Developmental Programming of Cardiac and Coronary Artery Dysfunction in Adult Male and Female Offspring Exposed to Prenatal Hypoxia

by

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

Doctor of Philosophy

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ABSTARCT

Cardiovascular disease is a leading cause of global mortality and a major contributor to morbidity worldwide. Early epidemiological studies demonstrated that pregnancy complications can impact the health of the offspring in adult life. Fetal hypoxia is one of the most common complications of pregnancy that leads to changes in fetal cardiovascular structure and function and predisposes the offspring to the development of cardiovascular dysfunction in later life; however, the mechanisms remain to be fully elucidated. This PhD thesis focussed on assessing the effect of prenatal hypoxia on offspring cardiovascular function in adult life with a focus on the endothelin-1 (ET-1) system. Moreover, prenatal hypoxia-induced placental oxidative stress contributes to fetal pathophysiology and subsequently predisposes the offspring to the development of cardiovascular dysfunction. Considering that treatment strategies during pregnancy can be complicated by the potential off-target adverse effects on the fetus, our laboratory has been exploring a placenta-targeted therapeutic strategy based on nanoparticle encapsulation of a mitochondrial-targeted antioxidant (nMitoQ). I also aimed to assess the long-term impact of a placenta-targeted treatment strategy during the hypoxic pregnancy on cardiac susceptibility of adult offspring to cardiac ischemia/reperfusion (I/R) injury in adult life. **I hypothesized** that the ET-1 system contributes to the development of cardiac and coronary artery dysfunction in adult prenatally-exposed hypoxic offspring. Also, prenatal nMitoQ treatment during a hypoxic pregnancy has a long-term beneficial effect on cardiac capacity to tolerate I/R insult in adult offspring.

To assess the effect of prenatal hypoxia on offspring cardiovascular function, pregnant Sprague–Dawley rats were divided into two groups: normoxic controls (housed at atmospheric oxygen throughout pregnancy: 21% O_2) or hypoxic dams (exposed to hypoxia from gestational day (GD) 15–21 by placing them in a hypoxic chamber: 11% O_2). The offspring born from those pregnancies were aged to 4- or 9.5 month of age. Cardiac function was assessed using isolated working heart preparation in 4-month-old offspring, while

coronary artery function was assessed with wire myography in 4- and ~9.5-month-old offspring. To assess the long-term effect of maternal treatment with nMitoQ on offspring cardiac susceptibility to I/R, pregnant Sprague–Dawley rats were intravenously injected via the tail vein with 100 µL of either saline or nMitoQ (125 µmol/L) on GD 15. Rats injected with nMitoQ or saline were then exposed to either hypoxia (11% O_2) from GD 15–21, or were housed at 21% O_2 throughout pregnancy. Cardiac function of 4-month-old offspring was assessed using isolated working heart preparation.

Prenatal hypoxia was associated with reduced cardiac levels of ET B receptor (ET_B) in female offspring, without changes in males. Infusion of ABT-627 (ET_A antagonist) before I/R insult tended to improve post-I/R recovery in prenatal hypoxia females; but surprisingly, ABT-627 prevented post-I/R recovery in the prenatal hypoxia males. In coronary arteries, at 4 months of age, constrictor responses to exogenous ET-1 were similar between groups. However, ET-1 levels were increased, and ET_B inhibition (with BQ788) tended to decrease ET-1-mediated responses in only prenatal hypoxic females. At 9.5 months of age, ET-1-mediated responses were decreased in only prenatal hypoxic females; with no effect with BQ788. Notably, prenatal hypoxia impaired endothelium-dependent vasodilation in both male and female offspring at 4- and 9.5-months of age that was attributed to an increased prostaglandin H synthase (PGHS)-dependent vasoconstriction.

Maternal nMitoQ treatment improved cardiac tolerance to an I/R insult in both male and female adult offspring born from pregnancies complicated with prenatal hypoxia; however, the calcium regulating mechanisms were sex-specific. In prenatally hypoxia male offspring, maternal nMitoQ treatment increased phospholamban (PLN) levels, tended to decrease phosphorylation of Ca^{2+}/calmodulin kinase δ and increased levels of protein phosphatase 2Ce. In female offspring born from hypoxic pregnancies, maternal nMitoQ resulted in increased phosphorylation of PLN and protein kinase Cε.

My studies suggests that prenatal hypoxia impacted the ET-1 pathway with sex specific differences in cardiac and coronary artery function. I also demonstrated that prenatal hypoxia impaired endothelial-dependent vasodilation in coronary arteries via a PGHS-dependent pathway. Maternal treatment strategies targeted against placental oxidative stress can improve offspring cardiac capacity to tolerate I/R insult by impacting the intracellular mechanisms of calcium cycling. Overall, these data show that understanding the impact of prenatal environment is necessary for precise treatment approaches throughout the lifecourse.

PREFACE

This thesis is an original work by Nataliia Hula. The research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, AUP #242 and #3692.

The research presented in the Chapter 5 of this thesis were conducted as part of an international research collaboration, led by Dr. Patrick C. Case at the University of Bristol, with Dr. Sandra T. Davidge being the lead collaborator at the University of Alberta. The University of Bristol has submitted a patent application for the nanoparticle formulation. The literature review in Chapter 1, the data presented in Chapters 3, 4, and 5 and the discussion section in the Chapter 6 are my original work.

A version of Chapter 3 of this thesis has been published as "*Hula, N., Vu J., Quon A., Kirschenman R., Spaans F., Liu R., Cooke C. M., and Davidge S. T. "Sex-Specific Effects of Prenatal Hypoxia on the Cardiac Endothelin System in Adult Offspring." American Journal of Physiology - Heart and Circulatory Physiology 322, no. 3 (Mar 2022): H442-H50."* Hula N. was responsible for concept formulation, the data collection and analysis as well as the manuscript composition; Vu J, contributed to data collection and analysis as well as the manuscript composition; Quon A., Kirschenman R. assisted with the data collection and contributed to manuscript edits; Spaans F. contributed to concept formulation and manuscript composition; Liu R. contributed to manuscript edits; Cooke C. M. contributed to manuscript edits; Davidge S. T. was the supervisory author and was involved in concept formulation and manuscript composition.

Chapter 4 of this thesis has been published as "*Hula, N., Liu R., Spaans F., Pasha M., Quon A., Kirschenman R., Cooke C. M., and Davidge S. T. "The Long-Term Effects of Prenatal Hypoxia on Coronary Artery Function of the Male and Female Offspring." Biomedicines 10, no. 12 (Nov 2022): 3019.* Hula N. was responsible for concept formulation, the data collection

and analysis as well as the manuscript composition; Liu R. contributed to data collection and analysis as well as the manuscript composition; Spaans F. contributed to concept formulation and manuscript composition; Pasha M. contributed to manuscript edits and data interpretation; Quon A. contributed to manuscript edits and data interpretation; Kirschenman R. assisted with the data collection and contributed to manuscript edits; Cooke C. M. contributed to concept formulation and manuscript edits; Davidge S. T. was the supervisory author and was involved in concept formulation and manuscript composition.

Chapter 5 of this thesis was published as *"Hula, N., Spaans F., Vu J., Quon A., Kirschenman R., Cooke C. M., Phillips T. J., Case C. P., and Davidge S. T. "Placental Treatment Improves Cardiac Tolerance to Ischemia/Reperfusion Insult in Adult Male and Female Offspring Exposed to Prenatal Hypoxia." Pharmacological Research 165 (Mar 2021): 105461."* Hula N. was responsible for concept formulation, the data collection and analysis as well as the manuscript composition; Spaans F. contributed to concept formulation and manuscript composition; Vu J, contributed to data collection and analysis as well as the manuscript composition; Quon A. and Kirschenman R. assisted with the data collection and contributed to manuscript edits; Cooke C. M. contributed to concept formulation and manuscript edits; Phillips T.J. and Case C.P provided the study materials and contributed to the manuscript edits; Davidge S. T. was the supervisory author and was involved in concept formulation and manuscript composition.

DEDICATION

This thesis is dedicated to my mom, dad, my brother and my grandparents.

ACKNOWLEDGMENTS

I believe that your success is the result of the combined efforts of yourself, and the efforts and support of people who were with you during that time.

I would like to take a chance to express my gratitude to my supervisor, Dr. Sandra Davidge for giving me this incredible opportunity to join her research group, a continuous trust and support of me during those years. Thank you for your patience, enthusiasm, and kindness during this time. I would like to thank Louanne Campbell (and Jim) for being amazing friends that always supported me, helped me, and gave me a guidance in many aspects of life. I would like to thank Dr. Jude Morton, Dr. Christy-Lynn Cooke for their support during my PhD. Also, I would like to thank Dr. Floor Spaans for her full-time (and overtime) help, support, and assistance with my studies, and more.

I would like to thank Dr. Alexander S. Khromov, Dr. Nataliya V. Dobrelya and Dr. Ievgen V. Strielkov for their support and inspiration at the beginning of my path in science and throughout all those years, their friendship and sincere love.

I would like to acknowledge my supervisory committee members, Dr. Stephane Bourque and Dr. Zamaneh Kassiri for their feedback, suggestions and thoughtful guidance during my PhD program. Thank you for your support during those years.

I am grateful to Anita Quon and Raven Kirschenman for their technical and overall support during the implementation of my studies into life. Thank you for sharing your expertise and skills with me throughout those years and making my experience in molecular analysis and work with animals possible and exciting.

I would like to recognize my lab mates for encouraging me, helping me, and sharing their knowledge and experience with me, and overall, being amazing friends and a team during those years: Esha, Tamara, Mazhar, Roberto, Amy, Mais, Murilo, Amanda and Paulami. Thank you for being with me during this time.

Also, I would like to express thanks to my summer/project students, Jennie and Ricky, for an amazing experience in the supervision and an overall, a fun time together.

Also, I would like to recognize my mom (Tatyana) and dad (Sergij), my brother (Vladimir) and his wife (Ania) and my grandparents for their motivation, support and encouragements during my path and their unconditional love.

I would also like to acknowledge the support of the following funding agencies that have supported my PhD work: the Women and Children's Health Research Institute (WCHRI), Canadian Institute of Health Research (CIHR), Faculty of Graduate Studies and Research (FGSR), Faculty of Medicine and Dentistry, Department of Physiology, and Department of Obstetrics and Gynecology.

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LIST OF ABBREVIATIONS

The following abbreviations have been used in the thesis.

Abbreviation	Meaning
ABT-627	Atrasentan hydrochloride
AC	Adenylyl cyclase
ACC	Acetyl-CoA carboxylase
ACh	Acetylcholine
AMPK	AMP-activated protein kinase
Ang II	Angiotensin II
ATP	Adenosine triphosphate
ATR	Ang II receptor
AUC	Area under the curve
BKCa	Large conductance Ca^{2+}-activated K^{+} channels
BSA	Bovine serum albumin
CaMK	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
Cat	Catalase
CF	Coronary flow
cGMP	Cyclic guanosine-3’,5’-monophosphate
CHD	Coronary heart disease
CK	Creatine kinase
CPI-17	Protein kinase C-potentiated myosin phosphatase inhibitor of 17 kDa
CV	Cardiovascular
DAG	Diacylglycerol

DAPI	4’,6’-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DOCA	Deoxycorticosterone acetate
DOHaD	Developmental Programming of Health and Disease
dP/d_{tmax}	Maximum derivative of change in systolic pressure over time
dP/dt_{min}	Minimum derivative of change in diastolic pressure over time
ECC	Excitation contraction coupling
ECE	Endothelin converting enzyme
EDCR	Endothelium-derived contracting factors
EDH	Endothelium-derived hyperpolarization
EDRF	Endothelium-derived relaxing factors
EDTA	Ethylenediaminetetraacetic acid
Egr-1	Early growth response factor-1
ELISA	Enzyme linked immunosorbent assay
E_{max}	Maximum relaxation response
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
ET-1	Endothelin-1
ET_A	Endothelin A receptor
ET_B	Endothelin B receptor
FABPpm	Fatty acid binding protein
FACS	Fatty acyl-CoA synthase
FATP	Fatty acid transport protein
FBF Amp	Fall in the amplitude of femoral blood flow
GLUT	Glucose transporter

GPCR	G-protein-coupled receptor
Gpx1	Glutathione peroxidase 1
GR	Glucocorticoid receptor
G_S	G stimulatory subunit
GTP	Guanosine-5′-triphosphate
H_2O_2	Hydrogen peroxide
HIF	Hypoxia-inducible factor
HR	Heart rate
Hsp70	Heat shock protein 70
I/R	Ischemia/reperfusion
IGF-IR	Insulin-like growth factor 1 receptor
IHD	Ischemic heart disease
IK_{Ca}	Intermediate-conductance Ca^{2+}-activated K^+ channel
IP	Cell surface PGI receptor
IP_3	Inositol-(1,4,5)-triphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IRE1	Inositol requiring enzyme 1
IVRT	Isovolumic relaxation time
JNK	c–Jun N–terminal kinase
K_{IR}	Inward rectify K^+-channels
L-NAME	Pan NOS inhibitor N(G)-nitro-L-arginine methyl ester hydrochloride
L-PAE	Phenylalanine ethyl ester
LAD	Left anterior descending artery
LDH	Lactate dehydrogenase
LTCC	L-type calcium channels
LV	Left ventricle

LVDP	Left ventricle developed pressure
LVEDP	Left ventricular end diastolic pressure
MAPK	Mitogen- activated protein kinase
MCh	Methylcholine
MEGJ	Myoendothelial gap junctions
MHC	Myosin heavy chain
MitoQ	Mitochondria-targeted ubiquinone
MLCP	Myosin light chain phosphatase
MMP-2	Matrix metalloproteinase-2
MVO2	Myocardial oxygen consumption
NADPH	Nicotinamide adenine dinucleotide phosphate
NCX	Na^+/Ca^{2+} exchanger
Nfe2l2	Nuclear factor erythroid 2-related factor 2
NHE	Na^+/H^+ exchanger
NO	Nitric oxide
O_2^-	Superoxide radical anion
OH•	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
p90RSK	90–kDa ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PBST	PBS with 0.1% Tween 20
PE	Phenylephrine
pEC_{20}	A negative log of the mean effective concentration that produces 20% of the maximal response
pEC_{50}	A negative log of the mean effective concentration that produces 50% of the maximal response

PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H synthase
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3'-OH kinase
PIP	Polyphosphoinositide
PKA	Protein kinase A
PKCδ	Protein kinase C delta
PKCε	Protein kinase C epsilon
PKG	Protein kinase G
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PP2Ce	Protein phosphatase 2C epsilon
PPARα	Peroxisome proliferator-activated receptor alpha
PSP	Peak systolic pressure
PSS	Physiological salt solution
RIPA	Radioimmunoprecipitation assay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPP	Heart rate-pressure product
RyR2	Ryanodine receptors type 2
SBP	Systolic blood pressure
SERCA2α	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SK_{Ca}	Small-conductance Ca^{2+}-activated K^+ channels
SNP	Sodium nitroprusside
SP1	Specificity protein 1
SR	Sarcoplasmic reticulum

Tm	Tropomyosin
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
TP	Thromboxane /prostaglandin receptor
TPP	Triphenylphosphonium
TT	Transverse tubules
TxA_2	Thromboxane A2
U46619	9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F_{2a}
UPR	Unfolded protein response
Vegf	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
β-AR	β-adrenergic receptor
γ-PGA	Poly γ-glutamic acid

CHAPTER 1

GENERAL INTRODUCTION

Pregnancy-related complications has been widely recognized as a critical factor that negatively impacts fetal development and affects the health and lifespan of the population worldwide. Fetal growth restriction is defined as a pathological inhibition of the fetal growth *in utero* and the failure to achieve its growth and developmental potential [1]. Various conditions experienced during pregnancy, including (but not limited to) hypertension [2], gestational diabetes [3], preeclampsia [4], obesity [5], maternal undernutrition [6] liver and kidney diseases [7,8], iron deficiency [9,10] lead to the fetal hypoxia, which is a common consequence of complicated pregnancies. Previous research has demonstrated that hypoxia, experienced during the prenatal life, impacts fetal cardiovascular (CV) development, thereby predisposing the offspring to development of CV dysfunction in adult life. My thesis is focused on understanding the impact of fetal hypoxia on cardiac and coronary artery function of the offspring in adult life.

1.1. Developmental Programming of Health and Disease (DOHaD)

The whole concept of "fetal programming" was first proposed by David Barker who demonstrated a strong association between disproportional fetal (human) growth (as a result of fetal undernutrition in the middle to late gestation) and predisposition to the development of coronary heart disease (CHD) [11]. In particular, Barker and his colleagues traced a population of men born in Hertfordshire during 1911-1930s and showed that those, whose weight in infancy (one-year-old) were 18 pounds or less had three times higher death rate from ischemic heart disease (IHD) compared to those who weighed 27 pounds or more [11]. Similar findings were later reported by Stein (in his study of Indian men and woman born between 1934 and 1954 [12]), Leon (in the cohort study of Swedish men and women born 1915-29 [13]), Forsén (in the cohort study of Helsinki woman born during 1924-33 [14]) and Eriksson (in longitudinal study of Helsinki men born during 1934-44 [15]). Mainly, a low birth weight, short birth length, small head circumference at birth and low ponderal index (birth weight/length^3; used to identify infants whose soft tissue mass is below normal for the stage

of skeletal development [16]) were associated with increased risk of development of CHD [12,15], while an increase in birth weight correlated with a proportional reduction in the rate of IHD and CHD [13,14]. Thus, it was proposed that among the recognized core health behaviors that are known to impair CV health (such as smoking [17], low physical activity [18], a high fat/sugar diet and obesity [19-21]), the quality of the prenatal period during fetal development can impact the health and lifespan of the offspring in later-life [22-24]. This theory was termed as a theory of Developmental Origins of Health and Disease, or DOHaD (Figure 1.1). There are many factors and conditions (including, but not limited to: undernutrition, pregnancy at high altitude, smoking, preeclampsia, placental insufficiency, iron deficiency) during pregnancy that can affect fetal growth, however a common feature of many of these conditions is prenatal hypoxia.

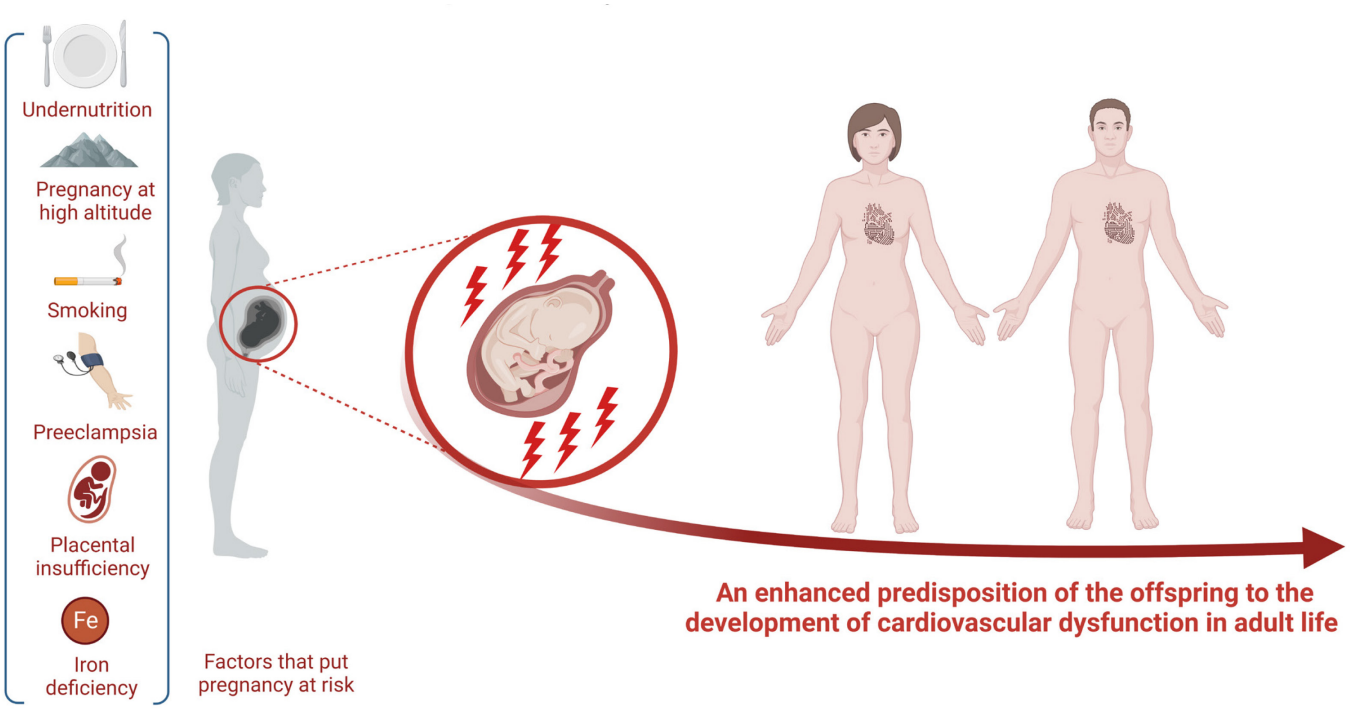

Figure 1.1. Schematic illustration of the theory of Developmental Origins of Health and Disease. *Created with BioRender.com.*

1.2. Pregnancy and fetal hypoxia

1.2.1. Origins of fetal hypoxia

There are several proposed models for the origins of fetal hypoxia, such as (1) *pre-placental hypoxia*, (2) *uteroplacental hypoxia* and (3) *post-placental hypoxia* [25].

(1) *Pre-placental hypoxia* is related to maternal conditions associated with reduction in the maternal oxygen uptake and oxygen delivery to the fetus. Pregnancies in high altitude settings are often associated with a decline in the birth weight [26] due to the direct negative impact on maternal CV adaptation in pregnancy (a reduction in uterine blood flow [27]; lack in the physiological fall of the blood pressure [28], reduction in cardiac output and stroke volume). Respiratory disorders during pregnancy may have a similar maternal-fetal consequences, such as an exposure to hypoxia [29]. For instance, asthma exacerbation during pregnancy increases the risk for development of complications, adverse perinatal outcomes and early childhood respiratory disorders [30]. Maternal hematological disorders, such as iron deficiency [31,32], hemoglobinopathies [33], sickle cell disease [34,35], thalassemia [36] may also directly affect oxygen transfer and impact systemic oxygen levels.

(2) *Uteroplacental hypoxia* refers to inability of oxygenated maternal blood to access the uteroplacental tissues due to abnormal placentation during gestation [37-39] and/or due to the placental vascular disease later in pregnancy [40,41]. Gestational vascular diseases such as preeclampsia, eclampsia or HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelet count) affects ~5-7% of pregnancies and is considered as a major cause of maternal and fetal morbidity and mortality [42-44]. Causes of its origins remain mostly unknown, however they are often associated with a systemic inflammation, systemic vascular resistance, endothelial dysfunction (responsible for the impaired production and action of vasodilators) and the activation of the coagulation system with an enhanced platelet aggregation [45-49]. Subsequently, those conditions can lead to intrauterine hypoxia.

(3) *Post-placental hypoxia* refers to the condition when only fetus becomes hypoxic due to fetal CV malformations and dysfunction or genetic abnormalities [50-53].

Because my thesis is focused on the effects of prenatal (fetal) hypoxia on offspring CV heath, it is essential to indicate key features of the hypoxia *in utero* (placental function and fetal CV development) before exploring its long-term impact on CV function of the offspring in adult life.

1.2.2. Placental dysfunction and fetal hypoxia

The placenta forms the interface between maternal and fetal physiological systems and provides oxygen and nutrient supply to the fetus [54,55], as such, its function is crucial for normal fetal development and growth. Previous study by Xu *et al.* have demonstrated that pregnancies complicated with the hypoxia lead to an impaired mitochondrial function in the trophoblast that may impact the nutrition and energy transfer to the growth restricted fetus [56]. Mitochondria is a major site for generation of reactive oxygen species (ROS) [57] and under normal (uncomplicated) physiological conditions, intracellular ROS production is balanced by antioxidants. However, if the production of ROS is excessive, it leads to the oxidative stress [58] that is linked to the development of various pregnancy pathologies [59].

Molecular oxygen is used to be a final electron acceptor in electron transport chain during mitochondrial oxidative phosphorylation. In response to the hypoxic insult, ROS are generated at mitochondrial complexes, that results in the stabilization of hypoxia-inducible factor (HIF)1α [60,61] and its translocation to the nucleus and dimerization with HIF1β subunit. HIF1α, through binding to the hypoxia response elements, is able to regulate the transcription of at least 70(+) different effector genes and thereby, impact physiological processes that intend to meet the metabolic and survival needs of the hypoxic cell [62,63]. Moreover, mitochondrial ROS are highly reactive and cause oxidative damage of macromolecules, including proteins (protein carbonylation and its cytotoxic effect [64]), lipids (contributes to the impairment of membrane function [65]) and nucleic acid [66], that result

in the mitochondrial dysfunction. Mitochondrial dysfunction and excessive ROS production has been associated with a development of placental dysfunction [67] due to the reduced trophoblast fusion [68] and impaired spiral artery remodelling, upregulation of soluble fms-like tyrosine kinase 1 (an anti-angiogenic factor, is increased in circulation in preeclampsia and FGR and contributes to the development of endothelial dysfunction) [69], an increase in the production/release of pro-inflammatory markers and a decrease in the production and release of anti-inflammatory cytokines [70]. Together, these changes contribute to the pathogenesis of fetal growth restriction.

1.2.3. Fetal cardiovascular consequences of hypoxia in utero

Hypoxia *in utero* drives a number of physiological adaptations in order to maintain fetal survival in unfavorable conditions *in utero*. In response to the hypoxic environment, fetal blood flow is redistributed away from the peripheral vascular bed towards circulation that perfuse brain (the term defined as a "brain sparing effect" [71,72]). Since oxygen delivery is associated with oxygen consumption, limiting the blood flow to peripheral vascular beds (such as fetal intestine and fetal limbs) allows to decrease the oxygen consumption by the fetal tissues during hypoxia [73,74]. Previous study by Gardner *et al.* demonstrated (in sheep fetus) that a reduction in hindlimb oxygen delivery is associated with an increased lactate output, that results in the acidification of fetal blood and enhanced unloading of oxygen from the hemoglobin to the fetal tissues [75]. The persistent redistribution of fetal cardiac output during the sustained reduction of oxygen and nutrient delivery to the peripheral organs, results in the asymmetrical fetal growth (i.e fetus being thin for its length or having a relatively normal sized head with a shorter body length [76-78]). Moreover, a persistent redistribution of fetal cardiac output from peripheral circulations also impact the development and function of other organ systems, including kidneys (impaired fetal kidney development and as a result, a reduced kidney weight and number of nephrons [79,80]) and pancreas (and subsequent reduced endowment and replication of pancreatic β-cells [81]). Further increase in fetal

peripheral vascular resistance increases fetal cardiac afterload that is often associated with aortic wall thickening [82], increased cardiac mass in relation to the body weight with a subsequent cardiac remodeling [83], as well as a decreased number of cardiomyocytes in the heart [84,85].

Fetal heart-sparing effect refers to the condition of the increased coronary blood flow as a result of further manifestation of the redistribution of ventricular output in the fetus. It was shown that growth restricted fetuses experience an increased tricuspid and mitral valve deceleration times that may be a result of impaired ventricular relaxation and decreased ventricular compliance [86]. Those changes in the cardiac performance also drive functional alterations in the fetal coronary circulation. [87]. In the fetus from normal (uncomplicated) pregnancy, the visualization of coronary blood flow is possible during the third trimester of pregnancy, however, in growth restricted fetuses, the visualization of coronary blood flow is possible at the earlier term (mean 25 weeks of pregnancy)[88]. This is because of myocardial hypoxia which is considered to be a strong stimulus for the coronary autoregulation that mediates an elevation in the coronary blood flow [89]. Thus, Chaoui have previously reported in (human) fetuses with severe FGR and heart-sparing effect, an increased systolic and diastolic velocities, suggesting a global increase in myocardial blood flow throughout the cardiac cycle [90]. Subsequently, all those changes in fetal CV physiology during adverse intrauterine conditions are often considered to be an origin for the development of "adult" CV diseases that can be traced in later-life [91-96]. Since my thesis is focused on adult offspring CV function, it is essential to understand adult cardiac physiology in order to explore the already known long-term impact of fetal hypoxia on the cardiac function in adult offspring.

1.3. Physiology of cardiac function in adult life

The heart pumps the blood into the systemic circulation through the generation of the force. Each coordinated contraction involves the modulation of electrical impulses and the muscle response. The normal cardiac cycle consists from the following phases: isovolumic

ventricular contraction (during which an influx of Ca^{2+} causes sarcomere contraction), ventricular ejection (during which the Ca^{2+} concentration remains high), ventricular relaxation (intracellular Ca^{2+} is removed by Ca^{2+} transporters) and ventricular filling [97].

1.3.1. Cardiac excitation-contraction coupling (ECC)

ECC is a physiological process that begins with the electrical excitation on the plasma membrane and results in the cardiac contraction (Figure 1.2) [98]. An initial depolarization of plasma membrane activates an inward Ca^{2+} current that drives the opening of L-type calcium channels (LTCCs) on the plasma membrane and transverse tubules (TT). Subsequently, Ca^{2+} ions enter the cell that results in the rise of intracellular concentration of calcium in the dyadic space (the space between sarcoplasmic reticulum (SR) and TT membranes). Early during depolarization, the membrane potential exceeds the membrane potential of Na^+/Ca^{2+} exchanger (NCX; a bidirectional ion transporter that catalyzes the exchange of Na^+ and Ca^{2+} depending on the electrochemical gradient [99]) that drives outward Na^+/Ca^{2+} current resulting in extrusion of Na^+ and entry of Ca^{2+} through NCX (reverse mode of operation), thereby contributing to the Ca^{2+} influx into the cell (coupling of Ca^{2+} influx with Na^+ efflux). An elevation of intracellular concentration of Ca^{2+} triggers an additional release of Ca^{2+} from a sarcoplasmic reticulum (SR), which is an intracellular store of calcium, via opening of ryanodine receptors type 2 (RyR2) on SR (a process defined as calcium-induced calcium release). Thereby, the combination of Ca^{2+} influx and Ca^{2+} release from SR increases intracellular concentration of calcium that modulates cardiac contraction.

Contraction of cardiomyocyte is based on the movement of the thick filament in respect to the thin filament (composed of actin, tropomyosin (Tm) and troponin (Tn) complex). Tropomyosin (Tm) is a regulatory protein of contraction that lies along the actin filament and blocks the sites of the interaction between myosin and actin [100]. Tn complex is composed of three types of proteins – cardiac troponin I (TnI) that binds directly to actin, cardiac

troponin T (TnT), that binds to Tm, and cardiac troponin C (TnC) that binds Ca^{2+}. When intracellular concentration of calcium increases, Ca^{2+} bind to TnC resulting in its conformational change and allowing Tm to move across the surface of actin, which uncovers the myosin-binding sites. To facilitate contraction, the myosin heads rotate out from the thick filament and form connections with actin (cross-bridges). Once attached, the myosin head flexes causing the actin to slide past the thick filament and subsequently resulting in contraction.

After contractile units are activated, the intracellular concentration of calcium must decline to allow dissociation of Ca^{2+} from cTnC to result in the relaxation. The calcium extrusion from the cytosol can be achieved by several mechanisms that involve NCX and sarcolemmal Ca^{2+}-ATPase that mediates Ca^{2+} removal out of the cell, mitochondrial Ca^{2+} uniport or the activity of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), by which the Ca^{2+} is pumped back into SR. SERCA transitions through multiple steps during the transport of Ca^{2+} through SR membrane [101]. Initial Ca^{2+} binding to the high-affinity sites on the cytoplasmic side of the SERCA results in the hydrolysis of adenosine triphosphate (ATP). For each molecule of ATP hydrolyzed, SERCA transports two Ca^{2+} ions into SR. There are a number of various factors that can influence SERCA activity, including the concentration of Ca^{2+} ions, the level of ATP, ATP binding and hydrolysis, pH, pump affinity to Ca^{2+}, its rate of phosphorylation, as well as the level and phosphorylation of phospholamban (PLN) or/and sarcolipin [102-105]. PLN diminishes the affinity of SERCA2α to Ca^{2+} in its dephosphorylated state by interaction with the calcium-free conformation of the SERCA2α and is released when it is converted to the calcium-bound state [106]. Previous studies *in vitro* have demonstrated that the addition of Ca^{2+} or phosphorylation of PLN (by protein kinase A (PKA), or Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) at Ser 16 or Thr 17, respectively) completely inhibits complex formation between PLN with SERCA2α [106,107]. Upon phosphorylation of PLN, the inhibition of SERCA2α relieves that thereby facilitates Ca^{2+} uptake

into the lumen of the SR and increases the rate of relaxation [108-110]. PLN phosphorylation is the main contributor to the positive inotropic and lusitropic effects of β-agonists (as more Ca^{2+} is accumulated in the SR lumen, a greater Ca^{2+} store is available for release for a subsequent beat, resulting in enhanced contractile force) [111-113]. The amount of Ca^{2+} extruded from the cell should be equal to the amount of Ca^{2+} that entered the cell per beat, otherwise the cell would not be able to reach a steady state [98]. Thereby, Ca^{2+} releases from the cTnC that returns to its initial conformational shape and releases TnI. TnI re-attaches to the actin filament that leads to the relaxation.

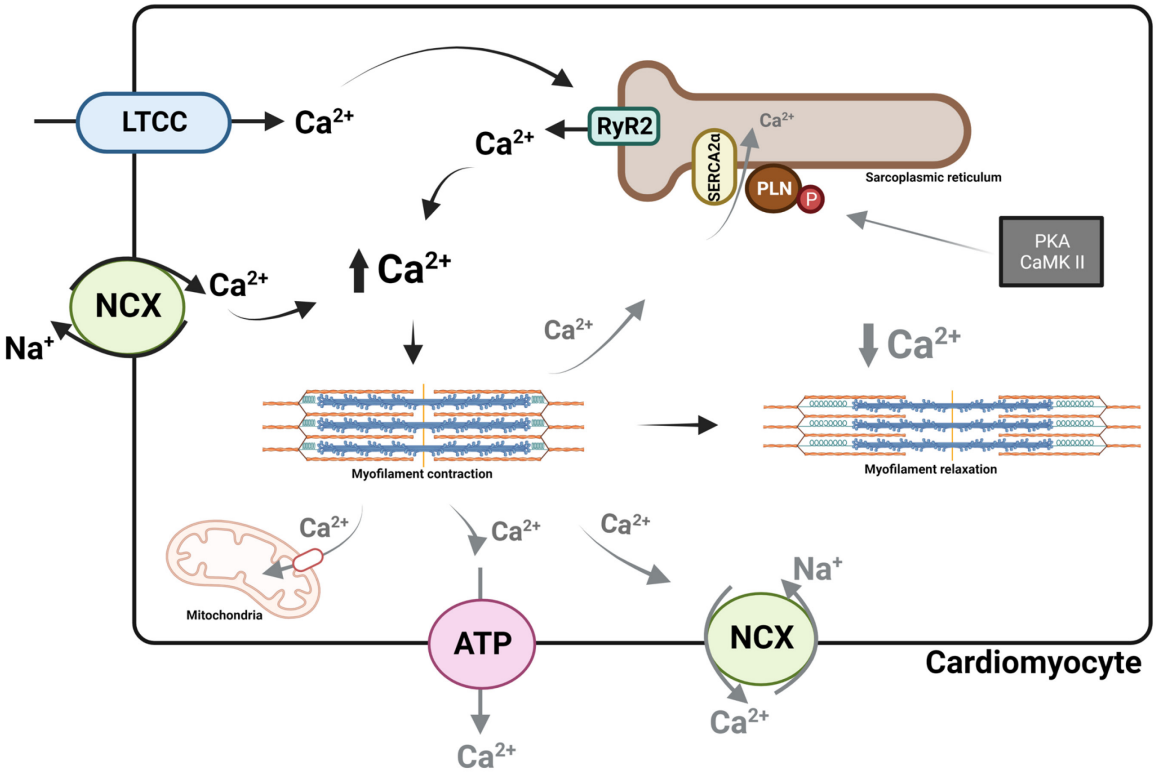

Figure 1.2. Cardiac excitation contraction coupling.

