THE LONG-TERM CARDIOVASCULAR AND BEHAVIOURAL CONSEQUENCES OF MATERNAL IRON RESTRICTION DURING GESTATION IN RAT OFFSPRING

by

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Abstract

Maternal and fetal stressors during development can permanently alter various physiological functions and impact long-term health. These alterations are said to be programmed because they persist long after the original insult. Current evidence indicates that iron deficiency (ID) during pregnancy can induce a host of long-term programming effects, including cardiovascular complications and behavioural deficits. Despite the relevance of ID as a model of developmental programming, these effects have not been extensively studied. The purpose of the present series of experiments was to develop a model of maternal ID throughout pregnancy to study its long-term cardiovascular and behavioural consequences in neonatal and adult offspring.

Female rats were fed either a low iron diet (3 mg/kg or 10 mg/kg Fe) or a control diet (> 225 mg/kg Fe) prior to and throughout gestation. At birth, all dams were fed a control diet (270 mg/kg Fe). This treatment caused altered growth trajectories which persisted in adulthood. Adult perinatal ID (PID) offspring, despite showing no signs of anemia at that time, had persistent elevations in arterial pressure (AP), as well as enhanced responsiveness of AP to high and low sodium intake. These animals also had altered responsiveness of renal medullary blood flow to changes in AP. PID offspring also had altered function of intrarenal and vascular nitric oxide signaling. Similar studies performed in acute ID animals revealed opposite trends in intrarenal and vascular NOS function, as well as in effects on the cardiovascular system.

In addition to the cardiovascular effects, adult PID male offspring exhibited a number of behavioural changes, as assessed by monitoring locomotor activity in their
home cages (by radiotelemetry) as well as in a novel environment. PID male offspring also performed poorly in a Morris water maze compared to controls. These differences were not observed in female PID animals.

In summary, these studies provide evidence that ID during gestation has deleterious effects on various aspects of the offspring’s physiology. Given the global incidence of ID, as well as its propensity to afflict pregnant women, developmental programming from this condition could have profound implications on global health.
Co-Authorship

The studies presented herein were performed by Stephane L. Bourque with the following co-authorships and technical assistance.

Chapter 2: Co-authored by Marina Komolova, Michael A. Adams and Kanji Nakatsu.

Technical assistance with the implantation of radiotelemetric transducers was provided by Corry Smallegange.

Chapter 3: Co-authored by Marina Komolova, Kanji Nakatsu and Michael A. Adams.

Technical assistance with the implantation of radiotelemetric transducers was provided by Corry Smallegange.

Chapter 4: Co-authored by Matt Twiddy, Michael A. Adams and Kanji Nakatsu. Figures 4.9 and 4.10 represent a re-analysis of a subset of data that were initially published in the Master’s thesis of Matthew R. Twiddy. Technical assistance with the implantation of radiotelemetric transducers was provided by Corry Smallegange.

Chapter 5: Co-authored by Umar Iqbal, James Reynolds, Michael A. Adams and Kanji Nakatsu.

Appendix 1: Co-authored by Carling D. Benjamin, Michael A. Adams, and Kanji Nakatsu. These data were published, in part, in the Master’s thesis of Carling D. Benjamin.
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# Table of Contents

Abstract .................................................................................................................................................. ii  
Co-Authorship ........................................................................................................................................ iv  
Acknowledgements .............................................................................................................................. v  
Table of Contents .................................................................................................................................. vi  
List of Abbreviations ........................................................................................................................... xi  
List of Figures ....................................................................................................................................... xiii  
List of Tables ......................................................................................................................................... xvi  
Chapter 1 General Introduction ......................................................................................................... 1  
1.1 Developmental Origins of Health and Disease ........................................................................... 1  
1.1.1 Overview of Developmental Programming .......................................................................... 1  
1.1.2 Genetic and Early Environmental Influences ..................................................................... 5  
1.1.3 Characteristic Features of Developmental Programming .................................................. 9  
  1.1.3.1 Altered Growth Trajectories: Low Birth Weight .......................................................... 9  
  1.1.3.2 Altered Growth Trajectories: Catch-Up Growth .......................................................... 10  
  1.1.3.3 Timing of Insult .............................................................................................................. 12  
  1.1.3.4 Sex Differences .............................................................................................................. 13  
1.2 Regulation of Arterial Pressure ................................................................................................. 14  
  1.2.1 Short and Long-Term Control of Arterial Pressure .......................................................... 16  
  1.2.2 The Pressure-Natriuresis Mechanism: Intrarenal Mechanisms ........................................ 19  
  1.2.3 NO Signaling in Arterial Pressure Regulation ................................................................... 25  
1.3 Iron Metabolism ......................................................................................................................... 29  
  1.3.1 Mechanisms of Iron Uptake, Transport and Storage ......................................................... 30  
  1.3.2 Regulation of Iron Uptake .................................................................................................. 35  
  1.3.3 Regulation of Iron Uptake in Pregnancy .......................................................................... 37  
1.4 Iron Deficiency ......................................................................................................................... 38  
  1.4.1 Overview ............................................................................................................................ 38  
  1.4.2 Iron Deficiency and Pregnancy ......................................................................................... 41  
  1.4.3 Iron Deficiency and the Developmental Origins of Health and Disease ......................... 43  
1.5 Statement of Hypotheses and Objectives .................................................................................. 46
4.2 Methods and Materials ................................................................. 106
  4.2.1 Animals and Treatments ......................................................... 106
  4.2.2 Tissue Collection and Analysis ............................................. 108
  4.2.3 Cardiovascular Assessments ................................................... 109
  4.2.4 Isolated Tissue Bath Assessments ........................................... 109
  4.2.5 NOS and sGC Activity Assays ............................................... 110
  4.2.6 Statistical Analyses .............................................................. 111
4.3 Results ....................................................................................... 112
  4.3.1 Study 1 Outcomes ................................................................. 112
  4.3.2 Study 2 Outcomes ................................................................. 114
4.4 Discussion .................................................................................. 123

Chapter 5 Perinatal Iron Deficiency Impacts Locomotor Behaviour and Water Maze Performance in Adult Male and Female Rats ......................................................... 132
  5.1 Introduction .............................................................................. 132
  5.2 Methods .................................................................................. 134
    5.2.1 Animals and Treatments ....................................................... 134
    5.2.2 Behavioural Testing ............................................................. 135
    5.2.3 Tissue Collection and Analysis ........................................... 137
    5.2.4 Statistical Analyses ............................................................. 138
  5.3 Results ...................................................................................... 139
    5.3.1 Maternal and Offspring Outcomes ...................................... 139
    5.3.2 Locomotor Behavioral Outcomes ....................................... 141
    5.3.3 Morris Water Maze Outcomes ......................................... 144
  5.4 Discussion ............................................................................... 148

Chapter 6 Summary and Discussion .................................................... 153
  6.1 Programming and Iron Deficiency ............................................ 153
  6.2 Cardiovascular Effects of Perinatal Iron Deficiency ..................... 155
  6.3 Behavioural Effects of Perinatal Iron Deficiency ....................... 165
  6.4 Concluding Remarks ............................................................... 167
References ...................................................................................... 169
Appendix A Lack of Hemodynamic Effects of Extended Heme Synthesis Inhibition by Succinylacetone in Rats
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2,3-bisphosphoglycerate</td>
<td>2,3-BPG</td>
</tr>
<tr>
<td>20-HETE</td>
<td>20-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AP</td>
<td>Arterial pressure</td>
</tr>
<tr>
<td>BPM</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Cp</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Dahl SS</td>
<td>Dahl salt-sensitive rat</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DcytB</td>
<td>Duodenal cytochrome B</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Distilled and dionized water</td>
</tr>
<tr>
<td>DEA-NONOate</td>
<td>Diethylamine-NONOate</td>
</tr>
<tr>
<td>DMT-1</td>
<td>Divalent metal transporter-1</td>
</tr>
<tr>
<td>DOHAD</td>
<td>Developmental origins of health and disease</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Median effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>Ferrous iron</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>Ferric iron</td>
</tr>
<tr>
<td>Fe-S</td>
<td>Iron-sulfur</td>
</tr>
<tr>
<td>FPN</td>
<td>Ferroportin</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Ht</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HCP-1</td>
<td>Heme-carrier protein 1</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazin-N’-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible factor-1α</td>
</tr>
<tr>
<td>HO</td>
<td>Heme oxygenase</td>
</tr>
<tr>
<td>Hp</td>
<td>Hephaestin</td>
</tr>
<tr>
<td>LHPA</td>
<td>Limbic-hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HS</td>
<td>High sodium treatment</td>
</tr>
<tr>
<td>ID</td>
<td>Iron deficiency</td>
</tr>
<tr>
<td>IDA</td>
<td>Iron deficiency anemia</td>
</tr>
<tr>
<td>IDNA</td>
<td>Iron deficiency non-anemic</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
</tbody>
</table>
i.p.    Intraperitoneal
IRE    Iron response element
LBW    Low birth weight
L-NMMA L-\(N^6\)-monomethyl-arginine citrate
L-NAME N-nitro-L-arginine methyl ester
LS    Low sodium treatment
LV    Left ventricle
MAP    Mean arterial pressure
MBF    Medullary blood flow
MWM    Morris Water Maze
NADPH Nicotinamide adenine dinucleotide phosphate, reduced form
NHE    Sodium hydrogen exchanger
nNOS    Neuronal nitric oxide synthase
NO    Nitric oxide
NOS    Nitric oxide synthase
NS    Normal sodium treatment
OF    Open Field
PE    Phenylephrine
PID    Perinatal iron deficiency
PP    Pulse pressure
PD    Postnatal day
RAS    Renin angiotensin system
RBF    Renal blood flow
RES    Reticuloendothelial system
RFC    In vivo renal function curve
RIHP    Renal interstitial hydrostatic pressure
ROS    Reactive oxygen species
RV    Right ventricle
SBP    Systolic blood pressure
sGC    Soluble guanylyl cyclase
SHR    Okamoto spontaneously hypertensive rat
STEAP-3 Six-transmembrane epithelial antigen of the prostate-3
TIBC    Total iron binding capacity
Tf    Transferrin
TfR    Transferrin receptor
TPR    Total peripheral resistance
VAT    Visceral adipose tissue
WHO    World Health Organization
WKY    Wistar Kyoto rat
List of Figures

Figure 1.1. Proposed mechanisms by which early developmental insults impact long-term health of an organism ................................................................. 3

Figure 1.2. Simplified diagrammatic representation of the short-term and long-term controllers of arterial pressure ......................................................... 17

Figure 1.3. Theoretical in vivo renal function curves of a (A) normotensive, salt-resistant patient; (B) normotensive, salt-sensitive patient; (C) hypertensive salt-resistant patient; and (D) hypertensive salt-sensitive patient .................. 20

Figure 1.4. Diagrammatic representation of the mechanisms by which various mediators of pressure-natriuresis exert their function .................................. 23

Figure 1.5. Distribution of functional, storage and transport iron in various tissue compartments ......................................................................................... 31

Figure 1.6. Proposed mechanisms of iron uptake and recycling in (A) reticuloendothelial macrophages, and (B) enterocytes ........................................... 32

Figure 1.7. Changes in tissue and circulating indices of iron status associated with the progression of negative iron balance ........................................ 40

Figure 2.1. Dam and offspring (A) hematocrit and (B) hemoglobin levels at postnatal days 1, 7, 14, and 21. ................................................................. 58

Figure 2.2. Effect of PID on offspring (A) liver iron concentration and (B) total liver iron content at postnatal days 1, 7, 14, and 21 .................................. 60

Figure 2.3. Relationship between hematocrit and total liver iron in control and PID offspring ......................................................................................... 62

Figure 2.4. Iron content of dams’ milk at postnatal days 1, 7, 14, and 21...................... 63

Figure 2.5. Effect of PID on (A) body weight, and (B-G) organ weights at postnatal days 1, 7, 14, and 21 ................................................................. 64

Figure 2.6. Correlation between body weight and hematocrit in control and PID offspring at 24 h ......................................................................................... 66

Figure 2.7. Correlation between organ weights and hematocrit in control and PID offspring at 24 h ......................................................................................... 67
Figure 2.8. Correlation between organ weight and body weight in control and PID offspring at 24 h ......................................................................................................................... 69
Figure 2.9. Effect of PID on locomotor activity in male offspring from 12 wk to 35 wk 73
Figure 3.1. Effect of PID on offspring body weights, expressed as (A) absolute weights, and (B) percentage of control body weight.................................................. 93
Figure 3.2. Effect of PID on (A) systolic blood pressure, mean arterial pressure (MAP), and diastolic blood pressure, and (B) heart rate, as assessed by radiotelemetry in 12 wk-old male offspring........................................................................... 95
Figure 3.3. Effect of PID on the relationship between the change in renal interstitial hydrostatic pressure and the change renal arterial pressure in 10 wk-old male offspring, expressed as (A) relative changes from baseline, and (B) absolute measurements................................................................. 97
Figure 3.4. (A) Effect of PID on (A) mean arterial pressure responsiveness to normal sodium, low sodium and high sodium intake in 15 wk-old male offspring... 99
Figure 4.1. Effect of PID on renal (A) nitric oxide synthase activity and (B) soluble guanylyl cyclase activity in neonatal offspring at postnatal days 1, 7, 14, and 21.................................................................................................................. 113
Figure 4.2. Effect of PID on renal (A) nitric oxide synthase and (B) soluble guanylyl cyclase activities in 12 wk-old male offspring....................................................... 115
Figure 4.3. Effect of PID on concentration-response relationships of rat aortic rings to (A) phenylephrine, (B) acetylcholine, and (C) diethylamine-NONOate in 14 wk-old male offspring......................................................................................... 116
Figure 4.4. Effect of low iron treatment in adult male rats on (A) hematocrit and (B) body weight........................................................................................................... 117
Figure 4.5. Effect of iron restriction in adult male rats on (A-G) hematological parameters and (H) liver iron......................................................................................... 119
Figure 4.6. Relationship between hematocrit and liver iron in adult male control, iron-deficient, and iron-replete rats ................................................................. 120
Figure 4.7. Effect of ID in adult male rats on renal (A) nitric oxide synthase activity, and (B) soluble guanylyl cyclase activity......................................................... 121
Figure 4.8. Effect of ID in adult male rats on concentration-response relationships of aortic rings to (A) phenylephrine, (B) acetylcholine, and (C) diethylamine-NONOate ......................................................................................................................... 122

Figure 4.9. Effect of ID on (A) mean arterial pressure, (B) heart rate, and (C) pulse pressure profiles during normal sodium, high sodium, and low sodium treatments, as assessed by radiotelemetry in 15 wk-old male rats.............. 124

Figure 4.10. Effect of ID in adult male rats on (A) net changes in mean arterial pressure associated with low sodium and high sodium intake, and (B) the resultant in vivo renal function curves derived from these data........................................................................... 125

Figure 5.1. Effect of PID on body weight (BW) of (A) male and (B) female Wistar rats ........................................................................................................................................................................ 142

Figure 5.2. Effect of PID on (A) distance traveled, (B) number of rearings, and (C) total time spent in center of an open-field apparatus of male and female Wistar rats at 12 wk........................................................................................................... 143

Figure 5.3. Effect of PID on path lengths to reach the hidden platform in a Morris water maze of male (A,B), and female (C,D) Wistar rats at 12 wk......................... 145

Figure 5.4. Effect of PID on spatial bias for the target quadrant in the hidden platform phase of a Morris water maze of male (A,B) and female (C,D) Wistar rats at 12 wk............................................................................................................ 146

Figure 6.1. Correlation between responsiveness of mean arterial pressure to dietary salt and visceral adipose tissue in male offspring at 36 wk......................... 161

Figure 6.2. Effect of PID on mean arterial pressure responsiveness to increasing doses of losartan, in 18 wk-old male offspring........................................................... 163
List of Tables

Table 2.1. Treatment regimen of dams and offspring in the pilot study, Study 1 and Study 2................................................................................................................................. 54
Table 2.2. Hematocrit and organ weights of control and PID offspring at 24 weeks...... 70
Table 2.3. Physical characteristics of male control and PID offspring at 36 weeks........ 72
Table 3.1. Summary of weights, hematological indices of iron status and pregnancy outcomes in control and iron-deficient dams. .......................................................... 91
Table 3.2. Control and PID offspring information at PD 1, 7, 14, 21. ......................... 92
Table 3.3. Physical and renal hemodynamics properties of control and PID offspring at 10 wk. .................................................................................................................... 96
Table 5.1. Iron status of control and PID offspring at 24 h and 24 wk....................... 140
Chapter 1

General Introduction

1.1 Developmental Origins of Health and Disease

1.1.1 Overview of Developmental Programming

It has become axiomatic that the intrauterine environment and immediate postnatal period can profoundly impact the long-term health of an individual. Although the influence of the early developmental environment on subsequent risk of disease has long been known,\(^1\),\(^2\) it is only within the last two decades that this knowledge has become widely accepted among the scientific community. The growing interest in the ‘developmental origins of health and disease’ (DOHAD), or simply ‘developmental programming’, stems primarily from evidence obtained by David Barker and colleagues that poignantly showed an inverse relationship between birth weight and mortality rate due to ischemic heart disease in later life.\(^3\) Since these ground-breaking studies, numerous research groups worldwide have demonstrated that stressors during development can increase the risk of developing cardiovascular and metabolic disturbances, as well as debilitating outcomes involving behavioural deficits and affective disorders.\(^4\) Though originally termed ‘fetal programming’, evidence now indicates that insults during any period that encompasses development, including the preimplantation phase,\(^5\) gestation,\(^6\) and the early postnatal period,\(^7\) can impact long-term health.

The observation that stressors of a maternal origin could impact the long-term health of the fetus has been known since the late 1930’s. These early epidemiological
studies revealed that conditions of adversity during gestation and early infancy were associated with decreased lifespan in these individuals. In the 1960’s, Widdowson and McCance conducted a series of experiments demonstrating that undernourishment in rats during the gestational period impacted body composition, central nervous system function, and susceptibility to infection in adulthood. However, the concept of developmental programming remained in relative obscurity until the seminal studies by David Barker and colleagues at the University of Southampton. These studies revealed that various indices of fetal and neonatal health, particularly birth weight, could be used to predict the incidence of future cardiovascular and metabolic disorders in populations in the United Kingdom. It is now widely acknowledged that adults that had low birth weight (LBW) have a greater risk of developing a number of diseases than their normal birth weight counterparts.

Developmental programming is defined as “the response to a specific challenge to the mammalian organism during a critical developmental time window that alters the trajectory of development qualitatively and/or quantitatively with resulting persistent effects on phenotype”. Thus, in response to early stressors such as a scarcity of nutrients, cardiometabolic, neurophysiological, and endocrine functions may be permanently altered; in certain scenarios, these adaptations prove to be maladaptive, and predispose the offspring to disease in later life (Figure 1.1). The propensity to develop long-term complications appears to be dependent on genetic susceptibility, as well as the nature, severity and timing of the perinatal insult. In combination with other risk factors (age, obesity, etc.), the effects manifest long after the original insult. It is important to
FETAL ADAPTATIONS TO INTRAUTERINE/EARLY POSTNATAL ENVIRONMENT

PERMANENT STRUCTURAL AND FUNCTIONAL ALTERATIONS IN ORGANS AND TISSUES

Pancreas
- ↓ β cell mass
- ↓ β cell insulin secretions

Liver
- ↑ gluconeogenesis

Skeletal Muscle
- ↓ skeletal muscle mass
- ↓ insulin sensitivity
- ↑ lipid oxidation

Adipose Tissue
- ↓ inhibition of lipolysis
- ↓ stimulated glucose uptake

Kidneys/Vasc.
- ↓ nephron number
- Altered intrarenal hemodynamics
- ↓ responsiveness to vasodilators

CNS
- ↑ responsiveness of LHPA axis
- Altered neurotransmitter profiles

Age

Obesity/Sedentarity

Long-term Outcomes
- Dyslipidemia
- Hypertension/CVD
- Insulin Resistance/Diabetes
- Cognitive/Behavioural Deficits

Figure 1.1. Proposed mechanisms by which early developmental insults impact long-term health of an organism. GC, glucocorticoids; LHPA, limbic-hypothalamic-pituitary-adrenal; CVD, cardiovascular disease. Adapted from Martin-Gronert et al.16
note that in the context of the DOHAD framework, the emphasis resides on supposed physiological processes in the fetus or the mother, rather than pathological or iatrogenic causes. Indeed, the developing embryo/fetus is known to be highly vulnerable to a number of extrinsic factors, including xenobiotics such as thalidomide, as well as ethanol, cocaine and other drugs of abuse. The scope of the present discussion has not included these influences.

Overall nutrient restriction, and imbalances in specific nutritional elements including protein, carbohydrates, fat, micronutrients such as calcium and iron, as well as exposure to glucocorticoids (either endogenous or synthetic) and prenatal hypoxia have all been shown to alter growth trajectories in the offspring and cause persistent deleterious effects. These long-term consequences include cognitive and behavioural deficits, such as affective disorders and impaired ability to cope with stress, neurotransmitter and hormonal dysregulation, as well as a host of cardiometabolic complications, including hypertension, glucose intolerance, dyslipidemia, and increased visceral fat deposition. The clustering of these cardiometabolic features is referred to as the metabolic syndrome. While the behavioural and cognitive deficits are potentially the most debilitating, the metabolic syndrome also has important implications for the individual and for public health issues in general. The presence of the metabolic syndrome doubles the risk of coronary heart disease, increases the incidence of death due to coronary heart disease by 3-4-fold, and increases the risk of type-II diabetes by 6-fold.
Although the developmental programming hypothesis has been verified by numerous epidemiological and experimental studies, little is known about the mechanisms that underlie these effects. It is still unclear whether perinatal stressors act via common pathways, or whether they act via independent mechanisms that ultimately manifest in similar ways. It appears however, that certain systems, such as the neuroendocrine, cardiovascular, and metabolic processes, are more plastic than others, and thus prone to perturbations by early insults. Furthermore, it is not clear whether physiological changes induced in fetus/offspring are a direct consequence of the stressors per se, or whether they reflect a deliberate adaptive mechanism to cope with insult as a means to ensure short and long-term survival. The ‘thrifty phenotype’ hypothesis, as well as the ‘predicted adaptive responses’ hypothesis, which are described in detail below, propose that the latter is a more reasonable explanation. An organism’s ability to adapt to its surroundings is essential for survival. Consequently, all organisms have evolved mechanisms to cope with environmental insult. The fetus is no exception.

1.1.2 Genetic and Early Environmental Influences

The classic model that persisted for a number of years was that genetic components determine the developmental limits of an organism, and the resulting phenotype is shaped by a multitude of environmental factors. However, it is becoming increasing clear that genetic factors work together with early environmental stimuli to determine the phenotypic outcome. The extent to which each component contributes is still a subject of intense debate. In the 1960’s, the concept of the “thrifty genotype” was first proposed by Neel, who suggested that certain populations had an inherently greater
propensity to develop insulin resistance. The hypothesis posited that random mutations leading to insulin resistance and decreased metabolic capacity could be beneficial to individuals born into environments with food shortages, leading to selection and propagation of the trait. Although various genetic causes have been shown to result in poor insulin secretion and altered glucose metabolism, evidence obtained in the last 20 years demonstrates that purely genetic factors, independent of environmental factors, do not have a strong enough correlation with long-term metabolic outcomes. Moreover, studies involving identical twins have shown that environmental effects that influence fetal growth predict metabolic complications independent of genetic factors.

In 1991, Hales and Barker proposed the ‘thrifty phenotype hypothesis’ as a means to explain the disparity between observed metabolic disease outcomes and those predicted based solely on genetic factors. The thrifty phenotype hypothesis proposed that environmental factors, rather than genetic factors, were the dominant cause of insulin resistance and other metabolic complications. Specifically, the hypothesis proposed that nutritional insufficiency would induce a state of fetal nutritional thrift, which would ensure adequate nutrient delivery to vital organs (i.e. brain, heart, reproductive organs) at the expense of others (pancreas, kidneys, skeletal muscle). Although this strategy would ensure immediate survival, the altered development and function of ‘non-vital’ organ systems would impact long-term metabolic function. Importantly, according to the hypothesis, the thrifty phenotype conferred upon the offspring does not invariably result in long-term disease. Indeed, the offspring would thrive in a postnatal environment wherein nutrients were scarce, since its metabolism has adapted to nutrient restriction.
However, where food is abundant and readily available in the post-development phase, the disparity between intrauterine and postnatal nutrition would lead to disease.\textsuperscript{52}

Although there are currently no data that contradict the key aspects of the thrifty phenotype hypothesis (see Hales \textit{et al.}\textsuperscript{52}), it fails to account for various observations. The thrifty phenotype hypothesis was originally proposed to explain the long-term metabolic consequences of protein insufficiency during gestation, although other nutritional deficiencies (e.g. iron deficiency (ID), caloric restriction) also fit nicely within this framework. The thrifty phenotype hypothesis does not, however, provide an adequate explanation for outcomes associated with other perinatal stressors, such as prenatal glucocorticoid exposure (either endogenous or synthetic) or models of nutritional excess (e.g. maternal high fat diet or high caloric intake during pregnancy). Furthermore, the thrifty phenotype fails to account for various observations, most notably the adaptive responses to stressors that do not confer an immediate survival advantage to the offspring. For example, early life stressors have been shown to persistently alter fluid homeostasis, without an apparent benefit on survival in early life.\textsuperscript{53}

Recently, Gluckman and colleagues proposed the ‘predictive adaptive responses’ hypothesis as a more encompassing model of developmental programming.\textsuperscript{39} The predictive adaptive responses hypothesis posits that early stressors lead to adaptive changes in function of key systems that do not necessarily confer any immediate benefit, but predict long-term outcomes with the intention of guaranteeing survival until reproductive age.\textsuperscript{54} According to this hypothesis, a number of traits are favored by early stressors such that they produce a phenotype that would have greater ability to survive in
later life. In the context of long-term survival, it is expected that growth, metabolism, cardiovascular function, and the stress response, would be highly responsive to programming stimuli. These traits include insulin resistance, which decreases energy investment into growth and metabolism; increased visceral fat deposition, as a means to store energy reserves; reduced skeletal muscle mass, which decreases energy demands, freeing up resources for survival and reproduction; decreased organ growth, which decreases energy requirements for growth and function; and reduced negative feedback of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis, which heightens the stress response resulting in increased vigilance in a nutrient deprived and predator-rich environment. Thus, the organism predicts the environment into which it will be born and grow; if it predicts correctly, the risk for long-term disease will be low. However, if stressors exist only during the prenatal period, and the fetus/neonate predicts that such an environment will persist, but is instead born into an environment wherein no such stressors exist, the incidence of long-term disease will be high. Interestingly, the predictive adaptive responses hypothesis has been proposed to explain a number of physiological outcomes, including the characteristic phenotype associated with Down syndrome, ovarian suppression and variation in menarcheal age.55-57

The mismatch between the intrauterine and post-developmental environments appears to be a prime determinant of long-term disease outcome in both the ‘thrifty phenotype’ and ‘predictive adaptive responses’ hypotheses. There is strong evidence to support this notion. For example, individuals born during the Dutch winter famine (1944-1945), in which starvation occurred for only one season, had an increased
propensity for glucose intolerance, insulin resistance, and dyslipidemia as adults.\textsuperscript{58} In contrast, individuals born during the famine of the siege of Leningrad (1941-1944), wherein starvation persisted for many years, showed no increased risk of long-term health complications.\textsuperscript{59} In the former case, the end of the war brought an end to the famine, and offspring who were nutrient restricted during pregnancy had a relative excess of nutrition in the period after development. Thus, the incorrect prediction of the postnatal environment by the fetus resulted in long-term disease. In the latter scenario, infants born during the famine continued to be malnourished in the ensuing period after development. Thus the adaptation to the predicted nutrient deprivation was ‘correct’, and consequently did not result in disease.

1.1.3 Characteristic Features of Developmental Programming

There are several features that appear to be common to all programming models. These include (1) altered growth trajectories, (2) critical time periods of susceptibility, and (3) sex-differences.

1.1.3.1 Altered Growth Trajectories: Low Birth Weight

Altered growth trajectories in early life, specifically those that involve the development and organization of key organs and tissues, appear to be key components in long-term programming. Birth weight, particularly LBW, has been used extensively as a surrogate measure for growth restriction in the fetus.\textsuperscript{60} Its use stems largely from the ease with which it can be obtained compared to more invasive measurements, such as hormone levels and tissue assessments. Despite its apparent usefulness in predicting the incidence of metabolic and cardiovascular complications in various populations, as
previously discussed, birth weight is a poor surrogate of overall growth and
development. LBW can reflect different patterns of fetal growth restriction. Indeed,
LBW can occur as a consequence of thinness at birth (as indicated by a low ponderal
index (weight/length$^3$)), which appears to be a predictor of long-term cardiovascular
disease in men. alternatively, LBW can reflect shortness at birth, which appears to be a
greater predictor of cardiovascular disease in women. In addition, birth weight fails to
take into account the relative contribution of lean body mass and fat mass, which are
important in the context of metabolic function. Finally, the reliance on deviation of
birth weight from norms as an indication of altered development may underestimate the
incidence of long-term developmental effects. That is, the magnitude of growth
restriction must be severe in order to manifest as LBW, and consequently more subtle
changes that do not affect birth weight, but only impact key organ systems, may be
overlooked. Indeed, the epidemiological data obtained from the initial studies of David
Barker and colleagues demonstrated that there was no threshold in birth weight that
predicted long-term programming effects. Instead, the relationship between birth weight
and long-term risk for metabolic disease appears to be continuous. Therefore,
although the mechanisms that alter growth patterns are greatest in newborns that have
LBW, they also operate in offspring whose weights are considered normal.

1.1.3.2 Altered Growth Trajectories: Catch-Up Growth

In addition to altered growth patterns in utero, evidence indicates that postnatal
growth patterns may be important for long-term programming effects. However, our
understanding of these events and processes is incomplete. In humans, rapid growth rates
in childhood appear to increase the risk of developing cardiovascular disease, obesity, glucose intolerance, and type II diabetes in later life, whereas slower growth rates are protective against these conditions. Similar results have been demonstrated in animal models; postnatal catch-up in the newborn rat results in earlier adult death due to cardiometabolic complications. Interestingly, it appears there is an opposite trend for neurodevelopment and cognition. In a recent study by Ehrenkrantz and colleagues, LBW infants (500-1000g) were divided into groups according to postnatal growth patterns; the risk of cerebral palsy and neurodevelopmental impairment were inversely correlated with growth rate. Similar results were obtained by Lucas and colleagues, who showed that higher protein intake in newborn infants increased postnatal growth and reduced neurodevelopment deficits; however, the same cohort showed unfavorable markers of insulin resistance in adolescence.

