EFFECTS OF HEME-L-ARGINATE ON L-NAME INDUCED HYPERTENSION

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ABSTRACT

N-ω-nitro-L-arginine methyl ester (L-NAME) has been used to induce experimental essential hypertension characterized by stimulation of the renin-angiotensin system (RAS) and oxidative system. Although the heme oxygenase (HO) system is known to suppress hypertension and the RAS, its effects on L-NAME-induced hypertension are poorly understood. Therefore, this study investigates the effects of heme-L-arginate (HA), a HO inducer, on L-NAME induced hypertension.

HA (15mg/kg/day) was administered for 4 weeks either during the development or after the establishment (4 weeks) of L-NAME-induced (60mg/kg/day) hypertension in Sprague-Dawley (SD) rats. Vehicle control groups were used. Co-treatment with HA prevented the development of L-NAME induced hypertension, (124 mmHg, n=13 vs 168; n=10, 11 weeks; $p<0.05$). After L-NAME-induced hypertension was established for 4 weeks, HA therapy reduced blood pressure to normotensive at 15 weeks (123 mmHg, n=8 vs. 190 mmHg, n=7; ($p<0.01$).

The prevention of hypertension was associated with increased HO-1 expression at 11 weeks (92.2±9.8 vs 15.9±9.9 HO-1/GAPDH %, n=4; $p<0.01$), reduction of heart Ang-II at 11 and 15 weeks (2.13±0.4 pg/mg, n=6 vs 4.06±0.4 pg/mg, n= 8; $p<0.05$ and 3.45±0.2 pg/mg, n=7 vs 4.23±0.2, n=7; $p<0.05$), respectively. HA co-treatment increased total antioxidant capacity (TAC) in heart tissue, the mesenteric artery and kidney.

We conclude that up-regulating the HO system with HA normalizes blood pressure and prevents the development of L-NAME induced hypertension by suppressing Ang-II and abating oxidative stress. HA may be explored in the prevention and management of other forms hypertension characterized by elevated Ang-II and excessive oxidative stress.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ABTS	2,2’-azino-di-[3-ethylbenzthiazoline sulphonate
AC	adenylate cyclase
ACE1	angiotensin converting enzyme type 1
ACE2	angiotensin converting enzyme 2
Ang (1-7)	angiotensin (1-7)
Ang I	angiotensin I
Ang II	angiotensin II
ANOVA	analysis of variance
AT_1	angiotensin type 1 receptor
ATP	adenosine triphosphate
BK_{Ca}	calcium activated potassium channel
BVR	biliverdin reductase
Ca^{2+}	cytosolic free calcium
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CO	carbon monoxide
COX	cyclooxygenase
cP450	cytochrome P450
CRP	C reactive protein
DAG	diacylglycerol
DiHEET	dihydroxyeicosatetraenoic acids

DOCA	deoxycorticosterone acetate
ECM	extracellular matrix
EDCF	endothelial derived contracting factors
EDRF	endothelial derived relaxing factor
EET	epoxyeicosatetraenoic acids
EIA	enzyme immunoassay
ET	endothelin
eNOS	endothelial nitric oxide synthase
g	grams
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine triphosphate
HA	heme-L-arginate
HCl	hydrochloric acid
HEET	hydroxyeicosatetraenoic acids
HO	heme-oxygenase
H_20_2	hydrogen peroxide
ICAM	intracellular adhesion molecules
iNOS	inducible nitric oxide synthase
ip	intraperitoneal
IP_3	inositol triphosphate
IRP	iron regulatory protein
K^+	potassium
NaOH	sodium hydroxide
N-LAME	N-ω-nitro-L-arginine methyl ester

M	molar
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
mg/kg/day	milligrams/kilogram/day
mmHg	millimeters of mercury
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NFκB	nuclear factor κB
O_2^-	superoxide anion
$ONNO^-$	peroxynitrite
PBS	phosphate buffered saline
PDE	phosphodiesterase
PKA	protein kinase A
PKG	protein kinase G
PLA_2	phospholipase A_2
PLC	phospholipase C
RAS	renin-angiotensin system
ROS	reactive oxygen species
rpm	revolutions per minute
SBP	systolic blood pressure
SD	Sprague Dawley
SEM	standard error of the mean
sGC	soluble guanylyl cyclase
SHR	spontaneously hypertensive rat

SNS	sympathetic nervous system
SOD	superoxide dismutase
SP-SHR	stroke prone-spontaneously hypertensive rats
TAC	total antioxidant capacity
TEAC	Trolox equivalent antioxidant capacity
TGR(Ren2)27	transgenic rat (Ren2)27
TNFα	tumor necrosis factor α
VCAM	vascular cell adhesion molecule
VSMC	vascular smooth muscle cell
1K1C	one-kidney one-clip
2K1C	two-kidney one-clip
2K2C	two-kidney two-clip

1. INTRODUCTION

1.1 Hypertension Overview

Blood pressure is a result of many interconnected factors. It is the product of cardiac output and total peripheral resistance, while cardiac output is the product of stroke volume and heart rate [1]. Nervous and endocrine systems as well as genetic and environmental factors work together in a complex system to determine an individual's blood pressure [2-4]. A measure of systolic and diastolic normal blood pressure is considered to be equal or less than 120/80 millimeters of Mercury (mmHg); in humans hypertension is defined as blood pressure equal to or above 140/90 mmHg [5]. Hypertension is considered to be a major risk factor for an array of cardiovascular and associated diseases, including heart failure, peripheral arterial disease and end stage renal disease [6]. Hypertensive states have also been linked to increased risk for type 2-diabetes and cardiometabolic syndrome [7, 8]. In fact, numerous studies have found a continuous relationship between elevated systolic and diastolic blood pressure and cardiovascular morbidity and mortality [5]. Early interventions and control of elevated blood pressure result in improved health outcomes [9]. However, despite current research and the large number of available treatments, hypertension remains a problem in both developed and developing nations and it is now being diagnosed in adolescents and children [10]. Corresponding with the aging population, Kearney reports that 26.4 % (972 million) of the world's population over the age of 20 had hypertension in 2000 and that will increase to 29.2% (1.56 billion) by 2025 [11]. Hypertension is a growing global problem and despite the decades of research its incidence continues to grow calling for more elucidation of its mechanisms and potential treatments.

1.2 Essential Hypertension

The most common and puzzling form of hypertension is essential or primary hypertension. Historically named 'essential' hypertension due to the theory that increases in total peripheral resistance and subsequently blood pressure were essential to aging. Presently, essential hypertension is defined as the rise of blood pressure due to an unknown cause [12]. Even more startling, essential hypertension is thought to account for up to 95% of hypertension cases [13]. While the complexities of its pathogenesis are not fully understood, it is considered to be multi-faceted involving neurogenic stimuli, endothelial dysfunction and the renin-angiotensin system (RAS) resulting in oxidative stress, remodeling and inflammatory processes [14, 15]. Moment to moment regulation of hypertension is regulated by neurogenic systemic peripheral vasoconstriction driven by the sympathetic nervous system (SNS), quickly resulting in endothelial dysfunction and activation of the RAS [16].

1.2.1 The Role of the Sympathetic Nervous System in Essential Hypertension

The sympathetic nervous system (SNS) is one component of the autonomic nervous system. The other component, the parasympathetic nervous system innervates the heart and a small number of blood vessels, while the SNS provides widespread direct and indirect control of cardiac and vascular function, innervating the heart, blood vessels, adrenal gland and kidneys. Responsible for the transient 'flight or fight' response, post-ganglionic sympathetic neurons release norepinephrine resulting in activation of the adrenergic pathways [17]. Directly connected to this response is the adrenal medulla, with its chromaffin cells acting as post-synaptic ganglion sympathetic neurons releasing epinephrine. The α_1-adrenoreceptors stimulate vasoconstriction and elevation of heart rate as well as stimulating the RAS by causing the release of renin [17]. Presynaptic α_2-adrenoreceptors provide a negative feedback response to the α_1-

adrenoreceptors by inhibiting the release of norepinephrine, while post-synaptic α_2-adrenoreceptors stimulate platelet aggregation and vasoconstriction [18]. In addition to the α-adrenoreceptors, the β-adrenoreceptors also contribute to the regulation of blood pressure. β_1-adrenoreceptor activation increases heart rate and contractility, while β_2- adrenoreceptors activation promotes vasodilation [19]. Moreover, under normal conditions the SNS rapidly responds to increases and decreases in blood pressure via arterial baroreceptors augmenting total peripheral resistance, heart rate and cardiac contractility [20]. The SNS also augments arterial blood pressure in response to chemoreceptors acting in response to the blood oxygen-carbon dioxide balance [20]. In fact, moment to moment changes in blood pressure are almost entirely mediated by the nervous system [21]. While the majority of anti-hypertensive research has focused on RAS targeting therapies, general consensus is that SNS over-activity is involved in the initiation and may contribute to sustaining essential hypertension [2]. SNS over-activity has been proven to cause elevated arterial pressure, the precise mechanism leading to the prolonged SNS over-activity is unclear [22]. Before the availability of antihypertensives clinicians recognized the importance of SNS in the control of long term blood pressure, using sympathectomies to reduce hypertension [23]. SNS over-activity has been directly involved in the increased morbidity in a number of pathophysiological conditions such as heart failure and end stage renal disease [24]. Working in conjunction with SNS activity, stimulation of endothelial activity and subsequent dysfunction contribute to the development and maintenance of essential hypertension.

1.2.2 The Role of Endothelial Dysfunction in Essential Hypertension

Endothelial dysfunction is defined as a functional and reversible alteration of endothelial cell relaxation, and while resulting from interplay of many factors, is often due to impaired nitric

oxide (NO) availability [25, 26]. Known as the primary endothelial derived relaxing factor (EDRF), the majority of the endothelial-dependent vasodilation has been attributed to NO [27]. Synthesis of NO from the oxidation of L-arginine by endothelial or inducible derived nitric oxide synthase (eNOS or iNOS, respectively) is stimulated by mechanical factors, such as pulsatile and shear stress, and hormonal signals [28]. In the blood vessels, NO binds to the intracellular enzyme soluble guanylyl cyclase (sGC), which then leads to a 200-fold increase in cyclic guanosine monophosphate (cGMP) from guanosine triphospate (GTP) [29]. cGMP then acts on protein effectors including; protein kinase G (PKG), cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), cyclic nucleotide-gated cation channels and phosphodiesterase (PDE) [28, 30]. The binding of cGMP to these proteins affects several physiological processes for instance, vascular relaxation and dilation, cardiac systole and immune mediated inflammation [30]. NO also acts independently of cGMP contributing further to vasodilation as well as regulating apoptosis, remodeling and angiogenesis; consequently, NO produced in the vasculature plays an important role in maintaining blood pressure [31]. In fact, inhibition of NOS results in elevation of peripheral vascular resistance, activation of the SNS and increased arterial blood pressure associated with subsequent structural and functional changes [32, 33]. Reduced NO itself has not only been linked to endothelial dysfunction, but to arterial stiffness, and in contrast a number of therapies have been shown to improve NO, endothelial function and reducing arterial stiffness [34].

In contrast to NO, endothelial derived contracting factors (EDCF) oppose the vasodilation of NO and exacerbate endothelial dysfunction. Endothelin (ET) is one of the most potent EDCF and is released continuously from endothelial cells [35]. Found in three isoforms, ET activates two subtypes of G-protein receptors leading to the formation of inositol triphosphate (IP_3) and subsequent vasoconstriction [36]. ET-1 in particular not only regulates vasoconstriction, but is

important in regulating processes such as remodeling, angiogenesis and extracellular matrix (ECM) synthesis [37]. ET-1 has been connected to both pulmonary and systemic hypertension. In fact, peripheral endothelial dysfunction has been thought to contribute to SNS over-activity and result in arterial hypertension [38]. Strong evidence supports the contribution of endothelial dysfunction to the development of essential hypertension. Endothelial dysfunction and blood pressure are further modulated by the activity of the RAS and its mediated effects as discussed below.

1.2.3 The Role of the Renin-Angiotensin System in Hypertension

Hormones play a crucial role in the regulation of blood pressure, including; vasopressin, aldosterone and the RAS. The RAS is a crucial effector of not only the cardiovascular system but of several organ systems. RAS controls blood pressure both through vascular tone and fluid-electrolyte balance. The synthesis and secretion of the hormone renin is the rate limiting step in the mobilization of the RAS [39]. Renin-synthesizing cells are found in the adult kidney where they respond to intrarenal baroreceptors, the sodium chloride load at the macula densa, SNS activity via β-adrenoreceptor activation and angiotensin II (Ang II) with interplay of second messengers, including: cAMP, cGMP and free cytosolic calcium [40, 41]. Activation of cAMP leads to phosphorylation of PKA or inhibition of PDE. Inhibition of PDE is responsible for the degradation of cAMP, and is a stimulator of renin secretion [39, 40]. In fact, many established activators of AC can result in increased renin secretion including activators of adrenoreceptors (catecholamines), and hormones, for example prostaglandins E_2 and I_2, and dopamine [40]. Renin levels are further modulated due to cGMP stimulated NO and subsequent inhibition of PDE via slowing of renin degradation [42]. Interestingly, cGMP can also inhibit renin secretion

rates through activation of a protein kinase [43]. Increases in cytosolic free calcium (Ca^{2+}) strongly inhibit renin release [44]. Decreases in glomerular filtration rate stimulate renin secretion, indicating pressure regulation at the renal level [39]. Over stimulation of renin synthesis and secretion is a primary cause for increased blood volume, hypertension and organ damage. Not only is renin the rate limiting enzyme of the RAS, it activates the pathway for the formation of the primary effecter peptide of the RAS, Ang II. Renin cleaves angiotensinogen, which primarily originates in the liver, to form angiotensin I (Ang I). Angiotensin converting enzyme type 1 (ACE1), the primary form of ACE, is produced predominantly from lung endothelium and hydrolyzes Ang I to form Ang II, an octapeptide, contributing to a vast array of actions. Interestingly, ACE1 also has the ability to hydrolyze bradykinin and kallidin, both known to lower blood pressure [45]. However, the net effect of ACE1 is to increase blood pressure by increasing a vasoconstriction [46]. In contrast to ACE1, angiotensin converting enzyme type 2 (ACE2) is an enzyme that primarily acts on Ang II. Found in several tissues, ACE2 cleaves Ang II resulting in the production of the heptapeptide angiotensin (1-7) (Ang (1-7) [39]. Ang (1-7) is an antagonist to Ang II and a potentiator of vasodilation through stimulation of prostaglandins, NO and bradykinin [47]. It should be noted that another enzyme, chymase, also acts on Ang I to form Ang II, but to a much lesser extent [48].

1.2.3.1 Angiotensin II and Hypertension

Ang II is the primary effector peptide of the RAS and controls much of the pressor, angiogenic and remodeling effects of the RAS [49]. Studies show that Ang II is distributed systemically as well as locally in tissues such as heart and kidney [50]. There are four known angiotensin receptors, but most actions of Ang II are mediated through G protein-coupled angiotensin type 1 (AT_1) receptor [46]. Through AT_1, Ang II exerts a multitude of effects which

contribute to hypertension including intermediate acting vasoconstriction, sodium reabsorption and stimulation of aldosterone synthesis [51]. Short term, Ang II is responsible for regulation of vascular tone through vasoconstriction [52]. The presence of Ang II immediately induces vasoconstriction via G proteins resulting in the activation of phospholipase C (PLC), leading to the hydrolysis of phosphatidyl inositol and subsequent formation of IP_3 and diacylglycerol (DAG) [53]. IP_3 results in increases in Ca^{2+} concentration by causing increased Ca^{2+} influx mobilizing intracellular Ca^{2+} the primary trigger for vascular contraction [54]. DAG together with Ca^{2+} activates PKC, further promoting vasoconstriction and vascular smooth muscle cell (VSMC) growth [55]. PKC mediated Ang II signaling also stimulates vasoconstriction via a sodium/hydrogen exchanger which changes intracellular pH (alkalinization) and modulates an actin-mysoin interaction [54]. PKC carries out its actions through phosphorylation of tyrosine kinases and mitogen-activated protein kinase (MAPK) signaling pathways [52]. Ang II also activates phospholipase A_2 (PLA_2) and the subsequent release of arachidonic acid (AA). AA is processed into many different eicosanoids by cyclooxygenase (COX), lipoxygenase or cytochrome P450 (cP450), influencing many vascular and renal actions [56]. For instance, COX metabolism of AA yields unstable and short lived endoperoxides which cause downstream generation of thromboxane A_2 and prostacyclin, leading to thrombosis or anti-platelet aggregation, respectively [57] Moreover, cP450 metabolizes AA to epoxyeicosatrienoic acids (EETs), dihydroxyeicosatetraenoic acids (DiHETEs) and hydroxyeicosatetraenoic acid (HETE), most notably 19 and 20 [58]. While EETs are vasodilators, 20-HETE is produced in VSMC and is a potent vasoconstrictor and promoter of angiogenesis [59]. HETEs are vasoconstrictors which decrease renal sodium excretion and increase fluid retention increasing blood pressure further [60].

