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The Role of NADPH Oxidase Isoform 1 (NOX1) in L-NAME-Induced Leukocyte-Endothelial Interactions in Rat Mesenteric Postcapillary Venules

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The Role of NADPH Oxidase Isoform 1 (NOX1) in L-NAME-Induced Leukocyte-Endothelial Interactions in Rat Mesenteric Postcapillary Venules

A thesis in vascular endothelial dysfunction by Alita Zabrecky

We have read and examined this manuscript and certify that it is adequate in scope and quality as a thesis for this MS degree.

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Abstract

Vascular endothelial dysfunction is a component of many chronic illnesses such as cardiovascular disease, diabetes, and hypertension. This dysfunction is initiated by an inflammatory response triggering an increase in leukocyte endothelial interactions. It is characterized by increased oxidative stress often associated with reduced endothelial-derived nitric oxide (NO) bioavailability. Activation of NADPH oxidase is a major source of oxidative stress. NADPH oxidase has five NOX and two Doux isoforms. NOX1 is expressed on vascular endothelial and smooth muscle cells, but is not expressed on neutrophils. Therefore, the role of NOX1 in inflammation is not completely clear. A NOX1 selective inhibitor, 2-acetylphenothiazine (ML171), was used to determine its effects on N^G-nitro-L-arginine methyl ester (L-NAME)-induced leukocyte endothelial interactions in rat post capillary venules using intravital microscopy. We found that 50 μM L-NAME, a NO synthase inhibitor, significantly increased leukocyte rolling, adherence, and transmigration after a 2 hour superfusion of post capillary venules compared to the Krebs' buffer control rats. However, ML171 was able to significantly attenuate L-NAME induced leukocyte-endothelial interactions compared to L-NAME alone. Superfusion of post capillary venules with L-NAME and 0.2 μM or 1.0 μM ML171 reduced the leukocyte rolling from 71±8 cells/minute to 19±5 and 25±5 cells/minute, respectively, adherence from 16±4 cells/minute to 3±1 cells/minute, and transmigration from 15±3 cells to 4±1 cells (both ML171 concentrations) over a 2 hour period. The results obtained from intravital microscopy were confirmed through

Hematoxylin & Eosin (H&E) staining of superfused mensenteric tissues from experimental groups. We found that post capillary venules superfused with L-NAME exhibited significantly more leukocyte vascular adherence and tissue transmigration compared to Krebs's control tissue. The addition of 0.2 μM or 1 μM ML171 during the superfusion was found to significantly attenuate both L-NAME induced adherence and transmigration, reducing cell attachment from 269±11 cells/mm^2 to 114±9 and 137±21 cells/mm^2, respectively, and transmigration from 505±60 cells/mm^2 to 171±6 and 202±19 cells/mm^2, respectively. Moreover, Hematoxylin & Eosin staining supported the observation that ML171 alone did not affect basal leukocyte-endothelial interactions. In summary, our data suggest that L-NAME induced leukocyte-endothelial interactions involves activation of NOX1. ML171 may mitigate vascular endothelial dysfunction induced-inflammatory responses and elicit protective effects against chronic inflammation in the pathogenesis of various disease.

Introduction

1. Vascular Endothelial Function

Vascular endothelium plays a crucial role in bodily homeostasis, providing a physical barrier between blood and tissues and regulating many physiological functions such as control of vasculature tone through the release of vasoactive molecules that can either relax or constrict the blood vessels (1,2). Vascular endothelium also plays a role in both innate and adaptive immunity through leukocyte-endothelial interactions involving leukocyte recruitment and transmigration in order to mitigate potential pathogens (1,2,3). Endothelial cells have the ability to metabolize, synthesize, and release a variety of substances, including vasoactive chemicals, nitric oxide (NO) and angiotensin II (Ang II), involved in vasodilation and vasoconstriction, respectively. Endothelial cells are able to upregulate substances participating in inflammatory responses such as P-selectin and ICAM-1 required to slow passing leukocytes and adhere them to the vasculature. Endothelial cells participate in the production of reactive oxygen species (ROS) such as superoxide (SO) produced by NADPH oxidase, uncoupled eNOS, xanthine oxidase, and the mitochondrial respiratory chain. SO produced under normal physiological conditions plays a role in endothelial cell signaling. Endothelial cells are also able to produce procoagulants such as Von Willebrand factor which plays a role in platelet adhesion, and anticoagulants such as prostacyclin and NO, which inhibit platelet activation and promote

vasodilation (1,2,4). In particular, endothelial-derived NO and NADPH oxidase play critical roles in normal vascular endothelial functions.

1.1) Endothelial-Derived NO

NO is an endothelium derived relaxing factor with profound vasodilatory effects that can affect both the function and structure of the underlying vascular smooth muscle (1). In the vasculature, NO is constitutively synthesized from L-arginine by nitric oxide synthase (NOS) (5). There are three isoenzymes of NOS: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), however, in the vasculature, the main NOS isoenzyme contributing to NO production is eNOS (6). NO production can be induced through increased blood flow (sheer stress) or the binding of vasoactive substances such as acetylcholine, bradykinin, and adenosine to endothelial cell receptors causing an increase in intracellular calcium levels (7). In response to elevated levels of intracellular calcium, calmodulin binds to eNOS, which then catalyzes the conversion of L-arginine to L-citrulline + NO (7). In order for this reaction to occur, a number of cofactors for eNOS must be present, including tetrahydrobiopterin (BH_4), FAD, FMN, and iron protoporphyrin IX (haem) (8). Arguably, BH_4 is most closely related to NO bioavailability due to its ability to stabilize the eNOS dimer formation. If the eNOS dimer is not stabilized by BH_4 it will favor the production of SO and thereby reduce the bioavailability of NO produced by eNOS (9). NO produced from eNOS diffuses locally in endothelial cells to the luminal surface where it activates guanylate cyclase in smooth muscle cells causing the conversion of GTP

to cGMP, resulting in the inhibition of calcium into the smooth muscle cell and ultimately leading to vasodilation mediated by the smooth muscle cell, as shown in figure 1 (7).

In addition to being a potent direct vasodilator, NO also indirectly promotes vasodilation through the inhibition of AT_1 receptor expression. NO and Ang II have several antagonist effects on each other. Ang II acts on its receptor AT_1 to stimulate NO production and in return NO down-regulates the AT_1 receptor indicating a balance between NO and Ang II (10). NO also is able to induce an anti-platelet effect through the reduction of intracellular calcium levels. Since platelet aggregation is dependent on an increase in intracellular calcium, the reduction of intracellular calcium in platelets, through increased levels of cGMP, ultimately results in an anti-platelet effect (11). Additionally, NO is able to produce an anti-inflammatory effect through its ability to inhibit leukocyte-endothelial interactions on the vascular wall (11). Leukocyte endothelial interactions are dependent on the expression of adhesion molecules on the vasculature cell surface. NO has been shown to prevent the expression of P-selectin on the vascular cell surface which is needed for leukocyte rolling to occur (12).

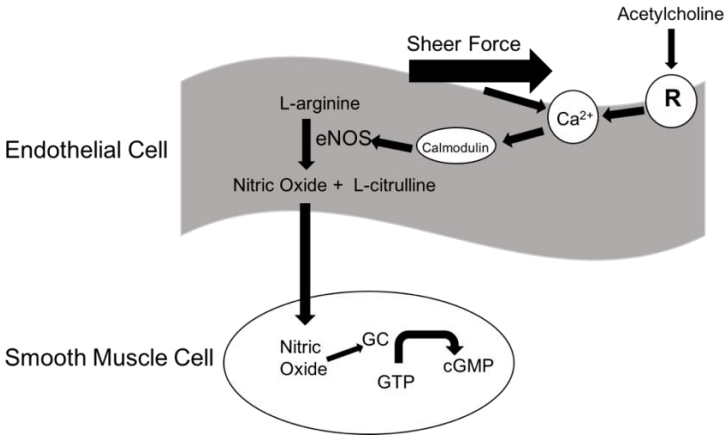


Figure 1: **Activation of eNOS**. Sheer force and the binding of acetylcholine to G-protein receptors causes an increase in intracellular calcium. Calcium is thereby able to activate calmodulin which binds to eNOS, activating it, and allowing the conversion of L-arginine to L-citrulline + Nitric Oxide (NO). NO is able to diffuse to smooth muscles cells activating guanylate cyclase causing the conversion of GTP to cGMP. cGMP further results in the inhibition of calcium into the smooth muscle cell, causing vasodilation. Figure adapted from (7).

