RECEPTOR FOR ADVANCED GLYCATION END-PRODUCTS BLOCKADE IMPROVES ENDOTHELIAL DEPENDENT VASCULAR FUNCTION IN ATHEROSCLEROTIC MICE

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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presented by Steven Ray McAfee,

a candidate for the degree Master of Science – Physiology

and hereby certify that, in their opinion, it is worthy of acceptance.

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To J.D., Mailey, and Evelyn

A.Y.K.I.L.

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Receptor For Advanced Glycation End-products Blockade Improves Endothelial Dependent Vascular Function In Atherosclerotic Mice

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Abstract

Atherosclerosis is a progressive inflammatory disease that is present in large vessels in the body. We hypothesized that either adiponectin treatment or soluble Receptor for Advanced Glycation Products (sRAGE) treatment would rescue the decreased endothelial function of aortae in apolipoprotein-E knockout (ApoE-/-) mice, a murine model of atherosclerosis. We examined endothelial-dependent vasorelaxation to acetylcholine (ACh) in aortae removed from ApoE-/- and control wild (WT) mice. Relaxation to ACh was blunted in ApoE-/- compared with WT controls while endothelial-independent vasorelaxation to sodium nitroprusside (SNP) was comparable. sRAGE improved ACh-induced vasorelaxation in ApoE-/- mice without affecting dilator response to SNP.

Adiponectin treatment did not show significant improvement of endothelial function in aortae of ApoE-/- mice. Dilation to ACh was significantly attenuated after administration of nitric oxide (NO) synthase inhibitor N^G-monomethyl L-arginine in WT mice, which indicates that vasodilation to ACh was NO mediated while L-NMMA did not further inhibit endothelial-dependent vasodilation in ApoE-/- mice. Immunostaining showed RAGE to co-localized with the endothelium in murine aortae. These results suggest that AGE/RAGE signaling may play a pivotal role in processes that lead to endothelial dysfunction in atherosclerosis.

Chapter 1: Introduction and Literature Review

Cardiovascular Disease and Inflammation

Cardiovascular disease, specifically heart disease and stroke, is the leading cause of death in the United States each year [1]. While the causes of heart disease and stroke are multi-factorial, there is a singular pathological condition common to all of these maladies: atherosclerosis (AS). Atherosclerosis itself is a complicated disease with environmental, epigenetic and genetic components. Despite the multivariable causes, atherogenesis tends to follow a particular course which includes a fatty streak that can progress to a luminal stenosis and ultimately vessel thrombosis. Early in the condition at and distal to its primary atheromatous lesion, there is development of endothelial dysfunction in both the macro- and microvascular networks. The endothelial dysfunction associated with a generalized inflammatory state can lead to further complications such as thromboses, that can result in myocardial infarction or stroke. The activation of the inflammatory response creates a milieu that is favorable for the development of the atherosclerotic disease and its complications. Where and when this inflammation becomes pathological during this process and whether or not it is possible to modulate this chronic state of inflammation remains to be elucidated.

Atherosclerosis – Development of Lesions

Relatively recently, the paradigm through which we view atherosclerosis has shifted; what was once thought to be a disease of stenoses and fluid mechanical effects in blood vessels is now understood to be a complex inflammatory disease whose complications extend beyond that of obstructed blood flow [1]. This is best demonstrated by noting the presence of vascular dysfunction throughout the arterial tree and not just at the site of the atherosclerotic lesion. While the entire mechanism for atherosclerotic lesion development remains unknown, lipids may be involved in processes that trigger atherogenesis. Atheromata begin development as "fatty streaks." Fatty streaks have been identified in very young animals and humans regardless of their lipidemic state [2]. For decades hypercholesterolemia has been identified as a significant risk factor in the development of atherosclerotic lesions [3]. Ross and Harkin demonstrated that hyperlipidemic subjects developed and maintained fatty lesions while their eulipidemic counterparts showed lesion regression in vessels denuded of the endothelium. Of particular interest to Ross and Harkin was the change in morphology of vascular smooth muscle cells (VSMC) within the lesions; these cells were replete with multiple "intracellular lipid inclusions" best described as "membrane bound and frequently contained concentric membranous whorls." [3] Further, the accumulation of lipid in the sub-endothelial layer of the vessel may initiate the inflammation that is currently believed to drive the development of AS. The accumulation of low-density lipoprotein (LDL) in the tunica media provides

an ideal environment for its oxidation to ox-LDL due to the presence of multiple generators of free radicals. Among the proteins responsible for the production of superoxide and subsequent oxidation of LDL is NADH oxidase, a conditionally assembled, membrane-associated, multi-subunit oxidase. There appear to be many cellular sources of NADH oxidase throughout the vessel; among them are vascular endothelial cells in the tunica intima, vascular smooth cells in the tunica media and in the tunica adventitia both macrophages and other connective tissue cells [4-7].

Role of Reactive Oxygen Species (ROS).

Once oxidized, LDL occurs in a milieu rich in receptors for which it is a ligand. Among the scavenger receptors known to bind oxLDL are CD36 and lectin-like oxidized LDL receptor-1 (LOX1). Several research groups identified cell lines that actively express CD36 and LOX1 and together with macrophages and endothelial cells play a prominent role in the development of atherosclerotic disease [8-11]. Depending on the cell line expressing the receptor that oxLDL binds, with varied outcomes, ranging from inducing endothelial dysfunction to stimulating macrophages to generate a pro-inflammatory milieu or stimulate proliferation of vascular smooth muscle cells or leukocytes within the vessel wall. The acute induction of endothelial dysfunction by oxLDL acting on its receptor appears to be mediated, at least in part by the formation of reactive oxygen species (ROS). This is due to the LOX1 dependent activation of NAD(P)H oxidase [12]. The increase in ROS leads to a concomitant decrease in the bioavailability of nitric oxide (NO). This is due to a reaction between superoxide

and NO that leads to generation of peroxynitrite and a reduction in endothelial dependent vasodilation as the NO is inactivated in a dose dependent manner by oxidation. While this provides an explanation for the acute dysfunction in vessels, oxLDL can trigger more chronic changes in the endothelium through its effects on a particularly potent, pro-inflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

The Role of Inflammatory Responses.

The activation of NF-κB appears to be a key event in the progression of atherosclerosis as an inflammatory disease. NF-κB is a transcription factor for a number of inflammatory cytokines; a key cytokine among them is TNF-α [13]. TNF-α signaling creates a positive feedback loop, whereby it activates NF-κB, begetting the synthesis of other pro-inflammatory cytokines including more TNF-α [13]. NF-κB is sequestered in its inactive state by IκK-ß, preventing its nuclear translocation and function as a transcription factor for inflammatory cytokines. Yang et al demonstrated that the addition of the IκK-ß inhibitor, sodium salicylate, decreased the expression of TNF-α, while genetically modified diabetic mice null for TNF-α exhibited decreased expression of NF-κB. These studies implicate TNF-α in the progression and maintenance of the atherosclerotic disease. Further, Tipping and Hancock observed that monocytes and macrophages isolated from atherosclerotic plaques removed during carotid endartectomy produced significantly more TNF-α when compared to monocytes in freshly collected blood [14]. Together, these studies provide evidence of a

positive feedback loop that has the potential to perpetuate a chronic inflammatory response.

To further explore the inflammatory response, one must address the increase in expression of cellular adhesion molecules that occurs in hyperlipidemic states and in the presence of the pro-inflammatory cytokine TNF-α. Collins and Cybulsky outline some key effects of NF-κB activation in atherosclerosis in their 2001 review. [15] “Multiple genes whose products are putatively involved in the atherosclerotic process are regulated by NF-κB. Leukocyte adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin, as well as the chemokines (chemoattractant cytokines) monocyte chemoattractant protein-1 (MCP-1) and IL-8, help recruit circulating mononuclear leukocytes to the arterial intima,” [15]. Vascular cell adhesion molecule (VCAM-1), of the immunoglobulin superfamily, has an expression that is inducible upon incubation with TNF-α, offering further support that TNF-α signaling and NF-κB activation are linked [16]. In the absence of VCAM-1, initial leukocyte adhesion is prevented and thus leukocytes that home to the inflamed endothelium blinded to the underlying lesion. VCAM-1 appears to be a part of a specific response to intimal inflammation, which aids in targeting inflammatory effector cells, particularly monocytes, to the early atherosclerotic lesion, promoting the amplification of errant inflammation in the developing atheroma. The atherosclerotic disease process further results in up-regulation of other endothelial targeting molecules such as P- and E-selectin.

The cellular adhesion molecules, VCAM-1 and ICAM-1 are not solely responsible for leukocyte rolling. Both P- and E-selectins, proteins that participate in the leukocyte rolling portion of the mechanism of extra-vasation, are found to be abnormally abundant in the mice with AS. Two studies from Wagner's laboratory [17,18] helped elucidate the roles of P- and E-selectins in AS. A P-selectin knockout was studied in LDL receptor (LDLR) deficient mice fed a high-fat diet to induce AS [17]. They observed that LDLR deficient mice exhibited leukocyte rolling on a high fat diet maintained from eight weeks to over eight months, a time when the progress of AS lesions was to the point of fibrous cap development signifying an advanced lesion. The rolling response was a crucial step in the process of leukocyte extravasation and furthered the inflammatory response characteristic of AS lesions. In their genetically modified, P-selectin deficient mice there was a significant decrease in leukocyte rolling along a presumably activated endothelium. Also, when measuring lesion area, LDLR mice deficient in P-selectin demonstrated lesion sizes approximately 20% smaller than their P-selectin wild-type littermates. Interestingly, P-selectin expressing mice developed advanced, fibrotic lesions whereas the double knockout mice developed lesions no more advanced than that of a fatty streak. Their following work using a triple knockout mouse deficient for P- and E-selectin and LDLR knockout. [18] These mice (triple knockout) were put on a high fat diet for eight weeks, at which time it was found they had considerably smaller atheromas in the selectin knockout models compared to their AS prone, but selectin intact littermates. Surprisingly, the cellular composition of the lesions from both

experimental groups was similar, despite the lack of proteins to permit leukocyte rolling. However they found that the overall number of lesion monocytes/macrophages was reduced, which the authors attributed to the decrease in lesion size and complexity in the triple knockout animals.

