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John Brian Clark Date

Role of Hydrogen Peroxide in the Development of Abdominal Aortic Aneurysms

By

John Brian Clark
Master of Science

Graduate Division of Biological and Biomedical Science
Molecular and Systems Pharmacology

_____

W. Robert Taylor, M.D., Ph.D.
Advisor

_____

Hanjoong Jo, Ph.D.
Committee Member

_____

Roy L. Sutliff, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Role of Hydrogen Peroxide in the Development of Abdominal Aortic Aneurysms

By

John Brian Clark

B.S., University of Alabama, 2008

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An abstract of

A thesis submitted to the Faculty of the

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Abstract

Role of Hydrogen Peroxide in the Development of Abdominal Aortic Aneurysms

By John Brian Clark

Abdominal Aortic Aneurysms (AAA) are a significant health problem in the United States and abroad. In men over the age of 55 it is estimated that the prevalence is between 5-9%. AAA rupture leads to thousands of deaths each year and is a leading cause of death in older men. Despite the significant clinical endpoints, very little is known about the initial events leading to the development of abdominal aortic aneurysms. The hallmarks of AAA are aortic diameters >50% normal, chronic inflammation of the abdominal aorta, matrix metalloproteinase (MMP) expression and elastin fragmentation. Many studies suggest a critical role for reactive oxygen species and oxidative stress in AAA development. The central hypothesis of this thesis is that a particular reactive oxygen species, hydrogen peroxide, plays a critical role in the development of AAA. To test this hypothesis we utilized several different murine models of AAA, ultrasound imaging to monitor aortic dilation, and Amplex Red to measure relative levels of hydrogen peroxide. Herein, we demonstrate that increases in hydrogen peroxide levels may be model dependent and commercially available antioxidant therapies fail to significantly lower hydrogen peroxide levels and have no effect on AAA development.

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Role of Hydrogen Peroxide in the Development of Abdominal Aortic Aneurysms

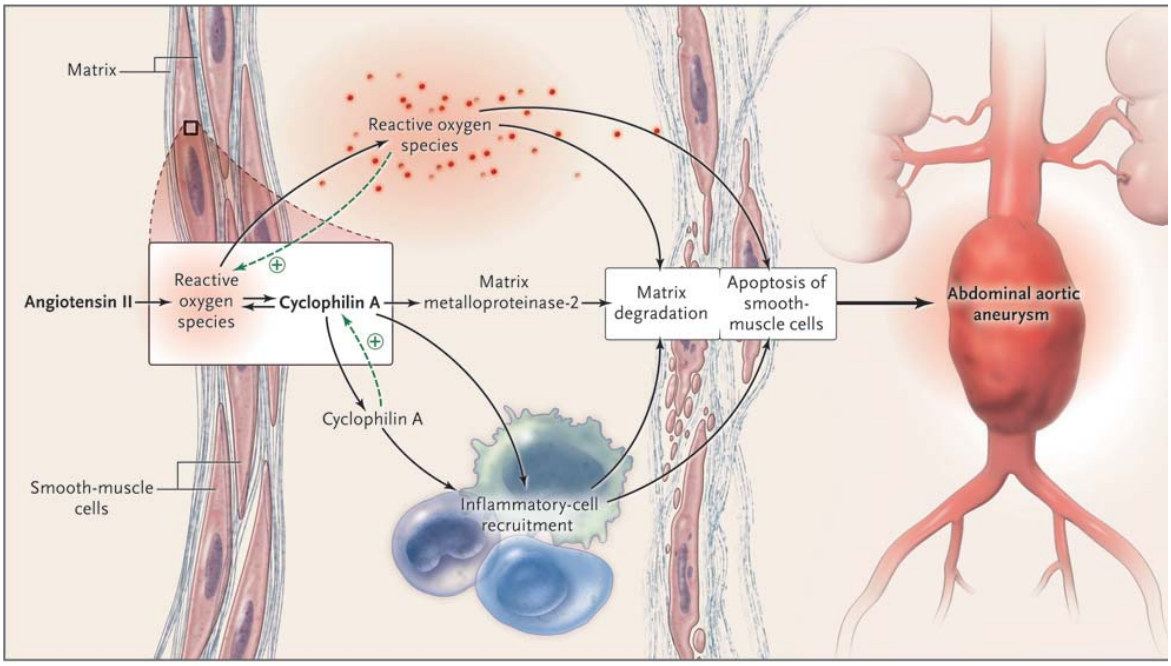
Chapter 1: General Introduction

a. Clinical Significance of Abdominal Aortic Aneurysms

Abdominal Aortic Aneurysm (AAA) rupture accounts for between 1.5-2% of deaths in men over the age of 55 [1, 2]. The death rate associated with AAA rupture has ranked it as high as 10th on the leading causes of death in men over 55 and 15th leading cause of death overall in the United States [3]. The prevalence of AAA is generally estimated at 5-9% of the male population over 65 years old but some reports estimate the prevalence to be as high as 12.6% [4, 5]. Much of the uncertainty regarding prevalence estimates is due to the fact that typically, AAAs are asymptomatic until rupture. These data demonstrate the significant clinical consequences of AAA and the need for additional research investigating the etiology, screening protocols and advanced treatment options for AAA.

AAA are characterized by a progressive and permanent dilation of the abdominal aorta. This dilation occurs in conjunction with thinning and weakening of the aortic wall. This is particularly dangerous because of the high pressures and stresses the aorta must endure. Recent work suggests aortic rupture depends on biological phenomena just as heavily as the biomechanical changes the aorta experiences as the aorta dilates and weakens [1]. These biological factors include expression of proteolytic enzymes and chronic inflammation.

A.)



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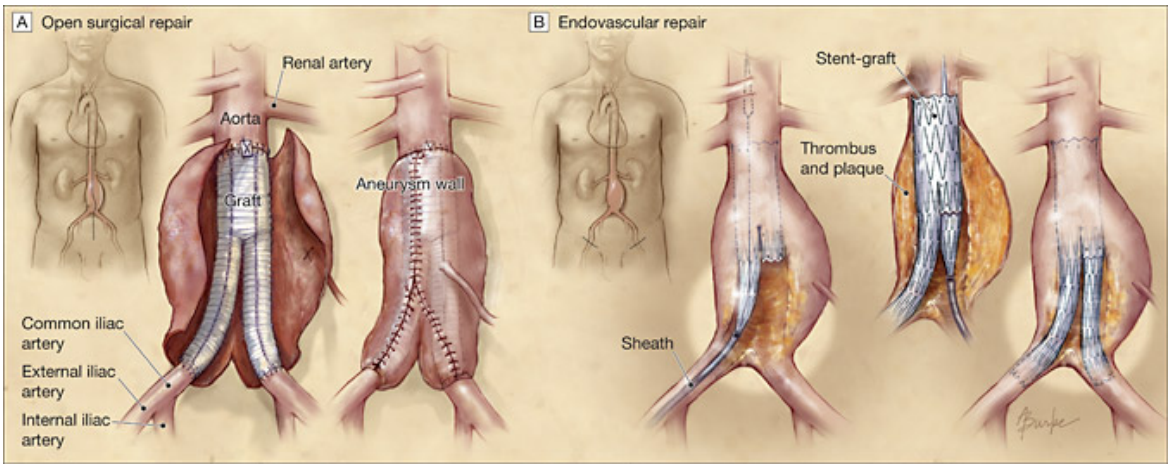


Figure 1.1 – Pathogenesis and Repair of AAA – Proposed mediators of human AAA based on angiotensin II model of AAA (A) and the surgical techniques to treat them (B) [6, 7].

Aneurysms are most often found in the arterial vasculature and rarely occur in the venous system [8]. Aneurysms can be localized to multiple regions of the body. These regions include the brain (cerebral aneurysms), carotid artery, popliteal artery, iliac artery and thoracic regions of the aorta. Abdominal aortic aneurysms are the most common location of aneurysms. In humans, 2/3 of aortic aneurysms occur in the abdominal region of the aorta [4]. Aortic aneurysms can also form in the thoracic region of the aorta. The pathophysiology of aneurysms can vary depending on their location and are typically classified by their location and shape. The two most common ways to describe aneurysm shape is fusiform or saccular. Saccular aneurysms are a focal enlargement of the aorta, which are described as looking similar to a bubble, or berry, bulging from the artery. Saccular AAAs can occur but are rare [9]. Fusiform aneurysms are the predominating form in the abdominal aorta. The fusiform classification is based on the circumferential dilation of the artery leading to expansion greater than 50% the normal diameter.

A major roadblock for studies into the mechanism of AAA development is that patients who present in the clinic with AAA are often in the later stages of disease progression. This inhibits the ability of scientific investigators to elucidate any early or causative mechanisms in the initial stages of disease progression. Many human studies are conducted on tissues obtained from surgical repair. These tissues provide valuable insights into the biological characteristics of AAA but little in terms of the initiating factors.

Although AAA is a multifactorial pathology, many risk factors have been associated with their development. Atherosclerosis is considered a major risk factor for AAA development. There is considerable debate to whether atherosclerosis is causal or merely positively associated with AAA development. A recent study suggests that atherosclerosis does not play a causal role in AAA [10]. An opposing theory suggests that the aortic remodeling caused by atherosclerosis

leads to AAA [11]. Other lifestyle risk factors positively associated with AAA include hypertension, age, body mass index (BMI), sex, smoking, and alcohol consumption [4, 12]. Hypertension has been consistently shown to be a major risk factor for AAA. One study showed that elevated diastolic blood pressure led to nearly a 40% increased risk of having a AAA [12]. Age (>55) and an elevated BMI were both positively associated with AAA [4]. In another study it was shown that men between the age of 65-79 are more than six times more likely to have a AAA than a woman of the same age [12]. Smoking has been associated with many diseases not just cardiovascular disease. As for AAA, ¾ of patients diagnosed with an AAA greater than or equal to 4cm had a history of smoking [13]. The risk seems to rise based on smoking intensity and less on longevity. Men who smoke greater than 25 cigarettes per day increased their risk of AAA by 15 times [4]. Some of these risk factors are preventable lifestyle choices which suggest a potential for daily lifestyle changes that can lower the likelihood of developing AAA. Interestingly, diabetes was shown to be inversely associated with AAA [13].

Often patients presenting to the hospital with a ruptured AAA were previously undiagnosed because most individuals with AAA are asymptomatic. This has led to an increased effort in screening programs for high risk populations. Screening programs have been shown to reduce the incidence of ruptured AAAs by 49% and are cost effective [6, 14]. The three noninvasive techniques most often used in these screening programs are ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI). These imaging modalities are necessary because physical examinations are not effective at identifying AAA [6]. An additional strategy to identify early AAAs and candidates for surgical repair is to find a circulating biomarker. Urbonavicius et al took on this challenge with a large scale meta-analysis [2]. Multiple biomarkers such as serum elastin peptides, plasmin-antiplasmin complexes, matrix metalloproteinase-9 and interferon

gamma have been found to potentially provide valuable biomarkers for predicting AAA expansion and rupture but must be further evaluated to confirm their findings [2, 15].

There is currently a lack of effective therapeutics to treat AAA. Small clinical trials and animal studies suggest antibiotics such as Roxithromycin (macrolide) or Doxycycline (tetracycline) inhibit growth of AAA [5]. In addition, HMG CoA Reductase Inhibitors, have been shown to attenuate AAA growth in two different observational studies [5]. These pharmaceuticals are believed to act through inhibition of matrix metalloproteinases (MMP). To prevent the necessity for high dose, systemic antibiotic treatments, preliminary site directed drug delivery methods are currently being investigated to treat AAA [16].