Ang II further regulates the fluid-electrolyte balance. Within the kidney, Ang II increases reabsorption of sodium and fluid in the proximal and distal tubules directly along with augmentation of the glomerular filtration rate [61]. Ang II also stimulates thirst and appetite for salt by acting on the hypothalamus [41]. Under normal physiological conditions Ang II increases glomerular permeability in the kidneys; however, it can also cause inflammation leading to tubulointerstitial damage and proteinuria of the kidneys [62]. Indirectly, Ang II also contributes to sodium and fluid reabsorption through stimulation of synthesis of a steroid hormone with mineralocorticoid activity, aldosterone [63].

Stimulation of mineralocorticoid receptor by aldosterone gives rise to the expression of proteins that stimulate multiple sodium transport mechanisms including a sodium-potassium adenosine triphosphate (ATP) pump leading to increased sodium and fluid retention in the distal tubule [48]. Mainly formed in the adrenal glomerulosa, aldosterone can exert effects on the cardiovascular and renal systems because of the wide distribution of its cytoplasmic mineralocorticoid receptor [63]. Recent studies show that aldosterone also promotes remodeling and inflammation of the vasculature [64]. Outside of its fluid balance role, aldosterone independently contributes to the long term deleterious effects of Ang II with the production of reactive oxygen species, inflammation, remodeling and fibrosis [65-67].

1.2.3.2 Oxidative Stress and Hypertension

While many of Ang II's damaging effects are direct, growing evidence indicates that a key component of Ang II mediated effects is generation of reactive oxygen species (ROS) [51, 68, 69]. Sustained over production of ROS and subsequent imbalance between oxidants and anti-oxidant capacity are considered to be oxidative stress [70]. All vascular cell types have the ability to produce ROS, which are associated with inflammatory responses [71]. While any

electron-transferring protein or enzyme can produce ROS, it is emerging that vascular ROS is primarily produced through the stimulation of vascular nicotinamide adenine dinucleotide phosphate (NAD(P)H)-oxidase with contributions from xanthine oxidase and uncoupled eNOS [15, 72]. NAD(P)H-oxidase is found in all vascular cell types, for instance endothelial cells, fibroblasts and VSMC [69]. Non-phagocytic NAD(P)H)-oxidase is regulated by many factors, including; hormones, growth factors and mechanical stimuli, but the pathway that is best characterized is regulation via AT_1 receptor activation by Ang II [72, 73]. Significantly, well unclear of exact mechanisms studies show that AT_1 receptor is activated in patients with essential hypertension and plays a role in ROS over-production [74]. Also, recent studies have indicated that hypertension with elevated endogenous levels of Ang II, resulting from RAS activity, resulted in increased NAD(P)H-derived ROS [54, 73]. Moreover, studies of animal models of Ang II-induced hypertension have revealed elevated expression of several NAD(P)H-oxidase subunits and increase activity of NAD(P)H-oxidase [62, 73]. Vascular NAD(P)H-oxidase activation, the major source of ROS in hypertension, results in the reduction of molecular oxygen to form superoxide anion (O_2^-) and a contributing source of hydrogen peroxide (H_20_2) [15, 68, 71].

O_2^- produced from the NAD(P)H-oxidase is unstable and is quickly reduced or oxidized [68]. O_2^- is considered one of the most potent ROS since it causes significant vascular injury as well as promotes damage carried out by an array of secondary products. The effects of O_2^- are thought to be local through internal signalling because it is hydrophilic and has a negative charge. One study showed Ang II-induced hypertension led to the doubling of O_2^- formed by NAD(P)H-oxidase [73]. In vascular endothelium, xanthine oxidase also contributes to the enzymatic formation of O_2^- [15]. In the endothelium, subsequent oxidization of O_2^- yields peroxynitrite ($ONNO^-$) by scavenging NO [71]. $ONNO^-$ is a strong oxidant, which has the ability to cause cell

damage by oxidizing proteins, lipids and nucleic acids [75]. Specifically, $ONNO^-$ has been shown to uncouple eNOS and iNOS, augmenting the antioxidant producing eNOS into a ROS producing enzyme, furthering the oxidative stress and activating the SNS [33, 76]. Interestingly, aldosterone further promotes NAD(P)H-oxidase activity through decreased protein expression as well as endothelial NOS uncoupling exacerbating the effects of Ang II [64] . Reduction of O_2^- by superoxide dismutase (SOD) results in the production of H_20_2. H_20_2 is a more stable ROS and has been implicated in the reduction of endothelial NOS and along with $ONNO^-$ contributes to increased oxidative stress [68, 77]. It is also an important ROS because of its lipophilic nature allowing it to cross cell membranes leading to a widespread effect. Also contributing to oxidative stress, the hydroxyl radical is a potent oxidant with an extremely short half life. It can be produced directly from water [78]. The production of these Ang II-mediated ROS influences inflammatory signaling pathways and related molecules leading to overall reduction in endogenous antioxidants [15]. In fact, hydroxyl radical scavengers are known to augment responses to Ang II in SHR [79]. Evidence suggest that ROS contribute significantly to the development of pathophysiological conditions such as hypertension, cardiovascular disease and renal damage [70].

It is important to note that the amount of oxidative damage is directly related to the availability of antioxidants. Antioxidants can be enzymatic or non-enzymatic in nature. One such enzymatic antioxidant is SOD. SODs are metalloenzymes whose role in the antioxidant defense mechanism is catalyzing the dismutation of the superoxide anion to molecular oxygen and H_20_2 at the cellular level. There are three isoforms of SODs in mammals: cytosolic and extracellular copper-zinc SOD and mitochondrial manganese SOD [80]. Copper-zinc SOD is the dominant isoform with high levels of expression in all cells and in particular vascular tissue [81]. Mitochondria are known producers of O_2^- and as such manganese SOD is a first line of defense

against oxidation. Catalase works in conjunction with SOD converting H_20_2 to benign oxygen and water [82]. Similar to catalase, glutathione peroxidase scavenges H_20_2 by oxidation of glutathione to glutathione disulfide and reduces it to water. Importantly, glutathione peroxidase is also able to reduce lipid peroxides and lipid alcohols.

Non-enzymatic antioxidants are crucial in the combating of oxidative stress. They include ascorbic acid, α-tocopherol, carotenoids, α-lipoic acid, polyphenols and tetrohydrocurcumin. In conjunction these antioxidants significantly protect against ROS and their damaging effects and have been noted to reduce hypertension, oxidative induced remodeling and atherosclerosis. The balance of ROS between antioxidants is very important for prevention of disease. With elevation of ROS in the cardiovascular system the resultant receptor activation, modulation of transcription factors and protein expression, leads to deleterious effects including vascular remodeling, endothelial dysfunction and hypertrophy [62, 72, 83].

1.2.3.3 Remodeling, Hypertrophy, Inflammation and Hypertension

Chronic hypertension can result remodeling, hypertrophy and increased total peripheral resistance [51, 84]. Vessels are composed of cells and ECM that are dynamic. Remodeling is associated with the reorganization of VSMC, changes in ECM composition and elastic fiber alteration [12]. While remodeling is considered a normal adaptive response to increased wall stress, it also plays a role in the maintenance of hypertension. Ang II is a powerful mediator of vascular remodeling through the stimulation of inflammatory molecules, growth factors and chemokines [85]. Ang II stimulates molecules such as prostaglandins and fibronectin, which are expressed in a variety of cardiovascular tissue including: VSMC, endothelial cells, cardiac fibroblasts and mesangial cells [86, 87]. Fibronectin, an ECM protein, binds to collagen and modulates fibrillogenesis, increasing collagen type 1 and decreasing collagen type 2 which are

responsible for stiffness and elasticity, respectively [88]. Furthering inflammation, Ang II enhances cellular adhesion through chemokines and cytokines.

Essential to Ang II-mediated effects, nuclear factor κB (NFκB) is particularly important in vascular inflammation, VSMC proliferation and migration as it controls many pro-inflammatory genes. Ang II, and Ang II-resultant ROS such as O_2^- and H_20_2, stimulate nuclear translocation, DNA binding and transcription of a NF-κB gene as well as protein expression of growth factors (tumor necrosis factor α (TNFα)), adhesion molecules [85, 89]. For example, selectins make the initial contact, leukocyte recruitment into the vessel wall is regulated through the stimulation of adhesion molecules, intracellular adhesion molecules (ICAM) 1 and 2 and vascular cell adhesion molecule-1 (VCAM-1) [62, 90]. The increase of ICAM-1 and VCAM-1 also involve activation of MAPK pathways, which are regulated by oxidation [91]. Also partially upregulated by H_20_2, MAPK growth signaling pathway is well characterized and leads to phosphorylation of proteins and subsequently influence cell cycle, apoptosis, differentiation and ultimately hypertrophy [90, 92]. After leukocyte recruitment takes place, chemokines play a key role in the migration of leukocytes into tissues [90, 91]. Once leukocytes move in to tissues they release metabolites and proteases that are toxic and may promote tissue damage. In chronic hypertension, inflammation is characterized by progressive replacement of leukocytes to mononuclear cells, subsequently undergoing transformation to macrophages which are phagocytic mediators of the tissue destruction, vascular proliferation, and fibrosis [93].

There are many markers of inflammation and vascular remodeling but one of the most powerful and stable is thought to be C reactive protein (CRP) [62]. Higher prevalence of hypertension has been correlated with CRP levels and evidence has suggested that CRP may be an independent risk factor for hypertension [94]. CRP is a direct participant in the Ang II-mediated response through the cascade production and stimulation of chemokines and adhesion

molecules [52]. Expression of CRP has been noted in VSMC and macrophages within atherosclerotic plaques [95]. In addition, CRP activation contributes to hypertension by inhibiting eNOS expression and activity [85]. The expression of CRP potentiates the actions of Ang II via increasing the expression of AT_1 [49].

The sum effect of the vasoconstriction, modulation of growth factors, adhesion molecule, and inflammatory process in hypertension is remodeling and hypertrophy. Remodeling is characterized by rearrangement of the ECM and VSMC, can be measured by a change in the ratio of medial thickness to lumen diameter. In hypertension, increased shear forces combined with the Ang II-mediated effects lead to vascular remodeling and ultimately atherosclerosis [96]. Hypertrophy refers to an increase size of cells or increase thickness of a vessel, without increases in cell number. Additional evidence linking Ang II to cardiovascular hypertrophy comes from studies of AT_1 receptor antagonists in animal models. Several AT_1 receptor antagonists have been reported to reduce left ventricular mass. By altering the functionality of tissues, remodeling and hypertrophy, stimulated by Ang II and its multitude of effects, leads to the development of sustained hypertension and a range of cardiovascular disorders and renal damage [51]. The severity and commonness of essential hypertension clearly indicate a need for further study on its driving mechanisms and possible therapies.

1.3 Animal Models of Hypertension

Animal models have proved to be useful in elucidating cause and progression of hypertension. Hypertension is diverse as the methods used to induce it in animals. Variables such as food and fluid intakes, structural alterations, environment, pharmacological intervention and importantly genetics have been manipulated to induce experimental hypertension in animals. Many models help to elucidate the many causes of hypertension in humans, such as the

spontaneously hypertensive rat (SHR), transgenic rat (Ren2)27 (TGR(Ren2)27, Goldblatt and deoxycorticosterone acetate (DOCA) hypertension models.

1.3.1 Spontaneously Hypertensive Rat

A genetic hypertensive model, the SHR is the most often used model of hypertension and it is the standard in research of essential hypertension [97]. Inbred from Wistar and Wistar-Kyoto non-hypertensive controls, SHR develop hypertension at 4-6 weeks of age without intervention [98]. In early stages of hypertension, SHR maintain total peripheral resistance, but have increased cardiac output. However, as hypertension is established hypertrophic vessels increase total peripheral resistance, cardiac output returns to normal and remodeling in the heart occurs [99, 100]. Like in human essential hypertension, the exact cause is unknown but SHR show changes in SNS activity, alterations in NO availability and endothelial dysfunction [101, 102]. Levels of sGC and cGMP were found to be significantly lower in young SHR compared with age-matched Wistar-Kyoto [103]. Increases in arterial wall renin have been observed in SHR. While increases in Ang II has not been noted, sensitivity to Ang II is seen in SHR [104].

1.3.2 TGR(Ren2)27 Hypertensive Rat

Like SHR, the (Ren2)27 TGR(Ren2)27 is an experimental model of essential hypertension that also shows sensitivity to Ang II. Created by Mullins et al., the TGR(Ren2)27 rat was developed through the introduction of the mouse Ren2 gene into rats [105]. It had been previously reported that injection of purified mouse renin elevated blood pressure and the Ren2 gene had shown high expression in a transgenic mouse model [106]. Homozygous TGR(Ren2)27 rats develop severe hypertension at an early age and reach maximum levels at 9 weeks of age. Similar to the SHR, the mechanisms behind the rise in blood pressure in the TGR(Ren2)27 rat

have not been clearly elucidated. Interestingly, research has noted that circulating renin levels remained unchanged or decreased as compared to heterozygous normotensive littermates [107]. Also, the adrenal gland of the TGR(Ren2)27 is hypertrophic along with large increases in local renin and aldosterone [108]. Ang II levels are found to be elevated, but more importantly the VSMC were shown to have increased sensitivity to Ang II, similar to observed changes in SHR [106]. In young, but not aged, TGR(Ren2)27 rats plasma steroid levels and secretion are enhanced and may be involved in the development of hypertension [108]. Aged TGR(Ren2)27 rats show diminished NO release suggesting endothelial dysfunction. The sum of these changes is remodeling, hypertrophy and end-organ damage. The development of TGR(Ren2)27 rats allow for further study of hypertension and its pathophysiology, but may not be representative of human hypertension due to its early on-set and severe nature [109]. As noted above, genetics play an important role in the development and sustaining of hypertension. However, structural changes and food and fluid intake are also integral factors in some forms of hypertension, as observed in the Goldbatt and DOCA animal models.

1.3.3 The Goldblatt Hypertensive Rat

The first animal model of hypertension was developed by Harry Goldblatt in 1934 when he clipped the renal artery of a dog and produced a hypertensive state [110]. As an experimental model of renal and secondary hypertension, it can include one of the following: two-kidney one-clip (2K1C) where both kidneys remain intact and one renal artery in constricted with a clamp, one-kidney one-clip (1K1C) where one kidney is removed and the renal artery of the remaining kidney is clamped or two-kidney two-clip (2K2C) where both kidneys are intact but either the aorta or both renal arteries are clamped. The clamps reduce renal perfusion pressure which in turn stimulates renin and Ang II synthesis [109]. This results in endothelial dysfunction,

increased peripheral resistance and subsequent increases in blood pressure. Unlike the 2K1C, the 1K1C model is considered to be sodium-fluid volume dependent because of the absence of the normal kidney and its absent compensatory elevated sodium and water excretion, leading to fluid retention [111]. Additionally, the 2K2C exhibit severe renal ischemia, as well as both increased RAS and SNS activity [109]. In rats, the Goldblatt hypertensive model induces a chronic hypertensive state with increased renin and subsequent Ang II, similar to that in humans with unilateral renal artery stenosis [97]. In contrast to the high renin levels noted in the Goldblatt hypertensive models, the administration of DOCA induces a low renin form of hypertension [112].

