ABSTRACT

Hypertension and salt-sensitivity are independent risk factors for cardiovascular disease. Although both conditions are idiopathic, they develop due to a complex interplay between susceptibility genes and environmental factors. Given that the kidney plays an important role in regulating blood pressure, in particular, by maintaining sodium and water balance via pressure-natriuresis, it is not surprising that disturbances in the proper functioning of this intrarenal mechanism have been linked to these conditions. Although direct coupling of changes in renal arterial pressure (RAP) to renal interstitial hydrostatic pressure (RIHP) and consequent sodium excretion is well established, few studies have characterized the moment-to-moment aspects of this process. Thus, the main focus of the research presented herein was to characterize the moment-to-moment RAP-RIHP relationship, and assess the functioning of this intrarenal mechanism in various animal models of genetic and environmentally-induced hypertension and/or salt-sensitivity.

In adult normotensive rats, the response time of RIHP to acute changes in RAP was rapid (<2 seconds), and the moment-to-moment RAP-RIHP relationship was linear over a wide range of pressures. Additionally, the functioning of this relationship was not affected by inhibition of the renin-angiotensin system and autonomic nervous system. Further, the acute RAP-RIHP relationship was impaired in hypertension and/or salt-sensitivity. Specifically, animals with a hypertensive phenotype (i.e. young spontaneously hypertensive rats [SHR] and pro-atrial natriuretic peptide gene-disrupted mice [ANP -/-]) displayed a rightward shift in the moment-to-moment pressure-natriuresis curve towards higher RAP. This rightward shift was associated with increased
structurally-based vascular resistance properties in the hindlimb of young SHR versus their normotensive controls. Salt-sensitive phenotypes were associated with a blunting of this acute mechanism. Specifically, this blunting was evident in both the ANP -/-, a transgenic model of salt-sensitive hypertension, and in adult perinatal iron deficient (PID) rats, a developmentally programmed model of salt-sensitivity. It appears that a blunting in the RAP-RIHP relationship is influenced by an imbalance of key blood pressure modulating factors (e.g. ANP). Further, visceral obesity was associated with salt-sensitivity in PID rats; however the mechanism(s) are yet to be elucidated. Novel methodologies (MRI, abdominal girth) were developed for non-invasive assessment of visceral obesity to aid future research.
CO-AUTHORSHIP

The studies presented herein were performed by Marina Komolova with the following co-authorships and technical assistance.

Chapter 2: Co-authored by Michael A. Adams.

Data acquisition: MK acquired all data. MK and MAA conceived and designed the research, analyzed and interpreted the data, performed statistical analysis, drafted and revised the manuscript.

Komolova M and Adams MA. Hypertension, Accepted with Minor Revision.

Chapter 3: Co-authored by Peter Friberg and Michael A. Adams.

Data acquisition: In vivo assessments of renal hemodynamic properties were performed by MK. Assessments of hindlimb vascular resistance properties were performed by PF and MAA. MK, PF, and MAA conceived and designed the research, analyzed and interpreted the data, performed statistical analysis, drafted and revised the manuscript.

Komolova M, Friberg P, and Adams MA. Submission to Hypertension.


Data acquisition: In vivo renal function assessments were performed by MK. Renal and adrenal renin-angiotensin system expression and peptide level assessments were performed by PFO. MK, PFO, MYT, SCP, and MAA conceived and designed the research, and drafted and revised the manuscript. MK, PFO, and MAA analyzed and interpreted the data, and performed statistical analysis.


Chapter 5: Co-authored by Stephane L. Bourque, Michael A. Adams, and Kanji Nakatsu.

Data acquisition: In vivo assessments of renal vascular properties, tissue and hematological assessments of adult offspring (10 weeks old) were performed by MK. Body weight, tissue and hematological assessments of dams and offspring, and conscious hemodynamic experiments via radiotelemetry were performed by SLB. SLB and MK performed salt challenge experiments. MK, SLB, KN, and MAA conceived and designed the research, analyzed and interpreted the data, performed statistical analysis, drafted and revised the manuscript. Technical
assistance with the implantation of radiotelemetric transducers was provided by Corry Smallegange.


**Chapter 6:** Co-authored by Stephane L. Bourque, Kanji Nakatsu, and Michael A. Adams. Data acquisition:

Assessments of body composition, physical characteristics, and locomotor activity via radiotelemetry were performed by MK. MK and SLB performed salt challenge experiments. MK, SLB, KN, and MAA conceived and designed the research, analyzed and interpreted the data, performed statistical analysis, drafted and revised the manuscript. Technical assistance with the implantation of radiotelemetric transducers was provided by Corry Smallegange.


**Appendix 1:** Co-authored by Tina M. Maio, Mark Jeronimo, Mark C. Blaser, Patrick W. Stroman, Johanna L. Hannan, and Michael A. Adams.

Data acquisition: Body composition and measurements were performed by MK (all animals), TMM (groups 1&2), and JLH (group 4). MRI optimization was performed by PWS. MRI data acquisition was performed by MK and TMM. Macros for Image-Pro MRI analysis were developed by MCB and MJ. MRI calibration experiments were performed by MK and MJ. MK and MAA conceived and designed the research. MK, MAA, and MJ analyzed and interpreted the data. MK and MAA performed statistical analysis, and drafted and revised the manuscript. Technical assistance with MRI data acquisition was provided by Sharon David. Igor Komolov provided the illustration.

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In Plato’s symposium, a story about soulmates was presented by Aristophanes… that once upon a time humans roamed the Earth with four legs, four arms, and two faces. Then one day, the humans upset Zeus, and he punished them by splitting them in half, so that they spent their lives in search of their other half – their soulmate. Words cannot describe how lucky I am to have found mine! Peter, you have been the most amazing life partner I could ever have asked for and more. You always know how to make my worst days better and my best days unforgettable. Thank you for being there for me in every possible way! You never cease to surprise and inspire me, and always keep me guessing about the next adventure we will have together exploring the unknown. I know that with my Choops, there is nothing I cannot accomplish, and I look forward to our next chapter in life… starting with the Galapagos. ;)

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This thesis is dedicated to my amazing Бабушка Рая
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>20-HETE</td>
<td>20-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>ANP +/-</td>
<td>pro-atrial natriuretic peptide gene-disrupted mouse</td>
</tr>
<tr>
<td>ANP +/-</td>
<td>Heterozygote atrial natriuretic peptide one-copy mouse</td>
</tr>
<tr>
<td>ANP +/+</td>
<td>Wild-type atrial natriuretic peptide two-copy mouse</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>AP</td>
<td>Arterial pressure</td>
</tr>
<tr>
<td>AT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Angiotensin type 1 receptor</td>
</tr>
<tr>
<td>AT&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Angiotensin type 2 receptor</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine 3’, 5’-cyclic monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CR</td>
<td>Caloric restriction</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome 450</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DOHaD</td>
<td>Developmental origins of health and disease</td>
</tr>
<tr>
<td>ECF</td>
<td>Extracellular fluid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Epoxyeicosatrienoic acid</td>
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<td>EPI</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HASTE</td>
<td>Half-Fourier acquisition single-spot spin-echo</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
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<tr>
<td>HS</td>
<td>High sodium diet</td>
</tr>
<tr>
<td>HW</td>
<td>Heart weight</td>
</tr>
<tr>
<td>ID</td>
<td>Iron deficiency</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<td>KW</td>
<td>Kidney weight</td>
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<td>LS</td>
<td>Low sodium diet</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MBF</td>
<td>Medullary blood flow</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NHE</td>
<td>Sodium hydrogen exchanger</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPRA</td>
<td>Natriuretic peptide receptor type A</td>
</tr>
<tr>
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<td>Normal sodium diet</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>pGC</td>
<td>Particulate guanylyl cyclase</td>
</tr>
<tr>
<td>PID</td>
<td>Perinatal iron deficiency</td>
</tr>
<tr>
<td>ppMC</td>
<td>Perfusion pressure at maximum constriction</td>
</tr>
<tr>
<td>ppMD</td>
<td>Perfusion pressure at maximum dilatation</td>
</tr>
<tr>
<td>RAP</td>
<td>Renal arterial pressure</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>RBF</td>
<td>Renal blood flow</td>
</tr>
<tr>
<td>RIHP</td>
<td>Renal interstitial hydrostatic pressure</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>RVR</td>
<td>Renal vascular resistance</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEE</td>
<td>Standard error of estimate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SHROB</td>
<td>Spontaneously hypertensive obese rat</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>UF</td>
<td>Urine flow</td>
</tr>
<tr>
<td>U_{Cl}V</td>
<td>Urinary excretion of Cl^-</td>
</tr>
<tr>
<td>U_{K}V</td>
<td>Urinary excretion of K^+</td>
</tr>
<tr>
<td>U_{Na}V</td>
<td>Urinary excretion of Na^+</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
</tr>
<tr>
<td>WC</td>
<td>Waist circumference</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto rat</td>
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</table>
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CHAPTER 1

General Introduction

1.1 Hypertension and Salt-Sensitivity: An Overview

Cardiovascular disease (CVD) is responsible for one third of deaths globally. Hypertension, typically an asymptomatic condition, affects approximately 1 billion people worldwide, and is a major risk factor for development of CVD. Essential hypertension, or elevated blood pressure with an unknown etiology, accounts for >90% of all cases of hypertension. Despite its idiopathic nature, there is no doubt that hypertension is polygenic and multifactorial. It is estimated that approximately 30-60% of blood pressure variability is genetically determined. Additionally, gene-environment interactions further contribute to the phenotypic expression of blood pressure, specifically in the critical periods of early life development (i.e. perinatal environment) and throughout the adult life (i.e. lifestyle factors). Furthermore, salt-sensitivity, or a heightened responsiveness of blood pressure to dietary salt intake, is found in approximately 50-70% of individuals with essential hypertension. Paradoxically, it must also be noted that approximately 26% of the human population is salt-sensitive in the absence of hypertension, suggesting that salt-sensitivity per se may be encoded separate genes from those encoding essential hypertension. Regardless, salt-sensitivity is also a risk factor for CVD independent of blood pressure. Although the etiology of both salt-sensitivity and hypertension is not fully understood, disturbances in the proper functioning of the kidney, specifically the ability to regulate blood pressure via sodium and fluid balance (i.e. pressure-natriuresis), have been linked to these conditions.
1.2 Control of Arterial Pressure

Regulation of arterial pressure (AP) is accomplished via the complex mixture of neural, hormonal and intrinsic factors involving the brain, heart, vasculature and the kidneys\textsuperscript{10}. Maintenance of AP around the operating point is not only necessary for adequate delivery of oxygen and nutrients to all tissues, but to also prevent aberrant AP deviations that may damage blood vessels and organs, and thereby lead to various cardiovascular pathologies (i.e. stroke, renal failure)\textsuperscript{10}. For this very reason, the body is equipped with an integrated, multifaceted system for AP regulation, which maintains pressure within an appropriate homeostatic range. This system consists of multiple modulators and mediators that function within specific timeframes i.e. short-term (second-to-second), intermediate (minutes to hours), and long-term (hours to days)\textsuperscript{10}.

Short-term mechanisms primarily originate from the autonomic nervous system (ANS), and affect total peripheral resistance and cardiac output (Figure 1.1). Examples of such mechanisms include the baroreceptor reflex, chemoreceptor reflex, and CNS ischemic response\textsuperscript{10,11}. These systems are a critical first line of defense against dramatic changes in AP, however they are unable to completely restore pressure to ‘normal’ levels since they reset to the new steady-state level of AP; specifically, they have a finite feedback gain. For example, the baroreceptor reflex acts within seconds to changes in AP and has a great capacity to oppose these changes in AP (i.e. gain); however its signaling diminishes dramatically over time, and finally resets to the new steady-state after a few days\textsuperscript{10} (Figure 1.2). Thus, these systems simply correct or buffer moment-to-moment changes in AP, rather than contribute to maintaining the long-term level of AP.
Figure 1.1: A simplified schematic representing the short-term and long-term controllers of arterial pressure. Solid arrows represent an agonistic effect and dashed arrows represent an inhibitory effect. TPR, total peripheral resistance; CO, cardiac output; ECF, extracellular fluid volume. Adapted from Guyton and Hall. 
Figure 1.2: Approximate degree of potency, expressed as feedback gain, of several arterial pressure control systems at various timeframes following an acute change in arterial pressure. See text for details. RAS, renin-angiotensin system. Adapted from Guyton and Hall.®
For example, ablation of the short-term regulatory systems (e.g. denervation of baroreceptors) increases the variance of AP over an acute period of time, but does not affect the long-term level of AP\(^{12}\).

Other pressure control mechanisms exhibit significant responses to AP changes within minutes to hours. These intermediate acting mechanisms include a number of humoral systems (e.g. renin-angiotensin system (RAS) and atrial natriuretic peptide (ANP)), as well as autoregulatory and capillary fluid shift mechanisms (Figure 1.2). These systems regulate blood flow by altering total peripheral resistance and vascular capacitance (i.e. ability of blood vessels to hold blood volume without an increase in pressure)\(^{10}\) (Figure 1.1). However, much like the short-term mechanisms, they too have a finite feedback gain, and eventually reset to steady-state AP levels, thereby diminishing the involvement of these mechanisms in regulating the long-term level of AP\(^{10, 13}\).

In order to maintain the long-term level of AP, a mechanism with an infinite feedback gain must be initiated following the decline of effectiveness of the aforementioned systems\(^{10, 14}\) (Figure 1.2). The kidney is not only a remarkable organ involved in excretion of waste, but also serves a critical role in the long-term regulation of extracellular fluid volume (ECF) and blood volume in response to changes in AP (Figure 1.1). The long-term regulation of AP is achieved by modifying sodium and water excretion in response to alterations in AP, a process referred to as pressure-natriuresis\(^{10, 11}\) (Figure 1.1). Specifically, increases in AP above the operating point, ‘jump-start’ the pressure-natriuresis mechanism to increase sodium and water excretion, and thereby eventually promote a lowering of AP via decreases in ECF and blood volume\(^{10, 11, 13, 15, 16}\) (6; 9-14). This mechanism also functions in the reverse to increase ECF and blood
volume in the face of decreases in AP below the operating point (i.e. it preserves sodium and water). The pressure-natriuresis mechanism is therefore considered to possess an infinite feedback gain due to its limitless capacity to adjust ECF and blood volume by maintaining a balance between fluid intake and output until AP is normalized to its operating point\textsuperscript{7, 8, 10, 11, 13, 14, 16, 17}. Thus, this intrinsic kidney mechanism plays a critical role in determining the long-term level of AP.

1.3 Pressure-Natriuresis: Role in the Long-Term Control of Arterial Pressure

Guyton and associates were first to propose that the kidney serves a critical role in the long-term regulation of ECF and AP, in part, via pressure natriuresis\textsuperscript{7, 10, 11, 13, 14, 16, 17}. Increases in renal arterial pressure (RAP), also denoted as renal perfusion pressure (RPP), reduce tubular reabsorption of sodium and water, and ultimately result in an increase in sodium and water excretion\textsuperscript{11}. Interestingly, this dynamic regulation of sodium and water occurs despite any discernible changes in either total renal blood flow (RBF) or glomerular filtration rate (GFR), which are autoregulated (i.e. remain constant over a wide range of RAP)\textsuperscript{11, 18, 19}. However, it should be mentioned that although approximately 90% of total RBF goes to cortical nephrons, the other 2-10% (species dependent) reaches juxtamedullary nephrons\textsuperscript{19, 20}. Blood flow through the latter structures has been proposed to be the driving force of pressure-natriuresis, since blood flow through the renal medullary circulation (i.e. vasa recta capillaries) has been shown to be poorly autoregulated\textsuperscript{15, 21, 22}. That is, changes in RAP produce proportional changes in medullary blood flow (MBF) (Figure 1.3). This is further supported by the anatomical discovery that some juxtamedullary nephrons have shunts from the pre-glomerular to the post-glomerular vasculature suggesting that these vessels bypass the glomerulus, and in
Figure 1.3: The proposed cascade of events in the pressure-natriuresis mechanism following changes in arterial pressure from its operating point. MAP, mean arterial pressure; RAP, renal arterial pressure; MBF, medullary blood flow; ECF, extracellular fluid volume.
turn, likely circumvent the autoregulatory mechanisms of the tubular glomerular feedback\textsuperscript{23}. While this proposed mechanism prevails, the degree to which MBF is autoregulated has been controversial\textsuperscript{18, 24}. The details of this controversy are beyond the scope of this review, and the reader is encouraged to seek out these excellent reviews\textsuperscript{11, 18}.

Thus, the proposed mechanism by which the kidney ‘senses’ fluctuations in AP is through the poorly autoregulated medullary circulation\textsuperscript{11, 19}, where changes in RAP are transmitted via the vasa recta capillaries into the renal interstitium. Specifically, changes in the perfusion of vasa recta capillaries are thought to proportionately change renal interstitial hydrostatic pressure (RIHP) by altering the Starling forces (i.e. balance between the hydrostatic and oncotic pressures within the capillaries and interstitium) inside the renal medulla\textsuperscript{18, 19, 27, 28} (Figure 1.3). These changes in RIHP of the medullary region are then transmitted uniformly throughout the kidney because of the renal capsule\textsuperscript{29, 30}, and consequently alter sodium reabsorption. Roman and colleagues\textsuperscript{22, 30} have provided evidence that renal medullary hemodynamics are closely linked to changes in RIHP, and also that changes in RIHP are associated with parallel changes in the sodium and water reabsorption in the proximal tubule and thin descending limb of deep nephrons\textsuperscript{31} (Figure 1.3). Although the precise manner by which RIHP mediates sodium and water homeostasis is not completely understood, multiple mechanisms have been proposed\textsuperscript{18}. It has been postulated that increases in RIHP result in enhanced passive sodium excretion by opposing the flux of sodium from tubules to the renal interstitium and enhancing the back-leak of sodium from the interstitium to the tubules\textsuperscript{18, 32-34}. Further, another possibility is that RIHP may affect the functioning of tubular sodium transporters (e.g. sodium-hydrogen exchanger (NHE), \text{Na}^+/\text{K}^+/2\text{Cl}^- \text{co-transporter, and
Na\textsuperscript{+}/K\textsuperscript{+}-ATPase), and therefore active sodium transport; although this is yet to be determined\textsuperscript{18}. Despite this, evidence strongly supports that RIHP is a necessary component of the pressure-natriuresis mechanism. For example, artificially increasing RIHP via direct intrarenal volume expansion (i.e. infusion of a protein solution into renal interstitium) has a strong natriuretic effect\textsuperscript{28,35,36}. Conversely, decapsulation of the kidney substantially attenuates both RIHP and the pressure-natriuretic response; however, it does not completely abolish it altogether\textsuperscript{28-30}. These manipulations alter renal interstitial compliance, defined as a change in local interstitial fluid volume divided by the corresponding change in RIHP. While evidence supports that MBF and RIHP are mediators of pressure-natriuresis, there are multiple intrarenal and extrarenal factors that can influence or modulate the pressure-natriuresis mechanism\textsuperscript{11,18}. These modulators can affect renal hemodynamics and/or tubular sodium transport to enhance or attenuate the efficiency of pressure-natriuresis.

Although neural and humoral factors can influence the pressure-natriuresis mechanism (discussed in more detail in later sections), in the long-term, pressure-natriuresis appears to be the dominant factor in sodium and water excretion\textsuperscript{7,10,11,13,14,16,17}. It is believed that this intrinsic renal mechanism is the principle determinant in setting the operating point of AP. The most convincing evidence for this concept comes from renal cross-transplant studies between normotensive and genetically hypertensive rats\textsuperscript{37-42}, as well as in human renal graft recipients\textsuperscript{43-46}. Briefly, it was found that hypertension can be transferred by renal transplantation from genetically hypertensive donors to normotensive recipients, and vice versa. These findings demonstrate that the AP operating point follows the transplanted kidney, and that altered renal function is a cause
rather than a consequence of hypertension. Further, the capacity of the transplanted kidney to dictate the operating point of AP is present despite that the transplanted kidney lacks neural input\(^4\), and exists in the hormonal milieu of the recipient.

1.3.1 The Operating Point of Arterial Pressure and Renal Function Curves

Guyton and colleagues were the first to describe how the kidney dictates the operating point of AP through *renal function curves*\(^6\) (Figure 1.4). As mentioned previously, the kidney regulates the long-term level of AP, in part, via pressure-natriuresis. The efficacy of pressure-natriuresis can be graphically represented using an *acute pressure-natriuresis curve*, where sodium excretion is on the y-axis, and AP on the x-axis\(^8,8\) (Figure 1.4A). From this curve, it is evident that alterations in AP induce proportional (i.e. near linear) changes in sodium excretion (e.g. ↑AP results in ↑ sodium excretion)\(^4\). Further, the *acute pressure-natriuresis curve* can also be extended to other intermediate components of this intrarenal mechanism (i.e. MBF, RIHP, and urine flow(UF)), since these components also respond proportionately to changes in AP\(^11,18,28\).

It should be noted that the *acute pressure-natriuresis curve* demonstrates the actions of pressure-natriuresis independent from neurohumoral influences, as shown by isolated, perfused kidney experiments\(^4\). However, in the whole organism, these neurohumoral systems, as well as conditions that alter salt intake, appear to modulate the steepness (i.e. slope) of the *acute pressure-natriuresis curve*\(^4\). Specifically, natriuretic factors or low sodium intake decrease the slope and shift the curve leftward, whereas anti-natriuretic factors or high sodium intake increase the slope and shift the curve rightward. Joining the equilibrium points at the various sodium intakes creates the *renal
Figure 1.4: Differences between the acute and chronic pressure-natriuresis curves at various sodium intakes. **Top panel:** The responses of the acute pressure-natriuresis mechanism (solid line) to various levels of sodium intake in the long-term (dashed lines) describe the chronic pressure-natriuresis curve (dotted line). Adapted from Montani and Van Vliet\(^49\). **Bottom panel:** Theoretical chronic pressure-natriuresis curves of normotensive, hypertensive and salt-sensitive individuals. Adapted from Guyton and Hall\(^8\).
function curve (or chronic pressure-natriuresis curve)\(^8,\)\(^{49}\) (Figures 1.4A and 1.4B). This curve reveals a very steep steady-state relationship at various sodium intakes, characterized by a minimal change in AP over a long period of time (i.e. AP is relatively salt insensitive). Together, the renal function curve encompasses the long-term actions of pressure-natriuresis coupled with adjustments from various neurohumoral systems\(^8,\)\(^{49}\).

As hypothesized by Guyton\(^7\)-\(^10\) and reinforced by extensive experimental and clinical data, renal mechanisms most likely play a primary role in the pathogenesis of hypertension. Disturbances in the proper functioning of renal function have been linked to hypertension and salt-sensitivity. The AP phenotype of an individual or organism can be determined from two characteristics of the renal function curve: 1) the salt insensitive (the positioning of the curve on the x-axis), and 2) the salt-sensitive (the steepness of the curve)\(^10,\)\(^{48}\) (Figure 1.4B). Accordingly, a parallel rightward shift in the renal function curve has been demonstrated in human and animal models of essential hypertension (e.g. spontaneously hypertensive rat [SHR])\(^11,\)\(^{50}-\)\(^{54}\). In this scenario, renal function appears to be normal with regard to its capacity to regulate sodium homeostasis, despite that the kidneys operate at a higher AP or operating point. Conversely, a blunting of the renal function curve slope has been associated with a salt-sensitive phenotype (e.g. Dahl salt-sensitive rat)\(^11,\)\(^{55,\)\(^{56}\). In this case, renal function appears to be normal until challenged with an abnormal sodium intake. Thus, in both conditions, an increase in AP is required in order to maintain sodium and water homeostasis. Further, it should be mentioned that these changes in the renal function curve are also reflected in the acute pressure-natriuresis curve, as well as in the RAP-MBF, RAP-RIHP and RAP-UF relationships\(^11,\)\(^{15,\)\(^{19,\)\(^{28,\)\(^{57-\)\(^{59}\).}
1.3.2 Pressure-Natriuresis: A Long-Term or Short-Term Mechanism?

Given that pressure-natriuresis has been regarded as a ‘long-term’ regulatory mechanism, it is not surprising that numerous studies have investigated the specific relationship between components of pressure-natriuresis (i.e. MBF, RIHP, UF, sodium excretion) and RAP over a prolonged time course. Specifically, these studies induced graded step changes in RAP for at least 30 minutes, and then recorded ‘steady-state’ changes in the intermediate components of pressure-natriuresis and the associated changes in sodium excretion\(^{22, 29, 30, 52, 59-65, 65-68}\). However, such assessments may not reflect true \textit{in vivo} control of AP, since AP in conscious animals does not change from one steady-state to another, but rather fluctuates spontaneously in varying degrees of magnitude around an AP operating point and over a wide range of time scales (frequencies)\(^{58}\). For example, oscillations in AP assessed over an acute timeframe tend to be more variable and of greater magnitude than AP fluctuations assessed over a prolonged timeframe. This suggests that the kidneys are exposed to greater AP variations over a shorter timeframe than have been examined using conventional pressure-natriuresis methodologies. Interestingly, only one study to date has described a statistically significant moment-to-moment correlation between spontaneous AP oscillations and RIHP within frequency windows of 1Hz to 0.01Hz\(^{58}\). This same group also found that UF, a variable downstream from RIHP, responded to changes in AP within ~6 seconds\(^{57}\). From these findings, they hypothesized that the long-term nature of the pressure-natriuresis mechanism is actually the cumulative effect of small, rapid changes in AP and UF that gradually induce homeostatically appropriate changes in blood volume and AP\(^{57}\). Thus, since changes in RIHP are an intermediate step in
pressure-natriuresis, there must be a shorter delay between AP and RIHP; however, the exact time course is yet to be elucidated.

1.3.3 Variations in the In Vivo Assessment of Pressure-Natriuresis: Focus on the RAP-RIHP Relationship

Numerous studies that have characterized the in vivo pressure-natriuresis mechanism, more specifically the intermediate step of this mechanism (i.e. RAP-RIHP relationship), adopted the auto-perfused rat kidney model proposed by Roman and Cowley. These studies describe the overall relationship between RAP and the components of the pressure-natriuresis mechanism as linear. Interestingly, Steele et al. (1993) suggest that acute increases above the AP point are dealt with more effectively than decreases below the operating point with respect to UF. Whether such a differential response to pressor and depressor stimuli exists with respect to RIHP is yet to be determined. Thus, a more physiological characterization of the RAP-RIHP relationship (i.e. alternating acute pressor and depressor stimuli around the AP operating point) is warranted.

Furthermore, due to the duration of the conventional ‘graded step’ methodology used to assess the pressure-natriuresis relationship, the influence of potential compensatory neuro-humoral factors had to be controlled for by various methods (e.g. renal denervation, uninephrectomy, and infusion of hormone cocktails consisting of any or all of the following: aldosterone, cortisol, norepinephrine, angiotensin II, and vasopressin). However, there has not yet been a systematic assessment of how various neuro-humoral systems impact on the RAP-RIHP relationship. Since there is evidence that RIHP may respond rapidly to changes in AP.
the need for hormonal cocktail infusions that fix or control the slower responding neuro-humoral control systems is questionable; however, this needs to be further investigated.

1.4 Neuro-Humoral Modulators of the Pressure-Natriuresis Mechanism

As mentioned previously, the sensitivity (i.e. slope) of the pressure-natriuresis mechanism is modulated, in part, via various neural and humoral factors. While the discussion of all neuro-humoral factors that may affect renal function is beyond the scope of this review (see 11,18), those relevant to this thesis (i.e. based on animal models and/or pharmacological treatments used) are discussed in the following sections.

1.4.1 Autonomic Nervous System: Focus on Renal Sympathetic Nerves

Sympathetic nerve fibers have been shown to innervate renal afferent and efferent arterioles, proximal tubules, distal tubules, loops of Henle, and juxtamedullary granular cells11. Because of this extensive renal sympathetic innervation, changes in sympathetic activity can modulate pressure-natriuresis under certain conditions. Activation of the sympathetic nervous system (e.g. due to a decrease in blood volume) stimulates renal sympathetic nerve activity and increases sodium and water reabsorption. This is achieved in the following manner: 1) the heavily innervated renal resistance vessels are vasoconstricted by norepinephrine (NE) release, and thereby reduce RBF and GFR; 2) NE acts on tubular epithelial cells to increase sodium reabsorption; and 3) NE stimulates renin release, and increases angiotensin II (Ang II) and aldosterone production, both of which further inhibit natriuresis (see below)10,69. Low-frequency electrical stimulation of renal nerves, which does not measurably affect RBF and GFR, has been shown to reduce natriuresis and appears to be a result of sympathetic effects on tubules70; although
the extent to which electrical stimulation of renal nerves simulates physiological levels of sympathetic activity is unknown. Further, supraphysiological infusion of epinephrine or bilateral carotid occlusion have also been shown to blunt the pressure-natriuresis relationship. Conversely, renal denervation or intrarenal α-adrenergic receptor antagonism results in natriuresis. These findings support the notion that renal sympathetic nerves play an important role in modifying the rate of achieving sodium balance. However, the fact that renal-denervated organisms survive and maintain sodium balance in the long-term demonstrates that sodium homeostasis can still be achieved in the absence of renal nerves. Thus, the principal function of renal nerves is to make short-term adjustments in sodium and water reabsorption rather than play a role in the long-term control of sodium and water balance. The effects of autonomic nervous system (ANS), particularly renal sympathetic nerve activity, on specific components of the pressure-natriuresis mechanism (i.e. RIHP) have not yet been elucidated.

