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CHRONIC LOW INTENSITY CONTINUOUS AND INTERVAL TRAINING PREVENT HEART FAILURE-RELATED CORONARY ARTERY STIFFNESS

DISSERTATION

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

By
An Ouyang

Lexington, Kentucky

Co-Directors: Dr. Lance Bollinger, Assistant Professor of Kinesiology and Health Promotion
and Dr. Jody Clasey, Associate Professor of Kinesiology and Health Promotion

Lexington, Kentucky
2019

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ABSTRACT OF DISSERTATION

CHRONIC LOW INTENSITY CONTINUOUS AND INTERVAL TRAINING PREVENT HEART FAILURE-RELATED CORONARY ARTERY STIFFNESS

Heart failure (HF) induced by aortic pressure over-load is associated with increased coronary artery stiffness. Perivascular adipose tissue (PVAT) and advanced glycation end products (AGE) both promote arterial stiffness. However, the mechanisms by which coronary PVAT promotes arterial stiffness and the efficacy of exercise to prevent coronary stiffness are unknown. The present study hypothesized both chronic continuous and interval exercise training would prevent coronary artery stiffness associated with inhibition of PVAT secreted AGE and the beneficial effects of interval exercise would be greater than continuous exercise.Yucatan mininature swine were divided into four groups: control-sedentary (CON), aortic-banded sedentary heart failure (HF), aortic-banded HF continuous exercise trained (HF+CONT), and aortic-banded HF interval exercise trained (HF+IT). Coronary artery stiffness was assessed by ex vivo mechanical testing and coronary artery elastin, collagen and AGE-related proteins were assessed by immunohistochemistry. HF promoted coronary artery stiffness with reduced elastin content and greater AGE accumulation which was prevented by chronic continuous and interval exercise training. HF PVAT secreted higher AGE compared with CON and was prevented in the HF+CONT and HF+IT groups. Young healthy mouse aortas cultured in HF PVAT conditioned media had increased stiffness, lower elastin content and AGE accumulation compared with CON, which was prevented by PVAT from the HF+CONT and HF+IT groups. HF coronary PVAT secreted greater interleukin-6 (IL-6) and IL-8 compared to CON which was prevented by both continuous and interval exercise training regimens. We conclude chronic continuous and interval exercise is a potential therapeutic strategy to prevent coronary artery stiffness via inhibition of PVAT-derived AGE secretion in a pre-clinical mini-swine model of pressure overload-induced HF.

Key Words**:** advanced glycation end products, perivascular adipose, inflammation, oxidative stress

An Ouyang

April 29, 2019

CHRONIC LOW INTENSITY CONTINUOUS AND INTERVAL TRAINING
PREVENT HEART FAILURE-RELATED CORONARY ARTERY STIFFNESS

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April 29, 2019
Date

Dedicated to my family, Xinjian Huang, Shanshan Ouyang, and Tianfei Hou

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CHAPTER I
Introduction

Stiffening of the large elastic arteries is an independent predictor of cardiovascular events, promotes hypertension, and is associated with incident heart failure (HF) [1]. HF is defined as dysfunction of ventricular blood filling or ejection due to any structural or functional impairment of the heart. [2] The two types of HF are HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF). HFpEF compromises almost 50% of HF patients [2]. In addition, no therapy has been shown to improve the survival rate in randomized control trial with HFpEF [3]. The increased afterload due to aortic stiffening and augmented pulse pressure contributes to the failure of the myocardium [4]. Recently, our laboratory reported increased coronary artery stiffness in an aortic-banded mini-swine model of pressure overload-induced HF [5], indicating the increased afterload also promotes coronary vascular dysfunction. Therefore, identifying mechanisms and interventions to reduce coronary artery stiffness may also alleviate additional stress on an already failing myocardium.

A key characteristic of arterial stiffening is remodeling of the extracellular matrix (ECM) within the vasculature. More specifically, reductions in elastin, increased collagen and a greater abundance of advanced glycation end products (AGE) collectively promote vascular stiffening [6]. AGE decreases arterial elastin and cross-links ECM proteins, in addition to influencing the artery through the receptor of AGE (RAGE)-mediated cellular signaling mechanisms [7]. Arterial AGE accumulation occurs in conditions where plasma glucose is elevated, such as diabetes. However, adipose tissue in non-diabetic conditions has also been

shown to secrete AGE [7]. Perivascular adipose tissue (PVAT) is an endocrine tissue shown to promote arterial dysfunction, including arterial stiffness [8, 9]. These findings are significant as recent evidence has shown PVAT surrounding the coronary arteries, which is associated with increased inflammation, predicts cardiac mortality [10]. Notably, both aging and HF promote PVAT oxidative stress and inflammation, which are important factors for AGE production in adipose tissue, suggesting PVAT may be a novel source of AGE contributing to arterial stiffness [5, 9, 11]. Currently, however, it is unknown if PVAT secretes AGE to promote arterial stiffening with HF.

Both chronic continuous and interval exercise training programs are effective at reducing blood pressure and de-stiffening large elastic arteries such as the aorta [12, 13]. However, the influence of exercise training on other conduit arteries, such as the carotid artery, is controversial. Continuous exercise training for 16-weeks did not decrease carotid artery stiffness in HFpEF patients [14]. While 16-weeks interval training prevented carotid artery stiffness of HFpEF mini-swine [15]. Thus, these data suggest interval exercise training may have greater beneficial effects on carotid artery stiffness compared to continuous exercise training. However, little is known about the efficacy of chronic continuous and interval exercise to prevent coronary artery stiffening in a translational large animal HF model.

The mechanisms by which exercise prevents and/or reverses central (aorta and carotid) arterial stiffness have been attributed to maintaining elastin and lowering collagen content [16-18], while suppressing oxidative stress and

inflammation [19]. Importantly, non-exercise interventions that reduce PVAT oxidative stress, inflammation and AGE accumulation are associated with reductions in aortic stiffness [20]. These findings collectively suggest exercise may prevent coronary artery stiffness by lowering PVAT-related AGE secretion.

The aim of this study was to determine the influence of both chronic continuous and interval exercise training on PVAT-related AGE secretion to promote coronary artery stiffness in a pre-clinical mini-swine model of pressure overload-induced HF. We hypothesize both continuous and interval exercise training will prevent coronary artery stiffness associated with ECM remodeling, AGE accumulation, oxidative stress and inflammation through a mechanism mediated by PVAT-related AGE secretion. We also hypothesize the beneficial effects of the interval exercise regimen will be greater than the continuous exercise program.

CHAPTER Ⅱ
Review of literature

Cardiovascular disease (CVD) is the leading cause of death in the United States and has a serious financial burden on national health care [21-23]. According to an American Heart Association (AHA) statement, 40.5% of the U.S. population is predicted to have some form of CVD and the medical cost estimation is expected to be $818 billion by 2030 [24]. CVD is referred to coronary artery disease, atherosclerosis, heart failure, hypertension, arterial stiffness, and other heart or blood vessel-related disease [25, 26]. Specifically,

arterial stiffness is associated with myocardial infarction and stroke which are the two leading causes of death in the developed world [27]. Aortic pulse wave velocity (aPWV) is the gold standard measurement of arterial stiffness in clinical settings. Importantly, aPWV is considered a strong predictor of mortality due to cardiovascular-related events and all-cause mortality [28-31]. Thus, it is of great importance to understand the mechanisms of arterial stiffness to develop potential treatment strategies.

Arterial stiffness

Definition

The most recent description of arterial stiffness is the reduced buffering capacity of arteries which is associated with decreased elasticity of the vascular wall [32, 33]. To better explain arterial stiffness, several models have been developed [34]. One of them is the Windkessel model [35] which describes the arterial system like a fire-hose system. The inverted air-filled dome, referred to large artery, was used to buffer the blood flow. The wide-bore hose was the conduit vessel. The fire hose nozzle was the peripheral artery/resistance [1]. However, elastic artery serves both “conduit” and “cushioning” functions. The Windkessel model ignores the “conduit” compliance while only focus on the peripheral resistance.

Artery compliance (C) was first proposed by Spencer and Denison in 1963 [36]. When given a certain amount of pressure to an artery, the degree of vessel volume change is termed as compliance [37]. The compliance can be calculated as $C=\Delta V/\Delta P$, where ΔV and ΔP are the volume and pressure changes in a

cardiac cycle during systole and diastole. Elastance is the reciprocal of compliance. Thus, stiffness can be quantified by elastic modulus/Young's modulus [38]. Compared to the Windkessel model, elastic modulus covers the elastic properties (elastin and collagen region) of the arterial wall [1]. Thus, arterial stiffness can be also defined as an artery with a reduced capacity to expand or recoil during the cardiac cycle. The above explanation of arterial stiffness is focused on the arterial wall component alterations, the two primary proteins collagen and elastin [39]. However, the factors contributing to arterial stiffness are not limited to collagen or elastin.

Measurement of arterial stiffness

Aortic pulse wave velocity (aPWV)

The aorta is the largest artery that contributes buffering effects to modulate blood pressure and maintain consistent blood flow to peripheral ends [32, 40-43]. Aortic stiffness affects blood pressure and blood flow. In addition, aortic stiffness is an independent predictor of CV events [28, 30, 44-47]. The most common and non-invasive clinical measurement of arterial stiffness is aortic pulse wave velocity (aPWV), which measures the pulse wave as it travels from carotid artery to femoral artery. Currently, pressure sensors (tonometer) which captures the wave form from pulse wave is a reliable and reproducible method to assess pulse wave velocity [48]. The PWV measurement system detects the time delay (Δt) between the feet of the two waveforms and records distance from carotid artery to femoral artery (D) [49, 50]. Then aPWV is calculated as D/Δt

[51]. Normal reference of aPWV value is about 6.2 m/s in less than 30 years old population and about 10.9 m/s in older than 70 years old population [52].

While aPWV is the gold standard assessment of arterial stiffness, this method does have disadvantages. The distance is measured on the body surface with a measurement tape by the investigator. In actuality, measured distance is an estimation over the true distance and is largely dependent on body shape [53]. Thus, systematic error in PWV estimation is dependent on distance measurement. To minimize the distance measurement error, some investigators recommend: 1) subtracting the distance from the carotid location to the sternal notch from the total distance or 2) subtracting the distance from the carotid location to the sternal notch from the distance between the sternal notch and the femoral site of measurement [50, 54]. In addition, heart rate and blood pressure are important confounders of PWV assessment. It has been shown that PWV increases with elevated blood pressure and increased heart rates [55, 56]. To minimize the blood pressure and heart rate influences, it is recommended that measurements be acquired in a quiet room with a minimal resting period of 10 minutes with no smoking, meals, alcohol, and beverage containing caffeine at least 4 hours prior to measurement [54].

Pulse wave analysis

Pulse wave analysis is a measurement that allows the accurate recording of peripheral pressure waveforms and the corresponding central waveform used to assess central hemodynamic parameters [57]. The SphygmoCor system

(AtCor Medical, Australia) is one of the most widely used devices to estimate central blood pressure which is correlated well with invasive catheter measurements of aortic blood pressure [58, 59]. The system can generate central hemodynamic parameters: systolic and diastolic pressure, average aortic hemodynamic pressure, aortic pulse pressure, augmentation index (AIx) [60].

Central pulse pressure

Central pulse pressure is the difference between systolic pressure and diastolic pressure which is an important marker of arterial stiffness. Central pulse pressure has two determinants: 1) ventricular ejection interacting with the viscoelastic properties of the aorta; and 2) wave reflection, or return wave from the distal site to heart [32, 61, 62]. Thus, increased arterial stiffness is associated with increased central pulse pressure. Increased pulse pressure is also a predictor of CV risk in subjects with myocardial infarction and congestive heart failure [63-65]. In addition, using pulse pressure to predict the risk of coronary heart disease is better than using systolic or diastolic pressure in middle-aged and elderly adults [66]. These findings demonstrate the central pulse pressure could be an important pathological index in clinical settings.

Augmentation index (AIx)

AIx is calculated as the difference in height between the first and second systolic peaks (augmentation pressure [AP]) in the aortic waveform which is expressed as a percentage of aortic pulse pressure: AP/PP *100% [67]. The

determinants of AIx is wave reflection during each cardiac cycle. Wave reflection is influenced by three major factors: 1) the distance to the reflecting site; 2) the speed of wave transmission, and 3) the magnitude of the reflection coefficient [68]. With increased PWV, the reflected wave arrives back to left ventricle earlier during diastole. Then the reflection wave leads to a secondary systolic pressure peak and increases central pulse pressure [68]. Previous research indicated that AIx is correlated with PWV in middle-aged men and women [69]. Thus, AIx could potentially serve as a predictor of CV events. As such, AIx is an independent risk factor for premature coronary artery disease in young subjects [70]. However, previous Framingham Heart Study showed that AIx increased in middle-aged individuals and then plateaued (in men) or declined (in women) beyond 60 years of age while aPWV continued to increase [71]. Furthermore, AIx was not associated with increased aPWV in patients with diabetes and during beta-adrenergic stimulation [72-74]. Therefore, additional consideration should be taken when using AIx as an assessment of arterial stiffness in special population groups.

Ex vivo mechanical testing

Although arteries share the same three layers which are the tunica intima, tunica media and tunica external (adventitial), the histological structure and composition of the arterial wall differs as does the function. [75]. In human beings, vessels more than 10 mm in diameter are typically considered elastic arteries. As such, the aorta is a large elastic artery that contains a high

percentage of elastic fibers in three layers. The elastic fibers allow the aorta to expand or recoil to maintain stable blood pressure and deliver blood to distal sites. Arteries with diameters between 0.1mm to 10mm are typically considered muscular arteries. Muscular arteries have fewer elastic fibers, which results in less ability to expand or recoil, and their major function is to distribute the blood to capillaries. Arteries with diameters less than 0.1mm are typically arterioles and function to provide resistance. Arterial stiffness assessed by PWV is quite different between these different arteries due to differences in wall structural components. The human coronary arteries are muscular arteries with an average diameter about 4mm, about 10-13 cm in length of the left anterior descending artery and 12-14 cm in length of the right coronary artery [76]. Because the length of the coronary artery is shorter, errors in assessing distance measurement will result in a larger mistake in calculating PWV. Therefore, PWV is not an ideal method to assess coronary artery stiffness *in vivo*.

The most common and easiest *ex vivo* mechanical test used to quantify arterial stiffness in the laboratory is the uniaxial tensile testing [77]. In this procedure an arterial segments are stretched in the horizontal plane at a constant displacement rate, while the force generated by artery is recorded. Testing is terminated at a yield point when tissue fails to generate force in response to increased stretch. The recorded displacement-force data are converted to a stress-strain curve. According to Young's Modulus, stiffness is quantified as the slope of the linear part of the stress-strain curve [77]. The toe part of the stress-strain curve represents elastin protein effects, which

responsible for expansion and recoil of the artery. While the heel part of the curve represents the collagen protein effects, which produces force against higher pressures. In summary, the most two common arterial stiffness assessments are PWV and ex vivo mechanical testing. The advantages of PWV are 1) non-invasive, 2) gold standard in clinical settings and 3) predictor of CV events. However, it cannot explain the arterial wall structural alterations. The advantages of mechanical testing are 1) application to all arteries and 2) explanation of the vascular wall component changes, but requires acquisition of arterial samples.

In this research project, the coronary artery stiffness was assessed by *ex vivo* mechanical testing with a Myograph (DMT 620, Denmark). The simplified description of the Stress-Strain curve is shown as Figure 2-1.

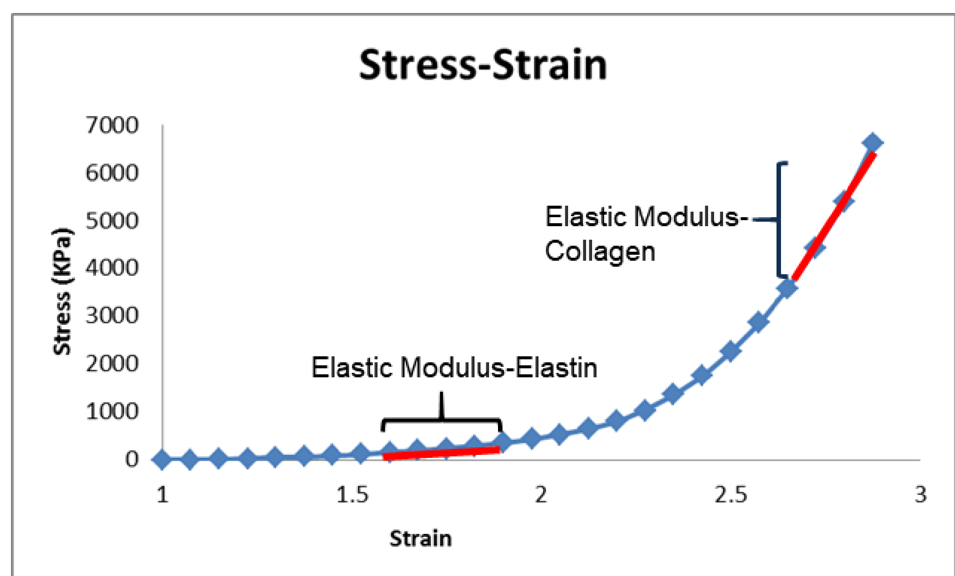


Figure. 2- 1 *Ex vivo* mechanical testing

Importance of arterial stiffness

Arterial stiffness is a strong predictor of not only CVD-related mortality, but also all-cause mortality. Particularly, increased aPWV has been shown as an independent predictor of coronary artery disease and stroke over a 10 year follow-up [78]. More studies have elucidated the importance of arterial stiffness. The estimation of myocardial infarction, unstable angina, heart failure or stroke is approximately twice in subjects with higher aPWV compared to subjects with a lower aPWV [79]. Another study conducted in well-functioning elders showed that higher aortic PWV was associated with CVD mortality and other disease mortality

[28]. A population-based study also showed that arterial stiffness is strongly associated with lipid plaques at various arterial tree sites [80]. An investigation on end-stage renal disease patients demonstrated that elevated aortic elastic modulus was associated with an increased hazard ratio of cardiovascular and all-cause mortality [81]. Importantly, angiotensin-converting enzyme inhibitor treatment on renal dysfunction patients increased survival rate associated with reduced arterial stiffness independent of blood pressure changes [82]. This study may suggest that, compared to blood pressure alone, arterial stiffness is more tightly related to survival in renal dysfunction patients.