It remains unclear how postnatal catch-up growth contributes to long-term programming. As previously discussed, an issue central to DOHAD is the disparity between the intrauterine environment and the postnatal environment into which the offspring is born. As such, neonatal catch-up growth may reflect a period of relative excess nutrient intake, with which the offspring’s thrifty metabolism is unable to cope. This would be expected to lead to fat deposition, glucose intolerance and insulin resistance. In this regard, the nature of the growth may be an important factor as well. While the accumulation of fat during early infancy may prove to be deleterious and may even propagate the metabolic syndrome, the accumulation of lean muscle mass may prove to be beneficial. However, the traits that are favored by early programming are
those that promote energy conservation, such as fat deposition and decreased lean muscle mass.\textsuperscript{52, 78}

1.1.3.3 Timing of Insult

Programming effects are associated with stressors that occur as early as the pre-implantation phase,\textsuperscript{5} as well as the gestational period\textsuperscript{6} and the subsequent postnatal development phase.\textsuperscript{7} Interestingly, the organism may show remarkable differences in susceptibility to an insult depending on the stage of development during which it occurs.\textsuperscript{79} This is perhaps best exemplified by the findings that glucocorticoid exposure in early gestation causes hypertension in adult sheep, whereas the same insult appears to have little, or even a lowering effect on arterial pressure (AP) when it occurs in late gestation.\textsuperscript{80, 81}

The reasons why certain periods of development are conducive to programming by stressors while others are not is not entirely clear, although it most likely reflects key periods of organ and tissue growth and differentiation. Beginning early in gestation, all cell types undergo numerous key periods of basic growth and differentiation that are highly dependent on activity and timing, whereas other periods of development are not as critically dependent. Consequently, even subtle interferences during specific periods can profoundly impact function of these organs, with no capacity for recovery once these periods have passed. Similarly, the placenta goes through various phases of development, which are characterized by periods of angiogenesis, trophoblast differentiation and invasion, and syncytium formation. Insults that disturb these normal
developmental patterns may disturb oxygen and nutrient delivery, further impacting growth and development of the fetus.\textsuperscript{82}

1.1.3.4 Sex Differences

Epidemiological studies suggest that males and females exhibit different susceptibilities to perinatal stressors. These may reflect, to some degree, inherent differences in normal birth weights and growth trajectories between males and females.\textsuperscript{83} More specifically, females tend to have lower birth weights, and undergo more rapid growth rates during the neonatal phase than males.\textsuperscript{83} Thus, LBW and subsequent rapid growth, which are known to increase the risk of metabolic syndrome (see sections 1.1.3.1 and 1.1.3.2) may be more important for male than female offspring. Despite the inherent differences in growth patterns between males and females, evidence obtained from various animal models indicates that males are more prone to maternal nutrient restriction and glucocorticoid exposure than females, independent of birth weight and postnatal growth pattern;\textsuperscript{80, 84-86} however both sexes are susceptible if the perinatal stressor is severe.\textsuperscript{22, 87} A notable exception is that female offspring appear to develop hypertension with increased maternal fat intake during pregnancy, whereas their male counterparts do not.\textsuperscript{88}

Based on current evidence, the most likely explanation for these sex differences is hormonal. More specifically, multiple programming effects appear to be mediated, at least in part, by testosterone. Indeed, castration at 10 weeks of age abolishes the hypertension observed in male growth restricted rats.\textsuperscript{89} Furthermore, testosterone has been shown to modulate activity of the renin-angiotensin system (RAS),\textsuperscript{90, 91} leptin\textsuperscript{92} and
insulin signaling, thereby implicating testosterone in the pathophysiology of metabolic syndrome. In contrast, estrogen is believed to have a protective role against hypertension, based on the observations that menopausal women exhibit increased cardiovascular risk associated with a decline in estrogen levels. Ovariectomy potentiates the programming of hypertension in growth restricted offspring, and these effects are reversed with estrogen replacement. These hormones have also been shown to modulate activity of multiple neurotransmitter systems as well as the LHPA axis in development, thereby implicating them in the sexually dimorphic behavioural and cognitive programming effects. Despite these data, the specific roles of testosterone and estrogen in the pathophysiology of developmental stressors are not presently clear.

The link between developmental insults and the risk of cardiovascular disease in later life has been well established. However, it remains to be determined how early stressors affect AP regulation, which may ultimately lead to cardiovascular disease. In order to elucidate these underlying mechanisms, a fundamental understanding of the systems that regulate AP is paramount. This section discusses the principal mechanisms that establish the long-term set point of AP. These mechanisms are also discussed in the context of various models of hypertension and salt sensitivity.

1.2 Regulation of Arterial Pressure

The global prevalence of hypertension, defined as a systolic blood pressure (SBP) greater than 140 mmHg or a diastolic blood pressure (DBP) greater than 90 mmHg, is almost 1 billion people. Nearly a third of people with hypertension live in industrialized
countries. Based on current projections, more than 1.5 billion people will have hypertension by the year 2025. In addition to being an important independent risk factor for cardiovascular disease, hypertension is also strongly associated with other cardiovascular risk factors as well, including obesity, dyslipidemia, renal dysfunction, and insulin resistance. Cardiovascular disease is the leading cause of mortality worldwide, accounting for half of all deaths in industrialized countries, and 23% of all deaths in developing countries. It is therefore not surprising that hypertension ranks among the World Health Organization’s (WHO) top ten global health risks.

Approximately 50% of hypertensive patients manifest exaggerated blood pressure changes in response to acute or chronic salt depletion or repletion; a condition known as salt-sensitivity. An additional 26% of the population is believed to have salt sensitivity in the absence of hypertension. The impact of salt sensitivity on cardiovascular morbidity and mortality were recently investigated in a landmark 27-year follow up study conducted by Weinberger et al. This study demonstrated that salt-sensitive hypertensive patients had lowest survival rate, and normotensive patients had the highest survival rates, as expected. Interestingly, normotensive patients with salt-sensitivity had a similar mortality rate as those with salt-resistant hypertension. These data indicate that salt sensitivity is an important independent risk factor for cardiovascular disease.
1.2.1 Short and Long-Term Control of Arterial Pressure

The capacity to oppose changes in AP is a critically important aspect of the cardiovascular system. Without adequate regulation, pressure could rise to levels that would damage blood vessels and organs, resulting in embolism, or end-organ pathology, such as renal failure or brain damage. Alternatively, AP could fall to levels that do not ensure adequate oxygen and nutrient delivery to organs. The systems responsible for maintaining AP within a homeostatic range include both short and long-term mediators. Short-term regulation occurs via the baroreceptor reflex, chemoreceptors, and the CNS ischemic response.106 These systems, but particularly the baroreflex, act primarily through efferent neural pathways of the sympathetic and parasympathetic nervous systems, which exert their effects by altering total peripheral resistance, as well as cardiac output (Figure 1.2). These systems’ capacity to oppose changes in AP, referred to as ‘gain’, is high, although their efficacy diminishes greatly with time.107 Even after a few minutes of stimulation, baroreceptor reflex signaling decreases dramatically, and continue to so over the course of days.108-111 These systems are therefore more important for moment to moment control, rather than long-term regulation of AP. Indeed, studies have shown that ablation of the short-term regulatory systems does not alter the long-term level of AP, but increases its variance over a given time period.112-115 This characteristic resetting of short-term regulatory mechanisms is in fact critical to maintain the functionality of the moment to moment regulation, irrespective of the set point of AP.106,116 Other mechanisms that act on a relatively short time scale (minutes to hours) include the autoregulatory and capillary fluid shift mechanisms, as well as local and
Figure 1.2. Simplified diagrammatic representation of the short-term and long-term controllers of arterial pressure. TPR, total peripheral resistance; CO, cardiac output. Adapted from Mohrman et al. 117
humoral factors; these systems control blood flow by altering peripheral resistance and capacitance. However, these systems cannot indefinitely oppose changes in AP, because they invariably lose gain over time. This characteristic alone precludes their involvement in dictating the long-term level of AP.

The long-term set point of AP is established by the kidney. The kidney accomplishes this by a renal feedback mechanism that regulates extracellular fluid and blood volume in response to changes in AP. That is, increased blood pressure causes the kidneys to excrete fluid and sodium, thereby reducing blood volume and restoring blood pressure. Likewise, reduced AP signals a decreased production in urine, which restores ECF and blood volume over time. With this mechanism, the body will remain in a state of negative fluid balance (net loss of sodium and fluid) when AP is above the operating set point. Similarly, the body will remain in a state of positive sodium balance (net intake of sodium and water) when AP is below the set point operating pressure. The pressure-natriuresis mechanism is therefore considered to have infinite gain since it will continue to influence tubular sodium and fluid excretion until AP is normalized. It is noteworthy that the efficacy of this system is infinite only over chronic periods; the system is limited on an acute basis by its capacity to regulate urine flow.

There are various lines of evidence that support the role of the kidney as the prime determinant of long-term AP. The most compelling data that support this concept were obtained from kidney cross-transplantation experiments. Transplantation of kidneys from genetically hypertensive animals (e.g. Okamoto spontaneously hypertensive rat (SHR))
confers upon the normotensive recipients (Wistar-Kyoto rats (WKY)) a hypertensive phenotype, and vice versa. Similar results have been obtained in humans; essential hypertension in patients can be corrected by receipt of a kidney from a normotensive donor. These data suggest that the level of blood pressure ‘follows the kidney’.

1.2.2 The Pressure-Natriuresis Mechanism: Intrarenal Mechanisms

The efficacy of the pressure-natriuresis mechanisms can be represented graphically by a renal function curve, depicting sodium balance at equilibrium on the ordinate and AP on the abscissa (Figure 1.3). Two characteristics are important in defining the pressure-natriuresis relationship. The first is the position of the renal function curve on the abscissa, which establishes the set point of long-term AP (compare curves A and C, or B and D in Figure 1.3). The second important characteristic of the renal function curve is steepness of the relationship, which defines the degree of AP sensitivity with respect to alterations in sodium balance (compare curves A and B, or C and D in Figure 1.3). Studies in humans support the involvement of an altered pressure-natriuresis relationship in both primary and secondary forms of hypertension, as well as salt sensitivity. Moreover, evidence indicates that a shift in the pressure-natriuresis relationship is a cause, not a consequence, of hypertension. Experimental models of genetic hypertension, such as the SHR, are associated with parallel right-shift in the pressure-natriuresis relationship, such that the set-point resides at a higher AP. However, the shape of the in vivo renal function curve (RFC) is similar, indicating that the same changes in AP are necessary to influence urinary output. These animals are considered hypertensive with minimal salt-sensitivity. In contrast, in genetic models of
Figure 1.3. Theoretical *in vivo* renal function curves of a (A) normotensive, salt-resistant patient; (B) normotensive, salt-sensitive patient; (C) hypertensive salt-resistant patient; and (D) hypertensive salt-sensitive patient.
salt sensitivity such as the Dahl salt-sensitive rat (Dahl-SS), the pressure-natriuresis relationship is blunted, such that greater changes in AP are required to influence urinary excretion and thus reduce overall blood volume.\textsuperscript{141} However, since the set point resides at a similar pressure as control rats, these animals exhibit normal AP, until challenged with abnormal sodium intake. These animals are considered normotensive with salt sensitivity.

The set point and shape of the RFC, and hence the characteristics of the pressure-natriuresis mechanism, are established by a number of intrarenal and extrarenal structural and functional components. Examples of extrarenal systems that act on the kidney include the renin angiotensin system (RAS), autonomic regulation, and several neurohumoral systems, including the kinin-kallikrein system, atrial natriuretic peptide, aldosterone, and vasopressin. Although these systems are critically important in AP regulation and sodium homeostasis, they are not discussed herein; for a review of this subject, see Cowley.\textsuperscript{106} One focus of our research has been on the role of intrarenal systems, in particular the involvement of medullary blood flow (MBF) in controlling renal sodium handling.

Total renal blood flow (RBF) and glomerular filtration rate (GFR) are highly autoregulated within a large physiological range of AP.\textsuperscript{142,143} This is an important feature of renal function, since the kidney controls the excretion of waste products somewhat independently of its role in controlling of AP. Despite this overall autoregulation, the kidney can still dynamically regulate urine flow in response to even subtle changes in renal perfusion pressure. There is no consensus on how the kidney
senses’ changes in AP, although multiple mechanisms have been proposed\textsuperscript{144} (Figure 1.4). This apparent contradiction may be reconciled by the observation that a small proportion (1-5\%) of total RBF, that which goes to the medulla, is poorly autoregulated.\textsuperscript{145, 146} Thus, AP is transmitted through the vasa recta of the juxtamedullary nephrons, which causes proportionate changes in renal interstitial hydrostatic pressure (RIHP).\textsuperscript{147} Since the kidney is encapsulated, changes in RIHP are transmitted throughout the kidney, which influence tubular sodium and water excretion.\textsuperscript{145, 146, 148} Thus, the kidney ‘senses’ changes in AP in the absence of noticeable changes in RBF and GFR.\textsuperscript{149}

The importance of the RAP-RIHP relationship in modulating the pressure-natriuresis mechanism is supported by numerous lines of evidence. Ablation of increases in RIHP interferes with the kidney’s ability to excrete urine in response to increases in blood pressure.\textsuperscript{150, 151} Similarly, increasing RIHP by administration of small volumes of fluids into the interstitium of the medulla induces a dramatic natriuretic response.\textsuperscript{150, 152} SHR have parallel right-shifted RAP-RIHP relationships compared to normotensive WKY, coincident with parallel shifts in their renal function curves.\textsuperscript{153, 154} Moreover, chronic inhibition of the RAS using ACE inhibitors causes a lowering of pressure, and these results are accompanied by leftward shift in their RFC, as well as their RAP-RIHP relationships. Interestingly, upon cessation of treatment, the AP, RFC and RAP-RIHP relationship remained permanently shifted, demonstrating that these relationships are inextricably linked.\textsuperscript{153, 155} Animal models of ‘salt-sensitivity’ have normally been characterized as having a blunted RAP-RIHP relationship, and in some cases, a rightward
Figure 1.4. Diagrammatic representation of the mechanisms by which various mediators of pressure-natriuresis exert their function. Solid lines indicate pathways that have been shown experimentally, and dotted lines indicate proposed pathways that have not yet been demonstrated. AP, arterial pressure; MBF, medullary blood flow; GFR, glomerular filtration rate; RBF, renal blood flow; RIHP, renal interstitial hydrostatic pressure; NO, nitric oxide; 20-HETE, 20-hydroxyeicosotetraenoic acid; EETs, epoxyeicosatrienoic acids; NHE, sodium-hydrogen exchanger. Adapted from Evans et al.144
shift of the renal function curve.\textsuperscript{156, 157} Taken together, these results implicate the regulation or dysregulation of RIHP as an important mediator of the pressure-natriuresis mechanism and hence long-term AP regulation.

Medullary blood flow (MBF) and RIHP play important roles in regulating pressure-natriuresis,\textsuperscript{158} although the precise mechanism by which they influence tubular sodium reabsorption/excretion and fluid homeostasis has not been elucidated. It is likely that RIHP influences tubular sodium and fluid dynamics via multiple mechanisms. Increases in RIHP appear to cause passive sodium excretion, not by washing out the intramedullary osmotic gradient,\textsuperscript{144} but by limiting nephron segments’ ability to reabsorb sodium through the backleak of sodium and water from the interstitium.\textsuperscript{159} Although this mechanism has been shown to occur,\textsuperscript{160, 161} there are likely other mechanisms at play as well, since the influence of RIHP on intramedullary and tubular gradients is insufficient to fully account for its capacity to alter sodium excretion.\textsuperscript{144} Therefore, RIHP may have a more direct effect on tubular sodium transporters (e.g. 3Na\textsuperscript{+}/2K\textsuperscript{+}/ATPase, Na\textsuperscript{+}/K\textsuperscript{+} 2Cl\textsuperscript{−} co-transporter, sodium-hydrogen exchanger [NHE]), although concrete evidence for this is lacking. However, the research group of Roman and colleagues has shown that RIHP influences the production of CYP450 metabolites 20-hydroxyeicosatrienoic acid (20-HETE), and epoxyeicosatrienoic acids (EET), which in turn impact on tubular sodium reabsorption.\textsuperscript{162, 163} This represents a mechanism by which RIHP alters sodium excretion independent of its impact on intramedullary osmolarity.

Experimental ablation of changes in RIHP substantially blunts the pressure-natriuresis mechanism, but does not abolish it altogether.\textsuperscript{150, 151} These studies
demonstrate that control of tubular sodium and fluid dynamics in response to changes in AP is mediated by mechanisms independent of changes in RIHP (Figure 1.4).

Alternative mechanisms include signaling by 20-HETE and EET,\textsuperscript{162} CO,\textsuperscript{164,165} as well as nitric oxide (NO).\textsuperscript{148} NOS is a particularly important signaling molecule in both acute and chronic regulation of AP. This system is discussed in more detail in the following section.

1.2.3 NO Signaling in Arterial Pressure Regulation

NO is synthesized endogenously from L-arginine by the enzyme nitric oxide synthase (NOS),\textsuperscript{166} and is a biologically active free radical that is known to exert a number of physiological functions (see below). It is a short lived molecule, with a biological half-life between 3-5 s.\textsuperscript{167} Its short half-life is a consequence of its spontaneous inactivation by oxidation to nitrite and nitrate, presumably via the actions of superoxide.\textsuperscript{168} NOS consists of endothelial, neuronal and inducible isoforms, which despite their nomenclature, have overlapping as well as distinct expression patterns.\textsuperscript{169} NO has two primary targets in the cell: cytochrome c oxidase and soluble guanylyl cyclase (sGC); NO activation of sGC is discussed in detail below. NO binding to cytochrome c oxidase is important for various cellular functions, including mitochondrial function and biogenesis, cellular respiration, cell signaling, and free radical production.\textsuperscript{170} Although the implications of NO binding to this enzyme are important, they will not be discussed here (for excellent reviews see the following\textsuperscript{170-172}). Various proteins may also be considered a target for NO-mediated S-nitrosylation; for example, ion channel conductance is modulated by oxidation reactions by reactive nitrogen species.\textsuperscript{173,174}
However, NO does not appear to contribute appreciably to protein-nitrosylation, since it is a poor nitrosating species; s-nitrosylation likely occurs by higher oxides of nitrogen (e.g. NO₂, N₂O₃, and ONOO⁻). In this regard, it is important to consider that the actions of NO are a continuum of various nitrogen and oxygen species.

The majority of the systemic effects of NO are mediated by activation of sGC, via binding to its regulatory heme moiety, which increases catalytic conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Cyclic GMP, in turn, results in activation of a number of signaling cascades via protein kinase G or various cGMP-dependent phosphodiesterases. In vascular tissues, cGMP activation of protein kinase G leads to intracellular calcium sequestration, resulting in vasodilation. NO’s actions on systemic vascular tone impacts total peripheral resistance, and therefore contributes to the regulation of AP on an acute basis. However, NO also affects the kidney independent of its effects on TPR. For instance, NO plays an important role in RBF and GFR autoregulation, and is involved in the tubuloglomerular feedback.

Studies have also shown that NO is important in regulating the pressure-natriuresis mechanism, as previously stated. Administration of NOS inhibitors, such as L-NAME or L-NNMA, at moderate to high doses produces hypertension. Although it was originally postulated that these effects were mediated by constriction of systemic blood vessels, localized blockade of medullary NOS could induce a salt sensitive hypertension independently of changes in GFR and total RBF. Subsequent studies demonstrated that intramedullary administration of NOS inhibitors in doses that do not affect vascular tone cause diminished MBF and reduced natriuresis,
hypertension, and salt sensitivity. Thus, although the systemic vasoconstriction (and hence increases in TPR) likely contribute to the hypertensive effect of NO blockade, these studies suggest that the major mechanism is by affecting pressure-natriuresis.

Given the importance of NO in modulating both acute and chronic mechanisms of AP regulation, it follows that hypertension and salt sensitivity may be associated with intrinsic reductions in intrarenal NOS activity. In models of genetic salt sensitivity such as the Dahl SS and Sabra rat, activity of intrarenal NOS systems, as well as its downstream mediators, has been shown to be attenuated. Moreover, administration of L-arginine, a precursor to NOS, has been shown to abrogate the salt sensitive phenotype. Interestingly, NOS activity does not appear to be a common factor in all models of cardiovascular dysregulation. In the SHR, NOS activity does not appear to be compromised. In fact, activity of renal and vascular NOS activity appears to be upregulated, likely as a compensatory mechanism to other etiological factors. However, it is noteworthy that the capacity of NO to elicit functional effects was not tested in these studies. It may be that despite NO upregulation, the bioavailability of NO is greatly reduced, thus compromising intrarenal NO function. Indeed, despite the increased NO production, SHR were associated with a reduced flow mediated dilatation.

The exact mechanism responsible for altered sodium regulation and increased AP due to diminished NO production is unknown. Studies have demonstrated the direct effects of NO on sodium reabsorption in tubular segments. Moreover, recent
evidence suggests that RIHP mediated alterations in sodium handling are dependent on NOS activity; thus, dogs treated with NOS inhibitors had blunted RAP-RIHP relationships, and decreased pressure-natriuresis relationships.\textsuperscript{206} There is evidence that NOS may act as a counter-regulatory mechanism, such that its presence prevents the actions of angiotensin II (AII).\textsuperscript{207} Support for this concept comes from studies showing that AII administration at subpressor doses induces upregulation of NO in the medullary interstitial fluid,\textsuperscript{208} which may be indicative of feedback regulation of AII by NO. This upregulation appears to be blunted in Dahl SS rats.\textsuperscript{197} NOS counterregulation has also been proposed for NE,\textsuperscript{209} as well as arginine vasopressin.\textsuperscript{210} In all, these studies indicate that NO-mediated responses play a key role in protecting the renal medulla from excessive vasoconstriction and chronic hypertension.\textsuperscript{148} It is noteworthy that there other mediators that have been found to play similar counter regulatory roles, including prostacyclin,\textsuperscript{211} and CO.\textsuperscript{212} These data further suggest there may be multiple redundant mechanisms that prevent excessive activities of AII, NE, and vasopressin.

As NOS and sGC function appears to be compromised in several genetic models of cardiovascular dysregulation, our research group has been interested in the contribution of decreases in hemoenzyme function in hypertension. As iron in the heme molecule is a critical component of NOS and sGC regulatory systems, it follows that iron dysregulation may play an important role in hypertension and cardiovascular disease, possibly by impacting the intrarenal hemodynamics and pressure-natriuresis. Moreover, inadequate iron delivery to the fetus during development and the neonatal phase is associated with cardiovascular dysregulation in adulthood,\textsuperscript{27, 213-215} further suggesting
iron may play a role in hypertension. To elucidate the potential involvement of iron in cardiovascular disease, an understanding of the fundamental aspects of iron metabolism is required. The following sections focus on iron metabolism, and its most common associated disorder, ID.

1.3 Iron Metabolism

Iron is an essential trace element that is required by all organisms, with the exception of a few species of bacteria. As the central component of the heme molecule, iron plays an important role in the function of numerous cellular processes, including oxygen transport (Hb, myoglobin), cellular respiration (cytochromes), signaling (NOS, HO, cyclooxygenase, sGC), protection against oxidative stress (catalases and hydroxylases), and xenobiotic metabolism (cytochromes). Although iron is most commonly associated with the heme molecule, it is also a constituent of various non-heme proteins, the most notable of these are iron-sulfur clusters found in dehydrogenases, oxidases and reductases. Iron’s involvement in myriad systemic functions stems from its high reduction-oxidation potential, and as such, it plays a critically important role in electron transfer reactions. Although this feature makes it important in biological functions, it also makes it potentially deleterious to the cell. In excess, iron can cause oxidative stress by creating reactive oxygen species (ROS), primarily via the Haber-Weiss-Fenton reaction. Because of this feature, iron uptake, transport and storage is highly regulated under normal circumstances.
1.3.1 Mechanisms of Iron Uptake, Transport and Storage

Approximately two thirds of total body iron is found either in erythrocytes, the bone marrow (where erythropoiesis occurs), or in the reticuloendothelial system (e.g. spleen, where breakdown of senescent erythrocytes occurs). The remaining third is distributed in tissues, with particularly high concentrations found in the liver (as ferritin) and skeletal muscle (as myoglobin) (Figure 1.5). Only a small fraction of total iron (<0.1%) is associated with transferrin (Tf), typically in transit between storage sites (e.g. liver) and tissues with high iron demands (e.g. bone marrow).

In the reticuloendothelial system, the iron liberated from the phagocytosis of erythrocytes is not excreted, but instead recycled and stored for future use (Figure 1.6A). Only minimal iron loss occurs through non-specific mechanisms, such as sloughing enterocytes and bile excretion. In total, approximately 2-3 mg of iron are lost daily. To recuperate this loss, iron is taken up from dietary sources, as either heme or non-heme iron. The former is taken up more efficiently than the latter, though it comprises a much smaller proportion of overall iron intake (approximately 10-15%). The uptake of iron occurs principally in the duodenum, through a coordinated series of protein interactions (Figure 1.6B). Heme is taken up via the apical heme-carrier protein-1 (HCP-1), and iron is then liberated in the cell through heme catabolism by heme oxygenase (HO). Inorganic iron is taken up by the divalent metal transporter-1 (DMT-1), which as its name implies, only transports metals in the 2+ oxidation state. Ferric iron is converted to ferrous iron prior to uptake by the ferrireductase duodenal cytochrome B (DcytB) in enterocytes, although other enzymes may carry out this function as well.
Figure 1.5. Distribution of functional, storage and transport iron in various tissue compartments. The size of the arrows denotes the relative iron trafficking between respective compartments. Values represent approximate values for an average 65 kg man. Tf, transferrin; Cyt, cytochromes; Fe-S, iron-sulfur cluster. Adapted from Finch et al.225
Figure 1.6. Proposed mechanisms of iron uptake and recycling in (A) reticuloendothelial macrophages, and (B) enterocytes. DcytB, duodenal cytochrome B; DMT-1, divalent metal transporter-1; HCP-1, heme-containing protein-1; HO, heme oxygenase; Hp, hephaestin; FPN, ferroportin; Tf, transferrin; TfR, transferrin receptor; STEAP-3, six-transmembrane epithelial antigen of the prostate 3. Adapted from Chua et al.222
Once inside the cell, iron forms a low molecular weight pool in transition between storage (bound to ferritin) and basolateral transport from the cell into the circulation. Iron extrusion from the enterocyte and transport to tissues occurs via a pathway that is common to all tissues studied thus far. Iron is transported across the basolateral membrane of the cell by ferroportin (FPN) (also referred to as iron regulatory protein-1 and metal transport protein-1). Once across the membrane, ferrous iron is oxidized to its trivalent state by hephaestin (Hp) or ceruloplasmin (Cp), enabling it to bind transferrin (Tf) for transport in the circulation.

Parenchymal cells of the liver are the main site of iron storage, although macrophages in the liver, spleen, and bone marrow are also capable of storing considerable amounts. Approximately 80% of the iron in the liver is stored as ferritin or hemosiderin, 5% is associated with Tf, and 2% is found in hemoenzymes. The remaining iron is located in labile iron pools. Ferritin is a water soluble protein that consists of 24 subunits, and is typically associated with several thousand iron atoms. Its synthesis is regulated by the presence of iron, via an iron response element (IRE), but has also been shown to be upregulated by inflammatory mediators such as interferon-γ, interleukin-1 and 6. Iron release from ferritin is poorly understood. Current evidence suggests that iron in ferritin is first oxidized by an as yet unidentified intracellular ferroxidase, and is released via proteolytic destruction of ferritin by the 20S proteosome. The quantity of iron released is based on cellular iron requirements. It is believed that hemosiderin, a water-insoluble proteinacious molecule, is formed by incomplete degradation of ferritin by the proteosome.
In its more common oxidized form (Fe\(^{3+}\)), iron’s solubility at physiological pH is approximately \(10^{-18}\) M.\(^{240}\) To overcome this poor solubility, iron is transported in the circulation bound to Tf, an 80kDa glycoprotein synthesized primarily by the liver,\(^{241}\) although it also synthesized in small amounts by the brain, testes, and mammary glands.\(^{242, 243}\) In addition to binding iron, Tf also binds various other metals (e.g. manganese, cobalt, copper, cadmium), albeit with lower affinity.\(^ {244}\) Tf can be present in the circulation in its apo, monoferric or diferric forms, depending on the plasma concentrations of iron. Typically, it remains 20-50% saturated with iron, providing a buffer range for acute increases in plasma iron levels. Thus, Tf acts as both an iron donor and acceptor, depending on the supply and demand. Uptake of Tf occurs via the Tf receptor (TfR) (Figure 1.6A). Currently there are two isoforms known, TfR1 and TfR2. TfR1 is expressed in all cells except mature erythrocytes, and is particularly high in developing erythrocytes, placental syncytiotrophoblasts, and rapidly growing cells.\(^ {245, 246}\) TfR1, like ferritin, is regulated by an IRE, which increases Tf expression in situations of low intracellular levels, and decreases expression in response to high intracellular levels.\(^ {247, 248}\) Tf also appears to be regulated by other transcription factors, including hypoxia-inducible factor-1\(\alpha\),\(^ {249}\) which may be important for iron mobilization during hypoxic conditions. The more recently identified TfR2 is localized primarily in the liver.\(^ {250}\) Unlike TfR1, TfR2 does not contain an IRE, and therefore mRNA expression is unaltered by cellular iron status.\(^ {251}\) Despite the lack of an IRE, TfR2 protein expression is altered in situations of iron overload and ID.\(^ {252}\) Recent evidence suggests that TfR2 may be regulated post-transcriptionally by diferric Tf levels.\(^ {253}\) Currently, the precise
role of TfR2 is unknown, but it does not appear to be able to compensate for a lack of TfR1, possibly as a consequence of its localization. Once Tf binds to its receptor, the complex is then taken up via clathrin-coated vesicles, which become acidified, and the iron is released from Tf. Iron is then reduced to its divalent state by an endosomal ferrireductase, STEAP-3 (six transmembrane epithelial antigen of the prostate 3), and subsequently released from the endosome through DMT1. At this stage, iron enters the cellular labile iron pool, where it may incorporated into ferritin, hemoenzymes or non-heme Fe-dependent enzymes, or may be transferred to subcellular compartments or organelles.