1.4.2 Renin-Angiotensin System: Focus on Angiotensin II

In the ‘classical’ descriptions of the renin-angiotensin system (RAS) (Figure 1.5), renin is said to be synthesized in the kidney and then released systemically following an appropriate stimulus (i.e. stimulation of renal sympathetic nerve activity, activation of baroreceptors in the afferent artery, or a decrease in sodium concentration of the tubular fluid in the macula densa). Although, the location was not described, the dogma indicated that renin cleaved hepatic angiotensinogen into Ang I, which was then subsequently converted into Ang II by angiotensin-converting enzyme (ACE), initially thought to be located in the lungs. Ang II remains the main effector of the RAS, and exerts its effects via two main G protein-coupled receptors: angiotensin receptor
Figure 1.5: Schematic of the classical renin-angiotensin system cascade. ACE, angiotensin-converting enzyme; AT₁, angiotensin type 1 receptor; AT₂, angiotensin type 2 receptor.
type I (AT\(_1\)) and type II (AT\(_2\)). These receptors differ in their distribution and action. AT\(_1\) is believed to mediate the vasoconstrictor and anti-natriuretic effects of Ang II, whereas AT\(_2\), which is mainly expressed during development, may mediate vasodilatory and natriuretic actions through NO and prostaglandins. In rodents, there are two AT\(_1\) receptor subtypes, AT\(_{1A}\) and AT\(_{1B}\), with AT\(_{1A}\) being the more common.

Recent advances in the understanding of the components and actions of RAS have extended the understanding of this ‘classical’ cascade of RAS. Specifically, several other components of the RAS have been discovered (e.g. Ang(1-9), Ang(1-7), ACE2; for a review see), and the presence of local tissue RAS was identified in various organs (e.g. kidney, adrenal glands, and heart). For example, the kidney has all of the components necessary for the production of Ang II. In fact, renal interstitial fluid levels of Ang II are 1000-fold higher than in plasma, and therefore, it is believed that 90% of Ang II found in the interstitium is formed within the kidney. Aside from its expression in the juxtamedullary apparatus, renin mRNA is also found in proximal tubules. Angiotensinogen mRNA has been measured in the proximal tubules of cortical and juxtamedullary nephrons with low levels in the medullary vasculature, and ACE is expressed in the brush border of the proximal tubule. Further, AT\(_{1A}\) is found throughout the kidney, including the vasa recta, whereas AT\(_2\) is expressed mainly in the vasculature at significantly lower levels than the AT\(_1\). The fact that the kidney has a functioning local RAS is important, since intrarenal actions of Ang II are most relevant in terms of how this peptide contributes to the regulation of the long-term level of AP.

The main functions of RAS is to regulate sodium and water homeostasis by adjusting Ang II levels in response to changes in AP, ECF, and sodium concentration.
The three main functions of Ang II are: 1) renal vasoconstriction, which occurs quickly (minutes to hours); 2) sodium reabsorption, which occurs over a period of hours to days; and 3) cardiovascular growth, which requires weeks to months to take effect\textsuperscript{10, 11, 81}. With respect to reabsorption of sodium and water, Ang II has direct and indirect effects in the kidneys that are concentration dependent\textsuperscript{82}. That is, AT\textsubscript{1} receptors on the proximal tubule are stimulated by concentrations of $10^{-13} - 10^{-12}$ M to promote sodium reabsorption via the Na\textsuperscript{+}/H\textsuperscript{+} antiporter\textsuperscript{82}. The adrenal glomerulosa cells require a 100-fold (of $10^{-11} - 10^{-10}$ M) greater concentration of Ang II to stimulate the release of aldosterone, which in turn also acts to enhance sodium reabsorption\textsuperscript{82}. Furthermore, $10^{-12} - 10^{-9}$ M of Ang II vasoconstricts the efferent arteriole, whereas the afferent arteriole and other intrarenal vessels require at least of $10^{-5}$ M of Ang II\textsuperscript{82}. Ang II modulates the pressure-natriuresis response mainly via these increases in tubular reabsorption of sodium and water. Ang II also interacts with the pressure-natriuresis response by decreasing MBF, and thereby promoting sodium and water reabsorption by the consequent reduction in RIHP\textsuperscript{11, 61, 68, 81}. The importance of Ang II in making the pressure-natriuresis mechanism more effective can be demonstrated by its effect on the \textit{renal function curve}\textsuperscript{10, 81-83} (Figure 1.6). When the RAS is fully functional, the curve is steep, indicating that very minor changes in AP are needed to maintain sodium homeostasis\textsuperscript{83}. However, when the dynamic regulation of the RAS is reduced by continuous infusion of Ang II, such that its levels are clamped at excessively high levels, then an elevated AP is needed to maintain sodium balance\textsuperscript{83}. This is depicted by the rightward shift of the \textit{renal function curve} towards greater AP and its blunted slope\textsuperscript{83}. Conversely, when the dynamic control of the RAS is reduced by either fixing Ang II levels at excessively low levels (i.e. ACE inhibition) or preventing its
Figure 1.6: Schematic illustrating the effects of losing dynamic function of the renin-angiotensin system on the pressure-natriuresis mechanism. Ang II, angiotensin II. Adapted from Hall et al. 83.
action on AT₁ (i.e. AT₁ antagonism), then a lower AP is needed to maintain sodium balance, despite the slope of the renal function curve also being blunted. These changes in the renal function curve indicate that if the dynamic regulation of RAS is abnormal, not only is the operating point of pressure-natriuresis altered, but the organism becomes salt-sensitive.

While the role of the RAS in the control of ECF and AP is widely accepted, there is evidence suggesting that Ang II is also a potent vascular growth factor, which is necessary for angiogenesis and nephrogenesis in early life. Studies on rodent models demonstrated that if the production or actions of Ang II are either blocked pharmacologically or via genetic manipulation during nephrogenesis, the animals display anatomical and functional renal abnormalities, including a blunting of the pressure-natriuresis mechanism. Thus, these findings suggest that an intact RAS in the neonate is a prerequisite for normal renal development and function.

Although Ang II appears to be important during development, persistent exposure to Ang II in adulthood can also induce vascular growth. Specifically, long-term exposure to Ang II has been shown to result in left ventricular hypertrophy, and changes in vascular structure. While elevated AP is known to also promote similar vascular structural changes, it has been shown that Ang II can promote these vascular alterations in the absence of an elevated AP. Changes in vascular structure have been associated with increases in AP, as well as a rightward shift in the pressure-natriuresis mechanism. That is, a greater AP is required to induce the same natriuretic effect.

Furthermore, although the long-term effects of Ang II on the RAP-RIHP relationship
have been characterized\textsuperscript{50, 61, 68, 90, 91}, the effects of Ang II on the functioning of the acute RAP-RIHP relationship have not been assessed.

1.4.3 Atrial Natriuretic Peptide

Atrial natriuretic peptide (ANP) is involved in the long-term regulation of AP\textsuperscript{11, 92-94}. ANP is synthesized, stored, and secreted by atrial myocytes in response to atrial distention (i.e. stretch) due to volume expansion\textsuperscript{11, 92-94}. Upon release, the peptide signals through the natriuretic peptide type A receptor (NPRA), a particulate guanylyl cyclase (pGC) receptor, to increase cyclic guanosine monophosphate (cGMP)\textsuperscript{93-95}. NPRA is present in target tissues such as blood vessels, heart and kidneys\textsuperscript{93, 94}. Specifically, within the kidney, NPRA is found in nearly every segment of the nephron, glomeruli, and preglomerular vessels; however, it is found in its highest levels in the inner medulla\textsuperscript{96}. The main actions of ANP are to counter increases in blood volume, and consequently AP, by: 1) stimulation of the pressure-natriuresis mechanism; 2) inhibition of the release and/or actions of other pressor neuro-humoral systems, including the RAS and catecholamines; and 3) vasodilation of blood vessels\textsuperscript{93, 94}.

ANP modulates pressure-natriuresis via both its direct tubular and renal hemodynamic effects\textsuperscript{11, 94}. Directly, ANP exerts its main effects in the inner medullary collecting duct via inhibition of amiloride-sensitive sodium channels, resulting in significant increases in sodium excretion\textsuperscript{97}. Although the inner medullary collecting duct is responsible for 2-5\% of the sodium reabsorption along the nephrons, this tubular segment is a very critical regulator of the final sodium concentration of urine\textsuperscript{97}.

The hemodynamic and indirect natriuretic effects of ANP appear to be largely mediated by its antagonistic effects on other vasoactive factors. In fact, the sensitization
of pressure-natriuresis by ANP is dependent on its ability to oppose the actions of the RAS\textsuperscript{98}. This is expected since ANP is thought to serve as a counter-regulatory system for the RAS. Specifically, ANP inhibits renin release\textsuperscript{99}, thereby decreasing circulating levels of Ang II and aldosterone\textsuperscript{99}. ANP also antagonizes Ang II-mediated vasoconstriction\textsuperscript{92}, and inhibits aldosterone synthesis directly in the adrenal cortex\textsuperscript{100}. Aside from its effects on the RAS, ANP also has potent sympatholytic effects. For example, ANP reduces the production and activity of tyrosine hydroxylase, a rate limiting step in catecholamine production, thereby decreasing norepinephrine synthesis and release from the adrenal medulla\textsuperscript{101,102}. ANP also was shown to inhibit $\alpha_1$ adrenoceptor-induced vasoconstriction\textsuperscript{103}. Together, these findings suggest that ANP exerts its natriuretic effects directly, via sodium channel inhibition in the inner medullary collecting duct, as well as indirectly, via its antagonistic effects on anti-natriuretic/vasoconstrictor systems.

Further, the vasodilatory properties of ANP may also mediate a portion of its natriuretic effects. Intravenous ANP infusion was shown to enhance the relationship between RAP and sodium excretion independent of any interactions with other neuro-humoral factors, such as the sympathetic nervous system and RAS\textsuperscript{11,104,105}. Furthermore, ANP has been shown to sensitize the pressure-natriuresis mechanism at high, but not low, pressures\textsuperscript{98}. A number of studies have shown that ANP induces sodium excretion by altering medullary hemodynamics\textsuperscript{11}. Specifically, it was found that infusions of ANP at levels that do not significantly influence RBF or GFR, increase MBF and vasa recta capillary pressure\textsuperscript{11,104,106-108}. Furthermore, infusion of ANP at supraphysiological levels result in an elevated RIHP\textsuperscript{109}; however, this increase in RIHP was not evident at physiological levels of ANP\textsuperscript{110}. Thus, it is not entirely clear whether ANP modulates the
RAP-RIHP response, specifically across a wide range of RAP. Further experiments are thus warranted.

1.4.4 Other Intrarenal Modulators

The effects of other vasoactive molecules (i.e. prostaglandins, endothelin peptides, bradykinins, cytochrome P450-dependent arachidonic acid metabolites (20-hydroxyeicosatetraenoic acid [20-HETE] and/or epoxyeicosatetraenoic acid [EET]), NO, superoxide [O₂⁻], hydrogen peroxide [H₂O₂], and cGMP) have also been assessed in terms of their modulatory effects on pressure-natriuresis (for reviews see ¹¹, ¹⁸, ¹¹¹). Briefly, prostaglandins (i.e. PGE₂; via an interaction with Ang II) ¹¹² and 20-HETE ¹¹³ were shown to be necessary for the full expression of the natriuretic effects of RIHP. Furthermore, increases in RAP result in increases in intrarenal NO (as a result of increases in MBF), and O₂⁻ and H₂O₂ (due to increased delivery of sodium through the medullary thick ascending limbs of Henle), where an imbalance in the production of these vasoactive molecules may lead to a blunted pressure-natriuretic response ¹¹¹, ¹¹⁴. The effects of NO or ANP (as described above) are mediated through cGMP. Interestingly, increases in RIHP have been associated with a rise in renal interstitial cGMP, which in turn, further increases RIHP via a positive feedback loop ¹¹⁵. Additionally, bradykinins can selectively increase perfusion of the medulla via activation of the NO system and calcium-dependent K⁺ channels ¹¹⁶. It has been suggested that the role of these paracrine or autocrine systems is to buffer the actions of vasoconstrictor hormones (i.e. Ang II and endothelin ¹¹⁷) so as to protect the renal medulla from ischemia ¹¹¹. Thus, an imbalance between the abovementioned vasodilator and vasoconstrictor molecules would result in an abnormal pressure-natriuretic response (i.e.
affect the slope of the *renal function curve*\(^{11,18,48}\). Furthermore, over long periods of time, vasoconstrictors (i.e. catecholamines\(^{118-121}\), Ang II\(^{122-126}\), and endothelin\(^{127-129}\)) have the ability to induce vascular growth, while vasodilators (i.e. NO\(^{124,125,130}\), bradykinin\(^{131-133}\), ANP\(^{134}\), PGE\(_2\)^{135}\) have been found to inhibit growth. Additionally, changes in AP due to an imbalance of these vasoactive agents have the ability to induce pressure-dependent adaptive changes in vascular structure, where increases in AP promote vascular growth, whereas decreases in AP inhibit vascular growth\(^{136,137}\). These changes can reset the AP operating point and thereby the pressure-natriuresis mechanism (i.e. shifting the *renal function curve* along the x-axis)\(^{11,18,48}\).

1.5 Vascular Structure: Influence on Arterial Pressure and Pressure-Natriuresis

It is well known that vascular structure is a determinant of the level of AP, such that changes in the vasculature cause a proportional change in AP. Folkow *et al* originally demonstrated that an upregulation of vascular structure plays an important role in the maintenance of hypertension\(^{138-140}\). That is, in response to hypertension (i.e. a long-term increase in AP), adaptive structural changes involving an increase in medial wall thickness and a narrowing of the lumen diameter occur to ensure that the blood vessels can withstand the increase in wall stress\(^{136-140}\). According to Poiseuille’s Law (\(R \propto 1/r^4\)), a small decrease in the radius (e.g. 8%) would result in a large increase in resistance (e.g. 39%)\(^{139}\) (Figure 1.7). Since AP is a product of resistance at a given blood flow (\(AP \approx \text{resistance} \times \text{flow}\)), then small changes in the lumen diameter would translate into greater changes in AP, further amplifying the already elevated AP.
Figure 1.7: According to Poiseulle’s law, small changes in the radius of the vascular lumen amplify the resistance properties of a vessel, and thereby pressure.
Indeed, vascular structural changes have been demonstrated in hypertensive humans and in experimental models of hypertension (i.e. SHR)\textsuperscript{141-150}. We and others have shown an altered vascular structure in SHR, both in conduit and resistance arteries, versus their normotensive control (i.e. Wistar Kyoto (WKY) rats)\textsuperscript{142, 148, 151, 152}. In fact, changes in vascular structure have been shown to occur prior to the full development of hypertension in SHR, and these vascular changes have been considered to contribute to the initiation and maintenance of hypertension\textsuperscript{142, 152-157}. Specifically, the types of vascular structural changes that lead to increases in vascular resistance, and thereby AP, are thought to involve either: i) hypertrophy of vascular smooth muscle cells (VSMC) in the medial layer, or ii) reorganization of VSMC in the media around a smaller lumen\textsuperscript{121, 141, 158}. Both of these changes result in an increased media to lumen ratio, and thereby increased vascular resistance\textsuperscript{158}. Due to the intimate nature of the interactions between vascular structure and AP, changes in both are often found to occur simultaneously. As a result, the cause-effect relationship between vascular structure and AP is difficult to establish. Specifically, with respect to development of hypertension, there is an ongoing debate regarding whether hypertension develops as a result of initial increases in AP or whether these increases are a result of changes in vasculature. Despite this controversy, it has been well characterized that changes in vascular structure are associated with changes in AP.

Given that the kidney serves a critical role in the regulation of AP, it is of no surprise that changes in renal vascular structure may be important in determining the long-term level of AP\textsuperscript{10, 11}. Göthberg and Folkow have shown that there is a structurally-based increase in the pre: post-glomerular resistance ratio in SHR\textsuperscript{159-161}. Furthermore,
morphometric assessments of pre-glomerular vessels also provided evidence for structural differences in renal vasculature in hypertensive versus normotensive rats\textsuperscript{157, 162, 163}. In fact, vascular wall thickening of renal vessels was found to precede the period of rapid AP increases in SHR\textsuperscript{152, 161, 164}. These findings suggest that an upregulation of renal vasculature may be responsible for the “resetting” of the pressure-natriuresis mechanism towards greater AP levels typically found in hypertension.

1.6 Genetic and Environmental Influences

Essential hypertension and/or salt-sensitivity develop due to a complex interplay between susceptibility genes and environmental factors. Four types of gene-environment interactions have been proposed: 1) the presence/absence of a critical gene in blood pressure regulation (e.g. ANP knockout mouse [ANP -/-]), where the endpoint phenotype exists in the absence of an environmental factor; 2) the presence of an environmental factor (e.g. lifestyle factors such as stress, smoking, diet, alcohol intake, physical activity), which impacts on the endpoint phenotype regardless of the genotype; 3) the presence of both genetic and environmental factors that produce an effect together (e.g. Dahl salt-sensitive rat), where the endpoint phenotype is expressed only in the presence of an environmental factor, and 4) when the presence of both genetic and environmental factors carry some risk for disease, and the combination has synergistic effects on the endpoint phenotype (e.g. genetic defects related to fat accumulation combined with diet and/or physical activity level)\textsuperscript{165}. In addition to these gene-environment interactions, the timing of exposure to environmental factors that trigger the various blood pressure phenotypes is also important; specifically, the effect of environmental insults during fetal growth and development versus adulthood\textsuperscript{165, 166}. 

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1.6.1 Genetic Factors

The significant role of genetic factors in the pathogenesis of hypertension has been suggested in a number of twin and family studies, where it is estimated that approximately 30-60% of blood pressure variability is genetically determined\(^4\). Most cases of hypertension involve a number of genes, which act either independently or in complex gene-gene interactions to generate the endpoint AP phenotype\(^{165}\). Consequently, there is no clear distinction between hypertension and normotension in the general population. Instead, the AP operating point typically follows a normal distribution. Given the genetic complexity of hypertension in humans, it is difficult to establish which genes contribute to the pathogenesis of hypertension.

The development of experimental models of hypertension allows for dissection and isolation of various hereditary traits and factors associated with AP regulation\(^{167, 168}\). There are two types of genetic animal models of hypertension that have been used to study hypertension: the phenotypic-driven and the genotypic-driven models\(^{167}\).

1.6.1.1 Hereditary Models of Hypertension: Focus on the Spontaneously Hypertensive Rat

The most abundantly used model for hypertension research has been the phenotype-driven (or hereditary) model, which takes advantage of selectively breeding animals (predominantly rats) that display the hypertensive phenotype. Experiments utilizing the phenotype-driven model allow for exploration of polygenic hypertension\(^{167, 168}\). There have been a number of hereditary models of hypertension, such as the Dahl salt-sensitive rat, Sabra model, Lyon hypertensive rat (for reviews see \(^{167, 168}\), however, the most widely used animal model is the SHR\(^{167, 168}\).
The SHR was developed by inbreeding Wistar rats with the highest blood pressure. The SHR model provides many similarities to human essential hypertension with respect to such important aspects as clinical course, pathophysiological development, and secondary diseases. At 5-6 weeks of age, blood pressure in the SHR starts to rise in comparison to their normotensive counterparts, the Wistar-Kyoto (WKY) rat. Similarly, human clinical studies have shown that young normotensive offspring of hypertensive parents have significantly higher blood pressure in comparison to young normotensive offspring with no familial background of hypertension. Furthermore, some hemodynamic alterations which occur during the development of hypertension in the SHR are similar to human essential hypertension (i.e. a high cardiac output early in life, and a normal cardiac output and an increased vascular resistance in adulthood). Lastly, the SHR develop many features of hypertensive end-organ damages (i.e. cardiac hypertrophy, heart failure, renal dysfunction), which lead to the development of secondary diseases (i.e. congestive heart failure and renal failure).

It has been well established that vascular abnormalities, such as increased vascular resistance, vascular reactivity to vasoconstrictors, and media-to-lumen ratio, are associated with hypertension in the adult SHR. However, despite the number of studies describing morphometrical and histological differences in the vessels in the early postnatal period and in weaning SHR (i.e. wall thickening), evidence linking AP elevation with vascular structural and functional changes is much more controversial in the neonatal and young SHR. Previously, we have reported longitudinally consistent differences between SHR and WKY rats, representing a 30-40% increase in SHR vasculature resistance properties throughout a broad interval in the
post-weaning period (4-50 weeks). Given that suckling (2 weeks) and weaning (~3-4 weeks) periods represent a time of intensive maturation of vascular structure in rats, it is of interest to assess whether alterations in structurally-based vascular resistance properties are present at these critical periods of development.

Further, since the kidney serves a critical role in the regulation of arterial pressure, it is not surprising that abnormalities specific to renal vascular resistance (RVR) properties have also been associated with blood pressure elevations in young and adult SHR. Roman and colleagues demonstrated that components of the pressure-natriuresis mechanism (i.e. MBF, UF, and sodium excretion) are shifted rightward towards greater RAP in 3-5, 6-9, and 12 week old SHR versus WKY. These findings suggest that alterations in medullary hemodynamics may participate in the development of hypertension in young SHR in order to maintain sodium homeostasis. Since RIHP is generated by MBF, then a similar rightward shift is expected in RIHP response to changes in RAP. Thus, to further expand on the work of others, it is of interest to assess the acute RAP-RIHP relationship in 4 week-old rats at a time when renal organogenesis is complete.

1.6.1.2 Transgenic Models of Hypertension: Focus on the Atrial Natriuretic Peptide Knockout Mouse

The genotype-driven model takes advantage of available transgenic techniques (typically in mice) to selectively delete or overexpress target genes. Studies using the genotype-driven model allow for exploration of specific susceptibility genes in the pathogenesis of hypertension, especially those known to be involved in the regulation of renal and vascular function (e.g. RAS, SNS, ANP; for review see). For example,
consistent with the known actions of ANP, the *proANP* gene-disrupted mouse (ANP -/-) is hypertensive and salt-sensitive, has high levels of circulating catecholamines, an abnormal regulation of systemic RAS, and cardiac hypertrophy\(^ {186-191}\). The heterozygous mouse (ANP +/-) develops hypertension and cardiac hypertrophy on a high salt diet\(^ {186, 187}\). Furthermore, the natriuretic deficit of ANP -/- mice was uncovered when acute volume expansion in mice fed a high salt diet attenuated sodium excretion via the medullary collecting duct (the principal site of ANP action)\(^ {192}\). Given that the main actions of ANP are to counter increases in blood volume, and thereby AP (discussed in 1.4.3), it is of interest to assess how a permanent removal of the ANP gene affects the pressure-natriuresis mechanism, more specifically the acute RAP-RIHP relationship.

**1.6.2 Environmental Factors**

It is often stated that “genes load the gun, and environment pulls the trigger”; that is, while the genotype predisposes an organism to a range of different phenotypes (as discussed above), the expression of these phenotypes is also largely influenced by the surrounding environment. Evidence with respect to poor lifestyle choices contributing to the onset and prevalence of disease is irrefutable. For example, evidence from an inter-population comparison of 32 nations (i.e. INTERSALT study) revealed that the incidence of hypertension is directly correlated with sodium intake. Interestingly, of the populations studied, the prevalence of hypertension was extremely low (<1%) among the Yanomami Indians in Brazil, who consume insignificant quantities of sodium in their diet\(^ {193}\). Further, in addition to sodium intake, countless other lifestyle factors have been shown to influence the likelihood of developing aberrant AP (i.e. diet, stress, sedentarity, smoking, and alcohol consumption)\(^ {3, 194}\). While environmental factors throughout life
have an influence on the development of disease, it is during the pre- and immediately postnatal period of life that the environmental influence on disease development is most potent (discussed below).

1.6.2.1 Developmental Origins of Health and Disease (DOHaD)

David Barker and colleagues were the first to propose the hypothesis of ‘fetal programming’\(^{195}\), which states that adverse events occurring in fetal life could have lifelong consequences on the health of the offspring in adulthood. The seminal studies by Barker and colleagues described an association between low birth weight and CVD later in life, and proposed that this was due to aberrant fetal development in response to maternal undernutrition\(^{196}\). These findings were subsequently explored and confirmed in numerous epidemiological and experimental studies\(^{197-205}\). From these studies, it was discovered that ‘programming’ is not exclusive to fetal life, and that insults during any period of development (i.e. including pre-implantation phase\(^{206}\), gestation\(^{207}\), and the early postnatal period\(^{208,209}\)) can also impact on long-term health – a concept termed ‘developmental programming’. The notion of developmental programming was further extended to address potential gene-environment interactions in the developing offspring. Coined as ‘developmental plasticity’, this concept explains how one genotype can give rise to a wide range of different phenotypes in response to environmental conditions during development\(^{198,199,210}\).

Given that the genetic background in animal models can be controlled, it is possible to examine the effects and influences of specific environmental changes during gestation and early postnatal life on long-term health. Various animal models have been established to study developmental programming. The most common models of maternal
and fetal insults include, but not limited to: i) undernutrition/overnutrition\textsuperscript{207, 211-213}; ii) imbalances of macronutrients (e.g. protein\textsuperscript{214-216}, fat\textsuperscript{217}, and carbohydrate\textsuperscript{218}); iii) micronutrient deficiencies (e.g. calcium\textsuperscript{219} and iron\textsuperscript{220-224}); iii) placental insufficiency\textsuperscript{207}; iv) fetal glucocorticoid exposure\textsuperscript{225}; v) prenatal hypoxia\textsuperscript{226, 227}; and vi) neonatal oxygen exposure\textsuperscript{228}. Interestingly, all of these models typically display similar characteristics, in that the long-term consequences of such insults in early life include a host of cardiometabolic risk factors for CVD in later life; specifically, hypertension, glucose intolerance, insulin resistance, dyslipidemia, and increased fat accumulation\textsuperscript{166, 197, 198, 229, 230}. Thus, it appears that both the cardiovascular and endocrine systems are particularly prone to developmental programming. However, the time course at which these insults induce the aberrant development of the aforementioned systems, as well as the mechanisms that underlie these cardiometabolic disorders are unknown.

The clustering of cardiometabolic disorders, such as hypertension, glucose intolerance, insulin resistance, dyslipidemia, and increased fat accumulation, is referred to as ‘metabolic syndrome’. Obesity, specifically of the visceral type, has been proposed to be the culprit in the development of this syndrome\textsuperscript{231}. Furthermore, risk estimates from the Framingham Study suggest that approximately 65-78\% of hypertension may be directly attributed to excess adiposity\textsuperscript{232}. The central feature of obesity-associated hypertension is related to alterations in proper sodium handling via abnormalities in key AP regulating systems, such as the SNS, RAS, ANP, and of course pressure-natriuresis\textsuperscript{233}. Although not yet characterized in any model of developmental programming, it has been shown the pressure-natriuresis mechanism is blunted in animal models of obesity (i.e. Wistar fatty rats\textsuperscript{214} and obese Zucker rats\textsuperscript{234}) suggesting that
obesity also plays a role in salt-sensitivity\textsuperscript{235}. Thus, these findings further suggests that the interplay between concomitant cardiometabolic risk factors in adulthood in various models of developmental programming limits the ability to distinguish whether specific insults in early life induce aberrant changes in all of these systems, or simply affect one (i.e. fat accumulation). Further research is warranted to unravel the potential mechanisms involved in the developmental programming of hypertension, specifically the effects of early life insults on body composition and pressure-natriuresis.

1.6.2.2 Perinatal Iron Deficiency

Consistent with other animal models of developmental programming, perinatal iron deficiency (PID) has been associated with various cardiometabolic complications in adulthood, such as hypertension\textsuperscript{220, 222, 223}, as well as altered glucose and lipid metabolism\textsuperscript{220}. Furthermore, PID offspring were found to have an abnormal renal morphology, specifically a reduced nephron endowment concomitant with increased AP\textsuperscript{224}. These data suggest that iron may be necessary for the proper renal development and function in regulating AP via pressure-natriuresis. Additionally, the presence of several components of metabolic syndrome in adult PID offspring suggests that these animals may have a detrimental body composition, characterized by excess accumulation of visceral adipose tissue. Thus, assessments of both the pressure-natriuresis mechanism and body composition are yet to be examined in this model of developmental programming.
1.7 Statement of Hypotheses and Objectives

Hypertension and salt-sensitivity are independent risk factors for the development of CVD\textsuperscript{2,236}. Although both salt-sensitivity and hypertension are idiopathic, these conditions develop due to a complex interplay between susceptibility genes and environmental factors. Given that the kidney serves a dominant role in the long-term regulation of AP\textsuperscript{7,10,13,14,17}, it is not surprising that disturbances in renal function, specifically the ability to regulate AP via sodium and fluid balance, have been linked to these conditions\textsuperscript{7-10}. While previous research has provided important foundational knowledge of renal regulation of AP in the long-term, the evidence with respect to renal function on a moment-to-moment basis has not been well studied. Thus, the main focus of the research presented herein is to characterize the moment-to-moment pressure-natriuresis mechanism (i.e. the RAP-RIHP relationship), and assess the functioning of this intrarenal mechanism in various animal models of hypertension and/or salt-sensitivity.

The overall working hypotheses are as follows:

1. The long-term nature of the pressure-natriuresis mechanism is the cumulative effect of small, rapid changes in AP and RIHP that gradually induce homeostatically appropriate changes in blood volume and AP (Figure 1.8).