1.3.4 Deoxycorticosterone acetate-salt Hypertensive Model

The DOCA-salt model uses synthetic mineralocorticoid steroids and sodium chloride to mimic aldosterone overload and induce volume overload and subsequent hypertension via retention of sodium and water [113, 114]. It is characterized by endothelial dysfunction, elevated RAS activity and oxidative stress [97, 115-117]. DOCA-salt rats exhibit low renin and do not respond well to RAS inhibitors, such ACE inhibitors or Ang II antagonists [97]. However, DOCA-salt hypertension does respond well to diuretics and aldosterone inhibitors [118]. DOCA-salt rats develop severe hypertrophy and end-organ damage [109]. This low renin, volume overload model of hypertension is valuable to study because it mimics the outcome of chronic human essential hypertension. The DOCA-salt hypertensive model and many other animal models allow further elucidation of the many unknown mechanisms driving human hypertension. Notably, a key element in all the experimental models above is the alteration in Ang II or the modulation of sensitivity to Ang II. Similar to these models is N-ω-nitro-L-arginine methyl ester (L-NAME)-induced hypertension.

1.4 N-ω-nitro-L-arginine methyl ester (L-NAME)-induced hypertension

1.4.1 Mechanisms of L-NAME-induced Hypertension

Experimental models of hypertension are extremely important in the exploration of hypertension. One such established model is L-NAME hypertension, a pharmacologically induced form of experimental hypertension [119]. Routes of effective administration include intravenous, intraperitoneal and oral. L-NAME produces a hypertensive state reflective of the dysfunction seen in essential hypertension via several mechanisms, including: inhibition of NO, SNS activity increasing total peripheral resistance, oxidative stress and arterial remodeling **(Figure 1.1)** [38]. Classically, L -NAME is known as an inhibitor of NOS leading to decreased NO, an important vasodilator [26]. Acute and chronic inhibition of NO produces endothelial dysfunction, which has been clearly demonstrated by L-NAME [26]. Moreover, the inhibition of NO by L-NAME in young SHR produce similar results to that of naturally aged SHR, supporting the role for NO inhibition in the study of essential hypertension [120]. In addition to the reduction of NOS activity by L-NAME, the production of O_2^- by L-NAME may decrease NO further through its $ONOO^-$ -mediated uncoupling of NOS [67]. While controversial, it has been reported that L-NAME may directly alter baroreceptor sensitivity in SNS leading to augmentation of blood pressure [121]. It is hypothesized that NO is an inhibitory modulator of SNS outflow [122]. Large volumes of evidence show that stimulation of SNS activity in L-NAME-induced hypertension can be modulated by reduced NO availability [38]. Despite the method of induction, SNS activity is a contributor to acute and chronic L-NAME-induced hypertension [121, 123]. In addition to the attenuation of NO, evidence has also shown that L-NAME stimulates the RAS [26, 119].

As in essential hypertension, RAS is stimulated at many levels by L-NAME. Studies of chronic L-NAME administration indicate increased mRNA expression of renin and, consequently; elevated levels and activity of renin have been reported in the plasma [124, 125]. In addition, modulation of ACE, the enzyme responsible for the conversion of Ang I to Ang II, by L-NAME has been observed [126]. Further evidence reveals that as duration of L-NAME treatment increases, ACE activity in plasma and tissue increases accordingly [126, 127]. Both, antagonists of AT_1 receptor and inhibitors of ACE prevented the development of L-NAME-induced hypertension revealing the importance of Ang II [128]. Not surprisingly, L-NAME administration results in augmentation of Ang II and its mediated effects [129]. In fact, recent reports show that plasma Ang II is elevated after just 3 weeks of treatment with L-NAME [124]. In addition to Ang II induction, the production of ROS and subsequently, oxidative stress is an important pathogenic factor in L-NAME induced hypertension [15, 74]. Vascular remodeling, fibrosis, inflammation and hypertrophy due to decreased NO, increased Ang II as well as increased ROS have all been noted in L-NAME induced hypertension [119]. Similarly to essential hypertensive states, L-NAME hypertension induces a complex, inter-connected pathophysiological response that requires an anti-hypertensive treatment capable of multiple causal and responsive factors.

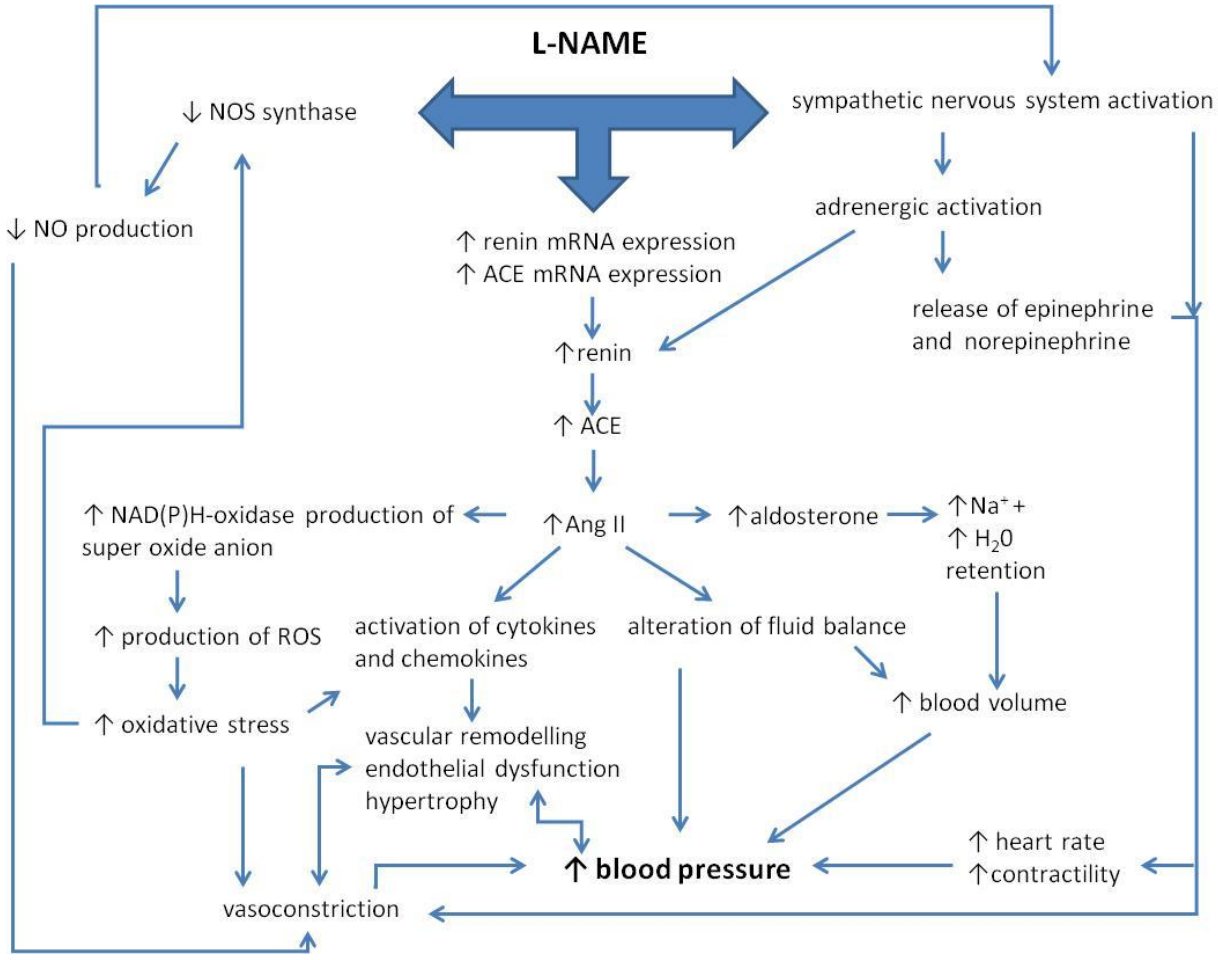

Figure 1.1. N-ω-nitro-L-arginine methyl ester modulation of blood pressure. L-NAME stimulates the uncoupling of NO leading to decreased NO and consequent vasoconstriction. Activation of the RAS by L-NAME leads to increase mRNA expression of renin and ACE. This results in the elevation of Ang II and it effects including increased NAD(P)H-oxidase production of super oxide anion and generation of ROS, increased aldosterone, alteration of fluid balance and activation of cytokines and chemokines. Ang II also directly stimulates vasoconstriction and increases in blood pressure. Stimulation of SNS activity results in increased heart rate, contractility and vasoconstriction. The sums of L-NAME's effects are endothelial dysfunction, remodelling and hypertrophy culminating with a hypertensive state. (N-ω-nitro-L-arginine methyl ester -L-NAME; Nitric Oxide- NO; Renin andgiotensin system- RAS; ACE- angiotensin converting enzyme; Ang II- Angiotensin II; NAD(P)H- nicotinamide adenine dinucleotide phosphate; ROS- reactive oxygen species); SNS- sympathetic nervous system

1.4.2 Current therapies used to counteract L-NAME-induced hypertension

Existing anti-hypertensive therapies that have been explored as potential treatments for L-NAME-induced hypertension are lacking in their ability to effectively combat all aspects of the experimental disease state, a similar predicament with anti-hypertensive therapies used to treat essential hypertension [119]. While preventing the diminished NOS activity, concurrent treatment with L-arginine, a substrate for NO synthesis, along with L-NAME did not prevent the establishment of hypertension [130]. Similarly, an anti-oxidant, N-acetylcysteine, failed to abolish hypertension, but reduced ROS production and augmented NOS activity [131]. One promising candidate for treating L-NAME-induced hypertension, hydralazine, was able to prevent the development of hypertension and restored vasodilation, but was unsuccessful in preventing the production of O_2^-, arterial fibrosis and inflammation [132]. Currently, chronic treatment with the ACE inhibitor, enalapril, or AT_1- receptor blocker, losartan, showed success in combating L-NAME-induced hypertension, but both treatments still need further research to ensure abrogation of all aspects of pathophysiology involved [133]. Recently, antioxidant therapies such as curcumin and tetrahydrocurcumin significantly suppressed blood pressure elevation and oxidative stress, but did not report changes in Ang II [129]. Consequently, the lack of successful therapies available to treat hypertension, like that of L-NAME-induced hypertension, demonstrates the need for new candidates that could potentially combat all aspects of this disease. One such candidate may be the heme-L-arginate, resulting in the upregulation of the heme-oxygenase (HO) pathway.

1.5 The Heme oxygenase system

1.5.1 Review of the Heme oxygenase system

Originally discovered in 1968 by Tenhunen and Schmidt, the HO system is a powerful generator of antioxidants, anti-inflammatory molecules, and vasodilation. HO is the initial and rate-limiting, microsomal enzyme in the pathway which degrades heme [134]. There are three isoforms of HO found in the body: HO-1, HO-2 and HO-3 [135]. HO-2 and, to a lesser extent, HO-3 are constitutively expressed and regulate normal cell function [136]. Conversely, HO-1 is not normally expressed in tissues, with the exception of the spleen, bone marrow and liver (which can contain specialized reticuloendothelial cells) [137]. HO-1, a heat shock protein (HSP32), expression is induced in response to oxidative stress, ischemia-reperfusion, hypoxia, hyperthermia, tissue inflammation and by a wide array of other diverse stimuli [138]. Importantly, increases in HO-1's enzymatic substrate (heme), promotes HO-1 protein expression and activity [136, 137]. Activation of HO-1 by its diverse array of stimulators rapidly increases widespread transcription and expression of the protein [134]. All forms of HO catalyze the oxidation of heme, a metalloporphyrin, which is a powerful oxidant and promoter of ROS generation and lipid peroxidation [139]. Free heme does not occur under normal conditions, but is deposited in tissues under pathological conditions. The catabolism of heme by HO leads to the liberation of biliverdin, carbon monoxide (CO) and free iron (in the ferrous form) through a coupled-oxidation mechanism. The coupled oxidation mechanism requires molecular oxygen and a NAD(P)H dependent reductase [134]. As such, the activity of HO not only protects cells from increased oxidation, but provides important sources of cytoprotection; CO, biliverdin, and free iron [134]. Moreover, benefits of HO protein expression are further driven by modulation of second messenger cascades such a cGMP and MAPK [140, 141]. There is a vast amount of evidence to the cytoprotective benefits of HO-1 upregulation. For instance, HO-1 regulation

protected rats from hypertensive renal damage and hypertrophy [114, 142]. While the benefits of HO-1 upregulation have been firmly established, more research must be done to clearly elucidate this pathway.

1.5.2 Carbon Monoxide

First identified as a toxic air pollutant, CO is a stable, lipid soluble gas and as such cannot be held within cell membranes [143]. A bi-product from all types of incomplete combustion with carbon-containing molecules, CO has a great affinity for hemoglobin and it can reduce blood oxygenation with the potential to result in hypoxia [144]. At its normal production rate of 16.4 µmol/h with daily production totaling up to 500 µmol in humans, CO has proven to be beneficial at endogenous levels and is commonly derived as a by-product of heme oxidation by NAD(P)H [134, 144]. CO is an important vasoactive signaling gas molecule that acts similarly to NO and is involved in regulating contractility and blood pressure in vascular tissues [145]. Similar to the actions of NO, evidence shows that CO stimulates the sGC resulting in the production of cGMP [146, 147]. cGMP is involved in vasodilation as well as other vascular functions, such as inhibition of platelet aggregation and SMC proliferation [140]. Independent of cGMP, CO has been shown to activate the calcium activated potassium channel (BK_{Ca}) directly increasing the outward potassium (K^+) current resulting in the hyperpolarization of the SMC and vasodilation [141]. It should be noted that functional endothelium are required to carry out CO benefits [139]. While stimulating a series of vasodilatory molecules and actions, CO simultaneously inhibits endothelin, cytochrome P450 enzyme activity and 20-HETE, all of which possess inflammatory and vasoconstrictive properties [137, 148]. CO contributes to the modulation of oxidative stress by inhibiting NAD(P)H-oxidase and the subsequent production of O_2^-, as well as increasing glutathione levels [149, 150]. In addition to CO, biliverdin, another product from the HO

pathway which is potentially toxic and formally known merely as a waste product, has been recognized as an important cytoprotective agent.

1.5.3 Biliverdin/Bilirubin

Biliverdin is a soluble greenish bile pigment which is primarily produced through the breakdown of heme by HO. It is quickly reduced to bilirubin by biliverdin reductase (BVR) [135]. Bilirubin is lipophilic, yellowish bile pigment and possesses the ability cross cell membranes [151]. Normally, the majority of bilirubin is derived from hemoglobin released from aging or damaged red blood cells [148]. This accounts for the basal expression of HO in the spleen and bone marrow. It is then conjugated and passes from the liver though bile and the feces to be excreted [134]. Interestingly, several studies have shown that elevated serum levels of bilirubin are related to a reduced risk of atherosclerosis, stroke and coronary artery disease [135, 136, 152]. Bilirubin, as a potent antioxidant and reducing agent, is able to scavenge ROS including H_2O_2 and O_2^- [135, 136]. Like CO, bilirubin is able to inhibit NAD(P)H-oxidase as well as PKC activity and Ang II induced vascular damage [140, 153]. Bilirubin also decreases arterial remodeling and inflammation through the reduction of several adhesion molecules and growth factors. HO derived bilirubin exerts cytoprotective properties on the cardiovascular system, which is manifested in individuals with Gilbert syndrome whom have higher than normal serum bilirubin levels and have decreased risk for coronary artery disease [150]. Many studies have shown the benefits of physiological bilirubin levels; however, it should be noted at high concentrations bilirubin itself can generate ROS [151].

1.5.4 Free Iron

Along with CO and biliverdin, free iron is released in the catabolism of heme. The free iron released is in the ferrous form, but molecular oxygen participating in the reaction results in the conversion to the ferric form [154]. The release of free iron in the catabolism of heme could be regarded as harmful and pro-oxidant, however; this iron is not allowed to accumulate as it is rapidly sequestered by ferritin. [135]. By subsequently binding to iron regulatory protein (IRP), free iron stimulates a pathway which leads to its sequestration and exportation [134]. In particular, this exportation is due to increased ferritin synthesis, an ubiquitous intracellular protein which acts as a reservoir for excess free iron and that has been linked to the propagation of HO-1 protection [134, 155]. The increased ferritin synthesis by high free iron levels enhances iron storage capacity within the cell leading to a decrease in iron's pro-oxidant capabilities. Therefore, the expression of ferritin remains a contributing factor to the cytoprotective benefits derived from the HO system. Additionally, unique to the free form of iron, synthesis of NO occurs via induction of NFκB which promotes the induction of NOS [154]. While individually CO, biliverdin and free iron have cytoprotective properties, evidence suggests that their coordination results in maximal cellular protection through mediating vasodilation and combating inflammation and oxidative stress [156].

