# THE EFFECTS OF EXERCISE ON ENDOPLASMIC RETICULUM STRESS-ASSOCIATED VASCULAR DYSFUNCTION

by
Junyoung Hong

A dissertation submitted to the Department of Health and Human Performance,
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Doctor of Philosophy

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Chair of Committee: Yoonjung Park, Ph. D

Committee Member: Daniel P. O'Connor, Ph. D

Committee Member: Melissa M. Markofski, Ph. D

Committee Member: Sean P. Marrelli, Ph. D

University of Houston
December 2019

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# ABSTRACT

Atherosclerosis and Alzheimer's disease (AD) constitute a high health threat worldwide. Endothelial dysfunction is generally known as an early pathogenic response in atherosclerosis and a major culprit for the development of AD. Chronic endoplasmic reticulum (ER) stress in endothelial cells contributes to endothelial dysfunction with an increase in oxidative stress, inflammation, and apoptosis, which leads to cell death. Alleviation of ER stress in the vascular system can be an important therapeutic strategy for retardation and treatment of pathophysiology response in atherosclerosis and AD. Exercise training is considered to be frontline therapy for prevention and treatment of vascular dysfunction through the mitigation of oxidative stress and the inflammatory and apoptotic response in cardiovascular and neurodegenerative diseases. However, the role of exercise training in ER stress-associated vascular dysfunction in different vascular beds and its underlying mechanism in atherosclerosis and AD is still largely unknown.

Therefore, the central aim of the dissertation study was to identify the underlying mechanisms by which exercise training mitigates ER stress-associated vascular dysfunction in different vascular beds and pathologies. To achieve the central aim of study, we investigated a comprehensive understanding of exercise effects on ER stress-associated endothelial dysfunction in different vascular beds (mesenteric artery and coronary arterioles) in atherosclerosis and on its molecular mechanisms. In addition, we explored the in-depth knowledge of the exercise effect on ER stress and purinergic receptor-associated cerebrovascular dysfunction using isolated intact posterior cerebral artery (PCA) from the transgenic mice model of AD and cultured human brain microvascular endothelial cells

(HBMECs). To measure vascular function, we isolated the mesenteric arteries and coronary arterioles from ApoE knock-out mice for atherosclerosis and PCA from APP/PS1 double transgenic mice for AD. Also, we utilized programmed treadmill running as an exercise training intervention. To find an in-depth understanding of the exercise-induced effects at the cellular level, HBMECs were exposed to laminar shear stress (LSS).

Our study demonstrates that ER stress impaired ACh-induced endothelial function in both mesenteric arteries and coronary arterioles of atherosclerosis through the reduction of eNOS expression and abnormal expression of ER stress markers, TXNIP/NLRP3 inflammasome, apoptosis, and oxidative stress. However, exercise training ameliorated ER stress-associated endothelial dysfunction in both vascular beds by reversing these abnormal molecular signaling pathways. In AD, ER stress impairs cerebrovascular function via the diminished eNOS expression, and increased ER stress markers and apoptosis expressions in AD mice brain, but exercise training improved cerebrovascular dysfunction by dampening these negative signaling cascades. Furthermore, ATP induced vasoconstriction in PCA from AD mice through the attenuation of eNOS and P2Y2 receptor expressions in AD mice brain. However, exercise training reversed ATP-induced vasoconstriction to vasodilation in PCA from AD mice with an increase in P2Y2 receptor-mediated eNOS signaling pathway in AD mice brain. Likewise, LSS upregulated the eNOS signaling and P2Y2 receptor expression in HBMECs, but inhibition of P2Y2 receptor blunted eNOS expression in LSS-induced HBMECs.

The findings of this study will provide novel insight into the protective and therapeutic effect of exercise on ER stress- and purinergic receptor-associated vascular disease in atherosclerosis and AD.

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# Chapter 1

## I. INTRODUCTION

Endothelial dysfunction is the initial pathophysiological response of atherosclerosis [1] and is highly associated with cerebrovascular dysfunction in the development of neurodegenerative disease [2] by multiple underlying mechanisms. Advanced atherosclerotic plaque formation by hyperlipidemia reduces blood flow and produces multiple pathological conditions. These alterations are primarily induced by endothelial dysfunction, which is tightly associated with oxidative stress, pro-apoptotic progression, and the inflammation response in athero-susceptible regions [3, 4]. Also, Aβ-induced neurotoxicity and aging impair endothelial and blood-brain barrier functions in cerebrovascular disease. Thus, impaired cerebral vascular function causes chronic hypoperfusion, in turn, accelerating the formation of Aβ, triggering the pathology of vascular dementia [5]. Accordingly, managing endothelial dysfunction has been recognized as an effective therapeutic strategy for cardiovascular and neurodegenerative diseases. Nevertheless, the underlying mechanisms still remain unclear.

It has been proposed that redox imbalance [6], the elevation of proinflammatory cytokines [7], and apoptotic response are highly associated with vascular dysfunction through the reduction of NO bioavailability [8]. Endoplasmic reticulum (ER) stress has been known as the key modulator in endothelial dysfunction in atherosclerosis [1, 9, 10] and is also profoundly involved with the etiology of AD [11, 12]. Prolonged activation of ER stress in endothelial cells by various pathologies including atherosclerosis and AD contribute to

endothelial dysfunction with the elevation of ER stress-associated oxidative stress (NADPH oxidase & UCP-2) [13, 14], inflammation (IL-1β & caspase-1) [15] and apoptosis (Bax & caspase-12) that can lead to cell death [16, 17]. Also, ER stress is tightly associated with the stimulation of thioredoxin interacting protein **(**TXNIP) and NLRP3 inflammasome which regulate oxidative stress and immune response. Furthermore, cerebrovascular dysfunction is one of the characteristics and the main contributor to the pathologic process of AD [18, 19]. Purinergic receptors play multiple roles in cellular homeostasis by regulating vasomotor tone in cerebral vessels [20, 21] and by modulating AD-like pathology and mortality [22]. However, limited studies have been conducted to identify the direct effect of ER stress-associated and purinergic receptor-mediated endothelial dysfunction and its cellular/molecular mechanisms in atherosclerosis and AD.

Exercise training has been regarded as a potential therapy for endothelial dysfunction through the inhibition of oxidative stress and inflammatory response in atherosclerosis [23] and the pathogenesis of AD [24]. The beneficial effect of exercise on vascular dysfunction in atherosclerosis and on cerebral pathology in AD has been well documented. Prolonged exercise training prevents the progression of atherosclerotic plaque in ApoE KO mice [25] and ameliorates endothelial dysfunction with the increase of anti-oxidant response (decreased NADPH and increased SOD) and NO bioavailability in aorta from LDL knock-out mice [26]. Moreover, treadmill exercise improves cognitive impairment, reduces Aβ load [27], decreases the expression of ER stress, TNF-α, and IL-1β in the hippocampus in AD mice [28]. In addition, regular exercise training positively regulates the response of ER stress signaling pathways in diverse tissues and disease models. Aerobic exercise training reduces the expression of ER stress signaling (PERK, IRE1, eIf2α, and CHOP) in adipose/hepatic tissues in obese rats [29],

cardiac tissue in a myocardial infarcted rodent model [30, 31], and in the hippocampus in transgenic AD mice [28]. However, few studies have reported the direct exercise effect on ER stress-associated endothelial dysfunction in the vascular system in atherosclerosis [32] and AD [33]. Accordingly, studies designed to determine the protective effect of exercise on ER stress-associated vascular dysfunction in atherosclerosis and AD and its underlying mechanisms are needed. Moreover, due to a heterogeneity of the endothelial phenotypes [34], the initiation of endothelial dysfunction is not uniformly exhibited in different vascular beds; small resistance vs large conduit vessels, and its response to local shear stress is different in disease states and vascular beds [35]. Therefore, a comprehensive understanding of endothelial diversity can provide better therapeutic strategies for patients with different vascular diseases.

Therefore, the primary aim of this study is to elucidate the underlying mechanism(s) of how exercise training regulates ER stress-associated vascular dysfunction in different vascular beds (mesenteric artery, coronary arterioles, and cerebral artery) and pathologies (atherosclerosis and AD), and the underlying mechanisms via oxidative stress, inflammation, apoptosis, purinergic receptors, and eNOS signaling. To test the central aim of study, we set up three independent studies; the first study identified the effects of exercise on ER stress-associated endothelial dysfunction in the mesenteric artery of atherosclerosis and its underlying mechanism of UCP-2, caspase-1, and NO signaling; the second study determined the comprehensive understanding of exercise effects on ER stress-associated endothelial dysfunction using different vascular beds (coronary arterioles) in atherosclerosis, and its underlying mechanisms of TXNIP (thioredoxin interacting protein), NLRP3 inflammasome, and NO signaling pathways; the third study investigated the exercise effects on ER stress-associated and purinergic receptor-mediated cerebrovascular dysfunction using posterior

cerebral artery (PCA) in AD mice and in cultured human brain microvascular endothelial cells (HBMECs), and on its underlying mechanism of apoptosis and NO signaling.

The specific aims of this proposal were addressed by the means of the following hypothesis:

## II. AIMS AND HYPOTHESES

### STUDY 1.

**Aim 1.** To determine the exercise effects on ER stress-associated endothelial dysfunction in the mesenteric artery in atherosclerosis and its underlying mechanisms

**Hypothesis 1. A.**

We hypothesize that ACh-induced endothelium-dependent vasodilation in the mesenteric arteries of ApoE KO mice will be impaired, but exercise training will improve it.

**Hypothesis 1. B.**

We hypothesize that ER stress stimulation in the mesenteric arteries of WT mice will reduce ACh-induced endothelium-dependent vasodilation, but ER stress inhibition in the mesenteric arteries of ApoE KO will ameliorate it.

**Hypothesis 1. C.**

We hypothesize that inhibition of caspase-1 will improve ACh-induced endothelium-dependent vasodilation in mesenteric arteries of ApoE KO, and inhibition of UCP-2 will decrease ACh-induced endothelium-dependent vasodilation in the mesenteric arteries of WT and ApoE KO with exercise (ApoE KO-EX) mice.

**Hypothesis 1. D.**

We hypothesize that the expression of ER stress markers, CHOP, will be higher in the mesenteric arteries of ApoE KO mice, but exercise training will reduce it.

## STUDY 2.

**Aim 2.** To investigate a crucial role of exercise training on ER stress-associated endothelial dysfunction in coronary arterioles in atherosclerosis and its cellular mechanisms

**Hypothesis 2. A.**

We hypothesize that ACh-induced endothelium-dependent vasodilation in coronary arterioles of ApoE KO mice will be impaired, but exercise training will alleviate it.

**Hypothesis 2. B.**

We hypothesize that activation of ER stress will decrease ACh-induced endothelium-dependent vasodilation in the coronary arterioles of WT and ApoE KO-EX mice, but not in ApoE KO mice.

**Hypothesis 2. C.**

We hypothesize that inhibition of UCP-2 will reduce ACh-induced endothelium-dependent vasodilation in coronary arterioles in WT and ApoE-EX mice, but no further decrease of ACh-induced vasodilation in ApoE KO mice.

**Hypothesis 2. D.**

We hypothesize that the expression of ER stress markers (GRP78, IRE1, eIF2$\alpha$, and CHOP), pro-apoptosis marker (Bax), TXNIP, and NLRP3 inflammasome (NLRP3, caspase-1, and IL-1$\beta$) will be increased in the heart of ApoE KO mice, but exercise training will decrease them.

**Hypothesis 2. E.**

We hypothesize that the expression of UCP-2 and NO production will be reduced in the heart of ApoE KO mice, but exercise training will elevate them.

## STUDY 3.

**Aim 3.** To elucidate the possible underlying mechanism of exercise training on ER stress-associated and purinergic receptors-mediated cerebrovascular dysfunction AD

**Hypothesis 3. A.**

We hypothesize that ATP-induced vasorelaxation will be reduced in PCA in AD mice, but exercise training reverses it.

**Hypothesis 3. B.**

We hypothesize that the P2Y2 receptor and p-eNOS expression will be diminished in the brain of AD mice, but exercise training will restore them.

**Hypothesis 3. C.**

We hypothesize that LSS will increase expressions of P2Y2 receptor, p-eNOS and t-eNOS in HBMECs, but inhibition of P2Y2 receptor in HBMECs will diminish the LSS effect.

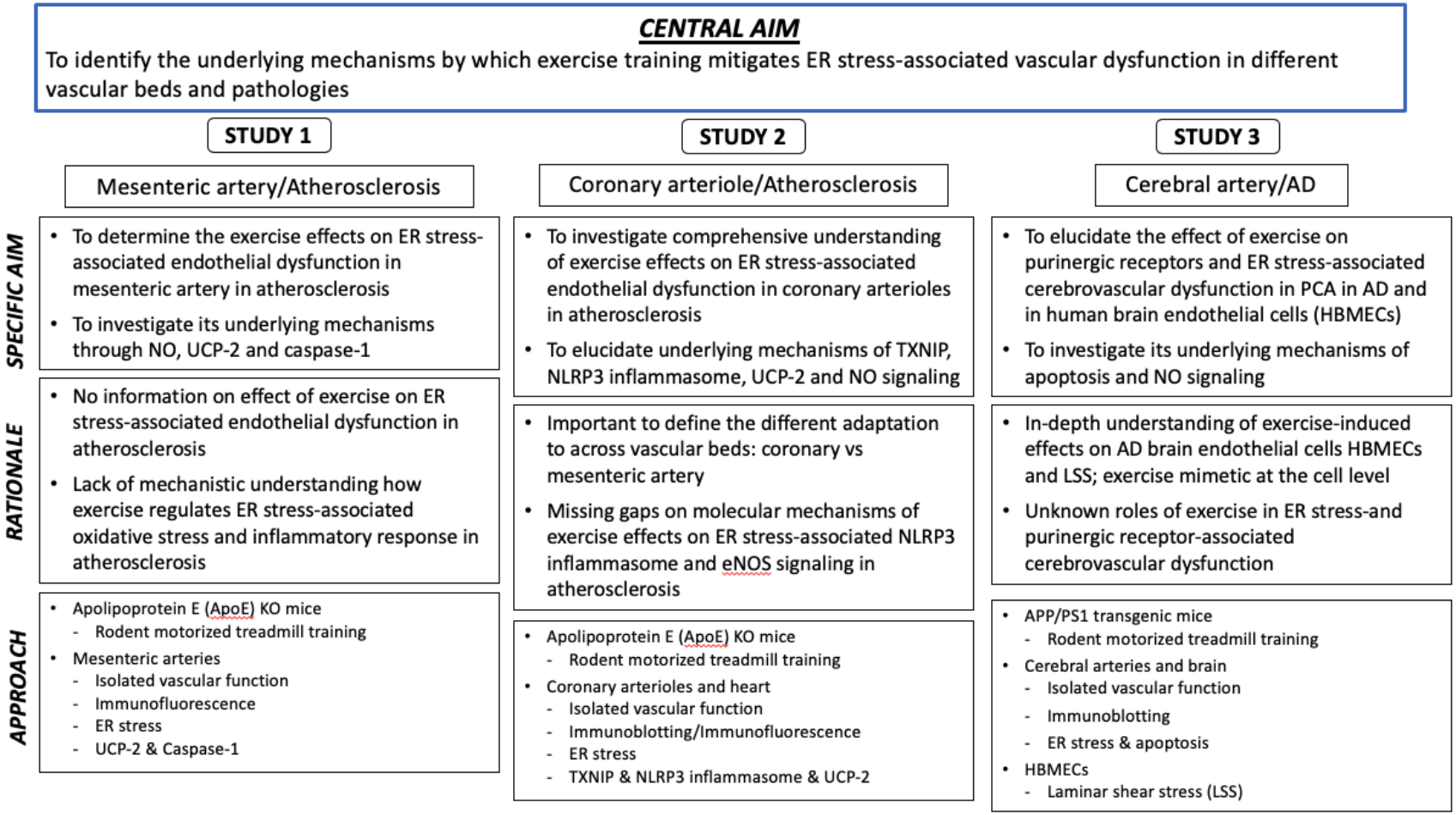
**Hypothesis 3. D.**

We hypothesize that the expression of APP and ER stress markers (IRE1, eIF2$\alpha$, and CHOP) will be elevated in the brain of AD mice, but exercise training will reduce them.

**Hypothesis 3. E.**

We hypothesize that the pro-apoptotic marker (Bax) will be increased and the anti-apoptotic marker (Bcl-2) will be diminished in the brain of AD mice, but exercise training will reverse those expressions.

**Figure 1. Dissertation overview**



## III. LITERATURE REVIEW

### 1. Atherosclerosis, Alzheimer's disease, and exercise

Cardiovascular disease is the leading cause of premature death in worldwide [36]. Atherosclerosis is one of the major pathogenetic factor of this disease, which is a chronic inflammatory disease of the small to large arteries [37]. Atherosclerosis is exacerbated by hyperlipidemia and hypercholesterolemia which impacts arteries by inducing atherosclerotic plaque formation, intimal thickening, and endothelial dysfunction [38]. Endothelial dysfunction is the initial detectable symptom during the progression of atherosclerosis accompanied by inflammation, thrombosis, and endothelial cell barrier dysfunction, all of which are promoted by the disturbed blood flow at atherosusceptible regions [3]. Continued plaque formation in intima ultimately triggers myocadiac infarction, ischemia, and stroke.

Among the many CVD risk factors, unhealthy diet, smoking, and lack of physical activity, sedentary lifestyle are now recognized as primary contributors accelerating atherosclerosis [23]. Conversely, regular physical activity is robustly associated with cardiovascular health and a reduction of cardiovascular risk factors [39]. Changes in plasma lipids by exercise may directly lower CVD risk by decreasing the levels of circulating lipoproteins and preventing the progression of atherosclerotic plaque formation [23]. Previous studies reported that prolonged exercise training reduces the development of atherosclerotic lesions in the aorta of LDL KO mice [40], and increased physical activity lowers the circulating lipoproteins level in dyslipidemia overweight men and women [41]. However, the underlying mechanism of the effect of exercise on the prevention of atherosclerosis is not fully understood yet.

Alzheimer's disease (AD) is the major cause of dementia with advancing age, and is responsible for 60-80% of all cases of dementia, nearly 5 million American are suffering from AD [42]. AD is a neurogenerative disease characterized by cognitive impairment and memory loss accompany the presence of extracellular amyloid peptide and neurofibrillary tau tangles, which lead to cerebrovascular dysfunction [18]. The pathogenic mechanisms of AD have been considered to coexist with vascular dementia and are highly associated with cerebral hypoxia and hypoperfusion [43]. Cerebral amyloid angiopathy (CAA) is induced by malfunction of the cerebrovascular system, which producing chronic impairment of cerebral blood flow in the brain that ultimately causes to neuronal injury/loss and the development of cognitive decline [44-46].

Although AD pathology and the underlying mechanisms have not been fully discovered to conquer this disease, exercise has been applied as a therapeutic strategy for reduction of the pathogenic phenotypes for AD [47]. Numerous studies have reported that physical activity has the beneficial effect on the cognitive decline in AD patients and the AD mouse model through a decrease in the accumulation of amyloid beta peptide and enhanced cerebrovascular health [44, 47-49]. However, a lack of evidence exists regarding the effect of exercise on cerebrovascular dysfunction in AD.