1.3.2 Regulation of Iron Uptake

Since mammals do not have specific mechanisms for excreting it, iron content is controlled at the level of uptake. Dietary iron absorption is regulated by three distinct mechanisms: (1) the erythropoietic regulator, (2) mucosal block, and (3) the stores regulator. The erythropoietic regulator stimulates iron uptake during severe blood loss or during ineffective erythropoiesis; this mechanism is independent of body iron stores. The erythropoietic regulator has the capacity to bring in large quantities of iron. For example, during iron deficiency anemia (IDA), iron uptake can increase ten-fold due to this mechanism. The identity of the erythropoietic regulator has not been established, although candidates include hepcidin (see below) and soluble TfR1. The second mechanism that regulates iron uptake is referred to as mucosal block. Large quantities of iron in the duodenum cause a down regulation of DMT-1 and DcytB, preventing the uptake of iron into the enterocyte; this occurs even in cases of ID. However, FPN
and Hp expression are not downregulated during mucosal block suggesting that uptake of iron into the enterocyte is inhibited. The mechanisms by which mucosal block occur are not well understood, but may involve signaling in response to increased Fe-bound ferritin. The third mechanism that controls iron uptake from the duodenum is the stores regulator. Although the erythropoietic regulator and mucosal block can dramatically impact iron uptake, these mechanisms do not play a significant role in normal circumstances; in general, systemic iron balance is controlled by the stores regulator. When body stores are low, the stores regulator can increase iron uptake from the duodenum by 2 to 3-fold. The mechanisms by which tissue iron stores regulate enterocyte uptake are not well established, although several hypotheses have been proposed. The most promising model, based on current knowledge, involves regulation by hepcidin. Hepcidin is a 25 amino-acid protein synthesized in the liver, and to a lesser extent in the kidneys and heart. Its synthesis is regulated by systemic iron levels, via interactions with the ‘IRE/iron response protein regulatory network’, as well as hypoxia. Hepcidin regulates iron flux by binding to FPN and enhancing its lysosomal degradation. Since FPN is the only transporter by which iron can cross the basolateral membrane of the enterocyte and enter the circulation, hepcidin mediated destruction of FPN effectively regulates iron uptake. Hepcidin expression is known to decrease during anemia and during pregnancy, resulting in diminished metabolism of FPN, and increased systemic delivery of Fe.
1.3.3 Regulation of Iron Uptake in Pregnancy

The maternal system undergoes a number of changes during pregnancy to ensure proper delivery of oxygen and nutrients to the developing fetus. Among these adaptive changes is an increased uptake and delivery of iron to the placental-fetal unit. The amount of iron transferred to the fetus throughout the course of pregnancy is estimated to be 1000 mg, which exceeds the amounts stored in maternal tissues. In humans, pregnancy is associated with a 9-fold increase in maternal iron absorption from the first to the third trimester. This increase in iron uptake likely reflects activity of the erythropoietic regulator, since maternal erythropoiesis is increased due to volume expansion. In rats, iron absorption increases dramatically from gestational day 16 to 21 and rapidly returns to normal after birth. These changes appear to stem from increased maternal expression DMT1, DcytB and FPN, coincident with decreased expression of hepcidin and TfR2 in maternal liver.

Iron uptake from the maternal circulation into the placenta and subsequent delivery to fetal tissues occurs by similar processes as in uptake and transport from the duodenum. A notable exception is that iron is delivered to the placenta bound to Tf, rather than in its elemental form, as in the gut. It is presently unclear whether heme can be taken up by the placenta. To prevent excess delivery of iron, iron uptake into the placenta is inhibited by hepcidin, likely by promoting lysosomal degradation of FPN as in the enterocyte, although this has yet to be confirmed. During inadequate delivery of iron (i.e. during ID), the placenta increases expression of TfR, as well as DMT1 as a means to increase delivery of iron to the fetus. Thus, the placenta serves as an
important regulator of fetal iron delivery during pregnancy. Interestingly, the placenta may have a protective effect for the fetus in response to maternal ID. Gambling and colleagues demonstrated that low iron treatment causes a 73% reduction in maternal liver iron stores, whereas fetal liver decreased by only 50%.\textsuperscript{281} Moreover, administration of iron (as supplements) to the mother increases iron transport to the fetus, even at the expense of the mother’s iron status.\textsuperscript{282}

Although the fetus is adept at sequestering iron from the maternal system to maintain erythropoiesis, ID is an inevitable consequence without adequate maternal intake during pregnancy. The following section discusses ID during pregnancy and its long-term consequences.

\textbf{1.4 Iron Deficiency}

\textbf{1.4.1 Overview}

ID is the most common nutritional deficiency in the world.\textsuperscript{283, 284} It is also the only nutritional disorder that is highly prevalent in both developing and affluent countries.\textsuperscript{102, 285} Despite this knowledge, the prevalence of ID is difficult to assess. This is because the presence of ID is confounded by many variables, including age, sex, race, socioeconomic status, and general health.\textsuperscript{286, 287} However, using anemia as an indirect indicator, the WHO estimates that 66-80% of the global population has some degree of ID (WHO, 2003). Based on these figures, the WHO has named ID as a top ten global health risk.\textsuperscript{102} The populations most at risk for ID, irrespective of geographical location, include pregnant women, women of childbearing ages, neonates and young children.\textsuperscript{286}
ID in pregnant women is of particular interest with respect to the present studies, and is discussed in detail below. In general, women of child bearing ages have lower iron stores than men, due to smaller livers and muscle mass, as well as periodic loss through menstrual flow. While men and non-menstruating women average 1-2 mg or iron loss per day, menstruating women can average an additional 10 mg per day, and has been reported to be as high as 42 mg per menstrual cycle in women with heavy blood flow.288

In general, ID is caused by an extensive period of negative iron balance, characterized by progressive depletion of iron stores. This negative iron balance can result from various factors, including inadequate iron content in the diet, diminished iron uptake from the diet, blood loss (e.g. hemorrhage, menstrual flow), increased iron demands due to growth and development, and/or due to pregnancy.289 Sequential changes occur in iron storage and handling as ID progresses (Figure 1.7). The first stage is characterized by a reduction of tissue iron stores, which is reflected by decreases in ferritin levels. Iron stores are reduced prior to manifestations of anemia because the body prioritizes iron utilization for erythropoiesis, in order to maintain tissue oxygenation. However, it is not currently known whether storage iron alone is compromised during this phase, or whether functional iron is also compromised to preserve circulating levels of Hb. After sustained negative iron balance, iron stores become depleted, and transport of iron from the gut and liver to the bone marrow diminish (hence decreased Tf saturation). At this stage, the lack of iron impacts erythropoiesis, although Hb levels may remain within a range that is deemed normal (see Killip et al.290 for details); this is referred to as iron deficient not anemic (IDNA) (Figure 1.7). This phase may last for an
Figure 1.7. Changes in tissue and circulating indices of iron status associated with the progression of negative iron balance. IDNA, iron deficient non-anemic. \(^\text{291}\)
extended period since the lifespan of an erythrocyte is approximately 120 days. In the final phase (IDA), the supply of iron to the bone marrow is unable to sustain erythropoiesis; this period is characterized by the presence of microcytic and hypochromic erythrocytes, reflecting reduced Hb levels in circulating erythrocytes. The reductions in Hb (and hematocrit) compromise oxygen delivery, and consequently, this phase is often associated with symptoms of fatigue, pallor, dyspnea, headache and concentration disorders.292

1.4.2 Iron Deficiency and Pregnancy

Pregnant women are especially prone to ID, due in large part to volume expansion (particularly during the second and third trimesters) as well as demands from the fetal-placental unit.274, 275 The incidence of maternal ID anemia during pregnancy is estimated to be over 50% in developing countries,286 and as high as 80% in certain southeastern Asian countries.293 In affluent countries, the estimated incidence of IDA during pregnancy is 30%, with teenagers, recent immigrants from developing nations, women of low socioeconomic status, and multiparous women with short inter-pregnancy intervals being most at risk.294 Moreover, despite iron supplementation, more than 30% of pregnant women have some signs of low iron status by the end of pregnancy in western countries.295

Maternal ID can have profound effects on pregnancy outcome. ID during pregnancy can lead to maternal and fetal death, and is strongly associated with premature labour.296-298 Moreover, even in the absence of severe birth related complications, maternal ID impacts fetal iron status,299-301 which has important implications on growth
and development, and thus long-term health. There is accumulating evidence that indicates that ID during pregnancy and during the immediate postnatal period can have deleterious effects on growth and development, which manifest in later life. Given the propensity of pregnant women and those of child bearing ages to be afflicted, maternal ID as a model of developmental programming is a highly relevant one; this is discussed in more detail in next section. In addition to maternal ID, several conditions during gestation, associated with decreased iron delivery or increased demands beyond the placenta’s capacity for delivery, can induce fetal ID. Intrauterine growth restriction, caused by maternal factors such as inadequate nutrition or hypertension during pregnancy, affects approximately 10% of all live births, and ID is associated with 50% of these cases. Gestational diabetes, which affects 5-10% of pregnancies, stimulates maternal and fetal metabolic rate and oxygen consumption. These increased oxygen demands stimulate erythropoiesis, which often cannot be met by uteroplacental transport of iron, and results in fetal ID. Studies have shown that storage and functional iron is depressed as much as 90% in liver, 55% in heart, and up to 40% in brain in pregnant women with poorly controlled diabetes mellitus. In both maternal hypertension and diabetes, iron delivery to the fetus is impaired due to compromised uteroplacental blood flow and/or placental vascular disease. Smoking can also cause fetal ID, due to diminished delivery of iron as a consequence of uteroplacental constriction caused by nicotine and catecholamines, and increased demands as a consequence of hypoxia-induced erythropoiesis.
The total body iron content of the fetus during the third trimester is approximately 75 mg/kg, with approximately 80% localized in erythrocytes, 10% in tissues as functional iron (e.g. myoglobin, cytochromes), and the remaining 10% stored in ferritin and hemosiderin. Sixty percent of this iron is acquired during the third trimester, making this an important period for iron accretion for use in the neonatal period. The neonatal period is characterized by a 30-50% decrease in Hb, due to cessation of erythropoiesis, lysis of senescent red blood cells, and expansion of blood volume. Thus, inadequate iron delivery from the maternal system during this period can be particularly influential on growth and development. For preterm birth, the risk of ID during the neonatal period is substantially greater, since it deprives the fetus of a very important phase of iron accretion, which will be needed in the neonatal period. Furthermore, catch-up growth, associated with either preterm birth or IUGR, places greater demands during the neonatal period, due to greater expansion of the blood volume.

1.4.3 Iron Deficiency and the Developmental Origins of Health and Disease

Numerous epidemiological studies have shown that ID, particularly during the first two years of life, impacts cognition and behaviour, as indicated by scholastic performance (see section 1.4.3.1). However, there are little available data in humans that show the impact of ID during pregnancy, either on cognition and behaviour, or on long-term health of the offspring. This is surprising, given that various hematological indices of iron status are obtained from pregnant women throughout pregnancy in routine clinical practice. The majority of available information on the long-term effects of
perinatal ID (PID) has been obtained from animal models, such as rodents and sheep. Evidence indicates that ID during the perinatal period can impact neurodevelopment, with long-term effects on behaviour and coping abilities.\textsuperscript{309} PID has also been shown to impact cardiovascular and metabolic function, with similar effects to those reported in other models of developmental programming. These two subjects are discussed separately in the following sections.

1.4.3.1 Perinatal Iron Deficiency and Neurodevelopment

Various laboratory groups have shown that PID can impact various facets of cognition and behaviour, including temperament,\textsuperscript{310} language ability,\textsuperscript{311} fine motor skills and traceability,\textsuperscript{311} and recognition memory processing,\textsuperscript{312} the latter observation persisted into infancy,\textsuperscript{313} even when liver iron stores were completely restored by that time.\textsuperscript{314} Similar results were obtained in rats and monkeys, using a barrage of cognitive function and behavioural tests, involving the Morris water maze, locomotor activity, elevated plus maze, among others.\textsuperscript{315-320}

The mechanisms by which PID impacts cognition and behaviour have not been elucidated, although studies in rats are revealing insights into these processes. It is known that iron is required for the synthesis of various neurotransmitters, metabolism, bioenergetics, and other processes. Thus, the persistent effects may stem from a number of potential metabolic, structural and electrophysiological changes that occur in response to early ID.\textsuperscript{321-323} Indeed, regions of the brain associated with cognitive processing, such as the hippocampus and striatum, appear to be particularly vulnerable.\textsuperscript{321-323} However, gaining insight into these mechanisms is complicated by the fact that acute ID can impact
numerous processes involved in learning, memory and behaviour.\textsuperscript{308, 324, 325} Moreover, these abnormalities may persist even when systemic iron stores are replenished.\textsuperscript{308, 324} This may occur because brain iron turnover has been reported to be extremely slow; thus, when brain iron levels are depleted, it may be difficult to return them to normal status. Consequently, long-term effects of PID may stem from persistent deficits in brain iron, versus the developmental structural and functional abnormalities that persist into adulthood. This issue was addressed in Chapter 5.

1.4.3.2. Perinatal Iron Deficiency and Cardiovascular Function

PID has been reported to induce hypertension in rats.\textsuperscript{27, 213-215} Despite being validated by a number of independent investigators, these findings have not been validated using direct hemodynamic measurements. This is an important caveat, since numerous artifacts have been associated with indirect tail cuff monitoring of AP.\textsuperscript{326} Moreover, numerous studies have shown that perinatal stressors can impact the offspring’s stress responses,\textsuperscript{327-330} which would likely be manifest during tail-cuff monitoring of AP. Thus, while the tail-cuff method may provide insight into the effects of a stress response on cardiovascular function, it is likely that these do not reflect actual physiological or pathophysiological conditions in the rat.

In addition, the underlying mechanisms by which PID produces persistent altered regulation of blood pressure are not known. Lisle and colleagues have shown that PID results in a reduced nephron endowment concomitant with elevated blood pressure.\textsuperscript{27} A decreased nephron endowment has been described in a number of programming models that have hypertension.\textsuperscript{331} It has been suggested that reduced filtration surface area may
lead to renal pathology due to glomerular hyperfiltration, which may impact AP regulation over time.\textsuperscript{331} This topic is being investigated by a number of groups. An alternative hypothesis is that these two events, though temporally associated, are independent phenomena that share a common etiological factor.

1.5 Statement of Hypotheses and Objectives

Cardiovascular disease and cerebrovascular disease are the leading causes of death in developing and industrialized countries.\textsuperscript{332} Hypertension, which is a significant risk factor for cardiovascular disease, affects more than 25\% of the global adult population.\textsuperscript{99} Although genetic and environmental factors are known to contribute, more than 90\% of cases of hypertension are of unknown cause.\textsuperscript{99, 333} Stressors that occur during gestation and during the immediate postnatal period are known to cause long-term programming of cardiovascular function. In particular, ID during pregnancy and during the subsequent neonatal period is known to cause long-term programming effects in the offspring. Given its prevalence and its propensity to afflict pregnant women,\textsuperscript{296} ID is a highly relevant model of developmental insult.

Little is known about the mechanisms by which ID impacts on long-term regulation of AP. The involvement of congenitally reduced nephron number has been proposed to be an etiological factor for hypertension,\textsuperscript{331} although a mechanistic link between the two phenomena has not been made. Given the importance of the kidney in the long-term regulation of AP, it is likely that fundamental changes in renal function,
which affect the pressure-natriuresis mechanism, are involved in some capacity in the pathogenesis of hypertension.

The specific hypotheses tested in the present series of studies were that:

1. Maternal ID alters the growth and developmental trajectories of offspring.
2. PID causes cardiovascular dysregulation in adult offspring, mediated by changes in intrarenal hemodynamics.
3. PID causes persistent alterations in hemoenzyme function, specifically within the vasculature and the kidney, which are functionally distinct from changes associated with acute ID.
4. PID impacts cognitive and behavioural function in adult males and females, and these effects occur in the absence of brain iron depletion.

The studies in which these hypotheses were tested include:

**Chapter 2: Perinatal Iron Deficiency in Wistar Rats: Characterization of the Experimental Model**

The objectives were:

1. To develop a rat model of PID that is relevant to the human population.
2. To characterize the model of PID with respect to birth weight, organ weights, iron status, growth patterns, and adult physical characteristics.
Chapter 3: The Long-Term Circulatory Consequences of Perinatal Iron Deficiency in Male Wistar Rats

The objectives were:

1. To determine, using radiotelemetry, whether PID causes persistent elevations in AP in adult male rats.
2. To determine whether PID impacts AP responses to low and high sodium intake.
3. To determine whether these changes are associated with intrinsic changes in intrarenal hemodynamics.

Chapter 4: The Impact of Perinatal and Acute Iron Deficiency on Intrarenal and Vascular Nitric Oxide Signaling in Male Wistar Rats

The objectives were:

1. To assess NOS and sGC activity in renal tissue extracts in newborn and adult male offspring.
2. To assess whether these animals have altered vascular responses to vasoconstrictors and vasodilators ex-vivo.
3. Develop a rat model of acute ID.
4. To assess NOS and sGC activity in renal medullary and cortical samples of acute iron deficient rats.
5. To assess vascular responses of acute iron deficient and replenished rats using known vasoconstrictors and vasodilators.
Chapter 5: Perinatal Iron Deficiency Impacts Locomotor Activity and Water Maze Performance in Adult Male and Female Wistar Rats

The objectives were:

1. To determine whether PID male and female adults display differential adverse cognitive and behavioural effects by testing using the Morris mater maze, and ambient locomotor activity apparatus.

2. To assess hematological and tissue iron levels in neonatal and adult offspring.
Chapter 2
Perinatal Iron Deficiency in Wistar Rats: Characterization of the Experimental Model

(Published in part in International Journal of Obesity (London) 2008; 32:1441-1444)

2.1 Introduction

Evidence obtained in the last two decades indicates that stressors that occur during pregnancy and the subsequent postnatal development phase, can impact various aspects of the offspring’s physiology. It is hypothesized that the fetus undergoes a number of adaptive changes to cope with the early stressor. Although they ensure survival of the developing offspring, these changes may be maladaptive for life after development, and thus impinge on normal systemic function in adulthood. These complications are said to be ‘developmentally programmed’ because they result from an early stressor, but persist long after the insult occurs.

Perinatal iron deficiency (PID) may be one of the most relevant programming models from a global health perspective. First, the incidence of iron deficiency (ID) and/or anemia has reached near epidemic proportions; according to the World Health Organization (WHO), an estimated 66-80% of the global population has some degree of ID (WHO, 2003). Second, ID is the only nutritional disorder that is highly prevalent in both industrialized and developing countries. This is in contrast to other models of perinatal insult, such as maternal malnutrition, which affects populations of developing countries, or maternal excess high-fat intake, which affects predominantly populations in...
affluent countries. Third, pregnant women are among the populations that are most at risk for ID. This group is particularly susceptible due to expansion of blood volume that accompanies normal pregnancy as well as demands from the fetal-placental unit.\textsuperscript{274, 275, 336} Women of child bearing ages are also at high risk, due to periodic loss of iron through menstrual flow\textsuperscript{288} (see section 1.4.1). Finally, given the myriad roles of iron in (i) oxygen and carbon dioxide transport (e.g. hemoglobin (Hb), myoglobin), (ii) circulatory function (e.g. NOS, sGC), (iii) neuromodulation (e.g. NO) (iv) xenobiotic metabolism (e.g. cytochromes), and (v) energy metabolism (cytochromes), early ID could have profound effects on many physiological systems. Ironically, the long-term consequences of PID have received relatively little attention.

Epidemiological studies have shown that ID during development can induce a number of long-term behavioural and cognitive deficits in infants and young children.\textsuperscript{337} Although these studies have confirmed a highly relevant problem, they have provided little insight into the underlying mechanisms. Furthermore, population-based studies are often confounded by genetic and environmental influences that cannot be controlled. The need for animal models is therefore essential to identify a causal relationship between developmental ID and its long-term adverse effects, and properly explore its underlying mechanisms.

In addition to confirming the cognitive and behavioural effects of PID, animal models have identified cardiovascular and metabolic related dysfunction associated with developmental ID.\textsuperscript{26, 27, 213, 215} Although these animal models have also begun to provide insight into the mechanisms by which PID impacts long-term health, this has been
particularly challenging; more challenging, it seems, than with other models of developmental insult. This undoubtedly stems from the infancy of the iron metabolism field, and thus from a lack of basic knowledge of iron handling in pregnancy and during early growth and development. Indeed, many important mediators of iron uptake and transport have been identified only within the last decade (e.g. ferroportin and hepcidin).

This lack of basic knowledge regarding iron utilization underscores the importance of proper characterization of animal models used in programming research. This chapter represents a compilation of data from one pilot study and two separate programming studies used to establish the conditions of maternal ID and offspring PID upon which future studies could be based. The purpose of this chapter was to document the iron status, growth and physical outcomes of offspring subjected to ID during gestation.

2.2 Methods and Materials

2.2.1 Animals and Treatments

All treatments described herein were approved by the Queen’s University Animal Care Committee. The data presented in this chapter were obtained from three studies: one pilot study and two independent studies, referred to in this chapter as ‘Study 1’ and ‘Study 2’. All rats were purchased from Charles River (Saint-Constant, QC), and housed in the Queen’s University Animals Care Facility, which maintained a 12 h/12 h light/dark cycle, and an ambient temperature of 23°C. All rats were given one week to acclimatize...
to their surroundings prior to experimentation. The purified diets (Research Diets Inc. New Brunswick, NJ) were based on the AIN-93G diet, which has been described elsewhere. All purified diets were identical in composition with the exception of the added ferric citrate, which was adjusted to obtain the iron concentrations described below. The non-purified diet (Lab Diet, Oakville, ON) was a grain-based rodent chow, and contained 270 mg/kg iron.

For the pilot study, 12 seven-week old and 12 five-week old female Wistar rats were used. Eight rats from each age group were randomly selected and fed the low iron purified diet (3 mg/kg Fe). The remaining 4 rats from each group were fed the control purified diet (225 mg/kg Fe). At 10 wk of age, the female rats were mated with age-matched males that were fed the non-purified control diet. Thus, the older group had a 2 wk pre-gestational treatment period, whereas the younger rats had a 4 wk pre-gestational treatment. All rats were maintained on their respective diets until postnatal day (PD) 12. Food intake prior to and throughout gestation was weighed 3 times per week. Within 24 h of birth, all litters were culled to 8 animals (4 males and 4 females) wherever possible.

Due to the impact this treatment regimen had on the survival of the offspring, alternate low-iron treatment protocols were adopted in subsequent studies (Study 1 and 2). In Study 1, dams were treated for 2 wk prior to pregnancy. Furthermore, dams were fed the non-purified control diet within 8 h of giving birth. A similar low iron treatment was used in Study 2, with the exception that the dams were fed a moderately low iron diet (10 mg/kg) during gestation. The treatments used in Studies 1 and 2 are described in
Table 2.1. Treatment regimen of dams and offspring in the pilot study, Study 1 and Study 2.

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
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<th>Gestation</th>
<th>Lactation</th>
<th>Post Weaning</th>
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ID; iron deficient; Ctl, control; 3, 3 mg/kg Fe; 10, 10 mg/kg Fe; 225, 225 mg/kg Fe, 270, 270 mg/kg Fe in diet. *denotes non-purified diet. See Methods and Materials section for details.
detail in Chapters 5 and 3, respectively. A summary of the treatment protocols used in the pilot study, as well as Studies 1 and 2 is presented in Table 2.1.

2.2.2 Tissue Collection and Analysis

Organs were collected as described in Chapter 3. Briefly, pups were killed by decapitation, and blood was collected (see below). Brains, hearts, livers, spleens, kidneys, and adrenals were rapidly excised and placed in ice-cold saline. Organs were then cleaned of extraneous connective tissue, blotted dry, and weighed. Milk was collected by dissecting the stomach, and removing the milk contents from within. All tissue samples were frozen in liquid nitrogen immediately after being weighed.

Hematocrit (Ht) was assessed by collecting a small volume (~70 μL) of blood into a heparinized microcapillary tube, and centrifuging at 1800 x g for 15 min; packed cell volume was then determined. A small blood sample (10-50 μL) was also collected in 250 volumes of Drabkin’s reagent (Sigma, St-Louis, MO), and hemoglobin (Hb) content was determined by the cyanomethemoglobin method. Tissue iron assessments were performed as described in Chapter 5. Briefly, frozen tissues were thawed and dried at 65°C for a minimum of 72 h, then ashed in a muffle furnace for 2 h at 200°C, and 18 h at 500°C. The residual ash was dissolved in hot nitric acid. The samples were subsequently diluted with ddH₂O. Iron content was assessed using a Varian SpectrAA-20 flame atomic absorption spectrometer (Varian Canada Inc., Ottawa, ON). Bovine liver standards (National Institute of Standards, 1577b) and blanks were included as quality controls.
2.2.3 Physical Characteristics and Locomotor Activity

Adult physical characteristics and locomotor activity were obtained as an extension of Study 2, and have been published elsewhere. Locomotor activity was assessed by radiotelemetry from 12 to 36 wk of age, as described in Chapter 3; locomotor activity was assessed while the rats were in their home cages. Body weight, naso-anal length and waist circumference (an indirect measure of abdominal obesity measured at the midpoint between tip of the sternum and penis in supine position), were assessed in offspring at 36 wk of age. Additionally, following anesthesia with sodium pentobarbital (120 mg/kg i.p.) and exsanguination, retroperitoneal, mesenteric, omental, and epididymal fat pads were removed from these animals. The sum of these major abdominal fat pads was considered total visceral adipose tissue (VAT).

2.2.4 Statistical Analyses

Ht, Hb levels, liver and milk iron content, neonatal and adult organ weights, and locomotor activity were analyzed by 2-way ANOVA, by treatment and time. Neonatal and adult organ weights from Study 1 were analyzed by treatment and sex. Where differences were observed, Newman-Keuls test or Student’s t-test with Bonferroni correction was used as a post-hoc test, as appropriate. Correlation analyses were performed using ordinary least squared regression. With the exception of correlation analyses, data obtained from the same litter were averaged and treated as a single value. Thus, n values represent the number of litters used. Grubb’s test was used to identify statistical outliers. Data are presented as mean ± SEM. P < 0.05 was considered statistically significant.
2.3 Results

2.3.1 Maternal Outcomes

Data from the pilot study are not presented herein because information pertaining to litter sizes and dams’ total weight gain throughout pregnancy could not be obtained; this is due to the fact that many ID dams would consume their offspring immediately after giving birth. The data presented in this section pertain to Study 2 unless otherwise specified. Maternal food consumption did not vary between groups fed the purified control and purified low iron diet in the periods prior to and throughout gestation (data not shown). However, in Study 1, the dams fed the non-purified diet consumed more food during all periods than the dams fed the purified control (P < 0.05) (data not shown). Consumption of a low-iron diet had a profound impact on iron status in the dams (Figure 2.1A, B). Twenty-four hours after delivery, dams fed the low iron diet had Ht and Hb levels both 28% lower than control values (P < 0.001 for both parameters). After being given the control diet, Ht and Hb levels recovered and reached control values within 7 days, and were not different thereafter (Figure 2.1A, B). Despite these differences, the low-iron treatment had no impact on weight gain during pregnancy, litter size, or ratios of male to female offspring (see Table 3.1).

2.3.2 Offspring Outcomes

Maternal ID had a profound impact on the offspring. In the pilot study, there were numerous still births and offspring deaths in the immediate postnatal period among ID litters. Moreover, the majority of surviving pups were often killed and eaten by the mother. Of the 6 dams that got pregnant in the group that was iron restricted for 2 wk
Figure 2.1. Dam and offspring (A) hematocrit and (B) hemoglobin levels at postnatal days 1, 7, 14, and 21. Data were obtained from Study 2. *P < 0.05, **P < 0.01, ***P < 0.001 compared to respective controls at the same postnatal day. †P < 0.05 compared to previous postnatal week in the same treatment group. Control offspring: n = 4-7; PID offspring: n = 5-7; control dams: n = 6-7; ID dams: n = 7-9. ID, iron-deficient; PID, perinatal iron-deficient.
prior to gestation, 4 litters were completely lost within the first 21 days after birth, and the remaining two litters had a minimum of 3 deaths in each. In the group that was iron restricted for 4 wk prior to gestation, 7 of 8 dams got pregnant, and a total of 7 pups from 3 litters survived until PD 21. In contrast, 7 of the 8 dams in the control group got pregnant, and only 1 offspring died between PD 1 and PD 21. Based on these findings, alternative treatment regimens were adopted for Studies 1 and 2, which markedly improved offspring survival in the ID groups. For details on offspring survival in these studies, see sections 5.3.1 and 3.3.1 respectively.

At PD 1, PID offspring Ht and Hb levels were 40% and 41% lower than control values, respectively (Figure 2.1A, B) (P < 0.001 for both parameters). In contrast to the ID dams, Ht in the PID offspring remained lower than controls until weaning (P < 0.05 at all postnatal days), and Hb levels were lower at all postnatal days except PD 21 (P < 0.05). Consistent with these findings, liver iron concentration and total liver iron content of PID offspring were both 89% lower than control values at PD 1 (P < 0.001 for both parameters), and remained lower throughout lactation (P < 0.05), except on PD 21 (Figure 2.2A, B). Ht, liver iron concentrations as well as total liver iron content in control pups decreased from PD 1 to PD 7 and PD 7 to PD 14 (P < 0.05 for both days); Hb levels decreased from PD 1 to PD 7, and there was a trend towards a decrease from PD 7 to PD 14 (P = 0.05; α = 0.025). In contrast, PID liver iron concentrations only declined between PD 1 and PD 7 (P < 0.05), and no further decreases were observed. Moreover, Ht and total liver iron content of PID pups increased from PD 7 to PD 14 (P < 0.05) and PD 14 to PD 21 (P < 0.05). The relationship between Ht and total liver iron
Figure 2.2. Effect of PID on offspring (A) liver iron concentration (per gram of tissue dry weight) and (B) total liver iron content at postnatal days 1, 7, 14, and 21. Data were obtained from Study 2. *P < 0.05, ***P < 0.001 compared to controls at the same postnatal day. †P < 0.05 compared to previous postnatal week in the same group. Note that in (A), the † at postnatal day 14 is associated with the control value. Control: n = 6-7; PID: n = 6-8.
in the offspring displayed a non-linear relationship ($R^2 = 0.72$), such that no changes in Ht were observed until total liver iron fell below approximately 50 μg (Figure 2.3A, B).