In lieu of efficient pharmacologic treatment for AAA, the currently accepted treatment options are open surgical repair or endovascular stenting. Nearly 30,000 AAA repairs are made annually in the United States [17]. Open repair requires surgical opening of the abdominal cavity, a longitudinal incision into the aneurysm, implantation of a graft inside the aneurysm and final closure of the aorta around the graft [17]. Endovascular AAA Repair (EVAR) is accomplished by inserting a catheter into the femoral artery, extending it up to the abdominal aorta, deploying a stent graft at the site of the aneurysm and final closure of the arteriotomy. EVAR has been shown to lower operative mortality, lower morbidity, shorten hospital stay, and provide a greater likelihood for the patient to be released to their home [6]. Although recent clinical trials show that there is no difference in total mortality between EVAR and open repair [18]. Many risk factors are associated with both surgical repair methods. These risk factors include endovascular leaks and complications from the invasiveness of open repair. This demonstrates the necessity of advanced understanding of the etiology of AAA to develop pharmaceutical targets that can treat AAA prior to requiring surgical repair or fatal rupture.

b. Pathophysiology of Abdominal Aortic Aneurysms

Through decades of human and animal studies many features of AAA have been elucidated. The definitive characteristic of AAA is the permanent dilation of the abdominal aorta to a diameter greater than 50% of the normal diameter. The biological explanation for this dilation is complex and multifactorial. From a gross anatomical perspective the outer two layers, the media and adventia, are most affected by AAA progression. These two regions undergo extensive remodeling and weakening stemming from extensive proteolysis, apoptosis, inflammation and oxidative stress [7]. Multiple different cells types contribute to the pathogenesis of AAA. Rizas et al. condensed the pathogenesis of AAA into four distinct contributing mechanisms: extracellular matrix degradation, apoptosis, inflammation and neovascularization [19]. This remodeling can lead to vast changes in the hemodynamic environment of the aorta. Multiple studies have investigated the localization and attempted to characterize the biomechanical modulation the aorta undergoes during aortic aneurysm development.

A common feature of aneurysmal tissue is extensive inflammatory cell infiltration. This inflammation can lead to protease expression and activation, reactive oxygen species generation and cytokine release. Macrophages, mast cells, T-cells, NKT cells and neutrophils have all been shown to contribute to AAA development [19]. These cells act in concert with resident cells such as vascular smooth muscle to activate a degenerative process within the aortic wall.

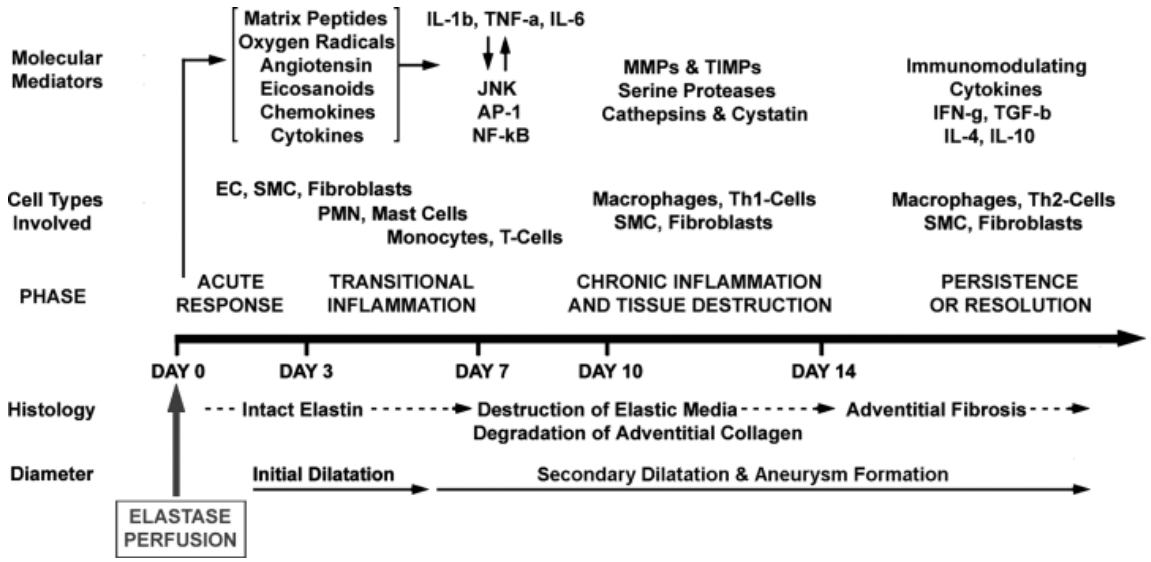


Figure 1.2: Pathogenesis of Elastase Induced AAA – This image provides a comprehensive overview of the known mediators of AAA in the elastase model [20]. Copyright (2006), with permission from Jon Wiley and Sons.

Apoptosis of vascular smooth muscle cells (vSMC) has been observed in human AAA tissue [21]. Although apoptosis occurs regularly in the body in the context of AAA, vSMC apoptosis can be accelerated by inflammatory cell secretion of apoptotic activating proteins such as fas and perforin [21].

The leading cause of structural instability within the aorta is due to proteolysis and degradation of the extracellular matrix (ECM). The two major components of the ECM are the elastin and collagen networks. Typically there is a dynamic process where there is a balance of healthy tissue remodeling where structural proteins are being produced and degraded at a controlled rate. During AAA development the balance is shifted to a degenerative phenotype where proteolysis predominates over protein production. It is hypothesized that elastin degradation leads to aortic dilation while collagen proteolysis is necessary for rupture [15]. Elastin fragmentation can occur from chronic hypertension as well as elastolytic enzymes digesting the elastin networks. As previously discussed, elastin peptides are a potential circulating biomarker for AAA screening [2, 15]. Collagen, mainly consisting of Type-I but also Type-3 collagen fibers in human aortas, can be readily digested by MMPs and cysteine proteases [15]. This highly efficient proteolytic process leads to tremendous weakening of the aortic wall and thus contributes to aneurysmal dilation and eventual rupture.

The enzymes responsible for the proteolytic cleavage of the structural proteins, elastin and collagen, are varied. The most highly studied of these enzymes are in the matrix metalloproteinase (MMP) family of enzymes. These proteolytic enzymes are often kept in balance with tissue inhibitors of matrix metalloproteinases (TIMPs) and are produced as propeptides which need to be activated before they can exert their proteolytic functions. Two heavily characterized enzymes associated with AAA development are MMP-2 and MMP-9.

MMP2 (also known as gelatinase A or 72-kDa gelatinase) can degrade both elastin and fibrillar collagen [22]. Vascular smooth muscle cells predominately produce this enzyme although macrophages and fibroblasts can also produce small amounts [22]. MMP-9 (also known as gelatinase B or 92-kDa gelatinase) is one of the major elastases in human AAA and is predominantly expressed in adventitial macrophages [22]. MMP-9 in the plasma has been shown to be elevated in patients with AAA [15]. Other MMPs such as MMP-12 (an elastase) and MMP-13 (a collagenase) have been found in human AAA tissue and are being investigated for their contribution to AAA development [22].

Matrix Metalloproteinases are not the only protelytic enzymes found in aneurysmal tissue. Cysteine proteases, such as the cathepsin family, and serine proteases are critical in AAA development. Cathepsin K is the most potent elastase and has additional collagenase activity [15]. Serine proteases, from the fibrinolytic system, are believed to play a large role in vessel wall destabilization in AAA [15].

From this general overview of the pathophysiology of AAA, one can see that it is a multifactorial pathology with many mediators of aortic wall degradation. MMP expression and aortic dilation are key components to this process but an initial event that triggers the inflammation and begins the process has eluded scientists thus far.

c. <u>Animal Models of Abdominal Aortic Aneurysms</u>

Since most human AAA samples are from advanced stages of disease, many animal models have been proposed to study the critical initiating steps in AAA development. One method to elucidate key mediators of AAA initiation is to use genetically manipulated mice. Most of these animals were targeted to disrupt the extracellular matrix, MMP expression, induce hyperlipidemia, or manipulate the renin-angiotensin system [23]. Another model of AAA in mice is angiotensin II infusion through an osmotic minipump. Various permutations of this

model have been used. A common approach is to give angiotensin II at a dose of 1000ng/kg/day to Apolipoprotein deficient (ApoE-/-) mice on a standard diet for 4 weeks. Another variation used in our lab is to administer angiotensin II at a dose of 500ng/kg/day in ApoE-/- mice while on an atherogenic diet. This reproducibly generates suprarenal aneurysms within 4 weeks. Much debate is circulating about the reliability of this model. The localization of the AAA to the suprarenal aorta does not correspond to the infrarenal aneurysms found in humans. It is hypothesized that this difference may be due to the hemodynamic differences between mice and humans. Additionally, this model does not produce a true aortic dilation characteristic of human AAA. The angiotensin II model produces a large aortic dissection that precedes aneurysm development [24]. This model is used because it is easy and reproducible although it is not the most accurate model of human AAA.

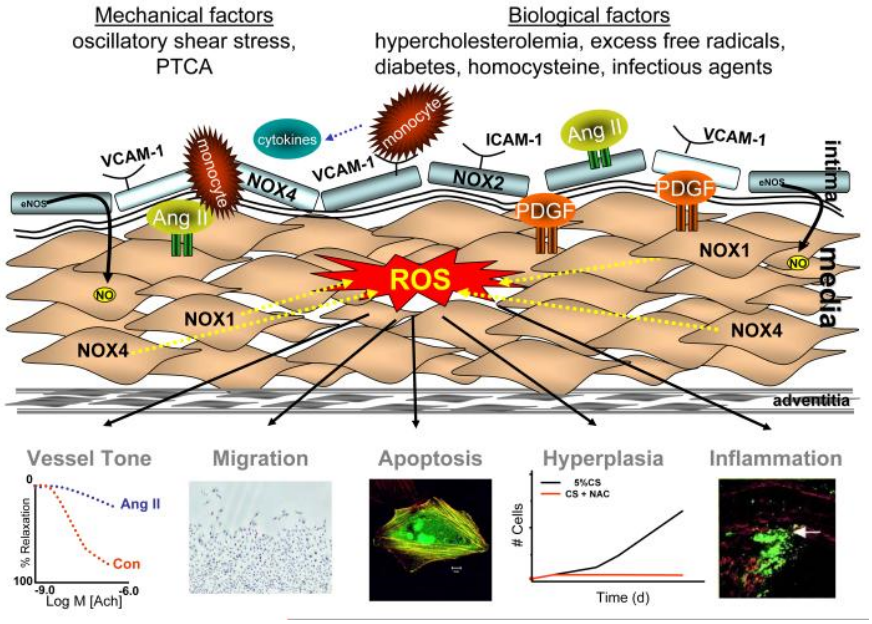
The two aneurysm models used in this thesis are the two chemically induced models of AAA in mice – periaortic calcium chloride application and intra-aortic elastase infusion. These models were used because of their similarity to the human AAA pathology. These similarities include localization of the aneurysm to the infrarenal aorta, a true aortic dilation without dissection, chronic inflammation and common protein expression profiles. The calcium chloride model consists of exposing the aorta from surrounding connective tissue and applying 0.25M calcium chloride to the infrarenal aorta for a period of 15 minutes. This model features a progressive dilation and chronic inflammation producing moderately sized aneurysms in the infrarenal aorta. The elastase infusion model is more complex but faster. In this model porcine pancreatic elastase is infused directly into the infrarenal aorta. During infusion the aorta will inflate and stretch the elastin networks so that the proteases have an opportunity to access the elastin proteins. Within 2 weeks an aneurysm will form. This model is often used in rats instead of mice because of the small size of the mouse aorta and high mortality rates.

d. Oxidative Stress in Cardiovascular Disease and AAA

It is well understood that reactive oxygen species (ROS) and oxidative stress play a critical role in cardiovascular disease. ROS is a general classification for highly reactive, radical and non-radical oxygen containing molecules. The most important in cardiovascular disease are superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite [25]. ROS and oxidative stress have been shown to have causal roles in the development of atherosclerosis and hypertension [26]. One source of ROS in the cardiovascular system is the NADPH Oxidase family of proteins [27].

