PROTEOMIC ANALYSIS OF THE SUPERIOR MESENTERIC GANGLION AND LIVER OF SPONTANEOUSLY HYPERTENSIVE RATS

by

Sarah M Svoboda

A thesis submitted to the Department of Anatomy and Cell Biology

In conformity with the requirements for

the degree of Master of Science.

Queen’s University

Kingston, Ontario, Canada

(October, 2009)

Copyright ©Sarah M Svoboda, 2009
Abstract

Spontaneously hypertensive rats (SHR) are a well accepted model of primary hypertension. Among other features common to human hypertension, these rats exhibit sympathetic hyperactivity. The neurons of the superior mesenteric ganglion (SMG) from SHR display enhanced collateral sprouting, higher firing rates and hyperinnervation of the mesenteric arteries compared to the SMG neurons from age-matched, normotensive Wistar-Kyoto (WKY) rats. Furthermore, SMG neurons in SHR are exposed to different conditions than are SMG neurons from WKY rats, including enhanced oxidative stress, increased afferent stimulation, and an altered hormonal environment. In order to identify proteins with potential involvement in the establishment or maintenance of peripheral sympathetic hyperactivity in SHR, we used proteomic techniques to search for differences in protein expression between the SMG of SHR and the SMG of WKY rats at 16 and 22 weeks of age. We found an upregulation of predominantly fetally expressed T1 domain and haptoglobin and a downregulation of serine protease inhibitor 2.1 in SHR relative to WKY rats at 16 and 22 weeks; Apolipoprotein-A1 was also found to be upregulated in 22 week SHR SMGs compared to age-matched WKY SMGs. These identifications improve our understanding of the ganglionic microenvironment in SHR and represent targets for the development of novel therapies to treat primary hypertension.

Hypertension is one of the defining components of the metabolic syndrome, together with insulin resistance, visceral adiposity and hyperlipidemia. Non-alcoholic fatty liver disease (NAFLD) is also a common feature of the metabolic disorder, and thus
primary hypertension and NAFLD are common comorbidities. Despite these clinical connections, very little is known about the effects of primary hypertension on hepatic physiology. We used proteomic techniques to search for evidence of significant involvement of the liver in SHR phenotype at the molecular level. We detected changes in the expression of several proteins involved in the regulation of oxidative stress and lipid metabolism which together show that the liver is strongly involved in the pathologies associated with hypertension. Our results suggest several novel mechanisms for the initiation of oxidative stress in SHR which could contribute to new advances in the treatment of metabolic abnormalities associated with hypertension.
Co-Authorship

The results of this thesis will be prepared in the form of two scientific papers, which will be submitted for publication. The first draft of these papers was written by Sarah Svoboda (with the exception of results found in Manuscript 1 which are not presented here; this draft was written by Todd McDonald), while final editorial recommendations and comments were provided by Dr. Michael D. Kawaja. Experimental designs and protocols were devised by Dr. Michael D. Kawaja and Sarah Svoboda and all experiments and data analyses were carried out by Sarah Svoboda.

Manuscript 1: Sarah Svoboda, Todd McDonald and Michael D. Kawaja. Proteomic analysis of the superior mesenteric ganglion and the superior mesenteric arteries in spontaneously hypertensive rats.

Acknowledgements

First of all, I would like to offer thanks to Dr. Michael D. Kawaja. By allowing me a huge degree of independence in navigating the course of my project, he taught me how to incorporate the scientific and the practical sides of research. I learned invaluable lessons from him regarding objective observation, ethics and scientific enthusiasm. Thanks to all his advice during the last two years, I have gained confidence and skills which will serve me well over the remainder of my education and career. And, of course, thank you for supporting me and, at times, humoring me while I pushed my submission deadline back and back as my thesis grew and grew. Now, let’s get those papers published!

To Todd McDonald (soon to be Dr. McDonald!), thank you for teaching me how to run gels, troubleshoot Westerns, run the mass spectrometer, and all the other techniques which were necessary to complete this thesis.

To Dave McLoed of the Protein Discovery and Function Laboratory at Queen’s University, thank you for answering my questions, digesting my samples and letting me use the mass spectrometer even when you weren’t sure I knew quite what I was doing!

To Janet Elliot and Verna Norkum, thank you for the amazing technical support and for taking care of all the behind the scenes jobs that keep a lab running efficiently (ordering, autoclaving, doing dishes, the list goes on and on). Special thanks also goes to Verna for doing a wonderful (and fast!) job cutting the tissue that I used in all of my immunohistochemistry experiments…Verna, I couldn’t have done it without you!

To Laura Smithson and Todd McDonald, thank you for making each day fun. It was a pleasure to share a lab with you!

To Dr. Cynthia Morton of Harvard University, thank you for proving the anti-pfetin IgG.

To Dr. Julie Chao of the Medical University of South Carolina, thank you for providing the anti-RKBP/Spi 2.2 IgG.

Finally, I would like to acknowledge the financial support of the Heart and Stroke Foundation of Ontario.
# Table of Contents

Abstract ............................................................................................................................................ ii  
Co-Authorship ................................................................................................................................ iv  
Acknowledgements .......................................................................................................................... v  
Table of Contents ........................................................................................................................... v  
List of Figures and Tables ................................................................................................................ viii  
List of Abbreviations ...................................................................................................................... ix  

**Chapter 1 General Introduction** ................................................................................................. 1  
  
  Introduction ................................................................................................................................... 1  
  Anatomy of the Sympathetic Nervous System ........................................................................... 2  
  Sympathetic Regulation of Blood Pressure .............................................................................. 6  
  Mechanisms Contributing to Sympathetic Hyperactivity ....................................................... 10  
  Spontaneously Hypertensive Rats as a Model of Primary Hypertension ............................ 13  
  Oxidative Stress and Sympathetic Hyperactivity in SHR ........................................................ 16  
  Non-Alcoholic Fatty Liver Disease ............................................................................................ 18  
  Common Features of Primary Hypertension and NAFLD ....................................................... 20  
  Evaluation of the Liver in SHR ................................................................................................. 26  
  Proteomics - the SHR Model ...................................................................................................... 27  
  Proteomics - Techniques ............................................................................................................ 30  

**Chapter 2 Proteomic Analysis of Sympathetic Ganglia of Spontaneously Hypertensive Rats** 38  
  
  Introduction ................................................................................................................................... 38  
  Methods ......................................................................................................................................... 42  
    Animals ....................................................................................................................................... 42  
    Tissue Collection ........................................................................................................................ 42  
    Proteomics .................................................................................................................................. 43  
      Isoelectric Focusing ...................................................................................................................... 43  
      2 Dimensional Gel Electrophoresis ........................................................................................ 44  
      Mass Spectroscopy .................................................................................................................. 45  
    Western Blots ............................................................................................................................. 46  
    Immunohistochemistry ............................................................................................................. 48  
  Results .......................................................................................................................................... 49  
  Discussion ...................................................................................................................................... 54
Chapter 3 Proteomic Analysis of the Liver of Spontaneously Hypertensive Rats

Introduction

Methods

Animals

Tissue Collection

Proteomics

Isoelectric Focusing

2 Dimensional Gel Electrophoresis

Mass Spectroscopy

Western Blots

Immunohistochemistry

Results

Discussion

Quinonoid Dihydropterine Reductase (QDPR)

Sulfite Oxidase (SOX)

Glutathione-S-Transferase μ1 and Glutathione-S-Transferase ω1 (GSTO1)

NADH Dehydrogenase Ubiquinone 1α Subcomplex 10 (NDUFA10)

Coproporphyrinogen Oxidase (CPOX)

TNF Receptor Associated Protein 1 (TRAP1)

Carboxylesterase ES-10 (ES-10) and Carboxylesterase ES-4 (ES-4)

Alcohol Dehydrogenase Iron-Containing 1 (ADHFe1)

17β-Hydroxysteroid Dehydrogenase (17BHSD)

Carbonic Anhydrase 2 (CA2)

Formiminotransferase Cyclodeaminase (FTCD)

MAWD Binding Protein (MAWDBP)

Halocacid Dehydrogenase Domain-Containing 3 (HDD3)

Chapter 4 General Discussion

References

Appendix A
List of Figures and Tables

Chapter 2

Figure 1: 2-Dimensional Gel Electrophoresis of the Superior Mesenteric Ganglion ……73
Figure 2: Representative Mass Spectra of Pfetin, Spi 2.1 and Haptoglobin ……………77
Figure 3: Pfetin Protein Abundance …………………………………………………………79
Figure 4: Pfetin Immunohistochemistry ……………………………………………………81
Figure 5: Spi 2.1 Protein Abundance ………………………………………………………83
Figure 6: Spi 2.1 Immunohistochemistry in the Superior Mesenteric Ganglion ……85
Figure 7: Spi 2.1 Immunohistochemistry in the Liver ……………………………………87
Figure 8: Haptoglobin Protein Abundance ………………………………………………89
Figure 9: Haptoglobin Immunohistochemistry …………………………………………91
Figure 10: Haptoglobin Immunohistochemistry in the Liver ……………………………93
Figure 11: Apolipoprotein A1 Protein Abundance ………………………………………95
Table 1: Protein Identifications in the Superior Mesenteric Ganglion …………………97

Chapter 3

Figure 12: 2-Dimensional Gel Electrophoresis (pH 3 – 11) of the Liver …………………150
Figure 13: 2-Dimensional Gel Electrophoresis (pH 6 – 9) of the Liver …………………152
Figure 14: Quantification of Differences in Protein Expression in the Liver Between
           SHR and WKY rats ……………………………………………………………………156
Figure 15: QDPR Spot Translocation ………………………………………………………162
Figure 16: QDPR Immunohistochemistry in the Liver …………………………………….164
Figure 17: Mechanism Depicting QDPR-Induced Hypertension ………………………166
Figure 18: SOX Protein Abundance ………………………………………………………168
Figure 19: SOX Immunohistochemistry in the Liver ………………………………………170
Figure 20: MAWDBP Immunohistochemistry in the Liver ………………………………172
Table 2: Protein Identifications in the Liver ……………………………………………….174

Appendix A

Figure A1: Quantification of Hepatic Haptoglobin Expression ………………………..205
List of Abbreviations

2D SDS-PAGE = 2 Dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
2DGE = 2 Dimensional gel electrophoresis
16wk = 16 weeks old
17BHSD = 17β-Hydroxysteriod dehydrogenase
22wk = 22 weeks old
ADH = Alcohol dehydrogenase
ADHFe1 = Alcohol dehydrogenase iron-containing 1
ALA = Aminolevulin acid
AngII = Angiotensin II
ApoA1 = Apolipoprotein A1
BH4 = Tetrahydropterine
BK = Bradykinin
BSA = Bovine serum albumin
CA2 = Carbonic anhydrase 2
CAPS = 3-(Cyclohexylamino)-1-propanesulfonic acid
CNS = Central nervous system
CPOX = Coproporphyrinogen oxidase
DAB = Diaminobenzidine
ES-4 = Carboxylesterase ES-4
ES-10 = Carboxylesterase ES-10
ETC = Electron transport chain
FAEE = Fatty acid ethyl esters
FFA = Free fatty acid
FTCD = Formiminotransferase cyclodeaminase
GSH = Glutathione
GSTM1 = Glutathione-S-transferase μ1
GSTO1 = Glutathione-S-transferase ω1
H$_2$O$_2$ = Hydrogen peroxide
Hb = Hemoglobin
HDD3 = Haloacid Dehalogenase-like Domain Containing 3
HDL = High density lipoprotein
Hp = Haptoglobin
IEF = Isoelectric focusing
IML = Intermediolateral column
IPG = Immobile pH gradient
Kv = K+ voltage-gated ion channels
LDL = Low density lipoprotein
MALDI-TOF = Matrix-assisted laser desorption ionization time of flight
MAWDBP = MAWD binding protein
MOPS = 3-[(n-morpholino) propanesulfonic acid
Mr = Molecular weight
MS = Mass spectroscopy
MTHFR = Methionine tetrahydrofolate reductase
NAFLD = Non-alcoholic fatty liver disease
NASH = Non-alcoholic steatohepatitis
NDUFA10 = NADH Dehydrogenase Ubiquinone 1α Subcomplex 10
NDS = Normal Donkey Serum
NE = Norepinephrine
NGF = Nerve growth factor
NO = Nitric oxide
NOS = Nitric oxide synthase
NPY = Neuropeptide Y
NTS = Nucleus tractus solitaries
O₂⁻ = Superoxide anion
PAH = Phenylalanine hydroxylase
PBG = Porphobilinogen
PBS = Phosphate buffered saline
Pfetin = Predominantly fetally expressed T1 domain
pI = Isoelectric point
PTM = Post-translational modification
PUFA = Polyunsaturated fatty acids
PVH = Paraventricular nucleus of the hypothalamus
QDPR = Quininoid dihydropterine reductase
Redox = Reduction/oxidation
RKBP = Rat kallikrein binding protein
ROS = Reactive oxygen speculate
RVLM = Rostral ventrolateral medulla
SCG = Superior cervical ganglion
Serpin = Serine protease inhibitor
SHR = Spontaneously hypertensive rat
SHRSP = Stroke-prone spontaneously hypertensive rat
SMG = Superior mesenteric ganglion
SNS = Sympathetic nervous system
Spi 2.1 = Serine protease inhibitor 2.1
Spi 2.2 = Serine protease inhibitor 2.2
SO2 = Sulpher dioxide
SO3−2 = Sulfite
SO4−2 = Sulfate
SOD = Sulfite oxidase deficiency
SOX = Sulfite oxidase
TBST = Tris buffered saline with Tween-20
TBX = Tris buffered saline with Triton-X
THF = Tetrahydrofolate
TRAP1 = Tumor necrosis factor receptor associated protein 1
WBS = western blocking solution
Chapter 1

General Introduction

Introduction: Hypertension

Hypertension, or increased blood pressure, affects millions of Canadians and is known to promote the development of coronary heart disease, arteriosclerosis, kidney disease and stroke. Primary hypertension was estimated to affect 600 million people and has been recorded as the cause of 5 million, or 13% of global fatalities (World Health Organization, 2002). Furthermore, cardiovascular disease is the leading cause of death in Canada and anti-hypertensive medications account for 40% of the 5 billion dollars spent on medications to treat cardiovascular illnesses each year (Jackevicius et al., 2009). Scientific research on the causes and effects of hypertension is therefore extremely important in order to develop therapies to treat this widespread disease.

Primary hypertension is defined as increased blood pressure that does not result from a pre-existing condition or disease. It is characterized by significantly increased blood pressure, increased vascular rigidity and sympathetic hyperactivity. Secondary hypertension, on the other hand, results from pre-existing conditions such as kidney disease or adrenal tumors, or from environmental factors such as poor diet and immobility. Hypertension often coexists with insulin resistance, hyperglycemia, hyperlipidemia and visceral adiposity, as a set of symptoms together known as the metabolic syndrome. Whether hypertension as part of the metabolic syndrome is primary or secondary depends on whether the syndrome as a whole is due to genetic predisposition or whether it results from environmental factors.
Anatomy of the Sympathetic Nervous System

Like other parts of the nervous system, the sympathetic nervous system (SNS) includes both motor and sensory modalities. The motor arm of the SNS begins centrally with neurons in the autonomic centers: hypothalamus and the brainstem (Robertson et al., 1996; Boron and Boulpaep, 2003; Guyenet, 2006; Low and Benarroch, 2008; Allen et al., 2009). The paraventricular nucleus of the hypothalamus (PVH) and nucleus tractus solitarii (NTS) in the medulla oblongata have received special attention for their role in the coordination and integration of incoming signals from higher centers in the cortex and limbic system as well as from afferents conveying sensory information from peripheral receptors (Aicher et al., 2003; Guyenet, 2006; Low and Benarroch, 2008; Allen, 2009). Neurons in the PVH and NTS project to the rostral ventrolateral medulla (RVLM), which is a crucial effector node for the regulation of blood pressure (Robertson et al., 1996; Guyenet, 2006; Hirooka, 2008; Low and Benarroch, 2008; Allen et al., 2009). RVLM neurons in turn project axons down through the spinal cord and synapse on the presynaptic sympathetic neurons located in the intermediolateral column (IML) in the thoracic and upper lumbar regions of the cord (T1 – L2/L3) (Reja et al., 2002; Boron and Boulpaep, 2003; Guyenet, 2006; Allen et al., 2009). Presynaptic neurons then project either through the white rami and synapse with post-synaptic neurons in the paravertebral ganglia of the sympathetic trunk, which are located parallel to the vertebral column, or through the splanchnic nerves to neurons in the prevertebral ganglia which are situated, in humans, on the ventral surface of the dorsal aorta (Robertson et al., 1996; Low and Benarroch, 2008). Finally, post-synaptic, or ganglionic, sympathetic neurons project
axons to and innervate target tissues (Robertson et al., 1996; Boron and Boulpaep, 2003; Low and Benarroch, 2008). These unmyelinated post-synaptic fibers can travel either inside peripheral nerves composed only of autonomic axons (such as cardiac branches), inside peripheral nerves made mostly of myelinated motor and sensory neurons, or as a relatively unorganized plexus of fibers conveyed along blood vessels. Sympathetically innervated tissues include vascular smooth muscle cells in blood vessels, the gastrointestinal tract, the bladder, the gallbladder, bronchioles and hair follicles, as well as organs including heart, kidney, liver, and various glands (Robertson et al., 1996; Boron and Boulpaep, 2003; Low and Benarroch, 2008). This primary neurotransmitter of presynaptic sympathetic neurons is acetylcholine while the primary neurotransmitter of postsynaptic sympathetic neurons is norepinephrine (NE).

The afferent limb of the SNS conveys signals from peripheral sensory receptors which allow the regulation of sympathetic activity in response to current physiological requirements. The most important autonomic sensory pathway for the maintenance of blood pressure is the baroreflex; peripheral baroreceptors located in the carotid sinus and aortic arch detect deformation of the blood vessel wall which denotes a change in intraluminal pressure. These receptors, whose firing rates are directly proportional to the degree of compression or shear stress within the vessel wall, send signals through afferent axons which project back to the cardiovascular centers in the brainstem, such as the NTS, that are crucially involved in the regulation of sympathetic tone (Robertson et al., 1996; Aicher et al., 2003; Boron and Boulpaep, 2003; Low and Benarroch, 2008). Increased aortic blood pressure, for example, results in heightened activity in the baroreflex afferents which in turn increases firing rates in the NTS. Inhibitory projections then
decrease activity in the RVLM and lead to a dampening of peripheral sympathetic drive to vascular smooth muscle. This results in decreased vasoconstriction and a compensatory reduction in arterial blood pressure (Robertson et al., 1996; Aicher et al., 2003; Boron and Boulpaep, 2003; Low and Benarroch, 2008).

The superior mesenteric ganglion (SMG) is one of the three prevertebral ganglia (the other two are the celiac and inferior mesenteric ganglia). It is located on the ventral surface of the dorsal aorta at the branch point of the superior mesenteric artery. The SMG receives innervation from pre-synaptic neurons in sections T5 – T12 of the IML in the spinal cord (Robertson et al., 1996; Low and Benarroch, 2008). Axons projecting from the neurons of the SMG travel in a complex, mesh-like network along the superior mesenteric artery as it branches and supplies the digestive tract. SMG neurons can largely be divided into two categories according to their targets and histological characteristics: vasomotor neurons innervate the smooth muscle of mesenteric blood vessels, as well as arterioles within the wall of the intestine, where they elicit contraction via the release of norepinephrine (NE), neuropeptide Y (NPY), and ATP, and have relatively simple dendritic trees (Robertson et al., 1996; Gibbins et al., 2003; Low and Benarroch, 2008). Intestinomotor neurons project axons into the wall of the digestive tract where they synapse with motor neurons in the myenteric plexus and secretomotor/vasomotor neurons in the submucosal plexus, which together make up the majority of the enteric nervous system (Low and Benarroch, 2008). Intestinomotor neurons inhibit the activity of enteric neurons, which decreases gut motility and mucosal secretion, have much more extensive dendritic trees than their vasomotor counterparts,
and can be identified by the coexpression of NE and somatostatin (Gibbins et al., 2003; Low and Benarroch, 2008).

The superior cervical ganglion (SCG) is the first and largest ganglion of the sympathetic trunk. It is bilaterally located near the bifurcation of the carotid artery into the external and internal carotid arteries. SCG neurons receive inputs from the pre-synaptic neurons in the IML of sections T1 – T3 of the spinal cord (Robertson et al., 1996; Low and Benarroch, 2008). Axons from SCG neurons travel along the carotid arteries and innervate targets in the head and neck. As in the SMG, these neurons can be divided into categories according to their targets and the neurotransmitters they express. Vasoconstrictor neurons innervate the smooth muscle surrounding blood vessels and express NE, NPY and ATP, while vasodilator neurons, which innervate the same targets, are negative for NE but express NPY and vasoactive intestinal peptide (Gibbins and Mathew, 1996; Low and Benarroch, 2008). Pilomotor neurons induce contraction of smooth muscle cells surrounding hair follicles and express NE and dynorphin, while sudomotor neurons inhibit secretion from salivary and lacrimal glands and express NE (Gibbins and Mathew, 1996; Robertson et al., 1996). Finally, motor neurons cause contraction of the pupil dilator muscle of the iris and the superior tarsal muscle of the eyelid and express NE, NPY and dynorphin (Robertson et al., 1996; Low and Benarroch, 2008).

In general, post-synaptic sympathetic neurons are multipolar with extensive dendrite networks. In the SMG, dendritic trees receive connections from incoming myelinated pre-synaptic axons, as well as from unmyelinated afferent axons from the enteric nervous system (Gibbins and Mathew, 1996; Gibbins et al., 2003). Sympathetic
ganglia thus contain a variety of glia cells including myelinating and unmyelinating Schwann cells as well as satellite cells which surround neuron somas and may regulate the ganglionic environment (Marcel et al., 1989). Since peripheral sympathetic ganglia lack a tight endothelial barrier separating neuropil from blood plasma as is found in the central nervous system, proteins and circulating factors have access to post-synaptic sympathetic neurons. Like any other tissue, ganglia contain populations of fibrocytes and immune cells as well as a system of capillaries.

There are several important differences between prevertebral ganglia like the SMG and paravertebral ganglia like the SCG (Low and Benarroch, 2008). As described above, neurons in these ganglia have very different target tissues and express different assortments of neurotransmitters (Gibbins and Mathew, 1996; Robertson et al., 1996; Gibbins et al., 2003). Another key difference is that input to prevertebral neurons is largely from afferent fibers arising in target tissues with relatively limited input from higher sources, while input to paravertebral neurons is mostly derived from the central nervous system (CNS) (Low and Benarroch, 2008). Consequently, prevertebral neurons are integrative in function and involved primarily in reflex pathways independent of the CNS, while paravertebral neurons function more as a relay for signals arising from higher centers (Low and Benarroch, 2008). Clearly, neurons in these two types of ganglia differ in many more respects than their location.

**Sympathetic Regulation of Blood Pressure**

It is well accepted that the SNS is involved in the regulation of blood pressure through a variety of mechanisms (Grassi, 1998; Boron and Boulpaep, 2003; reviewed in Guyenet,
Primary among these is the innervation of vascular smooth muscle cells in the intima media of resistance blood vessels. NE is released from varicosities along post-synaptic sympathetic axons into the extracellular milieu where it interacts with $\alpha_1$ adrenergic receptors on smooth muscle cells which induces contraction (Boron and Boulpaep, 2003; Guyenet, 2006). In arteries, the resulting increase in vascular tone directly maintains blood pressure by narrowing the space through which blood must travel and therefore increasing the total resistance to flow.

Arterial pressure can be increased either by decreasing blood vessel lumen diameter or by increasing the volume of fluid which passes through artery of a given diameter per unit time. Veins, as well as some specialized arteries such as the mesenteric arteries, can hold large amounts of blood at rest and thus function as reservoirs. In the event that blood pressure drops, however, sympathetically induced constriction of these vessels forces blood to move from the reservoir and into the arterial system, which raises arterial blood volume and corresponds to an increase in blood pressure.

The SNS also regulates blood pressure through innervation of the heart and kidney. Sympathetic activity in the heart influences cardiac output by increasing heart rate and cardiomyocyte contractility, both of which contribute to an increase in cardiac output which in turn increases arterial blood pressure (Iaccarino, 2001; Boron and Boulpaep, 2003; Guyenet, 2006). Renal sympathetic activity influences blood pressure through constriction of renal blood vessels which leads to a decrease in glomerular filtration and, consequently, a decrease in water loss (reviewed by DiBona 2002; Grisk, 2004). Activation of renal adrenergic receptors also promotes the reuptake of sodium and water in the nephron (DiBona 2002; Grisk, 2004) which, again, decreases water loss and
thus increases blood volume. This increase in blood volume in turn increases venous return, which improves cardiac output and raises arterial blood pressure. This illustrates that sympathetic control of blood vessel constriction, cardiac function and renal water resorption are mutually interrelated in blood pressure regulation.

Renal sympathetic activity also increases renin secretion from the granular cells of the juxtaglomerular apparatus (Boron and Boulpaep, 2003; Carey and Siragy, 2003). Renin enters circulation where it cleaves the pre-enzyme angiotensinogen and releases angiotensin I which is in turn cleaved again by angiotensin converting enzyme into the active peptide angiotensin II (AngII) (Boron and Boulpaep, 2003; Carey and Siragy, 2003; Allen et al., 2009). AngII increases blood pressure through a variety of mechanisms including increased activity in the central cardiovascular centers (Allen et al., 2009), augmented cardiac output (Dang et al., 1999; Carey and Siragy, 2003), blood vessel constriction (Viccari et al., 2008), and renal sodium and water resorption (Carey and Siragy, 2003).

Extensive evidence shows that increased sympathetic activity contributes to the development and maintenance of hypertension in humans and in animal models. In humans, sympathetic activity is measured directly through microneurography, which allows the recording of electrical activity in post-ganglionic efferent sympathetic axons travelling to the vasculature supplying skin or skeletal muscle (Grassi, 1998; Mancia et al., 1999; Esler, 2000; Guyenet, 2006). SNS activity can also be measured indirectly through plasma NE, which approximates regional levels of NE release from sympathetic varicosities by measuring the amount of NE which “spills over” into circulation before it can be taken up again by axon NE transporters (hence the name NE spillover) (Grassi,
Clinical studies have shown that sympathetic firing rates correlate directly with mean arterial pressure in young adult patients with moderate to severe primary hypertension compared to age-matched normotensive patients (Matsukawa et al., 1991; Grassi, 1998; Mancia et al., 1999; Esler, 2000; Guyenet, 2006). Interestingly, patients with secondary hypertension due to abnormal aldosterone secretion have sympathetic firing rates similar to normotensives, suggesting that SNS hyperactivity is a characteristic feature of specifically primary hypertension (Grassi 1998).

Hypertensive patients have also been shown to have increased NE spillover in the heart and kidney compared to age-matched, normotensive controls (Esler, 2000; Macia et al. 1999). Such patients, however, do not have augmented NE spillover in other organs, such as hepatomesenteric organs, which indicates that NE release is regulated separately for each target tissue (Esler 2000).

SNS hyperactivity contributes to several anatomical and physiological changes in the cardiovascular system; these changes are instrumental to the development and maintenance of hypertension. NE induces hyperplasia in vascular smooth muscle cells as well as myocyte hypertrophy in vitro (Grassi, 1998; Schiffrin, 2002) and animal studies have shown that increased NE spillover and sympathetic hyperinnervation of resistance arteries correlates with remodeling and thickening of the intima media, leading to a decrease in the lumen to wall ratio (Lee et al., 1983; Scott and Pang, 1983; Lee and Smeda, 1985; Donohue et al., 1988). NE has also been shown to promote collagen secretion and rearrangement of the extracellular matrix (O’Callaghan and Williams, 2002). Combined with the increased vascular tone due to contraction of these smooth
muscle cells, these structural changes contribute to increased vascular resistance and rigidity. Furthermore, these structural changes also promote the development of arteriosclerosis (Grassi, 1998; Mancia et al., 1999; Loria et al., 2008). Vascular remodeling and rigidity are also features of primary hypertension in humans (Grassi, 1998; Mancia et al., 1995, 1999; Et-Taouil et al., 2003), although no histological evidence of anatomical hyperinnervation of sympathetic targets in humans has been provided.

Finally, increased sympathetic drive to the heart has been shown to induce cardiac remodeling and left ventricular hypertrophy in humans and animal models (Dang et al., 1999; Mancia et al., 1999; Iaccarino, 2001), as well as increasing the risk of ventricular tachycardia in hypertensive patients (Meredith et al. 1991). Sympathetic hyperactivity also has numerous secondary negative effects on physiology resulting from restriction of blood flow and nutrient diffusion. For example, SNS activity contributes to the development of insulin resistance be retarding the delivery of glucose to skeletal muscle (Julius et al. 1992; Lembo et al., 1994). Vasoconstriction also impairs the clearing of lipids from the liver and thus predisposes patients to hepatic hyperlipidemia (Esler, 2000).

Mechanisms Contributing to Sympathetic Hyperactivity

The exact causes of this sympathetic hyperactivity are unknown, although several possibilities have been suggested. SNS activity seems to be at least partially genetically controlled (Elser, 2000) since normotensive patients with family histories of hypertension have higher rates of sympathetic firing than normotensives without such a family history
(Ferrier et al. 1993a). However, no specific gene influencing SNS activity in hypertension has been identified.

