2015

THE ROLE OF APOB-CONTAINING LIPOPROTEINS IN ABDOMINAL AORTIC ANEURYSM

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THE ROLE OF APOB-CONTAINING LIPOPROTEINS IN ABDOMINAL AORTIC ANEURYSM

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

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Lexington, Kentucky

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2015

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Abdominal aortic aneurysm (AAA) is a devastating disease that exhibits permanent lumen expansion typically in the infrarenal aorta. AAA is prevalent among aged population, especially in males. Despite the incidence in women is lower, studies indicate the tortuosity is more severe and aortic rupture risk is higher in women. In most patients, AAA remains asymptomatic until it ruptures leading to sudden and fatal hemorrhage.

To date, there is no proven medical therapy that can prevent the expansion or rupture. Human observational studies implicate the presence of AAA is associated with both high plasma low-density lipoprotein-cholesterol (HDL-C) and low plasma high-density lipoprotein-cholesterol (HDL-C) concentrations. To examine the role of specific lipoproteins in development of AAA, angiotensin (Ang) II-induced AAA was firstly determined in apolipoprotein AI deficient (apoAI -/-) mice in both C57BL/6 and LDL receptor deficient (LDL receptor -/-) backgrounds. The deletion of apoAI led to a significant decrease of HDL-C concentrations. However, we were unable to define any exacerbation of AngII-induced AAA in either normo- or hyperlipidemic mice with apoAI deficiency. Next we compared AngII-induced AAA formation using multiple mouse strains with dietary manipulation to generate different severities of hypercholesterolemia. We demonstrated the apolipoprotein B (apoB)-containing lipoproteins promoted the development of AngII-induced AAA. Moreover, ezetimibe administration significantly reduced both apoB-containing lipoproteins and AAA formation. Together, our studies demonstrate that elevated apoB-containing lipoproteins, contribute to the development of AngII-induced AAA.

To investigate the role of apoB-containing lipoproteins on established AAA, male LDL receptors -/- mice fed a Western diet were infused with AngII for 4 weeks to induced AAA. Then mice with AAA were stratified into either a group maintained on western diet or switched to a normal diet. AngII infusion was continued for an additional 8 weeks. The diet switch resulted in significantly reduced plasma cholesterol concentrations, which was attributable to the decrease of apoB-
containing lipoproteins. We found a profound inhibition of aneurysm progression in diet switched mice associated with attenuated macrophage accumulation and medial thickening. Collectively, our data demonstrate that apoB-containing lipoproteins promote the progression of established AAA.

KEYWORDS: Abdominal Aortic Aneurysm, Angiotensin II, ApoAl, ApoB-containing Lipoproteins, established aneurysm

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Student’s signature

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THE ROLE OF APOB-CONTAINING LIPOPROTEINS IN ABDOMINAL AORTIC ANEURYSM

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Acknowledgements

I would love to first express my appreciation to my mentor Dr. Alan Daugherty for giving me the opportunity to be a member of his laboratory and sincere help and guidance during my PhD study. He brought me into this field and encouraged me to think and work independently. His enthusiasm towards science has made him a fantastic mentor and an amazing role model for young scientists.

I would also love to thank all my committee members, Dr. Cassis, Dr. Smyth, Dr. van der Westhuyzen and Dr. Temel for their valuable discussion, suggestions and encouragement. These have been very helpful in my research progress.

I really appreciate the great help of our lab. Hong has shown her incredible patience and expertise with my work and always been there for support. I also thank Jess, Anju, Deborah and Deb for training me on performing important techniques in our lab and providing me technical support. I would like to express my appreciation to all present lab members who consistently help me in work. I wish to thank the previous lab for their contribution to my work.

I would love to thank our wonderful collaborator: Dr. Temel's lab for the help on lipoprotein isolation; Dr. Cassis's lab for analyzing renin concentrations; Merk company for providing ezetimibe and suggestion from Dr. Harry Davis; and Dr. Sorci-Thomas lab for providing ApoAI deficient mice.

I am also truly thankful for all my friends for their sincere friendship and support.

Finally, I would also like to thank my whole family, especially my parents, my baby girl Lexie and my husband Jing Wu. I could not go through all the difficulties without your support and encouragement. Your great love makes everything meaningful.
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Chapter one: Introduction

This chapter is based on a review published in “Curr Pharm Des. 2015;21(28):4035-48” with Jing Liu as the first author.

1.1 Human Abdominal Aortic Aneurysm

1.1.1 Epidemiology of Human AAA

Human abdominal aortic aneurysm (AAA) is the most common aneurysm of large arteries and it constitutes more than 60% of all cases. It most commonly occurs in the infrarenal aorta. The abdominal aorta is considered aneurysmal if it is at least 1.5 times the normal aortic diameter (1). The overall prevalence of AAA in developed countries is approximately 5%. The disease is more common in males, with a 4 times greater incidence than in women; while women afflicted with AAA have more severe tortuosity and a worse prognosis. During the 20th century, many developed countries have reported a consistent increase of incidence and mortality associated with AAA (2-6). Aortic aneurysms contribute to the 13th leading cause of death in the United States due to the fatal rupture. AAA mortality is greatest among those previously undiagnosed who present with rupture. These individuals have up to 90% mortality rate if rupture occurs outside the hospital (7-11). Even those undergoing surgical repair, only 50% of patients will survive beyond 30 days (12).

Given the adverse epidemiological trends, attempts have been made to improve the incidence of AAA in the past two decades. Recently, systematic evaluation of the global epidemiology of AAA has shown the decline of the disease burden. The age-specific prevalence rate per 100,000 ranged from 8.43 (40-44 years age group) to 2,422.53 (75-95 year age group) in 1990; while the corresponding range in 2010 was 7.88 to 2,274.82. Although the incidence and prevalence were higher in developed countries than developing countries, the rate within each stratum has decreased in the past two decades. Of note, the regional assessment reveals certain countries had a most appreciable improvement while others had increased incidence and
prevalence, which highlights the need for further surveillance efforts and intervention in these countries (13).

1.1.2 Risk Factors for Human AAAs

1.1.2.1 Risk factors associated with AAA development

Most patients with AAA are absent of clinical symptoms until AAA rupture that leads to sudden and fatal hemorrhage. The evaluation of risk factors is helpful in determining the risk of developing AAA, facilitating the early diagnosis and improving the prognosis. The identified risk factors include advanced age, male gender, smoking, family history and dyslipidemia. A number of factors such as coronary artery disease, atherosclerosis, hypertension, and ethnicity have also been identified as predisposing factors for AAA development.

**Age** Mean aortic diameter increases with age in both men and women (14). Clinically relevant aneurysms (at least 4 cm in diameter) are found in about 1 percent of men 55 to 64 years of age, and the prevalence increases by 2 to 4 percent per decade thereafter (13-16).

**Gender** Although women have a higher risk of rupture and worse prognosis, the prevalence of AAA is 4 times higher in men. Women less than 55 years of age rarely present with aneurysms. In addition, the age-related increase in men is more pronounced than that in women.

**Smoking** Smoking is one of the strongest risk factors that is independently associated with presence of human AAA. Current smokers were 7.6 times more likely to have an AAA while ex-smokers were 3 times more likely to have an AAA compared with the non-smokers (17). The association is closely related to the number of years of smoking and inversely related with the years of smoking cessation (18). The risk of AAA increases by 4% each year in smokers, while smoking cessation slowly decline in the risk of the AAA occurrence, approximately 1/30 of the original risk per year of smoking cessation (15, 17).
**Family history** Family history is another potential risk factor that has significantly increases the prevalence of AAA. The risk of AAA may increase 4 fold in a patient with a first-degree relative undergoing surgical intervention for an AAA (19).

**1.1.2.2 Risk factor associated with AAA rupture**

Rupture of AAA is a devastating event that occurs during progression of the disease. Many patients succumb pre or post-surgical repair, and many may not even reach the hospital. Only a few patients will survive after the surgery repair. The likelihood that an aneurysm will rupture is profoundly influenced by various factors including aneurysm size, expansion rate, current smoking status and gender (20). Other risk factors such as uncontrolled hypertension, shape, and intramural thrombus formation have also been reported which cause mechanical stress and hypoxia. These effects significantly increase the burden of the weakened AAA wall (21).

**Aneurysm size** Aneurysm size is the most robust predictor of the likelihood of rupture. The risk of rupture increases exponentially with elevated aneurysm diameter. Based on studies of the natural history of aneurysms prior to the elective repair, the estimated annual rupture risk according to AAA diameter is listed below (22, 23):

- <4.0 in diameter - 0%
- 4.0 cm-4.9 cm in diameter - 0.5-5%
- 5.0 cm-5.9 cm in diameter - 3-15%
- 6.0 cm-6.9 cm in diameter - 10-20%
- 7.0 cm–7.9 cm in diameter - 20-40%
- ≥8.0 cm in diameter - 30-50%

Importantly, the initial size of the aneurysms when first detected has been shown as an independent risk factor for rupture. In the UK Small Aneurysm
Trial, patients that had AAA rupture had larger mean initial abdominal aortic diameters compared to ones without rupture. The baseline diameter of AAA has also been associated with the expansion rate of AAA, which is an independent risk factor for AAA rupture (24). Every 10 mm larger diameter increment detected at baseline led to an additional 1.29 mm expansion.

**Expansion rate** Expansion rate is an important determinant in monitoring the risk of rupture (25, 26). Evidence indicates aneurysms with 0.5 cm expansion or more over a six month follow-up are more prone to rupture (27). Moreover, patients with ruptured AAA had over double the rate of expansion compared with individuals with non-ruptured AAA (28).

**Smoking** Smokers are at a great risk of a large spectrum of cardiovascular diseases. It has been consistently reported that smoking is not only a risk factor for the initiation of AAA, but also significantly contributes to the aneurysm expansion and rupture (21, 29). Current smoking has been shown to alter other independent risk factors that are associated with AAA rupture such as higher mean blood pressure and poor lung function (30, 31). Of note, only one manuscript reported that current smokers had a borderline significant increase of AAA rupture, which may indicate the inaccuracy of self-reporting of smoking status (20). Only after plasma cotinine (a long-lived metabolite of nicotine) was measured and used as the index of smoking, was the significant contribution of current smoking detected.

**Sex** Despite the prevalence of AAA being 4 times higher in men, evidence clearly indicates a 3 to 4-fold increased risk of rupture in women. The mean AAA diameter predisposed to rupture in women is 5 cm while the corresponding size in men is 6 cm (20, 32, 33). One possible explanation for the higher risk of rupture may be the smaller diameter of the normal aorta in women; male aortas are larger than their female counterparts. Currently, patients identified with small aneurysm (3.0-5.4 cm) are under regular
ultrasound surveillance. Those reaching the selected threshold (5.5 cm) are referred for aneurysm repair by open surgery or endovascular repair (33). Given the gender-specific baseline of aortic diameter and more compliant aortas in women, a lower intervention threshold for women has been suggested instead of this rigid criteria (22). The adjustment of the AAA diameter for body surface area and the ratio of infrarenal/suprarenal diameter also have been shown to be a better predictor of AAA rupture risk in women (34, 35).

1.1.3 Management of Human AAAs
The rupture of the asymptomatic AAA is the major consideration for this disease. Only 10 to 15 percent of patients survive a ruptured AAA. Of the patients reaching the hospital alive, only half will survive the emergency surgical repair (20). Although a few patients with AAA die from ruptured aneurysm, other cardiovascular diseases threaten their lives (36). The management options for AAA patients include screening and surveillance, risk factor reduction, medical therapy, surgery and endovascular stenting.

The one-time ultrasound screening has been recommended for all men over the age of 65, or 55 for those with a family history of AAA. Women over the age of 65 who have smoked, or have a family history should also have ultrasound screening. If a AAA is detected during screening, follow-up intervals are recommended: 5 years for patients with an AAA diameter between 2.6-2.9 cm; 3 years for patients with an AAA of 3.0-3.4 cm; 12 months for patients with an AAA diameter of 3.5-4.4 cm; and 6 months for patients with an AAA diameter of 4.4-5.4 cm (36).

Smoking has been shown consistently as the major contributor to aneurysm growth and rupture. Smoking increases the growth rate by 20 to 25 percent, which accelerates the risk of rupture for AAA patients (17, 24, 31, 37, 38). Data from 1743 patients demonstrated a more profound expansion (mean of 0.29 cm per year) in current smokers than in former smokers (mean of 0.25 cm per year) (15). The risk of rupture and rupture-related death are higher in current
smokers than former and non-smokers (20, 39). Therefore, the ACC/AHA guidelines recommend that smoking cessation should be advocated to all individuals with AAA or a positive family history of AAA (27).

Patients with AAA have markedly increased cardiovascular disease risks, which in turn worsen prognosis of this disease. During the surveillance period, the therapeutic goal is to prevent aneurysm from reaching a size with a high risk of rupture. Patients are encouraged to seek appropriate management for other risk factors such as hypertension, hypercholesterolemia, diabetes and atherosclerosis. Insufficient evidence supports the protective effects of doxycycline or roxithromycin in reducing AAA growth (36). Statins and angiotensin-converting enzyme (ACE) inhibitors are recommended given their broad protective benefits. Although animal studies have suggested beta-blockers protect against aneurysm expansion and rupture, three clinical trails that examine the effects of propranolol on AAA growth failed to detect significant benefits on the progression of AAA (40). Of note, two cohort studies and meta-analysis suggested statins, the lipid-lowering drug, were beneficial to reducing AAA growth.

The options for repair include surgical (transabdominal route or retroperitoneal route) and endovascular repair. Clinical trials indicated preventive endovascular or open surgical repair at early stage had no survival benefits compared to conservative treatment in combination with regular ultrasound, but associated with higher post-operative mortality (41). Based on these data, AAA repair is only recommended when the maximal diameter equals or exceeds 5.5 cm or when the growth rate of aneurysm is greater than 0.5 cm over 6 months. Endovascular repair is less invasive, associated with higher technical success rate (42-44) and improved mortality rate compared to elective surgical repair (45). However, studies failed to demonstrate long-term benefit of the endovascular approach versus surgical repair at the end of one or two years (46).
1.2 Overview of Animal Models of AAAs

1.2.1 Genetically induced AAA

**Blotchy** The blotchy mouse with alleles at the Mottled locus of the X chromosome have abnormal intestinal copper absorption that leads to a number of effects including a defect in the cross-linking of collagen and elastin. Elastin breakdown begins at an early age and progresses rapidly. Changes observed include replacement of elastin by fibroblasts and ground substance. Advanced lesions are characterized by infiltrates of inflammatory cells, hemorrhages and eventual ruptures of the aortic wall. As the disease progresses, observations include elastic fiber vacuolation and fragmentation, increased ground substance, fibroplasias and inflammatory cells infiltrates.

**THM** (Transgenic mice overexpressing renin and AngII) The Tsukuba hypertensive mouse was produced by cross-breeding strains that expressed transgenes of human renin and angiotensinogen. The double transgenic mice have modest elevated blood pressure. There are no overt vascular pathologies in the transgenic mice until they are provided 1 % sodium chloride in drinking water. Then there is a dramatic increase in death during the first 10 days of salt administration caused by aortic ruptures in both thoracic and abdominal regions. The increased incidence of rupture is not associated with changes in blood pressure.

**Lox deficiency** Lox deficient mice have an inability to crosslink both elastin and collagen. These mice develop to full term but are not viable because of aortic rupture.

1.2.2 Chemically induced AAA

**Elastase-induced AAA** To induce AAA by elastase, a catheter is introduced at the iliac bifurcation. Porcine pancreatic elastase is infused into the lumen and incubated within the infrarenal aortic segment for 5 minutes. Aortic wall diameter and structure remain stable for up to 7 days, after which a rapid and
significant increase occurs. By day 14, dilation is visible with extensive disruption of medial layers and profound inflammatory cell accumulation in the adventitial region. The AAA is defined by an increase in diameter of at least 100% greater than the pre-perfused aorta.

**Calcium chloride-induced AAA** To induce AAA by CaCl₂, the infrarenal abdominal aorta is exposed and gauze soaked in CaCl₂ is directly applied to the adventitia of this region for a period of time and then removed. CaCl₂-induced AAA formation has been reported to form between 2-12 weeks after application in rodents, dependent on mouse strains, exposure time and concentrations of CaCl₂. Histological examination of the aorta reveals disruption of the elastic network within the media by calcium precipitation. Peri-aortic application also induces inflammatory cell infiltration, including macrophages, neutrophils and lymphocytes to the aortic media and adventitia.

