players and cardioprotection. *Free Radic Res* **48**, 43-51, doi:10.3109/10715762.2013.825371 (2014).

275 Campos, J. C., Gomes, K. M. & Ferreira, J. C. Impact of exercise training on redox signaling in cardiovascular diseases. *Food Chem Toxicol* **62**, 107-119, doi:10.1016/j.fct.2013.08.035 (2013).

276 Neuhof, C. & Neuhof, H. Calpain system and its involvement in myocardial ischemia and reperfusion injury. *World J Cardiol* **6**, 638-652, doi:10.4330/wjc.v6.i7.638 (2014).

277 Schuler, G., Adams, V. & Goto, Y. Role of exercise in the prevention of cardiovascular disease: results, mechanisms, and new perspectives. *Eur Heart J* **34**, 1790-1799, doi:10.1093/eurheartj/eht111 (2013).

278 Green, D. J., Maiorana, A., O'Driscoll, G. & Taylor, R. Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiol* **561**, 1-25, doi:10.1113/jphysiol.2004.068197 (2004).

279 Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J. & Hintze, T. H. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res* **74**, 349-353 (1994).

280 Sindler, A. L., Delp, M. D., Reyes, R., Wu, G. & Muller-Delp, J. M. Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles. *J Physiol* **587**, 3885-3897, doi:10.1113/jphysiol.2009.172221 (2009).

281 Wang, J., Wolin, M. S. & Hintze, T. H. Chronic exercise enhances endothelium-mediated dilation of epicardial coronary artery in conscious dogs. *Circ Res* **73**, 829-838 (1993).

282 Rush, J. W., Turk, J. R. & Laughlin, M. H. Exercise training regulates SOD-1 and oxidative stress in porcine aortic endothelium. *Am J Physiol Heart Circ Physiol* **284**, H1378-1387, doi:10.1152/ajpheart.00190.2002 (2003).

283 Adams, V. *et al.* Impact of regular physical activity on the NAD(P)H oxidase and angiotensin receptor system in patients with coronary artery disease. *Circulation* **111**, 555-562, doi:10.1161/01.CIR.0000154560.88933.7E (2005).

284 Gunduz, F. *et al.* Exercise training enhances flow-mediated dilation in spontaneously hypertensive rats. *Physiol Res* **60**, 589-597 (2011).

285 Maeda, S. *et al.* Involvement of endothelin-1 in habitual exercise-induced increase in arterial compliance. *Acta Physiol (Oxf)* **196**, 223-229, doi:10.1111/j.1748-1716.2008.01909.x (2009).

286 Gliemann, L., Nyberg, M. & Hellsten, Y. Nitric oxide and reactive oxygen species in limb vascular function: what is the effect of physical activity? *Free Radic Res* **48**, 71-83, doi:10.3109/10715762.2013.835045 (2014).

287 Zguira, M. S. *et al.* Intense exercise training is not effective to restore the endothelial NO-dependent relaxation in STZ-diabetic rat aorta. *Cardiovasc Diabetol* **12**, 32, doi:10.1186/1475-2840-12-32 (2013).

288 Allen, J. D. *et al.* Diabetes status differentiates endothelial function and plasma nitrite response to exercise stress in peripheral arterial disease

following supervised training. *J Diabetes Complications* **28**, 219-225, doi:10.1016/j.jdiacomp.2013.08.002 (2014).

289 La Gerche, A., Connelly, K. A., Mooney, D. J., MacIsaac, A. I. & Prior, D. L. Biochemical and functional abnormalities of left and right ventricular function after ultra-endurance exercise. *Heart* **94**, 860-866, doi:10.1136/hrt.2006.101063 (2008).

290 Claessen, G. *et al.* Long-term endurance sport is a risk factor for development of lone atrial flutter. *Heart* **97**, 918-922, doi:10.1136/hrt.2010.216150 (2011).

291 Laher, I. *et al.* Short-term exercise worsens cardiac oxidative stress and fibrosis in 8-month-old db/db mice by depleting cardiac glutathione. *Free Radic Res* **47**, 44-54, doi:10.3109/10715762.2012.737463 (2013).

292 Siebel, A. L., Carey, A. L. & Kingwell, B. A. Can exercise training rescue the adverse cardiometabolic effects of low birth weight and prematurity? *Clin Exp Pharmacol Physiol* **39**, 944-957, doi:10.1111/j.1440-1681.2012.05732.x (2012).

