MACROPHAGE-DERIVED SUPEROXIDE DISRUPTS PERVASCULAR MESENTERIC ARTERIAL SYMPATHETIC NERVES IN A RAT MODEL OF DOCA-SALT HYPERTENSION

By

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ABSTRACT

MACROPHAGE-DERIVED SUPEROXIDE DISRUPTS PERVASCULAR MESENTERIC ARTERIAL SYMPATHETIC NERVES IN A RAT MODEL OF DOCA-SALT HYPERTENSION

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Major risks for stroke, heart, and kidney diseases have been linked to hypertension in more than 65 million Americans. Hypertension is a multi-organ disease that involves changes in nervous and immune system function. Sympathetic nerve activity is elevated in some hypertensive humans and in some animal models of hypertension, including the DOCA-salt model. DOCA-salt hypertension in rats is associated with the impairment of a α_2R function, and increased level of O_2^- and MΦ number in the MA adventitia. However, the relationships between MΦ infiltration, O_2^- production and α_2R impairment are unknown. This dissertation tested the hypothesis that as blood pressure increases in DOCA-salt rats, MΦ infiltrate into the adventitia of MA. MΦ then release O_2^- that disrupts α_2R function, causing an increase in NE release which further increases blood pressure.

A time-course study was used to determine the temporal relationship between impaired function of sympathetic nerve terminal α_2R and adventitial infiltration of pro-inflammatory MΦ in MA from DOCA-salt hypertensive rats. LEC was used to deplete adventitial MΦ. The results of these studies revealed that pro-inflammatory MΦ infiltration and increased O_2^- level in MA of DOCA-salt rats occurred after 10 days of

initial blood pressure increase, but α_2R impairment did not occur until a week after the infiltration of MΦ. Furthermore, LEC prevented the development of the later phases of DOCA-salt hypertension by blocking the infiltration of MΦ into the MA adventitia, reducing the O_2^- level in the MA, and preventing the α_2R dysfunction.

Focal nerve stimulation and amperometry with microelectrodes to measure NE oxidation currents at the adventitial surface of MA were used to elucidate the mechanism of α_2R function impairment. The results suggested that there is no alteration in the amount of NE release from the RRP vesicles, and in the activity of free $G_{\beta\gamma}$ function in tonic inhibition of voltage gated Ca^{+2} channels in DOCA-salt hypertensive MA. The α_2R function impairment occurs upstream from the $G_{\beta\gamma}$ protein. The balance between $G_{i/o}$ and G_s is shifted to the G_s, hence the increase in sensitivity of G_s and PKA activity in DOCA-salt hypertensive MA.

The novel aspect of this study is that it tested the hypothesis that MΦ-derived O_2^- disrupts α_2R function, which further contributes to the increase in blood pressure in DOCA-salt rats. Hypertension is a major public health concern. Therefore, clarifying the mechanism that leads to enhanced neurogenic vasoconstriction is important for new discoveries relevant to anti-hypertensive drug development.

DEDICATIONS

To my beloved wife KimHang whom I live, laugh and love with every moment of life together.

To my amazing son Isaiah whose smile outshines the sun and whose cheerfulness warms my heart.

To my respectful parent, Bay and Tuyet, who gave me the breath of life and taught me to be an indepdent thinker. (Đến bố mẹ kính mến của tôi, Bảy & Tuyết, người đã cho tôi hơi thở của cuộc sống và dạy tôi trở thành một nhà tư tưởng độc lập.)

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American Poet Haniel Long once said,

"*So much of what is best in us is bound up in our love of family, that it remains the measure of our stability because it measures our sense of loyalty.*"

Love and Gratitude

Loc Vinh Thang

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I feel it's only fitting to quote the great American Journalist, Dan Rather who once said, "*the dream begins, most of the time, with a teacher who believes in you, who tugs and pushes, and leads you onto the next plateau, sometimes poking you with a sharp stick called truth*."

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KEY TO ABBREVIATIONS

11β-HSD: 11β-hydroxysteroid dehydrogenase

20-HETE: 20-hydroxyeicosatetraenoic acid

3'UTR: 3' untranslated region

5-HT_{1A}: 5-hydroxytryptamine receptor 1A

ACTH: Adrenocorticotropic hormone

AME: Syndrome of apparent mineralocorticoid excess

Ang-II: Angiotensin II

APs: Aminopeptidases

AR: Adrenergic receptor

ASIC: Acid sensing ion channel

AT_1: Ang-II receptor type 1

AT_2: Ang-II receptor type 2

BMI: Body mass index

BP: Blood Pressure

CaMK II: Ca^{+2} calmodulin-dependent protein kinase II

CAPP: Ceramide-activated protein phosphatase

CHIF: Channel inducing factor

COX-2: Cyclooxgenase-2

CSP: Cysteine string protein

DAG: Diacylglycerol

DBP: Diastolic blood presure

DHE: Dihydroethidium

DM: Diabetes mellitus

DOPA: L-3,4-dihydroxyphenylalanine

DβH: Dopamine β-hydroxylase

ECE: Endothelin converting enzyme

EETs: Epoxyeicosatrienoic acids

EGFR: epidermal growth factor receptor

ELISA: Enzyme-linked immuno sorbent assay

ENaC: Amiloride-sensitive epithelial Na^+ channels

EO: Endogenous ouabain

EO: Endogenous Ouabain

EPHESUS: Epleronone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study

EPs: Endopeptidases

ERK: Extracellular signal-regulated kinase

ET-1: Endothelin-1

ET_A: Endothelin receptor type A

ET_B: Endothelin receptor type B

FAK: Focal adhesion kinase

G6PD: Glucose 6 phosphate dehydrogenase

GILZ: Glucocorticoid induced leucine-zipper protein

GIRK: G protein-coupled inwardly-rectifying potassium channels

GPCR: G-protein coupled receptor

GR: Glucocorticoid receptor

GRA: Glucocorticoid-remediable aldosteronism

GRK2: G-protein receptor kinase 2

INTERSALT: International Study of Salt and Blood Pressure

IP3: Inositol triphosphate

JG: Juxtaglomerular cells

JNK: c-jun N terminal kinase

KRH: Krebs-Ringers-HEPES

LEC: Liposomal-encapsulated clodronate

LHRH: Luteinizing hormone-releasing hormone

Lipo-PBS: Liposomal-encapsulated PBS

LPS: Lipoposaccharide

LTB4: Leukotriene B4

MA: Mesenteric arteries

MAP: Mean arterial pressure

MAPK: Mitogen-activated protein kinase

MnPO: Median preoptic nucleus

MR: Mineralocorticoid receptor

MR: Mineralocorticoid receptors

MΦ: Macrophages

NADPH: Nicotinamide adenosine dinucleotide phosphate

NE: Norepinephrine

Nedd4-2: Neural precursor cell-expressed, developmentally downregulated gene 4 isoform 2

NEP: Neprilysin

NET: NE transporter

NFκB: Nuclear factor kappa B

nNOS: Neuronal nitric oxide synthase

NO: Nitric oxide

NOS-1: Nitric oxide synthase 1

NR3C2: Nuclear receptor subfamily 3, group C, member 2

NSF: N-ethylmaleimide sensitive factor

NTS: Nucleus tractus solitarius

O_2^-: radical superoxide anion

OSAS: Obstructive sleep apnea syndrome

OVLT: Organum vasculosum of the lamina terminalis

PAI-1: Plasminogen activator inhibitor-1

PBS: Phosphate buffer saline

PDGF: Platelet-derived growth factor

PGE_2: Prostaglandin E_2

PKC: Protein kinase C

PLC: Phospholipase C

PTH: Parathyroid hormone

PTX: Pertussis toxin

RAAS: Renin Angiotensin Aldosterone System

RALES: Randomized Aldactone Evaluation Study

RAP: Right atrial pressure

ROS: Reactive oxygen species

RP: Reserved pool

RRP: Readily releasable pool

RVLM: Rostral ventrolateral medulla

SBP: Systolic blood pressure

SFO: Subfornical organ

SGK1: Serum-and glucocorticoid-regulated kinase 1

SGT: Small glutamine-rich protein

SHR: Spontaneously hypertensive rats

SNA: Sympathetic nervous system activity

SNAP-25: Synaptosomal associated protein of 25 kDa

SNAPs: Soluble NSF adaptor proteins

SNARE: SNAP receptor

SR: Salt resistant

SS: Salt sensitive

TGF-β: Tissue growth factor β

TNF-α: Tumor necrosis factor alpha

TPR: Total peripheral resistance

TRP: Transient receptor potential channel

VEGF: Vascular endothelial growth factor

VMAT: Vesicular monoamine transporter

WNK: With-no-lysine kinase

α_{2A}R: α_{2A}-adrenergic receptor

α_2R: α_2-adrenergic receptor

CHAPTER 1

GENERAL INTRODUCTION

Hypertension: An Epidemiological Perspective

Blood pressure is a measurement of the pressure exerted against the arterial walls as the heart pumps blood through the body. Hypertension is the term used to describe high blood pressure. There are 65 millions Americans with hypertension (1). This is a major health concern because hypertension is major risk factor for heart disease and stroke, which are the leading causes of death in the United States. In fact, for every 20 mmHg systolic or 10 mmHg diastolic increase in blood pressure there is a doubling of mortality from both ischemic heart disease and stroke (2). In the world, the prevalence of hypertension in adults is estimated at approximately 1 billion individuals, and approximately 7.1 million deaths per year may be attributable to hypertension (3). The prevalence of hypertension increases with advancing age; recent study showed that the lifetime risk of hypertension is about 90% for men and women who were non-hypertensive at age 65 years and survived to age 85 (4). DBP is a stronger cardiovascular risk factor than systolic blood pressure until age 50 (5); thereafter, SBP is more important.

Hypertension is defined as sustained SBP ≥ 140 mmHg or DBP ≥ 90 mmHg. Table 1 provides a classification of BP for adults (6). Pre-hypertensive individuals are those who have high risk of developing hypertension and are advised to modify their lifestyle in order to reduce the risk of developing hypertension in the future. The overall goal of hypertension management is to lower BP. Pre-hypertensive individuals should achieve this via lifestyle changes, and for all people with hypertension should be treated with drug therapy in addition to lifestyle modifications.

Table 1: Blood Pressure Classification

Blood Pressure Classification	Systole Blood Pressure	Diastole Blood Pressure
Normal	< 120 mmHg	and < 80 mmHg
Pre-hypertension	120-139 mmHg	or 80-89 mmHg
Stage 1 hypertension	140-159 mmHg	or 90-99 mmHg
Stage 2 hypertension	≥ 160 mmHg	or ≥ 100 mmHg

There are two categories of hypertension. Essential hypertension is high BP that has no identifiable causes, with it's the most common form, affecting about 95% of hypertensive individuals (7). High BP that is caused by another medical condition or medication is called secondary hypertension. Secondary hypertension may be due to chronic kidney disease, coarctation of the aorta, pheochromocytoma, Cushing's syndrome, hyperparathyroidism or medication such as birth control, diet pills, and some cold and migraine medications (8). A new class of hypertension is resistant hypertension. It's defined as the persistence of BP above 140/90 mmHg despite the treatment of three or more anti-hypertensive drugs from different classes at full concentration, one of which is a diuretic (9).

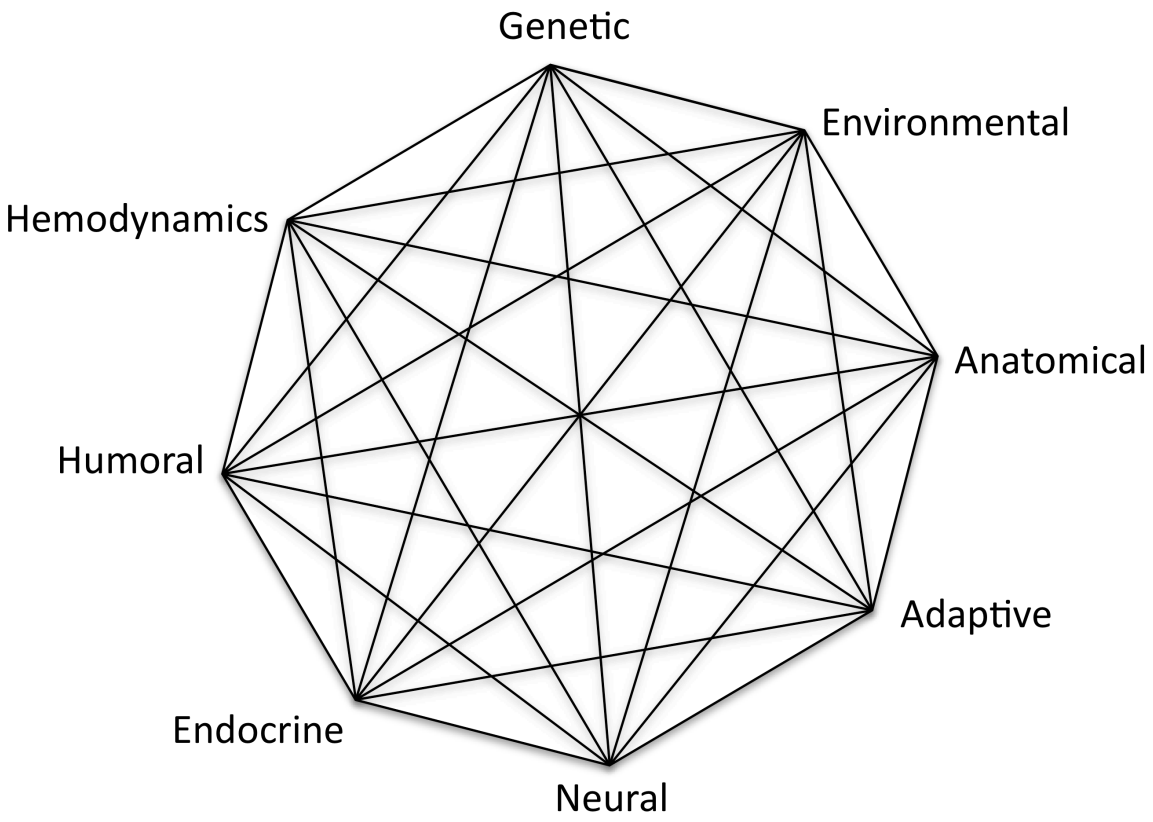
Considerable success has been achieved in the last five decades. The median SBP for individuals ages 60-74 declined by approximately 16 mmHg between 1960-1991 (10). Better treatment of hypertension has been associated with a significant reduction in the hospital case-fatality rate for heart failure. However, 30% of adults are still unaware of their hypertension and greater 40% of individuals with hypertension are not treated (6). Furthermore, the prevalence of resistant hypertension is increasing, and no new anti-

hypertensive drugs have been introduced in the last two-decades. Although the research in the past decades has significant impact in the treatments of hypertension, more research is still needed.

Blood Pressure Regulation: The Mosaic Theory of Hypertension

Hypertension is a complex medical condition with multi-factorial contributors. Dr. Irvine Page was one of the first pioneers who recognized and proposed that multiple “forces” interdigitate to cause hypertension. He later called this The Mosaic Theory of hypertension (Fig. 1), which states that the etiology of most cases of human hypertension is multi-factorial including genetics, environment, anatomical, adaptive, neural, endocrine, humoral and hemodynamics (11). In this theory, the crisscross of lines indicates that when a factor changes, some other must change concurrently to maintain the equilibrium and consequently the level of BP. While, the Mosaic Theory of hypertension is basic, it serves as a good framework for future studies to further understand the complex nature of the pathophysiology of hypertension.

Figure 1: The Mosaic theory proposed by Irvine Page M.D. in 1967



The etiology of most cases of human hypertension is multi-factorial, including genetics, environment, anatomical, adaptive, neural, endocrine, humoral, and hemodynamics.

Genetics of Hypertension

First-degree relatives of hypertensive indivuduals have twice the risk of developing hypertension compared to the general population, and the risk increases to four-fold when two or more family members have hypertension (12). Although BP has significant heritability (30-60%), the genes conferring susceptibility to hypertension remain largely unknown because hypertension is a heterogenous disease with multiple phenotypic and genotypic subtypes (13). The genetic determinants of hypertension are polygenic with each gene having only a small effect on the BP. However, the additive effect of these genes results in hypertension. Despite this, there are few cases of monogenic forms of human hypertension (Table 2). The most common form of monogenic human hypertension is GRA, an autosomal dominant disorder characterized by overproduction of aldosterone. People with GRA have two normal copies of aldosterone synthase gene and have moderate to severe hypertension. Cortisol, amiloride and spironolactone are effective treatments to lower BP in GRA (14). Various abnormalities of hydroxylase enzymes including 11β-hydroxylase and 17α-hydroxyase deficiencies cause hypertension in human. 11β-hydroxylase deficiency causes hypertension and hypokalemia because impaired conversion of 11-deoxycorticosterone to corticosterone results in the accumulation of 11-deoxycorticosterone, a potent mineralocorticoid.

Mutation causing 11β-hydroxylase deficiency cluster in exons 6-8 of the CYP11B1 gene (15). Similar to 11β-hydroxylase deficiency, 17α-hydroxyase deficiency is characterized by hypokalemia and hypertension. This disorder causes decreased and increased cortisol and 11-deoxycorticosterone production respectively. A large number of random mutations can cause 17α-hydroxyase deficiency (16).

Table 2: Monogenic Forms of Human Hypertension

Disorder	**Genes Alteration**
Glucocorticoid-remediable aldosteronism	Aldosterone synthase is under the control of CYP11B1 promoter, so aldosterone is also synthesized in *zona fasiculata*
11β-Hydroxylase, 17α-hydroxyase deficiencies	CYP11B1; CYP17
Hypertension exacerbated in pregnancy	Mineralocorticoid receptor
Liddle's syndrome	β or γ subunit of ENaC genes
Syndrome of apparent mineralocorticoid excess	11β-Hydroxysteroid dehydrogenase gene
Pseudohypoaldosteronism type II	With-no-lysine kinase (WNK)

Pregnancy-related hypertension is a serious health concern to the mother and baby. A S810L mutation in the MR causes early-onset hypertension and markedly exacerbated during pregnancy (17). This mutation results in constitutive MR activity, with progesterone--normally a MR antagonists--becoming a potent agonists. Liddle's syndrome, an autosomal dominant disorder, is characterized by excess sodium retention, low potassium and plasma-renin activity. The problem in Liddle's syndrome results from constitutive active ENaC on distal renal tubules. The defects have been localized to gene mutations on chromsome 16 that encode for β and γ subunits of ENaC (18). Cortisol and aldosterone can equally activate MRs *in vitro*; however, aldosterone is the primary activator of renal MR *in vivo*. Normally in the kidney, cortisol is metabolized to cortisone by 11β-HSD2, which prevents cortisol from activating MR. In states of 11β-HSD2 deficiency like that in AME, cortisol can reach and activate type I renal MR,

causing sodium retention, suppression of the RAAS and hypertension (19). Lastly, in the familial hypertension of pseudohypoaldosteronism type II, there are mutations in the genes encoding the WNK family causing overactivity of the thiazide-sensitive Na^+/Cl^- co-transporter in the distal nephron (20).

Environmental Factors in the Development of Hypertension

Genetic susceptibility accounts for much of the variation in blood pressure within population studies. However, environmental factors determine the variation in mean blood pressure between populations. Some of the most important environmental factors in the development of hypertension at the population level are excess calories intake, high sodium intake, low potassium intake, physical inactivity, heavy alcohol consumption, and psychosocial stress (21).

Obesity and Hypertension

Obesity is clinically defined as BMI ≥ 30 kg/m^2. In prospective studies, obesity has been shown to be an independent predictor of subsequent hypertension and weight reduction was an effective way to lower blood pressure (22). While obesity is strongly linked to hypertension, the causal relationship is not as clear. In fact, BMI alone is less tightly associated with cardiovascular disease risk than visceral fat mass. Using computer tomography, it is shown that visceral fat is much more metabolically active compared to subcutaneous fat. The mechanisms by which obesity and body fat distribution lead to higher BP are not well understood. However, increased fat distribution is usually associated with insulin resistance, which may contribute to the development of

hypertension (23). Multiple mechanisms have been proposed to explain a possible relationship between insulin resistance and hypertension, including increased sympathetic nervous activity, vascular smooth muscle hypertrophy, alteration of cation transport, and salt sensitivity (24). The relationship between insulin and BP is complex. Short-term insulin infusion raises catecholamines but not BP; and it causes vasodilation in humans and dogs (25). While the effect of chronic hyperinsulinemia is not known, ecological data do not support the relationship between insulin and hypertension. For example, Pima Indians and Mexican Americans have high rates of type 2 DM, hyperinsulinemia, and insulin resistance and yet have a lower prevalence of hypertension (26). Although, insulin resistance and hyperinsulinemia may be an important factor in the link between obesity and hypertension, the precise mechanisms have not been established. Perhaps, it is time to look at adipocytes from a different perspective. Recently, research has shown that adipose tissue functions not as a passive fat storage, but rather as an endrocine organ, producing factors that can affect appetite, energy storage, insulin signaling and sensitivity, inflammation, and vascular function (27).

Sodium and Hypertension

The relationship between sodium intake and BP has been extensively examined in adults and children by epidemiologic observations and experimental studies. For example, in the INTERSALT study that was conducted with 10,000 participants in 52 population samples and 32 countries demonstrated a highly significant relationship between sodium intake and BP as well as with increase in BP with age (28). Several meta-analyses of interventional studies of sodium intake in humans have demonstrated

significant but small reduction in BP (29). The magnitude of the BP reduction may be explained by the variation in the study. Early exposure to high sodium may be critical in the initiation of hypertension later in life. In a randomized study, sodium restriction in neonates has been shown to associate with lower blood pressure (30). In many sodium-loading studies in human, a progressive and significant rise in BP was observed when comparing the BP at the end of the study to the initial period of low sodium intake. In these studies, MAP of some individuals was increased by 5 mmHg, whereas others had a 35-mmHg increase (31). These observations suggest a difference in susceptibility to the BP-raising effect of sodium in the population. In fact, individuals with BP increases or decreases significantly in response to a positive or negative salt balance exhibited salt-sensitivity.

Salt-Sensitivity

The prevalence of salt-sensitivity increases in African American, elderly, obese, diabetes, and chronic renal failure populations. Salt-sensitive individuals have a worse cardiovascular prognosis than SR individuals, regardless of BP level (32). Linkage studies of gene polymorphisms about BP responses to salt support the genetic determination of SS hypertension, including studies about haptoglobin, adducin, α_2 and β-adrenergic receptors, β subunit of ENaC, angiotensinogen, and CYP4a11 (33). The best evidence of genetic determinant of SSBP comes from inbreeding rodents for SS including Dahl-SS, Milan-SS, and Sabra-SS rats. Abnormalities in renal regulatory mechanisms of salt and water can cause SS hypertension in animals (34). When the SR animals are given a salt-load, depressor and natriuretic mechanisms are activated

while pressor and antinatriuretic ones are simultaneously inhibited resulting in no change in BP. However, SS hypertension may result from abnormal responses to the depressor/natriuretic mechanisms and/or pressor/antinatriuretic mechanisms. Salt loading inhibits and salt deprivation stimulates RAAS, which prevent large changes in BP during salt intake. Keeping RAAS at very low or high level of Ang-II or aldosterone produces SS hypertesion in animals. ET-1 production is increased in the endothelium and the kidney in salt-dependent models of hypertension (35). ET-1 is a potent vasoconstrictor, a stimulator of cell growth and an elicitor of inflammatory responses by increasing oxidative stress in the vascular wall; it induces vascular remodeling and endothelial dysfunction. Antioxidant tempol attenuates the hypertension in the SHR, which supports that there is an imbalance between oxidant and antioxidant in SS hypertension. The production of nNOS-derived NO is increased in high salt condition. NO is a powerful vasodilator and a regulator of renal blood flow and natriuresis. Inhibition of nNOS leads to SS hypertension (36). With these combined factors, O_2^- generate during oxidative stress can deplete NO and lead SS hypertension. Salt loading increases the SNA and high SNA can impact renal and systemic hemodynamics, volume homeostasis and BP levels in SS hypertension (37). Vast amount of evidences support arachidonic acid products: cyclooxygenation, epoxygenation, and ω-hydroxylation as a major role in SS hypertension. Inhibition of cyclooxygenase 1 prevents the production of PGE_2, a vasodilatory and natriuretic active compound, in response to salt loading and BP becomes SS. Possible explanation for this is PGE_2 plays role in removing ENaC from the cell membrane of renal tubule cells for

proteasome recycling. Likewise, cyclooxygenase 1 knockout mouse and SS hypertensive individuals have a common feature of impairment BP reduction during sleep cycle. Salt loading increase the level of EETs, the products of epoxygenation of arachidonic acid, which inhibit distal sodium reabsorption altering the gating properties of ENaC. Reduction of EETs synthesis produces SS hypertension. 20-HETE, the major product of ω-hydroxylation of arachidonic acid, can reduce BP. Perhaps, this happens through the blockade of potassium channels, thereby inhibiting the action of $K^+/Na^+/2Cl^-$ co-transporter and Na^+/K^+ ATPase in the kidney. There is strong evidence that diminished level of renal 20-HETE is linked with SS hypertension (38). Like hypertension, SS hypertension is a complex medical condition with multi-factorial contributors.

Potassium and Hypertension

Many studies have shown that potassium intake is inversely related to systolic and diastolic blood pressure (39). In fact, in many of salt loading studies in humans, it was observbed that the net potassium loss occurred when sodium intake increased. In the same studies, when potassium was replaced, the sodium-induced hypertension was reduced compared to experiments without potassium supplement (31). Some of the proposed mechanisms of how potassium lowering BP are natriuretic effects, RAAS and SNA suppression, arterial vasodilation, baroreceptor function improvement, and effect on eicosanoids levels.

Physical Inactivity and Hypertension

The British Regional Heart Study of over 7000 middle-aged men found that there was significant inverse relationship between amount of physical activity, BP level and cardiovascular events (40). This association was independent of age, BMI, social class, smoking status, and lipid profile. There are numbers of suggested mechanisms linking physical activity and lower BP, including effects on body weight and insulin sensitivity, SNA and baroreceptor function, and vascular structure. Therefore, physical activity is an effective anti-hypertensive therapy; 150 minutes per week of moderate-intensity aerobic activity is recommended (6).

Alcohol Consumption and Hypertension

Cross-sectional epidemiologic studies have shown a direct relationship between excess alcohol intake (> 28g of ethanol/day, ~ 2, 12 oz of beer) and hypertension (41). Randomized trials and meta-analysis have shown that reduction in alcohol intake is associated with lowering BP (42). Each reduction by one drink per day reduces SBP and DBP by approximately 1 mmHg (43). Some of the proposed mechanisms of the hypertensive effects of alcohol are increase RAAS activity and SNA, depletion of NO, Ca^{+2} and Mg^{+2}, increase acetaldehyde, endothelin, and intracellular Ca^{+2} in vascular smooth muscle (44). Due to the negative effects of alcohol on BP and other health/psychosocial functions, it is recommended by health advisors to limit the maximum average intake of 2 drinks/day for men and 1 drink/day for women (6).

Psychosocial Stress and Hypertension

Stress is defined as a situation perceived as an uncontrollable threat to the individual's well being (45). Acute stress that occurs for example during fear or anxiety can cause

rapid, large and transient increase in BP and heart rate. The role of chronic stress in contributing to the development of hypertension is not clear. However there is evidence that stressful situations like a stressful job or living in poverty are associated with hypertension (46). People who work in high demands and low control jobs have elevated BP not only during the time at work, but also while at home during sleep. The mechanisms for how stress increases BP are not clear. Perhaps, stress increases the SNA or altering the levels of corticotropin hormones.

Hemodynamics in the Development of Hypertension

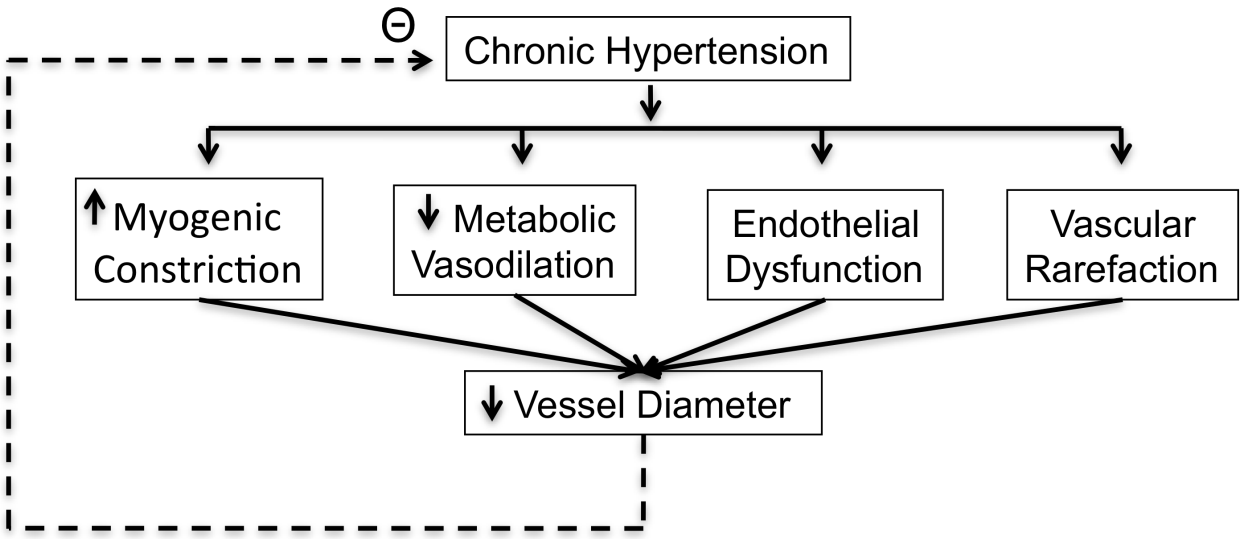
Contractions of the left ventricle propel blood into the systemic circulation that begins at the aorta and flows to the large arteries, small arteries, arterioles, capillaries, venules, veins and back to the right atrium. The aorta and large arteries have high elastic property, therefore they distend and recoil between each ventricular contractions providing a pulsatile pressure that drives blood further away from the heart. The small arteries and arterioles are known as the resistance vessels because of their thick smooth muscle walls that can contract and relax. They provide most of the total vascular resistance in the body. Capillaries are small and have thin walls, which allow for rapid exchange of nutrients and waste products. For this reason, they are called exchange vessels. Lastly, the venules and veins have larger diameters and thinner walls therefore they can hold a large amount of blood. These vessels are referred to as capacitance vessels. Compliance (C) = $\Delta V/(P_{inside} - P_{outside})$. Because the venous side of the systemic circulation is approximately 20 times more compliant than the arterial side, the drop in volume on the venous side has little effect on its pressure.

However, an equivalent increase in volume on the low compliant side, the arterial side, causes a 20-fold larger change in the arterial pressure. The left ventricle contractions create a pressure gradient in systemic circulation, which allows for the flow of blood. Although it does not exactly describe the blood flow through elastic, tapering blood vessels, Poiseuille's law ($Q = \Delta P \pi r^4 / 8\eta L$) can be used to understand blood flow. The flow (Q) is proportional to the pressure gradient (ΔP) between the two ends of the tube and inversely proportional to the TPR ($TPR = 8\eta L / \pi r^4$, r is the radius of the tube, L is its length, η is the viscosity of the fluid, and π is a geometric constant). In the body, changes in the radius of the small arteries and arterioles are responsible for most of the changes in the TPR, and flow (Q) is defined as cardiac output (CO) and ΔP = MAP – RAP where MAP is normally close to zero; Thus CO = MAP/TPR

Early hemodynamic changes in hypertension often include increased CO with normal TPR. However, in 60% of chronic human hypertension, TPR is increased and the elevation of TPR is uniformly distributed throughout the body (47). Although the TPR is increased in most patients with sustained hypertension, CO and blood flow in most tissues remain relatively normal. The local regulatory mechanisms including myogenic constriction, metabolic vasodilation, and endothelial-mediated relaxation may play a role in maintaining normal blood flow during increased BP (Figure 2) (48). Limited observational studies under the conjunctiva and nailfold indicate that the capillaries are narrowed and sparse in distribution. Many of hypertensive patients exhibit increased vascular reactivity to stressful stimuli and decreased responses to vasopressor agents. For example, exercise-induced vasodilation is impaired in essential hypertension (49).

There is also evidence that capacitance vessels may be contracted in chronic hypertension, which results in the redistribution of blood from the venous to arterial side and increased MAP (47). Moreover, hypertension is associated with the loss of arterial distensibility, which results in higher pulse pressure that is transmitted farther into the peripheral circulation.

Figure 2: Control mechanisms responsible for autoregulation in non-renal tissues during the development of hypertension



To maintain constant blood flow, myogenic tone is increase during high BP. Increased blood flow removes the metabolic vasodilators. High BP results in endothelial dysfunction and decrease vasodilation. Chronic hypertension leads to vasoconstriction and rarefaction.

Anatomical Factors in the Development of Hypertension

Coarctation of the Aorta

Aortic coarctation is a congenital condition whereby the aorta narrows to an abnormal width. There are three different types of hypertension associated with aortic coarctation: prerepair hypertension, postrepair paradoxical hypertension and late postrepair hypertension (50).

Mechanical, neural and renal artery stenosis are three main theories used to explain prerepair hypertension in aortic coarctation. Mechanical theory proposes that the increased BP proximal to the narrowed segment is because of high impedance to left ventricular emptying. The neural theory proposes that hypertension is the result of readjustment of the baroreceptors in the aortic arch, such that an increased proximal pressure becomes necessary to ensure an adequate blood flow to distal organs. Lastly, the renal artery stenosis theory explains that the narrowed aorta causes low renal perfusion, which stimulates RAAS activity and impairs salt/water homeostasis (51).

Postrepair paradoxical hypertension occurs during the first week after surgical repair of the coarctation. It has been postulated that loss of cardiopulmonary baroreflex afferent fibers lead to the activation of SNA and RAAS. Balloon angioplasty and β-blocker pretreatment can prevent postrepair paradoxical hypertension (52).

Late postrepair hypertension occurs much later after the surgical repair of the coarctation. It often shows in the form of upper extremity hypertension with treadmill exercise but not with arm exercise. There is an increased vascular reactivity to exogenous NE in the arm, normal vascular reactivity in the legs, and abnormal

aortocarotid baroreceptor activity. Different patterns of arterial remodeling, either caused by the narrowed aorta or differential postsurgical vascular remodeling in upper and lower extremity, may be the cause of postrepair hypertension (53).

Obstructive Sleep Apnea Syndrome and Hypertension

OSAS is defined as ≥ 15 apneas or hypopnea/hour of sleep plus daytime sleepiness. OSAS is associated with pulmonary and systemic hypertension, myocardial infarction, stroke and metabolic disorders (54). Peripharyngeal fat deposition, enlargement of the soft palate or tongue, craniofacial abnormalities and loss of respiratory muscle tone can cause partial or full collapse of the airway during sleep. OSAS triggers increase in SNA, and augmented SNA can be seen during sleep and during wakefulness when breathing is stable (55). High SNA increases RAAS activity and hypertension in individual with OSAS. Excessive aldosterone release may contribute to drug-resistant hypertension in these individuals (56). Suppression of RAAS activity by increasing salt intake can prevent the rise in BP of OSAS individuals. Chronic intermittent hypoxia in rats and in humans with OSAS can cause oxidative stress and vascular dysfunction specifically endothelium-dependent vasodilation impairment (57). Lastly, vascular remodeling is also documented in people with OSAS such as carotid intima media thickness, and arterial stiffness. The proposed mechanisms for the vascular remodeling include hypoxic stimulation of mitogenic factors such as ET-1, PGF, and VGEF (58).

Renal Stenosis and Hypertension

Renovascular hypertension is high blood pressure due to the narrowing of renal arteries or renal artery stenosis. Atherosclerotic disease of the renal artery, fibromuscular

dysplasia and narrowing at the anastomotic site of a transplanted kidney are some of the most common causes of renal artery stenosis. In animals, the two-kidney/one-clip (Goldblatt), the two-kidney/two-clip, and the one-kidney/one-clip are experimental models for renovascular hypertension. Initially, the narrowing of renal arteries decreases renal perfusion, which increases the activity of the afferent nerves, the baroreceptors, and the chemoreceptors in the kidney; together they lead to the activation of RAAS. Increased level of Ang-II promotes renal vasoconstriction and salt/water retention that result in the expansion of the extracellular and intravascular compartments. Volume expansion increases cardiac pre-load and stroke volume, which restores poststenoic perfusion pressure. Chronically, salt and water balance is restored but systemic BP is higher. Activation of renal chemoreceptors, baroreceptors and afferent nerves increase SNA and BP (59, 60).