2. The functioning of the moment-to-moment RAP-RIHP relationship reflects the AP phenotype set by genetic and environmental factors, such that a rightward shift in this relationship is indicative of hypertension and a blunting of the slope is indicative of salt-sensitivity.
3. The positioning of the moment-to-moment RAP-RIHP curve along the x-axis is primarily indicative of renal vascular resistance properties, whereas the steepness of the curve is modulated by neurohumoral systems and renal interstitial compliance.

**Figure 1.8:** Hypothetical functioning of renal interstitial hydrostatic pressure (RIHP) and the moment-to-moment pressure-natriuresis mechanism following acute changes in arterial pressure.
The studies in which these hypotheses were tested include:

**Chapter 2: Moment-to-Moment Characteristics of the Relationship Between Arterial Pressure and Renal Interstitial Hydrostatic Pressure**

The specific objectives were:

1. To develop an *in vivo* methodology to assess the moment-to-moment relationship between RAP and RIHP with all neurohumoral systems left intact.
2. To examine the time course of changes in RIHP following acute changes in RAP.
3. To determine whether RIHP responds differentially and linearly to acute changes in RAP above and below the AP operating point.
4. To assess whether neurohumoral systems, specifically the RAS and ANS, impact on the acute RAP-RIHP relationship.

**Chapter 3: Altered Vascular Resistance Properties and Acute Pressure-Natriuresis Mechanism in Young Spontaneously Hypertensive Rats**

The specific objectives were:

1. To determine whether structurally-based vascular resistance properties in young SHR are upregulated prior to the development of established hypertension.
2. To examine whether the acute pressure-natriuresis mechanism is altered in young SHR prior to the development of established hypertension.

**Chapter 4: Altered Regulation of Renal Interstitial Hydrostatic Pressure and the Renal Renin-Angiotensin System in the Absence of ANP.**

The specific objectives were:
1. To determine whether chronic ANP disruption results in altered functioning of the acute pressure-natriuresis mechanism.

2. To characterize whether ANP disruption affects renal and adrenal local expression of the RAS.

**Chapter 5: Long-Term Circulatory Consequences of Perinatal Iron Deficiency in Male Wistar Rats**

The specific objectives were:

1. To determine the long-term effects of PID on the circulatory phenotype using radiotelemetry.

2. To assess whether PID alters the functioning of the acute pressure-natriuresis mechanism.

3. To determine renal function by characterizing change in AP during low, normal, and high sodium intake.

**Chapter 6: Sedentariness and Increased Visceral Adiposity in Adult Perinatally Iron-Deficient Rats**

The specific objectives were:

1. To assess the effect of PID on visceral adiposity and physical activity.

2. To determine whether AP responsiveness to dietary sodium is associated with increased visceral adiposity in the adult PID.
CHAPTER 2

Moment-to-Moment Characteristics of the Relationship Between Arterial Pressure and Renal Interstitial Hydrostatic Pressure

(Komolova M and Adams MA. Hypertension, Accepted with Minor Revision)

2.1 Abstract

The kidney is a key controller of the long-term level of arterial pressure, in part, through pressure-natriuresis. Although direct coupling of changes in renal arterial pressure (RAP) to renal interstitial hydrostatic pressure (RIHP) and consequent sodium excretion is well established, few studies have characterized the moment-to-moment aspects of this process. These studies characterized the short-term hemodynamic component of pressure-natriuresis in vivo before and after autonomic nervous system (ANS) and renin-angiotensin system (RAS) inhibition. Changes in RIHP were determined over a range of RAP in Wistar rats receiving no treatment, a ganglionic blocker (hexamethonium; 20mg/kg/hr i.v.), or an angiotensin II type 1 receptor blocker (losartan; 10mg/kg/hr i.v.). Following a series of changes in RAP, a delay of only ~1s was found for the onset of RIHP responses that was independent of the stimulus magnitude and neurohumoral manipulation; however, completion of the full RIHP response was within ~15s for RAP changes of ≤30mmHg. The overall slope of the RAP-RIHP relationship (0.09±0.01) was also not affected by ANS and RAS inhibition despite decreasing RAP (decrease 40% and 28%, respectively). Separate assessment of this relationship above and below the prevailing arterial pressure revealed that the pressor versus the depressor portion was blunted (P<0.001); a difference that was abolished
following ANS and RAS inhibition. The results suggest that spontaneous changes in arterial pressure are coupled to moment-to-moment changes in RIHP over a wide range of pressures, emphasizing a likely role for the dynamic component of the RAP-RIHP relationship in the modulation of sodium excretion, and hence arterial pressure.

2.2 Introduction

The kidney plays an important role in regulating blood volume and arterial pressure, in particular via a pressure-dependent regulation of sodium and water balance, a process known as pressure-natriuresis\textsuperscript{7, 9, 11, 17}. Pressure-natriuresis acts through a final common pathway that directly couples renal arterial pressure (RAP) with renal interstitial hydrostatic pressure (RIHP) leading to changes in downstream sodium excretion (i.e. increases in RIHP lead to enhanced sodium excretion)\textsuperscript{11, 28, 34, 58, 237}.

Given that pressure-natriuresis has been regarded as a ‘long-term’ regulatory mechanism, it is not surprising that numerous studies have investigated the specific relationship between RAP and RIHP over a prolonged time course. Specifically, these studies induced graded step changes in RAP for at least 30min, and then recorded ‘steady-state’ changes in RIHP and associated changes in sodium excretion\textsuperscript{22, 30, 59-62, 65, 67, 91}. However, such assessments may not reflect true \textit{in vivo} control of arterial pressure, since arterial pressure in conscious animals does not change from one steady-state to another, but rather fluctuates spontaneously in varying degrees of magnitude around an operating point (baseline) and over a wide range of timeframes (frequencies)\textsuperscript{58}. For example, oscillations in arterial pressure assessed over an acute timeframe (Figure 2.1A) tend to be more variable and of greater magnitude than fluctuations assessed over a prolonged timeframe (Figure 2.1B). This suggests that kidneys are exposed to
Figure 2.1: Example tracings of a 24h mean arterial pressure (MAP) radiotelemetry profile from a conscious, freely-moving, 11 week-old male Wistar rat. Data points represent MAP A: over a 15s sampling period every 4min, and B: the average of the 4min data every 60min. It should be noted that rats are nocturnal animals (i.e. active at night), and as a result have elevated MAPs at night versus day. The 24h MAP average is represented by the black dashed line, the average night MAP is represented by the top grey dashed line, and the average day MAP is represented by the bottom dashed line. Insets: Frequency distribution plots for MAP in bins of 1mmHg.
greater arterial pressure variations over a shorter timeframe than have been examined using conventional pressure-natriuresis methodologies. Interestingly, only one study to date has described a statistically significant moment-to-moment correlation between spontaneous arterial pressure oscillations and RIHP within frequency windows of 1Hz to 0.01Hz\textsuperscript{58}. This same group also found that urine flow, a variable downstream from RIHP, responded to changes in arterial pressure within \textasciitilde6s\textsuperscript{57}. Since changes in RIHP are an intermediate step in pressure-natriuresis, there must be a shorter delay between arterial pressure and RIHP; however, the exact time course is yet to be elucidated.

Further, despite that previous studies using the conventional ‘graded step’ methodology describe the overall relationship between components of pressure-natriuresis (i.e. RIHP and sodium excretion) and arterial pressure as linear\textsuperscript{8,48}, a more physiological characterization of the RAP-RIHP relationship (i.e. alternating acute pressor and depressor stimuli around the arterial pressure operating point) is warranted. In fact, Steele \textit{et al.}\textsuperscript{57} suggest that acute increases above the arterial pressure operating point are dealt with more effectively than decreases with respect to urine flow. Whether such a differential response to pressor and depressor stimuli exists in regards to RIHP is yet to be determined.

Lastly, due to the duration of the conventional ‘graded step’ methodology, the influence of potential compensatory neurohumoral factors were typically controlled for by various methods (e.g. hormonal cocktails)\textsuperscript{22,30,59-62,65,67,91}. However, there has not been a systematic assessment of how various neurohumoral systems impact on the RAP-RIHP relationship.
Thus, given that in conscious, freely moving animals arterial pressure oscillations occur on a moment-to-moment basis, we sought to determine the renal hemodynamic responses (i.e. RIHP) associated with such changes. More specifically, our objectives were to: (i) examine the time course of changes in RIHP following acute changes in arterial pressure, (ii) determine whether RIHP responds differentially and linearly to acute changes in arterial pressure above and below its operating point, and (iii) assess whether neurohumoral systems, specifically the renin-angiotensin system (RAS) and autonomic nervous system (ANS), impact on the acute RAP-RIHP relationship.

2.3 Methods

2.3.1 Animals

Male Wistar rats (n=40; 300-400g; 11-12 weeks old) were obtained from Charles River. Rats were housed individually (21±1°C; 12h light/dark cycle) and acclimatized for at least 96h prior to experimentation. All rats were provided with standard rat chow (Purina; 0.4% Na+) and water ad libitum. All procedures followed guidelines of the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee.

2.3.2 Surgical Preparation

Rats were anesthetized with Rogarsetic (ketamine; 30mg/kg body wt i.p.) and Inactin (thiobarbital sodium; 100mg/kg body wt i.p.). Body temperature was monitored using a thermistor (model 402; Yellow Springs Instruments) and maintained at 37±1°C using a temperature controller (model 73A; Yellow Springs Instruments) connected to a heating pad and lamp. Additional anesthetic was given, as necessary, throughout experiment. Rats were tracheostomized (PE-240) and 95%O₂/5%CO₂ was passively
blown over the intake to assist respiration. A midline abdominal incision was made, and the right kidney removed. A saline-filled catheter was introduced into the inferior vena cava (at the level of right iliolumbar vein) for continuous infusion of saline (0.9% NaCl) at 33μl/min/100g body weight via a syringe pump (KD Scientific 220) to compensate for fluid loss during surgery. A second catheter was introduced just above the other for drug administration.

The modified *in vivo* assessment of renal vascular properties\textsuperscript{238, 239} was based on the technique described by Roman and Cowley\textsuperscript{59} and the direct determination of RIHP according to Ott *et al.*\textsuperscript{67, 240}; however, the neurohumoral control systems were left intact. The superior mesenteric artery was catheterized with heparinized saline-filled (50IU/ml) PE-50 tubing for continuous measurement of arterial pressure at the level of the renal artery (hereinafter referred to as RAP). Water-filled silastic balloon cuffs were placed between the celiac and superior mesenteric arteries, and 10mm distal from the left renal artery. The cuffs were connected to a syringe with a 3-way stopcock via a water-filled line, which allowed for manual manipulation and control of RAP over a wide range of pressures (i.e. pressor and depressor)\textsuperscript{91}. An electrosurgical unit (Elmed ESU30; Elmed Incorporated) was used to create a 3mm-hole into the longitudinal axis of the left kidney for insertion of a heparinized saline filled (50IU/ml) catheter for RIHP measurements (Tygon tubing (ID 0.64mm) fitted with 2-3mm long polyethylene matrix with 15-45μm pores; Porex)\textsuperscript{91, 238-240}. The catheter was held in place with cyanoacrylate glue. RAP and RIHP were continuously monitored via pressure transducers (CDX3, Cobe) connected to a PowerLab/8s (ADInstruments) data acquisition system with Chart v.4.2.2 software.
2.3.3 Experimental Protocol

After surgery, an equilibration period of ~15min was allowed prior to recording steady state baselines of RAP and RIHP. The acute RAP-RIHP relationship was determined via short-term manipulations of RAP (1-60s). These manipulations consisted of sequential pressor and depressor changes in RAP of various magnitudes around the operating point (baseline). Through visualization of the real-time RAP signal, an investigator manually generated a desired RAP within ~5s by inflating the appropriate cuff via the attached syringe and using the stopcock to keep the inflated cuff static at the desired RAP target. RAP changes that overshot or did not achieve the desired target within ~5s were not included in the analysis.

The groups were: Group 1 (n=19) received no treatment and Group 2 (n=14) were given a ganglionic blocker (hexamethonium; 20mg/kg/hr i.v.). Group 3 (n=7) were given an angiotensin II type 1 (AT$_1$) receptor blocker (losartan; 10mg/kg/hr i.v.) after an angiotensin II (Ang II; 250ng/kg bolus i.v.) pre-treatment. Ang II was also administered following losartan treatment to ensure sufficient blockade of AT$_1$-receptors. Additionally, control values were also obtained in half of the hexamethonium- and losartan-treated animals, such that paired analysis could be conducted.

Upon completion of the experiment, hematocrit was determined and rats were euthanized via exsanguination. Lastly, the left kidney was removed, and the position of the RIHP catheter was verified at the cortical-outer medullary junction.

2.3.4 Data Analysis

Baseline RAP and RIHP were determined by averaging the 60s period prior to starting RAP manipulations. The slopes were determined from steady-state values of
RAP and RIHP following manipulations of RAP (Figure 2.2A&B). The time delay between the initial RAP change and the onset of RIHP response was characterized as the difference between the first detectable change from the operating point following a manipulation (Figure 2.2A&B). The time delay between achieving steady-state RAP and RIHP was also characterized as the time difference when RAP and RIHP approach a plateau (Figure 2.2A&B). Although, not calculated for all data, we determined that when the variables reached a plateau, the rate of change at this point was not significantly different from the rate of change at steady-state (Figure 2.2C&D). Recovery from stimulus data (i.e. point at which cuff was deflated) was assessed in a similar fashion.

2.3.5 Statistical Analysis

All statistical calculations were performed and graphs constructed using GraphPad Prism 5 and/or Microsoft Excel. Linear regression analysis was used to calculate the overall, pressor, and depressor relationship between RAP and RIHP. Statistical significance of differences in values measured between untreated, hexamethonium-treated, and losartan-treated rats was determined using a one- or two-way analysis of variance (ANOVA) followed by a Newman-Keuls or Bonferroni post hoc test, where appropriate. All data are presented as means±SEM. P<0.05 was considered statistically significant.

2.4 Results

2.4.1 Baseline Characteristics and Intrarenal Hemodynamic Variables

A summary of body weights, hematocrit and intrarenal hemodynamic parameters assessed in control and pharmacologically manipulated animals is presented in Table 2.1. Body weights and hematocrit were not significantly different across groups. In the
Table 2.1: Baseline characteristics and intrarenal hemodynamics of 11-12 week old Wistar rats.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Control (n=19)</th>
<th>Hexamethonium (n=14)</th>
<th>Losartan (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (grams)</td>
<td></td>
<td>357.1 ± 5.1</td>
<td>358.3 ± 5.9</td>
<td>354.6 ± 3.0</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td></td>
<td>47.9 ± 0.6</td>
<td>48.3 ± 0.9</td>
<td>50.2 ± 0.5</td>
</tr>
<tr>
<td>Percent Change in RAP from Baseline</td>
<td></td>
<td>0.1 ± 0.9</td>
<td>-39.7 ± 3.9*</td>
<td>-28.2 ± 2.3*†</td>
</tr>
<tr>
<td>Percent Change in RIHP from Baseline</td>
<td></td>
<td>1.4 ± 3.3</td>
<td>-36.3 ± 7.9*</td>
<td>-15.0 ± 13.4</td>
</tr>
</tbody>
</table>

RAP, renal arterial pressure; RIHP, renal interstitial hydrostatic pressure. Values are mean ± SEM. * P < 0.001 vs. control group and † P < 0.05 vs. hexamethonium-treated group (One-way ANOVA followed by a Newman-Keuls post hoc test used to compare between groups).
control group, there was no difference between the baseline mean RAP and RIHP at the beginning of the experiment (94.2±3.3mmHg and 7.8±0.8mmHg, respectively) and the average RAP and RIHP throughout the experiment (94.1±3.1mmHg and 7.8±0.8mmHg, respectively). The mean RAP following hexamethonium and losartan treatments were approximately 40% (54.4±3.3mmHg) and 28% (65.2±3.7mmHg) lower than the pre-treatment baseline RAP, respectively (P<0.001). Further, there was a significant 36% decrease in mean RIHP following hexamethonium treatment (3.9±0.8mmHg; P<0.01), and a 15% decrease after losartan treatment (5.2±1.1mmHg).

As part of protocol, the losartan group was given a pressor dose of Ang II before and after losartan administration to confirm sufficient blockade of AT$_1$-receptors. Thus, whereas brief pretreatment with Ang II produced a rapid but transient increase in RAP (23%) and a decrease in RIHP (-15%); after losartan, Ang II did not produce any significant changes (data not shown).

2.4.2 In Vivo Characterization of the Acute RAP-RIHP Relationship

As described, a modified in vivo protocol was used to assess the acute RAP-RIHP relationship with all the neurohumoral systems left intact. In all animals, there was a clear, positive relationship between RAP and RIHP (Figures 2.2 & 2.3). Similar to previous studies showing a linear RAP-RIHP relationship over a prolonged timeframe, the overall acute RAP-RIHP responses were also found to be correlated linearly (i.e. response to stimulus slope was 0.09±0.01, $R^2$=0.94) (Figure 2.3A). In addition, the acute RAP-RIHP relationship during the recovery phase from each manipulation had a similar slope (i.e. recovery from stimulus slope was 0.09±0.01, $R^2$=0.95) (Figure 2.3B). However, separate analysis of the pressor and depressor components of this relationship revealed that the
Figure 2.2: Example of a pressor and depressor manipulation on RAP and RIHP changes in an untreated anesthetized rat. Change in RAP and RIHP following A: pressor and B: depressor manipulations. The pressure difference between corresponding arrows (i.e. black for RAP, grey for RIHP) represents values used to determine the slope of the RAP-RIHP relationship. The time delay between the initial RAP change and onset of RIHP response ($T_i$), and the time delay between achieving steady-state for RAP and RIHP ($T_s$) were characterized as shown. C: The rate of change (DP/dt) in RAP and RIHP following a pressor manipulation of ~26mmHg, which peaked at 8s and achieved steady-state (i.e. consecutive zero values) at 15 and 23s, respectively. D: The DP/dt in RAP and RIHP following a depressor manipulation of ~24mmHg, which peaked at 8 and 9s, respectively, and achieved steady-state at 11 and 20s, respectively. Data displayed are means obtained every second.
Figure 2.3: Changes in RIHP corresponding to induced changes in RAP in control (CTL; n=19), hexamethonium-treated (HEX; n=14), and losartan-treated (LOS; n=7) rats. Data displayed in bins of 10mmHg for A: response to stimulus and B: recovery from stimulus RAP-RIHP relationships. Insets: Slopes of the overall RAP-RIHP relationship. Values are means ± SEM. There were no significant differences in the slopes following treatments (One-way ANOVA followed by a Newman-Keuls post hoc test). Note: all points are bins of 10mmHg, except for RAP changes of -80 to -50mmHg and 30 to 50mmHg in CTL group, which were binned together due small amount of points in those pressure ranges.
pressor slope was substantially lower (-43%) than the depressor slope (P<0.001) (Figures 2.4A). A similar blunted pattern was present in the recovery phase following pressor manipulations (-39%) (P<0.001) (Figure 2.4B).

In all cases, RIHP was found to respond rapidly to changes in RAP (Figure 2.2). That is, following a change in RAP, initial changes in RIHP occurred with an average delay of only 1.1±0.2s. This response time was independent of the magnitude or type of change (pressor or depressor) in RAP (Figure 2.5A). Furthermore, the RIHP response time was the same for both the response to and recovery from RAP manipulations (Figure 2.5B). To characterize whether there was a lag in the overall RAP-RIHP time course, the time difference between achieving steady-state in RAP and RIHP was assessed. There was a clear impact of the magnitude of RAP change on the time difference to achieve steady-state between RAP and RIHP, as the time delay was found to be approximately 6s for RAP changes of ±0-10mmHg, whereas for greater pressure changes (±20-30mmHg) the delay was more than 2-fold longer (Figure 2.6A).

2.4.3 Role of Neurohumoral Systems on the Acute RAP-RIHP Relationship

As for the controls, the overall RAP-RIHP relationship was found to be linearly correlated following ANS and RAS inhibition (Figure 2.3). Additionally, there were no differences in the slopes for responses to stimulus (hexamethonium = 0.09±0.01, R²=0.91; losartan = 0.07±0.01, R²=0.95) and the recovery from stimulus (hexamethonium = 0.08±0.01, R²=0.92; losartan = 0.07±0.01, R²=0.94), for both between group comparisons and with respect to controls (Figure 2.3A&B). Further, paired analysis of data from animals that were initially controls but then received one of the treatments revealed there
Figure 2.4: **Average slopes of the RAP-RIHP relationship for increases and decreases in RAP from baseline.** Slopes for pressor and depressor manipulations in control (CTL; n=19), hexamethonium-treated (HEX; n=14), and losartan-treated (LOS; n=7) rats for A: response to stimulus and B: recovery from stimulus RAP-RIHP relationships. Two-way ANOVA showed significant overall differences between response and recovery slopes and treatments. Bonferroni post hoc testing showed significant differences between pressor and depressor manipulation slopes in the CTL group (** **P<0.001), between HEX and CTL pressor slopes (#P<0.05), and between HEX (ΨP<0.05), LOS (ΨP<0.05 and ΨΨP<0.01) and CTL depressor slopes. C: Induced changes in RAP around the average pressure operating point (larger squares) and the corresponding RIHP response in CTL (grey), HEX (white) and LOS (black) groups. Data displayed in bins of 10mmHg for RAP manipulations of ±30mmHg. Values are means ± SEM.
Figure 2.5: Time delay for RIHP to respond to a change in RAP. RIHP response times A: following absolute changes in RAP (10mmHg bins) in control rats B: in response to stimuli versus recovery from stimuli, and C: in untreated (CTL; n=19), hexamethonium-treated (HEX; n=14), and losartan-treated rats (LOS; n=7) following all types of RAP changes. Values are means ± SEM. One-way ANOVA showed no significant differences.
Figure 2.6: Time interval between achieving steady-state RAP and RIHP. Time delay by stimulus intensity (10mmHg bins) in A: control (n=19), B: hexamethonium-treated (n=14), and C: losartan-treated (n=7) rats. Values are means ± SEM. One-way ANOVA followed by a Newman-Keuls post hoc test showed significant differences between ±0-10 mmHg of change in RAP versus ±20-30 mmHg of change in RAP (*P<0.05), and ±10-20 mmHg of change in RAP versus ±20-30 mmHg of change in RAP (#P<0.05, ##P<0.01).
was no significant effect of the treatments on the overall slope of the response or recovery data (data not shown). However, the significant difference between the slopes of the pressor and depressor arms of RAP-RIHP relationship in controls was abolished after lowering the operating point with hexamethonium and losartan treatments (Figure 2.4A-C). In fact, this treatment-induced equalization of the pressor and depressor aspects of the RAP-RIHP relationship occurred for both the response and recovery slopes.

The time course for RIHP to respond to changes in RAP was assessed among the hexamethonium and losartan groups. Similar to controls, both treatments had a rapid RIHP onset time following a change in RAP, more specifically in the hexamethonium group there was an initial delay of 0.9±0.2s and in the losartan group an initial delay of 1.4±0.2s (Figure 2.5C). Also, the difference in the time delays between achieving steady-state in RAP and RIHP was not significantly different from controls in both treatment groups. Specifically, the time delay was 7.4±1.1s in the hexamethonium group and 6.7±1.1s in the losartan group for RAP changes of ±0-10mmHg, whereas for greater pressure changes (±20-30mmHg) the delay was approximately 2-fold longer (Figure 2.6B&C).

2.5 Discussion

Pressure-natriuresis is an important renal mechanism involved in the regulation of the long-term level of blood volume and arterial pressure. Investigations of pressure-natriuresis, specifically the RAP-RIHP relationship, have usually been conducted over time periods sufficient to induce measurable changes in urine production (e.g. ≥30min). While previous research has provided important foundational knowledge of renal mechanisms over this timeframe, regulation on a moment-to-moment basis has not been as well studied. The key findings of the present study were that the initial RIHP response
time to an acute change in RAP was approximately one second, regardless of the magnitude of RAP change and neurohumoral status of the animal, and that completion of the full RIHP response occurred within ~15s for RAP changes of ≤30mmHg. Further, the acute RAP-RIHP relationship correlated linearly over a wide range of arterial pressures, and the slope was not affected markedly by pharmacological antagonism of the ANS and RAS. A novel finding from a specific analysis of the RAP-RIHP relationship above and below the arterial pressure operating point revealed that the pressor component was not as steep as the depressor. However, following pharmacological inhibition of the ANS or RAS, this difference between the pressor and depressor components was abolished. These results suggest that this intrarenal hemodynamic mechanism, which is linked to downstream mechanisms regulating sodium excretion, has short-term baroreflex-like properties that may facilitate the regulation of arterial pressure around an operating point.

The time course of RIHP responses following rapid changes in arterial pressure has not been previously characterized. The present study is the first to reveal that the delay between an acute change in RAP and the onset of a RIHP response is approximately one second, regardless of the magnitude or direction of arterial pressure stimulus. These results are consistent with a prior study assessing the onset of changes in urine flow, a downstream step from RIHP in the pressure-natriuresis mechanism, where a 6s delay following various arterial pressure changes was documented\textsuperscript{57}. Furthermore, the time difference between achieving a steady-state RAP and RIHP was found to be dependent, in part, on the magnitude of arterial pressure change ranging from 6 to 12s for pressure changes of ±5-30mmHg. These findings suggest that the consequences of the
oscillations in RIHP (i.e. on sodium excretion) following spontaneous changes in arterial pressure could occur at a frequency of 5-10 changes/min. Given that arterial pressure fluctuates spontaneously and over a wide range of pressures, the observed baroreflex-like nature of RIHP suggests that this moment-to-moment aspect of pressure-natriuresis can act via progressive, cumulative effects on the level of arterial pressure. That is, moment-to-moment changes in RIHP may impact on urine formation, and the cumulative effect of these acute changes over time may account for the long-term aspect of pressure-natriuresis by inducing homeostatically appropriate changes in blood volume, and thereby returning arterial pressure towards its operating point\textsuperscript{57, 58}.

Similar to previous studies using conventional pressure-natriuresis methodology\textsuperscript{22, 30, 59-62, 65, 67, 91}, the overall acute RAP-RIHP relationship was found to correlate linearly and have a similar slope over a wide range changes in RAP. However, despite the numerous studies describing the RAP-RIHP relationship as linear, none assessed whether there is differential regulation above and below the arterial pressure operating point. The present findings demonstrate that the pressor slope, for RAP changes of 40mmHg above the baseline, was approximately half the depressor slope. Previously, it was shown that acute increases in arterial pressure above the operating point are dealt with more efficiently than decreases with respect to urine flow on a moment-to-moment basis\textsuperscript{57}. Since RIHP is an intermediate step to urine flow, it may be hypothesized that small, moment-to-moment increases in RIHP produce greater effects on sodium excretion than do decreases. This point will need to be determined in future studies, which assess both moment-to-moment changes in RIHP and urine flow. Further, we have previously shown that atrial natriuretic peptide knockout (ANP-/-) mice, which are salt-sensitive, have a
blunted pressor slope in comparison to their wild-type counterparts (Chapter 4). This data revealed that the functioning of the acute RAP-RIHP relationship appears to also reflect the salt-sensitive phenotype of the animal, but does not yet indicate it is causal. Despite this, it would be expected to find strain and species-specific differences in the renal mechanisms necessary to regulate sodium excretion, and thereby plasma volume and arterial pressure, under different physiological and pathophysiological conditions. Although it is speculation, the basis for the differences in pressor and depressor components may be due to mechanisms such as: i) myogenic response\textsuperscript{242}, ii) flow-mediated vasodilation\textsuperscript{243}, or iii) alterations in the functioning of certain neurohumoral systems\textsuperscript{18}. Given the blunted RIHP response following pressor manipulations, the myogenic response seems the more likely explanation, since the resulting increase in vascular tone should in theory decrease medullary blood flow (MBF), vasa recta hydrostatic pressure, and thereby RIHP; however MBF responses to spontaneous changes in RAP are yet to be examined. For example, this hypothesis is consistent with the finding that medullary interstitial NO does not rise substantially at RAPs of 110-140mmHg\textsuperscript{114}. Given that Lieb \textit{et al.} (2009) found that cyclic guanosine monophosphate (cGMP) levels increase following pressor stimuli of 30min, it may be that cGMP levels are increased after a sustained pressor response either through the NO-soluble guanylyl cyclase (GC) or ANP-particulate GC-cGMP pathways\textsuperscript{115}. However, the role of these and other vasoactive molecules (i.e. arachidonic acid metabolites, endothelins, etc.) in the functioning of the acute RAP-RIHP relationship needs to be examined in future studies\textsuperscript{18}. Lastly, since the full RIHP response is achieved in \textasciitilde15s, it is unlikely that slower acting neurohumoral mechanisms play a role in altering the acute functioning of the RAP-RIHP relationship.
relationship; although this also needs to be confirmed. Thus, although the basis for the disparity between the pressor and depressor slopes of RIHP and urine flow have not been fully resolved, one concept that appears certain is that the directionally opposing arms of the pressure-natriuresis relationship do not operate equivalently. Thus, future studies should assess these renal mechanisms in both directions from baseline in order to prevent masking the differences that may have occurred using conventional approaches.