Figure 1.2 legend: *LTCC, L-type calcium channel; NCX, Na^+/Ca^{2+} exchanger; SERCA2a, sarco/endoplasmic reticulum Ca^{2+} ATPase 2a; RyR2, ryanodine receptors type 2; PLN, phospholamban; PKA, protein kinase A; CaMK II, Ca^{2+}/calmodulin-dependent protein kinase II. Adapted and modified from ref [114]: this article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) License (http://creativecommons.org/licenses/by/4.0/). Created with BioRender.com.*

1.3.2. Cardiac structure

Cardiac sarcomeres are composed of thin (actin) and thick (myosin) filaments that show different mechanical properties. Myosin is a chemical-mechanical transducer of the motion that generates the movement by transferring energy from hydrolysis of ATP into the sliding of myofilaments. Each myosin consists of two myosin heavy chains (MHCs) and four myosin lights chains. In cardiomyocytes, MHCs are encoded by α and β genes and are developmentally and hormonally regulated [115]. In rodents, the expression of αMHC increases after the birth and becomes dominant isoform in the young adults, however, with ageing, the level of αMHC declines and βMHC becomes the major isoform expressed in the ventricle [115]. Cardiac functional properties are highly dependent on the expression of certain types of MHC isoforms. Rodent hearts have a predominant αMHC isoform that has a nearly three times higher cross-bridge cycling [116], higher ATPase activity [117] and have a greater capacity of power generation than βMHC isoform. As such, differences in motor performance within cardiac contractile system are directly attributed to basic differences between α and β MHC isoforms. Thus, the shift in MHC isoforms expression has been observed with the progression of the cardiac dysfunction [118].

1.3.3. Autocrine and paracrine regulation of cardiac function

A growing body of evidence supports an idea that the factors that are released by cardiac cells play an essential role in regulating cardiac function. Cardiac tissue consists of myocytes, fibroblasts, endothelial cells, vascular smooth muscle cells (VSMCs) and mast cells that are highly organized and are able to secrete autocrine and paracrine factors, thereby modulating the function of neighboring cells. An early study by Brutsaert *et al.* have demonstrated that cardiac contractile performance can be critically regulated by endocardium that modulates myocardium contractile performance and relaxation [119]. This observation was later also expanded for the vascular (coronary) endothelium in the heart, indicating its critical role in maintaining cardiac performance [120]. Vascular endothelial cells may also

indirectly affect cardiac function via changes in the coronary vascular tone by modulating changes in the myocardial blood supply (to be discussed in section 1.6).

Several studies have proved that endocardial and coronary endothelium regulate contractile state of cardiomyocytes by releasing biological active substances, including (NO), endothelin-1 (ET-1), prostacyclin (PGI_2), angiotensin II (Ang II) [120-123]. ET-1 exerts potent vasoconstrictor effects and has been frequently recognized as a potential therapeutic target for the treatment of a number of CV diseases. According to previous reports, ET-1 is also can be produced by other cardiac cell types, including cardiomyocytes, and there is now extensive evidence suggest that ET-1 function as a paracrine, autocrine, and intracrine regulator of cardiac performance with a potential contribution to the development of cardiac pathology [124-128]. However, its role in the development of cardiac and coronary artery dysfunction in adult offspring exposed to prenatal hypoxia was not previously evaluated and thus, it was one of the objectives in this thesis (Chapters 3 and 4). Thereby, the known effects of ET-1 system on the cardiac and coronary artery physiology are provided in sections 1.3.4 and 1.6 of this chapter.

1.3.4. Cardiac function and endothelin-1 (ET-1)

Transcription of the *edn1* gene yields an inactive preproET-1 peptide upon activation by various factors, including (but not limited to) thrombin [129], Ang II [130], oxidized low-density lipoprotein [131], hypoxia [132], insulin [133], glucose [134], shear stress [135], and various growth factors [136]. PreproET-1 is then processed to inactive bigET-1 by the endopeptidase, a furin convertase [137,138]. BigET-1 can be cleaved to active ET-1 by either endothelin-converting enzyme (ECE), matrix metalloproteinase-2 (MMP-2) or chymases [137,139]. Mainly, in the endothelium (coronary vascular endothelium and endocardial endothelium [140]), ET-1 is predominantly released abluminally (towards the VSMCs and cardiomyocytes), that indicates its paracrine role [141], however, its synthesis can be regulated in autocrine fashion, and therefore, ET-1 may also be produced by VSMCs [142],

leukocytes [143], macrophages [144], cardiomyocytes [145]. ET-1 elicits its effect by acting through its endothelin A (ET_A) and endothelin B (ET_B) receptors and has a potent positive inotropic effect on cardiac function. Both receptor isoforms are typical G-protein-coupled receptors (GPCRs) comprising 7α-helical transmembrane spanning domains, an extracellular N-terminal domain and intracellular C-terminal domain. The heart expresses a high density of ET_A and ET_B on cardiomyocytes [146]. Upon binding with ET-1, ET_A and ET_B are getting internalized and co-localized with the pericentriolar recycling compartment and can be recycled to the plasmalemma so that cellular ET-1 sensitivity can be maintained (in the case of ET_A) [147] or is targeted to lysosomes and degraded, thereby providing a mechanism for clearance of the peptide (in the case of ET_B) [148,149].

ET_A and ET_B interact with $G\alpha_{q/11}$ subunit that activates polyphosphoinositide (PIP) hydrolysis and production of inositol-(1,4,5)-triphosphate (IP_3), diacylglycerol (DAG) (Figure 1.3) [150-152]. DAG affects the release of Ca^{2+} ions from intracellular calcium stores indirectly by associating with PKC. Out of all DAG sensitive protein kinases, cardiomyocytes highly express PKC epsilon (PKCε) and PKC delta (PKCδ) [153], among which PKCε is preferentially activated by ET-1 [154]. Previous studies have demonstrated that ET-1 signalling is associated with the stimulation and activation of p42 and p44 mitogen- activated protein kinase (MAPK), c–Jun N–terminal kinases (JNKs) and p38-MAPKs [154-156] which function is currently under debate due to its relation to cytoprotection/preconditioning, hypertrophy and pro-apoptotic nature [157-160]. Moreover, ET-1 is able to rapidly activate Ras (upon activation of PKC [161]), which effectors include protein kinases of the Raf family and lipid kinases of the phosphoinositide 3'-OH kinase (PI3K) family. When Ras is activated, it binds to the Raf that initiates extracellular signal-regulated kinase 1/2 (ERK1/2) cascade (and subsequent modulation of 90–kDa ribosomal protein S6 kinases (p90RSKs)) thereby promoting cardiac hypertrophy [157]. p90RSKs are also known to phosphorylate the pro-

apoptotic Bcl-2 family protein (Bad) in cardiac myocytes, that potentially is able to reduce its activity [162,163].

ET-1 is also able to modulate cardiac contractility by affecting Na^+/H^+ exchanger (NHE) via PKC-, ERK1/2- and p90RSK-dependent mechanism [164-167], and/or PKC-mediated activation of NCX [168,169]. Because NHE extrudes H^+ ions in exchange to Na^+, it modulates inotropic effect by cytosolic alkalanization (that increases myofilament Ca^{2+} sensitivity) and by increasing intracellular concentration of calcium (via NCX reverse mode) [170]. ET receptors, phospholipase C (PLC)β and PKCε are also co-localized to the sarcolemma of the TT in ventricular myocytes, that brings them to the close proximity with LTCC, thereby facilitating calcium inflow and regulating the machinery underlying ECC [171]. LTCC activation can also occur secondarily through the ET-1-mediated generation of mitochondrial reactive oxygen species (ROS) [172]. In addition, ET-1 stimulates nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and production of ROS and in this way, participates in the regulation of cardiomyocyte contractility, pacemaker activity and remodelling [172,173]. IP_3 binds to IP_3R that stimulates intracellular Ca^{2+} release from SR. thereby supporting the development of cardiac inotropy as well as pro-arrhythmic Ca^{2+} signals in myocytes [174]. IP_3R also interacts with RyR2 upon stimulation with ET-1 that facilitates Ca^{2+} release from SR through RyR2 release clusters and enhances Ca^{2+} transients [175].

ET_B also interact with $G\alpha_{i/o}$ and $G\alpha_{13}$, which activation leads to the inhibition of cAMP accumulation. It was demonstrated that ET-1 binds in stoichiometric manner to its receptors [176], with a ratio of ET_A vs ET_B binding sites is 4:1 [177]. However, under the basal secretion of ET-1, it acts in autocrine way by binding directly to ET_B on endothelium, thereby modulating the release of dilator factors (including NO and PGI_2), rather than acting directly through ET_A and ET_B on cardiomyocytes and promoting myocardial inotropic effects. Previous study by Vaniotis *et al.* demonstrated that activation of ET_B on the nuclear membranes regulates the

production of NO in isolated cardiac nuclei and in the intact cardiomyocytes [178], that demonstrates a contribution of ET-1 in the cardiac production of NO.

In pathology (such as myocardial infarction, hypertrophy, and arrhythmia), the circulating and cardiac levels of ET-1 are increased [126,127,179-183]. Harzheim *et al.* reported in spontaneously hypertensive rats an elevated levels of IP_3R in cardiomyocytes that resulted in a greater rise in diastolic calcium following stimulation with ET-1 [184]. An elevation in the intracellular calcium leads to the activation of RyR2, thereby modulating an additional RyR2-dependent release of calcium from SR and promoting arrhythmia [185]. As such, blockers of ET-1 receptors have been used to ameliorate negative consequences of the upregulation of ET-1 system in pathological conditions [186-188].

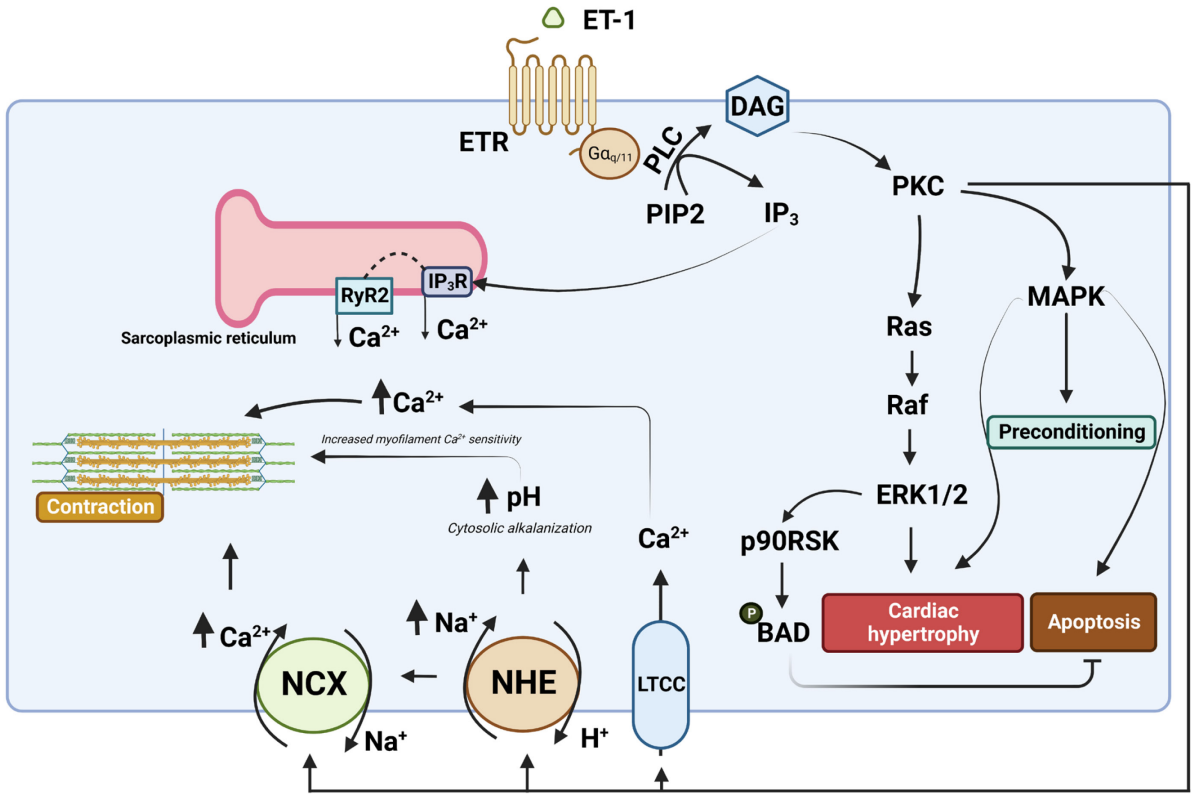

Figure 1.3. Main physiological effects of ET-1.

Figure 1.3 legend: *GPCR, G-protein-coupled receptor; ET-1, endothelin-1; ETR, endothelin receptor; $G\alpha_{q/q11}$, G protein α-subunit subtype q/q11; PLC, phospholipase C; PIP_2, polyphosphoinositide; IP_3, inositol-(1,4,5)-triphosphate; DAG, diacylglycerol; PKC, protein kinase C, Ras, small G–protein Ras; ERK1/2, extracellular signal-regulated kinase 1/2; Raf, initiator kinases for ERK1/2; p90RSK, 90–kDa ribosomal protein S6 kinase; BAD, pro-apoptotic Bcl-2 family protein; MAPK, mitogen-activated protein kinase; NHE, Na^+/H^+ exchanger; NCX, Na^+/Ca^{2+} exchanger; LTCC, L-type calcium channel; RyR_2, ryanodine receptor type 2; IP_3R, IP_3 receptor. Created with BioRender.com.*

1.4. Cardiac ischemia/reperfusion insult

Myocardial ischemia/reperfusion (I/R) injury is defined as a stress condition that occurs in the cardiac tissue after the restoration of blood flow. A number of clinical conditions feature the development of cardiac ischemia (includes but not limited to): angina and acute coronary syndromes, hypertension, cardiac arrest and silent ischemia [189-191]. There are various mechanisms known to contribute to the development of I/R-induced cardiac injury and functional dysfunction, including alterations in the cardiac metabolism and calcium overload.

To sustain its regular heartbeat, the heart needs to maintain a constant supply of the energy for its own contraction. This energy comes primarily from the hydrolysis of ATP, that can be generated within the cardiomyocyte by utilizing substrates and oxygen. Cardiac contractile function is directly influenced by cardiac metabolism, and in the situations of the limited ATP production (such as ischemia), cardiac contractile function declines [192]. Cardiac ischemia is a result of abolished delivery of substrates for energy metabolism and diminished oxygen supply within the myocardium. The loss of oxygen stimulates anaerobic glycolysis (owing its ability to generate ATP in the absence of oxygen) that increases lactate and proton (H^+) production, thereby resulting in the decreased intracellular pH within the ischemic myocardium [193]. Because pyruvate cannot be oxidized by mitochondria during ischemia, it is converted to lactate, that results in the excessive lactate accumulation within the myocardium. Also, in the presence of severe ischemia, the metabolic by-products of glycolysis are not removed, that inhibits glycolysis. Intracellular acidosis is able to impair myofilament responsiveness to Ca^{2+} and contribute to the loss of contractile force during ischemia [194]. These effects further aggravate disturbances in ionic homeostasis (discussed later). Clinical studies report that acute myocardial infarction is associated with an elevated circulating (plasma) levels of fatty acids [195,196]. Thereby, when an ischemic myocardium is reperfused, an increased delivery of nutrients and oxygen to the heart drives a rapid recovery of fatty acids oxidation [197] that inhibits the rate of the glucose oxidation to a much greater

extent than the rate of glycolysis, resulting in a marked uncoupling between the rates of glycolysis and glucose oxidation [198]. At the same time, the rate of glycolysis remains high during early period of reperfusion, resulting in its continued uncoupling, followed by production of H^+ ions and lactate. The continued production of H^+ ions during the reperfusion period contributes to altered ionic homeostasis and decreased cardiac efficiency [199,200].

A reduced ATP production during ischemia prevents Ca^{2+} reuptake by active transport, resulting in a sustained spike of the elevated Ca^{2+}. Moreover, an intracellular aggregation of H^+ ions during cardiac reperfusion results in the low intracellular pH, that leads to excessive Na^+ transport inside of cell due to the activation of NHE [201]. This drives a rapid increase in intracellular Na^+ that disbalances the sarcolemmal Na^+ gradient, thereby leading to the activation of NCX exchanger and an increased inflow of Ca^{2+} with a subsequent calcium overload [202]. I/R has been associated with a substantial damage of SR and its impaired Ca^{2+} uptake [203,204], as well as a reduction in protein contents for SERCA2α and Ca^{2+} release channels [205,206]. Talukder *et al.* reported an impaired postischemic myocardial relaxation and higher infarction in SERCA2α knockout mouse that indicates an importance of optimal SERCA2α levels during postischemic period [207]. Moreover, the dysfunction of SERCA2α exacerbates intracellular calcium overload during I/R injury, leading to activation of calcium-dependent proteases thereby modulating cardiac cell death and cleavage the cytoskeleton and membrane associated proteins [208]. Ischemia has been associated with a reduction in PLN phosphorylation [209,210], which ablation has been shown to exacerbate post-ischemic myocardial injury [211]. Akaike *et al.* have demonstrated a decreased calcium uptake by SR due to an enhanced activity of ROS-activated protein phosphatase during reperfusion period (that dephosphorylates PLN) that resulted in the impaired post-ischemic cardiac recovery [212]. An elevated concentration of calcium also results in the activation of CaMK that impacts the function of various proteins that are involved in cardiac EEC [213]. Mainly, CaMK II can increase Ca^{2+} entry through LTCC (that leads to the myocyte Ca^{2+} loading), SR Ca^{2+} leak and an increase in cytosolic Ca^{2+} levels due to the phosphorylation

of RyR (at Ser2814) [214-217]. CaMK II can also have beneficial effects during I/R by phosphorylating PLN at Thr 17 and thereby enhancing cytosolic Ca^{2+} clearance [218], however, it is important to consider that PLN phosphorylation may also lead to an opposite physiological consequence. On the one hand, it enhances the uptake of Ca^{2+} into SR and in this way, favors contractile recovery. However, in the other hand, it may lead to the increased SR Ca^{2+} load and lead to SR Ca^{2+} leak due to I/R-induced RyR2 dysfunction. As such, those factors need to be taken into consideration as they appear to play a crucial role in post-ischemic cardiac recovery. Bell *et al.* showed in *ex vivo* hearts subjected to ischemia that an inhibition of CaMK II (prior to global ischemia) abolished the incidence of ventricular tachycardia/fibrillation in reperfusion and overall, resulted in the improved post-ischemic cardiac performance [219]. The beneficial effects of CaMK II inhibition were also proved in the animal model of *in vivo* ischemia [220].

Myocardial reperfusion drives a massive production of ROS. There are multiple sources of ROS that has been detected in heart, such as xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS), NADPH oxidases and mitochondria. Reperfusion drives an increase in the production ROS, such as hydrogen peroxide (H_2O_2), the superoxide radical anion (O_2^-), the hydroxyl radical ($OH^\bullet$), and peroxynitrite anion ($ONOO^-$). Moreover, reactive nitrogen species (RNS), such as $ONOO^-$ generate RNS-damage, thus producing nitroxidative stress (due to the interaction between NO and the O_2^-). $ONOO^-$ causes structural damage and the impairment of myocardial function [221]. ROS and RNS react with cysteine residues on RyR2 and in this way, modulating RyR2 sensitivity to Ca^{2+} that contributes to Ca^{2+} leak from SR (a state of "leaky" RyR2) [222-224]. Pro-oxidant cellular environment can also increase CaMK II activity (even in the absence of a calcium influx) [225] that activates CaMK II pro-apoptotic signaling [226]. Thereby, the increase in ROS and RNS production during I/R may negatively impact the function of the proteins involved in the calcium cycling and apoptosis and thereby contribute to the development of cardiac dysfunction.

1.5. The long-term impact of prenatal hypoxia on cardiac function of adult offspring

As was indicated previously, prenatal hypoxia is associated with a reduction in the nutrient and oxygen supply to the fetus and drives unique changes in the cardiac function of the offspring. Because the long-term adaptations in the systemic (mesenteric arteries) and pulmonary vasculature in adult offspring are beyond the scope of the current thesis, I have focused my discussion on long-term impact of prenatal hypoxia on cardiac function of adult offspring. There is a literature base of animal studies that have focused on the impact of prenatal hypoxia on offspring cardiac health using a maternal exposure to hypoxia during pregnancy; an approach also used in my studies for this thesis. Those studies are described below and summarized in Table 1.1.

1.5.1. Prenatal hypoxia-induced changes in the cardiac structure of adult offspring

Substantial changes in the cardiac structure of the prenatally hypoxic offspring were previously reported by Wang *et al.* and Xu *et al.* who demonstrated an excessive accumulation of collagen I and collagen III in hearts of adult offspring (females were not assessed) [227,228], as well as a reduced cardiac MMP-2 levels of the offspring [227]. Also, prenatal hypoxia has been associated with an increased cardiac expression of insulin-like growth factor 1 receptor (IGF-IR) mRNA (that plays an essential role in mediating physiological cardiomyocyte hypertrophy [229]) and an increased β/α MHC ratio in adult offspring [227]. Alteration in the cardiac expression of MHC isoforms negatively impacts cardiac performance [118,230,231]. This transition in the expression MHC isoforms is considered to be a maladaptive response to negative insults, that may result in the slowdown of myocardial contraction due to a reduction in myofibrillar ATPase activity. Previous study by Bae *et al.* in rat model of prenatal hypoxia demonstrated an elevated HIF-1α protein level in fetal rat hearts [85]. HIF is a key regulatory factor that is involved in the cell response to a decreased oxygen levels [232]. It was shown (*in vitro*) that hypoxia-induced upregulation of βMHC is dependent

on the presence of HIF-1α [233], as such, it might be one of the potential mechanisms involved in the switch in β/αMHC ratio in adult offspring exposed to prenatal hypoxia.

1.5.2. Prenatal hypoxia-induced changes in the cardiac function of adult offspring

Further observations of the cardiac function in the offspring exposed to prenatal hypoxia demonstrate alterations in the basal cardiac performance. Mainly, prenatally hypoxic offspring experience an increase in dP/dt_{max} (defined as the maximal rate of rise of left ventricle (LV) pressure), increased cardiac contractility index and LVEDP (left ventricular end diastolic pressure; a reflection of ventricular compliance) [234,235] and overall, experience an enhanced cardiac sympathetic dominance. In particular, cardiac chronotropic and inotropic responses decrease (compared to normoxia controls) after the application of carbachol (a parasympathomimetic that mimics the effect of acetylcholine (Ach)), while the application of isoprenaline (β-adrenergic receptor (β-AR) agonist) has an opposite effect and increases cardiac contractility in prenatally hypoxic offspring (compared to respective controls) [24,234,235]. However, prenatal hypoxia did not alter cardiac protein levels of muscarinic receptors, as well as β_1-AR, but observed an increased the expression of SERCA2α [234], a key factor involved in calcium homeostasis. Moreover, a recent study by the same group revealed an increased mRNA levels of *Ryr2* and *PLN* in the hearts of adult prenatally hypoxic male offspring [235]. Previous studies in a chick model of fetal hypoxia demonstrated hypoxia-induced changes in β-AR pathway in hearts of adult chicken (5-weeks old). Mainly, fetal hypoxia resulted in the increased cardiac expression of G stimulatory (G_S) α ($G_s\alpha$) subunit (which activation generates cyclic adenosine monophosphate (cAMP) that activates PKA and subsequent phosphorylation of TnI, RyR2, LTCC, thereby modulating cardiac contractility [236]) and a systolic dysfunction [237]. Another study by Li *et al.* in a rat model of prenatal hypoxia demonstrated an increased β_2AR (but not β_1AR) and $G_s\alpha$ subunit of GPCRs in hearts of adult offspring [238]. βAR couple to $G_s\alpha$ thereby allowing the release of the Gβγ subunits of the hetero-trimeric G protein which provides a mechanism for amplification of signals from

the activated receptor. As such, prenatal hypoxia results in the upregulation of βARs and $G_s\alpha$ subunits of GPCRs, as well as alterations in the levels of proteins involved in the calcium cycling and homeostasis thereby modulating cardiac sympathetic dominance and subsequent enhanced cardiac contractility in adult offspring.

Studies have also demonstrated an enhanced cardiac susceptibility to I/R insult in adult offspring exposed to prenatal hypoxia [94,227,234,238-240]. Mostly, an impaired post-ischemic cardiac performance is associated with a decreased left ventricle developed pressure (LVDP), dP/dt_{max} and increased LVEDP as well as an impaired coronary flow post-ischemia [227,234,238,240]. An impaired post-ischemic recovery of the cardiac function has been linked to an increased release of creatine kinase (CK) and lactate dehydrogenase (LDH) into coronary circulation during the reperfusion period [227,234]. LDH and CK assays have been widely used as biochemical markers of cardiac injury [241]. Mainly, CK catalyses the reversible transfer of a high-energy phosphoryl group from ATP into creatine to form phosphocreatine (considered as an energy storage molecule, rapidly regenerating ATP during increased energy demand), while LDH participates in the conversion of pyruvate to lactate (in the absence of oxygen) in order to generate NADP to maintain glycolysis [242]. The study by Rueda-Clausen *et al.* have shown that prenatally hypoxic offspring had a significant increase in the amount of glucose that underwent glycolysis relative to the amount of glucose that was oxidized during reperfusion period [239]. An uncoupling of glucose metabolism is often results in the excessive accumulation of H^+ ions in the cytoplasm, that leads to the calcium overload and activation of SERCA2α. Subsequently, during the reperfusion period, the benefit of glycolytic pathway is diminished due to the increased cardiac metabolic demand.

The amount of glucose that can be used in glycolysis becomes available primarily in two ways: by regulation of glucose reuptake or by the regulation of the breakdown of glycogen. The transport of glucose inside of the cardiomyocyte is regulated by the transmembrane glucose gradient and by glucose transporters (GLUT) in the sarcolemma (mainly GLUT4). Cardiac gene expression of GLUT4 and the activation of AMP-activated

protein kinase (AMPK; a kinase that stimulates the translocation of GLUT into the sarcolemma), is elevated in both, male and female prenatally hypoxic offspring [84]. Moreover, prenatal hypoxia is also associated with an increased expression of peroxisome proliferator-activated receptor α (PPARα) as well as with an increase in the expression of fatty acid transport protein (FATP)1 and FATP6 that are involved in the transport of fatty acids through the plasma membrane [229]. In male (but not female) prenatally hypoxic offspring, the expression of long-chain fatty acyl-CoA synthase (FACS), AMPKα2, and acetyl-CoA carboxylase (ACC) were decreased [229], that suggests that male prenatally hypoxic offspring may have a gene expression profile that predisposes to altered lipid homeostasis.

Previous reports demonstrate an increased cardiac infarct size, the level (and activity) of caspases and DNA fragmentation in hearts of prenatally hypoxic offspring [238,240]. Novel PKCs, especially PKCδ and PKCε, play an essential role in myocardial I/R injury via regulation of various intracellular events, including cell death (necrosis and apoptosis), mitochondrial dysfunction and oxidative stress [243,244]. Prenatal hypoxia has been associated with a reduced cardiac levels and phosphorylation of PKCε in adult (male, no changes in female) offspring [238,240], as well as with a decreased PKCε mRNA levels in fetal (male; no changes in female) hearts [245]. Moreover, previous study in the rat model of prenatal hypoxia revealed an increased methylation of two specificity protein 1 (SP1) binding sites (−346 and −268) at the PKCε promoter in the fetal hearts of both sexes [245]. Here authors demonstrated (*in ex vivo* Langendorff preparation of male hearts) that the application of a selective PKCε activator peptide increased post-ischemic cardiac recovery and abolished the prenatal hypoxic effects (increased recovery of LVDP and decreased LVEDP and LDH release during the reperfusion period) [245]. In addition to the SP1 binding site, the same group demonstrated an increased methylation of early growth response factor-1 (Egr-1) binding site at the PKCε promoter in fetal male and female hearts that persisted in adult male (but not female) offspring [246]. Thereby, the deletion of SP1 or Egr-1 binding sites results in the

significant decline in the activity of PKCε promoter that contributes to the impaired cardiac capacity to tolerate I/R insult in adult prenatally hypoxic offspring.

Prenatal hypoxia is also associated with fetal programming of cardiac Ang II system in adult offspring [247]. Mainly, prenatal hypoxia increases levels of cardiac type 2 Ang II receptors (AT_2R) in males and AT_1R and AT_2R in female offspring and was associated with a decrease in protein abundance of total and nuclear glucocorticoid receptor (GR) in male and female offspring. GRs regulate the expression of both types of Ang II receptors [248], while prenatal hypoxia decreases GR binding at glucocorticoid response elements on AT_2R promoter, thereby proposing mechanism that contributes to the partial reversal of GR-dependent downregulation of Ang II receptors. The application of AT_2R antagonist (PD123,319; in *ex vivo* model of cardiac I/R insult) rescued prenatal hypoxia-mediated ischemic vulnerability in prenatally hypoxic males (increased LVDP recovery, and decreased LVEDP, LDH release and infarct size), that indicates an essential contribution of cardiac Ang II system to enhanced susceptibility of the offspring to cardiac I/R insult.

Table 1.1. Long-term effects of prenatal hypoxia on cardiac function of adult offspring

Animal model of prenatal hypoxia	Offspring (sex & age)	Main findings in prenatally hypoxic offspring	Ref.
Pregnant Wistar rats exposed to hypoxia (13% O_2) from day 6-20 of gestation	Males only (4-month-old)	↑ LVEDP and dP/dt_{max} ↓ CF ↓ Cardiac negative chronotropic and inotropic responses to carbachol ↑ Positive chronotropic and inotropic responses to isoprenaline ↓ Cardiac recovery from I/R insult ↑ Levels of CK and LDH post I/R ↑ Cardiac expression of SERCA2α	[234]
Pregnant Wistar rats exposed to hypoxia (10% O_2) from day 15-20 of gestation	Males only (4-month-old)	↑ Increased heart weight and left ventricle + septum weight ↑ Increased cardiac expression of eNOS	[249]
Pregnant Wistar rats exposed to hypoxia (13 % O_2) from day 6-20 of gestation	Males only (4-month-old)	↑ Cardiac dp/dt_{max} ratio, RPP and heart rate response to isoprenaline ↓ Heart rate response to carbachol	[24]
Pregnant Wistar rats exposed to hypoxia (13% O_2) from day 6-20 of gestation	Males only (4-month-old)	↑ Cardiac contractility index and inotropic sympathetic dominance and LVEDP ↑ Cardiac mRNA expression of *Nfe2l2*, *Gpx1*, *Cat*, *RyR2* and *Pln*	[235]
Pregnant guinea pigs exposed to hypoxia (12% O_2) from day 35-65 of gestation	Male (4-month-old)	↑ Cardiac expression of transcriptional regulator of FAs metabolism (PPAR-α) ↑ Regulation of genes responsible for the transport of FAs (FATP1, FABPpm, FATP6) ↓ Expression of genes involved in FAs activation and transport into the mitochondria (FACS, AMPK-α_2, ACC) ↑ Cardiac gene expression of glucose transporter (GLUT4)	[229]

		↑ mRNA expression of the physiological hypertrophy receptor (IGF-IR)	
	Female (4-month-old)	↓ Number of cardiomyocytes in LV ↑ Cardiac expression of transcriptional regulator of FAs metabolism (PPAR-α) ↑ Regulation of genes responsible for the transport of FAs (FATP1, ↑ FABPpm, FATP6) Cardiac gene expression of glucose transporter (GLUT4); ↑ Activity (phosphorylation) of AMPKα ↑ mRNA expression of the physiological hypertrophy receptors (IGF-IR)	
Pregnant Sprague–Dawley rats were exposed to hypoxia (11% O_2) from day 15-21 of gestation	Male (4-month-old)	No changes observed	[250]
	Male (12-month-old)	↑ Heart/body weight and LV/heart weigh ratios ↑ RV diameter in diastole ↑ End-systolic septal thickness ↓ LV end-systolic internal diameter ↑ Mitral E/A index ↑ Mitral deceleration time ↑ Mitral IVRT ↑ LVEDP	
	Female (4-month-old)	No changed observed	
	Female (12-month-old)	↑ Myocardial performance (Tei) index ↑ Mitral IVRT ↑ LVEDP ↓ LV dp/dt_{max}	
Pregnant Wistar rats exposed to hypoxia (10% O_2) from: 1) day 3-21 of gestation;	Males only (3-month-old)	1) ↑ Systolic blood pressure 2) ↑ Collagen I and III protein expression 3)	[228]

2) day 9-21 of gestation; 3) day 15-21 of gestation.		No changes in the systolic blood pressure or cardiac collagen content	
	Males only (5-month-old)	1) ↑ Systolic blood pressure ↑ Collagen I and III protein expression 2) ↑ Collagen I and III protein expression 3) No changes in the systolic blood pressure or cardiac collagen content	
Pregnant Sprague–Dawley rats exposed to hypoxia (10.5% O_2) from day 15 to day 21 of gestation	Male (3-month-old)	↓ Cardiac recovery after I/R insult ↑ Myocardial infarct size, post I/R LDH release ↓ Expression of total PKCε and phospho-PKCε ↓ Expression of total PKCδ protein, no changes in phospho- PKCδ	[240]
	Female (3-month-old)	No differences in cardiac recovery from I/R injury, myocardial infarct size, LDH release and PKCε, phospho-PKCε, PKCδ, and phospho-PKCδ levels	
Pregnant Sprague–Dawley rats were exposed to hypoxia (10.5% O_2) from day 15 to day 21 of gestation	Males only (6-month-old)	↓ Cardiac recovery from I/R injury ↓ LVDP, PRP, dP/dt_{max}, CF post ischemia ↑ LVEDP post ischemia ↑ Myocardial infarct size, myocyte apoptosis and DNA fragmentation in LV post I/R ↑ Level of caspase-3 cleaved form post I/R ↓ Hsp70 and eNOS protein expression in LV (non-ischemic) ↑ β_2AR and $G_s\alpha$ protein expression in LV (non-ischemic hearts)	[238]

		↓ $G_i\alpha$ protein expression in LV (non-ischemic)	
Pregnant Sprague–Dawley rats were exposed to hypoxia (10.5% O_2) from day 15 to day 21 of gestation	Males only (2-month-old)	↑ Increased LV myocyte cross-sectional area ↓ Total PKCε expression	[251]
Pregnant Sprague–Dawley rats were exposed to hypoxia (11% O_2) from day 15-21 of gestation	Male (4-month-old)	↓ Cardiac recovery from I/R injury ↑ Myocardial H^+ production (derived from glucose metabolism uncoupling) ↓ Myocardial energetic efficiency post I/R insult	[239]
	Male (12-month-old)	↓ Cardiac recovery from I/R injury ↑ Glycolysis rate relative to glucose oxidation rate during the reperfusion ↑ Myocardial H^+ production (derived from glucose metabolism uncoupling) ↓ Myocardial energetic efficiency post I/R insult	
	Female (4-month-old)	↓ Cardiac recovery from I/R injury ↑ Myocardial H^+ production (derived from glucose metabolism uncoupling) ↓ Myocardial energetic efficiency post I/R insult	
	Female (12-month-old)	↓ Cardiac recovery from I/R injury ↑ Myocardial H^+ production (derived from glucose metabolism uncoupling) ↓ Myocardial energetic efficiency post I/R insult	
Pregnant Sprague–Dawley rats were exposed to hypoxia (12% O_2) from day 15-21 of gestation	Males only (4- and 7-month-old)	↑ β/α MHC ratio ↓ MMP-2 level ↓ -dp/dt ↑ LVEDP ↓ Cardiac recovery from I/R injury ↓ CF post I/R	[227]

		↑ LDH release post I/R	
Pregnant Sprague–Dawley rats were exposed to hypoxia (11% O_2) from day 15-21 of gestation	Male (7-month-old)	↓ MV A wave ↑ E/A wave ratio (MV A and E/A wave index)	[252]
	Male (13-month-old)	Intraventricular septum in diastole, left ventricular posterior wall in systole ↑ MV E/A ratio	
	Female (7-month-old)	↑ (trend) E/A wave ratio	
	Female (13-month-old)	↓ MV A wave	
Pregnant Wistar rats were exposed to hypoxia (12% O_2) from day 10-20 of gestation	Males only (~3.2 month)	↑ PSP and developed pressure ↑ -dP/dt and RPP ↓ Capillary density (epicardium and endocardium) ↑ Oxygen diffusion distance ↓ Cardiac eNOS expression	[253]
Eggs were incubated in hypoxia conditions (14% O_2) from day 0-21 of gestation	Sex was not specified (5-weeks old)	↑ G protein α-subunit subtype ($G_{\alpha}s$) expression ↑ Systolic diameter ↑ FS upon stimulation with isoproterenol	[237]
Pregnant Sprague-Dawley rats were exposed to hypoxia (10.5% O_2) from day 15-21 of gestation	Males (3-month-old)	↑ Cardiac mRNA and protein AT_2R ↓ abundance GR abundance ↓ GRE binding at the AT_2R promoter ↑ Post-ischemic cardiac performance after application of selective AT_2R inhibitor: ↑ LVDP ↓ LVEDP ↓ Infarct size ↓ LDH release	[247]
	Females (3-month-old)	↑ Cardiac mRNA and protein AT_1R and AT_2R abundance	

		↓ GR abundance ↓ GRE binding at the AT_2R promoter Functional studies with AT_2R inhibitor were not performed	

↑ Increase

↓ Decrease

Table 1.1 legend: *LV, left ventricle; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, maximum derivative of change in systolic pressure over time; dP/dt_{min}, minimum derivative of change in diastolic pressure over time; CF, coronary flow; CK, creatine kinase; LDH, lactate dehydrogenase; SERCA2α, sarco/endoplasmic reticulum Ca^{2+}-ATPase 2α; eNOS, endothelial nitric oxide synthase; RPP, heart rate-pressure product; Nfe2l2, nuclear factor erythroid 2-related factor 2; Gpx1, glutathione peroxidase 1; Cat, catalase; RyR2, ryanodine receptor 2; PLN, phospholamban; PPAR-α, peroxisome proliferator-activated receptor alpha; FATP1, fatty acid transport protein 1; FABPpm, fatty acid binding protein; FATP, fatty acid transfer proteins; FACS, fatty-acyl CoA synthetase; AMPK-α_2, AMP-activated protein kinase α_2; ACC, acetyl-CoA carboxylase; GLUT4, glucose transporter type 4; IGFR, insulin-like growth factor 1 receptor; E wave, peak velocity of flow in early diastole; A wave, peak velocity of flow in late diastole that is produced by atrial contraction; IVRT, isovolumic relaxation time; PKCε, protein kinase C epsilon; PKCδ, protein kinase C delta; Hsp70, heat shock protein 70; β_2AR, β_2-adrenergic receptor; β/α MHC, β/α myosin heavy chain; MMP-2, matrix metalloproteinase-2; PSP, peak systolic pressure; FS, fractional shortening; $AT_{1,2}R$, type 1,2 angiotensin II receptors; GR, glucocorticoid receptor.*

1.6. Physiology of vascular function in adult life

Cardiac oxygen and nutrient supply is achieved via the coronary vasculature. A delicate balance between vasoconstrictor and vasodilator mechanisms are required to control vascular tone and, thus essential blood flow to the heart. Endothelium-derived contracting factors (EDCR) include (but not limited to) ET-1, thromboxane A2 (TxA2), Ang II and prostanoids, while endothelium-derived relaxing factors (EDRF) include NO, PGI and endothelium-derived hyperpolarization (EDH). Because ET-1 system plays an essential role in the development of various CV pathologies (including myocardial infarction and coronary artery dysfunction [127]), I have focused on its function in the vasculature. Also, I have included discussions on endothelial-dependent vasodilation (NO-, PGI- and EDH-mediated) as a counterbalance to vasoconstriction that are also potential modulators of ET-1 (Figure 1.4).