Endocrine and Hypertension

Mineralocorticoid

Aldosterone and its precursor 11-deoxycorticosterone are the mineralocorticoid in humans. Aldosterone is mainly synthesized by the *zona glomerulosa* of the adrenal cortex in response to Ang-II, ACTH, and hyperkalemia. The principal epithelial cells of the renal cortical collecting duct have been considered as the main cellular target of aldosterone. Under physiological conditions, glucocorticoid --cortisol--is approximately 1000 times more abundant than aldosterone and can also bind to MR; thus, the level of intracellular cortisol must be decreased in order for aldosterone to access MR. 11β-HSD2 enzymes confer this mechanism because they have high affinity for cortisol and

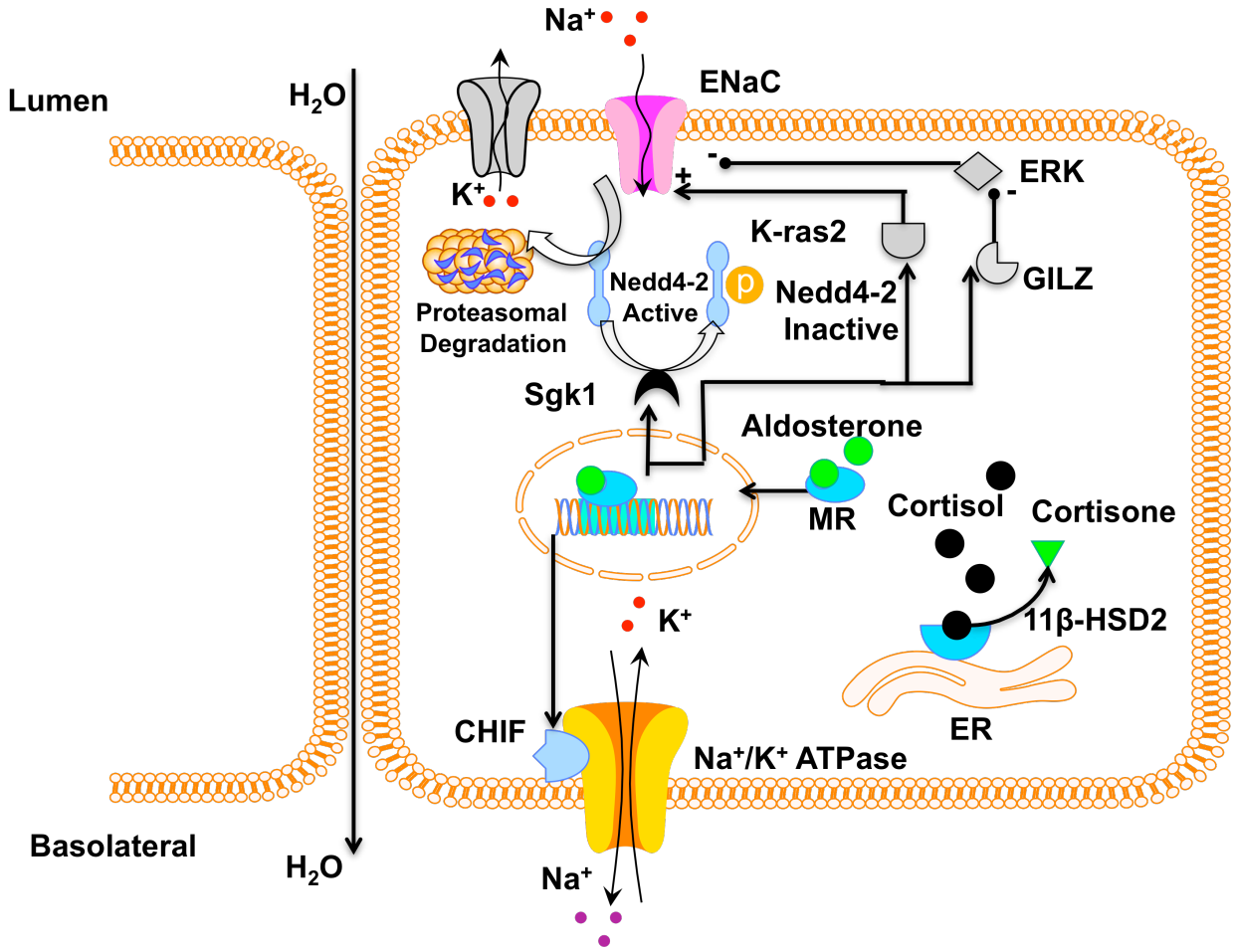
efficiently convert it to the inactive cortisone, which allow aldosterone to bind MR (61). If 11-HSD2 activity is reduced (licorice ingestion or by carbenoxolone administration), cortisol can activate MR and GR; in either case, it has indistinguishable effects on urinary electrolyte fluxes. The ability of GR to mimic the effects of MR suggests that the action of both receptors is mediated by a relatively nondiscriminating hormone response element. In addition to nondiscrimating hormone response element, MR also has specific mineralocorticoid response element. For example, MR activation by aldosterone leads to BP elevation; MR occupancy by corticosterone/cortisol, or by the MR antagonist RU28318, is without agonist effect but blocks the action of aldosterone. The findings that neither agonist nor antagonist effects via MR are mimicked or blocked by GR occupancy is interpreted as evidence for MR action via a specific mineralocorticoid response element. NR3C2, the systematic name of MR gene, encodes for a 107 KDa protein MR that shares a close homology with the GR (62).

Aldosterone Effects in Renal Epithelium

The putative genomic mechanisms of MR action are shown in Figure 3. Upon activation of MR by aldosterone, the receptor undergoes a conformational change and translocates into the nucleus where it functions as a transcriptional factor. MR activation leads to an enhanced activity of ENaC and Na^+/K^+ ATPase that results in sodium reabsorption and potassium excretion. MR activation increases the expression of SGK1, which results in the phosphorylation of ubiquitin-protein Nedd4-2 and consequently the inactivation of Nedd4-2. Thus, SGK1 activation results in decreased ubiquitin-mediated internalization and degradation of ENaC while an increased in its half-life at the plasma

membrane. Activated MR also up-regulates CHIF that enhances the affinity of Na^+/K^+ ATPase for Na^+ and K^+. Aldosterone has also been shown to induce the expression of the GTP dependent signaling molecule K-ras2, which enhances the opening probability of ENaC. The aldosterone-activated MR induced the expression of GILZ, and GILZ enhanced ENaC activity by antagonizing ERK signaling, a potent negative regulator of ENaC. Excess aldosterone induces hypertension due to volume expansion and activation of SNA. The most common cause of excess aldosterone is primary aldosteronism, which is also the most common form of secondary hypertension. However, mild elevations of aldosterone levels have also been shown in essential hypertension in humans (63).

Figure 3: Hormonal pathways of MR activation in renal cortical collecting duct cells. (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation)



11β-HSD2 converts cortisol into cortisone, which allows aldosterone to bind to the MR in renal cortical collecting cells. Aldosterone-induced Na^+ reabsorption by increases the activity Na^+/K^+ ATPase through the action of CHIF. It decreases ENaC degradation by the action of SgK1 and Nedd4-2, and enhances ENaC activity by stimulating K-ras 2 and GILZ. Overall, aldosterone increases the expression and activity of ENaC in renal cortical collecting cells.

Aldosterone Effects Outside Renal Epithelium

Besides the epithelial cells of the renal cortical collecting duct, aldosterone also works on non-epithelial tissues, such as the heart, the vessels, adipose tissue, and MΦ.

Aldosterone – Heart

The heart tissues produce aldosterone and express MR (64). Atrial MR expression is increased in patients with atrial fibrillation (65). It was suggested that aldosterone causes atrial fibrillation because it induces atrial cardiomyocytes hypertrophy, apoptosis and fibrosis. Spironolactone prevents atrial fibrosis and dilation in an experimental model of atrial fibrillation (66). Aldosterone-induced MR activation changes the intracellular Ca^{+2} signaling of cardiomyocytes and causes rhythm disorders by altering T-type Ca^{+2} channel expression, K^{+} and L-type Ca^{+2} channel, and ryanodine receptor activity (67). In fact, the RALES and EPHESUS showed that 50% of the benefits from blocking MR were related to the reduction of sudden death.

Aldosterone – Vasculature

In the blood vessels, aldosterone induces stress fiber formation and migration in smooth muscles by activating ERK, MAPK, c-Src, JNK, EGFR, and Rho-kinase (68). Furthermore, aldosterone increases aortic fibronectin expression without altering elastin and collagen density, which causes increased stiffness in large arteries (69). In fact, there is an inverse relationship between plasma aldosterone and large vessel compliance, independent of age and blood pressure, in hypertensive individuals. Interestingly, the effects of aldosterone on the cardiovascular system are dependent on

salt. For example, individuals with Gitelman or Bartter syndrome are characterized with high level of aldosterone and renal loss of sodium; they have normal BP and no vascular remodeling (70). Aldosterone also induces oxidative stress and inflammation in the vascular wall (71), endothelial cells, and vascular smooth muscles. In animal models, aldosterone infusion causes increased ICAM-1 (72), MCP-1, and TNF-α and infiltration of MΦ (73) and lymphocytes in the blood vessels. Aldosterone causes vascular oxidative stress by increasing NADPH-oxidase activity through c-Src activation (74). The increase in oxidative stress secondary to aldosterone infusion results in vascular hypertrophic remodeling and fibrosis. The generation of O_2^- by NADPH can reduce NO bioavailability and endothelial-dependent vasorelaxation in large and small arteries. However, aldosterone infusion also decreased vascular expression of G6PD, which may also play a role in endothelial dysfunction (75). Aldosterone can alter endothelium's electrolyte composition and increase endothelial cells' volume by affecting amiloride-sensitive sodium-proton exchanger (76). It also increases ET-1 expression in the blood vessels, which can cause inward hypertrophic remodeling of resistance arteries, vascular inflammation, and endothelial dysfunction (77). Recently, it has been shown that aldosterone exerts a synergistic effect with Ang-II and results in vascular smooth muscle proliferation and migration. What is interesting is that the effects of aldosterone and Ang-II exhibit a fast and slow non-genomic and genomic response. The genomic effects of aldosterone are all mediated via the activation of the MR whereas the non-genomic effects may be MR dependent or independent e.g. GPR30. In humans and animals, aldosterone injection is associated with an increased vascular resistance within 10 minutes (78). AT_1 and transglutaminase inhibitors prevent

this constrictive effect of aldosterone but not MR antagonist, which suggests the role of aldosterone in intracellular transglutaminase activity and AT_1 dimer formation independent of MR receptor (79). Similarly, aldosterone induces a rapid and concentration dependent increase in phospho-ERK in endothelium-denuded aortic rings, which was partly attenuated by the MR (eplerenon) and GPR30 (G15) antagonist (80). Lastly, aldosterone (independent of MR) has been shown to activate amiloride-sensitive Na^+/H^+ exchanger in vascular smooth muscles that causes increase intracellular IP_3 and Ca^{+2} levels (81).

Aldosterone – MΦ

MΦ expresses both MR and GR but not 11β-HSD2. Given that cortisol circulates at much higher concentrations than aldosterone, under normal circumstances the MR in MΦ would be occupied by cortisol (82). LPS stimulates the expression of GR but suppresses MR. This supports that GR is anti-inflammatory and MR is pro-inflammatory. Similarly, low concentration of corticosterone enhances peritoneal MΦ immune functions, whereas high concentrations are immune suppressive (83). The immuno-stimuatory effects produces by low dose of corticosterone are mediated via MR activity, whereas the immunosuppressive effects of high dose are produced by GR. Aldosterone enhances the expression of inflammatory and oxidative stress markers: $p22^{phox}$ and PAI-1 in isolated human MΦ (84). Treating ApoE-deficient mice with aldosterone increases oxidative stress in the MΦ (85). Blocking MR reduces MΦ

accumulation in a number of disease models, including peritoneal fibrosis, myocardial infarction, and vascular inflammation.

Glucocorticoid

Cortisol, the glucocorticoid in humans, is mainly synthesized by the *zona fasciculata* of the adrenal cortex in response to ACTH, and stress. Excess cortisol can increase BP as seen in Cushing's syndrome. The pathogenesis of glucorticoid-induced hypertension remains undetermined, although the increase in BP is independent of salt intake. Its been suggested that the following factors are involved in the pathogenesis of glucocorticoid-induced hypertension. Cortisol activates the RAAS by increasing circulating angiotensinogen. It also enhances NE and Ang-II pressor responses by altering number of receptors e.g. AT_1 receptor. However, it suppresses the depressor response of kallikrein-kinin systems and reduces the production of prostaglandins and NO (86).

Thyroid Hormone

Triiodothyronine (T_3) and thyroxine (T_4), the main thyroid hormones, are produced by the thyroid gland, which are primarily responsible for regulation of metabolism. T_4 is the major circulating thyroid hormone in the blood, and has a longer half-life than T_3. The ratio of T_4 to T_3 in the blood is roughly 20 to 1. The prevalence of hypertension in thyrotoxicosis is approximately 20%-30%. Systolic hypertension is the predominant finding because of the increase CO and cardiac contractility and decrease TPR. It also

been postulated that T_4 increases β-adrenergic receptors, which increases tissues e.g. the heart sensitivity to catecholamines. Similar to cortisol, thyroid hormones also activate RAAS by stimulate the synthesis and release of angiotensinogen (87). Unlike, thyrotoxicosis, hypothyroidism is associated with diastolic hypertension because of increased catecholamines and TPR (88).

Parathyoid Hormone

The chief cells of the parathyroid glands secrete PTH, an 84 amino acids long polypeptide. It acts to increase the concentration of Ca^{+2} in the blood. Excess PTH is associated with primary hyperparathyroidism (causes by adenoma or hyperplasia of the gland), pseudohypoparathyroidism (causes by resistance to PTH), or secondary hyperparathyroidism (causes by CKD). The prevalence of hypertension in primary hyperparathyroidism is approximately 10%-70% and in pseudohypoparathyroidism is approximately 40%-50%. The mechanisms for PTH-induced hypertension are not clear. Perhaps hypercalcemia increases free intracellular Ca^{+2}, which increases vascular smooth muscle contractility. However, individuals with pseudohypoparathyroidism have low Ca^{+2} but high PTH, suggesting that an increase in PTH itself may be responsible for hypertension. Many studies also show that RAAS activity is increased in hyperparathyroidism (89).

Humoral Agents in Hypertension

Studies of cross-circulation between hypertensive and normotensive animals reveal factors in blood that can increase BP. For example, when injecting plasma from dogs

with one-kidney Goldblatt hypertension or from essential or renovascular hypertensive patients into the jugular vein of a rat, the animal will slowly increases its BP (90, 91). Some of these humoral agents are endothelin, vasopressin, EO and members of RAAS.

Renin Angiotensin Aldosterone System

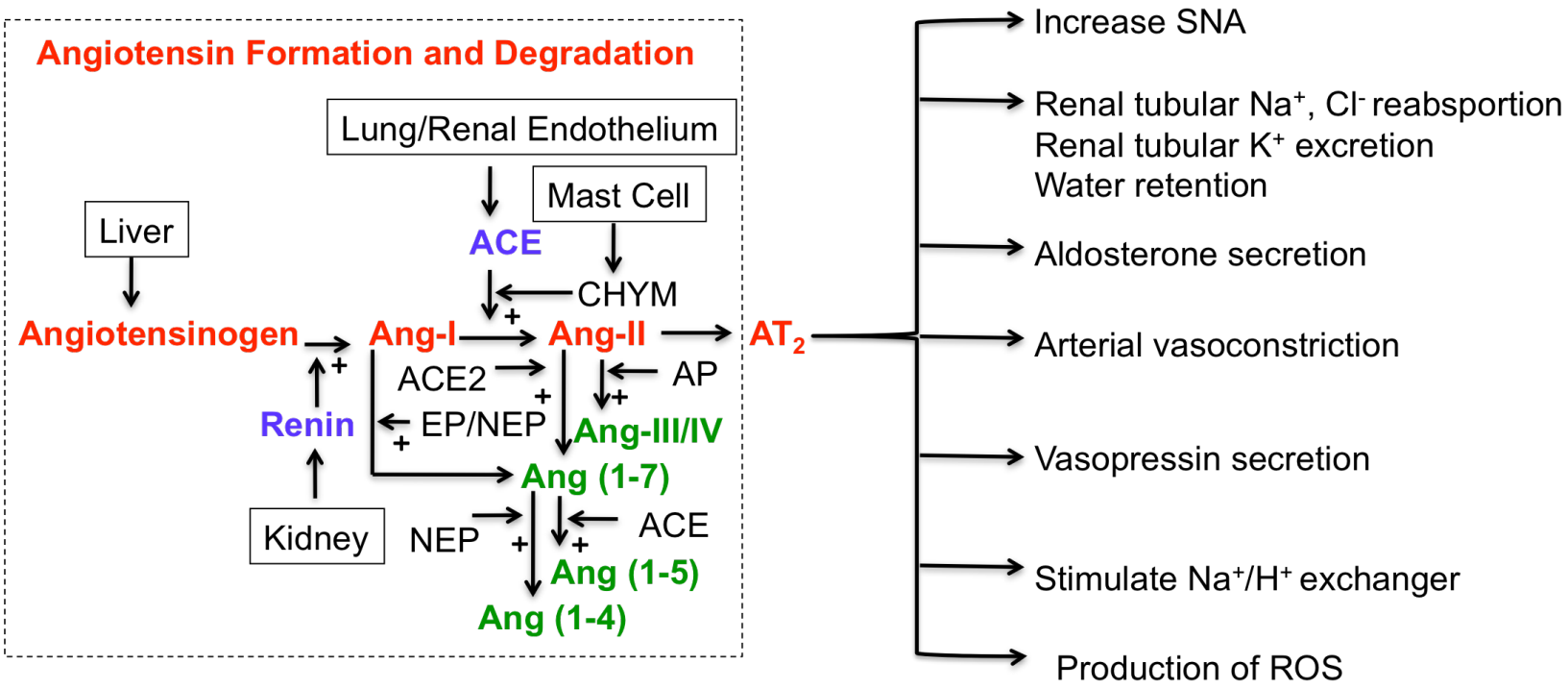
The renin angiotensin aldosterone system plays an important role in regulating blood volume and systemic vascular resistance, which together influence BP. There are three important components to this system: renin, angiotensin, and aldosterone. A schematic pathway and functions of RAAS is depicted in Figure 4. Renin is the rate-limiting step in the RAAS. It is an aspartyl proteolytic enzyme whose only known substrate is angiotensinogen. The JG cell in the kidneys is the primary location where renin is synthesized, stored and released. Renin is synthesized as a preprorenin (404 amino acids); in the rough endoplasmic reticulum, it is cleaved to prorenin (381 amino acids) which is transported to lysosomal granules and cleaved by cathepsin B to an active enzyme renin (339 amino acids). Prorenin is detected in circulation, and under basal conditions its concentration is 2-10 times greater than renin (92). It is also found in extra-renal tissues e.g. adrenal glands, pituitary, and submandibular glands. As an active area of curiosity, more research is needed to learn more about Prorenin. Sodium restriction and hormones e.g. androgens and T_4 have been shown to induce renin gene expression, whereas Ang-II suppressed renin expression (93). Renal intra-baroreceptors, chemoreceptors and renal sympathetic efferent nerves are the classic stimuli of renin release (94). On the one hand, reduction in renal perfusion pressure stimulates the JG cell's baroreceptors and increases its intracellular cAMP levels, which

triggers renin release. On the other hand, reduction in NaCl delivery to the macula densa stimulates the chemoreceptors and increases COX-2 production of PGE_2, which acts on the JG cells through PGE-4 receptor to increase cAMP production and consequently renin release (95). In addition to stimulating COX-2, chemoreceptors also stimulate NOS-1 production of NO and eventually cGMP, which acts on phosphodiesterase-3 in the JG cells to prevent the degradation of cAMP and sustains the stimulus for renin release (96). Sympathetic nerves directly innervate JG cells, and stimulation of renal efferent nerves releases NE that can activate β-adrenergic receptors on JG cells to increase cAMP production and renin release. Several central neural reflexes, including the cardiac baroreceptors, the aortocarotid baroreceptors and chemoreceptors, and the vagal afferent nerves can stimulate the renal sympathetic nerves. Unlike cAMP, intracellular Ca^{+2} in the JG cells causes decrease in renin release. Some humoral factors e.g. Ang-II, ET-1, atrial natriuretic peptide, adenosine, and thromboxane can inhibit renin secretion.

Angiotensinogen is the only known protein from which renin cleaves to generate a family of angiotensin: Ang-I, Ang-II, Ang-III, Ang-IV, Ang-(1-7), Ang-(1-5), and Ang-(1-4) (97). Figure 4 illustrates the enzymatic pathways responsible for the production and degradation of active angiotensins. The systemic source of angiotensinogen is from the hepatocytes but other tissues e.g. heart and MΦ can also synthesize angiotensinogen for local usage (98). The half-life of circulating angiotensinogen is about 16 hours. Several hormones e.g. cortisol, estrogens, T_4, insulin and selected cytokines can induce angiotensinogen expression in haptocytes. Renin cleaves circulating

angiotensinogen at its N-terminus to release Ang-I decapeptide. ACE converts Ang-I into active Ang-II octapeptide by cleaving the C-terminus dipeptide from Ang-I. However, ACE can also cleaves other peptides e.g. bradykinin (99), LHRH, enkephalins, and substance P. Therefore, ACE does not just generate vasoconstrictive Ang-II but it also removes vasodilator bradykinin. In fact, ACE has higher affinity for bradykinin than Ang-I. Most ACE is found on the plasma membrane of various cell types e.g. epithelial lining of small intestine, the choroid plexus, and the placenta. In the vascular beds, ACE is found in the plasma membrane of endothelial cells lining the lung, the retina, and the brain, but the renal proximal tubular brush border has 5-6 times more ACE per unit of wet weight (100). Chymase, mast cell-derived chymotrypsin-like serine protease, is another enzyme that can convert Ang-I into Ang-II; it provides another mechanism for the generation of Ang-II during chronic inhibition of ACE. Its roles in hypertension and especially in hypertensive patients who are taking ACE inhibitors are not clear; hence further research is needed. In addition to Ang-II, Ang-I can also be converted into Ang-(1-7) heptapeptide by EPs (101). Ang-(1-7) is a competitive inhibitor of Ang-II at AT_1 receptor but a substrate for AT_2 and mas receptor (102). In the fetus, AT_2 is the predominant subtype and plays an important role in renal development. In adult, AT_2 is found mostly in the brain, the uterus, adrenal medulla, and the kidneys. The functions of AT_2, a G_i-protein coupled receptor, are to antagonize those of AT_1, including vasodilation, natriuresis, and apoptosis (prevent growth and fibrosis). Mas is a GPCR whose signal transduction leads to protein phosphorylation and the release of NO and eicosanoids (103). Ang-(1-7) can also be formed when

Figure 4: Enzymatic pathways for Ang-II synthesis, metabolism, and functions.



Ang-II is formed from the hepatocyte-derived angiotensinogen through the actions of renin and ACE. Ang-II can be metabolized into Ang-III/IV, Ang-(1-7), Ang-(1-5), and Ang-(1-4) through the actions of CHYM, AP, EP/NEP and ACE. Ang-II has a wide range of functions including increase SNA, Na^+ reabsorption, vasopression secretion, ROS production, vasoconstriction, and stimulation of Na^+/H^+ exchanger.

ACE2 converts Ang-II into Ang-(1-7) or NEP converts Ang-I into Ang-(1-7); ACE2 is found in the heart, blood vessels, and the kidneys but NEP is found mostly in epithelial cells e.g. renal proximal tubule brush borders, fibroblasts, and neutrophils.NEP can also inactive Ang-II and Ang-(1-7) by converting them into inactive Ang-(1-4). Ang-(1-7) can also be inactivated by ACE by converting it to Ang-(1-5) (104). Inhibition of ACE has three main preventive effects: 1) the formation of vasoconstrictive Ang-II and 2) the degradation of Ang-(1-7), a vasodilation agent and 3) degradation of bradykinin. Together these two account for the BP-lowering effect of ACE inhibitors. Lastly, Ang-II can be metabolized into inactive Ang-III and Ang-IV by APs. The AT_1 is a $G_{q/11}$ protein coupled receptor. Upon activation of the receptor, the $G_{q/11}$ protein stimulates PLC to produce IP3 and DAG. IP3 binds to IP3 receptors and triggers the release of intracellular Ca^{+2} storage. AT_1 also triggers Ca^{+2} influx by open Ca^{+2} channels in the cell membrane. Together Ca^{+2} and DAG activate PKC, which phosphorylates and activates downstream signaling proteins to cause vascular smooth muscles contraction, aldosterone synthesis and release, increased SNA, ROS production and Na^{+}/H^{+} exchanger activation (105).

Endothelin

There are three endothelins found in mammals: ET-1, ET-2 and ET-3, and all three are 21-amino acids peptides (106). They are found in many different organs but often associated with vascular endothelial cells. They are important regulators of various organ systems including the cardiovascular, digestive, endocrine, nervous, renal and

genitourinary system. ET-1 is synthesized in endothelial cells as preproendothelin (203 amino acids), converted into proendothelin (183 amino acids), and sequentially cleaved by ECE into active ET-1 (39 amino acids) (107). Various factors e.g. Ang-II, vasopressin, catecholamines, TGF-β, and high pressure/low shear stress situation stimulate ET-1 secretion from endothelial cells (108). ET-1 can binds to ET_A and ET_B receptor, and both are GPCR. Upon activation by ET-1, G-protein stimulates PLC, induces intracellular Ca^{+2} mobilization (109), and activates PKC and MAPK, and also Na^{+}/H^{+} exchanger. ET-1 acts in a paracrine manner on the ET_A and ET_{B2} receptors on the vascular smooth muscle cell membrane to induce contraction, proliferation, migration, and cell hypertrophy (110). However, ET-1 can also activate endothelial ET_C and ET_{B1} receptors to induce the release of NO and prostacyclin which are vasodilators. ET-1 acts on ET_A receptors can also increase NADPH oxidase level and oxidative stress (111). In the heart ET-1 has positive chronotropic and inotropic effects on cardiomyocytes. In addition, ET-1 stimulates ET_A receptors on cardiomyocytes and fibrobasts to mediate extensive cardiac fibrosis and microvascular remodeling. In the kidney, endothelin receptors are mainly present in the blood vessels and the mesangial cells; and they stimulate the RAAS by increasing the levels of renin, Ang-II, and aldosterone (112). In the nervous system, through ET_B receptors, ET-1 modulates presynaptic neurotransmitters release, which increases central and peripheral SNA (113). The endothelin system is activated in many hypertensive models, including

DOCA-salt hypertension (114), Dahl salt-sensitive hypertension, Goldblatt hypertension, and cyclosporine-induced hypertension.

Endogenous Ouabain

45% of Caucasians who are diagnosed with essential hypertension have elevated EO in their blood. EO is a mammalian steroid counterpart of the plant glycoside ouabain. It specifically and reversibly binds to mammalian Na^+/K^+ ATPase with high-affinity. Upon binding, EO inhibits the Na^+/K^+ ATPase causes increase in intracellular Na^+, lowering the Na^+ gradient across the cell membrane and reduces the efflux of Ca^{+2} through Na^+/Ca^{+2} exchanger (115). This change in intracellular Ca^{+2} concentration may increase the arterial myogenic tone and the neurogenic activity (116). High salt intake raises plasma EO in both humans and rats (117). EO synthesis is mediated by the hormone aldosterone. In fact, approximately 50% of patients with primary hyperaldosteronism have elevated plasma EO. EO secretion is stimulated by Ang-II and during volume depletion. EO is an on going area of research. Rostafuroxin, currently in phase III trials for essential hypertension, blocks EO binding to Na^+/K^+ ATPase and reduces BP in hypertensive animal models.

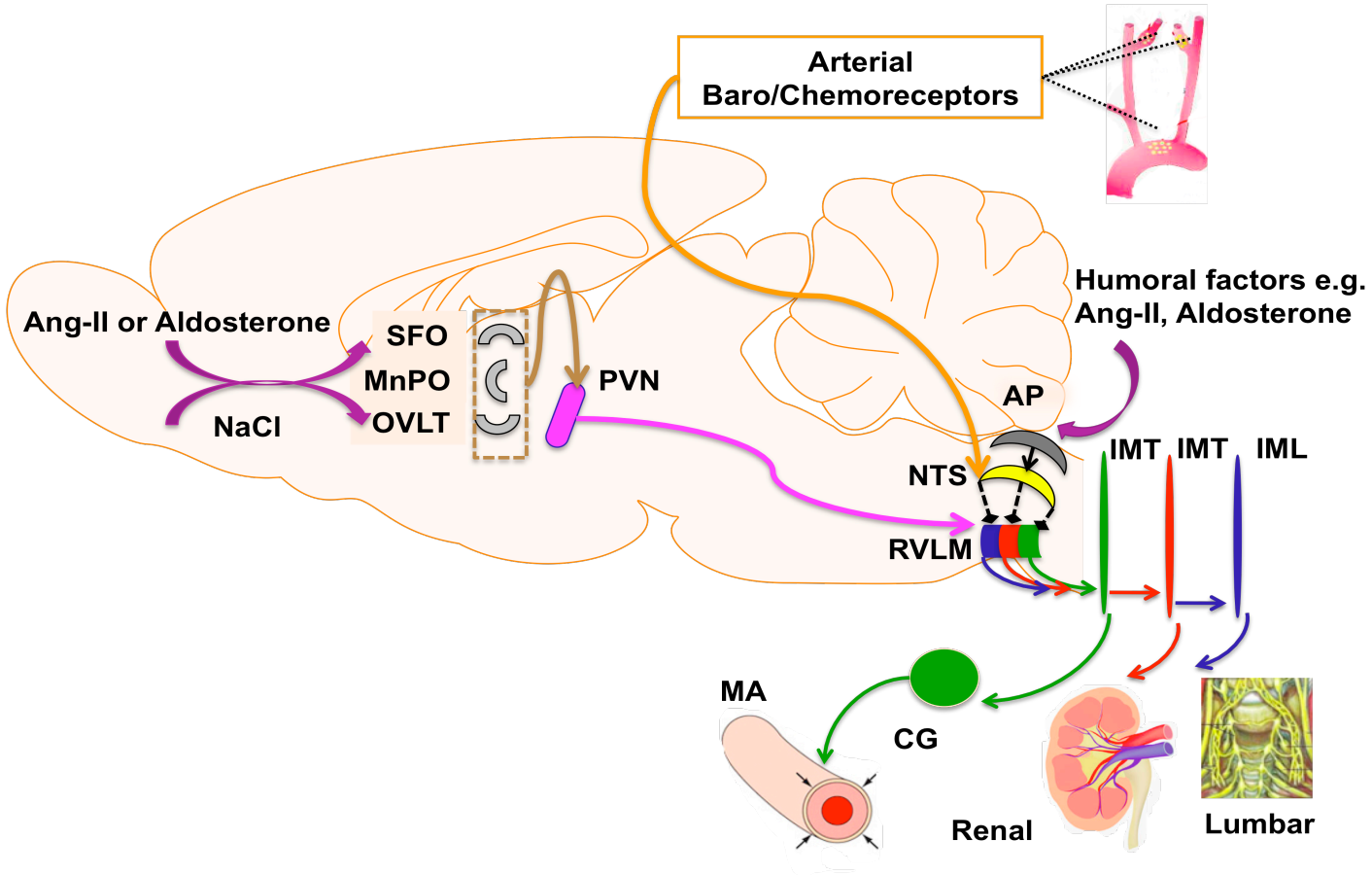
The Nervous System and Hypertension

The nervous system has short-term and long-term regulations on BP. In several animal models and in subsets of human essential hypertension, chronic activation of the nervous system appears to contribute to persistent hypertension (118). The sympathetic

nervous system and the neurohormonal system (primarily regulated by the hypothalamus) of the nervous system contribute the most to arterial blood pressure control. Figure 5 shows the central modulation of the sympathetic outflow by salt, circulating humoral factors e.g. Ang-II, and the afferent nerves. The brain continuously monitors BP through arterial baroreflex or stretch receptors attached to the vagal and glossopharyngeal axons innervating the carotid sinuses and the aortic arch. The mechanism of arterial baroreflex activation is thought to involve opening ENaC, ASIC, and TRP channels during vascular distention (119). The baroreflex provides second-to-second negative feedback to prevent too much BP fluctuation. In addition to responding to changes in pressure, it also has a tonic sympathoinhibitory effect during resting condition, because in the baroreceptor denervation condition, there is a profound transient increased sympathetic activity and BP but eventually BP returns back to normal. The mechanism underlying the normalization of BP after denervation is not entirely clear. Perhaps, loss of input from baroreceptor afferents may result in central remodeling of neural pathways. This is supported when destroying the NTS in baroreceptor-denervated animals produce no change in BP but in control animals it causes hypertension. Acute rise in BP initially increases baroreceptor activity, but within a minute the baroreceptor activity declines despite the elevated BP. This acute adaptation involves the activation of 4-aminopyridine-sensitive K^+ channels (120). In hypertension, the baroreceptors are reset to operate at higher-pressure levels. An acute resetting happens within the first 5-15 minutes after BP increased. This resetting is only partial because the increase in pressure threshold for baroreceptor activation represents a fraction of the total pressure increase. Furthermore, acute resetting doesn't

affect the sensitivity of the baroreceptor (121). However sustained increase in BP will lead to completed resetting in which the pressure threshold increase equals the total pressure increase, and the baroreceptor sensitivity is decrease. Baroreceptor resetting is caused in part by activation of an electrogenic Na^+ pump, which hyperpolarizes

Figure 5: The circuitry of central nervous system modulation of sympathetic outflow



RVLM generates tonic sympathetic activity output to different parts of the body via IMT/L regions of the spinal column or the prevertebral ganglions e.g. celiac ganglion. Afferent neuronal inputs from the arterial baro/chemoreceptors arrive to the NTS modulate RVLM activity. Similarly, PVN descending fibers can also modulate RVLM activity. Humoral factors e.g. salt and Ang-II can influence RVLM activity via the AP and circumventricular organ.

baroreceptor nerve endings. Humans with essential hypertension exhibit impaired baroreceptor reflex, and the baroreceptor is reset to higher-pressure levels (122). Humoral factors e.g. Ang-II acting at the area of postrema and circumventricular region of the brain can decrease the sensitivity and reset the baroreceptor independently of BP (123). ROS acting at the baroreceptor endings may also contribute to decreased baroreceptor sensitivity.

Integration and processing of afferent information from the baroreceptor is accomplished by the NTS. Its projections modify preganglionic sympathetic and parasympathetic activity and modulate the release of vasopression from the hypothalamus. Abnormality in NTS causes acute fulminating hypertension in rats, less severe hypertension in cats and dogs, and increased BP in humans because of higher SNA. The NTS neurons provide inhibitory inputs to the RVLM neurons. Cardiovascular neuronal projections of the NTS onto the RVLM are organized in distinct regions e.g. lumbar RVLM projection and renal RVLM projection. RVLM contains efferent neurons that provide tonic drive to the preganlionic efferent neurons that directly regulate peripheral sympathetic nervous system. Activation of the RVLM increases SNA and thereby vasoconstriction, cardiac contractility, and catecholamine release. Increased RVLM firing rate increases BP. RVLM is the hub for the regulation of SNA. It receives excitatory input from the PVN of hypothalamus. Salt and circulating humoral factors e.g. Ang-II, and aldosterone can stimulate neurons in the forebrain circumventricular organs (SFO and OVLT) and subsequently activate PVN neurons through direct or indirect projections via the MnPO. Benzamil-sensitive Na^+ channels are thought to be involved in this salt signaling. Like the NTS, PVN projections onto the RVLM are organized in

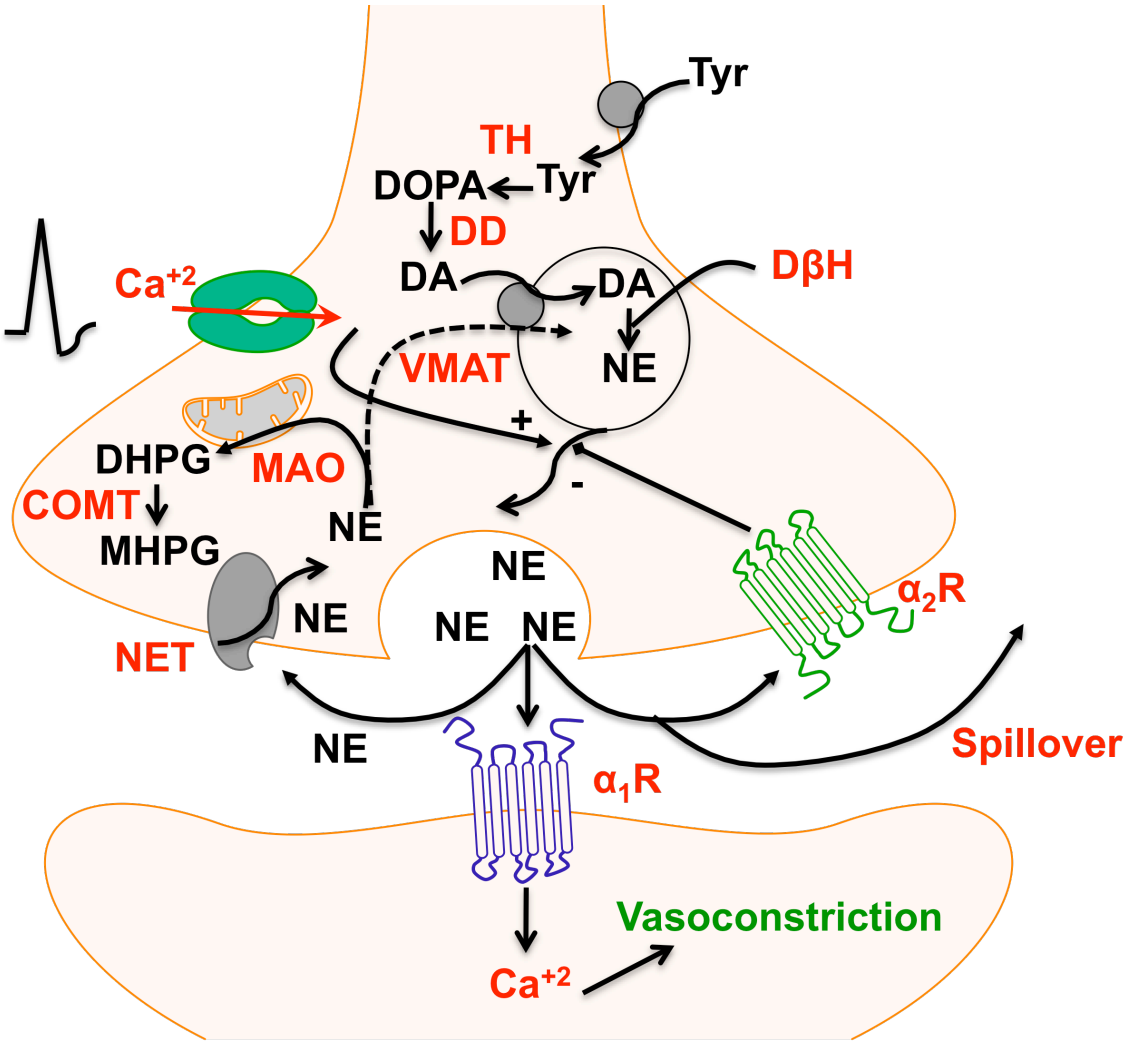
distinct areas (124). There is a hypothesis that in hypertension e.g. Ang-II-salt hypertension, there is an enhanced central drive of PVN onto the RVLM and it exhibits a differential pattern of sympathetic outflow such that splanchnic SNA is increase, renal SNA is decrease and lumbar SNA is not change. Sympathetic nerves originating from the celiac and superior mesenteric ganglia densely innervate the splanchnic vascular bed. Therefore, a high splanchnic SNA causes vasoconstriction in arterioles, and small veins, which contribute to increase TPR, decrease vascular capacitance, and increase BP (125). Furthermore, celiac ganglionectomy has been shown to protect against some form of hypertension (126). In some essential hypertensive patients, there is direct relationship between BP and SNA; the higher the SNA the higher the BP.