Based on these studies, the lesion area is primed to receive the inflammatory cells; most notably among them are monocytes, macrophages and T-cells. But what initially attracts these cells to the lesion area? A number of chemokines form a gradient that assists in homing leukocytes to inflammatory sites. However, which specific chemokines among the many are complicit in lesion formation is currently being investigated. Monocyte chemoattracant protein -1 (MCP-1) is thought to be a critical attractor of monocytes to the developing lesion. MCP-1 was identified to have a key role in the development of vascular dysfunction in a model of type II diabetes mellitus [19]. Inhibiting MCP-1 signaling via anti-MCP-1 antibody partially restored endothelial dependent vasodilation previously blunted by the diabetic disease. In addition, anti-MCP-1 treated animals showed a significant decrease in superoxide production, nitrotyrosine protein expression (an indirect indicator of peroxynitrite production), expression of VCAM, ICAM, E-selectin and NF-κB. This work demonstrated that either MCP-1 or TNF-α inhibition decreased macrophage infiltration into the vessel and decreased expression of a number of pro-inflammatory cytokines. Thus, MCP-1 appears to play a decidedly pro-inflammatory role within the vessel by attracting and aggravating macrophages. Quax and colleagues showed that in an AS prone, vein-grafted mice MCP-1 wass expressed on the vascular endothelium in

veins grafted onto the murine carotid arteries [20]. Soon after the development of the initial lesion, interstitial leukocytes produce their own MCP-1 for a limited time [20]. This creates yet another positive feedback loop, where inflammation begets more inflammation, pushing the growth and development of the initial fatty-streak toward the fibrous plaque stage. MCP-1, signaling through a member of the chemokine receptor family, chemokine (C-C motif) receptor 2 or CCR2, encourages monocyte adhesion to the vascular endothelium promoting emigration into the intima [20]. Once in the intima, monocytes undergo morphological changes and develop into mature macrophages. The macrophages begin ingesting lipids and develop into foam cells thus driving the preliminary fatty streak closer to a fibrous lesion.

Role of vascular smooth muscle cells.

The presence of vascular smooth muscle cells within the intima of the vessel is evidence of a more mature lesion. This condition is abnormal, as the elastin rich internal elastic lamina prevents migration of vascular smooth muscle cells out of the media and into the intima. Migration of smooth muscle cells is necessary for the lesion to reach its final mature morphology, that of a fibrous plaque protruding into the luminal space with a necrotic core of foam cells and non-cellular lipid droplets [21]. The presence of hyperlipidemia, induced by a fatty "Western" diet, is apparently sufficient to trigger smooth muscle cell proliferation. Using Watanabe heritable hyperlipidemic rabbits, a model similar to the LDLR knockout mice, Rosenfeld and Ross noted that on induction of a high fat diet there was an inverse correlation between the amount of radioactive thymidine

and ratio of the measured thickness tunica intima versus the tunica media in segments of the aorta [22]. Unfortunately, no real mechanism for increase in proliferation was addressed in these studies. However others pointed to a number of the inflammatory cytokines as possible mediators of VSMC proliferation. [23-25]

In a cell culture model activation of NF-κB, via a molecule known as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), activated VSMCs and caused proliferation through up-regulation of insulin-like growth factor-1 receptor (IGFR1) [23]. Inhibition of the NF-κB signal prevented TRAIL dependent IGFR1 expression. This work points to NF-κB as being vasoprotective in acute inflammation as IGFR1 is known to be anti-apoptotic but suggests a role for errant proliferation in chronic inflammation. This provides additional insight into a role for NF-κB within the vessel wall, beyond the traditional role in inflammation. There is ample evidence to show that the milieu of the atheroma is pro-inflammatory, ripe with a myriad of inflammatory cytokines, and shown to activate NF-κB. This mechanism highlighting IGFR1 induced VSMC proliferation offers new insight into how the atheroma progresses from a simple fatty streak to a fibrous plaque. In addition, there appears to be another mechanism by which inflammation can result in proliferation of VSMC. Matrix metalloprotease activation (MMP) via the CD40-CD40L signaling pathway can result in AS lesion development and maturation. CD40, a costimulatory molecule necessary for complete activation of antigen presenting cells, is expressed in a number of cell types such as platelets, monocytes/macrophages, endothelial cells, VSMC, B

and T lymphocytes and fibroblasts whereas CD40L is expressed in platelets, endothelial cells, monocytes/macrophages and activated T lymphocytes [24]. All of these cell types reside in normal blood vessels and those with atheromas. Chai, et al concluded that CD40/CD40L signaling caused an *in vitro* increase in both VSMC proliferation and migration [25]. There was also upregulation of both mRNA and protein for MMP-9 while MMP-2 showed decreases in both the mRNA and protein levels. Inhibition of MMP-9 by a neutralizing antibody blunted both proliferation and migration of VSMC. This points toward NF-κB dependent activation as a mechanism for proliferation and migration of VSMC in atheroma formation.

Inflammation and cellular adhesion plays a critical role in the development and progression of AS. Regulation of inflammation is critically important and several signaling pathways exist to maintain homeostasis. However, during AS, the normal homeostatic mechanisms fail and positive feedback loops become the dominant mechanism further worsening the milieu and thus improving the environment for inflammation and lesion progression.

Modulation of Inflammation

Inflammation plays a critical role in the development, progression and subsequent complications of atherosclerotic disease, as discussed above [71]. In order to develop diagnostic and therapeutic approaches to AS, it is critical that further mechanistic insight into the abnormal inflammatory responses be investigated. The study of obesity and diabetes mellitus may give rise to two important molecules involved in a native inflammatory response, adiponectin and

the signaling cascade downstream of the advanced glycation end-products (AGE) interactions with the receptor for advanced glycation end products (RAGE).

Adiponectin in atherosclerosis

As recently as the early 1990s, adipose tissue was thought of as a repository for excess calories in the form of lipids. During the mid-1990s, however, that paradigm began to shift with the discovery of leptin, a hormone produced in adipose tissue [26]. The discovery that adipose tissue functions as an endocrine organ led to further exploration of its physiological duties. The discovery of the hormone adiponectin, in 1996, was a key milestone for furthering our new view of adipose tissue as an endocrine organ [27-28]. Adiponectin (also known as AdipoQ, Acrp30, GPB28 and herein known as APN) is approximately 30 kD in size, with a linear sequence of 244 amino acids and is the most abundantly expressed protein produced by adipocytes. APN has been identified as a member of the complement 1 q (C1q) family of proteins. There is substantial conservation of structure between another member of the C1q family, TNF-α, and adiponectin [28]. The tertiary structures share so many features that Shapiro and Scherer hypothesize that the two molecules were products of divergent evolution from a common effector protein within the innate immune system. In earlier work by Scherer et al, they analyzed and described APN from primary to tertiary to structure [29]. There are four functional regions that comprise the adiponectin molecule: 1. The N-terminal signaling sequence followed by; 2. a short, non-homologous region of 27 amino acids which precedes; 3. a collagen-

like stalk region; and 4. the C-terminal globular head domain. Two major isoforms have been identified, globular adiponectin (gAPN) and full-length adiponectin. gAPN is reported to be a proteolytic cleavage product yielding the globular domain of the parent protein as an pharmacologically active agent [60]. Polyacrylamide gel electrophoretic analysis of plasma samples under non-reducing, non-denaturating conditions using antibodies targeted to APN revealed multiple bands. These bands correlated to the native forms of adiponectin and reflect its ability to oligomerize and for the oligomers to further oligomerize. A common oligomerization occurs when three full-length APN molecules associate and form a homotrimer, which is termed low molecular weight APN. Two homotrimers may then dimerize to form a medium molecular weight hexamer. The hexamers are formed through the formation of disulphide bonds between the two newly associated trimers. Hexamers either dimerize or trimerize to form 12- or 18-mers of high molecular weight (HMW) APN [30]. APN is robustly expressed by adipocytes and is abundant in the circulation with plasma concentrations reportedly at 2-10μg/mL in healthy human control subjects [31]. APN expression also exhibits a sexual dimorphism with females having slightly higher plasma concentrations than that of their male counterparts. This is interesting because multiple studies have demonstrated an inverse relationship between the concentration of plasma APN and adiposity [66,67]. The transcription factor, peroxisome proliferator-activator receptor – gamma (PPAR-γ), bears some control over the expression of APN [32, 33]. This supports the observation that patients treated with thiazolidendiones, known PPAR-γ agonists, to assist in the

management of diabetes mellitus show an increase in the plasma concentration of APN [70].