## 2. Vascular dysfunctions in atherosclerosis and Alzheimer's disease and a role of exercise

Endothelial dysfunction is historically accepted as one of the early events in the pathogenesis of atherosclerotic progression [50]. Endothelial cells (ECs) are the inner layer of a blood vessel, produces and releases a variety of substances including, NO, bradykinin, and prostacyclin to maintain and control fluid filtration, inflammation, oxidative stress, and

vascular reactivity, each of which is for vascular homeostasis [8]. Hypercholesteremia, hyperlipidemia, and hyperglycemia lead to vascular damage and initiate atherosclerotic lesion, which accelerates oxidative stress and inflammatory response that reduces nitric oxide (NO) bioavailability during the progression of atherosclerosis [51, 52]. NO is a potent vasodilator and is released from the ECs through the activation of nitric oxide synthase (eNOS), the major source of endothelium-derived NO, and plays an important role in inhibiting platelet aggregation and inflammatory response, and regulating cell survival and vascular tone, and proliferating smooth muscle cells [53]. During the progression of atherosclerosis, excessive oxidative stressors, such as, xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and mitochondrial superoxide are the major source of ROS generation, which directly reduce NO bioavailability and accelerate vascular dysfunction [6]. Therefore, imbalance of ROS production and the anti-oxidation system accompany the progression of atherosclerosis and induce endothelial dysfunction by reducing NO bioavailability [54]. In western diet (WD)-fed ApoE KO mice, endothelium-dependent vasodilation was impaired in resistance vessels [55, 56] (mesenteric arteriolar and coronary arterioles) and conduit vessels (aorta) [57] through elevated superoxide production ($O_2^-$), NADPH oxidase expression, and diminished NO bioavailability.

On the other hand, various inflammatory cytokines and chemokines are a major risk factor to lessen NO bioavailability by impairing endothelial function and accelerating the progression of atherosclerosis [58, 59]. Pro-inflammatory factors, such as nuclear factor kappa B (NF-κB), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-18 are produced in the endothelium and promote the expression of vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and monocyte chemoattractant protein-1

(MCP-1). The expression of these adhesion molecules are highly associated with inducing fibrotic/thrombotic processes, promoting the adherence and migration of monocytes, and dysregulating eNOS activity during atherosclerosis [60, 61]. Therefore, the inflammatory response in atherosclerosis is highly associated with the development of atherosclerotic lesions and endothelial dysfunction through decreasing NO bioavailability [61].

The numerous beneficial effects of regular physical activity and exercise training have been well established as an effective treatment for reducing cardiovascular risk factors. Recently, the American College of Cardiology (ACC) and American Heart Association (AHA) published a guideline for improving cardiovascular health. The guideline states that aerobic exercise training is recommended as a method for primary prevention and treatment for cardiovascular disease patients [36]. Vascular dysfunction in atherosclerosis is tightly associated with ROS generation via reduction of NO bioavailability, and regular exercise training mitigates the generation of superoxide and ROS synthesis through the elevation of anti-oxidant response, such as elevation of UCP-2, SOD expression, and reduction of NADPH oxidase production in vasculature, in turn, ameliorate vascular dysfunction in atherosclerosis [23, 36, 39]. Aerobic exercise training is a potential non-pharmacological treatment to improve endothelial dysfunction in thoracic aortic rings in LDL KO mice [26] and ApoE KO mice [40] that also reduces expression of NOX2 and ROS production, and increased the expression of SOD [62], and consequently increasing NO bioavailability. However, there is still insufficient information available to elucidate the effects of exercise training on the anti-oxidative mechanism in vascular dysfunction in different vascular beds in atherosclerosis.

Furthermore, exercise training not only positively regulates anti-oxidant response, but also modulates chronic systemic inflammation, such as NF-κB, IL-1β, and TNF-α, improving

endothelial dysfunction in cardiovascular disease [39, 60]. In ApoE KO mice, the expression of IL-6 and NF-κB are reduced by regular exercise training in serum [63] and in the aorta [64]. Lee et al [65] reported the direct effect of exercise on TNF-α and IL-6 mediate endothelial dysfunction in the coronary arterioles in diabetic mice with reduced expression of TNF-α in the heart and IL-6 in serum. However, the direct effect of exercise training on vascular dysfunction by inhibition of inflammatory cytokines in atherosclerosis and its underlying mechanism is still undiscovered.

The brain consumes the highest percentage of oxygen and nutrients during a resting state [66]. Continuous and well-regulated cerebral blood flow (CBF) is critical for brain function and cell survival [67]. Cerebral endothelial cells play a critical role in the regulation of the immune response, metabolic supply, amyloid beta peptide (Aβ), and CBF by releasing nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) [68]. AD is characterized by the accumulated neurofibrillary tangles in neurons and the elevated expression of amyloid β peptide (Aβ) in the brain that induce neuronal and vascular toxicity [69]. Reduced CBF is well-documented in patients with AD and cerebrovascular disease, which is highly associated with the pathogenesis of AD [68]. The impaired CBF by structural and functional alteration of brain vessels induces a permanent and irreversible neuronal injury/loss and the development of cognitive impairment in AD brain [18]. Hypertension and hypoxia are well-recognized major contributor for the pathologic process of AD, triggering cognitive impairment and degenerative changes in the brain with molecular and functional changes in the cerebral vasculature [69]. Accumulating evidence indicates that conduit to microvascular level in AD show reduction of density, length, and diameter of brain vessels that are highly associated with CBF dysregulation [68, 70]. Vascular endothelial growth factor

(VEGF) [71] and transforming growth factor-β (TGF-β) [72] are associated with vascular remodeling and angiogenesis, which elevated in patient with AD and vascular dementia and increased the risk factor of hypertension, and altered cerebrovascular structure and function. Furthermore, Aβ toxicity influences the impairment of the cerebrovascular function by narrowing or distorting the microvessels and controlling vascular reactivity [46]. In particular, a cerebrovascular function is also impaired in AD patients and AD mouse models. Specially, endothelium-dependent vasodilation measured by flow-mediated dilation in brachial artery is impaired in patients with AD [73], and acetylcholine (ACh)-induced vasodilation and its relaxation with L-NAME (endothelial nitric oxide synthase inhibitor) treatment are attenuated in the middle cerebral artery (MCA) in APP overexpression mice compared with normal control mice [74]. Furthermore, Aβ-induced oxidative stress exerts a deleterious effect on cerebral endothelial cells by producing excessive reactive oxygen species (ROS) which highly associated with NO bioavailability [66]. Anti-oxidant treatment (SOD & catalase) in isolated MCA from AD mice rescues ACh-induced vasodilatory function with increase the superoxide dismutase (SOD) expression level and NO bioavailability [74]. Moreover, eNOS plays an important protective role in the pathological mechanism against AD. Aβ is negatively associated with NO, a potent vasodilator, by reducing the expression/activity of eNOS in patients with AD [75] and AD-like pathology [76]. eNOS deficiency promotes the development of AD in genetically modified eNOS knock-out (KO) mice with cognitive impairment and the elevation of APP, BACE, and Aβ protein levels [77].

Over the decades, numerous clinical trials have been conducted to find a treatment to prevent or reduce risk factors of AD, but no pharmacological treatment has been approved yet for the disease. The neuroprotective benefits of exercise training have been well described in

both human and in animal models of AD. Exercise training reduces the RAGE, increases LRP expression, and induces angiogenesis in TgCRND8 mice [78]. The expression of $A\beta_{42}$, tau protein, and β-amyloid burden in the hippocampus are also reduced by exercise training accompany improved cognitive function in APP/PS1 mice [79, 80]. Also, exercise training positively regulates nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and SOD level in the hippocampus from the transgenic rat model of AD [81]. Elevated neuro-inflammatory cytokines is thought to be a key risk factor for the development of AD [82], but exercise training reduces the proinflammatory cytokines; IL-1β, TNF-α, NF-κB, IL-6, and vascular cell adhesion molecule (VCAM-1) in the hippocampus with a reduced level of interferon-gamma (IFN-γ) in Tg2576 AD mouse model [83, 84]. However, no study has been conducted to elucidate the direct effect of exercise on cerebrovascular dysfunction using isolated cerebral vessels in AD mice. Therefore, a more in-depth study is required to investigate the underlying mechanisms of exercise training effect on cerebrovascular dysfunction in AD.

**3. Uncoupling protein 2 (UCP-2)**

Excessive ROS production contributes to endothelial dysfunction and is a major factor in the pathogenesis of atherosclerosis. Uncoupling proteins (UCPs) are the member of the mitochondrial anion carrier proteins that are located within an inner mitochondrial membrane and play an important role in reducing the potential of mitochondrial membrane and dissipating metabolic energy, as well as preventing oxidative stress [14]. The important role of UCP-2 in cardiovascular disease has been highlighted as an anti-oxidant defense factor in vascular dysfunction [85, 86]. UCP-2 is expressed in most cell types and is involved in the antioxidant

mechanism regulating the balance mitochondrial-derived ROS production and reducing the pathogenesis of cardiovascular disease [14]. UCP-2 is strongly implicated in the progression of atherosclerosis; atherosclerotic lesions are significantly elevated in multiple regions in the aorta in atherogenic diet-fed UCP-2 knock-out mice [87]. Also, UCP-2 is involved in the vascular dysfunction in cardiovascular disease, that UCP-2 overexpression improves the endothelial dysfunction with elevation of eNOS and the reduction of ROS and ET-1 expressions in the aortas of LPS-induced mice [88] and in high glucose-induced obese mice [89]. A recent study also demonstrated the effect of UCP-2 in endothelial dysfunction in the coronary arterioles in HFD-induced ApoE KO mice, in which UCP-2 knock-out more severely exacerbated endothelial dysfunction with increased ROS production and decreased NO bioavailability compared to ApoE KO mice [90]. UCP-2 knock-down in LPS-treated human aortic endothelial cells (HAECs) leads to the further reduction of NO production and elevation of ROS/NADPH oxidase generation compared with LPS-treated HAECs [91]. However, ox-LDL-treated human umbilical vein endothelial cells (HUVECs) demonstrates an elevation of UCP-2 expression compared with control [90]. This elevated UCP-2 expression might be a defensive mechanism for increased oxidative stress in the cell. Although many previous studies demonstrate the role of UCP-2 as an anti-oxidant defender and a potential target to reduce vascular dysfunction in cardiovascular disease, the underlying mechanism of UCP-2 in atherosclerosis remains unsolved.

The expression of UCP-2 was positively regulated in response to exercise training in which treadmill exercise elevated UCP-2 expression with decreased expression of TNF-$\alpha$ in brown tissues in Zucker rats [92] and gastrocnemius muscles in rats with anti-apoptotic responses [93]. Also, exercise training elevates mRNA and protein level of UCP-2 in the brain

of rats [94, 95]. However, the effect of exercise training on UCP-2 is underestimated in endothelial dysfunction in atherosclerosis, so more mechanistic studies are necessary to determine the role of exercise training on UCP-2-mediated endothelia dysfunction in atherosclerosis.

**4. NLRP3 inflammasome**

A chronic inflammatory disorder is recognized as a major response during the progression of atherosclerosis [15]. The atheroprone regions in the arterial wall release pro-inflammatory cytokines that impair endothelial function through reduction of NO bioavailability and promote atherosclerosis [54]. Inflammasome, the multimolecular complex, has been recently highlighted as an important player in the innate immune response to defend from pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) [96]. Among the inflammasome families, NLRP3 inflammasome is the most characterized and has been implicated in critical pathogenesis for the progression of atherosclerosis on DAMP signals [97, 98]. These multimeric protein complexes are composed of nucleotide oligomerization domain-like receptor with pyrin domain containing 3 (NLRP3) inflammasome, the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)), and protease pro-caspase-1 [97]. The activated pro-caspase-1 induces the maturation and release of proinflammatory cytokines; IL-1β and IL-18, in turn, leading to cell death known as pyroptosis [96].

Numerous studies have been devoted to determine the causal relationship between NLRP3 inflammasome and the progression of atherosclerosis [15, 99]. Previous studies reported the linkage between inflammasome activation and the development of atherosclerotic

lesion, and that atherosclerotic lesion is relatively decreased in genetic ablation of NLRP3 inflammasome in hypercholesterinemia-induced bone marrow transplanted LDL KO mice compared to control mice [100]. In addition, IL-1β KO and IL-1β antagonist treatment in hypercholesterolemic mice diminish atherosclerosis compared to their controls [101-103]. Thus, caspase-1 KO in ApoE KO mice demonstrates the prevention effect on atherosclerosis development compared to ApoE KO mice in aorta [104, 105]. These evidences strongly suggest that activation of inflammasome is tightly associated with the development of atherosclerosis.

To discover the underlying mechanism of the NLRP3 inflammasome on endothelial dysfunction in atherosclerosis, isolated vessels and endothelial cells have been used in multiple studies [106]. Oxidized low-density lipoprotein (ox-LDL)-induced human aortic endothelial cells (HAECs) [107, 108] and HUVECs [108] show the elevated expression of NLRP3 and ASC in both protein and mRNA level and the downstream pathway of caspase-1, IL-1β, and IL-18 is also increased in protein and mRNA levels with increased apoptosis response. Additionally, endothelium-dependent vasodilation is impaired in coronary arterioles in acute hypercholesterolemia-induced mice, but not in acute hypercholesterolemia-induced NLRP3 knock out mice [109]. Similarly, ACh-induced endothelial function is attenuated in the aorta with an elevation of NLRP3, caspase-1, IL-1β, and IL-18 in high-fat diet (HFD)-induced mice, while incubation of the NLRP3 inhibitor alleviates endothelial dysfunction in an isolated aorta [110]. However, given the current knowledge of NLRP3 inflammasome on endothelial dysfunction is still insufficient to establish the underlying mechanism for the pathogenesis of vascular dysfunction in atherosclerosis, more empirical studies are required to determine the role of NLRP3 inflammation on endothelial dysfunction in atherosclerosis.

Furthermore, no studies have reported the direct effect of exercise training on NLRP3 inflammasome-mediated or associated endothelial dysfunction in atherosclerosis. Only a few studies have shown a positive effect of exercise training on the expression of NLRP3 inflammasome. The protein expressions of NLRP3, caspase-1, IL-1β, and IL-18 are reduced by exercise training in the hippocampus in OVX rat [111] and in the brain of diabetic [112], and mRNA level of NLRP3, IL-1β, and IL-18 are significantly diminished in adipose tissue in HFD-fed mice via exercise training [113]. Also, voluntary wheel running significantly decreases the protein expression of caspase-1, IL-1β in heart of HFD-fed mice [7]. Therefore, it is worthwhile to investigate the role of exercise training on NLRP3 inflammasome-mediated endothelial dysfunction to provide valuable insights on cardiovascular disease.

**5. Thioredoxin interacting protein (TXNIP)**

The regulation of oxidative stress is the key pathophysiological mechanism in cardiovascular disease including hypertension, heart failure, and atherosclerosis. The thioredoxin (TRX) system is comprised of TRX, TRX reductase, and NADPH [114]. TRX is ubiquitously expressed in most cells; TRX-1 is found in the nucleus and cytosol and TRX-2 is distributed in mitochondria [115]. The TRXs play a key role in regulating apoptosis, DNA synthesis/repair, and cellular oxidative stress [115]. The TRX becomes the reduced TRX after receiving an electron from NADPH through TRX reductase, in turn, the reduced TRX peroxidase scavenges ROS and transforms oxidized protein to reduced protein. Thioredoxin interacting proteins (TXNIP) is an endogenous inhibitor of TRX that negatively regulates the expression and activity of TRX, thereby impairing the cellular antioxidant system [116]. TXNIP may play a vital role in the inflammatory response, oxidative stress, and atherosclerosis

development [115]. Genetic ablation of TXNIP in ApoE KO mice displays a significant reduction of atherosclerotic lesions in the aortic root compared to ApoE KO mice [117]. Endothelial specific overexpression of TRX-2 in ApoE KO mice increases NO bioavailability and reduces atherosclerotic lesion area [118]. These results indirectly support the effect of TXNIP on atherosclerosis development. Normal blood flow decreases the TXNIP expression and increases TRX expression compared to low blood flow in a rabbit aorta [119]. Although accumulating evidence suggests that TXNIP is an essential step to activate NLRP3 inflammasomes in different cell lines and disease models [115, 120], no studies reported a direct role of TXNIP-mediated endothelial dysfunction with alteration of NLRP3 inflammasome expression in atherosclerosis. The expression of NADPH oxidase, NLRP3 inflammation, caspase-1, and IL-1β expression are reduced in aorta of high fat (HF) and low carbohydrate (LC) diet fed TXNIP knock-out mice compared to in the aorta of HP-LC diet fed mice [121]. In an acute oxidative stress condition, TXNIP interacts with NLRP3 inflammasome, inducing IL-1β [122, 123]. Furthermore, ER stress is associated with the stimulation of TXNIP expression and ER stress sensor proteins, PERK and IRE1, stimulating the expression of TXNIP and elevating caspase-1 and IL-1β [120, 124]. Given this collection of evidence, TXNIP might play key roles in the redox and inflammatory responses and development of atherosclerosis. However, more studies are required to identify the beneficial effect of inhibition of TXNIP on endothelial dysfunction in atherosclerosis.

## 6. Purinergic receptors in Alzheimer's disease

Adenosine triphosphate (ATP) is an extracellular nucleotide released from the peripheral and central nervous systems with the neurotransmitter noradrenaline (NA) and from

the endothelial cells in response to shear stress and hypoxia. The purinergic receptor is a plasma membrane receptor and plays a prominent role in immune cell recruitment, inflammation, neurotransmission, and regulation of vascular reactivity. These receptors react with adenosine and nucleotides for modulating vasodilation and vasoconstriction in the vasculature. Purinergic receptor exist as the three major subtypes; 1) P1 receptors have four subtypes which are mainly activated by adenosine, 2) P2X receptors have seven subtypes which are ionotropic receptors and interact with ATP, leading to calcium entry through P2X cation channels, membrane depolarization, and calcium influx through voltage-activated calcium channels, 3) G protein-coupled P2Y receptors have eight subtypes and induce ATP (nucleotides)-mediated vasodilation through calcium-activated NO generation.

In AD, the role of the P1 receptor is not well clearly defined, but the existing evidence indicates that the P1 receptor contributes the AD-associated pathologies by a neuroprotective mechanism by promoting α-secretase-mediated non-amyloidogenic sAPPα production [125, 126]. P2X receptor subtypes regulate a variety of pathological and physiological response in the central nervous system (CNS), especially, pain sensation and neuroinflammation [127-129].

In a vessel, ATP produces a biphasic response consisting of transient vasoconstriction through P2X1 receptors in SMC and subsequent vasodilation due to P2Y2 receptors in endothelial cells [20]. P2Y2 receptors have been linked to neuroinflammation, neuronal functions, the cerebral blood flow regulation, and the pathology of AD [22, 130, 131]. Upregulation of IL-1β in AD enhances the expression of P2Y2, consequently reducing Aβ accumulation [132]. In P2Y2 knockout in AD mice model, activation and upregulation of P2Y2 receptor shows a positive relationship between AD-like pathologies and mortality [133]. Also, the expression of P2Y2 receptor is lower in AD patients compared to age-matched

controls [131], and P2Y2 knockout mice show impaired vasodilation in the aorta leading to hypertension [134]. Furthermore, Aβ-incubated isolated rat penetrating arterioles from Tg2576 mice shows the enhanced ATP-induced vasoconstriction and the reduced vasodilation [135]. However, limited information is existed to demonstrate the role of P2Y2 receptor in vascular dysfunction in cerebral arteries in AD. Furthermore, the effect of exercise training on P2Y2 receptor in cerebrovascular dysfunction in AD is unconfirmed. Therefore, a more an in-depth study is required to determine the effect of exercise training on P2Y2 receptor-mediated cerebrovascular dysfunction in AD.

