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Effects of Raspberry Consumption on Angiotensin II-induced Hypertension and Expression of Antioxidant and Pro-oxidant Enzymes in the Brain of Rats

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This thesis: Effects of Raspberry Consumption on Angiotensin II-induced Hypertension and Expression of Antioxidant and Pro-oxidant Enzymes in the Brain of Rats

by Jasmynne Blacks

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induced oxidative stress in the subfornical organ in male Sprague-Dawley rats. *Curr Dev Nutr.* 2021;5(Suppl 2):298.

2. **Blacks J**, Najjar RS, Simecka C, Mu S, Feresin RG. Effects of raspberry on angiotensin II-induced oxidative stress, inflammation, and fibrosis in the heart of mice. *Curr Dev Nutr.* 2020;4(Suppl 2):370.

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ABSTRACT

Effects of Raspberry Consumption on Angiotensin II-Induced Hypertension and Expression of Antioxidant and Pro-Oxidant Enzymes in The Brain of Rats

By:

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Background: Angiotensin (Ang) II is a potent vasoconstrictor and inducer of oxidative stress. The subfornical organ (SFO) and organum vasculosum of lamina terminalis (OVLT) are circumventricular organs that lack the blood brain barrier and therefore are prone to oxidative stress. Raspberries (RB) are rich in polyphenols which have been shown to have great antioxidant capacity. Thus, the objective of this study is to determine whether RB mitigates blood pressure (BP) increases, attenuates the expression of pro-oxidant enzymes, and increases the expression of antioxidant enzymes induced by angiotensin Ang II in the SFO and OVLT of Ang-II infused rats.

Methods: Sprague Dawley rats were fed a diet with or without 10% w/w freeze-dried RB for seven weeks. At week 4, rats were implanted with subcutaneous osmotic minipumps that delivered 0.9% saline (Control) or Ang II (270 ng/kg body weight/min) for another three weeks. BP was measured at weeks 4, 5 and 6. Animals were sacrificed, and brains excised and stored for later analysis. Protein expression of NADPH oxidases, NOX2, NOX4, as well as superoxide dismutase (SOD) 1 and SOD2 were measured in the SFO and OVLT.

Results: Although not significantly, Ang II increased systolic BP while RB supplementation attenuated this increase. In the SFO, Ang II increased NOX2 and NOX4 expression. RB supplementation attenuated the increase in NOX2 expression. Ang II increased SOD1 while decreasing the SOD2 expression. RB supplementation increased SOD1 compared to control. Similarly, in the OVLT, Ang II increased NOX4 expression compared to control; however, no other significant changes were observed. **Conclusion:** Our preliminary findings suggest that RB may attenuate the Ang II-induced increases in BP and oxidative stress in the SFO. Future investigations are warranted to elucidate these effects.

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EFFECTS OF RASPBERRY CONSUMPTION ON ANGIOTENSIN II-INDUCED HYPERTENSION AND EXPRESSION OF ANTIOXIDANT AND PRO-OXIDANT ENZYMES IN THE BRAIN OF RATS

by

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ABBREVIATIONS

HTN Hypertension

ANG II Angiotensin II

RAAS Renin Angiotensin Aldosterone System

AT_1R Angiotensin Type 1 Receptor

AT_2R Angiotensin Type 2 Receptor

ROS Reactive Oxygen Species

NOX NAPDH Oxidases

SOD1 Superoxide Dismutase 1

SOD2 Superoxide Dismutase 2

SOD3 Superoxide Dismutase 3

BBB Blood Brain Barrier

CVO Circumventricular Organs

OVLT Organum Vasculosum of Lamina Terminalis

SFO Subfornical Organ

DBP Diastolic Blood Pressure

SBP Systolic Blood Pressure

MAP Mean Arterial Pressure

SHRs Spontaneously Hypertensive Rats

CHAPTER I

INTRODUCTION

Hypertension (HTN) is a major public health concern, accounting for 9.4 million deaths each year globally.[1, 2] In the United States (U.S.) alone, HTN affects an estimated 65 million individuals and contributes to the deaths of as many as 360,000 Americans every year.[2] There are various modifiable and non-modifiable risk factors that play into the development of HTN, and they arise from a combination of environmental, behavioral, and dietary factors.[3]

Angiotensin II (Ang) II is a vasoconstrictor and product of the renin angiotensin aldosterone system (RAAS). A dysregulation of the RAAS is closely tied to the development of HTN. [4, 5] Ang II increases sodium reabsorption and decreases sodium excretion by the kidneys via aldosterone and vasopressin. Synthesis of Ang II begins with the synthesis and processing of prorenin in the kidney's juxtaglomerular cells, eventually leading to the release of active renin into systemic circulation. [5] Renin is used to cleave angiotensinogen, which is formed by the liver, into Ang I. Lastly, angiotensin converting enzyme (ACE) produced by the lungs will cleave Ang I into Ang II. [5 6]

Ang II plays a critical role in the pathophysiological modulation of cardiovascular functions by binding to its receptors. Angiotensin type 1 receptor (AT_1R) AT_1R regulates a variety of Ang II effects, including vasoconstriction and increased blood pressure.[7] Angiotensin type 2 receptor (AT_2R) is believed to uphold opposing effects of AT_1R through vasodilation and hypotension.[7]High levels of Ang II can contribute to the overproduction of reactive oxygen species (ROS). [8] ROS plays an important role in immunity, cell growth, and cell signaling but in excess is lethal to cells, leading to a multitude of diseases.[8] This overproduction is known as oxidative stress which is an imbalance between the production of ROS and antioxidant defenses in your body.[8] Both exogenous and endogenous antioxidants counter ROS and may neutralize oxidative stress. Its overall effect is to increase blood pressure, body water, and sodium content.

Ang II-AT_1R binding creates a cascade of events, including activating NAPDH oxidases (NOX), leading to elevated levels of oxidative stress and formation of free radicals. [8] Excessive ROS, especially O_2^- play important roles in the pathogenesis of many cardiovascular diseases, including HTN. Ang II-AT_1R binding also reduces the expression of antioxidant systems such as superoxide dismutase's (SOD) which are detrimental in protection. SODs are the major antioxidant defense systems against O_2^- by catalyzing the conversion of $O_2^{\cdot -}$ to H_2O_2. SOD contains three isoforms, Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and extracellular Cu/ZnSOD (SOD3). [9, 10]

In exploration of the development and impact of oxidative stress in the brain we will focus on two key regions, organum vasculosum of lamina terminalis (OVLT) and subfornical organ (SFO). These regions are structurally unique as they lack the blood brain barrier (BBB), characterizing them as circumventricular organs (CVOs).[11, 12] The BBB

plays a critical role in protecting the brain and restricting access of Ang II.[4] The lack of a BBB allows the neurons in these regions to respond to osmotic pressure factors present in systemic circulation functioning in osmoregulation, cardiovascular regulation, and energy homeostasis. This can lead to a high response in the overproduction of Ang II affecting the brain, leading to high levels of ROS.[11, 12]

Red raspberries, are a common and important fruit in the western diet due to their content of essential nutrients and beneficial phytochemicals.[13] Their unique polyphenol profile and nutrient composition makes them a potential therapeutic method to increase antioxidants and reduce prooxidants in those regions that are more susceptible to high levels of circulating Ang II. The effects of raspberries on the brain, particularly those lacking the protective BBB, including the SFO and OVLT, in an Ang II-induced hypertensive model have not been previously evaluated. Thus, the objective of this study is to investigate whether raspberries can reduce blood pressure and attenuate the expression of cellular redox enzymes in the SFO and OVLT of rats in a model of Ang II-induced hypertension. We hypothesize that raspberry consumption will reduce the oxidative burden induced by Ang II by decreasing expression of pro-oxidant enzymes and increasing expression of antioxidant enzymes in regions of the brain while also mitigating the hypertensive effects of Ang II. To test this hypothesis, we propose the following specific aims:

Specific Aim 1: To assess whether raspberry supplementation attenuates blood pressure increases induced by chronic Ang II infusion. Sprague-Dawley rats will be fed a raspberry supplemented diet for four weeks prior to beginning Ang II infusion for an additional three

weeks. Blood pressure will be assessed at prior to Ang II infusion (week 4) and weeks 5 and 6.

Specific Aim 2: To determine whether raspberry consumption attenuates the expression of pro-oxidant enzymes while increasing the expression of antioxidant enzymes in the SFO and OVLT of Ang II-infused rats. Following the experimental period described in Aim 1, brains will be excised, and protein expression of NADPH oxidases and antioxidant enzymes (SOD) will be assessed via western blot.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Hypertension: Epidemiology and Classification

Hypertension (HTN) is a major public health concern, accounting for 9.4 million deaths each year globally.[1, 2] In the United States (U.S.) alone, HTN affects an estimated 65 million individuals and contributes to the deaths of as many as 360,000 Americans every year.[2] The global prevalence of HTN is believed to increase by 60% over the next decade despite the advancements in awareness.[14] HTN is characterized by a chronic elevation in arterial pressure and is a major risk factor for many common causes of morbidity and mortality, including: stroke, myocardial infarction, congestive heart failure, and end-stage renal disease.[15]

Blood pressure can be represented via systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP).[1] SBP represents contraction of the heart, while DBP represents relaxation.[16] Mean arterial pressure is the average arterial pressure throughout a cardiac cycle and is influenced by cardiac output and systemic vascular resistance.[14] Measuring blood pressure with a sphygmomanometer is the most common way to diagnose HTN. The American Heart Association (AHA) recommends SBP and DBP be less than 120/80 mm Hg and for regular blood pressure screenings if 20 years or older.[17] Nearly half of American adults have HTN; however, many do not even know they have it. Blood Pressure can be characterized as depicted in Table 1.