Increases in AngII have been hypothesized to contribute to the establishment of SNS hyperactivity. It is generally accepted that primary hypertension is associated with altered activity or sensitivity of the renin-angiotensin system (Mancia et al., 1999; Carey and Siragy, 2003; Weir, 2007). AngII has been shown to increase sympathetic firing and NE release, in addition to sensitization of adrenergic receptors in target organs, which augments the effect of a NE secretion (Saxena, 1992; Mancia, 1999). Evidence from animal models indicates that AngII concentration is elevated centrally and coincides with increased neural activity in brainstem nuclei involved in regulating sympathetic output such as the RVLM (Sved et al., 2003), and that this activity promotes increased peripheral sympathetic activity (Allen, 2001; DiBona and Jones, 2001; DiBona, 2002). AngII may therefore induce SNS hyperactivity through multiple mechanisms that act either peripherally at ganglionic neurons and target tissues, or centrally at the RVLM.

Hyperinsulemia has also been proposed as a factor contributing to the development of SNS hyperactivity. Insulin has been demonstrated to increase both sympathetic firing rates and NE secretion from nerve terminals (Anderson et al, 1991; Scherrer and Sartori, 1997; Tentolouris et al., 2008). Some evidence suggests, however, that increased activity occurs prior to insulin resistance in patients with primary, rather than secondary, hypertension (Mancia et al., 1999).

Multiple pieces of evidence indicate that SNS hyperactivity arises centrally in the hypothalamus or the brainstem (Esler, 2000; Colombari et al., 2001; Guyenet, 2006). Ferrier et al. (1993b) showed that hypertensive patients have increased NE spillover in
the subcortical forebrain regions, which include the hypothalamus, and that this spillover can be correlated with increased peripheral sympathetic firing rates. Furthermore, the injection of neurotransmitter antagonists in the NTS and RVLM results in decreases arterial pressure in rat models of hypertension (Smith and Baron, 1990; Allen, 2001; Colombari et al., 2001; Lin et al., 2005; Yajima et al., 2008). Increased oxidative stress (Hirooka, 2008; Nozoe et al., 2008; Chan et al., 2009) and altered gene expression (Reja et al., 2002; Aicher et al., 2003) in these brainstem nuclei have also been connected to increased arterial pressure in spontaneously hypertensive rats (SHR) compared to normotensive control rats.

High levels of sympathetic activity in hypertension may also result from impairment of peripheral sympathoregulatory pathways. The most notable example is the negative feedback provided by activation of high pressure arterial baroreceptors in the carotid sinus and aortic arch. As described above, under normal conditions an increase in blood pressure stimulates peripheral sensory receptors located in the artery wall which decreases firing rates in neurons of the RVLM, IML and peripheral ganglia. In order for hypertension to be sustained, this regulatory apparatus must be reset so that compensatory mechanisms are not triggered by increased arterial stretch (Biaggioni, 2003; Gracci 1998; Mancia et al. 1999). It remains unclear whether the baroreflex is dysfunctional in hypertensive patients or whether this reflex responds normally to changes in blood pressure but maintains “normal” pressure at higher set point. It is also unclear whether this change in responsiveness occurs prior to the development of hypertension (i.e. is genetically mediated) or in response to chronically elevated pressures (i.e. is environmentally mediated). Gracci et al. (1998) found no difference in baroreflex
responsiveness between normotensive and hypertensive patients, while other studies have found decreased baroreceptor inhibition of sympathetic activity in hypertensive humans (Matsukawa et al., 1991) and animals (Judy and Farrell, 1979; Thames et al., 1984).

SNS hyperactivity may also be due to decreased NE reuptake by the sympathetic nerve terminal. Studies have demonstrated decreased neuronal NE uptake in the heart of hypertensive patients (Esler et al., 2001; Biaggioni, 2003) and genetic polymorphisms have been found in the NE transporter in other autonomic disorders (Schroeder et al., 2002). Impaired NE uptake has also been shown in animal models including SHR (Cabassi et al., 2001). Although this would not explain increased rates of sympathetic nerve firing, it would contribute to NE spillover and to increased target tissue response to sympathetic stimulation.

Spontaneously Hypertensive Rats as a Model of Primary Hypertension

Spontaneously hypertensive rats (SHR) were established in 1963 by Okamoto and Aoki and have since become a well accepted model of primary hypertension. The strain was generated by breeding a male Wistar rat with spontaneous hypertension (150-175 mmHG) to a female rat with slightly elevated blood pressure (130-140 mmHG). F1 progeny showing spontaneous hypertension were then interbred. After several generations of selecting for high blood pressure, Okamoto and Aoki obtained a strain with approximately 100% occurrence of spontaneous hypertension by the F6 generation. As these animals were compared to a subset of Wistar rats maintained at the Animal Center Laboratory at Kyoto University, rather than to rats from the Wistar Laboratory,
experimental studies now compare SHR to normotensive control Wistar-Kyoto (WKY) rats instead of to standard Wistar rats.

SHR have become such an extremely valuable and widely used research model for genetically mediated hypertension because they exhibit many of the features which are characteristic of primary hypertension in humans, such as vascular rigidity, vascular remodeling, left ventricular hypertrophy, decreased NE reuptake, and increased peripheral and central sympathetic activity. SHR have increased sympathetic nerve firing rats (Judy et al. 1976; Judy and Farrell, 1979; Lundin et al., 1984; Cabassi et al., 2001) and elevated NE concentration in various tissues including mesenteric arteries (Head et al., 1985, 1989; Lee, 1985), kidneys (Donohue et al., 1988), skeletal muscle (Cabassi et al., 2001), and heart (Girouard et al., 2003) compared to normotensive WKY rats. Stimulation of nerves innervating the superior mesenteric arteries caused a greater increase in perfusion pressure and neurotransmitter release in isolated mesenteric artery beds from SHR than in beds from WKY (Byku et al., 2008), indicating that superior mesenteric nerve function is altered independently of signals from central sympathetic centers SHR. On the other hand, experimental procedures which inject neurotransmitter agonists into the brainstem to decrease sympathetic activity cause a greater decrease in blood pressure in SHR than in WKY rats (Smith and Baron, 1990; Colombari et al., 2001; Allen, 2001; DiBona and Jones, 2001; Shokoji et al., 2003; Lin et al., 2005; Guyenet, 2006; Yajima et al., 2008). This suggests that the elevated blood pressure in SHR is at least partially due to increased sympathetic drive originating from the CNS. Together, these results convincingly show that the sympathetic activity is increased
through both peripheral and central mechanisms in SHR and that this is important in the establishment and maintenance of hypertension in this model.

There is extensive evidence that this sympathetic hyperactivity contributes to the remodeling of resistance vessels in SHR. As in human hypertension, resistance vessels in hypertensive rats have a higher wall to lumen ration due to smooth muscle proliferation and thickening of the intima media (Scott and Pang, 1983; Intengen, 1999; Zhu et al., 2004). SHR have increased sympathetic innervation of these blood vessels prior to the establishment of hypertension (Lee et al., 1983; Scott and Pang, 1983; Head et al., 1989), and blood vessel denervation has been shown to lessen remodeling and moderate blood pressure (Cutilletta et al., 1977; Cutilletta and Oparil, 1980; Lee et al., 1987). One explanation for the increased sympathetic innervation of blood vessels in SHR is augmented expression of nerve growth factor (NGF) by vascular smooth muscle cells. NGF induces hypertrophy, neurite extension and sprouting of sympathetic neurons in vitro and is the primary growth factor responsible for guiding sympathetic fibers to their designated target tissues. Increased NGF protein (Donohue et al., 1989; Hamada et al., 1990) and mRNA (Falckh et al., 1992a, 1992b) synthesis has been shown in SHR mesenteric arteries compared to WKY arteries. The injection of anti-bodies against NGF lessens severity of the hypertensive phenotype in adult SHR (Cutilletta et al., 1977). Complete sympathectomy, achieved through the injection of both anti-NGF with guanethidine into neonatal rats, fully reduces blood pressure in SHR to control values and prevents blood vessel remodeling (Lee et al., 1987). In addition, Spitsbergen et al. (1995) found that cultured vascular smooth muscle cells from SHR secreted higher levels of NGF following stimulation than did similar cultures of WKY cells. Together, these
observations support the hypothesis that sympathetic hyperinnervation as a result of augmented NGF expression by resistance vessels plays an important role in the establishment of the hypertensive phenotype in SHR.

Oxidative Stress and Sympathetic Activity in SHR

The SHR model has allowed researchers to examine the causes of hypertension through experiments that could not be performed in humans, and as a result has greatly furthered our understanding of the molecular aspects of sympathetic hyperactivity. One example of this is the appreciation of the importance of oxidative stress in the development, maintenance and resulting tissue damage of hypertension. Oxidative stress is defined as an increase in the production of reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$) and superoxide anion (O$_2^-$), beyond the capacity of defensive antioxidant systems to neutralize and destroy these damaging molecules (Suematsu et al., 2002; Paravicini and Touyz, 2008; Puddu et al., 2008). This can be due to either an increase in ROS synthesis or a decrease in the activity of antioxidant enzymes such as catalase, superoxide dismutase, or glutathione reductase. ROS cause tissue damage through a number of mechanisms including, but not limited to, DNA mutation, protein modification, lipid peroxidation, and destabilization of signal transduction pathways that are dependent on the cytoplasmic reduction/oxidation (redox) balance (Masakitanito et al., 2004; Escobales and Crespo, 2005). In hypertension, the accumulation of these insults can lead to injury through increased apoptosis and necrosis (Kobayashi et al., 2003), smooth muscle hyperplasia (Escobales and Crespo, 2005), extracellular matrix remodeling in cardiovascular tissues (Lee and Greindling, 2008), vasoconstriction
(Nabha et al., 2004; MacArthur et al., 2007), sympathetic hyperactivity (Girouard et al.,
2003; Shokoji et al., 2003; MacArthur et al., 2008; Nozoe et al., 2008), mitochondrial
damage (Lopez-Carnpistrous et al., 2008; Puddu et al., 2007; Wei et al., 2008), and
inflammation (Suematsu et al., 2002; Biswas and Lopas de Faria, 2008; Sun et al., 2008).
It is well established that oxidative stress is increased in SHR relative to WKY rats
(Suematsu et al, 2002; Masakitanito et al., 2004; Nabha et al., 2004; Touyz, 2004; Biswas
and Lopas de Faria, 2008).

One example of a negative consequence resulting from increased oxidative stress
is the decrease in the bioavailability of nitric oxide (NO) (Nabha et al., 2004; Macarthur
et al., 2008; Paravicini and Touyz, 2008). NO is a signaling molecule which is present in
a wide variety of tissues and is involved in a variety of functions. Although a full review
of the importance of NO in hypertension is beyond the scope of this paper, a brief
summary of the role of NO in regulation of sympathetic activity seems warranted. NO is
released from endothelial cells and diffuses into smooth muscle cells where it promotes
relaxation and therefore counteracts the affects of NE released from sympathetic nerves
(Macarthur et al., 2007; Torok, 2008). Furthermore, Kolo and colleagues (2004) showed
that NO reacts with NE and prevents it from activating adrenergic receptors, which again
limits vasoconstriction following sympathetic nerve activation. Inhibition of nitric oxide
synthase (NOS), the enzyme with synthesizes NO, caused an increase in NE availability
following periarterial nerve stimulation in isolated mesenteric artery beds from WKY
rats, due either to an NO derived decrease in NE release from sympathetic nerve
terminals or promotion of NE degradation. Interestingly, this effect was not seen in
mesenteric artery beds from SHR. It was later shown that treating SHR mesenteric
vasculature with an antioxidant decreased NE release to levels similar to that of WKY tissues, and that the effect of the antioxidant was negated if it was used in combination with a NOS inhibitor (Macarthur et al., 2008). A plausible interpretation of these results is that the previously documented high levels of ROS in SHR mesenteric arterial arcades caused a decrease in levels of NO ($O_2^-$ reacts with NO to form peroxynitrite and NOS can become uncoupled in the presence of high ROS (Schulz et al., 2008)). Further inhibition of NOS, therefore, had no effect. Antioxidant treatment, however, lowered ROS and restored NO to control values, which allowed NO to counteract sympathetic activity by decreasing effective NE release as it had previously been shown to do in WKY arteries. Similar findings were reported by Girouard et al. (2003) who demonstrated that antioxidant treatment lowered plasma NE in SHR to values comparable to WKY, although no mechanism was provided. Gene transfer experiments have shown that over-expression of NOS controlled by a noradrenergic neuron specific promoter caused a decreased in NE release in atria from SHR compared to untreated WKY (Li et al., 2007); this suggests that NO modifies NE release from within sympathetic axons as well as from target tissues. Several studies have also provided evidence suggesting that increased oxidative stress and decreased NO bioavailability in the brainstem are involved in driving hyperactivity in the peripheral SNS (Hirooka, 2008; Nozoe et al., 2008; Torok, 2008; Chan et al., 2009). Together, these numerous studies exemplify the importance of oxidative stress in the regulation of sympathetic tone in SHR.

Non-Alcoholic Fatty Liver Disease
Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of excess triglyceride in the cytoplasm of at least 5% of hepatocytes with lipids accounting for 5-10% of total liver weight (normal is 1-2%) in patients who do not consume alcohol (Lonardo et al., 2005; Chueng and Sanyal, 2008; Wei et al., 2008; Paschos and Paletas, 2009). Histological features of NAFLD include cytoplasmic lipid droplets, hepatocyte ballooning, and enlarged mitochondria (Mehta et al., 2002). A more severe progression of this disease known as NASH (non-alcoholic steatohepatitis), which is seen in a minority of patients, is characterized by more severe lipid accumulation, enlarged mitochondria, inflammation in the form of Kupffer cell activation, hepatocyte necrosis and apoptosis, and fibrosis (Mehta et al., 2002; Paschos and Paletas, 2009). NAFLD is now generally accepted to be the hepatic manifestation of metabolic syndrome, which is a condition characterized by the combination of insulin resistance, visceral adiposity, hyperlipidemia, hyperglycemia, and hypertension. Insulin resistance and visceral adiposity are thought to be the most important factors contributing the development of NAFLD (Targher, 2007; Targher et al., 2008; Paschos and Paletas, 2009).

Insulin promotes the uptake of fatty acids from circulation and prevents fatty acid lipolysis in numerous cell types including hepatocytes, adipocytes, and skeletal muscle fibers. Following a meal, hepatocytes absorb circulating triglycerides, repackage them into very low density lipoproteins, and release them back into circulation for delivery to extra-hepatic tissues such as skeletal muscle and adipocytes (Boron and Boulpaep, 2003; Rector et al., 2008). During insulin resistance, however, adipocytes inappropriately maintain high postprandial levels of lipolysis and release of free fatty acids (FFA) (Rector et al., 2008; Paschos and Paletas, 2009). The ensuing hyperlipidemia exposes
hepatocytes to pathologically high concentrations of FFA (Targher et al., 2008; Paschos and Paletas, 2009), which causes these cells to take up triglycerides at a much faster rate than they can be repackaged and exocytosed (Mehta et al., 2007; Paschos and Paletas, 2009). As lipids are very hydrophobic, excess triglycerides coalesce into cytoplasmic droplets large enough to be viewed histologically and, in severe cases, grow so large as to displace hepatocyte nuclei and organelles to the periphery of the cell (Mehta et al., 2007). Large deposits of abdominal fat further exacerbate the problem since visceral fat is more biologically active and prone to insulin insensitivity than is subcutaneous fat (Rector et al., 2008; Targher et al., 2008; Paschos and Paletas, 2009). Furthermore, due its location, visceral fat releases large amounts of FFA directly into the portal vein, which exposes hepatocytes to even higher lipid concentrations (Nielsen et al., 2004; Paschos and Paletas, 2009).

Common Features of Primary Hypertension and NAFLD

Hypertension and NAFLD are both common features of the metabolic syndrome; patients with one of these pathologies are at a much higher risk than the general population of developing the other. 30% of people with hypertension exhibit signs of NAFLD as diagnosed by ultrasound and patients with hypertension are more likely to have hepatic steatosis than are normotensive controls, independent of insulin resistance (Donati et al., 2004; Brookes and Cooper, 2007). Patients with NAFLD exhibit vascular abnormalities normally associated with hypertension, such as thickening of the intima media of resistance vessels (Targher et al., 2006) and endothelial dysfunction (Villanova et al., 2005; Loria et al., 2008). Likewise, both primary hypertension and NAFLD are
associated with increased risk for developing more advanced forms of cardiovascular
disease, such as coronary artery disease, myocardial infarction and arteriosclerosis
(Villanova et al., 2005; reviewed in Targher, 2007; Loria et al., 2008). It has been
difficult to determine, however, whether hypertension and NAFLD are comorbidities
because they arise from common causes or whether there exists a more direct mechanistic
connection between increased blood pressure and hepatic steatosis.

Primary hypertension and NAFLD are currently thought to result from similar
physiological disturbances. Insulin resistance and visceral adiposity are strongly
connected to the development of both hypertension and hepatic steatosis (Targher et al.,
2008; Yanai et al., 2008; Paschos and Paletas, 2009). Furthermore, these two pathologies
have each been independently associated with sympathetic hyperactivity, oxidative stress,
mitochondrial dysfunction and inflammation. Increased sympathetic firing rates and
elevated tissue NE have been demonstrated in people with the metabolic syndrome,
especially in those with marked deposits of visceral fat (Tentolouris et al., 2008; Yanai et
al., 2008). Interestingly, sympathetic activity causes lipolysis in adipose tissue
(especially in visceral adipose which is more sensitive to NE than subcutaneous adipose),
which may contribute to hyperlipidemia and insulin resistance, although the mechanism
for this remains somewhat unclear (Tentolouris et al., 2008). SNS hyperactivity could
also potentially promote NAFLD through vasoconstriction of hepatic blood vessels,
which would decrease blood flow and consequently slow postprandial clearance of lipids.
Thus would theoretically maintain high lipid concentrations for longer time periods
(Esler, 2000).
Oxidative stress has been shown to play an important role in the development and progression of tissue damage in both primary hypertension and NAFLD. The effects of ROS in hypertension were described above; in NAFLD, elevated ROS production is a causative factor in the development of marked fibrosis and inflammation, which is characteristic of the transition from simple hepatosteatosis to steatohepatitis (Mehta et al., 2002; Videla et al., 2004a, 2004b; Albano et al. 2005; Cheung and Sanyal, 2008; Wei et al., 2008). Patients with NAFLD have increased protein oxidation and nitrosylation, which are markers of oxidative stress, compared to normal controls (Videla et al., 2004a, 2004b; Albano et al. 2005; Tilg and Moschen, 2008). NAFLD is also associated with lower overall plasma antioxidant capacity, decreased liver glutathione (GSH) content, and decreases in the activity of specific antioxidant enzymes such as superoxide dismutase and catalase (Videla et al., 2004b; Mehta et al., 2002). Biopsies taken from NAFLD patients have augmented hepatic lipid peroxidation, a common result of excess ROS, compared to controls (Videla et al., 2004a; Albano et al., 2005; Madan et al, 2006). Lipid peroxidation is associated with cell necrosis and selective decreases in polyunsaturated fatty acids (PUFA) compared to saturated fatty acids (Videla et al., 2004a). This last observation is important since PUFA play an important role in regulating lipid synthesis and storage in the liver through downregulation of enzymes involved in lipid synthesis and the upregulation of enzymes involved in FFA oxidation (Videla et al., 2004a). PUFA oxidation therefore represents a potential mechanism through which ROS overproduction could disrupt the regulation of lipid metabolism and induce lipid accumulation in hepatocytes. Oxidative stress and lipid peroxidation have also been shown to increase collagen synthesis and activation of Kupffer cells, which
together could be responsible for the cell necrosis, inflammation and fibrosis commonly seen in patients with NASH (Mehta et al., 2002; Videla et al., 2004a; Albano et al., 2005; Tilg and Moschen, 2008).

Mitochondrial dysfunction is a characteristic of both humans and animal models of NAFLD (Cheung and Sanyal, 2008; Wei et al., 2008). Liver biopsies present with ultrastructural abnormalities such as round mitochondria, low mitochondrial numbers and paracrystalline deposits in the mitochondrial matrix (Wei et al., 2008), as well as biochemical abnormalities such as decreased activity of mitochondrial respiratory enzymes and increased generation of O$_2^-$ (Cheung and Sanyal, 2008; Wei et al., 2008). As mitochondria are responsible for the majority of cellular FFA oxidation, impaired mitochondrial function leads to decreased FFA metabolism and, consequently, increased cytoplasmic lipid accumulation. It should be noted that not only are mitochondria a common source of ROS, both in health and disease, they are also extremely sensitive to oxidative stress. Mitochondrial DNA is not protected by histone proteins and is therefore prone to mutation (Puddu et al., 2007; Wei et al., 2008), which interferes with mitochondria replication and expression of mitochondrial proteins. Furthermore, the by-products of lipid peroxidation inhibit cytochrome c (Wei et al., 2008), which impedes activity of the respiratory enzymes residing in the inner mitochondrial membrane and increases the premature transfer of electrons to molecular oxygen, thus creating O$_2^-$.

Oxidative damage to mitochondria therefore initiates a positive feedback cycle which theoretically creates ever increasing amounts O$_2^-$ and culminates in physiological abnormalities and disease.
The presence of oxidative stress and mitochondrial dysfunction has been well established in human and animal hypertension, including the SHR model and oxidative stress in SHR was described in a previous section. ROS has been associated with vascular (Escobalas and Crespo, 2005; Kobayashi et al., 2005; Paravicini and Touyz, 2006) and renal damage (Touytz, 2004) and decreases in ROS in SHR are associated with decreased blood pressure and moderation of tissue damage (Shokoji et al., 2003; Nabha et al., 2005; Biswas and Lopas deFaria, 2007). Biochemical markers of mitochondrial dysfunction such as decreased abundance and activity of respiratory enzymes, decreased ATP synthesis and augmented mitochondrial generation of $O_2^-$ have also been experimental shown in SHR (Lopez-Campistrous et al., 2008; Puddu et al., 2008). Clearly, the SHR represents an effective model in which to study the connections between oxidative stress and disease.

Finally, an additional commonality between NAFLD and hypertension is the presence of inflammation. As has been previously mentioned, inflammation is a classic feature of NAFLD and is dramatically increased during the progression to NASH (Albano et al., 2005; Tilg and Moschen, 2008). Leukocyte infiltration and altered cytokine release have been documented in blood vessels in SHR (Suematsu et al., 2002; Biswas and Lopas deFaria, 2007) and markers of inflammation are increased in various tissues in SHR, including the liver (Sun et al., 2008).

Despite these similarities between NAFLD and primary hypertension, no clear mechanism linking the pathogenesis of NAFLD to hypertension has been established. Because of the inherent complexity of metabolic syndrome, it is very difficult to isolate cause and affect relationships between individual features of this disorder and clear
connections often remain elusive. The current thinking is that although primary hypertension and hepatic steatosis share many of the same predisposing factors, namely insulin resistance, oxidative stress, inflammation and sympathetic hyperactivity, hypertension does not directly cause NAFLD, and vice versa.

As previously described, SHR are a well accepted model of primary hypertension. Researchers studying the metabolic syndrome use different rat models, such as Zucker Fatty rats, obese stroke prone SHR (a cross between stroke prone SHR and Zucker Fatty rats) (Hiraoka-Yamamoto et al., 2003; Ueno et al., 2009), and ob/ob rats which lack leptin, which exhibit dramatic visceral adiposity, severe insulin resistance or diabetes, hyperlipidemia and NAFLD or NASH (Murase et al., 2005; Gudbrandsen et al., 2009). Several studies, however, indicate that hypertension, even “primary” hypertension, is inextricably linked to other aspects of the metabolic syndrome, both in humans (Donati et al., 2004; Brookes and Cooper, 2007; Cuspidi et al., 2008; Cheung, 2008; Fallo et al., 2008) and in animal models (Pravenec et al., 2004; Shimamoto and Ura, 2006). Furthermore, SHR have been shown to have insulin resistance (Shimamoto and Ura, 2006), excess visceral fat deposition, dyslipidemia and hepatic lipid accumulation (Rodriguez et al., 2008). Interestingly, some studies found plasma cholesterol to be lower in SHR (Kitts et al, 1998; Yuan and Kitts, 2003). These results suggest that the genetic features which cause hypertension in SHR may also contribute to other features of the metabolic syndrome (Pravenec et al., 2004).
Evaluation of the Liver in SHR

There have been very few studies regarding the effect of primary hypertension on the biology of the liver in SHR. Although it is generally accepted that SHR have altered hepatic lipid profiles and increased hepatic oxidative stress, there seems to be little interest in evaluating how these factors lead to alterations in gene expression or liver function in SHR relative to WKY. Ironically, changes in markers of hepatic lipid synthesis and oxidative stress are often used to quantitate the beneficial effects of novel therapies designed to treat hypertension (examples include Yahia et al., 2003; Gomez-Amores et al., 2006; Oh et al., 2007, 2008; Tada Y 2008), but these studies rarely include any comparison to WKY rats.

An exception to this paucity of information on liver function in SHR is the research regarding expression of transcription factors and enzymes involved in lipid synthesis as well as the activity of antioxidant enzymes. FFA transporter CD/36 (Bonen et al., 2009) was found to be upregulated in the liver and downregulated in skeletal muscle of SHR relative to WKY, while hepatic expression of lipogenic enzymes such as fatty acid synthase is increased in SHR (Ueno et al, 2009). Also, a mutation in the gene coding for SREBP1c (Pravenec et al., 2008), a transcription factor responsible for promoting synthesis of enzymes involved in cholesterol and triglyceride synthesis, was discovered in SHR. Surprisingly, this mutation led to decreased hepatic expression of SREPB1c as well as the enzymes it regulates (Pravenec et al., 2008). This may explain why SHR do not experience hypercholesteremia despite visible visceral adiposity. Lower levels of SREBP1c may also offer protection against hepatic steatosis and at least
partially explain the lack of histological characteristics of NAFLD in SHR despite the other predisposing factors already described.

Hepatic expression of antioxidant enzymes in the absence of complications of experimental therapies have only been characterized in of SHR and WKY rats in a few studies (Kitts et al., 1998; Yuan and Kitts, 2003). Catalase expression is increased in SHR, probably to compensate for increased production of $\text{H}_2\text{O}_2$, while there is no difference in levels of superoxide dismutase. Glutathione peroxidase expression is decreased, while glutathione reductase is increased, with no difference in total glutathione. This could represent an attempt to compensate for increased glutathione oxidation in a primarily oxidative environment.

*Proteomics – the SHR Model*

Clearly, primary arterial hypertension is a global condition with widely ranging effects in many, if not all, tissues and organ systems in the body. It is of especial importance to recognize this in SHR since this model differs from many other animal models since it is derived from a program of selective breeding for a specific phenotype rather than from a known genetic mutation or a physiological manipulation. This adds an additional level of complexity to SHR since there is likely to be a myriad of genetic changes which contribute to an accumulative phenotype. This view is supported by the fact that hypertension can be moderated or eliminated in SHR through a wide variety of treatments targeting the kidney, heart, vasculature or brain (Cutilletta et al., 1977; Lee et al., 1987; Dang et al., 1999; Shokoji et al., 2003; Lin et al., 2005; Yajima et al., 2008).

As a result of this complexity, there are probably numerous differences in the physiology...
and molecular biology between SHR and WKY rats which could not be predicted given our current understanding of the genetic factors contributing to primary hypertension. One strategy for discovering the unknown interconnections in a complex system is to use a technique which enables the analysis of global gene or protein expression without requiring that the investigator hypothesize which proteins will be altered. Such a technique is exemplified by proteomics.

Proteomics is the study of the complete protein content of a tissue or cell type. These methods allow investigators to simultaneously analyze the expression of hundreds to thousands of proteins without needing to pre-select which proteins to study. This allows the discovery of unexpected changes in undescribed or supposedly unrelated proteins. Proteomic studies are therefore a very useful means of improving our understanding of the more subtle and less obvious aspects of disease models.

Only a few studies have used proteomics to compare protein expression between tissues of SHR and WKY rats. Most of these projects have focused on the cardiovascular system; Jin and colleagues (2006) examined the hypertrophied left ventricle in SHR and WKY rats, as well as SHR treated with anti-hypertensive drugs, to isolate the protein changes associated with ventricular hypertrophy from genetic features of SHR that do not result directly from hypertension. Zhou and colleagues (2005) used a similar approach to determine changes in protein expression specifically dependent on ventricular hypertrophy by comparing the left ventricle proteomes from SHR, WKY and WKY with induced renovascular hypertension, which exhibit hypertension without hypertrophy. Both of these studies found evidence of a shift from oxidative to glycolytic metabolism, increased oxidative stress and altered mitochondrial function in SHR compared to WKY.
rats. Similarly, Junhong et al. (2008) compared the left ventricle of renovascular hypertensive rats with control rats and found similar changes in metabolic proteins and evidence for decreased energy production in hypertensive hearts, but failed to find evidence of increased oxidative stress as has been repeatedly shown in SHR. This indicates that elevated ROS production results from some physiological and/or genetic abnormality in SHR rather than simply from the mechanical stress inherent to increased blood pressure. Bian et al. (2008) examined protein expression in the aorta of SHR and WKY rats and found evidence for increased activation of angiotensin II receptors.