In general, healthy arteries expand and recoil during systole and diastole. The elastic properties of large arteries tends to decrease with aging and disease. Prevention strategies and treatments of arterial stiffness are critical to reduce CVD-related events and all-cause mortality. Thus, to understand the mechanisms of arterial stiffness is essential to support prevention and therapeutic strategies to lower CVD risk.

Contributors to arterial stiffness

Stiff arteries lose the elastic properties which is associated with vascular wall remodeling. Arterial stiffness is involved with complicated reactions at the cellular and molecular levels. These vasculature alterations are affected by blood flow shear force and extrinsic factors such as advanced glycation end products (AGE), oxidative stress, inflammatory response and metabolic dysfunctions of glucose and lipid.

Collagen and Elastin

Collagen and elastin are the two major scaffolding proteins which provide compliance, elasticity, and stability to the vascular wall [83]. Collagen is the most abundant protein in humans and is the main component of connective tissue [84]. The most abundant collagen subtypes in the normal human artery are type I (70-75%), type III (20-25%) and type V (1-2%) [85]. Type I and type III collagens are the major components of the three vessel layers, while type IV (comprise more than 90% of the total protein of the basal lamina) and type V collagens are in the basement membrane of smooth muscle cells of the intima and medial layers [86, 87]. Elastin is the other major extracellular matrix (ECM) protein of large arteries with characteristics of extensibility and elasticity [88]. Elastin formation is limited to the embryo stage and infancy [89]. Therefore, elastin content is greatest at birth and progressively decreases with aging. Accelerated elastin degradation and fragmentation due to some disease conditions will result in premature arterial stiffening [90].

The production and degradation of collagen and elastin are in balance in healthy conditions. Dysregulation of the balance caused by overexpression of abnormal collagen and degradation of normal elastin promotes vascular stiffness [6]. Increased collagen type I is associated with myocardial fibrosis in hypertensive heart disease patients. Another report shows that collagen type I overproduction is associated with increased aPWV in diabetes with coronary artery disease [91]. Moreover, in atherosclerotic lesions, type I and III collagens

are the major structural component while type IV and type V collagens are not [86]. Heart failure induced by hypertension and diabetes has been shown to stimulate excessive collagen production [92, 93]. Histological analyses of stiffened vessels indicate that the ratio of collagen to elastin was increased [94].

Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs

Collagen and elastin comprise a large portion of ECM and are regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) [95]. MMP are responsible for extracellular matrix degradation. Different MMPs subunits have different functions [96]. MMP-1 degrades types I, II, and III collagen; MMP-2 degrades type IV collagen and elastin; MMP-3 degrades laminin, fibronectin, elastin, gelatin, and proteoglycans, and MMP-9 degrades type IV collagen and elastin. A recent publication reported that increased MMP-2 and 9 levels were greater in hypertensive subjects and positively related to PWV [97]. Another study showed that deficiency of MMP-9 and MMP-12 protected ApoE $^{-/-}$ mice from atherosclerosis [98]. Moreover, MMP-1 was positively correlated with aPWV in both normotensive and hypertensive subjects [99].

The TIMP family is comprised of four MMP inhibitors: TIMP-1, 2, 3 and 4 [100]. A human study indicated that plasma TIMP-1 level was increased in hypertensive subjects [101]. An animal study demonstrated that hypertension induced by angiotensin II was prevented in TIMP 3$^{-/-}$ mice that had reduced collagen and elastin content in both the carotid and mesenteric arteries [102]. This finding shows that TIMP 3$^{-/-}$ has a preventative effect in hypertension but

with an adverse remodeling in the extracellular matrix. MMPs expression is also regulated by other cellular factors like, increased pro-inflammatory, cytokines, and oxidative stress which promote MMPs to induce less effective collagen and fragment elastin which in turn promotes vascular stiffness [103-105].

Advanced glycation end products (AGE)

Advanced glycation end products (AGE) are complex and heterogeneous groups of molecules which are associated with diabetes, cardiovascular disease, Alzheimer's disease and end stage renal disease [7].

AGE formation

Reducing sugars react with proteins, lipids, and nucleic acids following glycation, oxidation and/or carboxylation to produce AGE [7, 106]. In general, the highly reactive glucose aldehyde group with the amino group of proteins produce a Schiff's base, which spontaneously rearranges itself into an Amadori's product. After continued modifications Amadori's product becomes AGE. In addition, some highly reactive carbonyl agents like glyoxal and methyl-glyoxal can be produced by oxidized glucose, Schiff's base and then Amadori's product will react with free amino groups to form N-q-carboxy-methyl-lysine (CML). The slow extracellular glycoxidation reaction which produces CML [107] ranges from weeks to months, thus long-lived proteins, such as collagen are primary targets [108]. The major AGE chemical structures are: 2-(2-furoyl)-4(5)-furanyl-1H-

imidazole (FFI), 1-alkyl-2-formyl-3,4-diglycosyl pyrroles (AFGPs), N-ε-carboxy-methyl-lysine (CML), pyrraline and pentosidine [109].

Greater AGE concentrations are usually associated with increased plasma glucose and renal dysfunction [7]. AGE concentration was increased after a few weeks when animals had increased plasma glucose [110]. Compared to the normal conditions, circulating AGE increased approximately two fold in diabetic animals [111]. Since small soluble AGE peptides are cleared in the kidney, renal dysfunction contributes to AGE accumulation and leads to vascular disease [112]. Further, diabetic patients with end stage renal disease (ESRD) had up to 100 fold circulating AGE compared to diabetics with normal renal function patients [113, 114].

Lipids are involved in the glycation process as well. Quick lipid peroxidation with reactive oxygen species (ROS) produces free AGE or protein bound AGE in both intracellular and extracellular spaces [115, 116]. AGE reduces paraoxonase activity by 40% in type II diabetes and coronary artery disease patients [117] which in turn induces oxidized low-density lipoprotein (LDL) accumulation. In our previous study, we found AGE accumulated in the aorta with elevated plasma LDL [8]. In addition to the endogenous formation, AGE could also be accumulated in blood by food absorption [118, 119]. Plasma AGE level is related to aortic stiffness independent of age and blood pressure [120]. This finding indicates that adipose tissue is a potential source of AGE.

AGE effects in cellular and molecular levels

The pathogenic influence of AGE are via two different pathways. First, AGE form cross-links with proteins that results in an irreversible process which alters protein structures and functions [112]. Specifically, AGE cross-linking with type I collagen changes the protein structure [121]. AGE can form cross-links with collagen IV on the basement membrane to prevent normal network-like structures as well [122]. Increased stiffness in the vasculature and other tissues due to pathological alterations by AGE-collagen cross-link formation is common in diabetic and aging conditions [123].

AGE can also form cross-links with LDL through three-dimensional structural changes, which in turn, prevented recognition by cellular LDL receptor [124]. In addition, excessive AGE increases LDL susceptibility to oxidative modifications to promote oxidized LDL formation [125]. The oxidized LDL can react with free amino groups of proteins to form AGE products, including CML, CEL and others [126, 127].

In older mice, the increased aortic stiffness has been shown to be associated with less elastin per mm^2 of the vascular wall by AGE-elastin formation [128]. In addition, exogenous AGE administered to rodents promotes elastin disruption and fragmentation that is associated with arterial stiffness [129]. Previous research has indicated that cross-linking collagen promotes myocardial stiffness and cardiac chamber remodeling in hypertensive heart disease. In detail, spontaneously hypertensive rats had a decrease in soluble myocardial collagen concomitant with an increases in cross-linked myocardial collagen which in turn promoted the myocardial stiffness [130]. A histological study

conducted on human aorta illustrated a correlation between AGE accumulation and aortic stiffness, which is assessed by *ex vivo* mechanical testing [131-133]. Currently, adequate evidence supports the notion that collagen cross-linking is an important mechanism of decreased vascular and cardiac compliance.

Second, AGE binds to a cell membrane receptor to stimulate AGE related signaling. AGE have several receptors, the receptor for AGE (RAGE) is the most well characterized AGE receptor [134]. RAGE expression is increased in the blood and kidneys in those with diabetics compared to control [135]. RAGE has also been found on endothelial cells, specifically in the area of atherosclerosis [135]. *In vitro* investigations demonstrated that increased oxidative stress and activation of the Nuclear factor-kappa B (NF-κB) signaling was associated with AGE-RAGE binding on macrophage cells [136, 137]. We recently reported that the greater AGE accumulation in the vascular wall was associated with increased oxidative stress and NF-kB p65 subunit expression through RAGE expression was unchanged with HF [5].

AGE has been shown to increase intracellular oxidative stress. A previous study indicated that endothelial cells cultured with AGE increased thiobarbituric acid reactive substances, a byproduct of lipid peroxidation, and heme oxygenase mRNA expression [136]. Moreover, human endothelial cells cultured with AGE prompted intracellular generation of hydrogen peroxide and was prevented by soluble AGE, which blocks the RAGE receptor [138]. Further, AGE have been shown anti-oxidant properties resulting in a reduction of glutathione, vitamin C and nitric oxide leading to reduced vasodilation [139]. AGE accumulate in pro-

inflammatory sites and promote atherosclerotic lesions in non-diabetes as well [140]. A previous study showed that activated NF-kB induced by AGE released interleukin-1 (IL-1), IL-6 and TNF-a [141], in which IL-6 has been shown as a contributing factor to aortic stiffness [8]. Moreover, the AGE–RAGE mediated cellular signaling includes multiple intracellular signal transduction pathways like p21ras, mitogen-activated protein (MAP) kinases, Phosphoinositide 3 (PI3) kinase, cdc42/rac, Jak/STAT, NAD(P)H oxidase [138, 139, 142-146]

In summary, excessive AGE accumulation increases oxidative stress and inflammatory response leading to arterial stiffness, while the pathological changes are accelerated in diabetic and aging conditions [147].

Treatment targeting AGE

Therapeutic interventions focused on AGE formation, AGE cross-linking and AGE–RAGE interaction have been investigated. Aminoguanidine (AMG) is the first AGE inhibitor which functioned as nucleophilic hydrazine compound to prevent Amadori's products that react with proteins to form AGE [148]. AMG prevented albumin to form AGE with glucose and AGE cross-linking of collagen [148]. Evidence also demonstrated that AMG preserved arterial elasticity through inhibition of collagen cross-linking [149]. Further, AMG is the most promising agent which reduces the heart AGE levels, RAGE levels and collagen expression [150]. In addition, AMG reduces AGE accumulation absorbed from food [151].

Another agent was developed to remove the irreversibly bound AGE from connective tissues and matrix components [152]. Alagebrium chloride (ALT-711)

is a cross-link breaker compound which has been shown to decrease pulse pressure in elderly adults with vascular stiffening [153]. ALT-711 decreased large artery stiffness that was associated with reduced collagen deposition and attenuates left ventricle stiffness in diabetic and aging animal models [154, 155]. The development of AGE cross-link breakers may be a potential target for future therapy of isolated systolic hypertension and diastolic heart failure [123].

As mentioned above, AGE-RAGE mediated downstream cellular signaling promotes oxidative stress and inflammatory responses. This may suggest that interference with the AGE-RAGE interaction would interrupt or stop related cellular activation, and consequently ameliorate various chronic disorders [156, 157]. RAGE mRNA has two major truncated forms: N-terminal and C-terminal [158] which are named soluble RAGE. Soluble RAGE has been reported to dramatically reduce AGE accumulation and improve vascular function [159]. In addition, the administration of soluble RAGE to diabetic mice showed that inflammatory cells, mRNA levels of glomerular cytokines and extracellular matrix were decreased, which in turn reduced vascular lesion area and complexity [160, 161].

Insulin has also been proposed to contribute in AGE elimination through PI3-kinase pathway associated with nitric oxide production and resulting in vascular protective effects [162, 163]. This finding suggests activation of PI3-kinase not only promotes glucose metabolism but also AGE metabolism. In addition, the anti-oxidant agents Vitamin C, has been reported to prevent the oxidative conversion during AGE formation [164].

In summary AGE and AGE-mediated cellular signaling promote vasculature dysfunction and wall remodeling through cross-linking formation, oxidative stress, and inflammation. Comprehensive understanding of the mechanisms of AGE formation and regulated activity is critical to control or prevent cardiovascular disease.

Oxidative stress

Oxidative stress source

Oxidative stress is an imbalance between the overproduction of reactive oxygen species (ROS) and reduced antioxidant defenses [165, 166]. ROS are reactive chemicals with oxygen such as peroxides, superoxide, hydroxyl radical and single oxygen [167]. The major source of ROS comes from mitochondria where oxygen leaks during oxidative phosphorylation [168]. Mitochondrial DNA damage is greater in atherosclerotic human arteries compared to normal human arteries, in part, due to oxidative stress [169]. Mitochondrial DNA encodes portions of the electron transport chain [170], thus damage on mitochondrial DNA may cause more ROS leaking from mitochondria associated with structural damage. Mitochondria ROS are also regulated by mitochondria membrane potential, O_2 concentration, nuclear transcription factors, and cytokines [171].

Peroxynitrite is another ROS compound resulting from the reaction of superoxide and nitric oxide along with increased oxidative stress [172]. Peroxynitrite oxidizes lipoproteins and nitrate tyrosine residues in many proteins [173]. Since the production of peroxynitrite is difficult to assess, nitrosylation of

proteins have been considered as an indirect biological marker of oxidative stress [174]. Though nitrotyrosine is not detectable in the plasma of normal healthy subjects [175], the circulating nitrotyrosine concentrations are detectable and associated with cardiovascular disease [176].

Oxidative stress influences on cellular dysfunction

Increased ROS stimulates a pro-inflammation response and activates NF-κB related signaling [171, 177]. One previous study indicated that cells treated with agents that restored ROS prevents protein kinase C, AGE accumulation and NF-kB activation [178]. Activation of NACHT, LRR and PYD domains-containing protein 3 (NALP3) will trigger an inflammatory response, which is positively regulated by ROS [179, 180]. Recent studies showed that excessive ROS had an adverse effects on vascular function associated with cellular protein damage and an overall reduction in bioavailable endothelium-derived nitric oxide [181]. Moreover, reduced ROS accumulation decreased IL-6 production induced by endotoxin lipopolysaccharide [177]. Reduced IL-8 expression is associated with ROS inhibition in human aortic endothelial cells *in vitro* [182]. Several studies showed that excessive ROS accelerates atherosclerosis lesion formation. Deficiency of SOD2, a mitochondria-specific antioxidant enzyme, in apolipoprotein $E^{-/-}$ mice contributes to atherogenesis at arterial branching points [169]. Also, oxidative stress has been shown to dramatically increase in aortic-banded guinea pigs and is reduced by Vitamin E therapy [183]. However, clinical use of Vitamin E to prevent oxidative stress has not shown beneficial effects on

mortality compared to the control group [184]. Another long-term study showed that neither vitamin E nor vitamin C supplementation reduced risk of major cardiovascular events [185].

Oxidative stress has been shown to mediate collagen and collagen-related signaling. One study indicated that oxidative stress mediated the collagen type I expression and activation of NF-kB *in vitro* [186]. Another experiment showed that exposure to oxidative stress stimulated MMP expression and influenced the vascular remodeling [187].

Oxidative stress could influence vascular function by endothelial cells. Substantial evidence suggests that atherosclerotic lesion formation is associated with oxidative stress [188]. Another study shows that high blood glucose increases ROS, NF-κB activation, upregulation of endothelial cell adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), trans-endothelial migration of monocytes, and monocyte-endothelial adhesion in human coronary artery endothelial cells (140). In addition, over expression of thioredoxin-2 (Trx2), a mitochondrial antioxidant enzyme, attenuated endothelial function and prevented atherosclerotic lesions in $ApoE^{-/-}$ mice, which was associated with decreased oxidative stress and elevated NO bioavailability [189]. The above evidence suggests that attenuated oxidative stress protects the vasculature.

In summary, oxidative stress has been recognized as a crucial factor to vascular diseases and atherosclerosis development. Long-term studies have indicated that oxidative stress is a strong predictor of cardiovascular events in

coronary artery disease patients [190] and is strongly associated with arterial stiffness measured by PWV [191]. Thus, it is of importance to investigate the oxidative stress-related mechanisms of arterial stiffness in order to develop effective therapeutics.

Inflammation

Inflammation is part of biological responses to local injury [192]. The stimulated inflammatory response produces cytokines such as IL-1β, IL-6, tumor necrosis factor-α (TNF-α) [193, 194], and activates the NF-κB pathway [195]. C-reactive protein (CRP), an inflammation marker, has a direct role in promoting inflammatory response on human aortic endothelial cells which is associated with reduced antioxidant defense [196, 197]. Previous studies have shown that reduction of inflammation (CRP) reduced aPWV [198]. It suggests that CRP inhibition may decrease PWV. Moreover, arterial stiffness measured by aPWV is associated with inflammation-related signaling such as IL-6, TNF-α and CRP [199]. Another in vitro study indicated that inflammation promoted arterial stiffness associated with vascular wall remodeling through activated mitogen-activated protein kinases (MAPK) p38 controlled gene [200]. As mentioned in the "*Oxidative Stress*" section, angiotensin II increase ROS production and stimulates MMP expression through the expression of active transforming growth factor-β (TGF-β) [201] leading to vascular wall remodeling [202]. Another study suggested that macrophage, CD68, angiotensin II related proteins and enzymes activity were increased in older human thoracic aorta compared to young adults.