Many neurohumoral factors are known to modulate sodium balance, yet the precise mechanisms by which these factors may affect pressure-natriuresis, particularly in the short-term, have not been fully resolved. Nevertheless, most previous studies have tried to control for potential compensatory effects of neurohumoral factors. In the present study, inhibition of the ANS or RAS did not modify the overall slope of the moment-to-moment RAP-RIHP relationship or the time course of RIHP responses to changes in RAP despite the decrease in arterial pressure. However, unlike in untreated animals, the pressor and depressor slopes became similar following these treatments. Given that the treatments lowered the arterial pressure operating point, it appears that the moment-to-moment RAP-RIHP response has become a composite of the pressor and depressor arms in controls (i.e. increased pressor and decreased depressor slopes). In fact, these data support the hypothesis that a myogenic response may be responsible for the blunting of the pressor response in control animals. That is, animals treated with the depressor agents may have a dampened intrinsic myogenic response due to lower levels of wall stress, and thereby a heightened RIHP response. Future studies will have to determine whether lowering arterial pressure and/or inhibiting the ANS and RAS triggers
compensatory responses of vasoactive molecules (i.e. NO, arachidonic acid metabolites, etc.) that may impact on the functioning of the acute RAP-RIHP relationship\textsuperscript{18}.

Although the outcomes of these studies provide a better understanding of acute pressure-natriuresis mechanisms, there are some limitations that need to be addressed. Specifically, given the short-term and bi-directional design of the assessments, urine could not be collected. Further, it is unknown whether anesthesia and surgery could affect the acute RAP-RIHP relationship, however, given that the overall slope of the present experiments is comparable with those performed previously in conscious animals\textsuperscript{58}, the influence of these does not appear to be significant.

In summary, short-term changes in arterial pressure were found to be coupled to moment-to-moment changes in RIHP over a wide range of pressures. That is, this intrarenal mechanism is rapid (~1s) and linear, but different, over a wide range of acute pressor and depressor changes. Taken together, these findings suggest that this intermediate component of the pressure-natriuresis mechanism can respond rapidly and repeatedly to transient, and bidirectional changes in arterial pressure, thereby modulating sodium excretion in a cumulative manner.

2.6 Perspectives

The present study provides a new perspective on how the pressure-natriuresis mechanism, specifically the RAP-RIHP relationship, functions over a short-term timeframe. Pressure-natriuresis has been previously regarded as a ‘long-term’ controller of arterial pressure; however the findings from this study support the hypothesis that the long-term character of the pressure-natriuresis mechanism is established, in part, via cumulative moment-to-moment interactions between RAP and RIHP. Further, our results
indicate that the responsiveness of RIHP depends on the arterial pressure operating point of the organism. To further expand this concept, future studies should investigate the effects of various vasoactive factors and antihypertensive agents on this important component of the pressure-natriuresis mechanism, as well as how its functioning may be altered in hypertensive models.
CHAPTER 3

Altered Vascular Resistance Properties and Acute Pressure-Natriuresis Mechanism in Young Spontaneously Hypertensive Rats

(Komolova M, Friberg P, and Adams MA. Submission to Hypertension)

3.1 Abstract

Although they have been extensively scrutinized, the factor(s) involved in the initiation and development of hypertension in spontaneously hypertensive rats (SHR) remain unresolved. The present study assessed whether early in development the causal mechanism(s) involve an integration of two processes: upregulation of structurally-based vascular resistance properties and a rightward shift in the pressure-natriuresis relationship in young SHR versus Wistar-Kyoto rats (WKY). Mean arterial pressure (MAP) was determined in conscious 4 week-old SHR and WKY via previously implanted aortic catheters. Structurally-based hindlimb vascular resistance properties were assessed in 2 and 4 week-old SHR and WKY. Renal interstitial hydrostatic pressure (RIHP) was measured following short-term manipulations of renal arterial pressure (RAP) in 4 week-old, anesthetized rats. Although MAP in conscious SHR (113±5mmHg) and WKY (110±6mmHg) was not significantly different at 4 weeks of age, assessment of vascular resistance properties revealed that SHR at 2 and 4 weeks of age already had increases in structurally-based vascular resistance properties of ~30% above age-matched WKY. Further, the acute RAP-RIHP relationship was found to be linear in both strains and the temporal coupling of the stimulus-response was rapid; i.e. RIHP response for a change in RAP was <2s. Although the slope of the RAP-RIHP relationship was not significantly different between strains, the relationship was significantly shifted (18%) to higher RAP
in SHR. These results suggest that there is a probable mechanistic and temporal link between the alterations in vascular structure and in renal function in young SHR prior to elevations in MAP.

3.2 Introduction

To unravel key mechanisms and critical periods in the development of hypertension, studies utilizing animal models that mirror the course of human hypertension, such as the spontaneously hypertensive rat (SHR), are warranted. While the factors responsible for the initiation and development of genetic hypertension have been extensively studied in SHR, the basis and time course of blood pressure elevation remain enigmatic. Specifically, what remains controversial is whether the two most widely studied processes, vascular abnormalities and renal dysfunction, lead to increases in arterial pressure in SHR or vice versa.

Since the 1970s, it has been well established that vascular abnormalities, such as increased vascular resistance, increased vascular reactivity to vasoconstrictors, and increased media-to-lumen ratio, are associated with hypertension in the adult SHR. Furthermore, despite the number of studies describing morphometrical and histological differences in the vessels in the early postnatal period and in weaning SHR (i.e. wall thickening), evidence linking blood pressure elevation and vascular structural and functional changes remains unresolved in the neonatal and young SHR. The hypothesis that vascular structural changes antecede and initiate the rise in arterial pressure has been disputed for two main reasons: (1) the presence or lack of blood pressure elevation in studies of young SHR, and (2)
substantial differences between body weights in SHR and Wistar-Kyoto rats (WKY)\textsuperscript{154, 155}, or body weights not being reported\textsuperscript{178, 244, 245}.

Previously, we have reported\textsuperscript{142} longitudinally consistent differences between SHR and WKY rats, representing a 30-40\% increase in SHR vasculature resistance properties throughout a broad interval in the post-weaning period (4-50 weeks). In the present study we thought it of interest to extend these data by assessing hindlimb vascular resistance properties at 2 and 4 weeks of age in weight-matched SHR and WKY. These assessments fall into the putative pre-hypertensive stage of SHR\textsuperscript{142, 151-153}. Further, the suckling (2 weeks) and weaning (~3-4 weeks) periods represent a time of intensive maturation of vascular structure in rats\textsuperscript{180}. Thus, we wanted to test the hypothesis that vascular structural upregulation prevails already in this young SHR.

Given that the kidney serves a critical role in the regulation of arterial pressure\textsuperscript{15, 16}, it is not surprising that abnormalities specific to renal vascular resistance (RVR) properties have also been associated with blood pressure elevations in young SHR\textsuperscript{160, 161, 181-183}. Roman and colleagues\textsuperscript{52, 184} demonstrated that components of the pressure-natriuresis mechanism (i.e. medullary blood flow (MBF), urine flow, and sodium excretion) are shifted rightward towards greater renal perfusion pressures in 3 to 5 week old SHR. These findings suggest that alterations in medullary hemodynamics may participate in the development of hypertension in young SHR in order to maintain sodium homeostasis. Since transmission of RAP to vasa recta capillaries in the renal medulla is a critical mediator of the pressure-natriuretic response, and renal interstitial hydrostatic pressure (RIHP) is generated by MBF, then a similar rightward shift is expected in RIHP response to changes in RAP\textsuperscript{28}. Further, previously we demonstrated that there is an
underlying vascular basis to the acute RAP-RIHP relationship, as RIHP was shown to respond to changes in RAP on a moment-to-moment basis (Chapter 2). Thus, we wanted to test whether there is a rightward shift in the acute RAP-RIHP relationship, reflecting an upregulation of renal vasculature, in very young SHR versus WKY at a state when renal organogenesis is complete\(^{185}\).

### 3.3 Methods

#### 3.3.1 Animals

Male SHR and WKY (12-14d and 28d of age) obtained from the animal care facility of the Baker Medical Research Institute, Melbourne, Australia (colonies originating from a breeding nucleus provided by Dr. Y. Yamori, Japan) were housed in group cages either with their mother (2 weeks) or with their male littermates (4 weeks). These rats were used for the conscious cardiovascular profiling and hindlimb vascular resistance assessments.

Male SHR and WKY (21d of age; littermates) were obtained from Charles River Laboratories (Montreal, QC), and were acclimatized for 7d prior to experimentation. These rats were used for *in vivo* renal hemodynamic assessments.

Both colonies of SHR and WKY had a similar age-body weight (BW) relationship with the result bearing that no special pre-selection of rats was required to obtain age and weight matched pairs. Rats were housed individually (22±1°C; 12h light/dark cycle), with food and water provided *ad libitum*. All procedures followed guidelines of the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee.
3.3.2 Conscious Mean Arterial Pressure (MAP) and Heart Rate Assessments

MAP was measured directly in a group of 4 week old SHR (n=7) and WKY (n=6) surgically implanted previously (2-4d) with aortic cannulas under ketamine (70 mg/kg)/xylazine (5-10 mg/kg) anaesthesia246. MAP and heart rate were calculated from the average of values recorded every 15min for 3h after a one hour acclimatization period starting at 0900h.

3.3.3 Hemodynamic Analysis of Hindlimb Vascular Resistance Properties

SHR and WKY at 2 and 4 weeks of age were anesthetized with sodium pentobarbital (60mg/kg, i.p) and the lower abdominal aorta was exposed at the iliac bifurcation through a mid-line incision under microscopic examination. The middle caudal and the caudal mesenteric arteries were ligated and the rats heparinized (1000IU/kg, i.v.) prior to cannulation of the aorta using either a 25 or 23 gauge needle proximal to the bifurcation. Perfusion was started immediately after the spinal cord and vena cava were transected proximal to the cannula entrance. The perfusate (Tyrode-Dextran) and dual peristaltic pump delivery system was identical to that described previously142. Approximately 2-3min after the start of perfusion (1-2ml/min/100 g BW) and after the hindlimb vessels were cleared of blood, perfusate containing papaverine-HCL (1.5mg/kg) was given for several minutes to ensure maximum dilatation. Some 20min after washing out papaverine, perfusion pressure was measured at maximum dilatation (ppMD) in rats in which the pressure had remained constant over the preceding washout interval, thereby excluding edema formation. At this point a graded flow-pressure relationship was constructed (1, 2, 4, 6 and 8ml/min/100 g BW) to assess differences in flow-pressure responses in the pre-weanling SHR and WKY as calculated
from the slope of this line. Subsequently, maximum constrictor (ppMC) responses were obtained after an $\alpha_1$-adrenoceptor agonist, methoxamine (200µg/ml) was given alone and then after a cocktail containing supra-maximal concentrations of angiotensin II (20µg/ml) and vasopressin (2IU/ml).

### 3.3.4 In Vivo Assessment of Renal Hemodynamic Properties

SHR (n=9) and WKY (n=11) rats at 4 weeks of age were anesthetized with ketamine (30mg/kg, i.p.) and Inactin (thiobarbital sodium; 100mg/kg, i.p.). Body temperature was monitored using a thermistor (model 402; Yellow Springs Instruments) and maintained at 37±1°C using a temperature controller (model 73A; Yellow Springs Instruments) connected to a heating pad and lamp. Additional anesthetic was given, as necessary, throughout the experiment. The rats were tracheostomized (PE-90) and 95% O$_2$ / 5% CO$_2$ was passively blown over the intake to assist respiration (i.e. the source was not directly attached to the cannula). A midline abdominal incision was made, and the right kidney was removed. A saline-filled 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 (0.9% NaCl) at 33µl/min/100g BW via a syringe pump (KD Scientific 220 Multi-Syringe Pump; Fisher Scientific) to compensate for fluid loss during surgery.

The modified in vivo assessment of renal vascular properties was based on the technique described by Roman and Cowley$^{59}$ and the direct determination of RIHP according to Ott $et$ $al.$$^{67,240}$; however, the neurohumoral control systems were left intact. The superior mesenteric artery was catheterized with heparinized saline-filled (50IU/ml) polyethylene tubing (PE-10) for continuous measurement of arterial pressure at the level
of the renal artery (hereinafter referred to as RAP). A silastic balloon cuff was placed between the celiac and superior mesenteric arteries and another silastic balloon cuff was placed just below the left renal artery. The silastic balloon cuffs allowed for manipulation and control of RAP over a wide range of pressures (i.e. pressor and depressor). An electrosurgical unit (Elmed ESU 30; Elmed Incorporated) was used to create a 1.5mm-hole into the longitudinal axis of the left kidney for insertion of a catheter for RIHP measurements (PE-50 tubing fitted with 1.5mm long polyethylene matrix of 15-45μm pore size; Porex). The catheter was pre-flushed and pre-filled with heparinized saline (50IU/ml) and then held in place with cyanoacrylate glue. RAP and RIHP were continuously monitored via pressure transducers (model CDX3, Cobe) connected to a PowerLab/8s (ADInstruments) data acquisition system with Chart v. 4.2.2 software.

After surgery, an equilibration period of ~15min was allowed prior to recording steady state baselines of RAP and RIHP. The acute RAP-RIHP relationship was determined via short-term manipulations of RAP (1-60s) using the silastic balloon cuffs in encapsulated kidneys. These manipulations consisted of sequential pressor and depressor changes in RAP of various magnitudes (±30mmHg from baseline). Upon completion of the experiment, the kidneys were decapsulated, and after ~5min, baseline RAP and RIHP were recorded. Then, hematocrit was determined and the rats were euthanized via excision of the heart. Lastly, the left kidney was removed, and the position of the RIHP catheter was verified to be at the cortical-outer medullary junction.

Baseline values for RAP and RIHP were determined by averaging the 60s period prior to starting RAP manipulations. The overall, pressor, and depressor slopes of the acute RAP-RIHP relationship were determined from steady-state values of RAP and
RIHP following short-term manipulations of RAP. The time delay between the initial RAP change and the onset of RIHP response was characterized as the difference between the first detectable change in RAP and RIHP from the operating point following a manipulation.

3.3.5 Statistical Analysis

All statistical calculations were performed and graphs constructed using Prism 5 (GraphPad Software) and/or Microsoft Excel 2003. Linear regression analysis was used to calculate the slopes and y-intercepts of the hindlimb flow-pressure and the overall, pressor, and depressor relationship between RAP and RIHP$^{238, 239}$. Statistical significance of differences in values measured between WKY and SHR rats was determined using a Student’s $t$ test. Grubb’s test was conducted on all data sets to determine statistical outliers. All data are presented as means ± SEM. A level of $P < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Hindlimb Vascular Resistance Assessments in 2 and 4 Week Old Rats

Physical and Hemodynamic Characteristics:

The growth curves of the SHR and WKY colony from the Baker Institute have been shown to be almost identical$^{142}$. Accordingly, no special selection of animals was done to obtain rats of similar body weights at the predetermined ages of 2 weeks (SHR 24.0±0.2g; WKY 24.7±0.3g) and 4 weeks (SHR 66.9±0.8g; WKY 68.0±1.0g). In addition, we determined that SHR and WKY rats of 1-3 weeks of age have similar hindlimb weights. At 7-10d, hindlimb weight is 18% body weight, and at 28d, 26% of body weight (data not shown). Previous data has shown that the SHR in comparison to
WKY obtained from this colony have similar proportional hindlimb weights throughout their lifespan\(^1\). Further, at 4 weeks of age, there was no significant difference in conscious MAP (SHR 113.0±5.0mmHg; WKY 110.2±5.9mmHg), although heart rate was elevated by 16% in the SHR (459.1±9.5 beats/min) versus WKY (395.2±7.8 beats/min; P<0.001).

**Resistance Properties of the Neonatal Hindlimb Vasculature:**

Perfusion pressure at maximum dilatation (ppMD) is elevated ~30% in SHR at both 2 and 4 weeks of age (p≤0.05; Figure 3.1A). A similar increase in absolute ppMD between 2 and 4 weeks was found in both SHR and WKY, in accordance with the rapid normal maturation during this time (Figure 3.1A). The pressure responses, to acute changes of flow, in the vasculature of SHR at 2 and 4 weeks of age were significantly elevated at all flow rates tested (p<0.05; Figure 3.1B). Comparison of the y-intercepts of the lines representing the flow-pressure relationships indicated that there was ~35% increase in SHR (P<0.001; Figure 3.1B). The slope was ~1.2-fold greater in SHR (Figure 3.1B). Further, at both 2 and 4 weeks of age, the hindlimb vasculature of SHR generated a greater maximum contractile force using the constrictor cocktail, as indicated by the 30-40% greater perfusion pressure at maximum constriction (ppMC) than that of the WKY vasculature (p<0.01; Figure 3.1C). Comparison of 2 and 4 weeks results demonstrates that a rapid maturation (i.e. ~2-fold increase) of the maximal vasoconstrictor capacity of this vascular bed occurs in both SHR and WKY. Although the data is not presented, the maximum pressure response using methoxamine alone in both SHR and WKY was 80-90% of the total maximum pressure response observed with the supramaximal constrictor
**Figure 3.1:** A) Perfusion pressure at maximum dilatation (ppMD) produced by infusing papaverine at a flow rate of 4ml/min/100g BW, B) flow-pressure relationships at maximum dilatation, and C) perfusion pressure at maximum constriction (ppMC) produced by infusing supramaximal concentrations of angiotensin II, vasopressin, and methoxamine, at a flow rate of 4ml/min/100g BW, in weight-matched pairs of SHR and WKY rats at 2 (left; SHR n=8-11; WKY n=7-11) and 4 (right; SHR n=4-7; WKY n=6) weeks of age. **P<0.01 and ***P<0.001 vs. WKY, and ΨΨΨP<0.001 vs. elevations (y-intercepts) of WKY flow-pressure lines.
cocktail. The inability to produce the maximum pressure response with \( \alpha_1 \)-adrenoceptor activation alone was similar at the two ages.

**Attempt to Assess Resistance Properties in 1 Week Old Rats:**

Three series of experiments were performed to attempt to assess hindlimb vascular resistance properties in 6-8d old SHR and WKY weighing between 5-8g. The multiple attempts to perfuse the hindlimb vessels proved unsuccessful despite using microbore tubing. However, microscopic observation during the insertion of the small catheters revealed the major reason for the difficulty. Insertion of the small Teflon tubing into the aorta induced a rapid disintegration of the vascular wall with small fragments actually dissipating into the aortic lumen. These observations revealed the degree of immaturity of the structural elements of the aorta of the 1 week old rat and reinforced the marked difference even between the first and second post-natal week.

**3.4.2 In Vivo Renal Hemodynamic Assessments in 4 Week Old Rats**

**Physical and Hemodynamic Characteristics:**

Body weights of 4 week old SHR and WKY in the colony from Charles River Laboratories were not significantly different (Table 3.1), and did not significantly differ from age-matched rats of the Baker Institute. Consistent with the development of hypertension, kidney weight, kidney to body weight ratio, and the left ventricle to body weight ratio were significantly higher in the SHR (P<0.05; Table 3.1).

**In Vivo Renal Hemodynamic Properties of Neonatal Rats:**

Mean RAP was \(~18\%\) greater in anesthetized SHR compared with age-matched WKY (P<0.05), whereas RIHP was not different (Table 3.1). As a result, there was a rightward shift in the acute RAP-RIHP relationship of the SHR in comparison to WKY.
Table 3.1: Hemodynamic and physical characteristics of WKY and SHR rats at 4 weeks of age.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY n=11</th>
<th>SHR n=9</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAP (mmHg)</td>
<td>72.76±3.84</td>
<td>86.14±4.57</td>
</tr>
<tr>
<td>RIHP (mmHg)</td>
<td>3.13±0.59</td>
<td>3.03±0.48</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47.78±0.54</td>
<td>48.90±0.93</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>70.26±1.10</td>
<td>68.03±0.75</td>
</tr>
<tr>
<td>Right kidney weight (g)</td>
<td>0.37±0.02</td>
<td>0.46±0.004</td>
</tr>
<tr>
<td>Right kidney: body weight</td>
<td>0.005±0.0003</td>
<td>0.007±0.0001</td>
</tr>
<tr>
<td>Left ventricle weight (g)</td>
<td>0.22±0.01</td>
<td>0.23±0.004</td>
</tr>
<tr>
<td>Left ventricle: body weight</td>
<td>0.0031±0.0001</td>
<td>0.0033±0.0001</td>
</tr>
<tr>
<td>Right ventricle weight (g)</td>
<td>0.07±0.004</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>Right ventricle: body weight</td>
<td>0.0009±0.0001</td>
<td>0.0009±0.0001</td>
</tr>
</tbody>
</table>

RAP, renal arterial pressure; RIHP, renal interstitial hydrostatic pressure. Values are mean ± SEM. *P<0.05 and **P<0.01 vs. WKY.
Further, there were no differences in the slopes of the overall acute RAP-RIHP relationship between the two strains of rats (SHR 0.09±0.01; WKY 0.07±0.01; Figure 3.2A). However, there was a significant difference in the y-intercepts (P<0.001), and calculating RAP at 0mmHg RIHP also revealed a rightward shift of 20mmHg in the SHR (P<0.001). Comparison of the pressor versus depressor slopes revealed that in both SHR and WKY rats the pressor slope is ~2.5-fold higher than the depressor slope (P<0.01; Figure 3.2B). Similar to the overall assessment, there was a significant difference in the y-intercepts of the pressor and depressor relationships between SHR and WKY, as well as pressor versus depressor y-intercepts in both SHR and WKY (P<0.001). Calculating RAP at 0mmHg RIHP for the depressor relationships revealed that again there was a rightward shift in the RAP-RIHP relationship in the SHR (SHR 20.2mmHg; WKY 3.6mmHg). The overall time for RIHP to respond to any type of change in RAP in the SHR was 2-times quicker than WKY (P<0.001). A similar trend between the two strains was present when comparing pressor versus depressor times. Interestingly, pressor manipulations in RAP resulted in quicker RIHP response times than depressor manipulations in both strains (P<0.001; Figure 3.3). Comparison of the baseline RIHP and percent RIHP of RAP in encapsulated and decapsulated kidneys revealed that decapsulation decrease both variables in only the SHR, thereby indicating that the renal capsule plays a more prominent role in maintaining RIHP in SHR (P<0.05; Figure 3.4).
Figure 3.2: The A) overall and B) pressor versus depressor acute RAP-RIHP relationship in 4 week old SHR (n=9) and WKY (n=11) rats. Linear regression analysis revealed a significant difference in the elevations (y-intercepts) of the SHR and WKY renal hemodynamic relationships (P<0.001 vs. WKY), and the slopes of the pressor versus depressor relationships (**P<0.01 and ***P<0.001 vs. depressor). There was also a significant difference in RAP at all similar levels of RIHP (ΨΨΨP<0.001 vs. WKY). Data displayed in bins of 10mmHg for RAP manipulations of ±30mmHg around the operating point. Dashed lines represent the 95% confidence intervals for the overall acute RAP-RIHP relationship. Values are means ± SEM.
Figure 3.3: Time delay for RIHP to respond to a change in RAP in WKY (n=11) and SHR (n=9) at 4 weeks of age. ***P<0.001 vs. depressor RAP manipulations of 30mmHg in both WKY and SHR; and ψP<0.05 and ψψψP<0.001 vs. WKY at same magnitude of RAP manipulation. Data displayed in bins of 10mmHg for RAP manipulations of ±30mmHg. Values are means ± SEM.
Figure 3.4: Effects of kidney decapsulation on A) baseline RIHP, and B) percent RIHP of RAP at baseline in WKY (n=11) and SHR (n=9) at 4 weeks of age. *P<0.05 vs. encapsulated SHR kidney. Values are mean ± SEM.
3.5 Discussion

The major findings of the present study were that: 1) vascular resistance properties are higher in SHR compared to WKY at 2 and 4 weeks of age, suggesting vascular abnormalities are present prior to differences in conscious MAP between both strains; 2) renal hemodynamic properties are shifted towards greater pressures in 4 week old SHR; 3) the time delay for RIHP responses to acute changes in RAP are <2s, indicating that there is an underlying vascular basis to this component of the pressure-natriuresis mechanism; and 4) maintenance of RIHP in 4 week old SHR also depends, in part, on the renal capsule. Together, these findings suggest that during and following completion of postnatal vasculogenesis and renal organogenesis, young SHR are programmed susceptible to hypertension, in part, via phenotypic alterations in peripheral vasculature and renal function.

There has been a long-standing debate about whether elevations in arterial pressure precede alterations in vascular structure or vice versa. In our colony, conscious MAP was not different between SHR and WKY at 4 weeks of age; however, structurally-based vascular resistance properties were higher in both 2 and 4 week old SHR versus WKY. These findings extend our previous results in rats from the same colony, and suggest that vascular resistance properties differ to the same extent (~30%) in neonatal and adult SHR (up to 50 weeks). That is, since the full magnitude of the difference in SHR versus WKY vasculature already exists at 2 weeks of age, and there does not appear to be a critical period between 2 and 50 weeks during which SHR vessels function differently, these data do not support the concept that elevated arterial
pressure is a predominant factor in inducing further vascular structural and functional adaptation.

It is important that evidence for a prevailing difference in the configuration of blood vessel architecture in these neonatal SHR and WKY comes from three assessments. First, differences in ppMD in hindlimb vasculature of SHR elevate pressure ~30% more than the normotensive rat. According to Poiseuille’s Law, which states that resistance to flow is inversely proportional to radius to the fourth power, it can be calculated, that in the average hindlimb blood vessel of 2, 4, or even 50 week old SHR\textsuperscript{142}, the average lumen is ~7% smaller than in WKY either when there is no vasoconstrictor tone or at the same level of vessel contraction i.e. structurally determined. Secondly, assessments of perfusion pressure at different flow rates extend this concept further, where at all flow rates between 1 and 8 ml/min/100 g BW, resistance to flow is elevated such that there is a narrowing of the vessel lumen that is not dependent on the distending pressure. Lastly, ppMC in hindlimb vascular beds of young SHR demonstrate a greater contractile capacity compared to WKY. Mulvany and co-workers\textsuperscript{141} have demonstrated in small isolated segments of resistance vessels that differences in contractile capacity in SHR can be wholly accounted for by an increased muscle mass in the blood vessel wall. Accordingly, the increase in resistance/vasoconstrictor capacity of ~30% suggests that the average hindlimb blood vessel of the neonatal SHR relative to WKY has a smaller lumen and/or a thicker wall.

Similar to vascular resistance properties of peripheral resistance vessels (i.e. hindlimb arteries), previous findings show that renal vascular resistance (RVR) is elevated in young “prehypertensive” SHR (i.e. ≤4 weeks of age)\textsuperscript{160, 161, 181-183}, and
continues to be elevated into adulthood (i.e. 16 weeks of age)\textsuperscript{160, 161, 181, 182}. Given that transmission of RAP to vasa recta capillaries in the renal medulla is a critical mediator of the pressure-natriuretic response, an elevated RVR would require increased RAP to induce homeostatically-appropriate changes in sodium excretion\textsuperscript{15, 27}. Corroborating previous findings\textsuperscript{52, 184}, the present study demonstrates that although the overall slope of the acute RAP-RIHP relationship is not different, there is a rightward shift towards greater RAP in 4 week old SHR relative to WKY. This finding not only supports the proposed cascade of players in pressure-natriuresis (i.e. MBF, RIHP, urine flow and sodium excretion)\textsuperscript{18}, but also the hypothesis that medullary vascular resistance properties are elevated even in young SHR following completion of postnatal renal organogenesis (i.e. postnatal day 30)\textsuperscript{52, 185}. Additionally, the moment-to-moment nature of RIHP responses (i.e. <2s) to changes in RAP further suggests that there is underlying vascular basis for this component of the pressure-natriuresis mechanism.

In an animal model of salt-sensitivity and hypertension (i.e. atrial natriuretic peptide knockout mouse; Chapter 4), we previously demonstrated that the acute RAP-RIHP relationship responds differentially to pressor versus depressor stimuli\textsuperscript{239}. Specifically, in addition to a rightward shift towards greater RAP (i.e. indicative of greater structurally-based vascular resistance properties), there was also a blunting of the pressor slope (i.e. indicative of salt-sensitivity)\textsuperscript{48}. Herein, young SHR display a parallel shift in both pressor and depressor components to a greater RAP, and interestingly, in both SHR and WKY, the pressor slope is steeper than the depressor. The role for this differential response to pressor versus depressor stimuli is not fully understood and requires further investigation; however, given that there is no change in slope in both
strains, this suggests that these animals are not likely salt-sensitive at 4 weeks of age. Furthermore, the time delay for initial RIHP response to a pressure change is markedly shorter following pressor stimuli, indicating that these rats are not salt-sensitive, since elevations in RAP are dealt with more efficiently than decreases.

