

SYMPATHETIC NERVOUS SYSTEM IN THE DEVELOPMENT OF MILD DOCA-SALT
HYPERTENSION

By

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ABSTRACT

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Understanding mechanisms leading to essential hypertension has been challenging even after several decades of research. Long-term regulation of arterial pressure is generally linked to the ability of the kidneys to maintain volume homeostasis by altering the renal excretion of salt and water in response to changes in arterial pressure. But there is also compelling evidence for a role of the central nervous system in long-term regulation of arterial pressure, primarily by modulating sympathetic nerve activity. Sympathetic nervous system activation contributes to the pathogenesis of human hypertension. Recent evidence points to mineralocorticoids as a cause of sympathetic overactivity in hypertension. In the studies for this dissertation, low dose deoxycorticosterone acetate along with high salt in drinking water with both kidneys intact was used as a model (mild DOCA-salt hypertension model) to understand the role of the sympathetic nervous system in hypertension caused by mineralocorticoids. My studies indicate hypertension development is not associated with global increases in sympathetic activity. Contrary to the classical thought about importance of renal nerves, I found that renal nerves are not essential for hypertension development. In fact, my studies indicate that regional sympathetic activity specifically to splanchnic vascular bed

is required for full development of hypertension. I concluded that sympathetically mediated vasoconstrictor effects to the splanchnic organs are likely enhanced in mineralocorticoid hypertension due to increased responsiveness of the splanchnic blood vessels to norepinephrine. The data in this dissertation suggest that splanchnic sympathetic activity and splanchnic vascular reactivity are important in regulation of blood pressure in mineralocorticoid-salt induced hypertension.

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LIST OF ABBREVIATIONS

AR	adrenergic receptor
ATP	Adenosine-5'-triphosphate
CG	celiac ganglion
CGX	celiac ganglionectomy
CNS	central nervous system
DOCA	deoxycorticosterone acetate
ET	endothelin
FX	fractional extraction
HR	heart rate
IP	intraperitoneal
IV	intravenous
MAP	mean arterial pressure
MR	mineralocorticoid receptors
NE	norepinephrine
NPY	neuropeptide Y
PA	primary aldosteronism
RDX	renal Denervation

SC	subcutaneous
SHR	spontaneously hypertensive rat
SNA	sympathetic nerve activity
SNS	sympathetic nervous system
TPR	total peripheral resistance

INTRODUCTION

1. Essential hypertension

Hypertension is a major public health problem irrespective of age, race, or gender; it affects approximately 50 million individuals in the United States and approximately 1 billion worldwide (Chobanian et al., 2003b). Hypertension is a condition featuring persistent and non-physiologic elevation of arterial blood pressure. The operational definition of hypertension by the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure is systolic blood pressure of 140 mmHg or higher and/or diastolic blood pressure of 90 mmHg or higher (Chobanian et al., 2003b). Hypertension is a major risk factor for all forms of thrombotic and atherosclerotic cardiovascular disease. Even within the so called “normal” range, higher blood pressure is associated with increased risk for cardiovascular and non-cardiovascular disease in a continuous and graded fashion. High blood pressure increases risk for total mortality, cardiovascular mortality, heart failure, myocardial infarction, stroke, atrial fibrillation, left ventricular hypertrophy, and renal failure.

The etiology of 90-95% cases of hypertension remains unknown. This form of hypertension is called primary or essential hypertension. The other form making up 5-10% of all cases is referred to as secondary hypertension. Unlike essential hypertension, secondary hypertension has an identifiable cause. Some of the identifiable causes of secondary hypertension include sleep apnea, renovascular

disease, chronic renal disease, primary aldosteronism, pheochromocytoma, Cushing's syndrome, chronic steroid therapy, thyroid or parathyroid disease, coarctation of the aorta, and drug-induced hypertension (Chobanian et al., 2003a).

After several decades of research, the exact cause of hypertension is not known. Even though blood pressure is influenced by many factors, they may or may not serve as the mechanisms involved in the long-term regulation of blood pressure. A general thought is that mechanisms regulating blood pressure must have the ability to sense or detect changes in blood pressure and also the ability to correct these changes. Currently there are two major hypotheses about long-term pressure regulation. One is that the kidneys regulate blood pressure by adjusting blood volume via the pressure-diuresis mechanism. The other hypothesis is that the brain senses changes in blood pressure and modifies pressure primarily by adjusting sympathetic outflow to peripheral organs. The former hypothesis is based on the concept that changes in arterial pressure (AP) are detected by the kidney, and that these changes cause the kidney to excrete a volume of urine equivalent to daily water intake, thus maintaining total body fluid homeostasis (Coleman et al., 1975; Guyton, 1989). According to this concept, the only way the blood pressure can change over a long period of time is when the renal function curve (that relates AP to urinary volume output) is shifted to a higher AP. Such a shift in the renal function curve (caused for example by hormones, sympathetic activity or loss of renal mass) results in water (and sodium) retention and increases blood volume. This causes an increase in cardiac output and blood pressure, and restoration of fluid homeostasis (Guyton, 1989). The main argument against this concept is that total blood

volume is normal or reduced in established hypertension in humans (Ulrych et al., 1969; Schmieder et al., 1995) and in experimental hypertension (Ackermann and Tatemichi, 1983; Evenwel et al., 1983). However, the theory also states that whole-body autoregulation, produced by adjustments of blood flow within individual vascular beds in response to changes in tissue perfusion, can convert increased cardiac output into the raised total peripheral resistance (TPR) that is typically observed in hypertension. This could produce sustained increases in AP even in the face of reduced total circulating blood volume (Guyton, 1989).

The other school of thought is that blood pressure is regulated by the central nervous system (CNS), mainly but not exclusively by modulating efferent sympathetic nervous system activity (SNA) (Osborn et al., 2005). The CNS consists of a network of neurons that interprets and responds to various humoral and neurochemical signals. The CNS 'senses' factors that can influence blood pressure – extracellular fluid volume, osmolality, concentration of circulating hormones – and most of these factors are in turn affected by changes in pressure (Sved et al., 2000; Osborn et al., 2005; Guyenet, 2006). Also, one mechanism by which AP is sensed by the CNS is through the baroreceptors. Cardiopulmonary baroreceptors in the heart and great vessels, and arterial baroreceptors in the aortic arch and carotid sinus, respond to changes in vascular stretch and thereby detect changes in AP. Increased AP causes an increase in afferent signals from the baroreceptors to the CNS, leading to feedback inhibition of efferent sympathetic tone (Sved et al., 2000; Guyenet, 2006). This sympathetic-

baroreflex feedback loop was once believed to be incapable of long-term blood pressure regulation because of its tendency to “reset” (i.e. exhibit gradually reduced feedback responses during sustained elevations in AP) (Igler et al., 1981; Cowley, 1992). However, recent studies have proved otherwise (Lohmeier et al., 2004; Thrasher, 2005). In a recent review, Osborn proposed that a central baroreceptor also exists that is capable of sensing blood pressure within the CNS (Osborn, 2005). Regardless of the way CNS senses blood pressure, it is now generally agreed that the sympathetic nervous system plays an important role in the long-term regulation of blood pressure (Esler, 2000; Guyenet, 2006).

2. Sympathetic nervous system and control of blood pressure

The sympathetic preganglionic neurons arise in the ventral intermediolateral columns of the spinal cord in the thoracic and lumbar regions and synapse in the prevertebral and paravertebral sympathetic ganglia. Sympathetic post-ganglionic fibers innervate target organs and release norepinephrine (NE) at nerve terminals upon activation (Elfvig et al., 1993). In the heart, NE acts on β -adrenergic receptors to increase rate, myocardial contractility, electrical conduction velocity and the rate of myocardial relaxation. NE also causes vasoconstriction by acting on α -adrenergic receptors. Arterial constriction leads to an increase in total peripheral resistance. Constriction of veins decreases venous compliance and capacitance, and redistributes blood out of the venous circulation into the arterial circulation thereby increasing the total amount of blood in the arterial circulation. Sympathetic overactivity has been implicated in the pathogenesis of

hypertension. Evidence includes the following: 1. Successful therapeutic use of central and peripheral sympatholytic drugs and adrenergic receptor blockers to lower blood pressure in hypertensive humans (Guthrie et al., 1984; Izzo et al., 1987; Schulte et al., 1987). 2. Surgical interventions to sympathetically denervate organs attenuate hypertension in experimental models and humans (Grimson et al., 1949; Jacob et al., 2005; King et al., 2007; Krum et al., 2009). 3. Increased plasma NE and total-body NE spillover levels (indicative of elevated sympathetic activity) in hypertensive individuals and experimental models of hypertension (Cabassi et al., 2002; Schlaich et al., 2004; King et al., 2008). But the most conclusive evidence comes from the direct single unit and multi-unit recordings of SNA in human hypertensive patients (Grassi et al., 1998; Greenwood et al., 1999), where increased SNA is consistently found within at least a subset of patients. SNA is regionally differentiated, with activation in one region sometimes accompanied by inhibition or no change in others (Esler, 2009). For example, radioisotope dilution measures of NE spillover from heart and kidneys are increased in human hypertension (Esler et al., 1984a; Esler et al., 1989; Esler, 2000). Experimental work also has implicated increased sympathetic outflow to kidneys, heart, and splanchnic organs (Bell and McLachlan, 1979; Jacob et al., 2005; King et al., 2007). The importance of regionally specific increases in sympathetic activity as a cause of hypertension was highlighted by a recent study performed in treatment-resistant, human hypertensive subjects. Catheter-based renal denervation proved successful in bringing about a sustained reduction (over one year) in blood pressure and muscle SNA (Krum et al., 2009). When combined with the voluminous literature on the effects of renal denervation in experimental hypertension (DiBona and Kopp, 1997; DiBona and Esler,

2010), this result suggests a compelling way to link the two main theories of hypertension pathogenesis, i.e. impaired renal function and sympathetic overactivity.

The experimental work described in this thesis is predicated on this idea that *non-uniform changes in sympathetic outflow are critical to understanding the pathogenesis of neurogenic hypertension and identifying better strategies for its treatment or cure.*

Multiple mechanisms probably cause increased SNA in essential hypertension, including diminished arterial baroreflex buffering of sympathetic nerve traffic (Matsukawa et al., 1991). It is believed that SNA is partially heritable. Skeletal muscle sympathetic nerve firing rates were found to be almost identical in monozygotic twins (Wallin et al., 1993). Also, normotensive young men with a family history of hypertension have higher NE spillover rates than young men with a negative family history of hypertension (Ferrier et al., 1993). Some specific genes regulating SNA have been identified (Milsted et al.; Beetz et al., 2009; Grassi, 2009; Ueno et al., 2009), but their individual contribution to overall SNA in humans is small. There is also substantial evidence that stress is related to increases in SNA in human hypertension (Sherwood et al., 1995). For example, it has been demonstrated in epidemiological studies that migration causes elevation in blood pressure (Poulter et al., 1990). A particularly important and prevalent factor associated with increased SNA in humans is increased energy storage as fat. Landsberg (Landsberg, 1986) hypothesized that increased dietary intake results in positive energy balance which in turn stimulates SNA and elevates AP. Calorie restriction has shown to reduce SNA and blood pressure (Jung et

al., 1979). Interestingly, there is selective activation of sympathetic outflow to the kidneys and skeletal muscle vasculature in even normotensive, obese humans, but suppression of cardiac SNA (Vaz et al., 1997). However, in obesity-related hypertension, there is increased renal SNA without suppression of cardiac SNA. In fact, cardiac SNA is more than twice the level found in normotensive obese subjects and about 25% higher than in healthy individuals (Rumantir et al., 1999). Finally, there is a possible link between physical inactivity and increased SNA in hypertensive patients. It has been reported that the antihypertensive effect of exercise is most probably caused by inhibition of the SNS, especially in the kidneys (Jennings et al., 1986; Meredith et al., 1991).

Circulating hormones and other endogenous substances can affect blood pressure and SNA. Angiotensin II is linked to the development of hypertension and sympathoactivation by its actions in the CNS (Ferguson and Washburn, 1998; Osborn et al., 2007). Increased circulating leptin levels are associated with sympathetic activation in obesity-related hypertension (Dunbar et al., 1997; Rahmouni and W, 2002). Chronic inflammation is also linked to hypertension development because plasma cytokine levels are elevated in hypertensive individuals (Bautista et al., 2005). For example, data suggest that IL-6 is associated with, and often predictive of hypertension development with increased serum IL-6 levels (Stumpf et al., 2005). Also, insulin-mediated sympathetic activation is reported to be associated with essential hypertension (Lembo et al., 1992; Landsberg, 1996).

Finally, it is important to note that sympathetic augmentation can occur in hypertension even in the presence of *normal* sympathetic activity. That is, during the process of sympathetic neurotransmission at the neuroeffector junction there can be a functional uncoupling between nerve firing rate and NE release, or between NE release and NE concentration at the receptor, or between NE concentration at the receptor and end organ response (Esler et al., 1990).

3. Sympathetic neurotransmission to the vasculature

The postganglionic nerves supply visceral structures of the thorax, abdomen, head, and neck. These nerves release NE, ATP (Adenosine-5'-triphosphate) and neuropeptide Y (NPY) at their nerve terminals (Goodman et al., 2006). In the preganglionic neuron, tyrosine, which is a precursor of NE, is sequentially hydroxylated and decarboxylated to form dopamine by the actions of tyrosine hydroxylase and DOPA decarboxylase respectively. The hydroxylation step is the rate limiting step in the biosynthesis of NE (Zigmond et al., 1989). Dopamine then enters small vesicles via vesicular monoamine transporter-2 (Schuldiner, 1994). Inside the vesicle, dopamine is converted to NE by the action of dopamine β -hydroxylase and is stored as granules. Vesicles also contain ATP and NPY, in the same or different vesicles (Racchi et al., 1999). Increase in intracellular Ca^{2+} due to generation of action potential occurs via voltage gated Ca^{2+} channels and is necessary for NE release. Once released, NE can act at various places. It acts on $\alpha 1$ - and $\beta 1/\beta 2$ adrenergic receptors (ARs) located on smooth muscle cells of the blood vessels. NE also acts presynaptically on $\alpha 2$ - and on $\beta 2$ - ARs located on the nerve

terminal to modulate its own release (Huidobro-Toro and Donoso, 2004). The majority of released NE is taken up into the nerve by the neuronal norepinephrine transporter (NET). NE that enters the cell is recycled back into the vesicle or is metabolized by mitochondrial monoamine oxidase to 3,4 dihydroxyphenylglycol or DHPG. NE is also taken up in non-neuronal tissue and is metabolized to normetanephrine by the action of catechol-O-methyltransferase or COMT (Esler et al., 1990). A small fraction of released NE escapes into the plasma and the rate of this release is referred to as NE spillover rate (Esler et al., 1990). NE spillover rate depends on the nerve firing rate, blood flow, NE reuptake, and capillary permeability to NE, and the surface area of the microcirculation available for exchange. NE spillover rate can be influenced by incongruities between sympathetic nerve firing rates and rates of NE synthesis, release, and overflow to plasma.

There are two types of α -ARs: α 1- and α 2-ARs (Goodman et al., 2006). They are expressed by vascular smooth muscle cells and the relative contribution of each receptor type to vasomotor responses is specific to the vascular bed studied. It has been demonstrated that arteries and veins express different α -ARs in the same vascular bed (Fowler et al., 1984; Itoh et al., 1987). In the murine mesenteric vasculature, veins are more sensitive than arteries to the constrictor effects of α -AR agonists and veins are resistant to desensitization by adrenergic agonists (Perez-Rivera et al., 2004), possibly because α 2-adrenoceptors potentiate constrictions mediated by α 1-adrenoceptors in mesenteric veins but not mesenteric artery (Perez-Rivera et al., 2007).

4. Aldosterone – role in sympathetic activation and human hypertension

Aldosterone is a mineralocorticoid hormone secreted by the zona glomerulosa of the adrenal cortex. First isolated in 1953, aldosterone is the major mineralocorticoid hormone in humans on the basis of its potent effects on unidirectional transepithelial sodium transport (Simpson et al., 1953; Crabbe, 1961). Aldosterone secretion is stimulated by multiple factors such as angiotensin II, adrenocorticotrophic hormone, plasma potassium, and lipid soluble factors present in adipose tissue (Sowers et al., 2009; Funder, 2010b). There is also ectopic secretion of aldosterone in the heart (Takeda et al., 2000a; Takeda et al., 2000b), blood vessels (Takeda et al., 1995), and the brain (Gomez-Sanchez et al., 1997). Aldosterone acts on target tissues via mineralocorticoid receptors (MR) located in the kidneys (Wrange and Yu, 1983), heart (Pearce and Funder, 1987), blood vessels (Funder et al., 1989), salivary gland (Sheppard and Funder, 1987), distal colon (Funder, 2010a), and regions of the brain such as the hippocampus, hypothalamic periventricular structures, and the amygdala (Gomez-Sanchez, 1997). MR are present in the cytosol and are members of the steroid nuclear transactivator family. Upon activation by aldosterone, MR translocate to the nucleus and affect gene transcription and translation of effector proteins involved in regulating tissue functions, for example sodium and potassium balance across renal tubular epithelial cells (Odermatt and Atanasov, 2009).

It has long been known that excessive endogenous secretion of aldosterone or other mineralocorticoids (Fardella et al., 2000), or administration of high doses of exogenous

MR agonists (Pirpiris et al., 1994; O'Donoghue and Brooks, 2006), cause hypertension, particularly in a setting of high intake of salt (sodium chloride). Blood pressure elevations in response to excess aldosterone have traditionally been understood to result from salt and water retention by the kidneys, leading to increases in intravascular blood volume and cardiac output (August et al., 1958; Distler et al., 1973; Tarazi et al., 1973; Montani et al., 1989). Cardiac output then usually declines while total peripheral resistance increases, perhaps due to whole body autoregulation. There is strong evidence however that effects of MR activation in the vasculature and the CNS also contribute to mineralocorticoid induced hypertension. For example, mineralocorticoids alter ion transport in vascular smooth muscle and increase contractile responsiveness to pressor agents such as NE, angiotensin II, serotonin, and tyramine even before systemic blood pressure increases (Berecek and Bohr, 1978; Garwitz and Jones, 1982). In the brain, ablation of the anteroventral third ventricle (AV3V) region prevents the development of mineralocorticoid hypertension. Also, infusion of the MR antagonist RU28318 selectively into the brain via the cerebroventricles blocks aldosterone-salt hypertension (Gomez-Sanchez et al., 1992).

Primary aldosteronism (PA) is a condition with excess circulating aldosterone due to autonomous production of aldosterone by the adrenal gland (Conn, 1960). Not so long ago, PA was considered a rare cause of hypertension, accounting for less than 1% of hypertensives (Kaplan, 1994). However, there are several groups (**Table 1**) from all around the world reporting the estimated prevalence of PA ranging anywhere between 8-32% of hypertensive patients (Stowasser, 2001). In addition, excessive activation of

MR is now recognized as an important factor in treatment-resistant human hypertension (Acelajado and Calhoun, 2010). Thus, there is renewed interest in elucidating the mechanisms by which mineralocorticoids cause hypertension. Most early workers concluded that increased SNA is not a factor in mineralocorticoid hypertension in humans (Miyajima et al., 1991; Pirpiris et al., 1994). However, a recent study showed that individuals with aldosterone-producing adenoma have sympathetic overactivity which is reversed by surgical removal of the adenoma (Kontak et al., 2010). And another recently published study showed that lowering AP in hypertensive patients with a MR antagonist causes less sympathoexcitation than when AP is lowered a similar amount with a thiazide diuretic (Wray and Supiano, 2010). Collectively these findings suggest that activation of MR leads to hypertension in humans in part by increasing SNA.

Table 1. Prevalence of primary aldosteronism among patients with hypertension.

Investigator	Country	Prevalence of primary aldosteronism
(Gordon et al., 1994)	Australia	8.5%
(Gordon et al., 1993)	Australia	12%
(Lim et al., 1999)	UK	16%
(Lim et al., 1999)	UK	14%
(Loh et al., 2000)	Singapore	18%
(Rayner et al., 2000)	South Africa	32%
(Fardella et al., 2000)	Chile	9.5%

Source: Stowasser M, J Hypertens. 2001 Mar;19(3):363-6

5. DOCA-salt hypertension

Deoxycorticosterone acetate or DOCA is a precursor of aldosterone and is commonly used to create experimental hypertension. Generally, DOCA-salt hypertension is induced by administering DOCA, along with high salt in the drinking water, to uninephrectomized rats. Because high salt intake accelerates hypertension development and produces higher absolute levels of AP when compared to results seen in animals on normal salt intake, DOCA-salt treatment is referred to as a “salt-sensitive” model of hypertension (Gomez-Sanchez et al., 1996). Hypertension can be associated with an increase in cardiac output, total peripheral resistance, or both (Miller et al., 1979; Yamamoto et al., 1983). It is important to note however that hypertension develops fully even when total peripheral resistance is prevented from increasing (Huang et al., 1992a; Huang et al., 1992b).

As noted earlier, DOCA acts in the kidneys to cause water and salt retention via increased expression of epithelial sodium channels on the luminal membranes of tubular cells in the collecting ducts (Schenk and McNeill, 1992). Along with high salt intake this results in suppression of renin release and low plasma angiotensin II levels, so DOCA-salt hypertension is referred to as “renin-independent”. The model is also described as “volume dependent” on the assumption that increased blood volume and/or extracellular fluid volume is a primary cause of the increased AP. However, more recent data do not support the idea that DOCA-salt rats have increased blood volume (Fink et al., 2000; Obst et al., 2004).

Considerable evidence suggests that DOCA-salt hypertension is caused by increased SNA or increased target organ responsiveness to normal SNA (Clarke et al., 1970; de Champlain et al., 1989). Plasma catecholamines are elevated (Bouvier and de Champlain, 1986) and ganglionic blockade causes a larger depressor response in DOCA-salt rats compared to that seen in normotensive control rats (Fink et al., 2000). Central administration of 6-hydroxydopamine prevents DOCA-salt induced rises in blood pressure and associated increases in plasma NE levels (Reid et al., 1975), suggesting that central catecholaminergic pathways are involved in the peripheral sympathoexcitation. In DOCA-salt hypertension, there is increased NE release from sympathetic nerves associated with mesenteric arteries and veins along with increased NET expression in mesenteric veins (Luo et al., 2003). Increased sympathetic neurotransmission is also found in DOCA-salt hypertension (Park et al., 2010). These data indicate that sympathetic neurotransmission is significantly altered in DOCA-salt hypertension, especially in the mesenteric vascular bed. Also, the prejunctional α_2 -ARs at the sympathetic nerve terminal mediate feedback inhibition of NE release (Langer, 1980). Data from various studies indicate that α_2 -AR function is impaired in human hypertension (Damase-Michel et al., 1992; Damase-Michel et al., 1993) and animal models of hypertension, including DOCA-salt (Tsuda et al., 1989b; Moreau et al., 1995; Zugck et al., 2003; Luo et al., 2004).

There is also considerable evidence supporting the role of vasopressin in the pathogenesis of DOCA-salt hypertension. Plasma vasopressin levels are reported to

increase 10-fold during the onset of malignant DOCA-salt hypertension (Mohring et al., 1976; Mohring et al., 1977). In vasopressin-deficient rats, DOCA-salt hypertension is attenuated compared to vasopressin-synthesizing rats (Kunes et al., 1989; Zicha et al., 1989). There is a small but significant elevation of plasma NaCl and osmolality in DOCA-salt hypertension (Simon, 2003; O'Donoghue and Brooks, 2006). This may participate in the pathogenesis of hypertension by causing vasopressin release and increased SNA (Brooks et al., 2005; O'Donoghue et al., 2006). DOCA-salt hypertension is also associated with increased endothelin release, and oxidative stress in the sympathetic nervous system. Endothelin plays an important role in the development and maintenance of hypertension in DOCA-salt model by increasing both peripheral resistance and venomotor tone (Fink et al., 2000; Johnson et al., 2001). Bosentan, an ET receptor antagonist, reduced AP in DOCA-salt hypertension and this reduction was further enhanced by administration of a vasopressin antagonist (Yu et al., 2001). ET-1 production is increased in the endothelium and the kidney in DOCA-salt hypertension, which elicits an inflammatory response by increasing oxidant stress in the vascular wall. Oxidative stress is increased in DOCA-salt hypertension and causes nitric oxide inactivation, release of inflammatory cytokines, vascular smooth muscle proliferation, and neutrophil infiltration (Beswick et al., 2001a; Beswick et al., 2001b). Superoxide is produced mainly by reduced NADPH oxidase, which in turn contributes to decreased bioavailability of nitric oxide (NO), causing endothelial dysfunction (Schiffrin, 2005). Superoxide anion production is increased in the sympathetic ganglia of DOCA-salt hypertensive animals via increased activity of NADPH oxidase (Dai et al., 2004; 2006).

However, the impact of oxidative stress on sympathetic nerve function is not yet well understood.

6. The role of vascular capacitance and splanchnic circulation in blood pressure regulation

Vascular capacitance is the relationship between contained volume and the distending pressure of a segment of the vasculature (Rothe, 1993; Pang, 2000). It is essentially the “blood holding capacity” of the vasculature (Fink, 2009). Vascular compliance is the ratio of a change in volume to a change in the transmural distending pressure (Pang, 2000) and is a measure of elasticity of the vascular bed. Since the veins have much thinner walls and larger lumens than the arteries, venous capacitance is much higher than the arteries. About 70% of the total blood volume is contained in the systemic veins (Rothe, 1986; Schmitt et al., 2002). Venous compliance is estimated to be 30 times greater than arterial compliance in humans (Gelman, 2008) so the overall vascular capacitance is largely determined by venous structure and function.

It is known that systemic vascular resistance is increased in established hypertension. However, total systemic and venous compliance are also reduced (Safar and London, 1987). Increased venous tone in the peripheral veins (outside the thoracic cavity) redistributes blood towards the heart, resulting in a transient increase in cardiac output (Rothe, 1993). This shifts a small quantity of blood into the arterial circulation, thereby

leading to an increase in AP. This decrease in vascular compliance is most marked in the splanchnic circulation (Nyhof et al., 1983; Fink, 2009).

The splanchnic vasculature contains about 25% of the total blood volume and is richly innervated by sympathetic nerves (Greenway, 1983; King et al., 2007). Sympathetically mediated venoconstriction is an important determinant of splanchnic and global venomotor tone (Rothe, 1993; Pang, 2001). Veins are more sensitive to sympathetic stimulation than are arteries (Hottenstein and Kreulen, 1987; Luo et al., 2003). Therefore, even small changes in SNA that do not affect arterial function can significantly affect splanchnic vascular capacitance and cause a change in blood volume distribution. Studies in anesthetized cats demonstrate that the splanchnic vascular bed mobilizes about 65% of the blood volume lost during hemorrhage and pools the same amount if excess blood is infused (Greenway and Lister, 1974). This is consistent with splanchnic nerve stimulation studies showing increased mobilization of blood from splanchnic vascular bed causing an increase in portal venous pressure, cardiac output and blood pressure (Greenway and Innes, 1980). Even though these studies do not differentiate the individual contribution of splanchnic arteries and veins, they do suggest that control of vascular capacitance by sympathetic outflow to the splanchnic circulation is critical in blood pressure regulation.