1.2) NADPH Oxidase

The NADPH oxidase family of enzymes, also known as NOX have 7 different isoforms: NOX1, NOX2, NOX3, NOX4, NOX5, Duox1, and Duox2 (13). The expression of the NOX isoforms varies throughout the body, with NOX1-5 known to be expressed on the endothelial cells, NOX2 being expressed on leukocytes and Duox1 and Duox2 being expressed on epithelium (14). NOX enzymes are a multisubunit enzyme, consisting of cytosolic subunits, NOXO1 (organizer subunit), NOXA1 (activator subunit) and RAC1 (regulator), as well as membrane bound subunit, phox22, and membrane bound catalytic unit NOX (figure 2). Under normal physiological conditions, the NOX enzymes are responsible for catalyzing the transfer of an electron from NADPH to O_2 resulting in the production of SO (14). The SO produced from the NOX enzyme have different functions depending on the location of the NOX isoenzyme, but is known to be involved in the respiratory burst in phagocytes as well as involved in a myriad of signaling pathways such as cell migration and upregulation/surface expression of epidermal growth factor receptor (14). The O_2^- produced from the NOX enzymes is unstable and reacts quickly with superoxide dismutase (SOD) to form the more stable hydrogen peroxide (H_2O_2). In select

NOX isoforms, such as NOX4, H_2O_2 is a direct product from the catalytic subunit (15). H_2O_2 is able to further act as a signaling molecule and is able to diffuse from the cell, unlike SO, rendering it useful in normal physiological conditions, however, can also lead to lipid peroxidation of cell membranes under excessive productions such as ischemia-reproduction injury (16).

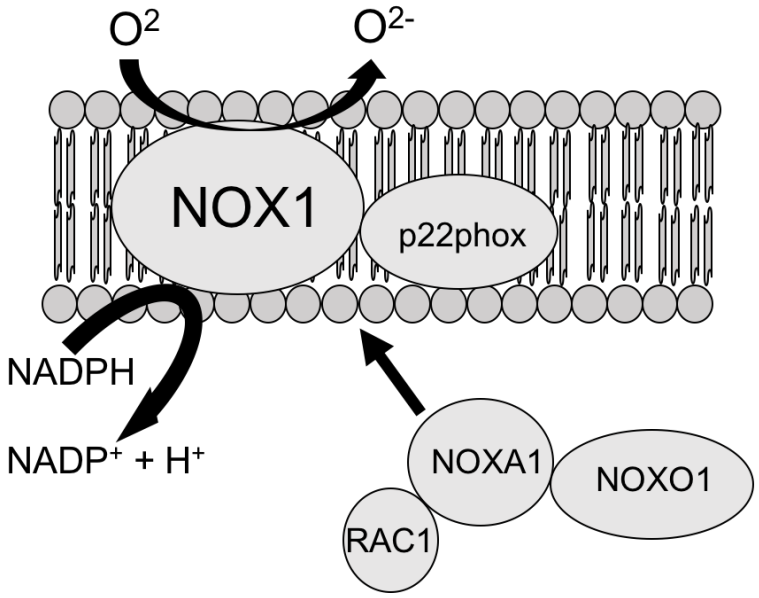


Figure 2. **NADPH oxidase structure**. NADPH oxidase activation requires the assembly of cytosolic subunits (RAC1, NOXA1, and NOXO1) and membrane subunits (p22phox and the catalytic subunit [NOX1]) on the cell membrane. All subunits facilitate the transfer of an electron from NADPH to O_2 to produce superoxide (O^{2-}). Figure adapted and modified from (17).

2. Endothelial Dysfunction

Vascular endothelial dysfunction is considered an essential clinical event in many illnesses such as cardiovascular disease, diabetes, and hypertension. Therefore, it is important to understand the underlying mechanisms causing vascular endothelial dysfunction in order to provide new clinical treatments (1).

In a state of dysfunction, the endothelium becomes activated and predisposed to vasoconstriction, leukocyte adhesion, and inflammation due to loss of balance in homeostasis, leading to the pathogenesis of cardiovascular disease (2). The major characteristics of vascular endothelial dysfunction are increased oxidative stress and/or reduced endothelial-derived NO bioavailability.

Oxidative stress occurs when the amount of ROS present in the cell outweighs the cells ability to convert the ROS into less harmful substances, such as SO. Normally, SO is then converted to H_2O_2 via SOD, and the further conversion of H_2O_2 to H_2O via catalase. Inappropriate amounts of ROS present in a cell can cause alterations in membranes, DNA, and proteins as well as interfere with intracellular cascades resulting in altered protein activity which can be implicated in the pathophysiology of disease (18). During vascular damage in hypertension, ROS influences vascular remodeling by causing an increase in proteins, such as collagen, to be present on the extra cellular matrix (ECM) (19). Overproduction of SO can further be implicated in vascular endothelial dysfunction through direct quenching of endothelial-derived NO to form peroxynitrite ($ONOO^-$). There are four major sources of oxidative stress: mitochondrial respiratory chain, xanthine oxidase, uncoupled eNOS, and NADPH oxidase.

2.1) Mitochondrial Respiratory Chain

In the mitochondrial respiratory chain, SO is produced through the reduction of O_2, caused by electron leakage from the oxidative phosphorylation pathway (20). Under

physiological conditions, SO is produced in small amounts mainly from complex I and complex III of the mitochondrial respiratory chain as electrons "leak" to O_2 creating SO (21). The SO is quickly converted via SOD to H_2O_2. However, under some pathological conditions, such as ischemia, SO production can be increased in the respiratory chain from complex III, possibly due to the inhibition of complex IV. In other conditions where NADPH is activated, mitoK_{ATP} channels are opened, resulting in mild mitochondrial uncoupling (21). The increase in SO production from the mitochondrial respiratory chain will cause the further upregulation of NADPH oxidase and continue the cycle of increased SO production.

2.2) Xanthine Oxidase

Xanthine oxidase is expressed on the luminal surface of epithelial cells and is another source of SO produced as a byproduct of the conversion of hypoxanthine into urate (18). Xanthine oxidase is a unique source of SO because the enzyme is usually present as xanthine dehydrogenase which does not generate SO, however, it can be converted to xanthine oxidase through oxidation which can occur in the presence of cellular oxidative stress, thereby, amplifying SO production (18).

2.3) eNOS Uncoupling/Downregulation

Uncoupling of eNOS is marked by a decrease in essential cofactor, tetrahydrobiopterin BH_4, a critical cofactor in stabilizing the eNOS dimer. BH_4 bioavailability can be affected by increased oxidative stress resulting in the oxidation of BH_4 to dihydrobiopterin (BH_2), making the cofactor unusable to eNOS for the production of NO (22). Uncoupled eNOS further promotes the production of SO, rather then the wanted NO, by not allowing the conversion of L-arginine to L-citrulline, ultimately increasing oxidative stress (22). When there is an abundance of SO in the presence of NO, the two will react to form a peroxynitrite ion (13). The peroxynitrite ion increases the uncoupling of eNOS through the conversion of BH_4 to BH_2 resulting in further reduction in the production of NO (13).

In pathological conditions, eNOS can be downregulated by inflammatory cytokines such as TNF-α through the inhibition of eNOS promoter activity and mRNA destabilization (23). TNF-α also leads to suppressed eNOS activity through the prevention of degradation of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS (23).