The Properties of Adrenergic Receptors in Cardiovascular System

NE Synthesis, Release, Reuptake, and Metabolism

Figure 6 illustrates the enzymatic pathways for NE synthesis, release, reuptake and metabolism. The enzymatic rate-limiting step in NE synthesis is the conversion of tyrosine into DOPA by tyrosine hydroxylase. DOPA is converted into dopamine by dopamine decarboxylase. Dopamine is transported into the vesicle by VMAT, and inside the vesicle DβH catalyzes the conversion of dopamine into NE (127).

Figure 6: Enzymatic pathways for NE synthesis, release, reuptake, and metabolism



NE is synthesized from tyrosine through a series of enzymes, including TH, DD and DβH. It is transported into the vesicles by VMAT. Upon stimulus of Ca^{+2}, NE containing vesicles are exocytosis. NE are reuptake mostly by NET and broken down by MAO and COMT. Once NE is released, it binds to α_1R on the smooth muscle cells and α_2R on the presynaptic terminals causing vasoconstriction and inhibition of further release of NE, respectively.

Release of NE from sympathetic nerve endings is by vesicles exocytosis. It involves a series of steps, including docking of synaptic vesicles at the active zone, priming the vesicles ready for release, and fusion of the vesicle and plasma membranes. Figure 7 shows the schematic overview of vesicular trafficking and release. Vesicles in the synaptic terminal are grouped into three pools: the RRP, the recycling pool, and the RP. The RRP are docked to the cell membrane and activated for exocytosis and are the first group of vesicles to be released upon stimulation. The recycling pool is close to the cell membrane; under moderate stimulation, these vesicles are cycling between exocytosis and endocytosis. The RP constitutes the vast majority of vesicles in the nerve terminal where they cluster farther away from the plasma membrane. Synapsins are a family of neuronal phosphoproteins that function to cluster the vesicles into the RP (128). Under dephosphorylated state, synapsins form homodimers and heterodimers to anchor the vesicles into a cluster farther away from the membrane forming a RP. Upon phosphorylation by PKA, MAPK, and CaMK II, phosphorylated synapsins mobilize the vesicles to the RRP (129).

Before the vesicles can be exocytosis, they must dock to the active zone on the presynaptic terminal. At the plasma membrane, vesicle docking involves a large tethering complex called the exocyst, which comprises eight proteins: Sec3p, Sec5p, Sec6p, Sec8p, Sec 10p, Sec15p, Exo70p, and Exo84p (130). At the vesicular membrane, CSP_{α}/SGT/Hsc70 molecular chaperone facilitate the interaction of the vesicle with the plasma membrane bound SNAP-25 (131). The interaction between RIM and GTP bound Rab3A may also contribute to the docking reaction. At this stage, Munc18 is associated with the closed conformation of syntaxin-1. GTP hydrolysis

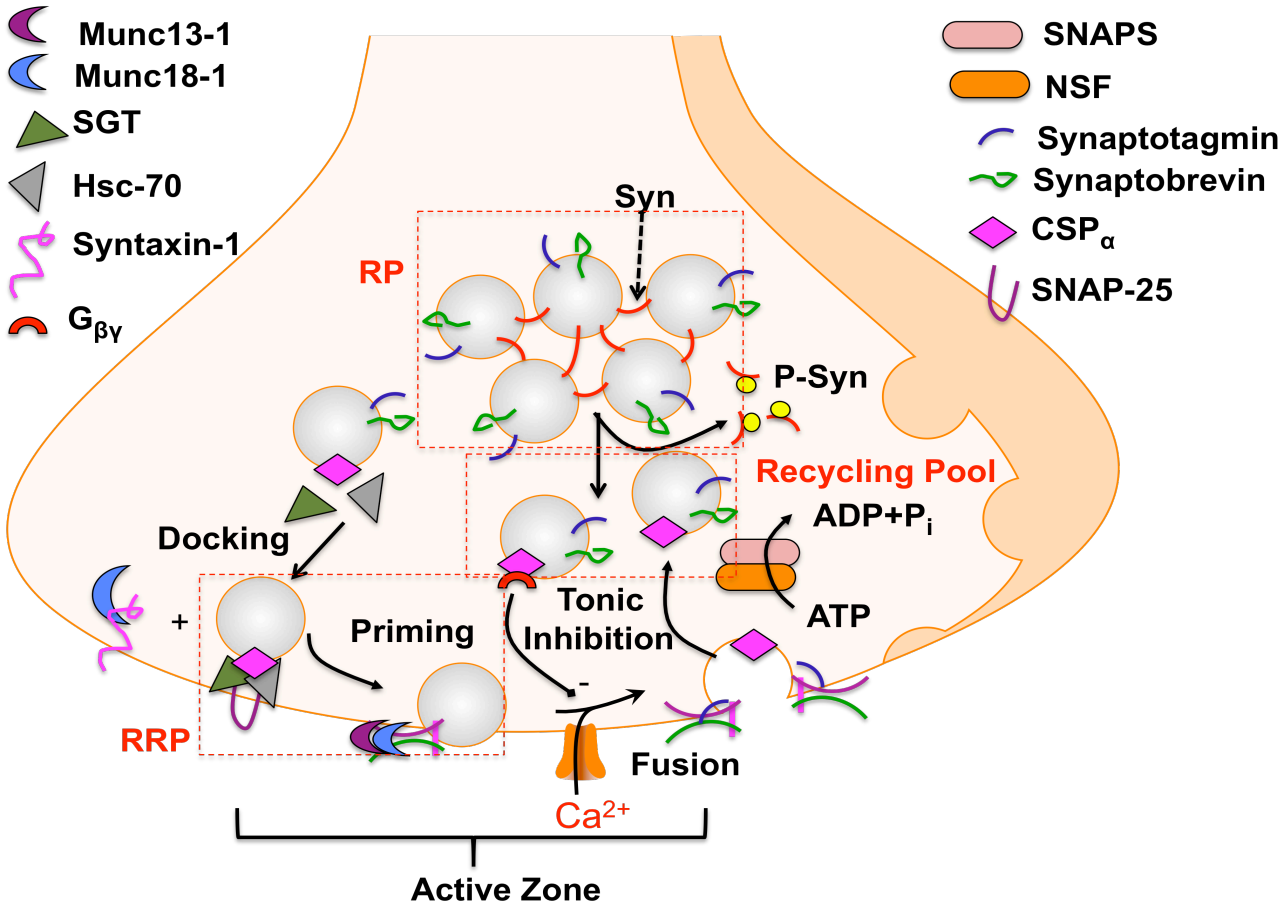
causes the dissociation of Rab3A from RIM and the synaptic vesicle. RIM then binds Munc13 and displaces Munc18 from syntaxin-1, which changes syntaxin-1 into the open state. Once the syntaxin-1 is in its open state, vesicular priming begins with syntaxin-1, synaptobrevin, and SNAP-25 assembles into the *trans*-SNARE complex, which pulls synaptic vesicle and plasma membrane into close contact (132). Finally, complexin binds to the fully assemble SNARE complex stabilizes the primed vesicle. The influx of Ca^{+2} triggers the binding of synaptotagmin to the SNARE complex and penetration of synaptotagmin into the plasma membrane, leading to membrane fusion. After fusion, the *cis*-SNARE complex is dissociated by NSF/α-SNAP, and the SNARE proteins are recycled for more exocytosis (133).

Protein phosphorylation is an important regulatory mechanism that controls the secretory pathway. Phosphorylation of syntaxin-1 by PKA and PKC and desphosphorylation by CAPP inhibits and stimulates the assembly of SNAP-25 and syntaxin-1 complex, respectively. Even so, PKA was shown to phosphorylate threonine-138 of SNAP-25 in adrenal chromaffin cells, which is required for the RRP of vesicles to be in a primed and releasable state. PKA phosphorylation of SNAP-25 activates the re-filling of recycling pool vesicles and increases the size of the RRP. Munc18 plays a central role in membrane fusion through its interaction with syntaxin-1. Munc18 binds tightly to syntaxin-1 holding it in a closed formation that prevents assembly into a SNARE complex. PKA and PKC phosphorylate Munc18 and inhibit it from binding to syntaxin-1. Thus, the phosphorylation of Munc18 may release syntaxin-1 from an inhibitory interaction in order to promote fusion and increase exocytosis (134). In addition to phosphorylation, G proteins interaction with vesicle fusion machinery can

also regulate secretory pathway. It has been shown that $G_{\beta\gamma}$ subunits interact with CSP and N-type Ca^{+2} channels resulted in a tonic G protein inhibition of the channels (135). Similarly, $G_{\beta\gamma}$ subunits directly bind SNAP-25 and interfere with the binding of SNAP-25 to synaptotagmin and prevent vesicle fusion (136).

After vesicular exocytosis, NE is reuptake into the presynaptic terminal using NET, and this is the main mechanism of inactivation of NE released from sympathetic nerves. Cocaine and tricyclic antidepressants e.g. Desipramine block NET. Most of NE taken up into the synaptic terminals is transported back into the vesicles by VMAT, and reserpine inhibits VMAT. A small fraction of the non-reuptake NE spills into the circulation, but the majority are metabolized by MAO and COMT into DHPG and MHPG, respectively (127).

Figure 7: Vesicular trafficking and release in presynaptic nerve terminal



Vesicles exocytosis involves a series of steps, including docking, priming, and fusion of the vesicles. Vesicles in the synaptic terminal are grouped into 3 pools: the RRP, the recycling pool, and the RP. Synapsins are important proteins that hold the vesicles in the RRP. SNARE proteins e.g. synaptobrevin, synaptotagmin, SNAP-25 and syntaxin-1 and SNARE chaperone proteins e.g. CSP-α, HSC-70 and SGT, and SNARE accessory proteins e.g. MUNC18-1 and MUNC13-1 are some of the major components of the exocytosis machinery. SNAPS and NSF are two important proteins involve in the recycling of the vesicles. $G_{\beta\gamma}$-CSPα has a tonic inhibition on the Ca^{+2} channels.

Adrenergic Receptors

Adrenergic receptors were originally divided into two different classes: α-adrenergic and β-adrenergic. In 1948, Ahlquist was the first to observe the opposing effects of catecholamine in smooth muscle cells, and he suggested that the excitatory effects were mediated by α-adrenergic receptors, whereas the inhibitory actions were caused by β-adrenergic receptors (137). Now through pharmacological, molecular, and cloning techniques, we know there are three types and each has 3 subtypes: α_1-adrenergic receptors (α_{1A}, α_{1B}, α_{1D}), α_2-adrenergic receptors (α_{2A}, α_{2B}, α_{2C}), and β-adrenergic receptors (β_1, β_2, β_3) (138). All three α_1-adrenergic receptors couple to the G_q pathway, resulting in stimulation of PLC, generation of IP_3 and DAG, mobilization of intracellular Ca^{+2}, activation of PKC, Na^+/H^+ exchanger and Na^+/Ca^{+2} exchanger, and inhibition of K^+ channel. All three α_2-adrenergic receptors are couple to Gi/o, which decreases cAMP production, inhibition of Ca^{+2} channels and PKA (139). All three β-adrenergic receptors are couple to G_s, which increase cAMP and activate PKA. The functions of adrenergic receptors are listed in Table 3. In addition to type- and subtype-specific signaling, adrenergic receptor signaling pathway is a complex multidimensional "signalome" (140). Adrenergic receptors can form homodimers or heterodimers. They can couple to multiple G-proteins, signaling pathways, and scaffold proteins in a temporally and spatially regulated manner. Together these result in different pharmacological and functionally distinct receptor populations (141).

Table 3: Tissue Distribution and Functions of Adrenergic Receptor Subtypes

Receptor	Tissue	Response
$\alpha_{1A,B,D}$	Smooth muscle: vascular, iris, ureter, uterus, sphincters of bladder and GI	Contraction
	Smooth muscle of GI	Relaxation
	Heart	Positive inotropic, cell growth, hypertrophy
	Salivary, sweat gland	Secretion
	Adiocytes	Glycogenolysis
	Kidney	Gluconeogenesis, sodium reabsorption
$\alpha_{2A,B,C}$	Presynaptic sympathetic nerve terminals	Inhibition of NE release
	Platelets	Aggregation, granule release
	Pancreas	Inhibition of insulin release
	Adiocytes	Inhibition of lipolysis
	Vascular smooth muscle	Contraction
	Kidney	Inhibition of renin release
β_1	Heart	Positive inotropic and chronotropic, cell growth and hypertrophy
	Adiocytes	Lipolysis
	Kidney	Release rennin
β_2	Hepatocytes	Glycogenolysis, gluconeogenesis
	Skeletal muscle	Glycogenolysis, lactate release
	Smooth muscle: vascular, bronchi, uterus, GI	Relaxation
	Pancreas	Insulin secretion
	Salivary glands	Amylase secretion
β_3	Adipocytes	Lipolysis
	Skeletal muscle	Thermogenesis

α_2-adrenergic Receptors

Stimulation of α_2R elicits a wide range of effects including hypotension, bradycardia, analgesia, hypothermia, sedation, hypnosis, and anesthetic-sparing (142). α_{2B} plays an important role in the development of the placenta and the lungs during embryonic development (143). It also regulates the vascular tone. α_{2C} were identified as the major feedback receptors of catecholamine release from chromaffin cells in the adrenal medulla (144). , α_{2A} inhibit insulin release from pancreatic islets, facilitate working memory, sedation, hypnosis, and mediates hypotension, bradycardia, and modulate baroreflex sensitivity (145). The completed knockout of α_2R is embryonic lethal (146).

α_{2A}-adrenergic Receptors and Cardiovascular Functions

Data from genome-wide association study have recently linked a single nucleotide polymorphism within the 3'UTR of the human α_{2A}-adrenergic receptor gene ADRA2A with increase BP (147). α_{2A}R activation inhibits voltage-gated Ca^{+2} channels, activates GIRK, and MAPK in sympathetic neurons, which results in presynaptic inhibition of neurotransmitter release. α_{2A}R deficient animals were more susceptible to the development of cardiac fibrosis, hypertrophy, and heart failure in chronic cardiac pressure overload condition (148). Furthermore, high level of NE concentrations in the synaptic cleft activates smooth muscle and cardiac myocyte adrenergic receptors, which cause hypertension. Numerous studies have shown that α_2R function is impaired

in human hypertension and in DOCA-salt hypertension (149, 150). Nevertheless, the exact mechanism that causes the receptor impairment is unknown. Clinically, α_2R agonist clonidine is used to lower BP and treat hypertension.

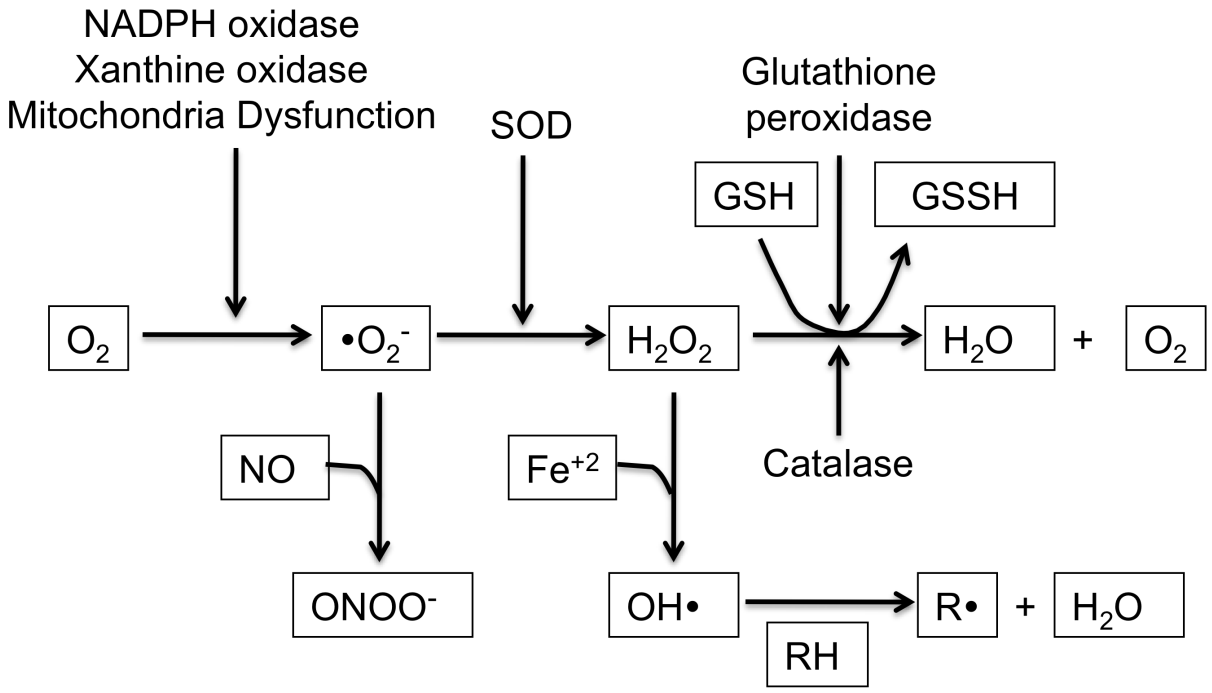
Following stimulation, α_{2A}R is desensitized and downregulated. A major mechanism for desensitization is initiated by GRK2 where it binds to activate α_{2A}R and phosphorylates several serine residues within the third intracellular loop (151). PKC-dependent phosphorylation is also implicated in α_{2A}R desensitization (152). Phosphorylated α_{2A}R recruits β-arrestins, which uncouples the receptors from the G-proteins and induces receptor endocytosis. Spinophilin was shown to block GRK2 interaction with α_{2A}R and prevent β-arrestin signaling. α_{2A}R in sympathetic neurons have a high receptor reserve and therefore, changes in the receptor density in sympathetic neurons do not correlate with functional changes (153).

Oxidative Stress, Inflammation and Hypertension

Reactive Oxygen Species

Reactive oxygen species are metabolites of oxygen that possess an unpaired electron in their outer orbital. These molecules include the radical superoxide (O_2^-), hydroxyl radical (HO•), and nitric oxide (NO•), and non-radical hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Figure 8 depicts the pathways for ROS synthesis and metabolism.

Figure 8: Enzymatic pathways for ROS synthesis and metabolism



ROS are metabolites of oxygen that possess an unpaired electron; they include $\bullet O_2^-$, HO•, NO•, H_2O_2, $ONOO^-$ and are generated from xanthine oxidase, NADPH oxdiase, and mitochondrial dysfunction. ROS can be removed by catalase or glutathione peroxidase.

Enzymes that generate cellular O_2^- include NAPDH oxidase, xanthine oxidase, NOS, heme oxygenase, cyclooxygenase, lipoxygenase peroxidases, and mitochondrial oxidases (154). In cardiovascular biology, low ROS levels modulate vascular tone and structure (155). Under physiological conditions, ROS are maintained at a low level by the enzymes superoxide dismutase (SOD), catalase, thioredoxin, and glutathione peroxidase. Perturbation of the balance between ROS production and removal results in oxidative stress. In many pathological conditions there is an increase ROS which up-regulates many signaling pathways including those that involve in smooth muscle cells growth, endothelial dysfunction, extracellular matrix deposition, angiogenesis and inflammation (156). Uncontrolled ROS causes cellular damage and eventually apoptosis because ROS can damage proteins, lipids, and DNA (157). Interestingly, there is a large body of literature correlating ROS to hypertension.. The SOD mimetic, tempol, lowers blood pressure and sympathetic nerve activity in DOCA-salt hypertension (158). A major source of O_2^- is a multi-subunit enzyme, NADPH oxidase. In phagocytes such as MΦ, NADPH oxidase has intracellular $p47^{phox}$, $p67^{phox}$, $p4O^{phox}$ subunit and a cytochrome b558 catalytic core composed of membrane bound subunits Nox-2/$gp91^{phox}$ and $p22^{phox}$ (159). Although NADPH oxidases were originally found in phagocytic cells, the discovery of $gp91^{phox}$ homologs indicates that there is an entire family of NADPH oxidases. The family includes Nox1, 2, 3, 4, 5, Duox 1 and 2, which is also found in many tissues and mediates diverse functions. Hypertensive stimuli up-regulate $p22^{phox}$ subunit and enzyme activity. Mice deficient of Nox-1 have blunted

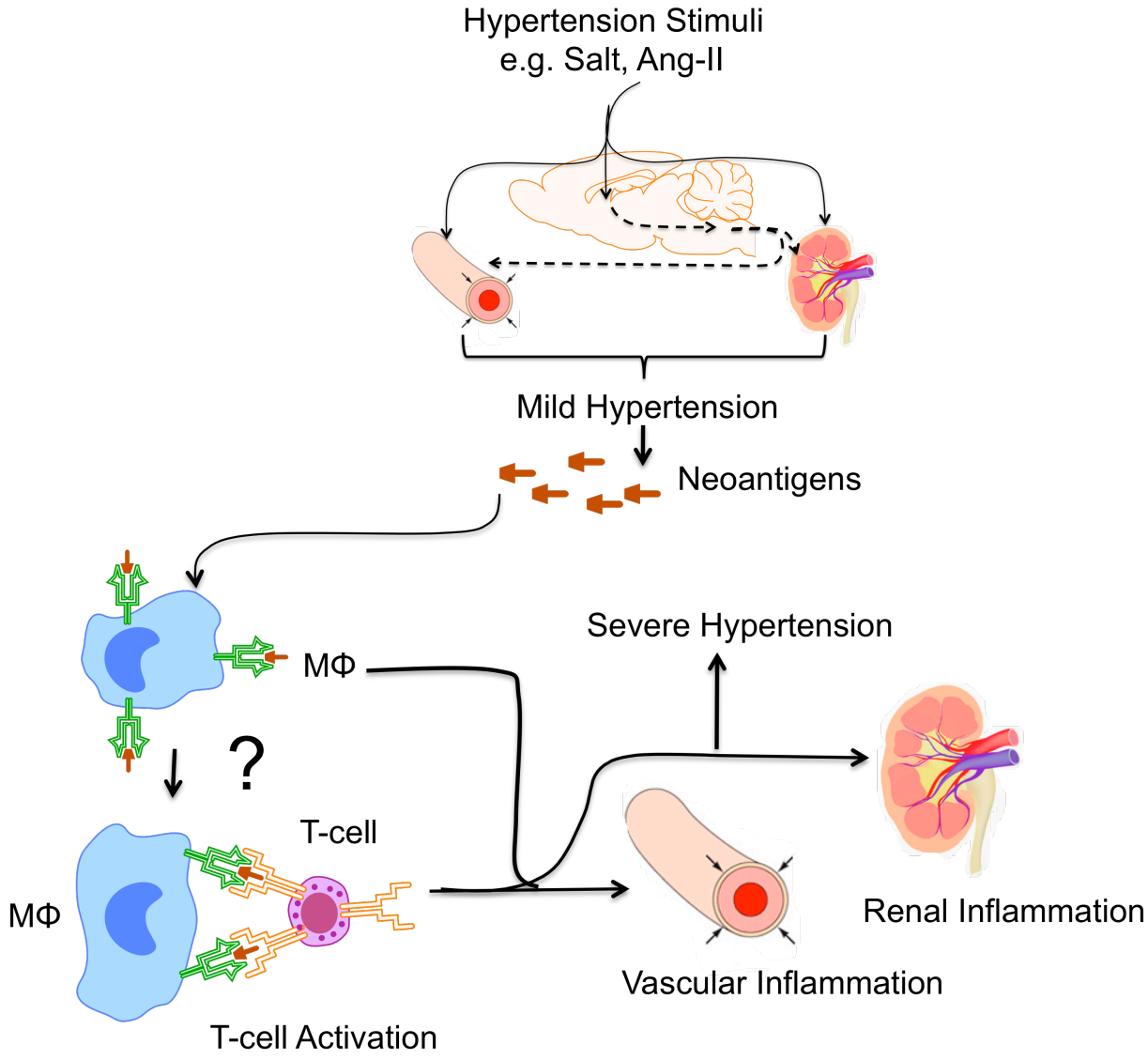
DOCA-salt hypertension (160). In the brain, increased NADPHD oxidase activity in the circumventricular organs and the NTS increase sympathetic outflow and inhibits its activity in the same brain area prevent hypertension. Despite the ample of evidence suggesting that ROS contributes to hypertension, there is not a clear understanding of exactly how this happens. Furthermore, treatment with antioxidant e.g. vitamin C and E has been found to be effective in some animal models and small clinical trials but data from large clinical studies have not demonstrated benefits (161).

Inflammation and Hypertension

Many academic publications indicate that inflammation may be involved in the development of hypertension. Transferring of splenic cells from DOCA-salt hypertensive and renal hypertensive rats into normotensive rats caused hypertension in the recipient rats (162). Animals that have thymectomy also have blunted delayed phase of DOCA-salt hypertension (163). Interestingly, a recent analysis of 6000 people with AIDS showed that they have lower prevalence of hypertension comparing to the general population. In addition, further treatment with retroviral therapy for two years restored the prevalence of hypertension to that of the control population (164). Immunosuppressive therapy e.g. mycophenolate mofetil prevents hypertension in some animal models (165). Inhibition of proinfammatory transcription factor, NFκB, prevents nitric oxide inhibitor-induced hypertension. Etanercept, a TNF-α antagonist, prevents hypertension and vascular dysfunction in Ang-II induced hypertension and fructose-fed hypertension (166). IL-17 has been shown to induce chemokines and adhesion molecules in tissues; mice lacking IL-17 have reduced BP when compared to wild-type mice treated with Ang-II (167). A hypothesis for mechanism underlying inflammation-

induced hypertension is the formation of a neo-antigen (168). Perhaps, the modest increase in BP during early stage of hypertension causes some cellular damage, neo-antigen formation, and inflammatory response that serve as stimuli to recruit immune cells e.g. MΦ and T-cells. Hypertensive animal models that lack the immune components e.g. $RAG^{-/-}$ mice lacking lymphocytes (169), and Op/Op mice lacking MΦ only increase BP to about 135 mmHg (170), (modest hypertension) even when treated with maximal stimuli e.g. Ang-II and DOCA-salt. Rats that are immune tolerant to HSP70 developed minimal renal inflammation and were protected from the development of salt-sensitive hypertension (171). This supports the hypothesis which suggests that inflammation plays role in the development of hypertension. Figure 9 illustrates the hypothesis for the underlying role of the immune system in hypertension.

Figure 9: Proposed hypothesis describing the role of adaptive immunity in hypertension



Humoral factors e.g. salt and Ang-II elevate BP. This increased in BP causes tissue damage and the formation of neo-antigen, which triggers the adaptive immune system activation. Activated MΦ and T-cells infiltrate into the blood vessels and kidneys cause further damage and severe hypertension.

Lymphocytes and Hypertension

Although it is known that hypertension involves inflammation, most studies focuse on the contribution of lymphocytes. Mice lacking T and B cells have blunt hypertension and do not develop vascular remodeling during angiotensin II infusion or DOCA-salt treatment; adoptive transfer of T, but not B cells restored these changes (169). Furthermore (WC), T cells express angiotensinogen, angiotensin I-converting enzyme, and renin. AT1 receptors are expressed intracellularly by T-cells and angiotensin II activates T-cells (172). However, it is unclear how angiotensin II activates T cells in DOCA-salt hypertension when DOCA-salt hypertension is characterized by low levels of circulating angiotensin II. Even so, none of the studies involving lymphocytes focuse on how these cells affect the periarterial sympathetic nerve function in hypertension.

MΦ and Hypertension

The role of inflammation through recruitment, activation and proliferation of MΦ in the vascular adventitia has been recognized in hypertension (173). In some models of hypertension MΦ/monocyte infiltrate into the arterial wall (174). Alterations in the number of circulating monocytes and their activation occur in hypertensive patients and animals (175, 176). It is unclear how MΦ is activated. Leukocyte adhesion molecules, chemokines, specific growth factors and endothelin-1 and angiotensin II can modulate MΦ activity (177). An interesting question is whether circulating monocytes can sense increased blood pressure. Adventitial MΦ in MA is exposed to increased cyclic mechanical strain by high blood pressure. Cyclic strain on in vitro monocytic cells induces transcription of cytokine IL-8, NF-κB-inducible IEX-1, and an apoptosis related

PAR-4, in an amplitude-dependent manner (178). This suggests that high blood pressure can induce the expression of cytokines and adhesion molecules in the vascular wall causing MΦ infiltration. It is logical then to ask what is/are the source(s) of infiltrating MΦ. A current paradigm states that monocytes circulate freely and patrol blood vessels but differentiate irreversibly into MΦ or dendritic cells. A recent publication showed that bona fide undifferentiated monocytes reside in the spleen and outnumber their equivalents in the circulation. The reservoir monocytes assemble in clusters in the cords of subcapsular red pulp. In response to ischemic myocardial injury, splenic monocytes increase their motility, exit the spleen and accumulate in injured tissue (179). Whether a similar pathophysiology in which splenic monocytes leave the spleen and infiltrate the adventitia of MA in hypertensive animal is unknown. The focus of the contribution of macrophages to the pathophysiology of salt-sensitive hypertension is a novel aspect of this proposal.

CD163 and Its Biological Functions

CD163 is a member of a scavenger receptor cysteine-rich super family class B (180). In human, it is mapped to the region p13 on chromosome 12 (181). In rats, CD163 has been identified as ED2 antigen and found on the rat chromsome 6 (182). CD163 gene encodes for 1076 amino acid protein with 1003 amino acids as the extracellular part, 24 amino acids as a single transmembrane segment, and 49 amino acids as cytoplasmic domain (183). In general, CD163 is exclusively found on cells of monocytes MΦ lineage, but only 5-30% of monocytes experesses the receptor (184). CD163 is expressed at high levels in mature tissue MΦ e.g. splenic red pulp MΦ, Kupffer cells, dust cells, lymph node MΦ, thymic MΦ, and peritoneal MΦ (180). CD163 have been

shown to be involved in pro- and anti-inflammation. TNF-α Interferon-γ, LPS, or TGF-β causes decrease in the CD163 expression, whereas glucocorticoids, IL-6, or IL-10 induces the increase of CD163 expression (185-189). Some studies show that soluble CD163 may inhibit human T lymphocytes activation and proliferation in vitro (190). However, there are studies reporting that cross-linking human CD163 either with antibodies or hemoglobin-haptoglobin complexes induces the production of NO, IL-1β, IL-6 and TNF-α (191). The specific function of CD163 on MΦ is still unclear. It has also been showed that CD163 is involved in the adhesion of monocytes to endothelial cells (192). The best-known function of CD163 is the clearance of fell free hemoglobin from circulation (193). Oxidative stress or inflammatory stimuli e.g. activation of TLR2 or 5 causes the extracellular domain of this receptor to shed from the cell surface and generate a soluble CD163 or sCD163 (194). sCD163 is a new class of MΦ specific biomarkers to the detection of coronary artery disease, transplantation, atherosclerosis, and rheumatoid arthritis (195). It has been documented in inflammatory conditions characterized by monocytic infiltration.

CD11b and Its Biological Functions

CD11 is an integrin α_M subunit that forms the heterodimeric integrin $\alpha_M\beta_2$ molecule. It is also known as Mac-1 or CR3. $\alpha_M\beta_2$ belongs to the β_2 subfamily integrins and is expressed on the surface of many leukocytes, including monocytes, MΦ, granulocytes, and natural killer cells (196). The α_M subunit of $\alpha_M\beta_2$ integrin is directly involved in causing the adhesion and spreading of the cells but alone, without the β_2 subunit, it

cannot mediate cellular migration (197). $\alpha_M\beta_2$ integrins are stored in intracellular granules and are rapidly translocated to the cell membrane during cell activation, significantly increasing their expression on the cell surface. Different agonists are able to induce $\alpha_M\beta_2$ integrin expression, including phorbol esters, bacterial formylated peptides, TNF-α, C5a, and LTB4 (198, 199). In addition, the signal-transduction of L- and E-selectin can also induce $\alpha_M\beta_2$ integrin expression by neutrophils (200). The ligands for $\alpha_M\beta_2$ integrin are ICAM-1, fibrinogen and iC3b. $\alpha_M\beta_2$ is involved in the phagocytosis of particles that are coated with iC3b. Under inflammatory condition, the level of ICAM-1 expression on endothelial cells, MΦ, and lymphocytes are upregulated. Integrins are a very important family of cell adhesion molecules involved in both extracellular matrix/cell and cell/cell interactions. As a result, $\alpha_M\beta_2$ integrin is under strict control with multiple levels of regulation. The first level of regulation is the translocation of $\alpha_M\beta_2$ integrin from the granules to the cell membrane. The second level of regulation is through increase avidity in which the receptors are clustered to a specific cell membrane microdomain and activated to increase its affinity for the ligand (201). Both α_M and β_2 subunit have intracellular tails that interact with cytoskeleton components and cytoplasmic receptors and allows $\alpha_M\beta_2$ integrin to function as a signal-transduction receptor. Two main pathways are triggered through $\alpha_M\beta_2$ are activation of protein kinases (e.g. Src kinases, FAK, Syk-Zap-70 family) and activation of MAPK (202-204). The binding of $\alpha_M\beta_2$ integrin to its ligand triggers MAPK signaling pathways

and results in the activation of different transcriptional factors e.g. AP-1 and NFκB (205). These transcriptional factors can upregulate pro-inflammatory cytokines such as IL-1 and TNF-α (206). $\alpha_M\beta_2$ mediates inflammation through its ability to regulate leukocyte chemotaxis, phagocytosis, cell-mediated cytotoxicity, and activation.

Deoxycorticosterone acetate (DOCA)-salt Hypertension

The proposed studies will use the DOCA-salt model of hypertension in rats. This model was chosen because the sympathetic nervous system participates in the etiology of hypertension and there is oxidative stress (elevated O_2^-) in the vasculature of DOCA-salt rats, with important consequences for the development of hypertension. Interestingly, studies of DOCA-salt hypertension have resulted in controversies because there are different determinants at different phases of the hypertension (207). In order to resolve some of this controversy, we propose to study different phases of hypertension. In addition, DOCA-salt treatment reliably produces hypertension in rodents, but has a gradual onset, allowing the investigation of “pre-hypertensive” changes in blood pressure control systems. We will capitalize on this latter characteristic extensively in our research. Finally, current research indicates excessive mineralocorticoid action contributes to a larger number of cases of clinical hypertension than generally appreciated. The increase in sympathetic nerve activity in DOCA-salt hypertension suggests that there may be alterations in the local mechanisms that modulate sympathetic neurotransmission (208, 209). There is an impaired function of α_2R on sympathetic nerves associated with MA in DOCA-salt hypertension (149, 150). In addition, the function of the NE transporter (NET) from sympathetic nerves associated

with MA is impaired in DOCA-salt hypertension resulting in an increase in neurogenic constrictions (210). Lastly, purinergic neurotransmission to MA in DOCA-salt hypertensive rats is also impaired due to decreased ATP bioavailability in periarterial sympathetic nerves (211). Recently, a growing number of studies indicate that vascular inflammation may be involved in both the initiation and development of hypertension (212-214). The Nox-based NADPH oxidases produce reactive oxygen species, especially O_2^-, contributing to hypertension (215, 216). In a flow-augmented common carotid artery model, there is an increase in MΦ infiltration into the sustained high blood flow artery and MΦ depletion reduced vascular remodeling (217). Mice deficient in MΦ colony-stimulating factor exhibit reduced vascular inflammation and they are protected against damage caused by DOCA-salt hypertension (170). However, it is unclear whether high blood pressure induces MΦ infiltration into the adventitia of MA and if infiltrated MΦ can release O_2^- that disrupts sympathetic nerve functions causing further increase in blood pressure. This issue will be addressed in the proposed studies.

G-Protein-Coupled Receptors

Guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs) are the largest and most versatile group of cell surface receptors. GPCRs are a superfamily of integral membrane proteins, and possess seven transmembrane domains, three extracellular loops, and three cytosolic loops. They have periplamisc N-terminal domain, and cytosolic C-terminal domain (218).