1.6.1. Endothelium-derived contracting factor: ET-1

In the vasculature, the production and release of ET-1 is similar to ET-1 production in the heart (see section 1.3.4), however, the ET_A are primarily expressed on VSMCs, while ET_B are expressed on endothelial cells and VSMCs (Figure 1.4). On VSMCs, the activation of ET_AR and ET_BR increase the intracellular calcium levels due to the activation of PLC, and subsequent cleavage PIP2 to IP_3 and DAG. IP_3 binds to its receptors on the SR and induces Ca^{2+} release from intracellular calcium stores, thereby increasing its intracellular levels [254,255], while DAG causes activation of PKC and subsequent modulation of VSMCs contraction via phosphorylation of MLCK. Moreover, activation of PKC leads to the activation of several other target proteins promoting VSMCs contraction, such as ERK1/2, Rho-kinase and CaMK II. In the form of a Ca^{2+}-calmodulin complex, an activation of MLCK results in the induction of contraction via formation of the actin-myosin cross bridges. Additionally, various calcium channels are activated upon stimulation with ET-1, including voltage-operated, receptor-operated, store-operated calcium channels and calcium permeable nonselective cation channels, that modulate an increase in the intracellular concentration of calcium [256-259].

VSMCs contraction also occurs independently of intracellular calcium concentration, via PKC-dependent phosphorylation of CPI-17 (a protein kinase C-potentiated myosin phosphatase inhibitor of 17 kDa) that leads to inactivation of myosin light chain phosphatase (MLCP) [260].

ET_B activation of endothelial cells causes an increase in intracellular concentration of calcium which activates NOS and prostaglandin H synthases (PGHS) and results in the production of NO and PGI, respectively, as well as modulates EDH [261-263]. The ET_B receptor-mediated release of vasodilator factors may account for the transient vasodilator action of ET-1. Study by Tirapelli *et al.* showed that the removal of functional endothelium increases vascular response to ET-1 [264] that indicates a substantial contribution of endothelial-dependent vasodilation to ET-1-mediated contractile function. Also, previous studies have shown that ET-1 production and activity can be inhibited by NO via inhibition of preproET-1 and ECE mRNA synthesis and/or release of endothelin converting enzyme (ECE), as well as due to NO-mediated dissociation of ET-1 from ETR [265-268].

1.6.2. Endothelium-derived relaxing factors: nitric oxide (NO), prostaglandin H Synthase (PGHS) pathway, and endothelium-derived hyperpolarization (EDH)

Mechanisms of EDRF-induced vasodilation are based on the activation of guanylyl cyclase (GC)—cyclic guanosine-3',5'-monophosphate (cGMP) or adenylyl cyclase (AC)— cyclic adenosine monophosphate (cAMP) pathways, or by activation of membrane potential of VSMC thereby inducing hyperpolarization (Figure 1.4).

NO is a free radical, freely diffusible, and permeable to cell membranes. It is synthesized from L-arginine by NOS in the presence of various co-factors, including NADPH, flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin, and calmodulin. Once NO is produced, it rapidly diffuses across cell membranes and binds to the heme cofactor of sGC thereby increasing cGMP levels (by conversion of guanosine-5'-triphosphate (GTP) into cGMP and pyrophosphate [269]). cGMP activates targeted kinases (including protein kinase G (PKG), cGMP-dependent protein kinase) that phosphorylate various substrates (PLN, ion

channels, heat shock protein 20, phosphodiesterase V and vasodilator-stimulated phosphoprotein) [270]. cGMP dependent relaxation is based on the dephosphorylation of myosin light chain due to activation of MLCP, decreased intracellular calcium [via inhibition of IP_3R and phosphorylation of PLN, decrease Ca^{2+} inflow through LTCC; activation of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca})], reduction in the number of focal adhesions (due to phosphorylation of vasodilator-stimulated phosphoprotein) and a reduced sensitivity of the vascular contractile system to calcium [271]. The vascular effects of NO are also based on the inhibition of VSMCs proliferation, suppression of platelet aggregation and an inhibition of leukocyte adhesion [272-274].

PGI production is regulated by the availability of arachidonic acid and the activity of PGHSs. Once liberated from membrane-bound lipids, arachidonic acid is available for metabolism by PGHS (in the literature PGHS is also referred as cyclooxygenase) to be converted to prostaglandin H_2 (PGH_2). PGHS is present in two isoforms (PGHS-1 and PGHS-2), among which PGHS-1 is constitutively expressed, while PGHS-2 expression is induced in sites of inflammation [275]. PGH_2 is a substrate for a range of downstream prostaglandin synthase enzymes, which actions result in the formation of various prostaglandins, including PGI (due to activity of prostacyclin synthase) and TXA_2 (due to activity of TXA_2 synthase). The interaction of PGs with their specific receptors generates different actions on the target cell. For instance, PGI acts on the cell surface prostacyclin receptors (IP), which activation results in G-protein-mediated activation of AC, and subsequent formation of cAMP with phosphorylation of PKA, reduction of intracellular concentration of calcium (due to the activation of SERCA2α, increased Ca^{2+} extrusion and reuptake), activation of various K^+ channels (that leads to VSMC membrane hyperpolarization), decreased Ca^{2+} influx through LTCC, and subsequent relaxation of VSMCs [276-278]. However, the stimulation of the thromboxane /prostaglandin receptors (TP) promotes constriction. Briefly, TP can couple with multiple G proteins, and the signaling through G_q and $G_{12/13}$ has been widely reported [279,280]. Coupling to PLC evokes the biosynthesis of DAG and IP_3 that leads to activation of

PKC and accumulation of intracellular calcium, respectively. Those are primary events in TP receptor signaling and, via PKC, TP signalling leads to the induction of platelet aggregation [281].

In many types of blood vessels, endothelium-dependent relaxation can be accompanied by hyperpolarization of VSMCs. With a decrease in the vessel size, the contribution of EDH to endothelial-dependent vasorelaxation increases [282]. Following stimulation of endothelial cells with an agonist (such as ACh or bradykinin) there is a transient increase in the intracellular concentration of calcium that results in the activation of Ca^{2+}-dependent K^+ channels (small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) and/or intermediate-conductance Ca^{2+}-activated K^+ channel (IK_{Ca})) thereby resulting in membrane hyperpolarization. EDH-mediated responses relate to the mechanism on which this endothelial cell hyperpolarization is transferred to VSMCs. Hyperpolarization of the VSMC is enabled by the function of myoendothelial gap junctions (MEGJ) and results in the decrease in intracellular concentration of calcium and subsequent vessel dilatation. Electrical coupling through MEGJ serves to conduct electrical changes from the endothelium to VSMCs and may mediate or propagate hyperpolarization. Increasing the intracellular concentrations of calcium (in endothelial cells) opens not only SK_{Ca} and IK_{Ca} channels (that result in the accumulation of K^+ in the myo-endothelial space) but also leads to the activation of various enzymes including phospholipases and the metabolism of arachidonic acid by cytochrome P450 epoxygenases (prerequisite for the generation of epoxyeicosatrienoic acids that diffuse from the endothelium and hyperpolarize VSMCs by activating BK_{Ca}). Other endothelium-derived factors, including C-type natriuretic peptide and H_2O_2 can also be attributed to EDH. C-type natriuretic peptide acts via a specific C-subtype of natriuretic peptide receptor followed by activation of inward rectify K^+-channels (K_{IR}) and Na^+/K^+-ATPase in VSMC, thereby leading to hyperpolarization and subsequent relaxation [283]. H_2O_2 induces dimerization of 1α-isoforms of PKG thereby enhancing the kinase activity through phosphorylation [284]. PKG1α stimulates K^+ channels with subsequent hyperpolarization and vasodilatation [285].

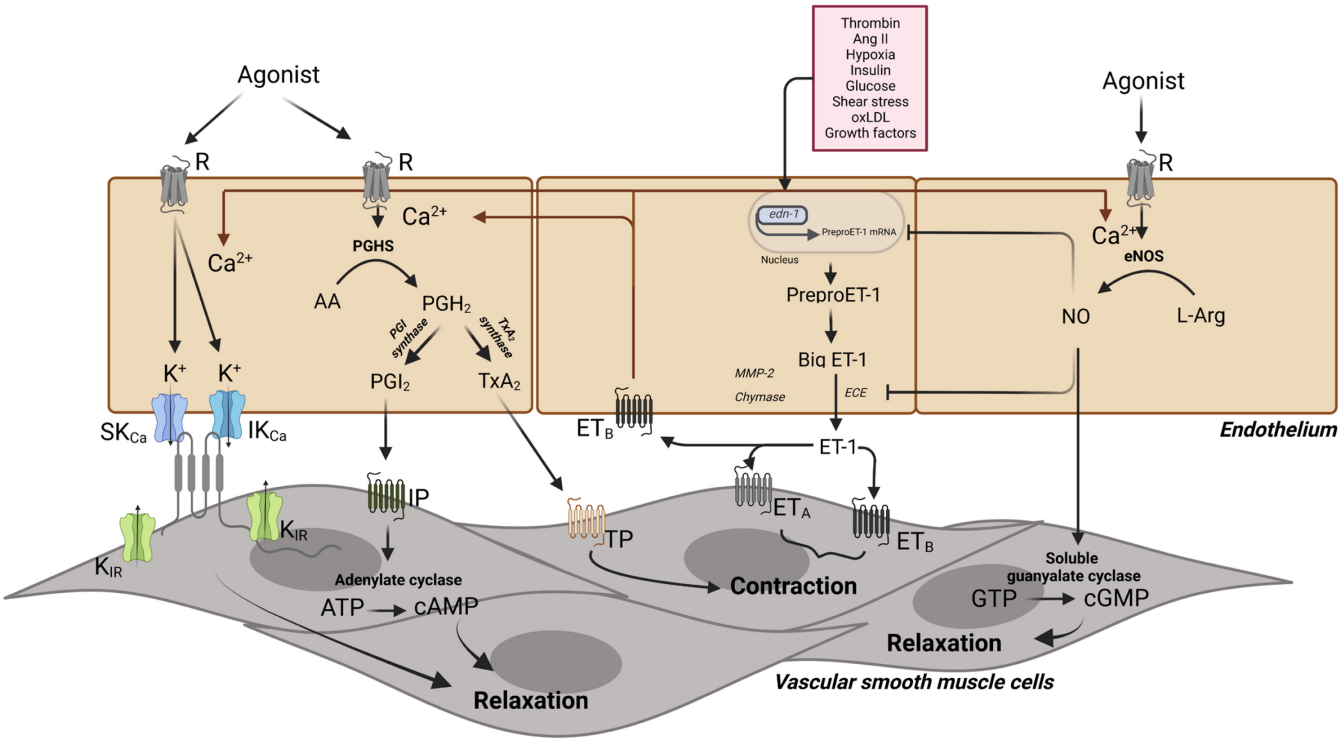

Figure 1.4. Vasodilator and vasoconstrictor pathways.

Figure 1.4 legend: *R, receptor; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; AA, arachidonic acid; PGHS, prostaglandin H synthase; PGH_2, prostaglandin H_2; PGI, prostacyclin; TxA_2, thromboxane A_2; SK_{Ca}, small conductance Ca^{2+}-activated potassium channels; IK_{Ca}, intermediate conductance Ca^{2+}-activated potassium channels; K_{IR}: inward-rectifying K^+ channels; IP, cell surface PGI receptor; TP, TxA_2/PGH_2 receptor; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PIP, phosphatidylinositol phosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; edn-1,endothelin-1 gene; ET-1, endothelin-1; $ET_{A,B}$, endothelin A, B receptors; Ang II, angiotensin II; oxLDL, oxidized low-density lipoprotein; MMP-2, matrix metalloproteinase-2; ECE, endothelin converting enzyme. Adapted and modified from ref [286]: this article is an open access article distributed under the terms and*

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1.6.3. Coronary circulation

The coronary circulation provides blood supply only to the myocardium. The right and left main coronary arteries originate above the right and left cusps of the aortic valves. The left anterior descending artery (LAD) provides diagonal and interventricular septal branches, whereas left circumflex coronary artery ends as a posterior LV branch after providing a left marginal branch, thereby supplying with a blood the left side of the heart.

1.6.3.1. ET-1

The role of ET-1 in the coronary circulation has been extensively discussed in the literature. Previous studies in human coronary arteries revealed that the expression of ET_A predominates in the coronary arteries compared to ET_B, and ET_A is the primary receptor that mediates coronary vasoconstriction to ET-1 [287,288]. ET-1 effects on vascular tone *in vivo* are the result of the balance between the contraction (mediated by ET_A and ET_B on VSMCs) and the dilatation (mediated by ET_B on endothelium). Because the coronary circulation is characterized by unique hemodynamic features (the blood flow in the coronary arteries appears during diastolic phase, while during the systolic phase the blood flow is at its lowest level due to contracting myocardium that squeezes the subendocardial coronary arteries), coronary arteries is a prime site for the development of endothelial dysfunction. Interestingly, it was revealed that the coronary artery endothelium has 5-fold reduced eNOS mRNA levels (compared to the aorta), while ET-1 expression was 2.5-fold higher [289]. Thereby, this pattern of eNOS *vs* ET-1 expression in the coronary vasculature may predispose coronary arteries to the development of endothelial dysfunction and atherosclerosis that later can negatively impact overall cardiac performance.

1.6.3.2. Endothelium-derived relaxing factors

Previous studies have demonstrated that NO production is primarily responsible for eliciting epicardial coronary vasodilation (to endothelium-dependent agonists) and showed

that the inhibition of NO synthesis results in the substantial reduction in coronary diameter [290-294]. Coronary blood flow increases with rise in cardiac metabolic demand [295]. An increase in cardiac myocardial oxygen consumption (MV_{O2}) was shown to be associated with an increase in plasma levels of oxidative products of NO in coronary sinus blood in healthy subjects, thereby suggesting that an increase in coronary NO release contributes to coronary flow response to cardiac metabolic demand [296]. Also, it was shown that NO is entirely responsible for the epicardial coronary vasodilation, and significantly contributes to microvascular vasodilation under metabolic demand in patients free of risk factors for atherosclerosis and without endothelial dysfunction [297]. However, in patients with risk factors, the vasodilation to acetylcholine was shown to be impaired together with a reduced contribution of NO to coronary epicardial and microvascular vasodilation [297].

The role of PGHS pathway in the regulation of coronary circulation has been broadly discussed in the literature. For instance, the study by Edlund *et al.* showed no changes in basal coronary tone or the coronary response to exercise in man after oral administration of non-selective inhibitor of PGHS (ibuprofen) [298], or the study by Dai and Bache that showed no alterations in coronary blood flow upon inhibition PGHS (with indomethacin) in dogs [299]. However, in models of coronary artery disease, the blockade of PGHS has been shown to alter coronary artery diameter and coronary flow. Altman *et al.* showed that the application of aspirin (that inhibits prostaglandin synthesis by covalently acetylating a serine in the catalytic pocket of the PGHSs) results in the constriction of collateral vessels (a vessel type that develops in response to coronary occlusion) in dogs [300], thereby indicating that PGHS pathway is involved in the mechanism of tonic vasodilation in coronary collateral vasculature. Also, other animal studies report an essential role of PGHS in the regulation of coronary circulation during hypoxia [301] and coronary artery occlusion [302]. For instance, Afonso *et al.* demonstrated a decreased coronary blood flow responses to hypoxia after administration of indomethacin (an inhibitor of both PGHS-1 and PGHS-2) [301], while study by Friedman

showed an increased coronary vascular resistance and decrease in coronary blood flow in patients with coronary artery disease, that was mediated due to the blockade of synthesis of vasodilatory PGI [303]. Subsequently, it appears that coronary circulation relies on PGHS-mediated mechanism of vasodilation mostly in pathology or in the states of cardiac metabolic load.

In addition to NO and PGI_2, the coronary vascular endothelium is also able to induce EDH that contributes to the regulation of vascular tone [304], thereby, an agonist- or flow-induced vasodilation that is independent of endothelium-derived NO and PGI_2 accounts to EDH of the underlying VSMCs. The complexity of endothelium-depended control of coronary blood flow is complicated due to complementary as well as inhibitory interaction between NO and EDH. For instance, previous research have shown that NO inhibits EDH (via a negative feedback pathway that is based on NO-mediated inhibition of cytochrome P450 [305,306]), and therefore it appears that when NO synthesis is impaired, an alleviation of this NO-mediated intrinsic inhibition may maintain endothelial vasodilator function. As such, previous study by Miura *et al.* have shown that NO contributes to flow-induced vasodilation in coronary arteries of patients without CAD, however, in patients with CAD, authors showed a substantial involvement of cytochrome P450 metabolites to flow-induced vasodilation, while the inhibition of NO and PGIs had no effect on coronary vasodilatory response [307].

It could be suggested that under uncomplicated physiological conditions, NO is a predominant factor that contributes endothelium-dependent vasodilation, however in pathological conditions, a decreased NO-stimulated vasodilation is often associated with an enhanced contribution of PGIs and EDH to vasodilatory mechanisms in coronary circulation.

1.7. The long-term impact of prenatal hypoxia on coronary artery function in adult offspring

Despite adverse long-term effects of prenatal hypoxia on the offspring systemic vascular function as well as cardiac performance that has been previously reported [24,227,234,239,245,247,250,252,308-316], the long-tern impact of prenatal hypoxia on adult offspring coronary artery function is currently the least investigated. Previous study by Kono *et al.* (in sheep model of pregnancy in high altitude) demonstrated a decreased intracellular concentration of calcium and a reduced tension response to calcium in LADs of adult offspring (sex of the offspring was not specified) [317]. In rat model of prenatal hypoxia Chen *et al.* demonstrated that the male offspring (females were not assessed) have an impaired coronary artery contractility to serotonin and a decreased coronary artery response to PKC agonist (phorbol 12, 13-dibutyrate) [318]. Further assessment of the mechanisms for impaired coronary artery contractility revealed a reduced PKCβ phosphorylation (at Ser660), decreased LTCC currents and a reduced expression of *Cacna1c* gene (that encodes the LTCC, $Ca_V1.2$ subunit). Moreover, the stimulation of VSMCs with caffeine uncovered an attenuated peak height, departure velocity, and return velocity of calcium transients, thereby indicating a reduced capability of calcium release from SR in coronary arteries of the prenatally hypoxic offspring [318]. However, it is currently unknown the effects of prenatal hypoxia on the mechanisms of coronary artery vasodilation or ET-1 mediated constriction in adult offspring.

1.8. Systemic therapeutic interventions during pregnancies complicated with prenatal hypoxia and offspring health

Various therapeutic strategies have been tested in animal models of prenatal hypoxia in order to improve offspring health [24,234,235,249,319-324]. Due to the fact that prenatal hypoxia is associated with excessive oxidative stress, those interventions are frequently aimed to reduce ROS production during complicated pregnancies. As such, several studies

have been demonstrating a successful application of those therapeutic interventions during hypoxic pregnancies and its beneficial effects on offspring health (Table 1.2).

Previous study by Hansell *et al.* in the rat model of prenatal hypoxia demonstrated a beneficial effect of maternal supplementation with melatonin on offspring health. Mainly, the application of melatonin during hypoxic pregnancy reduces hyper-reactivity of systemic arteries (mesenteric arteries) to phenylephrine and to the thromboxane mimetic (U46619) in adult offspring [249]. Another prenatal strategy of the application of allopurinol (a xanthine oxidase inhibitor) during hypoxic pregnancy showed encouraging results [234]. In particular, Niu *et. al.* reported that the supplementation with allopurinol to hypoxic dams ameliorates the long-term negative impact of prenatal hypoxia on offspring cardiac function by increasing offspring cardiac capacity to tolerate I/R insult and by decreasing cardiac sympathetic drive [234]. Maternal supplementation with Vitamin C during hypoxic pregnancies has also been tested and showed encouraging results [24,320,321,323]. Mainly, Vitamin C supplementation during hypoxic pregnancy enhanced systemic NO biovailability and endothelial-dependent vasodilation, and reduced mean arterial blood pressure in adult offspring (Table 1.2). Furthermore, the application of Vitamin C restored cardiac baroreflex gain, decerased cardiac sympathetic dominance, as well as ameliorated an adverse effect of prenatal hypoxia on hippocampal atrophy and memory loss in the adult offspring [24,320,321,323]. Recent studies have also employed a therapy with a mitochondria-targeted antioxidant (MitoQ) during hypoxic pregnancies [235,319]. For instance, maternal treatment with MitoQ during the hypoxic pregnancy increased NO-mediated vasodilation in systemic arteries, lowered mean arterial pressure and decreased cardiac sympathetic dominance and contractility in the offspring, thus proving the concept that mitochondria plays a key role of in developmental programming of offspring CV dysfunction [235,319].

Table 1.2. Systemic therapeutic interventions during hypoxic pregnancies and long-term offspring health.

Animal model of prenatal hypoxia	Treatment strategy	Offspring (sex & age)	Treatment outcomes in adult offspring	Ref.
Pregnant Wistar rats exposed to hypoxia (13 % O_2) from day 6-20 of gestation	Maternal treatment with melatonin (provided in drinking water)	Males only (4-month-old)	↓ Constrictor sensitivity to PE in mesenteric arteries ↓ Constrictor sensitivity to U46619 in mesenteric arteries	[249]
Pregnant ewes exposed to hypoxia (10% O_2) from day 105-138 of gestation	Maternal treatment with MitoQ (*iv* injection every day for 33 days)	(9-month-old)	↓ Mean arterial pressure and diastolic arterial pressure ↑ Femoral relaxation to SNP ↑ NO-dependent dilatation in femoral arteries	[319]
Pregnant Wistar rats exposed to hypoxia (13-14 % O_2) from day 6-20 of gestation	Maternal treatment with MitoQ (provided in drinking water)	Males only (4-month-old)	↓ Femoral blood flow amplitude ↓ SBP responses to PE in femoral arteries ↓ FBF Amp responses to PE and peak FBF Amp response in femoral arteries ↓ Cardiac contractility index, LVEDP and cardiac inotropic sympathetic dominance ↓ Cardiac *Cat* mRNA level	[235]
Pregnant Wistar rats exposed to hypoxia (13% O_2) from day 6-20 of gestation	Maternal treatment with vitamin C (provided in drinking water)	Males only (4-month-old)	↑ Relaxant response to Methacholine and the contribution of NO-independent mechanism ↑ Contribution of NO-independent mechanisms — Vasodilation response to SNP	[24]

Pregnant ewes exposed to hypoxia (10% O_2) from day 105-138 od gestation	Maternal treatment with vitamin C (*iv* injections)	Females only (9-month-old)	↑ Femoral vascular resistance following L-NAME treatment (greater circulating NO biovariability)	[320]
Pregnant Wistar rats exposed to hypoxia (13 % O_2) from day 6-20 of gestation	Maternal treatment with vitamin C (provided in drinking water)	Male only (4-month-old)	↓ Mean arterial pressure ↓ Low frequency / high frequency power ratio (an indicator of sympathetic to parasympathetic dominance) Restored the baroreflex gain towards control values	[323]
Pregnant Wistar rats exposed to hypoxia (13 % O_2) from day 6-20 of gestation	Maternal treatment with allopurinol (provided in jelly)	Male only (4-month-old)	↓ LVEDP, dP/dt_{max} ↓ Ratio of HR and LVDP response to maximal dose of isoprenaline and carbachol ↑ Cardiac tolerance to I/R insult ↓ Relative change of CK and LDH post I/R ↓ Cardiac SERCA2α expression	[234]

↑ Increase

↓ Decrease

Table 1.2 legend: *PE, phenylephrine; U46619, thromboxane A_2-mimetic (9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F_{2a}); SNP, sodium nitroprusside; SBP, systolic blood pressure; FBF Amp, fall in the amplitude of femoral blood flow; LVEDP, left ventricle end diastolic pressure; Cat, catalase; NO, nitric oxide; LVEDP, left ventricular end diastolic pressure; HR, heart rate; LVDP, left ventricular developed pressure; CK, creatine kinase; LDH, lactate dehydrogenase.*

1.9. Placenta-targeted strategies and maternal nMitoQ treatment during hypoxic pregnancies

The above discussed studies demonstrate that prenatal therapies that aimed to prevent oxidative stress during pregnancy are beneficial for long-term offspring health. Considering a central role of the placenta in the fetal development and growth, there are multiple therapeutic strategies that have been developed (in various animal models of pregnancy complications) for specifically targeting the placenta [325-328], in order to prevent any unforeseen off-target effects on the fetus.

In collaboration with Dr. Phillips (Cardiff University, UK) and Dr. Case (University of Bristol, UK), our laboratory has been exploring the effects of the placenta-targeted therapeutic strategy (with a mitochondria-targeted ubiquinone, MitoQ, encapsulated into nanoparticles (nMitoQ)) on placental, fetal and offspring outcomes in the rat model of prenatal hypoxia [252,329-332].

MitoQ is a commercially available antioxidant that is produced by linking ubiquinone to a positively charged lipophilic cation. Mitochondrial ubiquinone is a respiratory chain component that accepts two electrons from complexes I or II becoming reduced to ubiquinol, which then donates electrons to complex III [333-335]. Because the membrane potential of mitochondria is negative inside (130-150 mV), lipophilic cations have been used as a drug delivery system inside of the mitochondria due to its positive charge and its ability to pass through the hydrophobic barrier of the lipid bilayer [336]. Subsequently, ubiquinone antioxidant is covalently linked with lipophilic triphenylphosphonium (TPP^+) moiety. Positively charged TPP^+ cation enables MitoQ to rapidly permeate through the phospholipid bilayers of the plasma membrane and accumulate within the inner mitochondrial matrix in response to the large negative mitochondrial membrane potential. Previous research highlight a beneficial effect of application of MitoQ in animal models of cardiac dysfunction [337,338], sepsis [339], renal and brain [340,341] injury, endothelial dysfunction [342], hypertension [343] and liver

disease [344]. As was previously mentioned, the therapeutic potential of MitoQ was also explored in animal models of pregnancy complications [235,319,345]. The application of MitoQ during the hypoxic pregnancy increases placental volume and fetal capillary surface area (in the labyrinthine zone), expands maternal blood space, reduces mitochondrial stress [345] and improves offspring CV outcomes (Table 1.2, [235,319]).

Although MitoQ is able to cross the placenta (and has been detected in the fetal liver [345]), a novel technique that involves the attachment of MitoQ to biodegradable nanoparticles (that are composed of a poly γ-glutamic acid (γ-PGA) hydrophilic outer shell and an phenylalanine ethyl ester (L-PAE) hydrophobic inner core [346-348]) has been developed in order to specifically target the placenta and avoid direct fetal exposure and potential off-target effects [332]. These nanoparticles are not able to cross the outer syncytial membrane due to their large size (~180nm), negative zeta potential (-20mV) and their hydrophilic outer surface composition (that facilitates cellular uptake) [332]. Using fluorescent tagging, these nanoparticles were observed in placental tissue (after maternal *iv* infusion of nMitoQ) but were not detected in the fetus (fetal cortex or fetal liver) [332]. The authors also reported that nanoparticles were detected in the maternal liver, particularly in Kupffer cells and hepatocytes, as well as in the maternal brain. An increased oxidative stress in the maternal liver was no longer observed after nMitoQ treatment during the hypoxic pregnancy [332].

The Davidge lab with their collaborators showed that maternal nMitoQ treatment during hypoxic pregnancy rescued (increased) offspring birth weight, reduced placental oxidative stress and ameliorated some of the long-term effects of gestational hypoxia on the offspring brain [332]. Later, our laboratory explored sex-specific effects of maternal nMitoQ treatment on the placental mitochondria function and mitochondrial fusion [330]. Mainly, prenatal hypoxia reduced activity of the complex IV in the placenta from the male fetuses, that was rescued by nMitoQ treatment. Furthermore, prenatal hypoxia increased the

contribution of the N-pathway (through complex I) and decreased the contribution of the S-pathway (through complex II) in the placenta from female fetuses, however, nMitoQ treatment did not alter those functional changes, but increased the expression of the protein involved in mitochondrial fusion [330]. The assessment of fetal/placental outcomes revealed that maternal nMitoQ treatment reduces hypoxia in the placenta and liver (from the female fetuses) and hearts (from the male fetuses) in the rat model of prenatal hypoxia [329]. Moreover, nMitoQ treatment increases placental expression of vascular endothelial growth factor (Vegf) A and CD31 (markers of angiogenesis) and fetal blood space area of the female fetuses [329].

It was shown that maternal nMitoQ treatment is able to influence placenta-derived factors, that are linked with changes in the gene expression in the fetal brain during the hypoxic pregnancy [332]. In our lab, Ganguly *et al.* have demonstrated that a placenta-derived factors during the hypoxic pregnancy are also able to impair maturation and growth of fetal cardiomyocytes [331]. Mainly, conditioned medium from prenatally hypoxic placenta reduced the percentage of mononucleated cardiomyocytes and increased the percentage of binucleated cardiomyocytes (for both, male and female sex) [331]. The transition from mononucleate to binucleate state is associated with a decrease in the proliferative capacity of the heart due to the terminal differentiation [349]. Conditioned media from hypoxic placenta treated with nMitoQ increased the percentage of mononucleated cardiomyocytes to the control levels [331], thereby suggesting that strategy of maternal nMitoQ treatment has a potential to improve fetal cardiomyocyte development during pregnancies complicated with hypoxia.

The long-term impact of maternal nMitoQ treatment on offspring CV function has also been assessed in our lab [252]. Mainly, in 7-month-old male offspring, maternal nMitoQ treatment resulted in the increased pulmonary valve peak velocity, decreased mesenteric artery contractile capacity (to phenylephrine), increased NO modulation of vasoconstrictor responses (to phenylephrine) and decreased the contribution of NO to endothelial dependent

vasodilation, while in the females, nMitoQ treatment resulted in the decreased mitral valve E/A ratio and increased stroke volume [252]. In 13-months-old male offspring exposed to prenatal hypoxia, maternal nMitoQ treatment increased mitral valve deceleration time, increased mesenteric artery sensitivity to methacholine and phenylephrine. In females, there was decreased internal diameter in systole and LV volume in diastole, and an increased mitral valve E wave velocity, ejection fraction, fractional shortening and mesenteric artery sensitivity to methacholine [252]. Thus, these data suggest that maternal placental-targeted nMitoQ treatment during hypoxic pregnancy has potential benefits in offsetting the CV pathologies manifested in adult offspring. However, it is still unknown whether maternal nMitoQ treatment rescues impaired cardiac tolerance to I/R insult in adult male and female offspring.

To summarize, prenatal hypoxia is associated with early onset CV disease and increased susceptibility to cardiac I/R injury, however the mechanisms are not fully explored. Prenatal systemic interventions show promising results in the prevention of fetal programming of enhanced susceptibility to I/R insult in adult offspring, however, the long-term effects of a placenta-targeted treatment have not been fully explored. My studies are aimed to expand our knowledge on understanding the mechanisms of prenatal-hypoxia induced cardiac and coronary artery dysfunction in adult male and female offspring, and the effect of the placenta-targeted treatment on adult offspring cardiac health.