Regulation of the splanchnic circulation by SNA is important in human hypertension, as shown by studies demonstrating that splanchnic nerve section is an effective means to

lower blood pressure in hypertensive patients (Grimson et al., 1949; Grimson et al., 1953). A critical experimental proof-of-principle study was conducted in 1953: it demonstrated that chronic stimulation of splanchnic nerves in conscious dogs could produce sustained hypertension (Kubicek et al., 1953). Venomotor tone is elevated in DOCA-salt, SHR, angiotensin II-salt and several other experimental models of hypertension (Yamamoto et al., 1981; Martin et al., 1998; Fink et al., 2000; King and Fink, 2006). This increase in venomotor tone is mediated, at least in part, by SNS activation (Willems et al., 1982; Fink et al., 2000; King and Fink, 2006). In DOCA-salt hypertension, the increase in venomotor tone is also mediated through the action of ET-1 on subtype A receptors (Fink et al., 2000). It has been hypothesized that a primary cause of hypertension in DOCA-salt rats is a relative redistribution of blood from the venous to the arterial circulation (Fink et al., 2000). There is some evidence that this redistribution is driven by an increase in SNA to the veins of the splanchnic organs (Xu et al., 2007).

Most of the sympathetic outflow to splanchnic organs is derived from neurons with their cell bodies in the celiac ganglion as shown in **Figure 1** (Trudrung et al., 1994; Hsieh et al., 2000; Quinson et al., 2001). The celiac ganglion is a prevertebral ganglion and has more complex presynaptic inputs and a greater degree of neuronal heterogeneity than the simpler paravertebral ganglia (Sejnowski, 1982; Carroll et al., 2004). Prevertebral sympathetic ganglia receive projections from the interemediolateral column of the spinal cord, dorsal root ganglion, the vagus nerve, myenteric ganglia, and other sympathetic ganglia (Szurszewski, 1981; Elfvin et al., 1993; Carroll et al., 2004). Prevertebral ganglia

also differ from the paravertebral ganglia in being more sensitive to development of neuroaxonal dystrophy in diabetes (Carroll et al., 2004), in part because of their increased sensitivity to oxidative injury (Low et al., 1997; Obrosova, 2002; Carroll et al., 2004). Thus, the celiac ganglion is well-positioned to act as a regulator of the intensity and pattern of sympathetic outflow to the splanchnic circulation. Importantly, celiac ganglionectomy was shown to be an effective means to lower AP in humans with hypertension (Heuer, 1936; Grimson et al., 1949; Grimson et al., 1953).

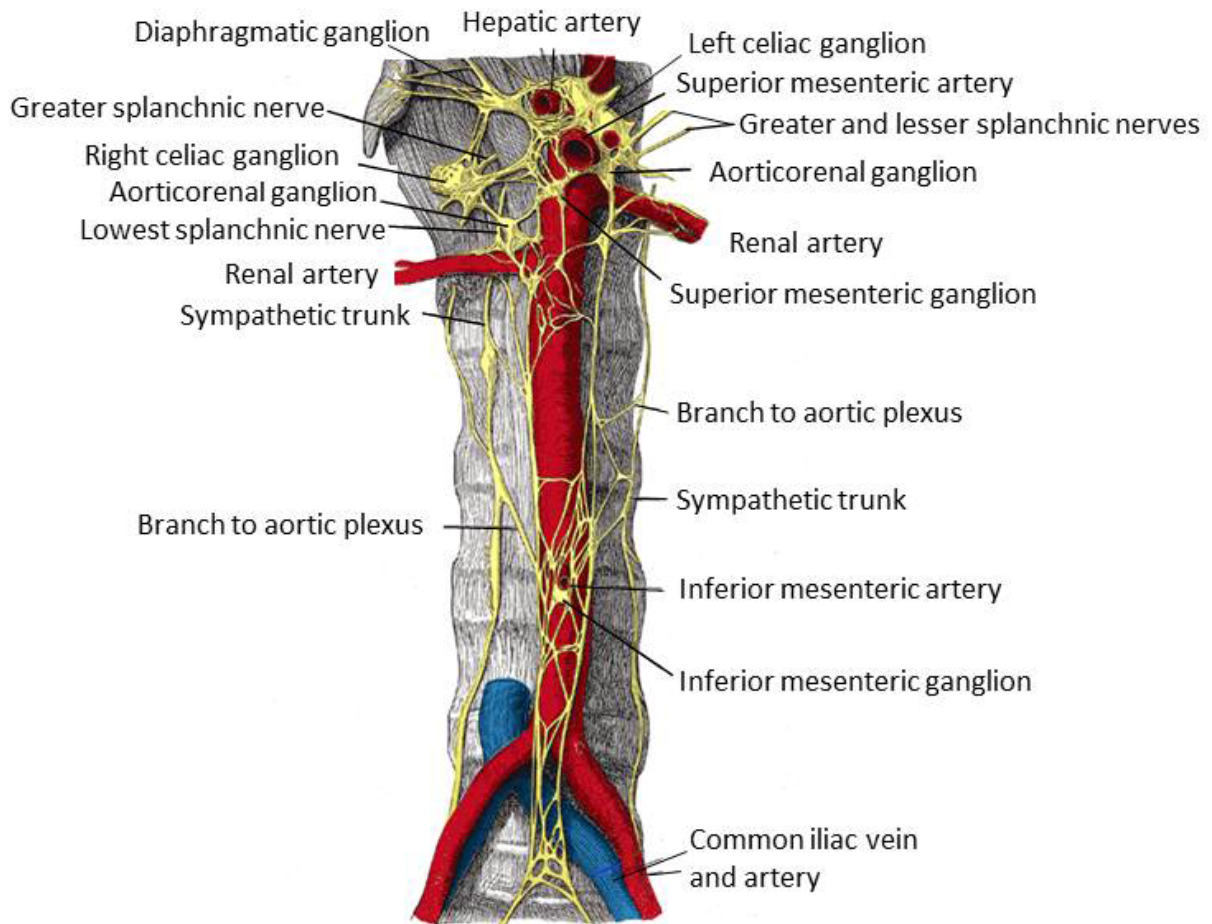


Figure 1. Abdominal portion of the sympathetic trunk showing the celiac and hypogastric plexuses.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. Source: Anatomy, descriptive and surgical by Henry Gray.

7. Mild DOCA-salt hypertension model

Administration of deoxycorticosterone acetate (DOCA) plus high salt intake (DOCA-salt hypertension) to the rat has been extensively studied as an experimental animal model of mineralocorticoid-dependent hypertension. The majority of these studies have employed an experimental paradigm consisting of a uninephrectomized rat being treated with a high dose of DOCA (150-200 mg/kg) and being given a drinking solution containing 0.9-1.0% NaCl in water (with or without supplemental potassium chloride). This model has some deficiencies when used to probe specific causes of the development of human hypertension. Severe hypertension, with MAPs in the range of 180-200 mmHg, is seen within a few weeks. Thus both the rate and magnitude of hypertension development are significantly greater than the slower developing and more modest increases in blood pressure observed in most human hypertensives—even individuals with secondary hypertension (e.g. primary aldosteronism). Very high blood pressure in the standard DOCA-salt model contributes to the rapid onset of extensive end-organ damage (Gavras et al., 1975; Wada et al., 1995). For example, glomerular sclerosis, interstitial fibrosis, cell proliferation and inflammation are seen in the kidney (Elmarakby et al., 2008; Jadhav et al., 2009). Hypertrophy and fibrosis in the heart leads to cardiac dysfunction (Loch et al., 2006). Vascular injury and inflammation occur as revealed for example by vascular fibrosis, increased endothelin and superoxide production, and reduced nitric oxide bioavailability (Fujita et al., 1995; Schiffrin et al., 1995; Beswick et al., 2001a; Xu et al., 2005). There is considerable evidence suggesting vascular remodeling in DOCA-salt hypertension (Ko et al., 2007). The media-to-lumen ratio, a marker of vascular hypertrophy, is increased in coronary

and mesenteric arteries from DOCA-salt hypertensive rats (Pu et al., 2002; Millette et al., 2003). Similar changes are also observed in the abdominal aorta and cerebral blood vessels (Takaoka et al., 2001; Dorrance et al., 2006). Typically, a significant loss of total body fat and lean body mass is seen (Titze et al., 2005). Some of these *responses* to high blood pressure (particularly vascular remodeling and impaired renal function) can become mechanisms serving to maintain or even amplify the hypertension. Moreover, the engagement of *many primary causative mechanisms* (renal, neural, and hormonal) is likely to be required to initially raise blood pressure to the very high levels seen in the model. Together these factors make it difficult to use the standard DOCA-salt model to dissect out the relative contribution of any one mechanism to hypertension development.

8. Overall hypothesis and specific aims

The overall hypothesis of this project is that sympathetic nervous system activity is elevated in the development of DOCA-salt hypertension, specifically in the splanchnic region (**Figure 2**), and that this is one cause of the hypertension.

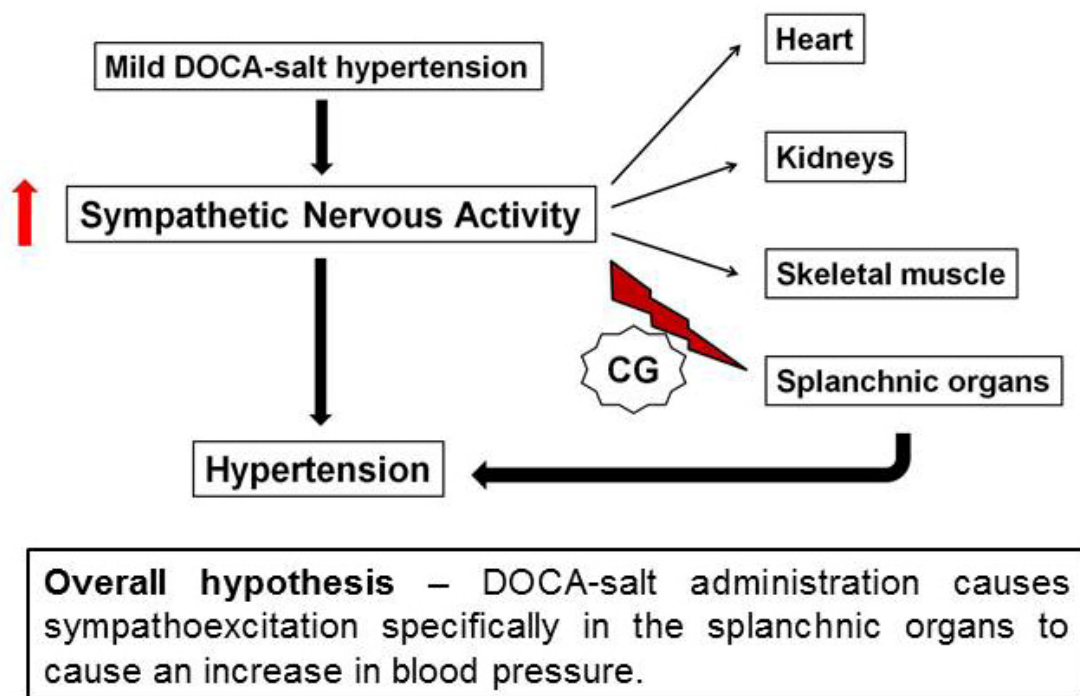


Figure 2. Overall hypothesis of the project.

The following specific aims are addressed in this dissertation to test the overall hypothesis.

Specific aim 1

Characterize an experimental model for studying mild DOCA-salt hypertension.

Specific aim 2

Estimate global SNA in the development of mild DOCA-salt hypertension using the total-body NE spillover technique.

Specific aim 3

Investigate the role of renal nerves in the development of mild DOCA-salt hypertension by selective renal denervation.

Specific aim 4

Investigate the role of splanchnic SNA in the development of mild DOCA-salt hypertension by selective splanchnic denervation.

Specific aim 5

Study the effect of celiac ganglionectomy on whole-body NE spillover.

Specific aim 6

Assess splanchnic SNA by non-hepatic splanchnic NE spillover in the development of mild DOCA-salt hypertension.

Specific aim 7

Investigate the effect of celiac ganglionectomy on non-hepatic splanchnic NE spillover.

The purpose of the work presented in this dissertation is to evaluate the mechanisms by which the sympathetic nervous system is involved in long-term control of blood pressure in a model that is closely related to human hypertension. I used a DOCA-salt rat model because an elevation in circulating mineralocorticoids has been linked to essential hypertension. Rats are the smallest species I could use to perform all the necessary physiological experiments proposed in this dissertation and there is a large body of literature on hypertension research in rat models. Because previous studies have indicated possible roles for altered vascular capacitance and splanchnic SNA in hypertension, the major focus of my work was to understand how splanchnic SNA supplying the high capacitance splanchnic vascular bed is linked to hypertension development.

EXPERIMENTAL DESIGN AND METHODS

1. Animals

Male Sprague Dawley rats (Charles River Laboratories, Portage, MI) weighing 225-275g were used for all experiments. All protocols were approved by the Michigan State University Institutional Animal Care and Use Committee. Rats were housed in a plastic cage upon arrival. Prior to any experiments, rats were acclimatized to the animal room for 7 days under controlled temperature and humidity conditions with alternate 12 hour light-dark cycle. At this time they were allowed free access to water and standard rat chow containing 0.4% sodium and 1% potassium (Harlan Laboratories, IN).

2. Anesthesia

General anesthesia was induced by placing rats in a chamber containing 4% isoflurane in oxygen, while 2% isoflurane was administered using a nose cone for maintenance of anesthesia during all surgical procedures. Toe pinch reflex, respiratory rate, and movements were used as indicators to assess the depth of anesthesia.

3. Analgesia and antibiotics

Post-surgical analgesia was achieved by carprofen (5 mg/kg, SC). Meloxicam (1 mg/kg, PO) was administered daily for 3 additional days after surgery. Ticarcillin-clavulanate (60 mg/kg, IV) and enrofloxacin (5 mg/kg, IV) were administered daily for the entire

duration of experiment to achieve anti-microbial prophylaxis in animals with chronically implanted catheters.

4. Mild DOCA-salt hypertension

Rats were acclimatized to water containing 1% NaCl and 0.2% KCl for 7 days before surgery. Under isoflurane anesthesia, rats were then instrumented with a radiotelemeter or exteriorized catheters for hemodynamic measurements, sampling, or infusion purposes. After a 7 day recovery and a 3 day control period, a DOCA pellet (50 mg/kg) was implanted subcutaneously under isoflurane anesthesia in one group of rats while the other group underwent sham implantation surgery with both kidneys left intact. During the entire experimental period, rats received standard rat chow (Harlan Laboratories, IN). The model used to induce mild-DOCA salt hypertension is shown in **Figure 3.**

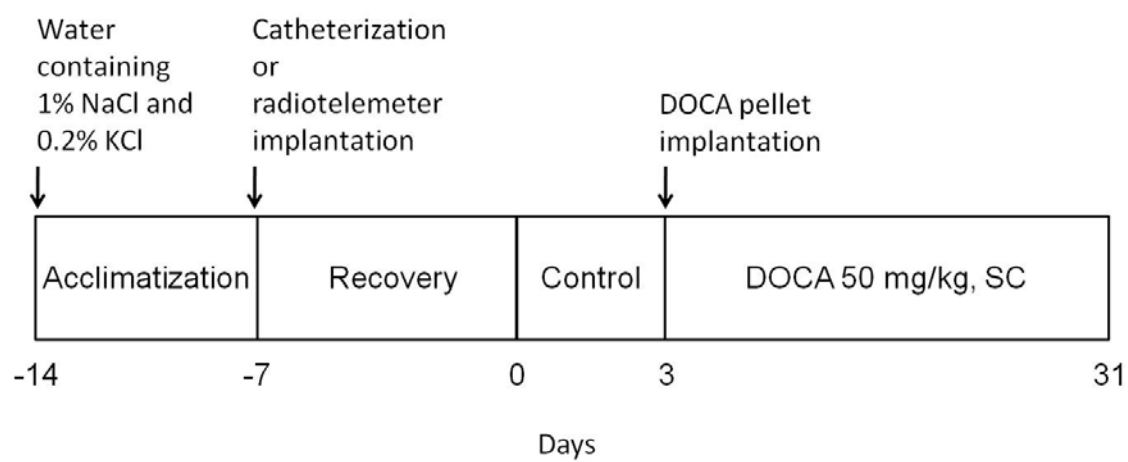


Figure 3. Mild DOCA-salt hypertension protocol.

5. Arterial catheterization

A TecoFlex® polyurethane catheter with outer diameter tapered from 0.055 in to 0.030 in and inner diameter tapered from 0.025 in to 0.015 in (Strategic Applications Inc.) was implanted into the abdominal aorta through the left femoral artery. The catheter was tunneled subcutaneously to the back and exteriorized at the neck between the scapulae. The free end was then passed through a stainless steel spring attached to the rat by a loosely fitting nylon harness (Instech Solomon). The other end of the spring was attached to a swivel to allow the rat free movement in a plastic cage. Rats were allowed free access to water and food, and allowed to recover for 7 days. The catheter was flushed and refilled daily with heparin-saline (100 U/ml).

6. Venous catheterization

6.1 Femoral vein: A silicone catheter with inner diameter 0.020 in and outer diameter 0.037 in (Dow Corning) was placed into the abdominal vena cava through left femoral vein. The catheter was tunneled subcutaneously to the back and exteriorized at the neck between the scapulae. The free end was then passed through a stainless steel spring attached to the rat by a loosely fitting nylon harness (Instech Solomon). The other end of the spring was attached to a swivel to allow the rat free movement in a plastic cage. Rats were allowed free access to water and food, and allowed to recover for 7 days. The catheter was flushed and refilled daily with heparin-saline (100 U/ml).

6.2 Portal vein: A ventral midline incision was made to expose the abdominal cavity. The intestines were retracted to one side to allow visualization of the portal vein.

A small branch of the portal vein was carefully dissected free of connective tissue. A silicone catheter with inner diameter 0.020 in and outer diameter 0.037 in from (Dow Corning) was placed into the portal vein through this branch such that the tip of the catheter was close the entry of the portal vein into the liver, without obstructing portal blood flow. The catheter was then tunneled subcutaneously to the back, exteriorized at the neck and passed through a stainless steel spring tether as described earlier. The abdominal incision was closed in layers. The catheter was flushed and refilled daily with heparin-saline (100 U/ml).

7. Radiotelemetry Transmitter Implantation

A TA11-PA-C40 radiotelemetry transmitter (Data Sciences International) was used for the measurement of blood pressure and HR in some protocols. The tip of the transmitter catheter was placed in the abdominal aorta through the femoral artery under general anesthesia. The body of the transmitter was placed in a subcutaneous pocket in the abdomen. Enrofloxacin (5 mg/kg) was administered once for anti-microbial prophylaxis and post-surgical analgesia was achieved by carprofen (5 mg/kg, SC).

8. Hemodynamic measurements

8.1 Exteriorized arterial catheters: AP was measured by connecting the arterial catheter to a pressure transducer (TDX-300, Micro-Med) that senses changes in AP and relays signals to a digital pressure analyzer (BPA-400, Micro-Med). Mean arterial

pressure (MAP), systolic pressure, diastolic pressure and heart rate (HR) were sampled at a rate of 1000 Hz. The pressure analyzer was linked to a computer where the data was analyzed by data acquisition software (DMSI-400, Micro-Med). The pressure transducers were calibrated at the beginning of the experiment using a sphygmomanometer and balanced daily against a water column located at the level of rat's heart. AP and HR were measured daily for an hour and recorded as 1 minute averages.

8.2 Radiotelemetry: The implanted radiotelemeter (TA11PA-C40, Data Sciences International) transmits signals to a plate receiver (RPC-1, Data Sciences International) placed under the rat's cage, which then relays the signals to a computerized data acquisition program (Dataquest ART 4.1, DSI). Hemodynamic measurements were sampled 24 hours a day at a scheduled sampling interval of 10 seconds every 10 minutes for the duration of the experiment. Data are reported as 24 hour averages.

9. Celiac ganglionectomy (CGX)

Laparotomy was performed via a ventral midline incision. After the abdomen was exposed, the intestines were retracted and soaked with warm saline gauze for the entire duration of surgery. The CG plexus was visualized at the junction of the aorta and celiac artery, dissected and removed. The intestines were placed back into the

abdominal cavity and lavaged with warm saline. Sham surgery was performed by exposing and visualizing the CG after laparotomy. The incision was closed in layers.

10. Renal denervation (RDX)

A ventral midline laparotomy was performed to expose the abdominal cavity. Bilateral RDX was performed as described previously by Trostel et al. (Trostel and Osborn, 1992). Briefly, renal vessels were exposed and stripped of fat, connective tissue and nerves. The vessels were then painted with 10% phenol to ensure destruction of any remaining intramural nerve fibers. Sham surgery was performed by visualizing the renal nerves after laparotomy (SHAM-DX).

11. Confirmation of denervation

Rats were euthanized with an intraperitoneal injection of pentobarbital (100 mg/kg) at the end of the experiment. Depending on the region denervated, splanchnic organs and/or kidneys were harvested from each animal, frozen in liquid nitrogen, and stored at -80°C for later analysis. Tissue NE content of the samples was measured by high performance liquid chromatography analysis with electrochemical detection as previously reported (King et al., 2007). Data are reported as nanograms of NE per gram of tissue.

12. Plasma NE measurements

Blood sampling: One ml of blood was collected from the arterial catheter into a 1ml syringe containing 25 µl of an EGTA (9 mg/ml) and reduced glutathione (6 mg/ml) solution. The blood was centrifuged at 4°C for 15 minutes at 14000 rpm and the plasma was collected and stored at -80°C until analysis.

Plasma NE: Plasma NE was measured by batch alumina extraction followed by separation using high performance reversed-phase liquid chromatography with coulormetric detection (HPLC-CD, ESA Biosciences Inc.). Quantification was accomplished using a modified method originally reported by Holmes et al. (Holmes et al., 1994).

NE extraction: In a 1.5ml plastic tube, 100 µL freshly thawed plasma, 10 mg of acid washed alumina (EcoChrom MP Alumina A, MP Biomedicals, Germany), 15 µL of DHBA internal standard and 400 µL of 2M TRIS/0.5M EDTA buffer (pH 8.1) were added. After shaking for 25 min on a vortex mixer, the samples were briefly centrifuged and the supernatant discarded. The alumina pellet was then washed with D.I. water (18 MΩ), mixed for 15 s and then again centrifuged; this step was repeated twice. Catecholamines and metabolites were then eluted from alumina with 100 µL of 0.04 M phosphoric acid - 0.2 M acetic acid (20:80, v/v). The eluate was then directly injected onto the HPLC column (10 - 40 µL injection).

High performance reversed-phase liquid chromatography with coulometric detection:

HPLC-CD was performed using a commercial system (ESA Biosciences, Inc, Chelmsford, MA) consisting of a solvent delivery module (model 584), an autosampler (model 542) cooled to 4°C and a Coulochem III detector which was equipped with a 5021A conditioning cell (electrode I) and a 5011A high sensitivity analytical cell (electrode II and III). Both cells use flow-through porous graphite electrodes. The high surface area of the detection electrodes results in an almost 100% reaction of the electroactive compound. Hydrodynamic voltammograms were obtained to determine the optimum potential for detection. The highest signal-to-noise results were obtained when the electrode I was set at +200 mV, the electrode II at +100 mV and electrode III at -280 mV. Chromatograms were obtained by monitoring the reduction current for working electrode III. The catecholamines and metabolites were separated on an HR-80 (C18, 3 µm particle size, 80 mm length x 4.6 mm I.D.) reversed-phase column (ESA Biosciences, Inc.). The mobile phase was a commercial Cat-A-Phase II (ESA Biosciences, Inc.) that consisted of a proprietary mixture of acetonitrile, methanol, phosphate buffer and an ion pairing agent (ca. pH 3.2). The optimum flow rate for the separation was 1.1 mL/min. The separation column was maintained at 35°C.

13. Total body NE spillover

Total body NE clearance and spillover were measured by an established method described previously (Keeton and Biediger, 1988; King et al., 2008). Catheters were

implanted into the abdominal aorta and vena cava through left femoral artery and vein respectively for infusion and sampling purposes, as described earlier.

Radiolabeled NE infusion: Tracer amounts of Levo-[ring-2,5,6-³H]-NE (³H-NE, PerkinElmer) were infused intravenously at $0.13 \mu\text{Ci} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ using an infusion pump to administer 16 $\mu\text{l}/\text{min}$ for 90 minutes to produce a steady-state plasma concentration of ³H-NE. The infusion solution was prepared by adding 500 μl acetic acid (0.2 mol/L), 50 μl sodium sulfite (100 mg/ml), 350 μl reduced glutathione (6 mg/ml), along with an amount of ³H-NE that depended on the weight of the rat. The final volume of the solution was brought to 10 ml by adding 0.9% saline.

Blood sampling: After achieving steady state concentration of ³H-NE by a 90-minute infusion, 1 ml of blood was collected from the arterial catheter into a 1ml syringe containing 25 μl of an EGTA (9 mg/ml) and reduced glutathione (6 mg/ml) solution. The blood was centrifuged at 4°C for 15 minutes at 14000 rpm and the plasma was collected and stored at -80°C until analysis.

Radiolabeled NE concentration: After chromatographic analysis, the NE fraction was collected and ³H-NE was quantified by liquid scintillation counting.

NE spillover: NE clearance and spillover were calculated using the following formulae (Keeton and Biediger, 1988):

NE clearance (ml/min) = $^3\text{H-NE}$ infusion rate (dpm/min)/Steady state $^3\text{H-NE}$ (dpm/ml)

NE spillover (ng/min) = NE clearance (ml/min) $\times$ Plasma NE concentration (ng/ml)

14. Non-hepatic splanchnic NE spillover

NE clearance and spillover were measured by an established method described previously (Eisenhofer, 2005). Catheters were implanted in the femoral artery, vein, and portal vein, as described earlier.

Radiolabeled NE infusion: Intravenous infusion of tracer amounts of Levo-[ring-2,5,6- ^3H]-NE ($^3\text{H-NE}$, PerkinElmer) was administered at $0.13 \mu\text{Ci} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ using an infusion pump at a rate of $16 \mu\text{l/min}$ for 90 minutes to produce a steady-state plasma concentration of $^3\text{H-NE}$. The infusion solution was prepared by adding $500 \mu\text{l}$ acetic acid (0.2 mol/L), $50 \mu\text{l}$ sodium sulfite (100 mg/ml), $350 \mu\text{l}$ reduced glutathione (6 mg/ml), along with an amount of $^3\text{H-NE}$ that depended on the weight of the rat. The final volume of the solution was brought to 10 ml by adding 0.9% saline.