G Proteins

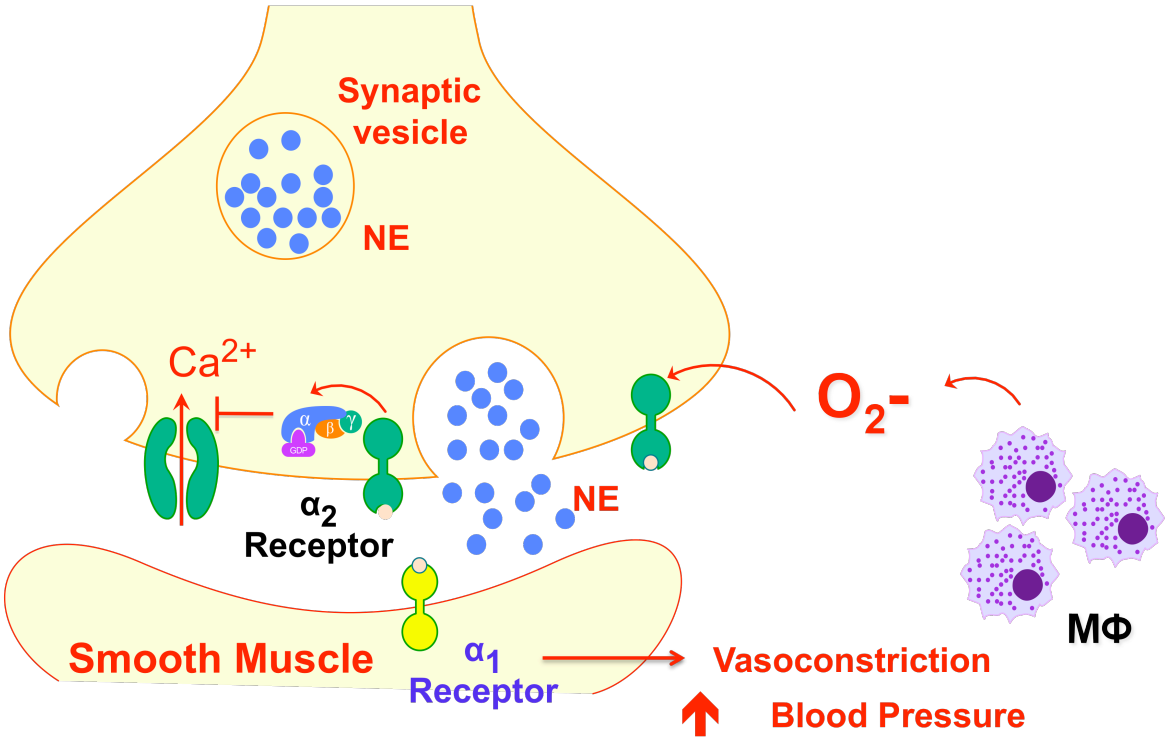
The G proteins are a family of signal-coupling proteins that play key roles in many signal transduction processes in cell. They consist of three polypeptides: a guanyl-nucleotide binding α chain (39-52 kDa), a β chain (35-36 kDa), and a γ chain (8 kDa). G proteins are cycle between an inactive GDP state and an active GTP state. When GDP is bound, α associates with β and γ to form a $G_{\alpha\beta\gamma}$ complex that is membrane bound. When GTP is bound, the G_{α}-GTP dissociates from the $G_{\beta\gamma}$. The conversion from GDP to GTP state is slow in the absence of the activated receptor. The activated receptor stimulates the G protein to speed up the rate of exchange of GTP for bound GDP. The α chain is also a GTPase that converts bound GTP to GDP to terminate activation. The role of Gβγ is to bind Gα-GDP to the receptor.

G proteins are classified based on their function and α subunits. The G_s proteins couples to a stimulatory α-subunit which activates adenylyl cyclase, leading to an increase in cAMP. G_s are ADP-ribosylated by cholera toxin, and G_s alone suffice to propagate the ligand signal from the receptor to the effector. ADP-ribosylation of G_s by cholera toxin inhibits the hydrolysis of bound GTP to GDP, keeping the Gs in its activated state. Activation of G_s by GTP analog or AlF_4^- causes reversible dissociation of the βγ subunit from the activated α subunit. This increase in βγ subunit slows down the activation of α subunit. The G_i and G_o proteins ($G_{i/o}$) proteins activates inhibitory α-subunit that blocks adenylyl cyclase. The inhibitory action of $G_{i/o}$ is mediated by both α and βγ subunits. $G_{i/o}$ are abundant G protein in the brain and G_o comprises about 1% of

the membrane bound protein in the brain. $G_{i/o}$ are targets of PTX ADP-ribosylated. However, G_i is more rapidly ADP-ribosylated by PTX than G_o, and G_o hydrolyzes GTP more rapidly than does G_i. $G_{i/o}$ proteins ADP-ribosylated by PTX are permanently trapped in the GDP inactive state because they are unable to bind to the excited receptor. $G_{q/11}$ proteins activate PLC and its downstream effectors. Lastly G_{12} proteins cause activation of the Rho small G-protein. The β chains of G proteins are nearly identical, whereas their γ chains show some differences. The βγ subunits of G_s, G_i, G_o, are functionally interchangeable (219).

RESEARCH GOALS & SPECIFIC AIMS

Figure 10: The Proposed Hypothesis



As BP increases in DOCA-salt rats, MΦ infiltrate into the adventitia of mesenteric arteries. MΦ release O_2^- that disrupts α_2R function causing increased NE release and further increases in blood pressure.

Research Goals

More than 65 million Americans have hypertension, which is a major risk for stroke, heart and kidney disease. Hypertension is a complex medical condition but sympathetic nerve activity is elevated in some hypertensive humans and in some animal models of hypertension including the DOCA-salt model. The DOCA-salt model is a salt dependent but rennin-angiotensin independent model of hypertension. In the DOCA-salt model in rats, the function of the α_2R regulating NE release from sympathetic nerves supplying arteries is impaired. The cause of this impairment is unknown but this impairment contributes to the increase in sympathetic activity in this model.

Overall hypothesis

The proposed studies will test the hypothesis that as BP increases in DOCA-salt rats, MΦ infiltrate into the adventitia of mesenteric arteries. MΦ release O_2^- that disrupts α_2R function causing an increase in NE release and further increaseing the blood pressure.

This study is novel because it is the first to examine the relationship between MΦ-derived O_2^- and the impairment of the α_2R function. The overall hypothesis will be tested in 3 specific aims:

Specific aim 1

These studies will test the hypothesis that there is a time dependent infiltration of activated MΦ into the adventitia of MA of DOCA-salt hypertensive rats. This infiltration is associated with a progressive increase in O_2^- and impaired α_2R function beginning in phase 2.

Measurements of O_2^-, MΦ infiltration and activation and sympathetic neuroeffector transmission will be made in rats in the pre-hypertensive stage: Phase 1, Phase 2 and established hypertension. These studies are important for two reasons. Firstly, they will establish when α_2R function is impaired. Does this occur early in the onset of DOCA-salt hypertension and therefore is a contributor to hypertension progression or does it occur later in hypertension and therefore is a consequence of the blood pressure increase? Preliminary data indicate that α_2R impairment will be first detected during Phase 2 hypertension. Secondly, these studies will establish a relationship between MΦ infiltration into the vasculature and the development of hypertension. These studies will determine if the vascular inflammatory response is a cause or a consequence of hypertension.

Specific aim 2

These studies will test the hypothesis that blockade of MΦ infiltration into MA reduces blood pressure in phase 2 and established hypertension and preserves α_2R function in established hypertension.

The studies in Aim 2 will show that there is a requirement for MΦ infiltration into the vasculature for development of the late phases of DOCA-salt hypertension and impairment of α_2R function. These studies will use liposomal clodronate to deplete MΦ beginning at the start and throughout the period of development of DOCA-salt hypertension. Radiotelemetry will be used to continuously monitor blood pressure in controls and DOCA-salt rats. Measurements of O_2^-, MΦ infiltration and activation and sympathetic neuroeffector transmission will be made in untreated and liposomal clodronate treated DOCA-salt rats at day 28 (established hypertension).

Specific aim 3

These studies will test the hypothesis that O_2^- uncouples the receptor from its G-protein.

The studies in Aim 3 will show that O_2^- disrupts sympathetic nerve function in DOCA salt rats by uncoupling the receptor from the target G-protein. Idazoxan, cocaine, and M119, Gβγ inhibitor will be used to show NE vesicular filling/trafficking and $G_{\beta\gamma}$-Ca^{+2} channel coupling is not affected in DOCA-salt rats at day 28 (established hypertension).

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CHAPTER 2

EXPERIMENTAL DESIGN & METHODOLOGIES

Animals

All animals use were approved by the Institutional Animal Care and Use Committee at Michigan State University. Male Sprague-Dawley rats weighing 200 g (Charles River Laboratories, Portage, MI) were acclimated for 5 days before entry into experimental protocols.

General Anesthesia Induction and Maintenance

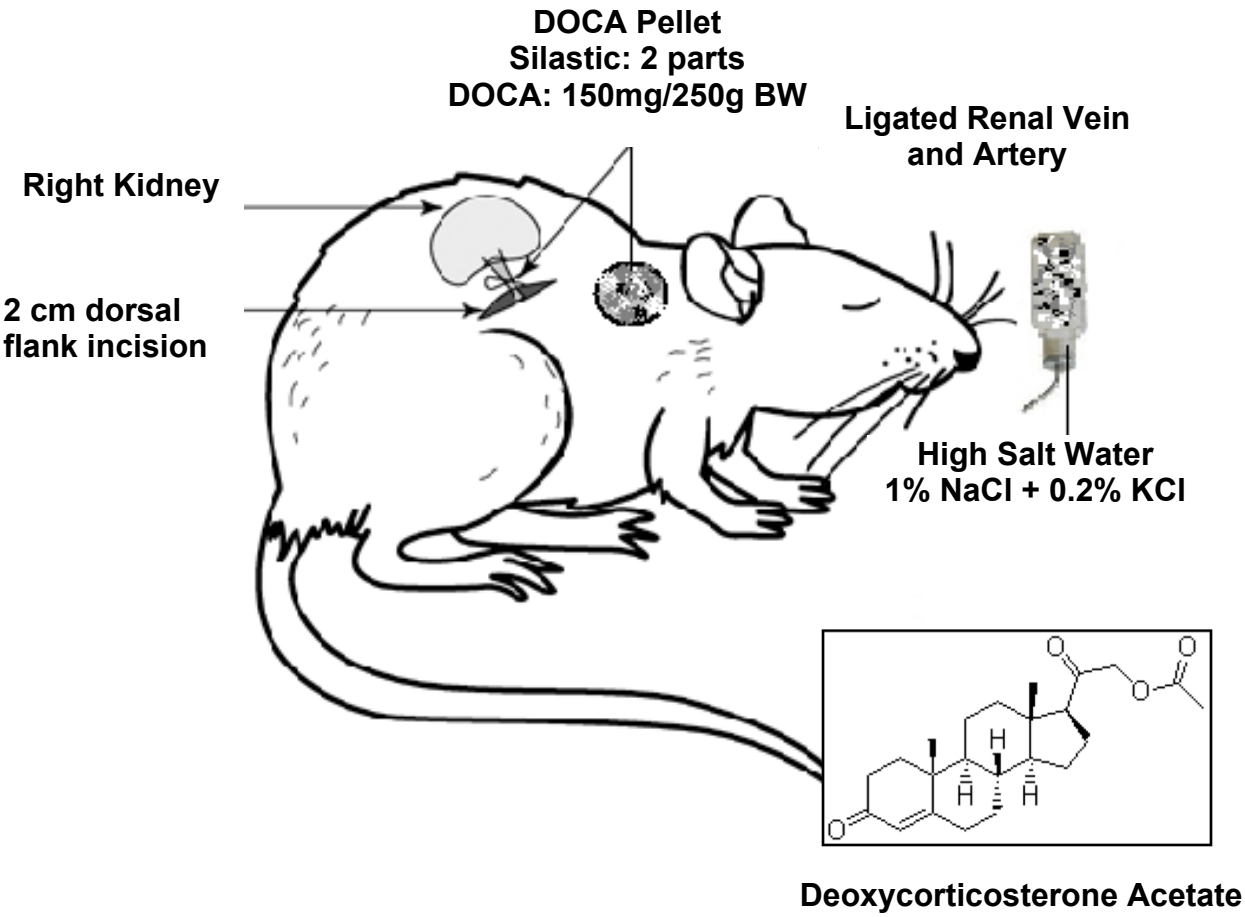
The rat was placed in an anesthesia chamber and allowed to breathe a mixture of 2-4% isoflurane in oxygen (2.0 L/min) until a surgical plane of anesthesia is achieved. The animal was positioned on a heated surgical field in dorsal recumbency with its nose inserted into an anesthesia mask and allowed to breathe a mixture of 0.5-2% isoflurane and oxygen (2.0 L/min). The animal was maintained on this anesthesia protocol for the duration of the surgery and closely monitored for the depth of anesthesia.

Deoxycorticosterone Acetate (DOCA)-salt Hypertension Induction

The animal's fur on the left flank and the back of the neck, between shoulder blades, was shaved and the skin was disinfected with chlorhexadine scrub and alcohol. A 1.5 cm vertical incision was made at the left flank, through the skin and underlying muscle just caudal to the rib cage. The left kidney was exteriorized and perirenal fat was separated from renal blood vessels. The renal artery, vein and ureter were ligated with 5-0 silk sutures and the kidney was removed. The muscle and skin layers were closed separately with 5-0 silk sutures. A 0.5-1.0 cm incision was made in the shaved area on the back of the neck, and the DOCA-pellet was implanted subcutaneously. Control animals only had uninephrectomy without DOCA-pellet implantation. The DOCA pellet

is made up of silastic and DOCA powder in a ratio of two parts silastic to one part DOCA. The DOCA pellet administered is 150 mg per implant per Kg body weight. Post surgery, animals received enrofloxacin antibiotic (5 mg/Kg, i.m.) and carprofen analgesic (5 mg/Kg, s.c.). Rats were housed under standard conditions for 4 weeks. All rats received standard rat chow (Harlan/Teklad 8640 Rodent Diet); however, DOCA and control rats received (ad libitum) salt water (1% NaCl + 0.2% KCl), and distilled water, respectively. Figure 11 summarizes the DOCA-salt hypertension experimental design.

Figure 11: Deoxycorticosterone Acetate-Salt (DOCA)-Salt Model



The chemical structure at the bottom right is that of DOCA. Male Spraque-Dawley rats are uninephrectomized and received DOCA pellet 150 mg/implant/250 g body weight. Control animals only had uninephrectomy without DOCA pellet implantation. DOCA and control rats received (*ad libitum*) salt water (1% NaCl + 0.2% KCl), and distilled water, respectively for 4 weeks.

Blood Pressure Measurement

Tailed-Cuff Plethysmography: Blood pressure in conscious rats were measured using the CODA rat tail-cuff system (Kent Scientific Corporation, Torrington, CT). The animals were restrained and allowed to sit quietly to pre-warm for 10 minutes. The entire measurement process was automated via a computer program that controlled the system. The tail-cuff was inflated five times to 250 mmHg and slowly deflated over a period of 15 seconds. Blood pressure was obtained during each inflation cycle by a volume recording sensor. The reported blood pressure was the average of the five readings.

Radiotelemetry: Rat was anesthetized following the protocol in the General Anesthesia Induction and Maintenance section. The anesthetized rat's fur over the ventral abdomen and left inner thigh region was shaved and the skin was surgically prepared with 3 alternating rounds of chlorhexadine scrub and alcohol. The surgical site was draped with sterile gauze. The catheter of a radiotelemetry-based pressure transmitter (TA11PA-D70, DSI, St. Paul, MN) was implanted into the femoral artery and the body of the transmitter placed subcutaneously at the inner thigh. Rats were allowed 4 days to recover postoperatively; with free access to food and water, each rat was housed in individual cages on top of a radiotelemetry receiver (RPC-1, DSI) that was connected to a data exchange matrix and computerized data acquisition program (Dataquest ART 3.0, DSI) to monitor arterial pressure remotely. Mean arterial pressure was sampled for 10 seconds every minute for the 24 hours period.

Immunohistochemistry

Second order MA from DOCA-salt and sham rats were excised and cleaned of perivascular fat. MA (1 cm) were fixed in 4% paraformaldehyde overnight at 4°C, blocked with 0.1% Triton X-100 blocking serum for 1 hour. All incubations were done at room temperature, incubated with appropriate 1°antibodies for 2-hour followed by a 1-hour incubation with appropriate 2nd antibodies (Table 4). MA were washed with 0.01 M PBS (composition mM: NaCl 13, KCl 2.7, Na_2HPO_4 10, and KH_2PO_4 2) between each incubation. MA were mounted under a glass coverslip with anti-fade gold solution (Vector Laboratories, Burlingame, CA), and images were acquired using confocal microscope (Leica Microsystems, Buffalo Grove, IL).

Table 4: Source of Primary and Secondary Antibodies and the Working Dilutions

Primary Antibodies				
Antigen	*Target*	*Source*	*Host*	*Dilutio*
CD163	MΦ	AbD Serotec	Mouse	1:200
p22Phox	NADPH Oxidase	Santa Cruz	Rabbit	1:400
TNF-α	Inflammatory cytokine	Santa Cruz	Goat	1:200
NPY	Sympathetic nerves	Amersham Biosciences	Rabbit	1:200
Secondary Antibodies				
Target Species	*Host Species*	*Conjugated to*	*Dilution*	
Mouse	Donkey	FITC	1:50	
Rabbit	Donkey	Cy3	1:400	
Goat	Donkey	Cy3	1:400	

Dihydroethidium (DHE) Staining

When DHE reacts with O_2^-, ethidium bromide is formed and intercalated into DNA yielding a red fluorescent signal when excited at 488 nm. MA were removed from euthanized rats in chilled KRH solution (composition mM: NaCl 130, KCl 1.3, Ca_2Cl_2 2.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 1.0, glucose 0.09; pH = 7.4). Blood vessels were incubated with 2 μM DHE solution for 1 hour at 37°C. Following DHE incubation, blood vessels were washed with KRH solution and mounted for microscopy. Confocal fluorescence images were obtained with 488 nm excitation wavelength and collected at >560 nm wavelengths.

Amperometric Measurement of Norephinephrine

Carbon Fiber Microelectrode Preparation

Heat-treated (3000°C) pitch-base type carbon fiber (Specialty Materials Incorporation, Lowell, MA), with nominal diameter of 35 μm, were attached to a copper wire (2 inches) by silver epoxy. The carbon fiber-copper wire assembly was then inserted into a polypropylene pipette tip (PF2411; Dot Scientific, Burton, MI) and the tapered end was carefully heated in a micropipette puller. The heat softened the polypropylene which caused it to flow over the carbon fiber surface insulating the electrode. The resulting microelectrode possessed a cylindrical architecture with an exposed length of 700-900 μm. Ionic composition in Krebs solution can deteriorate the surface of carbon fiber microelectrode. The oxidation current is found to decay over time after used for several

days. The microelectrode may require reconditioning with purified isopropyl alcohol to improve microelectrode sensitivity.

Focal Stimulation and Real-time Measurement of NE from Perivascular Nerves

Secondary or tertiary MA were isolated, cleaned of fat and connective tissue; they were placed into a small silicone chamber and perfused with 37°C, oxygenated Krebs' buffer (composition mM: NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; dextrose, 11) at flow rate 4 ml/min. Tissues were allowed to equilibrate for 1 hour before beginning experiments. If the experiments required drug treatment, the drug solution was perfused for 40 minutes prior to the recording of NE oxidation currents. The carbon fiber electrode was fixed to a micromanipulator and positioned parallel to the blood vessel so that it detected the NE flux from nearby release sites on its surface. Ag-AgCl reference electrode (Cypress Systems Inc., Fresno, CA) was positioned in the chamber and oxidation currents were recorded with a BioStat Multi-mode potentiostat (ESA Products, Chelmsford, MA). 600 mV applied potential was used to detect NE, and short train of electrical stimulation (10Hz, 5 s, 80V, Grass Instruments; Quincy, MA) was applied to trigger NE release using a bipolar focal stimulation electrode (2 AgCl-coated Ag wires inserted into a double-barreled capillary glass wit tip diameter ~180 μm) positioned along the surface of the vessel. Figure 12 illustrates the experimental set up for the focal stimulation and real-time measurement of NE.

Figure 12: Experimental set up for the focal stimulation and real-time measurement

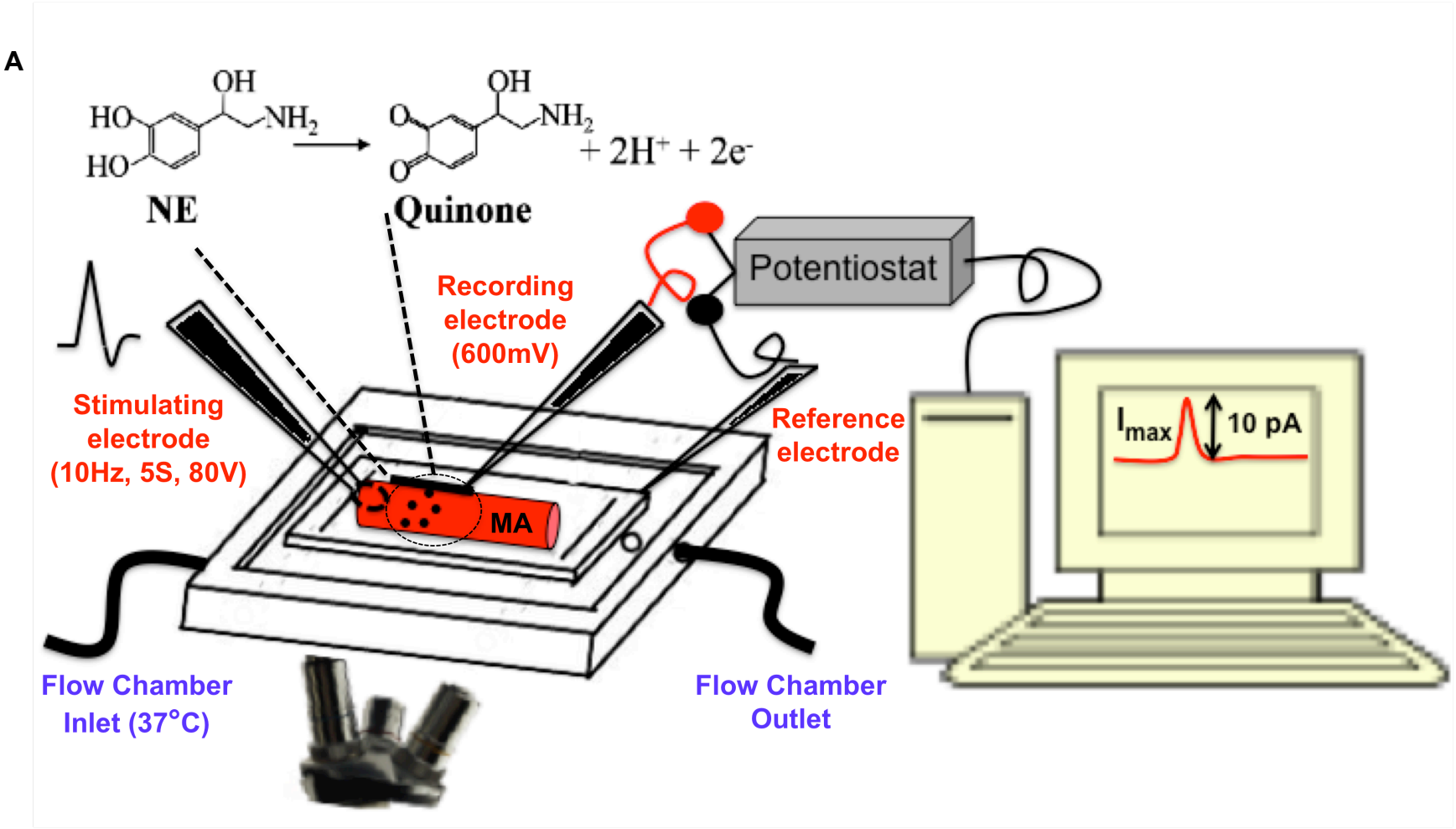
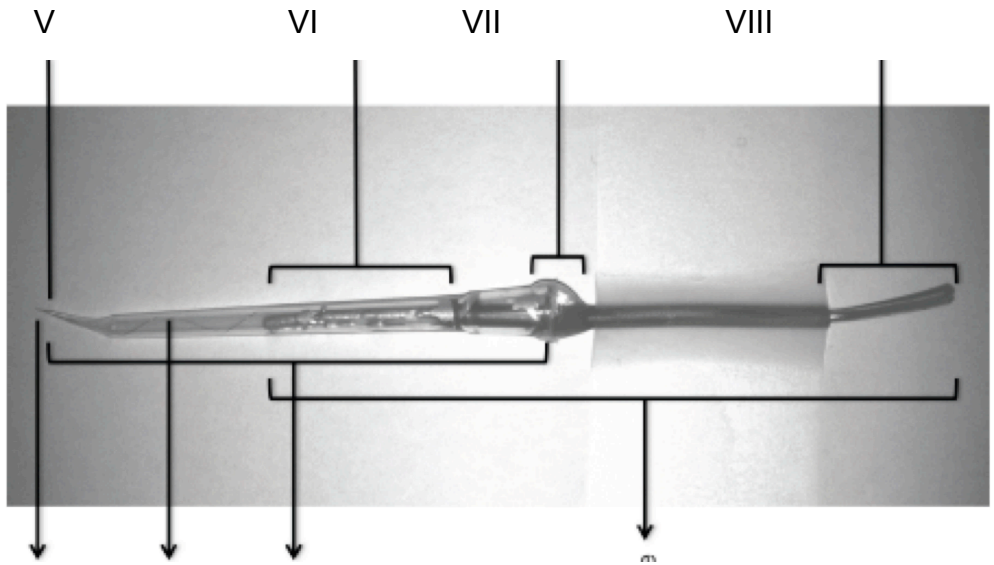


Figure 12 (cont'd)

B



I: 700-900 μm tip

II: carbon fiber

III: polypropylene pipet tip

IV: silver wire

V: fiber carbon electrode tip

VI: silver epoxy

VII: expoxy glue

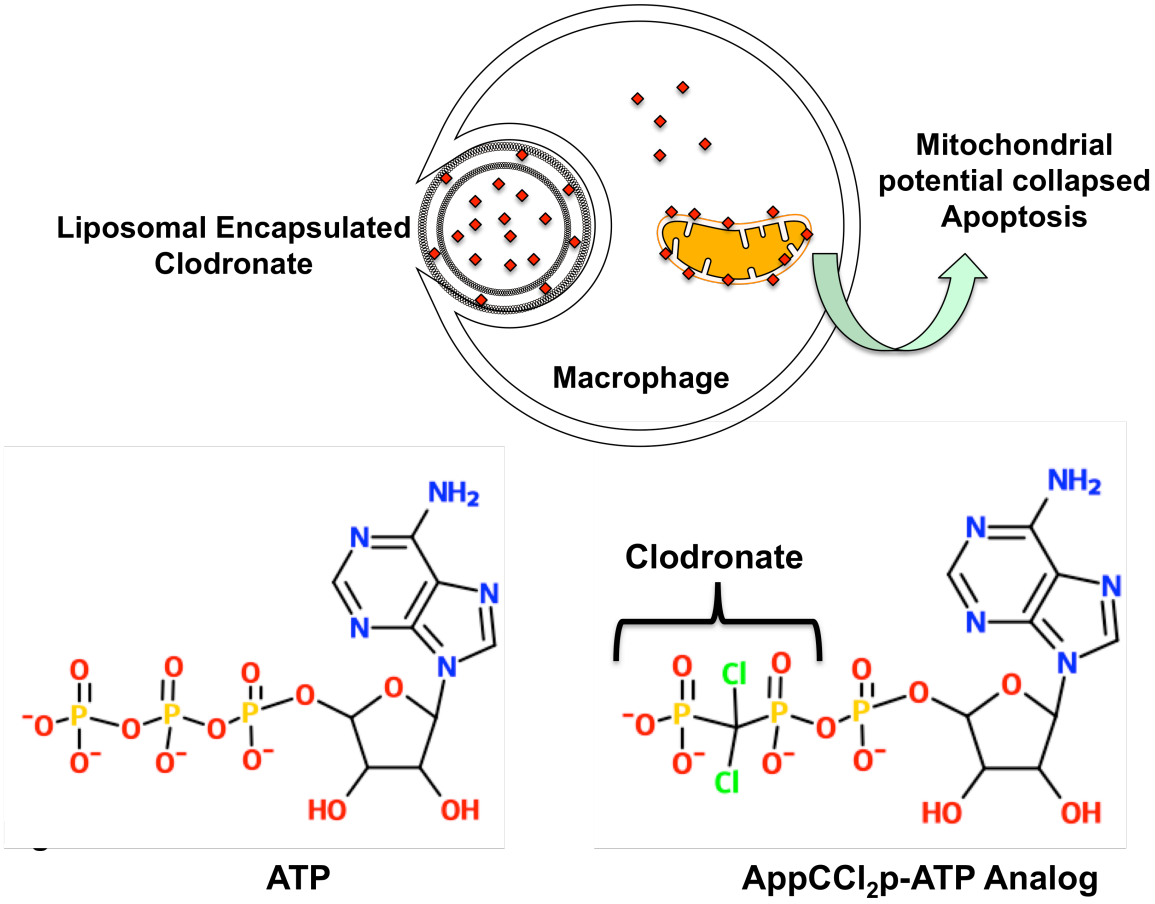
VIII: un-insulated silver wire

A) MA is superfused with 37 ◦C in a flow chamber. A bipolar stimulating electrode (10Hz, 5S, 80V) is used to evolk NE release from perivascular nerves. A recording electrode (holding at 600 mv) is used to detect the oxidation current of NE. The potentiostat converts the current into digital signal, which is displayed on the computer screen. B) The picture of a representive fiber carbon electrode and its parts.

Liposomal-encapsulated Clodronate (LEC) Depletion of MΦ:

Clodronate is a member of the family of bisphosphonates that lacks a nitrogen group at the R_2 position, and it is considered the best drug to deplete MΦ from organs and tissues in vivo because of its maximum efficacy and minimal toxicity. Clodronate can formed an AppCCl_2p-ATP analog intracellularly, which is cytotoxic to MΦ in vitro. AppCCl_2p-ATP analog inhibits mitochondrial oxygen consumption by a mechanism that involves competitive inhibition of the ADP/ATP translocase, and eventually causes the collapse of the mitochondrial membrane potential. By itself clodronate is not a toxic drug; free clodronate will not easily pass phospholipid bilayers. However, when capsulated in a liposome, MΦ will phagocytose clodronate and it will not escape from phagocytic cells. Once the LEC are inside the MΦ lysosomal phospholipases disrupt the membrane and the clodronate accumulates intracellularly until a threshold concentration is reached where the cell is irreversibly damaged initiating apoptosis. Free clodronate released from dead MΦ has very short half-life (15 minutes) in the circulation and it is quickly removed by the kidney. In short, clodronate can be used to remove MΦ in the liver, spleen, lung, peritoneal cavity, and lymph nodes (1). Systemic injections of LEC in mice deplete 90% of peripheral monocytes and tissue MΦ within 24 hours, and MΦ reappearance does not take place until around 1 week later (2). Moreover, LEC appears to have a very selective effect on MΦ and phagocytic dendritic cells, and not on neutrophils and lymphocytes (3, 4). Figure 13 shows the structure of clodronate, its AppCCl_2p-ATP analog, the mechanism of cellular damage, and apoptosis.

Figure 13: Liposomal-encapsulaed clodronate structure and cellular mechanism of apoptosis



Clodronate, a member of non-nitrogenated bisphosphonates, is encapsulated inside a double lipid bilayer. Upon macrophage phagocytosis of the LEC, clodronate is release by phospholipases. Inside the cell, it forms an AppCCl2p-ATP analog that disrupts the ADP/ATP translocase and causes apoptosis.

LEC Prepration: Clodronate was a gift of Roche Diagnostics GmbH. Clodronate (0.6 M) or PBS are encapsulated in liposomes composed of phosphatidylcholine (100 mg/ml) and cholesterol (0.8 mg/ml)(52). ~1% of the clodronate will be encapsulated in the liposomes. The non-encapsulated clodronate will be removed by centrifugation. After washing with sterilized PBS, the LEC are re-suspended into sterilized PBS at 5 mg clodronate/ml.

MΦ Depletion Treatment Plan: The treatment groups to be used for the MΦ-depletion experiment are outlined in table 5. To remove peritoneal MΦ, rats were treated with LEC. Control groups received liposome-encapsulated PBS (lipo-PBS). After the animals have been implanted with a radiotemeter for 1 week, DOCA-salt animals received subcutaneously (250 mg/kg) DOCA pellets + either intravenously LEC or lipo-PBS; and similarly sham animals received sham pellets + either LEC or lipo-PBS. The initial dose was 5 mg clodronate/ml per 100 g body weight. Thereafter 2.5 mg clodronate/ml per 100 g body weight was administered intraperitoneally every 7 days. All rats received standard rat chow; however, DOCA, and sham rats received (*ad libitum*) salt water (1% NaCl + 0.2% KCl), and distilled water, respectively.

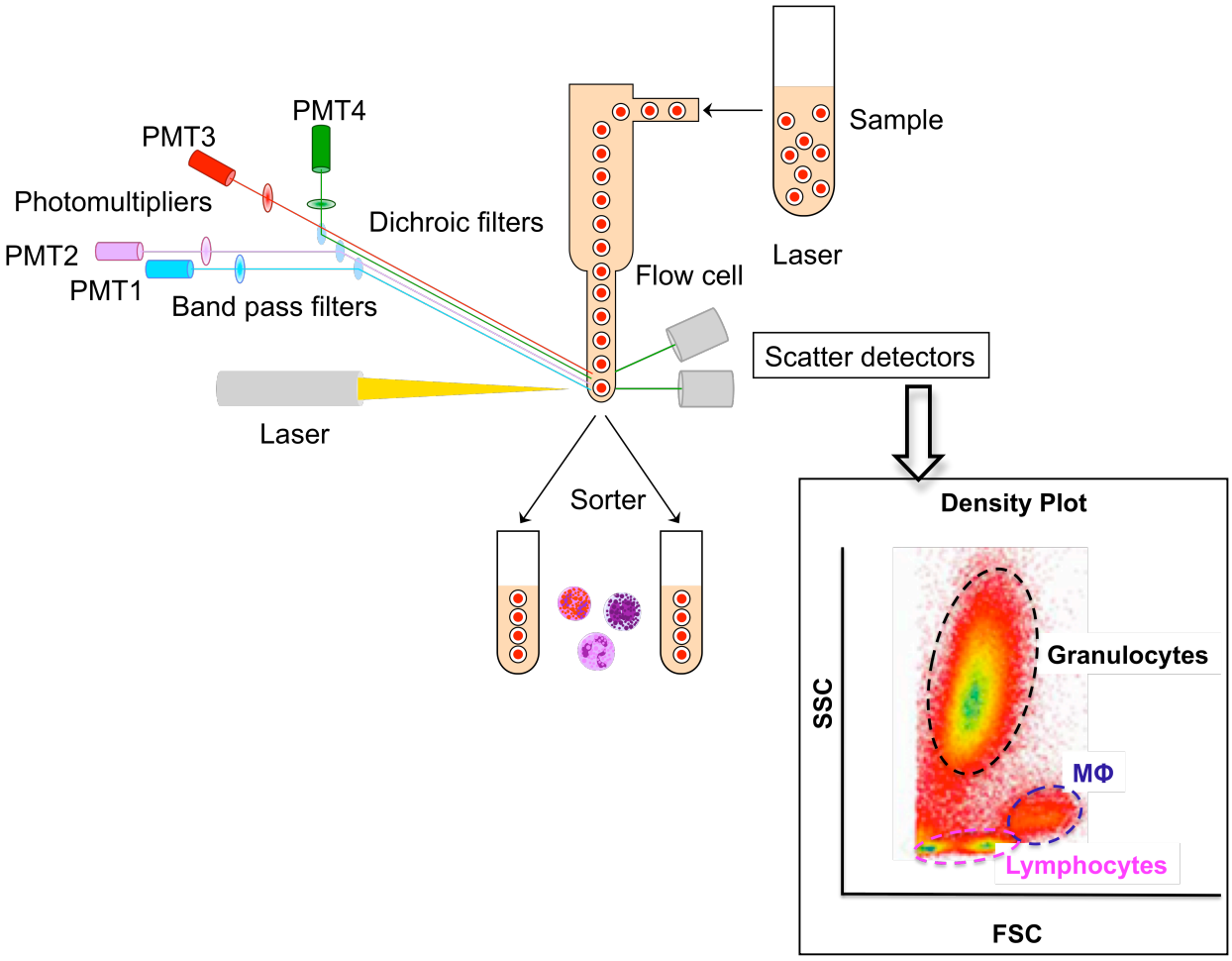
Table 5: The Treatment Groups for the MΦ-depletion Experiment

Primary	Secondary Treatment
Sham	PBS liposomes
Sham	Clodronate liposomes
DOCA-salt	PBS liposomes
DOCA-salt	Clodronate liposomes

Flow-cytometry

After anesthesia, the rat abdomen was exposed and peritoneal cells were collected with 1x HBSS calcium free (Invitrogen). The cells were spun-down at 300-xg centrifugation in 4°C. Erythrocytes were removed by ACK lysing buffer (Invitrogen). Total number of viable leukocytes was determined using Trypan Blue and Automatic Cell Counter (Bio-Rad). In 1 million cells suspension, the Fc receptors were block with Fc Block (BD Pharmingen), and MΦ were identified with a CD11b-FITC and CD163-PE antibody (AbD Serotec). Lastly, cells were fixed with Cytofix (BD Biosciences). Data was acquired on an LSRII flow cytometer (BD Biosciences). Figure 14 illustrates the mechanisms and principles of how flow-cytometry identified subtypes of leukocytes.

Figure 14: Mechanisms and principles of flow-cytometry



Forward Scatter Channel (FSC) describes the light that is scattered in the forward direction, typically up to 20° offset from the laser beam's axis and its intensity roughly equates to the cell's size. Whereas, the Side Scatter Channel (SSC) defines the light that is measured approximately at a 90° to the excitation line, and it provides information about the granular content within a cell. Base on these two parameters, we could identify types of leukocytes: granulocytes, MΦ, or lymphocytes. In addition, using fluorescent-labeled antibodies e.g. CD11b-FITC and CD163-PE, we could identify the specific subpopulation within a type of leukocytes, and the intensity of the fluorescent also measures the levels of that protein.