The maintenance of RIHP in young SHR to comparable levels of WKY does not only depend on the level of RAP, but also the function of the renal capsule. The capsule aids in the distribution of hydrostatic pressure generated by MBF throughout the renal interstitium\textsuperscript{28,30}. Decapsulation has been shown to blunt RIHP in adult normotensive rats\textsuperscript{28}, however it has never been investigated in young rats. For the first time, the present study demonstrates that decapsulation in young SHR results in a marked blunting of baseline RIHP, whereas WKY remains unaffected. This suggests that following completion of renal organogenesis, the capsule is programmed such that it aids in maintaining similar basal RIHP levels to WKY. This may also explain why the initial RIHP response time following RAP changes in encapsulated kidneys is quicker in the SHR than WKY; however this speculation requires further investigation. Interestingly, by adulthood, the capsule appears to lose its function in SHR, and conversely becomes necessary in WKY for maintenance of RIHP\textsuperscript{64}. Consequently, RIHP is blunted in adult SHR, and the natriuretic response is no longer sensitive to decapsulation, indicating that the animal is salt-sensitive (i.e. blunted slope of RAP-RIHP relationship)\textsuperscript{48,64}. Together, it can be speculated that the newly developed kidneys of young SHR maintain sodium and fluid homeostasis, in part, due to a less elastic or tougher capsule, and by adulthood, these properties are abolished leading to salt-sensitivity.
Although the outcomes of these studies provide a better understanding of vascular structure and renal function in young SHR versus WKY, there are some limitations that need to be addressed. First, presence of potential unanticipated or uncontrollable environmental factors during critical periods of perinatal development may have influenced the programming of the phenotypes expressed in these rats. Further, comparisons of hindlimb and renal hemodynamic assessments might be complicated since these studies were conducted in two separate colonies; however, the rats displayed similar body weight and left ventricle to body weight ratio suggesting that the growth rates of these two colonies are comparable\textsuperscript{142}. Lastly, rats had to be anesthetized for the renal hemodynamic assessments. Given that it has been previously demonstrated that SHR are less sensitive to certain anesthetics than WKY, this may have affected the outcome\textsuperscript{247}. Despite this caveat, the results of the present study are comparable to that of others\textsuperscript{52, 184}.

Taken together, these data indicate that the SHR may be programmed susceptible to developing hypertension via a combination of increased structurally-based vascular resistance properties and a rightward shift in acute renal hemodynamic properties following critical periods of postnatal development. Furthermore, these results suggest that there is a probable mechanistic and temporal link between the alterations in vascular structure and in renal function in SHR. Given the importance of the kidney in the regulation of arterial pressure, the findings suggest that the renovascular component of the acute pressure-natriuresis mechanism may be the key functional alteration generating the susceptibility to hypertension in young SHR.
CHAPTER 4

Altered Regulation of Renal Interstitial Hydrostatic Pressure and the Renal Renin-Angiotensin System in the Absence of ANP


4.1 Abstract

**Background:** Although it has been well established that atrial natriuretic peptide gene-disrupted (ANP -/-) mice are a useful model of salt-sensitive hypertension, surprisingly little is known about the control of their intrarenal renin-angiotensin system (RAS) and pressure-natriuresis mechanism – key components in blood pressure, fluid and electrolyte regulation. Thus, the aim of this study was to determine whether ANP disruption results in changes of the renal and adrenal local RAS and the acute pressure-natriuresis mechanism.

**Methods:** Renal and adrenal renin, AT\(_1\) (A and B) and AT\(_2\) receptor mRNA expression levels were determined by Northern blotting and/or RT-PCR. Plasma aldosterone and renal and adrenal angiotensin II (Ang II) peptide levels were determined via radioimmunoassay. To examine the acute pressure-natriuresis response, changes in renal interstitial hydrostatic pressure (RIHP) were assessed following manipulations of renal arterial pressure (RAP) in anesthetized mice.

**Results:** Renal and adrenal renin mRNA and Ang II levels were lower in ANP -/- and +/- mice compared to the +/- mice. ANP -/- mice also had greater renal AT\(_{1A}\) and adrenal AT\(_2\) mRNA levels compared to the other genotypes. RAP and RIHP were significantly
higher in -/- mice compared with +/+ mice. Furthermore, there was a blunted slope of the RAP-RIHP relationship following increases in RAP in the ANP -/- mice.

**Conclusions:** These data indicate that ANP disruption results in a blunting of the dynamic properties of the acute pressure-natriuresis mechanism at increased levels of RAP, as well as a reduced expression of renal and adrenal local RAS.

### 4.2 Introduction

The important role of atrial natriuretic peptide (ANP) in the maintenance of blood pressure homeostasis was confirmed by the generation, in 1995, of the *proANP* gene-disrupted mouse by John *et al.*[^186]. ANP -/- mice are hypertensive[^186], have high levels of circulating catecholamines[^188], display cardiac hypertrophy and are salt-sensitive[^186]. The normotensive heterozygotes (ANP +/-) also display salt-sensitivity, becoming hypertensive on a high salt diet[^186]. This commonality of salt-sensitivity in animals completely or partially lacking ANP is not fully understood and requires further investigation.

ANP is a well known antagonist of the renin-angiotensin system (RAS)[^248]; although in the absence of ANP, the expression of the RAS is not fully understood. Melo *et al.* have postulated that abnormal RAS activity may be responsible for the salt-sensitive phenotype in animals lacking ANP[^189,190]. This is consistent with findings in some salt-sensitive variants of human[^5,249] and animal hypertension[^250-252], where the salt-sensitivity was attributed to aberrant RAS activity. However, it is not entirely known whether alterations in RAS activity are present in the *proANP* gene-disrupted mouse model, and thus a direct genotypic comparison of the baseline RAS expression in this model is warranted.
ANP sensitizes the pressure-natriuresis relationship, an effect dependent on ANP’s antagonism of the RAS\textsuperscript{98}. Regulation of the pressure-natriuresis mechanism in animals lacking ANP has never been examined; although in other models of salt-sensitivity and hypertension (i.e. Dahl salt-sensitive rat and the spontaneously hypertensive rat (SHR)), the pressure-natriuresis mechanism was found to be ‘blunted’\textsuperscript{50, 63, 90}. That is, a higher arterial pressure is required to achieve sodium homeostasis than in the normotensive control\textsuperscript{53, 56, 253}. Given that there are no differences in chronic sodium or chloride excretion among mice with or without ANP\textsuperscript{187}, it can only be speculated that animals lacking ANP also maintain sodium balance via a chronic increase in arterial pressure due to the ‘blunting’ of their pressure-natriuresis mechanism. Furthermore, pharmacological blockade of the RAS in the SHR has been shown to markedly increase the sensitivity of the pressure-natriuresis mechanism\textsuperscript{50, 91}, such that there are alterations in the essential intermediate step of the pressure-natriuresis mechanism - the relationship between renal arterial pressure (RAP) and renal interstitial hydrostatic pressure (RIHP)\textsuperscript{11}. The existence of such a ‘blunting’ in this mechanism and whether the RAS affects the pressure-natriuresis relationship (and its components) has not yet been established in the ANP gene-disrupted mouse and requires further examination.

We hypothesize that the generation of a salt-sensitive circulation in mice lacking ANP is due to decreased activity of the RAS, a blunting of the RAP-RIHP component of the pressure natriuresis mechanism, or the combination of both. In the present study, we analyze the expression pattern of the local renal and adrenal RAS in ANP +/+, +/- and -/- mice to determine if altered levels of tissue renin, Ang II or circulating aldosterone are an element of the salt-sensitive phenotype of the \textit{proANP} gene-disrupted mouse.
Furthermore, for the first time in mice, we characterize the responsiveness of RIHP to changes in RAP, which provides a quantitative assessment of a key component of the pressure-natriuresis mechanism, in mice with (+/+) and without (-/-) ANP.

4.3 Materials and Methods

4.3.1 Animals

Experimental protocols pertaining to the use of mice in this study have been approved by the Animal Care Committee of Queen’s University in accordance with the guidelines of the Canadian Council on Animal Care. ANP gene-disrupted mice (C57BL/6J genetic background) originally described by John et al. were bred and maintained in our Animal Care Facility (Queen’s University). Genotyping of animals was performed as previously described. Animals were housed, 1-3 per cage and kept at 21 ± 1°C in a room with a 12:12-hr light-dark schedule. Mice were maintained on a pellet Lab Diet™ (Mouse Diet 5015; Na⁺ 0.44%; Cl⁻ 0.73%; K⁺ 0.72%; Brentwood, MO) and water was provided ad libitum. For molecular analyses, three groups of 12 week old male ANP 2-copy (+/+), 1-copy (+/-) and 0-copy (-/-) mice (n=5) were used. Mice were anesthetized with sodium pentobarbital (65 mg·kg⁻¹ body wt; i.p.) and killed by cardiac puncture and exsanguination. The kidneys and adrenal glands were carefully removed, decapsulated, weighed and rapidly snap frozen in liquid nitrogen and stored at -70°C until use. The heart was removed and weighed. Femur bones were isolated with articulating surfaces intact. Residual ligaments and skeletal muscle were removed by immersion in 1M NaOH for 48 hours. After all remaining skeletal muscle and ligaments were removed the femur length was measured with digital callipers.
4.3.2 Urine Collection and Electrolyte Measurements

Urine was collected from proANP gene-disrupted mice over a 24-hour period in metabolic cages (Nalgene®; Nalge Nunc International, Rochester NY). Urine volume, food and water intake were recorded during this time. Urinary electrolyte concentrations were measured using ion-selective electrodes at the Clinical Chemistry Core Lab; Department of Pathology and Molecular Medicine; Kingston General Hospital; Kingston, Ontario, Canada.

4.3.3 RNA Isolation and Northern Blot Analysis

Total RNA was extracted from frozen tissue samples by a modification of the acid-guanidinium-phenol-chloroform method (Trizol, Molecular Research Corp.) as per manufacturer’s instructions. Precipitated RNA was resuspended in RNase-free (DEPC-treated) water and stored at -70°C until use. Northern blotting for renin, angiotensin type 1A (AT\textsubscript{1A}) and type 2 (AT\textsubscript{2}) receptors was performed as previously described\textsuperscript{254} using 5µg of total RNA. Northern blots were scanned and densitometric analysis was performed using AlphaEaseFC™ Software (Alpha Innotech; San Leandro, CA). Intensity of the specific mRNA bands were normalized with those of the ribosomal 18S RNA. Results were expressed as mean ± SEM. Differences between groups were determined by ANOVA analysis and the Tukey’s post-hoc test using the GraphPad PRISM program (GraphPad, San Diego). \( P \) values < 0.05 were considered statistically significant.

4.3.4 Real Time PCR Analysis

Expression of renin and angiotensin type 1B (AT\textsubscript{1B}) receptor mRNA was examined in the kidney and/or adrenals of proANP gene-disrupted mice. First strand cDNA was produced from 1-2µg of total RNA by using the Omniscript™ Reverse
Transcriptase Kit (Qiagen Inc., Mississauga Ontario, Canada) as per manufacturer’s instructions. Primers for PCR amplification were designed using the PRIMER DESIGNER 2.01 program against DNA sequences obtained from the GenBank database. The sequences of each gene-specific primer pair are as follows:

Renin (S): 5’-ATGGAGAATGGAGACGAC-3’
Renin (AS): 5’-CCAGGTTGAAGGAGATGT-3’;
AT$_{1B}$ (S): 5’-TCGATCGCTACCTAGCCA-3’
AT$_{1B}$ (AS): 5’-ACTAGCCAAGCCAGCCA-3’;
GAPDH (S): 5’-ATGGTGAAAGGTCGGTG-3’
GAPDH (AS): 5’-TTCTCGGCCCTTGACTGTG-3’

Real time quantitative PCR was performed using the Rotor-Gene 3000 Thermal Cycler (Corbett Research, Montreal Biotech Inc., Montreal QU, Canada), with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. The annealing temperature of each primer pair was 62°C. PCR amplicons were detected by fluorescent detection of SYBR Green 1 (Invitrogen, Burlington, ON). Standard curves were created for each primer pair in each tissue to be analyzed. Following cycling, the melt curve of the resulting amplicon was analyzed to ensure that a single product was detected. Quantification of mRNA (copy number) was performed using the respective standard curves with manufacturer’s software (Rotor-gene 6; Corbett Research). Values were expressed as a ratio of the gene of interest : GAPDH in each sample. Results were expressed as means ± SEM. Differences between groups were determined by ANOVA analysis and the Tukey’s post-hoc test using the GraphPad PRISM program (GraphPad, San Diego). \( P \) values < 0.05 were considered statistically significant.
4.3.5 Angiotensin II Radioimmunoassay (RIA)

Kidney and adrenal tissue was prepared for extraction by Sep-Pak C₁₈ cartridges (Waters Ltd., Mississauga, ON), as previously described²⁵⁵ [22]. The purified eluted samples were lyophilized and stored at -70°C until RIA. The RIA method employed in this study has been previously described²⁵⁴,²⁵⁵. The Ang II antiserum (Peninsula Laboratories Inc., Belmont, CA) was used at a dilution of 1:50,000. All tubes were counted on a Beckman Gamma 5500B gamma counter (Beckman Coulter Canada Inc.) A detection limit of 0.8 pg per tube and the 50% binding value of 12.5 pg/tube were achieved.

4.3.6 Aldosterone Radioimmunoassay

Aldosterone levels in plasma of proANP gene-disrupted mice were determined using a RIA kit following the manufacturer’s instructions (Coat-a-Count®, Diagnostic Products Corporation; Los Angeles, CA). The assay sensitivity was 11 pg/ml.

4.3.7 Evans Blue Dye Experiments

ProANP gene-disrupted mice (n=5) and wildtype controls (n=5) were anesthetized with ketamine/xylene anesthetic (100 and 25 mg/kg). A modified version of the protocol by Kaufman et al.²⁵⁶ was used in this study to examine intravascular plasma volume. An aqueous solution of Evans blue dye (Fisher Scientific, Toronto, ON), at a final concentration of 25 mg/kg, was injected into the external jugular vein and allowed to circulate for 4 min. Mice were sacrificed by cardiac puncture and exsanguination. Blood samples were added immediately to pre-chilled tubes containing EDTA (1mg/mL) and centrifuged for 10 min at 4000g to isolate plasma. Plasma samples were diluted 1:100 in water and Evans blue content was determined spectrophotometrically (610nm).
Calculations of plasma volume were extrapolated using a standard curve for Evans blue dilution.

4.3.8 In Vivo Assessment of Renal Function

Young adult mice (14-16 weeks old) were anesthetized with sodium pentobarbital (90 mg/kg body wt, i.p). Body temperature was monitored using a thermistor (model 402; Yellow Springs Instruments, Yellow Springs, OH) and maintained at 37 ± 0.5°C using a controller (model 73A; Yellow Springs Instruments), a heat lamp, and a warming pad. After tracheostomy (PE-90 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 containing bovine serum albumin (2.25%) and glucose (1%) at 33µL/min/100g body wt via a syringe pump (KDS220, Fisher). Ligatures were tied around the non-renal arteries of the left renal artery and the celiac artery. The superior mesenteric artery was cannulated with PE-10 tubing for continuous mean arterial pressure measurements, hereinafter referred to as RAP. A Silastic balloon cuff was placed around the abdominal aorta between the celiac trunk and superior mesenteric arteries, and a snare clamp was placed around the aorta distal to the left renal artery. The balloon cuff and clamp 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 (~1.5 mm in length) into the longitudinal axis of the left kidney for insertion of a catheter for RIHP measurements. The RIHP catheter was made of PE-50 tubing (O.D. of 0.965mm) fitted with 1.5mm 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 minute 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 seconds) using the Silastic cuff and clamp. These acute manipulations of RAP resulted in a reliable and stable change in RIHP, as consistent with previous studies\(^{58}\). Upon completion of the experiment, hematocrit levels were measured and the mice were killed. 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.

### 4.3.9 Statistical Analysis

All data were represented as means ± SEM. Linear regression analysis was performed by the least-squares method to calculate the relationship between RIHP and RAP for each manipulation in each animal\(^{30}\). Statistical significance of differences in values measured between +/+ and +/- mice was determined using an unpaired Student’s \(t\) test. Throughout this manuscript, \(P < 0.05\) was accepted as a statistically significant difference.

### 4.4 Results

No differences in body weight, kidney weight or ratio of kidney-to-body weight were observed between ANP +/-, +/-, or +/+ mice (Table 4.1). Consistent with the hypertension of the ANP +/- mice, absolute heart weight, heart-to-body weight and heart-to-femur length ratios were significantly greater in the +/- as compared to the +/- and +/+ mice (Table 4.1).
Table 4.1: Physical parameters of proANP gene-disrupted mice.

<table>
<thead>
<tr>
<th>ANP genotype</th>
<th>+/-</th>
<th>+/-</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>36.3 ± 1.7</td>
<td>35.7 ± 2.3</td>
<td>37.0 ± 0.8</td>
</tr>
<tr>
<td>Left Kidney Weight (mg)</td>
<td>206 ± 12</td>
<td>213 ± 8</td>
<td>214 ± 9</td>
</tr>
<tr>
<td>LKW:BW (mg/g)</td>
<td>5.8 ± 0.5</td>
<td>6.1 ± 0.5</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>161 ± 10</td>
<td>168 ± 4</td>
<td>208 ± 9*</td>
</tr>
<tr>
<td>HW:BW (mg/g)</td>
<td>4.5 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>5.6 ± 0.3*</td>
</tr>
<tr>
<td>HW:FL (mg/mm)</td>
<td>10.2 ± 0.7</td>
<td>10.5 ± 0.1</td>
<td>12.5 ± 0.5*</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n=4-6). * Significant difference (P<0.05 by ANOVA) as compared to ANP +/-. FL, femur length.
To determine if proANP gene-disruption is associated with alterations in locally-produced Ang II, the expression of components of the renal RAS was analyzed. Renin mRNA levels were significantly less in ANP -/- and +/- mice than in +/+ mice (Figure 4.1A). Ang II peptide levels were also significantly less in -/-, but not +/- kidneys, as compared to wild-type mice (Figure 4.1B). The AT1A mRNA levels were significantly greater in -/- versus +/+ controls (Figure 4.1C). The AT2 mRNA levels were not significantly different between genotypes (Figure 4.1D).

As observed in the kidney, adrenal renin mRNA levels in -/- and +/- ANP mice were significantly less than those of +/+ mice (Figure 4.2A). Adrenal Ang II levels in -/- mice were less than the other genotypes but this did not reach statistical significance (P = 0.06) (Figure 4.2B). Angiotensin type 1A and 1B receptor mRNA levels were not significantly different between genotypes (Figure 4.3A&B). Adrenal AT2 mRNA levels were significantly greater in -/- mice as compared to +/- and +/+ (Figure 4.3C). Plasma aldosterone levels did not differ significantly between ANP genotypes (Table 4.2), as seen previously in high salt-treated ANP mice, despite the alterations in the adrenal RAS presented here. In addition, the 24 hour urinary excretion of electrolytes did not differ significantly between genotypes (Table 4.2), nor did the food and water intake.

Initial baseline values for RAP and RIHP were measured under pentobarbital anesthesia following a 15-20 minute equilibration period. Mean RAP was significantly higher in -/- mice (97.9 ± 2.1 mmHg) compared with +/- mice (83.4 ± 2.5 mmHg) (P<0.01) (Figure 4.4A). ANP -/- mice also had a significantly higher RIHP (8.8 ± 0.8 mmHg) in comparison to +/- mice (5.3 ± 0.3 mmHg) (P < 0.01) (Figure 4.4B). Hematocrit levels were significantly different between groups
Figure 4.1: Renal renin-angiotensin system expression in proANP gene-disrupted mice. Renin mRNA levels in both +/- and -/- mice are significantly less than in control +/+ mice (A). Renal Ang II levels in -/- mice are significantly less than in +/- or +/+ mice (B). AT$_{1A}$ mRNA is greater in -/- mice as compared to heterozygotes (C). No significant differences were seen in AT$_{2}$ mRNA expression between genotypes (D). Values represent means ± SEM (n=4-6). *$P < 0.05$ vs. ANP +/++; § $P < 0.05$ vs. ANP +/-; ANOVA. (A.U. = arbitrary units).
Figure 4.2: Adrenal renin mRNA expression and angiotensin II levels in proANP gene-disrupted mice. Renin mRNA expression was significantly decreased in both +/- and -/- mouse adrenals relative to +/- (A). Adrenal Ang II levels are not significantly different between genotypes (B). Values represent means ± SEM. (n=4-5). *P < 0.05 vs. ANP +/-; ANOVA. (A.U. = arbitrary units).
Figure 4.3: Adrenal angiotensin receptor mRNA expression in proANP gene-disrupted mice. There were no significant differences seen in angiotensin type 1A receptor (AT$_{1A}$) or type 1B (AT$_{1B}$) mRNA expression between genotypes (A, B). Angiotensin type 2 (AT$_2$) receptor mRNA was significantly greater in the -/- adrenals relative to both +/- and +/- (C). Values represent means ± SEM. ($n$=4-5). * $P < 0.05$ vs ANP +/+; § $P < 0.05$ vs. ANP +/-; ANOVA. (A.U. = arbitrary units).
Table 4.2: Aldosterone and electrolyte levels in proANP gene-disrupted mice.

<table>
<thead>
<tr>
<th>ANP genotype</th>
<th>+/-</th>
<th>+/-</th>
<th>-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Aldosterone (pg/ml)</td>
<td>341 ± 36</td>
<td>561 ± 62</td>
<td>417 ± 84</td>
</tr>
<tr>
<td>Urine Output (ml/24 hrs)</td>
<td>1.29 ± 0.35</td>
<td>1.58 ± 0.35</td>
<td>1.27 ± 0.27</td>
</tr>
<tr>
<td>U_{Na}V (mmol/24 hrs)</td>
<td>0.28 ± 0.05</td>
<td>0.24 ± 0.04</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>U_{Cl}V (mmol/24 hrs)</td>
<td>0.28 ± 0.04</td>
<td>0.26 ± 0.04</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>U_{K}V (mmol/24 hrs)</td>
<td>0.36 ± 0.03</td>
<td>0.36 ± 0.06</td>
<td>0.37 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n=4-6). U_{Na}V, U_{Cl}V, U_{K}V, urinary excretion of Na^+, Cl^- and K^+ respectively.
(+/+ = 40.4 ± 1.2 %; -/- = 35.0 ± 0.9 %) (P < 0.01), as seen previously. In addition, according to the Evan’s Blue dilution studies, plasma volume normalized to body weight was significantly greater in the ANP knockout mice than in the wildtypes (-/- = 3.96 ± 0.25 mL/100g vs. +/+ = 3.21 ± 0.06 mL/100g) (P<0.05).

Although the baseline operating values of RIHP were significantly increased in the -/- animals, there was no significant difference in the slopes of the depressor RAP-RIHP relationship between the two strains of mice (+/+ = 0.06 ± 0.01 mmHg of RIHP/mmHg of RAP; -/- = 0.07 ± 0.01 mmHg of RIHP/mmHg of RAP) of the RAP-RIHP relationship (Figures 4.5A&B). It is important to note that the average RAP decreases were equivalent (+/+ = -19.6 ± 2.1 mmHg; -/- = -20.3 ± 2.8 mmHg). In contrast to a similar downward relationship, there was a significantly decreased slope of the pressor portion of the RAP-RIHP relationship in the -/- animals (0.07 ± 0.01 mmHg of RIHP/mmHg of RAP) compared to the +/+ animals (0.14 ± 0.02 mmHg of RIHP/mmHg of RAP) following comparable increases in RAP (+/+ = 20.1 ± 1.0 mmHg; -/- = 19.2 ± 0.7 mmHg) (P<0.01) (Figures 4.5A&B).
Figure 4.4: Hemodynamic variables in 14-16 week old +/+ and -/- mice under pentobarbital anesthesia. A: Renal arterial pressure (RAP) obtained via direct superior mesentery artery cannulation; B: Renal interstitial hydrostatic pressure (RIHP) at baseline RAP. Values are means ± SEM (n = 5). * P < 0.01 vs. +/+ mice; unpaired Student’s t test.
Figure 4.5: Relationship between RAP and RIHP of 14-16 week old +/+ (A) and -/- mice (B) under pentobarbital anesthesia. Values are means ± SEM ($n = 5$). Inserts: raw RAP-RIHP relationship of an individual +/+ mouse (A) and an individual -/- mouse (B).
4.5 Discussion

In this study we have characterized mechanisms by which animals lacking ANP may be predisposed to a salt-sensitive and hypertensive phenotype. Commonly, alterations in the RAS, vasodilatory mechanisms and renal medullary hemodynamics are associated with hypertension and salt-sensitivity in animal models. In the human population, children of normotensive parents have higher circulating ANP levels than children of hypertensive parents, especially in response to an increase in dietary salt\textsuperscript{258},\textsuperscript{259}, suggesting that the dysregulation of ANP can cause blood pressure to become salt-sensitive. Using an animal devoid of this vasodilator-natriuretic substance, the present study has examined two important controllers of blood pressure and electrolyte homeostasis, the RAS and the acute pressure-natriuresis mechanism. Consistent with the salt-sensitive phenotype, the results revealed for the first time that the RAP-RIHP relationship, a key component of the pressure-natriuresis mechanism, is blunted in hypertensive ANP -/- mice during pressor, but not depressor, challenges. Furthermore, the finding that renin and Ang II levels in the kidney and adrenal gland of ANP -/- mice were already downregulated at baseline, suggests that these animals have multiple phenotypes that would confer salt-sensitivity. Taken together with previous findings, the salt-sensitive phenotype of the ANP gene-disrupted mouse appears to comprise both a decreased expression and responsiveness of the RAS\textsuperscript{189,190} and the acute pressure-natriuresis mechanism, more specifically the RAP-RIHP relationship.

Previously, Melo et al. postulated that decreased responsiveness of the RAS may be responsible, at least in part, for the salt-sensitive phenotype of ANP -/- mice\textsuperscript{189,190}. Further, these combined results suggest that the dynamic capacity of the RAS may be
attenuated in these animals, as both Ang II levels and renin expression in kidney and adrenal glands of ANP gene-disrupted mice are depressed as compared to the wildtype. These findings are consistent with the literature regarding other models of salt-sensitive hypertension such as the Dahl rat \(^{260-262}\) and NPRA \(-/-\) \(^{263, 264}\). Indeed, the downregulation of the renal and adrenal RAS was observed previously in the Dahl rat \(^{260-262}\) and presently in the ANP gene-disrupted mouse, indicates that a chronic alteration in baseline levels of a system critical to sodium homeostasis can predispose an organism to salt-sensitivity. Furthermore, it may be that local tissue changes in RAS activity (or lack thereof) are more relevant to the salt-sensitive phenotype than are changes in the circulating system \(^{254}\). In a situation in which there is already low baseline levels of renin, it may not be possible to markedly depress renin release in response to a salt load \(^{265}\), thereby exposing the salt-sensitivity of the gene-disrupted mice. Indeed, many salt-sensitive models are characterized by a low-renin state \(^{260, 264, 266-268}\).

The presence of a salt-sensitive and hypertensive phenotype in the proANP gene-disrupted mice confirms that ANP is an important player in the control of sodium excretion and the resulting impact on arterial pressure. Consistent with previous reports in SHR \(^{63}\) and Dahl salt-sensitive rats \(^{269}\), these data show that the slope of the RAP-RIHP curve is blunted in ANP \(-/-\) as compared to the ANP \(+/+\) mice. The characteristics of the relationship between RAP and RIHP have been found to play an integral role in pressure-natriuresis \(^{11, 28, 34, 51, 237}\), where the sensitivity of RIHP to changes in RAP has a profound effect on the level of sodium excretion and in turn, the regulation of blood volume and pressure. Interestingly, ANP has previously been demonstrated to be able to sensitize pressure-natriuresis at high, but not low, pressures \(^{98}\). To account for these divergent
actions in the present study, for the first time, the pressor and depressor changes in the RAP-RIHP relationship were characterized separately. Indeed, the present findings confirm that the RIHP of animals lacking ANP are less sensitive to increases in RAP, as shown by the decreased slope of the RAP-RIHP curve, whereas the sensitivity of RIHP to decreases in RAP is similar between both mice with and without ANP.

The present results revealed that there is a selective blunting of the pressor component of the RAP-RIHP relationship in the gene disrupted animals. A decrease in responsiveness of RIHP to changes in RAP has previously been linked with increased compliance of the renal interstitium\textsuperscript{270} and/or increased resistance of the medullary circulation\textsuperscript{19}. For example, increased renal interstitial compliance, such as during pregnancy\textsuperscript{270} or following renal decapsulation\textsuperscript{29}, results in a blunting of the RAP-RIHP relationship and a decrease in baseline RIHP. However, in the present study there was an increase in RIHP at baseline in the ANP -/- mice, a phenomenon that has also previously been reported in volume expanded animals\textsuperscript{271}. The finding that the ANP -/- mice are, in fact, hypervolemic, provides a basis for the high baseline RIHP. Whether an increased baseline RIHP changes renal interstitial compliance or the responsiveness of the RAP-RIHP relationship in the volume-expanded state remains unknown. Based on previous studies in the SHR\textsuperscript{54,91,159}, an alternative explanation for the blunted RAP-RIHP relationship could be increased medullary vascular resistance (MVR) in the ANP -/- mice. It may be that the high circulating\textsuperscript{188,272} and renal\textsuperscript{273} catecholamine levels found in ANP -/- mice contributes to the increased MVR. Overall, the present findings suggest that long-term changes in AP may be a direct result of alterations intrinsic to the kidney.
A link between changes in RAS activity, or lack thereof, and the long-term pressure-natriuresis mechanism is well established in models of salt-sensitivity. In particular, a loss of dynamic control of the RAS is known to attenuate the ability of the organism to respond efficiently to chronic changes in salt intake. It may thus be that the antagonism of the RAS by ANP is important in long-term sodium homeostasis and AP control. The present findings suggest that ANP disruption results in both a decrease in the renal actions of the RAS, as well as blunting of the acute pressure-natriuresis mechanism. Both of these results are consistent with the chronic changes in sodium homeostasis that are observed in the ANP gene-disrupted mouse.