Table 1: Blood Pressure Categories

Blood Pressure Categories	Systolic, mm Hg		Diastolic, mm Hg
Normal	< 120	and	< 80
Elevated	120-129	and	< 80
High Blood Pressure (HTN) stage 1	130-139	or	80 - 89
High Blood Pressure (HTN) stage 2	≥ 140	or	≥ 90
Hypertensive Crisis	> 180	and/or	> 120

2.2 Etiology and Pathophysiology of Hypertension

There are various modifiable and non-modifiable risk factors that play into the development of HTN and they come from a combination of environmental, behavioral, and dietary factors.[3] Non-modifiable contributing factors include family history, age, gender, and race.[18] Modifiable factors are lack of physical activity, sedentary lifestyle, excessive sodium intake, excessive alcohol consumption, high cholesterol, diabetes, low potassium intake, low calcium intake, smoking/tobacco use, depression, and stress. [3, 18]

A dysregulation of the Renin Angiotensin Aldosterone System (RAAS) is closely tied to the development of HTN. RAAS plays an important physiological function in the regulation of water and electrolyte balance, systemic vascular resistance, blood pressure and cardiovascular homeostasis. [4, 5] Angiotensin (Ang) II is a major byproduct of RAAS and is implicated in driving HTN. Synthesis of Ang II begins with the synthesis and processing of prorenin in the kidneys juxtaglomerular cells, eventually leading to the release of active renin into systemic circulation.[5] Renin is used to cleave angiotensinogen, which is formed by the liver, into Ang I. Lastly, angiotensin converting enzyme (ACE) produced by the lungs will cleave Ang I into Ang II.[5] Ang II is a hormone which increases sodium reabsorption and decreases sodium excretion by the kidneys via aldosterone and vasopressin. The adrenal cortex, responsible for producing aldosterone known for salt

balance in the blood is located on top of the kidney. Ang II will act on the adrenal cortex releasing aldosterone, a hormone that causes the kidneys to retain sodium and lose potassium. [6]

2.3 Angiotensin II in the Vasculature

Angiotensin type 1 receptor (AT_1R) is the primary receptor known for mediating majority of the physiological actions of Ang II. Ang II causes hypertension by stimulating vasoconstriction and sodium reabsorption in the kidneys. Ang II increases NADPH oxidase (NOX) activity in endothelial cells (ECs) enhancing reactive oxygen species (ROS) production primarily via AT_1R. ROS production promotes inflammatory signaling via mitogen activated protein kinase (MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). [19]

Angiotensin type 2 receptor (AT_2R) is another Ang II receptor; however, it is believed to have protective effects. Evidence has shown this receptor opposes functions mediated by AT_1R, [4,5,20] as AT_2R promotes vasodilation, natriuresis, antiangiogenesis, antiproliferation, and decreases fibrosis.[4,5] This receptor is highly expressed within the fetus and decreases to low levels soon after birth, with AT_1R being the dominant receptor. If Ang II binds to AT_2R, it will inhibit the sodium hydrogen exchange pump (NA^+/H^+ exchange), leading to high levels of hydrogen in the vascular smooth muscle cells (VSMCs), promoting bradykinin-mediated dilation.[20,21] However, AT_2R also increases endothelial nitric oxide synthase (eNOS) activity leading to more nitric oxide (NO) production in ECs. NO opposes the effects of endothelium-derived vasoconstrictors by diffusing to the VSMCs and activating guanylate cyclase (GC). Guanylate Cyclase is an enzyme that converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate

(cGMP) and pyrophosphate and when activated GC will increase intracellular cGMP, causing vasodilation.[21]

2.4 Circumventricular Organs (CVOs)

The BBB contains blood vessels that tightly regulate the movement of ions, molecules, and cells between the blood and the brain. Vessels deliver oxygen and nutrients to a variety of tissues and organs, and the BBB are the blood vessels that surround the central nervous system. This tight regulation also protects the brain toxins and pathogens.[22]

Found in the midline of the brain and grouped around the third and fourth ventricles, CVOs are growing in research interest.[23] CVOs are structurally unique regions of the brain that lack the BBB, a critical component to protect the brain and restrict access of Ang II to the brain.[4, 24] There are seven major CVOs divided into two forms, secretory and sensory. Secretory CVOs include, the pineal gland, median eminence, neurohypophysis, and subcommisural organ, while the sensory CVOs include the SFO, area postrema, and the OVLT.[24] Functionally, sensory organs are defined as they contain neurons that can receive chemical inputs from the bloodstream and secretory organs are responsible for secreting hormones and glycoproteins into the peripheral blood using feedback from both the brain and external stimuli.

In exploration of the impact of systemic Ang II infusion in the brain, we will focus on two key regions, OVLT and SFO.

2.4.1 Organum Vasculosum of the Lamina Terminalis (OVLT)

Within the lamina terminalis is an important CVO known as the organum vasculosum of lamina terminalis, also referred to as the vascular organ of lamina terminalis or supraoptic crest (Figure 1). This is one of the four sensory CVOs providing information

to other brain regions. The OVLT forms the anterior wall of the third ventricle. The third ventricle has an anterior, posterior, medial, and lateral side. In-between the optic chiasm and the anterior side of the third ventricle is the lamina terminalis and the OVLT lies in front of the lamina terminalis. Capillaries of the OVLT do not have a BBB; however, neurons of the OVLT are involved in controlling thirst, blood volume regulation, and vasopressin release. The lack of a BBB allows the neurons in this region to respond to osmotic pressure factors present in systemic circulation. It functions as an osmoreceptor and contains high densities of AT_1R. If osmolarity of the serum rises, it will communicate with the supraoptic nucleus, causing it to release antidiuretic hormone (ADH). Secretion of renin by the kidneys leads to secretion of Ang II, which leads to stimulation of OVLT receptors.[11]

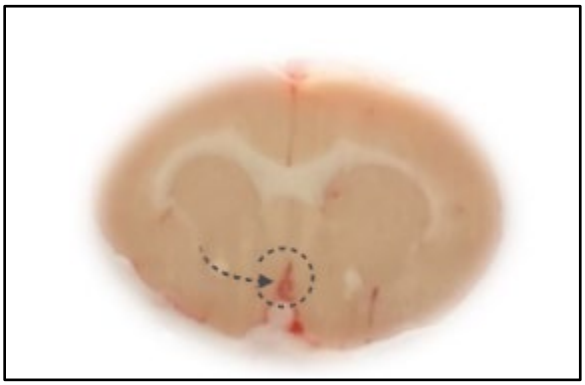

Figure 1: Coronal View of the Organum Vasculosum of Lamina Terminalis (OVLT). Created with Biorender.com.

2.4.2 Subfornical Organ (SFO)

The SFO is located on the inferior surface of the anterior column fornix, which interconnect the lateral and third ventricles (Figure 2). The SFO expresses AT_1R, therefore, when these receptors are stimulated by Ang II, they will communicate with the supraoptic nucleus, causing it to release ADH. The SFO also communicates with the OVLT, which

also contains the AT_1R and mineralocorticoid receptor (MR) that can relay the signals of circulating Ang II to downstream nuclei such as the PVN and RVLM. The SFO plays a role in many bodily processes including osmoregulation, cardiovascular regulation, and energy homeostasis. This region is well-vascularized and like all CVOS its capillaries have fenestrations, thus lacking a BBB and increasing capillary permeability. The lack of a BBB makes the SFO and other CVOs structurally unique. The SFO is also considered a sensory CVO due to its responsiveness to a wide variety of hormones and neurotransmitters, opposed to secretory organs. Neurons within the SFO have receptors for many hormones that circulate in the blood and do not cross the BBB such as Ang II and atrial natriuretic peptide (ANP). Some of these neurons are osmoreceptors, being sensitive to the osmotic pressure of the blood.[12, 25]

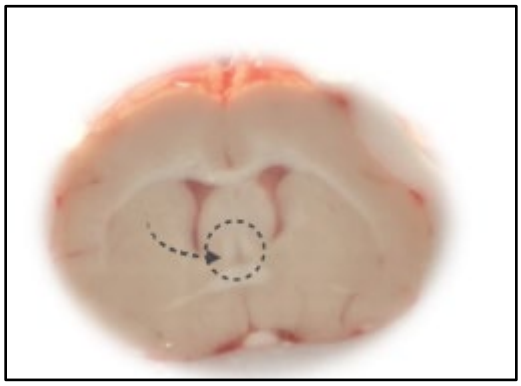

Figure 2: Coronal View of the Subfornical Organ (SFO). Created with Biorender.com.

