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The Role of Endothelial Nitric Oxide Synthase (eNOS) Uncoupling on Leukocyte-Endothelial Interactions in Rat Mesenteric Postcapillary Venules

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THE ROLE OF ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) UNCOUPLING ON LEUKOCYTE-ENDOTHELIAL INTERACTIONS IN RAT MESENTERIC POSTCAPILLARY VENULES

A Thesis in Endothelial Dysfunction by Maria Kern

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We the undersigned duly appointed committee have read and examined this manuscript and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

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Abstract

Endothelial derived nitric oxide (NO) is essential in the regulation of blood pressure and attenuates leukocyte-endothelial interactions associated with vascular injury. However, when endothelial-derived NO is decreased, endothelial dysfunction results and promotes inflammation characterized by increased leukocyte-endothelial interactions. Under normal conditions, eNOS produces NO in the presence of an essential cofactor, tetrahydrobiopetrin (BH_4) by facilitating the reduction of molecular oxygen to *L*-arginine oxidation and generation of *L*-citrulline. Whereas uncoupled eNOS refers to the electron transfer that becomes uncoupled to *L*-arginine oxidation and therefore superoxide (SO) is generated when the dihydrobiopetrin (BH_2) to BH_4 ratio is increased. SO is subsequently converted to hydrogen peroxide as a result. However, the role of uncoupled eNOS promoting endothelial dysfunction and leukocyte-endothelial interactions in not well characterized *in vivo*.

This study examined the role of eNOS uncoupling by superfusing BH_2 (100 or 200 µM) by itself and BH_2 (100 µM) combined with BH_4 (100 or 250 µM) in rat mesenteric venules on leukocyte rolling, adherence, and transmigration by using intravital microscopy. The effects of BH_2 were compared to Krebs' buffer, to NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 50 µM), and to the combination of BH_2/BH_4. We found that superfusion of BH_2 (100 µM n=6, 200 µM n=6, both $P<0.05$) significantly increased leukocyte rolling, adherence, and transmigration, similar to L-NAME (n=6, $P<0.01$), within a 2 hr period compared to Krebs' buffer (n=6) control rats on these three parameters. The BH_2 induced response was significantly attenuated by BH_4 (100 µM n=6, 250 µM n=6, both $P<0.05$) dose dependently.

These results were further confirmed by hematoxylin/eosin staining which showed significantly decreased leukocyte adherence and transmigration in BH_4 treated rats compared to BH_2 (100 µM n=6, 200 µM n=6, both $P<0.05$) controls or L-NAME (n=6, $P<0.01$). The data suggest that eNOS uncoupling may be an important mechanism mediating inflammation-induced vascular injury.

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Introduction

Endothelial Function

Endothelial function plays a vital role in the normal physiological process. Endothelium contains a monolayer of endothelial cells that acts as a barrier and is present between the blood vessel wall and the circulating blood. This tissue functions as a barrier by transferring molecules through this semi-permeable layer as well as maintaining vascular homeostasis [1, 2, 3]. In addition to serving as a barrier, these cells recruit and modulate leukocytes which may result in cell activation. Normal endothelium has an anti-thrombotic surface that prohibits circulating leukocytes from adhering to the vasculature. This allows ample blood flow by normalizing vasomotor tone, thrombosis, and immune and inflammatory responses by regulating leukocyte interactions in the vessel wall [2]. Endothelial cells produce nitric oxide (NO) which serves to mediate the anti-inflammatory and anti-thrombotic properties and prevent damage to normal vascular endothelium. Impaired NO production and release contributes to endothelial dysfunction that can lead to inflammation-mediated vascular injury. This makes it an ideal target in new therapies for diseases such as atherosclerosis, hyperglycemia, shock, diabetes, and ischemia-reperfusion (I/R) injury [2, 4].

Nitric Oxide/Endothelial Nitric Oxide Synthase (eNOS)

Nitric oxide is present in all blood vessels and is involved in regular vascular function. It is a key cellular signaling molecule that has a vital role in many biological processes. Because NO is a major mediator of endothelium-dependent vasodilation, it acts as an important determinant of cardiovascular health [1]. NO is made in endothelial

cells from *L*-arginine through the action of the membrane bound enzyme endothelial NO synthase (eNOS). Calcium is released from the sarcolemma storage site from activating stretch and/or Gq coupled receptors which results in an increase in intracellular calcium. Thereafter, calcium associates with calmodulin to activate the enzyme eNOS which produces NO from its precursor *L*-arginine forming *L*-citrulline as a byproduct.

Tetrahydrobiopterin (BH_4) and nicotinamide-adenine-dinucleotide phosphate (NADPH) are both essential cofactors in this electron chain reaction [5, 6, 7, 8]. NADPH begins the electron transport chain which binds flavin adenine dinucleotide and flavin mononucleotide to the prosthetic heme group. This is then activated by BH_4 in the presence of molecular oxygen to produce NO and this is referred to as eNOS coupling. Whereas when the dihydrobiopterin (BH_2), the oxidized form of BH_4, to BH_4 ratio increases, the product profile of eNOS switches from NO to superoxide (SO). BH_2 competes with BH_4 binding at the oxygenase domain of eNOS with equal affinity [9]. In the presence of BH_2, the electron transfer becomes uncoupled to *L*-arginine and eNOS utilizes molecular oxygen instead to produce SO [8]. This is referred to as eNOS uncoupling as seen in Figure 1.

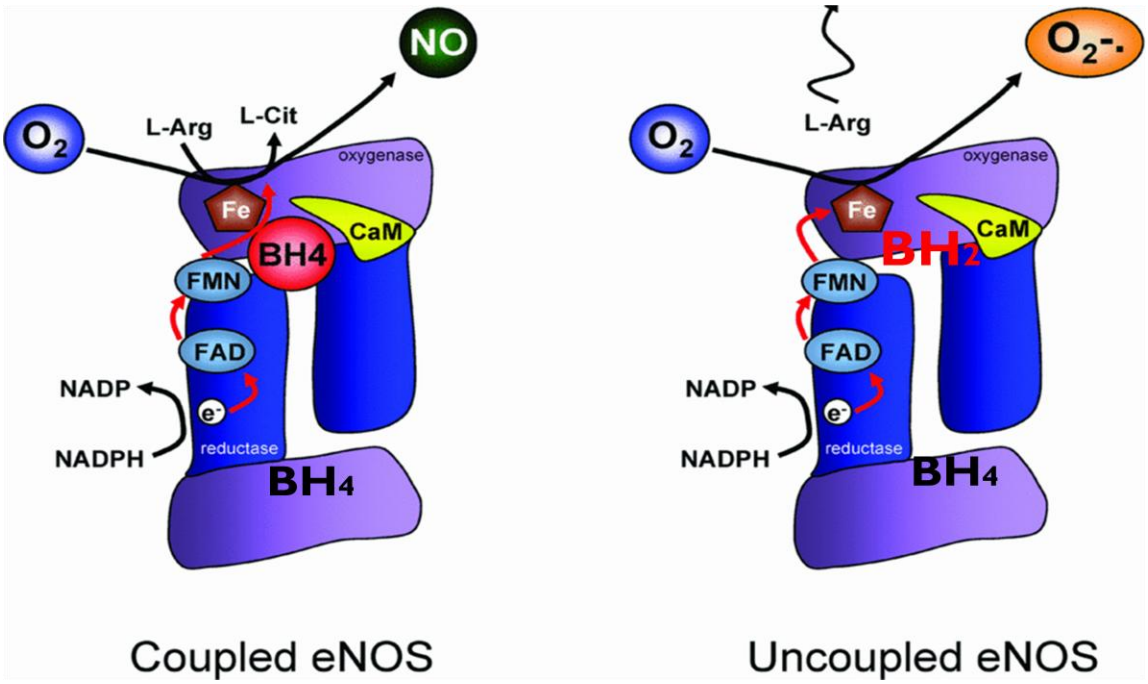

Figure 1: Illustration of the coupled eNOS and uncoupled eNOS [Adapted from 8].

In the normal physiological process, after NO is produced as a byproduct from *L*-arginine, NO diffuses into vascular smooth muscle cells adjacent to the endothelium where it binds to and activates the enzyme guanylyl cyclase which catalyzes the dephosphorylation of guanosine triphosphate to cyclic guanosine monophosphate, an important second messenger that induces smooth muscle relaxation as seen in Figure 2 [3, 10].

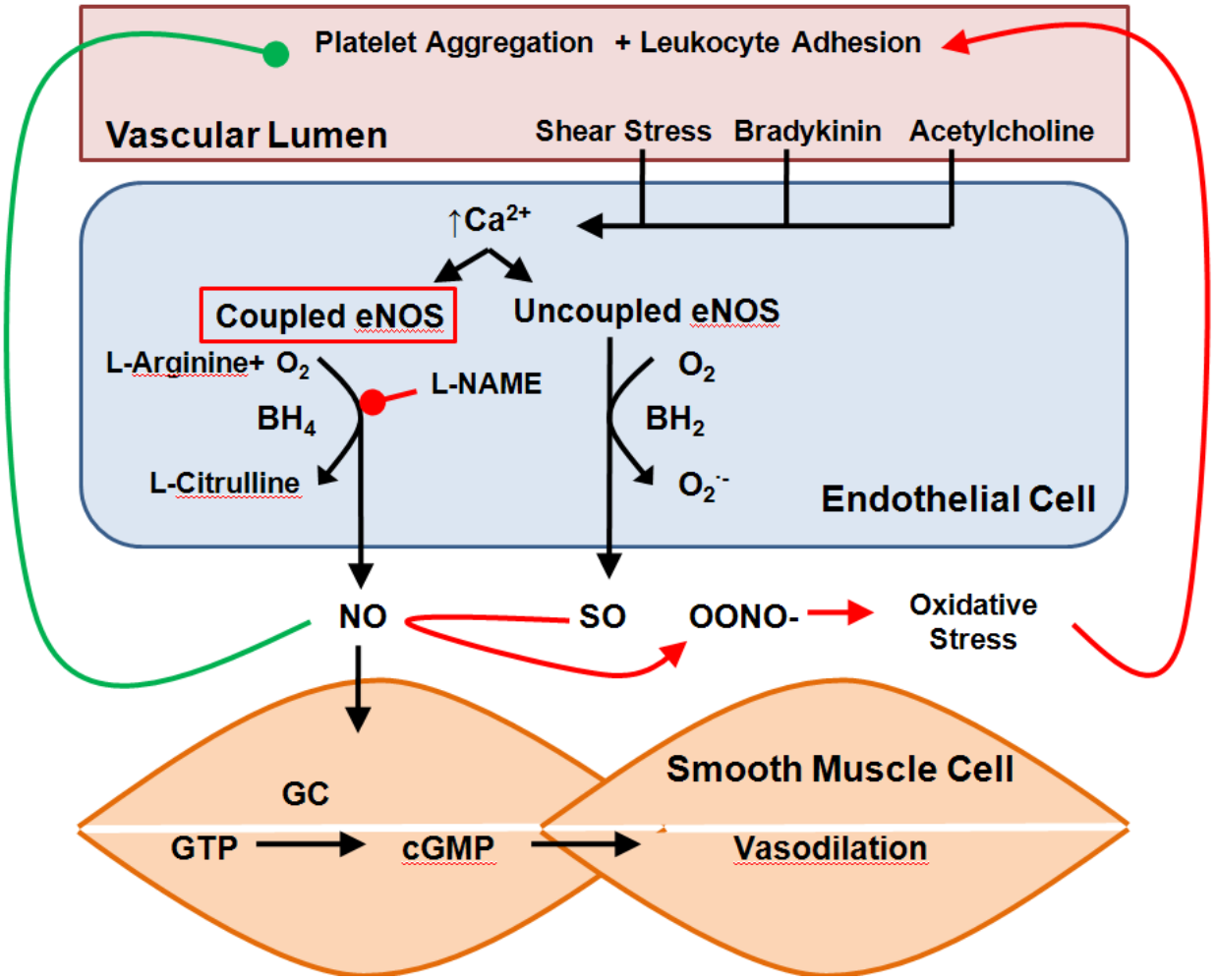

Figure 2: Illustration of the mechanism of action of nitric oxide displaying how it maintains endothelium normal function.