**1.2.3 AngII-induced AAA**
The AngII induced AAA model is currently the most commonly used animal model. AngII (1,000 ng/kg/min) is infused subcutaneously via mini-pumps for 28 days. The initial studies were performed in hypercholesterolemic mice that were either LDL receptor -/- mice fed a western diet or apoE -/- mice fed either normal or a western diet. Subsequent studies have also demonstrated AngII infusion also could induce AAA formation in normolipidemic mice, however at much lower incidence than hypercholesterolemic mice. AngII-induced AAA formation is independent of the modestly increased systolic blood pressure during AngII infusion. AAA induced by chronic AngII infusion is exclusively located at the suprarenal aortic region. It exhibits progressive luminal expansion, macrophage accumulation throughout the aneurismal tissues, elastin fiber disruption accompanied by leukocytes infiltration, disorganized extracellular matrix deposition and aortic wall remodeling.

**1.2.4 Novel Mouse Model of AAA**
Recently, new mouse models of AAAs have been reported. (47-49) Significant calcification deposits have been found in CaCl₂-induced AAA mouse model with calcium phosphate (CaPO₄) as the major component. The sequential application of CaCl₂ and phosphate buffered saline (PBS) to the adventitial surface facilitate the CaPO₄ crystals deposition in aortas which is associated with fragmentation of elastin layer and enhancement of apoptosis in the medium. This AAA model creates rapid and robust aneurysmal dilation and displays similar pathological and histological characteristics as CaCl₂ but at a higher magnitude. (47)

Another simplified but highly reproducible and less challenging AAA mouse model is peri-adventitial elastase model. The aorta is exposed and bathed in elastase for 10 minutes. Early dilation is observed and ensures adequate exposure to elastase. At day 14, significant expansion of the aorta is found in the area that was exposed to elastase and it is associated with degradation of elastin fibers, accumulation of macrophage (48).

AngII-dependent TGF-β activity has been extensively investigated in experimental Marfan syndrome while its role in the AAA has not been elucidated. A newly developed AAA mouse model demonstrated the neutralization of TGF-β made disease resistant C57BL/6 mice susceptible to AngII-induced AAA formation. This mouse model indicates the protective role of TGF-β activity during development of AAA.

1.3 Characteristics of AngII-induced AAAs
There is evidence that the renin angiotensin system contributes to experimental AAAs. Inhibition of angiotensin-converting enzyme (ACE) or AT1 receptors reduces elastase-induced AAAs (50-53) or decellularise aortic xenograft-induced AAAs (54) in rats and multiple mouse models (55). These findings highlight effects of the endogenous activation of AngII in AAA pathogenesis.
The most direct evidence from animal models is that AngII infusion leads to AAAs and aortic rupture in mice (56, 57), and conversely, AngII type 1 (AT1) receptor inhibition diminishes AngII-induced AAAs (58-60). In the AngII-induced AAA mouse model, constant AngII delivery was achieved by implanting mini osmotic pumps into the subcutaneous area of the flank. A range of AngII infusion rates, from 500 - 2,500 ng/kg/min, have been used to induce AAAs (56, 57, 61, 62). However, the most frequently used AngII infusion rate in hypercholesterolemic mice is 1,000 ng/kg/min (or 1.44 mg/kg/day) for a duration of 28 days (or 4 weeks) (57, 63), while a prolonged infusion for 84 days (or 12 weeks) were also reported (64-66). Since the initial report, this mouse model has been reproduced by more than 40 laboratories. One significant feature of AngII-induced AAAs in mice is its location in the suprarenal aortic region. This location was also noted in other chemically induced experimental AAAs such as hypercholesterolemia and aldosterone (7, 67, 68), and some genetically manipulated mouse models (69). This differs from the infrarenal location that is most common in humans. The mechanistic basis for the location of AAAs is unclear in both humans and mice. Although it is unknown why AAAs locate to the suprarenal aortic region in mice have not been defined, the potential hemodynamic differences between mice and humans may be attributed to the different locations since humans are biped, whereas mice are quadruped.

AngII-induced AAAs recapitulate many pathological features of human AAAs including elastin breaks, extracellular matrix degradation, inflammatory cell accumulation and aortic rupture. One apparent pathological difference is that intraluminal thrombus is present in human AAAs, whereas intramural hemorrhage has been observed in AngII-induced AAAs in mice (70, 71).

1.3.1 Pathological Features of AngII-induced AAAs

Human AAAs are heterogeneous both within a single aneurysmal tissue and at different stages during its development. Consistent with human AAAs, AngII-induced AAAs are highly heterogeneous, thereby providing an important tool to
understand potential processes from the initiation to advanced stages of AAAs (72). In the past decade, understanding of the complex pathologies of AngII-induced AAAs in mice has provided insights into defining the complex mechanisms of AAAs in humans.

There are a scant number of macrophages in the adventitia and the presence of this cell type is very rare in the medial layers of a normal aorta in mice. As early as 48 hours after starting infusion of AngII, profound accumulation of macrophages in both adventitial and medial layers are detected that are frequently accompanied by elastin fiber breaks (64). At the same time, diminished vessel wall compliance and significant luminal dilation are also observed by ultrasonography (73). The rapid expansion phase of AAA progression occurs within 10 days (64, 73). Another significant feature in this period is frequent aortic rupture (approximately 10 - 30%) due to transmural medial rupture that fails to constrain blood within the adventitia. If bleeding is constrained by the adventitia, thrombi form surrounding the ruptured media. In addition to pronounced accumulation of red blood cells, accumulation of macrophages at the site of medial rupture and the surrounding adventitia is also profound. Thrombi have largely resolved after 28 days of AngII infusion, accompanied by remarkable remodeling that is characterized by continuous leukocyte infiltration, disorganized collagen deposition, and formation of neomicrovessels (64, 65). Both T and B lymphocytes are also detected in aneurysmal tissues (57, 64).

Continuous AngII infusion beyond the initial rapid lumen expansion that occurs in the initial 10 days leads to slowly progressive luminal expansion as demonstrated by both real-time in vivo measurements using ultrasonography and ex vivo measurement after termination (64-66, 73). After infusion of AngII for 84 days, thrombi have usually resolved completely, while remodeling of extracellular matrices, expanded lumen, and thinner aortic walls are more pronounced. Aortic rupture rate is also increased at more advanced stages.
(>20% after 28 days of infusion as noticed in our recent study) (65). Also similar as reported in humans, atherosclerotic lesions are frequently detected in hypercholesterolemic mice with prolonged AngII infusion. Of note, adventitial thickening with normal or even smaller luminal diameter both proximally and distally around intact media is apparent. The heterogeneity of AngII-induced AAAs requires careful consideration in selecting tissue sections to provide insights into mechanisms.

1.3.2 Associations of AngII-induced AAAs with Risk Factors Identified in Humans

1.3.2.1 Gender
In agreement with the male gender preference in humans, the incidence of AngII-induced AAAs is 4-fold higher in male mice (74-78). It appears that androgen augments AngII-induced AAAs, whereas effects of estrogen in protection of AngII-induced AAAs remain to be clarified (74-77, 79). Castration of male apolipoprotein E (apoE) -/- mice to remove endogenous androgen resulted in reductions of AngII-induced AAAs that was comparable to age-matched AngII-infused female apoE -/- mice (74, 75). Particularly interesting, adult females had high incidence of AngII-induced AAAs that was equivalent to male mice if they were administered testosterone on the first day after birth (77). Conversely, exogenous estrogen administration to AngII-infused male apoE -/- mice reduced incidence of AngII-induced AAAs (79). However, diminished endogenous estrogen by ovariectomy in female AngII-infused apoE -/- mice did not augment AAAs (74). Although manipulations of sex hormones in AngII-infused mice provide insights into understanding effects of sex hormones on AAA development, molecular mechanisms that contribute to this effect are unclear.

1.3.2.2 Aging
Since one initial study used apoE -/- mice at the age of 6 months (57), many of the published studies also used this mouse model at similar ages. Infusion of AngII at 1,000 ng/kg/min for 28 days in male apoE -/- mice at the age of 6
months or older led to AAA formation at 80 - 100%. In later experiments, investigators reported that AngII-induced AAA incidence was also above 80% in apoE -/- mice at the age of 2 - 3 months (80, 81). Therefore, currently there is no literature evidence that aging is a risk factor for AngII-induced AAAs.

1.3.2.3 Smoking
Smoking is associated with the presence of AAAs in humans (82). Epidemiological studies have demonstrated consistently that smoking is a positive risk factor for AAAs in humans (83-87). Molecular mechanisms by which smoking augments AAAs were explored using the AngII-infused mouse model. In one study, apoE -/- mice were exposed to cigarette mainstream smoking that mimics the human heavy smoker status (88). Mice were infused with AngII 1000 ng/kg/min for 4 weeks, and then separated into two groups to receive AngII 500 ng/kg/min and either control (no smoking) or cigarette mainstream smoking for another 4 weeks. Therefore, contribution of smoking to progression, not the initiation of AAAs, was studied. At the endpoint, AAA incidence was not significantly different between mice with and without smoking exposure (88). This study did not report aortic rupture rate or compare maximal aortic diameters between mice not or exposed to smoking. A later experiment used a subcutaneous pellet to release nicotine, a putative major toxin in cigarettes (89), and found that it augmented AngII-induced AAAs via regulating microRNA (miR)-21 that act as a key modulator of proliferation and apoptosis of vascular smooth muscle cells. A single study also reported that subcutaneous infusion of nicotine alone induced AAAs in apoE -/- mice that had comparable pathological changes as AngII-induced AAAs, although the incidence is much lower (90). In addition to nicotine, 3,4-benzopyrene, another compound in cigarette smoke, was found to augment AngII-induced AAAs in C57BL/6 mice (91). It is unclear why single components in cigarette smoke augmented AngII-induced AAAs, while entire components did not have this augmentation effect. In the initial study using cigarette mainstream smoking (88), there are two potential differences from the other studies. First, this experiment determined whether smoking augmented the progression, not the formation, of AngII-
induced AAAs. Second, it is unclear whether AAA incidence was different when mice were grouped to receive cigarette smoking, or not, since no ultrasononography was used to stratify mice. Further studies are necessary to both replicate these experiments and explore potential discrepancies on smoking effects between human AAAs and AngII-induced AAAs in mice.

1.3.2.4 Obesity

The literature has been inconsistent regarding associations between AAAs and obesity in humans, with some showing potential associations (92-96), and some failing to define significant associations (97, 98). Adipokines, including adiponectin and leptin, regulate functions of adipose tissues, and impairment of adipokines results in dysfunction of adipose tissues in obese individuals. Adiponectin, an anti-inflammatory adipokine, is solely derived from adipocytes. In apoE -/- mice infused with AngII 1,000 ng/kg/min, deficiency of adiponectin augmented AngII-induced AAAs without affecting body weight (99). Different from adiponectin, leptin is closely associated with body weight change. Leptin deficiency leads to profound body weight gain in mice (100). Leptin deficiency augmented AngII-induced AAAs in C57BL/6 mice (101). However, it appears that this is not a leptin-specific effect since diet-induced obesity also augmented AngII-induced AAAs in C57BL/6 mice that was comparable to AAAs in leptin-deficient mice (101, 102). In contrast to the effects of whole body deficiency of leptin, local application of leptin surrounding the abdominal aorta augmented AngII-induced AAAs (103). These findings implicate that leptin plays differential roles in AngII-induced AAAs locally versus systematically. Therefore, similar to the confusing associations in human AAAs, contributions of obesity as well as its related inflammatory changes to AAAs require systematic determination in future studies.

1.3.2.5 Dyslipidemia

In several small observational studies in humans, AAAs showed positive associations with plasma LDL-cholesterol concentrations and negative associations with plasma high-density lipoprotein (HDL)-cholesterol
concentrations (104, 105), whereas other studies did not find associations of AAAs with either LDL- or HDL-cholesterol concentrations (106-109). Although there are no established associations between dyslipidemia and AAAs, administration of statins, a commonly used class of drugs to reduce plasma LDL cholesterol, to patients with AAAs reduced aortic expansion rate in observational studies (110-112). Therefore, the literature supports potential contributions of dyslipidemia to AAA development in humans.

Infusion of AngII to promote AAAs was first reported in LDL receptor -/- mice fed a diet enriched with both saturated fat and cholate (56). The infusion rate of AngII was 500 ng/kg/min. Subsequently, it was found that infusion of AngII into apoE -/- mice fed a normal laboratory diet also led to AAAs (57). Apolipoprotein (apo)B-containing lipoproteins are the predominant lipoprotein in these hypercholesterolemic mouse models. However, later studies have demonstrated that AngII-induced AAAs also occur in normocholesterolemic mice (113-116). Although there are no side-by-side comparisons, it appears that the incidence is approximately 5-fold or higher in hypercholesterolemic mice than in normocholesterolemic mice (113-116). One speculation regarding high incidence of AngII-induced AAAs in hypercholesterolemic mice is that atherosclerosis is associated with augmentation of AAAs, since the association between atherosclerosis and AAAs were also implicated in human observational studies. However, multiple genetic mouse models have provided evidence about the dissociation between atherosclerosis and AngII-induced AAAs (61, 78, 117).

Although deficiency of apoE promotes AngII-induced AAAs in mice, associations between genotypes of apoE and AAAs in humans are uncertain. In one study that screened apoE genotypes in 57 men with small AAAs, patients with E3E4 genotype had less aortic expansion, compared to patients with E3E3 genotype during an interval of 2-4.5 years (118). However, a study having investigated apoE genotypes in 640 men with small AAAs failed to
detect associations between apoE genotypes and growth of AAAs in a 4-year follow-up (119). These two studies did not compare plasma cholesterol concentrations in patients with different apoE genotypes.

Statins do not change plasma cholesterol concentrations in mice. One study reported that simvastatin reduced AngII-induced AAAs in apoE-/- mice (120) fed a saturated fat-enriched diet without changing plasma cholesterol concentrations. In contrast, other studies found that simvastatin, rosuvastatin, or atorvastatin had no significant effects on AAAs in AngII-infused apoE -/- mice or LDL receptor -/- mice (121-123).

Fenofibrate is used to reduce plasma triglycerides concentrations in humans. One study reported that fenofibrate reduced AngII-induced AAAs in apoE -/- mice (122). In addition to associations of AngII-induced AAAs with hypercholesterolemia, a recent study has provided evidence that administration of native or reconstitutive HDL diminishes AngII-induced AAAs in apoE -/- mice (124). This study infers a potentially important role of this apoAI-containing lipoprotein in development of AngII-induced AAAs.

The current literature implicates potential and complex associations between dyslipidemia and AngII-induced AAAs. It will be important to explore roles of both apoAI- and apoB-containing lipoproteins using appropriate genetic and pharmacological approaches in AngII-infused mice.

1.3.3 Potential Protective Factor for AngII induced AAAs

1.3.3.1 Diabetes

Diabetes contributes to augmentation of atherosclerosis. In contrast, several studies have described diabetes being negatively associated with the presence of human AAAs (125). The mechanisms of reduced AAAs in diabetes have not been determined. There is only one study that has investigated the contribution of diabetes to AngII-induced AAAs (126). ApoE -/- mice were injected with streptozotocin intraperitoneally to induce hyperglycemia. Subsequently, AngII
(1,000 ng/kg/min) was infused for 28 days. There was a modest reduction at day 14 and 21 of AngII-induced aortic dilation in mice with hyperglycemia, as determined by ultrasonography (126). Mechanisms by which hyperglycemia reduced aortic dilation have not been explored.

### 1.3.3.2 Irradiation

Irradiation suppresses immune response, and has been demonstrated to attenuate inflammation in humans (127). Studies by Gavish et al. have provided evidence that irradiation diminishes AngII-induced AAAs in apoE -/- mice that were associated with enhanced extracellular matrix (ECM) reinforcement and modification in response to the inflammatory process (128, 129).

### 1.3.4 Genetic and Pharmacological Manipulations of AngII-induced AAAs

The renin angiotensin system contributes to development and progression of AAAs in mice. Using AngII-induced AAA mouse model, it has been defined that excessive AngII leads to activation of AngII type 1 (AT1) receptor. In mice, AT1 receptor has two subtypes, AT1a and AT1b. There have been consistent demonstrations that AT1 receptor antagonism, using pharmacological approaches, reduces AngII-induced AAAs (58, 60, 130). This is attributed to interactions between AngII and AT1a receptor in mice because whole body deficiency of AT1a receptor ablates the development of AngII-induced AAAs (131), whereas AT1b receptor (132) or angiotensin II type 2 (AT2) receptor (133) deficiency had no effects on AngII-induced AAAs. Studies of mice that had AT1a receptor depletion on leukocytes, endothelial cells, or smooth muscle cells did not define a single cell type that played a critical role in AngII-induced AAA development (131, 134), implicating orchestrated contributions of multiple cell types in the interaction of AngII and AT1a receptor to promote AAAs.

AngII interacts with AT1a receptors to promote AngII-induced AAAs. As reviewed by Mehta and Griendling (135), AngII binding to AT1a receptors activates a broad spectrum of downstream cascades that influence cardiovascular pathophysiology. Many of these downstream signalings have
been involved in molecular mechanisms of AAAs, such as activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate reactive oxygen species (ROS) or mitogen-activated protein kinase (MAPK) signaling pathway including extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) (55).