Other research groups have focused on subsets of the left ventricular proteome such as cardiac mitochondria (Jullig et al., 2008) or phosphorylated proteins (Jin et al., 2008). Changes in cardiovascular protein expression have also been studied in other models of genetically or pharmacologically induced arterial hypertension (Cheon et al., 2008; Delbosc et al., 2008; Addabo et al., 2009).

Two other studies have used proteomics to look at protein expression in SHR outside the cardiovascular system: Lopez-Campistrous and colleagues (2008) found evidence of mitochondrial damage in an analysis of brain mitochondria and Tyther and colleagues (2007) examined protein nitrosylation in the SHR kidney. Tian and coworkers (2008) also investigated the renal proteome of Dahl salt-sensitive rats, which is another model of genetic hypertension. Surprisingly, there has not been any proteomic evaluation of whole tissue protein expression in the kidney of SHR and WKY rats. A few studies have compared protein expression between SHR and stroke-prone SHR (SPSHR), a strain of rat derived from SHR (Sironi et al., 2001, 2004; Chiga et al., 2008). Overall, the
analysis of SHR protein expression using proteomic techniques has been surprisingly limited.

Proteomics – Techniques

Proteins are the crux of functional biochemistry and the final products of gene expression. A tissue or cell proteome is the collection of all proteins expressed by that unique tissue or cell type under a given set of conditions; it encompasses all the information passed down through transcription, mRNA processing, translation and post-translational modifications, as well as information from the extracellular environment that reaches the cytoplasm through signal transduction events. All forms of regulation imposed upon any of these processes, including chromatin remodeling, alternative splicing and protein phosphorylation, are theoretically detectable in and exert their influence at the level of the cellular proteome. Knowledge of the proteome of an isolated cell type therefore imparts invaluable information regarding cellular phenotype and function.

Proteomics is the study of proteomes, be they from cells or tissues, from organelles or intact cells, or from plants, animals, or microbes. Proteomic methods can be used to characterize a sample by listing the proteins which are present, or, more commonly, these techniques can be used to compare protein expression in two similar samples derived from different genetic or environmental backgrounds. Examples include comparisons between healthy and diseased tissue, between transgenic and wild type animal stains, between different developmental stages, or between cell cultures raised under variant conditions.
The most important strength of proteomics lies in its ability to detect changes in protein expression without having to predict which proteins will be affected by the experimental conditions (Rabilloud, 2002; Gorg et al., 2004; Rabilloud et al., 2008). This feature enables the discovery of novel protein functions, interactions, or modifications not previously known to be involved with the research model in question. Proteomics also allows the analysis of large numbers of proteins simultaneously (Rabilloud, 2002; Gorg et al., 2004; Rabilloud et al., 2008). In this regard, proteomics is very similar to genomics, which is the study of the gene expression through the analysis of total mRNA. Proteomics, however, is able to detect aspects of protein regulation that cannot be studied through analysis of gene expression alone. As proteins, rather than mRNA, mediate cellular functions, studies which focus on mRNA make the inherent assumption that protein expression mirrors mRNA synthesis (Faber et al., 2006). Although this position is true in broad strokes, it does not take into consideration numerous regulatory processes which modify protein expression downstream of transcription such as different rates of protein translation, variations in mRNA stability, co-translational or post-translational modification of proteins, or targeted protein degradation. Proteomic methods are therefore required to gather information regarding the complete set of factors which come together to influence protein, and thus cellular, physiology.

There are other fundamental differences between proteomic and genomic studies which are important when deciding how best to analyze a sample or when comparing the results of different experiments run on similar tissues. Primary among these differences is the fact that proteomics examines what is currently present within a sample while genomics examines what is currently being synthesized. This is an important distinction,
especially when examining tissues at different developmental stages where temporal regulation of gene expression is of utmost importance. Very stable proteins with low turnover rates would be well represented in proteomics datasets but may be omitted from genomic datasets. Likewise, secreted proteins which are no longer present in the sample but synthesized at a high rate would be included in genomic datasets but maybe not in proteomic datasets (such proteins would appear in the proteomes, but not the transcriptomes, of the tissues in which they accumulate). It is therefore necessary to use both genomic and proteomic strategies to get a complete picture of the gene expression in a sample.

Another key difference between proteomic and genomic experiments lies in use of PCR to amplify mRNA signal. This allows a large signal to be attained from a very small amount of sample. As there is no analogous means of amplifying protein (Rabilloud, 2002; Gorg et al., 2004), much larger amounts of tissue are required for proteomics than for genomics. This is of especial importance when examining low abundance proteins such as signaling molecules and transcription factors.

One of the original and most popular techniques developed for the analysis of proteomes is 2-dimensional gel electrophoresis (2DGE) (reviewed in Rabilloud, 2002; Gorg et al., 2004; Vercauteren et al., 2007). This method couples the separation of proteins according to isoelectric point (pI) in the first dimension to separation according to molecular weight (M_r) in the second dimension. The result is a 2-dimensional grid of spots in which each spot theoretically represents a unique protein. The utility of 2DGE was greatly enhanced through developments in protein identification using mass spectrometry (MS).
2DGE begins with tissue homogenization using chemical and mechanical means such as osmotic cell lysis and tissue grinding in liquid nitrogen, respectively. Proteins are solubolized using denaturing buffers which maintain protein charge while disrupting the hydrogen bonds and disulfide bridges which maintain protein-protein interactions and tertiary structure. The extracted proteins are then loaded onto an immobilized pH gradient (IPG) gel strip and separated according to pI by running an electrical current across the strip, a process known as isoelectric focusing (IEF). The focused strip is then laid across the top of an acrylamide gel and a current is again used to transfer the proteins from the strip to the gel and to separate the proteins according to Mr, just as is done in traditional 1-dimensional electrophoresis. The gels are then stained to visualize the protein spots and allow comparison of gels displaying different samples. The protein located in a specific spot is identified by manually excising the spot from the stained gel, digesting the protein in the gel plug to its component peptides, and analyzing the peptides using MS.

Like any technique, 2DGE has specific strengths and weaknesses which must be taken into consideration when planning an experiment (Graham et al., 2005a; Faber et al., 2006; Vercauteren et al., 2007). One of its most important strengths is that 2DGE allows quantitative assessment of protein abundance rather than qualitative detection of protein presence or absence. This allows researchers to examine changes in protein expression which are more subtle than the turning on or off of a gene.

Another key advantage of 2DGE over other proteomic techniques is the ability to detect post-translational modifications (PTMs) (Rabilloud, 2002; Gorg et al., 2004; Graham et al., 2005a). PTMs represent a powerful biological mechanism for regulating
protein activity, structure and function. Examples of common modifications include polypeptide cleavage or the covalent attachment of functional groups such as phosphorylation, glycosylation, methylation, ubiquitination, or acetylation. Any modification which alters the pI or the Mr of the protein will be viewed as a shift in spot location in either the horizontal or vertical axis, respectively. Following 2DGE, modified forms of the protein can be excised and analyzed using tandem MS to determine the nature and location of the PTM within the amino acid sequence (Graham et al., 2005a).

One of the main limitations of 2DGE is the low solubolization of hydrophobic proteins (Rabilloud, 2002; Gorg et al., 2004; Vercauteren et al., 2007; Rabilloud et al., 2008). Because homogenization buffers must maintain protein charge in preparation for IEF, these buffers must employ relatively mild denaturing agents and detergents. Urea, the most popular IEF-compatible denaturing agent, effectively disrupts hydrogen bonds but is much less effective at interrupting hydrophobic protein interactions (Gorg et al., 2004). SDS, on the other hand, which is a powerful anionic detergent that coats hydrophobic proteins with negative charges and pulls them into solution, cannot be used since it interferes with IEF. Due to these limitations, hydrophobic, integral membrane proteins often fail to be extracted from sample tissues and are consequently dramatically under-represented in 2D gels. In an attempt to address this issue, extensive research has gone into the identification of other denaturing agents which improve solubolization of membrane proteins without altering protein charge. Rabilloud and colleagues (2008) have shown that combining thiourea with urea in homogenization buffers improves the disruption of hydrophobic interactions. Zwitterionic detergents such as CHAPS and sulfobetaines have also been shown to improve representation of membrane proteins on
2-D gels (Gorg et al., 2004, Rabilloud et al., 2008). Despite these developments, analysis of membrane proteins using 2DGE remains incomplete.

The apparent incompatibility of IEF with the solubolization of hydrophobic membrane proteins has inspired the development of gel-free proteomic procedures that eliminate IEF altogether (reviewed in Graham et al., 2005a; Roe and Griffin, 2006). Mudpitt, a technique which couples biphasic liquid chromatography to MS/MS has been successfully used to analyze protein expression in shotgun proteomics experiments. Advantages of Mudpitt compared to 2DGE include higher representation of membrane proteins, identification of a greater number of proteins, and greater potential for automation. When combined with isotope-coded affinity tags, Mudpitt allows the quantitative assessment of protein abundance. However, as Mudpitt requires that proteins be digested into their composite peptides prior to analysis, it is not able to detect PTMs.

Another challenge associated with 2DGE is the visualization of low abundance proteins. The difference in concentration between the most abundant protein and the least abundant protein, known as dynamic range, is much larger in cells and tissues (there are around $10^5$ molecules per cell of the most concentrated protein for every one molecule of the least concentrated protein) than even the most sensitive stains used in 2DGE can detect (silver stain and Deep Purple fluorescent stain both have a dynamic range of $10^4$) (Rabilloud, 2002). Furthermore, even if low abundance proteins are seen as faint spots on 2D gels, there is often too little protein present to identify the spot using MS. The result is that expression of low abundance proteins, such as transcription factors and signaling molecules, are very difficult to analyze using 2DGE. One way to overcome
this limitation is to fractionate the sample proteins according to hydrophobicity, subcellular localization, or protein pI (Rabilloud, 2002; Gorg et al., 2004; Graham et al., 2005a). For example, a sample can be consecutively treated with two or three different homogenization buffers containing progressively stronger detergents to separate hydrophilic cytoplasmic proteins from hydrophobic membrane proteins. One can also fractionate a sample by isolating a specific organelle through centrifugation on a sucrose gradient. Mitochondrial proteomes, for example, have been extensively studied using this technique. These types of prefractionation simplify the examined proteome and selectively enrich the sample for groups of proteins with common characteristics. Because the number of different proteins is decreased, low abundance proteins within a fraction represent a greater percentage of the total protein load and are therefore easier to detect using 2DGE.

Another way to fractionate a sample is by running it on multiple narrow range IPG strips (Rabilloud, 2002; Gorg et al., 2004). IPG strips can be made for wide pI ranges (e.g. pH 3-10) which essentially encompass the entire physiological protein pH range, medium ranges (e.g. pH 4-7 or pH 7-11) which cover either the acidic or basic end of the proteome, narrow ranges (e.g. pH 4.7-5.9 or pH 6.3-8.3) or ultra-narrow ranges (less than one pH unit). Narrow range strips allow higher protein loads than wide range strips since only a small portion of the total protein will actually be focused on the gel during IEF. By enriching for a small portion of the proteome, researchers are able to zoom in on a subset of proteins, which amplifies the signal of each individual spot and increases protein separation. One drawback of fractionation is that it requires large
amounts of tissue, which makes in depth proteome analysis impractical for certain tissues.

Despite these limitations, 2DGE is a powerful tool for proteome analysis. It allows simultaneous visualization of the relative abundance of hundreds to thousands of proteins and is well suited for the discovery of unexpected changes in protein expression. For these reasons, we used 2DGE coupled with MS to compare the proteomes of the superior mesenteric ganglion (chapter 2) and the liver in (chapter 3) from SHR and WKY rats.
Chapter 2
Proteomic Analysis of Sympathetic Ganglia of Spontaneously Hypertensive Rats

Introduction

Hyperactivity of the sympathetic nervous system (SNS) plays a crucial role in the pathogenesis of primary, or “essential”, hypertension in humans. The SNS is composed of both central and peripheral circuits. Neurons in the brainstem and hypothalamus project down to pre-synaptic neurons in the intermediolateral column of the spinal cord, which in turn project axons to post-synaptic neurons located outside the CNS in sympathetic ganglia (Robertson et al., 1996; Reja et al., 2002; Boron and Boulpaep, 2003; Guyenet, 2006; Low and Benarroch, 2008; Allen et al., 2009). These ganglionic neurons then send processes out to a variety of target tissues including blood vessels, the kidney, and the heart. The SNS regulates blood pressure through heart rate elevation (Iaccarino, 2001; Boron and Boulpaep, 2003; Guyenet, 2006), increased renal reabsorption of sodium and renin secretion (DiBona 2002; Grisk, 2004), and constriction of blood vessels (Boron and Boulpaep, 2003; Guyenet, 2006).

Sympathetic hyperactivity in human hypertension is well documented. SNS activity can be assessed clinically through measurement of firing rates in peripheral sympathetic efferent nerve fibers innervating the vasculature that supplies skin or skeletal muscle using a technique known as microneurography (Grassi, 1998; Mancia et al., 1999; Esler, 2000; Guyenet, 2006). Sympathetic activity can also be indirectly evaluated by determining the amount of norepinephrine (NE), the primary neurotransmitter released from sympathetic nerves, in blood draining a specific body region (Grassi, 1998; Mancia
et al., 1999; Esler, 2000; Guyenet, 2006). This second method has been useful in assessing sympathetic activity to vital organs where direct measurement of sympathetic firing rates would be too invasive. Clinical studies have shown that patients with primary hypertension have significantly increased sympathetic firing rates compared to normotensive controls as well as increased NE spillover to the heart and kidney (Matsukawa et al., 1991; Grassi, 1998; Mancia et al., 1999; Esler, 2000; Guyenet, 2006). Additionally, sympathetic firing rates are increased in young, normotensive patients with a family history of hypertension compared to age-matched normotensive patients with no family history of cardiovascular disease (Ferrier et al. 1993a). These observations suggest that sympathetic activity has a genetic component and may be a reasonable predictor of the likelihood of developing hypertension in the future, although no long-term studies have been carried out. Together, these results illustrate the importance of studying the SNS as a means of understanding hypertension.

Elevated SNS activity has also been shown in animal models of primary hypertension such as the spontaneously hypertensive rat (SHR). As in humans, SHR exhibit increased sympathetic firing rates (Judy et al. 1976; Judy and Farrell, 1979; Lundin et al., 1984; Cabassi et al., 2001) and high tissue concentration of NE (Head et al., 1985, 1989; Lee, 1985; Donohue et al., 1988; Girouard et al., 2003) compared to normotensive, control Wistar-Kyoto (WKY) rats. Increased activity has also been documented in regions of the medulla oblongata which are known to regulate sympathetic activity, such as the caudal pressor area and solitarius nucleus, in SHR relative to WKY (Smith and Baron, 1990; Colombari et al., 2001; Allen, 2001; DiBona
and Jones, 2001; Shokoji et al., 2003; Lin et al., 2005; Guyenet, 2006; Yajima et al., 2008), which may explain the source of increased sympathetic drive.

SHR exhibit an increased density of sympathetic fibers innervating muscular resistance blood vessels (Lee et al., 1983; Scott and Pang, 1983; Head et al., 1989). This hyperinnervation is established prior to elevations in blood pressure and is associated with blood vessel remodeling through hyperplasia of the smooth muscle of the intima media, resulting in an increased wall to lumen ratio, as well as changes in the organization of extracellular matrix proteins (Lee et al., 1983; Scott and Pang, 1983; Head et al., 1989; Integen, 1999; Zhu et al., 2004). Denervation of blood vessels in SHR has been shown to prevent or even reverse these anatomical changes, as well as to avoid increased blood pressure relative to WKY (Cutilletta et al., 1977; Cutillet and Oparil, 1980; Lee et al., 1987). It is important to note that vasomotion of these resistance arteries represents the main effector organ of the baroreflex, which regulates blood pressure in the short term, and possibly the long term as well (Robertson et al., 1996; Aicher et al., 2003; Boron and Boulpaep, 2003; Low and Benarroch, 2008). These findings suggest that remodeling is a direct result of hyperinnervation, although they do not eliminate the possibility of other contributing factors.

In SHR, peripheral sympathetic neurons are exposed to several physiological factors different from those experienced by peripheral neurons of WKY rats. These factors include, but are not limited to, increased stimulations from sympathetic centers in the brain and spinal cord, hormonal differences (increased renin and angiotensin II, for example) (Allen, 2001; DiBona and Jones, 2001; DiBona, 2002), higher levels of oxidative stress (Suematsu et al, 2002; Masakitanito et al., 2004; Nabha et al., 2004;
Touyz, 2004; Biswas and Lopas de Faria, 2008), increased action potential firing rates of the peripheral neurons themselves, and axonal sprouting associated with target tissue hyperinnervation. It is therefore reasonable to suspect that these environmental differences either derive from or result in changes in the synthesis or activity of certain proteins in the SHR peripheral sympathetic neurons compared to WKY neurons.

The superior mesenteric ganglion (SMG) is located in the abdominal cavity and innervates the smooth muscle in the mesenteric arteries as well as the neurons of the enteric nervous located in the wall of the digestive tract. The mesenteric arteries are important for the regulation of blood pressure because these blood vessels can serve as reservoirs during rest and hold large volumes of blood; constriction of the mesenteric arteries, as would occur in response to sympathetic hyperactivity in the SMG, moves blood from this reservoir into the arterial vascular system and thus increases arterial blood pressure (Robertson et al., 1996; Low and Benarroch, 2008). Furthermore, the hyperinnervation of the mesenteric arteries in SHR is assumed to be due to sprouting of axons projecting from SMG neurons. The SMG therefore represents a population of postsynaptic sympathetic neurons which are important for the regulation of blood pressure and experience hyperactivity in the SHR model. For these reasons, the SMG was used to search for differences between the ganglionic proteomes between SHR and WKY rats using two-dimensional gel electrophoresis (2DGE) and mass spectrometry. The proteome of the superior cervical ganglion (SCG) of SHR and WKY rats was also examined to indicate if changes were specific to the neurons of the SMG or were more broadly expressed in multiple sympathetic ganglia.
Methods

Animals

A total of twenty-five male SHR and twenty-five male WKY rats were purchased from Charles River Incorporated. Sixteen rats of each genotype were aged to 16 weeks and the remaining nine rats of each genotype were aged to 22 weeks. The 16wk old rats were ordered in two separate cohorts of eight rats each (per genotype) and the 22wk old rats were ordered as a single cohort. Animals were housed in pairs under low stress conditions and were fed standard rat chow. All methods were approved by the Queen’s University Animal Care Committee, following the guidelines set forth by the Canadian Council on Animal Care.

Tissue Collection

Sixteen 16 week old (16wk) SHR and sixteen 16wk WKY rats were anesthetized using cocktail of ketamine (5.6mg/kg), Rompun (4mg/kg) and acepromazine (0.75mg/kg). Eleven rats of each strain (six from the first cohort and five from the second cohort) were transcardially perfused with 200 mL of phosphate buffered saline (PBS) or until buffer ran clear. The SMG and SCG of each animal were removed and snap frozen in liquid nitrogen (-196°C). Tissue was stored at -80°C until further processing for proteomics. Five 16wk rats of each strain (two from the first cohort and three from the second cohort) were transcardially perfused with 200 mL 4% (w/v) paraformaldehyde in PBS for immunohistochemistry. The SMG and SCG were removed and placed in 4% (w/v) paraformaldehyde overnight and then transferred to 20% sucrose for 48 hours. Cryo-protected tissues were then snap frozen and cut in 20 μm sections for immunostaining.
Livers were also collected from ten 16wk rats of each genotype, including rats from both cohorts, in anticipation of the project described in Chapter 3.

Six 22 week old (22wk) SHR and six 22wk WKY rats were sacrificed as described above and transcardially perfused with PBS for proteomics. The SMG and SCG were removed from each animal and snap frozen in liquid nitrogen. Three 22wk rats of each genotype were sacrificed and transcardially perfused with 4% (w/v) paraformaldehyde in PBS for immunohistochemistry. The SMG and SCG were removed from each animal and processed as described above.

**Proteomics**

*Isoelectric Focusing*

Frozen SMGs were pooled to maximize protein yield according to genotype and age of rats. For the 16wk rats, ganglia from rats of different cohorts were processed separately. Ganglia were homogenized using a glass tissue grinder in whole tissue homogenization buffer containing 8M urea, 2M thiourea, 4% w/v CHAPS in 15 mM Tris (pH 7.0). Samples were centrifuged for 15 minutes at 170,000 g and supernatants were collected and stored at -80°C. SCGs were then pooled and processed in exactly the same way. Protein concentration of each sample was determined using the 2-D-Quantification Kit (Amersham Biosciences, Baie d’Urfe, QC, Canada) and these values were verified using a Western blot for actin (see below for procedure). 250 ug of sample protein was reduced using 1% (w/v) DTT for 15 minutes and diluted to 450 uL in rehydration buffer containing 8M urea, 2M thiourea, 4% (w/v) CHAPS, 2% (v/v) HED and 0.5% ampholytes (pH 4-7). 24cm (pH range 4-7) immobilized pH gradient (IPG) strip gels
(Amersham) were rehydrated overnight at room temperature in the buffer containing the sample protein. Isoelectric focusing was carried out on a Protean IEF cell machine (BioRad; Mississauga, ON, Canada) according to the following program: 8 hrs at 250V; 1 hr at 500V; 1 hr at 1000V; 3 hr linear ramping to 10,000V; 65 kVhr at 10,000V. Focused IPG strips were stored at -80°C to prevent protein diffusion. A minimum of six IPG strips were prepared for each sample.

2 Dimensional Gel Electrophoresis

2-D sodium dodecyl sulfate polyacrylamide gel electrophoresis (2-D SDS-PAGE) was carried out according to Graham et al. (2005b). Focused IPG strips were treated for 20 minutes in equilibration buffer containing 8M urea, 30% (v/v) glycerol and 2% SDS in 50 mM Bis-Tris (pH 8.8), supplemented with 1% (w/v) DTT and again for 20 minutes in equilibration buffer supplemented with 2.5% (w/v) iodoacetamide. Equilibrated strips were placed on top of 1mm thick 10% acrylamide gels (with Bis-Tris (pH 7.4)) and embedded using 0.5% (w/v) low-melt agarose in electrophoresis buffer. Large format 2-D SDS-PAGE electrophoresis was carried out in a DALT6 apparatus (Amersham) in MOPS (3-(n-morpholino) propanesulfonic acid) electrophoresis buffer and run at 80V at 4°C overnight. Except where described below, gels were visualized using the silver stain according to Shevchenko et al. (1996) and scanned using a UMAX Powerlook2100XL. Gels were dried between porous cellophane sheets (Biorad) for spot excision and long-term storage.
Mass Spectrometry

Protein samples were prepared for mass spectroscopy according to McDonald et al. (2008). All consistent spot differences between gels from the SMG or SCG of SHR and WKY rats (i.e. spot changes found in every gel from every animal of a given phenotype) were manually excised from the gels, robotically rehydrated and destained with aqueous 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. Gel plugs digested with 6 ng/mL trypsin (sequencing Grade-modified trypsin; Promega, Napean, ON, Canada) for 5 hours at 37°C. Peptides were extracted using 1% formic acid/2% acetonitrile followed by 50% acetonitrile. Extraction solutions were combined and evaporated down to 10 uL. Peptides were then spotted onto a MALDI target plate on top of a recrystallized spot of alpha-cyano-4-hydroxy-cinnamic acid matrix and spectra were obtained using a Voyager DE-Pro matrix-assisted laser desorption ionization time of flight mass spectrometer (MALDI-TOF MS; PerSeptive Biosystems, Framingham, MA, USA). The resulting peaks were analyzed using Data Explorer software; spectra were adjusted to baseline, noise filtered, externally calibrated against a spectrum of a known 4-peptide mix and internally calibrated against Trypsin I and Trypsin III peaks, when present (see green stars in Figure 2). Peptide masses were manually selected and the resulting mass lists were searched against the NCBI database using the peptide mass fingerprinting MASCOT PMF search engine (http://www.matrixscience.com). Spectra which did not contain adequate peaks to get a statistically significant hit (significance as determined by MASCOT) were ZIP-TIPPED (Millipore Corporation, Bedford MA, USA) and eluted into 70% acetonitrile in 0.1% TFA in ddH2O to purify and concentrate the peptides and new MS spectra were collected. Only protein identifications with statistically significant
MASCOT scores and whose predicted molecular weight and pI values approximately matched those of the spot in question were included.

*Western Blot*

For 1D western blots, 5 μg of protein per sample was diluted to 25 μL in loading buffer and reduced using DTT for 15 minutes. Samples were then loaded onto 10% acrylamide gels overlayed with 5% acrylamide stacking gel. Gels were run in MOPS buffer using the Mini-Protean tetra-Electrophoresis system (Biorad) at 100V at room temperature until the dye front reached the bottom of the gel. The Precision Plus All-Blue molecular weight ladder (Biorad) was used to determine protein molecular mass. Protein was then transferred onto nitrocellulose membranes in CAPS (3-(Cyclohexylamino)-1-propanesulfonic acid) buffer in the Mini-TransBlot cell (Biorad) overnight at 23V at 4°C. Membranes were blocked in Western Blocking Solution (WBS; Amersham) for one hour at room temperature and then incubated in one of the following primary antibodies at room temperature for two hours: goat anti-actin (1/10,000; Santa Cruz Biotechnologies, Santa Cruz USA), rabbit anti-rat serine protease inhibitor 2 (Spi 2.1/2.2; 1/10,000; obtained from Dr. Chow), rabbit anti-mouse haptoglobin (Hp; 1/10,000; CalBioreagents, San Mateo, CA, USA), goat anti-apolipoprotein A-1 (ApoA1; 1/1000; Santa Cruz) for one hour at room temperature, or with rabbit anti-predominantly fetally expressed T1 domain (pfetin; 1/1000; obtained from Dr. Morton) for 12 hours at room temperature. Membranes were then washed in TBST and incubated in WBS containing one of the following secondary antibodies for one hour at room temperature: goat anti-rabbit (1/10,000; HRP conjugated; Jackson Immunoresearch, West Grove, PA, USA), rabbit
anti-goat (1/10,000; HRP conjugated; Jackson Immunoresearch). Membranes were then treated with Chemi-Illuminescence (Amersham) according to the manufacturer’s protocol.

For 2D Western blots showing expression patterns of pfetin and Hp in the SMG, 20μg of protein per sample was diluted to 450ul in rehydration buffer and run out on 24-cm IPG strips (pH 4-7; Amersham) as described above. A 7.0cm section of the focused, equilibrated strip which contained the spots identified as pfetin, as well as actin, was cut out and laid on top of a 7cm long, 1mm think 10% acrylamide gel and embedded with 0.5% low melting point agarose in MOPS buffer. Gels were run at 100V as described and transferred onto nitrocellulose membranes. Membranes were blocked and treated with anti-pfetin, anti-Hp and anti-rabbit antibodies as described.

For 2D Western blots showing expression patterns of Spi 2.1 in the SMG, the top right corner of silver stained, large format gels containing the spots identified as Spi 2.1 and Spi 2.2 was cut out and destained (citation). Protein on the destained gels was then transferred to nitrocellulose membrane and exposed to anti-Spi 2.1/2.2 IgG as described.

As Spi 2.1, Hp and ApoA1 are synthesized and secreted by the liver, liver samples were collected and prepared for proteomic analysis as described above. Protein expression was analyzed using 1D and 2D blots using the same antibodies as were used for SMG Westerns. For 2D Western blots of Spi 2.1 and Hp, 30ug of protein was focused on IPG strips (24cm pH 4-7) and 7cm sections covering approximately pH 4.5-5.5 were run on 10% acrylamide gels and transferred to nitrocellulose membranes.
**Immunohistochemistry**

For validation of proteomic data, fluorescent immunostaining was used to visualize and localize the expression of pfetin, RKBP and ApoA1, while chromogenic immunostaining was used to visualize expression of Hp. SMGs and SCGs were placed in 4% (w/v) paraformaldehyde overnight and then transferred to 20% sucrose for at least 48 hours. Cryo-protected tissues were then snap frozen and cut in 20um sections. Sections were stored at -20C until further use. For fluorescent immunostaining, SMG and SCG slides were blocked for 1 hour in 10% Normal Donkey Serum (NDS; Jackson Immunoresearch) in 0.25% TBX and then incubated with rabbit anti-pfetin (1/1000), rabbit anti-Spi 2.1/2.2 (1/5000), or goat anti-ApoA1 (1/200) in 3% NDS for 72 hours at room temperature. Each antibody was used to stain tissue from a minimum of three unique animals with at least one animal from each cohort. Slides were washed for 5 minutes in 0.1M Tris buffer and incubated in donkey anti-rabbit IgG (1/200 FITC-conjugated; Jackson) in 3% NDS for two hours at room temperature. The specificity of all antibodies was tested by running slides under the following control conditions: primary IgG without secondary IgG; secondary IgG without primary IgG; correct primary IgG with incorrect secondary IgG. Coverslips were applied using DAPI-conjugated mounting media (Vector Laboratories; Burlington, ON, Canada) to visualize nuclei and images were acquired using a Zeiss fluorescence microscope and Axiovision software.