The pathological alterations with inflammation increased vascular wall thickness [203]. Also, monocyte chemoattractant protein-1/C-C chemokine receptor type 2 (MCP-1/CCR2) pathway has an important role in arterial inflammation to induce IL-1 and IL-6 expression [204] which resulted in vascular remodeling and left ventricular hypertrophy [205]. MCP-1 induces vascular smooth muscle cell proliferation and activates NF-kB signaling [206]. In summary, substantial evidence demonstrates that inflammation influences arterial stiffness, suggesting treatments targeting reductions in the inflammatory response will prevent arterial stiffness.

Perivascular adipose tissue (PVAT)

PVAT characteristics

In the past decade, much interest has been focused on adipose tissue, which surrounds the blood vessels. Recent investigations have demonstrated that PVAT is an endocrine tissue and can transfer signals to adjacent blood vessels [207]. There is no fascial layer or elastic lamina between PVAT and the vasculature, where PVAT interlaces with the adventitial layer [208, 209]. In addition, the vasa vasorum within PVAT proliferates when inflammation and injury occurrs [210-212], resulting in the release of mediators from PVAT to the vascular system. The vasa vasorum are tiny blood vessels which supply blood to the outer layers of the artery [213]. Secretomes from PVAT are different from visceral and subcutaneous adipose tissue [214]. Thus, these characteristics

suggest that PVAT may have a unique role in influencing the vasculature [215, 216].

PVAT and cytokines

One of the key features of PVAT is regulation of inflammation. Cytokines like IL-6, IL-8, leptin, MCP-1, and resistin released from PVAT induces endothelial cell dysfunction, increases oxidative stress, and causes smooth muscle cell proliferation and migration [217-219]. One recent study demonstrated that inflammatory cells were increased in PVAT surrounding the atherosclerotic aorta compared to the normal aorta [220]. Other similar studies revealed that mRNA and protein concentrations of IL-1β, IL-6, MCP-1, and TNF-α were elevated and adiponectin protein level was lower in PVAT diseased coronary arteries [221, 222]. Wild type mouse aorta cultured with LDL receptor$^{-/-}$ PVAT which secreted greater IL-6, promoted aortic stiffness [8]. Another animal study indicated that IL-8, IL-6 and MCP-1 mRNA level and protein expressions were higher in PVAT compared to peri-renal and subcutaneous adipose tissue [208], thus confirming PVAT's specific role in inflammatory responses. Another research investigation reported that adrenomedullin which was a vasodilator and anti-oxidative peptide was expressed in epicardial adipose tissue [223]. Thus, PVAT released pro-inflammatory cytokines may serve as a protective mediator.

Recent studies reported that epicardial adipose tissue size was positively related to coronary artery disease, which was associated with insulin resistance and inflammation [224-227]. However, the influence of PVAT on the vasculature

is not completely understood. Yudkin et al. proposed a “vasocrine” model in that cytokines were directly released from PVAT to the vasculature, and thereby regulated vascular function [228]. The above studies proposed that mediators from PVAT were directly released into adjacent blood vessels. PVAT has been shown to affect smooth muscle cells as well. Animal studies have showed that PVAT releases cytokines which stimulated smooth muscle cell proliferation [218]. Conditioned media from cultured PVAT of aging and obese rats promotes human aortic smooth muscle cell proliferation [229]. In summary, cytokines released from PVAT contributes to vasculature modifications, but the mechanism is still not understood.

PVAT and oxidative stress

Another feature of PVAT is promoting oxidative stress. Animal study showed that mice with characteristics of metabolic syndrome had mesenteric artery remodeling associated with increased superoxide production and NADPH oxidase activity in PVAT [230]. A similar study concluded that diet induced obese mice had vascular endothelial dysfunction and an increased pro-inflammatory response associated with increased oxidative stress in PVAT [231]. To confirm that oxidative stress promotes aortic stiffness, PVAT from old mice was transplanted into healthy young mice which increased PWV and decreased elastic modulus. In addition, the increased stiffness was abolished by 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), a superoxide scavenger [232]. In summary, substantial published research supports the role for PVAT as an

important regulator of inflammatory responses and oxidative stress. Thus, PVAT may be potential novel therapeutic target for improving coronary artery stiffness.

Hypertension and arterial stiffness

Blood pressure is an important physiological and pathological predictor of cardiovascular events [32]. Previous studies have shown that patients with isolated hypertension have higher aPWV [233]. In addition, a large population study indicated that higher aortic stiffness was associated with an increased risk of incident hypertension [234]. These findings revealed the connection between arterial stiffness and hypertension. However, it is controversial which comes first: arterial stiffness or hypertension [235]. One theory is that vascular wall stiffness precedes elevated blood pressure resulting in hypertension. Obese mice fed a diet of high fat and high sucrose showed increased aPWV within 1 to 2 months which preceded development of hypertension at 5 months [236]. An alternative theory is that elevated blood pressure precedes vascular wall alterations: hypertension deteriorates vascular wall pathology changes leading to stiffer arteries [237]. This long-term study revealed that hypertension exacerbated aortic stiffness. Moreover, individuals with well-controlled blood pressure had less progression of arterial stiffening assessed by aPWV than individuals poorly controlled blood pressure [238]. Recently, numerous studies investigated the cellular and molecular mechanisms of arterial stiffening and its relationship with the development of hypertension. The elastin knockout ($Eln^{-/-}$) mouse model has greater arterial stiffening than wild- type mice. Until postnatal day 1, the blood

pressure in $Eln^{-/-}$ mouse was about twice that of wild-type, and the aortic stiffness was further increased. $Eln^{+/-}$ mouse had increased arterial stiffness by postnatal day 7, whereas systolic blood pressure increased until postnatal day 14 [239].

Treatments for hypertension have been shown to work for attenuation of arterial stiffness. Anti-hypertension drugs targeting the renin-angiotensin system and aldosterone have been shown to prevent arterial stiffness associated with decreased collagen accumulation [240]. However, isoflavones, compounds with antioxidant properties, [241] reduced arterial stiffness associated with suppressed vascular cell adhesion molecule-1 level but did not affect blood pressure [242]. In summary, it is important to better understand the association of arterial stiffness and hypertension.

Aging and arterial stiffness

Aging is defined as the age-related reduction in physiological functions essential for survival and fertility [243]. It is well established that aging is associated with increased aortic stiffness [244]. Moreover, age-related changes in vascular structure and function increase the risk of CVD [245].

Age-related metabolic syndrome promotes arterial stiffness

The risk of acquiring metabolic syndrome components, such as obesity, dyslipidemia, insulin resistance, and hyperglycemia, increases with aging. Substantial evidence indicates that aging related metabolic syndrome promotes

arterial stiffness and accelerates the development of hypertension [246-249]. In particular, diabetes and dyslipidemia leads to endothelial dysfunction, increased oxidative stress and inflammation leading to arterial stiffening [243]. Metabolic syndrome also interacts with inflammatory responses [250, 251]. Therefore, aging promotes activation of inflammatory responses resulting in increased pro-inflammatory cytokines, which in turn produces more cytokines, resulting in endothelial dysfunction and increased oxidative stress [252]. The increased oxidative stress and inflammatory responses collectively contribute to arterial stiffening.

Aging increases MMPs expression

Aging is also associated with over expression of MMPs to promote elastin degradation and collagen accumulation [253]. MMP-2 is associated with the aging process. Over expression of MMP-2 changes the collagen and elastin ratio, and activates TGF-β signaling. Increased MMP-2 expression promotes arterial stiffness which was associated with vascular wall remodeling and calcium content [254]. Inhibition of MMPs prevented age-related arterial inflammation, which was associated with preservation of elastin, reduction of collagen leading to prevent high blood pressure [253]. Interestingly, it appears that carotid artery properties improve with lessening metabolic syndrome severity which suggest that arterial stiffness may be reversible with metabolic syndrome [248].

Oxidative stress and other factors in aging

It is evident that oxidative stress occurs with aging. An animal study showed that superoxide production was increased in the mesenteric artery in aging rats [255] and NO bioavailability was decreased in aging-related hypertensive rats [256]. Moreover, recent studies confirmed that aging-related endothelial dysfunction and oxidative stress promotes vascular wall remodeling and stiffness [257-259]. Therefore, aging-related oxidative stress promotes endothelial dysfunction and activation of inflammatory response leading to vascular remodeling [260-263].

Aging has been shown to induce aldosterone dysregulation, which contributes to aging-related inflammatory response [264]. Thus, these findings suggest age-related salt sensitivity may be regulated by inflammation and contributes to arterial stiffness. It is well known the nervous system is important in regulating peripheral artery resistance and blood pressure. A recent study showed increased sympathetic nerve activity in older women contributes to the development of hypertension but not in young women [265]. Another paper demonstrated that reduced sympathetic baroreflex sensitivity promotes arterial stiffness and hypertension in elderly adults [266]. The above findings may suggest arterial stiffness in aging may be influenced by the nervous system as well.

Aging genetic influences in CV events

Recent interests have been focused on age-related gene changes in vascular function and hypertension. Klotho gene is an anti-aging gene which

extended life span [267]. In mice where the klotho gene is overexpressed, oxidative stress and insulin resistance were attenuated [267]. Greater expression of klotho gene also reduced blood pressure and restored kidney damage in spontaneously hypertensive rats [268]. However, the klotho gene expression prevented IL-10 production and superoxide production [268]. To confirm the influence of klotho on blood pressure, another research group showed that klotho gene deficiency caused salt-sensitive hypertension via CCR2 and MCP-1 [269]. Since arterial stiffness and hypertension are influenced by oxidative stress and inflammation, it would be logical to predict that anti-aging genes have an important role in modulating age-related vascular structural and functional alterations.

Genetic base of arterial stiffness

Arterial stiffness is an independent predictor of cardiovascular mortality after adjustment for traditional risk factors like age, sex, and mean blood pressure [44, 45, 270]. Moreover, carotid artery stiffness was greater in teenagers with a parental history of myocardial infarction or diabetes compared to the teenager without such parental history [271]. A similar study showed that offspring had increased AIx in families which had a history of hypertension [272]. Thus, these findings suggest that genetic factors are a potential factor related to arterial stiffness.

Genetic mutation of elastin and collagen

Gene mutations in elastin and collagen also contributes to arterial stiffness. Genetic research on elastin indicated A allele of the Ser422Gly polymorphism, which is A-to-G nucleotide change, was positively associated with carotid stiffness [273]. Another study showed genetic mutation of fibrillin-1 is associated with large artery stiffening and increased pulse pressure in coronary artery disease patients [274]. In addition, MMP-3 genotyping promoted aging-related aortic stiffness associated with vascular remodeling [275]. Though some investigations demonstrated phenotype of some genes promoting arterial stiffness, the underlying signaling pathway is not well understood.

Genetic mutation of renin-angiotensin system

Genotype-phenotype studies focused on the renin-angiotensin system showed that angiotensin II type 1 receptor gene polymorphism was correlated with aortic stiffness [276]. Another large population research concluded that angiotensin II type 1 receptor genotype could influence arterial stiffness in aging hypertensive patients [277]. A similar study performed in hypertensive patients and genotyped mice showed that angiotensinogen gene is a genetic marker for arterial stiffness, whereas in genotyped mice with high concentration of angiotensinogen had increased arterial stiffening [278]. These data reinforce that renin-angiotensin system is another key factor in arterial stiffness.

Heart failure and coronary artery disease

Heart failure (HF) is a life-threatening disease [279] affecting about 5.7 million adults in the United States [280]. The risk of death is about 35% in the first year after diagnosis with HF [281]. The most common risk factor of HF is coronary artery disease (CAD) [279]. Maintenance of coronary artery function is critical because the coronary arteries are responsible in the transport of blood and nutrition to heart. From the 2016 American Heart Association annual report, more than half of all cardiovascular events in both men and women is due to CAD [282]. Moreover, the prevalence of CAD will increase about 18% by 2030 based on projections [282]. CAD due to HF has been well established. However, the influence of HF on coronary artery stiffness is largely unknown [283].

Heart failure

HF is a syndrome which includes symptoms such as: shortness of breath at rest and exercise, fatigue, pulmonary congestion and abnormal heart structural and functional changes [284]. By 2030 more than 8 million adults will be expected to have HF in the United States [280]. HF is the underlying cause of 1 in 9 deaths [285]. Patients with HF have lower stroke volume and cardiac output, which results in overstimulation of the sympathetic nerve system and renin-angiotensin aldosterone system leading to vasoconstriction, and sodium and fluid retention. Elevation of blood pressure and increased afterload cause ventricular wall remodeling [286]. A long-term study involving with 5143 subjects concluded that hypertensive subjects had a greater risk of HF compared to normotensive subjects [287]. Thus, the above evidence suggests that hypertension is the most

common risk factor of HF. Given the lack of effective therapies [3], research about exercise interventions to treat HF has been popular in recent years.

Coronary artery disease

Pathology of coronary artery disease (CAD) is an imbalance between myocardial oxygen demand and supply due to the narrow or blockage in coronary arteries [288]. One possible mechanism is lipid deposits on coronary artery which results in artery stiffening or atherosclerosis [289]. CAD contributes to more than half of all cardiovascular events in individuals less than 75 years old [290]. Additionally, CAD is the underlying cause of one in seven deaths [280]. However, the effects of HF induced by increased central pressure, on coronary artery stiffness has not been examined.

Physical activity/exercise influences on cardiovascular disease

Definition

Physical activity is defined as any bodily movement produced by skeletal muscles that results in energy expenditure [291]. Exercise is a subset of physical activity that is planned, structured, and repetitive and has as a final or an intermediate objective the improvement or maintenance of physical fitness [291].

Physical activity/exercise benefits

Physical activity is a non-invasive prevention and treatment strategy for obesity, diabetes, hyperlipidemia and CVD [292-294]. Physical activity has been

shown to effectively control body weight, lower cholesterol, decrease blood pressure and improve cardiovascular fitness, reduce collagen accumulation [295] and suppress inflammatory response [296-300]. It is generally accepted that physical activity decreases the risk of cardiovascular disease, diabetes, colon and breast cancers [301]. Evidence suggests that physically active individuals have lower coronary heart disease risk compared to less physically active individuals [302-307]. Physical activity has been reported to reduce and treat many atherosclerotic risk factors, such as high blood pressure, insulin resistance, high triglyceride concentration and low high-density lipoprotein (HDL) concentration [308]. Atherosclerotic lesions progression can be prevented or slowed and O_2 uptake increased during one year treadmill exercise [309]. A meta-analysis report showed that after 12 weeks of exercise subjects had increased HDL and decreased LDL [310]. A large population study indicated moderate intensity exercise help reduces systolic and diastolic blood pressure 3.4 and 2.4mmHg respectively [311]. In addition, mean systolic and diastolic blood pressure reduction in hypertensive subjects were 7.4 and 5.8mmHg respectively and in normotensive subjects were 2.6 and 1.8mmHg respectively. These results suggest that moderate intensity exercise decreases high blood pressure and serves as an effective therapy for hypertensive patients. Chronic moderate exercise has also been reported to attenuate blood glucose concentrations in type II diabetics [312].

Influence of exercise on arterial stiffness

It has been shown that 30 minutes of moderate cycling causes acute reductions in arterial stiffness [313] and 60 minutes of maximal treadmill exercise decreases lower-limb vascular stiffness [314]. Investigators proposed that reduction in arterial stiffness was due to exercise-related incremental increases of NO production from the vascular endothelium. Twelve weeks of exercise training increased superoxide dismutase activity, plasma nitrite and nitrate levels leading to decrease oxidative stress with increased vasodilation [315]. However another experiment concluded that low intensity cycling which reduced PWV was not associated with NO production [316]. Animal microarray results showed that aorta prostaglandin receptor, C-type natriuretic peptide and endothelial nitric oxide synthase genes were overexpressed with reduced aPWV after 4 weeks treadmill running in rats [317]. These findings suggest that exercise-related attenuations of arterial stiffness may be associated with improved endothelial function with decreased oxidative stress.

Furthermore, 3-week moderate exercise reduced carotid and femoral artery stiffness in type 2 diabetics with improved insulin resistance [318]. Another study indicated nutrition and exercise interventions improved radial artery elasticity index with decreased blood glucose, cholesterol and C-reactive protein [319]. In summary, it is reasonable to conclude that exercise attenuates arterial stiffness with decreased oxidative stress. However, the exercise effects on arterial stiffness via reductions in inflammation need further investigation.

Exercise treatment in coronary artery disease

Exercise is a treatment for patients with coronary heart disease. Low intensity exercise improves endothelial function and is associated with increased phosphorylation of endothelial nitric oxide (NO) synthase in coronary artery disease patients [320]. Exercise rehabilitation after myocardial infarction helps reduce cardiovascular mortality, sudden death and fatal or nonfatal re-infarction [321]. Clinical meta-analysis suggests cardiovascular death was lower in exercise rehabilitation patients [322]. A large population study shows old (~63 y) CAD patients had lower risk of all-cause mortality after 5 years light or moderate-intensity activity compared to the sedentary control group [323]. Another study examining exercise training effects on old or young CAD patients indicates that 12 weeks moderate-intensity exercise improved both groups' plasma lipid level, indices of obesity and exercise capacity [324].