In addition to understanding the renin angiotensin system related mechanisms, AngII-induced AAAs have been used to explore molecular mechanisms by multiple genetic or pharmacological manipulations and seek for potential non-invasive therapeutic strategies. Microarray analysis discovered that a complex network profiling is involved in AngII-induced AAAs. This section focuses on mechanistic explorations including inflammation, disrupted homeostasis of extracellular matrix related factors, vascular oxidative stress and newly emerged mechanisms such as microRNA-mediated changes.

1.3.4.1 Inflammation Related Factors

1.3.4.1.1 Macrophage and Related Inflammatory Components
Many inflammatory cell types have been identified in AAA tissues, and macrophages are the largest subpopulation of leukocytes detected through all stages of AAAs (70). In mice infused with AngII, macrophage accumulation in both medial layers and the adventitia is observed within 48 hours after initiating AngII infusion, and is persistent from the initial stage through advanced stages (64, 65, 102, 103). The spleen is proposed to act as a reservoir for mobilizing monocytes/macrophages into the circulation in response to AngII stimulation. Impaired mobilization and accumulation of monocytes protected against AngII-induced AAA development (136). Infiltration of macrophages from the peripheral blood into the aortic wall requires passing through the endothelial barrier. CD31, an immunoreceptor that is abundant on endothelial cells, plays a critical role in monocyte-endothelial adhesion. Inhibition of CD31 by administering a murine CD31-derived peptide profoundly reduced incidence of AngII-induced AAAs in apoE -/- mice, which suppressed macrophage activation
(137). CCR2 and monocyte chemoattractant protein-1 (MCP-1) interactions are important for monocyte chemotaxis and many macrophage-mediated inflammatory responses. Deficiency of CCR2 globally, or in bone marrow-derived cells in mice, diminished AngII-induced AAAs (55). Infusion of everolimus, a rapamycin inhibitor, suppressed CCR2-expressing monocytes, and reduced AngII-induced AAAs in apoE -/- mice (138). While no studies have been performed using the available MCP-1 deficient mice, inhibition of whole body MCP-1 signaling through plasmid delivery of a MCP-1 dominant negative form did not reduce AngII-induced AAAs (139). MKEY is a mouse CCL5-based synthetic cyclic peptide that inhibits the proinflammatory interaction between CCL5 and CXCR4 thereby attenuating monocyte recruitment (140). Administration of MKEY only reduced maximal aortic diameter on day 3 during AngII infusion as measured by ultrasound (60, 141), but had no effects on incidence of AngII-induced AAAs at the endpoint in apoE -/- mice (60), implicating rapid and transient effects of CCL5-CXCR4 interaction in AngII-induced AAAs.

Macrophage accumulation is accompanied by activation of many inflammatory factors that are involved in immune responses. Myeloid differentiation factor 88 (MyD88) is an important adaptor protein that regulates macrophage functions in innate immunity. Genetic deficiency of this protein globally or in bone marrow-derived leukocytes reduced AngII-induced AAAs in both LDL receptor -/- and apoE -/- mice (81), CD14 is a glycoposphatidylinositol-linked surface protein pattern recognition receptor, acting via MyD88 and other proteins to activate the innate immune system. Comparable to MyD88 deficiency, deficiency of CD14 diminished aortic macrophage infiltration and AngII-induced AAA formation in apoE -/- mice (142). Telomerase reverse transcriptase (TERT) is the enzyme that stabilizes telomeres, and is abundant in macrophages. TERT deficiency in macrophages attenuated AngII-induced AAAs in mice (143). Syndecan-1 is a cell surface heparan sulfate proteoglycan that modulates proinflammatory and proteolytic processes within the vascular wall. Deficiency
of syndecan-1 led to profound increases of AAA incidence and rupture rate in apoE -/- mice infused with AngII at 500 ng/kg/min for 2 weeks (144). Notch-1 encodes a single-pass transmembrane receptor. Notch-1 haploinsufficiency or pharmacological inhibition of Notch-1 by DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) reduced AngII-induced AAAs in apoE -/- mice (145). Notch-1 haploinsufficiency in bone marrow-derived cells reduced AngII-induced AAAs (145). Dibenzazepine, a Notch γ-secretase inhibitor, also reduced AngII-induced AAAs in apoE -/- mice (146).

Transforming growth factor (TGF)-β is a critical cytokine in many inflammatory processes that is secreted from many cells types. Cyclosporine A has strong anti TGF-β effects. In an AAA patient treated with cyclosporine A, greatly accelerated progression of aortic expansion was noted (147), implicating potential detrimental effects of TGF-β inhibition on the progression of AAAs. Consistent with this clinical case report, systemic blockade of TGF-β activity augmented AngII-induced AAAs in mice and was associated with enhanced vascular inflammation (49). Depletion of monocytes in circulation by clodronate-containing liposomes in mice administered AngII and TGF-β neutralizing antibody attenuated AAA formation and aortic rupture (49), implicating important roles of monocytes in AAAs induced by AngII infusion with simultaneously inhibiting TGF-β.

These studies, using genetic and/or pharmacological manipulations, demonstrate that both the presence and function of macrophages are critical in the development of AngII-induced AAAs; however, it is unclear whether inhibition of macrophage numbers or specifically targeting certain components to inhibit macrophage functions would prevent or improve AAAs in humans.

1.3.4.1.2 Lymphocytes and Related Inflammatory Components
Lymphocytes are present in human AAAs (148). Both T and B lymphocytes are also detected in AngII-induced AAAs (57, 64). Recombination-activating gene (Rag-1 and -2) proteins are crucial for immunoglobulin and T-cell receptor gene
recombination. Genetic deletion of either Rag in mice leads to deficiency of mature T and B lymphocytes. Despite striking effects of Rag-1 deficiency on lymphocyte functions, it did not influence AngII-induced AAAs in apoE -/- mice (114). In contrast, Rag-2 deficiency reduced AngII-induced AAA formation (136). It should be noted that these two Rag proteins have different expression levels during selected stages of T and B lymphocyte antigen receptor assembly (149, 150), implicating potentially different effects in AngII-induced immune responses, thereby differential effects on AngII-induced AAAs. In addition to general changes of lymphocytes, changes in number or function of some subtypes of lymphocytes also influenced the development of AngII-induced AAAs. For example, CD4+CD25+ regulatory T cells are a small subpopulation of T cells that play a critical role in maintaining immune homeostasis. Deficiency of this subtype of T lymphocytes, despite representing a small portion, led to multiple pathological immune responses (151). Consistent with the critical role of this T lymphocyte subtype in immunological tolerance, depletion of regulatory T cells using an anti-CD25 monoclonal antibody significantly increased the incidence and severity of AngII-induced AAAs in C57BL/6 mice (152), while transfer of regulatory T cells into apoE -/- mice attenuated AngII-induced AAAs (153).

Roles of many cytokines and chemokines secreted by lymphocytes have been implicated in the development of AAAs. Th1 cells secrete interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α and interferon γ, and Th2 cells secrete IL-5. TNF-α is one of the landmark cytokines in many inflammatory responses. TNF-α is increased in plasma and AAA tissues from patients with AAAs (154-157). TNF-α-converting enzyme (TACE or ADAM17) and osteoprotegerin, a secreted glycoprotein member of the TNF receptor superfamily, are enhanced in human AAAs (158). Although the presence of TNF-α related components implicates an important role of TNF-α and its related factors in AAAs, deficiency of the p55 TNF receptor did not influence AngII-induced AAAs in LDL receptor -/- mice (159).
Interferon-γ and CXCL10, an interferon-γ inducible T-lymphocyte chemoattractant, were increased in aneurysmal tissues of AngII-infused mice (160). Deficiency of either component led to augmentation of AngII-induced AAAs (160). Consistent with these findings, deficiency of a critical molecule in interferon-γ signaling, signal transducer and activator of transcription (STAT)1, also augmented formation of AngII-induced AAAs (161). In mice with CXCL10 deficiency, concomitant decreases of T lymphocytes and interferon-γ production were noticed in aneurysmal tissues, accompanied by increases of TGF-β. In agreement with these results, administration of an anti-TGF-β neutralizing antibody reduced AngII-induced AAAs in CXCL10 deficient mice (160). It is unclear why inhibition of TGF-β in mice with CXCL10 deficiency yielded conflicting findings with inhibition of TGF-β augmenting AngII-induced AAAs in wild-type mice (49).

IL-5 is a cytokine secreted by Th2 cells. AngII infusion into apoE -/- mice led to a profound and peak increase of IL-5 in the aortic wall within 7 days of AngII infusion, and then slowly decreased back to the baseline level during 28 days of AngII infusion (162). It appears that this initial, transient increase of IL-5 is critical to AngII-induced AAA formation because administration of TRFK-5, a monoclonal antibody against IL-5, into AngII-infused apoE -/- mice led to reductions of AAAs (162).

In general, effects of cytokines and chemokines produced or stimulated by lymphocytes on AngII-induced AAAs, as reported in the literature, are either modest or not consistent with their roles in inflammation.

1.3.4.1.3 Orchestrations of Multiple Cell Types or inflammatory components
In addition to leukocyte infiltration, roles of mast cells have been recognized in AAA tissues from humans (163, 164) and animal models (165). This cell type synthesizes and releases multiple proteases and inflammatory mediators,
thereby playing a critical role in inflammation and immunity. Kit\textsuperscript{W-sh/W-sh} mice are considered a mast cell deficient mouse model, although these mice have multiple issues in addition to deficiency of mast cells. A recent study found that incidence and severity of AngII-induced AAAs were profoundly lower in Kit\textsuperscript{W-sh/W-sh} mice with an apoE \textsuperscript{-/-} background (166). Particularly interesting, adoptive transfer of bone marrow-derived mast cells from CCR2 and apoE compound deficient mice retained the protective effects on AngII-induced AAAs in Kit\textsuperscript{W-sh/W-sh} mice, whereas adoptive transfer of bone marrow-derived mast cells from apoE \textsuperscript{-/-} mice led to augmented infiltration of macrophages, T lymphocytes, and MHC class II-positive cells in Kit\textsuperscript{W-sh/W-sh} mice, and thereby augmentation of AngII-induced AAAs (166).

Immunoglobulin E (IgE) activates mast cells. Pharmacological inhibition of IgE or whole body deficiency of its receptor Fc\textsubscript{R1} led to profound reductions of AngII-induced AAAs (167). In addition to mast cells, interactions of IgE with Fc\textsubscript{R1} activated macrophages and CD4\textsuperscript{+} T cells (167). In agreement with crucial roles of Fc\textsubscript{R1} in these cell types, adoptive transfer of mast cells, macrophages, or CD4\textsuperscript{+} T cells individually into Fc\textsubscript{R1} \textsuperscript{-/-} mice did not change AngII-induced AAAs (167).

STAT3 is a transcription factor that can be activated by multiple proinflammatory factors in many types of leukocytes. AngII infusion led to T helper 17 (Th17) cell recruitment into the aortic wall, which was mediated by activation of IL-6-STAT3 signaling, and IL-17A was one major cytokine secreted by Th17 cells (168). As a consequence, deficiency of IL-6, genetic or pharmacological inhibition of IL-17A, or pharmacological inhibition of STAT3 reduced AngII-induced AAAs (168). In contrast to this finding, another study reported that deletion of STAT3 signaling via overexpression of suppressor of cytokine signaling 3 (SOCS3) reduced IL-17, but augmented AAAs induced by AngII infusion and injections of TGF\textbeta neutralizing antibody (169). It remains to
be defined whether these conflicting findings are due to different mechanisms between AngII alone versus AngII-TGFβ interaction.

Complement activation through multiple pathways plays an important role in immune responses. The formation of membrane attack complex is the result product of complement activation. CD59 inhibits the membrane attack complex. A recent study found that overexpression of CD59 protected against AngII-induced AAAs in apoE−/- mice (170), associated with changes of matrix metalloproteases (MMP)2 and 9 as well as attenuated inflammation.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcriptional factors with multiple functions in energy metabolism and vascular biology. GW501516, an activator of PPARδ, reduced AngII-induced AAAs in apoE −/- mice (171). Drugs in the class of thiazolidinediones such as pioglitazone and rosiglitazone are used to reduce blood glucose in diabetic patients. It is also well known that these drugs activate PPARγ. Pioglitazone or rosiglitazone reduced AngII-induced AAAs in apoE −/- mice (122, 172). However, administration of pioglitazone to LDL receptor −/- mice did not attenuate AngII-induced AAAs (173). In accord with this finding, deficiency of PPARγ in smooth muscle cells did not affect AngII-induced AAAs (173). It is unclear whether PPARγ expressed in other cell types would contribute to AngII-induced AAAs since systemic administration of pioglitazone did not attenuate AngII-induced AAAs in mice with smooth muscle cell-specific PPARγ deficiency (173). Although thiazolidinediones activate PPARγ, there is no direct evidence that this class of drugs attenuates AngII-induced AAAs by activating PPARγ. It is also unclear why pioglitazone reduced AngII-induced AAAs in apoE −/- mice (122), but not in LDL receptor −/- mice (173), although the dose of pioglitazone used in LDL receptor −/- mice was lower (20 mg/kg/d in diet) (173) than in apoE −/- mice (50 mg/kg/d in drinking water) (122).
Mesenchymal stem cells are a small population of non leukocytic stem cells. These cells suppress immunoreactivity of T cells and inhibit macrophage-mediated inflammation. Application of bone marrow-derived mesenchymal stem cells through laparotomy or intravenous injections attenuated AngII-induced AAAs (174, 175).

Overall, the evidence is consistent with inflammatory cells and responses play a crucial role in development of AngII-induced AAAs. However, currently there has no clinical evidence whether inhibition of inflammatory cell number or functions would improve AAAs in humans. Using cell-specific deficient mice that had AT1a receptor depletion on leukocytes, endothelial cells, or smooth muscle cells did not define a single cell type that played a critical role in AngII-induced AAA development (131, 134), implicating orchestrated contributions of multiple cell types in the interaction of AngII and AT1a receptor to promote AAAs.

1.3.4.2 Extracellular Matrix Related Factors

Extracellular matrix (ECM), composed of collagen and elastin fibers, are essential in maintaining normal aortic structure. Mechanisms by which ECM related factors contribute to AAAs as implicated in reported studies include disrupted homeostasis of ECM degradation and subsequent loss of vessel wall integrity. Inflammatory responses are also pronounced during changes of vessel wall integrity. Many components in the ECM family have been studied in AAA formation in AngII-infused mice. Osteopontin is a secreted extracellular matrix protein present in aortic tissue (176). Deficiency of osteopontin globally or specifically in bone marrow-derived cells reduced AngII-induced AAAs (62). MMPs and tissue inhibitors of metalloproteinases (TIMPs) maintain structural integrity of the aortic wall. Several MMPs are present in human AAAs. In a recent meta-analysis, 8 case-control studies were pooled to determine whether circulating MMP9 concentrations were associated with AAA presence in humans (177). Although higher plasma MMP9 concentrations were associated with AAA presence, it is worth noting that there were broad value ranges of
MMP9 for both AAAs (30 - 750 ng/L) and non-AAAs (9 - 680 ng/L) (177). Many studies reported that AngII infusion increased MMP9 in aortic tissues (a few examples (66, 114, 138, 178)). Although deficiency of MMP9 prevented elastase-induced [19] and calcium chloride-induced AAAs in mice (179), deficiency of MMP9 in AngII-induced AAAs in mice has not been reported. MMP2 deficiency in C57BL/6 mice did not affect AngII-induced AAAs (180). In contrast, TIMP3 augmented AngII-induced AAAs in C57BL/6 mice, and MMP2 deficiency beyond TIMP3 deficiency further augmented AngII-induced AAAs in C57BL/6 mice (181). Currently, there has no specific pharmacological inhibitors for MMP9 or MMP2. Doxycycline is an antibiotic, which is also known as a pharmacological inhibitor of a broad range of MMPs. Preventive administration of this drug either reduced (78, 182) or had no effect (60) on AngII-induced AAAs in mice.

Cathepsins K, L, and S are elastases that are activated in remodeling of arterial wall. These cathepsins have been implicated in human AAAs (183). Deficiency of cathepsin S reduced AngII-induced AAAs in apoE/-/- mice (184), whereas deficiency of cathepsin K had no effects on AngII-induced AAAs (185). Cystatin C is an endogenous inhibitor of cysteinyl cathepsins. Deficiency of cystatin C augmented AAAs in AngII-infused mice (186).

Caspases and calpains belong to the family of cysteine proteases. Pharmacological inhibition of either caspase [163] or calpain (187, 188) reduced AAAs in AngII-infused mice, implicating that these cysteine proteases are contributors to AngII-induced AAAs.

Granzyme B, chymase, and urokinase-type plasminogen activator (uPA) are serine proteases. Deficiency of granzyme B (189) or pharmacological inhibition of chymase attenuated AngII-induced AAAs in apoE -/- mice (190). Plasminogen activator inhibitor-1 (PAI-1) is a natural inhibitor of uPA. One study reported that increased PAI-1 prevented AngII-induced AAAs in apoE/-/-
mice through local delivery recombinant adenovirus containing human PAI-1 gene (intra-adventitial injection), but not through systemic delivery (tail vein injection) (191). The mechanism leading to the different response to local versus systemic administration of PAI-1 was not defined. Additionally, effects of uPA on AngII-induced AAAs have not been consistent. An initial study found that uPA deficiency attenuated AngII-induced AAAs in both apoE -/- mice and C57BL/6 mice (113). Conversely, neither uPA nor uPA receptor deficiency changed aortic dilation in AngII-infused LDL receptor -/- mice (114). In contrast, uPA deficiency led to increased aortic rupture in LDL receptor -/- mice (114). It appears that this finding is not due to different mouse strains used in the two studies since our study also found that deficiency of uPA had no effects on AngII-induced AAAs in C57BL/6 mice.

It is well known that ECM related factors play essential roles in the development and progression of AAAs. However, findings using AngII-induced AAAs have not consistently defined specific factors that would provide benefits in the treatment of AAAs. The disappointing findings in the recent reported clinical trial (192) that doxycycline did not improve AAAs in humans provides a caveat in the current mechanistic explorations and we have a long way to discover or develop ideal targets to treat patients with AAAs (193).

1.3.4.3 Phospholipid Related Factors
Phospholipids contribute to maintaining cell membrane structure. This class of lipids also plays important roles in inflammation. Non-selective pharmacological inhibition of phospholipases reduced AngII-induced AAAs (194). Several critical components of arachidonic acid cascade have been studied in AngII-induced AAAs. 5-lipoxygenase (5-LO) is key component in biosynthesis of leukotrienes by catalyzing arachidonic acid. Deficiency of 5-LO had no effect on AngII-induced AAAs in mice (195). A pharmacological inhibitor of 5-LO, MK-0591, did not change AngII-induced AAAs in apoE-/- mice (195), whereas another 5-LO inhibitor, LP105, reduced severity, but not aortic diameters of AngII-induced AAAs in apoE -/- mice (196). Leukotriene B4,
produced through 5-LO pathway, exerts its effects through a G protein-coupled receptor BLT1. Genetic deficiency (197) or pharmacological inhibition (198) of BLT1 attenuated AngII-induced AAAs. Considering the complexity of 5-LO signaling pathway, it is possible that BLT1 is a better target for inhibiting AngII-induced AAAs. Cyclooxygenase (COX) and its downstream pathway to generate prostaglandin E2 (PGE2), have also been detected in human AAAs (199). Genetic deficiency of COX-2 either reduced (200) or had no effects on AngII-induced AAAs (201). A specific pharmacological inhibitor of COX-2, celecoxib, reduced AngII-induced AAAs (115, 202). Microsomal PGE2 synthase-1 (mPGES-1) is the enzyme to generate PGE2. Deficiency of mPGES-1 reduced AngII-induced AAAs (203). Pharmacological inhibition (AE3-208) (201, 204) or genetic depletion (EP4 +/-) of EP4, a PGE2 receptor, attenuated AAA formation in AngII-infused apoE-/- mice (204). However, deficiency of EP4 in bone marrow-derived cells increased AngII-induced AAAs (205).

Phospholipases A2 (PLA2) are a group of enzymes that cleave phospholipids to generate lysophospholipids and free fatty acids. Group V and group X secretory PLA2 have been studied in AngII-induced AAAs. Genetic deficiency of group X, but not group V, secretory PLA2 attenuated AngII-induced AAAs in apoE -/- mice (206, 207). Plasma phospholipid transfer protein (PLTP) is a member of lipid transfer/lipopolysaccharide-binding proteins. Whole body deficiency of PLTP reduced AngII-induced AAAs in apoE -/- mice, which was not attributed to its deficiency in bone marrow-derived cells (208).

Although many phospholipid related factors are present in AAA tissues, there are conflicting findings regarding their roles in AngII-induced AAAs. The associations between phospholipid related factors and the formation of AAAs remain to be unraveled.
1.3.4.4 Oxidation Related factors

Oxidation contributes to a spectrum of diseases. AngII promotes and augments oxidation in multiple cell types (209). Critical enzymes in the homeostasis of oxidation include catalase and NADPH oxidases (NOX). Reactive oxygen species are present in either circulation or diseased tissues in patients with AAAs (210, 211). Vitamin E, a recognized antioxidant that inhibits NOX, reduced AngII-induced AAAs in 24-week-old apoE-/- mice (212). However, this effect was not significant in 50-60-week-old apoE -/- mice when both vitamin E and vitamin C were administered (59). Later, studies using genetic mouse models found that global deficiency of p47phox, a cytosolic subunit of NOX, in apoE -/- mice (213), global or bone marrow cell specific deficiency of NOX2 in LDL receptor -/- mice (178) or global deficiency of NOX1 in C57BL/6 mice (214) reduced AngII-induced AAAs. In contrast to potential effects of NADPH on augmentation of AngII-induced AAAs, deficiency of catalase, an antioxidant enzyme, did not influence AngII-induced AAAs (215). Overall, similar to other mechanisms explored in AngII-induced AAA mouse model, contributions of oxidation to AAAs and the potential therapeutic effects have not been defined.

1.3.4.5 MicroRNAs

Since the discovery of microRNAs (miR), this class of single-strand small RNA molecules have been reported in many disease processes. Multiple miRs were detected in circulation and aneurysmal tissues of patients with AAAs (216-218). One initial study reported that inhibition of miR-29 by locked nucleic acid-modified antisense oligonucleotides prevented aortic dilation in 18-month-old C57BL/6 mice or 6-month-old apoE -/- mice within 7 days of AngII. However, inhibition of miR-29 did not maintain this beneficial effect after 28 days of AngII infusion (219). In subsequent studies from two research groups (216, 217) inhibition of a miR-29 subtype, miR-29b, did not prevent aortic dilation within 10 - 14 days of AngII infusion, whereas this inhibition led to a modest reduction of luminal diameter, as measured by ultrasound or MRI, after 28 days infusion of AngII in apoE -/- mice at 10 weeks of age. These findings were consistent with
overexpression of miR-29b modestly augmenting AngII-induced luminal dilation during 14 and 28 days of AngII infusion (216).

miR-21 induces cell proliferation and diminishes apoptosis in AngII-induced AAAs (89). Overexpression of miR-21 prevented AAA formation, and inhibition of miR-21 augmented AAA formation (89). miR-24 suppresses chitinase 3-like 1 mediated cytokine synthesis in macrophages and macrophage interaction with vascular smooth muscle cells and endothelial cells (220). Inhibition of miR-24 augmented AngII-induced AAAs, whereas overexpression of miR-24 reduced AngII-induced AAAs (220). miR-712, a murine-specific miR, and its human homolog miR-205 were upregulated in endothelial cells isolated from abdominal aortas in AngII-infused mice (218). Inhibition of miR-712 or miR-205 reduced AngII-induced AAAs in apoE -/- mice. In general, recent studies have provided insights into understanding contributions of miRs in the development of AAAs. Mechanisms involve orchestration of inflammation, cell-cell interactions, extracellular matrix protein degradation, oxidation, and many other signaling pathway (221). It would be important to explore potential therapeutic benefits of these miRs.

1.3.5 Regression of AngII-induced AAAs

In the clinical environment, treatment is initiated in patients following detection of an established AAA. The mechanisms or therapeutics discussed above are mostly manipulations that prevent AAA initiation, in which manipulations were performed prior, or simultaneous to initiation of AngII infusion. One decade ago, a single study reported that SP600125, an inhibitor of JNK, regressed established AAAs induced by AngII infusion in apoE -/- mice (222) ApoE -/- mice were infused with AngII 1,000 ng/kg/min for 4 weeks, and then stratified by ultrasound into two groups to receive either vehicle or SP600125 for 8 weeks. Luminal diameter, as measured by ultrasound, was profoundly reduced by JNK inhibition (222). Unfortunately, despite the striking effects of JNK inhibition, this study has not been followed up by other research groups or translated into clinical investigations.
Recently several studies have explored therapeutic potentials in mice with established AAAs using similar method reported by the above study (222). In some studies, apoE-/- mice were infused with AngII 1,000 ng/kg/min for 4 weeks to induce AAAs. Mice were then stratified based on ultrasound measurements of suprarenal aortic diameters. One study reported that a peptide against the leucine-serine-lysine-leucine (LSKL) sequence in thrombospondin prevented progression of AngII-induced AAAs (223). Another study reported that aliskiren administration, a renin inhibitor (50 mg/kg/d), led to attenuated progression of AngII-induced AAAs (224). In this study, in the absence of AngII infusion, suprarenal aortas expanded continuously, which contradict recent studies showing that AAAs did not progressively expand if AngII infusion was discontinued after 4 weeks of AngII infusion (65, 223). Considering that accuracy of ultrasonographic measurements is largely user-dependent, further experiments are necessary to directly measure maximal diameters of the suprarenal aorta using ex vivo images.

There are another two recent studies (225, 226) that used similar method to induce AAAs. However, mice were not stratified using ultrasound measurements, but were randomly assigned to different groups. In one study, after the 4-week infusion of AngII, bone marrow-derived mesenchymal stem cells were injected intravenously into apoE-/- mice (225). Compared to control groups, aortic diameters were much smaller in mice injected with bone marrow-derived mesenchymal stem cells after 2 or 4 weeks. However, aortic diameters were equally large between control and bone marrow-derived mesenchymal stem cells injected mice after 8 weeks (225). In another study, apoE-/- mice was infused with AngII 1,000 ng/kg/min for 45 days. These mice were subsequently grouped randomly to receive normal laboratory diet, diet containing α-tocopherol or β-carotene, or both for 60 days. α-tocopherol and β-carotene are considered to have anti-oxidation effects. Individual or combination of these two reagents led to significantly smaller diameters of
suprarenal aortas in apoE -/- mice (226). Since it is unclear whether mice had equally established AAAs among study groups, it is hard to provide evaluation whether manipulations reported in these two studies had potential therapeutic benefits on established AAAs.

While the above studies implicate potential beneficial effects of treating established experimental AAAs, two recent studies (66, 202) failed to show beneficial effects of two different treatment on established AAAs. One study used apoE -/- mice infused with AngII 750 ng/kg/min for 3 weeks prior to celecoxib administration, a COX-2 inhibitor, for 5 weeks when AngII was infused continuously. Compared to mice infused with AngII for 8 weeks alone, mice receiving celecoxib had smaller aortic diameters. However, this parameter in mice receiving celecoxib was not different from those infused with AngII for 3 weeks alone, implicating that celecoxib does not regress established AAAs (202). In another study, LDL receptor -/- mice fed a Western diet were infused with AngII 1,000 ng/kg/min for 4 weeks, and then ultrasound were performed. Mice with established AAAs were stratified into two groups to receive either plain drinking water or doxycycline (100 mg/kg/d) in drinking water when AngII infusion was continued for another 8 weeks. Doxycycline had no effects on the progression of AngII-infused AAAs (66). This result was supported by the recent clinical trial reporting that doxycycline had no beneficial effects on established AAAs in humans (192). Compared to studies showing “positive” results, these two “negative” studies performed continuous AngII infusion during the treatments. Taken the findings of these current studies together, although there are hopes from these animal studies to treat established AAAs in humans, it would be important to follow standard protocols and well-designed experiments with proper controls and accurate measurements to ensure capability to replicate experimental findings before we can move forward with clinical trials.
Chapter Two: ApoB-containing Lipoproteins Augment AngII-induced AAAs in Male Mice

This chapter is based on a manuscript published in “Arterioscler Thromb Vasc Biol. 2015 August;35(8):1826-34”, with Jing Liu as the first author.

2.1 Introduction
Abdominal aortic aneurysms (AAAs) are permanent dilations that portend the devastating consequence of aortic rupture. AAA prevalence and severity have been associated with increased plasma cholesterol concentrations (84, 227). Several studies demonstrated that prevalence of AAAs was correlated positively with plasma low-density lipoprotein (LDL)-cholesterol concentrations and negatively correlated with plasma high-density lipoprotein (HDL)-cholesterol concentrations (14, 104, 105, 107, 228, 229). However, other studies failed to demonstrate a relationship between either LDL- or HDL-cholesterol concentrations and AAAs (106, 107, 109, 230). Overall, hypercholesterolemia has a poorly defined role in human AAAs and further clarification is needed on the function of specific lipoprotein fractions. Regardless of an undefined association between hypercholesterolemia and AAAs, statin administration is common for patients afflicted with AAA to reduce aortic expansion, which has beneficial effects in preventing progression of AAAs or improving related cardiovascular events (110, 112).

Chronic subcutaneous infusion of angiotensin II (AngII) into mice is a commonly used model of AAAs (231). Initial studies using this model were performed in hypercholesterolemic mice that were either LDL receptor-/- fed a Western diet (56) or apolipoprotein (apo)E-/- fed either a normal or Western diet (57, 58). In addition to a substantial number of studies of AngII-induced AAAs using hypercholesterolemic mice (232) a small number of studies have demonstrated that AngII infusion induces AAA formation in normocholesterolemic mice (49, 113-115, 232, 233), albeit at much lower incidence than in hypercholesterolemic mice. Despite the potential role of hypercholesterolemia in formation of AngII-induced AAAs, effects of specific lipoprotein fractions in
experimental AAA development have received sparse attention. One study reported that increased plasma HDL-cholesterol concentrations by daily subcutaneous administration of human HDL (10 mg/kg/day of apoAI) reduced AngII-induced AAAs in apoE-/- mice (124). Injection of reconstituted HDL, composed of human apoAI and phosphatidylcholine, also decreased AAA formation in both AngII-infused hypercholesterolemic apoE-/- mice and calcium chloride administered normcholesterolemic C57BL/6 mice (124). Findings from this study implicate potential associations between apoAI and AAAs.

To determine roles of specific lipoprotein fractions in development of AngII-induced AAAs, we used multiple mouse strains with dietary and pharmacological manipulations that resulted in different forms and severity of hypercholesterolemia. The occurrence of AngII-induced AAAs in these mice was contrasted to the formation of atherosclerosis, which has been strongly associated with plasma apoB-containing lipoprotein concentrations in mouse studies. These studies demonstrated that development of AngII-induced AAAs was augmented by elevation of apoB-containing lipoproteins, but not by deficiency of apoAI, the major structural apolipoprotein of HDL. However, unlike atherosclerosis, there was no simple concentration-dependent association of plasma apoB-containing lipoproteins with AngII-induced AAAs.

2.2 Methods

2.2.1 Mouse Housing Condition and Diets

C57BL/6J, apoA-I -/-, LDL receptor -/-, and apoE -/- mice were purchased from The Jackson Laboratory (Stock # 0664, 2055, 2207, and 2052, respectively; Bar Harbor, ME, USA). Breeding pairs of apoA-I +/- x LDL receptor +/- and apoA-I -/- x LDL receptor -/- mice were developed by Dr. Sorci-Thomas. Mice were maintained in individually vented cages (maximally 5 mice/cage) on a light:dark cycle of 14:10 hours. The cage bedding was Teklad Sani-Chip bedding (Cat # 7090A, Harlan Teklad, Madison, WI, USA). Mice were fed a normal rodent
laboratory diet (Diet # 2918, Harlan Teklad; Madison, WI, USA) and given
drinking water from a reverse osmosis system ad libitum. During experiments,
mice were either continuously fed the normal diet or a Western diet (Diet #
TD.88137; Harlan Teklad) containing 21% (wt/wt, which equals 42%
calories/calories) saturated fat extracted from milk, 48.5% (wt/wt) carbohydrate,
17.3% (wt/wt) protein, and 0.2% (wt/wt) cholesterol (0.15% supplemented, and
0.05% from the fat source) 1 week prior to and during 4 weeks of AngII
infusion. Ezetimibe was provided by Merck & Company, Inc. Diets containing
ezetimibe (50 mg/kg) were customized by Harlan Teklad. All procedures were
performed with the approval of the University of Kentucky Institutional Animal
Care and Use Committee.

2.2.2 Osmotic Mini Pump Implantation and AngII Infusion
AngII (1,000 ng/kg/min or 500 ng/kg/min; Cat# H-1706; Bachem; Torrance, CA,
USA) was infused subcutaneously via Alzet osmotic mini pumps (Alzet Model #
2004;Durect; Cupertino, CA, USA) as described previously. Only apoAI +/+ and
-/- mice in an LDL receptor -/- background fed a Western diet were infused with
AngII 500 ng/kg/min. Mice were sedated with isoflurane and pumps were
implanted subcutaneously on the right flank of each mouse. Surgical staples
were used to close the incision site and a topical anesthetic cream (LMX4;
Ferndale Laboratories; Ferndale, MI) was applied immediately after surgery to
relieve pain.