When NO forms, it has a very short half life (seconds) and can react with SO to form peroxynitrite, which in turn can generate damage to the cell membrane causing lipid peroxidation. SO can quench NO thus reducing NO bioavailability and induce endothelial dysfunction. N^G-nitro-*L*-arginine methyl ester (L-NAME) inhibits NO synthesis by competing with *L*-arginine. L-NAME is used to cause endothelial dysfunction and

consequently induce leukocyte-endothelial interactions which can simulate early reperfusion events [4, 11].

Furthermore, NO regulates vascular tone, controls mitochondrial oxygen consumption, and inhibits leukocyte adhesion, aggregation, and activation [12, 13]. When normal NO production is impaired, cardiovascular diseases such a hypertension, atherosclerosis, angiogenesis-associated disorders, diabetes, and hypercholesterolaemia may occur [6, 12].

Endothelial Dysfunction

Endothelial dysfunction is an early and frequent characteristic of cardiovascular disease conditions that refers to the decreased bioavailability of NO and prostacyclin [14]. This term is used to describe an impairment of endothelium-dependent vasorelaxation caused by a reduction in NO activity in the vasculature or increased SO release from leukocytes and vascular endothelium. This decrease in NO bioavailability may also transpire if eNOS is not fully expressed or activated due to the lack of *L*-arginine or the increased BH_2 to BH_4 ratio. Additionally, reactive oxygen species (ROS) may increase NO degradation [15]. Endothelial dysfunction often is associated with shock, atherosclerosis, I/R, hypertension, diabetes, and other vascular pathophysiological conditions [11, 15].

Oxidative Stress/Reactive Oxygen Species (ROS)

Oxidative stress causes cellular damage due to the unrestrained action of ROS molecules [16]. These molecules are derived from oxygen and its' derivatives which

include the following free radicals: the SO anion, the hydroxyl radical, and large quantities of NO (i.e. micromolar amounts) derived from inducible NO synthase. Additionally, hydrogen peroxide (H_2O_2), peroxynitrite, and hypochlorous acid possess analogous oxidizing capabilities [1, 15, 16, 17]. ROS generation occurs under normal physiological conditions as well as pathophysiological conditions. ROS production in conjunction with endothelial activation and inflammatory cell recruitment creates an equilibrium between the oxidizing agent formation and the protective antioxidant mechanism removal. The balance between oxidant and antioxidant functions controls how much ROS is produced by endothelial cells, and the imbalance between these processes will inhibit normal endothelial function [1, 17]. It is termed oxidative stress when this balance shifts in favor of ROS production [17]. This action is stimulated in the presence of cardiovascular risk factors such as hypertension, diabetes, hypercholesterolemia, stroke, chronic smoking, and peripheral vascular diseases [16, 18].

In the cardiovascular system, three main sources of ROS production that inactive NO performance have been studied. These include xanthine oxidase, hydrogenated nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and eNOS [1, 16, 17, 18]. Xanthine oxidoreductase is a molybdenum-conjugated enzyme that exists in two interconvertible forms: xanthine dehydrogenase or xanthine oxidase – both of which catalyze xanthine to hypoxanthine and urate during purine metabolism. Xanthine dehydrogenase reduces NAD+, while xanthine oxidase prefers molecular oxygen leading to production of the SO anion and subsequently, H_2O_2 via superoxide dismutase (SOD) [15, 16].

NADH/NADPH oxidase is the enzyme that predominately drives SO radical production especially in leukocytes [15, 16]. It is also known to reside in vascular cells (particularly endothelial cells) and smooth muscle cells [16]. NADH/NADPH oxidase is regulated by cytokines and hormones that activate coupled Gq receptors and have been identified throughout the pathogenic process involved in vascular conditions. Most importantly, current data suggests that activation of SO from NADH/NADPH oxidase can initiate endothelial dysfunction by decreasing the bioavailability of NO [15].

The enzyme eNOS has been known to be a third source of ROS production in vascular tissues [7]. The mechanism behind this enzyme requires the cofactor BH_4 bound near the heme group to transfer electrons to *L*-arginine in order to produce NO as seen in Figure 1 [15, 16]. In the presence of BH_2, eNOS uncouples and as a result produces SO which causes a subsequent increase in H_2O_2. The reduction in NO levels in addition to the increase in SO levels further intensifies any oxidative stress present [16]. Recent preliminary studies have also shown cytochrome p450 to be an important source of SO and contribute to the inactivation of NO [1, 15]. Moreover, incomplete oxidative phosphorylation from mitochondria may contribute to additional oxidation of BH_4 to BH_2 intracellularly. Consequently, evidence suggests that endothelial dysfunction may be caused by the accelerated inactivation of NO by ROS via the previously mentioned major enzyme systems and this has been further implicated by numerous pathophysiological conditions [15, 16, 18].

Leukocyte-Endothelial Interactions

Leukocyte-endothelial cell adhesion is primarily generated by shear stress and decreased endothelial-derived NO which is caused by blood flow in postcapillary venules. The stress on the walls of the venules establishes the amount of leukocyte rolling and adhesion, while increases in the movement of blood are likely to counteract leukocyte-endothelial cell adhesion [19]. Leukocytes adhere to the side of the postcapillary venules and pass across the border in a process called diapedesis, or transmigration as seen in Figure 3 [9, 11]. Postcapillary venules are the preferred site of migration of blood not only because they begin the venous system, but also because they lack a significant muscle cell layer, possess a larger lumen than capillaries, and highly express cellular adhesion molecules (CAMs) [17]. CAMs defend the host cell by supplying the basis for cell communication, trafficking, and immune surveillance [17, 20].

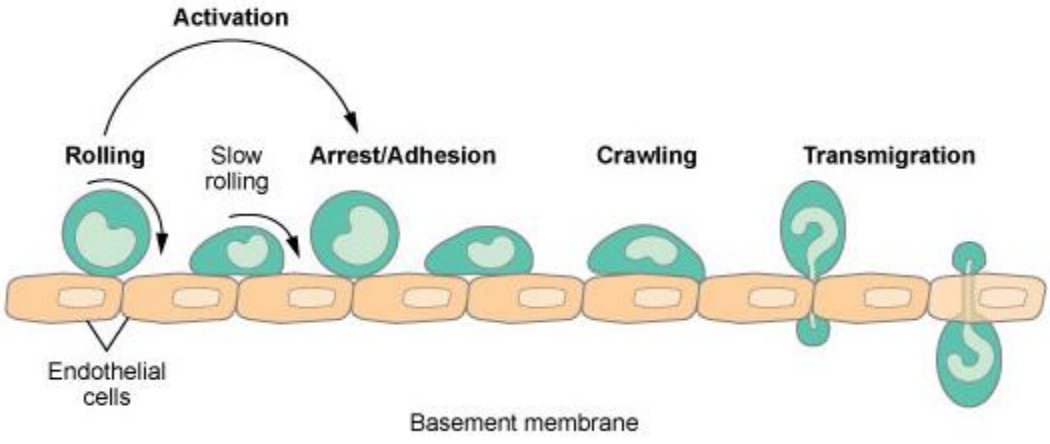

Figure 3: The leukocyte-endothelial interaction cascade [21].

CAMs are known to facilitate blood-endothelial cell interactions that can take place in all areas of the microvasculature under specific physiological and pathophysiological conditions. Disease may occur due to the loss of endothelial NO since the dysfunctional endothelium expresses a proinflammatory phenotype exemplified by atherogenic CAMs [22]. Selectins, integrins, and supergene immunoglobins make up the different families of adhesions molecules involved in the three stages of leukocyte recruitment (i.e. rolling, adherence, and transmigration) [23].

P-selectin is a CAM expressed on the surface of activated endothelial cells and platelets. When inactive, P-selectin in found in the cytoplasm of endothelial cells referred to as Weibel-Palade bodies and in alpha granules in platelets [23, 24]. It is activated and mobilized to the cell membrane within minutes of cytokine recognition via thrombogenic and inflammatory mediators. Decreased endothelial-derived NO bioavailability induces the upregulation of P-selectin. Because P-selectin expression is short-lived (i.e. minutes), it makes an ideal candidate to induce leukocyte-endothelial interactions [24]. During inflammation, endothelial P-selectin recruits leukocytes into postcapillary venules, whereas platelet-associated P-selectin promotes the aggregration of leuckocytes with platelets to form thrombi [23]. Generally, P-selectin has been noted to be the primary CAM affiliated with leukocyte rolling along the venular wall [24, 25].

Five members of the immunoglobin superfamily act as adhesion molecules during leukocyte-endothelial interactions [23]. Intracellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) are the most extensively studied [20]. ICAM-1 is constitutively expressed on the surface of vascular endothelial cells at low levels. Expression can be increased by stimulation from interleukins, tumor

necrosis factor alpha, interferon gamma, lipopolysaccharide, and decreased endothelial-derived NO bioavailability and increased SO/H_2O_2 release [20, 26, 27]. It is an essential signaling molecule connecting leukocyte adhesive interactions with downstream endothelial cell actions [28]. ICAM-1 serves to arrest leukocyte rolling and mediates firm adhesion between leukocytes and the vascular endothelium in postcapillary venules. The leukocyte integrin CD11a/18b binds to ICAM-1 to promote firm adhesion. This action precedes PECAM-1-dependent transmigration between endothelial cells [19].

PECAM-1, another member of the immunoglobulin superfamily, is an adhesion molecule that is constitutively expressed on leukocytes, platelets, and endothelial cells. It is mostly found at the intracellular borders of adjoining cells and is involved in neutrophil recruitment to inflamed sites in the body [9, 23, 29]. PECAM-1 can mediate adhesion of leukocytes through hemophilic or heterophilic interactions [23]. Most importantly, it is required for leukocyte transmigration, or diapedesis, through the perivascular subendothelial basal lamina [9, 23]. After transmigration, leukocytes can release harmful molecules (i.e. SO) and enzymes (i.e. elastase) which cause cell and tissue injury. Figure 4 depicts the steps and CAMs involved in leukocyte-endothelial cell recruitment.