2.4) Activated NADPH Oxidase

Activated NADPH oxidase is another source of SO acting by facilitating the transfer of an electron from NADPH to O_2. While NADPH has a role in normal physiological conditions, in pathological conditions, NADPH oxidase is upregulated to produce more SO. Upregulation of NADPH oxidase occurring in pathological conditions begins with cytokines binding to G-protein receptors causing an increase in de novo synthesis of DAG.

Increase in PKC activity is further increased by diacylglycerol (DAG) and allows PKC to activate the RAC subunit of NADPH oxidase, facilitating the assembly and activation of the NOX enzyme. Under basal conditions, the SO produced from the NOX enzyme is able to be converted to less harmful substances such as H_2O_2, however, under pathological conditions, through the upregulation of NOX, there is a marked increase in SO production, outweighing the cells ability to convert it to a less harmful substance. SO, readily reacts with other ROS, mainly NO, to form the peroxynitrite ion, resulting in the uncoupling of eNOS, promoting the production of SO rather then the desired NO through the inability to convert L-arginine to L-citrulline + NO (22). Therefore, the increase in NOX activation continues to contribute to the pathological state, and ultimately the continued upregulation of NOX, forming a deleterious cycle.

3. Inflammation

3.1) Acute Inflammation

Acute inflammation begins immediately following an injury and is marked by increased blood flow, increased permeability of the capillaries, and migration of neutrophils from capillaries and venules into the interstitial space. The migration of

neutrophils is marked by leukocyte-endothelial interactions through adhesion molecules promoting leukocyte rolling, adherence, and transmigration. Leukocyte-endothelial adhesion molecules are placed into one of three categories: selections, integrins, and the immunoglobulin superfamily. (24,25). Cytokines involved in acute inflammation such as IL-β, IL-8, and TNF-α, are able to upregulate processes needed for the migration of leukocytes into interstitial space.

The first stage of leukocyte migration is leukocyte rolling, mediated by a family of glycoproteins, termed selectins. On leukocytes, L-selectin is constitutively expressed and is vital to leukocyte rolling on the inflamed or activated endothelium (25). On the endothelial side, P-selectin is located in Weibel-Palade bodies of endothelium cells and can be upregulated to the exterior of the cell by humoral activators present during inflammation such as thrombin, histamine, and platelet factors (25). The interaction between L-selectin and P-selectin causes a rolling effect on the leukocytes, slowing them down in the blood stream, thereby rendering the endothelium sticky to passing leukocytes.

Once leukocytes are slowed, they can begin adhering to the endothelium. L-selectin is shed from neutrophils and the CD11b/CD18 integrin is then upregulated to the neutrophil surface (25). Cd11b/CD18 then interacts with ICAM-1, constitutively expressed on the surface of endothelial cells, thus creating a firm adherence to the vascular cell wall. While ICAM-1 is constitutively expressed to a degree, it can be strongly upregulated by cytokines including IL-β, IL-8, and TNF-α (25).

Once leukocytes are firmly adhered they can then begin transmigrating through the vascular wall. Transmigration of leukocytes through the vascular cell wall is believed to

be mediated by platelet-endothelial cell adhesion molecule, PECAM-1, which is constitutively expressed on the surface of both leukocytes and the vascular cell wall, with its density concentrated in cell junctions (25). When leukocytes transmigrate into interstitial spaces, they then release ROS to kill invading pathogens. While these steps are vital to combatting infection, they are also responsible for vascular endothelial dysfunction and inflammation.

3.2) Chronic inflammation

Chronic inflammation is characterized by the immune system's inability to resolve a pathogen, a state mimicked in diseases in which vascular endothelial dysfunction is present. Numerous studies have shown that chronic inflammation contributes in the pathogenesis of various vascular diseases, such as hypertension, vascular complication of diabetes, ischemia/reperfusion (I/R) injury, and atherosclerosis. While in these diseases there is no pathogen to kill, the body mimics the conditions as if there was one, including the presence of cytokines in the blood. One of the main contributors to prolonged endothelial dysfunction is the cytokine TNF-α. TNF-α has been shown to be associated with a decrease in NO produced from eNOS, a main contributor to endothelial dysfunction (23). TNF-α works to inhibit eNOS promoter activity as well as mRNA destabilization. Moreover, TNF-α upregulates NADPH oxidase as well as induces CAM expression on the vascular cell surface, further promoting the transmigration of leukocytes into the interstitial space (figure 3) (23). The upregulation of NOX in states of chronic inflammation is

extremely harmful as it potentiates further endothelial dysfunction due to the excessive production of SO, creating a vicious cycle. Therefore, studies have begun to look into NOX inhibition in the vasculature in order to attenuate vascular endothelial dysfunction. Studies into NOX2, an isoform present in both the vasculature and leukocytes, have found that blocking the catalytic subunit of the NOX enzyme is able to attenuate leukocyte endothelial interactions present in endothelial dysfunction such as rolling, adherence and transmigration (26). However, in the case of chronic inflammatory diseases, inappropriate leukocyte transmigration and release of ROS leads to tissue and organ damage. Therefore, when treating chronic inflammatory disease, it may be potentially beneficial to prevent inappropriate leukocyte rolling, adherence, and transmigration, in an effort to prevent unwanted tissue damage, such as the case when using a NOX2 inhibitor (25,26). Additionally, inhibition of other NOX isoforms could be potentially beneficial in preventing endothelial dysfunction in chronic inflammatory diseases.

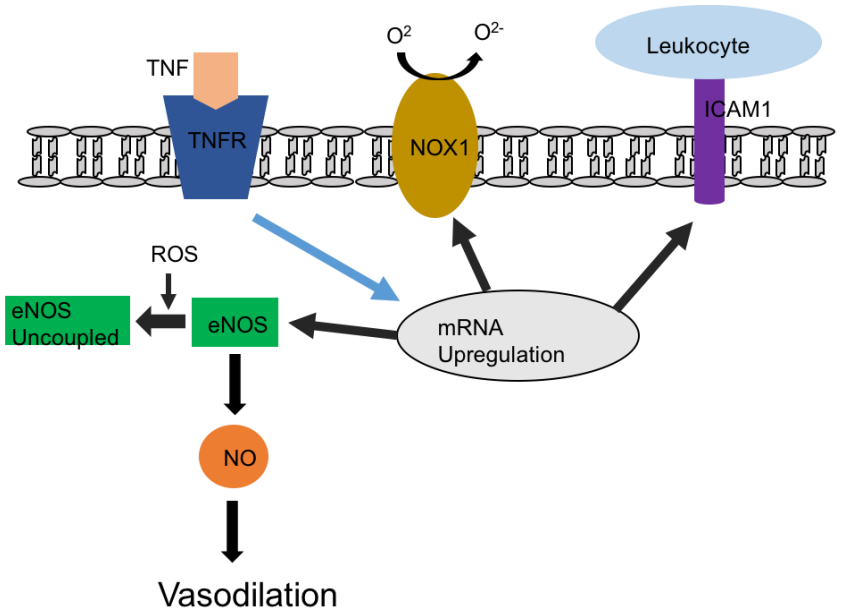


Figure 3: **Mediators of endothelial dysfunction in inflammatory diseases**. Binding of Tumor Necrosis Factor Alpha (TNF-α) to its receptor, Tumor Necrosis Factor Receptor (TNFR), leads to reduced eNOS protein expression by suppression of promoter activity and destabilized mRNA. TNFR increases expression of cellular adhesion molecules (ICAM-1) and NOX1. mRNA upregulation is mediated by ROS. eNOS uncoupling, mediated in part by ROS, is associated with reduced NO production and enhanced generation of ROS. Figure modified and adapted from (23).

4. Summary

In summary, what is not clear: The role of NOX1 in vascular endothelial dysfunction. NOX1 is an NADPH oxidase isoform known to be present in the colon, vascular smooth muscle cells, and endothelial cells (14). NOX1 is upregulated at the mRNA level and activated by vascular pathological stimuli, making it worthy to study in cases of vascular endothelial dysfunction. NOX1 is a multisubunit enzyme, consisting of cytosolic subunits: NOXO1 (organizer subunit), NOXA1 (activator subunit) and RAC1 (regulator), as well as membrane bound subunit, phox22, and membrane bound catalytic subunit NOX1 (27). NOX1 is responsible for transferring an electron from NADPH to O_2 resulting in the formation of SO. Moreover, ROS generated by NOX1 has been reported to contribute to a number of diseases involving inflammation, hypertension, and atherosclerosis (27).