The iron content of the milk obtained from the stomachs of culled pups between PD 1 and PD 21 is presented in Figure 2.4. Two-way ANOVA revealed no treatment or time effects, although there was an overall interaction ($P < 0.01$). Subsequent analysis revealed that milk in the stomachs of PID offspring on PD 1 contained 26% less iron than controls ($P < 0.001$), although these differences were not observed at any other postnatal day. In fact, there was a trend for higher iron content in the milk of iron deficient dams at PD 7 and PD 14 ($P = 0.05$ and $P = 0.06$ respectively).

In Study 1, assessment of male and female offspring organ weights, obtained at 24 h postpartum, revealed no sex-based differences. The organs weighed included hearts, brains, livers, spleens and kidneys (data not shown). Consequently, in Study 2, female offspring were preferentially culled over males to assess growth patterns. This was done to maximize the number of male offspring available for future experiments. Body and organ weights from Study 2, obtained at PD 1, 7, 14, and 21 are presented in Figure 2.5. PID caused decreased birth weights ($P < 0.05$), and these decreases persisted throughout the lactation phase (Figure 2.5A), and even into adulthood (see Figure 3.1A). It is noteworthy that while PID caused similar reductions in the birth weight in Study 1, these differences did not persist in adulthood (see Figure 5.1). PID heart weights (normalized to body weight; Figure 2.5B) were 29% higher at birth ($P < 0.001$), and reached a maximum of 34% higher at PD 7 ($P < 0.001$), and remained elevated until PD 21 ($P < 0.05$). Even when expressed as absolute weights, hearts were 24% larger at PD 1
Figure 2.3. Relationship between hematocrit and total liver iron in control and PID offspring. Data from postnatal days 1, 7, 14, and 21 are (A) not distinguished and (B) distinguished from one another. Data from PD 1 were obtained from Studies 1 and 2; data from PD 7-21 were obtained only from Study 2. Each data point represents an offspring chosen at random from each litter; each litter is represented only once. The thinner, dashed lines in (A) denote 95% confidence intervals.
Figure 2.4. Iron content of dams’ milk (expressed per gram dry weight) at postnatal days 1, 7, 14, and 21. Data were obtained in Study 2. ***P < 0.001, §P < 0.06 compared to control at the same postnatal day. Control: n = 3-7; PID: n = 6-8.
Figure 2.5. Effect of PID on (A) body weight, and (B-G) organ weights at postnatal days 1, 7, 14, and 21. Data reflect predominantly female offspring values. All organ weights are normalized to body weight. *P < 0.05, **P < 0.01, ***P < 0.001 compared to controls at the same postnatal day; †P < 0.05 compared to the previous postnatal week in the same treatment group. Control: n = 5-7; PID: n = 6-8.
compared to controls ($P < 0.001$). Brain weights (normalized to body weight) were 9% larger in the PID group compared to controls, reaching a maximum of 26% at PD 7. However, these differences likely reflect the changes in body weight, since there were no differences in absolute brain weights at any of the postnatal days. Two-way ANOVA also revealed a treatment effects in liver weights ($P < 0.05$); post hoc analysis revealed that PID livers were 6% higher than controls at PD 14 ($P < 0.05$). However, like brain weights, these differences likely reflect differences in body weights, since absolute liver weights were not different at any postnatal day. All other organs assessed were not different between treatment groups. All organs weights normalized to body weight varied with postnatal time ($P < 0.001$), and tended to regress toward PD 21. Spleen growth from PD 1 to PD 7 was lower in the PID offspring compared to controls. More specifically, spleen weights of control animals increased 7.4-fold from PD 1 to PD 7, whereas spleen weights of PID rats only increased 5.4-fold increase ($P < 0.05$); even when spleen weights were normalized to body weights, spleen weights increased 3.1-fold and 2.7-fold in the control and PID offspring, respectively ($P < 0.05$).

The relationship between body weight and Ht at 24 h is presented in Figure 2.6; this is a compilation of data obtained from Studies 1 and 2. A positive correlation between body weight and Ht was found in the PID group ($r^2 = 0.21; P < 0.001$), whereas no such correlation was found in the control offspring. The relationship between various organ weights and Ht at 24 h is presented in Figure 2.7. In the PID offspring, a positive correlation was found between Ht and brain weights, spleen weights, kidney weights, and liver weights ($P < 0.05$ for all parameters) whereas no correlation was found between
Figure 2.6. Correlation between body weight and hematocrit in control and PID offspring at 24 h. Data were obtained from Studies 1 and 2. Data reflect predominantly female offspring values. Control: 57 pups from 13 litters; PID: 72 offspring from 14 litters. The thinner dashed lines denote 95% confidence intervals.
Figure 2.7. Correlation between organ weights and hematocrit (Ht) in control and PID offspring at 24 h. Organ weights are presented as absolute weights (not normalized to body weight). Data were obtained from Studies 1 and 2; adrenal weights were obtained only from Study 2. Data reflect predominantly female offspring values. *P < 0.05 compared to control slope. †Comparisons could not be made because slopes were so different. Control: 56 offspring from 13 litters; PID: 73 offspring from 14 litters; for adrenals, control: 24 offspring from 6 litters; PID: 33 offspring from 8 litters. NA, not applicable.
Ht and heart and adrenal weights. In contrast, a negative correlation was found between Ht and kidney weight in the control offspring (P < 0.05), and no correlations were observed between Ht and heart, brain, spleen, liver or adrenal weights. The relationship between these organ weights and body weights were also examined (Figure 2.8 A-F). All organ weights, with the exception of adrenal glands, were positively correlated with body weight in both the control and PID groups (P < 0.001). PID caused a blunting in the association, as determined by slope, between heart weight and body weight (control: 6.7 ± 1.1 vs. PID: 3.4 ± 1.0; P < 0.05) as well as brain weight and body weight (control: 35.8 ± 4.1 vs. PID: 25.9 ± 2.6; P < 0.05).

2.3.3 Adult Offspring Outcomes

In Study 1, both male and female adult offspring were killed at 24 wk; their body weights, Ht, and organ weights are presented in Table 2.2. No differences between control and PID offspring were observed in any of these variables at this age when normalized to body weights, although differences were observed between the males and females for all organ weights, as expected. There was an interaction between treatment and sex in the case of brain weight (P < 0.01); again these results likely reflect the subtle differences in body weight, since there were no differences in absolute brain weights. The results obtained in this study were similar to those obtained in Study 2, with the following two exceptions. First, in Study 1, differences in body weight between control and PID offspring during lactation did not persist into adulthood (Figure 5.1), whereas in study 2, body weights remained lower in male PID offspring (Figure 3.1) (note that only males were studied in adulthood in Study 2). Second, RV weights from Study 2 were
Figure 2.8. Correlation between organ weight and body weight in control and PID offspring at 24 h. Organ weights are presented as absolute weights (not normalized to body weight). Data were obtained from Studies 1 and 2. Data reflect predominantly female offspring values. *P < 0.05 compared to control slope. †Comparisons could not be made because slopes were so different. Control: 56 offspring from 13 litters; PID: 73 offspring from 14 litters. Adrenal weights were obtained only Study 2 (control: 24 offspring from 6 litters; PID: 33 offspring from 8 litters). NA, not applicable.
Table 2.2. Hematocrit and organ weights of control and PID offspring at 24 wk.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Males</th>
<th>Females</th>
<th>2 way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PID</td>
<td>Control</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.46 ± 0.01</td>
<td>0.46 ± 0.00</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>707.6 ± 28.6</td>
<td>612.0 ± 31.7</td>
<td>362.8 ± 9.9</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>1.53 ± 0.03</td>
<td>1.54 ± 0.03</td>
<td>1.41 ± 0.03</td>
</tr>
<tr>
<td>LV (g)</td>
<td>1.40 ± 0.04</td>
<td>1.33 ± 0.05</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>RV (g)</td>
<td>0.38 ± 0.01</td>
<td>0.37 ± 0.02</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>1.48 ± 0.11</td>
<td>1.34 ± 0.09</td>
<td>0.83 ± 0.03</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>2.67 ± 0.21</td>
<td>2.42 ± 0.18</td>
<td>1.40 ± 0.04</td>
</tr>
<tr>
<td>Brain/BW (g/kg)</td>
<td>2.15 ± 0.14</td>
<td>2.56 ± 0.29</td>
<td>3.90 ± 0.32</td>
</tr>
<tr>
<td>LV/BW (g/kg)</td>
<td>2.04 ± 0.29</td>
<td>2.20 ± 0.22</td>
<td>2.83 ± 0.18</td>
</tr>
<tr>
<td>RV/BW (g/kg)</td>
<td>0.54 ± 0.07</td>
<td>0.60 ± 0.05</td>
<td>0.78 ± 0.08</td>
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<tr>
<td>Spleen/BW (g/kg)</td>
<td>2.17 ± 0.21</td>
<td>2.20 ± 0.30</td>
<td>2.30 ± 0.23</td>
</tr>
<tr>
<td>Kidney/BW (g/kg)</td>
<td>3.81 ± 0.58</td>
<td>3.97 ± 0.41</td>
<td>3.87 ± 0.22</td>
</tr>
</tbody>
</table>

Data were obtained from Study 1. **P < 0.01, ***P < 0.001. Controls: n = 6; PID: n = 6. PID, perinatal iron-deficient; Int., interaction; LV, left ventricle; RV, right ventricle; BW, body weight; NS, not significant.
greater in the PID offspring compared to controls (0.54 ± 0.03 mg/kg (control) vs. 0.61 ± 0.04 mg/kg (PID), P < 0.01); this was not the case in Study 1 (Table 2.2).

A number of physical characteristics were assessed at 36wk in a subset of the male offspring from Study 2; these data are presented in Table 2.3. The differences in body weight between PID and control offspring that were present at birth persisted until 36 wk of age (P < 0.05), although hematological indices if iron status were no longer different at that time. PID offspring body lengths were approximately 4% lower than control body lengths (P < 0.05). Individual visceral fat pad weights, as well as their combined weights (VAT) were similar in both groups, as was abdominal circumference. VAT as a percentage of body weight was 18% greater in PID rats compared to controls (P < 0.05). Locomotor activity, assessed in the rats’ home cages, was lower in the PID group at all ages (P < 0.001) (Figure 2.9). The overall mean locomotor activity in adulthood was 25% lower in the PID group compared to controls (P < 0.001) (Figure 2.9, inset). In both PID and control groups, locomotor activity declined with age (P < 0.001).

2.4 Discussion

The purpose of this chapter was to document the characteristics of programming due to PID in a Wistar rat model. There were numerous findings in the present study. First, an extended maternal low iron treatment from 2 to 4 wk prior to and 2 wk following pregnancy, proved to be too severe to ensure survival of the offspring. Second, maternal ID up until delivery had a profound impact on pups’ iron status, as well as body and organ growth. Third, overall iron status and iron utilization differed between control and
Table 2.3. Physical characteristics of male control and PID offspring at 36 wk.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>PID</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit</td>
<td>0.47 ± 0.02</td>
<td>0.47 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>779.4 ± 17.8</td>
<td>677.2 ± 9.6</td>
<td>*</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>27.8 ± 0.2</td>
<td>26.8 ± 0.0</td>
<td>*</td>
</tr>
<tr>
<td>Waist Circumference. (cm)</td>
<td>24.2 ± 0.5</td>
<td>23.3 ± 0.19</td>
<td>NS</td>
</tr>
<tr>
<td>Omental fat (g)</td>
<td>2.93 ± 0.35</td>
<td>3.45 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td>Epididymal fat (g)</td>
<td>18.80 ± 0.89</td>
<td>19.20 ± 1.57</td>
<td>NS</td>
</tr>
<tr>
<td>Mesenteric fat (g)</td>
<td>15.95 ± 0.68</td>
<td>16.45 ± 0.77</td>
<td>NS</td>
</tr>
<tr>
<td>Retroperitoneal fat (g)</td>
<td>22.14 ± 1.39</td>
<td>22.07 ± 1.27</td>
<td>NS</td>
</tr>
<tr>
<td>Visceral Adipose Tissue (g)</td>
<td>59.81 ± 2.85</td>
<td>61.17 ± 2.02</td>
<td>NS</td>
</tr>
<tr>
<td>Visceral Adipose Tissue/Body Weight (%)</td>
<td>7.65 ± 0.22</td>
<td>9.01 ± 0.25</td>
<td>*</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>10.23 ± 0.29</td>
<td>9.47 ± 0.13</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data were obtained from Study 2. *P < 0.05. Visceral adipose tissue reflects combined omental, epididymal, mesenteric and retroperitoneal fat pads. Control: n = 5; PID: n = 3. PID, perinatal iron-deficient; NS, not significant.
Figure 2.9. Effect of PID on locomotor activity in male offspring from 12 wk to 35 wk. Data are summarized over the entire recording period (inset). Data were obtained in Study 2. Activity was assessed in rats’ home cages, using a radiotelemetry data acquisition system; see methods section for details. ***P < 0.001 compared to control at the same time interval. Control: n = 7; PID: n = 6. Note that the telemetry units were turned off between 21 and 22 wk to preserve battery life.
PID offspring during the neonatal period. Finally, PID impacts long-term locomotor activity, as well as visceral adipose tissue accumulation in adult offspring, suggesting potential long-term consequences on metabolic function. Taken together, these data provide insight into importance of iron during development, and suggest that inadequate iron delivery to the developing offspring impacts body and organ growth trajectories, which may have important implications for long-term health in these animals.

The finding that mothers from the iron restricted and control purified diet groups did not consume different quantities of food is important in characterizing an animal model of developmental insult. Indeed, there is substantial evidence that indicates either restricted or excess maternal intake of protein or energy content (fat and/or carbohydrate) may cause long-term programming effects of cardiometabolic function, and can have detrimental effects on cognition and behaviour. In the present model, since there were no differences in overall maternal consumption of the purified diet throughout the study, the long-term health consequences observed in these offspring can be ascribed primarily to a lack of iron during their development.

The purified diet used in the present studies was based on the AIN-93G diet, which has been shown to contain the necessary nutritional components to optimize growth and development. However, the standard iron content in this diet is only 48 mg/kg. Studies have shown that animals fed 50 mg/kg do not have maximum storage iron levels, and a level of between 50 mg and 250 mg was required. In the present studies, we used a control diet that consisted of 225 mg/kg, to ensure control rats had sufficient iron for optimal growth and delivery. Although ferric iron was used in the
purified diets (which is known to be less bioavailable than ferrous iron salts), ferric-citrate has been shown to be more readily taken up than other ferric salts, such as ferric orthophosphate.

In Study 1, we compared this control diet to a standard grain-based rodent chow on its ability to provide the necessary iron to the pregnant dams. Although the dams fed the non-purified control diet consumed more food than dams fed the purified control diet, the pups from these two groups had no differences in hematological indices of iron status, liver iron content, birth weights, or organ weights (see Chapter 5). It is therefore unlikely that the differences in quantities of food consumed contributed to any long-term programming effects; rather, it is more likely that the non-purified rodent diet was less efficiently metabolized by these animals. This hypothesis is consistent with our observation that these animals produced more feces than the both groups fed the purified diets. It was concluded that the purified diet with 225 mg/kg ferric citrate is an ideal control diet for use in these studies. In the interest of simplicity, the non-purified control group was excluded from Study 2.

In the pilot study conducted prior to Studies 1 and 2, dams were fed the low iron diet until PD 12, with the intention of restricting iron during the entire period of kidney and brain growth and differentiation (both of which continue until approximately the second postnatal week in the rat). However, these treatment regimens proved to be too severe to ensure survival of the ID pups. The majority of pups in the 4 wk pretreatment group were found dead in their cages within the first 8 h of birth. The 2 wk pretreatment group had improved survival in the immediate postnatal period, although the
majority of these animals were killed by their mothers in the ensuing three weeks. Consequently, in subsequent studies, only a 2 wk pretreatment period was used, and iron was restricted only during the gestational period; all dams were placed on the non-purified control diet within 8 h of giving birth. This treatment replenished maternal Ht and Hb levels within seven days, and dramatically improved offspring survival between PD 1 and PD 21.

Despite improving maternal indices of iron status, feeding the dams the control diet immediately after birth did little to improve Ht, Hb levels or liver iron status in the offspring. In fact, control offspring’s iron status steadily declined throughout the lactation phase. This may be due to the low amount of iron available in the dam’s milk, which is unlikely able to meet the demands of the offspring during this phase of early growth and development. Instead, the offspring likely derive the majority of their iron from the liver stores. The observation that control pups’ liver iron concentration and total liver iron content decreased dramatically between PD 1 and PD 7, and continued to drop until PD 14 is consistent with this notion. Thus, the increased iron mobilization from the liver likely minimized the decreases in the Hb levels so as to ensure tissue oxygen delivery. Indeed, neonatal animals, like adults, appear to prioritize iron utilization for Hb synthesis.

It is perplexing that, despite increasing body weights, the PID offspring did not show dramatic decreases in liver iron content or in hematological indices of iron status during the neonatal period, as in the case of the control pups. It was expected that the PID offspring would grow and the resultant hemodilution would cause further decreases
in Ht, Hb levels and liver iron. However, PID offspring show a proportionately greater increase in body weight between PD 7 and PD 14 compared to control offspring (16.4 ± 0.9 g to 39.0 ± 1.4 g (control) vs. 10.9 ± 1.1 g to 28.0 ± 1.9 g (PID)), and simultaneously increase total liver iron content as well as Ht. Although it has been reported that ID offspring consume more milk (per g body weight) during the lactation phase, it is unlikely that this would be sufficient to provide the necessary iron for the increased erythropoiesis associated with growth and volume expansion. It may be that PID offspring have adapted means of mobilizing iron from other sources (e.g. brain) to maintain Hb synthesis, although this hypothesis requires further investigation.

The degree of ID used in Studies 1 and 2 was sufficient to cause intrauterine growth restriction and impact organ growth. The organs that were affected by ID were heart and brain, both of which displayed higher weights relative to body weight compared to controls (Figure 2.5). The increased brain weights suggest a brain sparing effect, which has been extensively reported, since absolute brain weights were not different between groups, and brain weights are expected to correlate with body weight in both control and PID groups (Figure 2.7). The increased heart weights are suggestive of a cardiac hypertrophy, rather than a sparing effect; PID heart weights are larger than controls even when not normalized to body weight. These may reflect a hyperkinetic circulation, as a consequence of the ID. This is discussed in more detail in Chapter 3. The remaining organs tended to decrease over time, reflecting a proportionately greater increase in body weight than organ weight. Interestingly, spleen weights increased substantially within the first 7 days before following a similar trend of diminishing over
time. This may reflect the transition between fetal and normal Hb utilization, and an associated increased activity of the reticuloendothelial system scavenging fetal erythrocytes.

An examination of body and organ weights and their respective growth trajectories was performed in order to assess whether birth weight could be used as a surrogate marker of altered organ growth and development. Although birth weight has been described as a poor surrogate marker of altered growth trajectories (see sections 1.1.3.1), it has been used with relative success to predict the increased incidence of metabolic and cardiovascular complications in developmental programming models of maternal nutrient restriction. The ease by which it can be obtained compared to Ht (the latter requires killing the animal) makes it a particularly attractive as a potential surrogate for insult in this model of developmental programming. The finding that body weights vary with Ht only in the iron restricted offspring suggests that there may be a threshold Ht above which no decreases in body weight occur, and below which body weight is expected to drop in proportion to Ht. Alternatively, these data may indicate that Ht, independent of tissue iron levels, is not a determinant of body weights. Irrespective, the rather modest reductions in birth weight, and the degree of overlap between the control and PID offspring birth weights, in spite of the severity of anemia suggest birth weight is not a suitable indication of the degree of insult in this particular model of developmental programming.

The observation that Ht were positively correlated with kidney weights in the PID offspring is of interest, given that Lisle et al. have shown that PID impacts kidney
development and causes reductions in nephron endowment. Greater reductions in kidney weights would therefore be anticipated with a reduced nephron mass. In contrast, the observation that the correlation between Ht and kidney weight is negative in the control offspring is difficult to rationalize. It may be, in the case of the control offspring that reduction in Ht leads to increased kidney weights due to a hyperkinetic circulation, analogous to the situation with heart weights. In contrast, this trend may not have been observed on the PID animals because a lack of tissue iron in some way prevents compensatory growth. Nevertheless, based on analysis of organ growth trajectories, it is clear that ID impacts body weights and organ weights at birth. Whether these altered growth trajectories directly impact immediate or long-term function remains to be determined.

Despite the dramatic impact on body and organ weight growth patterns at birth and during lactation, these overt differences do not persist in adulthood. However, consistent with the “thrifty phenotype” hypothesis, PID results in developmental adaptations that predispose the offspring to increased visceral adiposity in adulthood. These results are of particular interest, because recent evidence suggests models of developmental programming may not accumulate visceral adipose tissue unless challenged with a high fat diet. This is because the chows fed to laboratory animals are specifically designed to minimize the development of obesity. Thus, the metabolic dysfunction does not become manifest until the system is challenged with high caloric or high fat intake. Herein, increased VAT was apparent even when animals were fed the standard grain-based chow, suggesting a profound alteration in energy metabolism.
The etiology of obesity, in particular VAT deposition, is multifactorial and complex.\textsuperscript{361, 362} The increased VAT in PID offspring is associated with reduced locomotor activity. This finding is corroborated by the results presented in Chapter 5, where adult male PID rats exhibited reduced exploratory behaviour when left in an open-field apparatus.\textsuperscript{363} Studies have also shown that PID results in decreased activity, albeit in young rats, over a short-term time course, in a novel environment, and in paradigms designed to determine exploration and anxiety-like behaviours.\textsuperscript{315} Our long-term, continuous assessment of activity suggests that PID programs the offspring to be less physically active throughout adulthood, thereby potentially contributing to their obese-phenotype. This observation is supported by clinical studies where VAT is greater among sedentary individuals, and a reduction in VAT is achieved with increased physical exercise (for review see Janiszewski \textit{et al.}\textsuperscript{364}).

In summary, the data obtained in the present series of experiments identify a complex scenario wherein iron restriction in early development can alter the trajectory of growth and development. Interestingly, control and PID rats vary substantially in their utilization of iron in the neonatal period, and this may underlie certain adaptive changes that impact metabolic and cardiovascular function in adulthood. Despite seeing no differences between males and females in growth restriction, organ weights or iron status, it is well established that the sexes differ in their susceptibilities to programming effects, including in response to PID.\textsuperscript{365} Although males were preferentially selected for long-term cardiovascular studies, the investigation of sex-differences may provide a new perspective into the mechanisms of PID-induced morbidities.
Chapter 3
The Long-Term Circulatory Consequences of Perinatal Iron Deficiency in Male Wistar Rats

(Hypertension 2008;51:151-159)

3.1 Introduction

Iron-deficiency ranks among the World Health Organization’s (WHO) top ten global health risks, and is considered a significant health risk in both developing and industrialized countries. This is not surprising given that the worldwide incidence of iron deficiency (ID) is estimated to be 66-80% (WHO, 2003). Although ID significantly affects all populations, the group most at risk is pregnant women. The enhanced risk profile in pregnancy is a consequence of increased erythropoiesis due blood volume expansion in the mother, and increased iron utilization from the growing fetal-placental unit. Overt iron-deficiency manifests as anemia in more than half of pregnant women in developing countries, and 20-40% of women in western countries.

Perinatal iron-deficiency (PID) can adversely impact the growth and development of the offspring, resulting in cardiovascular complications in later life. Specifically, studies in rats have shown that inadequate iron supply during early development can produce hypertension, even when iron levels are subsequently normalized. In fact, Lisle and colleagues demonstrated that PID produced both a nephron deficit and elevated blood pressure in adult offspring. Although the effects of PID on renal function was not investigated, the study by Lisle and colleagues implicates the developing kidney as a potential target for perinatal insult.
There is a large body of evidence that suggests that the kidney plays a critical role in establishing the long-term set point of arterial blood pressure, by modulating sodium and water excretion (and hence blood volume). The most compelling evidence for this hypothesis involves the transplantation of kidneys from hypertensive animals into normotensive animals, which confers upon the recipient the hypertensive phenotype. Furthermore, we have shown, using similar kidney-cross transplant experiments that pharmacological manipulations that persistently alter the renal vascular structure and function are sufficient to confer long-term changes in blood pressure, independent of changes in systemic vasculature. Thus, changes in renal vascular resistance properties, at least in part, are likely to play a crucial role in determining the set-point of blood pressure control.

Together, these studies provide a clear rationale for investigating the role of the kidney in the development of PID-induced hypertension. The objective of this study was threefold: (1) to determine the long-term effects of PID on the circulatory phenotype using direct measurements of blood pressure by radiotelemetry, (2) to determine the impact of PID on the intrinsic hemodynamic properties of the kidney, and (3) to assess renal function by characterizing changes in AP during low, normal and high sodium intake.
3.2 Methods and Materials

3.2.1 Animals and Treatments

The experimental protocols described herein were approved by the Queen’s University Animal Care Committee. Eighteen 6 wk-old female Wistar rats were purchased from Charles River (Saint-Constant, QC) and housed in the Queen’s University Animal Care Facility. Dams were housed in individual plastic cages with a stainless steel mesh covering, which held their food and water bottle. Rats had ad libitum access to food and water. The Animal Care Facility maintained a 12 h/12 h light/dark cycle and an ambient temperature of 23°C. Animals were allowed to acclimatize for one week prior to experimentation.

All purified diets were obtained from Research Diets Inc. (New-Brunswick, NJ). The diets used prior to and throughout gestation were based on the AIN-93G rodent diet, which has been described elsewhere. The control and low-iron diets were identical in composition, with the exception of the amount of added ferric citrate, which was adjusted to obtain the following iron concentrations: control diet: 225 mg/kg; low-iron diet: 3 mg/kg (no added ferric citrate); moderately low-iron diet: 10 mg/kg. The standard grain-based rodent chow (Lab Diet, St-Louis, MO) had an iron concentration of 270 mg/kg.

During the acclimatization period, all dams were placed on the purified control diet. Ten females were then randomly selected and placed on the low-iron diet, while the remaining eight females were maintained on the control diet. After two weeks on their respective diets, dams were bred naturally (i.e. without synchronization of estrus) to 9 wk old male Wistar rats fed the standard grain-based rodent chow. This was accomplished
by housing one male with each dam for 4 consecutive days; those that did not mate within this period were excluded from the study. Beginning at the time of mating, all dams in the low-iron group were then changed to the moderately low-iron diet. This was done to ensure that the dams in this group were not so iron-deficient as to compromise the survival of the offspring.\textsuperscript{369} At birth (postnatal day (PD) 0), all dams were placed on the grain-based rodent chow. At PD 21, the offspring were separated from their mothers, and weaned onto the grain-based rodent chow. Post-weaning food consumption and body weights were monitored twice weekly.

3.2.2 Tissue Collection and Analysis

Hematocrits (Ht) and hemoglobin (Hb) levels were measured weekly in the dams during the 2 wk period prior to conception and during the three week period between parturition and weaning. No measurements were taken throughout gestation to eliminate potential confounding factors associated with anaesthetizing pregnant animals. At 24 h postpartum, all litters were reduced to 10 males to standardize postnatal conditions; in litters that contained fewer than 10 males, the difference was made up with females. One control litter only consisted of a total of 9 pups and was thus excluded from the chronic study, although the data for this dam and litter are included in Table 3.1. At PD 7, PD 14 and PD 21, two animals per litter were sacrificed, with females again being preferentially selected. Ht, Hb levels, heart weights and kidney weights were obtained from culled pups. Hearts and kidneys were excised, rinsed in ice-cold saline, cleaned of extraneous connective tissue, blotted dry and weighed. Adult rats were anaesthetized under isoflurane, and blood was collected from a toe-clip. Blood samples were collected into
heparinized microcapillary tubes and subsequently centrifuged (1800 x g for 15 min), and packed cell volume was determined. A small blood sample (10-50 μL) was also collected into 250 volumes of Drabkin’s reagent (Sigma, St-Louis, MO), and hemoglobin content was determined by the cyanomethemoglobin method.339

3.2.3 Conscious Hemodynamic Assessments

Starting at 10 wk of age, mean arterial pressure (MAP) and systolic blood pressure (SBP) were continuously monitored in the offspring using radiotelemetry data acquisition (Data Sciences International, St. Paul, Minnesota), as previously described.370 Briefly, male Wistar rats were anesthetized with isoflurane (induction: 5%, maintenance: 2% in O2). A fluid filled telemetric catheter was introduced into the lower abdominal aorta, such that it was positioned approximately 1 cm below the left renal artery. The body of the transducer was sutured to the muscular layer of the abdominal wall to prevent device movement. All animals were allowed to recover for 10 days before recording blood pressures. Animals were housed in individual cages that were placed on Model RPC-1 receivers (Data Sciences International, St-Paul, MN), which convert the radio telemetric waveform into a digital signal. This information was then transmitted via a BCM100 consolidation matrix to a computer based Dataquest IV acquisition system (version 3.1; Data Sciences International) located in an adjacent room. DBP, MAP, SBP and HR measurements were recorded for 15 s every 5 min for each animal. The daily hemodynamic measurements presented in this study is a mean of all data points collected between 2100 h and 0400 h (72 total recordings per day); this period corresponds to the 6 h interim period of the night cycle in the animal care facility at Queen’s University,
which cycles from night to day and vice versa at 0700 h and 1900 h, respectively. Night-time measurements were used in this study because this time-period corresponds to the active component of the rats’ activity cycle. Following the study, the radiotelemetric transducers were surgically removed and verified for accuracy. This was accomplished by calibrating the transducers to a standard pressure to calculate offset. Baseline hemodynamic recordings and subsequent sodium challenge measurements began immediately after the 10 day recovery from implantation surgery, to ensure minimal drift in the transmitter recordings.

For the sodium challenge experiments, blood pressure from rats (13 wk of age) implanted with radiotelemetric transducers were recorded at baseline levels (normal sodium intake) for 5 days, placed on a low sodium treatment regimen for 5 days, and were subsequently placed on a high-sodium treatment regimen for 5 days. The low sodium regimen consisted of \textit{ad libitum} access to a low sodium (0.04% Na\textsuperscript{+}) purified diet (Research Diets Inc. New Brunswick, NJ), based on the AIN-76A rodent diet, as well as tap water. The high salt treatment regimen consisted of \textit{ad libitum} access to the standard grain-based rodent chow described above (0.4% Na\textsuperscript{+}), as well as drinking water supplemented with 1% NaCl (w/v). The normal salt treatment consisted of the grain-based rodent chow and tap water. Body weights, as well as food and water intake were monitored daily during these treatments.