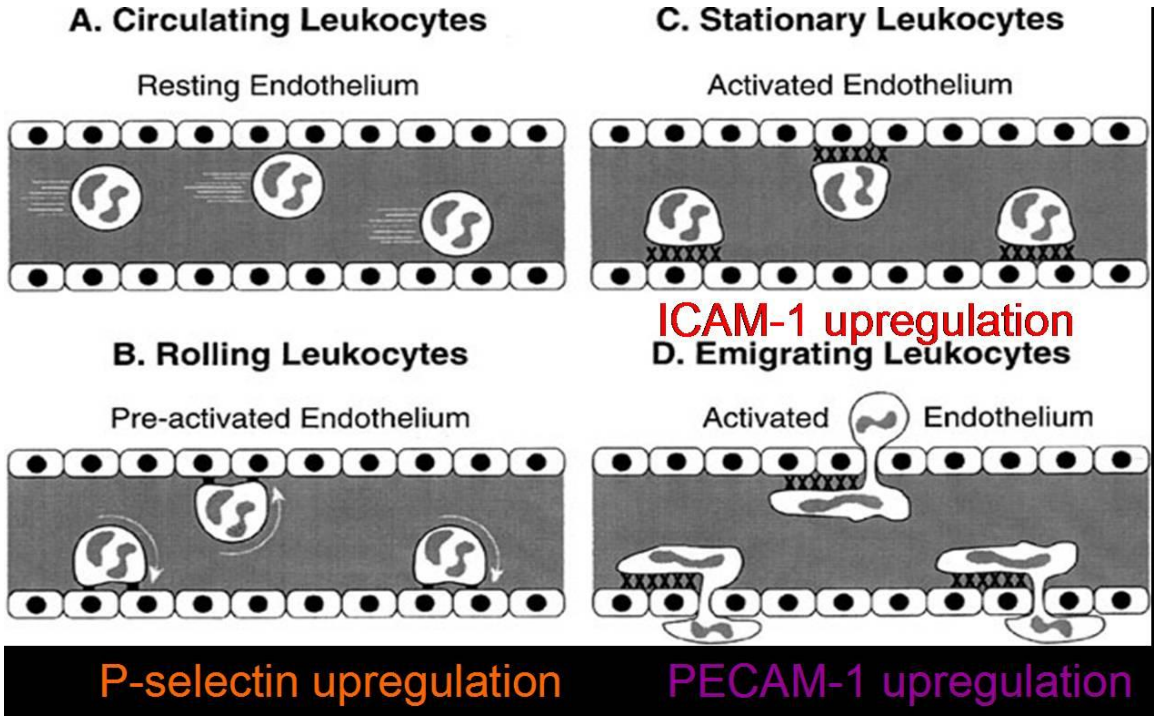

Figure 4: Steps and CAMs involved in leukocyte-endothelial cell recruitment [Adapted from 23].

Endothelial Dysfunction in Pathological Conditions

Diabetes

It is estimated that 25.8 million people in the United States suffer from diabetes, up from 23.7 million in 2007. The majority of diabetics suffer vascular complications associated with the disease which include blindness, kidney failure, hypertension, accelerated atherosclerosis, heart disease, and stroke [30, 31]. Change of diabetic microvasculature due to increased permeability to macromolecules and inflammation is known to cause diabetes complications. A number of studies have shown that increased endothelial permeability is an early sign of endothelial dysfunction displayed in diabetes and hyperglycemia found in both humans and animals [31, 32]. Both acute and chronic hyperglycemia increases leukocyte adhesion to the vascular walls of endothelium in

postcapillary venules and as a result decreases NO production [32]. Vascular alterations are the most frequent causes of morbidity and mortality in patients with diabetes which directly correlates with leukocyte adhesion in postcapillary venules associated with endothelial dysfunction [31, 33].

Ischemia-Reperfusion (I/R) Injury

Ischemia is a restriction of blood and oxygen to an organ which results in tissue damage or dysfunction and can eventually lead to cell necrosis [17]. Reperfusion refers to the restoration of blood flow to vessels and organs after a period of ischemia and can induce additional leukocyte-mediated injury. I/R produces an inflammatory response that both increases local injury and leads to systemic damage [7]. Although there are numerous improvements in interventional techniques, imaging, and drugs, morbidity from reperfusion remains remarkably high. The pathophysiological mechanisms involved as well as the development of potential therapeutic strategies need to be further studied in order to decrease the death rate [17]. A major event leading to endothelial dysfunction is the loss of endothelial cell release of NO which happens a few minutes after blood flow has been reestablished to an ischemic organ. This dysfunction is followed by neutrophil amplication which leads to capillary plugging and edema formation which in turn reduces coronary blood flow [19]. I/R is responsible for the complex interplay between local and systemic inflammation present in endothelial dysfunction that leads to numerous cardiovascular conditions.

Atherosclerosis

Vascular endothelium dysfunction which is associated with increased oxidative stress and decreased NO production is considered as an early and vital event in the pathogenesis of countless clinical cases involving atherosclerosis [11]. Atherosclerosis is a chronic disease that involves inflammatory mechanisms in which artery walls thicken due to a build-up of lipids [34]. The beginning stages are classified by endothelial cell injury characterized by recruitment of blood leukocytes which is associated with numerous predisposing risk factors for atherosclerosis including diabetes, smoking, hypertension, and hypercholesterolemia [1, 34]. Lipid accumulation then occurs in the arterial walls along with increased adherence of monocytes, macrophages, and T lymphocytes. Eventually, these cells transmigrate and release cytokines and growth factors that contribute to neointimal lesion formation [35]. Increasing evidence suggests that both inflammation and immune processes play a role in the acute thrombotic event of atherosclerosis [34].

Hypertension

Hypertension is widely known to be a risk factor of coronary disease and is found in over 20% of Western adult populations. It is defined as a systolic and/or diastolic pressure greater than or equal to 140 and/or 90 mmHg, or if the patient is being treated with anti-hypertensive drugs [16, 36]. NO is produced by endothelial cells to maintain vascular tone and normal function. Increased oxidative stress in hypertensive states has been known to lead to a reduced bioavailability of NO [1]. This decrease in NO bioavailability and therefore shift towards ROS has been shown to act as the central

method through which endothelial dysfunction occurs in hypertensive patients. In spontaneously hypertensive rats, the administration of pharamacological BH_4 supplementation exhibits ameliorated endothelial dysfunction [8]. Regardless of whether the origin is essential or secondary to endocrine or renal processes, endothelial dysfunction is now certainly recognized to be imperative in the pathophysiology of hypertension [16, 37].

Tetrahydrobiopterin (BH_4)/Dihydrobiopterin (BH_2)

When BH_4 is present, eNOS generates NO by converting *L*-arginine into *L*-citrulline. However, when BH_4 is oxidized to BH_2 under conditions of oxidative stress, such as I/R, the ratio of BH_2 to BH_4 can be increased. Under these conditions, BH_2 competes with BH_4 for binding at the oxgenase domain of eNOS with equal affinity causing eNOS to use molecular oxygen as a substrate instead of *L*-arginine to produce SO [6, 8, 37]. Oxidative stress and reduced NO bioavailability, brought on by SO production from the addition of BH_2, comprises a distinctive characteristic and common generic marker for endothelial dysfunction in chronic vascular diseases, such as atherosclerosis and hypertension [9, 37]. Moreover, reduced serum BH_4 levels are associated with coronary artery disease, hypertension, and atherosclerosis [36]. The reduction of NO bioavailability under these conditions can often foreshadow undesirable cardiovascular outcomes, such as increased leukocyte-endothelial interactions, which can lead to microvascular plugging and an overall reduction in perfusion to tissue [7, 11, 19].

The availability of BH_4 to function as an essential cofactor in order to facilitate eNOS to produce NO is well described regarding *in vitro* systems [6]. BH_4 functions to

aid in the coupling of electron transfer at the heme domain of eNOS to cause chemical reduction of molecular oxygen and *L*-arginine oxidation to generate NO. Under normal physiological conditions BH_4 is required for eNOS to produce NO which is important to maintain normal blood flow and diastolic blood pressure [38]. However, incomplete oxidative phosphorylation in dysfunctional mitochondrial endothelium may lead to the initial oxidation of BH_4 resulting in increased BH_2 to BH_4 ratio leading to eNOS principally producing SO instead of NO [6, 22]. Uncoupled eNOS can then generate additional SO to increase oxidation of BH_4 and BH_2/BH_4 ratio [7].

Previous studies have revealed that BH_4 dose-dependently increases NO release in non-ischemic rat aortic segments as well as in femoral I/R *in vivo*. Whereas, BH_2 dose-dependently decreases NO release in these preparations [7, 11, 39]. Furthermore, BH_2 resulted in cardiac contractile dysfunction associated with increased leukocyte adherence and transmigration in myocardial I/R compared to BH_4 treatment. By contrast, BH_4 significantly reduced leukocyte adherence/transmigration and increased postreperfused left ventricular developed pressure in isolated rat hearts compared to control and BH_2 treated hearts [7, 11, 39]. These results collectively suggest that BH_2 may induce leukocyte rolling, adherence and transmigration in rat mesenteric venules via uncoupled eNOS by reducing NO production which is similar to previous studies utilizing L-NAME, a NOS inhibitor, to induce leukocyte-endothelial interactions by intravital microscopy. Nevertheless, the role of eNOS uncoupling in regulating leukocyte-endothelial interactions has not been confirmed *in vivo* in real-time.

Therefore, BH_2 and BH_4 serve as pharmacological tools to stimulate and attenuate leukocyte-endothelial interactions using intravital microscopy to monitor these responses

in mesenteric venules of anesthetized rats. Adhesion molecule, such as P-selectin and ICAM-1, expression will be further determined in this setting. This project will establish the basis for the future study of combining an eNOS activity enhancer or inhibitor with BH_2 to facilitate or attenuate leukocyte-endothelial interactions in the presence/absence of BH_4. The long-term goal of this project will provide experimental evidence to support novel pharmacological intervention in the settings of vascular endotheial dysfunction associated with ischemic heart disease, coronary bypass/angioplasty, and organ transplantation.

Hypothesis

The present study uses intravital microscopy to observe, in real time, leukocyte-endothelial interactions at the cellular level within rat mesenteric post capillary venules [4, 7, 11]. BH_2 was administered in order to reduce the cellular BH_4/BH_2 ratio and facilitate eNOS uncoupling to decrease NO bioavailability and increase leukocyte-endothelial interactions. Moreover, the effects of BH_2 to enhance leukocyte-endothelial interactions will be compared to tradition tools such as L-NAME to delineate the importance of eNOS uncoupling compared to inhibiting eNOS activity with a methyl ester analog of *L*-arginine. It was hypothesized that administration of BH_4 to increase the BH_4/BH_2 ratio will attenuate the leukocyte-endothelial interactions induced by BH_2. The proposed project serves as a basis for future study of drugs that relate to pathophysiological conditions, such as I/R, stroke, and cardiovascular diseases [37].

Methods

Intravital Microscopy

Vascular endothelium plays an essential role in maintaining the homeostatic properties of the cardiovascular system in the human body [3]. Intravitral microscopy provides a means to explore the leukocyte-endothelial interactions occurring at a microscopic level. Although often associated with a mild inflammatory response, it is most advantageous to analyze vascular endothelium *in vivo*. This quantitative and qualitative technique provides direct observation of leukocyte-endothelial interactions in real time [11, 40]. It allows the supervision of microcirculation changes in live animals. Additionally, it clarifies results that were acquired through *in vitro* experiments.

Experimental Setup

The Institutional Animal Care and Use Committee of the Philadelphia College of Osteopathic Medicine have approved all animals used in this study. Animals were allowed free access to food and water throughout the entire study and were housed in an animal facility that maintained 12 hour light-dark cycle conditions. Male Sprague-Dawley rats, weighing 275 to 325g, were anesthetized with 60 mg/kg pentobarbital sodium intraperitoneally (i.p., induction anesthesia) and maintained via 30 mg/kg pentobarbital sodium (i.p.) as previously described [11]. A PE-50 polyethylene catheter was inserted in the left carotid artery for monitoring of mean arterial blood pressure (MABP). The abdominal cavity was opened via a midline laparotomy, and a loop of ileal mesentery was exteriorized and placed in a temperature controlled Plexiglas chamber for

observation of leukocyte-endothelial interactions via intravital microscopy as shown in figure 5 (Nikon Corp., Tokyo, Japan).

