A Dissertation

entitled

The Role of the Innate Immune System and Resolution of Inflammation in Microvessels from Hypertensive Animals

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in

Biomedical Sciences

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August 2021

An Abstract of

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Approximately half of the American population has high blood pressure, or hypertension. Hypertension is a multifactorial medical condition that greatly increases the risk factor for heart, brain, kidney, and other life-threatening diseases. As blood pressure is described as the force exerted by circulating blood against the walls of the body's arteries, it is of grave importance to identify novel therapeutic targets for treatment of hypertension.

A critical barrier in the development of novel therapeutic approaches for hypertension is the gap in understanding of a precise cause-effect mechanism. Recently, immune mechanisms have been implicated in the pathophysiology of hypertension. Innate immune system activation, and subsequent inflammatory events, have been proposed to cause injury and dysfunction in the cardiovascular system leading to hypertension. However, it is still unclear if the activation of the innate immune system precedes hypertension. To examine the role of the innate immune system in hypertension, we targeted a pattern recognition receptor, the formyl peptide receptor-1 and its agonists in microvessels. Our results identified a role for the formyl peptide receptor-1 in the genesis

of salt-sensitive hypertension. Specifically, we showed that cell injury, and subsequently, mitochondrial-derived peptides activate formyl peptide receptor-1 and are responsible for vascular remodeling and the premature elevation in blood pressure in Dahl salt-sensitive rats, independent of high salt diet.

Continuing our investigation into mechanisms of pathophysiology in microvessels, we observed regular vascular dysfunction in resistance arteries from hypertensive rats. We observed arterial contraction from a widely used pharmacological vasodilator, acetylcholine. Acetylcholine binds to G-protein coupled muscarinic receptors that mediate a transient elevation in intracellular, free calcium. This intracellular rise in calcium is triggers several cellular responses, including the synthesis of nitric oxide, endothelium-derived hyperpolarizing factor, and eicosanoids derived from polyunsaturated fatty acids. The eicosanoids derived from polyunsaturated fatty acids, like arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid, can be vasodilatory or vasoconstrictive. The metabolism of these fatty acids is also important for mediating inflammatory responses. As a result, acetylcholine-induced contraction due to fatty acid metabolism is a commonly observed feature of endothelial dysfunction and vascular inflammation in hypertension. In opposition of investigating the role of the inflammation in and vascular dysfunction in hypertension, we investigated the resolution of inflammation in hypertension.

Resolution of inflammation is an active phenomenon to switch off the inflammatory processes and facilitate the return to homeostasis. Increasing the levels of pro-resolving mediators to promote the resolution of inflammation is emerging as a novel therapeutic approach. The previously mentioned polyunsaturated fatty acids are substrates to produce

the pro-resolving lipid mediators. However, it is unknown if these mediators can ameliorate dysfunction in arteries from hypertensive animals. Therefore, we hypothesized that pro-resolving lipid mediators decrease acetylcholine-induced contractions in arteries from spontaneously hypertensive rats (SHR). To examine the role of pro-resolving lipid mediators in vascular function, we incubated mesenteric resistance arteries from male SHR and Wistar Kyoto (WKY) with either pro-resolving lipid mediators or vehicle for 1 hour prior to concentration response curves to acetylcholine or phenylephrine. Our results showed that incubating arteries with pro-resolving lipid mediators reduced acetylcholine-induced contraction in arteries from hypertensive arteries, suggesting that resolution of inflammation and the pro-resolving lipid mediators may be used as a new therapeutic tool to improve vascular function in hypertension. Our studies thus far identify roles for the innate immune system and the resolution of inflammation, specifically in microvessels, to be possible novel therapeutic approaches for the treatment of hypertension.

This dissertation is dedicated to my parents, Bobby and Yolanda Edwards, because without them, I would not be here. No matter what it was, they always made a way to give me exactly what I needed to move forward. All the good times and bad times, they were the first ones that I called to share. Thank you for molding me into a woman with resilience, courage, and determination. Thank you for wiping my tears and pushing me to keep going no matter how hard things seemed to be. With all of me, thank you for everything!

Acknowledgments

I would like to acknowledge all members of the Wenceslau Laboratory, affectionately known as the Wenceslau Gang. They all have played a part in my journey and I am grateful to know every one of them.

I would like to acknowledge my mentoring circle begun at The University of Toledo: Amanda Bryant-Friedrich, Ph.D., Anita Easterly, Ph.D., Flapp Cockrell, Ph.D., Kimberly Jenkins, M.D., and Cyndee Gruden, Ph.D. I am beyond grateful for their dedication to research, medicine, and students and their unwavering support during my time in this program.

I would like to acknowledge the entire Northern Ohio Alliance-Alliances for Graduate Education and the Professoriate funded by the National Science Foundation. A special thank you goes to Susan Pocotte, Ph.D., UToledo Principal Investigator, Teri Green, Site Coordinator, and the members of my cohort: Shermel Sherman, Ph.D., La'Nese Lovings, Ph.D., Ethel Tackie-Yarboi, Ph.D., Maura Graves, and David Baliu-Rodriguez. They have been on this journey with me from the beginning and were another source of unconditional support that could not be replaced.

Last, but certainly not least, I would like to acknowledge my fiancé, Martez Glenn. He has been my shoulder to cry on, my grammar and spell checker, and avid listener without complaint. His love and support are unwavering, and I am glad to have had him on this journey and for the rest of our life's journeys.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AA.....arachidonic acid
Ach.....Acetylcholine
AMO .....amoxicillin

BMDM.....bone marrow-derived macrophage
BP.....blood pressure

CSA.....cross sectional area
CsH .....Cyclosporin H
COX .....cyclooxygenase
CYP450.....cytochrome P450

DAMPs .....damage associated molecular patterns
DHA.....docosahexaenoic acid

EDH .....endothelium-dependent hyperpolarization
EDHF .....endothelium-derived hyperpolarizing factor
EETs.....epoxyeicosatrienoic acids
eNOS.....endothelial nitric oxide synthase
EPA.....eicosapentaenoic acid

fMLP .....N-formylmethionine-leucyl-phenylalanine
FPR .....formyl peptide receptor

HAEC.....human aortic endothelial cells
HS .....high salt

KCl.....potassium chloride

LS.....low salt
LOX .....lipoxygenase
LV .....left ventricle
LXA_4.....Lipoxin A_4

mAChRs.....muscarinic receptors

MRA .....mesenteric resistance arteries

NFPs.....N-Formyl Peptides
NaCl .....sodium chloride
NO.....nitric oxide

PE.....phenylephrine
PG .....prostaglandin
PGI_2.....prostacyclin

R.....Dahl salt-resistant rat
ROS.....reactive oxygen species
RvD1 .....Resolvin D1
RvE1 .....Resolvin E1

S .....Dahl Salt-sensitive rat
SHR.....Spontaneously Hypertensive Rats
SPMs.....Specialized pro-resolving mediators

TL.....tibia length
TXA .....thromboxane

VSMCs.....vascular smooth muscle cells

WKY .....Wistar Kyoto Rats

List of Symbols

kg .....kilogram
mg ..... milligram
ml ..... milliliter
mm ..... millimeter
mmHg ..... millimeter of mercury
mN..... milliNewtons
µg ..... microgram
µl ..... microliter
nM..... nanomolar
µM..... micromolar
> ..... greater than
< ..... less than
= ..... equal to
α ..... Alpha
β ..... Beta
ω..... Omega

Chapter 1

1. Introduction

1.1 Objectives

Hypertension is defined as high or elevated blood pressure (BP) above 130 mmHg systolic BP over 80 mmHg diastolic BP [1]. There are two types of hypertension in humans. Essential, or primary, hypertension is the most common accounting for 95% of hypertensive cases but has no known medical cause [2]. Secondary hypertension is much less common and has a known medical cause such as obesity, kidney disease, or diabetes [2]. With no warning signs or symptoms, primary hypertension is known as the “silent killer.” Hypertension is a multifactorial disorder that can be influenced by many environmental and genetic factors. Factors that increase the risk of hypertension development include increased salt intake and poor diet choices, stress, smoking, infection, and lack of physical activity [1]. Hypertension development risk can be decreased with healthier lifestyle choices, but sometimes that is not enough. Even after lifestyle change and therapeutic intervention, blood pressure can still be uncontrolled. Uncontrolled high blood pressure increases the risk of cardiovascular disease. As our population ages,

increases in hypertension prevalence may be due to a decrease in vascular compliance due to arterial stiffening [3]. The occurrence of vascular aging in all ages could be contributing to hypertension incidence and cardiovascular risk. There are still many other mechanisms that contribute to hypertension development and maintenance. With our research, we hope to shed a new light on new molecular pathways associated with the vascular system in the pathophysiology of hypertension.

1.2 Hypertension: A Brief History

The first person to measure arterial blood pressure directly was Reverend Stephen Hales in a horse in 1733 [4]. His famous experiment showed that blood rose to 8 feet and 3 inches in a glass tube that was placed in the horse artery [5,6]. About one hundred years later, noninvasive devices called sphygmographs, were created to measure blood pressure [6]. However, these devices were not sensitive enough to gain accurate blood pressure readings in humans.

Along with the measuring of blood pressure, the processes and mechanisms by which hypertension occurs have been studied since the early 1870s when a medical resident at Guy's Hospital in London named Frederick Mahomed started measuring blood pressure in the general population [3]. He made a portable, spring-based version of the sphygmograph that was used previously and discovered that a subset of the population had high blood pressure independent of kidney disease and proteinuria as previously observed [7]. Even with Mahomed's discovery, blood pressure measurement did not become common practice until the 1890s when the sphygmomanometer, a combined BP cuff and mercury manometer, was created and the determination of diastolic BP was discovered [4,

8-9]. Young Russian surgeon Nikolai Korotkoff is credited with discovering the characteristic sounds during inflation and deflation using a stethoscope and correlating it with the associated pressure [4]. Due to that discovery, the pressure during the sound of inflation and deflation are now termed as systolic and diastolic BP, respectively [4].

The sphygmomanometer was brought to the United States by Theodore Janeway and Harvey Cushing. Janeway's early studies revealed that systolic BP was rarely above 140mmHg in adults under 65 years old. For adults older than 65 years old, BP was closer to 160 mmHg [10]. In the early 1900s insurance companies recognized that patients with hypertension had increased mortality [10]. Eventually the measurement of BP became a standard procedure by physicians with 140/90 mmHg being defined as hypertension [11]. Hypertension was also found to increase risk for chronic kidney disease, congestive heart failure, and stroke [1]. It is projected that by 2025, hypertension will be the most common noncommunicable disease, affecting 1.5 billion people [12].

1.3 Hypertension Epidemiology

Hypertension is one of the leading causes of death worldwide. A little over a decade ago, 1.38 billion people had hypertension [4]. These cases were based on hypertension being defined as systolic BP greater than 140mmHg or diastolic BP greater than 90 mmHg [4]. However, a recent update in the 2017 Guidelines for the detection, evaluation, and treatment of hypertension changed the criteria for hypertension. Currently, any person with systolic BP greater than 130 mmHg and/or diastolic BP greater than 80 mmHg is considered to have hypertension [1]. This change means that approximately 50% of the American population now has high blood pressure.

According to the most recent National Health and Examination Survey, hypertension control has dropped from 53.8% to 43.7% [13]. Not only is there a lack in controlling blood pressure, there is also a drop in the knowledge of having hypertension. The number of adults who know they have hypertension declined from 84.7% to 77%. In line with that, the number of people taking antihypertensive medications dropped from 92.7% to 88.2%. This data is corroborated by a report from the American Heart Association [14]. These statistics elude to the lack of proper surveillance and treatment of hypertension in the American population and in turn increases medical procedures and economic burden over time [14].

Hypertension disproportionately affects different races and genders and increases with age. The prevalence of hypertension is higher in males than females (51.7% vs 42.8% respectively). Hypertension prevalence increases with age group; age group 20-34 (male-29.0%, female-13.6%), 35-44 (male-48.1%, female-30.3%) 55-64 (male-68.3%, female-62.8%), 65-74 (male-67.5%, female-75.7%), and 75 and over (male-83.6%, female-84.5%). As age increases, there is a switch in prevalence from males to females. In 2018, hypertension prevalence was also higher among adult non-Hispanic black Americans (males-58.3%, females-57.6%) than non-Hispanic white Americans (males-51.0%, females-40.5%), non-Hispanic Asians (males-50.1%, females-42.1%) or Hispanic (males-50.6%, females=40.8%) [14].

The American hypertension prevalence is striking but prevalence is not only rising in the United States. Currently, hypertension is considered a global epidemic. As the world is aging and there is more exposure to unhealthy life choices, the number continue to rise. Diets higher in sodium and decreased physical activity are becoming more normal and

increasing numbers. Surprisingly, the changes in prevalence are not equal amid all countries. Low- and middle-income countries have an experienced increase in hypertension prevalence, whereas high-income countries have seen a decrease in hypertension in the last twenty years [14]. As evidenced, hypertension is a complex disease influenced by a number of environmental, genetic, and socio-economic factors.

1.4 Rat Models for Hypertension Research

Animal models have long served as valuable tools to understand the physiology and pathophysiology of diseases in laboratory settings. They also allow us the opportunity to uncover mechanisms to develop new therapeutics for many diseases. Animal models used in research include fish, mice, rats, birds, primates, cats, dogs, pigs, horses, amphibians, reptiles, and other mammals [15]. Among these animals, the rat model has been popular due to the availability of different inbred strains and characteristics and reproducibility.

There are multiple rat strains commercially available to study hypertension. Rat strains exhibiting hypertension include Dahl Salt-Sensitive Rats (S), Spontaneously Hypertensive Rats (SHR), Milan hypertensive rats, and Lyon hypertensive rats. Majority of these hypertensive rats were derived from outbred Wistar or Sprague-Dawley rats that were selectively bred for hypertension-related traits [16]. Kyoto, Japan is the origin of the SHR that exhibited a spontaneous elevation in BP [17]. This model is used as a model for primary hypertension and is useful for studying vascular function, renal function, and the genetics of primary hypertension among others [16].

Dahl salt-sensitive and salt-resistant (R) rats were developed from Sprague-Dawley rats that were observed to have blood pressure changes respond or not respond to dietary

sodium, respectively [18]. S rats develop hypertension on a low salt diet, but a high salt diet expedites the hypertension development rate. These rats were selectively bred to create a strain of rats that are completely salt sensitive. In opposition, R rats were bred to be resistant to salt-induced hypertension. Dahl rats were originally maintained as outbred stocks but were later developed into inbred stocks at the University of Toledo College of Medicine and Life Sciences, formally known as the Medical College of Ohio, by Dr. John P. Rapp [19]. Although many other institutions have Dahl rat colonies, the University of Toledo rat colony has been termed as the only one true genetically and phenotypically consistent S rat colony [19].

Other hypertensive rat models include Milan rats that were selectively bred from Wistar rats for BP, like Dahl rats, which resulted in Milan hypertensive strain (MHS) and the Milan normotensive strain (MNS) [20]. These rats are also used to study primary hypertension. Lyon rats were selectively outbred Sprague-Dawley rats for elevated, normal, or decreased BP and named Lyon hypertensive (LH), Lyon normotensive (LN), or Lyon low blood pressure (LL) rats, respectively [21]. The differences in groups can be observed as early as 5 weeks and are used in renal, metabolic, cardiac, and genetic studies [16, 22].

1.5 Vascular Function Changes in Hypertension

As stated previously, hypertension is a multifactorial disease whose overall origin has yet to be elucidated. Dr. Irvine Page is one of pioneers of hypertension research and produced an octagonal diagram that lays out just how many factors can cause hypertension [23]. This diagram evolved over time but still shows that alterations in vascular elastance,

neural and chemical perturbations, blood volume, and cardiovascular reactivity among other things contribute to hypertension development [24-25]. Although elevated blood pressure has detrimental effects on numerous systems in the body, it is well known that the kidney plays a major role in blood pressure regulation [24, 26-29] . Paired with the kidney, the vasculature plays an equally important role. Systemic vascular resistance is increased in adults with hypertension and the most common treatment is vasodilators [24]. A recent review proposed four vascular deviations that happen in and contribute to hypertension [24]. One, the increase in vasoconstrictive substance that lead to vascular dysfunction and promote vasoconstriction over vasodilation. Second is vascular structure changes in both micro- and macro-vessels. Third is the stiffening of the aorta. Last, the vasculature is a target and source of immune activation due to the crosstalk between immune cells and the endothelium [24]. All these roles of vasculature in hypertension are cause for research to continue to uncover the mechanisms of action in vessels to be targeted for treatment.

1.6 Vascular Remodeling in Hypertension

Increased peripheral resistance to blood flow is associated with hypertension [30]. A specific group of arteries called resistance arteries are vessels with lumen diameters smaller than 400μm when fully relaxed [31]. Resistance arteries include small arteries and arterioles which are abundant in the body. Due to the abundance, small arteries have been implicated in the pathogenesis of hypertension [32]. The cause for this implication is due to decreases in lumen diameter directly increase vascular resistance. According to Poiseuille's Law, any small change in blood vessel radius is raised to the fourth power

which will cause an exponential effect on resistance which in turn results in a dramatic change in blood flow through the vessel [32].

Changes in vascular function in the pathophysiology of hypertension are described above. Paired with functional changes, there are also structural changes in arteries associated with hypertension. Specifically, resistance arteries mainly undergo a reduction in lumen diameter collectively named remodeling [33]. Vascular remodeling is an active process that involves changes in cell death, cell growth, cell migration, and the creation or degradation of the extracellular matrix [33]. It has been shown that small artery remodeling is the first manifestation in hypertension [34-36]. The Schiffrin group has shown that approximately all of stage I hypertensive patients present with small artery remodeling [34]. Arterial remodeling in resistance arteries is a complex system of interactions that have yet to be defined. These interactions include, but are not limited to, genetic factors, adaptions to mechanical conditions, humoral response, and other local trophic influences [36]. There is a gap in the literature as to what factor is triggering the remodeling of arteries in hypertension leading to vascular dysfunction. Although remodeling was first defined as hypertrophic due to increased cross sectional area and smaller lumen size [37], the Mulvany group proposed that vascular remodeling should encompass all diameter changes observed in relaxed vessels [38-41]. A general overview of the different types of vascular remodeling are pictured below.

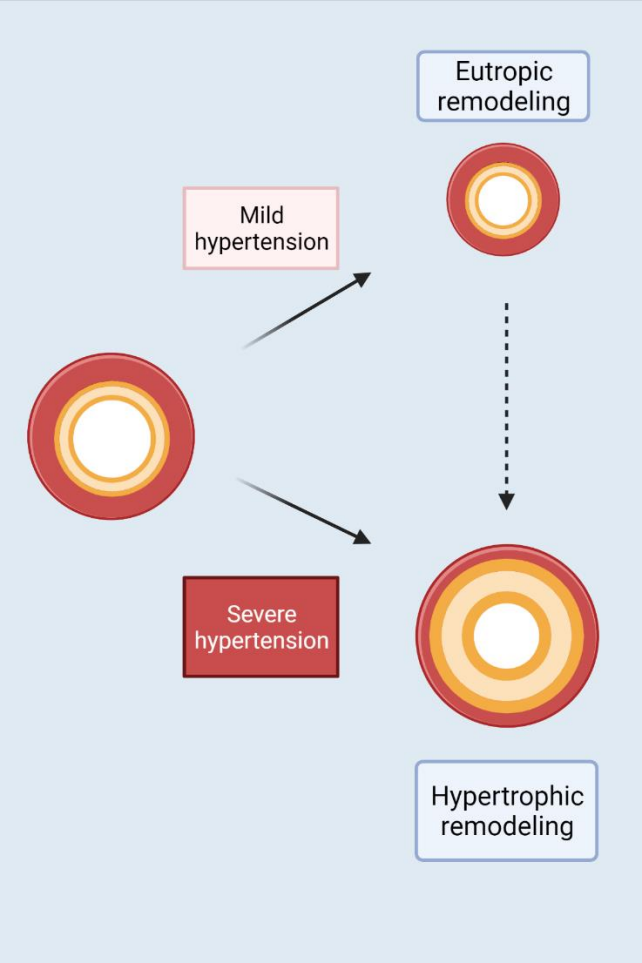


Figure 1.1. Different types of vascular remodeling in hypertension. Eutrophic and hypertrophic remodeling are the two main types of vascular remodeling in hypertension. Eutrophic remodeling is mainly observed in mild, essential hypertension. Hypertrophic remodeling is mainly observed in more severe cases of hypertension like renovascular hypertension.

Although there are multiple types of vascular remodeling, structural changes in resistance arteries in hypertension involve a combination of eutrophic and hypertrophic remodeling [42]. In eutrophic remodeling, the lumen diameter and outer diameter are decreased while the cross-sectional area of the vessel remains unchanged which results in a greater media-

lumen ratio [38]. This type of remodeling is observed in SHR and in humans with mild, essential hypertension [43-47]. Hypertrophic remodeling involves the increase of the outer diameter paired with decreased lumen diameter which causes an increase in the media to lumen ratio and cross-sectional area [32]. Hypertrophic remodeling is observed in severe hypertension like deoxycorticosterone (DOCA)-salt induced hypertension and Dahl salt-sensitive hypertension also in humans with renovascular hypertension [48-50]. Both types of remodeling can be due to abnormalities in endothelial or vascular smooth muscle cells, cell adhesion, and the extracellular matrix [32]. It has also proposed that by some unidentified mechanisms, eutrophic remodeling progresses into hypertrophic remodeling. Understanding these vascular changes and the mechanisms by which they occur may offer insight into the development of therapeutics to prevent vessel-initiated end-organ damage in CVD. The combination of vascular function impairment and vascular remodeling leading to increase peripheral resistance and ultimately their effect on blood pressure shows why microvessels continue to be investigated in hypertension.

1.7 Innate Immune System and Hypertension: Introduction to the Formyl Peptide Receptor-1

Recently, immune mechanisms have been implicated in hypertension [51-52]. In fact, immune system activation and inflammation have been proposed to link the kidneys, cardiovascular system, and the gut together mechanistically in hypertension development [51-53].

The formyl peptide receptor (FPR) is a pattern recognition receptor that functions in the innate immune system. The FPR family is a group of G-protein coupled receptors and is comprised of 3 members (FPR-1, FPR-2, and FPR-3) in humans and 8 in mice [54-55]. Each member of the FPR family has a different binding affinity and elicits different responses. FPR-1 has a high binding affinity for N-formyl peptides (NFPs) like N-Formylmethionine-leucyl-phenylalanine, produced by bacterial degradation. Bacteria is one of two sources of NFPs in the body and the formyl group at the N-terminus is recognized by FPR [56]. The other source of NFPs detectable by FPR-1 is mitochondria [57] (Figure 1.2). The NFPs are recognized as pathogens and play a role in initiation of inflammation. We previously observed that FPR is not only in sentinel cells but also in endothelial cells and VSMCs [58-60]. A slow and sustained contraction in airways and vascular leakage is induced by the activation of FPR and systemic FPR activation leads to systemic inflammation [58, 60]. As FPR-1 elicits a defensive response against endogenous and exogenous dangers, it is an attractive target for therapeutics.

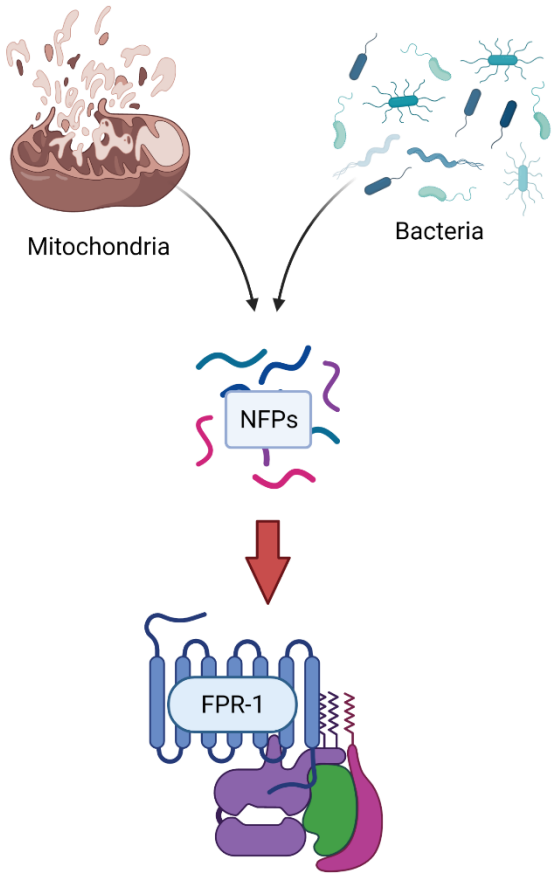


Figure 1.2. Sources of NFPs that activate FPR-1. Mitochondria and bacteria release molecules called N-formyl peptides that activate FPR-1 to elicit numerous cellular responses.

The interaction between gut microbiota and blood pressure have been studied more extensively in recent years. Gut microbiota contributes to blood pressure homeostasis and the pathogenesis of hypertension through the maintenance of microbial-derived bioactive metabolites [61]. Gut dysbiosis has been implicated as having a negative effect on hypertension, demonstrated by weakened intestinal barrier and increased occurrence of kidney disease [62-65]. The disruption of the intestinal barrier, another characteristic of gut dysbiosis, does not allow proper protein fermentation which allows translocation of

endotoxin and bacterial metabolites into the circulation which contributes to a myriad of problems including cardiovascular disease [66]. Although there have been some advancements in studying the two independently, it is plausible that there is still a more mechanistic link between blood pressure, the vasculature, and the gut.