Blood sampling: After achieving a steady state concentration of $^3\text{H-NE}$ by a 90-minute infusion, 1 ml of blood was collected simultaneously from arterial and portal venous catheters into two 1 ml syringes containing $25 \mu\text{l}$ of an EGTA (9 mg/ml) and reduced

glutathione (6 mg/ml) solution each. The blood was then centrifuged at 4°C for 15 minutes at 14000 rpm and the plasma was collected and stored at -80°C until analysis.

Plasma NE concentration: Plasma NE concentration was determined as previously described.

Radiolabeled NE concentration: After chromatographic analysis, the NE fraction was collected and $^3\text{H-NE}$ was quantified by liquid scintillation counting.

NE spillover: NE clearance and spillover were calculated using the following formulae (Eisenhofer, 2005).

$$\text{NE spillover} = [(F_X \times \text{NE}_A) + (\text{NE}_V - \text{NE}_A)] \times \text{PF}$$

$$F_X = \frac{{}^3\text{H-NE}_A - {}^3\text{H-NE}_V}{{}^3\text{H-NE}_A}$$

NE_A – Arterial plasma NE concentration

NE_V – Portal venous plasma NE concentration

PF – Plasma flow in the portal vein

F_X – Fractional extraction of NE during its passage through the splanchnic bed

${}^3\text{H-NE}_A$ – Arterial ${}^3\text{H-NE}$ concentration

${}^3\text{H-NE}_V$ – Portal venous ${}^3\text{H-NE}$ concentration

15. Animal euthanasia

After the completion of studies, rats were euthanized by administration of sodium pentobarbital (100 mg/kg). This approach is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

16. Data analysis and statistics

Within group hemodynamic differences were assessed by repeated measures ANOVA with Bonferroni's multiple comparisons test. Between groups hemodynamic differences were analyzed by two-way ANOVA followed by Bonferroni's test. When only two groups were compared, Student's t-test was used. A p-value of < 0.05 was considered significant. Data are presented as mean $\pm$ SEM.

CHAPTER ONE: DEVELOPMENT OF AN EXPERIMENTAL MODEL FOR STUDYING MILD DOCA-SALT HYPERTENSION

In an attempt to avoid complications related to the traditional DOCA-salt hypertension model as discussed earlier, I sought to create a modified model of DOCA-salt hypertension in which the rate and magnitude of hypertension development was reduced compared to the traditional model. Others have reported successful implementation of such models, generally by using older rats, eliminating nephrectomy and/or reducing the dose of DOCA administered (Karen et al., 1977; Bell and McLachlan, 1979; DiPette et al., 1989; Chen et al., 1996). I chose to not perform nephrectomy and use a lower dose of DOCA. I theorized that a hypertension that was more modest in magnitude and developed more slowly than in the standard DOCA-salt model would allow a more clear identification of mechanisms that serve as a primary cause of hypertension development.

Methods

Male Sprague Dawley rats (250-275 g) received free access to water containing 1% NaCl and 0.2 % KCl and were acclimatized to the housing facility for a week. Rats were divided into 7 groups (n=3). A radiotelemeter was implanted into the abdominal aorta via the femoral artery in all groups to measure MAP. Rats in one group were uninephrectomized and to create the traditional DOCA-salt hypertension model. After a 5 day recovery and a 4 day control period, DOCA-pellets were implanted

subcutaneously in all rats. DOCA was administered at doses of 5, 15, 25, 50, 100 and 150 mg/kg in 6 different groups with two-kidneys and at 150 mg/kg in uninephrectomized animals. MAP was recorded for a period of 4 weeks.

Results

MAP during the control and DOCA periods is shown in **Figure 4**. During the control period, MAP in all groups was similar. MAP increased by 15-20 mmHg compared to control period values in rats receiving 50, 100, and 150 mg/kg DOCA. There was little or no change in MAP in rats receiving 5, 15 and 25 mg/kg DOCA compared to control period values. The group with one kidney receiving 150 mg/kg had a rapid increase in blood pressure (59.22 ± 4.9 mmHg increase) which was significantly higher than the rest of the groups.

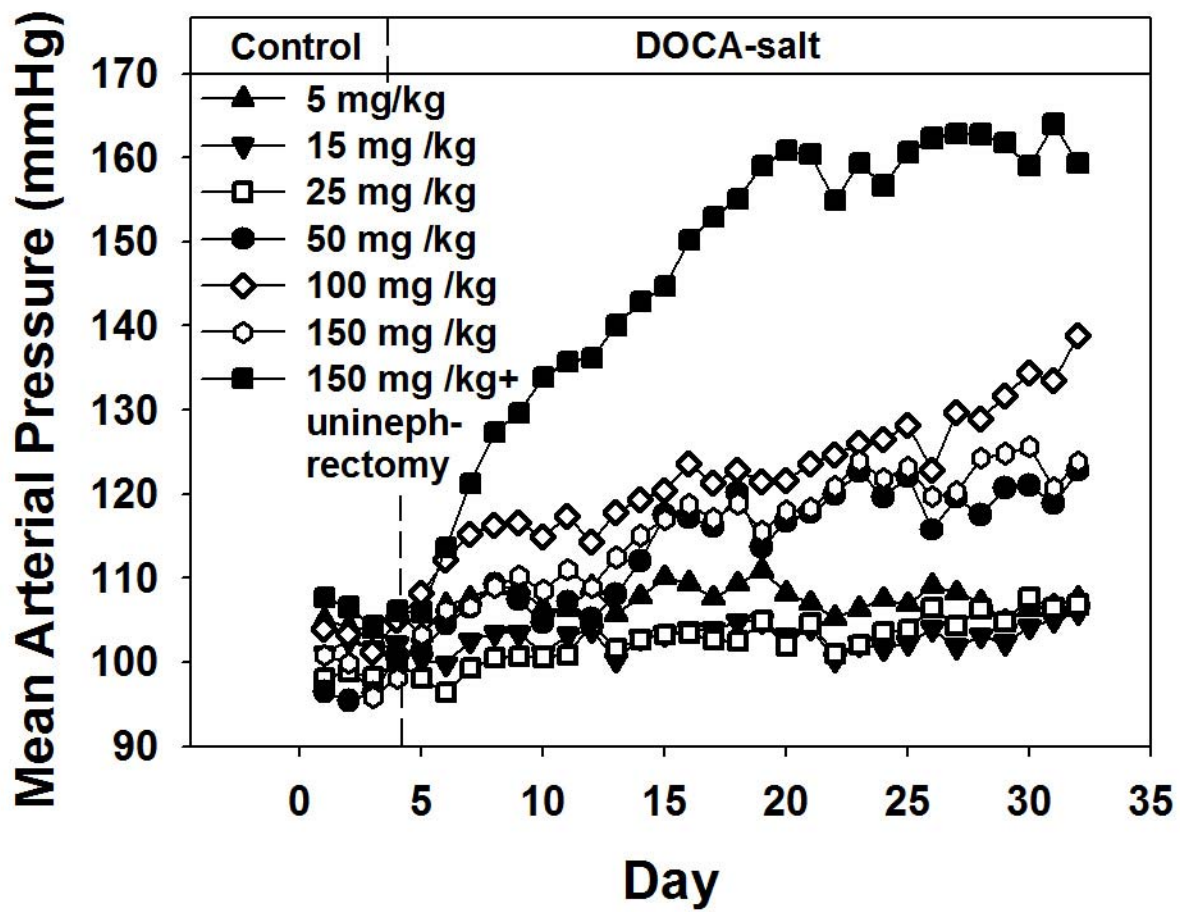


Figure 4. MAP response to different doses of DOCA (n=3).

Discussion

The results of this experiment led me to choose a 50 mg/kg dose of DOCA for inducing moderate hypertension in my additional studies. There were several reasons for choosing this dose. The most important was that it was the smallest dose required to induce a measureable elevation in blood pressure. Administration of 100 and 150 mg/kg doses of DOCA caused similar increases in blood pressure to those I observed with the 50 mg/kg dose. I chose to use the lower dose to exclude any additional actions that might occur at the higher doses but that were not required to produce hypertension. The second reason I chose to use the 50 mg/kg dose is that it caused a 15-20 mmHg increase in blood pressure. The diagnosis of hypertension is made in humans when systolic and/or diastolic AP hypertension exceeds 15 mmHg (Chobanian et al., 2003b). Furthermore, most hypertensive patients have mild elevations in AP, on the order of 15-25 mm Hg (Chobanian et al., 2003b; Egan et al., 2010). Thus, the magnitude of increase in AP I observed in rats receiving 50 mg/kg DOCA was equivalent to that seen in most humans with hypertension. Another advantage of having a mild increase in AP is that it would be less likely to induce “reactive” physiological changes, such as vascular remodeling and renal injury, that help sustain elevated AP, but obscure the true original causes of hypertension development. It is the latter that I am trying to identify in my studies. The third reason I chose the 50 mg/kg dose is that AP increased gradually; in fact AP did not show measureable increases until approximately 7 days after initiating DOCA treatment. There are two advantages to having hypertension develop slowly: it resembles the clinical scenario, where initiating causes presumably exist for months or years before hypertension is actually diagnosed; and it allows for

more ready separation of factors causing hypertension (e.g. increased SNA) from those that are instead a consequence (e.g. increased oxidative stress) of high blood pressure. Changes in causal physiological factors should be identifiable before the occurrence of frank hypertension.

OVERALL CONCLUSION: Administering 50mg/kg DOCA plus saline drinking fluid to male rats with intact kidneys produces a useful experimental model of mild DOCA-salt hypertension.

CHAPTER TWO: GLOBAL SYMPATHETIC ACTIVITY IN THE DEVELOPMENT OF MILD DOCA-SALT HYPERTENSION

A large body of evidence suggests that increased SNA is involved in the pathophysiology of hypertension, particularly in the developmental phase (Guyenet, 2006). There are many potential causes of sympathetic overactivity in hypertension, but recent studies have created renewed interest in the possibility that mineralocorticoids may be one such cause (Provoost and De Jong, 1978; Yemane et al., 2009; Kontak et al., 2010). There is compelling evidence that increased SNA contributes to traditional DOCA-salt hypertension. Plasma catecholamines are increased in DOCA-salt rats (Bouvier and de Champlain, 1986) and increased depressor responses to ganglionic blockade are seen in DOCA-salt rats compared to the normotensive control rats (Fink et al., 2000). Also, central administration of 6-hydroxydopamine, which causes destruction of central catecholaminergic neurons, prevented DOCA-salt induced rises in blood pressure and associated increases in plasma NE (Reid et al., 1975; Lamprecht et al., 1977). Finally, in one earlier study in which DOCA-salt hypertension was produced in rats with two kidneys and receiving a low dose of DOCA (similar to my model), sympathectomy prevented the development of hypertension (Bell and McLachlan, 1979). However, there is also evidence against increased SNA as a cause of DOCA-salt hypertension. For example, neonatal sympathectomy was shown to actually accelerate, rather than impair, the development of DOCA-salt hypertension (Provoost and De Jong, 1978). Also, whole body NE spillover was found to be decreased in mineralocorticoid induced hypertension in man (Pirpiris et al., 1994).

Radioisotope dilution measures of NE spillover rate into the plasma provide an accurate estimate of sympathetic nerve activity (Esler et al., 1990; Eisenhofer, 2005). Rather than the rate of release of NE from the sympathetic nerve terminals, NE spillover provides the rate at which released NE enters plasma. Thus, it factors into consideration both the release of NE and clearance before NE escapes into plasma. In order to evaluate the role of global (i.e. overall or total body sympathetic activity as opposed to regional) sympathetic activity in mild DOCA-salt hypertension, I measured whole-body NE spillover during the development of hypertension. I hypothesized that global sympathetic activity is increased during the development of mild DOCA-salt hypertension. Hence, I expected that whole body NE spillover would increase during DOCA-salt treatment.

Methods

Experimental protocol

Catheters were implanted into the abdominal aorta and vena cava through the left femoral artery and vein respectively to monitor blood pressure and HR. After a 7 day recovery from catheterization and a 3 day control hemodynamic measurement period, DOCA pellets (50 mg/kg, SC) were implanted in one group of rats while the other group underwent SHAM implantation surgery. Plasma NE, clearance and spillover were measured on control day 2, and days 7 and 14 after DOCA implantation. The experimental protocol is shown in **Figure 5**.

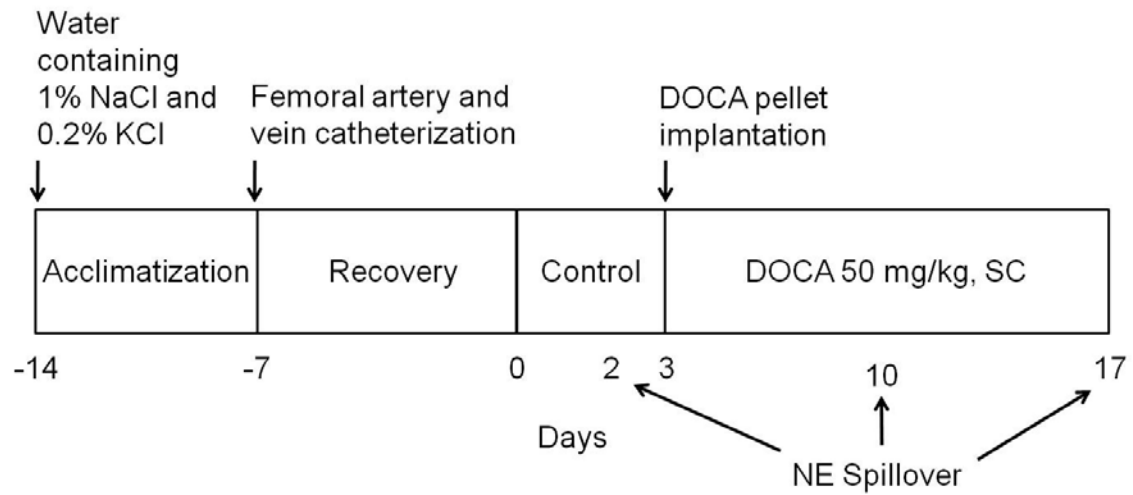


Figure 5. Experimental protocol for measuring whole-body NE spillover in the development of mild DOCA-salt hypertension.

Results

MAP and HR responses to chronic DOCA treatment are shown in **Figure 6** and **Figure 7**. During the control period, MAP was similar in SHAM (105 ± 3.3 mmHg) and DOCA (110 ± 4.3 mmHg) rats. HR was also not different between the two groups. MAP significantly increased in the DOCA group compared to the SHAM group (Day 14: SHAM = 109 ± 5.3 , DOCA = 128 ± 3.6 mmHg) during the treatment period (**Figure 6**). Changes in HR were not different between the two groups (**Figure 7**). Whole body NE spillover was measured after a 90-min infusion of ^3H -NE. This infusion did not alter blood pressures in either group. Total plasma NE concentration, NE clearance and NE spillover (SHAM: 31 ± 3 , DOCA: 39 ± 6 ng. $\text{min}^{-1} \cdot \text{kg}^{-1}$) were not different during the control period between the two groups. Total plasma NE concentration (**Figure 8**), NE clearance (**Figure 9**) and NE spillover (**Figure 10**) were not different in the DOCA and SHAM group on days 7 and 14 of DOCA treatment.

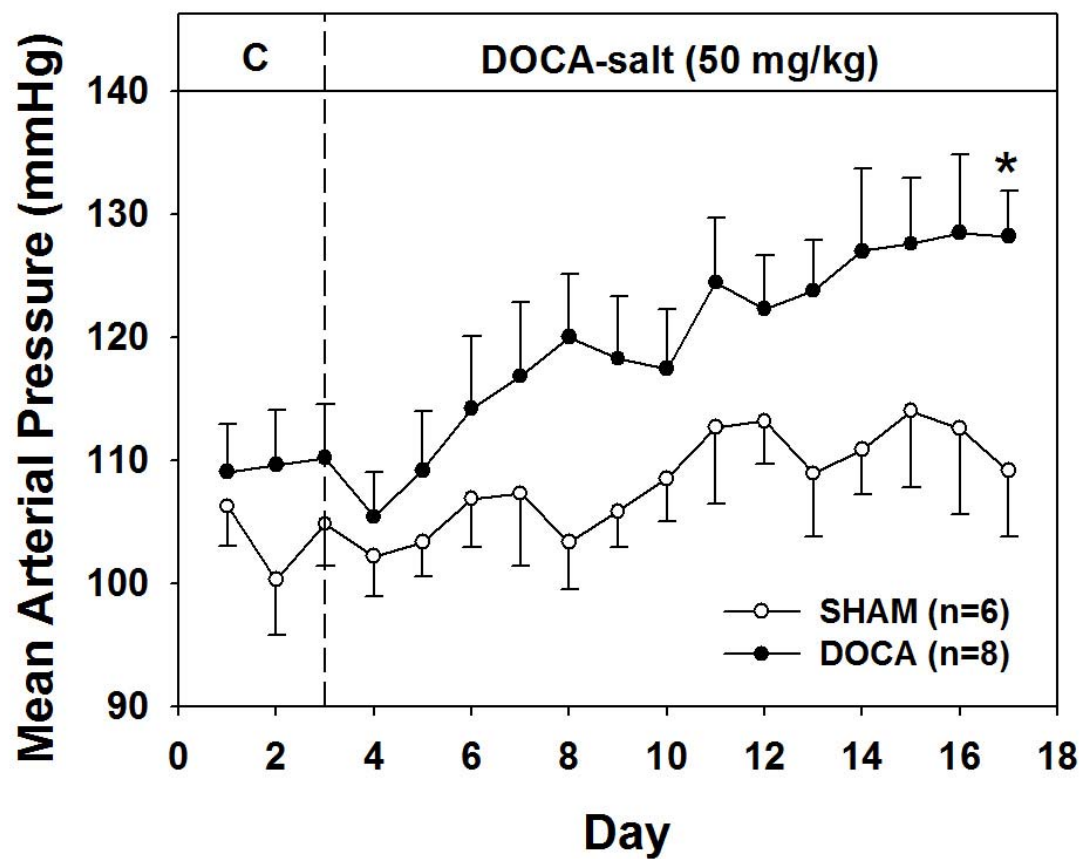


Figure 6. MAP during the control and DOCA treatment periods in SHAM and DOCA groups.

Asterisk (*) indicates a significant difference within group from day 3 (control period) values. C - control.

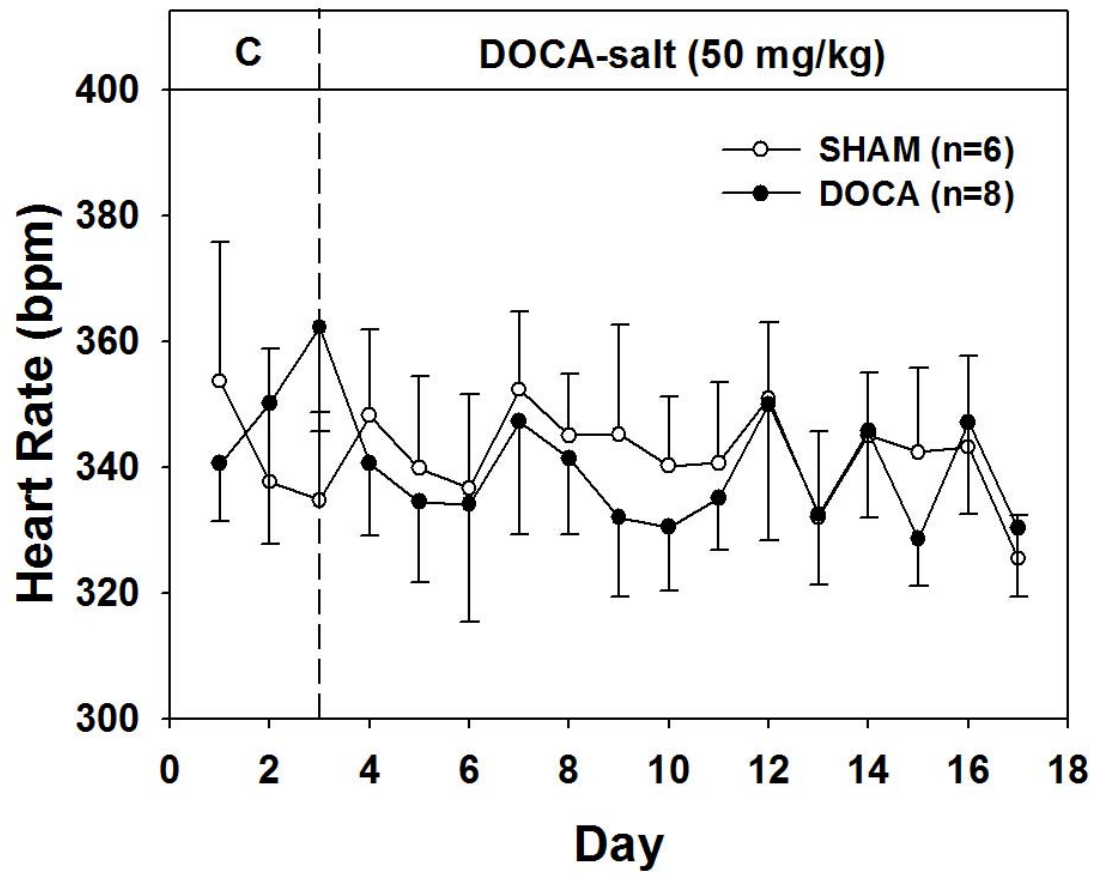


Figure 7. HR during the control and DOCA treatment periods in SHAM and DOCA groups.

C indicates control.

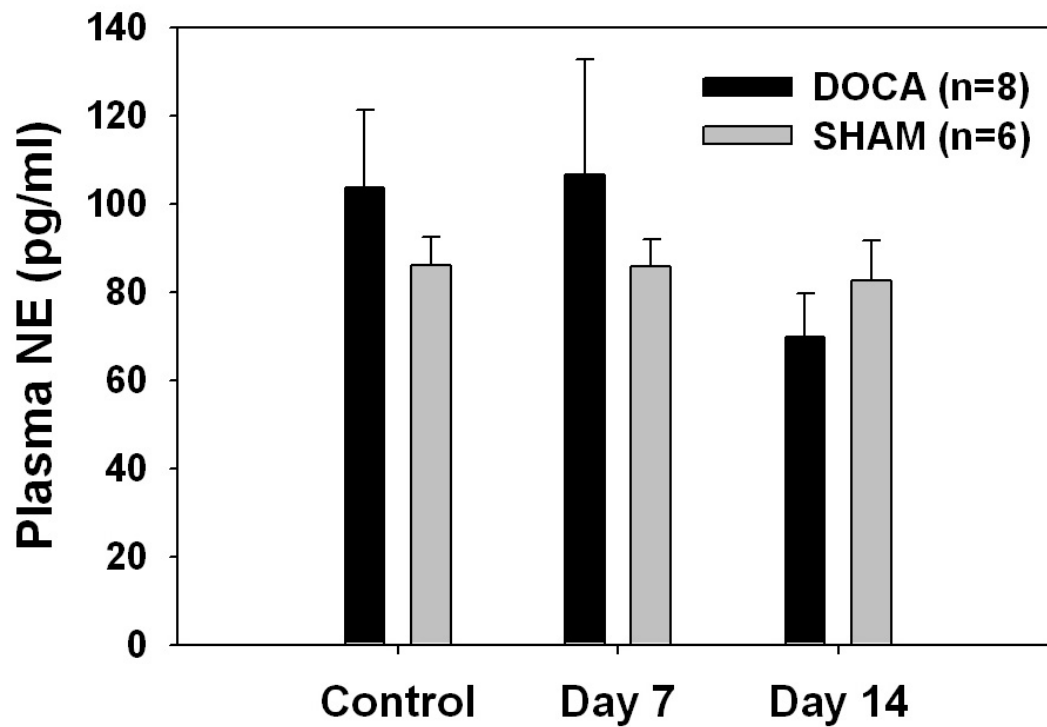


Figure 8. Plasma NE in SHAM and DOCA-salt hypertensive rats on control day 2, and days 7 and 14 after DOCA treatment.

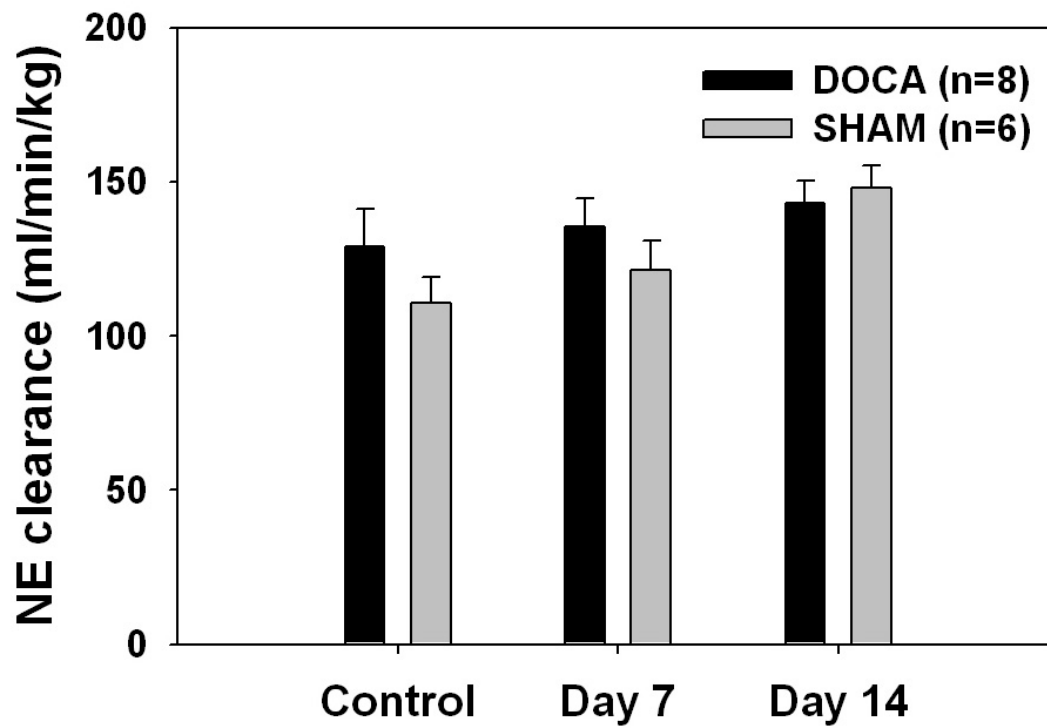


Figure 9. NE Clearance in SHAM and DOCA-salt hypertensive rats on control day 2, and days 7 and 14 after DOCA treatment.