293 Rogers, M., Fay, T. B., Whitfield, M. F., Tomlinson, J. & Grunau, R. E. Aerobic capacity, strength, flexibility, and activity level in unimpaired extremely low birth weight (<or=800 g) survivors at 17 years of age compared with term-born control subjects. *Pediatrics* **116**, e58-65, doi:10.1542/peds.2004-1603 (2005).

294 Clemm, H. *et al.* Aerobic capacity and exercise performance in young people born extremely preterm. *Pediatrics* **129**, e97-e105, doi:10.1542/peds.2011-0326 (2012).

295 Chifamba, J., Chakanyuka, K. E., Longo-Mbenza, B., Mahachi, C. B. & Mufunda, J. Comparison of exercise-induced hypertension in low birth weight and normal birth weight young black adults in Zimbabwe. *High Blood Press Cardiovasc Prev* **19**, 123-127, doi:10.2165/11632150-000000000-00000 (2012).

296 Chifamba, J. *et al.* Exercise-induced cardiac fatigue in low and normal birth weight young black adults. *Cardiol Young* **25**, 481-484, doi:10.1017/S1047951114000158 (2015).

297 Miles, J. L., Huber, K., Thompson, N. M., Davison, M. & Breier, B. H. Moderate daily exercise activates metabolic flexibility to prevent prenatally induced obesity. *Endocrinology* **150**, 179-186, doi:10.1210/en.2008-1035 (2009).

298 Laker, R. C. *et al.* Exercise early in life in rats born small does not normalize reductions in skeletal muscle PGC-1alpha in adulthood. *Am J Physiol Endocrinol Metab* **302**, E1221-1230, doi:10.1152/ajpendo.00583.2011 (2012).

299 Laker, R. C. *et al.* Short-term exercise training early in life restores deficits in pancreatic beta-cell mass associated with growth restriction in adult

male rats. *Am J Physiol Endocrinol Metab* **301**, E931-940, doi:10.1152/ajpendo.00114.2011 (2011).

300 Garg, M. *et al.* Early exercise regimen improves insulin sensitivity in the intrauterine growth-restricted adult female rat offspring. *Am J Physiol Endocrinol Metab* **296**, E272-281, doi:10.1152/ajpendo.90473.2008 (2009).

301 Oliveira, V. *et al.* Influence of aerobic training on the reduced vasoconstriction to angiotensin II in rats exposed to intrauterine growth restriction: possible role of oxidative stress and AT2 receptor of angiotensin II. *PLoS One* **9**, e113035, doi:10.1371/journal.pone.0113035 (2014).

302 Wadley, G. D., Laker, R. C., McConell, G. K. & Wlodek, M. E. Endurance training in early life results in long-term programming of heart mass in rats. *Physiol Rep* **4**, doi:10.14814/phy2.12720 (2016).

303 Class, Q. A., Rickert, M. E., Lichtenstein, P. & D'Onofrio, B. M. Birth Weight, Physical Morbidity, and Mortality: A Population-based Sibling-Comparison Study. *Am J Epidemiol* **179**, 550-558, doi:10.1093/aje/kwt304 (2014).

304 Shay, C. M., Gooding, H. S., Murillo, R. & Foraker, R. Understanding and Improving Cardiovascular Health: An Update on the American Heart

Association's Concept of Cardiovascular Health. *Prog Cardiovasc Dis* **58**, 41-49, doi:10.1016/j.pcad.2015.05.003 (2015).

305 Arida, R. M., Scorza, C. A., da Silva, A. V., Scorza, F. A. & Cavalheiro, E. A. Differential effects of spontaneous versus forced exercise in rats on the staining of parvalbumin-positive neurons in the hippocampal formation. *Neurosci Lett* **364**, 135-138, doi:10.1016/j.neulet.2004.03.086 (2004).

306 Leasure, J. L. & Jones, M. Forced and voluntary exercise differentially affect brain and behavior. *Neuroscience* **156**, 456-465, doi:10.1016/j.neuroscience.2008.07.041 (2008).

307 Lesage, J. *et al.* Perinatal malnutrition programs sympathoadrenal and hypothalamic-pituitary-adrenal axis responsiveness to restraint stress in adult male rats. *J Neuroendocrinol* **14**, 135-143 (2002).

308 Jendzjowsky, N. G. & DeLorey, D. S. A prospective evaluation of non-interval- and interval-based exercise training progressions in rodents. *Appl Physiol Nutr Metab* **36**, 723-729, doi:10.1139/h11-092 (2011).