## 7. ER stress in atherosclerosis and Alzheimer's disease

Endoplasmic reticulum (ER) is an intracellular organelle that regulates protein synthesis, calcium homeostasis, and lipid biosynthesis for maintaining cellular homeostasis [1]. ER stress is the results of various pathological factors, such as redox imbalance, hyperlipidemia, and viral infection disturb ER homeostasis, initiating prolonged unfolded protein response (UPR) in ER lumen. ER stress contributes to the pathogenesis of the cardiovascular and neurodegenerative disease [1, 2]. In excessive and prolonged ER stress, the three sensors of ER membrane-associated proteins, PERK (PKR-like eukaryotic initiation factor 2α kinase), IRE1 (inositol requiring enzyme 1), and ATF6 (activating transcription factor-6) are dissociated from GRP78 (glucose-regulated protein 78), subsequently activating ER stress-mediated apoptosis and ER stress-mediated inflammation leading to cell death [136]. In atherosclerosis, ER stress-mediated CHOP (CCAAT-enhancer-binding protein homologous protein) induces apoptosis signaling that regulating antiapoptotic Bcl-2 family and proapoptotic Bax/bak members and caspase-12 [32, 137]. Also, the expression of NF-kB,

TNF-α, IL-6/8, MCP-1, caspase-1, IL-1β, NLRP3 inflammasome, and TXNIP are elevated through IRE1α complex with increase of ER stress markers expression (PERK, p-IRE1α, IRE1α, ATF6, CHOP, p-eIF2α (eukaryotic translation initiation factor 2 alpha) in atherosclerotic lesions [124, 136, 138].

The direct effect of ER stress on the etiology of AD has been well corroborated and is regarded as a possible therapeutic intervention to conquer AD [12, 139]. The association between ER stress and AD pathology has been postulated as Aβ-induced cytotoxicity altering ER calcium homeostasis and Tau tangles blocking the ERAD (ER-associated protein degradation), which lead to a misfolded protein in the ER lumen [140, 141]. The increase in GRP78 and phosphorylated PERK, IRE1α, eIF2α are markedly elevated in AD brain when compared to age-matched controls [12, 142], and CHOP-mediated proapoptotic makers are also found in AD [142]. Aβ peptide and its accumulation are highly associated with the increased level of GRP78, p-PERK, p-eIF2α, p-IRE1, ATF4, CHOP, and caspase-12 in AD which is a likely cause of cognitive impairment and neuronal damage [142, 143]. Furthermore, genetically knocking-out IRE1α in an AD mouse model leads to the reduction of Aβ accumulation and the improvement of cognitive impairment [143], and PERK knock-out in AD mice model prevents synaptic plasticity and spatial memory compared with AD mice [144, 145]. Thus, IRE1/TRAF2 (TNF receptor-associated factor 2)/ASK1 (apoptosis signal-regulating kinase 1) complex promotes the expression of proinflammatory cytokines, such as IL-6/8 and TNF-α, and TXNIP-mediated NLRP3 inflammasome [124], caspase-1, and IL-1β [138], which is associated with AD-related memory deficits and cell damage [142, 146].

Therefore, the contribution of ER stress to the pathogenesis of atherosclerosis and AD is concisely defined from previous studies. Modulating ER stress brings a beneficial effect in

the prevention of atherosclerosis and AD pathologies. However, there is a lack of evidence to demonstrate the ER stress-associated vascular dysfunction in atherosclerosis and AD.

**8. The role of ER stress in vascular dysfunction and exercise**

The initial step of ER stress is an adaptive mechanism and subsequent prolonged response that implicates a pro-inflammatory and pro-apoptotic signaling pathway. These pathways are crucial in the pathophysiology of cardiovascular disease and neurodegenerative disease in atherosclerosis [4] and AD [12]. Regular exercise training has been well-known for therapeutic treatment and intervention in reducing the mortality and morbidity of cardiovascular disease [32] and AD [24]. The effectiveness of exercise for vascular dysfunction and cardiovascular homeostasis occurs via an improvement of the antioxidant response, abatement of an inflammatory response, and anti-apoptotic process [10, 32]. However, the beneficial effect of exercise training on ER stress-mediated vascular dysfunction in cardiovascular disease and neurodegenerative disease still contains insufficient data.

Inhibition of the ER stress normalizes myogenic response and ACh-induced endothelium-dependent vasodilation with reduction of Bip, p-IRE1$\alpha$, p-PERK, CHOP, and p-eIF2$\alpha$ in the coronary artery of diabetic mice [147] and a hypertensive mouse model [148]. Also, phenylephrine-induced vasoconstriction and endothelium-derived relaxation in mesenteric arteries in hypertensive rats are diminished by ER stress inhibition with decreased ER stress markers (GRP78 & CHOP) and ROS production [149]. Few studies have investigated the positive effect of exercise on ER stress-associated vascular dysfunction in atherosclerosis and especially in AD.

Treadmill exercise attenuates the ER stress markers (GRP78, p-PERK, p-eIf2α, XBP-1, and CHOP) and cleaved caspase-3, leading to improvement of cardiac function and reduction of cardiac infarction [30, 31]. Aerobic exercise training alleviates endothelial dysfunction in the aorta and the mesenteric arteries via the reduction of oxidative stress and elevation of NO bioavailability through PPAR-γ-dependent mechanism in diabetic and HFD-induced obese mice [150]. Furthermore, treadmill exercise alleviates GRP78, p-PERK, p-eIF2α, ATF6α, XBP-1, CHOP, and pro-apoptotic markers (procaspase-3/12 & Bax), and TNF-α and IL-1α, as well as Aβ accumulation in the hippocampus in AD transgenic mouse model [28]. However, limited information is currently available to define the role of exercise training on ER stress-associated endothelial dysfunction in atherosclerosis and AD. Therefore, further studies are required to find the missing knowledge gaps on the underlying mechanism of the effect of exercise on ER stress-associated vascular dysfunction in atherosclerosis and AD.

# Chapter 2: Exercise training ameliorates ER stress-mediated vascular dysfunction in mesenteric arteries in atherosclerosis

## 2. 1. ABSTRACT

Endoplasmic reticulum (ER) stress is closely associated with atherosclerosis, but the effects of exercise on ER stress-mediated endothelial dysfunction in atherosclerosis is not yet fully understood. We assessed endothelium-dependent vasodilation in isolated mesenteric arteries from wild type (WT), WT with exercise (WT-EX), ApoE knockout (ApoE KO), and ApoE KO mice with exercise (ApoE KO-EX). Vasodilation to acetylcholine (ACh) was elicited in the presence of inhibitors of ER stress, eNOS, caspase-1, and UCP-2 (Tudca, L-NAME, AC-YVARD-cmk, and Genipin, respectively) and the ER stress inducer (Tunicamycin). Immunofluorescence was used to visualize the expression of CHOP, as an indicator of ER stress, in superior mesenteric arteries (SMA). Dilation to ACh was attenuated in ApoE KO but was improved in ApoE KO-EX. Incubation of Tudca and AC-YVARD-cmk improved ACh-induced vasodilation in ApoE KO. L-NAME, tunicamycin, and Genipin attenuated vasodilation in WT, WT-EX and ApoE KO-EX, but not in ApoE KO. Exercise training reversed the increase in CHOP expression in the endothelium of SMA of ApoE KO mice. We conclude that ER stress plays a significant role in endothelial dysfunction of resistance arteries in atherosclerosis and that exercise attenuates ER stress and regulates its critical downstream signaling pathways including eNOS, UCP-2 and caspase-1.

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Chapter 2 is reproduced based primarily upon "Exercise training ameliorates endoplasmic reticulum stress-mediated vascular dysfunction in mesenteric arteries in atherosclerosis", DOI: 10.1038/s41598-018-26188-9 [151]

## 2. 2. INTRODUCTION

The endoplasmic reticulum (ER) is an organelle that modulates various physiological processes including protein folding, calcium homeostasis, and lipid biosynthesis for maintaining cellular homeostasis. Multiple pathologic factors, such as hyperlipidemia, oxidative stress, and calcium imbalance trigger prolonged disturbances or perturbations on ER homeostasis, leading to the unfolded protein response (UPR) pathway in the ER lumen which is known as ER stress. In prolonged ER stress, the three arms of UPR (PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6)) are activated together by the dissociation of ER chaperone BiP (GRP78) from the three sensors to restore cellular homeostasis or to activate the inflammatory pathways and apoptotic cell death [16]. ER stress is closely linked to atherosclerosis [136]; studies have reported that ER stress markers including GRP78, CHOP (CCAAT-enhancer-binding protein homologous protein), p-IRE-1, XBP-1, ATF-6, p-PERK, and p-elf2, were elevated in atherosclerotic aorta in ApoE KO mice [152, 153].

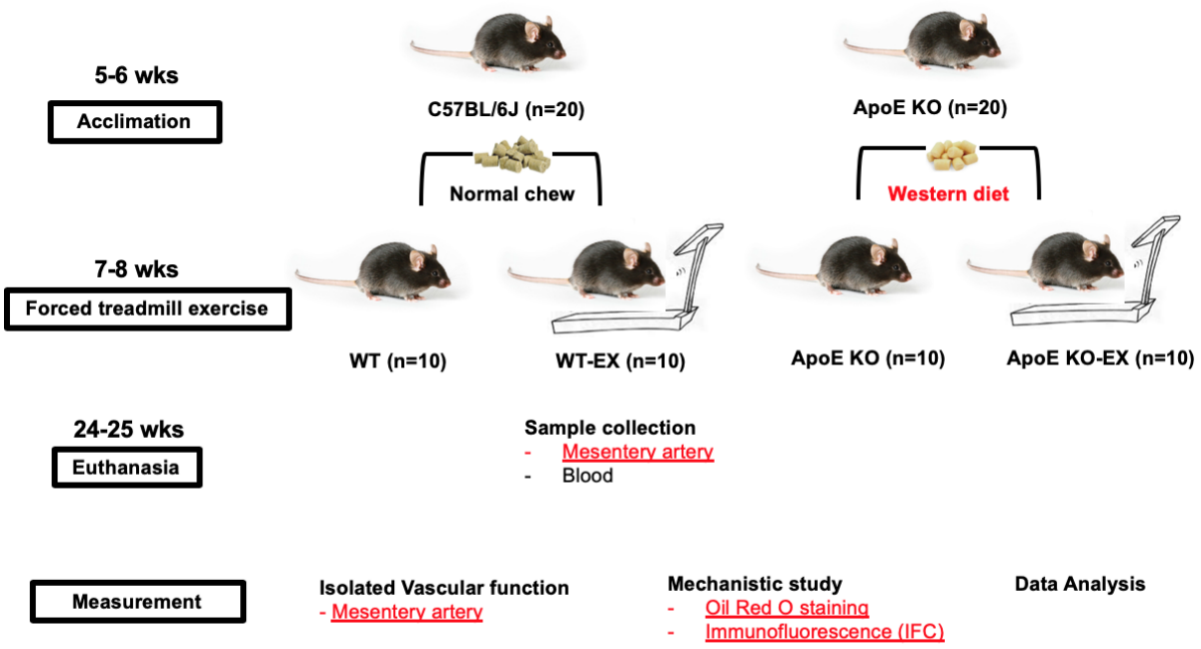
The endothelium is recognized as a crucial site for maintaining vascular homeostasis and regulating vascular reactivity, and endothelial dysfunction is recognized as the first step toward atherosclerosis [154]. It is known that ER stress is associated with endothelial dysfunction by reduced endothelial nitric oxide synthase (eNOS) signaling [155] and endothelium-dependent vasodilation [148], but a comprehensive understanding of how ER stress induces endothelial dysfunction in atherosclerosis, particularly through inflammation and oxidative stress, is currently lacking. Caspase-1, which is known as the precursor of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18 [104, 156] is highly expressed in human atherosclerotic plaque [157], plays a critical role in endothelial dysfunction in

atherosclerosis via ER stress [104, 109]. As mentioned, oxidative stress is a major risk factor for inducing ER stress and endothelial dysfunction [158]. Uncoupling protein-2 (UCP-2), which regulates mitochondrial ROS generation, may prevent a process of atherosclerosis and mitigate endothelial dysfunction with an increase in NO production in atherosclerosis and metabolic disorder [89, 159]. Collectively, the ER stress-associated risk factors, inflammation and oxidative stress, causing endothelial dysfunction suggest that ER stress could be an important potential therapeutic target for vascular diseases.

It is well established that exercise has beneficial effects on endothelial function in atherosclerosis. Aerobic exercise training reversed the endothelial dysfunction observed in the aortas of LDL receptor knockout mice by increased NO bioavailability, enhanced p-eNOS (phosphorylated eNOS) expression, and down-regulation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-derived superoxide production [26]. Furthermore, the suppression of ER stress, PERK, IRElα, and ATF6 by treadmill exercise, which also ameliorated endothelial dysfunction in diabetic aortas and mesenteric arteries, suggests that exercise improves endothelial dysfunction induced by ER stress [150]. Furthermore, regular exercise has been shown to reduce caspase-1, IL-1β, and IL-18 expression in ovariectomized rat hippocampus [111], and increase UCP-2 expression in aged rat aorta [160]. However, the direct effects of exercise on ER stress-mediated endothelial dysfunction and on ER stress-associated caspase-1 and UCP-2 signaling in atherosclerosis merit further examination. Thus, the main objective of this study is to investigate the effects of exercise training on ER stress-mediated endothelial dysfunction in atherosclerosis and the underlying mechanisms. Our study focuses on endothelial function of mesenteric arteries to determine whether atherosclerosis impairs endothelial function in resistance vessels which are central in blood pressure regulation

## 2. 3. METHODS

**Figure 2. 1. Experimental design overview.**




### Animal models

Male wild type mice (5 wks of age, WT, C57BL/6) and ApoE knockout mice (6 wks of age, ApoE KO, ApoE$^{tm1Unc}$) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in an animal facility with controlled temperature (22-23°C) and 12 hours light/dark cycles, and allowed free access to water and chow. WT and ApoE KO mice were randomly divided into exercise training (EX) and sedentary groups. WT and WT-EX mice were fed a normal chow and ApoE KO and ApoE KO with EX mice were fed a western atherogenic diet (0.2% cholesterol, 42% Kcal from fat, TD.88137, ENVIGO) for 17 wks to

accelerate atherosclerosis development in the ApoE KO model. All experiments on animals were performed in accordance with the approved animal care, relevant guidelines, and regulation of the Institutional Animal Care and Use Committee at the University of Houston (14-009).

**Exercise training protocols**

Briefly, WT-EX and ApoE KO-EX mice groups were randomly assigned and acclimated to run on a motorized rodent treadmill (Columbus Instruments, Columbus, OH) prior to exercise training for 1 week. The exercise training was performed at 7-8 wks of age, and the exercise training protocol consisted of up of 1 hour of running on a rodent motor-driven treadmill at 15m/min at a 5% grade (60~80% of $VO_2$ max), 5 days/week for 15-16 wks. The daily exercise protocol consisted of warm-up (5 min), running (50 min), and cool-down (5 min). Non-exercise groups of mice were housed in the same room and under the same conditions, but without the exercise training. At 24-25 wks of age, mice were sacrificed within 24 hrs after the final training day.

**Functional assessment of isolated mesenteric arteries of ApoE KO mice**

After euthanasia, the mesenteric artery bed was rapidly excised and placed in a cold dissection chamber (~ 4°C) with cold physiological saline solution (PSS) containing 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.2 mM $NaH_2PO_4$, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1% of BSA at pH 7.4. The second or third branch of mesenteric artery was carefully isolated, and the connective and fatty tissues were removed from the surrounding artery under a dissecting microscope (Nikon SMZ1000).

A single isolated mesenteric artery (180-220 μm in internal diameter and 0.5-1.0 mm in length) was transferred to a Lucite chamber containing PSS, cannulated with glass micropipettes filled with PSS-albumin solution, and secured with surgical nylon sutures. The chamber was transferred to a stage of an inverted microscope (Nikon Eclipse Ti-S) and the cannulated artery was pressurized to 80cm$H_2O$ intraluminal pressure without altering the intraluminal flow. Temperature was maintained at 36.5°C. Vascular diameter changes were monitored by inverted microscope with video caliper (Colorado Video, Boulder, CO, USA), as previously described by our laboratory [161]. After equilibrium (1 hr), the isolated mesenteric artery was pre-constricted with phenylephrine (PE, 1-5μmol/L) and the vasodilation function was tested by different stimuli. If the vessel constricted less than 20% of initial diameter, it was discarded. To determine whether atherosclerosis and exercise regulated vascular function in ApoE KO mice, the dose–dependent-diameter changes were established with endothelium-dependent vasodilator, ACh (0.1 nmol/L to 10 μmol/L) and endothelium-independent vasodilator, sodium nitroprusside (SNP, 0.1 nmol/L to 10 μmol/L) in mesenteric arteries from WT, WT-EX, ApoE KO, and ApoE KO-EX. Tudca (ER stress inhibitor, 10 μmol/L), Tunicamycin (ER stress inducer, TM, 10 μmol/L), AC-YVAD-cmk (caspase-1 inhibitor, 10 μmol/L), and genipin (UCP-2 inhibitor, 10 μmol/L) were each incubated with the isolated vessel for 20-30 min before measuring ACh-induced endothelium-dependent vasodilation. The contribution of NO to vasodilation was assessed by incubating the vessels with the *N*G-nitro-L-arginine methyl ester (L-NAME; NO synthases inhibitor; 10 μmol/L, 20 min). Tudca and TM were dissolved in 0.1% DMSO and all other drugs were dissolved in PSS and then extraluminally administrated into the PSS in the chamber before measuring the vascular function.

**Atherosclerotic lesions analysis**

Oil Red O staining was used measure and visualize the severity of the atherosclerotic lesion. The aorta was dissected from ascending (thoracic aorta) to descending aorta (femoral artery), and adipose and connective tissue were removed and fixed in 4% of paraformaldehyde for overnight at 4°C. The dissected samples were placed into a tube filled with 400 μm of Sudan red Vb (5mg/mL in 70% isopropanol) and each tube was incubated in a water bath for 40 min at 37°C. After incubation, the samples were washed with the 70% isopropanol. Total 3-4 mice per group were utilized and one whole image per mice was taken for atherosclerotic lesions analysis. The extent of the staining area was assessed under a dissecting microscope, and the percentage of plaque per total area was measured by an image analyzer (image J software, NIH, MA).