3.2.4 \textit{In Vivo} Assessment of Renal Vascular Properties

Intrarenal hemodynamic assessments were performed in anesthetized 10 wk old male PID and control offspring, based on a method previously described.\textsuperscript{371} Briefly,
young adult rats (10-13 wk old) were anaesthetized with ketamine (30 mg/kg body weight, i.p.; Rogar/STB, London, ON) and Inactin (thiobarbital sodium; 100 mg/kg body weight, i.p.; Sigma, St-Louis, MO). Body temperature was monitored using a thermistor (model 402; Yellow Springs Instruments, Yellow Springs, OH) and maintained at 37 ± 0.5°C using a temperature controller (model 73A; Yellow Springs Instruments) coupled to a heat lamp, and a warming pad. After tracheotomy (PE-240 tubing), a steady stream of 95% O₂/5% CO₂ was provided. A midline abdominal incision was made, and the right kidney was removed. A catheter was introduced into the inferior vena cava (at the level of the right iliolumbar vein; secured with cyanoacrylate glue) for continuous infusion of saline at 33µL·min⁻¹·100g body wt⁻¹ via a syringe pump (KDS220, Fisher Scientific, Ottawa, ON). Ligatures were tied around the branches of the left renal and celiac arteries. The superior mesenteric artery was cannulated with PE-50 tubing to determine AP continuously as a direct index of renal artery perfusion pressures (RAP). Silastic® balloon cuffs were placed around the abdominal aorta between the celiac trunk and superior mesenteric arteries and around the aorta distal to the left renal artery. The balloon cuffs allowed for the manipulation and control of RAP over a wide range of pressures. An electrocautery (Bovie, World Precision Instruments, Inc.) was used to make a small hole (~3 mm in length) in the longitudinal axis of the left kidney for insertion of a catheter for renal interstitial hydrostatic pressure (RIHP) measurements. The RIHP catheter was made of PE-50 tubing (outer diameter of 0.965 mm) fitted with 1.5 mm long polyethylene matrix (15-45 μm pore size; Porex, Fairburn, GA), and was secured with a drop of cyanoacrylate glue. RAP and RIHP were monitored via pressure
transducers (model CDX3, Cobe) connected to a PowerLab/8s (ADInstruments, Colorado Springs, CO) data acquisition system. A 15-20 min period was given for equilibration and to obtain RAP and RIHP baselines after completion of the surgery. The RAP-RIHP relationship was then determined via manipulations of RAP (<10 s) using the Silastic® cuffs. These acute manipulations of RAP resulted in a reliable and stable change in RIHP, consistent with previous studies. Upon completion of the experiment, Ht were measured and the rats were killed via excision of the heart, which was then weighted. The kidney was removed, cut in half, and the location of the RIHP catheter was confirmed to be at the corticomedullary junction in all animals.

3.2.5 Statistical Analyses

Neonatal offspring Ht, Hb levels, and organ weights from each litter were averaged and mean values for litters in each experimental group were calculated and presented as a single value. Neonatal male and female data were analyzed separately, and then pooled when no gender-differences were observed. Female offspring data were not included beyond PD 21. All information pertaining to the dams and offspring intrarenal hemodynamic parameters were compared using a Student’s t test. All time-dependent measurements (MAP and HR data obtained by radiotelemetry) were analyzed using a repeated measures 2-way analysis of variance (ANOVA); renal function curves were analyzed by 2-way ANOVA; when significance was found, 1-way ANOVA with Newman-Keuls post-hoc test or Student’s t test was conducted on data sets, as appropriate. For intrarenal hemodynamic assessments, linear regression analysis was performed by the ordinary least-products method to calculate the relationship between
RIHP and RAP for each manipulation in each animal. RAP-RIHP slopes between groups were compared by unpaired Student’s t test. For the sodium challenge experiments, ‘normal sodium’ MAP values were calculated as a 5-day average. High and low sodium MAP values represent highest and lowest 1-day average MAP measurement within the corresponding treatment period, respectively. Grubb’s test was conducted on data sets to determine statistical outliers. All data are presented as mean ± SEM.

3.3 Results

3.3.1 Maternal and Offspring Outcomes

Food consumption during the pre-gestational treatment period and throughout pregnancy was not different between the control and ID dams (data not shown). A summary of hematological values and pregnancy outcomes pertaining to the dams is presented in Table 3.1. Weight-gain before and throughout pregnancy (normalized to the number of pups born) was not affected by the low-iron treatment. Additionally, there were no differences between groups in the number of pups born per litter or the proportion of males and females. There were however clear signs of negative impact in the PID group. For example, one litter in the PID group did not survive 24 h. Additionally, there were 2 deaths in 2 separate PID litters within the first 14 days. Tissues from these animals were excluded from subsequent analysis. In contrast, there were no perinatal deaths in the control group.

During the two week treatment period prior to conception, dams fed the low-iron diet had a modest decrease in Ht (93% of control; P < 0.05) and Hb levels (91% of
control; P < 0.05) (Table 3.1). Ht and Hb levels fell to less than 75% (P < 0.001) of controls 24 h after parturition but had returned to control levels within 7 days. Conversely, Ht and Hb levels in pups of iron deficient dams were 60% (P < 0.001) and 59% (P < 0.001) of control values at birth respectively, and remained decreased until after PD 14 (Table 3.2). At PD 21, Ht in the PID offspring remained more than 10% below controls (P < 0.05), but Hb levels were no longer depressed. Control pups, but not PID pups, had decreases in Ht and Hb levels after birth (P < 0.01 at all times compared to PD 1).

Body weights of offspring in the PID group were more than 10% lower than control throughout the study period (P < 0.05) (Figure 3.1A). Following a marked decrease in relative body weight during the first post-natal week (Figure 3.1B), PID pups underwent two periods of ‘catch-up’ growth (when absolute weight gain was greater in the PID group); one period during the pre-weaning (PD 10-PD 21) phase and one period during the post-weaning phase (beyond PD 24). Heart weights (normalized to body weight) were 29% higher in the PID offspring at PD 1 compared to control offspring (P < 0.01) (Table 3.2). These differences persisted until PD 21. There were no observed differences in kidney weights (normalized to body weight) between the control and PID offspring between PD 1 and PD 21 (Table 3.2).

3.3.2 Systemic and Intrarenal Hemodynamic Outcomes

Arterial pressures, expressed as SBP, MAP, starting at approximately 11 wk of age (following a 10-day recovery period after surgery), were moderately but consistently
Table 3.1. Summary of weights, hematological indices of iron status and pregnancy outcomes in control and iron-deficient dams.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Iron-Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (7 wk) (g)</td>
<td>195.6 ± 3.9</td>
<td>188.0 ± 3.1</td>
</tr>
<tr>
<td>BW gain (pre-pregnancy) (g)</td>
<td>60.7 ± 4.3</td>
<td>58.3 ± 2.8</td>
</tr>
<tr>
<td>BW gain (pregnancy) (g/pup)</td>
<td>6.7 ± 0.4</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>Pups per litter</td>
<td>15.1 ± 1.9</td>
<td>16.1 ± 0.9</td>
</tr>
<tr>
<td>Percentage of male pups</td>
<td>46.8 ± 3.0</td>
<td>46.4 ± 3.0</td>
</tr>
<tr>
<td>Ht (pre-pregnancy)</td>
<td>0.47 ± 0.01</td>
<td>0.44 ± 0.01*</td>
</tr>
<tr>
<td>Hb (pre-pregnancy) (g/L)</td>
<td>134 ± 5</td>
<td>121 ± 3*</td>
</tr>
<tr>
<td>Ht (1 day after parturition)</td>
<td>0.41 ± 0.01</td>
<td>0.30 ± 0.01**</td>
</tr>
<tr>
<td>Hb (1 day after parturition) (g/L)</td>
<td>114 ± 5</td>
<td>82 ± 5***</td>
</tr>
<tr>
<td>Ht (7 days after parturition)</td>
<td>0.47 ± 0.01</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>Hb (7 days after parturition) (g/L)</td>
<td>123 ± 7</td>
<td>128 ± 3</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 compared to control values. Control: n = 7; PID: n = 9. BW, body weight; Ht, hematocrit; Hb, hemoglobin.
Table 3.2. Control and PID offspring information at PD 1, 7, 14, and 21.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PD 1</th>
<th>PD 7</th>
<th>PD 14</th>
<th>PD 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Ht</td>
<td>0.39 ± 0.01</td>
<td>0.33 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>PID Ht</td>
<td>0.23 ± 0.01***</td>
<td>0.21 ± 0.01**</td>
<td>0.25 ± 0.01**</td>
<td>0.27 ± 0.01*</td>
</tr>
<tr>
<td>Control Hb (g/L)</td>
<td>106 ± 3</td>
<td>80 ± 3</td>
<td>71 ± 2</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>PID Hb (g/L)</td>
<td>62 ± 3***</td>
<td>54 ± 2***</td>
<td>56 ± 2***</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>Control Body Weight (g)</td>
<td>6.1 ± 0.1</td>
<td>16.4 ± 0.9</td>
<td>39.0 ± 1.4</td>
<td>67.8 ± 1.7</td>
</tr>
<tr>
<td>PID Body Weight (g)</td>
<td>5.4 ± 0.3</td>
<td>10.9 ± 1.1</td>
<td>28.0 ± 1.9</td>
<td>52.9 ± 3.0</td>
</tr>
<tr>
<td>Control Heart Weight/BW (mg/kg)</td>
<td>5.7 ± 0.2</td>
<td>6.5 ± 0.3</td>
<td>4.9 ± 0.1</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>PID Heart Weight/BW (mg/kg)</td>
<td>7.4 ± 0.3**</td>
<td>8.7 ± 0.4***</td>
<td>6.4 ± 0.2***</td>
<td>5.6 ± 0.2**</td>
</tr>
<tr>
<td>Control Kidney Weight/BW (mg/kg)</td>
<td>5.1 ± 0.2</td>
<td>6.0 ± 0.2</td>
<td>5.1 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>PID Kidney Weight/BW (mg/kg)</td>
<td>5.1 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>5.4 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001 compared to control values at same postnatal day. Data reflect predominantly female offspring values. Control: n = 4-7; PID: n = 6-8. PD, postnatal day; PID, perinatal iron deficient; Ht, Hematocrit; Hb, hemoglobin; BW, body weight.
Figure 3.1. Effect of PID on offspring body weights, expressed as (A) absolute weights, and (B) percentage of control body weight. *P < 0.05 compared to controls at the same postnatal week. Control: n = 7; PID: n = 9.
elevated in the PID group compared to controls over a 10-day period (Figure 3.2). DBP, as well as HR, were not significantly different between treatment groups. The mean six-hour values for the control and PID animals, respectively, over the 10-day period were: MAP: 105.8 ± 0.8 mmHg (control) vs. 110.7 ± 1.5 mmHg (PID) (P < 0.05); SBP: 124.0 ± 0.7 mmHg (control) vs. 129.3 ± 2.0 mmHg (PID) (P < 0.05); DBP: 93.1 ± 1.4 mmHg (control) vs. 96.6 ± 2.0 (PID); HR: 405.6 ± 5.8 bpm (control) vs. 410.4 ± 8.2 bpm (PID).

A summary of intrarenal haemodynamic parameters assessed in control and PID animals at approximately 10 wk of age is presented in Table 3.3. The mean renal arterial pressure (RAP) in the PID group under anesthesia was found to be approximately 12 mmHg higher than controls (P < 0.05). Consistent with the elevated pressure, left ventricular weights (normalized to body weight) were 10.4% larger in the PID group compared to controls (P < 0.05); right ventricular weights (normalized to body weight) were not statistically different between groups. Despite the increased RAP, the resting mean RIHP was not different. In contrast, the slope of the overall $\Delta$RAP-$\Delta$RIHP relationship (Figure 3.3) was blunted by 41% in the PID group (0.062 ± 0.005; $r^2 = 0.87$) compared to controls (0.10 ± 0.002; $r^2 = 0.95$) (P < 0.01). Likewise, assessment of the slope of the $\Delta$RAP-$\Delta$RIHP relationship between the more physiologically relevant RAP interval of -25 to 25 mmHg revealed a similar blunting of 45.5% in the PID animals (0.054 ± 0.006; $r^2 = 0.76$) compared to controls (0.099 ± 0.010; $r^2 = 0.80$) (P< 0.01) (data not shown). The slope of the RAP-RIHP relationship, when not normalized to baseline pressures, was 24% blunted in the PID offspring (0.098 ± 0.004; $r^2 = 0.85$) compared to controls (0.075 ± 0.005; $r^2 = 0.73$) (P < 0.01) (Figure 3.3B).
Figure 3.2. Effect of PID on (A) systolic blood pressure (SBP), mean arterial pressure (MAP), and diastolic blood pressure (DBP), and (B) heart rate (HR), as assessed by radiotelemetry at 12 weeks. *P<0.05 over the 10-day period. Control: n = 9; PID: n = 11.
Table 3.3. Physical and renal hemodynamics properties of control and PID offspring at 10 weeks.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>PID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>486.0 ± 18.0</td>
<td>396.4 ± 16.5**</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.54 ± 0.02</td>
<td>0.50 ± 0.01</td>
</tr>
<tr>
<td>Left Ventricular Weight/BW (g/kg)</td>
<td>1.70 ± 0.052</td>
<td>1.95 ± 0.10*</td>
</tr>
<tr>
<td>Right Ventricular Weight/BW (g/kg)</td>
<td>0.55 ± 0.05</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Kidney Weight/BW (g/kg)</td>
<td>3.88 ± 0.13</td>
<td>3.70 ± 0.30</td>
</tr>
<tr>
<td>Baseline RAP (mmHg)</td>
<td>102.1 ± 3.5</td>
<td>113.9 ± 3.4*</td>
</tr>
<tr>
<td>Baseline RIHP (mmHg)</td>
<td>6.96 ± 0.30</td>
<td>5.90 ± 0.55</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01 compared to control values. Control: n = 5; PID: n = 5. PID, perinatal iron-deficient; BW, body weight; RAP, renal arterial pressure; RIHP, renal interstitial hydrostatic pressure.
Figure 3.3. Effect of PID on the relationship between the change in renal interstitial hydrostatic pressure (RIHP) and the change renal arterial pressure (RAP) in 10 wk-old male offspring, expressed as (A) relative changes from baseline, and (B) absolute RAP and RIHP measurements. ***P < 0.001 for PID slope compared to control slope. Circles represent individual manipulations made on each animal (n = 5 in each group).
In Figure 3.4, analysis of the MAP profiles in response to changes in dietary salt (normal, 5 day low and 5 day high) revealed that the PID animals were more responsive to the extremes of dietary sodium (P < 0.05). In particular, the greatest differences was in the transition from normal salt to low salt intake, where MAP changes were nearly two-fold greater in PID animals compared to controls (4.3 vs. 8.5 mmHg; P < 0.05), and the slope of the sodium-intake-MAP relationship was blunted by 24% in the low to normal sodium portion (P < 0.05).

### 3.4 Discussion

The major findings in the offspring following the maternal iron restriction intervention during pregnancy include: (1) severe decreases in hematological indices, (2) marked cardiac hypertrophy, (3) a moderate but persistent elevation in AP, (4) alterations in the hemodynamic properties of the kidney, and (5) an increased sensitivity of AP to changes in dietary sodium intake. These findings suggest that iron-deficiency during periods of growth and development has a detrimental impact on circulatory function that persists in adulthood and which may, at least in part, be mediated by changes in renal function.

The treatment paradigm adopted in this study was one in which iron–deficiency was induced primarily during the gestational period, as the dams were placed on an iron-replete diet immediately after giving birth, allowing them to recover Hb levels and Ht within 7 days (Figure 3.1). With this approach, we avoided confounding factors
Figure 3.4. (A) Effect of PID on (A) mean arterial pressure (MAP) responsiveness to normal sodium (NS), low sodium (LS) and high sodium (HS) intake in 15 wk-old male offspring. Dotted lines represent transitions to the various sodium treatments. Inset: Summarized changes in MAP during altered sodium intake. ‘Total’ represents the MAP change from low to high sodium treatments; low sodium (LS) represents the MAP change from normal to low sodium treatments; high sodium (HS) represents the MAP change from normal to high sodium treatments. (B) In vivo renal function curves obtained from data in (A). *P < 0.05 compared to control on same treatment; †P < 0.05 for PID slope between LS and NS compared to control slope in the same interval. Control: n = 6-8; PID: n = 7.
associated with continued anemia in the mothers during the nursing phase. Specifically, milk production in ID dams appears to be adversely affected with respect to iron, energy and fat content.\textsuperscript{357} Despite feeding the ID dams an iron-replete diet after birth, their pups remained anemic for the entire fostering phase. This is consistent with previous reports that rat milk is low in iron content, even in mothers with normal iron status.\textsuperscript{374} Indeed, Ht and Hb levels in control animals steadily decreased throughout lactation, suggesting diminished iron supply during the fostering phase in these animals as well. It may be that progressive iron-deficiency in the control offspring in the immediate postnatal period is part of the natural pattern of development, although the mechanisms have not been investigated. In the present studies, although the magnitude of the iron-deficiency was greater in the PID neonates, the specific impact of this period of development remains to be elucidated.

The enlarged hearts in the PID animals during the neonatal period is consistent with reports by others,\textsuperscript{213-215,375} and may be an adaptive response to anemia during gestation and the neonatal periods. Indeed, fetal anemia has been shown to increase heart weight and cardiac output in sheep.\textsuperscript{376} In the present study, the increased cardiac weight (which may result from hyperplastic and/or hypertrophic cardiomyocyte growth) may be linked to increases in cardiac output; a circulatory change that would facilitate perfusion of fetal tissues during development. As suggested by Lewis \textit{et al.},\textsuperscript{375} this adaptation would be expected to limit the generation of hypoxia.

Consistent with many models of fetal programming, the PID offspring had lower birth weights than controls. Interestingly, the PID pups underwent two periods of ‘catch-
up growth’—one during the pre-weaning phase and one in the post-weaning phase (Figure 3.1B). These periods of ‘catch-up growth’ in the PID offspring have been proposed to be an important predisposing factor for long-term cardiovascular disease associated with fetal programming.214 However, similar iron-deficiency induced-fetal programming effects have been reported by others26,213 in the absence of this ‘catch-up growth’ phase. Regardless, it is clear that there is decreased growth in the PID animals during the first two weeks (when renal maturation is completed), and this may have further adversely affected the circulation. As indicated above, the precise role of these postnatal changes is presently unresolved.

The finding, using radiotelemetry, that AP was elevated in the adult offspring following PID confirms previous results in which SBP was assessed using the indirect tail-cuff method.26,27,213-215 These results are corroborated by the presence of left ventricular hypertrophy, but not right ventricular hypertrophy, in the 10 wk old animals; an adaptive response normally associated with arterial hypertension. The magnitude of the increase in AP found in the adult PID offspring is modest compared to those observed by other investigators, who reported increases in the adult offspring between 18 mmHg27,213 and 30 mmHg26 using the indirect tail-cuff method. These discrepancies may be due to a number of factors, including (1) differences in timing and degree of ID in the mothers and offspring,369 (2) strain-specific differences377 (e.g. Rowett Hooded-Lister,213 Sprague Dawley,214 and Wistar,26,27,215), and most importantly, (3) systolic blood pressure measured via the tail-cuff methodology is affected by restraint and thermal stress.326 Indeed, it may be that programmed animals are more responsive to such
stressors compared to non-programmed animals.\textsuperscript{327, 378, 379} In light of this evidence, the current validation of this cardiovascular phenotype in this model of programming using direct conscious, chronic determinations of AP is an important foundation for future studies.

The key finding that PID altered the intrarenal hemodynamic properties—namely the RAP-RIHP relationship—may explain, in part, the long-term elevations in AP observed in these animals. The kidney is fundamental in establishing the set point of long-term AP by regulating sodium and fluid balance.\textsuperscript{118} Fluctuations in AP around the long-term level induce changes in perfusion of the poorly-autoregulated medullary vessels and consequently cause changes in RIHP, which ultimately influence the set-point of AP at which sodium and water balance occur.\textsuperscript{372, 380} That is, a decrease in the responsiveness and set-point of the RAP-RIHP relationship can impact the pressure-natriuresis mechanism, such that greater changes in AP are required to generate corresponding changes in RIHP to regain sodium and water balance.\textsuperscript{381}

The blunting of the RAP-RIHP relationship may also explain, in part, the altered responsiveness in handling low and high sodium intake in the PID animals. As depicted in the dietary sodium-MAP relationship (Figure 3.4B), control offspring will increase sodium and fluid excretion in response to minor changes in AP.\textsuperscript{382} However, adult PID offspring would require greater changes in MAP to regain sodium balance. Indeed, in other rodent models in which blood pressure is salt-sensitive (e.g. neonatal RAS-inhibited rat, SHR, Dahl salt-sensitive rat, ANP/- mouse), a similar blunting of the RAP-RIHP relationship is observed.\textsuperscript{151, 199, 383, 384}
Although the specific mechanisms by which PID adversely impacts intrarenal hemodynamics and kidney function are beyond the scope of this study, decreased responsiveness of RIHP to changes in RAP has previously been linked to alterations in renal interstitial compliance as well as changes in the medullary circulation.\textsuperscript{146,385} PID could potentially affect the development of the renal interstitium and medullary vessels during development via changes in overall growth, nephron endowment, development of the renal tubules and associated vasculature, expression of tubular transporters (e.g. Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, Na\textsuperscript{+}-H\textsuperscript{+} co-transporter), modified renin-angiotensin system activity, and changes in expression and activities of vasoactive species (e.g. NOS, sGC, 20-HETE, etc.). Indeed, as iron is an integral component of numerous signaling and effector molecules, it’s likely that the etiology of the adverse programming effects observed in the present study is multifaceted and complex.

3.5 Perspectives

The adverse programming effects of iron-deficiency, solely during the perinatal period, on the long-term circulatory phenotype further demonstrate the importance of the developmental origins of health and disease. The concept emphasized by the present study is that subtle changes in the status of maternal nutrition during pregnancy can influence the long-term health of the offspring in postnatal life. Although programming effects have been associated with a number of macro and micronutrient deficiencies, given the worldwide prevalence of ID as well as its propensity to afflict pregnant women, it may represent an especially important risk factor for long-term cardiovascular disease.
4.1 Introduction

Evidence obtained in the last 2 decades indicates that stressors of a maternal or placental origin can have profound effects on the developing fetus and neonate. The organism may undergo a number of metabolic, cardiovascular, and neuroendocrine changes in response to stressors, which impact on health status in adulthood. These adaptive changes are said to be programmed, because they persist long after the original stimulus.\(^52,334\) Iron deficiency (ID) during gestation has been shown to induce a number of long-term deleterious effects on the fetus.\(^386-390\) As a stimulus for developmental programming, ID may be particularly relevant because of its tendency to affect pregnant women,\(^274,275\) and newborn children.\(^391\) Moreover, it is the only nutritional deficiency that has a significant prevalence in both affluent and developing countries,\(^285\) making this model of perinatal programming a global health concern.

We have previously reported that perinatal ID (PID) can induce permanent changes in the regulation of arterial pressure (AP). Specifically, we observed subtle, albeit persistent increases in AP, as well as exaggerated AP responses in response to changes in sodium intake.\(^392\) Subsequent analyses revealed that these persistent cardiovascular changes were associated with alterations in intrarenal hemodynamics,\(^392\) providing the first evidence that the cardiovascular dysregulation associated with PID
may be due to suboptimal transmission of changes in AP to changes in medullary blood flow. Despite this evidence, the underlying mechanisms leading to changes in renal control of AP have not been fully described.

Iron, particularly as a component of the heme molecule, is an important regulatory cofactor of numerous enzymes. In the cardiovascular system, two important heme containing enzymes are nitric oxide synthase (NOS) and its downstream mediator soluble guanylyl cyclase (sGC). NOS is responsible for the catalytic conversion of L-arginine and molecular oxygen to L-citrulline and nitric oxide (NO). NO is known to play an important role in mediating vascular tone, and hence AP, by binding to activating sGC, although sGC-independent mechanisms are also known. Substantial evidence indicates that NO and sGC are also important in modulating the intrinsic pressure-natriuresis system, the key determinant of long-term arterial blood pressure control. Indeed, interference with intrarenal NOS activity using inhibitors such as L-NAME and L-NMMA blunts the pressure-natriuresis relationship, and impacts on the organisms’ ability to efficiently excrete sodium and water; these results were observed during both acute and chronic treatment with NOS inhibitors. Granger et al. have shown that increases in medullary blood flow and the resultant changes in renal interstitial hydrostatic pressure are also highly dependent on NOS activity.

Collectively, these studies have led us to hypothesize that that intrinsic reduction in renal NOS activity could be responsible, at least in part, for the altered intrarenal hemodynamics and altered pressure-natriuresis observed in adult PID offspring. Thus, the purpose of Study 1 was to examine NOS and sGC activity in renal tissues as well as
test vascular responses to endothelium dependent and independent vasodilators from tissues of adult PID offspring that were previously reported to have cardiovascular dysregulation.

To gain insights into the possible mechanisms by which ID could impact long-term cardiovascular function, in a separate series of experiments, in vivo hemodynamics assessments and NO signaling were examined in adult rats made severely iron deficient. Although various effects of PID have been reported, the impact of acute ID on cardiovascular function has not been adequately characterized. Given the importance of NO signaling in regulating vascular tone and renal function, we hypothesized that diminished iron supply for heme synthesis would decrease the body’s capacity to produce NO, and thus cause cardiovascular dysregulation. If these effects occurred early in life, this could represent a potential mechanism by which PID permanently alters cardiovascular regulation. The purpose of the Study 2 was to determine whether severe ID could alter hemoenzyme activity, in particular renal and vascular NOS and sGC activity, and impact cardiovascular regulation in adult male rats.

4.2 Methods and Materials

4.2.1 Animals and Treatments

The methods described herein were approved by the Queen’s University Animal Care Committee. Wistar rats were purchased from Charles River (Saint-Constant, QC), and housed individually in the Animal Care Facility at Queen’s University. Rats were given 1 wk to acclimatize to their surroundings before treatments began. The purified
diets used in the present studies were obtained from Research Diets Inc. (New Brunswick, NJ). The purified diets were based on the AIN-93G diet and were identical in composition, with the exception of added ferric citrate, which was adjusted to obtain the following concentrations of iron: the low iron diet contained of 3 mg/kg Fe; the moderately low iron diet contained 10 mg/kg Fe, and the control contained 225 mg/kg Fe. The non-purified, grain-based diet was obtained from Lab Diets Inc. (St-Louis, MO), and it contained 270 mg/kg Fe. All rats had ad libitum access to food and water.

For Study 1, animals were treated as described in Chapter 3. Briefly, 18 female Wistar rats, aged 7 wk, were fed either the purified low-iron diet (n = 10) or the purified control diet (n = 8) for 2 wk prior to mating with age-matched, non-purified control diet-fed male Wistar rats. After mating, the group initially fed the low-iron diet was fed the moderately low-iron diet to ensure the pregnant dams did not become too severely ID that survival of the pups was compromised. Twenty-four hours after giving birth, litters were reduced to 10 pups to standardize postnatal conditions. At each of the postnatal days 7, 14, and 21, an additional 2 pups were selected for culling, and tissues were collected as described below. Female pups were preferentially selected over males during the neonatal period to maximize the number of males available for future studies.

For Study 2, 14 Wistar rats, aged 7-wk, were fed either the purified low-iron diet (n = 7) or the purified control diet (n = 7) for 5 wk. Once a week, a small volume of blood was collected from a toe-clip to assess Ht and Hb levels, as described below. After 5 wk on their respective diets, the rats were killed, and organs were collected.
4.2.2 Tissue Collection and Analysis

For Studies 1 and 2, blood was collected by two methods. For non-terminal surgeries (i.e. weekly monitoring of rats’ iron status), rats were anesthetized isoflurane (inhaled), and blood was obtained from a toe clip. Blood was collected into a microcapillary tube, and centrifuged at 1800 x g, and packed cell volume was determined. Additionally, a small volume of blood was collected into 250 volumes of Drabkin’s reagent (Sigma, St-Louis, MO), and Hb levels were assessed spectrophotometrically, as previously described.339 For terminal surgeries (i.e. during organ collection) adult rats were anesthetized with sodium pentobarbital (120 mg·kg\(^{-1}\) i.p.). Blood was collected from the vena cava into both EDTA and silicon-coated Vacutainer® tubes (BD Scientific, Oakville, ON), and sent to Kingston General Hospital for analysis (using a Sysmex XE Roche HST analyzer; Roche Diagnostics, Mississauga, ON).

Organs were collected as described in Chapter 3. Briefly, after blood collection, adult rats were injected with 100 units of heparin. A section of the thoracic aorta was then removed for testing in an isolated tissue bath. Rats were then perfused through the descending abdominal aorta with ice-cold saline using a peristaltic pump (90 mL·min\(^{-1}\)). Kidneys, aortas, and livers were then removed, rinsed and cleaned of extraneous connective tissue in ice-cold saline, frozen in liquid nitrogen and stored at -80°C until analysis. Liver iron analysis was performed as described in Chapter 5. In Study 1, neonatal pups were killed by decapitation, and organs were collected as described in Chapter 3.
Tissue homogenates for the NOS and sGC activity assay were prepared as previously described. Briefly, kidneys were separated into cortical and medullary fractions and were homogenized in 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA, 21 μM leupeptin and 1 mM dithiothreitol. Tissues were homogenized on ice using a sonic dismembrator (Fisher Scientific, Ottawa, ON) at an approximate power output of 15W for 2 min. Homogenates were then centrifuged at 1800 x g for 20 min at 4°C to remove cell debris, and half of this sample was frozen in liquid nitrogen, and stored at -80°C. The remaining homogenate was then centrifuged at 100,000 x g for 60 min at 4°C. The supernatant was then frozen and stored at -80°C. Prior to activity assays, an aliquot was thawed, and protein content was assessed by the Biuret method.

4.2.3 Cardiovascular Assessments

Cardiovascular assessments were made by radiotelemetry, as described in Chapter 3. The impact of acute ID on hemodynamics was previously reported. See statistical methods section for details pertaining to analysis.