1.10. Hypotheses

- Enhanced susceptibility to cardiac I/R insult in adult prenatally hypoxic offspring is associated with upregulation of cardiac endothelin system, while a selective blockade of ET_A improves post ischemic cardiac performance in prenatally hypoxic offspring (Chapter 3).
- Prenatal hypoxia impairs coronary artery function in adult offspring by reducing endothelium-dependent vasodilation and ET-1-mediated constriction in the adult (4- and 9.5-month-old) male and female offspring (Chapter 4).

- Treating the placenta with a mitochondria-targeted antioxidant (nMitoQ) improves cardiac tolerance to ischemia/reperfusion insult in prenatally hypoxic offspring (Chapter 5)

1.11 Aims

- To assess the contribution of endogenous (local) cardiac endothelin system to an impaired post-ischemic cardiac recovery in 4-month-old male and female offspring.
- To assess the effect of prenatal hypoxia on vasoconstrictive and vasodilative mechanisms in left anterior descending coronary arteries of 4- and 9.5-month-old male and female offspring.
- To assess the effects on placenta-targeted treatment with a mitochondria-targeted antioxidant (nMitoQ) during hypoxic pregnancy on cardiac susceptibility to I/R insult in adult male and female offspring.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animal ethics approval

The animal study protocol was approved by the Animal Care and Use Committee Health Sciences 2 (Animal Policy and Welfare Committee of the University of Alberta) in accordance with the Canadian Council on Animal Care and the "Guide for the Care and Use of Laboratory Animals" (AUP #242 and #3692).

2.2. Rat model of prenatal hypoxia

Three-month old female Sprague-Dawley rats (Charles River, Kingston, NY; Raleigh, NC; Hollister, CA, USA) were mated overnight by housing with young Sprague-Dawley males. Pregnancy was confirmed the following morning by the presence of sperm in the vaginal smear (designated as gestational day [GD] 0). Throughout the pregnancy period, rats were single housed in standard rat cages under a 10:14h light-dark cycle and fed standard rat chow *ad libitum*. Pregnant dams were randomly divided into two groups (normoxia and prenatal hypoxia (p-Hypoxia)). Rats were exposed to either hypoxia (11% O_2; p-Hypoxia group) from GD 15-21 by placing them in a Plexiglas hypoxic chamber or were housed at atmospheric oxygen (21% O_2) throughout pregnancy as a normoxic control group. Rats were removed from the hypoxic chamber on GD 21 (GD 22=term) and were allowed to give birth in standard housing and oxygen conditions. At birth, litter size was decreased to 8 pups/litter (4 males and 4 females) to standardize postnatal conditions. At postnatal day 21, the offspring were weaned, sex-matched and double housed at standard housing conditions until euthanasia/experiments at 4- and/or 9.5 months of age.

2.3. Assessment of cardiac function using *ex vivo* isolated working heart technique

At 4 months of age, offspring were anesthetized by inhaled isoflurane (pre-oxygenation with oxygen alone for 4-5 breaths, start off with 0.5% isoflurane and increase by 0.5% increments every few breaths to a maximum of 4%) and cardiac function was assessed using an *ex vivo* isolated working heart preparation. Hearts were excised, kept in

ice-cold modified Krebs-Henseleit solution (mmol/L: 120 NaCl, 25 $NaHCO_3$, 5 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 1.25 $CaCl_2$; pH 7.4) and directly mounted onto an aortic cannula and ligated with silk sutures. For ~10 min, hearts were perfused in retrograde Langendorff mode with modified Krebs-Henseleit solution (37°C, pH 7.4, gassed with 95% O_2 and 5% CO_2). During this time, the left atrium was cannulated and ligated with silk sutures. Afterwards, the anterograde flow was opened, and the retrograde flow was closed, so hearts were set up in isolated working heart mode. Hearts were perfused in anterograde mode with modified Krebs-Henseleit solution containing 1.2 mmol/L palmitate fatty acid. This concentration of fatty acids in the perfusion buffer was used because a high plasma concentration of fatty acids has been previously reported in patients during cardiac bypass surgery and patients after acute myocardial infarction [195,350]. Unlike the Langendorff preparation (that relies only on the retrograde perfusion of the heart through the coronary vasculature), in the isolated working heart set up the myocardial perfusion is achieved during the course of ventricular relaxation when the aortic hydrostatic pressure leads to perfusion of the coronary vasculature of the heart [351,352]. The pressure at the cannulated left atrium is equivalent to 11.5 mmHg, the buffer passes to LV, from which it is spontaneously ejected through the aortic cannula against a pressure equivalent to 80 mmHg (afterload). As such, isolated working heart represents a more physiological assessment of cardiac function. Cardiac data was recorded using Hugo Sachs Elektronic data acquisition system and Isoheart Software for Windows 10 (Harvard Apparatus, Germany). Cardiac function was reported as cardiac power (J min-1 (g dry weight) -1) and calculated as: [(peak systolic pressure, mmHg−maximal preload, mmHg) × cardiac output, mL/min × 0.13]/dry weight in g (joules/min/g dry weight). At the end of the experiments, the LV with septum was separated and stored at −80°C for further molecular analysis.

2.3.1. Protocol for assessment of cardiac function (Chapter 3)

Measurements of cardiac function were carried out throughout the whole duration of the experiment. The protocol for cardiac I/R challenge consisted of 30 min aerobic perfusion with 25 min global ischemia followed by 40 min of aerobic reperfusion or hearts were aerobically perfused for 95 min (aerobic control). To assess the contribution of ET_A to the development of cardiac dysfunction (Chapter 3), a selective ET_A antagonist, Atrasentan hydrochloride (ABT-627, 5 μmol/L, Sigma-Aldrich, Burlington, MA, USA; CAS#: 195733-43-8,) [187], in dimethyl sulfoxide (DMSO; Sigma-Aldrich; CAS#67-68-5)) was infused for 20 min. before the ischemic insult and recirculated in the system during reperfusion.

2.3.2. Protocol for assessment of cardiac function (Chapter 5)

Measurements of cardiac function were carried out throughout the whole duration of the experiment. The protocol for cardiac I/R challenge consisted of 30 min aerobic perfusion with 20 min global ischemia followed by 40 min of aerobic reperfusion.

2.4. Assessment of coronary artery vascular function by wire myography

After collection of the heart, the left descending coronary artery (LADs; 150–250 μm) was isolated and cleaned from the myocardium in ice-cold physiological salt solution (PSS; in mmol/L: 10 HEPES, 5.5 glucose, 1.56 $CaCl_2$, 4.7 KCl, 142 NaCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, pH 7.5) and divided into ~1-2 mm segments. Vascular function was assessed *ex vivo* using wire myography. The successful dissection and isolation of these vessels allowed to obtain 1-3 segments of LAD per one heart. Subsequently, we used one to two offspring (of the same sex) per one dam in order to perform all the vascular function experiment. The data are presented based on the dam as the experimental unit; thus, duplicate curves from 2 offspring from the same dam were averaged. The segments of LAD were mounted onto a wire myograph system (620M DMT, Copenhagen, Denmark) using 25 μm tungsten wires. Isomeric tension of the vessels was recorded using LabChart software (version 8.1.13; AD Instruments; Colorado Springs, CO, USA). All vessels were normalized through a series of stepwise

increases in diameter to reach their optimal resting tension: 0.8 × IC100; 13.3 kPa (the internal circumference equivalent to a transmural pressure of 100 mmHg). After normalization, the vessels were exposed to the first wake up dose of the thromboxane A_2 receptor agonist 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α (U-46619; 1 μmol/L; Sigma-Aldrich, St. Louis, MO, USA; CAS# 56985-40-1) for 5 min. After washing with PSS thrice and 10 min rest, the vessels were exposed to a second wake up dose of U-46619 followed by a single dose of methylcholine (MCh; 3 μmol/L; Sigma-Aldrich; CAS# 62-51-1) to confirm endothelial cell function. If LAD segments developed spontaneous sustained vasoconstriction during the experiment, the data obtained from those segments were excluded. After washing and 30 min of rest, vascular responses to MCh were assessed using a cumulative concentration response curve (CCRC; 0.001 μmol/L to 100 μmol/L MCh; doses were added in 2-minute intervals) after pre-constriction with EC_{80} concentration of U-46619 (0.2 μmol/L; the mean effective concentration that produces 80% of the maximal response). After washing with PSS thrice and 30 min of rest, vasoconstriction responses to ET-1 were assessed in LAD of 4-month-old and 9.5-month-old offspring using CCRCs to ET-1 (CCRC; 0.0001 μmol/L to 0.3 μmol/L; Sigma-Aldrich; CAS# 117399-94-7) in the presence or absence of ET_A (BQ-123; 10 μmol/L; MilliporeSigma; CAS# 136553-81-6) or ET_B (BQ-788; 10 μmol/L; MilliporeSigma; CAS# 156161-89-6) receptor antagonists [353]. As we observed impaired vasodilation to MCh at 4-months, which was maintained at 9.5 months of age, specific vasodilation pathways potentially involved in the impaired endothelium-dependent vasodilation were assessed in the 9.5-month-old offspring. CCRCs to MCh were performed in the absence or presence (30 min pre-incubation prior to U-46619 EC_{80} dose) of the following specific inhibitors: NOS was inhibited with the pan NOS inhibitor N(G)-nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μmol/L; Sigma-Aldrich; CAS# 51298-62-5) [354], PGHS was inhibited with meclofenamate (10 μmol/L; Sigma-Aldrich; CAS# 6385-02-0) and EDH-mediated vasodilation was inhibited with a combination of apamin (0.5 μmol/L; Sigma-Aldrich; CAS# 24345-16-2) [355] which blocks small-conductance Ca^{2+}-activated potassium

channels (SK), and 1-(2-chlorophenyl)diphenylmethyl-1H-pyrazole (Tram-34; 0.4 μmol/L; Sigma-Aldrich; CAS# 289905-88-0) [355], an intermediate-conductance Ca^{2+}-activated potassium channel (IK) inhibitor. Simultaneously, a CCRC to the NO donor sodium nitroprusside (SNP CCRC; 0.0001 μmol/L to 10 μmol/L, Sigma-Aldrich; CAS# 13755-38-9; doses were added in 2-minute intervals) was conducted in one of the LAD segments to assess endothelium-independent relaxation. All data were presented as the maximum relaxation response to MCh (E_{max}), the negative log of the mean effective concentration that produces 20% and 50% of the maximal response (pEC_{20} and pEC_{50}, respectively), as a measure of vasodilator sensitivity, or as area under the curve (AUC).

2.5. Assessment of cardiac protein levels with Western blotting

Frozen LV tissues (50-100 mg/sample) were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 10 mmol/L NaF) (Chapter 3) or lysis buffer (20 mmol/L Tris (pH 7.4), 5 mmol/L EDTA, 10 mmol/L Tetrasodium pyrophosphate, 100 mmol/L Sodium Fluoride, 1% NP-40 (Chapter 5) with 2 mmol/L Sodium orthovanadate and 1X Protease Inhibitor (SIGMAFAST™ Protease Inhibitor Cocktail Tablets, EDTA-Free; Sigma-Aldrich Canada Co., Oakville, ON, Canada) and 20 μg/ml phenylmethylsulfonyl fluoride (PMSF; MilliporeSigma; CAS#329-98-6).

Total protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Total protein (50-100 μg) was loaded and separated on SDS polyacrylamide gels for 2-3 hours at 4°C. The resolved proteins were then transferred onto nitrocellulose membranes (0.2 μm: Biorad, Mississauga, ON, Canada). Equal protein loading was confirmed by incubation of the membrane with α-tubulin or total protein staining. For the total protein staining, membranes were incubated with Revert™ total protein stain (LI-COR Biotechnology, Lincoln NE, USA) for 5 min while gently shaking, and then rinsed with a wash solution (6.7% glacial acetic acid and 30% methanol) twice. Membranes were imaged in the

700 "red" channel using the LI-COR Odyssey Imaging Systems v3.0 (LI-COR Biosciences, Lincoln, NE, USA). After imaging the blots, membranes were incubated with a stain reversal solution (0.1 mol/L sodium hydroxide, 30% methanol), two times for 5 min, while gently shaking. The membranes were rinsed briefly in deionised, distilled water followed by incubation with 50-100% blocking reagents (Rockland Immunochemicals, Inc., Pottstown, PA, USA) for 1.5-2 hours at room temperature. After washing in PBS with 0.1% Tween 20 (PBST), the membranes were incubated overnight at 4°C with primary antibodies (in PBST). The following day, after washing 3 times with PBST for 10 min each, membranes were probed with secondary antibodies in 25% blocking reagent for 1 hour at room temperature with agitation. After the secondary antibody incubation, the membranes were washed with PBST (3X10 min), then rinsed with distilled water. Protein bands were imaged, and densitometry analysis was performed using the Li-Cor Odyssey Imaging Systems v3.0 (LI-COR Biosciences).

2.5.1. Primary and secondary antibodies (Chapter 3)

Total protein (50 µg/sample) was loaded and separated on 10% SDS polyacrylamide gel for 1.5-2 hours at 4°C. Nitrocellulose membranes were incubated overnight at 4 ºC with primary antibodies (Table 2.1). The following day, after washing 3 times with PBST for 10 min each, membranes were probed with secondary antibody IRDye™ 800CW-labeled donkey anti-rabbit (1:10 000; LI-COR Biosciences; Cat# 926-32213) in 25% blocking reagent for 1 h at room temperature with agitation.

Table 2.1. List of primary antibodies used in Chapter 3

Antibody	Dilution	Company
rabbit polyclonal anti-ET_A	1:5000	Thermo Fisher Scientific, Waltham, MA, USA; Cat# PA3-065
rabbit polyclonal anti-ET_B	1:7500	Bioss, Woburn, MA, USA; Cat# bs-4198R

2.5.2. Primary and secondary antibodies (Chapter 5)

Total protein (50-100 µg/sample) was loaded and separated on 7.5% for PKCε [pPKCε Ser729], 11% for SERCA2α, PLN [pPLN Ser16/Thr17] and PP2Ce, and 12% for CaMK2δ [pCaMK2δ Thr287] for 2-3 hours at 4°C. Equal protein loading for PKCε was confirmed using α-tubulin (1:10,000 mouse monoclonal, GenScript, Piscataway NJ, USA; Cat# A01410). Nitrocellulose membranes were incubated overnight at 4 ºC with primary antibodies (Table 2.2).

Table 2.2. List of primary antibodies used in Chapter 5

Antibody	Dilution	Company
rabbit polyclonal anti-SERCA2α	1:1000	Abcam, Waltham, MA, USA; Cat# ab137020
mouse monoclonal anti-PLN	1:250	Cell Signaling Technology, Danvers, MA, USA; Cat# cs8496
rabbit polyclonal anti-pPLN Ser16/Thr17	1:250	Thermo Fisher Scientific, Cat# MA3922
mouse monoclonal anti-CaMK IIδ	1:100	Novus Biologicals, Centennial, CO, USA; Cat# H0000817-M02
rabbit polyclonal anti-pCaMK IIδ Thr287	1:100	Thermo Fisher Scientific, Cat# PA5-37833
mouse monoclonal anti-PP2Ce	1:500	Bio-Techne, Minneapolis, MN, USA; Cat# MAB6914
mouse monoclonal anti-PKCε	1:10	Santa Cruz, Piscataway, NJ, USA; Cat# sc56944
rabbit polyclonal anti-pPKCε Ser729	1:100	Santa Cruz, Cat# ab63387

The following day, after washing 3 times with PBST for 10 min each, membranes were probed with secondary antibodies (all at 1:10,000; Li-Cor Biosciences) were: IRDye™ 680RD-labeled donkey anti-mouse (Cat# 926-68072) for PLN, CaMK IIδ, PP2Ce, α-tubulin; IRDye™ 680RD-labeled donkey anti-rabbit (Cat# 926-68073) for pPKCε Ser729; IRDye™ 800CW-labeled donkey anti-mouse (Cat# 926-32212) for PKCε and IRDye™ 800CW-labeled donkey anti-rabbit (Cat# 926-32213) for SERCA2α, pPLN Ser16/Thr17, pCaMK IIδ Thr287.

2.6. Assessment of coronary artery protein levels with immunofluorescent detection

The main left coronary artery (from 4- and 9.5-month-old offspring) was isolated and immediately snap frozen in optimal cutting temperature compound (Tissue-Tek®, Sakura Finetek, Inc.Torrance, CA, USA) for further molecular analysis. Cryo-sections (9 µm) of coronary arteries of the 4- and 9.5-month-old offspring were prepared using a cryostat (SLEE medical GmbH, Nieder-Olm, Germany) for immunofluorescent detection of ET-1, endothelin A (ET_A), and endothelin B (ET_B) receptors, and for eNOS, PGHS-1, and PGHS-2 in coronary arteries of the 9.5-month-old offspring. Sections were fixed in ice cold methanol (-20°C for 10 min), then acetone (-20°C for 5 min), air-dried, and washed in PBS (pH: 7.5; 3 × 5 min). Afterwards, sections were subjected to an autofluorescence reduction treatment by incubating with $NaBH_4$ (MilliporeSigma, CAS# 16940-66-2) in PBS (1 mg/mL) for 10 min at room temperature and washed with PBS (3 x 10 min). After incubation with blocking solution (PBS supplemented with 2% donkey serum and 1% bovine serum albumin (BSA; MilliporeSigma; CAS# 9048-46-8) with 0.1% Triton X-100 in PBS) for 1 h at room temperature, sections were washed with PBS (3 x 10 min) and incubated overnight at 4°C in 1% BSA/PBS with the primary antibodies (Table 2.3).

Table 2.3. List of primary antibodies used in Chapter 4

Antibody	Dilution	Company
rabbit polyclonal anti-ET-1	1:50	Bioss, Cat# bs-0954R
rabbit polyclonal anti-ET_A	1:300	Thermo Fisher Scientific; Cat# PA3–065
rabbit polyclonal anti-ET_B	1:50	Bioss, Cat# bs-4198R
mouse monoclonal anti-eNOS (NOS Type III)	1:50	BD Biosciences, Mississauga, ON, Canada; Cat# 610297
rabbit polyclonal anti-COX-1	1:100	Abcam; Cat# ab53766
rabbit polyclonal anti-COX-2	1:50	Abcam, Cat# ab52237
mouse monoclonal anti-PECAM-1	1:200	BD Pharmingen, Mississauga, ON, Canada; Cat# 550300

The next day, sections were washed with PBS (3 x 5 min) and incubated with secondary antibodies (all at 1:250; Thermo Fisher Scientific) with 4',6'-diamidino-2-phenylindole (DAPI; 1:500 Thermo Fisher Scientific, Cat# D3571) in 1% BSA/PBS for 1 h at RT in the dark: donkey-anti-mouse Alexa Fluor™ 488 (Cat # A-21202) for eNOS, donkey-anti-mouse Alexa Fluor™ 546 (Cat # A10036) for CD31, and donkey-anti-rabbit Alexa Fluor™ 488 (Cat # A-21206) for ET-1, ET_A, ET_B, PGHS-1, and PGHS-2. After washing in PBS (3 x 10 min), mounting medium containing DAPI was added (Vector Laboratories, Burlingame, CA, USA), and slides were stored in the dark and left to dry overnight.

Immunofluorescent images were obtained the next day using a confocal Zeiss LSM 700 microscope with Zen Black software (version 8.1.6.484; Zeiss, Toronto, ON, Canada). Gains for AF546, AF488, and DAPI were set using the blank sections (i.e., incubated with secondary antibody only). Fluorescent images of ET-1, ET_A, ET_B, eNOS, PGHS-1, and PGHS-2 staining in the coronary arteries were analyzed using FIJI ImageJ software (version 1.53n; Wayne Rasband NIH, Bethesda, MD, USA). The total vessel area was selected for ET-1, ET_A, eNOS, PGHS-1, and PGHS-2, the vessel area outside of the endothelium (the area negative for CD31) was selected for ET_B (ET_B that are located on VSMCs mediate vasoconstriction [356]), and the mean fluorescent intensity was measured.

2.7. Enzyme Linked Immunosorbent Assay (ELISA)

2.7.1. Molecular assessment of plasma levels of ET-1 with ELISA

Plasma was collected using ethylenediaminetetraacetic acid (EDTA) anticoagulant from 4- and 9.5-month-old offspring, centrifuged and stored at -80 °C for assessment of ET-1 levels. Plasma levels of ET-1 were assessed using a rat endothelin-1 ELISA kit (Boster Bio, Pleasanton, CA, USA; Cat# EK0952), according to the manufacturer's instructions. Briefly, on the day of experiment, plasma samples were thawed on ice and vortexed. All reagents and working standards of the ELISA kit were prepared on the same day as the assay procedure.

After adding all standards, controls, and samples in duplicate, plates were sealed and incubated for 90 min at 37°C. The plates were emptied, and each well was washed with washing solution 3 times. Next, biotinylated anti-rat Edn1 antibody was added in each well, sealed, and incubated for 60 min at 37°C. Afterwards, the plates were emptied and washed 3 times with washing buffer. Avidin-biotin-peroxidase complex was added in each well, and the plate was sealed and incubated for 30 min at 37°C. Plates were emptied, and each well was washed with washing solution 5 times. Color developing reagent was added to each well, and plates were sealed and incubated in the dark for 20 min at 37°C. Stop solution was added, and the absorbance of each well was measured under 450 nm wavelength within 30 min with multimode reader (BioTek Synergy HTX, Santa Clara, CA, USA). To determine the amount of ET-1 in the plasma sample, a standard curve was generated by plotting the average absorbance (450 nm) obtained for each of the eight standard ET-1 concentrations provided. The absorbance value was calculated for each standard and sample well, and all absorbance values (average of duplicates) were subtracted by the average of the zero-standard value (i.e., the blank). The levels of ET-1 in each plasma sample were calculated using the standard curve.

2.7.2. Molecular assessment of cardiac tissue levels of ET-1 with ELISA

LV with septum from aerobically perfused hearts or hearts, subjected to I/R protocol, was separated and stored at –80°C for further assessment of cardiac levels of ET-1 using a rat endothelin-1 ELISA kit (MyBioSource, San Diego, CA, USA; Cat# MBS774571), according to the manufacturer instructions. Frozen LV tissue was weighed and thawed in PBS (pH 7.4) at 2-8 ºC and homogenized thoroughly. Samples were centrifuged at 3000 RPM for 20 min. and supernatant was collected. All reagents of the ELISA kit and the samples were prepared on the same day as the assay procedure. Before the procedure, washing solution was added to each well and incubated for 1-2 min, and this process was repeated for 5 times. After adding all standards and samples in duplicate, the HRP-conjugate reagent was added to each

well, the plate was covered with sealing membrane and gently shaken and mixed for 60 min. at 37 ºC incubation. After the sealing film was removed, the plate was emptied, and each well was washed with washing solution, as described previously. For color developing, chromogen solution A and chromogen solution B were added in each well and the plate was gently shaken to mix. The plate was incubated for 15 min. at 37 ºC, in the dark. Stop solution was added to each well and the absorbance of each well was measured under 450 nm wavelength within 10 min. To determine the amount of ET-1 in the sample, a standard curve was generated by plotting the average absorbance (450 nm) obtained for each of the six standard ET-1 concentrations on the y-axis versus the corresponding concentration on the x-axis. Afterwards, the mean absorbance value was calculated for each standard and sample well, and all absorbance values were subtracted by the mean value of the blank value. The amount of ET-1 in each sample was calculated using the standard curve. Intra-assay coefficient of variability was 3.23% (inter-assay % coefficient of variability of less than 7% was considered an acceptable value, according to the manufacturer's instructions). According to the manufacturer, the ET-1 antibody from the ELISA kit does not cross react with either preproET-1 and big-ET-1, while information is not available for ET-3.

2.8. Maternal treatment during hypoxic pregnancy and preparation of nMitoQ

In Chapter 5, pregnant normoxic and hypoxia dams were randomly divided into two groups (control or treatment) and intravenously injected via the tail vein with 100 µL of either saline (control) or nMitoQ (125 µmol/L; treatment) on GD 15 before hypoxia exposure. The dose of nMitoQ was chosen based on the previous study by Phillips *et al.* [332]. Experimental offspring groups consisted of: Normoxia/Saline, Normoxia/nMitoQ, p-Hypoxia/Saline, p-Hypoxia/nMitoQ.

The preparation of nMitoQ has been previously described [332]. Briefly, mitochondrial antioxidant, MitoQ, was adsorbed by hydrophobic and electrostatic interaction with nanoparticles consisting of an amphiphilic copolymer of poly(γ-glutamic acid) and L-

phenylalanine ethylester (γ-PGA-Phe), that were synthesized as described previously by Kim *et al.* [346]. γ-PGA-Phe (10 mg/mL) was dissolved in dimethyl sulfoxide (DMSO), added to equivalent volume of 0.15 mol/L NaCl and dialyzed against distilled H_2O. The solution was freeze-dried and resuspended in phosphate buffered saline (PBS) solution (10 mg/mL). γ-PGA-Phe nanoparticles were mixed with MitoQ (2 mg/mL) at equivalent volume in 0.2 mol/L NaCl, and afterwards were incubated at 4°C for 12 h. Nanoparticles were centrifuged, washed, and resuspended in PBS to 10 mg/mL. The amount of MitoQ that was absorbed to nanoparticles (278 nm) was evaluated by UV absorption measurement as was previously described [332].

2.9. Statistical analysis

2.9.1. Statistical analysis for Chapter 3

Offspring were randomized for each experimental procedure with n=1-2 offspring/sex/dam. Data were analyzed by two-way ANOVA with Sidak`s post hoc test (GraphPad Prism, version 9.1.2.; San Diego, CA, USA; https://www.graphpad.com/scientific-software/prism/), with 2 between-subject factors: prenatal hypoxia and sex (for cardiac levels of ET_A and ET_B), prenatal hypoxia and I/R (for cardiac ET-1 levels) or prenatal hypoxia and ABT-627 (for assessment of cardiac function). Data were presented as means ± standard error of the mean (SEM); $p<0.05$ was considered statistically significant.

2.9.2. Statistical analysis for Chapter 4

Data from the dose response curves were fitted to the Hill equation, and pEC_{50} and pEC_{20} values were calculated. The pEC_{50} and pEC_{20} values are defined as the negative logarithm of the EC_{50} and EC_{20} (the concentration of agonist that provokes a response 50% and 20% of maximum response, respectively) and represent receptor sensitivity to the agonist. For MCh (in the presence or absence of L-NAME) and ET-1 (in the presence or absence of BQ123 or BQ788) dose response curves, the area under the curve (AUC) was calculated as

a cumulative measurement of an inhibitor effect. Data were analyzed by student t-test or two-way ANOVA with Sidak's post-hoc test (GraphPad Prism, version 9.1.2.; San Diego, CA, USA; https://www.graphpad.com/scientific-software/prism/). Data were presented as means ± SEM; $p<0.05$ was considered statistically significant.

2.9.3. Statistical analysis for Chapter 5

Offspring were randomized for each experimental procedure with one or two offspring/sex/dam. Male and female offspring were assessed separately. A two-way ANOVA with Sidak`s post hoc analysis was used for assessment of the effect of prenatal hypoxia and our prenatal intervention (nMitoQ treatment). Welch's two-sample t-test (also referred to as the unequal variance t-test) was used to determine statistical differences in the variance in cardiac recovery to baseline between the Hypoxia/Saline and Hypoxia/nMitoQ groups. Data were statistically analyzed using GraphPad Prism 8 (San Diego, CA, USA; https://www.graphpad.com/scientific-software/prism/) and presented as means ± SEM; $p\leq0.05$ was considered statistically significant.

CHAPTER 3

SEX-SPECIFIC EFFECTS OF PRENATAL HYPOXIA ON THE CARDIAC ENDOTHELIN SYSTEM IN ADULT OFFSPRING

A version of this chapter has been published:

Hula, N., Vu J., Quon A., Kirschenman R., Spaans F., Liu R., Cooke C. M., and Davidge S. T. "Sex-Specific Effects of Prenatal Hypoxia on the Cardiac Endothelin System in Adult Offspring." American Journal of Physiology - Heart and Circulatory Physiology 322, no. 3 (Mar 2022): H442-H50. https://dx.doi.org/10.1152/ajpheart.00636.2021.

Author contributions: N.H., conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved final version of manuscript; **J.V.,** performed experiments, analyzed data, edited and revised manuscript, approved final version of manuscript; **A.Q.,** interpreted results of experiments, edited and revised manuscript, approved final version of manuscript; **R.K.,** performed experiments, edited and revised manuscript, approved final version of manuscript; **F.S.,** conceived and designed research, interpreted results of experiments, drafted manuscript, edited and revised manuscript, approved final version of manuscript; **R.L.,** drafted manuscript; edited and revised manuscript, approved final version of manuscript; **C.M.C.,** edited and revised manuscript, approved final version of manuscript; **S.T.D.,** conceived and designed research, interpreted results of experiments, drafted manuscript, edited and revised manuscript, approved final version of manuscript.

3.1. Introduction

CV disease is a leading cause of morbidity and mortality in the world and is recognized as a major concern for global health worldwide [357]. There is now a substantial body of evidence showing that the quality of the prenatal period of fetal development can determine the health of the offspring later in life [22-24]. Fetal hypoxia impairs fetal development and growth and is associated with an increased risk of fetal morbidity and mortality [1]. In a long-term perspective, fetal hypoxia has been show to result in elevated blood pressure [358,359], an impaired nitric oxide-dependent vasorelaxation (in femoral [24], mesenteric [311] and pulmonary [360] arteries resistance), changes in cardiac structure [227], cardiac diastolic dysfunction [252], increased myocardial contractility with sympathetic dominance [24,234] and increased susceptibility to cardiac I/R insult [227,234,239,247,361] in adult offspring.

Previous research demonstrated a direct correlation between the hypoxic status of the fetus and elevated levels of ET-1 in the fetal circulation [362], thus suggesting that high levels of ET-1 play an essential role in maintenance of fetal circulatory homeostasis. Hypoxia is a major regulator of ET-1 synthesis due to the presence of HIF-1α binding site in the 5' promoter region of *edn1* gene [363], and is able to increase ET-1 production and ET-1 responsiveness in cardiomyocytes [364]. Indeed, previous reports have shown a direct link between fetal hypoxia and the ET-1 system. For instance, Bae *et al.* demonstrated that fetal hearts from hypoxia-exposed pregnant rats express higher levels of HIF-1α compared to the normoxic controls, implying that the fetal myocardium was hypoxic [85], and in a follow-up study, the same group reported increased preproendothelin-1 mRNA levels in fetal rat cardiomyocytes from pregnant rats exposed to hypoxia compared to normoxic controls [365]. Importantly, ET-1 treatment of fetal rat cardiomyocytes decreased cardiomyocyte proliferation, increased the percent binucleate cells and DNA methylation [365], and induced changes in the fetal cardiomyocyte proteome [366], thus suggesting that ET-1 may be involved in a premature

transition of terminal differentiation of cardiomyocytes, which, subsequently, may result in a reduced number of cardiomyocytes and altered cardiac growth after birth [365,366].

Cardiac-derived ET-1 functions as a paracrine, autocrine, and intracrine regulator of normal cardiac performance, with the potential to contribute to the development of different CV pathologies (reviewed in [136]). Extensive clinical studies demonstrated that ET-1, being a potent vasoconstrictive agent, plays an essential role in the development and progression of different CV diseases, including hypertension [367,368], atherosclerosis [369,370], pulmonary arterial hypertension [371,372], coronary artery disease [127,373,374], heart failure [375,376] and myocardial infarction [127,181]. In addition, cardiac ET-1 content was shown to be increased in the myocardium after I/R compared to non-ischemic tissue [377], indicating that I/R alone induces biosynthesis of ET-1. Active ET-1 exerts its effects through ET_A and ET_B [363], and an inhibition of ET_B has been shown to impair post-ischemic cardiac recovery [187], whereas targeted inhibition of ET_A or dual inhibition of ET_A and ET_B has been shown to improve post-ischemic cardiac performance in various animal models of myocardial ischemia [187,378-382]. Our laboratory previously showed an enhanced reactivity to the (inactive) ET-1 precursor, bigET-1, in systemic (mesenteric) arteries from adult offspring exposed to prenatal hypoxia, suggesting that prenatal hypoxia may have long-term effects on the vascular ET-1 system in the offspring [314]. However, the link between prenatal hypoxia and offspring cardiac ET-1 system has not been previously investigated. Thus, in the current study, we hypothesized that prenatal hypoxia alters the cardiac ET-1 system in adult male and female offspring by increasing ET-1 levels and ET_A expression, thus contributing to the development of cardiac dysfunction, while selective blockade of ET_A improves cardiac performance after I/R insult.

3.2. Results

3.2.1. Assessment of cardiac function using ex vivo *isolated working heart technique*

In male offspring, infusion of ABT-627 (a selective ET_A antagonist) did not affect pre-ischemic cardiac performance in either the normoxic or prenatally hypoxic groups (Figure 3.1, A. *i*; *ii*). Infusion of ABT-627 did not alter the percent recovery of baseline in the normoxia male offspring (control: 56.8±12.3% vs ABT-627: 88.8±6.3%; p=0.18), while in prenatally hypoxia males, pre-incubation with ABT-627 prevented the recovery from ischemia (Figure 3.1. A. *iii*). In female offspring, pre-ischemic cardiac power was similar between groups (Figure 3.1. B. *i*; *ii*). The percentage recovery from baseline was not altered by ABT-627 in hearts from female normoxia offspring (control: 55.4±10.5% vs ABT-627: 68.9±6%; p=0.55), but the percent recovery after ischemia tended to be increased after pre-incubation with ABT-627 in hearts of female offspring exposed to prenatal hypoxia (p=0.0528; Figure 3.1. B. *iii*).

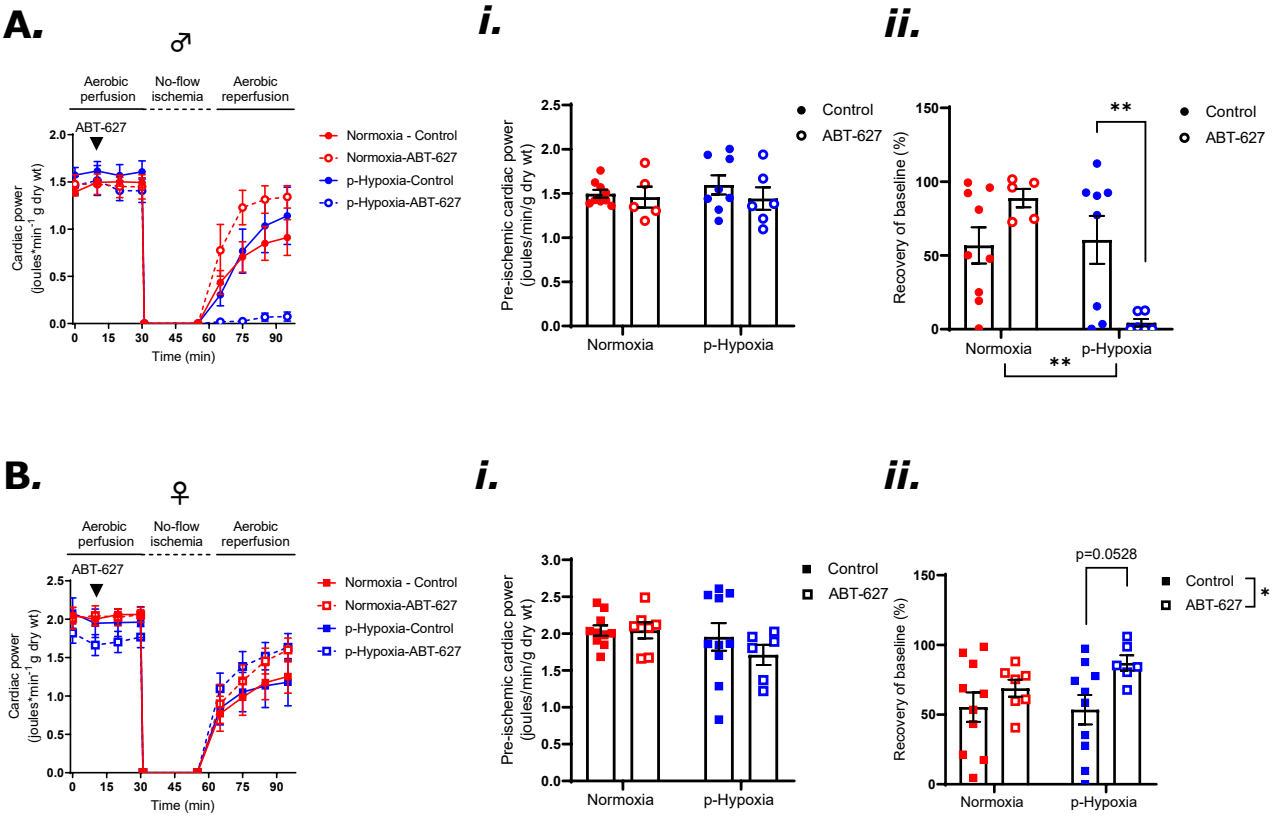


Figure 3.1. Cardiac power development *ex vivo* in adult male and female offspring.