**Immunofluorescence staining**

Superior mesenteric arteries were stained by immunofluorescence to visualize and localize the ER stress maker with markers of the endothelial cells. Briefly, isolated superior mesenteric arteries were fixed in 4% paraformaldehyde solution in PBS overnight, and the fixed tissue was perfused in 30% sucrose in PBS overnight at 4°C. Once the superior mesenteric artery was completely perfused, the tissue was embedded into tissue-tek O.C.T. compound, snap frozen at -60°C, and stored at -80°C. The frozen samples were sectioned at 5 μm thick using cryostat (Leica), the sections were placed on microscope slides, and the slides were dried at room temperature (RT) for 10-15 min and washed 3 times in PBS (each 10 min) to remove O.C.T compound. Heat induced epitope retrieval (sodium citrate buffer, pH 6.0) was performed on the washed slides for 20 mins, and the slides were cooled for 20 min and

then washed 3 times in PBS. For cell permeabilization, the slides were incubated in 1% of triton X-100 for 15 min and washed 3 times in PBS. Blocking buffer (5% donkey serum in PBS) was then applied for 30 min at RT. Primary antibodies, diluted in blocking buffer, were incubated: mouse IgG CHOP (CST, #2895, 1:100), and goat IgG PECAM-1 (Santa Cruz, M-20, 1:400) overnight at 4°C. After washing the slides 3 times in PBS, secondary antibodies, diluted in PBS, were incubated: FITC (Donkey anti-goat IgG, Alexa 488-conjugated) or Texas Red (donkey anti-mouse, Alexa 594-conjugated) for 2h at RT and washed 3 times in PBS. After washing, the slides were mounted in mounting media with DAPI (ProLong® Diamond Antifade Mountant with DAPI, Thermo Fisher Scientific) with a cover slide in the darkroom. Stained tissues were observed with a fluorescence microscope (Olympus BX51) and 20X and 100X objective, imaged with a color camera (Olympus DP73), and the digital images acquired with image software (Olympus cellSens Dimension). For negative controls, the primary antibody was omitted in the protocol and samples imaged using the same parameters. For quantification, images were acquired from 3-4 mice per group with 20X objective lens, and cells were quantified using the cell counting function of the NIH Image J software. Total 3-4 mice per group were taken and one image per mice was used for immunofluorescence. Typical ranges of total cell count per tissue were 10-20 in the endothelial cell layer.

**Data analysis**

All diameter changes to pharmacological agonists were normalized to the control diameter. Normalized diameters were averaged at each concentration of agonist used and shown as mean ± SEM. Statistical comparisons of vasoreactivity responses between groups were performed with two-way analysis of variance (ANOVA) for repeated measure and

intergroup differences were tested with Fisher's protected LSD test. The significance of intergroup differences observed in body weight and vessel diameters were analyzed with one-way analysis of variance (one-way ANOVA) using software SPSS 22.0. Significance was accepted at $p < 0.05$.

## 2. 4. RESULTS

### *Animal and vascular characteristics*

Body weight was measured at 7-8 wks of age and after 15-16 wks of exercise training (24-25 wks of age). Although body weight at 7-8 wks of age was not different among the groups, it significantly increased in ApoE KO and ApoE KO-EX ($p < 0.05$) compared with WT and WT-EX at 24-25 wks (Table 2. 1). This result demonstrates that ApoE gene knockout fed western diet (WD) increased the body weight regardless of the exercise training. Heart weight and heart/body weight ratio were evaluated at 24-25 wks of age and they were significantly higher in ApoE KO ($p < 0.05$) compared with all other groups. The result suggests that atherosclerosis may induce cardiac hypertrophy, and that exercise training prevented it (Table 2. 1). Maximal intraluminal diameters of mesenteric arteries were not significantly different in WT and atherosclerotic ApoE KO vessels with or without exercise training (Table 2). No significant difference was observed in initial diameter and percentage constriction tone of mesenteric arteries in all groups (Table 2. 2).

**Table 2. 1. Animal characteristics**

| | | Sedentary | | Exercise | |
|---|---|---|---|---|---|
| | | **WT** | **ApoE KO** | **WT-EX** | **ApoE KO-EX** |
| **N** | | 9 | 10 | 9 | 8 |
| **Body Weight (g)** | **Pre** | 23.52±0.62 | 24.92±1.13 | 24.17±0.90 | 26.03±0.59 |
| | **Post** | 31.32±0.71 | 35.37±1.26*† | 29.62±0.65 | 35.94±1.37*† |
| **Heart weight (mg)** | | 150.41±6.73 | 191.41±4.69*† | 142.83±3.98 | 155.64±5.85# |
| **Heart weight/Body weight (mg/g)** | | 4.81±0.19 | 5.45±0.18*† | 4.84±0.19 | 4.32±0.18# |

Body weight was significantly increased in ApoE KO and ApoE KO-EX compared with WT and WT-EX. Heart weight and heart/body weight were significantly elevated in ApoE KO mice compared with the other groups. Values are means ± SEM. *$p<0.05$ vs. WT, †$p<0.05$ vs. WT-EX, #$p<0.05$ vs. ApoE KO.

**Table 2. 2. Vessel characteristics (Mesenteric arteries).**

| | Sedentary | | Exercise | |
|---|---|---|---|---|
| | **WT** | **ApoE KO** | **WT-EX** | **ApoE KO-EX** |
| **N** | 7 | 10 | 8 | 8 |
| **Mesentery arteries constriction tone, %** | 32.14±6.08 | 33.70±4.57 | 26.88±3.15 | 27.38±2.06 |
| **Mesentery arteries initial lumen diameter, μm** | 157.57±19.38 | 138.30±7.82 | 149.13±6.23 | 158.13±8.62 |
| **Mesentery arteries maximal lumen diameter, μm** | 237.43±17.10 | 213.10±10.27 | 205.63±11.80 | 218.38±10.33 |

Exercise training had no effect on the constriction tone, initial lumen, and maximal lumen of mesenteric arteries.

***Exercise training decreases atherosclerotic lesion in aorta***

Atherosclerotic lesion was evaluated to demonstrate the effectiveness of ApoE gene knockout fed with WD and exercise training on the development of atherosclerosis in aorta. The accumulation of atherosclerotic lesion in aortic roots was significantly higher in ApoE KO compared with WT ($p < 0.05$), but was significantly reduced by exercise training in ApoE KO-EX ($p < 0.05$; Figure 2. 2A & B). It tended to be lower in WT-EX mice compared to WT mice, but it was not statistically different between the groups ($p = 0.157$; Figure 2. 2A & B).

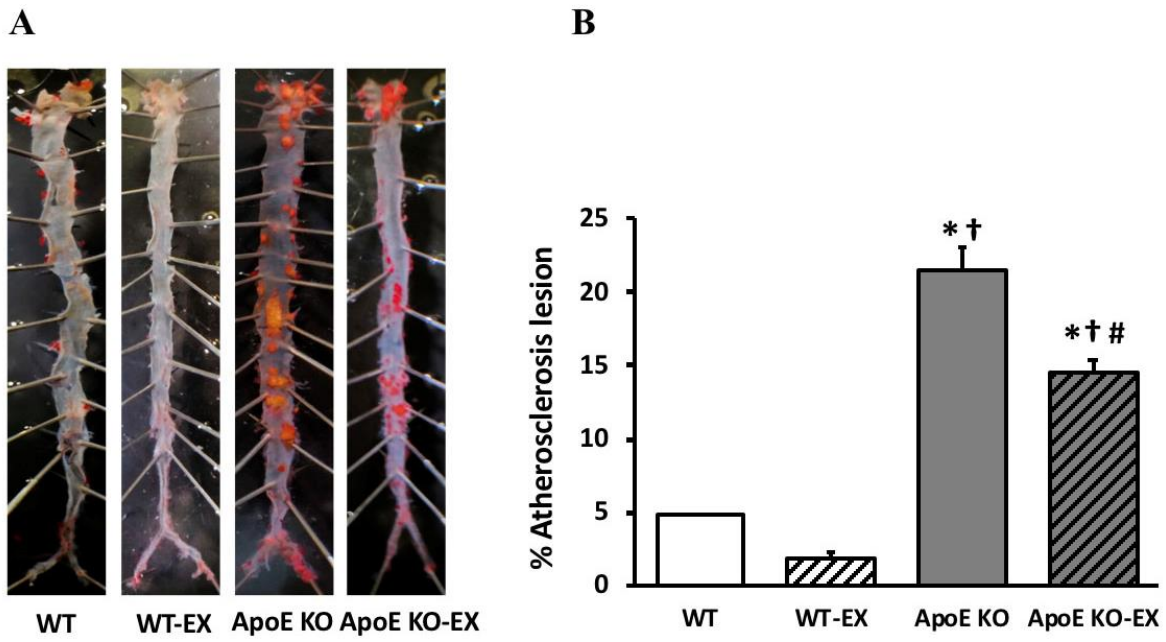



**Figure 2. 2. Atherosclerosis in aorta.**

**A.** Atherosclerosis lesions development in aorta from four groups of mice. **B.** Atherosclerosis lesion was significantly higher in ApoE KO (n=3) compared with WT (n=2), but was significantly reduced in ApoE KO-EX compared with ApoE KO. Values are means ± SEM. *p<0.05 vs. WT, †p<0.05 vs. WT-EX, #*p*<0.05 vs. ApoE KO.

***Exercise training ameliorates endothelial dysfunction in mesenteric arteries of ApoE KO mice***

To evaluate the beneficial effects of exercise training on endothelium-dependent vasodilation in atherosclerosis, ACh-induced vasodilation was measured in the isolated mesenteric arteries. ACh-induced vasodilation was significantly attenuated in mesenteric arteries of ApoE KO ($p < 0.05$) compared with WT mice, but exercise training markedly enhanced ACh-induced vasodilation in ApoE KO mice (Figure 2. 3A). These findings suggest that atherosclerosis impairs endothelial function in mesenteric arteries, and that exercise training ameliorates the impairment. However, vasodilation in response to endothelium-independent vasodilator (SNP; NO donor), showed no difference in the isolated mesenteric arteries among all groups (Figure 2. 3B). These results suggest that the alterations of vascular function by atherosclerosis and exercise training are endothelium dependent.

In addition, ACh-induced vasodilation was measured in the isolated mesenteric arteries in the presence of NOS inhibitor (L-NAME), to examine whether the NO contribution of the vasodilatory response is altered in mesenteric arteries of ApoE KO mice. Incubation with L-NAME significantly decreased ACh-induced vasodilation in the WT and ApoE KO-EX mice ($p < 0.05$), but not in ApoE KO mice (Figure 2. 3C & D). These results suggest that the atherosclerosis-induced endothelial dysfunction in ApoE KO and the improved endothelial function in ApoE KO mice by exercise training are a NO-dependent mechanism in mesenteric arteries.

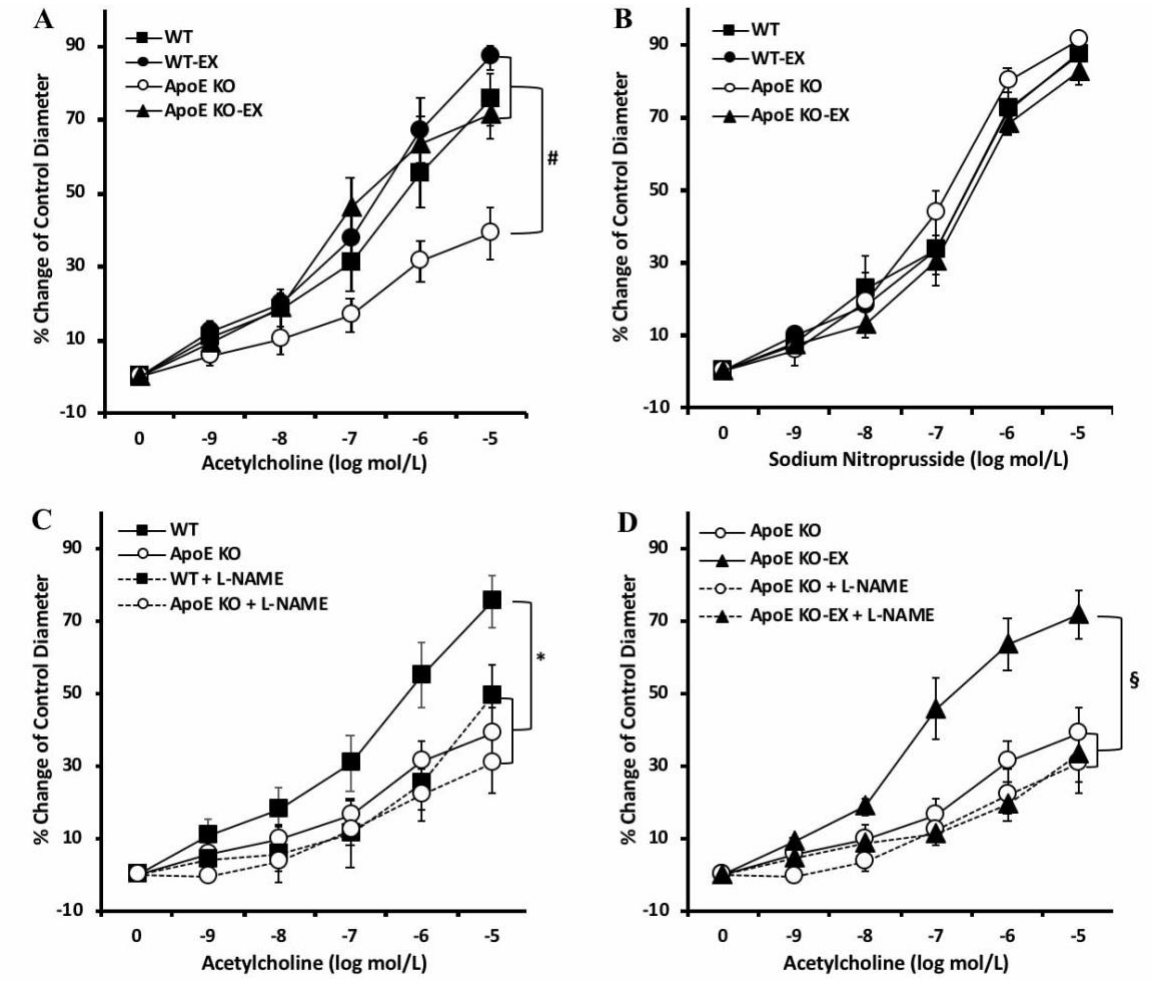



**Figure 2. 3. Effect of exercise training on endothelial dysfunction in mesenteric arteries of ApoE KO mice.**

**A.** Isolated mesenteric arteries from WT (n=7), WT-EX (n=8), ApoE KO (n=10), and ApoE KO-EX (n=8) measured the response of ACh in a dose-dependent manner. ACh-induced vasodilation was significantly attenuated in the mesenteric arteries of ApoE KO mice compared with WT mice. However, exercise ameliorated the ACh-induced endothelial dysfunction in ApoE KO. **B.** SNP-induced, the endothelium-independent vasodilation, showed no difference among the groups (WT+SNP (n=9), WT-EX+SNP (n=9), ApoE KO+SNP (n=9), ApoE KO-EX (n=8)). **C & D.** Incubation of L-NAME, the eNOS inhibitor, significantly

abolished endothelium-dependent vasodilation in WT and ApoE KO-EX; WT+L-NAME (n=7), ApoE KO+L-NAME (n=8), and ApoE KO-EX (n=8). Values are means ± SEM. $^{*}p<0.05$ vs. WT, $^{\#}p<0.05$ vs. ApoE KO, $^{\S}p<0.05$ vs. ApoE KO-EX.

***Exercise training alleviates ER stress-mediated endothelial dysfunction in mesenteric arteries of ApoE KO mice***

To determine the contribution of ER stress to the vascular dysfunction in mesenteric arteries of ApoE KO mice, ACh-induced vasodilation was measured following treatment with an ER stress inducer (Tunicamycin) and an ER stress inhibitor (Tudca). ACh-induced vasodilation with incubation of Tunicamycin was significantly attenuated in WT indicating that ER stress can directly impair endothelium-dependent vasodilation (Figure 2. 4A) but tunicamycin had no additional effect on ACh-induced vasodilation in ApoE KO mice. In contrast, exercise treatment restored ACh-induced vasodilation in ApoE KO, and this restored vasodilatory function was abolished by Tunicamycin treatment (Figure 2. 4B). Furthermore, treatment of Tudca had little improvement of ACh-induced vasodilation in WT, but it was significantly enhanced ACh-induced vasodilation in ApoE KO ($p < 0.05$) to a comparable extent as exercise in ApoE KO-EX (Figure 2. 4C & D). These findings directly suggest that ER stress is acutely causative of endothelial dysfunction in the mesenteric arteries of ApoE KO mice. Importantly, exercise training appears to reverse the ER stress-mediated endothelial dysfunction in these arteries.

Immunofluorescence analysis showed that ER stress marker, CHOP (red, Figure 2. 5A), was expressed in the vascular wall and co-localized with the vascular endothelial cell (green, Figure 2. 5A) of mesenteric arteries. The expression of CHOP was significantly elevated in the

vascular wall and in the mesenteric endothelial cells of ApoE KO compared with WT and WT-EX (Figure 2. 5B), but was significantly decreased in ApoE KO-EX (Figure 2. 5B). These results demonstrate that ER stress is highly expressed in the endothelium and is associated with endothelial dysfunction, and that exercise training ameliorates ER stress-mediated endothelial dysfunction via the diminished expression of CHOP in the endothelial cells of mesenteric arteries of ApoE KO mice.

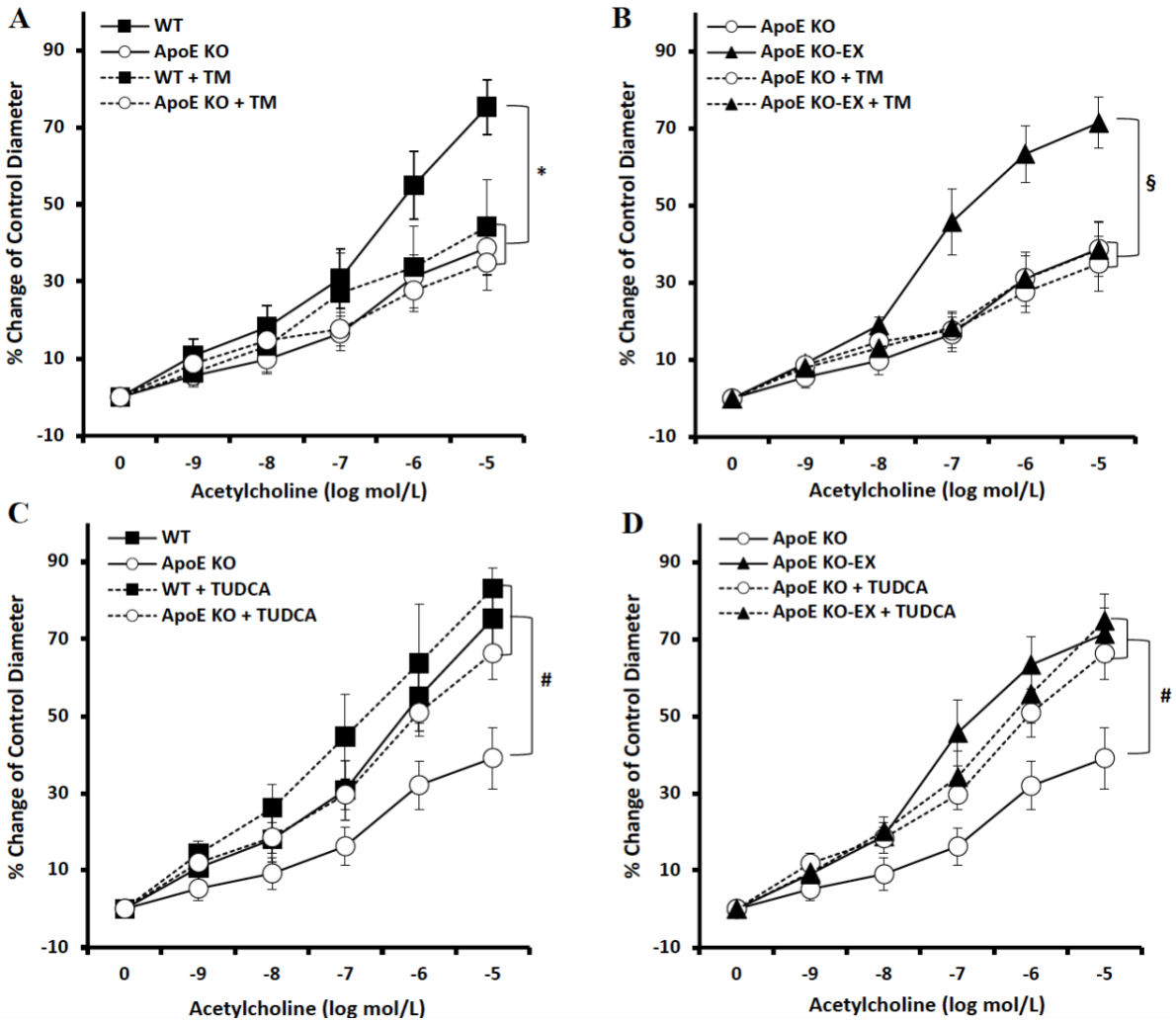



**Figure 2. 4. Effects of exercise training on ER stress-mediated endothelial dysfunction in mesenteric arteries of ApoE KO mice.**

**A & B**. Incubation of the ER stress inducer, Tunicamycin (TM), in the isolated mesenteric arteries significantly attenuated endothelial-dependent vasodilation in WT+TM (n=7) and ApoE KO-EX+TM (n=7). C & D. Incubation of the ER stress inhibitor, Tudca, in the isolated mesenteric arteries and ACh-induced vasodilation were significantly augmented in ApoE KO+Tudca (n=9). Values are means ± SEM. $*p< 0.05$ vs. WT, $\#p<0.05$ vs. ApoE KO, $\S p<0.05$ vs. ApoE KO-EX.