5. ML171

ML171: 2-acetylphenothiazine, referred to as ML171 (MW=241.31 g/mol, IC50 for NOX1=0.25 μM), is a specific NOX1 inhibitor (28) (Figure 4). While the exact mechanism

of action for ML171 is not known, studies have shown that an over expression of the NOX1 catalytic subunit in the presence of ML171 can recover ROS production (27). This result is not seen with an over expression of other NOX1 subunits, such as NOXO1 or NOXA1, suggesting that the method of action is a direct block on the NOX1 catalytic subunit (27). ML171 exhibits a 30-fold selectivity over other NOX isoforms, making it ideal to study the effects of blocking NOX1 in vascular endothelial dysfunction (27).

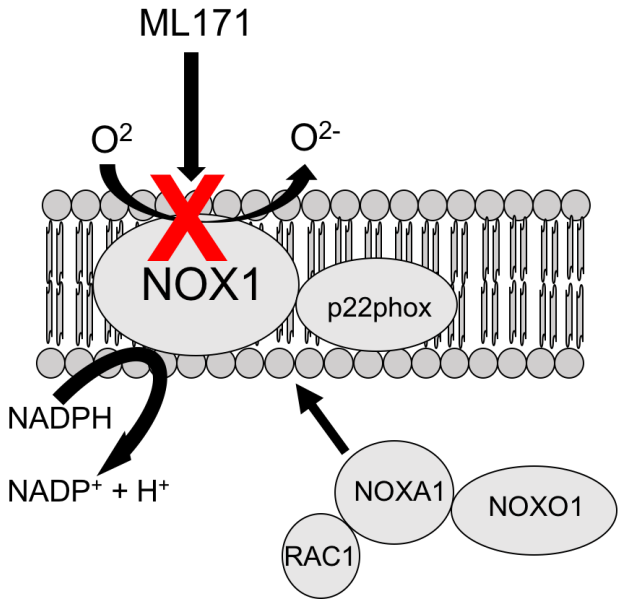


Figure 4: **ML171 Effects on NOX1**. ML171 inhibits the catalytic NOX1 subunit and attenuates SO release. Figure adapted and modified from (17).

6. Hypothesis

This study utilized intravital microscopy to analyze, in real time, in vivo, leukocyte endothelial interactions in postcapillary venules. Interactions observed included leukocyte rolling, adherence, and transmigration. Endothelial dysfunction was

induced using L-NAME, a non-selective NOS inhibitor. It is hypothesized that: 1) The use of ML171, at varying doses, will significantly reduce the number of leukocyte rolling, adherence, and transmigration in the post capillary venule in which vascular endothelial dysfunction has been induced by L-NAME. 2) This reduction in leukocyte-endothelial interactions will be confirmed using H&E staining on the superfused mesenteric tissue.

Methods

1. Intravital Microscopy

Endothelial dysfunction plays a role in many chronic inflammatory diseases, therefore, the need for a real time, in vivo, method to study leukocyte-endothelial reactions in microcirculation is necessary. Intravital microscopy provides a qualitative and quantitative technique for the study of leukocyte-endothelial interactions (29). Intravital microscopy also provides one of the best techniques to study authentic microcirculation interactions due to the tissue being left mainly intact, with only minor disruption caused by the surgical procedure necessary to unveil the tissue used. Utilization of this technique allows for the induction of various pathological conditions and conversely the study of how various drugs might affect those pathological conditions, as well as the effects various drugs may have.

2. Experimental Setup

The use of rat mesenteric tissue in this study has been approved by the Institutional Animal Care and Use Committee of Philadelphia College of Osteopathic. Male Sprague-Dawley rats (Charles River, Springfield, NH), ranging from 275-325g in weight, were anesthetized with 60 mg/kg pentobarbital sodium through an intraperitoneal (i.p.) injection. They were maintained using 30 mg/kg doses of pentobarbital sodium injected i.p. as needed. The left carotid artery was isolated and a PE-50 polyethylene catheter was inserted into the artery to allow for monitoring of mean arterial blood pressure (MABP) during the procedure via a BP-1 Pressure Monitor (World Precision Instruments (WPI), Sarasota, FL). The abdominal cavity was then opened via a midline laparotomy. A loop of ileal mesentery was selected and placed on a temperature controlled Plexiglass chamber, at 37^{o}C, held at a pH ranging from 7.3 -7.4. The tissue was superfused, with one of the experimental solutions outlined below, to allow for drug and buffer to be absorbed by the mesenteric tissue and enter into the vasculature. Mesenteric tissue that was subsequently placed over the Plexiglass pedestal was monitored for post capillary venule leukocyte-endothelial interactions via intravital microscopy (Nikon Corp., Tokyo, Japan); leukocyte trafficking was recorded using Image-Pro (Media Cybernetics, Inc., Bethesda, MD). The experimental set up is pictured below in figure 5.

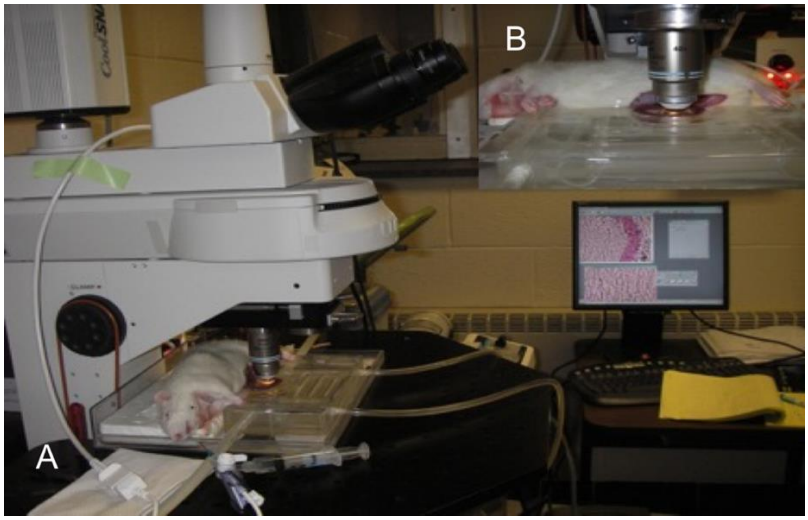


Figure 5: **Intravital microscopy experimental set-up.** Side view (A) and front view (B) of experimental setup for intravital microscopy.

The Krebs' buffer was composed of the following in mmol/L: 118 NaCl, 4.47 KCL, 2.45 $CaCl_2$, 12.5 $NaHCO_3$, and 1.19 KH_2PO_4. The buffer was aerated with 95% N_2 and 5% CO_2, and maintained at a pH of 7.3-7.4 at 37^oC. Leukocyte-endothelial interactions were recorded in 2-minute intervals, beginning with a baseline, and then every 30 minutes after for 2 hours. The recorded video was analyzed using the Image-Pro recording software (Media Cybernetics, Inc., Bethesda, MD). Leukocyte-endothelial interactions were assessed according to the following criteria: 1) Leukocytes were considered rolling if they were moving at a speed noticeably slower than that of red blood cells within the venule. 2) Leukocytes were considered adherent if they remain in a stationary position for >30 seconds in the length of a 100 μm venule. 3) Leukocytes were considered transmigrated if they were located in an area 20 x 100 μm^2 adjacent to the postcapillary venule. The numbers of leukocytes within each of these three categories were then quantified.

3. Treatment Groups

The experimental design consisted of five treatment groups (with 5-9 subjects in each group) for recording of leukocyte-endothelial interactions.