Exercise treatment in heart failure

Exercise is also helpful to treat patients with heart failure. Exercise used to be restricted in HF patients due to the fear of sudden cardiac death during exercise. Until the past few decades, numerous studies have revealed exercise testing and training are safe for heart failure patients [325]. Exercise is beneficial for heart failure in several ways. First, exercise helps increase peak oxygen uptake between 12% and 31% [326]. Second, exercise improves cardiac output [327], increases skeletal muscle mitochondrial size and density [328], and attenuates endothelial dysfunction [329]. Third, exercise increases NO synthase activity and expression and prevents endothelial dysfunction [329]. Recent

research indicates that long term moderate-intensity exercise training improves chronic HF patients' quality of life and longevity [330]. Notably, Wisløff et al. compared the cardiovascular effects of continuous and interval exercise training in heart failure patients [331]. After 12 weeks of training, 3 times per week, interval exercise trained subjects had greater aerobic capacity that was associated with attenuation of LV remodeling compared to continuous exercise trained subjects. Moreover, LV end-diastolic and end-systolic volumes decreased with interval

Training, which was associated with preservation of LV ejection fraction and reduced of pro-brain natriuretic peptide. This study suggests interval exercise training may have greater beneficial effects compared to continuous exercise training in patients with HF.

Mechanisms of exercise effects on arterial stiffness in physiological settings

Low intensity chronic exercise reduces blood pressure and increases survivability in HF rats induced by hypertension [332]. In addition, moderate-intensity exercise reduces myocardial stiffness with reduced cross-linking of collagen in older rats [333]. Other animal studies showed that exercise interventions reduce carotid artery stiffness [334], attenuate myocardial oxygen balance and diastolic function in swine [335] and improve survival rats with HF [336]. The underlying mechanisms of exercise on physiological adaption has been, in part, related to reducing oxidative stress. Moreover, an animal study

showed that exercise increased mouse circulating endothelial progenitor cells and reduced endothelial progenitor cell apoptosis dependent on NO synthesis [337]. Another animal research showed 8 weeks moderate treadmill walking increased anti-oxidant enzyme activity and prevented lipid oxidation in multiple organs [338].

Exercise has also been shown to prevent inflammatory responses in CVD-related conditions. Exercise had protective effects in the development of atherosclerosis or regression of atherosclerotic lesions [339]. Another study indicated that hypertensive women had improvements in immune function, reduction of IL-1α, TNF-α and elevation of athero-protective cytokines IL-4 and IL-10 after 6 months moderate exercise [19].

This emerging evidence indicates that exercise is beneficial for apparently healthy and patient populations. However, no direct evidence demonstrates the influence of exercise on AGE in HF conditions. An animal study conducted in an obesity rat showed that exercise prevented AGE accumulation [340], but the mechanisms were not examined.

High intensity exercise

In addition to the beneficial effects of exercise, there are potential risks such as injury when performing physical activity and exercise. Risk of injury increases with greater volume of exercise [341] and intensity [342]. High intensity exercise dramatically increased the risk of sudden cardiac death [343, 344] and myocardial infarction [345, 346] among individuals with cardiovascular events.

Previous research has reported that improvements of peak oxygen uptake, left ventricle remodeling, left ventricle function and endothelial function were greater with high intensity exercise than moderate intensity exercise [347]. But the underlying mechanisms remain unknown.

Summary

In summary, arterial stiffness is an independent risk factor and predictor of cardiovascular events and all-cause mortality. Thus, investigating the mechanisms of arterial stiffness to establish effective therapeutic interventions is clinically significant. AGE and PVAT related oxidative stress, and inflammation are potential risk factors for arterial stiffness. However, the influence of PVAT-derived AGE on arterial stiffness has not been explored. While numerous studies focused on arterial stiffness with hypertension, few have assessed arterial stiffness in HF model. Moreover, no evidence is available to reveal the mechanisms of AGE accumulation with coronary artery dysfunction in non-diabetic conditions. Regular moderate-intensity physical activity has been determined as a useful strategy to attenuate HF and coronary heart disease. However, the mechanisms of exercise on AGE and its related signaling in non-diabetic model are poorly understood. Therefore, it is of great importance to verify the efficacy of chronic exercise effects on coronary artery stiffness with heart failure via PVAT-related AGE secretion.

CHAPTER III
Method

Aortic Banding and Exercise Training Protocols

The animals in the present study are the same animals used in recently published work from our laboratory in the *Journal of the American Heart Association* [334] and the *Journal of Applied Physiology* [348, 349]. Left ventricular hypertrophy/heart failure was induced by aortic banding for a period of 24 weeks using methods previously published by our laboratory [334, 335, 350-352]. Moreover, the animal model showed key characteristics of HFpEF with an increased end-diastolic P-V slope, increased lung weight, increased LV natriuretic peptide levels, and normal resting ejection fraction [334, 348, 349].

Male Yucatan mininature swine (29-32 kg; 8 months old) were assigned into 4 groups (n=7 for all groups): sedentary control (CON), aortic-banded sedentary (HF), aortic-banded chronic continuous exercise trained (HF+CONT) and aortic-banded chronic interval exercise trained (HF+IT). Aortic bands were placed around the ascending aorta (proximal to the brachiocephalic artery), and a systolic transstenotic gradient of ~70 mmHg was established (74±2 , 74±2, and 72±1 mmHg for HF, HF+IT, and HF+CONT, respectively, P= not significant [NS]) under anesthesia using phenylephrine (I.V. 1-3 ug/kg/min) to maintain a distal peripheral vascular mean aortic pressure (MAP) of approximately 90 mm Hg (90 ± 1, 91 ± 2, and 89 ± 1 for HF, HF+IT and HF+CONT, respectively, P=NS) at a heart rate of 100 beats/min (104 ± 5, 99 ± 8, and 106 ± 5 for HF, HF+IT and HF+CONT, respectively, P=NS) as previously reported [348, 349, 353].

Transverse aortic constriction (TAC) is the most commonly used approach to develop pressure overload induced HF in mice [354]. However, cardiac hypertrophy is induced within 48 hours after TAC in mice [355]. This acute pathological alteration in the heart induced by pressure overload does not mimic the gradual changes of myocardial remodeling. [356]. While the mini-swine model of pressure overload-induced HF in this study better represents the human HF condition. Moreover, HF mice induced by TAC cannot finish the 17 weeks exercise training regimen since the 8 week survival rate is less than 50% [357].

Animals began chronic exercise training consisting of treadmill running 55 min/day, 3 days/week, for 17 weeks with gradually increases in intensity using the following previously published protocols: continuous exercise 1) a 5 min warm up at 1.5 mph; 2) 45 min at 2.5 mph; and 3) a 5 min cool down at 1.5 mph [349, 353]; interval exercise 1) a 5-min warm up at 2 mph, 2) six 5-min sessions at 3 mph with five 3-min intervals at 4 mph in between, and 3) a 5-min cool down at 2 mph [335, 348, 358, 359]. This protocol is based off previous findings in young, healthy miniswine that underwent a VO_2max test. The VO_2max of young healthy miniswine is about 60ml/kg/min which is similar to a young, physically fit adult male [360]. Due to the aortic banding, the intensity used in the current study was set at ~30%-40% of VO_2max for the healthy miniswine. These exercise training protocols have been used in both healthy and HF miniswine, which has shown to preserve left ventricular function and improve coronary micro-circulation function [361-364]. A table with summarized animal characteristics is attached for reference (Table 3-1). Animals were fed a standard diet averaging 15–20 g/kg

once daily, and water was provided ad libitum. All animal protocols were in accordance with the “Principles for the Utilization and Care of Vertebrate Animals Used in Testing Research and Training” and approved by the University of Missouri Animal Care and Use Committee.

Table 3- 1. Animal Physiological Characteristics

	CON	HF	HF+CONT	HF+IT
BW, kg	46±3	48±2	48±1	49±3
SBP, mmHg	82±7	103±8*	83±7	82±6
DBP, mmHg	61±8	80±5	68±4	67±5
LV SV, ml	66±6	67±4	72±3	74±5
LV EF, %	64±3	66±3	65±3	65±4

Table 3-1. Animal Physiological Characteristics. Values are Mean±S.E. n=7 for each group. CON, sedentary control; HF, aortic-banded heart failure sedentary; HF+CONT, aortic-banded heart failure continuous exercise trained; HF+IT, aortic-banded heart failure interval exercise trained. BW-body weight; SBP-systolic blood pressure; DBP-diastolic BP; LV SV-left ventricular stroke volume; LV EF-LV ejection fraction. *vs. all, $P<0.05$.

Mechanical Stiffness Testing

Arterial stiffness was assessed as previously described [5, 8, 20, 365]. The left circumflex (LCX) and right coronary artery (RCA) were cleaned of the surrounding adipose tissue and cut into ~1.5mm segments. The coronary arterial ring was placed in a preheated 37°C Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) solution in Myograph (DMT 620). The artery segment was stretched 1mm every 3 minutes until mechanical failure. The elastic modulus was calculated from the stress-strain curve as previously described [5, 8, 20, 365]. In brief, one-dimensional stress (t) was calculated as: $t = \lambda L/2HD$, and strain (λ) defined as: $\lambda = \Delta d/d(i)$, where L = one-dimensional load applied, H = wall thickness, D = length of vessel, Δd = change in diameter, and d(i) = initial diameter. Coronary diameter and wall thickness were assessed in histological sections, and length was measured under a dissecting microscope using calipers. The elastic modulus was determined as the greatest r^2 value from the stress-strain curve as described previously [5]. The elastin region, coinciding with the elastin elastic modulus (EEM), was determined as the transition point between the toe and heel regions of the stress-strain curve, and collagen region, coinciding with the collagen elastic modulus (CEM), was the determined greatest r^2 value prior to mechanical failure (Figure. 3-1) [5].

Figure 3- 1. Representative of Stress-Strain curve.

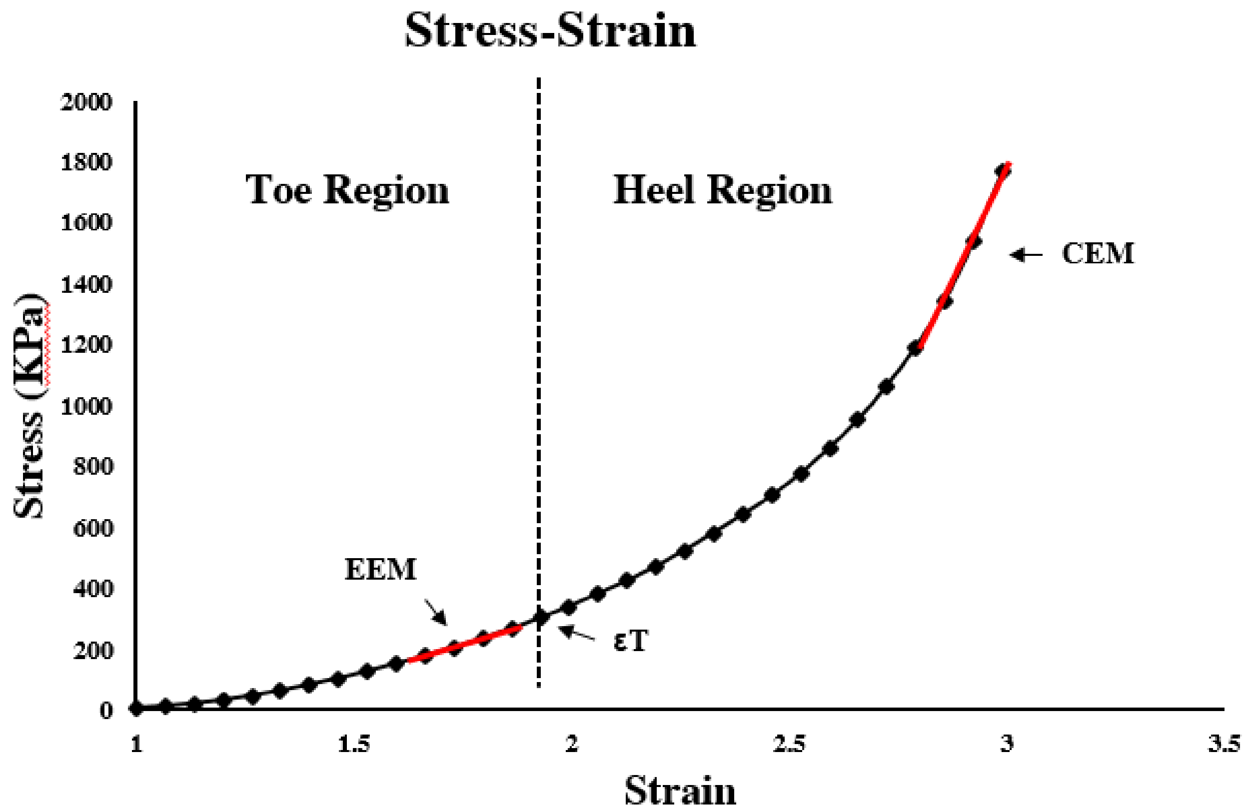


Figure 3-1. Representative of Stress-Strain curve. True stress is plotted on the y-axis and true strain on the x-axis. Transition point (εT) is determined between the toe and heel regions. The elastin elastic modulus (EEM) is the slope of the four points prior to εT and the collagen elastic modulus (CEM) is the slope of the last four points before the yield point.

Coronary Perivascular Adipose Conditioned Media Experiments

The conditioned media study was performed as described previously by our lab [5, 8, 366]. Briefly, adipose tissue surrounding the coronary artery was removed and cultured for 24 hours in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (20mg fat/100μl DMEM) at 37° C and 5% CO_2. The coronary adipose conditioned media (10% of total volume) was heat inactivated by placing in a heat block for 30 minutes at 56° C. Aortas (without the surrounding adipose tissue) from young (4-6 month old), wild-type C57BL/6 mice (The Jackson Laboratory) were cultured in the coronary conditioned media with or without 1 mM AGE inhibitor (aminoguanidine, AMG) for 72 hours at 37° C and 5% CO2. The conditioned media was changed daily. After treatment, aortic stiffness testing was performed.

Immunohistochemistry (IHC)

IHC was performed by standard procedures as previously described [5, 8]. Segments of LCX, RCA and mouse aortas were embedded with optimal cutting temperature (O.C.T.) and frozen. Arteries were cross-sectioned (8μm) , fixed on glass slides with acetone and stained using Dako Envison+ system HRP-DAB kit (Agilent). The following primary antibodies were used: collagen (abcam), elastin (abcam), AGE (abcam), receptor of AGE (RAGE, abcam), nitrotyrosine (EMD), NF-κB p65 (abcam) and scavenger receptor A (SRA, TransGenic) were applied on slides separately and incubated at 4°C overnight with pre-optimized dilutions (Table. 3-1). The secondary HRP conjugated labeled polymer was applied for 30

minutes at room temperature followed diaminobenzidine (DAB) application for approximately 2 minutes until appropriate darkness achieved. Coronary PVAT was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned in 5μm thickness. Slides were deparaffinized with xylene and proteinase K was used for antigen retrieval. Slides were stained with pre-optimized diluted primary antibodies (Table. 3-1). Images were acquired using a Nikon 80i microscope using a 4x, 10x and 20x objectives for the swine coronary artery, mouse aorta and adipose tissue, respectively. Densitometry analysis was performed by ImageJ (NIH) on the medial layer of the artery, and the data are presented as relative density in arbitrary units (AU).

Table 3- 2. Immunohistochemistry Primary Antibody Concentration

Primary antibody	Coronary artery	PVAT	Mouse aorta
Collagen	1:400		
Elastin	1:300		1:200
AGE	1:400	1:200	1:150
RAGE	1:200	1:150	
Nitrotyrosine	1:300	1:50	1:100
NF-κB p65	1:300	1:50	1:100
SRA	1:100	1:50	

Adipose Morphology

PVAT surrounding coronary arteries was fixed in 4% paraformaldehyde, embedded in paraffin, and cross-sectioned (5µm). After deparaffinized, adipose

tissue was stained with hematoxylin and eosin using standard procedures [5, 8, 367]. Digital images were acquired with a Nikon 80i microscope with 20x objective lens in an uncompressed tiff format. Adipocyte area (μm^2) and diameter (μm) were averaged from at least 100 cells from 3 tissue sections for each sample using ImageJ (NIH). The data for each sample was averaged, and used to calculate the mean for each group.

Enzyme-linked Immunosorbent Assay (ELISA)

Peri-coronary adipose tissue was removed and cultured in serum-free DMEM for 24 h at 37°C and 5% CO2 at a concentration of 20mg fat/100μl. AGE concentration in swine coronary perivascular adipose conditioned media was assessed by ELISA kit per the manufacturer's protocol (Cell Biolabs).

Cytokine Array

Cytokines in the peri-coronary adipose conditioned media were assessed by Porcine Cytokine Antibody Array Kit (Abcam, ab197479). Ten cytokines targets (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, GM-CSF, IFNγ, TGFβ1, TNFα) were assessed. However, only IL-6 and IL-8 were detectable. Measurement was performed per product's manual. Array fluorescence signals were scanned by a laser scanner equipped with Cy3 wavelength (Typhoon 9500, GE, USA). Dots densitometry were analyzed by ImageJ (NIH). The quantitative of cytokines were calculated based on standard curves.

Statistical Analysis

All data analyses were performed with Graphpad Prism 7.0. Group mean comparisons for coronary stiffness, IHC analyses, the AGE ELISA and cytokines array were analyzed by one-way ANOVA. Coronary PVAT culture studies were analyzed by two-way ANOVA. Fisher's LSD post-hoc analyses were performed when appropriate. All data were presented as Mean±S.E. Statistical significance was reported as $P<0.05$.

Chapter IV
Result

LV Remodeling and Function

A thorough investigation of myocardial remodeling and LV function was provided for the same animals used in the current study in recently published work from our laboratory in the *Journal of Applied Physiology* [334, 348, 349]. In brief summary, these studies demonstrated the therapeutic efficacy of chronic exercise training on coronary vascular, isolated cardiomyocyte, and cognitive function in a translational pressure-overload model of heart failure with potential relevance to human HF. Aortic banding caused symptoms of heart failure in the HF group including increased lung weight and left ventricular natriuretic peptide levels, global concentric hypertrophic remodeling at the gross and cellular level, and diastolic dysfunction (increased slope of the end diastolic pressure-volume relationship). Echocardiography and pressure-volume analysis indicated ejection fraction, fractional shortening, and stroke volume at rest were normal in HF compared with CON animals. Chronic exercise training had a positive impact at both the organ and molecular level in a number of different functional systems including isolated cardiomyocyte calcium handling and contractile function, large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel-mediated coronary vascular function, and peripheral arterial stiffness and cognition, and we refer the reader to these published studies for specifics [334, 348, 349].

Exercise Prevents Coronary Artery Stiffness, ECM Remodeling, Oxidative stress and Inflammation

Compared to CON, the combined EEM of the LCX and RCA was lower in HF group (284±28 vs. 444±38 kPa, $P<0.05$; Figure 4-1A), which was associated with reduced medial elastin protein content ($P<0.05$; Figure 4-1B). No differences were observed between the CON and HF groups for either the CEM (9324±1319 vs. 10563±1272 kPa, $P>0.05$; Figure 4-1C) or collagen content ($P>0.05$; Figure 4-1D). The 16-week HF+CONT and HF+IT exercise training regimens prevented the decrease in coronary EEM (474±38 and 511±56 kPa, respectively, $P<0.05$; Figure 4-1A) and elastin content ($P<0.05$; Figure 1B), without influencing CEM or collagen content ($P>0.05$; Figure 1C, 1D).

Figure 4- 1. Coronary artery stiffness and arterial elastin and collagen content

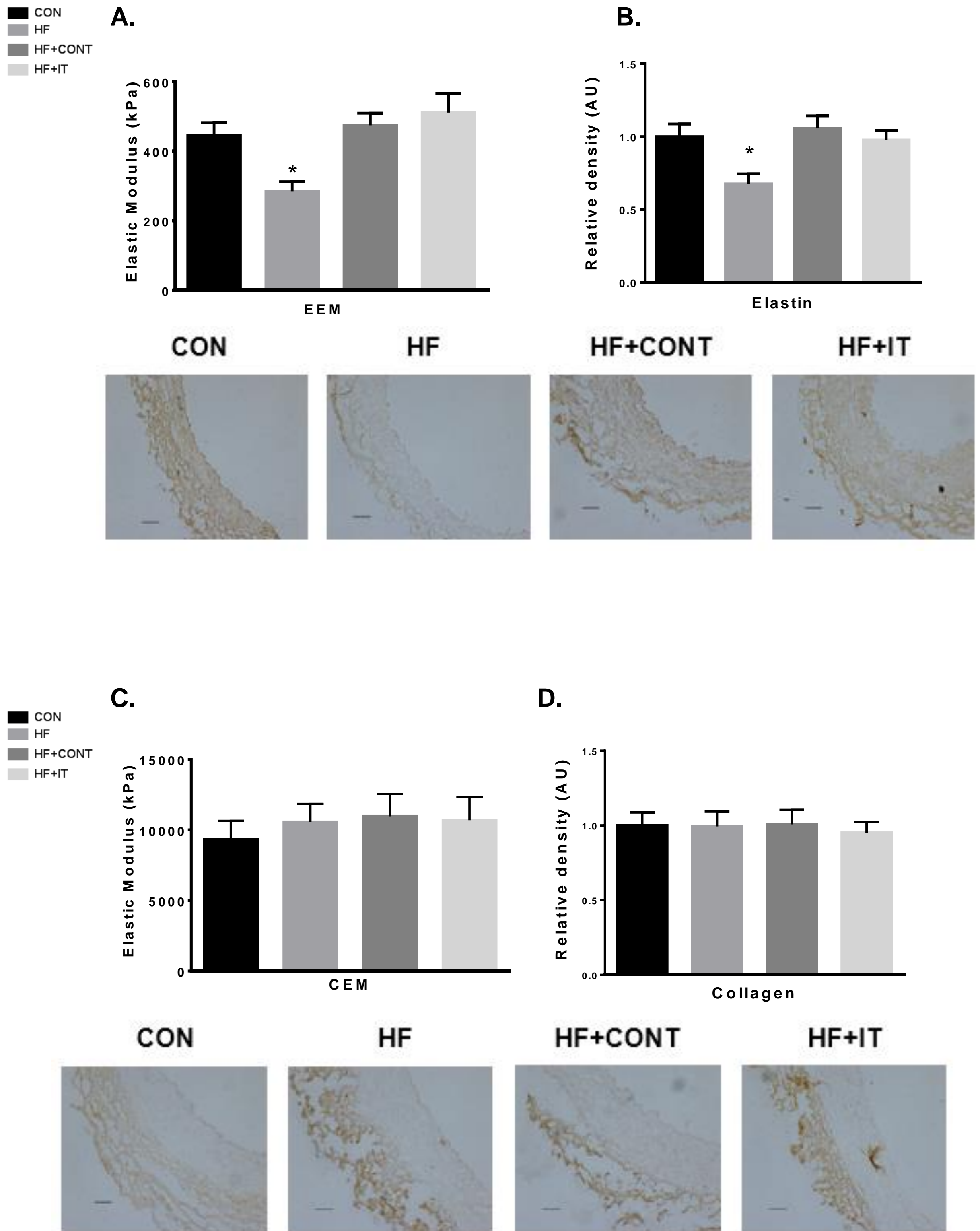
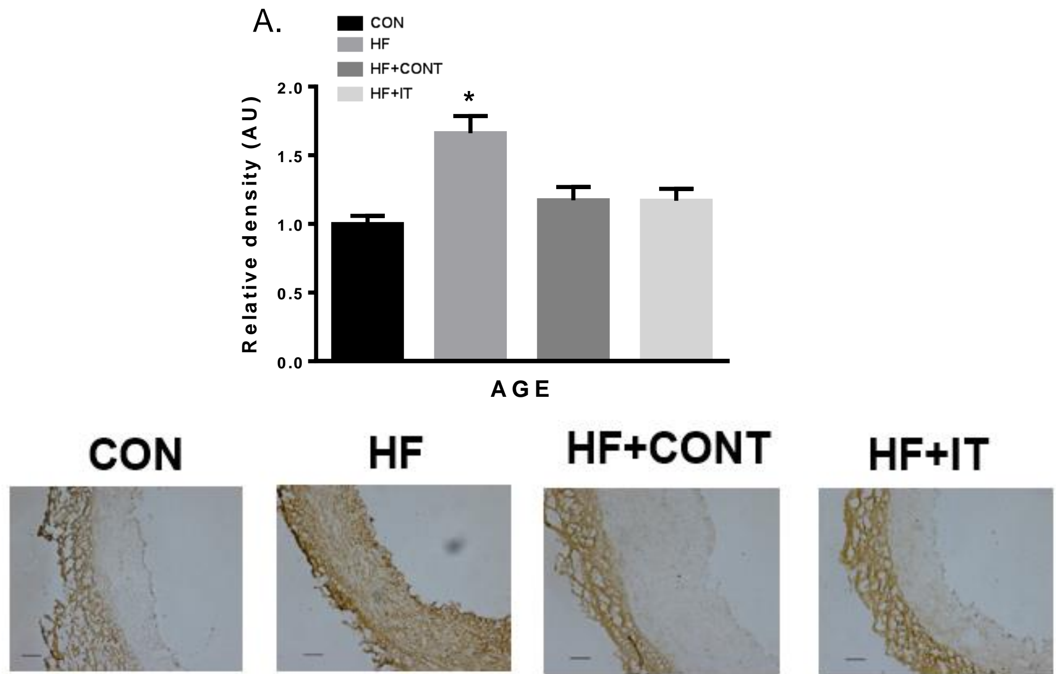
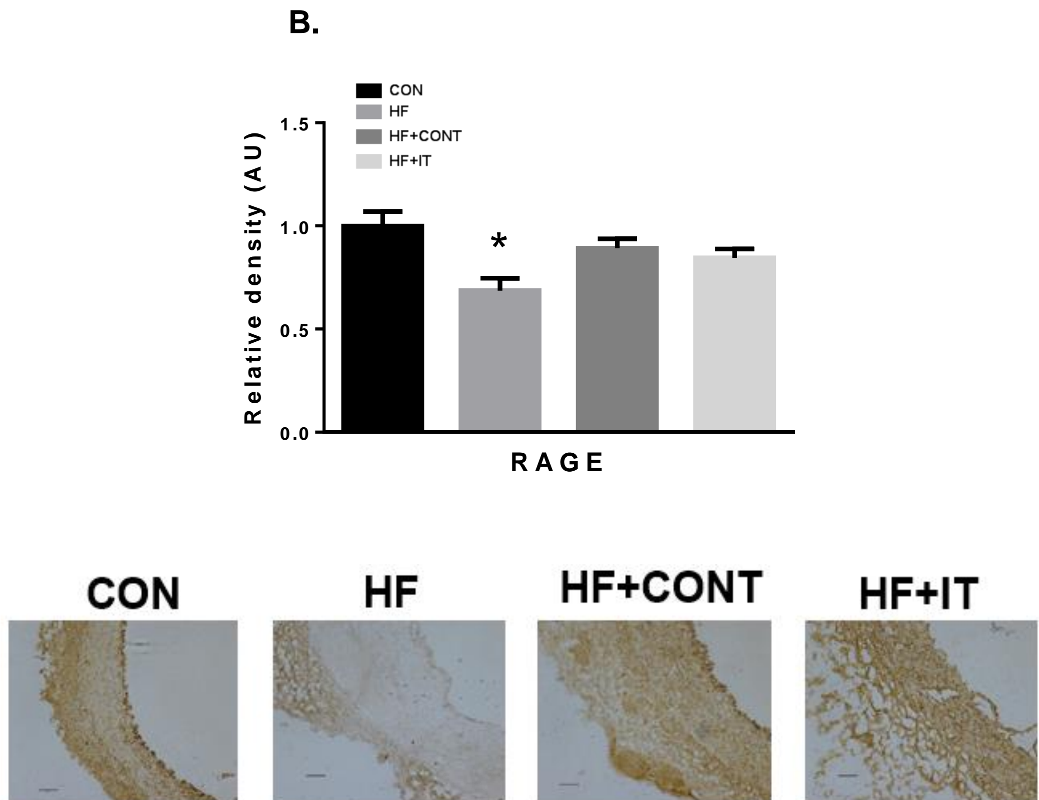


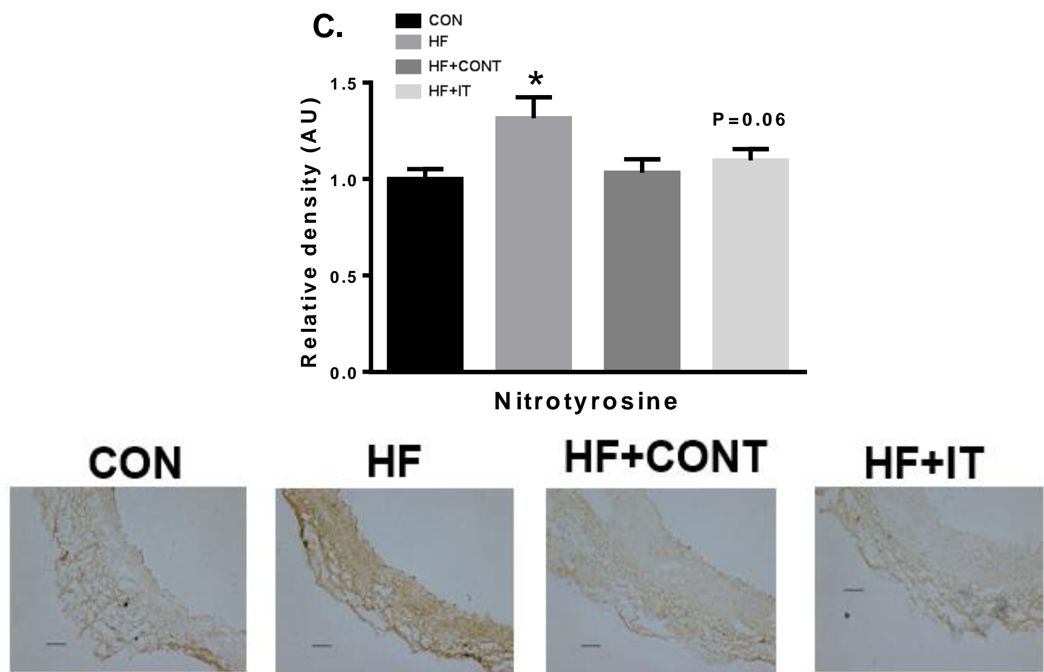
Figure 4-1. Coronary artery stiffness and arterial elastin and collagen content. Reduced elastin elastic modulus (EEM) (**A**) and elastin content (**B**) in HF swine coronary was prevented by exercise training. Collagen elastic modulus (CEM) (**C**) and collagen deposition (**D**) were not different between groups. (Data are Mean ± S.E., * vs. all, $P<0.05$). Representative immunohistochemistry images of coronary artery showing medial elastin and collagen. *Abbreviation: control (CON), heart failure (HF), HF continuous exercise trained (HF+CONT), HF interval exercise trained (HF+IT).*

Increased coronary stiffness with HF was associated with greater arterial AGE abundance, and increased nitrotyrosine, NF-κB p65 and SRA relative to CON ($P<0.05$; Figure 4-2A, 4-2C-E). Arterial RAGE was lower in HF group compared to CON group ($P<0.05$; Figure 2B). Both HF+CONT and HF+IT prevented the increase of AGE, nitrotyrosine, NF-κB p65 and SRA ($P<0.05$; Figure 4-2A, 4-2C-E), and the decrease in RAGE (Figure 4-2B; $P<0.05$). These findings demonstrate that a chronic continuous or interval exercise training regimen prevents coronary artery stiffness and vascular wall ECM remodeling in a pre-clinical mini-swine model of pressure overload-induced HF.

Figure 4- 2. Immunohistochemistry analysis of AGE, RAGE, oxidative stress and inflammation level in coronary artery







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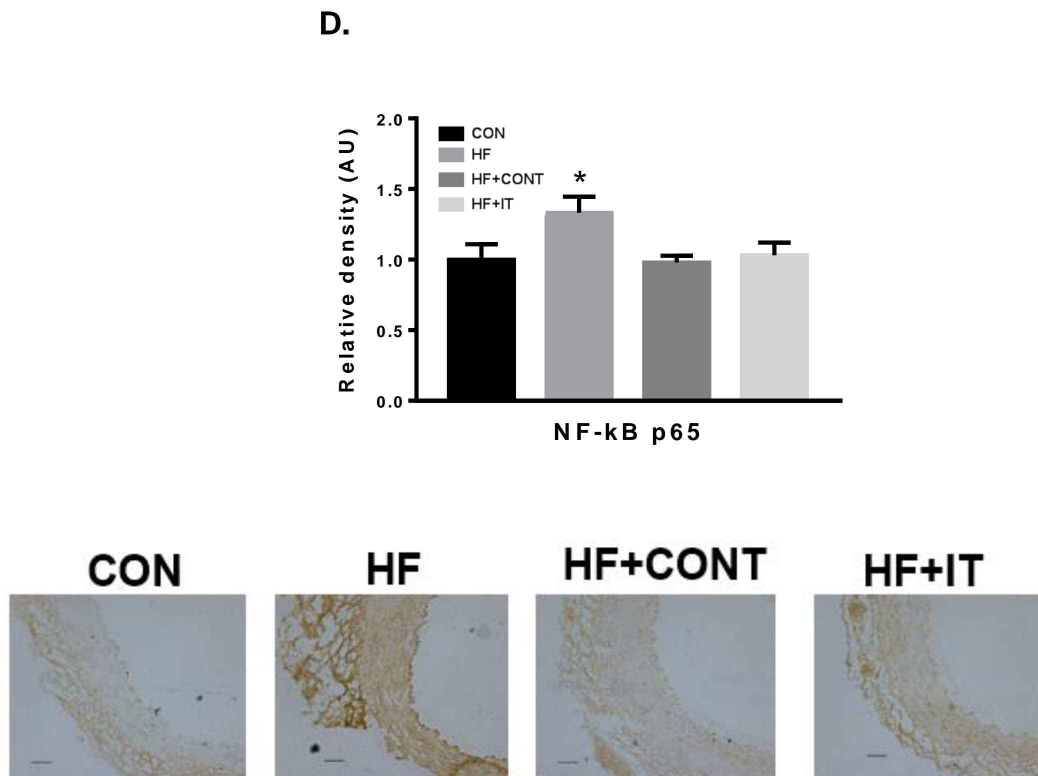


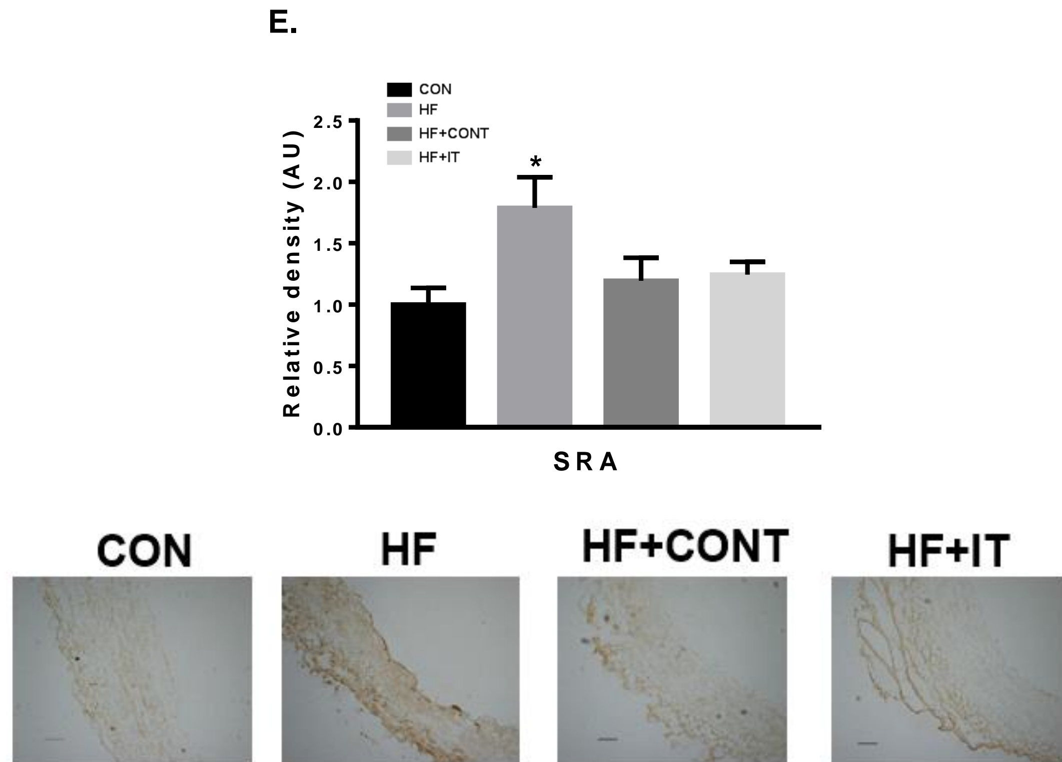


Figure 4-2. Immunohistochemistry analysis of AGE, RAGE, oxidative stress and inflammation level in coronary artery. Greater AGE (**A**), nitrotyrosine (**C**), NF-κB p65 (**D**) and scavenger receptor A (SRA) (**E**) in HF compared with CON, which was attenuated by continuous and interval exercise training. (**B**) Receptor of AGE (RAGE) was lower in HF compared to control, HF+CONT and HF+IT. (Data are Mean ± S.E., * vs. all, $P<0.05$. The nitrotyrosine abundance in HF+IT showed decreased trending compared to HF, P+0.06). Representative immunohistochemistry images of coronary artery showing AGE, RAGE nitrotyrosine, NF-κB p65 and SRA alterations. *Abbreviation: control (CON), heart*

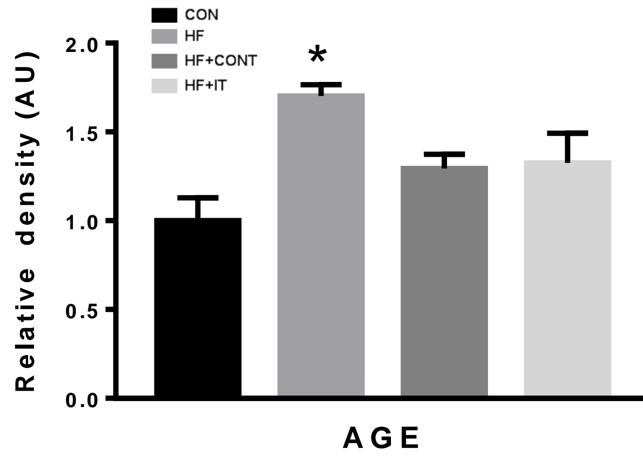
failure (HF), HF continuous exercise trained (HF+CONT), HF interval exercise trained (HF+IT).

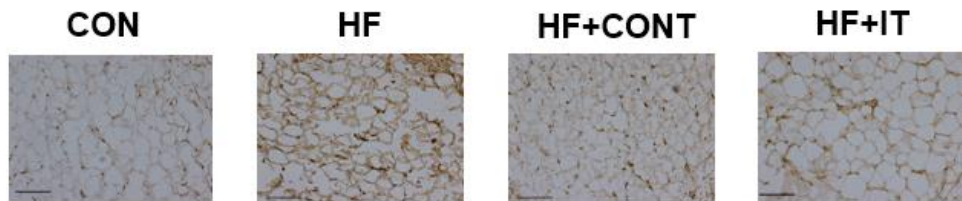
PVAT-related AGE Secretion, Arterial Stiffening and ECM Remodeling are Prevented by Exercise Training

Compared with CON, HF had greater PVAT AGE abundance ($P<0.05$; Figure 4-3A) and concentrations of AGE in PVAT-conditioned media (1.404±0.44 vs. 0.39±0.07µg/mL, $P<0.05$; Figure. 4-3B). To determine the influence of PVAT-derived AGE on arterial stiffness, mouse aortas were cultured with the conditioned media and stiffness testing was performed. The EEM was reduced after exposure to HF compared with CON PVAT-conditioned media (368±20 vs. 582±29 kPa, $P<0.05$; Figure 4-3C). Both HF+CONT and HF+IT exercise attenuated or prevented the HF-related AGE accumulation in PVAT ($P<0.05$; figure 4-3A), AGE secretion from PVAT (0.27±0.06 and 0.23±0.04 µg/mL, respectively, $P<0.05$; Figure. 4-3B) and the PVAT-induced reduction in EEM (542±58 and 518±22 kPa, respectively, $P<0.05$; Figure 4-3C). The AGE inhibitor, AMG, prevented the PVAT-related reduction in EEM with HF (643±35 kPa, $P<0.05$; Figure 4-3C). AMG also increased the EEM in HF+CONT aortic segments (772±101 kPa, $P<0.05$; Figure 4-3C).

Figure 4- 3. AGE secretion from peri-coronary adipose tissue and PVAT derived AGE effects on aortic stiffness

A.





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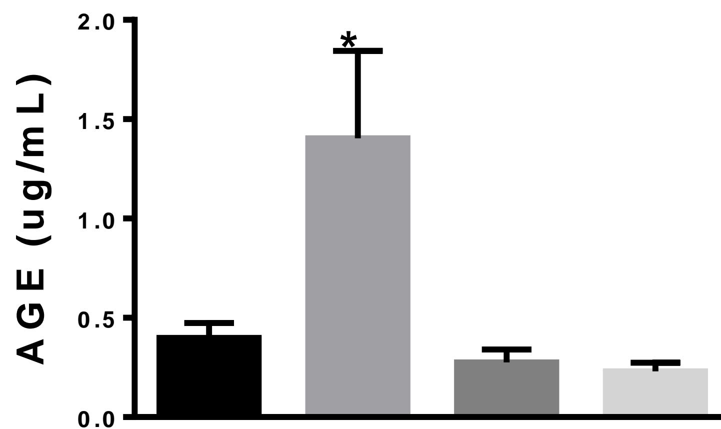

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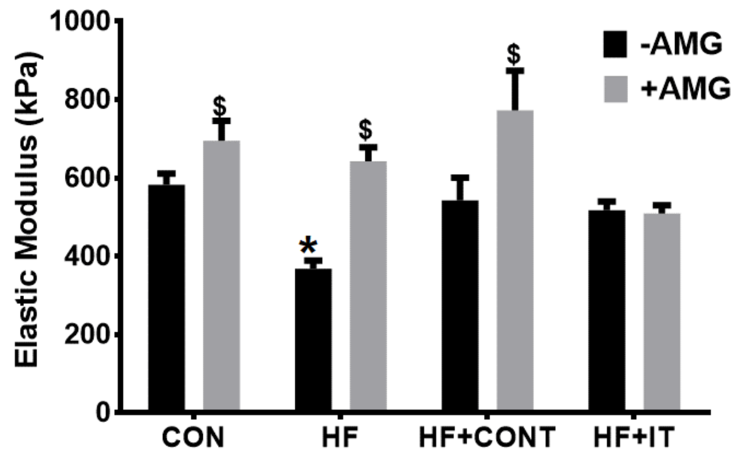
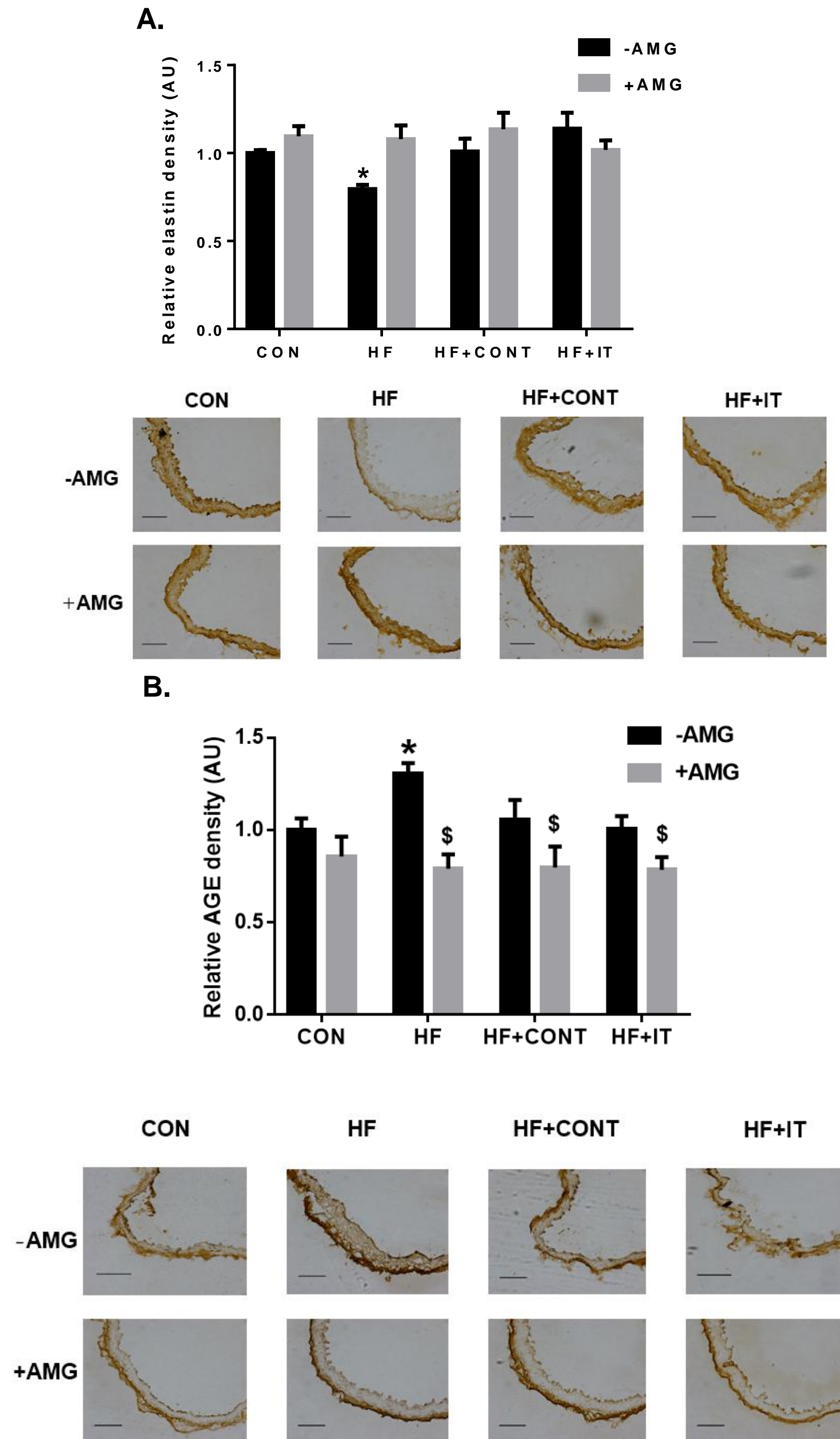


Figure 4-3. AGE secretion from peri-coronary adipose tissue and PVAT derived AGE effects on aortic stiffness. Greater peri-coronary adipose tissue AGE (**A**) accumulation in HF compared with CON, HF+CONT and HF+IT. **(B**) AGE concentration in HF swine coronary PVAT conditioned media was higher compared to control and exercise training groups. **(C**) Exposure to HF swine coronary conditioned media increased mouse aortic stiffness and AGE inhibitor, AMG prevented the increased aortic stiffness. (Data are Mean ± S.E., * vs. all, P<0.05; $ vs. same group -AMG, P<0.05.) *Abbreviation: control (CON), heart failure (HF), HF continuous exercise trained (HF+CONT), HF interval exercise trained (HF+IT), aminoguanidine(AMG).*

Effects of peri-coronary adipose conditioned media on aortic extracellular matrix, oxidative stress and inflammatory.

To determine if coronary PVAT-derived AGE contributes to reductions in arterial elastin and AGE accumulation, mouse aortas treated with PVAT conditioned media were analyzed. Elastin content was decreased ($P<0.05$; Figure 4-4A) and AGE was increased ($P<0.05$; Figure 4-4B) after culture in HF-conditioned media. HF+CONT and HF+IT exercise training regimens preserved elastin content and prevented AGE accumulation in arteries cultured in HF PVAT-conditioned media (Figure 4-4A, 4-4B; $P<0.05$). AMG prevented the HF-induced reductions in arterial elastin ($P<0.05$; Figure 4-4A) and vascular AGE accumulation ($P<0.05$; Figure 4-4B). Arterial segments treated with AMG had lower nitrotyrosine and NF-κB p65 subunit expressions compared with segments not treated ($P<0.05$, main effect of drug; Figure 4-4C, D). Collectively, these data indicate AGE associated with PVAT promotes arterial stiffness via reductions in elastin functionality and content, which is prevented by exercise training of two different intensities.

Figure 4- 4. Influence of coronary adipose conditioned media on aortic extracellular matrix proteins



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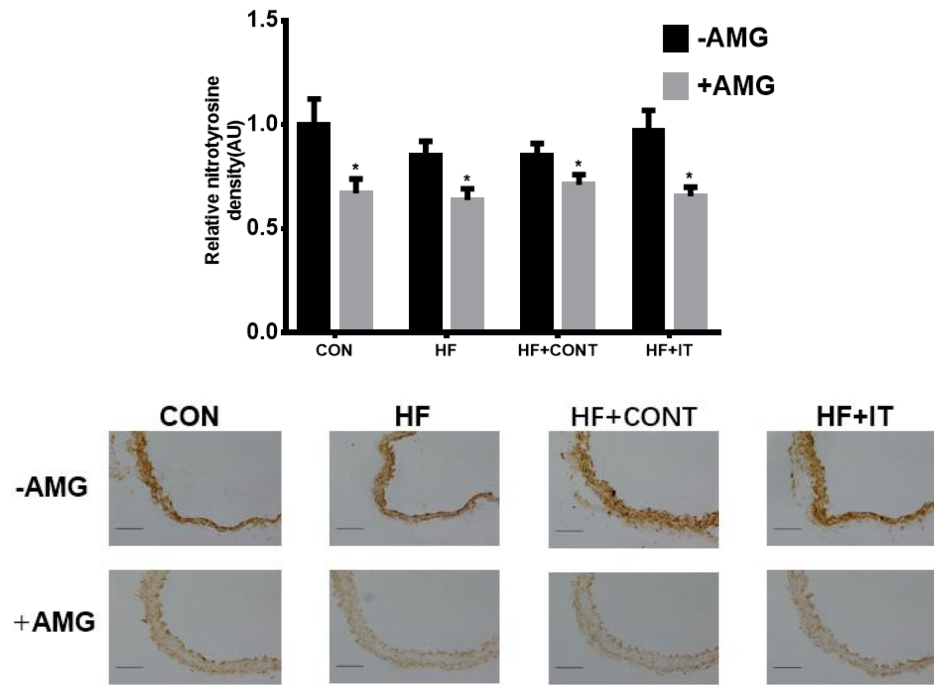

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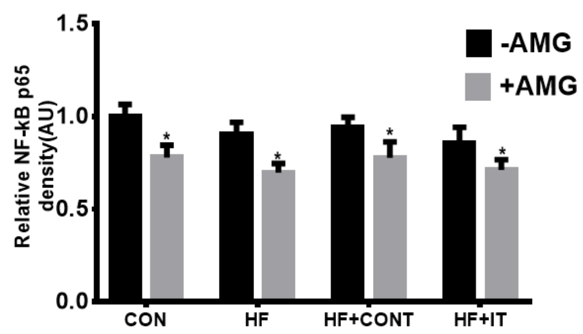