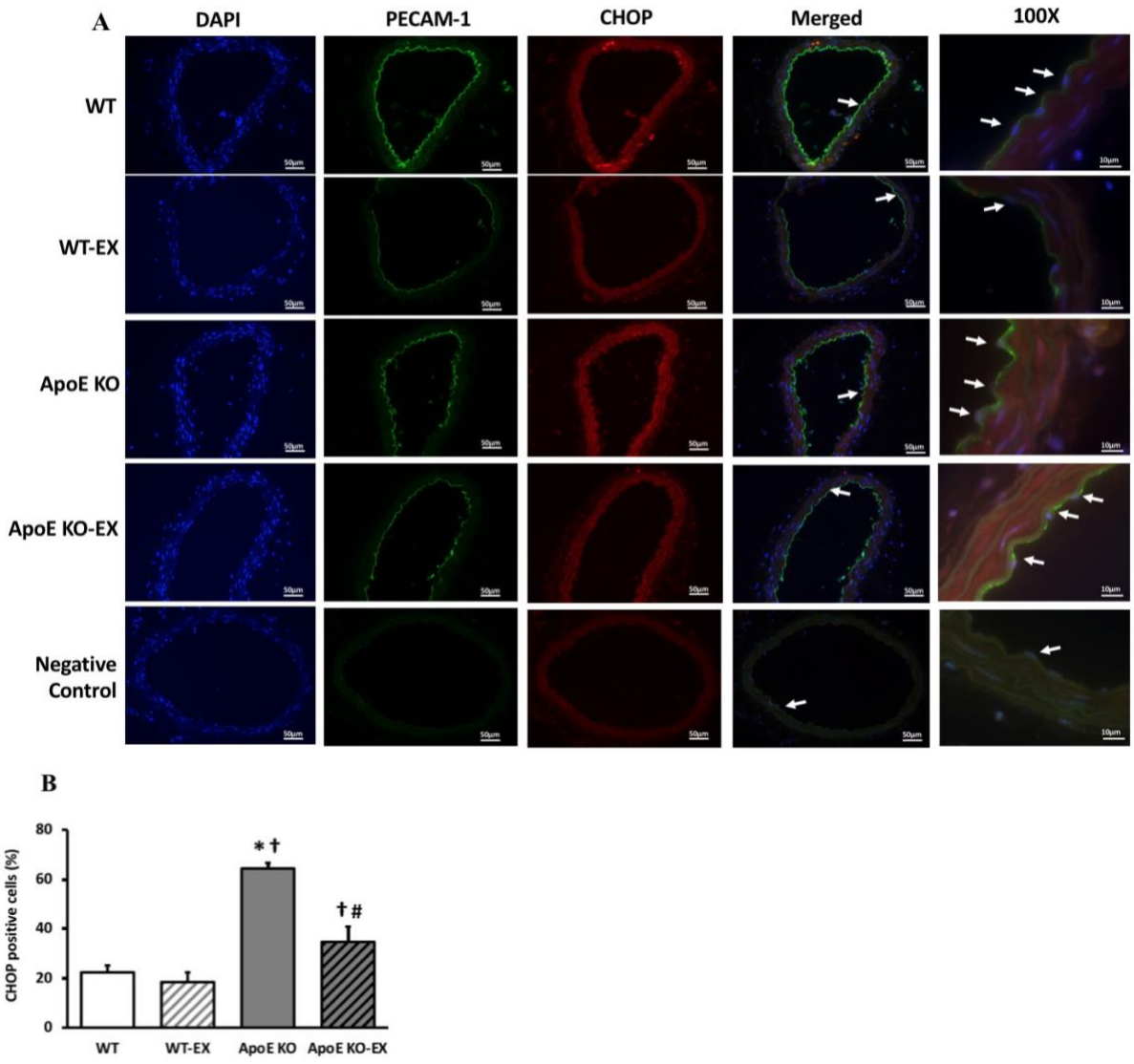


**Figure 2. 5. Expression and localization of CHOP with endothelial cells in the isolated superior mesenteric arteries of ApoE KO mice.**

**A.** Immunofluorescence of DAPI (blue) CHOP (red), and PECAM-1 (green) in WT, WT-EX, ApoE KO, and ApoE KO-EX in the isolated superior mesenteric arteries. CHOP is colocalized with endothelial cells in the isolated superior mesenteric arteries. Dual staining of CHOP (red) and endothelial cells (green) in mesenteric arteries from all mice. Negative control with an absence of staining with primary antibodies. Arrows show colocalization of CHOP in endothelial cells. **B**. CHOP positive-stained mesenteric arteries were counted per section (n=3); magnification is X 20 (scale bar; 50 μm) and X 100 (scale bar; 10 μm). $^{*}p< 0.05$ vs. WT, $^{\dagger}p<0.05$ vs. WT-EX, $^{\#}p<0.05$ vs. ApoE KO, $^{\S}p<0.05$ vs. ApoE KO-EX.

***Exercise training ameliorates oxidative stress and inflammation in mesenteric arteries of ApoE KO***

To establish the role of oxidative stress in endothelial dysfunction in atherosclerotic mesenteric arteries, arteries of all groups were incubated with, genipin, a UCP-2 inhibitor. In the presence of genipin, ACh-induced vasodilation was significantly attenuated in WT and ApoE KO-EX ($p < 0.05$), but not reduced further in ApoE KO (Figure 2. 6A). However, genipin did effectively abolish the restored ACh-induced vasodilation in the the ApoE KO-EX mice (Figure 2. 6B). These results suggest that ER stress-associated oxidative stress in atherosclerotic mesenteric arteries impairs endothelial-dependent vasodilation, and that exercise training ameliorates endothelial dysfunction in the mesenteric arteries of ApoE KO mice.

To determine the contribution of caspase-1-mediated inflammation to endothelial dysfunction in mesenteric arteries of ApoE KO mice, arteries of all groups were incubated with, caspase-1 inhibitor, AC-YVAD-cmk. Treatment of AC-YVAD-cmk in the mesenteric arteries of ApoE KO mice significantly restored ACh-induced vasodilation ($p < 0.05$) which was comparable to WT and ApoE KO-EX (Figure 2. 6C & D). This result demonstrates that caspase-1 is involved in the mechanism of endothelial dysfunction in mesenteric arteries of ApoE KO mice, and that exercise alleviates caspase-1-associated endothelial dysfunction in ApoE KO mice.

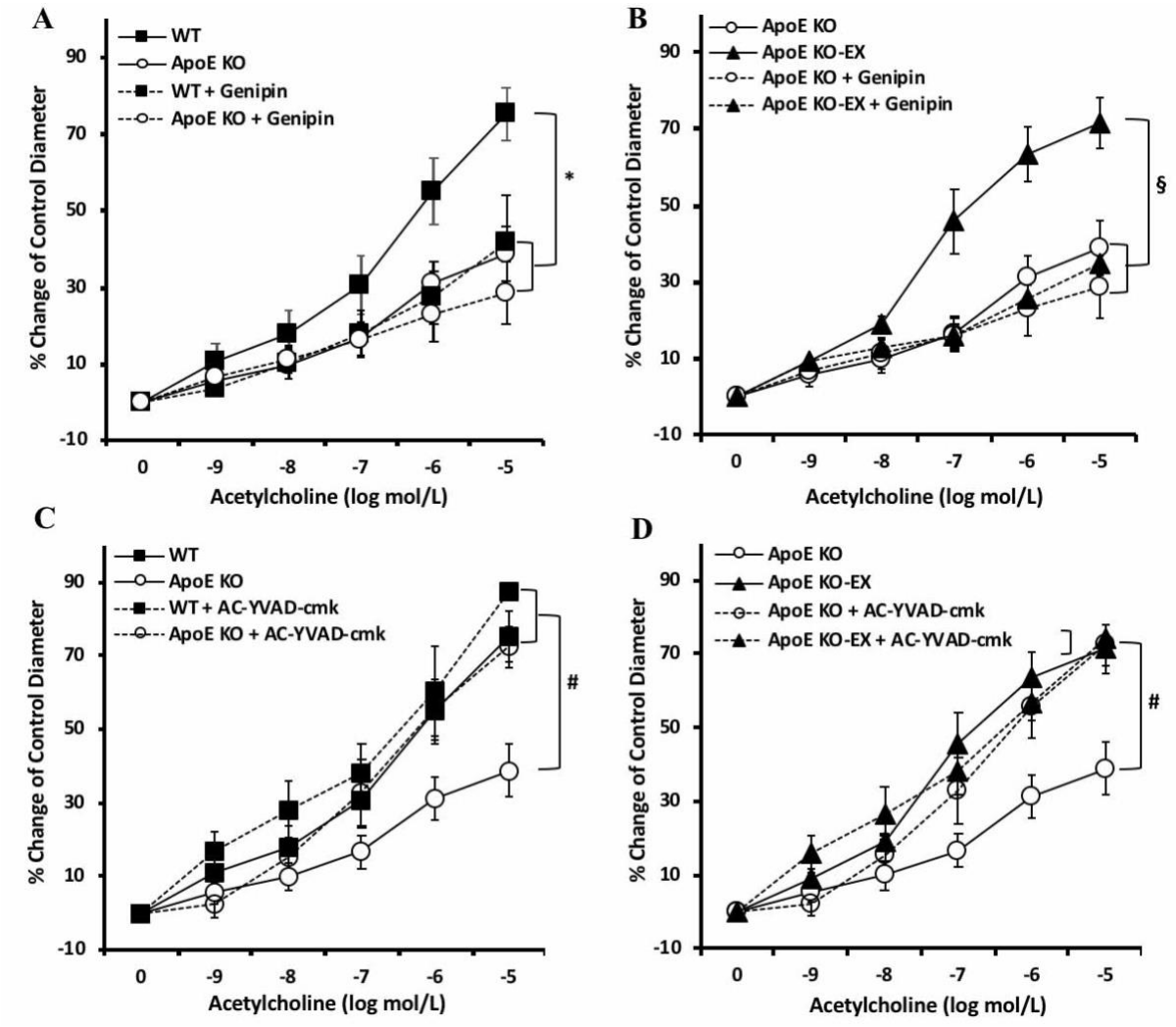


**Figure 2. 6. Role of caspase-1 and UCP-2 in endothelial dysfunction in mesenteric arteries of ApoE KO mice.**

**A & B.** Incubation of the UCP-2 inhibitor, genipin, in isolated mesenteric arteries diminished ACh-induced vasodilation in WT+genipin (n=7) and ApoE KO-EX+genipin (n=7). **C & D.** Anti-caspase-1 treatment in the isolated mesenteric arteries improved endothelium-dependent vasodilation in ApoE KO+AC-YVAD-cmk (n=7). Values are means ± SEM. $*p<0.05$ vs. WT, $\#p<0.05$ vs. ApoE KO, $\S p<0.05$ vs. ApoE KO-EX.

## 2. 5. DISCUSSION

The present findings show the contribution of ER stress to the endothelial dysfunction and the effects of exercise training to reverse ER stress-mediated vascular dysfunction in mesenteric arteries of ApoE KO mice. The key findings are as follows: 1) Exercise training normalized heart/body weight ratio and atherosclerotic region in ApoE KO mice; 2) ACh-induced endothelium-dependent vasodilation was impaired in mesenteric arteries of ApoE KO mice and was ameliorated by exercise training through restored endothelial NO signaling; and 3) The changes mediated by ER stress were associated with caspase-1 and UCP-2. Taken as a whole, the results of this study suggest that exercise training acts as a significant moderator of endothelial dysfunction by attenuating ER stress and ER stress-associated inflammation and oxidative stress in mesenteric arteries of ApoE KO mice.

Atherosclerosis is associated with pathological cardiac hypertrophy by increased aortic stiffness and prolonged hypertension [162], but exercise training could normalize this pathological cardiac hypertrophy as evidenced by reduced LV wall thickness, cross sectional area of cardiomyocyte, and heart/body weight ratio [163, 164]. In accordance with previous findings, these new findings reinforce that heart weight and heart/body weight ratio significantly increased in WD fed ApoE KO mice compared to other groups (Table 2. 1), and that exercise training significantly decreased heart weight and heart/body weight ratio in ApoE KO, which was comparable to the other groups. This finding provides direct evidence of the effectiveness of exercise as a therapeutic intervention (Table 2. 1), and demonstrates that exercise training may normalize pathological cardiac hypertrophy in ApoE KO mice. Furthermore, exercise training significantly reduced the elevated atherosclerotic lesion in the aorta of WD fed ApoE KO (Figure 2. 2A & B). Previous studies reported that swimming and

aerobic exercise training markedly alleviated atherosclerosis in the diminished atherosclerotic lesions area, fatty streak formation, and fibrofatty plaques of aorta in the atherosclerosis mouse models [62, 165].

NO, a potent vasodilator released from endothelial cells, is associated with the mechanism of atherosclerosis initiation and progression. Endothelial dysfunction is an initial parameter of atherosclerosis and the impaired endothelial function of resistance vessels in the ApoE KO mice is characterized by reduced NO bioavailability [56]. In this study, ACh-induced vasodilation was impaired in the mesenteric arteries of ApoE KO (Figure 2. 3A), but no difference was observed in SNP-induced endothelium-independent vasodilation (Figure 2. 3B). Furthermore, incubation with L-NAME did not alter endothelium-dependent vasodilation in ApoE KO, while it drastically reduced it in the other groups (Figure 2. 3C & D). These findings suggest that atherosclerosis impairs the vascular function of mesenteric arteries in an endothelium-dependent and NO-mediated manner.

Although the effectiveness of exercise on atherosclerosis-associated vascular dysfunction has been widely studied on conduit vessel, to our knowledge the present study is the first to demonstrate that exercise training restores NO-mediated endothelial dysfunction in mesenteric arteries, which are resistance arteries. Notably, exercise training restored ACh-induced vasodilation in the mesenteric arteries of ApoE KO mice (Figure 2. 3A), whereas no difference was found in SNP-induced vasodilation (Figure 2. 3B). Moreover, L-NAME treatment in the isolated mesenteric arteries of ApoE KO-EX drastically reduced ACh-induced vasodilation, but produced no further reduction in the ApoE KO mice (Figure 2. 3C & D). Our work aligns with two earlier findings: swimming exercise training, which positively regulated ACh-induced endothelial dysfunction in the aorta of ApoE KO [166], and treadmill exercise,

which prevented endothelial dysfunction with the increased expression of p-eNOS, NO production, and antioxidant protein in the thoracic aorta of LDL-deficient mice [26]. Accordingly, the reduction of NO production in mesenteric arteries of ApoE KO mice in our study may also be through decreased production and/or activity of eNOS, with reversal by exercise training.

The inhibition of ER stress restored endothelial dysfunction with the increase in p-eNOS, and NO production in aorta and mesentery arteries of the hypertensive [167] and diabetic mice [150]. The present study found that ACh-induced endothelial dysfunction in ApoE KO mice was ameliorated by Tudca, the ER stress inhibitor (Figure 2. 4C), and Tunicamycin, the ER stress inducer, directly impaired endothelium-dependent vasodilation in the WT and ApoE KO-EX mice, but not in ApoE KO mice (Figure 2. 4A & B). It reinforces that ER stress tightly correlates with endothelial dysfunction in the mesenteric arteries of ApoE KO. Furthermore, exercise training has been accepted as an effective treatment to reduce ER stress, but more studies are needed to confirm the beneficial effects of exercise on ER stress-associated vascular dysfunction [32]. Recently, Cheang et al. reported that treadmill exercise ameliorated endothelial dysfunction via the PPAR-$\delta$ dependent mechanism with a decrease in the expression of p-eIF2$\alpha$, ATF3 & 4 and an increase in intracellular NO production in the aorta and mesentery artery of db/db mice [150]. Our studies showed that reduced ACh-induced vasodilation in ApoE KO-EX mice by Tumicamycin is similar to the ApoE KO mice, and the incubation of Tudca in the ApoE KO mice enhances the vasodilatory function to the level of ApoE KO-EX (Figure 2. 4). These findings suggest that exercise training may restore the impaired endothelium-dependent vasodilation in mesenteric arteries of ApoE KO mice by attenuating ER stress-mediated mechanisms.

Our immunostaining data indicated that CHOP was expressed in the vascular wall, including the endothelium of mesenteric arteries and its expression was significantly elevated in ApoE KO mice (Figure 2. 5A & B), which suggests that the augmented CHOP-associated ER stress in the mesenteric artery contributes to endothelial dysfunction. This result is consistent with the earlier findings that CHOP expression was augmented in smooth muscle cells of the isolated coronary arteries of hypertensive rats [148] and in endothelium of the aorta of ApoE KO [168], and they were associated with the CHOP-mediated apoptotic signaling, which induces vascular damage [153]. Furthermore, we showed that exercise training significantly reduced the elevated CHOP expression in the mesenteric arteries of ApoE KO mice (Figure 2. 5A & B), which aligns with the previous findings that regular exercise training decreased the expression of CHOP in myocardial infarction rats [30] and CHOP-mediated apoptosis proteins, Bcl-2 & Bax in obese mice [169]. The changes of CHOP expressions in endothelial cells, which may be responsible for the impaired ACh-induced endothelial function in the ApoE KO mice and decreased protein level of CHOP in mesenteric arteries by exercise training in atherosclerotic mice, may indicate a shift from a pro-apoptotic to a pro-survival UPR signal.