For chromogenic immunostaining, ganglia and liver sections were fixed for 30 minutes in 4% (w/v) paraformaldehyde in PBS. Slides were then treated for 1 hour in 0.3% (v/v) hydrogen peroxide and blocked for 1 hour in 10% bovine serum albumin (BSA) in 0.25% TBX. Tissue sections were then incubated with rabbit anti-mouse Hp
IgG for 72 hours at room temperature. Slides were next incubated in goat anti-rabbit biotinylated IgG (1/200; Jackson Immunoresearch) in 3% BSA for two hours at room temperature. The specificity of the Hp antibody was tested by running slides under the following control conditions: primary IgG without secondary IgG; secondary IgG without primary IgG; correct primary IgG with incorrect secondary IgG. Slides were then treated for two hours with ABC (Vector Laboratories) for two hours, followed by DAB for three minutes. Slides were finally dehydrated in an ethanol series followed by washes in HistoClear®. Coverslips were applied using Permount mounting media and images were acquired using a Zeiss microscope and Axiovision software. Sections of liver tissue were also chromogenically stained with the goat anti-ApoA1 IgG and rabbit anti-Spi 2.1/2.2 as described to serve as controls for the effectiveness of these antibodies.

**Results**

No spot differences were observed between SMG and SCG gels (allowing for a normal degree of inter-gel variation) for both the WKY and SHR phenotypes. The term SMG will henceforth be used to refer to results found in both the SMG and the SCG except where specified.

Thirteen spot differences were identified between SMG gels from 16wk SHR and WKY rats (Figure 1A, B); all of the differences found between 16wk SHR and WKY SMG gels were also found in 22wk SMG gels (Figure 1C, D). Example MS spectra for the proteins identified from 16wk gels are shown in Figure 2. These differences translated to three unique protein identifications: predominantly fetally expressed T1 domain containing protein (pfetin, two spots; Figures 3 and 4), serine protease inhibitor 2.1 (Spi
2.1, eight spots arranged in a line; Figures 5, 6 and 7) and haptoglobin (Hp, three spots; Figures 8, 9 and 10). Protein identification data is presented in Table 1. Five additional spots could not be identified due to low abundance (Figure 1A, B, box 2 and box 4). Because of similarities in spot size and intensity, two pairs of spots appear to represent proteins that shift location due to changes in pI.

There were seven additional spot changes on the 22wk gels not present on the 16wk gels (Figure 1C, D); one of these spots was successfully identified as apolipoprotein A1 (ApoA1; Figure 1C, D, box 7 and Figure 11). The protein abundance of several of the other spot differences was too low for reliable MS analysis (Figure 1C, D, box 5 and box 6).

The difference in expression of pfetin in the SMG of 16wk SHR and WKY rats was confirmed using 1D and 2D Western blots. As shown in Figure 3B, two bands are immuno-reactive to the anti-pfetin IgG, one at 40 kDa and the other at 60 kDa. The signal for the 40 kDa band is stronger in SHR than in WKY while there is no difference in the signal strength of the 60 kDa band between the two phenotypes. Quantification of the 40 kDa band (Figure 3C) showed that mean pfetin expression is almost 6.5-fold greater in SMGs of SHR than SMGs of WKY. The 2D Western blot in Figure 3C shows that there are, in fact, three immuno-reactive pfetin isoforms, one at 60 kDa and two at 40 kDa. The difference in expression observed on the 1D Western blot is due to a dramatic increase in the more acidic (lower pI) 40 kDa isoform in SHR relative to WKY.

This difference in pfetin expression in the SMG was further verified using fluorescence immunohistochemistry. Figure 4 shows an increase in staining intensity in 16wk SHR compared to 16wk WKY. Staining seems to localize either to the neuron cell
membrane, dendritic process or satellite cells. Electron microscopy with immuno-gold staining would be required to further determine the location of pfetin expression within the SMG.

Two trains of protein spots were observed in the upper left (pI ~4, 65 kDa) corner of WKY SMG gels, while only one train of spots was present in SHR SMG gels. The bottom string of spots, which was found in both phenotypes, was identified as serine protease inhibitor 2.2 (Spi 2.2), while the upper line of spots, found only in WKY gels, was identified as serine protease inhibitor 2.1 (Spi 2.1). Spi 2.2 (also know as rat kallikrein binding protein) has been previously shown to be decreased in various tissues of SHR compared to WKY rats and to be involved in blood pressure regulation through an unknown mechanism. As our results differed from those previously reported (we found no difference in the intensity of spots identified as Spi 2.2), we obtained the anti-Spi 2.2 IgG created by the lab responsible for the previous work. As shown in Figure 5, this IgG reacts with both Spi 2.1 and Spi 2.2 (and is thus hence forth referred to as anti-Spi 2.1/2.2 IgG). This blot confirms that Spi 2.1 is absent in the SHR SMG, while there is no detectable difference in the expression of Spi 2.2 between the WKY and SHR SMGs.

As Spi 2.1 and Spi 2.2 are both synthesized in the liver, expression of these proteins was analyzed in the liver using 2D Western blots (Figure 5) and immunohistochemistry (Figure 7). 2D Western blots confirmed that Spi 2.1 and Spi 2.2 are both synthesized in WKY but only Spi 2.2 is synthesized in SHR. Spi 2.1/2.2 staining was localized to both the hepatocytes and the hepatic triads. There was no difference in staining intensity using immunohistochemistry.
Haptoglobin (Hp) was identified in three spots which had greater protein abundance in SHR SMG gels than in WKY SMG gels. These changes were verified using 1D and 2D Western blot (Figure 8). As shown by the 2D Western blot (Figure 8D), there exist multiple spots immunoreactive to the Hp-IgG which are distributed in three horizontal lines at approximately 35 kDa, 50 kDa and 65 kDa, as well as a single spot around 45 kDa. The 35 kDa, 45 kDa and 50 kDa spots correspond to the anticipated molecular weights of the Hp \( \beta \) chain, preprohaptoglobin and prohaptoglobin, respectively (Hanley et al., 1983). It is not surprising that the Hp antibody, which is a polyclonal IgG directed against the Hp \( \beta \) chain, binds preprohaptoglobin and prohaptoglobin since these molecules contain the entire \( \beta \) chain sequence (Hanley et al., 1983). Multiple spots are seen at 35 kDa and 50 kDa because the Hp \( \beta \) chain and prohaptoglobin undergo several glycosylations; each spot represents a unique glycosylation product. The Hp \( \beta \) chain isoforms, three of which correlate with the spots identified with 2DGE (arrows in Figure 8D), have a much higher signal intensity in SHR. The spots located at 50 kDa are absent in SHR but present in WKY, while there was no difference in staining at 65 kDa on 2D Western blots (Figure 8D). The staining at 65 kDa seen in Figure 8B and 8D represents cross reactivity between the Hp-IgG and albumin. Interestingly, this cross-reactivity was consistently stronger in SHR than in WKY tissue when viewed by 1D blot (Figure 8B). Albumin is normally present as string of spots, most of which are too basic to be viewed in Figure 8D.

Cellular localization of Hp was investigated using chromogenic immunochemistry (Figure 9). In the ganglion, Hp appears to be found in the extra-cellular spaces between the neurons. This observation is consistent with descriptions of Hp as a blood borne
protein that is synthesized in the liver and accumulates in extrahepatic tissues. Hp was found at much higher levels in the interstitial space of the SMG of SHR relative to WKY rats (Figure 9A, B, D, E). A similar increase was seen in the microvasculature of adipose tissue Figure 9G, J). Staining of the superior mesenteric arteries showed that Hp is found in the adventitia but is absent from the intima media, with no difference in staining intensity between SHR and WKY (although Hp was found to be higher in SHR arteries using 2DGE according to unpublished results). Surprisingly, no difference in Hp accumulation was found between the SCG of SHR and WKY rats. This was the only difference between the SMG and the SCG detected in this study.

Although Hp is synthesized by a variety of tissues, the majority of Hp is synthesized by hepatocytes and released into circulation (Asleh and Levy, 2005). For this reason, hepatic expression of Hp was analyzed. Hp immunoblotting revealed similar patterns of isoform molecular weight and abundance according to 1D and 2D Western blot with the exceptions that prohaptoglobin appears to be more abundant in the liver than it is in SMGs and that the difference in the amounts of the Hp β chain isoforms at 35 kDa between SHR and WKY extracts was of much smaller magnitude than the difference observed in the SMG (Figure 8D). Quantification of the 1D blot (Figure 8C) showed that the β chain (35 kDa) is upregulated almost 4-fold in SHR relative to WKY (p≤ 0.001), while prohaptoglobin (50 kDa) is downregulated over 3-fold in SHR relative to WKY (p≤ 0.005) (data shown in Appendix A). Despite this, there was no difference in staining intensity between SHR and WKY using immunohistochemistry (Figure 10). Hp staining was found both in hepatocytes and in the connective tissue surrounding the hepatic triad. Hepatocyte staining intensity increased around the central veins, with a characteristic spot
pattern which appears to outline the basolateral membranes where the hepatocytes are in contact (Figure 10A-D). Staining was lighter in the hepatocytes surrounding hepatic triads; however, clusters of blotches, for lack of a better term, could be found with a loose proximal association with hepatic triads (Figure 10E-H). The identity of these “blotches” is unknown, although they resemble Kupffer cells.

ApoA1 was found to be upregulated in SMGs from 22wk SHR compared to SMGs from 22wk WKY rats. This observation was verified using a Western blot (Figure 11); quantification of this Western blot using PD Quest software showed a ~40% increase in protein abundance in SHR. SMG sections were also treated with anti-ApoA1 IgG for fluorescence immunohistochemistry, as were liver sections which served as a control for the antibody since it is well established that the liver expresses high levels of ApoA1. Although there was a general increase in staining intensity in the SMG from SHR compared to the SMG from WKY rats, staining was very weak and had a hazy quality so that cytolocalization of ApoA1 expression was not possible (data not shown). Furthermore, the antibody failed to produce a positive signal in the liver. For these reasons, it was determined that the anti-ApoA1 IgG could not be reliably used for immunostaining following our established protocol.

**Discussion**

This study is the first proteomic assessment of differences in protein expression between sympathetic ganglia from hypertensive rats and ganglia from normotensive control rats. Hyperactivity of both central and peripheral components of the sympathetic nervous system is well documented in the SHR model of primary hypertension, and normalization
of sympathetic firing rates, NE release, and target tissue innervation are associated with moderation of blood pressure, decreased blood vessel remodeling and less end-organ tissue damage (Cutilletta et al., 1977; Cutillet and Oparil, 1980; Lee et al., 1987). This study was designed to identify proteins with potential involvement in the establishment or maintenance of peripheral sympathetic hyperactivity and blood vessel hyperinnervation in SHR.

It should be noted that definitively establishing that these protein changes are in fact dependent on hypertension or sympathetic hyperactivity is beyond the scope of this project. The discussions of the individual proteins which follow are based on the assumption that these changes are significantly involved in the development, maintenance, or compensation to primary hypertension. Although it is possible to speculate and hypothesize as to why the expression of the proteins listed below is altered in SHR, extensive further research is required to determine whether the ideas put forth here are accurate.

Four differentially expressed proteins were identified: predominantly fetally expressed T1 domain, serine protease inhibitor 2.1, haptoglobin and apolipoprotein A1.

*Predominantly Fetally Expressed T1 Domain (Pfetin)*

Pfetin was found to be upregulated in SHR sympathetic ganglia compared to WKY sympathetic ganglia (Figures 3, 4). Pfetin was recently characterized by Resendes and colleagues (2004) during a search for genes involves in genetically acquired deafness. Pfetin was identified as being upregulated in the human fetal cochlea compared to the fetal brain using a subtractive analysis of cDNA libraries. The pfetin cDNA was
sequenced and then used to determine the amino acid sequence. Low levels of pFetin expressions were found in numerous murine tissues, including the kidney, thymus, lung, heart and skeletal muscle, but much higher expression was found in neural tissue, namely the brain and cochlea. P Fetin is developmentally regulated in that it was found to be much more abundant in fetal tissues than in adult tissues.

Analysis of the pFetin amino acid sequence showed that it contains a portion very similar to the T1 domain of voltage-gated K\(^+\) ion (Kv) channels, yet it does not include any sequences which resembled transmembrane domains (Resendes et al., 2004). The T1 domain is a cytosolic structure which is attached by a short linker sequence to the end of the first transmembrane domain in the Kv channel subunit. In a mature Kv channel, four identical subunits, each with six transmembrane domains, are arranged around a central pore. The T1 domains, located in the N-terminus of each subunit, hang below the membrane and aggregate into a tetramer that has been described as a “hanging gondola” (Kobertz et al., 2000). Although the precise function of the T1 domain is a matter of debate, it is clear that this domain plays a central role in the regulation and function of Kv channels (Sewing et al., 1996; Strang et al., 200; Gu et al., 2003; Rivera et al., 2005; Wang and Covarrubias, 2006). The T1 domain is important in channel tetramerization and ensures that all channels are homotetramers (Strang et al., 2001). The T1 domain was shown to be involved in axon targeting and surface expression of the Kv1.2 (Gu et al., 2003) and Kv1.3 channels (Rivera et al., 2005). Furthermore, the T1 domain was shown to be involved in voltage gating of Kv4.2 channels (Wang and Covarrubias, 2006). It is possible that this voltage gating activity is mediated through interactions with Kv channel B subunits, which are separate, cytosolic proteins that regulate voltage gating and
have been shown to interact with the assembled T1 tetramer (Sewing et al., 1996). It is also of interest that the T1 domain can adopt a protein fold known as the BTB/POZ fold (Strang et al., 2001; Resendes et al., 2004), which is a relatively common fold found in numerous proteins that is often involved for protein-protein interactions and oligomerizations (Collins et al., 2001).

We found pfetin to be upregulated by almost 6.5-fold in the SMG from SHR compared to WKY rats. As shown in Figure 3, this change was due to the increased expression of a specific pfetin isoform with no change in expression of the other two isoforms. Because the function of pfetin has not been experimentally tested, it is difficult to speculate as to why pfetin expression would be increased in the sympathetic ganglia of a model of neurogenic hypertension. It possible that pfetin is associated with the regulation of ion concentrations and neural excitability. The presence of a T1 domain without any transmembrane domains could mean that pfetin interacts with ion channels (Kv family members or others), possibly by interacting with the “hanging gondola” or β-subunits, while it itself is not inserted into the membrane. Any involvement in regulation of K⁺ currents would certainly have an impact on neural activity. Likewise, pfetin may be expressed in glia cells, in this case stallelite cells, which participate in the regulation of extracellular ion concentrations in neural tissues. The hypothesis that pfetin is involved in the regulation of ion concentrations is supported by the fact that type I fibrocytes in the spiral ligament were found to have the highest pfetin expression in the cochlea (Resendes et al., 2004). These cells are involved in K⁺ recycling and maintenance of ion concentrations in endolymph. It is also possible that what was identified as a T1 domain
actually functions as a BTB/POZ fold and thus may be involved in protein interactions unrelated to ion channels.

It should be noted that two pfetin transcripts were previously identified (Resendes et al., 2004) which may explain the presence of multiple protein isoforms. It is also possible that the immunoreactive spot at 60 kDa represents an early form of pfetin which then undergoes additional post-translational processing into either of the two 40 kDa isoforms. Further research is necessary to elucidate the interconnections between the three pfetin isoforms identified in this study.

Serine Protease Inhibitor 2.1 (Spi 2.1)

A string of protein spots that was present in WKY SMG gels, but absent in SHR SMG gels were identified as Spi 2.1. Spi 2.1 is one of three structurally similar proteins that are synthesized in the liver, secreted into the serum, and collected in the extracellular spaces of various extrahepatic tissues throughout the body (Pages et al., 1990). These three proteins, named Spi 2.1, Spi 2.2, and Spi 2.3, are members of a class of proteins known as serpins (Serine Protease Inhibitors) that inhibit serine proteases through formation of the Michaelis complex (reviewed in Ye et al., 2001). Briefly, the inhibitor mimics the protease’s normal substrate so that the protease cleaves a specific peptide bond in the reactive site of the inhibitor. The protease then makes a stable covalent complex with the inhibitor by forming a new peptide bond and replacing the peptide which was just cleaved off. The serpin therefore traps and deactivates the protease. Other members of the serpin protein family include anti-trypsin, anti-chymotrypsin, and protease C inhibitor.
Expression of the genes encoding Spi 2.1 and Spi 2.2 is increased following stimulation with growth hormone or thyroid stimulating hormone and is decreased during inflammation due to glucocorticoids (Yoon et al., 1987; Pages et al., 1990; Thomas et al., 1995). The specific function of the Spi 2.1 protein, however, is completely unknown; the assumption that it inhibits a serine protease is based only on the observation that its nucleotide sequence is similar to other serpins. Fortunately, more research has gone into determining the function of the very similar protein Spi 2.2. As these two proteins share 90% sequence homology and are expressed under similar conditions (Yoon et al., 1987; Pages et al., 1990), observations regarding Spi 2.2 are relevant to a discussion of Spi 2.1.

Spi 2.2, also known as rat kallikrein binding protein (RKBKP) has been found to form a covalent complex with human and rat tissue kallikrein (Chao et al., 1986). Kallikreins are serine proteases which act on kininogen to release small biologically active peptides known as kinins (reviewed in Sharma, 2006). It is well accepted that activation of the kallikrein-kinin system causes a general decrease in systemic arterial blood pressure. Tissue kallikreins cleave low molecular weight kininogens to release bradykinin (BK) or kalliden. Kinins have varied physiological functions including vasodilation, smooth muscle contraction or relaxation, altered electrolyte and glucose transport, and pain sensitization (Chao, et al., 1990; Sharma, 2006) and BK specifically increases general vasodilation and renal salt excretion and can moderate the development of left ventricular hypertrophy in models of secondary hypertension (Sharma, 2006).

Excretion of tissue kallikreins has been found to be lower in hypertensive patients (Sharma, 2006) and animals (Wang et al., 1995) compared to normotensive controls and transgenic mice over-expressing human kallikrein are hypotensive, an effect which was
reversed through the injection of RKBP (Ma et al., 1995). Likewise, injection of kallikrein has also been found to decrease blood pressure in SHR (Wang et al., 1995).

RKBP has been shown to be decreased in SHR compared to WKY (Chao et al., 1990). Also, RKBP isolated from SHR serum fails to make a covalent complex with tissue kallikrein as is formed between kallikrein and RKBP from WKY (Chao and Chao, 1988). These observations seem counterintuitive; a decrease in RKBP aught to be associated with low blood pressure due to increased release of kinins. However, it was also found that transgenic mice over expressing RKBP were hypotensive (Chen et al., 1996) and that RKBP induced vasodilation of aortic rings independent of the BK pathway (Chao et al., 1997). These results suggest a duel function for RKBP; in an abundance of tissue kallikrein, RKBP will increase blood pressure by countering kallikrein activity. In the absence of excess kallikrein, RKBP acts as a vasodilator through alternative unknown mechanisms (Chao et al, 1997).

RKBP/Spi 2.2 was identified as a string of protein spots just underneath the string of spots identified as Spi 2.1. The individual spots within the strings presumably differ in the level of glycosylation (Yoon et al., 1987; Pages et al., 1990). We did not observe any difference in the abundance of RKBP/Spi 2.2 between SHR and WKY rats using 2D Western blots (Figure 5) or immunohistochemistry (Figure 6). As the same results were obtained in the liver (Figure 5 and Figure 7), it can be concluded that the observations in the SMG are due to a lack of Spi 2.1 synthesis and not to an inability of Spi 2.1 to accumulate in the ganglionic interstitial space. We also found that the anti-RKBP IgG was not specific to RKBP/Spi 2.2 but instead bound to both Spi 2.1 and Spi 2.2. As this result was obtained using the same antibody used in the studies performed above.
(generously supplied by Dr. Julie Chao), it is possible to conclude that the previously observed differences in RKBP abundance and binding activities between SHR and WKY protein extracts are actually due to the difference in expression of Spi 2.1. It can be assumed that any experiment performed by the Chao lab which used purified RKBP protein or detected RKBP with this antibody would have actually purified or detected a mixture of Spi 2.1 and Spi 2.2. It is tempting to hypothesize that it is Spi 2.1, not RKBP/Spi 2.2, which complexes with tissue kallikrein. This would explain no high molecular weight complex was formed between kallikrein and RKBP/Spi 2.2 from SHR, as was detected in WKY (Chao and Chao, 1988; Chao et al., 1990). Interestingly, RKBP does not have an optimal active site for tissue kallikrein, which prefers to cleave a peptide bond following a basic amino acid (lysine or arginine) (Chai et al., 1993). The amino acids on either side of the cleavage site in RKBP are leucine – lysine, while the complementary amino acids for Spi 2.1 are arginine – arginine (Yoon et al, 1987; Pages et al, 1990). Although this is merely circumstantial evidence, it does support the hypothesis that Spi 2.1 is capable of forming a covalent complex with tissue kallikrein.

It should be made clear that we are not disputing the observation that RKBP induces hypotension or vasodilation since transgenic mice over-expressing RKBP were made from a construct specifically containing the Spi 2.2 gene. Nor are we suggesting that the dual functions previously assigned to RKBP are in fact split between Spi 2.1 and Spi 2.2. If that were the case and Spi 2.1 were the sole inhibitor of tissue kallikrein, the profound decrease in Spi 2.1 observed in SHR would promote hypotension as opposed to hypertension. Rather, we are proposing that the functions of Spi 2.1 are similar to those of RKBP/Spi 2.2 and that it is possible that Spi 2.1 is capable of forming a covalent
complex with kallikrein. It should be stressed that further research is required to test the validity of these speculations in order to determine if Spi 2.1 and RKB/Spi 2.2 do in actuality share functions and that Spi 2.1 is involved in the regulation of blood pressure.

Haptoglobin (Hp)

Three spots upregulated on SHR gels compared to WKY gels were identified as Hp. Hp is synthesized in the endoplasmic reticulum of hepatocytes as preprohaptoglobin (Mr = ~40 kDa) which is cotranslationally processed into prohaptoglobin (Mr = ~45 kDa), which undergoes several glycosylations (Hanley et al., 1983). Each molecule of prohaptoglobin is then cleaved into an α chain (Mr = ~ 15 kDa) and a β chain (Mr = ~ 35 kDa), which are associated into αβ dimers by the formation of a single intermolecular disulfide bond (Hanley et al., 1983; Asleh and Levy, 2005). These heterodimers then associate into larger complexes through an additional disulfide bridge between two α chains and are released into circulation (Langlois and Delanghe, 1996; Asleh and Levy, 2005). Mature Hp scavenges free hemoglobin (Hb) and thus prevents the iron containing haem group from causing oxidative damage to blood vessels (Langlois and Delanghe, 1996; Asleh and Levy, 2005). The free Hb and iron released from the spontaneous rupture of erythrocytes are potent pro-oxidant molecules that induce the formation of hydroxyl radicals and promote lipid peroxidation, which enhances other forms of oxidative tissue damage and causes endothelial dysfunction through the oxidation of nitric oxide (Langlois and Delanghe, 1996; Asleh and Levy, 2005; Dayan et al., 2009). Furthermore, free Hb may enter the vessel wall through areas of minor endothelial damage and cause local oxidative damage which hastens the development of these
regions into arteriosclerotic plaques (Langlois and Delanghe, 1996). Hp prevents this oxidative damage by binding Hb and carrying it to the liver where the Hp-Hb complex is removed from circulation and degraded.

Hp is also an acute phase protein whose expression increases several fold during inflammation (Langlois and Delanghe, 1996; Asleh and Levy, 2005; Nakagawa et al., 2008). Hp in turn limits inflammation by inducing synthesis of the anti-inflammatory cytokine IL10 and decreasing production of pro-inflammatory prostaglandins. Hp is also important in wound healing; it scavenges Hb released from crushed erythrocytes which would otherwise cause oxidative tissue damage and promotes angiogenesis in regenerating tissue following injury (Nakagawa et al., 2008). Finally, Hp-Hb complexes interact with macrophages and other immune cells through the CD163 receptor and alter cytokine production and cell adhesion (Asleh and Levy, 2005).

The physiological importance of Hp is illustrated by the different phenotypes associated with the three Hp genotypes in humans (there is only one genotype in rodents). There are two α chain alleles known as Hp1 and Hp2 which combine to generate three distinct types of Hp known as Hp1-1, Hp2-1 and Hp2-2 (Langlois and Delanghe, 1996; Asleh and Levy, 2005). Since Hp1 α chains form bridges with one β-chain and one α-chain so that Hp1-1 only exists as a dimer of dimers (\((\alpha_1\beta)_2\)) with a total molecular weight of 86 kDa. Hp2 α chains, on the other hand, are capable of binding to one β-chain and two other α chains so that circulating Hp2-2 forms huge aggregates of dimers (\((\alpha_2\beta)_n\)) with molecular weights ranging from 170 – 900 kDa. Hp2-1, which is a mixture of Hp1 and Hp2 α chains, forms linear aggregates (\((\alpha_1\beta)_2 + (\alpha_2\beta)_n\)) with molecular weights ranging from 86 – 300 kDa.
Hp1-1 and Hp2-2 have been shown to have very different functional characteristics. Hp2-2 is a much weaker antioxidant than Hp1-1 and binds Hb with a much lower affinity (Langlois and Delanghe, 1996; Asleh and Levy, 2005; Nakagawa et al., 2008; Dayan et al., 2009). Also, because Hp2-2 forms such large multimers, it is incapable of extravasation and therefore cannot bind free Hb which has already diffused out of the blood vessel lumen (Langlois and Delanghe, 1996). Hb2-2 therefore offers little protection against oxidative damage to blood vessels and having this genotype is considered a risk factor for kidney disease, retinopathy and myocardial infarction in diabetics (Langlois and Delanghe, 1996; Asleh and Levy, 2005; Nakagawa et al., 2008; Dayan et al., 2009). Clearly, Hp is important in protecting the body from oxidative damage and maintaining proper blood vessel function.

Hp has also been found to be produced by neural tissues. Hp was found in reactive astrocytes following ischemia (Lee et al., 2002) and in the neural retina in diabetics (Chen et al., 1998). Hp can thus be synthesized in extra-hepatic tissues and has multiple incompletely understood functions which extend beyond those described above.

Hp was found at much higher levels in the SMG of SHR relative to WKY rats (Figure 8) and immunolocalization showed that Hp is found in the interstitial space between the neurons (Figure 9). The increased Hp in the SMG is due either to 1) increased production within the ganglion or due to 2) increased accumulation of Hp from circulation. The second possibility seems much more likely given the absence of Hp inside neuron cell bodies. Interestingly, although an increase in Hp was found in the liver of SHR compared to WKY by Western blot (Figure 8C, D), the magnitude of this difference was not as great as that seen in the SMG (Figure 8B, D), which implies that
the greater ganglionic accumulation of Hp is not due solely to an increase in Hp concentration in plasma. Rather, this observation suggests that Hp is moving out of the vasculature more efficiently in SHR than in WKY tissues, which is consistent with “leaky” blood vessels in SHR. This conclusion is further supported by greater amount of albumin in the SHR ganglion than in the WKY ganglion, according to cross-reactivity with the Hp anti-body (Figure 8B), and by the presence of higher levels of Hp were found in the capillary system of adipose tissue (Figure 9G, J) and in the adventitia of the mesenteric arteries of SHR than those of WKY rats using 2DGE (unpublished results from our laboratory). Furthermore, ApoAI, another blood protein which is synthesized in hepatocytes, was also found at higher levels in the SHR SMG than the WKY SMG (Figure 11). This increase in capillary permeability could be due to numerous factors such as increased endothelial modifications in response to inflammatory cytokines or physical breakdown of the structural components of the blood vessel wall. Outside of the general implications of leaky vessels, Hp accumulation in the interstitial space may have several physiological effects in sympathetic ganglia. Increased Hp would decrease the oxidative stress and inflammation which could otherwise increase sympathetic activity. Hp could thus function as a protective mechanism which prevents an even greater stress to the neural environment than is already seen. Alternatively, Hp could have undescribed functions within the ganglion which promote changes in sympathetic function.

The 50 kDa Hp isoforms corresponding to prohaptoglobin were present in WKY SMG and liver but absent in SHR SMG and liver (Figure 8D). This may indicate that Hp synthesis is more efficient in SHR such that a greater proportion of Hp is processed to
maturity before being released into circulation. This would account for at least a portion of the increase in Hp-β chain abundance.

Hp was also identified in three other proteomic studies of comparing SHR and WKY tissues: Hp was found to be increased in the aorta (Bian et al., 2007) and heart (Jin et al., 2006) of SHR while the amount of Hp in the serum of stroke-prone SHR rats progressively decreased with the development of hypertension with no change in the serum of SHR or WKY rats with age (Kiga et al., 2008). In addition, this third study suggests that concentration of Hp is higher in WKY than in SHR. All of these studies agree with the results presented above in that they show an increase in Hp in extra-hepatic tissue in SHR compared to WKY.