1.6 Role of the heme oxygenase pathway in hypertension

The potential therapeutic benefits of HO activity are of clinical interest. Over the years many studies have investigated the effects of the HO pathway on hypertension. Recently, genetic polymorphisms of HO have been implicated in human susceptibility of essential hypertension [157]. A variety of HO-1-inducers and heme substrates such as metalloporphyrins like hemin, heme-L-lysinate and heme-L-arginate have been explored as modulators of hypertension [148,

158, 159]. The induction of the HO system has proven effective in treating many forms of experimental hypertension. In fact recent evidence reveals that the HO inducer, hemin, successfully combated DOCA–salt induced hypertension while increasing anti-oxidant capacity, reducing cardiac hypertrophy and a host of other related pathology associated with severe hypertension [160]. Hemin also attenuates acute phenylephrine-induced renal hypertension in stoke prone-spontaneously hypertensive rats (SP-SHR) [138]. Heme-L-arginate, in which the heme molecule is stabilized with three molecules of arginine, has been shown to be effective in lowering blood pressure in SHR [158]. In addition, research shows that HO-inducers normalize blood pressure in adult SHR with established hypertension after just three weeks of treatment [113]. Furthermore, induction of HO-1 gene expression by retroviruses result in a decrease of mean arterial pressure after introduction of Ang II [147]. Interestingly, a marked reduction of blood pressure in SHR after just four days was noted after treatment with heme-L-arginate [158]. In contrast, the absence of HO-1 expression in mice resulted in elevated blood pressure, cardiac hypertrophy and renal failure [150]. Overall, evidence suggests that HO-inducers are effective in abrogating not only hypertension, but also the pathophysiology that accompany it.

1.6.1 Implications of heme oxygenase inducers as treatment in L-NAME-induced hypertension

In particular, HO-inducers have the potential to be effective in treating L-NAME-induced hypertension. While past and current proposed therapies have only partially combated the many aspects of essential hypertension, HO-inducers and their subsequent by-products may be able to treat all aspects of this complex disease. HO-inducers have the ability to cause direct vasodilation in vasculature through the release of CO which may be sufficient to negate the effect of NO inhibition and RAS caused by L-NAME [137]. Also, stimulation of cGMP leads to further

vasodilation and inhibition of NAD(P)H-oxidase and contributes to the powerful therapeutic value of HO-inducers [138, 140]. HO-inducers, including heme-L-arginate, have also successfully suppressed fibrosis and hypertrophy in animal hypertensive models [161, 162]. Importantly, heme-L-arginate reduced PLC and oxidative stress in mesenteric arteries of DOCA-salt hypertensive rats [163]. The reduced production and scavenging of ROS and free radicals, further support the hypothesis that HO-induction is a potentially viable therapy for hypertension. With the widespread distribution of HO-1, there are many benefits to be explored in the administration of heme-L-arginate including the possible reduction of a hypertensive state.

2) Rationale, Hypothesis and Objectives

2.1) Rationale

Essential hypertension is a complex disease state that increases risk for morbidity and mortality. The involvement of the SNS, endothelial dysfunction and the RAS are all known to play a part in the development and maintenance of hypertension. Despite decades of research the mechanisms driving essential hypertension have yet to be fully elucidated. Studying hypertension through the use of animal models has and will continue to uncover many underlying contributors of hypertension.

One such model is L-NAME induced hypertension. Similar to the pathophysiology seen in essential hypertension, L-NAME has more than just one mechanism driving its on-set of hypertension. L-NAME induces hypertension through the inhibition of NO synthesis, the stimulation of both SNS and the RAS, particularly increasing Ang II along with the formation of ROS [26, 74]. Further investigations are required to more clearly elucidate the mechanisms by which L-NAME induces hypertension.

The need for an effective well rounded anti-hypertensive therapy to combat L-NAME-induced hypertension has yet to be discovered. The role of the HO system in hypertension is continuing to be explored in many models. It has shown notable effects in SHR and DOCA animal models, not only lowering blood pressure, but ablating hypertrophy and remodeling [142, 164]. Taking these observations into account, treatment of L-NAME induced hypertension with an HO-inducer seems valid. It has been established that HO inducers promote the production of anti-inflammatory and anti-oxidative compounds through the release of CO, bilirubin and free iron from heme, effectively decreasing hypertension and its deleterious effects [148, 165]. The effects of the HO-inducer, heme-L-arginate, on Ang II levels and related oxidative stress in L-NAME-induced hypertension are unknown to date. Consequently, the mechanisms by the HO

inducer, heme-L-arginate, interacts with L-NAME-induced hypertension and its deleterious effects will be explored.

2.2) Hypothesis

Administration of heme-L-arginate abrogates the development and diminishes the establishment of L-NAME-induced hypertension via upregulation of heme-oxygenase-1, the reduction of angiotensin II levels and oxidative stress.

2.3 Objectives

2.3.1 To determine the effect of heme-L-arginate on the development of L-NAME induced hypertension

To determine the effect of heme-L-arginate on the development of L-NAME induced hypertension, heme-L-arginate was administered simultaneously along with L-NAME. Vehicle groups were also included to identify any additional effects. Systolic blood pressure was measured throughout the study in conjunction with regular assessment of body weight, fluid intake and urine excretion. At 15 weeks food intake was assessed. Upon termination, heart weight was assessed. At the molecular level, the effect of heme-L-arginate on the HO system was evaluated by the determination of the protein expression of HO-1. In addition, quantification of Ang II and total antioxidant capacity was carried out to determine the effect of heme-L-arginate on L-NAME-induced pathology.

2.3.2 To determine the effect of heme-L-arginate on established L-NAME induced hypertension

Heme-L-arginate was administered to animals with established L-NAME induced hypertension. Systolic blood pressure was measured throughout the study in conjunction with regular assessment of body weight. Upon termination, heart weight was also assessed. Similar to the first study, exploration of HO-1 protein expression, modulation of Ang II and total antioxidant capacity was done to determine the effect of heme-L-arginate on L-NAME-induced pathology.

3. Materials and methods

3.1 Animal care and handling

All Animals were housed at room temperature with 12 hour light/dark cycles. Animals had *ad libitum a*ccess to standard rodent chow and drinking water. This work was approved by the University of Saskatchewan Standing Committee on Animal Care and Research Ethics board and adhered to the Canadian Council on Animal Care.

3.2 Experimental Design

3.2.1 Treatment Protocol

61 male Sprague Dawley (SD) rats, age 6 weeks, were purchased from Charles River (St. Constant, QB, Canada) and were acclimatized for 7 days in the housing facility. At 7 weeks, pre-treatment systolic blood pressure was determined and body weight was recorded. Animals were divided into groups with similar mean body weight. In study one animals (L-NAME, n=10) received L-NAME, at a dose of 60 milligrams/kilogram/day (mg/kg/day) via intraperitoneal (ip) injection. In another group, (L-NAME + HA, n=13) L-NAME (60 mg/kg/day) was given simultaneously with heme-L-arginate (HA) (15 mg/kg/day) via two ip injections **(Figure 3.1)**. Sterile deionized water, the solvent used to dissolve L-NAME, was given via ip injection, (Vehicle 1, n=6). Animals (L-NAME + Vehicle 2, n=12) received L-NAME (60 mg/kg/day) along with sterile phosphate buffered saline (PBS), the solvent used in the preparation of heme-L-arginate. All treatments above were given for 4 weeks. For study 2, the remaining 20 animals were treated with L-NAME for 4 weeks with L-NAME (60 mg/kg/day) via ip injection, after 4 weeks treatment animals were divided into two groups with similar mean body weight. Subsequently, animals continued to receive L-NAME (60 mg/kg/day) via ip injection (L-NAME,

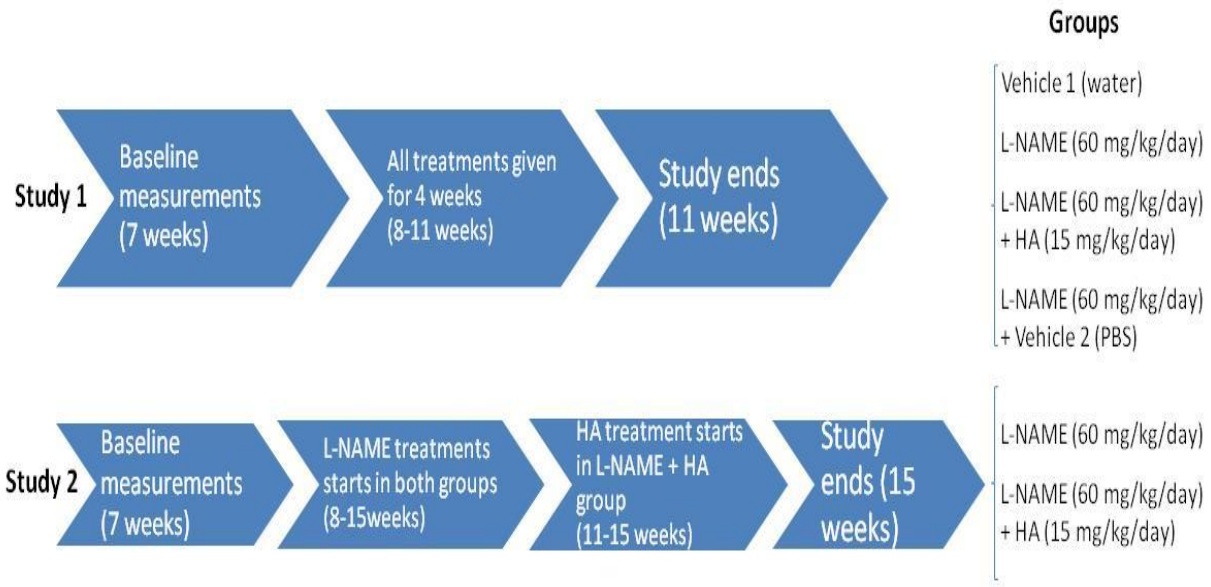

Figure 3.1 Study timeline. Animals in study 1 were divided into 4 groups. Baseline measurements were taken at 7 weeks followed by 4 weeks of treatment for all groups. Study 1 was terminated at 11 weeks. Study was comprised of 2 groups. After baseline measuments were taken at 7 weeks, L-NAME treatment began and continued for the duration of the study. From 11 to 15 weeks, animals in the L-NAME + HA received in addition to L-NAME. N-ω-nitro-L-arginine methyl ester- L-NAME; Heme-L-arginate- HA.

n=10) or heme-L-arginate (15 mg/kg/day) via i.p. injection was given separately, but in addition to L-NAME (60 mg/kg/day), (L-NAME + HA, n=10), until age 15 weeks.

3.2.2 Preparation of solutions

3.2.2.1 Preparation of L-NAME

Sterile water was used to dissolve L-NAME (Sigma-Aldrich, St. Louis, MO, USA, Product No. N5751). The solution was then titrated to a pH of 7.4 using 0.1 molar (M) hydrochloric acid (HCl) and 0.1 M sodium hydroxide (NaOH). Sterile phosphate buffered saline (PBS) was subsequently used to dilute the solution to the desired concentration. The L-NAME was solution was made weekly. No quality control was done to ascertain dissolution of L-NAME.

3.2.2.2 Preparation of Heme-L-Arginate

A solution of heme-L-arginate was prepared under sterile conditions dissolving equal parts hemin (Sigma-Aldrich, Product No. 51280) in 0.1 M NaOH and L-arginine (Sigma-Aldrich, Product No. A5006) in sterile water. The solutions of hemin and L-arginine were then combined and then titrated to a pH of 7.4 using 0.1 M HCl. Following titration, the solution heme-L-arginate was diluted using sterile PBS to reach the desired concentration. Importantly, the volume of NaOH did not exceed greater than 10 % of the final volume of the solution [159]. The heme-L-arginate solution was stored in a sterilized glass container at 4^{o}C. No quality control was done to ascertain dissolution the formation of heme-L-arginate or the presence of hemin or free L-arginine.

3.2.3 Measurement of Systolic Blood Pressure

Animals were acclimatized for 5 days to an appropriate sized restrainer and tail cuff prior to the measurement of systolic blood pressure. Thereafter, blood pressure was measured weekly until the termination of the study, using the standard non-invasive tail cuff method (Model 29 SSP, Harvard Apparatus, Montreal, Canada). Animals were placed in a temperature-controlled environment maintained at 21 degrees Celsius, and experiment data was acquired. The MP100 data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA) was used to obtain blood pressure readings, the mean of five readings was taken as the final value.

The following protocol was used to measure systolic blood pressure (SBP). After allowing the animals to acclimatize to the restrainer, the pressure of the tail cuff was increased to above tail artery occlusion pressure. Then pressure was released steadily from the tail cuff until no cuff pressure remained. This procedure was repeated every 30-60 seconds to ensure tail artery rest between readings. For each reading the corrected SBP was obtained. Raw SBP was obtained at the point where deflections in the pulse channel resumed, as the cuff pressure was released. Following the collection of raw SBP, baseline pressure is obtained by taking the mean value of the 5 seconds before the reading was initiated. From the raw and baseline pressures the corrected SBP was calculated as follows:

Corrected pressure = Raw systolic blood pressure –Baseline pressure.

3.2.4 Recording of food, fluid intake and urine output

Fluid intake and urine output were monitored weekly for all groups and recorded as ml/kg/day. At 15 wks food intake (mg/kg/day) was monitored for L-NAME and L-NAME + HA. Fluid and food intake readings were taken by changes in mass from individually housed animals

during normal housing, while urine output was measured in metabolic cages over a period of 18 hours. This was done to assess the possible effects of heme-L-arginate on appetite and fluid balance. It should be noted that spillage was not accounted for.

3.2.5 Assessment of body and organ weight

Body weight was assessed weekly throughout the duration of treatment. Upon termination of treatment animals were fasted in metabolic cages for 18 hours and body weights (in grams) were recorded. After anaesthetization, animals were sacrificed by decapitation method using guillotine. It should be noted that animals were not perfused and as such organs contained some blood. The organs were immediately extracted and cleaned in ice-cold PBS. Upon isolation, the heart, kidneys and adrenal glands were blotted dry and weighed using an analytical balance (Precisa XR 205SM-DR, Precisa Instruments Ltd, Switzerland). The organ weight-to-body weight ratios were then calculated. Left-to-right ventricle weight was also determined by isolating each ventricle and then weighing them. All organs were stored in -80°C until needed for further use.

3.2.6 Determination of Heart HO-1 expression via western blot analysis

Isolated ventricle tissue was homogenized using in 10 mM Tris-buffered saline (20 mM Tris-HCl of pH 7.4, 0.25 M sucrose, and 1 mM Ethylenediaminetetraacetic acid (EDTA)) along with a cocktail of protease inhibitors containing 1 µg/ml antipain hydrochloride, 1 µg/ml leupeptin hemisulfate, 1 µg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl floride. Samples were then centrifuged at 21,000 x g for 10 minutes at 4ºC. The supernatant was decanted and protein concentration was determined using the Bradford method. Aliquots of 80 µg of protein

were mixed with a reducing buffer and denatured by heating. Samples were subsequently loaded on to a 12% SDS-polyacrylamide gel. The fractionated proteins were subsequently transferred by electrophoresis to nitrocellulose membrane. Non-specific binding was blocked by washing the membrane for 2 hours in filtered 2.5% non-fat milk, followed by incubating the membranes for 48 hours with primary antibody HO-1 (Affinity BioReagents, USA) at a dilution of 1:2000. After thorough washing in PBS with 0.1 % Tween 20 (Sigma), the blot was incubated with secondary antibody, a peroxidase conjugate (Sigma) at a dilution of 1:10,000. The blot was then washed again and the immuno-reactivity visualized with enhanced horseradish peroxide/luminol chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA, USA). Relative densitometry analyses of respective bands of blots were carried out using UN-SCAN-IT software (Silk Scientific, Utah, USA). A monoclonal antibody raised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control due to stable basal levels in the heart.