The ex vivo *experimental design consisted of 30 min of aerobic perfusion followed by 25 min no-flow ischemia with 40 min of aerobic reperfusion (i). Summarized as cardiac power development pre-ischemia (ii) and the percentage of cardiac recovery of the baseline after ischemia/reperfusion insult (iii) in hearts from male (circles; A) and female (squares; B) offspring exposed to normoxia (red symbols) or prenatal hypoxia (blue symbols), without (solid symbols, solid line) or with (open symbol, dashed line) pre-infusion of the ET_A antagonist ABT-627. Data are presented as mean±SEM and were analyzed with two-way ANOVA and Sidak`s post hoc analysis, n=4-6 dams/group; 1-2 offspring/dam. *p<0.05, **p<0.01.*

3.2.2. Cardiac ET-1 content

Cardiac levels of ET-1 were increased after I/R compared to aerobically perfused hearts in both male (0.22±0.012 pg/µg vs 0.31±0.022 pg/µg; p=0.002) and female (0.24±0.021 pg/µg vs 0.36 ±0.027 pg/µg, p=0.001) offspring, with no differences between offspring exposed to normoxia compared to prenatal hypoxia (Figure 3.2. A, B).

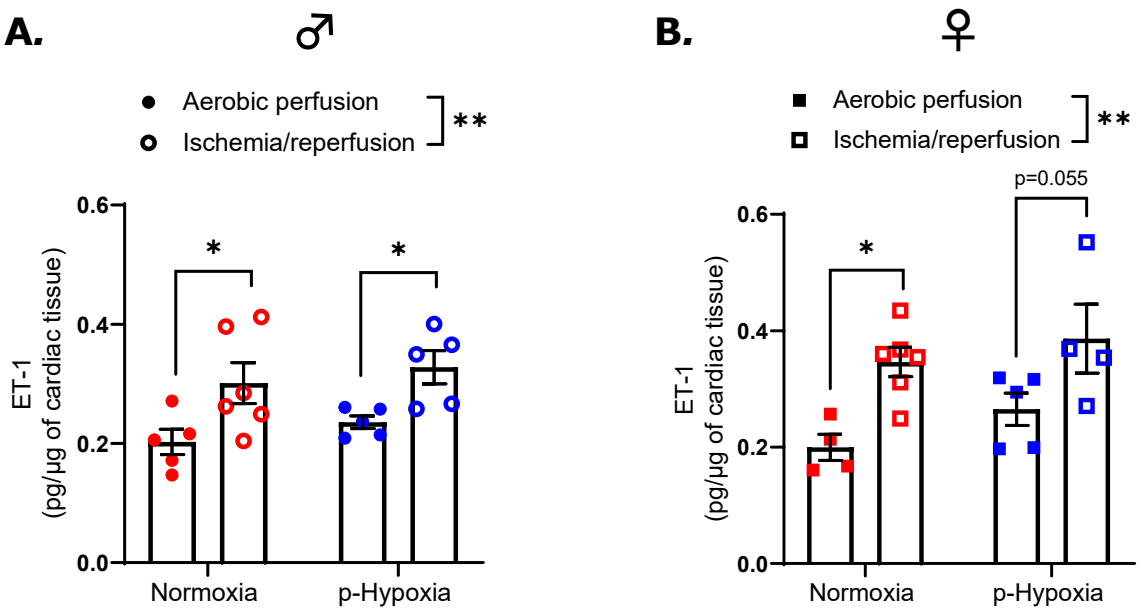


Figure 3.2. *Cardiac ET-1 content.*

*Endothelin-1 (ET-1) levels in aerobically perfused (solid symbols) or post-ischemic (open symbols) left ventricular tissues of adult male (circles; A) and female (squares; B) offspring exposed to normoxia (black symbols) or prenatal hypoxia (grey symbols). Summary graphs are presented as pg ET-1 per µg cardiac tissue, shown as mean±SEM and analyzed with two-way ANOVA and Sidak`s post hoc analysis, n=4-6 dams/group; 1 offspring/dam. *p<0.05, **p<0.01.*

3.2.3. Cardiac levels of endothelin receptors

Cardiac ET_A levels (expressed as a single band at ~50 kDa) were not different between male and female offspring, or between the normoxia and prenatal hypoxia-exposed groups (Figure 3.3. A. B). ET_B blots showed two bands, at molecular weights of ~60 kDa (Figure 3.3. B. *i*) and ~50 kDa (Figure 3.3. B. *ii*) which denote receptor isoforms C and A, respectively [383]. Protein levels of isoform C were decreased in the prenatal hypoxia female group only and were not altered by prenatal hypoxia in the male offspring (Figure 3.3. B. *i*). In contrast, cardiac levels of isoform A were not significantly altered in either group (i.e. an overall effect of p-Hypoxia in both sexes: p=0.064) (Figure 3.3. B. *ii*).

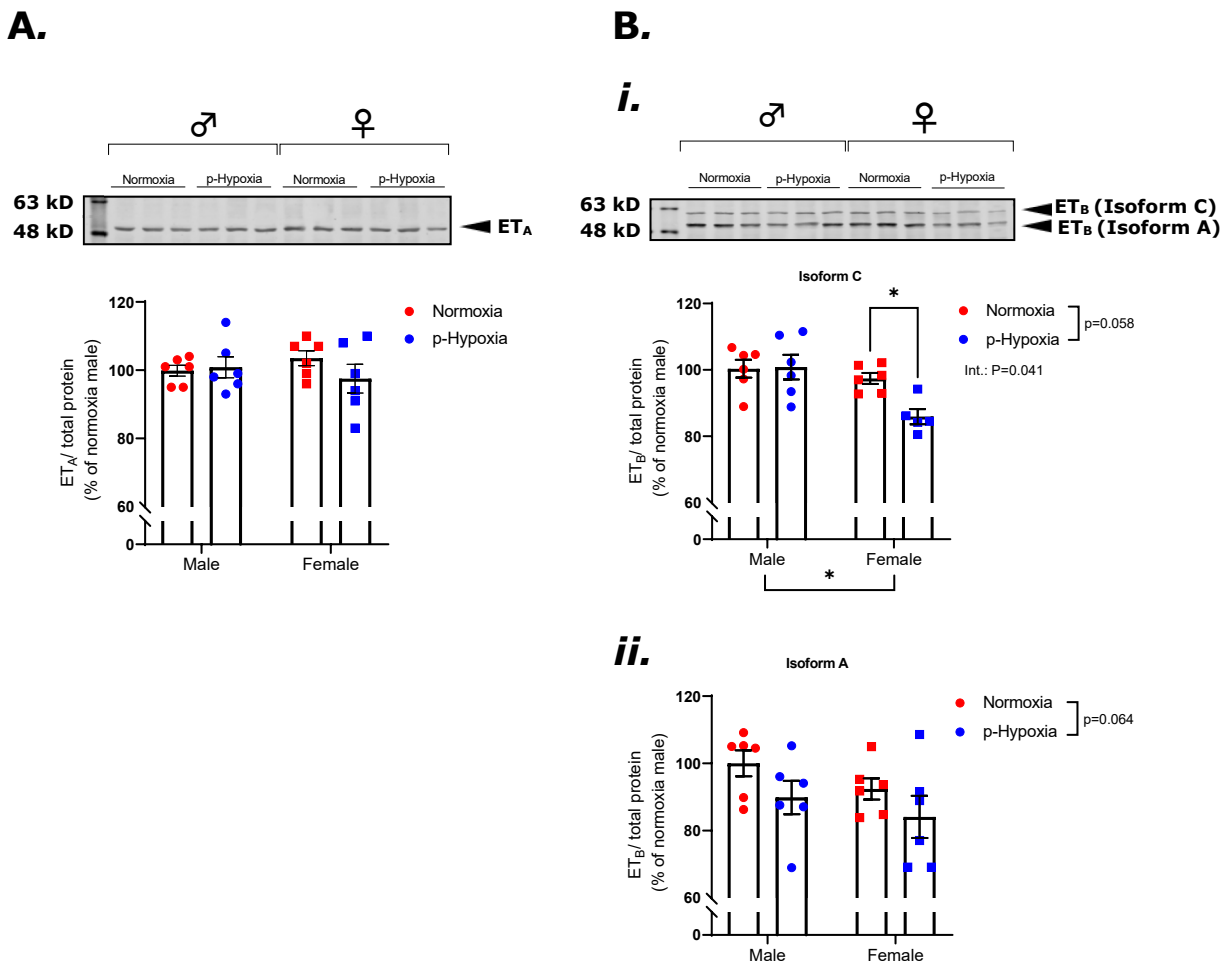


Figure 3.3. *Cardiac levels of endothelin receptors*

*Protein levels of endothelin A receptors (ET_A) (A) and endothelin B receptors (ET_B) (B: isoform C (i) and isoform A (ii)) in non-ischemic left ventricle tissues of adult male (circles) and female (squares) offspring exposed to normoxia (red symbols) or prenatal hypoxia (blue symbols). Representative immunoblots are shown above the graphs. Summary graphs are presented as percentage difference from the mean of the normoxia males (mean value set to 100%), shown as mean±SEM and analyzed with two-way ANOVA with Sidak`s post hoc analysis, n=5-6 dams/group; 1 offspring/dam. *p<0.05, **p<0.01.*

3.3. Discussion

We and others have previously shown that prenatal hypoxia enhances the susceptibility to the development of cardiac dysfunction in adult offspring [239,240,384]. In the current report, we further investigated the potential mechanisms for this increased risk, with a focus on ET-1. We showed that selective blockade of ET_A during I/R prevented the recovery of cardiac function in prenatally hypoxic male offspring, while ET_A blockade in hearts from prenatally hypoxic females tended to improve post-I/R cardiac performance. Prenatal hypoxia also decreased cardiac ET_B levels (isoform C in females only), without affecting tissue levels of ET-1 and ET_A. In summary, we observed sex-specific effects of prenatal hypoxia on the cardiac ET-1 system and its contribution to the development of cardiac dysfunction in adult offspring.

In the current study, we did not observe major changes in the recovery of the baseline between normoxic and prenatally hypoxic groups, as we reported previously [227,239,385]. One of the potential explanations could be a different duration of *ex vivo* experimental design (the timing of aerobic perfusion, ischemia insult and the reperfusion periods), that substantially impaired a recovery of the baseline in the normoxic group. As such, the difference in the percentage of cardiac recovery of the baseline (in the absence of ABT-627) between normoxic and prenatally hypoxic groups were not evident in the current study. Here we showed that inhibition of ET_A before I/R challenge did not alter post-ischemic cardiac recovery in normoxic male and female offspring. However, in contrast to our expectations, inhibition of ET_A in offspring exposed to prenatal hypoxia prevented the recovery of cardiac function post-ischemia in males, while it improved recovery in female offspring. ET-1 receptor antagonists (ET_A-specific or dual) have been widely used in animal models of cardiac dysfunction and clinical trials [187,386-388], however, the effect of these antagonists on cardiac function has been shown to vary. For instance, in rat models of myocardial infarction, inhibition of ET_A impaired scar healing and LV dilatation and dysfunction [388,389], while

others reported that ET_A inhibition improved post-ischemic recovery of cardiac function [187,390]. These contradictory results may be due to differences in experimental protocols, or the chemical nature of the used antagonists (which may display different degrees of selectivity against ET_A). Mechanistically, ET-1-mediated stimulation of ET-1 receptors on cardiomyocytes has been shown to result in stimulation of PLC and accumulation of DAG and subsequent activation of PKC (reviewed in [391]). Moreover, selective inhibition of specifically PKCε in males was shown to result in a significant decline of post-ischemic cardiac performance [392] suggesting a vital role of PKCε in cardiac tolerance of I/R insult in the male sex (females were not assessed). Prenatal hypoxia reduces PKCε mRNA and protein levels in fetal cardiomyocytes [245] and adult (male) offspring hearts [240,393], which subsequently was associated with a decreased cardiac tolerance to I/R compared to offspring born from uncomplicated (normoxic) pregnancies. Thus, it may be speculated that the striking functional outcomes we observed after inhibition of ET_A in the male prenatally hypoxic offspring could be attributed to inhibition of crucial ET_A-mediated PKCε-dependent recovery of cardiac function.

We did not find any differences in cardiac ET-1 levels between normoxia or prenatal hypoxia offspring (in either sex), while we did observe increased cardiac tissue levels of ET-1 after I/R challenge in all groups (male and female), which is in line with earlier reported findings [126,377,394-397]. Previous research in a rat model of chronic intermittent hypoxia reported increased ET-1 levels and ET_A expression in coronary arteries, and decreased ET_B expression in (male) rats which resulted in a decreased LVDP and augmented coronary vascular resistance [398]. Here, we reported similar levels of ET_A in adult offspring of both sexes (regardless of prenatal exposure) and decreased cardiac ET_B expression (isoform C) after prenatal hypoxia exposure in females only. Despite the relative lack of knowledge of the effect of prenatal hypoxia on the cardiac ET-1 system of adult offspring, it has been shown that in hypoxic conditions, ET_B may play a negative role. For instance, ET_B inhibition in mice

resulted in an improved cardiac performance, decreased blood lactate levels and ameliorated excessive erythrocytosis under hypoxia [399]. Moreover, under extreme hypoxia (i.e. 5% O_2 for 30 min.), ET_B knockout resulted in an improved cardiac performance and lower cardiac tissue hypoxia in mice [400]. Interestingly, despite this favorable effect of ET_B knockout under hypoxic settings, under normoxic conditions, cardiac contractility was reported to be enhanced, indicating that ET_B knockout may compromise cardiac performance in a non-hypoxic state [400]. Thus, it may be speculated that the lower cardiac ET_B expression observed in the adult female offspring exposed to prenatal hypoxia is the result of a compensation for an adverse hypoxic environment as part of the early-life cardiac adaptations of the fetus.

Previous research suggested that application of the selective ET_A antagonist (BQ-123) decreases the post-ischemic cardiac perfusion flow rate (thus implying that ET-1 was able to exert its vasoconstrictive effects via ET_B alone) [401], while others report no alterations in coronary vascular tone [402]. Therefore, the role of ET_B in the cardiac susceptibility of adult offspring exposed to prenatal hypoxia would be interesting to assess. Thus, in future studies, specific ET_B antagonist, as well as dual ET_A and ET_B receptor antagonists, may be employed to determine the receptor-specific effects of ET-1 in cardiac susceptibility to I/R challenge of adult prenatally hypoxia offspring.

Our findings offer new insights into the sex differences in the role of ET-1 in the cardiac capacity to recover after I/R in adult offspring exposed to prenatal hypoxia. Despite the fact that we did not observe any changes in ET-1 and ET_A levels due to prenatal hypoxia, in males, ET-1 signaling appears to be essential for cardiac ability to tolerate I/R, while in females, activation of ET_A seems to contribute to the development of cardiac dysfunction. Our data suggest that developmental influences and sex differences need to be taken into consideration during the development and implementation of potential therapeutic interventions for the prevention of CV disease in adult offspring born from complicated pregnancies.

CHAPTER 4

THE LONG-TERM EFFECTS OF PRENATAL HYPOXIA ON CORONARY ARTERY FUNCTION OF THE MALE AND FEMALE OFFSPRING

A version of this chapter has been published:

Hula, N., Liu R., Spaans F., Pasha M., Quon A., Kirschenman R., Cooke C. M., and Davidge S. T. "The Long-Term Effects of Prenatal Hypoxia on Coronary Artery Function of the Male and Female Offspring." Biomedicines 10, no. 12 (Nov 2022): 3019. https://www.mdpi.com/2227-9059/10/12/3019.

Author contributions: N.H., conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved final version of manuscript; **R.L.,** performed experiments, analyzed data, edited and revised manuscript, approved final version of manuscript; **F.S.,** conceived and designed research, interpreted results of experiments, drafted manuscript, edited and revised manuscript, approved final version of manuscript; **M.P.,** interpreted results of experiments, edited and revised manuscript, approved final version of manuscript; **A.Q.,** interpreted results of experiments, edited and revised manuscript, approved final version of manuscript; **R.K.,** performed experiments, edited and revised manuscript, approved final version of manuscript; **C.M.C.,** conceived and designed research, edited and revised manuscript, approved final version of manuscript; **S.T.D.,** conceived and designed research, interpreted results of experiments, drafted manuscript, edited and revised manuscript, approved final version of manuscript.

4.1. Introduction

Pregnancy-related complications severely impact the CV health and lifespan of the offspring [403]. However, little is known regarding the long-term effects of complicated pregnancies on coronary artery function of adult offspring. Taking into account that the coronary circulation is essential in maintaining proper cardiac performance and represents ~5% of total cardiac output [404], the dysfunction of coronary arteries has been attributed to the development of myocardial ischemia [405] and severe clinical outcomes.

The vascular endothelium plays a major role in maintaining vascular function and homeostasis. Primarily, the vascular endothelium is involved in the production and release of endothelium-derived relaxing factors (such as NO, PGI_2, and EDH), and endothelium-derived constricting factors (such as ET-1 and TxA_2)) [406]. An imbalance between vasodilation and vasoconstriction has been implicated in the development of endothelial dysfunction [407] resulting in CV dysfunction [408]. Previous reports suggest that offspring exposed to prenatal hypoxia are more prone to the development of hypertension [320], accompanied by an enhanced vasoconstrictor reactivity to big ET-1 (a precursor of ET-1) and an impaired NO-dependent endothelial function in the systemic circulation [314,320]. However, the link between prenatal hypoxia and coronary artery function in the adult male and female offspring has not been fully explored. In the current study, we hypothesized that prenatal hypoxia impairs coronary artery function in adult offspring by reducing endothelium-dependent vasodilation and constrictor capacity in the adult (4- and 9.5-month-old) male and female offspring.

4.2. Results

4.2.1. Coronary artery endothelium-dependent and endothelium-independent vasodilation responses

4.2.1.1. Coronary artery endothelium-dependent and endothelium-independent vasodilation responses in 4-month-old offspring

Coronary artery vasodilation responses to MCh (a muscarinic receptor agonist) were decreased in p-Hypoxia males, while the sensitivity to MCh was comparable between Normoxia and p-Hypoxia groups. In females, MCh-induced vasodilation responses and sensitivity to MCh tended to decrease in p-Hypoxia group compared to Normoxia control (Figure 4.1. A, B). In contrast, no changes in the vascular responses to the NO donor (SNP) were observed in coronary arteries of 4-month-old male (E_{max}: Normoxia: 98.95 ± 1.11 vs p-Hypoxia: 94.11 ± 1.51, n=6-8 dams/group) or female offspring (E_{max}: Normoxia: 98.56 ± 0.79 vs p-Hypoxia: 96.83 ± 1.75, n=6-8 dams/group).

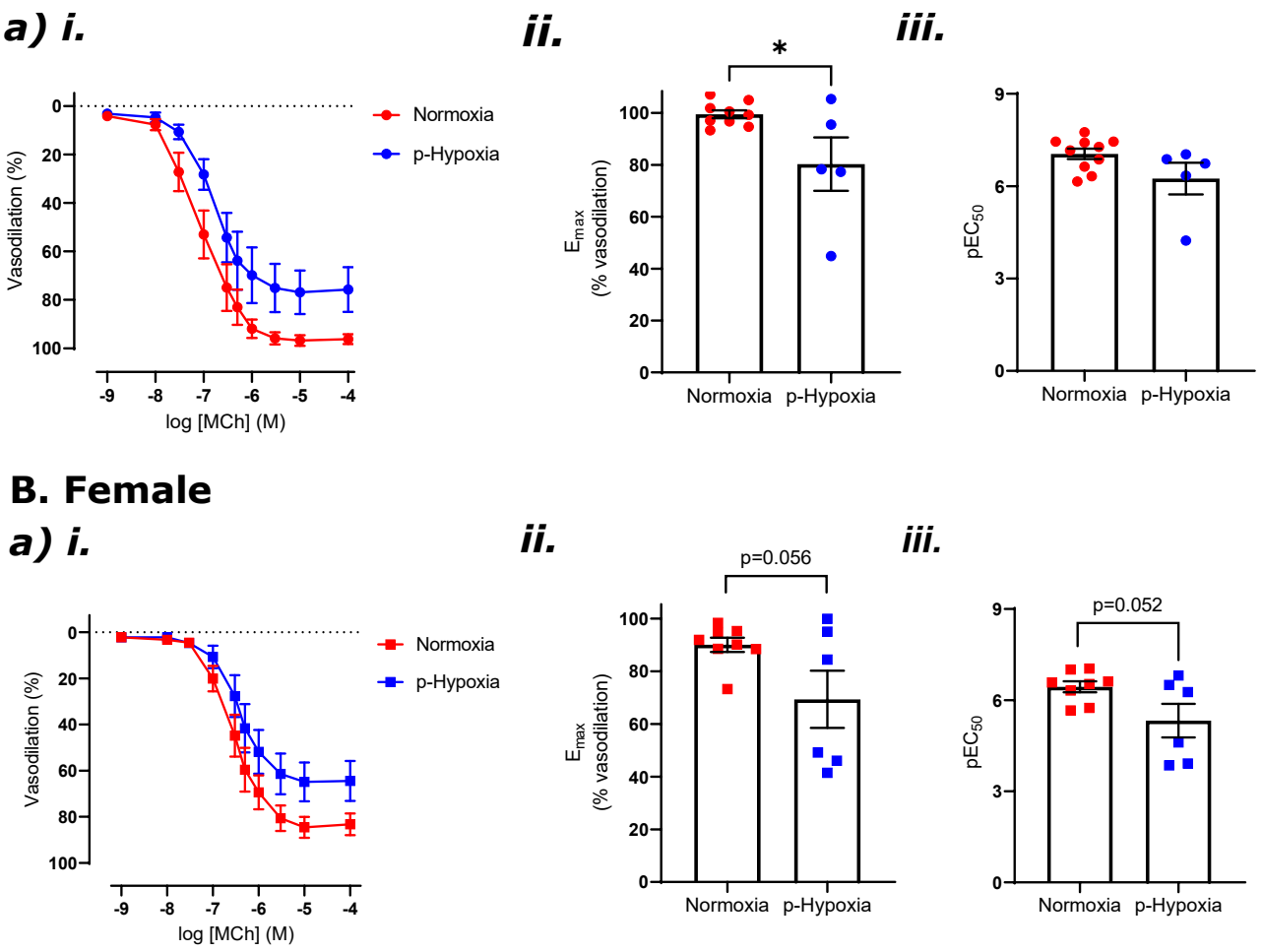


Figure 4.1. *Endothelium-dependent vasodilation in 4-month-old male (A) and female (B) offspring exposed to prenatal hypoxia.*

*(a. i) Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in left descending coronary arteries of Normoxia (red) and p-Hypoxia (blue) male (circles) and female (squares) 4-month-old offspring. (ii) Data were summarized as maximal vasodilation to MCh (E_{max}) and (iii) the sensitivity to MCh (the negative logarithm of the EC_{50}, pEC_{50}), n=5-10 dams/group. Data are presented as mean ± SEM; analyzed by t-test; *p<0.05. **p<0.01.*

4.2.1.2. Coronary artery endothelium-dependent and endothelium-independent vasodilation responses in 9.5-month-old offspring

With an advancement in age, at 9.5 months of age, maximal vasodilation responses to MCh and the sensitivity to MCh were decreased in coronary arteries of p-Hypoxia males and females (Figure 4.2. A, B). SNP-mediated vasodilation responses were similar between Normoxia and p-Hypoxia males (E_{max} Normoxia: 100.02 ± 0.37 vs p-Hypoxia: 98.47 ± 1.37, n=6-8 dams/group) and females (E_{max} Normoxia: 98.95 ± 0.66 vs p-Hypoxia: 97.73 ± 2.65, n=6-8 dams/group).

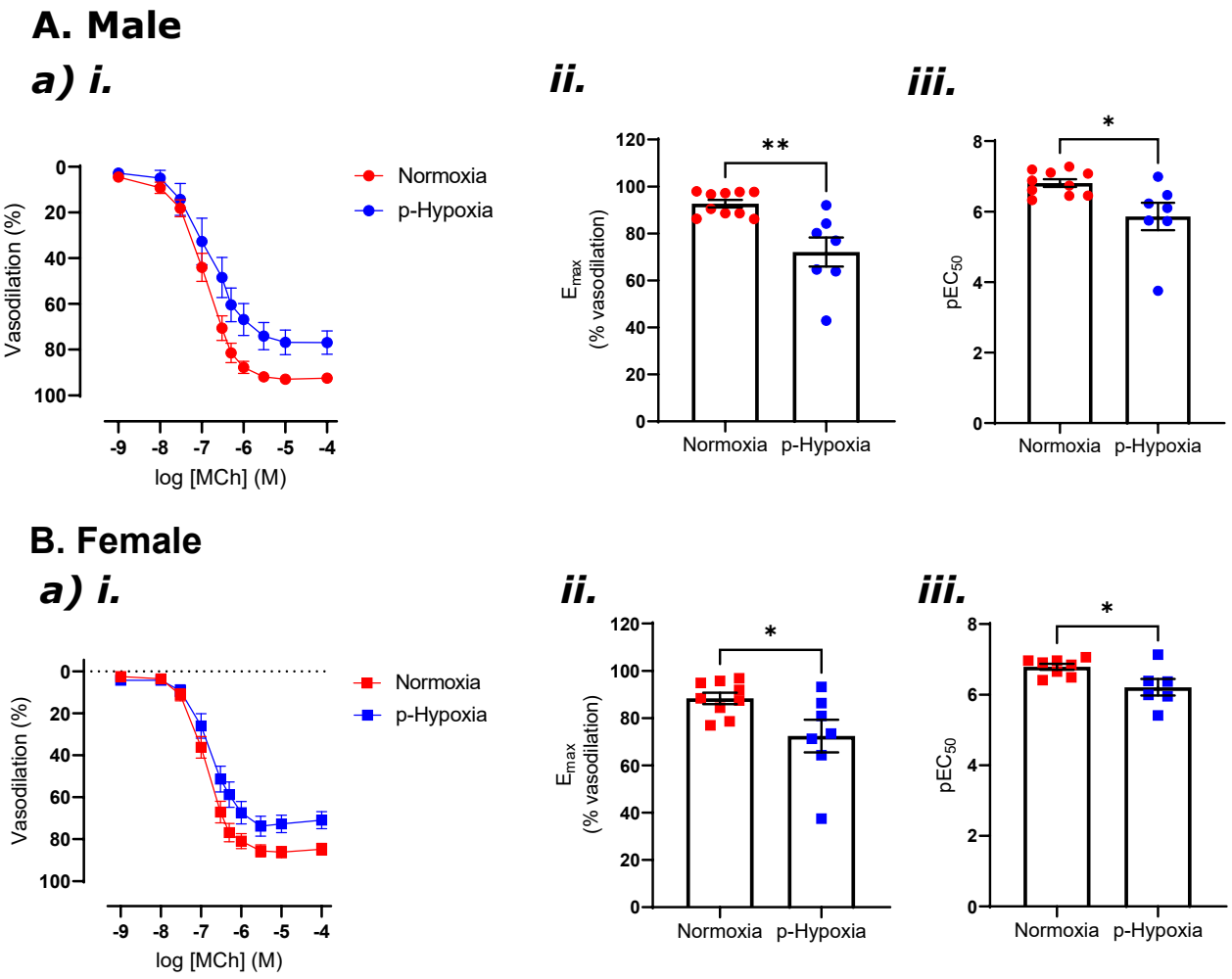


Figure 4. 2. *Endothelium-dependent vasodilation in 9.5-month-old male (A) and female (B) offspring exposed to prenatal hypoxia.*

*(a. i) Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in left descending coronary arteries of Normoxia (red) and p-Hypoxia (blue) male (circles) and female (squares) 4-month-old offspring. (ii) Data were summarized as maximal vasodilation to MCh (E_{max}) and (iii) the sensitivity to MCh (the negative logarithm of the EC_{50}, pEC_{50}), n=5-10 dams/group. Data are presented as mean ± SEM; analyzed by t-test; *p<0.05. **p<0.01.*

Because an impairment in MCh-induced vasodilation was maintained at 9.5 months of age in both, male and female offspring, specific vasodilation pathways potentially involved in the impaired endothelium-dependent vasodilation were assessed in the 9.5-month-old offspring.

4.2.1.2.1. NO synthase pathway is a major contributor to coronary artery endothelium-dependent vasodilation in male and female offspring

Pre-incubation with L-NAME (a NOS inhibitor) prevented MCh-induced vasorelaxation in Normoxia and p-Hypoxia male offspring (Figure 4.3 A. a, *i*, *ii*). Because eNOS is a predominant NOS isoform in the vasculature and is responsible for most of the NO produced, we assessed eNOS protein expression in the coronary artery tissue of the offspring. eNOS expression was similar between Normoxia and p-Hypoxia male offspring (Figure 4.3. A. b). Similar to the male offspring, in the coronary arteries of female offspring, pre-incubation with L-NAME prevented MCh-induced vasorelaxation in Normoxia and p-Hypoxia groups ($p<0.0001$; Figure 4.3 B. a. *i*, *ii*). eNOS levels were similar between Normoxia and p-Hypoxia groups (Figure 4.3. B. b).

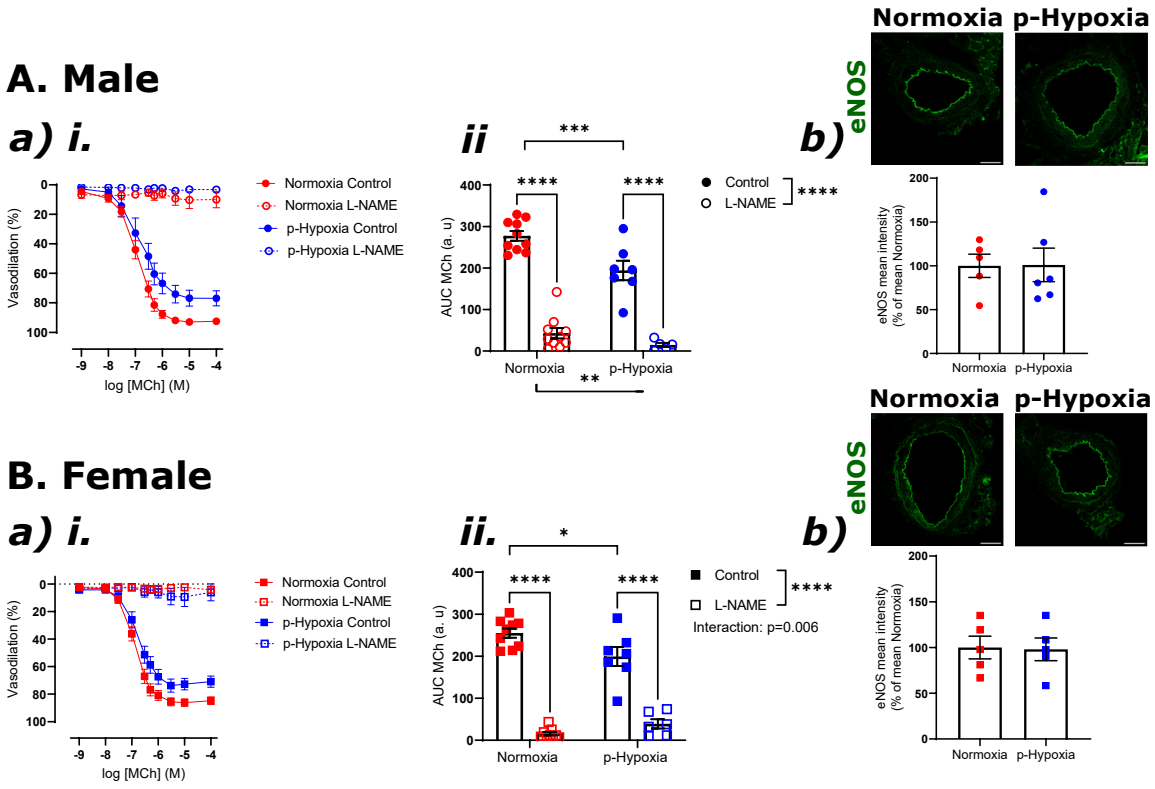


Figure 4.3. *Contribution of nitric oxide (NO) to coronary artery endothelium-dependent vasodilation in 9.5-month-old male and female offspring.*

*(a. i) Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in the presence or absence of L-NAME (NOS inhibitor N(G)-nitro-L-arginine methyl ester hydrochloride) in left descending coronary arteries of Normoxia (red) and p-Hypoxia (blue) male (A; circles) and female (B; squares) 9.5-month-old offspring. (ii) Data were summarized as area under the curve (AUC) in the absence or presence of L-NAME, n=5-10 dams/group. (b) Representative confocal images of immunofluorescence staining of eNOS (green) and quantitative analysis of eNOS expression in coronary arteries of Normoxia (red symbol) and p-Hypoxia (blue symbol) male (A; circles) and female (B; squares) 9.5-month-old offspring; n=5-6 dams/group. Data are presented as mean ± SEM; analyzed by two-way ANOVA with Sidak's multiple comparison post-hoc test or t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar for all images is 100 µm.*

4.2.1.2.2. Enhanced contribution of the PGHS pathway to coronary artery endothelium-dependent vasodilation in prenatally hypoxic offspring

In the male offspring, pre-incubation with meclofenamate did not affect MCh-induced vasorelaxation in the Normoxia group, while meclofenamate (a PGHS inhibitor) increased max MCh-induced vasodilation in the p-Hypoxia offspring (Figure 4.4 A. a, *i*, *ii*). Since PGHS-1 and PGHS-2 are key enzymes involved in the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids, coronary artery tissue expression of PGHS-1 and PGHS-2 was assessed. Coronary artery PGHS-1 expression was similar between the Normoxia and p-Hypoxia male offspring, while the expression of PGHS-2 was lower ($p=0.050$) in the p-Hypoxia group compared to the Normoxia male offspring (Figure 4.4 A. b. *i*, *ii*). Similar to the male offspring, in females, pre-incubation with meclofenamate tended to increase ($p=0.06$) MCh-induced vasorelaxation in only the p-Hypoxia offspring (Figure 4.4 B. a. *i*, *ii*). Coronary artery expressions of PGHS-1 and PGHS-2 were not different between the Normoxia and p-Hypoxia groups (Figure 4.4 B. b).