There are three known receptors for APN, Adiponectin Receptor 1 (AR1), Adiponectin Receptor 2 (AR2) and T-cadherin. T-cadherin is believed to be largely involved in angiogenesis, though its role remains unclear [63-65]. AR1 and AR2 have been characterized as seven pass transmembrane receptors after examination of a human cDNA library [33]. Unlike other seven transmembrane domain receptors, AR1 and AR2 present their C-terminus on the extracellular surface of the cell while the N-terminus remains in the cytoplasmic compartment. Further examination points to specialization of the receptors, both in their tissue-specific expression and regarding the ligand that they bind most effectively [33]. Between AR1 and AR2 approximately two thirds of their amino acid sequence is homologous [69]. AR1 appears to be the predominant APN receptor, expressed in nearly all tissues. AR1 shows a strong expression in skeletal and cardiac muscle, while AR2 is predominantly expressed in the liver. [69] The expression of both AR1 and AR2 in the vessels of mice has been observed; however, the role of dual expression in the vasculature remains unclear at this point [61]. There is also a difference in ligand affinity exhibited by each receptor. AR1 has a much greater affinity for gAD than for full-length APN. In contrast, AR2 has demonstrated equivalent affinity for both gAD and full-length APN.

Numerous investigators have explored the downstream signaling of APN receptors. Conserved between the two receptors are two major signaling activities, one involves the activation of 5’ adenosine monophospate-activated

kinase (AMPK) while the other is activation of PPAR- γ [32, 35]. Of note, is PPAR-γ's role as a transcription factor leading to the expression of APN [32]. Evidence also suggests that APN signaling activates PPAR-γ both directly and indirectly through its AMPK mediated mechanism [33]. This presents the opportunity for a positive-feedback mechanism ensuring that APN begets more APN. With regard to the vasculature, AMPK is a known activator/phosphorylator of endothelial nitric oxide synthase (eNOS) [35]. Thus AMPK activation increases the amount NO produced by the endothelium leading to greater dilation in response to endothelium-dependent vasodilators, such as acetylcholine (ACh). Additionally, APN has been shown to directly interfere with TNF-α signaling [34,35].

APN has a marked effect on inflammation in many tissues, but in particular on blood vessels. Full-length APN prevents the activation of NF-κB in *in vitro* and in *in vivo* models [30,36,37]. In contrast gAPN does not but it increases the expression of p65 as opposed to full-length APN's action. This finding lends support to the observation that administration of full-length APN can attenuate the expression of a number of inflammatory cytokines and adhesion molecules. APN knockout animals show an increased expression of many cytokines, chemokines, and adhesion molecules, such as TNF-α, MCP-1, VCAM-1 and ICAM-1 respectively, when compared with their wild type littermates [38]. This phenotype is partially reversible with the administration of murine recombinant APN. These findings provide some mechanistic insight into observations that APN is vasoprotective and anti-atherosclerotic in nature.

Okamoto et al examined the effect of APN on atheroma formation using Apolipoprotein E knockout (ApoE-/-) mice [39]. After transfection with virus expressing human APN, they were able to draw several major conclusions. Lesion size, as assessed by measurements of cross-sectional area in aortae, was reduced in the animals over-expressing APN, as was the size of intravascular lipid droplets. mRNA expression of a known receptor for ox-LDL, the class A scavenger receptor, was blunted along with expression of VCAM-1 and TNF-α. This is likely due, in part, to the known suppression of NF-κB signaling by APN. Hattori et al reported that both gAPN and HMW APN increase NO production in vascular endothelial cells [40,41]. This increase in NO production by HMW APN is due to the activation of AMPK and its subsequent phosphorylation and activation of eNOS. Interestingly, Hattori et al also found that adiponectin interferes with TNF-α induced, NF-κB activation in addition to demonstrating a mild activation of NF-κB itself. The activation of NF-κB by APN has been reported for both gAPN and HMW APN, but it remains unclear as to the purpose or effect of this activation [30,41]. In another study using APN knockout mice, the investigators examined the effect of APN on leukocyte-endothelial cell interactions in the peri-intestinal venules of mice and found that APN deficiency leads to increases in VCAM-1 and E-selectin, in addition to an increase in leukocyte adhesion and rolling [41]. These biochemical interactions create the proper milieu for vascular fatty streak development and atheroma formation [42]. These pathways were confirmed in another series of experiments using human aortic endothelial cells *in vitro* [43]. Ouchi et al showed that APN *in vitro* prevents

human macrophage differentiation to foam cells by preventing the accumulation of lipids, a critical step in atheroma progression [44]. In addition, scavenger receptor A expression was reduced resulting in blunted macrophage uptake of lipids on treatment with APN. Work in our laboratory investigated the role of APN in the evolution of atherosclerotic disease [45]. We administered recombinant human gAD to ApoE-/- mice to test the hypothesis that there is "reciprocal association between adiponectin and lectin-like oxidized LDL receptor-1 (LOX-1) [which] contribute to the regulation of aortic endothelial dysfunction in atherosclerosis." In ApoE-/- mice we found that APN limited endothelial dysfunction associated with AS and reduced both the expression of LOX-1 and the formation of ROS in the aortic endothelium [45]. Taken in total, these works demonstrate that there is a clear role for AD in AS which needs to be further examined.

AGE/RAGE in atherosclerosis

RAGE is an extracellular receptor for a number of ligands, among them are advanced glycation end products (AGE). AGEs are proteins and lipids that have acquired glucose moieties in a non-enzymatic fashion. Other RAGE ligands are members of the s100/calgranulin family, high mobility group box (HMGB-1) proteins and advanced oxidation protein products such as ox-LDL [46]. RAGE was first cloned in 1992 by Neeper et al who was searching for a human analog to the bovine lung protein that acted as a receptor for the ligand AGE [47]. Human RAGE is encoded by a cDNA of 1406 base pairs yielding a primary sequence of 404 amino acids for the mature protein [47]. Native RAGE has a

molecular weight of approximately 35 kD [47]. It has been identified as a member of the immunoglobulin (Ig) superfamily [47]. Multiple isoforms of RAGE exist. Isoforms termed soluble indicate that they are no longer anchored to the cytoplasmic membrane exist and among the isoforms known to be soluble are endogenous secretory RAGE (esRAGE), and soluble RAGE (sRAGE) [47]. Recently, Hudson's group studied the presence of splice variants of RAGE in various mice tissues [48]. They identified a number of tissue and disease dependent variants in the mouse. Interestingly, in all of the conditions studied they were unable to detect sRAGE, even in diabetic animals where they hypothesized it would be expressed in the greatest amounts. Because the soluble isoforms can be produced through either alternative splicing or perhaps by enzymatic cleavage, as suggested by Kalea et al and Zhang et al, it is possible that their disease model did not meet the conditions necessary to produce this response [48,49]. To produce soluble RAGE, both alternative splicing and proteolysis produce an N-terminus/ligand binding domain free from both the transmembrane and cytocplasmic/signaling domain. This positions the soluble RAGE isoforms to act as buffers by competitively inhibiting RAGE signaling by binding and making unavailable the many ligands for the full-length RAGE molecule.

RAGE is expressed in a variety of tissues under normal physiological conditions. It plays a role in the regulation of neuronal development but also is found expressed on the endothelium of non-diseased animals [50]. Signaling downstream of RAGE ultimately terminates in the activation of NF-κB [50].

Another of the downstream mediators activated during RAGE signaling is NAD(P)H oxidase, generating ROS contributing to endothelial dysfunction by inactivating the NO present. This was noted in the presence of RAGE ligands as shown by Gao et al in a murine model of type II diabetes mellitus [51]. Gao et al also showed that RAGE inhibition, via the null receptor sRAGE, partially restored the vasodilation and decreased mRNA expression of NAD(P)H oxidase in coronary arterioles of diabetic mice. Finally, Gao et al reported that sRAGE treatment in diabetic animals decreased the production of ROS, leading us to conclude that the improvement in dilation is due, at least in part, to an increase in the NO bioavailability. Those studies with RAGE inhibition also demonstrated decreased protein expression of p65/NF-κB, TNF-α and RAGE itself. These findings provide support for the idea that RAGE signaling creates a positive feedback loop of inflammation by increasing production of inflammatory cytokines such as TNF-α via an NF-κB dependent mechanism. This mechanism also serves to increase the expression of RAGE, as NF-κB is a known transcription factor of RAGE [52]. Since RAGE may function as both cause and effect of inflammation in the vasculature, it may prove to have a critical role in the development of AS.

Since AGE were the first ligands identified for RAGE, much of the early work regarding RAGE was performed in models of diabetes mellitus. Recently, in the early 2000's, Schmidt's laboratory began investigating the role of RAGE in atherosclerosis in the development of atheromata [53]. They found that sRAGE

administration to ApoE-/- mice with a streptotozocin induced type one diabetes mellitus developed larger atheromata as compared to their non-diabetic littermates. RAGE blockade with sRAGE slowed the progression of AS lesions with mice having 4 fold smaller lesions as well as significantly fewer complex lesions than vehicle treated littermates. Further, this work demonstrated a decreased expression of RAGE within the lesion, as well as smooth muscle α-actin (a marker of VSMC proliferation) and CD68 indicating a decrease in macrophage/foam cell infiltration into the lesion. The protein expression of MCP-1 and VCAM-1 also were decreased, demonstrating less opportunity for monocytes/macrophages to migrate into the developing atheroma. This work illustrated that RAGE may play a role in the development of AS however the presence of hyperglycemia in this model does confound the results. To that end, ApoE-/- mice were bred to mice expressing: 1) dominant negative RAGE which expresses the extracellular and transmembrane domain of RAGE but not the cytoplasmic domain; or 2) RAGE knockout (RAGE -/-) animals to assess how RAGE mediates the progression of atherosclerosis [54]. Both RAGE mutants with ApoE-/- had decreased expression of a variety of pro-inflammatory molecules such as s100b, HGMB-1, VCAM-1 and MCP-1 as compared with their non-mutant RAGE littermates. Interestingly, RAGE signaling is shown to be initiated by both s100b, a product of macrophages and ox-LDL, a known resident of atheromas. Both ligands are readily available to RAGE expressed within and on the endothelium overlying the AS lesion.