3.2.7 Quantification of angiotensin II levels

Cardiac tissue was homogenized in a 5 mM potassium phosphate buffer, (pH 7.4 containing 0.9% sodium chloride and 0.1% glucose) along with the protease cocktail mentioned in section 3.2.6 and centrifuged at 10,000 x g for 15 minutes at 4ºC. The supernatant was then collected and the protein was determined using the Bradford method. Plasma was collected at the time of sacrifice, using EDTA (1.75 mg/ml) and centrifuging for 10 minutes at 3000 x g. Subsequently, angiotensin II concentration was quantified by enzyme immunoassay (EIA), (Cayman chemical Company, Ann Arbor, MI, USA) [166]. Using a fixed amount of monoclonal antibody the assay is based on competition between angiotensin II and a tracer (acetylcholinesterase-labelled monoclonal antibody (mAb)). A standard curve was established using known concentrations of angiotensin II which had been previously extracted using

methylene chloride. Standards were obtained from the absorbance recorded at 412 nm with a microplate reader (SpectraMax 340PC, Molecular Device, CA, USA). Plasma angiotensin II levels were calculated (pg/ml). In the case of cardiac tissue, angiotensin II levels were then standardized using sample protein content, (pg/mg of protein).

3.2.8 Determination of total antioxidant capacity

Using Antioxidant Assay kit (Cayman Chemical, Ann Arbor, MI, USA), total antioxidant capacity (TAC) was measured in the heart, kidney and mesenteric artery. The total antioxidant capacity is the sum of all endogenous and food-derived antioxidants present in the tissues, revealing the ability of the antioxidants to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate (ABTS) to ABTS plus metmyoglobin. Cardiac tissue was homogenized in a 5mM potassium phosphate buffer, (pH 7.4 containing 0.9% sodium chloride and 0.1% glucose) along with protease inhibitors mentioned in section 3.2.6 and was centrifuged at 10, 000 x g for 15 minutes at 4ºC. The supernatant was then collected and the protein was determined using the Bradford method. Samples or trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), were added to each well, followed by metmyoglobin and chromogen. Hydrogen peroxide was used to initiate the reaction and sample absorbance was read after 5 minutes at 750 nm using Synergy Microplate Reader (BioTek Instruments, Inc Vermont, USA) with Gen5 Data Analysis Software. The TAC was standardized using sample protein content and was shown as Trolox equivalent antioxidant capacity (TEAC) per mg protein [167].

3.2.9 Statistical Analysis

All statistical analyses were conducted using one way ANOVA for repeated measures. T-tests were used for comparing single measurements. All data was expressed as mean ± standard error of mean (SEM). P values of $p<0.05$ were considered statistically significant.

4. Results

4.1 Effects of heme-L-arginate on systolic blood pressure in L-NAME-induced hypertension

Mean SBP in SD rats was measured by the standard tail-cuff method to assess the effect of treatment with L-NAME and heme-L-arginate in **Figure 4.1.1**. After 4 weeks of L-NAME treatment with simultaneous co-administration of heme-L-arginate mean SBP remained normotensive (126 ± 1 mmHg; n=13, 11 weeks) [168]. Furthermore, at 11 weeks mean SBP in vehicle 1 group and did not significantly differ from the animals in the L-NAME + Heme-L-arginate groups (125 ± 1 mmHg, n=6). Following treatment with L-NAME, 11 week old animals in the L-NAME and the L-NAME + vehicle 2 groups had significantly elevated mean SBP compared to age-matched vehicle ($p<0.05$), 168 ±2 mmHg (n=10) and 154 ± 3 mmHg (n=12), respectively. Furthermore, co-treatment with heme-L-arginate, after 4 weeks of L-NAME pre-treatment, significantly lowered hypertensive mean SBP to normotensive levels (190 ± 1 mmHg, n=7 to 123 ± 1 mmHg, n=8; 15 weeks; $p<0.01$), as demonstrated in **Figure 4.1.1 and 4.1.2** [169]. Unfortunately animals were lost during the study. During the final weeks of treatment, week 14 until 15, 2 animals from the heme-L-arginate + L-NAME groups and 3 from the animals L-NAME group were lost. The reason was not determined, but may be due to extreme elevation of SBP. However, a gradual decrease in mean SBP in the heme-L-arginate treated animals was observed over the duration of the study. Another contributing factor may be toxicity; however, past studies have shown heme substrates to not be toxic [162]. After one week of the addition heme-L-arginate therapy to L-NAME induced hypertension, mean systolic blood pressure significantly was decreased compared to L-NAME alone group (176 ± 1 mmHg vs. 200 ± 1 mmHg; n=10, $p<0.05$). In the second week the trend remained similar to that observed in the first week, as the mean systolic blood pressure in heme-L-arginate treated animals continue to decrease, while the mean systolic blood pressure of L-NAME group remained elevated

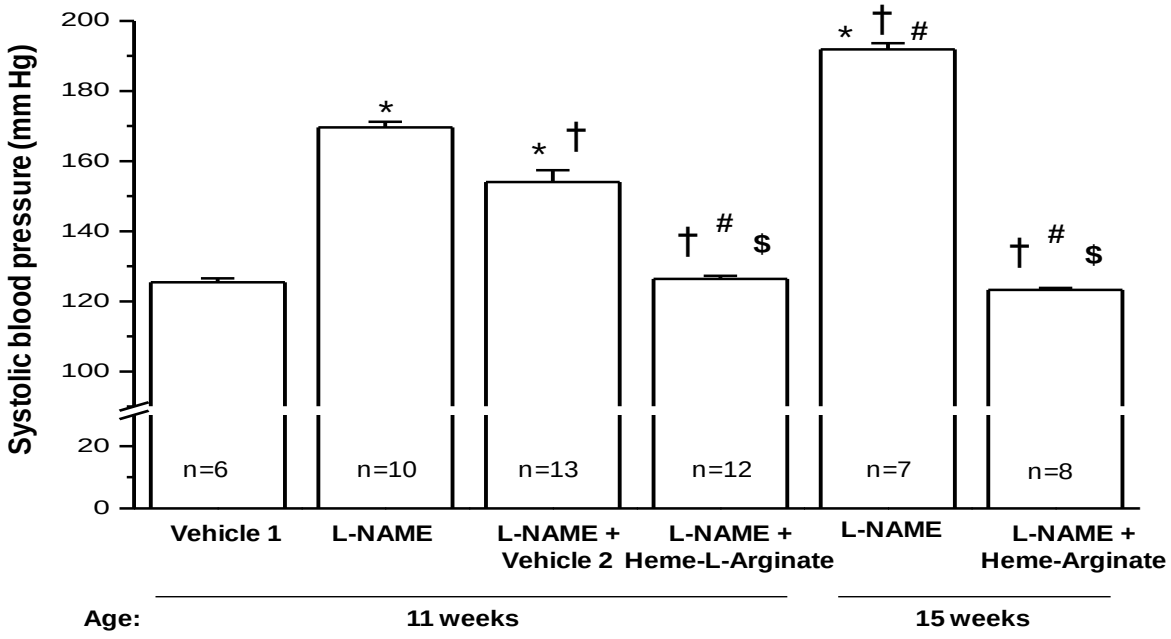

Figure 4.1.1. The effect of heme-L-arginate on systolic blood pressure of L-NAME-induce hypertension. The mean systolic blood pressure of vehicle controls remained in the normal physiological range. Treatments started at 8 weeks of age in all animals. At 11 weeks after 4 weeks of treatment, L-NAME and L-NAME+ Vehicle 2 groups showed significantly raised mean systolic blood pressure when compared Vehicle 1. Co-administration of heme-L-arginate therapy along with L-NAME for 4 weeks prevented the development of hypertension at 11 weeks resulting in a normotensive systolic blood pressure. L-NAME-induced hypertension was established during 4 weeks of treatment. Systolic blood pressure continued to increase during the next 4 weeks of L-NAME treated, while mean systolic blood pressure of animals co-treated with heme-L-arginate returned to normal. Data is expressed as mean ± SEM, * $p<0.05$ vs Vehicle 1, † $p<0.01$vs L-NAME (11 wks), # $p<0.05$ vs L-NAME + Vehicle 2 (11wks), $ $p< 0.05$ vs L-NAME (15 wks).

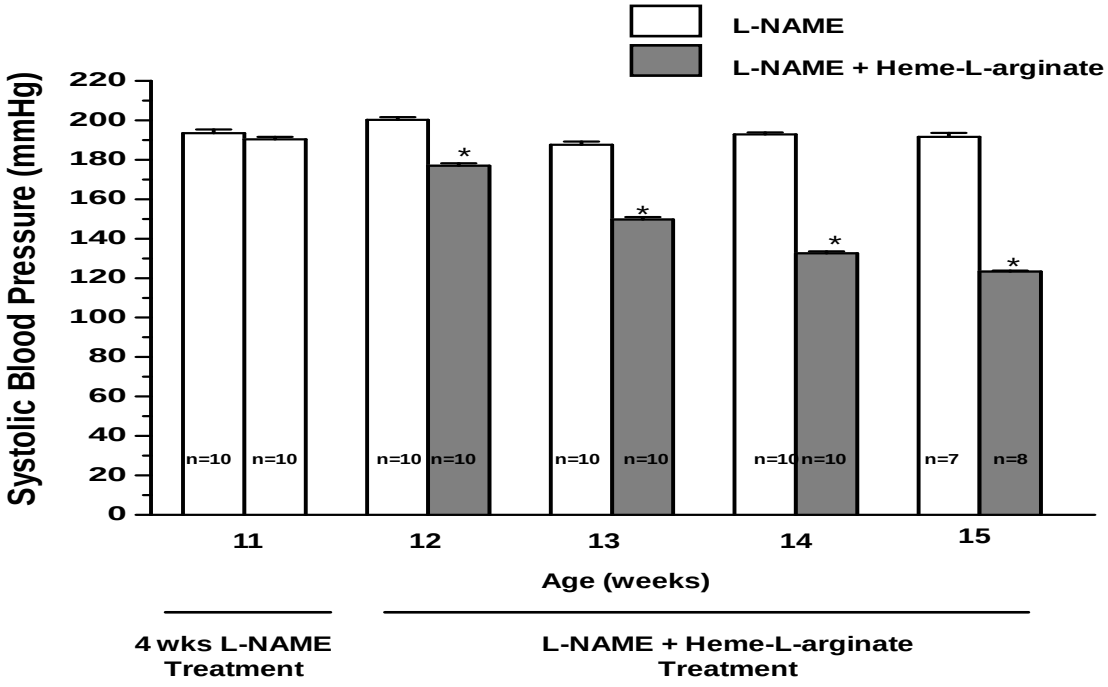

Figure 4.1.2. The effect of heme-L-arginate on systolic blood pressure in established L-NAME-induced hypertension. After 4 weeks of L-NAME treatment mean systolic blood pressure was similar in groups at 11 weeks. The addition of heme-L-arginate therapy to established L-NAME induced hypertension from 11 weeks to 15 weeks significantly reduced systolic blood pressure. After 4 weeks of heme-L-arginate therapy mean systolic blood pressure returned to a normotensive range. Data is expressed as mean ± SEM, * $p<0.01$ vs L-NAME.

(149 ± 1 mmHg vs. 188 ± 2 mmHg; n=10 $p<0.001$). At the end of third week of treatment mean systolic blood pressure of the heme-L-arginate treated group was 132.52 ± 1.01 mmHg (n=8) compared to that of aged matched L-NAME (193.20 ± 0.92 mmHg n=8).

4.2 The effect of heme-L-arginate on food intake, water intake and urine output on L-NAME induced hypertension.

Throughout the study water intake was monitored to assess the effect of L-NAME and heme-L-arginate alterations in fluid balance **(Figure 4.2.1)**. L-NAME significantly reduced water intake at two and four weeks treatment compared to baseline (2.93 ± 0.36 ml/kg/hr, n=6 and 1.36 ± 0.27 ml/kg/hr, n=5 vs. 3.93 ± 0.42 ml/kg/hr, n=6; $p<0.05$). Interestingly, the addition of heme-L-arginate to L-NAME treatment eliminated this significant difference through the first three weeks of co-treatment. However, after four weeks of co-treatment with both L-NAME and heme-L-arginate there was again a significant reduction in water intake when compared to baseline (1.11 ± 0.32 ml/kg/hr; n=5, $p<0.05$). When comparing the effect in animals co-treated with heme-L-arginate and L-NAME to age matched animals treated with only L-NAME, no significant change was seen until 4 weeks of co-treatment (1.11 ± 0.32 ml/kg/hr, n=5 vs 2.15 ± 0.13 ml/kg/hr, n=6; $p<0.01$).

Urine output was also monitored to allow the observation of further alterations to the fluid balance **(Figures 4.2.2)**. With the exception of three weeks of co-treatment with heme-L-arginate and L-NAME there were no significant changes when compared to baseline or age matched L-NAME treated animals (2.27 ± 0.34 ml/kg/hr vs. 3.93 ± 0.42 ml/kg/hr and 3.24 ± 0.98 ml/kg/hr). Animals showed normal fluid intake and urine output when compared literature references, with the exception of the animals in group of heme-L-arginate + L-NAME at 15 weeks.

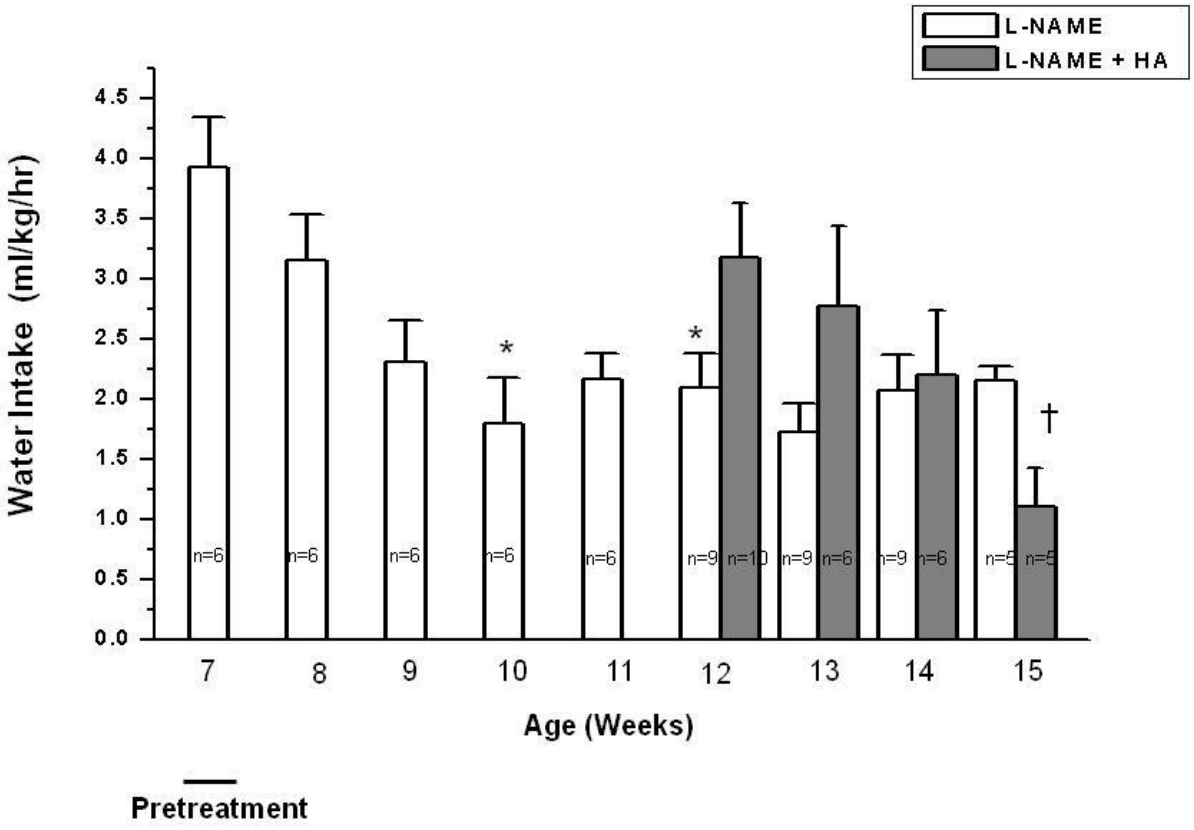

Figure 4.2.1. Water intake in rats treated with L-NAME and Heme-L-arginate. After establishing pretreatment values at 7 weeks L-NAME treat was started. During the initial four weeks of L-NAME treatment the trend of water intake showed a decline, reaching significance at 2 weeks and 4 weeks of treatment compared to baseline intake (* $p<0.05$). The addition of heme-L-arginate to L-NAME at 11 weeks did not significantly alter water intake until the fourth week of combined treatment, when compared to age matched L-NAME animals († $p<0.01$). Data is expressed as mean ± SEM.