2.5 Ang II and Oxidative Stress

Evidence has accumulated regarding Ang II-induced reactive oxygen species (ROS) generation in the pathogenesis of hypertension [26] ROS plays important roles in immunity, cell growth, and cell signaling.[8] It is needed for normal body functions, but in excess ROS is lethal to cells and leads to a multitude of diseases. This overproduction is

known as oxidative stress.[27] Oxidative stress is an imbalance between the production of ROS and antioxidant defenses in your body. The brain is particularly prone to oxidative damage due to its high level of oxygen consumption often resulting in chronic inflammatory responses that can contribute to neuronal damage. [28] Ang II plays a critical role in the pathophysiological modulation of cardiovascular functions and in excess it can contribute to the overproduction of ROS.[8] Its overall effect is to increase blood pressure, body water and sodium content. By Ang II binding to AT_1R, it creates a cascade of events, including activating NOX, leading to elevated levels of oxidative stress and formation of free radicals.[8] NOX is currently viewed as the predominant source of Ang II-derived ROS production in the brain. [26]

NOX participates in the reaction of electron transfer from NADPH to oxygen molecule to produce superoxide anions (O_2^-) or hydrogen peroxide (H_2O_2). NOX1, NOX2, and NOX4 play diverse roles in cardiovascular diseases. NOX2 AND NOX4 are homologues abundantly expressed in forebrain CVOs.[29] ACE is a major component of RAAS, and ACE inhibitors have been found to be beneficial in reducing morbidity and mortality in HTN. ACE is believed to be the primary enzyme leading to Ang II production. ACE homologues include ACE1 and ACE2.[7, 30] ACE1 converts the hormone Ang I to the active vasoconstrictor Ang II. ACE2, a carboxypeptidase that is insensitive to ACE1, cleaves one amino acid from either Ang I or Ang II, decreasing circulating Ang I levels, and increasing the metabolite Ang (1-7), which binds to the Mas receptor and is believed to be beneficial in cardiovascular diseases[7, 30] and considered a putative target for the development of new cardiovascular drugs. [7, 30]

Excessive ROS, especially O_2^- play important roles in the pathogenesis of many cardiovascular diseases, including HTN. Ang II binding to AT_1R reduces the expression of antioxidant systems such as superoxide dismutase's (SOD) which are detrimental in protection. SODs are the major antioxidant defense systems against O_2^- by catalyzing the conversion of $O_2^{\cdot -}$ to H_2O_2. SOD contains three isoforms, Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and extracellular Cu/ZnSOD (SOD3). SOD1 is the major intracellular SOD. SOD1 is mainly localized in the cytosol, smaller fraction in the mitochondria, nuclei, lysosomes, peroxisomes, and a white distribution in a variety of cells. Overexpression of SOD1 can elevate H_2O_2 levels, leading to toxicity. SOD2 is a mitochondrial manganese containing enzyme and localized in the mitochondrial matrix. It is synthesized in the cytoplasm and its active site shows no relation to SOD1.[9] Unlike SOD1, SOD2 does not exhibit product inhibition by H_2O_2. SOD3, otherwise known as extracellular SOD, is the most important SOD in extracellular including the lymphatic fluid, synovial fluid, and plasma.[10]

Nuclear factor related factor 2 (Nrf2) is a nuclear transcription factor, and its activity increases antioxidant enzyme espression.[31] Heme oxygenase-1 (HO-1) is a Nrf2-regulated enzyme that plays a critical cytoprotective role under conditions of cell stress. This form of HO is responsible for the oxidative cleavage of heme groups, and has antioxidant and anti-inflammatory effects. [32] NAD(P)H dehydrogenase quinone 1 (NQO1) has SOD like activity and work on detoxification pathways that are induced by many stress conditions, including oxidative stress. The protection against oxidative stress may involve its substrates including UBQ, vitamin E quinone, and superoxide. Glutathione peroxidase

1 (GPx1) functions in the detoxification of H_2O_2 to H_2O. It is widespread in many tissues, including the brain where it protects cells from oxidative stress.

2.6 Therapeutic Modalities of Hypertension

Pharmacological therapies to treat HTN typically target RAAS, including, ACE inhibitors (ACEIs), Ang receptor blockers (ARBs), and direct renin inhibitors. ACEIs are known for antagonizing RAAS by preventing the conversion of Ang I to Ang II, and can actively reduce sympathetic outflow, while ARBs prevent Ang II from binding to AT_1R [33]. Additionally, calcium channel blockers and diuretics, may also be used.[33] Calcium channel blockers and diuretics are both direct renin inhibitors that are most effective in volume-mediated HTN. Calcium channel blockers can lower blood pressure by preventing calcium from entering smooth muscle cells in the heart and arteries. Diuretics, otherwise known as water pills, helps your body to eliminate sodium and water by increasing urine output, thereby reducing blood pressure.

Typically, in neurogenic HTN, renal efferent nerves stimulate renin secretion and inhibit sodium excretion, therefore, it is believed blood pressure should respond to agents such as ACEIs and ARBs. However, there has been a failure to effectively control blood pressure in patients with resistant HTN, high blood pressure that does not respond well to aggressive medical treatment. Additionally, α- and β-adrenergic receptor blockers block the effects of sympathetic stimulation of the two limbs of the sympatho-adrenal system, the adrenal limb that stimulates adrenal secretion of epinephrine, and the neural limb that stimulates secretion of norepinephrine at sympathetic nerve endings in arterial walls.[33] Additionally, β-blocker monotherapy lowers blood pressure by inhibiting renin secretion.[33]

Targeted inhibition of inflammatory and oxidative stress-associated proteins downstream of AT_1R are also of therapeutic relevance. Ablation of the NOX subunit p22(phox) from the SFO prevents Ang II-induced inflammatory responses in the peripheral vasculature. [28] Further, NF-κB inhibition in the PVN also abrogates ROS production, which reduces inflammation in the hypothalamus and attenuates Ang II-dependent HTN.[34] Animal studies indicate that systemic administration of ARB has anti-hypertensive effects and also prevent SNS hyperactivity, reducing heart rate and blood pressure.[35]

Even though we have all these medications to control blood pressure, the American Heart Association as well as the American College of Cardiology recommend the first line of treatment for pre- or stage 1-hypertensive individuals to be lifestyle changes. This includes dietary changes such as decreasing sodium intake and increasing intake of fruits and vegetables, exercising, stop drinking and smoking, and weight loss in the case of overweight or obese individuals.

2.7 Dietary Therapies in Hypertension

The possibility of certain foods and their bioactive compounds to reverse or prevent the progression of various diseases has attracted significant research interest.[36] Dietary guidelines around the world recommend the increased consumption of fruits and vegetables.[13] Consuming a diet rich in fruits and vegetables is associated with a reduced risk of several age and lifestyle related diseases, including HTN.[36]

A study consisting of 71,910 female and 37,725 male participants were surveyed for their fruit and vegetable consumption in relation to overall health. A food frequency questionnaire (FFQ) was used to collect and quantify consumption of fruits and vegetables by participants and the study found that total fruit and vegetable intake was inversely

associated with risk for cardiovascular disease.[37] An analysis including 134,796 Chinese adults in Shanghai was completed in a prospective cohort study. They also utilized a validated FFQ and assessed baseline through in-person interviews. Overall, it was found the fruit and vegetable intake, especially in the intake of cruciferous vegetables, was inversely associated with risk of total mortality in both women and men in a dose response manner.[38]

Another prospective population-based cohort study was conducted from 1993 and 1997 with 20,069 men and women aged 20 to 65 yrs. free of cardiovascular diseases at baseline. A validated FFQ was used in this study and the mean follow up was 10.5 yrs. The risk of CHD incidence was 34% lower for participants with high intake of raw and processed fruit and vegetables compared to the participants with low consumption.[39] Lastly, in the United States 9,608 adults aged 25-74 yrs. free of cardiovascular diseases participated in a National Health and Nutrition Examination Survey Follow-up study. Baseline data were collected between 1971 to 1975 and fruit and vegetable intake were measured using an FFQ. Incidence of cardiovascular disease was gathered from medical records and death certificates. The consumption of fruits and vegetables led to a 27% lower stroke incidence, 42% lower stroke mortality, 24% lower ischemic heart disease mortality, 27% lower cardiovascular disease mortality, and a 15% lower all-cause mortality, showing the well-established inverse relationship of fruit and vegetable intake in association with the risk of cardiovascular disease and all-cause mortality in the general US population.[40]

2.8 Polyphenols

Polyphenols are organic compounds abundantly found in plants and the most prominent antioxidants in our diet. They are reducing agents that protect the body's tissues

against oxidative stress, inflammation, and associated pathologies such as coronary heart disease. The biological properties, bioavailability, antioxidant activity, specific interactions with cell receptors and enzymes, are related to the chemical structure of polyphenols.[41]

2.8.1 Polyphenol Classes and Structure

Four principles classes of polyphenols include, phenolic acids, flavonoids, stilbenes, and lignans.[42-44] Berries are rich in phenolic acids and flavonoids; thus, we will focus on these two classes.