309 Baserga, M. *et al.* Uteroplacental insufficiency affects kidney VEGF expression in a model of IUGR with compensatory glomerular hypertrophy and hypertension. *Early Hum Dev* **85**, 361-367, doi:10.1016/j.earlhumdev.2008.12.015 (2009).

310 Rogers, J. M. *et al.* Elevated blood pressure in offspring of rats exposed to diverse chemicals during pregnancy. *Toxicol Sci* **137**, 436-446, doi:10.1093/toxsci/kft248 (2014).

311 Boaventura, G. *et al.* Effects of running wheel training on adult obese rats programmed by maternal prolactin inhibition. *J Endocrinol* **219**, 29-37, doi:10.1530/JOE-13-0102 (2013).

312 Desai, M., Gayle, D., Han, G. & Ross, M. G. Programmed hyperphagia due to reduced anorexigenic mechanisms in intrauterine growth-restricted offspring. *Reprod Sci* **14**, 329-337, doi:10.1177/1933719107303983 (2007).

313 Fukami, T., Sun, X., Li, T., Desai, M. & Ross, M. G. Mechanism of programmed obesity in intrauterine fetal growth restricted offspring: paradoxically enhanced appetite stimulation in fed and fasting states. *Reprod Sci* **19**, 423-430, doi:10.1177/1933719111424448 (2012).

314 Wang, X. *et al.* Temporal proteomic analysis reveals defects in small-intestinal development of porcine fetuses with intrauterine growth restriction. *J Nutr Biochem* **25**, 785-795, doi:10.1016/j.jnutbio.2014.03.008 (2014).

315 Payne, J. A., Alexander, B. T. & Khalil, R. A. Reduced endothelial vascular relaxation in growth-restricted offspring of pregnant rats with reduced uterine perfusion. *Hypertension* **42**, 768-774, doi:10.1161/01.HYP.0000084990.88147.0C (2003).

316 Mazzuca, M. Q., Wlodek, M. E., Dragomir, N. M., Parkington, H. C. & Tare, M. Uteroplacental insufficiency programs regional vascular dysfunction and alters arterial stiffness in female offspring. *J Physiol* **588**, 1997-2010, doi:10.1113/jphysiol.2010.187849 (2010).

317 Thakor, A. S., Herrera, E. A., Seron-Ferre, M. & Giussani, D. A. Melatonin and vitamin C increase umbilical blood flow via nitric oxide-dependent mechanisms. *J Pineal Res* **49**, 399-406, doi:10.1111/j.1600-079X.2010.00813.x (2010).

318 Kane, A. D., Herrera, E. A., Hansell, J. A. & Giussani, D. A. Statin treatment depresses the fetal defence to acute hypoxia via increasing nitric oxide bioavailability. *J Physiol* **590**, 323-334, doi:10.1113/jphysiol.2011.217968 (2012).

319 Herrera, E. A. *et al.* A role for xanthine oxidase in the control of fetal cardiovascular function in late gestation sheep. *J Physiol* **590**, 1825-1837, doi:10.1113/jphysiol.2011.224576 (2012).

320 Batinic-Haberle, I. *et al.* Pure MnTBAP selectively scavenges peroxynitrite over superoxide: comparison of pure and commercial MnTBAP samples to MnTE-2-PyP in two models of oxidative stress injury, an SOD-specific Escherichia coli model and carrageenan-induced pleurisy. *Free Radic Biol Med* **46**, 192-201, doi:10.1016/j.freeradbiomed.2008.09.042 (2009).

321 Stewart, K. G., Zhang, Y. & Davidge, S. T. Aging increases PGHS-2-dependent vasoconstriction in rat mesenteric arteries. *Hypertension* **35**, 1242-1247 (2000).

322 Ozaki, T., Nishina, H., Hanson, M. A. & Poston, L. Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. *J Physiol* **530**, 141-152 (2001).

323 Mazzuca, M. Q. *et al.* Uteroplacental insufficiency programmes vascular dysfunction in non-pregnant rats: compensatory adaptations in pregnancy. *J Physiol* **590**, 3375-3388, doi:10.1113/jphysiol.2012.230011 (2012).

324 Yen, M. H., Yang, J. H., Sheu, J. R., Lee, Y. M. & Ding, Y. A. Chronic exercise enhances endothelium-mediated dilation in spontaneously hypertensive rats. *Life Sci* **57**, 2205-2213 (1995).