**Apolipoprotein A1 (ApoA1)**

ApoA1 was found to be expressed at a higher level in the sympathetic ganglia of 22wk SHR than those of 22wk WKY rats using 2DGE and Western blots (Figure 11). ApoA1 and ApoAII are the two major apolipoproteins associated with targeting and trafficking of high density lipoprotein (HDL) particles (Boron and Boulpaep, 2003). HDL particles, commonly known as “the good cholesterol”, are higher in protein and lower in triglycerides than their infamous counterpart low density lipoprotein (LDL), and low LDL/HDL ratios are associated with decreased risk of hypertension and cardiovascular disease. HDL particles scavenge excess cholesterol from plasma and extrahepatic tissue and then transporting that cholesterol back to the liver for repackaging (Boron and Boulpaep, 2003). Indeed, genetic polymorphisms in the ApoA1 gene have been linked to increased risk of hypertension and obesity in several populations (Chen et al., 2009).
ApoA1 has also been identified in neural tissue (Boyles et al, 1985, 1989; Fujii et al., 2002; Lemieux et al., 1996; Ito et al., 2006; Simo et al., 2009). Together with ApoE, ApoA1 is one of the most widely expressed apolipoproteins in the brain and human cerebrospinal fluid (Boyles et al, 1989; Fujii et al., 2002). Both proteins are thought to play a role in lipid and cholesterol trafficking in both the peripheral and central nervous system through their association with HDL (Boyles et al., 1989; Lemieux et al., 1996; Ito et al., 2006; Simo et al., 2009). One application of this is the repackaging and targeting of lipids released during myelin remodeling, as ApoA1 expression is significantly increased during periods of myelination in the developing (Lemieux et al., 1996) and regenerating (Boyles et al., 1989) sciatic nerve. ApoA1 (Ito et al., 2006) and ApoE (Boyles et al., 1985) have also been localized to glial cells where, again, they are proposed to function in cholesterol trafficking. Furthermore, evidence suggests that ApoA1 is capable of stabilizing microtubule formation in astrocytes and of targeting signal transduction molecules involved in lipid exocytosis to the membrane or cytoskeleton (Ito et al., 2006). This has been interpreted as a potential mechanism for enabling intracellular transport of cholesterol droplets to the plasma membrane for export.

There are several possible explanations as to why ApoA1 may be more abundant in 22wk SHR SMGs compared 22wk WKY SMGs. For example, additional ApoA1 accumulation in the SHR SMG may be due to a higher concentration of ApoA1 in circulation due to increased production of ApoA1 in the liver. Alternatively, ApoA1 may diffuse into the ganglion extracellular space more readily in SHR due to structural differences in the capillaries as was hypothesized for Hp (see above). As ApoA1 is
involved in the scavenging of free cholesterol, the increase in ApoA1 may represent a compensatory mechanism to counter hyperlipidemia, and may help prevent hypercholesteremia in SHR. If, on the other hand, ApoA1 expression is enhanced cells residing in the ganglion, such as neurons, satellite cells, Schwann cells or fibroblasts, then this change in expression could possibly reflect an effort to counter altered lipid profiles in the interstitial space. Alternatively, it is possible, albeit less likely, that the increase in ApoA1 is due to enhanced neural plasticity. As ApoA1 has been shown to be increased in regenerating peripheral nerves, and has been hypothesized to be involved in myelin trafficking during remyelination, a high level of synapse formation and elimination could be associated with elevated synthesis of ApoA1. Enhanced neural plasticity may be expected in the SMG of SHR because of the sympathetic hyperactivity which has already been shown to exist in this strain. Unfortunately, cytolocalization of ApoA1 expression within the SMG was not successful (see results) so it is not possible to differentiate between these possibilities at this time. Further research is required to determine the source of increased ApoA1 expression in SHR and to determine if it is involved in the pathogenesis of hypertension.

Peripheral sympathetic hyperactivity is a well documented phenomenon in both human and animal hypertension. This increase in activity is manifested through increased firing rates, hyperinnervation of target organs, and augmented NE spillover. In SHR, hyperinnervation of the mesenteric arteries by neurons in the SMG has been shown to induce blood vessel remodeling prior to the development of hypertension. Given these differences in function, we expected to find numerous changes in protein expression in
the sympathetic ganglia of SHR relative to those of WKY rats. To the contrary, only eight protein differences (three identified) were found in 16wk ganglia and an additional seven (one identified) differences in 22wk ganglia. Overall, the proteomes of the sympathetic ganglia from these two strains were remarkably similar. This could be due to 1) the limitations of 2DGE, 2) the source of sympathetic hyperactivity, or 3) developmental period examined.

1) 2DGE has become a standard technique for the visualization of proteomes. However, there are several limitations in this procedure which must be taken into consideration when planning an experiment and analyzing data. First, since the buffers used to solubolise proteins must maintain protein charge for isoelectric separation in the first dimension, hydrophobic proteins do not readily enter solution and are underrepresented on the final gels (Rabilloud, 2002; Gorg et al., 2004; Rabilloud et al., 2008). Most often, these hydrophobic proteins are integral membrane proteins. Although certain modifications to the buffers were made to maximize membrane protein solubility in the preparation of the SMG ganglia, such as the use of thiourea in combination with urea rather than pure urea to denature protein (Rabilloud, 2002; Rabilloud et al., 2008), the visualization of hydrophobic membrane proteins remains a challenge in proteomics. Because of this, differences in proteins such as ion channels between SHR and WKY neurons would not necessarily be detected using 2DGE. This problem could be partially overcome using other proteomic approaches such as Mudpitt or iTRAQ chromatography (Roe and Griffin, 2006), although these methods have their own drawbacks, such as the inability to detect protein modifications (see proteomics section of literature review for more detail).
Another limitation of 2DGE is the inability to visualize the entire dynamic range of protein concentration within a tissue (Rabilloud, 2002; Gorg et al., 2004). The difference between the most abundant protein and the least abundant protein, known as dynamic range, is much larger in cells and tissues (around $10^6$ molecules per cell) than even the most sensitive stains used in 2DGE can detect (silver stain and Deep Purple fluorescent stain both have a dynamic range of $10^4$) (Rabilloud, 2002). The result is that low abundance proteins, such as transcription factors and signaling molecules, are very difficult to detect. Thus, any changes in synthesis or modifications of such proteins in the SHR SMG may not have been identified in this study. One way to overcome this limitation is through sample fractionation according to hydrophobicity, subcellular location, or protein pI (see proteomics section of literature review for more detail). One drawback of fractionation is that it requires large amounts of sample, which is difficult when studying small structures, such as rodent ganglia.

Finally, proteins cover a huge breadth of molecular weights, ranging from huge membrane proteins at 500 kDa or more to tiny signaling peptides less than 5 kDa (Rabilloud, 2002; Gorg et al., 2004). The standard 10% acrylamide gel used in this study satisfyingly separates proteins ranging from 150 kDa to 15 kDa. As many transmembrane receptors are very large and most neuropeptides are quite small, these two classes of proteins would not be adequately separated for changes in expression to be reliably observed using the standard 2DGE methods described above. This limitation can be avoided by using several sets of gels with different concentrations of acrylamide, or by using gradient gels where the percentage of acrylamide changes along the y-axis (Rabilloud, 2002; Gorg et al., 2004). It may thus be beneficial to analyze protein
expression in the SMG in SHR and WKY rats using with a variety of acrylamide percentages to target the underrepresented extremes of protein molecular weights in the ganglionic proteome. This problem could also be avoided through the use of liquid chromatography, especially for large proteins which tend to be hydrophobic (Gorg et al., 2004).

It is therefore possible that there are changes in the expression of membrane proteins and signaling factors between the SMGs of SHR and WKY rats which this study was not designed to detect.

2) Although sympathetic hyperactivity is normally measured in peripheral nerves, evidence suggests that augmented sympathetic drive originates in the hypothalamus and brainstem of the central nervous system (Smith and Baron, 1990; Colombari et al., 2001; Allen, 2001; DiBona and Jones, 2001; Shokoji et al., 2003; Lin et al., 2005; Guyenet, 2006; Yajima et al., 2008). It is therefore possible that the peripheral ganglia, such as the SMG and SCG, process and transmit this augmented signal without being greatly affected at the level of protein expression by the increase in magnitude or frequency.

3) This study examined the proteome of the SMGs from 16wk and 22wk SHR and WKY rats. At these ages, SHR already reliably exhibit the hypertensive phenotype and show anatomical signs of tissue remodeling in the resistance arteries and left ventricle. Sympathetic hyperactivity and hyperinnervation of the blood vessels, including the mesenteric arteries, is already established (Lee et al., 1983; Scott and Pang, 1983; Head et al., 1989). It is possible that the majority of the changes in protein expression in the sympathetic ganglia of hypertensive rats are detectable during the development of
hyperinnervation but subside after these structures are in place. In this case, an
examination of the proteome of sympathetic ganglia of neonatal or 5wk SHR rats may
yield more numerous alterations in protein expression.

In conclusion, four proteins were identified as having altered expression in the
SMG of SHR compared to the SMG of WKY rats at 16wk to 22wk of age. Of these,
only one protein, pfetin, is of neural origin while the other three proteins are blood
proteins which are circulating proteins that are most likely accumulating in the ganglia.
Although this small number of proteins could be easily dismissed as “too few” changes,
any one of these four proteins can potentially influence neural activity and deserves
further investigation in SHR. Pfetin is of special interest since this relatively unknown
protein shares sequence homology with voltage-gated K⁺ channels and thus could
potentially regulate neuron excitability. Another important point to take from these
results is that most of the differences between the SHR and WKY sympathetic ganglia
are in the environment that the neurons reside, rather than in the neurons themselves.
This environment could very likely influence neural activity and may go a long way to
explaining functional variations in SMG physiology between normotensive and
hypertensive research models. Finally, it must be mentioned that, despite the fact that
increased mesenteric expression of NGF has been hypothesized as driving sympathetic
hyperinnervation of these arteries, none of the proteins normally associated with
augmented NGF exposure of sympathetic neurons, such as tyrosine hydroxylase and
TrkA, were identified in this study. This suggests that these neurons are not exposed to
increased NGF, as previous publicationed, and that sympathetic hyperinnervation of the
mesenteric arteries of SHR is due to an as yet unidentified source.
2-Dimensional Gel Electrophoresis (2DGE) was used to compare the whole tissue proteome of the superior mesenteric ganglion (SMG) from 16 week old WKY (A), 16 week old SHR (B), 22 week old WKY (C), and 22 week old SHR (D). Spot changes between WKY and SHR of the same age are emphasized in boxes. In gels from 16 week old rats (A, B), box 1 shows the spots identified as Spi2.1 (top line) and spots identified as Spi2.2 (bottom line; box 2 shows an unidentified spot which appears to undergo a shift in location; box 3 shows both the spots identified as pfetin and those identified as haptoglobin; box 4 shows another spot which appears to undergo a shift in spot location. These gels show proteins with isoelectric points within the pI range of 4 – 7. Proteins were visualized using the silver stain (Shevchenko et al., 1996). For magnification of the spots that were identified as pfetin, Spi 2.1, haptoglobin, and apolipoprotein A1, please see Figures 3, 5, 8, and 11, respectively.
Figure 1 continued: 2-Dimensional Gel Electrophoresis (2DGE) was used to compare the whole tissue proteome of the superior mesenteric ganglion (SMG) from 16 week old WKY (A), 16 week old SHR (B), 22 week old WKY (C), and 22 week old SHR (D). Spot changes between WKY and SHR of the same age are emphasized in boxes. For 22 week old rats (C, D), box 5 and box 6 show unidentified spot changes and box 7 shows the spot identified as apolipoprotein A1. These gels show proteins with isoelectric points within the pI range of 4 – 7. Proteins were visualized using the silver stain (Shevchenko et al., 1996). For magnification of the spots that were identified as pfetin, Spi 2.1, haptoglobin, and apolipoprotein A1, please see Figures 3, 5, 8, and 11, respectively.
Figure 2: Representative Mass Spectra of Pfetin, Spi 2.1 and Haptoglobin
Predominantly Fetally Expressed T1 domain (pfetin) (A), serine protease inhibitor 2.1 (Spi 2.1) (B), and haptoglobin (C), where identified using MALDI-TOF mass spectrometry from spots excised from gels of 16 week old SHR and WKY SMGs. Red stars mark the peaks which were used to positively identify these proteins; green starts mark the peaks from self-digestion of trypsin which are used to calibrate the spectra.
A  Pfetin

B  Spi 2.1

C  Haptoglobin
Figure 3: Pfetin Protein Abundance

Predominantly Fetally Expressed T1 domain (pfetin) was found to be upregulated in SMGs from 16wk SHR relative to SMG from 16wk WKY rats.  A) Two spots which had greater protein abundance on SHR SMG gels (circles) were identified as pfetin using MALDI-TOF mass spectrometry.  B) The increase in pfetin expression was verified using a Western blot.  The 40kDa pfetin isoform was found to have higher signal intensity in SMGs from 16wk SHR (lanes 2 and 4) than in SMGs from 16wk WKY (lanes 1 and 3).  WKY1 and SHR1 indicate ganglia from rats of cohort 1; WKY2 and SHR2 indicate ganglia from rats of cohort 2.  C) Quantification of the 40 kDa band shown in (B) shows a 6.5 fold increase in pfetin abundance in SHR SMGs relative to WKY SMGs.  D) C) A 2-D Western blot was used to further examine pfetin expression in the SMG of SHR and WKY rats.  Three isoforms of pfetin are present.  The change in protein abundance is primarily due to an upregulation of the more acidic 40kDa isoform (arrow) in SHR relative to WKY.
Pfetin Abundance

WKY        SHR

WKY1  SHR1  WKY2   SHR2

Anti - β-Actin

Anti - Pfetin

60 kDa

40 kDa

C

Pfetin Abundance

60 kDa

40 kDa

% Actin Expression

WKY        SHR

D

WKY        SHR

60 kDa

40 kDa
Figure 4: Pfetin Immunohistochemistry
Predominantly Fetally Expressed T1 (pfetin) immunostaining in the SMG of WKY and SHR. Pfetin signal (green) was much stronger in the SMG of SHR (B, D, E, F) than in the SMG of WKY (A, C). Images (C) and (D) are high magnifications of the boxed regions in images (A) and (B), respectively. This staining (arrowheads) suggests that pfetin localizes to the dendritic processes and possibly to satellite cells of post-synaptic sympathetic neurons in the SMG. Scale bars = 50 μm.
Figure 5: Spi 2.1 Protein Abundance

Serine Protease Inhibitor 2.1 (Spi 2.1) was found to be present in the SMG of WKY rats but absent in the SMG of SHR. **A)** Two trains of spots were observed on WKY gels while **B)** only one train of spots was observed on SHR gels. **C, D)** The upper and lower of these trains were identified using MALDI-TOF mass spectrometry as Spi 2.1 (white circles) and Spi 2.2 (blue circles), respectively. **E, F)** 2D Western blots verified these observations using an antibody which does not distinguish between Spi 2.1 and Spi 2.2 (Anti-Spi 2.1/2.2). **G, H)** 2D Western blots of liver tissue, which secretes Spi 2.1 and Spi 2.2, showed that Spi 2.1 is synthesized in WKY but not in SHR.
WKY

A

SHR

B

75 kDa

50 kDa

60 kDa

C

D

75 kDa

50 kDa

60 kDa

E

F

60 kDa

G

H

60 kDa

Anti – Spi 2.1/2.2

Anti – Spi 2.1/2.2
Figure 6: Spi 2.1 Immunohistochemistry in the Superior Mesenteric Ganglion

Serine Protease Inhibitor 2.1 (Spi 2.1) and Spi 2.2 immunostaining (the antibody doesn’t differentiate between these two proteins) in the SMG of WKY and SHR. Combined Spi 2.1 and Spi 2.2 signal (green) was similar in the SMG of WKY (A, C) and in the SMG of SHR (B, D). This staining indicates that Spi 2.1 and Spi 2.2 are found in the interstitial spaces between the neurons in the SMG. Controls were treated with the primary antibody and the incorrect secondary antibody (E, F). Scale bars = 50 μm.
Figure 7: Spi 2.1 Immunohistochemistry in the Liver

Because serine Protease Inhibitor 2.1 (Spi 2.1) and Spi 2.2 are synthesized and secreted by hepatocytes, chromogenic immunostaining was conducted in the liver. Combined Spi 2.1 and Spi 2.2 signal (brown) was similar in the liver of WKY (A, C, E) and in the SMG of SHR (B, D, F). Spi 2.1 and Spi 2.2 were found in hepatocytes and in the connective tissue surrounding the hepatic triad (A, B, C, D). There was no difference in the staining intensity in hepatocytes surrounding the hepatic triads compared to hepatocytes surrounding the central veins (E, F). Control sections are shown for WKY (G) and SHR (H). A, B) Scale bars = 100 μm. C, D, E, F) CV = central vein; HA = hepatic artery; HV = hepatic vein; Scale bars = 50 μm.
Figure 8: Haptoglobin Protein Abundance

Haptoglobin (Hp) was found to be upregulated in SMGs from 16wk SHR relative to SMG from 16wk WKY rats. A) Three spots which had greater protein abundance on SHR SMG gels (circles) were identified as Hp using MALDI-TOF mass spectrometry. B) The increase in haptoglobin expression was verified using a 1D Western blot. The 35 kDa haptoglobin isoform has dramatically higher signal intensity in SMGs from SHR than it does from SMGs from WKY rats. The staining at 60 kDa represents cross-reactivity of the anti-Hp IgG with albumin. C) Expression of haptoglobin in the liver was determined by 1D Western blot. The 35 kDa haptoglobin isoform is at a higher abundance in SHR livers, while the 50 kDa isoform is at a higher abundance in WKY livers. See Appendix A for quantification. D) 2-D Western blots were used to further examine haptoglobin expression in the SMG (top row) and liver (bottom row) of SHR and WKY rats. Again, the 35 kDa isoforms are much more abundant in the SMGs from SHR than from WKY rats. The 50 kDa isoforms are much more abundant in the SMGs and livers from WKY rats than from SHR. The arrows correspond to the three identified spots emphasized in (A). The faint spots at 65 kDa are most likely cross reactivity with albumin breakdown products.
Figure 9: Haptoglobin Immunohistochemistry

Haptoglobin immunostaining in various tissues of WKY and SHR. Haptoglobin signal (brown) was stronger in the SMG of SHR (D, E) than in the SMG of WKY (A, B). The pattern of staining suggests that haptoglobin accumulates in the interstitial spaces of the ganglion. Control sections are shown for WKY (C) and SHR (F). Haptoglobin staining in the microvasculature of adipose tissue from SHR (J) is also stronger than staining in microvasculature of adipose tissue from WKY (G). There is no qualitative difference in staining in the superior mesenteric artery (H, K) or in the superior cervical ganglion (I, L) between tissues from SHR and WKY rats. Scale bars = 50 μm.
Figure 10: Haptoglobin Immunohistochemistry in the Liver

Haptoglobin immunostaining in the liver of WKY and SHR. Haptoglobin signal (brown) was similar in the liver from SHR (right column) and WKY rats (left column). Haptoglobin staining was stronger in hepatocytes surrounding the central veins (A, B) than in hepatocytes surrounding the hepatic triads (E, F). The small boxes in these images are blown up (C, D, G, H) to show patterns of haptoglobin staining. Lines of dark spots (arrowheads) are seen approximately outlining the basolateral membranes of the hepatocytes near the central veins (D, E), while dark areas representing Kupffer cells (arrowheads) are visible proximal to the hepatic triads (G, H). Control sections are shown for WKY (I) and SHR (J). CV = central vein; HA = hepatic artery; Scale bars = 50 μm.
Apolipoprotein A1 (ApoA1) was found to be upregulated in SMGs from 22wk SHR relative to SMG from 22wk WKY rats. A) One spot which had greater protein abundance on SHR SMG gels (circles) was identified as ApoAI. B) The increase in ApoA1 expression was verified using a Western blot. C) Quantification of the signal intensity of ApoA1 staining showed that there is a 40% increase in ApoA1 in the SMG of SHR relative to the SMG of WKY rats.
A

WKY  SHR

25 kDa

B

Anti – ApoA1

WKY  SHR

25 kDa

Anti – β-Actin

WKY  SHR

42 kDa

C

% of Actin Intensity

WKY  SHR
Table 1: Protein Identifications in the Superior Mesenteric Ganglion

<table>
<thead>
<tr>
<th>Protein</th>
<th>Acronym</th>
<th>MASCOT</th>
<th>Identifier</th>
<th>MW</th>
<th>Seq. Cov</th>
<th>pI</th>
<th>SHR/WKY†</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 weeks old</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Hp</td>
<td>118</td>
<td>60097941</td>
<td>39052</td>
<td>20%</td>
<td>6.10</td>
<td>↑</td>
</tr>
<tr>
<td>Predominantly Fetally Expressed T1 Domain</td>
<td>Pfetin</td>
<td>121</td>
<td>50400892</td>
<td>36155</td>
<td>27%</td>
<td>5.63</td>
<td>↑</td>
</tr>
<tr>
<td>Serine Protease Inhibitor 2.1</td>
<td>Spi 2.1</td>
<td>104</td>
<td>2507387</td>
<td>46419</td>
<td>19%</td>
<td>5.48</td>
<td>↓</td>
</tr>
<tr>
<td>22 weeks old</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein A1</td>
<td>ApoA1</td>
<td>75</td>
<td>6978515</td>
<td>30100</td>
<td>16%</td>
<td>5.52</td>
<td>↑</td>
</tr>
</tbody>
</table>

Molecular weight (MW) and isoelectric point (pI) are the values found in the NCBI databank, rather than the MW and pI at which the spot was identified in this project.
† Arrows indicate the change in protein expression in SHR relative to WKY.
Chapter 3
Proteomic Analysis of the Liver of Spontaneously Hypertensive Rats

Introduction

Primary, or “essential”, hypertension is a sustained increase in mean arterial blood pressure that does not result from a pre-existing pathology, such as kidney disease. However, it must be kept in mind that high blood pressure does not occur spontaneously. The development of primary hypertension in humans and animals is associated with predisposing factors such as sympathetic hyperactivity, insulin resistance, visceral adiposity, hyperlipidemia, oxidative stress, inflammation, and augmented production of various hormones such as angiotensin II (Guyenet, 2006; Shimamoto and Ura, 2006; Chueng, 2008; Yanai et al., 2008). Clinically, hypertension is one component of the metabolic syndrome, which is a disorder characterized by the combination of insulin resistance, central obesity, hyperlipidemia, and hyperglycemia. Furthermore, 30 – 50% of patients diagnosed with primary hypertension are found to also have other aspects of the metabolic syndrome upon closer investigation (Chueng, 2008; Cuspidi et al., 2008; Fallo et al., 2008; Yanai et al., 2008). In the spontaneously hypertensive rat (SHR), a well accepted research model of primary hypertension, increased arterial blood pressure is associated with mild insulin resistance and visibly augmented visceral fat deposition compared to normotensive, control Wistar-Kyoto (WKY) rats (Shimamoto and Ura, 2006). Clearly, primary hypertension cannot be treated as an isolated phenomenon.

Non-alcoholic fatty liver disease (NAFLD), or hepatic steatosis, has come to be accepted as the hepatic manifestation of the metabolic syndrome. NAFLD is defined as
the accumulation of excess triglyceride in the cytoplasm of at least 5% of hepatocytes with lipids accounting for 5-10% of total liver weight (normal is 1-2%) in patients who do not consume alcohol (Lonardo et al., 2005; Chueng and Sanyal, 2008; Wei et al., 2008; Paschos and Paletas, 2009). Histological features of NAFLD include cytoplasmic lipid droplets, hepatocyte ballooning, and enlarged mitochondria (Mehta et al., 2002). Insulin resistance, visceral adiposity, inflammation and mitochondrial dysfunction are thought to be the most important factors contributing to the development of hepatic steatosis (Targher, 2007; Targher et al., 2008; Paschos and Palets, 2009).

NAFLD and primary hypertension are common comorbidities; NAFLD is present in 70 – 90% of patients with obesity (Targher, 2006) and in 40 – 50% of patients with hypertension (Fallo et al., 2008). Although no direct mechanism connecting the pathogenesis of hypertension with that of NAFLD has been convincingly shown, both conditions result from similar predisposing factors. It is therefore reasonable to expect that a person or animal with hypertension would have altered hepatic physiology reminiscent of NAFLD at either the histological or molecular level.

Despite extensive epidemiological and clinical data connecting NAFLD, hypertension, and cardiovascular disease, very little research has been done examining the effect of primary hypertension on the molecular biology of the liver. In experiments investigating SHR, the liver is commonly used as an indicator of systemic oxidative stress and lipid metabolism in response to novel anti-hypertensive therapies (Yahia et al., 2003; Gomez-Amores et al., 2006; Oh et al., 2007, 2008; Tada Y 2008), yet it has rarely been compared to WKY livers in the absence of any treatment. There are several
exceptions; various lipogenic enzymes, such as fatty acid synthase (Ueno et al., 2009) and the free fatty acid transporter CD/36 (Benen et al., 2009), have been shown to be upregulated in the SHR liver, and SHR were shown to have dyslipidemia and increased hepatic lipid accumulation compared to WKY rats (Rodriguez et al., 2008). Antioxidant enzymes such as catalase and glutathione reductase are increased in SHR while glutathione peroxidase is decreased and superoxide dismutase is unchanged (Kitts et al., 1998; Yuan and Kitts, 2003). Finally, various markers of inflammation and neutrophil infiltration are augmented in several SHR tissues, including the liver (Sun et al., 2008).

Two-dimensional gel electrophoresis (2DGE) is a powerful tool for the analysis of the proteome, or the complete protein content, of a sample. Proteomic methods have been used to study the liver from many disease models including high fructose-induced hepatic steatosis (Zhang et al., 2008), insulin resistance and dyslipidemia (Morand et al., 2005), mitochondria under oxidative stress (Lee et al., 2008), cirrhosis (Molleken et al., 2009), hepatocellular carcinoma (Kim et al., 2002), hepatitis C infection (Diamond et al., 2007) and others. To our knowledge, this is the first proteomic study which examines the livers of hypertensive SHRs to age-matched normotensive WKY rats. We used 2DGE to compare whole tissue protein expression in the SHR and WKY livers in an attempt to better understand the molecular biology of the liver in this model. Numerous changes in protein abundance and post-translational modifications were identified in proteins whose functions relate to oxidative stress, mitochondrial activity, lipid metabolism, and other processes pertinent to the pathophysiology of primary hypertension.
Methods

Animals

A total of ten 16 week old male SHR and ten 16 week old male WKY rats were purchased from Charles River Incorporated. Rats were ordered in two separate cohorts to avoid clonal effects associated with littermates. Animals were housed in pairs under low stress conditions and were fed standard rat chow. All methods were approved by the Queen’s University Animal Care Committee, following the guidelines set forth by the Canadian Council on Animal Care.

Tissue Collection

Rats were anesthetized using cocktail of ketamine (5.6mg/kg), Rompun (4mg/kg) and acepromazine (0.75mg/kg). Seven SHR and seven WKY rats were transcardially perfused with 200 mL of phosphate buffered saline (PBS) or until buffer ran clear. Portions of the liver of each animal was removed and snap frozen in liquid nitrogen (-196°C). Tissue was stored at -80°C until further processing for proteomics. Three rats of each strain were transcardially perfused with 200 mL 4% (w/v) paraformaldehyde in PBS for immunohistochemistry. Livers were removed and placed in 4% (w/v) paraformaldehyde overnight and then transferred to 20% sucrose for 48 hours. Cryo-protected tissues were then snap frozen and cut in 20 μm sections for immunostaining.

Proteomics

Isoelectric Focusing
Frozen livers were individually ground into a powder in liquid nitrogen using a mortar and pestle and then further homogenized using a glass tissue grinder in whole tissue homogenization buffer containing 8M urea, 2M thiourea, 4% w/v CHAPS in 15 mM Tris (pH 7.0). Samples were centrifuged for 15 minutes at 170,000 g and supernatants were collected and stored at -80°C. Protein concentration was determined using the 2-D-Quantification Kit (Amersham Biosciences, Baie d’Urfe, QC, Canada) and these values were verified using a Western blot for actin (see below for procedure). 250 μg of sample protein was reduced using 1% (w/v) DTT for 15 minutes and diluted to 450 μL in rehydration buffer containing 8M urea, 2M thiourea, 4% (w/v) CHAPS, 2% (v/v) HED and 0.5% ampholytes (pH 3-11). 24-cm (pH range 3-11) immobilized pH gradient (IPG) strip gels (Amersham) were rehydrated overnight at room temperature in the buffer containing the sample protein. Isoelectric focusing was carried out on a Protean IEF cell machine (BioRad; Mississauga, ON, Canada) according to the following program: 8 hrs at 250V; 1 hr at 500V; 1 hr at 1000V; 3 hr linear ramping to 10,000V; 65 kVhr at 10,000V. Focused IPG strips were stored at -80°C to prevent protein diffusion. A minimum of four IPG strips were prepared for each sample.

For MS/MS, a very large protein sample is required to obtain reliable data regarding post-translational modifications (PTMs). To gather this sample, ten IPGs strips were loaded with 3 mg of liver protein per strip for each strain. For spots located in the acidic range of the proteome, protein was loaded onto IPG strips (24-cm, pH range 4-7) using in gel rehydration as described above. For spots in the basic range of the proteome, protein was loaded using the paper bridge method according to Kane et al (2006).
Briefly, strips (24-cm, pH range 6-9) were rehydrated overnight in rehydration buffer not containing sample protein. The following day, a volume of sample containing 3mg of protein was reduced with DTT and diluted to a total of 275 μL. The sample was then transferred to thick filter pad (3-cm long by 0.4-cm wide by 0.3-cm thick) and the pad was placed at the anodic end of the rehydrated strip in the IEF machine with approximately 0.5-cm of overlap between the strip and the pad. The electrode was then placed at the far end of the filter pad. A set of empty cups (normally used for cup-loading) was placed over the overlapping region to ensure sufficient contact between strips and filter pads. The 24-cm strips were trimmed at the cathodic end as necessary. Isoelectric focusing for both acidic and basic strips was carried out according to the following program for both strip lengths: 16 hrs at 350V; 2 hr at 500V; 2 hr at 1000V; 3 hr linear ramping to 10,000V; 100 kVhr at 10,000V. Pads were left in place for the duration of the focusing to maximize protein transfer.