Drugs:

Idazoxan, UK 14304, H-89, cocaine, forskolin, PTX, NaF and $AlCl_3$ were obtained from Sigma Chemical (St. Louis, MO). M119 was obtained from the chemical diversity set from the Developmental Therapeutics Program from the NCI/NIH. M119 is referenced as compound NSC 119910 within that series. Idazoxan, H-89, cocaine, M119 and NaF and $AlCl_3$ were diluted in deionized water, while UK-14304 and forskolin were dissolved in DMSO to make a concentrated stock solution. Working solution of UK-14304 contained <0.01% of DMSO. Final solutions were made in Kreb's buffer at the time of experiment.

For the PTX experiments, 60 µl of the stock PTX (dissolved in water) is mixed with 2 ml OPTI-MEM buffer to make a PTX working solution (50 µg/500 µl). The MA with its perivascular fats removed is pinned down and incubated in the PTX working solution at 37°C for 2 hours prior to experimental studies.

Statistics

Data are presented as mean ± SEM and "n" is the number of animals from which the data were obtained. Data were analyzed with Graphpad Prism using Student's t test, paired t test, one- or two-way ANOVA with Bonferonni's post hoc test, and nonparametric data were analyzed with Mann-Whitney or Kruskal-Wallis' test. For multivariate analysis of flow cytometry data, Flowjo 8.8.6 was used for probability binning comparison. Probability binning comparison is nonparametric test designed specifically for analysis of flow-cytometry, particularly when multiple parameters are

measured simultaneously. In order to carry out probability binning comparison, multivariate data must first be divided into multidimensional bins such that the control sample has the same number of events in each bin. Therefore, when selecting an event at random from the control population, there is an equal probability that it will fall into any given bin. The bins defined by the control population are then applied to a comparison sample. The number of events falling within each bin is determined, and the normalized chi-squared value is calculated (5). For all tests, differences were considered significant when $P<0.05$.

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CHAPTER 3

ADVENTITIAL MACROPHAGE INFILTRATION INTO MESENTERIC ARTERIES AND α_2 ADRENERGIC AUTORECEPTOR IMPAIRMENT DURING DOCA-SALT HYPERTENSION DEVELOPMENT

Abstract

DOCA-salt hypertension in rats is associated with the impairment of α_2-adrenergic autoreceptor (α_2R) function, and increased levels of superoxide (O_2^-) and macrophage (MΦ) number in the mesenteric arterial (MA) adventitia. However, the relationship between MΦ infiltration, O_2^- production and α_2R impairment are unknown. This study tests the hypothesis that there is a time dependent infiltration of activated MΦ into the MA adventitia of DOCA-salt hypertensive rats. This infiltration is associated with an increase in O_2^- and impaired α_2R function in later phases of hypertension development in rats. DOCA-salt hypertension developed biphasically with phase 1 occurring during days 5-10 and phase 2 after day 10. O_2^- levels in DOCA-salt MA were twice that in control MA during day 10-28. MΦ numbers in MA from DOCA-salt rats were 4-5x higher than in controls during the same time period. There was no detection of MΦ infiltration into skeletal muscle arteries from DOCA-salt rats. Impairment of α_2R function did not occur until after day 18. Infiltrated MΦ were in close apposition with perivascular sympathetic nerves, and they expressed TNF-α and the $p22^{phox}$ subunit of NADPH oxidase. Finally peritoneal MΦ were activated beginning at day 10; thus, they could be the source of MΦ in the MA adventitia. The data show that as DOCA-salt hypertension develops peritoneal MΦ were recruited to the MA adventitia where they release O_2^-. O_2^- disrupts the α_2R function causing increased NE release further increasing blood pressure.

Introduction

One billion people worldwide have hypertension and 60% of Americans are pre-hypertensive (1). This is a major health concern because hypertension increases the risk for cardiovascular diseases (2). The causes of hypertension are complex, but sympathetic nerve activity is elevated in many hypertensive humans and some animal models of hypertension including the deoxycorticosterone acetate (DOCA)-salt model (3). Increased sympathetic nerve activity in DOCA-salt hypertension suggests that there may be alterations in the local mechanisms that modulate sympathetic neurotransmission (4, 5). α_2- adrenergic autoreceptor (α_2R) function is impaired at sympathetic nerve endings of mesenteric arteries (MA) in DOCA-salt hypertension (6, 7). Interestingly, there is some controversy about the mechanisms causing DOCA-salt hypertension as these vary over the time course of hypertension development (8).

Reactive oxygen species (ROS) particularly O_2^- contribute to hypertension development. This conclusion is supported by studies showing that the superoxide dismutase mimetic, tempol, lowers blood pressure and sympathetic nerve activity in DOCA-salt hypertension (9). A major source of O_2^- is a multi-subunit enzyme, Nox-based nicotinamide adenosine dinucleotide phosphate (NADPH) oxidases, which contributes to hypertension (10, 11). In phagocytes such as macrophages (MΦ), NADPH oxidase has an intracellular $p47^{phox}$ subunit and a cytochrome b558 catalytic core composed of membrane bound subunits, Nox-2/$gp91^{phox}$ and $p22^{phox}$ (12). Hypertensive stimuli up-regulate $p22^{phox}$ subunit expression, and enzyme activity.

Interestingly, mice deficient in this enzyme have a blunted level of DOCA-salt hypertension (13) and inhibition of this enzyme prevented up-regulation of ICAM-1, and MΦ infiltration into the aorta of Angiotensin II-induced hypertensive rats (14). Despite the ample evidence that ROS contributes to hypertension, the source of O_2^- is not entirely clear.

Vascular inflammation may be involved in both the initiation and development of hypertension (15, 16, 17). Although it is known that hypertension involves inflammation, most studies focus on the contribution of lymphocytes (18) and there have been no studies of how these cells might interact with periarterial sympathetic nerves. Thus, there is a need to understand the interaction between inflammatory mechanisms and sympathetic nerve function in controlling blood pressure. Recruitment, activation and proliferation of MΦ in the vascular adventitia occurs in hypertension and this response may contribute to the vascular consequences of hypertension (19, 20). For example, mice deficient in MΦ colony-stimulating factor exhibit reduced vascular inflammation and they are protected against damage caused by DOCA-salt hypertension (21). However, it is unclear whether high blood pressure induces MΦ infiltration into the adventitia of MA and if infiltrated MΦ release O_2^- to disrupt sympathetic neuroeffector transmission causing further increases in blood pressure. This study tests the hypothesis that there is a time dependent infiltration of activated MΦ into the adventitia of the MA of DOCA-salt hypertensive rats. This infiltration is associated with a progressive increase in O_2^- and impaired α_2R function, which contributes to increased NE release and further increased blood pressure.

Results

Time dependent MΦ infiltration and O_2^- production in MA of DOCA-salt rats

The number of CD163 positive MΦ in DOCA-salt MA adventitia was higher than in control MA (Fig. 15A-B). MΦ infiltration began at day 10-13 and remained high to day 28 (Fig. 15C). Some MΦ (Fig. 15E) were located spatially close to perivascular sympathetic nerve fibers (Fig 15D & F). While we detected sympathetic nerve fibers in arteries that supply abdominal skeletal muscle (Fig. 15G), there were no CD163 positive MΦ (Fig. 15H-I).

The relative level of O_2^- in the adventitia of MA from DOCA-salt rats (Fig. 16A) was significantly higher compared to controls (Fig. 16B). Semi-quantification of O_2^- levels using DHE fluorescence intensity, we found that O_2^- level increased in DOCA-salt rats relative to controls at day 10 through day 21 (Fig. 16C).

α_2R Impaired Function Begins at Day 18 in DOCA-salt Hypertensive Rats

Electrical stimulation and amperometry were used to measure real-time NE release from MA. Idazoxan, an α_2R antagonist, caused an increase in NE oxidation current equally in DOCA and control during day 3-5 (Fig. 17A) and 10-13 (Fig. 17B). However, during day 18-21, it was not able to increase the NE oxidation current to the level of the controls (Fig. 17C). UK 14304, an α_2R agonist, decreased the normalized NE current equally in DOCA and controls during day 3-5 (Fig. 17D) and 10-13 (Fig. 17E); however, the normalized NE current in DOCA-salt was significantly higher than

that of controls during day 18-21 (Fig. 174F). This pharmacological data suggest that the impairment of α_2R occurred during day 18-21 which was much later than the infiltration of MΦ.

Time course of MΦ activation in DOCA-salt hypertensive rats.

Using flow-cytometry we found that there are three peritoneal MΦ populations: those with low (200<CD11b$^{\text{fluorescent intensity}}$<2,000), intermediate (2,000<CD11b$^{\text{fluorescent intensity}}$<20,000) and high CD11b expression (CD11b$^{\text{fluorescent intensity}}$>20,000) (Fig. 18A). Between days 3-5 there were no differences in the percentage of MΦ with low, intermediate or high levels of CD11b expression (Fig. 18B). However, between days 10-21 control rats had a significantly lower percentage of CD11b$^{\text{high}}$ and a higher percentage of CD11b$^{\text{intermediate}}$ MΦ, respectively (Fig. 18B). Furthermore, when comparing control rats to DOCA-salt rats, CD11b$^{\text{high}}$ MΦ expressed significantly higher levels of CD163 (Fig. 18C). CD11b and CD163 are cell membrane integrin and hemoglobin-haptoglobin scavenger receptors, respectively. These two markers are elevated in activated MΦ (22, 23).

Activated MΦ express high levels of TNF-α and p22^{Phox} subunit of NADPH oxidase

The number of infiltrating MΦ in DOCA-salt was significantly higher in the adventitia of DOCA-salt compared to control rats (Fig. 19A & D). Furthermore, MΦ in DOCA-salt rats expressed higher levels of the pro-inflammatory cytokine, TNF-α (Fig. 19B, E & G). MΦ (Fig. 20A) found in the adventitia of MA from DOCA-salt rats

expressed higher levels of $p22^{Phox}$ subunit of NADPH oxidase compared to those in control rats (Fig. 20B-C).

The impairment of α_2R function, and the elevation of BP, MΦ number, and O_2^- level required the synergistic effect of both DOCA and high salt

When DOCA-treated or sham-treated rats were placed on distilled water and high salt water, respectively, they did not have elevated BP compared to DOCA-treated rats placed on high salt (Fig. 21A). In addition to not having high BP, these animals also did not have increased numbers of MΦ infiltration in their MA adventitia (Fig. 21B). Similarly, the levels of O_2^- in the MA adventitia of these animals were also not different than that of control animals. Idazoxan, an α_2R antagonist, increased the NE oxidation current equally in shams on high salt (Fig. 22A) or DOCA on distilled water (Fig. 22C) when compared to control animals. UK 14304, an α_2R agonist, decreased the NE oxidation current equally in shams on high salt (Fig. 22B) or DOCA on distilled water (Fig. 22D) when compared to control animals.

Figure 15: Identification of MΦ and sympathetic perivascular nerve in rat MA but not skeletal muscle arteries

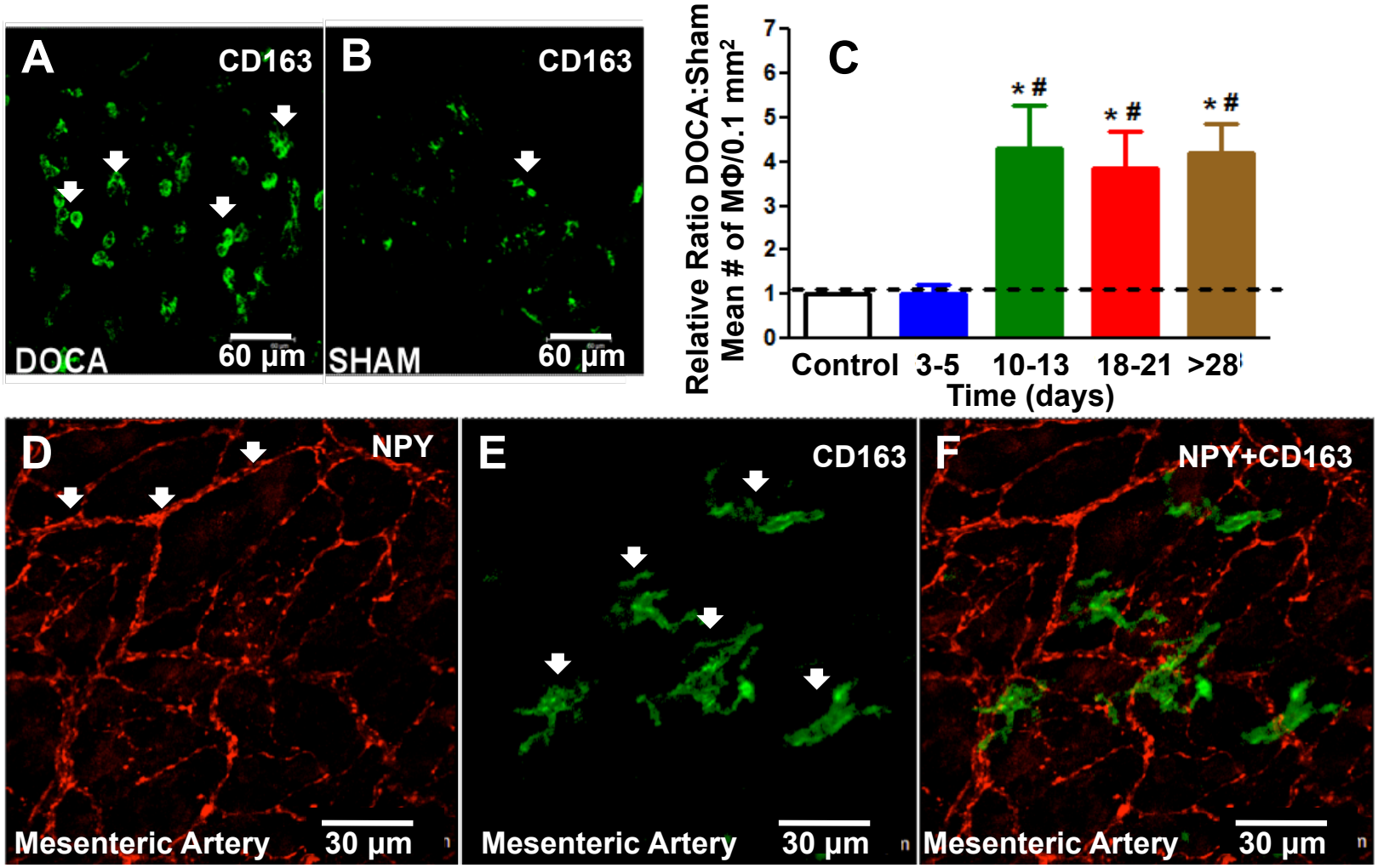
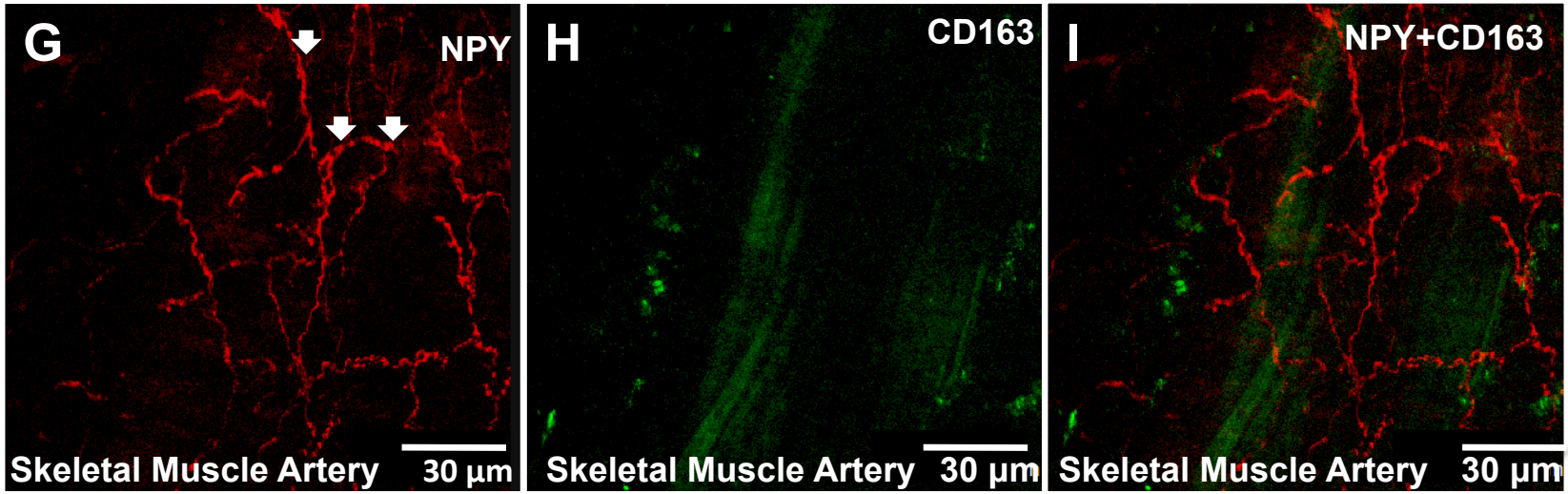
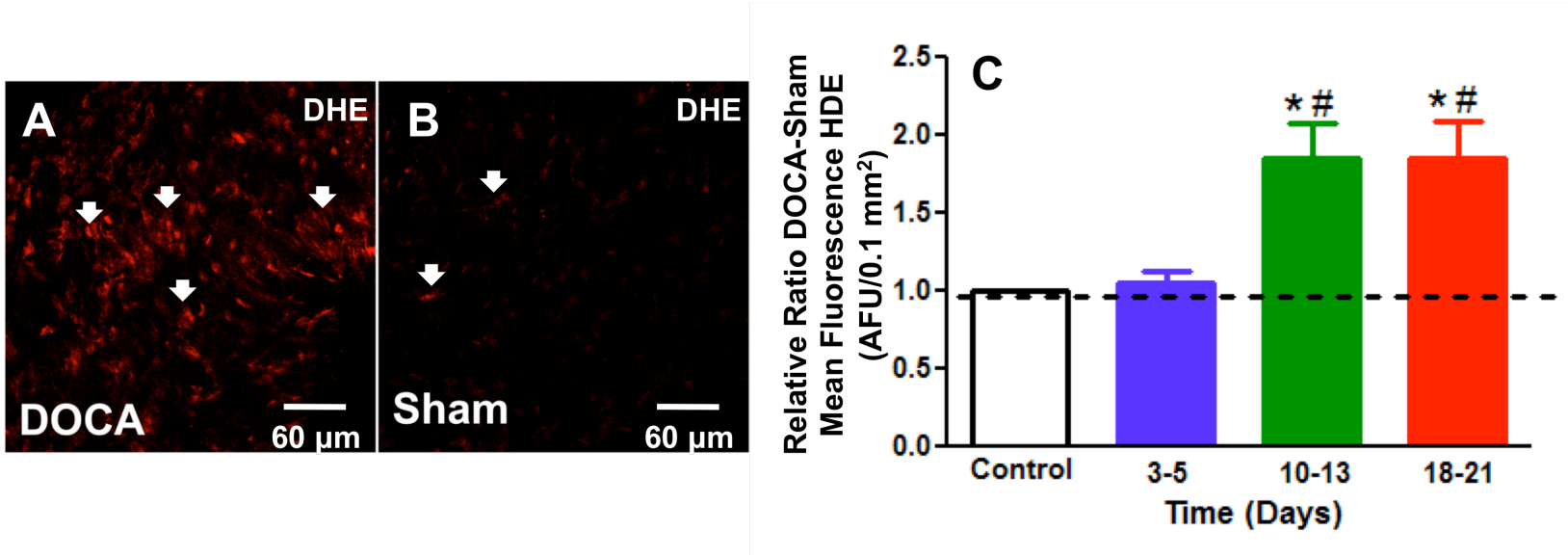


Figure 15 (cont'd)



(A) and control MA adventitia (B). Normalized mean number of MΦ /0.1 mm^2 showing 4-5X more MΦ in DOCA-salt MA adventitia compared to control starting day 10-28 DOCA-salt hypertension (C). The mean number of MΦ was calculated from 5 areas of 0.1 mm^2. Data are mean ± SEM and analyzed by one-way ANOVA and Bonferonni's post hoc test. * P<0.05 vs. Control, # P<0.05 vs. Day 3-5, (n=5). Whole-mount immunohistochemical labeling of perivascular sympathetic nerve (arrows) (D), MΦ (E) in DOCA-salt MA adventitia. F) Overlay of photomicrographs in D and E show a close spatial relationship between MΦ and sympathetic nerves. Whole-mount immunohistochemical labeling of perivascular sympathetic nerve (arrows) (G), but no MΦ (H) in arteries supplying abdominal skeletal muscle arteries a. I) Overlay of photomicrographs G and H.

Figure 16: Detection of superoxide anions in rat MA adventitia



Photomicrographs showing fluorescence of dihydroethidium (DHE) (arrows) in DOCA (A) and Sham rat MA adventitia (B). Normalized mean fluorescence intensity of DHE/0.1 mm^2 showing 2X higher in DOCA MA adventitia comparing to control starting day 10-21 DOCA-salt hypertension (C). The mean fluorescence intensity of DHE was calculated from 5 areas of 0.1 mm^2. Data are mean ± SEM and analyzed by one-way ANOVA & Bonferonni's post hoc test. * $P<0.05$ vs. Control, # $P<0.05$ vs. Day 3-5, (n=5).

Figure 17: α_2R is impaired during DOCA-salt day 18-21

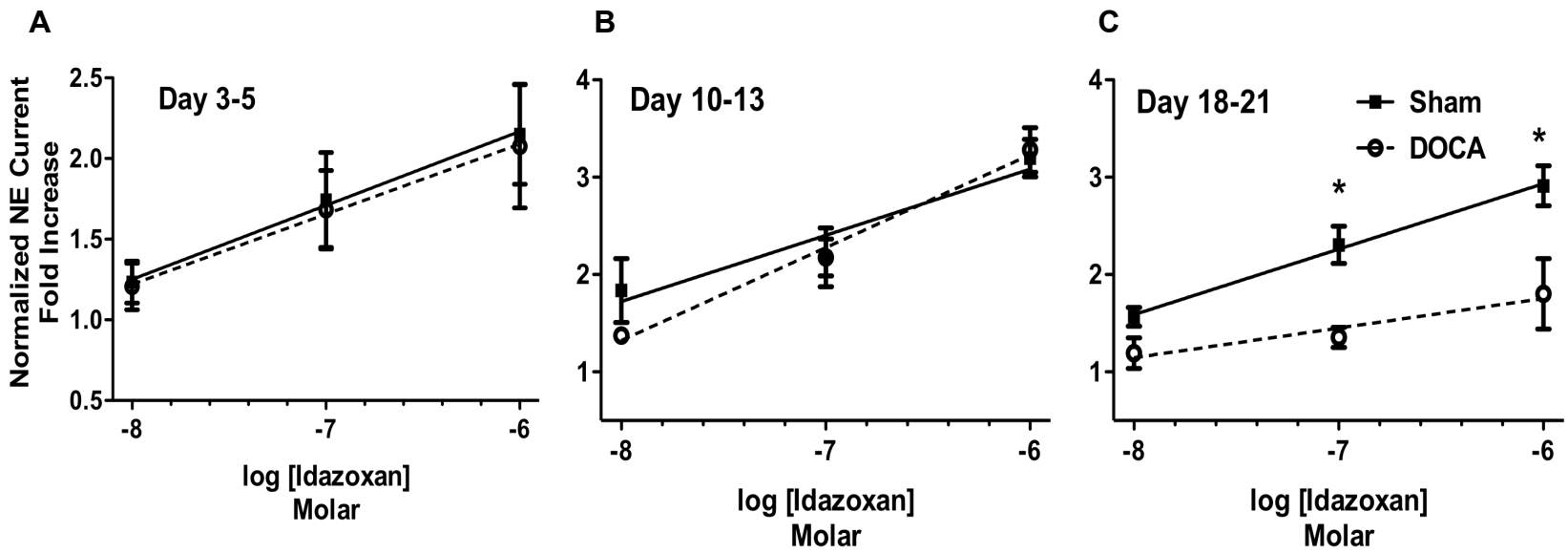
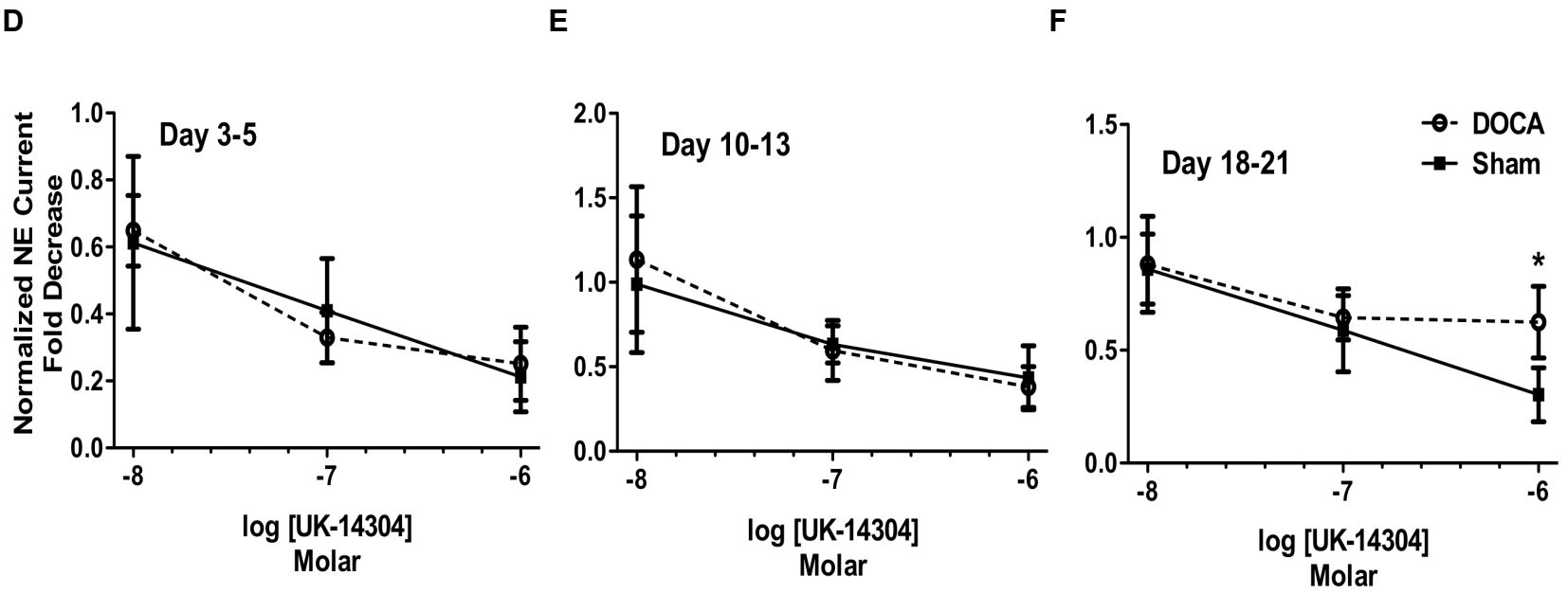


Figure 17 (cont'd)



Idazoxan, an α_2R antagonist, increased the normalized NE current equally in DOCA and control during DOCA-salt day 3-5 (A) and 10-13 (B), however, DOCA normalized NE current was significantly less than that of control during DOCA-salt day 18-21 (C). UK 14304, an α_2R agonist, decreased the normalized NE current equally in DOCA and control during DOCA-salt day 3-5 (D) and 10-13 (E), however, DOCA normalized NE current was significantly higher than that of control during DOCA-salt day 18-21 (F). Data are mean ± SEM and analyzed by two-way ANOVA & Bonferonni's post hoc test. * $P<0.05$ vs. Control, (n=5).

Figure 18: Time-course of peritoneal MΦ activation in DOCA-salt hypertensive rats

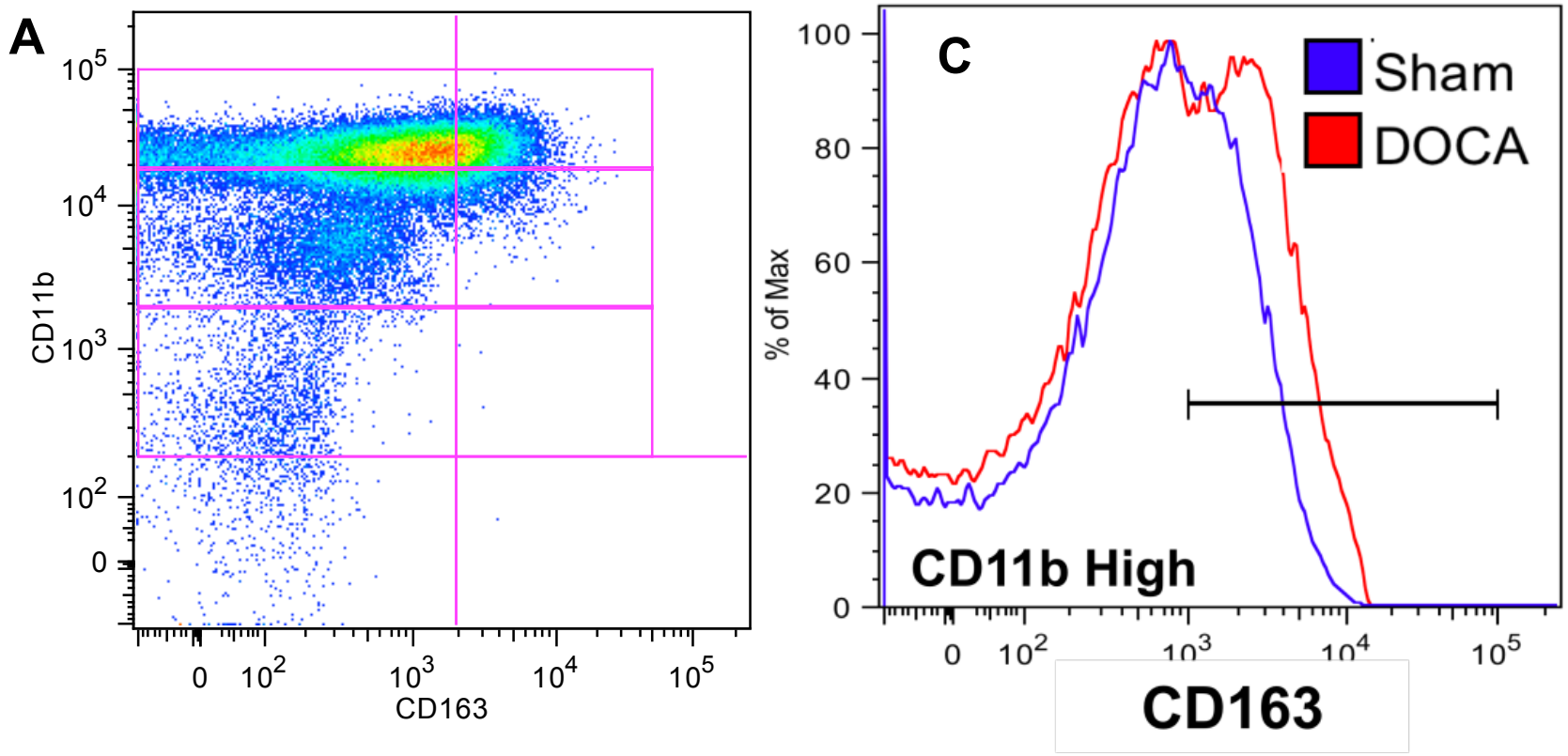
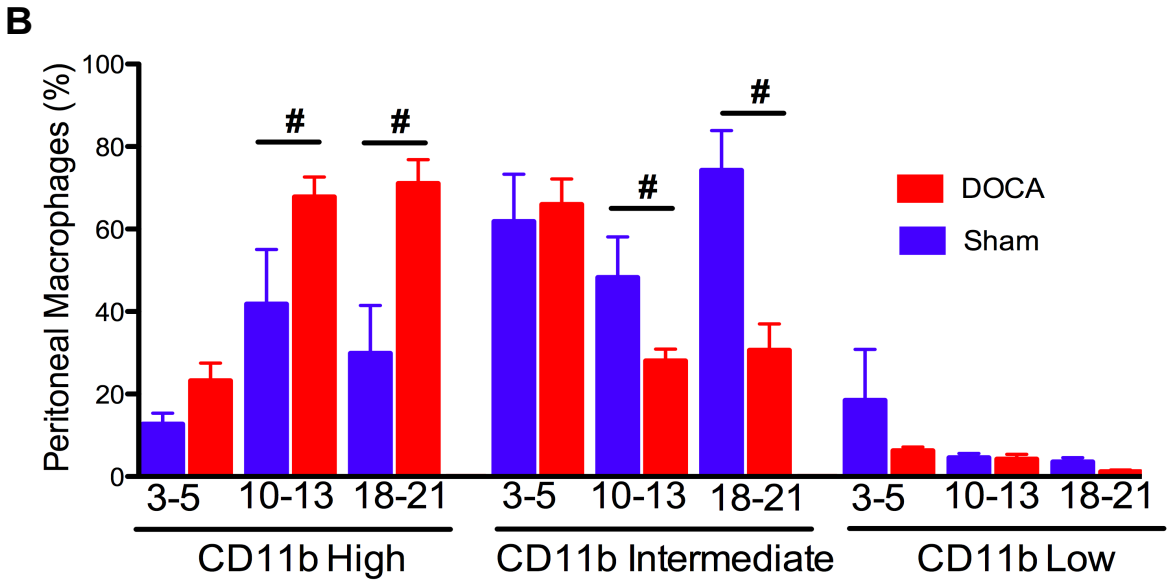


Figure 18 (cont'd)



Dot-plot of Day 28 DOCA-salt hypertensive peritoneal MΦ, showing three different populations of MΦ: CD11b low, intermediate, and high (A). Percentage of $CD11b^{high}$ MΦ was significantly higher in DOCA than control during DOCA-salt day 10-13 and 18-21 (B). In contrast, percentage of $CD11b^{intermediate}$ was significantly lower in DOCA than control during the same time periods (B). There were no significant changes in the $CD11b^{low}$ MΦ population. Data are mean ± SEM and analyzed Kruskal-Wallis' test. # $P<0.05$ vs. Control, (n=5). CD163 Fluorescence intensity histogram of $CD11b^{high}$ MΦ population showed a significantly higher express CD163 in DOCA comparing to control (C). Data are analyzed with Flowjo 8.8.6 by using probability binning comparison

Figure 19: Co-localization of MΦ and TNF-α in rat MA adventitia at day 28

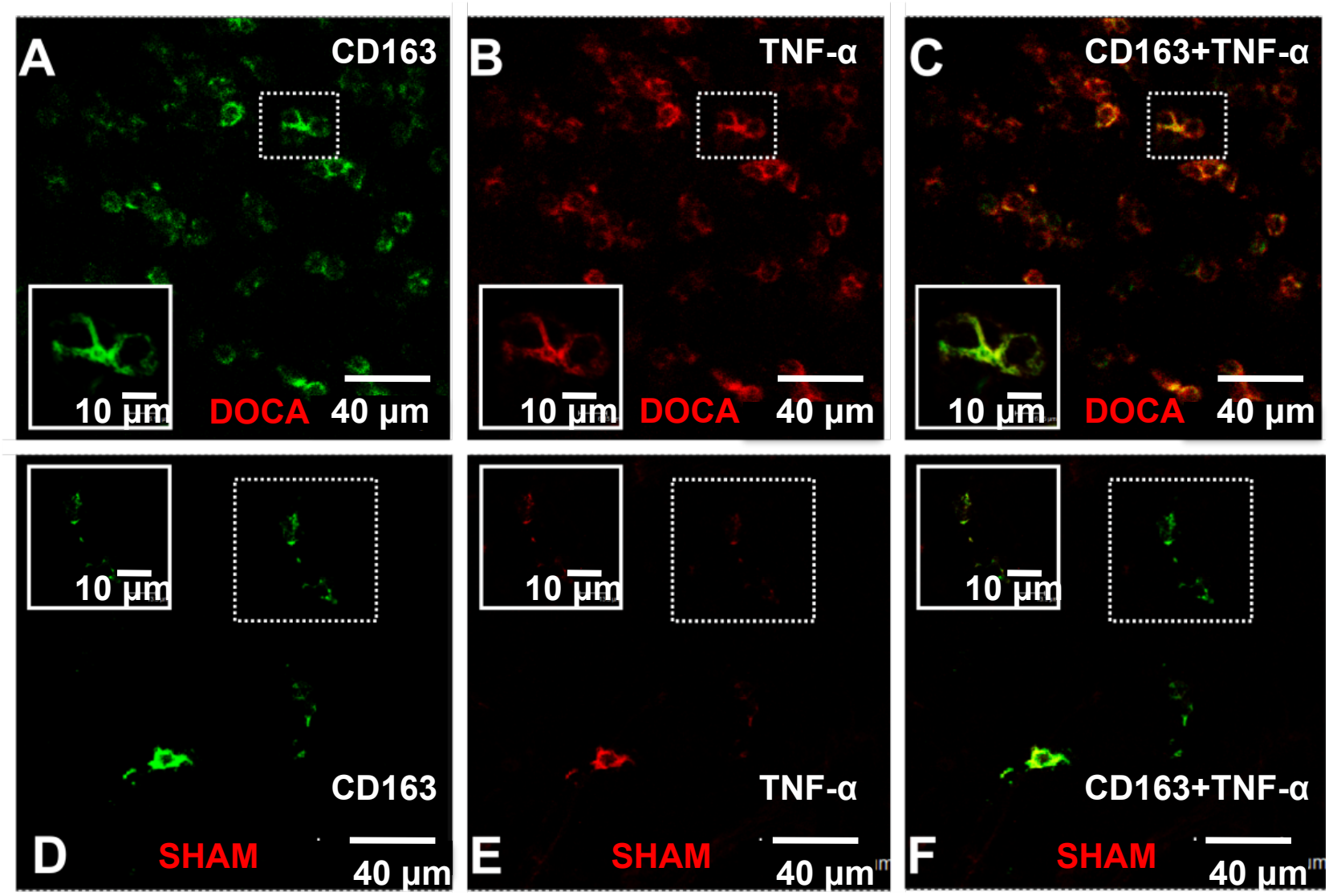


Figure 19 (cont'd)

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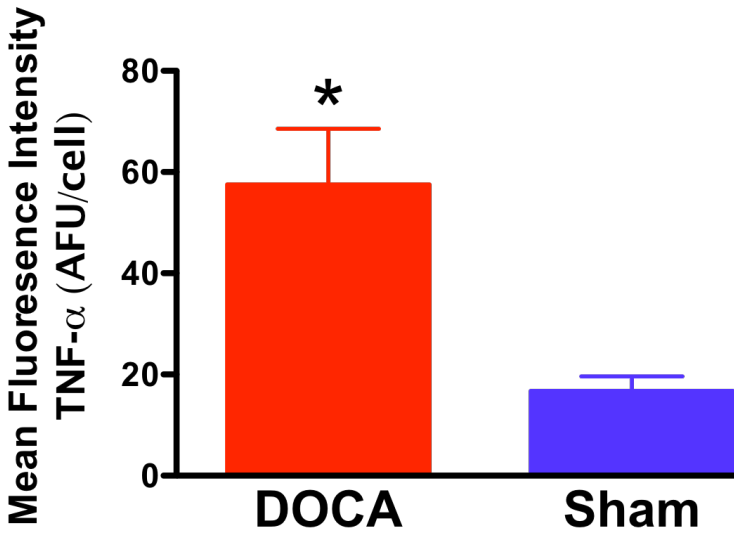

Whole-mount immunohistochemical labeling of CD163 positive MΦ (A), TNF-α (B), and overlay of MΦ with TNF-α (C) in Day 28 DOCA-salt hypertensive rat. Whole-mount immunohistochemical labeling of CD163 positive MΦ (D), TNF-α (E), and overlay of MΦ with TNF-α (F) in normotensive rat. (G) Mean MΦ TNF-α fluorescence intensity showing 3X higher in DOCA MA adventitial MΦ comparing to control. Data are mean ± SEM and analyzed by paired t test. * $P<0.05$ vs. Control, (n=5).