Treatment groups:

1. Krebs' control: Krebs' buffer was superfused for 2 hours following baseline recording to monitor basal leukocyte-endothelial interactions.
2. Krebs' + ML171 (1 μM): 1 μM ML171 in Krebs' buffer was superfused after baseline recording to determine the effects of ML171 on basal leukocyte-endothelial interactions.
3. L-NAME (50 μM): L-NAME was superfused for 2 hours following baseline recording to determine if attenuation of vascular NO production could increase leukocyte-endothelial interactions.
4. L-NAME (50 μM) + ML171 (0.2 μM): 50 μM of L-NAME with 0.2 μM ML171 was superfused after baseline recording to monitor the effect of a lower dose of ML171 on L-NAME induced-endothelial interactions.
5. L-NAME (50 μM) + ML171 (1 μM): 50 μM of L-NAME with 1 μM ML171 was superfused after baseline recording to monitor the effect of a higher dose of ML171 on L-NAME induced-endothelial interactions.

4. Hematoxylin and Eosin Staining

At the end of the experiment, a section of the loop of ileal mesentery that was superfused was removed and stored in 4% paraformaldehyde for later histological analysis.

Three representative sections of ileal mesentery from each experimental group (Krebs' control, Krebs + 1 μM ML171, L-NAME, L-NAME + 0.2 μM ML171, and L-NAME + 1 μM ML171) were selected for histological analysis. The sections were chosen based on the leukocyte-endothelial interactions data which was closest to the mean values of the entire group. The tissue was embedded in paraffin and sectioned into 4.5 μm serial sections and placed onto glass slides. Sections were deparaffinized and rehydrated, then stained with H&E. Under light microscopy, areas containing venules/arterioles in the serosa connected to the mesentery were counted for leukocyte vascular adherence and transmigration into the tissue and expressed as adhered and transmigrated leukocytes/mm^2.

5. Statistical Analysis

All data is represented as means ± SEM. The comparison of more than two groups was analyzed by ANOVA using post hoc analysis with the Bonferroni Dunn test to detect the differences among experimental groups within each aim. Probability values of <0.05 are considered to be statistically significant.

Results

1. Leukocyte-endothelial interactions via intravital microscopy

1.1) Rolling

Figure 6 illustrates leukocyte rolling along the post capillary venule among the experimental groups. There was no significant difference between baseline leukocyte rolling interactions among experimental groups. The Krebs' buffer did not significantly increase leukocyte rolling during the 120 min observation period and was maintained around 13±5 cells/minute (n=7). By contrast, L-NAME significantly increased leukocyte rolling beginning at the 30-minute time period and lasting the remainder of the time interval. At the end of the 2-hour time period, leukocyte rolling was increased to 71±8 cells/minute (n=9, $p<0.01$) compared to the Krebs' control. The use of ML171, was able

to significantly attenuate the increase in leukocyte rolling induced by L-NAME. The L-NAME + ML171 0.2 μM group significantly attenuated leukocyte rolling at the 30-minute time period, and continued to attenuate the leukocyte rolling throughout the 2-hour time interval, reducing rolling to 19±5 cells/minute (n=5, $p<0.05$). In addition, the L-NAME + ML171 1.0 μM group was able to significantly attenuate leukocyte rolling beginning at the 60-minute time period, and continued to attenuate the rolling throughout the 2-hour time interval, reducing rolling to 25±5 cells/minute (n=5, $p<0.05$). In order to determine the whether ML171 directly influences basal leukocyte rolling, ML171(1 μM) was dissolved in Krebs' buffer and superfused across the rodent mesentery. There was no significant change in leukocyte rolling during the 2-hour observation when ML171 was administered in the absence of L-NAME.

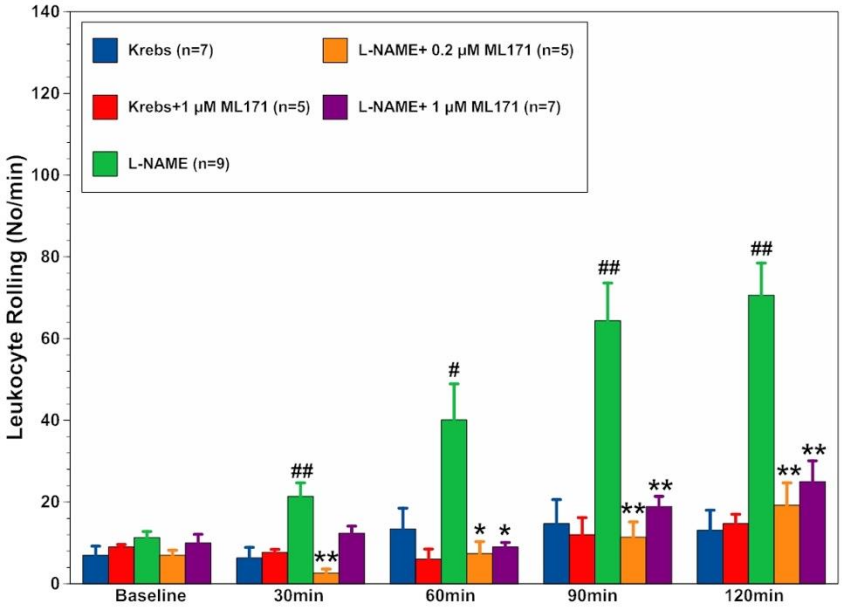


Figure 6: **Leukocyte rolling among different experimental groups.** L-NAME (50 μM) significantly increased leukocyte rolling from 30 min (#p<0.05, ##p<0.01 from Krebs'). ML171 (0.2 μM and 1.0 μM) significantly attenuated L-NAME induced leukocyte rolling (*p<0.05, **p<0.01 from L-NAME).

1.2) Adherence

Figure 7 illustrates leukocyte adherence to the post capillary venule among the experimental groups. There was no significant difference between baseline leukocyte adherence among the experimental groups. The Krebs' buffer did not significantly increase leukocyte adherence during the 120 min observation period and was maintained around 2±1 cells/100 μm (n=7). By contrast, L-NAME significantly increased leukocyte adherence at the 60-minute time period and lasting the remainder of the time interval. At the end of the 2-hour time period, leukocyte adherence was increased to 16±4 cells/100 μm (n=9, p<0.01) compared to the Krebs' control. The use of ML171 (both 0.2 μM and 1 μM), significantly attenuated L-NAME induced leukocyte adherence from 60-minute throughout the rest of experiment. Leukocyte adherence was 3±1 cells/100 μm [n=5, (p<0.05 for 0.2 μM;p<0.01 for 1.0 μM)]. There was no significant change in leukocyte adherence during the 2-hour observation when ML171 (1.0 μM) was administered in the absence of L-NAME.

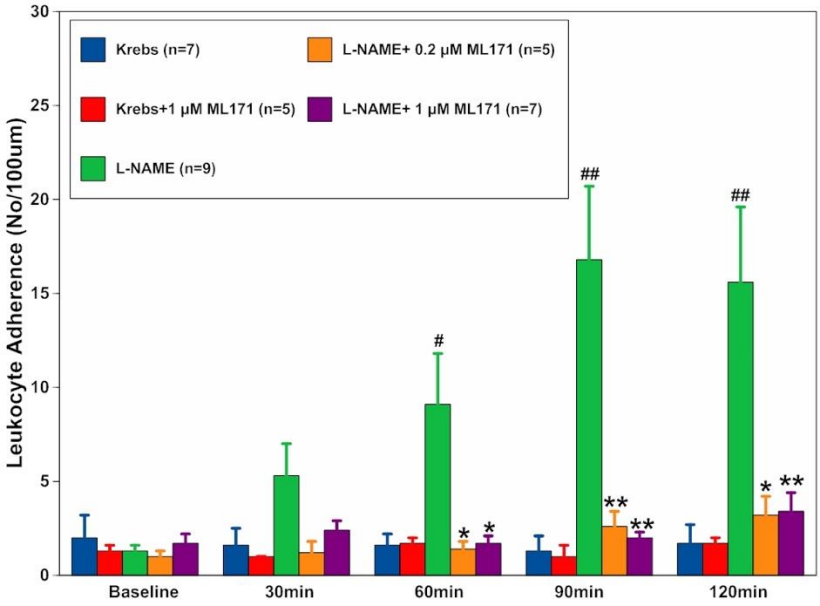


Figure 7: **Leukocyte adherence among different experimental groups.** L-NAME (50 µM) significantly increased leukocyte adherence from 60 min (#p<0.05, ##p<0.01 from Krebs'). ML171 (0.2 µM and 1.0 µM) significantly attenuated L-NAME induced leukocyte adherence (*p<0.05, **p<0.01 from L-NAME).