Within the context of AAA formation, ROS are believed to play a role in activating MMPs, inducing vSMC apoptosis, and propagating a chronic inflammatory response. It was found that tissue from human AAA had 2.5 times higher superoxide levels than adjacent, non-aneurysmal tissue [26]. Macrophages, a mediator of AAA, are known to release ROS extracellularly which can activate MMPs critical to AAA development [25]. Reactive oxygen species were shown to induce vSMC apoptosis [28].Using the elastase model of AAA in rats, it was shown that elastase treatment elevated both Mn-superoxide dismutase (SOD2), which is known to convert superoxide to hydrogen peroxide, and MMP-9 levels [29]. Other studies have shown inhibition or deletion of NADPH oxidases can attenuate AAA and aortic dissection [30-32]. Gavrila et al. demonstrated a potential role for vitamin E, a dietary antioxidant, to prevent AAA formation [33].

A.)



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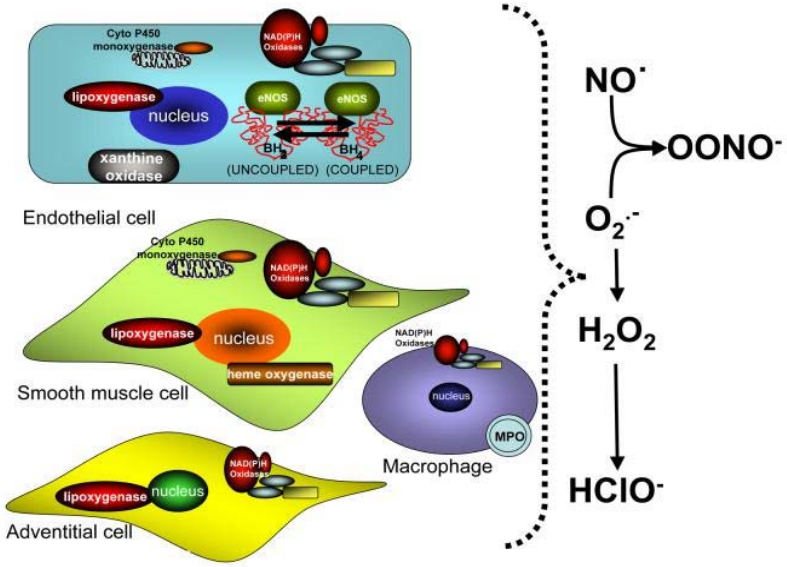

Figure 1.3: Sources of ROS – Reactive oxygen species can be produced by various cell types and is often mediated by the NOX family of enzymes. These images show the role of ROS in response to injury (A) and the vascular sources of ROS (B) [34]

Previous work focuses primarily on the role of superoxide alone or the presence of general markers of oxidative stress and ROS. To date very little is known about the contributions of hydrogen peroxide specifically to AAA development. It has been shown that abrupt changes in shear stress produces hydrogen peroxide in human arteries [35]. With the extensive remodeling and changes in hemodynamic forces observed in AAA development it would not be surprising to see increases in hydrogen peroxide production. At concentrations of 1μM, hydrogen peroxide stimulated activity of purified MMPs [36]. The actions of MMPs activated by hydrogen peroxide stimulate adhesion molecules such as VCAM-1 to allow leukocyte migration into the aortic wall [36]. Additionally, evidence suggests hydrogen peroxide can induce vSMC apoptosis [28]. In terms of inflammation, hydrogen peroxide may propagate the inflammatory response via activation of NF-ϰB [37]. With this evidence, we developed the hypothesis that hydrogen peroxide plays a critical role in AAA development.

Some of the major difficulties in studying ROS are the ability to reliably measure and scavenge these molecules at their site of action/production [38]. These difficulties are present due to the accessibility, localization within the body and source of ROS production within a cell. For this reason, much research is focused on developing highly specific and sensitive assays as well as targeted antioxidant therapies. These therapies could focus on scavenging of ROS already produced or inhibition of the source of ROS. The metalloporphyrin class of compounds has been shown to readily scavenge ROS. More specifically, Manganese tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP)and Manganese tetrakis (4-benzoic acid) porphyrin (MnTBAP) were shown to catalytically dismutate hydrogen peroxide [39]. Zhou et al. showed that Manganese (MnTMPyP) was able to signifcanty reduce ROS levels following Angiotensin II treatment in cardiomyocytes [40]. Previous work in our division showed Ebselen, a glutathione

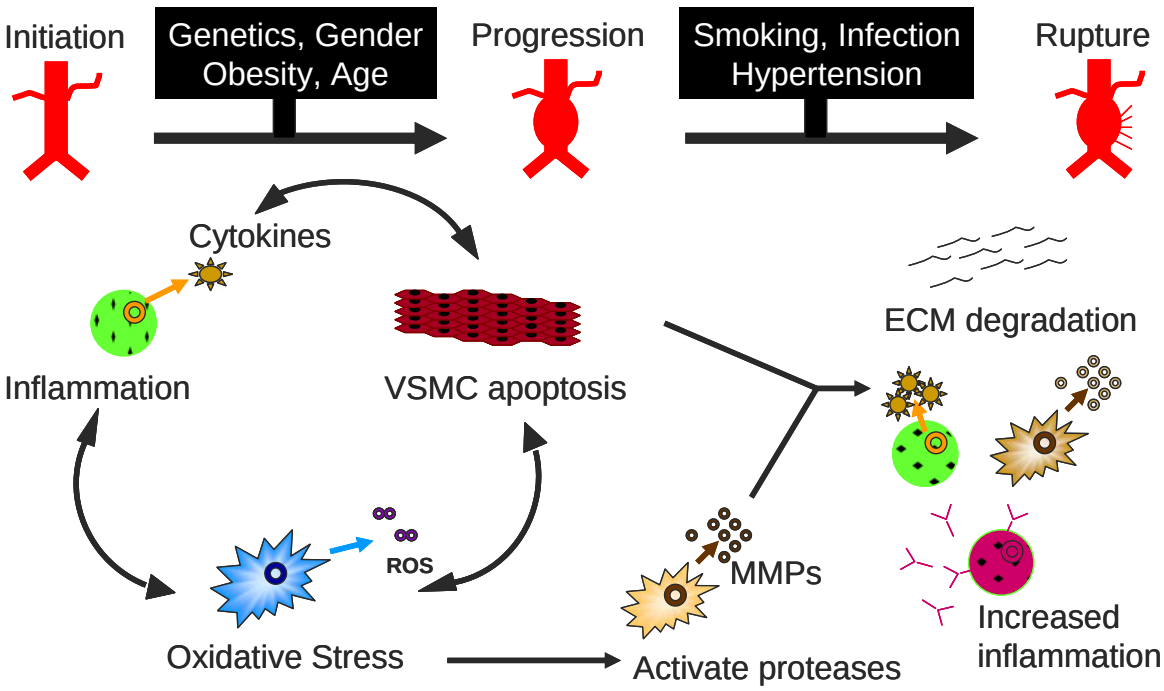

Figure 1.4: Pathogenesis of AAA. Many factors contribute to the pathogenesis of AAA. This schematic represents the complex nature of multiple risk factors, inflammation, apopotosis and oxidative stress to activate proteases and degrade the aortic wall. Recreated from [41]. Drugs News Perspect 2008; 21(3): 142-148.

peroxidase mimetic, to lower hydrogen peroxide production in vivo [42]. Despite the fact that oxidative stress has been shown numerous times to play a role in cardiovascular pathology, antioxidant based clinical trials have seen little success. It is proposed that these results could be caused by poor timing, inappropriate patient selection and use of ineffective antioxidants [43].

Here we worked to elucidate the contribution of hydrogen peroxide in AAA development. In order to accomplish this most effectively we measured extracellular hydrogen peroxide levels in three commonly used models of AAA. These data were then used with in vivo aortic diameter measurements to indicate AAA formation. We show that hydrogen peroxide is elevated in all three models of AAA. To determine the specific role for hydrogen peroxide in this process we used MnTMPyP, MnTBAP and Ebselen in an attempt to lower hydrogen peroxide and attenuate AAA formation.

Chapter 2: Calcium Chloride stimulates hydrogen peroxide production and AAA

a. Introduction

One animal model for AAA is periaortic application of calcium chloride ($CaCl_2$). This model was introduced in 1988, where calcium chloride was applied to the carotid artery of rabbits to produce an aneurysm [44]. Since then, the model has been used to produce aortic aneurysms in mice [45]. Calcium chloride application leads to a progressive dilation of the abdominal aorta and significant differences in aortic diameters are apparent within 4 weeks [46]. This model was shown to elicit a chronic inflammatory response, leading to disruption of the elastic networks and vSMC apoptosis [46]. Regarding the role of ROS and oxidative stress in this model, Xiong et al demonstrated an attenuation of AAA formation by apocynin, an NADPH oxidase inhibitor [32]. Some of the advantages of this model over other AAA models are the localization of AAA to the infrarenal aorta, the circumferential dilation of the aorta, the lack of dissection and thrombus formation, and the relative ease at which one can perform the surgery. Manganese tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) was used to scavenge hydrogen peroxide in order to elucidate a role for hydrogen peroxide in this model. MnTMPyP was selected as a candidate antioxidant due to its selectivity for hydrogen peroxide and solubility for administration through osmotic minipumps [47, 48].

b. Materials and Methods

Calcium Chloride Model of AAA – Nine week old, Male, C57/Bl6 mice were anesthetized using inhalation of isoflurane at an induction rate of 5% and a maintenance dose of 1.5%. Under aseptic conditions, the skin of the abdomen was shaved and prepped with betadine. With sterile instruments an incision in the midline of the abdomen was made to expose the abdominal cavity. Retractors were used to hold the skin open and expose the contents of the abdomen. The bowels were retracted to the upper left quadrant and wrapped in moistened gauze. The bladder

and seminal vesicles were gently pushed deep into the pelvis to expose the bifurcation of the aorta. The abdominal cavity was kept moist with saline. The aorta was then isolated from the inferior vena cava (IVC) using fine forceps. Once the aortic bifurcation was located the connective/adipose tissue surrounding it was removed. Sterile gauze was soaked in 0.25M calcium chloride or sterile saline (negative control) for 20 minutes. Once the aorta was isolated from the IVC a small piece of soaked gauze was applied to the external surface of the aorta for a period of 15 minutes. Following application of calcium chloride to the aorta, the gauze was removed and excess calcium chloride solution was absorbed with sterile gauze. The bladder and seminal vesicles were moved back to their correct anatomic position to prevent obstruction of the urinary tract. The bowels were returned to their proper anatomic position to prevent intestinal ischemia. The abdominal incision was closed with 4-0 sutures. First the abdominal wall was closed with continuous sutures in one layer and then the skin was closed in another layer using interrupted 4-0 sutures. Buprenex was given for post operative analgesia at a dose of 0.01mg. The mouse was then placed on a heating pad to recover. Depending on the amount of blood loss during the procedure a subcutaneous injection of 1mL saline was given. Animals were monitored daily to check for post operative complications. The final time point for aneurysm development in this model was 8 weeks following application of calcium chloride. All surgical procedures were approved by the IACUC at Emory University.