Flavonoids contains 15 carbon atoms, consisting of two phenyl rings and heterocyclic ring and include six subclasses: flavones, flavanols, flavanols, flavanones, isoflavones, and anthocyanins.[45] Berries are rich in anthocyanins, flavonols, and flavanols. Anthocyanins create the blue, violet, or red pigment in plants/ many berries and red fruits including grapes, blackberry, cranberries, and raspberries. They possess antioxidant activities that are implicated in various health effects.[46] Flavonols are known for their antioxidant, anti-inflammatory, and vasodilatory properties. They are widely distributed in fruits and vegetables.[47] Quercetin, a type of flavonol is found in onions, green tea, apples, berries.[48] Research suggests it may prevent the most common forms of cardiovascular disease.[47] Flavanols include catechins and procyanidins and are found in high concentration in grapes, red wine, cocoa, and tea and exhibit high antioxidant activity.[49]

Phenolic acids can be divided in two subclasses: hydroxycinnamic and hydroxybenzoic acids. The latter include coumaric, ferulic, sinapic, caffeic, chlorogenic, and rosmarinic acid.[50] The former includes p-hydroxybenzoic acid, vanillic, syringic, protocatechuic, gallic, ellagic, caffeic acids among others.[51, 52] They contain one of more aromatic rings with a carboxylic acid. Gallic acid is present in common foods, blueberry,

cashew nut, and tea. Grapes, raspberries, and pecans are some foods that contain ellagic acid.

2.8.2 Polyphenol Metabolism

Bioavailability of dietary polyphenols varies within the gastrointestinal tract with their chemical structure having the greatest impact. Most polyphenols must be hydrolyzed before being absorbed.[53, 54] Digestion and absorption of polyphenols may occur via hydrolyzation through the action of B-glucosidase in the saliva or oral microbiota. Poor absorption occurs in the small intestine, 10-20% with lactase phlorizin hydrolase present on the brush border of epithelial cells.[53, 54] The small intestine can absorb forms of polyphenols such as aglycones, monomeric, and dimeric structures.[55]

Most of the absorption occurs in the colon (large intestine) where the polyphenols will meet colonic microflora. The gut microbiota hydrolyzes glycosides into aglycones by opening the heterocycle production of smaller molecules that can be absorbed if reaching the liver. In hepatocytes, polyphenols encounter conjugation, resulting in methyl, glucuronide, or sulfate derivatives.[43, 56-58] [54, 59] In blood circulation, metabolites will reach other organs. Conjugation prepares the polyphenols for elimination through bile and urine. [55] Higher conjugation will be carried back to the small intestine through bile excretion and it may reach the colon. For smaller conjugates it will mainly be excreted through the urine[54, 57, 59, 60].

2.9 Dietary Polyphenols, Oxidative Stress, and Inflammation

2.9.1 Human Studies

Dietary polyphenols can contribute to the reduction of chronic disease as they can neutralize ROS and inhibit inflammatory signaling. A significant inverse association was

found between anthocyanin intake (avg. 24.17 ± 0.32 mg/day) and systolic blood pressure. Anthocyanin intake was recorded using a 24-h diet recall and top sources of anthocyanin were from blackberry (5-65%), cherry (2-24%), blueberry (2-13%), and raspberries (3-12%).[61] Weasel et al. [62] completed a four-week intervention with a red berry juice (700 mL), containing red raspberry juice, which improved levels of glutathione and reduced DNA oxidative damage in healthy adult males. Another study examining anthocyanin intake, arterial stiffness, and blood pressure in women aged 18 – 75 also showed an inverse relationship between higher anthocyanin intake being associated with a significantly lower central SBP.[63] A six-month study was conducted by Romero-Prado et al.[64] in hypertensive young patients within the ages of 20 – 55 years. They found that the combination of antihypertensive treatment ACE inhibitor, captopril, or ARB, telmisartan, and dietary flavonoids (daily dose: 425.8 ± 13.9 mg epicatechin equivalent) elicited a significant reduction in blood pressure.

A three-arm double blind randomized controlled crossover study was conducted in 10 healthy males consuming 200 and 400 g of red raspberries containing 201 or 403 mg of total polyphenols, or a matched control drink. Flow mediated dilation (FMD) was significantly improved by 1.2 and 0.7% at 2 and 24 hours, respectively post- consumption of both the 200 and 400 g raspberry drinks in comparison to the control[65] Additionally, a 12-week randomized crossover study was conducted in two phases to examine the effects of daily dietary raspberries in obese adults with type 2 diabetes. The first phase included a postprandial phase of "acute raspberry supplementation" on two separate days at least one week apart, followed by a 1-week washout phase. Then, there was a 10-week "diet supplement phase" with and without raspberry supplementation periods of four weeks

each, separated by two-week washout phase. For serum inflammatory markers, interleukin (IL)-6 and high-sensitivity tumor necrosis factor alpha (hsTNF-α) this study showed significantly lower levels of expression 4 h postprandial after raspberry vs. control phase in obese adults with type 2 diabetes and systolic blood pressure showed a decreasing trend as well.[66]

2.9.2 Animal Studies

A study investigated the effects of chronic treatment with epicatechin on blood pressure, endothelial function, and oxidative stress in deoxycorticosterone acetate (DOCA) salt-induced hypertension rats. The rats were treated for five weeks with either 2 or 10 mg kg^{-1} day^{-1} of epicatechin, a flavanol. The high dose 10 mg/kg/day of epicatechin prevented both the increase in systolic blood pressure and the proteinuria induced by the DOCA-salt.[67] Kluknavsky et al. examined the effects of sub chronic treatment with 100 mg/kg/day of epicatechin for two weeks on locomotor activity and HTN development in young SHRs. Epicatechin significantly prevented the development of HTN and increased serum total antioxidant capacity. Epicatechin increased gene expression of neuronal NOS (nNOS) in the brainstem and cerebellum and eNOS protein expression in the cerebellum with no effect on overall NOS activity.[68]

In Ang II-induced HTN and vascular remodeling in C57BL/6J mice over a two-week study, 5 or 20 mg/kg/day of gallic acid, a phenolic acid significantly ameliorated Ang II-induced HTN, vascular inflammation, and cardiac fibrosis.[69] In another study, HTN was induced in rats with 60 mg/kg/day N_w-Nitro-L-arginine methyl ester hydrochloride, an eNOS inhibitor, for six weeks. In SHRs administered on average 320 mg of gallic acid per day for 16 weeks, attenuation systolic blood pressure through the inhibition of vascular

contractility and components of renin-Ang II system were observed. Gallic acid also reduced aortic wall thickness and body weight in SHRs.[70]Lastly, another phenolic acid, ellagic acid (10 or 30 mg/kg/day), was co-administered between the second and sixth week. Treatment with both doses of ellagic acid reduced HTN.[71]

2.10 Red Raspberries

Red raspberries are a commonly consumed and important fruit in the western diet due to their content of essential nutrients and beneficial phytochemicals.[13] Berries contain a unique composition of macronutrients and micronutrients that contribute to the overall health benefit (Table 2 and 3). Macronutrients are required in large amount in the diet and consists of protein, fats, and carbohydrates. Micronutrients are essential elements needed by life in small quantities, including vitamins and minerals. [72]Some key micronutrients that were found within red raspberries include, vitamin C (26.2 mg), vitamin A (33 IU), potassium (151 mg), phosphorous (29 mg), magnesium (22 mg), calcium (25 mg), and folate (21 ug) (Table 3). Red raspberries also contain 6.5 grams of fiber for ½ a cup. [73, 74] Each macro and micronutrient have individual health benefits. Vitamin C and vitamin A both function as antioxidants to neutralize free radicals [75, 76] Potassium are one of the nutrients that were found to be in low consumption and most do not meet the recommended intake. It is found to work opposite from sodium and there is a moderate body of evidence of the association between potassium intake and blood pressure reduction in adults.[77] Phosphorous and magnesium are also known for their role in blood pressure regulation.[78, 79] Calcium focuses on muscle and bone health, folate contributes to the production of healthy red blood cells, and fiber helps with digestive health.[80-82]

Table 2: Macronutrient Composition of Red Raspberries

Nutrient	Amount in 100 g (½ cup)
Energy, kcal	52
Protein, g	1.2
Carbohydrates, g	11.9
Fat, g	0.65
Fiber, g	6.5

Table 3: Micronutrient Composition of Red Raspberries

Nutrient	Amount in 100 g (½ cup)
Calcium, mg	25
Iron, mg	0.069
Magnesium, mg	22
Phosphorous, mg	29
Potassium, mg	151
Sodium, mg	1
Zinc, mg	0.42
Copper, mg	0.09
Manganese, mg	0.67
Selenium, mg	0.2
Vitamin C, mg	26.2
Thiamin, mg	0.032
Riboflavin, mg	0.038
Niacin, mg	0.598
Pantothenic Acid, mg	0.329
Folate, ug	21
Choline, mg	12.3
Vitamin A, IU	33
Vitamin E, mg	0.87