1.3) Transmigration

Figure 8 illustrates leukocyte transmigration from the post capillary venule into the tissue among the experimental groups. There was no significant difference in leukocyte transmigration among the baseline recordings among the experimental groups. The Krebs' buffer did not significantly change leukocyte transmigration during the 2-hour recording period. L-NAME significantly increased leukocyte transmigration beginning during the 60-minute time interval and lasting the remainder of the 2-hour time period. L-NAME increased transmigration 15±3 cells/20 x 100 μm^2 (n=7, p<0.01) at the 2-hour time mark from the Krebs' control 1±1 cells/minute (n=7, p<0.01). The use of ML171

significantly attenuated the increase of leukocyte transmigration caused by L-NAME at the 90-minute time period for both the 0.2 μM and 1.0 μM. The L-NAME + ML171 (0.2 μM and 1.0 μM) groups were able to attenuate the transmigration of leukocyte 4±1 cells/20 x 100 μm^2 compared to L-NAME [n=5, ($p<0.01$ for 0.2 μM; $p<0.05$ for1.0 μM)].). There was no significant change in leukocyte transmigration during the 2-hour observation when ML171 was administered in the absence of L-NAME.

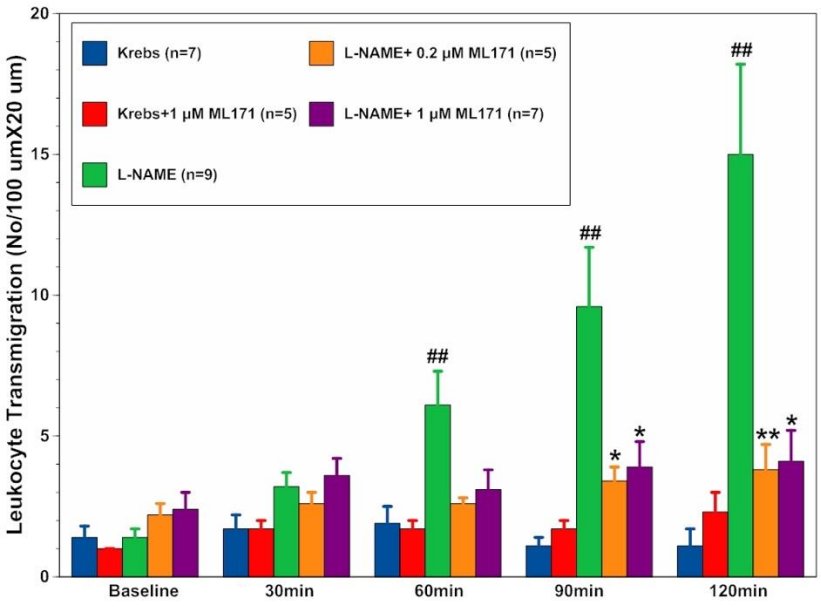


Figure 8**. Leukocyte transmigration among different experimental groups.** L-NAME (50 μM) significantly increased leukocyte transmigration from 60 min (##$p<0.01$ from Krebs'). ML171 (0.2 μM and 1.0 μM) significantly attenuated L-NAME induced leukocyte transmigration (*$p<0.05$, **$p<0.01$ from L-NAME).

2. Leukocyte Adherence and Transmigration via Hematoxylin and Eosin Staining

The superfused rat mesenteric tissue that were harvested at the end of the experiment were assessed via hematoxylin and eosin (H&E) staining to calculate the density of leukocyte vascular adherence and tissue transmigration. Figure 9 illustrates the representative leukocyte vascular adherence and tissue transmigration from experimental groups. Figure 10 shows images of assessed tissues from each experimental group. Results of the H&E staining were consistent to those obtained by in vivo intravital microscopy. There was low leukocyte adherence (84±9 cells/mm^2) and transmigration (129±35 cells/mm^2) in the Krebs' control group. By contrast, the 50 µM L-NAME group exhibited a 3-fold increase in leukocyte adherence (269±11 cells/mm^2, $p<0.01$) and 4-fold increase in leukocyte transmigration (505±59 cells/mm^2, $P<0.01$) compared to Krebs' buffer control. 50 µM L-NAME in the presence of 1 µM ML171 however, reduced leukocyte adherence (137±21 cells/mm^2) and transmigration (207±19 cells/mm^2). Similar results were observed for 0.2 µM ML171 adherence (114±9 cells/mm^2) and transmigration (171±6 cells/mm^2).

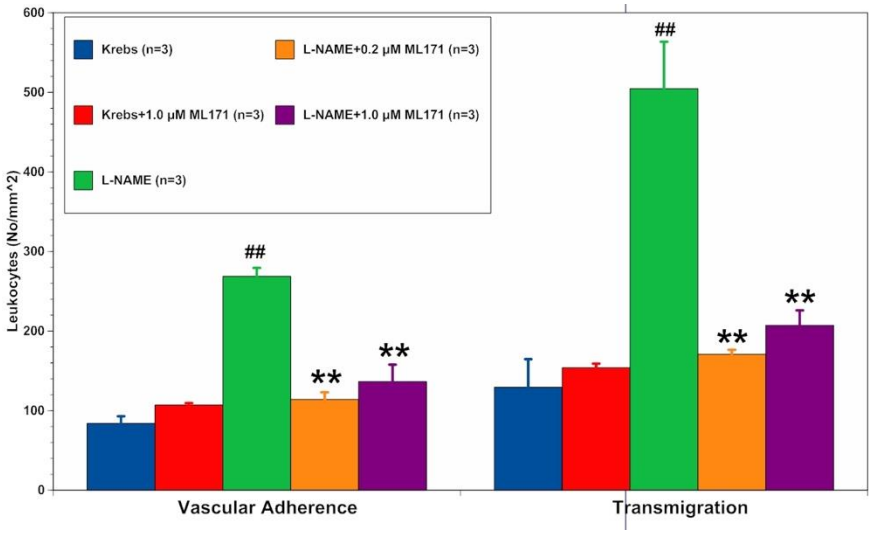
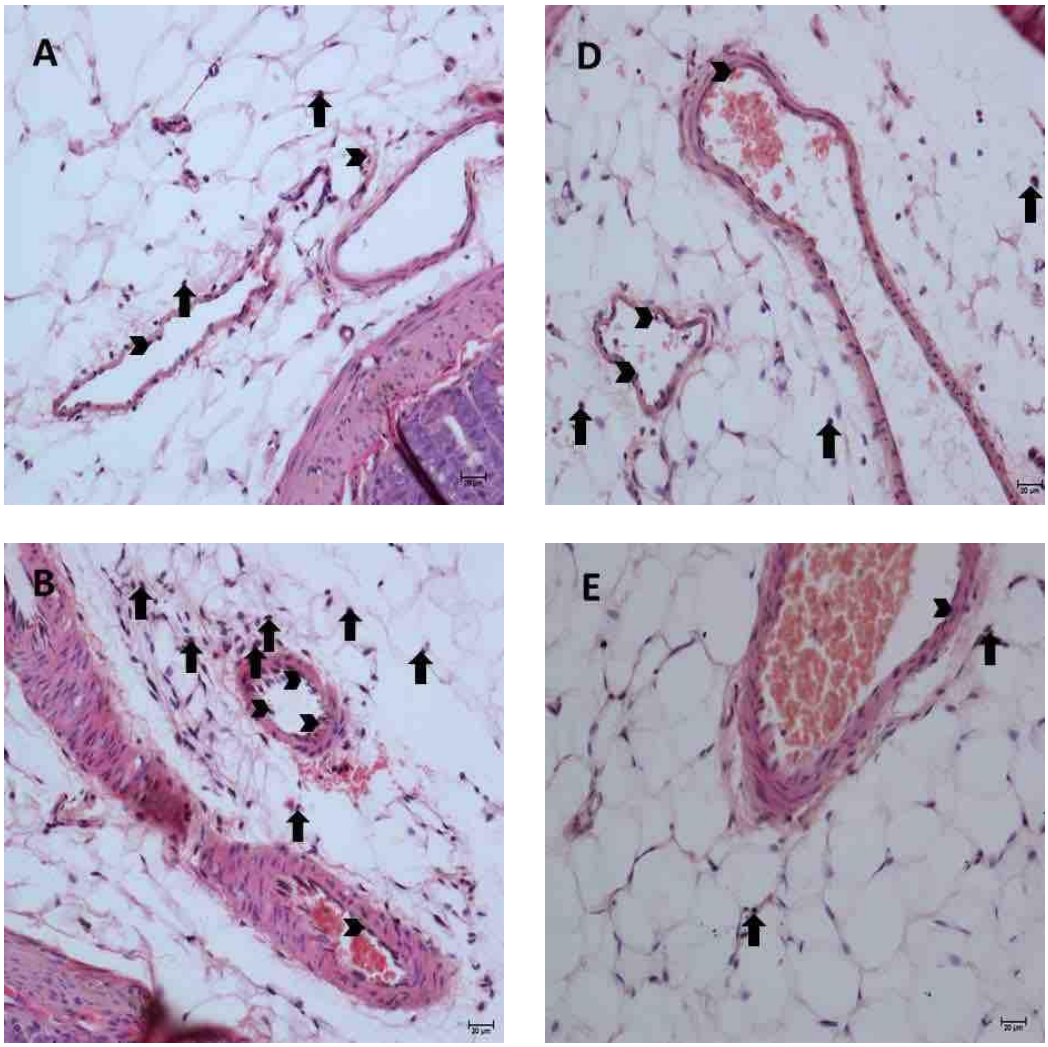