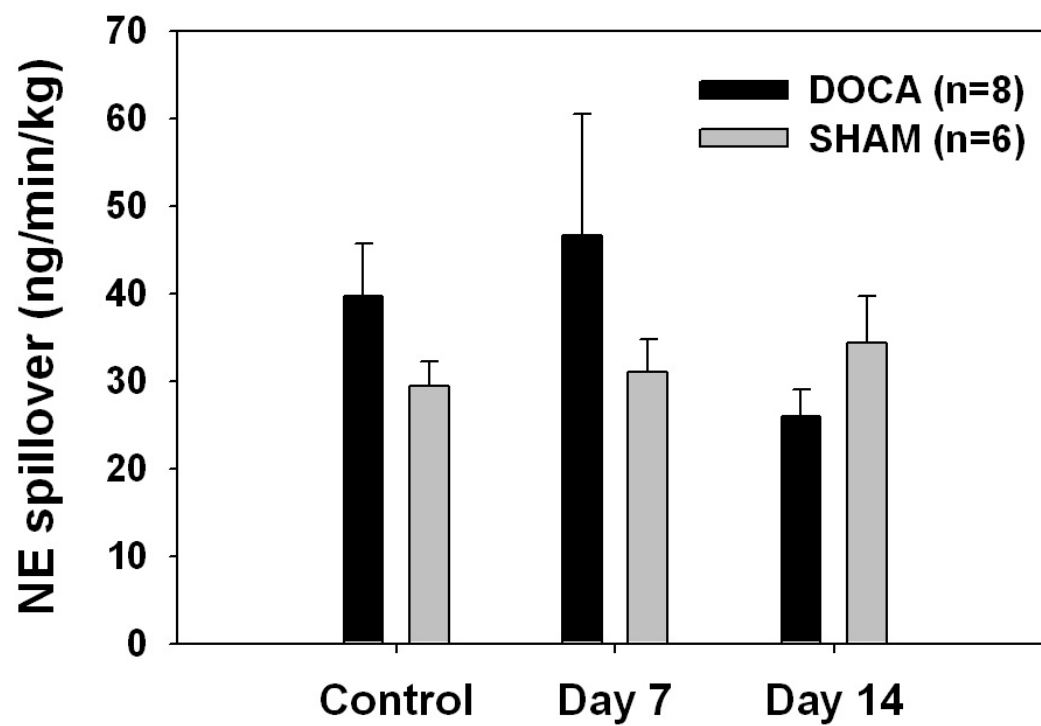


Figure 10. NE Spillover in SHAM and DOCA-salt hypertensive rats on control day 2, and days 7 and 14 after DOCA treatment.

Discussion

The major finding of this part of my project was that global sympathetic activation does not occur in mild DOCA-salt hypertension. Whole body NE spillover was not different between the hypertensive and the normotensive groups on any experimental day. Although previous work in experimental DOCA-salt hypertension suggests that sympathetic activity is increased, most of the studies were done using indirect measures of sympathetic nerve activity (Iriuchijima et al., 1975; de Champlain et al., 1987; Takata et al., 1988). For example, plasma NE was reported to be higher in DOCA-salt hypertensive rats compared to normotensive controls (de Champlain et al., 1987). I used the radioisotope dilution technique to measure NE spillover, which is a more accurate method for assessing neurotransmitter release than measuring plasma NE alone because NE spillover takes into consideration the NE release and clearance before NE enters plasma (Eisenhofer, 2005). In one previous study using the spillover technique, whole-body NE spillover was found to be elevated in anesthetized rats with standard DOCA-salt hypertension, and this increase was proportional to the increase in blood pressure (Bouvier and de Champlain, 1985). My study did not confirm this finding in conscious, unrestrained animals. One explanation could be that the model used in my study is different from standard DOCA-salt hypertension; and sympathoexcitation only occurs in the standard model. This could be due, for example, to the lower dose of DOCA I used or the presence of 2 kidneys in my model. Or sympathoexcitation could be caused by tissue injury secondary to the rapid and severe hypertension seen in the standard model. Finally, of course, it may simply be that sympathetic activity is not

increased in DOCA-salt hypertension, or that it is increased only in specific target regions.

One of the limitations of the spillover technique is that continuous measurements are not possible: it estimates sympathetic activity at a single point in time. Thus, increases in sympathetic activity occurring only at night, for example, would be missed. Nevertheless, the results are convincing because the NE spillover values were similar in the two groups on any experimental day and were quite reproducible. The results of this experiment are consistent with a study performed in conscious sheep showing that mild mineralocorticoid hypertension is not associated with global sympathoexcitation (May, 2006). Furthermore, NE spillover and muscle sympathetic nerve activity have been reported by some investigators to be decreased in humans with mineralocorticoid hypertension (Miyajima et al., 1991; Pirpiris et al., 1994). It is worth noting, however, that in one of these studies (Pirpiris et al., 1994), mineralocorticoid-induced increases in blood pressure were very modest and well within the normotensive range according to the current definition of hypertension.

The NE spillover technique estimates the release rate of NE from nerve terminals. As reviewed earlier in this dissertation, that process is determined by the net effect of 1) nerve firing rate, 2) amount of NE released per nerve impulse, and 3) amount of NE metabolized or removed from the neuroeffector junction before it can reach post-junctional receptors. There is evidence that α -2 adrenergic receptor mediated feedback

inhibition of prejunctional release of NE is impaired in hypertension, including in the DOCA-salt model. Nerve stimulation evoked NE release was enhanced in arteries from SHAM rats treated with yohimbine (α -2 adrenergic receptor antagonist), and was decreased after treatment with UK-14,304 (α -2 adrenergic receptor agonist). These effects were absent in arteries from DOCA-salt rats (Luo et al., 2004). Similarly, intravenous administration of yohimbine augmented plasma NE levels in normotensive control animals, but a similar result was not observed in DOCA-salt treated animals (Moreau et al., 1995). Alpha-2 adrenergic receptor dysfunction is also responsible for altered purinergic neurotransmission in DOCA-salt hypertension (Demel and Galligan, 2008). Thus, although it is possible that enhanced release of NE contributes to increased sympathetic pressor effects in DOCA-salt hypertension, this should have been detectable using the NE spillover method. But in my study an increase in whole body NE spillover was not found. Therefore, in mild DOCA-salt hypertension, α -2 adrenergic receptor function may be normal, or dysfunction may only occur in some vascular beds. Alternatively, α -2 adrenergic receptor function may be impaired, but this is balanced by either reduced nerve activity or increased removal of NE from the neuroeffector junction.

The majority of NE released from sympathetic nerve terminals is taken up by the prejunctional NET (Eisenhofer, 2001). Previous reports indicate that neuronal NE reuptake may be impaired in hypertension due to altered NET function (Esler et al., 1980; Esler et al., 1981; Eisenhofer, 2001). However, NET protein is elevated in the vasculature and sympathetic ganglia of DOCA-salt hypertensive animals compared to

those from normotensive, sham-operated control animals (Luo et al., 2003). Since NE clearance was not different between the hypertensive and normotensive animals in this study, it appears that NET function in mild DOCA-salt hypertensive animals was not impaired. Further studies are necessary to confirm this hypothesis. It also is important to note that there is some controversy about the relative importance of neuronal vs. non-neuronal uptake of NE (Eisenhofer et al., 1996; Eisenhofer, 2005).

Finally, it is possible that, as reported previously (Berecek and Bohr, 1978; Xu et al., 2007), vascular reactivity to NE is increased in this model and that a “neurogenic pressor effect” is occurring with normal sympathetic activity and normal amounts of NE in the neuroeffector junction. In other words, the degree to which blood pressure is affected by sympathetic activity is increased.

OVERALL CONCLUSION: Generalized sympathoexcitation is not required for the development of mild DOCA-salt hypertension.

CHAPTER THREE: ROLE OF RENAL NERVES IN MILD DOCA-SALT HYPERTENSION DEVELOPMENT

Introduction

Even if global sympathetic activity is not measurably increased, it is possible that selective increases in SNA to key target organs could be a key factor in development of hypertension. Many investigators have proposed that the renal sympathetic nerves are the most important component of the autonomic nervous system involved in the maintenance and development of hypertension. This hypothesis provides an attractive link between the two major theories for hypertension pathogenesis, i.e. renal dysfunction and sympathetic overactivity. Changes in renal SNA could affect AP regulation by influencing renal vascular resistance, renin release, and/or sodium and water balance (DiBona and Kopp, 1997). The ability of renal SNA to affect sodium and water balance is particularly key according to Guyton's well-known theory of long-term blood pressure regulation (Guyton, 1989). However, the role of the renal nerves in hypertension has been controversial. Surgical renal denervation is a commonly used technique to study the effects of the renal nerves on hypertension. Many investigators have reported that renal denervation attenuates or prevents experimental hypertension development (DiBona, 2003). For example, prior renal denervation completely prevents obesity-induced hypertension in the dog (Kassab et al., 1995). Similarly, renal denervation attenuates hypertension development in spontaneously hypertensive rats (Kline et al., 1980; Lee and Walsh, 1983; Yoshida et al., 1995). Renal NE spillover is

increased in human essential hypertension compared to normotensive controls suggesting that renal SNA is elevated (Schlaich et al., 2004). Also, it has recently been reported that catheter-based renal denervation by radiofrequency ablation caused a substantial decrease in blood pressure in human hypertensive patients with resistant hypertension (Krum et al., 2009).

Evidence against an important role for the renal nerves in hypertension comes from other studies showing no effect of renal denervation on hypertension development in various animal models of hypertension. Studies indicate that renal nerves do not contribute to hypertension development in Dahl salt-sensitive rats (Wyss et al., 1987; Osborn et al., 1988; Iwata et al., 1991). Renal denervation performed in animals with angiotensin II induced hypertension also suggest that renal nerves are not important in the pathogenesis of hypertension (Vari et al., 1987; King et al., 2007).

Osborn and colleagues demonstrated in the traditional DOCA-salt hypertension model that renal SNA is crucial for the development of hypertension because renal denervation significantly attenuated hypertension development (Jacob et al., 2005). On the contrary, Katholi et al. showed that renal nerves are only important in the early established phase of DOCA-salt hypertension and play a diminished role during the later phase (Katholi et al., 1983). Dzielak and colleagues concluded that intact renal nerves are not necessary for the development or maintenance of DOCA-salt hypertension in rats (Dzielak and Norman, 1985).

Not only are the efferent renal nerves implicated in regulation of cardiovascular function, but several studies have provided functional evidence for the role of afferent renal nerves. Renal afferent nerves directly influence sympathetic outflow to the kidneys and other organs by modulating posterior hypothalamic activity (Calaresu and Ciriello, 1981; Campese and Kogosov, 1995). Sympathetic outflow was normalized after bilateral nephrectomy in patients with end-stage renal disease indicating that afferent signaling via renal sensory nerves modulate sympathetic drive (Schlaich et al., 2009b). In animals, renal denervation also produced a decrease in peripheral sympathetic activity (Katholi et al., 1982a), suggesting the importance of afferent renal nerves. These data suggest that renal afferents project centrally and modulate peripheral sympathetic activity.

In order to examine the role of renal nerves in my model of mild DOCA-salt hypertension, bilateral renal denervation was performed in rats prior to the induction of hypertension. I hypothesized that if renal nerves (afferent or efferent) contributed to hypertension development, then rats with renal denervation should exhibit delayed or impaired increases in AP during DOCA-salt treatment.

Experimental protocol

Male Sprague Dawley rats (225-275g) were used for all experiments. Bilateral RDX along with a radiotelemeter implant was performed in a group of rats as described previously. The other group underwent sham denervation surgery (SHAM-DX). Rats

were allowed to recover for seven days. After 3 days of control hemodynamic measurements, a DOCA-pellet (50 mg/kg, SC) was implanted in both RDX and SHAM-DX rats. Rats received free access to water containing 1% NaCl and 0.2% KCl and AP was measured throughout the period of the experiment. Acute hemodynamic responses to hexamethonium (30 mg/kg, IP) were measured on days 14 and 21 following DOCA treatment. Saline intake was also monitored during the control period, and on days 7, 14 and 21 after DOCA administration. The experimental protocol is shown in **Figure 11**.

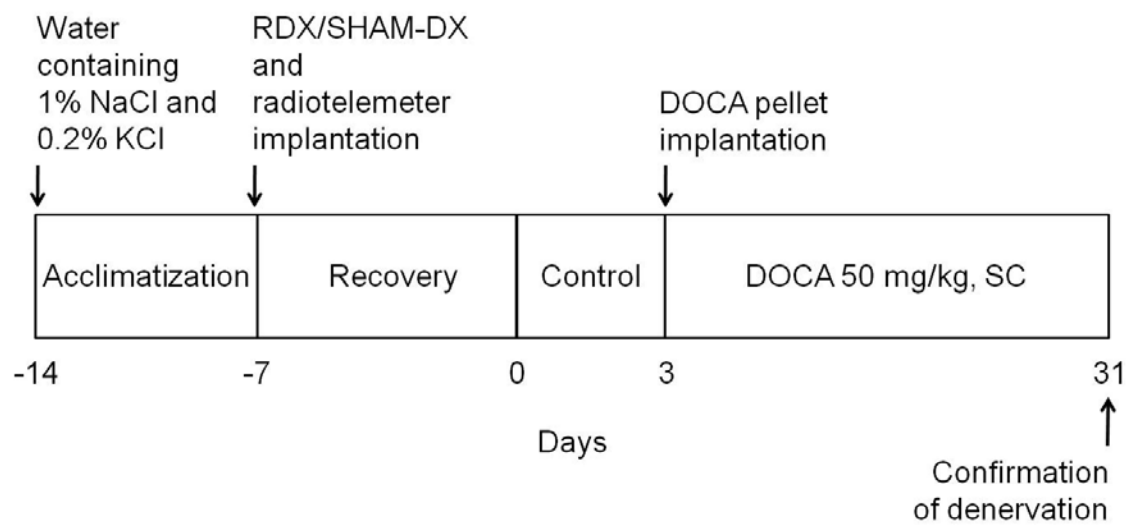


Figure 11. Experimental protocol for renal denervation in the development of mild DOCA-salt hypertension.

Results

MAP and HR in RDX and SHAM-DX rats are shown in **Figures 12 and 13**. During the control period, MAP was slightly lower in RDX (100 ± 1.4 mmHg) compared to SHAM-DX rats (103 ± 1.6 mmHg). After DOCA administration the MAP increased significantly in both groups to a similar degree. At no time point was the difference in MAP between the two groups significant. Both groups of rats had similar HR and exhibited identical falls in HR during the course of the experiment. MAP responses to acute ganglion blockade on days 14 and 21 following DOCA treatment are shown in **Figure 14**. On day 14, there was no significant difference between the groups in the peak fall in MAP following hexamethonium injection. On day 21 after DOCA administration, however, the fall in blood pressure was significantly attenuated in RDX rats compared to sham rats. Both the RDX and SHAM-DX groups consumed similar quantities of saline during the control period, and days 7, 14, and 21 after DOCA treatment (**Figure 15**). **Figure 16** shows that total renal NE content was significantly lower in both kidneys of rats with RDX compared to kidneys from SHAM-DX rats.

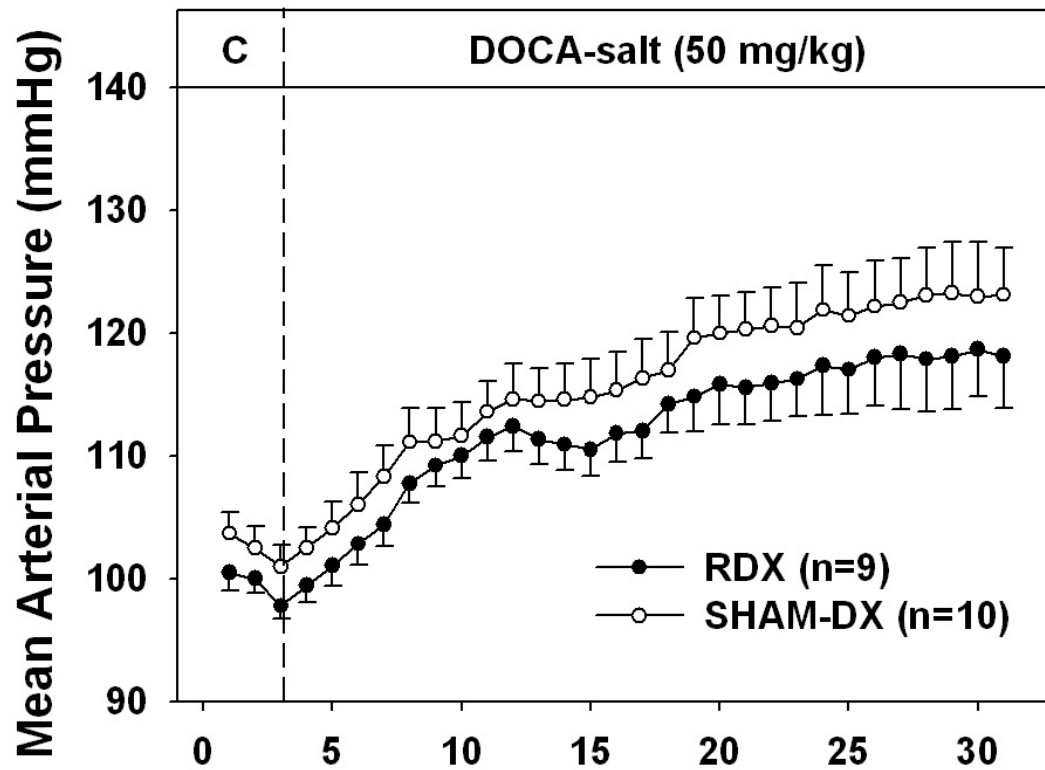


Figure 12. MAP in renal denervated (closed circles) and SHAM rats (open circles) during control (C) and DOCA period.

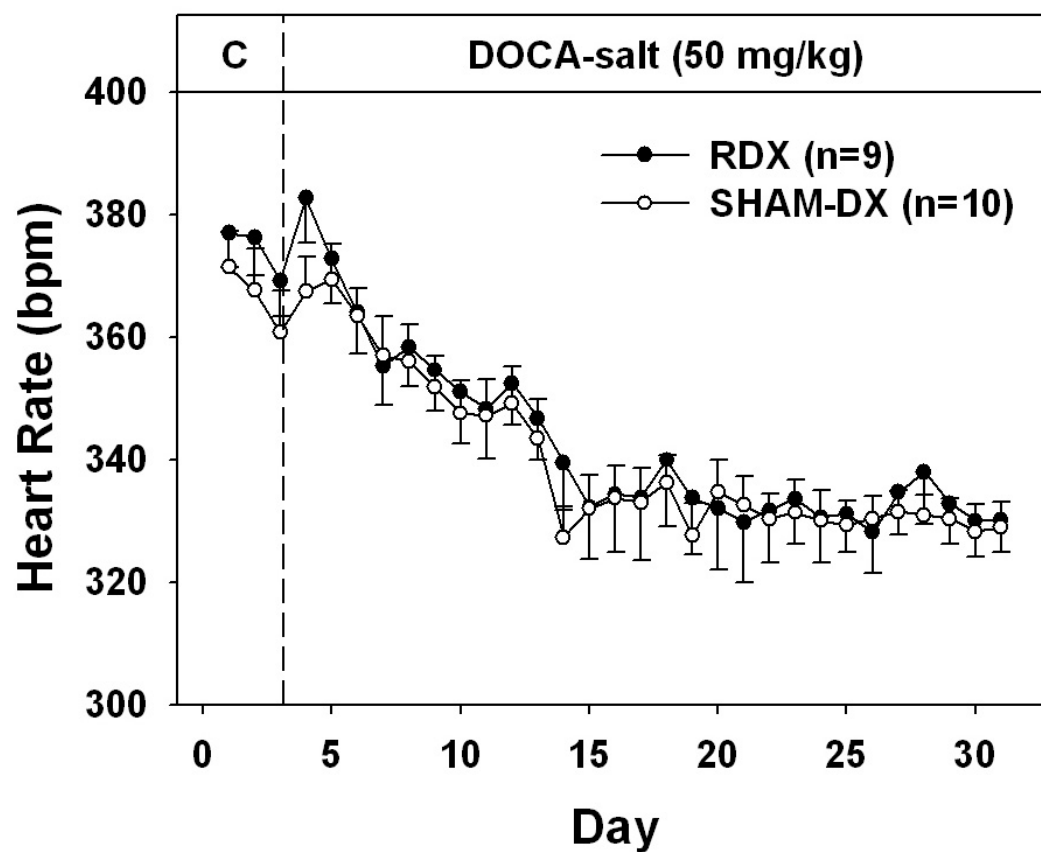


Figure 13. HR in renal denervated (closed circles) and SHAM rats (open circles) during control (C) and DOCA period.

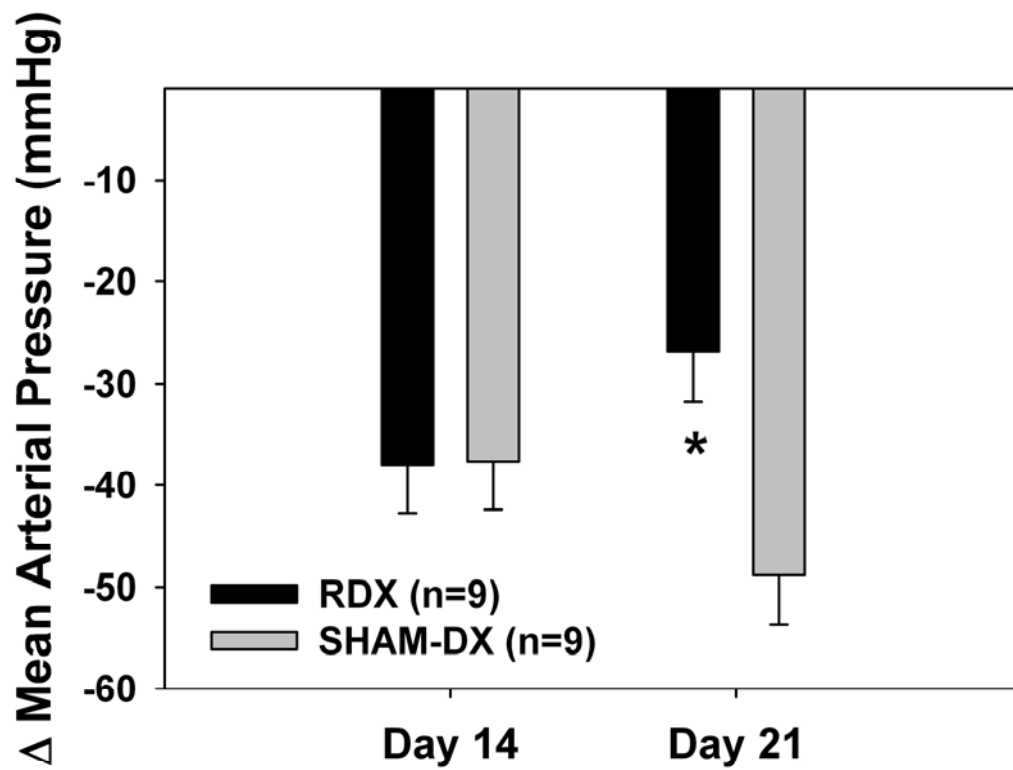


Figure 14. Peak fall in MAP following acute administration of hexamethonium (30 mg/kg) in renal denervated (black bars) and SHAM rats (gray bars) on days 14 and 21 following DOCA administration.

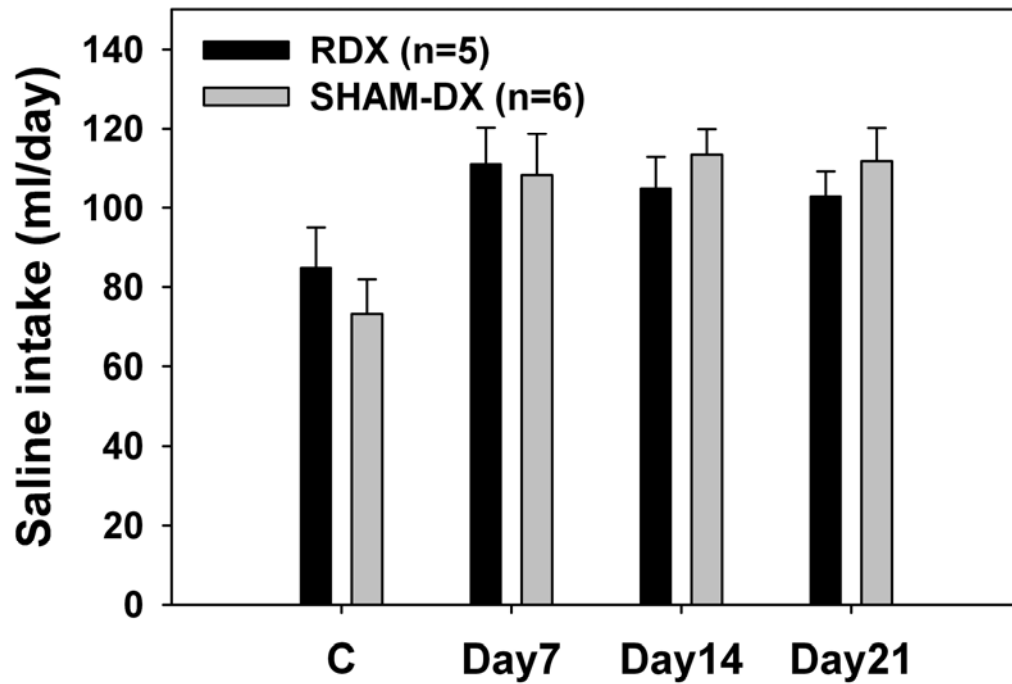


Figure 15. Saline intake (24-hour average) during the control period (C), and days 7, 14, and 21 after DOCA treatment in renal denervated (black bars) and SHAM rats (gray bars).

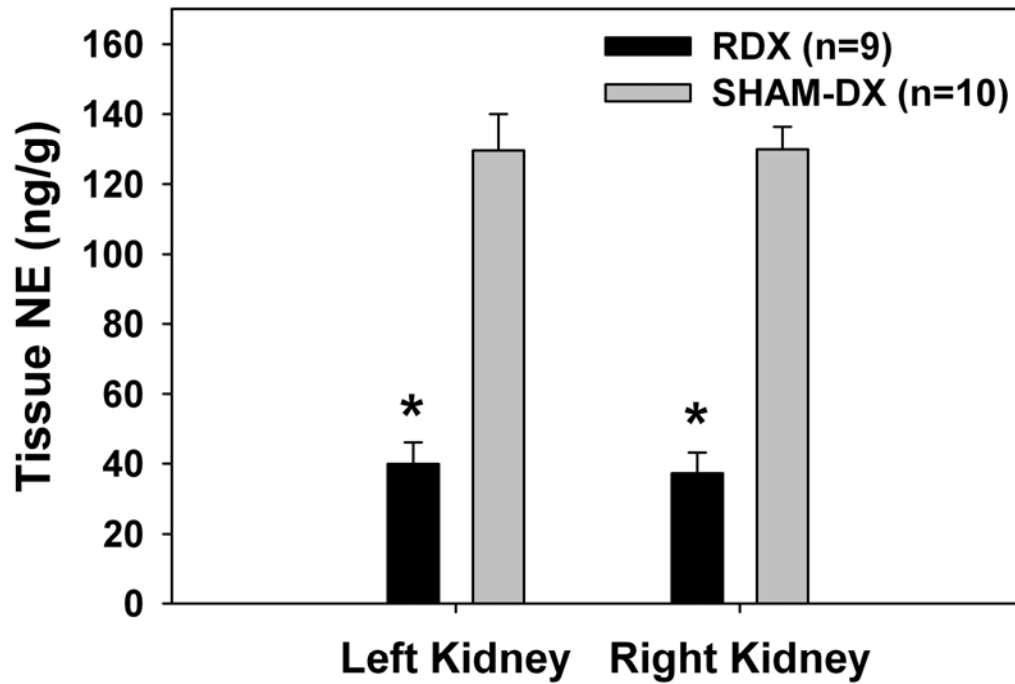


Figure 16. Tissue NE content in the left and right kidneys of renal denervated (black bars) and SHAM rats (gray bars) after 4 weeks of DOCA administration.

Discussion

The major findings of this part of my project were:

- a) Intact renal nerves are not essential for development of mild DOCA-salt hypertension and
- b) Renal denervation attenuates neurogenic pressor activity in the established phase of mild DOCA-salt hypertension.

During the pretreatment period, blood pressure was slightly lower in the RDX group compared to SHAM-DX (~3 mmHg) but the difference between the groups was not statistically significant. It has been asserted that renal nerves are important in setting the basal level of blood pressure in normotensive individuals. One study showed that renal denervation chronically lowered resting blood pressure in normotensive rats; the authors concluded that renal nerves influence resting levels of AP (Jacob et al., 2003). Many other investigators have not observed a significant difference in blood pressure between intact and renal denervated normotensive animals (Katholi et al., 1980; Takahashi et al., 1984; Ichihara et al., 1997). Jacob et al. argued that their use of radiotelemetric pressure measurements allowed detection of a small effect of renal denervation not shown by less precise measurement techniques. I used radiotelemetry in my studies, but found that blood pressure during the control period was not different between the RDX and SHAM-DX groups. One possible explanation is that in my study the degree of renal denervation (assessed from renal NE content) was less than

reported by Jacob et al. It remains unclear whether or not renal nerves contribute to the maintenance of resting blood pressure in normotensive animals.

More importantly, renal denervation did not affect the development of hypertension during DOCA treatment. This finding is consistent with one earlier study showing that renal nerves are not necessary for the development or maintenance of standard DOCA-salt hypertension in rats (Dzielak and Norman, 1985). Katholi et al. (Katholi et al., 1983) also showed that renal nerves are only important in the early established phase of standard DOCA-salt hypertension and play a diminished role during the later phase. Hence, I conclude that renal nerves are not critical to the development of DOCA-salt hypertension.

Chronic renal nerve stimulation has been shown to induce hypertension (Kottke et al., 1945), providing proof-of-principle that increased renal nerve activity can affect AP regulation. Electrical stimulation of renal sympathetic nerves causes graded increases in renin secretion rate, urinary sodium excretion, and renal blood flow. At lower frequency ranges, there is stimulation of renin secretion rate (RSR), without effects on sodium excretion, renal blood flow, or glomerular filtration rate (La Grange et al., 1973; Echtenkamp and Dandridge, 1989; Kopp and DiBona, 1993). At slightly higher frequencies, there is stimulation of RSR along with decreases in urinary sodium excretion without changes in renal blood flow and glomerular filtration rate (Kubicek and Kottke, 1946; Poucher and Karim, 1991). At higher frequencies, there is increased RSR, antinatriuresis, and a decrease in both renal blood flow and glomerular flow rate

(Hermansson et al., 1981; Kon and Ichikawa, 1983). This variation in response to different levels of renal nerve activity could explain why renal nerves contribute to hypertension development in certain models and not in others. For example, in traditional DOCA-salt hypertension, several factors may act in concert to increase sympathetic outflow to the kidneys, and the resulting higher levels of renal nerve activity could account for why some investigators find that renal denervation attenuates the development of hypertension in that model. However, in mild DOCA-salt hypertension there may be a lesser increase in renal sympathetic activity, insufficient to engage the key renal mechanisms affecting systemic AP. This hypothesis needs to be investigated further.

In one previous study it was concluded that attenuation of DOCA-salt hypertension development by renal denervation was due in part to reductions in sodium and water intake (Jacob et al., 2005). They found that renal denervated rats drank less saline during DOCA-salt treatment than did non-denervated rats. Restricting saline intake in rats with intact renal nerves to an amount identical to that seen in renal denervated rats resulted in similar impairment of hypertension development. The authors speculated that interruption of renal *afferent* nerve traffic accounted for the reduced saline intake in renal denervated rats. In my study I found that rats in both groups drank similar amounts of saline during the control period, and on days 7, 14 and 21 after DOCA administration. The reason for the differences in the two models is not clear. It is possible that renal injury associated with the traditional DOCA-salt model (Li et al., 1996; Hartner et al., 2003) is responsible for activating renal afferents. Jacob et al. also

reported that rats with renal denervation showed reduced sodium and water retention during DOCA-salt treatment. This implies that the magnitude of hypertension in the traditional DOCA-salt model might also be related to renal sympathetic control of sodium and water balance. I did not assess sodium and water balance in my study.

Previous reports indicate that renal denervation decreases peripheral sympathetic activity in hypertensive animals (Katholi et al., 1982a; Katholi et al., 1982b). And catheter-based renal denervation in a human patient with drug-resistant hypertension decreased renal NE spillover, whole body NE spillover, and directly recorded muscle sympathetic nerve activity (Schlaich et al., 2009a). The authors of that study concluded that since the patients experienced pain during the procedure, ablation not only disrupts the efferent nerves, but also the renal afferents. They hypothesized that interruption of renal afferent nerve traffic was responsible for reduced systemic sympathetic activity after renal denervation; and furthermore suggested that the antihypertensive response to renal denervation could be explained by reduced sympathetic activity to non-renal, cardiovascular targets (i.e. reduced neurogenic pressor activity).

To study the effect of renal denervation on neurogenic pressor activity (the net effect on blood pressure of autonomic nervous system activity) in SHAM-DX and RDX groups, I measured changes in MAP to acute ganglionic blockade with hexamethonium. The magnitude of the acute depressor response was taken as an index of overall neurogenic pressor activity. I observed that the depressor response was similar in both groups on day 14 but was significantly attenuated on day 21 after DOCA treatment in

the RDX group compared to SHAM-DX rats. I conclude that neurogenic pressor activity is decreased in the established phase of hypertension in RDX animals compared to SHAM-DX rats. A possible explanation for this finding is that renal denervation attenuates afferent renal nerve activity, and therefore decreases global SNA. It has been reported that afferent signals from the kidney project centrally and play an important role in modulating peripheral SNA (Campese and Kogosov, 1995; DiBona and Kopp, 1997; Schlaich et al., 2009a). If increased afferent renal activity stimulates peripheral sympathetic outflow in DOCA-salt animals, then disruption of renal nerves should result in attenuation of peripheral sympathetic activity. Further studies are necessary to confirm that idea. Nevertheless, considering that blood pressure was similar in SHAM and RDX rats treated with DOCA-salt, I conclude that increased neurogenic pressor activity is not essential to maintain hypertension in my model. Presumably other blood pressure control mechanisms compensated for loss of neurogenic pressor activity after renal denervation. The decrease in blood pressure observed in human hypertensive patients after renal denervation, however, indicates that this compensation does not always occur.

In summary, the data presented in this study are inconsistent with the idea that renal nerves are essential for development of DOCA-salt hypertension. Taking into account differences in DOCA-salt models, however, it is possible that sympathetic outflow to the kidneys, and/or sensory afferent signals from the kidneys, is important in more severe and drug-resistant forms of hypertension. Understanding the mechanism by which renal

denervation attenuates neurogenic pressor activity might prove useful in treating drug-resistant hypertension.

OVERALL CONCLUSION: Renal nerves are not essential for development of mild DOCA-salt hypertension. RDX may decrease peripheral sympathetic activity during the established phase of mild DOCA-salt hypertension by eliminating renal afferent activity.

CHAPTER FOUR: THE EFFECT OF SELECTIVE SPLANCHNIC DENERVATION ON MILD DOCA-SALT HYPERTENSION DEVELOPMENT AND WHOLE BODY NOREPINEPHRINE SPILLOVER

Introduction

Thus far my previous studies have led to the conclusions that 1) global sympathetic activity, as measured by whole body NE spillover, is unchanged in the development of mild DOCA-salt hypertension, and 2) renal nerves are not essential for hypertension development in this model. In an attempt to identify a role for increases sympathetic actions in other organ systems in my model, I decided to study splanchnic SNA.

What is the evidence that splanchnic SNA might be a cause of hypertension? A very critical proof-of-principle experiment was published in 1953: chronic stimulation of the splanchnic nerves in conscious dogs was shown to cause sustained hypertension (Kubicek et al. 1953). Splanchnic organs receive approximately 95% of their postganglionic sympathetic innervation from neurons in the celiac ganglion plexus (Trudrung et al., 1994; Hsieh et al., 2000; Quinson et al., 2001). In the late 1940s and early 1950s, surgical removal of celiac ganglion plexus (celiac ganglionectomy, CGX) performed in humans, proved beneficial in treating hypertension (Grimson et al., 1949; Grimson et al., 1953). Recent studies performed in the angiotensin II-salt model of experimental hypertension also demonstrated that CGX significantly attenuates hypertension development (King et al., 2007). Selective activation of the celiac ganglion, assessed by increased tyrosine hydroxylase activity, was reported in pre-

hypertensive young spontaneously hypertensive rats (Nakamura and Nakamura, 1977a; Nakamura and Nakamura, 1977b). The investigators also reported that increases in plasma NE levels in these rats (compared to the normotensive Wistar Kyoto rats) were normalized after bilateral removal of the celiac ganglia (Nakamura and Nakamura, 1977a). These data suggest that 1) sympathetic activation in the splanchnic region precedes hypertension development in the spontaneously hypertensive rats, and 2) that denervation of splanchnic region via CGX reduces global sympathetic activity. Similarly, in borderline hypertensive (pre-hypertensive) individuals hepatosplanchnic vascular resistance was preferentially increased, while total peripheral resistance was unchanged, suggesting that hemodynamic changes may occur preferentially in the hepatosplanchnic circulation in the early stage of essential hypertension development (Sugawara et al., 1997).

I performed CGX in mild DOCA-salt hypertension to assess the role of splanchnic SNA in the development of hypertension. I hypothesized that splanchnic SNA is increased in this model, and that CGX will prevent this increase and thereby lower AP. Since a large fraction of total body sympathetic outflow is directed toward mesenteric organs (Aneman et al., 1996), I also hypothesized that CGX would remove the measureable splanchnic contribution to whole body NE spillover.

Experimental protocols

Effect of CGX on hypertension development: The experimental protocol is shown in **Figure 17**. Male Sprague Dawley (250-275g) rats were used for all experiments. Celiac ganglionectomy was performed on one group of rats (CGX, n=10) while the other group underwent sham surgery (SHAM-GX, n=10). A radiotelemeter was implanted for hemodynamic measurements. MAP and HR recordings were started after a 7 day post-surgical recovery period. After 5 days of control recordings, rats were allowed free access to water containing 1% NaCl and 0.2% KCl for the entire duration for experiment. After a 7 day period of salt treatment, a DOCA pellet (50 mg/kg, SC) was implanted in both CGX and SHAM-GX groups and blood pressure was recorded for 4 more weeks. Acute hemodynamic responses to hexamethonium (30 mg/kg, IP) were measured on day 25 following DOCA treatment. Twenty four hour saline intake was also measured for two days at the end of the experiment before the rats were sacrificed for harvesting splanchnic organs to measure tissue NE content.

Effect of CGX on whole body NE spillover: The experimental protocol is shown in **Figure 18**. To study the effect of CGX on whole body NE spillover, some rats underwent celiac ganglionectomy (DOCA-CGX) while the others had sham operation (DOCA-SX) surgery. These groups are essentially the same as rats in the previous protocol except that they had externalized catheters instead of a radiotelemeter. After a 7 day recovery period, catheters were implanted to measure whole body NE spillover as described earlier. Five days after catheter implantation, control hemodynamic measurements were made for 3 days followed by a subcutaneous DOCA pellet implant

in both groups. Blood pressure and HR were measured for an hour every day for another 14 days. Whole body NE spillover was measured on control day 2, and days 3, 7 and 14 following DOCA treatment.

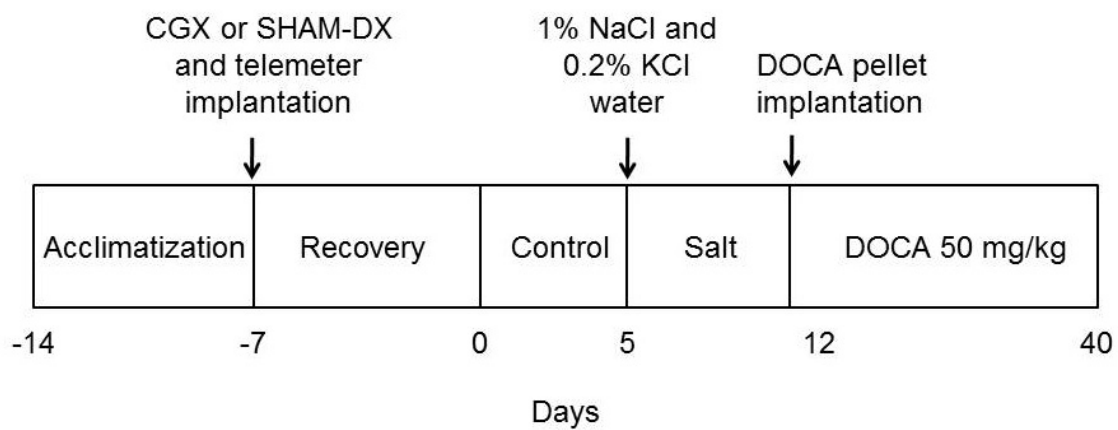


Figure 17. Protocol for the effect of CGX on the development of mild DOCA-salt hypertension.

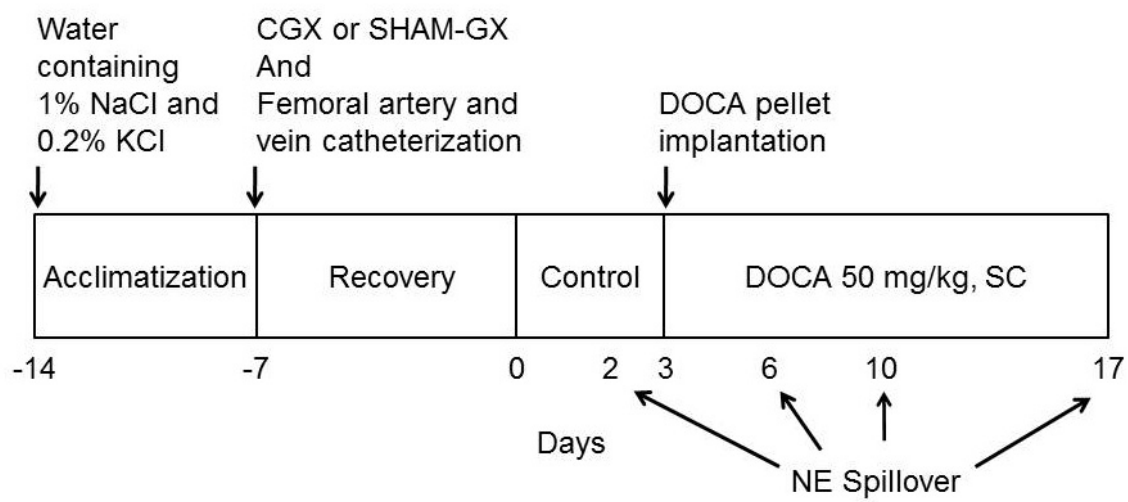


Figure 18. Protocol for the effect of CGX on whole body NE spillover.

Results

Effect of CGX on hypertension development: MAP during the development of DOCA-salt hypertension in CGX and SHAM-GX rats is shown in **Figure 19**. During the control period, CGX rats had a slightly lower MAP than normotensive rats; however this difference was not statistically significant. Salt alone did not change MAP in either CGX or SHAM-GX groups. Upon DOCA administration, the increase in MAP was significantly attenuated in CGX compared to the SHAM-GX group (15.6 ± 2.2 vs. 25.6 ± 2.2 mmHg, day 28 after DOCA treatment). HR was significantly lower in both CGX and SHAM-GX groups during high salt intake and during DOCA treatment than their respective control period values, but the change in HR between the two groups was not significantly different at any time during the experiment (**Figure 20**). Tissue NE content of selected splanchnic organs measured at the end of the experiment is shown in **Figure 21**. Tissue NE content in the CGX group compared to SHAM-GX group was 30-40% lower in the kidneys, 81% lower in liver, 67% lower in small intestine, and 94% lower in the spleen. Peak falls in MAP after administration of the ganglionic blocker hexamethonium are shown in **Figure 22**. Values were similar in the CGX and SHAM-GX groups. Salt intake, measured at the end of the experiment, was not different between the two groups (**Figure 23**).

Effect of CGX on whole body NE spillover: **Figure 24** and **Figure 25** show the MAP and HR response during the development of mild DOCA-salt hypertension in ganglionectomized (DOCA-CGX) and sham operated (DOCA-SX) animals. During the

control period, MAP was slightly lower in DOCA-CGX than DOCA-SX rats but the difference was not significant. After DOCA administration, both groups became hypertensive but the increase in MAP was slightly attenuated in the DOCA-CGX group. However, the absolute MAP was not statistically different between the two groups. No significant difference was observed in HR between the two groups. During the control period, plasma NE and NE spillover were significantly higher in DOCA-SX rats compared to DOCA-CGX animals, but were similar in both groups on days 3, 7, and 14 of DOCA treatment (**Figure 26, Figure 27, and Figure 28**). NE clearance was not different in the two groups on control day 2, or on days 3, 7, and 14 of DOCA treatment.

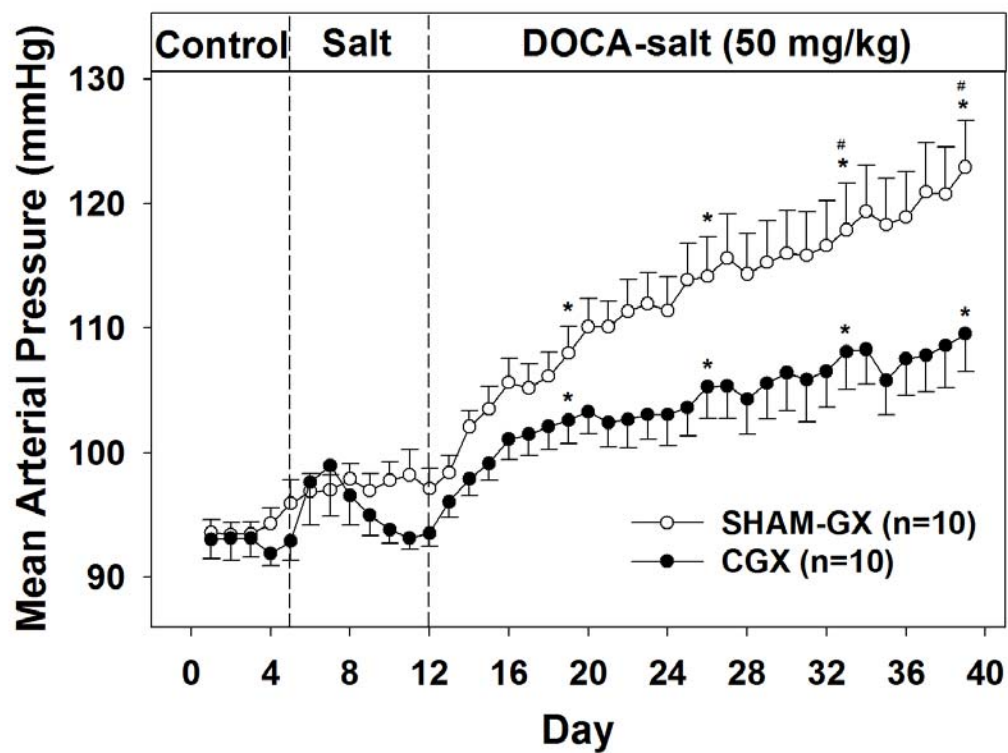


Figure 19. MAP during the control, high salt, and DOCA treatment periods in SHAM-GX and CGX groups.

Asterisk (*) indicates a significant difference from day 3 (control period) values. Pound sign (#) indicates difference.

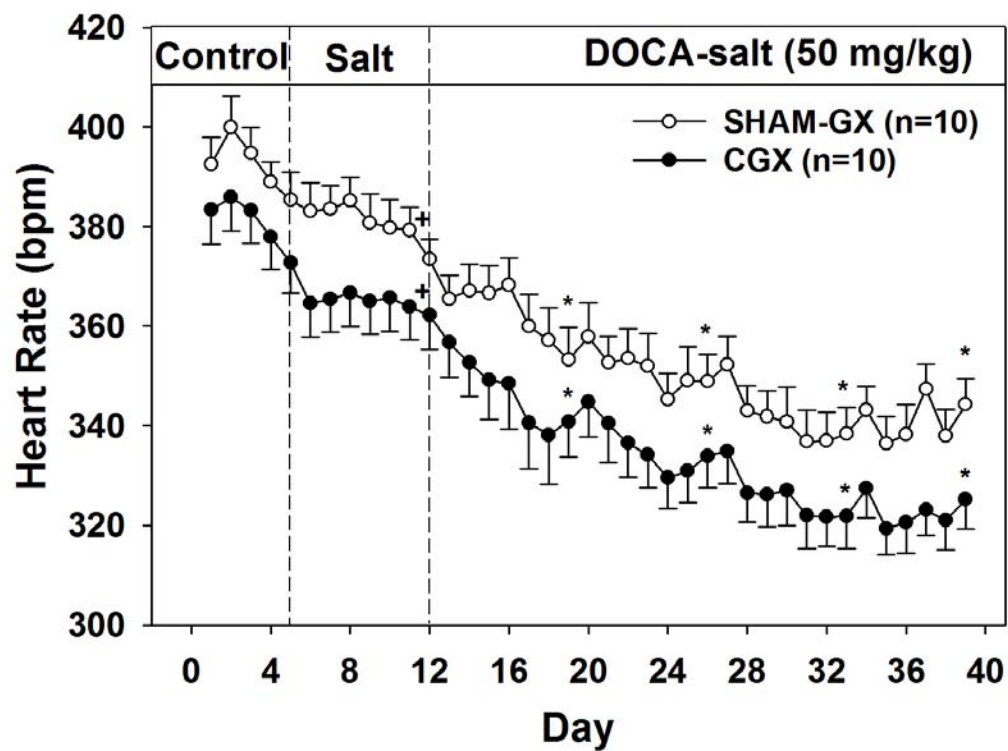


Figure 20. HR response during the control, high salt, and DOCA treatment periods in SHAM-GX and CGX groups.

Asterisk (*) indicates a significant difference from day 3 (control period) values.

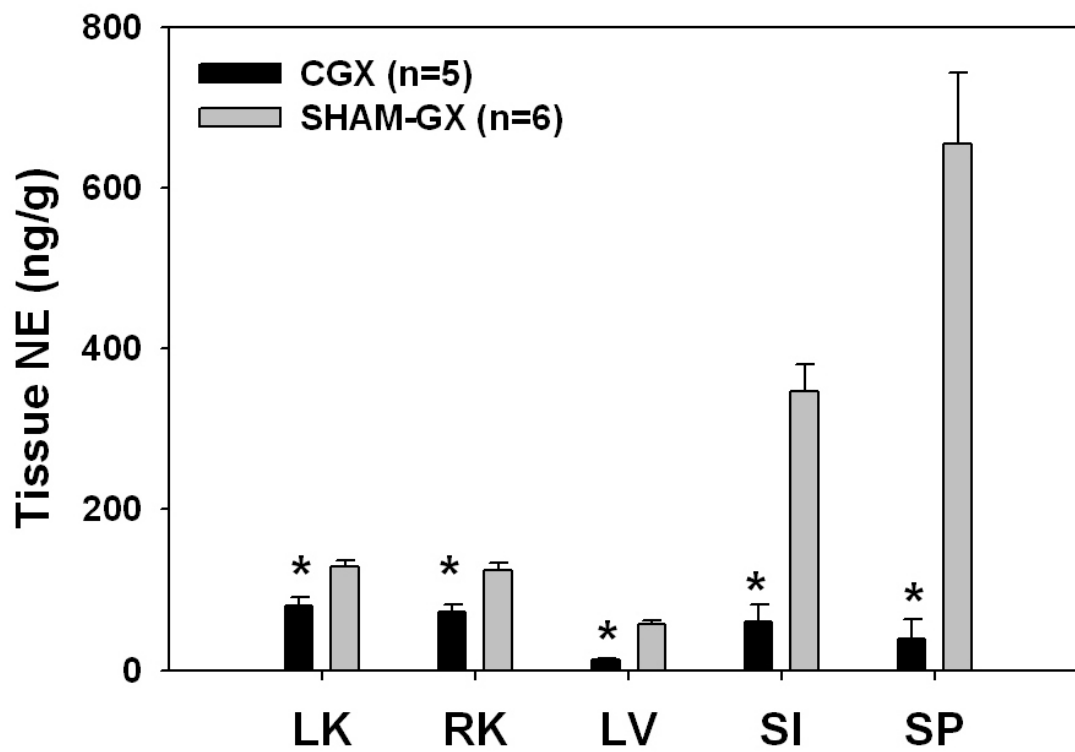


Figure 21. Tissue NE content in the splanchnic organs after 4 weeks of DOCA administration.

LK – left kidney, RK – right kidney, LV – liver, SI – small intestine, SP – spleen.

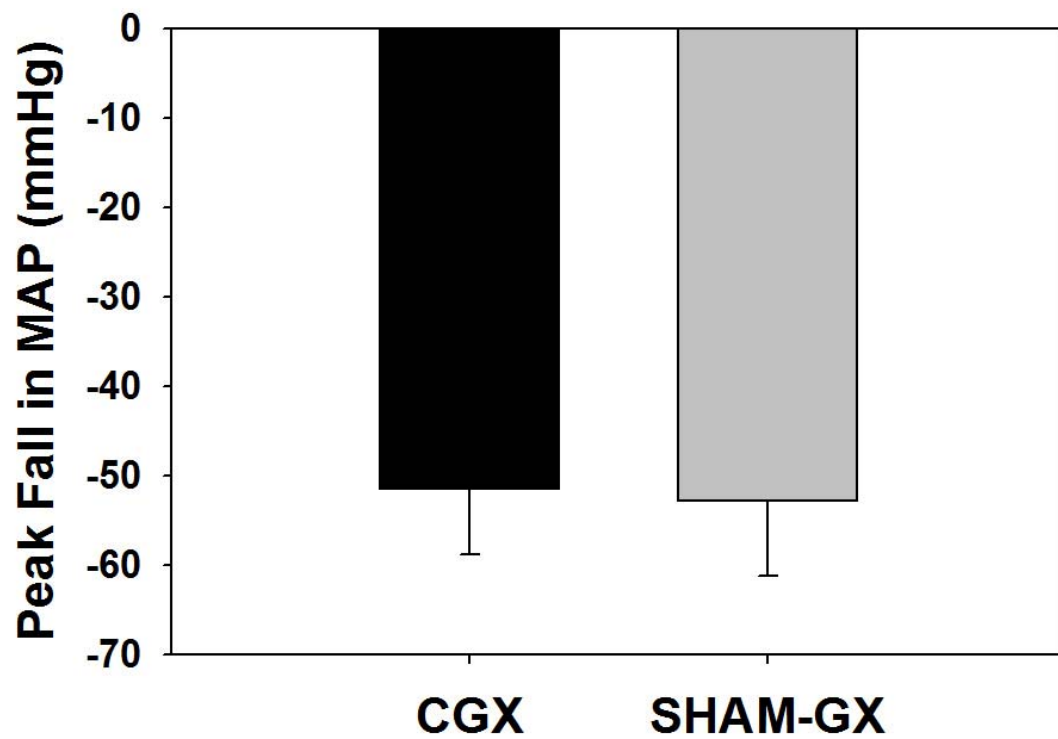


Figure 22. Peak fall in MAP following acute administration of hexamethonium (30 mg/kg) in celiac ganglionectomized rats (black bars) and sham operated rats (gray bars).

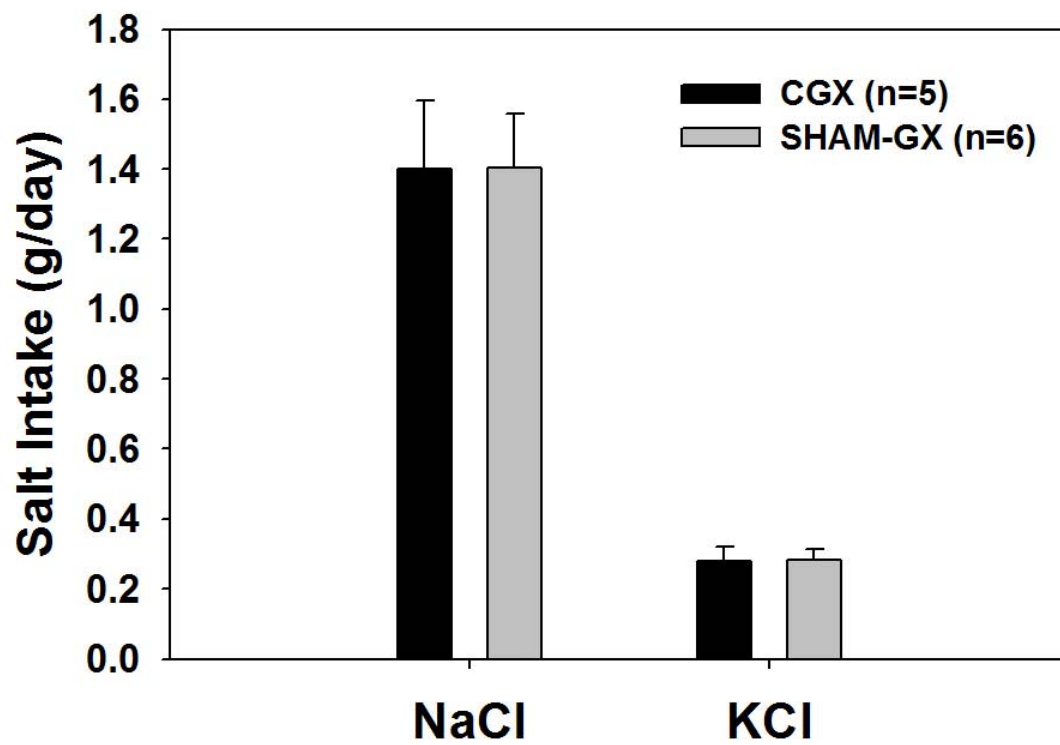


Figure 23. Salt intake (24-hour average) measured in CGX rats (black bars) and SHAM-GX rats (gray bars).

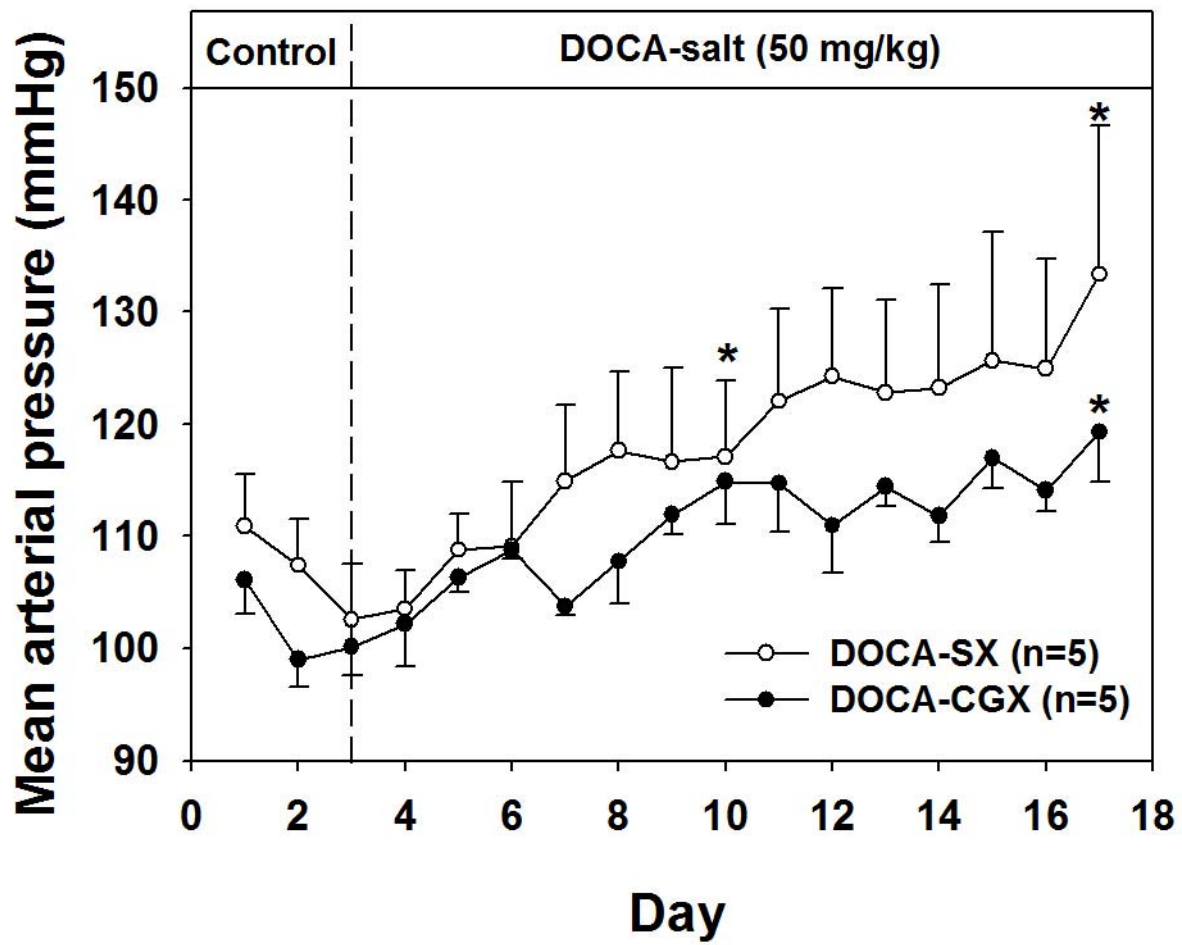


Figure 24. MAP during the control and DOCA treatment periods in DOCA-SX (sham operated) and DOCA-CGX (ganglionectomized) groups.

Asterisk (*) indicates a significant difference from day 3 (control period) values.

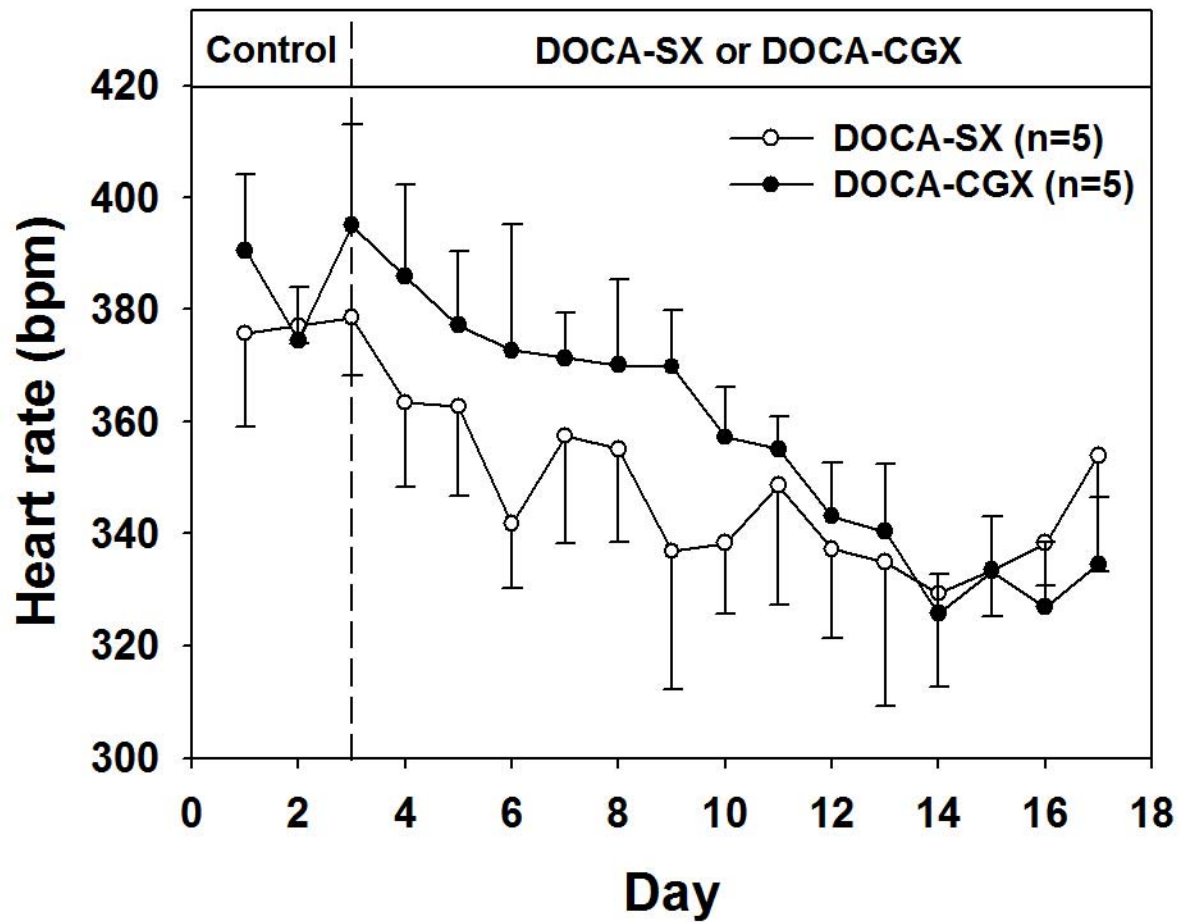


Figure 25. HR during the control and DOCA treatment periods in DOCA-SX (sham operated) and DOCA-CGX (ganglionectomized) groups.

Asterisk (*) indicates a significant difference from day 3 (control period) values.

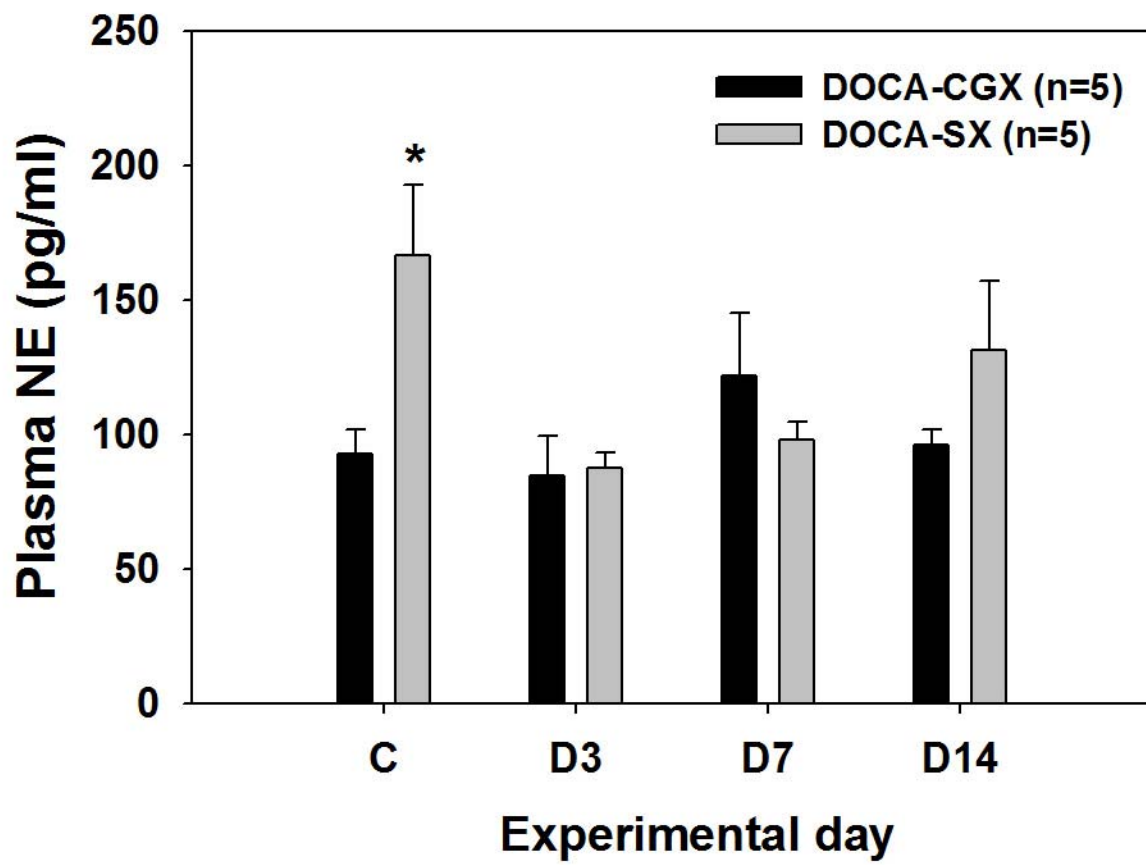


Figure 26. Plasma NE in DOCA-SX and DOCA-CGX on control day 2, and days 3, 7 and 14 after DOCA treatment.

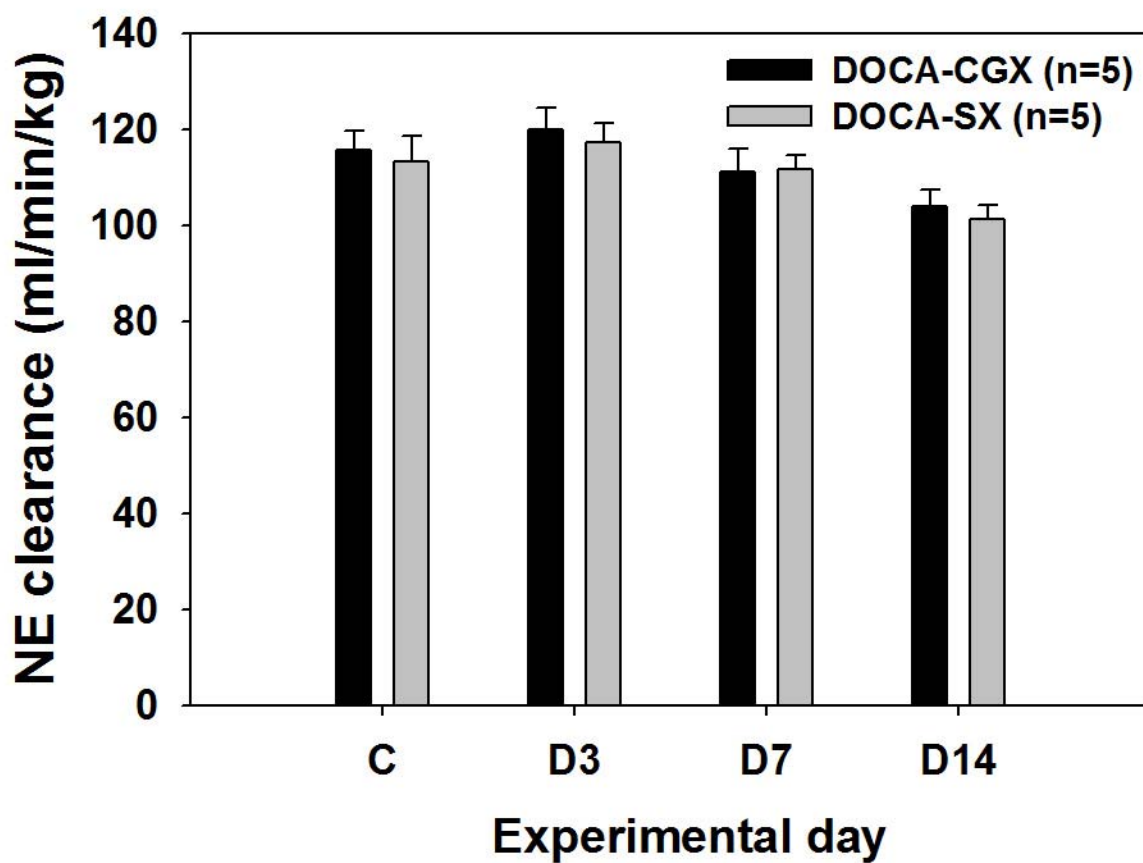


Figure 27. NE Clearance in DOCA-SX and DOCA-CGX on control day 2, and days 3, 7 and 14 after DOCA treatment.

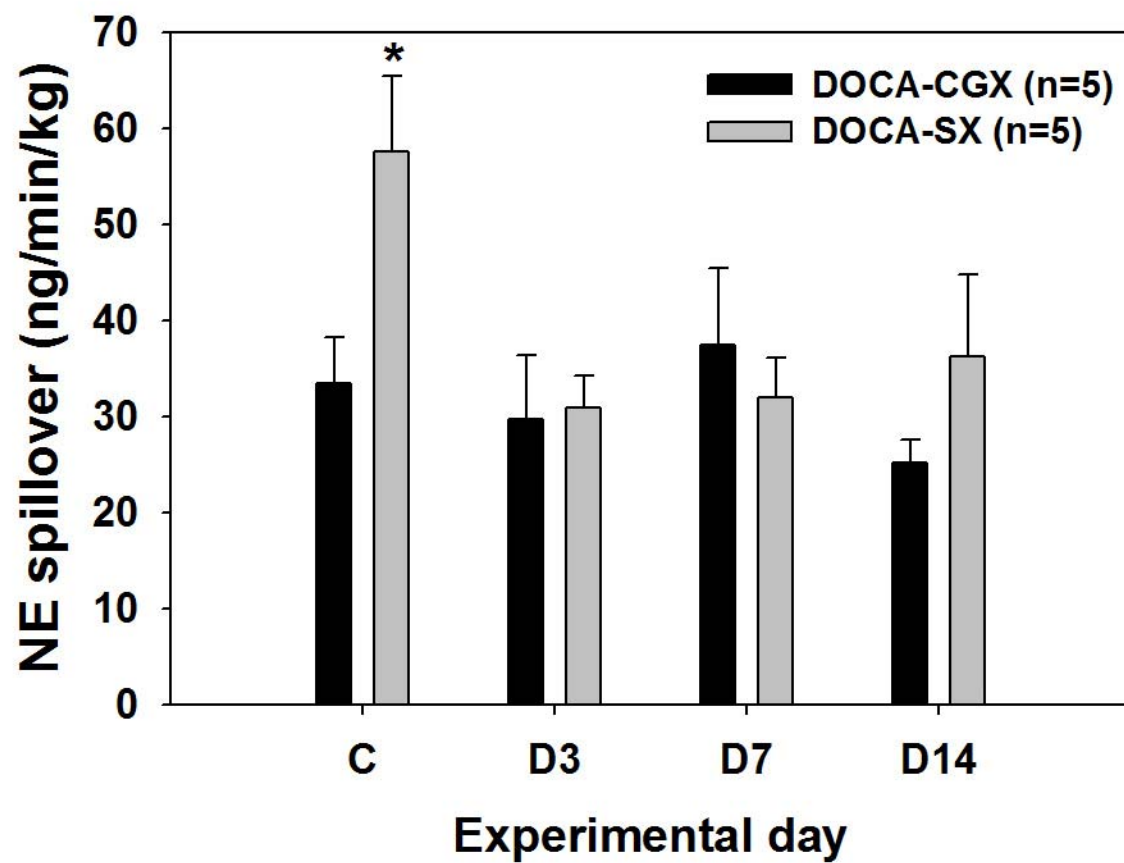


Figure 28. NE Spillover in DOCA-SX and DOCA-CGX on control day 2, and days 3, 7 and 14 after DOCA treatment.

Discussion

In this study, I evaluated the effect of splanchnic sympathectomy (via CGX) on blood pressure and global sympathetic activity (measured using NE spillover) during the development of mild DOCA-salt hypertension. My main findings were that CGX 1) effectively denervates the splanchnic organs, 2) attenuates the development of mild DOCA-salt hypertension, 3) does not affect neurogenic pressor activity assessed by acute ganglionic blockade, and 4) does not affect global sympathetic activity as measured by either plasma NE or whole body NE spillover.

NE content in the splanchnic organs was measured at the end of the study using HPLC. A decrease in the tissue NE content even 7 weeks after CGX provided confirmation that denervation was achieved successfully. NE content in the splanchnic organs was significantly attenuated, with splenic content reduced the most (by 94%). This is consistent with previous findings showing an approximately 85% reduction in splenic NE content (Bellinger et al., 1989; Li et al., 2010) after CGX or selective denervation of the spleen. Previous reports indicate that sympathetic postganglionic nerves show regeneration after chemical or surgical sympathectomy (Hill et al., 1985; Li et al., 2010). This regeneration of postganglionic neurons can re-establish neuroeffector transmission at the nerve terminal. It is possible that some regeneration might have occurred during this study. However, the effect does not appear to be large because NE content of splanchnic organs was still very low at the end of study, and blood pressure was attenuated throughout the experimental period. There was a 30-40% reduction in NE

content in the kidneys. This is not surprising because 20-25% of the post-ganglionic neurons supplying the kidneys originate in the celiac ganglion, while the majority (~80%) of them come from the paravertebral sympathetic chain (Ferguson et al., 1986; Sripairojthikoon and Wyss, 1987; Chevendra and Weaver, 1991). However, as discussed in the previous chapter, renal nerves are not essential for hypertension development in this model. Thus, the effects of CGX on AP in my model are due to sympathetic denervation of non-renal splanchnic organs.

By what mechanism did splanchnic sympathectomy impair hypertension development in this study? The most obvious mechanism by which SNA to the splanchnic organs affects AP regulation is through altering vascular tone. Postganglionic sympathetic nerves originating from the CG innervate both arteries and veins. Retrograde tracing studies have been performed by the uptake of tracers from the postganglionic nerve endings that innervate the mesenteric vessels to localize the neurons innervating arteries and veins. One such study in rats reported that 54% of the neurons from CG innervate both the mesenteric arteries and veins (Hsieh et al., 2000). Neurons that singly innervate the arteries are 41% while only 5% of the neurons innervate just the veins. Therefore, it is possible that splanchnic SNA could affect arterial and venous function selectively. But at the present time there is little evidence supporting that idea, and splanchnic SNA probably affects arterial and venous function in a concerted fashion. However, it is worth pointing out that low levels of SNA can produce substantial constriction of mesenteric veins with little effect on arterial tone (Hottenstein and Kreulen, 1987; Luo et al., 2003; Park et al., 2007).

Increased vascular resistance in mineralocorticoid hypertension is particularly marked in the splanchnic organs (Yates and Hiley, 1979; May, 2006). Sympathetic tone to arterial resistance vessels (as assessed by ganglion blockade) in the splanchnic organs (mesenteric artery) is increased in conscious rats with traditional DOCA-salt hypertension (Shimamoto and Iriuchijima, 1987). This could be due to increased splanchnic SNA, but another possibility is suggested by the finding that mesenteric arterial constrictor responsiveness to NE is increased during the development of traditional DOCA-salt hypertension (Tsuda et al., 1986). Enhanced sympathetic neurotransmission to mesenteric arteries also has been found in established DOCA-salt hypertension (Park et al., 2010). In these situations even normal splanchnic SNA could produce an increase in splanchnic arterial vasoconstriction. Therefore CGX may have attenuated hypertension development in my study by reducing sympathetically mediated increases in splanchnic vascular resistance.

Rats with established traditional DOCA-salt hypertension have an increase in venous tone mediated in part by the sympathetic nervous system (Fink et al., 2000; Xu et al., 2007). This appears to be caused mainly by increased reactivity of mesenteric veins to released NE (Xu et al., 2007). Therefore, it is possible that neurogenically mediated venous tone is increased in mild DOCA-salt hypertension. As discussed earlier, this could contribute to the development of hypertension by translocation of blood from the venous to the arterial compartment. If so, CGX may impair mild DOCA-salt hypertension by decreasing venous tone and increasing vascular capacitance.

It is also important to consider other possible effects of CGX on neurally mediated normal physiological functions of the splanchnic organs such as intestinal motility (Fukuda et al., 2005), secretion (Fandriks and Jonson, 1990; Hansen et al., 2004), hepatic glucose metabolism (Nonogaki and Iguchi, 1997), and insulin secretion (Hell and de Aguiar Pupo, 1979; Brockman and Halvorson, 1982) . Chronic splanchnic nerve stimulation in rats has shown to reduce food intake, increase metabolic rate, and improve body composition (Wu et al., 2009). One previous report indicated that CGX in dogs caused intractable diarrhea along with a 20% weight loss (Dayton et al., 1984). This observation was not found in my study. The animals did not show any signs of illness or distress. A previous study also reported that bilateral splanchnic nerve section in rats did not cause any changes in behavior, food intake, abdominal fat, body weight, brown adipose tissue, abdominal organ weight, plasma leptin concentration, or hypothalamic neuropeptide Y level, compared to sham operated animals (Furness et al., 2001). Nevertheless, I cannot rule out the possibility that the effect of CGX on DOCA-salt hypertension is caused by interruption of sympathetic control of non-vascular functions of the splanchnic organs.

The finding that splanchnic sympathectomy with CGX impaired hypertension development is surprising since in earlier studies I found, using both plasma NE levels and whole-body NE spillover as indices of SNA, no evidence for increased SNA during the development of my model of DOCA-salt hypertension. One interpretation is that increased sympathetic pressor effects in the splanchnic bed do not require actual increases in splanchnic SNA (as discussed in previous paragraphs). Another

interpretation, however, is that splanchnic SNA is not reflected in whole-body assessments of sympathetic activity. This might seem unlikely since a study using a more complicated total body NE spillover technique reported that a large fraction (~37%) of total sympathetic outflow is directed toward splanchnic organs (Aneman et al., 1996). But NE released into the blood from the splanchnic bed must pass through the liver before reaching the systemic circulation, and the vast majority (~86%) is extracted there. Thus, non-hepatic splanchnic NE spillover is obscured when using standard methods of measuring whole-body NE spillover (Aneman et al., 1996). Therefore, I conducted studies to determine if removal of sympathetic input to splanchnic organs by CGX would be detectable as attenuation in whole body NE spillover.

During the control period of that study, plasma NE and NE spillover were in fact significantly lower in CGX versus SHAM rats. On the surface this finding appears to support the conclusion that CGX causes a measureable decrease in global SNA assessed from whole-body NE spillover or plasma NE levels. However, there was no change in plasma NE or whole body NE spillover in CGX rats on days 3, 7, and 14 after DOCA treatment; and both measures were comparable in CGX and SHAM rats throughout the remainder of the study. I conclude that the higher values of plasma NE and NE spillover in SHAM rats during the control period were most likely due experimental error related to the blood sampling technique in that particular group. This is further supported by the fact that plasma NE and whole-body NE spillover in the

SHAM rats during the control period in this study were markedly higher than I measured in my earlier study (**Figure 8, Figure 10**).

There was no difference in whole-body NE clearance between the SHAM and CGX groups during the control period. I conclude that neuronal reuptake in the splanchnic organs makes a negligible contribution to overall removal of NE from plasma. This is somewhat surprising since the neuronal NE transporter is clearly functional in sympathetic nerves of the rat mesenteric circulation (Park et al., 2006). However, the transporter may primarily clear NE released from sympathetic nerve endings rather than circulating NE (Venning and de la Lande, 1988). And as discussed earlier most NE clearance from the splanchnic bed is due to hepatic metabolism.

NE clearance was unchanged during the development of mild DOCA-salt hypertension (**Figure 27**) in both SHAM and CGX rats. This indicates that overall non-neuronal NE uptake and metabolism were not impaired by DOCA-salt treatment. Previous investigators also concluded that non-neuronal uptake of NE was not altered in the mesenteric vasculature of DOCA-salt hypertensive rats (Longhurst et al., 1988).

Overall, I conclude that the effects of CGX on splanchnic sympathetic nerve activity are not revealed by measurements of plasma NE levels or whole-body NE spillover. In order to address this issue and further evaluate the role of splanchnic SNA in the pathogenesis of hypertension, regional measures of NE spillover are necessary. Those studies are described in the next chapter.

I measured the acute depressor response to ganglionic blockade toward the end of the study as an index of neurogenic pressor activity. The peak fall in blood pressure was similar in both groups suggesting that neurogenic pressor activity was not changed by CGX. This result differs from a previous finding in angiotensin II-salt hypertension (King et al., 2007), where CGX attenuated hypertension development and neurogenic pressor activity. The reason for the difference is not clear, but it is important to note that, unlike in AngII-salt hypertension, whole body NE spillover was not increased in my DOCA-salt model during the development of hypertension. Thus, this result supports the conclusion that global sympathetic nerve activity is not increased during the development of mild DOCA-salt hypertension.

Sensory nerves are closely localized with the sympathetic nerves in the splanchnic region (Galligan et al., 1988; Davies and Campbell, 1994). Spinal sensory neurons have their cell bodies in the dorsal root ganglia, but only 10-15% supply visceral tissues (Holzer, 2001). The spinal afferents reach the gastrointestinal tract through the splanchnic and pelvic nerves in which they constitute 10-30% of all nerve fibers (Holzer, 2001). Certain spinal afferents play an efferent-like function by releasing transmitters like calcitonin gene-related peptide, substance P, neurokinin A, nitric oxide and/or ATP from their peripheral endings and induce functional changes affecting motility of intestines, secretions in the gut, and dilation of arterioles and increase in venular permeability. In this study, CGX likely removed the sensory innervation along with the sympathetic supply to the splanchnic organs (Li et al., 2010). Therefore, it is possible that removal of sensory nerves plays a role in the effects of CGX on AP regulation.

Finally, salt and water intake were monitored in this study and found to be similar in both groups during the experimental period. This is consistent with a previous finding showing that extrinsic denervation of small intestine does not alter water and electrolyte absorption (Duininck et al., 2003). This is important because reduced salt intake will attenuate DOCA-salt hypertension development (Theriot et al., 2000; O'Donoghue and Brooks, 2006), and at least one previous study showed that regional denervation (Jacob et al., 2005) slowed DOCA-salt hypertension development exclusively by decreasing salt and water intake.

OVERALL CONCLUSION: Splanchnic SNA is critical for the development of mild DOCA-salt hypertension. The effects of CGX on splanchnic sympathetic nerve activity are not revealed by measurements of plasma NE levels or whole-body NE spillover.

CHAPTER FIVE: NON-HEPATIC SPLANCHNIC NOREPINEPHRINE SPILLOVER IN THE DEVELOPMENT OF MILD DOCA-SALT HYPERTENSION

Introduction

The findings of my previous studies indicate that splanchnic SNA is critical in the development of mild DOCA-salt hypertension. They show in addition that the standard whole body NE spillover technique is not suitable to detect changes in splanchnic SNA, most likely because the liver is highly efficient in extraction of plasma NE (Aneman et al., 1996). Hence, any increase (or decrease) in splanchnic NE spillover will not be reflected in whole body NE spillover measurements. Whole body NE spillover has some other limitations as well. Sympathetic outflow to individual tissues and organs is not uniform. For example, sympathetic activity assessed by measuring plasma NE concentrations obtained from veins in the limbs may not reflect changes in sympathetic activity elsewhere in the body (Esler et al., 1984a). To avoid this limitation, tracer dilution techniques to estimate regional NE spillover can be used (Esler et al., 1984a; Kopin et al., 1998; Rumantir et al., 1999). Regional NE spillover methods have been used to assess regional sympathetic activity during exercise (Hasking et al., 1988; Coker et al., 1997), meal ingestion (Cox et al., 1995), disease states (McCance and Forfar, 1989), and to study the effects of drugs (Goldsmith et al., 1998; Aggarwal et al., 2003; Johansson et al., 2003). I chose to use this method in the my study because: 1) regional NE spillover accurately predicts changes in regional SNA, and 2) the rate of regional NE spillover into the venous drainage of individual organs is proportional to their rate of sympathetic nerve firing (Esler et al., 1990). Numerous studies have

demonstrated this correlation using electrical stimulation of sympathetic nerves to organs such as liver, kidney, heart, pancreas, spleen, and skeletal muscle (Yamaguchi et al., 1975; Yamaguchi and Garceau, 1980; Blombery and Heinzow, 1983; Bradley and Hjemdahl, 1984; Kahan et al., 1984; Havel et al., 1988).

Applying regional NE spillover technique in clinical studies, with sampling of blood from arterial and hepatic venous sites, indicated a 9% contribution of hepatomesenteric organs to the total body NE spillover (Esler et al., 1984a; Esler et al., 1984b). However, sampling from arterial, hepatic, and portal venous sites indicated that mesenteric organs made a 37% contribution and the liver a 5% contribution to total body NE spillover (Aneman et al., 1996). This is consistent with my finding that splanchnic SNA is not revealed by whole NE spillover measurements. Previous reports indicate that there is increased NE release in the mesenteric vasculature in traditional DOCA-salt hypertension (Tsuda et al., 1989b; Luo et al., 2003). There is also increased sympathetically mediated vasoconstrictor tone in the mesenteric circulation in conscious rats with established DOCA-salt hypertension (Shimamoto and Iriuchijima, 1987). Large mesenteric arteries from traditional DOCA-salt hypertensive rats are more sensitive to the contractile effects of exogenously applied NE (Longhurst et al., 1988; Perry and Webb, 1988; Suzuki et al., 1994). Similarly mesenteric veins are more reactive to NE in traditional DOCA-salt hypertension (Xu et al., 2007). These data suggest that in traditional DOCA-salt hypertension, there is increased NE release in the splanchnic vasculature and the splanchnic vasculature is more sensitive to circulating NE. An increase in NE levels in the splanchnic circulation – due to increased splanchnic SNA or

to increased release of NE – could cause significant vasoconstriction of the splanchnic vasculature resulting in increased vascular resistance and/or decreased vascular capacitance. Taking into consideration my finding that CGX attenuates the development of DOCA-salt hypertension, I hypothesized that either splanchnic SNA or prejunctional release of NE is increased, and that this would be reflected in increased NE spillover from the splanchnic region during the onset of hypertension. Therefore, I measured splanchnic NE spillover during the development of mild DOCA-salt hypertension. I performed blood sampling from the portal vein to avoid hepatic NE extraction; thus, strictly speaking, I measured non-hepatic splanchnic NE spillover.

Experimental protocols

Non-hepatic splanchnic NE spillover: The experimental protocol is shown in **Figure 29**. Male Sprague Dawley rats weighing 250-275g were used for this experiment. Catheters were implanted in the femoral artery and vein, and the portal vein for chronic infusion and sampling purposes. After a 7 day recovery period, control hemodynamic measurements were made for 3 days. A DOCA pellet was then implanted subcutaneously to induce hypertension development. MAP and HR were measured for another 3 weeks. Non-hepatic splanchnic NE spillover was measured on control day 2, and days 7, 14, and 21 following DOCA treatment.

Effect of CGX on non-hepatic splanchnic NE spillover: The experimental protocol is shown in **Figure 30**. CGX surgery was performed on a group of rats and catheters were

implanted to measure non-hepatic splanchnic NE spillover. Rats were allowed to recover for 10 days. Hemodynamic measurements were made for 3 days following recovery. Non-hepatic splanchnic NE spillover was then measured in all rats.

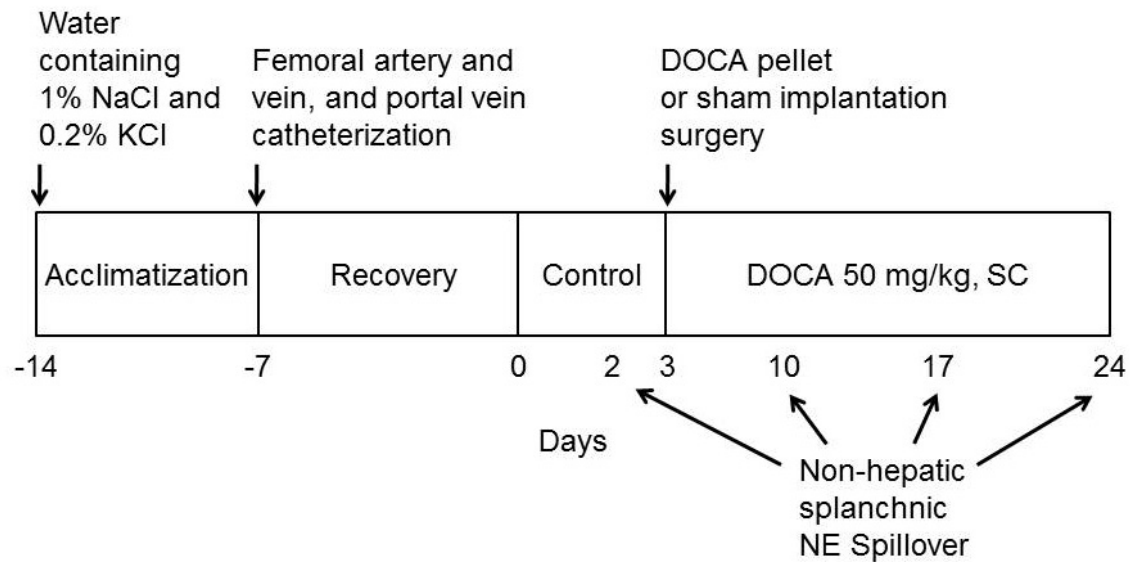


Figure 29. Protocol for non-hepatic splanchnic NE spillover in the development of mild DOCA-salt hypertension.

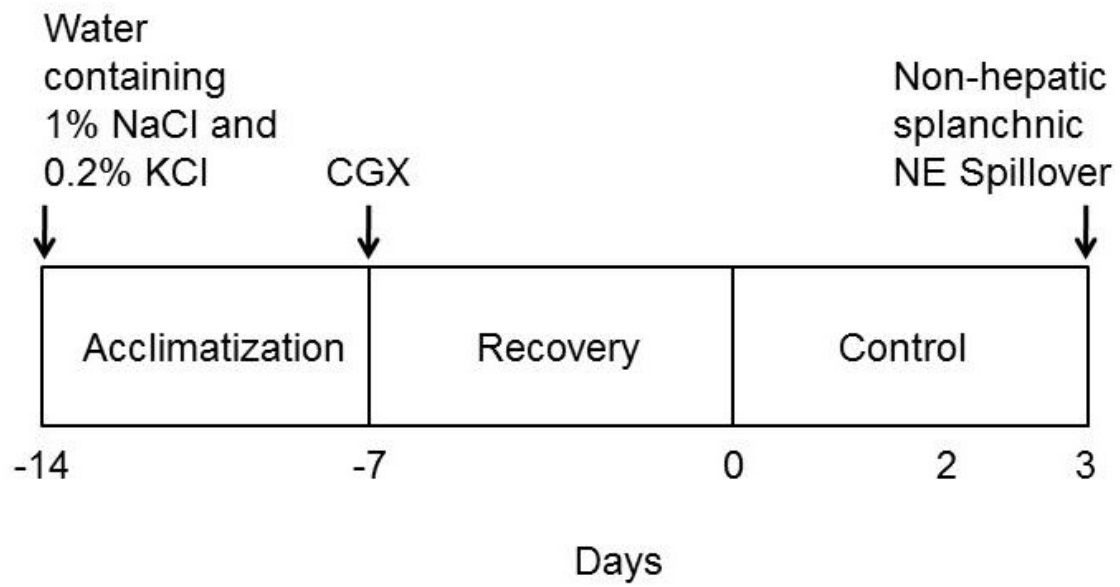


Figure 30. Protocol for the effect of CGX on non-hepatic splanchnic NE spillover.

Results

Non-hepatic splanchnic NE spillover: MAP was similar in DOCA and SHAM rats during the control period. DOCA rats became significantly hypertensive after DOCA treatment while the SHAM rats remained normotensive throughout the experimental period (**Figure 31**). There was no difference in HR between the two groups at any time during the experimental period (**Figure 32**). Arterial (**Figure 33**) and portal venous (**Figure 34**) plasma NE were not different at any time in DOCA versus SHAM rats. NE concentration was higher in portal vein plasma than in arterial plasma in all groups, but the difference was not statistically significant on all experimental days. Fractional extraction of NE was also similar in both groups (**Figure 35**). Hence, non-hepatic splanchnic NE spillover was not different at any time in DOCA versus SHAM rats (**Figure 36**).

Effect of CGX on non-hepatic splanchnic NE spillover: MAP before measuring non-hepatic splanchnic NE spillover in CGX rats was 110.1 ± 1.7 mmHg. Non-hepatic splanchnic NE spillover after CGX was 0.5 ± 0.53 ng/min/kg, which was not significantly different from zero (**Figure 37**)

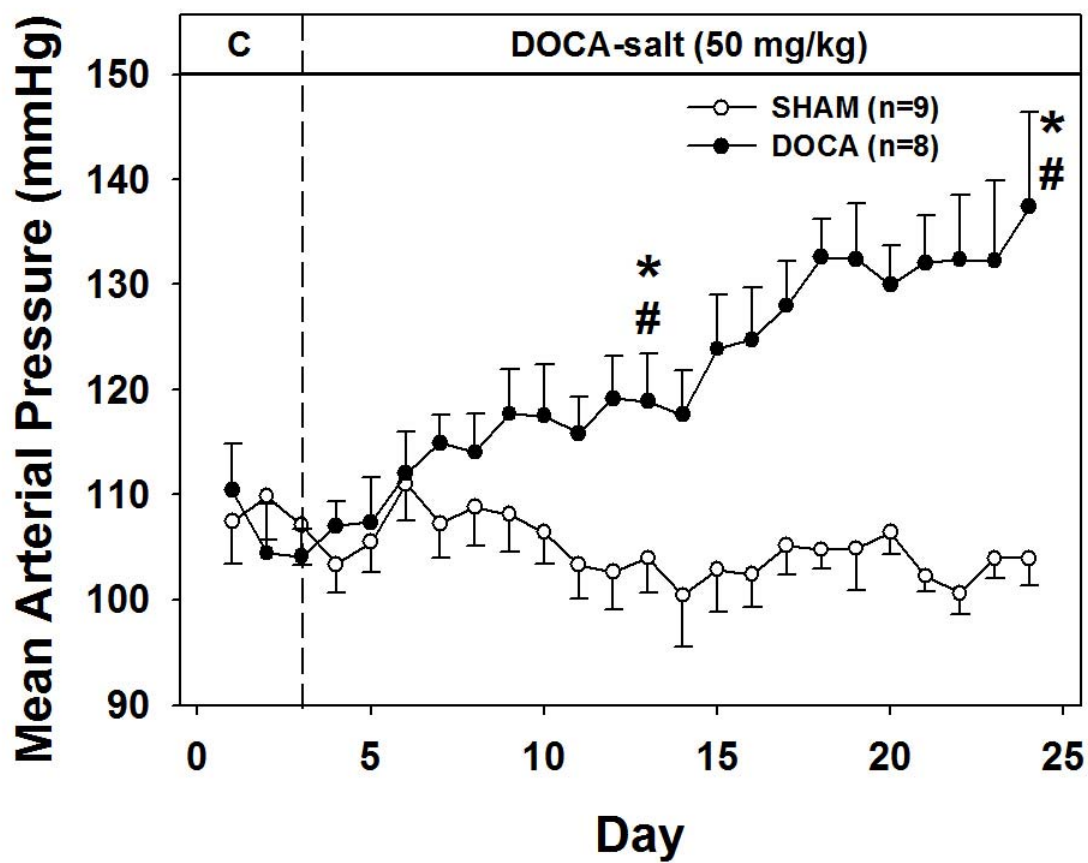


Figure 31. MAP during the control and DOCA treatment periods in DOCA and SHAM rats.

Asterisk (*) indicates a significant difference from day 3 (control period) values. Pound sign (#) indicates difference between the two groups. 'C' indicates control.

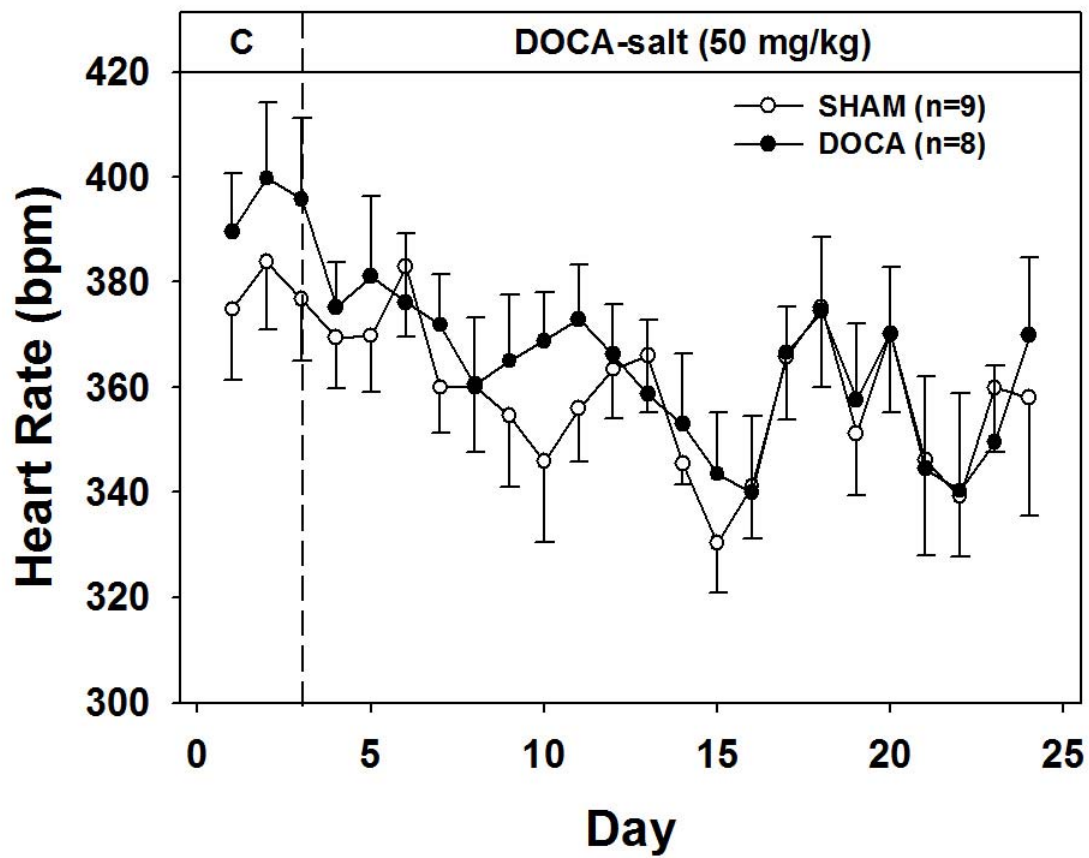


Figure 32. HR during the control and DOCA treatment periods in DOCA and SHAM rats.

'C' indicates control.

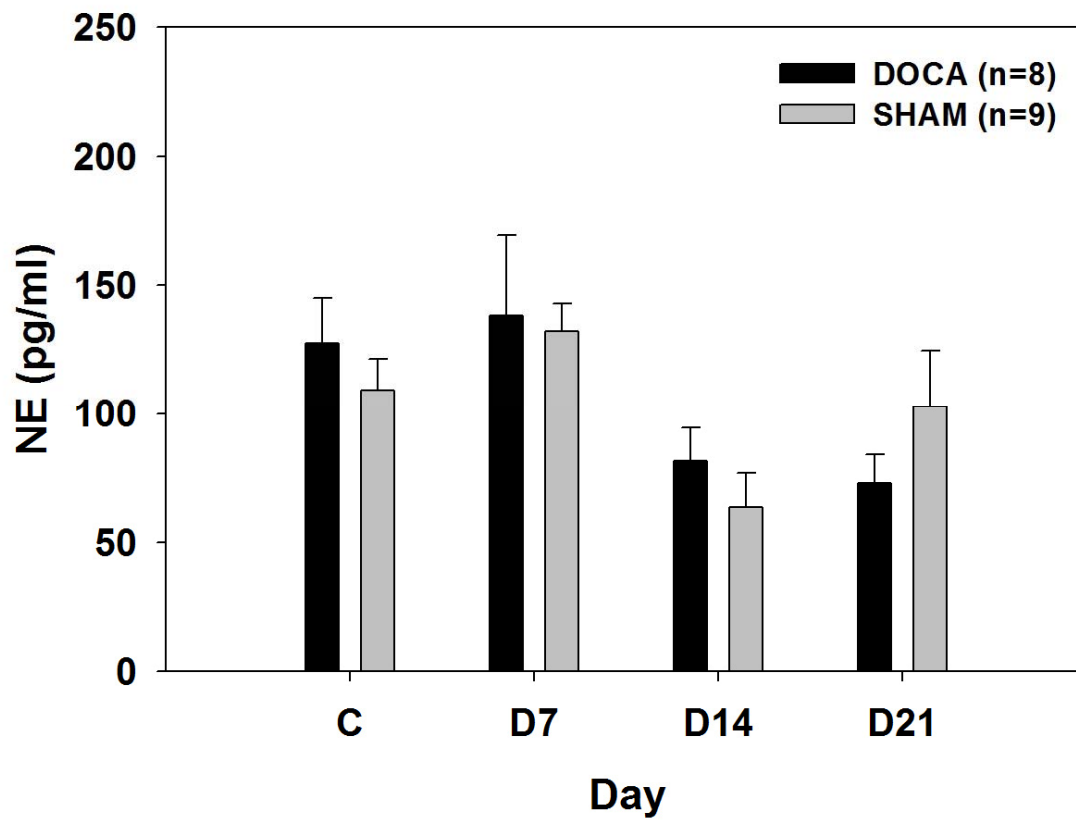


Figure 33. Arterial plasma NE in DOCA and SHAM rats on control day 2, and days 7, 14 and 21 after DOCA treatment.