Berries are also a particularly rich source of polyphenols compared with other fruits and are associated with a reduced risk of HTN (Table 4) [13]. They possess phenolic acids, tannins, and a variety of anthocyanins that offer antioxidant, anti-inflammatory, and potential direct effects on the brain. [83, 84] Their total polyphenolic and antioxidant capacity is approximately four times greater than other fruits, 10 times greater than vegetables, and 40 times greater than cereals.[85] Raspberries, in particular have the highest antioxidant capacity amongst seven commonly consumed fruits and vegetables including strawberries,

kiwi, broccoli, leek, apple, and tomato. The polyphenolic profile of red raspberries is comprised of 50% ellagitannins and 25% anthocyanins.[36] Ellagitannins are the other major polyphenol group in red raspberries and is the primary hydrolysable tannin.[36] Some other flavonoids found in smaller quantities in raspberries include, quercetin-3-glucuronide, kaempferol-3-glucuronide, catechins, p-hydroxybenzoic acid, as well as derivatives of hydroxycinnamic acids, including caffeic acid, ferulic acid, synaptic acid, p-coumaric acid, cinnamic acid, and vanillic acid. A more thorough breakdown is listed in tables 2-4 below.[73, 74, 86]

Table 4: Polyphenol Profile of Red Raspberries

FLAVONOIDS	**Amount (mg/100 g)**
Anthocyanins	
Cyanidin	0.53
Cyanidin 3-O-glucoside	14.89
Cyanidin 3-O-glycosyl-rutinoside	7.06
Cyanidin 3-O-rutinoside	5.20
Cyanidin 3-O-sophroside	37.61
Delphinidin 3-O-glucoside	0.21
Malvidin 3-O-glucoside	0.62
Pelargonidin 3-O-glucoside	1.65
Pelargonidin 3-O-glucosyl-rutinoside	0.82
Pelargonidin 3-O-rutinoside	0.42
Pelargonidin 3-O-sophoroside	3.46
Flavanols	
(+)-Catechin	0.58
(-)-Epicatechin	5.05
Procyanidin dimer B2	0.10
Flavonols	
Kaempferol	2.14e-03
Kaempferol 3-O-glucoside	1.03
Quercetin	0.02
Quercetin 3-O-glucoside	3.58
Quercetin 3-O-glucuronide	0.63
Quercetin 3-O-rutinoside	11.00
PHENOLIC ACIDS	
Hydroxybenzoic Acids	
Ellagic acid	2.12
Ellagic acid acetyl-arabinoside	0.20

Ellagic acid acetyl-xyloside	0.36
Ellagic acid arabinoside	2.27
Lambertianin C	30.84
Sanguiin H-6	76.10
Hydroxycinnamic acids	
5-Caffeoylquinic acid	0.57
p-Coumaric acid	2.30e-04
p-Coumaric acid 4-O-glucoside	0.32

The unique polyphenol profile and nutrient composition of red raspberries makes them a potential nutritional therapeutic. Based on compelling animal data and limited human findings, berries or specific phenolic compounds found in red raspberries tend to reduce HTN. However, the effects of raspberries on the brain, particularly those lacking the protective BBB such as SFO and OVLT, in an Ang-II-induced HTN model have not been previously evaluated.

CHAPTER III

METHODS

3.1 Reagents

Radioimmunoprecipitation assay (RIPA) buffer, phosphatase inhibitor cocktail 1 and 2 and protease inhibitor cocktail (Sigma Aldrich); Rabbit polyclonal antibodies against: NOX2 (Cat#: ab180642; Abcam), NOX4 (Cat# NB110-58849; Novus Biologicals), SOD1 (Cat# NBP2-24915; Novus Biologicals), and SOD2 (Cat# D3X88F; Cell Signaling), GAPDH (Cat# 14C10; Cell Signaling). Secondary antibodies against rabbit IgG (Cat#: 65-6120; Invitrogen), mouse IgG (Cat#: 7076S; Cell Signaling) and goat IgG (Cat#: HAF017; R&D Systems). Immobilon Forte Western HRP Substrate (EMD Millipore).

3.2 Study Design

Eight-week-old male Sprague Dawley rats were purchased from Envigo (Indianapolis, IN). Upon arrival, rats were singly housed in an environmentally controlled animal care facility and maintained on 12-hr light/dark cycles. Rats were given free access to water and maintained on a semi-purified casein-based diet (AIN-93M) in which soybean oil was replaced with corn oil since soybean oil contains phenolic compounds. After seven days of acclimation, rats were randomized to one of three groups (n=10 per group): 1) control 2) Ang II and 3) Ang II + raspberry (RB)

Animals in the control and Ang II groups consumed the control diet AIN-93M diet while animals in Ang II + RB consumed a diet supplemented with 10% freeze-dried raspberry powder for the entire duration of the study. The 10% raspberry diet is equivalent to approximately four servings (three cups) of fresh raspberries per day for humans. This is based on body surface area and corresponds to a dose of approximately 333 mg total phenolics/kg body weight/day.[87] The control diet was isocaloric and matched for macronutrients, fiber, and micronutrients such as potassium and sodium. Food intake and body weight was monitored weekly throughout the study. At week four, rats were implanted with subcutaneous osmotic minipumps that delivered 0.9% saline (control group) or 270 ng/kg body weight/min of Ang II (Ang II and Ang II + RB groups) for another three weeks.

3.3 Blood Pressure Measurements

Blood pressure was measured using the CODA High Throughput Non-Invasive Blood Pressure system (Kent Scientific, Torrington, CT) in up to four rats simultaneously according to Daugherty *et al*.[88] All rats were encouraged to walk into the restraint tubes, and all tubes were adjusted to prevent excessive movement. Occlusion cuffs were placed at the base of the tail, and Volume Pressure Recording (VPR), measuring blood volume changes over the animal's tail. The cuffs were placed approximately 2 mm adjacent to the occlusion cuffs. The rats were acclimated to the tubes and heating pad for 10 min prior to the start of the experiment, and tail temperatures were checked before and during blood pressure readings. Tail temperatures remained between 32 to 35°C. Occlusion cuffs were inflated to 250 mm Hg and deflated over 20 secs. The minimum volume changes as sensed by the VPR cuff was set to 15 μL. Each recording session consisted of 25 inflation and

deflation cycles with the first five cycles marked as “acclimation” and not considered in the final analysis. Blood pressure measurements were performed at weeks 4, 5 and 6.

3.4 Tissue Dissection and Region-Specific Tissue Punches

At week four, following sacrifice by CO_2 and decapitation, brains were removed from the rat skull, placed into liquid nitrogen, and stored in -80°C freezer. A Leica Cryostat (CM3050 S) set at [-18 OT and -23 CT] was utilized to slice brain sections, until reaching the proper region. Brain region-specific tissue punches were collected from flash-frozen sections containing the OVLT and SFO. A 1-mm puncher was used to gather 6-10 punches per brain region. The punched tissues were saved in properly labeled tubes in a -80°C freezer prior to analysis.

3.5 Western Blot Analysis

Brain punches were homogenized utilizing 200 µl of RIPA supplemented with protease and phosphatase inhibitors. Lysates were sonicated and centrifuged at 16,000 x g for 20 min. Protein was quantified and normalized via the DC protein assay kit (BioRad Laboratories, Hercules, CA). In preparation for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, 20 µg of samples were mixed with 4x laemmli buffer + 5% 2-mercaptoethanol (BioRad Laboratories, Hercules, CA). Samples were then briefly vortexed and centrifuged, then heated for 10 min at 70°C in a dry heating block and loaded onto a polyacrylamide gel for electrophoresis. Following electrophoresis, gels were transferred to polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in TBS-T + 5% non-fat dry milk (NFDM) and washed in TBS-T (3 x 5 min). Each region was probed with the antibodies described above.

3.6 Statistical Analysis

Blood pressure data were analyzed by two-way ANOVA and are represented as mean ± standard error of the mean (SEM). Protein expression data were normalized to control group by fold-change. Data were analyzed for normality using Shapiro-Wilk test. If the data were normally distributed, one-way analysis of variance (ANOVA) followed by Tukey posthoc test was performed. Otherwise, Kruskal-Wallis test was used. Data are presented as mean ± 95% confidence interval (CI). Significance was determined at $P \leq 0.05$.