Krebs' buffer alone, Krebs' buffer containing 100 or 200 µM concentrations of BH_2 (MW= 239.2; Cayman Chemicals) alone or in the presence of 100 or 250 µM BH_4 (MW=314.2; Cayman Chemicals), as well as L-NAME, was used to superfuse the rat mensentery for 2 hours. The Krebs' buffer has the following composition (in mmol/l):17 dextrose, 120 NaCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, 0.5 EDTA, 5.9 KCl, and 1.2 $MgCl_2$. The solution was aerated with 95% N_2-5% CO_2 and equilibrated at a pH of 7.3-7.4 at 37 degrees celsius. The leukocyte-endothelial interactions were recorded for a 2 minute observation period during baseline and at 30 minute intervals after superfusion of Krebs' buffer or drugs. The image was analyzed by Image-pro (Media Cybernetics, Inc., Bethesda, MD).

Figure 5: Experimental setup for intravital microscopy. Inserted picture in upper right → Exteriorized loop of mesenteric tissue undergoing superfusion of test solution.

Analysis of leukocyte-endothelial interactions was based on the following criteria:1) Leukocytes will be considered to be rolling if they are moving at a velocity significantly slower than that of red blood cells and expressed as the number of rolling leukocytes per minute. 2) Leukocytes are judged to be adherent if they remain stationary for >30 seconds. Adherence is expressed as the number of leukocytes adhering to the endothelium/100 μm of vessel length. 3) The number of extravasated leukocytes in the rat mesenteric tissue is counted in the tissue area adjacent to the postcapillary venules and normalized with respect to perivascular area (20x100 μm) [4].

Treatment Groups

The experimental design consists of seven groups (with six subjects in each group) for recording leukocyte-endothelial interactions by intravital microscopy and performing hematoxylin/eosin staining on the excised mesenteric tissue thereafter. The proposed concentration of the drugs are based on their dose-response effects from previous cardiac function, aortic segment and femoral I/R studies [7, 11, 22]. Part of the superfused mesenteric tissue during the intravital microscopy experiment was preserved in 4% paraformaldehyde solution in PBS to be later used for hematoxylin/eosin staining. The remaining tissue sample was quickly frozen with liquid nitrogen and stored it at -80 degrees celsius. The stored mesenteric tissue from different experimental groups will be used to investigate the expression of P-selectin and ICAM-1 by western blot and/or immunohistochemistry in future studies [22].

Treatment Groups:

1. Control: Superfusion of Krebs' buffer alone to determine if the animal surgical preparation and the physiological buffer can cause an increase in leukocyte-endothelial interactions.
2. L-NAME: Superfusion of 50 µM to determine if this non-selective NOS inhibitor can cause an increase in leukocyte-endothelial interactions.
3. BH_2: Superfusion of 100 µM to determine the dose-dependent response by increasing the BH_2/BH_4 ratio to induce leukocyte-endothelial interactions.

4. BH_2: Superfusion of 200 µM to determine a dose-dependent response by further increasing the BH_2/BH_4 ratio that can induce leukocyte-endothelial interactions.
5. BH_4: Superfusion of 250 µM to determine if BH_4 alone exerts any effect on leukocyte-endothelial interactions compared to the Krebs' buffer control.
6. $BH_2 + BH_4$: Superfusion of 100 µM BH_2 + 100 µM BH_4 to determine the dose-dependent anti-BH_2 induced inflammation response if there is an increase in the BH_2/BH_4 ratio to 1:1.
7. $BH_2 + BH_4$: Superfusion of 100 µM BH_2 + 250 µM BH_4 to determine the dose-dependent anti-BH_2 induced inflammation response if a further increase in the BH_2/BH_4 ratio to 1:2.5.

Hematoxylin and Eosin Staining

After the experiment, the loop of the ileal mesentery that was superfused with test solutions was removed and quickly placed in a 4% paraformaldehyde solution in PBS for future histological analysis of leukocyte-endothelial interactions via hematoxylin/eosin staining. At a later time, three representative sections of the ileal mesenteric tissue were selected from the Krebs' control, 50 µM L-NAME, 100 µM BH_2, 200 µM BH_2, 250 µM BH_4, 100 µM BH_2/100 µM BH_4, and 100 µM BH_2/250 µM BH_4 groups for histological analysis. The three samples for each group were chosen based on how close their leukocyte-endothelial interaction intravital microscopy data was to the mean value of the group. Each tissue sample was embedded in paraffin wax, cut into 4.5 µM serial sections,

and placed onto 25 x 75 x 1.0 mm Colorfrost® microscope slides. These sections were later deparaffinized and rehydrated, stained with hematoxylin and eosin, and observed using light microscopy. Leukocyte adherence and transmigration into the tissue were counted in areas containing venules and arterioles in the serosa and mesentery. These numbers were expressed as adhered and transmigrated leukocytes/mm^2 [7, 11].

Statistical Analysis

All data will be represented as means ± SEM and analyzed by ANOVA using post hoc analysis with the Fisher's PLSD test to detect differences among experimental groups within each aim. Probability values are considered to be statistically significant when $P<0.05$.

Results

The initial MABP recordings were not significantly different among the seven experiment groups. Throughout the entire observation time, the MABP stayed within a normal range with mean values of 112 ± 5 mmHg at baseline and 108 ± 3 mmHg at 2 hours. Blood flow within the selected venules was not directly measured, however throughout the experiment there were no apparent observable changes.

Leukocyte-Endothelial Interactions via Intravital Microscopy

Rolling

Among the seven experimental groups, leukocyte rolling showed no significant difference at baseline (Fig. 6). Krebs' buffer was maintained around 14±2 cells/minute during the entire observation period; consequently leukocyte rolling did not significantly change. By contrast, 50 μM L-NAME generated a significant increase in rolling beginning at the 60 minute interval and continuing throughout the rest of the observation period. At the 2 hour time point, leukocyte rolling was significantly increased to 47±4 cells/minute ($n=6$, $P<0.05$) compared to the Krebs' buffer control. BH_2 (100 μM) generated a significant increase in rolling throughout with entire 2 hour time period that finally increased to 51±3 cells/minute ($n=6$, $P<0.01$) at the last time interval. Similarly, when 200 μM BH_2 was administered, rolling increased throughout all time periods with the mean being 59±10 cells/minute (results not shown in graph) at the 2 hour time period and was not different from 100 μM BH_2. Thereafter, the 100 μM of BH_2 was used to induce leukocyte-endothelial interactions in subsequent groups.

In order to determine the dose-dependent response in the BH_2/BH_4 ratio in leukocyte-endothelial interactions, both 100 µM BH_2/100 µM BH_4 and 100 µM/250 µM BH_4 were used. In regards to the 1:1 ratio of BH_2/BH_4, leukocyte rolling significantly decreased in comparison to 100 µM BH_2 at 30 minutes with 17±1 cells/minute to 60 minutes with 16±2 cells/minute ($n=6$, $P<0.05$), but significantly increased rolling at later time points compared to Krebs' buffer ending with 43±9 cells/minute ($n=6$, $P<0.05$) at the 120 minute interval. By contrast, decreasing the BH_2 to BH_4 ratio from 1:1 (i.e. 100 µM BH_2/100 µM BH_4) to 1:2.5 (i.e. 100 µM BH_2/250 µM BH_4), significantly decreased BH_2-induced rolling throughout the entire 2 hour observation period ending with 26±4 cells/minute ($n=6$, $P<0.01$) at 120 minutes, which was not different from Krebs' buffer. In regard to 250 µM of BH_4, it was not significantly different from Krebs' buffer throughout the entire observation period. However, it did show a modest decrease in leukocyte rolling at the 30, 60, 90 and 120 minute time periods with 13±1 cells/minute, 9±1 cells/minute, 8±1 cells/minute, and 14±1 cells/minute respectively, in comparison to baseline with 22±1 cells/minute. Figure 6 illustrates leukocyte rolling among the given experimental groups.

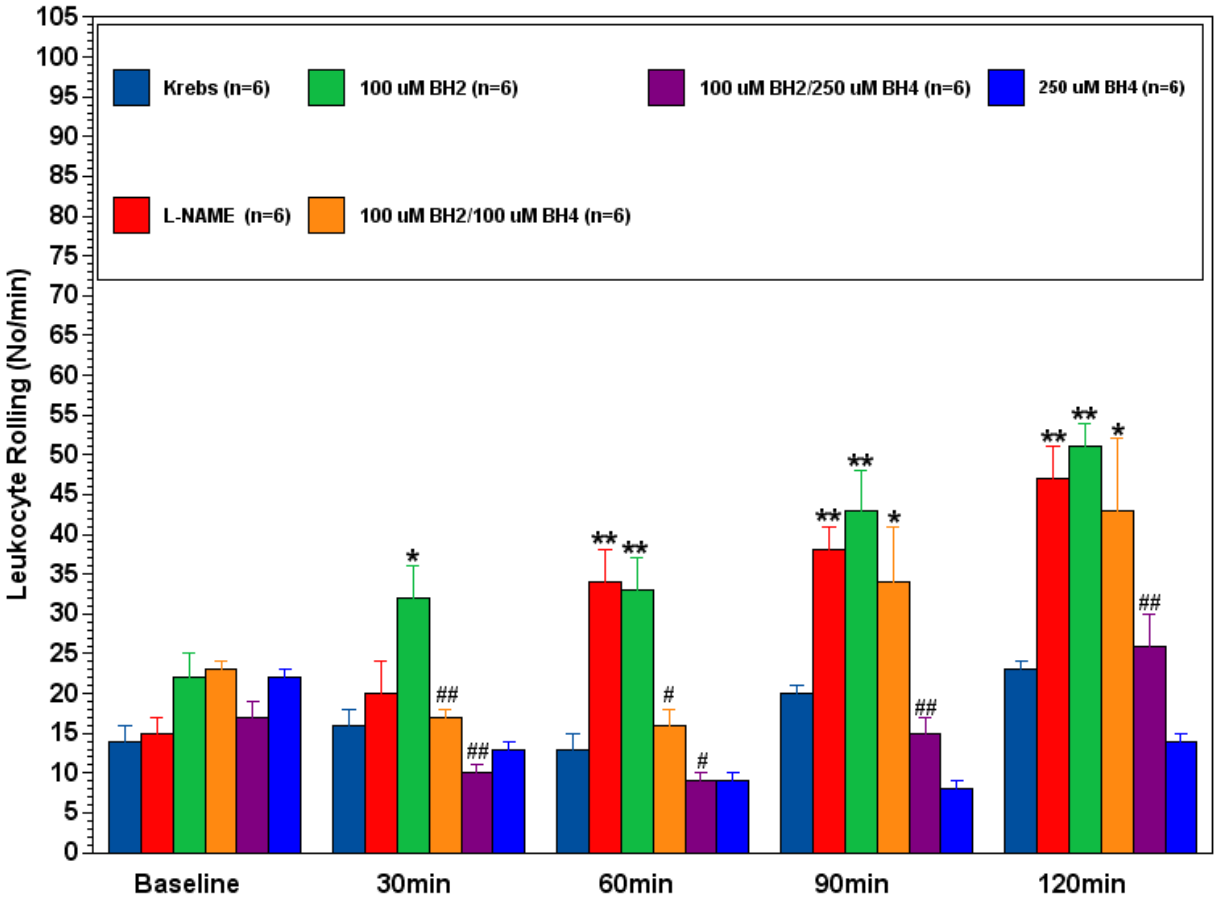

Figure 6: Leukocyte rolling among different experiment groups. L-NAME and 100 µM BH_2 significantly increased leukocyte rolling (*P<0.05, **P<0.01 from Krebs'). By contrast, BH_2/BH_4 dose-dependently decreased leukocyte rolling (#P<0.05, ##P<0.01 from 100 µM BH_2).