2.2.3 Systolic Blood Pressure Measurements
Systolic blood pressure was measured using a standardized protocol described
Previously (105) on conscious mice for 3 consecutive days prior to (baseline)
and during the last week of AngII infusion by a non-invasive tail-cuff system
(Coda 8; Kent Scientific; Torrington, CT, USA).

2.2.4 Measurement of Plasma Components
Mice were anesthetized using ketamine/xylazine cocktail at termination. Blood
samples were harvested by right ventricular puncture with EDTA (final concentration: 1.8 mg/ml) and then centrifuged at 400 g x 20 minutes at 4 °C to prepare plasma.

Plasma cholesterol concentrations were measured using an enzymatic kit (Cat # 439-17501; Wako Chemicals USA, Richmond, VA, USA). Plasma lipoprotein distributions were resolved by size exclusion gel chromatography followed by enzymatic measurement of cholesterol in each collected fraction, as described previously (104).

Plasma renin concentrations were measured using a method described in our previous publication (234). Briefly, plasma samples harvested with EDTA were incubated in an assay buffer (Na₂HPO₄ 0.1 M; EDTA 0.02 M, maleate buffer pH 6.5, phenylmethylsulfonyl fluoride 2 μl; total volume of 250 μl) with an excess of rat angiotensinogen at 37 °C for 30 minutes. Rat angiotensinogen was obtained through partial purification of nephrectomized rat plasma. Reactions were terminated by placing samples at 100 °C for 5 minutes. AngI generated in each sample was quantified by radioimmunoassay using a commercially available kit (Cat # 1553; DiaSorin, Stillwater, MN, USA).

2.2.5 Quantification of Aortic Pathologies
At termination, after blood collection, the right atrium was cut open, and saline was perfused through the left ventricle to remove blood from the systemic circulation. Subsequently, aortas were dissected and placed in 10% neutrally buffered formalin overnight at room temperature. After fixation, periaortic adventitia was carefully removed.

Necropsies were performed for mice that died during AngII infusion. Aortic rupture was defined as observation of blood clots in either the thoracic cavity (thoracic aortic rupture) or retroperitoneal cavity (abdominal aortic rupture). Maximal outer diameter of the suprarenal aorta was measured ex vivo as a
parameter for abdominal aortic aneurysm (AAA) quantification using Image-Pro software (Version 7; Media Cybernetics; Bethesda, MD, USA). Thoracic aortas were cut open and pinned for quantification of intimal area and atherosclerotic lesion area in a region including ascending, arch and 3 mm of descending aorta using an en face technique (107, 228). Atherosclerosis was compared between groups with percent lesion area (lesion area/intimal area x 100%).

### 2.2.6 Statistical Analysis

Data were analyzed using SigmaPlot version 12 (SYSTAT Software Inc., Chicago, IL, USA). Data are represented as means ± standard error of means (SEM). To compare two study groups of each experiment on a continuous variable, unpaired two-tailed Student’s t test was performed for normally distributed and equally variant values, and Mann-Whitney rank sum test were used for non-normally distributed variables. Systolic blood pressure was analyzed using two-way repeated measures ANOVA. P < 0.05 was considered statistically significant.

### 2.3 Results

#### 2.3.1 Western Diet Had No Effects on AngII-induced AAA Formation in Male C57BL/6 Mice

Prolonged feeding of a high fat diet (60% calories from saturated fat) augments AngII-induced AAAs in normocholesterolemic mice that have become obese (101). Therefore, in the initial experiment we determined whether Western diet (42% calories from milk fat) per se had effects on AngII-induced AAAs in wild-type C57BL/6J mice. Male C57BL/6J mice were fed either a normal or Western diet and infused with AngII (1,000 ng/kg/min) for 4 weeks. Western diet feeding started 1 week prior to AngII infusion and was maintained during AngII infusion. There was no significant body weight gain difference between mice fed normal versus Western diet. Western diet feeding modestly increased plasma total cholesterol concentrations in C57BL/6 mice (Figure 2.1A). With no overt presence of apoB-containing lipoproteins, HDL was the predominant lipoprotein in these mice fed either diet as defined by size exclusion chromatography.
There were no differences of body weight or LDL/HDL ratio between C57BL/6 mice fed normal versus Western diet (Table 2.1 and 2.2). No discernable atherosclerotic lesions were detected in these mice. One of 10 mice (10%) from each group died of aortic rupture. There were no significant differences in maximal outer diameter of suprarenal aortas between mice fed these two diets (Figure 2.1C).

2.3.2 Deficiency of ApoAI Did Not Exacerbate AngII-induced AAA Formation

HDL is the major lipoprotein fraction in plasma of male C57BL/6 mice (Figure 2.1B), and apoAI is the predominant structural apolipoprotein of HDL. To determine whether low HDL augmented AngII-induced AAAs, we compared AngII-induced AAA formation between male apoAI+/+ and -/- mice in a C57BL/6 background fed the normal laboratory diet and infused with AngII (1,000 ng/kg/min) for 4 weeks. There were no differences of body weight between two apoAI+/+ and -/- mice (Table 2.1). Deficiency of ApoAI led to significant reductions of plasma cholesterol concentrations (Figure 2.2A) due to reductions of HDL-cholesterol concentrations (Figure 2.2B). One of 10 mice (10%) from each group died of aortic rupture. Deficiency of ApoAI did not augment AngII-induced AAAs in C57BL/6 background (Figure 2.2C).

Effects of apoAI deficiency were also studied in male LDL receptor-/- mice. In the first experiment, mice were infused with 1,000 ng/kg/min of AngII and fed the normal laboratory diet. Plasma total cholesterol or apoB-containing lipoprotein concentrations were not significantly different between the two apoAI genotypes (Figure 2.3A and 2.3B), whereas plasma HDL-cholesterol was barely detectable in mice with apoAI deficiency fed the normal laboratory diet (Figure 2.3B). Atherosclerotic lesions were minimal and not significantly different between the two genotypes (Figure 2.3C). Consistent with findings in C57BL/6 mice, apoAI deficiency in LDL receptor-/- mice had no effects on AngII-induced AAA formation (Figure 2.3D). Since apoAI deficiency was hypothesized to enhance AngII-induced AAA formation, infusion rates of AngII
were selected to create a low incidence of AAAs in apoAI+/+ mice to enable demonstration of enhanced AAAs in apoAI-/- mice. Subsequently, we compared AngII-induced AAAs using an infusion rate of 500 ng/kg/min between apoAI+/+ and -/- mice with LDL receptor-/- background that were fed the Western diet. As with normal diet,apoAI deficiency led to profound reductions of plasma cholesterol concentrations with barely detectable HDL with Western diet (Figure 2.4 A and 2.4B). Atherosclerotic lesions were modestly reduced in mice with apoA-I deficiency (Figure 2.4C). In agreement with the other studies described above, apoAI deficiency in LDL receptor-/- mice fed the Western diet did not exacerbate AngII-induced AAA formation (Figure 2.4D). One of 12 mice that were wild type for apoAI died of abdominal aortic rupture, whereas no apoAI-/- mice died of aortic rupture.

2.3.3 Hypercholesterolemia Augmented AngII-induced AAA in Male LDL Receptor -/- Mice

LDL receptor-/- mice fed normal laboratory diet are modestly hypercholesterolemic with comparable cholesterol distributions between apoB-containing lipoproteins and HDL. Previous studies have reported high susceptibility to AngII-induced AAA in this mouse model fed Western diet to AngII-induced AAAs (114, 235). However, AngII-induced AAAs have not been reported in this mouse model fed normal laboratory diet. To determine whether augmented hypercholesterolemia influenced AAA formation in LDL receptor-/- background, male mice of this genotype were fed either normal or Western diet and infused with AngII 1,000 ng/kg/min for 4 weeks. Western diet feeding greatly increased plasma total cholesterol concentrations compared to mice fed normal laboratory diet (Figure 2.5A). These increases were solely attributed to increased apoB-containing lipoproteins that included chylomicrons, chylomicron remnants, very low density lipoprotein (VLDL), and LDL (Figure 2.5B). Body weight was not significantly different between two groups (Table 2.1). LDL/HDL ratios were increased in male LDL receptor-/-mice fed Western diet, compared to those fed a normal laboratory diet (Table 2.2) As expected, the greatly augmented hypercholesterolemia promoted by Western diet feeding profoundly
increased atherosclerotic lesions (Figure 2.5C). Western diet also significantly increased AAA formation, as defined by maximal outer diameters of suprarenal aortas (Figure 2.5D). No deaths due to aortic rupture occurred in these mice.

Male sex has been demonstrated to enhance AngII-induced AAA formation in apoE-/- mice (74, 75, 77). To determine whether hypercholesterolemia has similar effects in both sexes, female LDL receptor-/- mice fed either normal or Western diet were infused with AngII 1,000 ng/kg/min for 4 weeks. As with males, body weight was not significantly different between two groups (Table 2.2). Western diet feeding profoundly increased plasma cholesterol concentrations due to increased apoB-containing lipoprotein cholesterol concentrations (Figure 2.6A and 2.6B). Consistent with data from male LDL receptor-/- mice, Western diet feeding augmented atherosclerotic lesions (Figure 2.6C). Despite equivalent elevations in plasma cholesterol concentrations as male mice, Western diet feeding did not augment AngII-induced AAAs in female LDL receptor-/- mice (Figure 2.6D). No deaths due to aortic rupture occurred in female mice.

2.3.4 Comparable AngII-induced AAA Formation in Male ApoE-/- Mice Fed Normal versus Western Diet

Although there have been no direct comparisons, it is inferred from the literature that AngII-induced AAAs are equivalent between apoE-/- mice fed normal versus Western diet (57, 58). To compare directly, male apoE-/- mice were fed either normal or Western diet and infused with AngII 1,000 ng/kg/min. Body weight was not significantly different between two groups (Table 2.1). ApoE-/- mice fed a normal laboratory diet had plasma cholesterol concentrations of 347 ± 38 mg/dl, and Western diet feeding led to profound increases (1260 ± 121 mg/dl) due to increased apoB-containing lipoprotein cholesterol concentrations (Figure 2.7A and 2.7B). In agreement with findings in LDL receptor-/- mice, Western diet feeding significantly increased atherosclerotic lesions (Figure 2.7C). However, there was no difference in
AngII-induced AAAs (Figure 2.7D) or death due to aortic rupture (60% versus 40%) between mice fed normal and Western diets.

2.3.5 Reduction of Plasma ApoB-containing Lipoproteins Attenuated AngII-induced AAA Formation in Male ApoE -/- Mice Fed Normal Diet

Results from LDL receptor-/- mice fed Western diet and apoE-/- mice fed either diet implicate that high concentrations of plasma apoB-containing lipoproteins contribute to augmentation of AngII-induced AAAs. ApoE-/- mice are endogenously hypercholesterolemic even when fed the normal laboratory diet. Therefore, to determine whether reduced apoB-containing lipoprotein concentrations attenuate development of AngII-induced AAAs in male apoE-/- mice, we administered an intestinal cholesterol absorption inhibitor, ezetimibe, started one week prior to initiating AngII infusion (1,000 ng/kg/min). Body weight was not significantly different between two groups (Table 2.1). In male apoE-/- mice fed Western diet, plasma cholesterol concentrations were reduced compared to mice not administered the drug. However, this concentration (546 mg/dl) was still high, with a predominance of high plasma apoB-containing lipoproteins (Figure 2.8A and 2.8B). This reduction was associated with attenuation of atherosclerosis (Figure 2.8C), whereas dilation of suprarenal aortas (Figure 2.8D) and death due to aortic rupture (control versus ezetimibe: 30% versus 40%) were not significantly different.

The same dose of ezetimibe decreased plasma cholesterol concentrations from 451 ± 8 mg/dl to 204 ± 18 mg/dl in male apoE-/- mice fed a normal laboratory diet (Figure 2.9A) and infused with AngII (1,000 ng/kg/min). This is similar to plasma cholesterol concentrations in LDL receptor-/- mice fed the same diet. Body weight was not significantly different between two groups (Table 2.1). This reduction was due to decreased plasma apoB-containing lipoproteins (Figure 2.9B). Atherosclerotic lesion sizes were minimal in both groups (Figure 2.9C). Aortic rupture rate was comparable between two groups (control versus ezetimibe: 30% versus 20%). However, administration of ezetimibe decreased
AngII-induced AAA formation as determined by maximal outer diameters of suprarenal aortas (Figure 2.9D).
2.4 Discussion
Aberrant metabolism of specific lipoprotein fractions, particularly LDL and HDL, is associated with atherosclerotic diseases and modulation of their plasma concentrations is a tenet of therapeutic strategies (236). Dysfunctional lipoprotein metabolism has also been implicated in human AAA formation, although associations have not been studied extensively (227). In this study, AngII-induced AAAs in multiple mouse models with different plasma lipoprotein distributions were used to determine whether facets of dyslipidemia directly associated with AAA formation. We were unable to define any effect of reduced HDL-cholesterol concentrations, promoted by apoAI deficiency, on AngII-induced AAA formation in two mouse strains with dietary manipulations. However, our findings clearly demonstrated that hypercholesterolemia augmented the development of AngII-induced AAAs in mice. Changes in large sized lipoprotein particles were associated with augmentation of AngII-induced AAAs. The only feature in common for these lipoproteins was the presence of apoB. However, unlike atherosclerosis in which plasma apoB-containing lipoprotein concentrations closely correlated with lesion size, AAAs were augmented with modestly hypercholesterolemic states, but not further enhanced with progressive increases in hypercholesterolemia.

HDL-cholesterol has been associated with AAAs in humans in a limited number of observational studies (104, 105, 107, 109, 237, 238). Recent experimental evidence consistent with this association demonstrated that administration of exogenous native or reconstituted HDL reduced AngII-induced AAAs (124). We performed the converse study in which plasma HDL concentrations were reduced markedly by genetic deficiency of apoAI. ApoAI deficiency did not augment AngII-induced AAAs in C57BL/6 mice fed a normal laboratory diet. This lack of effect was also demonstrated in LDL receptor-/- mice fed either normal or Western diet. Therefore, depletion of endogenous apoAI had no discernable effect on AngII-induced AAAs. The mechanisms by which exogenously delivered apoAI and its endogenous reduction have differential
effects on AngII-induced AAAs are unclear. A similar contrast of HDL supplementation versus genetic deficiency has been observed in atherosclerosis studies, with overexpression of apoAI reducing atherosclerosis (239, 240), but apoAI deficiency did not augment atherosclerosis (241, 242). ApoAI deficiency was reported to be associated with (243) or have no correlations with (244) coronary heart disease in humans. Although a few studies suggested that low plasma apoAI concentrations were associated with AAAs in patients (107, 238, 245), no studies reported whether apoAI deficiency was associated with the development of AAAs.

Chronic AngII infusion into normocholesterolemic mice induces AAAs at a rate that has ranged from 5% to 40% (49, 101, 113-115). In contrast, the incidence of AngII-induced AAAs in hypercholesterolemic mice has routinely been over 70% (79, 81, 113, 114). Hypercholesterolemic mice in these previous studies have included LDL receptor-/- mice fed Western diet or apoE-/- mice fed either normal or Western diet (57, 58). Ezetimibe lowered plasma cholesterol concentrations in apoE -/- mice fed normal laboratory diet to approximately 200 mg/dl. This reduction in plasma apoB-containing lipoproteins significantly attenuated abdominal aortic dilation. Ezetimibe also reduced the extent of hypercholesterolemia in apoE-/- mice fed Western diet. While this reduction decreased atherosclerosis, it did not attenuate aortic dilation. This result demonstrated that reduced AAAs in apoE-/- mice administered ezetimibe and fed normal laboratory diet was not attributed to a direct effect of the drug. These findings from male LDL receptor-/- or apoE-/- mice fed normal versus Western diet, coupled with results using ezetimibe, demonstrate that substantial reductions in apoB-containing lipoproteins are required to attenuate AngII-induced AAAs in mice. Consistent with our findings, increased concentrations of plasma LDL, a major class of apoB-containing lipoproteins, have been associated with AAAs in humans (105, 227, 246, 247).
ApoB-containing lipoproteins encompass a heterogeneous mix of lipoprotein particles with a range of lipid and apolipoprotein compositions. One study reported that LDL incubation with cultured human smooth muscle cells increased AT1 receptor mRNA and inferred this was the cause of increased response to AngII in hypercholesterolemic patients (248). However, although whole body depletion of AT1a receptors ablates AngII-induced increases of AAAs in hypercholesterolemic mice (131), deletion of AT1a receptors specifically in smooth muscle cells had no effects on AngII-induced AAAs (134, 249). One mechanism of AngII-induced AAAs is that AngII promotes leukocyte infiltration both systemically and locally into the aortic wall (64, 65, 81). Hypercholesterolemia also promotes leukocyte infiltration in arteries (250) or facilitates AngII-induced leukocyte mobilization into the arterial wall (136). In our future studies, it will be important to explore mechanisms by which hypercholesterolemia and AngII have synergistic effects on leukocyte infiltration to promote AAA formation.