Thus our hypotheses are twofold: 1. Treatment with either sRAGE or AD will improve the endothelial dysfunction observed in the aortae of ApoE-/- mice fed a Western diet; and 2. RAGE, like AD, co-localizes with the endothelium of the murine aorta.

Chapter 2: Methods

Animal Models.

All animal use was performed in direct compliance with the guidelines set forth by the Animal Care and Use Committee of the University of Missouri – Columbia. Male mice were purchased from the *Jackson Laboratory* (Bar Harbor, ME USA, http://www.jax.org) at 5 to 6 weeks of age. Animals purchased included wild type controls (WT, C57Bl/6J), and an atherosclerotic model, the apolipoprotein E deficient mouse (ApoE-/-). To accelerate atheroma formation and the associated vascular dysfunction, ApoE-/- animals were provided with a high-fat, "western diet" (adjusted calories diet; Harlan Teklad TD88137; 42% from milk fat, 0.15% cholesterol) for 12 weeks, as well as water ad libidum. Wild type controls were provided a standard chow and water ad libidum on arrival to the Dalton Center for Cardiovascular Research. No animals were fasted prior to use. All animals were maintained in a climate controlled, clean environment with 12 hour light/dark cycles.

Measurement of Blood Compounds

Mice were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection. 0.5-1 cc of heparin (1%) was injected into the parenchyma of the lung to prevent coagulation and heparinized syringes were used for blood collection. On sacrifice, whole blood was collected from the inferior vena cava into a heparinized syringe and placed immediately into a 1.5 ml centrifuge tube. Blood was then centrifuged at 10,000 RPM for 10 minutes at

4° C and the plasma separated from the cells and stored in another centrifuge tube at -20° C for future testing and analyses.

A. **Blood Glucose.** Glucose concentration was measured on fresh, whole blood by a LifeScan OneTouch Ultramini glucometer (Milpitas, CA USA).

B. **Plasma Adiponectin Measurements.** Estimations of plasma adiponectin were made using western blot analysis.

C. **Plasma Lipid Concentrations.** Due to limited quantities of serum, high density lipoprotein, low density lipoprotein and triglycerides were not measured. The ELISA kits all required greater amounts of serum than was available from these experiments. Previously, measurements of total cholesterol by our group showed no significant difference among the WT, ApoE-/- and ApoE-/- + Adiponectin treated groups, while others reported no differences between Zucker obese diabetic rats or Zucker obese diabetic rats treated with sRAGE [45,55].

Adiponectin and soluable RAGE (sRAGE) Treatment

Following twelve weeks on the Western diet, Apolipoprotein E deficient mice were selected for one of three possible treatments. Group 1 (n=8, ApoE-/-) was used as a non-treated, diseased control group, group 2 (n=4, Adipo) was treated with recombinant human globular adiponectin [15 μg/day/mouse, subcutaneously daily at 4pm for 8 days] purchased from Peprotech (Rocky Hill, NJ USA) and group 3 (n = 4, sRAGE) was treated with soluble RAGE [60 mg/day/mouse, intraperitoneally at 4:00 pm daily for three days] which was

graciously provided by Dr. Anne Marie Schmidt of Columbia University (New York, NY USA). Untreated wild type mice (n=12, WT) served as normal control animals.

Functional Assessment of Murine Thoracic Aortae

Shortly after anesthesia, the hearts and aortae of the experimental mice were exposed. Prior to pericardiectomy, the parenchyma of the lungs was injected with 0.5-1 cc of 1% heparin sulfate to prevent excessive coagulation in the lumen of the aorta. Following pericardiectomy, the aortae were excised superior to the aortic valve to the region proximal to the bifurcation of the aorta into the common iliac artery and rinsed in ice cold, modified Kreb's solution (PSS; composition 119.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \bullet 7H_2O$, 2.5 mM $CaCl_2 \bullet 2H_2O$, 14.9 mM $NaHCO_3$, 5.5 mM D-Glucose, and 0.03 mM EDTA). While in the ice cold PSS, the excess connective tissue and adipose were removed from the vessel. The aortae were sectioned into approximately 2 mm rings and mounted onto a DMT model 610M myograph (Georgia, USA) for assessment of contractile function. The organ bath containing PSS was maintained at 37°C and bubbled with a 95% O_2/ 5% CO_2 mixture. Every 15 minutes, the organ bath was emptied and fresh PSS was added to the chamber. The organ bath was emptied and fresh PSS was added immediately prior to concentration-response studies for acetylcholine (ACh) or sodium nitroprusside (SNP). A passive tension of 15 mN was applied to the vessels over 15 minutes and they were allowed to equilibrate for 30 minutes after passive tension reached 15 mN. After equilibration the vessels were preconstricted with

phenylephrine (PE; 1 μM). A concentration-response curve was generated using cumulative, stepwise addition of either ACh or SNP from 1 nM to 10 μM in log molar increments for each compound [45,56]. To determine the extent of relaxation, the force at each concentration was measured and represented as a percentage of maximum force in response to PE. To assess the vasorelaxation contributed by nitric oxide (NO) to agonist induced relaxation, a competitive endothelial nitric oxide synthase (eNOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) was added to each organ bath at a concentration of 100 μM 30 minutes prior to the concentration-response curve. During this time the organ bath solution was changed, and each time new 37º C PSS was added to the bath, another dose of 100 μmolar L-NAME was added to inhibit NO synthase. After completing the functional assessments, aortae were flash frozen in liquid nitrogen and stored with the previously excised and frozen portion of the aorta not used in the ring studies for later investigations.

Immunofluorescent Assessment of RAGE

Hearts and aortic segments (arches and thoracic segments distal to the arch) were rinsed in ice cold PSS and embedded in Sakura Finetek Optimal Cutting Temperature (Torrance, CA, USA) compound and frozen at -27ºC and stored at -80ºC for use later. Blocks were placed onto and cut using a Leica cryostat (Wetzler, Germany) to a thickness of 5 μm and allowed to air dry at room temperature for 25 minutes. After drying, the slides were fixed in acetone at 4ºC for 10 minutes. After fixation, the slides were again allowed to air dry and then were washed three times for 5 minutes in room temperature National Institutes of

Health phosho-buffered saline (PBS). The composition of the PBS was 5mM Na_2HPO_4, 1 mM KH_2PO_4 and 153 mM NaCl with a pH adjusted to 7.4. Next excess 0.3% Triton X-100 was applied to the slides for 10 minutes. The slides were again washed with PBS 3 times for 5 minutes each time to remove detergent. Next, the slides were blocked in a solution of serum dissolved in 1% bovine serum albumin (BSA). There serum used in the solution was matched to the animal that produced the secondary antibody. After blocking for approximately 30 minutes, a solution of primary antibody in 1% BSA in PBS was prepared and slides were incubated at 4ºC overnight. Post incubation, the slides were washed 3 times for 5 minutes each. Next the secondary antibody was applied, again in BSA and allowed to incubate for one hour at room temperature. The cells were again washed and cover-slips were applied to preserve the slides for microscopic review. The primary antibodies used in these included antibodies against the endothelial protein von Willebrand Factor (AbCam, Cambridge, MA, USA - ab6994, 1:1000), alpha actin (AbCam - ab5694, 1:1000) which was utilized as our smooth muscle cell marker, and RAGE (AbCam - ab7764, 1:250). The secondary antibodies used included a Santa Cruz (Santa Cruz, CA, USA) donkey anti-goat FITC conjugated antibody (sc-2024, 1:1000) and a donkey anti-rabbit Texas Red conjugated secondary antibody from AbCam (ab6883, 1:1000).

Statistical Analyses

All data presented are mean±SEM, unless otherwise noted. The number of animals for each group are as follows WT=12; ApoE-/-=8; ApoE-/- + Adipo=4; ApoE-/- + sRAGE=4. For functional assessment, the n number for each group

represents the number of aortic ring segments used to assess vasorelaxation. The WT group provided 21 rings, the ApoE-/- group 16 rings, the ApoE-/- + Adipo group yielded 6 rings and the ApoE-/- + sRAGE group was composed of 8 rings. To determine statistical significance, one way ANOVA was performed. If the F-test proved significant, Fisher's Least Significant DIfference post-hoc analysis was performed. A P value of < 0.05 was considered statistically significant. EC_{50} calculations were performed using GraphPad Prism 5.0 and statistical analyses of those measurements were performed in the same program utilizing Dunnett's mean separation post-hoc analysis of the ANOVA. Both EC_{50} calculations and maximal relaxation values were obtained from experimental data. All other statistics were performed using SPSS 12.0.

Chemicals and Supplies

Unless otherwise noted, all chemical compounds were obtained from Sigma Aldrich (St. Louis, Missouri, USA).

Chapter 3: Results

Basic Animals Parameters and Physical Characteristics (See Table 1)

Physical characteristics were measured at the time of sacrifice (18-22 weeks of age and a minimum of 12 weeks after beginning the high-fat "western" diet for ApoE-/- and ApoE-/- + adiponectin or sRAGE mice) for all animals. Body mass, abdominal girth and whole blood glucose were similar between WT and ApoE-/- animals (Table 1). Further, treatment with either adiponectin or sRAGE had no effect on weight, abdominal girth or blood glucose concentrations. Plasma adiponectin of animals was measured utilizing western blot and yielded no difference among the groups (data not shown).