Adherence

At baseline, there was no statistical significance among any of the experimental groups (Fig. 7). Krebs' buffer maintained similar cell adhesion throughout the entire time period, with a slight increase towards the later time intervals, from baseline with 3±1 cells/minute versus the 90 minutes with 5±0 cells/minute and 120 minutes with 6±1 cells/minute. However, both L-NAME and 100 µM BH_2 increased leukocyte adherence from baseline at all time points and became statistically significant at 90 and 120 minute

time periods with 10±2 cells/minute (n=6, P<0.01) and 17±2 cells/minute, respectively (n=6, P<0.01) for L-NAME and 9±1 cells/minute (n=6, P<0.05) and 11±1 cells/minute, respectively (n=6, P<0.05) for 100 μM BH_2, both in comparison to Krebs' buffer.

For the 1:1 ratio of BH_2/BH_4 leukocyte adherence was similar to Krebs' at baseline with 5±1 cells/minute and did not increase during the first 60 minutes. However, leukocyte adherence increased to 7±1 cells/minute and 9±2 cells/minute at 90 and 120 minutes, respectively. The adherence data was not significantly different from BH_2-induced leukocyte adherence at these time points. By contrast, decreasing the BH_2 to BH_4 ratio from 1:1 (i.e. 100 μM BH_2/100 μM BH_4) to 1:2.5 (i.e. 100 μM BH_2/250 μM BH_4), significantly decreased BH_2-induced adherence at the 120 minute time period ending with 4±1 cells/minute (n=6, P<0.01), and was not different from Krebs' buffer throughout the 2 hour time period. Regarding 250 μM of BH_4, it was not significantly different from Krebs' buffer in leukocyte adherence throughout the entire observation period. The differences in leukocyte adhesion among the different experimental groups are illustrated in Figure 7.

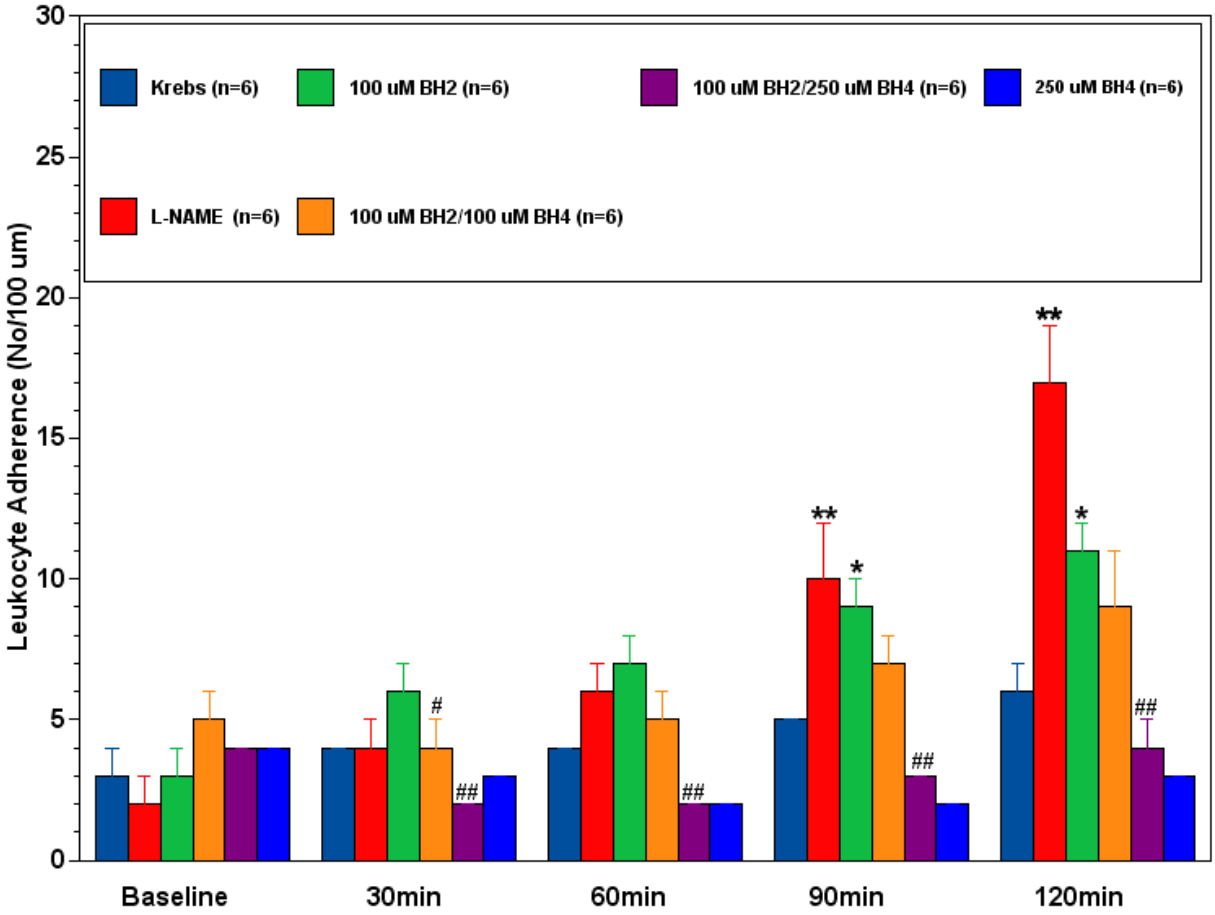

Figure 7: Leukocyte adherence among different experiment groups. L-NAME and 100 µM BH_2 significantly increased leukocyte adherence (*P<0.05, **P<0.01 from Krebs'). By contrast, BH_2/BH_4 dose-dependently decreased leukocyte adherence (#P<0.05, ##P<0.01 from 100 µM BH_2).

Transmigration

Leukocyte transmigration was similar to rolling and adherence in that there were no significant changes at baseline among the experimental groups (Fig. 8). Over the 120 minute observation period, Krebs' buffer did not show any significant changes in leukocyte transmigration. In comparison to Krebs' buffer, both L-NAME and 100 µM BH_2 significantly increased throughout all time periods with L-NAME having 3±0

cells/minute (n=6, P<0.01) at 30 minutes and 10±1 cells/minute (n=6, P<0.01) at 120 minutes, and 100 µM BH_2 having 3±0 cells/minute (n=6, P<0.05) at 30 minutes and 7±0 cells/minute (n=6, P<0.01) at 120 minutes.

When 100 µM BH_2/100 µM BH_4 was superfused at 30 minutes, there was a significant decrease in leukocyte transmigration with 2±0 cells/minute (n=6, P<0.01) in comparison to 100 µM BH_2. This 1:1 combination lost its' significant in comparison to BH_2-induced leukocyte transmigration at all of the later time points although it was not different from Krebs' buffer at any time point during the 2 hour observation period. In contrast, the 1:2.5 BH_2/BH_4 ratio significantly decreased BH_2-induced leukocyte transmigration at all time points within the 2 hour observation period beginning with 2±0 cells/minute (n=6, P<0.05) at 30 minutes and finishing up with 2±0 cells/minute (n=6, P<0.01) at 120 minutes, in comparison to 100 µM BH_2 and was not different from the Krebs' control. In regard to 250 µM of BH_4, it was not significantly different from Krebs' buffer in leukocyte transmigration throughout the entire observation period. Leukocyte transmigration is illustrated among the different experimental groups in Figure 8.

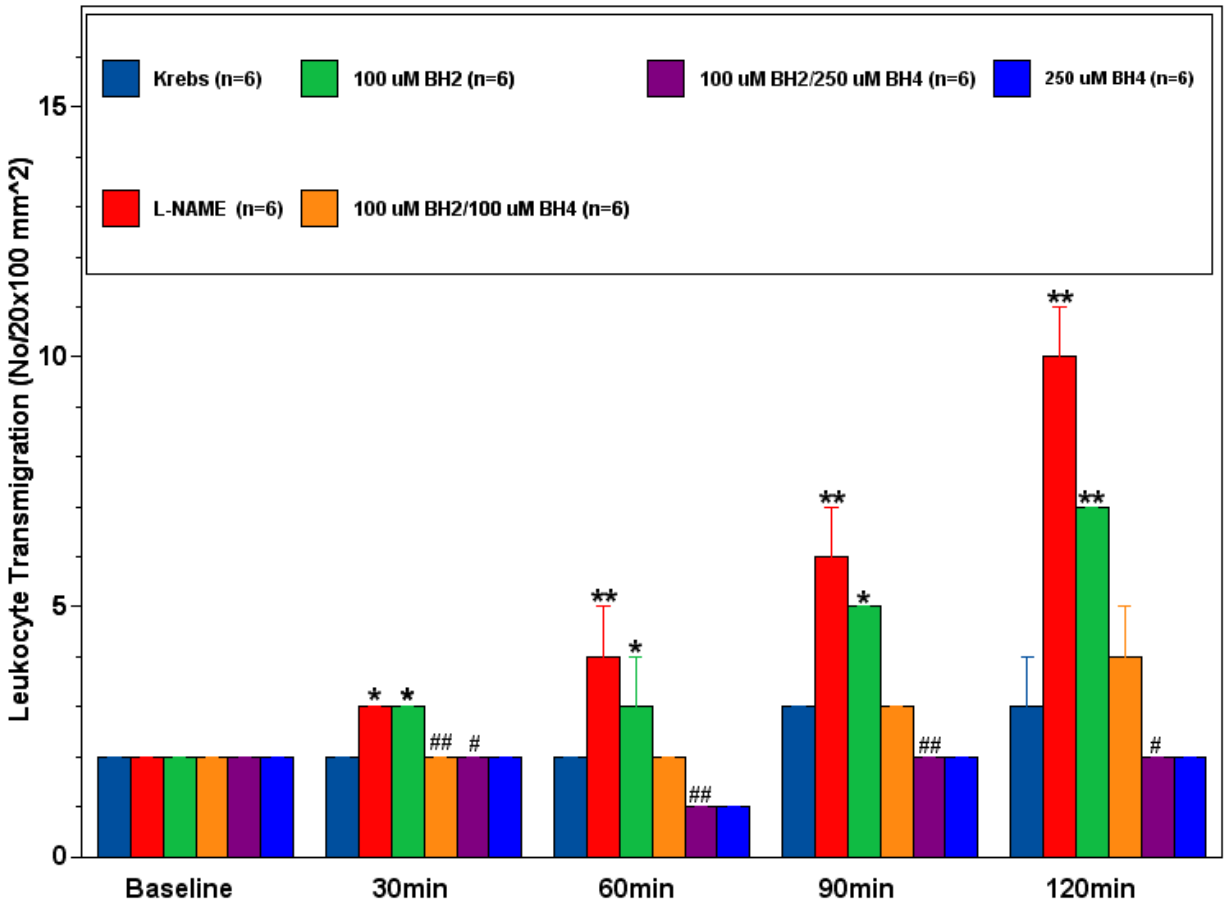

Figure 8: Leukocyte transmigration among different experiment groups. L-NAME and 100 µM BH_2 significantly increased leukocyte transmigration (*P<0.05, **P<0.01 from Krebs'). By contrast, decreasing the BH_2/BH_4 ratio dose-dependently decreased BH_2-induced leukocyte transmigration during all time periods. (#P<0.05, ##P<0.01 from 100 µM BH_2).