Figure 9**. H&E Staining Data Summary.** Summarized leukocyte adherence and transmigration by H&E staining of mesenteric tissues from all control groups**.** Compared to Krebs' control, the L-NAME treatment group significantly increased adherence and transmigration in leukocytes (##p<0.01 from Krebs'). By contrast, both ML171 groups (0.2 µM and 1.0 µM) were able to significantly attenuate the increase in adherence and transmigration initiated by L-NAME. (**p<0.01 from L-NAME).



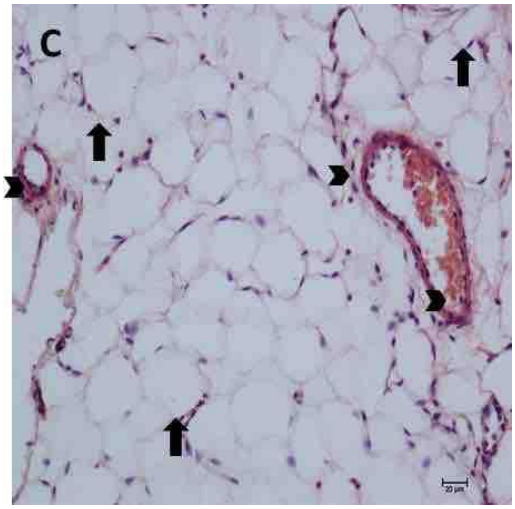


Figure 10. **H&E Staining Images.** Representative pictures (20x, scale bar: 20 μm) of leukocyte vascular adherence and tissue transmigration in mesenteric tissue from Krebs' control (A), L-NAME (B), 1.0 μM ML171 (C), L-NAME+ 0.2 μM ML171 (D) L-NAME+ 1.0 μM ML171 (E) by H&E staining. > denotes adhered leukocyte. ↑ denotes transmigrated leukocyte. Arrows are representative leukocytes.

Discussion

1. Summary of Major Findings

The major findings of this study are as follows: 1) 50 μM L-NAME significantly increased leukocyte-endothelial interactions compared to the Krebs' control group. 2) The administration of ML171 in a concentration of either 0.2 μM or 1.0 μM, significantly attenuated the leukocyte rolling, adherence, and transmigration induced by L-NAME, which is further confirmed by results via H&E staining. 3) ML171 did not affect the basal leukocyte-endothelial interactions.

2. Effects of L-NAME on Leukocyte-Endothelial Interactions

The superfusion of the Krebs' buffer alone was not able to significantly increase leukocyte rolling, adherence, and transmigration suggesting that the surgical procedure

performed on the rodent to expose the mesenteric tissue did not initiate an intense inflammatory response. By contrast, the addition of 50 μM of L-NAME to the Krebs' buffer solution was able to significantly increase the leukocyte rolling, adherence, and transmigration beginning between the 30-60 minute intervals compared to the Krebs' solution alone. L-NAME competes with L-arginine to bind to eNOS, preventing the conversion of L-arginine to L-citrulline + NO via eNOS, ultimately leading to a decrease in NO bioavailability. The use of L-NAME simulates the pathological state of endothelial dysfunction in vivo. Previous studies by Lefer et al. have shown that the use of L-NAME upregulated P-selectin on endothelial cells from ~10% in untreated tissue to 70-80% with the use of L-NAME, confirmed through immunostaining (15). Once P-selectin is upregulated on the endothelial cells, leukocytes can begin to slow down due to the interaction of P-Selectin with the L-selectin constitutively expressed on leukocytes. Once this interaction occurs, leukocytes are slowed and L-selectin is shed, causing the upregulation of Cd11b/CD18. Cd11b/CD18 interacts with ICAM-1, constitutively expressed on the surface of endothelial cells, to create a firm adherence to the vascular cell wall. While ICAM-1 is constitutively expressed, it can be upregulated in L-NAME induced inflammation by cytokines such as IL-β, IL-8, and TNF-α (25). Previous studies in our lab found that superfusion of L-NAME for 2 hours significantly increased the expression of ICAM-1 on endothelial cells (26). Therefore, the use of L-NAME in vivo induces a state of endothelial dysfunction, characterized by a marked increase in leukocyte-endothelial interaction, initiating a vicious inflammatory cycle.

3. Effects of ML171 on Leukocyte-Endothelial Interactions

We did not observe any effect of ML171 on basal leukocyte-endothelial interactions suggesting that the constitutive activity of NOX1 has no significant impact on basal vascular endothelial function. Under basal conditions, it does not appear that inhibition of NOX1 will disrupt cellular function, and the small amount of SO produced by NOX1 under basal conditions may mainly involve the signal transduction in vasculature, such as angiogenesis. We found that ML171 significantly reduced L-NAME induced leukocyte-endothelial interactions. L-NAME induces leukocyte-endothelial interactions through competitive inhibition of L-arginine, thereby, reducing NO bioavailability and inducing endothelial dysfunction like conditions. Additionally, SO derived from NOX1 has been shown to accelerate the translocation of PKC which phosphorylates NOXO1, a cytosolic subunit of NOX1, thereby, increasing the amount of SO produced from NOX1 (30). Furthermore, the SO produced by NOX1 has the ability to uncouple eNOS through the formation of the peroxynitrite ion. The peroxynitrite ion then oxidizes BH_4 to BH_2 resulting in the inability of eNOS to use its cofactor BH_4 (13). When the BH_4 to BH_2 ratio is increased, the product profile of eNOS is shifted to produce SO more so than NO, further reducing the bioavailability of NO, thereby, increasing endothelial dysfunction. Our findings suggest that NOX1 may be upregulated during inflammation and promote eNOS uncoupling in addition to more PKC activation, resulting in further NADPH oxidase upregulation and subsequently more SO release. Therefore, blocking the movement of an electron from NADPH to O_2 via ML171, may be

able to attenuate the inflammatory cycle present in the pathological state of endothelial dysfunction. Additionally, a study by Weaver et. al found that the use of ML171 can produce anti-inflammatory effects in beta cells through the inhibition of cytokine induce production of ROS produced by NOX1 (23). Cytokines play important role in upregulation of NOX1 assembly which can further amplify the damage of inflammation.