Baseline parameters

	Body Mass (g)	Abdominal Girth (cm)	Glucose (mg/dl)
WT (n=12)	31.8±1.8	8.5±0.4	198±25
ApoE-/- (n=8)	36.3± 5.1	8.0±1.0	189±27
Adipo (n=4)	34.2±4.0	8.4±1.7	197±35
sRAGE (n=4)	33.2±1.1	8.8±1.1	166±47

Data represent mean± SD. P=NS

Table 1. Physical characteristics of the animals are included. No significant difference was noted amongst groups on any parameters presented. WT = wild type, ApoE-/- = Apolipoprotein E knockout, Adipo = ApoE-/- treated with adiponectin, sRAGE = ApoE-/- treated with soluble RAGE.

Effect of Adiponectin and sRAGE Treatment on Endothelial Dependent Vasorelaxation (See Table 2 and Figures 1 – 3)

Vasorelaxation in response to the endothelial dependent vasorelaxing agent acetylcholine (ACh) in atherosclerotic ApoE-/- animals was impaired compared to the values for WT littermates (Figure 1). The maximal relaxation observed at 10^{-5} M ACh in WT animals was 71±2.0% whereas ACh induced relaxation in aortae from ApoE-/- mice was significantly lower (45±9.0%; $P<0.05$). Treatment of ApoE-/- mice with sRAGE significantly increased maximal relaxation in response to ACh (79±3.0%; $P<0.05$)) compared to untreated ApoE-/- (Figure 2).

In contrast, treatment with adiponectin in ApoE-/- mice did not significantly improve relaxation in response to ACh when compared to untreated ApoE-/- mice (see Figure 3).

Calculated EC_{50} (see Table 2) from Ach concentration-response curves from adiponectin treated ApoE-/- mice was -7.9±0.2 compared to that calculated in WT animals of -7.1±0.2 ($P<0.05$). The precontractile force generated by 1 μM phenylephrine was not statistically different among the groups (P=NS).

General Responses of Aortic Ring Segments

	Force after PE (mN)	% Response to ACh -5	ACh EC50 (log M)
WT (n=21 rings)	6.4±2.4	71.4±1.9	-7.1±0.2
ApoE-/- (n=16 rings)	27.7± 2.6	45.3±8.9*	-7.4±0.1
Adipo (n=6 rings)	31.8±3.6	53.2±9.0*	-7.9±0.2*
sRAGE (n=8 rings)	34.1±3.2	79.4±3.3	-7.3±0.2

Data represent mean± SD. # P< 0.05 vs. WT group, * P <0.05 vs. ApoE KO group

Table 2. Responses of Aortic Ring Segments. The values are means ± SD of force developed with the addition of 1μM PE to the organ bath, the percent of relaxation after a dose of 10^{-5} acetylcholine, and the acetylcholine EC_{50} for each group.

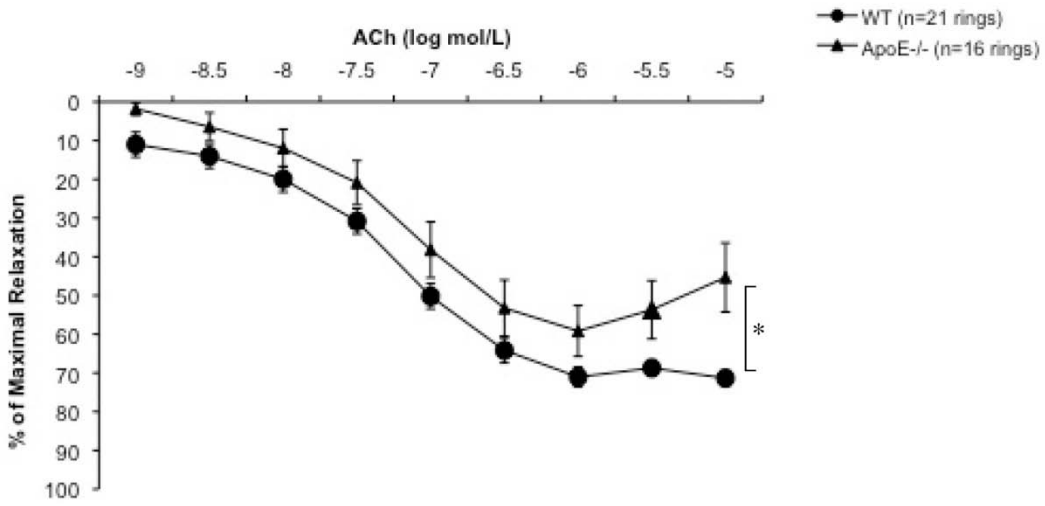

Figure 1. Vasorelaxation Concentration-Response Curves for ACh. Aortae from untreated WT and genetically modified to be Apolipoprotein E deficient mice were exposed to increasing concentrations of ACh. The aortae from ApoE-/- animals demonstrates blunted endothelial dependent vasorelaxation to ACh when compared to WT littermates at all concentrations. *P < 0.05 vs. WT.

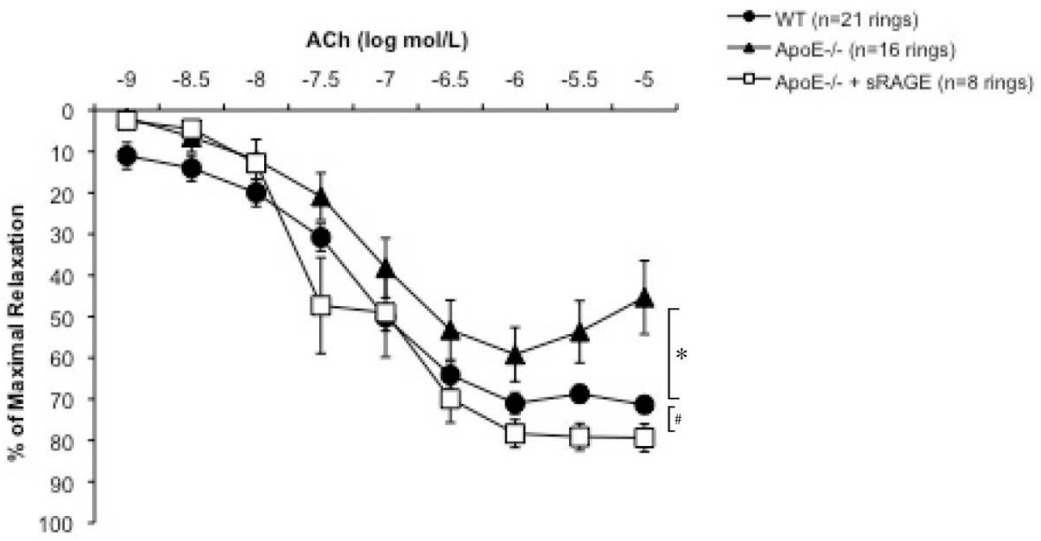

Figure 2. sRAGE Treated Animals Show Improved Vasorelaxation To ACh When Comapred to ApoE-/- Animals. Treatment of ApoE-/- animals with soluble RAGE shows improved vasorelaxation to increasing concentrations of ACh compared to untreated ApoE-/- animals. *P < 0.05 vs WT; $^{\#}$P < 0.05 vs ApoE$^{-/-}$.

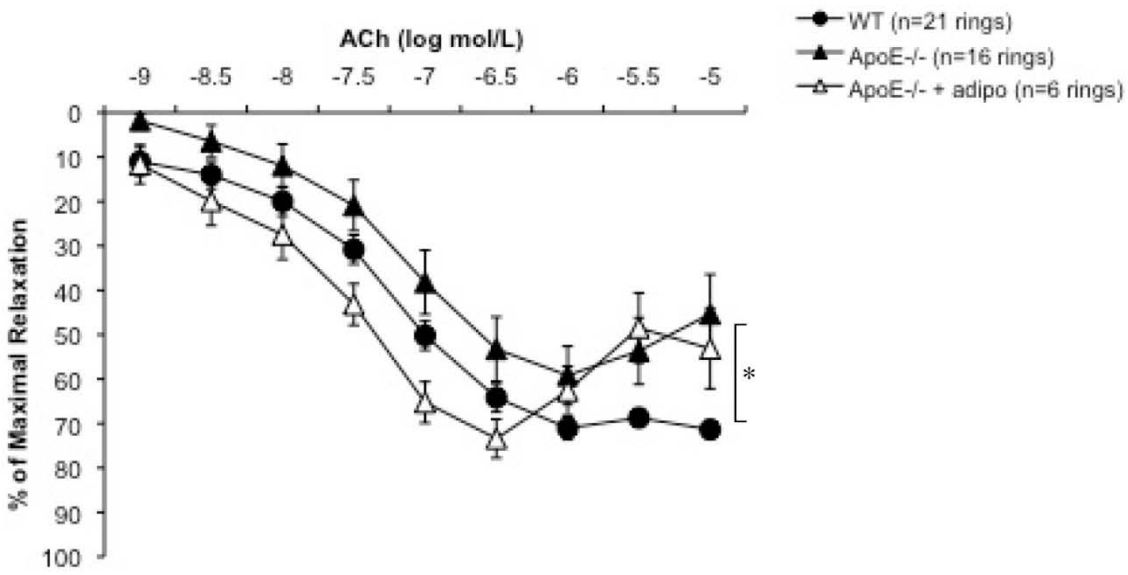

Figure 3. Adiponectin Treatment Does Not Improve Endothelial Dependent Vasorelaxation. Adiponectin treated ApoE$^{-/-}$ mice did not exhibit an improved endothelial dependent vasodilation compared to untreated ApoE$^{-/-}$ mice (P = 0.073). *P < 0.05 vs WT.