Antioxidant Therapy – Manganese tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) (Enzo Life Sciences. Plymouth Meeting, PA) at a dose of 10mg/kg/day (dissolved in saline) was delivered subcutaneously via 28 day osmotic minipumps (Alzet. Cupertino, CA). Two days prior to calcium chloride application, animals had the minipumps implanted subcutaneously. Pumps were replaced at the 4 week time point. To implant the pumps, animals were anesthetized as described above, a small incision was made on the back of the animal and the pump was inserted

into the region just under the skin. The surgical wound was closed with 4-0 monofilament suture. After the surgery animals were kept on heating pads to recover.

Aortic Diameter Measurements – Aortic diameters were measured using two different methods: ultrasound imaging and direct imaging/computer analysis. Noninvasive, ultrasound imaging was performed on a Visual Sonics (Toronto, Canada) Vevo 770 High Resolution Micro-imaging system running software version 2.3.0. Animals were anesthetized with isoflurane at an induction dose of 5% and a maintenance dose of 1.5%. After being placed on the ultrasound platform the animal's abdomen was shaved and prepped for the ultrasound probe. A generous amount of Aquasonic 100 Ultrasound gel was applied to the abdomen of the animal and the probe was positioned above the lower abdominal region. The abdomen was scanned using the B-Mode function. The infrarenal aorta was found based on its proximity to the kidneys, Inferior Vena Cava, and if possible, the aortic bifurcation. To confirm the vessel was truly the aorta, Doppler velocity profiles were used to show a strong pulsatile flow in the region of interest. The program was then switched to M-Mode and the Vevo 770 software's built-in measuring function was used to measure the inner aortic diameter. The measurements were made prior to surgery, at 4 weeks and at 8 weeks after calcium chloride application.

The other method of measuring aortic diameter was in vivo microscopic imaging. Prior to surgical manipulation of the aorta a picture was taken of the infrarenal aorta with a QImaging 3.3 micropublisher RTV camera mounted on an Olympus SZ61 Microscope. The camera was connected to a computer running QCapture v2.81.0 image processing software. For a reference of scale, a small, sterile ruler was placed adjacent to the aorta. This image was then uploaded into the ImageJ image processing and analysis program. ImageJ allows the user to measure aortic diameters based on the scale set by the ruler and the number of pixels across the external surface of the aorta.

Hydrogen Peroxide Measurements – Amplex Red (Invitrogen, Carlsbad, CA) fluorescent assay was used to quantify extracellular hydrogen peroxide. Krebs-Ringer Phosphate (KRPG) Buffer (pH 7.35) was prepared fresh prior to tissue harvesting. To harvest aortas for this assay, euthanized mice were perfused with cold KRPG buffer injected into the left ventricle of the heart. Adipose and connective tissue was cleared from the aorta. The infrarenal or suprarenal aorta was then dissected from the animal and placed in cold KRPG buffer. The aortas were then cut into 2mm segments for tissue normalization. After preparing the Amplex Red working solution (per aorta: 300μL KRPG buffer, 1.5μL Amplex red, 3μL Horseradish Peroxidase) each aorta was incubated for 50 minutes at 37°C in the cell culture incubator. Samples were read on a Cytofluor Multiplate Reader Series 4000 at an excitation wavelength of 530nm and emission wavelength of 580nm in duplicate. The mean fluorescence was analyzed on Cytofluor software version 4.2.1.

Statistical Analyses – Students T-test, one-way analysis of variance (ANOVA) with Tukey post hoc tests and two-way ANOVA with Bonferroni post hoc analysis were performed on GraphPad Prism version 5.01.

c. Results

Preliminary studies with the calcium chloride model were to confirm reproducibility and accuracy in measuring aortic diameters. This was accomplished by measuring the aortic diameter with ultrasound (Echo) imaging software or Image J analysis software as described in the materials and methods portion of chapter 2. Figure 2.1 validates the use of either ultrasound or in vivo imaging to readily measure aortic diameter.

Utilizing the calcium chloride model of AAA, we began to investigate the role of hydrogen peroxide in AAA and the potential of MnTMPyP to inhibit AAA formation. Figure 2.2 demonstrates significant increases in aortic diameter 8 weeks following calcium chloride

administration. Additionally, MnTMPyP did not attenuate aortic dilation following $CaCl_2$ application. Ultrasound imaging was used to monitor aortic diameter differences at 4 weeks (Figure 2.4).

Since MnTMPyP did not attenuate AAA formation, we elected to investigate the efficacy of of MnTMPyP in lowering hydrogen peroxide levels in the infrarenal aorta. Figure 2.3 shows that there was no significant differences between MnTMPyP treated or calcium chloride alone groups compared to saline controls in hydrogen peroxide 3 days following $CaCl_2$ treatment. Additionally, at day 5 hydrogen peroxide levels were significantly increased in $CaCl_2$ treated animals with or without MnTMPyP compared to saline control.

d. <u>Conclusions</u>

The results of the calcium chloride set of experiments provided evidence that hydrogen peroxide may play a role in AAA formation. It was seen that hydrogen peroxide was elevated at day 5 in mice treated with $CaCl_2$. MnTMPyP at a dose of 10mg/kg/day did not attenuate this elevation at day 5.

This set of experiments was important in establishing the $CaCl_2$ model in our lab. This is the first time we have successfully induced AAA with $CaCl_2$ application. This was confirmed in Figure 2.2 where aortic diameters were significantly dilated at week 8. We also showed the feasibility of either ultrasound or in vivo imaging to accurately and reproducibly measure aortic diameters in this model. Addtionally, we show that MnTMPyP was unable to inhibit hydrogen peroxide in this model or attenuate AAA formation.

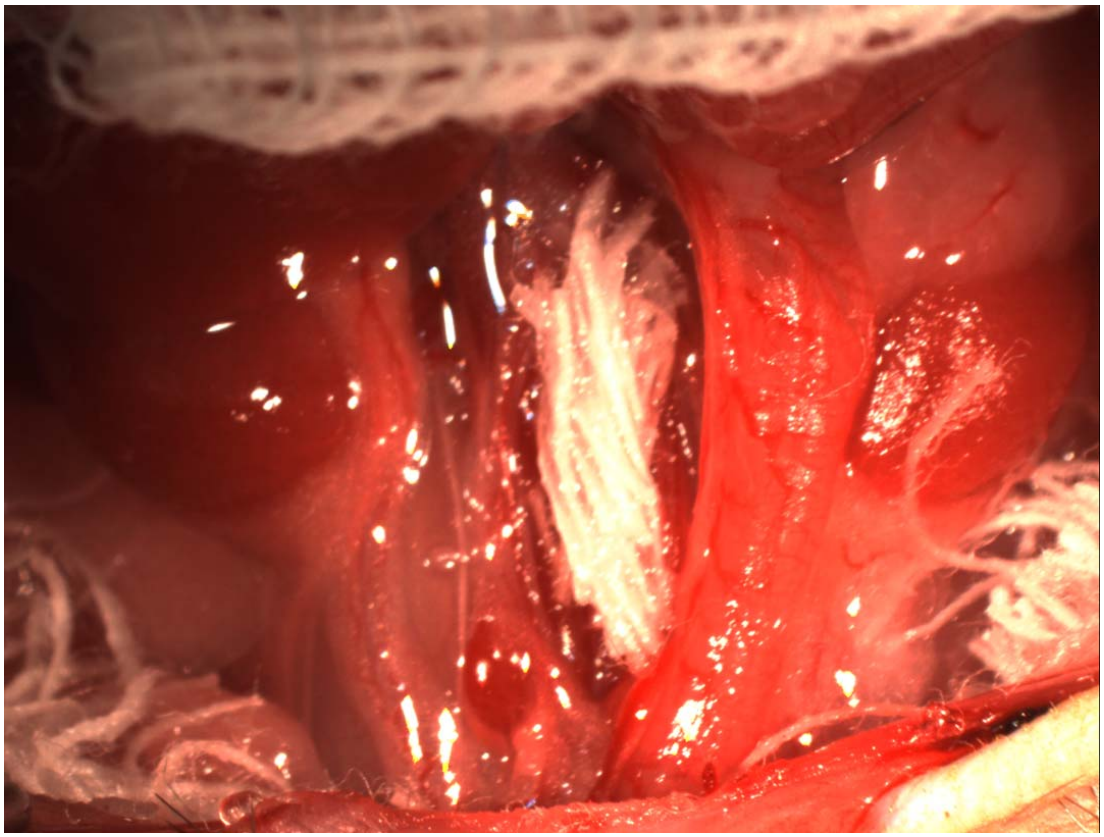

Figure 2.1: Calcium Chloride Surgical Procedure. This is an image of the calcium chloride soaked gauze being placed on the aorta for a period of 15 minutes. Below the gauze was the aorta, which was isolated from the inferior vena cava prior to calcium chloride application.

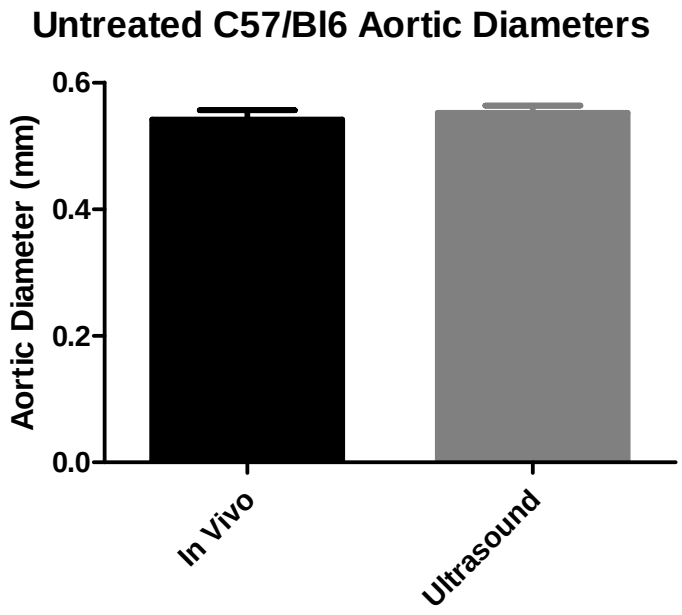

Figure 2.2 – Ultrasound vs. In vivo. Nine week old, male, C57/Bl6 mice were subjected to two different aortic diameter measurements. The data here validates ultrasound and in vivo imaging as reliable ways to measure aortic diameter.