2 Dimensional Gel Electrophoresis

2 dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2-D SDS-PAGE) was carried out according to Graham et al. (2005b). Focused IPG strips were treated for 20 minutes in equilibration buffer containing 8M urea, 30% (v/v) glycerol and 2% SDS in 50 mM Bis-Tris (pH 8.8), supplemented with 1% (w/v) DTT and again for 20 minutes in equilibration buffer supplemented with 2.5% (w/v) iodoacetamide. Equilibrated strips were placed on top of 1mm thick 10% acrylamide gels (with Bis-Tris (pH 7.4)) and embedded using 0.5% (w/v) low-melt agarose in electrophoresis buffer.
Large format 2-D SDS-PAGE electrophoresis was carried out in a DALT6 apparatus (Amersham) in MOPS (3-(n-morpholino) propanesulfonic acid) electrophoresis buffer and run at 80V at 4°C overnight. Except where described below, gels were with silver stained according to Shevchenko et al. (1996) and scanned using a UMAX Powerlook2100XL. Gels were dried between porous cellophane sheets for spot excision and long-term storage.

For quantification of spot differences, two SHR and two WKY homogenized liver samples were run out on 2D SDS-PAGE gels as described and stained with Deep Purple Fluorescent stain (GE Healthcare Bio-Sciences Corp.; Piscataway, NJ, USA) according to the manufacturer’s protocol. Gels were imaged using a Typhoon scanner (GE Healthcare) and 8 bit grayscale images were analyzed using PD-Quest software (Biorad). Spot intensities were normalized to the general background staining intensity of the entire gel, spots were manually delineated and total spot intensities were determined. Spot intensities were graphed in Microsoft Excel and interpreted as relative differences in protein abundance.

Gels for tandem MS were stained with colloid Blue Silver according to Candiano et al. (2004). Briefly, gels were fixed for one hour in 50% (v/v) methanol, 10% (v/v) acetic acid followed by a ten minute wash in 50% (v/v) methanol and two ten minute washes in double distilled H₂O. Gels were then immersed in Blue Silver dye for a minimum of 72 hours. Gels were then destained with several water washes to remove background. Stained gels were scanned as dried as described above and spots were manually excised for tandem MS analysis.
Mass Spectrometry

Protein samples were prepared for mass spectroscopy according to McDonald et al. (2008). All consistent spot differences between gels from the livers of SHR and WKY rats (i.e. spot changes found in every gel from every animal of a given phenotype) were manually excised from the gels, robotically rehydrated and destained with aqueous 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. Gel plugs digested with 6 ng/mL trypsin (sequencing Grade-modified trypsin; Promega, Napean, ON, Canada) for 5 hours at 37°C. Peptides were extracted using 1% formic acid/2% acetonitrile followed by 50% acetonitrile. Extraction solutions were combined and evaporated down to 10 μL. Peptides were spotted onto a MALDI target plate on top of a recrystallized spot of alpha-cyano-4-hydroxy-cinnamic acid matrix and spectra were collected using a Voyager DE-Pro matrix-assisted laser desorption ionization time of flight mass spectrometer (MALDI-TOF MS; PerSeptive Biosystems, Framingham, MA, USA). Peaks were analyzed using Data Explorer software; spectra were adjusted to baseline, noise filtered, externally calibrated against a spectrum of a known 4-peptide mix and internally calibrated against Trypsin I and Trypsin III peaks, when present. Peptide masses were manually selected and the resulting peak mass lists were searched against the NCBI database using the peptide mass fingerprinting database search tool MASCOT PMF search engine (http://www.matrixscience.com). Spectra which did not contain adequate peaks to get a statistically significant hit (significance as determined by MASCOT) were ZIP-TIPPED (Millipore Corporation, Bedford MA, USA) and eluted into 70% acetonitrile in 0.1%
TFA in ddH₂O to purify and concentrate the peptides and new MS spectra were collected. Only protein identifications with statistically significant MOWSE scores and which approximately matched the predicted molecular weight and pI values were included.

**Western Blot**

For 1D western blots, 15μg of protein per sample was diluted to 25μL in loading buffer and reduced using DTT for 15 minutes. Samples were then loaded onto 10% acrylamide gels overlaid with 5% acrylamide stacking gel. Gels were run in MOPS buffer in the Mini-Protean tetra-Electrophoresis system (Biorad) at 100V at room temperature until the dye front reached the bottom of the gel. Protein was then transferred onto nitrocellulose membranes in 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer in the Mini-TransBlot cell (Biorad) overnight at 23V at 4°C. Membranes were blocked in Western Blocking Solution (WBS; Amersham) for one hour at room temperature and then incubated in one of the following primary antibodies at room temperature for two hours: goat anti-actin (1/10,000; Santa Cruz Biotechnologies, Santa Cruz USA), goat anti-sulfite oxidase (SO; 1/5000; Santa Cruz), rabbit anti-quininoid dihydropterine reductase (QDPR; 1/2000; Santa Cruz) or mouse anti-MAWDBP (1/500; Abnova, Neihu District, Taipei City, Taiwan). Membranes were then washed in TBST and incubated in WBS containing one of the following secondary antibodies for one hour at room temperature: goat anti-rabbit (1/10,000; HRP conjugated; Jackson Immunoresearch, West Grove, PA, USA), rabbit anti-goat (1/10,000; HRP conjugated; Jackson Immunoresearch) or goat anti-mouse (1/10,000; HRP conjugated; Jackson Immunoresearch). Membranes were then
treated with Chemi-Illuminescence according to the manufacturer’s protocol. Signal intensity was quantified using PD Quest software where indicated. Statistical significance was determined with the student’s t-test using Microsoft Excel, 2007.

For 2D blots, 30μg of protein was loaded onto IPG strips (24-cm pH, range 3-10) using in-gel rehydration and isoelectrically focused according to the program previously described for 250μg. The locations of the proteins of interest were estimated using dried gels and two 7-cm sections, one extending approximately from pH 4.5 to pH6 and the other extending from pH6 to pH9, were cut out of the strips. The strips were carefully lined up so that the cut were always made in the same places to facilitate comparisons. Strip pieces were placed on top of 10% acrylamide gels, embedded in 0.5% agarose in MOPS and run as described for 1D Westerns. Protein was then transferred to nitrocellulose membrane and exposed to antibodies against sulfite oxidase, QDPR and MAWDBP.

**Immunohistochemistry**

For further validation of protein expression, liver sections were prepared and cut at 20 μm on a cryostat. For chromogenic immunostaining, liver sections were fixed for 30 minutes in 4% (w/v) paraformaldehyde in PBS. Slides were then treated for 1 hour in 0.3% (v/v) hydrogen peroxide in 0.1M Tris buffer and then blocked for 1 hour in 10% bovine serum albumin (BSA) in 0.25% TBX. Tissue sections were then incubated with one of the following antibodies in 3% BSA for 72 hours at room temperature: goat anti-arginase 1 (1/500; Santa Cruz Biotechnologies); rabbit anti QDPR (1/100); goat anti-SO (1/100);
mouse anti-MAWDBP (1/100). Slides were then incubated in goat anti-rabbit biotinylated IgG (1/200; Jackson Immunoresearch), rabbit anti-goat biotinylated IgG (1/200; Jackson Immunoresearch) or horse anti-mouse biotinilated IgG (1/200; Jackson Immunoresearch) in 3% BSA for two hours at room temperature. The specificity of all antibodies was tested by running slides under the following control conditions: primary IgG, without secondary IgG; secondary IgG, without primary IgG; correct primary IgG with incorrect secondary IgG. Slides were then treated for two hours with ABC (Vector Laboratories, Burlington, ON, Canada) for two hours according to manufacturer’s protocol, followed by diaminobenzidine (DAB) staining for three minutes. Slides were then dehydrated in an ethanol series followed by washes in HistoClear®. Coverslips were applied using Permount mounting media and images were acquired using a Zeiss fluorescence microscope and Axiovision software.

**Results**

Eighteen consistent spot differences were identified between gels made from SHR and WKY liver tissue (Figure 12). These spot were shown using MALDI-TOF MS to represent twelve unique proteins. Protein identification data is summarized in Table 2. Five proteins (formiminotransferase cyclodeaminase, glutathione-S-transferase μ1, MAWDBP, NADH dehydrogenase ubiquinone 1α subcomplex 10 and quininoid dihydropterine reductase) shifted position on the SHR liver gels relative to WKY liver gels without a noticeable change in protein abundance, while one protein (carboxylesterase ES-10) shifted position in combination with an increase in spot
intensity in SHR gels. Three protein spots (alcohol dehydrogenase iron containing 1, carbonic anhydrase II and haloacid dehalogenase-like domain containing 3) showed increased spot intensity in SHR gels relative to WKY gels, while three protein spots (carboxylesterase ES-4, glutathione-S-transferase ω1 and sulfite oxidase) showed increased spot intensity in WKY gels relative to SHR gels.

Shifts in spot location are most likely due to differences in post-translations modification (PTMs) of the protein between SHR and WKY rats. In order to identify the specific modifications involved in the spot translocations shown in Figure 12, large amounts of each protein had to be isolated. This was done by running 3 mg of protein on 24-cm strips (pH 4-7 and pH 6-9) and pooling the spots from many gels which were then stained with Coomassie Blue Silver. As a result of increasing protein load and narrowing the pH range of the strips, three additional spot changes were observed on these narrow pI range gels which had not been visible on the wide pI range gels used previously (Figure 13). 17β-hydroxysteroid dehydrogenase, coproporphyrinogen oxidase and tumor necrosis factor type 1 receptor associated protein were all found to be increased in SHR relative to WKY. These spots were later quantified using the Deep Purple fluorescent stain. (Unfortunately the tandem MS data analysis on the PTMs is incomplete at this time.) Quantification of the differences in protein spot intensity for all changes was carried out using Deep Purple fluorescent stain (Figure 14).

The changes in expression of QDPR (Figures 15, 16 and 17) and SOX (Figures 18 and 19) were chosen from the above proteomic data for verification using Western blots and immunohistochemistry; these proteins were selected because their potential role in
the generation of ROS makes them especially interesting and relevant to the SHR model (see Discussion). The shift in location of the QDPR spot in SHR gels relative to WKY gels was confirmed using a 2D Western blot (Figure 15). The QDPR spot in SHR is at a higher pI than the QDPR spot in WKY rats; there is no difference in molecular weight. As expected, there is no difference in protein abundance between strains according to 1D Western blot (data not shown).

Quantification of SOX expression by 1D Western blot showed that SHRs had a slight but significant decrease in total SOX protein compared to WKY rats (Figure 18C). When expressed as percent of actin signal intensity, SHR livers had a mean±standard error of 56.5%±12 and WKY had a mean of 75.6%±10. 2D Western blots showed that there are two SOX immunopositive spots in WKY but only one spot in SHR. It is unclear whether this decrease in total SOX protein in SHR is due more to the absence of the less abundant isoform or to a decrease in expression of the more abundant isoform. Overall, the 2D Western blots shown in Figure 18 confirm the proteomic observations described above.

Immunohistochemistry was used to analyze the cellular localization of QDPR and SOX expression in the rat liver. As shown in Figure 16A and B, QDPR was found to be moderately expressed in hepatocytes and highly expressed in the tissue surrounding the hepatic triads. There also seems to be some QDPR staining in the endothelium lining hepatic arteries and central veins (Figure 16C, D). To further determine which layer of blood vessels (i.e. endothelium, intima media or adventitia) expressed QDPR, aortas from SHR and WKY rats were processed and stained. Staining was present in the adventitia
and in the endothelium, but absent in the intima media (Figure 16D, E). Although patterns of QDPR expression in the aorta are not necessarily directly applicable to hepatic triads, it seems likely that QDPR is also expressed in endothelial cell and fibrocytes, but not in smooth muscle cells, in the vessels found within the liver and possibly throughout the body. No difference in staining intensity or distribution was observed between SHR and WKY rats in any of the tissues examined.

Cellular localization of SOX followed a very different pattern; SOX was found in small cells scattered throughout the liver, located in either the sinusoids or the Space of Disse (Figure 19). No staining was found in hepatocytes or in cells associated with blood vessels or bile ducts. This pattern resembles stellate cells (Higashi and Senoo, 2003), or Kupffer cells. As for QDPR, there was no difference in staining intensity or pattern of localization between the livers of SHR and WKY rats.

The difference in MAWDBP expression was also chosen for validation since a decrease in MAWDBP protein abundance was found in the kidney of SHR with no change in spot location (unpublished results from our laboratory). MAWDBP immunostaining was found to be evenly distributed throughout the hepatocytes with a spotty staining pattern in the connective tissue surrounding the hepatic triads. Unfortunately, MAWDBP immunoblotting was not possible due to a lack of antibody specificity on 1D and 2D Western blots (data not shown).
Discussion

This study represents the first proteomic comparison of the liver of SHR relative to the liver of WKY rats. Primary hypertension and NAFLD share many molecular and clinical features including, but not limited to, increased oxidative stress, mitochondrial dysfunction, the presence of inflammatory markers, altered hepatic lipid metabolism and handling, and increased risk of advanced cardiovascular disease. Furthermore, the risk of developing hypertension and/or NAFLD is greatly enhanced by insulin resistance or visceral adiposity. Despite these profound similarities, very little work has been done investigating the effects of hypertension on the molecular biology of the liver. This study was designed to assess how hepatic protein expression is altered in a model of primary hypertension. We hope to gain insight into the type of changes that are occurring in the liver of SHR, to hypothesize on how these changes are involved in the pathogenesis and maintenance of primary hypertension, and to suggest mechanistic similarities between hepatic function in hypertension and in NAFLD.

It should be noted that the purpose of this study was to identify proteins with a potential involvement in the pathophysiology of the SHR model; establishing that these protein changes are in fact dependent on hypertension as opposed to unrelated phenomena associated with this model is beyond the scope of this project. The following discussion of the individual proteins is based on the assumption that these changes in expression are significantly involved in the development, maintenance, or compensation to chronically elevated arterial blood pressure. Although it is possible to speculate and hypothesize as to why the expression of the proteins listed below are affected in SHR,
extensive further research is required to determine whether the ideas put forth here are accurate.

Fifteen proteins were identified as having altered expression in the liver of SHR compared to WKY. These proteins can be divided into the categories of 1) proteins involved in regulation of ROS synthesis or mitochondrial function, 2) proteins related to lipid metabolism, and 3) proteins with other functions connected to hypertension. Overall, these results support the generally accepted hypothesis that there is enhanced oxidative stress and altered lipid metabolism in the liver of SHR. However, as many of the specific proteins identified in this study have not been previously associated with hypertension or with the SHR model, the results presented here provide novel mechanisms for the development or maintenance of oxidative stress and dyslipidemia.

Of the fifteen proteins listed in Table 1, seven identifications are either mitochondrial proteins or proteins closely related with the regulation of oxidative stress. They are quininoid dihydropterine reductase (QDPR), sulfite oxidase (SOX), glutathione-S-transferase μ1 (GSTM1), GST ω1 (GSTO1), and NADH dehydrogenase ubiquinone 1α subcomplex 10 (NDUFA10), coproporphyrinogen oxidase (CPOX) and tumor necrosis factor receptor associated protein 1 (TRAP1).

Quininoid Dihydropterine Reductase (QDPR)

The QDPR protein spot was observed at different pI values on the gels made from SHR and WKY livers. This shift is presumably due to a PTM present in one strain but not in the other. QDPR was found to be expressed in the endothelium lining the hepatic arteries
and, possibly, in the central veins and in the connective tissue surrounding the hepatic triads, as well as in hepatocytes. A difference in QDPR regulation and activity is potentially relevant to vasoconstriction, NO signal transduction, and oxidative stress in hypertension.

QDPR, also known as dihydrofolate reductase, is one of two enzymes that catalyze the reduction and regeneration of the tetrahydropterin (BH4) cofactor (Scriver, 1994). BH4 is required for the activity of the enzymes phenylalanine, tyrosine, and tryptophan hydroxylase in amino acid metabolism and neurotransmitter synthesis, for all isoforms of nitric oxide synthase (NOS) in signal transduction, and for glyceryl-ether monooxygenase in lipid metabolism (Thony et al., 2000). When a given molecule of BH4 is used by one of these enzymes, it becomes oxidized and must be reduced by QDPR before it is again available to act as a cofactor (Scriver, 1994). It is important to note that, without this cyclic regeneration of BH4, the entire population of BH4 would become oxidized and thus unable to support enzyme activity. In this situation, even though total BH4 is not changed, BH4 activity is effectively decreased since levels of bioavailable BH4 are significantly diminished. Thus, it is the pool of reduced-BH4 which is required for the reactions catalyzed by the above enzymes to proceed at normal rates, rather than the total pool of BH4.

Nitric oxide (NO), the product of NOS, is an important signaling molecule involved in intracellular and paracrine communication in a variety of tissues. The most well known of these functions is the vasodilation of blood vessels through the relaxation of smooth muscle cells (Boron and Boulpaep, 2003; Torok, 2008). NO synthesized in
endothelial cells diffuses across the basement membrane to the intima media where it mediates smooth muscle relaxation, decreases extracellular matrix synthesis, prevents smooth muscle cell proliferation, and decreases leukocyte and platelet adhesion to blood vessel walls (Landmesser et al., 2003; Torok, 2008). These functions of NO are important in maintaining vascular health and decreased NO bioavailability has been associated with several cardiovascular pathologies including primary hypertension. Decreased NO synthesis and/or increased NO degradation has been shown in several hypertensive animal models (reviewed by Torok, 2008).

Decreased bioavailability of BH4 has been connected with loss of NOS activity and increased oxidative stress in models of primary hypertension (Hong et al., 2001; Landmesser et al., 2003; Alp and Channon, 2004; Chalupsky and Cai, 2005; Shulz et al., 2008). BH4 is a powerful superoxide anion (O$_2^-$) scavenger and exogenous BH4 has been shown to decrease oxidative stress (Thony et al., 2000). However, when endogenous BH4 acts as an antioxidant, it becomes oxidized and must be regenerated before it can function as a cofactor. In hypertensive blood vessels, the high levels of ROS oxidize BH4 and thus decrease the pool of BH4 capable of supporting NOS activity (Hong et al., 2001; Landmesser et al., 2003; Alp and Channon, 2004; Chalupsky and Cai, 2005; Shulz et al., 2008). When BH4 availability decreases in this way, NOS becomes uncoupled and electrons are passed to molecular oxygen instead of to arginine (Schulz et al., 2008). This causes NOS to produce O$_2^-$ instead of NO (Landmesser et al., 2003). This further promotes oxidative stress not only by increasing ROS concentrations but also by decreasing NO bioavailability, which indirectly leads to vasoconstriction and blood
vessel remodeling (Figure 17). The half-life of NO is determined by the oxidative levels of the cytoplasm since NO reacts with $O_2^-$ to form peroxynitrite which is an example of a reactive nitrogen species (RNS) and causes protein nitrosylation (Hong et al., 2001; Shulz et al., 2008; Torok, 2008). NOS uncoupling and BH4 oxidation has been observed in DOCA-induced models of hypertension, which led to increased ROS and decreased NO (Landmesser et al., 2003). Additionally, overexpression of NOS has been shown to worsen oxidative tissue damage in animal models (Shulz et al., 2008). Increased NOS has also been demonstrated in the brainstem of SHR and may be involved in the signaling cascades that lead to the establishment of neurogenic hypertension (Waki et al., 2006; Hirooka, 2008). Upregulation of NOS has been interpreted as a compensatory mechanism to counter the rapid degradation of NO in the presence of $O_2^-$. 

SHR have decreased amounts of BH4 in blood vessels relative to WKY rats and treatment with exogenous BH4 cause decreases in oxidative stress, improved vessel relaxation and lowered blood pressure in SHR with no effect in WKY rats (Hong et al., 2001). This evidence supports the hypothesis that oxidative stress decreases the pool of available BH4 and thus leads to a vicious cycle that promotes further oxidative damage, decreases NO abundance and increases blood pressure (see Figure 17). Furthermore, NOS inhibition led to a decrease in blood pressure in SHR, suggesting that eNOS expression actually contributes to ROS, rather than preventing it (Waki et al., 2006). This probably occurs as a result of NOS uncoupling, potentially due to decreased levels of reduced BH4.
The regulation of QDPR represents a potential mechanism through which BH4 bioavailability can be controlled. Chalupsky and Cai (2005) found that angiotensin II induced hypertension was associated with an H2O2-dependent decrease in QDPR protein and that inhibition of QDPR resulted in a decrease in BH4. This study also showed that overexpression of QDPR prevented the decrease in NO normally seen in this model of hypertension.

Decreased NO bioavailability has also been shown to have a cytotoxic effect on hepatocytes following liver ischemia and reperfusion (Liu et al., 1998; Abe et al., 2009). This effect is mediated by upregulation of ROS synthesis, similar to that described above, which may activate signal transduction pathways that lead to apoptosis (Abe et al., 2009). The decrease in NO also enhances the inflammatory reaction initiated by Kupffer cells, promotes neutrophil infiltration, and augments the “oxidative burst” in inflammatory cells that results in a huge release of ROS into the extracellular space (Liu et al., 1998; Shulz et al., 2008; Abe et al., 2009). NO production is therefore important in the liver as well as the vasculature.

We observed a shift in location of the QDPR spot in SHR liver relative to WKY liver using 2DGE (Figure 14) which was verified using Western blots (Figure 15). Unfortunately, it is unknown whether the PTM that caused this spot translocation results in an increase, decrease, or no change in QDPR activity. Furthermore, it is not possible to determine whether this modification occurs in hepatocytes, endothelial cells and fibroblasts or whether it is specific to one or two cell types; however, it should be noted that, because the spot change shown in Figure 15 involves the majority of the QDPR
protein found in the liver, it is likely that the modification is found in all QDPR expressing cells. Due to these unknown factors, it is very difficult to hypothesize as to the biological relevance of this difference in QDPR expression between SHR and WKY rats and it is important to keep these unanswered questions in mind throughout the following discussion.

Since BH4 has been shown to be decreased in SHR compared to WKY rats, and because QDPR has been shown to be decreased in another model of hypertension, it is very tempting to assume that the QDPR PTM lowers this enzyme’s activity. As shown by the hypothetical mechanism presented in Figure 17, one would expect a decrease in QDPR activity to result in impaired regeneration of oxidized-BH4 which would severely limit BH4 availability, as previously observed in SHR and DOCA rats (Hong et al., 2001; Landmesser et al., 2003). Consequently, synthesis of NO would be hampered and NOS uncoupling would augment production of $O_2^-$. This increased ROS has been shown to disrupt cellular physiology through numerous mechanisms such as initiating chain reactions (such as lipid peroxidation), tissue damage (such as mitochondrial damage and cytotoxicity), and positive feedback cycles (such as NOS uncoupling) that perpetuate ROS synthesis, all of which contribute to a loss of homeostasis and establishment of the condition known as oxidative stress. In addition, lower NO bioavailability leads to vasoconstriction, blood vessel remodeling, inflammatory cell infiltration, sympathetic hyperactivity and interference of signal transduction cascades, which together have been shown to promote hypertension. Downregulation of QDPR therefore represents a
potential mechanism through which oxidative stress could be initiated in either the liver or the vasculature of SHR.

It is also possible that downregulation of QDPR activity is the result of increased ROS production (Chalupsky and Cai, 2005) rather than the cause. In this scenario, QDPR itself would part of the positive feedback cycle depicted in Figure 17. The modification responsible for the change in QDPR pI may be due to oxidative damage to the enzyme or may be the result of signal transduction cascades initiated by H₂O₂. Both options would have the same effect on oxidative stress and tissue damage.

Another potential mechanism is that reduction in BH4 bioavailability mediates hypertension through an increase in testosterone synthesis. Fortepiani and Reckelhoff (2005) found that BH4 supplementation drastically lowered plasma testosterone in addition to decreasing blood pressure in intact SHR male rats. Furthermore, BH4 failed to lower blood pressure in castrated male rats or in rats given with exogenous testosterone. As there was no decrease in testosterone upon sodium nitrite supplementation, regulation androgen synthesis does not seem to be mediated by NO status. Testosterone seems to increase blood pressure through parallel increases in oxidative stress (Fortepiani and Reckelhoff, 2005; Sullivan et al., 2006; Kienitz and Quinkler, 2008), which would in turn lead to NO uncoupling as described above (Figure 17). This hypothesis is especially interesting since 17β-hydroxysteroid dehydrogenase, the enzyme which converts inactive androgen precursors to testosterone, was found to upregulated in SHR relative to WKY rats (Figure 14). For more on testosterone and hypertension, see the section on 17BHSD below.

119
One caveat to this hypothesis is that the requirement of BH4 for phenylalanine hydroxylase (PAH) is actually higher than for NOS (Thony et al., 2000) yet there is no sign of phenylketouria in SHR. One would expect that, if this downregulation of QDPR did in fact translate into a significant reduction in available BH4, SHR would have severe metabolic intolerance to phenylalanine. Mutations in the QDPR gene have been shown to cause decreased PAH activity and are responsible for one third of cases of BH4 deficiency in humans (Dianzani et al., 1998). One solution to this problem would be for the increase in NOS activity following BH4 administration to be mediated by a drop in testosterone. Alternatively, it is possible that PAH has a stronger affinity for BH4 than does NOS and thus has preferential access to the limited available BH4.

Sulfite Oxidase (SOX)

Two SOX spots were observed in WKY gels while only one of these spots was present in SHR and total SOX protein was found to be significantly decreased in SHR compared to WKY (Figure 18). Altered SOX activity is potentially important to in the formation of ROS in mitochondria.

SOX oxidizes toxic sulfite (SO$_3^{2-}$) to sulfate (SO$_4^{2-}$) as the final step in processing sulfur containing amino acids (cysteine and methionine) and detoxification of other sulfur containing compounds (Feng et al., 2008). SOX is located in the intermembranous space of the mitochondrion where it transfers electrons from sulfur compounds to cytochrome c in the electron transport chain (Feng et al., 2008). SOX is important for preventing O$_2^-$ production and cytotoxicity associated with sulfite exposure (Herken et al., 2008;
This function is illustrated by sulfite oxidase deficiency (SOD), a rare autosomal recessive mutation in SOX that results in severely decreased enzyme activity (Karakas et al., 2005). SOD rats are distinctly sensitive to SO$_3^{2-}$, an effect which seems to be mediated by an accumulation of ROS (Chiarani et al., 2008; Herken et al., 2008; Niknahad and O’Brian, 2008).

SOX may also be directly associated with regulation of vasomotor tone. Sulfite treatment of aortic rings from SOD rats decreased the degree of relaxation in response to acetylcholine (Nacitarhan et al., 2008). This effect was credited to an increase in production of O$_2^-$ and its interaction with NO since normal relaxation could be rescued through treatment with superoxide dismutase or L-arginine. Interestingly, exogenous delivery of sulfur dioxide (SO$_2$) has been shown to be capable of decreasing hypertension in SHR, while decreased endogenous production of SO$_2$ in normotensive rats led to increased blood pressure, smooth muscle proliferation and increased intima media thickness in aortic rings (Jin et al., 2007). This is relevant since SO$_2$ is endogenously produced by cardiovascular tissues (Jin et al., 2007) and is converted to SO$_3^{2-}$ in aqueous solutions (Herken et al., 2008). Since endogenous SO$_2$ should be metabolized by SOX, it follows that altered SOX activity could influence both the half-life and potency SO$_2$. It remains to be shown whether the observed SOX spot change is specific to the liver or if it is also found in extra-hepatic tissues, or whether a change in liver SO activity is capable of influencing sulfite levels in cardiovascular organs.

The lesser abundant SOX isoform in WKY was found to be absent in SHR; it is unclear whether there is also a decrease in expression in the more abundant isoform. As
this smaller spot represents a small portion of total SOX protein, and because the nature of the structural difference between these two isoforms is unknown, it is unclear whether and how the absence of this protein modification affects SOX activity. This change could lead to an increase, a decrease, or no change in SOX activity in SHR tissues. Conveniently, either an increase or a decrease in SOX can potentially be related to hypertension in SHR. A deficiency in SOX activity in SHR could lead to an increase in ROS production which has been shown, as described above, to be instrumental in the pathogenesis of hypertension in this model. It would then be possible that decreased SOX function would promote the establishment of oxidative stress in prehypertensive rats in response to exogenous SO₂ encountered through air pollution or through its use as a food preservative. The hypothesis that SOX function is lower in SHR is supported by the fact that SHR are more sensitive to oxidative stress and tissue damage due to SO₂ exposure in a model of bronchitis than are normotensive control rats (Kodavanti et al., 2006).

SOX hyperactivity, on the other hand, may promote rapid metabolism of endogenous SO₂ and thus limit vasodilation and promote vascular remodeling. Obviously further investigation is required to determine whether there is a difference in SOX activity between SHR and WKY and whether either of the described scenarios can be experimentally supported.
Glutathione-S-Transferase M1 (GSTM1) and GST Ω1 (GSTO1)

GSTM1 was found to be located in two spots in SHR liver gels but only one spot in WKY liver gels. Approximately 75% of the GSTM1 protein was shifted to a higher pI in SHR with no change in total protein abundance (Figure 14). GSTO1, on the other hand, was significantly downregulated in SHR relative to WKY. GST enzymes are involved in detoxification and biotransformation of toxins and metabolic by-products as well as deactivation of ROS, through conjugation with glutathione (GSH) (Sheehan et al., 2001; Hayes et al., 2005). The oxidation of GSH represents one of the body’s main defenses against oxidative stress in normal and disease conditions (Wu and Juurlink, 2002). There are five families of GST enzymes and family members differ in their substrate specificity, reaction kinetics, tissue distribution or structure (Sheehan et al., 2001; Hayes et al., 2005).