In summary, the present study contains novel findings revealing that there is a selective impact on the pressor portion of the RAP-RIHP relationship in a mouse model of hypertension resulting from the absence of ANP. Taken together with previous studies, it also appears that the ability of ANP gene-disrupted mice to respond to a salt challenge is enhanced by the pre-existing low RAS activity. Given the importance of these systems in the renal regulation of arterial pressure, the exact quantitative contribution of these two mechanisms that result in the hypertensive and salt-sensitive phenotype remains to be established.
CHAPTER 5

The Long-Term Circulatory Consequences of Perinatal Iron-Deficiency in Male Wistar Rats

(Bourque SL, Komolova M, Nakatsu K, and Adams MA. Hypertension 2008; 51:151)

5.1 Abstract

Perinatal iron-deficiency (PID) has been reported to induce developmental abnormalities, including cardiovascular complications in rats. These complications are believed to be “programmed” by an aberrant perinatal environment since the changes persist long after the insult is corrected (i.e. despite subsequent iron replenishment). Little is known about the mechanisms by which PID affects blood pressure in the offspring, although the kidney is likely to play a central role. The objective of this study was to investigate the circulatory complications of PID and the putative role of the kidney involved therein. Prior to and throughout gestation, female Wistar rats were fed either a low-iron diet (3ppm/10ppm Fe) or an iron-enriched diet (225ppm Fe). After giving birth, all dams were placed on a standard grain-based diet. At 24hours postpartum, hematocrit and hemoglobin levels from offspring of iron-deficient mothers were 60% and 59% of control values, respectively. Adult PID animals had greater mean arterial pressure (110±2 vs. 106±1 mmHg) and systolic blood pressure (129±2 vs.124±1 mmHg) than controls, as assessed by radiotelemetry. The relationship between renal arterial pressure and renal interstitial hydrostatic pressure, assessed in anaesthetized rats, was blunted by 41% in the PID group compared to controls. Additionally, arterial pressure changes were significantly greater in response to altered sodium in the PID animals compared to
controls. These data confirm that PID adversely affects blood pressure control which appears to be mediated, at least in part, by altered intrarenal hemodynamic properties.

5.2 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 is estimated to be 66-80% (WHO, 2003). Although iron deficiency significantly affects all populations, the group most at risk is pregnant women. The enhanced risk profile in pregnancy is a consequence of increased erythropoeisis due to 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 were 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 arterial pressure during low, normal and high sodium intake.

5.3 Methods

5.3.1 Animals and Treatments

The experimental protocols described herein were approved by the Queen’s University Animal Care Committee. Eighteen 6-week 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 12h/12h 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 iron-deficient 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: 225ppm (Cat#: D03072504); low-iron diet: 3ppm (no added ferric citrate) (Cat# D03072501); moderately low-iron diet: 10ppm (Cat#: D03072505). The standard grain-based rodent chow (Lab Diet, St-Louis, MO, Cat#: 5001) had an iron concentration of 270ppm.

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 week-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. At birth (postnatal day (PD) 0), all dams were placed on the grain-based rodent chow. At PD21, the offspring were separated from their mothers, and weaned onto the grain-based rodent chow. Food consumption and body weights were monitored twice weekly.
5.3.2 Tissue Analysis

Hematocrits (Hct) and hemoglobin (Hb) levels were measured weekly in the dams during the two-week 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 hours 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 1. At PD7, PD14 and PD21, two animals per litter were sacrificed, with females again being preferentially selected. Hct, 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.

5.3.3 Conscious Hemodynamic Assessments

Starting at 10 weeks 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. 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. Nighttime 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 weeks of age) implanted with radiotelemetric transducers was 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 *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. The high salt treatment regimen consisted of *ad libitum* access to the standard grain-based rodent chow described above (0.4% Na\(^+\)), 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.

5.3.4 In vivo assessment of renal vascular properties

Intrarenal hemodynamic assessments were performed in anesthetized 10-week old male PID and control offspring, based on a method previously described\(^{284}\). 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 ± 1°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% O\(2\)/5% CO\(2\) 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/100g body wt 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 electrosurgical unit (Elmed ESU30; Elmed Incorporated) 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.

5.3.5 Data Analyses

Neonatal offspring Hct, Hb levels, and organ weights from each litter were pooled and means 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 PD21. All information pertaining to the dams (Table 5.1), and intrarenal hemodynamic parameters (Table 5.2) 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 regular 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\textsuperscript{241}. 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 means ± SEM.

### 5.4 Results

Food consumption during the pre-gestational treatment period and throughout pregnancy was not different between the control and iron-deficient dams (data not shown). A summary of hematological values and pregnancy outcomes pertaining to the dams is presented in Table 5.1. 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
Table 5.1: 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 Wks) (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>Hct (pre-pregnancy)</td>
<td>0.47 ± 0.01</td>
<td>0.44 ± 0.01*</td>
</tr>
<tr>
<td>Hb (pre-pregnancy) (g/dL)</td>
<td>134 ± 5</td>
<td>121 ± 3*</td>
</tr>
<tr>
<td>Hct (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/dL)</td>
<td>114 ± 5</td>
<td>82 ± 5†</td>
</tr>
<tr>
<td>Hct (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/dL)</td>
<td>123 ± 7</td>
<td>128 ± 3</td>
</tr>
</tbody>
</table>

Control: n=7; PID: n=9. *P<0.05; †P<0.001 compared to control values. BW, body weight; Hct, hematocrit; Hb, hemoglobin.
impact in the PID group. For example, one litter in the PID group did not survive 24
hours. 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 2-week treatment period prior to conception, dams fed the low-iron
diet had a modest decrease in Hct (93% of control; P<0.05) and Hb levels (91% of control; P<0.05) (Table 5.1). Hct and Hb levels fell to less than 75% (P<0.001) of controls 24 hours after parturition but had returned to control levels within 7 days (Table 5.1). Conversely, Hct 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 significantly decreased until after PD14 (Table 5.2). At PD21, Hct in the PID offspring remained more than 10% below controls (P<0.05), but Hb levels were no longer significantly depressed. Control pups, but not PID pups, had significant decreases in Hct and Hb levels after birth (P<0.01 at all times compared to PD1).

Body weights of offspring in the PID group were more than 10% lower than control throughout the study period (P<0.05) (Figure 5.1). Following a marked decrease in relative body weight during the first post-natal week (Figure 5.1, inset), PID pups underwent two periods of ‘catch-up’ growth (when absolute weight gain was greater in the PID group); one pre-weaning (PD10-PD21) and one post-weaning (beyond PD24). Heart weights (normalized to body weight) were 29% higher in the PID offspring at PD1 compared to control offspring (P<0.01) (Table 5.2). These differences persisted until PD21. There were no observed differences in kidney weights (normalized to body weight) between the control and PID offspring between PD1 and PD21 (Table 5.2).
Table 5.2: Neonatal Offspring Information at PD1, 7, 14, 21.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PD1</th>
<th>PD7</th>
<th>PD14</th>
<th>PD21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctl Hct</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 Hct</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>Ctl Hb (g/dL)</td>
<td>106 ± 3</td>
<td>80 ± 3</td>
<td>71 ± 2</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>PID Hb (g/dL)</td>
<td>62 ± 3 ‡</td>
<td>54 ± 2 ‡</td>
<td>56 ± 2 ‡</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>Ctl HW/BW (g/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 HW/BW (g/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>Ctl KW/BW (g/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 KW/BW (g/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>

Control (Ctl): n=4-7 litters, PID: n=6-8 litters. *P<0.05; †P<0.01; ‡P<0.001 compared to control values at same postnatal day. PD, postnatal day; PID, perinatal iron deficient; Hct, Hematocrit; Hb, hemoglobin; HW, heart weight; KW, kidney weight.
Figure 5.1: PID offspring (○) (n=9 litters) had lower body weight compared with control offspring (●) (n=7 litters) throughout the entire study period. The same data are expressed as a percentage of control offspring body weight (inset). *P<0.05 compared to controls at same postnatal day.
Blood pressure data expressed as MAP and SBP, starting at approximately 11 weeks of age (following a 10-day recovery period after surgery), were moderately but significantly elevated in the PID group compared to controls over a 10-day period (Figure 5.2). The mean six-hour MAP values for the control and PID animals over the 10-day period were 105.8 ± 0.8 vs. 110.7 ± 1.5 mmHg, respectively (P<0.05); the average SBP values were 124.0 ± 0.7 vs. 129.3 ± 2.0 mmHg, respectively (P<0.05).

A summary of intrarenal haemodynamic parameters assessed in control and PID animals at approximately 10 weeks of age is presented in Table 5.3. The mean renal arterial pressure (RAP) in the PID group under anaesthesia was found to be approximately 12mmHg 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 ΔRAP-ΔRIHP relationship (Figure 5.3) was blunted by 41% in the PID group (0.062 ± 0.005; r²=0.87) compared to controls (0.100 ± 0.002; r²=0.95) (P<0.01). Likewise, assessment of the slope of the ΔRAP-ΔRIHP 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; r²=0.76) compared to controls (0.099 ± 0.010; r²=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; r²=0.85) compared to controls (0.075 ± 0.005; r²=0.73) (P<0.01) (Figure 5.3, inset).
Figure 5.2: SBP and MAP profiles are higher in adult PID offspring (○, ○, ○) (n=9) compared to controls (●, ●, ●) (n=11) (P<0.05 for both parameters) as assessed by radiotelemetry. SBP, systolic blood pressure; MAP, mean arterial pressure.
Table 5.3: Physical and Renal Properties of Offspring at 10 Weeks of Age.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>PID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body 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>LV/BW (g/Kg)</td>
<td>1.70 ± 0.05</td>
<td>1.95 ± 0.10*</td>
</tr>
<tr>
<td>RV/BW (g/Kg)</td>
<td>0.55 ± 0.05</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>KW/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>

Control: n=5; PID: n=5. *P<0.05; †P<0.01 compared to control values. RAP, renal arterial pressure; RIHP, renal interstitial hydrostatic pressure; LV, left ventricle; RV, right ventricle; KW, kidney weight; BW, body weight.
Figure 5.3: The relationship between the change in renal interstitial hydrostatic pressure and the change in renal arterial pressure is significantly blunted in adult male offspring of the PID group (○) compared to controls (●) (P<0.001). The same data are expressed as absolute RAP and RIHP measurements (inset). Circles represent individual manipulations made on each animal (n=5 animals in each group). *P<0.001 for PID slope compared to control slope. RAP, renal arterial pressure; RIHP, renal interstitial hydrostatic pressure.
In Figure 5.4, 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 significantly more responsive to the extremes of dietary sodium (F(1,36) = 10.0, 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).
Figure 5.4: Extended low- and high-sodium treatment had a more profound impact on MAP in adult PID offspring (−O−) (n=6) compared to controls (−●−) (n=7) (upper panel). The absolute changes in MAP are summarized in the inset of the upper panel; ‘Total’ representing the MAP change from low to high sodium treatments; low sodium (LS) representing the MAP change from normal to low sodium treatments; high sodium (HS) representing the MAP change from normal to high sodium treatments. The resultant renal function curves obtained from these data are shown in the lower panel. For details on the protocol, see methods section. *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.
5.5 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 arterial pressure, (4) alterations in the hemodynamic properties of the kidney, and (5) an increased sensitivity of arterial pressure 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 hemoglobin levels and hematocrits within 7 days (Figure 5.1). With this approach, we avoided confounding factors associated with continued anemia in the mothers during the nursing phase. Specifically, milk production in the iron-deficient mother appears to be adversely affected with respect to iron, energy and fat content\textsuperscript{285}. Despite returning the iron-deficient dams to 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{286}. Indeed, Hct 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{221-223, 287}, 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{288}. 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{287}, this adaptation would be expected to limit the generation of hypoxia.

Consistent with most 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 5.1, inset). 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\textsuperscript{222}. However, similar iron-deficiency induced-fetal programming effects have been reported by others\textsuperscript{221, 223} 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 nephrogenesis 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 arterial pressure was significantly elevated in the adult offspring following perinatal iron deficiency confirms previous results in
which systolic blood pressure was assessed using the indirect tail-cuff method\textsuperscript{220-224}. These results are corroborated by the presence of left ventricular hypertrophy, but not right ventricular hypertrophy, in the 10-week old animals; an adaptive response normally associated with arterial hypertension. The magnitude of the increase in arterial pressure found in the adult PID offspring is modest compared to those observed by other investigators, who reported increases in the adult offspring between 18 mmHg\textsuperscript{223, 224} and 30 mmHg\textsuperscript{220} using the indirect tail-cuff method. These discrepancies may be due to a number of factors, including (1) differences in timing and degree of iron deficiency in the mothers and offspring\textsuperscript{282}, (2) strain-specific differences\textsuperscript{289} (e.g. Rowett Hooded-Lister\textsuperscript{223}; Sprague Dawley\textsuperscript{222}, and Wistar\textsuperscript{220, 221, 224}), and most importantly, (3) systolic blood pressure measured via the tail-cuff methodology is affected by restraint and thermal stress\textsuperscript{290}. Indeed, it may be that programmed animals are more responsive to such stressors compared to non-programmed animals. In light of this evidence, the current validation of this cardiovascular phenotype in this model of programming using direct conscious, chronic determinations of arterial pressure is an important foundation for future studies.

The key finding that perinatal iron deficiency altered the intrarenal hemodynamic properties—namely the RAP-RIHP relationship—may explain, in part, the long-term elevations in arterial pressure observed in these animals. The kidney is fundamental in establishing the set point of long-term arterial pressure by regulating sodium and fluid balance\textsuperscript{13}. Fluctuations in arterial pressure 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 arterial pressure at which sodium
and water balance occur\textsuperscript{58, 60}. 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 arterial pressure are required to generate corresponding changes in RIHP to regain sodium and water balance\textsuperscript{64}.

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 5.4, lower panel), control offspring will increase sodium and fluid excretion in response to minor changes in arterial pressure\textsuperscript{30}. 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{63, 85, 239, 269}.

Although the specific mechanisms by which perinatal iron deficiency 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{19, 270}. Perinatal iron deficiency 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 is likely that the
etiology of the adverse programming effects observed in the present study is multifaceted and complex.

5.6 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. Although programming effects have been associated with a number of macro- and micro-nutrient deficiencies, given the worldwide prevalence of iron deficiency as well as its propensity to afflict pregnant women, it may represent an especially important risk factor for long-term cardiovascular disease.
CHAPTER 6

Sedentariness and Increased Visceral Adiposity in Adult Perinatally Iron-Deficient Rats


6.1 Abstract

Background: Perinatal iron deficiency (PID) adversely programs offspring resulting in alterations in adult cardiometabolic function. Increased visceral adiposity is the proposed culprit for these sequelae, and may be potentiated by decreased physical activity. Herein, we determined (i) the effect of PID on visceral adipose tissue (VAT) and locomotor activity, and (ii) whether increased VAT is associated with blood pressure responsiveness to increased dietary sodium.

Methods and Results: Dams were fed a low iron diet (<10 mg/Kg Fe) prior to and throughout gestation. From 12-35 weeks of age, locomotor activity (assessed by radiotelemetry) in PID offspring was 25% lower compared to control offspring (P<0.001). At 36 weeks of age, PID offspring had 15% more VAT than controls (P<0.05). Furthermore, the elevation of mean arterial pressure (measured by radiotelemetry) in response to increased sodium intake was ≈ 2-fold greater in the PID offspring (P<0.05).

Conclusions: PID results in increased visceral adiposity, which was associated with enhanced blood pressure responsiveness to dietary salt, perhaps due to programmed sedentary behaviour.
6.2 Introduction

Iron deficiency (ID) may affect approximately 4-5 billion people worldwide, with pregnant women being most susceptible\textsuperscript{276}. In animal studies, perinatal ID (PID) results in cardiometabolic complications in adulthood\textsuperscript{220, 222, 223, 238}. Hales and Barker coined the term “thrifty phenotype” for individuals ‘programmed’ to develop cardiovascular and metabolic risk factors in adulthood, such as hypertension and abdominal obesity, in response to nutritional deficiencies in fetal and early life\textsuperscript{291}. Hypertension occurs in adult PID rats\textsuperscript{220, 222, 223, 238}, and we recently showed that these animals have exaggerated blood pressure responsiveness to changes in sodium balance\textsuperscript{238}. Given that obesity has been implicated in the development of salt-sensitivity and hypertension\textsuperscript{233, 292}, we investigated whether the exaggerated blood pressure responsiveness to dietary salt in PID offspring is associated with increased adiposity. Moreover, other rat models of maternal undernutrition (calorie or protein) are associated with abdominal obesity in adulthood, which was attributed to programmed sedentary behaviour\textsuperscript{212-214}. However, in the adult PID rats, physical activity and adiposity have not been characterized. Thus, the aim of this report was to determine whether PID offspring have (i) programmed sedentary behavior, and (ii) whether increased visceral adipose tissue (VAT) is associated with the enhanced blood pressure responsiveness to increased dietary salt.

6.3 Methods

The current study is an extension of prior work that investigated effects of PID on intrarenal hemodynamic properties and blood pressure\textsuperscript{238}. Control (n=6) and PID (n=4) rats were used to determine physical characteristics, locomotor activity, visceral adiposity, and blood pressure responsiveness to sodium intake.
6.3.1 Animals and Treatments

All procedures followed guidelines of the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee. Seven week-old female Wistar rats purchased from Charles River (Saint-Constant, QC) were randomly assigned to either a low-iron diet (3 mg/Kg Fe) or a control diet (225 mg/Kg Fe) for 2 weeks prior to mating. At time of mating, all dams on the low-iron diet were fed a moderately low-iron diet (10 mg/Kg Fe) to ensure offspring survival\textsuperscript{282}. Purified diets were from Research Diets Inc. (New-Brunswick, NJ), and based on the AIN93G diet\textsuperscript{281}. At birth, all dams were fed a non-purified diet which contained 270 mg/Kg Fe (Lab Diet, St-Louis, MO). At 24 hours postpartum, litters larger than 10 pups were culled to 10 (litters with <10 pups were not used). At postnatal day (PD) 21, all offspring were separated from their mothers and were fed a non-purified diet. For this study, 1-2 male offspring were selected randomly from litters. If 2 offspring were taken from the same litter, then their data were pooled and treated as a single value. All rats had \textit{ad libitum} access to food and water, and were maintained on a 12h/12h light/dark cycle at 23˚C.

6.3.2 Locomotor Activity and Blood Pressure Monitoring for Salt Challenge

Locomotor activity and mean arterial pressure (MAP) were assessed in consciously free moving rats by a system consisting of an implantable radio-telemetry device, receiver, and computer-based data acquisition system (Data Sciences International)\textsuperscript{238}. Following implantation of radio-telemetry units, rats were allowed to recover for 10 days before recording of data. MAP was obtained from the aortic probe and locomotor activity was obtained from the movement of the animal (i.e. radio-telemetry device in abdomen) across the receiver positioned under the cage. Data were
sampled every 5 minutes for 15 seconds, and reported as an average of all data points collected at night (2200h to 0400h), the most active period of the day. Locomotor activity was assessed from 12-35 weeks of age. MAP was assessed starting at 24-26 week of age during a normal sodium regimen (non-purified diet; 0.4% Na⁺; Laboratory Diet) for 5 days, on a low-sodium regimen (LS; 0.04% Na⁺; Research Diets Inc.) for 5 days and on a high-sodium regimen (HS; drinking water supplemented with 1% NaCl (wt/vol) and non-purified diet; 0.4% Na⁺; Laboratory Diet) for 5 days.

**6.3.3 Physical Characteristics and Visceral Fat Pad Assessments**

Body weight (BW), naso-anal length and waist circumference (measured at the midpoint between tip of the sternum and penis in supine position), were determined in offspring at 36 weeks of age. Additionally, following anesthesia with sodium pentobarbital (120 mg/Kg i.p.), retroperitoneal, mesenteric, omental, and epididymal fat pads were removed; the sum of these major abdominal fat pads was considered as VAT.

**6.3.4 Statistical Analysis**

Physical characteristics, VAT, and overall locomotor activity were analyzed by Student’s t-test, and locomotor activity over time was assessed via a 2-way ANOVA. Linear regression analysis was used to calculate the relationship between percent VAT per BW and difference in MAP on a HS versus LS diet. Grubb’s test was conducted on data sets to determine statistical outliers. Data are presented as means ± SEM; n= number of litters (1-2 offspring/litter). P<0.05 was considered statistically significant.
6.4 Results

The number of pups per litter was found to not be significantly different between PID and control rats (control = 15.1±1.9; PID = 16.1±0.9)\(^{238}\); however neonatal BW of offspring in the PID group were 10% lower than control values at PD 1 (P<0.05). Growth restriction persisted in adulthood; PID animals were 21% lighter and significantly shorter than controls (Table 6.1; P<0.05). Despite this growth restriction, VAT weights were similar in both groups, as were waist circumferences (Table 6.1). Accordingly, VAT as a percentage of BW was significantly greater in PID rats compared to controls (Table 6.1; P<0.05).

In both PID and control groups, locomotor activity declined with age, with activity in the PID group being lower than controls at all ages (Figure 6.1A; P<0.001). The overall mean locomotor activity in adulthood was 25% lower in the PID group compared to controls (Figure 6.1A inset; P<0.001).

The effect of dietary salt on MAP (as measured by the difference in MAP on HS versus LS) was almost 2-fold greater in adult PID offspring compared to control offspring (PID: 13.5 ± 1.9 mmHg; control: 7.7 ± 0.5 mmHg; P<0.05). This heightened MAP sensitivity to changes in sodium balance was correlated with increased VAT (Figure 6.1B; \(r^2= 0.7\); P<0.05). There were no differences in normal, LS, or HS food intake (normalized to BW) at 24-26 weeks of age.
Table 6.1: Physical Characteristics and Visceral Adipose Tissue Weight of Offspring at 36 Weeks of Age.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=5)</th>
<th>PID (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>779.4 ± 19.2</td>
<td>677.2 ± 10.8 *</td>
</tr>
<tr>
<td>Body Length (cm)</td>
<td>27.8 ± 0.2</td>
<td>26.9 ± 0.1 *</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>24.2 ± 0.5</td>
<td>23.3 ± 0.2</td>
</tr>
<tr>
<td>VAT (g)</td>
<td>59.8 ± 3.1</td>
<td>61.17 ± 2.3</td>
</tr>
<tr>
<td>Percent VAT to BW (%)</td>
<td>7.7 ± 0.3</td>
<td>9.0 ± 0.3*</td>
</tr>
</tbody>
</table>

Abbreviations: BW, body weight; VAT, visceral adipose tissue; PID, perinatally iron-deficient; n, number of litters. Values are means ± SEM. *P<0.05 vs. control by unpaired t-test.
Figure 6.1: Effect of perinatal iron-deficiency on activity and visceral adiposity. A) Solid bars depict locomotor activity (mean ± SEM) in control (n=5) and open bars in PID (n=3) rats from 12-35 weeks of age. Throughout adulthood, PID rats had significantly lower activity than control (***P<0.001). Note: telemetry units were turned off from 21-22 weeks to preserve battery life. Inset: Mean ± SEM locomotor activity in adulthood (12-35 weeks of age). B) Correlation between visceral adiposity and responsiveness of mean arterial pressure (MAP) to dietary salt (high salt (HS) versus low salt (LS) diet). There was a significant (P<0.05) linear correlation between percent visceral adipose tissue (VAT) to body weight (BW) and change in MAP following the salt challenge collapsed across the whole group (control = black circles; PID = white circles). Average MAP responsiveness following salt challenge and percent VAT to BW were significantly higher in PID (white box) versus control (black box) animals (P<0.05). Boxes represent group means and error bars represent ± SEM.
6.5 Discussion

The major findings of the present study are that adult PID rats were less physically active, had increased VAT, and had enhanced responsiveness of MAP to alterations in sodium homeostasis. These observations extend prior studies which revealed that maternal ID results in hypertension\textsuperscript{220, 222, 223}, as well as altered glucose and lipid metabolism\textsuperscript{220} in adult offspring. The present observations suggest that PID can lead to abdominal obesity, possibly via programming of sedentary behaviour, which may be associated with altered cardiovascular regulation in adulthood. This phenotype may be particularly relevant given the prevalence of ID amongst pregnant women in both developing and industrialized countries\textsuperscript{276}, and the global rise of obesity with its associated diseases\textsuperscript{293}.

Consistent with the “thrifty phenotype” hypothesis, PID results in developmental adaptations that predispose the offspring to increased visceral adiposity in adulthood\textsuperscript{291}. The etiology of obesity, in particular VAT deposition, is multifactorial and complex\textsuperscript{233, 293}. Herein, the data show that increased VAT in PID offspring is associated with reduced locomotor activity. The latter finding is corroborated by previous results\textsuperscript{294, 295}, in which young adult male PID rats exhibited reduced exploratory behaviour when left in an open-field apparatus. The present findings, involving long-term assessments in home cages, reveal that these responses, at least in part, likely relate to physical inactivity rather than just anxiety-like behaviors. Further, these activity-related changes probably contribute to the 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\textsuperscript{296}).
Given that visceral adiposity is an independent risk factor for hypertension\(^{297}\), it is not surprising that PID animals are predisposed to both conditions. Obesity-induced hypertension appears to be related to abnormalities in sodium handling resulting in amplified MAP responsiveness to alterations in sodium equilibrium\(^{233}\). In the present study, we confirm that adult PID rats have increased MAP responsiveness to changes in sodium balance, which is associated with increased visceral adiposity. Thus, one potential mechanism whereby obesity may induce MAP sensitivity to changes in sodium balance is by resetting the pressure-natriuresis mechanism, a renal feedback system that maintains fluid and electrolyte balance around a blood pressure set-point\(^{297}\). In fact, this mechanism is blunted in ‘Wistar fatty rats’\(^{292}\) and obese Zucker rats\(^{234}\), such that a higher arterial pressure is required to excrete the same amount of sodium as controls. Interestingly, we previously reported a blunting of pressure-natriuresis in this particular model of PID\(^{238}\).

In conclusion, a specific micronutrient deficiency involving iron during pregnancy and immediate postnatal life of the offspring may be important in generating a phenotype characterized by sedentariness, increased visceral adiposity, and altered blood pressure control. Given that pregnant women are highly susceptible to ID\(^{276}\), maintaining proper maternal iron status throughout pregnancy and in the postnatal period may be critical in ensuring proper development of the offspring, and thus reducing cardiometabolic disease risk in adulthood.
CHAPTER 7

General Discussion

Guyton and associates were the first to propose that the kidney serves a critical role in the long-term regulation of ECF and AP, in part, via pressure-natriuresis\textsuperscript{7, 10, 11, 13, 14, 16, 17}. It is well regarded that AP fluctuates spontaneously in varying degrees of magnitude around an operating point and over a wide range of time scales (frequencies)\textsuperscript{11, 58}. These changes in AP are ‘sensed’ by the kidney through the poorly autoregulated medullary circulation. For example, increases in AP result in parallel increases in MBF and RIHP, which ultimately lead to sodium excretion\textsuperscript{11, 21, 27, 28}. While the functioning of the pressure-natriuresis mechanism in the long-term has been well characterized in normotensive, hypertensive and salt-sensitive models, whether the kidney ‘senses’ moment-to-moment fluctuations in AP is not well known, and warrants characterization. This is especially important since the kidneys are exposed to greater AP variations over a shorter timeframe (second-to-second) than have been previously examined using conventional pressure-natriuresis methodologies (i.e. minute-to-minute; hour-to-hour; day-to-day) (Figure 2.1). Thus, the focus of the studies presented herein was to characterize the moment-to-moment function of the pressure-natriuresis mechanism, specifically the RAP-RIHP relationship, and assess its functioning in various animal models of hypertension and salt-sensitivity. In order to accomplish these objectives, we developed a new \textit{in vivo} methodology for assessing the RAP-RIHP relationship in a more physiological manner (i.e. mirroring spontaneous oscillations of AP above and below the operating point, and with all the neurohumoral systems left intact). The major findings reveal that changes in RAP are ‘sensed’ by the kidney through RIHP on a moment-to-
moment basis, and that the functioning of this intrarenal hemodynamic mechanism (i.e. the acute RAP-RIHP relationship) is impaired in hypertensive and salt-sensitive organisms.

7.1 The Moment-to-Moment Pressure-Natriuresis Mechanism and its Role in Hypertension and Salt-Sensitivity

Findings from the Britton laboratory\textsuperscript{57, 58} were the first to suggest that pressure-natriuresis acts dynamically, such that spontaneous changes in AP are rapidly transmitted to the renal interstitium, inducing moment-to-moment changes in RIHP and thus UF (Figure 1.8). Since this hypothesis was originally proposed more than 15 years ago, there have not been any follow-up studies to fully characterize the functioning of this acute intrarenal mechanism. The results presented in Chapter 2 are the first to fully characterize how the kidney ‘senses’ changes in AP on a moment-to-moment basis, specifically with respect to RIHP. Consistent with the previous finding that UF, a variable downstream from RIHP, responds to changes in AP within ~6 seconds\textsuperscript{57}, it was found that RIHP responds to changes in AP on a second-to-second basis in adult normotensive rats. A similar time course assessment on data from young WKY and SHR rats (Chapter 3) further supports these findings, in that the RIHP response time was also found to function rapidly. Thus, these findings support the aforementioned hypothesis that components of the pressure-natriuresis mechanism function dynamically and on a moment-to-moment basis.