Mechanism of Endothelial Dysfunction (see Figures 4 and 5)

To test whether impaired vasorelaxation in ApoE-/- animals was due to abnormalities in endothelium-derived eNOS, two additional experiments were performed. In the first set of experiments, the competitive inhibitor eNOS, L-NAME (100 μM), was added to the organ bath and the vessel was incubated for 30 minutes. An ACh concentration-response curve experiment was performed and showed there was complete inhibition of the Ach-induced vasodilation and no significant difference between the WT and ApoE-/- littermates was observed (See Figure 4).

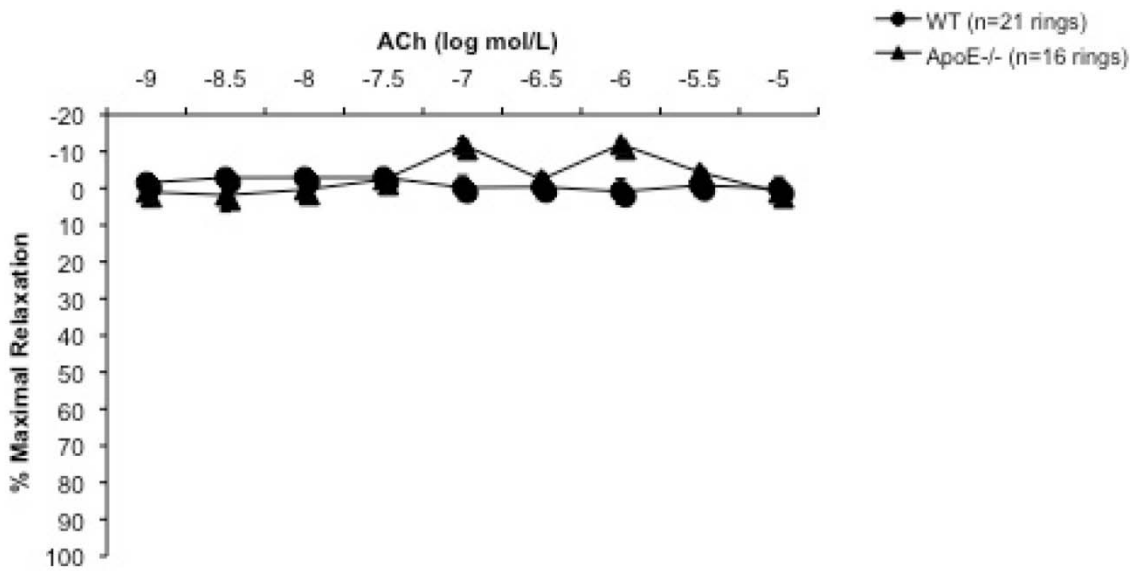

Figure 4. L-NAME Abolishes ACh Dependent Vasorelaxation. Incubation with the competitive eNOS inhibitor L-NAME abolishes endothelial dependent vasorelaxation in both WT and ApoE$^{-/-}$ animals. P=NS

The second experiments were designed to test whether the impaired relaxation responses to ACh were due to vascular smooth muscle cell dysfunction. After washing the aortic rings in PBS, they were constricted with PE and a concentration-response curve using the NO donor, sodium nitroprusside (1nM to 10 μM) was performed in both WT and ApoE-/- groups of mice. Aortic rings from WT mice maximally relaxed (100.5±0.8%) with a 10^{-5} M dose of SNP (Figure 5). Aortic rings from ApoE-/- mice produced a similar response to maximum dose SNP (104.7±2.7%; P=NS). Thus impairment of Ach responses in ApoE-/- test of VSMC function showed no significant differences between the diseased and non-diseased animals in this experiment. This indicates that the

difference in relaxation observed with ACh was due to something other than VSMC dysfunction.

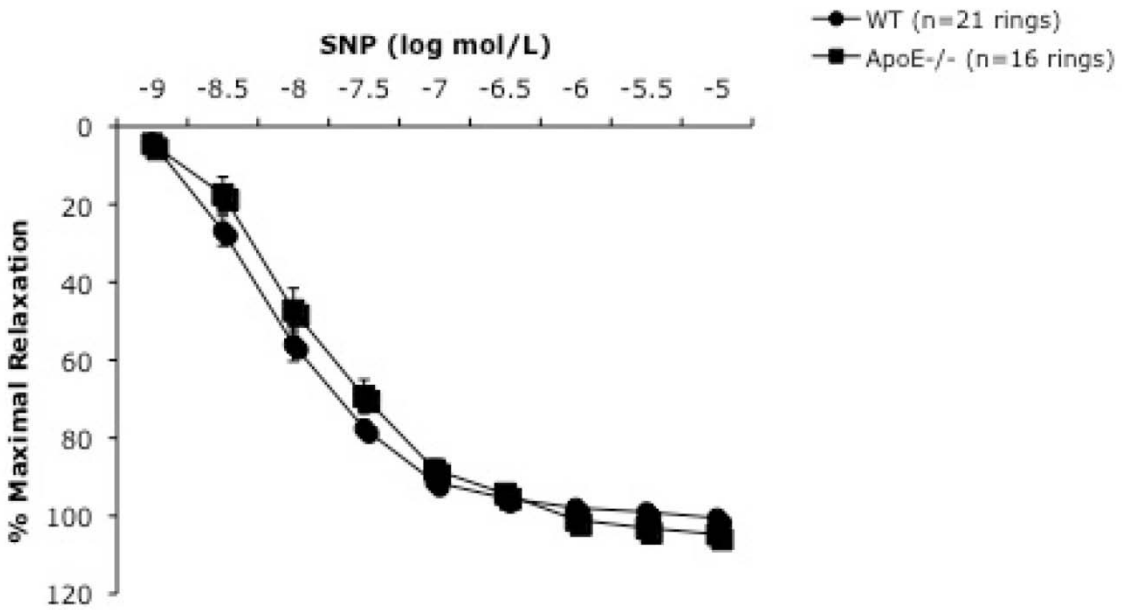

Figure 5. SNP Mediated Relaxation Is Similar Between WT and ApoE-/-. Relaxation to the endothelial independent NO donor, SNP, is similar between both WT and ApoE$^{-/-}$ animals at all concentration. P=NS.

Co-localization of RAGE with Endothelium (see Figure 6)

We used immunofluorescent (IFC) staining to visualize the location of RAGE within the aortae from two groups of mice. The results in Figure 6 show RAGE co-localized with the tunica media of the aorta, but there is a strong co-localization of RAGE with the endothelium. This finding is similar to work that showed adiponectin was strongly co-localized with aortic endothelial cells [45]. For this experiment, no primary antibody was used as a negative control.

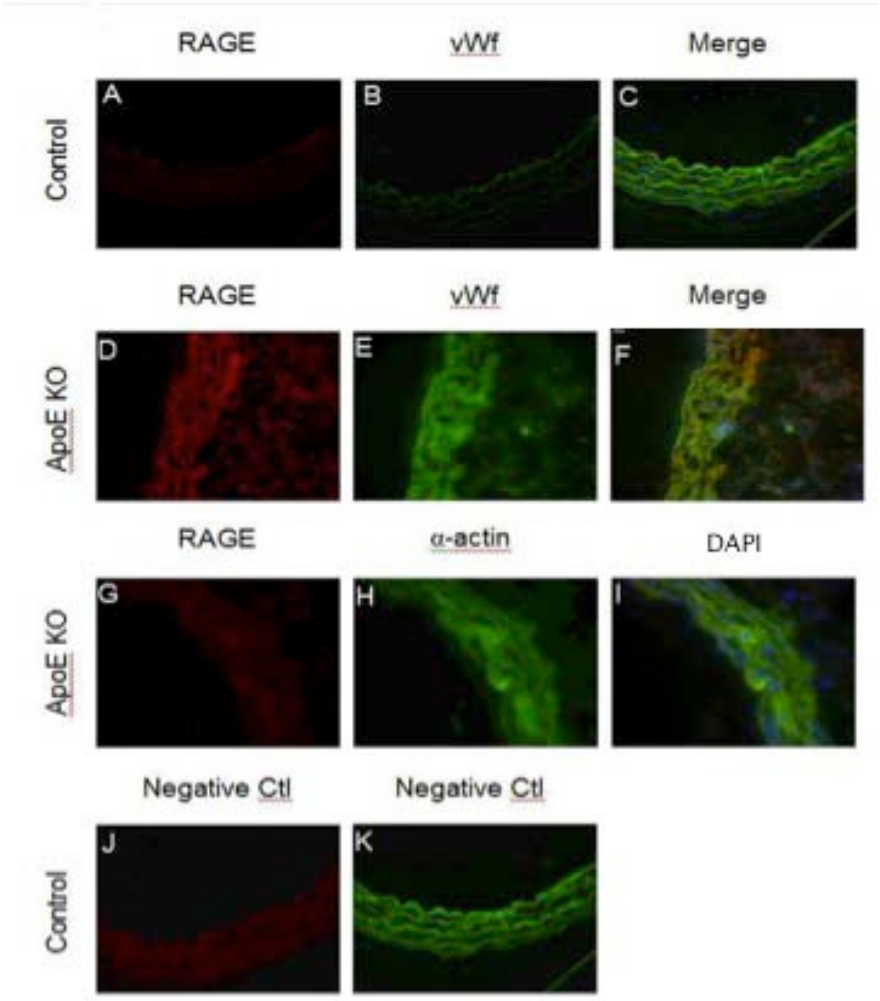

Figure 6. IFC reveals RAGE co-localization with vWF in the endothelium of murine aortae. in both diseased and healthy controls. Panels A, D, and G demonstrated a RAGE signal in the wall of the aorta. Von Willebrand Factor (vWF) is visualized on the endothelium in the same slides and panels C and F show the merged images and the co-localization of RAGE with the endothelium. Panel H shows the location of α-actin on the vascular smooth muscle and I is the merge of G and H. Note the apparent lack of co-localization. J and K are negative controls for this experiment and were performed in the absence of primary antibody. Scale bar = 50μm.