325 Minami, A. *et al.* Exercise training improves acetylcholine-induced endothelium-dependent hyperpolarization in type 2 diabetic rats, Otsuka Long-Evans Tokushima fatty rats. *Atherosclerosis* **162**, 85-92 (2002).

326 Mokelke, E. A. *et al.* Altered functional coupling of coronary K+ channels in diabetic dyslipidemic pigs is prevented by exercise. *J Appl Physiol (1985)* **95**, 1179-1193, doi:10.1152/japplphysiol.00972.2002 (2003).

327 Milkau, M., Kohler, R. & de Wit, C. Crucial importance of the endothelial K+ channel SK3 and connexin40 in arteriolar dilations during skeletal

muscle contraction. *FASEB J* **24**, 3572-3579, doi:10.1096/fj.10-158956 (2010).

328 Reyes, L. M., Morton, J. S., Kirschenman, R., DeLorey, D. S. & Davidge, S. T. Vascular effects of aerobic exercise training in rat adult offspring exposed to hypoxia-induced intrauterine growth restriction. *J Physiol*, doi:10.1113/jphysiol.2014.288449 (2015).

329 Xu, Y. *et al. AT1 and AT2 receptor expression and blockade after acute ischemia-reperfusion in isolated working rat hearts*. Vol. 282 (2002).

330 Galland, B. C., Taylor, B. J., Bolton, D. P. & Sayers, R. M. Heart rate variability and cardiac reflexes in small for gestational age infants. *J Appl Physiol (1985)* **100**, 933-939, doi:10.1152/japplphysiol.01275.2005 (2006).

331 Danielson, L., McMillen, I. C., Dyer, J. L. & Morrison, J. L. Restriction of placental growth results in greater hypotensive response to alpha-adrenergic blockade in fetal sheep during late gestation. *J Physiol* **563**, 611-620, doi:10.1113/jphysiol.2004.080523 (2005).

332 Duncker, D. J., Haitsma, D. B., Liem, D. A., Verdouw, P. D. & Merkus, D. Exercise unmasks autonomic dysfunction in swine with a recent myocardial infarction. *Cardiovasc Res* **65**, 889-896, doi:10.1016/j.cardiores.2004.12.010 (2005).

333 Tsutsumi, T. *et al.* Modulation of the myocardial redox state by vagal nerve stimulation after experimental myocardial infarction. *Cardiovasc Res* **77**, 713-721, doi:10.1093/cvr/cvm092 (2008).

334 Telford, R. D. *et al.* Echocardiographic dimensions in trained and untrained 12-year-old boys and girls. *J Sports Sci* **6**, 49-57, doi:10.1080/02640418808729793 (1988).

335 Barbier, J., Ville, N., Kervio, G., Walther, G. & Carre, F. Sports-specific features of athlete's heart and their relation to echocardiographic parameters. *Herz* **31**, 531-543, doi:10.1007/s00059-006-2862-2 (2006).

336 McDonald, M. W., Hall, K. E., Jiang, M., Noble, E. G. & Melling, C. W. Ischemia-reperfusion injury and hypoglycemia risk in insulin-treated T1DM rats following different modalities of regular exercise. *Physiol Rep* **2**, doi:10.14814/phy2.12201 (2014).

337 Oparil, S., Bishop, S. P. & Clubb, F. J., Jr. Myocardial cell hypertrophy or hyperplasia. *Hypertension* **6**, III38-43 (1984).

338 Ponnusamy, M., Li, P. F. & Wang, K. Understanding cardiomyocyte proliferation: an insight into cell cycle activity. *Cell Mol Life Sci*, doi:10.1007/s00018-016-2375-y (2016).

339 Mustonen, E. *et al.* Tumour necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor Fn14 during cardiac remodelling in rats. *Acta Physiol (Oxf)* **199**, 11-22, doi:10.1111/j.1748-1716.2010.02080.x (2010).

340 Chorianopoulos, E. *et al.* FGF-inducible 14-kDa protein (Fn14) is regulated via the RhoA/ROCK kinase pathway in cardiomyocytes and mediates nuclear factor-kappaB activation by TWEAK. *Basic Res Cardiol* **105**, 301-313, doi:10.1007/s00395-009-0046-y (2010).

341 Novoyatleva, T., Janssen, W., Wietelmann, A., Schermuly, R. T. & Engel, F. B. TWEAK/Fn14 axis is a positive regulator of cardiac hypertrophy. *Cytokine* **64**, 43-45, doi:10.1016/j.cyto.2013.05.009 (2013).