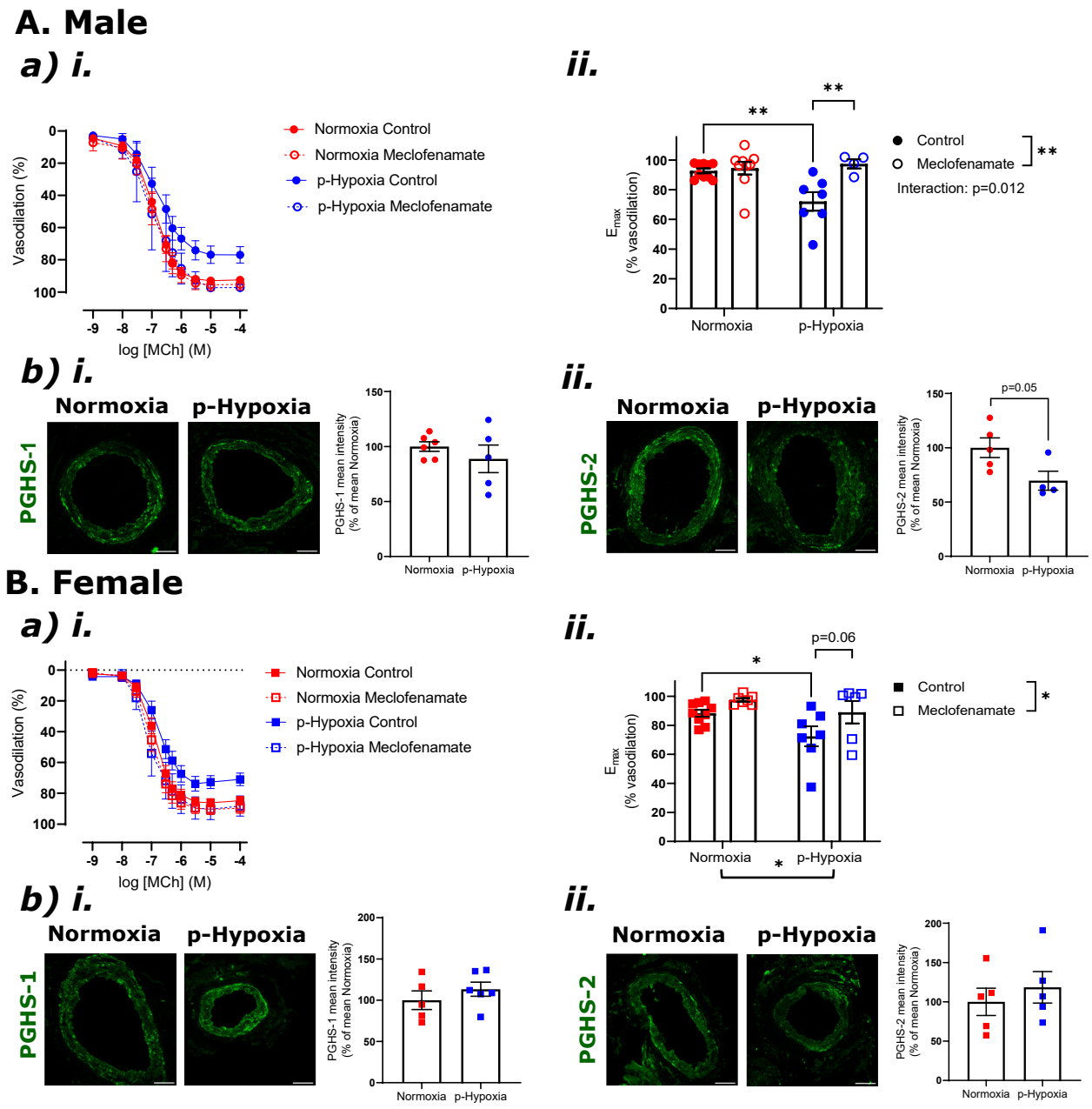


Figure 4.4. *Contribution of the prostaglandin-H synthase (PGHS) pathway to endothelium-dependent vasodilation in 9.5-month-old male and female offspring.*

(a. i) Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in the presence or absence of meclofenamate (a PGHS inhibitor) in left descending coronary arteries of Normoxia (red) and p-Hypoxia (blue) male (A; circles) and female (B; squares) 9.5-month-old offspring. (ii) Data were summarized as maximum vasodilation

*response (E_{max}) in the absence or presence of meclofenamate; n=4-10 dams/group. (b. i, ii) Representative confocal images of PGHS-1 (green) and PGHS-2 (green) expression, and quantitative analysis of immunofluorescence staining for PGHS-1 and PGHS-2 in coronary arteries of Normoxia (red) and p-Hypoxia (blue) male (circles) and female (squares) offspring; n=4-6 dams/group. Data are presented as mean ± SEM; analyzed by t-test or two-way ANOVA with Sidak's multiple comparison post-hoc test, *p<0.05, **p<0.01. Scale bar for all images is 100 μm.*

4.2.1.2.3. The contribution of EDH to endothelium-dependent vasodilation is enhanced in coronary arteries of prenatally hypoxia male and female offspring

In the males, pre-incubation with apamin and Tram-34 (small- and intermediate-conductance Ca^{2+}-activated potassium channels inhibitors) decreased coronary artery sensitivity to MCh in the p-Hypoxia group only (Figure 4.5. A. a). In females, apamin and Tram-34 did not alter coronary artery sensitivity to MCh in Normoxia group, while decreased coronary artery sensitivity to MCh in the p-Hypoxia offspring (Figure 4.5. B. a).

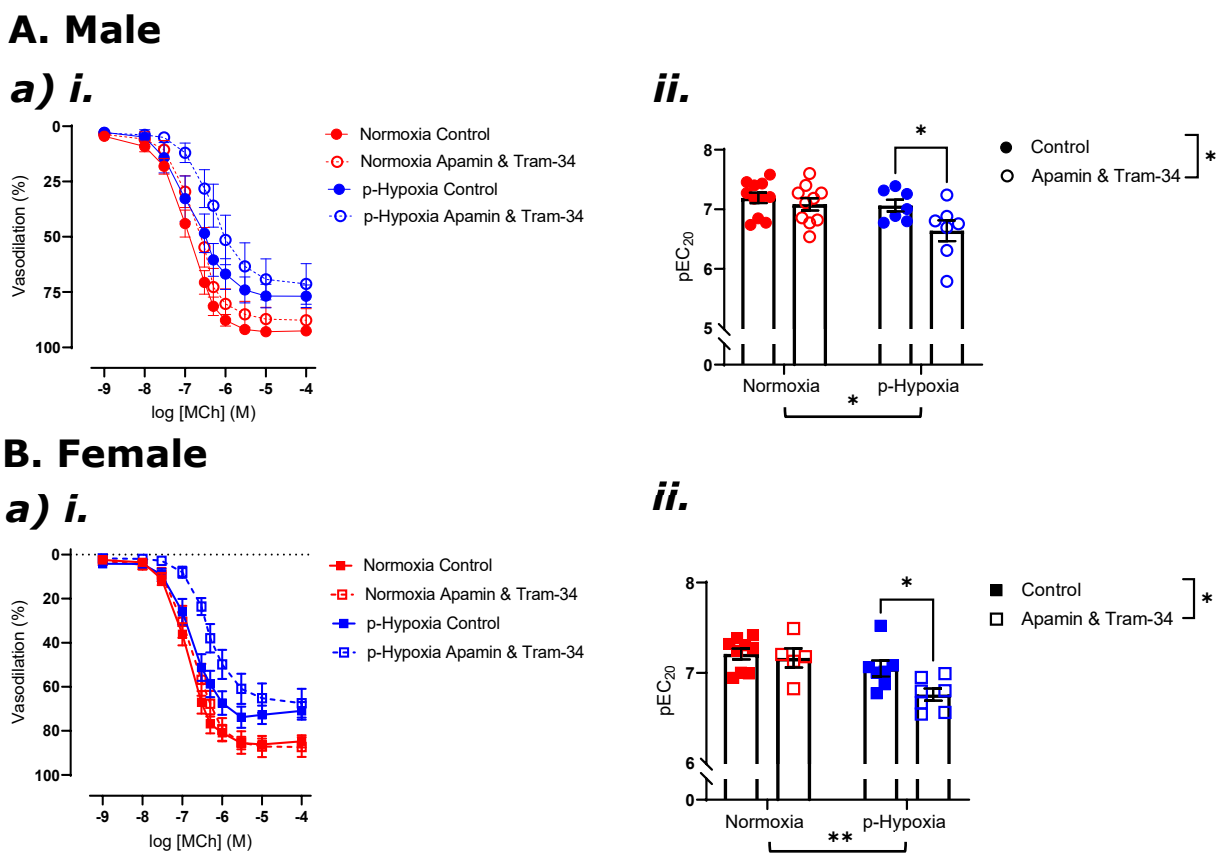


Figure 4.5. *Contribution of endothelium-derived hyperpolarization (EDH) to endothelium-dependent vasodilation in 9.5-month-old male and female offspring.*

(a. i) Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in the presence or absence of apamin and Tram-34 (small- and intermediate-conductance Ca^{2+}-activated potassium channels inhibitors) in left descending coronary

*arteries of Normoxia (red) and p-Hypoxia (blue) male (A; circles) and female (B; squares) 9.5-month-old offspring. (ii) Data were summarized as the sensitivity to MCh (the negative logarithm of the EC_{20}, pEC_{20}); n=5-9 dams/group. Data are presented as mean ± SEM; analyzed by two-way ANOVA with Sidak's multiple comparison post-hoc test, *p<0.05, **p<0.01.*

4.2.2. Coronary artery responses to ET-1 and the contribution of ET_A and ET_B

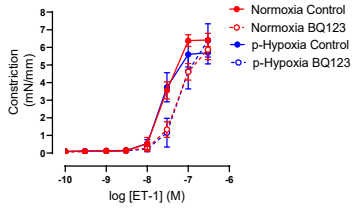
4.2.2.1. Increased contribution of ET_B receptors to ET-1 mediated vasoconstriction in 4-month-old female offspring.

In 4-months-old male offspring, ET-1-mediated coronary artery responses and ET-1 expression were similar between the Normoxia and p-Hypoxia groups (Figure 4.6. A. a, b, c. *i*). Moreover, ET-1 plasma levels were comparable between Normoxia and p-Hypoxia males (Normoxia: 1.40 ± 0.19 pg/ml vs p-Hypoxia: 1.13 ± 0.36 pg/ml, n=6-7 dams/group). BQ123 (a competitive antagonist of ET_A) decreased vasoconstriction responses to ET-1 to a similar extend in both the Normoxia and p-Hypoxia males (Figure 4.6. A. a). Coronary artery ET_A expression was similar between Normoxia and p-Hypoxia male offspring (Figure 4.6. A. c. *ii*). Pre-incubation with BQ788 (a non-competitive antagonist of ET_B receptors) did not alter ET-1-mediated vasoconstriction in the Normoxia group and p-Hypoxia group (Figure 4.6. A. b). ET_B expression was not different between Normoxia and p-Hypoxia male offspring (Figure 4.6. A. c. *iii*).

In 4-month-old females, ET-1-mediated coronary artery constriction responses were similar between the Normoxia and p-Hypoxia groups (Figure 4.6. B. a, b). ET-1 expression was increased in the p-Hypoxia females compared to Normoxia group (Figure 4.6. B. c. *i*), while plasma ET-1 levels were similar between the groups (Normoxia: 2.55 ± 0.35 pg/ml vs p-Hypoxia: 1.60 ± 0.48 pg/ml, n=7-8 dams/group). BQ123 decreased ET-1-mediated vasoconstriction in both the Normoxia and p-Hypoxia offspring, while no significant differences in ET_A expression were observed between the groups (Figure 4.6. B. a, c. *ii*). BQ788 did not alter ET-1-mediated coronary artery responses in the Normoxia offspring, while ET-1-mediated vasoconstriction tended to be decreased (p=0.058) by BQ788 in the p-Hypoxia offspring (Figure 4.6. B. b). However, ET_B expression was similar between the Normoxia and p-Hypoxia groups (Figure 4.6. B. c. *iii*).

A. Male

a) i.



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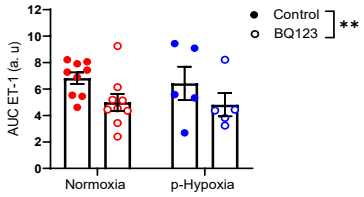
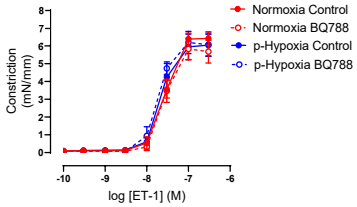
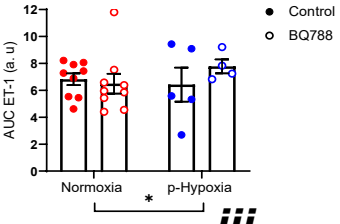


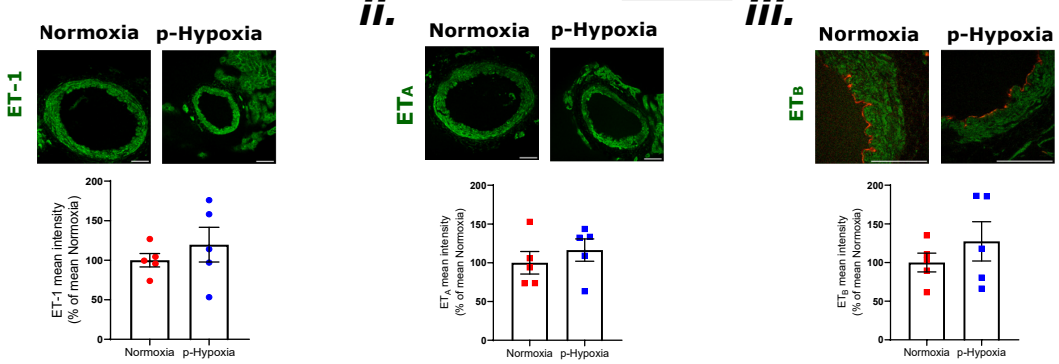
b) i.



ii.

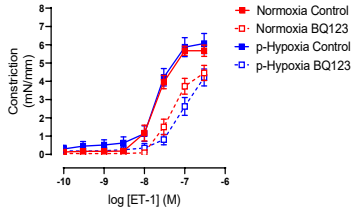


c) i. ***ii.*** ***iii.***

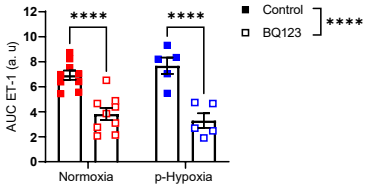


B. Female

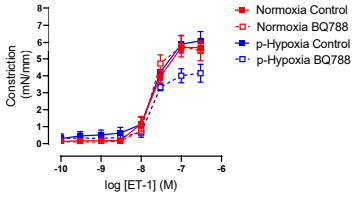
a) i.



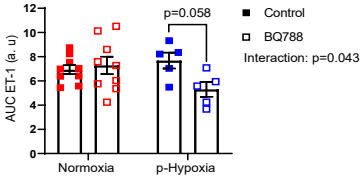
ii.



b) i.



ii.



c) i. ***ii.*** ***iii.***

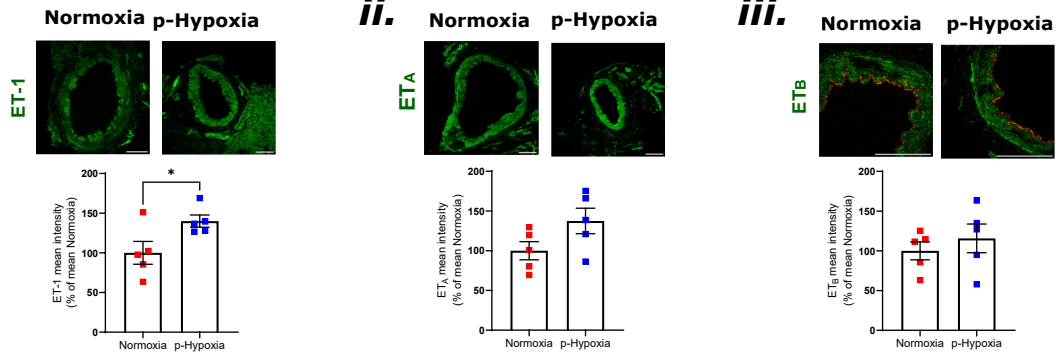


Figure 4.6. *ET-1-mediated vasoconstriction, contribution of ET_A and ET_B to ET-1-mediated response and coronary tissue levels of ET-1, ET_A and ET_B in 4-month-old male and female offspring.*

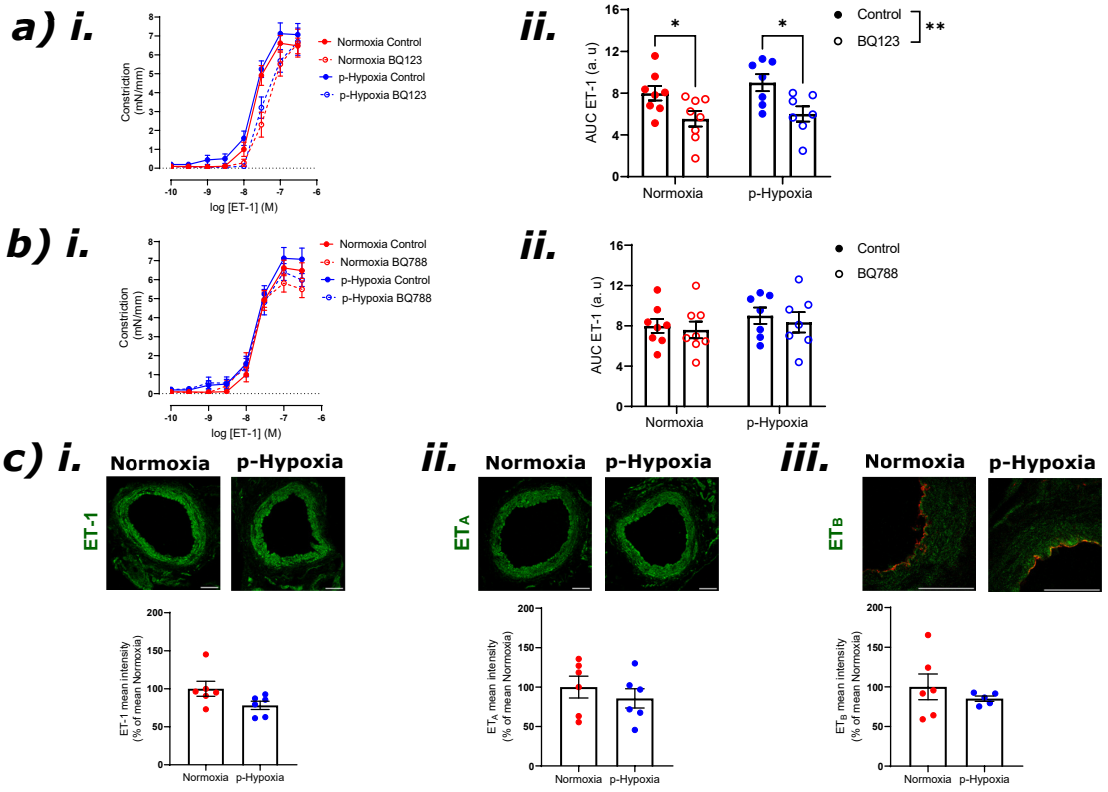
*(i) ET-1-mediated vasoconstriction in the presence or absence of (a) BQ123 (a competitive antagonist of ET_A) or (b) BQ788 (a selective ET_B receptors antagonist) in left descending coronary arteries of Normoxia (red) and p-Hypoxia (blue) male (A; circles) and female (B; squares) 4-month-old offspring. (ii) Data were summarized as area under the curve (AUC); n=4-9 dams/group. (c) Representative confocal images of (i) ET-1 (green), (ii) ET_A (green) and (iii) ET_B (green) co-stained with CD31 (endothelial cell marker; red) and quantitative analysis of immunofluorescence staining in Normoxia (red symbol) and p-Hypoxia (blue symbol) male (circles) and female (squares) offspring; n=4-6 dams/group. Data are presented as mean ± SEM; analyzed by t-test or two-way ANOVA with Sidak's multiple comparison post-hoc test, *p<0.05, **p<0.01, ****p<0.0001. Scale bar for all images is 100 µm.*

4.2.2.2. An impaired ET-1 mediated vasoconstriction in 9.5-month-old female offspring.

In 9.5-months-old male offspring, ET-1 mediated coronary artery responses and coronary tissue expression of ET-1 were similar between the Normoxia and p-Hypoxia groups (Figure 4.7. A. a, b, c. *i*). Plasma levels of ET-1 were similar between Normoxia and p-Hypoxia male offspring (Normoxia: 4.65 ± 0.86 pg/ml vs p-Hypoxia: 4.11 ± 0.67 pg/ml, n=7 dams/group). BQ123 (a competitive antagonist of ET_A) decreased to a similar extend vasoconstriction responses to ET-1 in both the Normoxia and p-Hypoxia males (Figure 4.7. A. a). ET_A expression was similar between Normoxia and p-Hypoxia males (Figure 4.7. A. c. *ii*). Pre-incubation with BQ788 (a selective ET_B antagonist) did not alter ET-1-mediated coronary artery responsiveness in the Normoxia and p-Hypoxia males, and coronary expression of ET_B was similar between Normoxia and p-Hypoxia groups (Figure 4.7. A. b, c. *iii*).

In female offspring, ET-1 mediated responses were reduced in the p-Hypoxia group compared to the Normoxia controls (Figure 4.7. B. a, b). ET-1 tissue levels were similar between Normoxia and p-Hypoxia groups (Figure 4.7. B. c, *i*), while plasma levels tended to be lower in p-Hypoxia groups compared to Normoxia (Normoxia: 10.47 ± 0.82 pg/ml vs p-Hypoxia: 7.67 ± 1.02 pg/ml; p=0.065, n=6-8 dams/group). Pre-incubation with BQ123 (a competitive antagonist of ET_A receptors) decreased vasoconstriction responses to ET-1 in the Normoxia and p-Hypoxia females, while coronary tissue levels of ET_A were similar between the Normoxia and p-Hypoxia groups (Figure 4.7. B. a, c. *ii*). Pre-incubation with BQ788 (a selective ET_B antagonist) did not alter ET-1-mediated coronary artery responsiveness in the Normoxia and p-Hypoxia females, and coronary expression of ET_B was similar between Normoxia and p-Hypoxia groups (Figure 4.7. B. b, c. *iii*).

A. Male



B. Female

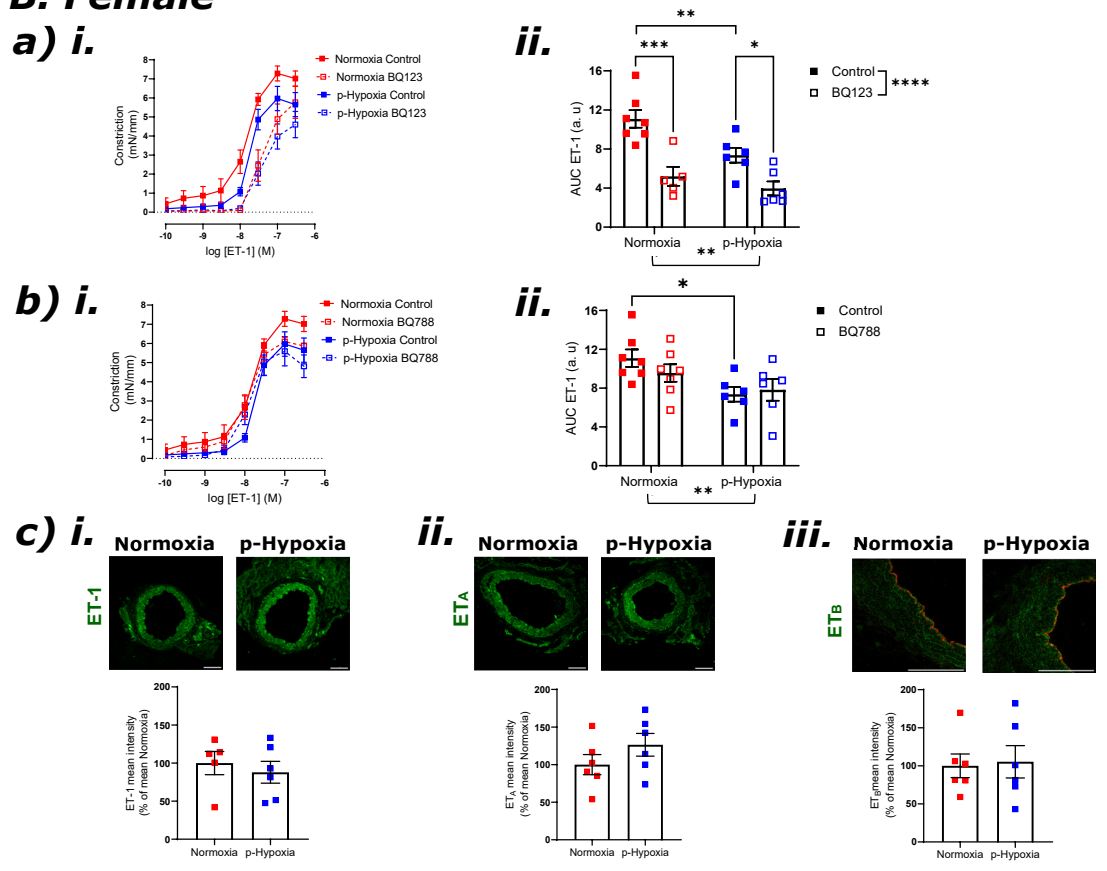


Figure 4.7. *ET-1-mediated vasoconstriction, contribution of ET_A and ET_B to ET-1-mediated response and coronary tissue levels of ET-1, ET_A and ET_B in 9.5-month-old male and female offspring.*

*(i) ET-1-mediated vasoconstriction in the presence or absence of (a) BQ123 (a competitive antagonist of ET_A) or (b) BQ788 (a selective ET_B receptors antagonist) in left descending coronary arteries of Normoxia (red) and p-Hypoxia (blue) male (A; circles) and female (B; squares) 9.5-month-old offspring. (ii) Data were summarized as area under the curve (AUC); n=5-8 dams/group. (c) Representative confocal images of (i) ET-1 (green), (ii) ET_A (green) and (iii) ET_B (green) co-stained with CD31 (endothelial cell marker; red) and quantitative analysis of immunofluorescence staining in Normoxia (red symbol) and p-Hypoxia (blue symbol) male (circles) and female (squares) offspring; n=5-6 dams/group. Data are presented as mean ± SEM; analyzed by t-test or by two-way ANOVA with Sidak's multiple comparison post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar for all images is 100 μm.*

4.3. Discussion

In the current study, we demonstrated that hypoxia, experienced during prenatal life, is associated with changes in vasoconstrictor and vasodilator capacity of coronary arteries in the adult male and female offspring. We showed that prenatal hypoxia impairs endothelium-dependent vasodilation in both males and females at 4- and 9.5-month of age. Nitric oxide was the predominant vasodilatory pathway in the coronary circulation. However, in both males and females exposed to prenatal hypoxia, the impaired endothelium-dependent vasodilation appeared to be mediated via enhanced prostaglandin H-synthase-dependent vasoconstriction. Moreover, a higher contribution of endothelium dependent hyperpolarization to coronary artery vasodilation was observed in prenatally hypoxic males and females. In 4-month-old offspring, prenatal hypoxia did not affect ET-1-mediated responsiveness but tended to increase the contribution of ET_B to ET-1 vasoconstriction, in female offspring only. With advancement in age, vasoconstriction responses of the coronary arteries to ET-1 were reduced by prenatal hypoxia in only the female offspring, without effects of prenatal hypoxia on ET_B-mediated responses in either sex.

4.3.1. The effect of prenatal hypoxia on coronary artery endothelium-dependent and endothelium-independent vasodilation in adult offspring

To the best of our knowledge, we showed for the first time that prenatal exposure to hypoxia impairs endothelium-dependent vasodilation in coronary arteries of male and female offspring at 4- and 9.5-month of age. The coronary endothelium regulates vascular tone by releasing vasoconstricting and vasodilating factors, and an impairment in the endothelium-dependent vasodilation has been shown to contribute to the development of various CV pathophysiological states [409], such as hypertension [410], atherosclerotic vascular disease [411], and congestive heart failure [412]). We, and others, have previously reported an impaired cardiac tolerance to I/R injury in the offspring, exposed to prenatal hypoxia [227,234,240,250]. As impaired vasodilation of the coronary microcirculation has been

associated with defects in myocardial perfusion (suggestive of myocardial ischemia) [413], it may be speculated that the impaired functional properties of the coronary arteries, observed in the current study, are a significant contributor factor to the development of cardiac dysfunction of adult offspring.

Further assessment of the potential mechanisms of the impaired endothelium-dependent vasodilation in the 9.5-month-old offspring revealed that, in the LAD, endothelium-dependent vasodilation is predominantly mediated via NO. A significant role of NO in modulating epicardial coronary vasomotor tone was previously reported in several studies [290,293,414]. NO is produced in the tissue by conversion of L-arginine to L-citrulline by NOS [415]. eNOS is critical for normal vascular homeostasis and is responsible for generating endothelium - derived NO [416]. Previous studies reported that coronary artery endothelial dysfunction may be associated with an increase [417], as well as decrease [418,419] expression of eNOS. In the current study, however, we did not observe any changes in eNOS expression in either males or females due to prenatal hypoxia.

Interestingly, in 9.5-month-old adult male and female offspring, we observed that inhibition of PGHS improved the impairment in MCh-induced vasorelaxation induced by prenatal hypoxia. PGHS is an enzyme involved in the production of PGs, which include vasodilator PGI_2, as well as the vasoconstrictor compounds (such as PGH_2, PGF2α and TXA_2). PGs are critical modulators of vascular tone in both physiological and pathophysiological conditions (reviewed by [275,420]). Because the inhibition of PGHS improved MCh-induced vasorelaxation in prenatally hypoxia males and females, PGHS expression was assessed in the coronary artery tissue. Prenatal hypoxia did not alter PGHS-1 and PGHS-2 expression in females, and PGHS-1 expression in males, which suggests that not the levels but the activity of the PGHS pathway that is involved in the production and action of constrictive PGs or TXA_2 may be enhanced by prenatal hypoxia, thereby contributing to an impaired vasodilation. In contrast to the females, the expression of PGHS-2 was decreased in the prenatally hypoxic

male offspring. Although PGHS-2 expression and activity is enhanced in various pathophysiological conditions such as preeclampsia, hypertension and ageing (reviewed in [275]), previous research demonstrated an essential role of PGHS-2 in postnatal CV maturation (the transition of the cardiopulmonary circulation at birth) [421], in the maintenance of normal renal architecture (progression of the renal dysplasia seen in COX (PGHS)-2–deficient mice) [422] and PGHS-2 downregulation has been shown to contribute to the development of kidney pathologies due to intrauterine growth restriction [423]. Thus, it may be suggested that a lower expression of PGHS-2 in coronary arteries of the adult male offspring may be an indication of an early-life adaptation of the coronary circulation to adverse intrauterine environment during pregnancies complicated with prenatal hypoxia.

We observed that in normoxic and prenatally hypoxic male and female offspring, endothelial hyperpolarization through SK_{Ca} and IK_{Ca} channels appears to contribute to endothelium-dependent vasodilation. Both SK_{Ca} and IK_{Ca} channels play an important, but channel-specific, role in endothelium-dependent vasodilation (reviewed by [424,425]). For instance, SK_{Ca} are mainly expressed in caveolae, and are important for activation of NOS, while IK_{Ca} can be mainly found within myoendothelial gap junction-associated endothelial cell projections [426]. SK_{Ca} and IK_{Ca} channel activation generates an endothelium-dependent hyperpolarization that is conducted along the endothelium and into the smooth muscle cell layer leading to smooth muscle cell hyperpolarization and subsequent inhibition of vascular tone [427]. An impaired function of SK_{Ca} and IK_{Ca} channels has been observed in various CV pathologies [428,429]. Moreover, it has been reported that hypoxia *per se* leads to a reduction in the expression level of SK_{Ca} and IK_{Ca} as well as a reduction in endothelial K^+ currents via SK_{Ca} and IK_{Ca} channels in porcine coronary arteries (in either sex) [430]. Thus, it may be suggested that the EDH pathway is maintained in prenatally hypoxic males and females as an additional mechanism to compensate for an impaired relaxation capacity of coronary arteries, thus maintaining coronary artery vasorelaxation in males and females.

4.3.2. The effect of prenatal hypoxia on coronary artery ET-1 system in adult offspring

At 4-months of age, ET-1 tissue levels were increased in coronary arteries from prenatal hypoxia females only, while ET-1-mediated vasoconstriction and plasma levels of ET-1 were not affected by prenatal hypoxia in either sex. Previous research in adult rat model of chronic intermittent hypoxia reported an enhanced ET-1 expression in coronary vessels that was accompanied with an enhanced ET-1-mediated response [398]. Thus, it can be suggested that prenatal hypoxia may have an adaptive response that although there is an increase in ET-1 expression, this was not accompanied by an increased ET-1-mediated coronary artery responsiveness.

The biological effects of ET-1 are achieved via activation of the ET_A and ET_B. The upregulation of ET_B on VSMCs is often observed in atherosclerosis [431] and IHD [432] as their activation potentiates ET-1-mediated vasoconstriction. On the VSMCs, ET_B activation induces PLCβ activity which results in the increase in IP_3 and DAG. DAG activates PKC, which phosphorylates the actin-binding protein calponin or leads to phosphorylation of caldesmon, thereby increasing the myofilament force sensitivity to Ca^{2+}, resulting in constriction [356]. Also, previous research has demonstrated a sex-specific function of ET_B [433]. Thus, in men, the activation of ET_B mediates tonic vasoconstriction in blood vessels of the skin, while in females, results in tonic vasodilation in the same type of blood vessels [433]. We demonstrated that in 4-month-old prenatally hypoxic male offspring, inhibition of ET_B receptors did not alter ET-1-mediated vasoconstriction, while in prenatal hypoxic female offspring, ET_B inhibition tended to decrease ET-1-mediated vasoconstriction. In a rat model of prenatal hypoxia, Chen *et al.* previously showed an attenuated constriction of coronary arteries of male offspring, which was associated with a decreased PKCβ Ser660 phosphorylation [318]. However, as our functional results were not associated with changes in ET_B expression, it may be speculated that it is the downstream signaling of ET_B receptors

that was impacted by prenatal hypoxia in female offspring, which resulted in sex-specific changes in an ET_B-dependent functional response.

In 9.5-month-old offspring, prenatal hypoxia decreased ET-mediated coronary artery responsiveness in female offspring and tended to decreased plasma levels of ET-1 (likely due to reduced ET-1 release or an enhanced ET-1 clearance from the circulation). While the long-term effect of prenatal hypoxia on the coronary artery ET-1 system is currently unknown, a decreased ET-1-mediated vasoconstriction in coronary arteries has been previously reported in obese rats, and this was associated with uncoupling of $[Ca^{2+}]_i$ signaling, while ET-1, ET_A and ET_B expressions were not changed [434]. Moreover, a reduction in ET-1-induced coronary artery contraction has been observed in deoxycorticosterone acetate (DOCA)-salt hypertensive rats that was associated with uncoupling of ET-1-receptors and an impaired $[Ca^{2+}]_i$ signaling [435]. Thus, it may be that the reduction in ET-1-mediated vasoconstriction in 9.5-month-old females is attributed to an impaired $[Ca^{2+}]_i$ signaling, however, further studies are warranted**.**

In the current study, we showed that prenatal hypoxia has long-term and sex-specific effects on the mechanisms of coronary artery vasodilation and vasoconstriction. As coronary artery function is essential in maintaining cardiac performance, coronary artery dysfunction may contribute to the development of cardiac dysfunction and an impaired cardiac tolerance to I/R insult in adult offspring that has been previously reported [227,234,240]. Understanding the mechanistic pathways involved in the programming of CV disease allows for the development of future prenatal and postnatal therapeutic interventions.