Evidence from studies utilizing the conventional ‘graded-step’ methodology\textsuperscript{22, 29, 30, 52, 59-65, 65-68} suggests that the relationship between AP and components of the pressure-natriuresis mechanism (i.e. MBF, RIHP, UF, and sodium excretion) is linear (Chapter
1.3.3). Using a novel methodology, we confirm that the overall relationship between changes in RAP and acute responses in RIHP are also linear in normotensive rats (Chapter 2). However, upon examination of the RAP-RIHP relationship according to pressor versus depressor stimuli, it was discovered that the slope was lower for increases versus decreases in AP. Since no other study has ever assessed whether a differential regulation of this mechanism above and below the AP the operating point exists, we can only speculate that the basis for the aforementioned differences may be due to the following mechanisms: i) myogenic response\textsuperscript{242}, ii) flow-mediated vasodilation\textsuperscript{243}, or iii) alterations in the functioning of certain neurohumoral systems\textsuperscript{18}. Since there is a blunted RIHP response following pressor stimuli, mechanisms that increase vascular tone/resistance seem more likely to be the culprit, as in theory they would decrease MBF, and thereby RIHP; however this is purely speculative and warrants further investigation. Aside from potential mechanisms that may target intrarenal vascular resistance properties, another possibility may be mechanisms that affect renal interstitial compliance (e.g. the elasticity of the renal capsule)\textsuperscript{29,66}.

It is well accepted that a resetting of the pressure-natriuresis curve towards a higher AP is associated with hypertension (Chapter 1.3.1)\textsuperscript{10,11}. Corroborating this, evidence from Chapters 3-5 reveals that a rightward shift in the acute RAP-RIHP relationship exists in all models of hypertension studied. In fact, assessment of this acute RAP-RIHP relationship with our novel methodology has proven to be sensitive enough to detect moderate hypertension (i.e. ~5-10mmHg) (Chapter 5). Additionally, in young SHR, a rightward shift in this acute intrarenal mechanism coincided with an upregulation of structurally-based vascular resistance properties, thereby suggesting that there is a
probable mechanistic and temporal link between the alterations in vascular structure and renal function (Chapter 3). The rapid response time of RIHP further supports this theory, as many slower acting neurohumoral systems are not able to function within this timeframe. Together, these findings add to the notion that the AP operating point of an organism is set by the kidney (i.e. its structurally-based vascular resistance properties), as was previously demonstrated by renal cross-transplantation studies.\textsuperscript{42, 44, 280, 298}

Conversely, in salt-sensitivity, the pressure-natriuresis curve appears to be blunted (i.e. there is an exaggerated AP responsiveness to changes in sodium balance) (Chapter 1.3.1)\textsuperscript{10, 11}. We demonstrate that salt-sensitivity is also reflected in the moment-to-moment functioning of RIHP (Chapters 4 and 5). The blunting of this intrarenal hemodynamic process is also corroborated by long-term salt challenge experiments in both the ANP \(-/-\) mouse model\textsuperscript{186, 187} (Chapter 4) and the adult PID rat (Chapter 5). Particularly of interest, following an assessment of pressor versus depressor responses in the acute RAP-RIHP relationship, there appears to be a selective blunting of the pressor component in ANP \(-/-\). These data are consistent with previous findings that ANP is able to sensitize pressure-natriuresis at high, but not low, pressures.\textsuperscript{98} The specific mechanism(s) responsible for this blunting in ANP \(-/-\) are unknown, however it has been proposed that a decrease in responsiveness of RIHP may be associated with alterations in the compliance of the renal interstitium.\textsuperscript{270} Renal interstitial compliance is affected in three ways: 1) changes in the gel matrix of the interstitium; 2) the elasticity of the renal capsule; and 3) volume expansion.\textsuperscript{66} Given that we find an elevated baseline RIHP in the ANP \(-/-\), as well as hypervolemia and a lower hematocrit, the latter scenario appears to be likely. Further, taking into consideration the finding that renal decapsulation in the
young SHR results in a blunting of the moment-to-moment RAP-RIHP relationship (Chapter 3), an alternative explanation may be that there are changes in the elasticity of the capsule in ANP -/-; although, this is yet to be determined.

Many neurohumoral factors are known to modulate sodium balance, yet the precise mechanisms by which these factors may affect pressure-natriuresis, particularly in the short-term, have not been fully resolved\textsuperscript{18}. In Chapter 2, inhibition of the ANS or RAS did not modify the overall slope of the moment-to-moment RAP-RIHP relationship or the time course of RIHP responses to changes in RAP, despite the decrease in AP. However, unlike in untreated animals, the pressor and depressor slopes became similar following these treatments. The reason for these differences may be due to the fact that the acute antihypertensive effects of RAS and ANS inhibition simply shifted the AP operating point further down the acute RAP-RIHP curve (Figure 2.4C). As a result of this, the equivalent pressor and depressor slopes may reflect a composite of the pressor and depressor arms in controls (i.e. increased pressor and decreased depressor slopes). Thus, it appears that short-term manipulation of the RAS and ANS does not influence the moment-to-moment functioning of the RAP-RIHP relationship, and further supports the theory that this intrarenal mechanism is primarily a vascular phenomenon.

Conversely, it is well known that long-term aberrations in the functioning of certain neurohumoral factors can induce structural changes (Chapters 1.4 and 1.5). Given that in the ANP -/- mice there is a permanent removal of ANP, the rightward shift in the moment-to-moment functioning of the RAP-RIHP relationship may be explained by altered renal vascular structure in these animals. Although this was not directly studied, it is well known that ANP -/- mice have high circulating catecholamine levels\textsuperscript{191}, thus this
imbalance in pressor (trophic) and depressor (anti-trophic) systems may result in an upregulated renal vascular structure\textsuperscript{136, 137}. However, this is speculation and therefore further experimentation is required to deduce the mechanisms responsible for the rightward shift in pressure-natriuresis in this animal model. Interestingly, it has previously been shown that following long-term RAS inhibition in adult SHR, the pressure-natriuresis mechanism is persistently shifted leftward upon cessation of therapy\textsuperscript{50, 91}. Furthermore, studies conducted in our laboratory\textsuperscript{280, 298} demonstrate that RAS inhibition in adult SHR results in a persistent lowering of AP, and transplantation of a treated kidney into an untreated SHR results in a similar persistent lowering of AP. Together, these findings suggest that long-term changes in certain neurohumoral factors can induce changes in renal structurally-based vascular resistance properties, and thereby alter the AP operating point of the organism.

Furthermore, studies on various rodent models demonstrated that if the production or actions of Ang II are either blocked pharmacologically or via genetic manipulation during nephrogenesis, then the animals display anatomical and functional renal abnormalities\textsuperscript{84}, including a blunting of the pressure-natriuresis mechanism\textsuperscript{85}. In light of this evidence, it may be postulated that the blunting in the RAP-RIHP relationship in the ANP -/- mice may be due to the permanent removal of ANP throughout critical periods of fetal and postnatal development (i.e. nephrogenesis); however, this is yet to be determined.

In addition to genetic influences, it should be mentioned that environmental influences, especially during critical periods in early life development can also play a role in impairing pressure-natriuresis. In line with the concept of DOHaD (Chapter 1.6.2.1), it
is well known that maternal and fetal environmental stressors (e.g. nutrition) can program the offspring to be susceptible to hypertension in adult life\textsuperscript{166, 197, 198, 229, 230}. Indeed, we demonstrate that PID, which is induced within the period of nephrogenesis (gestational day 12 to postnatal days 7-10\textsuperscript{84}), results in moderate hypertension, increased AP responsiveness to dietary sodium intake, and a blunting in the acute RAP-RIHP relationship in adulthood (Chapter 5). Although the mechanisms by which PID induces salt-sensitivity are not entirely clear, and thus further investigation is warranted.

7.2 Role of Visceral Adiposity on the Functioning of Pressure-Natriuresis

In addition to salt-sensitivity, we demonstrate that PID animals have 2 phases of catch-up growth (Chapter 5). Interestingly, in humans and animal models where the maternal and fetal insult results in altered growth trajectories (i.e. low birth weight and catch-up growth), there appears to be an increased risk of developing metabolic syndrome (i.e. hypertension, glucose intolerance, insulin resistance, dyslipidemia, and increased fat accumulation)\textsuperscript{166, 197, 198, 229, 230}. Given that obesity, specifically of the visceral type, is the proposed culprit in the development of this syndrome\textsuperscript{231}, it was of interest to assess whether PID offspring are also predisposed to visceral adiposity in adulthood. Indeed, we demonstrated that PID results in increased visceral adiposity, in part, due to programmed sedentary behavior. Interestingly, this increase in adiposity was associated with enhanced AP responsiveness to dietary sodium (Chapter 6). While the mechanisms by which PID predisposes the offspring to visceral adiposity are unknown, it is well known that obesity is associated with hypertension. Risk estimates from the Framingham Study suggest that approximately 65-78% of hypertension may be directly attributed to excess adiposity\textsuperscript{232}. Additionally, a number of studies suggest that
abdominal obesity, or an increase in visceral adipose tissue (VAT), is more closely associated with the presence of hypertension than total or peripheral obesity. It has been proposed that the central feature of obesity-associated hypertension is related to alterations in proper sodium handling via abnormalities in key AP regulating systems, such as the SNS, RAS, ANP, and of course pressure-natriuresis. However, the specific mechanisms by which VAT contributes to hypertension and salt-sensitivity are not completely understood. Certainly, there is a need for more basic research, particularly using animal models, to ascertain the time course of VAT accumulation, as well as VAT-specific mechanisms involved in the pathogenesis of hypertension and salt-sensitivity. Currently there is a lack of adequate tools available to conduct *in vivo* longitudinal assessments of VAT in animal models. Thus, we developed two techniques (i.e. water-suppressed MRI and abdominal girth), which offer reliable, non-invasive, non-terminal and rapid means of measuring VAT (Appendix 1).

**7.3 Summary of Findings and Future Perspectives**

The studies presented herein demonstrate that the moment-to-moment relationship between RAP and RIHP, which is linked to downstream mechanisms regulating sodium excretion, has short-term baroreflex-like properties that may facilitate the regulation of AP around an operating point. Furthermore, it appears that aberrant functioning of this intrarenal mechanism predicts the hypertensive and/or salt-sensitive phenotype. Specifically, there can be a shift along the x-axis and/or a change in the responsiveness of RIHP to changes in RAP (i.e. slope) (Summarized in Table 7.1). The hypertensive phenotype typically has a rightward shift along the x-axis in the moment-to-moment RAP-RIHP relationship towards greater RAP, and this change appears to be primarily of
### Table 7.1: Summary of main findings with respect to the functioning of the acute RAP-RIHP relationship in Chapters 2-6.

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Moment-to-Moment RAP-RIHP Relationship</th>
<th>Potential Mechanism(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adult Wistar Rat</strong>&lt;br&gt; (normotensive)&lt;br&gt; Control vs. Losartan-treated and Hexamethonium-treated</td>
<td>Pressor vs. Depressor Slope&lt;br&gt;Changes in RAP (i.e. shift along x-axis)</td>
<td>Myogenic response following pressor stimuli in control (?)&lt;br&gt;Antihypertensives shift the operating point down the moment-to-moment RAP-RIHP curve of controls, thus no changes in pressor and depressor slopes</td>
</tr>
<tr>
<td><strong>Young Rats (pre-hypertensive)</strong>&lt;br&gt; Spontaneously hypertensive rats (SHR) vs. Wistar-Kyoto rats (WKY)</td>
<td>Pressor slope steeply than depressor slope in both strains&lt;br&gt;Rightward shift towards greater RAP in SHR vs. WKY</td>
<td>Rightward shift may be due to increase renal structurally-based vascular resistance properties (?)&lt;br&gt;Renal capsule maintains RIHP in SHR to levels of WKY</td>
</tr>
<tr>
<td><strong>Adult ANP -/- Mice</strong>&lt;br&gt; (salt-sensitive and hypertensive)&lt;br&gt; ANP -/- vs. ANP +/-</td>
<td>Pressor slope in ANP-/- blunted vs. ANP +/-&lt;br&gt;Depressor slopes are similar&lt;br&gt;Rightward shift towards greater RAP in ANP-/- vs. ANP+/-</td>
<td>Rightward shift due to increased renal structurally-based vascular resistance properties (?)&lt;br&gt;Hypervolumia elevates RIHP in ANP-/-&lt;br&gt;Slope is blunted due to lack of ANP</td>
</tr>
<tr>
<td><strong>Adult Perinatal Iron-Deficient (PID) Rats</strong>&lt;br&gt; (salt-sensitive and moderately hypertensive)&lt;br&gt;Control vs. PID</td>
<td>Both pressor and depressor slopes are blunted in PID vs. controls&lt;br&gt;Moderate rightward shift towards greater RAP in PID vs. controls</td>
<td>Rightward shift due to increased renal structurally-based vascular resistance properties (?)&lt;br&gt;Blunted slope due to visceral adipose tissue and changes in neurohumoral systems (?)&lt;br&gt;Moment-to-moment profiles reflect long-term renal function curves</td>
</tr>
</tbody>
</table>
vascular origin (i.e. due to structural changes). Conversely, a blunting in the slope of this acute mechanism is characteristic of the salt-sensitive phenotype, and appears to be primarily influenced by an imbalance of key blood pressure modulating factors (e.g. due to functional changes resulting from vasoactive systems [e.g. ANP]) and/or changes in renal interstitial compliance (e.g. renal capsule or volume status of animal).

These findings provide a new perspective on how the pressure-natriuresis mechanism, specifically the RAP-RIHP relationship, functions over a short-term timeframe. Pressure-natriuresis has been regarded as a long-term controller of AP; however, the findings from the studies presented herein support the hypothesis that the long-term character of the pressure-natriuresis mechanism is established, in part, via cumulative moment-to-moment interactions between RAP and RIHP. Further, the responsiveness of RIHP depends on the AP operating point, the phenotype, and whether there are vascular structural, renal interstitial compliance or neurohumoral changes in the organism (Summarized in Table 7.1). Future studies are warranted to expand on these findings now that a novel methodology to study the moment-to-moment functioning of this key intrarenal mechanism is available (e.g. how other vasoactive factors and antihypertensive agents can influence this important intermediate component of pressure-natriuresis). For example, infusion of pharmacological agents into the renal interstitium can aid in unraveling which blood pressure control systems act to modulate the slope of the acute RAP-RIHP relationship above and below the AP operating point (e.g. infusion of a calcium channel blocker into the renal interstitium to examine whether the myogenic response is responsible for the blunted slope in the acute RAP-RIHP relationship following pressor stimuli). Additionally, power spectrum analysis could be conducted on
the data set already collected through these moment-to-moment studies to characterize and assess the role of various buffering systems involved in regulating AP variability. That is, via power spectrum analysis it is possible to examine the buffering capacity and frequency ranges within which various control systems may impact on the moment-to-moment functioning of RIHP and RAP. Lastly, concomitant risk factors for hypertension and salt-sensitivity, such as visceral obesity, appear to influence AP responsiveness to sodium intake, and therefore require further investigation with respect to how these risk factors may influence the moment-to-moment RAP-RIHP relationship.
REFERENCES


APPENDIX 1

*In Vivo* Assessment of Visceral Adipose Tissue in Rats Using Water Suppressed MRI and Abdominal Girth

Abstract:

**Background:** While accumulation of visceral adipose tissue (VAT) has been linked to morbidity and mortality, little is known about the role of VAT in pathogenesis of disease. Development of tools that allow researchers to conduct rapid *in vivo* longitudinal assessments of VAT in response to growth, aging, disease progression, and interventions is warranted.

**Objective:** The study herein sought to develop and evaluate techniques that allow for practical, non-invasive, non-terminal, cost-effective and rapid quantification of VAT.

**Methods:** Two tools were developed, specifically water suppressed MRI (using a 3-Tesla scanner) and abdominal girth. MRI data were analyzed by: i) conventional threshold determination from histograms, and ii) calibration from water/peanut oil emulsions. Girth was assessed in conscious and anesthetized rats. VAT estimates from these methods were compared to dissected VAT (sum of retroperitoneal, omental, mesenteric, and epididymal fat pads). Additionally, different rat strains (Sprague-Dawley, Spontaneously hypertensive rat obese (SHROB), and Wistar), ages (30-36wks and 50wks), and diets (*ad libitum* and caloric restriction) were used (total n=82).

**Results:** MRI-based determination of VAT strongly associated (r=0.95-0.97) and agreed (ρ=0.91-0.93) with dissected VAT (P<0.0001), despite estimating higher VATs than excised. The calibrated approach was superior in estimating a wide range of VATs over the conventional, which tended to underestimate VAT in leaner rats. With respect to abdominal girth, intra- and inter-observer comparisons revealed minimal bias (<6%), and excellent degrees of agreement and association (P<0.0001). Despite significant differences in various physical characteristics among the rats studied, there was no
significant effect of strain and age on the relationship between girth and excised VAT, where girth accounted for 74% of the variance in VAT (P<0.0001).

**Conclusion:** Both MRI and abdominal girth demonstrate a strong relationship with dissected VAT, thereby offering reliable, non-invasive, non-terminal and rapid tools for researchers to conduct *in vivo* longitudinal assessments of VAT in animal models.
Introduction:

Abdominal obesity, specifically accumulation of visceral adipose tissue (VAT), is strongly associated with morbidity and mortality risk\(^1\). Despite this recognition, there is still insufficient information regarding the processes that link VAT with disease. Certainly, there is a need for more basic research, particularly using animal models, to ascertain the time course of VAT accumulation, as well as VAT-specific mechanisms involved in the pathogenesis of disease.

Currently, the only published methodologies for quantifying VAT in animal models involve the direct surgical removal and measurement of visceral fat pads\(^2\)-\(^4\) and the indirect estimation of VAT via imaging techniques (i.e. computed tomography (CT) and magnetic resonance imaging (MRI))\(^2, 3, 5-7\). All of these techniques have inherent limitations, especially with respect to animal studies. Surgical excision of VAT is time-consuming and terminal, thus making longitudinal assessments of VAT not possible. Imaging techniques are expensive, require trained technicians, can be time-consuming (10-30 minutes per animal for data acquisition), and use anesthesia to immobilize the animals\(^2, 3, 5, 6, 8\). Use of anesthesia, even intermittently, can reduce food intake and eventually alter body composition, especially in longitudinal studies requiring repeated assessments\(^8, 9\). Furthermore, although it is not known whether radiation exposure with CT affects body composition and health, it is certain that this pitfall imposes restrictions on the frequency of measurements with this technology\(^10\).

Conversely, in addition to the long data acquisition times and anesthesia of animals, previous MRI estimation of VAT was limited to the standard T\(_1\)-weighted imaging protocol\(^2, 3, 5, 6\), which suffers from low lipid:water contrast, use of arbitrary
thresholding techniques for fat segmentation, and motion artifacts from cardiac, respiratory and bowel motion\textsuperscript{11}. All of these issues can result in poor image quality and hamper proper estimation of VAT. To improve on fat segmentation techniques, a recent study used localized proton magnetic resonance spectroscopy (MRS) to evaluate lipid:water content from a standardized, manually-selected intra-abdominal region of interest (ROI)\textsuperscript{7}. However, MRS can be compromised by the ROI selection (i.e. shape, size, and potential contamination by surrounding tissues), long acquisition times, low spatial resolution, and field inhomogeneity\textsuperscript{8,12}. MR relaxometry has also been used to separate water and lipid content less ambiguously in biological tissue according to the different T\textsubscript{2} and /or T\textsubscript{1} relaxation times\textsuperscript{8}. Although MR relaxometry allows for rapid data acquisition in conscious rodents, this technique has only been used for quantification of total, not regional, body fat composition\textsuperscript{8}. With advancements in MRI technology, researchers have obtained fat-only intra-abdominal images in humans using water suppression/saturation protocols, which have rapid imaging times, and therefore are less sensitive to motion artifacts, as well as provide improved lipid:water contrast, thus making fat quantification much easier and more reliable\textsuperscript{11,13}. Surprisingly water suppression protocols have not yet been implemented in animal studies, and thus it was an objective of the current study.

In human studies, anthropometric measurements, such as BMI and waist circumference (WC), are often used as indices of obesity with WC being by far the most widely used index of regional adipose tissue distribution (i.e. abdominal obesity)\textsuperscript{14-16}. In fact, clinically, WC accounts for approximately 77-94\% of the variance in VAT quantified by reliable imaging techniques (e.g. CT and MRI)\textsuperscript{14,17}. Given this strong
association between WC and VAT in humans, as well as the rapidity, ease and cost effectiveness of this method, it was of interest to determine whether measurements of abdominal girth could also predict VAT in rats.

There is a clear need for practical, non-invasive, non-terminal, cost-effective and rapid methodologies that allow for longitudinal assessment of VAT following experimental manipulations in animal models. In the present study, two indirect methods were developed and compared for rapid in vivo estimation of VAT in rats, specifically water suppressed MRI and physical measurements (e.g. abdominal girth, which is equivalent to WC in humans). VAT measurements from these indirect methodologies were compared to those obtained from the direct surgical excision of VAT.
Methods:

Animals:

Male rats were obtained from Charles River Laboratories. Rats were housed individually (21±1°C; 12hr light/dark cycle) and acclimatized for at least 96 hours prior to experimentation. All rats were provided with water and standard rat chow (Purina LabDiet 5001) *ad libitum*, unless otherwise specified. All procedures followed guidelines of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee.

Groups:

**Group 1: Sprague-Dawley rats (30-35 weeks old; n=48)**

There were three subgroups within this group: i) control *ad libitum* group (n=27), ii) aggressive calorically restricted (i.e. calorically restricted (CR) from 10 weeks of age to maintain body weight at 10 week level; n=15), and iii) moderate CR (i.e. from 10 weeks of age CR to maintain body weight between *ad libitum* and aggressive CR groups; n=6). Sprague-Dawley rats were studied because they are a widely used strain of rats in various research areas. Rats were CR to study a wide range of VAT amounts without adding other potential confounders (e.g. age).

**Group 2: Spontaneously Hypertensive Rats Obese (SHROB; 30-35 weeks old; n = 19)**

There were two subgroups within this group: i) control *ad libitum* group (n=10), and ii) aggressive CR (i.e. CR from 10 weeks of age to maintain body weight at 10 week level; n=9). SHROBs were used to assess whether genetic predisposition to obesity (i.e. mutation of the leptin receptor gene) affects the variables studied.

**Group 3: Wistar rats (36 weeks old; n=7)**
Wistar rats were studied to determine the effects of another commonly used strain of rats on the variables studied.

**Group 4: Sprague-Dawley rats (50 weeks old; n = 8)**

To study the effects of age on the variables, *ad libitum* fed Sprague-Dawley rats at 50 weeks of age were compared to those at 30-35 weeks of age from *Group 1*.

**MRI Data Acquisition:**

In approximately half the rats from *Groups 1* (n=25) and 2 (n=9), MRI images were acquired using a Siemens Magnetom Tria 3-Tesla MRI scanner fitted with a twelve-channel head matrix coil. Prior to imaging, the rats were fasted overnight to remove potential fat signals from the food in the bowel. For imaging, conscious rats were restrained in Plexiglas tubes with holes for air circulation (Harvard Apparatus) and placed into cage units fitted with HEPA filter tops. After running a localizer sequence, half-Fourier acquisition single-shot turbo spin-echo (HASTE) normal and HASTE water suppressed sequences with a 403 ms repetition time (TR), 33 ms echo time (TE), and 119° flip angle were used for all acquisitions. The image matrix was 128 x 128 within a 128-mm field of view giving 1-mm pixels. Sixty transverse slices (2mm in thickness) were acquired over the entire length of the animal, excluding the tail. There was a 1-mm inter-slice gap to prevent signal crossover from adjacent sections. Rat restraining and imaging time was 2-5 minutes per rat. To confirm water suppression, two plastic tubes were filled to capacity, one with peanut oil and the other with water, and placed on either side of the rat during data acquisition (Figure 1A).
Figure 1: A: Transverse magnetic resonance images (MRI) acquired from the abdominal region (level of the kidneys) of male Sprague-Dawley rat using a Siemens 3-Tesla scanner. Half-Fourier acquisition single-shot turbo spin-echo (HASTE) normal (left) and HASTE water suppressed sequences (middle) were used for data acquisition. Fat and water are represented by the bright pixels in the HASTE normal scan, which are confirmed by the water- and oil-filled control tubes (on the left and right of the rat, respectively). Water suppression is confirmed in the HASTE water suppressed scan by the disappearance of the water-filled tube. Visceral adipose tissue (VAT) is defined as the bright area outlined by a muscular wall, where manual selection of the region of interest allows for VAT segmentation (right). B: Calibration plot derived from a HASTE water suppressed scan of peanut oil in water emulsion phantoms (12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100% peanut oil). The equation was derived from linear regression analysis of three consistently chosen points on each phantom. The equation was used to determine the percentage of fat in each pixel based on the pixel intensity (arbitrary unit). C: A schematic illustrating where abdominal girth and length measurements were obtained on each rat. Abdominal girth is the midpoint between the tip of the sternum and the top of the iliac crest, and length is the distance from the tip of the nose to the anus.
**MRI analysis:**

Image analysis was performed by a blinded investigator using semiautomatic and interactive segmentation algorithms developed for Image-Pro Plus 6.0 (Media Cybernetics). Segmentation of VAT required manual selection of the region of interest (ROI) from HASTE water suppressed images. Specifically, slices from the diaphragm to the top of testes were visually determined and used. VAT was defined by the bright area outlined by a muscular wall consisting of the abdominal, oblique, and back muscles (Figure 1A). The HASTE normal images aided in confirming the anatomy, VAT segmentation, and water suppression in the HASTE water suppressed images, especially in lean rats where the adipose tissue signal tends to be less intense.

Segmentation of adipose tissue pixels was conducted by two methods:  i) the *conventional approach* of using a threshold value determined from an intensity histogram of the ROI (usually pixel intensities of 100+ arbitrary units)\(^2,3,5,11,13,18\), and ii) a novel *calibrated approach* using water/peanut oil emulsion phantoms. More specifically, the conventional approach designates a threshold from the intensity histogram of the water suppressed ROI as the nadir between the low intensity first peak, representing the muscle and background pixel intensities, and the higher intensity peak, representing the fat pixel intensities\(^2,3,5,11,13,18\). Conversely, the calibrated approach uses an equation (pixel intensity = 2.89 x % fat – 38.7, r=0.98) derived from the water suppressed images of tubes containing water/peanut oil emulsions (0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100% peanut oil) to determine the percentage of fat in each pixel based on the pixel intensity and the corresponding percentage of fat from the emulsions (Figure 1B). VAT volume (cm\(^3\)) was determined from the sum of all adipose tissue pixels multiplied by the
slice thickness. To determine adipose tissue weight, the total volume was multiplied by the average adipose tissue density of 0.9255g/ml. Inter-technique variability was measured between the conventional and calibrated approaches.

**Body Measurements:**

Body weights, naso-anal lengths and abdominal girths (measured at the midpoint between tip of the sternum and the top of iliac crest) were assessed in all rats (i.e. Groups 1-4). Naso-anal lengths were determined in prone position from conscious restrained rats and supine position in anesthetized rats (Figure 1C). Abdominal girth was determined in an upright position in conscious restrained rats, and in supine and upright positions in anesthetized rats (Figure 1C). All measurements were done in triplicates by two investigators and typically done in less than 5 minutes per animal. Intra-observer variability was measured from the first two measurements by the most experienced investigator. Inter-observer variability was measured between the average values of two investigators. Inter-technique variability was measured between the three different abdominal girth measurements and the two length measurements.

**Direct Fat Pad Assessment:**

Following anesthesia with sodium pentobarbital (120 mg/kg i.p.) and exsanguination, retroperitoneal, mesenteric, omental, and epididymal fat pads were dissected and weighed from all rats. Retroperitoneal fat was defined as the fat surrounding the kidneys, abdominal aorta and vena cava, and the spinal muscles, which run from the diaphragm down as far as the inguinal region. Mesenteric and omental fat was defined as the fat attached to the mesentery and omentum, respectively. Epididymal
fat was separated from the epididymis, epididymal vessels, and vas deferens. The sum of these major abdominal fat pads was considered total VAT.

**Statistics:**

All statistical calculations were performed and graphs constructed using GraphPad Prism and/or Microsoft Excel. Pearson’s correlation coefficient (r) was used to assess the degree of association between variables. Lin’s concordance correlation coefficient (p-c) and Bland-Altman method were used to evaluate the degree of agreement on continuous measurements obtained by the most experienced investigator (intra-observer variability), two investigators (inter-observer variability), or two methods (inter-technique variability). The Lin’s concordance correlation coefficient evaluates the degree of agreement between two variables by taking into account the precision (i.e. Pearson’s correlation coefficient, r) and the accuracy (i.e. deviation of the line of best fit from the 45° line through origin) of the data. Like the Pearson’s correlation coefficient, the Lin’s concordance correlation coefficient ranges from zero (no agreement) to one (perfect agreement). The Bland-Altman method was used to assess the bias (i.e. overestimation or underestimation) between variables, as well as the limits of agreement at 95%. Slopes and intercepts of linear regression models were tested for significant differences from the identity line (y=x). Linear regression analysis was also used to generate a predictive equation for VAT from abdominal girth measurements. Statistical significance was determined by either a Student’s t-test or one-way ANOVA, where appropriate. Data were presented as means ± s.e.m. P< 0.05 was considered statistically significant.
Results:

*Characteristics of rats used in intra-observer, inter-observer, and inter-technique comparisons*

Physical characteristics of Sprague-Dawley and SHROB rats at 30-35 weeks of age are presented in Table 1. With two exceptions (i.e. percent VAT relative to body weight and naso-anal length measured in conscious rats between the *ad libitum* and moderate CR rats), there was a significant difference in all variables between the subgroups (i.e. *ad libitum* > moderate CR > aggressive CR) in the Sprague-Dawley group (P<0.01). Of particular interest, VAT of *ad libitum* fed rats was ~1.5-fold and 4-fold greater than the moderate and aggressive CR rats, respectively. Correspondingly, abdominal girth of *ad libitum* fed rats was 11% and 27% larger than the moderate and aggressive CR rats, respectively. In the SHROB group, there were significant differences in all variables between *ad libitum* fed and aggressive CR rats (P<0.05), except for percent VAT relative to body weight. VAT was 27% greater and abdominal girth was 10% larger in the *ad libitum* versus the aggressive CR rats.