342 Jain, M. *et al.* A novel role for tumor necrosis factor-like weak inducer of apoptosis (TWEAK) in the development of cardiac dysfunction and failure. *Circulation* **119**, 2058-2068, doi:10.1161/CIRCULATIONAHA.108.837286 (2009).

343 Shi, J. *et al.* PGC1alpha plays a critical role in TWEAK-induced cardiac dysfunction. *PLoS One* **8**, e54054, doi:10.1371/journal.pone.0054054 (2013).

344 Sun, L. *et al.* Cardioprotection by acetylcholine: a novel mechanism via mitochondrial biogenesis and function involving the PGC-1alpha pathway. *J Cell Physiol* **228**, 1238-1248, doi:10.1002/jcp.24277 (2013).

345 Barker, D. J. *et al.* Fetal nutrition and cardiovascular disease in adult life. *Lancet* **341**, 938-941 (1993).

346 Kantor, P. F., Dyck, J. R. & Lopaschuk, G. D. Fatty acid oxidation in the reperfused ischemic heart. *Am J Med Sci* **318**, 3-14 (1999).

347 Bartkevics, M. *et al.* Efficacy of mechanical postconditioning following warm, global ischaemia depends on circulating fatty acid levels in an isolated, working rat heart modeldagger. *Eur J Cardiothorac Surg* **49**, 32-39, doi:10.1093/ejcts/ezv008 (2016).

348 Frasier, C. R., Moore, R. L. & Brown, D. A. Exercise-induced cardiac preconditioning: how exercise protects your achy-breaky heart. *J Appl Physiol (1985)* **111**, 905-915, doi:10.1152/japplphysiol.00004.2011 (2011).

349 Ji, L. L., Gomez-Cabrera, M. C. & Vina, J. Exercise and hormesis: activation of cellular antioxidant signaling pathway. *Ann N Y Acad Sci* **1067**, 425-435, doi:10.1196/annals.1354.061 (2006).

350 Radak, Z., Chung, H. Y., Koltai, E., Taylor, A. W. & Goto, S. Exercise, oxidative stress and hormesis. *Ageing Res Rev* **7**, 34-42, doi:10.1016/j.arr.2007.04.004 (2008).

351 Wilmore, J. H. *et al.* Relationship of changes in maximal and submaximal aerobic fitness to changes in cardiovascular disease and non-insulin-dependent diabetes mellitus risk factors with endurance training: the HERITAGE Family Study. *Metabolism* **50**, 1255-1263 (2001).

352 Hanson, M., Godfrey, K. M., Lillycrop, K. A., Burdge, G. C. & Gluckman, P. D. Developmental plasticity and developmental origins of non-communicable disease: theoretical considerations and epigenetic

mechanisms. *Prog Biophys Mol Biol* **106**, 272-280, doi:10.1016/j.pbiomolbio.2010.12.008 (2011).

353 Sengupta, P. The Laboratory Rat: Relating Its Age With Human's. *Int J Prev Med* **4**, 624-630 (2013).

354 Chen, M., Xiong, F. & Zhang, L. Promoter methylation of Egr-1 site contributes to fetal hypoxia-mediated PKCepsilon gene repression in the developing heart. *Am J Physiol Regul Integr Comp Physiol* **304**, R683-689, doi:10.1152/ajpregu.00461.2012 (2013).

355 Marcoux, S., Brisson, J. & Fabia, J. The effect of leisure time physical activity on the risk of pre-eclampsia and gestational hypertension. *J Epidemiol Community Health* **43**, 147-152 (1989).

356 Dempsey, J. C. *et al.* A case-control study of maternal recreational physical activity and risk of gestational diabetes mellitus. *Diabetes Res Clin Pract* **66**, 203-215, doi:10.1016/j.diabres.2004.03.010 (2004).

357 Muktabhant, B., Lawrie, T. A., Lumbiganon, P. & Laopaiboon, M. Diet or exercise, or both, for preventing excessive weight gain in pregnancy. *Cochrane Database Syst Rev*, CD007145, doi:10.1002/14651858.CD007145.pub3 (2015).

358 Moyer, C., Reoyo, O. R. & May, L. The Influence of Prenatal Exercise on Offspring Health: A Review. *Clin Med Insights Womens Health* **9**, 37-42, doi:10.4137/CMWH.S34670 (2016).