We did not find any dose-dependent effects of ML171 (0.2 µM-1.0 µM) on L-NAME induced leukocyte rolling, adherence, or transmigration. However, in other studies, like the study mentioned above by Weaver et. al, a dose dependent effect of ML171 was observed. Weaver et. al found that inflammatory cytokine induced beta cell death was reduced maximally at 10 µM and had a much more minimal effect at a dose of 0.1 µM. However, their study worked directly with cell models as well as a proinflammatory cytokine cocktail, providing a difficult comparison to our in vivo model. The lack of a dose dependent effect could be due to a multitude of different factors such as using a live animal or the inability to determine the actual amount of drug concentration absorbed through the mesenteric tissue during each experiment.

4. H&E Staining Compared to In Vivo Recording

Data collected from the H&E staining shows a greater number of leukocyte transmigration among experimental groups than the number of leukocytes adhered, an opposite finding compared to that of the in vivo data in this study. For leukocyte vascular adherence and tissue transmigration by H&E staining, our lab collected the

superfused mensenteric tissue and all types of blood vessels were included. By contrast, in vivo, our lab record leukocyte-endothelial interactions in real-time by observing only one mesenteric postcapillary venule throughout experiments. These differences in observation may have attributed to the differences among the data.

5. Limitations

The use of L-NAME in this study, a non selective NOS inhibitor inhibited all three forms of NOS (iNOS, nNOS, and eNOS), providing limitations to the study which aimed to induce vascular endothelial dysfunction by mainly reducing eNOS activity. However, it is worth noting that within a 2.5-hour experimental period, iNOS can not be induced and nNOS has minimal contribution to normal vascular endothelial function, therefore, the L-NAME induced inflammation was mainly caused by the inhibition of eNOS. Moreover, L-NAME is a chemical and is not a disease related condition. It has been well established that use of L-NAME provides a model of vascular endothelial dysfunction in vivo to study the underlying mechanisms involved in the subsequent inflammation. Further limitations are encountered through the experimental model. The superfusion of L-NAME only induces acute inflammatory conditions, whereas, diseases such as diabetes, hypertension, and atherosclerosis are chronic inflammatory diseases.

Therefore, it proves difficult to study long-term effects of the inhibition of ML171 using this model.

6. Future studies

Future studies are needed to learn more about NOX1's role in processes involved in leukocyte-endothelial interactions, such as upregulation of adhesion molecules, and changes of PKC and other NOX isoforms

7. Summary and Significance of Major Findings:

In summary, our data demonstrates that the inhibition of NOX1 using ML171 is able to attenuate L-NAME induced leukocyte-endothelial interactions. The mechanism of this action may be associated with reduced levels of SO, which help restore vascular endothelial function (37). Our data suggest that the use of ML171 may be useful in attenuating vascular endothelial dysfunction and subsequent inflammation in chronic inflammatory diseases such as diabetes, hypertension and I/R injury.

References

1. Su JB. Vascular endothelial dysfunction and pharmacological treatment. World J Cardiol. 2015;7(11):719-41.
2. Favero G, Paganelli C, Buffoli B, Rodella LF, Rezzani R. Endothelium and its alterations in cardiovascular diseases: life style intervention. Biomed Res Int. 2014;2014:801896.
3. Deanfield J, Halcox J, Rabelink T. Endothelial Function and Dysfunction Testing and Clinical Relevance. Circulation American Heart Association. 2007; 115: 1285-1295
4. Eroschenko VP, Fiore MS. DiFiore's Atlas of Histology with Functional Correlations. Lippincott Williams & Wilkins; 2013.
5. Cardiovascular Physiological Concepts. c1998-2016. Richard E. Klabunde PhD. Accessed May 5, 2016 http://www.cvphysiology.com/Blood%20Flow/BF011.htm
6. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012;33(7):829-37, 837a-837d.
7. Galley HF, Webster NR. Physiology of the endothelium. Br J Anaesth. 2004;93(1):105-13.
8. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357(Pt 3):593-615.
9. Yang YM, Huang A, Kaley G, Sun D. eNOS uncoupling and endothelial dysfunction in aged vessels. Am J Physiol Heart Circ Physiol. 2009;297(5):H1829-36.
10. Ampofo E, Widmaier D, Montenarh M, Menger MD, Laschke MW. Protein Kinase CK2 Regulates Leukocyte-Endothelial Cell Interactions during Ischemia and Reperfusion in Striated Skin Muscle. Eur Surg Res. 2016;57(1-2):111-124.

11. Gewaltig MT, Kojda G. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. Cardiovasc Res. 2002;55(2):250-60.
12. Wallace JL. Nitric oxide as a regulator of inflammatory processes. Mem Inst Oswaldo Cruz. 2005;100 Suppl 1:5-9.
13. Ago T, Kuroda J, Kamouchi M, Sadoshima J, Kitazono T. Pathophysiological Roles of NADPH Oxidase/NOX Family Proteins in the Vascular System. *Circulation Journal.*
14. NOX proteins in signal transduction
15. Nisimoto Y, Diebold BA, Cosentino-gomes D, Constentino-gomes D, Lambeth JD. Nox4: a hydrogen peroxide-generating oxygen sensor. Biochemistry. 2014;53(31):5111-20.
16. Perkins KA, Pershad S, Chen Q, et al. The effects of modulating eNOS activity and coupling in ischemia/reperfusion (I/R). Naunyn Schmiedebergs Arch Pharmacol. 2012;385(1):27-38.
17. Sirker A, Zhang M, Shah AM. NADPH oxidases in cardiovascular disease: insights from in vivo models and clinical studies. Basic Res Cardiol. 2011 Sep;106(5):735-47
18. Ray R, Shah AM. NADPH oxidase and endothelial cell function. Clin Sci. 2005;109(3):217-26.
19. Xu S, Touyz RM. Reactive oxygen species and vascular remodelling in hypertension: still alive. Can J Cardiol. 2006;22(11):947-51.
20. Gutterman DD. Mitochondria and reactive oxygen species: an evolution in function. Circ Res. 2005;97(4):302-4.
21. Dikalov S. Cross talk between mitochondria and NADPH oxidases. Free Radic Biol Med. 2011;51(7):1289-301.
22. Mogensen CE, Christensen CK, Vittinghus E. The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. Diabetes. 1983;32 Suppl 2:64-78.
23. Steyers CM, Miller FJ. Endothelial dysfunction in chronic inflammatory diseases. Int J Mol Sci. 2014;15(7):11324-49.
24. Langer HF, Chavakis T. Leukocyte-endothelial interactions in inflammation. J Cell Mol Med. 2009;13(7):1211-20.
25. Lefer AM, Lefer DJ. The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. Cardiovasc Res. 1996;32(4):743-51.
26. Pham H, Barsotti R, Koon A, Rueter B, Young L, Chen Q, The Role of NAPDH Oxidase on L-NAME-Induced Leukocyte-Endothelial Interactions in Rat Mesenteric
27. Gianni D, Taulet N, Zhang H, et al. A novel and specific NADPH oxidase-1 (Nox1) small-molecule inhibitor blocks the formation of functional invadopodia in human colon cancer cells. ACS Chem Biol. 2010;5(10):981-93.
28. Remarcke R, Patel S, Stutzman D, et al. The Cardio Protective effects of a NOX1 Inhibitor, ML171, on Myocardial Ischemia/Reperfusion (I/R) Injury. The FASEB Journal. 2015;29(1):635.3

29. Gavins FN, Chatterjee BE. Intravital microscopy for the study of mouse microcirculation in anti-inflammatory drug research: focus on the mesentery and cremaster preparations. J Pharmacol Toxicol Methods. 2004;49(1):1-14.
30. Inoguchi T, Sonta T, Tsubouchi H, et al. Protein kinase C-dependent increase in reactive oxygen species (ROS) production in vascular tissues of diabetes: role of vascular NAD(P)H oxidase. J Am Soc Nephrol. 2003;14(8 Suppl 3):S227-32.