CHAPTER 5

PLACENTAL TREATMENT IMPROVES CARDIAC TOLERANCE TO ISCHEMIA/REPERFUSION INSULT IN ADULT MALE AND FEMALE OFFSPRING EXPOSED TO PRENATAL HYPOXIA

A version of this chapter has been published:

Hula, N., Spaans F., Vu J., Quon A., Kirschenman R., Cooke C. M., Phillips T. J., Case C. P., and Davidge S. T. "Placental Treatment Improves Cardiac Tolerance to Ischemia/Reperfusion Insult in Adult Male and Female Offspring Exposed to Prenatal Hypoxia." Pharmacological Research 165 (Mar 2021): 105461. https://dx.doi.org/10.1016/j.phrs.2021.105461.

Author contributions: N.H., conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved final version of manuscript; **F.S.,** conceived and designed research, edited and revised manuscript, approved final version of manuscript; **J.V.,** performed experiments, analyzed data, edited and revised manuscript, approved final version of manuscript; **A.Q.,** performed experiments, interpreted results of experiments, edited and revised manuscript, approved final version of manuscript; **R.K.,** performed experiments, edited and revised manuscript, approved final version of manuscript; **C.M.C.,** conceived and designed research, edited and revised manuscript, approved final version of manuscript; **TP;** conceived and designed research, provided study materials, edited and revised manuscript, approved final version of manuscript; **CPC;** conceived and designed research, provided study materials, edited and revised manuscript, approved final version of manuscript; **S.T.D.,** conceived and designed research, interpreted results of experiments, drafted manuscript, edited and revised manuscript, approved final version of manuscript.

5.1. Introduction

Prenatal hypoxia leads to placental oxidative stress (an abnormal increase in ROS [436]) which in turn induces tissue damage and impairs placental function [437]. Mitochondria are one of the major sources of ROS in the placenta and therefore a mitochondrial antioxidant treatment may provide a potential therapeutic strategy to improve fetal/offspring outcomes. MitoQ is a commercially available mitochondrial antioxidant that targets oxidative stress [335]. Since maternal treatments during pregnancy are often associated with negative off-target effects on fetal/offspring health (reviewed in [438]), we have been using the strategy of encapsulation of MitoQ into nanoparticles (nMitoQ) to specifically target the placenta, and in this way prevent direct fetal exposure [332]. Our laboratory previously reported the effectiveness of nMitoQ treatment (please refer to Chapter 1, section 1.9), however, the long-term effects of maternal nMitoQ treatment on the cardiac capacity to tolerate a negative insult such as I/R in the adult offspring, and the potential mechanisms, have not been assessed.

Cardiac I/R injury is characterized by enhanced cardiac oxidative stress, an acidic intracellular pH and subsequent Ca^{2+} overload. These events drive intracellular Ca^{2+} oscillations and promote an excessive contractile activity. Hypercontractures of the cardiac muscle *per se* are able to disrupt cellular architecture and prime sarcolemmal rupture and cell death. SR, being the main intracellular Ca^{2+} store in the heart, mediates excitation-contraction coupling via rapid uptake of Ca^{2+} by SERCA2α and release of Ca^{2+} ions by RyR2 (reviewed in [439]). SERCA2α activity can be inhibited by PLN, and when intracellular Ca^{2+} concentrations are low, PLN interacts with SERCA2α and decreases its affinity to Ca^{2+} [439]. Previous research showed the importance of activation of protein kinases involved in the regulation of PLN activity during an I/R insult on post-ischemic recovery of cardiac function [103]. Dysregulated Ca^{2+} handling by the SR leads to cardiomyocyte necrosis and is shown to be a main trigger for development of cardiac failure [440]. At high Ca^{2+} concentrations, the inhibitory function of PLN is reduced due to activation of CaMK II, a serine/threonine-specific

protein kinase that phosphorylates PLN at the threonine17 (Thr17) residue. Protein phosphatase 2C epsilon (PP2Ce) is able to dephosphorylate PLN at Thr17 site and increased PP2Ce level has been associated with the development of cardiac dysfunction in transgenic rat animal model [212]. At the same time, the novel Ca^{2+}-independent PKCε was found to be protective, when activated during preconditioning [441], and is able to both increase and decrease PLN phosphorylation at Ser16 residue [441,442]. Therefore, as Ca^{2+}signalling is essential in the establishment of I/R-induced cardiac dysfunction, it is likely to be one of the potential mechanisms involved in the development of cardiac dysfunction in offspring exposed to prenatal hypoxia. In the current study we tested the hypothesis that treating the placenta using nMitoQ improves cardiac tolerance to I/R in prenatal hypoxic-exposed adult male and female offspring.

5.2. Results

5.2.1. Adult offspring characteristics

At 4 months of age, in the male but not the female offspring, body weights were lower in the hypoxic group compared to normoxia control offspring, while there was no effect of maternal nMitoQ treatment. There were no differences in heart weight and heart/body weight ratios in any of the groups in male or female offspring (Table 5.1).

Table 5.1. Effect of prenatal exposure to hypoxia and treatment with nMitoQ on offspring biometrics at 4 months of age.

Offspring biometrics (4 months of age)							
	Normoxia		p-Hypoxia		Two-way ANOVA		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Int.
MALE							
Body weight (g)	**733.6±10.5**	**712.1±31.5**	**682.2±6.6**	**678.8±14.1**	*	-	-
Heart weight (g)	2.12±0.06	2.16±0.15	1.98±0.07	2.02±0.07	-	-	-
Heart/body weight ratio (mg/g)	2.9±0.1	2.8±0.1	2.9±0.1	2.9±0.1	-	-	-
FEMALE							
Body weight (g)	362.3±9.5	360.8±15.4	346.7±9.4	360.7±15.5	-	-	-
Heart weight (g)	1.22±0.03	1.18±0.05	1.26±0.06	1.16±0.04	-	-	-
Heart/body weight ratio (mg/g)	3.4±0.1	3.3±0.1	3.5±0.1	3.3±0.1	-	-	-

Table 5.1 legend: *data are presented as mean ± SEM and were analyzed with two-way ANOVA with Sidak's multiple comparisons post hoc test. *p≤0.05 group effect of prenatal environment or treatment; n=12-14 offspring/1-2 offspring per dam/group.*

5.2.2. Assessment of cardiac function using *ex vivo* isolated working heart technique

In male offspring, pre-ischemic cardiac power during aerobic perfusion was not different between the groups (Figure 5.1. A). Post-ischemic cardiac power was decreased in male offspring exposed to prenatal hypoxia compared to normoxic control offspring. Maternal nMitoQ treatment did not affect post-ischemic cardiac performance in normoxic offspring, while maternal nMitoQ treatment increased post-ischemic cardiac power in the offspring exposed to prenatal hypoxia (Figure 5.1. A. *i*). The percentage of cardiac recovery of the baseline was lower in male prenatally hypoxic offspring compared to the normoxic group, while maternal nMitoQ treatment increased cardiac recovery from baseline in prenatally hypoxic male offspring only (Figure 5.1. A. *ii*).

In females, pre-ischemic cardiac power was not different between the groups (Figure 5.1. B). Although post-ischemic cardiac power was not different (Figure. 5.1. B. *i*), the percentage of recovery of the baseline was reduced in female offspring exposed to prenatal hypoxia compared to normoxic controls (Figure 5.1. B. *ii*). Moreover, maternal nMitoQ treatment improved the recovery of the baseline in prenatally hypoxic female offspring but did not affect the recovery of cardiac power in the normoxic group (Figure 5.1. B. *ii*).

Intriguingly, there was significantly less variability in cardiac recovery of baseline after I/R within the prenatal hypoxia male and female offspring after maternal treatment with nMitoQ compared to offspring from saline treated dams (Figure 5.1. A and B. *ii*; Welch's two-sample t-test).

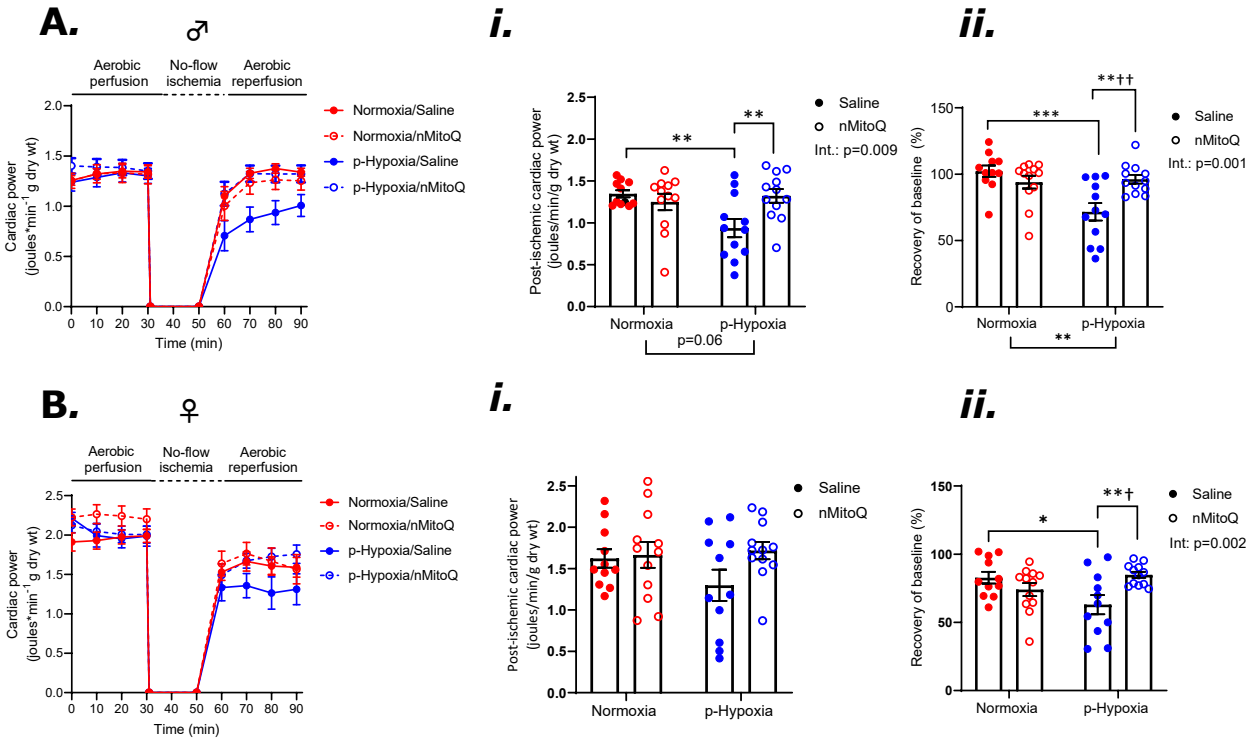


Figure 5.1. *Cardiac power development during ischemia/reperfusion insult in adult male and female offspring.*

*Cardiac power development during 30 min of aerobic perfusion followed by 20 min no-flow ischemia with 40 min of aerobic reperfusion in adult male (A) and female (B) offspring exposed to normoxia (red) or hypoxia (p-hypoxia; blue) during pregnancy and after receiving maternal treatment with saline (closed circles) or nMitoQ (open circles). Summary graphs show: (i) the mean cardiac power during the aerobic reperfusion phase and (ii) the percentage of cardiac recovery from baseline. Data are presented as mean ± SEM and were analyzed with two-way ANOVA with Sidak's multiple comparisons post-hoc test (*p≤0.05; **p≤0.01; ***p≤0.001), and Welch's two-sample t-test for Hypoxia/Saline and Hypoxia/nMitoQ groups (†p≤0.05, ††p≤0.01). n=12-14 offspring/1-2 offspring per dam/group.*

5.2.3. Molecular analysis of cardiac intracellular proteins involved in the regulation of calcium cycling

5.2.3.1. Cardiac sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase protein levels after ischemia/reperfusion insult in adult offspring

SERCA2α content in LV tissues from adult male offspring exposed to prenatal hypoxia was not different compared to normoxic control offspring and no changes were observed after maternal nMitoQ treatment (Figure 5.2. A). However, in females, cardiac SERCA2α content was lower in prenatally hypoxic group compared to normoxic controls that was not altered by maternal nMitoQ treatment (Figure 5.2. B).

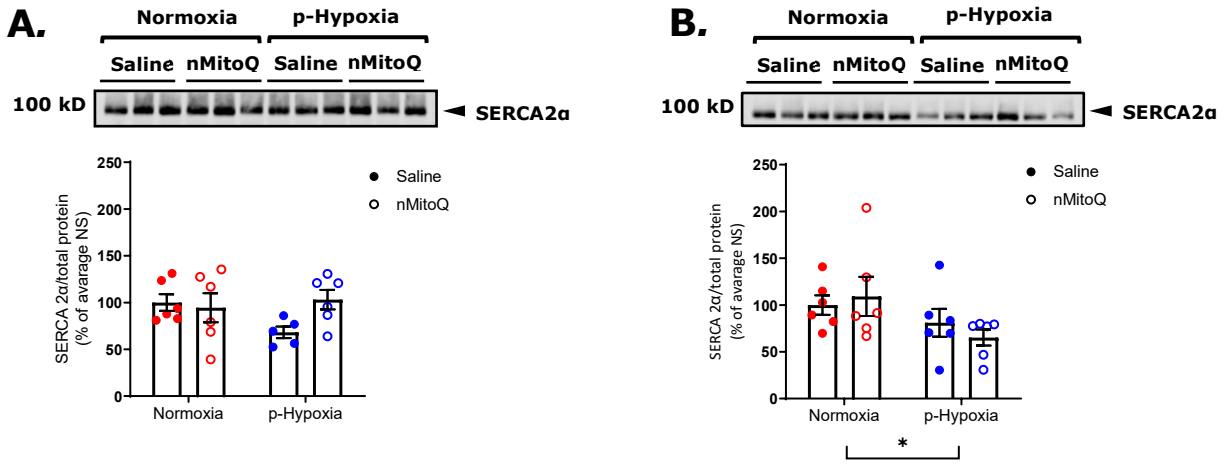


Figure 5.2. *Cardiac sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2α) protein levels after ischemia/reperfusion insult in adult offspring.*

*Cardiac SERCA2α protein content in 4-month-old male (A) and female (B) offspring exposed to normoxia (red) or hypoxia (p-hypoxia; blue) during pregnancy and after receiving maternal treatment with saline (closed circles) or nMitoQ (open circles). Representative immunoblots are shown above the graphs; protein band intensities were normalized towards total protein. Data are presented as the percentage of the mean of the Normoxia/Saline control group (NS), shown as mean ± SEM and were analyzed with two-way ANOVA with Sidak's multiple comparisons post hoc test. *p≤0.05; n=5-6 offspring/1 offspring per dam/group.*

5.2.3.2. Cardiac phospholamban protein levels and phosphorylation after ischemia/reperfusion insult in adult offspring

In hearts from male offspring, PLN levels were not different between normoxic and prenatally hypoxic offspring (Figure 5.3. A. *i*). However, after maternal nMitoQ treatment, cardiac PLN protein content was elevated in male offspring from both normoxic and hypoxic pregnancies in comparison to maternal saline treatment (Figure 5.3. A. *i*). No differences in PLN phosphorylation (pPLN Ser16/Thr17/ total PLN ratio) were found in LV cardiac tissues from male offspring (Fig 5.3. A. *ii*). In female offspring, cardiac PLN protein levels were not affected by prenatal hypoxia or maternal nMitoQ treatment (Figure 5.3. B. *i*), however, PLN phosphorylation was enhanced in both normoxic and hypoxic female offspring after maternal nMitoQ treatment (Figure 5.3. B. *ii*).

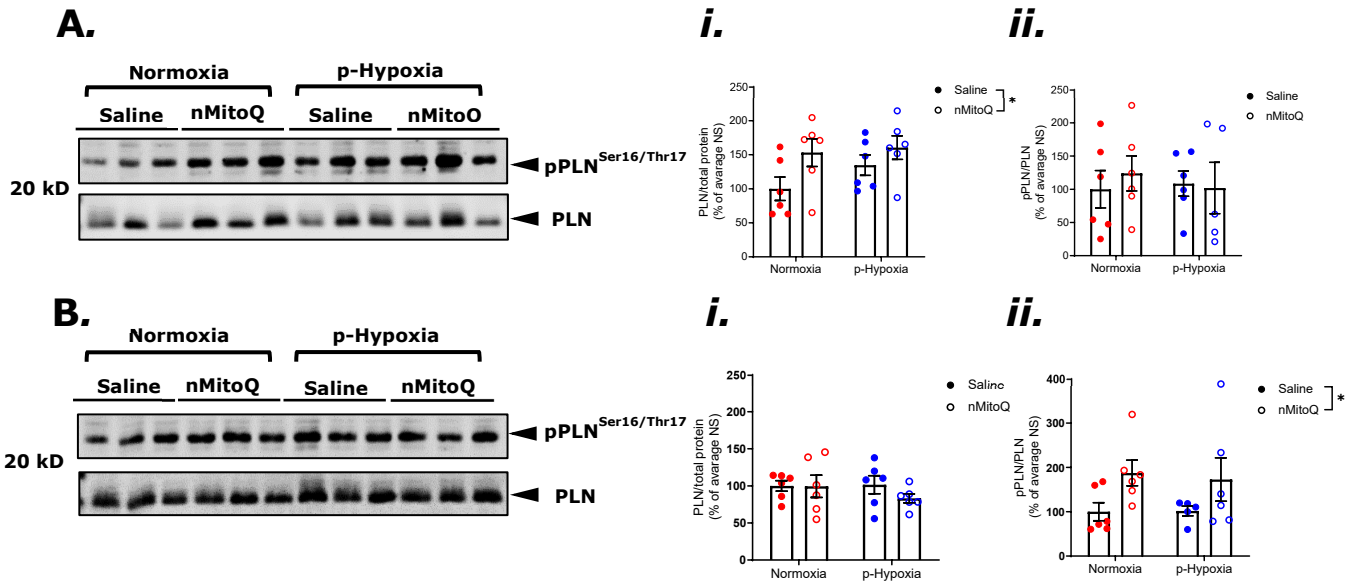


Figure 5.3. *Cardiac phospholamban (PLN) protein levels and phosphorylation after ischemia/reperfusion insult in adult offspring.*

*Cardiac PLN protein content (i) and phosphorylation at Ser 16/Thr17 residues (ii) in 4-month-old male (A) and female (B) offspring exposed to to normoxia (red) or hypoxia (p-hypoxia; blue) during pregnancy and after receiving maternal treatment with saline (closed circles) or nMitoQ (open circles). Representative immunoblots are shown above the graphs; protein band intensities were normalized using total protein. Data are presented as the percentage of the mean of the Normoxia/Saline control group (NS), shown as mean ± SEM and were analyzed with two-way ANOVA with Sidak's multiple comparisons post hoc test. *p≤0.05; n=5-6 offspring/1 offspring per dam/group.*

5.2.3.3. Cardiac Ca^{2+}/calmodulin kinase δ protein levels and phosphorylation after ischemia/reperfusion insult in adult offspring

Cardiac levels of CaMK IIδ were not different in male offspring exposed to either normoxia or prenatal hypoxia, or after nMitoQ treatment (Figure 5.4. A. *i*). Moreover, phosphorylation of CaMK IIδ (pCaMK Thr287/total CAMK IIδ) was not changed by prenatal hypoxia in males but tended to be decreased in hypoxic group after maternal nMitoQ treatment (Figure 5.4. A. *ii*). No changes in cardiac levels or phosphorylation of CaMK IIδ were observed in female offspring between the groups (Figure 5.4. B. *i*, *ii*).

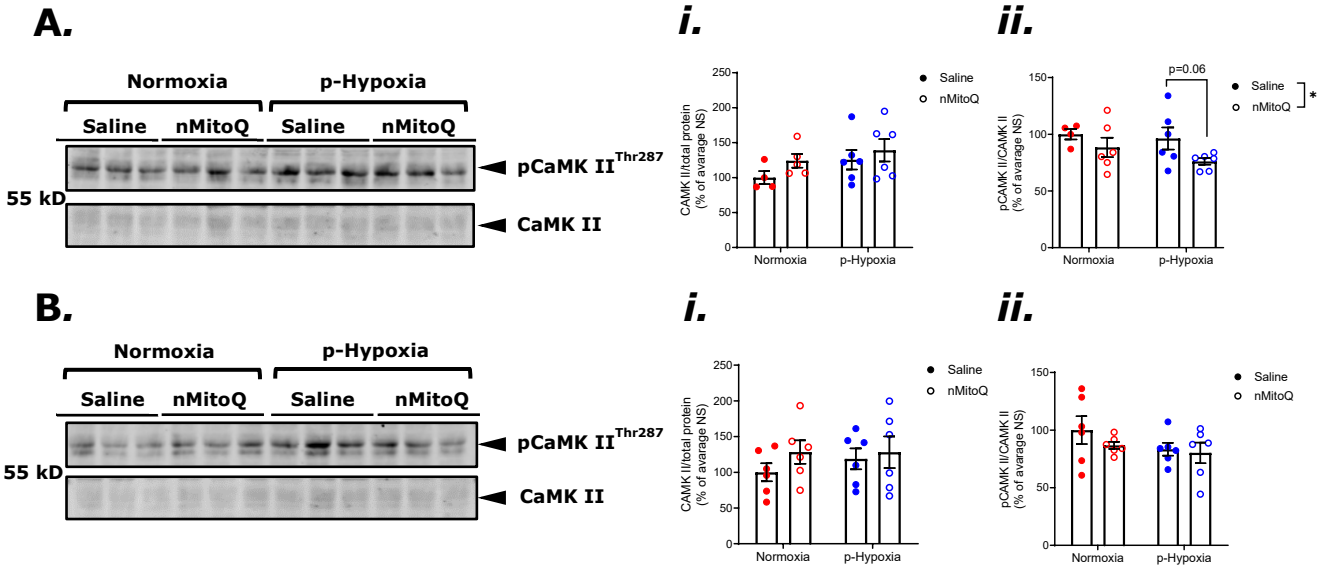


Figure 5.4. *Cardiac Ca^{2+}/calmodulin kinase δ (CaMK IIδ) protein levels and phosphorylation after ischemia/reperfusion insult in adult offspring.*

Cardiac CaMK IIδ protein content (i) and phosphorylation at Thr 287 residue (ii) in 4-month-old male (A) and female (B) offspring exposed to normoxia (red) or hypoxia (p-hypoxia; blue) during pregnancy and after receiving maternal treatment with saline (closed circles) or nMitoQ (open circles). Representative immunoblots are shown above the graphs; protein band intensities were normalized towards total protein. Data are presented as the percentage of the mean of Normoxia/Saline control group, (NS), shown as mean ± SEM and were analyzed

*with two-way ANOVA with Sidak's multiple comparisons post hoc test. *p≤0.05; n=4-6 offspring/1 offspring per dam/group.*

5.2.3.4. Cardiac protein phosphatase 2Ce protein levels after ischemia/reperfusion insult in adult offspring

Cardiac PP2Ce levels were not different between normoxic and hypoxic male offspring (Figure 5.5. A). However, in prenatally hypoxic male offspring, PP2Ce levels were elevated after maternal nMitoQ treatment, compared to saline treatment (Figure 5.5. A). In female offspring, neither prenatal hypoxic exposure nor maternal nMitoQ treatment affected PP2Ce protein content (Figure 5.5. B).

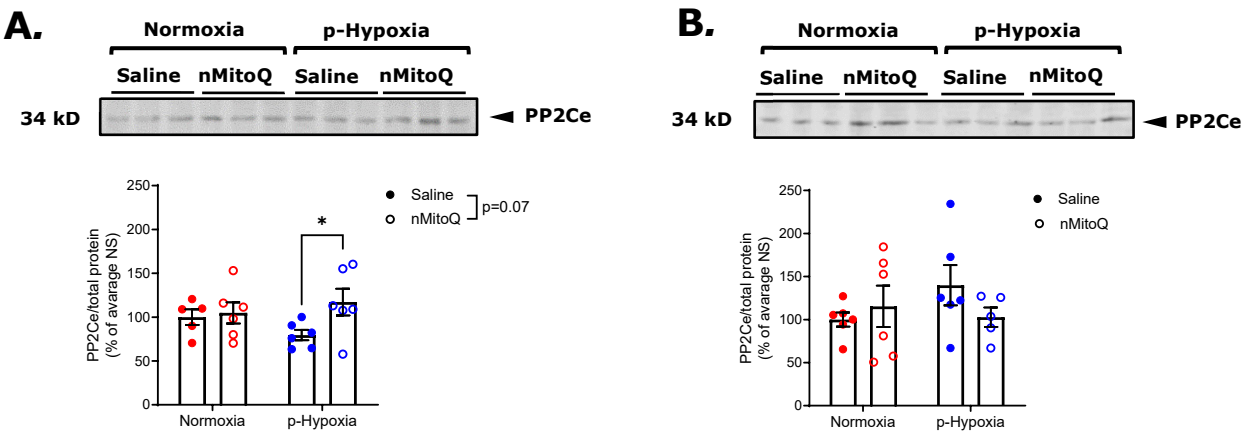


Figure 5.5. *Cardiac protein phosphatase 2Ce (PP2Ce) protein levels after ischemia/reperfusion insult in adult offspring.*

*Cardiac PP2Ce level in 4-month-old male (A) and female (B) offspring exposed to normoxia (red) or hypoxia (p-hypoxia; blue) during pregnancy and after receiving maternal treatment with saline (closed circles) or nMitoQ (open circles). Representative immunoblots are shown above the graphs; protein band intensities were normalized towards total protein. Data are presented as the percentage of the mean of Normoxia/Saline control group, (NS), shown as mean ± SEM and were analyzed with two-way ANOVA with Sidak's multiple comparisons post hoc test. *p≤0.05; n=5-6 offspring/1 offspring per dam/group.*

5.2.3.5. Cardiac protein kinase Cε protein levels and phosphorylation after ischemia/reperfusion insult in adult offspring

Cardiac levels of PKCε were not different between the groups in male offspring (Figure 5.6. A. *i*). However, in adult hypoxic male offspring, the phosphorylation of PKCε (pPKCε Ser729/total PKCε ratio) was decreased compared to normoxic controls (Figure 5.6. A. *ii*). In the normoxic male offspring, PKCε phosphorylation was decreased after maternal nMitoQ treatment, while in the offspring exposed to prenatal hypoxia PKCε phosphorylation appeared to be higher compared to saline treatment (significant interaction; Figure 5.6. A. *ii*). In female offspring, PKCε levels were not different between the groups, however, in female offspring exposed to prenatal hypoxia, phosphorylation of PKCε was increased after maternal nMitoQ treatment compared to saline treatment (Figure 5.6. B. *i*).

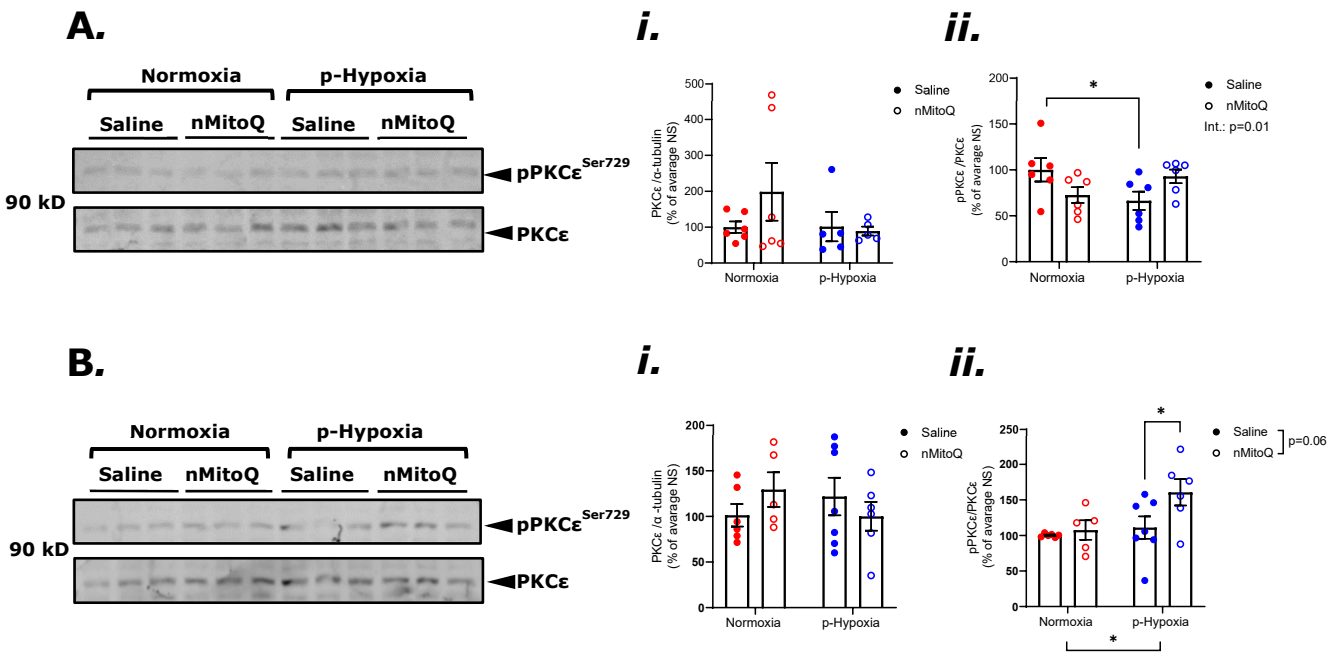


Figure 5.6. *Cardiac protein kinase Cε (PKCε) protein levels and phosphorylation after ischemia/reperfusion insult in adult offspring.*

*Cardiac PKCε protein content (i) and phosphorylation at Ser 729 residue (ii) in 4-month-old male (A) and female (B) offspring exposed to normoxia (red) or hypoxia (p-hypoxia; blue) during pregnancy and after receiving maternal treatment with saline (closed circles) or nMitoQ (open circles). Representative immunoblots are shown above the graphs; protein band intensities were normalized using α-tubulin levels. Data are presented as the percentage of the mean of Normoxia/Saline control group (NS), shown as mean ± SEM and were analyzed with two-way ANOVA with Sidak's multiple comparisons post hoc test. *p≤0.05; n=5-7 offspring/1-2 offspring per dam/group.*

5.3. Discussion

In the current study we demonstrated that a placenta-targeted treatment with a mitochondrial antioxidant, nMitoQ, improves long-term cardiac performance by increasing cardiac tolerance to I/R insult in both adult male and female offspring born from pregnancies complicated by prenatal hypoxia. Interestingly, we showed a latent sex-specific (i.e. similar outcome is achieved through distinct underlying mechanisms in males and females) effect of maternal nMitoQ treatment on intracellular proteins that can be involved in the regulation of intracellular calcium cycling. In adult male offspring born from hypoxic pregnancies, we found that maternal nMitoQ treatment increased PLN levels (an inhibitor of SERCA2α activity), tended to decrease CaMK IIδ phosphorylation and increased PP2Ce levels (kinases involved in dephosphorylation of PLN). In contrast, in female offspring born from hypoxic pregnancies, maternal nMitoQ was accompanied by increased phosphorylation of PLN and PKCε. Our data suggest that, while resulting in a similar functional improvement of cardiac tolerance to I/R, there are sex-specific differences in the effects of nMitoQ treatment on cardiac proteins and kinases involved in the regulation of calcium cycling in offspring born from hypoxic pregnancies.

We used an *ex vivo* I/R insult as a tool to unmask an impairment in cardiac function in adult offspring exposed to prenatal hypoxia [227,234,239,385,443,444], and to assess the potential effect of maternal nMitoQ treatment. Indeed, in line with previous research [227,234,239,240,385,444] cardiac recovery after I/R was lower in offspring that experienced prenatal hypoxia compared to offspring from normoxic controls. Moreover, to the best of our knowledge, we are the first to show that a prenatal treatment specifically targeting the placenta (i.e. reducing placental oxidative stress), and not the fetus, during hypoxic pregnancies was able to improve cardiac tolerance to I/R insult in adult offspring. Several prenatal (not placenta-specific (reviewed in [438])) and postnatal interventions have shown capacity to improve post-ischemic cardiac performance in offspring exposed to prenatal

hypoxia. For instance, our lab previously showed that postnatal supplementation with resveratrol is able to improve cardiac function in adult intrauterine growth restricted offspring fed with high-fat diet [385,444]. Improved cardiac recovery after I/R insult was accompanied with increased cardiac phospho-adenosine monophosphate kinase and superoxide dismutase levels in adult (female) offspring and a decreased post-ischemic cardiac superoxide level in both male and female adult offspring [385,444]. Moreover, maternal supplementation with vitamin C improved (increased) cardiac chronotropic responses to carbachol (acetylcholine receptor agonist) and decreased prenatal hypoxia-induced elevated cardiac responses to isoprenaline (β adrenoreceptor agonist) in adult male offspring [24]. Maternal treatment with allopurinol (an effective inhibitor of the enzyme xanthine oxidase) has been shown to increase cardiac tolerance to I/R insult (by decreasing lactate dehydrogenase and creatine kinase levels), and ameliorate the enhanced sympathetic dominance in the heart (by decreasing SERCA2α expression) in adult male offspring exposed to prenatal hypoxia [234]. In the current study, we explored the effect of a placenta-targeted treatment (a mitochondrial antioxidant encapsulated into nanoparticles) as a prenatal strategy aimed to improve offspring outcomes (in offspring born from hypoxic pregnancies) without direct fetal drug exposure. We previously showed that these nanoparticles target the placenta and do not cross the basal membrane to the fetus [332]. Moreover, we now showed that treatment of the placenta during a complicated pregnancy has (indirect) effects on fetal health and has the ability to prevent long-term effects of complicated pregnancies on the cardiac tolerance to a negative insult (such as I/R injury) in the adult offspring. Together with previous studies, our current findings indicate that maternal pre- and postnatal interventions can improve offspring health in adult life and have the potential to decrease the risk of adult-onset CV disease.

Interestingly, in both male and female adult offspring, there was high variability in the cardiac recovery after I/R within the hypoxic group, while maternal nMitoQ treatment during hypoxic pregnancy not only improved cardiac tolerance to I/R insult, but also decreased the

variability in cardiac recovery in prenatally hypoxia groups. One explanation for this could be that hypoxia differently affects offspring within a litter in rats (i.e. depending on where the pups are located within the uterine horn), while maternal nMitoQ treatment appears to distribute equally across all placentas and thus equally improves cardiac performance in the offspring.

There are a number of intracellular and extracellular mechanisms involved in development of cardiac dysfunction caused by I/R injury (extensively reviewed in [445]). In general, an I/R insult results in Ca^{2+} overload that leads to intracellular Ca^{2+} oscillations which promotes an excessive contractile activity, disruption of intracellular architecture and cell death [446]. One of the key kinases involved in the regulation of intracellular Ca^{2+} cycling is SERCA2α, which is responsible for actively transporting Ca^{2+} from the cytoplasm to the SR during myocardial diastole [447]. SR Ca^{2+} uptake (through SERCA2α) and release (through RyR2) has been known to play an essential role in the pathophysiology of I/R injury [448,449]. A recent study by Talukder *et al.* demonstrated that mice expressing lower levels of SERCA2α experience a decreased post-ischemic contractile function and excessive myocardial infarction compared to wild-type mice [450], thus indicating that maintaining optimal SERCA2α levels is critical for myocardial protection from I/R injury and post-ischemic cardiac functional recovery. Although previous research did not observe any sex differences in SERCA2 protein abundance in non-ischemic (rat) hearts [451], our molecular analysis of cardiac tissue after I/R insult showed a reduction in SERCA2α levels only in female offspring exposed to prenatal hypoxia. The exact cause for this remains unclear and further studies will be necessary to determine what type of proteases may be involved in the sex-dependent degradation of SERCA2α during I/R insult in offspring exposed to prenatal hypoxia. Interestingly, maternal nMitoQ treatment did not affect cardiac SERCA2α levels in both sexes and thus changes in SERCA2α expression may not be part of the mechanism by which nMitoQ treatment improved

cardiac tolerance to I/R (in either male or female offspring). However, prenatal nMitoQ treatment could be involved in regulating SERCA2α activity.