Leukocyte Adherence and Transmigration via Hematoxylin and Eosin (H&E) Staining

Hematoxylin and eosin were used to stain the nucleus and cytoplasm, respectively, in rat mesenteric tissue which was superfused by the test solutions throughout the experimental observation phase to further quantify leukocyte vascular adherence and transmigration. Neutrophils composed the majority of the adhered and

transmigrated leukocytes as seen under the light microscope, although a small percentage were found to be monocytes. Figures 9, 10, and 11 show representative H&E staining in the Krebs' control, 50 μM L-NAME, 100 μM BH_2, 200 μM BH_2, 250 μM BH_4, 100 μM BH_2/100 μM BH_4, and 100 μM BH_2/250 μM BH_4 groups.

Similar to leukocyte-endothelial interactions observed by intravital microscopy, there was low leukocyte adherence with 51 cells/mm^2 and transmigration with 52 cells/mm^2 in the Krebs' control group (Figure 12). By contrast, the 50 μM L-NAME group exhibited a five-fold increase in leukocyte adherence of 244 cells/mm^2 ($P<0.01$) and transmigration of 286 cells/mm^2 ($P<0.01$) compared to the Krebs' buffer control. Results for both the 100 μM BH_2 and 200 μM BH_2 were similar to L-NAME with the adhered leukocytes containing 271 cells/mm^2 ($P<0.01$) and 261 cells/mm^2 ($P<0.01$), respectively, and the transmigrated containing 237 cells/mm^2 ($P<0.01$) and 256 cells/mm^2 ($P<0.01$), respectively.

For the 100 μM BH_2/100 μM BH_4 ratio, leukocyte adherence was similar to BH_2-induced adherence with 203 cells/mm^2 ($P<0.01$) and transmigration with 177.8 cells/mm^2 ($P<0.01$) compared to Krebs' buffer which is consistent with the intravital microscopy data at the 2 hour time period. Although similar to BH_2, there was a modest, yet significant decrease in leukocyte adherence and transmigration compared to BH_2. By contrast, decreasing the BH_2/BH_4 ratio (i.e. 100 μM BH_2/250 μM BH_4) resulted in significantly lower leukocyte adherence and transmigration with 28 cells/mm^2 ($P<0.01$) and 21 cells/mm^2 ($P<0.01$), respectively compared to BH_2 which is also consistent with intravital microscopy data. The 250 μM BH_4 group showed a decreased leukocyte adherence and transmigration with 29 cells/mm^2 that adhered and 22 cells/mm^2 that

transmigrated, but it was not statistically different from the Krebs' control which corresponds with intravital microscopy data. The summary data among the experimental groups is illustrated in Figure 12.

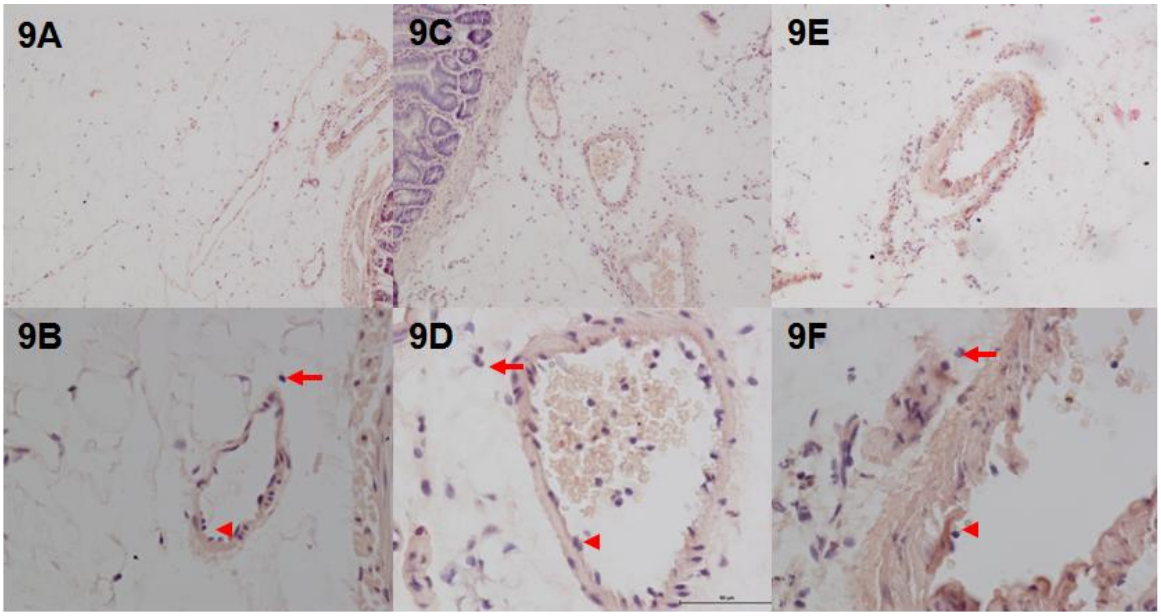

Figure 9: Representative pictures of leukocytes by H&E staining of mesenteric tissues from Krebs' control (9A & 9B), 50 μM L-NAME (9C & 9D), and 100 μM BH_2 (9E & 9F). 9A, 9C, & 9E are at 10x while 9B, 9D, and 9F are at 40x. Compared to Krebs' control, the L-NAME and 100 μM BH_2 treated groups exhibited a marked increase in adhered and transmigrated leukocytes. Arrow head indicates leukocyte adherence and full arrow indicates leukocyte transmigration. Scale bar: 50 μm.

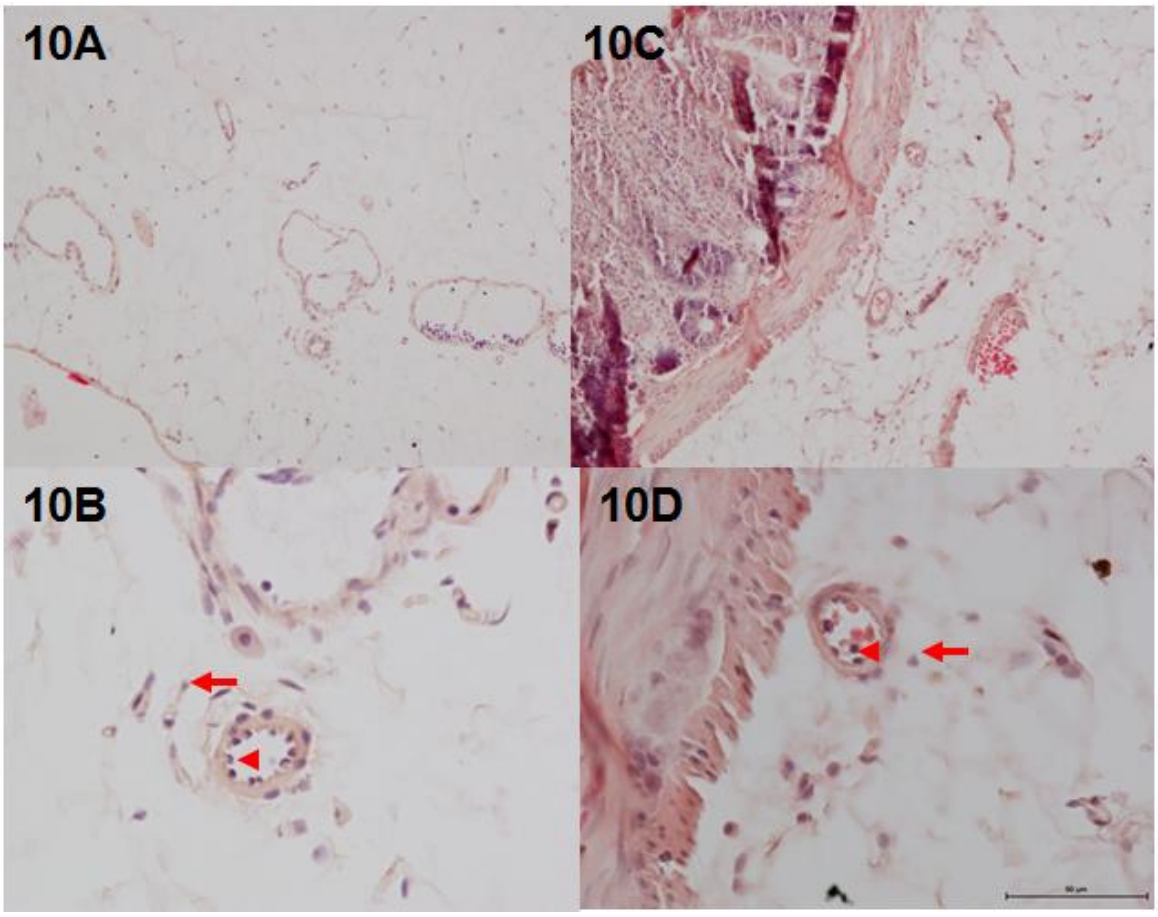

Figure 10: Representative pictures of leukocytes by H&E staining of mesenteric tissues from 200 μM BH_2 (10A & 10B) and 250 μM BH_4 (10C & 10D). 10A & 10C are at 10x while 10B & 10D are at 40x. Compared to both concentrations of BH_2 (100 μM BH_2 in figure 9E & 9F), the BH_4 treated group exhibited a marked decrease in adhered and transmigrated leukocytes. Arrow head indicates leukocyte adherence and full arrow indicates leukocyte transmigration. Scale bar: 50 μm.