4.2.4 Isolated Tissue Bath Assessments

Isolated aortas were used to evaluate concentration-dependent effects of phenylephrine (PE), acetylcholine (ACh), and diethylamine NONOate (DEA-NONOate), as previously described (Appendix 1). Briefly, thoracic aortas were cut into rings 4 mm in width and mounted in 10 mL tissue baths, containing Krebs’ bicarbonate solution aerated with 95% O₂-5% CO₂ at 37°C (see Hussain et al. for details). Data were obtained using a Powerlab® data acquisition system (ADInstruments) and displayed
using Chart (version 5) software. Tissues were allowed to equilibrate at a resting tension of 9.8 mN for one hour, with rinses every 15 min, prior to testing. Cumulative concentration-response relationships were obtained for phenylephrine (PE), acetylcholine (ACh) and diethylamine NONOate (DEA-NONOate) by adding increasing doses to the bath. In the case of ACh and DEA-NONOate, the aortic rings were pre-contracted (50-80% of maximal contraction) with PE prior to administration of vasodilators. Tissues were rinsed and allowed to return to baseline over a 30 min period between PE, ACh and DEA-NONOate testing.

4.2.5 NOS and sGC Activity Assays

Tissue homogenates were assayed for NOS activity by the conversion of radiolabeled L-Arginine to L-Citrulline, as previously described. Briefly, kidney homogenates (0.5 mg/mL) were preincubated for 10 min in 50 mM HEPES (pH 7.4) containing 1 mM EDTA, 2.0 mM NADPH, and 1.25 mM CaCl₂, after which time 30 μL of 0.18 mM 14C-L-Arginine solution (0.063 μCi·mmol⁻¹) was added (final reaction volume was 200 μL). After 60 min of incubation, the reaction was stopped by the addition of 1.0 mL of 20 mM HEPES buffer (pH 5.5) containing 2mM EDTA. The L-citrulline produced was separated from the residual L-arginine chromatographically using columns of 1.0 mL (1:2 w:v) of Amberlite IRP-69 ion-exchange resin (100-500 wet mesh) (Aldrich, St-Louis, MO) suspended in distilled and deionized water; water was also used as the mobile phase. The radioactivity contained within the eluate was quantified by liquid scintillation counting.
Soluble GC activity was assessed in kidney cytosolic fractions, as previously described. Briefly, kidney homogenate samples were incubated at a final protein concentration of 0.15 mg/mL in 50 mM HEPES buffer (pH 7.4) containing 2 mg/mL bovine serum albumin, 6 mM MgCl₂, 1 mM L-cysteine, 2 mM isobutyl methylxanthine, 5 mM benzamidine HCl, and 100 μM sodium nitroprusside. The reaction was initiated by adding 25 μL of 2 mM GTP (final reaction volume was 250 μL), and was carried out for 30 min. The reaction was terminated by the addition of 125 μL of 125 mM zinc acetate and 125 μL of 125 mM sodium carbonate. Samples were then centrifuged at 5000 x g for 10 min at 4°C. The cGMP formed was then quantified in the supernatant by enzyme immunoassay (EIA) using a Kit (Cayman Chemical Co. Ann Arbor, MI) according to the manufacturer’s instructions.

4.2.6 Statistical Analyses

In Studies 1 and 2, NOS and sGC activity were analyzed by 2-way ANOVA. Tissue bath data from Studies 1 and 2 were analyzed by unpaired t-test at each of the concentrations; logEC₅₀ values were obtained by interpolating a sigmoid curve for each animal, and calculating the mean value among all the animals in the group. For Study 1, N values represent number of litters used; that is, if 2 littermates were used, these data were combined and treated as a single value.

For Study 2, hematology and liver iron data were assessed by unpaired Student’s t-test. The relationship between Ht and liver iron was assessed by non-linear regression. The telemetry data included herein represent rats from the ID group that had Ht below 0.38; 3 rats from this group were therefore excluded from the original analysis.
obtained by radiotelemetry were assessed by repeated measures 2-way ANOVA, for time and treatment. The summarized salt-sensitivity data were analyzed by repeated measures 2-way ANOVA, for iron treatment and salt treatment. Where 2-way ANOVA revealed significant differences, 1-way ANOVA with Newman-Keuls or Student’s t-test were performed as post-hoc tests, as appropriate. In vivo renal function curves (RFC) were obtained from each individual animal, and the average slopes and RFC positions (on the abscissa) were compared by unpaired Student’s t-test. Data are presented as Mean ± SEM. P < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Study 1 Outcomes

The effects of maternal iron restriction prior to and throughout pregnancy on neonatal and adult offspring iron status as well as the long-term cardiovascular outcomes are presented in detail in Chapter 3.

The impact of PID on neonatal intrarenal NOS and sGC activities are presented in Figure 4.1. During the lactation period, although the 2-way ANOVA revealed no main effect of time or treatment alone on NOS activity, there was a significant interaction (P < 0.05). That is, in controls, NOS activity was increased on PD 14 and PD 21 compared to PD 1 and PD 7. In the PID offspring, NOS activity was increased on PD 14 compared to PD 1 and PD 7 (P < 0.05), but decreased again on PD 21 (P < 0.05). To determine whether the interaction existed within the treatment effects as well, comparisons were made between treatment groups at all postnatal days. NOS activity was reduced in the
Figure 4.1. Effect of PID on renal (A) nitric oxide synthase (NOS) activity and (B) soluble guanylyl cyclase (sGC) activity in neonatal offspring at postnatal days 1, 7, 14, and 21. Data reflect predominantly female offspring values. sGC activities at postnatal day 1 could not be assessed due to a lack of tissue. Different letters denote significant differences between postnatal days in the same group (P < 0.05). Control: n = 5-7; PID: n = 4-7.
PID offspring compared to controls at PD 21 (P < 0.05); there were no differences at any other postnatal day. There was no overall treatment effect in sGC activity in the neonatal period, although there was a significant day effect (P < 0.05) (Figure 4.1B); PD 14 was higher than PD 7 and PD 21 in the control group (P < 0.05). No such differences were observed in the PID group. In adult offspring, NOS activity was decreased in medullas of PID offspring compared to controls (P < 0.05), although no treatment effects were observed in the cortex (Figure 4.2A). There was more NOS activity (per gram of tissue) in medulla than in cortex (P < 0.05). In contrast, there were no differences in sGC activity between treatment groups, or between tissues (Figure 4.2B).

PID caused a left-ward shift in the concentration response curves of PE, as indicated by a 2.5 fold decrease in EC50 (P < 0.05) (Figure 4.3A). Additionally, blood vessels from PID offspring had diminished sensitivity to ACh, as indicated by a 2.5-fold increase in EC50 (P < 0.05) (Figure 4.3B). Similarly, the aortas of PID offspring had decreased sensitivity to DEA-NONOate, as shown by a 3.2-fold increase in EC50 compared to control animals (P < 0.01) (Figure 4.3C).

4.3.2 Study 2 Outcomes

After the first week on low iron treatment, ID rats had lower Ht than control rats (P < 0.05) (Figure 4.4A). At five weeks on the low iron treatment, the Ht of the ID group was 60% lower than controls (P < 0.001), and had decreased by 35% compared to the baseline measurements (P < 0.001); controls’ Ht increased by 14% compared to baseline measurements (P < 0.001). The low iron treatment did not impact body weight (Figure 4.4B). Following the induction of ID, a thorough analysis of iron status was performed
Figure 4.2. Effect of PID on renal (A) nitric oxide synthase (NOS) and (B) soluble guanylyl cyclase (sGC) activities in 12 wk-old male offspring. *P < 0.05 compared to controls. ‡P < 0.001 compared to medulla in the same treatment group. Control: n = 7; PID: n = 8.
Figure 4.3. Effect of PID on concentration-response relationships of rat aortic rings to (A) phenylephrine (PE), (B) acetylcholine (ACh), and (C) diethylamine-NONOate (DEA-NONOate) in 14 wk-old male offspring. *P < 0.05, **P < 0.01 compared to controls. Control: n = 6; PID: n = 7.
Figure 4.4. Effect of low iron treatment in adult male rats on (A) hematocrit and (B) body weight. *P < 0.05 compared to controls at same week; †P < 0.025, ‡P < 0.001 compared to previous week in the same treatment group. Control: n = 7; ID: n = 7. ID, iron-deficient.
Five weeks of low iron treatment caused reductions on all hematological indices assessed (P < 0.001) (Figure 4.5 A-F), with the exception of total iron binding capacity, which was elevated by 62% (P < 0.001) (Figure 4.5G). Liver iron concentrations in the ID group were reduced to less than 19% of control values (P < 0.001) (Figure 4.5 H). The non-linear relationship between Ht and liver iron is presented in Figure 4.6. These data consist of control and ID rats, as well as those made iron-deficient and subsequently fed the purified control diet for either 4, 7 and 14 days. The relationship between Ht and liver iron concentration displays a non-linear relationship (R² = 0.69) (Figure 4.6).

Acute ID impacted renal NOS production (Figure 4.7A). Medullary NOS activity was increased by 25% in the ID group (P < 0.05), and increased by 21% in the cortex (P < 0.05). As in Study 1, medullary NOS activity was found to be higher in medullary tissue than cortical tissue in both treatment groups (P < 0.001). There was no interaction between these tissue and treatment variables. Interestingly, ID caused a reduction in renal sGC activity (P < 0.05) (Figure 4.7B). Post-hoc analysis revealed that medullary sGC activity was 32% lower in the ID group compared to controls (P < 0.05); the differences in cortical sGC activity between treatment groups were not significant.

ID did not significantly impact vascular responses to PE (Figure 4.8A), or ACh (Figure 4.8B). Although EC₅₀ values for DEA-NONOate concentration response curves were not different, addition of NO-donor at doses from 3x10⁻⁹ M to 1x10⁻⁸ M caused greater vascular relaxation in the ID group compared to controls (P < 0.05) (Figure 4.8C).
**Figure 4.5.** Effect of iron restriction in adult male rats on (A-G) hematological parameters and (H) liver iron. ***P < 0.001 compared to controls. Control: n = 7; ID: n = 7.
Figure 4.6. Relationship between hematocrit and liver iron in adult male control, iron-deficient, and iron-replete rats. Open shapes represent rats that were made iron-deficient, and subsequently fed the control diet for the indicated number of days prior to sampling. n = 7 for all groups.
Figure 4.7. Effect of ID in adult male rats on renal (A) nitric oxide synthase (NOS) activity, and (B) soluble guanylyl cyclase (sGC) activity. *P < 0.05 compared to controls. †P < 0.05, ‡P < 0.001 compared to medulla in the same treatment group. Control: n = 6; ID: n = 6.
**Figure 4.8.** Effect of ID in 19 wk-old male rats on concentration-response relationships of aortic rings to (A) phenylephrine (PE), (B) acetylcholine (ACh), and (C) diethylamine-NONOate (DEA-NONOate). *P < 0.05 compared to controls at the same dose. LogEC$_{50}$ values presented in each graph reflect averaged LogEC$_{50}$ values obtained for each animal. Control: n = 4; ID: n = 4.
The iron status of animals used for cardiovascular assessments in both the control and iron restricted groups were similar to those used for tissue analysis, as assessed by Ht and serum iron levels (data not shown); these animals underwent a similar iron restriction treatment beginning at the same age (7 wk) for a period of 10 wk before telemetry data were obtained. The impact of ID on cardiovascular parameters during normal sodium, low sodium and high sodium intake is presented in Figure 4.9. ID had no effect on MAP, HR or pulse pressure during the normal, low sodium or high sodium intake phases. Sodium intake did impact AP (P < 0.001), as well as heart rate (P < 0.001), but did not affect pulse pressure. The summary of AP changes associated with sodium challenge, and the in vivo renal function curves derived from these data are presented in Figure 4.10. Two-way ANOVA revealed no main effects of ID or sodium alone on MAP, but there was a significant group by salt treatment interaction (P < 0.05); control rats had significant changes in MAP in response to low and high salt intake (P < 0.05), whereas ID rats did not. There were no differences in the in vivo renal function curves between the control and ID groups.

4.4 Discussion

The objective of Study 1 was to determine whether PID causes persistent alterations in intrarenal and vascular NO signaling. In summary of Study 1, PID was found to cause: (i) altered renal NOS and sGC activity at PD 21, (ii) diminished medullary NOS activity, but not sGC activity in adult offspring, and (iii) alterations in endothelium-dependent and -independent relaxation in thoracic aorta of adult offspring.
Figure 4.9. Effect of ID in adult male rats on (A) mean arterial pressure, (B) heart rate, and (C) pulse pressure profiles during normal sodium (NS), high sodium (HS) and low sodium (LS) treatments, as assessed by radiotelemetry. These data represent a subset of data that have been published elsewhere;\textsuperscript{402} see section 4.2.6 for details. Control: n = 6; ID: n = 4.
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Log [Na⁺ Intake/BW (g/g)]

Figure 4.10. Effect of ID in adult male rats on (A) net changes in mean arterial pressure (MAP) associated with low sodium (LS) and high sodium (HS) intake, and (B) the resultant in vivo renal function curves derived from these data. †P < 0.05, ‡P < 0.001 compared to control LS. In (A), LS data were calculated as the net change in MAP between normal sodium intake (NS) and LS; HS data were calculated as the net change in MAP between HS and normal sodium; Total was calculated as the net change in MAP between LS and HS. These data represent a subset of data that have been published elsewhere; see section 4.2.6 for details. Control: n = 6; ID: n = 4.
Subsequently, we developed a model to investigate the impact of acute ID on NOS signaling in the kidney and aorta. In adult animals (iv) a 5 wk treatment of iron restriction caused severe ID anemia. ID in turn, caused (v) increased medullary and cortical NOS and (vi) decreased medullary and cortical sGC activity; (vii) alterations in responsiveness to endothelium independent vasodilation, and finally (viii) no significant alterations in the magnitude of the AP response to normal, low and high sodium intake.

The findings that adult PID offspring have reductions in medullary NOS capacity provide further evidence that renal function and structure are altered in these animals. Although nephron number was not assessed in these animals, reduced medullary NO synthesis was expected based on the findings of Lisle et al. that showed a reduced nephron endowment in PID offspring.

The left and right-shifted phenylephrine and ACh concentration response curves, respectively, in isolated aortic rings are consistent with the notion that intrinsic alterations in NO signaling are present in this model of developmental programming. In addition, we have shown that the vasomotion responses of aortas and renal arteries from PID rats in the presence of normal and low oxygen conditions is compromised, providing further support for intrinsic alterations in NOS-mediated signaling. Altered vascular NO signaling has also been reported in other models of programming, including offspring of mothers that were hypoxic during pregnancy, but not of nutrient restricted dams, suggesting that this outcome is characteristic of only certain models of programming. The findings that aortic responses to DEA-NONOate are less sensitive in the PID group are interesting, given the lack of altered sGC activity in kidney cortical and medullary
fractions. Although it may be that, unlike in the kidney, sGC activity is compromised in vascular tissues, it could also reflect a number of cGMP-independent mechanisms by which NO mediates its vascular effects; these include calcium dependent K channels, as well as activation of SERCA. Another possibility is that the changes in vascular reactivity to the administered agonists stem from alterations in smooth muscle function, rather than endothelial dysfunction. That is, impairment of the vascular smooth muscle contractile mechanism could result in altered functionality of these vessels’ response to administered ACh, PE, and DEA NONOate, despite no changes in NOS expression patterns or NO production. Yet another possibility is that PID has influenced another system, such as the HO/CO pathway, which could affect the interaction between NO and sGC at the level of the smooth muscle. Analysis of aortic NOS and sGC activity and expression patterns will provide insight into these mechanisms.

We hypothesized that the reductions in medullary NOS may be a contributing factor in the altered cardiovascular function we previously reported in these animals. This notion is compelling, given the importance of NOS activity to the pressure-natriuresis mechanism. Specifically, transmission of changes in AP to medullary blood flow, and the consequent alterations in RIHP appear to depend, in part, on medullary NOS activity. Moreover, NOS has been shown to have a direct influence on tubular sodium excretion, further implicating reductions in NO production in the development of salt sensitive hypertension and altered pressure-natriuresis. However, the studies that demonstrate the reliance of renal control mechanisms on NOS, using inhibitors such as L-NAME have shown reductions in NOS activity on the order of 80%. It was therefore
unclear whether more subtle alterations in NOS signaling could account for the observed cardiovascular consequences of PID. We therefore sought to develop alternative experimental models to characterize the involvement of hemoenzyme activities in AP control.

Despite the impact of severe ID on NOS and sGC activity, due to various confounding factors it is not an ideal model to examine the impact of hemoenzyme function in AP (see below). In a separate series of experiments, we used a model of heme depletion using an irreversible inhibitor of heme biosynthesis, succinylacetone (SA; 4,6-dioxoheptanoic acid) (Appendix 1). This treatment caused reductions in systemic NOS, as well as renal NOS and sGC activity (Appendix 1, Figure 1). Furthermore, alterations in vascular responses to endothelium-dependent and independent vasodilators were similar in this model as in the PID animals, thus providing a different model in which to examine the effects of hemoenzyme reduction. Although there were hematological manifestations of heme synthesis inhibition (Appendix 1, Table 1), it is unlikely these were severe enough to impact oxygen delivery. Despite the similarities in altered NOS signaling between heme synthesis inhibited animals and PID animals, no observable changes in baseline cardiovascular function was observed in the former group. Moreover, when these animals were challenged with low and high sodium, their cardiovascular responses were improved compared to control animals (Appendix 1, Figure 4B). These data suggest that the modest reductions in NOS activity are not sufficient to impact circulatory function. It is therefore likely that reductions in NOS activity in the PID offspring alone are insufficient to account for the observed changes in
arterial pressure in those animals. However, it would be of interest, in both PID and SA-treated rats, to assess whether the reduced NO production reflects diminished vascular NOS activity (i.e. reduced eNOS function, which is strictly expressed in vascular endothelial cells) or a reduction in nephron content (i.e. diminished nNOS activity, which is highly expressed in nephrons). These experiments will be critical in evaluating the relative contribution of altered NOS signaling to the cardiovascular function and dysfunction in both SA-treated and PID rats.

The impact of acute ID on renal sGC capacity supports the hypothesis that reduced iron status impacts hemoenzyme function. The reduction in sGC activity as a consequence of diminished iron is a more plausible explanation than a hypoxia mediated effect, because the latter has been shown to have no effect in aortic rings. Although Ni and colleagues reported increased cGMP in the kidney, these values likely reflect increased systemic production of NOS, rather than sGC activity per se within the kidney. The findings that medullary sGC activity was affected by ID suggest that medullary hemoenzyme function is not spared in iron deficiency, as previously reported. Although cortical sGC was not affected, these results may reflect a more subtle effect of ID in this tissue, which is expected given its intrinsically lower sGC activity than the medulla. Interestingly, aortic sensitivity to DEA-NONOate was increased, which is opposite the effects observed in the medulla. These data could potentially be explained on the basis of diminished heme catabolism by HO, and hence diminished CO production, which has been shown to mitigate the vasorelaxation effects of NO. Thus, it could be that ID and the consequent reduced heme production,
increases NO binding to sGC by removing the inhibitory effects of CO. However, this hypothesis assumes a role for endogenous CO in the regulation of vascular tone, which has not been directly demonstrated.

In contrast to the reduced sGC activity, medullary and cortical NOS capacity were increased, which was opposite to the predictions based on other studies that showed ID causes reduced hemoenzyme function, including NOS, in tissues such as the gut.411 However, the increased NOS capacity in the present study is consistent with reports from other investigators that have shown that acute ID causes increased NOS expression and activity in the aorta.409 It is not clear why the induction of acute ID induced upregulation of NOS capacity in the kidney and vasculature. Ni and colleagues suggested that anemia would increase cardiac output, and thereby increase NO synthesis as a function of the increased blood delivery to tissues and organs.409 However, this is unlikely to be the cause of the increased NOS activity in the present study, since no changes in either heart rate or pulse pressure were observed in the ID rats, suggesting cardiac output was not altered. An alternative explanation is that the increased NOS production in response to decreased oxygen delivery causes vascular relaxation, and thus facilitates the delivery of blood to tissues.412 Indeed, hypoxia has been reported to upregulate eNOS activity.413, 414 In this scenario, an increased blood flow to tissues could occur without concomitant increases in cardiac output or AP. However, this concept is complicated by the observations that vascular responses to ACh were not changed in the ID group. Nevertheless, assessment of resistance vessels, rather than a conduit artery such as the aorta, may provide more valuable insight into these mechanisms. Although it may be that
a lack of sensitivity to cholinergic stimulation is opposite to what would be expected as a result of the increased eNOS expression, increased vascular relaxation to administered ACh would not be expected to be altered if other signaling processes are limiting (e.g. Ca\(^{2+}\) binding to calmodulin to activate NOS). Given iron’s involvement in many physiological and pathophysiological functions (see section 1.3), it is likely that the vascular effects of iron deprivation are complex and multifactorial, such that many mediators need to be considered. It is clear from these results that further investigation is needed to fully appreciate the complexity associated with iron deficiency and how it impacts cardiovascular function.

Although the model of acute ID may not be ideal for examining the effects of hemoenzyme function in cardiovascular regulation, it provides insight into the mechanisms by which early ID could mediate its long-term programming effects. First, these data demonstrate that the long-term cardiovascular effects are not due to a cardiovascular effect induced by ID *per se*, but more likely to the persistent effects of the various systems (e.g. brain, kidney), during development (see Chapter 5). Secondly, examination of the systems affected by ID may provide insight into the events that occur during pregnancy and postnatal periods of development. For example, based on the finding that ID causes upregulation of NOS activity in the kidney in adult animals, but caused no overt changes in NOS or sGC activity between PD 1 and PD 14 suggests ID impacts systems differently during development compared to adulthood.
Chapter 5
Perinatal Iron Deficiency Impacts Locomotor Behaviour and Water Maze Performance in Adult Male and Female Rats

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5.1 Introduction

Iron deficiency (ID) is the most widespread single nutritional disorder in the world. It is the only global nutritional health risk that has a significant prevalence in populations of both developing and industrialized nations.\textsuperscript{102,285} Although ID is common among the general population, there is a preponderance of pregnant women that are affected by this condition, due in part to an expansion in blood volume and increased erythropoiesis, as well as increased iron demands from the fetal-placental unit.\textsuperscript{336} According to conservative estimates, more than half of pregnant women in developing countries\textsuperscript{296} and between 20-40\% of women in western industrialized countries are anemic, due mainly to ID.\textsuperscript{296}

ID during and shortly after pregnancy can impact multiple facets of cognition and behaviour in the offspring, including learning, conduct, motivation, and attentiveness. These deficits persist even after the insult has been corrected (i.e. despite subsequent iron replenishment). This “developmental programming” of cognition and behaviour has been reported in intervention and non-intervention studies involving newborn infants and young school children throughout a number of geographical regions (for review, see Grantham-McGregor \textit{et al.}.\textsuperscript{337}). Most of these studies were epidemiological, and therefore
of limited use in elucidating the specific mechanisms involved in this model of
developmental plasticity. Consequently, the use of rodent models of perinatal ID (PID)
has been quintessential in characterizing the biochemical and neurophysiologic changes
that underlie these cognitive and behavioural deficits.

Independent from the programming effects described above, ID can induce
cognitive and behavioural deficits in adults.\textsuperscript{415} Depletion of brain iron appears to impact
on numerous neurotransmitter systems which appear to be linked to reduced learning
capacity and locomotor activity.\textsuperscript{324,325} Interestingly, in certain studies, these effects
persist even when the ID is corrected.\textsuperscript{324} In light of these reports, in some cases it may be
difficult to distinguish between the programming effects of developmental ID versus the
effects of acute brain iron deprivation. Studies in which iron is restricted during gestation
and through the subsequent postnatal period may induce, in addition to the fetal-
programmed behavioural effects, persistent decreases in brain iron that also influence
cognition and behaviour. In the studies by Felt \textit{et al.}\textsuperscript{319} and Kwik-Uribe \textit{et al.},\textsuperscript{416} ID was
induced in the mother during gestation and continued throughout the postnatal phase,
resulting in persistent decreases in brain iron at the time of behavioural testing. In a more
recent study, Felt and colleagues demonstrated that maternal ID throughout pregnancy
and lactation resulted in persistent neurophysiological changes in the offspring at 12 wk
of age, despite brain iron levels being normalized at that time.\textsuperscript{317} Behavioral deficits
were also observed in the offspring of ID mothers at 5 wk of age, although brain iron
levels at that time were not reported.\textsuperscript{317}
To gain insights into the nature of the behavioural deficits previously reported in adult PID rats, we conducted spontaneous locomotor and Morris water maze (MWM) testing in offspring of dams that were iron-restricted during the gestational period. Our overall objective was to determine whether behavioural and cognitive differences persisted in adulthood in the absence of changes in brain iron content. Furthermore, previous work done with PID offspring in the MWM has largely focused on males rather than females. The present studies were therefore intended to also identify sex-differences in behavioural outcomes due to PID. Finally, unlike other behavioural studies involving PID offspring, the MWM testing protocol used in the present study is the first to include several design elements that control for potential non-behavioural interferences which could influence performance; these include, for example, sensorimotor deficits, random encounters with the platform and differences in swim speeds.

5.2 Methods

5.2.1 Animals and Treatments

The experimental protocols described herein were approved by the Queen’s University Animal Care Committee. Sixteen 8-wk old female Wistar rats were purchased from Charles River (St-Constance, QC) and housed separately in plastic cages in the Queen’s University Animal Care Facility, which maintained a 12 h/12 h light/dark cycle and an ambient temperature of 23°C. All rats were given one week to acclimatize to the novel surroundings before beginning treatment.
All purified diets (Research Diets Inc., New Brunswick, NJ) were based on the AIN-93G diet, which has been described elsewhere. The purified diets were identical in composition, with the exception of added ferric citrate, which was adjusted to obtain the following iron concentrations: 225 mg/kg in the control diet (Cat# D03072504), and 3 mg/kg in the low iron diet (Cat# D03072501). The non-purified diet (Lab Diets, Oakville ON; Cat# 5001), which has been described elsewhere, contained 270 mg/kg iron. All rats had ad libitum access to food and water.

Following the acclimatization period, eight female rats were randomly chosen and assigned to the low iron diet, while four different females were randomly selected and assigned to the purified control diet. The remaining 4 dams were administered the non-purified diet. After two weeks on their respective diets, all dams were mated to 10 wk old male Wistar rats that were fed the non-purified diet. All rats remained on their respective diets throughout pregnancy. Within 8 h after giving birth, all dams were given the non-purified control diet. Food consumption and body weights of offspring were monitored every 2 days until postnatal day (PD) 35 and weekly thereafter. At 24 h postpartum, litters were culled to 8 rats (4 males and 4 females) to standardize postnatal conditions. At weaning (PD 21), the remaining pups were separated from their mothers and given the non-purified diet. Behavioral testing began when rats reached 12 wk of age. Adult offspring were euthanized at 24 wk of age.

5.2.2 Behavioural Testing

All behavioural testing was done between 1200 h and 1600 h. At approximately 12 wk, adult offspring were tested for spontaneous locomotor activity as previously
described. Rats were left in the open-field (OF) apparatus for a total of 20 min. Outcome measures obtained included distance traveled, distance traveled in the center of the apparatus, time spent at rest, and time spent hyperactive (defined as movement faster than 15cm/s).

For MWM testing, Swim path, speed, and distance to reach platform were obtained using the Videomot® computer program (TSE Systems Inc.) and recorded for off-line analysis. Escape latency was also measured and analyzed; however distance to platform (path length) was used instead for statistical comparisons and is presented in this study because it is deemed to be a more reliable indicator of overall performance.

Day 1: Visible Platform Phase: A black, circular escape platform (dia. 12 cm) was placed in the center of a quadrant of a circular pool (dia.1.8 m), filled with water (maintained at 26 ± 1°C) made opaque with non-toxic white paint. The platform protruded 1-2 cm above the water’s surface. The top of the visible platform (as well as the hidden platform-see below) was covered with a fine rubber mesh to facilitate the rats’ climbing onto it. Rats were trained in blocks, each block consisting of 4 consecutive trials. Each trial in turn lasted a maximum of 60 s. If the rat could not locate the platform in 60 s, the experimenter showed the rat the location by placing it on the platform. All rats remained on the platform for 15 s between each trial. A different release point (from the intersection of each of the 4 quadrants near the wall) was used for each trial. Rats received 2 blocks of 4 consecutive trials, separated by a resting period of 5 min. After each block, rats were towel-dried and returned to their cages, which were warmed by a heat lamp.
**Day 2 to 5: Hidden Platform and Probe Trial Phase:** A white circular escape platform (dia. 12 cm) was submerged 1-2 cm below the surface of the water in the same pool. The location of the hidden platform was the middle of the quadrant adjacent to the quadrant where the visible platform had been previously located. The hidden platform did not change locations from day 2 to day 5. The same testing pattern was used for the hidden platform phase as was used for the visible platform phase with the following exception: following the two blocks of testing, the hidden platform was removed, and after a 5 min rest period, rats were placed in the center of pool and released; this “probe trial” lasted 60 s.

**5.2.3 Tissue Collection and Analysis**

Pups that were culled at 24 h were killed by decapitation, and blood samples were collected in heparinized micro-capillary tubes. The tubes were then centrifuged (11,500 x g for 15 min) and packed cell volume was determined as a measure of hematocrit (Ht). Organs were excised into ice-cold saline, cleaned of extraneous connective tissue, blotted dry, weighed and snap frozen in liquid nitrogen and stored at -80°C until processing. Adult rats (24 wk of age) were first anaesthetized with sodium pentobarbital (90 mg kg⁻¹ body weight, i.p.). Blood samples were subsequently obtained from the inferior vena cava, and Ht was assessed as described above. Adult rats were then perfused through the aorta with ice-cold saline using a peristaltic pump to remove blood, and tissues were collected as described above.

For tissue iron analysis, frozen tissues were thawed and dried at 65°C for a minimum of 72 h. Dried tissues were ashed in a muffle furnace at 200°C for 2 h and at
500°C for 18 h. The ash was then dissolved in hot, concentrated nitric acid and then diluted with distilled and deionized water. Iron concentrations were determined using a Varian SpectrAA-20 flame atomic absorption spectrophotometer (Varian Canada Inc., Ottawa, ON). Bovine liver standards (National Institute of Standards, 1577b) and blanks were included as quality controls.