A significant novel finding of the current study is that the inhibition of caspase-1 alleviates the impaired ACh-induced vasodilation in atherosclerotic mesentery arteries (Figure 2. 6C & D), which suggests that caspase-1 is responsible for endothelial dysfunction in atherosclerosis possibly through an ER stress-dependent manner. The cleaved caspase-1 is activated by NLRP3 inflammasome, and is a biologically active form with proteolytic actions protein substrates, including pro-inflammatory cytokine interleukin (IL)-1β [170], which associates with endothelial dysfunction and injury [171]. Caspase-1 deficiency in ApoE KO

mice alleviates atherosclerosis through the reduced atherosclerotic plaque area and plasma level of inflammation (IL-1β, IL-1α, IL-6 and TNF-α) [104, 156]. Furthermore, the inhibition of caspase-1 ameliorated the impaired endothelium-dependent vasodilation via upregulation of eNOS activity in the coronary arteries of hypercholesterolemia [109] and homocysteinemia [171]. Induction of ER stress augmented the expression of caspase-1 p20 and IL-1β, but the inhibition of ER stress reduced the caspase-1 expression in the livers of ob/ob mice [172]. Wang et al. showed that treadmill exercise reduced the caspase-1 expression along with a decrease in expression of NLRP3/ASC and IL-1β/18 in the hippocampus of ovariectomy-induced depression-like mice [111]. However, the direct effects of exercise on caspase-1 in the vascular function have not been clearly established. Our finding indirectly suggested that exercise training may ameliorate endothelial dysfunction possibly via caspase-1 in the mesentery arteries of ApoE KO (Figure 2. 6C & D), but further study is needed to confirm the clear link between caspase-1 and ER stress in the endothelial dysfunction in atherosclerosis, and the specific effects of exercise training on the caspase-1 expression in the mesentery arteries of ApoE KO mice.

The UCP-2 is the mitochondrial anion carrier protein, which is a critical factor regulating a normal range of mitochondrial ROS production, and in turn maintains NO homeostasis to protect endothelial function [173] in atherosclerosis [89, 173]. Tian et al. found that UCP-2 deficiency impaired endothelium-dependent vasodilation with an elevated ROS and a decrease in p-eNOS expression in the aortae and mesenteric arteries, but that UCP2 overexpression by adenoviral infection of UCP2 restored the impaired endothelium-dependent vasodilation, elevated p-eNOS, and reduced ROS [89]. Figure 2. 6A & B concurs with a previous finding that incubation of Genipin decreases ACh-induced endothelium-dependent

vasodilation in WT and ApoE KO-EX, but not in ApoE KO, which indicates that the increase in ROS by UCP-2 inhibition exacerbates endothelial dysfunction in mesenteric arteries of ApoE KO mice. Moreover, it is known that the inhibition of UCP-2 increases the expression of Bip and CHOP, and IRE1α, PERK, ATF6 with the elevation of NADPH oxidative activity in macrophages [174] and mouse aorta endothelial cells (MAECs) [91]. It has been shown that exercise training alleviates endothelium-dependent vasodilation via an increase in UCP-2 expression, i.e., chronic exercise training ameliorated ACh-induced endothelial dysfunction with the elevated UCP-2 expression [160]. However, no study has evaluated the exercise effects on UCP-2 in vascular dysfunction in any pathology including atherosclerosis. Figure 2. 6B shows that the Genipin eliminates the exercise effect on endothelium-dependent vasodilation in the mesentery artery of ApoE KO mice (ApoE KO-EX), which is similar to the ApoE KO mice. This finding suggests that improved endothelial function in the ApoE KO mice by exercise training occurs in a UCP-2 dependent manner in mesenteric arteries of ApoE KO mice. However, further study is required to define the protective role of exercise in UCP-2-mediated vascular dysfunction in atherosclerosis.

A limitation of the current study is the absence of cellular and molecular expressions data of ER stress markers and key factors such as caspase-1, UCP-2, and eNOS affected by atherosclerosis and exercise training. This is mainly due to the small amount of tissue available and long experimental time course. Future studies would be required to more directly test the exercise effect on specific component of ER stress-associated endothelial dysfunction in atherosclerotic mesenteric arteries. Despite the limitation, this study is novel to report how of exercise ameliorate ER stress-mediated mesenteric vascular dysfunction within mesenteric resistance vessels in atherosclerosis.

# Chapter 3: Aerobic exercise training mitigates ER stress-associated coronary vascular dysfunction in atherosclerosis: a potential role of TXNIP/ NLRP3 inflammasome and UCP-2

## 3. 1. ABSTRACT

Endoplasmic reticulum (ER) stress and uncoupling protein-2 (UCP-2) are a key modulator for endothelial dysfunction in atherosclerosis, but the effects of exercise on ER stress and UCP-2 in endothelial dysfunction in atherosclerosis is not yet fully understood. We used 4 groups of mice; wild type (WT), WT with exercise training, running on the treadmill for 12 weeks (WT-EX), apolipoprotein E knockout (ApoE KO) and ApoE KO with exercise training (ApoE KO-EX). We measured endothelium-dependent acetylcholine (ACh)-induced vasodilation using isolated and pressurized coronary arterioles. ACh-induced vasodilation was elicited in the presence of an inhibitor of eNOS and UCP-2 (L-NAME and genipin) and the ER stress inducer (tunicamycin). Immunoblotting was performed to measure the protein expression of ER stress markers, NLRP3 inflammasome signaling, Bax, TXNIP, and UCP-2 in the heart. The expression of $p67_{phox}$ and superoxide were visualized using immunofluorescence and DHE staining in coronary arterioles. NO production was measured by nitrate/nitrite assay.

ACh-induced endothelium-dependent vasodilation was attenuated in coronary arterioles of ApoE KO, but was improved in ApoE KO-EX. L-NAME, tunicamycin, and Genipin significantly attenuated vasodilation in WT, WT-EX and ApoE KO-EX, but not in ApoE

KO. Exercise training reduced the expressions of ER stress (GRP78, p/t-IRE1, p-eIF2α, and CHOP), NLRP3 inflammasome (TXNIP, caspase-1 p20, and IL-1β), Bax, superoxide production, and NADPH oxidase $p67_{phox}$, and increased NO production in ApoE KO-EX mice.

In conclusion, exercise training alleviates endothelial dysfunction in atherosclerotic coronary arterioles through the NOS, UCP-2, and ER stress signaling pathways including TXNIP/NLRP3 inflammasome and oxidative stress.

## 3. 2. INTRODUCTION

Atherosclerosis is a major risk factor for cardiovascular disease and is one of the leading causes of death in the United States and Worldwide. Atherosclerosis has been known for a chronic inflammatory disease of small to large arteries with lipid accumulation in vessel wall [8]. Endothelial dysfunction is considered the initial hallmark in the development and progression of atherosclerosis accompanied by the reduced NO bioavailability and the increase of inflammation, reactive oxygen species (ROS), and apoptosis in atherosclerotic lesion [37].

Emerging evidence reported that endoplasmic reticulum (ER) stress is involved in the initiation and progression of atherosclerosis [175] and is accepted as a potent modulator for endothelial dysfunction by regulating eNOS signaling pathway [176]. ER homeostasis is impaired in hypercholesteremia and hyperlipidemia by increasing the protein folding demand in ER lumen, and then triggers unfolded protein response (UPR) cascades, which activating PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). These altered UPR, called ER stress, can trigger oxidative stress, IRE1-mediated inflammation, and CCAAT-enhancer-binding protein homologous protein (CHOP)-mediated apoptosis signaling pathways [175].

Excessive ROS production directly damages a vascular endothelium and then induces endothelial dysfunction by reducing NO bioavailability in atherosclerosis [14]. Uncoupling protein-2 (UCP2) is as an antioxidant defender to balance mitochondrial-derived ROS production and prevent the development of atherosclerosis [173], as well as, ameliorates endothelial dysfunction by increase of NO bioavailability in metabolic disorder [177].

Recently, it is reported that thioredoxin-interacting protein (TXNIP), which is key factor linking ER stress to oxidative stress and inflammation, and also is associated with endothelial dysfunction and the development of atherosclerosis [114]. The nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing 3 (NLRP3) inflammasome consisted of multimeric protein complex; NLRP3, apoptosis-associated speck-like protein containing (ASC), and caspase-1, which is a pivotal regulator for initial immune response [96] and is activated by TXNIP in response to ER stress [99]. Activation of NLRP3 promotes the maturation and secretion of IL-1β and IL-18 in a caspase-1 dependent manner [178], which implicated in endothelial dysfunction [109, 178] and atherosclerosis pathogenesis [15]. However, little is known about the pathological linkage of ER stress, TXNIP/NLRP3 inflammasome and UCP-2 on coronary vascular dysfunction in atherosclerosis.

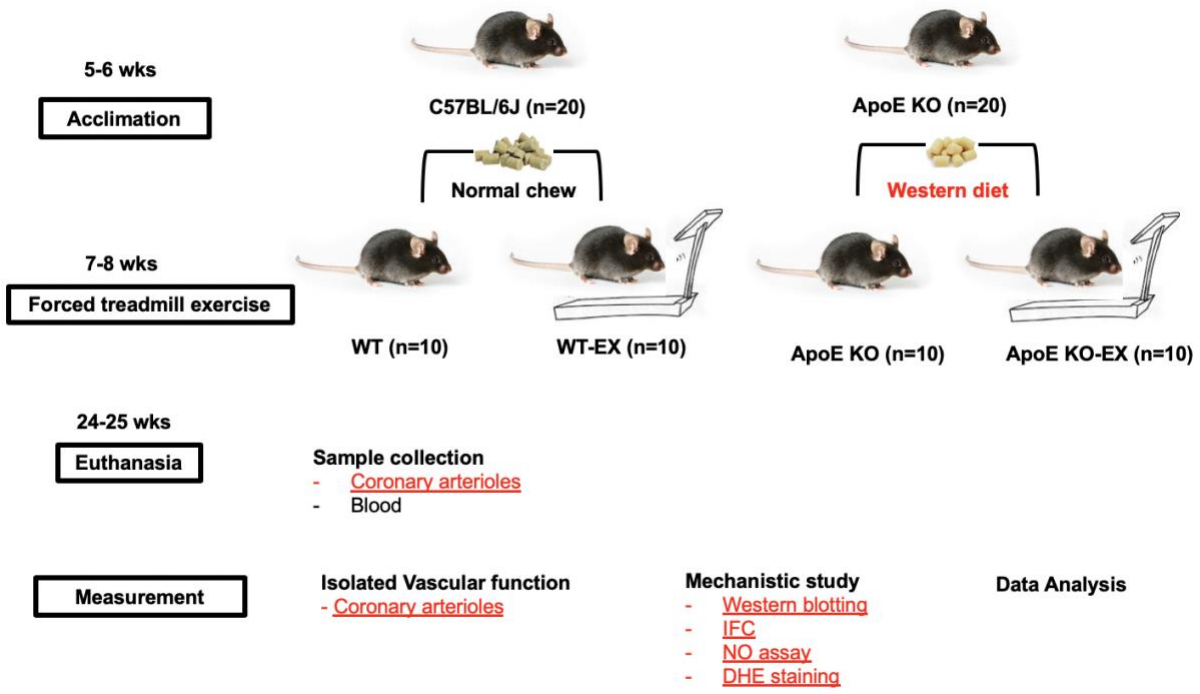
Exercise training has been extensively documented the vasoprotective outcome on endothelial dysfunction, inflammation, and oxidative stress in atherosclerosis [58]. A few studies have reported the effect of exercise training on ER stress-associated endothelial dysfunction with the reduced expression of ER stress markers in aorta and mesenteric arteries of diabetes [150] and atherosclerosis [151]. Also, the positive effect of exercise training on UCP-2-mediated endothelial dysfunction in mesenteric artery of ApoE KO mice [151] and vascular protective effect of voluntary wheel running on NLRP3 inflammasome-associated vascular dysfunction in obesity [7] have been recently reported. However, there are very limited approaches to determine the beneficial effect of exercise training on ER stress and UCP-2 associated endothelial dysfunction in coronary microcirculation in atherosclerosis.

Therefore, the main purpose of the current study is to investigate the effect of exercise training on ER stress-and UCP-2-associated coronary vascular dysfunction in atherosclerosis

and its underlying mechanisms. To answer these questions, we evaluated whether exercise training ameliorates coronary arterioles dysfunction via regulation of ER stress, TXNIP/NLRP3 inflammasome, and UCP-2 signaling cascade in atherosclerosis.

## 3. 3. METHODS

**Figure 3. 1. Experimental design overview.**




### Animal models

Five weeks of wild type male (WT, C57BL/6 mice) and 6 weeks of apolipoprotein knock out mice (ApoE KO, $ApoE^{tm1Unc}$) were supplied from the Jackson Laboratory (Bar Harbor, ME), and were housed in an animal holding facility with controlled temperature (22-23°C) with an alternating 12 hours light/dark cycles, and allowed free access to water and chow. WT and ApoE KO mice were randomly divided into exercise training (EX) or sedentary groups. WT and WT-EX mice were fed a normal chow and ApoE KO and ApoE KO-EX mice were fed a western atherogenic diet (0.2% cholesterol, 42% Kcal from fat, TD.88137, ENVIGO) for 17 wks to accelerate atherosclerosis development in the ApoE KO model. All

EX and sedentary animals were sacrificed at the age of 24-25 wks old. The procedure was performed in accordance with the approved guidelines by the Institutional Animal Care and Use Committee at the University of Houston (14-009).

**Exercise training protocols**

The exercise training protocol was previously described in detail [151]. Briefly, WT-EX and ApoE KO-EX mice were acclimated to run on a motorized rodent treadmill (Columbus Instruments, Columbus, OH) prior to exercise training for 1 week, and were initiated exercise training at 7-8 wks of age. The exercise training protocol consisted of up of 1 hour of running on a rodent motor-driven treadmill at 15m/min at a 5% grade (60-80% of $VO_2$ max), 5 days/week for 15-16 wks. The daily programmed exercise protocol consisted of warm-up (5 min), running (50 min), and cool-down (5 min). Sedentary groups of mice were housed in the same room and under the same conditions, but without the exercise training. At 24-25 wks of age, mice were sacrificed within 24 hrs after the final training day.

**Functional assessment of isolated mesenteric arteries of ApoE KO mice**

After euthanasia, the heart was removed and rapidly placed with cold (4°C) physiological saline solution (PSS). Each isolated coronary arteriole (60-100 μm in internal diameter and 0.5-1.0 mm in length) was cannulated with glass micropipettes filled with PSS-albumin solution, and secured with surgical nylon sutures. The chamber was transferred to a stage of an inverted microscope (Nikon Eclipse Ti-S) and the cannulated arteriole was pressurized to 60cm$H_2O$ intraluminal pressure without the flow. After developing a stable basal tone (1 hr), the vasodilation function was tested by different stimuli, as previously

described [179]. The dose–dependent-diameter changes were established with endothelium-dependent vasodilator, ACh (0.1 nmol/L to 10 µmol/L) in coronary arterioles from WT, WT-EX, ApoE KO, and ApoE KO-EX. Tunicamycin (ER stress inducer, TM, 10 µmol/L), and genipin (UCP-2 inhibitor, 10 µmol/L) were each incubated with the isolated vessel for 20-30 min before measuring ACh-induced endothelium-dependent vasodilation. The contribution of NO to vasodilation was assessed by incubating the vessels with the *N*G-nitro-L-arginine methyl ester (L-NAME; NO synthases inhibitor; 10 µmol/L, 20 min). TM was dissolved in 0.1% DMSO and all other drugs were dissolved in PSS and then extraluminally administrated into the PSS in the chamber before measuring the vascular function. At the end of each experiment, endothelium-independent vasodilator, sodium nitroprusside (SNP, 0.1 nmol/L to 10 µmol/L) was applied to the vessel to obtain smooth muscle-dependent vasodilation.

**Cardiomyocyte cross-sectional analysis**

Membrane specific dye, wheat germ agglutinin (WGA, W11262, Thermo Fischer Scientific, Waltham, MA) was used to test ApoE KO induced myopathies as described elsewhere[180]. Frozen left ventricle was cut 10 µm thickness and fix in cooled acetone (-20ºC) for an hour. The slides were then washed three times in PBS for 5 minutes and incubated with WGA for 10 minutes at room temperature. Section was washed and mounted using ProLong gold antifade mounting media. Sections were visualized with fluorescence microscope (Aixiophot2; Carl Zeiss, Thornwood, NY) using 16x objective (NeoFluar, NA: 0.5). Cross-sectional area (CSA) was obtained by tracing the outlines using NIH-Image J (NIH, Bethesda, MD, USA) of minimum 150-200 random cardiomyocytes from 5 different fields of view from two different animals per groups.

**Measurement of intracellular superoxide ($O_2^-$)**

To evaluate the production of superoxide in coronary arterioles, the oxidative fluorescent dye dihydroethidium (DHE) was used. Frozen cardiac tissue embedded in OCT compound was sectioned at 10 μm thick at -25°C using cryostat (Leica CM 1950, Leica Biosystems Inc., Buffalo Grove, IL), the sections were placed on microscope slides, and the slides were dried at room temperature (RT) for 15 min. The slides in PBS were incubated in bacteria incubator at 37°C with 95% $O_2$ and 5% $CO_2$ for 1 hr, and then added 5μm DHE and incubated for 15 mins at RT in dark room. The slides were washed 3 times in cold PBS (each 5 min) and were mounted with a cover slide in the darkroom. DHE stained images were visualized by an Olympus BX51 fluorescence microscope and acquired with 20X objective lens at an excitation peak of 545nm with an emission spectral peak of 610nm. Fluorescence intensity was measured by Image J.

**Measurement of total nitrate/nitrite concentration in heart tissue**

Myocardial nitrate/nitrite concentration was estimated using a Colorimetric Assay Kit (78001, Cayman Chemical, Ahn Arbor, MI), as per the manufacturer's instruction. To determine the total nitrate/nitrite level in the supernatant solution, the samples were duplicated and measured by a colorimetric microplate reader at 540nm**.**

**Immunoblotting**

Heart tissue was homogenized and lysed in RIPA buffer with protease/phosphatase inhibitor cocktails, and then centrifuged 15,000 *g* for 20 mins at 4°C and protein concentration

was measured by BCA assay. Proteins from brain and cell lysates (30μg per samples) were resolved by Tris-glycine SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membrane. Subsequently, the membrane was blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (0.05% TBST) for 30 mins at room temperature (RT) and incubated overnight with respective primary antibodies. The primary antibodies were purchased from the following sources: TXNIP (Santa Cruz; sc-271237), NLRP3 (Abcam; ab4207), caspase-1 (Abcam; ab108362), IL-1β (Abcam; ab9722), p-IRE1 (Abcam; ab48187), IRE1 (Abcam; ab37073), p-eIF2α (Invitrogen; 44728G), eIF2α (Cell Signaling; 9722), CHOP (Invitrogen; MA1-250), Bax (Cell Signaling; 2772), UCP-2 (Santa Cruz; sc-390189), and GAPDH (Cell Signaling; sc47778). The membrane was then washed three times in TBST and incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies for two hours at RT followed by washing three times with TBST. The blot bands were developed with enhanced chemiluminescence (Thermo Fisher Scientific Inc., MA, USA) for visualization, detected by ChemiDoc™ MP imager (Bio-Rad, CA), and quantified using NIH ImageJ software for densitometric analysis. Protein expression was normalized to the internal control, GAPDH.