*Intra-observer and inter-observer variability*

Comparison of intra-observer (i.e. most experienced investigator) and inter-observer measurements via the Bland-Altman method, Lin’s concordance correlation, and Pearson’s correlation are presented in Supplement Tables 1 (Sprague-Dawley) and 2 (SHROB). Briefly, there was a minimal degree of bias (0.01- 0.2%) in the first versus second measurement of abdominal girth and length made by the most experienced investigator, with the exception of the conscious length measurement in the SHROBs (1.3±4.6%; likely due to differences in restraint between investigators).
Table 1: Physical characteristics of male Sprague-Dawley and SHROB rats at 30-35 weeks of age fed *ad libitum* and calorically restricted (moderately and/or aggressively).

<table>
<thead>
<tr>
<th></th>
<th>Sprague-Dawley</th>
<th>SHROB</th>
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<tr>
<td></td>
<td><em>Ad Libitum</em></td>
<td>Moderate CR</td>
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<tr>
<td>Body Weight (g)</td>
<td>673.7±12.0</td>
<td>533.7±18.5***</td>
</tr>
<tr>
<td>VAT (g)</td>
<td>44.1±2.6</td>
<td>29.6±4.0**</td>
</tr>
<tr>
<td>VAT/Body Weight (%)</td>
<td>6.5±0.3</td>
<td>5.5±0.7**</td>
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<tr>
<td>Abdominal Girth (cm)</td>
<td></td>
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<tr>
<td><em>Anesthetized (supine)</em></td>
<td>22.5±0.3</td>
<td>20.2±0.4***</td>
</tr>
<tr>
<td><em>Anesthetized (upright)</em></td>
<td>21.3±0.5</td>
<td>18.6±0.3***</td>
</tr>
<tr>
<td><em>Conscious (upright)</em></td>
<td>21.9±0.4</td>
<td>19.6±0.5***</td>
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<tr>
<td>Length (cm)</td>
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<tr>
<td><em>Anesthetized (supine)</em></td>
<td>27.6±0.1</td>
<td>26.6±0.3***</td>
</tr>
<tr>
<td><em>Conscious (prone)</em></td>
<td>27.3±0.2</td>
<td>26.8±0.3</td>
</tr>
<tr>
<td>Body Weight/Length (kg/m)</td>
<td>2.5±0.1</td>
<td>2.0±0.1***</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>8.9±0.1</td>
<td>7.6±0.1***</td>
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</table>

SHROB, spontaneously hypertensive rat obese; CR, caloric restriction; VAT, visceral adipose tissue; BMI, body mass index. *P<0.05, **P<0.01, and ***P<0.0001 vs. *ad libitum*. ††P<0.01 and †††P<0.0001 vs. moderate CR. Values are mean ± s.e.m.
Supplement Table 1: Comparison of intra-observer, inter-observer, and inter-technique measurements in male Sprague-Dawley rats at 30-35 weeks of age using the Bland-Altman method, and Lin’s concordance and Pearson’s correlation coefficients.

<table>
<thead>
<tr>
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<th>Bland-Altman</th>
<th>Lin’s concordance</th>
<th>Pearson’s</th>
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<tbody>
<tr>
<td></td>
<td>Bias±SD (%)</td>
<td>95% limits of agreement</td>
<td>ρ-c</td>
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<tr>
<td><strong>Intra-observer</strong></td>
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<tr>
<td>Abdominal Girth</td>
<td></td>
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<tr>
<td>A. Anesthetized (supine)</td>
<td>-0.02±0.82</td>
<td>-1.62, 1.58</td>
<td>0.998</td>
</tr>
<tr>
<td>B. Anesthetized (upright)</td>
<td>-0.17±1.20</td>
<td>-2.52, 2.18</td>
<td>0.997</td>
</tr>
<tr>
<td>C. Conscious (upright)</td>
<td>-0.06±0.86</td>
<td>-1.76, 1.64</td>
<td>0.998</td>
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<tr>
<td>Length</td>
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<tr>
<td>A. Anesthetized (supine)</td>
<td>0.01±0.82</td>
<td>-1.60, 1.62</td>
<td>0.979</td>
</tr>
<tr>
<td>B. Conscious (prone)</td>
<td>-0.12±0.55</td>
<td>-1.21, 0.96</td>
<td>0.993</td>
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<tr>
<td><strong>Inter-observer</strong></td>
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<tr>
<td>Abdominal Girth</td>
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<tr>
<td>A.</td>
<td>1.61±2.85</td>
<td>-3.98, 7.20</td>
<td>0.963</td>
</tr>
<tr>
<td>B.</td>
<td>4.08±3.21</td>
<td>-2.21, 10.40</td>
<td>0.940</td>
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<tr>
<td>C.</td>
<td>5.44±3.70</td>
<td>-1.81, 12.70</td>
<td>0.897</td>
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<td>Length</td>
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<tr>
<td>A.</td>
<td>1.48±1.58</td>
<td>-1.62, 4.58</td>
<td>0.849</td>
</tr>
<tr>
<td>B.</td>
<td>-1.02±2.00</td>
<td>-4.94, 2.90</td>
<td>0.923</td>
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<tr>
<td><strong>Inter-technique</strong></td>
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<tr>
<td>Abdominal Girth</td>
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<tr>
<td>A vs. B</td>
<td>-6.57±2.55</td>
<td>-11.60, 1.57</td>
<td>0.893</td>
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<tr>
<td>A vs. C</td>
<td>-3.35±3.08</td>
<td>-9.39, 2.68</td>
<td>0.939</td>
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<tr>
<td>B vs. C</td>
<td>-3.19±3.33</td>
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<td>0.952</td>
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<tr>
<td>Length</td>
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<tr>
<td>A vs. B</td>
<td>-1.82±3.19</td>
<td>-8.08, 4.44</td>
<td>0.729</td>
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<tr>
<td>MRI</td>
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<tr>
<td>Calibrated vs. Conventional</td>
<td>-11.1±14.1</td>
<td>-38.7, 16.4</td>
<td>0.989</td>
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SD, Standard deviation; CI, Confidence interval.
**Supplement Table 2:** Comparison of intra-observer, inter-observer, and inter-technique measurements in male SHROB rats at 30-35 weeks of age using the Bland-Altman method, and Lin’s concordance and Pearson’s correlation coefficients.

<table>
<thead>
<tr>
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<th>Lin’s concordance</th>
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<tr>
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<td>Bias±SD (% mean difference)</td>
<td>95% limits of agreement</td>
<td>ρ-c</td>
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<tr>
<td><strong>Intra-observer</strong></td>
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<tr>
<td>Abdominal Girth</td>
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<tr>
<td>D. Anesthetized (supine)</td>
<td>0.08±0.78</td>
<td>-1.45, 1.61</td>
<td>0.991</td>
</tr>
<tr>
<td>E. Anesthetized (upright)</td>
<td>-0.11±0.74</td>
<td>-1.57, 1.35</td>
<td>0.996</td>
</tr>
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<td>F. Conscious (upright)</td>
<td>-0.05±0.62</td>
<td>-1.26, 1.17</td>
<td>0.994</td>
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<tr>
<td><strong>Length</strong></td>
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<tr>
<td>C. Anesthetized (supine)</td>
<td>-0.16±0.99</td>
<td>-2.11, 1.79</td>
<td>0.966</td>
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<tr>
<td>D. Conscious (prone)</td>
<td>1.25±4.58</td>
<td>-7.74, 10.20</td>
<td>0.467</td>
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<td><strong>Inter-observer</strong></td>
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<tr>
<td>Abdominal Girth</td>
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<tr>
<td>D.</td>
<td>3.70±2.74</td>
<td>-1.68, 9.08</td>
<td>0.782</td>
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<tr>
<td>E.</td>
<td>4.52±4.46</td>
<td>-4.22, 13.30</td>
<td>0.741</td>
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<tr>
<td>F.</td>
<td>4.63±2.71</td>
<td>-0.69, 9.94</td>
<td>0.637</td>
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<td><strong>Length</strong></td>
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<tr>
<td>C.</td>
<td>1.52±1.36</td>
<td>-1.15, 4.19</td>
<td>0.851</td>
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<tr>
<td>D.</td>
<td>-1.20±2.89</td>
<td>-6.85, 4.46</td>
<td>0.638</td>
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<tr>
<td><strong>Inter-technique</strong></td>
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<tr>
<td>Abdominal Girth</td>
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<tr>
<td>A vs. B</td>
<td>-8.28±3.54</td>
<td>15.20, 1.34</td>
<td>0.530</td>
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<tr>
<td>A vs. C</td>
<td>-7.60±3.45</td>
<td>14.40, 0.84</td>
<td>0.442</td>
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<tr>
<td>B vs. C</td>
<td>-0.69±4.79</td>
<td>-10.10, 8.71</td>
<td>0.741</td>
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<tr>
<td><strong>Length</strong></td>
<td></td>
<td></td>
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<tr>
<td>A vs. B</td>
<td>0.11±2.09</td>
<td>-4.00, 4.21</td>
<td>0.805</td>
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<tr>
<td><strong>MRI</strong></td>
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<tr>
<td>Calibrated vs. Conventional</td>
<td>12.4±1.4</td>
<td>9.8, 15.1</td>
<td>0.669</td>
</tr>
</tbody>
</table>

SHROB, spontaneously hypertensive rat obese; SD, Standard deviation; CI, Confidence interval.
The degree of agreement ($\rho_{c}=0.97-1.00$) and association ($r=0.97-1.00$) for all measurements, except for conscious length measurements in the SHROBs, was excellent. Comparison of the inter-observer measurements revealed that the least amount of bias and highest degree of agreement was among the abdominal girth measurements conducted in anesthetized rats in the supine position in both Sprague-Dawley and SHROB rats. Further, although there was the least amount of bias in the conscious versus anesthetized length measurement in both strains, the difference was marginal (i.e. within 0.5%). In both conscious and anesthetized length measurements of the Sprague-Dawleys, there was substantial agreement ($\rho_{c}=0.99$ and 0.98, respectively) and association ($r=0.99$ and 0.98, respectively); in SHROBs the agreement ($\rho_{c}=0.85$) was still reasonable. Even with a less experienced investigator, the error in assessment of abdominal girths was less than 5.4% and length was less than 1.5%.

Inter-technique variability

Inter-technique comparisons were conducted for abdominal girth and length measurements, as well as VAT quantification using the conventional and calibrated MRI analysis approaches in Sprague-Dawley (Supplement Table 1) and SHROB (Supplement Table 2) rats. Given that there were marginal differences in the range of VAT weights between the *ad libitum* and aggressive CR SHROB versus Sprague-Dawley rats, inter-technique comparisons were also conducted by pooling all the data from both strains (Figure 2). Within the abdominal girth measurements, there was 1.5±4.0% bias, and an excellent degree of agreement ($\rho_{c}=0.92$) and association ($r=0.94$) between the anesthetized/supine and conscious/upright measurements (Figure 2A). Comparisons with anesthetized/upright measurements were not done as a major disadvantage to this
Figure 2: Comparison of inter-technique measurements in male Sprague-Dawley and SHROB rats at 30-35 weeks of age fed ad libitum and calorically restricted. Pearson’s correlation plot (left) demonstrating the degree of association, and Lin’s concordance correlation plot (left) and Bland-Altman plot (right) demonstrating the degree of agreement between: A: abdominal girth measurements conducted on anesthetized rats in supine position and conscious rats held upright, B: length measurements conducted on rats in supine (anesthetized) and prone (conscious) positions, and C: MRI quantification of VAT using the calibrated and conventional analysis approaches. All analysis was conducted on values from both Sprague-Dawley and SHROB rats. The dotted lines in the correlation plots represent the identity line, and the solid and dotted lines in the Bland-Altman plots represent the percent difference from mean (bias) and the 95% confidence intervals, respectively. SHROB, spontaneously hypertensive rat obese; VAT, visceral adipose tissue; SD, Standard deviation; ρ-c, Lin’s concordance correlation coefficient; CI, confidence interval.
approach was that the anesthetized rats lacked abdominal muscular tone, and thus the abdominal contents descended when held upright, thus underestimating girth measurements. With respect to length measurements, there was a 0.7±2.6% bias with an excellent degree of agreement (ρ-c=0.96) and association (r=0.96) (Figure 2B).

Comparison of VAT quantification via the conventional and calibrated MRI analysis approaches revealed a 4.9±16.0% bias, where the conventional analysis underestimated the calibrated analysis, especially in leaner rats with small amounts of VAT, as displayed by the Bland-Altman plot. However despite these differences, there was a near perfect degree of agreement (ρ-c=0.97) and association (r=0.98) between the two approaches (Figure 2C).

**Association of excised VAT to water suppressed MRI quantification of VAT**

Quantification of VAT from both the calibrated and convention MRI analysis approaches resulted in excellent degrees of association (r=0.95 and 0.97, respectively) and agreement (ρ-c=0.91 and 0.93) to excised VAT weights. Further, the calibrated approach had 18.4±17.3% bias and the conventional approach had 12.1±18.8% bias in quantifying VAT versus the excised VAT. Although both MRI analyses indicated that the amount of VAT was greater than the VAT amount excised, it was evident from the Bland-Altman plots that the conventional approach underestimated the amount of VAT in leaner rats (Figure 3). Slope and intercept comparison of the dissected values versus MRI approaches to the identity line (y=x) further revealed that the calibrated approach is not significantly different from the identity line, whereas the conventional approach is nearly different in the slope (P=0.06) and significantly different in the intercept (P<0.05).
Figure 3: Association and agreement between excised VAT and MRI quantified VAT in male Sprague-Dawley and SHROB rats at 30-35 weeks of age fed *ad libitum* and calorically restricted. Pearson’s correlation plot (left) demonstrating the degree of association, and Lin’s concordance correlation plot (left) and Bland-Altman plot (right) demonstrating the degree of agreement between excised VAT and MRI quantified VAT using the **A:** calibrated, and **B:** conventional analysis approaches. The dotted lines in the correlation plots represent the identity line, and the solid and dotted lines in the Bland-Altman plots represent the percent difference from mean (bias) and the 95% confidence intervals, respectively. All analysis was conducted on values from both Sprague-Dawley and SHROB rats. SHROB, spontaneously hypertensive rat obese; VAT, visceral adipose tissue; ρ-c, Lin’s concordance correlation coefficient; CI, confidence interval; SD, standard deviation.
Associations of VAT to abdominal girth, length, body weight to length and BMI measurements

There was a significant degree of association between excised VAT and all physical characteristics \((r=0.90-0.94; P<0.0001)\) in the Sprague-Dawley group (Figure 4A-D), with abdominal girth having the highest degree of association and accounted for 89% of the variance in VAT (Figure 4A). In the SHROB group (Figure 4E-H), there was also a significant degree of association between all these variables \((P<0.001)\), however BMI had the highest degree of association with VAT \((r=0.81)\), which explained 66% of the variance in VAT (Figure 4F). This difference between Sprague-Dawley and SHROB rats, specifically the lower degree of association, may be partly explained by the narrow range of VAT weights achieved in the SHROB \((i.e.\ 38.4-73.4g)\) versus Sprague-Dawley \((i.e.\ 4.1-81.7g)\) group, despite the aggressive CR in the SHROBs.

Effects of strain and age on the physical characteristics

Table 2 presents the physical characteristic of Sprague-Dawley (30-35 and 50 weeks old), SHROB (30-35 weeks old) and Wistar (36 weeks old) rats fed ad libitum. Comparison among the strains revealed that body weight was the largest in Wistar rats, followed by Sprague-Dawley rats, and lastly SHROB rats. VAT was 37% greater in the Wistar versus Sprague-Dawley rats \((P<0.0001)\), but similar to SHROB rats. Therefore, there was no significant difference in percent VAT relative to body weight between Wistar \((7.6\pm0.5\%)\) and Sprague-Dawley \((6.5\pm0.3\%)\) rats, but a significant percent VAT difference in these strains to SHROBs \((11.6\pm0.4\%; P<0.0001)\). Abdominal girth was similar in Wistar and SHROB rats, and was approximately 2cm greater than that of the Sprague-Dawley rats \((P<0.05)\). However, naso-anal lengths were similar in
Figure 4: Correlations of VAT to physical characteristics of male Sprague-Dawley (A-D) and SHROB (E-H) rats at 30-35 weeks of age fed *ad libitum* and calorically restricted. A, E: Correlation of VAT to abdominal girth measured on anesthetized rats in supine position. Insets: Comparison of correlations of VAT to abdominal girth measured in anesthetized rats in supine position (S), anesthetized rats held upright (UPa), and conscious rats held upright (UPc). Correlations of VAT to B, F: BMI, C, G: body weight to length ratio, and D, H: body weight. SHROB, spontaneously hypertensive rat obese; VAT, visceral adipose tissue; BMI, body mass index.
Table 2: Physical characteristics among various rat strains and ages fed *ad libitum*.

<table>
<thead>
<tr>
<th></th>
<th>Strains (♂ 30-36wk)</th>
<th>Age (Sprague-Dawley)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD</td>
<td>Wistar</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>673.7±12.0</td>
<td>786.1±18.3***</td>
</tr>
<tr>
<td>VAT (g)</td>
<td>44.1±2.6</td>
<td>60.3±4.9**</td>
</tr>
<tr>
<td>VAT/Body Weight (%)</td>
<td>6.5±0.3</td>
<td>7.6±0.5</td>
</tr>
<tr>
<td>Abdominal Girth (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anesthetized (supine)</td>
<td>22.5±0.3</td>
<td>24.4±0.4*</td>
</tr>
<tr>
<td>Anesthetized (upright)</td>
<td>21.3±0.5</td>
<td>-</td>
</tr>
<tr>
<td>Conscious (upright)</td>
<td>21.9±0.4</td>
<td>-</td>
</tr>
<tr>
<td>Length (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anesthetized (supine)</td>
<td>27.6±0.1</td>
<td>27.8±0.3</td>
</tr>
<tr>
<td>Conscious (prone)</td>
<td>27.3±0.2</td>
<td>-</td>
</tr>
<tr>
<td>Body Weight/Length (kg/m)</td>
<td>2.5±0.1</td>
<td>2.8±0.1***</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>8.9±0.1</td>
<td>10.2±0.2***</td>
</tr>
</tbody>
</table>

SD, Sprague-Dawley; SHROB, spontaneously hypertensive rat obese; VAT, visceral adipose tissue; BMI, body mass index. *P<0.05, **P<0.01, ***P<0.001 vs. SD (♂ 30-35wk). †P<0.05, ††P<0.01, †††P<0.0001 vs. Wistar. Values are mean ± s.e.m.
Sprague-Dawley and Wistar rats, and approximately 5cm longer than the SHROBs (P<0.0001). Lastly, BMI was similar among Wistar and SHROB rats, and significantly larger than the Sprague-Dawley rats (P<0.0001). Comparison of the 30-35 and 50 week old Sprague-Dawley rats revealed that body weight, VAT, and naso-anal lengths were significantly greater in the 50 week old rats (P<0.05), however there was no significant difference in the percent VAT, abdominal girths, body weight/length, and BMI.

Effects of strain and age on the association of VAT to abdominal girth

The contribution of rat strain and age to the variance between VAT and abdominal girth was assessed. There was no significant effect of rat strain on the association between VAT and abdominal girth as assessed by linear regression comparisons of slopes and y-intercepts. Grouping the data from all of the strains at 30-36 weeks of age revealed that abdominal girth accounted for 77% of the variance in VAT. Additionally, age did not contribute significantly to the unexplained variance between VAT and abdominal girth, and combination of the data from the two age groups of Sprague-Dawley rats studied (30-35 and 50 week olds) revealed that abdominal girth accounted for 71% of the variance. Further, multivariate regression analysis revealed that addition of strain and age to the predictors of VAT did not at all increase the accuracy of the estimate of VAT from abdominal girth (data not shown).

Generation and validation of the predictive equation for estimating VAT from abdominal girth

To generate the predictive equation for VAT from abdominal girth measurements a linear regression analysis was conducted on all data from male Sprague-Dawley, Wistar and SHROB rats of all ages studied that were fed ad libitum (Figure 5A). The resultant
equation was as follows: predicted VAT = 7.4 x abdominal girth – 121.3, where abdominal girth explains 74% of the variance in VAT (P<0.0001). Validation of the VAT equation yielded a marked degree of association (r=0.86) and agreement (ρ-c=0.85) between the excised and predicted VAT (Figure 5B). Slope and intercept comparison of the excised versus predicted VAT to the identity line (y=x) revealed that this relationship is not significantly different from the identity line. In fact, there was only a 1.1±14.5% bias between the excised and predicted VAT (Figure 5C).
Figure 5: Generation and validation of the predictive equation for VAT from abdominal girth measurements in male rats at 30-50 weeks of age. **A:** Linear regression analysis demonstrating the predictive equation for VAT estimation from abdominal girth measurements conducted on anesthetized rats in supine position. **B:** Validation of the predicted VAT from abdominal girth measurements to excised VAT via Pearson’s and Lin’s concordance correlation coefficients. **C:** Bland-Altman plots of percent difference from mean versus mean of predicted and excised VAT values. The dotted line in the correlation plot represents the identity line, and the solid and dotted lines in the Bland-Altman plots represent the percent difference from mean (bias) and the 95% confidence intervals, respectively. VAT, visceral adipose tissue; ρ-c, Lin’s concordance correlation coefficient; CI, confidence interval.
Discussion:

The main findings of this study are that both water suppressed MRI and abdominal girth offer reliable, non-invasive, non-terminal, and rapid methods for \textit{in vivo} assessments of VAT. Both of these techniques demonstrate strong relationships between surgically dissected VAT and their predicted VAT measurements.

\textbf{Water Suppressed MRI}

Existing imaging protocols allow for quantification of VAT, but they may suffer from the following limitations: long data acquisition times, expense, susceptibility to motion artifacts, requirement for anesthesia, low lipid:water contrast, exposure to radiation (with CT), use of arbitrary thresholding techniques for fat segmentation, and restrictions to whole body, rather than regional, composition. Water suppressed MRI protocols have been shown to be effective in acquiring VAT images in human studies\textsuperscript{11, 13}, and until the present study, had not been adopted in animal studies. The protocol used herein utilized the HASTE water suppressed sequence to acquire VAT images in rats. HASTE sequences have been routinely used in abdominal imaging due to its short acquisition time; making images less susceptible to motion artifacts, especially in uncooperative subjects\textsuperscript{21}. It was found that the HASTE water suppressed sequence allowed for rapid data acquisition in 2-5 minutes versus previous studies that required anywhere from 10-30 minutes\textsuperscript{2, 3, 5, 6, 8}, making the HASTE protocol more suitable for high-throughput studies in animal studies. Due to the rapid data acquisition time and the animals’ innate behaviour to seek out and hide in dark burrows (i.e. the restraining tubes)\textsuperscript{8}, we did not require the use of anesthesia, especially after acclimatizing the rats to the restraining tubes a few times prior to the imaging day. Further, the water suppressed
mode was shown to be effective in eliminating the water signal from the control water-filled tubes and fluid-filled organs (i.e. bladder), as well as facilitating fat segmentation due to improved lipid:water contrast.

Water suppressed MRI-derived VAT measurements agreed and associated closely with surgically dissected VAT weights; although the MRI-based determination of VAT indicated that there was more VAT than was excised. This latter result is in agreement with previous studies using T\textsubscript{1}-weighted MRI protocols\textsuperscript{2,3,5}, and may be in part explained by the technical difficulty of ensuring complete excision of VAT. Furthermore, the calibrated approach followed this pattern consistently throughout the range of VATs studied, whereas the conventional approach (i.e. thresholding method) tended to underestimate VAT in leaner rats, and conversely overestimate VAT in heavier rats. This can be explained by the fact that the conventional approach does not take into account the possibility of partial volume artifacts (i.e. when a voxel signal represents an average of different tissues, which results in a loss of resolution)\textsuperscript{22}. Specifically, even though water-suppressed images provide mainly the fat signal and eases the determination of the segmentation threshold from the grey-scale histogram, certain fat-containing voxels may have lower signal intensities due to ‘marbling’ with other tissues in the abdomen that may be omitted in quantification using the conventional approach. Given that a leaner animal may have more partial volume artifacts because they have less VAT and inevitably more voxels with a mixture of different tissue types, this might explain why the conventional approach fails to properly estimate VAT content in leaner rats. The calibrated approach, on the other hand, takes this into account by measuring the percent of fat in a voxel, regardless of its makeup, and thus allows for a more accurate estimation of VAT.
Further, since storage lipids make up >80% of the mass of adipocytes depending on the physiological condition of the organism (i.e. the more obese the organism the more lipids are stored in adipocytes)\(^{23,24}\), using the calibrated approach, we can more accurately determine the true lipid content in leaner versus obese animals since their fat voxel signals would be less intense than in the obese. This is especially important because MRI actually measures the resonance signal of the protons in the –CH\(_2\) groups of lipids, not whole adipocytes\(^7,24\). Additionally, the calibrated approach can be modified and applied to estimating lipid accumulation in other organs, such as liver and muscle, which may aid in determining the role of ectopic fat deposition in metabolic disturbances. Therefore, the calibrated approach appears to be superior to the conventional approach in accurately estimating a wide range of VAT amounts using the HASTE water suppressed mode.

**Abdominal Girth**

Although MRI allows for accurate VAT estimation, the low availability, high cost, and need for trained MRI technicians can make this technique unaffordable and impractical for some researchers. For the first time ever, we demonstrate a rapid (i.e. <5 minutes), inexpensive, and simple alternative to assessing abdominal obesity and predicting VAT, specifically abdominal girth measurements, which are comparable to that of the widely used waist circumference measurements in humans. In fact, abdominal girth seems to be an excellent predictor of VAT, explaining 74% of the variance in VAT, independent of age and strain of rats studied. It should be noted that these measurements were conducted on animals that reached maturity (i.e. 30-50 weeks of age), when growth typically occurs at a slower rate\(^{25}\), and therefore more studies are necessary to assess abdominal girths across a wider range of ages to accurately determine the effects of age
on visceral adiposity. Eliminating the effects of age and strain by simply investigating the group with a greater range of VAT amounts and number of rats (i.e. among 30-35 week old, male, Sprague Dawley rats), abdominal girth predicted 89% of the variance in VAT. These data are consistent with the waist circumference measurements conducted in clinical studies, in that waist circumference accounts for 77-94% of the variance in VAT quantified by reliable imaging techniques\textsuperscript{14, 17}. Furthermore, this technique proved to be very reliable, in that the intra- and inter-observer bias was very low (<6%), and the degree of agreement and association between measurements was generally excellent.

Further, we have conducted abdominal girth measurements in three different ways: anesthetized/supine, anesthetized/upright, and conscious/upright. Not surprisingly, it was found that the least amount of bias and highest degree of agreement was among the abdominal girth measurements conducted in anesthetized rats in the supine position. However, investigators that do not want anesthetize their animals can use the conscious/upright method, as inter-technique comparisons between these two measurements revealed a small amount of bias (~1.5%) and an excellent degree of agreement. It is strongly advised that the investigators should practice taking measurements on conscious rats, thereby also acclimatizing the rats to the handling, and ensure that both investigators are consistently and similarly restraining the rats to prevent introduction of variability in measurements. It is also recommended that investigators do not use the anesthetized/upright approach, as a major disadvantage to this approach is that the anesthetized rats lack abdominal muscular tone, and thus the abdominal contents tend to descend when held upright, thereby causing an underestimation of abdominal girth.
As indicated above, using abdominal girth as a predictor of VAT provides a rapid, inexpensive and easy alternative to MRI, however it cannot discriminate between visceral and subcutaneous abdominal fat. Despite this, abdominal girth still reflects the regional measurement of abdominal obesity rather than the distribution of ‘heaviness’ across the body like other physical measurements (i.e. BMI and body weight to length). In fact, Janssen et al. (2004) showed that waist circumference is a better predictor of VAT associated co-morbidities than BMI in humans. Thus, abdominal girth measurements may be superior to other physical measurements (i.e. BMI) in assessing VAT-related risk of disease, although more animal studies are warranted to support this speculation.

**Conclusion**

Given that abdominal obesity is strongly associated with morbidity (i.e. cardiovascular disease, stroke, type II diabetes, certain cancers, non-alcoholic fatty liver disease, osteoarthritis, and Alzheimer’s disease) and mortality, it is important that basic research develop techniques, as have been described herein, to further the understanding of the role of VAT in the pathogenesis of disease. Although each of the described technique has its associated advantages and disadvantages, they provide researchers with the tools necessary to conduct rapid in vivo longitudinal assessments of VAT in animal studies in response to growth, aging, disease progression, and pharmacological, nutritional and exercise interventions.
References:


