THE ROLE OF INFLAMMATION AND OXIDATIVE STRESS ON ALPHA 2-ADRENERGIC RECEPTOR-MEDIATED INHIBITION OF CALCIUM CHANNELS IN DOCA-SALT AND OBESITY-RELATED HYPERTENSION

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ABSTRACT

THE ROLE OF INFLAMMATION AND OXIDATIVE STRESS ON ALPHA 2-ADRENERGIC RECEPTOR-MEDIATED INHIBITION OF CALCIUM CHANNELS IN DOCA-SALT AND OBESITY-RELATED HYPERTENSION

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DOCA-salt and obesity-related hypertension are associated with inflammation and sympathetic nervous system hyperactivity. Prejunctional α_2-adrenergic receptors (α_2ARs) provide negative feedback to norepinephrine release from sympathetic nerves through inhibition of N-type Ca^{2+} channels. Increased neuronal norepinephrine release in DOCA-salt and obesity-related hypertension occurs through impaired α_2AR signaling, however, the mechanisms involved are still unclear. Mesenteric arteries are resistance arteries that receive sympathetic innervation from the superior mesenteric and celiac ganglia (SMCG). We tested the hypothesis that macrophages and oxidative stress impair α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons in hypertension development and maintenance in DOCA-salt hypertensive rats and obesity-related hypertensive Sprague Dawley (SD) and Dahl salt-sensitive (DahlS) rats. DOCA-salt treated rats developed severe hypertension. On a high fat diet (HFD), SD rats developed obesity with mild hypertension while Dahl S rats developed obesity with severe hypertension. Only DOCA-salt hypertension was associated with increased vascular macrophage accumulation in mesenteric arteries and increased SMCG mRNA levels of monocyte chemoattractant protein-1 and tumor necrosis factor-α. Impaired α_2AR-mediated inhibition of Ca^{2+} currents occurred in SMCG neurons from DOCA-salt hypertensive rats and to a milder extent, in obese mildly hypertensive SD rats. This did not occur in SMCG neurons from

obese hypertensive DahlS rats. In DOCA-salt hypertension, α_2AR dysfunction was not associated with changes in receptor expression, GRK-mediated desensitization, or downstream signaling. In DOCA-salt treated rats, macrophage depletion using clodronate-liposomes preserved α_2AR function in SMCG neurons and protected against hypertension development. Oxidative stress, specifically thiol-oxidation, impaired α_2AR-mediated inhibition of Ca^{2+} currents in SMCG neurons and resulted in receptor internalization to the perinuclear cytoplasm in HEK-293T cells. These results suggest that macrophage-mediated α_2AR dysfunction may be more relevant in states of mineralocorticoid-salt excess. Furthermore, oxidative stress may impair α_2AR signaling by promoting mechanisms of receptor internalization.

This thesis is dedicated to the many positive role models in my life, both past and present. When you have the right people supporting you, anything and everything becomes possible.

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KEY TO ABBREVIATIONS

α_1AR- α_1 adrenergic receptor

α_{2A}- α_2 adrenergic receptor subtype 2A

α_{2B}- α_2 adrenergic receptor subtype 2B

α_{2C}- α_2 adrenergic receptor subtype 2C

α_2AR- α_2 adrenergic receptor

Ach- Acetylcholine

ANG-II – Angiotensin II

AP- Area postrema

AT1R- Angiotensin 1 receptor

ATP- Adenosine triphosphate

BBB-Blood brain barrier

CRP- C-reactive protein

DahlS- Dahl salt-sensitive

DCT- Distal convoluted tubule

DOPA- Dihydroxyphenylalanine

eNOS- Endothelial nitrogen oxide synthase

GM-CSF- granulocyte-macrophage colony-stimulating factor

GppNHp- 5’-guanylyl imidodiphosphate

GPCR- G protein-coupled receptor

GRK- G protein-coupled receptor kinase

HA- Hemagglutinin

HBSS- Hank’s buffered salt solution

HFD- High fat diet

IML- Intermediolateral

ICAM-1- Intracellular adhesion molecule 1

JAM-1- Junctional adhesion modlecule-1

IL-6- Interleukin 6

M3 – Muscarinic 3 receptor

MCP-1- Monocyte chemoattractant protein-1

NADPH- nicotinamide adenine dinucleotide phosphate

NE- Norepinephrine

NET- Norepinephrine transporter

NFD- Normal fat diet

NO- Nitric oxide

NPY- Neuropeptide Y

NSF- N-ethyl-maleimide-sensitive factor (NSF)

NTS- Nucleus of the solitary tract

OP- Obesity-prone Sprague Dawley

OR- Obesity-resistant Sprague Dawley

OVLT- Organum vasculosum of the lamina terminalis

PPARγ- Peroxisome proliferator-activated receptor

PVN- Paraventricular nucleus

RAAS- Renin-angiotensin-aldosterone system

RAS- Renin-angiotensin system

ROS- Reactive oxygen species

RVLM- Rostral ventrolateral medulla

SD- Sprague-Dawley

SFO- Subfornical organ

SHR- Spontaneously hypertensive rat

SCG- Superior cervical ganglia

SMCG- Superior mesenteric and celiac ganglia

SNA- Sympathetic nerve activity

SNAP- Soluble N-ethyl-maleimide-sensitive factor attachment protein

SNAP-25- 25 kDa synaptosome-associated protein

SNARE- N-ethylmaleimide sensitive factor attachment protein receptor

SNS- Sympathetic nervous system

TNFα- Tumor necrosis factor-α

t-SNARE- Target membrane N-ethylmaleimide sensitive factor attachment protein receptor

TTX- Tetrodotoxin

v-SNARE- Vesicular membrane N-ethylmaleimide sensitive factor attachment protein receptor

WK- Wistar Kyoto

CHAPTER 1: GENERAL INTRODUCTION

1.0 Human Hypertension:

1.1 Blood Pressure Relationships

Blood pressure is a measurement of force exerted against the vascular wall of large elastic arteries that is proportional to cardiac output and peripheral vascular resistance to blood flow [1], as depicted in Figure 1. Cardiac output is the product of heart rate and stroke volume, the latter of which is influenced by myocardial contractility, preload, and afterload [2]. Cardiac output receives innervation from both the sympathetic (increases cardiac output) and parasympathetic (decreases cardiac output) nervous system [3]. Peripheral vascular resistance is contributed by small muscular resistance arteries, where resistance is inversely proportional to radius to the fourth power [1]. The majority of blood vessels receive innervation only from the sympathetic nervous system [3].

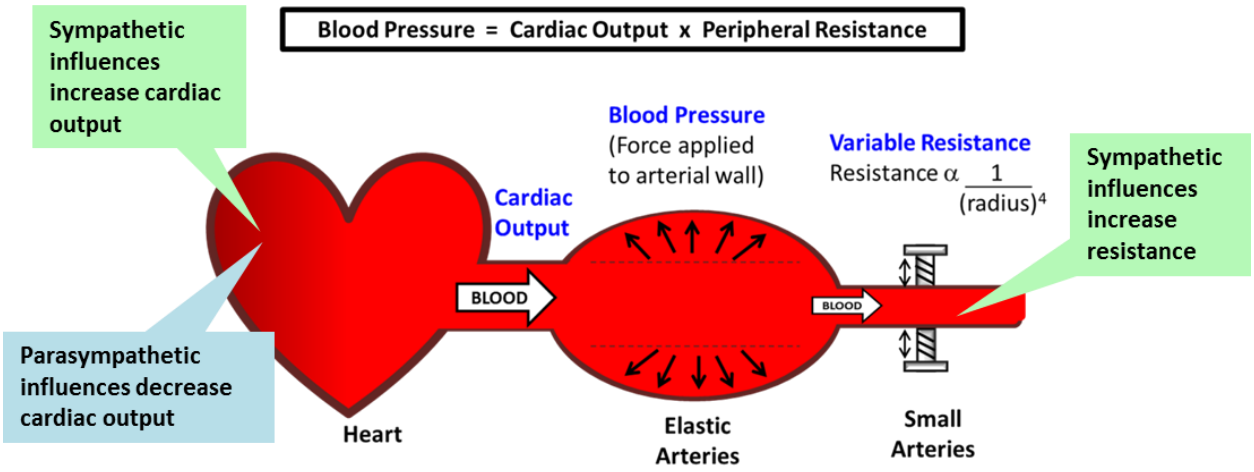

Figure 1. Relationship between blood pressure, cardiac output, and peripheral vascular resistance. Increases in cardiac output and/or decreases in diameter of small resistance arteries result in the accumulation of blood in large elastic arteries, which increases blood pressure.

1.2 Hypertension: Definition and Conventional Treatment Strategies

Definition: In humans, hypertension is defined in adults by systolic blood pressures equal or greater to 140 mmHg and/or diastolic blood pressures equal or greater to 90 mmHg. Hypertension development does not usually present with clinical symptoms [4], however, leads to significant morbidity and mortality as a major risk factor for stroke, heart disease, and kidney failure [5]. The risk of cardiovascular disease begins to increase as early as 115/75 mmHg [6, 7], suggesting that there is benefit in reducing blood pressure even before hypertension begins.

Treatment: Hypertension is the leading cause of preventable death in the United States [8] and therefore, requires aggressive diagnosis and treatment. Hypertension is the most common chronic condition managed by health care providers [9]. Unfortunately, clinical management success rates remain poor [10, 11] and novel approaches and therapeutic targets are needed. Pre-hypertension (where blood pressures can range from 120/80-139/89 mmHg) is typically managed by lifestyle modifications. Exercise, weight loss, and decreasing dietary salt are commonly encouraged to delay or prevent hypertension development [9]. When hypertension develops, treatment warrants pharmacological intervention, usually involving more than one antihypertensive drug consisting either an angiotensin converting enzyme antagonist (or angiotensin receptor blocker), a calcium channel blocker, or a diuretic [9]. Lifestyle modifications are equally important in hypertension management. Salt intake and obesity can affect hypotensive therapy [12-14] and are therefore important therapeutic targets.

Resistant hypertension: A significant proportion of hypertensive patients are resistant to conventional therapy. Resistant hypertension occurs when hypertension is sustained despite the use of three anti-hypertensive drugs of different classes [15]. Resistant hypertension affects

5-30% of hypertensive patients [16] and furthers the risk of adverse cardiovascular-related complications already associated with hypertension [17, 18]. Although universal therapeutic recommendations are not yet determined, the addition of aldosterone antagonists in the treatment of resistant hypertension appear to quite effective [19].

1.3 Hypertension: Prevalence and Risk Factor-Related Trends

The prevalence of human hypertension generally increases with age, typically beginning after 25 years and becoming apparent after 50 years [20, 21]. Although hypertension development is often regarded as a normal process of aging, the high prevalence of hypertension cannot be explained only by an aging population. It is now clear that hypertension is associated with common risk factors like high dietary salt intake and obesity [9, 22]. While the clinical manifestations of hypertension become evident mainly in adulthood, factors that contribute to its development may be rooted in childhood. Although hypertension cut-offs are not clearly defined in children, higher blood pressures in childhood are able to predict hypertension development and severity in adulthood [23-25]. Interestingly, the prevalence of childhood and adolescent hypertension is on the rise and may be a attributed to the increased consumption of processed foods and beverages, which leads to earlier exposure to high dietary salt intake and obesity rates in children [26-31].

1.4 Hypertension: Categorizing Beyond Primary and Secondary Classifications

Primary versus secondary hypertension: The majority of human hypertension cases (~95%) are classified as primary (essential, idiopathic) hypertension, where there is sustained hypertension without an identifiable cause [9]. As a non-specific diagnosis, essential hypertension is difficult to treat and often results in suboptimal management that may involve inappropriate choice of anti-hypertension medication [32]. This is in contrast to secondary hypertension (~5% of cases), where increased blood pressure arises due to an underlying and often correctable medical condition [33]. Examples of secondary hypertension include hyperaldosteronism, renal artery stenosis, thyroid disorders, pheochromocytoma, and Cushing syndrome. Secondary hypertension can also result as a side effect from using medications such as oral contraception, decongestants, steroids, and amphetamines [33].

Although hypertension is heterogeneous in nature, identifying subgroups of hypertensive patients with common features may be useful in understanding the complex mechanisms that underlie hypertension development and maintenance.

Inflammation and increased SNA: The most consistent findings in human hypertension appear to be enhanced SNA and inflammation. With improvements in detection techniques, it is now clear that the majority of hypertensive patients, especially during the development phase, display enhanced SNA [34]. Inflammation is evidenced by levels of the inflammatory marker C-reactive protein (CRP), which predictably increase from normotension to pre- and overt hypertension, and identify those at risk of cardiovascular sequelae [35-38].

Salt-sensitivity: A significant subset of hypertensive patients is salt-sensitive. Although a universally defined definition is still lacking, it is generally accepted that salt-sensitivity reflects

a 5-10% increase in blood pressure to salt intake [39]. Salt-sensitivity affects roughly half of all essential hypertensive patients and a quarter of normotensive individuals, and may be due to underlying genetic predisposition [40, 41].

Obesity: It is estimated that the majority of hypertension cases are attributed to obesity [42, 43]. In humans, obesity is defined as body mass index greater or equal to 30 kg/m^2. Increases in body mass index is associated with hypertension development and blood pressure significantly decreases with weight loss [42].

Low-renin and aldosterone-excess: A percentage of hypertensive patients (25-33%) have low-renin hypertension [44, 45]. Of these, approximately 60-70% have resistant hypertension [44]. Low-renin hypertension is higher among blacks and the elderly, and is typically salt-sensitive [45]. Low-renin may be accompanied by high circulating levels of aldosterone (a mineralocorticoid) [44, 46], which may be due to idiopathic autonomous aldosterone hypersecretion [47, 48]. Primary hyperaldosteronism is the leading cause of secondary hypertension and is evident in conditions like Conn's syndrome, where aldosterone is produced in excess by the adrenal gland [49].

The effects of sympathetic hyperactivity, inflammation, salt, obesity, low-renin status and mineralocorticoids are all clinically relevant and are therefore exploited interventionally to induce hypertension development in experimental animal models to better understand human hypertension (see section 3- animal models for more detail).

2.0 Blood Pressure Regulation via the Sympathetic Nervous System

2.1 General Introduction to Sympathetic Blood Pressure Regulation

The sympathetic nervous system modulates both short and long term regulation of the cardiovascular system without conscious control [50, 51]. Sympathetic neuroeffector transmission increases blood pressure either by increasing cardiac output and by promoting vasoconstriction of resistance arteries [52]. Increased peripheral vascular resistance is the prominent hemodynamic disturbance in human hypertension as cardiac output is usually relatively within normal limits, possibly due to parasympathetic compensation [53, 54].

Sympathetic regulation of the splanchnic circulation: The splanchnic circulation provides the majority of the body's vascular resistance through mesenteric resistance arteries. Mesenteric resistance arteries are innervated by sympathetic neurons of the superior mesenteric and celiac ganglion (SMCG) [55, 56], are not innervated by parasympathetic neurons, and have profound influences on hemodynamics and blood pressure [57-60]. Sympathetic hyperactivity in the splanchnic circulation contributes to hypertension development. Splanchnic denervation decreases blood pressure in normal [61] and hypertensive rats [59, 60, 62, 63]. Furthermore, the density of sympathetic innervation to mesenteric resistance arteries is similar to other vascular beds, such as cerebral resistance vessels and femoral arterial beds [64, 65]. Therefore, investigating neurovascular mechanisms of blood pressure regulation in the splanchnic circulation has been the focus of many experimental hypertension studies and these approaches may have relevance in other vascular systems that are more difficult to study.

Neurogenic hypertension: Evidence suggests that a large component of essential hypertension development and maintenance is neurogenic [66]. The immediate effector response occurs at the neuroeffector junction. For instance in resistance arteries, depolarization of post-ganglionic sympathetic neurons causes the release of neurotransmitters from nerve terminals onto vascular smooth muscle. Baseline sympathetic nerve activity preserves vascular homeostatic tone while increased sympathetic firing rate increases blood pressure by vasoconstriction [52], as depicted in Figure 2. It is well established that sympathetic hyperactivity occurs in human essential hypertension as well as experimental hypertension animal models [67, 68]. However, in order to study potential mechanisms that underlie sympathetic alterations in hypertension, it is first important to understand how SNA is modulated at different levels of regulation, including both the peripheral nervous system (PNS) and central nervous system (CNS).

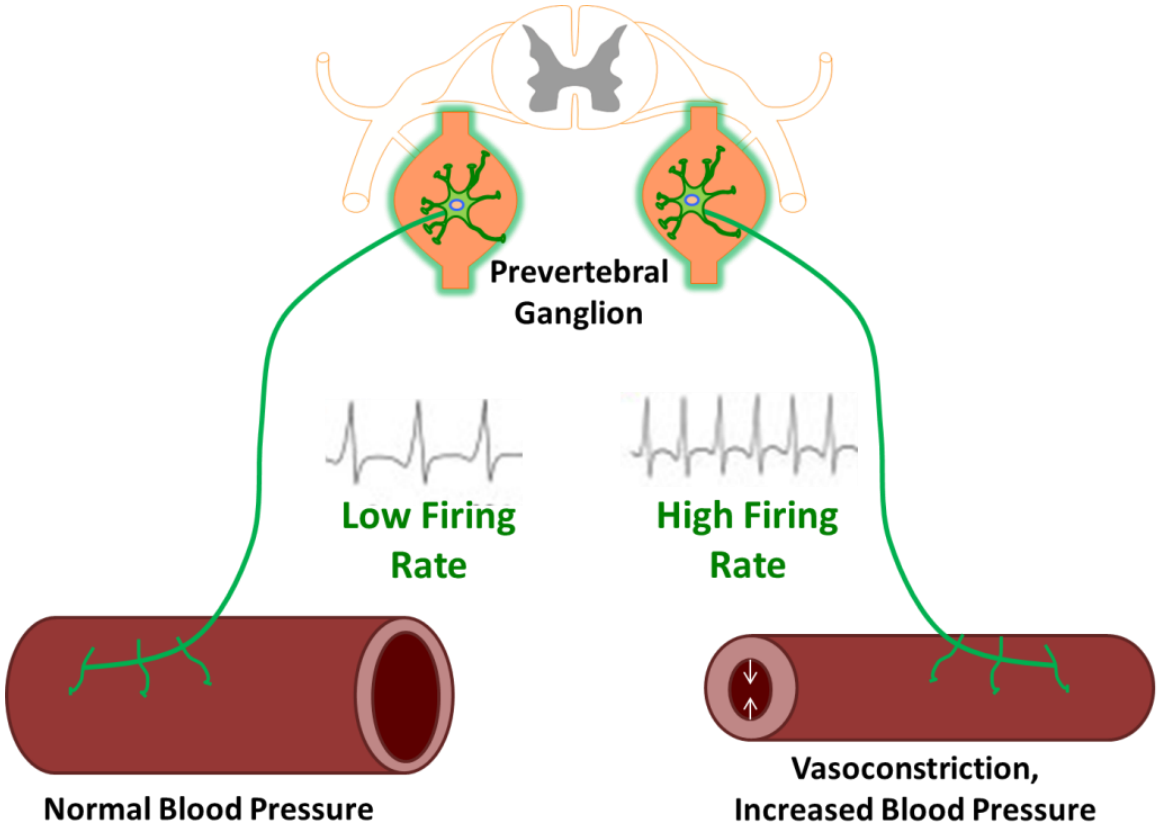

Figure 2. Arterial diameter and blood pressure is modulated by sympathetic nerve activity. Higher frequency of sympathetic firing results in increased neuroeffector transmission, resulting in the constriction of small resistance arteries and an increase in blood pressure.

2.2 Central Regulation of Sympathetic Nerve Activity

Sympathetic firing rate is largely determined by input received and processed by circumventricular organs (CVOs) in the central nervous system (CNS) as well as through autonomic reflexes. These are shown in Figure 3.

CVOs and autonomic regulatory centers: CVOs are specialized autonomic control centers. They are extensively vascularized without a proper blood-brain barrier because they monitor and respond to variations in systemic circulation composition, from basic blood chemistry to hormone levels. CVOs include the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and the area postrema (AP).

Sensory information received and interpreted by the CVOs provide feedback via distinct neuroanatomical connections to autonomic regulatory centers in the hypothalamus and brainstem. These centers include the paraventricular nucleus (PVN), the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM) [69]. Eventually, all transmitted information reaches the RVLM, which is the main driver that increases sympathetic outflow.

RVLM neurons activate preganglionic sympathetic neurons, whose cell bodies are mostly located in the intermediolateral (IML) nucleus of the thoraco-lumbar spinal cord. These neurons project either directly to glands such the adrenal medulla, or to paravertebral and prevertebral sympathetic ganglia, where they synapse onto post-ganglionic sympathetic neurons. Post-ganglionic sympathetic neurons innervate target organs of the cardiovascular and renovascular system [70], which are key players in blood pressure regulation.

A classic example of central mediated enhancement of SNA is hypernatremia, which can result from increased salt consumption [71]. OVLT and SFO neurons contain osmoreceptors

that can detect changes in osmotic pressure via osmosis. Hyperosmotic challenges activate these neurons. Increased glutamate and decreased GABA release activates neurons of the PVN and subsequently the RVLM [71-74]. RVLM efferent neurons activate peripheral sympathetic neurons, resulting in an increase in peripheral vascular resistance.

Autonomic Reflexes: Efferent sympathetic activity is also regulated by autonomic reflexes. Although often described as short-term mechanisms of blood pressure regulation, these reflexes can be modified to operate at higher blood pressures if hypertension is sustained for a long period of time. These reflexes involve sensory input from afferent fibers of the cranial nerves, which are then transmitted to the hypothalamus and brainstem [51].

Baroreceptor Reflex: The most classic example is the baroreceptor reflex. Baroreceptors are sensory mechanoreceptors located on vagal and glossopharyngeal nerves that innervate the carotid sinuses and aortic arch. Baroreceptors function to monitor and regulate acute changes in blood pressure and are tonically sympathoinhibitory at rest. Increased blood pressure from baseline stretches the vascular wall, resulting in increased baroreceptor sensory transmission to the NTS. This Information is transmitted to the RVLM, which decreases output to peripheral sympathetic neurons that innervate the heart and resistance arteries, allowing blood pressure to decrease back to baseline [51]. Similarly, decreases in blood pressure from baseline (such as with standing) reduces vascular stretch, decreasing baroreceptor sensory input to the NTS, resulting in an increase in sympathetic output [51]. Baroreceptors can adapt to prolonged changes in blood pressure by resetting their baseline response to operate at higher blood pressures. Studies of the baroreceptor reflex by phenylephrine bolus delivery (high pressure induction) and by inhalation of amyl nitrite (low pressure induction) suggest

impaired baroreceptor sensitivity in hypertensive individuals and even in normotensive individuals that have a family history of hypertension [75, 76].

Chemoreceptor Reflex: Another autonomic reflex is the chemoreceptor reflex. Chemoreceptors are also located in the carotid sinuses and aortic arch. Chemoreceptors activate when O_2 delivery and CO_2 removal is reduced, which occurs as a result of poor tissue perfusion when blood pressure falls (usually < 80 mmHg). Activated chemoreceptors transmit signals to the RVLM, resulting in enhanced sympathetic outflow to the cardiovascular and renovascular system [77, 78].

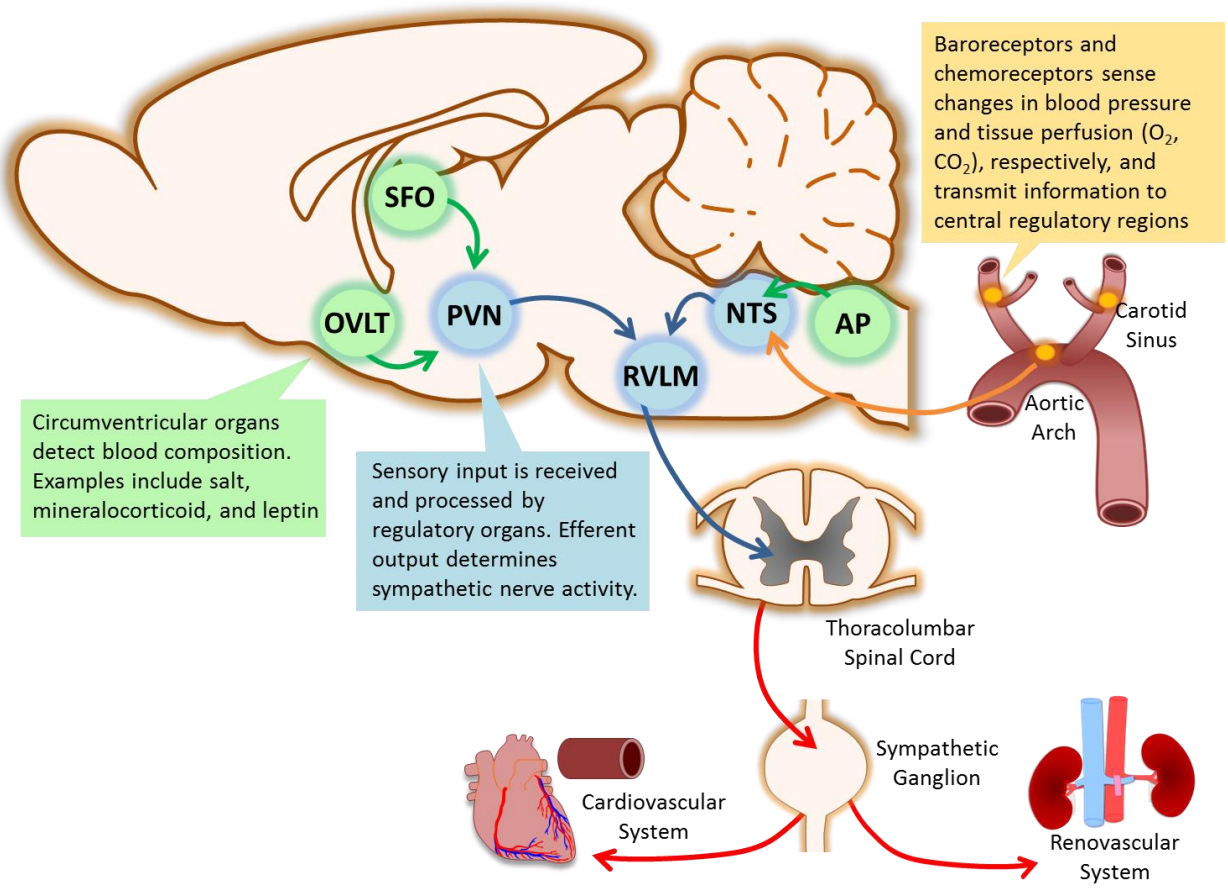

Figure 3. Central regulation of sympathetic nerve activity. Circumventricular organs, which include the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and area postrema (AP) monitor blood composition. This information is transmitted to regulatory organs, which include the paraventricular nucleus (PVN), the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM). Information eventually reaches the RVLM, which contains efferent neurons that synapse onto pre-ganglionic sympathetic neurons located in the intermediolateral nucleus of the thoracolumbar spinal cord. Preganglionic sympathetic neurons synapse onto post-ganglionic neurons in sympathetic ganglia that innervate cardiovascular and renovascular systems to regulate blood pressure. Sympathetic nerve activity is also regulated by autonomic reflexes. Baroreceptors and chemoreceptors in the carotid sinus and aortic arch transmit sensory information regarding blood pressure changes to the NTS, resulting in effector responses that attempt to restore blood pressure back to baseline.

2.3 Effects of Renovascular SNA on Blood Pressure

Renin-angiotensin-aldosterone system: Sympathetic regulation of renal function is important in blood pressure control. The kidney is innervated by sympathetic nerves and renal denervation attenuates increased blood pressure in the majority of salt-sensitive hypertension models [79]. Vasoconstriction from enhanced SNA to the kidney has consequences on Na^+ and water balance through the renin-angiotensin-aldosterone system (RAAS), as shown in Figure 7.

Firstly, renal SNA to the proximal tubules activates α_1ARs on renal tubular cells resulting in increased Na^+ and water reabsorption, and α_1ARs on renal afferent arterioles, potentiating vasoconstriction and reduced renal plasma flow [80, 81].

Secondly, Renal SNA also increases blood pressure indirectly through members of RAAS [82, 83]. Juxtaglomerular (JG) cells within renal afferent arterioles are innervated by sympathetic neurons. Enhanced SNA to JG cells activates β_1-adrenrgic receptors (β_1AR), resulting in release of the enzyme renin. Renin catalyzes the first step in the synthesis of angiotensin II (ANG-II). Through the angiotensin type I (AT1) receptor, ANG II increases blood pressure directly through vasoconstriction and by increasing Na^+ and water reabsorption by modulating ion transporter activity in the proximal tubule [84]. ANG-II also indirectly increases blood pressure by enhancing aldosterone secretion from the adrenal cortex. Aldosterone increases blood pressure through the cytosolic mineralocorticoid receptor by increasing Na^+ and water reabsorption at the expense of K^+ in the distal nephron. This is accomplished by increasing apical permeability through upregulation of epithelial sodium channels (ENaC) and by upregulating Na^+/K^+ ATPases in the basolateral membrane. Basolateral Na^+/K^+ATPase upregulation keeps intracellular levels of Na^+ low and K^+ high, which sets up a gradient for more

Na^+ (and water) reabsorption from the tubular lumen. K^+ efflux into the tubule counterbalances N^+ entry, and is the reason why states of mineralocorticoid excess are often associated with hypokalemia [82, 83].

The influences of RAAS on hypertension development extend beyond the renal system. ANG-II has been implicated in enhanced neurotransmission both in the central and peripheral nervous system. Staining for c-FOS shows that ANG-II infusion activates the PVN, SFO, and NTS [85], and microdialysis studies show that intracerebroventricular administration of ANG-II increases NE release in the hypothalamus [86], specifically in the PVN [87]. Furthermore, ANG-II may also be increasing SNA through oxidative stress production by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases in CVOs, which contributes to hypertension development [88]. In the periphery, ANG II has been shown to increase release of NE in cardiovascular tissues [89-92] and EP from the adrenal gland [93].

In addition to the kidney, aldosterone increases blood pressure through Na^+ and water reabsorption in the colon [94], and also by mediating cardiovascular tissue injury through inflammation and tissue remodeling [95, 96]. Finally, volume expansion from increased RAAS further increases blood pressure through local vasoconstriction that occurs independently of central control [97]. This local reflex is often overlooked, but can be quite powerful as seen in the development of recurrent intradialytic hypertension in patients [98].

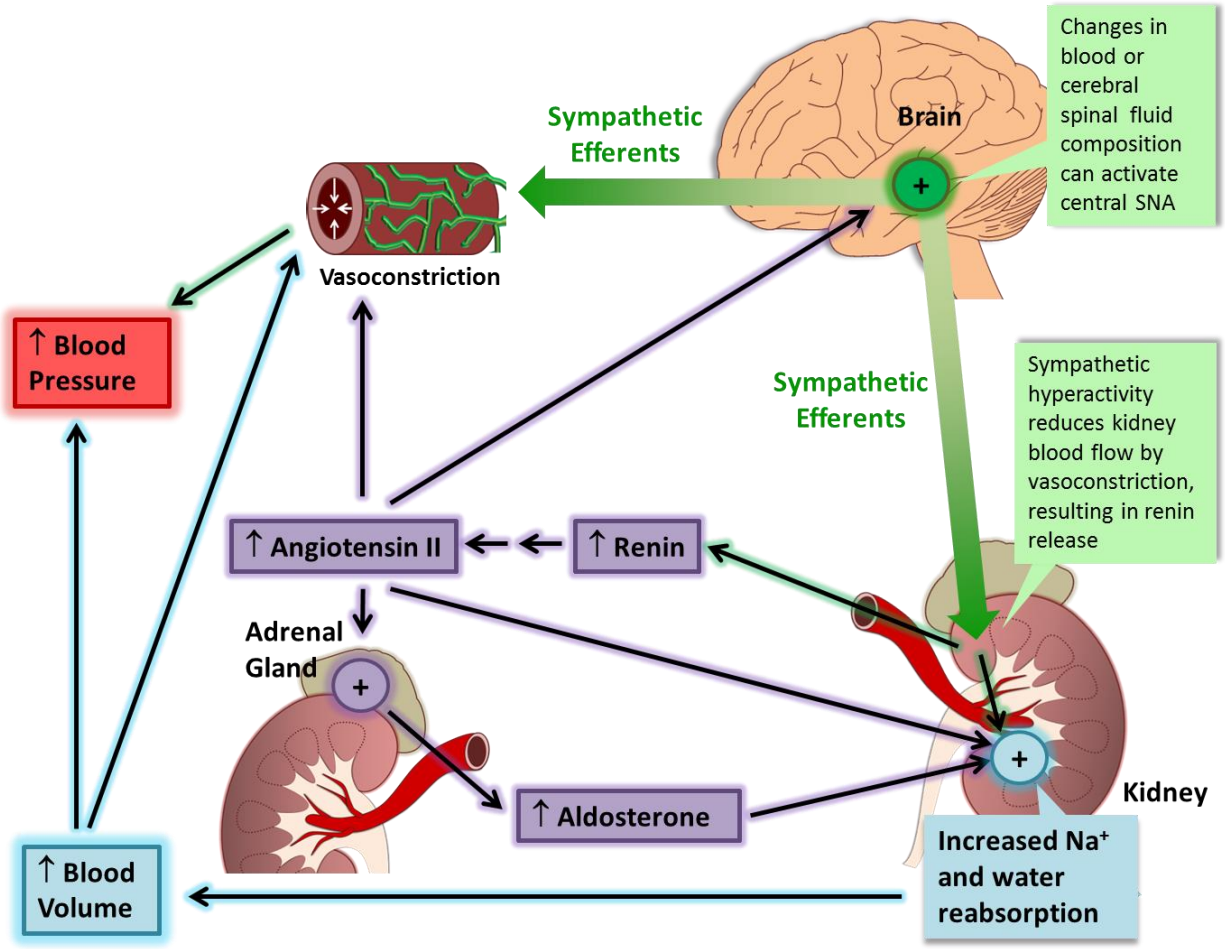

Figure 4. Enhanced SNA to the kidney increases blood pressure. Increased renal SNA enhances renin release from juxtaglomerular cells of renal afferent arterioles, leading to increased angiotensin II (ANG-II) production. ANG-II causes vasoconstriction, increases Na^+ and water reabsorption from the proximal tubule, and activates circumventricular organs in the brain, which further increases SNA. ANGII also increases secretion of aldosterone from the adrenal gland, which causes increased Na^+ and water reabsorption in the distal nephron. Volume expansion potentiates local vasoconstrictor responses that occur independent of central control. Increases in SNA, total peripheral vascular resistance, and volume expansion all combine to increase blood pressure.

Pressure-natriuresis: Renal Na^+ and water reabsorption is regulated by renal perfusion pressure. This pressure-natriuresis relationship prevents sustained fluctuations in blood pressure. This occurs because increases in blood pressure elevate renal perfusion pressure, which causes the kidneys to excrete more Na^+ and water [99]. Dehydration returns blood pressure back to baseline [78]. In early humans, this mechanism likely evolved for the opposite scenario, where decreases in blood pressure from severe dehydration or blood loss enabled survival by facilitating Na^+ and water retention [99, 100]. This mechanism is mediated in large by RAAS activity. JG cells secrete renin in response to a drop in renal perfusion pressure, which is sensed by a decrease in vascular stretch by mechanoreceptors [99].

Blood osmolarity affects the pressure-natriuresis curve and Na^+ intake is met with compensatory Na^+ clearance. JG cells regulate renin secretion also in response to feedback received by neighboring macula densa (MD) cells of the distal convoluted tubule (DCT), which contain chemoreceptors that sense tubular NaCl concentration. JC cells secrete renin when MD cells sense low NaCl in the DCT [101]. Therefore, high salt intake (which increases DCT NaCl) results in a left-shift in the pressure-natriuresis curve while low salt intake (which decreases DCT NaCl) results in a right-shift in the pressure-natriuresis curve. Salt intake also affects the slope of the pressure-natriuresis curve so that Na^+ regulation can occur over a wider span of blood pressures (Figure 8A).

Factors that determine the homeostatic set-point value of blood pressure are not completely understood. However, it is conceivable that for hypertension to occur and be maintained, this homeostatic set-point must be reset to operate at higher blood pressures. This results in a right-shift in pressure-natriuresis [102] (Figure 8B). Interestingly, obesity also results

in a right-shift in pressure-natriuresis (Figure 8C) and it is suggested that this occurs due to increased Na^+ and water reabsorption as a result of activation of the sympathetic nervous system and RAAS [103]. Salt-sensitive hypertension not only causes a right-shift in pressure-natriuresis, but also a decreases the slope [102] (Figure 8D). The steepness of the pressure-natriuresis curve reflects salt-sensitivity of blood pressure.

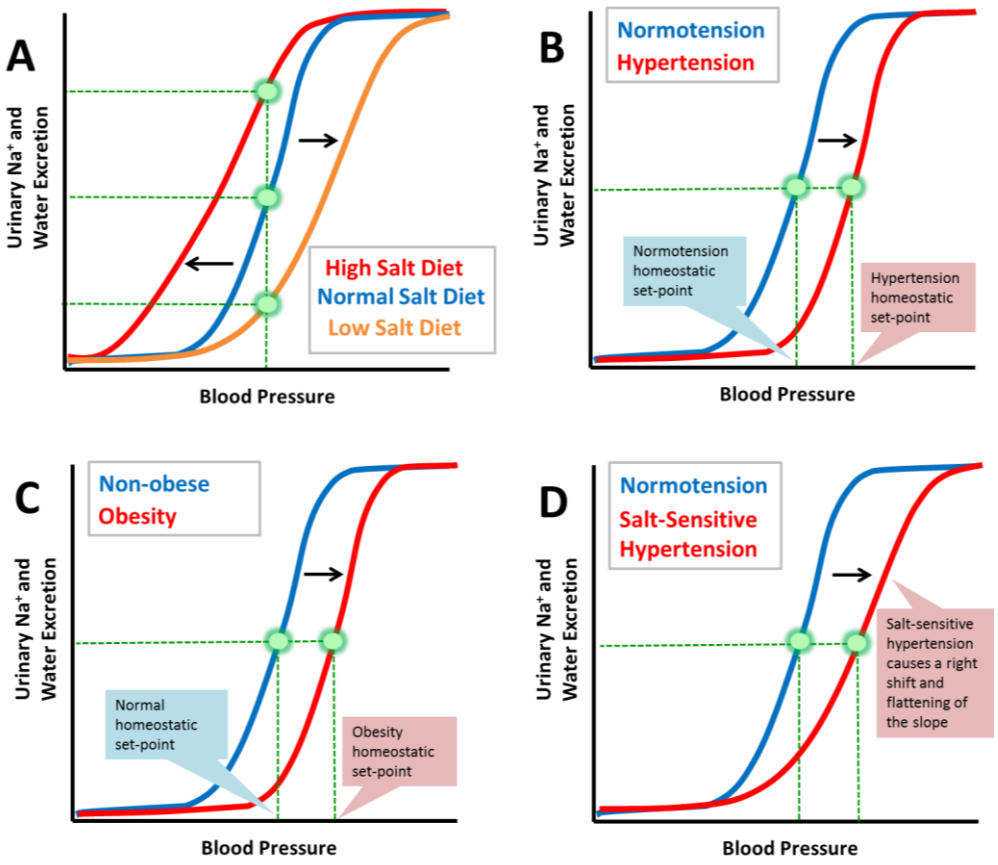

Figure 5. Pressure-natriuresis relationships. ***A:*** Salt affects slope. To maintain blood pressure homeostasis, high dietary salt results in increased urinary Na^+ and water excretion (↓RAAS activity) and low dietary salt results in decreased Na^+ and water excretion (↑RAAS activity). ***B:*** In normotension, increases in blood pressure are accompanied by increases in urinary Na^+ and water excretion to maintain homeostasis. In hypertension, homeostatic set points are reset, shifting the pressure-natriuresis curve to the right so that natriuretic responses now operate at higher blood pressures. ***C:*** Obesity also causes a right-shift in pressure-natriuresis (↑SNA and RAAS activity). ***D:*** Salt-sensitive hypertension causes a right-shift and flattening of slope.

2.4 Peripheral SNA at the Neuroeffector Junction

Mechanisms that contribute to enhanced sympathetic outflow also occur at sites of neurotransmitter release, either via synapses between pre and post-ganglionic neurons or at neuroeffector junctions between post-ganglionic neurons and non-neuronal target tissues. During their course to the splanchnic circulation, pre-ganglionic sympathetic nerve fibers originating in the IML column project to prevertebral ganglia like the SMCG and release acetylcholine (Ach) onto nicotinic Ach receptors on post-ganglionic sympathetic neurons. Postganglionic sympathetic neurons project to targets like mesenteric resistance arteries. Since the sympathetic effector response is directly determined by neurotransmitter release and action on target tissue, the neuroeffector junction represents that last regulatory level of SNA.

At the neuroeffector junction, neurotransmitters are released in a similar manner to paracrine secretion, where target effector cells are located at greater distances (up to thousands of nanometers) from nerve terminal release sites [104]. Neurotransmitters are stored in vesicles contained in varicosities, which are swellings along sympathetic nerve terminals that give the appearance of beads on a string [105]. Norepinephrine (NE) is the main neurotransmitter released from post-ganglionic sympathetic neurons [51] and is co-released with other sympathetic effector molecules like adenosine triphosphate (ATP) and neuropeptide Y (NPY) [106]. The sympathetic effector responses of NE and ATP are similar, resulting in vasoconstriction through α_1/α_2 adrenergic receptors and P2X receptors, respectively. NPY mediates vasoconstriction through NPY Y1 receptors, however, may serve a larger role in regulating the extent of NE and ATP release [106-108].

2.5 NE Synthesis, Release, Reuptake and Metabolism

NE synthesis, release, reuptake, and metabolism all take place in postganglionic sympathetic neurons. A simplified summary of these events are depicted in Figure 4.

NE synthesis: Synthesis of NE occurs first by the enzyme tyrosine hydroxylase, which catalyzes the conversion of tyrosine to dihydroxyphenylalanine (DOPA) in the cytoplasm. The enzyme DOPA decarboxylase then converts DOPA to dopamine, which is packaged into vesicles. In vesicles, the enzyme dopamine β-monooxygenase converts dopamine into NE [109].

Vesicular docking and release: These vesicles are then docked to the target membrane in active zones, which are sophisticated compartments that contain all the constituents needed for rapid exocytosis in response to electrical signals [110]. In brief, vesicular docking and fusion to the presynaptic membrane is achieved through organized interaction between soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) found in vesicular membranes (v-SNAREs) or target presynaptic membranes (t-SNAREs). The v-SNARE proteins include synaptotagmin and synaptobrevin, and the t-SNARE proteins include syntaxin and 25 kDa synaptosome-associated protein (SNAP-25). Synaptotagmin is a Ca^{2+} binding/sensing and membrane trafficking protein that first guides secretory vesicles to active zones at the presynaptic membrane [111], in part, by interacting with SNAP-25 [112]. Once correctly positioned, synaptobrevin binds syntaxin and SNAP-25, forming a “core SNARE complex” that solidifies vesicular-to-presynaptic membrane interaction [113].

Exocytosis of neurotransmitters is achieved by the influx of Ca^{2+} ions during depolarization due to the opening of voltage-gated N-type Ca^{2+} channels, which allows intracellular concentrations to reach 1-300 μM [110, 114, 115]. Exocytosis occurs because in its

Ca^{2+}-bound state, synaptotagmin binds phosphatidylinositol 4,5-bisphosphate (PIP2) lipids in the presynaptic membrane, leading to rapid membrane fusion, membrane penetration, and neurotransmitter release [116].

NE reuptake and metabolism: When NE is released into the neuroeffector junction, its action is limited in part by reuptake into the cytoplasm through the prejunctional NE-transporter (NET) and through degradation via catechol-O-methyltransferase [117, 118]. Once returned to the cytoplasm, NE is either recycled back into vesicles or is metabolized by monoamine oxidase (MOA) [118].

Enhanced neuroeffector transmission: An increase in neuroeffector transmission could be achieved by mechanisms that increase NE synthesis and release or by mechanisms that decrease NE reuptake and degradation. For instance, some studies suggest that impaired neuronal uptake of NE contributes to enhanced sympathetic nerve activity in human essential hypertension [119, 120].

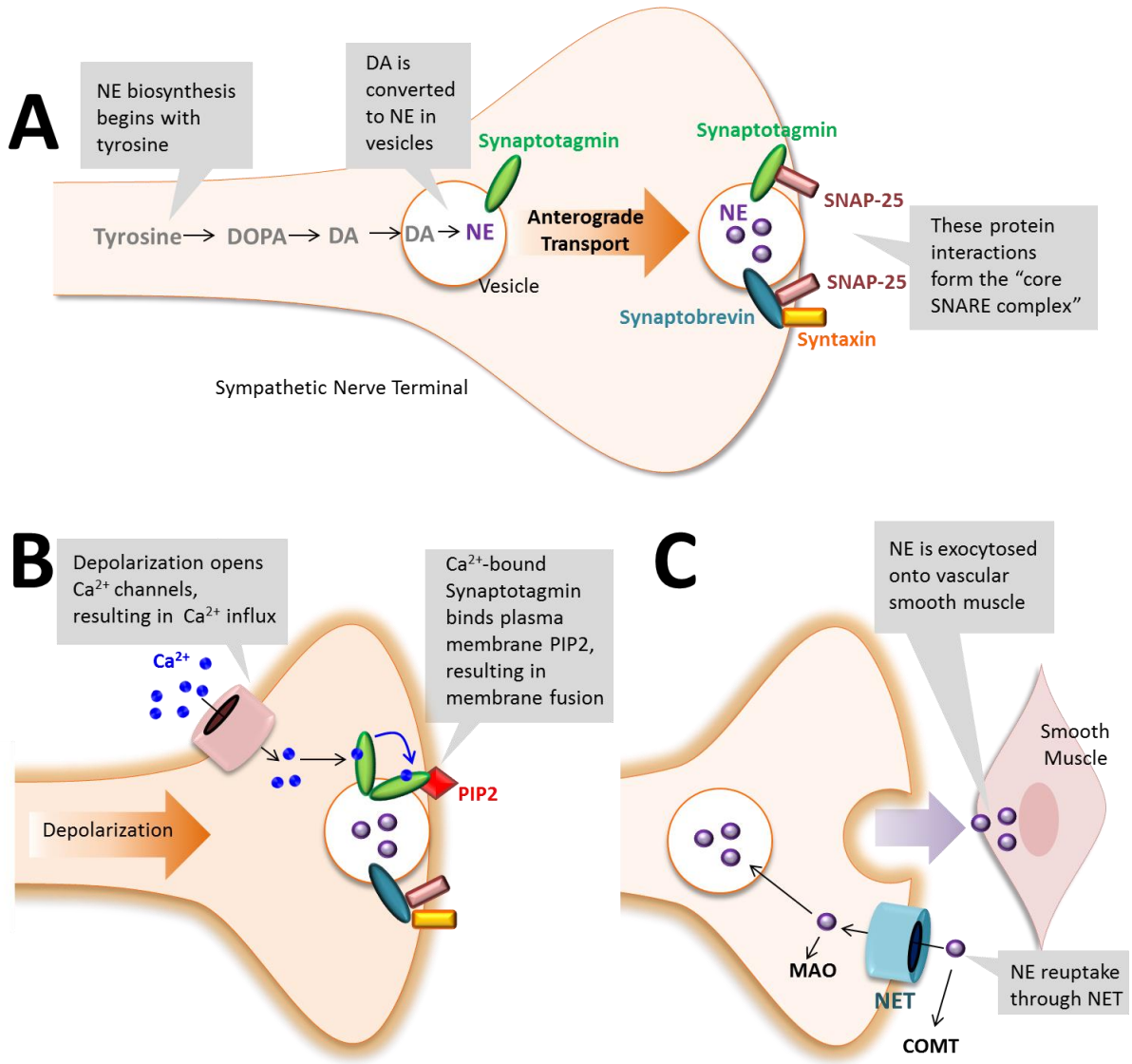

Figure 6. NE synthesis, release, reuptake and metabolism in sympathetic neurons. A: Tyrosine is converted into DOPA by tyrosine hydroxylase, which is converted into dopamine (DA) by DOPA decarboxylase. DA is taken up into vesicles and converted into norepinephrine (NE) by dopamine β-monooxygenase. Synaptotagmin assists in transporting NE-containing vesicles to active zones near the presynaptic membrane. Binding between synaptotagmin and SNAP-25 and synaptobrevin with SNAP-25 and syntaxin forms the "core SNARE complex", solidifying vesicular and presynaptic membrane interaction. **B:** Depolarization opens voltage-gated N-type Ca^{2+} channels, resulting in Ca^{2+} influx. Ca^{2+} binds synaptotagmin and promotes its interaction with phosphatidylinositol 4,5-bisphosphate (PIP2) in presynaptic membranes. This results in rapid membrane penetration, fusion, and exocytosis. **C:** NE is exocytosed onto effector targets like vascular smooth muscle. NE action is limited by reuptake back into the cytoplasm, which occurs through the NE transporter (NET) and through degradation via catechol-O-methyltransferase (COMT). Once re-uptake occurs, NE is either repackaged into vesicles or degraded by monoamine oxidase (MOA).

2.6 Negative Feedback Regulation Through the α_2-Adrenergic Receptor

The α_2-adrenergic receptor (α_2AR) regulates neurotransmitter release from nerve terminals in the central and peripheral sympathetic nervous system [121, 122]. In peripheral tissues such as resistance arteries, sympathetic neuroeffector transmission results in blood pressure elevation through the activation of postsynaptic α_1-adrenregic receptors (α_1ARs) (and to a lesser extent, α_2ARs) on vascular smooth muscle cells. This potentiates intracellular Ca^{2+} release and activation of protein kinase C, resulting in vascular smooth muscle contraction [123].

The extent of neuroeffector transmission is regulated by mechanisms of negative feedback that include signaling through the prejunctional α_2-adrenergic receptors (α_2AR), as depicted in Figure 5A. The α_2AR is an autoreceptor that couples via G proteins of the $G\alpha_{i/o}$ family to facilitate $G\beta\gamma$ dimer-dependent inhibition of voltage-gated N-type calcium channels [115, 124, 125], as depicted in Figure 5B. This mechanism prevents sustained increases in blood pressure [126-129]. Importantly, prejunctional α_2AR signaling provides negative feedback not only to NE release, but also functionally similar neurotransmitters like ATP [130]. Furthermore, mechanisms of α_2AR signaling and negative feedback on neurotransmitter release are conserved in adrenergic neurons of the CNS [131-133]. However, prejunctional α_2AR signaling at the neuroeffector junction represents the last line of defense to counteract enhanced sympathetic output originating from the CNS and is the focus of the majority of our work.

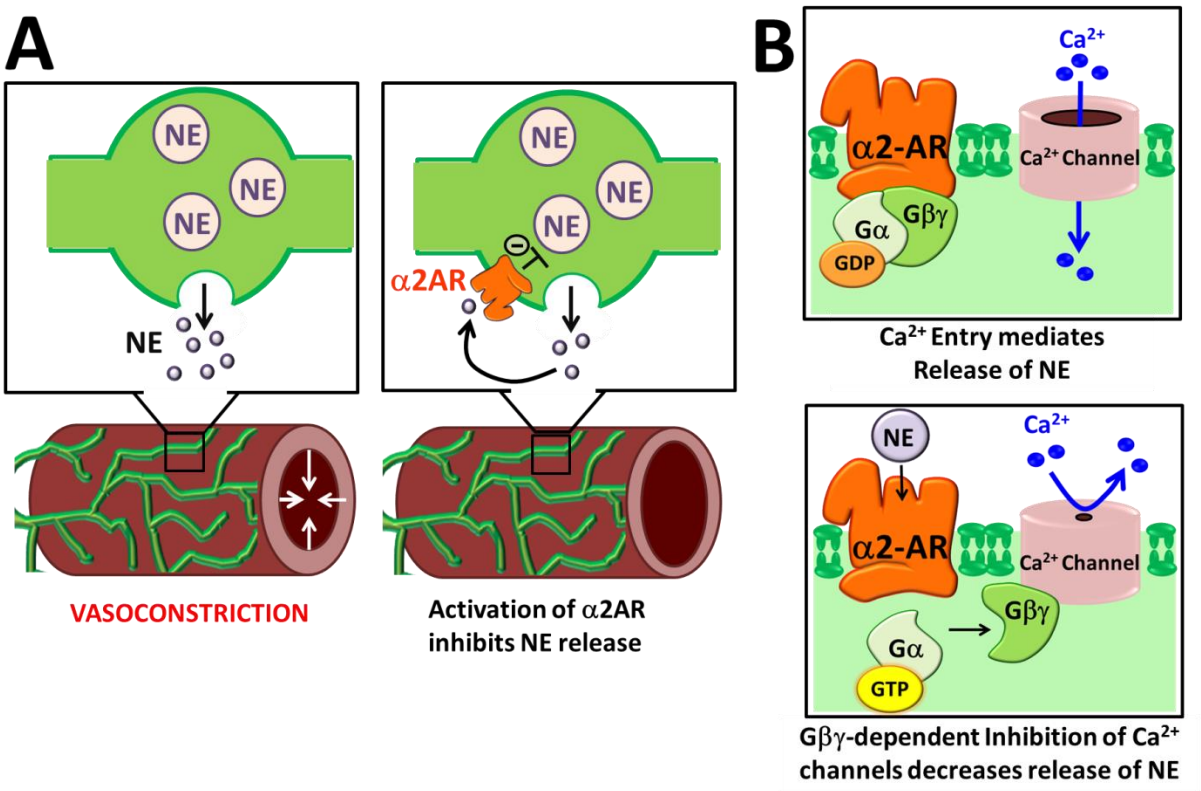

Figure 7. **Mechanism of negative feedback through the prejunctional α_2-adrenergic receptor (α_2AR).** ***A:*** Activation of the α_2AR by norepinephrine (NE) provides feedback inhibition to nerve terminal NE release. By decreasing NE bioavailability in the neuroeffector junction, vascular smooth muscle cells relax and blood pressure returns back to baseline. ***B:*** In its inactive state, the α_2AR associates with GDP-bound Gα-proteins at the membrane with sequestered G$\beta\gamma$ subunits. In its inactive state, voltage-dependent Ca^{2+} influx occurs undisturbed, facilitating NE release (*top*). When activated, Gα exchanges GDP for GTP, which releases G$\beta\gamma$ subunits that directly inhibit voltage-gated N-type Ca^{2+} channels. This attenuates NE release (*bottom*).

2.7 Physiological Relevance of α_2AR Subtypes

To date, three homologous subtypes of α_2ARs have been identified and cloned; subtype α_{2A}, α_{2B}, and α_{2C}. Disappointingly, pharmacological agents and ligands have not been selective enough to fully investigate subtype-specific properties and function [134]. Fortunately, the use of α2AR subtype-specific knockout mice has improved our understanding regarding the physiological differences in how α_2AR subtypes affect blood pressure regulation.

Knock out studies suggest that α_2AR subtypes do not participate equally in hypertension development. Evidence for this is supported in the subtotal nephrectomized mouse model of salt-induced hypertension. Wildtype mice develop hypertension in response to chronic dietary salt-loading similar to α_{2A} and α_{2C} knockout mice. However, α_{2A} knockout mice start out with higher baseline blood pressure and plasma catecholamine levels, and when salt-loaded, develop hypertension at a faster rate (2 versus 4-5 weeks of salt treatment) than wildtype and α_{2C} knockout mice. In contrast, mice heterozygous for α_{2B} are resistant to hypertension development despite chronic dietary salt loading [135, 136].

Feedback inhibition of NE release is associated mainly with α_{2A} and α_{2C} [133, 137-140]. Studies using α_2AR subtype-specific knockout mice suggest that inhibition of NE release from peripheral sympathetic nerves occurs mostly through α_{2A} as significant increases in plasma and urine NE levels occurred only in α_{2A} knockout mice [139]. Transgenic rescue of α_{2A} under in $\alpha_{2A/2C}$ double knockout mice restores plasma NE levels comparable to wildtype mice [141]. In contrast, α_{2C} knockout mice have normal plasma and urine NE levels but have significantly elevated plasma and urine epinephrine (EP) levels. Experiments performed in isolated chromaffin cells suggest that only α_{2C} is required for regulating EP release in the adrenal

medulla [139]. Tissue-specific contributions are also evident in the pancreas, where the inhibition of insulin secretion in pancreatic islet cells is mediated mostly by α_{2A} [142]. The individual contributions of α_{2A} and α_{2C} autoregulation may also be dependent on physiological conditions. Negative feedback through α_{2A} is faster than α_{2C} and is most influential at higher frequencies of nerve stimulation [137], suggesting that tonic SNA may be regulated by α_{2C} while increased SNA may be regulated by α_{2A}. While expression of all three subtypes can be detected in postganglionic sympathetic neurons [133], in the neuroeffector junction, it suggested that α_{2B} plays a larger role in vascular smooth muscle, mediating vasoconstriction like the α_1AR [140, 143].

2.8 Mechanisms of Prejunctional α_2AR Regulation

Receptor internalization: Activity of the α_2AR is regulated by ligand-occupied desensitization, which results from receptor endocytosis [141, 144]. Gene knock-in strategies using epitope-tagged receptors, such as hemagglutinin (HA)-tagged α_{2A}, have made it easier to monitor receptor trafficking and evaluate functional consequences of internalization. The characteristics of α_{2A} internalization and degree of receptor desensitization appear to be ligand-specific [144]. Clonidine and guanfacine are two α_2AR agonists that have been used clinically to treat hypertension [145]. Clonidine-occupied α_{2A} internalizes faster and to a larger extent than guanfacine-occupied α_{2A}. This may be due to phosphorylation, which occurs to a higher degree when the α_2AR is activated by clonidine. Furthermore, unlike guanfacine-occupied α_{2A}, clonidine-occupied α_{2A} internalizes as large puncta that localize predominantly in the perinuclear cytoplasm. Taken together, these data may explain why clonidine potentiates a larger and faster desensitization of α_2AR-mediated inhibition of Ca^{2+} current in cultured superior cervical ganglion (SCG) neurons than guanficine [144].

G protein-coupled receptor kinases: Receptor desensitization and internalization of GPCRs in the active state is mediated through phosphorylation by members of the G protein-coupled receptor kinase (GRK) family. Phosphorylation of GPCRs results in G protein uncoupling and arrestin-mediated receptor endocytosis [146-148]. Once internalized, receptors are either recycled back to the plasma membrane or downregulated through lysosomal proteolytic degradation [149]. These processes are depicted in Figure 6 using the α_2AR as an example.

For the α_2AR, GRK phosphorylation [150], arrestin binding [151], and G protein activation [152] all occur within the third intracellular loop. GRK-induced desensitization may

also involve subtype-specific characteristics. GRK 2 and GRK 3, but not GRK 5 have been shown to phosphorylate α_{2A} and α_{2B}. Agonist-occupied α_{2C} does not undergo phosphorylation [153-156]. Furthermore, α_2AR subtypes have selective binding to different arrestin family members. Internalization of α_{2B} involves arrestin-2 and arrestin-3 while internalization of α_{2C} involves arrestin-3. By contrast, internalization of α_{2A} occurs independently of these arrestins [157].

Relevance of GRK to hypertension: Most importantly for blood pressure control, receptor internalization is an effective way of desensitizing the α_2AR in postganglionic sympathetic neurons. Concanavalin A treatment, which prevents receptor internalization, reduces clonidine-induced α_2AR desensitization of Ca^{2+} current inhibition to near completion in SCG neurons [144]. Furthermore, upregulation of GRK 2 in the adrenal medulla dramatically reduces negative feedback on catecholamine release through the α_2AR [158]. Conditions that alter GRK 2 expression may also affect α_2AR signaling and blood pressure regulation. Non-canonical roles of GRK 2 involve the production of reactive oxygen species [159, 160] and GRK 2 is upregulated in inflammatory conditions that are associated with increased blood pressure [161, 162]. Whether GRK 2 upregulation increases blood pressure via oxidative stress and/or by increasing adrenergic drive through a reduction in α_2AR signaling is not known.

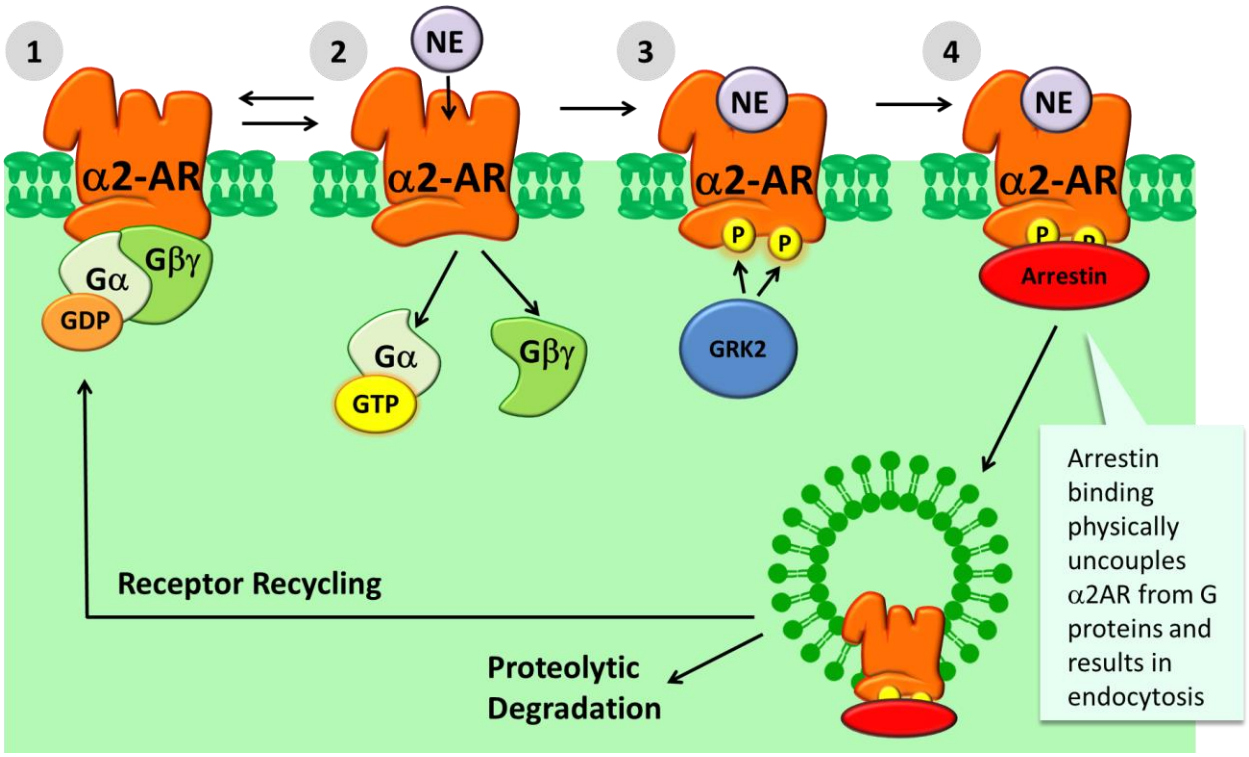

Figure 8. GRK-dependent desensitization and internalization of ligand-activated α_2AR. When activated by NE, α_2AR dissociates from G proteins (1-2). This vacates the third intracellular loop of α_2AR enabling GRK2 phosphorylation (3). Arrestin binds the phosphorylated form of α_2AR, which prevents G protein-receptor re-association and results in receptor endocytosis (4). Once internalized, α_2AR undergoes lysosomal proteolytic degradation (down-regulation) or is recycled back to the plasma membrane.

Receptor modification: Covalent protein modifications can also alter protein function. Cysteine residues in particular, are important for proper α_2AR signaling. G protein coupling to the α_2AR involves a conserved cysteine in the C-terminal region of $G\alpha_{i/o}$ proteins. Treatment with pertussis toxin (PTX) results in ADP-ribosylation of this cysteine, which impairs α_2AR signaling by uncoupling the receptor from its G protein [163]. Furthermore, treatment with phenylmercuric chloride, which reacts with sulfhydryl groups on cysteine residues, significantly decreases α_2AR-ligand affinity [164]. Finally, the C-terminal cysteine of the α_2AR is associated with mechanisms of downregulation [165]. These results suggest that modifications to cysteine residues on the G protein and receptor may affect α_2AR signaling.

3.0 Increased SNA in Human and Experimental Hypertension

3.1 Increased SNA Human Hypertension

It is now well accepted that sympathetic hyperactivity contributes to hypertension development, maintenance, and cardiovascular-related complications in humans [67, 68]. In fact, sympathetic hyperactivity is also associated with prehypertension as well as normotension with a positive family history of hypertension, suggesting a possible genetic component [166-168]. Evidence of enhanced SNA in human hypertension studies has been mainly demonstrated using techniques that include NE spillover and microneurography [34].

NE spillover: Due its relative ease and broad applicability, adrenergic drive has been evaluated indirectly through measurement of plasma NE. The use of radiolabeled NE has enabled the net quantification of enhanced NE spillover from nerve terminal release after taking into account NE clearance from the blood [169, 170] and has validated numerous observations that plasma NE increases in hypertensive versus normotensive subjects is due to enhanced adrenergic drive [171]. Furthermore, the use of catheter placement has enabled the detection of NE spillover in specific vascular regions that include the heart and kidney [169, 172]. Enhanced NE nerve terminal release in these organs occur in subjects with even just borderline blood pressure elevation [173].

Microneurography: Enhanced SNA in human hypertension has also been determined using microneurography, which involves the intra-neuronal recording of postganglionic sympathetic nerve traffic to skeletal muscle [34]. Microneurography is a favored technique as it is both direct and highly reproducible [34, 172], and is correlated with region-specific NE spillover in the heart [174] and kidney [175]. When corrected for heart rate, muscle

sympathetic nerve traffic significantly increases as individuals progress from normotension to pre-hypertension, from borderline hypertension to severe hypertension, and from severe hypertension to resistant hypertension [176]. These results may suggest that increased adrenergic drive precedes different stages of hypertension development.

Other techniques: Finally, it is worth mentioning that enhanced SNA has also been studied with some success in humans through either heart rate monitoring, or device-assisted approaches that target SNA. Heart rate, much like plasma NE is easy to measure, however it may not be as easy to interpret. Heart rate alone cannot truly reflect adrenergic drive because of parasympathetic (cholinergic) influences [3, 34]. This may explain why increased plasma NE and muscle sympathetic nerve traffic does not always correlate with increased heart rate [172]. Therefore, caution should be used when interpreting heart rate as an index of adrenergic drive.

The contribution of enhanced SNA in human hypertension has most recently been demonstrated using renal artery denervation in clinical studies and standard practice to treat resistant hypertension. Earlier studies using catheter-based radiofrequency denervation of the renal arteries showed success in sustainably decreasing blood pressure in patients with resistant hypertension [177, 178]. Furthermore, renal denervation has been shown to decrease plasma NE and muscle sympathetic nerve activity [179, 180]. However, newer investigations designed to be more rigorous (SYMPLICITY HTN-3) [181] by including a larger sample sizes, a blinded approach, and a sham control failed to show a decrease in blood pressure following renal artery denervation [182]. Until more investigations are carried out, the benefits of renal denervation remain unclear.

Limitations of human studies: Data from multiple human hypertension studies suggest that increased SNA in hypertension involves increased postganglionic sympathetic nerve firing and release of NE. Unfortunately, the mechanisms and precipitating factors that result in enhanced adrenergic drive in human hypertension are not completely understood, largely because essential hypertension is multifactorial in origin. Furthermore, there are only a handful of techniques that are ethical for use in human subjects. Therefore, research investigations have relied on animal models to study precipitating factors and underlying mechanisms that contribute to enhanced SNA and hypertension in humans.

3.2 Increased SNA in Experimental Hypertension

A series of different experimental hypertension animal models can be useful in understanding specific etiological factors that contribute to human hypertension development and maintenance. Genetics, salt, mineralocorticoid, and obesity are all risk factors of hypertension that involve increased sympathetic nerve activity in human hypertension.

3.2.1 Enhanced SNA in Genetic Hypertension

Many genetic rat models of hypertension were derived from variations of inbred strains. These include the spontaneously hypertensive (SH) and dahl salt-sensitive (DahlS) rat.

The SH rat model: The SH rat is a derivative of Wistar Kyoto (WK) rats that develops hypertension at 4-6 weeks of age even in the absence of experimental intervention [183] and are prone to stroke-related death [184]. In the SHR model, hypertension development and associated sequelae are more severe when put on diets consisting of high salt or fat [185, 186], which is similar in human hypertension [187]. The SH rat has been useful in determining if enhanced SNA participates in the initiation of essential hypertension. In young SH rats, microdiaylsis probe measurements detect enhanced NE release in vascular beds of skeletal muscle and subcutaneous fat [188], field stimulation of atrial preparations show enhanced cardiac NE release [189], and sympathectomy protects against hypertension development [190]. Increased NE release in young SH rats may be the result of prejunctional α_2AR dysfunction. In mesenteric arteries of young SH rats, electrical stimulation-evoked NE release is enhanced versus WK rats. Furthermore, when mesenteric arteries are treated with the α_2AR antagonist yohimbine, NE release is sustained in young SH rats but increased in WK rats [191].

Furthermore, ganglionic blockade with high doses of hexamethonium (nicotinic Ach receptor antagonist) results in a greater reduction in arterial pressure and renal SNA recordings in SH versus WK rats [192].

Finally, enhanced SNA has also been suggested to occur centrally via inflammation in the NTS, where expression of the leukocyte adhesion molecule JAM-1 is higher in SH rats versus control WK rats [193].

The DahlS rat model: The DahlS rat is a derivative strain of Sprague Dawley (SD) rats that has genetic predisposition to salt-sensitive hypertension associated with low renin secretion. This is in contrast to Dahl-resistant (DahlR) rats, which do not develop salt-sensitive hypertension or impaired renin secretion [194]. In the DahlS model, hypertension is achieved even on high-normal (1%) and low (0.3-0.4%) salt diets [195], but occurs faster on diets that higher in salt [196] and fat [197].

Early studies showed that hypertension development in DahlS rats involved impaired barorereceptor reflex control. In DahlS rats, phenylephrine-induced increases in blood pressure does not increase multifiber afferent aortic baroreceptor discharge [198] and dextran-induced volume overload does not reduce splanchnic SNA [199] as effectively as DahlR rats.

Newer studies suggest that central sympathetic activation results from increased levels of Na^+ in cerebrospinal fluid (CSF). CSF Na^+ levels are increased in high salt fed-DahlS rats [200], which activates central sympathetic efferent regulatory regions. In this model, CSF Na^+ is contributed by dietary salt and defective transport of Na^+ in CSF [201]. Central SNA may also be enhanced by oxidative stress production, which occurs from salt loading and is increased in the hypothalamus of high salt-fed DahlS rats [202]. Infusion of the antioxidant tempol into

intracerebral ventricles attenuates hypertension and renal SNA, as assessed by abdominal aorta catheterization and renal nerve multifiber discharge recordings, respectively [202].

Hypertension in the DahlS rat may also involve enhanced nerve terminal release of NE release. This was observed in early studies of salt-induced vasoconstriction, which was found to be enhanced in high salt-DahlS versus DahlR rats. Interestingly, DahlR rats produced vasodilation in response to salt loading [203]. Experiments measuring vascular resistance from lumbar sympathetic nerve stimulation in perfused hindquarters show enhanced vasoconstrictor response in DahlS rats that do not involve increased vascular reactivity to NE. This suggests that hypertension in DahlS rats may involve impaired negative feedback to nerve terminal release of NE [204]. It should be noted however that salt loading in DahlS rats does not increase neurogenic mechanisms of enhanced renal SNA [205, 206] and therefore, enhanced neuroeffector transmission in the DahlS may be organ-specific. Whether mechanisms that increase nerve terminal release of NE involve impaired α_2AR signaling is unknown.

3.2.2 Enhanced SNA in Salt and Mineralocorticoid Hypertension

In salt-sensitive hypertension, salt and water is retained, resulting in a rightward shift in pressure-natriuresis that is contributed in part by enhanced SNA [207]. Salt-sensitivity can be influenced by genetic factors, as seen in the DahlS rat, but also through acquired deficits that interfere with excretory mechanisms of the kidney. Examples include sympathetic hyperactivity, salt-loading, and mineralocorticoid excess [79].

Salt: High dietary salt is a risk factor for hypertension and blood pressure is proportional to body Na^+. Exactly how salt leads to the development of salt-sensitive hypertension is still unclear but may involve mechanisms in the CNS and renal system. Hypernatremia from increased salt consumption can activate osmosensory neurons in the OVLT and SFO, leading to downstream activation of PVN neurons and subsequent activation of the RVLM neurons and sympathetic efferents [72-74]. To some extent, this transient increase in blood pressure is buffered by the kidney through pressure-natriuresis via decreased RAAS activity. However, the homeostatic set-point of pressure-natriuresis can be reset, perhaps due to prolonged blood pressure elevation from increased adrenergic drive. This may also occur through acquired or pre-existing impairments in sodium excretion, leading to higher than normal Na^+ and water retention [208]. Volume expansion is also met with a regional vasoconstriction response that is independent of central control, which further increases blood pressure [97]. For these reasons, salt-loading has been commonly used to study increased SNA in salt-sensitive hypertension.

Aldosterone: As previously discussed, aldosterone contributes to increased blood pressure mostly in the periphery by increasing Na^+ and water reabsorption in the distal nephron [82] and colon [94], and through cardiovascular tissue injury from inflammation, oxidative stress production and tissue remodeling [95, 96, 209]. Activation of aldosterone-sensitive neurons in the brain increases blood pressure, however, this occurs only with direct aldosterone infusion into the cerebral ventricles [210, 211] and cannot be reproduced with systemic infusion. This may be because aldosterone, despite its lipophilicity, does not easily cross the blood brain barrier like other steroid hormones (corticosterone, progesterone,

estradiol, and testosterone) [212]. An indirect mechanism by which aldosterone may increase SNA is through mechanisms that increase salt appetite [213, 214].

The effects of salt and mineralocorticoid signaling: Aldosterone and salt work synergistically in the development of salt-sensitive hypertension, possibly via oxidative stress production and damage to the cardiovascular and renovascular system. This may occur because salt enhances MR signaling. This is evident in the salt-loaded DahlS rat where treatment with the MR blocker eplerenone improves proteinuria and cardiac function [215]. However, this is counterintuitive given that the DahlS rat is a model of low aldosterone. This can be explained by salt-induced activation of MR signaling independent of aldosterone, which in salt-loaded DahlS rats, occurs through Rac1, a member of the Rho family of GTPases [216] that has been implicated in oxidative stress production through the activation of NADPH oxidase. NADPH oxidases contribute oxidative stress by catalyzing the conversion of dioxygen to superoxide [217]. Therefore, in hypertension, oxidative stress at least in in part, may occur through mineralocorticoid signaling. Mechanisms of Rac1 activation in hypertension remain unclear, but may be due to mechanical stretch in vascular endothelial cells, which increases in hypertension [216, 218]. Rac1-induced activation of MR is also increased by aldosterone [219], which may make hypertension more severe in models where salt loading is accompanied by mineralocorticoid-excess. These findings are important and suggest that salt can enhance MR signaling in hypertension, possibly via oxidative stress and is relevant even in models where RAAS activity is low.

***The DOCA-salt rat model*:** The study of salt and mineralocorticoid status in hypertension has been mostly investigated using the deoxycorticosterone (DOCA, a mineralocorticoid

derivative of aldosterone)-salt hypertensive rat model. Here, DOCA administration (20-150 mg/kg) and 0.6-1% NaCl drinking water in uninephrectomized rats leads to the development of low-renin hypertension [220]. Low-renin hypertension is commonly found in patients with salt-sensitive hypertension, which account for about half of the cases of essential hypertension [40, 41, 221, 222]. Enhanced SNA has been studied extensively in DOCA-salt hypertension and involves impairment of the baroreceptor reflex, enhanced brain renin-angiotensin system (RAS), systemic oxidative stress production, enhanced lumbar, splanchnic and renal SNA, and dysfunction of the prejunctional α_2AR. Interestingly, many of these factors are salt-dependent, suggesting that mineralocorticoid and salt act synergistically to enhance SNA.

Enhanced SNA in DOCA-salt hypertension begins with functional impairment of the baroreceptor reflex, which can be detected even before hypertension develops. Activation of the baroreceptor reflex using aortic depressor nerve stimulation generates reduced sympathoinhibitory responses as measured via splanchnic nerve recordings in DOCA-salt rats versus uninephrectomized (SHAM, control) rats after only 5 days of treatment. These deficits are central as aortic depressor nerve stimulation initiates the baroreceptor reflex independent of arterial baroreceptors [223].

Central mechanisms of enhanced SNA in DOCA-salt hypertension may result from increased activation of RAAS and oxidative stress production in the brain. Although DOCA-salt hypertension is characterized by low plasma renin and ANG II, increased levels of ANG II have been detected in CSF [224]. In DOCA-salt hypertension, ANG II activation of AT1Rs is enhanced in the SFO [225] and possibly other CVOs [226]. ANG II infusion has been shown to activate neurons within the PVN, SFO, and NTS [85]. Furthermore, oxidative stress production may also

enhance central SNA. In DOCA-salt rats, superoxide levels in the hypothalamus are increased and intracerebroventricular delivery of tempol (an antioxidant) decreases blood pressure and renal SNA to a greater extent in DOCA-salt versus SHAM rats [227]. These effects do not occur in DOCA-treated rats with access to normal water [227]. Plasma NaCl, which is mildly elevated in DOCA-salt rats, may be sufficient to enhance sympathetic outflow. Nerve recordings show significantly enhanced lumbar SNA in DOCA-salt versus SHAM rats. This effect does not occur in DOCA-treated rats with access to normal drinking water [228].

Enhanced SNA has also been suggested by experiments using ganglionic blockade, celiac renal denervation and celiac ganglionectomy. Ganglionic blockade by hexamethonium decreases blood pressure to a greater extent in DOCA-salt versus SHAM rats [63, 227]. These effects are not observed in DOCA-treated rats with access to normal water [227]. Renal denervation can be achieved by renal vessel dissection followed by application of 10-20% phenol in alcohol solution. The effects of renal denervation on the development of DOCA-salt hypertension have been mixed. Renal nerve recordings performed in DOCA-salt rats show that renal SNA is increased versus SHAM rats [229], and that renal denervation significantly reduces, although not to completion, DOCA-salt induced elevation of blood pressure [229-231]. These effects may be salt-dependent, as they do not occur in DOCA-treated rats with access to normal drinking water [227]. Furthermore, enhanced renal SNA may involve inflammation and oxidative stress. Increased levels of inflammatory mediators that include chemokine ligand, monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) in the kidney occur in DOCA-salt hypertension and are significantly reduced by renal denervation [229]. Furthermore, intracebroventricular administration of tempol lowers renal SNA to a greater extent in DOCA-

salt versus SHAM rats and DOCA-treated rats with access to normal water [227]. On the other hand, in another study, renal denervation was not shown to reduce or delay DOCA-salt hypertension development at any time point, suggesting that intact sympathetic innervation to the kidney is not important in DOCA-salt hypertension [232]. Furthermore, a study performed in mild DOCA-salt hypertension (50 mg/kg, 1% salt water, no nephrectomy) also showed that renal denervation had no effect on DOCA-salt induced elevation of blood pressure versus non-denervated DOCA-salt rats [233]. Interestingly, using the same model, splanchnic denervation through celiac ganglionectomy reduced the extent (although not fully) of DOCA-salt induced elevation of blood pressure versus non-denervated DOCA-salt treated rats [59]. These results suggest that at least in mild DOCA-salt hypertension, increased SNA may be regionally-specific to the splanchnic circulation.

Finally, DOCA-salt hypertension is associated with functional impairment of prejunctional α_2AR signaling. In DOCA-salt rats, basal levels of plasma NE are increased [129, 227] and unchanged after administration of the α_2AR-selective antagonist yohimbine [129]. Similarly, nerve stimulation-evoked NE release detected from mesenteric arteries using amperometry show that NE release is enhanced in mesenteric arteries from DOCA-salt rats. Furthermore, inhibition of NE release is blunted when artery preparations are treated with yohimbine [234].

3.2.3 Enhanced SNA in Obesity-Related Hypertension

Obesity is an independent risk factor of hypertension and its increasing prevalence is changing the overall cause of human hypertension. It is now estimated that more than half of human hypertension cases are directly linked to obesity [42, 43]. Obese subjects have increased levels of plasma NE [235] and increased muscle SNA [236] versus non-obese subjects. This suggests that obesity, much like salt, contributes to hypertension by increasing SNA. The development of obesity-related hypertension is multifactorial with some mechanisms are likely conserved with other forms of hypertension, such as salt-sensitive hypertension. However, great effort must be made to properly identify these similarities as well as potential differences that may be used to develop more effective clinical treatment strategies. Experiments performed in animal models suggest that obesity-related hypertension involves an increase in SNA, RAAS activation, inflammation, and oxidative stress production [237].

***The HFD-SD model*:** Obesity-related hypertension can be studied through high dietary fat consumption in animals such as Sprague-Dawley (SD) rats. SD rats are not predisposed to hypertension development and like humans, show individual variations. In SD rats, a moderately HFD (32% kcal fat, a typical Western diet) induces obesity that is associated with mild hypertension (termed obesity-prone, OP) after 10 weeks of feeding. Remaining animals exhibit normal body weight and blood pressure (termed obesity-resistant, OR) similar to SD rats fed a normal fat diet (NFD, 11% kcal fat) [238]. This is characteristic of human obesity, which is also bimodal in distribution [239]. Obesity-related hypertension in HFD-OP rats is associated with an increase in vascular hypertrophy, plasma renin activity, circulating cholesterol, and lipid peroxides versus HFD-OR rats and NFD-rats [238]. Lipid peroxides result from the oxidative

degradation of lipids and it is suggested that this might occur via increased lipolysis, secondary to sympathetic activation [238, 240]. When carried out to 16 weeks of feeding, blood pressure, plasma renin activity and lipid peroxide levels are still markedly elevated in HFD-OP rats versus HFD-OR rats and NFD-SD rats. Furthermore, in HFD-OP rats, superoxide levels are significantly increased in aortic rings versus HFD-OR rats and NFD-SD rats [240]. Given increased plasma renin activity, it is suggested that SNA and ANG-II production through RAAS could be enhanced. This is important as enhanced SNA increases renin release from JG cells and members of the RAAS like ANG-II and aldosterone can enhance oxidative stress production [95, 96, 240, 241].

In a recent study, hypertension development in this model was suggested to occur through inflammation-induced impaired baroreceptor regulation of renal SNA, as measured from renal nerve recordings in response to changes in blood volume. 8 weeks of HFD (45% kcal fat) resulted in obese and hypertensive rats with higher levels of TNFα and IL-6 in the plasma and kidney, and lower levels of urinary output and Na^+ clearance. Furthermore, renal SNA responses to the baroreceptor reflex initiated by phenylephrine (high pressure) and sodium nitroprusside (low pressure) bolus infusion were attenuated in HFD-rats versus NFD-rats. Interestingly, treatment of HFD-rats with the tacrolimus (an immunosuppressant) restored renal SNA responses to the baroreceptor reflex. Tacrolimus also preserved blood pressure, reduced inflammation, and improved urinary output and Na^+ excretion [242]. Taken together, these findings suggest that HFD and obesity may contribute to enhanced SNA (and possibly RAAS activity) through chronic inflammation.

Approaches to study obesity and salt: Dietary salt and obesity are risk factors for hypertension development [81, 243], and dietary salt and hypertension are also risk factors for

the development of obesity [244, 245]. Reductions in dietary salt improves blood pressure to a greater extent in obese versus non-obese adolescents [246] suggesting the need to investigate this relationship early in development. It has been suggested that mechanisms of salt-sensitivity and obesity work synergistically to increase blood pressure by increasing SNA [81, 243], possibly through insulin signaling [247], inflammation, and oxidative stress [81].

Strategies to investigate the effects and relationships of obesity and salt-sensitivity on hypertension development can involve interventions that combine a HFD with high salt. Interestingly, the combination of a high fat (32% kcal fat) and high salt (2-4%) diet in OP rats accelerates the onset of hypertension, increases superoxide production in the aorta, increases adipocyte size, and increases leptin production versus HFD containing normal salt (0.8%)-OP rats [248]. However, the magnitude of hypertension was not affected by salt and may be explained by a lack of salt-sensitivity in all SD rats. Therefore, a more suitable approach would be to use a HFD in a salt-sensitive hypertension model, like the DahlS rat.

HFD-DahlS model: In the DahlS model, hypertension is achieved even on normal (1%) and low (0.3-0.4%) salt diets, but occurs faster when given a diet that is high fat [197]. DahlS rats on a low salt-HFD (0.3% salt, 45% kcal fat) for 8 weeks develop hypertension (that can be detected as early as 2 weeks) associated with significantly increased weight and visceral adiposity versus NFD (0.3% salt, 10% kcal fat) rats. These rats also develop hyperinsulinemia, hyperglycemia, and higher urinary NE. Furthermore, replacing the low salt-HFD diet with a high salt-HFD (8% salt, 45% kcal fat) for an additional 8 weeks worsens the degree of hypertension and causes significant proteinuria [197], suggesting that salt aggravates mechanisms of hypertension development in this model. This seems plausible as after 5 months of low salt

HFD-feeding, DahlS rats develop hypertension with proteinuria [249]. It has been suggested that increased insulin in this model may be a reflection of enhanced SNA and that salt loading, which causes insulin resistance even in NFD-DahlS rats, plays an important role [197, 247, 250].

Inflammation plays a role in this model. Four weeks on a normal salt-HFD (0.8% NaCl, 60% kcal fat) results in hypertension associated with significant weight gain, increased visceral adiposity (without changes in leptin or cholesterol), and increased CD3+T-lymphocytes accumulation in glomerular and medullary tissues versus low salt-NFD (0.4% NaCl, 14% kcal fat). Treatment with the immunosuppressant mycophenolate mofetil attenuated hypertension development and weight gain, reduced visceral adiposity, and prevented CD3+ T-lymphocyte infiltration in the glomerulus [251].

Closely related to inflammation, oxidative stress also plays a role in this model. In a 20 week study, a low salt-HFD (0.3% NaCl, 35% kcal fat) not only induced hypertension with weight gain in DahlS rats, but also increased aortic levels of superoxide versus low salt-NFD (0.3% NaCl, 5% kcal fat). Interestingly, although the DahlS rat is a model of low-renin/ANG-II, blockage of the AT1 receptor with daily olmesartan attenuated HFD-induced hypertension and vascular superoxide production, suggesting that ANG-II contributes to hypertension development in this model through mechanisms that may involve oxidative stress [252].

4.0 Inflammation and Oxidative Stress in Hypertension

In all the animal models previously discussed, mechanisms of inflammation and oxidative stress production appear to be common features that contribute to hypertension development. Inflammation is associated with human hypertension. The inflammatory marker CRP predictably increases as normotensive subjects develop prehypertension and are highest in subjects with overt hypertension [35-38], which suggests that mechanisms of inflammation may come into play before hypertension development [253].

4.1 Vascular Injury in Hypertension

Vascular protection against pressure overload: In addition to the mechanisms previously discussed, vascular tissue injury can occur from pressure-overload. In arteries, layers of vascular smooth muscle and adventitia provide mechanical support to the vascular endothelial layer, which is in direct contact with friction caused by blood flow. Small changes in blood flow occur all the time, from the pulsatile force of a beating heart to physiological responses to for instance, exercise. Frictional force usually poses no threat to the endothelium, which counteracts wall shear stress by releasing the potent vasodilator nitric oxide (NO) [254].

NO is made by the enzyme endothelial NO synthase (eNOS). Activation of muscarinic-3 (M3) receptors by Ach on smooth muscle of arteries (unlike the gut and bronchi) causes dilation. Vasodilation requires eNOS. Mice lacking eNOS develop hypertension from impaired Ach-induced relaxation [255]. In further support, the use of L-NAME, which inhibits NOS and impairs NO production, is commonly used to induce experimental hypertension. Interestingly, hypertension persists even once L-NAME treatment stops [256]. This may suggest that vascular

damage sustained from a reduction in NO may be robust enough to maintain high blood pressure even when NO is restored.

Vascular injury and effects of ROS: Decreased NO bioavailability affects vascular function and predisposes the endothelium to tissue injury during hypertension, where there is sustained increase in mechanical loading from increasing blood pressure and flow. This affects all layers of the blood vessel [257]. Evidence suggests that oxidative stress may play an important role [257, 258]. Increased circumferential stretch increases oxidative stress production in vascular smooth muscle and endothelium [259, 260]. ROS like superoxide can decrease NO bioavailability by combining with NO to form peroxynitrite, which is a very strong oxidizing agent [257, 261]. Vascular oxidative stress production in hypertension occurs through NADPH oxidases [261] via Rac1 activation, possibly through mechanical stretch from vascular endothelial cells during pressure-overload [216, 218]. Normal mechanisms that regulate levels of ROS such as antioxidants like glutathione and enzymes like superoxide dismutase may be insufficient in counteracting the increasing levels of oxidative stress [262].

4.2 Lymphocytes and Macrophages in Hypertension

Immune cell recruitment: Although commonly described in the pathogenesis of disease, inflammation actually serves as a protective response to injury. Immune cells infiltrate into sites of injury, attempt to eliminate the causative agent, and repair tissue damage. This process involves the release of pro-inflammatory mediators, which in excess, can add to disease progression and modify the microenvironment by changing cellular and protein function [253]. Immune cells such as lymphocytes and macrophages have been implicated in hypertension development and maintenance in both humans and experimental animal models.

4.2.1 Lymphocytes

Lymphocytes play a role in adaptive immunity where inflammatory effector responses involve specific antigen-receptor interactions that mostly occur after the initiation of innate immunity [263]. T-lymphocytes mature in the thymus to become either CD8+ T-cells (cytotoxic) or CD4+ T-cells (helper). CD4+ T-cells help other leukocytes mediate immune responses, such as the activation of macrophages. CD8+ T-cells effectively eliminate cells by degranulation of cytotoxic contents, by apoptosis through the Fas pathway, or by cytokine release [263, 264]. B-lymphocytes undergo maturation in secondary lymphoid tissues such as the spleen and lymph nodes. B-lymphocytes mediate humoral immune responses either as plasma cells, which secrete antibodies against foreign antigens, or memory B cells, which release antibodies only when antigens from a previous exposure are encountered [265].

Lymphocytes and hypertension: Lymphocytes have been implicated in human hypertension. Mycophenolate mofetil is an immunosuppressant that suppresses T-lymphocyte

growth. Hypertensive patients taking mycophenolate mofetil for psoriasis or rheumatoid arthritis (autoimmune conditions) develop significant reductions in blood pressure [266].

In animal models, T-lymphocytes appear to be more important than B-lymphocytes in hypertension development. RAG1 knockout mice lack T and B-lymphocytes and are resistant to DOCA-salt and ANG-II-induced hypertension, increased levels of vascular superoxide, and vascular dysfunction. Adoptive transfer of T-lymphocytes (but not B-lymphocytes) into these animals restores susceptibility to hypertension development, increased vascular superoxide and vascular dysfunction [267]. Similarly in DahlS rats (4% salt diet), lymphocyte depletion via targeted depletion of RAG1 attenuates lymphocyte infiltration in the kidney, proteinuria, tubular and glomerular damage, and hypertension development [268]. Taken together, these results suggest that T-lymphocytes are required for the development of hypertension and play a role in oxidative stress and tissue damage.

4.2.2 Macrophages

Macrophages are phagocytes that are capable of secreting cytokines during innate immune responses and during tissue repair [269]. Simplistically, once monocytes infiltrate into tissues, they can differentiate into either M1 or M2 macrophages. M1 macrophages are associated with pro-inflammatory oxidative stress production and release of cytokines such as tumor necrosis factor-α (TNF α). M2 macrophages are associated with tissue repair and can release anti-inflammatory cytokines such as transforming growth factor-β (TGFβ). Macrophages can fluctuate between M1 and M2 phenotypes [269]. Such adaptations may involve salt, which attenuates the M2 phenotype. *In vitro*, salt has been shown to increase pro-inflammatory

cytokine production either through activation of mitogen-activated protein kinase [270] or by attenuating IL-4 and IL-13 signals which promote the M2 phenotype [271]. The effect of salt on macrophages is so profound that pro-inflammatory cytokine production occurs even in the absence of stimulation [272]. This also affects T-lymphocytes, as salt prevents macrophage suppression of T-lymphocyte proliferation [271].

Macrophages and human hypertension: Macrophages play an important role in hypertension development and maintenance. In humans, circulating pre-activated monocytes (as determined by in vitro stimulation with ANG-II) and macrophage-related markers (MCP-1, soluble ICAM-1, E-selectin, GM-CSF) are increased in hypertensive versus normotensive subjects [273-275].

Macrophages and Experimental hypertension: Animal studies have enabled a better understanding of how macrophages contribute to increase SNA in the brain and peripheral blood vessels. These mechanisms are summarized in Figure 9.

During inflammation, monocytes infiltrate into injured tissue via chemotaxis through MCP-1 [269, 276]. An increase tissue macrophage infiltration is associated with the development of hypertension in numerous models that include DOCA-salt [127, 277] and DahlS hypertension [278]. Macrophage suppression attenuates hypertension development in these models [127, 278]. In DOCA-salt and ANG-II-induced hypertension, blocking MCP-1 receptors on macrophages with INCB3344 also prevents macrophage infiltration and protects against hypertension development. This may suggest that tissue injury from DOCA-salt and ANG-II treatment alone is insufficient in causing hypertension [279].

Macrophages and vascular injury: Unopposed pro-inflammatory cytokine release and oxidative stress production from macrophages increases the severity of tissue injury. Macrophage-derived ROS decreases NO bioavailability by reacting with NO (as previously described) [257, 261]. Macrophage-derived TNFα decreases NO bioavailability directly by reducing eNOS activity [280], and indirectly by activating NADPH oxidase [281], which further enhance vascular oxidative stress production. This promotes vascular dysfunction and remodeling, which can increase vascular resistance. DOCA-salt treated mice that lack monocyte colony stimulating factor (m-CSF) do not develop DOCA-salt related vascular remodeling, dysfunction, or increased NADPH oxidase-derived superoxide in mesenteric arteries and are protected from hypertension [282].

Macrophages and neuro-inflammation: Macrophages may also contribute to enhanced SNA through mechanisms of neuro-inflammation [269, 283, 284]. Leukocytes in the blood have limited access to the brain due to the blood brain barrier. Microglia are specialized resident brain macrophages that are replenished by local proliferation and function mostly independently of other immune cells [285, 286]. In hypertension induced by L-NAME and ANG-II infusion, microglia release pro-inflammatory mediators in autonomic brain centers that are associated with enhancement of SNA. Targeted microglial depletion using the transgenic CD11b-diptheria toxin receptor (DTR) mouse decreases neuro-inflammation, glutamate receptor expression in the PVN, and blood pressure in these models [285].

Furthermore, a recent study has demonstrated that hypertension is associated with pro-inflammatory status in the bone marrow, and that some of these immune cells extravasate into the PVN to become microglial cells. Normotensive WK rats reconstituted with bone marrow

derived from SH rats develop hypertension that is associated with peripheral and central inflammation. Specifically in the PVN, these animals had higher microglia number and percent activation versus non-reconstituted WK rats. The authors of this study suggested that extravasation may have occurred through the CSF, as CSF levels of the MCP-1 were elevated in reconstituted rats [284]. This is supported by another study that found in SH rats, there is a hypertension-related increase in junctional adhesion modlecule-1 (JAM-1) expression in the brainstem, which enables recruitment of leukocytes to vascular endothelium [287]. Similarly, hypertension was reduced in SH rats reconstituted with bone marrow from normotensive WK rats and these animals had lower microglia number and percent activation in the PVN versus non-reconstitued SH rats. [284]. In all rats, hypertension could be reduced by administering minocycline, which is a brain-permeable antibiotic with anti-inflammatory properties, which have also been effective in decreasing blood pressure in hypertensive patients [283, 284].

Macrophages and neuroeffector transmission: Finally, macrophage-related neuro-inflammation may also contribute to enhanced SNA at the neuroeffector junction. We have recently shown that in DOCA-salt hypertension, prejunctional impairment of α_2AR signaling involves macrophage-derived inflammation and oxidative stress as clodronate depletion of macrophages reduces superoxide, preserves α_2AR function, and protects against the late phase of DOCA-salt hypertension development [127].

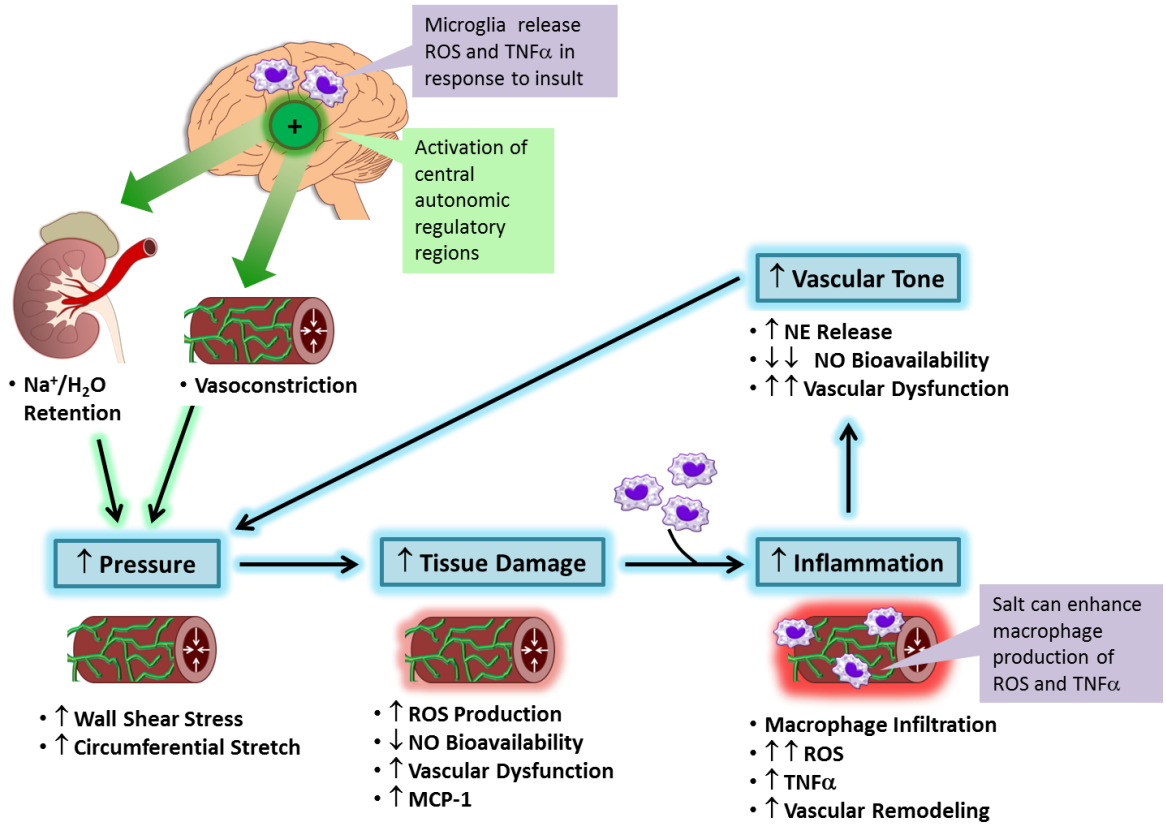

Figure 9. Macrophages contribute to enhanced SNA in hypertension. Microglia in the brain secrete ROS and cytokines onto central autonomic regulatory regions that increase sympathetic output to the kidney and resistance arteries, potentiating volume expansion (by increasing Na^+/H_2O reabsorption) and vasoconstriction, respectively. This increases wall shear stress to the endothelium and circumferential stretch to vascular smooth muscle and adventitial layers. To a point, these effects can be buffered by the release of NO, which causes vasodilation. However, prolonged stress and stretch increases ROS, which reduces NO bioavailability, resulting in vascular dysfunction that makes the endothelium more susceptible to injury from pressure-overload. Tissue injury increases MCP-1 expression, which enables monocytes in circulation to infiltrate via chemotaxis and differentiate into mature macrophages. Macrophages increase ROS and cytokine production, which can be further aggravated by salt. As ROS and TNFα levels continue to rise, NO levels become depleted. Furthermore, the prejunctional α_2AR becomes impaired, resulting in enhanced NE release. These effects combine to increase vascular tone, which leads to greater pressure-overload.

4.2.3 Sympathetic Modulation of Macrophages

Adrenergic drive also affects immune cell function. Lymphocytes and macrophages express adrenergic receptors that can be activated by both NE and EP [288]. In general, it is supported that activation of β1AR promotes pro-inflammatory responses while β2AR and α_2AR promote anti-inflammatory responses [288, 289]. However, the expression of adrenergic receptors on immune cells is dynamic and their immunological influences may be dependent on specific tissue environment. Therefore, it is important to investigate the effects of adrenergic activation in the context of specific inflammatory conditions.

This is especially true for monocytes and macrophages, which respond rapidly to inflammatory signals [288]. Activated macrophages increase synthesis and release of NE, which contributes to inflammation and tissue damage in acute lung injury models. Interestingly, antagonism of α_2AR (RX 821002), but not α_1ARs (prazosin) or βARs (metoprolol and ICI 118551) significantly suppresses pulmonary immune cell infiltration, fibrin and hemorrhage. These effects were reproduced by blocking tyrosine hydroxylase (α-methyltyrosine) and could be reversed by activating α_2AR with UK14304 [290]. This suggests that activation of α_2AR on macrophages increases NE bioavailability and induces a pro-inflammatory phenotype.

Furthermore, the effects of adrenergic activation may be cell-type specific. Whereas β-AR activation favors CD8+ T-lymphocyte cell differentiation and enhances cytokine production [291], in macrophages, proinflammatory status is suppressed by β-ARs and enhanced by α_2ARs [292]. Consistent with this, activation of α_2AR on macrophages leads to increased production of TNFα [293]. However, evidence also suggests that β2AR-specfiic activation can increase pro-

inflammatory cytokine production and release in macrophages even in the absence of pro-inflammatory stimuli [294].

Taken together, these data suggest that in macrophages, adrenergic drive in general, may be pro-inflammatory, thus, supporting a possible interdependent relationship between inflammation and enhanced SNA.

CHAPTER 2: RESEARCH AIMS AND HYPOTHESES

1.0 Research Goals

The goal of our research was to compare the effects of salt, mineralocorticoid, and HFD on inflammation and hypertension development as it relates to prejunctional α_2AR function. Salt, mineralocorticoid, and obesity are all risk factors of hypertension. Activation of the prejunctional α_2AR results provides negative feedback to NE release from nerve terminals by inhibiting Ca^{2+} channels and represents the last line of defense to counteract enhanced SNA in hypertension. Macrophage-derived superoxide impairs signaling through the α_2AR in DOCA-salt hypertension, which is a model of inflammation, oxidative stress, and enhanced sympathetic nerve activity. However, the mechanisms of this dysfunction remain unclear. It is also unclear if similar deficits and inflammation occur in other types of hypertension, such as salt-sensitive (with low mineralocorticoid) and obesity-related hypertension.

In our study, we evaluated inflammation and α_2AR function in 3 models of hypertension. The effects of mineralocorticoid and salt-sensitivity were studied using the DOCA-salt rat. To study obesity-related hypertension, we induced hypertension and obesity using a low-salt/HFD (0.3% salt, 60% kcal fat) in Sprague Dawley (DS) and Dahl salt-sensitive (DahlS) rats. SD rats were selected because like humans, display great variability and are not uniformly predisposed to developing salt-sensitive hypertension or obesity. By contrast, the DahlS rat is genetically salt-sensitive, and like the DOCA-salt hypertensive rat, is a model of low renin-hypertension.

Ultimately our goal was to perform the same experiments on a series of different animal models in order to decipher specific or common etiological factors that contribute to human hypertension development and maintenance.

2.0 Overall Hypotheses

We will test the hypothesis that salt, mineralocorticoid, and high fat contribute to hypertension development through increased inflammation and macrophage-associated impairment of prejunctional α_2AR-mediated inhibition of Ca^{2+} channels. We will also test the hypothesis that the functional deficits lie at the receptor level and occur through oxidative stress. Finally, we will test the hypothesis that macrophage depletion in these animal models preserves α_2AR function and attenuates hypertension development. Hypotheses as well as specific aims 1-3 (which reflect chapters 4-6, respectively) are presented in Figure 10.

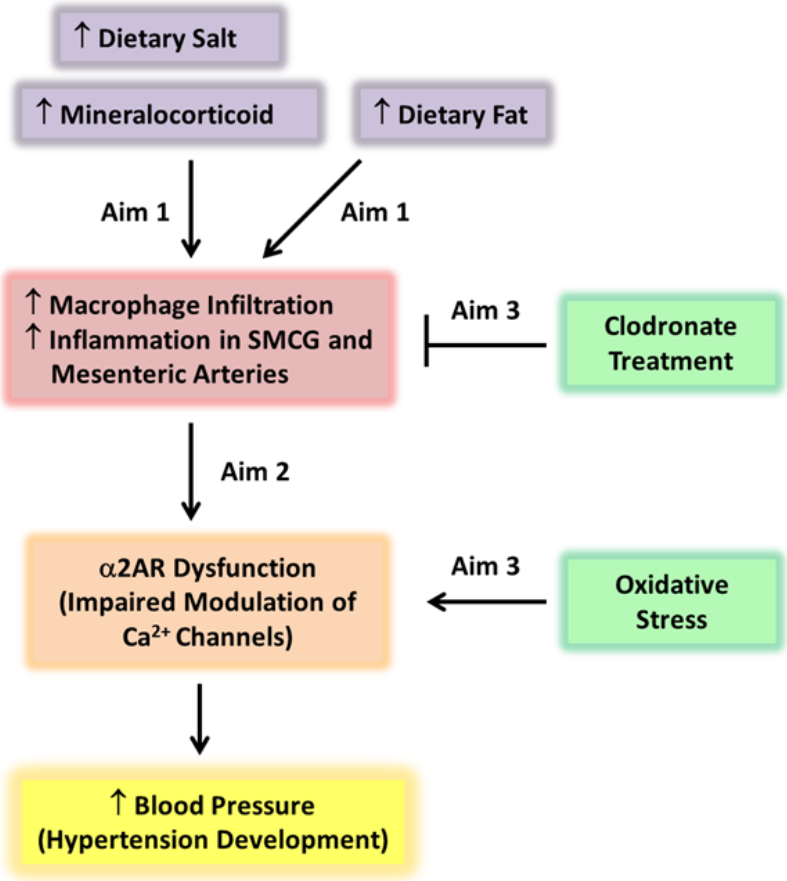

Figure 10. Hypotheses and specific aims of our study. Aim 1 is presented in Chapter 4 and investigates if hypertension is associated with increased inflammation in SMCG tissue and vascular macrophage infiltration in mesenteric arteries. Aim 2 is presented in Chapter 5 and investigates if hypertension is associated with α_2AR dysfunction in SMCG neurons. Aim 3 is presented in Chapter 6 and investigates if macrophage depletion preserves α_2AR function and attenuates hypertension and investigates how oxidative stress relates to α_2AR dysfunction.

3.0 Specific Aims

Aim 1: To test the hypothesis that increased adventitial macrophage infiltration in mesenteric arteries and an increase in MCP-1 and TNFα expression in SMCG tissue is associated with mineralocorticoid-salt and obesity-related hypertension. Vascular macrophage infiltration will be determined by immunohistochemical localization of CD163 on fixed whole-mounts of mesenteric arteries as an average of macrophage number per 1 mm^2 of tissue. Inflammation in SMCG tissues will be assessed by qRT-PCR for gene expression levels of MCP-1 and TNFα. In some animals, these experiments will be performed at different time points to capture changes in inflammation that might occur during different developmental stages of hypertension.

Aim 2: To test the hypothesis that impaired α_2AR-mediated inhibition of Ca^{2+} channels is associated with mineralocorticoid-salt and obesity-related hypertension. Whole-cell patch clamp methods will be used to evaluate α_2AR-mediated inhibition of Ca^{2+} channels, Ca^{2+} channel activity, current-voltage relationships, and cell membrane capacitance in acutely dissociated SMCG neurons. In some animals, these experiments will be performed at different time points to capture changes that might occur during different developmental stages of hypertension. Levels of α_2AR and GRK subtype mRNA expression will be evaluated by qRT-PCR to verify if results occur due to changes in receptor expression or desensitization. GppNHp will be used to evaluate Ca^{2+} channel inhibition independent of α_2AR activation. Mechanisms of receptor desensitization will be further evaluated using patch clamp and pharamocological blockade of GRK.

Aim 3: To test the hypothesis that macrophage depletion preserves α_2AR function and attenuates hypertension development, and to test the hypothesis that oxidative stress impairs α_2AR function by promoting thiol-dependent receptor internalization. Liposome-encapsulated clodronate will be used to deplete animals of macrophages. Blood pressures will be measured by the tail-cuff method. Macrophage depletion will be verified by immunohistochemical localization of CD163 in whole-mounts of mesenteric arteries and by spleen weight. Whole-cell patch clamp methods will be used to evaluate α_2AR-mediated inhibition of Ca^{2+} channels, Ca^{2+} channel activity, current-voltage relationships, and cell membrane capacitance in acutely dissociated SMCG neurons. The effects of non-specific oxidative stress (H_2O_2, t-BOOH) and diamide (thiol-specific oxidant) on α_2AR function will be evaluated using patch-clamp on SMCG neurons and G1A1 cells (HEK293-T cells that express Ca^{2+} channels) co-transfected with α_2AR and pertussis-insensitive (PTXi) Gαo (Cys351Gly). The effects of oxidative stress on receptor localization will be evaluated by membrane co-localization of F-actin and α_2AR in HEK293-T cells transfected with the α_2AR.

CHAPTER 3: EXPERIMENTAL DESIGN AND METHODS

1.0 General Experimental Design

An algorithm of the experimental design used in these studies is depicted in Figure 10. In brief, hypertension associated with inflammation and α_2AR dysfunction will be further explored by studies that involve macrophage-depletion, determine the level of dysfunction, and determine how oxidative impairs α_2AR function. In the absence of inflammation and α_2AR dysfunction, the next steps will depend on the degree of hypertension. In models with mild hypetension, treatment time will increase to see if α_2AR dysfunction occurs when hypertension is prolonged and/or more severe. In models with more severe forms of hypertension, α_2AR function will be assessed at earlier time points to determine if α_2AR dysfunction particpiates more in the developmental phase of hypertension.

We will explore α_2AR dysfunction further by determining if deficits lie at the level of the receptor or through downstream signaling and investigate if oxidative stress impairs α_2AR function and localization.

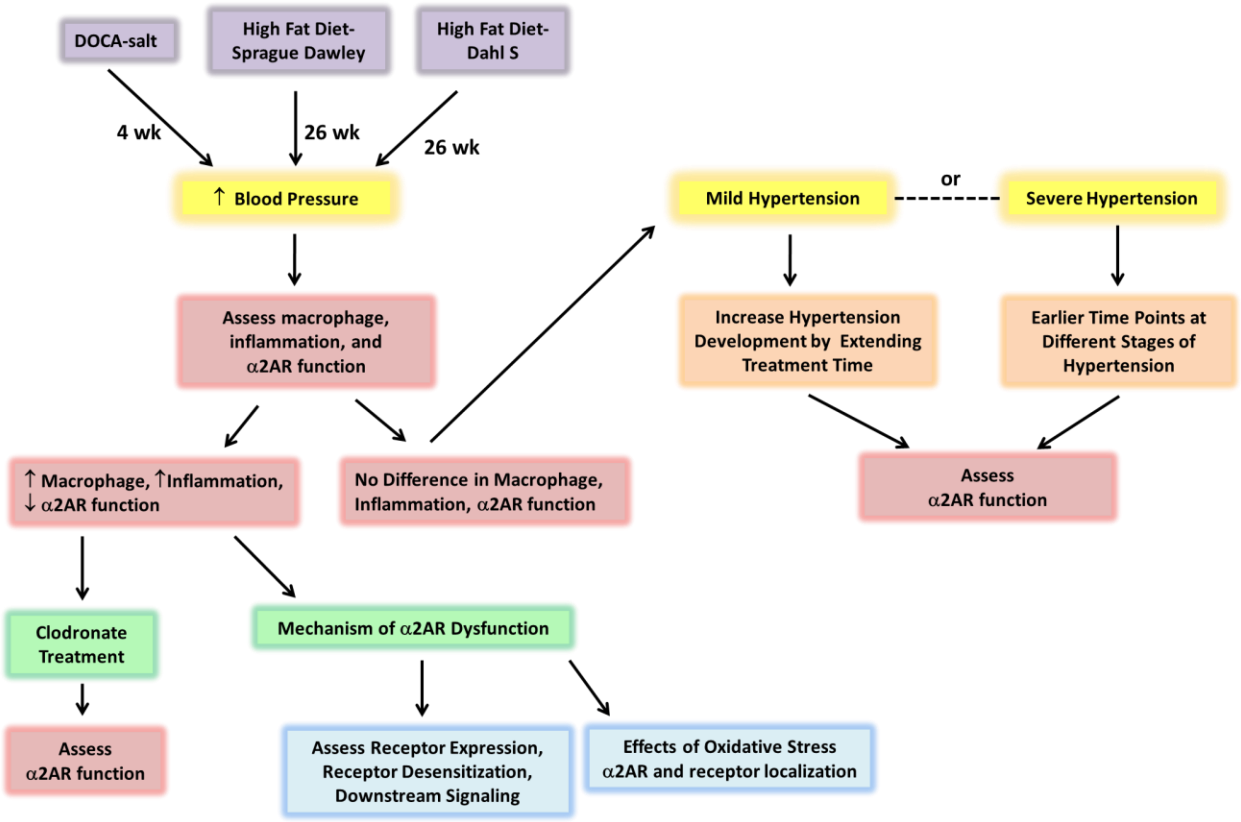

Figure 11. Experimental design. We will ssess hypertension development and α_2AR function following administration of DOCA-salt or high fat diet. Experiments in models that do not involve α_2AR dysfunction will be repeated at earlier (for severe hypertension) or extended (for mild hypertension) time points.

2.0 Materials and Methods

DOCA-Salt hypertensive rats: All animal use protocols were approved by the Institutional animal Care and Use Committee of Michigan State University. Male SD rats (Charles River Laboratories, Portage, MI) were delivered at 8 weeks of age and allowed to acclimate for 5 days before surgical procedures. Rats were anesthetized via isofluorane inhalation (0.5-2%) on a heating pad in dorsal recumbent position. The left kidney was exteriorized and the renal artery, vein, and ureter were ligated with 5.0 silk before removing the kidney. After uninephrectomy, some of the rats were given normal tap water *ad libitum* (SHAM). The remaining rats received DOCA-salt treatment that consisted of a silastic pellet containing 150 mg per 250 g bodyweight DOCA, which was implanted s.c in between the scapulae and high salt/potassium drinking water (1% NaCl, 0.2% KCl) *ad libitum*. Following surgery all rats were given penicillin (5mg/kg, i.m.) and carprofen (5 mg/kg, s.c.) and housed for 4 weeks on standard rat chow (Harlan/Tekland 8640 Rodent Diet).

Systolic blood pressure was measured in conscious rats using the automated CODA tail-cuff system (Kent Scientific, Torrington, CT). Rats were restrained in holding chambers and allowed to acclimate for 10 minutes before measurements were taken. Tail cuffs were inflated to 250 mmHg and slowly deflated over a period spanning 15 seconds. Systolic blood pressure was calculated by the average of 5 consecutive measurements using a volume recording sensor.

In some experiments, rats were depleted of macrophages using clodronate embedded liposomes (ClodronateLiposomes, Amsterdam, The Netherlands), prepared as previously described [295]. After uninephrectomy, rats were injected intravenously via the tail vein with either clodronate embedded liposomes at 50 mg/kg of rat from a 5 mg/mL suspension

(clodronate-liposomes), or an equivalent volume of PBS containing empty liposomes (PBS-liposomes). Thereafter, all rats were injected intraperitoneally once a week with clodronate-liposomes at 25 mg/kg from a 5 mg/mL suspension, or an equivalent volume of PBS-liposomes until the date of sacrifice.

Retrograde labeling: For non-macrophage depletion studies, experiments were performed on mesenteric artery-projecting SMCG neurons that were labeled with 1% cholera toxin subunit B conjugated to Alex Fluor 555 (CTB-555, Thermo Fisher Scientific, Waltham, MA). This procedure was done midway in the final week of DOCA-salt treatment. Rats were anesthetized via isofluorane inhalation (0.5-2%) on a heating pad in dorsal recumbent position. Following laparotomy, a loop of small bowel was exteriorized, spread, and secured by gauze soaked in sterile pre-warmed HBSS. Three secondary branches of mesenteric arteries were cleanly dissected from perivascular fat and encased in longitudinally cut silicone tubing. Once placed around the artery, the tubing was sealed with silicone at both ends. Using a pipetteman, 30 μL of CTB-555 was transferred directly onto arteries and allowed to incubate for 1 hour. The tubing was then removed and washed with sterile HBSS. The bowel was undraped and returned into the abdomen and moistened with 3 ml of HBSS. Rats received penicillin (5 mg/kg i.m.) and carprofen analgesic (5 mg/kg, s.c.), and were housed for 4 days before the 4 week end point. Immunohistochemistry was performed to determine labeling efficiency in the SMCG and in fixed SMCG neurons. Retrogradely labeled SMCG tissue was fixed in in Zamboni fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) before embedding in O.C.T compound (VWR, Randor, PA). 40 μM sections were made using a HM 550 Cryostat and placed onto superfrost plus microscope slides (Thermo Fisher Scientific, Waltham, MA). Slides were washed

with 0.1 M phosphate buffer (pH 7.4) and incubated in blocking buffer (0.1 M phosphate buffer, 4% donkey serum, 4% goat serum, 0.2% Triton X-100) for 1 hr at room temperature. Samples were incubated overnight at 4 °C in blocking buffer containing primary antibody against tyrosine hydroxylase (TH) (BML-TZ1010-0050, Enzo Life Sciences, Farmingdale, NY). Samples were washed with 0.1 M phosphate buffer and incubated in blocking buffer containing secondary donkey anti-rabbit Alexa Fluor 488 (1:1000, Thermo Fisher Scientific, Waltham, MA) for 1 hr at room temperature. Samples were washed with 0.1 M phosphate buffer and mounted with mounting media containing DAPI (Thermo Fisher Scientific, Waltham, MA). Images were taken using a Nikon TE2000-U inverted fluorescent microscope. SMCG neurons were stained with the same protocol described above and imaged using an Olympus FluoView FV1000 confocal laser scanning microscope.

HFD-induced hypertension: All animal use protocols were approved by the Institutional animal Care and Use Committee of Michigan State University. Beginning at 3 weeks of age, male Sprague-Dawley (SD) and Dahl salt-sensitive (DahlS) rats (Charles River Laboratories, Portage, MI) were fed either a low salt-normal fat containing diet (NFD, 20 kcal% protein, 70 kcal% carbohydrate, 10 kcal% fat, 0.3% salt from Research Diets, #D12450J) or a low salt-high fat containing diet (HFD, 20 kcal% protein, 20 kcal% carbohydrate, 60 kcal% fat, 0.3% salt from Research Diets, #D12492) with access to tap water *ad libitum* for 6 months. Rats were weighed once a week. Blood pressure was continuously monitored by radiotelemetery (TA11PA-C40, DSI). This was accomplished by catheterization of the femoral artery with transmitter placed subcutaneously in the inner thigh. Rats were housed in separation on top of a radiotelemetry receiver (RPC-1, DSI) and blood pressure was continuously monitored through a data exchange

matrix and computer based data-acquisition system (Dataquest ART 4.36, DSI). Rats were housed for 6 months. A separate batch of DahlS rats were analyzed at 2 and 4 months, representing different stages of hypertension development. A separate batch of SD rats (Harlan Laboratories, Haslett, MI) were analyzed after 12 months of feeding in order to allow more time for obesity and hypertension to develop. A separate batch of obesity-prone (OP) and obesity-resistant (OR) SD rats (Charles River Laboratories, Portage, MI) were analyzed after 6 months weeks of feeding. A separate batch of female SD rats (Charles River Laboratories, Portage, MI) were analyzed after 10 months of feeding in order to determine any sex-related effects.

Dissociated SMCG neurons: Rats were euthanized with sodium pentobarbital (50 mg/kg, *i.p.*) or by decapitation after isofluorane inhalation (0.5-2%). SMCG tissues were removed, dissected, cut into small pieces, and transferred into 3 mL of Earle's balanced salt solution (supplemented to 3.6 mg/mL glucose and 10 mM HEPES) containing 0.6 mg/ml collagenase D (Roche, Indianapolis, IN), 0.4 mg/ml trypsin (Worthington, Lakewood, NJ), and 0.1 mg/ml DNAse (Sigma, St. Louis, MO) at 35 ^{o}C for 1 hour in a shaking water bath. 6 mL of minimal essential medium (MEM, Thermo Fisher Scientific, Waltham, MA), containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA), 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA), and 1% L-glutamine (Thermo Fisher Scientific, Waltham, MA) was added to the reaction before dispersion using fire-polished Pasteur pipettes. Cells were centrifuged twice at 70 x g, plated in MEM (containing 10% FBS, 1% penicillin/streptomycin) onto poly-L-lysine coated 12 mm coverslips (VWR, Randor, PA), and stored in a humidified incubator (95% air and 5% CO_2) at 37 ^{o}C for 3-10 hours.

Whole-cell patch clamp recordings. Whole-cell patch clamp was used to measure Ca^{2+} currents and α_2AR-mediated inhibition of Ca^{2+} channels at room temperature (20-24 ^{o}C) using an Axopatch 200A voltage clamp amplifier (Molecular Devices, Union City, CA) and a Digidata 1322A (Axon Instruments, Union City, CA) A/D converter. Schott 8250 capillary glass (World Precision Instruments, Sarasota, FL) was used to generate recording electrodes (2-4 MΩ) using a P-97 micropipette puller (Sutter Instrument Co., Novato, CA). Electrodes were fire-polished and filled with internal pipette solution that contained (in mM): CsCl 143, MgCl2 1, EGTA 11, HEPES 10, MgATP 4, Na_2GTP 0.3, adjusted to PH 7.2 with CsOH and 295 mosmol/kg with sucrose. When appropriate, 5'-guanylyl imidodiphosphate (GppNHp, Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 500 μM. The external recording solution contained (in mM): NaCl 97, TEA-Cl 20, CsCl 4.7, $CaCl_2$ 5, MgCl2 1.2, HEPES 10, glucose 11, tetrodotoxin (TTX) 0.00001, adjusted to pH 7.4 with CsOH and 310 mosmol/kg with CsCl. Drugs to evaluate α_2AR function included NE, UK14304, and yohimbine and were manually administered into the chamber. For GRK2 inhibition experiments, cells were pretreated with 30 μM paroxetine (GRK2 inhibitor) or fluoxetine (control) in MEM for 50-60 minutes. For oxidative stress experiments, cells were pretreated with 150 μM H_2O_2, 150 μM t-BOOH, or 50 μM diamide in MEM for 30-40 minutes. Ca^{2+} current recordings were low-pass-filtered at 5 kHz. Data were evaluated as maximal current inhibition and were analyzed using pCLAMP version 10.2 software (Molecular Devices, Sunnyvale, CA).

qRT-PCR. Total RNA was extracted with the MagMax mirVana kit (Thermo Fisher Scientific, Waltham, MA) and reverse transcribed into cDNA with the High capacity RNA to cDNA kit (Thermo Fisher Scientific, Waltham, MA). Taqman probes (Thermo Fisher Scientific,

Waltham, MA) for all assessed genes (Table 1) were used and were processed in the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Reactions were prepared with Taqman Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA). Relative gene transcript levels were calculated by the cycle threshold and normalized with endogenously expressed GAPDH.

Table 1. Taqman probes for genes assessed in the SMCG.

Gene ID	Assay ID
Adr2a	Rn00562488_s1
Adr2b	Rn00593312_s1
Adr2c	Rn00593341_s1
Grk2	Rn00563688 m1
Grk3	Rn00562822 m1
Grk5	Rn00578086 m1
TNFa	Rn01525859_g1
MCP1	Rn00580555_m1
Gapdh	Rn01775763_g1

Immunohistochemical localization of macrophages and sympathetic nerve fibers. Second order mesenteric arteries were carefully dissected from perivascular fat and fixed overnight in Zamboni fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Samples were washed with 0.1 M phosphate buffer (pH 7.4) and incubated in blocking buffer (0.1 M phosphate buffer, 4% donkey serum, 4% goat serum, 0.2% Triton X-100) for 1 hour at room temperature. Samples were incubated overnight at 4 °C in blocking buffer containing primary antibodies against CD163 (1:200; Cat #: MCA342R, BIORAD, Hercules, CA) and tyrosine hydroxylase (TH) (BML-TZ1010-0050, Enzo Life Sciences, Farmingdale, NY). Samples were washed with 0.1 M phosphate buffer and incubated in blocking buffer containing secondary

donkey anti-rabbit Alexa Fluor 594 (1:1000, Thermo Fisher Scientific, Waltham, MA) and goat anti-mouse Alexa Fluor 488 (1:1000, Thermo Fisher Scientific, Waltham, MA) antibodies for 1 hour at room temperature. Samples were washed with 0.1 M phosphate buffer and mounted with mounting media containing DAPI (Thermo Fisher Scientific, Waltham, MA). Images were taken using an Olympus FluoView FV1000 confocal laser scanning microscope.

Transfection and α_2AR localization in HEK-293T cells. All cells and DNA constructs were kindly gifted to us by Richard Neubig. The human embryonic kidney (HEK-293T) cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA), containing 10% FBS (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA) in a humidified incubator (95% air and 5% CO_2) at 37 ^{o}C. Cells were transfected with the porcine α_{2A} adrenergic receptor in the pEGFP vector [296] using Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions 24 hours before cells were treated with 150 μM H_2O_2, 150 μM t-BOOH, or 50 μM diamide in DMEM (45 minutes). F-actin was stained with Alexa Fluor 594 phalloidin (Thermo Fisher Scientific, Waltham, MA) and receptor membrane localization was evaluated using an Olympus FluoView FV1000 confocal laser scanning microscope and by Image J using Pearson's correlation coefficient of co-localization. G1A1 are HEK 293 cells that stably express the α_{2B}, β_1, and $\alpha_{2\delta}$ subunits of N-type Ca^{2+} channels that have been used to study α_2AR-induced modulation of Ca^{2+} currents [297]. G1A1 cells were maintained in MEM (Thermo Fisher Scientific, Waltham, MA), containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA) in a humidified incubator (95% air and 5% CO_2) at 37 ^{o}C. Cells were co-transfected with the α_{2A} adrenergic receptor in the

pEGFP vector [296] and the pertussis toxin (PPTX)-insensitive murine $G\alpha_o$ (PTXi-$G\alpha_o$) in the pCI vector [298] using Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions 24 hours before cells were treated with 150 μM H_2O_2, 150 μM t-BOOH, or 50 μM diamide in DMEM (45 minutes). All constructs were kindly gifted from Dr. Richard Neubig's laboratory.

Statistical Analysis. All data are represented as mean ± standard error of the mean (SE), where n equals animal number or number of rats or cells from which all the data were obtained. Data were analyzed with Prism software using the unpaired two sample-student's t-test.

CHAPTER 4: ADVENTITIAL MACROPHAGE ACCUMULATION AND SMCG INFLAMMATION OCCUR IN DOCA-SALT BUT NOT HIGH FAT DIET-INDUCED HYPERTENSION

1.0 Abstract

Macrophages are associated with mineralocorticoid, salt, and obesity-related hypertension. We have previously shown that macrophage-associated neurovascular inflammation occurs in mesenteric arteries of DOCA-salt hypertensive rats. However, it is unclear if inflammation occurs in the SMCG that provides sympathetic innervation to mesenteric arteries, or if this mechanism is relevant to other models of hypertension, such as obesity-related hypertension. Therefore, we investigated if adventitial macrophage accumulation in mesenteric arteries and SMCG inflammation were increased in DOCA-salt hypertensive rats and high fat diet (HFD)-induced hypertensive Sprague Dawley (SD) and Dahl salt-sensitive (DahlS) rats. DOCA-salt treatment resulted in hypertension development that was associated with increased adventitial macrophage infiltration and SMCG inflammation. After 6 months, a HFD was associated with mild hypertension without significant weight gain in SD rats versus normal fat diet (NFD)-fed rats. 6 months of HFD induced hypertension with significant weight gain in DahlS rats versus NFD-fed rats, which became significant at 4 months and continued to increase until the 6 month time point. A HFD was not associated with increased adventitial macrophage infiltration and SMCG inflammation in SD and DahlS rats. These results suggest that neurovascular inflammation in the splanchnic circulation may only be relevant to states of mineralocorticoid-salt excess. These results may be explained by animal model differences.

2.0 Introduction

Macrophages play an important role in human hypertension. Circulating pre-activated monocytes and macrophage-related markers are increased in hypertensive subjects compared to normotensive subjects [273-275]. Organs that are associated with hypertension development include the peripheral vasculature, kidney and brain; organs all of which are enriched with macrophages in many experimental models of hypertension [54, 299]. However, the mechanisms that trigger monocyte recruitment and the role that macrophages play in the pathogenesis of hypertension are still unclear. Studies using macrophage depletion strategies suggest a large part involves pro-inflammatory cytokine and oxidative stress production [269].

In hypertension, vascular monocyte recruitment is largely dependent on chemotaxis via the MCP-1/CCR2 system as blocking CCR2 (MCP-1 receptor) on macrophages attenuates vascular macrophage accumulation and hypertension development caused by DOCA-salt and ANG-II-infusion [279]. MCP-1 is upregulated during tissue injury and blood monocytes are on constant patrol for endothelial damage [300, 301]. In hypertension, vascular tissue injury occurs first in the endothelial layer, which is the only vascular layer that comes into direct contact with blood flow. Normal increases in friction and wall shear stress are counteracted by endothelial release of NO, which causes vascular smooth muscle relaxation to accommodate mild and transient increases in blood pressure [255]. However, persistent increases in pressure or decreases in NO bioavailability can cause endothelial dysfunction that makes the blood vessel less flexible and more prone to injury [254, 257]. Enhanced SNA contributes to increased mechanical loading and wall stress either directly by vasoconstriction or indirectly by activating RAAS and increasing renal reabsorption of Na^+ and water. Reductions in NO bioavailability are

mediated by ROS members like superoxide, which combines with NO to form peroxynitrite [257, 261]. Enhanced vascular oxidative stress production occurs with prolonged mechanical stretch to endothelial cells through Rac1 activation of NADPH oxidases [216, 218]. Therefore, increases in intensity and duration from pressure-overload increases vascular ROS production, depletes NO bioavailability, and makes blood vessels more prone to injury. Tissue injury promotes upregulation of MCP-1, which recruits blood monocytes [279].

Macrophage accumulation and pro-inflammatory activation in vascular and neuronal tissue play a significant role in the pathogenesis of hypertension. Macrophages contribute to endothelial dysfunction and vascular remodeling that leads to an increase in vascular resistance and stiffness, providing further support to tissue injury [302]. Macrophage-derived TNFα and superoxide deplete NO by reducing eNOS and activating NADPH oxidase, and reacting to form peroxynitrite, respectively. This increases endothelial dysfunction, leading to tissue injury progression and unopposed influence by endothelial-derived contracting factors such as endothelin-1 [261, 280, 281, 303]. Macrophages also participate in flow-induced vascular remodeling in hypertension. Remodeling involves not only the production of inflammatory mediators, but also matrix metalloproteinases [304], which degrade extracellular matrix and modulate cytokine and chemokine activity [305, 306]. Taken together, these results suggest that macrophage-related endothelial dysfunction and vascular remodeling play a large role in increasing vascular resistance.

We have recently shown that macrophage-derived superoxide contributes to hypertension development in the DOCA-salt rat by impairing negative feedback of NE nerve

terminal release through the prejunctional α_2AR in mesenteric arteries [127]. These results suggest that macrophage-related neuro-inflammation play a role in modulating SNA.

Enhanced production of macrophage-derived inflammatory mediators are common features of mineralocorticoid, salt-sensitive, and obesity-related hypertension. Under all of these conditions, macrophage recruitment and activation appear to depend on peroxisome proliferator-activated receptor (PPARγ, glitazone receptor) [307, 308], which when activated by metabolites of arachidonic (15-HETE) and linoleic (9-HODE, 13-HODE) acid, enter the nucleus to potentiate gene transcription of inflammatory proteins [307, 309, 310]. Salt has been shown to directly enhance mineralocorticoid signaling in macrophages through Rac1 activation, which leads to activation of NADPH oxidases and enhanced oxidative stress production. This can occur independently with either salt or aldosterone, but is enhanced in the presence of both [216, 219]. In obesity, leptin is increased and has been shown to enhance macrophage proliferation and production of TNFα and IL-6 [311].

Taken together, macrophages may play a role in mineralocorticoid, salt, and obesity-related hypertension by affecting both vascular and neuronal tissue. Both can be investigated in the splanchnic circulation, which provides most of the body's vascular resistance through mesenteric resistance arteries. Splanchnic denervation decreases blood pressure in normal [61] and hypertensive rats [59, 60, 62, 63]. Mesenteric arteries are innervated by sympathetic neurons of the superior mesenteric and celiac ganglion (SMCG) [55, 56]. Therefore, we investigated if increased macrophage infiltration in mesenteric arteries and inflammation in the SMCG was associated with hypertension caused by mineralocorticoid, salt, and obesity. Mineralocorticoid-salt was investigated using the DOCA-salt rat model, which is a hypertension

model characterized by mineralocorticoid excess, salt-sensitivity, and low-renin hypertension. We attempted to induce obesity-related hypertension in Sprague Dawley (SD) and Dahl salt-sensitive (DahlS) rats through a low salt (0.3%) and high fat (60% kcal fat) diet. Unlike SD rats, DahlS rats are associated with salt-sensitive and low-renin-hypertension. This enabled us to study the effects of obesity in consideration of other hypertension-associated factors that might be relevant to human hypertension.

3.0 Results

Effects of DOCA-salt treatment on blood pressure and inflammation. At the 4 week end point, mean systolic blood pressure was significantly higher in DOCA-salt rats compared to SHAM rats (Figure 12A). SMCG and mesenteric artery tissues were collected to evaluate inflammation by qRT-PCR and macrophage infiltration, respectively. In the SMCG, levels of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNFα) mRNA were significantly higher in DOCA-salt rats compared to SHAM rats (Figure 12B). In mesenteric arteries, adventitial macrophage infiltration was evaluated by CD163 immunoreactivity and was significantly higher in DOCA-salt rats compared to SHAM arteries (Figure 12, C and D). Adventitial macrophages were in close proximity to perivascular sympathetic nerve fibers identified by TH immunoreactivity (Figure 12C). Although adventitial macrophage infiltration was generally increased in DOCA-salt hypertension, this occurred to variable degrees. Mesenteric arteries displayed areas of low as well as high levels of adventitial macrophage infiltration (Figure 13, A and B).

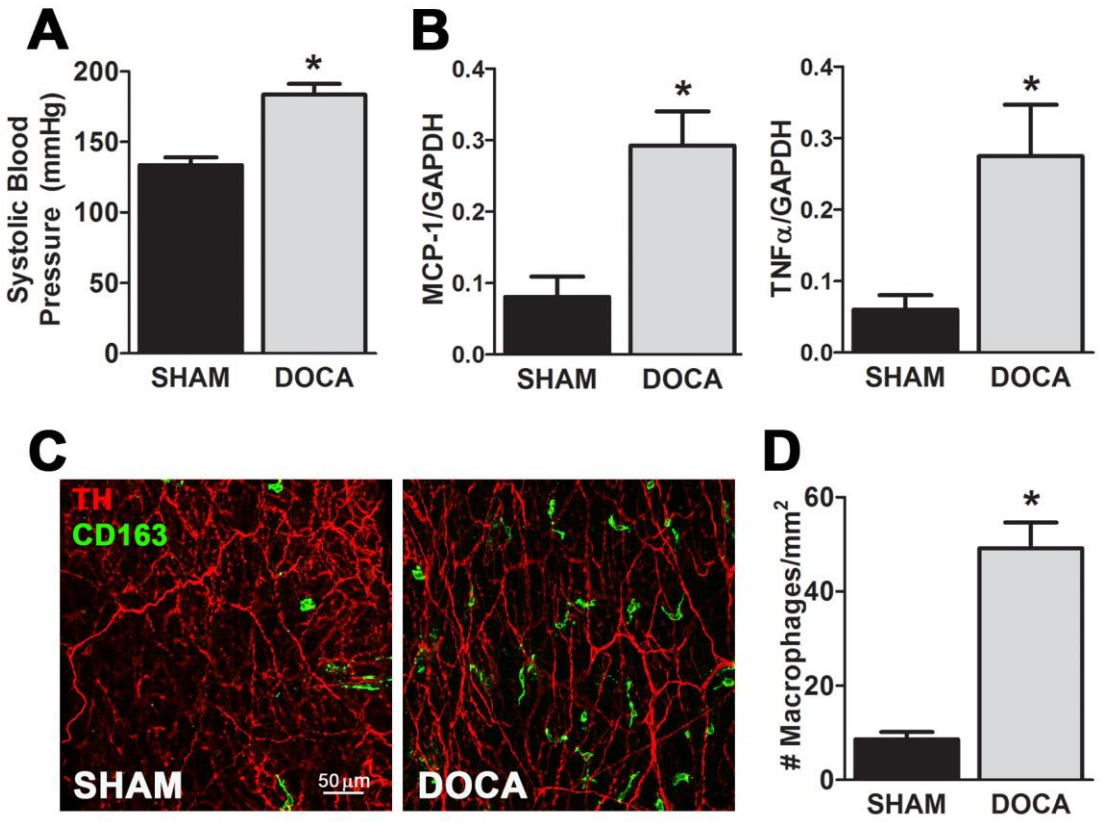

Figure 12. DOCA-salt hypertension is associated with inflammation in SMCG and mesenteric arteries. *A*: Mean systolic blood pressure was increased in DOCA-salt rats compared to SHAM rats; *n* = 7 rats/group. ***B*:** mRNA levels of monocyte chemoattractant protein -1 (MCP-1, *left*) and tumor necrosis factor-α (TNFα, *right*) were higher in SMCG from DOCA-salt rats compared to SHAM rats; *n* = 4 rats/group. ***C*:** Immunohistochemical labeling of CD163-positive macrophages and tyrosine hydroxylase (TH)-positive sympathetic nerve fibers in whole-mounts of mesenteric arteries. ***D*:** Mean adventitial macrophage number per mm^2 was higher in mesenteric arteries from DOCA-salt rats compared to SHAM rats; *n* = 7 rats/group. Data are means ± SE, *P < 0.05 vs. SHAM.

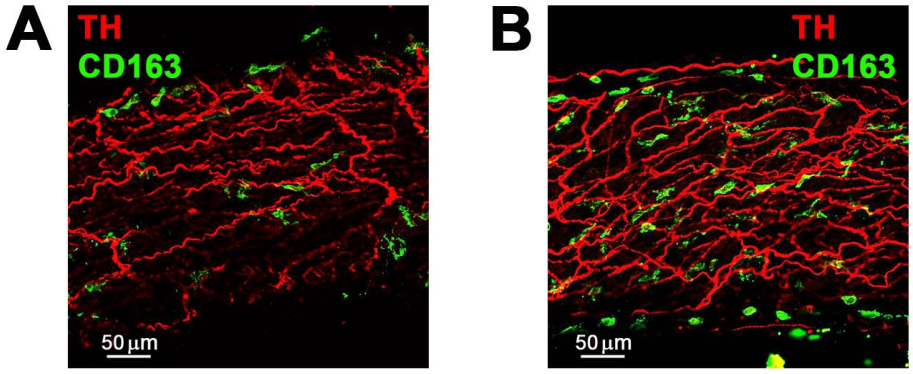

Figure 13. Low and high adventitial macrophage infiltration in mesenteric arteries from DOCA-salt rats. Immunohistochemical labeling of CD163-positive macrophages and tyrosine hydroxylase (TH)-positive sympathetic nerve fibers in whole-mounts of mesenteric arteries. Mesenteric artery showing **A:** lower and **B:** higher degree of macrophage accumulation.

Effects of HFD on obesity, hypertension and inflammation in SD and DahlS rats. SD rats fed a high fat diet (HFD) for 6 months did not develop significant weight gain but developed mild hypertension compared to normal fat diet (NFD)-fed SD rats (Figure 14, A and B). DahlS rats fed a HFD developed significantly higher weight gain and systolic blood pressure at 4 and 6 month endpoints compared to NFD-fed DahlS rats (Figure 15, A and B). SMCG and mesenteric arteries were collected to evaluate inflammation by qRT-PCR and macrophage infiltration, respectively. SMCG mRNA levels of MCP-1 and TNF α were similar in NFD and HFD-fed SD rats at 6 months (Figure 14C) and DahlS rats at 2 months (Figure 16A), 4 months (Figure 16D), and 6 months (Figure 16G). Similarly, macrophage accumulation in mesenteric arteries, as evaluated by CD163 immunoreactivity, was similar regardless of diet in SD rats at 6 months (Figure 14, D and E) and DahlS rats at 2 months (Figure 16, B and C), 4 months (Figure 16, E and F), and 6 months (Figure 16, H and I).

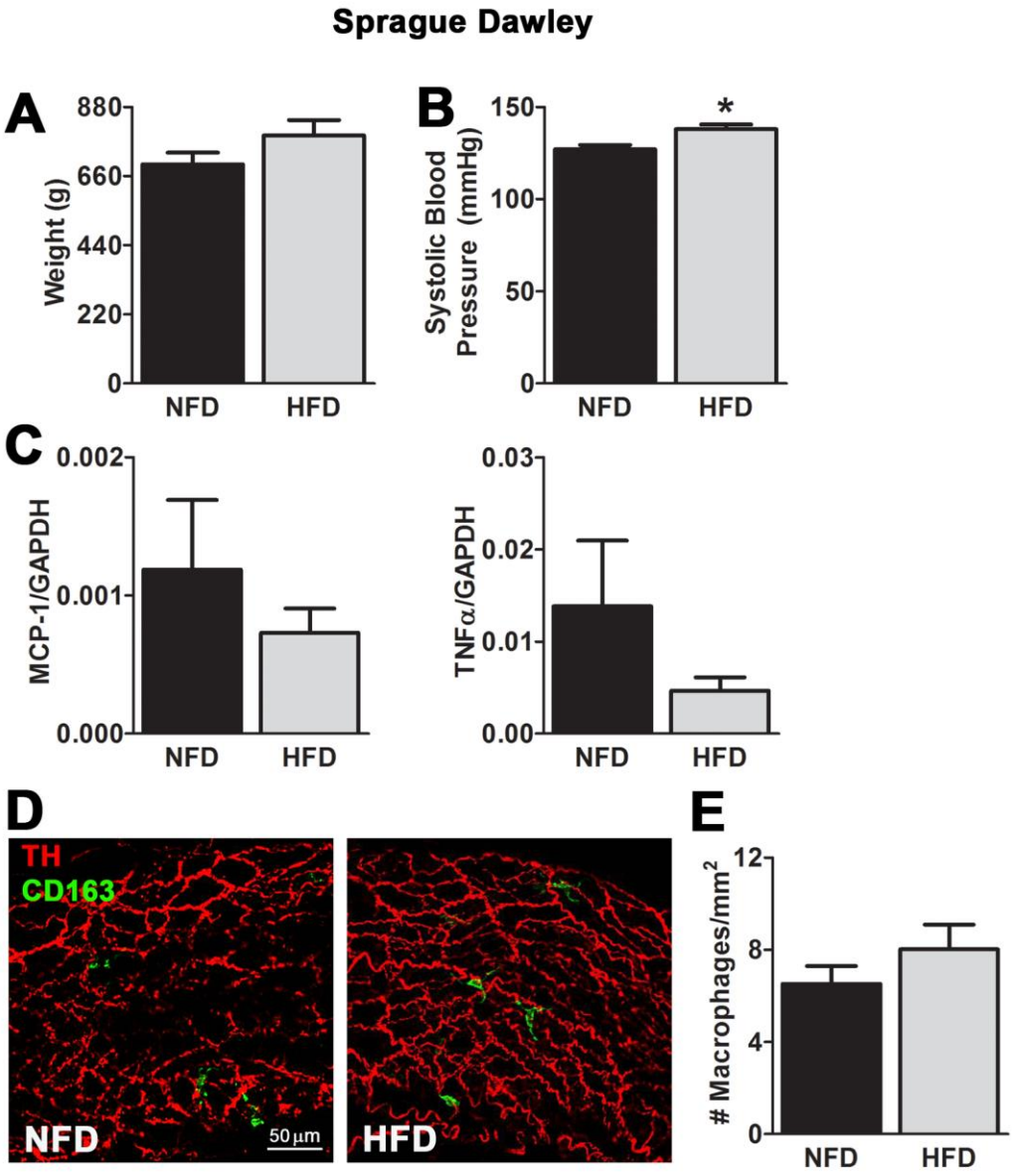

Figure 14. Hypertension development in Sprague Dawley (SD) rats fed a HFD for 6 months is not associated with obesity or SMCG and mesenteric artery inflammation. *A*: Body weight was not increased in SD rats fed a HFD rats compared to NFD rats; *n* = 5 rats/group. ***B*:** Mean systolic blood pressure was mildly increased in HFD rats compared to NFD rats; *n* = 5 rats/group. ***C*:** mRNA levels of chemoattractant protein -1 (MCP-1, *left*) and tumor necrosis factor-α (TNF-α, *right*) in SMCG tissue were similar in all rats; *n* = 3 rats/group. ***D*:** Immunohistochemical labeling of CD163-positive macrophages and tyrosine hydroxylase (TH)-positive sympathetic nerve fibers in whole-mounts of mesenteric arteries from HFD rats and NFD rats. ***E*:** Mean adventitial macrophage number per mm^2 was similar in mesenteric arteries from HFD rats and NFD rats; *n* = 5 rats/group. Data are means ± SE , *p<0.05.

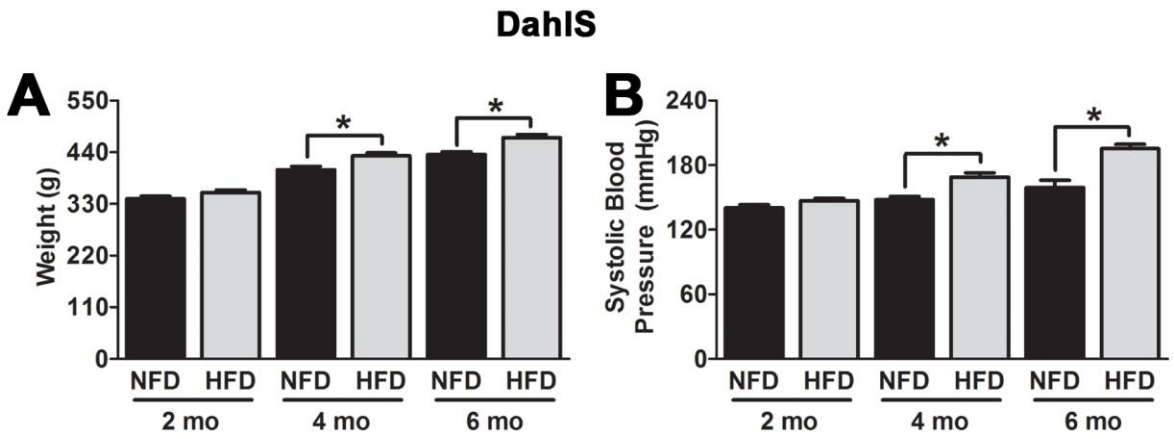

Figure 15. Obesity-related hypertension development in HFD-fed DahlS rats. *A*: Mean weight was significantly increased at 4 and 6 months in HFD-DahlS rats compared to NFD-DahlS rats. ***B*:** Mean systolic blood pressure was significantly increased at 4 and 6 months in HFD DahlS rats compared to NFD DahlS rats. Data are means ± SE, *P < 0.05, *n* = 5 rats/group.

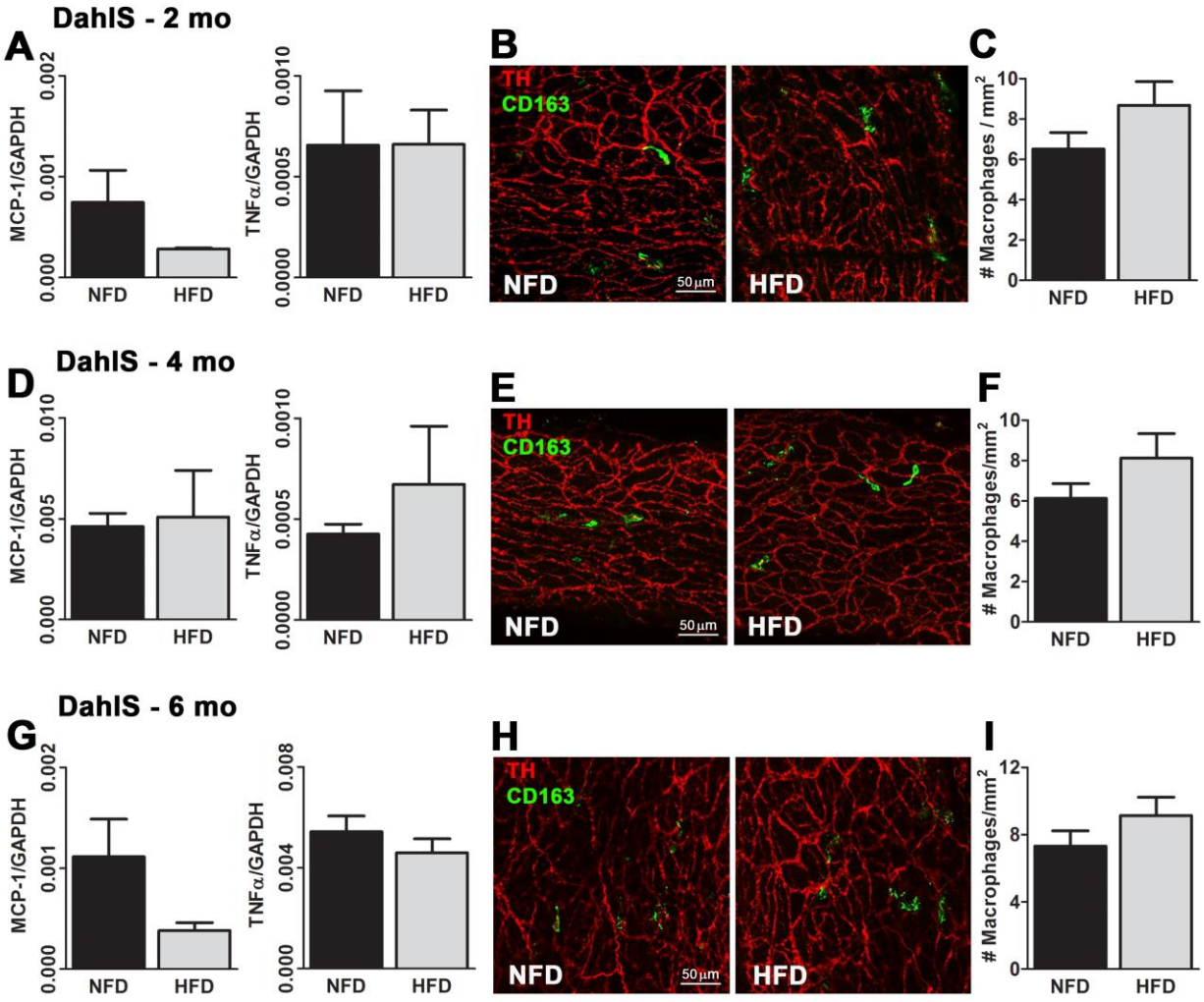

Figure 16. Obesity-related hypertension in HFD DahlS rats is not associated with inflammation in SMCG and mesenteric arteries at 2 (*top row*), 4 (*middle row*), and 6 months (*bottom row*). ***A, D, G*:** mRNA levels of monocyte chemoattractant protein -1 (MCP-1, *left*) and tumor necrosis factor-α (TNFα, *right*) in SMCG tissue were similar in all rats; *n* = 3 rats/group. ***B, E, H*:** Immunohistochemical labeling of CD163-positive macrophages and tyrosine hydroxylase (TH)-positive sympathetic nerve fibers in whole-mounts of mesenteric arteries from HFD and NFD DahlS rats. ***C, F, I*:** Mean adventitial macrophage number per mm^2 was similar in mesenteric arteries from HFD compared to NFD DahlS rats; *n* = 5 rats/group.

4.0 Discussion

The main findings of this chapter are: 1) DOCA-salt treatment results in hypertension that is associated with inflammation in mesenteric artery and SMCG tissue. 2) HFD induces mild hypertension without significant weight gain in SD rats. 3) HFD induces hypertension with significant weight gain in DahlS rats. 4) HFD-induced hypertension is not associated with inflammation in mesenteric artery and SMCG tissue.

Accumulation of peripheral vascular macrophages contributes to hypertension by mechanisms that are not fully understood [299]. Adventitial immune cell infiltration has been suggested to occur bi-directionally, from both luminal ("inside-out") and adventitial ("outside-in") ends [304, 312]. Mechanisms of chemotaxis via MCP-1 may be important in hypertension as blocking MCP-1 receptors reduces vascular macrophage accumulation and blood pressure [279]. There are also interactions between vascular tissue injury, oxidative stress, and cytokine production that can increase blood pressure [253, 313]. We found increased adventitial macrophage accumulation in mesenteric arteries from DOCA-salt but not HFD-fed hypertensive rats. There are differences in the animal models that can explain this result.

Firstly, monocyte recruitment in DOCA-salt rats may occur in response to vascular injury sustained by accelerated hypertension development and excessive mineralocorticoid and salt. The DOCA-salt hypertensive rat is a model of cardiovascular inflammatory and oxidative stress [262] that is associated with significant endothelial dysfunction and arterial hypertrophy [314, 315]. This is commonly observed in animal models that develop severe hypertension [316] and makes the blood vessel less flexible and more prone to injury [254, 257]. Early arterial remodeling observed in this model suggests pronounced mechanical stress and strain to the

arterial wall and endothelium [257, 316, 317] as well as stretch from pressure-overload which enhances vascular production of superoxide, MCP-1, and endothelin-1 [259, 260, 318]. Designated blood monocytes specifically patrol for vascular damage, and have been observed to display crawling behavior along the endothelium of mesenteric arteries. Following tissue injury, these monocytes infiltrate through the endothelium, increase cytokine production, and differentiate into macrophages [300, 301]. In addition, macrophages may be directed to the adventitia from perivascular tissue. This may occur due to increased MCP-1 and ROS production in the adventitia and media layers from pressure-overload [312]. For these reasons, the accelerated rate and magnitude of DOCA-induced hypertension are likely to cause an increase in vascular injury and macrophage infiltration.

In contrast, HFD-induced hypertension developed over a period of months, not weeks. Therefore, vascular injury may be less severe in these models due to adaptations that are able to preserve vascular wall structure during small and steady increases in vascular wall stress and stretch. However, it is interesting that macrophage accumulation did not change between HFD-SD rats and HFD-DahlS rats, considering the mild hypertension in SD rats versus more severe hypertension in DahlS rats that was detected even at 4 months. This may be due to observations that obesity in DahlS rats protects against obesity-related vascular dysfunction [249], which occurs in other models like the SD rat [319]. This paradoxical advantage of obesity may provide an additional level of protection against pressure-induced vascular injury in DahlS rats that is not present in SD rats or DOCA-salt rats.

Furthermore, in DOCA-salt hypertension, vascular injury and monocyte recruitment may be enhanced by elevated mineralocorticoid and salt. DOCA is a precursor for aldosterone

synthesis. Aldosterone increases vascular macrophage accumulation [96, 320], possibly by upregulating endothelial expression of intracellular adhesion molecule-1 [321]. Furthermore, aldosterone enhances production of inflammatory mediators in arteries [313, 322-324] and macrophages [313, 325]. In addition, salt enhances oxidative stress production through Rac1 activation of NADPH oxidase and these effects are augmented in the presence of aldosterone [216, 219]. Therefore, DOCA-salt may provide additional feedforward support for vascular (and neuronal, discussed below) tissue injury and monocyte recruitment that is not present in HFD-induced hypertension.

Secondly, while obesity is associated with vascular inflammation [326], it is possible that in HFD-fed rats, macrophages accumulate more in mesenteric adipose tissue versus arteries. Our macrophage analysis did not include perivascular fat, where inflammation is likely to be increased due to continuous adipocyte turnover and expansion [327, 328]. Therefore, it is possible that macrophages are retained in the adventitia of mesenteric arteries in DOCA-salt hypertension while macrophages are retained in expanding perivascular fat in HFD-induced hypertension. Furthermore, perivascular fat may also be limited in DOCA-salt hypertension due to weight loss and reductions in perivascular fat size [329].

Finally, vascular macrophages were identified using CD163, which is a scavenger receptor for the hemoglobin-haptoglobin complex expressed in mature tissue macrophages. Activation of CD163 induces expression of pro-inflammatory cytokines, that include TNF-α [330]. We have previously shown that macrophage expression of CD163 in DOCA-salt rats coincides with increases in TNFα, $p22^{phox}$ and CD11b [127] which we did not measure in the

present study. Therefore, we cannot rule out whether macrophage infiltration in obesity-related hypertension involves a different subset of macrophages.

Similar to vascular inflammation, neuro-inflammation in the SMCG was increased in DOCA-salt but not HFD-induced hypertensive rats. Inflammation was evidenced by an increase in TNFα and MCP-1 mRNA expression. These data support previous observations that superoxide is increased in SMCG tissues of DOCA-salt hypertensive rats [331] suggesting that sympathetic neuro-inflammation may be directly linked to activation of postganglionic sympathetic neurons. Macrophage density in SMCG tissues in general is low compared to mesenteric arteries (data not shown) and could not be objectively measured. An increase in sympathetic ganglionic macrophages can be detected in severe neuronal injuries such as complete nerve transection where there is extensive neuronal cell death [332]. The injury sustained in hypertension development or maintenance is likely less severe. Nevertheless, this makes it difficult to speculate the source and cause of increased TNFα and MCP-1. It is possible that these results may be due to tissue injury caused directly by mineralocorticoid-salt and/or due to mineralocorticoid-salt induced pro-inflammatory activation of macrophages.

What is clear from these findings is that mesenteric artery and SMCG inflammation are associated with DOCA-salt hypertension. The DOCA-salt hypertension model is associated with enhanced SNA [220]. SMCG nerve fibers terminate in the adventitia, in close apposition to macrophages. It is tempting to speculate that some form of neuro-immuno-vascular interaction may contribute to hypertension development in this model. Whether vascular tone is increased due to sympathetic modulation of macrophage activity or macrophage-directed modulation of neuroeffector transmission, or both, remains to be determined.

CHAPTER 5: ALPHA 2-ADRENERGIC RECEPTOR-MEDIATED INHIBITION OF CALCIUM CHANNELS IN DOCA-SALT AND OBESITY-RELATED HYPERTENSION

1.0 Abstract

DOCA-salt and obesity-related hypertension are associated with sympathetic nervous system hyperactivity. Mesenteric arteries are resistance arteries that receive sympathetic innervation from the superior mesenteric and celiac ganglia (SMCG) and have profound influences on blood pressure. Prejunctional α_2-adrenergic receptors (α_2ARs) provide negative feedback to norepinephrine release from sympathetic nerve terminals through inhibition of N-type Ca^{2+} channels. Increased neuronal norepinephrine release in DOCA-salt and obesity-related hypertension occurs through impaired α_2AR signaling, however, the mechanisms involved are unclear. We tested the hypothesis that α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons is impaired in DOCA-salt and obesity-related hypertension. DOCA-salt hypertension was induced after 4 weeks of DOCA-salt treatment. Obesity-related hypertension was induced using a high fat diet (HFD). Obesity-related hypertension developed in SD rats after 12 months of HFD. Obesity-prone, but not obesity-resistant SD rats developed obesity-related hypertension after 6 months of HFD. DahlS rats developed obesity-related hypertension after 4 months of HFD. Whole cell patch clamp methods were used to record Ca^{2+} currents from SMCG neurons maintained in primary culture. We found that α_2AR-mediated inhibition of Ca^{2+} channels was impaired in DOCA-salt hypertension, and that these results were not due to differences in receptor expression or downstream factors of signaling. We also found that α_2AR-mediated inhibition of Ca^{2+} channels was impaired in obese mildly hypertensive SD rats at 12 months and in normotensive obesity-resistant SD rats, but not in obese hypertensive DahlS rats. These results suggest that enhanced SNA in hypertension include the neuroeffector

junction, possibly via dysfunction of negative feedback through the α_2AR. This is especially relevant to mineralocorticoid-salt excess.

2.0 Introduction

Enhanced SNA plays an important role in hypertension development, maintenance, and progression [333]. Central mechanisms of increased SNA have been studied extensively in the majority of hypertension animal models. Sympathetic effector responses however, may be determined by alterations in peripheral SNA [334] in vascular beds such as mesenteric resistance arteries. Peripheral sympathetic nerve activity (SNA) increases in experimental hypertension [335] including DOCA-salt hypertension [336], where uninephrectomy, the mineralocorticoid derivative deoxycorticosterone (DOCA), and salt loading increase SNA and blood pressure [337]. Sympathetic nerve terminal release of NE onto vascular smooth muscle causes vasoconstriction. The prejunctional α_2-adrenergic receptor (α_2AR) inhibits NE release by coupling via $G\alpha_{i/o}$ proteins to inhibit voltage-gated N-type calcium channels that control NE release [125, 338]. This negative feedback regulates sympathetic tone and prevents sustained increases in blood pressure [127, 129]. Of the three α_2ARs, α_{2A}AR and α_{2C}AR couple to feedback inhibition of NE release, with α_{2A}AR being more predominant at higher levels of sympathetic nerve activity [137].

Receptor desensitization and internalization of activated α_2AR is mediated through phosphorylation by members of the G protein-coupled receptor kinase (GRK) family. Phosphorylation of the α_2AR occurs through GRK 2 and GRK 3 but not GRK 5, resulting in G protein uncoupling and arrestin-mediated receptor endocytosis [146-148, 153-156]. GRK 2 is upregulated in inflammatory conditions that are associated with increased blood pressure [161, 162]. Whether GRK 2 upregulation increases blood pressure by reducing prejunctional α_2AR signaling is not known.

Hypertension is associated with region-specific enhancement of SNA [233, 339]. Therefore, it is important to separately investigate mechanisms of SNA control in vascular beds of specific organ systems. In DOCA-salt hypertension, peripheral mechanisms that enhance neurotransmission include impaired inhibition of NE release caused by prejunctional α_2AR activation in mesenteric arteries [127, 340, 341]. Mesenteric arteries are major resistance arteries with profound influences on hemodynamics and blood pressure. Mesenteric arteries receive sympathetic innervation largely from the superior mesenteric and celiac ganglion (SMCG). Enhanced splanchnic SNA contributes to hypertension and splanchnic denervation decreases blood pressure in normal [61] and hypertensive rats [60, 62, 63]. We have recently shown that prejunctional impairment of α_2AR signaling in mesenteric arteries contributes to the late phase of DOCA-salt hypertension development [127]. It is unclear if these results are due to receptor impairment or downstream signaling, or if these deficits occur in other models of hypertension, such as obesity-related hypertension. Blunted responses to the α_2AR antagonist, yohimbine, on plasma NE suggest α_2AR impairment in obesity-association hypertension in dogs [342], however it is not known if this is attributed to presynaptic or central influences, or if NE release is augmented in splanchnic circulation.

Therefore, we tested the hypothesis that α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons is impaired in DOCA-salt and obesity-related hypertension. Whole cell patch clamp methods were used to record Ca^{2+} currents from SMCG neurons maintained in primary culture in order to evaluate α2AR function and determinants of downstream signaling. DOCA-salt hypertension was induced by 4 weeks of DOCA-salt treatment. Obesity-related hypertension was induced using a HFD at several time points in SD rats and DahlS rats.

Investigations that use a series of different animal models to decipher specific and common etiological factors of human hypertension may lead to a better understanding of human hypertension and how to improve current treatments.

3.0 Results

3.1 Retrograde Labeling and Recording from Dissociated SMCG Neurons in Culture

Isolation of SMCG neurons and retrograde labeling using CTB-555. The SMCG contains a heterogeneous mixture of sympathetic neurons and satellite glial cells (Figure 17). Sympathetic neurons were identified by TH but also by distinctively large and rounded nuclei with prominent nucleoli compared to nuclei of satellite glial cells, which in general were smaller and irregularly shaped. CTB-555 is retrogradely transported from mesenteric arteries to the cell bodies of SMCG neurons. CTB-555 is detectable in a small percentage of SMCG neurons (Figure 18). Retrograde labeling efficiency was reached by 4 days post-labeling. CTB-555 was detectable in a small percentage of dissociated SMCG neurons. Enzyme and mechanical dissociation ensured that SMCG neurons were isolated away from satellite glial cells (Figure 18).

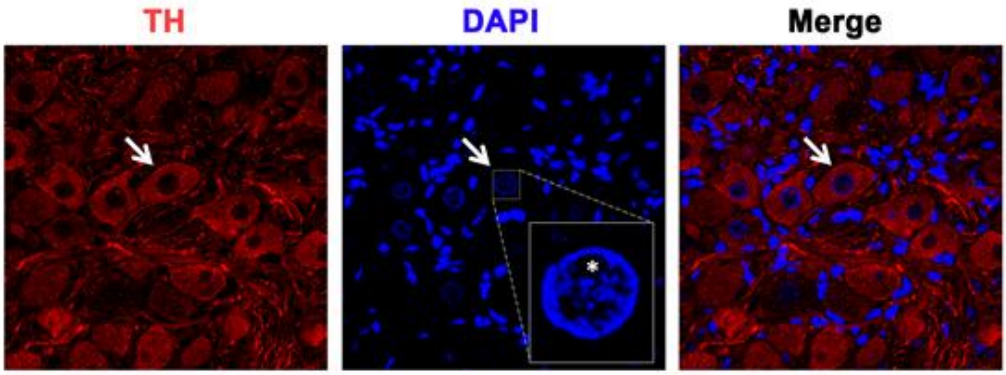

Figure 17. Immunohistochemical localization of sympathetic neurons in the SMCG (white arrow). SMCG neurons were identified by tyrosine hydroxylase (TH, *left image*). These neurons had large nuclei (*middle image)* with distinguishable nucleoli structures (asterisk). DAPI-staining revealed many smaller nuclei, which corresponded to satellite glial cells. Merged image (*right*) shows that satellite glial cells surrounded neuron cell bodies in the SMCG.

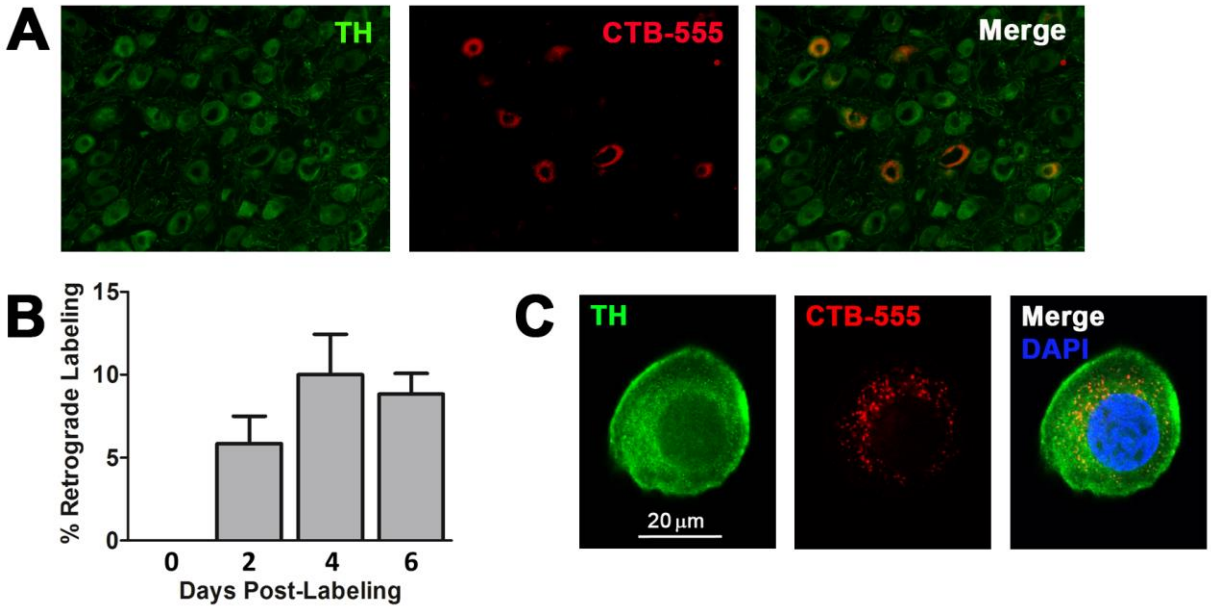

Figure 18. Detection of retrograde labeling with CTB-555 in SMCG section (*A, B*) and dissociated SMCG neurons (*C*). ***A:*** CTB-555 and tyrosine hydroxylase (TH) positive neurons can be detected in low numbers in SMCG sections. These are SMCG neurons that innervate mesenteric arteries. ***B:*** Retrograde labeling efficiency is reached at 4 days post-labeling; n=1-3 rats/group. ***C:*** CTB-555 can be detected in dissociated SMCG neurons that are positive for TH. Enzyme and mechanical dissociation methods can isolate SMCG neurons away from satellite glial cells (evidenced by a lack of surrounding satellite glial nuclei). Data are means ± SE.

Verification of pure Ca^{2+} currents recorded from SMCG neurons. Na^{+} channels were blocked by 10 nM TTX. K^{+} channels were blocked by 20 mM TEA-Cl and 143 mM CsCl. Ca^{2+} currents were detectable in SMCG neurons bathed in standard external solution (Figure 19A). Ba^{2+} has higher affinity for Ca^{2+} channels that Ca^{2+}, and therefore augments the magnitude of current detected. Exchanging external solution that contained Ba^{2+} instead of Ca^{2+} significantly increased currents recorded from SMCG neurons (Figure 19B). Addition of 1 mM CdCl to inhibit Ca^{2+} channels completely attenuated currents (Figure 19C).

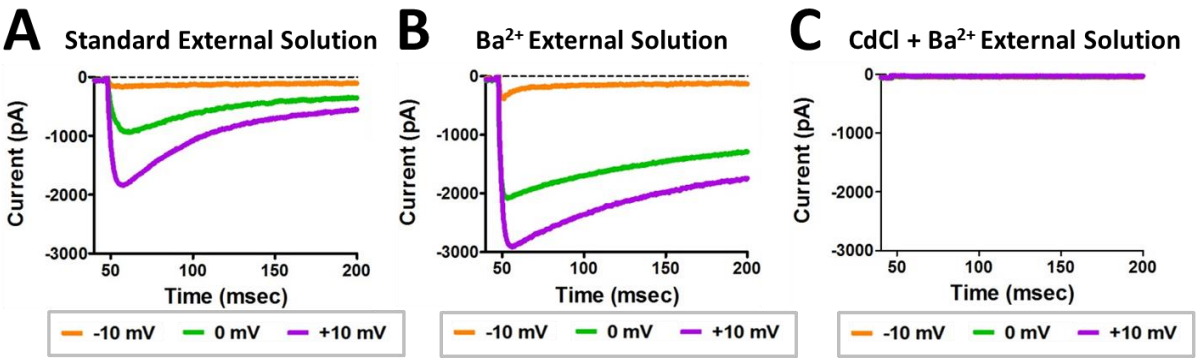

Figure 19. Representative pure Ca^{2+} and Ba^{2+} currents recorded from SMCG neurons. ***A:*** Ca^{2+} currents recorded at -10, 0, and +10 mV. ***B:*** When standard external solution was exchanged for external solution containing 10 mM $BaCl_2$, current magnitude increased considerably. ***C:*** Ba^{2+} currents were attenuated when Ca^{2+} channels were inhibited with 1 mM CdCl.

3.2 Investigating α_2AR Function in DOCA-Salt Rats

Effects of DOCA-salt treatment on α_2AR function in mesenteric artery-projecting SMCG neurons. Whole-cell recordings were performed on acutely dissociated mesenteric artery-projecting SMCG neurons identified by CTB-555 to evaluate α_2AR function. Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons (Figure 20, A and B). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE and the α_2AR-specific agonist UK14304 (each at 1 μM) at +10 mV. Inhibition of Ca^{2+} current was significantly reduced in SMCG neurons from DOCA-salt rats compared to SHAM rats (Figure 20D). Pretreatment of neurons with the α_2AR antagonist, yohimbine (10 μM, 1 min), blocked Ca^{2+} current inhibition caused by NE (Figure 20E).

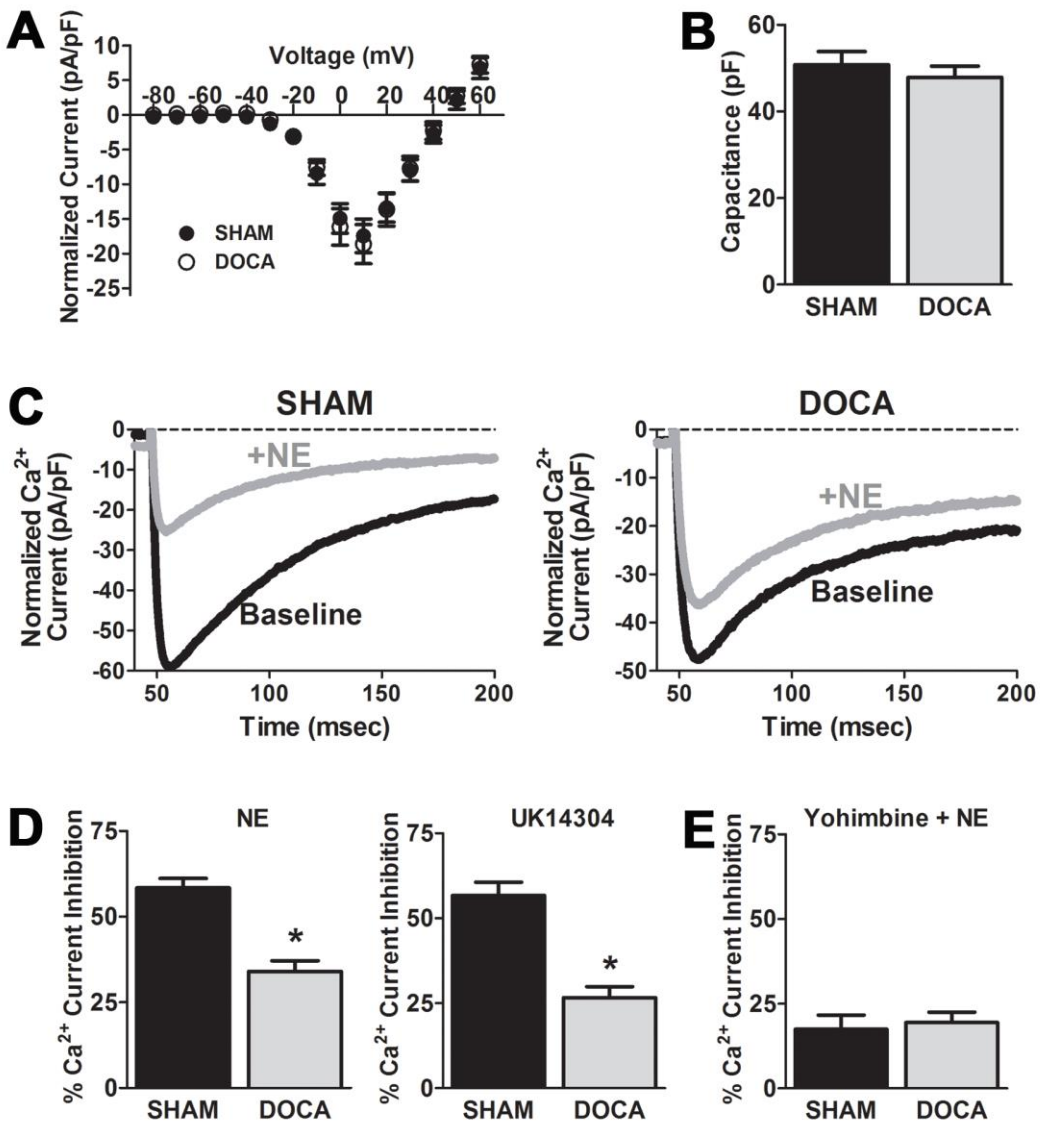

Figure 20. DOCA-salt hypertension is associated with impaired α_2AR-mediated inhibition of Ca^{2+} channels in acutely dissociated mesenteric artery-projecting SMCG neurons. *A*: Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 22-25 cells. ***B*:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 33-36 cells. ***C*:** Represent traces of Ca^{2+} current at baseline and when α_2AR was activated by 1 μM NE in SMCG neuron from SHAM (*left*) and DOCA-salt (*right*) rat. ***D*:** Ca^{2+} current inhibition was impaired in SMCG neurons from DOCA-salt rats when α_2AR was activated by NE (1 μM) (*left*) and UK14304 (1 μM) (*right*); *n* = 9-11 cells. ***E*:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons; *n* = 7-9 cells. Data are means ± SE, *P < 0.05.

Mechanisms that impair α_2AR function do not involve downstream signaling in DOCA-salt hypertension. We investigated if impaired α_2AR signaling was due to differences in α_2AR expression and/or desensitization. mRNA levels of α_2AR subtypes 2A, 2B, and 2C were equal in SMCG samples from DOCA-salt and SHAM rats (Figure 21A). G protein-coupled receptor kinases (GRKs) phosphorylate activated G protein-coupled receptors, resulting in arrestin-dependent receptor desensitization [343]. mRNA levels of GRK 2, but not GRK 3 or GRK 5, were higher in SMCG samples from DOCA-salt rats compared to SHAM rats (Figure 21B). In order to determine if GRK 2 contributed to impaired α_2AR signaling in DOCA-salt hypertension, we measured α_2AR-mediated inhibition of Ca^{2+} currents by NE (1 μM) in SMCG neurons pretreated (50-60 min) with the GRK2 inhibitor paroxetine (30 μM) versus fluoxetine (control, 30 μM). Paroxetine did not increase α_2AR-mediated inhibition of Ca^{2+} currents recorded from DOCA-salt neurons or SHAM neurons compared to fluoxetine (Figure 21C). In order to investigate if impaired α_2AR signaling was due to a reduced ability of G-proteins to inhibit Ca^{2+} channels, we dialyzed the non-hydrolyzable GTP analog, 5'-guanylyl imidodiphosphate (500 μM, GppNHp) directly into SMCG neurons. GppNHp inhibited Ca^{2+} currents similarly in all SMCG neurons (Figure 21D).

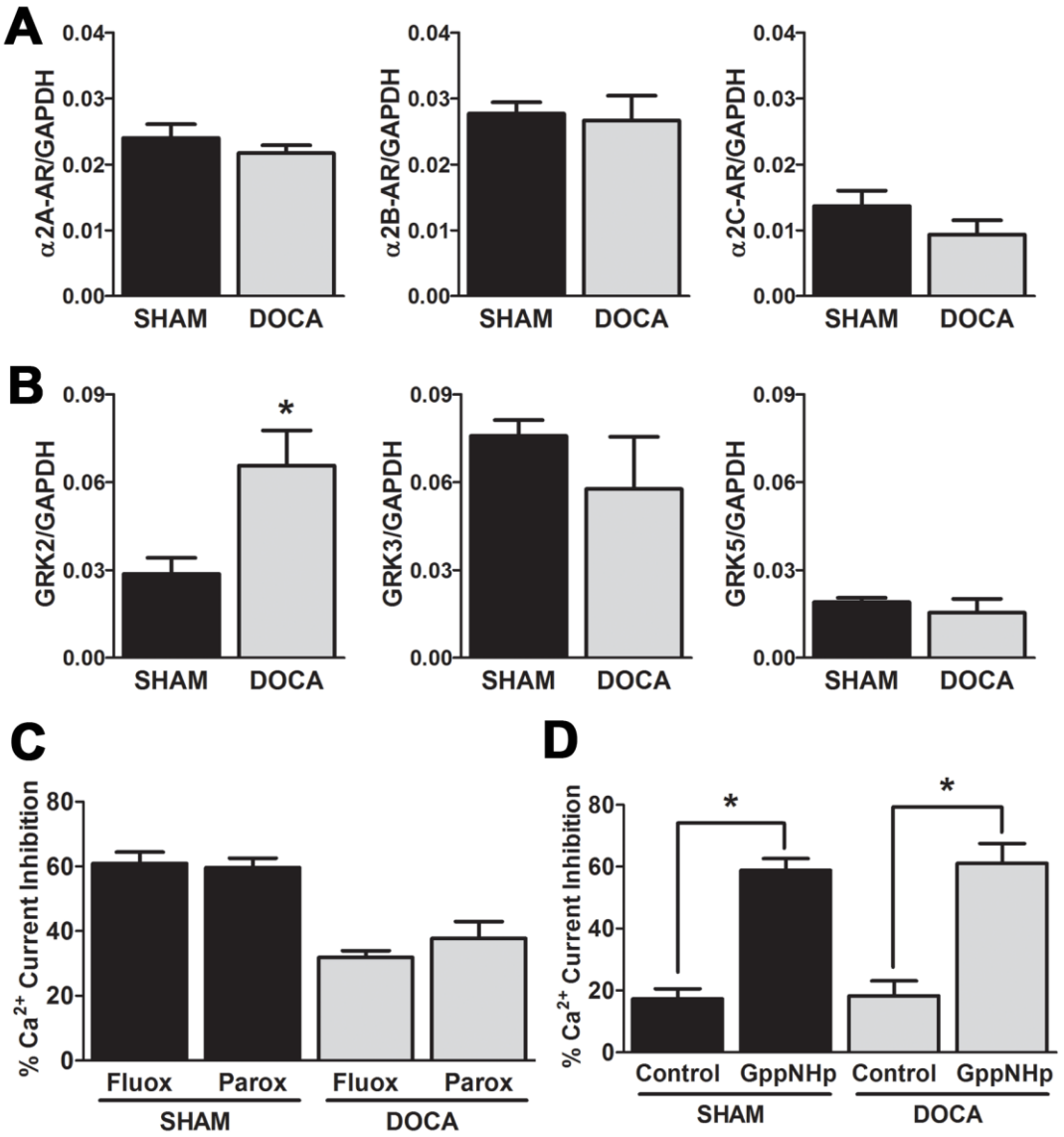

Figure 21. Impaired α_2AR-signaling in DOCA-salt hypertension does not involve changes in receptor expression, receptor desensitization, or G-protein signaling to inhibit Ca^{2+} channels. ***A*****:** mRNA expression of α_2AR subtypes 2A (*left*), 2B (*middle*), and 2C (*right*) were similar in SMCG from DOCA-salt rats compared to SHAM rats; *n* = 4 rats. ***B*****:** mRNA expression of G-protein receptor kinase (GRK) subtype 2 (*left*), but not 3 (*middle*) and 5 (*right*), were increased in SMCG from DOCA-salt rats compared to SHAM rats; *n* = 4 rats. ***C*****:** Treating SMCG neurons with the GRK2 inhibitor paroxetine (30 μM) did not increase α_2AR-mediated inhibition of Ca^{2+} channels compared to fluoxetine (30 μM); *n* = 6-8 cells. ***D*****:** The non-hydrolyzable GTP analog, 5'-guanylyl imidodiphosphate (GppNHp, 500 μM) caused similar inhibition of Ca^{2+} channels in all SMCG neurons; *n* = 6-8 cells. Data are means ± SE, *P < 0.05.

3.3 Investigating α_2AR Function in HFD-Fed Sprague Dawley (SD) Rats

Effects of HFD on obesity and hypertension development in SD rats. SD rats fed a HFD for 6 months developed mild hypertension without significant weight gain compared to NFD-SD rats (Figure 22, A and B). We extended feeding time to 1 year in order to determine if increasing the duration of a HFD led to hypertension with obesity. SD rats fed a HFD for 12 months developed mild hypertension that was associated with significant weight gain compared to NFD-SD rats (Figure 22, C and D).

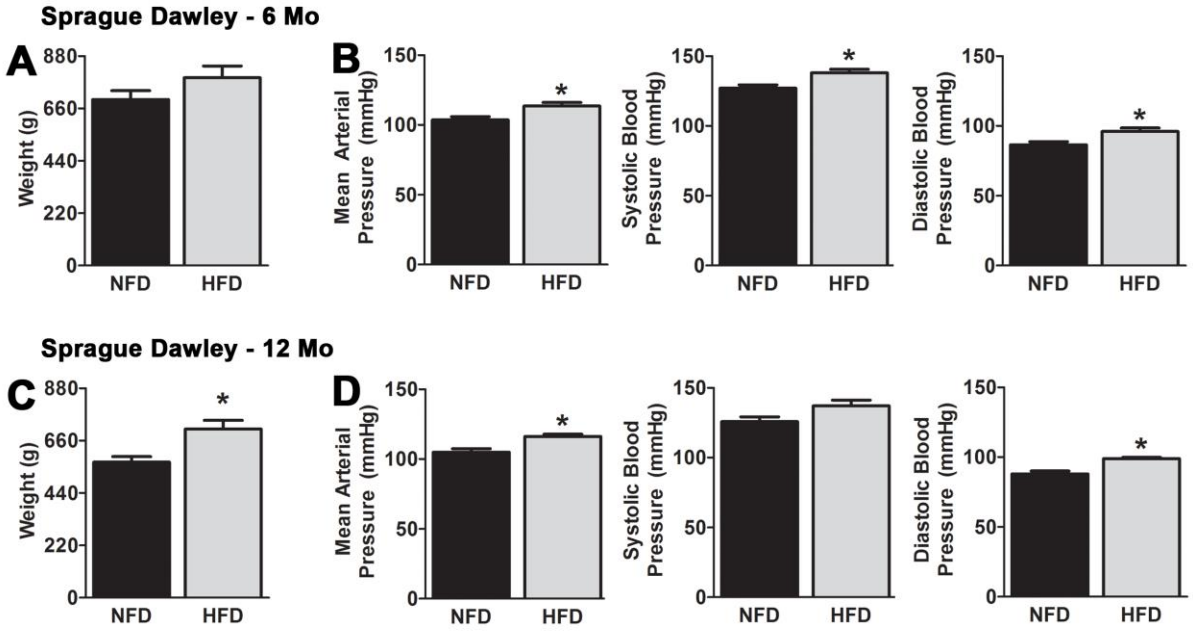

Figure 22. Effects of HFD on weight and blood pressure in SD rats. *A and B*: 6 months of HFD feeding does not cause weight gain but induces mild hypertension (increase in mean, systolic, and diastolic pressure) compared to NFD rats; n=4-5 rats/group ***C and D*:** 12 months of HFD feeding induces obesity with mild hypertension (increase in mean and diastolic pressure) compared to NFD rats; n=5 rats/group. Data are means ± SE, *P < 0.05.

Effects of 6 months of HFD feeding on α_2AR function in SMCG neurons isolated from non-obese and mildly hypertensive SD rats. Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons regardless of diet (Figure 23, A and B). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE and the α_2AR-specific agonist UK14304 (each at 1 μM) at +10 mV. Inhibition of Ca^{2+} current was similar in all SMCG neurons in non-obese mildly hypertensive SD rats fed a HFD for 6 months versus NFD rats (Figure 23D). Pretreatment of neurons with the α_2AR antagonist, yohimbine (10 μM, 1 min), blocked Ca^{2+} current inhibition caused by NE (Figure 23E). These results are not associated with changes in α_2AR or GRK mRNA expression (Figure 24, A and B).

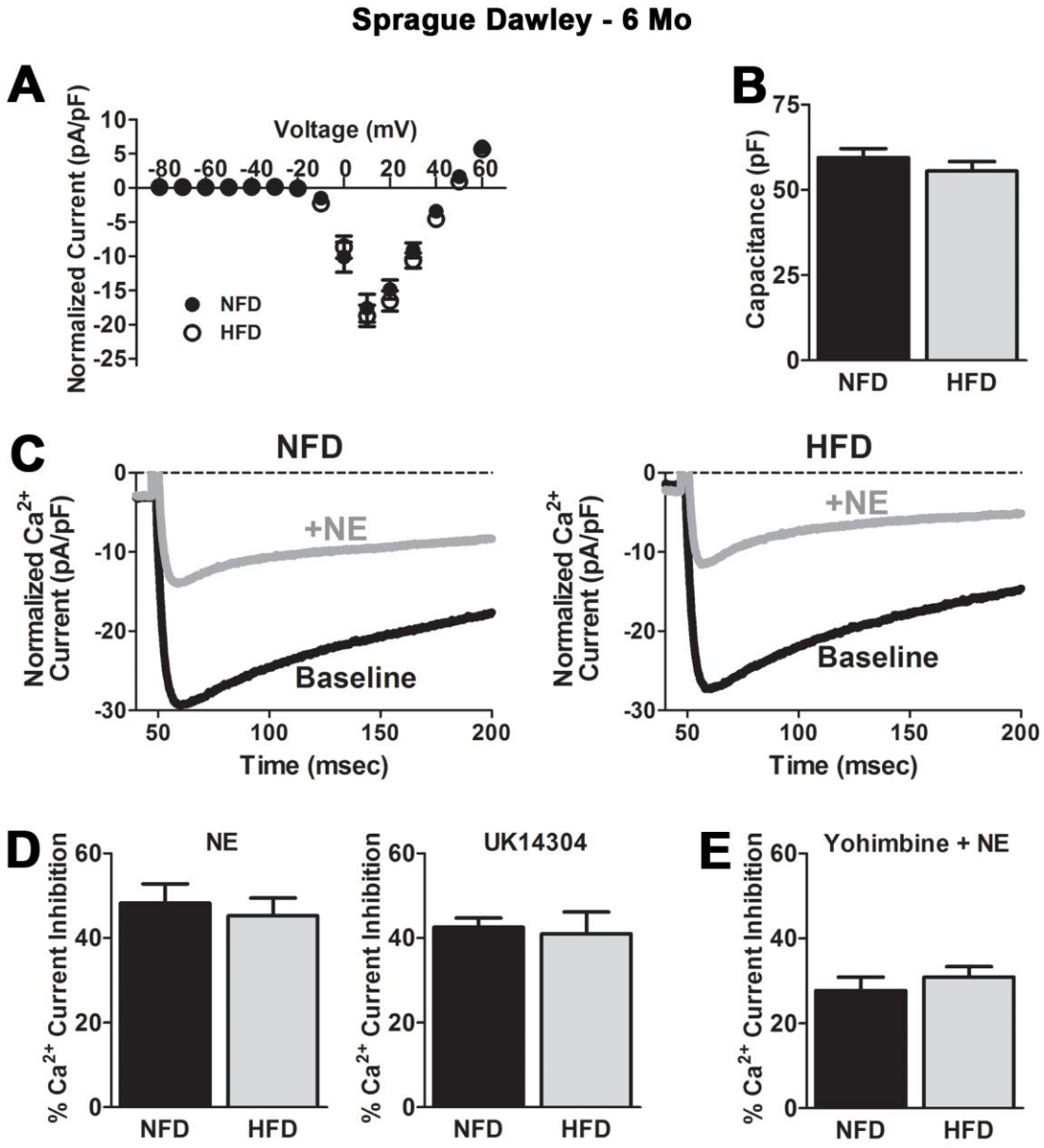

Figure 23. HFD-induced hypertension in non-obese SD rats (6 months) is not associated with impaired α_2AR-mediated inhibition of Ca^{2+} channels in acutely dissociated SMCG neurons. *A*: Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 24-26 cells. ***B*:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 26-29 cells. ***C*:** Represent traces of Ca^{2+} current at baseline and when α_2AR was activated by 1 μM NE in SMCG neuron from NFD (*left*) and HFD (*right*) rat. ***D*:** Ca^{2+} current inhibition was similar in all SMCG neurons when α_2AR was activated by NE (1 μM) (*left*) and UK14304 (1 μM) (*right*); *n* = 7-9 cells. ***E*:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons; *n* = 7 cells. Data are means ± SE, *P < 0.05.

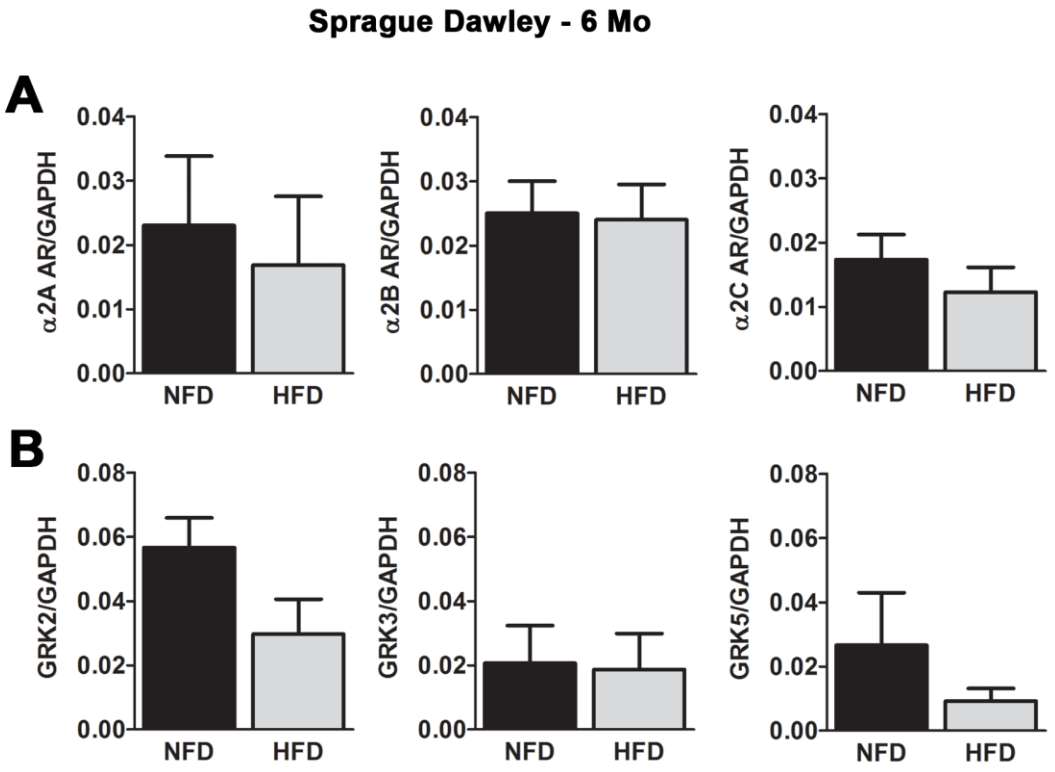

Figure 24. SMCG mRNA expression of α2AR and GRK subtypes are not changed after 6 months of HFD in SD rats. ***A*****:** mRNA expression of α_2AR subtypes 2A (*left*), 2B (*middle*), and 2C (*right*) were similar in SMCG tissues; *n* = 3 rats. ***B*****:** mRNA expression of G-protein receptor kinase (GRK) subtype 2 (*left*), 3 (*middle*) and 5 (*right*), were similar in SMCG tissues; *n* = 3 rats.

Effects of 12 months of HFD feeding on α_2AR function in SMCG neurons isolated from obese mildly hypertensive SD rats. Similar whole-cell patch clamp approaches were used to determine whether a longer period of HFD feeding affected α_2AR function. Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons regardless of diet (Figure 25, A and B). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE and the α_2AR-specific agonist UK14304 (each at 1 μM) at +10 mV. Inhibition of Ca^{2+} current was reduced in SMCG neurons in obese mildly hypertensive SD rats fed a HFD for 12 months versus non-obese normotensive NFD rats (Figure 25D). Pretreatment of neurons

with the α_2AR antagonist, yohimbine (10 μM, 1 min), blocked Ca^{2+} current inhibition caused by NE (Figure 25E).

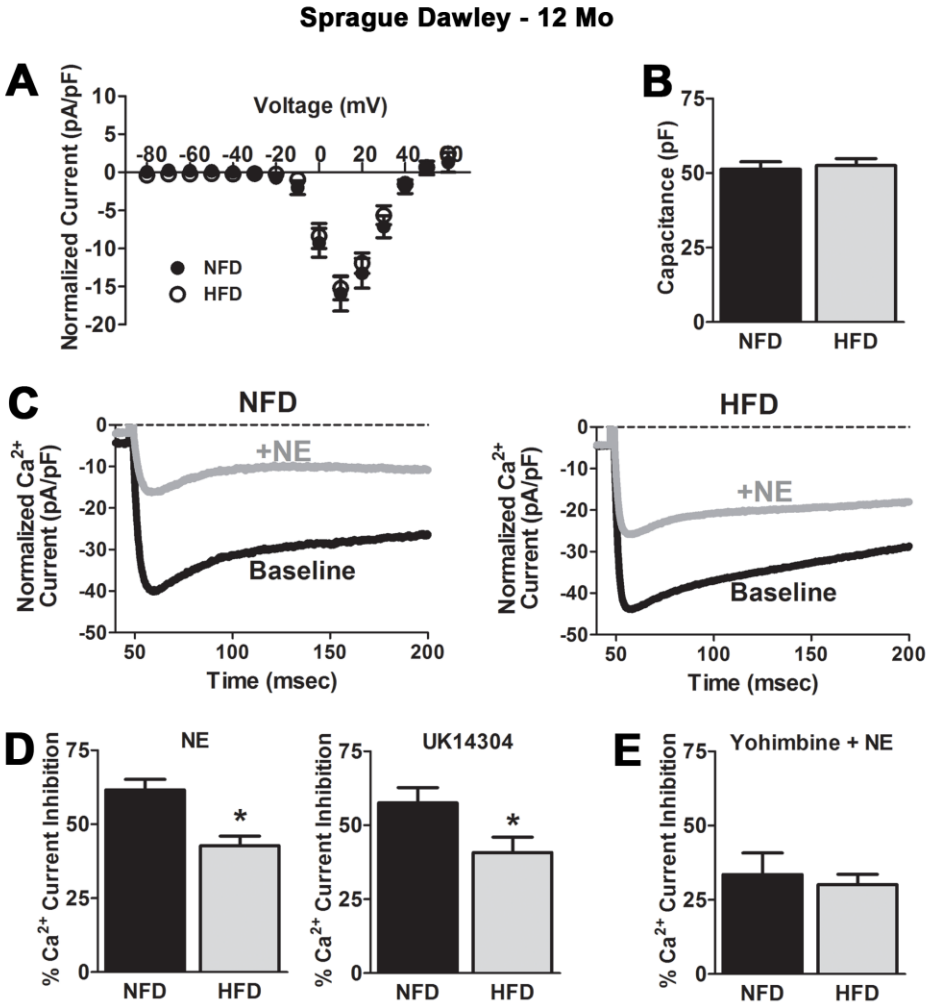

Figure 25. HFD-induced hypertension in obese SD rats (12 months) is associated with impaired α_2AR-mediated inhibition of Ca^{2+} channels in acutely dissociated SMCG neurons. *A*: Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 23-24 cells. ***B*:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 23-24 cells. ***C*:** Represent traces of Ca^{2+} current at baseline and when α_2AR was activated by 1 μM NE in SMCG neuron from NFD (*left*) and HFD (*right*) rat. ***D*:** Ca^{2+} current inhibition was reduced in SMCG neurons from HFD-rats versus NFD rats when α_2AR was activated by NE (1 μM) (*left*) and UK14304 (1 μM) (*right*); *n* = 7-10 cells. ***E*:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons; *n* = 6-7 cells. Data are means ± SE, *P < 0.05.

Effects of 6 months of HFD feeding on weight, blood pressure and, α_2AR function in SMCG neurons isolated from obesity-prone (OP) and obesity-resistant (OR) rats. In order to determine if α_2AR dysfunction in 12-month HFD SD rats was due to age, obesity or prolonged exposure to HFD, we performed a similar 6 month study using OP and OR-SD rats.

A HFD for 6 months induced mild hypertension with obesity in OP rats (Figure 26A) but had no effect on weight gain and blood pressure in OR rats (Figure 26B).

Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons regardless of diet or susceptibility to obesity (Figure 27; A, B, F, and G). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE (1 μM) at +10 mV. Ca^{2+} current inhibition was similar in all SMCG neurons regardless of diet in OP rats (Figure 27D). Inhibition of Ca^{2+} current was reduced in SMCG neurons from HFD-OR rats versus NFD-OR rats (Figure 27I). Pretreatment of neurons with the α_2AR antagonist, yohimbine (10 μM, 1 min), blocked Ca^{2+} current inhibition caused by NE (Figure 27, E and J).

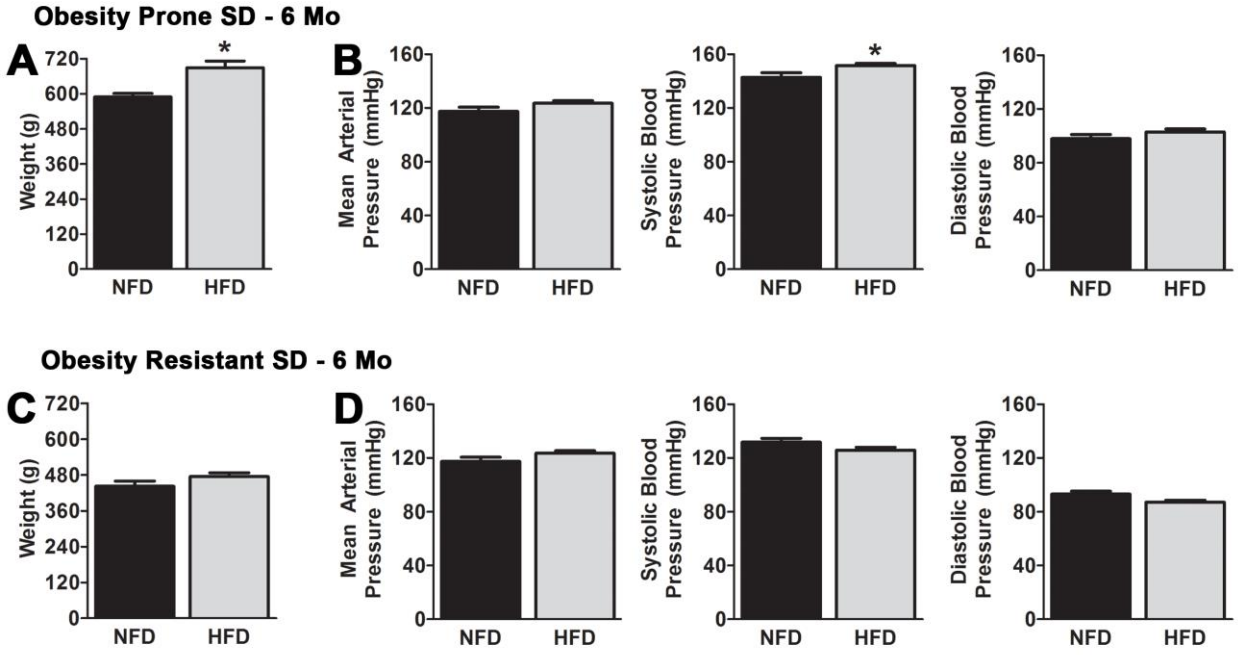

Figure 26. Effects of HFD on weight and blood pressure in obesity-prone (OP) and obesity-resistant (OR) SD rats. ***A and B*****:** HFD induced obesity with mild hypertension (increase in systolic blood pressure) in OP rats compared to NFD; n=5-6 rats. ***C and D*****:** HFD had no effect on weight or blood pressure in OR rats. N=4-5 rats/group. Data are means ± SE, *P < 0.05.

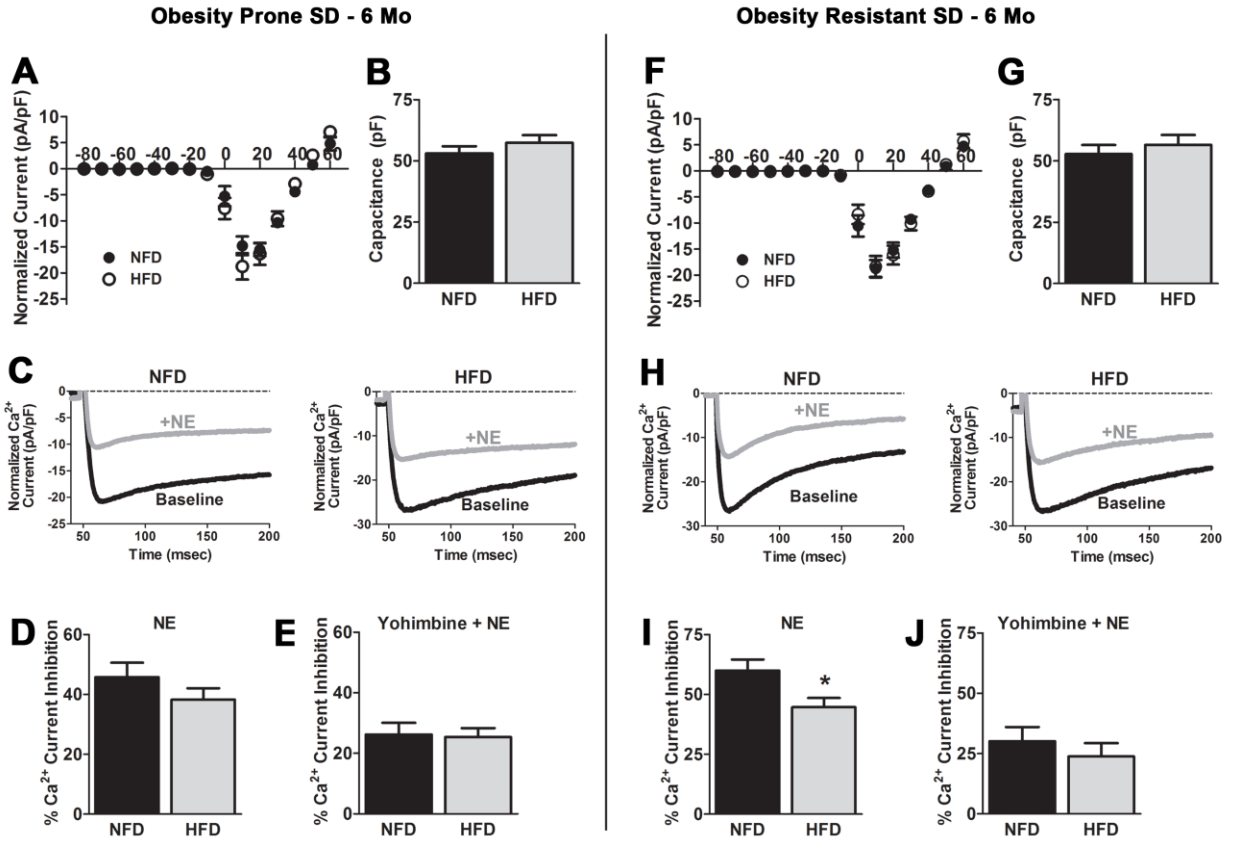

Figure 27. Effects of HFD on α_2AR-mediated inhibition of Ca^{2+} channels in acutely dissociated SMCG neurons from obesity-prone (*left side*) and obesity-resistant (*right side*) SD rats. *A and F*: Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 14-17 cells. ***B and G*:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 16-18 cells. ***C and H*:** Represent traces of Ca^{2+} current at baseline and when α_2AR was activated by 1 μM NE in SMCG neuron from NFD (*left*) and HFD (*right*) rat. ***D*:** Ca^{2+} current inhibition was similar in all SMCG neurons from OP rats regardless of diet; *n* = 6-7 cells. ***I*:** Ca^{2+} current inhibition was significantly reduced in SMCG neurons from HFD-OR rats versus NFD-OR rats; *n* = 8 cells. ***E and J*:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons; *n* =5-6 cells. Data are means ± SE, *P < 0.05.

3.4 Investigating α_2AR Function in HFD-Fed Dahl Salt-Sensitive (DahlS) Rats

Effects of 2, 4, and 6 months of HFD feeding on body weight and hypertension. In order to investigate the effects of HFD in a model that is salt-sensitive, we performed a 6 month study in DahlS rats. DahlS rats fed a HFD developed significantly higher weight and blood pressure at 4 and 6 month endpoints compared to NFD-fed DahlS rats (Figure 28, A-D).

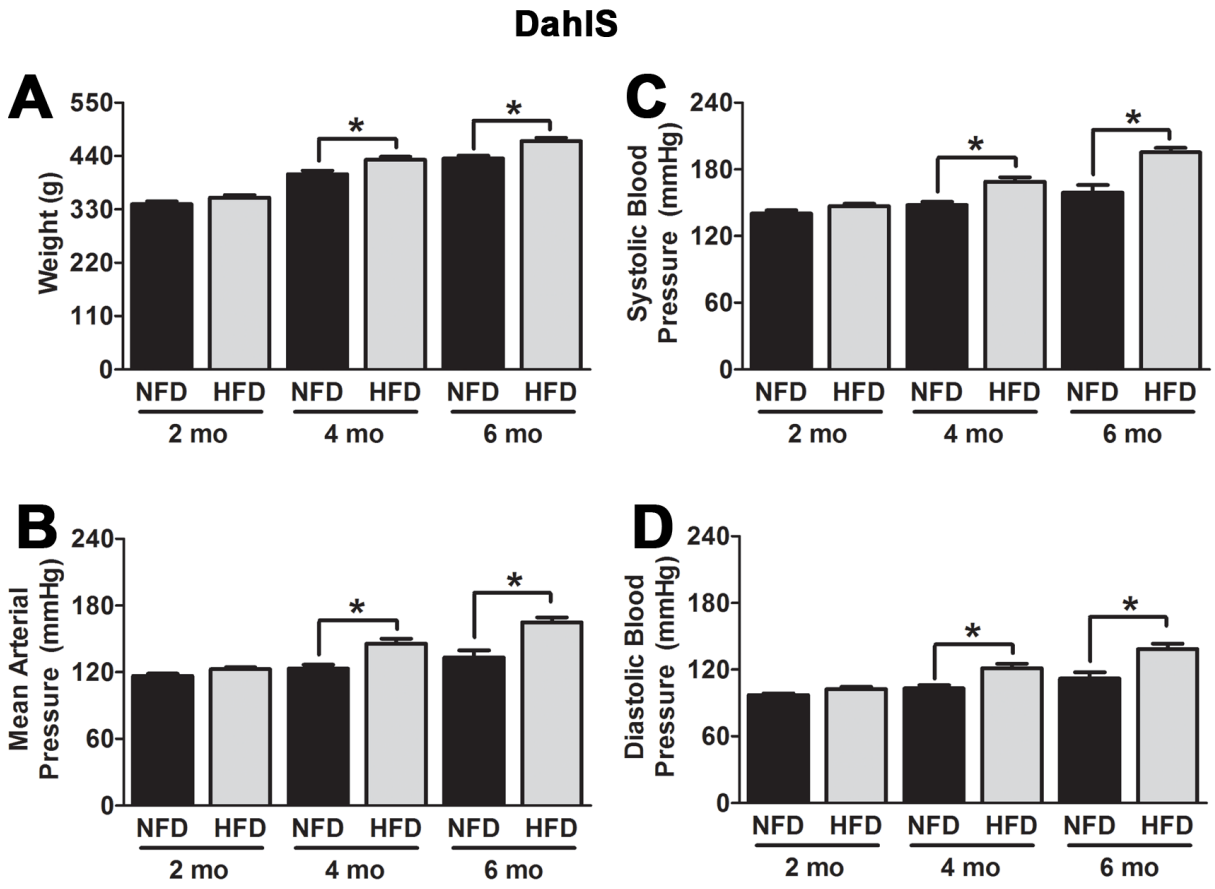

Figure 28. Obesity-related hypertension development in HFD-fed DahlS rats. *A*: Mean weight was significantly increased at 4 and 6 months in HFD-DahlS rats compared to NFD-DahlS rats. ***B-D*:** Mean, systolic, and diastolic blood pressure was significantly increased at 4 and 6 months in HFD DahlS rats compared to NFD DahlS rats. Data are means ± SE, *P < 0.05, *n* = 5 rats/group.

Effects of 2, 4, and 6 months of HFD feeding on α_2AR function in SMCG neurons isolated from DahlS Rats. We investigated the effects of HFD on α_2AR function in DahlS rats at 2, 4, and 6 month time points, which represent different stages of hypertension development. All the data from patch clamp studies at these time points are presented in Figure 29 and Figure 30. Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons regardless of diet and time point (Figure 29; A, B, D, E, G, and H). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE and the α_2AR-specific agonist UK14304 (each at 1 μM) at +10 mV. Inhibition of Ca^{2+} current was similar in all SMCG neurons regardless of diet or time point (Figure 30; A, C, and E). Pretreatment of neurons with the α_2AR antagonist, yohimbine (10 μM, 1 min), blocked Ca^{2+} current inhibition caused by NE (Figure 30; B, D, and F). These results are not associated with changes in mRNA expression of α_2AR (Figure 31; A, B and C) or GRK (Figure 32; A, B and C).

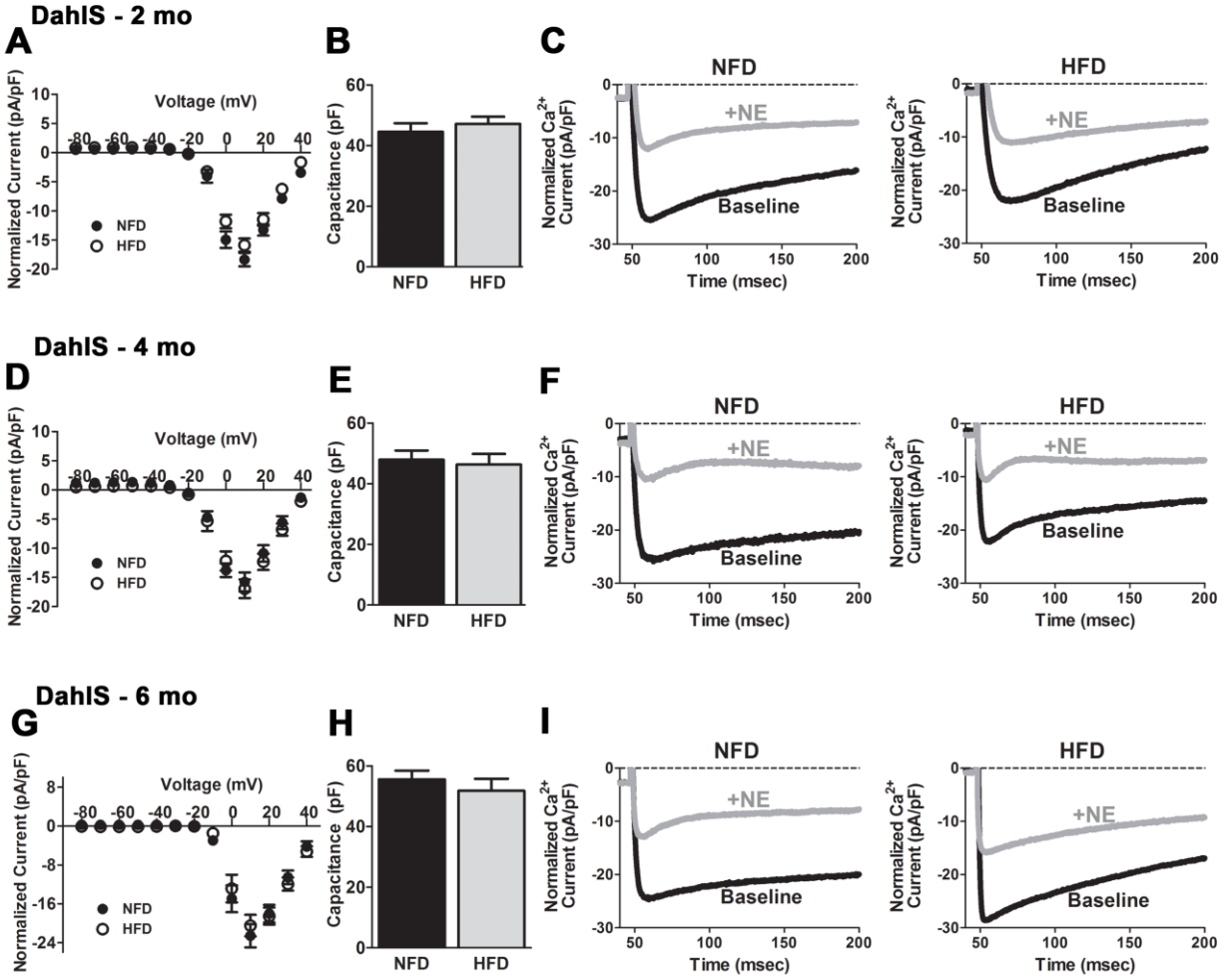

Figure 29. Obesity-related hypertension in HFD-fed DahlS rats is not associated with changes in current-voltage relationships, cell capacitance, or Ca^{2+} current inhibition in acutely dissociated SMCG neurons at 2 (*top row*), 4 (*middle row*), and 6 months (*bottom row*). ***A, D, G:*** Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 17-28 cells. ***B, E, H*:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 25-33 cells. ***C, F, I*:** Represent traces of Ca^{2+} current at baseline and when α_2AR was activated by 1 μM NE in SMCG neuron from NFD (*left*) and HFD (*right*) rat. *Note: Data continued in Figure 30.*

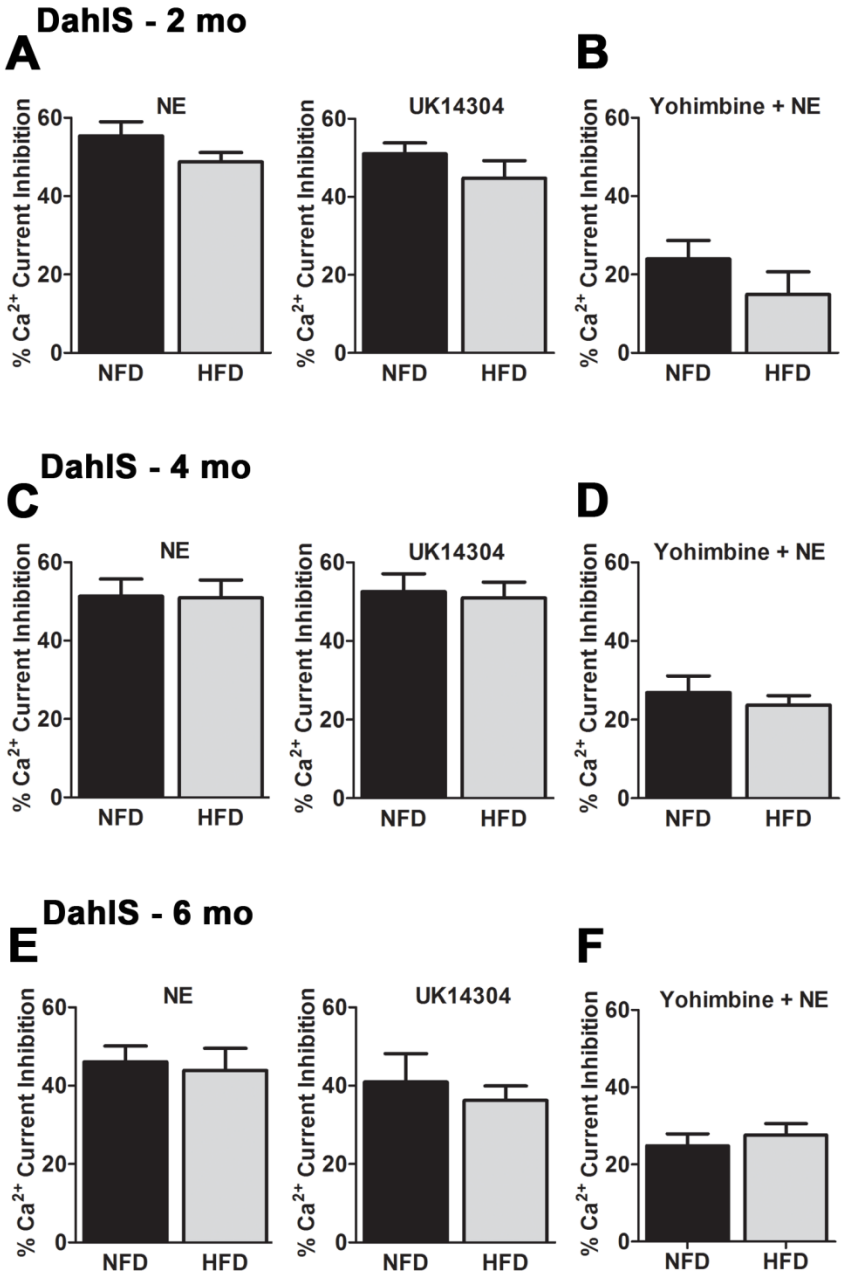

Figure 30. Obesity-related hypertension in HFD-fed DahlS rats is not associated with impaired α_2AR-mediated inhibition of Ca^{2+} channels in acutely dissociated SMCG neurons at 2 (*top row*), 4 (*middle row*), and 6 months (*bottom row*). ***A, C, E*:** Ca^{2+} current inhibition when α_2AR was activated by NE (1 μM) (*left*) and UK14304 (1 μM) (*right*) was similar in all SMCG neurons; *n* = 7-8 cells. ***B, D, F*:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons *n* = 6-7 cells. Data are means ± SE.

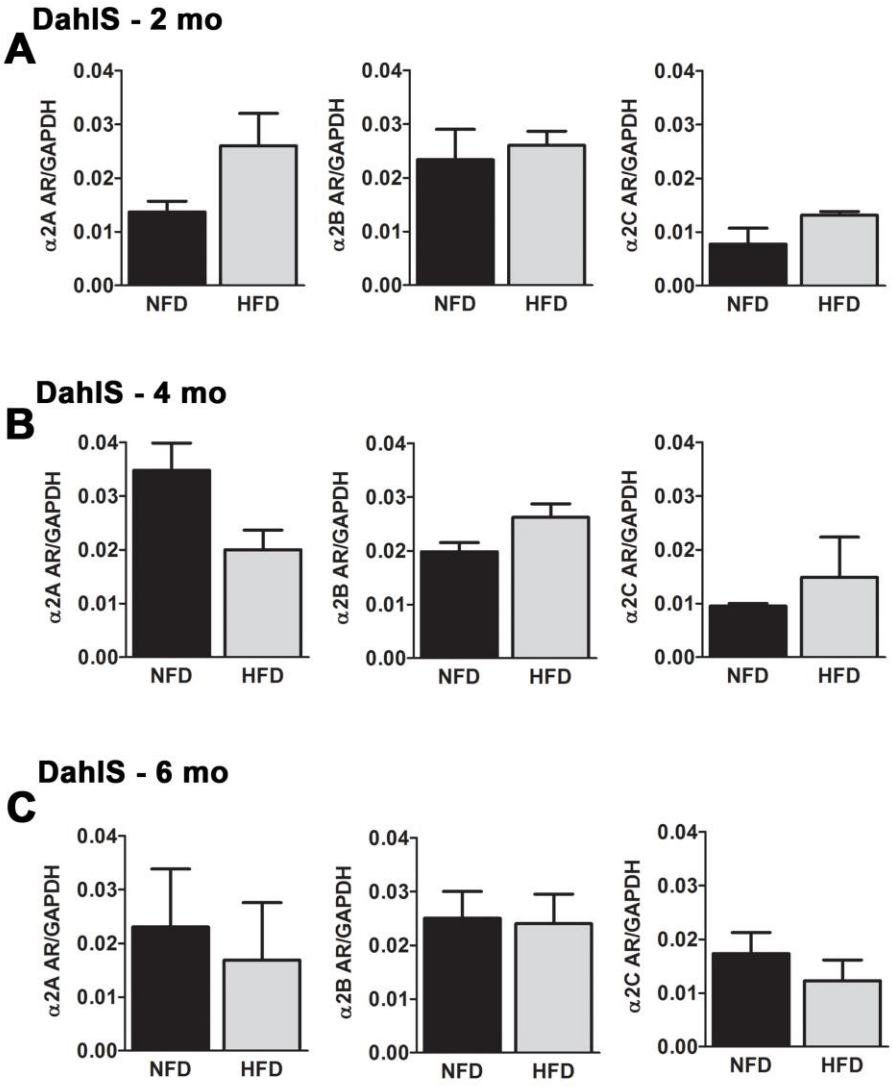

Figure 31. Obesity-related hypertension in HFD-fed DahlS rats is not associated changes in receptor expression in SMCG. There was similar mRNA expression of α_2AR subtype 2A (left), 2B (middle) and 2C (right) in SMCG regardless of diet at **A:** 2 months, **B:** 4 months, and **C:** 6 months; *n* = 3 rats. Data are means ± SE.

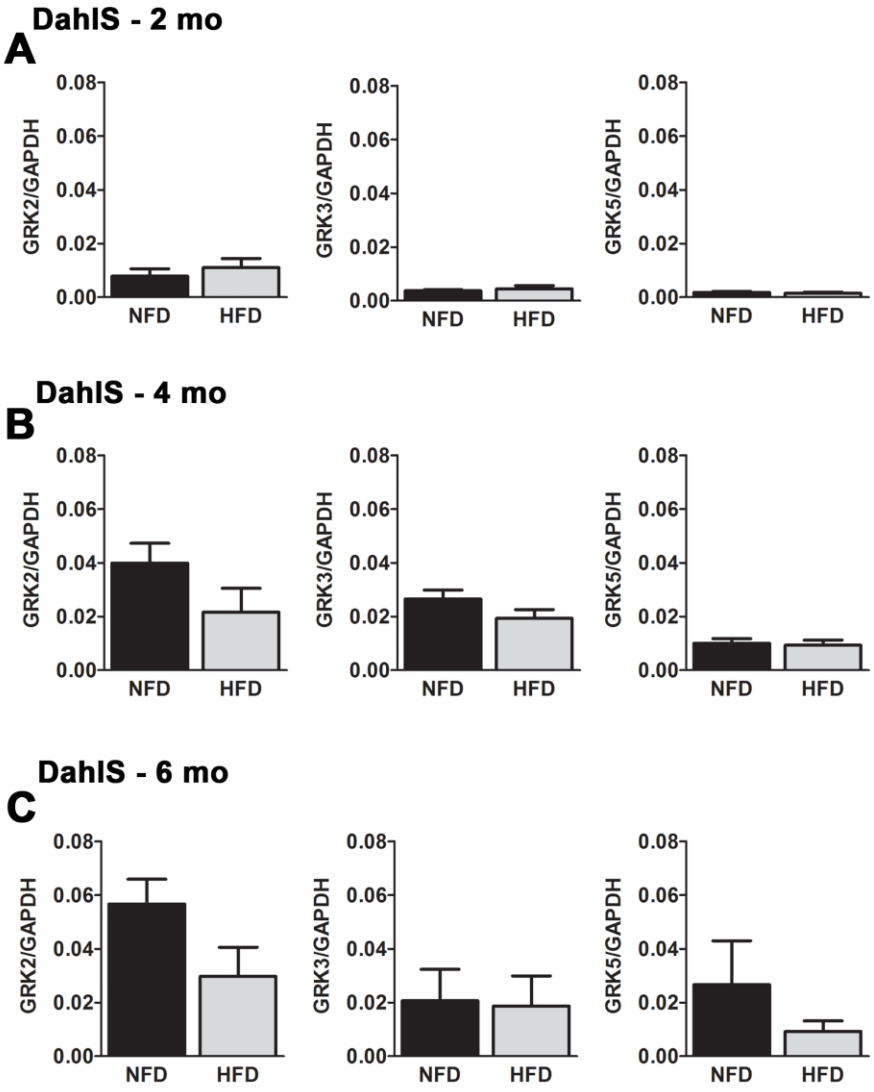

Figure 32. Obesity-related hypertension in HFD-fed DahlS rats is not associated changes in GRK expression in SMCG. There was similar mRNA expression of GRK subtype 2 (left), 3 (middle) and 5 (right) in SMCG regardless of diet at **A:** 2 months, **B:** 4 months, and **C:** 6 months; *n* = 3 rats. Data are means ± SE.

4.0 Discussion

In the present study, we used whole-cell patch clamp to directly evaluate α_2AR-mediated inhibition of Ca^{2+} channels in dissociated SMCG neurons of DOCA-salt and HFD-induced hypertensive rats. The main findings are: 1) DOCA-salt hypertension is associated with impaired α_2AR function. 2) In SD rats, 6 months of HFD induces mild hypertension without obesity that is not associated with impaired α_2AR function. 12 months of HFD induces mild hypertension with obesity that is associated with impaired α_2AR function. 3) In obesity-prone SD rats, HFD-induces mild hypertension with obesity that is not associated with impaired α_2AR function. In obesity-resistant SD rats, HFD does not cause hypertension or obesity but is associated with impaired α_2AR function. 4) In DahlS rats, HFD rapidly induces hypertension with obesity that is not associated with impaired α_2AR function.

4.1 DOCA-salt hypertension

We found that α_2AR-mediated inhibition of Ca^{2+} channels was significantly impaired in DOCA-salt hypertensive rats. These results may explain previous findings that NE release is increased in mesenteric arteries of DOCA-salt hypertensive rats [127]. We further showed that deficits in α_2AR signaling did not involve changes in receptor mRNA expression, Ca^{2+} channel density or voltage-dependent activation, or G-protein inhibition of Ca^{2+} channels using GppNHp activation. Taken together, these results suggest that impaired Ca^{2+} channel modulation is likely due to changes in α_2AR function.

The DOCA-salt rat is a model of neurogenic hypertension that results from enhanced SNA. This is evidenced by impaired central regulation of the baroreceptor reflex [223], central activation of CVOs through brain RAAS and oxidative stress [85, 225-227], salt-dependent enhanced lumbar SNA [228], salt-dependent enhanced renal SNA [229-231] and enhanced splanchnic SNA [59]. Taken together with our results, DOCA-salt hypertension is associated with central and peripheral-driven mechanisms that enhance SNA. The purpose of prejunctional α_2AR signaling is to counteract enhanced sympathetic outflow. We previously showed that impaired α_2AR signaling begins during the late phase of DOCA-salt hypertension (week 3-4) [127]. Therefore, central mechanisms of enhanced SNA resulting from mineralocorticoid-salt may precede peripheral α_2AR impairment [337]. This suggests that prejunctional dysfunction of α_2AR enhances the effects of central sympathetic activation to effector organs and contributes to sustained increases in blood pressure in this model. This dual synergism may explain why hypertension development in this model is so robust and rapid versus other models where hypertension development is either milder or slower.

While exploring mechanisms of receptor desensitization, we found that mRNA levels of GRK2 were higher in SMCG from DOCA-salt hypertensive rats. GRK2 is upregulated in inflammatory conditions associated with hypertension and results in a loss of α_2AR sympatho-inhibitory function in the adrenal medulla [158, 162]. However, the GRK2 inhibitor paroxetine did not improve α_2AR-mediated Ca^{2+} current inhibition in SMCG neurons of DOCA-salt rats. This suggests that GRK2-mediated α_2AR desensitization may not be relevant in SMCG neurons, or that due to its short half-life [344], prolonged incubation of neurons in the absence of agonist resulted in GRK2 degradation beyond the detection limits of our assay.

Interestingly, our findings were associated with an increase in SMCG inflammation and adventitial macrophage infiltration (presented in Chapter 4). However, whether inflammation and macrophages impair α_2AR function remains unknown (but are investigated in Chapter 6).

4.2 Obesity-related hypertension in SD and DahlS rats

Blunted responses to the α_2AR antagonist, yohimbine, on plasma NE suggest presynaptic α_2AR impairment in obesity-related hypertension in dogs [342]. However, these studies have not been performed in rats and are unable to determine if enhanced NE release occurs in the splanchnic circulation. Unlike DOCA-salt hypertension, the relationship between obesity-related hypertension and α_2AR function is not as straightforward and is not associated with increased SMCG inflammation or adventitial macrophage infiltration (presented in Chapter 3). Interestingly, mild hypertension in obese SD rats was associated with α_2AR impairment while severe hypertension in obese DahlS rats was not associated with α_2AR impairment. These results may be explained by differences in animal model and by duration exposed to a HFD.

Obesity-related hypertension in SD rats: At 6 months, a HFD induced mild hypertension without weight gain in SD rats that was not associated with α_2AR dysfunction or differences in receptor and GRK mRNA expression. This suggests that impaired α_2AR signaling does not play a role in the developmental phase of HFD-induced hypertension in SD rats.

However, increasing HFD exposure to 12 months resulted in the development of mild hypertension with obesity that was associated with impaired α_2AR-mediated inhibition of Ca^{2+} channels. This suggests that either obesity or prolonged exposure to HFD, or both, was associated with α_2AR dysfunction. Therefore, we performed the same 6 month study in OP-SD rats, which are more susceptible to developing early obesity-related hypertension [238]. In agreement with previous reports, 6 months of HFD induced obesity and mild hypertension in OP-SD rats. However, α_2AR dysfunction was not associated with the development of obesity-

related hypertension in OP-SD rats. These results suggest that it may be the duration of exposure to a HFD and not obesity per se that contributes to α_2AR dysfunction in SD rats.

Interestingly, 6 months of HFD feeding in OR-SD rats resulted in α_2AR dysfunction despite a lack of increased body weight or hypertension. Taken together with our observations that HFD-induced obesity in mildly hypertensive OP-SD rats was not associated with α_2AR dysfunction, it is tempting to speculate that obesity, at least in the short term may help preserve α_2AR function. High dietary fat induces oxidative stress production in adipose tissues [345] and blood vessels [346] by upregulating NADPH oxidase. It is possible that in the initial stages of obesity development, adipocytes surrounding blood vessels are able to counteract ROS by shielding the blood vessel or through secretion of anti-inflammatory adipokines, such as adiponectin. Adiponectin is recognized for its anti-inflammatory and insulin sensitizing properties. On a HFD, OP but not OR-SD rats, develop glucose intolerance and insulin resistance until about 6 months [347]. The anti-inflammatory effects of adiponectin activity correlate with high glucose and insulin insensitivity, which include suppressing ROS production in blood vessels [348]. Therefore, it is possible that OR-SD rats (and α_2AR) are more susceptible to ROS-induced by a HFD, at least during the first 6 months of a HFD. However, more studies are needed to substantiate this hypothesis.

The relevance of α_2AR dysfunction in obesity-related hypertension development in SD rats remains unclear. In SD rats, α_2AR dysfunction detected after 12 months of a HFD was mild compared to DOCA-salt hypertension, and did not worsen the severity of hypertension versus 6 months of a HFD diet. In fact, systolic blood pressure in 12 month HFD-fed SD rats was not significantly different than NFD, suggesting that blood pressure may actually be improving.

Therefore, it is possible that in chronically obese SD rats, α_2AR dysfunction may develop only after hypertension has established, as a mechanism that maintains blood pressure. This is observed in the SH rat, where there is an age-dependent decline in adrenal α_2AR expression that coincides with impaired inhibition of nicotinic-evoked EP secretion [349].

Obesity-related hypertension in SD rats: HFD fed DahlS rats developed obesity-related hypertension, which was detectable as early as 4 months. The degree of hypertension was more severe than SD rats, resembling DOCA-salt hypertension. However, unlike DOCA-salt hypertension, α_2AR dysfunction was not detected in SMCG neurons from DahlS rats at any of the time points studied. This suggests that α_2AR dysfunction does not play a role in DahlS obesity-related hypertension. This may reflect the lack of increased TNFα and MCP-1 mRNA expression in the SMCG and a lack of increased vascular macrophage accumulation in mesenteric arteries. This suggests that neurovascular damage sustained in obesity-related hypertensive DahlS rats is not as severe as DOCA-salt hypertensive rats. This may seem counterintuitive as salt-induced hypertensive DahlS rats develop vascular and endothelial dysfunction like DOCA-salt hypertensive rats, which occurs even in normotensive DahlS rats [249, 350, 351]. However, we used a low salt/HFD in our study, and thus, the effects of salt are less influential versus studies that used high salt to induce hypertension. Furthermore, diet-induced obesity in DahlS rats protects against vascular dysfunction [249], possibly because obesity is unable to increase RAAS activity [249, 350]. This paradoxical advantage of obesity may provide an additional level of protection against neurovascular injury that preserves α_2AR function in DahlS rats despite a degree of hypertension that is comparable to DOCA-salt rats. Like DahlS rats, RAAS activity is also suppressed in the DOCA-salt rat. This suggests that α_2AR

dysfunction may be primarily caused by exogenous mineralocorticoid. This may also help explain α_2AR dysfunction observed in obese hypertensive SD rats (12 month HFD) as obesity increases RAAS in SD rats [238].

Mechanisms of enhanced SNA in obesity-related hypertension: Experimental and human obesity-related hypertension is associated with sympathetic hyperactivity [34, 352]. However, we suggest that these mechanisms differ from those in DOCA-salt hypertension. Oxidative stress has been associated with sympathetic hyperactivity in obesity-related hypertension. Adipose tissues may be releasing pro-inflammatory molecules that enhance central sympathetic nerve activity [353, 354] as pro-inflammatory molecules can access CVOs [355, 356]. Furthermore, obesity-related hyperleptinemia and insulinenemia can also directly activate central mechanisms that increase SNA. Intravenous and intracerebroventricular administration of leptin increases renal and lumbar sympathetic activity [357, 358] and insulin infusion increases lumbar sympathetic nerve activity by increasing glutamatergic drive to the rostral ventrolateral medulla [359].

Compared to SD rats, DahlS rats were more susceptible to the development of obesity-related hypertension. In our 6 month studies, a HFD induced a mild increase in blood pressure without significant weight gain in SD rats and pronounced hypertension with obesity in DahlS rats, which was detected as early as 4 months. Although SD rats are commonly used to study obesity and hypertension, only a subset of SD rats are susceptible to diet-induced obesity-related hypertension [238]. In contrast, DahlS rats are genetically predisposed to salt-sensitive hypertension [360], and mild increases in blood pressure occurs even on a low-salt (0.3%) diets [195]. Furthermore, there is also evidence that leptin and insulin synergistically increase

sympathetic outflow [361] and that in DahlS rats, insulin and leptin exaggerate obesity-induced blood pressure responses to salt in central cardiovascular regulatory centers [197].

Interestingly, like obesity, salt-sensitive hypertension is associated with insulin resistance [362]. Hyperinsulinemia occurs in the DahlS rat [363] and augments DOCA-salt hypertension [364]. Therefore, there are common mechanisms of enhanced SNA in both salt-sensitive and obesity-related hypertension, however, we suggest that they do not include impairment of α_2AR function in SMCG neurons.

CHAPTER 6: MACROPHAGES CONTRIBUTE TO HYPERTENSION DEVELOPMENT IN DOCA-SALT RATS BY IMPAIRING ALPHA 2-ADRENERGIC RECEPTOR FUNCTION POSSIBLY VIA OXIDATIVE STRESS-INDUCED RECEPTOR INTERNALIZATION

1.0 Abstract

DOCA-salt hypertension is associated with inflammation, oxidative stress, and sympathetic nervous system hyperactivity. Prejunctional α_2-adrenergic receptors (α_2ARs) feedback inhibit norepinephrine (NE) release from sympathetic nerves by coupling via $G\alpha_{i/o}$ to inhibit Ca^{2+} channels. Increased neuronal NE release in DOCA-salt occurs through impaired α_2AR signaling; however, the mechanisms involved are unclear. Mesenteric arteries are resistance arteries that receive sympathetic innervation from the superior mesenteric and celiac ganglia (SMCG). We tested the hypothesis that macrophages impair α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons obtained from DOCA-salt hypertensive rats. Whole cell patch clamp methods were used to record Ca^{2+} currents from SMCG neurons maintained in primary culture. Clodronate-liposomes were used to deplete animals of macrophages. In Chapters 4 and 5, we showed that DOCA-salt treatment increased adventitial macrophage accumulation in mesenteric arteries, increased SMCG mRNA levels of monocyte chemoattractant protein-1 and tumor necrosis factor-α, and impaired α_2AR-mediated inhibition of Ca^{2+} currents in SMCG neurons. In the present study, we show that macrophage depletion preserves α_2AR function and protects against hypertension development in DOCA-salt treated rats. Macrophages mediate inflammatory responses by oxidative stress production. Oxidative stress is associated with adrenergic dysfunction and $G\alpha_{i/o}$ are targets of oxidative stress. We tested the hypothesis that oxidative stress impairs α_2AR function and found that oxidative stress impaired α_2AR-mediated inhibition of Ca^{2+} currents in SMCG neurons. Inhibition of α_2AR-$G\alpha_{i/o}$ coupling following pertussis toxin (PTX) treatment occurs due to ADP-ribosylation on the C-terminal cysteine (position 351) of $G\alpha_{i/o.}$ It is not known if thiol-oxidation

of this residue results in similar uncoupling. Therefore, we tested the hypothesis that oxidizing agents impair α_2-AR function by thiol-oxidation of Cys351 on $G\alpha_o$. The thiol-specific oxidant diamide impaired α_2AR function equally in G1A1 cells co-transfected with α_2AR and PTXi-$G\alpha_o$ (PTX-insensitive due to a Cys351Gly mutation). Oxidative modification can also affect protein trafficking. We tested the hypothesis that thiol-oxidation affects receptor localization and found that oxidative stress resulted in significant α_2AR internalization to the perinuclear cytoplasm in HEK-293T cells. These results suggest that macrophages contribute to the development of DOCA-salt hypertension by impairing α_2AR function through oxidative stress-induced receptor internalization.

2.0 Introduction

Inflammation and oxidative stress play a role in hypertension development and sympathetic hyperactivity in salt-sensitive hypertension [127, 185, 202, 227, 365]. Reactive oxygen species (ROS) and immune cell infiltration can be detected in mesenteric arteries early in hypertension development [127, 366-368]. This is especially relevant in the DOCA-salt hypertensive rat, which is a model of cardiovascular inflammation and oxidative stress [262]. Oxidative stress from peripheral inflammation contributes to centrally-mediated enhancement of SNA [369], but can also increase peripheral sympathetic outflow by directly affecting NE release from nerve terminals.

The prejunctional α_2-adrenergic receptor (α_2AR) inhibits NE release by coupling via $G\alpha_{i/o}$ proteins to inhibit voltage-gated N-type calcium channels that control NE release [125, 338]. This negative feedback regulates sympathetic tone and prevents sustained increases in blood pressure [127, 129]. In mesenteric arteries of DOCA-salt hypertensive rats, inflammation and oxidative stress production enhance neuroeffector transmission, which contributes to increased blood pressure [127, 370, 371]. In agreement with these findings, we showed that DOCA-salt hypertension was associated with an increase in adventitial macrophage accumulation (Chapter 4) and impairment of α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons that did not involve α_2AR expression, GRK-dependent desensitization, or downstream signaling factors (Chapter 5). Therefore, the primary focus of this study was to determine if macrophages contributed to DOCA-salt hypertension by impairing α_2AR function in SMCG neurons.

Macrophages are major sources of oxidative stress, which can affect cell signaling and neuroeffector transmission [313]. Oxidative protein modification can affect protein structure, function and localization [372, 373]. Oxidative targets include amino acids like cysteine, whose thiol groups are oxidation-sensitive [372], as depicted in Figure 33. Cysteine residues are important for proper α_2AR signaling. G protein coupling to the α_2AR involves a conserved cysteine in the C-terminal region of $G\alpha_{i/o}$ proteins. Treatment with pertussis toxin (PTX) causes ADP-ribosylation of this cysteine, resulting in α_2AR-G protein uncoupling [163]. It is unknown if cysteine oxidation impairs α_2AR signaling in DOCA-salt hypertension. Therefore we tested the effects of oxidative stress, specifically with respect to thiol-oxidation, on α_2AR function and localization in SMCG neurons and HEK-293T cells.

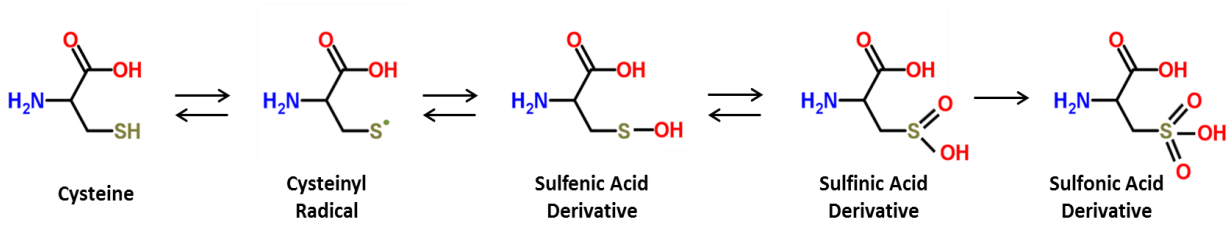

Figure 33. Thiol-oxidation of cysteine residues. Oxidative modifications can affect protein structure, function and localization. Oxidation reactions are reversible with the exception of the formation of sulfonic acid derivatives.

3.0 Results

Effects of clodronate-liposomes on macrophage infiltration. Macrophage infiltration of mesenteric arteries was evaluated by CD163 immunoreactivity, which was significantly higher in mesenteric arteries from DOCA-salt compared to SHAM rats treated with PBS-liposomes. Clodronate-liposomes prevented macrophage accumulation in mesenteric arteries from all rats (Figure 34, A and B). The effectiveness of clodronate-liposomes in depleting macrophages was also confirmed by a significant reduction in spleen weight compared to PBS-liposome treatment (Figure 34C).

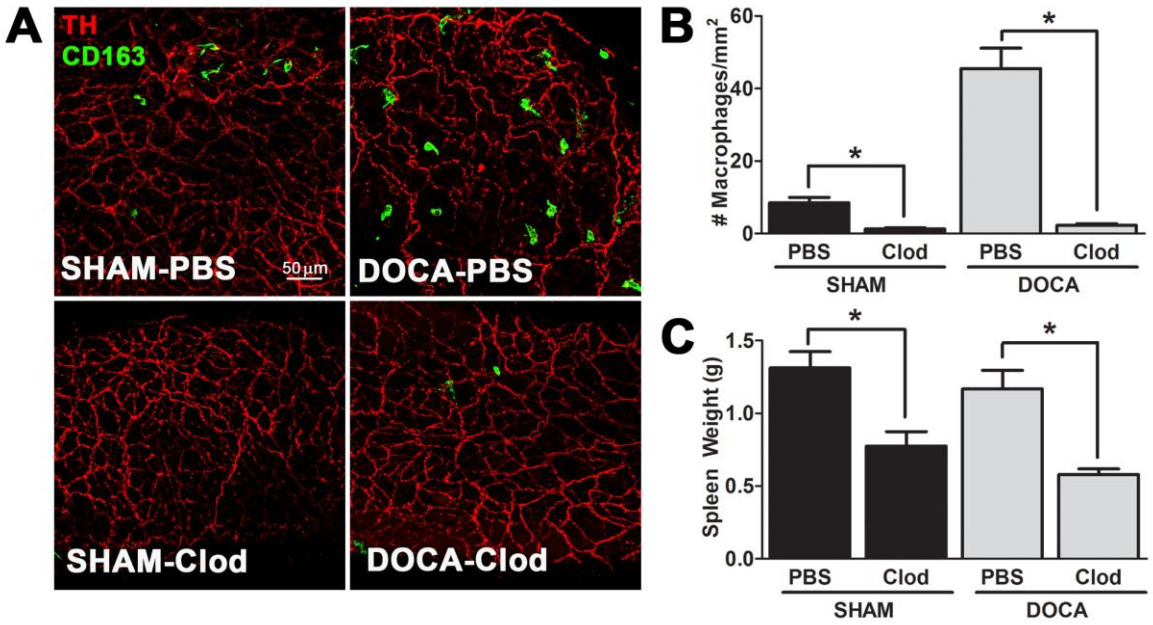

Figure 34. Clodronate-embedded liposome treatment attenuates adventitial macrophage infiltration. ***A*****:** Immunohistochemical labeling of CD163-positive macrophages and tyrosine hydroxylase (TH)-positive sympathetic nerve fibers in whole-mounts of mesenteric arteries from DOCA-salt rats and SHAM rats treated with clodronate-liposomes (Clod) or PBS-liposomes (PBS). ***B*****:** Mean adventitial macrophage number per mm^2 was higher in DOCA-salt rats compared to SHAM rats treated with PBS and significantly reduced in all rats treated with Clod; *n* = 5-7 rats/group. ***C*****:** Mean spleen weight was reduced in rats treated with Clod; *n*= 3-5 rats/group. Data are means ± SE, *P < 0.05.

Effects of macrophage depletion on α_2AR function and blood pressure in DOCA-salt rats. Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons (Figure 35, A and B). Peak current occurred at +10 mV in SMCG neurons (Figure 35A). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE and UK14304 (each at 1 μM) at +10 mV. In SMCG neurons from DOCA-salt rats treated with clodronate-liposomes, Ca^{2+} current inhibition was significantly higher when α_2AR was activated with 1 μM NE (Figure 37A) and UK14304 (Figure 37B) versus neurons from DOCA-salt rats treated with PBS-liposomes. Pretreatment with yohimbine (10 μM) reduced α_2AR function to similar levels in all SMCG neurons (Figure 37C). Clodronate-liposome treatment prevented significant increases in systolic blood pressure in DOCA-salt treated. Mean systolic blood pressure in DOCA-salt rats receiving clodronate-liposomes was comparable to SHAM rats (Figure 37D).

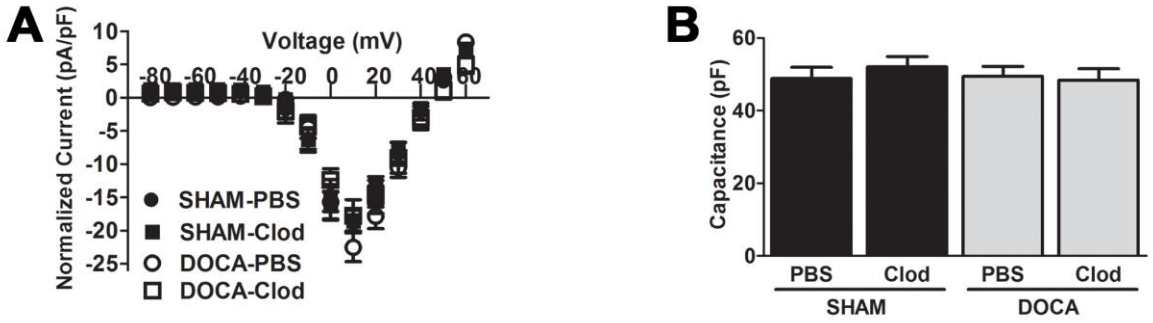

Figure 35. Macrophage depletion with clodronate-liposomes (Clod) does not affect current-voltage relationships and cell size versus PBS-liposomes (PBS). *A*: Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 19-23 cells. ***B*:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 27-36. Data are means ± SE.

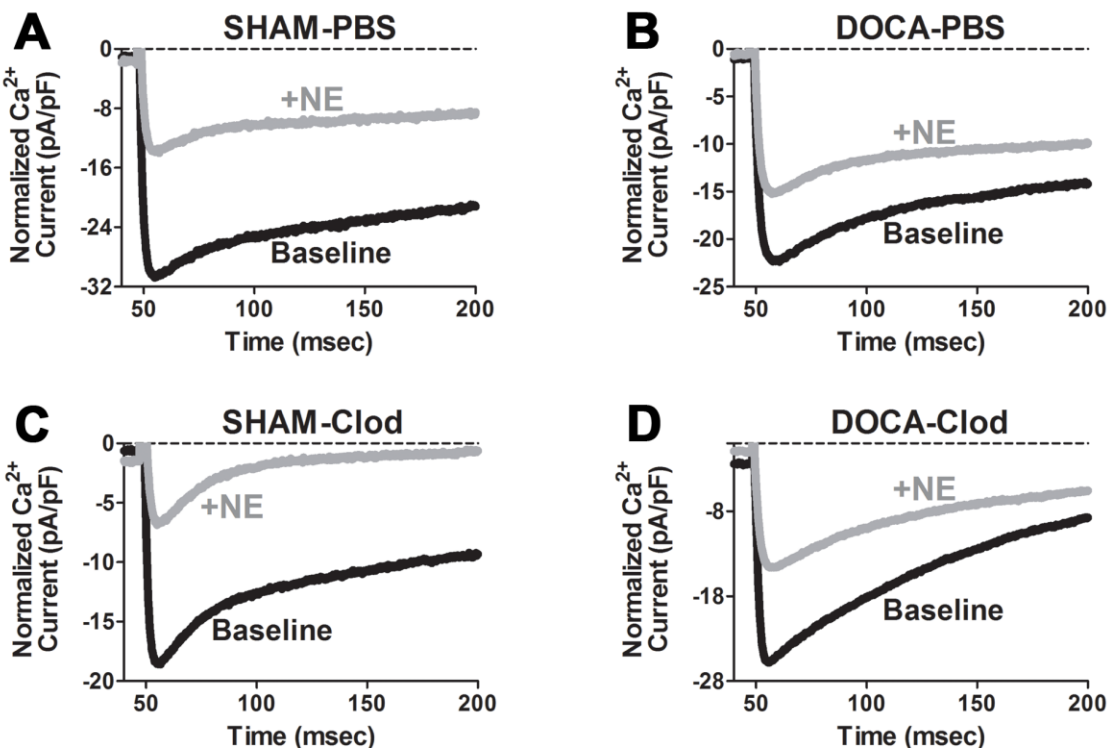

Figure 36. Representative traces of Ca^{2+} current inhibition following macrophage depletion with clodronate-liposomes (Clod) versus PBS-liposomes (PBS). ***A*****:** SHAM PBS-liposomes ***B*****:** DOCA PBS-liposomes ***C*****:** SHAM clodronate-liposomes ***D*****:** DOCA clodronate-liposomes.

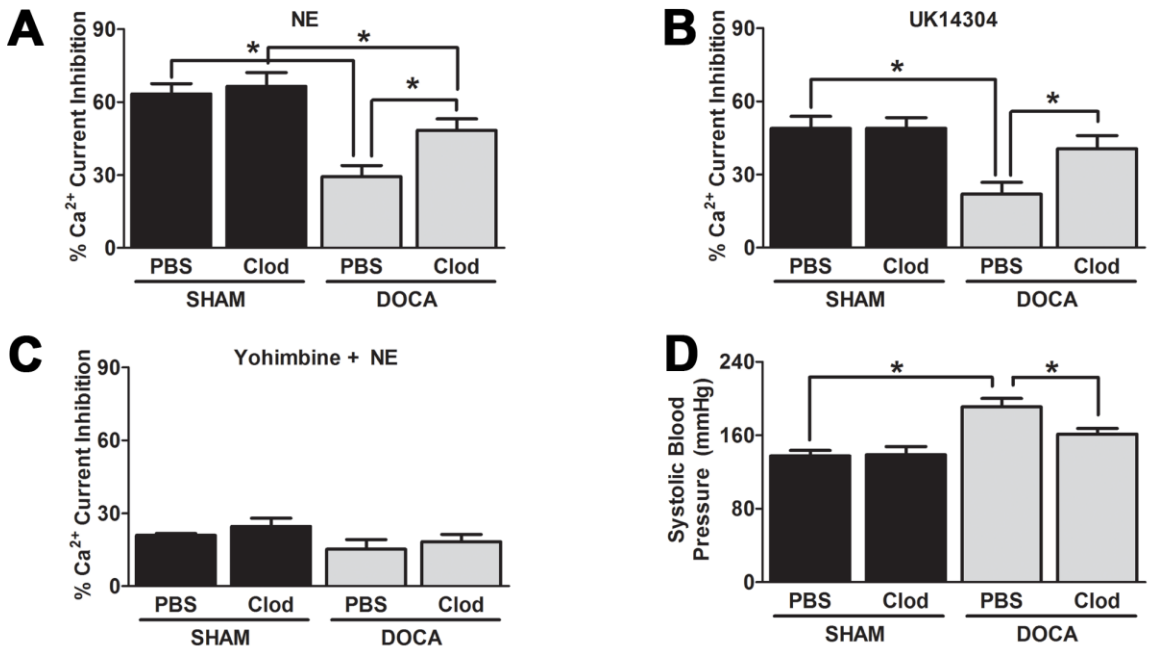

Figure 37. Macrophage depletion with clodronate-liposomes (Clod) preserves α_2AR function and protects against hypertension development in DOCA-salt rats versus PBS-liposomes (PBS). ***A*****:** When activated with NE (1 μM), α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons was higher in DOCA-salt rats treated with Clod compared to DOCA-salt rats treated with PBS, but less than SHAM rats treated with Clod; *n* = 8-9 cells. ***B:*** When activated with UK14304 (1 μM), α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons was higher in DOCA-salt rats treated with Clod compared to DOCA-salt rats treated with PBS, similar to SHAM rats treated with Clod; *n* = 6-7 cells. ***C*****:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons *n* = 5-6. ***D:*** Blood pressure was lower in DOCA-salt rats treated with Clod compared to DOCA-salt rats treated with PBS; *n* = 5-7 rats / group. Data are means ± SE, *P < 0.05.

Effects of oxidative stress on a_2AR function and localization. DOCA-salt hypertension is associated with oxidative stress in celiac ganglia [331] and mesenteric arteries [127]. Therefore, we investigated if oxidative stress impaired α_2AR function. SMCG neurons were pretreated with the non-specific oxidants H_2O_2 and t-BOOH (each at 150 μM), and the thiol-specific oxidant diamide (50 μM) for 30-40 min prior to activating α_2AR with NE (1 μM). H_2O_2, t-BOOH and diamide significantly reduced α_2AR-mediated Ca^{2+} current inhibition in SMCG neurons (Figure 38A). ADP-ribosylation of Cys351 of the $G\alpha_o$ protein by PTX attenuates α_2AR signaling [163]. The effects of Cys351 are currently unknown. Therefore, we wanted to determine if thiol-oxidation of Cys351 affected α_2AR function. This was investigated in G1A1 cells transfected with α_2AR and PTXi $G\alpha_o$. The PTXi $G\alpha_o$ protein harbors a Cys351Gly mutation that does not affect signaling [298]. Cells expressing the α_2AR also expressed PTXi $G\alpha_o$ (Figure 38B). Diamide treatment (50 μM) significantly reduced α_2AR-mediated inhibition of Ca^{2+} channels in cells transfected with α_2AR and PTXi $G\alpha_o$ (Figure 38C).

Protein oxidation can affect protein localization. Therefore, we investigated the effects of oxidative stress on α_2AR receptor localization by treating α_2AR-transfected HEK-293T cells with H_2O_2, t-BOOH or diamide for 40 min. In untreated cells, α_2AR was predominantly localized to the membrane as indicated by co-localization with F-actin (Figure 39, A and B). Oxidative stress significantly reduced the fraction of membrane-bound receptor and increased the fraction localized to the perinuclear cytoplasm (Figure 39, A and B).

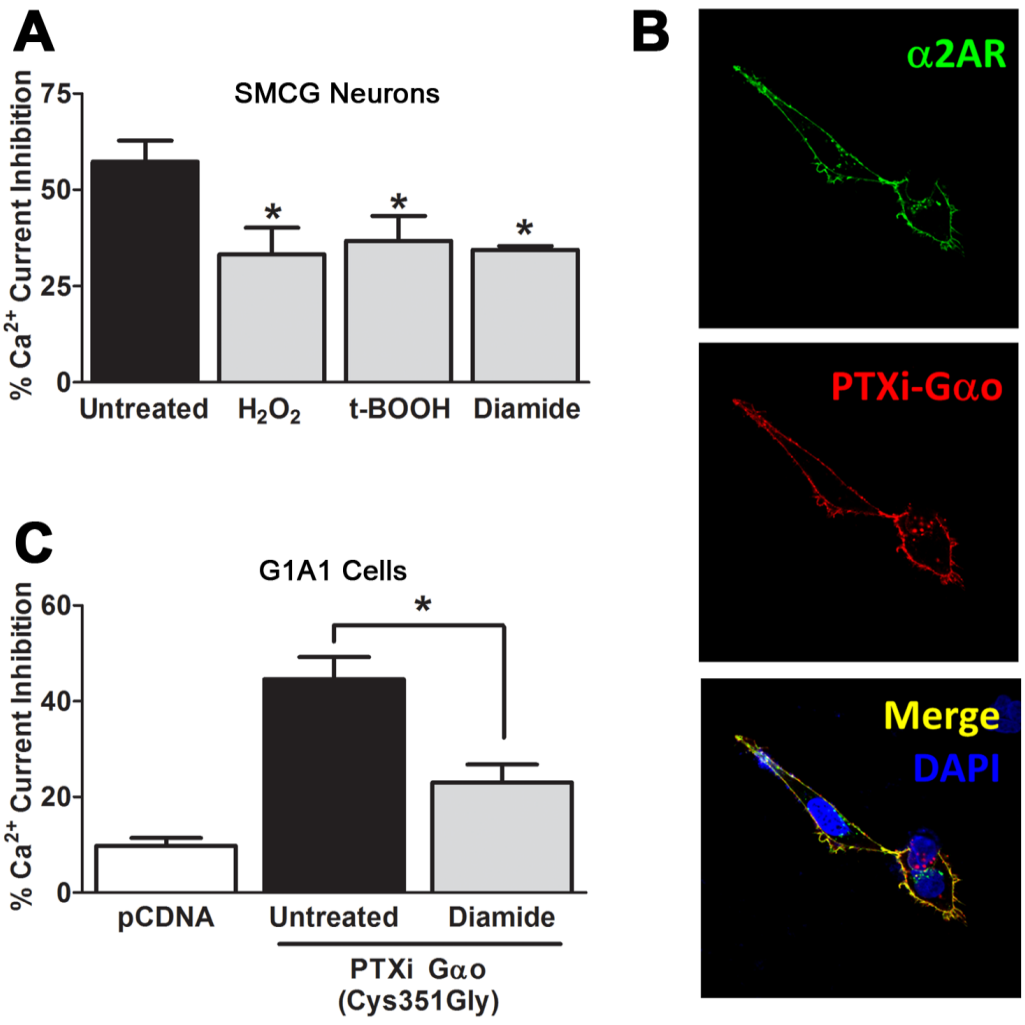

Figure 38. Oxidative stress impairs α_2AR function in SMCG neurons and G1A1 cells. ***A:*** α_2AR-mediated inhibition of Ca^{2+} channels was impaired in acutely dissociated SMCG neurons treated with H_2O_2 (150 μM), t-BOOH (150 μM), and diamide (50 μM); *n* = 7-10 cells. ***B:*** G1A1 cells co-transfected with α_2AR and PTXi$G\alpha_o$, which contains a Cys351Gly mutation. ***C:*** Diamide (50 μM) impairs α_2AR-mediated inhibition of Ca^{2+} channels in G1A1 cells co-transfected with PTXi $G\alpha_o$; n = 3-6 cells. Data are means ± SE, *P < 0.05 vs. untreated.

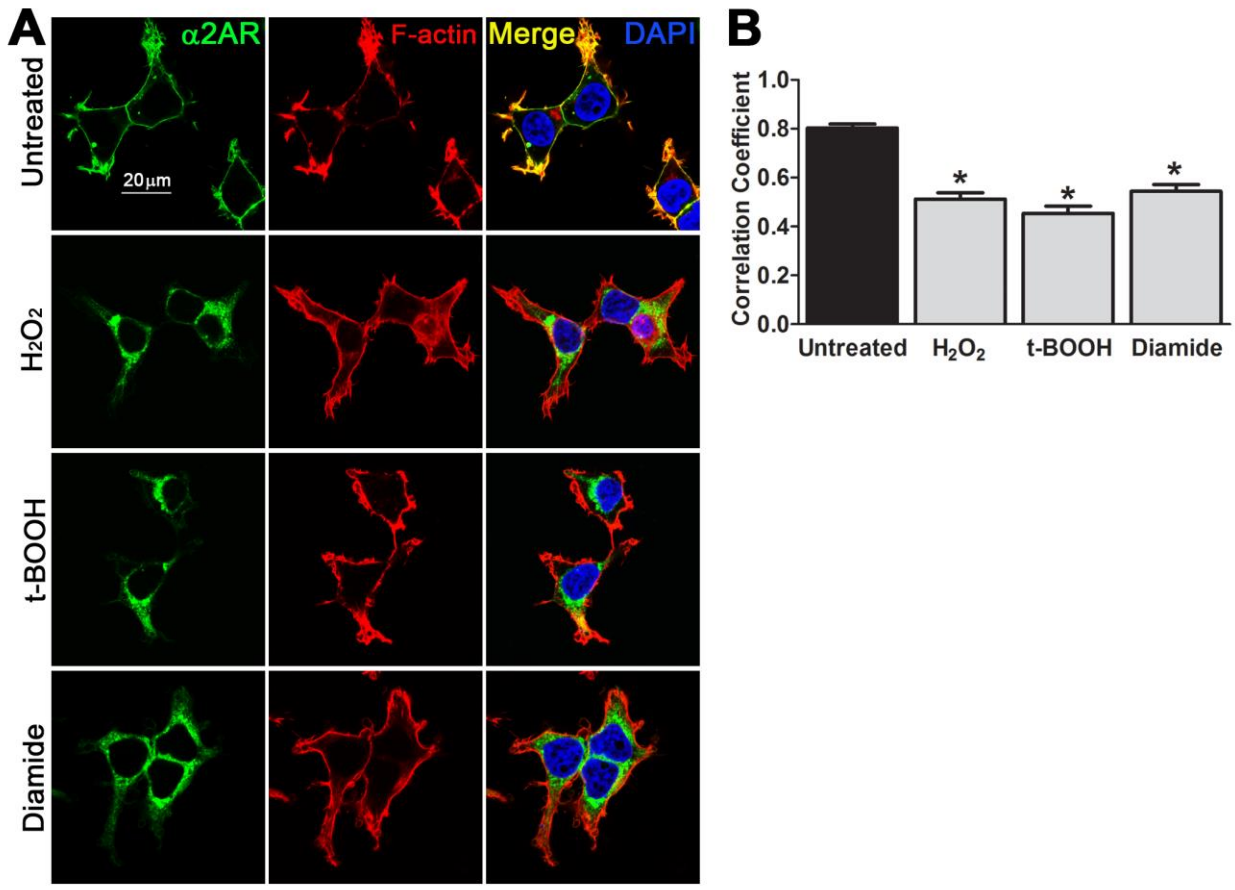

Figure 39. Oxidative stress results in receptor localization to the perinuclear cytoplasm. *A*: Treatment of α_2AR-GFP transfected HEK 293T cells with H_2O_2 (150 μM), t-BOOH (150 μM), and diamide (50 μM) resulted in receptor internalization from the membrane to the perinuclear cytoplasm (*left column*). Alexa Fluor 594 phalloidin was used to stain F-actin and localize the cell membrane (*middle column*). Membrane localization of α_2AR was highest in untreated cells (*right column*). ***B*:** Pearson's correlation coefficient of co-localization between α_2AR and membrane F-actin decreased in α_2AR-GFP transfected HEK 293T cells treated with H_2O_2, t-BOOH, and diamide; *n* = 16-18 cells. Data are means ± SE, *P < 0.05 vs. untreated.

4.0 Discussion

The main findings of this chapter are: 1) Macrophages contribute to DOCA-salt hypertension by impairing α_2AR function. 2) Thiol-oxidation impairs α_2AR function in SMCG neurons. 3) Cys351 of the $G\alpha_o$ protein does not participate in oxidative stress induced-inhibition of α_2AR signaling in G1A1 cells. 4) Thiol-oxidation results in α_2AR mislocalization to the perinuclear cytoplasm in HEK-293T cells.

The DOCA-salt rat is a model of oxidative stress and inflammation [262]. Neuronal superoxide levels are increased in celiac ganglia of DOCA-salt hypertensive rats [331]. In agreement with these observations, we detected significantly higher mRNA expression of TNFα and MCP-1 in SMCG. SMCG neurons innervate mesenteric arteries, which together with previous work, were found to be enriched with adventitial macrophages, TNFα, and superoxide [127]. These results suggest that peripheral vascular and neuro-inflammation from macrophages may be important in the development of DOCA-salt hypertension. Macrophages and oxidative stress are associated with sympathetic hyperactivity in DOCA-salt hypertensive rats [127, 227]. A better understanding of these relationships may lead to improvements in the clinical management of hypertension.

In the present study, we used whole-cell patch clamp to directly evaluate α_2AR-mediated inhibition of Ca^{2+} channels in dissociated SMCG neurons of DOCA-salts receiving either clodronate-liposomes (to deplete macrophages) or PBS-liposomes (vehicle). We found that α_2AR-mediated inhibition of Ca^{2+} channels was significantly impaired in PBS-liposome treated DOCA-salt rats versus SHAM rats. Furthermore, in DOCA-salt treated rats, we found that macrophage depletion preserved α_2AR function and protected against increases in blood

pressure. These results are in agreement with previous observations that macrophage-derived superoxide impairs α_2AR signaling [127].

Macrophages promote oxidative stress that contributes to vascular injury and remodeling [374, 375], and we add to this list impaired α_2AR function. In DOCA-salt hypertension, antagonism of the MCP-1 receptor with INCB3344 attenuates vascular macrophage accumulation and reverses hypertension [279]. This is in agreement with our findings that macrophage depletion preserves blood pressure in DOCA-salt rats. Whether macrophage depletion affects other mechanisms that contribute to DOCA-salt hypertension, such as vasopressin, salt and endothelin-1 [337] remain to be determined. Interestingly, α_2AR activation in macrophages is associated with increased TNFα production [293]. It is tempting to speculate that increased NE release from nerve terminals through impaired α_2AR function may enhance macrophage pro-inflammatory capacities, suggesting possible neuro-immune synergism that may contribute to hypertension. Therefore, our findings support the use of therapeutic approaches that target macrophages in the clinical management of hypertension. This may be relevant to patients with resistant hypertension, who often have salt-sensitivity, low-renin and increased mineralocorticoid status [44]; all of which are hallmarks of the DOCA-salt hypertensive rat. This, in addition to the successful use of mineralocorticoid antagonists like spironolactone in patients with resistant hypertension [376] demonstrates that the DOCA-salt rat may be a suitable model to study human resistant hypertension.

Macrophages are major sources of oxidative stress that may affect cell signaling and neuroeffector transmission by oxidative protein modifications that alter protein structure, function and localization [313, 372, 373]. Oxidative stress has been implicated in modulating G

protein-coupled receptor signaling [377, 378] and oxidative targets include amino acids like cysteine, whose thiol groups are oxidation-sensitive to almost all forms of ROS [372, 379]. Cysteine residues are important for proper α_2AR signaling. The most notable of these involves a conserved cysteine in the C-terminal region of $G\alpha_{i/o}$ proteins. Treatment with pertussis toxin (PTX) causes ADP-ribosylation of this cysteine, resulting in α_2AR-G protein uncoupling [163]. In our study, non-specific oxidants (H_2O_2, t-BOOH) and the thiol-specific oxidant diamide impaired α_2AR function in SMCG neurons similar to DOCA-salt challenge. However, diamide also impaired α_2AR signaling in G1A1 cells transfected with PTXi $G\alpha_o$, which harbors a Cys351Gly mutation. Therefore, these results suggest that oxidative modification of other cysteine residues could be contributing to α_2AR dysfunction. Cysteine modifications by phenylmercuric chloride have been shown to decrease α_2AR-ligand affinity [164] and the C-terminal cysteine of the α_2AR is associated with mechanisms of downregulation [165]. Additional studies are needed to identify the cysteine residues responsible for oxidation-mediated α_2AR dysfunction.