N=18 per group. Students T-test *$p<0.05$ ±SEM

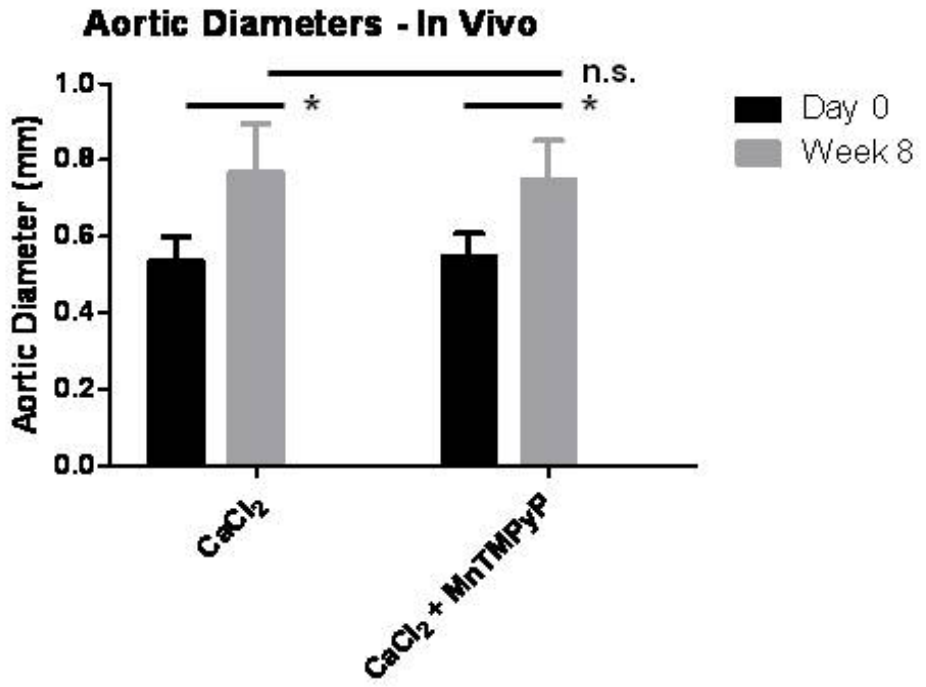

Figure 2.3 – Calcium Chloride Aortic Diameters. Nine week old, male, C57/Bl6 mice underwent periaortic $CaCl_2$ application. One group was administered MnTMPyP for the duration of the experiment. The data here demonstrate reproducibility in producing AAA with $CaCl_2$ after 8 weeks. Imaging performed was in vivo imaging with IMageJ analysis. Additionally, MnTMPyP did not significantly reduce aortic dilation 8 weeks after $CaCl_2$. N=9 per group. Two Way ANOVA with Bonferroni *$p<0.05$ ±SEM

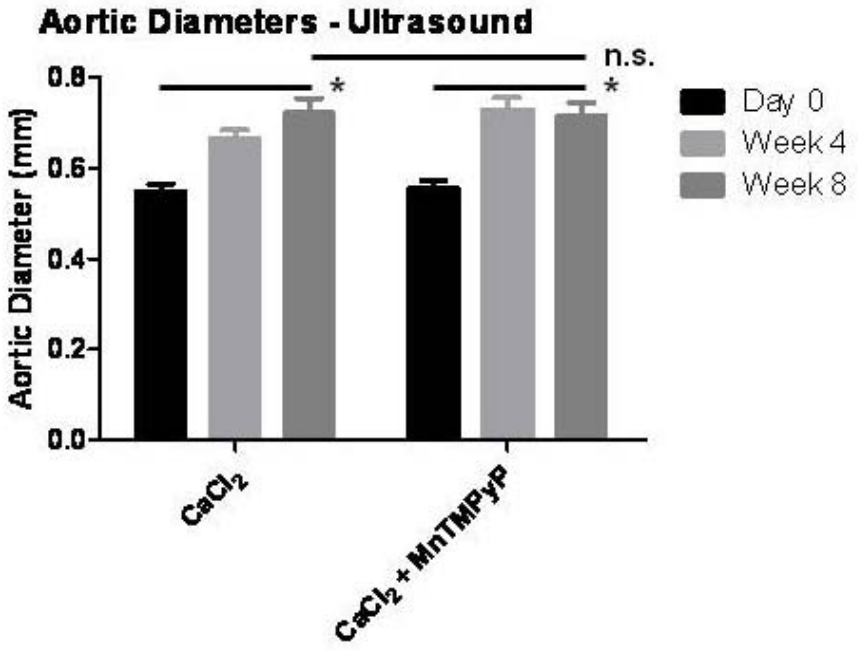

Figure 2.4 – Calcium Chloride AAA Growth Over Time. Nine week old, male, C57/Bl6 mice underwent periaortic $CaCl_2$ application. One group was administered MnTMPyP for the duration of the experiment. Ultrasound imaging was used to measure aortic diameter at all time points. Additionally, MnTMPyP did not significantly reduce aortic dilation 8 weeks after $CaCl_2$. N=9 per group. Two Way ANOVAwith Bonferroni *$p<0.05$ ±SEM

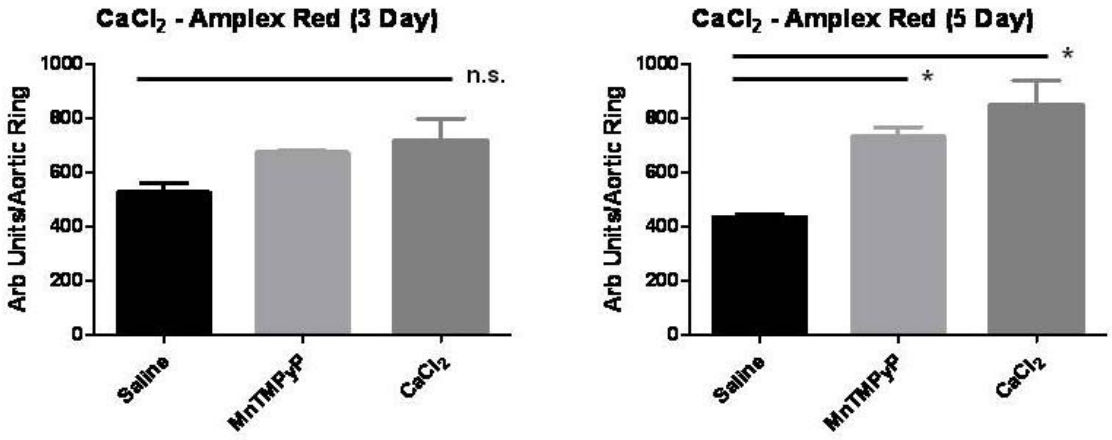

Figure 2.5 – Calcium Chloride Amplex Red. Nine week old, male, C57/Bl6 mice underwent periaortic $CaCl_2$ application. Control animals received saline instead of $CaCl_2$. One group was administered MnTMPyP for the duration of the experiment. The data here demonstrate that there was a significant increase in hydrogen peroxide production over baseline on day 5 and not day 3. MnTMPyP did not significantly reduce H2O2 concentrations.

N=4 per group. One Way ANOVA with Tukey *$p<0.05$ ±SEM

Chapter 3: Elastase treatment reliably produces AAA

a. Introduction

The elastase surgical model of AAA was developed in the early 1960's [46]. Pyo et al described the first protocol for producing aneurysms in mice with this method [49]. Since then, multiple publications have characterized this model. A comprehensive review of the elastase model in mice was published in 2006 [20]. This review demonstrates the reproducible characteristics of this technically challenging model. As with the calcium chloride model of AAA, the elastase model produces an infrarenal AAA, with circumferential dilation of the aorta, chronic inflammation, dependence on MMP expression and elastin degradation. Multiple studies suggest a role for oxidative stress in the elastase model in rats [50, 51]. Ebselen was selected as a candidate antioxidant due to its selectivity for hydrogen peroxide and previous research showing biological effects in cardiovascular applications [47, 48].

b. Materials and Methods

Elastase Model of AAA – Nine week old, Male, C57/Bl6 mice were anesthetized using inhalation of isoflurane at an induction rate of 5% and a maintenance dose of 1.5%. Under aseptic conditions, the skin of the abdomen was shaved and prepped with betadine. With sterile instruments an incision in the midline of the abdomen was made to expose the abdominal cavity. Retractors were used to hold the skin open and expose the contents of the abdomen. The bowels were retracted to the upper left quadrant and wrapped in moistened gauze. The bladder and seminal vesicles were gently pushed deep into the pelvis to expose the bifurcation of the aorta. The abdominal cavity was kept moist with saline. The aorta was then isolated from the inferior vena cava (IVC) using fine forceps. Once the aortic bifurcation was located the connective/adipose tissue surrounding it was removed. The IVC and aorta were then separated 3-4mm above the bifurcation and a loosely placed 6-0 suture was wrapped around the aorta.

The branches of the aorta were then isolated and tied off with 10-0 sutures. The pre-infusion image was then taken with the same microscope imaging system described in Chapter 2b. To prepare for the aortotomy, one vascular microclamp (Fine Science Tools) was placed just above the bifurcation and another just distal to the left renal vein. Using a 30 gauge needle an aortotomy was made between the distal microclamp and the 6-0 suture. A small PE-10 catheter stretched at one end to decrease outer diameter was attached to a 30 gauge needle. This needle was then attached to a 10mL syringe filled with Type I Porcine Pancreatic Elastase (Sigma) at a dose of 0.414U/mL Elastase in sterile saline solution. The syringe was placed in a single chamber syringe pump (Harvard Apparatus model 11 plus) to regulate flow of elastase into the aorta. The stretched end of the catheter was then placed into the aorta and the 6-0 suture gently secured the catheter in place. Once the catheter was secured, the aorta was infused with elastase at a rate of 2.00ml/hr until an aortic diameter of 1.10mm was achieved. The aorta was maintained at a minimum of 1.10mm for an additional 5 minutes and 30 seconds. Following infusion the 6-0 suture and catheter was removed. The aortotomy was closed with a single interrupted stitch with 10-0 suture. The distal microclamp was removed first and then the proximal microclamp was removed. To prevent bleeding from the aortotomy site slight pressure was applied to the site with a cotton tipped applicator. The aorta was gently massaged for a period of 5 minutes to prevent clotting in the aorta. The bladder and seminal vesicles were moved back to correct anatomic position to prevent obstruction of the urinary tract. The bowels were returned to their proper anatomic position to prevent intestinal ischemia. The abdominal incision was closed with 4-0 sutures. First the abdominal wall was closed with continuous sutures in one layer and then the skin was closed in another layer using interrupted 4-0 sutures. Post-operative analgesia, buprenex (0.01mg) was given and the mouse was placed on a heating pad to recover. Depending on the amount of blood loss during the procedure a subcutaneous

injection of 1mL saline was needed. Animals were monitored daily to check for post operative complications. Impaired hind limb mobility was one assessment made for post-operative complications. The final time point for aneurysm development in this model was 14 days following surgical infusion of elastase. All surgical procedures were approved by the IACUC at Emory University.

Antioxidant Therapy – Ebselen (Cayman Chemical) was administered at a dose of 10mg/kg/day subcutaneously via osmotic minipumps (Alzet. Cupertino, CA). Ebselen solution was prepared by initially dissolving it in 100% DMSO. That solution was then diluted by half with saline. Two days prior to elastase infusion, animals had the minipumps implanted subcutaneously. The implantation procedure was identical to that described in Chapter 2b

Aortic Diameter Measurements – Aortic measurements were made from ultrasound and ImageJ analyses of images taken of the aorta in the same way as found in Chapter 2b.

Hydrogen Peroxide Measurements – The Amplex Red (Invitrogen) fluorescent hydrogen peroxide detection assay was used to measure relative amounts of hydrogen peroxide in the same manner as it was described in Chapter 2b. The only difference was the time points chosen.