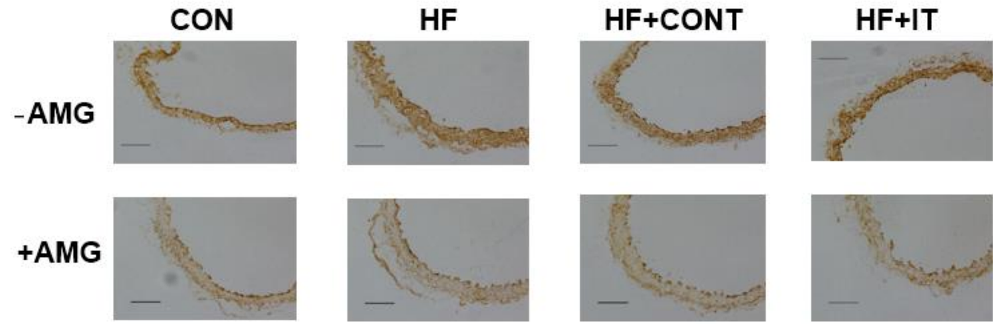


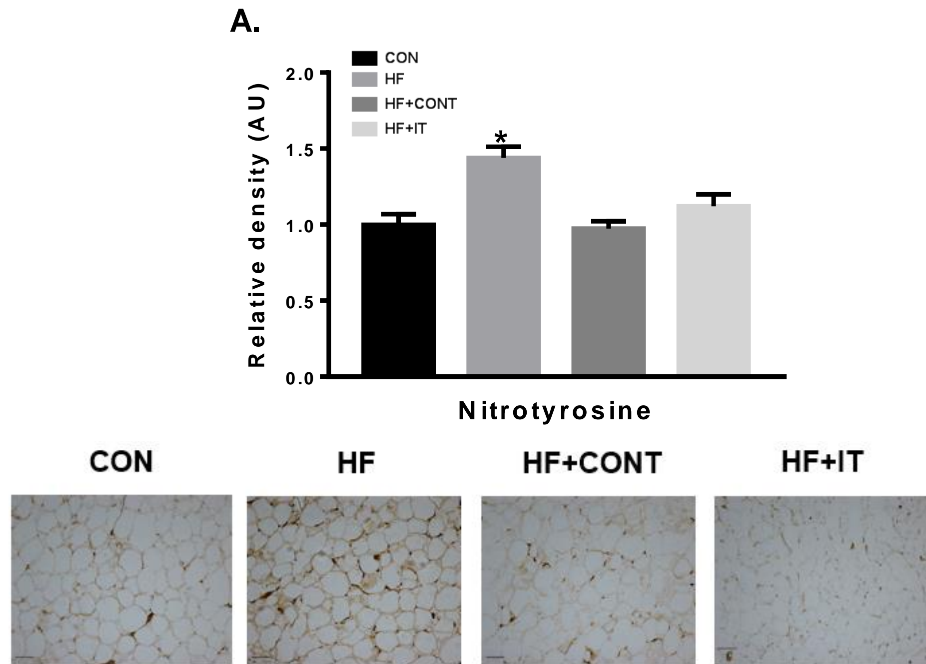
Figure 4-4. Influence of coronary adipose conditioned media on aortic extracellular matrix proteins, oxidative stress and inflammation. The HF coronary adipose conditioned media decreased mouse aorta elastin content (**A**) and increased AGE (**B**) compared to other groups while AMG prevented AGE accumulation. (Data are Mean±S.E., * vs. all, $P<0.05$; $ vs. same group -AMG, $P<0.05$). Nitrotyrosine (**C**) and NF-κB p65 (**D**) were unchanged after cultured in coronary adipose conditioned media, but the AGE inhibitor AMG decreased nitrotyrosine and NF-κB p65 level. (Data are Mean±S.E., * vs. all, $P<0.05$; $ vs. same group -AMG, $P<0.05$, two-way ANOVA-Interaction effects). Representative immunohistochemistry images of mouse aorta showing medial elastin, AGE alterations, nitrotyrosine and NF-κB p65 level. *Abbreviation: control (CON), heart failure (HF), HF continuous exercise trained (HF+CONT), HF interval exercise trained (HF+IT), aminoguanidine(AMG).*

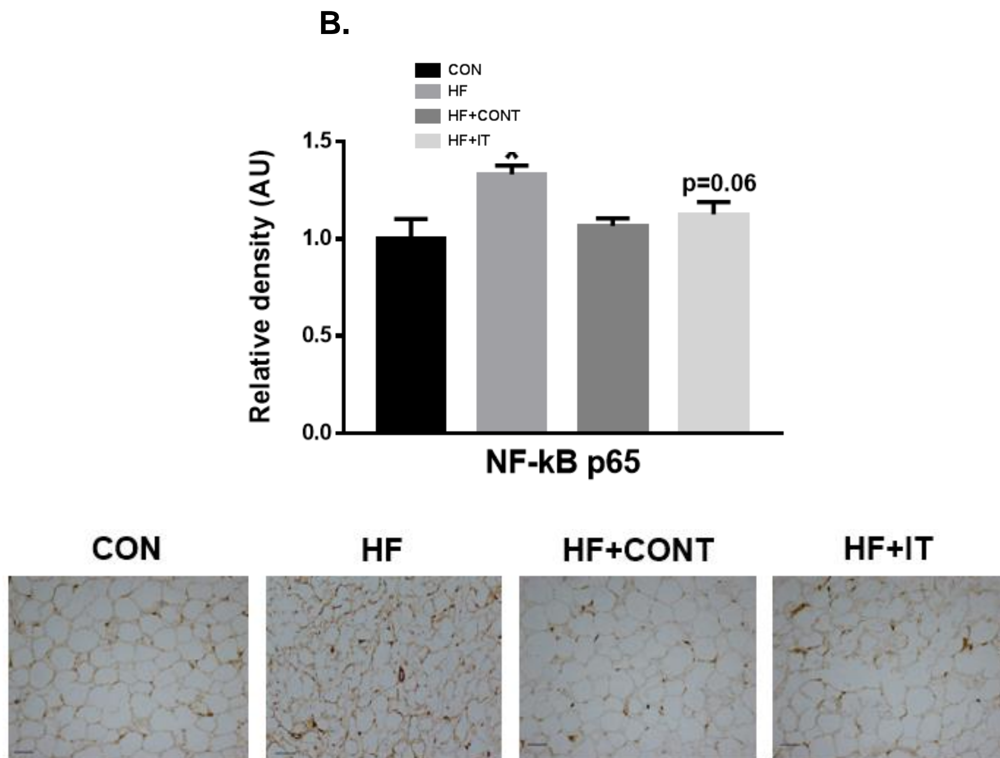
Exercise Prevents Coronary PVAT Oxidative Stress, Inflammation and Cytokines

Coronary PVAT nitrotyrosine and NF-κB p65 subunit were increased in the HF compared with CON (P<0.05; Figure 4-5A, 5B). No differences were observed for coronary PVAT SRA (P>0.05,Figure 4-5C) or RAGE (P>0.05, Figure 4-5D) between the CON and HF groups. Both exercise regimens prevented the increase in nitrotyrosine (P<0.05; Figure 4-5A). Further, HF+CONT prevented NF-κB p65 subunit expression (P<0.05; Figure 4-5B) while reductions with HF+IT approached significance (P=0.06; Figure 4-5B). Peri-coronary adipose secreted more Interleukin-6 (IL-6) and IL-8 in HF conditioned media compared to CON (265.88±30.85 vs. 146.02±30.68 pg/mL; 720.93±212.26 vs. 297.08±25.60 pg/mL, respectively, P<0.05; Figure 4-5E, F). In addition, both continuous and interval exercise training prevented IL-6 (167.74±31.97 and 180.44±24.11 pg/mL, respectively; P<0.05; Figure 4-5E) and IL-8 (347.02±59.15 and 296.95±24.10 pg/mL, respectively; P<0.05; Figure 4-5F) secretions in

conditioned media. Other cytokines are not detectable. PVAT cell area or diameter were not influenced by HF or exercise training (Table 4-1).

Figure 4- 5. Immunohistochemistry analysis of oxidative stress and inflammation in peri-coronary adipose tissue and cytokines





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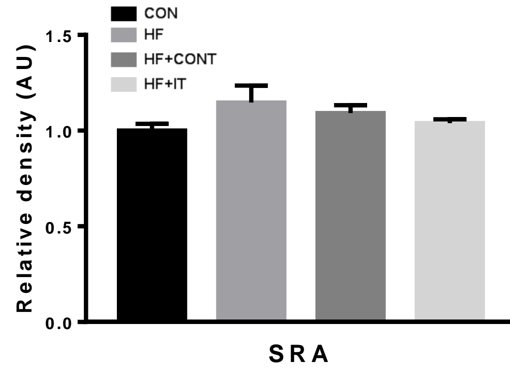

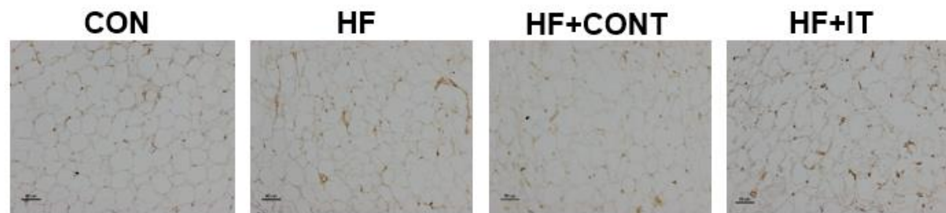

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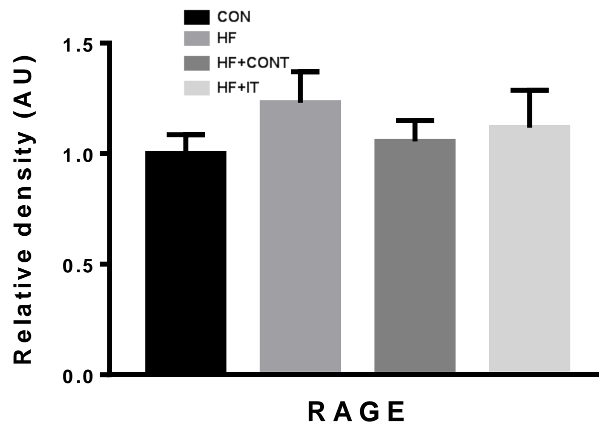

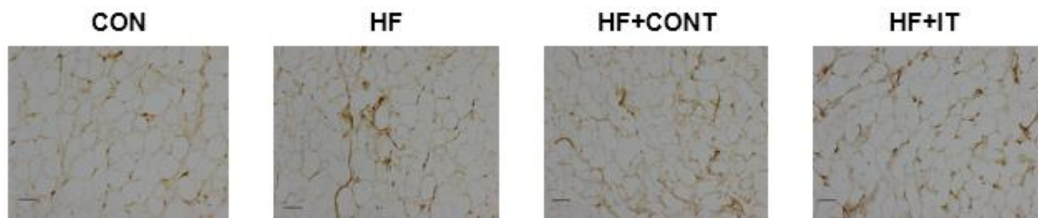

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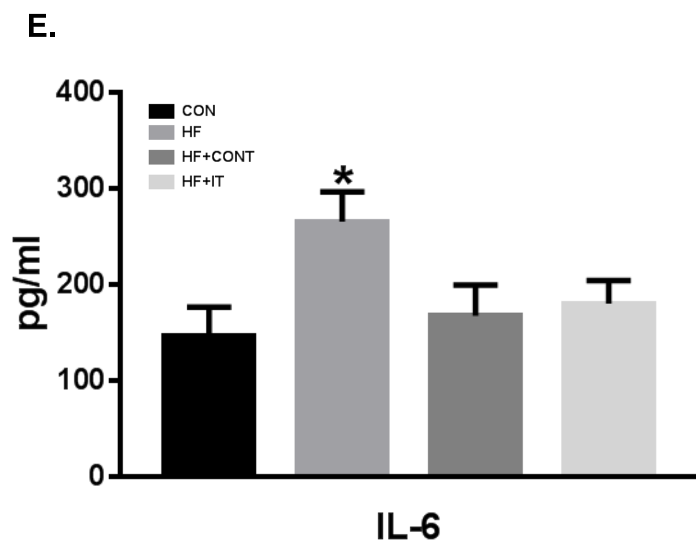

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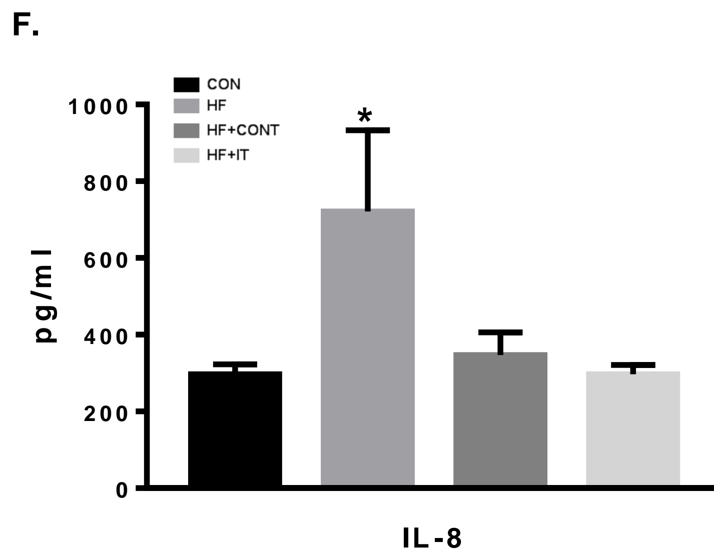

Figure 4-5. Immunohistochemistry analysis of oxidative stress and inflammation in peri-coronary adipose tissue. Greater nitrotyrosine (**A**) and NF-κB p65 (**B**) in HF was attenuated by exercise training. RAGE (**C**) and SRA (**D**) level were unchanged in all groups. Excessive Interleukin-6 (IL-6) (**E**) and IL-8 (**F**)

secretion in HF conditioned media were prevented by HF+CONT and HF+IT. (Data are Mean±S.E., * vs. all, P<0.05; $ vs. same group -AMG, P<0.05). (Data are Mean±S.E., * vs. all, P<0.05). Representative immunohistochemistry images of coronary artery showing peri-coronary adipose tissue nitrotyrosine, NF-κB p65, SRA and RAGE alterations. *Abbreviation: control (CON), heart failure (HF), HF continuous exercise trained (HF+CONT), HF interval exercise trained (HF+IT).*

Table 4- 1. Peri-coronary Adipose Morphology

	CON	HF	HF+CONT	HF+IT
Size (um^2)	1349 ± 99	1218 ± 61	1180 ± 49	1347 ± 80
Diameter (um)	40.34 ± 1.38	38.49 ± 0.94	37.78 ± 0.80	40.08 ± 1.14

Chapter V
Discussion

In this study, we examined the efficacy of, and identified a mechanism by which, a 16-week continuous or interval exercise training regimen prevents coronary artery stiffness in a clinically relevant, pressure overload model of HF. The primary findings from this study include: 1) both continuous and interval exercise training prevent coronary artery stiffness, which is associated with preservation of arterial elastin content, and prevention of arterial AGE accumulation; 2) PVAT-related AGE secretion promotes arterial stiffness, which was prevented by both exercise interventions; and 3) PVAT is a source of AGE secretion contributing to decrements in arterial elastin and AGE accumulation. These findings provide preliminary evidence for the efficacy for two unique exercise regimens to prevent coronary vascular stiffening with HF, and identify PVAT-derived AGE secretion as a novel target to prevent arterial stiffening.

Exercise Training Prevents Coronary Stiffening

A thorough investigation of cardiac function and coronary micro-circulation in the animal model used in current study was demonstrated and published in Journal of American Heart Association and *Journal of Applied Physiology* by our team [334, 348, 349]. Briefly, both continuous and interval exercise training attenuated HF with decreased lung weight and LV brain natriuretic peptide mRNA level. Moreover, both exercise training interventions improved coronary blood flow and vasodilatory response [334, 348, 349].

A primary finding of the present study is that both chronic continuous and interval exercise training prevents coronary artery stiffness associated with pressure overload-induced HF. To our knowledge this is the first study demonstrating an effect of exercise to prevent coronary artery stiffness and the associated ECM remodeling. These findings complement recently published work from our group in these same animals that demonstrated chronic exercise training also preserved coronary microvascular vasodilatory function via a BK_{Ca} channel-mediated mechanism [348] and prevented stiffening of the carotid artery [334], suggesting exercise can prevent pathological adaptations not only to the peripheral vasculature but also to the coronaries all along the vascular tree (i.e. both macro- and microvessels) in the presence of chronic pressure overload. Aerobic exercise training has been shown to reduce both central (aortic) and peripheral conduit artery stiffness in older and diseased adults [368-370], which are supported by animal models providing insight to the structural changes by which exercise attenuates arterial stiffening [16-18]. Moreover, 12 months Tai Chi prevents AGE accumulation in healthy subjects [371] and 12 weeks moderate-intensity exercise training prevents AGE accumulation in healthy women [372]. Similar to these prior studies, our findings demonstrate an effect of exercise to preserve elastin content, and prevent AGE accumulation [17, 18, 340, 373]. Notably, we extend these findings to demonstrate similar effects for both chronic continuous and interval exercise to prevent coronary stiffness and ECM remodeling. Our findings are supported by previous investigations demonstrating that continuous and interval exercise training prevent arterial stiffness in healthy

and hypertensive subjects [12, 374]. Due to a lack of effective treatment strategies for HF patients [3], our findings lend support for exercise training to lower coronary artery stiffness, which in combination with previous observations of improved coronary vascular conductance in these same animals [348] implies an overall effect of exercise to prevent increases in vascular resistance throughout the coronary arterial tree. Importantly, these data also show the efficacy of chronic exercise training as a therapeutic option for treating coronary vascular dysfunction in a setting of pressure overload-induced heart failure, using an intensity tolerable to HF patients.

Prevention of Coronary Artery Stiffness due to Preservation of ECM

The ECM proteins elastin and collagen are primarily responsible for contributing to mechanical stiffness of arteries [83]. Our data indicate HF promotes coronary artery stiffness by reducing arterial elastin content, without influencing collagen. Prior studies demonstrate other pathological conditions, such as obesity and hyperlipidemia, as well as the elastin deficient mouse promote arterial stiffness by reducing elastin content and/or function [8, 365, 375]. As such, the elastin elastic modulus was decreased with HF suggesting that in addition to lower elastin content, elastin function is also diminished. This is the first time to our knowledge it has been shown chronic continuous and interval exercise training programs of an intensity tolerable to HF patients prevents decrements in elastin content and function in the coronary vasculature of a pre-clinical model of pressure overload-induced HF. Previous investigations in rats

have indicated the potential for this effect in the aorta following exercise training, although these protocols used a higher training intensity and frequency compared to the current study [17, 18].