**Immunofluorescence**

Immunofluorescence experiments were performed as previously described with some modifications [7, 181], to determine $p67_{phox}$ localization on the endothelium of coronary arterioles with diameters ranging from 50 to 100 μm. In brief, the freshly frozen heart was sectioned at 10 μm. After 1 h acetone permeabilization, slides were incubated with a blocking solution (10% goat serum in PBS) for 30 min at room temperature after three PBS washes. A

mouse on mouse (M.O.M., Vector Laboratories, Burlingame, CA) kit was used to prevent non-specific binding between testing tissue and an antibody from mouse host. The sections were then incubated for 1 h (room temperature) in blocking buffer with primary antibodies, including PECAM1 (1:100, Abcam, Cambridge, MA, #ab32457) and anti-$p67_{phox}$ (1:100, BD Biosciences, San Jose, CA #610913). After three washes in PBS, sections were incubated with the fluorescence-conjugated secondary antibody, Alexa Fluour® 488 and Alexa Fluor® 594 (Thermo Fischer Scientific, Waltham, MA) and mounted with ProloLong antifade solution. Negative controls were performed with the use of mouse $IgG_{2b}$ and rabbit IgG (Santa Cruz Biotechnology, Dallas, TX). Images were taken with an Olympus BX41 fluorescence microscope, using 40x objective (Uplan, NA: 1.3). Microscope and camera settings were kept consistent throughout the process for obtaining image data for all group of samples.

**Data analysis**

All diameter changes to pharmacological agonists were normalized to the control diameter. Normalized diameters were averaged at each concentration of agonist used and shown as mean ± SEM. Statistical comparisons of vasoreactivity responses between groups were performed with two-way analysis of variance (ANOVA) for repeated measure and intergroup differences were tested with Fisher's protected LSD test. The significance of intergroup differences observed in body weight, vessel diameters, and protein expression were analyzed with one-way analysis of variance (one-way ANOVA) using software SPSS 22.0. Significance was accepted at $p < 0.05$.

## 3. 4. RESULTS

***Animal and vascular characteristics.***

The animal set for this study was same as our previous study [151]. The data from our previous study showed that the initial body weight at 7-8 wks of age was not different among the groups, but it was significantly elevated in ApoE KO and ApoE KO-EX groups at 24-25 wks of age [151] (Table 2. 1). However, initial and maximal intraluminal diameters of coronary arterioles were not significantly different in WT and atherosclerotic ApoE KO vessels and exercise training did not affect both diameter changes (Table 3. 1).

**Table 3. 1. Vessel characteristics (Coronary arterioles).**

| | Sedentary | | Exercise | |
|---|---|---|---|---|
| | **WT** | **ApoE KO** | **WT-EX** | **ApoE KO-EX** |
| **N** | 10 | 9 | 6 | 7 |
| **Coronary arterioles initial lumen diameter, µm** | 81.88 ± 6.04 | 75.70 ± 11.33 | 95.53 ± 6.70 | 91.78 ± 8.24 |
| **Coronary arterioles maximal lumen diameter, µm** | 125.89 ± 11.31 | 116.99 ± 8.87 | 126.14 ± 7.68 | 119.79 ± 7.79 |

The initial lumen and maximal lumen of coronary arterioles were measured at 24-25 wks of age.

***Exercise training prevents pathological myocardial hypertrophy in heart of ApoE KO mice.***

To test whether exercise training inhibits pathological myocardial hypertrophy induced by atherosclerosis, the cross-sectional area (CSA) was analyzed for each animal. The cardiomyocyte CSA was significantly increased in the heart of ApoE KO mice compared with WT mice ($p < 0.05$; Fig. 3. 2A & B). However, exercise training reduced the cardiomyocyte

CSA in the heart of ApoE KO-EX mice compared with ApoE KO mice ($p < 0.05$; Fig. 3. 2A & B). These results suggest that atherosclerosis-induced pathological cardiac hypertrophy is prevented by exercise training in ApoE KO mice.

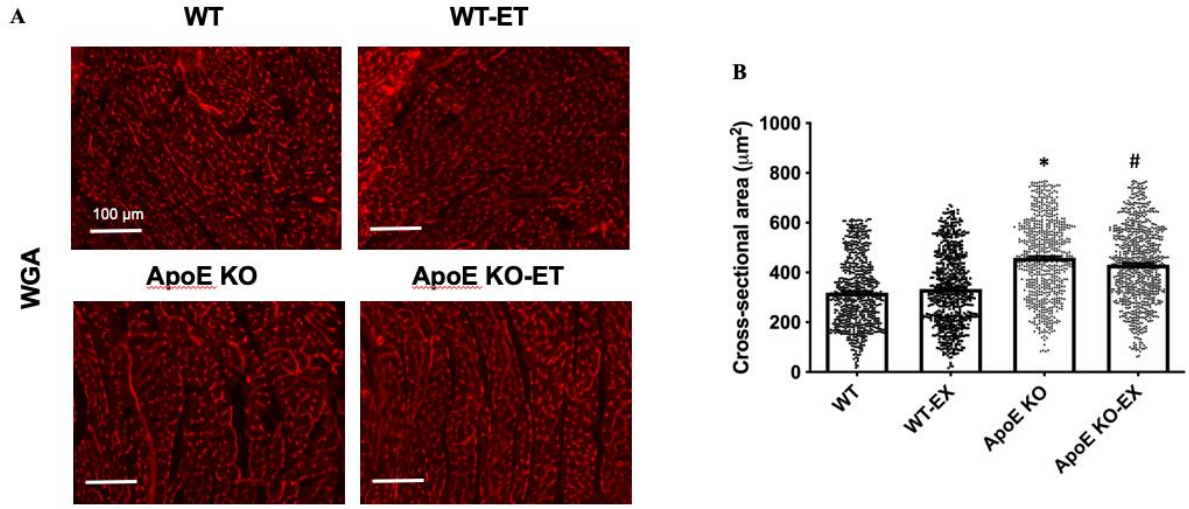



**Figure 3. 2. Effect of exercise training on cardiomyocyte cross sectional area in the heart of ApoE KO mice.**

**A:** Representative WGA staining for cardiomyocyte cross sectional area from each group. B: Quantitative analysis of cardiomyocyte cross sectional area (n=2, 150-200 random cardiomyocytes from 5 different fields of views from each mice). Values are means ± SEM. $^{*}p<0.05$ vs. WT, $^{\#}p<0.05$ vs. ApoE KO.

***Exercise training ameliorates endothelial dysfunction via increase of NO bioavailability in coronary arterioles of ApoE KO mice.***

ACh-induced endothelium-dependent vasodilation was significantly attenuated in coronary arterioles in ApoE KO mice compared with WT mice ($p < 0.05$; Fig. 3. 3A). However, aerobic exercise training significantly improved ACh-induced vasodilation in coronary

arterioles of ApoE KO-EX mice compared to ApoE KO mice ($p < 0.05$; Fig. 3. 3A). To confirm whether the impaired coronary vascular function is endothelium-dependent or not, SNP-induced endothelium-independent relaxation was examined in coronary arterioles. Fig. 3. 3B showed that SNP-induced vasodilatory response was identical in coronary arterioles of all mice groups ($p > 0.05$).

Treatment of L-NAME on isolated coronary arterioles significantly attenuated vasodilation in response to ACh in the WT and ApoE KO-EX mice ($p < 0.05$), but not in ApoE KO mice (Fig. 3. 3C & D). Furthermore, the absolute concentration of total nitrate/nitrite level was significantly reduced in the heart of ApoE KO compared with WT ($p < 0.05$), but its expression was significantly elevated by exercise training in the heart of ApoE KO-EX mice compared with ApoE KO mice (Fig. 3. 3E). These data suggest that exercise training improves ACh-induced endothelial dysfunction in coronary arterioles through NO-dependent manner.

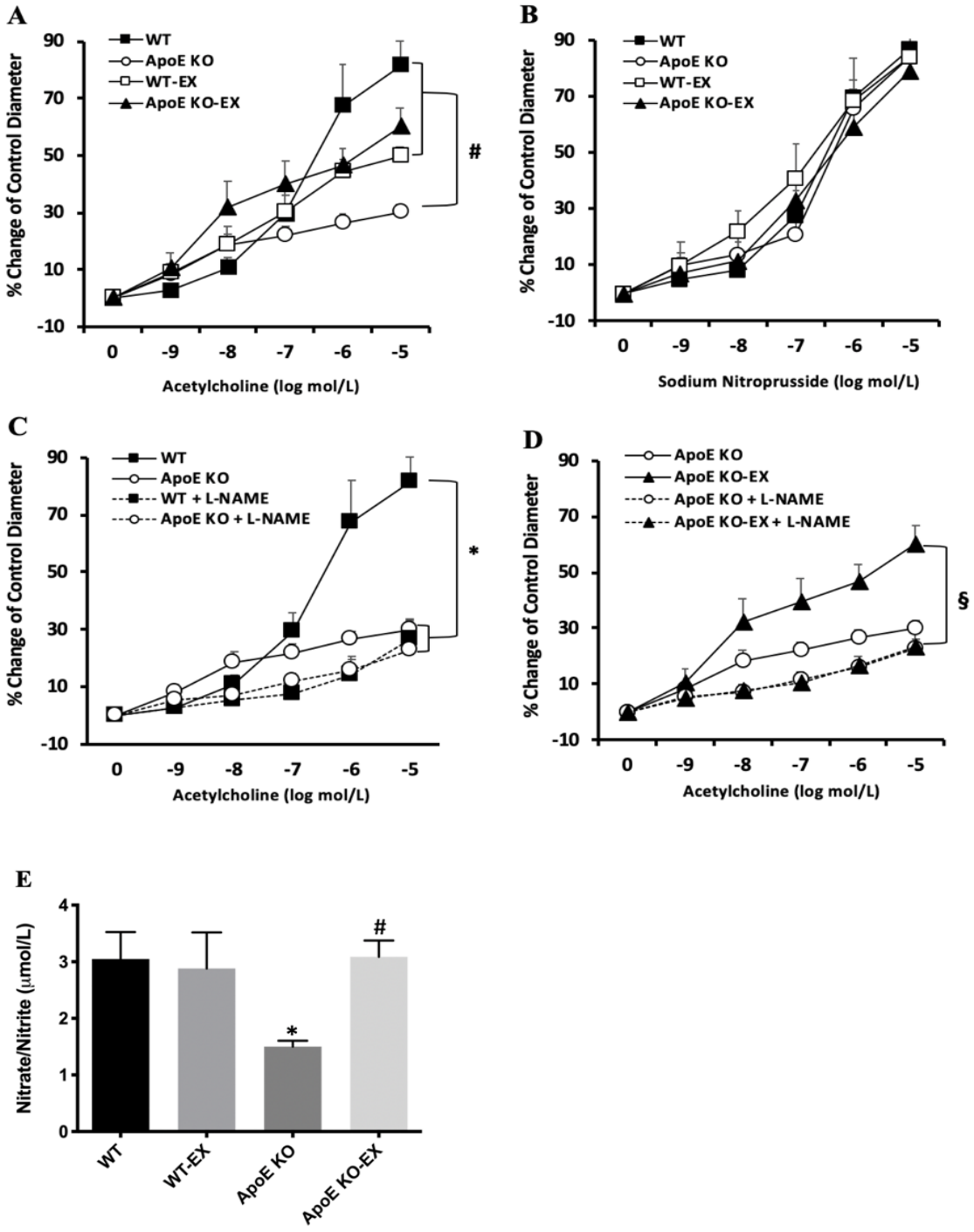



**Figure 3. 3. Effect of exercise training on endothelial dysfunction and NO production in ApoE KO mice.**

**A**: Isolated coronary arterioles from WT (n=4), WT-EX (n=4), ApoE KO (n=5), and ApoE KO-EX (n=4) were measured the response of ACh in a dose-dependent manner. **B**: Endothelium-independent vasodilation was measured in isolated coronary arterioles from WT+SNP (n=5), WT-EX+SNP (n=4), ApoE KO+SNP (n=5), ApoE KO-EX (n=4). **C & D:** ACh-induced vasodilation in coronary arterioles was measured in the presence of L-NAME, the eNOS inhibitor, from WT+L-NAME (n=4), WT-EX (n=4), ApoE KO+L-NAME (n=5), and ApoE KO-EX (n=4). **E**: Total nitrate/nitrite concentration was analyzed in mice heart from each group (n=4-5/groups). Values are means ± SEM. $^{*}p<0.05$ vs. WT, $^{\dagger}p<0.05$ vs. WT-EX, $^{\#}p<0.05$ vs. ApoE KO.

***Exercise training alleviates ER stress-mediated coronary vascular dysfunction in ApoE KO mice***.

Fig. 3. 4A & B showed that incubation of ER stress inducer (Tunicamycin) significantly impaired ACh-induced vasodilation in coronary arterioles in both WT and ApoE KO-EX mice, whereas it had no effect on coronary arterioles in ApoE KO mice ($p < 0.05$). Likewise, the expression of ER stress markers, including GRP78, p/t-IRE1, p/t-eIF2$\alpha$, and CHOP were significantly increased in the heart of ApoE KO mice compared with WT mice ($p < 0.05$; Fig. 3. 4C-H), but up-regulated expression of these ER stress markers were significantly reduced by exercise training in the heart of ApoE KO-EX mice ($p < 0.05$; Fig. 3. 4C-H). Thus, elevated the expression of pro-apoptotic marker, Bax, in the heart of ApoE KO mice was also significantly diminished by exercise training ($p < 0.05$; Fig. 3. 4I).

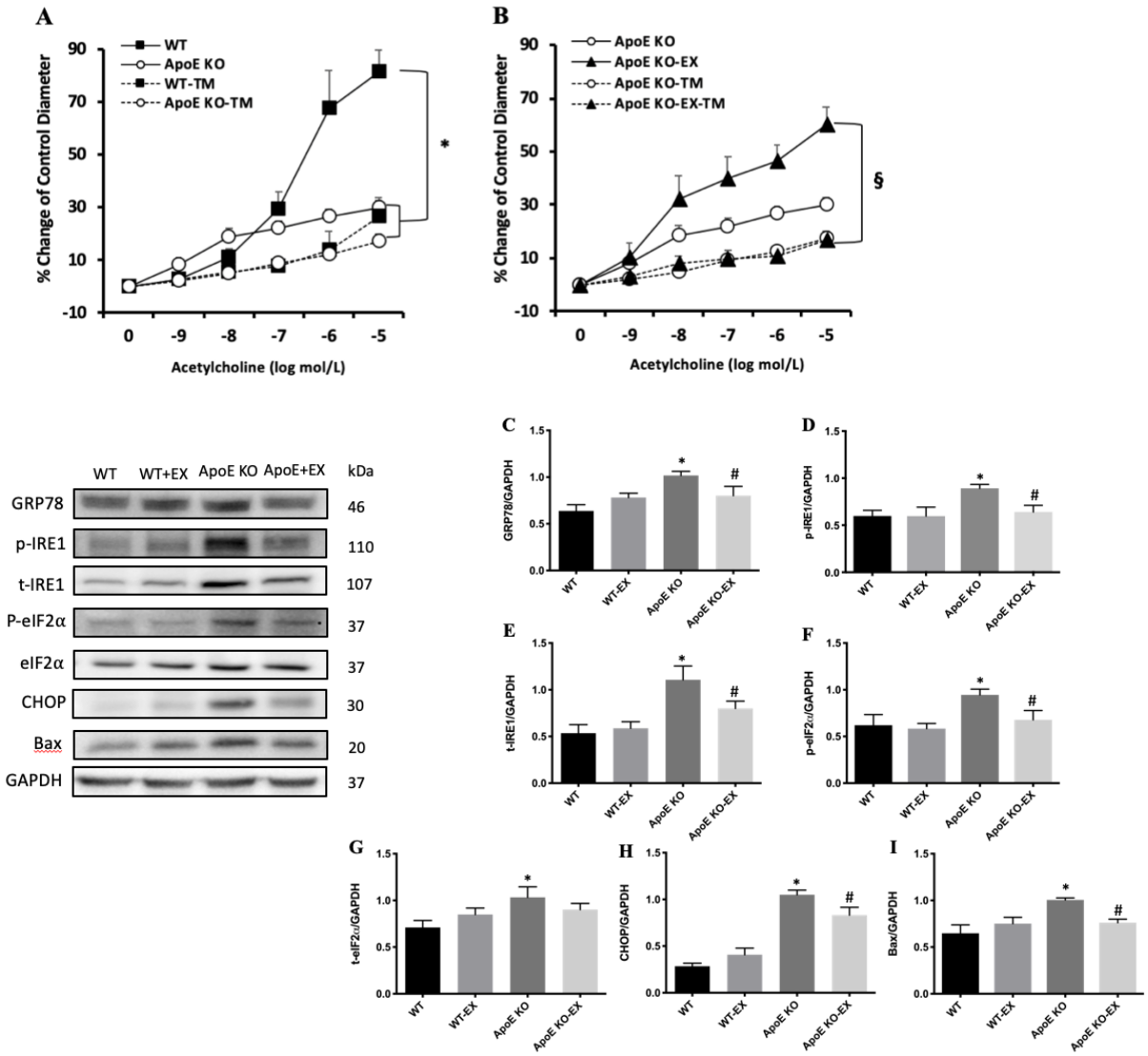



**Figure 3. 4. Effect of exercise training on ER stress-associated endothelial dysfunction in coronary arterioles and the expression of ER stress and apoptosis in the heart of ApoE KO mice.**

**A & B**: ACh-induced vasodilation of isolated coronary arterioles from WT+TM (n=4), WT-EX+TM (n=4), ApoE KO+TM (n=5), and ApoE KO-EX+TM (n=4) were measured in the presence of ER stress inducer (tunicamycin). **C-I**: Protein expression of ER stress markers; GRP78 (C), p-IRE1 (D), t-IRE1 (E), p-eIF2α (F), t-eIF2α (G), and CHOP (H) and pro-

apoptotic marker, Bax (I) were measured by western blotting (n=5-8/groups). Values are means ± SEM. *$p$<0.05 vs. WT, #$p$<0.05 vs. ApoE KO.

***Exercise training reduces the expression of TXNIP/NLRP3 inflammasome in the heart of ApoE KO mice.***

Chronic vascular inflammation is one of the prominent factors inducing endothelial dysfunction in atherosclerosis [182]. The expression of TXNIP, NLRP3, pro-caspase-1, caspase-1 p20, and IL-1β were significantly elevated in the heart of ApoE KO mice compared with WT mice, and exercise training drastically reduced the expression of TXNIP, caspase-1 p20, and IL-1β expression in the heart of ApoE KO-EX mice ($p$ < 0.05; Fig. 3. 5A-E).

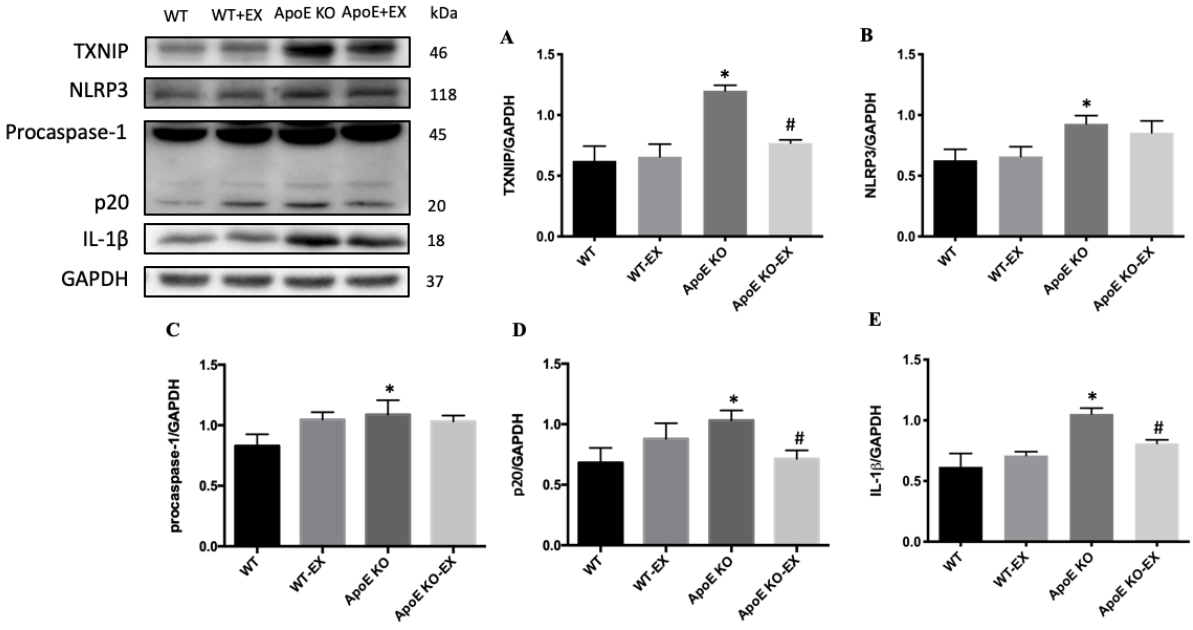



**Figure 3. 5. Effect of exercise training on TXNIP/NLRP3 inflammasome in the heart of ApoE KO mice.**

**A-E**: The protein expression of TXNIP (A), NLRP3 (B), pro-caspase-1 (C), caspase-1 p20 (D), and IL-1β (E) were measured by immunoblot analysis from each group (n=3-6/groups). Values are means ± SEM. $^{*}p< 0.05$ vs. WT, $^{\#}p<0.05$ vs. ApoE KO.