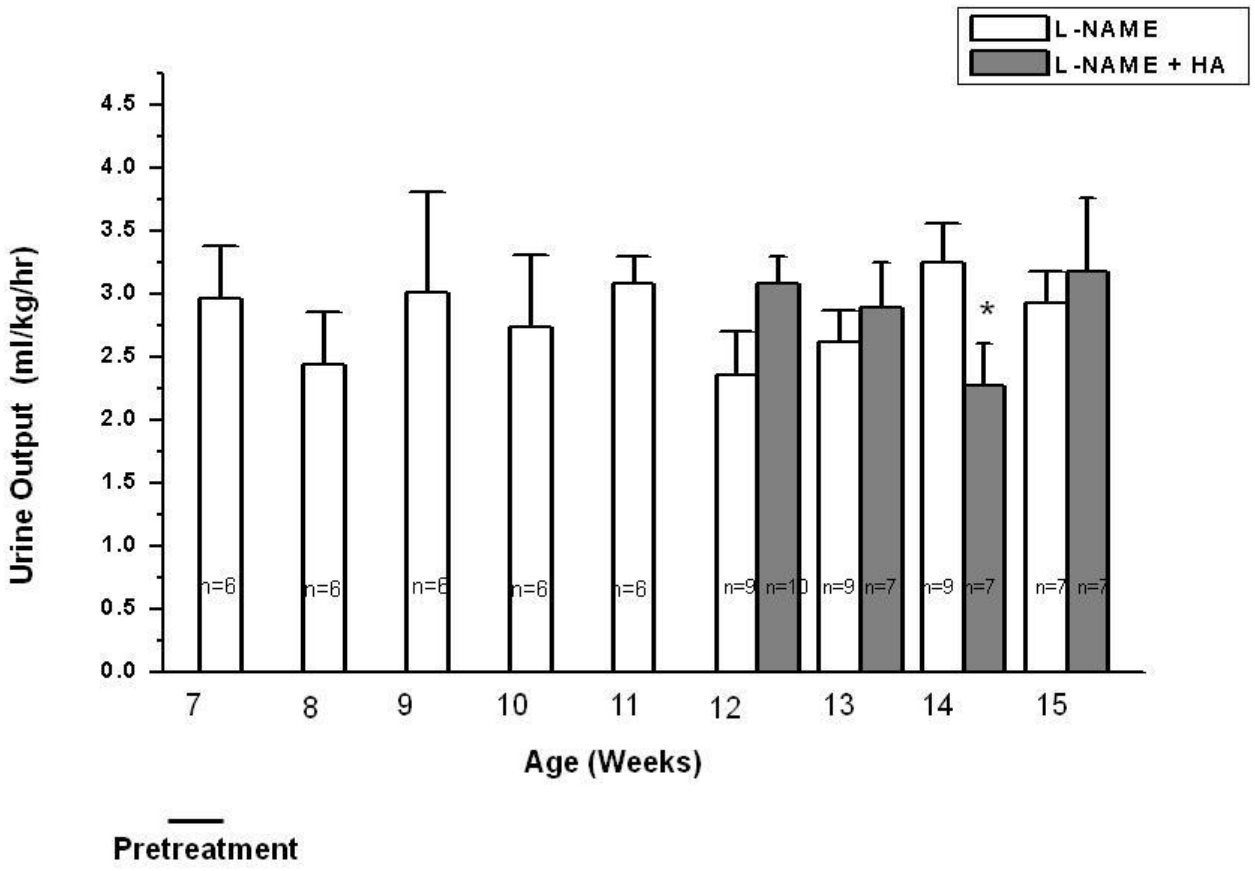

Figure 4.2.2. The effect of L-NAME and Heme-L-arginate on urine output. After establishing pretreatment values at 7 weeks L-NAME treat was started. Urine output is unchanged throughout 8 weeks of L-NAME treatment including after the addition of heme-L-arginate co-treatment. The addition of heme-L-arginate from 12 to 15 weeks did not significantly alter urine output when compared to age matched L-NAME animals, except during the third week of treatment (*$p<0.05$). Data is expressed as mean ± SEM

Again, variation in sample size was noted and was due to a several reasons. Fluid intake sample size variation was primarily due to error in collection or excess spillage from animals. Urine output sample size variation was due to limited number of metabolic cages at our disposal.

4.3 Assessment of gross body and organ weight

Table 1 illustrates the effects of L-NAME and heme-L-arginate on body and organ weight. Wet heart was examined in order to assess simply if there was any evidence of hypertrophy, as it may have been a possible outcome. At 11 weeks, animals treated with heme-L-arginate in addition to L-NAME had significantly reduced wet heart weight compared to all other groups (295.62 ± 6.93 g; n=13, $p<0.05$). Wet heart weight was unchanged in animals treated with L-NAME (1.25 ± 0.05 g, n=10) and L-NAME + Vehicle 2 (1.21 ± 0.03 g, n=12) when compared to the animals treated with vehicle 1 (1.22 ± 0.05 g, n=6). At 15 weeks animals co-treated with L-NAME and heme-L-arginate (1.35 ± 0.05 g, n=8) showed no change in heart weight when compared to age matched animals treated with L-NAME alone (1.37 ± 0.05 g, n=7). Despite the changes in wet heart weight, when heart-to-body weight ratio is examined, animals treated with heme-L-arginate showed a significant increase ($p<0.05$) in comparison with the all other groups at both 11 and 15 weeks. The addition of heme-L-arginate to L-NAME caused a decreased body weight upon termination at 11 weeks (295.62 ± 6.93 g; n=13, $p< 0.05$) in relation to all other groups: Vehicle 1 (382.58 ± 4.64 g, n=6), L-NAME (391.70 ± 9.97 g, n=10), L-NAME + Vehicle 2 (382.58±3.59 g, n=12). It should be noted that while the addition of heme-L-arginate resulted in a significant decreased body weight, it did not induce weight loss. Animals treated with heme-L-arginate consistently gained weight throughout the study, but a slower rate than animals in all other groups.

Group	Age (weeks)	Mean Body Wt. at time 0 (g)	Mean Body Wt. (g)	Heart Wt. (g)	Heart-to-Body Wt. Ratio (g/ Kg)
Vehicle 1 (n=6)	11	238.209 ± 2.09	382.58 ± 4.64	1.22±0.05	3.23±0.12
L-NAME (n=10)		254.00 ± 3.68*	391.70 ± 9.97	1.25±0.05	3.22±0.11
L-NAME + Vehicle 2 (n=12)		240.58 ± 2.89$^{\#}$	382.58±3.59	1.21±0.03	3.15±0.05
L-NAME + HA (n=13)		242.78 ± 2.61$^{\#}$	295.62 ± 6.93$^{*\#\dagger}$	1.07±0.02$^{*\#\dagger}$	3.64±0.08$^{*\#\dagger}$
L-NAME (n=7)	15	280.64± 3.48*†‡	474.00±21.28$^{*\#\dagger\ddagger}$	1.37±0.05*†‡	2.90±0.06$^{*\#\dagger\ddagger}$
L-NAME + HA (n=8)		264.30± 5.49$^{*\#\dagger\ddagger\$}$	388.38±10.12$^{\ddagger\$}$	1.35±0.05†‡	3.50±0.20$^{*\dagger\ddagger\$}$

Table 1. The effect of heme-L-arginate and L-NAME on body and organ weight. Initially, animals were grouped according to similar mean group body weights. At 11 weeks after 4 weeks of co-treatment with heme-L-arginate and L-NAME animals had a significantly lower body weight than all other age matched groups. After L-NAME is established for 4 weeks the addition of heme-L-arginate from 12 to 15 weeks also results in a significantly reduced body weight. The assessment of raw heart weight, after isolation and blotting, showed significantly reduced raw heart weight at 11 weeks, but not at 15 weeks. Animals treated with heme-L-arginate both during (11 weeks) and after the establishment (15 weeks) of L-NAME-induced hypertension had a significant increase in heart weight-to- body weight ratio. Data is expressed as mean ± SE. * $p<0.001$ vs Vehicle 1, # $p<0.01$vs L-NAME (11 wks), † $p<0.05$ vs L-NAME + Vehicle 2 (11wks), ‡ $p<0.05$ vs L-NAME + HA (11 wks), $ $p< 0.05$ vs L-NAME (15 wks)

Animals treated with heme-L-arginate consistently gained weight throughout the study, but a slower rate than animals in all other groups.

At 15 weeks, after addition of heme-L-arginate for 4 weeks after the L-NAME induced hypertension is established animals had a significantly reduced body weight (388.38 ± 10.12 g, n=8, $p<0.05$) when compared to age matched animals treated with L-NAME alone (474.00±21.28, n=7).

At the end of the study food intake was assessed to determine the effect of heme-L-arginate on appetite **(Figure 4.3.1)**. After four weeks of treatment there was no significant difference in food intake between animals co-treated with heme-L-arginate and L-NAME for 4 weeks between 12 and 15 weeks when compared to those treated with L-NAME alone (40.23 ± 6.29 g/kg/hr vs. 21.99 ± 4.03 g/kg/hr, n=6); however, animals treated with heme-L-arginate displayed a possible trend of increased food intake (P=0.12). This could suggest possible changes to metabolism.

4.4 The effect of heme-L-arginate on the expression of HO-1 in the heart

HO-1 protein expression in heart tissue was quantified by Western immunoblot and compared to the relative percentage GAPDH. The expression of HO-1 was significantly ($p<0.01$) increased in animals 11 weeks of age treated with heme-L-arginate in addition to L-NAME compared to those treated with L-NAME alone (92.16 ± 9.86 vs. 15.98 ± 9.97 HO-1/GAPDH %, n=4) **(Figure 4.4.1)**. In comparison to previously reported HO-1 expression levels in control SD rats, the L-NAME did not show increases; however, heme-L-arginate group has elevated expression [142]. Smaller sample sizes of this assay are due to uncontrollable technical difficulties involving shipping errors and contaminated materials (water source).

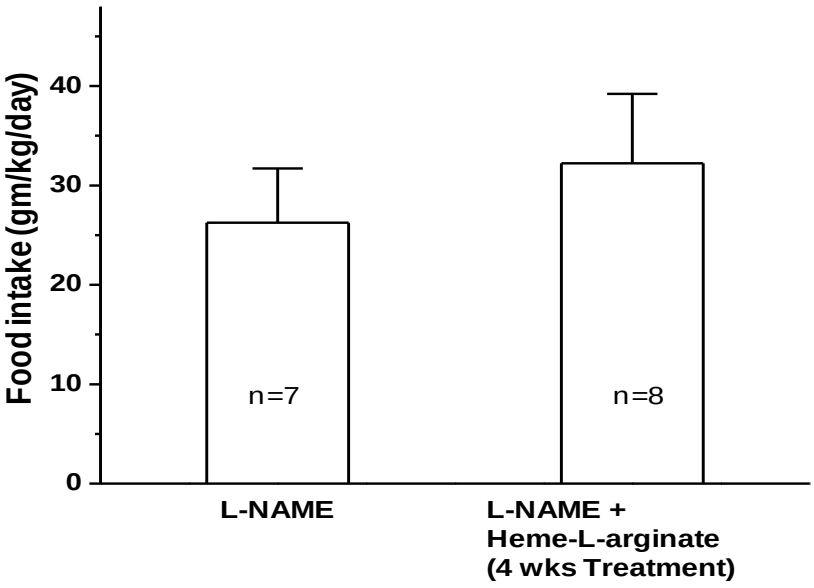

Figure 4.3.1. The effect of Heme-L-arginate on food intake in L-NAME induced hypertension. At 15 weeks food intake was unchanged after 4 weeks of heme-L-arginate L-NAME induced hypertension (P=0.12). Data is expressed as mean ± SEM.

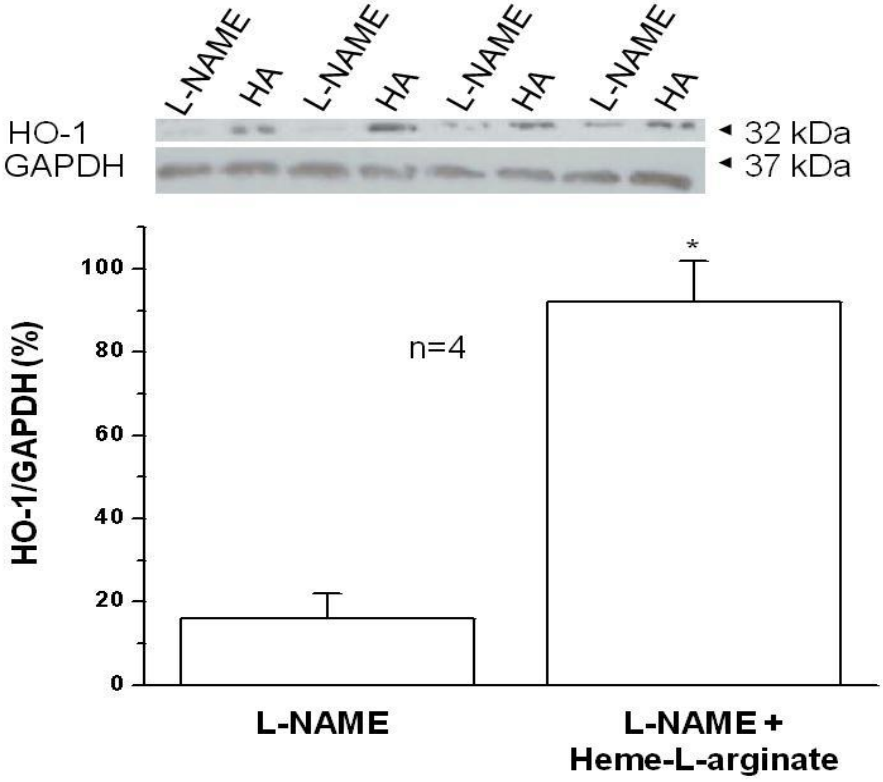

Figure 4.4.1. The effect of heme-L-arginate and L-NAME on heme-oxygenase 1 (HO-1) protein expression against GAPDH using representative western blot and densitometry analysis in heart tissue. At 11 weeks, after 4 weeks of co- treatment with L-NAME and Heme-L-arginate the expression of HO-1 is significantly elevated above that of rats treated with L-NAME alone. Data is expressed as mean ± SEM, *p=0.01.

4.5 Modulation of Angiotensin II by L-NAME and heme-L-arginate

Administration of L-NAME caused significant elevation in heart Ang II after 4 weeks (87.5 ± 6.2 pg/mg, n=8) and 8 weeks (89.5 ± 10.4 pg/mg, n=8) of treatment compared to the animals in the vehicle 1 (2.55 ± 0.38 pg/mg, n=6), as shown in **Figure 4.5.1.A.** The addition of vehicle 2 to L-NAME treatment had no effect on heart Ang II levels after 4 weeks in comparison to the other L-NAME groups (84.9 ± 5.0 pg/mg, n=12). 4 weeks heme-L-arginate significantly suppressed Ang II levels in the heart when co-administered with L-NAME (70.7 ± 1.9 pg/mg, 11 weeks; n=6, $p<0.05$) and attenuated Ang II levels subsequent to the establishment of L-NAME-induced hypertension (65.5 ± 4.7 pg/mg, 15 weeks; n=7, $p<0.05$) in contrast to L-NAME groups. While not significant ($p=0.08$), Ang II plasma levels followed the trend of Ang II levels in the heart **[Figure 4.5.1.B]**. Animals in the L-NAME-treated group (15 weeks) had slightly elevated levels of Ang II (58.12 ± 9.26 pg/ml) when compared to animals treated with heme-L-arginate in addition to L-NAME (39.77 ± 2.75 pg/ml).