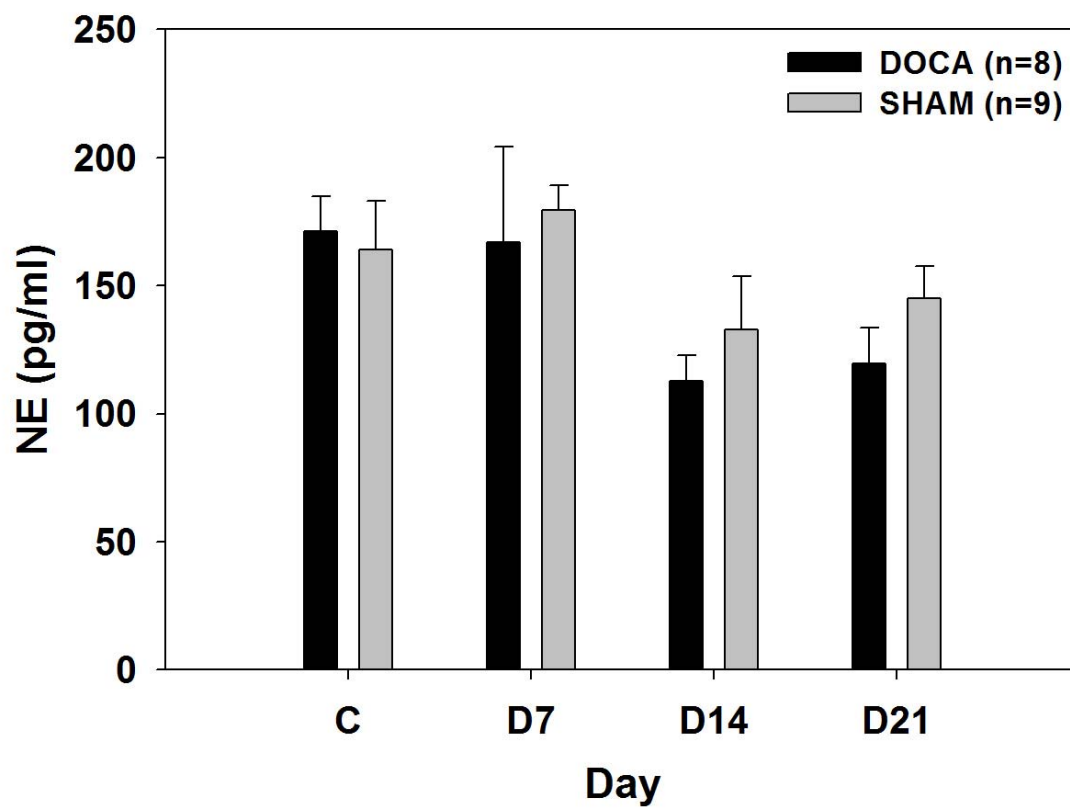


Figure 34. Portal venous plasma NE in DOCA and SHAM rats on control day 2, and days 7, 14 and 21 after DOCA treatment.

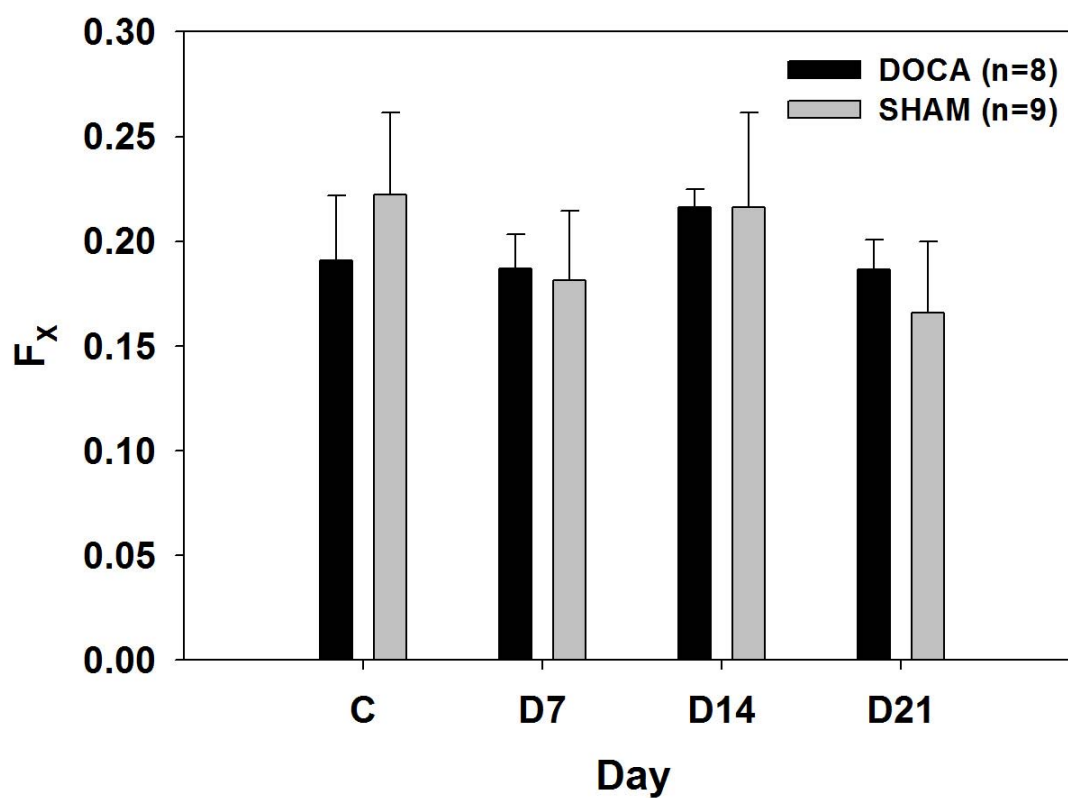


Figure 35. Fractional extraction of NE (FX) in DOCA and SHAM rats on control day 2, and days 7, 14 and 21 after DOCA treatment.

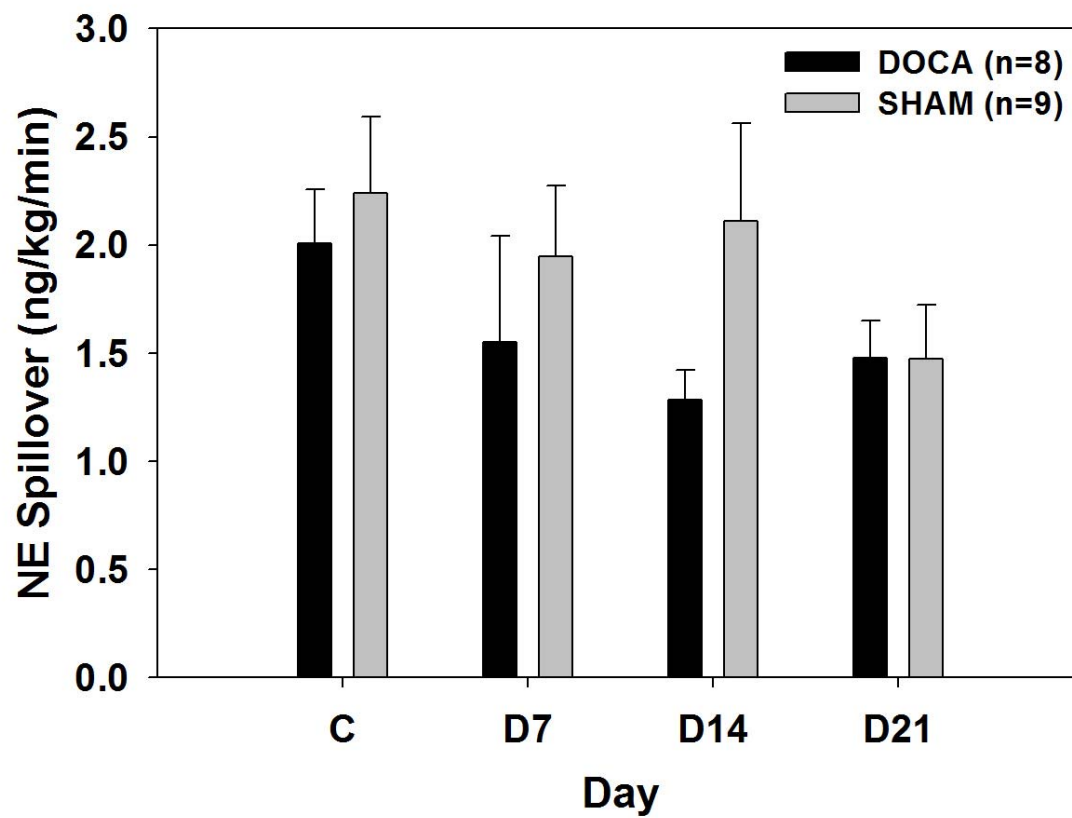


Figure 36. Non-hepatic splanchnic NE spillover in DOCA and SHAM rats on control day 2, and days 7, 14 and 21 after DOCA treatment.

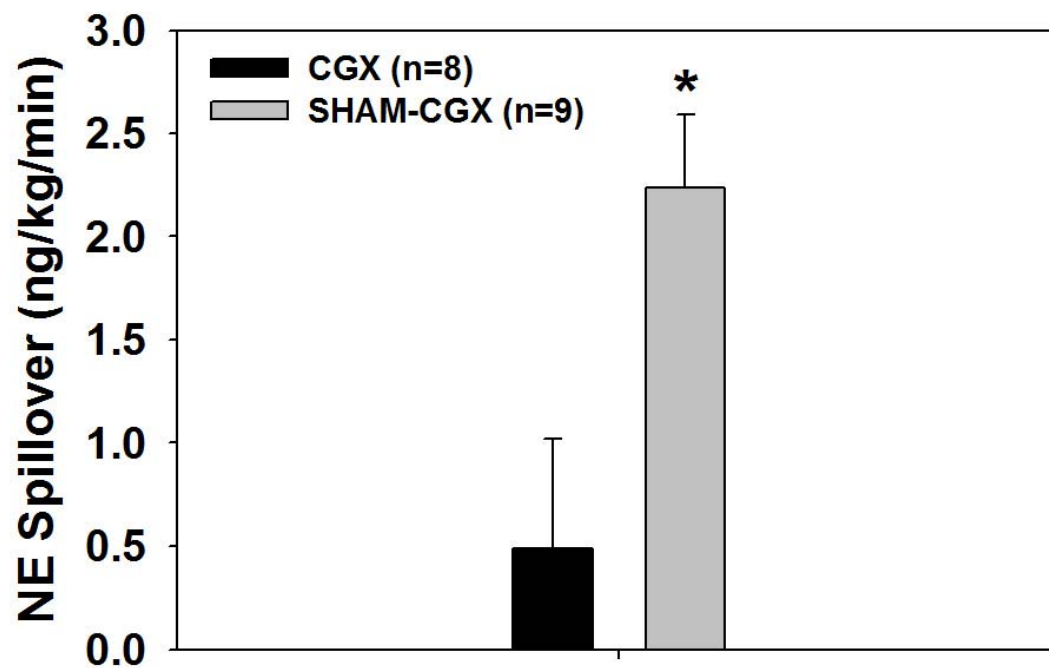


Figure 37. Non-hepatic splanchnic NE spillover in CGX and SHAM-CGX rats.

Asterisk indicates a value that is significantly different from zero.

Discussion

In this experiment, I assessed splanchnic SNA during the development of mild DOCA-salt hypertension using the radioisotope dilution technique to measure the rate of NE release from non-hepatic splanchnic organs (i.e. non-hepatic splanchnic NE spillover). I also studied the effect of splanchnic denervation (via CGX) on non-hepatic splanchnic NE spillover. My major findings were that 1) splanchnic SNA, as measured by non-hepatic splanchnic spillover, is unchanged in mild DOCA-salt hypertension development, 2) CGX is an effective technique to denervate splanchnic organs, and 3) the non-hepatic splanchnic NE spillover method can detect large changes in splanchnic SNA.

Due to inaccessibility of splanchnic venous drainage in humans, most of the studies examining NE kinetics have been performed from samples from arterial and hepatic venous plasma (Esler et al., 1984a; Henriksen et al., 1987; Cox et al., 1995). These measurements are of limited value for several reasons : 1) liver and splanchnic organs are in series and this does not allow accurate estimation of NE spillover by non-hepatic splanchnic organs, 2) the hepatic vein receives venous drainage from several organs such as the gastro-intestinal tract, liver, spleen, and pancreas, 3) the liver receives its blood supply from two sources, hepatic artery and portal vein, which makes estimation of splanchnic NE release rate rather difficult and 4) the liver is highly efficient in extracting NE that is released into the portal circulation by splanchnic organs and only a small fraction that exits through hepatic vein is measured as spillover when sampling is

done from the hepatic vein. In order to avoid these issues, sampling of portal venous blood is ideal and provides direct estimate of the amount of NE released by splanchnic organs before it enters the liver. Non-hepatic splanchnic NE measurements have been reported in anesthetized patients and animals (Ipp et al., 1991; Aneman et al., 1995; Aneman et al., 1996), and in conscious large animals (Coker et al., 1997). This is the first study to report non-hepatic splanchnic NE spillover in conscious, undisturbed rats.

Non-hepatic splanchnic NE spillover rate can be derived by measuring radiolabeled and endogenous NE concentrations in the arterial and portal venous plasma, and the portal venous plasma flow. It has been reported that mesenteric blood flow did not change during chronic administration of aldosterone for 28 days (Yamamoto et al., 1984; Huang et al., 1992b; May, 2006). For this reason, I assumed constant plasma flow in DOCA and SHAM rats. The value of portal venous plasma flow was obtained from a previous study performed in our laboratory (unpublished data).

Consistent with the whole body NE spillover experiment, arterial plasma NE concentration was similar in DOCA and SHAM groups throughout the experimental period (**Figure 33**). This again confirms our previous finding that global sympathetic activity is unchanged during hypertension development.

It has been reported that portal venous plasma NE concentrations are two-fold or higher than arterial plasma NE concentrations indicating that mesenteric organs release substantial amount of NE into the portal vein (Aneman et al., 1995; Aneman et al., 1996). In this study, although not consistently statistically significant, there was a tendency for portal venous plasma NE concentrations to be higher than arterial plasma NE concentration. This agrees with previous findings and supports the idea splanchnic organs are exposed to relatively high SNA under normal circumstances.

Fractional extraction (F_X) is the fraction of NE extracted from plasma during passage through splanchnic organs. F_X is largely dependent upon regional blood flow. An increase in blood flow reduces F_X and vice versa (Eisenhofer, 2005). In this study, there was no difference in F_X between the two groups at any time during the experiment. This suggests that mild DOCA-salt hypertension is not associated with altered NE extraction in the splanchnic organs. As discussed earlier, there is evidence both for and against alterations in NE removal from the neuroeffector junction in the mesenteric bed of rats with traditional DOCA-salt hypertension.

Non-hepatic splanchnic NE spillover was not different in the SHAM and DOCA groups during the control or DOCA treatment periods. I conclude that splanchnic SNA is not increased during the development of mild DOCA-salt hypertension. This is surprising because selective splanchnic denervation via CGX caused a significant attenuation of hypertension. There are several possible explanations for this finding. It has been

reported that mesenteric vascular responsiveness to NE is increased in traditional DOCA-salt hypertension (Masuyama et al., 1984; Tsuda et al., 1986). This increased vascular responsiveness is Ca^{2+} -dependent. It is possible that administration of DOCA and salt “primes” the mesenteric vasculature for the actions of NE by altering Ca^{2+} -signaling (Tsuda et al., 1989a). Therefore, even normal levels of sympathetic activity are sufficient to produce vasoconstriction in the splanchnic bed. Similarly, another study found increased reactivity to NE released from sympathetic terminals in mesenteric veins of traditional DOCA-salt rats (Xu et al., 2007). This increased reactivity was responsible for vascular smooth muscle cell depolarization and an increase in venomotor tone. Several investigators have reported increased release of NE from sympathetic nerve terminals in the mesenteric circulation of traditional DOCA-salt hypertensive rats (de Champlain et al., 1989; Tsuda et al., 1989b; Park et al., 2010). However, increased NE release should have been detectable with the spillover method, but was not found in my studies. This could be explained by a difference between my model and the standard model.

Finally, a mismatch can exist between sympathetic nerve firing rate and NE synthesis, release, and overflow (Esler et al., 1990). There could be a mismatch between NE synthesis and release, sympathetic firing rate and NE release, and rates of NE release and overflow (Dubocovich and Langer, 1976; Majewski et al., 1982; Chang et al., 1986; Jie et al., 1987). This mismatch in NE kinetics could result in normal spillover rates in the presence of increased sympathetic activity. Therefore, it is possible that non-hepatic splanchnic spillover could be unchanged even when sympathetic outflow to the

splanchnic bed is increased. Direct recording of splanchnic SNA would be necessary to address this possibility.

Catheter-based renal denervation in resistant hypertension attenuated renal NE spillover by 47% (Krum et al., 2009). To test whether splanchnic denervation attenuates splanchnic NE spillover and to validate the non-hepatic splanchnic NE spillover technique, I performed CGX in a group of rats and then measured non-hepatic splanchnic NE spillover. I expected that CGX, by interrupting the majority of sympathetic outflow to splanchnic organs, would attenuate the release of NE into the splanchnic venous drainage. This would result in a decrease in measured non-hepatic splanchnic spillover. I found that CGX completely abolished the release of NE from the splanchnic bed as measured using this method. This suggests that not only is CGX successful in denervating splanchnic organs, but that the non-hepatic splanchnic NE spillover technique can detect changes in release of NE from splanchnic nerves.

OVERALL CONCLUSIONS: Splanchnic SNA, as measured by non-hepatic splanchnic NE spillover, is not increased in the development of mild DOCA-salt hypertension. Hence, normal levels of SNA to splanchnic organs appear to be sufficient to maintain hypertension. The non-hepatic splanchnic NE spillover technique can detect large changes in splanchnic NE spillover. CGX virtually eliminates sympathetic activity to the splanchnic region.

CHAPTER SIX: GENERAL CONCLUSIONS

The purpose of the work presented in this dissertation was to evaluate the mechanisms by which the sympathetic nervous system is involved in long-term control of blood pressure. I used a DOCA-salt rat model because an elevation in circulating mineralocorticoids has been linked to essential hypertension and increased SNA in human patients. I sought to create a modified model of DOCA-salt hypertension in which the rate and magnitude of hypertension development was reduced compared to the traditional model, to make the model more closely resemble human hypertension; and to allow a more clear identification of factors involved in the development (versus maintenance) of hypertension. Because previous studies have indicated a possible role for both *altered renal function* and *vascular capacitance* in hypertension, the major focus of my work was to understand how *renal* and *splanchnic* SNA contribute to hypertension development.

I established a DOCA-salt model of hypertension that met my objectives, i.e. blood pressure rose slowly and peaked at approximately 15-20 mmHg above basal values. I found that global sympathetic activity was unchanged during the development of hypertension. I found no evidence that renal SNA is essential for hypertension development, but my results did suggest that renal afferents could increase SNA to other cardiovascular targets once hypertension was established. I found evidence that splanchnic SNA is important in hypertension development--splanchnic sympathectomy

using CGX prior to DOCA-salt treatment reduced the magnitude of the hypertension. However, my studies suggest the rate of NE release from splanchnic sympathetic nerves was not increased during hypertension development.

My overall conclusion is that *increased sympathetically mediated vasoconstrictor effects to the splanchnic organs likely occurs in mineralocorticoid hypertension due to increased responsiveness of the splanchnic blood vessels to norepinephrine, and that this contributes significantly to the raised arterial pressure.*

There are, however, a number of possible caveats to this conclusion. It is possible that my failure to see an increase in whole body NE spillover could be due to subtle increases in global sympathetic activity which are below the detection limits of the technique. However, whole body NE spillover is currently the best method available to assess overall sympathetic activity in humans and experimental models. Another limitation of the whole body NE spillover technique is that it does not measure regional sympathetic activity. Several studies in humans and animals models have suggested that sympathetic activity in hypertension is not uniform; it varies in different organs. Thus it is possible that hypertension could be driven by an increase in SNA to only one specific target, e.g. the kidney.

I used two approaches to address the possibility that increases in SNA to only specific organs would be sufficient to influence hypertension development. First, I employed region-specific surgical sympathectomy. Renal denervation indicated that renal nerves

are not essential for hypertension development. One important limitation to this conclusion is that renal denervation might not have been complete. Renal NE content in the RDX rats was decreased by 70%. This reduction was less than previously reported studies which had about 95% reduction in renal NE content. It has been demonstrated in the dog, that renal denervation reduced tissue NE content to zero when first measured at 2 weeks with recovery to 5% at 6 weeks, 42% at 12 weeks, and 96% at 16 weeks (Mogil et al., 1969; Mogil et al., 1970). And the plasma renin response to sodium depletion returned substantially toward normal when renal tissue NE content was 40% of control. Similarly, renal NE concentration in renal denervated SHR was 13, 25, 33, and 35% of that found in sham-operated SHR at 2, 4, 6, and 8 weeks, respectively, after denervation (Kline et al., 1980). The hypertension development was delayed but renal denervation did not prevent the development of hypertension. In renal-denervated WKY rats, the renal NE concentration was 11, 24, 32, and 34% of controls but AP was not significantly altered. Together, these data suggest that even incomplete denervation can have a marked effect on renal function. It seems likely that the >70% denervation in my study would be sufficient to affect the development of hypertension. Additional experiments with more thorough renal denervation could address this issue.

I did find that renal denervation attenuated the neurogenic depressor response to acute hexamethonium in the established phase of hypertension, suggesting a role of afferent renal nerves in modulating peripheral sympathetic activity. Previous reports indicate that renal denervation decreases peripheral sympathetic activity in hypertensive animals via disruption of renal sensory afferent nerves (Katholi et al., 1982a; Schlaich et al., 2009a).

It has been demonstrated that dorsal root rhizotomy, which selectively disrupts renal afferent nerves on the side of clipped kidney, attenuated 1K1C renovascular hypertension (Wyss et al., 1986). The same procedure on the side of the removed kidney did not alter hypertension. In order to assess the role of renal afferent nerves in my model, dorsal root rhizotomy could provide additional information. Another way to test the role of afferent renal nerves is by intrathecal administration of capsaicin which causes a selective destruction of renal afferent nerves in the spinal cord (Burg et al., 1994). However, it has been demonstrated in the traditional DOCA-salt hypertension model that renal afferent denervation by intrathecal capsaicin did not alter hypertension development (Burg et al., 1994).

The major finding of my study was that CGX attenuated DOCA-salt hypertension development. Both measurements of tissue NE content and non-hepatic splanchnic NE spillover indicated that CGX was effective in eliminating SNA to the splanchnic circulation. This strongly supports the importance of splanchnic SNA in the development of DOCA-salt hypertension. Nevertheless, non-hepatic splanchnic spillover did not change during hypertension development. This implies that neither splanchnic nerve firing rate nor NE release rate were increased. However, unchanged non-hepatic splanchnic spillover can occur even in the presence of increased splanchnic SNA. There could be a mismatch between splanchnic SNA (i.e. firing rate), NE release, and NE spillover. Hence, it is also possible that splanchnic SNA is actually increased but it is not detectable by the non-hepatic splanchnic spillover method. A superior approach to addressing this issue would be to use direct recording of splanchnic SNA before and

during hypertension development. I have worked towards mastering that technique, but do not yet have data on this key point.

As discussed previously, splanchnic neurotransmission is enhanced in traditional DOCA-salt hypertension. Taken together with my data, this suggests that in my model too *even normal levels of splanchnic SNA* also may be sufficient to increase sympathetic vasoconstriction in the splanchnic bed. Hypertension development could be due to increased splanchnic vascular resistance or capacitance as a result of increased *vascular responsiveness* to NE. This could be readily tested in my model using the same techniques that have been applied previously to study sympathetic neurotransmission and vascular responsiveness of mesenteric vessels in traditional DOCA-salt hypertension.

Another possibility is that CGX alters blood pressure by disrupting visceral sensory afferents. Activation of abdominal visceral afferents has been demonstrated to cause profound cardiovascular responses that include increases in blood pressure, HR, and cardiac contractility (Longhurst and Ibarra, 1982; Cervero, 1994; Pan et al., 1996). Activation of visceral afferents not only increased blood pressure, but also caused regional hemodynamic changes in the splanchnic vasculature and a differential increase in the sympathetic outflow to the splanchnic viscera, but not to the heart and somatic tissues (Pan et al., 2001). Thus, it is likely that blood pressure lowering effects of CGX in hypertension development could be mediated by visceral sensory afferent nerves. Recent studies demonstrate that selective ablation of sympathetic preganglionic

neurons is now possible using a ribosomal inactivating protein Saporin, that binds to and inactivates ribosomes (Lujan et al., 2009; Lujan et al., 2010). When conjugated with retrogradely transported molecule cholera toxin B subunit, which binds to specific membrane components of sympathetic preganglionic neurons, the complex caused cell death by inducing apoptosis (Bolognesi et al., 1996; Lujan et al., 2009). This technique could be used to selectively target sympathetic preganglionic neurons in the CG.

Splanchnic SNA could also contribute to the development of hypertension by mechanisms other than those affecting splanchnic blood vessels. Insulin is a vasodilator and the sympathetic nervous system plays a major role in insulin secretion; increased SNA tonically inhibits insulin secretion (Curry, 1983; Lee et al., 1993). Thus CGX, by disrupting the sympathetic supply to the pancreas, could increase insulin secretion which would in turn lower blood pressure. This possibility could easily be addressed by measuring insulin in CGX and SHAM rats.

It is possible that CGX affects water and salt absorption leading to a decrease in blood volume and blood pressure. I did not measure blood volume in my CGX rats, but other studies from our lab have not found a consistent effect of CGX on blood volume. Sodium and water balance experiments using metabolic cages could be performed to determine if CGX alters renal function.

A recent study found that CGX resulted in a 50% decrease in the epinephrine and NE responses to insulin-induced hypoglycemia suggesting that sympathoadrenal responses

to hypoglycemia were attenuated by CGX (Fujita and Donovan, 2005). The authors concluded that this effect of CGX was due to disruption of sympathetic efferents to the adrenal glands. Even though retrograde tracer studies of the adrenal medulla have failed to demonstrate any labeling of sympathetic efferent cells within the CG (Kesse et al., 1988), it is possible that some adrenal denervation might have occurred during the surgery. Therefore, CGX may have attenuated the release of epinephrine from adrenal gland and caused a reduction in blood pressure. Surgical removal of the adrenal gland during established DOCA-salt hypertension decreased blood pressure (De Champlain and Van Ameringen, 1972). Rats that were adrenalectomized and subsequently DOCA-salt treated did not develop hypertension (Moreau et al., 1993). Furthermore, adrenal medullae contribute more to the maintenance of blood pressure in DOCA-salt hypertension in male rats than female rats (Lange et al., 1998). Unpublished data from my lab found no changes in circulating NE or epinephrine after CGX in rats, but these measurements have not been performed in DOCA-salt hypertension.

Finally, it is possible that NE might not be the primary neurotransmitter involved in sympathetic neurotransmission in the mesenteric arteries of mild DOCA-salt hypertension. Therefore, the assays measuring NE spillover into the plasma would provide insufficient information about important aspect of splanchnic sympathetic activity in the development of hypertension. It has been reported that purinergic transmission is altered in DOCA-salt hypertension (Demel and Galligan, 2008; Demel et al., 2010; Park et al., 2010). Also, there is evidence suggesting the importance of NPY in neurotransmission in DOCA-salt hypertension (Moreau et al., 1992). Measurement of

portal venous NPY and/or purine levels could be used to investigate whether other neurotransmitters play a major role in sympathetic neurotransmission in hypertension development.

It is worth considering possible hemodynamic mechanisms that might link increased sympathetic vasoconstriction in the splanchnic region and hypertension development. An important factor leading to my central hypothesis was the possibility of blood volume redistribution due to changes in splanchnic vascular capacitance. Measuring venomotor tone using mean circulatory filling pressure during hypertension development could address this issue. Likewise, measuring splanchnic blood flow and calculating splanchnic vascular resistance during hypertension development would test the role of splanchnic arteries in the model. Making measurements in rats with CGX would allow assessment of the contribution of splanchnic SNA to any vascular changes observed.

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