Coronary artery stiffness mediated by AGE

In addition to changes in the ECM, we observed greater AGE accumulation in the coronary artery of HF animals. Chronically increased plasma glucose concentrations is a well-recognized factor contributing to AGE formation. It is interesting to note the increase in coronary vascular AGE levels in the current study was observed in an experimental setting absent metabolic comorbidities. This finding recapitulates recently published observations from our laboratory in the same swine model, which we previously attributed to increased oxidative stress related to chronic pressure overload [5]. AGE cross-links proteins within the arterial wall, which includes elastin and collagen, to promote arterial stiffness [123, 376]. Further, exogenous AGE administered to rodents has shown to promote elastin disruption and fragmentation that is associated with arterial stiffness [129]. Thus, the increased coronary AGE may contribute to the reductions in elastin content and impaired functionality observed with HF. Elastin content and function were preserved with both continuous and interval exercise programs in this study, consistent with prior work related to aortic stiffness in aged Fisher 344 rats exercised daily at relatively low intensity [373, 377]. Collectively, these data indicate exercise training prevents coronary AGE accumulation thereby preserving elastin content and function in a translational swine model of heart failure.

An important downstream effect of AGE is to signal via RAGE that, in turn, contributes to arterial oxidative stress and inflammation. As such, we observed an increase in nitrotyrosine abundance, and NF-κB p65 and SRA protein in the coronary arteries of HF swine. Oxidative stress and inflammation are important signaling mechanisms contributing to aortic stiffness both in animal models of aging and disease [20, 365] as well as in older adults [370, 378]. More specifically, oxidative stress activates a pro-inflammation response via the NF-κB signaling pathway [171, 177] which in turn influences the ECM leading to arterial stiffness [379]. Our data suggests AGE accumulation with HF increases nitrotyrosine, NF-κB and SRA reducing elastin content and function leading to arterial stiffness. In support of the beneficial therapeutic effects of exercise, both chronic continuous and interval training prevented pressure overload-induced increases in nitrotyrosine, NF-κB and SRA in the coronary arteries, suggesting the prevention of AGE accumulation by exercise also precludes the arterial oxidative stress and inflammatory response induced by HF.

Coronary PVAT and Arterial Stiffness

PVAT promotes age- and disease-related arterial stiffening and coronary PVAT has been shown to predict cardiac mortality, yet the identification of factors secreted from this fat depot still need to be elucidated [10, 380]. Consistent with our previous findings, perivascular AGE accumulation was increased in PVAT from HF animals [5, 20]. We also demonstrate PVAT from HF swine secretes increased AGE that promotes impaired elastin function *in vitro* in isolated aortas

from healthy mice similar to what was observed with the coronary artery. To our knowledge, this is the first evidence to demonstrate AGE secretion from coronary PVAT in a pre-clinical swine model of pressure overload-induced heart failure, revealing a novel source of AGE production. Notably, the current study extends our previous observations by demonstrating the mechanistic involvement of PVAT-derived AGE in the reduced arterial elastin content and increased AGE accumulation in the mouse aorta experiments, providing evidence for this fat depot to promote ECM remodeling consistent with what was observed in the coronary artery of HF animals. Continuous and interval exercise prevented PVAT AGE expression and secretion, as well as the impairments to mouse aortas exposed to PVAT-conditioned media from HF animals under our *in vitro* experimental conditions including decreased elastin content, function, and increased AGE accumulation. Use of the AGE inhibitor AMG also prevented the detrimental impact of PVAT-conditioned media from the HF group on mouse aortic stiffness and ECM remodeling, suggesting a mechanistic flux point by which chronic exercise training exerts its protective effect on pressure overload-induced coronary arterial stiffness is by reducing PVAT-related AGE secretion and associated inflammation. These findings imply further interrogation of AGE inhibition is warranted regarding its potential as a useful therapeutic strategy to combat coronary arterial stiffness and dysfunction in heart failure.

Exercise Training Effects Comparable to AGE Inhibitor

While AMG severed as prevention of AGE formation, it will probably not be effective in patients with a long history of disease that already resulted in extensive tissue AGE accumulation. Combined with previous findings that AMG has been shown to prevent age-related arterial stiffness and cardiac hypertrophy in the absence of changes in collagen and elastin content [381]. This may suggest that the effect of AMG is related to a decrease in the AGE-induced cross-linking of the extracellular matrix. Our PVAT study indicates that AMG cannot preserve elastin content in CON, HF+CONT and HF+IT groups compared to no AMG groups which is similar to previous finding. It would be logical to predict that the chronic continuous and interval exercise training prevent the arterial stiffness through inhibition of AGE cross-linking ECM. We may conclude that arteria collagen is not formed cross-linking with AGE.

Effects of Oxidative Stress and Inflammation on Coronary Artery Stiffness

In the present study, we observed increased coronary PVAT nitrotyrosine and NF-κB in the HF group which is consistent with the hypothesis that AGE formed in adipose tissue are a result of increased oxidative stress and inflammation [7, 127, 382, 383]. Moreover, the cytokine array results indicated that HF promoted coronary PVAT IL-6 and IL-8 secretions. The increased IL-6 level in conditioned media was similar to our previous findings that in pathological conditions PVAT secreted excessive IL-6 which plays a key role in regulation of arterial stiffness [6]. Oxidative stress has been shown as a regulator of IL-8 expression [51] and oxidized lipid induces greater IL-8 accumulation [52]. Thus,

the PVAT with nitrotyrosine abundance could be a source of IL-8 expression. Further NF-kappa B and IL-6 family has been shown to interact with transcriptional activation of the IL-8 gene [53]. It would be reasonable to conclude that increased oxidative stress and NF-κB in PVAT promoted the cytokines secretion which finally result in arterial stiffness. Exercise training prevented the HF-related increase in PVAT nitrotyrosine abundance, NF-κB p65 subunit expression and cytokines secretions, suggesting these processes may indeed promote AGE production in PVAT. It is noteworthy, however, that PVAT-conditioned media from the HF group did not promote arterial oxidative stress and inflammation in the mouse aorta, yet inhibition of AGE did lower normal levels of these endpoints on a global fashion across all experimental groups suggesting other factors such as IL-6 and IL-8 may promote vascular oxidative stress and inflammation. However additional research is needed to verify the effects of IL-6 and IL-8 on vascular oxidative stress and inflammation.

In addition, AGE mediated cellular signaling via oxidative stress and inflammation is another factor to contribute arterial stiffness. In the present study we observe that increased oxidative stress and inflammation in coronary and PVAT which is similar to our previous data in the same animal model [5]. Considering that AGE formation in adipose tissue are a result of increased oxidative stress and inflammation [5, 51-53]. It would be logical to predict that the interaction between AGE and oxidative stress and inflammation are, at least, partial key features to promote coronary artery stiffness in HF. We observed that both chronic continuous and interval exercise prevent the oxidative stress and

inflammation in coronary artery and PVAT which may explain that exercise prevent AGE accumulation through inhibition of oxidative stress and inflammation.

Limitations and Future Directions

There are several limitations of the current study. 1) As mentioned above, arterial stiffness could be influenced by total elastin and collagen content. AGE formed cross-linking could also contribute to stiffening arteries. It is noteworthy to discriminate that how the elastin, collagen and cross-link affect the stiffness; 2) in the current study we investigated AGE accumulation in coronary arteries and PVAT as well as AGE secretion from PVAT. Further we added AGE inhibitor into PVAT conditioned media and found that with AGE inhibition the stiffness was prevented. With these results we conclude that PVAT secreted AGE is a key factor in arterial stiffness. However, it is unknown if we will observe the similar results when culturing the healthy artery and fat depot directly with AGE. It is noteworthy to run these experiments to elucidate whether HF has the similar effects as AGE influences on coronary artery stiffness. In addition, we discussed the association between oxidative stress and AGE formation. But we still need further investigation to determine the PVAT secreted AGE is formed from oxidized lipids; 3) this study has 4 groups: CON, HF, HF+CONT and HF+IT. Due to the funding and experiment limitations, CON+CONT and CON+IT are not included. Though we do not observe any differences between HF+CONT and

HF+IT, whether there is any differences or changes between CON, CON+CONT and CON+IT is unclear;

and 4) women have lower risk rate of cardiovascular diseases at all ages compared to men, however the low estrogen level due to post-menopausal will dramatically increase the risk of CV events [384, 385]. It is noteworthy to consider the sex difference influences on coronary artery stiffness with heart failure. Thus, the future study design is focused on aortic-banding and ovariectomy effects on female mini-swine to mimic the HF- and aging-related women. Previous findings indicated that 8 weeks exercise preserved endothelial function and nitric oxide to reduce arterial calcification in ovariectomized rats [386]. This provides support that exercise potentially is a therapeutic strategy for after menopause women in cardiovascular diseases.

Perspectives

The prevalence of HF is expected to increase 46% by 2030 and given the lack of effective therapies, our findings suggest that chronic continuous and interval exercise training could be a therapeutic strategy for reducing arterial stiffness in HF patients [3, 387]. Recently we reported chronic exercise training preserved coronary microvascular vasodilatory function [348] and prevented carotid artery stiffness [334] in this swine model of pressure overload-induced heart failure. Our current data extend these findings demonstrating the efficacy of chronic continuous and interval exercise to prevent conduit coronary artery stiffness. The therapeutic benefits of both modes of chronic exercise were

through the preservation of elastin function in the coronary artery, which was due to reduced PVAT-derived AGE. Thus, our study provides support for chronic continuous and interval exercise training as a therapeutic option for HF patients at an intensity that is tolerable for this population.

In summary, we provide novel evidence for chronic continuous and interval exercise to preserve normal coronary arterial stiffness associated with ECM remodeling in a pre-clinical mini-swine model of pressure overload-induced HF. We elucidate that PVAT derived AGE plays a key role in coronary artery stiffness associated with vascular wall remodeling, oxidative stress and inflammation, which is prevented by chronic continuous and interval exercise. Previous findings (Table 5-1) are summarized to demonstrate that continuous and interval exercise training improves cardiac and coronary artery function and prevents carotid artery stiffness [334, 348, 349].The primary findings (Table 5-2) and proposed mechanisms (Figure 5-1) are summarized demonstrating the effects of chronic low intensity continuous and interval exercise training to prevent coronary artery stiffness with HF via inhibition of PVAT derived AGE. Collectively, our data lend support that the therapeutic potential of exercise intervention to prevent coronary artery stiffness with tolerable intensity to HF patients, and suggest the beneficial effects via PVAT AGE inhibition.

Table 5- 1. Continuous and interval exercise training improves cardiac and coronary artery functions and prevents carotid artery stiffness in HF miniswine induced by aortic-banding

	CON	HF	HF+CONT	HF+IT
LV BNP mRNA	Normal	Increased	Attenuated	Attenuated
LV fibrosis area and density	Normal	Increased	Attenuated	Attenuated
LV collagen area	Normal	Increased	Attenuated	Attenuated
Carotid Artery PEM	Normal	Increased	Attenuated	Attenuated
Carotid Stiffness Index	Normal	Increased	Attenuated	Attenuated
CBF	Normal	Decreased	Preserved	Preserved
CVC	Normal	Decreased	Preserved	Preserved

Table 5-1. CON, sedentary control; HF, aortic-banded heart failure sedentary; HF+CONT, aortic-banded heart failure continuous exercise trained; HF+IT, aortic-banded heart failure interval exercise trained; LV BNP-left ventricular brain natriuretic peptide; PEM-Peterson elastic modulus; CBF-coronary blood flow; CVC-coronary vascular conductance.

Table 5- 2. Continuous and interval exercise training prevents coronary artery stiffness with the reduction of PVAT derived AGE

	CON	HF	HF+CONT	HF+IT
Coronary EEM, kPa	284±28	444±38*	474±38	511±56
Coronary CEM	9324±1319	10563±1272	10958±1582	10684±1623
Coronary Elastin‡	1	0.67	1.057	0.97
Coronary Collagen‡	1	0.9	1.008	0.95
Coronary AGE‡	1	1.66	1.173	1.17
Coronary Nitrotyrosine‡	1	1.31	1.03	1.09
Coronary NFkB‡	1	1.33	0.98	1.03
Coronary PVAT AGE‡	1	1.70	1.29	1.32
PVAT secreted AGE, μg/mL	0.39±0.07	1.404±0.44*	0.27±0.06	0.23±0.04

Table 5-2. CON, sedentary control; HF, aortic-banded heart failure sedentary; HF+CONT, aortic-banded heart failure continuous exercise trained; HF+IT, aortic-banded heart failure interval exercise trained; EEM-elastin elastic modulus; CEM-collagen elastic modulus; PVAT-perivascular adipose tissue; AGE-advanced glycation end products. ‡-fold change relative to CON. *vs. all, $P<0.05$.

Figure 5- 1. Proposed mechanisms by which HF and exercise training influence coronary artery stiffness

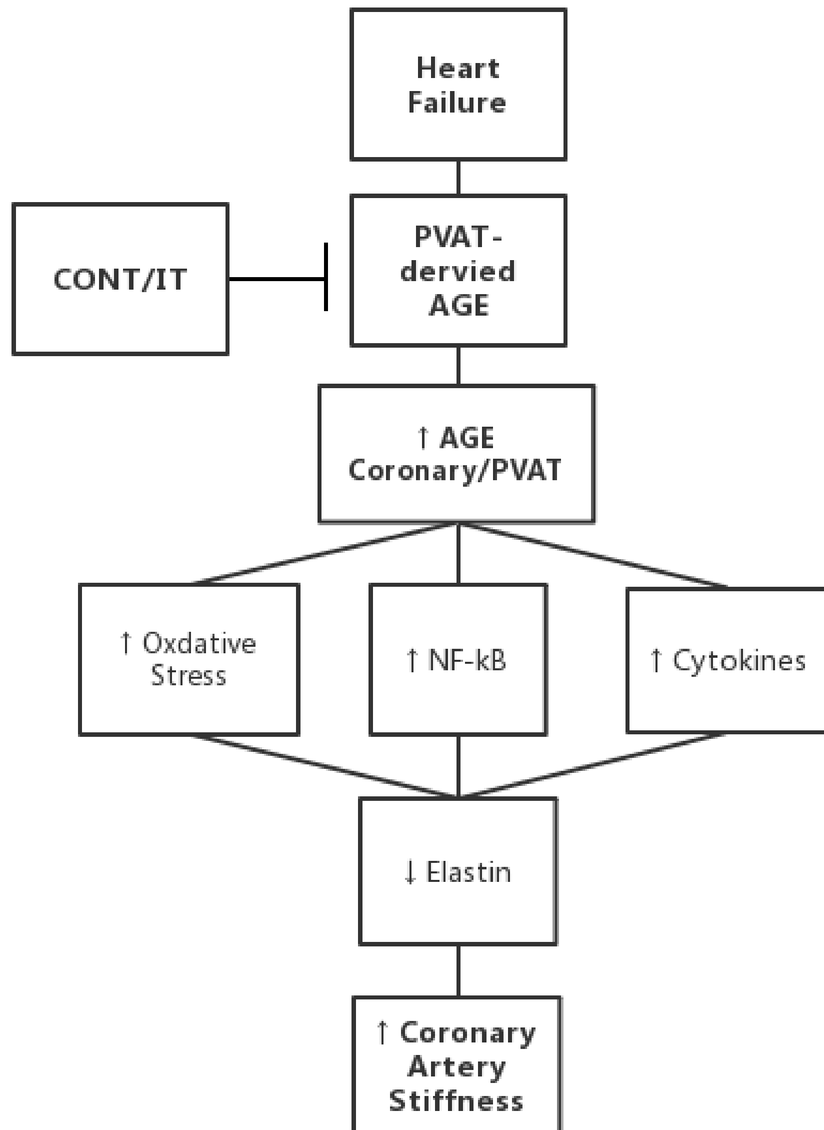

Figure 5-1. CONT-continuous exercise training; IT-interval exercise training; PVAT-perivascular adipose tissue; AGE-advanced glycation end products.

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Publications

1. Campbell, M. S., **Ouyang, A.**, Krishnakumar, I. M., Charnigo, R. J., Westgate, P. M., & Fleenor, B. S. (2019). Influence of enhanced bioavailable curcumin on obesity-associated cardiovascular disease risk factors and arterial function: a double-blind, randomized-controlled trial. *Nutrition* (2019).
2. Fleenor, B. S., **Ouyang, A.**, Olver, T. D., Hiemstra, J. A., Cobb, M. S., Minervini, G., & Emter, C. A. (2018). Saxagliptin Prevents Increased Coronary Vascular Stiffness in Aortic-Banded Mini Swine. Hypertension, 72(2):466-75.
3. **Ouyang, A.**, Garner, T. B., & Fleenor, B. S. (2017). Hesperidin reverses perivascular adipose-mediated aortic stiffness with aging. Experimental Gerontology, 97, 68-72.
4. Garner, T., **Ouyang, A.**, Berrones, A. J., Campbell, M. S., Du, B., & Fleenor, B. S. (2017). Sweet potato (Ipomoea batatas) attenuates diet-induced aortic stiffening independent of changes in body composition. Applied Physiology, Nutrition, and Metabolism, 42(8), 802-809.
5. Du, B., **Ouyang, A.**, Eng, J. S., & Fleenor, B. S. (2015). Aortic perivascular adipose-derived interleukin-6 contributes to arterial stiffness in low-density lipoprotein receptor deficient mice. American Journal of Physiology-Heart and Circulatory Physiology, 308(11), H1382-H1390.
6. Wang, H., Wang, X. T., Tong, H. Q., **Yang-An, O. U.**, & Jiang, C. M. (2014). "The comparative validity of three accelerometers during several activities." China Sport Science, 34(5), 45-50.