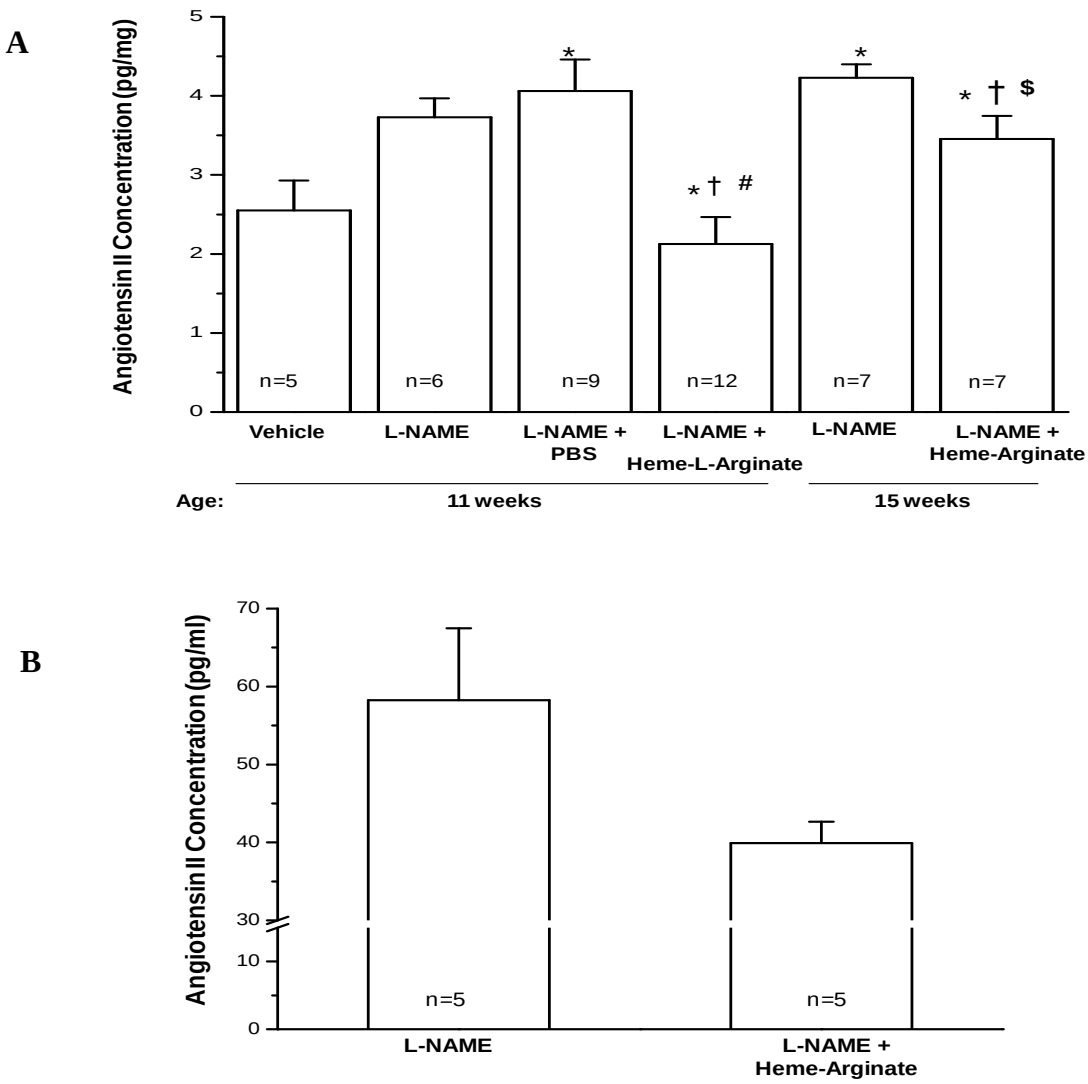

Figure 4.5.1. The effect of heme-L-arginate and L-NAME on Angiotensin II in heart and plasma. **A.** At 11 weeks, after 4 weeks of treatment with L-NAME and L-NAME + Vehicle 2 significantly elevated Angiontensin II levels in the heart when compared to Vehicle 1 and L-NAME + heme-L-arginate ($p<0.05$). At 11 weeks, co-treatment with L-NAME and Heme-L-arginate for weeks maintains angiotensin II levels similar to those of normal levels (Vehicle 1). Treatment with L-NAME for 8 weeks did not significantly increase Ang II levels in the heart when compared L-NAME treatment for 4 weeks. However, the addition of heme-L-arginate to L-NAME for 4 weeks after the establishment of L-NAME –induced hypertension significantly lowered Ang II levels. * $p<0.05$ vs Vehicle 1 (11wks), † $p<0.01$vs L-NAME (11 wks), # $p<0.05$ vs L-NAME + Vehicle 2 (11wks), $ $p< 0.05$ vs L-NAME (15 wks) **B.** At 15 weeks after 4 weeks of co-administration of heme-L-arginate therapy in addition to L-NAME, after L-NAME induced hypertersion was established for 4 weeks, did not significantly alter plasma levels of angiotensin II when compared with animals treated with L-NAME alone ($p=0.08$). Data is expressed as mean ± SEM.

4.6 Effects of heme-L-arginate on the total antioxidant capacity

TAC was assessed in heart **[Figure 4.6.1]**. While all treatment groups have significantly reduced TAC in relation to Vehicle 1 (0.93 ± 0.2mmol TEAC/mg of protein, n=6), treatment with heme-L-arginate partially maintains and restores heart TAC, 0.61 ± 0.07 mmol of TEAC/ mg of protein (n=8, 11 weeks) and 0.55 ± 0.04 mmol of TEAC/ mg of protein (n=8, 15 weeks) ($p<0.05$). Marked decreases in heart TAC were seen subsequent to L-NAME treatment: L-NAME (0.31 ± 0.04 TEAC/mg of protein; n=7, 11 weeks), L-NAME + Vehicle 2 (0.37 ± 0.06 TEAC/mg of protein; n=12, 11 weeks) and L-NAME (0.37 ± 0.06 TEAC/mg of protein, 15 weeks) ($p<0.05$ versus vehicle 1).

Similar preservation of the TAC was seen in the kidney and the mesenteric artery **[Figure 4.6.3]**. The mesenteric artery of animals co-treated with heme-L-arginate had significantly higher TAC than that of animals treated only with L-NAME (0.18 ± 0.04 TEAC/mg of protein, n=6 vs. 0.10 ± 0.03 TEAC/mg of protein, n=6, 11 weeks). At 11 weeks, co-treatment of heme-L-arginate along with L-NAME showed significant elevation of TAC in the kidney (1.18 ± 0.13 TEAC/mg of protein vs. 0.79 ± 0.03 TEAC/mg of protein, n=5) ($p<0.05$).

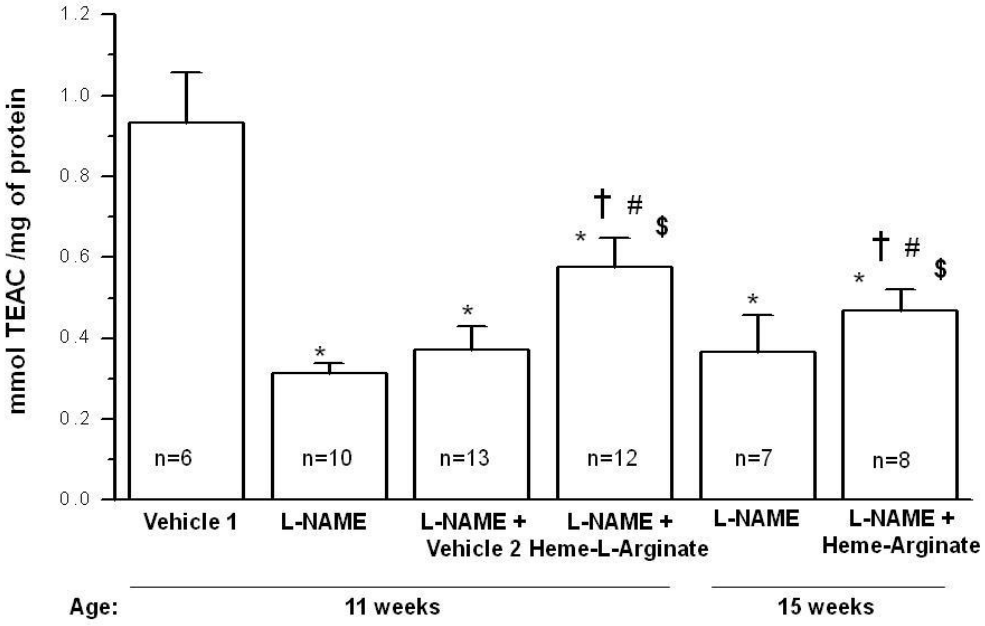

Figure 4.6.1. The effect of heme-L-arginate and L-NAME on Total Antioxidant Capcity in the heart. Treatment with L-NAME reduced TAC in the heart compared to age matched vehicle controls ($p<0.05$). The addition of heme-L-arginate to L-NAME during the development of hypertension significantly abrogated the reduction in TAC. The elevation of TAC with heme-L-arginate is also seen in established L-NAME hypertension. * $p<0.05$ vs Vehicle 1, † $p<0.01$vs L-NAME (11 wks), # $p<0.05$ vs L-NAME + Vehicle 2 (11wks), $ $p< 0.05$ vs L-NAME (15 wks). Data is expressed as mean ± standard SEM.

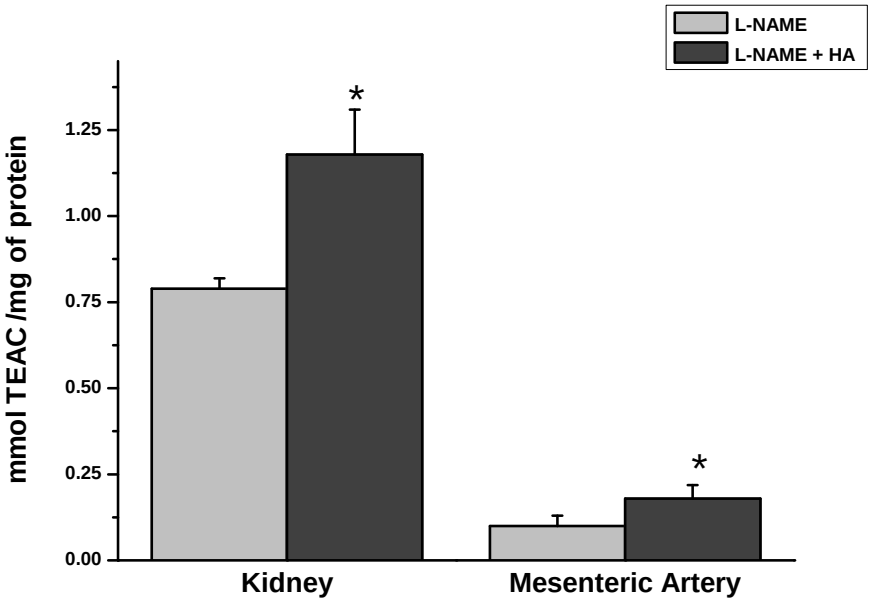

Figure 4.6.3. The effect of heme-L-arginate and L-NAME on Total Antioxidant Capcity in kidney and mesenteric artery. The co-treatment of heme-L-arginate along with L-NAME significantly abrogated the reduction in TAC in the kidney (n=5) and mesenteric artery (n=5 and n=6) compared to age matched L-NAME treated animals ($p<0.05$, 11 weeks). Data is expressed as mean ± SEM.

5. Discussion

5.1 The consequences of L-NAME-induced hypertension and the benefits of induction of the heme oxygenase pathway

L-NAME-induced hypertension represents a complex experimental model of human essential hypertension [124]. The SHR model of hypertension is considered the standard essential hypertension animal model; however, L-NAME also exhibits similar pathophysiology to that described in essential hypertension. Classically, L-NAME is known to induce hypertension via inhibition of NOS, which subsequently results in the depletion of NO [32]. More recently, it has been reported that L-NAME also upregulates several key components of the RAS and activates the SNS [119, 170]. Research has revealed renal and vascular injury as well as vasoconstriction and endothelial dysfunction after chronic L-NAME administration [124, 133, 171]. Given these characteristics, this study explored changes in mean SBP after chronic L-NAME treatment utilizing the standard tail-cuff method [127]. The administration of L-NAME to SD rats resulted in significant increase in mean SPB. Consistent with literature, throughout the course of L-NAME treatment mean SBP continually rises, due to modulation of several factors, including increasing modulation of RAS activity [172, 173]. In order to identify the causative factors of this augmentation of mean SBP, Ang II and TEAC levels were determined. Data showed that rats treated with L-NAME have elevated Ang II levels in heart tissue. One may postulate that the increased mean systolic blood pressure could be a result of direct and in-direct actions by Ang II [119]. L-NAME treatment also resulted in decreased TAC indirectly indicating that oxidative stress is increased as well. Importantly, treatment with a heme substrate, heme-L-arginate, maintained normal mean systolic blood pressure during simultaneous administration with L-NAME and abrogated established L-NAME-induced hypertension. Moreover, these

changes were accompanied by significant increases in HO-1 expression, reduction of tissue Ang II levels and elevation of TAC.

5.2 Induction of hypertension after L-NAME administration

Mean SBP was obtained by non-invasive tail cuff method throughout the duration of the study. In animals treated with L-NAME, an increase in mean SBP was observed after just one week of treatment. A similar trend followed for the first five weeks of treatment with L-NAME; after which a hypertensive state was established and mean SBP reached a plateau, remaining in a hypertensive state for the remainder of the study. After four weeks of treatment mean systolic blood pressure in animals was significantly elevated compared to normotensive age-matched Vehicle 1 treated animals [174]. While not clearly elucidated research indicates that the effect of L-NAME on blood pressure can be attributed to depletion of NO, activation of SNS and stimulation of the RAS [119, 170]. L-NAME has been shown to stimulate several components of the RAS including, renin, ACE and Ang II leading to vasoconstriction, alterations in fluid balance, oxidative stress and activation of remodeling and hypertrophy [51, 124, 125]. Interestingly, previous research indicates that Ang II may be the primary target of L-NAME. The pressor effect of Ang II showed marked and significant increase after exposure to L-NAME; an effect not seen with epinephrine or norepinephrine [175]. The effect of L-NAME on Ang II and the inter-connections with NO, the SNS and RAS were also previously explored by examining the effects of L-NAME [176]. The authors demonstrated that L-NAME induced vasoconstriction is potentiated with α-adrenoreceptor blockade and abrogated after Ang II receptor antagonist administration [176]. While not clearly elucidated, recent data suggests key interactions occur between Ang II, NO and SNS activity after L-NAME exposure. It is also likely that Ang II acts on NO and ET-1 while contributing to endothelial dysfunction through the production of O_2^-,

followed by NOS uncoupling and decreased NO [71]. Research suggests that chronic hypertension may be potentiated by both decreased NO and increased SNS activity [18]. Moreover while the degree of involvement varies, both SNS activity and local RAS are known to be fundamental mechanisms in promoting essential hypertension, cardiovascular and renal damage [120].

5.3 Modulation of systolic blood pressure in L-NAME induced hypertension with heme-L-arginate

In this study, simultaneous co-administration with heme-L-arginate had a much greater impact and completely abolished the development of L-NAME induced hypertension, similarly to previous published HO modulation of other hypertensive models [142, 146]. After eight weeks of L-NAME treatment mean SBP is further increased from that of four weeks of L-NAME treatment. Initiating heme-L-arginate treatment after L-NAME induced hypertension is established also abated mean SBP to a normotensive range, not significantly different from that of normotensive animals (Vehicle 1) or animals treated with heme-L-arginate during the development of hypertension with L-NAME administration [177]. This data corresponds to previous reported data in which heme substrates have led to normotensive blood pressure in SHR and DOCA-salt hypertensive rats [103, 114]. Unlike in other models, such as SHR, age and duration of hypertension does not seem to affect the effectiveness of heme-L-arginate therapy [141]. Interestingly, in this study the addition of Vehicle 2 (PBS) to L-NAME for four weeks significantly reduced mean systolic blood pressure by 12% from that of animals treated with L-NAME alone. While not known, this observation may indicate that PBS has some contribution to the regulation of mean SBP or it may be due to biological variation. These reductions in mean SBP resulting from heme-L-arginate were accompanied by increased HO-1 expression and

elevated TAC, suggesting increased HO system activation and reduction of oxidative stress [163].