PLN is one of the main proteins that regulates SERCA2α activity in the heart. In its unphosphorylated state PLN decreases SERCA2α sensitivity to Ca^{2+} ions and decreases Ca^{2+} reuptake to SR. nMitoQ treatment increased PLN levels in normoxic and hypoxic male offspring, without changes in PLN phosphorylation, which may indicate an enhanced inhibition of SERCA2α. Previous research demonstrated that delayed phosphorylation of PLN in the presence of an abnormally high cytosolic Ca^{2+} concentration during reperfusion (Ca^{2+} overload) resulted in effective cytosolic Ca^{2+} extrusion and contributed to cardioprotection in post-conditioned male hearts [452]. In females, however, we found an enhanced phosphorylation of PLN after maternal nMitoQ treatment, without alterations in PLN protein level *per se*, which may contribute to decreased inhibition of SERCA2α activity. This increased PLN phosphorylation could help improve the recovery from I/R insult in female offspring, as early research in transgenic animal model demonstrated an essential role of PLN phosphorylation at the Thr17 residue in the recovery of cardiac function after an I/R insult [453]. For instance, mice mutant for phosphorylation residues (Ser16 and Thr17) of PLN experienced a decreased recovery of contractility and Ca^{2+} transient amplitude compared to wild-type mice however, sex-differences were not assessed [453].

CaMK IIδ is a kinase involved in the phosphorylation of PLN at the Thr17 site, however, the role of CaMK IIδ during I/R is somewhat controversial. Previous research demonstrated beneficial as well as negative effects of increased CaMK IIδ activity during an I/R insult (reviewed in [454]). We found no effects of prenatal hypoxia on CaMK IIδ expression or phosphorylation in both sexes, while a trend to a decreased CaMK IIδ activity was observed in adult prenatally hypoxic male offspring after maternal nMitoQ treatment. CaMK IIδ is highly sensitive to cytosolic Ca^{2+} levels and regulates a variety of channels and transporters implicated in the steps leading to Ca^{2+} overload, which suggests that CaMK IIδ is likely to

contribute to the cascade of events leading to ischemic injury. For instance, CaMK II is able to activate LTCC and Na^+ channels that are able to increase in intracellular Ca^{2+} associated with I/R [455,456]. Experimental evidence indicates that inhibition of CaMK II is protective in the irreversible I/R injury [457] and isolated rat hearts subjected to post-conditioning were shown to have a better recovery of cardiac function after ischemic insult, which was accompanied by a decreased CaMK II activity (decreased autophosphorylation at Thr287) [452]. It is important to mention that CaMK II contribution to I/R-induced cardiac dysfunction can also be attributed to induction of cardiac apoptosis and necrosis as the inhibition of CaMK II results in decreased caspase-3 activity, Bax/Bcl-2 ratio, Ca^{2+} -induced mitochondrial swelling and decreased release of lactate dehydrogenase, respectively [457]. In our study, CaMK IIδ phosphorylation tended to be lower after nMitoQ treatment in prenatally hypoxic males, which thus may have contributed to the improved cardiac function after I/R injury.

Further assessment of post-ischemic cardiac tissue showed elevated PP2Ce levels (a kinase that dephosphorylates PLN at the Thr17 residue [212]) in adult hypoxic male offspring after maternal nMitoQ treatment. PP2Ce specifically colocalizes with SERCA2α on the SR membrane [212]. During stimulation with a β-adrenoreceptor agonist, PP2Ce on the SR was shown to have a significant effect on Ca^{2+} homeostasis by reducing the intracellular peak Ca^{2+} amplitude and slowing the transient Ca^{2+} decline rate, in line with its targeted dephosphorylation of PLN [212]. Therefore, it may be suggested that enhanced PP2Ce content, together with a trend to a decreased phosphorylation of CaMK IIδ, in hypoxic male offspring after maternal nMitoQ treatment could lead to enhanced inhibition of SERCA2α. Interestingly, in human hearts with ischemic cardiomyopathy or dilated cardiomyopathy, PP2Ce levels were elevated in comparison to non-failing control subjects, while transgenic mice with enhanced PP2Ce expression experienced exacerbated cardiac injury after I/R insult [212]. In contrast, a recent study demonstrated an important role for PP2Ce in the regulation of endoplasmic reticulum (ER) stress [458]. I/R injury is a cellular stress condition that leads

to the depletion of ER Ca^{2+} stores, exposure to free radicals, and accumulation of unfolded/misfolded proteins, thereby disrupting proper function of the ER and inducing ER dysfunction [459]. When ER stress is excessive, unfolded protein response (UPR) cell-signaling may initiate apoptosis and subsequent cell death. PP2Ce has highly specific activity towards inositol requiring enzyme 1 (IRE1) and has been reported to regulate the IRE1-mediated UPR by suppressing basal and ER-stress induced IRE1 activity leading to loss of UPR and stress response [458]. Although the current study was not focused on ER stress during I/R insult, our results suggest that assessing a possible contribution of ER stress in manifesting cardiac dysfunction of the offspring exposed to prenatal hypoxia in future studies would be an interesting avenue to pursue.

Previous research has shown substantial sex-differences in the level/phosphorylation of PKCε [392]. For instance, female hearts have higher levels of the active form of PKCε (pPKCε Ser 729) than male hearts, while reperfusion increased pPKCε Ser 729 in female but not male hearts, suggesting that increased PKCε is likely to play an important role in protecting female hearts from I/R and to contribute to the sex differences in cardiac vulnerability to I/R injury. However, prenatal exposure to hypoxia resulted in similar enhanced susceptibility in hearts of both male and female adult offspring, but with latent sex differences in the molecular mechanism. Our results showed enhanced phosphorylation of PKCε in post-I/R cardiac tissue of adult (female) hypoxic offspring after maternal nMitoQ treatment which was accompanied by enhanced phosphorylation of PLN. Thus, maternal nMitoQ treatment may have improved cardiac recovery after I/R insult in adult (female) offspring by increasing the activity of cardioprotective PKCε. Previous research in a rat model of prenatal hypoxia showed decreased levels and activity of cardiac PKCε in adult (male) offspring [240] which contributed to enhanced susceptibility to cardiac I/R. Active PKCε is known to play an important role in cardioprotection (by phosphorylating pro-apoptotic proteins, inducing opening of mitochondrial ATP-sensitive K^+ channels [460], inhibiting Ca^{2+}-induced opening of

mitochondrial permeability transition pores (reviewed in [244]). Although PKCε is able to phosphorylate cardiac regulatory proteins [461,462], the literature related to its contribution to phosphorylation of PLN varies [442,463]. For instance, Yamamura *et al.* demonstrated PKCε-mediated dephosphorylation of PLN at Ser16 site in adult rat cardiomyocytes [463]. While recently, selective silencing of PKCε was shown to attenuate PLN phosphorylation (at Ser16; by novel cAMP sensor proteins), thus indicating a contribution of PKCε to PLN phosphorylation [442]. In line with previous research [240], we observed decreased cardiac PKCε phosphorylation in male offspring exposed to prenatal hypoxia. Maternal nMitoQ treatment resulted in decline of PKCε phosphorylation in normoxic males but appeared to increase in the prenatally hypoxic group. This indicates that nMitoQ treatment may differently impact intracellular mechanisms of cardiac function in adult offspring depending on the prenatal exposure.

Our study highlights the importance of early-stage interventions focused on specifically targeting the placenta during complicated pregnancies and emphasizes latent sex differences in treatment effects on long-term offspring cardiac health. Moreover, we show that later-life CV health of the offspring can be improved by treating the placenta (and preventing direct off-target effects on the fetus) during complicated pregnancies. Therefore, a placenta-targeted treatment strategy may offer new opportunities for the development of efficient treatment strategies in complicated pregnancies to prevent developmental programming of cardiac dysfunction of the offspring in adult life.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1. Summary of the thesis

Prenatal hypoxia has been recognized as a major consequence of complicated pregnancies that can impact fetal development and growth [464-466]. I used a well-established rat model of pregnancy complication with hypoxia, where pregnant rats were subjected to the hypoxia insult (11% O_2) during the last trimester of pregnancy. Previous research from the Davidge lab has shown that the hypoxia exposure on GD15-21 is associated with adverse pregnancy outcomes and has a long-term negative impact on offspring CV function [227,252,330]. In the current thesis I aimed to assess the mechanisms of the impaired post-ischemic cardiac performance in adult offspring with the focus on the local cardiac ET-1 system. Moreover, because coronary artery function is essential in providing proper oxygen and nutrient supply to the myocardium, I aimed to evaluate a long-term impact of prenatal hypoxia on coronary artery function in adult offspring. Owing the fact that the placenta is a main site for oxygen and nutrient supply to the fetus, it is essential in fetal development and growth, and thus, plays a significant role in developmental programming of health and disease [467,468]. As placental oxidative stress is important in the pathophysiology of many pregnancy complications, including fetal growth restriction and developmental programming of diseases [469,470], our lab have been establishing a placenta-targeted treatment with an antioxidant to improve placental, fetal and offspring outcomes [252,329-332]. Thus, I also aimed to evaluate the long-term impact of placenta-targeted treatment during hypoxic pregnancy on cardiac susceptibility to a secondary hit (such as I/R injury) in adult offspring.

6.1.1. Summary and key findings: Role of ET-1 pathway in susceptibility of adult prenatally hypoxic offspring to cardiovascular complications

6.1.1.1. Male offspring: role of ET-1 in cardiac and coronary artery function

I focused on cardiac ET-1 system that has been shown to be upregulated in pathological conditions, and the blockade of ET-1 receptors has been used to ameliorate its effects [471]. With the application of ABT-627 (a selective ET_A antagonist), I showed that ET_A receptors are essential in maintaining recovery of the cardiac function post-ischemia in adult male offspring exposed to prenatal hypoxia. This was a surprising finding as my data showed no recovery with ET_A receptor inhibition. Cardiac tissue levels of ET-1 (that acts through ETR) increased with I/R challenge that has been previously reported in the literature [377,394], however, prenatal hypoxia did not alter this change; and thus, the level of ET-1 post I/R was comparable between normoxia and prenatal hypoxia groups. Also, the levels of ET_A and ET_B in the heart of the male prenatally hypoxic offspring were not different from the normoxia control. Cardiac function is extremely dependent on proper coronary artery function and thus, using the same animal model, I further explored the effects of prenatal hypoxia on ET-1-mediated coronary artery contractile capacity, as well as vasodilatory mechanisms, that balance contractile agents in the vasculature. ET-1-mediated coronary artery reactivity was not affected by prenatal hypoxia in 4- and 9.5-month-old offspring, as well as tissue levels of ET-1, ET_A and ET_B. However, here we report an impaired coronary artery endothelium-dependent vasodilation, while endothelium-independent mechanism of vasodilation was not affected. Further assessment of the mechanisms of impaired endothelium-dependent vasodilatory function revealed a major contribution of NO to vasodilation regardless of prenatal exposure, and an increased contribution of EDH to vasodilatory response in prenatally hypoxic male offspring. However, inhibition of PGHS pathway improved coronary artery dilation to MCh in the prenatal hypoxic offspring, indicative of an enhanced contribution of the vasoconstrictor part of the PGHS pathway to the coronary artery dilatory response.

6.1.1.2. Female offspring: role of ET-1 in cardiac and coronary artery function

In female prenatally hypoxic offspring, ABT-627 (the ET_A receptor blocker) tended to improve post-ischemic cardiac recovery. The assessment of cardiac tissue levels of ET-1 showed similar results that were obtained in males. Mainly, I/R challenge increased the production of ET-1, however, the prenatal hypoxia did not alter this change and it was comparable with the normoxia group. The levels of ET_A were not different from the normoxia control, while levels of ET_B (isoform C) were decreased by prenatal hypoxia. Further assessment of prenatal hypoxia-induced changes in coronary artery function revealed no alterations in ET-1-mediated vasoconstriction in 4-month-old females, despite elevated coronary tissue levels of ET-1. A selective blockade of ET_B showed an increased contribution of ET_B to ET-1-mediated response. With advancement in age, by 9.5-month of age, coronary artery constriction to ET-1 was reduced in prenatally hypoxic offspring. In addition, in 4- and 9.5-month-old offspring, there was an impairment in endothelium-dependent, but not in endothelium-independent, vasodilation. Similar to males, the mechanistic pathways include an enhanced contribution of EDH to endothelium-dependent vasodilation and an enhanced contribution PGHS-dependent vasoconstriction in prenatally hypoxia group.

In summary, prenatal hypoxia impacted the ET-1 pathway with sex specific differences in both cardiac and coronary artery function. Interestingly, coronary artery endothelium-dependent vasodilation was impaired due to an enhanced PGHS-dependent vasoconstrictor that was a similar finding in both male and female offspring. Thus, understanding mechanisms for impaired cardiac and coronary artery function are critical for developing appropriate intervention strategies depending on prenatal history and sex.

6.1.2. Discussion: Role of ET-1 pathway in susceptibility of adult prenatally hypoxic offspring to cardiovascular complications

In the rat model of maternal hypoxia, we previously showed an increased binucleation and decreased proliferation of fetal cardiomyocytes upon its incubation with a placenta conditioned media from the hypoxic dams [331]. Hypoxia is a potent inducer of *edn* gene expression [472,473] and a previous study in goats demonstrated that maternal hypoxemia (a decreased oxygen content in the blood) results in increased plasma levels of ET-1 in the fetus [474], indicating a negative correlation between the level of oxygen and the level of ET-1 in fetal plasma. Recent study by Paradis *et al.* reported an elevated level of prepro ET-1 mRNA in fetal hearts from a hypoxic pregnancy, and exposure cardiomyocytes *in vitro* to high levels of ET-1 is accompanied with a reduced cardiomyocytes proliferation and differentiation [475]. Thus, I investigated an impact of fetal hypoxia on cardiac and coronary artery ET-1 system in adult male and female offspring.

First of all, ET-1 acts in paracrine and autocrine fashion and has been recognized as a potent modulator of cardiac contraction (please refer to Chapter 1). The ET-1 system has been shown to be involved in the pathophysiology of CV diseases due to an enhanced local and systemic (in circulating plasma) production of ET-1, due to an enhanced responsiveness of targeted cells to ET-1 or due to a reduction of counterbalanced mechanisms (for instance, a reduced production or action of dilatory factors). Ischemia increases synthesis and release of ET-1 (in the tissue and circulation), and inhibitors of ET-1 biosynthesis or action has been shown to significantly reduce I/R-induced cardiac damage [126,187,476-478]. Similarly, in my *ex vivo* heart preparation, I/R doubled cardiac tissue levels of ET-1 (in comparison to aerobically perfused hearts), however, prenatal hypoxia did not impact this change. An inhibition of ET_A did not significantly impact post-ischemic cardiac recovery in the normoxia group but prevented post-ischemia cardiac recovery in the prenatal hypoxia males. Taking into account that no changes in the levels of ET_A and ET_B were observed due to the prenatal

hypoxia, I can suggest that the downstream ET-1-mediated signalling may be essential for cardiac recovery of prenatal hypoxia male offspring. As was shown in the Chapter 1, activation of ET_A can be associated with the activation of cardioprotective PKCε (which levels are shown to be downregulated in prenatally hypoxia males [240]) and the activation of MAPK or ERK1/2 that involved in the preconditioning and inhibition of apoptosis. We may speculate that those factors may play a critical role in maintaining cardiac function of prenatally hypoxic male offspring, and its inhibition (via ET_A inhibition) is detrimental for post-I/R cardiac recovery.

In contrast to males, in prenatally hypoxic female offspring, an application of ET_A antagonist had a beneficial effect. We also showed that prenatal hypoxia is associated with a reduction in ET_B in the female hearts. Previous study by Udpa *et al.* reported ET_B as a top candidate gene that modulates high altitude adaptation in Ethiopians [479]. Further research by Stobdan *et al.* in mice demonstrated a major advantage of decreased cardiac ET_B levels under hypoxic conditions [400]. Mainly, ET_B knockout mouse experienced better cardiac performance under hypoxia exposure (higher dP/dt_{max} at any level of hypoxia exposure), lower level of blood lactate and lower tissue staining for hypoxia (after tissue was harvested in sever hypoxic environment – 5% O_2) [400] thus suggesting that low levels of ET_B seems to be beneficial in protecting cardiac function in moderate and severe hypoxia, that could be a result of fetal adaptation to low oxygen environment *in utero.* However, overall, ET_B are involved in cardiac release of NO and play an important role in ET-1 clearance [178,356], thus ET_B were shown to be beneficial in maintaining cardiac recovery from I/R [187]. Previous study by Yamamoto *et al.* have shown that ET_B–deficient rats experience an impaired post-ischemic recovery of cardiac function that was improved after application of selective antagonist of ET_A (ABT-627), thereby suggesting a major role of ET_A, rather than ET_B in post-ischemic cardiac recovery [187]. Prenatal hypoxia is associated with an impaired cardiac recovery from I/R insult in female offspring, and here, despite decreased ET_B levels, ABT-627 improved cardiac performance post I/R, thus suggesting that ET_A is a major contributor to the development of

cardiac dysfunction in prenatally hypoxic female offspring. The mechanisms remain to be investigated, as the cardiac tissue levels of ET-1 was comparable between normoxia and prenatal hypoxia groups, that may indicate an involvement of ET_A-mediated signalling. ET-1 has been shown to have direct pro-ischemic effect (independently of coronary vasoconstriction) by increasing intracellular calcium levels (please refer to Chapter 1). Thus, if ET-1 is altering intracellular calcium homeostasis, it may reflect its effects on SR and mitochondria that play pivotal roles in basal cardiac function, as well as during I/R, and as such, further studies are warranted.

The next step was to understand whether prenatal hypoxia impacts coronary artery ET-1-mediated reactivity and vasodilatory mechanisms (that counterbalance ET-1) in adult offspring. We showed no significant changes in the coronary contractile capacity to ET-1, systemic (plasma) levels of ET-1, ET_A and ET_B expression in the prenatally hypoxic offspring. However, prenatal hypoxia was associated with increased tissue levels of ET-1 and major contribution of ET_B to ET-1-mediated vasoconstriction (in 4-month-old female offspring). It was suggested that ET_A rather than ET_B play a more prominent role in coronary vasoconstriction induced by ET-1 [480], however, in pathology, enhanced coronary vasoconstriction to ET-1 can be mediated through ET_B [481]. For instance, Handai *et al.* showed *in vitro* that the inhibition ET_B in hypercholesterolemic pigs attenuates coronary artery contractile responses to ET-1 [481], suggesting its pivotal role in modulating contractile function of ET-1. The endothelium is able to impact ET-1-mediated vasoconstriction by secreting not only relaxing, but also contracting factors. Here we also showed that prenatal hypoxia leads to the impaired MCh—induced coronary vasodilation (an endothelium-dependent vasodilation) in male and female offspring. Subsequently, a decline in ET-1-mediated vasoconstriction after inhibition of ET_B may be caused or by decreased release of endothelium-derived vasoconstrictors, or increased release of endothelium-derived vasorelaxant factors. Taking into account that the application of meclofenamate (a PGHS

inhibitor) improved MCh-induced vasorelaxation (in both sexes), we may consider a potential contribution of vasoconstrictor agents derived from PGHS pathway to the impairment in endothelium-dependent vasodilation. Moreover, as was described in the Chapter 1, ET_B are also located on endothelium whose activation facilitates the activity of PGHS and a subsequent release of its products. Thus, we may speculate that the application of BQ788 inhibited ET_B on both sites, VSMCs and endothelium, and thereby prevented the activation of PGHS and the release of PGHS-mediated contractile factors that could contribute to ET-1-mediated contractile function in prenatally hypoxic offspring. Our results did not show any changes in tissue levels of PGHS-1 or PGHS-2 in the females, and as such, the question regarding its activity in prenatal hypoxic offspring remains open. By 9.5-month of age, ET-1-mediated responsiveness declined in prenatally hypoxic females, without a specific contribution of ETR. An impaired ET-1-mediated coronary vasoconstriction has been previously demonstrated in deoxycorticosterone acetate (DOCA)-salt hypertensive rats [435] and obesity [434]. Based on those reports, a decline in ET-1-mediated constriction was not associated with changes in the expression of the ETR, but mostly with an enhanced ET_B-mediated generation of NO (that can be excluded in our study as the inhibition of ET_B did not alter ET-1-mediated vasoconstriction in 9.5-month-old female offspring), diminished elevation in $[Ca^{2+}]_i$ and an uncoupling of ETR [434,435]. A previous study by *Chen et al.* demonstrated in coronary arteries from prenatally hypoxic offspring an impaired coronary artery contraction to serotonin and PKC agonist that was associated with a reduced capability of calcium release from SR [318]. Thus, we may speculate that an overall impairment in ET-1-mediated responsiveness in coronary arteries from adult prenatally hypoxic females could be, at least in part, due to alterations in intracellular regulation of calcium homeostasis, however, further studies are warranted to confirm this hypothesis.

We also used L-NAME (an inhibitor of NOS) and apamin and Tram-34 (an inhibitors of SK_{Ca} and IK_{Ca} channels, respectively) to evaluate the potential contribution of other pathways

to an impaired endothelium-dependent vasodilation in adult prenatally hypoxic offspring. Abundant data from previous studies exist regarding the contribution of alterations in NO-dependent pathway (decreased synthesis or bioavailability of NO) in the endothelium to the development of coronary artery pathology, including acute coronary syndrome, coronary heart disease and atherosclerosis [482-484]. L-NAME completely prevented MCh-induced vasodilation in LADs, thereby suggesting its major role in endothelium-dependent vasodilation. We further assessed coronary tissue levels of eNOS, however, no changes were observed due to the prenatal hypoxia. Further *in vitro* studies are warranted to evaluate possible changes in this pathway due to the prenatal hypoxia, potentially by simultaneous inhibition of PGHS and EDH pathways during *in vitro* assessment of coronary artery function with wire myography, and by assessing the activity (or phosphorylation status) of NOS and NO bioavailability in the coronary artery tissue as well as levels of oxidative products of NO in the coronary circulation. We also demonstrated an enhanced contribution of EDH (via SK_{Ca} and IK_{Ca} channels) to endothelium-dependent vasodilation in prenatally hypoxic male and female offspring (as the application of apamin and Tram-34 decreased coronary artery sensitivity to MCh (pEC_{20})). Previous study by Yada *et al.* reported (in animal model of coronary occlusion) an impaired NO mediated vasodilatations of small coronary arteries that were compensated by EDH [485], giving an essential protective role of EDH in pathological condition. I assume that EDH may serve as an additional endothelium-dependent mechanism to compensate for an impaired vasodilation in the coronary vasculature of prenatally hypoxic offspring.

6.1.3. The long-term effect of placenta-targeted treatment on offspring cardiac tolerance of ischemia/reperfusion injury

Prenatal hypoxia has been associated with a developmental programing of CV dysfunction in the offspring in adult life (please refer to Chapter 1; table 1.1). Thus, prenatal period (before birth) has been recognized as a "window of opportunity" for interventional

strategies that can impact fetal development and growth, and potentially ameliorate negative consequences of pregnancy complications. Our laboratory previously reported a beneficial effect of maternal nMitoQ treatment during hypoxic pregnancy on placental and fetal outcomes as well as CV function in adult life (Chapter 1; section 1.9). Because an enhanced cardiac susceptibility to I/R insult of prenatally hypoxia offspring is a common complication (Chapter 1, table 1.1), I assessed the impact of placental-targeted treatment on later-life offspring (male and female) susceptibility to I/R injury by assessing calcium regulating pathways due to their critical importance to cardiac contractility.

My data demonstrated that adult offspring exposed to hypoxia in utero are susceptible to cardiac I/R insult, however, the intracellular proteins that are involved in EEC and calcium cycling revealed sex-specific differences (Figure 6.1). Sex differences in SR calcium loading upon cardiac stimulation with isoproterenol was previously reported by Chen *et al* [486], where in males, isoproterenol significantly increased SR Ca^{2+} (compared to females). Subsequently, we may expect that this may be one of the mechanisms that leads to different calcium load in male *vs.* female, and thus, the expression in intracellular proteins that are involved in the regulation of calcium cycling can be also sex specific. I showed that maternal nMitoQ treatment increased PLN and PP2Ce levels and decreased cCaMK II/CaMK II ratio that may prevent activation of SERCA2α and active calcium reuptake to SR, aiming to reduce intracellular calcium level. This can be one among several mechanisms that is involved in the regulation of intracellular concentration of calcium. We still don't know regarding other mechanisms, such as the level and activity of Ca^{2+} ATPase, mitochondrial Ca^{2+} uptake or NCX activation in prenatally hypoxic males. The last one has been shown to be involved in the development of calcium overload during I/R, and its inhibition or knockout (in mice) was shown to be beneficial during I/R [487,488].

In contrast to prenatally hypoxic males, maternal nMitoQ treatment during hypoxic pregnancy was associated with increased pPLN/PLN ratio and increased phosphorylation of

PKCε, which shown to be cardioprotective during I/R insult [489-491]. Among many mechanisms that can be involved in PKCε-mediated cardioprotection, previous study by Baines *et al.* have shown that PKCε activation increases mitochondrial co-localization of PKCε with MAPKs, enhances phosphorylation of mitochondrial MAPKs, and mediates the formation of mitochondrial PKCε-MAPK signaling modules that are related to the inhibition of pro-apoptotic molecules and the genesis of a cardioprotective phenotype [492]. Moreover, PKCε was shown to be also involved in the phosphorylation of PLN [442] that may contribute to enhanced pPLN/PLN ratio and potentially modulate Ca^{2+} uptake by SR. Interestingly that prenatal hypoxia was not associated with changes in phosphorylation of PKCε in females, which is in line with previous research (Chapter 1, table 1 [240]); thus, PKCε may not be a part of the mechanism of impaired cardiac tolerance to I/R, but it may be one of the mechanisms involved in improved post-ischemic cardiac recovery in the nMitoQ treated group.

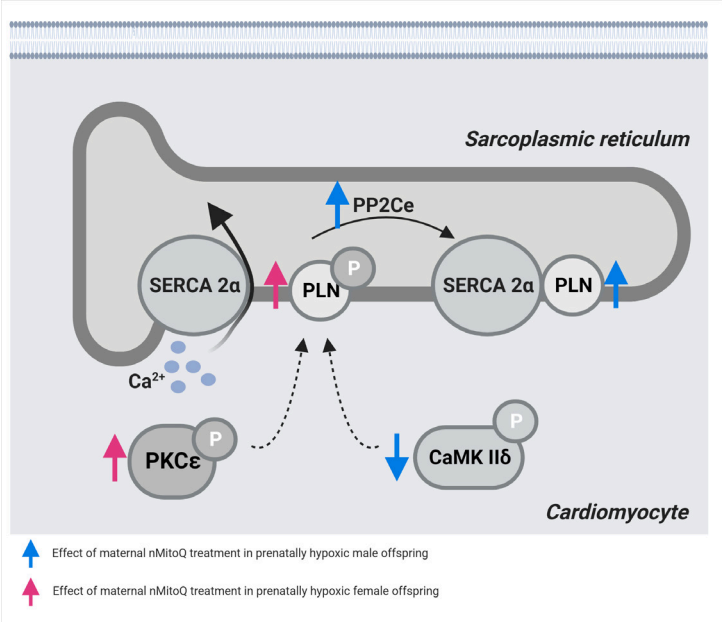


Figure 6.1. Schematic summary of key findings: the effect of maternal nMitoQ treatment during hypoxic pregnancies on cardiac proteins involved in regulation of calcium cycling after ischemia/reperfusion insult in adult offspring.

Figure 6.1. legend: *SERCA2α, sarco/endoplasmic reticulum Ca^{2+}-ATPase; PP2Ce, protein phosphatase 2Ce; PLN, phospholamban; PKCε, protein kinase C epsilon; CaMK IIδ, Ca^{2+}/calmodulin-dependent protein kinase IIδ. Adapted from ref [393]: this article was published in Pharmacological Research, 165, Nataliia Hula, Floor Spaans, Jennie Vu, Anita Quon, Raven Kirschenman, Christy-Lynn M. Cooke, Tom J. Phillips, C. Patrick Case, Sandra T. Davidge, Placental treatment improves cardiac tolerance to ischemia/reperfusion insult in adult male and female offspring exposed to prenatal hypoxia, 105461, Copyright Elsevier Ltd. (2021).*

6.2. Project limitations

To address our research question, we used a rat model of prenatal hypoxia that was previously established in our laboratory by exposing pregnant dams to hypoxia during the last third of gestation (GD 15-21; term: 22 days). Therefore, this model system represents a reliable tool to study the effects of decreased oxygen supply to the fetus on its development and growth, as well as what are the potential mechanisms of impaired function of different organ systems (including the CV system) in adult offspring. One of the limitations is based on the systemic maternal exposure to the hypoxia by placing pregnant rat to the hypoxia chamber. This may drive various maternal adaptations (for instance, cardiac and pulmonary) as rats are subjected to chronic hypoxia that may have an additional impact on maternal CV system. Subsequently, this hypoxic exposure does not fully represent clinical condition of pregnancy complication as pregnancy complications in human are frequently associated with a placental insufficiency, but not with the systemic hypoxia exposure.

Another limitation of the last study (Chapter 5) is a time of the intervention. First, *iv* infusion of nMitoQ was given before the hypoxia insult, and as such, is considered to be a preventative therapy. In human, the determination of the time for intervention is challenging and can be implemented at any trimester, depending on the clinical situation. Subsequently, the application of nMitoQ at different time points after the start of the hypoxia exposure could be considered for future research. Moreover, the duration of human pregnancy is longer compared to rats, as such, the number of doses of nMitoQ should be modified accordingly.

In clinical conditions, the application of ET_A antagonists are usually associated with enhanced activation of ET-1 system (increased systemic levels of ET-1) or in patients with severe chronic conditions (including, but not limited to heart failure and coronary artery disease [493,494]) that was not evident in our 4-month-old offspring (for example, no changes in plasma levels of ET-1 were observed) before our cardiac or coronary artery

experiments and as such, ABT-627 was used only as a tool to evaluate an overall contribution of ET_A to an impaired cardiac tolerance to I/R insult in adult offspring.

In Chapters 3 and 4 we evaluated the systemic and tissue levels of ET-1. The ET-1 levels assessment is based on the detection of specific amino acid residues (1-21), however, this amino acid sequence can be a part of the big ET-1 (1-38 amino acid residues) as well as preproET-1 (1- 212 amino acid residues) peptides, and as such, the question regarding the activity of detected ET-1 remains open. Another aspect that I would like to mention are the *ex vivo* and *in vitro* assessments of cardiac and vascular function, respectively, that could be a potential limitation of my project. These types of preparations are important to evaluate local changes within heart or vascular bed, separately from the systemic immune, humoral and neuronal effects. However, those systems can substantially influence the function of the CV system in *in vivo* conditions, thereby, this fact should be taken into consideration for future research.

6.3. Future directions

In this thesis I demonstrated that maternal hypoxia exposure alters the cardiac ET-1 system and leads to different sex-specific effects of ET_A antagonist on post-ischemic cardiac performance in adult offspring. Taking into account that maternal nMitoQ treatment during hypoxic pregnancy improved cardiac capacity to recover in both, male and female offspring, further studies on evaluating its effects on cardiac ET-1 system would be an interesting avenue to pursue. Mainly, it would be important to know whether nMitoQ treatment prevents a hypoxic phenotype of the lethal cardiac outcomes of inhibition of ET_A (in males) during I/R challenge, as well as the potential changes in cardiac expression of ETR and its downstream signalling. Also, our laboratory has previously shown a beneficial effect of maternal nMitoQ treatment during hypoxic pregnancy on the placental mitochondrial function [330]. Subsequently, it would be important to evaluate the effects of nMitoQ treatment during the hypoxic pregnancy on the placental metabolism.

There are various epigenetic mechanisms that has been shown to impact ET-1 pathway, including DNA methylation and histone modification [495,496]. Prenatal hypoxia is a common form of intrauterine stress that has been shown to result in DNA methylomic and transcriptomic changes in the fetal hearts, and to have a delayed and lasting effect on the adult offspring hearts [497,498]. As such, further studies are warranted to evaluate prenatal hypoxia-induced epigenetic changes in ET-1 pathway in adult offspring.

In Chapter 4 I assessed the mechanisms of the impaired endothelial-dependent vasodilation in adult offspring. Because NO is a major vasodilatory factor in the coronary circulation, an application of the L-NAME (a NOS inhibitor) prevented MCh-induced vasodilation in male and female normoxia and prenatal hypoxia offspring. Subsequently, to evaluate the effect of prenatal hypoxia on NO-mediated vasodilatory pathway in the coronary circulation, a simultaneous application of meclofenamate and apamin and Tram-34 (inhibitors of PGHS and small- and intermediate- conductance Ca^{2+}-activated potassium channels) in the absence of L-NAME is warranted. Moreover, despite the fact that we observed no changes in the tissue levels of NOS due to prenatal hypoxia, it may not express the full picture of the effects on prenatal hypoxia on NO pathway in offspring coronary circulation. eNOS activity depends on the availability of substrates and cofactors, as well as eNOS phosphorylation (phosphorylation of Ser617, 635, and 1179 results in the activation of eNOS, while the phosphorylation of Ser116 and Thr497 reduces eNOS function [499]) and posttranslational modifications (acylation, S-nitrosylation, palmitoylation). Thereby, the assessment of those factors in prenatally hypoxic offspring would be interesting avenue to pursue in the future.

In the Chapter 5, I evaluated the effects of prenatal hypoxia and prenatal intervention on offspring cardiac function and intracellular proteins involved in the intracellular calcium homeostasis. Changes in the Ca^{2+} steady-state is one of the major mechanisms of cardiac post-ischemic reperfusion injury [446,500]. Subsequently, further assessment of calcium

transients in isolated cardiomyocytes of prenatally hypoxic offspring is necessary in order to evaluate the mechanisms of the enhanced susceptibility of adult offspring to cardiac I/R insult.

I also demonstrated that prenatal hypoxia is associated with an impaired coronary artery endothelium-depended vasodilation in both male and female offspring, thus, it would be important to evaluate the effect of maternal placenta-targeted antioxidant treatment on offspring coronary artery function. Moreover, because prenatal hypoxia was associated with changes in coronary artery function, it would be interesting to assess whether those changes impact cardiac oxygenation under basal or loaded (for example with exercise or hypoxia) conditions. Also, during the isolation of the coronary arteries, I observed an enhanced vascularization of the heart in the prenatal hypoxic group (that was distinct from the control group), however, those observations were qualitative, thus further studies specifically designed to assess cardiac vascularization are warranted. Previous studies demonstrate that the hypoxia is a critical factor that drives neovascularization in order to provide oxygen supply to the heart (reviewed here [501]), subsequently, despite the fact that adult offspring were not exposed to the hypoxia in postnatal life, changes that occurred during the fetal life could persist in adult life and impact coronary artery structural, and also functional properties. As such, the evaluation of cardiac vascularization and coronary artery structure in prenatally hypoxia offspring would be essential in understanding the effects of prenatal hypoxia on developmental programing of coronary artery dysfunction.

6.4. Significance

Overall, my studies have shown sex distinct differences in response to prenatal hypoxia on later-life CV function. I also demonstrated 'proof of principle' that treating the placenta can improve later-life cardiac tolerance to secondary insults (such as I/R injury). Moreover, my data highlight the need to understanding mechanisms of developmental origins of CV disease in order to have appropriate and precise therapy based on sex and prenatal history.

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