GSTM1 is one of the most important cytosolic GSTs in humans and has wide tissue distribution (Sheehan et al., 2001). There are four GSTM1 alleles in humans and 50% of Caucasians carry a null allele and consequently do not have any detectable GSTM1 activity (Izzotti et al., 2001; Sheehan et al., 2001). The GSTM1-null allele has been found to increase the risk of cardiovascular disease in smokers, drug induced liver disease and various types of cancer (Izzotti et al., 2001; Sheehan et al., 2001; Schneider et al., 2004).

Several different GST enzymes have been found to be downregulated in SHR or stroke-prone SHR (SHRSP) in the kidney, heart, skeletal muscle or liver (Wu and Juurlink, 2002; Okuda et al., 2002; McBride et al., 2005; Zhou et al., 2007). For
example, GSTM1 mRNA was found to be significantly decreased the kidney of SHRSP relative to WKY and there are several polymorphisms in the GSTM1 promoter region which are shared by SHR and SHRSP but not WKY rats (McBride et al., 2005). Such observations are generally interpreted as a decrease in the antioxidant capacity of the given tissue.

In the present study, we found that most of the GSTM1 protein had shifted location on the SHR gels, presumably due to a PTM that does not occur in WKY, with no change in total GSTM1 protein. The reasons for the discrepancy between these results and those of previous studies are unknown. The most likely explanation is that expression of GSTM1 is regulated differently in the liver and the kidney. This may indicate that GSTM1 serves different functions in these two organs. It would be interesting to determine whether this modification of GSTM1 is specific to the liver or whether it is present in other tissues. If it is in fact a liver specific phenomenon, this would call into question the practice of using the liver as a measure of total body oxidative stress. Another important question posed by these results is whether this modification affects the antioxidant or detoxification functions of GSTM1, and thus whether it contributes to the establishment of, or is a counter measure against, oxidative stress. Further research is needed to better understand the specific functions of GSTM1 in the kidney, liver and other tissues in hypertension.

Another GST family member, GSTO1, was found to be present in the WKY liver gels and absent in the SHR liver gels. GSTO1 is structurally distinct from other GST enzymes in that it has a cysteine in the active site where other GSTs have a tyrosine or
phenylalanine (Whitbread et al., 2003, 2005). This cysteine imparts on GSTO1 thiotransferase activity which is not shared by other GST enzymes and allows GSTO1 to interact with a unique range of substrates (Board and Anders, 2007; Board et al., 2007). For example, GSTO1 has dehydroascorbate reductase activity, giving it the ability to rejuvenate ascorbate, an important neural anti-oxidant, to its active, reduced form (Li et al., 2003; Schmuck et al., 2005; Whitbread et al., 2005). GSTO1 is therefore able to promote the neutralization of ROS through a mechanism independent of GSH transferase activity. Furthermore, GSTO1 polymorphisms have been linked to the early-onset Alzheimer’s and Parkinson’s disease, two diseases associated with neural oxidative stress, possibly due to decreased ascorbate activity (Li et al., 2003; Schmuck et al., 2005). Decreased GSTO1 protein therefore potentially dampens the anti-oxidant activity of ascorbate in the SHR liver and hence reduces the tissues’ defenses against oxidative stress.

GSTO1 was identified in another proteomic study to be increased in the heart of SHR compared to WKY rats (Jin et al., 2006). One possible explanation for the completely opposite results between Jin and colleagues and the present study is that GSTO1 undergoes a PTM in either SHR or WKY rats. Because of the different spot distribution in liver and heart proteomes, the SHR spot could have been covered in this study and the WKY spot covered in the previous study. The discrepancy between these two proteomic data sets emphasizes the need to validate observations using immunoblotting (as was done for QDPR and SOX in this study) before making conclusions as to their biological significance.
**NADH Dehydrogenase Ubiquinone 1α Subcomplex 10 (NDUFA10)**

The NDUFA10 spot was found to shift positions to a lower, more acidic pI in SHR liver gels compared to WKY liver gels (Figure 14). NDUFA10 is one of the subunits of Complex I, also known as NADH dehydrogenase, of the electron transport chain (ETC) in the inner mitochondrial membrane (Fearnley et al., 1991; Remacle et al., 2008). NADH dehydrogenase accepts electrons from NADH and then transfers those electrons to quinone at a lower oxidation state (Remacle et al., 2008). The energy released during this electron transfer is used to transport protons across the inner mitochondrial membrane into the intermembrane space and thus build an H+ concentration gradient which will later be used to synthesize ATP. The specific function NDUFA10 subunit within the entire holoenzyme is unknown, although it is thought to have regulatory function since it undergoes phosphorylation (Schulenberg et al., 2003). It has been suggested that NDUFA10 influences the catalytic activity of Complex I by regulating its affinity for NADH (Jin et al., 2006).

Complex I is one of the primary sources of O$_2^-$ production in the mitochondria in health as well as during the induction of apoptosis (Li et al., 2003; Grivennikova and Vinogradov, 2006; Hirst et al., 2008). Current models hypothesize that when enzymes of the ETC are impaired, electrons can accumulate on Complex I and be transferred from the flavin moiety directly to molecular oxygen, thus generating O$_2^-$ (Grivennikova and Vinogradov, 2006; Hirst et al., 2008). Inhibition of Complex I has been shown to cause dramatic upregulation of ROS synthesis (Li et al., 2003). Furthermore, decreased ETC
respiratory enzyme activity, impaired electron coupling and diminished protein abundance have been experimentally shown in SHR (Jullig et al., 2008; Lopez-Campistrous et al., 2008; Chan et al., 2009). Together, these findings indicate that regulation and function of Complex I are of great importance to mitochondrial physiology.

Changes in NDUFA10 expression have also been identified in two other proteomic studies done on mitochondrial isolates from cardiomyocytes of SHR and WKY rats. In the first study, two NDUFA10 spots were found in SHR gels while a single separate spot was found in WKY gels (Meng et al., 2008). In the second study NDUFA10 showed a 10% increase in total spot intensity in SHR with no observed change in spot location (Jullig et al., 2008). A third proteomic study examining a whole tissue extract of the left ventricle found a shift in the location of the NDUFA10 spot between gels from WKY and from SHR (Jin et al., 2006), similar to the observations reported above. The reason for the discrepancies between these studies and the results presented here are unknown. Taken together, the results of these four experiments indicate that there is some change in either the quantity or modification of NDUFA10 in SHR compared to WKY rats, which may have implications for Complex I function. Further research is warranted to determine the relevance of this protein to the pathogenesis of hypertension.
Coproporphyrinogen Oxidase (CPOX)

CPOX was identified in a spot which was present on SHR liver gels and absent on WKY liver gels (Figure 14). CPOX is the sixth enzyme in the synthesis of haem; it converts coproporphyrinogen III to protoporphyrinogen IX (Doss et al., 1999; Gross et al., 2002; Bonkovsky, 2005; Herrick and McColl, 2005). CPOX is located in the intermembrane space of the mitochondrion with approximately half of the protein attached to the inner mitochondrial membrane and half freely soluble within the space (Doss et al., 1999; Bonkovsky, 2005; Herrick and McColl, 2005).

CPOX deficiency is responsible for a condition known as hereditary coproporphyria which belongs to a class of diseases known as porphyrias (Laiwah et al., 1985; Doss et al., 1999; Gross et al., 2002; Bonkovsky, 2005; Herrick and McColl, 2005). Porphyrias are autosomal dominant genetic disorders which result from a deficiency in one of the enzymes of the haem biosynthesis pathway. These conditions can be divided into hepatic and erythropoietic porphyrias, depending on which tissue expresses the enzyme in question. Hereditary coproporphyria is an acute hepatic porphyria; all acute porphyrias are characterized by attacks which present with severe abdominal pain, vomiting, constipation, neurogenic pain, paresis, tachycardia and systemic arterial hypertension. Patients are asymptomatic between attacks and most people harboring the mutation are unaware of their condition. The diagnosis of an acute porphyric attack is confirmed by the presence of high levels of haem precursors in the feces and urine or by a biochemical test for enzyme function (Doss et al., 1999; Gross et al., 2002; Bonkovsky, 2005; Herrick and McColl, 2005). All acute porphyrias are...
characterized by the urinary accumulation of aminolevulin acid (ALA) and porphobilinogen (PBG) (Gross et al., 2002; Bonkovsky, 2005; Herrick and McColl, 2005).

All symptoms of an acute attack are thought to derive from the interaction of haem precursors, specifically ALA, with the neuromuscular system (Laiwah et al., 1985; Emanuelli et al., 2001). Acute attacks are associated with peripheral neuropathy, autonomic deregulation and psychosis (Doss et al., 1999; Gross et al., 2002; Bonkovsky, 2005; Herrick and McColl, 2005). Sympathetic hyperactivity and parasympathetic hypoactivity are together responsible for tachycardia, vasoconstriction and decreased intestinal tone (Laiwah et al., 1985; Cutler et al., 1990; Bonkovsky, 2005). Abdominal pain is considered to be a result of inadequate mesenteric blood supply, although this has not been systematically investigated. Tachycardia is due primarily to vagal neuropathy (Laiwah et al., 1985). Although the precise mechanisms remain unclear, these neuropathic symptoms may be a result of increased oxidative stress (Montiero et al., 1989; Hermes-Lima, 1995; Juknat et al., 2003; Lelli et al., 2005), interference of GABAergic synaptic transmission (Emanuelli et al., 2001) and peripheral demyelination (Laiwah et al., 1985; Felitsyn et al., 2008).

ALA accumulation has been shown to significantly increase ROS and free radical formation; this is associated with increased protein carbonylation, lipid peroxidation, DNA damage and other common results of oxidative stress (Montiero et al., 1989; Hermes-Lima, 1995; Juknat et al., 2003; Rocha et al., 2003; Lelli et al., 2005). Glial cells seem to be especially sensitive to ALA, and peripheral demyelination has been suggested
to result from oxidative damage to Schwann cells (Felitsyn et al., 2008; Juknat et al., 2003). Furthermore, porphyria carriers have upregulated antioxidant enzymes, indicating that their cells have adapted to a highly oxidative environment (Montiero et al., 1989). Augmented ALA has also been shown to cause decreased activity of gluconeogenic and glycogenolytic enzymes in animal models, probably though oxidative damage to the enzyme structure itself (this does much to explain why glucose supplementation is a successful therapy for mild porphyric attacks) (Lelli et al., 2005). ALA has also been shown to cause damage to the iron-binding protein ferritin, and thus promotes the circulation of free iron, which is highly oxidative (Rocha et al., 2003). Finally, hepatic porphyrias are associated with morphologically abnormal mitochondria and high concentrations of ALA have been shown to cause permabilization of the inner mitochondrial membrane, as well as other indications of mitochondrial dysfunction (Hermes-Lima, 1995). Clearly, inhibition of haem synthesis and accumulation of haem precursors is capable of promoting oxidative stress.

Porphyric attacks can also be elicited by conditions which dramatically stress the haem synthesis pathway in the absence of any genetic mutation (Doss et al., 1999). These secondary coproporphyrias are caused by a huge upregulation in ALA-synthase such that ALA is synthesized faster than the other enzymes in the pathway can process it, causing it to accumulate. Interestingly, secondary coproporphyrias are associated with high urinary coproporphyrin, hence the name, as is seen in CPOX deficiency in hereditary coproporphyria. This may indicate that CPOX becomes the new rate-limiting enzyme in this situation. Secondary coproporphyria can be induced by intoxication,
diabetes, liver disease, fatty liver, myocardial infarction, and many other factors (Doss et al., 1999).

CPOX was found to be increased in the liver of SHR rats relative to the liver of WKY rats according to 2DGE. The functional relevance of this, however, is very difficult to interpret. Although hereditary coproporphyria is always associated with decreased CPOX activity, CPOX protein abundance may be increased or decreased, depending on the mutation (Gross et al., 2002). Also, very little is known about the regulation of CPOX expression; haem synthesis normally is controlled through regulation of the rate limiting enzyme, ALA-synthase (Doss et al., 1999; Gross et al., 2002; Bonkovsky, 2005; Herrick and McColl, 2005). It is only possible to speculate on the significance of these observations in relation to information available on hereditary coproporphyria. Given the similarities of sympathetic hyperactivity, oxidative stress and mitochondrial abnormalities between acute porphyrias and the SHR model, it is tempting to suggest that CPOX expression is upregulated either in an effort to compensate for the accumulation of haem precursors as is seen in secondary coproporphyria, possibly due to a decrease in CPOX enzymatic efficiency, or to meet an increased demand for haem production. It is worth noting that, since several antioxidant and electron transport enzymes require haem, oxidative stress, which can damage or increase expression of these enzymes, may itself stress the haem biosynthesis pathway and cause an upregulation of CPOX and/or ALA-synthase. Additional research is needed to answer the many questions posed by the observation of increased abundance of CPOX in the SHR liver.
Tumor Necrosis Factor Receptor Associated Protein 1 (TRAP1)

TRAP1 (also HSP75/mtHSP70/Grp75/Ssc1/mortalin) was found to be upregulated on the gels from SHR liver relative to the gels from WKY liver (Figure 14). TRAP1 is a ubiquitous protein which is localized mainly to mitochondria with some expression also reported in the endoplasmic reticulum, cytosolic vesicles, plasma membrane and cytosol (Voos et al., 1999; Felts et al., 2000; Liu et al., 2005; Montesano Gesualdi et al., 2007; Im et al., 2007; Williamson et al., 2008; Xu et al., 2009). Within the mitochondrion, TRAP1 is found inside the matrix with a subset associated with the inner mitochondrial membrane. TRAP1 is an ATPase with a high level of homology with HSP90, although it fails to interact with any of the targets of this cytoplasmic chaperone (Felts et al., 2000). Rather, TRAP1 functions as a chaperone in the translocation of proteins through the inner mitochondrial membrane (Ostermann et al., 1990; Voos et al., 1999; Liu et al., 2005; Williamson et al., 2008). Proteins destined for the mitochondrion are translated in the cytosol in an unfolded, immature state. These pre-proteins carry an N-terminus targeting sequence which allows them to pass through the translocase complex in both the outer and inner mitochondrial membranes (Voos et al., 1999). TRAP1 binds the targeting sequence as soon as it passes through the inner membrane, and “pulls” the peptide through the translocase machinery in a directional, ATP dependent manner (Voos et al., 1999). TRAP1 then assists in refolding proteins within the matrix in cooperation with the major mitochondrial chaperone, HSP60 (Ostermann et al., 1990; Voos et al., 1999; Williamson et al., 2008). Proteins processed in this way either reside in the matrix, such
as fatty acid $\beta$-oxidation enzymes, or are reinserted into the inner mitochondrial membrane, such as ETC enzymes (Ostermann et al., 1990).

TRAP1 has been found to provide protection from several different cellular insults including ischemia/reperfusion, glucose depletion, radiation and oxidative stress (Liu et al., 2005; Montesano Gesualdi et al., 2007; Im et al., 2007; Williamson et al., 2008; Costantino et al., 2009; Xu et al., 2009). In addition, TRAP1 was found to be upregulated in cell lines adapted to mild oxidative stress (Montesano Gesualdi et al., 2007) as well as in many human tumors (Costantino et al., 2009). Over-expression of TRAP1 is associated with decreased mitochondrial ROS generation (Liu et al., 2005; Montesano Gesualdi et al., 2007; Im et al., 2007; Williamson et al., 2008; Xu et al., 2009), maintenance of electron transport chain protein function (Williamson et al., 2008; Xu et al., 2009), decreased lipid peroxidation (Xu et al., 2009) and improved cell viability under stressful conditions (Liu et al., 2005; Montesano Gesualdi et al., 2007; Im et al., 2007; Williamson et al., 2008; Costantino et al., 2009; Xu et al., 2009). In support of the view of TRAP1 as a stress-induced protein, upregulation of TRAP1 has very little effect on mitochondrial parameters under basal conditions (Williamson et al., 2008; Xu et al., 2009). One study found that TRAP1 transfected cells have higher levels of GSH, which is indicative of a more reducing cellular environment compared to control, untransfected cell lines (Montesano Gesualdi et al., 2007).

The role of TRAP1 in reducing oxidative stress has received extensive attention. The mechanism through which this is achieved is not completely clear; one likely possibility is that, through its role in protein translocation and refolding, TRAP1 replaces
damaged mitochondrial matrix and inner membrane proteins and protects them from oxidative conditions (Williamson et al., 2008). This would then prevent ETC proteins, which are highly sensitive to ROS due to their iron-sulpher reactive centers, from becoming uncoupled and thus producing increasing quantities of superoxide anion. This hypothesis is supported by the fact that TRAP1 over-expression is associated with maintained complex III and complex IV function during ischemia/reperfusion (a condition known to produce damaging levels of ROS) (Williamson et al., 2008; Xu et al., 2009), and by the observation that TRAP1 is required for the import of cytochrome c under normal conditions (Ostermann et al., 1990).

The upregulation of TRAP1 in the liver of SHR is very revealing. This observation further supports the now oft repeated idea that there is increased oxidative stress and mitochondrial dysfunction in SHR relative to WKY tissues. Furthermore, this indicates that the cells are adapting to this oxidative environment and employing protective mechanisms to maintain homeostasis. It remains to be determined whether TRAP1 expression is also altered in human hypertension or whether this is unique to the SHR model. If found only in SHR, TRAP1 upregulation may explain why the hepatic anatomy and physiology is reasonably well preserved with no clinical signs of NAFLD, despite the presence of insulin resistance and visceral adiposity, as is commonly seen in human hypertension and metabolic syndrome. If TRAP1 is upregulated in human hypertension as well as in SHR, it would lend support to the hypothesis that oxidative stress is central to the establishment of arterial hypertension in humans as well as in
animal models and will provide insight into the molecular changes associated with hypertension in human tissues.

Three of the proteins identified are involved in metabolic activities of the liver which have potential connection to NAFLD.

*Carboxylesterase ES-10 (ES-10) and Carboxylesterase ES-4 (ES-4)*

ES-10 was identified in two spots on the SHR liver gels but only one of these spots was present on the WKY liver gels (Figure 14). Furthermore, the ES-10 spot which is present only in SHR has much higher signal intensity than the ES-10 spot present in both SHR and WKY. The spot identified as ES-4 is present on the WKY gels and absent in the SHR gels.

ES-10 and ES-4 are two of the five carboxylesterases found in the rat liver, and together these two enzymes are responsible for 85% of hepatocyte carboxylesterase activity (Mello et al., 2008). In general, hepatic carboxylesterases interact with a wide variety of substrates involved in multiple functions including ethanol catabolism (Diczfalusy et al., 2001), lipid metabolism (Soni et al., 2004), vitamin A storage (Mello et al., 2008), and drug detoxification (Robbi et al., 1996).

ES-10 has been shown to have a role in ethanol catabolism through the synthesis of fatty acid ethyl esters (FAEE) (Diczfalusy et al., 2001; Pfutzer et al., 2002). In response to high blood alcohol content, ES-10 processes ethanol by esterifying it to a fatty acid; the resulting FAEE are released back into the blood stream where they mediate many of
the long-term physiological effects of chronic ethanol consumption (Diczfalusy et al., 2001; Salem et al., 2006). Interestingly, ES-4 has the opposite function in FAEE metabolism compared to ES-10; ES-4 has been shown to hydrolyze FAEE in the rat liver (Diczfalusy et al., 2001). The results of this study suggest that ES-10 is increased while ES-4 is simultaneously decreased in the liver of SHR relative to the liver of WKY rats; this may imply a significant metabolic shift toward increased FAEE synthesis in SHR.

FAEE are of clinical importance since evidence suggests that they are responsible for a large portion of the organ damage associated with alcoholism (Diczfalusy et al., 2001; Pfutzer et al., 2002; Bhopale et al., 2006; Salem et al., 2006). Augmented levels of FAEE in the liver are associated with lipid accumulation (hepatic steatosis) and inflammation (steatohepatitis) that is histologically indistinguishable from NAFLD (Bhopale et al., 2006). Interestingly, FAEE have also been shown to induce mitochondrial dysfunction (Lange and Sobel, 1983). Furthermore, ES-10 is upregulated during chronic ethanol exposure in rats, which first lends support to the hypothesis that ES-10 is a potential source of FAEE in alcohol-induced liver disease (Pfutzer et al., 2002), but also helps inspire the idea that SHR livers are undergoing the initial molecular events which could eventually lead to histologically recognizable NAFLD. It is tempting to suggest that, if such shift in FAEE metabolism does occur in SHR as these preliminary results suggest, then altered expression of carboxylesterases ES-10 and ES-4 may represent a mechanism connecting NAFLD to genetic arterial high blood pressure. However, extreme caution must be taken until these speculations have been experimentally supported, both in SHR and in humans. Further investigations are
required to determine whether FAEE synthesis is enhanced in human and animal hypertension and how this relates to liver health.

**Alcohol Dehydrogenase Iron-Containing 1 (ADHFE1)**

The spot identified as ADHFE1 had a higher signal intensity on gels made from the SHR liver compared to gels made from the WKY liver (Figure 14). Alcohol dehydrogenase (ADH) enzymes catalyze the conversion of alcohols into ketones and aldehydes. There are three classes of ADH enzymes: 1) medium chain Zn containing; 2) short chain Zn lacking; 3) Fe-containing (Reid and Fewson, 1994). Until recently, only the first two classes had been described in mammals while Fe-containing ADH enzymes were thought to be restricted to bacteria and archaea. This changed, however, when ADHFE1 was identified in human fetal brain and adult human liver (Deng et al., 2002). This enzyme retains ~50% homology with invertebrate Fe-containing ADH enzymes, especially in the active sites. ADHFE1 has since been found to also be expressed in the heart, kidney and adipose tissue of mice (Kim et al., 2007).

Beyond clues gleamed from its nucleotide sequence, the function of ADHFE1 in most tissues is unknown. There is evidence suggesting that ADHFE1 is important in the differentiation and phenotype of both white and brown adipocytes (Kim et al., 2007). Changes in ADHFE1 protein levels parallel those of other well known markers of adipocyte differentiation, such as PPARγ, which is considered the “master” adipogenic transcription factor. Expression of ADHFE1 is dependent on the PI3K signaling pathway
but is not influenced by TNFα, insulin, MEK1, p38-MAPK or rapamycin (Kim et al., 2007).

Any connection made between these findings and the SHR model are completely speculative. It is possible that the increase in ADHFE1 expression in SHR is somehow related to lipid metabolism in hepatocytes. However, it must also be kept in mind that ADHFE1 may have entirely different functions in hepatocytes than in adipose tissue. Furthermore, it should be noted that ADHFE1 was found to have mitochondrial localization (Kim et al., 2007), and thus represents yet another example of a mitochondrial protein whose expression is different between SHR and WKY rats.

In humans, ADH enzymes convert ethanol into acetaldehyde as part of ethanol detoxification in the liver. They therefore compete with FAEE synthase enzymes such as ES-10 for substrate. If ADHFE1 retains alcohol dehydrogenase activity as its sequence suggests, it is possible that the upregulation of ADHFE1 in SHR is somehow associated with the previously described changes in expression of carboxylesterases ES-10 and ES-4. Before making any hypotheses regarding this observation, however, it would be necessary to show that ADHFE1 has alcohol dehydrogenase activity. Again, it must be emphasized that such hypotheses are highly speculative and extensive further research is necessary to provide them with experimental support.

The remaining proteins are involved in other mechanisms which are potentially related to hypertension.
17β Hydroxysteroid Dehydrogenase (17BHSD)

17BHSD was found to be upregulated in SHR liver gels relative to WKY liver gels (Figure 14). 17BHSD catalyzes the final steps in the production of biologically active androgens and estrogens. Twelve unique 17BHSD enzymes have been identified which vary in their catalytic properties, their affinities for different steroid precursors, as well as their patterns of expression throughout the body. Some 17BHSD subtypes also exhibit additional functions ranging from fatty acid oxidation in mitochondria to regulation of glucocorticoid synthesis (Mindnich et al., 2004). All of these enzymes are in the short-chain dehydrogenase/reductase superfamily with masses around 30kDa, with the exception of type 5 which is in the aldo-ketoreductase superfamily which have masses around 37kDa (Mindnich et al., 2004). Because the protein spot identified as 17BHSD also had significant sequence similarities with an aldo-ketoreductase enzyme and had a mass closer to 37kDa than 30kDa, it seems likely that the protein in this spot is 17BHSD type 5. However, the Mascot search engine used to search MS peak lists did not specify which subtype had been identified.

The risk of developing cardiovascular disease exhibits surprising sexual dimorphism; men have higher rates of hypertension, arteriosclerosis, myocardial hypertrophy, kidney disease, peripheral arterial disease and stroke compared to age matched women independent of environmental risk factors such as diet and smoking (Liu et al., 2003, Ng, 2007). A similar phenomenon is seen in all rat models of hypertension (including SHR, Dahl salt-sensitive, and New Zealand genetically hypertensive rats) with males having higher blood pressures than females (Reckelhoff et al., 1999; Liu et al.,
The cause of this dimorphism in humans and animals is only partially determined. There is evidence suggesting that the high plasma testosterone concentration and tissue expression of the androgen expression found in men, but not in women, are required to mediate this increase in risk of cardiovascular problems (Reckelhoff et al., 1999; Liu et al., 2003; Ng, 2007). In SHR, castration lowers blood pressure to the level of normally found in female rats, while ovariectomy has no effect, and testosterone supplementation in castrated males and females raises blood pressures to values seen in intact males (Reckelhoff et al., 1999). Furthermore, expression of dominant-negative androgen receptor also ablates the difference in blood pressure between male and female rats (Liu et al., 2003). These observations suggest that testosterone itself is involved in the increased incidence of hypertension in men.

The mechanisms through which testosterone increases blood pressure are unknown. Several hypotheses have been proposed which focus on the effect of testosterone on renal function; testosterone decreases excretion of sodium and water, possibly through acting on the proximal tubule, and thus increases blood volume (Reckelhoff and Granger, 1999; Kienitz and Quinkler, 2008). There is also evidence suggesting that testosterone increases the activity of the renin-angiotensin system. Men have 27% higher renin than women and inhibition of the angiotensin converting enzyme lowers blood pressure to the same values in male and female SHR (Reckelhoff and Granger, 1999; Kienitz and Quinkler, 2008). Alternatively, testosterone may increase blood pressure through increases in oxidative stress. Men have higher oxidative stress than women (Fortepiani and Reckelhoff, 2005a) and castration of male SHR lowers
urinary H$_2$O$_2$ levels to levels seen in female SHR (Sullivan et al., 2006). Tempol, a superoxide mimic, lowered blood pressure in male SHR but had no effect in male WKY or female SHR if given after hypertension was established (Fortepiani and Reckelhoff, 2005a). Interestingly, testosterone and H$_2$O$_2$ have very similar effects on renal sodium resorption and blood flow (Makino et al., 2003), which has led to the hypothesis that androgen-dependent control of blood pressure is mediated by regulation of ROS formation.

Despite the extensive work showing that testosterone contributes to high blood pressure in SHR, no study, to the best of my knowledge, has determined whether there is a difference in testosterone levels or activity between male SHR and WKY rats. The increase in 17BHSD protein abundance identified in this study suggests that SHR may have higher rates of testosterone synthesis than WKY rats, which may contribute to the altered renal physiology and oxidative stress seen in SHR. Further research is necessary to determine the biological significance of this observation.

*Carbonic Anhydrase II (CA2)*

The spot identified as CA2 was present on the gels made from SHR liver and absent on gels made from WKY liver (Figure 14). Since CA2 is known to be expressed at high levels in the normal liver, it is likely that there exists another CA2 spot that is present on both SHR and WKY liver gels and that the identified CA2 spot results from a PTM that occurs in SHR but not in WKY rats.
CA2 is the most active cytosolic CA enzyme in humans. It is present at high concentrations in kidney, liver, and red blood cells and at lower concentrations in many tissues throughout the body. CA2 is required for the regulation of blood pH and osmotic balance (Boron and Boulpaep, 2003; Purkerson and Schwartz, 2007). In the kidney, it is found at the highest levels in the epithelial cells of the proximal convoluted tubule where it plays a crucial role in the resorption of NaHCO3 and H2O from the glomerular filtrate (Purkerson and Schwartz, 2007). In the liver, CA2 is expressed in greater amounts in perivenous hepatocytes than in periportal hepatocytes and has been suggested to be involved in gluconeogenesis, fatty acid synthesis and urea production (Dodgson et al., 1984, 1993). In red blood cells, CA2 activity maintains blood pH and prevents acidosis (Boron and Boulpaep, 2003).