CHAPTER IV

RESULTS

4.1 BLOOD PRESSURE

4.1.1 Effects of Ang II and Raspberry Supplementation on Blood Pressure

Blood pressure results are displayed in Figure 3. There were no significant differences in SBP between groups prior to Ang II infusion though SBP in raspberry supplemented rats appeared lower in the raspberry group at baseline (146.4 ± 8.7-fold, n=5, vs. 144.7 ± 26.2, n=6 vs. 136.1 ± 20.8, n=12) (Figure 3A). As expected, blood pressure steadily increased in Ang II infused rats (144.7 ± 26.2 vs. 161.5 ± 20.8, n=6), though not significantly ($p = 0.6$) (Figure 3A). Raspberry supplemented rats exhibited a lower increase in SBP compared to Ang II infused rats (152.8 ± 23.9, n=12 vs. 161.5 ± 20.8, n=6) (Figure 3A). As seen in Figure 3B, raspberry supplementation attenuated the blood pressure increase induced by Ang II, albeit not significantly (10.5 ± 17.4, n=13 vs. 12.3 ± 27.7, n=9, $p = 0.9$).

4.2 SUBFORNICAL ORGAN (SFO)

4.2.1 Effects of Raspberry Consumption on the Expression of NADPH Oxidases in the SFO of Ang II-Treated Rats

Ang II treatment significantly increased the expression of NOX4 compared to control, as seen in Figure 4 (2.11 ± 1.2-fold, n=10, vs 1.00 ± 0.08-fold, n=5, $p = 0.02$). While not significantly, raspberry supplementation appeared to decrease NOX4 expression compared to Ang II alone (1.32 ± 0.39-fold, n=9, $p = 0.16$) and expression of NOX4 in the raspberry supplemented group did not differ significantly from control.

In a similar fashion, Ang II treatment increased NOX2 expression, approaching the level of significance (Figure5) compared to control (1.21 ± 0.2-fold, n=9, vs 1.00 ± 0.09-fold, n=6, p = 0.06). Additionally, raspberry supplementation significantly decreased Ang II-induced increases in NOX2 (0.83 ± 0.17-fold, n=9; p = 0.0002).

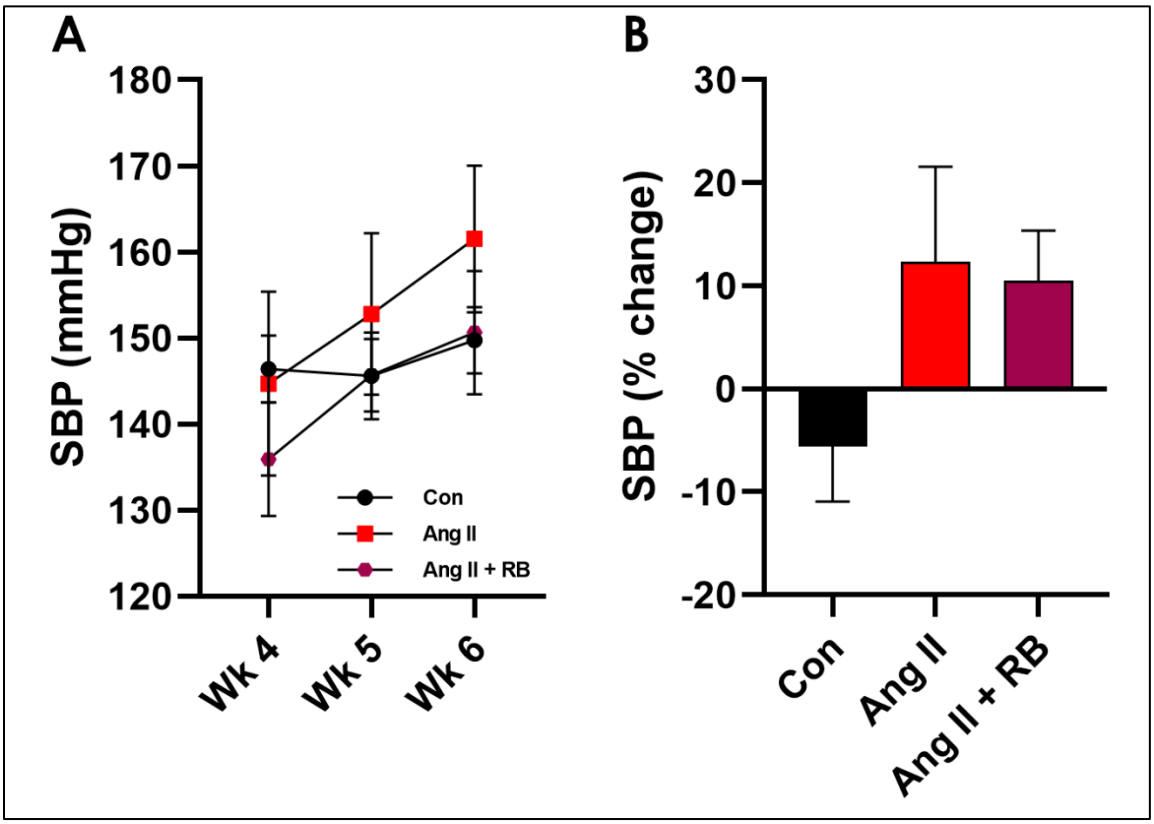

Figure 3. Effects of Ang II and Raspberry Supplementation on Blood Pressure. Systolic blood pressure (SBP) was measured using tail-cuff plethysmography at weeks 4, 5 and 6 (A). Changes in SBP from week 4 (B). Control: n = 10; Ang II: n = 9; Ang II + RB: n = 13.

4.2.2 Effects of Raspberry Consumption on the Expression of Antioxidant Enzymes in the SFO of Ang II-Treated Rats

As shown in Figure 5C, SOD1 expression was increased in animals infused with Ang II, with and without raspberry supplementation (1.63 ± 0.25-fold, n=9, p = 0.001 vs. 1.45 ± 0.46-fold, n=10, p = 0.0394 vs. 1.00 ± 0.15-fold, n=6, p = 0.005) compared to control.

Interestingly, SOD2 expression (Figure 5D) was decreased in response to Ang II infusion compared to control (0.74 ± 0.16, n=9, vs 1.00 ± 0.07-fold, n=6, p = 0.009). Raspberry supplementation acted to prevent this decrease as the raspberry supplemented group did not differ from control (0.86 ± 0.17-fold, n=9, p = 0.2).

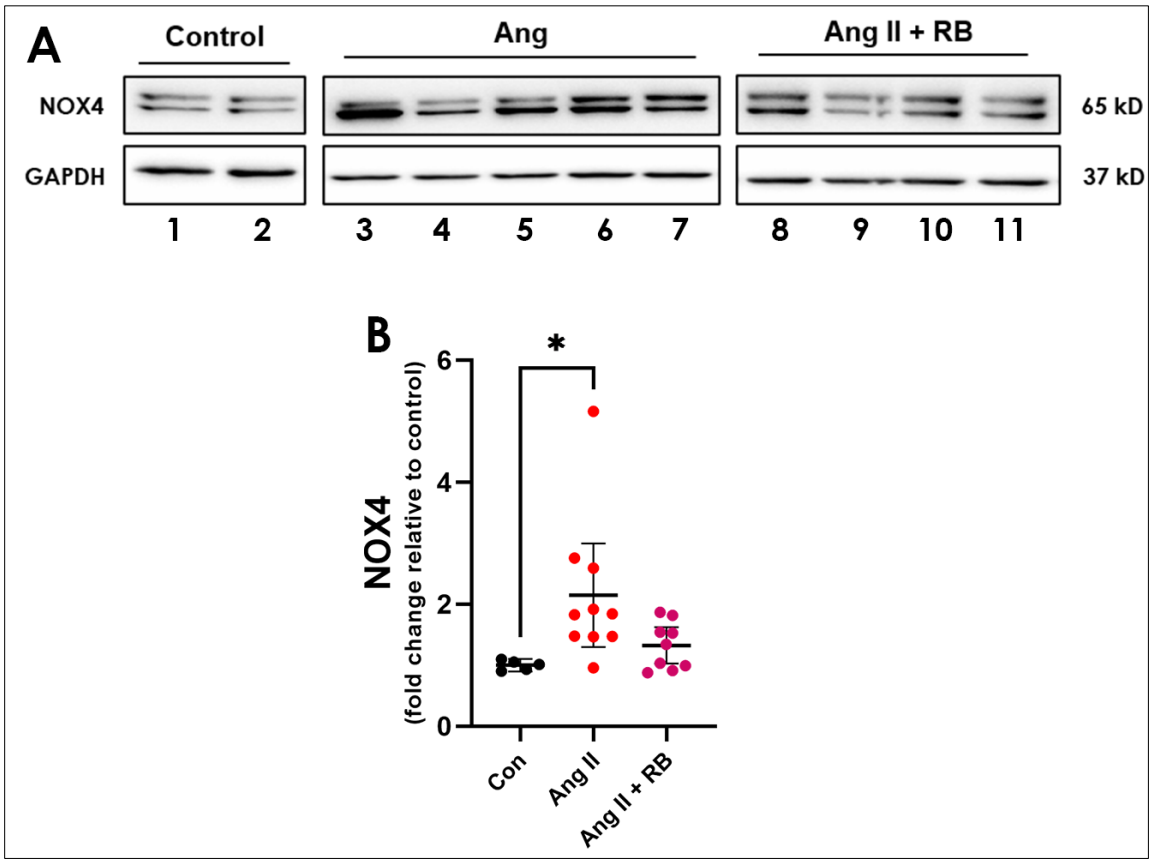

Figure 4. Ang II increases NOX4 Expression in the Subfornical Organ of Rats. Male Sprague-Dawley rats were fed either AIN-93M diet alone or supplemented with freeze-dried raspberry (RB) powder (10% w/w) for seven weeks. At week 4, rats were either infused with 0.9% saline (control) or Ang II (270 ng/kg body weight/min). Brain region-tissue punches were collected from flash frozen sections containing the SFO. Protein expression was determined using western blot (A). Quantification of NOX4 (B) was performed using Image J. Results were normalized to control and are expressed are means ± 95% CI. (Control, n=5 and Ang II groups, n=10)

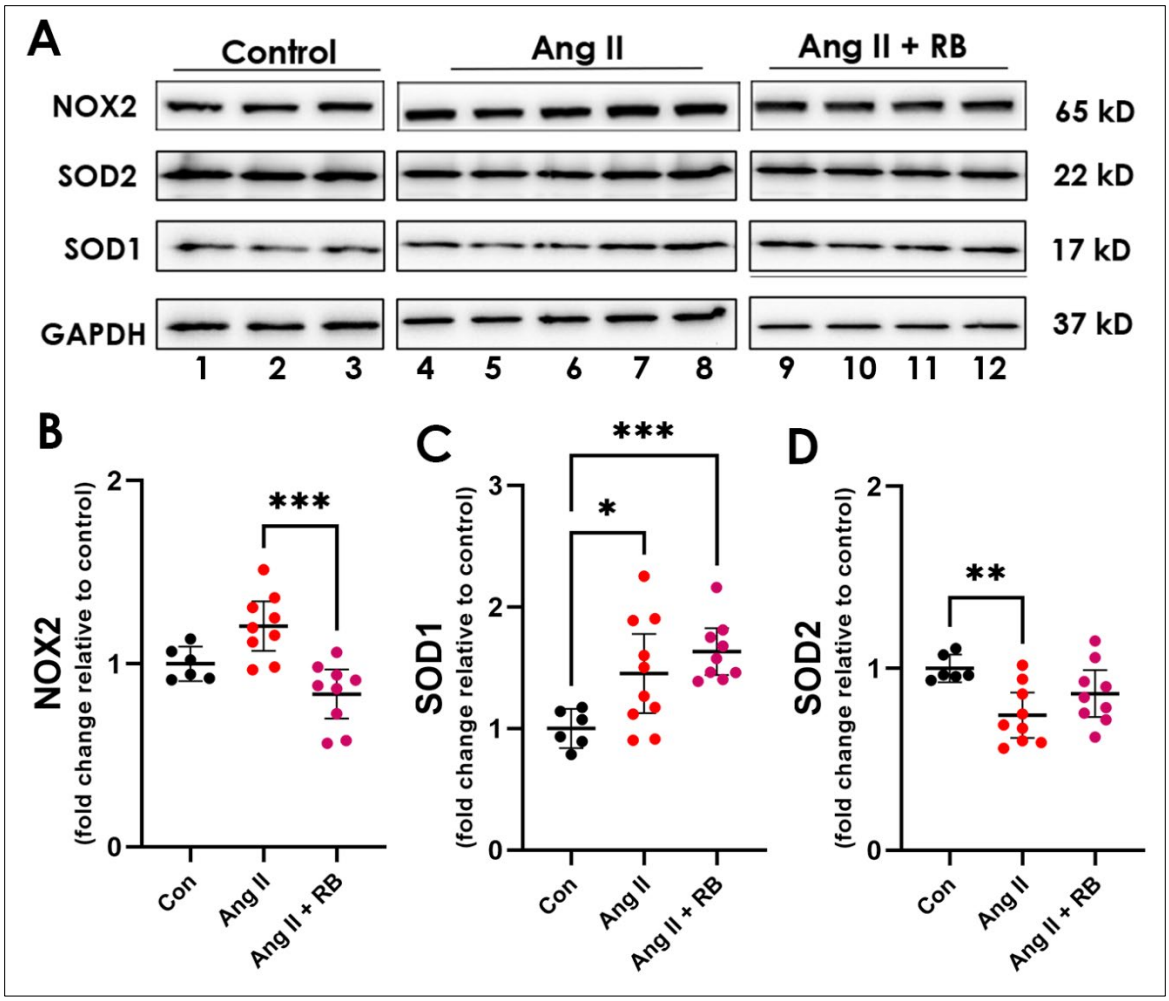

Figure 5. Raspberry Consumption decreases NOX2 expression while increasing SOD1 expression in the Subfornical Organ of Rats. Male Sprague-Dawley rats were fed either AIN-93M diet alone or supplemented with freeze-dried raspberry (RB) powder (10% w/w) for seven weeks. At week 4, rats were either infused with 0.9% saline (control) or Ang II (270 ng/kg body weight/min). Brain region-tissue punches were collected from flash frozen sections containing the SFO. Protein expression was determined using western blot (A). Quantification of NOX2 (B), SOD1 (C), SOD2 (D) was performed using Image J. Data were normalized to control and are expressed are means ± 95% CI. (Control, n=6 and Ang II groups, n=9)

4.3 ORGANUM VASCULOSUM OF LAMINA TERMINALIS (OVLT)

4.3.1 Effects of Raspberry Consumption on the Expression of NADPH Oxidases in the OVLT of Ang II-Treated Rats

Ang II infusion significantly increased the expression of NOX4 compared to control (1.59 ± 0.48-fold, n=9 vs. 1.00 ± 0.29-fold, n=7, p = 0.03), as seen in Figure 6 However, in the OVLT of those rats supplemented with raspberry, NOX4 expression was similar to control (1.80 ±_1.03-fold, n=8, p = 0.2). Interestingly, there were no changes in NOX2 expression in response to Ang II infusion or raspberry supplementation (1.44 ± 0.59-fold, n=9 vs. 1.01 ± 0.18, n=9, p = 0.2) when compared to control.

4.3.2 Effects of Raspberry Consumption on the Expression of Antioxidant Enzymes in the OVLT of Ang II Treated Rats

As shown in Figure 6D, no significant changes were observed for SOD1 expression after treatment with Ang II compared to control (1.20 ± 0.18-fold, n=4 vs. 1.00 ± 0.12-fold, n=3, p = 0.3), or in response to raspberry supplementation (1.06 ± 0.15-fold, n=4, p = 0.5) compared to Ang II. As seen in Figure 6E, the expression of SOD2 in the OVLT of rats infused with Ang II was unchanged, regardless of raspberry consumption compared to control (1.06 ± 0.21-fold, n=4 vs. 1.05 ± 0.06-fold, n=4 vs. 1.00 ± 0.16-fold, n=4, p = 0.9).

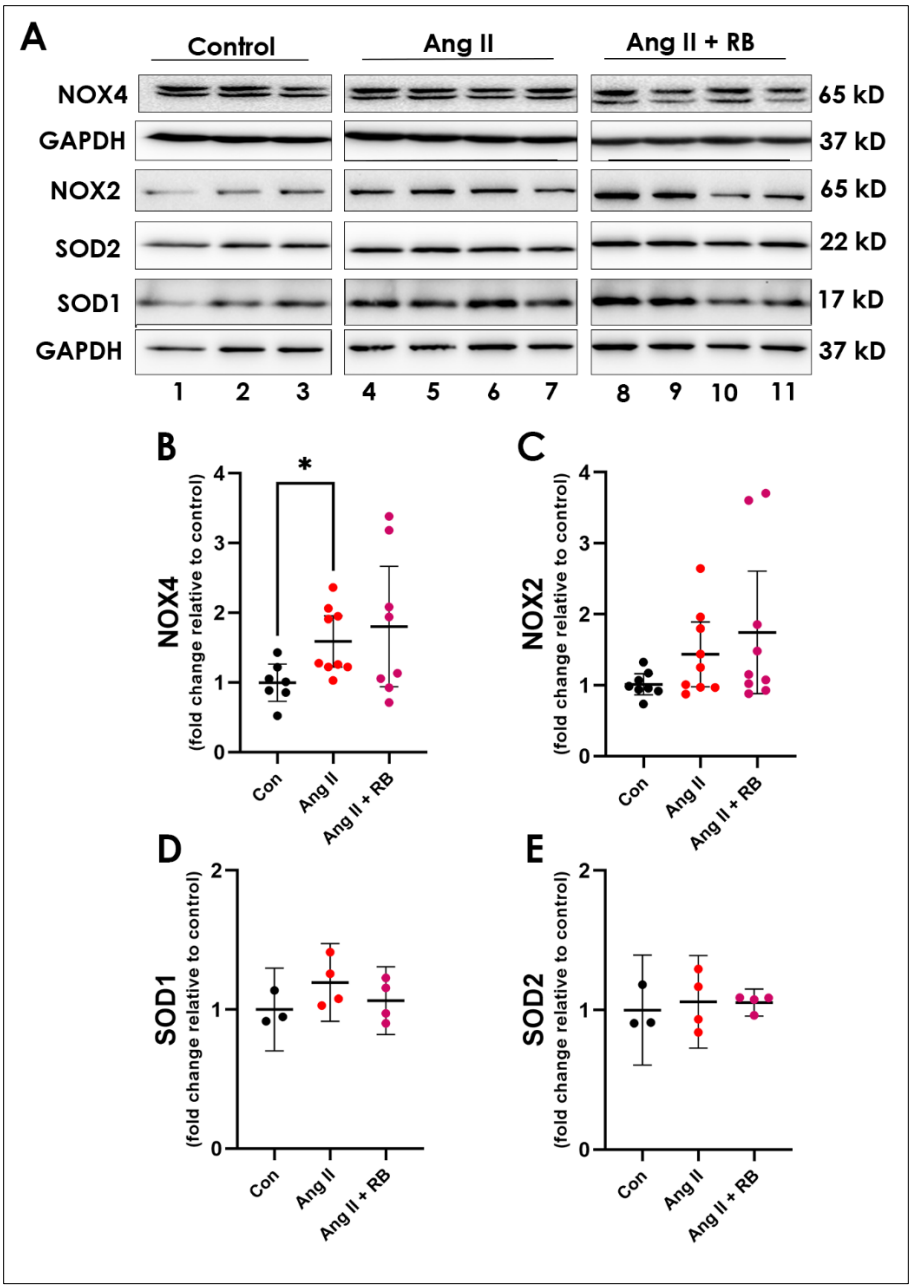

Figure 6. Ang II increases the expression of NOX4 in the Organum Vasculosum of Lamina Terminalis (OVLT) of Rats. Male Sprague-Dawley rats were fed either AIN-93M diet alone or supplemented with freeze-dried raspberry (RB) powder (10% w/w) for seven weeks. At week 4, rats were either infused with 0.9% saline (control) or Ang II (270 ng/kg body weight/min). Brain region-tissue punches were collected from flash frozen sections containing the OVLT. Protein expression was determined using western blot (A). Quantification of NOX4 (B), NOX2 (C), SOD1 (D), SOD2 (E) was performed using Image Data were normalized to control and are expressed are means ± 95% CI. (Control, n=3-7 and Ang II groups, n=4-9).

CHAPTER V

DISCUSSION

Hypertension is a major public health concern, accounting for millions of deaths.[1, 2] Both modifiable and non-modifiable risk factors can play a role in development.[3] Dysregulation of the RAAS plays a significant role in the development of HTN, specifically through the production of the peptide hormone, Ang II.[4, 5] Ang II has been implicated in driving HTN and contributing to oxidative stress, both known to contribute to the development of a number of chronic conditions.[5-7]

To determine the overall impact of Ang II on the development of HTN, we assessed BP prior to beginning Ang II infusion (week 4) and following the start of Ang II infusion (week 5 and week 6). Raspberry was able to mitigate Ang II-hypertensive effects as SBP were similar to control at weeks 5 and 6. While the results are not statistically significant, likely due to variation, they may be of clinical and physiological relevance. It has been documented that lowering blood pressure reduces cardiovascular diseases.[89] More importantly, linear relationships were found between reductions in SBP and the risk of cardiovascular disease and mortality.[89]

A previous eight-week study conducted in our lab indicates that a raspberry (10% w/w) supplemented diet attenuates Ang II-induced increases in blood pressure in mice.[87] The study included 4 weeks of chronic Ang II infusion (1 μg/kg body weight/day) via a subcutaneous osmotic minipump.[87] No other studies have examined the effects of raspberry on blood pressure. However, the effects of blueberry have been investigated

. One study reported that a diet supplemented with 3% freeze-dried blueberry for eight weeks lowered blood pressure in spontaneously hypertensive rats (SHRs).[90] Another study indicate that consumption of 2% w/w blueberry enriched diet for six or twelve weeks reduced blood pressure.[91]

CVOs are structurally unique as they lack the BBB. The BBB typically protects the brain and restricts levels of circulating compounds from impacting the tissue. As such, circulating levels of Ang II have been shown to impact these regions, such as the SFO and OVLT, of the brain.[4, 11, 12, 24, 25] As a product of RAAS, Ang II is a peptide hormone that not only acts to regulate blood pressure through vasoconstriction and fluid retention, but also is known to activate and regulate expression of pro-oxidant enzymes contributing to oxidative stress.[4, 5, 8, 26] The brain is particularly prone to oxidative stress which can lead to neuronal damage.[28] Ang II binding to its receptor, AT_1R, instigates a cascade of events, including activation of NOXs which produce ROS in significant amounts in the brain.[8] In response to Ang II infusion, NOX2 and NOX4 expression were increased in the SFO while only NOX4 expression increased in the OVLT. Ang II induced different effects in NOX2 and NOX4 expression in differing regions of the brain, this may be related to the localization of these two isoforms as NOX2 is reportedly localized with the plasma membrane or perinuclear membrane whereas NOX4 may be localized to focal adhesions, the perinuclear endoplasmic reticulum, or the nucleus.[92] Even though NOX2 and NOX4 are found in the brain, they respond differently to Ang II due to their subcellular localization.[92] In fact, genetic silencing of NOX2 and NOX4 indicates that both have a role in the direct impact of Ang II in the brain; however, NOX2 was the only isoform that when silenced it showed Ang II effects that led to increases in water intake for the rats. [29]

In addition to initiating increases in the pro-oxidant NOX isoforms, Ang II infusion had a direct effect on the expression of antioxidant enzymes in the SFO but not in the OVLT. Ang II induced expression of SOD1 but decreased expression of SOD2. This may be due to the localization of each of the isoforms within the cell. SOD1 is located within the cytoplasm and is Cu/Zn dependent while SOD2 is found in the mitochondria.[93] Interestingly, a study analyzing the overexpression of SOD1 in the OVLT showed a reduction in Ang II-induced hypertensive effects of blood pressure.[94] Zimmerman et al. also looked at $O_2^{\cdot-}$ production in the central nervous system. An Ang II slow pressor model was used in mice and $O_2^{\cdot-}$ levels were replicated in the CVO via delivery of SOD. SOD was delivered either in the cytoplasm (CuZnSOD) or the extracellular matrix (ECSOD). Successful attenuation was found through the overexpression of CuZnSOD in the SFO, but not the ECSOD.[95]

To our knowledge, no studies have assessed the effects of raspberry in the SFO or OVLT of hypertensive rats. Nonetheless, based on the nutrient and polyphenol breakdown of raspberries, we expected them to uphold the same quality as an antioxidant which would neutralize free radicals and reduce oxidative stress. These beneficial effects are expected from raspberries over other fruits and vegetables due to its unique polyphenol profile. Here, we showed the ability of raspberries to mitigate the effects of Ang II in the SFO. Specifically, raspberry supplementation reduced NOX2 expression while also increasing SOD1 expression compared to animals infused with Ang II alone and control, respectively. These promising results show the potential for raspberries to shift the oxidative stress burden induced by Ang II in the SFO.

The SFO communicates with the OVLT, which also contains the AT_1R receptor.[12, 25] The OVLT is one of the four sensory organs and also characterized as a CVO as it lacks the BBB.[11] Neurons of OVLT are responsible for controlling thirst, blood volume regulation, and vasopressin release.[11] Previous studies have shown that both the SFO and OVLT are responsive to Ang II.[96, 97] When superoxide levels ($O_2^{\cdot -}$) levels are overproduced it is found to play an important role in the development of Ang II dependent hypertension.[94] Additionally, the overexpression of CuZnSOD in the OVLT was found to attenuate the increase in MAP induced by Ang II infusion.[94] These authors have shown that the SFO is necessary to achieve full hypertensive response to the Ang II infusion.[98, 99] Questions have emerged on which of CVOs function as the primary site that drives the hypertensive response.[94] The OVLT did show minor changes, but the significant data came from the SFO, indicating the possibility that the SFO is one of the more important CVOs to target for therapeutic methods and Ang II induced oxidative stress.

This study has limitations. First, the sample size for OVLT was small which may have affected our ability to see statistically significant changes. Additionally, blood pressure was taken through a non-invasive method via a tail-cuff system at one-time point within the day. Perhaps use of the gold standard and radio-telemetry would have elicited different results as it would allow for blood pressure to be monitored 24 h per day for over two weeks. [100]

It is important to mention the level of raspberry supplementation used in the present study, 10% w/w, would equate to approximately three cups of raspberries daily. For a fresh cup of raspberries, this would equate to 123 g and 233.7 mg of polyphenols. It is possible that a lower dose would be more efficient as a previous from our laboratory study

examining the effects of two different doses of strawberries, 25 and 50 g, in hypertensive postmenopausal women found significant reduction with the group who consumed 25 g.[101]

In summary, our findings indicate the potential role raspberries may play as a nutritional strategy to mitigate BP and factors contributing to oxidative stress in the areas of the brain that lack the protective BBB. More specifically, the SFO may be a key target for this therapeutic strategy. Future investigations in humans are warranted to confirm these effects.

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