359 Vega, C. C. *et al.* Exercise in obese female rats has beneficial effects on maternal and male and female offspring metabolism. *Int J Obes (Lond)*, doi:10.1038/ijo.2013.150 (2013).

360 Volpato, G. T. *et al.* Oxidative stress status and placental implications in diabetic rats undergoing swimming exercise after embryonic implantation. *Reprod Sci* **22**, 602-608, doi:10.1177/1933719114556485 (2015).

361 Mueller, P. J. Exercise training and sympathetic nervous system activity: evidence for physical activity dependent neural plasticity. *Clin Exp Pharmacol Physiol* **34**, 377-384, doi:10.1111/j.1440-1681.2007.04590.x (2007).

362 Carter, J. R. & Ray, C. A. Sympathetic neural adaptations to exercise training in humans. *Auton Neurosci* **188**, 36-43, doi:10.1016/j.autneu.2014.10.020 (2015).

363 Hsu, Y. C. *et al.* Chronic treadmill running in normotensive rats resets the resting blood pressure to lower levels by upregulating the hypothalamic GABAergic system. *J Hypertens* **29**, 2339-2348, doi:10.1097/HJH.0b013e32834c628f (2011).

364 Fisher, J. P., Kim, A., Hartwich, D. & Fadel, P. J. New insights into the effects of age and sex on arterial baroreflex function at rest and during dynamic exercise in humans. *Auton Neurosci* **172**, 13-22, doi:10.1016/j.autneu.2012.10.013 (2012).

365 McElroy, C. L., Gissen, S. A. & Fishbein, M. C. Exercise-induced reduction in myocardial infarct size after coronary artery occlusion in the rat. *Circulation* **57**, 958-962 (1978).

366 Bersohn, M. M. & Scheuer, J. Effect of ischemia on the performance of hearts from physically trained rats. *Am J Physiol* **234**, H215-218 (1978).

367 Bowles, D. K., Farrar, R. P. & Starnes, J. W. Exercise training improves cardiac function after ischemia in the isolated, working rat heart. *Am J Physiol* **263**, H804-809 (1992).

368 Vidavalur, R., Swarnakar, S., Thirunavukkarasu, M., Samuel, S. M. & Maulik, N. Ex vivo and in vivo approaches to study mechanisms of cardioprotection targeting ischemia/reperfusion (i/r) injury: useful techniques for cardiovascular drug discovery. *Curr Drug Discov Technol* **5**, 269-278 (2008).

369 Prabhu, S. D. & Frangogiannis, N. G. The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. *Circ Res* **119**, 91-112, doi:10.1161/CIRCRESAHA.116.303577 (2016).

370 Dobaczewski, M., Gonzalez-Quesada, C. & Frangogiannis, N. G. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. *J Mol Cell Cardiol* **48**, 504-511, doi:10.1016/j.yjmcc.2009.07.015 (2010).

371 Frangogiannis, N. G., Smith, C. W. & Entman, M. L. The inflammatory response in myocardial infarction. *Cardiovasc Res* **53**, 31-47 (2002).

372 Droste, S. K. *et al.* Effects of long-term voluntary exercise on the mouse hypothalamic-pituitary-adrenocortical axis. *Endocrinology* **144**, 3012-3023, doi:10.1210/en.2003-0097 (2003).

373 Brown, D. A. *et al.* Short-term treadmill running in the rat: what kind of stressor is it? *J Appl Physiol (1985)* **103**, 1979-1985, doi:10.1152/japplphysiol.00706.2007 (2007).

374 Gerdes, J. *et al.* Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* **133**, 1710-1715 (1984).

375 Gratzner, H. G. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* **218**, 474-475 (1982).

376 van Dierendonck, J. H., Wijsman, J. H., Keijzer, R., van de Velde, C. J. & Cornelisse, C. J. Cell-cycle-related staining patterns of anti-proliferating cell nuclear antigen monoclonal antibodies. Comparison with BrdUrd labeling and Ki-67 staining. *Am J Pathol* **138**, 1165-1172 (1991).

377 Drewinko, B., Yang, L. Y., Barlogie, B. & Trujillo, J. M. Cultured human tumour cells may be arrested in all stages of the cycle during stationary

phase: demonstration of quiescent cells in G1, S and G2 phase. *Cell Tissue Kinet* **17**, 453-463 (1984).

378 Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* **182**, 311-322, doi:10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9 (2000).