5.4 Heme-L-arginate upregulates expression of HO-1

After four weeks of co-administration of heme-L-arginate along with L-NAME the expression of HO-1 was significantly elevated in the heart compared to age matched animals treated with L-NAME alone. Due to time constraints and unforeseen circumstances evaluation of HO-1 expression in other groups was not possible. However, in comparison to previously reported HO-1 expression levels in control SD rats, the animals treated with L-NAME did not show significant increases [142]. Distribution of HO-1 has been previously detected by Western blot in the heart, and is significantly elevated by HO- inducers, while under normal conditions it is barely detectable [144]. Earlier studies have shown that HO-1 inducers, including heme-L-arginate, have resulted in enhanced activity of the HO system [178]. Based on previous studies the magnitude of HO-1 expression, stimulated by heme-L-arginate, may have been sufficient enough to trigger the HO system [163, 179]. In fact, in this study hypertensive animals treated with heme-L-arginate had a 70% increase in HO-1 expression when compared to those treated with L-NAME alone. The products of heme catabolism, CO, bilirubin and free iron, are widely known to be cytoprotective, anti-inflammatory, anti-oxidative as well as anti-hypertensive. One of the driving mechanisms behind these changes is the release of CO. CO is an important vasodilator which acts to abrogate hypertension and exerts homeostatic control on cardiovascular functions including contractility and oxidation [145]. In particular, HO-1 derived CO mediated vasorelaxation has been shown repeatedly [148]. While not specifically characterized in this study, it has been demonstrated that heme substrates increase HO system activity through HO-1 expression subsequently increasing levels of sGC and cGMP contributing to a reduction of blood

pressure in both SHR and DOCA [159, 163]. While mechanisms are not clearly elucidated, CO is known to activate BK_{Ca} and to stimulate cGMP, both of which contribute to vasodilation [141]. Additionally, CO in co-ordination with bilirubin, inhibits NAD(P)H-oxidase which contributes to overall lower oxidative stress [149]. Bilirubin further decreases oxidative stress by scavenging H_2O_2 and O_2^- [135]. Oxidative stress is also combated by HO-1 derived free iron, its stimulation of ferritin synthesis and its subsequent sequestration of cellular free iron [134]. Elevation of ROS and oxidative stress in hypertensive animals and humans has been well documented [180]. It has also been linked to elevated RAS activity and Ang II levels.

5.5 Alterations of Ang II levels by L-NAME and heme-L-arginate

Stimulation of the RAS and it damaging effects has been previously noted in L-NAME induced hypertension [124]. Several key components of the RAS involvement in L-NAME induced hypertension have been documented including elevation of renin, increased ACE activity and Ang II. Ang II has been implicated in L-NAME via research showing the effectiveness of various angiotensin receptor blockers in decreasing the severity of L-NAME's deleterious effects [174]. Ang II is primarily responsible for the actions of the RAS [49]. Ang II has been implicated in several experimental models of hypertension including SHR, Gold-Blatt hypertensive model and the TGR(mRen2)27 rat model suggesting it importance [97, 104, 106]. Its distribution has been noted locally in the heart as well as the kidney [50]. The heart tissue Ang II levels of the Vehicle 1 group were similar to those seen in the Wistar rat reported in early studies [181]. In our study after 4 weeks of L-NAME, Ang II in heart tissue was significantly elevated. The elevation of Ang II may be a major contributor to the development of L-NAME induced hypertension by augmenting local vascular tone, oxidative stress, secondary inflammatory processes and vasoconstriction all of which can contribute to increases in blood

pressure. Despite continued elevation of mean systolic blood pressure, at 8 weeks of L-NAME there were no further increases in Ang II in the heart. This be attributed to oxidative stress, endothelial dysfunction and secondary remodeling as a result of elevated transcription factors, adhesion molecules and inflammation as seen previously [51]. Interestingly, a significant reduction of Ang II levels was seen in the heart, but not in the plasma, after heme-L-arginate co-treatment. While not reduced to that of the Vehicle 1 group, the changes in Ang II levels indicate that heme-L-arginate acts on Ang II and it mediated effects to contribute to reducing L-NAME-induced hypertension [62, 74, 93].

Stimulation of local HO systems after a heme substrate treatment has been noted in earlier studies, including the heart and may be the primary mechanism through which Ang II is reduced [161, 163, 179]. HO inducers have reportedly alleviated the effects of Ang II, including; reducing cardiac lesions, decreasing activity of molecules like NFκB and TGF-β in addition to preventing hypertrophy [160, 161]. However, Ang II levels in plasma were not significantly diminished; which may be due to the insufficient magnitude of HO system activation needed to counteract positive feedback loops in the RAS [182]. Furthermore, the possible contribution of L-arginine to the significant reduction in mean SBP needs to be explored. The potency of heme-L-arginate has been noted previously and may be attributed the presence of bound L-arginine, a substrate of NOS [141]. The release of L-arginine may contribute to elevated NO release, vasodilation and reduction of blood pressure. While L-arginine has been reported to reduce blood pressure through the generation of NO in SHR, L-arginine was not successful in combating blood pressure in L-NAME induced hypertension [158]. The abrogation of Ang II by heme-L-arginate in L-NAME induced hypertension is novel and the mechanisms driving these changes need to be more clearly elucidated with further study.

5.6 Antioxidant status altered by heme-L-arginate in L-NAME induced hypertension

L-NAME indirectly and directly contributes to the elevation of oxidative stress. The implications of elevated Ang II, not only in L-NAME induced hypertension, but in a wide range of hypertensive states have been clearly demonstrated. The production of oxidants and promotion of oxidant forming molecules by Ang II is also well known [68, 113]. Ang II is largely responsible for the production of O_2^- via NAD(P)H-oxidase [73]. Subsequently, there is increased production of $H_2 O_2$ which independently contributes to vascular pathophysiology [67, 77]. Ang II mediated oxidative stress has been implicated in end-stage organ damage and as such we assessed tissue Ang II in the heart [183]. Additionally, L-NAME directly inhibits NOS which can lead to the development of $ONOO^-$ [67]. In this study we observed a depletion of TAC in heart tissue in L-NAME induced hypertension indirectly indicating the rise of ROS and oxidative stress. This depletion of TAC suggests a rise in oxidative stress which may be correlated to L-NAME induced rise in Ang II as mentioned above. Additionally, increased oxidation due to mechanical forces may contribute to hypertension development independently of Ang II [15]. In contrast, simultaneous treatment with heme-L-arginate improved TAC, as well as treatment initiated after hypertension was established. In fact, TAC was improved in not only heart tissue but also kidney and mesenteric artery of heme-L-arginate treated animals compared to animals treated with L-NAME alone. The kidney regulates fluid balance while the mesenteric artery is closely tied to total peripheral resistance; both are important factors in the maintenance of blood pressure. With the elevation of oxidative stress damage to tissue could lead to retention of sodium and fluid in the kidney along vasoconstriction and remodeling in both the kidney and mesenteric artery. Consistent with previous studies, heme-L-arginate protected the kidney and mesenteric artery against hypertension related oxidation and subsequent lesions [114, 163]. These changes could be a reflection of the HO-pathway stimulation and by the cytoprotective

properties of its products: CO, bilirubin and free iron. Released by the catabolism of heme; these products work together to neutralize ROS. Specifically, bilirubin inhibits NAD(P)H oxidase leading to decrease O_2^- production [153]. Moreover, bilirubin has been inversely related to atherogenic risk and can protect against endothelial damage while its precursor biliverdin interacts with vitamin E to provide anti-oxidative protection [152, 184]. Adding to the cytoprotective effect of bilirubin; the release of free iron promotes the induction of ferritin synthesis. Ferritin has been independently linked to cytoprotection through the sequestration of free iron [140]. It has been also suggested that activation of the HO-system may also stimulate efflux of intracellular free iron, in that it was found to be inversely related to intracellular free iron concentrations [185]. The combination of CO, bilirubin and free iron provide antioxidative and cytoprotective effects result in the efficacy of using the HO system as a tool to combat hypertension.

5.7 Heme-L-arginate altered rate of body weight gain and fluid balance, but not food intake or wet heart weight

To assess the effect of L-NAME and heme-L-arginate on health and metabolism body weight, and food intake was monitored. Treatment with heme-L-arginate resulted in a diminished to a significant decrease in body weight at the time of sacrifice, at both 11 and 15 weeks. In order to assess the possible reason for this change, food intake was assessed. However, animals treated with L-NAME and heme-L-arginate did consume more food, indicating that the diminished rate of body weight gain was due to either toxicity or change in metabolism. While time did not allow for assessment of either of these parameters recent published data has shown that after chronic treatment with hemin, another heme substrate, no markers of liver toxicity were identified while reduction in insulin signaling was noted indicating

increased insulin sensitivity [169]. This data suggests that heme substrates, including heme-L-arginate, may have the ability to alter metabolism and consequently affect body weight.

Additionally, throughout the study water intake and urine output were monitored in order to observe any alterations in fluid balance that may be caused by either L-NAME and/or heme-L-arginate. During the development of L-NAME induced hypertension there were some significant alterations in water intake at weeks three and four. Water intake was reduced when compared to baseline water intake which may be a result of diminished thirst induced by L-NAME as blood pressure rose noted in previous study [186, 187]. Interestingly, the addition of heme-L-arginate does not further modulate water intake until the fourth week of treatment at which point it is significantly reduced. This significant reduction may be attributed to reduction of Ang II and possible subsequent diminished thirst and altered fluid balance. In addition to gain a better understanding of the fluid balance urine output was assessed. However, urine output was unchanged from baseline values for the duration of the study, during the development of L-NAME induced hypertension and subsequent co-administration of heme-L-arginate. Treatment with heme-L-arginate did however reduce urine output after two weeks compared to age matched animals treated with L-NAME alone, but the trend did not continue in the rest of the study.

The presence of hypertrophy in L-NAME-induced hypertension is controversial [172, 188, 189]. In this study, wet heart weight was obtained to assess for hypertrophy. Evaluation of heart weight alone as a measure of hypertrophy showed only slight reduction in wet heart weight in heme-L-arginate treated animals compared to all other groups. However, when the heart weight-to-body weight ratio was calculated the animals co-treated with heme-L-arginate and L-NAME had significantly increased values. This is due the significant diminished rate of body weight gain with heme-L-arginate treatment and subsequent reduction in body weight at the end of the study. Unfortunately, we were unable to assess for changes in cardiac and vascular

remodeling. Studies have documented modulation in fibrosis and collagen deposition after chronic L-NAME administration [130, 174]. Interestingly, heme substrates have proven to be effective in abrogating both hypertrophy and remodeling in different animal models [161, 190]. This area will require further study in the future to elucidate the effect of heme substrates on hypertrophy and remodeling in L-NAME-induced hypertension.

5.8 Limitations of this Study

In this study there were several limitations. Firstly, one limitation was the indirect measurement of blood pressure using the standard non-invasive tail cuff method. Direct measurement of blood pressure uses an implanted radiotelemetric device allowing measurement while the animal is ambulatory. In comparison, the standard non-invasive tail cuff method requires the animal to be restrained and warmed. These requirements may result in a stressed animal and subsequent elevation in blood pressure. Also, this method also only obtains a very small sample of cardiac function compared to that of direct methods. However, following several guidelines can virtually eliminate these factors [191]. Proper acclimatization of the animal to the restrainer and proper temperature control may reduce an animal's stress response. In order to obtain accurate results it is critical to not only acclimatize animals, but use the proper size of tail cuff depending on the size of the animal. Also, having the same researcher measure blood pressure at the same time of day in a quiet, darkened room throughout the study will ensure further consistency of results. In addition, cleaning the equipment, including the restrainer, to remove possible blood or foreign scents may reduce animal stress and result in more consistent measurement of blood pressure. In this study all of the above considerations were taken.

Also, in this study only systolic blood pressure was measured. Measuring diastolic blood pressure and/or mean arterial pressure (MAP) would have also been valuable. Measuring

diastolic blood pressure in addition to systolic blood pressure would allow for the determination of pulse pressure and estimation of MAP (MAP≈ $P_{diastolic}$ + 1/3 ($P_{systolic}$ – $P_{diastolic}$). MAP is another important measurement to assess systemic vascular function and is affected by important vascular factors such as cardiac output, systemic vascular resistance, and central venous pressure. Both measurements would allow further indirect assessment of arterial changes induced by L-NAME and heme-L-arginate.

As noted several times throughout this document, variation in sample size occurred both in and between samples. This is partially due to extra animals shipped with order, but also due loss of animals during the study. These losses may be due to sustained hypertension which is known to increase morbidity and mortality. During molecular analysis several factors impacted sample size. When analyzing HO-1 expression through western blot, I had a great degree of difficulty due to impure water that was not previously detected as well as poor antibody quality due to prolonged exposure to higher temperatures than recommended due to personnel issues. Issues due to the strike also affected the Ang II and TAC results due to issues with shipping.

Adding to these difficulties was the short amount of time given to complete my studies due to funding difficulties. This also hindered my ability to measure a number of markers like HO-1 activity, other components of the RAS, NO availability and other indicators of oxidative stress that would have been helpful in assessing to what extent heme-L-arginate impacts L-NAME induced hypertension.

6. CONCLUSIONS

The results from this study indicate that heme-L-arginate therapy might prove to be useful in combating hypertension. Co-treatment with heme-L-arginate, alongside of L-NAME, prevented the development of hypertension. Interestingly, treatment with heme-L-arginate after the establishment of L-NAME induced hypertension effectively lowered mean systolic blood pressure back to that of normotensive animals. Reduction in hypertension was also accompanied with elevation of HO-1 expression, indirectly indicating upregulation of the HO system. Along with HO system activation, reduction in local heart Ang II levels was seen. Importantly, heme-L-arginate therapy significantly abrogated oxidative stress reflected in the elevation of TAC.

7. Perspectives

Based on literature, it can be speculated that by providing a heme substrate, such as heme-L-arginate, results in the stimulation the HO-system and the catabolism of heme. Together the products of the heme catabolism contribute to cytoprotection, decreased inflammation and a reduction in hypertension. CO is an important vasodilator which acts to abrogate hypertension [145]. In particular, HO-1 derived CO mediated vasorelaxtion has been shown repeatedly [148]. While mechanisms are not clearly elucidated, CO is known to activate BK_{Ca} and to stimulate cGMP, both of which contribute to vasodilation [141]. Additionally, CO in co-ordination with bilirubin, inhibits NAD(P)H-oxidase which contributes to overall lower oxidative stress [149]. Bilirubin further decreases oxidative stress by scavenging H_2O_2 and O_2^- [135]. Oxidative stress is also combated by HO-1 derived free iron, its stimulation of ferritin synthesis and its subsequent sequestration of cellular free iron [134]. As a whole, the HO-pathway leads to increased TAC by reducing the amount of pro-oxidants in addition to increasing the supply of antioxidants. Oxidative stress is lowered in heme-L-arginate treatment along with its damaging inflammatory processes.

8. FUTURE DIRECTIONS

1. This study showed the promising effect of heme-L-arginate in L-NAME-induced hypertension model for the prevention and treatment of hypertension. However, its mechanisms need to be clearly elucidated including how it affects SNS activity.

2. Decreases in the tissue levels of angiotensin-II were noted in heart tissue, possibly due to the activity of the HO system. Further exploration of Ang II and other components of the RAS need to be evaluated in other tissues, including kidney and other vasculature.

3. Increased expression of HO-1 in the heart was observed in the present study. Further investigation of HO-1 distribution is necessary. The effect of the upregulation of HO-1 and the related mechanisms behind the decrease in systolic blood pressure, including HO activity, sGC and cGMP activity also need to be explored to more concretely establish the effect of the HO system.

4. It needs to be determined if the effect of heme-L-argninate on L-NAME-induce hypertension is in part because of the NO derivative L-arginine working in cooperation with the HO system.

5. The characterization of different molecular changes within the HO system, the remodeling and hypertrophic processes including markers of extracellular matrix remodeling and fibrosis needs to explored.

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