5.2.4 Statistical Analyses

All offspring data from the three diet groups were initially compared by 2-way ANOVA. Given that there were no behavioural or biochemical differences between offspring born to dams fed the control purified diet and control non-purified diets, these groups were combined for all subsequent statistical analyses. Body weights, brain weights, Ht, and tissue iron levels were analyzed by 2-way ANOVA (by treatment and sex) with Student’s $t$ test as post hoc analysis. Spontaneous locomotor activity, as well as MWM swim speeds and thigmotactic behaviour were analyzed by repeated measures 2-way ANOVA (by treatment and time), with Student’s $t$-test (for between treatment group comparison) or 1-way ANOVA with Newman-Keuls (for between time comparisons). MWM path lengths and spatial bias data were analyzed by repeated measures ANOVA with Dunnett post-hoc test (for comparisons between the first block and subsequent blocks within a particular testing phase; e.g. blocks H2-H8 were compared to block H1). This comparison was done because a 2-way ANOVA using all the data from a testing phase would mask treatment effects that only occur during particular intervals within that testing phase (i.e. delayed acquisition). No comparisons were made between males and females to identify treatment effects in behaviour because of the extensive body of work
showing that males perform substantially better than females in the water maze task (for review see D’hooge et al.421). Data were analyzed using Graph Pad Prism software (Version 5). Offspring data from each litter were pooled, and each n value refers to number of litters. Data were analyzed for homogeneity of variance using Bartlett’s test for samples of unequal size; all data sets compared were found to be homogeneous. Results are presented as mean ± SEM. P < 0.05 was considered statistically significant.

5.3 Results

5.3.1 Maternal and Offspring Outcomes

The quantities of food ingested in the period before and throughout pregnancy did not differ among treatment groups (data not shown). Maternal ID did not impact on pregnancy success rate (87.5% in both groups), or the number of pups per litter (14.3 ± 0.9 (ID) vs. 15.4 ± 0.5 (controls), n =  6-7 per group). The maternal ID did compromise offspring; one litter was completely lost, and a total of seven pups from the remaining litters also perished within the first 24 h of birth. In contrast, only one pup from the control group died. The low iron treatment markedly affected iron status of the offspring at birth (Table 5.1). Ht were 32% lower (P < 0.001), liver iron was 83% lower (P < 0.001), and brain iron was 38% lower (P < 0.001) in the PID offspring compared to controls. There were no sex differences or treatment by sex interactions in any of these parameters. There were no treatment effects in Ht, brain iron or liver iron in adult offspring (24 wk of age) in either males or females; however there were sex-based differences in liver iron content (P < 0.001) (Table 5.1).
Table 5.1. Iron status of control and PID offspring at 24 h and 24 wk of age.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hematocrit</th>
<th>Liver Iron (μmol/g)</th>
<th>Brain Iron (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>PID</td>
</tr>
<tr>
<td>M (24 h)</td>
<td>0.39 ± 0.01</td>
<td>0.27 ± 0.01***</td>
<td>29.6 ± 2.9</td>
</tr>
<tr>
<td>F (24 h)</td>
<td>0.40 ± 0.02</td>
<td>0.26 ± 0.01***</td>
<td>29.4 ± 2.3</td>
</tr>
<tr>
<td>Pooled M &amp; F (24 h)</td>
<td>0.39 ± 0.01</td>
<td>0.26 ± 0.01***</td>
<td>30.1 ± 2.4</td>
</tr>
<tr>
<td>M (24 wk)†</td>
<td>0.46 ± 0.02</td>
<td>0.46 ± 0.01</td>
<td>7.8 ± 0.5‡</td>
</tr>
<tr>
<td>F (24 wk)</td>
<td>0.45 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>21.2 ± 0.8</td>
</tr>
</tbody>
</table>

**P < 0.01, ***P < 0.001 compared to controls of the same sex at the same age; †P < 0.001 compared to females at the same age in Liver iron content; ‡P < 0.001 compared to females of the same group at the same age. Control: n = 4-7; PID: n = 7. PID, perinatal iron-deficient; M, males; F, females.
PID male body weights were lower than controls between PD 5 and PD 15 (P < 0.05) (Figure 5.1A); these data are also expressed as a fraction of control male body weights (Figure 5.1A, inset). PID females had reduced body weights compared to controls between PD 1 and PD 9 (P < 0.05) (Figure 5.1B); these data are also expressed as a fraction of control body weight (Figure 5.1B, inset). Sex-based differences in body weight were present from PD 29 onward (P < 0.05). No treatment by sex interactions were observed at any postnatal day. There were no treatment effects or sex differences in brain weights at birth (data not shown). Adult brain weights were not different between PID and control rats at the time of sacrifice, although there were sex-based differences (P < 0.001) at that time; males had larger brain weights than females, as expected.

5.3.2 Locomotor Behavioral Outcomes

Adult males and females from the PID group had markedly decreased spontaneous locomotor activity and exploratory behaviour in the OF compared to adult controls (Figure 5.2). PID males, compared to control males, traveled less total distance (P < 0.01) (Figure 5.2A) and had fewer rearings (P < 0.001) (Figure 5.2B). These animals also spent more time at rest (P < 0.01) and spent less time hyperactive (P < 0.05) (data not shown). Additionally, PID males spent less time in the center of the apparatus compared to control offspring (P < 0.01) (Figure 5.2C). PID females, in turn, traveled less total distance (P < 0.05) (Figure 5.2A) and had fewer rearings (P < 0.01) (Figure 5.2B); they also spent less time hyperactive (P < 0.05), and there was a trend for more time spent at rest (P < 0.07). In contrast, PID did not impact the total amount of time spent in the center of the apparatus in the females (Figure 5.2C). Total distances traveled,
Figure 5.1. Effect of PID on body weight (BW) of (A) male and (B) female Wistar rats. Data are also expressed as percentage of control BW (insets). *P < 0.05 compared to controls at the same postnatal day. Male BW was different from female BW from PD 29 onward (P < 0.05). Control: n = 6; PID: n = 6.
Figure 5.2. Effect of PID on (A) distance traveled, (B) number of rearings, and (C) total time spent in center of an open-field apparatus of male and female Wistar rats at 12 wk. *P < 0.05 compared to controls at the same time interval. Control: n = 5-6; PID: n = 5-6.
percent time hyperactive, and number of rearings in all males and females decreased as
time progressed (P < 0.001 for all parameters). There were no treatment by time
interactions in any of the parameters analyzed in either male or female offspring.

5.3.3 Morris Water Maze Outcomes

PID treatment had no impact on path length to reach the escape platform during
the pre-training/visible platform phase of testing (Day 1) in either the males or females
(data not shown). In the hidden-platform phase, control males showed immediate
improvement in path length to reach the platform compared to block H1 (P < 0.001)
(Figure 5.3A). In contrast, PID males did not show significant improvement until block
H4 (second day of the hidden-platform phase) (P < 0.01) (Figure 5.3B). While PID
females showed improvement in performance as early as block H2 (P < 0.05) (Figure
5.3D), performance in the control females did not improve until block H3 (P < 0.05)
(Figure 5.3C), albeit there was a trend for improved performance in block H2 (P < 0.07).

Control males demonstrated improvement in spatial bias for the target quadrant
(defined as the propensity to remain in the quadrant wherein the platform is located) on
block H2 (P < 0.05), and remained elevated thereafter (Figure 5.4A). In contrast, PID
males did not improve until block H5 (third day of the hidden platform phase) (P < 0.05)
(Figure 5.4B). Control females showed improvement in spatial bias performance in
block H5 (P < 0.05) (Figure 5.4C), whereas PID females showed signs of improved
performance in block H6 (P < 0.01) (Figure 5.4D). There were no differences in spatial
bias between treatment groups during the visible platform phase or probe trials (data not
shown).
**Figure 5.3.** Effect of PID on path lengths to reach the hidden platform in a Morris water maze of male (A,B), and female (C,D) Wistar rats at 12 wk. Shaded areas in the background represent different days of testing. *P < 0.05 compared to block H1 in the same treatment group. Control: n = 6; PID: n = 6.
Figure 5.4. Effect of PID on spatial bias for the target quadrant (expressed as a percentage of total distance travelled) in the hidden platform phase of a Morris water maze of male (A,B) and female (C,D) Wistar rats at 12 wk. Shaded areas in the background represent different days of testing. *P < 0.05 compared to block H1 in the same treatment group. Control: n = 6; PID: n = 6.
There was a trend for males in the PID group to travel an overall greater proportional distance in the thigmotaxis zone (defined as the outer tenth of the perimeter of the pool) compared to control rats during the probe trials (16.5 ± 2.5% (PID) vs. 13.0 ± 2.1% (control), n = 6 per group; P < 0.06). There was also a treatment by time interaction in the males (P < 0.05), although there were no time effects observed. Conversely, PID females showed no differences in thigmotactic behaviour in the probe trials (21.6 ± 3.8 % (PID) vs. 21.5 ± 4.3 % (control), n = 6 per group). There was an effect of time (P < 0.001) in the females, although there was no treatment by time interaction. Thigmotactic behaviour was not assessed in the visible or hidden platform phases of the MWM task because there are inherent limitations of this component of the analysis (the release point is within the thigmotactic boundary, and therefore inappropriately impacts the proportion of distance traveled in that region).

Overall, there were no differences in swim speeds between the PID and control males throughout MWM testing (29.6 ± 0.3 cm/s (PID) vs. 29.9 ± 0.7 cm/s (control), n = 6 per group). In contrast, PID females swam slower than control females (31.0 ± 0.5 cm/s (PID) vs. 33.0 ± 0.5 cm/s (control), n = 6 litters; P < 0.001). There were also treatment by time interaction in the swim speeds of both males (P < 0.05) and females (P < 0.01).
5.4 Discussion

The major findings in the offspring following maternal iron-restriction during pregnancy include marked reductions in hematocrits and tissue iron levels at birth, and overall reduced exploratory behaviour in an open field (OF) apparatus in adulthood. Additionally, PID males, but not females, tended to avoid the center of the OF apparatus and had overall poorer performance in the Morris water maze (MWM) compared to control males. PID females in turn, had markedly slower swim speeds than controls. Taken together, these results suggest that (i) PID induces long-term changes in behaviour despite subsequent iron replenishment after birth, and (ii) male and female rats may have different susceptibilities to these adverse programming effects.

The treatment paradigm used in the present study was intended to restrict iron in the gestational and immediate postnatal period. Dams were given a high-iron diet immediately after giving birth, which replenishes maternal Ht and Hb levels within 7 days. However, whether this is sufficient time to replenish the iron in maternal milk is not known. Consequently, the supply of iron to the pups may remain inadequate throughout the nursing period, despite recovered hematological indices in the mothers. Indeed, in the present study, PID rats’ weights continued to drop until the second postnatal week, indicating that nutrition during lactation was inadequate for optimal growth (Figure 5.1). Moreover, Beard and colleagues recently showed that exploratory deficits in an OF apparatus are reversed when ID pups are out-fostered to control fed dams within days of birth, suggesting that iron restriction during the postnatal period is important for certain behavioural deficits in this model of developmental programming.
Nevertheless, the present treatment regimen produced marked reductions in tissue iron concentrations and hematocrits at birth that did not persist in adulthood, when testing revealed the behavioural abnormalities. That is, behavioural differences were evident in the PID adult male and female rats despite the lack of PID impact on adult tissue levels of iron. Thus, these data reveal the programming effects of PID and not changes associated with reduced brain iron per se, an alteration in adults which is known to cause impaired task acquisition in behavioural studies.\textsuperscript{324}

In the OF apparatus, both PID males and females exhibited reduced exploratory behaviour. However, only PID males spent less time in the center of the OF apparatus compared to their respective controls, a behaviour that has been interpreted as a sign of anxiety and fear.\textsuperscript{422, 423} The finding that the PID females did not display the same aversion to the center of the OF apparatus suggests that their reduced exploratory behaviour is not a consequence of an elevated response to stress, but may be due motor deficits instead. This hypothesis is supported by the observation that PID females had slower swim speeds compared to controls in all phases of the MWM.

The MWM has been important in the study of behavioural neuroscience, particularly as an indicator of altered function of the hippocampus, among other brain regions.\textsuperscript{424} Improvement in the MWM requires numerous integrated processes, including task acquisition, spatial navigation, and implementation of search strategies in addition to memory consolidation.\textsuperscript{421} Thus, disturbances in any of these processes could potentially affect performance. Consequently, analysis of the behavioural patterns in the MWM can provide valuable information about the underlying functional deficits. In the present
study, we used a pre-training phase, involving a visible platform task, to habituate the rats to the novel water environment. Lack of this pre-training has been shown to mask subtle differences in treatment groups. That is, habituation or pre-training to the water maze is important in controlling for other factors that can be misinterpreted as learning or memory deficits (e.g. learning to swim away from the wall to escape the pool).

Furthermore, having a visible platform phase allows for identification of sensorimotor deficits that could impact performance in the MWM. This concept is important given that the present data suggest that there are motor deficits in the PID females, and both Felt et al. and Beard et al. reported that PID rats show signs of sensorimotor deficits using the forelimb placement test.

PID males displayed less improvement in reaching the hidden platform compared to control males in the first 2 days of the invisible platform phase of testing; a finding which indicates delayed task acquisition. This hypothesis is further supported by the finding that PID males displayed no improvement in spatial bias toward the target quadrant until the third day of the invisible platform phase compared to the control males; this analysis controls for the element of chance encounters with the platform. Moreover, despite being critically important in MWM testing as stated above, Iqbal et al. previously demonstrated that the pre-training/visible platform phase can mask the degree of impairment in a different model of fetal insult. Therefore, the true extent of impairment might be revealed by a more complex MWM protocol.

The delayed task acquisition observed in the male PID offspring, much like the differences observed in the OF, may be related to increased anxiety and fearfulness.
Stress has been shown to contribute to learning and memory impairment in the MWM. This notion is further supported by the finding that PID males had increased thigmotaxis (wall seeking behaviour) during the probe trials of the MWM. Increased thigmotactic behaviour has also been interpreted as a sign of anxiety and fear, which would likely be manifested when the platform is removed (i.e. probe trials). Indeed, altered limbic-hypothalamic-pituitary-adrenal (LHPA) axis responsiveness has been reported in a PID model of developmental plasticity.

The underlying mechanisms that are responsible for the locomotor deficits in the females, and the proposed elevated state of anxiety in the PID male offspring are beyond the scope of present study. Nevertheless, numerous alterations in central neurotransmitter signaling have been implicated, in addition to altered growth and development of brain regions involved in influencing behaviour. Interestingly, despite evidence showing an apparent altered responsiveness of the LHPA axis in PID males (i.e. increased anxiety and fear), these same studies indicate that there are no long-term differences in circulating levels of corticotrophin-releasing hormone or corticosterone. It may be that enhanced LHPA axis responses results from an increased density of receptors without a change in hormone levels. Indeed, Iqbal et al. have shown that increased glucocorticoid receptor densities in the hippocampus produce enhanced glutamate release in response to a standard dose of dexamethasone in a guinea pig model of chronic prenatal ethanol exposure.

In summary, we have characterized the behavioural outcomes in a model of developmental plasticity due to PID to provide an important foundation for ongoing
mechanistic studies. An important concept emphasized herein is that changes in maternal iron intake during pregnancy can impact long-term behaviour in the adult offspring, even without persistent differences in brain iron levels in adulthood. Adverse programming effects have been associated with a number of macro and micronutrient deficiencies; however, the worldwide prevalence of ID in humans, especially in pregnant women, makes this an especially relevant form of developmental insult to study.
Chapter 6
Summary and Discussion

6.1 Programming and Iron Deficiency

Twenty-five to 30 years ago, the etiology of disease was though to comprise of two distinct components: genetic factors and environmental factors. The general consensus was that genetic factors, in large part, dictated the predisposition to a number of long-term diseases, and environmental risk factors would potentiate their onset and severity. The developmental origins of health and disease have provided a new paradigm, in which early environmental factors contribute substantially to the initial risk of developing long-term disease. These programming effects are hypothesized to occur via structural and functional changes, and/or epigenetic mechanisms that occur in response to stressors during early development. David Barker’s thrifty phenotype hypothesis and Peter Gluckman’s predictive adaptive responses hypothesis provide a conceptual framework by which these events could take place.\textsuperscript{52, 334}

As discussed previously, iron deficiency (ID) is a global epidemic. Its capacity to impinge on quality of life, and its high prevalence in both industrialized and developing countries help to explain why it ranks among the WHO’s top ten global health risks, and 9\textsuperscript{th} of 26 on the list of Global Disease risk factors.\textsuperscript{285} Indeed, ID accounts for 841,000 deaths and approximately 35,000,000 disability-adjusted life years (DALY) lost per year (DALY are a summation of the years lost to premature death and the years of healthy life “lost” to living in a state of less-than-perfect health).\textsuperscript{332} However, its tendency to afflict
pregnant women and those of child-bearing ages, as well as neonatal infants, make it an especially important model of developmental programming. Indeed, it is in these populations where ID will impact long-term health of the developing offspring, and hence where the most debilitating, and consequently the most expensive outcomes in terms of global health burden, will occur. Hypertension alone accounts for nearly 8,000,000 deaths, and over 90,000,000 DALY per year. Thus, as perinatal ID (PID) increases the risk of hypertension and other cardiovascular risk factors (salt sensitivity, increased visceral adiposity, etc.) the economic impact of its prevention could be vast. This is not even taking into account the potential impact of behavioural and cognitive dysfunction, the impact of which on quality of life could be just as pervasive.

The present series of studies was intended to identify the long-term deleterious effects of PID and provide insights into their mechanisms. The results obtained herein demonstrate that PID causes persistent effects on cardiovascular function (Chapters 3 and 4), as well as on behaviour (Chapter 5). Amidst these studies, a large amount of data was collected pertaining to animals’ growth rates, organ weights, and iron status throughout development and in adulthood. These data formed the basis of Chapter 2. Our initial pilot experiment and two subsequent programming studies (Studies 1 and 2 in Chapter 2) provided the necessary information to develop a suitable model of developmental ID. We believe that the degree of ID in our experimental model closely resembles a large cohort of pregnant women in the population. The two week iron restriction phase prior to gestation was intended to mirror the phase of negative iron balance that is common in women of child bearing ages as previously discussed (see section 1.4.1). This treatment
did not cause overt symptoms of anemia (see Chapter 3), but was designed to partially diminish iron stores prior to pregnancy. Additionally, our final study utilized a moderately low iron diet during gestation, to provide at least minimal iron necessary for growth and development of the embryo or fetus. Although the degree of maternal ID was severe throughout pregnancy, it did not substantially compromise offspring survival (see sections 3.3.1 and 5.3.1). Furthermore, although some offspring did succumb to ID, anemia is also known to be a cause of perinatal mortality in humans,\textsuperscript{297, 298} thus confirming the relevance of our experimental model.

6.2 Cardiovascular Effects of Perinatal Iron Deficiency

The results presented in Chapter 3 are the first data to demonstrate, using direct blood pressure assessments by radiotelemetry, that PID causes persistent elevations in arterial pressure (AP) in adult offspring. Although the observed elevations were modest (~5 mmHg), they are nevertheless relevant from a pathophysiological perspective. Clinical trials, such as the DREAM, HOPE, and ADVANCE studies have demonstrated remarkable reduction in cardiovascular morbidity and mortality associated with small reductions (2-3 mmHg) in AP.\textsuperscript{435-437} Perhaps most interesting was that adult PID offspring exhibited, in addition to increased AP, salt sensitivity, a feature that has been independently associated with increased risk of cardiovascular disease.\textsuperscript{105} Moreover, the fact that these observations were made in young rats, aged approximately 11-16 wk of age, is particularly important. Based on the findings that adult PID offspring accumulate visceral adipose tissue in later life (see Chapter 2), and both age and obesity are
independent risk factors for cardiovascular disease, there is a high probability that the cardiovascular consequences of PID will worsen over time. Interestingly, ageing is associated with impairment of endothelial function as well as intrinsic alterations in alpha-receptor mediated vasoconstriction; this is of interest since these vascular functions are altered in PID offspring even at a young age (Figure 4.3), and will likely worsen over time. Together, these cardiovascular effects further emphasize the importance of PID as a model of developmental programming.

The results presented in Chapter 3 are also the first to demonstrate that intrinsic alterations in intrarenal hemodynamics may underlie the cardiovascular effects in this model of programming, and thus the first to demonstrate a definitive link between PID and alterations in kidney function. Lisle and colleagues have shown that nephron endowment and total glomerular filtration area are reduced as a consequence PID. However, as previously discussed, there are no studies that unequivocally link nephron endowment with hypertension. In fact, several programming models that exhibit hypertension have only modest decreases in nephron number, or none at all. Moreover, some models have found a reduced AP associated with decreased nephron number. Although various perinatal stressors cause a reduction in nephron number as well as cardiovascular dysfunction, these may occur independently of one another by a common etiological factor, thus giving the appearance of causality. Interestingly, uninephrectomy immediately after birth in normal rats leads to eventual salt sensitivity and renal disease. Although these data suggest a potential mechanistic link between nephron endowment and hypertension, the salt sensitivity could also be a
consequence of other renal structures or mediators removed during the nephrectomy at birth.

The data from Chapter 3 nevertheless demonstrate the involvement of the kidney in the long-term programming of cardiovascular dysfunction by PID. Based on various independent lines of evidence that demonstrate that nitric oxide synthase (NOS) activity, particularly within the kidney, plays an important role in regulating AP (see Chapter 1, section 1.2.3), we chose to investigate nitric oxide (NO) signaling as a potential mechanism of cardiovascular dysfunction in PID offspring. This approach was also based, in part, on the observations that acute ID causes alterations in NOS and soluble guanylyl cyclase (sGC) signaling (Chapter 4, Study 2). However, no such increases in NOS activity were observed in the neonates, despite being severely anemic. In fact, NOS activity was found to be decreased at PD 21. From these data, it may be concluded that altered NOS signaling during development is not responsible for the long-term programming effects of PID. Nevertheless, data have shown that the relative contributions from the NOS isoforms (eNOS, nNOS and iNOS) vary considerably throughout the neonatal period, with potential developmental consequences. Thus, although it appears that overall NOS is not affected, the relative expression patterns of the various isozymes may be altered, with important implications on the generation of hypoxia, and reactive oxygen species (ROS), as well as controlling other systems, such as the RAS. NOS signaling was found to be altered in the kidney and vascular tissues in adult PID offspring. However, evidence obtained from an experimental model of heme-
deficiency (Appendix 1) suggests that the model reductions in intrarenal NOS activity *per se* are insufficient to account for the cardiovascular effects observed in PID animals.

It is possible that the altered NOS signaling may not be a causal factor in the cardiovascular dysfunction, but may instead be a consequence of an underlying metabolic dysfunction. The most interesting results obtained from Chapter 2, from a potential etiological standpoint, are those that pertain to the increased visceral adipose tissue (VAT) deposition and diminished locomotor activity in the PID offspring. It is particularly noteworthy that these outcomes were observed without feeding the offspring a high fat diet. The standard rodent chow given to these animals consists of only 13.5% fat energy, which is likely designed to prevent obesity in these animals. In several programmed models, feeding the offspring a high-fat diet (which is more akin to an American diet that consists of 35-45% fat energy) accentuated the differences in metabolic function, causing greater fat accumulation in programmed animals than controls. Consequently, if the present animals had been fed a high-fat diet in adulthood, it is anticipated that the obesity would have been more pronounced, along with the deleterious cardiovascular effects that accompany increased visceral adiposity (see below).

At its most fundamental level, increased fat deposition represents an imbalance between energy intake and energy expenditure. Thus, the increased VAT in the PID offspring suggests intrinsic alterations in energy homeostasis. This imbalance could occur either at the level of central nervous system, wherein appetite as well as energy storage and utilization is controlled, or within the periphery via altered signaling of
various hormones (leptin, insulin, etc.). However, the idea that there may be intrinsic alterations in mitochondrial biogenesis and function in PID animals is a particularly intriguing hypothesis that warrants further investigation. In obese human subjects, as well as genetic models of obesity (leptin-deficient mice \(ob/ob\)), altered mitochondrial function and biogenesis, particularly in tissues such as skeletal muscle and adipose tissue, has been implicated. It has been suggested that diminished mitochondrial oxidative respiration, and in turn decreased systemic energy availability leads to sedentarity, increased food consumption and increased fat storage, thus contributing to the pathophysiology of metabolic syndrome. Indeed, Taylor et al. have shown that certain models of developmental programming (offspring born to dams fed a high-fat diet during pregnancy) have reduced metabolic rates and mitochondrial copy number. Whether this is a common outcome in programmed offspring, or unique to this particular model is not presently known. Interestingly, several lines of evidence, including studies with knockout mice, indicate that eNOS has an important role as a regulator of mitochondrial biogenesis. The findings that NOS activity in the kidney and in the vasculature is functionally altered suggests a potential link between PID and long-term metabolic dysfunction through altered mitochondrial biogenesis. A reduction in mitochondrial biogenesis and function, and the consequent lack of energy production could account for the diminished locomotor activity observed in these animals.

Although the involvement of reduced mitochondrial biogenesis and function in conditions in which there is VAT accumulation is purely speculative, the pathophysiological consequences of abdominal obesity are well known. Results from the
Framingham study suggest that 78% of essential hypertension in men, and 65% in women have a component that can be directly attributed to obesity. Furthermore, weight loss in obese patients nearly always causes a reduction in AP. Obesity is also known to impact renal medullary structure and function, presumably by infiltration of perirenal fat. Furthermore, fat accumulation has been shown to impact the pressure-natriuresis mechanisms, thus altering of sodium reabsorption and blood pressure regulation. This may explain why obesity is very closely associated with salt sensitivity. In the PID model, the increased adiposity was correlated with the degree of salt sensitivity (Figure 6.1), providing further support for this concept. However, it is impossible to establish causality in the current model, because it is not presently known whether the cardiovascular events preceded or occurred subsequent to the increased visceral adiposity in the PID offspring.

Another system that has not been examined in the present studies but warrants brief discussion is the renin-angiotensin system (RAS). Various programming models, including offspring of dams prenatally treated with glucocorticoids, as well as protein-restricted dams, are associated with alterations in both peripheral and central nervous system RAS activity. These changes are believed, by some, to underlie the hypertension in these models, based on observations that postnatal treatment with angiotensin converting enzyme inhibitors prevents the development of hypertension. However, due to conflicting results and temporal discordances in effects, the role of the RAS is not presently clear. In adult PID offspring, altered activity of the RAS has been reported. In our own studies, pharmacological inhibition of the RAS using
Figure 6.1. Correlation between responsiveness of mean arterial pressure (MAP) to dietary salt (high salt [HS] versus low salt [LS] diet) and visceral adipose tissue (VAT) (normalized to body weight [BW]) in 36 wk-old offspring. Average MAP responsiveness following salt challenge and percent VAT to BW was significantly higher in PID (open square) versus control (closed square) animals (P < 0.05).
AT1 receptor antagonist losartan caused a more pronounced lowering of AP in PID animals, although this effect was not quite significant (P = 0.07), possibly due to the number of animals used (Figure 6.2). These data suggest the possibility of enhanced RAS activity, although this will require confirmation and further investigation.

Disturbances in intrarenal RAS may also be a potential mechanism for PID induced programming, as it is known to play an important role in kidney development. Rats that are treated with angiotensin converting enzyme inhibitors during the neonatal period develop gross morphological and functional changes in the kidney, including altered vascular development, decreased tubular formation, and papillary atrophy, as well as severely diminished capacity to concentrate their urine. Interestingly, these animals develop subtle alterations in circulatory function, characterized by alterations in renal interstitial hydrostatic pressure and a blunted in vivo renal function curve, akin to those observed in the present PID model. As alterations in nephrogenesis have been reported in this model, it may be that certain perinatal stressors, such as ID, cause down regulation of intrarenal RAS, which impacts kidney development, and hence long-term renal function. Offspring of protein restricted dams exhibit reduced intrarenal RAS immediately after birth, and have reduced nephron numbers and hypertension in adulthood. Both tissue specific and systemic RAS are now known to control erythropoiesis. Although the impact of ID on RAS activity has not been described, it is reasonable to assume that the RAS system would be responsive to such a stimulus if it does indeed play an important role in controlling erythropoiesis. Alternatively, an
Figure 6.2. Effect of PID on mean arterial pressure (MAP) responsiveness to increasing doses of losartan (Los), an AT1 receptor antagonist, in 18 wk-old male offspring. (A) MAP profiles, and (B) summarized MAP changes from baseline, assessed in animals treated as described in Chapter 3. Repeated measures 2-way ANOVA revealed an overall losartan dose effect (P < 0.001), and a trend towards a PID treatment effect (P = 0.07). Data are mean ± SEM. Control: n = 6; PID: n = 4.
upregulation of RAS activity during renal development could also be implicated. It may be that ID during early development causes over expression of RAS, exacerbating the intrarenal hypoxia caused by anemia,\textsuperscript{470} and contributing to ROS production,\textsuperscript{471} thereby affecting kidney development and long-term function.\textsuperscript{472} However, the effect of ID on RAS activity in either adult or neonatal rats has not been studied.

Another mechanism by which maternal ID could impact long-term cardiovascular function in the offspring is by generating fetal hypoxia. Prolonged maternal iron restriction causes fetal anemia, which indicates that there is insufficient iron delivery to maintain fetal erythropoiesis. Nevertheless, Lewis et al. have shown that PID was not associated with upregulated hypoxia-inducible factor 1α (HIF-1α),\textsuperscript{375} suggesting oxygen delivery was not compromised in these animals. However, it is noteworthy that only gene expression was examined in this study; protein expression levels would have provided greater insight into this model.

It is not presently clear what degree of fetal anemia is necessary to compromise oxygen delivery, although it is likely that this will vary depending on the stage of gestation. Adult hemoglobin (e.g. HbA) is capable of increasing oxygen delivery to tissues in cases of anemia; this cooperativity is mediated by increased binding of 2,3-bisphosphoglycerate (2,3-BPG), which associates with Hb to reduce its affinity for oxygen. In contrast, fetal hemoglobin (HbF) does not associate with 2,3-BPG, as a means to maximize oxygen sequestration from maternal Hb during gestation. Thus, during anemia, lack of increased cooperativity mediated by 2,3-BPG prevents increased
delivery of oxygen to fetal tissues. Therefore, the relative amounts of HbF and HbA (or other adult Hb) will likely impact the degree of hypoxia during fetal anemia.

Fetal hypoxia has been shown to cause persistent changes in cardiac and vascular function in rats and sheep.\textsuperscript{405, 473-476} Although the precise mechanisms by which fetal hypoxia causes programming effects remain elusive, potential candidates include altered gene expression, disturbed angiogenesis, and generation of reactive oxygen species, in addition to those described above. These mechanisms will not be discussed herein; for reviews, see References 477 and 478.