Chapter 4: Discussion

Major Findings

The major findings from this work include: 1. Treatment of ApoE-/- with sRAGE improved vascular responsiveness to the endothelial dependent vasorelaxing agent acetylcholine. 2. Treatment of ApoE-/- with gAD did not demonstrate improvement in vascular function. 3. RAGE co-localizes with the vascular endothelium of the murine aorta in $ApoE^{-/-}$ mice.

Methodological Considerations

The major methods used in this work are functional assessment of aortic rings, immunofluorescent staining of frozen sections of murine aortae, and measurement of plasma adiponectin concentration by western blot analysis. These methods have strengths and weaknesses when evaluating responses in ApoE -/- mice which will be further discussed below.

Functional Assessment of the Murine Aorta

Functional studies in the murine aorta are a major component of the results in this study. While relatively easy to perform, there are features which can alter and significantly affect the validity of the results. Care must be taken to assure that the endothelium remains intact as you isolate, mount and measure force of the aorta using a myograph. A number of earlier studies have demonstrated the importance of careful isolation and mounting of large vessels on myograph set-ups; most notably the pioneering work of Dr. Robert F. Furchgott demonstrating the contribution of an intact and function endothelium. [62] With care and training, stable and repeatable results are expected. However,

the translation of in vitro force measurements into in vivo vascular reactivity is uncertain and limited. Measurement of force generation neglects measurements of more physiologically relevant data such as vessel diameter or flow. Isolation of the aorta, cutting the aorta into rings and mounting them onto a force transducer allows for direct injury to the aortic rings. In particular, the tunica media is subjected to possible damage as connective tissue is stripped away. This may impair the vascular reactivity and necessitate the exclusion of vessels from data sets because of undue damage. This can be evidenced by the excision of a vessel and noting variable function after intentional damage is done to the tunica media (personal observation). Further, cutting the vessel into rings and mounting the vessel on the pins of the myograph may lead to damage of the vessel that necessitates exclusion of vessels from analysis. It is critical that the force transducer and myograph be carefully controlled and calibrated. A major advantage of using aortic rings is that it does provide meaningful data on macrovessels with atherosclerosis. While endothelial dysfunction appears in vessels of all sizes, atheroma formation is reserved for large vessels and tension myography allows for some insight into the functionality of those vessels.

Immunofluorescent Staining.

Immunofluorescent staining is a powerful technique that allows for visualization of the location of proteins in their native tissues. One limitation is that a particular tissue or cell type may not express the target protein in quantities necessary to be visualized. This can be improved by increasing the number of sections stained to increase the likelihood of finding a section expressing the

target protein. The technique is heavily dependent upon antibodies with a high sensitivity and a high specificity. In short, the antibody must be able to detect small amounts of protein throughout a sample but also must bind only to its target protein. This makes selection and testing of the antibodies used in the experiments critical. Optimizing methods that can increase the signal to noise ratio in the experiment is another necessary step in using immunofluorescence. Both of the above concerns can be ameliorated through the use of adequate controls, optimizing antibody concentration and minimizing exposure to light. A potential major limitation is the specificity of the antibody. To establish controls for immunofluorescence staining, positive as well as a negative controls must be considered. Typically, a tissue section shown to express the protein of interest while no expression is seen in a negative control(Reword). An experiment which serves as a negative control is either an experiment performed in the absence of the primary antibody or an experiment utilizing an antibody which is not to designed to react with the protein of interest. The results of the positive control should assure the researcher that the antibody is detecting the appropriate protein, though this is not guaranteed. Further, the negative control will demonstrate the ability of the secondary antibody to selectively recognize its target and hopefully help to minimize noise in the experiment. Additionally, using the minimal amount of primary antibody helps to maintain the antibody sensitivity without washing out the image with noise arising from non-specific staining. Lastly, the secondary antibodies contain the fluorescent molecules and must be shielded from light to ensure the molecule is able to respond to excitation at the

appropriate time and thus produce the desired signal. Exposure to light weakens the signal of that provided by the primary seeking secondary antibody and thus makes detection of the target molecule unnecessarily difficult. If the above conditions are met, immunofluorescent staining can be a powerful tool to identify the relative physical location of a target molecule within a section of tissue.

Western Blot Analysis of Plasma Proteins.

Western blot analysis can also be a very useful tool in measuring the relative amount of a protein of interest in a given tissue. This method has both strengths and weaknesses. While it does allow for some quantification of molecular data, it is better served for comparing the relative concentrations of proteins. As with immunofluorescent staining, antibody sensitivity and specificity necessitates important controls and calibration of the antibodies being used. Unlike immunofluorescence, the chance of detecting the target protein is higher in a given tissue sample since the sample may be larger than a single slide. In our case in the aorta, the entire aorta is homogenized and used for experiments where with immunofluorescence only 5 μm thick cross-sections are used in staining. Further, as cells are lysed intracellular protein is released and made available for antibody mediated reactions whereas immunofluorescence may not make every pool of protein available to the antibody. Unfortunately, this decreases the utility of western blotting as it destroys some of the compartmentalization available in staining making determinations of expression with respect to physical location difficult. However, it is easier to establish a positive control because one well of a gel may be loaded with purified or

recombinant target protein to ensure the primary antibody is performing adequately. Additionally, proper inhibition of non-specific protein binding interactions and use of the optimal concentrations of antibody are necessary in western blotting, as they are in staining, to ensure the noise to signal ratio is minimized.

These techniques, while they do have their advantages and disadvantages are staples of modern laboratory work and when taken in context can provide powerful information to investigators.

Comparison of Results to Others

As previously published by our lab, we found significant impairment of endothelial dependent vasorelaxation in the ApoE -/- (AS) aorta when compared to wild type control mice [45]. Further, these findings agree with previous reports associating endothelial dysfunction with atherosclerosis [57]. We also found very little vasorelaxation to ACh after incubation with L-NAME in either WT or ApoE-/- mice. Like our previous work, this indicates that the impairment appears to be due to impaired function of the endothelium in the vessels of diseased mice. Further, the function of VSMC in our experiments, as measured by the vasorelaxation induced by SNP, aligns with our previously reported data. There is one contradiction regarding the ApoE-/- and WT groups between this work and our previously reported data; our WT animals did not exhibit the same degree of vasorelaxation as previously reported. Compared to prior work, our WT animals demonstrated impaired endothelial dependent vasorelaxation when compared with previously studied WT animals. [45] However, data from ApoE-/- animals

closely resembles our previously reported work. Interestingly, our ApoE-/- animals treated with AD did not exhibit a significant improvement in endothelial performance compared with their untreated, diseased littermates. This is in contrast to our most recent work on AS, in which ApoE-/- + AD animals demonstrated a partial rescue of their vascular function in the aortic rings tested [45]. Experimental methodolgies remained consistent between the two studies, suggesting another cause for the noted difference in performance of the vessels. One possible explanation related to our differences with adiponectin treatment and responses was that adiponectin concentration in the plasma was not different amongst groups, as has been previously observed. [45] Previous work indicated that ApoE-/- mice demonstrated a decreased level of plasma adiponectin. Perhaps the functional data reflect the absence of decreased APN expression in ApoE-/- animals. While treatment with gAD has been previously shown to rescue impaired vessel function, the result of this study yielded no difference in plasma adiponectin expression among treated or untreated groups. The data from sRAGE treated animals compared favorably with previously reported functional data from murine aortae [54]. These mice demonstrated a relaxation that appears slightly impaired from the data previously reported in ApoE-/- mice. However those animals were genetically modified $ApoE^{-/-}$ that expressed either no RAGE molecule or a modified RAGE that did not contain the portion responsible for intracellular signaling, whereas our animals had only been treated with RAGE blockade for 3 days. Furthermore, the diseased animals, treated and untreated, were provided *ad libidum* access to the high fat Western

diet versus the normal rodent chow used for all animals in Harja, et al. The purpose of the Western diet is to increase the development of atherosclerotic lesions and this may have provided our animals with more advanced disease impairing their endothelial function moreso than animals in other studies. [54] Lastly, the co-localization of RAGE with the aortic endothelium is reflective of work previously reported [48,49]. RAGE is expressed in a variety of cells types and this is evident in our work where cells of the tunica media exhibit some expression but the strongest signal produced originates from the aortic endothelium.

Limitations of the study

There are several limitations to the study described above. 1. Concerns regarding the statistical power of the experiment; 2. Lack of data on plasma lipids; and 3. Need for mechanistic information.

Lack of Statistical Power.

The most significant limitation to this study is the lack of power for this study. The data discrepancies with prior works may revolve around the small number of treated animals available in this study. Due to the cost concerns regarding both APN and sRAGE, only four animals for each respective group were treated. Thus, variation from methodological inconsistencies and natural biological variation could not be overcome. It is this lack of power that we believe explains, in part, the difference in our functional data regarding ApoE-/- mice treated with AD in this work and in our last paper. A statistical analysis was performed to determine the necessary sample size to detect a difference

between only the untreated ApoE$^{-/-}$ mice and the adiponectin treated group. Using the mean relaxation produced at an ACh concentration of 10^{-5} for each group, a pooled standard deviation for the two groups, and assuming a β of 0.2 and an α of 0.05, the necessary sample size is 42 rings per group. Because 2 rings were collected from most animals, this translates to a requirement for 21 animals per group. The necessary n is in stark contrast to our previous work, where a statistically significant difference was noted with no more than 7 animals per group [45]. This analysis suggests that methodological considerations were a substantial contributor to the variation seen among animals within each group. From each animal treated with either AD or sRAGE 2 rings were assessed for vascular function, giving a total of 8 rings per group. In the AD treated group, two of the rings were excluded due to a lack of responsiveness to ACh during assessment of endothelial function which suggested the rings were harmed during isolation, removal of connective tissue, or mounting. This reduced power is associated with an increase in the standard error of the data points. In addition, in the experiments to measure serum adiponectin, blood was obtained from only 2 ApoE-/- animals treated with APN. This may have contributed to the lack of difference in APN expression across the groups. Further, blood was only able to be collected from four of the untreated ApoE-/- animals.