Regulation of CA2 activity can potentially influence blood pressure through multiple mechanisms. Changes in renal CA2 function, for example, could alter total blood volume by increasing or decreasing resorption of bicarbonate and water. CA regulates intraocular pressure through a similar mechanism (Sugrue, 2000; Kehler et al., 2007). Under normal conditions, CA activity causes bicarbonate and H2O to enter the anterior chamber, thereby increasing the volume of aqueous humour. Under conditions of increased intraocular pressure, as is seen in glaucoma, CA inhibition is used to decrease humour production and thus decrease intraocular volume and pressure (Sugrue, 2000). Alternatively, CA2 may control blood pressure by controlling arteriole vasoactivity. Inhibition of CA, or of CA2 specifically, has been shown to induce local vasodilation and thus prevent pulmonary (Swenson, 2006) and ocular hypertension.
(Sugrue, 2000; Kehler et al., 2007) in high altitude pulmonary edema and glaucoma, respectively. CA inhibition was also shown to cause renal vasodilation and to improve renal blood flow in patients with primary hypertension with no change in systemic blood pressure (Horita et al., 2006). Likewise, activation of CA through application of prostaglandin F2 promotes vasoconstriction (Puscas and Coltau, 1995). In isolated retinal arteries, an increase in local tissue pH induces vasodilation through moderation of smooth muscle contraction (Kehler et al., 2007). Acidosis has likewise been found to promote relaxation of smooth muscle in bronchioles and arterioles (Faisy et al., 2007). On the contrary, acidosis has been shown to induce contraction of the aorta in SHR and WKY rats, with a greater degree of contraction seen in SHR (Rohra et al., 2003).

Several questions must be answered in order for the physiological repercussions of this shift in the CA2 spot location to be fully understood. It remains to be determined whether CA2 activity is significantly changed in the SHR liver and whether this translates to a change in local pH. Depending on which cells express CA2 within the liver, an increase or decrease in CA2 activity may cause an increase or decrease, respectively, in local tissue pH, which may in turn influence enzyme function or change hepatic blood flow through either vasoconstriction or vasorelaxation. Furthermore, it is not known whether this observation is restricted to the liver or whether it CA2 function is modified in other tissues. Additional investigations are required to answer the multitude of questions posed by the observed change in the location of the CA2 spot on the SHR liver gels.
Formiminotransferase Cyclodeaminase (FTCD)

The spot identified as FTCD shifts to a slightly higher pI value on the SHR gels compared to the WKY gels. FTCD is a bifunctional enzyme involved in the transfer of one-carbon units in the form of the formimino group (-CH=NH) from histidine into the pathways of tetrahydrofolate (THF) dependent one-carbon metabolism (Gao et al., 1998; Mao et al., 2004; Stipanuk, 2006). The formiminotransferase (FT) and cyclodeaminase (CD) domains are functionally distinct yet physically connected enzymes that were probably joined by some kind of genetic reorganization. The FT domain transfers the formimino group from N-formiminoglutamate, an intermediate in histidine catabolism, to THF, thus generating N5-formiminoTHF and glutamate. N5-formiminoTHF is then immediately passed to the CD portion of the enzyme which converts it to 5,10-methenylTHF. 5,10-methenylTHF is the converted into 5,10-methyleneTHF, which is positioned at the crossroads of purine, methionine and thymidylate metabolism. 5,10-methyleneTHF is the substrate of the enzyme methyleneTHF reductase (MTHFR), mutations of which have been associated with increased risk of cardiovascular disease (see below).

FTCD has additional functions seemingly unassociated with its enzymatic activity. FTCD is found either free in the cytosol or very closely associated with the Golgi apparatus and cycling of FTCD between the Golgi and other components of the secretory pathway have been documented (Gao et al., 1998; Hennig et al., 1998; Mao et al., 2004). As FTCD has been shown to associate with microtubules (Hennig et al., 1998) and to promote the formation of vimentin bundles initiating from the Golgi (Mao et al., 2004).
2004), it has been proposed that FTCD is involved in establishing physical associations between the Golgi apparatus and the cytoskeleton which are required for intracellular vesicular trafficking.

FTCD and the metabolic pathways summarized above have been indirectly connected to hypertension. Several polymorphisms in MTHFR have been linked to an increased risk for hypertension, arteriosclerosis and heart disease in various populations (Moat et al., 2003; D’Angelo et al., 2004; Stanger et al., 2004). These polymorphisms cause varying degrees of MTHFR protein thermolability and a resulting decrease in activity, which causes a decreased renewal of 5,10-methyleneTHF and therefore decreased conversion of homocysteine to methionine (Stanger et al., 2004). The resulting accumulation of homocysteine in plasma is thought to promote the development of cardiovascular disease through the promotion of altered vascular morphology, endothelial dysfunction culminating in arteriosclerosis, hepatic steatosis and a prothrombotic environment (Moat et al., 2003; D’Angelo et al., 2004; Stanger et al., 2004; Wu et al., 2009). Evidence suggests that these clinical effects of hyperhomocysteinuria may be due to increased oxidative stress and inflammation (Moat et al., 2003; Stanger et al., 2004; Wu et al., 2009). It should be noted that elevated plasma homocysteine is a common condition, found in 10% of the general population and 40% of people with vascular disease, which can be caused by a variety of environmental and genetic factors in the absence of a detected MTHFR mutation (Stanger et al., 2004). Since FTCD is required for the same metabolic pathways as MTHFR, it is conceivable that a PTM which alters FTCD activity may also influence metabolic pathways controlling plasma homocysteine
levels or have some other metabolic effect which influences cardiovascular physiology or sympathetic activity.

*MAWD Binding Protein (MAWDBP)*

The MAWDBP spot was found to shift to a slightly higher pI in the SHR liver gels compared to the WKY liver gels, presumably due to a difference in PTMs. The function of MAWDBP is primarily unknown. This protein was initially described in liver as a binding partner of MAWD, which is a MAPK activating protein with WD repeats which may be involved in carcinogenesis (Iriyama et al., 2001; Herde and Blankenfeldt, 2006). MAWDBP is expressed in variety of tissues including the kidney, heart, pancreas, and brain (Iriyama et al., 2001) and its expression has been found to be modified in several proteomic and genomic studies. MAWDBP is increased in the kidney in hypotension (Westhoff et al., 2005) as well as in the liver during insulin resistance (Solomon et al., 2005). In a proteomic study of the liver in folate deficiency, two MAWDBP spots were identified, one of which was present only in the control liver cells while the other spot was present in both proteomes but increased in folate deficiency (Chanson et al., 2005). Interestingly, MAWDBP was also found to decrease in the kidney of SHR compared to WKY rats in a yet unpublished study in our laboratory.

Together, these observations show that the regulation of MAWDBP is complex. Although these studies suggest that MAWDBP is involved in the regulation of blood pressure in the kidney, they give very little indication of this protein’s function in the liver. It is of interest that hepatic MAWDBP was found to be increased in insulin
resistance, as SHR show moderate insulin insensitivity. Given this information, the best hypothesis which can be made is that MAWDBP is a signal transduction protein involved in the regulation of metabolic pathways, and that the change in protein pI described here may mark a modification in the regulation of these pathways. These scant results suggest at the existence of an important pathway which warrants further investigation and could improve our understanding of the factors controlling metabolism in hypertension.

_Haloacid Dehydrogenase Domain-Containing 3 (HDD3)_

HDD3 seems to be an undescribed protein. Highest levels of expression are found in the parathyroid, thyroid, intestine, heart, ovary and ascites (peritoneal cavity fluid). HDD3 was found to be up-regulated in the gels from the SHR liver relative to gels from the WKY liver.

The results of this study support two main conclusions. First of all, several of the proteins described above lend further support to the already generally accepted idea that there is greater oxidative stress and altered mitochondrial function in various tissues of SHR compared to WKY rats. However, despite the numerous publications devoted to the molecular sources and effects of ROS in the various pathologies including hypertension, most of the proteins identified here have not been previously associated with cardiovascular disease or the SHR model. Thus, the observations presented here suggest new avenues for research into the mechanisms involved in the production of ROS and effects on hepatic physiology.
Several of the proteins identified above are involved in hepatic metabolism; these results support the hypothesis that the hepatic physiology of SHR shares similarities with that observed in hepatic steatosis. This was not unexpected given the wealth of clinical evidence showing that primary hypertension and NAFLD are common comorbidities, as well as the frequent experimental use of hepatic expression of specific enzymes involved in lipid metabolism as indicators of total body lipid status. Changes in the expression of protein such as carboxylesterase ES-10, ES-4 and ADHFE1 hint at mechanisms that may be altered in SHR and provide conceptual mechanistic connections between hypertension and NAFLD. Even MAWDBP, which is increased in the liver in a model of insulin resistance (Solomon et al., 2005), may be involved in NAFLD-like metabolic processes occurring in hypertensive models. The results presented above are not complete and much more work is required to fully understand their significance. What can be taken from these preliminary findings is that there are many complex molecular events occurring in the liver of SHR and that a significant portion of these events associated with mechanisms which could eventually lead to pathologies similar to NAFLD.

Many of the protein changes identified here may also occur in tissues outside the liver. QDPR, for example, is expressed in both hepatocytes and blood vessels and may thus be involved in the vascular pathologies of SHR. The changes in mitochondrial protein such as SOX and NDUFA10 may be found throughout the body and would therefore be especially relevant to energy-rich tissues such as cardiac and skeletal muscle. It remains to be determined whether these protein changes are widely expressed or are specific to the liver. A broad proteomic analysis of SHR tissues is required to fully
understand how the proteins described there fit into the complete picture of molecular events inherent to the establishment of hypertension.

The molecular biology of the liver in hypertension has been largely overlooked by researchers in favor of more obviously affected organs such as the kidney, heart, blood vessels and the nervous system. This may be due to a view of primary hypertension as being separate from other components of the metabolic syndrome. Studies such as this, which show that metabolic organs are altered in well accepted hypertensive models, help illustrate that hypertension is more complex than spontaneously increased blood pressure; rather, it is a system condition resulting from anatomical, physiological and molecular events which can generate an abnormal environment to which all tissues must adapt. Beyond the specific proteins detailed above, these results show that the liver deserves more attention for its role in the pathophysiology of primary hypertension.
2-Dimensional Gel Electrophoresis (2DGE) was used to compare the whole tissue proteome of the liver from 16wk WKY (A) and 16wk SHR (B). Spot changes between WKY and SHR are emphasized in boxes. Box 1 shows spots identified as sulfite oxidase and formiminotransferase cyclodeaminase; box 2 shows spots identified as carboxylesterase ES-4 and carboxylesterase ES-10; box 3 shows spots identified as NADH Dehydrogenase Ubiquinone 1α Subcomplex 10; box 4 shows spots identified as Alcohol Dehydrogenase Iron Containing 1; box 5 shows spots identified as MAWD binding protein; box 6 shows spots (from left to right) identified as glutathione-S-transferase omega1, haloacid dehalogenase-like domain containing 3, quinonoid dihydroprioterine reductase and carbonic anhydrase 2; box 7 shows spots identified as glutathione-S-transferase mu1. These gels show proteins with isoelectric points within the pI range of 3 – 10. Proteins were visualized using the silver stain (Shevchenko et al., 1996). For magnification and quantification of the spot changes, please see Figure 14.
Figure 13: 2-Dimensional Gel Electrophoresis (pH 6 – 9) of the Liver

Narrower ranges of the liver proteome which contained the changes in protein expression were run on 24 cm strips with a higher protein load (3 mg) to better view differences in protein expression. The pI range 4 – 7 in WKY (A) and SHR (B) and the pI range 6 – 9 in WKY (C) and SHR (D) are shown. Spot changes which were previously identified are outlined in dotted boxes while new spot changes which were not previously identified are emphasized in solid boxes. In A) and B), box 1 shows the spot identified as TNF receptor associated protein 1; box 2 shows spots identified as sulfite oxidase and formiminotransferase cyclodeaminase; box 3 shows spots identified as carboxylesterase ES-10; box 4 shows spots identified as NADH Dehydrogenase Ubiquinone 1α Subcomplex 10; box 5 shows spots identified as MAWD binding proteína; box 6 shows a change in expression of an unidentified protein. For magnification and quantification of the spot changes, please see Figure 14.
**Figure 13 continued:** Narrower ranges of the liver proteome which contained the changes in protein expression were run on 24 cm strips with a higher protein load (3 mg) to better view differences in protein expression. The pI range 4 – 7 in WKY (A) and SHR (B) and the pI range 6 – 9 in WKY (C) and SHR (D) are shown. Spot changes which were previously identified are outlined in dotted boxes while new spot changes which were not previously identified are emphasized in solid boxes. In C) and D), box 7 shows the spot identified as 17β Hydroxysteroid Dehydrogenase; box 8 shows the spot identified as Coproporphyrinogen Oxidase; box 9 shows the isoform of quinoid dihydropterine reductase that is more prevalent in WKY; box 10 shows the spot identified as carbonic anhydrase 2; box 11 shows the isoform of quinoid dihydropterine reductase that is more prevalent in SHR; box 12 shows the spots identified as glutathione-S-transferase Mu1.
Figure 14: Quantification of Differences in Protein Expression in the Liver between SHR and WKY rats

Differences in protein expression between the liver of SHR and of WKY rats. Quantification of protein abundance is shown on the right. Blue circles and bar graphs represent spots which have higher signal intensities in WKY while red circles and bar graphs represent spots which have higher signal intensities in SHR. ADHFe1 = alcohol dehydrogenase iron-containing 1; CA2 = carbonic anhydrase 2; ES-4 = carboxylesterase ES-4; ES-10 = carboxylesterase ES-10; FTCD = formiminotransferase cyclodeaminase.
Figure 14 continued: Differences in protein expression between the liver of SHR and of WKY rats. Quantification of protein abundance is shown on the right. Blue circles and bar graphs represent spots which have higher signal intensities in WKY while red circles and bar graphs represent spots which have higher signal intensities in SHR. GSTM1 = glutathione-S-transferase μ1; GSTO1 = glutathione-S-transferase ω1; HHD3 = haloacid dehalogenase-like domain containing 3; MAWDBP = MAWD binding protein; NDUFA10 = NADH dehydrogenase ubiquinone 1α subcomplex 10.
GSTM1

GSTO1

HDD3

MAWDBP

NDUFA10

WKY

SHR

WKY

SHR

WKY

SHR

WKY

SHR

WKY

SHR

WKY

SHR

WKY

SHR
**Figure 14 continued:** Differences in protein expression between the liver of SHR and of WKY rats. Quantification of protein abundance is shown on the right. Blue circles and bar graphs represent spots which have higher signal intensities in WKY while red circles and bar graphs represent spots which have higher signal intensities in SHR. QDPR = quinonoid dihydropterine reductase; SOX = sulfite oxidase; 17BHSD = 17β Hydroxysteroid Dehydrogenase; CPOX = coproporphyrinogen oxidase; TRAP1 = TNF receptor associated protein 1.
Figure 15: QDPR Spot Translocation

Quinoid dihydropterine reductase (QDPR) was found to be upregulated in the liver from 16wk SHR relative to the liver from 16wk WKY rats. **A)** One spot which was present only on gels from SHR livers and another spot which was present only on gels from WKY livers were identified as QDPR (circles) using MALDI-TOF MS. **B)** The shift in location of the QDPR spot was verified using a 2D Western blot. This change represents an increase in pI in the SHR isoform relative to the WKY isoform.
Figure 16: QDPR Immunohistochemistry in the Liver

Quinoid dihydropterine reductase (QDPR) immunostaining in the liver of 16wk WKY and 16wk SHR. QDPR signal (brown) was similar in the liver from SHR (right column) and WKY rats (left column). QDPR staining was found in hepatocytes and in the connective tissue surrounding the hepatic triads (A, B). Light staining was also observed in the endothelium lining the central vein (arrowheads) (C, D). In the aorta, QDPR staining is seen in the adventitia (arrows) and endothelium (arrowheads) with no staining in the intima media (E, F). Control sections are shown for WKY (G) and SHR (H). CV = central vein; HA = hepatic artery; Scale bars = 50 μm.
Figure 17: Mechanism Depicting QDPR-Induced Hypertension

The proposed mechanism connecting a decrease in quinoid dihydropterine reductase (QDPR) to an increase in oxidative stress and hypertension. Purple arrow represents a decrease in QDPR activity; green arrows represent changes in the concentration of a substance. Solid lines represent mechanisms which have been experimentally confirmed in previous studies. Dotted lines represent mechanisms which are being suggested based on the protein differences found in the liver of SHR compared to that of WKY rats in this study. 17BHSD = 17β Hydroxysteroid Dehydrogenase; BH4 = reduced tetrahydropterin cofactor; NO = nitric oxide; O$_2^-$ = superoxide anion; ROS = reactive oxygen species.
Arterial remodeling
Inflammation
Vasoconstriction
Sympathetic hyperactivity
Altered signal transduction

Hypertension
Figure 18: SOX Protein Abundance

Sulfite oxidase (SOX) was found to be upregulated in the liver from 16wk SHR relative to the liver from 16wk WKY rats. A) One spot which was present on gels from WKY livers and absent on gels from SHR livers (circles) was identified as QDPR using MALDI-TOF MS. B, C) A significant decrease in SOX protein abundance ($p \leq 0.05$) was detected and quantified. D) A 2D Western blot shows that there are SOX isoforms in WKY livers but only one SOX isoform in SHR livers.
A

WKY  SHR


B

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th></th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Anti – SOX

Anti – β-actin

65 kDa

42 kDa

C

SOX Protein Abundance

D

WKY  SHR

Anti – SOX

65 kDa
Figure 19: SOX Immunohistochemistry in the Liver
Sulfite oxidase (SOX) immunostaining in the liver of WKY and SHR. SOX signal (brown) was similar in the liver from SHR (right column) and WKY rats (left column). SOX staining showed a speckled pattern in amongst the hepatocytes that resembles stellate cells (A, B). This pattern is shown in a higher magnification in (C, D). Staining was also found in the endothelium lining hepatic arteries but not in the endothelium lining central veins (E, F). No staining was seen in hepatocytes or connective tissue. Control sections are shown for WKY (G) and SHR (H). CV = central vein; HA = hepatic artery; Scale bars (A, B) = 50 μm; Scale bars (all others) = 100 μm.
Figure 20: MAWDBP Immunohistochemistry in the Liver

MAWD binding protein (MAWDBP) immunostaining in the liver of 16wk WKY and 16wk SHR. MAWDBP signal (brown) was similar in the liver from SHR (right column) and WKY rats (left column). MAWDBP staining was found uniformly distributed in hepatocytes (A, B) with minimal staining in the connective tissue surrounding the hepatic triads (C, D). Control sections are shown for WKY (G) and SHR (H). CV = central vein; HA = hepatic artery; Scale bars = 100 μm.
### Table 2: Proteins identified as having differential expression between the livers of SHR and WKY rats.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Acronym</th>
<th>MASCOT</th>
<th>Identifier</th>
<th>MW</th>
<th>Sequ. Cov</th>
<th>pI</th>
<th>SHR/WKY†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTMs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxylesterase ES-10</td>
<td>ES10</td>
<td>136</td>
<td>57013350</td>
<td>62393</td>
<td>27%</td>
<td>6.10</td>
<td>2.31</td>
</tr>
<tr>
<td>Forminotransferase Cycledeaminase</td>
<td>FTCD</td>
<td>320</td>
<td>16758338</td>
<td>59504</td>
<td>48%</td>
<td>5.80</td>
<td>1.68</td>
</tr>
<tr>
<td>Glutathione-S-Transferase μ1</td>
<td>GSTM1</td>
<td>283</td>
<td>8393502</td>
<td>26068</td>
<td>70%</td>
<td>8.27</td>
<td>1.25</td>
</tr>
<tr>
<td>Quinoid Dihydropteridine Reductase</td>
<td>QDPR</td>
<td>231</td>
<td>11693160</td>
<td>25764</td>
<td>83%</td>
<td>7.67</td>
<td>0.59</td>
</tr>
<tr>
<td>MAWD Binding Protein</td>
<td>MAWDBP</td>
<td>184</td>
<td>19743770</td>
<td>31982</td>
<td>64%</td>
<td>6.54</td>
<td>0.82</td>
</tr>
<tr>
<td>NADH Dehydrogenase Ubiquinone 1α Subcomplex 10</td>
<td>NDUFA10</td>
<td>181</td>
<td>170295834</td>
<td>40753</td>
<td>42%</td>
<td>7.64</td>
<td>2.20</td>
</tr>
<tr>
<td><strong>SHR &gt; WKY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17β Hydroxysteroid Dehydrogenase</td>
<td>17BHSD</td>
<td>154</td>
<td>84993586</td>
<td>37503</td>
<td>36%</td>
<td>6.47</td>
<td>N/A‡</td>
</tr>
<tr>
<td>Alcohol Dehydrogenase Iron Containing 1</td>
<td>ADHFe1</td>
<td>75</td>
<td>70794742</td>
<td>50707</td>
<td>16%</td>
<td>7.25</td>
<td>2.50</td>
</tr>
<tr>
<td>Carbonic Anhydrase II</td>
<td>CA2</td>
<td>161</td>
<td>9506445</td>
<td>29267</td>
<td>65%</td>
<td>6.89</td>
<td>10.40</td>
</tr>
<tr>
<td>Coproporphyrinogen Oxidase</td>
<td>CPOX</td>
<td>316</td>
<td>79749365</td>
<td>49817</td>
<td>66%</td>
<td>8.80</td>
<td>N/A‡</td>
</tr>
<tr>
<td>Haloacid Dehalogenase-like Domain Containing 3</td>
<td>HDD3</td>
<td>76</td>
<td>157824168</td>
<td>28004</td>
<td>25%</td>
<td>6.50</td>
<td>7.30</td>
</tr>
<tr>
<td>TNF Receptor Associated Protein 1</td>
<td>TRAP1</td>
<td>80</td>
<td>84781723</td>
<td>80639</td>
<td>13%</td>
<td>6.56</td>
<td>N/A‡</td>
</tr>
<tr>
<td><strong>WKY &gt; SHR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxylesterase ES-4</td>
<td>ES4</td>
<td>112</td>
<td>157073955</td>
<td>62634</td>
<td>22%</td>
<td>6.29</td>
<td>0.02</td>
</tr>
<tr>
<td>Glutathione-S-Transferase ω1</td>
<td>GSTO1</td>
<td>110</td>
<td>12585231</td>
<td>27936</td>
<td>29%</td>
<td>6.25</td>
<td>0.17</td>
</tr>
<tr>
<td>Sulfite Oxidase</td>
<td>SOX</td>
<td>144</td>
<td>74024923</td>
<td>61167</td>
<td>17%</td>
<td>6.32</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Molecular weight (MW) and isoelectric point (pI) are the values found in the NCBI databank, rather than the MW and pI at which the spot was identified in this project. †For PTMs, where there are two isoforms, this value represents the ratio of the total signal intensity of both isoforms in SHR to the total signal intensity of both isoforms in WKY. ‡Numerical representation of protein abundance for several identifications is unavailable since these spot changes could not be seen on wide pH range gels (pH3-10) which were used for spot quantification.
Chapter 4

Future Directions

This study presented a collection of proteins whose abundance or chemical characteristics, namely pI or Mr, are different in the tissues of SHR compared to WKY rats. This data, however, is only the first step on the path to understanding whether these proteins are relevant to hypertension and how their function influences normal physiology. It remains to be determined whether these changes in proteins expressions are specific to hypertension, what is causing these changes in protein expression, and how these changes influence protein function and tissue physiology.

The discussions in the previous two chapters were designed based on the assumption that the changes in protein expression are relevant to the SHR phenotype. Unfortunately, this is by no means certain and the relevance of these protein changes must be experimentally verified. The first step is to validate the 2DGE data itself; differences in protein abundance or spot location must be verified using 1D and 2D Western blots as was done for pfetin, Spi 2.1, haptoglobin, ApoA1, QDPR and SOX in this study. This gives a more complete impression of how the expression of a specific protein is altered between the SHR and WKY rat tissues than is possible using proteomics alone. For example, proteins which appeared to be present in one phenotype and completely absent in the other phenotype might turn out to be merely more abundant and less abundant, respectively, or, as was seen with SOX, there may be additional isoforms of the protein which were not noticeably different between the gels from SHR and WKY rats and thus not identified in the initial 2DGE dataset. Immunohistochemistry
can also be used to verify proteomic data. This strategy has the additional, very important benefit of localizing protein expression and to a specific cell type or tissue region, which may give clues as to the protein’s function. It must be kept in mind, however, that immunohistochemistry only shows quantitative differences in protein expression when there is a very large difference in protein abundance, as was seen in haptoglobin in the SMG (Figure 9) and thus should be used in conjunction with immunoblotting.

Once the initial data has been confirmed, it must be determined whether the protein is involved in hypertension or whether it represents an unrelated phenomenon of the SHR genotype. This can be done by comparing the tissue proteomes of WKY rats with artificially induced hypertension (such as two-kidney one-clip Goldblatt rats) and SHR rats with chemically ameliorated hypertension (such as SHR on angiotensin converting enzyme inhibitors) with the tissue proteomes from untreated SHR and WKY rats. This strategy was exemplified by other proteomic studies of the hearts of SHR and WKY rats (Zhou et al., 2005; Jin et al., 2006). Briefly, if hypothetical protein PRO is found to increase in the proteome of SHR compared to WKY rats, and also increases in the proteome of hypertensive Goldblatt WKY rats, it is likely, although still not certain, that the given protein is involved in the tissue’s response to increased blood pressure, whether it be related to tissue damage and reorganization, altered oxidation status or compensation to such physiological shifts. Likewise, if PRO decreases in the proteome of SHR treated with anti-hypertensive drugs relative to untreated SHR, the increase in PRO expression was likely to be a result of hypertension.
If expression of the protein is not influenced by either of these treatments, there are two possible conclusions: either the protein is unrelated to hypertension or it is involved in the development and establishment of hypertension, rather than the reaction to hypertension. This second option can first be investigated by determining of PRO, to continue with the example introduced above, is increased in young, pre-hypertensive SHR compared to age-matched WKY rats. If this yields positive results, one can further test the hypothesis that an increase in PRO promotes the development of increased arterial blood pressure by treating pre-hypertensive SHR with PRO siRNA or anti-PRO IgG, or by injecting young WKY rats with purified exogenous PRO protein or viral vectors containing the PRO DNA. If such treatments prevent the establishment of hypertension in SHR or promote the establishment of hypertension in WKY rats, this is an indication that PRO is an important player in the pathogenesis of hypertension and deserves further study. Given these results, it would not be surprising that PRO expression failed to respond to the treatments of adult rats described above since the change in PRO expression occurs prior to the elevation of arterial blood pressure, rather than being dependent on it. If, on the other hand, enhancement or depletion of PRO expression fails to influence the development of hypertension, it is unlikely that PRO plays an important role in blood pressure regulation.

Once it has been shown that a specific change in protein expression is indeed involved in either the development or the reaction to hypertension, it is time to determine the mechanisms through which the protein influences blood pressure. Here, the appropriate strategies are so dependent on the specific characteristics of the protein in
question that the PRO example is no longer informative. Suffice to say that such experiments would probably draw on *in vitro* as well as well as *in vivo* models and be aimed at answering questions pertaining to the protein’s cellular function and mechanism of action.

An obvious problem to the strategies outlined above is that most of the proteins described in this study underwent spot shifts rather than, or in addition to, changes in abundance. How does one artificially induce a PTM? Initially, the answer most likely lies in the effect of the PTM on protein function, rather than on the replication of the PTM itself. The first step should be to determine whether the activity of the modified version of the protein is increased, decreased or unchanged relative to the unmodified version. Although there are several possible approaches to answer this question, the most direct method would be to develop an assay for measuring the protein’s relative activity in SHR and WKY rats. If, for example, the hypothetical protein MOD, which exhibits a change in pI, turns out to be a synthetic enzyme, one could search for differences in the abundance of its product between the two phenotypes. If the product is more abundant, the modification seems to increase MOD activity and can be treated as if it were equivalent to an increase in MOD abundance in experiments described previously. Although it is necessary to determine MOD’s function, if unknown, prior to any attempt at elucidating how its activity is altered by the modification, investigations into the protein’s role in hypertension can follow as described above once this has been achieved.

Eventually, it becomes useful to know the chemical characteristics of the modification in question. Near the end of this study, we attempted to use tandem MS to
determine the identity and location of any and all covalent attachments or differences in
sequence between the WKY and SHR isoforms of all proteins which exhibited shifts in
spot location on the gels of the WKY and SHR liver proteomes. (The gels shown in
Figure 13, which contain a high protein load of 3 mg spread over a relatively narrow pH
range were done in preparation for this PTM analysis.) The resulting data is not
presented here since a thorough analysis remains to be done. We hope that we will soon
be able to report the cause of these spot shifts; this should facilitate the discovery of their
potential role in hypertension by potentially suggesting the effect that modification has on
protein activity, aiding identification of the same modification in future experiments and
by allowing the synthesis of molecules which mimic or antagonize the activity of the
modified protein to discover the details of how it functions within the cell and within the
intact animal.

Finally, the results presented in the preceding chapters describe collection of
proteins which were identified as being differentially expressed in the tissues of SHR
compared to the tissues of WKY rats. Although the full physiological significance of
these findings is not known, many of these proteins are of potentially great significance
to the pathology of hypertension and deserve further study.
References


194


Appendix A

Quantification of Hepatic Haptoglobin Expression

Hp in the Liver

Figure A1: Quantification of haptoglobin (Hp) expression in the superior mesenteric ganglia (SMG) (A) and liver (B) from the 1-dimensional Western blots shown in Figure 8B and Figure 8D of the preceding text, respectively. In the SMG (A), Hp (35 kDa) abundance is increased almost 4-fold in SHR relative to WKY rats. In the liver (B), Hp at 35 kDa increases over 3-fold (* = p ≤ 0.005) in SHR, while Hp at 50 kDa decreases almost 4-fold († = p ≤ 0.001) in SHR compared to WKY rats.