Furthermore, in α_2AR-transfected HEK-293T cells, oxidative stressors that included diamide caused receptor internalization to the perinuclear cytoplasm. Oxidation of proteins can occur directly from ROS or through secondary oxidation processes like lipid peroxidation. Oxidation of proteins is important in the pathological progression of disease because it is associated with protein downregulation. Mechanisms of repair for oxidized proteins are not as frequently reported and characterized in comparison to mechanisms that repair oxidized lipids and nucleic acids [379]. Although there are disulfide reductases that can reduce oxidized cysteines (before conversion to sulfonic acids) [380], it is generally accepted that oxidized proteins are targeted for degradation by the proteasome system [381] or chaperone-mediated

autophagy with incomplete degradation within lysosomes [382]. Therefore, it is possible that oxidation-mediated internalization of the α2AR impairs α2AR signaling through mechanisms of receptor downregulation/degradation.

Recently, there has been growing evidence supporting that some oxidative protein modifications do not result in degradation, rather, occur in order to regulate cell signaling [383]. Our findings are not in disagreement with this notion either. In fact, internalization-mediated downregulation is a natural mechanism that regulates α2AR signaling. Interestingly, the perinuclear distribution of internalized α_2AR in our studies bears a close resemblance to α_2AR internalization following prolonged clonidine-activation [144]. Agonist-mediated receptor internalization is an efficient method of receptor desensitization. Therefore, we suggest that sulfhydryl-oxidation of cysteines to sulfenic acids may promote mechanisms of α_2AR desensitization by uncoupling α_2AR from its G-protein through receptor internalization. Oxidative stress has been shown to modulate signaling of the D1-dopamine receptor via G-protein uncoupling [384] and this mechanism may be responsible for α_2AR dysfunction in DOCA-salt hypertension.

CHAPTER 7: ALPHA 2-ADRENERGIC RECEPTOR DYSFUNCTION IN HIGH FAT DIET-INDUCED OBESE HYPERTENSIVE FEMALE SPRAGUE DAWLEY RATS

1.0 Abstract

In humans, blood pressure and hypertension prevalence tend to be higher in males than females until the age of 65. Testosterone may promote mechanisms that contribute to hypertension development while estrogen may provide protection against hypertension development. Many experimental models suggest that hypertension induced by ANG-II, L-NAME, mineralocorticoid-salt and obesity are more severe in males. We previously showed (Chapter 5) that long term HFD feeding induces obesity-related to mild hypertension in male SD rats that is associated with prejunctional α_2AR dysfunction in SMCG neurons. Given the sex-differences in susceptibility to hypertension development, we wanted to investigate if a HFD induced obesity, hypertension, and impaired α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons from female SD rats. Short term HFD feeding (4 months) did not cause weight gain, hypertension or prejunctional α_2AR dysfunction in female SD rats. Long term HFD feeding (10 months) caused obesity, increased diastolic blood pressure and mild impairment of α_2AR-mediated inhibition of Ca^{2+} in SMCG neurons. The results of our short term study suggest that SD female rats are more resistant to HFD-induced hypertension development than SD male rats. The results of our long term study suggest that similar to SD males, SD females develop HFD-induced obesity related to mild hypertension and α_2AR dysfunction. It is not known if these results are due to obesity itself or from prolonged exposure to a HFD.

2.0 Introduction

In humans, hypertension prevalence and blood pressures in general tend to be higher in males versus females independent of race or ethnicity [385]. This difference occurs until about age 65 [386, 387] and may be explained by findings that age-associated blood pressure occurs later and more rapidly in females (typically around age 50-60) compared to males [388]. While sex differences in blood pressure are not large and are usually less than 10/5 mmHg (systolic/diastolic), they nevertheless suggest that females may be less susceptible to hypertension development in early adulthood before menopause.

Sex-differences in blood pressure are also evident in experimental models of hypertension. Hypertension development is more severe in males versus females in ANG-II induced hypertension [389], L-NAME-induced hypertension [390], mineralocorticoid-salt hypertension [391, 392], and salt-induced DahlS hypertension [393]. These effects may be attributed to estrogen. Aldosterone infusion and high salt induces higher blood pressure in males that is associated with increased Fra-like activity (indicator of chronic neuronal excitation) in the PVN. Interestingly, ovariectomy increases PVN Fra-like activity and attenuates protection against hypertension development in female rats [391] .

However, estrogen is not able to explain all hypertension-related sex differences. In HFD-induced hypertension, increased blood pressure appears to depend on testosterone. Castrated but not ovariectomized rats are protected from HFD-induced hypertension. Administration of exogenous testosterone restores susceptibility in castrated male rats and augments hypertension development in ovariectomized female rats [394]. Hypertension development in the SH rat also appears to be androgen-dependent [395, 396]. Urinary Na^+

excretion measurements suggest that this may involve impairment of pressure-natriuresis, which occurs in male SH rats but not female or castrated SH rats. Testosterone supplementation in castrated and ovarietomized SH rats attenuates protection against impaired pressure-natriuresis [396].

Understanding sex differences in blood pressure may lead to a better understanding of hypertension pathogenesis as well as mechanisms that might protect against hypertension development. This could lead to the development of better therapeutics that are tailored specifically to patient backgrounds. Therefore, we wanted to determine if females were protected from α_2AR defects in hypertension development. We selected to study sex-specific effects using the HFD-induced obesity/hypertension model in SD rats (at 4 month and 10 month time points).

3.0 Results

Effects of HFD on obesity and hypertension development in female SD rats. A HFD did not increase body weight or blood pressure at 4 months (Figure 40, A and B). We also determined if increasing the duration of a HFD led to the development of obesity-related hypertension. Female SD rats fed a HFD for 10 months did not develop increased systolic or mean arterial blood pressure, but did develop increased diastolic pressure and body weight gain compared to NFD-SD rats (Figure 40, C and D).

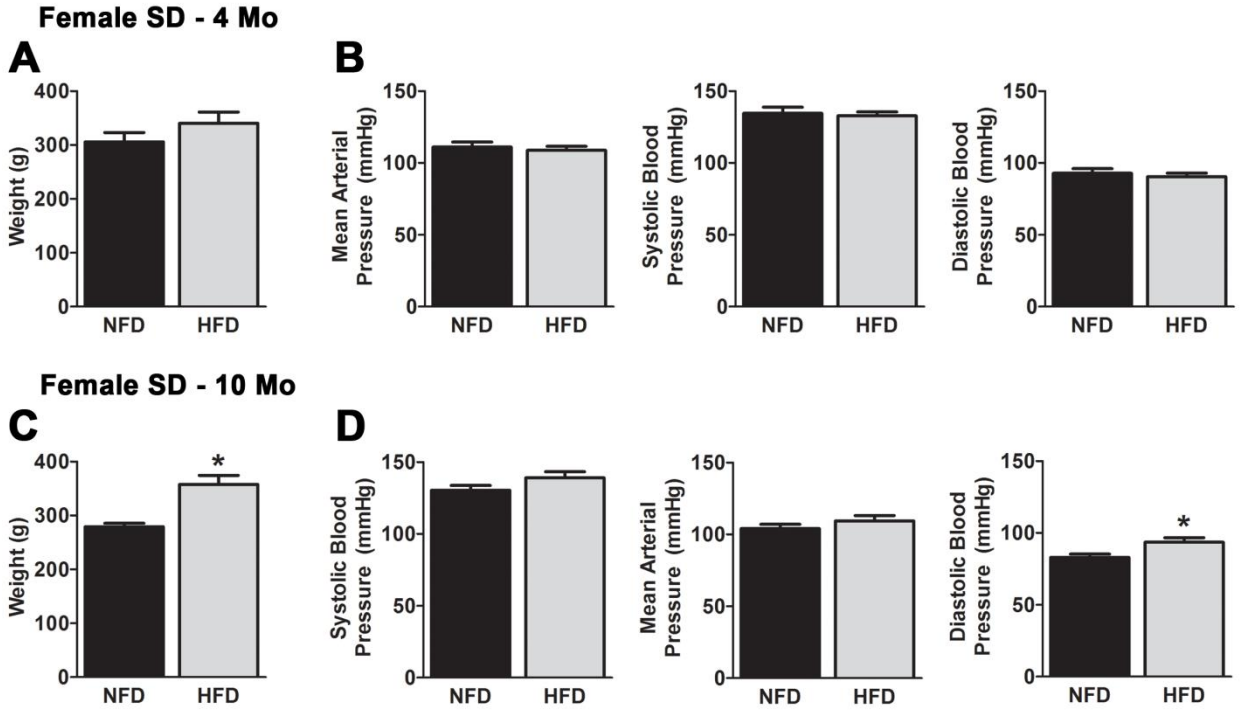

Figure 40. Effects of HFD on body weight and blood pressure in female SD rats. *A and B*: 4 months of HFD feeding does not increase body weight or blood pressure; n=5 rats/group. ***C and D*:** 10 months of HFD feeding caused weight gain and an increase in diastolic pressure compared to NFD rats; n=4-5 rats/group. Data are means ± SE, *P < 0.05.

Effects of 4 months of HFD feeding on α_2AR function in SMCG neurons. Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons regardless of diet (Figure 40, A and B). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE and the α_2AR-specific agonist UK14304 (each at 1 μM) at +10 mV. Inhibition of Ca^{2+} current was similar in all SMCG neurons (Figure 40D). Pretreatment of neurons with the α_2AR antagonist, yohimbine (10 μM, 1 min), blocked Ca^{2+} current inhibition caused by NE (Figure 40E).

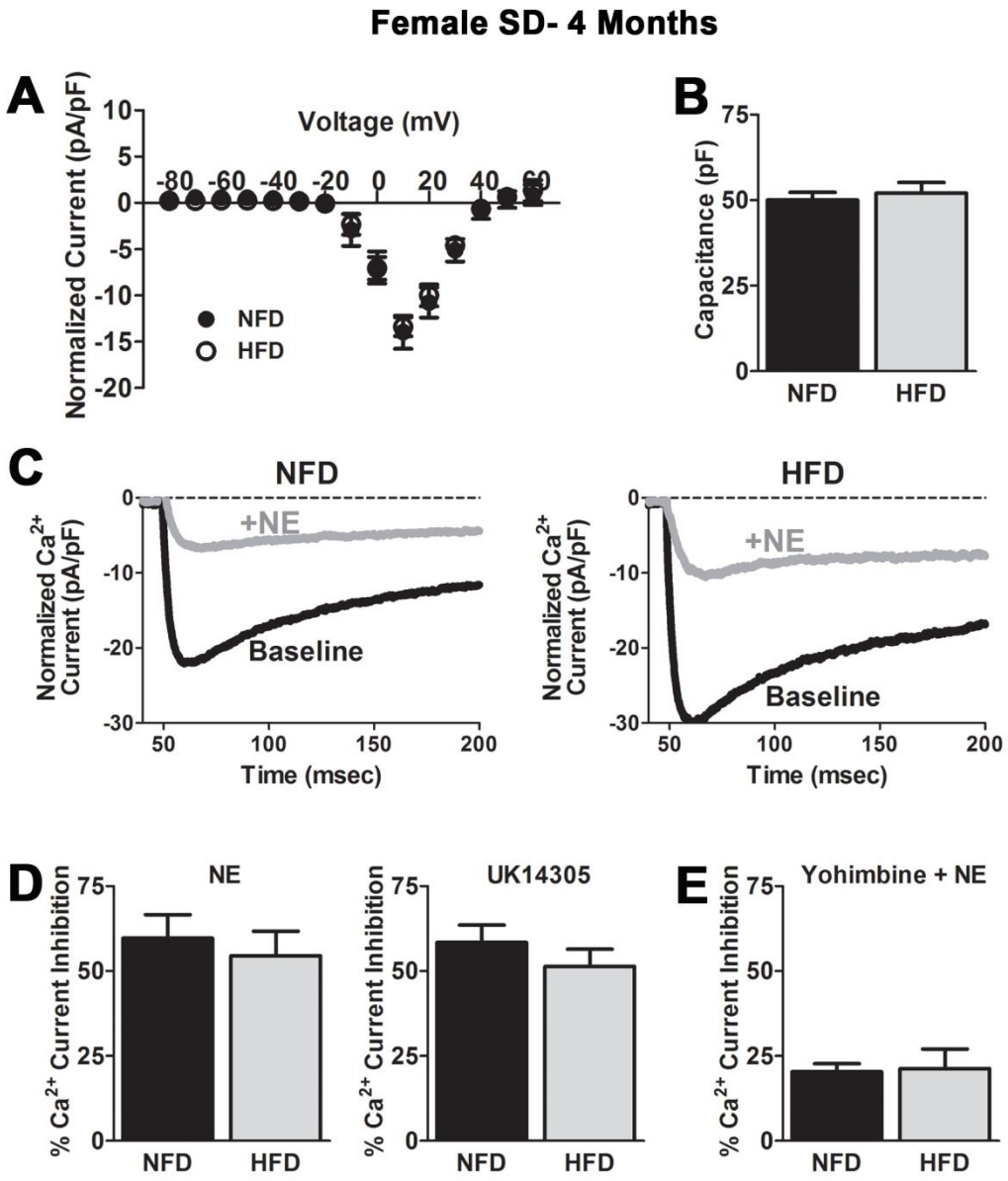

Figure 41. 4 months of HFD does not impair α_2AR-mediated inhibition of Ca^{2+} channels in acutely dissociated SMCG neurons. ***A*****:** Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 25-31 cells. ***B*****:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 37-43 cells. ***C*****:** Represent traces of Ca^{2+} current at baseline and when α_2AR was activated by 1 μM NE in SMCG neuron from NFD (*left*) and HFD (*right*) rat. ***D*****:** Ca^{2+} current inhibition was similar in all SMCG neurons when α_2AR was activated by NE (1 μM) (*left*) and UK14304 (1 μM) (*right*); *n* = 5-8cells. ***E*****:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons; *n* = 5-6 cells. Data are means ± SE.

Effects of 10 months of HFD feeding on α_2AR function in SMCG neurons. Normalized Ca^{2+} current-voltage relationships and cell membrane capacitance were similar in all SMCG neurons regardless of diet (Figure 42, A and B). Ca^{2+} current inhibition via activation of α_2AR was evaluated using NE and the α_2AR-specific agonist UK14304 (each at 1 μM) at +10 mV. Inhibition of Ca^{2+} current was reduced when α_2AR was activated by NE in SMCG neurons of HFD-fed female SD rats (Figure 42D). Pretreatment of neurons with the α_2AR antagonist, yohimbine (10 μM, 1 min), blocked Ca^{2+} current inhibition caused by NE (Figure 42E).

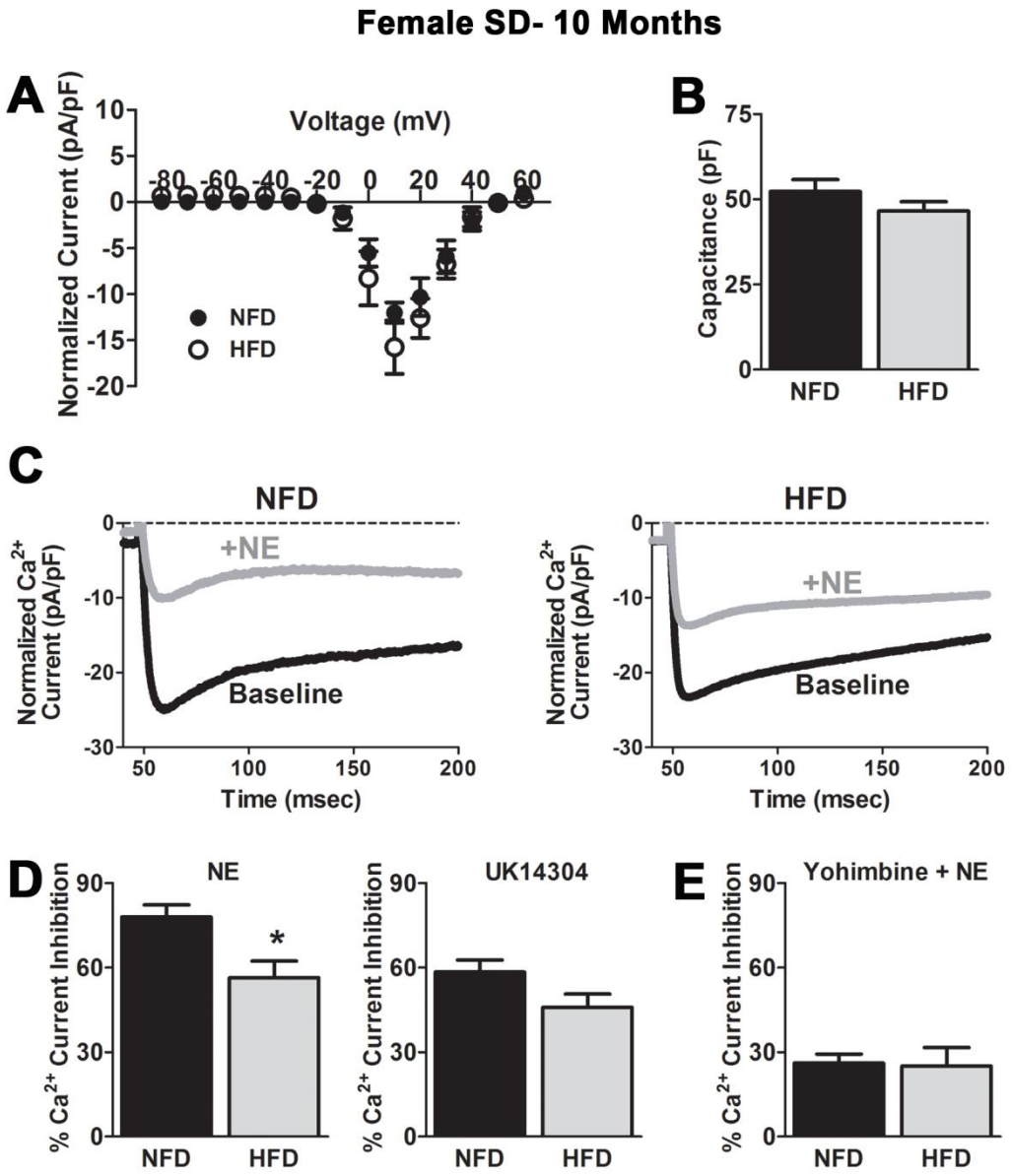

Figure 42. 10 months of HFD impairs α_2AR-mediated inhibition of Ca^{2+} channels by NE activation in acutely dissociated SMCG neurons. ***A*****:** Normalized Ca^{2+} current-voltage relationships were similar in all SMCG neurons; *n* = 16-17 cells. ***B*****:** Mean cell membrane capacitance was similar in all SMCG neurons; *n* = 30 cells. ***C*****:** Represent traces of Ca^{2+} current at baseline and when α_2AR was activated by 1 μM NE in SMCG neuron from NFD (*left*) and HFD (*right*) rat. ***D*****:** Ca^{2+} current inhibition was reduced when α_2AR was activated by NE (1 μM) (*left*) but not UK14304 (1 μM) (*right*) in SMCG neurons of HFD-fed female SD rats; *n* = 5-6 cells. ***E*****:** Pre-treatment with yohimbine (10 μM) attenuated α_2AR function equally in all SMCG neurons; *n* = 3-5 cells. Data are means ± SE.

4.0 Discussion

We wanted to investigate if α_2AR impairment observed in obese hypertensive male SD rats was sex-specific. Therefore, we performed a similar short term (4 month) and long term (10 month) HFD study in female SD rats. The major findings of this study are: 1) A HFD induced obesity and increased diastolic pressure only during longer exposure to a HFD. 2) Long term HFD-induced obesity and increased diastolic blood pressure was associated with mild impairment of α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons.

Before the age of 65, females appear to be less susceptible to higher blood pressure and hypertension development versus males [385-387]. This has been largely attributed to female sex hormones, which may be cardioprotective [397]. However, the prevalence of hypertension and related sequelae in females may be changing due to increasing rates of obesity [398-400]. Obesity is a major risk factor for hypertension [401] and females are more prone to obesity than males [398, 402]. In general, males tend to have higher SNA than premenopausal females of comparable age [403, 404]. However, whether sex affects obesity-related enhancement of SNA is still undetermined. Therefore, in the present study, we investigated the effects of HFD, obesity, and hypertension on α_2AR function in SD females.

In male SD rats, we previously showed that short term exposure to HFD resulted in mild hypertension (mean arterial, systolic, and diastolic blood pressure) without significant weight gain that was not associated with α_2AR dysfunction in SMCG neurons. In the present study, similar to male SD rats, short term HFD feeding in female SD rats did not increase body weight or impair α_2AR function. However, unlike male SD rats, short term HFD feeding in female SD rats did not cause an increase in mean arterial, systolic or diastolic blood pressure. These

results suggest that in the short term, females may be protected from HFD-induced hypertension development. Protection against diet-induced hypertension in female rats has been reported in several experimental models that include diets consisting of high fat [394] and high fructose [405]. Interestingly, ovariectomy increases susceptibility to fructose-induced hypertension, but not HFD-induced hypertension [394, 405]. This suggests that estrogen does not play a role in HFD-induced hypertension. Furthermore, in HFD-induced hypertension, castration protects against hypertension development and exogenous testosterone administration restores susceptibility to hypertension in castrated male rats and augments hypertension in ovariectomized females [394]. Therefore, in our study, SD female rats may be protected from HFD-induced hypertension development because of lower levels of androgen compared to SD male SD rats.

In male SD rats, we previously showed that long term exposure to a HFD leads to obesity with mildly elevated mean arterial and diastolic blood pressure that is associated with α_2AR dysfunction (Chapter 5). In the present study we found that this was similar in female SD rats, although to a milder degree. In female SD rats, only diastolic blood pressure increased and α_2AR dysfunction was detected only when activated by NE and not the α_2AR-specific agonist UK14304. It should be noted that UK14304 mediated inhibition of Ca^{2+} channels tended to be lower in SMCG neurons from HFD-fed rats versus NFD-fed rats (P=0.08) and may have become significant if we increased cell number. It was less likely that NE was mediating a response through other adrenergic receptors as the α_2AR antagonist yohimbine attenuated the effects of NE equally in all neurons. Therefore, we suggest that differences in NE-mediated inhibition of Ca^{2+} channels are attributed to altered α_2AR function.

Evidence suggests that obesity-related impairment in autonomic control of the cardiovascular system is sex-specific [406]. Although our study captures only a small picture of total SNA, our results demonstrate that α_2AR dysfunction may participate differently in obese males and females following long term exposure to a HFD. We suggested that impaired α_2AR signaling did not play a role in the developmental phase of HFD-induced hypertension in male SD rats because short term HFD-fed male rats were hypertensive without a change in α_2AR function. However, in female SD rats, we detected α_2AR impairment as diastolic blood pressure began to increase after long term exposure to a HFD. Therefore, it may be possible that in female SD rats, α_2AR dysfunction plays a role in hypertension development unlike males, where α_2AR may play a role in hypertension maintenance during long term exposure to a HFD. However, a more thorough analysis of α_2AR function at different time points is needed to fully substantiate this hypothesis.

CHAPTER 8: GENERAL SUMMARIES/CONCLUSIONS AND DISCUSSION

1.0 Mineralocorticoid-Salt Hypertension

The DOCA-salt hypertensive rat is characterized by cardiovascular inflammation, oxidative stress, and enhanced SNA [220]. The majority of studies have focused on central mechanisms that enhance sympathetic output. However, even enhanced activation of post-ganglionic sympathetic nerves from higher centers can be counteracted by the prejunctional α_2AR. Activation of the prejunctional α_2AR provides immediate feedback inhibition to NE (and ATP) release from nerve terminals. In this study, we show that DOCA-salt hypertension is associated with functional impairment of the prejunctional α_2AR.

DOCA-salt treatment resulted in severe hypertension that was associated with an increase in neuro-inflammation in the SMCG, an increase in adventitial vascular macrophage infiltration, and impaired α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons. Deficits in α2AR signaling did not involve mechanisms of receptor expression, GRK-dependent desensitization, or downstream signaling and likely involved receptor function.

Furthermore, macrophages contributed to this dysfunction as macrophage depletion in DOCA-salt treated rats preserved α_2AR-mediated inhibition of Ca^{2+} channels and protected against hypertension development. Finally, oxidative stress, specifically thiol-oxidation, impaired α_2AR signaling by promoting receptor internalization to the perinuclear cytoplasm. These results may suggest that macrophage-derived ROS impairs prejunctional α2AR signaling by promoting mechanisms of receptor internalization and desensitization/degradation.

Common features of resistant hypertension include salt-sensitivity, low-renin and increased mineralocorticoid status [44]. These are all hallmarks of the DOCA-salt hypertensive rat [220]. This, in addition to the successful use of mineralocorticoid antagonists like

spironolactone in patients with resistant hypertension [407] suggests that the DOCA-salt hypertensive rat is a suitable model to study resistant hypertension. Furthermore, our results suggest that there may be benefits of targeting oxidative stress and macrophage-derived inflammation in human resistant hypertension either directly or as adjunctive therapy.

Conclusion: Macrophages contribute to DOCA-salt hypertension by impairing prejunctional α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons through mechanisms of oxidative stress-induced receptor internalization.

2.0 Obesity-Related Hypertension

Unlike DOCA-salt hypertension, the development of obesity-related hypertension was not clearly associated with macrophages and prejunctional α_2AR impairment in mesenteric arteries. This was consistently observed in both HFD-fed SD rats and also DahlS rats. We used these rats specifically to determine how a HFD and obesity affect blood pressure regulation in rats with different backgrounds. SD rats are not predisposed to hypertension development, have normal RAAS responses, and like many humans, show great variability [408]. By contrast, DahlS rats are predisposed to developing salt-sensitive and low-renin hypertension [409]. Studying the effects of obesity and HFD using different animal models is important because of the variability in susceptibility and onset of obesity-related hypertension in humans.

On a high fat diet (HFD), SD rats slowly developed obesity with mild hypertension over the course of a year while Dahl S rats developed obesity with severe hypertension by 6 months. Obesity-related hypertension was not associated with neuro-inflammation in the SMCG or an increase in adventitial macrophage infiltration in SD and DahlS rats. This may have been attributed to compensatory mechanisms that could respond to gradual increases in blood pressure (versus rapid onset like in DOCA-salt hypertension), thus, preventing tissue injury.

Mild but significantly impaired α_2AR-mediated inhibition of Ca^{2+} currents occurred in SMCG neurons from obese mildly hypertensive SD rats (male and female) after prolonged exposure to HFD feeding (10-12 months). In male SD rats, this impairment did not augment the severity of hypertension versus 6 months of HFD feeding, suggesting that α2AR dysfunction may serve more in hypertension maintenance rather than development in this model. In female SD rats, this impairment occurred at the same time as increased diastolic pressure, and may

play a role in hypertension development after prolonged exposure to a HFD. Impaired α_2AR function did not occur in DahlS rats at any of the time points studied. Prejunctional α_2AR dysfunction and enhanced NE release has been suggested to occur in salt-induced hypertensive DahlS rat [204]. Therefore, the lack of α_2AR dysfunction in our study may reflect the fact that HFD, and not salt was used to induce hypertension.

There are several explanations to account for differences in hypertension and susceptibility to α2AR dysfunction within animal models in our HFD study and in contrast to findings from our DOCA-salt study. All diets used in the HFD study consisted of 0.3% salt. While this is relatively low for the rat, it significant for DahlS rats, which have been shown to develop increased blood pressure even on a 0.3-0.4% salt diet [195]. Therefore, salt, at least in part, may explain the quicker onset and degree of hypertension developed in HFD-fed DahlS rats versus HFD-SD rats. Furthermore, obesity increases RAAS activity [410], which likely occurred more in SD rats considering DahlS rats are genetically predisposed to low-renin secretion [409]. From our DOCA-salt study, α_2AR dysfunction was associated with mineralocorticoid excess and it is possible that aldosterone played a similar role in SD but not DahlS rats. Furthermore, despite a more severe form of hypertension in obese DahlS rats, diet-induced obesity in DahlS rats protects against vascular dysfunction [249], possibly because obesity is unable to increase RAAS activity [249, 350]. This may protect against vascular injury, thus, explaining why macrophage infiltration was not increased and why α_2AR function was not altered in obese hypertensive DahlS rats compared to DOCA-salt hypertensive rats.

The effects of obesity on α_2AR function, unlike DOCA-salt, are complex and remain elusive. This was further exemplified by our paradoxical findings that following 6 months of a

HFD, OP-SD rats, which developed obesity and hypertension faster than unspecified SD rats, were not associated with α_2AR dysfunction while α_2AR dysfunction occurred in non-obese normotensive OR-SD rats. We speculate that in the short term, obesity may protect against HFD-induced oxidative stress production [345], which we know from our DOCA-salt study, impairs α2AR function. In obese OP rats, production of anti-inflammatory adipokines like adiponectin may buffer ROS from blood vessels. Glucose intolerance and insulin resistance occur in OP-SD but not OR-SD rats [347] and adiponectin suppresses vascular ROS production during states of hyperglycemia and insulin-insensitivity [348].

However, what is clear from our combined studies is that hypertension alone is not associated with vascular macrophage infiltration or α_2AR dysfunction. The use of multiple hypertension models is helpful in studying the contributions of selected precipitating factors to specific pathological processes observed in human hypertension. Blood pressure-dependent associations with α_2AR dysfunction in our studies likely involved mineralocorticoid and salt, but not obesity. The only association detected was between mild α_2AR impairment and a HFD (prolonged exposure in SD rats and short term exposure in OR-SD rats). Therefore, we suggest that in obesity-related studies, careful attention should be placed on the choice of diet and duration of exposure, and not just on the development (or lack) of obesity itself.

Conclusion: Unlike DOCA-salt hypertension, macrophage-associated impairment of α_2AR-mediated inhibition of Ca^{2+} channels in SMCG neurons does not occur in obesity-related hypertension. Prolonged HFD-feeding leads to obesity with mild hypertension and impaired prejunctional α_2AR function in male and female SD rats. In the SD rat, α_2AR dysfunction is more associated with a HFD than obesity, and in the short term, obesity may protect against HFD-

induced α_2AR impairment. A HFD causes obesity and severe hypertension development in DahlS rats but does not alter prejunctional α_2AR function in SMCG neurons. Therefore, DahlS rats are more susceptible than SD rats to HFD-induced obesity-related hypertension; however, the underlying mechanisms for this do not involve impairment of the prejunctional α_2AR.

3.0 Macrophages in DOCA-Salt and HFD-Induced Hypertension

It is important to appreciate that macrophage number alone does not provide adequate information regarding the inflammatory status of macrophages, which is likely more important for hypertension development. Macrophage depletion in our DOCA-salt studies showed that macrophages contribute to hypertension development by impairing α_2AR function. However, macrophage depletion was not performed in our obesity-related studies because it was not practical to administer clodronate-liposomes for the extended time it would take to develop obesity-related hypertension in SD rats (months to a year in HFD-SD rats versus weeks in DOCA-salt rats). Therefore, we cannot rule out whether macrophages impaired α_2AR dysfunction in our aged obese and mildly hypertensive SD rats. Future experiments could involve administering drugs that suppress macrophage-derived inflammatory mediators or inhibit MCP-1 to clarify whether this relationship also exists in some forms of obesity-related hypertension.

Macrophages without question play a role in obesity-related disorders. Obesity is associated with chronic low-grade inflammation and macrophages are the main leukocytes in adipose tissue [310, 411]. However, it is reasonable that the pro-inflammatory status of macrophages in DOCA-salt hypertension was more severe than obesity-related hypertension. This may be explained not only by the rate of hypertension development which may cause vascular damage in DOCA-salt rats, but enable time for compensatory mechanisms to protect against vascular damage in slow developing obesity-related hypertension. Rather, the use of mineralocorticoid and salt, which are precipitating factors that drive rapid hypertension development in the DOCA-salt model, also directly promote pro-inflammatory polarization of macrophages. Salt increases pro-inflammatory cytokine production by activating mitogen-

activated protein kinase [270] and by attenuating IL-4 and IL-13 signals that favor the M2 phenotype [271]. The effect of salt on macrophages is so profound that pro-inflammatory cytokine production occurs even in the absence of macrophage stimulation [272]. Furthermore, mineralocorticoid signaling also enhances pro-inflammatory polarization of macrophages [412, 413] as well as vascular macrophage recruitment [321]. Selective deletion of the MR from macrophages protects DOCA-salt treated rats from hypertension and cardiovascular remodeling [414]. Therefore we suggest that macrophages are likely relevant to both obesity-related and DOCA-salt hypertension, however, macrophages may play a more dominant role, especially with respect to adrenergic signaling when hypertension develops rapidly due to mineralocorticoid-salt excess.

Our macrophage depletion study suggested that macrophages impair α_2AR. Whether this occurs directly or indirectly cannot be fully addressed from this approach alone. Although it is well supported that macrophages play a role in DOCA-salt hypertension development [127, 279, 307, 415], T-lymphocytes have also been implicated as well [267]. RAG1 knockout mice lack T and B-lymphocytes and are resistant to DOCA-salt hypertension, increased levels of vascular superoxide, and vascular dysfunction. Adoptive transfer of T-lymphocytes (but not B-lymphocytes) into these animals restores susceptibility to hypertension development, increased vascular superoxide and vascular dysfunction [267]. Furthermore, lymphocyte suppression using mycophenolate mofetil protects against the development of DOCA-salt hypertension [416]. Our results do not suggest that T-lymphocytes do not play a role and we suggest that they are likely just as important as macrophages in hypertension development. In general, T-lymphocytes play a role in adaptive immunity where inflammatory effector responses involve

specific antigen-receptor interactions that mostly occur after the initiation of innate immunity [263]. Macrophages participate in both innate and adaptive immune responses. In adaptive immunity, macrophages present antigens to CD4+ helper T-lymphoctes, which then activate CD8+ cytotoxic T-lymphocytes [417]. Macrophages have also been shown to directly stimulate and enhance cytotoxic capacities of CD8+ T lymphocytes [418]. On the other hand, CD4+ T-lymphocytes also modulate the pro-inflammatory status of macrophages, which largely forms the basis of tissue transition from a pro-inflammatory state to one of repair [419]. Given the interdependent functional relationship between macrophages and T-lymphocytes, it is conceivable that suppression of one cell type would ultimately affect the other.

4.0 Limitations of Whole-Cell Approach

In the present study, we used whole-cell patch clamp in order to directly evaluate α_2AR-mediated inhibition of Ca^{2+} channels in dissociated SMCG neurons of DOCA-salt and obesity-related hypertension. Whole-cell approaches are used to study α_2AR signaling in nerve terminals [124, 125, 338, 420], which are too small for direct electrophysiological recordings. While this approach is beneficial as it allows investigating pre-junctional α_2AR responses independent of post-junctional α_2AR responses, our results need to be interpreted in light of several considerations.

We evaluated α_2AR function in the cell bodies of SMCG neurons while hypertension-related changes occur in nerve terminals. In our studies, we showed that SMCG inflammation was associated with α_2AR dysfunction in SMCG neurons. Sympathetic neurons in prevertebral ganglia are affected in models where inflammation occurs in distal target tissues [421, 422]. However, we cannot be sure that our findings occur at the nerve terminal using data from our assay alone. In DOCA-salt hypertension, these α_2AR deficits have been verified in mesenteric artery preparations using nerve stimulation and amperometric measurements of NE release [127]. These experiments will be performed in our obesity-related hypertension models in the near future.

Furthermore, analysis of α_2AR function in dissociated SMCG neurons occurs hours after tissues are removed from the rat and incubated in artificial supportive media. Therefore, the damage sustained to the α_2AR in our hypertension models likely needs to be severe or permanent enough to be detected by our assay. It is possible that α_2AR dysfunction occurs in obesity-related hypertension but this impairment is able to recover when SMCG neurons are

cultured. Therefore we can only suggest that based on our results, α_2AR dysfunction does not seem to play a role in obesity-related hypertension development. Ultimately, these findings will need to be verified using *in vivo* approaches.

5.0 Other Considerations of α_2AR Dysfunction in DOCA-Salt Hypertension

This work has largely focused on prejunctional α_2AR function with applications on nerve terminal NE release from SMCG neurons onto mesenteric arteries. It is possible that α_2AR dysfunction occurs in other tissues and systems that may also contribute to DOCA-salt hypertension development.

Impairment of endothelial α_2AR. Activation of endothelial α_2ARs causes vasodilation through increased NO release onto vascular smooth muscle [423, 424]. In rat mesenteric arteries, this occurs independently of cAMP as treatment with forskolin (inhibitor of cAMP production) does not attenuate endothelial α_2AR-mediated NO release [425]. However, this effect is inhibited by PTX treatment, suggesting that coupling to $G\alpha_{i/o}$ proteins activates NO synthase in the vascular endothelium [426, 427]. Activation of endothelial α_2ARs has been shown to reverse endothelial dysfunction during reperfusion injuries [428, 429] and may also serve in counteracting increased vascular permeability from cytokine-related disruption of tight junctions, which can leads to enhanced immune cell infiltration [430-433]. Therefore, endothelial α_2ARs provide a mechanism that counteracts vasoconstriction from post-junctional α_1/α_2 AR activation and vascular immune cell infiltration. It is possible that like SMCG neurons, α_2AR dysfunction might also occur in the endothelium of DOCA-salt treated rats, which would additionally contribute to hypertension development/maintenance as well as vascular injury.

Impairment of central α_2AR. Activation of central α_2ARs (primarily through the α_{2A} and α_{2C} subtypes) results in sustained decreases in blood pressure associated with SNA suppression [434, 435]. Interestingly, from antisense gene knock down studies, activation of the α_{2B} subtype

appears to cause salt-sensitive hypertension [436]. However, α_{2A} is the most abundant subtype expressed in cardiovascular control centers [437] and likely plays a larger role than α_{2B} on SNA regulation. Macrophage-associated neuro-inflammation occurs in the brain. This occurs via activation of microglia, which are specialized brain macrophages that release pro-inflammatory mediators in autonomic brain centers that are associated with increased glutamate-signaling and enhanced of SNA [285]. Even though clodronate liposomes do not easily pass through the BBB [438], we cannot rule out the possibility that DOCA-salt hypertension may also result from microglial-mediated impairment of central α_2ARs. Furthermore, it has been recently shown that in hypertension, activated bone marrow precursors contribute to an increase in microglia number and activation via MCP-1-mediated extravasation into the brain from CSF [284]. Therefore, it is possible that peripheral immune cells (which are susceptible to clodronate-liposomes) may influence activity in CNS cardiovascular centers in DOCA-salt rats.

Prejunctional α_2AR impairment on kidney function. The prejunctional α_2AR also regulates SNA to the kidney. Enhanced SNA to the kidney affects blood pressure through increased renin release (enhancing RAAS) and reabsorption of Na^+ and water. Although renal SNA studies have yielded mixed results, some studies suggest that DOCA-salt hypertension results from enhanced renal SNA that is associated with increased chemokine ligand, MCP-1 and interleukin-6 (IL-6) [229]. We did not consider the effects of α_2AR dysfunction or macrophage depletion on renal mechanisms of blood pressure regulation. However, these mechanisms are important given that hypertension was induced specifically by administering mineralocorticoid and salt. Considering the profound renovascular macrophage-related inflammation that occurs in DOCA-salt treated rats, it is also likely that clodronate treatment in

our study affected kidney function. Therefore, our findings that macrophage-depletion protected against blood pressure in DOCA-salt treated rats are likely not attributed only to decreased sympathetic drive to mesenteric arteries, and likely involve other organ systems that include the kidney.

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