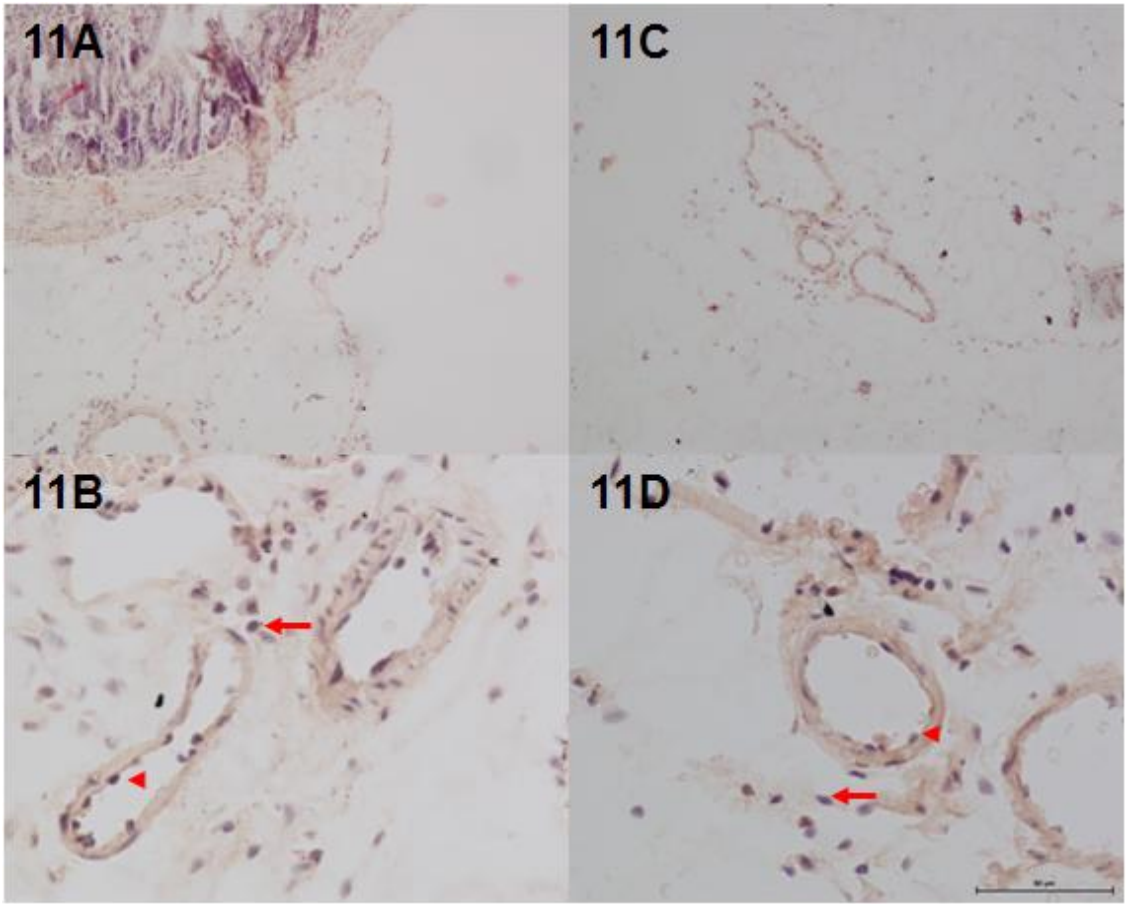

Figure 11: Representative pictures of leukocytes by H&E staining of mesenteric tissues from 100 μM BH_2/100 μM BH_4 (11A & 11B), and 100 μM BH_2/250 μM BH_4 (11C & 11D). 11A & 11C are at 10x while 11C & 11D are at 40x. Compared to 100 μM BH_2, the 100 μM BH_2/100 μM BH_4 treated group exhibited a decrease in adhered and transmigrated leukocytes, and the 100 μM BH_2/250 μM BH_4 showed a further decrease in induced leukocyte-endothelial interactions. Arrow head indicates leukocyte adherence and full arrow indicates leukocyte transmigration. Scale bar: 50 μm.

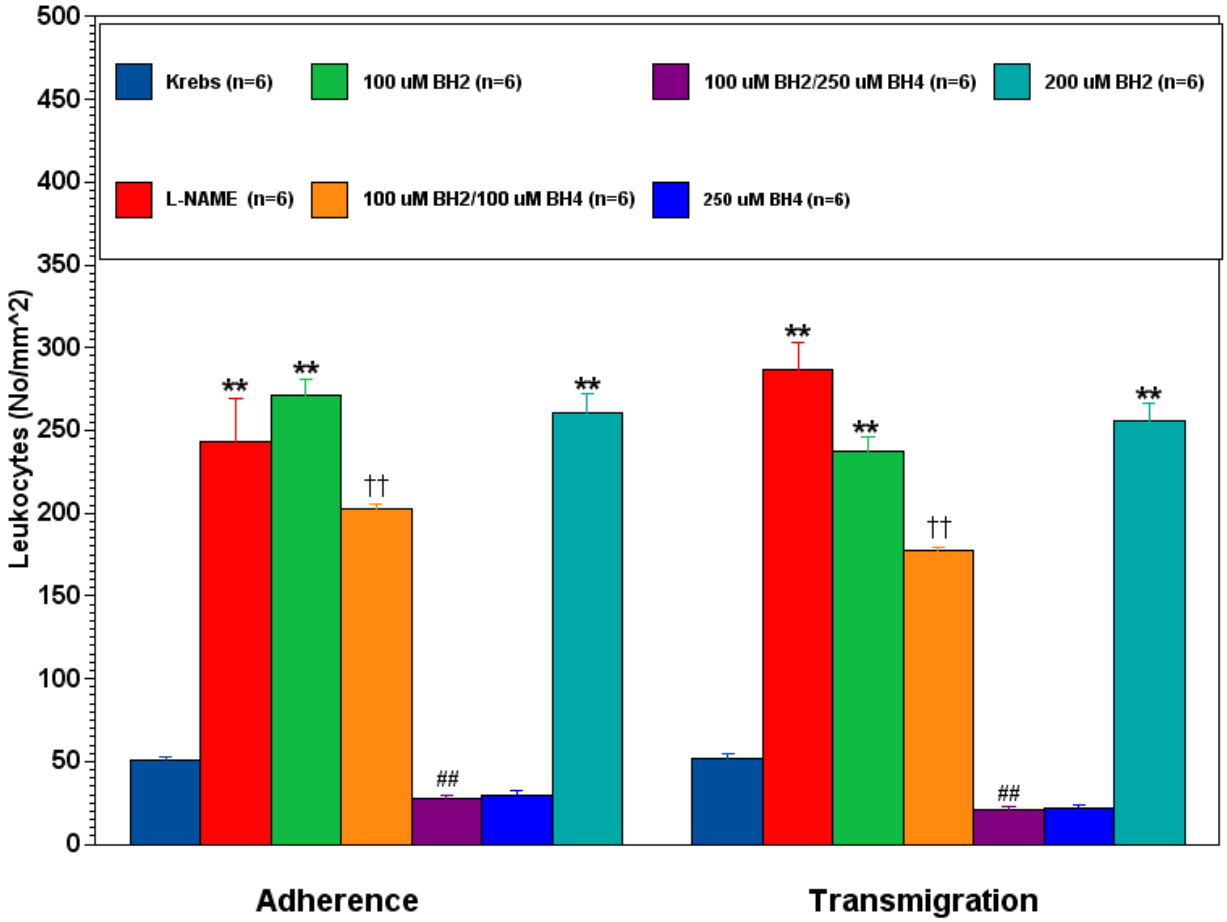

Figure 12: Leukocyte adherence and transmigration by H&E staining of mesenteric tissues from Krebs' control, 50 µM L-NAME, 100 µM BH_2, 200 µM BH_2, 250 µM BH_4, 100 µM BH_2/100 µM BH_4, and 100 µM BH_2/250 µM BH_4. Compared to Krebs control, the L-NAME and 100/200 µM BH_2 treated groups exhibited a significant increase in adhered and transmigrated leukocytes (**P<0.01 from Krebs'). By contrast, 100 µM BH_2/100 µM BH_4 (††P<0.01 from 100 µM BH_2, Krebs', and 100 µM BH_2/250 µM BH_4) and 100 µM BH_2/250 µM BH_4 (##P<0.01 from 100 µM BH_2) significantly decreased leukocyte adherence and transmigration compared to 100 µM BH_2, whereas the 100 µM BH_2/100 µM BH_4 also significantly increased leukocyte adherence and transmigration in comparison to Krebs' buffer and 100 µM BH_2/250 µM BH_4.

Discussion

Summary of Major Findings

The major findings in this study were as follows: 1.) 50 µM L-NAME, 100 µM BH_2, and 200 µM BH_2 similarly and significantly increased leukocyte-endothelial interactions compared to Krebs' buffer control group. 2.) The administration of the BH_2/BH_4 dose-dependently (i.e. 1: 2.5 ratio compared to 1:1 ratio) attenuated the leukocyte-endothelial interactions in comparison to the 100 µM BH_2. 3.) H&E staining of the experimentally superfused mesenteric tissue from 50 µM L-NAME, 100 µM BH_2, and 200 µM BH_2 groups showed a significant increase in leukocyte vascular adherence and transmigration compared to the Krebs' buffer control group. This result confirmed the inflammatory effects of the BH_2 and L-NAME observed via intravital microscopy. 4.) Furthermore, H&E staining of the experimentally superfused mesenteric tissue from 250 µM BH_4, 100 µM BH_2/100 µM BH_4, and 100 µM BH_2/250 µM BH_4 showed a significant decrease in leukocyte vascular adherence and transmigration compared to the 100 µM BH_2. Increasing the BH_4 to BH_2 ratio correlated with better attenuation of leukocyte-endothelial interactions suggesting that promoting coupled eNOS activity contributed to the anti-inflammatory effects of BH_4. Therefore, this study supports the anti-inflammatory effects of BH_4 when the vascular endothelium becomes dysfunctional by promoting eNOS coupled status.

Effects of L-NAME on Leukocyte-Endothelial Interactions

Superfusion of Krebs' buffer alone for 2 hours did not significantly increase leukocyte-endothelial interactions. The data suggests that the animal preparation and the

superfusion of a physiological solution (i.e. Krebs' buffer) for 2 hours did not significantly induce an inflammatory response. By contrast, L-NAME significantly increased leukocyte rolling, adherence, and transmigration during 2 hours of superfusion compared to the Krebs' control group. This result is consistent with previous studies that found 50 µM L-NAME to consistently increase the number of rolling, adhered, and transmigrated leukocytes [11, 41]. A study conducted by Kubes et. al. also discovered that L-NAME-induced leukocyte vascular adherence can be completely overturned by increasing the concentration of exogenous *L*-arginine by five times. [42]. This suggests the increase in L-NAME-induced leukocyte-endothelial interactions transpires because L-NAME competes with *L*-arginine for the active site in eNOS resulting in decreased NO release. The decrease in NO release in turn leads to endothelial dysfunction and inflammation [11, 41]. Thus, this project further supports that L-NAME is an effective agent that synthetically reproduces endothelial dysfunction and causes leukocyte-endothelial interactions in rat mesenteric tissue.

BH_2 Effects Compared to L-NAME on Leukocyte-Endothelial Interactions

BH_2 and L-NAME both showed an increase from baseline in leukocyte-endothelial interactions throughout the entire observation period. Because L-NAME is a nonselective NOS inhibitor, it competes with *L*-arginine to inhibit NO production, causing SO to be released instead of NO [6]. As a result, L-NAME caused an increase in leukocyte rolling, adherence, and transmigration during the intravital microscopy experiments, which is additionally well-noted in previous literature [11, 41]. By contrast, BH_4 or BH_2 serve as cofactors to determine eNOS production of NO or SO, respectively.

Normally, BH_4 binds to the oxygenase domain of eNOS and facilities the eNOS coupled status and generates NO. Whereas when BH_4 becomes oxidized to BH_2, the electron transfer becomes uncoupled to the *L*-arginine oxidation. Consequently, the ferrous-dioxygen complex dissociates and SO is generated from the oxygenase domain from molecular oxygen [8]. Similar to L-NAME, BH_2 administration resulted in an increase in leukocyte-endothelial interactions at all time points throughout the 2 hour observation period. BH_2 was superfused to induce inflammation in this study and change the intracellular BH_2/BH_4 ratio causing eNOS uncoupling which simulates the eNOS changes under some pathophysiological conditions such as I/R or hyperglycemia. Thus, L-NAME cannot change the eNOS coupled or uncoupled status compared to modulating the ratio of the BH_4 to BH_2. It is known that reduced serum BH_4 levels are associated with coronary artery disease and hypertension [36]. This study suggests that BH_2-induced inflammation model is more suitable to test the effects of eNOS regulation under various pathophysiological conditions.

Effects of BH_2/BH_4 on Leukocyte-Endothelial Interactions

At baseline, there was no significant difference among any of the experimental groups in this study for rolling, adherence, transmigration, or mean arteriolar blood pressure (MABP). This finding denotes that the initial conditions after preparation of the rat were similar among the various experimental groups. In contrast to the L-NAME- and 100 μM BH_2-induced increases in leukocyte-endothelial interactions, decreasing the BH_2/BH_4 ratio dose-dependently attenuated leukocyte rolling, adherence, and transmigration in rat postcapillary venules. The 1:1 ratio (i.e. 100 μM BH_2/100 μM BH_4)

significantly reduced leukocyte rolling, adherence, and transmigration only within the first 60 minutes of superfusion, whereas the 1:2.5 ratio decreased leukocyte rolling, adherence, and transmigration throughout the entire 2 hour period. For the 1:1 ratio, this may be due to increased oxidation of BH_4 later in the observation period which would in turn increase the BH_2 to BH_4 ratio and promote rather than attenuate leukocyte-endothelial interactions. Determination of the BH_2 to BH_4 ratio by high performance liquid chromatography (HPLC) in tissue obtained at the end of the experiment will address this issue in future studies.