As mentioned above, our group has shown that innate immune system activation decreases vascular function and FPR is indeed present in vasculature [52, 59]. From the previously mentioned reports and our data, a possible mechanism could be that bacterial metabolites from the gut released into circulation cause inflammation in the resistance arteries. This proposed pathway has not been studied. With this knowledge, it is plausible to investigate FPR's role in resistance arteries and if elimination of bacteria from the gut will ameliorate vascular dysfunction in hypertensive subjects.

Many discoveries surrounding the immune system have been focused on the adaptive immune system and its ability to lead to inflammation and vascular dysfunction. However, the innate immune system does not get the same attention so its role in unknown. We need a functioning innate immune system to respond to foreign invaders as well as host DAMPs in the body to regulate normal functions in the body but also continue to test whether there is a chronic inflammatory state where the body is not able to combat the invaders properly. Studies are needed to continue to bridge the immune system and the vasculature together to potentially identify a new way to use the innate immune system to improve vasculature function. Although this study is focused on hypertension, the immune-vasculature interactions could be applied to a plethora of other inflammatory associated diseases.

Chapter 2

Formyl Peptide Receptor-1 Activation Promotes Spontaneous, Premature Hypertension in Dahl Salt-Sensitive Rats

Edwards JM, Roy S, Galla S, Tomcho J, Bearss N, Waigi E, Mell B, Cheng X, Saha P, Vijay-Kumar M, McCarthy CG, Joe B, & Wenceslau CF. Formyl Peptide Receptor-1 Activation Promotes Premature, Spontaneous Hypertension in Dahl Salt-Sensitive Rats. Hypertension. 2021 Apr;77(4):1191-1202. doi: 10.1161/HYPERTENSIONAHA.120.16237. Epub 2021 Mar 1. PMID: 33641367; PMCID: PMC7946782. Permission has been granted by the publishing journal to reprint as a part of my dissertation.

2.1. Abstract

Cell death has long been a characteristic phenotype of organ damage in hypertension and recently, leaky gut has been revealed as a novel hypertensive phenotype. However, despite the increase in bacterial and damaged mitochondrial products in the circulation of hypertensive patients and animals, the mechanistic contribution of these two phenomena to hypertension pathophysiology is unknown. Mitochondria and bacteria both start protein translation with an N-formyl methionine residue and thus are the only sources of N-formyl peptides (NFPs) which activate the formyl peptide receptor-1 (FPR-1). We hypothesized that the synergistic action of bacterial and mitochondrial N-formyl peptides would cause the spontaneous elevation of blood pressure and vascular remodeling in male Dahl salt-sensitive (S) rats via FPR-1. We observed that mitochondria-derived peptides originating from cell death in the kidneys are responsible for FPR-1-induced vascular hypercontractility and remodeling and premature elevation of BP in Dahl S rats fed a low salt diet. However, a high-salt diet leads to gut barrier disruption and, subsequently, a

synergistic action of mitochondria and bacterial-derived leaky gut NFPs leads to a severe and established hypertension. Administration of an FPR-1 antagonist lowered blood pressure in Dahl S rats on a low salt diet, but amoxicillin administration did not. These results reveal for the first time that cell death can be a cause of hypertensive pathophysiology, whereas leaky gut is a consequence.

2.2. Graphical Abstract

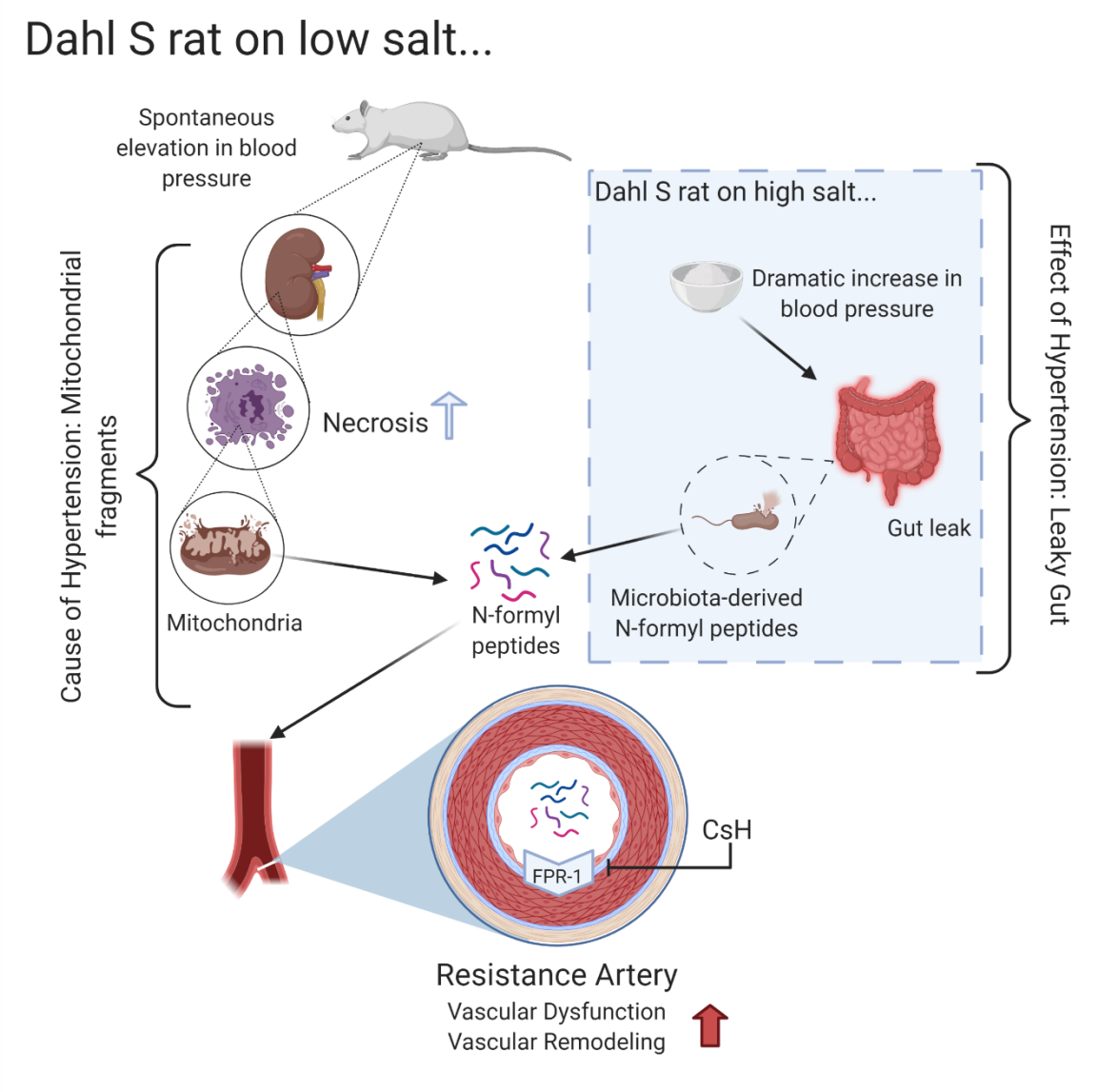


Figure 2.0. Graphical Abstract for chapter 2.

2.3. Introduction

Hypertension is a global health concern (1). Although newly updated guidelines for hypertension diagnosis promote early intervention (2), there are a number of patients with resistant hypertension, whose elevated blood pressure remains uncontrolled despite therapy (3). A critical barrier in advancing the development of novel therapeutic approaches for hypertension is the gap in understanding of the precise cause-effect mechanism. Recent evidence implicates immune mechanisms in the pathophysiology of hypertension (4-6). In fact, immune system activation and inflammation have been proposed as a unifying mechanism linking the major organ systems involved in the development of hypertension – the cardiovascular system, the kidneys, the autonomic nervous system (7,8), and more recently, the gut (9). However, it is still unclear if the activation of the innate immune system precedes hypertension.

The formyl peptide receptor (FPR-1) is a pattern recognition receptor that plays a crucial role in the function of the innate immune system. FPR-1 is a G-protein-coupled receptor that can bind N-formyl peptides such as N-formylmethionine-leucyl-phenylalanine (fMLP), produced by bacterial degradation (10-15). Interestingly, mitochondria carry hallmarks of their bacterial ancestry including the usage of N-formyl-methionyl-tRNA as an initiator of protein synthesis (10-15). Any injury that causes plasma membrane lysis *in vivo* can lead to the release of mitochondrial N-formyl peptides (NFPs). Consequently, both mitochondrial and bacterial-produced peptides have a formyl group at their N-terminus. Therefore, NFPs, regardless of origin, are recognized by FPR-1 and thus play a role in the initiation of inflammation.

One of the most potent inducers of actin polymerization is FPR-1 (10, 16). Interestingly, FPR-1 is also expressed in endothelial and vascular smooth muscle cells (15, 17), and it is important for vascular remodeling and motility via actin polymerization, independent of bacteria or mitochondrial fragments (15). This role is like the one observed in sentinel cells of the innate immune system, such as neutrophils. In physiological conditions, FPR-1 responds to tension and contributes to arterial myogenic tone (18). Nonetheless, the precise mechanism linking FPR-1 activation in the vascular-immune network in hypertension remains unknown.

Hypertensive patients are known to have “leaky gut” (19-22), whereby circulating levels of bacterial products are increased in hypertension. Therefore, we postulated that both leaky gut-derived bacterial NFPs (from microbiota) and cell damage-derived mitochondrial NFPs (from the host) could cause vascular dysfunction and remodeling in hypertension. Specifically, we hypothesized that mitochondrial-derived NFPs and FPR-1 activation would lead to vascular remodeling and the genesis of high BP. A high-salt diet would further exacerbate this response by causing gut barrier disruption (23). Subsequently, a synergistic action of mitochondria and bacteria-derived leaky gut NFPs would be the major driving force in the maintenance of hypertension.

2.4. Materials and Methods

2.4.1 Animals

All animals were from a colony maintained at the University of Toledo College of Medicine and Life Sciences. All animal procedures and protocols used were approved by the University of Toledo Institutional Animal Care and Use Committee (IACUC protocol

approval numbers 108854, 104573, 108390). Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines. Male inbred Dahl salt-resistant (R) and salt-sensitive (S) rat strains were from the University of Toledo College of Medicine and Life Sciences. All rats were weaned at 4 weeks of age and maintained on a 12-hour light cycle with water *ad libitum* and a low-salt diet (0.3% NaCl, Harlan Teklad diet TD 7034; Madison, WI) until 5 weeks of age. At this time, the R and S were divided into two groups high-salt diet (2% NaCl, Harlan Teklad diet; TD 94217; Madison, WI) or maintained on a low-salt diet for a further 5-6 weeks.

2.4.2 Telemetry

Male rats (~ 10-11 weeks old) were surgically implanted with radiotelemetry transmitters (HDS10) (Data Science International, St Paul, MN) as previously described (24-26). For this, all rats were anesthetized with isoflurane (2% in 100% O_2 administered via nose cone) and implanted with S10 radiotelemetry transmitters via the femoral artery. Rats under anesthesia were given analgesics (buprenorphine, 0.05mg/kg) as approved by the IACUC. Post-surgery, rats were individually housed and allowed to recover from surgery for four days before baseline BP was recorded (24-26). Post-operative care was provided as per the approved protocol and euthanasia was via carbon dioxide inhalation.

2.4.3 *In vivo* treatments

2.4.3.1 Antibiotic treatment

After the baseline BP was recorded, rats received either normal drinking water (control) or water supplemented with amoxicillin (50 mg/kg/day, Sigma-Aldrich, St. Louis, MO) for 2 weeks. Water bottles were replenished once a week.

2.4.3.2 Cyclosporin H (CsH) treatment

In another experiment, a group of rats (Dahl R and S on low salt diet) were administered FPR-1 antagonist, cyclosporin H (CsH, 0.3 mg/kg/day), or vehicle by osmotic minipump for 2 weeks. For this, CsH was prepared at the desired concentration (based on animals' body weight) in the required volume of ethanol (15%) and water. The volume of 100 µl of CsH solution was loaded into the osmotic minipumps (Azlet, model 1002, Cupertino, CA) as directed by the manufacturer using the blunt needle provided. The flow moderator was inserted into the pump, and the osmotic minipumps were stored aseptically in saline in an incubator overnight until implantation into rats. Rats were randomized to receive either CsH or vehicle minipumps.

For mini-pump implantation, rats were anesthetized (2% isoflurane in 100% O_2 administered via nose cone). A patch of skin was exposed by shaving fur from the flank using a clipper. The surgical site was disinfected with isopropyl alcohol and iodine swabs. Under sterile conditions, 2-3 cm skin incision was made between the shoulder blades. Blunt artery forceps were inserted into the incision to make a subcutaneous pocket. The osmotic mini pump was inserted into the pocket with the flow moderator away from the incision and the incision was closed using wound clips. Antibiotic ointment was applied to the outside of the incision. While rats were anesthetized, they were given analgesics

(meloxicam, 2 mg/kg) as approved by the IACUC policy. Rats were watched closely until they recovered from anesthesia.

2.4.4 Survival Curve

Starting from birth, mortality was reported in R and S rats on low and high salt diet. Age at time of death (in days) was entered into analysis software and mortality percentage was calculated.

2.4.5 Necrosis and Apoptosis Assay

Bone marrow-derived macrophage (BMDM), whole kidney cells, and whole mesenteric resistance artery cells were isolated from 7-week old male Dahl R and S rats and were used to measure the basal level of necrosis and apoptosis. For this, we used the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 and Propidium Iodide (PI) (Invitrogen™, Eugene, OR) according to the manufacturer's instruction. Briefly, cells ($2.0x10^6$) were resuspended in Annexin V binding buffer (1X) in 100 µl per assay. Alexa Fluor® 488 Annexin V (5 µl) and PI (1 µl of 100 µg/ml) working solution was added to each 100 µl of cell suspension and incubated the cells at room temperature for 15 min without light. After the incubation period, 400 µl of 1X annexin-binding buffer was added to each tube and mixed gently and kept the samples on ice. The % necrosis and apoptosis cells were measured via flow cytometry (Accuri C6, BD Biosciences, San Jose, CA) and analyzed using the BD Accuri C6 Software (Becton Dickinson, Canaan, CT). Results were presented as the percentage of (i) early apoptotic cells: Annexin-V single-positive cells;

(ii) late apoptotic cells: Annexin V+ propidium iodide (PI) double-positive cells and (iii) necrotic cells: PI single-positive cells.

2.4.6 Tissue Collection

After treatment, rats were weighed and euthanized by thoracotomy and exsanguination via cardiac puncture under isoflurane anesthesia (5% in 100% O_2 administered via nose cone). Whole blood was first collected from the abdominal aorta and centrifuged. For serum, whole blood was centrifuged for 15 minutes at 2000 rcf in a 4°C centrifuge and the supernatant was collected. For plasma, whole blood was centrifuged for 15 minutes at 1500 rcf in a 4°C centrifuge and the supernatant was collected. Subsequently, mesenteric resistance arteries whole hearts, kidneys, and tibias were all harvested. The left and right ventricles were dissected from the whole hearts and weighed. Lungs were measured wet immediately after removal and allowed to dry in a 37°C oven for a minimum of 4 days and re-weighed.

2.4.7 Left ventricle mass measurements

Body weight is correlated nonlinearly (cubically) with tibia length (TL), but linearly with TL^3 (27). The linear relation with the left ventricle (LV) and TL^3 showed that LV weight crossed the x-axis at TL^3 -26.7^3 (95% CI -29.8^3; -23.4^3). Therefore, TL and LV weight were indexed by dividing the weights by $26.7^3 + TL^3$ (27).

2.4.8 Vascular Function

Third or fourth order MRA, 2 mm in length, were mounted on DMT wire myographs (Danish MyoTech, Aarhus, Denmark) and kept in a Krebs solution (Table 1). The MRA were normalized to their optimal lumen diameter for active tension development, as described previously by our group (28, 29). To test vascular smooth muscle cell integrity, the arteries were initially contracted with 120 mmol/L potassium chloride (KCl). Concentration-response curves to acetylcholine (ACh, 1 nmol/L to 10 µmol/L) after initial contraction to phenylephrine (PE, 30 µmol/L) was performed to evaluate relaxation. Relaxation responses to ACh are shown as a percent of the initial PE contraction (30 µmol/L). Concentration-response curves to PE (10 nmol/L to 10 µmol/L) were also performed (force, mN).

2.4.9 Vascular Structure

Fifth to seventh order MRA were mounted on DMT pressure-culture myographs (Danish MyoTech, Aarhus, Denmark) to test the mechanical properties of the arteries as previously described by our group (29). Briefly, arteries were mounted on glass cannulas and tied down with nylon suture. Intraluminal pressure was slowly raised to 160 mmHg and the artery was stretched to obtain the optimal parallel view of the walls. The MRA was equilibrated at 60 mmHg in filtered Krebs for 30 min. After testing vascular smooth muscle cell integrity with 120 mmol/L KCl, the extraluminal Krebs was exchanged with high Ca^{2+} Krebs (2.5 mM). Intraluminal pressure was dropped to 3 mmHg and a pressure curve was obtained by increasing the intraluminal pressure in 20 mmHg steps to 160 mmHg with a two-minute equilibration time at every step. After this curve, the same procedure was followed using an extraluminal buffer with zero Ca^{2+} Krebs buffer. DMT data capture

software was used to capture videos and/or images and subsequently analyzed with the VasoTracker Offline Diameter Analyzer for accurate inner and outer diameter measurements (30). From the internal and external diameter measurements in the passive conditions, structural parameters were calculated as previously described (29, 31). See all Krebs solutions in Table S1.

2.4.10 Leaky gut assay

Zonulin concentration was determined using a sandwich ELISA (MyBioSource, San Diego, CA, Catalog # MBS2606662) following the manufacturer's protocol.

Samples and the detecting antibody were added into the ELISA plate wells and placed in a 37°C incubator for 90 min. The plate was washed with 1X phosphate buffer saline (PBS) 3 times. The enzyme-conjugate was added to each well and placed in a 37°C incubator for 30 min. The plate was washed with 1X PBS 5 times. The color reagent was added to the wells and the plate was placed in a dark 37°C incubator. When the highest concentration for the standard curve darkened and the color gradient appeared, the plate was removed from the incubator. Color reagent C was then added to each well and mixed well. The plate was read immediately. The intensity of the color was inversely proportional to the Zonulin concentration in the samples, which were read at 450 nm.

2.4.11 Real-time PCR

Total RNA was extracted from aortas using the Qiagen RNeasy Mini Kit following the manufacturer's protocol (Qiagen, Hilden, Germany). Nucleic acid concentration was quantified with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific,

Wilmington, DE). After isolation, cDNA was generated by reverse transcription using the Applied Biosystems cDNA synthesis kit (Cat No. #: 4368814, Darmstadt, Germany). The thermal-cycling settings used were as follows: Step 1: 25°C for 10 minutes, Step 2: 37°C for 120 minutes, Step 3: 85°C for 5 minutes, and Step 4: 4°C for no more than 1 hour. After thermal-cycling, the sample was diluted with 80 µL of RNAse-Free dH_2O and stored in −20°C freezer until further analysis with qRT-PCR (23). PCR amplification of cDNA was performed by quantitative RT-PCR with the TrueAmp SYBR Green qPCR SuperMix (Smart Bioscience, Maumee, OH) (25). The annealing temperature for PCR was 56°C and 26 cycles were carried out. Relative gene expression was calculated using the ΔΔCt method with GAPDH as an internal control. Primers were designed using the National Center for Biotechnology Information primer blast software (Bethesda, MD) or previously published (32). Primer sequences are listed in Table S2.

2.4.12 Immunoblotting

Mitochondrial N-formylated protein (NADH dehydrogenase 6) was measured in plasma from all groups. Plasma was diluted (1:20) and 25 uL of the solution was loaded and proteins were separated by SDS-polyacrylamide electrophoresis as previously described (14). PVDF membranes were incubated overnight at 4°C with a primary antibody raised against rat NADH dehydrogenase 6 (ND6, 1:1000, Sigma). Ponceau was used as a loading control.

2.4.13 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 8.4.2 (La Jolla, CA, USA). Data are presented as mean ± standard error of the mean (SEM) and statistical significance was set at $p<0.05$ unless noted otherwise. Procedures used include Student's unpaired t-test, one-way, and two-way analysis of variance (ANOVA), non-linear regression analysis ($LogEC_{50}$ and Emax). Tukey's post-hoc testing and the Bonferroni post-hoc testing were used in one-way ANOVA and two-way ANOVA respectively. For the survival curve, we used the Mantel-Cox test. The number (n) of animals per group is described within the graphs.

2.5. Results

2.5.1 Survival curve

In Figure 2.1A, the probability of survival in animals in low and high salt diets was observed. There was no mortality among Dahl R rats (LS and HS diets) and Dahl S-LS. On the other hand, Dahl S-HS rats began to die around 86 days of age.

2.5.2 Increases in circulating levels of NFPs and, subsequently FPR-1 activation, contribute to the genesis and maintenance of hypertension

Dr. John Rapp (33) observed that the premature spontaneous elevation of BP occurs in inbred Dahl S rats fed a low salt diet (younger 12 weeks old), but not in Dahl R rats. This phenomenon occurred due to unknown genetic influences, and independent of environmental factors. High-salt diet accelerates this process leading to a malignant

hypertension (33). Here, we observed that mitochondrial NFPs were increased in the plasma of Dahl S-LS rats at 7-9 weeks of age and in established models of hypertension (Dahl S-HS) (Figure 2.1B and C).

Due to the dual source of NFPs (from microbiota and host mitochondria), we postulated that leaky gut-derived bacterial NFPs (from microbiota) could also play a role in the genesis of hypertension. However, this hypothesis was refuted given that zonulin (biomarker for leaky gut) was not increased in the circulation of Dahl R-LS, Dahl R-HS, and Dahl S-LS (Figure 2.1D). On the other hand, zonulin was significantly increased in Dahl S-HS (Figure 2.1D). These data suggest that high-salt diet leads to leaky gut and the synergistic action of mitochondria and bacterial-derived leaky gut NFPs only maintains hypertension.

In the Figure 2.1E, we observed that arteries from male Dahl S-LS present increased FPR-1 mRNA expression. These data suggest that FPR-1 expression in Dahl S is independent of high salt. However, high salt diet also induced FPR-1 expression (Figure 2.1E) in normotensive animals.

Given that leaky gut is not present in animals fed a low salt diet, suggesting that gut microbiota-derived fragments may not play a role in the genesis of hypertension in Dahl S, we decided to investigate whether increased FPR-1 expression and mitochondrial NFPs play a role in the genesis of hypertension. Therefore, the results demonstrated from this point were collected only from Dahl-R and S on a low salt diet to avoid leaky gut and a possible increase in levels of microbiota-derived NFPs in the circulation. However, for scientific rigor, we still treated one group of Dahl S and R rats with antibiotics to investigate (1) the possible effect, if any, of gut microbiota, but independent of leaky gut, in the genesis

of the premature elevation of blood pressure in Dahl S fed a low salt diet; (2) the possible role of microbiota from another source outside of the gut; and (3) possible pleiotropic effect of antibiotics on the cardiovascular system.

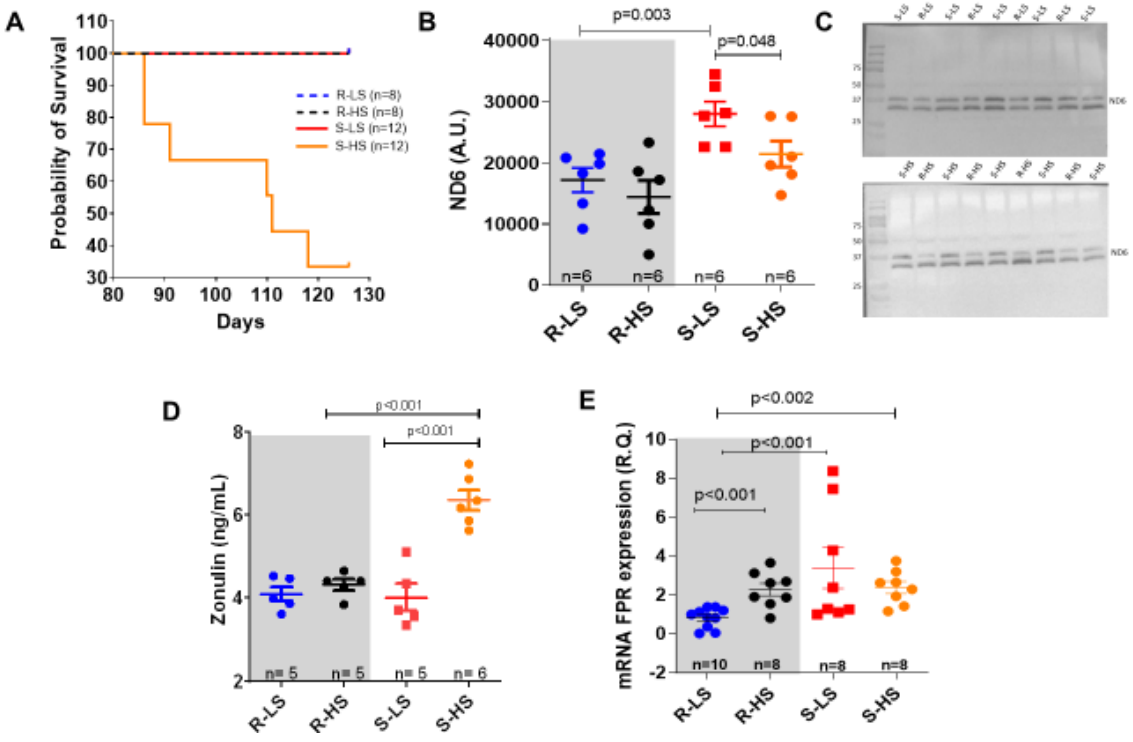


Figure 2.1: Increased circulating levels of NFPs and, subsequently FPR-1 activation, contribute to the genesis and maintenance of hypertension. Survival curve (**A**), serum zonulin levels **(B)**, mitochondrial ND6 expression in plasma analysis and representative images (**C and D**), and FPR-1 gene expression in arteries (**E)** from male Dahl salt resistant (R) and Dahl salt sensitive (S) rats on low salt (LS, 0.3%) and high (HS, 2%) diet. Data presented in mean ± SEM. Statistics: t-test or One- ANOVA: Number of animals and p value are indicated on graphs.

2.5.3 Cell death derived mitochondrial NFPs are present prior to the onset of hypertension

We observed that Dahl S-LS present with cell necrosis in the kidney when compared to Dahl R-LS (Figure 2.2A). No differences were observed in cell necrosis from bone marrow derived macrophages (BMDM) and mesenteric resistance arteries (Figure 2.2 B and C). No differences were observed in early and late cell apoptosis from all tissues evaluated (Figure 2.2A-C). These data suggest that low-grade trauma is inherited and independent of high salt diet. Further, cell necrosis is present during the development of hypertension and kidneys are the possible initial source of mitochondrial NFPs in the circulation. See Figure S1 and Table S4.

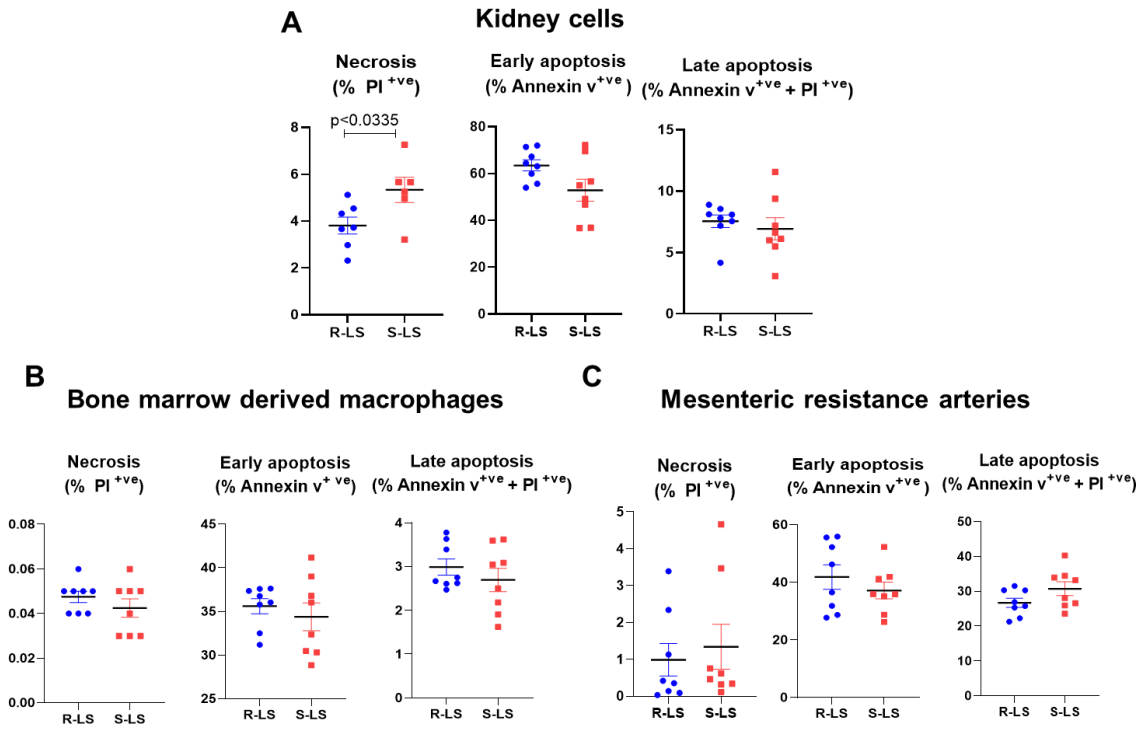


Figure 2.2: Cell death-derived mitochondrial NFPs are present prior to the onset of hypertension. Cell necrosis, early apotosis, and late apoptosis measured by flow cytometry in kidney cells (**A**), bone marrow derived macrophages (**B**), and mesenteric resistant

arteries cells (**C**) from male Dahl salt resistant (R) and Dahl salt sensitive (S) rats on low salt (LS, 0.3%) diet. Data presented in mean ± SEM. Statistics: t-test. Each dot represents an individual rat and p values are indicated on graphs.

2.5.4 Formyl peptide receptor-1 antagonist prevents BP increase in Dahl-S fed a low salt diet

FPR-1 antagonist, CsH, prevents the increase of diastolic BP in Dahl S-LS after 4 days of treatment with CsH (Figure 2.3A). Although, there was a tendency to prevent an increase in systolic BP after treatment with CsH, no statistical differences were observed (Figure 2.3B). On the other hand, antibiotic amoxicillin (AMO) did not change diastolic and systolic BP in Dahl S-LS diet (Figure 2.3C and D). These data confirm that leaky gut is not associated with the genesis of premature spontaneous elevation of BP in Dahl S-LS rats. Both CsH and AMO did not change BP in normotensive animals (Dahl R-LS) (Figures 2.3A-D).

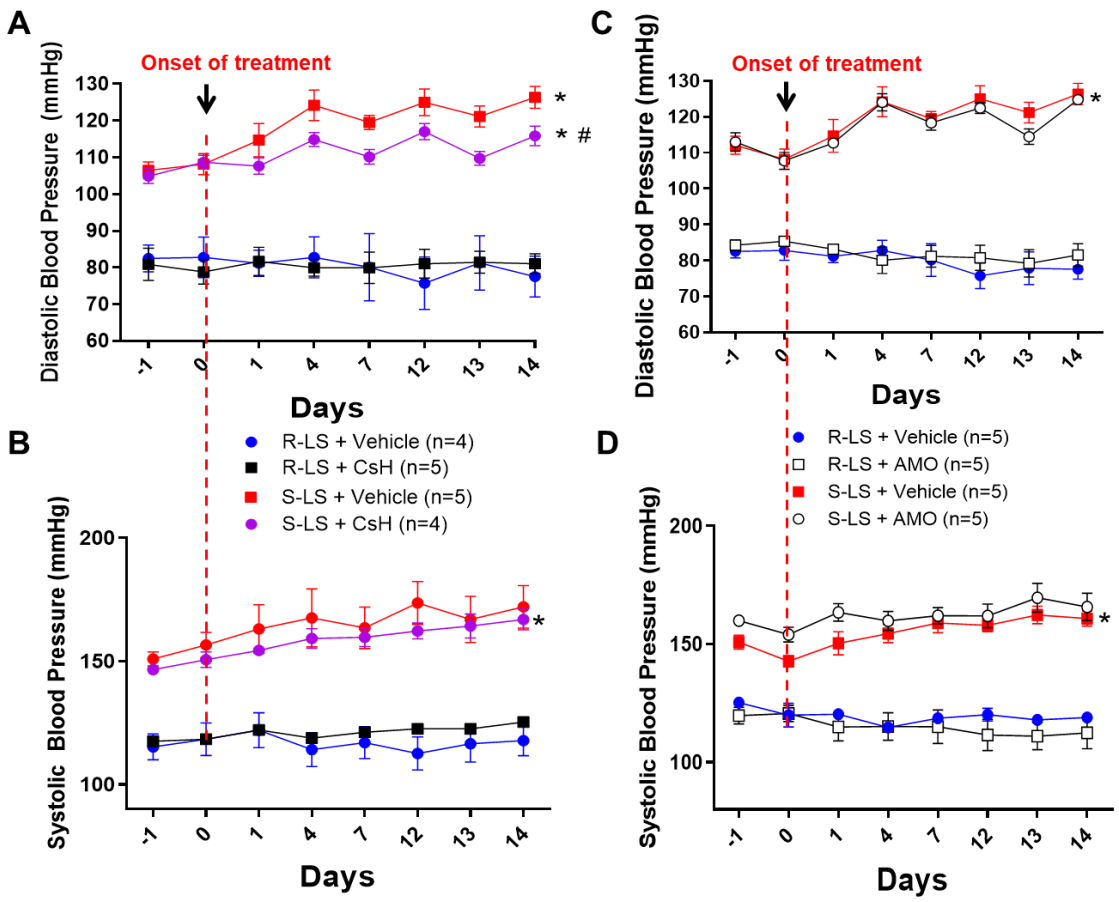


Figure 2.3: Formyl peptide receptor-1 antagonist prevents BP increase in Dahl-S fed a low salt diet. Diastolic and systolic blood pressure, measured by telemetry, from male Dahl salt resistant (R) and Dahl salt sensitive (S) rats on low salt (LS, 0.3%) diet treated with cyclosporin H (CsH) (**A and B**) or amoxicillin (AMO) (**C and D**) for 14 days. The first point (-1) on the graph is the baseline average of BP for 14 days. Arrows indicate the start of the treatment. Data presented in mean ± SEM. Number of animals are indicated on graphs. P value <0.05. Statistics: Two-way ANOVA, * vs. R-LS and R-LS + CsH; # vs. S-LS.

2.5.5 FPR-1 antagonist treatment decreases heart left ventricle (LV) mass and vascular cross-sectional area (CSA) of resistance arteries

Similar to hypertensive animals, we observed that Dahl S-LS presented with a significant increase in LV mass and CSA of the mesenteric resistance arteries (Figure 2.4A-D), which suggests vascular hypertrophy, when compared to Dahl R-LS. Treatment with CsH reduced both parameters (Figure 2.4A, B and D). Interestingly, although AMO treatment did not affect BP in normotensive and hypertensive animals, this antibiotic decreased LV mass in Dahl S-LS animals (Figure 2.4A). On the other hand, AMO did not change vascular CSA (Figure 2.4C and D). Both treatments did not affect LV mass and CSA from normotensive animals (Figure 2.4A-D). There were no changes in lung wet to dry mass ratio, showing no lung edema (Table S2).

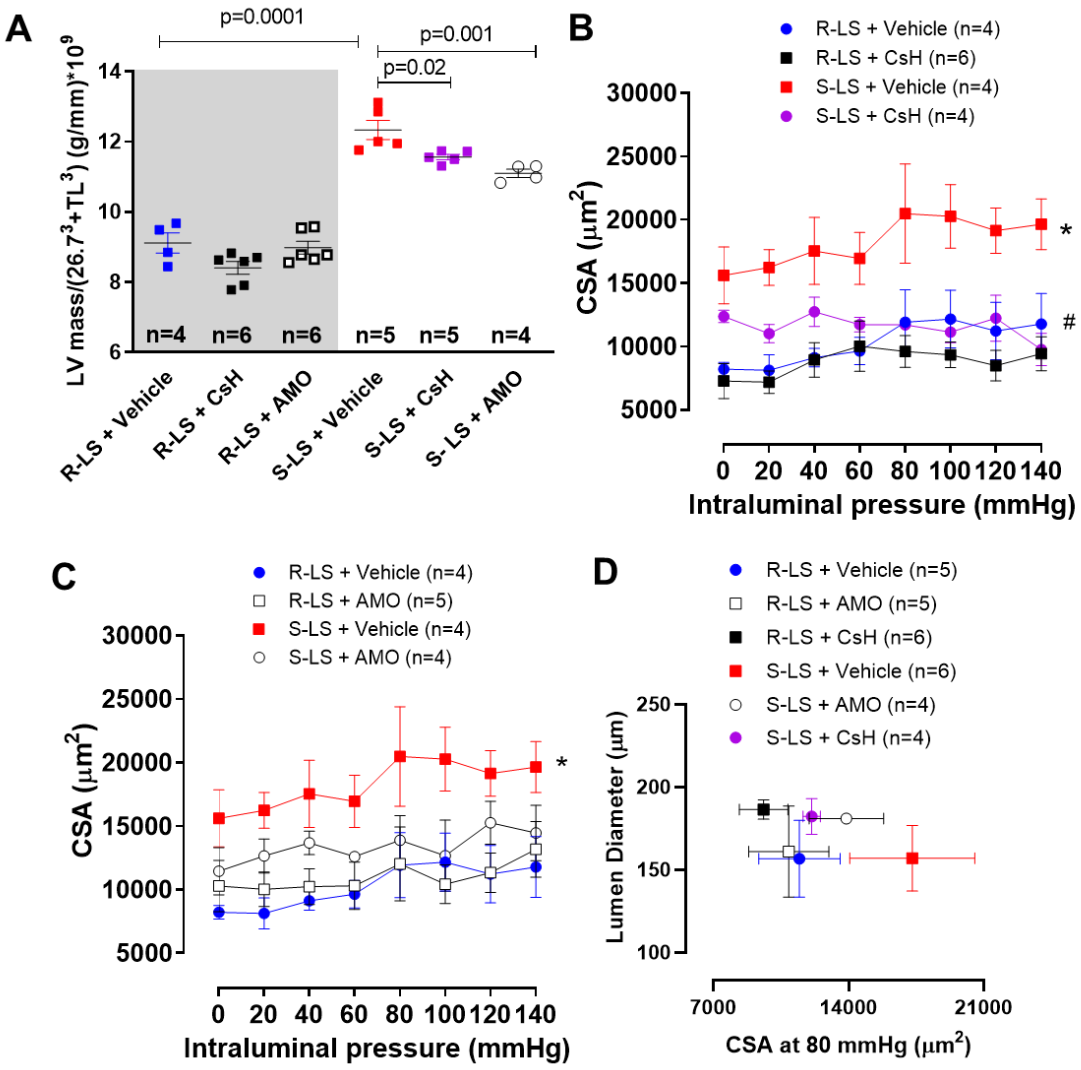


Figure 2.4: FPR-1 antagonist treatment decreases heart left ventricle (LV) mass and vascular cross-sectional area (CSA) of resistance arteries. Left ventricle (LV) mass normalized by tibia length $(TL)^3$ (**A**). For this, the linear relation with LV and TL^3 showed that LV weight crossed the x-axis at TL^3 -26.7^3 (95% CI -29.83; -23.43). Therefore, TL and LV weight were indexed by dividing the weights by $26.7^3 + TL^3$. Cross-sectional area (CSA) measured with increasing intraluminal pressure in mesenteric resistance arteries from male Dahl salt resistant (R) and Dahl salt sensitive (S) rats on low salt (LS, 0.3%) treated with cyclosporin H (CsH) (**B**) or amoxicillin (AMO) (**C**) for 14 days. Mesenteric

resistance arteries at 80 mmHg from all groups are plotted CSA vs. lumen diameter (**D**), suggesting vascular hypertrophy. Data presented in mean ± SEM. Number of animals and p value are indicated on graphs, otherwise p value <0.05. Statistics: One or two- way ANOVA; * vs. R-LS; # vs. S-LS.

2.5.6 Vascular Function

After FPR-1 antagonist, antibiotic and vehicle treatment, we evaluated vascular function in mesenteric resistance arteries from all groups. We observed that KCl-induced contraction was increased in arteries from Dahl S-LS compared to Dahl R-LS (Figure 2.5A). Cyclosporine H, but not AMO, decreased this response (Figure 2.5A). A similar pattern was observed in phenylephrine induced contraction (Figures 2.5B and C). There were no significant changes in contraction to phenylephrine in arteries from Dahl R-LS treated with CsH or AMO (Figures 2.5A-C).

Arteries from hypertensive animals displayed an endothelium-dependent acetylcholine-induced contraction when compared to normotensive (Figures 2.6A and B). Interestingly, AMO treatment significantly decreased the late-phase contraction to acetylcholine (Figure 2.6B), while CsH treatment was not able to improve endothelial function (Figure 2.6A). No treatment differences were observed in the concentration response curves to acetylcholine in arteries from Dahl R-LS (Figure 2.6A and B).

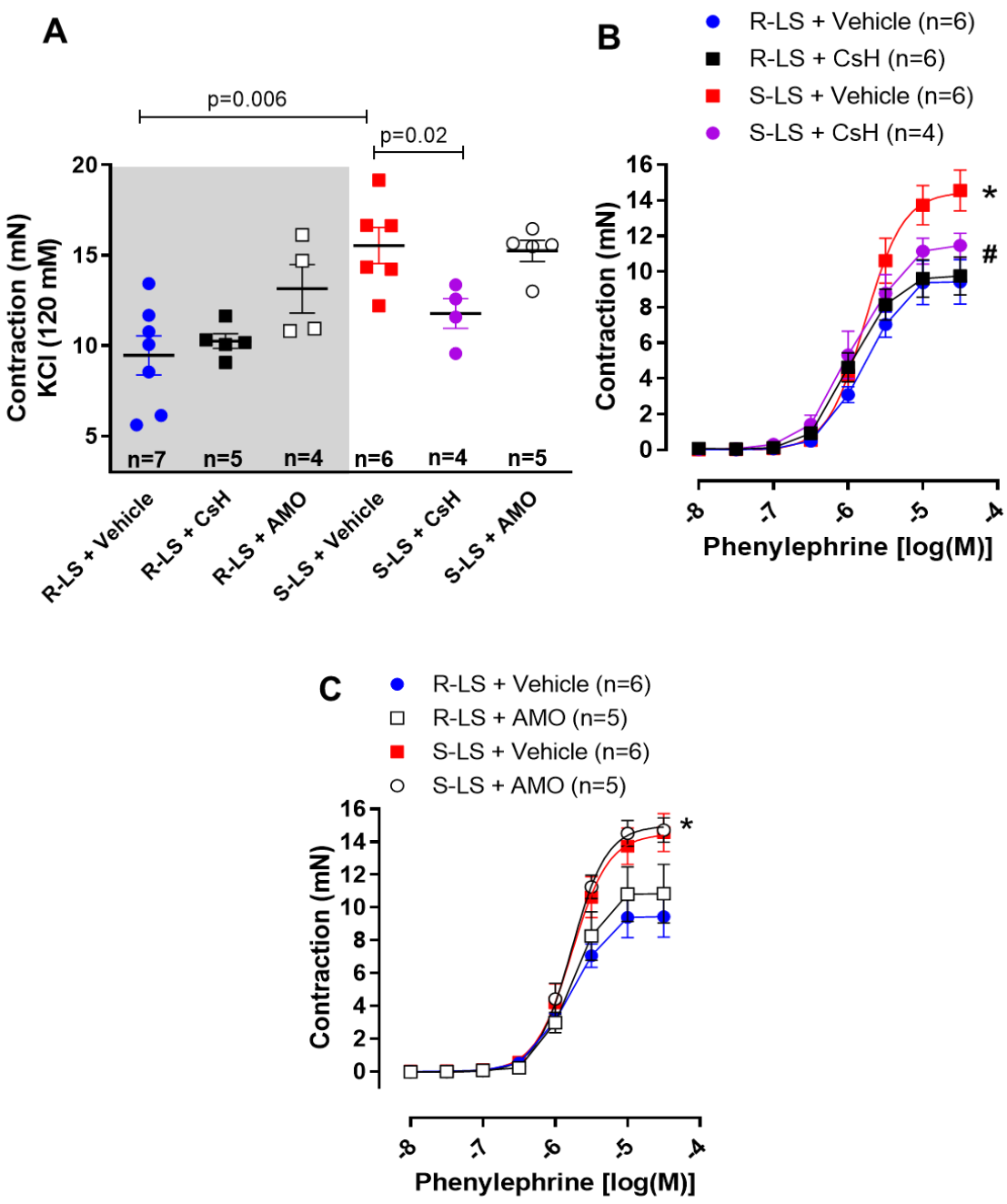


Figure 2.5: FPR-1 antagonist decreases hypercontractility in arteries from hypertensive rats. KCl (120 mM) (**A**) and phenylephrine (**B and C**)-induced contraction in mesenteric resistance arteries from male Dahl salt resistant (R) and Dahl salt sensitive (S) rats on low salt (LS, 0.3%) treated with cyclosporin H (CsH) or amoxicillin (AMO) for 14 days. Data presented in mean ± SEM. Number of animals and p value are indicated on

graphs, otherwise p value <0.05. Statistics: One or two- way ANOVA; * vs. R-LS; # vs. S-LS.

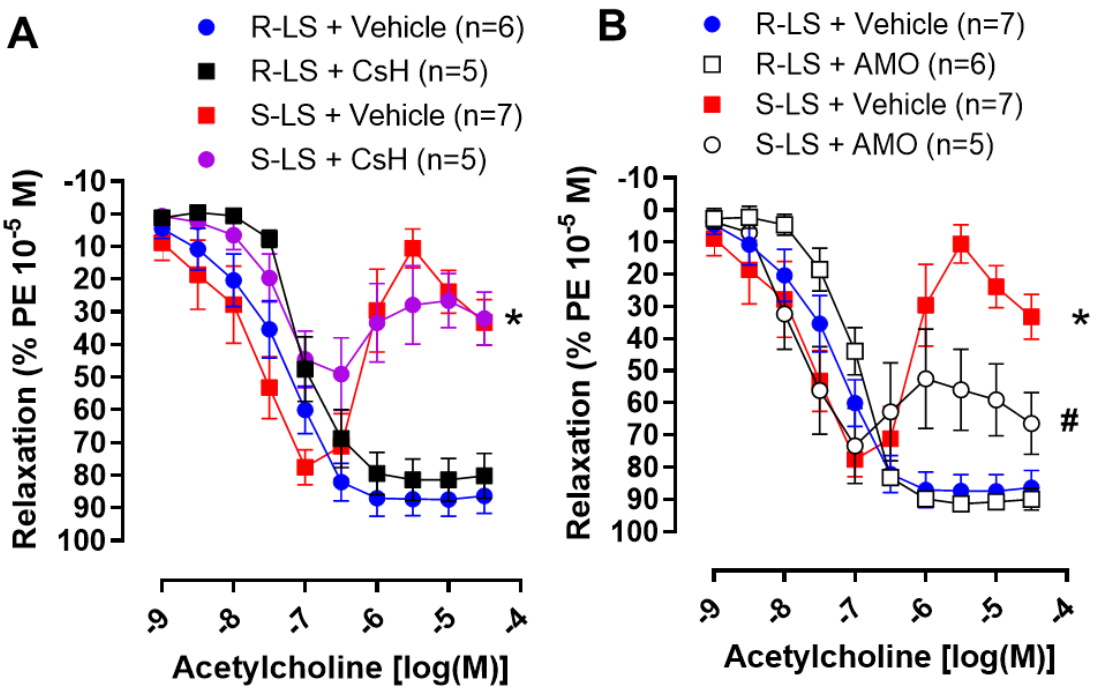


Figure 2.6: FPR-1 antagonist does not improve endothelium-dependent relaxation in arteries from hypertensive rats. Concentrations response curves to acetylcholine in mesenteric resistance arteries from male Dahl salt resistant (R) and Dahl salt sensitive (S) rats on low salt (LS, 0.3%) treated with cyclosporin H (CsH) (A) or amoxicillin (AMO) (B) for 14 days. Arteries were pre-contracted with phenylephrine (PE). Data presented in mean ± SEM. Number of the animals are indicated on graphs. P value <0.05. Statistics: Two- way ANOVA; * vs. R-LS; # vs. S-LS.

2.6. Discussion

Hypertension has been a topic of concern since its discovery over a century ago. Diagnostic guidelines have evolved over time which has led to over half of the American population being considered hypertensive (2). Many scientists debated whether mild hypertension should be treated until the 1960s when mild hypertension was shown to lead to the same negative outcomes as severe hypertension (34).

Inbred Dahl salt-sensitive rats, developed by Dr. John Rapp at the University of Toledo (33), mimic human salt-sensitive hypertension (35). Salt-sensitive hypertension is considered a hallmark of hypertension in the Black population, including African Americans, given that it is found in 73% of male and female hypertensive Blacks (36). Dr. Rapp (33) observed that the premature, spontaneous elevation of BP occurs in Dahl S rats fed a low salt (LS) diet, but not in Dahl R rats, due to unknown genetic influences and independent of environmental factors such as dietary salt. Specifically, premature, spontaneous elevations in BP begin in animals younger than 12 weeks of age (33). A high-salt diet can accelerate this process leading to a malignant hypertension, similar to humans. Here, we also observed that the Dahl S-LS presents premature, spontaneous elevation of BP, vascular dysfunction and increased LV mass. High salt diet worsened these parameters (Figure S2 and 2.3A-B). These inbred animals are a unique model, because although they present similarities with spontaneous hypertensive rats (SHR), such as cardiovascular injury prior the establishment of hypertension, they are also salt sensitive. Therefore, unknown genetic influences, rather than high salt diet, are the trigger of the vascular injury and genesis of elevated BP and a high salt diet accelerates and exacerbates these phenotypes.

Santisteban et al. (21) have provided provocative data demonstrating that in established hypertension, there is decreased expression of several tight junction proteins in the gut and a concomitant increase in intestinal permeability. In addition, alterations in human gut microbiota have been reported in the prehypertensive state (19, 20), suggesting that changes in the gut microbiota precede the onset of hypertension. This led us to test for intestinal gut leak in normotensive and hypertensive rats fed a low and high salt diet. We measured levels of serum zonulin, which is an intestinal permeability indicator (37, 38). There are multiple stimuli that can cause zonulin (39). One of them is gut dysbiosis which can cause increased amounts of zonulin production and secretion into circulation (40). Surprisingly, we observed that zonulin was not increased in the circulation of Dahl R-LS and HS, and Dahl S-LS. These data suggest that leaky gut-derived fragments are not the cause of the premature spontaneous elevation of BP in Dahl S-LS, despite the elevated BP presented in these rats. However, zonulin is significantly increased in Dahl S-HS, suggesting that high-salt diet, in an animal that is sensitive to salt, leads to gut barrier disruption (23). Therefore, gut disruption, and subsequently, leaky gut-derived fragments, are associated with accelerated and exacerbated cardiovascular injury, and they are important for the maintenance of established hypertension. Given that we did not observe leaky gut in Dahl S-LS animals, we questioned what factors were leading to the development of hypertension in the absence of high salt diet. Because we observed that FPR-1 mRNA expression was increased in arteries from Dahl S-LS, we suggested that mitochondria NFPs could be the factor that initiates immune activation and vascular injury via FPR-1. It has been previously shown that hypertensive animals have increased cell death and subsequently, increased levels of mitochondria fragments in the circulation (28).

Therefore, we hypothesized that mitochondrial-derived NFPs and FPR-1 activation lead to vascular dysfunction and remodeling and the genesis of elevated BP. Indeed, here we observed that the mitochondrial protein ND6 is increased in the circulation in hypertensive rats, confirming that gut-independent NFPs would be the activator of FPR-1. In general, cell necrosis can be due to both external and internal forces which target the structure of the cell and its energy provider, the mitochondrion, and causes them to swell and explode releasing their contents, leading to an inflammatory response (41, 42). In opposition, apoptosis is programmed cell death that shrinks cells and leaves mitochondria in normal condition. The different cellular contents are phagocytized, and minimal inflammatory response is initiated (43). We observed an increase in cell necrosis in the kidneys of Dahl S rats. There were no changes in the apoptosis measurements in both normotensive and hypertensive subjects. In opposition to our findings, it has been shown that Dahl S rats on a high salt diet have severe kidney injury including tubular atrophy, tubular cell loss, intraluminal cast formation, and expansion of the interstitium (44) along with increased apoptosis in the kidney cortex, glomerular, and tubular components of the kidney (45,46). This suggests that the kidney, as one of the contributors of cell death in salt-sensitive hypertension, serves as a possible source of mitochondrial NFPs due to necrosis and warrants further investigation in rats on low and high salt.

Both mitochondrial and bacterial NFPs bind the FPR-1 to induce vascular stiffness (14, 47). Here, an FPR-1 antagonist, CsH, decreased the spontaneous elevation of BP seen in Dahl S-LS. We decided to use cyclosporine H (CsH), because this FPR-1 inhibitor, unlike cyclosporin A, does not induce an immunosuppressant response nor bind cyclophilin (48, 49). Along with FPR-1 blockade, we also used antibiotic treatment to

indiscriminately reduce bacteria in hypertensive rats. Although we observed that these rats do not have a leaky gut, we investigated the effects that other microbiota sources or gut dysbiosis (in the absence of leak gut), would have on BP. However, no changes in BP were observed with AMO treatment in Dahl-S LS.

Arteries from animals with mild hypertension exhibit vascular hypercontractility and vascular remodeling (50, 51). Previously, we observed that the absence of FPR-1 decreases vascular contraction in intrarenal resistance arteries and aorta (15). Direct induction of actin polymerization ameliorates this response (15). In the present study, arteries from Dahl S-LS presented with greater KCl- and phenylephrine-induced contraction and an increase in vascular cross-sectional area when compared to Dahl R-LS. Treatment with FPR-1 antagonist decreased these parameters. These results suggest that FPR-1 activation, via mitochondria-derived NFPs, initiates vascular dysfunction and remodeling in Dahl S-LS. On the other hand, FPR-1 antagonist did not improve endothelium-dependent relaxation, suggesting that FPR-1 activation plays a role mainly in vascular smooth muscle cells. Surprisingly, antibiotic treatment improved endothelium-dependent relaxation in arteries and decreased LV mass from Dahl S-LS. These data suggest that microbiota and/or fragments from microbiota, which are not originating from and thereby are independent of a leaky gut, may act on the endothelium to induce vascular dysfunction in hypertension. This may lead to an increase in vascular resistance and subsequently, an increase in LV mass. Another possible explanation for this phenomenon may be that antibiotics, independently, act on the cardiovascular system impact hypertension.

2.7. Supplemental Material

Supplemental Figures

Table S1. Krebs Buffer Recipes (all in mM) used for vascular function and vascular structure tests.

	Regular Krebs Buffer (wire myograph)	HEPES Krebs Buffer (pressure myograph infusion)	Myogenic Tone Krebs Buffer (pressure myograph superfusion)	Calcium-free Krebs Buffer (wire and pressure myograph)
NaCl	130	118.4	115	115
KCl	4.70	4.70	4.70	4.70
KH_2PO_4	1.18	1.20	1.20	1.20
$MgSO_4$	1.18	1.20	1.20	1.20
$NaHCO_3$	14.9	4.00	25.0	25.0
EDTA	0.026	-	0.010	0.010
Glucose	5.50	6.00	11.1	11.1
$CaCl_2$	1.56	2.00	2.50	-
HEPES	-	10.0	-	-

Table S2. Primer sequences for rt-PCR

Product	Forward	Reverse
Fpr1	CATGAACAAGTCTGCAGTGAACCT	AGGTTTATGTCTATTACAGTATAT
GAPDH	GTGAACGGATTTGGCCGTATCG	ATCACGCCACAGCTTTCCAGAGG

Table S3. Lung mass ratio of Dahl salt resistant (R) and Dahl salt sensitive fed a low salt (LS) diet. Animals were treated with cyclosporine H (CsH), amoxicillin (AMO) or vehicle.

	Lung (g-wet:dry)
R-LS + Vehicle	3.23 ± 0.08 (n=6)
R-LS + AMO	3.36 ± 0.03 (n=6)
R-LS + CsH	3.37 ± 0.09 (n=6)
S-LS +Vehicle	3.23 ± 0.03 (n=5)
S-LS + AMO	3.29 ± 0.08 (n=5)
S-LS + CsH	3.32 ± 0.04 (n=5)

Table S4. Summary chart of the % apoptosis or necrosis were measured via flow cytometry derived from Figure S1.

Bone marrow derive macrophages

	UL (necrotic cells, PI single positive, %)	LR (early apoptotic cells, Annexin-V single positive, %)	UR (late apoptotic cells, Annexin-V+PI double positive, %)
R1	0.1	31.7	3.4
R2	0.0	37.3	2.8
R3	0.0	37.6	3.6
R4	0.0	36.8	2.6
S1	0.0	36.0	2.5
S2	0.0	32.4	1.6
S3	0.0	39.0	3.0
S4	0.1	30.3	3.6

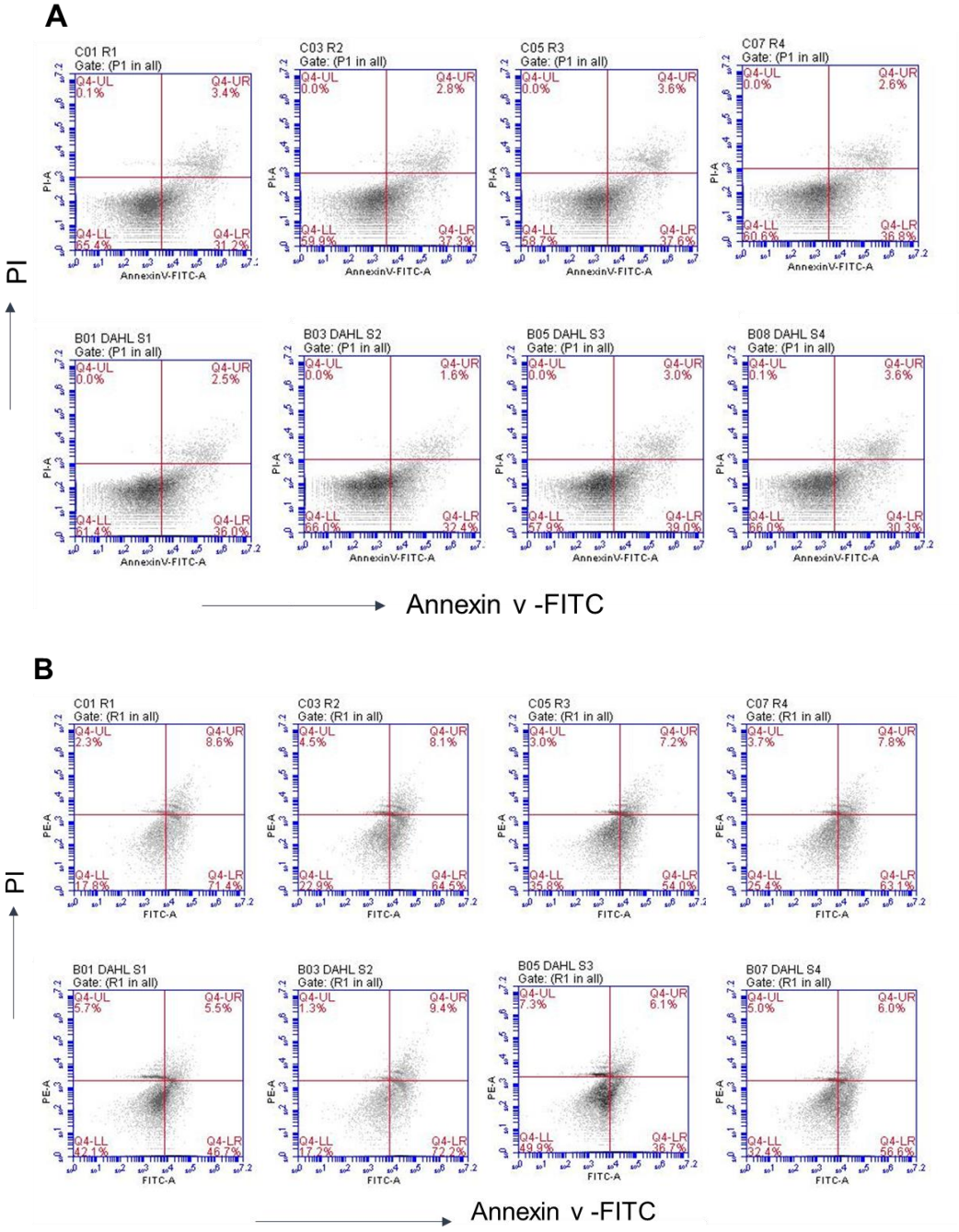
Kidney

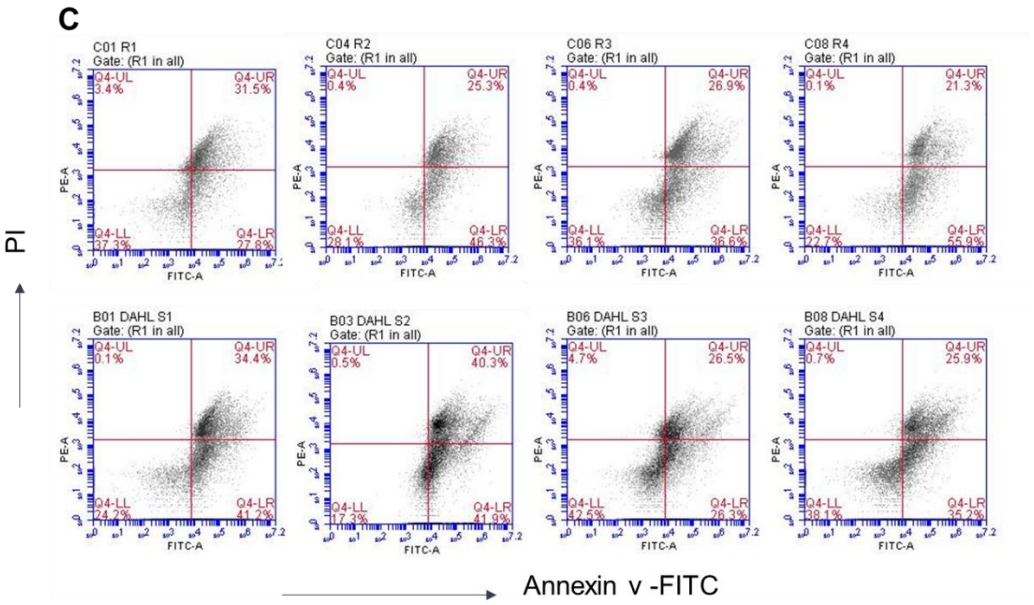
	UL (necrotic cells, PI single positive, %)	LR (early apoptotic cells, Annexin-V single positive, %)	UR (late apoptotic cells, Annexin-V+PI double positive, %)
R1	2.3	71.4	8.6
R2	4.5	64.5	8.1
R3	3.0	54.0	7.2
R4	3.7	63.1	7.8
S1	5.7	46.7	5.5
S2	1.3	72.2	9.4
S3	7.3	36.7	6.1
S4	5.0	56.6	6.0

Mesenteric resistant arteries

	UL (necrotic cells, PI single positive, %)	LR (early apoptotic cells, Annexin-V single positive, %)	UR (late apoptotic cells, Annexin-V+PI double positive, %)
R1	3.4	27.8	31.5
R2	0.4	46.8	25.3
R3	0.4	36.6	26.9
R4	0.1	55.9	21.3
S1	0.1	41.2	34.4
S2	0.5	41.9	40.3
S3	4.7	26.3	26.5
S4	0.7	35.2	25.9

Figure S1. Representative dot plots to measure cell apoptosis and necrosis

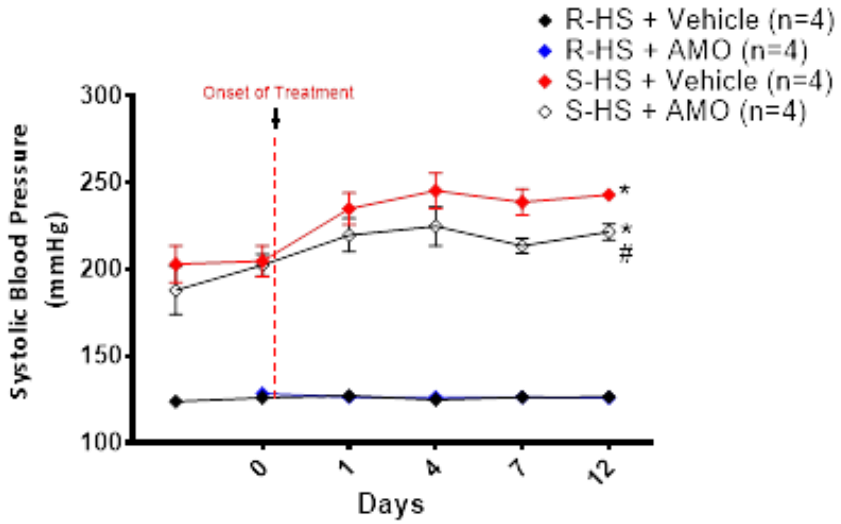




Representative dot plots to measure cell apoptosis and necrosis in (**A**) bone marrow derived macrophages, (**B**) kidney, and (**C**) mesenteric resistant arteries cells from Dahl resistant (R) and sensitive (S) rats. Annexin V Alexa Fluor™ 488 and Propidium Iodide (PI) (Invitrogen™, Eugene, OR) were used to detect cell apoptosis. The % apoptosis or necrosis were measured via flow cytometry. Results were presented as the percentage of (i) early apoptotic cells: Annexin-V single-

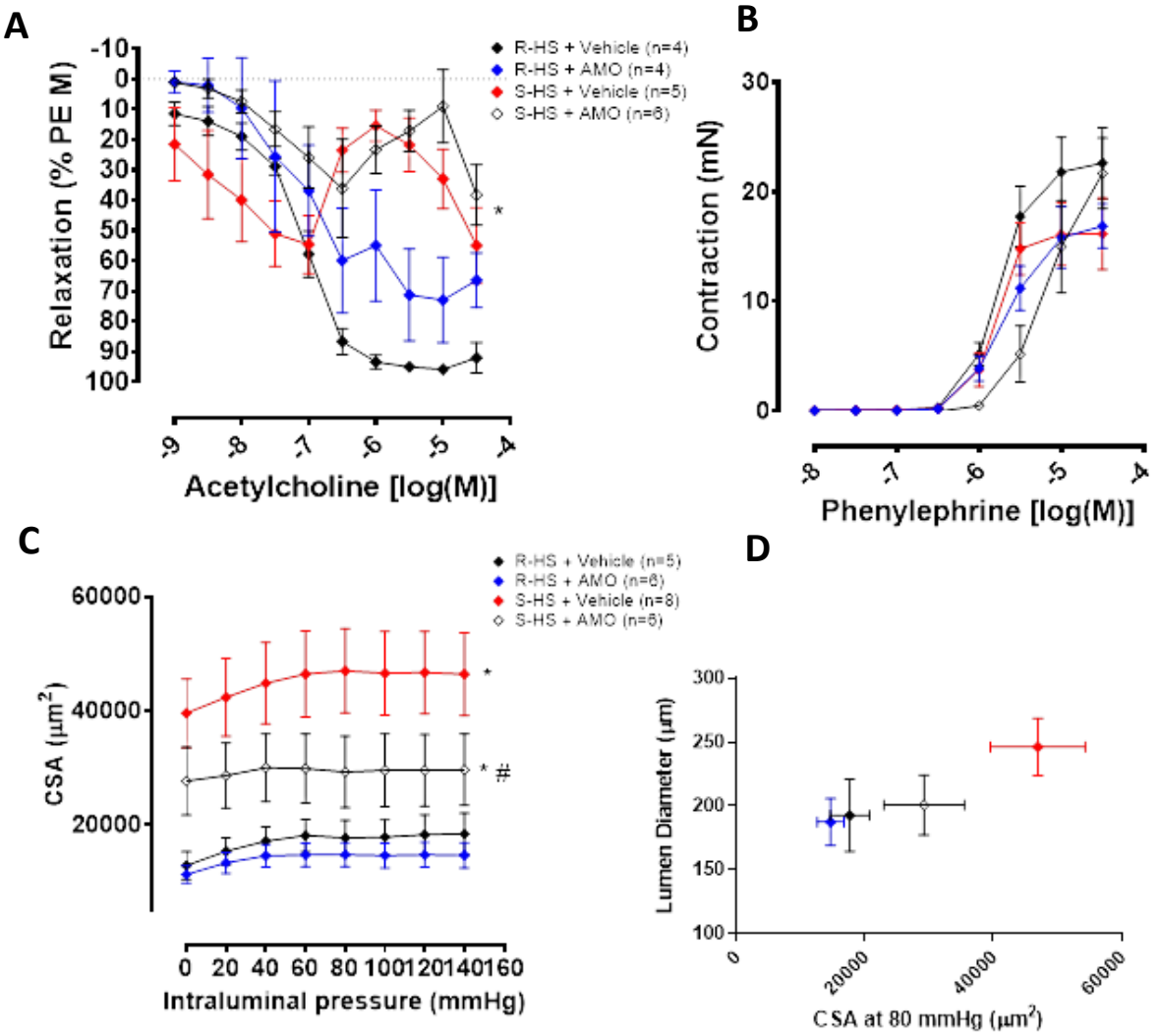
positive cells; (ii) late apoptotic cells: Annexin-V+ propidium iodide (PI) double-positive cells and (iii) necrotic cells: PI single-positive cells.n=6-8.

Figure S2. Systolic blood pressure from male Dahl resistant salt sensitive rats on high salt diet treated with amoxicillin



Systolic blood pressure, measured by telemetry, from male Dahl resistant (R) salt sensitive (S) rats on high salt (HS, 2%) diet treated with amoxicillin (AMO) for 12 days. The first point on the graph is the baseline average of BP for 14 days. Arrows indicate the start of the treatment. Data presented in mean ± SEM. Number of the animals are indicated on graph. P value <0.05. Statistics: Two-way ANOVA, * vs. R-HS, # vs. S-HS.

Figure S3. Vascular function and structure in mesenteric resistance arteries from male Dahl resistant salt sensitive rats on high salt diet treated with amoxicillin



(**A**) Concentrations response curves to acetylcholine in mesenteric resistance arteries from male Dahl salt resistant (R) and Dahl salt sensitive (S) rats on low salt (HS, 2%) treated with amoxicillin (AMO) for 14 days. Arteries were pre-contracted with phenylephrine (PE). (**B**) Concentration response curves to PE in mesenteric resistance arteries from male Dahl R and Dahl S rats on high salt (HS, 2%) treated with amoxicillin (AMO) for 14 days. (**C**) Cross-sectional area (CSA) measured with increasing intraluminal pressure in mesenteric resistance arteries from male Dahl R and Dahl S rats on high salt (HS, 2%)

treated with amoxicillin (AMO) for 14 days. (**D**) Mesenteric resistance arteries at 80 mmHg from all groups are plotted CSA vs. lumen diameter, suggesting vascular hyperthrophy. Data presented in mean ± SEM. Number of the animals and p value are indicated on graphs, otherwise p value <0.05. Statistics: One- or two- way ANOVA; * vs. R-HS; # vs. S-HS.

Chapter 3

The obligatory role of the acetylcholine-induced endothelium-dependent contraction in hypertension: Can arachidonic acid resolve this inflammation?

Edwards JM, McCarthy CG, & Wenceslau CF. The obligatory role of the acetylcholine-induced endothelium-dependent contraction in hypertension: Can arachidonic acid resolve this inflammation? Curr Pharm Des. 2020;26(30):3723-3732. doi: 10.2174/1381612826666200417150121. PMID: 32303165; PMCID: PMC7542659. Permission has been granted by the publishing journal to reprint as a part of my dissertation.

3.1 Abstract

The endothelium produces many substances that can regulate vascular tone. Acetylcholine is a widely used pharmacological tool to assess endothelial function. In general, acetylcholine binds to G-protein coupled muscarinic receptors that mediate a transient elevation in intracellular, free calcium. This intracellular rise in calcium is responsible for triggering several cellular responses, including the synthesis of nitric oxide, endothelium-derived hyperpolarizing factor, and eicosanoids derived from arachidonic acid. Endothelial arachidonic acid metabolism is also an important signaling pathway for mediating inflammation. Therefore, in conditions with sustained and excessive inflammation such as hypertension, arachidonic acid serves as a substrate for the synthesis of several vasoconstrictive metabolites, predominantly via the cyclooxygenase and lipoxygenase enzymes. Cyclooxygenase and lipoxygenase products can then activate G-protein coupled receptors expressed on vascular smooth muscle cells to causes contractile responses. As a result, acetylcholine-induced contraction due to arachidonic acid is a

commonly observed feature of endothelial dysfunction and vascular inflammation in hypertension. In this review, we will critically analyze the literature supporting this concept, as well as address the potential underlying mechanisms, including the possibility that arachidonic acid signaling is diverted away from the synthesis of pro-resolving metabolites in conditions such as hypertension.

3.2 Introduction

Endothelial health has been a major topic of research since the 20th century. The endothelium plays an intricate role in vascular homeostasis by synthesizing and secreting several vasoactive factors, such as pro- and anti-inflammatory lipid mediators. In a healthy state, acute vascular inflammation is necessary for immune surveillance to eliminate pathogens and host-derived threats and to repair the associated tissue injury. It is a self-limiting process, leading to complete resolution that allows a return to homeostasis after threat eradication and subsequent damage repair [1]. The concern is when there is prolonged or excessive inflammation, which can exacerbate the vascular damage and contribute to the genesis and/or progression of diseases. Accordingly, low-grade, chronic vascular inflammation is broadly accepted as a common factor in many cardiovascular diseases, including hypertension. Among various inflammatory mediators implicated in hypertension, metabolites and enzymes involved in arachidonic acid (AA) metabolism are of greater significance. Accordingly, arteries from hypertensive patients and animals present with a late phase acetylcholine-induced endothelium-dependent contraction (Figures 3.1-3.3) due to AA-derived biologically active products. In contrast with other

reviews about decreased acetylcholine-induced relaxation in hypertension, this mini-review will focus on the involvement of AA-derived lipids mediators in acetylcholine-induced endothelium-dependent contraction. Furthermore, given that AA-derived lipids also are crucial for “switching-off” inflammation, this mini-review will briefly address the possible mechanisms underlying the resolution of inflammation in vascular tissue.

3.3 Acetylcholine-induced endothelium-dependent contraction

In physiological conditions, vasculature oscillates between contraction and relaxation to maintain vascular tone and homeostasis. Over the last four decades, we have come to understand that the endothelium plays a major role in the regulation of vascular function. The endothelium produces many substances that can regulate vascular smooth muscle contraction, proliferation, migration, inflammation, and platelet function [2]. In general, vascular biology research laboratories use acetylcholine as a pharmacological tool to assess endothelial function (and dysfunction). Acetylcholine binds to G-protein coupled muscarinic receptors (mAChRs) on the endothelial cell membrane. These receptors are comprised of five subtypes (M1-M5). M1, M3, and M5 are coupled with the Gq protein and induce cytosolic calcium transients via phospholipase C signaling pathway. M2 and M4 are coupled with the Gi protein, which inhibits adenylyl cyclase [3]. A transient elevation of the intracellular, free calcium concentration is considered a key mechanism in triggering several cellular responses, such as the synthesis of nitric oxide (NO),

endothelium-derived hyperpolarizing factor (EDHF) and eicosanoids derived from arachidonic acid (AA) [4-9]. Overall, healthy, isolated, resistance and conductance arteries release both vasodilator and vasoconstrictor factors, but the relative number of vasodilators is greater, leading to a full relaxation for long periods. In diseases states this homeostatic scenario changes, resulting in an exacerbated production of vasoconstrictors molecules. For further details on how the endothelium regulates vascular function, please see references [10-11].

One of the major pathophysiological signatures in cardiovascular diseases is the presence of endothelial dysfunction, regardless of artery diameter (i.e., conductance and resistance arteries). Diseased conductance arteries are stiffer and larger than their normal controls which leads to increase systolic pressure. Diseased resistance arteries have several types of alterations that contribute to cardiovascular disease (i.e., reduced lumen diameter and hypertrophy of the vascular wall). Endothelium dysfunction is a result of an imbalance between endothelium-derived relaxing factors and endothelium-derived contracting factors. Endothelial dysfunction may lead to several abnormalities in the vasculature, such as the increase in maximum response and/or sensitivity to contractile factors, the decrease in maximum response and/or sensitivity to endothelium-dependent vasodilators, vascular remodeling, etc. In the case of hypertension, especially in early stages or “mild” hypertension, a phenomenon known as acetylcholine-induced endothelium contraction occurs (Figure 3.1 and 3.2). Endothelium-dependent contractions were first observed in isolated dog veins [12], although most studies today are performed on arteries. In 1981, Dr. Paul Vanhoutte was the first to report endothelium-derived vasoconstriction, showing that an unknown factor was being produced by the endothelium to cause increased vascular

tone. In physiological conditions, it has been shown that stretch applied to isolated canine basilar arteries caused the development of active tension in rings with endothelium but not in those in which the endothelium had been removed [13]. The authors suggested that the endothelium may contribute to the autoregulation of cerebral blood flow during increases in transmural pressure by the increased production and/or release of prostaglandins, which causes activation of the underlying vascular smooth muscle [13]. However, more probable is that the incidence of endothelium-dependent contractions is pathological, as they are prominent in arteries from animals that present vascular diseases, such as hypertension. In line, in 1986, Dr. Vanhoutte, along with Dr. Thomas Luscher, set out to investigate the decreased relaxation to acetylcholine in spontaneously hypertensive rats (SHR). Unexpectedly, they observed that acetylcholine caused endothelium-dependent contractions in arteries from SHR but not in those from Wistar Kyoto (WKY) rats [14]. In this study, aortas from hypertensive animals possessed a contraction to acetylcholine in concentrations ranged from ~100 nM – 10 μM. It was uncovered that the contraction to acetylcholine was also a characteristic of endothelium dysfunction [15-16].

Significant to the pathogenesis of hypertension, acetylcholine-induced contraction is not only unique to SHR (Figure 3.1). In our laboratory, we routinely observe acetylcholine-induced contraction in resistance and conductance arteries from Dahl salt-sensitive (S) rats (Figures 3.2 and 3.3). Salt-sensitivity is considered a hallmark of hypertension in blacks, including African Americans, given that it is found in 73% of hypertensive blacks [17]. Using inbred Dahl S rats, Dr. John Rapp [18] observed that the spontaneous elevation of blood pressure occurs in Dahl S rats fed a low salt diet, but not in Dahl salt-resistant (R) rats, due to unknown genetic influences and independent of

environmental factors. According to Dr. Rapp's measurements, spontaneous elevations in blood pressure from Dahl S rats begin at about ~4 weeks of age [18], and a high-salt diet accelerates this process leading to malignant hypertension, similar to humans. Corroborating, Dr. Rapp's findings, we observed that arteries from young Dahl S rats (~8 weeks old) fed a low-salt diet also present acetylcholine-induced contractions (Figure 2), but arteries from Dahl S fed a high salt diet (~15 weeks old), did not worsen this phenotype (Figure 3.3). These data suggest that Dahl S already has endothelial dysfunction prior to malignant hypertension. Therefore, the genesis of acetylcholine-induced contraction may be, at least in part, due to genetic influences. Another characteristic that we frequently observe is resistance arteries from SHR or Dahl S high-salt start relaxing to acetylcholine at lower concentrations (100 nM-1 μM), which means that they are more sensitive to acetylcholine when compared to the arteries from normotensive animals (Figure 3.3). However, at higher concentrations (0.3 μM), these arteries start contracting as previously described [13-14]. It is important to note that 0.3 μM acetylcholine, arteries should be relaxing back to baseline; however, hypertensive arteries have relaxed and start to contract again. Although acetylcholine-induced endothelium-dependent contraction was also observed in other conditions, such as aging and diabetes [19], it seems that this phenomenon is predominant in hypertension.

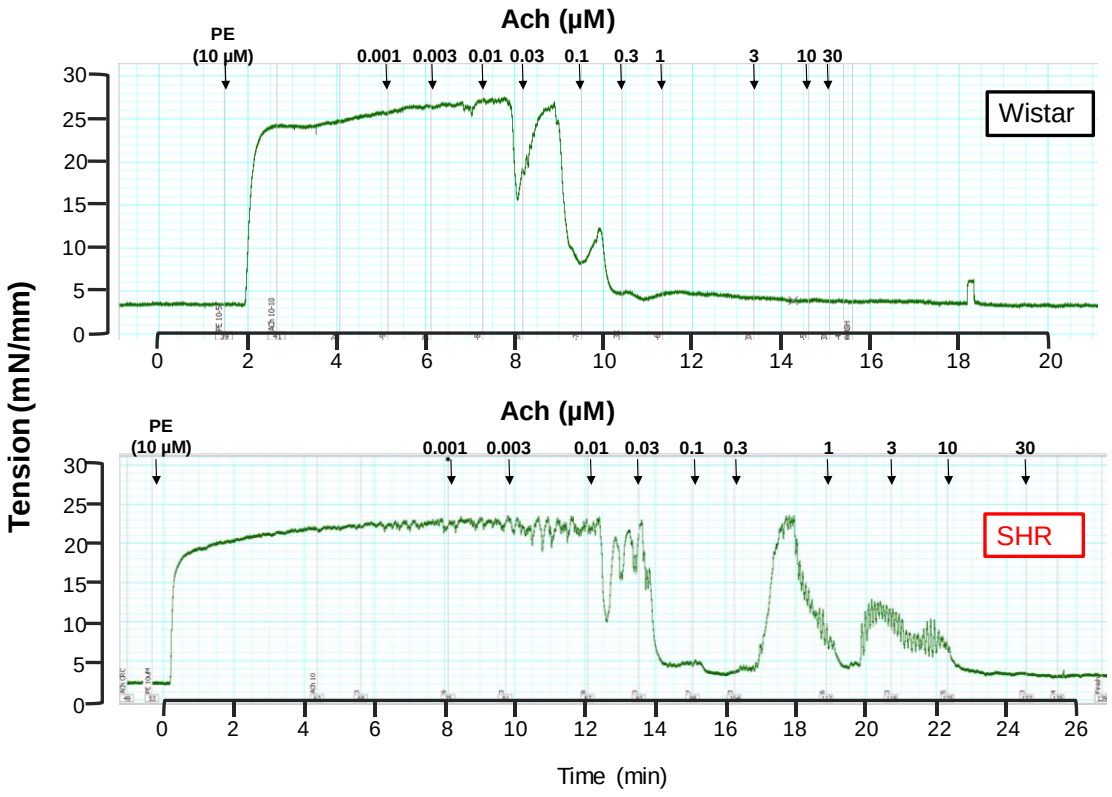


Figure 3.1. Typical traces represent concentration response curve to acetylcholine (1nM -30 µM) in mesenteric resistance arteries from 12-weeks old spontaneously hypertensive rats (SHR) and normotensive Wistar rats. The arteries were initially contracted with phenylephrine (10 µM). Please note that at the concentration of 0.03 µM acetylcholine, arteries from SHR are completely relaxed when compared to arteries from Wistar rats. This is a representative illustration of traces observed in our laboratory. However, similar findings are quantified in multiple studies [35, 47].

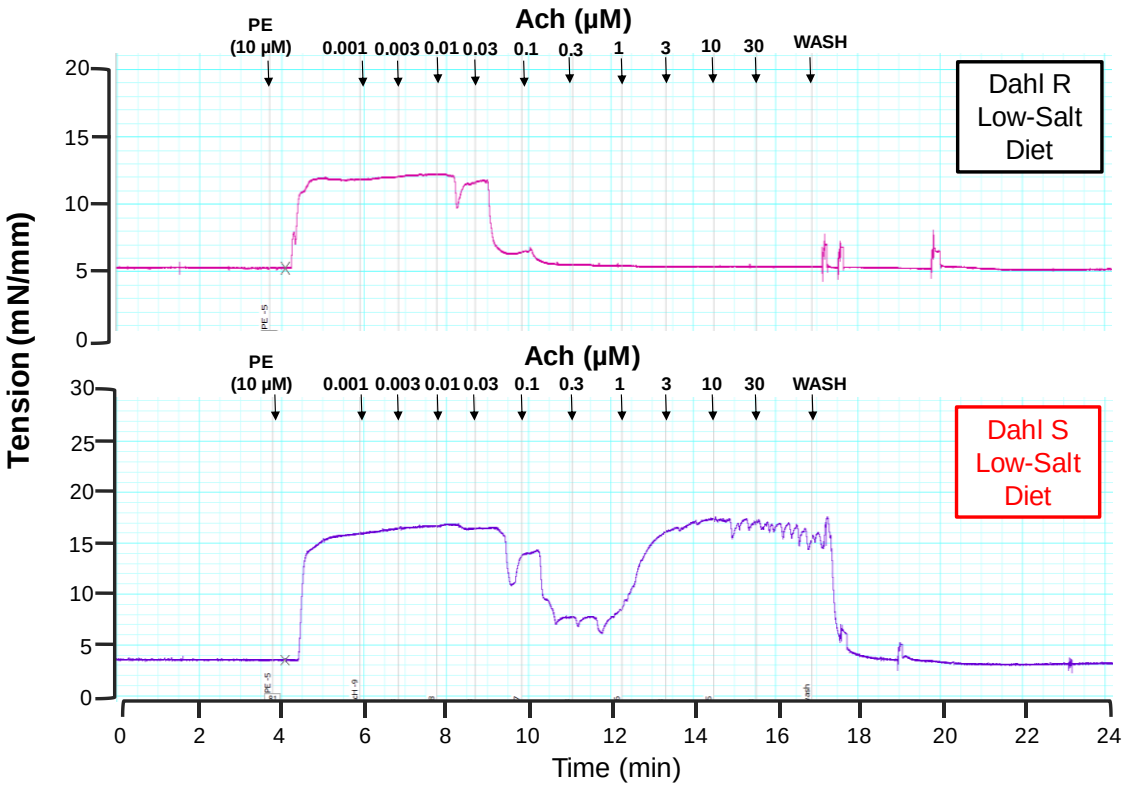


Figure 3.2. Typical traces represent concentration response curve to acetylcholine (1nM -30 μM) in mesenteric resistance arteries from 8-weeks old Dahl salt (S) sensitive and resistant (R) rats fed a 0.3% low-salt diet. The arteries were initially contracted with phenylephrine (10 μM). This is a representative illustration of traces observed in our laboratory. However, similar findings are quantified in multiple studies [13-14].

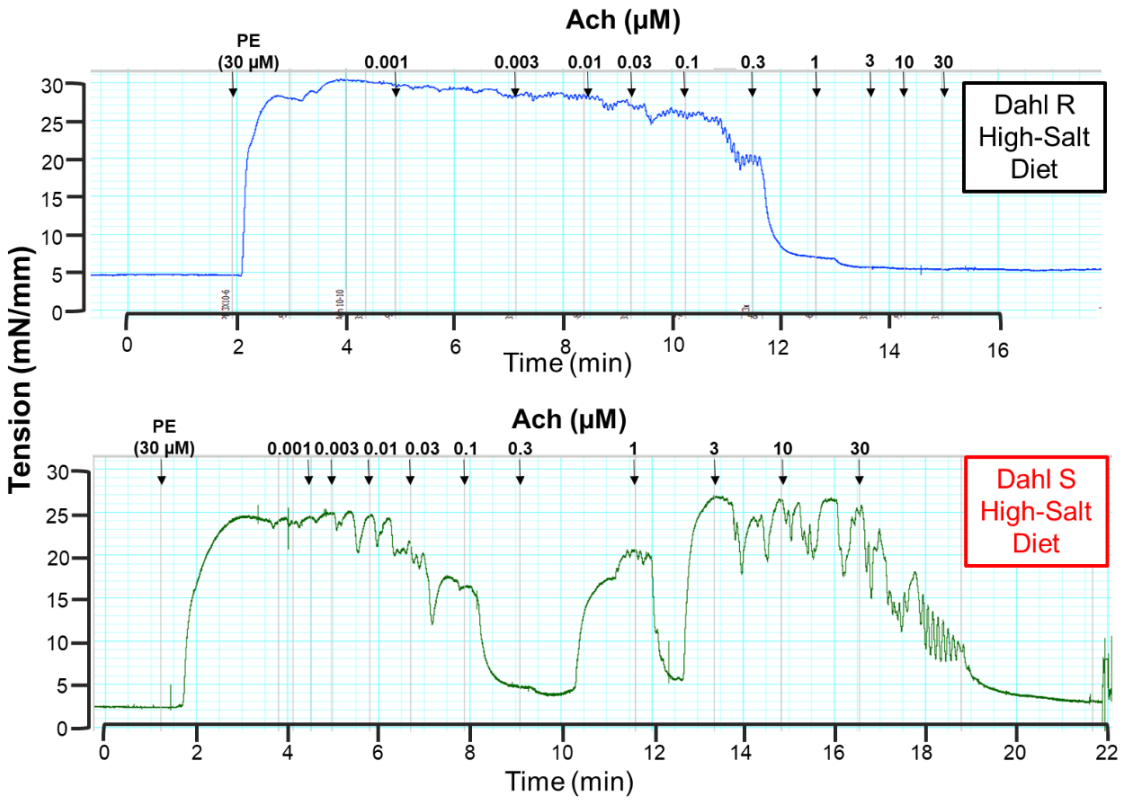


Figure 3.3. Typical traces represent concentration response curve to acetylcholine (1nM -30 μM) in mesenteric resistance arteries from 15-week old Dahl salt (S) sensitive and resistant (R) rats fed a 2% high-salt diet for 8 weeks. The arteries were initially contracted with phenylephrine (10 μM). Please note that at the concentration of 0.3 μM acetylcholine, arteries from Dahl S are completely relaxed when compared to arteries from Dahl R. This is a representative illustration of traces observed in our laboratory. However, similar findings are quantified in multiple studies [13-14].

3.4 Culprits of acetylcholine-induced contraction in hypertension

Multiple studies have demonstrated contractions to endothelium-derived vasoactive factors in isolated arteries from animals with spontaneous hypertension and salt-sensitive hypertension [14, 20-24]. After that initial observation and many years of research later, mechanistic studies began uncovering possible "culprits" to this phenomenon. In line, it has been shown that endothelium pathways for reactive oxygen species (ROS), cyclooxygenase (COX) and lipoxygenase (LOX) products are the instigators and/or contributors to acetylcholine-induced endothelium-dependent contractions in hypertension [25-26].

In hypertension, there is no doubt that ROS, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide are increased [27], and contribute to amplified contractile responses. ROS can increase contraction by quenching the bioavailability of NO [28] and also by uncoupling endothelial nitric oxide synthase (eNOS) (which itself can be a self-sustained source of ROS) [27]. ROS also leads to increased smooth muscle contraction and proliferation, platelet aggregation, and expression of adhesion molecules [29]. Moreover, ROS can depolarize vascular smooth muscle cells by inhibiting potassium channels and induce calcium sensitization, via activation of RhoA and Rho kinase activity [30]. However, while it is clear that ROS participates in acetylcholine-induced endothelium-dependent contractions, whether ROS are the primary instigators of this phenomenon is debatable. For example, it has been established that acetylcholine-induced endothelium-dependent contractions are decreased by superoxide dismutase, which breaks down superoxide anions to hydrogen peroxide, but not by catalase or deferoxamine, which scavenge hydrogen peroxide and hydroxyl radicals, respectively [31]. These observations

suggest an important role for superoxide anions in acetylcholine-induced contraction [31-33].

On the other hand, it is also known that COX, a major downstream enzyme of AA signaling, is a significant source for ROS [34]. Specifically, we have observed that the incubation with the specific inhibitor of COX-2 decreased ROS generation in isolated arteries from ouabain-treated hypertensive rats [34]. While these rats present with malignant hypertension and their arteries were hyperreactive to noradrenaline and had oxidative stress, acetylcholine-induced contraction was not observed. These findings suggest that ROS generation is not the primary instigator of acetylcholine-induced endothelium-dependent contraction and that ROS only indirectly contribute to this phenomenon by reducing the bioavailability of NO and/or activating COX in the vascular smooth muscle cells [35]. Therefore, it is still unresolved whether ROS are a “cause” or an “effect” of endothelium-dependent, acetylcholine-induced contractions in hypertension. On the other hand, as it will be discussed below, it is established that AA-derived biologically active products are the cause of endothelium-dependent contractions elicited by acetylcholine.

3.4.1 Arachidonic acid-derived bioactive lipids

AA is the most common precursor of biologically active lipids metabolites [26], and it plays a vital role in the initiation of inflammation and the resolution of inflammation in physiological states. AA is the preferred substrate for the COX, LOX, and cytochrome P450 (CYP450) pathways to produce prostaglandins, leukotrienes, and

epoxyeicosatrienoic acids (EETs), respectively [26, 36]. These lipid mediators are collectively referred to as eicosanoids, and these pathways are the target of approved drugs for the treatment of pain, allergies, inflammation, asthma, cardiovascular disorders, and, most recently, to inhibit tumor-associated inflammation [37]. A proper balance of substrate availability, enzyme expression and activity, and non-enzymatic oxidation is important for maintaining homeostasis. However, in hypertension, an unbalance in eicosanoids occurs that has a causative-effect in the genesis of acetylcholine-induced endothelium-dependent contractions predominantly through the COX, LOX, and CYP450 pathways that will be discussed below (Figure 3.4).

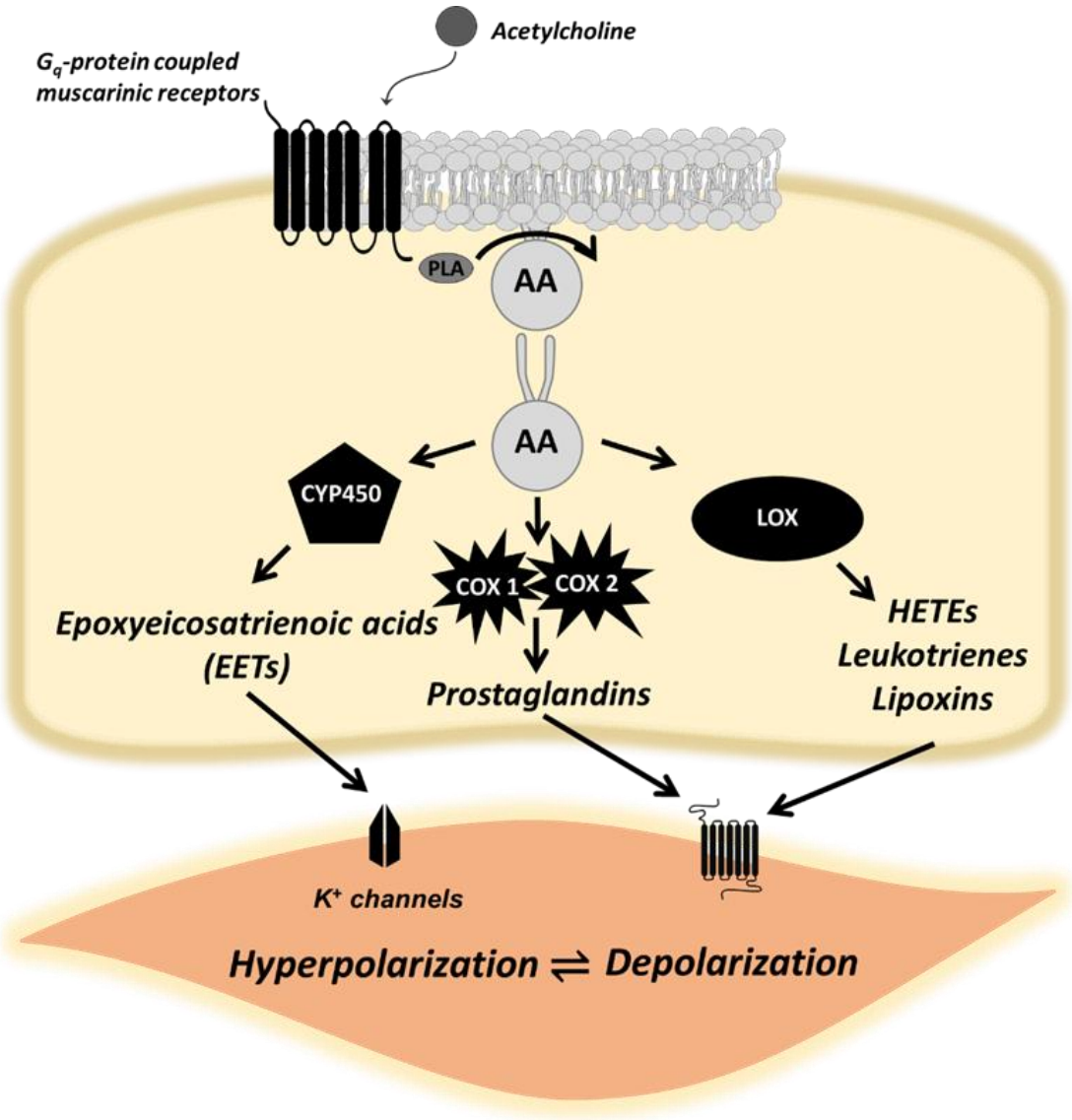


Figure 3.4. The Janus face of acetylcholine and endothelium-dependent, arachidonic acid signaling. In physiological conditions, acetylcholine promotes the synthesis of vasodilating eicosanoids and other endothelium-derived hyperpolarizing factors (e.g., nitric oxide). However, in pathophysiological conditions such as hypertension, there is aberrant arachidonic acid signaling due to excessive inflammation. As a result, acetylcholine promotes the synthesis of vasoconstrictor metabolites, predominantly from cyclooxygenase and lipoxygenase that can act on vascular smooth muscle cells. As a result,

acetylcholine causes endothelium-dependent contractile responses, as opposed to vasodilation.

3.4.1.1 Cyclooxygenases

The COX pathways produce pro-inflammatory or pro-resolving lipid mediators from the COX-1 or COX-2 isoforms. Although COX-1 and COX-2 are 65% homologous, they are regulated differently and can function independently within the same cell [38]. COX-1 uses fatty acids like AA preferentially, while COX-2 uses fatty acids and a glycerol, because of this COX-2 is capable of making mediators that COX-1 cannot [39]. COX-1 is constitutively expressed in tissues but can also be overexpressed [40]. COX-2 is constitutive as well, but its activity is induced mainly in inflammatory states. However, it has also been shown that COX-2 participates in physiological states. Specifically, it has been shown that cultured bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) exposed to steady fluid shear stress of 10 dyn/cm2 for five hours upregulates COX-2 and prostacyclin (a potent vasodilator) [41]. The authors observed the relationship between the mechanosensor platelet endothelial cell adhesion molecule-1 (PECAM-1) and the intracellular mechanoresponsive molecules phosphatidylinositol 3-kinase (PI3K), focal adhesion kinase (FAK), and mitogen-activated protein kinase p38 in the fluid shear stress induction of COX-2 expression and PGI_2 release. Knockdown of PECAM-1 expression inhibited fluid shear stress-induced activation of $\alpha5\beta1$-integrin, upregulation of COX-2, and release of PGI_2 [41]. In addition, there is a third distinct COX isozyme, called COX-3, whose discovery explained, in part, the

pharmacology of acetaminophen [42]. Less is known about COX-3, which seems to be found in the cerebral cortex and cardiac tissue and appears to be involved in centrally mediated pain. COX-3 is a splice variant for COX-1. Therefore, drugs that favorably block COX-1 also appear to act at COX-3.

The COX pathway converts AA to endoperoxides that are then converted by their respective synthases into five main prostaglandins (PGs): PGD2, PGE2, PGF2α, PGI2 (prostacyclin), and thromboxane (Tx)A2. Because prostanoids are a family of lipid mediators produced from fatty acids, and hence are generally regarded as hydrophobic compounds, it was thought in earlier times that they were incorporated into the cell membrane and exerted their action by perturbing lipid fluidity [43]. However, each prostanoid has a unique activity profile, not exactly overlapping with others, which indicates that each prostanoid has a specific site of action. Hence, the concept of prostanoid action via prostanoid receptors gradually appeared [43]. Supporting the concept that prostanoids indeed act via receptor, it was shown that some prostanoid actions induce changes in intracellular cAMP levels, phosphatidylinositol (PI) turnover, and free Ca2+ concentrations [43]. Currently, we know that each prostanoid (TxA2, PGI2, PGE2, PGF2, and PGD2) has a specific receptor (TP, IP, EP, FP, and DP, respectively) [43]. These receptors are G protein-coupled receptors, and there are eight types and subtypes that are encoded by different genes, but as a whole constitute a subfamily in the superfamily of the rhodopsin-type receptors [43]. Prostanoid receptors have been classically grouped into three categories, depending on the type of G-protein they bind to: the contractile receptors EP1, FP and TP are coupled to Gq and activate phospholipase C, leading to intracellular calcium increases; the relaxant type, which comprises IP, DP1, EP2 and EP4, signal

through Gs to induce adenylate cyclase activity; and the inhibitory receptor EP3 couples to Gi, leading to a decrease in intracellular cAMP content [44]. Promiscuously, prostanoids can bind to multiple receptors or receptor isoforms and are therefore able to activate diverse G-proteins and signal transduction pathways. Accordingly, it has been shown that in aorta from SHR and WKY, different prostaglandins studied, TXA2, PGH2, PGF2α, PGE2, PGD2, PGI2 as well as 8-isoprostane, were able to induce vascular contraction via TP receptors, given that the contractions were abolished by S18886, a specific antagonist for the TP receptor. [45].

In hypertension, COX-1 and 2 expression and activity are enhanced in conductance and resistance arteries [34, 46], consequently leading to an increase in prostanoids production. Incubation with non-selective (indomethacin) or selective inhibitors of COX-1 and -2, such as N[-2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS398) or 2-[(1-oxopenytyl)oxy]-benzoic acid (valeryl salicylate) prevented acetylcholine-induced endothelium-dependent contractions [48]. As mentioned above, TXA2 is a potent vasoconstrictor, and its synthesis is increased in endothelial cells from hypertensive animals [36]. However, the use of a selective inhibitor of TXA2 synthase only partially prevented the endothelium-dependent contractions indicating that TXA2 is only one factor contributing to contraction [47-52]. On the other hand, acetylcholine-induced endothelium-dependent contractions were completely inhibited by treatment with a TXA2/PGH2 antagonist (O NO-3708), but not with a TXA2 synthase inhibitor (OKY-046) [53]. The authors suggested that since these contractile responses were completely suppressed by TXA2/PGH2 antagonist, but were not affected by a TXA2 synthase inhibitor, they are most likely mediated by endothelium-derived PGH2 [53]. There was also a statistically

significant correlation between the acetylcholine-induced contractions and blood pressure. However, in vivo administration of another TXA2 /PGH2 antagonist (ONO-8809) (10 or 30 micrograms per body per day) for 3 weeks (5-8 weeks of age) did not affect blood pressure in SHR or WKY. These observations suggest that the increase in endothelium-derived contractile factors in SHR is more likely to be a result than a cause of hypertension in SHR [53].

Another prostaglandin associated with endothelium-dependent contractions in hypertension is PGI2. Notably, PGI2 is the most abundant prostaglandin in hypertensive endothelial cells [49-52]. In physiological and acute conditions, the induction of COX-2 promotes the production of PGI2, which is a vasodilator, however in chronic inflammatory conditions, as seen in hypertensive animals, COX-2 upregulation induces endothelium-dependent contractions via PGI2 [36]. In hypertension, PGI2 synthase activity and expression are increased in endothelial cells, which leads to an exacerbated production of PGI2 via activation of TP receptors to induce contraction [20, 49-50]. Exogenous prostacyclin induced a concentration-dependent relaxation in mesenteric resistance arteries from WKY rats. However, in arteries from non-treated SHR and both WKY and SHR treated with aldosterone (0.05 mg/kg/day) for 3 weeks, prostacyclin concentrations below 0.1 μM induced concentration-dependent relaxation whereas concentrations equal or above 0.1 μM have a biphasic effect, characterized by an initial contractile response followed by a relaxing phase [54]. These authors also observed that the treatment with aldosterone-induced an exacerbated COX-2 expression in both strains. However, chronic aldosterone administration did not significantly modify blood pressure levels. These suggest that

another mechanism, different from high blood pressure, maybe the initiator of endothelial-induced contraction in hypertension.

3.4.1.2 Lipoxygenases

The LOX pathway produces hydroperoxides from AA [55]. LOX enzymes are expressed in several cells, such as immune, epithelial, and tumor cells that display a variety of physiological functions, including inflammation, skin disorder, and tumorigenesis. In humans, there are five LOXs: the 5S-(arachidonate: oxygen 5-oxidoreductase), 12R-(arachidonate 12-lipoxygenase, 12R-type), 12S-(arachidonate: oxygen 12-oxidoreductase), and two distinct 15S-(arachidonate: oxygen 15-oxidoreductase) LOXs that oxygenate arachidonic acid in different positions along the carbon chain and form the corresponding 5S-, 12S-, 12R-, or 15S-hydroperoxides, respectively [56]. In general, 15-LOX synthesizes anti-inflammatory 15-hydroxyeicosa-tetraenoic acid (HETE), 5-LOX and 12-LOX induce pro-inflammatory mediators, and 5-LOX produces 5-HETE and leukotrienes, which are potent chemoattractants.

Lipoxygenase inhibitors have been reported to reduce the angiotensin II-induced contraction on isolated arteries. Specifically, contractions induced by angiotensin II in isolated human internal mammary artery were inhibited by phenidone 100 μM (cyclooxygenase and lipoxygenase inhibitor), baicalein 100 μM (5-, 12- and 15-lipoxygenases inhibitor), AA861 10 μM (5-lipoxygenase inhibitor) and MK571 1 μM (CysLT1 receptor antagonist) [57]. Interestingly, increases in the systolic pressor response to angiotensin II were reduced by two different LOX inhibitors, baicalein (30 mg/kg) and

esculetin (60 mg/kg) in rats, but not by the cyclooxygenase inhibitor indomethacin, suggesting that LOX pathway may have an important role in mediating the pressor effect of angiotensin II [58]. DelliPizzi et al. [59] demonstrated that in cytosolic fractions of aortae taken from aortic coarctation-induced hypertensive rats, 12-lipoxygenase protein was increased as compared to normotensive controls. Aortic rings from hypertensive, but not from normotensive rats, exhibited a basal tone that was reduced by the lipoxygenase inhibitors cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC,10 μM) and 5,8,11-eicosatriynoic acid (ETI, 10 uM) [59]. Corroborating previous studies, these authors observed that CDC (8mg/kg administered subcutaneously) did not affect the blood pressure of normotensive rats but decreased that of hypertensive rats. Only a few studies evaluated the possible mechanisms of LOX-derived products and endothelium-dependent contraction in hypertension [59]. Accordingly, it has been shown that SHR and WKY orally treated for 3 weeks with cysteinyl leukotrienes biosynthesis inhibitor, MK-886 (0.1 mg/ml), did not present changes in blood pressure. However, in arteries incubated with NOS inhibitor N-nitro-L-arginine (L-NNA; 100 μM) and contracted to prostaglandin F2α (PGF2α)-induced tone, acetylcholine was able to evoke concentration-dependent contractions in SHR aorta [60]. Further, incubation with MK-886 (10 μM), the 5-lipoxygenase (5-LO) inhibitor AA861 (10 μmol/l), or the cysteinyl leukotriene receptor antagonist MK571 (1 μmol/l) was able to decrease these acetylcholine-induced contractions. Overall this suggests that LOX-derived products may be associated in the endothelium-dependent contraction to acetylcholine, but not in the regulation of blood pressure in SHR [60].

As previously described, AA is also a substrate for LOX to produce leukotrienes and lipoxins [26, 36]. Leukotrienes and lipoxins are two functionally different classes of

LOX-derived eicosanoids. Leukotrienes are potent pro-vasodilatores mediators and directly and indirectly stimulate fibroblast chemotaxis, proliferation, and collagen synthesis. On the other hand, lipoxins counter-regulate the pro-inflammatory actions of leukotrienes and activate resolution of the inflammatory response [61]. Lipoxin A4 (LXA4) was discovered in 1984 through interaction(s) between the 5- and 15-lipoxygenase pathways in human leukocytes [62]. The generation of LXA4 is a very rapid process that aspirin does not inhibit. In fact, aspirin has been shown to trigger the production of LXA4 through acetylation of COX-2 that metabolizes arachidonic acid to 15(R)-hydroxyeicosatetraenoic acid. This metabolite is then converted via lipoxygenase to LXA4, also known as 'aspirin-triggered lipoxin.' This process is augmented during inflammation, atherosclerosis, and thrombosis [63]. Interestingly, LXA4 binds the formyl peptide receptor-2 (FPR2), a subfamily of G protein-coupled receptors. FPR activation in the cardiovascular system has been shown to have functional implications, such as modulation of vascular tone and blood pressure [64]. Specifically, we observed that LXA4 induced concentration-dependent contractions via FPR-2 activation, and both RhoA/Rho kinase inhibitor (Y-27632, 1 μm) and ROS scavenger (tempol, 1mM) decreased this contraction. Also, endothelium removal, COX and NAD(P)H oxidase inhibitors (indomethacin and apocynin, respectively, both 10μM) attenuate the LXA4-induced contraction. LXA4 potentiated phenylephrine-induced contraction and inhibited acetylcholine-induced relaxation. In another study, we observed that FPR-1 activation induces actin polymerization in vascular smooth muscle cells. Absence of FPR-1 in the vasculature significantly decreased vascular contraction and induced loss of myogenic tone to elevated intraluminal pressures via disruption of actin polymerization [65]. Therefore, it is possible

to infer that LXA4 may contribute to further acetylcholine-induced contraction in conditions where its production is exacerbated.

3.4.1.3 Cytochrome P450

Endothelial and vascular smooth cells also generate CYP450 metabolites from AA that modulates endothelial cell function and vascular homeostasis. CYP450 produces two main eicosanoid products, EETs, formed by CYP epoxygenases, and HETEs, formed by CYP hydroxylases [66]. Similar to PGI2, EETs are recognized as endothelium-derived relaxing factors [26]. Actually, EETs have been proposed to be the unidentified endothelium-derived hyperpolarizing factor because they hyperpolarize and relax vascular smooth muscle cells by activating calcium-sensitive potassium (KCa2+) channels [66]. On the other hand, 20-HETE is the predominant CYP hydroxylase synthesized by vascular smooth muscle cells [67]. 20-HETE generation is directly correlated with myogenic tone in renal and cerebral arteries [68]. Once formed, 20-HETE increases smooth muscle contraction by inhibiting large-conductance potassium channels (BKCa2+), inducing depolarization, and further increasing intracellular calcium [68]. The elevation of 20-HETE production in malignant hypertension was first reported in 1989 [69]. 20-HETE inhibition decreases blood pressure in angiotensin II and endothelin-induced hypertension [70-71]. Interestingly, it has been shown that Dahl S rats present a decrease in 20-HETE production, leading to salt-sensitive hypertension development [72]. As described above, it is well known that the hyperpolarization in response to acetylcholine in small arteries is closely regulated by CYPP450–dependent enzymes [73]. However, it is currently unknown

whether CYP450 products may be associated with acetylcholine-induced endothelium-dependent contractions in hypertension.

3.5 Can we resolve acetylcholine-induced endothelium-dependent contractions?

Excessive production of inflammatory mediators, such as leukotrienes and prostaglandins, in damaged tissue, triggers the transition from acute inflammation to chronic inflammation [74]. Failure to resolve inflammation or chronic activation of the inflammatory response accelerates tissue injury and can eventually develop into disease. As complex as inflammation is, resolution of inflammation is a physiological response to "switch off" the inflammatory cascade. Cessation of acute inflammation is an active process that involves the biosynthesis of specialized pro-resolving mediators [1]. Pro-resolving mediators are endogenously derived from the AA, omega-6 fatty acid, and omega-3 fatty acids, including eicosapentaenoic acid and docosahexaenoic acid. As described above, AA-bioactive lipids play a fundamental role in acetylcholine-induced endothelium-dependent contraction in hypertension; therefore, it is possible that unresolved vascular inflammation may also play a role in this process. Unfortunately, there is little or no evidence beyond expert opinion to support this concept, so, as a result, we will briefly discuss about pro-resolving mediators that were shown to be involved in vascular function, such as LXA4 and resolvins, and only infer that acetylcholine-induced endothelium-dependent contraction may also occur because the mechanisms behind the resolution of inflammation failed to resolve this process in hypertension.

3.5.1 Resolution of Inflammation

Specialized pro-resolving mediators can be anti-inflammatory at transcriptional and translation levels and are capable of activating NO and PGI2 production, phagocytosis, and efferocytosis [75]. Although there are four identified specialized pro-resolving lipid mediators: resolvins, protectins, LXA4, and maresins, this review will focus on lipoxins and resolvins.

LXA4 is the most studied and characterized of the lipoxin family. As described above, LXA4 is biosynthesized from AA, and it has potent anti-inflammatory and resolution capabilities [76]. Interestingly, aspirin triggers the production of LXA4 through acetylation of COX-2 that metabolizes arachidonic acid to 15(R)-hydroxyeicosatetraenoic acid. This acid is quickly converted to LXA4 by LOX [64]. In arteries, LOX-5 biosynthesizes LXA4, and in platelets, LOX-12 biosynthesize LXB4 [74, 77]. The formation of LXA4 within the vascular lumen and wall during inflammation places this lipid in a strategically advantageous site for modulation of vascular function. Accordingly, von der Weid et al. [78] demonstrated that LXA4 induces endothelium-dependent relaxation in mesenteric arteries and aortic segments. This study showed that LXA4 (1 μmol/l) in rat aortic rings contracted with phenylephrine resulted in relaxation [78]. In opposition, Feuerstein and Siren [79] showed that intravenous LXA4 concentration-dependently constricted mesenteric arteries, but did not alter blood pressure or heart rate. Supporting these data, we showed that LXA4 induces contraction in aortic rings via FPR-2 and RhoA activation in the vascular smooth muscle cells [64]. Nascimento-Silva et al. [80] demonstrated that LXA4 suppresses NADPH oxidase-mediated ROS generation in endothelial cells. Also, another study demonstrated that LXA4 attenuates

lipopolysaccharide-induced intracellular ROS in microglia cells by inhibiting cytoplasmic NADPH oxidase subunit p47(phox) translocation to the cell membrane and NADPH oxidase activity [81]. Recently it was observed that plasma LXA4 levels were higher in preeclamptic women compared to in non-pregnant and normotensive pregnant women. Nonetheless, endogenous LXA4 concentration seems to be insufficient to attenuate inflammation in preeclampsia because these women still showed features of systemic inflammatory response syndrome despite the increased levels of LXA4 [82].

Resolvins, previously referred to as bioactive lipid signals [83], are also lipid mediators that inhibit neutrophil transmigration in between endothelial cells, among other functions. Resolvins are derived from omega-3 polyunsaturated fatty acids. Humans obtain eicosapentaenoic acid and docosahexaenoic acid from marine oils that are eaten or supplemented [83-84]. Resolvins were originally isolated from murine dorsal air pouches treated with aspirin. They were also isolated from co-cultured endothelial cells and neutrophils [83]. Transcellular formation of resolvins can occur with the formation of 18R-hydroxyeicosapentaenoic acid by endothelial cells expressing COX-2 and treated with aspirin [85]. Interestingly, selective COX-2 inhibitors block resolvins formation, but neither indomethacin nor acetaminophen can.

Similar to LXA4, resolvins also attenuate nuclear factor kappa B activation and ROS generation [86-87], stimulate macrophages to phagocytose apoptotic neutrophils [88], and inhibit cytokine release and cell migration [89-90]. These lipids also modulate pro-inflammatory leukocyte expression and disrupt TXA2-mediated platelet aggregation [91]. Interestingly, a study conducted by Rathod et al. found that young women are protected

against systemic inflammation-induced endothelial dysfunction due to accelerated resolution of inflammation, which is mediated by the D-series resolvin pathway [92].

To summarize these data, it is clear that LXA4 and resolvins are important pro- and anti-inflammatory mediators and play a role in vascular health. However, there is still a gap in the literature in understanding the mechanism associated with resolution of inflammation, vascular dysfunction, and hypertension. Specifically, it is unclear if disturbances in LXA4 and resolvins pathways could be associated and, perhaps, a cause of vascular inflammation and, subsequently, acetylcholine-induced- endothelium-dependent contractions in hypertension.

3.6 Conclusion and Perspectives

Acetylcholine-induced endothelium-dependent contractions are considered a hallmark for the most studied models of hypertension, such as SHR and Dahl S rats. However, this phenomenon's precise mechanism is still debated, which makes it unclear if this process is fundamental for the genesis of hypertension or if it is exacerbating vascular tissue injury leading to malignant hypertension. Conflicting arguments are suggesting that elevations of blood pressure may be or may not be the cause of this phenomenon. However mechanistically, it is acceptable that AA and its metabolites play critical roles in this process. With this thought, we question whether a decrease in resolution of inflammation would participate in the genesis and/or maintenance of endothelium-dependent contraction to acetylcholine.

Chapter 4

Resolution of Inflammation improve vascular function in Resistance Arteries from Spontaneously Hypertensive Rats

4.1 Abstract

It is well known that low-grade chronic inflammation induces vascular dysfunction and contributes to hypertension. On the other hand, resolution of inflammation is an active phenomenon to switch off the inflammatory processes once the harmful stimuli are removed and facilitate the return to homeostasis. Increasing the levels of pro-resolving mediators to promote the resolution of inflammation is emerging as a novel therapeutic approach. Arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid are substrates for the production of the pro-resolving lipid mediators lipoxin A_4 (LXA_4), resolvin D1 (RvD1), and resolvin E1 (RvE1), respectively, through the 5-lipoxygenase enzymatic pathway.

Although is well know that these lipid mediators decreases low-grade chronic inflammation, it is unknown if pro-resolving lipid mediators can ameliorate dysfunction in arteries from animals that present low-grade chronic inflammation, such as hypertensive animals.

A common signature seen in arteries from hypertensive animals is that high concentrations of acetylcholine leads to vascular contraction, mainly via activation of cyclooxygenase 2, and, subsequently, the release of prostaglandins. Therefore, based on the anti-

inflammatory action of the resolvins, we hypothesized that pro-resolving lipid mediators are decreased in hypertension, and acute exposure of these molecules will decrease acetylcholine-induced contractions in arteries from SHR.

To test this hypothesis, we first performed lipidomic analysis to understand if the precursors of resolvins were decreased in hypertension. Indeed, we observed that tt RvD1 and LXA4 decreased in plasma from SHR. After, we mesenteric resistance arteries (MRA, lumen diameter ~250 μm) from male 14-week-old SHR and Wistar Kyoto (WKY) were collected to assess vascular function via wire myograph. MRA were incubated with either RvD1, RvE1, or LXA4 or vehicle prior to concentration response curves to acetylcholine or phenylephrine. As expected, low concentrations (≤ 100 nM) of acetylcholine induced relaxation in arteries from both groups, however high concentrations (≥ 1 μM) of acetylcholine induced contraction in arteries from SHR only. Treatment with the pro-resolving lipid mediators did not change acetylcholine-induced relaxation in arteries from WKY. However, incubation with RvD1 and LXA4 abolished acetylcholine induced contraction in arteries from SHR and promoted relaxation. Overall, these results suggest that resolution of inflammation are decreased in SHR because the fatty acids that they are derived from have low bioavailability when compared to WKY. Direct treatment with the end-stage resolvins improves endothelium-dependent relaxation in arteries from hypertensive animals. Our work suggests that the RvD1 or LXA4 may also be used as a new therapeutic tool to specifically improve vascular function in hypertension.

4.2 Introduction

Chronic, low-grade inflammation that leads to vascular dysfunction is a common characteristic of hypertension. Although acute inflammation is necessary to protect the body from internal and external dangers, excessive inflammation is a problem and often pathological. Therefore, a process of resolving inflammation, especially acute inflammation, is mandatory to limit the development of chronic inflammation [1]. It was previously believed that inflammatory processes passively disappeared when a danger was eradicated. However, it is now accepted that the resolution of inflammation is an active process that creates specific stops signals for inflammation to return to a state of homeostasis [2]. Any imbalance in the resolution of inflammation may contribute to chronic inflammation like that observed in cardiovascular diseases.

Specialized pro-resolving mediators (SPMs) derived from ω-3 or ω-6 polyunsaturated fatty acids have an important role in the resolution of inflammation. There are four identified specialized pro-resolving lipid mediator groups: resolvins, protectins, lipoxins, and maresins. SPMs can be anti-inflammatory at transcription and translation levels and are capable of activating nitric oxide (NO) and prostacyclin (PGI_2) production, phagocytosis, and efferocytosis [3]. Interestingly, SPMs also have important roles in resolving inflammation either acting on their own GPCRs or modulating GPCRs for other fatty acids [4]. For instance, Lipoxin A4, derived from ω-6 polyunsaturated fatty acid arachidonic acid, and Resolvin D1, derived from ω-3 polyunsaturated fatty acid docosahexaenoic acid, bind to the ALX/FPR2 receptor [5-6]. Resolvin E1, derived from ω-3 polyunsaturated fatty acid eicosapentaenoic acid binds to the ChemR23 or BLT1 receptor [7]. After binding their receptors, they act to enhance phagocytosis, prevent neutrophil migration through

endothelial cells into damaged tissue, and stimulate macrophages [8]. These lipid mediators also modulate pro-inflammatory leukocyte expression and disrupt thromboxane (TXA_2)-mediated platelet aggregation [9].

The resolvin effects in the endothelium are described as limiting transendothelial migration and cell-cell interactions with leukocytes to generate resolvins or decreasing leukocyte adhesion [10]. Resolvins have also been found to regulate microvasculature permeability [10]. Interestingly, young women are protected against systemic inflammation-induced endothelial dysfunction due to accelerated resolution of inflammation, which is mediated by the D-series resolvin pathway [11]. A study has recently shown that Resolvin D1 and Resolvin E1 have a relaxant effect on rat and human arteries [12]. Although the endothelium has emerged as a therapeutic target and resolvins have been shown to inhibit arterial contraction, the role of resolvins in endothelium biology and function in cardiovascular diseases, such as hypertension, is not yet understood.

We and others previously observed that resistance arteries from hypertensive rats have a characteristic contraction to the known vasodilator acetylcholine [13-16]. Acetylcholine is a pharmacological tool used to assess endothelial function. In arteries from naïve animals, acetylcholine binds to G-protein coupled muscarinic receptors on the endothelial cell membrane which causes an increase in intracellular calcium levels. Increasing in calcium levels will lead to various cellular responses, such as NO and eicosanoid production [17-22], and subsequently, complete vascular relaxation. In hypertension, a biphasic response is observed in acetylcholine-induced relaxation. For instance, at lower concentrations of acetylcholine, the arteries relax as expected mainly due to the release of vasodilators NO and/or PGI_2 [23]. However, at high concentrations, the

arteries start to contract [13]. This phenomenon has been observed since the 1980s [24]. These contractions have been attributed in part to multiple contractile mediators derived from ω-6 polyunsaturated fatty acids, such as TxA_2, leukotrienes, reactive oxygen species (ROS) and other prostaglandins [25-26]. As described above, healthy arteries release both vasodilatory and vasoconstrictive mediators when activated by acetylcholine, but the vasodilatory are more abundant and lead to full relaxation. Endothelial dysfunction is observed when there is an imbalance between the vasodilatory and vasoconstrictive mediators [27-28]. Based on these premises, and on the fact that lipid mediators also disrupt thromboxane TXA_2-mediated platelet aggregation, as described above, we questioned if (1) precursors of resolvins are decreased in hypertension, and (2) enhancement of resolution of inflammation would decrease acetylcholine-induced contraction in arteries from hypertensive animals. Specifically, given that low-grade chronic inflammation is present in hypertension, it is unclear if disturbances in SPM pathways could be associated with acetylcholine-induced contractions in hypertension. This study attempts to distinguish the role of pro-resolving mediators in endothelium-dependent contraction in resistance arteries from spontaneously hypertensive rats.

4.3 Materials and Methods

4.3.1 Animals

All animals were from a colony maintained at the University of Toledo College of Medicine and Life Sciences. All animal procedures and protocols used were approved by the University of Toledo Institutional Animal Care and Use Committee (IACUC protocol approval numbers 108854, 104573, 108390). Experiments were conducted in accordance

with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines. Male Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) rat strains were from the University of Toledo College of Medicine and Life Sciences. All rats were weaned at 4 weeks of age and maintained on a 12-hour light cycle with water ad libitum and a maintained on a normal chow diet (0.3% NaCl, Harlan Teklad diet TD 7034; Madison, WI) until euthanasia at 13-15 weeks of age.

4.3.2 Tissue Collection

After reaching the appropriate age for hypertension development, rats were euthanized by thoracotomy and exsanguination via cardiac puncture under isoflurane anesthesia (5% in 100% O2 administered via nose cone). Whole blood was first collected from the abdominal aorta and centrifuged. For serum, whole blood was centrifuged for 15 minutes at 2000 rcf in a 4°C centrifuge and the supernatant was collected. For plasma, whole blood was centrifuged for 15 minutes at 1500 rcf in a 4°C centrifuge and the supernatant was collected. After euthanasia, mesenteric resistance arteries (MRA) were harvested, as described before [29].

4.3.3 Vascular Function

Third or fourth order MRA, 2 mm in length, were mounted on DMT wire myographs (Danish MyoTech, Aarhus, Denmark) and kept in a Krebs solution (Table 1). The MRA were normalized to their optimal lumen diameter for active tension development, as described previously by our group [29]. To test vascular smooth muscle cell integrity, the arteries were initially contracted with 120 mmol/L potassium chloride (KCl). Arteries were then incubated with either vehicle (10% Ethanol), Resolvin D1

(10nM), Resolvin E1 (10nM), or Lipoxin A4 (10nM) for 1 hour. Concentration-response curves to acetylcholine (ACh, 1 nmol/L to 10 μmol/L) after initial contraction to phenylephrine (PE, 30 μmol/L) was performed to evaluate relaxation. Relaxation responses to ACh are shown as a percent of the initial PE contraction (30 μmol/L). Concentration-response curves to PE (10 nmol/L to 10 μmol/L) were also performed (force, mN).

4.3.4 Thromboxane A_2 and Prostaglandin E_2 Measurements in Mesenteric Resistance Arteries

To evaluate thromboxane A2 (TXA2) and prostaglandin E2 (PGE2) in MRA , we followed our previously published method [30]. Briefly, the mesenteric bed from each strain was perforated and divided into separate 1.5mL microcentrifuge tubes, as shown in Figure X. One mL of fresh oxygenated Krebs solution was added to the tubes. One tube was left as a control and the other tubes were treated with 10nM Resolvin D1 or LXA4. The tubes were treated with 10 μM PE. After 5 minutes, the tubes were treated with 10 μM ACh for 5 minutes. The supernatant was collected from the vials and flash frozen in liquid nitrogen and stored at -80°C until analysis. To detect TXA2 or PGE2 in the supernatant, we used the monoclonal ELISA kit for thromboxane B2 (TXB2, Catalog No. 501020, Cayman Chemical, USA) or PGE2 (Catalog No. 514010, Cayman Chemical, USA) according to the instructions provided by the manufacturer. TXB2 is a stable biologically inert metabolite formed from the non-enzymatic hydrolysis of TXA2, which has 30 second half-life. For both kits, all samples were run in triplicate.

4.3.5 Lipidomic Analysis

Mesenteric resistance arteries and plasma from WKY and SHR rats were collected as described above. Samples were flash-frozen in liquid nitrogen, and then sent to Creative Proteomics (Shirley, New York, USA) for comprehensive eicosanoid lipid panel analysis. Samples were tested for approximately 150 eicosanoids in both tissues following the company's procedure. Briefly, MRA samples were homogenized in 1000 µL 10% MeOH/PBS. Then, 100 µL internal standard mix was added to each sample. EIC were purified by SPE: Strata-x polymeric reverse phase columns (8B-S100-UBJ Phenomenex). Samples were reconstituted in 50 µL Buffer A (63% H20, 37% ACN, 0.02% Acedic Acid). The chromatographic system was a Waters ACQUITY UPLC. The Mass spectrometer is AB Sciex 6500 Qtrap. Data analysis software used are Analyst and Multiquant. The LC column was a Waters BEH-Shield 2.1x100 mm 1.7 µm.

4.3.6 Cell Culture

Human aortic endothelial cells were purchased from Lonza (Walkersville, MD). The normotensive cells were from a healthy, 53-year-old male, and the hypertensive cells were from a 55-year-old male. Cells were maintained at 37 °C in a 5% CO_2 humidified incubator. Cells were cultured with Endothelial Cell Basal Medium-2 (EBM-2, Lonza, Walkersville, MD) supplemented with human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), R3-Insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, hydrocortisone, human fibroblast growth factor-beta (hFGF-β), heparin, fetal bovine serum (FBS) and gentamicin/amphotericin-B (GA-1000). Medium was changed 18-24 hours after plating and replaced with fresh medium. Medium was changed every 48 hours and cells were split when they reached 70-80% confluence.

4.3.7 Nitrate/Nitrite Measurement in HAEC and Mesenteric Resistance Arteries

To evaluate nitrate/nitrite levels in MRA and cell culture supernatant, we used the supernatant collected from cells and arteries that were treated with vehicle, RvD1, or LXA4. Briefly, the mesenteric bed from each strain was perforated and divided into separate 1.5mL microcentrifuge tubes. One mL of fresh oxygenated Krebs solution was added to the tubes. One tube was left as a control and the other tubes were treated with 10nM Resolvin D1 or LXA_4. The tubes were then treated with 10 µM PE for 5 minutes. The tubes were then treated with 10 µM ACh for 5 minutes. The supernatant was then collected from the vials and flash frozen in liquid nitrogen and stored in multiple aliquots at -80°C until analysis. The same procedure was completed in the supernatant collected from a plate of confluent human endothelial cells from a normotensive and a hypertensive donor treated with vehicle, RvD1 (10nM), or LXA_4 (10nM) for 24 hours.

To detect total nitrate/nitrite in the supernatant from the MRA and HAEC, we used the nitrate/nitrite colorimetric assay kit (Catalog No. 780001, Cayman Chemical, USA) according to the instructions provided by the manufacturer. All samples were run in duplicates. This kit measures nitrite and nitrate which are the final products of NO *in vivo*. The measurement of total nitrite and nitrate is the best index of total NO production.

4.3.8 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 9.1.1 (La Jolla, CA, USA). Data are presented as mean ± standard error of the mean (SEM) and statistical significance was set at $p<0.05$ unless noted otherwise. Procedures used include Student's

unpaired t-test, one-way, and two-way analysis of variance (ANOVA),and non-linear regression analysis (LogEC50 and Emax). Tukey's post-hoc testing and the Bonferroni post-hoc testing were used in one-way ANOVA and two-way ANOVA respectively. The number (n) of independent animals per group is described within the graphs or in captions.

4.4 Results

4.4.1 Incubation with LXA4 and RvD1 decreases acetylcholine induced contraction in mesenteric resistance arteries from spontaneously hypertensive rats

As expected, we observed acetylcholine-induced contraction in arteries from SHR and total relaxation to acetylcholine in arteries from WKY (Figure 4-1A, B, and H). Incubation with both LXA4 and RvD1 improved these responses (Figure 4-1A-B) as indicated by the significant increase in AUC (Figure 4.1C-D). Incubation with RvE1 was not able to improve the endothelium dysfunction in SHR arteries (Figure 4-1G). There were no changes observed in PE-induced contraction in arteries from WKY, SHR, or any of the resolvin incubations (Figure 4-1E-F).

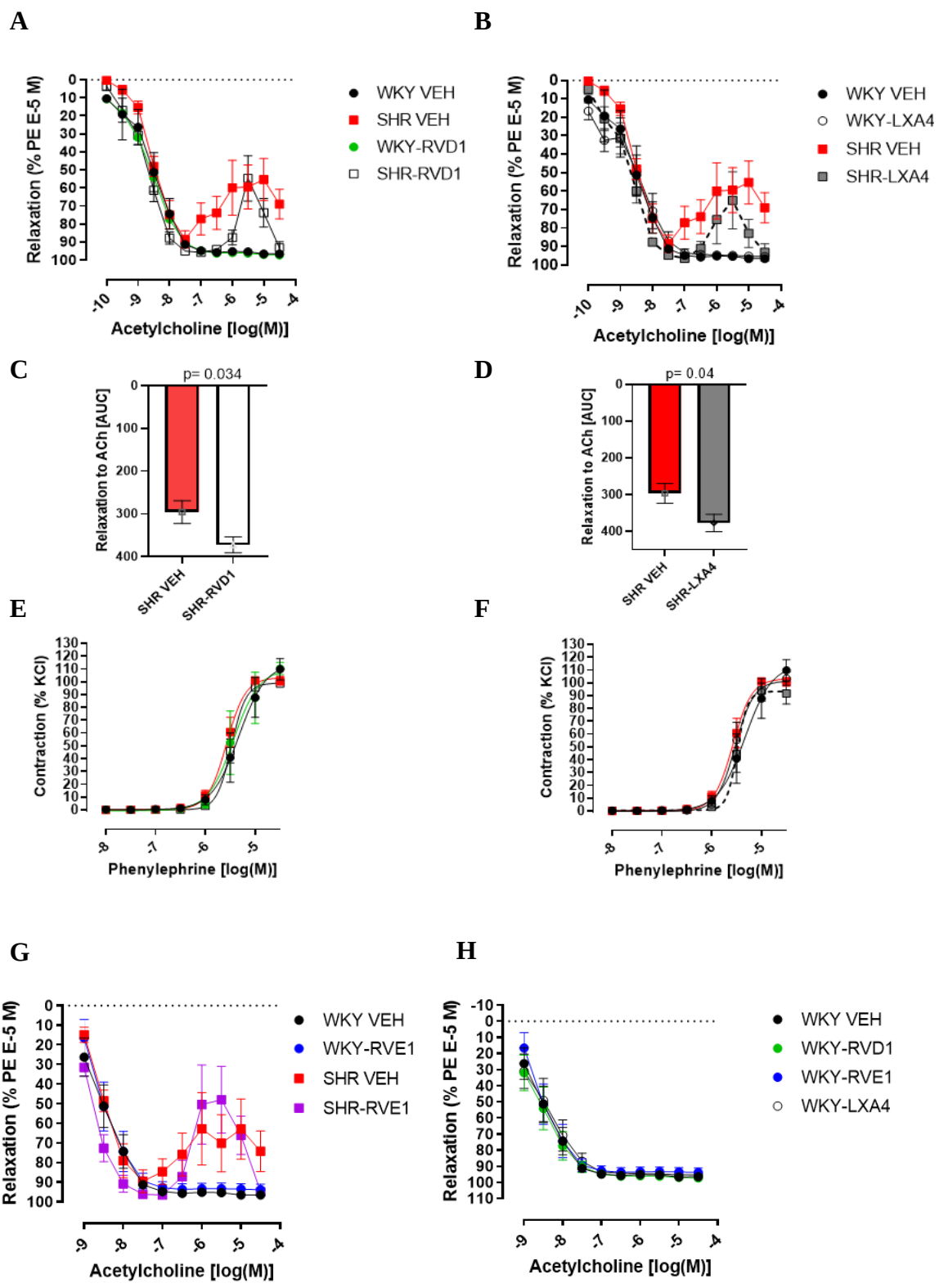
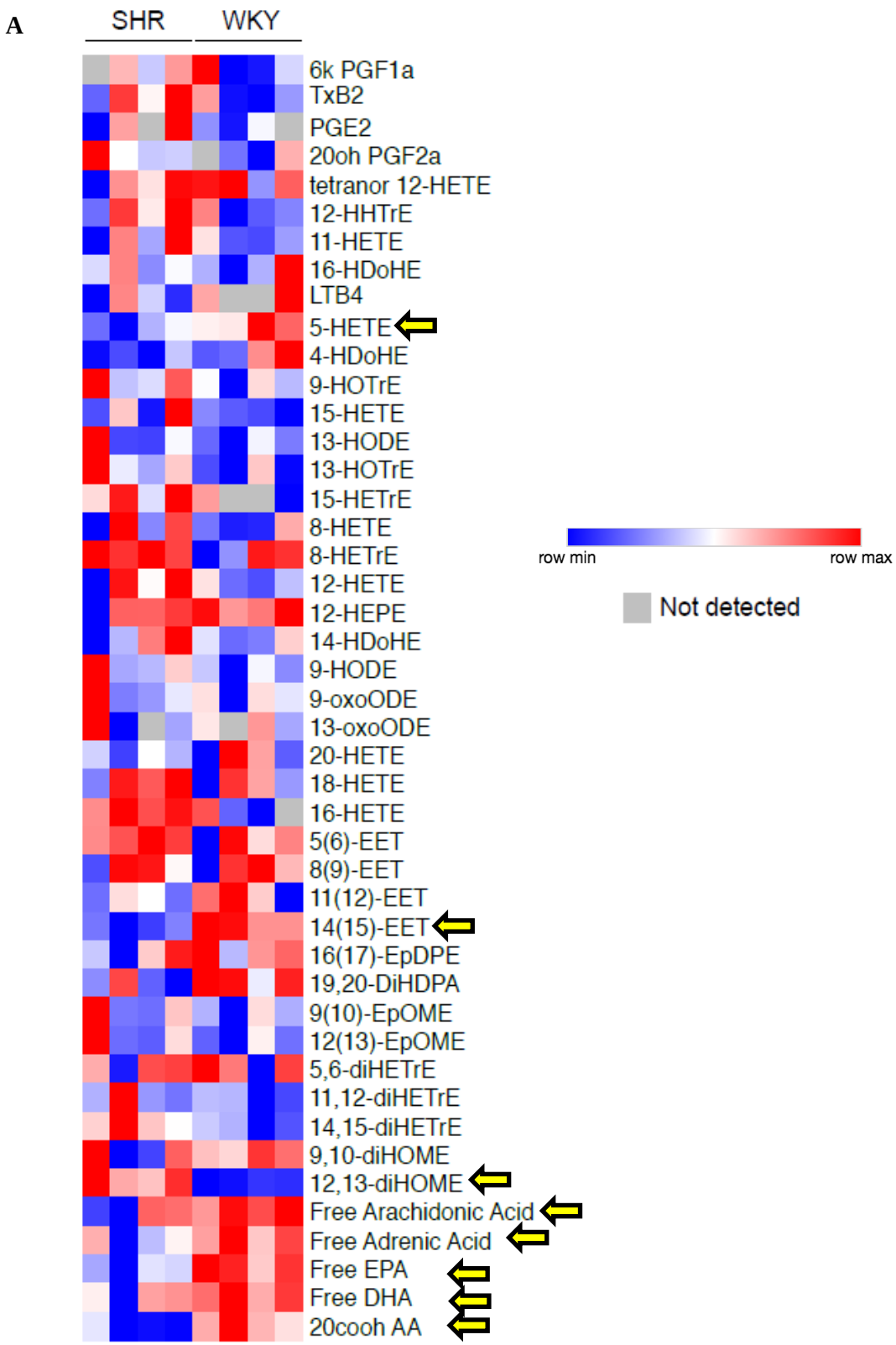

A
Relaxation (% PE E-5 M)
Acetylcholine [log(M)]
WKY VEH
SHR VEH
WKY-RVD1
SHR-RVD1
B
Relaxation (% PE E-5 M)
Acetylcholine [log(M)]
WKY VEH
WKY-LXA4
SHR VEH
SHR-LXA4
C
p= 0.034
Relaxation to ACh [AUC]
SHR VEH
SHR-RVD1
D
p= 0.04
Relaxation to ACh [AUC]
SHR VEH
SHR-LXA4
E
Contraction (% KCl)
Phenylephrine [log(M)]
F
Contraction (% KCl)
Phenylephrine [log(M)]
G
Relaxation (% PE E-5 M)
Acetylcholine [log(M)]
WKY VEH
WKY-RVE1
SHR VEH
SHR-RVE1
H
Relaxation (% PE E-5 M)
Acetylcholine [log(M)]
WKY VEH
WKY-RVD1
WKY-RVE1
WKY-LXA4

Figure 4.1. RvD1 and LXA4 decrease acetylcholine-induced contraction in resistance arteries from hypertensive rats. Concentration response curves to acetylcholine in mesenteric resistance arteries from WKY and SHR rats incubated with vehicle (VEH), RvD1, LXA4, or RvE1 **(A, B, and G)**. Arteries were precontracted with phenylephrine. Area under the curve obtained from concentration response curves to acetylcholine in mesenteric resistance arteries from SHR incubated with RvD1 **(C)** and LXA4 **(D).** Phenylephrine-induced contraction in mesenteric resistance arteries from WKY and SHR **(E and F).** Concentration response curves to acetylcholine in mesenteric resistance arteries from WKY rats incubated with vehicle (VEH), RvD1, LXA4, or RvE1 **(H).** Data presented in mean ± SEM. N=6-8 for acetylcholine concentrations response curves and AUC. N=4 for phenylephrine concentration response curves. P value <0.05. Statistics: t-test. P-value is indicated on graphs.

4.4.2 Precursors for resolvins are decreased in plasma from SHR

Given that LXA4 and RvD1 are end-products of different fatty acids and utilize different enzymatic pathways, we investigated the levels of over 150 eicosanoids in MRA and plasma from WKY and SHR. To present the level of global changes of differential eicosanoids, a heat map was generated to show the relative level of eicosanoids in WKY and SHR (**Figure 4A**). From the heat map, we chose 8 eicosanoids that showed differences to further quantify. We observed no significant difference in free AA and free DHA in plasma between the groups, but both are trending toward a decrease in SHR (**Figure 4.2B and C**). However, both free EPA and free adrenic acid are decreased in plasma from SHR (**Figure 4.2D and E**). Adrenic acid is an ω-6 polyunsaturated fatty acid (PUFA), derived

from arachidonic acid [31]. 20-COOH-AA, a metabolite of 20-HETE produced from arachidonic acid by cytochrome P450 ω-oxidases [32] , is decreased in SHR plasma (**Figure 4.2F**). Likewise, 5-HETE, an oxylipid derived from AA by 5-lipoxygenase [33-34], is also decreased in SHR plasma (**Figure 4.2G**). 14(15)-EET, a cytochrome p450 product of AA [35], is also decreased in SHR plasma (**Figure 4.2H**). Interestingly, 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DiHOME), a cytochrome P450-derived linoleic acid metabolite [36], is increased in SHR plasma (**Figure 4.2I**).


A
SHR
WKY
6k PGF1a
TxB2
PGE2
20oh PGF2a
tetranor 12-HETE
12-HHTrE
11-HETE
16-HDoHE
LTB4
5-HETE
4-HDoHE
9-HOTrE
15-HETE
13-HODE
13-HOTrE
15-HETrE
8-HETE
8-HETrE
12-HETE
12-HEPE
14-HDoHE
9-HODE
9-oxoODE
13-oxoODE
20-HETE
18-HETE
16-HETE
5(6)-EET
8(9)-EET
11(12)-EET
14(15)-EET
16(17)-EpDPE
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14,15-diHETrE
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Free Arachidonic Acid
Free Adrenic Acid
Free EPA
Free DHA
20cooh AA
row min
row max
Not detected

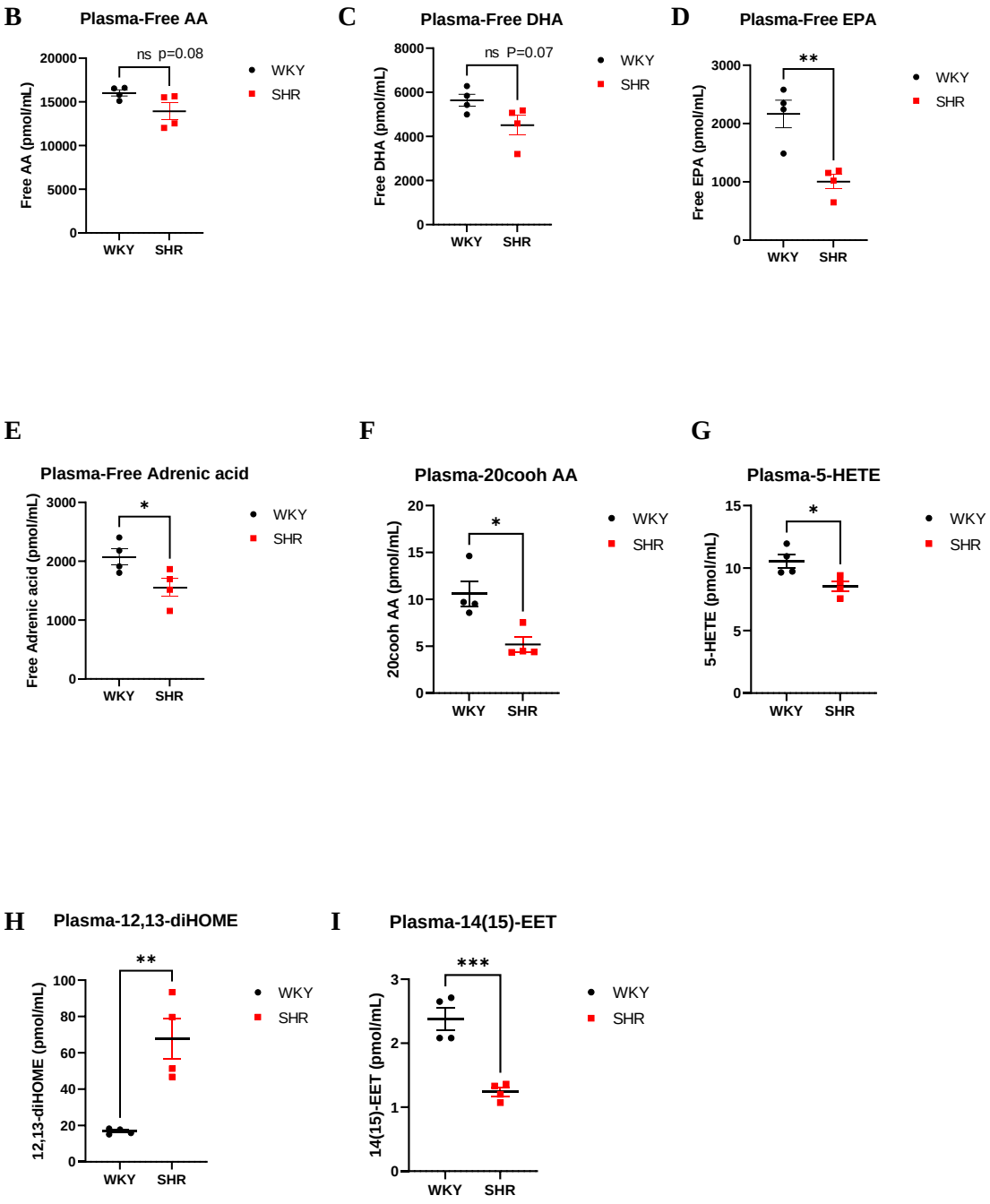

Figure 4.2. Lipidomic analysis of eicosanoids. (A)Heat map analysis of the potential biomarkers among WKY and SHR. The x-axis represents the different groups, and the y-axis represents the different eicosanoids. Yellow arrows denote eicosanoids chosen for further analysis. Polyunsaturated fatty acids, AA (**B**), DHA (**C**), EPA (**D**), and adrenic acid

(**E**) levels in plasma from WKY and SHR. Other eicosanoid intermediates 20-COOH-AA (**F**), 5-HETE (**G**), 12,13-DiHOME (**H**), and 14(15)-EET (**I**) levels are also shown in plasma from WKY and SHR. Data presented in mean ± SEM. N=3-4 for all groups. P value <0.05 unless noted on graph. Statistics: t-test; * vs. WKY.

4.4.3 Release of prostaglandin E2 and thromboxane A2 is the same in arteries from WKY and SHR

As we only observed a decrease in acetylcholine-induced contraction in arteries incubated with RvD1 and LXA4, we moved forward with investigating the mediators released from these arteries. As previously described, we infer that TxA_2 levels would be elevated in arteries from SHR to facilitate the late-phase contraction. Surprisingly, no changes were observed in the release of PGE_2 (**Figure 4.3A**) nor TXB_2 (**Figure 4.3B**).

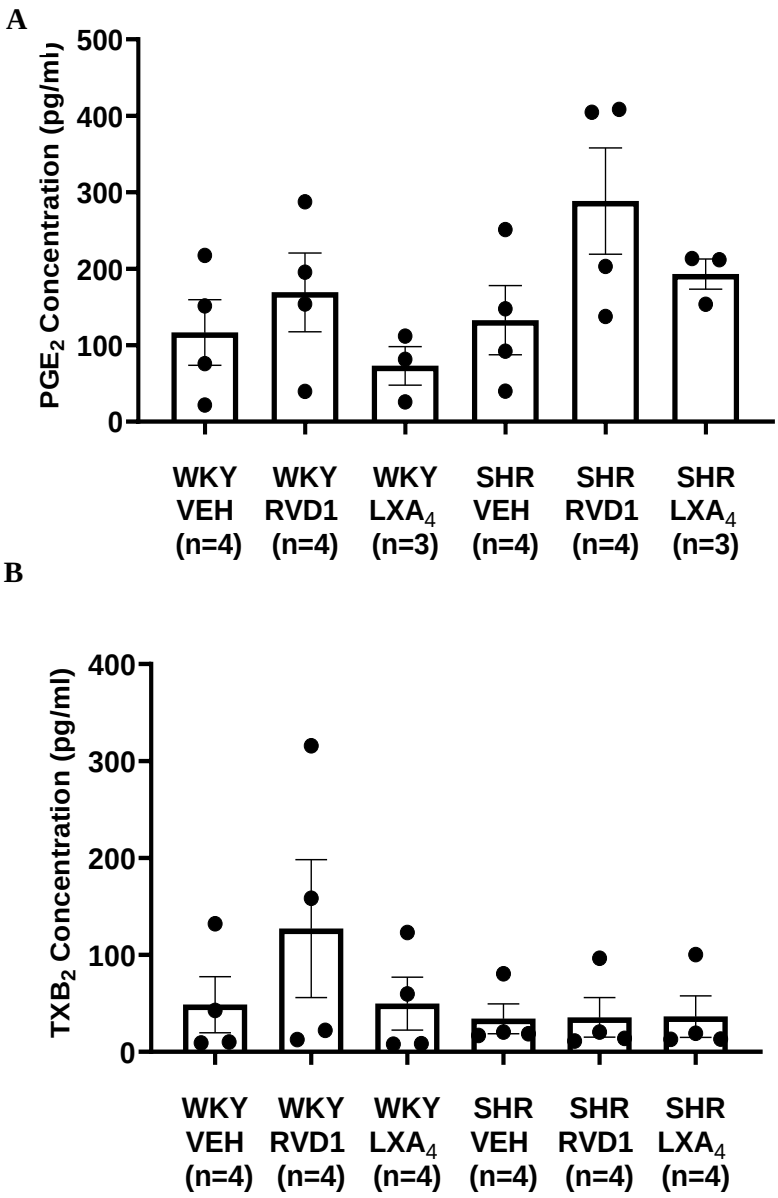

Figure 4.3. Release of prostaglandin E2 and thromboxane A2 is the same in arteries from WKY and SHR. Measurements of **(A)** prostaglandin E_2 (PGE2) and **(B)** thromboxane B_2 (TXB_2) in the supernatant from mesenteric resistance arteries from WKY and SHR incubated with vehicle, RvD1, or LXA4. Arteries were stimulated with phenylephrine and acetylcholine to mimic concentration response curves. Number of animals are indicated in the graphs. Data are presented as mean ± SEM.

4.4.4 RvD1 treatment increased NO released from hypertensive endothelial cells

One of the main vasoactive molecules produced to cause arterial dilation is NO [23] in large arteries and endothelium-dependent hyperpolarization (EDH) and NO in resistance arteries. Since the arteries from SHR decreased contraction to acetylcholine after incubation with RvD1 and LXA_4, we investigated if it was due to increase NO release and/or bioavailability from the arteries. To answer this question, we measured to nitrate/nitrite in the supernatant from resistance arteries. As nitrate and nitrite are the final products of NO in vivo, total nitrate and nitrite is the best index of total NO production [37]. There were no differences in the amount of NO released from arteries. Next, we went further and tested HAEC from hypertensive and normotensive patients themselves to investigate if there were changes in NO production. We observed that there was a substantial decrease in NO production in HAEC from hypertensive patients when compared with normotensive patients. Although the production of NO in hypertensive cells is almost nonexistent, we observed that treatment with RvD1 is trending toward increasing NO production in these cells.

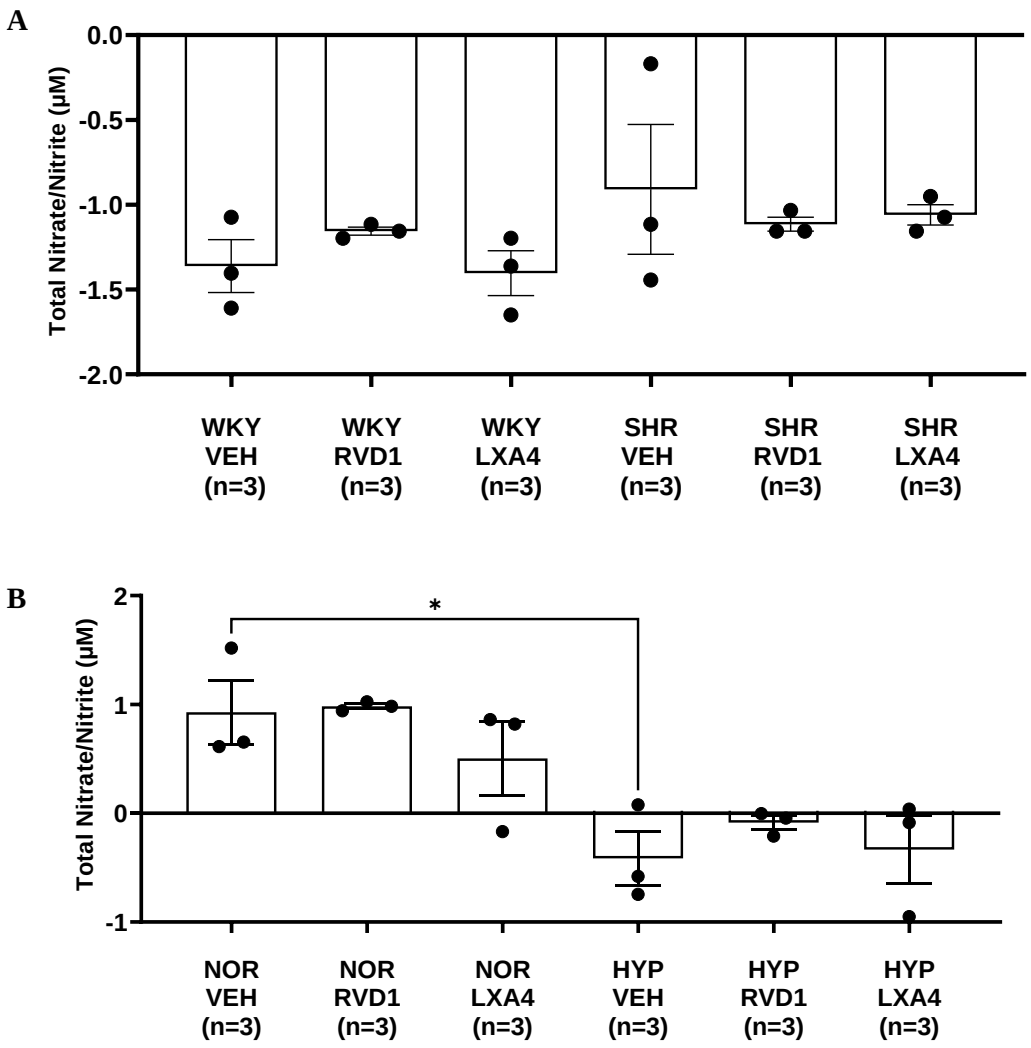

Figure 4.4. RvD1 treatment increased NO released from hypertensive endothelial cells. Measurements of total nitrite/nitrate in the supernatant of WKY and SHR mesenteric resistance arteries (**A**) and human aortic endothelial cells from normotensive (NOR) and hypertensive (HYP) donors (**B**) incubated with vehicle, RvD1, or LXA4. Number of animals or replicates are indicated on the graphs. Data are presented as mean ± SEM; * vs. NOR-VEH.

4.5 Discussion

Uncontrolled, or chronic, inflammation is associated with many chronic diseases, including hypertension. On the other hand, resolution of inflammation is an important active process that works to prevent acute inflammation from progressing to chronic inflammation. Any disruption to this process can lead to chronic inflammatory states [38]. Increased inflammatory response contributes to endothelium dysfunction by increasing endothelial permeability, cell adhesion, and production of inflammatory cytokines [39]. However, there are other aspects of endothelium dysfunction that have been attributed to pro-inflammatory molecules. The synthesis and release of TxA_2 and prostaglandins via the COX pathways and leukotrienes via the LOX pathway from AA can cause resistance arteries to contract [25]. In healthy conditions, these molecules are released but they are outnumbered by the counteracting molecules that cause arteries to relax. However, in disease, exacerbated production of pro-inflammatory molecules leads to an increase in vascular contraction [13,25].

In opposition, ω-3 polyunsaturated fatty acids produce pro-resolving, sometimes anti-inflammatory, lipid mediators that aid in reversing endothelium dysfunction [40-41]. The role of the pro-resolving molecules in the vascular function of resistance arteries has not been uncovered. This led us to question if the direct application of SPMs would improve the function of resistance arteries in hypertension, a chronic inflammatory state.

Essential ω-3 fatty acids are recommended by the American Heart Association for their beneficial cardiovascular effects [42-44]. Resolvins, derived from ω-3 polyunsaturated fatty acids, inhibit neutrophil transmigration in between endothelial cells, among other functions. Humans obtain eicosapentaenoic acid and docosahexaenoic acid

from marine oils, either be eating seafood or by supplementation [44]. Resolvins were originally isolated from aspirin treated murine dorsal air pouches and later isolated from co-cultured endothelial cells and neutrophils [45]. Aspirin treated cells expressing cyclooxygenase (COX)-2 can form resolvins transcellularly from 18R-hydroxyeicosapentaenoic acid [46].

Another SPM, LXA4, is biosynthesized from AA through 5- or 15-lipoxygenase [47]. It has potent anti-inflammatory and resolution capabilities [48]. Since LXA4 is synthesized within the vascular lumen and wall during inflammation, it is placed advantageously for modulation of vascular function. LXA4 induces endothelium-dependent relaxation in mesenteric arteries and aortic segments [49].

Corroborating these data, our study shows that LXA4 and RvD1 improve endothelium function in MRA from SHR. Incubating arteries for 1 hour with only nM concentrations, physiological concentration, of these SPMs partially inhibited the acetylcholine-induced contractions in hypertensive arteries. On the other hand, no changes were observed in phenylephrine-induced contraction. These data suggest that the beneficial effects exerted on the arteries by LXA4 and RvD1 are endothelium dependent.

After observing the beneficial effects of RvD1 and LXA4 on the arteries, we took a step back to examine whether there were differences in eicosanoid levels between WKY and SHR. We ran a full eicosanoid lipidomic panel in the plasma from both strains and found that SHR had lower levels of EPA and adrenic acid and that there was a trend towards decreased AA and DHA. This suggests that the bioavailability of these precursors to synthesize resolvins and lipoxins is lower in SHR when compared to WKY. Of note, adrenic acid has recently been implicated to block the production of leukotriene B4 by

neutrophils and enhance resolution of inflammation *in vivo* [50]. Lipidomic analysis also revealed that metabolites 20-COOH-AA, 5-HETE, and 14(15)-EET synthesized from AA were also decreased in SHR plasma regardless if they were derived by cytochrome P450 or 5-lipoyxygenase. This could be due to the decrease bioavailability of AA and/or a decrease in AA catabolism. Only one metabolite was increased in SHR plasma, 12,13-DiHOME which has been shown to increase fatty acid uptake [51-52]. This metabolite could be one of the reasons that we have observed overall decreased levels of polyunsaturated fatty acids in this study, but further studies are required to explore that possibility. Corroborating these data, it has been shown that a marked alteration in the cardiac metabolite profile with notable increases in DiHOME in wild-type hearts following LPS administration [53]. It was found that DiHOMEs triggered pronounced mitochondrial structural abnormalities, which also contributed to the development of extensive mitochondrial dysfunction in cardiac cells. Accumulation of DiHOMEs may represent an intermediate mechanism through which LPS-induced acute inflammation triggers deleterious alterations in the myocardium in vivo and cardiac cells in vitro [53]. Further, DiHOMEs were observed to cause dysfunction in endothelial cells through disruption of endothelial barrier function and inducing more IL-6 expression [54]. Furthermore, DiHOMEs seem to promote proinflammatory cascades and disrupt numerous cellular processes [54]. Therefore, increasing in 12,13-DiHOME may be associated with cardiovascular dysfunction in SHR. Regarding vascular tissue, another important point is that cytochrome P450 generates EDH, an important vasodilator factor that is increases in hypertension, to compensate reduction in the synthesis and/or bioavailability of NO. EDH induces vasodilation via activation potassium channels, and subsequently

hyperpolarization. EDH is more pronounced in small arteries, and it is increased in diseases, as a possible compensatory mechanism. All these inferences will be investigated in future studies.

The first step of resolution of inflammation is the initiation phase where polymorphonuclear neutrophils infiltrate tissue which leads to an increase in PE2 and G2 formation [55]. PGE2 is a potent proinflammatory mediator that is produced from AA by COX-2 [56]. A recent review suggested that lipid mediator imbalance leads to inflammation that enhances the production of COX-derived prostaglandins over resolvins that ultimately leads to blood pressure increases through endothelial dysfunction [57]. This led us to test the levels of prostaglandins produced by resistance arteries. Although there was not a significant increase in PGE2 levels, we did observe that both WKY and SHR resistance arteries treated with RvD1 had an overall increase in PGE2 levels which could indicate the beginning of resolution of inflammation. Depending on the situation, PGE2 can be a vasoconstrictor or a vasodilator [58]. Further studies will need to be completed to determine which effect PGE2 is eliciting in these arteries. Nonetheless, since activation of the endothelium with acetylcholine was only five minutes, it is possible that we were observing the initiation phase of resolution of inflammation in these arteries. Like PGE2, TxA_2 is a known vasoconstrictor that is exacerbated in arteries from hypertensive animals [59]. Accordingly, we questioned if resolvins would decrease the release of Txa2 in arteries from hypertensive animals. Interestingly, we were not able to detect differences between control and hypertensive animals in basal levels nor after treatment with resolvins.

After the initiation phase of resolution of inflammation, there is a lipid mediator class switch that stimulates the upregulation of 15-LOX which switched production of

proinflammatory mediators to lipoxins leading to resolution of inflammation [55]. Lipoxins and resolvins stimulate the production of endothelial NO and PGI_2. Although NO and PGI_2 have been studied in resolution of inflammation and found to be antiadhesive and antithrombotic, they are also able to cause relaxation in arteries [8, 23]. In normotension, when acetylcholine binds muscarinic receptor, NO is released which ultimately causes vascular smooth muscle to relax [25]. Conversely, in hypertension, the pathway is interrupted and leads to the production of COX-derived prostaglandins that overwhelm the vascular smooth muscle cells with calcium which ultimately causes contraction [25]. Further, an increase in ROS production leads to a decrease of NO bioavailability. Therefore, this led us to question if resolvins would ameliorate vascular function via increase of NO production and/or bioavailability. Corroborating the literature, we observed a decrease in NO production in human endothelial cells from hypertensive patients when compared to their normotensive control. However, RvD1 treatment increased NO production in human endothelial cells from hypertensive patients. These data suggest that RvD1 is restoring balance to the production of NO that could contribute to the decrease in acetylcholine-induced contraction in hypertensive resistance arteries. No changes were observed in HAEC treated with LXA_4.

4.6 Conclusion

Collectively our data show for the first time that RvD1 and LXA4 can improve endothelial function in arteries and endothelial cell from hypertensive animals and patients at nanomolar concentrations. We have also shown that the levels of free ω-3 and ω-6 polyunsaturated fatty acids are decreased in the plasma from SHR, limiting the production

of lipoxins and resolvins in vivo. Multiple downstream intermediates of AA are also decreased in SHR plasma

We were not able to uncover what the exact mechanisms are behind the improvement of endothelium function due to RvD1 and LXA4, or if these resolvins act via a receptor : 1)Different than TxA_2, the generation of ROS could be causing arteries from hypertensive animals to contract to acetylcholine and the SPMs are reverting that. 2) SPMs could be exerting anti-inflammatory effects to enhance relaxation via inhibition of secretion of TNFα and IL-1β as well as increasing the production of IL-10 and TGF-β [60], 3) the SPMs could be acting directly on receptors in the endothelium to increase the production of vasodilatory molecules, such as NO and EDH,decreasing arterial contraction. Overall, this study suggests that RvD1 and LXA4 could be revealed as a new therapeutic agent to improve vascular function in arteries from hypertensive rats.

Chapter 5

Future Directions

The first half of this dissertation examined the role of the innate immune system in microvessels from hypertensive rats. The pattern recognition receptor associated with the innate immune system, FPR-1, was shown to have a vital role in the premature elevation of blood pressure in Dahl salt-sensitive rats.

Are there sex differences in the role of FPR-1 in hypertension?

We did not include females in the present study. However, it is well established that there are sex differences in the prevalence of hypertension. Therefore, a follow up study should consider understanding whether FPR-1 activation plays a role in sex differences, vascular remodeling, and hypertension.

What is the mechanism behind the pathophysiological role of FPR-1 in hypertension?

The next logical step is to study the mechanism for FPR-1's critical role in the pathophysiology of salt-sensitive hypertension. Whether pharmacological methods are used to block FPR-1 or used to decrease NFPs' activating ability, additional studies need to be conducted to target this receptor in hypertension treatment. Second to this, a future study should be conducted to investigate the benefit FPR-1 blockade in younger animals.

We observed that CsH caused a therapeutic decrease in blood pressure in S rats, but it could also prevent an overall increase in S rats given CsH immediately after weaning. We also observed that cell death is increased in kidney cells from S rats. Future studies should investigate which kidney cells are the source of cell death that are activating FPR-1 in S rats.

In the second half of this dissertation, the promotion of the resolution of inflammation was investigate for the treatment of resistance arteries from SHR. Our results show a beneficial role for LXA_4 and RvD1 in vascular function. Also, our results show that SHR have decreased levels of eicosanoids and major precursors for SPM synthesis.

How do LXA4 and RvD1 improve vascular function in resistance arteries from SHR?

Although we were not able to uncover the mechanism of action for the improvement from SPMs in SHR arteries, there are other pathways that can be studied. It is well established that ROS are generated in hypertension and that they can cause vascular dysfunction. Accordingly, the generation of reactive oxygen species could be causing hypertensive arteries to contract to acetylcholine while SPMs are inhibiting or eliminating ROS generation. Two, SPMs could be exerting anti-inflammatory effects to enhance relaxation. Linking together both of these potential causes, a review by Renna et al suggests that oxidative stress and the activation of genes involved in inflammation are involved in vascular damage [1]. Lastly, the SPMs could be acting directly on receptors in the endothelium to increase the production of vasodilatory molecules decreasing arterial contraction. Both LXA_4 and RvD1 bind to the ALX/FPR2 receptor. RvD1 also binds to the

GPR32 receptor [2-3]. Both receptors are expressed on human endothelial cells [4-5]. These two receptors should be investigated for ligand-receptor interaction with LXA_4 and RvD1 as well as downstream signaling.

Conclusion

I hope that this dissertation will contribute to further understanding of the role that the innate immune system plays in hypertension, exclusively in the microvessels. We have identified one new potential target for blood pressure management after proper explanation of its mechanism of action. Likewise, I hope that this dissertation opens a broader view on targeting the resolution of inflammation to be a therapeutic approach for hypertension, specifically in microvessels. It will be important to investigate the systemic application of SPMs in hypertensive animals to uncover the other possible vascular benefits.

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Chapter 1

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Chapter 5

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Appendix A

Publications and Presentations

Publications:

1. **Edwards JM**, Roy S, Tomcho J, Schreckenberger Z, Chakraborty S, Bearss N, Saha P, McCarthy CG, Vijay-Kumar M, Joe B, & Wenceslau CF. Microbiota are critical for vascular physiology: Germ-free status weakens contractility and induces sex-specific vascular remodeling in mice. Vascul Pharmacol. 2020 Feb-Mar;125-126:106633. doi: 10.1016/j.vph.2019.106633. Epub 2019 Dec 13. PMID: 31843471; PMCID: PMC7036036.
2. **Edwards JM**, McCarthy CG, & Wenceslau CF. The obligatory role of the acetylcholine-induced endothelium-dependent contraction in hypertension: Can arachidonic acid resolve this inflammation? Curr Pharm Des. 2020;26(30):3723-3732. doi: 10.2174/1381612826666200417150121. PMID: 32303165; PMCID: PMC7542659.
3. Joe B, McCarthy CG, **Edwards JM**, Cheng X, Chakraborty S, Yang T, Golonka R, Mell B, Yeo J, Bearss N, Furtado J, Saha P, Yeoh B, Vijay-Kumar M, & Wenceslau CF. Microbiota introduced to germ-free rats restores vascular contractility and blood pressure. Hypertension. 2020 Dec;76(6):1847-1855. doi: 10.1161/HYPERTENSIONAHA.120.15939. Epub 2020 Oct 19. PMID: 33070663; PMCID: PMC7666075.
4. Roy S, **Edwards JM**, Tomcho J, Schreckenberger Z, Bearss N, Zhang Y, Morgan E, Cheng X, Spegele A, Vijay-Kumar M, McCarthy CG, Koch L, Joe B, & Wenceslau CF. Intrinsic exercise capacity rat models with discordant mitochondrial DNA leads to opposing vascular-associated risks. Function (Oxf). 2021;2(1):zqaa029. doi:

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5. Ashraf UM, Abokor AA, **Edwards JM**, Waigi E, Royfman RS, Hasan SA, Smedlund KB, Hardy AM, Chakravarti R, & Koch LG. SARS-CoV-2, ACE2 expression, and systemic organ invasion. Physiol Genomics. 2021 Feb 1;53(2):51-60. doi: 10.1152/physiolgenomics.00087.2020. Epub 2020 Dec 4. PMID: 33275540; PMCID: PMC7900915.
6. **Edwards JM**, Roy S, Galla S, Tomcho J, Bearss N, Waigi E, Mell B, Cheng X, Saha P, Vijay-Kumar M, McCarthy CG, Joe B, & Wenceslau CF. Formyl Peptide Receptor-1 Activation Promotes Premature, Spontaneous Hypertension in Dahl Salt-Sensitive Rats. Hypertension. 2021 Apr;77(4):1191-1202. doi: 10.1161/HYPERTENSIONAHA.120.16237. Epub 2021 Mar 1. PMID: 33641367; PMCID: PMC7946782.
7. Aradhyula V, Waigi E, Bearss NR, **Edwards JM**, Joe B, McCarthy CG, Koch LB, Wenceslau CF. Intrinsic exercise capacity induces divergent vascular plasticity via arachidonic acid-mediated inflammatory pathways in female rats. Vascul Pharmacol. 2021 Apr 16:106862. doi: 10.1016/j.vph.2021.106862. Epub ahead of print. PMID: 33872803.
8. Cheon S, Tomcho JC, **Edwards JM**, Bearss NR, Waigi EW, Joe B, McCarthy CG, & Wenceslau CF. Opioids cause sex-specific vascular remodeling via Cofilin-ERK signaling: Female mice present higher risk of developing morphine-induced vascular dysfunction than male mice. J. Vas. Res. Accepted 2021 June 2.

Presentations:

Published Abstracts:

1. Selected for Oral: **Edwards, J.,** Galla, S., McCarthy, C.G., Bearss, N., Roy, S., Joe, B. and Wenceslau, C.F. (2019), Activation of Formyl Peptide Receptor Precedes the Onset of Hypertension in Dahl Salt Sensitive Rats: Effects of Microbiota and Salt. The FASEB Journal, 33: 836.7-836.7. https://doi.org/10.1096/fasebj.2019.33.1_supplement.836.7.

2. Selected for poster: **Edwards, J.,** Waigi, E., McCarthy, C., Joe, B. and Wenceslau, C. (2021), Pro-Resolving Lipid Mediators Reduce Acetylcholine-Induced Contractions in Resistance Arteries from Hypertensive Rats. The FASEB Journal, 35:. https://doi.org/10.1096/fasebj.2021.35.S1.03567.

Invited Speaker:

1. **Competitively selected for Oral Presentation, Gull Lake Hypertension Meeting** Gull Lake, Michigan 05/03/2019 "Mitochondrial and Bacterial N-formyl Peptides Activate Formyl Peptide Receptor Prior to the Onset of Hypertension in Dahl Salt Sensitive Rats"
2. **Competitively selected for Oral Presentation, American Heart Association-Council on Hypertension Meeting** New Orleans, Louisiana 09/08/2019 "Low-Grade Chronic Infection Induces Vascular Dysfunction and Remodeling in Salt Sensitive Hypertension"
3. **Competitively selected for Oral Presentation, American Heart Association-Council on Hypertension Meeting** Virtual-09/13/2020 "Formyl Peptide Receptor-1 Activation is Crucial for the Cause of Spontaneous Hypertension in Dahl Salt Sensitive Rats"
4. **Competitively selected for Oral Presentation, University of Toledo Graduate Research Forum: Placed Second.** Virtual-03/25/2021 "Pro-Resolving Lipid Mediators Reduce Acetylcholine-Induced Contractions in Hypertensive Resistance Arteries"

Poster Presentations:

1. **Graduate Research Forum,** University of Toledo College of Medicine and Life Sciences. 03/20/2019. "Increased Circulating Levels of Mitochondrial Fragments Leads to Innate Immune System Activation Prior to the Onset of Hypertension in Dahl Salt Sensitive Rats."
2. **Experimental Biology 2019.** Orlando, Florida. 04/09/2019. "Activation of Formyl Peptide Receptor Precedes the Onset of Hypertension in Dahl Salt Sensitive Rats: Effects of Microbiota and Salt."
3. **National Science Foundation-National AGEP Conference,** Virtual. 03/11/2021. "Formyl Peptide Receptor-1 Activation is Crucial for the Spontaneous and Salt-Induced Hypertension in Dahl Salt Sensitive Rats: Mitochondria vs. Microbiota."

4. **Experimental Biology 2021**. Virtual. 4/27/21. “Pro-Resolving Lipid Mediators Reduce Acetylcholine-Induced Contractions in Resistance Arteries from Hypertensive Rats.”

Press Release:

1. Story in the Toledo Blade newspaper, Jonnelle M. Edwards, “UT research aims to get blood pressure under control.” November 2020. Press. Web.