Statistical Analyses – Students T-test, One-way and two-way ANOVA with Bonferroni post hoc analyses were performed on GraphPad Prism version 5.01.

c. <u>Results</u>

Figure 3.1 confirms the development of AAA following elastase infusion into the infrarenal aorta. Saline infused animals did not display significant dilations of the aorta. Following the confirmation of the model, it was necessary to test whether hydrogen peroxide was involved in the model. Figure 3.2 demonstrates that both saline and elastase infused models have significantly elevated hydrogen peroxide levels at days 5 and 14. In an attempt to attenuate AAA formation, elastase treated mice we treated with ebselen or vehicle control (DMSO). Figures 3.4

and 3.5 show that ebselen was not able to lower hydrogen peroxide levels at day 5 or AAA by day 14, respectively.

d. **Conclusions**

This was the first time the elastase model was successfully performed in our lab. From this work we confirmed that elastase infusion can produce AAA while saline infusion does not, as previously shown. Following confirmation of the model, Amplex Red demonstrated that hydrogen peroxide levels were elevated at days 5 and 14, regardless of the solution infused into the aorta. Although we expected that the surgery might initially cause an increase in hydrogen peroxide production because of the extensive manipulation of the aorta, we thought by 14 days the elevation would be dependent on elastase infusion and the pathological features of AAA. Day 5 was selected because of the results of the $CaCl_2$ experiments and other cardiovascular pathology models used in our lab. Day 14 was selected because at the end of the experiment we expected to see a difference in aneurysmal tissue and non aneurysmal tissue. I also confirmed that Ebselen was not able to significantly inhibit AAA or lower hydrogen peroxide compared to vehicle controls. These data suggest the role of hydrogen peroxide may be model dependent and the complex elastase model may not be the best model to elucidate the role of hydrogen peroxide in AAA.

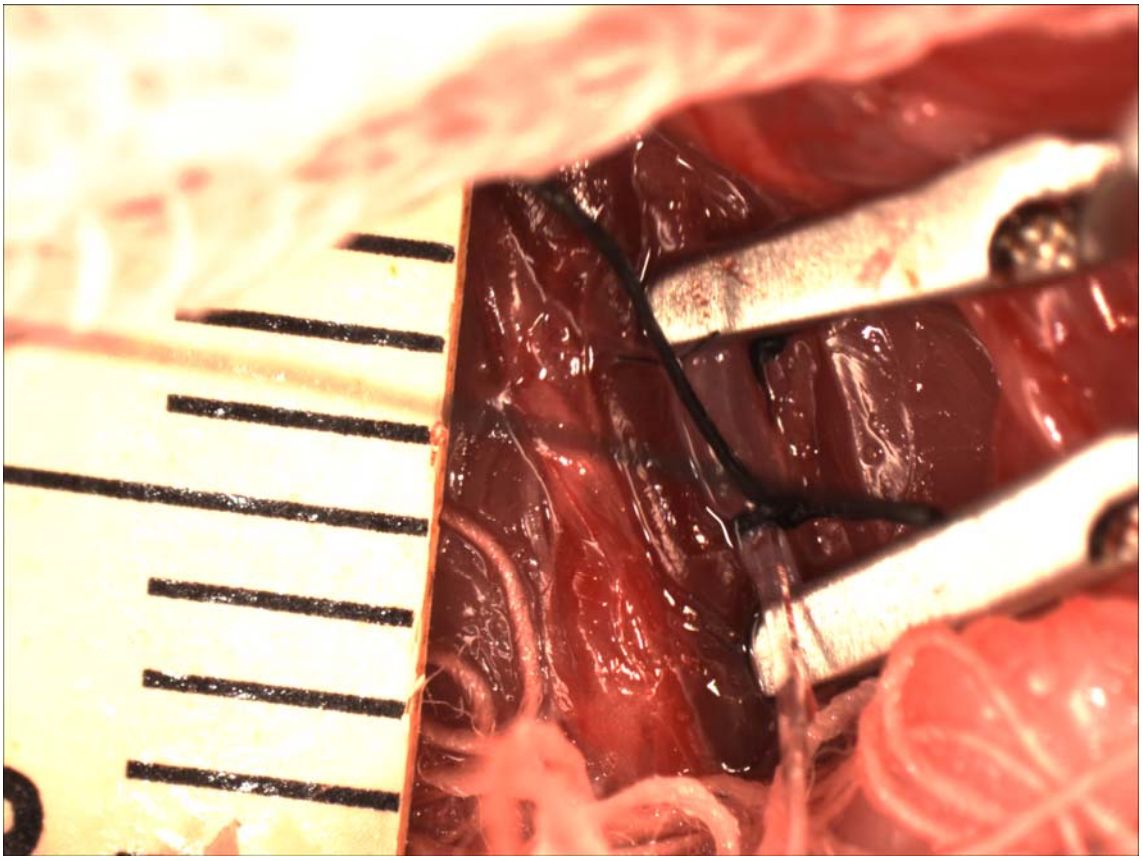

Figure 3.1 - Elastase Surgical Procedure. This image was taken during the inflation phase of the elastase surgery. It is clear that the aorta is inflated with elastase to a diameter nearly double its normal size. Additionally in the image are the two vascular microclamps, the clear catheter going into the aorta and the multiple branches of the aorta which are tied off. You can also appreciate the small size from the ruler where the space between each black line is only 1mm.

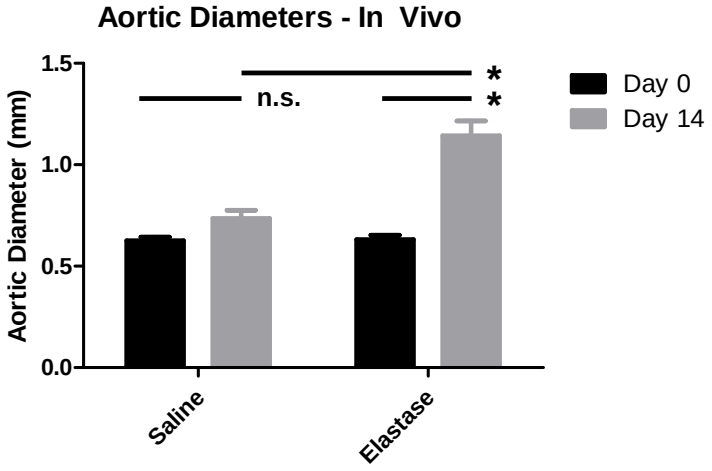

Figure 3.2 – Elastase Aortic Diameters. Nine week old, male, C57/Bl6 mice underwent saline or elastase infusion into the infrarenal aorta. The data here demonstrate the development of AAA in elastase treated mice but not in saline treated controls.

N=4 per group. Two Way ANOVA with Bonferroni *$p<0.05$ ±SEM

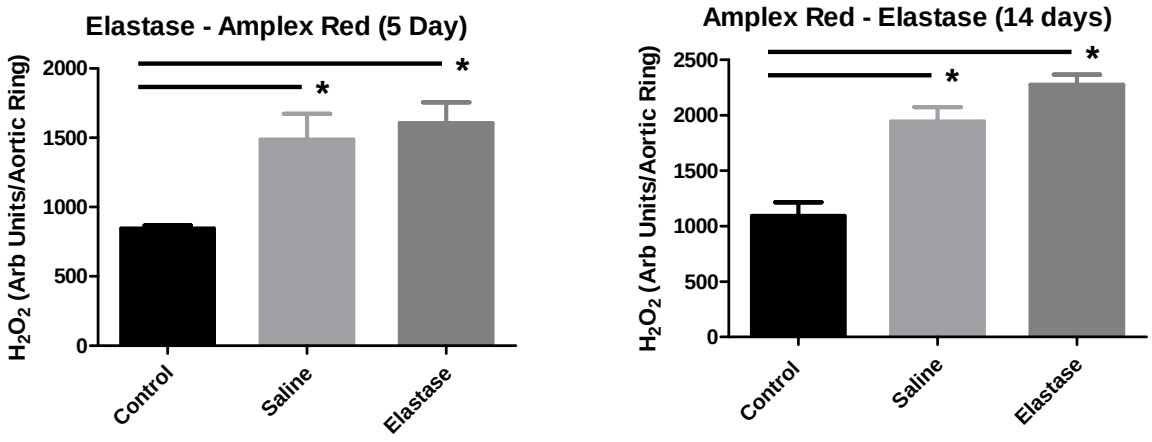

Figure 3.3 – Elastase Amplex Red. Nine week old, male, C57/Bl6 mice underwent saline or elastase infusion into the infrarenal aorta. Controls were untreated C57/Bl6 mice. The data here demonstrate that hydrogen peroxide production occurs regardless of elastase or saline infusion. N=6 (5day) or N=4 (14 day) per group. One Way ANOVA with Bonferroni *$p<0.05$ ±SEM

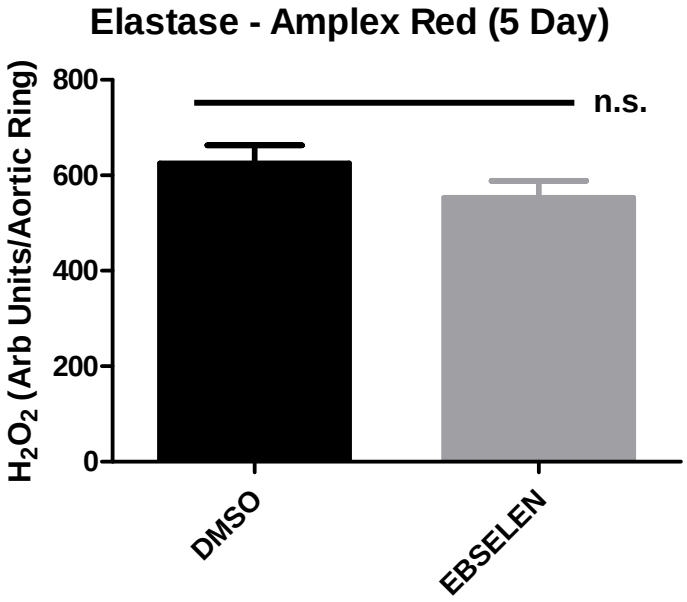

Figure 3.4 – Ebselen (Elastase) Amplex Red. Nine week old, male, C57/Bl6 mice underwent elastase infusion into the infrarenal aorta. One group received 10mg/kg/day Ebselen the other received vehicle control. The data here demonstrate that hydrogen peroxide levels at day 5 could not be significantly changed by Ebselen treatment following Elastase infusion.