We have demonstrated previously that AngII-induced AAAs in apoE-/- mice have a strong sexual dimorphism with males being much more susceptible to aortic expansion (78). This dimorphism is ablated by orchidectomy (74, 75, 77). The current study demonstrated this dimorphism also exists in LDL receptor-/- mice and that Western diet feeding increased plasma cholesterol concentrations to a similar magnitude in both sexes and led to equivalent atherosclerotic development. However, there was no difference on AngII-induced AAA formation in female mice fed the Western versus normal diet. Mechanisms of male gender preference in hypercholesterolemic mice remain to be defined.

In summary, this study demonstrated that increased plasma concentrations of apoB-containing lipoproteins are associated with augmentation of AngII-induced AAAs in a male-gender specific manner. Most interestingly, unlike
atherosclerosis, AngII-induced AAAs were not further augmented with progressive increases of plasma apoB-containing lipoprotein concentrations.
Figure 2.1 Western diet did not augment AngII-induced AAA formation in male C57BL/6 mice.  (A) Plasma cholesterol concentrations.  Histobars are means and error bars represent SEM.  * denotes P=0.03 by Mann-Whitney Rank Sum Test.  N=9 per group.  (B) Plasma lipoprotein distributions were resolved by size exclusion chromatography. Circles and error bars are means ± SEM.  N=6-8 per group.  CM: chylomicrons, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein.  (C) Maximal outer diameters of suprarenal aortas. Triangles are values from individual mice.  Circles represent means and error bars are SEM.  P=0.6 by Mann-Whitney Rank Sum Test.  N=9 per group.
Figure 2.2 Deficiency of ApoAI in male C57BL/6 mice did not exacerbate AngII-induced AAA formation. (A) Plasma cholesterol concentrations. Histobars are means and error bars represent SEM. * denotes P<0.001 by Student’s t test. N=9 per group. (B) Plasma lipoprotein distributions were resolved by size exclusion chromatography. Circles and error bars are means ± SEM. N=4 per group. CM: chylomicrons, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein. (C) Maximal outer diameters of suprarenal aortas. Triangles are values from individual mice. Circles represent means and error bars represent SEM. P=0.2 by Mann-Whitney Rank Sum Test. N=9 per group.
Figure 2.3 Deficiency of ApoAI had no effect on AngII-induced AAAs in male LDL receptor -/- mice fed a normal laboratory diet. (A) Plasma cholesterol concentrations. Histobars are means and error bars represent SEM. N=5-6 per group. (B) Plasma lipoprotein distributions resolved by size exclusion chromatography. Circles and error bars are means ± SEM. N=3 per group. (C) Percent atherosclerotic lesion area in the aortic arch regions. (D) Maximal outer diameters of suprarenal aortas. Triangles are values from individual mice, and circles represent means and error bars represent SEM (C and D). N=7 per group.
Figure 2.4 Deficiency of ApoAI did not exacerbate AngII-induced AAAs in male LDL receptor −/− mice fed Western diet. (A) Plasma cholesterol concentrations. Histobars are means and error bars represent SEM. N=11-13 per group. * denotes P< 0.001 by Mann-Whitney Rank Sum Test. (B) Plasma lipoprotein distributions resolved by size exclusion chromatography. Circles and error bars are means ± SEM. N=4-5 per group. (C) Percent atherosclerotic lesion area in the aortic arch regions. N=10-13 per group * denotes P= 0.03 by Student’s t test. (D) Maximal outer diameters of suprarenal aortas. N=11-13 per group. * denotes P=0.01 by Mann-Whitney Rank Sum Test. Triangles are values from individual mice, and circles represent means and error bars represent SEM (C and D).
Figure 2.5 Hypercholesterolemia increased AngII-induced AAA formation in male LDL receptor −/− mice. (A) Plasma cholesterol concentrations. Histobars are means and error bars represent SEM. * denotes P<0.001 by Mann-Whitney Rank Sum Test. N=10 per group. (B) Plasma lipoprotein distributions were resolved by size exclusion chromatography. Circles and error bars are means ± SEM. N=10 per group. CM: chylomicrons, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein. (C) Atherosclerosis in the aortic arch region. Triangles are values from individual mice. Circles represent means and error bars represent SEM. * denotes P<0.001 by Mann-Whitney Rank Sum Test. N=10 per group. (D) Maximal outer diameters of suprarenal aortas. Triangles are values from individual mice. Circles represent means and error bars represent SEM. * denotes P=0.002 by Mann-Whitney Rank Sum Test. N=10 per group.
Figure 2.6 Hypercholesterolemia did not increase AngII-induced AAA formation in female LDL receptor -/- mice. (A) Plasma cholesterol concentrations. Histobars are means and error bars represent SEM. N=10 per group. * denotes P<0.001 by Student’s t test. (B) Plasma lipoprotein distributions resolved by size exclusion chromatography. Circles and error bars are means ± SEM. N=5-7 per group. (C) Percent atherosclerotic lesion area in the aortic arch regions. N=10 per group. * denotes P<0.001 by Mann-Whitney Rank Sum Test. (D) Maximal outer diameters of suprarenal aortas. N=10 per group.
Figure 2.7  Modest and severe hypercholesterolemia had equivalent effects on AngII-induced AAA formation in male ApoE-/- mice.  (A)  Plasma cholesterol concentrations.  Histobars are means and error bars represent SEM.  * denotes P=0.01 by Mann-Whitney Rank Sum Test.  N=4-6 per group.  (B)  Plasma lipoprotein distributions were resolved by size exclusion chromatography.  Circles and error bars are means ± SEM.  N=4-5 per group.  CM: chylomicrons, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein.  (C)  Atherosclerosis in aortic arch region.  Triangles are values from individual mice.  Circles represent means and error bars represent SEM.  * denotes P=0.02 by Mann-Whitney Rank Sum Test.  N=4-6 per group.  (D)  Maximal outer diameters of suprarenal aorta.  Triangles are values from individual mice.  Circles represent means and error bars represent SEM for each group.  P=0.7 by Student’s t test.  N=4-6 per group.
Figure 2.8 Administration of ezetimibe reduced atherosclerosis, but not AngII-induced AAA formation in male ApoE-/- mice fed Western diet. (A) Plasma cholesterol concentrations. Histobars are means and error bars represent SEM for each group. * P=0.001 by Mann-Whitney Rank Sum Test. N=6-7 per group. (B) Plasma lipoprotein distributions were resolved by size exclusion chromatography. Circles and error bars are means ± SEM. N=4-7 per group. CM: chylomicrons, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein. (C) Atherosclerosis in the aortic arch region. Triangles are values from individual mice. Circles represent means and error bars represent SEM. N=6-8 per group. (D) Maximal outer diameters of suprarenal aortas. Triangles are values from individual mice. Circles represent means and error bars represent SEM. *P=0.965 by Student's t test. N=6-8 per group.
Figure 2.9 Administration of ezetimibe reduced AngII-induced AAA formation in male ApoE-/- mice fed normal laboratory diet. (A) Plasma cholesterol concentrations. Histobars are means and error bars represent SEM. * denotes P<0.001 by Student’s t test. N=6-8 per group. (B) Plasma lipoprotein distributions were resolved by size exclusion chromatography. Circles and error bars are means ± SEM. N=4-7 per group. CM: chylomicrons, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein. (C) Atherosclerosis in aortic arch regions. Triangles are values from individual mice. Circles represent means and error bars represent SEM. P=0.256 by Student’s t test. N=6-8 per group. (D) Maximal outer diameters of suprarenal aortas. Triangles are values from individual mice. Circles represent means and error bars represent SEM. P=0.009 by Student’s t test. N=6-8 per group.
Table 2.1 Characteristics of normal diet and Western diet fed mice

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<th>Strain</th>
<th>Diet</th>
<th>Gender</th>
<th>Body Weight</th>
<th>Systolic BP (mmHg)</th>
<th>Renin (ng/ml/30 min)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
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<td>C57BL/7</td>
<td>Normal Diet</td>
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<tr>
<td></td>
<td>Western Diet</td>
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<td>25.7 ± 0.4</td>
<td>187 ± 5</td>
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<td>Normal Diet</td>
<td>Male</td>
<td>25.6 ± 0.6</td>
<td>26.1 ± 0.6</td>
<td>189 ± 5</td>
</tr>
<tr>
<td>ApoA-I +/-</td>
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<td>23.5 ± 0.8</td>
<td>24.5 ± 0.6</td>
<td>182 ± 5</td>
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<tr>
<td>LDLr +/-</td>
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<tr>
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<td>LDLr +/-</td>
<td>Normal Diet</td>
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<td>19.6 ± 0.2</td>
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<td>19.8 ± 0.4</td>
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<td>24.1 ± 0.3</td>
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<td>Normal Diet + EZ</td>
<td>Male</td>
<td>24.3 ± 0.5</td>
<td>27.1 ± 0.7</td>
<td>185 ± 7</td>
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<tr>
<td>ApoE +/-</td>
<td>Western Diet</td>
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<td>24.9 ± 0.6</td>
<td>27.2 ± 0.8</td>
<td>167 ± 11</td>
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<tr>
<td>ApoE +/-</td>
<td>Western Diet + EZ</td>
<td>Male</td>
<td>24.5 ± 0.4</td>
<td>26.9 ± 0.4</td>
<td>187 ± 7</td>
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Table 2.2 Ratio of LDL/HDL in male mice infused with AngII for 28 days

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<tr>
<th>Mouse Strain</th>
<th>Normal diet</th>
<th>Western diet</th>
<th>P value</th>
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<tr>
<td>C57BL/6</td>
<td>0.15 ± 0.02</td>
<td>0.12 ± 0.02</td>
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<tr>
<td>LDL receptor -/-</td>
<td>1.38 ± 0.21</td>
<td>2.98 ± 0.35</td>
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</tbody>
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Chapter Three: ApoB-containing Lipoproteins Promote the Progression of Established AAAs in AngII Infused Mice

3.1 Introduction
Abdominal aortic aneurysm (AAA) is a devastating disease that exhibits gradual and permanent lumen expansion that is typically presenting in the infrarenal region. Population-based screening studies demonstrate that AAA is prevalent among aged population especially in males. As an AAA progresses, it remains asymptomatic until rupture occurs leading to sudden and fatal hemorrhage. Since the AAA size is the major predictor of rupture, attempts should be made to slow the progressive expansion of AAA. To date, there is no proven medical therapy that can prevent either dilation or rupture of AAA.

Experimental mouse models have demonstrated that hypercholesterolemia enhances development of AAA and there is accumulating evidence that it is also a contributor to human AAA pathologies (105, 227, 246, 251). Chronic infusion of angiotensin II (AngII) induces AAA formation in normocholesterolemic mice (49, 110, 113-115, 233) with a much lower incidence than in hypercholesterolemic mice (56-58). Human observational studies demonstrated a positive correlation of plasma LDL-C concentrations and the presence of human AAA (105, 227, 246, 251).

Although numerous pharmacological targets have been explored to address mechanisms contributing to the initiation of AAAs, few studies have investigated pharmacological approaches that would inhibit the progression of established AAA. A recent study demonstrated prolonged infusion of AngII into hypercholesterolemic mice promoted further lumen expansion (65) which implicated a potential role of hypercholesterolemia in the progression of established AAA induced by AngII infusion. In line with this finding, a meta-analysis including more than 9,000 patients from 7 high-quality observational studies demonstrated a statistically significant benefit of statin therapy for growth rate in patients with small AAAs. Although the mechanism underlying
lipid-lowering treatment is undefined, statins are commonly administered to AAA patients to reduce aortic expansion (110, 112).

To determine the role for hypercholesterolemia in progression of established AAA in mice with prolonged infusion of AngII, we performed a diet switch of Western to normal diet that led to a quick and profound reduction in plasma cholesterol concentration within one week. Consistent with previous study, lumen expansion progressed in mice maintained on Western diet; while no further expansion was detected in mice that were switched to normal diet.

3.2 Methods

3.2.1 Mouse Housing Condition and Diets

Male LDL receptors -/- mice (8 weeks of age) were purchased from The Jackson Laboratory (Stock # 2207; Bar Harbor, ME, USA). Mice were maintained in individually vented cages (maximally 5 mice/cage) on a light:dark cycle of 14:10 hours. The cage bedding was Teklad Sani-Chip bedding (Cat # 7090A, Harlan Teklad, Madison, WI, USA). Mice were fed a normal rodent laboratory diet (Diet # 2918, Harlan Teklad; Madison, WI, USA) and given drinking water from a reverse osmosis system ad libitum. To induce AAAs, all mice were infused with AngII (1,000 ng/kg/min) for 4 weeks and fed a Western diet supplemented with saturated fat extracted from milk (21 %, wt/wt) and cholesterol (0.15 % wt/wt supplemented, 0.05 % wt/wt from the fat source; Diet # TD.88137; Harlan Teklad). On day 27 of AngII infusion, lumen diameter of suprarenal aortas was measured using noninvasive high frequency ultrasound (Vevo 2100, Visualsonics, Toronto, Ontario, Canada). AAAs were defined as 50% increase of maximal lumen diameter compared to baseline in the suprarenal aortas. Based on lumen diameter, mice exhibiting AAAs were evenly stratified into two groups, that were either continuously fed western diet or switched to a normal diet. All mice were infused with AngII for an additional 8 weeks.
3.2.2 Osmotic Mini Pump Implantation and AngII infusion
AngII (1,000 ng/kg/min; Cat# H-1706; Bachem; Torrance, CA, USA) was infused subcutaneously via Alzet osmotic mini pumps (Alzet Model # 2006; Durect; Cupertino, CA, USA) for 42 days. Pumps were pre-incubated in saline at 37°C for 60 hours. All mice were implanted with new mini pumps (Alzet Model # 2006) at day 43 to permit continuous delivery of AngII for another 6 weeks. Mice were sedated with isoflurane and pumps were implanted subcutaneously on the right flank of each mouse. Surgical staples were used to close the incision site and a topical anesthetic cream (LMX4; Ferndale Laboratories; Ferndale, MI) was applied immediately after surgery to relieve pain.

3.2.3 Systolic Blood Pressure Measurements
Systolic blood pressure was measured using a standardized protocol described previously on conscious mice for 3 consecutive days by a non-invasive tail-cuff system (Coda 8; Kent Scientific; Torrington, CT, USA). Systolic blood pressure was measured one week before mini pump implantation (baseline) and repeated on week 9 during AngII infusion.

3.2.4 Ultrasound Measurement and 3-dimensional Imaging
Reconstruction
Lumen diameter of suprarenal aortas was measured using the Vevo 2100 ultrasound imaging system (Visualsonics, Toronto, Ontario, Canada). Mice were anesthetized with isoflurane and restrained in a supine position. Short-axis scans (100 frames of cine loops) were acquired from the level of the left renal artery moving cephalically to the suprarenal region. The maximal lumen diameter of the suprarenal region was measured on collected images during the aortic dilation phase. To reconstruct three-dimensional (3D) images, mice were whole-body fixed by 10% naturally buffered formalin perfusion in vivo to preserve aortic structure. Aortas were subsequently dissected and placed in formalin overnight at room temperature. Periaortic adventitia was then carefully removed. Cleaned aortas were submerged in saline solution. Ex vivo 3D mode
was performed on abdominal aortas to obtain sequential 2D cross-section images. Vevo software was used to assemble the serial scans into 3D images and for quantification of total and lumen volumes of AAA. Vascular volumes of AAA were calculated by subtracting lumen volumes from total volumes. To compare volumes between two groups, we reconstructed 15 mm length of AAA segments with the most dilated lumen at the center to minimize variations caused by heterogeneity of AAA pathologies.

3.2.5 Measurement of Plasma Components
Detail was described in Chapter 2 (2.2.4).

3.2.6 Quantification of Aortic pathologies
Detail was described in Chapter 2 (2.2.5).

3.2.7 Histological Staining and Immunohistology
We chose whole-body perfusion fixed mice with suprarenal aortic diameters that were similar to the mean for each group for histological analysis of AAAs. 4 mm above right renal artery of suprarenal aortas were carefully excised and placed in OCT. Serially cross-sections were performed (10 μm thick/section, series of 10 slides with 9 sections/slide) from the proximal to the distal as described previously (64, 65). H&E and Movat’s pentachrome stainings were used to determine morphological characteristics and elastin fibers. Oil Red-O staining was used to examine the neutral lipid and triglyceride deposition. Immunostaining was performed to identify macrophages and smooth muscle cells as described previously (252). The following antibodies were used: α-actin for smooth muscle cells (Cat # ab5694; Abcam; Cambridge, MA) and CD68 for macrophages (Cat#MCA1957GA, Serotec; Raleigh, NC); Biotin labeled secondary antibodies (Cat # BA-4001 and BA-6000; Vector Laboratories; Burlingame, CA); horseradish peroxidase ABC kits (Peroxidase Elite Standard; Cat # PK-6100; Vector Laboratories; Burlingame, CA) and 3-amino-9-ethylcarbazole for chromogen (ImmPACT™; Cat # SK-4205; Vector Laboratories; Burlingame, CA). Non-immune primary antibodies, secondary only, and no primary or secondary antibody slides were run as negative
controls to confirm specificity of primary and secondary antibodies, and ablation of endogenous tissue peroxidase, respectively.