Lack of Lipid Measurements.

The lack of data on plasma lipids is another limitation of our study. While lipids were not measured in these experiments, previous work in our laboratory has measured lipids in the ApoE -/- mouse and those have compared favorably

to prior studies in other laboratories [45]. Assessment of plasma lipid levels is important because intravascular inflammation in these animals can be driven, in part, by the formation of LDL sequestered in the tunica media from anti-oxidants leading to the formation of ox-LDL.

Need for Mechanistic Studies.

While studies were designed to provide mechanistic insight into the abnormalities that exist between the ApoE -/- and WT mice, the small sample sizes prevents complete investigation of these hypotheses (see Future Directions below). This limits the interpretation to a more superficial understanding and not the deep mechanistic understanding that was desired. The small group sizes also presented challenges and difficulty in obtaining reliable blood and tissue samples in some of the studies.

Future Directions (see Figure 7)

To further expand on this work, I propose a mechanistic study. Adiponectin increases the phosphorylation of AMPK and AMPK is a known kinase for eNOS, which increases its activity [40,41]. Thus studies on AMPK and the role of AD would be of value. In addition, the effect of sRAGE treatment on AMPK phosphorylation and eNOS phosphorylation/activity remain unclear in the atherosclerotic aorta and should be investigated. Western blotting uses homogenates of the murine aortae to measure the ratio of phopho?? -AMPK to total AMPK and phoshpo?? -eNOS to total eNOS could provide some useful data to explain the improvement in vascular function of aortae from mice treated with sRAGE. In the current study, these experiments could not be done due to lack of

available tissue. Beyond this, adiponectin has a demonstrated ability to activate its own transcription factor, PPAR-γ both directly and via an AMPK dependent process. If RAGE blockade does increase AMPK phophorylation, measurement of adiponectin protein and mRNA expression may prove fruitful. sRAGE treatment increasing the expression of adiponectin may offer more insight into its ability to improve vascular function. Further, an increase in NO whether actual or indirect may provide a valid explanation for the improvement in vascular function in atherosclerotic mice treated with sRAGE. To determine whether the improvement in endothelial dependent vasorelaxation is due to an increase in eNOS activity or a reduction in ROS generation, several experiments should be performed. First, dihydroethidium staining, a compound used to detect the presence of superoxide radicals, such as those produced by NAD(P)H oxidase, is excited by the reaction of $O_2^{\cdot}$ with dihyroethidium to produce ethidium bromide (57,58). Ethidium bromide will fluoresce red, indicating the presence of ROS. Blunting RAGE signaling has been shown to decrease NAD(P)H oxidase expression. However the role of adiponectin in the expression of NAD(P)H oxidase is unclear [51]. Western blotting and real-time quantitative polymerase-chain reaction to determine the protein and mRNA expression respectively, may assist and help define adiponectin's role in reducing vascular dysfunction, via a RAGE mediated mechanism. This is of interest as adiponectin has been shown to interfere with NF-κB signaling, and NF-κB is a known transcription factor for RAGE. If adiponectin treatment reduces the production of ROS by desensitizing tissues to RAGE ligands, we expect a reduction in both the mRNA and protein

level expression of RAGE. Lastly, a measurement of lipids must be made to ensure that our effects are not due to an alteration in plasma lipid content and hence an decrease in ox-LDL and decrease in RAGE signaling by an alternative pathway.

The role of RAGE in atherosclerosis needs further clarification. The atherosclerotic disease is driven, at least in part, by inflammation and the effector cells of inflammation and understanding the mechanisms by which they are modulated (see Figure 7) is crucial to the development of better treatments for the disease. The ability to halt the progression or prevent formation of atheroma may lie in the inflammatory pathways above or those not yet identified. A further understanding of the role of adiponectin and RAGE in atherosclerosis may be critical for understanding mechanisms of disease and designing treatments for those who suffer from atherosclerosis.

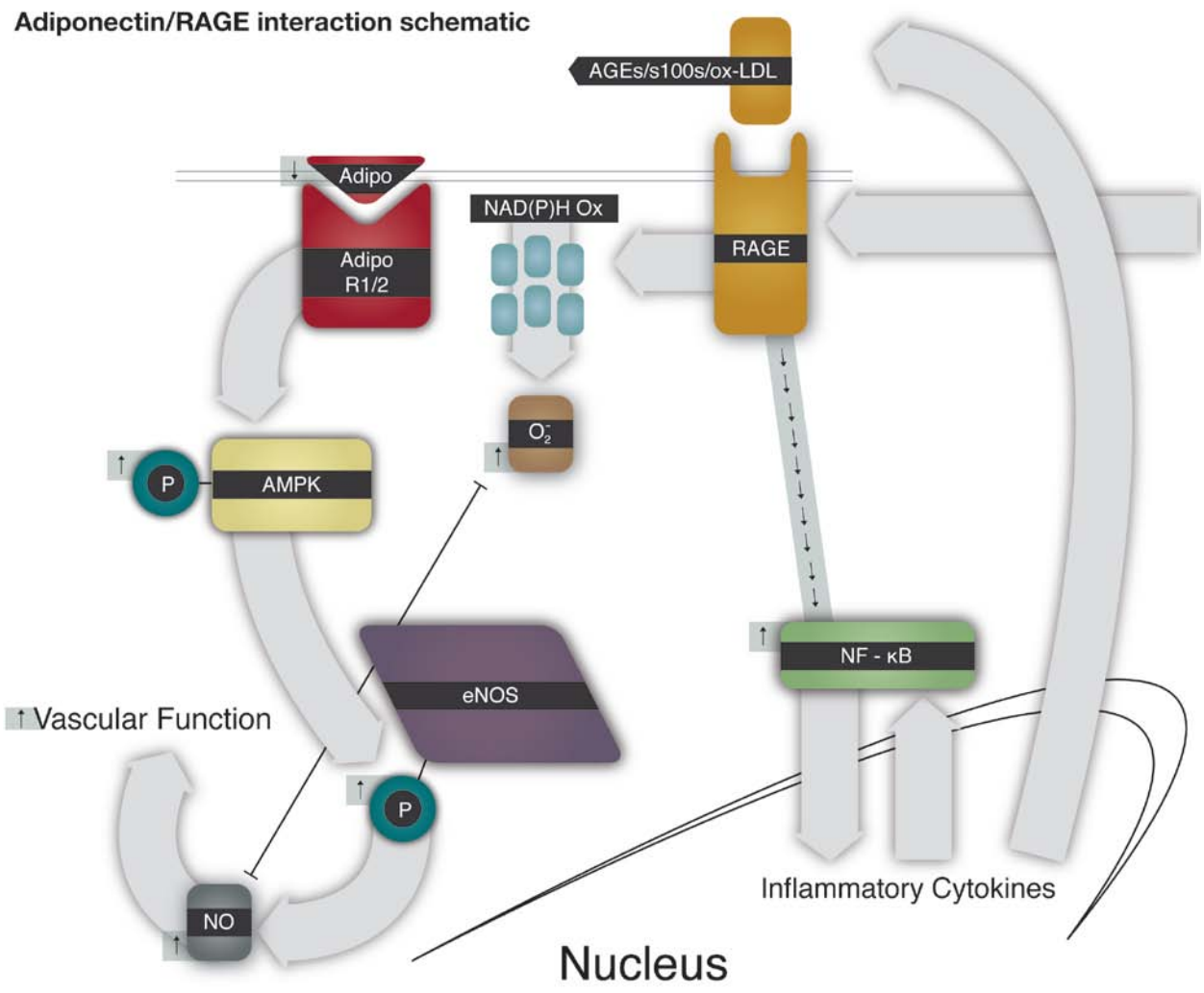

Figure 7. Putative diagram of the potential interaction between RAGE and adiponectin signaling within the endothelium.

Conclusions

In conclusion, sRAGE does improve vascular function in the form of endothelial dependent vasorelaxation in the aortae of ApoE-/- mice. Further, RAGE is preferentially expressed on the endothelium of the murine aorta. Adiponectin did not improve the endothelial dependent vasodilation in ApoE-/- mice; however conclusions regarding these findings are limited by the statistical power of the data analyses. These results suggest that preferential expression of adiponectin on the endothelium and that of RAGE provide a possibility for interaction. To clearly define this hypothesis will require further studies.

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VITA

Steven Ray McAfee is the first of two sons born to Dennis Ray and Deborah Ann McAfee. He was raised in Hannibal, MO, along with his brother Dennis Eugene “Denny” McAfee. In August of 2003, he enrolled at the University of Missouri. In 2008, he graduated with a Bachelor of Science in Biology while maintaining employment throughout his undergraduate career. He enrolled in the Medical Pharmacology and Physiology department’s Master of Science program immediately afterward. In 2010, he matriculated to the University of Missouri School of Medicine with hopes of practicing medicine in Missouri after the completion of a residency in a field of his choosing. Steve is an avid weightlifter and enjoys spending time with his wife Danielle, a 2010 graduate of the University of Missouri School of Law, and their dogs.