N=8 (Ebselen) N=9 (DMSO) per group. Students T-Test *$p<0.05$ ±SEM

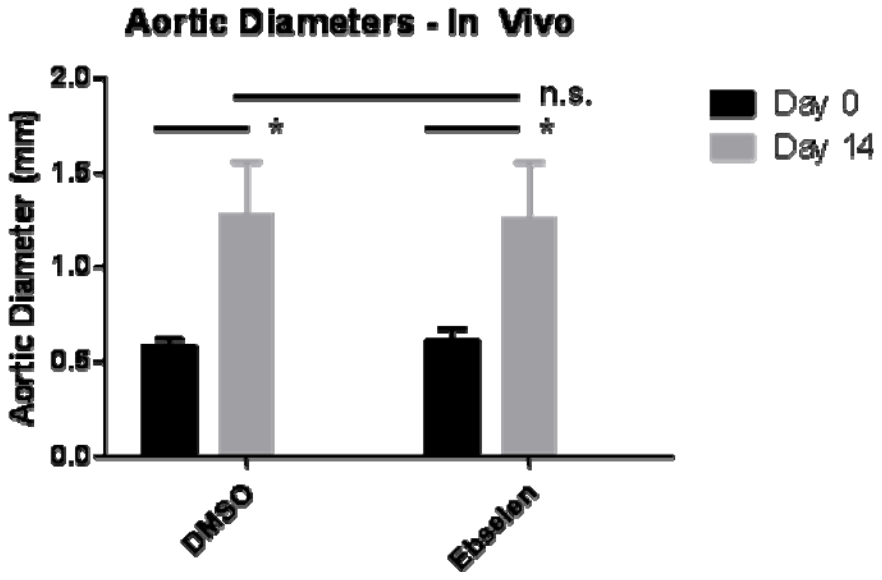

Figure 3.5 - Ebselen treatment (Elastase) Aortic Diameters. Nine week old, male, C57/Bl6 mice underwent elastase infusion into the infrarenal aorta. One group received 10mg/kg/day Ebselen the other received vehicle control. The data here demonstrate that Ebselen treatment following Elastase infusion does not inhibit AAA development.

N=7 per group. Two Way ANOVA with Bonferroni *$p<0.05$ ±SEM

Chapter 4: Antioxidants fail to lower hydrogen peroxide in Angiotensin II model

a. Introduction

Angiotensin II administration is known to produce ROS via NADPH oxidases [52]. Our lab has used angiotensin II in mice extensively and is familiar with the critical time points of maximal ROS production. I used this angiotensin II model to screen multiple antioxidant therapies using Angiotensin II as a positive control for ROS production in the mouse suprarenal aorta. The three antioxidants were selected on their published selectivity for hydrogen peroxide [39, 48]. In addition to MnTMPyP and Ebselen, Manganese tetrakis (4-benzoic acid) porphyrin (MnTBAP) was investigated based on its ability to scavenge hydrogen peroxide in cell culture [39].

b. Materials and Methods

Administration of Angiotensin II – Nine week old, Male, C57/Bl6 mice were anesthetized using inhalation of isoflurane at an induction rate of 5% and a maintenance dose of 1.5%. Under aseptic conditions, osmotic mini pumps (Alzet. Cupertino, CA) were implanted subcutaneously to deliver continuous angiotensin II (0.75mg/kg/day). The procedure was identical to the one described in Chapter 2b in the antioxidant therapy subsection. All surgical procedures were approved by the IACUC at Emory University.

Antioxidant Therapy – Multiple antioxidants that have been shown to target hydrogen peroxide were screened in mice administered Angiotensin II. Ebselen (50% DMSO:50% Saline) (Cayman Chemical) was administered at a dose of 10mg/kg/day, Manganese tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) (Enzo Life Sciences. Plymouth Meeting, PA) was administered at a dose of 10mg/kg/day (dissolved in saline), and Manganese tetrakis (4-benzoic acid) porphyrin (MnTBAP) (Enzo Life Sciences. Plymouth Meeting, PA) was administered at a dose of 50mg/kg/day (dissolved in saline) were all delivered subcutaneously via osmotic minipumps

(Alzet. Cupertino, CA). Antioxidant containing pumps were implanted 3 days prior to Angiotensin II administration. The implantation procedure was identical to that described in Chapter 2b

Hydrogen Peroxide Measurements – The Amplex Red (Invitrogen) fluorescent hydrogen peroxide detection assay was used to measure relative amounts of hydrogen peroxide as described in Chapter 2b.

Statistical Analyses – Two way ANOVA with Bonferroni was performed on GraphPad Prism version 5.01

c. Results

Figure 4.1 shows the results of multiple experiments screening antioxidant therapies, where angiotensin II was used to induce hydrogen peroxide production. The only significant difference in hydrogen peroxide was between control (untreated) and AngII treated animals. All other treatments failed to reach a statistically significant difference from the Angiotensin II positive control. MnTMPyP and MnTBAP had similar levels of hydrogen peroxide as the Angiotensin II group. The vehicle control for Ebselen was 50% DMSO. The DMSO group had hydrogen peroxide levels trending downward, along with the Ebselen treated group but no statistical significance was reached.

d. Conclusions

The data from the Angiotensin II antioxidant screen shows that three commercially available small molecule hydrogen peroxide scavengers were unable to lower hydrogen peroxide levels in the suprarenal aorta at day 7. The 7 day time point was selected based on extensive experience in our lab that shows hydrogen peroxide production peaks between 7 and 9 days following implantation of Angiotensin II. These data are surprising because MnTMPyP, MnTBAP, and

Ebselen have all been previously shown to scavenge hydrogen peroxide at the dose used in these experiments.

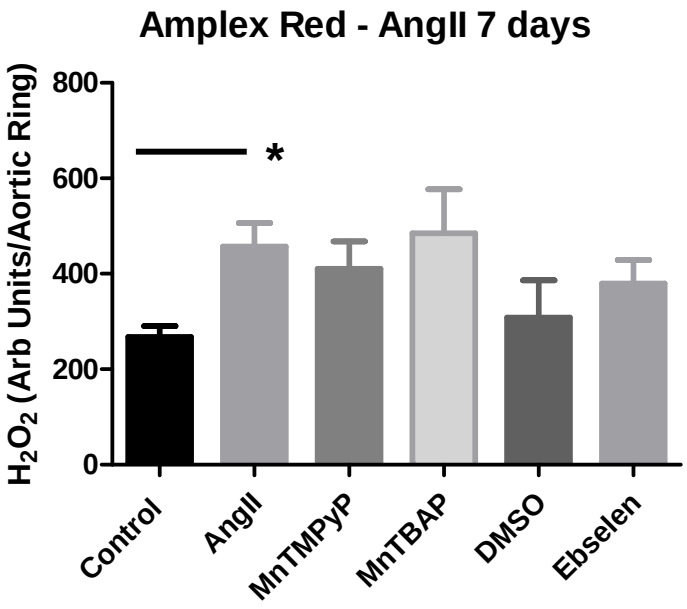

Figure 4.1 – Angiotensin II Antioxidant Screen Amplex Red. Nine week old, male, C57/Bl6 mice were administered angiotensin II and antioxidant treatment. Controls were untreated C57/Bl6 mice. The data here demonstrate that hydrogen peroxide production could not be inhibited by MnTMPyP, MnTBAP or Ebselen at day 7. N=10 (Control, AngII); N=6 (MnTMPyP); N=5 (MnTBAP); N=3 (DMSO, Ebselen) per group. One Way ANOVA with Tukey *$p<0.05$ ±SEM

Chapter 5: Discussion

a. Summary and Conclusions

I began with calcium chloride as a model of AAA because of the pathological features of the model. I was able to confirm both AAA development and increases in hydrogen peroxide when $CaCl_2$ was applied to the aorta. This demonstrates $CaCl_2$ application as a reliable model to study AAA and oxidative stress. Elastase infusion as a model of AAA is technically challenging but provides a reliable model to study AAA pathogenesis. It was unexpected that there was not a significant difference in hydrogen peroxide between elastase and saline infused mice. The cause of this is likely due to the invasive and aggressive nature of the surgical procedure. It has been shown that stretch can cause increases in ROS [53]. Additionally, we studied the Angiotensin II model of AAA and showed that hydrogen peroxide also increases in this third model of AAA. We used this model to screen three commercially available antioxidants and showed no significant difference in hydrogen peroxide levels.

It was surprising that three commercially available antioxidants previously shown to target hydrogen peroxide were unable to significantly lower hydrogen peroxide levels in the aorta. Even after trying multiple time points and models, these small molecules were unable to scavenge hydrogen peroxide despite their reported efficacy in the literature.

b. Interpretations

The ultimate goal of this work was to establish a role for hydrogen peroxide in the development of AAA. With three out of three AAA models showing increases in hydrogen peroxide we believe hydrogen peroxide does play a role, but may be model specific. The one caveat is that in the elastase model saline treatment does not produce AAA but does increase hydrogen peroxide. This observation suggests that hydrogen does not play a causal role in this model. A major contributing factor is that stretching the aorta, as is necessary in the elastase

surgery, could lead to increases in hydrogen peroxide. One explanation for the variability between models is the role of hydrogen peroxide and atherosclerosis. Previous work in our lab shows that increases in smooth muscle specific catalase, which results in lower hydrogen peroxide, attenuates atherosclerosis. Since AAA is often associated with, but not believed to be caused by, atherosclerosis the lack of atherosclerosis in the calcium chloride and elastase models could impact the growth of the AAA.

One of the most pressing questions in terms of impact is why didn't the antioxidant therapies work? There are multiple reasons for these observations. The first would be that the drugs were not reaching the aorta. In order to lower hydrogen peroxide levels in the aorta, the drug must be present, in an active state and at high enough levels to adequately scavenge the hydrogen peroxide. The fact that the drugs were given subcutaneously may have inhibited the drugs from reaching the aorta in sufficient biologically active levels. Another explanation for the lack of efficiency seen in these experiments could be due to shortcomings of the Amplex Red assay. Although a standard method for quantifying relative levels of hydrogen peroxide, it only detects extracellular hydrogen peroxide and may not be sensitive enough to detect the potentially small differences produced by the antioxidants. A third explanation for the lack of antioxidant therapies from attenuating AAA formation, could be based on the molecule being targeted, hydrogen peroxide. Since there are no reports to directly associate hydrogen peroxide to AAA formation, we may be investing the wrong ROS. Another explanation could be due to incorrect dosing. Although the doses used were based off of previously reported results, a larger dose may have been necessary to lower hydrogen peroxide levels in these relatively aggressive ROS producing models.

The data here shows that hydrogen peroxide is involved in AAA development although it may be model specific. If our hypothesis is correct, the calcium chloride model may be the best

model to study role of hydrogen peroxide because by day 5 there is a significant difference between calcium chloride treated and saline controls. Additionally, if our hypothesis is correct, the elastase surgery may not be the best model to study a role for hydrogen peroxide in AAA because of the invasive nature of the surgery. This is not too surprising because previous reports show stretching of vSMC can cause ROS generation. Differences in models are an issue often seen in basic science research. We try to generate the best model we can to study a particular disease, in this case AAA, but rarely can we perfectly recapitulate the human condition. With most of our understanding of the initial mediators of AAA coming from animal models we are limited from making the jump back to the human pathology. This work gives rise to important questions about the role of hydrogen peroxide in human AAA. We show that hydrogen peroxide goes up in the three most common mouse AAA models but more work must be done. We also generated a significant amount of data showing no effect of antioxidant therapies on hydrogen peroxide production. This is surprising but could have been caused by multiple different factors include dose, detection and route of administration.

c. <u>Future Directions</u>

This work opens the door for multiple future directions. The first is to characterize the $CaCl_2$ model more effectively. Part of this would be to run a full time course investigating peak hydrogen peroxide levels. Once peak hydrogen peroxide levels were determined one could attempt to correlate MMP activity to hydrogen peroxide levels by enzymography or vSMC apoptosis using a live/dead assay. Another future direction would be to investigate AAA formation in catalase overexpressing animals. All three models of AAA discussed here, $CaCl_2$, Elastase and Angiotensin II all show significant production of hydrogen peroxide. With elevated endogenous catalase you would expect that hydrogen peroxide levels would decrease and attenuate AAA formation.