3.2.8 Primary Aortic Smooth Muscle Cell Culture
Primary aortic smooth muscle cells isolated from C57BL/6 (Cell Biologics; Cat # C57-6080; Chicago, IL) were grown in a 5% CO2 atmosphere at 37 °C in DMEM supplemented with 1% nonessential amino acids, 20% FBS and 1% penicillin and streptomycin. Cells were grown to confluence and serum starved for 24 hours before use.

3.2.9 Isolation of Plasma Lipoproteins
To prepare LDL and VLDL, blood from western diet fed LDL receptor -/- mice (N=10) was collected by right ventricular puncture with syringes containing EDTA (final concentration: 1.8 mg/ml) and pooled, then centrifuged at 400 g x 20 minutes at 4 °C to separate plasma. LDL and VLDL were isolated from plasma samples by sequential ultracentrifugation. Briefly, plasma samples were carefully loaded into 3.5 ml Quick-Seal centrifuge tubes partially filled with saline (1.006 g/ml) and centrifuged for 3 hours at 100 K rpm (TLA 100.3 rotor, Beckman instrument) to float up the VLDL. Then the density of remaining plasma was adjusted to 1.063 g/ml with solid KBr and carefully loaded under a layer of 1.061 g/ml KBr solution. Ultracentrifugation was performed at 100 K rpm for 3 hours. Isolated LDL and VLDL were placed into dialysis bags (Molecular weight cutoff: 12-14 kD; Spectra; Cat # 132676) and placed in 1 liter of cold EDTA saline solution in the cold room. Dialysis fluid was changed every 1 hour for two times.

3.2.10 RNA Isolation and Real-time PCR
Total RNA in primary aortic smooth muscle cells was extracted with a RNaseasy Mini Kit (Qiagen), and then reversely transcribed with an iScript™ cDNA Synthesis Kit (Cat#170- 8891; Bio-Rad, Hercules, CA). Real time PCR was performed to quantify mRNA abundance using a SsoFast™ EvaGreen® Supermix kit (Cat# 172-5203; Bio-Rad) on a Bio-Rad CFX96 cycler. Data were analyzed using ∆∆Ct method, normalized to β-actin abundance. Primers used
for AT1a Receptor and β-actin in real-time PCR were 5’-ACTCACAGCAACCCTCCAAG, 5’ ATCACCACCAAGCTGTTTCC and 5’-GCCTTCCTTTGGGTATGG, 5’-GCACTGTGTTGGCATAGAGG, respectively.

3.2. 11 Statistical Analysis
Data were analyzed using SigmaPlot version 12 (SYSTAT Software Inc., Chicago, IL, USA). Data are represented as means ± standard error of means (SEM). To compare three study groups in each experiment on a continuous variable, one way ANOVA was performed for normally distributed and equally variant values, and Kruskal-Wallis One Way Analysis rank sum test were used for non-normally distributed variables. Systolic blood pressure was analyzed using two-way repeated measures ANOVA. P < 0.05 was considered statistically significant.

3.3 Results
3.3.1 Characteristics of Study Mice
To induce AAA, eighty male LDL receptor -/- mice were fed Western diet one week prior to and four weeks during AngII infusion (1,000 ng/kg/min). During the 28-day infusion, 9 mice died of aortic rupture. On day 27, the abdominal aortas of remaining mice were scanned by ultrasound to determine presence of AAA. Mice with AAAs were randomly stratified and divided into two groups based on their abdominal aortic lumen diameter. After stratification, mice either remained on the Western diet (n=17) or were switched to a normal laboratory diet (n=17). AngII infusion was continued for an additional 8 weeks. The experimental design for this study is illustrated in Figure 3.1. Body weight was not significantly different between the mice either remained on Western diet or switched to a normal diet (Table 3.1). During the prolonged interval, 1 mouse died of aortic rupture in the group remaining on Western diet and 2 mice died of aortic rupture in the diet reversal group, respectively.
3.3.2 Western Diet Withdrawal Led to Rapid Reductions of Plasma Total Cholesterol Concentrations

Mice fed Western diet were significantly hypercholesterolemic compared with the baseline (1469 ± 59 mg/dl versus 250 ± 9 mg/dl). In order to determine the effect of reduction of plasma cholesterol concentrations on established AAA progression, hypercholesterolemia was reversed by switching Western diet to a normal laboratory diet after stratification. Strikingly, diet reversal resulted in significantly reduced plasma cholesterol concentrations within one week (535 ± 31 mg/dl versus 1621 ± 62 mg/dl) which were maintained for the remaining interval (two-week diet switch: 382 ± 22 mg/dl versus 1845 ± 69 mg/dl; at termination: 1661 ± 54 mg/dl versus 312 ± 12 mg/dl; Figure 3.2A). The lipoprotein profile was determined by size exclusion chromatography at study termination. The reduction of plasma cholesterol concentrations were solely attributed to decreased apoB-containing lipoproteins that included chylomicrons, chylomicron remnants, very low density lipoprotein (VLDL), and LDL (Figure 3.2B).

3.3.3 Decreased ApoB-containing Lipoproteins Attenuated Lumen Expansion of AngII-induced AAA

Lumen diameter of the suprarenal region was monitored by ultrasound during the study. Consistent with our previous report (65, 66), in mice maintained on Western diet, lumen diameter increased progressively with protracted AngII infusion. In contrast, further expansion of abdominal aortic lumen diameter was not detected in mice switched to a normal diet. This significant attenuation measured with ultrasound was confirmed at study termination by ex vivo measurement of suprarenal aortas (Figure 3.3A and B).

3.3.4 Reductions of ApoB-containing Lipoproteins Contributed to Reduced Aortic Remodeling

The profound reduction of apoB-containing lipoproteins resulted in decreased lumen and external diameter in diet reversal mice. To better understand and provide more insight into the disease, we performed three-dimensional (3D)
AAA reconstruction imaging. We reconstructed 15 mm length of AAA with the most dilated lumen at the center to minimize the variations caused by the heterogeneity of AAA pathologies (Figure 3.4A). We quantified the total aortic volumes, lumen volumes and vascular volumes which were calculated by subtracting the lumen volumes from the total aortic volumes. The diet reversal mice with profound reductions of apoB-containing lipoproteins exhibited lower total aortic volumes, lumen volumes and vascular volumes (Figure 3.4B).

3.3.5 Reduction of ApoB-containing Lipoproteins Attenuated Medial Thickening and Inflammation

Pathologies of AngII-induced AAA exhibit high heterogeneity and complex features even along the length of a single aneurysm (64, 65). To determine whether profound reductions of apoB-containing lipoproteins influenced AAA pathological characteristics, entire AAA segments were serially cross-sectioned and examined at 100 μm interval throughout the aneurysmal tissue to account for the considerable heterogeneity along the length of the aorta. Prolonged AngII infusion in mice maintained on Western diet resulted in significant lumen expansion associated with profound medial thickening around the AAA segment. Adjacent to the dilated region, adventitia was also thickened. In contrast, in the diet reversal mice, the adventitia and medial thickening were significantly attenuated and had less macrophage accumulation in the media (Figure 3.5).

3.3.6 Effects of ApoB-containing Lipoproteins on AT1a Receptor in Aortic Smooth Muscle cells

Elevated concentrations of native LDL (253) have been reported to upregulate gene expression of the AT1 receptors and its functional response to the stimulation of AngII both in vitro and in vivo. (253, 254) A human study also demonstrated hypercholesterolemia induced AT1 receptor overexpression and enhanced biological effects of AngII in men (255). Therefore, we examined whether apoB-containing lipoproteins promoted expansion of AAA through the interaction between AngII and the upregulated AT1a receptor. Since LDL and VLDL are the major components of apoB-containing lipoproteins and the
inflammatory response was the most prominent in the media, we investigated the effects of LDL and VLDL on AT1a receptor gene expression in cultured primary aortic vascular smooth muscle cells (VSMCs). Cells were grown to confluency and serum was removed from the culture medium 24 hours before initiation of experimental treatment. Reports indicate that AT1 receptor mRNA significantly increases 12 hours after exposure to LDL and this increase is sustained for up to 24 hours (253). To determine LDL-induced enhancement of AT1a receptor expression, cells were incubated for 24 hours with 0, 25, 50, 100, and 200 ug/ml LDL or VLDL. However, neither LDL nor VLDL significantly altered AT1a receptor expression in the mouse primary aortic smooth muscle cells (Figure 3.6A and B).
3.4 Discussion

Dysregulated lipoprotein fractions, especially apoB-containing lipoproteins are associated with development of human AAA. A few studies also implicated statins, the lipid-lowering drugs, were beneficial of reducing AAA growth rate. The rupture of AAA is a devastating event that occurs during the progression of this disease with the aneurysm size is the most robust predictor. The risk of rupture increases exponentially with elevated aneurysm diameter. In this study, we determined the influence of apoB-containing lipoproteins in mice with established AAA. To achieve this, AAA was induced by 28 days AngII infusion in western diet fed mice. Then we evenly stratified the mice with AAA into two groups based on their lumen diameter. They were either maintained on Western diet or switched to a normal laboratory diet for an additional 8 weeks of AngII infusion. Of note, significantly decreased plasma cholesterol concentrations were noted within one week after diet switching and they were maintained for the remaining study interval. Significant attenuation of established AAA progression in diet switched group was detected by ultrasound during the study and this observation was confirmed by ex vivo measurement of suprarenal region at termination. This profound attenuation was associated with less vascular remodeling and inflammatory accumulation.

Hypercholesterolemia reversal has been achieved in several mouse models. Microsurgical transplantation of arterial segments containing atherosclerotic plaques from hypercholesterolemic mice into normocholesterolemic counterparts has been used to investigate the plaque regression (264-266). Hsiao and colleagues developed “Reversa mice” in which using Cre/loxP techniques, the conditional alleles of microsomal triglyceride transfer protein (Mttp) were switched off in the liver. They bred LDL receptor -/- mice that were homozygous for an apoB-100-only allele with mice that were homozygous for a conditional Mttp allele and Mxl-Cre transgene. Offspring were bred to produce mice that were homozygous for all 4 alleles (LDL receptor -/- apoB^{100/100} Mttp^{fl/fl} Mxl-Cre^{+/+}) To inactivate Mttp, Cre expression was induced by intraperitoneal
injections of polyinosinic-polycytidylic ribonucleic acid (pi-pC) every other day for a total of 4 injections (267). Another study conducted by Maganto-Garcia et al also reversed just by simply switched the diet fed LDL receptor -/- mice. They designed a study in which mice were fed a cholesterol diet for 4 weeks and then a cholesterol-free diet for an addition 4 weeks to reverse the hypercholesterolemia. The diet switch significantly decreased plasma cholesterol concentration to the level of mice on control diet. Considering the practical limitation of time and money-consuming for the complex mouse breeding and technical issues for the transplantation surgery, in our study, the reduction of apoB-containing lipoprotein was achieved by diet switch. We found statistically significant reduction of plasma cholesterol concentrations as early as day 3 of diet switch. So our diet switching protocol might be a potentially easier and less costly procedure design for investigating reversal of hypercholesterolemia.

Nickenig and colleagues demonstrated that elevated native LDL upregulates the AT1a receptor gene expression and its biological functions. In this regard, we performed the similar experiments-we isolated the mice LDL and VLDL and cultured them with primary SMCs. However, we were unable to identify upregulation of AT1a receptors in mouse primary aortic smooth muscle cells. It is known that the expression pattern of AT1a receptors along the entire aorta is different and the abdominal region is more susceptible to AAA formation. Therefore, the isolation of smooth muscle cells from abdominal aorta might be more relevant. We previously demonstrated although the whole body AT1a receptor deficiency attenuated AngII-induced AAA, the deletion of AT1a receptors on single type did not prevent aneurysm formation. This implicating orchestrated contributions of multiple cell types in the interaction of AngII and AT1a receptor to promote AAAs. Therefore, multiple cell type co-incubations should be optimal to investigate regulation of AT1a receptors and interactions and cross-talking among different vascular cell types in a hypercholesterolemic environment.
Figure 3.1 Overall experimental design. Male LDL receptors -/- mice fed western diet were infused with AngII for 28 days to induce AAAs. On day 27, the abdominal aortas of all mice were scanned by ultrasound to determine the presence of AAAs. Mice with AAAs were randomly stratified and divided into two groups which remained on western diet or were switched to a normal diet. AngII infusion was continued for another 8 weeks for all mice.
Figure 3.2 Diet reversal significantly reduced plasma cholesterol concentrations in mice with established AAA. (A) Plasma cholesterol concentrations. Circles and error bars are means ± SEM. * denotes P< 0.001 by Two-way repeated measurement ANOVA. N=9-16 per group. (B) Plasma lipoprotein distributions were resolved by size exclusion chromatography at study termination. Circles and error bars are means ± SEM. N=11 per group. CM: chylomicrons, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, and HDL: high-density lipoprotein.
Figure 3.3 Profound reduction of apoB-containing lipoproteins by diet switch attenuated further lumen expansion in mice with established AAA. (A) Suprarenal aortic lumen diameter measured by ultrasound at various time points (Left). Ultrasound images of representative aortas in each group measured at study termination illustrated less expansion in diet switch group (Right). Circles and error bars are means ± SEM. * denotes P< 0.001 by Two-way repeated measurement ANOVA. N=15-16 per group. (B) Maximal outer diameters of suprarenal aortas. Triangles are values from individual mice. Circles and error bars are means ± SEM. * denotes < 0.001 by Mann-Whitney Rank Sum Test. (C) Aortas from mice in each group.
Figure 3.4 Profound reduction of apoB-containing lipoproteins by diet switch attenuated aortic remodeling. (A) Three-dimensional (3D) images of a representative AAA from a mouse in each group. (B) Volumes of suprarenal aortas were measured on 3D reconstructed images of ex vivo AAAs. Triangles are values from individual mice. Circles and error bars are means ± SEM. * denotes P< 0.05 Student’s t test.
Figure 3.5 Representative serial cross-sections from abdominal aortas were immunostained for SMCs (α-actin) and macrophages (CD68). Red color indicates positive staining.
Figure 3.6 AT1a receptor mRNA did not increase in primary aortic smooth muscle cell cultured with various LDL (A) and VLDL (B) concentrations. Histobars are means and error bars represent SEM. N=2 per group.
Table 3.1 Characteristics of mice maintained on Western diet or switched to normal diet with continuous AngII infusion

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Body weight</th>
<th>Systolic BP (mmHg)</th>
<th>Renin (ng/ml/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Stratification</td>
<td>Final</td>
</tr>
<tr>
<td>Western diet</td>
<td>16</td>
<td>22.7 ± 0.3</td>
<td>26.4 ± 0.3</td>
<td>27.4 ± 0.7</td>
</tr>
<tr>
<td>Diet switch</td>
<td>15</td>
<td>23.1 ± 0.4</td>
<td>27.0 ± 0.5</td>
<td>28.1 ± 0.6</td>
</tr>
</tbody>
</table>
Chapter Four: General Discussion and Future Directions

4.1 Section One
Aberrant lipoprotein metabolism, particularly LDL and HDL fractions, is associated with many cardiovascular diseases. Dysfunctional lipoprotein metabolism has also been implicated in human AAA formation, although associations have not been studied extensively. In Chapter 2, we used multiple mouse strains with dietary and pharmacological manipulations to create different forms and severities of dyslipidemia to determine whether specific lipoprotein fractions directly contributed to AngII-induced AAA formation. We were unable to identify any effects of reduced HDL-cholesterol concentrations, promoted by apoAI deficiency, on AngII-induced AAA formation in two mouse strains with dietary manipulations. However, our findings clearly demonstrated that hypercholesterolemia, especially the large sized lipoprotein particles, augmented the development of AngII-induced AAAs in mice. The only feature in common for these lipoproteins was the presence of apoB. However, unlike atherosclerosis in which plasma apoB-containing lipoprotein concentrations closely correlated with lesion size, AAAs were augmented under modestly hypercholesterolemic states, but were not further enhanced with progressive increases in hypercholesterolemia.