Differing cellular adhesion molecule regulation may play a role in the dose-dependent effects of the BH_2/BH_4 ratio on leukocyte-endothelial interactions. In the case of this study, the 250 µM concentration of BH_4 in combination with 100 µM BH_2 attenuated BH_2-induced leukocyte-endothelial interactions and was more effective than 100 µM BH_4. It is also probable that the more effective dose-dependent response of BH_4 on leukocyte transmigration is due to the cumulative attenuation of leukocyte rolling and adherence before diapedesis occurs.

The 100 µM BH_2 concentration was chosen to use in the BH_2/BH_4 ratio instead of the 200 µM BH_2 concentration because results from both concentrations were almost identical. Moreover, in light of the current data, one would have to use a higher dose of BH_4 (i.e. 500 µM) to overcome the BH_2 effect. Previous studies also used this concentration (i.e. 100 µM BH_2) effectively to increase H_2O_2 release in femoral I/R and to decrease NO release [7]. Additionally, the 250 µM BH_4 concentration was chosen to use in the BH_2/BH_4 ratio with 100 µM BH_2 because significance was lost after 60 minutes for rolling and adhered leukocytes while superfusing the 100 µM BH_4

concentration [7]. Once the higher concentration of BH_4 was used, significance returned at all of the time points throughout the 2 hour period in comparison to the 100 µM BH_2. Moreover, the 250 µM of BH_4 correlates with previous femoral I/R *in vivo* studies that showed decreased H_2O_2 and increased NO release [7].

Although research on the effects of the BH_2/BH_4 ratio has been limited, previous studies have noted that this ratio may be the determining factor in causing endothelial dysfunction. Vasquez-Vivar et. al. showed that increasing the ratio between BH_2 and BH_4 tightly regulates SO formation from eNOS [6]. They also note that the augmentation of BH_4 should increase NO formation; while the high turnover of BH_4 leading to an increase in oxidized BH_4 (i.e. BH_2) causes SO production by eNOS [43]. Therefore, the results from this *in vivo* study support the concept that increasing the BH_2/BH_4 ratio promotes endothelial dysfunction and thereby increases leukocyte-endothelial interactions observed via intravital microscopy. These results also indirectly support the concept of previous *in vitro* studies suggesting that increasing the BH_2/BH_4 ratio results in increased vascular oxidative stress leading to enhanced inflammation responses [7].

Clinical studies have found a positive correlation between the BH_4/BH_2 ratio and flow mediated vasodilatation (FMD). FMD is induced by reactive hyperemia (via the release of a blood pressure cuff) and is considered a method to assess the relationship between coronary risk factors and endothelial dysfunction. This study also found that patients with multiple coronary risk factors had a decreased serum BH_4/BH_2 ratio and those that underwent treatment with statins had improved FMD with an increased BH_4/BH_2 ratio. Furthermore, this clinical research shows plasma pteridine levels (i.e.

BH_4/BH_2 ratio) can be used as a biomarker to evaluate endothelial dysfunction in the absence of evident systemic inflammation [36].

H&E staining of the mesenteric tissue superfused with 100 µM BH_2 and 200 µM BH_2 showed a significant increase in leukocyte vascular adherence and transmigration in comparison the Krebs's buffer. By contrast, increasing the BH_4/BH_2 from 1:1 to 2.5:1 exhibited a dose-dependent and marked decrease in leukocyte adherence and transmigration in comparison to BH_2 alone. This decrease in the number of leukocytes in the BH_2/BH_4 ratio test solution groups confirms the dose-dependent and anti-inflammatory effects of BH_4 observed during intravital microscopy.

Mechanism of Action Related to Anti-Inflammatory Effects of Reducing the BH_2/BH_4 Ratio

The mechanism of increasing SO production to induce leukocyte rolling and adherence due to oxidative stress, and reversing those leukocyte-endothelial interactions via superoxide dismutase has been well established in the scientific community [42]. However, this is the first study to show that eNOS uncoupling can induce inflammation *in vivo* by intravital microscopy in the rat mesenteric circulation. There are numerous mechanisms involved in the effect of eNOS uncoupling on NO bioavailability in vascular conditions. The uncoupling of eNOS may be an important mechanism mediating inflammation-induced vascular injury. One of the most important roles is played by BH_4 where it acts to regulate NO and SO production by eNOS [44]. NO is produced from coupled eNOS as a dimer under normal conditions where BH_4 acts an essential cofactor stabilizing the eNOS dimer and facilitates the electron transfer. BH_4 promotes eNOS

dimerization, protein stability, and NO synthesis making it a modulator of eNOS function [45].

Heitzer et. al. demonstrated the that positive effects of acute BH_4 administration in chronic smokers was most likely by way of a direct effect on eNOS coupling versus merely an antioxidant effect. This was noted because a control infusion of tetrahydroneopterin (NH_4), another reduced pteridine, had no effect on endothelial function despite having a comparable capacity to scavenge SO anions like BH_4 *in vitro* [44, 46]. As a result, BH_4 can be directly linked to these positive findings improving vasodilation in chronic smokers. Because BH_4 can be oxidized into BH_2 when oxidative stress exists, the BH_2/BH_4 ratio plays an essential role in mediating inflammation-induced vascular injury. Our study is unique in that altering the cofactor status can be just as effective at studying inflammation and may be more clinically relevant than synthetically inhibiting *L*-arginine.

Additionally, several studies have shown that administration of ascorbate (vitamin C) may be an important aspect in maintaining BH_4 levels in the presence of oxidative stress. It has been shown to ameliorate endothelial dysfunction in patients with diabetes, hypercholesterolemia, overt coronary artery disease, as well as those who smoke [44]. Supplementation with vitamin C increased the BH_4/BH_2 ratio and NO bioavailability in apolipoprotein E knock-out mice in a study conducted by d'Uscio et. al [47]. They found that there is a dose-dependent effect of ascorbate on BH_4 levels and NO synthesis in cultured endothelial cells, although this mechanism is not fully understood [44, 47]. Current research indicates that maintaining BH_4 levels in endothelium is essential in regulating the NO balance and SO synthesis by eNOS [44].

The pro-inflammatory or anti-inflammatory effects of the BH_2/BH_4 ratio have been proposed in numerous review articles and research studies. There have not been many *in vivo* experiments conducted in order to further prove this finding; yet future studies aim to confirm these results. Specifically, measurement of BH_4/BH_2 in tissue samples from clinical and basic research may confirm the significance of uncoupled eNOS as a major source of oxidative stress in pathophysiological conditions such as diabetes, atherosclerosis, hypertension, and I/R injury. This current project is one of the few that established an innovative animal model which functions by altering the BH_2/BH_4 ratio thus investigating the role of eNOS uncoupling in vascular conditions. An early characteristic of all pathophysiological vascular diseases and risk factors associated with them is endothelial dysfunction, resulting in abnormal homoeostatic pathways which will exacerbate disease processes such as inflammation, thrombosis and oxidative stress [8]. The BH_2/BH_4 ratio is one area that can be targeted in preventing and improving inflammation-induced vascular injury.

Clinical Relevance

Data suggest that eNOS uncoupling may be an important mechanism mediating inflammation-induced vascular injury. Decreased leukocyte-endothelial interactions by BH_4 correlates with previous studies in our lab which include improved post-reperfused cardiac function (*ex vivo*), reduced H_2O_2 release and increased NO release in femoral I/R (*in vivo*), increased NO release from aortic segments (*in vitro*) [7]. This data in conjunction with the present study suggests that adding BH_4 to pathological conditions may lessen the effects of vascular injury associated with I/R, hypertension, diabetes,

atherosclerosis, and hyperglycemia. Additionally, this study established an innovative BH_2-induced leukocyte-endothelial interactions in an animal model by modulating the ratio between BH_2 /BH_4, exploring the role of eNOS uncoupling under pathophysiological conditions, as well as assisting in discovering potential therapeutic strategies.

Limitations/Future Studies

Three main limitations were present in this study. First, our lab was unable to acquire an optical Doppler velocimeter to observe erythrocyte velocity in order to determine the venular shear rates. Nevertheless, we were able to monitor the blood flow throughout the experiment using intravital microscopy and no significant changes were detected among any of the experimental groups. Moreover, there were no significant differences in MABP among all groups at baseline, suggesting that blood flow would not be significantly different in any group. In addition, it has been found that superfusion of some of the treatment groups (i.e. Krebs', L-NAME) in this study do not cause significant effects on venular shear rates [48]. Therefore, given these conditions, shear rate most likely does not play a major role in increasing leukocyte-endothelial interactions.

Secondly, this research project was one of the first of its kind to increase the ratio of BH_2/BH_4 to simulate vascular endothelial dysfunction *in vivo* making it a pilot study. We chemically induced endothelial dysfunction by administering L-NAME and BH_2. To relate a more pathological condition to the BH_2/BH_4 ratio, future studies are aimed at inducing superior mesenteric artery ischemia/reperfusion to induce endothelial

dysfunction and subsequent endothelial-leukocyte interactions and to see if this effect can be attenuated or exacerbated by BH_4 or BH_2 respectively. These future experiments are planned in order to compare the results from these endothelial dysfunction-causing agents to those found under experimental I/R conditions regarding the BH_2/BH_4 ratio. Thirdly, although specific concentrations of the test solutions were superfused over the mesentery, there is no real way to know the absolute concentrations of BH_4/BH_2 inside the mesenteric venule circulation, versus the amount administered. Therefore, future studies are planned to detect BH_4/BH_2 levels using HPLC in tissue obtained at the end of intravital microscopy. One would speculate that the effects of the higher dose of BH_4 (i.e. 250 μM) would at the very least to show a 1:1 ratio or better. Conversely, the lower dose of BH_4 (i.e. 100 μM) most likely would detect an increased BH_2 to BH_4 ratio rather than the 1:1 ratio that was used at the beginning of the experiment.

Future challenges include identifying new strategies that target BH_4 supplementation, synthesis, and/or stabilization with sufficient potency and specificity in long term clinical studies. In the near future, our lab has plans to use BH_2 combined with an eNOS activator (i.e. protein kinase C epsilon activator) to exacerbate BH_2-induced leukocyte-endothelial interactions. Whereas, increasing the BH_4/BH_2 ratio combined with the eNOS activator should attenuate the BH_2-induced effects. Additionally, we plan to use an eNOS inhibitor (i.e. protein kinase C epsilon inhibitor, or PKCε-) that theoretically should attenuate the BH_2-induced leukocyte-endothelial interactions in contrast to L-NAME since it will not compete with *L*-arginine to induce its' effects. However, the effects of PKCε- may not be ameliorated in the case of BH_4.

Summary and Significance of Major Findings

In summary, our data demonstrates that administration of BH_4 dose-dependently attenuated BH_2-induced leukocyte-endothelial interactions. The mechanism of this anti-inflammatory action is associated with reduced oxidative stress and the restoration of vascular endothelial function. Our data provides indirect evidence to support that the BH_2/BH_4 ratio may be a potential strategy to attenuate endothelial-dysfunction induced inflammatory responses in different diseases such as diabetes, I/R injury, atherosclerosis, and hypertension.

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