Figure 20: Co-localization of MΦ and p22Phox subunit of NADPH Oxidase in rat MA adventitia at day 28

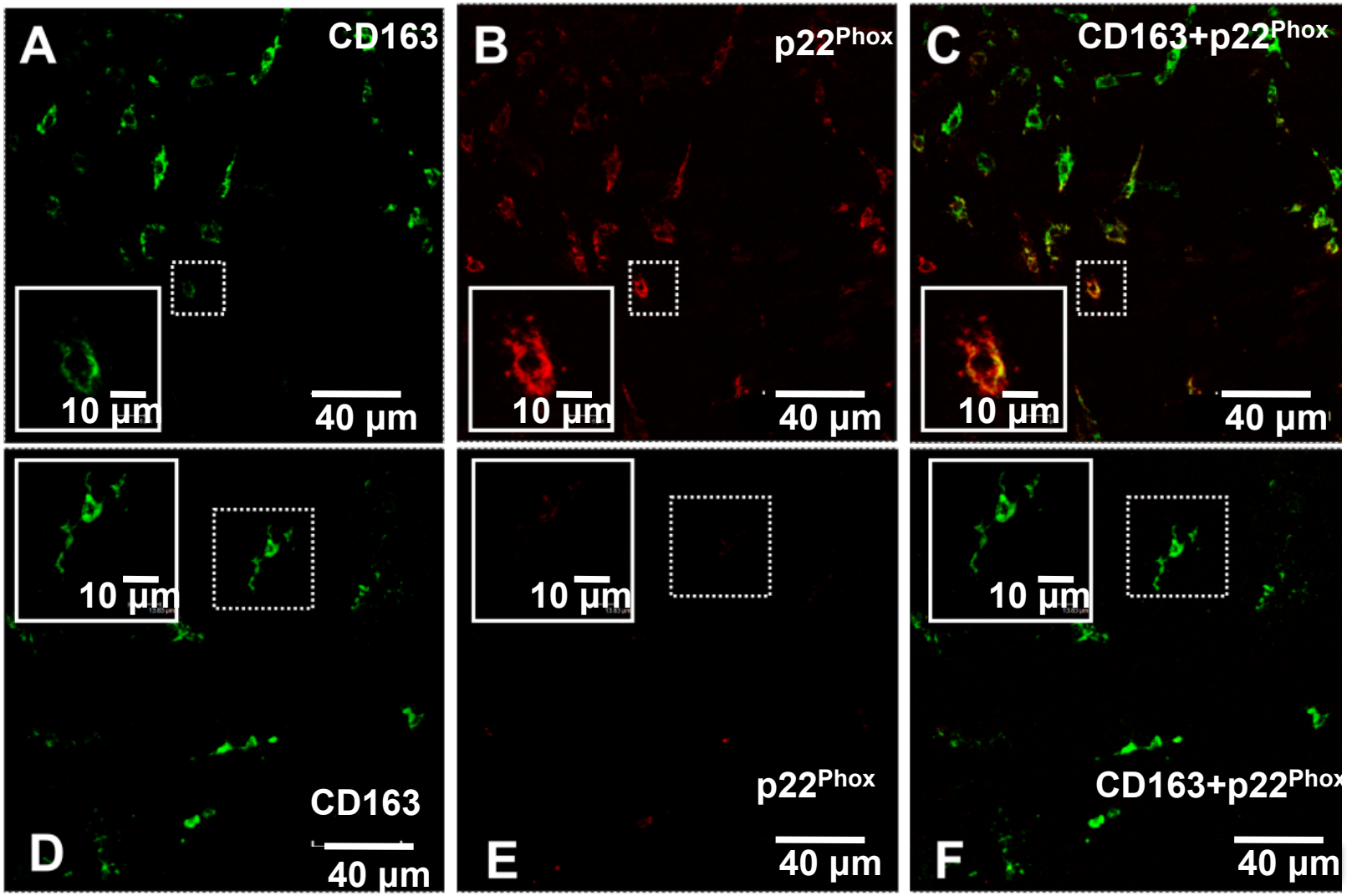

Figure 20 (cont'd)

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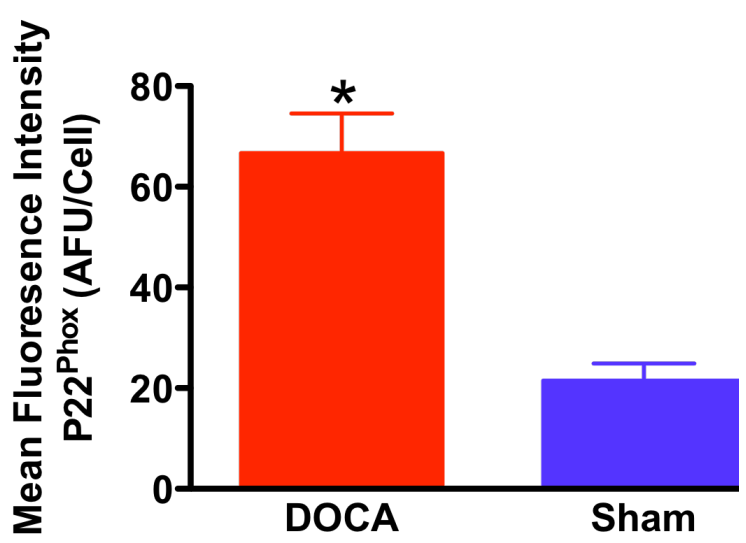


Whole-mount immunohistochemical labeling of CD163 positive MΦ (A), p22Phox (B), and overlay of MΦ with $p22^{Phox}$ (C) in Day 28 DOCA-salt hypertensive rat. Whole-mount immunohistochemical labeling of CD163 positive MΦ (D), $p22^{Phox}$ (E), and overlay of MΦ with $p22^{Phox}$ (F) in normotensive rat. (G) Mean macrophage $p22^{Phox}$ fluorescence intensity: showing 3X higher in DOCA MA adventitial MΦ comparing to control. Data are mean ± SEM and analyzed by paired t test. * $P<0.05$ vs. Control, (n=5).

Figure 21: The elevation of blood pressure, MΦ number, and O_2^- level required the synergistic effect of both DOCA and high salt

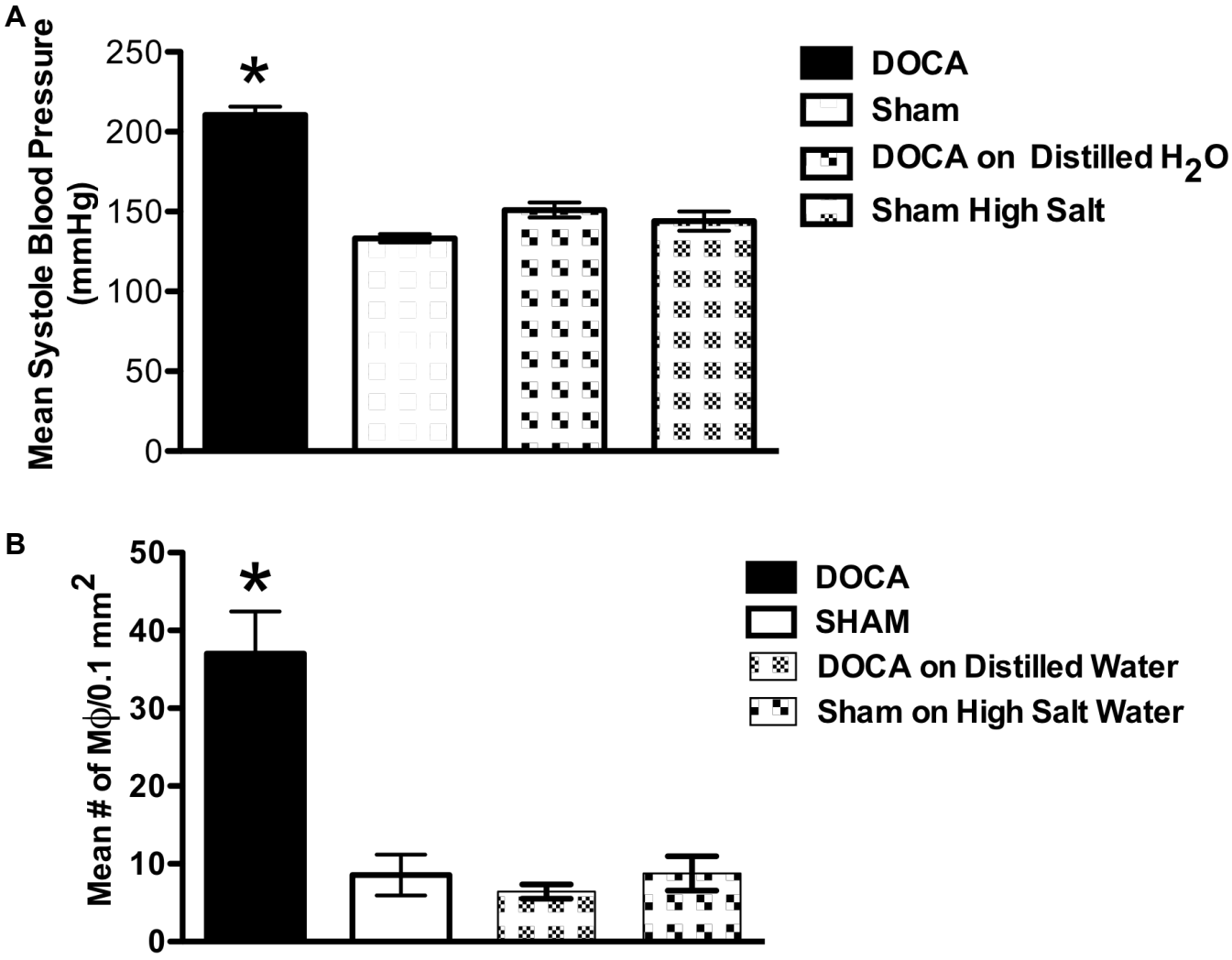
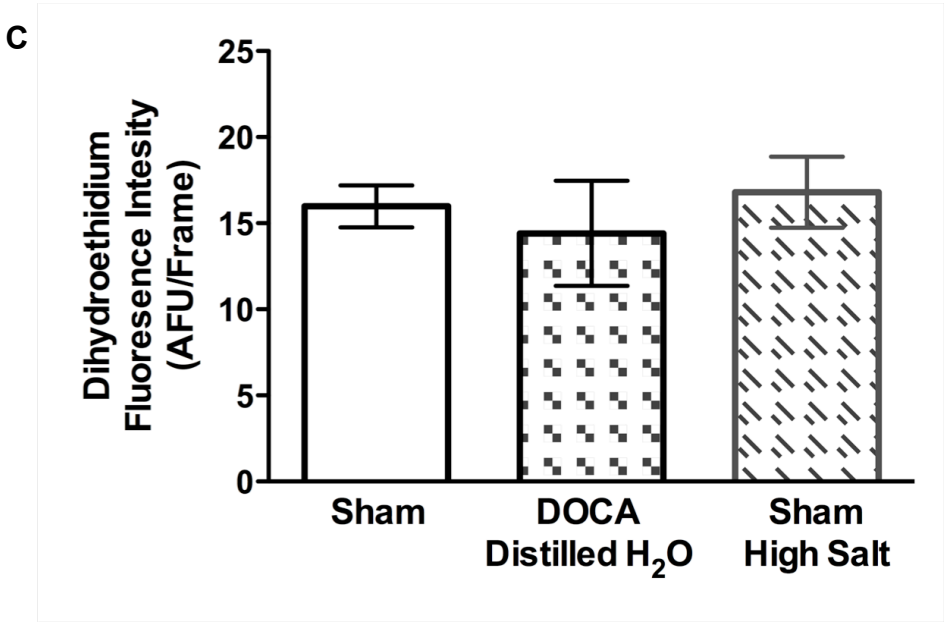


Figure 21 (cont'd)



Rats that had DOCA pellet implantation or high salt water alone did not show an increase in BP **(A)**. Similarly, treating with DOCA or high salt alone did not show an increase in the number of MΦ **(B)** or O_2^- level **(C)** in MA adventitia. * P<0.05 vs. Sham, (n=5).

Figure 22: The impairment of α_2R required the synergistic effect of both DOCA and high salt

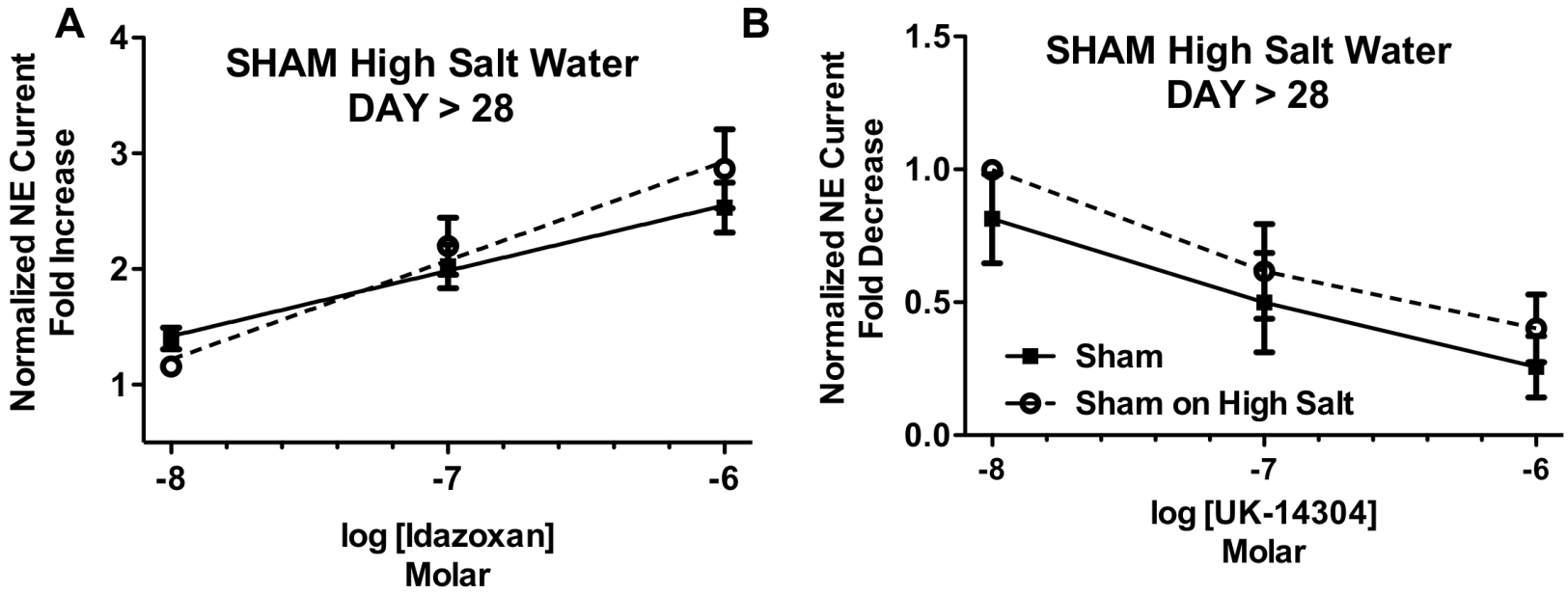
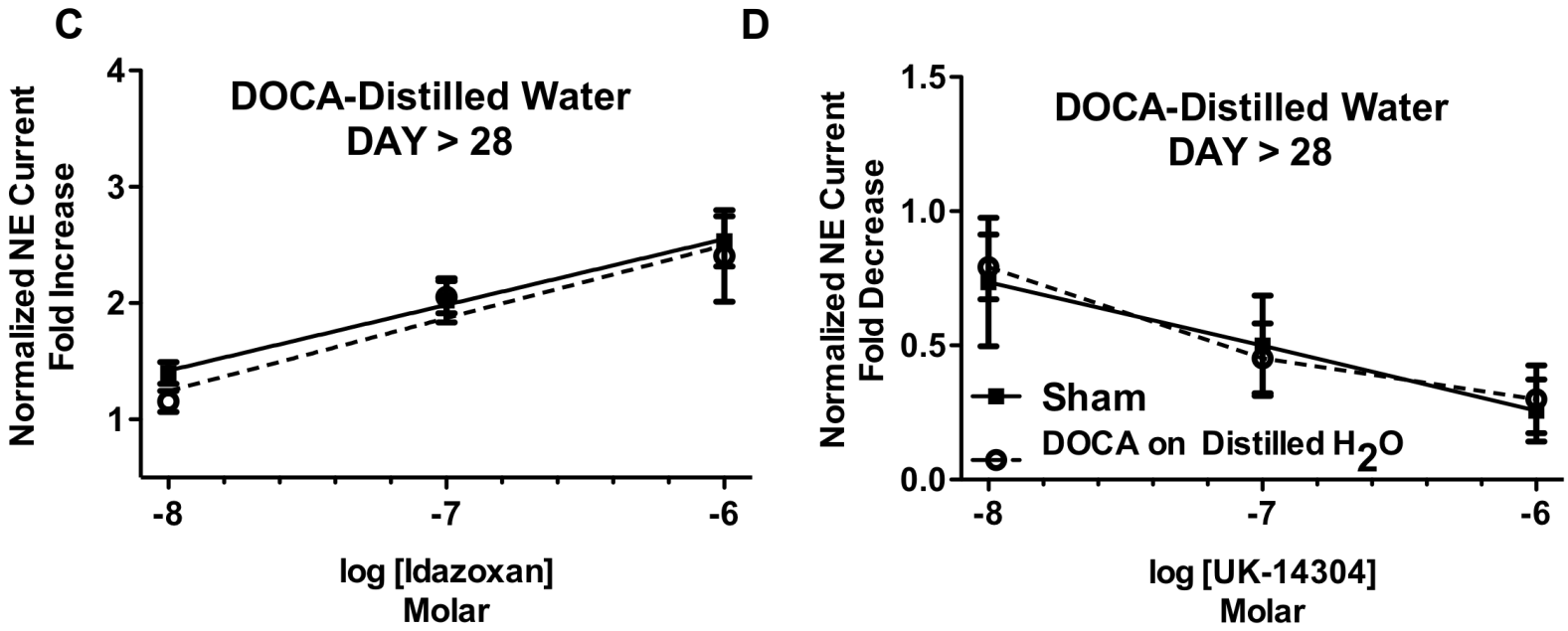


Figure 22 (cont'd)



Idazoxan, an α_2R antagonist, increased the NE oxidation current equally in shams on high salt (A) or DOCA on distilled water (C) when comparing to shams. UK 14304, an α_2R agonist, decreased the NE oxidation current equally in shams on high salt (B) or DOCA on distilled water (D) when comparing to shams. Data are mean ± SEM and analyzed by two-way ANOVA & Bonferonni's post hoc test, n = 4.

Discussion

Our data show that: 1) pro-inflammatory MΦ infiltrate in the adventitia of MA after the initial BP increase, 2) high-levels of MΦ derived O_2^- disrupts α_2R function leading to further increases in BP, 3) peritoneal MΦ are activated and may be the source of MA adventitial MΦ 4) lastly, the impairment of α_2R function, the elevated BP, the number of MΦ, and O_2^- level required the synergistic effects of DOCA and high salt.

Previous studies showed that there is an increased sympathetic nerve activity in DOCA-salt hypertension. In addition to increased sympathetic nerve activity, there is impaired function of α_2R on sympathetic nerve terminals, which provides feedback inhibition of NE release (7, 24). α_2R impairment may be due to the effects of vascular oxidative stress, particularly O_2^- (25). Many human and animal studies show an increase in ROS production during hypertension (9, 10, 11, 12, 13). ROS causes lipid peroxidation, receptor uncoupling and cellular damage. Vascular ROS are primarily derived from NADPH oxidase, an enzyme catalyzing O_2^- production (26). MΦ are a major source of NADPH oxidase derived O_2^- (10, 11). This study established the association between MΦ-derived O_2^- and α_2R function on perivascular sympathetic nerve terminals.

Our time-course study shows that there are two phases to DOCA-salt hypertension development. Perhaps, the initiation of hypertension is triggered by sodium retention, which then activates the sympathetic nervous system (27). This modest increase in BP could lead to cellular damage, neo-antigen formation, and

inflammatory response that further increase BP. This model is supported by studies done in rats that were immuno-tolerant to HSP70 and that developed minimal renal inflammation and were protected from the development of salt-sensitive hypertension (28). Possibly the formation of neo-antigen e.g. damaged-HSP70 is the signal that recruits MΦ into the MA adventitia of DOCA-salt rats. These MΦ are pro-inflammatory and capable of releasing O_2^- because they express a high level of NADPH oxidase. Although, our time-course study suggests O_2^- is derived from MΦ, there is evidence suggesting that NADPH oxidase-derived O_2^- is a stimulus that recruits MΦ into the aorta of Ang II-induced hypertensive rats (14). However, DOCA-salt model is a rennin-angiotensin independent model of hypertension. Perhaps, ET-1 is the humoral factor that enhances vascular NADPH oxidase activity and causes a low-level oxidative stress. This low-level of oxidative stress triggers activated MΦ infiltration and further releases of O_2^- that contribute to cellular damage in MA of DOCA-salt rats.

O_2^- is a reactive and short-lived molecule. However, we show that some infiltrated MΦ come in close contact with the perivascular sympathetic nerves, which could allow the O_2^- to disrupt α_2R function. Damage to G-protein receptor signaling by oxidative stress has been demonstrated in renal proximal tubules in primary culture (29). Thus, a possible mechanism of O_2^- disruption of α_2R function is through uncoupling the G-protein from its receptor. It appears that the infiltration of MΦ is location specific because we only found MΦ in the MA and not in skeletal muscle arteries. Perhaps, the reason for this is because the MA bed is located in the peritoneal

cavity. We also found that the peritoneal MΦ expresses high level of the adhesion molecule, CD11b. This transmembrane receptor may mediate the attachment of MΦ to the MA adventitia. Previously, we showed that rats treated with apocynin had reduced vascular oxidative stress and improved α_2R function (25). Hence, the later phases of DOCA-salt hypertension may be due to oxidative damage of α_2R on the perivascular sympathetic nerves. The data support the hypothesis that MΦ NADPH oxidase is the source for vascular O_2^-.

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CHAPTER 4

MACROPHAGE DEPLETION REDUCES VASCULAR OXIDATIVE STRESS, RESTORES α_2 ADRENERGIC AUTORECEPTOR FUNCTION AND ATTENUATED HYPERTENSION DEVELOPMENT IN DOCA-SALT HYPERTENSION

Abstract

There is temporal relationship between impaired function of sympathetic nerve terminal α_2-adrenergic autoreceptor (α_2R) and adventitial infiltration of pro-inflammatory macrophages (MΦ) in mesenteric arteries (MA) from DOCA-salt hypertensive rats. We tested the hypothesis that MΦ release O_2^-, which disrupts α_2R function causing increased norepinephrine (NE) release and further increase of blood pressure in DOCA-salt rats. Liposome-encapsulated clodronate (LEC) was used to deplete adventitial MΦ in rat MA, (average # MΦ/0.1 mm^2, DOCA PBS: DOCA LEC, 39.96: 4.16, $p<0.05$). MΦ depletion reduced vascular O_2^- (measured using dihydroethidium) (Δ_σ DOCA PBS-DOCA LEC: 23.85, $p<0.05$). α_2R function was also restored in MΦ depleted animals. To establish this we used focal nerve stimulation and amperometry with microelectrodes to measure NE oxidation currents at the adventitial surface of MA in the presence α_2R agonist, UK 14304, and antagonist, idazoxan. (Normalized 1μM UK 14304 and idazoxan NE current fold changes respectively, DOCA LEC: DOCA PBS, 0.326: 0.611, & 2.74: 2.18, $P<0.05$). Lastly, MΦ depletion attenuated DOCA-salt blood pressure development. (MAP on day 25-28,DOCA LEC: DOCA PBS, 154.8 mmHg: 197.4 mmHg, $p<0.05$). These data support the hypothesis that MΦ increased blood pressure in DOCA-salt rats by releasing O_2^-, which disrupted α_2R function and enhanced sympathetic nerve activity.

Introduction

Blood pressure (BP) regulation is complex but the nervous system plays an essential role, and the sympathetic nerves innervating the splanchnic circulation are particularly important (1). In the periphery, norepinephrine (NE) is released from postganglionic sympathetic nerves and binds to postjunctional α_1-adrenergic receptor causing vasoconstriction. It also binds to α_2-adrenergic autoreceptor (α_2R), a $G_{i/o}$-protein coupled receptor, on the prejunctional sympathetic nerve terminal inhibiting further release of NE (2). In the DOCA-salt model in rats, the function of α_2R regulating NE release from sympathetic nerves supplying arteries is impaired (3). The cause of this impairment is unknown, but this impairment contributes to the increase in sympathetic activity in this model.

Reactive oxygen species (ROS) have been shown to impair G-protein coupled receptor function by uncoupling the receptor from its G-protein (4). Perhaps, vascular ROS e.g. O_2^- impairs the function of α_2R by a similar mechanism. One of the major sources of O_2^- is macrophage (MΦ). The recruitment, activation and proliferation of MΦ in the vascular adventitia have been recognized in hypertension (5). In some models of hypertension MΦ infiltrate into the arterial wall (6). Alterations in the number of circulating monocytes and their activation occur in hypertensive patients and animals (7, 8). Mice deficient in MΦ colony-stimulating factor exhibit reduced vascular inflammation and they are protected against damage caused by DOCA-salt hypertension (9). Furthermore, INCB3344, an antagonist of CCR2, prevented the infiltration of vascular

MΦ and reduced blood pressure in DOCA-salt mice (10). However, it is unclear whether MΦ-derived O_2^- can disrupt α_2R and further increase BP in DOCA-salt model. This study tests the hypothesis that MΦ depletion blocks the infiltration of MΦ into mesenteric arteries (MA) adventitia, reduces vascular O_2^-, preserves the α_2R function and prevents the development of BP in the later phases of DOCA-salt hypertension.

Results

LEC attenuated the development of later phases of DOCA-salt hypertension.

Using radiotelemetry we examined the effect of LEC on the development of DOCA-salt hypertension. Development of DOCA-salt hypertension was biphasic, phase 1: day 5-18 and phase 2: day >18 (Fig. 23A). Mean arterial pressure (MAP) of DOCA-salt rats treated with LEC was significantly lower during days 23-28 compared to DOCA-salt rats treated with lipo-PBS (Fig. 23A). LEC treatment did not affect the MAP of sham rats (Fig. 23A). Furthermore, there was no difference in heart rate between the two treatment groups of DOCA and of Sham groups (Fig. 23B). These in vivo data show that depletion of perivascular MΦ prevented the development of the late phase of DOCA-salt hypertension.

LEC depleted activated peritoneal MΦ.

MΦ can phagocytose cellular debris and pathogens. We capitalized on this characteristic and injected LEC into our DOCA-salt rats to deplete the MΦ. Upon encountering LEC, MΦ engulfed the molecules, and the cells were killed by clodronate via apoptosis. Using flow-cytometry we found that the percentage of peritoneal MΦ in DOCA-salt rats treated with LEC was significantly lower than in DOCA-salt rats treated Lipo-PBS (Fig. 24A & B). Further gating on the peritoneal MΦ population, we found that LEC depleted the activated, $CD11b^{+}/CD163^{+}$, peritoneal MΦ from DOCA-salt rats treated with LEC (Fig. 24C & D).

LEC depleted MΦ in the MA adventitia of DOCA-salt rats.

Using whole-mount immunohistochemistry, we found that the number of CD163 positive MΦ in the MA adventitia of DOCA-salt rats treated with LEC was significantly lower than DOCA-salt rats treated with lipo-PBS (Fig. 25A, B & D). This validated that depletion of peritoneal MΦ would reduce perivascular MΦ in DOCA-salt rats. Most interestingly, in the MA adventitia of DOCA-salt rats treated with LEC, we detected CD163 positive apoptotic MΦ cell ghosts (Fig. 25C), and we did not find any MΦ cell ghosts in DOCA-salt rats treated with lipo-PBS (Fig. 25A).

LEC reduced the levels of O_2^- in the MA adventitia of DOCA-salt rats.

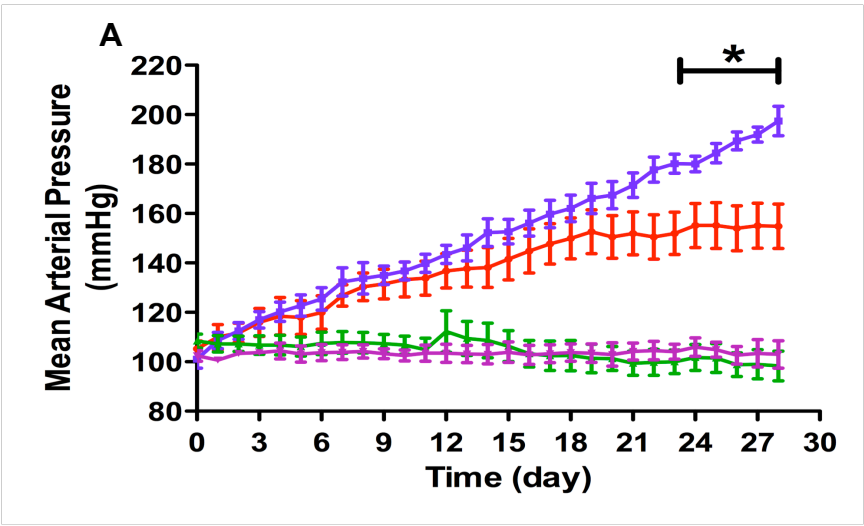
Using DHE-fluorescence we found that relative levels of O_2^- in MA of DOCA-salt rats treated with LEC was much lower than in DOCA-salt rats treated with lipo-PBS (Fig. 26A & B). Semi-quantification of O_2^- levels revealed that the levels of O_2^- in MA of DOCA-salt rats treated with LEC was significantly lower than in DOCA-salt rats treated with lipo-PBS. Furthermore, LEC also reduced the baseline levels of O_2^- in control rats (Fig. 26C). Thus, the depletion of perivascular MΦ reduced the levels of O_2^- in DOCA-salt and sham rats.

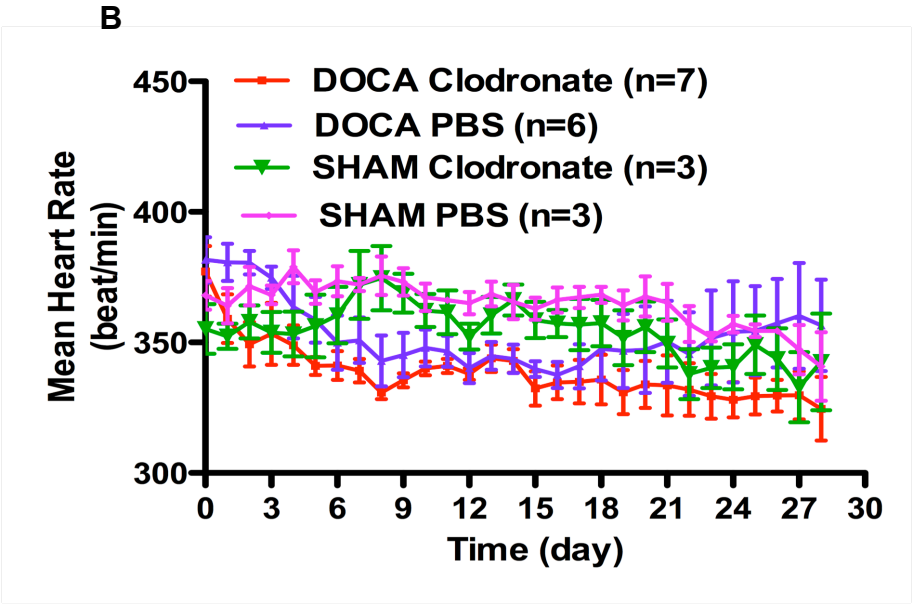
LEC prevented the dysfunction of α_2R in DOCA-salt rats.

Previously, we have shown that α_2R on sympathetic nerve terminals regulating feedback inhibition of NE release in MA is impaired. Using electrical stimulation and amperometry, we measured real-time NE release from MA. Idazoxan, an α_2R

antagonist, failed to increase the NE oxidation current in DOCA-salt rats treated with lipo-PBS (Fig. 27B & C). However, in the DOCA-salt rats that were treated with LEC, idazoxan increased the NE oxidation current (Fig. 27A & C). UK 14304, an α_2R agonist, decreased the NE oxidation current in DOCA-salt rats treated with LEC (Fig. 27D & F) but failed to reduce the current in DOCA-salt rats treated with lipo-PBS (Fig. 27E & C). These pharmacological data confirmed that the depletion of perivascular MΦ prevented α_2R dysfunction in DOCA-salt rats.

Figure 23: MΦ depletion lower blood pressure during the late phase of DOCA-salt hypertension





A) MAP of DOCA-salt rats treated with LEC was significantly lower during day 23-28 comparing to DOCA-salt rats treated with lipo-PBS. LEC treatment did not affect the blood pressure in Sham groups. B) There was no significant change in heart rate between two treatment groups of DOCA, and similarly in Shams groups. Data are mean ± SEM and analyzed by two-way ANOVA & Bonferonni's post hoc test. * $P<0.05$ vs. LEC, (n=6-7).