4.1.1 Dysregulation of Lipoproteins in Human AAA
There is accumulating evidence of dysregulated lipoproteins, particularly HDL and LDL fractions, being critical contributors to the prevalence and severity of human AAA. To address this issue, the concentrations of blood lipids and apolipoproteins in subjects were measures in the reported studies. The first study published by Norrgard and colleagues (104), reported an association between human HDL-C and AAA. They observed the concentration of HDL-C in serum was lower in patients than in the healthy controls. However, a subsequent study conducted by Louwrens et al (106) reported AAA patients had a higher HDL-C concentration compared with the control. In contrast with this latter study, several studies subsequently showed that the presence of
human AAA was negatively associated with the level of both HDL-C or apoAI, the predominant structural apolipoprotein of HDL (14, 105, 107, 109, 227, 237, 238, 246, 256). In addition, a study by Femke et al found a negative association between serum HDL-C with human aneurysm size (257). A later study demonstrated serum apoAI is inversely associated with AAA size and thrombus volume in patients. Of note, this study also demonstrated HDL-C negatively correlated with growth rate of AAA and the need for later repair (245).

Besides abnormal HDL-C, the contribution of dysregulated LDL-C to human AAA has also drawn attention for many decades. It was observed that AAA patients had higher (VLDL+LDL)-cholesterol or total cholesterol concentrations than healthy controls (104, 227, 258). However, several studies identified LDL-C as the only independent predictor of the presence of human AAA (105, 247, 251). Of note, although the above studies implicate the alteration of plasma lipids might contribute to the AAA, one single study identified the size of LDL was independently associated with the presence of human AAA (109). In contrast to these findings, a study conducted by Golledge (237) reported the concentration of LDL-C was not directly associated with the presence of human AAA, which was confirmed by a subsequent meta-analysis (246). Regardless of this undefined association, statins, the lipid-lowering drugs, are commonly used for AAA patients to reduced aortic expansion. In line with this, a recent meta-analysis published by a Japanese group (259) demonstrated a statistically significant benefit of stain therapy for growth rate in patients with small AAA.

Overall, the non-consistent findings and limited number of studies impede the understanding of the impact of dysregulated lipoprotein fractions on human AAA. The interpretation of the results from those studies could be greatly confounded by the coexistence of other cardiovascular diseases and the use of multiple medications. Given this undefined association and complex situation in humans, using our in vivo system with genetic and pharmacological manipulations in multiple mouse models, we overcame these issues and
provided direct evidence for an association between dysregulated lipoproteins and the development of AAA. On the basis of our data, generated from multiple mouse models to manipulate apoAI- or apoB-containing lipoproteins, we conclude that apoB-containing lipoproteins augmented the development of AngII-induced AAA. However, unlike the linear correlation between atherosclerotic size and plasma apoB-containing lipoproteins, AAA was exacerbated by modestly increased apoB-containing lipoproteins but not enhanced with further increase of hypercholesterolemia. This result clearly demonstrated the divergent mechanisms underlying the pathological processes of atherosclerosis and AAA which require further elucidation. One interesting question remains whether this “threshold” effect of AAA development observed in our mouse studies also applies to observational human studies. One indication reported by Forsdahl et al is the increased risk of AAA was only observed in subjects with the highest reading (highest quartile) of total cholesterol; however, no statistically significant association was observed in other quartiles (227). If this “threshold” effect in mouse also applies to human AAA, the conflicting results might be explained as different criteria of plasma cholesterol concentrations in different studies. If the level of plasma cholesterol concentrations in the study is below or above the threshold, no direct correlation would be observed. So further investigation is needed to elucidate the association between apoB-containing lipoproteins and human AAA.

4.1.2 Role of HDL in AngII-induced AAA
There is a large body of evidence of abnormal HDL-C being a major contributor of numerous cardiovascular diseases. HDL plays a pivotal role in reverse cholesterol transport (RCT), a pathway by which cholesterol is transported from peripheral tissues to the liver where it is secreted through biliary excretion. RCT is a key pathway in maintaining cholesterol balance. In addition to its role in promoting the RCT pathway, HDL possesses anti-oxidative, anti-inflammatory, anti-thrombotic, anti-apoptosis and anti-platelet activity properties (260). This broad spectrum of effects for HDL and the similarity of risk factors between
AAA and other cardiovascular diseases provide the basis for potential therapeutic benefits of HDL in the treatment of AAA. In line with this, one study reported by Torsney et al demonstrated that exogenous administration of pooled native HDL or reconstituted HDL reduced AngII-induced AAA in both AngII and CaCl\textsubscript{2} mouse models (124). The reconstituted HDL, CSL111, is composed of human apoAI and soybean phosphatidylcholine thereby resembling native HDL. It has been demonstrated that these nascent particles will be rapidly remodeled with endogenous lipoproteins into the mature HDL particles within one hour after intravenous injection (261). Another recent study conducted by Burillo et al reported reduction of AAA development by administration of ApoAI mimetic peptide D4F (245). In their study, an apoAI mimetic peptide was intraperitoneally injected into AngII infused apoE -/- mice during progression of the disease. The development of AAA was monitored by high frequency ultrasound with a VEVO770 system. They observed a decreased lumen expansion in the D4F treated group compared with control counterparts.

To better understand why C57BL/6 mice with HDL fractions as the predominant lipoproteins were protected from AngII-induced AAA formation, we performed the converse study in which plasma HDL concentrations were reduced markedly by genetic deficiency of apoAI. ApoAI deficiency significantly reduced HDL-C while it did not augment AngII-induced AAAs in C57BL/6 mice fed a normal laboratory diet. Effects of apoAI deficiency were also studied in male LDL receptor-/- mice. Since apoAI deficiency was hypothesized to enhance AngII-induced AAA formation, infusion rates of AngII were selected to create a low incidence of AAAs to demonstrate the enhancement of AAAs in apoAI-/- mice. We compared AngII-induced AAAs using an infusion rate of 1000 ng/kg/min between apoAI +/+ and -/- mice fed normal diet and a rate of 500 ng/kg/min between apoAI +/+ and -/- mice fed western diet. Consistent with finding in C57BL/6 mice, apoAI deficiency in LDL receptor-/- mice did not exacerbate AngII-induced AAA formation.
The mechanisms by which exogenously delivered ApoAI and its endogenous reduction have differential effects on AngII-induced AAAs are unclear. A critical question is whether HDL or its major apolipoprotein ApoAI involved during the formation of AngII-induced AAA? It is noteworthy that HDL is more complex than originally proposed. In addition to the major apolipoproteins in HDL, it has been reported that there are over 40 different proteins and various potent bioactive signaling molecules, such as paraoxonase and vitamin E, associated with HDL particles (262). These proteins and molecules have a large spectrum of functions involving inflammation, proteolysis and oxidation pathways. For example, paraoxonase is an enzyme carried and transported by HDL particles that could neutralize oxidized lipids. It is known that overexpression of this enzyme decreased atherosclerosis while the deficiency exacerbated the atherosclerosis. So this implicates many of these beneficial roles of HDL were likely to be indirect consequences of the interaction between HDL on cells and possibly related to its ability to transport some of these proteins and molecules. Of note, although administration of reconstituted HDL prevented the formation of AngII-induced AAA and attenuated the expansion of AAA in CaCl₂ model, the elevation of HDL in this study was only minimal which might suggest that the protective role of HDL could be mediated by those transported proteins.

ApoAI consists of 243 amino acids and its secondary structure contains 10 amphipathic α-helices that are critical for the interaction with lipids. Studies on HDL/apoAI-based-therapy provide convincing evidence on their protective role in atherosclerosis. However, a major practical limitation of these therapies is the large quantity of HDL that is needed for treatment. In this regard, considerable investigations have been made to develop apoAI mimetic peptides containing the α-helix that mimics functional properties of apoAI. The D4F peptide is the most widely studied apolipoprotein mimetic peptide. In the Burillo et al study, although HDL-C and apoAI concentrations were not reported after the administration of D4F, another study has demonstrated that
administration of D4F did not affect plasma or HDL cholesterol levels (263). If the HDL or apoAI are critical in the development of AAA, it is interesting to answer why administration of D4F profoundly attenuated the AngII-induced AAA formation without overt effects on HDL-C level? It has been noticed that administration of D4F increased pre-β HDL which was enriched in paraoxonase activity and led to increased HDL anti-oxidant capacity and decreased atherosclerosis (263).

Taken together, these two reports (124) did not contradict with our findings. Instead, these results suggest that the investigation of HDL function as a carrier for other critical proteins and signaling molecules other than the actual concentration of HDL or apoAI might be more interesting.

4.2 Section Two
Since clinical intervention would be initiated following detection of an established AAA, in Chapter 3, we determined the influence of apoB-containing lipoproteins in mice with established AAA. To achieve this, AAA was induced by 28 days AngII infusion in western diet fed mice. Then we evenly stratified the mice with AAA into two groups based on their lumen diameter. They were either maintained on Western diet or switched to a normal laboratory diet for an additional 8 weeks of AngII infusion. Significantly decreased plasma cholesterol concentrations were noted within one week after diet switching and they were maintained for the remaining study interval. Significant attenuation of established AAA progression in diet switched group was detected by ultrasound during the study and this observation was confirmed by ex vivo measurement of suprarenal region at termination. This profound attenuation was associated with less vascular remodeling and inflammatory accumulation. We concluded Chapter 3 by demonstrating apoB-containing lipoproteins contributed to the progression of established AAA and provided evidence on the potential therapeutic benefit of a lipid-lowering treatment during the progression of established AAA.
4.2.1 Plasma Cholesterol Concentration Reversal

The hypercholesterolemic mouse models have become a critical platform for atherosclerosis research. Studies with these mice have explored the impacts of genes and pharmacological manipulations on the development of atherosclerosis and other related cardiovascular diseases thereby facilitating mechanistic understanding of interventions during disease progression. The reversal of hypercholesterolemia is also important to investigate during plaque regression, delayed progression, and remodeling and stabilization of atherosclerotic lesion.

The microsurgical transplantation of arterial segments has been used in numerous rodent models. Syngeneic transplantation of descending thoracic aortas containing advanced atherosclerosis from hypercholesterolemic mice into normocholesterolemic counterparts have been used to determine the development of advanced atherosclerosis. In one report, thoracic aortic segments containing preexisting atherosclerotic lesions from aged apoE -/- mice were transplanted into the infrarenal abdominal aortas of wild-type normolipidemic recipients. The marked regression of atherosclerotic lesion was observed (264). In another study, thoracic aortas from apoE -/- mice were transplanted in apoE -/- mice that expressed human ApoAI. The elevated human HDL in the recipients halted progression of established atherosclerosis (265). Subsequently, elegant work by Chereshnev and colleagues provided a new mouse model using heterotopic aortic arch transplantation with more severe lesion compared with segments of descending aortas and demonstrated this was a useful alternative model for the investigation (266).

In developing a hypercholesterolemic mouse model in which plasma cholesterol concentrations could be reversed with a simple intervention, Hsiao and colleagues developed “Reversa mice” in which using Cre/loxP techniques,
the conditional alleles of microsomal triglyceride transfer protein (Mttp) were switched off in the liver. They bred LDL receptor -/- mice that were homozygous for an apoB-100-only allele with mice that were homozygous for a conditional Mttp allele and Mxl-Cre transgene. Offspring were bred to produce mice that were homozygous for all 4 alleles (LDL receptor -/- apoB100/100 Mttpfl/fl Mxl-Cre+/+) and named “Reversa mice”. To inactivate Mttp, Cre expression was induced by intraperitoneal injections of polyinosinic-polycytidylic ribonucleic acid (pI-pC) every other day for a total of 4 injections. Hypercholesterolemia could be reversed in these mice without intervention of diet or lipid-lowering medication (267).

Another study conducted by Maganto-Garcia et al also reversed hypercholesterolemia in order to investigate regulatory T cells function (268). In their study, they just simply switched the diet fed LDL receptor -/- mice. They designed a study in which mice were fed a cholesterol diet for 4 weeks and then a cholesterol-free diet for an addition 4 weeks to reverse the hypercholesterolemia. The diet switch significantly decreased plasma cholesterol concentration to the level of mice on control diet.

Consistent with the Maganto-Garcia study, investigating the role of apoB-containing lipoproteins on established AAA, we also reversed hypercholesterolemia by simply switching the Western diet to normal laboratory diet. We found within one week of diet switching, plasma cholesterol concentration was profoundly reduced and maintained for the remaining interval. There is no doubt that reversal of hypercholesterolemia by gene switch off and surgery are useful mouse models. However, the practical limitations are the time and money-consuming for the complex mouse breeding and technical issues for the transplantation surgery. So our diet switching protocol might be a potentially easier and less costly procedure design for investigating reversal of hypercholesterolemia.
4.2.2 Effects of LDL on AT1a Receptor Regulation

AngII is the main bioactive peptide of the RAS system. Its principle functions are involved in the modulation of blood pressure, maintaining water and sodium homeostasis, neuronal function and other neurohumoral systems (269, 270). Although initial investigation with AngII focused on its role in the pathogenesis of hypertension, emerging evidence has indicated that AngII is a critical effector in development of atherosclerosis, myocardial infarction, vascular remodeling and heart failure (271-274). Effects of AngII are mediated through the interaction with its receptors. The main AngII receptors are G protein-couple receptors designated as angiotensin receptor type 1 (AT1) and angiotensin receptor type 2 (AT2). There are two subtypes of the AT1 receptors (AT1a and AT1b) in rodents.

There is evidence that the renin angiotensin system contributes to experimental AAAs. Inhibition of angiotensin-converting enzyme (ACE) or AT1 receptors reduces elastase-induced AAAs (50-53) or decellularise aortic xenograft-induced AAAs (54) in rats and multiple mouse models (55). Besides this evidence that implicates the endogenous activation of AngII in the pathogenesis of AAA, more direct evidence from animal models is AAA formation and rupture in mice infused with AngII. Moreover, the whole body deletion of AT1a receptor diminishes AngII-induced AAA (131). In the AngII-induced AAA mouse model, chronic AngII infusion into normocholesterolemic mice induces AAAs has ranged from 5% to 40% (49, 101, 113-115). In contrast, the incidence of AngII-induced AAAs in hypercholesterolemic mice has routinely been over 70% (81, 101, 113-115). Although this enhancement of AngII-induced AAA formation by hypercholesterolemia has been observed, the underlying mechanism is still poorly defined.

Studies by Nickenig and colleagues demonstrated that elevated native LDL upregulates the AT1a receptor gene expression and its biological functions in both in vitro and in vivo. They also observed overexpression of AT1 receptor
and enhanced biological effects in hypercholesterolemic men when stimulated by AngII infusion. The elevated AT1a receptor by hypercholesterolemia might be a potential mechanism to enhance AAA formation in response to AngII stimulation. So we performed similar experiments using LDL and VLDL isolated from hypercholesterolemic mice and cultured with mouse primary aortic smooth muscle cells. However, we were unable to detect upregulation of AT1a receptors in primary aortic smooth muscle cells stimulated by co-culture with LDL.

It is worth noting that in the Nickenig study, smooth muscle cells were isolated from rat thoracic aorta and co-cultured with LDL isolated from human normolipidemic subjects. This method raises several questions that remain to be elucidated. Firstly, the expression pattern of AT1a receptors along the entire aorta is different and the region is more susceptible to AAA formation. Therefore, the isolation of smooth muscle cells from thoracic aorta might not be relevant. Secondly, it has been reported that LDL receptors from different animal species have different affinities and binding patterns with human apoB-100, the major apolipoprotein recognized by LDL receptors (275). In Nickenig’s study, to investigate whether LDL receptors from different animal species recognizes the binding domain on human apoB-100, the interaction of LDL from healthy controls and familial binding defective apoB-100 (FDB) with cultured cells were examined. They found rat cells failed to distinguish between normal and FDB LDL and the binding affinity was 4-8 times lower than the human LDL receptor. The use of antibody Mb47 which is capable of blocking the binding of human LDL to human cells failed to inhibit the binding of human LDL to rat cells. These findings indicate that rat LDL receptors recognize human apoB different from the human receptor binding domain. The lack of effects we observed is possibly due to the distinct recognition pattern and different affinity between the interactions of human LDL to rat cells and mouse LDL to mouse cells. Besides, as mentioned previously, there have been consistent demonstrations that AT1 receptors antagonism reduces AngII-induced AAAs
which is attributed to interactions between AngII and AT1a receptors in mice because whole body deficiency of AT1a receptors ablates development of AngII-induced AAAs (131). However, although bone-marrow transplantation study indicated the role of AT1a receptor on resident cell of aortic wall are important, using cell-specific deficient mice that had AT1a receptor depletion on leukocytes, endothelial cells, or smooth muscle cells did not define a single cell type that played a critical role in AngII-induced AAA development (131, 134), implicating orchestrated contributions of multiple cell types in the interaction of AngII and AT1a receptor to promote AAAs. So the elevation of AT1 receptor driven by hypercholesterolemia in single cell type might not account for the exacerbation of AAA in response to AngII infusion. Therefore, in the future, multiple cell type co-incubations should be optimized to investigate regulation of AT1a receptors and interactions and cross-talking among different vascular cell types in a hypercholesterolemic environment.
References


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**Conference Poster**