6.3 Behavioural Effects of Perinatal Iron Deficiency

As previously discussed, although the cardiovascular effects may have a profound impact on the global health, the long-term behavioural and cognitive dysfunction associated with perinatal stressors are potentially the most debilitating. Indeed, PID impacts cognition, attention and focus, and may also impact a person’s capacity to cope with stress.\textsuperscript{320, 390, 430, 479} Although Chapter 5 did not dissect any mechanism associated with the behavioural deficits, it demonstrated that PID deficiency causes long-lasting effects in the absence of persistent reduced levels of brain iron. This was important, because studies have shown the lasting effects of brain iron depletion in adult animals.\textsuperscript{324} In addition, this study demonstrated that the behavioural effects of PID are sexually dimorphic, which is a common theme in the developmental programming literature (see section 1.1.3.4). Finally, these data provide support for the involvement of altered limbic-hypothalamic-pituitary-adrenal (LHPA) axis function, which has been previously
reported in this developmental programming model. A heightened stress response could have important implications for the health of the offspring. Indeed, enhanced anxiety in the PID offspring could explain, in part, the exaggerated differences in blood pressures reported by other groups using the tail-cuff, which as described in Chapter 3, is highly influenced by restraint and thermal stress.

The specific mechanisms by which PID impacts LHPA axis function axis are currently unknown, although reports have shown that acute ID can alter its regulation. Whether iron restriction impacts directly on this system during development, or whether it is due to an indirect effect of ID (e.g. hypoxia) requires further investigation. Nevertheless, the programming effects of increased maternal LHPA activity are well known and have received considerable attention, largely due to clinical relevance of this model of maternal insult; for a review on this subject see Seckl. Synthetic glucocorticoids, such as dexamethasone and beclometasone, are used in cases of impending or imminent pre-term delivery, to promote maturation of critical organs (e.g. lungs). The outcomes of prenatal glucocorticoid excess include cognitive dysfunction, altered responsiveness to stress, as well as metabolic and cardiovascular outcomes. Other insults, including maternal psychological stress, nutrient restriction, alcohol exposure, and immune challenge, are also associated with increased LHPA activity during pregnancy, with similar consequences in offspring, demonstrating that these effects are not solely iatrogenic. An alteration of the LHPA axis as a potential mechanism for programming by stressors is compelling for a number of reasons. First, glucocorticoids are involved in the development and maturation of neural, metabolic, and
cardiovascular circuits during gestation and after birth. Second, during development, the LHPA axis is known to be highly plastic, thus prone to perturbations by maternal insults. Finally, steroids in general are known to account for sexually dimorphic phenotypes. Thus, alterations in this system could represent a convergent mechanism by which various maternal stressors impact development of critical organs and systems. This could explain, at least in part, the similarities among programming insults.

6.4 Concluding Remarks

A theme that invariably arises in DOHAD is the remarkable similarity between long-term programming outcomes, despite the apparent differences in the nature of the perinatal stressors. As discussed in the preceding sections, proof of the involvement of common mechanisms, including the LHPA axis and the RAS, may represent an elegant solution to this conundrum. Thus, ID, maternal nutrient imbalances, prenatal glucocorticoid treatment, maternal preeclampsia, neonatal hypoxia, and possibly maternal gestational diabetes, may represent different stimuli that impact function of a limited number of maternal or fetal systems during pregnancy and development, thereby resulting in a limited number of long-term programming outcomes.

Although these various programming models share many features, there is one important distinction. Unlike maternal glucocorticoid exposure, maternal nutrient restriction, preeclampsia, gestational diabetes, pregnancy induced hypertension, ID is preventable. Since PID does not appear to have any logical benefits, as in the case of prenatal glucocorticoid treatment, its prevention could be instituted with minimal effort.
The high proportion of pregnant women that are ID in industrialized countries is an obvious target if the goal is to prevent a large burden on future health care. Rather than coping with the incidence of cardiometabolic disease caused by PID, it would be far more cost effective to systematically begin iron supplementation before pregnancy or early in gestation. Iron supplementation with 40 mg/day has been shown to reduce ID during pregnancy in >90% of women in developed countries.\textsuperscript{492} Even in full term infants, iron supplementation with either iron containing foods or vitamins, is recommended beyond 6 months due to the relatively low levels of iron in the breast milk (<1 mg Fe/L).\textsuperscript{294} Furthermore, beginning iron supplementation after 1 month reduced the risk of ID at 13 months, and improved neurodevelopment indices at 13 months.\textsuperscript{493}

Current practices rely on hematological indices of iron status. However, use of these markers may not be useful since the fetus and neonate, like adults, prioritize iron for erythropoiesis, at the expense of tissue iron levels\textsuperscript{294} (Figure 2.3). Thus, the fetus can experience severe iron depletion, without manifestations of overt anemia. Other markers of iron status (such as zinc protoporphyrin, soluble TfR, ferritin levels, hepcidin and prohepcidin levels) may not be adequate either, because they may reflect periods of enhanced intrauterine erythropoiesis, rather than iron status.\textsuperscript{494} Thus, it is likely a combination of various markers would provide better indices. However, without an awareness of the potential deleterious effects of PID, clinicians may be less inclined to perform proper assessments of maternal and fetal iron status.
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Appendix A

Lack of Hemodynamic Effects of Extended Heme Synthesis Inhibition by Succinylacetone in Rats

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Abstract

Heme is a key component of numerous enzymes involved in arterial pressure (AP) regulation, including nitric oxide synthase (NOS) and its downstream mediator soluble guanylyl cyclase (sGC). As NOS and sGC mediate vasodilation, it was hypothesized that interference with the heme supply to these enzymes would result in hypertension. The purpose of the present study was to determine if an extended treatment with succinylacetone (SA) would impact the cardiovascular capacities of NOS and sGC and result in the induction of a measurable hypertensive response. Male, Sprague-Dawley rats were treated with SA (40mg/kg i.p.) for 14 days. This treatment resulted in decreased levels of circulating heme (e.g. hematocrits, hemoglobin levels, mean corpuscular volume, P < 0.001), as well as diminished heme content in the kidney, liver, spleen and mesenteric vasculature (P < 0.05). Systemic NOS activity was reduced by 37% after 10 days of treatment (P < 0.05), and 60% by 14 days of treatment. Renal NOS and sGC activities in vitro were decreased by 32% (P < 0.05) and 38% (P < 0.01) after SA treatment, respectively. SA administration also compromised the ex vivo sensitivity of aorta to endothelium-dependent and -independent vasodilation (P < 0.001). SA treatment failed to induce any change in AP at any time during the 14 day treatment, as assessed by radiotelemetry. Moreover, AP profiles in the SA treated animals were less responsive to altered sodium intake, as well as NOS inhibition. The present results demonstrate that extended inhibition of heme synthesis with SA impacts hemoenzyme function, albeit without consequent effects on AP regulation and sodium excretion.

Key Words: Heme; Nitric Oxide; Guanylate Cyclase; Blood Pressure; Kidney.
Introduction

Heme is an important component of many enzymes, several of which are involved in controlling arterial pressure (AP), and include, for example, nitric oxide synthase (NOS), soluble guanylyl cyclase (sGC), heme oxygenase (HO), and cytochromes P450. In the context of hypertension, the NOS/sGC system is particularly interesting because it comprises a major dilatory mechanism for blood vessels. NOS employs heme at its active site to convert L-arginine to L-citrulline, releasing nitric oxide (NO) as a co-product. sGC utilizes heme as a regulatory prosthetic group allowing it to be activated by NO.

On the basis of these underlying mechanisms, we proposed that experimental conditions that render the cardiovascular system heme deficient would lead to a hypertensive tendency. Indeed, it is well established that certain clinical conditions in which heme synthesis is disturbed are associated with elevated blood pressure. For example, more than half of patients who suffer from porphyria, a genetic disease in which one or more enzymes required for heme biosynthesis are impaired, are also hypertensive. Similarly, in cases of lead poisoning, wherein lead toxicity disrupts heme biosynthesis, hypertension is a common outcome.

The purpose of the present studies was to examine the effects of systemic heme synthesis inhibition, with a particular emphasis on the kidney. It is widely acknowledged that the kidney plays a critical role in establishing the long-term set-point of arterial blood pressure by regulating sodium and water balance, and therefore blood volume. Hemoenzyme activities within the kidney, such as NOS, have an important role in
regulating medullary hemodynamics to control the intrinsic pressure-natriuresis system. Indeed, in models of salt-sensitivity (e.g. Dahl-SS rat), where the pressure-natriuresis relationship is known to be blunted, there is reduced medullary activity of NOS, and other hemoenzymes. Moreover, administration of the NOS substrate, L-arginine, has been shown to abrogate this salt-sensitive phenotype, suggesting that diminished production of NO may be a causal factor.

In a previous study, we found that a four-day treatment of rats with succinylacetone (SA), an irreversible inhibitor of δ-aminolevulinic acid dehydratase (ALA-D), caused substantial depletion of heme in various organs (liver, kidney, aorta, mesentery) and partially disrupted the function of hemoenzymes important in blood pressure control. Nevertheless, we did not observe the development of elevated blood pressure in these animals. We reasoned that the degree of SA-mediated inhibition was not sufficient to elicit a cardiovascular response, and undertook the present studies to determine the effects of extended treatment with SA. Thus, the purpose of the present study was to explore the hypothesis that extended heme depletion impairs hemoenzyme function and alters blood pressure regulation in vivo. We investigated the effects of a ≥14-day treatment with SA on (i) heme levels and hemoenzyme function in organs with a particular emphasis on the kidney, (ii) the ex vivo responsiveness of blood vessels to NOS-dependent and –independent agonists, (iii) resting hemodynamic parameters, and (iv) arterial pressure responsiveness to alterations in salt intake and NOS inhibition.
Methods and Materials

Animals and Treatments

Study 1: Heme content, hemoenzyme activity and ex vivo assessment of vascular function studies.

Adolescent male Sprague-Dawley rats (150-175g) were obtained from Charles River Canada (Montreal, QC), and housed in Queen’s University Animal Care Facility on a 12 h light/dark cycle (07:00 h and 19:00 h). Rats had ad libitum access to a standard rodent diet (Lab Diet, St-Louis, MO, No. 5001) and water. Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocols were approved by the Queen’s University Animal Care Committee. After one week of acclimatization, rats were injected twice daily with SA for 14 days (40 mg/kg, i.p., q12h @ 10:00 h and 22:00 h). This dosage was shown to produce >90% inhibition of ALA-D activity in rat liver. On day 15, rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.), blood was collected from the lower vena cava into EDTA-coated Vacutainer® tubes (BD Medical, Franklin Lakes, NJ), for hematological assessments (Kingston General Hospital). Rats were then heparinized and perfused with ice-cold saline until livers and kidneys were blanched. Kidneys, liver, spleen and mesenteric vessels were excised, cleaned of extraneous connective tissue, weighed, and snap frozen in liquid nitrogen and stored at -80°C until processed. Aortas were also removed and used immediately for assessment of responsiveness to vascular agonists as described below.
For systemic nitrate assessments, rats were placed in metabolic cages after 4, 7, 10 and 14 days of SA treatment and urine was collected for 24 h. Urine samples were centrifuged at 10,000xg, and the supernatants were treated with 2-propanol (6.5% v/v) to prevent bacterial growth, snap-frozen in liquid nitrogen, and stored at -80°C.

**Study 2: Arterial pressure responsiveness to salt and NOS inhibition**

A separate cohort of rats (150-175 g), after acclimatization, were surgically instrumented with radiotelemetric pressure transducers, as previously described.19 Rats were then given 10 days to recover, after which began a four-day baseline recording period, prior to the subsequent SA treatment. SA was administered twice daily (40 mg/kg, i.p., q12 h @ 10:00 and 22:00) via an indwelling catheter (to reduce stress responses associated with intraperitoneal injections) beginning when the rats were approximately 10 weeks of age. After the first 14 days of treatment with SA, manipulations of sodium intake began, consisting of a 4-day low-sodium treatment regimen, followed by a 5-day high-sodium treatment regimen. For the low-sodium regimen, rats had *ad libitum* access to a low-sodium (0.04% Na⁺) purified diet (Research Diets Inc. New Brunswick, NJ) based on the AIN-76A rodent diet, as well as tap water. For the high-sodium treatment regimen, rats had *ad libitum* access to the standard grain-based rodent diet described above (0.4% Na⁺), as well as drinking water supplemented with 1% NaCl (w/v). After five days of high-sodium intake, the drinking water was supplemented with L-NAME in addition to the 1% NaCl, such that the animals ingested L-NAME at 1mg/kg for 3 days, 3mg/kg for 3 days, and 30mg/kg for 2 days. Body
weights, as well as food and water intake were monitored daily during these treatments. Upon completion of the telemetry studies, the rats were euthanized, and organs were collected as described above.

**Measurement of heme depletion and hemoenzyme activity**

Rat tissues were homogenized in ice-cold 20 mM phosphate buffer pH 7.4 with a Complete Mini®, EDTA-free protease inhibitor tablet (Roche Diagnostics, Laval, QC). Samples were centrifuged for 10 min at 10,000 x g to remove cellular debris, frozen in liquid nitrogen, and stored at -80°C until analyzed. Heme content in the liver, kidney, mesentery, and spleen homogenates were assessed using the fluorometric method of Morrison et al.²⁰ Hematocrits were assessed by collecting a small sample of blood (50μL) into a heparinized microcapillary tube, followed by centrifugation at 11,500 x g, for determination of packed erythrocyte content.

Urine nitrate levels were quantified by a colourimetric non-enzymatic NO assay kit (No. NB-88, Cedarlane Labs Ltd., Burlington, ON). Optimized *in vitro* activities of NOS were assessed in kidney homogenates by the radiometric assay of Kimura et al.²¹ Soluble GC activity was assessed in kidney cytosolic fractions as described by Kinobe et al.²² using an enzyme immunoassay (EIA) cGMP detection kit (Cayman Chemical Company, Ann Arbor, MI). For sodium nitroprusside (SNP)-induced sGC activity, kidney cytosolic fractions were preincubated with 100μM SNP for 10 min.
*Ex Vivo Assessment of Vascular Function*

Rat, isolated aortas obtained as described under *study 1* were used to evaluate concentration-dependent effects of phenylephrine (PE), acetylcholine (ACh), and methylamine hexamethylene methylamine NONOate (MAHMA-NONOate). Thoracic aortas were cut into rings 4 mm in width and mounted in 10 mL tissue baths, containing Krebs’ bicarbonate solution aerated with 95% O₂-5% CO₂ at 37°C (see Ref. 23 for recipe). Data were obtained using a Powerlab® data acquisition system (AD Instruments) and displayed using Chart (version 5) software. Tissues were allowed to equilibrate at a resting tension of 9.8 mN for one hour, with rinses every 15 min, prior to testing. PE concentration-response relationships were obtained by adding increasing doses of PE to the bath (100pM to 30μM, in approximately 3-fold increments). Aortic rings were then rinsed until they returned to baseline. For concentration-response relationships for ACh and MAHMA-NONOate, aortic rings were submaximally contracted (50-80%) with PE prior to administration of vasodilators. After steady precontraction, increasing concentrations of vasodilators (100pM to 300μM for ACh, and 1pM to 30μM for MAHMA NONOate, in approximately 3-fold increments) were then added to the tissue baths. Tissues were rinsed and allowed to return to baseline over a 30 min period between ACh and MAHMA NONOate testing.

*Statistical Analyses*

Heme content, and enzyme activities were analyzed by unpaired Student’s *t* test. Changes in BP due to sodium manipulations were also analyzed by Student’s *t* test for
each treatment. All time-dependent measurements (i.e. systemic nitrate levels, as well as data obtained by radiotelemetry) as well as telemetry data consisting of increasing doses of L-NAME, were analyzed using a repeated measures 2-way analysis of variance (ANOVA); when significant differences were found, 1-way ANOVA with Newman-Keuls post-hoc test or Student’s t test was conducted on data sets, as appropriate. MAP profiles assessed by radiotelemetry are presented as the difference from the last day of baseline, to account for differences in baseline recordings between animals and groups. Telemetry data presented herein were obtained between the hours of 22:00 and 04:00, which corresponds to the night phase of the light cycle in the Queen’s Animal Care Facility, and hence the period of greatest activity in rats. For ex vivo assessment of vascular function, exclusion criteria were established prior to experimentation to ensure that the tissues were viable. Thus, aortic rings that did not achieve an initial minimum of contraction of 19.6 mN or did not achieve a minimum of 50% relaxation with ACh were deemed to be compromised, and were excluded from all analyses. Data are presented as mean ± SEM. Grubb’s test was conducted on data sets to identify statistical outliers. N values represent number of animals used. P < 0.05 was considered statistically significant.

Results

Depletion of heme by succinylacetone (study 1)

SA treatment for 14 days caused a significant decrease in heme content in all tissues analyzed (Table 1); heme concentration was diminished in liver by 63% (P < 0.05),
spleen by 47% (P < 0.05), kidney by 58% (P < 0.001) and mesenteric vessels by 31% (P < 0.05). SA treatment also induced decreases of 14%, 16% and 7.5% of Ht, Hb levels, and mean corpuscular volume (MCV), respectively (P < 0.001 for all parameters). All other hematological parameters assessed, including counts for erythrocytes, leukocytes, and platelets, as well as circulating iron levels, were not significantly different from untreated controls. SA-treated rats also had significantly reduced body weights at the end of the 14-day treatment period (Control: 324.1 ± 3.9g vs. SA: 300.6 ± 8.2g; P < 0.05).

After seven days of SA treatment, urinary output of nitrate, a surrogate for NOS, was significantly diminished and by day 14 urine nitrate output was 72% lower than controls (P < 0.001) (Figure 1A). Similarly, NOS activity as assessed in kidney homogenates, was 32% lower in rats treated with SA for 14 days compared to saline treated controls (P < 0.05) (Figure 1B). Basal and SNP-induced sGC activity in cytosolic fractions of SA-treated animals were 38% (P < 0.01) and 47% (P < 0.001) lower than controls, respectively (Figure 1C). In both SA-treated and control samples production of cGMP after stimulation with SNP was approximately 5-fold higher than basal production levels (P < 0.001).

SA treatment had no effect on the sensitivity of the rat aorta to PE as the two concentration response curves were superimposable (Figure 2A). In contrast, blood vessels of SA-treated animals showed diminished sensitivity to ACh, as indicated by an approximate 12-fold rightward shift in EC50 (P < 0.001) (Figure 2B). Similarly, the aortas of SA-treated animals had decreased sensitivity to MAHMA-NONOate, as shown
Table 1. Effect of two-week succinylacetone treatment on tissue heme content and hematology in rats.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver heme (mg/g)</td>
<td>0.51 ± 0.11</td>
<td>0.19 ± 0.03*</td>
</tr>
<tr>
<td>Spleen heme (mg/g)</td>
<td>23.5 ± 4.5</td>
<td>12.5 ± 1.8*</td>
</tr>
<tr>
<td>Kidney heme (mg/g)</td>
<td>0.45 ± 0.054</td>
<td>0.19 ± 0.02†</td>
</tr>
<tr>
<td>Mesenteric heme (mg/g)</td>
<td>0.029 ± 0.002</td>
<td>0.020 ± 0.001*</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.439 ± 0.009</td>
<td>0.376 ± 0.011†</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.99 ± 0.31</td>
<td>12.6 ± 0.32†</td>
</tr>
<tr>
<td>Mean corpuscular volume (fL)</td>
<td>61.7 ± 1.5</td>
<td>57.1 ± 0.8†</td>
</tr>
<tr>
<td>Erythrocytes (x10^12/L)</td>
<td>7.13 ± 0.19</td>
<td>6.60 ± 0.23</td>
</tr>
<tr>
<td>Leukocytes (x10^9/L)</td>
<td>10.9 ± 1.7</td>
<td>7.8 ± 2.4</td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td>905 ± 55</td>
<td>902 ± 100</td>
</tr>
<tr>
<td>Iron (mol/L)</td>
<td>31.6 ± 4.7</td>
<td>38.6 ± 3.1</td>
</tr>
<tr>
<td>Total iron binding capacity (mol/L)</td>
<td>80.8 ± 3.0</td>
<td>79.9 ± 2.5</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>39.8 ± 6.8</td>
<td>49.0 ± 5.47</td>
</tr>
</tbody>
</table>

Control: n=7; SA treated: n=8. *P < 0.05, †P < 0.001 vs. controls. SA, succinylacetone.
Figure 1. Impact of two-week succinylacetone (SA) treatment on (A) urine nitrate excretion, (B) nitric oxide synthase activity in kidney homogenates, and (C) soluble guanylyl cyclase activity in kidney cytosolic samples. Control: n = 7; SA-treated: n = 7. *P < 0.05, **P < 0.001 vs. controls; †P < 0.05 vs. day 4 in SA-treated rats.
Figure 2. Effect of 2 wk succinylacetone (SA) treatment did not alter the sensitivity of rat aorta to (A) phenylephrine (PE), but altered sensitivity to (B) acetylcholine (Ach) and (C) methylamine hexamethylene methylene NONOate (MAHMA-NONOate). Rats were treated with SA for two weeks; controls received saline vehicle. For details on protocol, see the methods section. *P < 0.05, ***P < 0.001 vs. controls. Control: n = 7; SA-treated: n = 7.
by a 3.5-fold increase in EC₅₀ compared to control animals (P < 0.001) (Figure 2C).

**Hemodynamic assessments (study 2)**

The effect of SA treatment on MAP, obtained by radiotelemetry, is presented in Figure 3. These data represent night-time assessments (see methods section) although similar effects were observed during the daytime recordings, as well as when data were assessed as 24h mean values (data not shown). SA treatment did not impact MAP levels during the initial 14-day treatment period (P > 0.05). Low sodium treatment caused a significant decrease in MAP (compared to the net change from baseline on days 11-14 of SA-treatment) in the SA-treated group (P < 0.025; α = 0.025), although this decrease was not quite significant in the control group (P = 0.025; α = 0.025). High sodium treatment caused a significant increase in MAP in both treatment groups (P < 0.01 for both groups; α = 0.025). There were, however, no significant differences in MAP responses with low salt or high salt intake between treatment groups (Figure 4A). Interestingly, SA-treated animals had a trend for diminished MAP responses with increased sodium intake (P = 0.08), as well as the total MAP change from low sodium to high sodium (P = 0.08). The resultant in vivo renal function curve derived from these data, which takes into consideration the amount of sodium consumed, revealed a significantly steeper slope in the SA treated animals compared to controls (P < 0.05) (Figure 4B). Combination of 30mg/kg L-NAME with high sodium intake caused a significant increase in arterial pressure (compared to the net change from high sodium treatment) (P < 0.001), although there were no differences between treatment groups (Figure 5).
Figure 3. Effect of succinylacetone (SA) treatment (40 mg/kg, i.p. twice daily) or saline control on mean arterial pressure (MAP), and responses to changes in sodium intake and nitric oxide synthase inhibition, as assessed by radiotelemetry. Data represent MAP differences from baseline. SA, succinylacetone treatment phase; LS, SA treatment with low sodium; SA + HS, SA treatment with high sodium; +1L, SA treatment with high sodium, and 1 mg/kg L-NAME; +3L, SA treatment with high sodium and 3 mg/kg L-NAME; +30L, SA treatment with high sodium and 30mg/kg L-NAME. Note that controls were treated with saline instead of SA during all periods. Control: n = 7; SA-treated: n = 7.
Figure 4. Impact of succinylacetone (SA) treatment on (A) mean arterial pressure (MAP) responses to changes sodium intake, and (B) the resultant in vivo renal function curves derived from these data. Data represent changes in hemodynamic parameters assessed at night (see methods for details). All bars in (A) are significantly non-zero (i.e. changes with sodium intake are different from baseline) *P < 0.05 compared to control slope. †P < 0.05, ‡P < 0.01 compared to baseline value. Control: n=5; SA-treated: n=6.
Figure 5. The impact of succinylacetone (SA) treatment on mean arterial pressure (MAP) responses to high-sodium intake plus increasing doses of L-NAME. Control: n=5; SA-treated: n=6. HS, high sodium; HS+1, high sodium + 1mg/kg L-NAME; HS+3, high sodium + 3mg/kg L-NAME; HS+30, high sodium + 30mg/kg L-NAME.


Discussion

In the present study, extended treatment with SA (≥14 days) was used to inhibit heme synthesis and produce a state of marked heme deficiency to characterize the role of hemoenzymes in cardiovascular function. This study tested the hypothesis that extended heme depletion would impair hemoenzyme function and alter blood pressure regulation in vivo. The major findings of this study were that SA treatment for ≥14 days resulted in (i) reduction in tissue and blood heme concentration, (ii) impairment of systemic and renal NOS and sGC activity (iii) reduced ex vivo sensitivity of blood vessels to NOS-dependent and -independent vasodilators. Despite the impact of SA treatment on systemic and intrarenal hemoenzyme function, no cardiovascular dysregulation was observed, either under normal circumstances, or when challenged with low and high sodium intake, or when challenged with high sodium and L-NAME treatment. In fact, AP was less responsive to altered sodium intake in the SA-treated group compared to controls.

This extended treatment with SA (≥14 days) resulted in greater depletion of heme throughout the body compared to a four day treatment. The impact on hemoenzyme activities was also greater in the present study. Although SA treatment for four days did not alter urinary nitrate/nitrite levels, there was some degree of inhibition at seven days and even more after 14 days (Figure 1A). As urinary nitrate/nitrite output reflects systemic and renal NO production, it appears that prolonged inhibition of heme synthesis is required to observe significant effects on blood pressure. This concept is supported by finding that SA impacted negatively sGC activity in the kidney; whereas four days of SA
17 14 days of SA treatment resulted in significantly impaired sGC activity whether the activity was measured with or without SNP stimulation.

The hypothesis presented herein predicted that blood vessels from SA-treated animals will be less sensitive to heme-dependent vasodilators. The blunted sensitivity of aortic rings from SA-treated rats to a NOS-dependent vasodilator (ACh) is consistent with the notion that there was reduced NOS and/or sGC activity in these tissues. Moreover, the reduced sensitivity to the NO-like donor MAHMA-NONOate, which acts via direct activation of sGC, suggests reduced sGC activity due to SA treatment. The greater effect of SA treatment on ACh sensitivity compared with MAHMA-NONOate follows since ACh-induced responses require two heme-containing enzymes, while MAHMA-NONOate requires only one. These observations are taken as an indication of altered endothelial and vascular smooth muscle function induced by SA treatment, which could impact cardiovascular function.24-26

The lack of changes in AP observed after extended inhibition of heme biosynthesis is interesting, given the critical role of intrarenal hemoenzymes in arterial pressure regulation. Indeed, chronic administration of L-NAME causes dramatic, sustained elevations in AP.27,28 Furthermore, administration of even subconstrictor doses of NOS inhibitors into the medulla has been shown to influence urinary output as well as AP,29-31 clearly implicating NOS in the control of pressure-natriuresis mechanism. The present observations suggest that there may be compensatory mechanisms that act after prolonged heme synthesis inhibition, that abrogate changes blood pressure.
Alternatively, our data may indicate that there is considerable redundancy in hemoenzyme function, such that greater than 32% and 38% of NOS and sGC respectively, are required to elicit larger changes in blood pressure. Where Newcomer *et al.* observed robust elevations in blood pressure, they also observed similarly robust decreases (80%) in NOS capacity.\(^{27}\)

Despite its clear involvement in the pressure-natriuresis mechanism, genetic models of salt sensitivity, such as the Dahl salt-sensitive rat, which are associated with reduction in intrarenal NOS activity,\(^{12}\) do not manifest elevated AP until challenged with high or low sodium intake. In these animals, the deficits in NOS signalling result in a blunting of the pressure-natriuresis relationship,\(^{32}\) such that exaggerated changes in AP are required to maintain sodium balance when challenged with either high or low sodium intake. It was therefore hypothesized that the impact of SA treatment would become manifest with alterations in Na intake. Interestingly, SA-treated animals were associated with a steeper in vivo renal function curve, indicating these animals have lesser changes in MAP associated with altered sodium intake. Moreover, co-treatment with high sodium and L-NAME did not reveal any SA-mediated effects, suggesting that the reliance on NOS systems is similar in both groups. These data further support the notion that the NOS activity is highly redundant, and can function normally despite the reductions we induced in these animals.

An alternative mechanism that warrants discussion is the possibility that SA induces functionally antagonistic effects that oppose the changes induced by the reduction of intrarenal and vascular NOS and sGC. For example, circulating heme has
been reported to be highly cytotoxic,\textsuperscript{33,34} and may contribute to oxidative stress in the organs, which has been shown to impair vascular relaxation.\textsuperscript{35,36} It may be that the reduction in heme organ heme content by SA treatment was associated with reduced oxidative stress, and improved function. These effects would be hypothesized to mitigate the functional consequences of reduced hemoenzyme function in the kidney and vasculature.

The concepts explored above relating decreased heme availability for hemoenzymes have correlates in a number of human conditions. Clinical cases of heme disruption, such as the porphyrias or lead intoxication, are often associated with altered regulation of blood pressure, as mentioned above. Such cases are consistent with idea that adequate heme supplies are important for the proper function and regulation of the cardiovascular system. Moreover, it has been reported that treatment of spontaneously hypertensive rats with hemin produces substantial decreases in blood pressure.\textsuperscript{37} Similar results have been demonstrated in the clinical setting, wherein hypertensive patients administered δ-ALA, a precursor to heme, show dramatic AP lowering.\textsuperscript{38} It is presently unclear whether administration of hemin or δ-ALA elicit their effects by replenishing heme stores or whether the addition of these pro-oxidant molecules induce other changes that in turn lower blood pressure. Nevertheless, the data from the present study demonstrate the potential deleterious consequences of inadequate heme production, and raise the question whether this could be relevant in the etiology of primary hypertension.
**Perspectives**

It is well established that heme dependent enzymes constitute important mechanisms for AP control. The results obtained in the present study demonstrate that inhibition of heme synthesis and the consequent diminished function of these enzymes do not impact on AP regulation. These data suggest that these systems have a considerable amount of functional redundancy, and/or there are multiple compensatory systems that prevent loss of complete regulatory function in the cardiovascular system. Indeed, upregulation of compensatory mechanisms have been documented in various experimental models where primary regulatory systems are compromised. The concepts that have arisen emphasize the importance of integrative physiological studies and may help to identify new therapeutic strategies for essential hypertension and its sequelae.
References