In terms of the elastase model, treatment with enzymatic antioxidants such as catalase may be necessary to see a difference in AAA development. If the treatment with catalase was shown to attenuate AAA formation without significantly lowering hydrogen peroxide, this would suggest a role for ROS at a microenvironment level. This means that there may be small pockets, or pools, of hydrogen peroxide that cause large pathological phenomena in the aorta but cannot be readily detected via Amplex Red.

Since all of the work we do relates back to a human pathology it would be important to study the levels of hydrogen peroxide in human aneurysmal tissue. At the time of surgical repair a piece of aneurysmal tissue and non-aneurysmal adjacent tissue could be taken and subjected to Amplex Red. If our hypothesis is correct the results would show increases in hydrogen peroxide compared to the non-aneurysmal tissue. Another negative control for this experiment would be to collect non-aneurysmal tissue from autopsy samples.

This work demonstrates a role for hydrogen peroxide in the pathogenesis of AAA. We also demonstrate that MnTMPyP, MnTBAP and Ebselen fail to inhibit hydrogen peroxide in all three models of AAA. Based on previous studies, it is not surprising that hydrogen peroxide is elevated in all three models of AAA. We show that hydrogen peroxide production in AAA may be model specific because in the elastase model saline and elastase both caused significant increases in hydrogen peroxide production. Additional studies are needed to elucidate a causative mechanism for hydrogen peroxide in AAA.

References

1. Choke, E., et al., *A review of biological factors implicated in abdominal aortic aneurysm rupture.* Eur J Vasc Endovasc Surg, 2005. **30**(3): p. 227-44.
2. Urbonavicius, S., et al., *Potential circulating biomarkers for abdominal aortic aneurysm expansion and rupture--a systematic review.* Eur J Vasc Endovasc Surg, 2008. **36**(3): p. 273-80; discussion 281-2.
3. Silverberg, E., C.C. Boring, and T.S. Squires, *Cancer statistics, 1990.* CA Cancer J Clin, 1990. **40**(1): p. 9-26.
4. Wong, D.R., W.C. Willett, and E.B. Rimm, *Smoking, hypertension, alcohol consumption, and risk of abdominal aortic aneurysm in men.* Am J Epidemiol, 2007. **165**(7): p. 838-45.
5. Assar, A.N., *Pharmacological therapy for patients with abdominal aortic aneurysm.* Expert Rev Cardiovasc Ther, 2009. **7**(8): p. 999-1009.
6. Schermerhorn, M., *A 66-year-old man with an abdominal aortic aneurysm: review of screening and treatment.* JAMA, 2009. **302**(18): p. 2015-22. Copyright © 2009 American Medical Association. All Rights Reserved.
7. Weintraub, N.L., *Understanding abdominal aortic aneurysm.* N Engl J Med, 2009. **361**(11): p. 1114-6. Copyright (2009), with permission from New England Journal of Medicine.
8. Gabrielli, R., et al., *Our experience of popliteal vein aneurysm.* Interact Cardiovasc Thorac Surg, 2010.
9. Taylor, B.V. and P.G. Kalman, *Saccular aortic aneurysms.* Ann Vasc Surg, 1999. **13**(6): p. 555-9.
10. Johnsen, S.H., et al., *Atherosclerosis in abdominal aortic aneurysms: a causal event or a process running in parallel? The Tromso study.* Arterioscler Thromb Vasc Biol, 2010. **30**(6): p. 1263-8.
11. Golledge, J. and P.E. Norman, *Atherosclerosis and abdominal aortic aneurysm: cause, response, or common risk factors?* Arterioscler Thromb Vasc Biol, 2010. **30**(6): p. 1075-7.
12. Vardulaki, K.A., et al., *Quantifying the risks of hypertension, age, sex and smoking in patients with abdominal aortic aneurysm.* Br J Surg, 2000. **87**(2): p. 195-200.
13. Lederle, F.A., et al., *The aneurysm detection and management study screening program: validation cohort and final results. Aneurysm Detection and Management Veterans Affairs Cooperative Study Investigators.* Arch Intern Med, 2000. **160**(10): p. 1425-30.
14. Wilmink, T.B., et al., *The influence of screening on the incidence of ruptured abdominal aortic aneurysms.* J Vasc Surg, 1999. **30**(2): p. 203-8.
15. Hellenthal, F.A., et al., *Biomarkers of AAA progression. Part 1: extracellular matrix degeneration.* Nat Rev Cardiol, 2009. **6**(7): p. 464-74.
16. Sho, E., et al., *Continuous periaortic infusion improves doxycycline efficacy in experimental aortic aneurysms.* J Vasc Surg, 2004. **39**(6): p. 1312-21.
17. Aziz, F. and H. Kuivaniemi, *Role of matrix metalloproteinase inhibitors in preventing abdominal aortic aneurysm.* Ann Vasc Surg, 2007. **21**(3): p. 392-401.
18. Greenhalgh, R.M., et al., *Endovascular versus open repair of abdominal aortic aneurysm.* N Engl J Med, 2010. **362**(20): p. 1863-71.

19. Rizas, K.D., N. Ippagunta, and M.D. Tilson, 3rd, *Immune cells and molecular mediators in the pathogenesis of the abdominal aortic aneurysm.* Cardiol Rev, 2009. **17**(5): p. 201-10.
20. Reprinted from Ann N Y Acad Sci, **1085**, Thompson, R.W., et al., *Pathophysiology of abdominal aortic aneurysms: insights from the elastase-induced model in mice with different genetic backgrounds.*, 59-73, Copyright (2006), with permission from Jon Wiley and Sons.
21. Henderson, E.L., et al., *Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms.* Circulation, 1999. **99**(1): p. 96-104.
22. Kadoglou, N.P. and C.D. Liapis, *Matrix metalloproteinases: contribution to pathogenesis, diagnosis, surveillance and treatment of abdominal aortic aneurysms.* Curr Med Res Opin, 2004. **20**(4): p. 419-32.
23. Daugherty, A. and L.A. Cassis, *Mouse models of abdominal aortic aneurysms.* Arterioscler Thromb Vasc Biol, 2004. **24**(3): p. 429-34.
24. Saraff, K., et al., *Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2003. **23**(9): p. 1621-6.
25. Griendling, K.K. and G.A. FitzGerald, *Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS.* Circulation, 2003. **108**(16): p. 1912-6.
26. McCormick, M.L., D. Gavrila, and N.L. Weintraub, *Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms.* Arterioscler Thromb Vasc Biol, 2007. **27**(3): p. 461-9.
27. Brandes, R.P. and K. Schroder, *Composition and functions of vascular nicotinamide adenine dinucleotide phosphate oxidases.* Trends Cardiovasc Med, 2008. **18**(1): p. 15-9.
28. Li, P.F., R. Dietz, and R. von Harsdorf, *Reactive oxygen species induce apoptosis of vascular smooth muscle cell.* FEBS Lett, 1997. **404**(2-3): p. 249-52.
29. Sinha, I., et al., *Differential regulation of the superoxide dismutase family in experimental aortic aneurysms and rat aortic explants.* J Surg Res, 2007. **138**(2): p. 156-62.
30. Thomas, M., et al., *Deletion of p47phox attenuates angiotensin II-induced abdominal aortic aneurysm formation in apolipoprotein E-deficient mice.* Circulation, 2006. **114**(5): p. 404-13.
31. Gavazzi, G., et al., *NOX1 deficiency protects from aortic dissection in response to angiotensin II.* Hypertension, 2007. **50**(1): p. 189-96.
32. Xiong, W., et al., *Inhibition of reactive oxygen species attenuates aneurysm formation in a murine model.* Atherosclerosis, 2009. **202**(1): p. 128-34.
33. Gavrila, D., et al., *Vitamin E inhibits abdominal aortic aneurysm formation in angiotensin II-infused apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2005. **25**(8): p. 1671-7.
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35. Leopold, J.A. and J. Loscalzo, *Oxidative risk for atherothrombotic cardiovascular disease.* Free Radic Biol Med, 2009. **47**(12): p. 1673-706.
36. Cook-Mills, J.M., *Hydrogen peroxide activation of endothelial cell-associated MMPs during VCAM-1-dependent leukocyte migration.* Cell Mol Biol (Noisy-le-grand), 2006. **52**(4): p. 8-16.
37. Oliveira-Marques, V., et al., *Role of hydrogen peroxide in NF-kappaB activation: from inducer to modulator.* Antioxid Redox Signal, 2009. **11**(9): p. 2223-43.
38. Dikalov, S., K.K. Griendling, and D.G. Harrison, *Measurement of reactive oxygen species in cardiovascular studies.* Hypertension, 2007. **49**(4): p. 717-27.
39. Day, B.J., I. Fridovich, and J.D. Crapo, *Manganic porphyrins possess catalase activity and protect endothelial cells against hydrogen peroxide-mediated injury.* Arch Biochem Biophys, 1997. **347**(2): p. 256-62.
40. Zhao, Z., et al., *Angiotensin II induces afterdepolarizations via reactive oxygen species and calmodulin kinase II signaling.* J Mol Cell Cardiol, 2010.
41. Boddy, A.M., et al., *Basic research studies to understand aneurysm disease.* Drug News Perspect, 2008. **21**(3): p. 142-8. Drugs News Perspect 2008; 21(3): 142-148. Copyright © 2008 Prous Science, S.A.U. or its licensors. All rights reserved..
42. Laude, K., et al., *Hemodynamic and biochemical adaptations to vascular smooth muscle overexpression of p22phox in mice.* Am J Physiol Heart Circ Physiol, 2005. **288**(1): p. H7-12.
43. Griendling, K.K. and G.A. FitzGerald, *Oxidative stress and cardiovascular injury: Part II: animal and human studies.* Circulation, 2003. **108**(17): p. 2034-40.
44. Gertz, S.D., A. Kurgan, and D. Eisenberg, *Aneurysm of the rabbit common carotid artery induced by periarterial application of calcium chloride in vivo.* J Clin Invest, 1988. **81**(3): p. 649-56.
45. Chiou, A.C., B. Chiu, and W.H. Pearce, *Murine aortic aneurysm produced by periarterial application of calcium chloride.* J Surg Res, 2001. **99**(2): p. 371-6.
46. Trollope, A., et al., *Animal models of abdominal aortic aneurysm and their role in furthering management of human disease.* Cardiovasc Pathol, 2010.
47. Tojo, T., et al., *Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia.* Circulation, 2005. **111**(18): p. 2347-55.
48. Schewe, T., *Molecular actions of ebselen--an antiinflammatory antioxidant.* Gen Pharmacol, 1995. **26**(6): p. 1153-69.
49. Pyo, R., et al., *Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms.* J Clin Invest, 2000. **105**(11): p. 1641-9.
50. Yajima, N., et al., *Oxidative stress is involved in the development of experimental abdominal aortic aneurysm: a study of the transcription profile with complementary DNA microarray.* J Vasc Surg, 2002. **36**(2): p. 379-85.
51. Kalyanasundaram, A., et al., *Simvastatin suppresses experimental aortic aneurysm expansion.* J Vasc Surg, 2006. **43**(1): p. 117-24.
52. Satoh, K., et al., *Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms.* Nat Med, 2009. **15**(6): p. 649-56.

53. Birukov, K.G., *Cyclic stretch, reactive oxygen species, and vascular remodeling.* Antioxid Redox Signal, 2009. **11**(7): p. 1651-67.