Figure 24: Flow-cytometry dot-plots of peritoneal MΦ

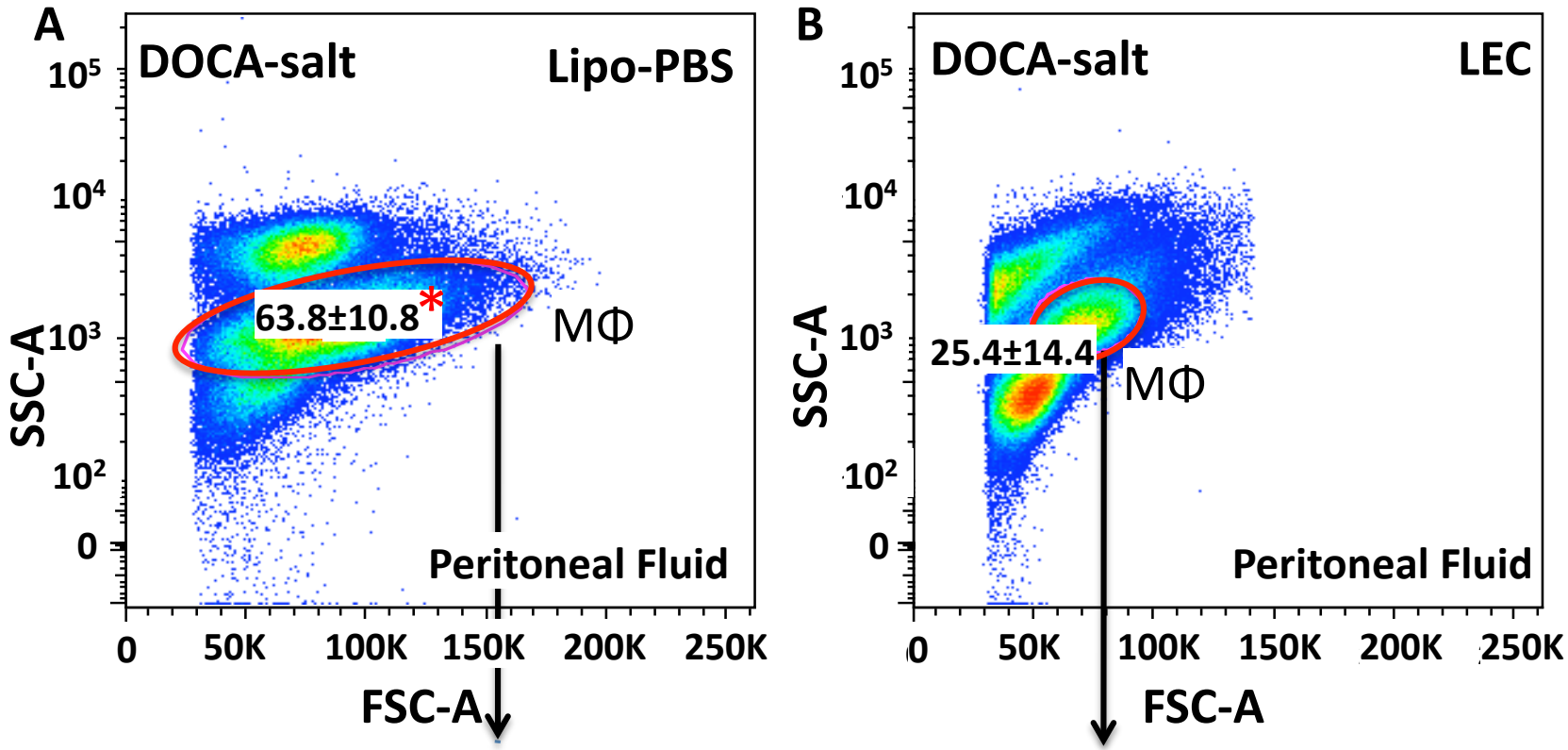
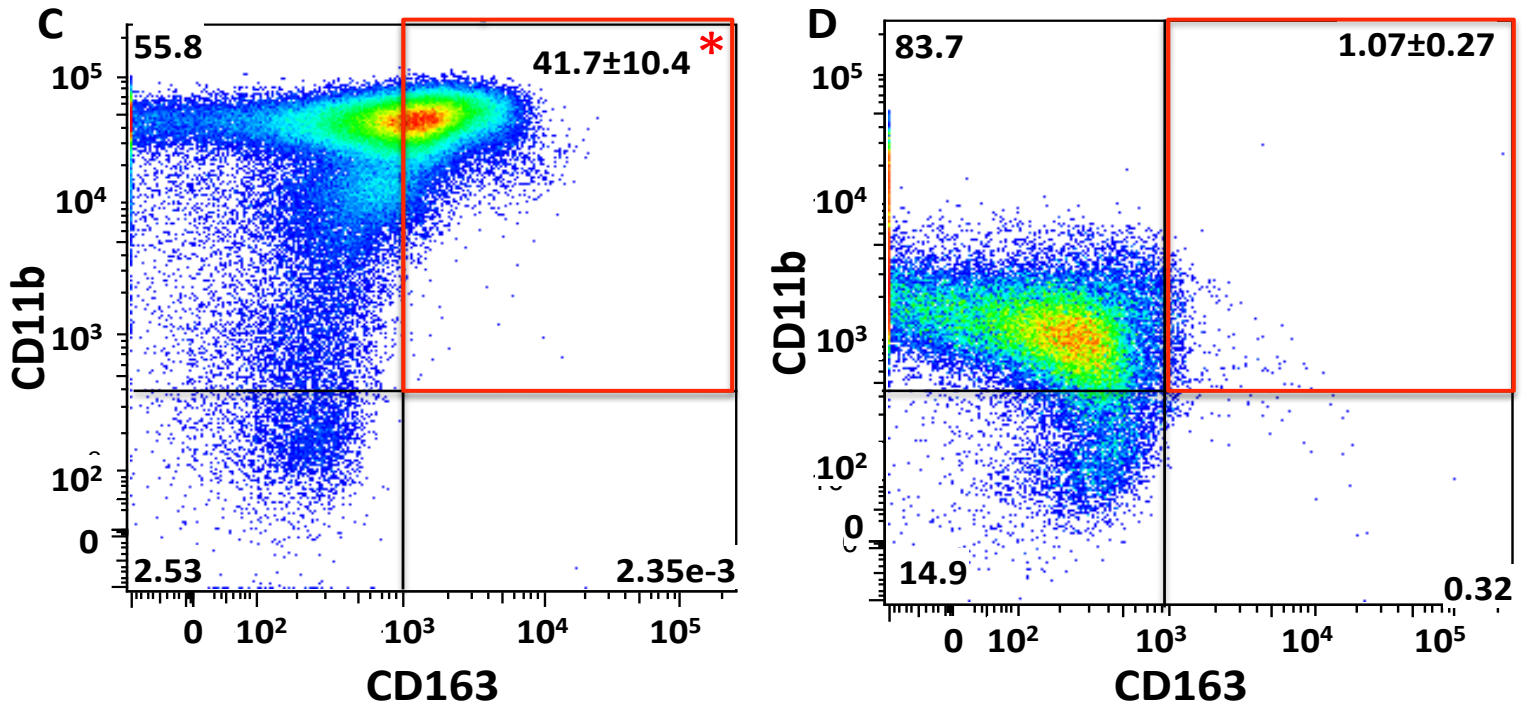


Figure 24 (cont'd)



A) Peritoneal fluid from DOCA-salt rats treated with lipo-PBS and B) DOCA-salt rats treated with LEC. The percentage of peritoneal MΦ was significantly decreased. Dot-plots of C) peritoneal MΦ of DOCA-salt rats treated with lipo-PBS and D) DOCA-salt rats treated with LEC. The percentage of activated, $CD11b^{+}/CD163^{+}$, peritoneal MΦ was significantly lower in LEC treated rats. Data are mean ± SEM and analyzed Kruskal-Wallis' test. *P<0.05 vs. LEC, (n=5). Dot-plots depicted are concatenated analysis of at least five animals.

Figure 25: Identification of MΦ and apoptotic MΦ cell ghosts

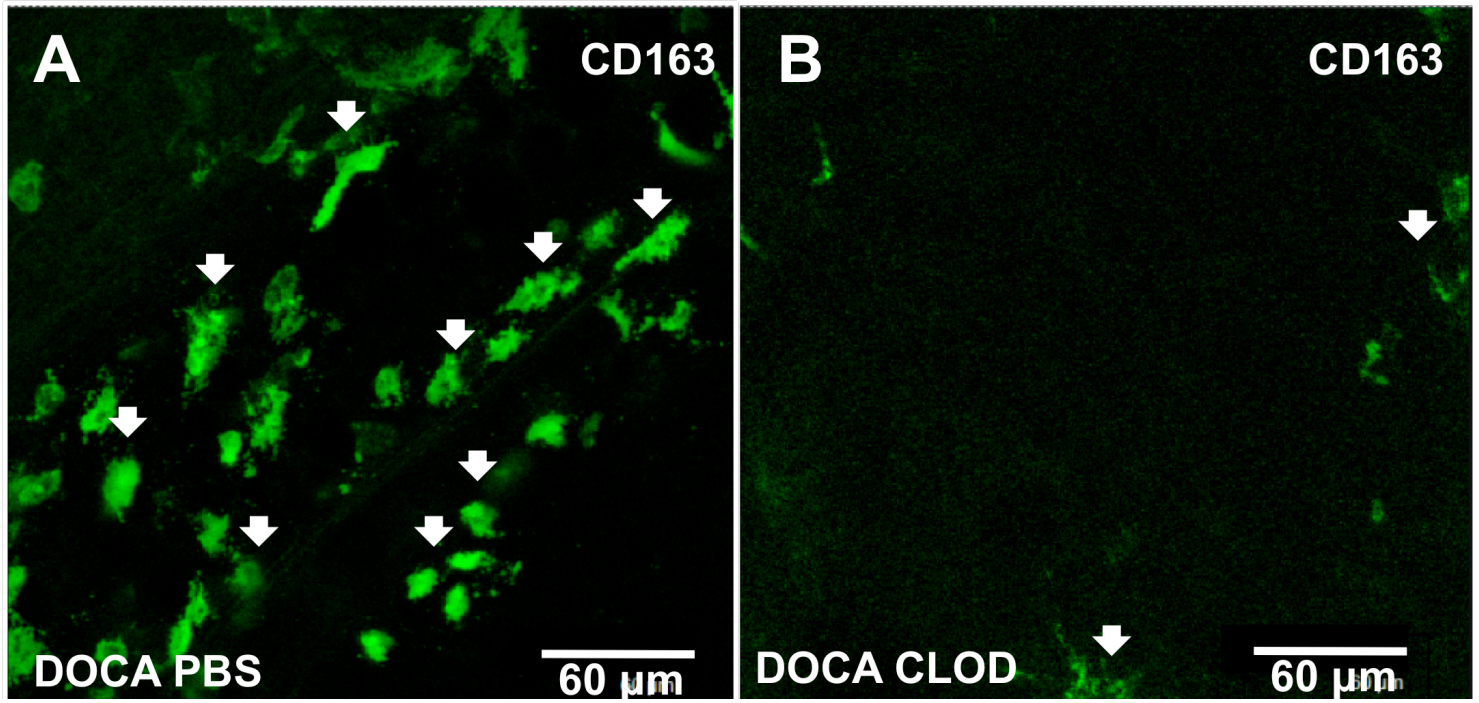
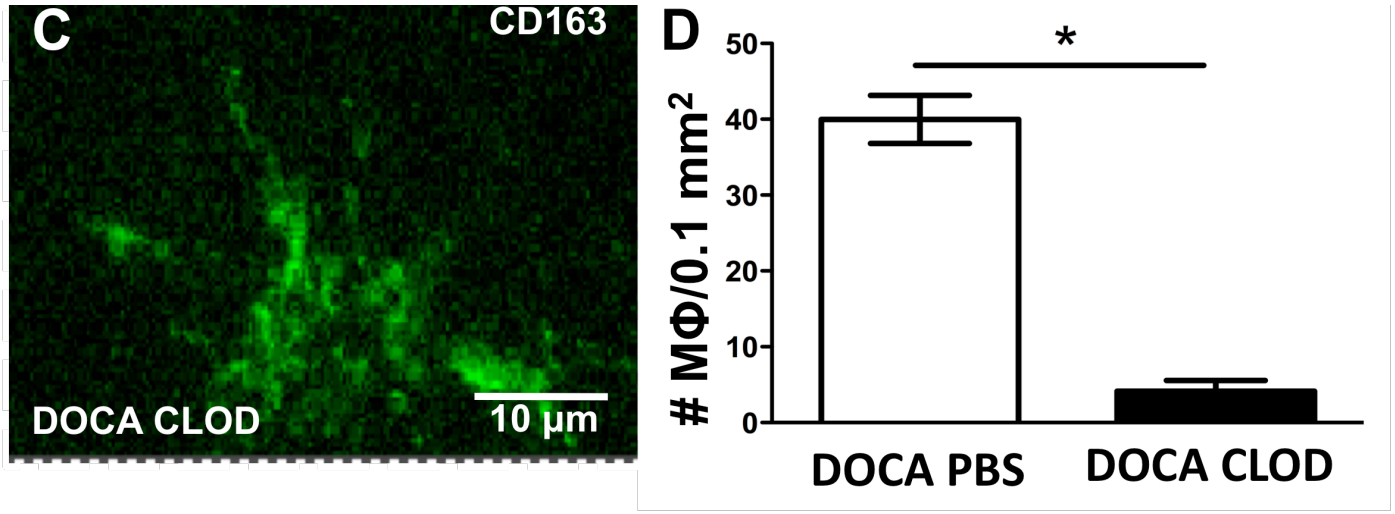
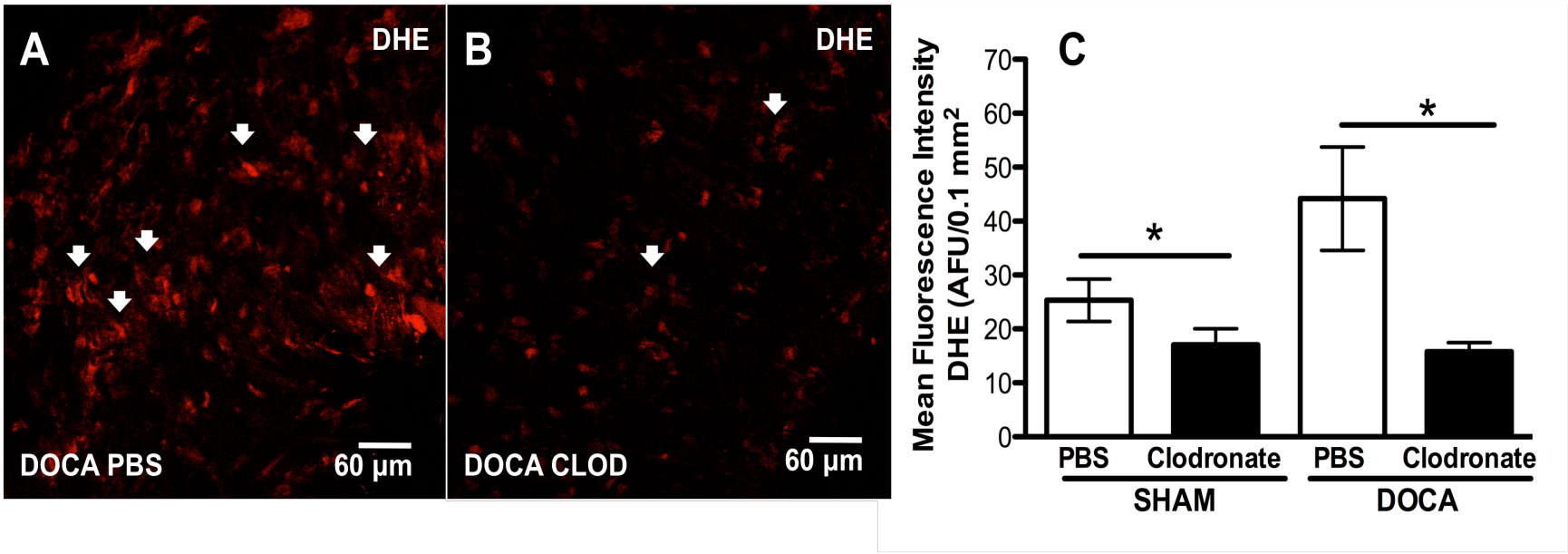


Figure 25 (cont'd)



A) Whole-mount immunohistochemical labeling of high number of CD163 positive MΦ (arrows) in MA adventitia of DOCA-salt rats treated with lipo-PBS, B) but there were much fewer MΦ (arrows) in MA adventitia of DOCA-salt rats treated with lipo-LEC. C) CD163 positive apoptotic MΦ cell ghosts in DOCA-salt treated with LEC. D) Mean number of MΦ /0.1 mm^2: showing a significant reduction in the number of MΦ in groups treated with clodronate comparing to groups treated with PBS. The mean number of MΦ was calculated from 5 areas of 0.1 mm^2. Data are mean ± SEM and analyzed by Mann-Whitney Test. * P<0.05 vs. DOCA PBS, (n=5).

Figure 26: Detection of O_2^- in MA adventitia of DOCA-salt rats



A) Photomicrograph showing fluorescence of dihydroethidium (DHE) (arrows) in MA adventitia of DOCA-salt rats treated lipo-PBS, and B) DOCA-salt treated with LEC. C) Mean DHE fluorescence intensity of DHE/0.1 mm^2 showing a significant reduction in the levels of O_2^- in groups treated with clodronate comparing to groups treated with PBS in both SHAM and DOCA-salt animals. The mean fluorescence intensity of DHE was calculated from 5 areas of 0.1 mm^2. Data are mean ± SEM and analyzed by paired t-test. * $P<0.05$ vs. PBS, (n=5).

Figure 27: Analysis of α_2R function in DOCA-salt rats

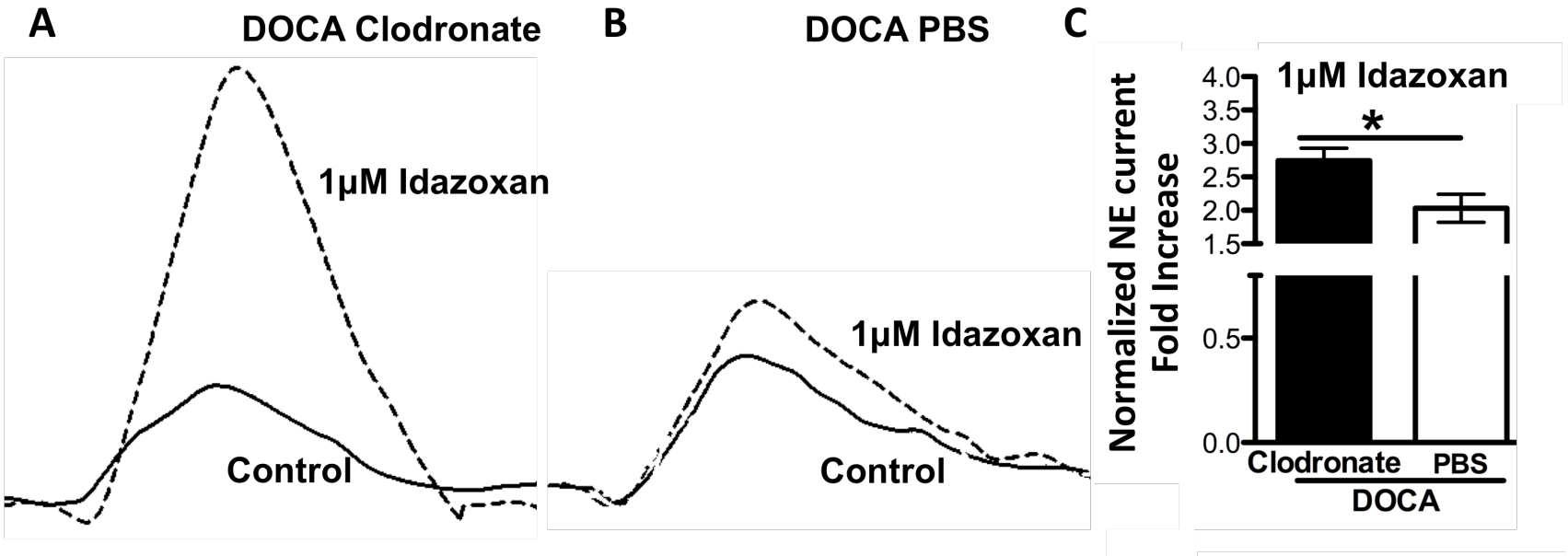
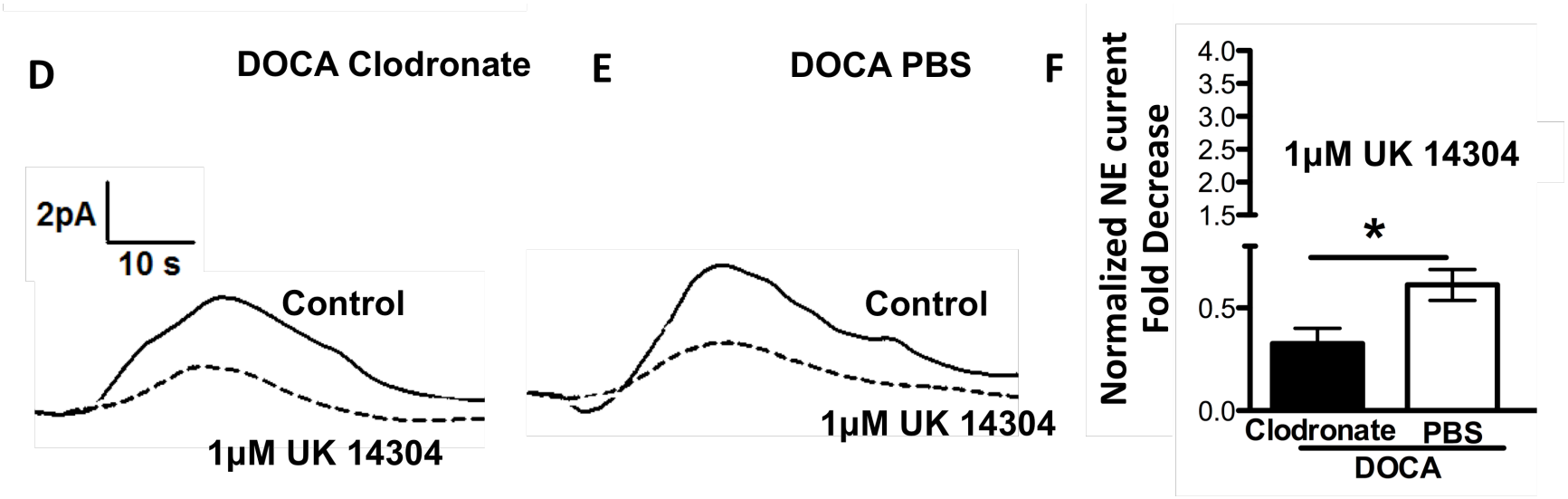


Figure 27 (cont'd)



A) Idazoxan (1 μM) increased the NE oxidation current in DOCA-salt rats treated with LEC but B) failed to raise the current in DOCA-salt rats treated with lipo-PBS. D) UK 14304 (1 μM), an α_2R agonist, decreased the NE oxidation current in DOCA-salt rats treated with LEC but E) failed to reduce the current in DOCA-salt rats treated with lipo-PBS. C) The idazoxan (1 μM)-induced increase in the normalized NE oxidation current was significantly higher in DOCA-salt rats treated with LEC compared to DOCA-salt rats treated with lipo-PBS. F) However, the normalized NE oxidation current in the presence of UK 14304 (1 μM) was significantly lower in DOCA-salt rats treated with LEC comparing to DOCA-salt rats treated with lipo-PBS. Data are mean ± SEM and analyzed by Student's t-test. * $P<0.05$ vs. PBS, (n=5).

Discussion

The results from this study indicate that LEC: 1) prevents the development of the later phases of DOCA-salt hypertension, 2) depletes activated peritoneal MΦ, 3) blocks the infiltration of MΦ into the MA adventitia, 4) reduces the levels of vascular O_2^-, 5) restores the function of α_2R. Taken together, these results are consistent with the hypothesis that MΦ-derived O_2^- disrupts α_2R and further increases BP in the late phase of DOCA-salt hypertension.

Hypertension is a multiorgan disease that involves changes in nervous (1) and immune system function (11). Sympathetic nerve activity is elevated in some hypertensive humans and in some animal models of hypertension including the DOCA-salt model (12). In DOCA-salt rats, the function of the α_2R regulating NE release from sympathetic nerves supplying MA is impaired and this impairment contributes to the increased sympathetic activity (3). A large body of literature indicates that vascular inflammation may be involved in the development of hypertension. Mice lacking T and B cells have blunted hypertension and these mice do not develop vascular remodeling during angiotensin II infusion or DOCA-salt treatment; adoptive transfer of T, but not B cells restored these changes (13). Furthermore, T cells expressed angiotensinogen, angiotensin I-converting enzyme, and renin. AT_1 receptors are expressed intracellularly by T-cells and angiotensin II activates T-cells (14). In experimental animals, target-organ damage in hypertension involves MΦ infiltration into the blood vessels, the brain, the heart, and the kidneys (15). Particularly, MΦ accumulation in the vascular wall

during hypertension contributes to oxidative stress, endothelial dysfunction, and vascular inflammation. Although it is known that hypertension involves inflammation and periarterial sympathetic nerve dysfunction, the interaction between inflammatory mechanisms and sympathetic nerve function (particularly at the vascular neuroeffector junction) and hypertension is not clear. This study is the first to show that MΦ contribute to the impairment of α_2R in the periarterial sympathetic nerves in DOCA-salt rats.

MΦ are phagocytic cells that produce and release O_2^- in response to a variety of stimuli (16). Our study shows that perivascular MΦ contributes to the increased levels of O_2^- in the adventitia of MA. Depletion of these MΦ reduces the vascular oxidative stress in DOCA-salt rats. Perhaps, the modest increase in BP during the early stage of DOCA-salt hypertension causes some cellular damage, neo-antigen formation, and inflammatory response that serve as stimuli to recruit MΦ. For example, rats that are immune tolerant to HSP70 developed minimal renal inflammation and were protected from the development of salt-sensitive hypertension (17). In MΦ NADPH oxidase is a major source of O_2^-. Under physiological conditions, O_2^- is maintained at a low level by the enzymes superoxide dismutase, catalase, and glutathione peroxidase. However, in many pathological conditions e.g. hypertension there is an increase O_2^- which activates many signaling pathways including those involved in smooth muscle cell growth and inflammation (18). Uncontrolled O_2^- causes cellular damage and eventually apoptosis because O_2^- can damage proteins, lipids, and DNA (19). This study supports the hypothesis that MΦ-derived O_2^- disrupts the α_2R function because depletion of MΦ

restores its function. Perhaps, a possible mechanism of O_2^- disruption of α_2R function is through uncoupling the G-protein from its receptor. H_2O_2 causes uncoupling of D1 dopamine receptors from target G-proteins in cell culture (4). The role of inflammation through recruitment, activation and proliferation of MΦ in the vascular adventitia has been recognized in hypertension (20). Mice deficient in MΦ colony-stimulating factor exhibit reduced vascular inflammation and they are protected against damage caused by DOCA-salt hypertension (9). Our data suggests that one possible protection mechanism is by preventing the impairment of α_2R function.

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CHAPTER 5

MECHANISMS OF PRESYNAPTIC NOREPINEPRHINE RELEASE AUTOREGULATION ALTERATION IN SYMPATHETIC PERIVASCULAR NERVES SUPPLYING MESENTERIC ARTERIES FROM DOCA-SALT HYPERTENSIVE RATS

Abstract

DOCA-salt hypertension in rats is associated with the impairment of α_2-adrenergic autoreceptor (α_2R), and increased levels of superoxide (O_2^-). Many studies have shown that α_2R function is impaired in human hypertension and in DOCA-salt hypertension. Previous studies in our lab showed that that α_2R impairment was due to the effects of vascular oxidative stress, particularly O_2^-. However, the mechanism of impairment is unknown. This study tests the hypothesis that α_2R function impairment is due the uncoupling of the $G_{i/o}$-protein from the α_2R but the downstream effectors from the G-protein is not altered in presynaptic sympathetic perivascular nerves of DOCA-salt hypertensive MA. To study this we used focal nerve stimulation and amperometry with microelectrodes to measure NE oxidation currents at the adventitial surface of MA. The frequency response curves of NE oxidation currents in presence of PTX, idazoxan, and cocaine from DOCA-salt hypertensive and control MA showed no significant difference at each frequency between the two groups. Using M119, a $G_{\beta\gamma}$ subunit inhibitor, we found that M119 increased NE oxidation equally in DOCA-salt hypertensive and control MA. Furthermore, in the presence of M119, idazoxan increased NE oxidation in DOCA-salt hypertensive MA to the same level of that in control MA. High concentration AlF_4^-, a stimulus of $G_{i/o}$ proteins, decreased the NE oxidation currents in DOCA-salt hypertensive MA much more compared to control MA, and this effect was blocked by PTX. Low concentration AlF_4^-, a stimulus of G_s proteins, increased the NE oxidation

currents in DOCA-salt hypertensive but not control MA; and this effect was not blocked by PTX. Furthermore, the level of forskolin-induced increased NE oxidation current was much higher in DOCA-salt hypertensive MA than control MA. The H-89 concentration response curves for DOCA-salt hypertensive MA was shifted rightward compared to the control MA. These data support that G-protein is not affect in DOCA-salt hypertensive MA and the mechanism of α_2R function impairment was between the α_2R and its G-protein. In addition, there was an increase in the expression or the activity of $G_{i/o}$ and G_s proteins in sympathetic perivascular nerves of DOCA-salt hypertensive MA.

Introduction

In the world, the prevalence of hypertension in adult is estimated at approximately 1 billion individuals, and approximately 7.1 million deaths per year may be attributable to hypertension (1). This is a major health concern because hypertension increases the risk for cardiovascular diseases (2). The causes of hypertension are complex but sympathetic nerve activity is elevated in many hypertensive humans and some animal models of hypertension including the deoxycorticosterone acetate (DOCA)-salt model (3). Increased sympathetic nerve activity in DOCA-salt hypertension in rats suggests that there may be alterations in the local mechanisms that modulate sympathetic neurotransmission (4, 5). α_2R, a $G_{i/o}$-protein-coupled autoreceptor, is found in presynaptic sympathethic nerve terminals. Stimulation of the α_2R inhibits further release of NE from the sympathetic nerve terminals. Data from genome-wide association study have recently linked a single nucleotide polymorphism within the 3'UTR of the human α_{2A}-adrenergic receptor gene ADRA2A with increase BP (6). This suggests α_2R plays an important role in regulating BP and alteration of its function causes hypertension. In fact, studies have shown that α_2R function is impaired in human hypertension and in DOCA-salt hypertension (7, 8). Previous studies showed that α_2R impairment is due to the effects of vascular oxidative stress, particularly O_2^- (9). However, the mechanism of impairment is unknown.

Reactive oxygen species (ROS) has been shown to impair G-protein-coupled-receptor by uncoupling the receptor from its G-protein (10). Perhaps, vascular ROS e.g. O_2^-

impairs the function of α_2R by the similar mechanism. This study tests the hypothesis that α_2R function impairment is due to the uncoupling of the $G_{i/o}$-protein from the α_2R but the downstream effectors from the G-protein is not altered in presynaptic sympathetic perivascular nerves of DOCA-salt hypertensive MA.

Results

The amount of NE release from the RRP vesicles was the same in sympathetic perivascular nerves from DOCA-salt hypertensive and control MA.

When $G_{i/o}$ proteins were inactivated with PTX, α_2R were inhibited with idazoxan, and the NE transporter were blocked with cocaine. Then, the amount of NE released from sympathetic perivascular nerves of DOCA-salt hypertensive MA was not different than in the control MA (Fig. 28). This suggested that the amount of NE in the RRP vesicles was not affected in sympathetic perivascular nerves supplying MA of DOCA-salt hypertensive rats.

Inhibition of $G_{\beta\gamma}$ equally increased NE release from sympathetic perivascular nerves from DOCA-salt hypertensive and control MA.

M119 inhibited the $G_{\beta\gamma}$-dependent calcium release (11). It has been shown that free $G_{\beta\gamma}$ subunits interact with CSP, a SNARE chaperone protein, and N-type Ca^{+2} channels results in a tonic G protein inhibition of the channels (12). Here we showed blocking $G_{\beta\gamma}$ from interacting with its downstream effectors increased NE release (Fig. 29A). Furthermore, M119 was equally effective in increase NE release from both sympathetic perivascular nerves of DOCA-salt hypertensive and control MA (Fig. 29A). This data suggested that there was no alteration in the activity of free $G_{\beta\gamma}$ in DOCA-salt hypertensive MA.

α_2R function impairment occurred upstream from the G-protein.

Idazoxan, an α_2R antagonist, increased NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA. However, idazoxan increased the release of NE much higher in control MA when compared to DOCA-salt hypertensive MA (Fig. 29B). This data suggested that the α_2R function is impaired. In the presence of M119, idazoxan increased the release of NE from sympathetic perivascular nerves of DOCA-salt hypertensive MA to the same level of that in control MA (Fig. 29B). This suggested that the impairment of the α_2R function is upstream of the $G_{\beta\gamma}$.

Low concentration of AlF_4^- increased NE release from sympathetic perivascular nerves of DOCA-salt hypertensive but not control MA and this effect was not block by PTX.

It has been shown that low concentration of AlF_4^- stimulated the G_s protein (13). Here we showed low concentration of AlF_4^- increased NE release from sympathetic perivascular nerves of DOCA-salt hypertensive but no control MA (Fig. 30A). This effect was not block by PTX (Fig. 30B). This supported that there was an increase in the expression or the activity of G_s protein in sympathetic perivascular nerves of DOCA-salt hypertensive MA.

High concentration of AlF_4^- decreased NE release from sympathetic perivascular nerves from DOCA-salt hypertensive much more when compared to control MA and this effect was block by PTX.

High concentration of AlF_4^- stimulated the $G_{i/o}$ proteins population instead of G_s proteins (13). Here we showed that high concentration of AlF_4^- decreased NE release from MA sympathetic perivascular nerves of both DOCA-salt hypertensive and control MA (Fig. 30B). However, DOCA-salt hypertensive MA was more sensitive to the effect of AlF_4^- on the inhibition of NE release, and PTX was able to block this effect in both DOCA-salt and control MA (Fig. 30B). This data supported that there was an increase in the expression or the activity of $G_{i/o}$ protein in sympathetic perivascular nerves of DOCA-salt hypertensive MA.

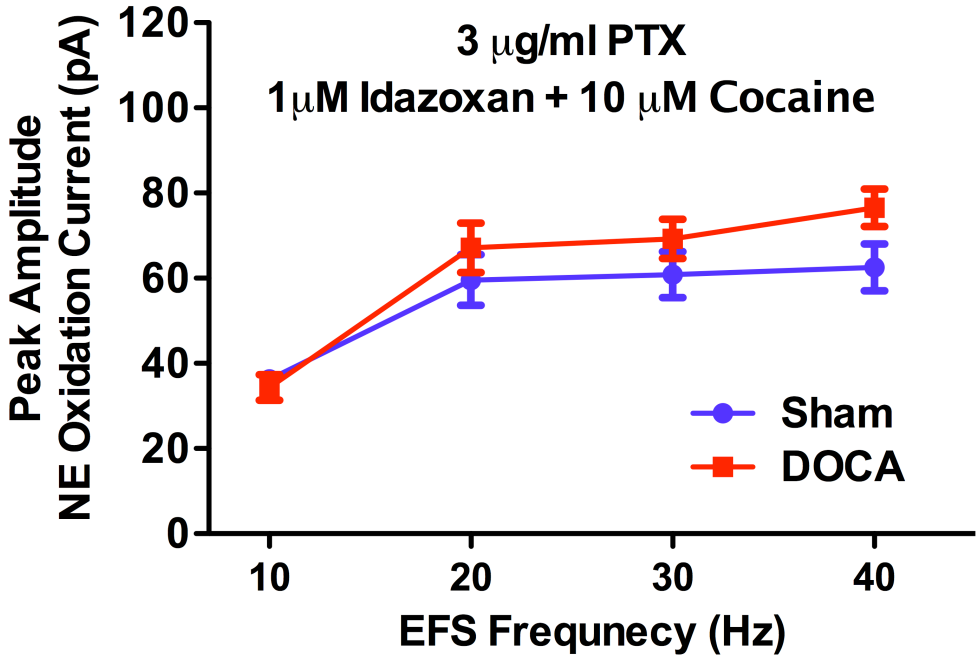
Forskolin increased the release of NE much more in sympathetic perivascular nerves from DOCA-salt hypertensive compared to control MA.

Forskolin, an adenylyl cyclase activator, increased the release NE from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA. However, forskolin caused a much higher amount of NE release from DOCA-salt hypertensive MA than that of control MA (Fig. 31A). Forskolin increases activity of adenylyl cyclase leads to increased levels of cAMP and subsequently PKA activity. PKA phosphorylation has been shown to promote and increase exocytosis (14, 15). This data suggested that there was a much higher expression or activity of adenylyl cyclase from sympathetic perivascular nerves of DOCA-salt hypertensive MA.

H-89 was less effective in inhibiting NE release from sympathetic perivascular nerves of DOCA-salt hypertensive compared to control MA.

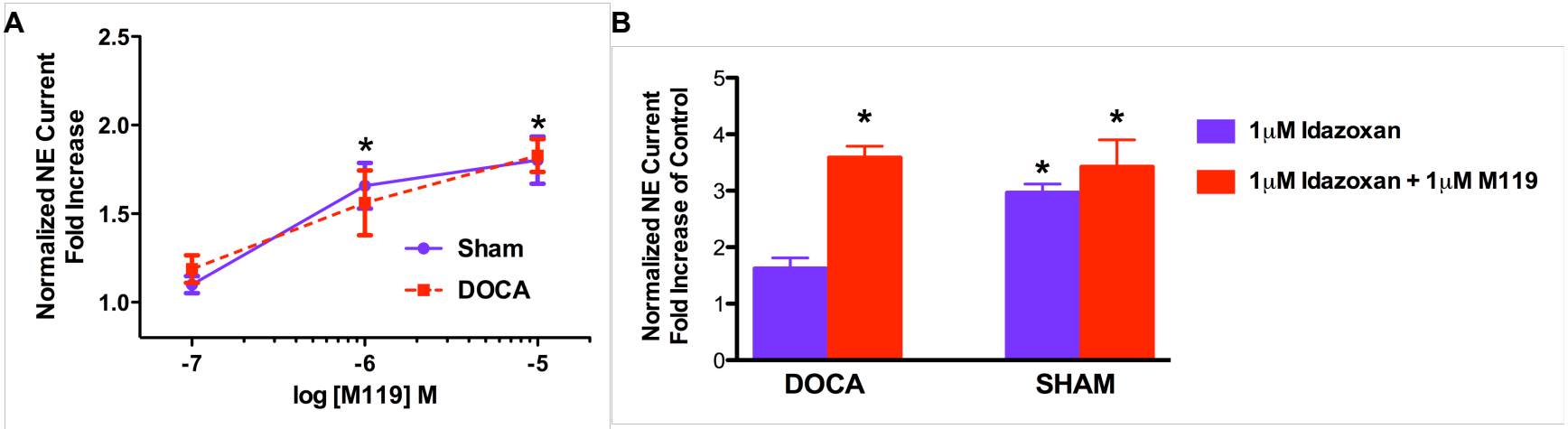
At low concentration of H-89, a competitive inhibitor of PKA, it inhibited the NE release from sympathetic perivascular nerves of control MA but not DOCA-salt hypertensive MA (Fig. 31B). However, at high concentration, H-89 inhibited NE release in DOCA-salt hypertensive MA but the effect was much less compare to control MA (Fig. 31B). This data supports that there is a higher level of PKA activity in DOCA-salt hypertensive rats hence H-89 is less effective in lower this effect.

Figure 28: Frequency-response-curves for NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA in the presence of PTX, idazoxan, and cocaine



The amount of NE release was measured as NE oxidation current. There is no significant different in the amount of NE release at each frequency of stimulation between DOCA-salt hypertensive comparing to control MA in the presence of PTX, idazoxan and cocaine. This suggested that the amount of NE in the readily releasable pool vesicles was the same between presynaptic sympathetic perivascular nerves of DOCA-salt hypertensive and control MA. Data are mean ± SEM and analyzed by two-way ANOVA.

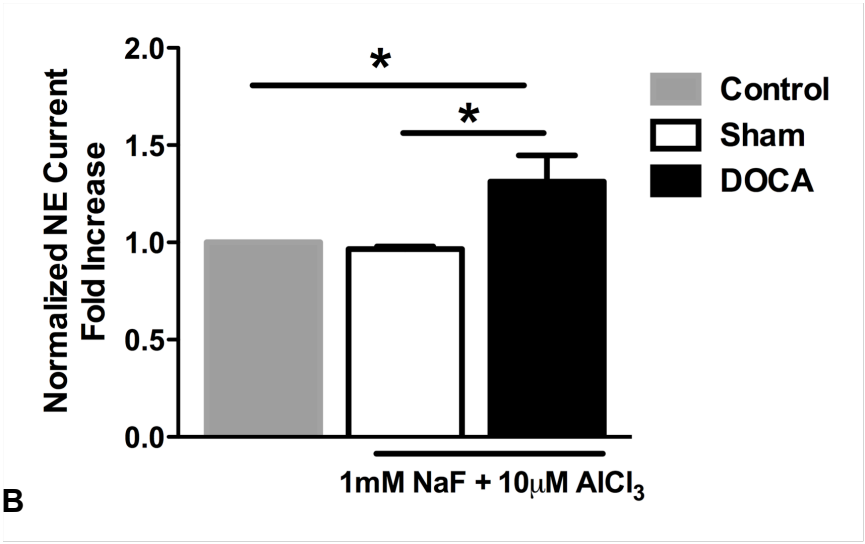
Figure 29: The effects of M119, a $G_{\beta\gamma}$ inhibitor, on NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA



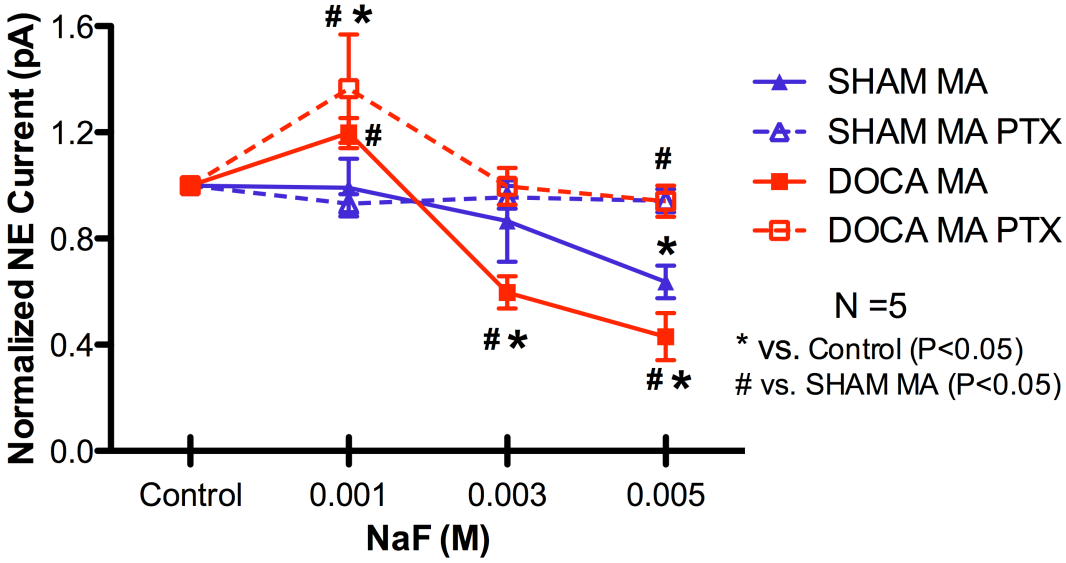
A) M119 blocked the effect of $G_{\beta\gamma}$ and increased NE release; this was equally effective in DOCA-salt hypertensive and control rats. Data are mean ± SEM and analyzed by two-way ANOVA. * $P < 0.05$ vs. 1E-7 M, n = 6. This suggested that there was no impairment in the activity of free $G_{\beta\gamma}$ on tonic inhibition of voltage gated calcium channel. B) Idazoxan, an α_2R antagonist, was not as effective in increase NE release in DOCA-salt hypertensive MA when compared to control MA. However, in the presence of M119, idazoxan was effectively increased NE release in DOCA-salt hypertensive MA and this was equal to that of NE release in control MA. This data suggested the impairment of α_2R in DOCA-salt hypertensive MA was upstream to the G-protein. Data are mean ± SEM and analyzed by one-way ANOVA. * $P < 0.05$ vs. DOCA 1 μM idazoxan, n = 6

Figure 30: The effects of AlF_4^- on NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA

A

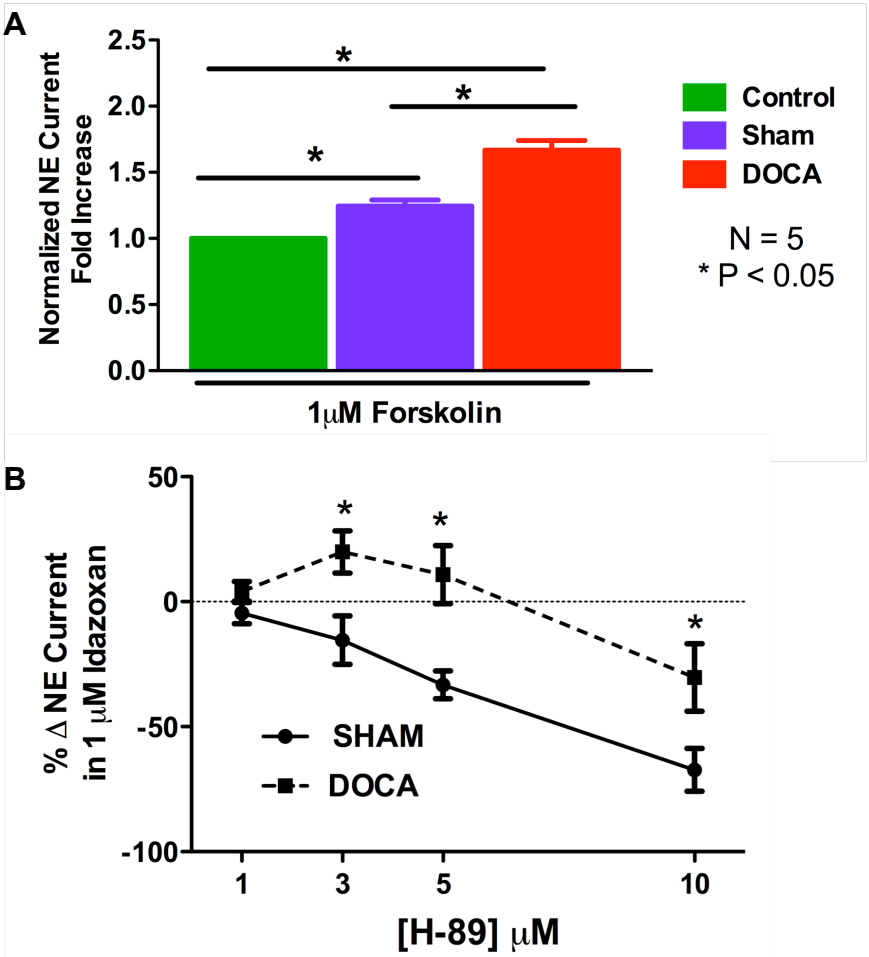


B



A) Low concentration of AlF_4^- increased NE release from sympathetic perivascular nerves of DOCA-salt hypertensive but not control MA. Data are mean ± SEM and analyzed by one-way ANOVA. * P < 0.05 vs. control, n = 5. B) High concentration of AlF_4^- decreased NE release from sympathetic perivascular nerves from both DOCA-salt hypertensive and control MA. However, DOCA-salt hypertensive MA was more sensitive to the effect of AlF_4^- on the reduction of NE release. PTX blocked the effects of high concentration AlF_4^- on decreasing NE release from DOCA-salt hypertensive and control MA. However, PTX did not block the effect of low concentration AlF_4^- on increasing NE release from DOCA-salt hypertensive MA. Data are mean ± SEM and analyzed by two-way ANOVA. * P < 0.05 vs. control, # p < 0.05 vs. SHAM MA, n = 5.

Figure 31: The effects of forskolin and H-89 on NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA



A) Forskolin increased NE release from sympathetic perivascular nerves from both DOCA-salt hypertensive and control MA. However, the level of forskolin-induced NE release was much higher in DOCA-salt hypertensive compared to control MA. Data are mean ± SEM and analyzed by one-way ANOVA. * $P < 0.05$ vs. control, n = 5. B) H-89 (3-5 μM) inhibited NE release in control but not DOCA-salt hypertensive MA. At high concentration H-89 (10 μM), it inhibited NE release from both DOCA-salt hypertensive and control MA, but the inhibition was greater in the control compared to DOCA-salt hypertensive MA. Data are mean ± SEM and analyzed by two-way ANOVA. * $P < 0.05$ vs. SHAM, n = 5.

Discussion

The depolarization of presynaptic sympathetic perivascular nerve terminals activates voltage gated Ca^{+2} channels and Ca^{+2} influx, which triggers exocytosis of NE vesicles. NE binds to postsynaptic α_1R receptors on smooth muscle cells and causes vasoconstriction. NE also binds to the α_2R autoreceptors on presynaptic sympathetic nerve terminals and causes inhibition of further NE release. Previously, we have shown that α_2R function is impaired in MA from DOCA-salt hypertensive rats and the impairment is due to the effects of vascular oxidative stress, particularly O_2^-. It is possible that the expression of α_2R is down-regulated in DOCA-salt hypertensive MA. However, it is less likely because α_2R in sympathetic neurons have a high receptor reserve and therefore, changes in the receptor density in sympathetic neurons do not correlate with functional changes (16). In this study we show that amount of NE released is not different between DOCA-salt hypertensive and control MA when the major regulation mechanisms of NE release are blocked. This suggests that the amount of NE in the RRP vesicles is not affected in presynaptic sympathetic perivascular nerves supplying MA from DOCA-salt hypertensive rats. Our data also show that blocking $G_{\beta\gamma}$ subunit with M119 equally increases the amount of NE from presynaptic sympathetic perivascular nerves of both DOCA-salt hypertensive and control MA. It has been shown that the receptor independent-$G_{\beta\gamma}$ subunits interact with CSP, a SNARE chaperone protein, and N-type Ca^{+2} channels resulted in a tonic G-protein inhibition of the

channels (13). Similarly, receptor dependent-$G_{\beta\gamma}$ subunits directly bind SNAP-25 and interfere with the binding of SNAP-25 to synaptotagmin and prevent vesicle fusion (17). Thus, the increase in NE release in the presence of M119 may be due to the decrease in the tonic $G_{\beta\gamma}$-dependent inhibition of Ca^{+2} influx and vesicles fusion. Interestingly, idazoxan, an α_2R antagonist, causes a 3-4 fold increase in NE release from control MA but only 1-2 fold from DOCA-salt MA. This suggests that α_2R is impaired. However, in the presence of M119, idazoxan causes a 3-4 fold increase in NE release from DOCA-salt hypertensive MA, which is similar to the level of control MA. This result suggests that the impairment of the α_2R function is upstream to the $G_{\beta\gamma}$ subunit. Furthermore, it supports that NE inhibits exocytosis by $G_{\beta\gamma}$. Different concentrations of AlF_4^- are used to bypass the receptor and stimulate the G-proteins directly. It has been shown that low and high concentration of AlF_4^- stimulates G_s and $G_{i/o}$, respectively (13). We also show that direct stimulation of $G_{i/o}$ proteins with AlF_4^- causes decrease in NE release from both DOCA-salt hypertensive and control MA. However, DOCA-salt MA was more sensitive to the simulation of AlF_4^-, which suggests that the $G_{i/o}$ protein is not impaired and there may be an increase in $G_{i/o}$ expression or activity in DOCA-salt hypertensive MA. It is more likely that there is an increase in the expression of $G_{i/o}$ protein in presynaptic sympathetic perivascular nerves from DOCA-salt hypertensive MA to compensate for the uncoupling of the G-proteins from their receptors. Other studies in our lab show that the uncoupling of the receptor from its G-protein is not specific to just

the α_2R but also the Adenosine$_{1A}$ receptor and the 5-HT$_{1A}$ receptor are impaired in DOCA-salt hypertensive MA. The common feature of these three receptors is that they all coupled to the $G_{i/o}$ protein. The effect of AlF_4^- in the inhibiting NE release is block with PTX, which supports the role of $G_{i/o}$ protein subtype.

Low concentration AlF_4^- increases NE oxidation currents in DOCA-salt hypertensive but not control MA. This effect is not block by PTX and supports that there is an increase in the expression or the activity of G_s protein in DOCA-salt hypertensive MA. Perhaps the increase in G_s activity is due reduction in $G_{i/o}$ activity because the uncoupling of α_2R and Adenosine$_{1A}$ receptor slows down the rate of $G_{i/o}$ protein cycling from inactive state into active state. NE retards the refilling of the RRP vesicles via $G_{\alpha i/o}$ subunits in INS 832/13 cell, a pancreatic β-cell line (18). Thus, the uncoupling of the $G_{i/o}$ protein from the α_2R increases the refilling of the RRP vesicles and higher NE release in the presence of low AlF_4^- concentration. PKA is shown to phosphorylate threonine-138 of SNAP-25 in adrenal chromaffin cells, which is required for the RRP of vesicles to be in a primed and releasable state. In addition, PKA phosphorylation of SNAP-25 activates the re-filling of recycling pool vesicles and increases the size of the RRP (14, 15). We also show that the level of increase in NE release in the presence of forskolin is higher in DOCA-salt hypertensive compared to control MA. Furthermore, H-89 was less effective in inhibiting NE release from DOCA-salt hypertensive compared to control MA. This

supports that a higher activity PKA in DOCA-salt MA. In summary, our data indicate that the α_2R function impairment occurs between the receptor and its G-protein.

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CHAPTER 6

GENERAL CONCLUSION AND PERSPECTIVES

Summary

The first aim of this study shows that DOCA-salt hypertension in rats is associated with pro-inflammatory MΦ infiltration into the MA adventitia, vascular oxidative stress and α_2R function impairment. MΦ infiltration and vascular oxidative stress occur 1 week before the impairment of the α_2R function. However, the inflammation and α_2R function impairment does not occur until mean arterial blood pressure elevates to approximately 135 mmHg. This study also shows that the inflammatory response is not due to the high salt diet or DOCA treatment alone but the synergistic effect of both is required for pro-inflammatory MΦ infiltration, oxidative stress, and α_2R function impairment in MA of rats. Furthermore, the BP is not affected by either treatment alone but it requires both, DOCA and high salt, as well to cause hypertension. Perhaps, the initial elevation in BP is what initiates the inflammatory effects. Our time course study also shows that peritoneal MΦ became activated in the similar temporal order as the infiltration of MΦ. It suggests that the peritoneal MΦ might be the source for the MΦ that infiltrated into the MA adventitia of DOCA-salt hypertensive rats.

The second aim of this dissertation shows that the depletion of MΦ using LEC attenuates the later phases of DOCA-salt hypertension development in rats without affecting the heart rate. LEC removes the pro-inflammatory MΦ from the peritoneal cavity and it blocks the infiltration of MΦ into the MA adventitia of DOCA-salt hypertensive rats. Blocking the MΦ infiltration into the MA adventitia of DOCA-salt rats

also lowered the level of vascular O_2^- in those animals. Furthermore, blocking the infiltration of MΦ into the MA adventitia of DOCA-salt rats prevents the α_2R function impairment. Perhaps, one possible mechanisms of how MΦ contributes to the development of later phase DOCA-salt hypertension is through the releasing of O_2^- that leads to the disruption of the α_2R function.

The third aim of this dissertation shows that if $G_{i/o}$ proteins are inactivated with PTX, α_2R are inhibited with idazoxan, and the NE transporters are blocked with cocaine then the amount of NE release from sympathetic perivascular nerves of DOCA-salt hypertensive MA is not different than in the control MA. This suggests that the amount of NE released from the RRP vesicles is not affected in DOCA-salt hypertensive MA. M119, a $G_{\beta\gamma}$ inhibitor, blocks the effect of $G_{\beta\gamma}$ and equally increases NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control rats. It has been shown that free $G_{\beta\gamma}$ subunits interact with CSP, a SNARE chaperone protein, and N-type Ca^{+2} channels results in a tonic G protein inhibition of the channels. This data suggest that there is no impairment in the activity of free $G_{\beta\gamma}$ on tonic inhibition of voltage gated calcium channel in sympathetic perivascular nerves of DOCA-salt hypertensive MA. Idazoxan, an α_2R antagonist, is not as effective in increasing NE release in DOCA-salt hypertensive MA when compared to control MA. However, in the presence of M119, idazoxan is effective in increasing NE release from sympathetic

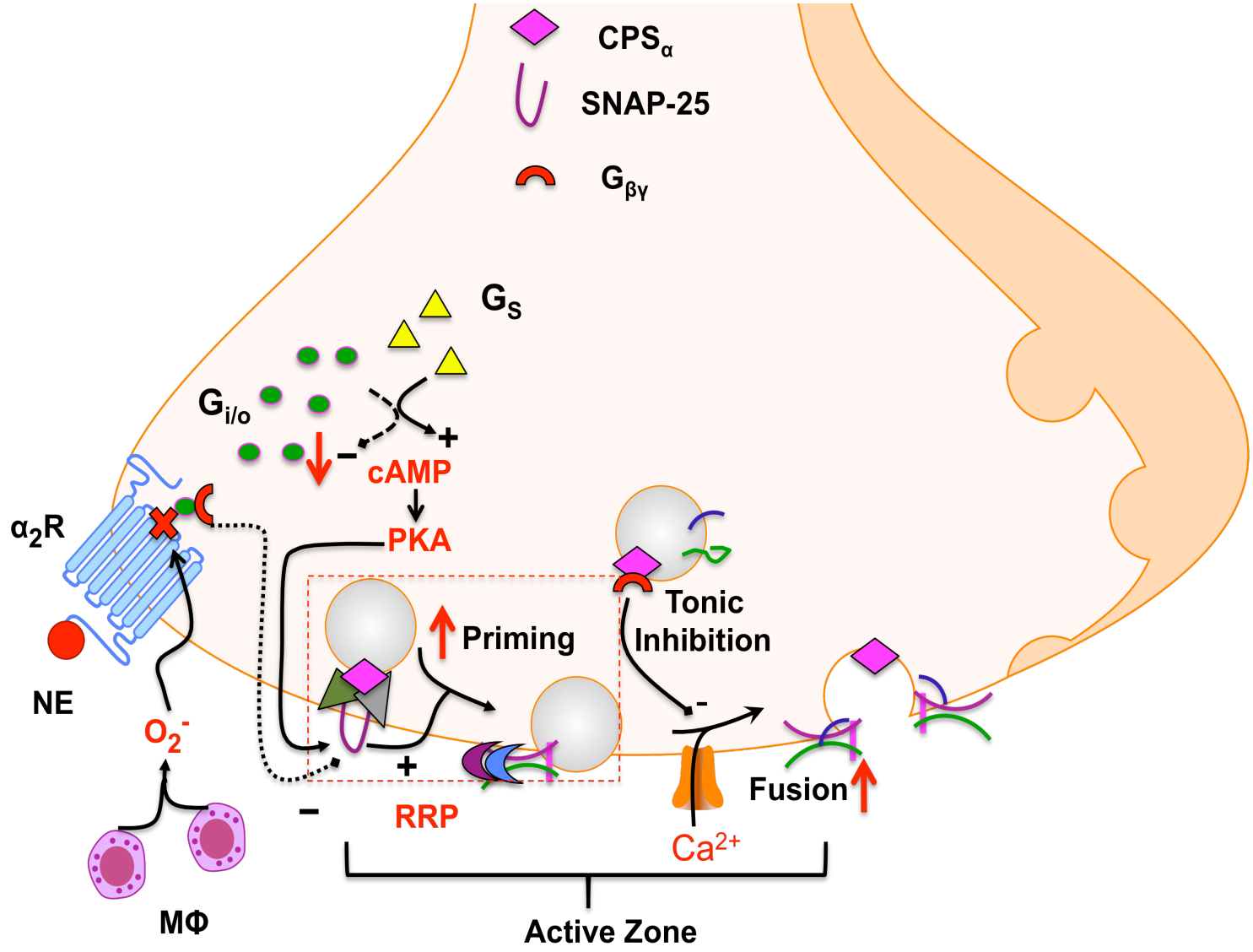
perivascular nerves of DOCA-salt hypertensive MA and this is equal to that of NE release in control MA. The data suggested the impairment of α_2R in DOCA-salt hypertensive MA is upstream to the G-protein. Low concentration of AlF_4^- increases NE release from MA sympathetic perivascular nerves of DOCA-salt hypertensive rats but not in control rats. It has been shown that low concentration and high concentration of AlF_4^- stimulates G_s and $G_{i/o}$ protein, respectively. Higher G_s protein function activates the production cAMP, and the increased cAMP level stimulates PKA activity. Protein phosphorylation is an important regulatory mechanism that controls the secretory pathway. PKA phosphorylation has been shown to promote and increase exocytosis. Thus, DOCA-salt MA is more sensitive to increased activity of G_s protein.

However, high concentrations of AlF_4^- decreases NE release from MA sympathetic perivascular nerves of both DOCA-salt hypertensive and control rats. However, DOCA-salt hypertensive MA is more sensitive to the effect of AlF_4^- on the reduction of NE release. The data support that the impairment in the α_2R function occurs upstream of the G-protein because directly stimulate $G_{i/o}$ protein results in reduction in NE current. It also suggests that there is a compensation increase in the expression or the activity of the Gi/o protein in DOCA-salt hypertensive MA. PTX blocks the effects of high concentration AlF_4^- on decreasing NE release from DOCA-salt hypertensive and control MA. However, PTX does not block the effect of low concentration AlF_4^- on increasing NE release from DOCA-salt hypertensive MA. This

supports that the low concentration of AlF_4^- stimulates G_s protein and the high concentration stimulates $G_{i/o}$ protein because $G_{i/o}$ protein is sensitive to PTX. Forskolin, an adenylyl cyclase activator, increases NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA. However, forskolin-induced increase in NE release is much higher in DOCA-salt hypertensive MA than that of control MA. Forskolin increases activity of adenylyl cyclase leads to increased levels of cAMP and subsequently PKA activity. The forskolin data support the low concentration AlF_4^- data where both, forskolin and low concentration AlF_4^-, increase NE release in DOCA-salt hypertensive MA. However, AlF_4^- stimulates more upstream from where forskolin stimulation occurs thus its effect is less than that of forskolin and is not able to increase NE release in control MA. Taking together the forskolin and low concentration AlF_4^- data suggests that there is also an increase in the level or the activity of G_s protein in sympathetic perivascular nerves of DOCA-salt hypertensive MA. However, it is more likely that there is an increase in the expression of $G_{i/o}$ protein to compensate for the uncoupling of the G-proteins from their receptors in presynaptic sympathetic perivascular nerves from DOCA-salt hypertensive MA. Our lab also finds that two other $G_{i/o}$ receptors on the presynaptic sympathetic nerves from DOCA-salt hypertensive MA are impaired as well. At lose concentration of H-89, a competitive inhibitor of PKA, it inhibits the NE release from sympathetic perivascular nerves of control MA but not DOCA-salt hypertensive MA. However, at high concentration, H-89 could inhibit NE

release in DOC-salt hypertensive MA but the effect is much less compare to control MA. The data support that there is a higher level of PKA activity in DOCA-salt hypertensive rats hence H-89 is less effective in lower this effect.

Figure 32: Proposed mechanisms of how MΦ-derived superoxide anions disrupt α_2R function in presynaptic sympathetic perivascular nerve termials from DOCA-salt hypertensive MA



As blood pressure increases MΦ is recruited into the MA adventitia. The MΦ release superoxide anions and the superoxide anions uncouple the α_2R from its G-protein. Uncoupling the G-protein from the receptors causes the reduction in the rate of G-protein activation, decreases the ability of $G_{\beta\gamma}$-subunit from interact with SNAP-25 to inhibit exocytosis. Furthermore, α_2R uncoupling also reduces $G_{i/o}$ activity, which shift the equilibrium toward G_s activity. Higher Gs activity leads to higher PKA activity, increased phosphorylated SNAP-25 and better priming of the NE vesicles for exocytosis. Take it all together, uncoupling of α_2R increases the amount of NE release and higher vasoconstriction in DOCA-salt hypertensive MA.

Novelty and Significance

What is relevant?

- DOCA-salt hypertension is associated with vascular inflammation and oxidative stress.
- α_2R function is impaired in DOCA-salt hypertensive rats.

What is New?

- MΦ infiltration and vascular O_2^- elevation in MA adventitia and α_2R function impairment in DOCA-salt hypertensive rats is time-dependent.
- MΦ infiltration and vascular oxidative stress occur 1 week before the impairment of the α_2R function.
- Peritoneal MΦ activation occurs during the same time period at that of MΦ infiltration into the MA adventitia of DOCA-salt hypertensive rats. Perhaps peritoneal MΦ is the source for the MΦ that is in the MA adventitia.
- MΦ infiltration, vascular oxidative stress, and α_2R function impairment is dependent on the synergistic effect of both DOCA and high salt. Similarly, blood pressure elevation is dependent on both DOCA and high salt diet.
- Liposomal-encapsulated clodronate depletes activated peritoneal MΦ and blocks MΦ infiltration into the MA adventitia of DOCA-salt rats.

- Blocking MΦ infiltration into the MA adventitia reduces vascular O_2^- level and prevents the impairment of α_2R function.

- Depletion of MΦ attenuates the later phase of DOCA-salt hypertension development in rats.

- If $G_{i/o}$ proteins are inactivated with PTX, α_2R are inhibited with idazoxan, and the NE transporter are blocked with cocaine then the amount of NE release from sympathetic perivascular nerves of DOCA-salt hypertensive MA is not different than in the control MA. This suggests that the amount of NE release from the RRP vesicles is not altered in presynaptic sympathetic perivascular nerve from DOCA-salt hypertensive MA.

- M119, a $G_{\beta\gamma}$ inhibitor, block the effect of $G_{\beta\gamma}$ and equally increases NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control rats. This supports that there is no alteration in the activity of free $G_{\beta\gamma}$ function in tonic inhibition of voltage gated Ca^{+2} channels.

- In the presence of idazoxan, the level of NE release is much lower in DOCA-salt hypertensive compared to control MA. However, in the addition of M119, idazoxan equally increases NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA. The data show that the α_2R function impairment occurs upstream from the $G_{\beta\gamma}$ protein.

- Low concentration of AlF_4^- increases NE release from MA sympathetic perivascular nerves of DOCA-salt hypertensive rats but not in control rats; and this effect is not block by PTX. Low concentration AlF_4^- stimulates G_s protein directly. Thus, this shows that G_s protein is functional and performed at a higher level in DOCA-salt hypertensive compared to control MA.
- High concentration of AlF_4^- decreased NE release from MA sympathetic perivascular nerves of both DOCA-salt hypertensive and control rats. However, DOCA-salt hypertensive MA is more sensitive to the effect of AlF_4^- on the reduction of NE release and PTX is able to block this effect in both DOCA-salt and control MA. High concentration AlF_4^- stimulates $G_{i/o}$ protein directly. Thus, this shows that $G_{i/o}$ protein is functional and performed at a higher level in DOCA-salt hypertensive compared to control MA.
- Forskolin, an adenylyl cyclase activator, increases NE release from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA. However, forskolin-induced increase in NE release is much higher in DOCA-salt hypertensive MA than that of control MA.
- Low concentration of H-89 (3-5 μM) inhibits the release of NE from sympathetic perivascular nerves of control MA but not DOCA-salt hypertensive MA.
- High concentration of H-89 (10 μM) inhibits the release of NE from sympathetic perivascular nerves of DOCA-salt hypertensive and control MA, but the inhibition

was significantly higher in control MA compared to DOCA-salt hypertensive MA. Together with the forskolin, it suggests that the activity of PKA is higher in DOCA-salt hypertensive compared to control MA.

- All together, it suggests that the uncoupling of the α_2R from the $G_{i/o}$ protein decreases the cyclying rate of $G_{i/o}$ protein from the inactive to active state and shifts the equilibrium toward the inactive state. This tilts the balance between $G_{i/o}$ and G_s to G_s, hence the increased sensitivity of G_s in DOCA-salt hypertensive MA.

What is Novel?

My study is the first to focus on the interaction between the systemic inflammatory responses and the perivascular sympathetic nerves as a contributing factor to the evolution of chronic hypertension. Specifically, it is the first to exam the relationship between MΦ-derived O_2^- and the impairment of the α_2R. It is also the first to show the possible mechanism(s) of α_2R impairment in perivascular sympathetic nerves terminals from DOCA-salt hypertension MA.

Limitations of Experiments

The results of these studies reveal that pro-inflammatory MΦ infiltration and increased O_2^- level in MA adventitia of DOCA-salt rats occur after 10 days of initial blood pressure increase, but α_2R impairment does not occur until a week after the infiltration of MΦ. O_2^- is a reactive and a short-lived molecule. I show that MΦ and perivascular sympathetic nerves are found in the same 10 μM thick section of the adventitia of MA from DOCA-salt hypertensive rats. Although, the technique is not able to calculate the exact distance between the MΦ and varicosities of the nerve endings, we can use the Z-section to further narrow down the distance between the MΦ and the varicosities.

Most of the neurotransmitter synaptic transmission studies are done at large presynaptic nerve endings with direct intracellular or whole-cell patch recordings. Perivascular nerves of the MA are not as large or densely innervated. Thus direct intracellular and whole-cell patch recordings cannot be used. However, amperometry allows for the precise spatial and temporal measurement of vesicular NE release from perivascular nerves of the MA (1-2). Although, we can measure the amount of NE released, this technique does not measure the amount of the receptors at the presynaptic nerve endings. In this dissertation, I show that the α_2R from MA of DOCA-salt rats generate less response to its agonist, UK 14304, and antagonist, idazoxan, than from the control MA. This suggests that the α_2R is impaired. However, it is also important to measure the amount of α_2R in the presynaptic nerve endings from the MA.

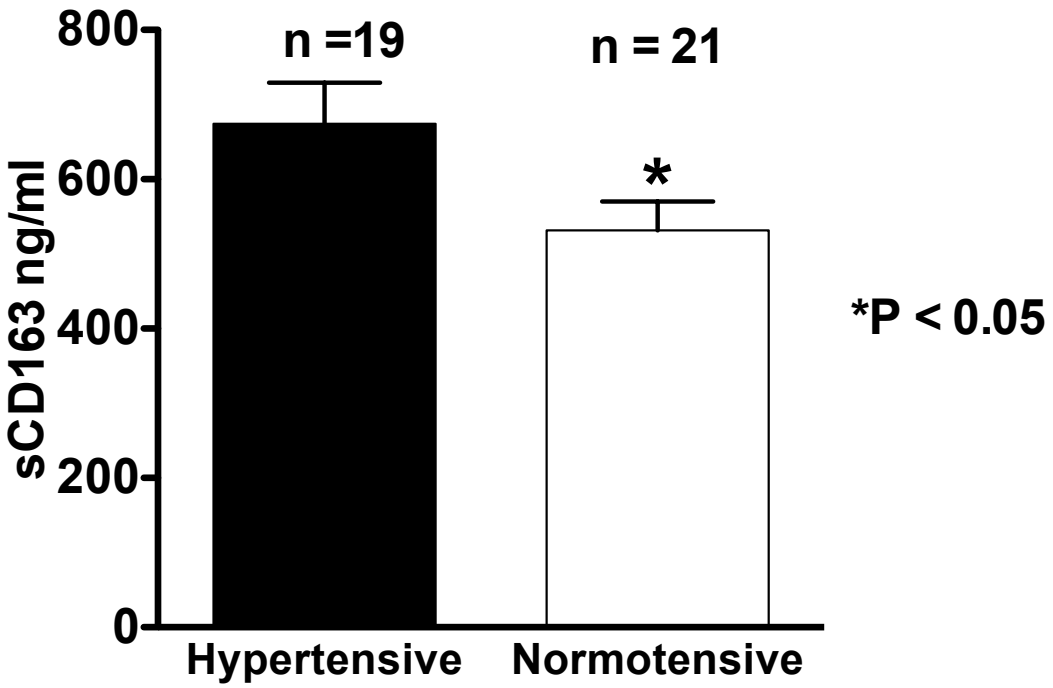
A limitation is that the amount of nervous tissue relative to others e.g. smooth muscles in the MA is very small, α_2R is found in both presynpatic sympathetic perivascular nerve endings and smooth muscles. Therefore, Western Blot cannot distinguish between the two sources if whole MA is used to do the Western Blot. Instead of measuring the α_2R protein directly we could measure its mRNA from the celiac ganglion. However, neurons in the celiac ganglion have projections to many different places not just the MA. This may mask any small differences that may exist in the α_2R mRNA levels between hypertensive and normotensive neurons that project to the MA. Retrograde tracer can also be used to measure the other important proteins e.g. PKA, $G_{i/o}$ and G_s in sympatheic perivascular nerve supplying MA that appear to be affected in DOCA-salt hypertension.

Our studies show that LEC prevent the development of the later phases of DOCA-salt hypertension by blocking the infiltration of MΦ into the MA adventitia, reducing the O_2^- level in the MA, and preventing the α_2R dysfunction. However, MΦ also play an important role in regulating T-cells, and T-cells have been shown to play a role in experimental hypertension. Therefore, it is possible that depletion of MΦ alters the activation of T-cells. Furthermore, pro-inflammatory cytokines e.g. TNF-α have been implicated in the development of hypertension and MΦ is one of the sources of TNF-α release. Hence, depletion of MΦ could block the effects of TNF-α on the development of DOCA-salt hypertension.

Perspectives

Hypertension is a major risk factor for heart disease and stroke, which are leading causes of death in the United States. The good news is the average blood pressure of the population has steadily declined over the last five decades. However, the bad news is the prevalence of resistant hypertension is increasing. Furthermore, during the last two decades, no new anti-hypertensive drugs have been introduced. Therefore, clarifying the mechanisms that leads to enhanced neurogenic vasoconstriction is important for anti-hypertensive drug discovery. This study indicates that MΦ-derived O_2^- disrupts α_2R and further increases blood pressure in DOCA-salt rats. It is possible that vascular MΦ markers could be used in the future to identify patients at risk for long-term changes in sympathetic nerve function at the first diagnosis of high blood pressure. This dissertation shows that the expression of CD163, a MΦ specific hemoglobin-heptoglobin scavenger receptor, is increased in DOCA-salt hypertensive rats. Oxidative stress or inflammatory stimuli e.g. activation of TLR2 or 5 cause the extracellular domain of this receptor to shed from the cell surface and generate a soluble CD163 or sCD163. The plasma of a group of subjects who have hypertension and normal BP is measured for sCD163 using ELISA. Figure 33 shows that hypertensive subjects have higher level of sCD163 in their plasma compared to normotensive subjects. Thus, sCD163 may be the vascular MΦ markers that could be used in the future to identify patients at risk for long-term changes in sympathetic nerve function at the first diagnosis of high blood pressure. Finally, this disseration also highlights MΦ as a new promising target for the next generation of anti-hypertensive drugs.

Figure 33: The level of sCD163 in human subjects with hypertesion and normotension



sCD163 was measured in the plasma of 19 hypertensive patients and 21 nomotensive subjects using ELISA. Patients with hypertension had higher level of sCD163 in their plasma compared to normotensive subjects. Data are mean ± SEM and analyzed by student t-test, $p < 0.05$, n=19-21.

Future Work

For the future, it would be important to find out the level of mRNA and/or protein expression of the α_2R, $G_{i/o}$, G_s, and PKA in MA specific neuron population in the celiac ganglia. Although neurons in the celiac ganglion project to many different locations, it is possible to use retrograde tracers to label specific MA subpopulation of neurons in the celiac ganglion. Flow-cytometry can be used to sort out the positive labeled neurons from the negative neurons, and RT-PCR and Western Blot can be used to measure the mRNA and protein level, respectively of only the positive neurons in the celiac ganglion. Using combination of these techniques will increase the sensitivity in detecting the potential differences of the mRNA and/or protein levels between hypertensive and normotensive state.

In this dissertation, I show that there is an infiltration of MΦ into the MA adventitia of DOCA-salt hypertensive rats. However, it is unclear what is the source of the infiltrated MΦ and from which route they arrive to the adventitia. These MΦ can infiltrate through the tunica intima and media to get to the tunica adventitia. However, we also show that the peritoneal MΦ are activated and the MA are located inside the peritoneal cavity. It is possible that these MΦ get onto the adventitia of MA from the peritoneal cavity. Furthermore, we did not find any MΦ in the adventitia of skeletal muscle arteries from the DOCA-salt hypertensive rats. This evidence supports the out-side-in mechanism in which MΦ arrive to the MA adventitia from the peritoneal cavity. In order to answer the question about the source and the route of infiltration, we can use the GFP-transgenic rats. We could harvest the GFP-peritoneal MΦ and intravenously or

intraperitoneally inject them into the non GFP DOCA-salt rats and track the paths of the GFP-peritoneal MΦ. Using GFP MΦ we can detemine how and where these MΦ get into the MA adventitia of DOCA-salt hypertensive rats.

We establish that the level of CD163 expression on the MΦ is increased in DOCA-salt rats and hypertensive patients have higher of sCD163 in their plasma. However, it is unclear how this receptor contributes to the development of blood pressure. Cross-linking of rat CD163 on peritoneal macrophages with anti-CD163 antibodies induces production of pro-inflammatory mediators, including NO, IL-1β, IL-6 and TNF-α. Furthermore, the level of sCD163 is increased in the plasma of patients with peripheral artery disease. Using anti-CD163 anitbodies we can test the function of CD163 in DOCA-salt hypertension model in rats. It would be important to elucidate the acute effect of CD163 on the blood pressure of established hypertensive animals, and also the chronic effect of CD163 on the development of DOCA-salt hypertension in rats. After the administration of anti-CD163 antibodies, inflammatory and anti-inflammatory mediators should be measured to excess the inflammatory effects of CD163 in DOCA-salt hypertension.

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