***Exercise training mitigates UCP-2-mediated endothelial dysfunction in coronary arterioles and oxidative stress in heart of ApoE KO mice***.

To investigate the critical role of oxidative stress, especially mitochondria-derived ROS generation, in coronary vascular dysfunction in atherosclerosis, isolated coronary arterioles were incubated with genipin (UCP-2 inhibitor) and measured vasodilatory function. Fig. 3. 6A & B showed that ACh-induced vasodilation was significantly attenuated in genipin-incubated coronary arterioles in WT and ApoE KO-EX, but no further impairment was shown in coronary arterioles in ApoE KO mice. Thus, protein expression of UCP-2 was significantly reduced in the heart of ApoE KO mice compared to WT mice, but its expression was increased by exercise training ($p < 0.05$; Fig. 3. 6C).

To establish the cellular/molecular mechanisms of ROS involved in endothelial dysfunction in coronary arterioles of ApoE KO mice, the production of superoxide ($O_2^-$) and NADPH oxidase expression in coronary arterioles were measured. The $O_2^-$ production was highly increased in coronary arterioles of ApoE KO heart compared to WT, but was diminished by exercise training shown by DHE staining ($p < 0.05$; Fig. 3. 6D & E). NADPH oxidase is the major source of superoxide generation, our immunofluorescence image demonstrated that NADPH oxidase subunit $p67_{phox}$ was abundantly expressed in endothelial cells of coronary arterioles in ApoE KO mice, but its expression appeared to be reduced by exercise training in ApoE KO-EX (Fig. 3. 6F). Our current findings imply that elevated ROS in atherosclerosis

might be implicated in coronary endothelial dysfunction in ApoE KO mice, but exercise training improves it via down-regulation of ROS generation in coronary arterioles in ApoE KO-EX mice.

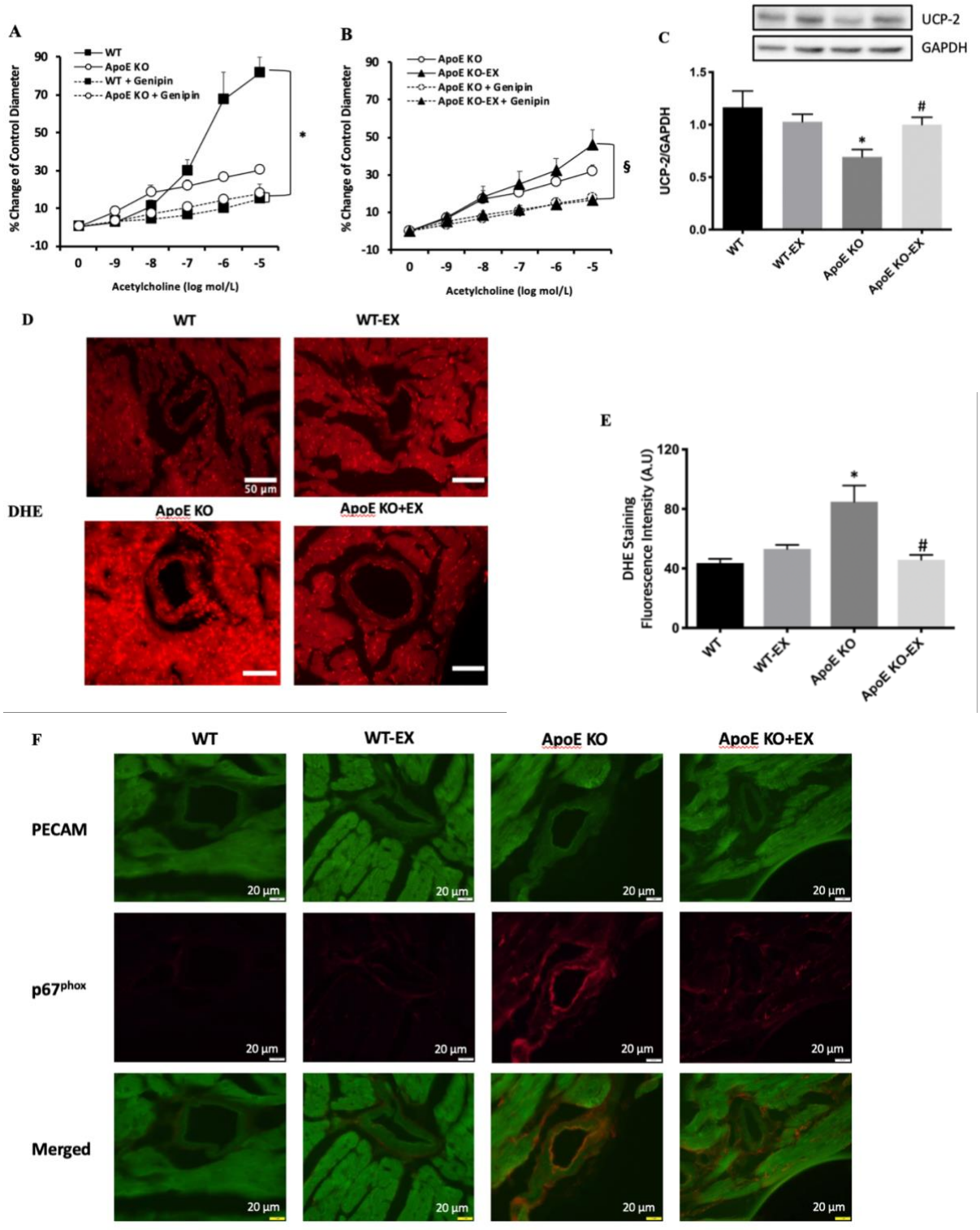



**Figure 3. 6. Effect of exercise training on UCP-2 mediated endothelial dysfunction in coronary arterioles and oxidative stress in the heart of ApoE KO mice.**

**A & B**: ACh-induced vasodilation of isolated coronary arterioles from WT+genipin (n=4), WT-EX+genipin (n=4), ApoE KO+genipin (n=5) and ApoE KO-EX+genipin (n=4) were measured in the present of UCP-2 inhibitor (genipin). **C**: The protein expression of UCP-2 was measured by immunoblot analysis from each group (n=4/groups). **D.** Representative images of DHE staining from each group. **E:** Quantified DHE intensity from four independent experiments using the different samples (n=4/groups). **F**: Immunofluorescence image of NADPH oxidase subunit p67phox in the coronary arterioles. Values are means ± SEM. $*p<0.05$ vs. WT, $\#p<0.05$ vs. ApoE KO.

## 3. 5. DISCUSSION

The major findings of this study are 1) exercise training prevented pathological myocardial hypertrophy, 2) exercise training improved endothelial dysfunction in coronary arterioles of ApoE KO mice through NO-dependent signaling pathways, 3) exercise training ameliorated endothelial dysfunction in coronary arterioles in ApoE KO mice with subsequent reduction of ER stress signaling cascade, 4) exercise training suppressed the expression of TXNIP/NLRP3 inflammasome signaling pathway in the heart of ApoE KO mice, 5) exercise training rescued UCP-2-mediated endothelial dysfunction in coronary arterioles of ApoE KO via down-regulation of ROS and up-regulation of UCP-2 expressions.

Exercise training has been extensively implicated in atherosclerosis as a therapeutic strategy to prevent the progression of atherosclerosis [62, 165], protect pathological cardiac hypertrophy [25], and ameliorate of endothelial dysfunction by an increase in NO bioavailability [26, 166]. Previous studies have suggested that exercise training antagonized pathological cardiac hypertrophy by reducing left ventricle (LV) thickness, myocardial CSA, and the ratio of heart/body weight in cardiovascular disease [183, 184]. The current study confirms the direct cardioprotective effect of exercise training on pathological cardiac hypertrophy in atherosclerosis with a reduction in volume overload-induced pathological myocardial hypertrophy (Fig 2. 2A & B) and the heart/body weight (Table 2. 1) in ApoE KO mice. More importantly, our results showed that exercise training improved the ACh-induced endothelial dysfunction in coronary arterioles of ApoE KO mice (Fig. 2. 3A-E) through endothelium-dependent NO signaling pathways. These NO-mediated beneficial effects of

exercise on the vascular dysfunction in atherosclerosis have been well-matched with a number of previous studies [26, 166, 185].

It has been well defined that ER stress is a crucial factor for modulating endothelial dysfunction in atherosclerosis [16] and exercise training has been implicated as a therapeutic intervention to reduce the ER stress burden in diverse disease models [32]. Our study results demonstrated that exercise training effectively suppressed all elevated ER stress markers in the heart of ApoE KO mice (Fig. 3. 4C-H). We then showed that ER stress induction (tunicamycin) directly impaired endothelial function in coronary arterioles of WT and ApoE KO-EX mice, but no further impairment was shown in ApoE KO mice (Fig. 3. 4A & B), which provide solid evidence that exercise training is the protective modulator against ER stress-associated endothelial dysfunction in atherosclerosis. Furthermore, exercise training positively counteracted the expression of CHOP-mediated pro-apoptosis, Bax, in the heart of ApoE KO mice (Fig. 3. 4I), suggesting that exercise training induces cellular survival signal from the stress. The vascular functional studies reported that ER stress induction (tunicamycin) directly impaired ACh-induced vasodilation with reduction of eNOS expression and NO production in tunicamycin-treated mouse aorta [186] and in the mesenteric arterioles of ApoE KO mice [151], but tudca treatment (ER stress inhibitor) improved endothelial dysfunction with elevation of p-eNOS and NO production. Also, exercise training ameliorates endothelial dysfunction with the reduction of ER stress markers; p-eIF2$\alpha$, ATF3/6, and XBP-1, CHOP and JNK, as well as the elevation of NO production in aorta of diabetic [150] and mesenteric arterioles of ApoE KO mice [151], which suggest that inhibition of ER stress is tightly associated with eNOS signaling and exercise training might protect ER stress-associated endothelial dysfunction through the elevation of eNOS signaling. These previous results support our finding, in part,

that exercise training ameliorated ER stress-associated coronary arterioles dysfunction possibly through increased NO bioavailability (Fig. 3. 3E).

Compelling evidence has indicated that ER stress-induced inflammatory response in endothelial cells is mediated through the activation of TXNIP/NLRP3 inflammasome signaling cascade[187, 188]. TXNIP/NLRP3 inflammasome signaling pathway is highly associated with the initiation and progression of atherosclerosis [103, 189] and is directly linked to endothelial dysfunction by activation of caspase-1, IL-1β, and IL-18 in endothelium [108, 190, 191]. Previous studies reported that the protein expression of TXNIP, NLRP3, pro-caspase-1, IL-1β are increased in ox-LDL-treated ECs [108] and ER stress-induced ECs [190, 191], which aligned with our findings. The protein expressions of TXNIP, NLRP3, active caspase-1 (p20), pro-caspase-1, and IL-1β were significantly elevated (Fig. 3. 5A-E) with ER stress markers (Fig. 3. 4C-H) in the heart of ApoE KO mice, suggesting that ER stress-induced TXNIP/NLRP3 inflammasome signaling pathways might impair coronary endothelial function in ApoE KO mice. Furthermore, exercise training has been well-known effective intervention to ameliorate inflammation-induced vascular dysfunction by promoting anti-inflammatory milieu in cardiovascular disease [192]. However, very limited evidence is currently available to establish the effect of exercise training on TXNIP/NLRP3 inflammasome signaling pathways in atherosclerosis. Voluntary wheel running reduces the expression of caspase-1 and IL-1β in the heart of HFD-induced obese mice model [7]. Here, we also showed that exercise training decreased the elevated expressions of TXNIP and of NLRP3 inflammasome signalings (caspase-1 p20, IL-1β) (Fig. 3. 5A-E), and elevation of NO production (Fig. 3. 3E) in the heart of ApoE KO-EX compared with ApoE KO. Blocking of NLRP3 increases the reduced eNOS expression and NO production by showing diminished expression of NLRP3, caspase-1, and

IL-1β in coronary arterioles of hypercholesterolemia [109] and high fat & glucose-exposed HUVECs [110], suggesting that the alleviation of TXNIP/NLRP3 inflammasome by exercise training might be the key sequence to improves coronary endothelial dysfunction via elevation of NO bioavailability in ApoE KO mice.

Accumulating evidences have strongly defined the beneficial effect of exercise training on oxidative stress-associated vascular dysfunction in atherosclerosis. Exercise training improves endothelial dysfunction through reduction of superoxide production and increase of anti-oxidant protein expression, which consequently elevates NO bioavailability. UCP-2 is one of the major antioxidant proteins dampening the mitochondria-derived ROS production[5], which regulates the initiation and progression of atherosclerosis [173] and endothelial dysfunction [14, 89, 193]. Previous studies reported the positive effect of exercise training on the regulation of UCP-2 expression showing that exercise training improved UCP-2 mediated endothelial dysfunction in mesenteric arterioles of ApoE KO mice [151] and increased UCP-2 expression in the brain of adults rats [94, 95] and in aorta of aged rats [160]. However, there is lack of approaches to define the beneficial effect of exercise on UCP-2 mediated-vascular dysfunction in cardiovascular disease, including atherosclerosis. Here, we demonstrated that exercise training increased the protein expression of UCP-2 in the heart of ApoE KO-EX mice compared to ApoE KO mice (Fig. 3. 6C). Thus, genipin incubation abolished the improved vasodilation by exercise training in coronary arterioles of ApoE KO-EX, which is comparable with ApoE KO (Fig. 3. 6A & B). These results suggest that exercise training rescues UCP-2 mediated endothelial dysfunction possibly through elevation of UCP-2 expression. Also, our data showed that exercise training reduced the elevated superoxide and NADPH oxidase expression in coronary arterioles of ApoE KO-EX mice with the increase of UCP-2 expression

and NO production in the heart of ApoE KO mice, which suggest that upregulation of UCP-2 might ameliorate endothelial dysfunction in coronary arterioles of ApoE KO mice possibly through regulating UCP-2-derived ROS production and NO bioavailability [89] in atherosclerosis. Furthermore, UCP-2 is tightly associated with the regulation of ER stress, based on the studies showing that UCP-2 inhibitor increases the expression of mitochondrial-specific ROS, NADPH oxidase, and mRNA/protein level of ER stress markers; Bip, IRE1, PERK, ATF6, CHOP with reduction of NO production in MAECs [91] and UCP-2 KO macrophages [174].

In conclusion, our study reinforces that exercise training is the profound effective therapy for the treatment and prevention on ER stress-and UCP-2 associated coronary arterioles dysfunction in atherosclerosis via regulation of inflammation and oxidative stress including TXNIP/NLRP3 inflammasome and UCP-2-mediated ROS generation. However, further mechanical studies are required to establish the causal effect of exercise training on ER stress-mediated TXNIP/NLRP3 inflammasome and UCP-2-mediated endothelial dysfunction in atherosclerosis.

# Chapter 4: Exercise training ameliorates cerebrovascular dysfunction in Alzheimer's disease: a role of P2Y2 receptor and ER stress

## 4. 1. ABSTRACT

Cerebrovascular dysfunction is a critical risk factor for the pathogenesis of Alzheimer's disease (AD). Purinergic P2Y2 receptor and ER (endoplasmic reticulum) stress are tightly associated with vascular dysfunction and pathogenesis of AD. However, the protective effect of exercise training on P2Y2 receptor-and ER stress-associated cerebrovascular dysfunction in AD has been largely unknown. Control (C57BL/6, CON) and AD (APP/PS1, AD) mice were either underwent the treadmill exercise training (EX) or remain sedentary for 10-12 weeks. ATP (2-MeS-ATP)-induced dose-dependent vasoreactivity were determined using pressurized posterior cerebral artery (PCA) at 10-12 months of age. Human brain micro endothelial cells (HBMECs) were exposed to laminar shear stress (LSS) at 20 dyne/cm$_2$ for 30 mins, 2 hrs, and 24 hrs. Western blotting was utilized to analyze the expression of P2Y2 receptors, endothelial nitric oxide synthesis (eNOS), and ER stress signaling. Notably, exercise training reversed ATP-induced vasoconstriction in PCA from AD mice to vasodilation (AD+EX) comparable to CON mice. Exercise training reduced the expression of APP expression and increased the P2Y2 receptor and Akt/eNOS expression in AD mice brain. Also, LSS increased the expression of P2Y2 receptor and eNOS in HBMECS, but these increases were blunted by P2Y2 receptor inhibitor (AR-C) in HBMECs. Exercise training normalized the abnormal expression of ER stress markers; p-IRE1, p/t-eIF2$\alpha$, CHOP, and ER stress-associated apoptosis; Bax and Bcl-2 in AD mice brain. Taken together, the current study provides the first

evidence that exercise training improves the cerebrovascular dysfunction in AD possibly through P2Y2 receptor-and ER stress-dependent endothelial dysfunction.

## 4. 2. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and one of the top leading causes of death in the United States in 2017 [194]. A growing body of recent evidence strongly suggested that cerebral hypoperfusion is observed prior to the onset of AD [195] and the reduced cerebral blood flow (CBF) initiates the accumulation of amyloid-beta (Aβ), which ultimately induces irreversible neuronal injury/loss and the development of cognitive impairment [19, 43, 196]. Furthermore, cerebrovascular amyloid deposition, which called cerebral amyloid angiopathy (CAA), promotes endothelial dysfunction by reduction of eNOS activity and NO bioavailability that reduces cerebral blood flow in AD brain [197, 198]. Evidence suggests that cerebrovascular dysfunction might be a critical implication in AD pathophysiology, but the precise underlying mechanism for cerebrovascular dysfunction-associated AD pathologies is still unclear.

Adenosine triphosphate (ATP) is an essential regulator of vascular tones in cerebral vessels by acting on vascular purinergic receptors. It is triggered and released by hypoxia and shear stress to regulate cerebral blood flow [20]. Extracellular ATP evokes the biphasic response via purinergic receptors; an initial transient constriction through smooth muscle P2X1 receptor and the subsequent dilation via endothelial P2Y2 receptor [20, 199]. Stimulation of P2Y2 receptor releases nitric oxide (NO) [200, 201] through eNOS signaling pathway. Although P2Y2 receptors have been known as a key factor in cerebrovascular function [200, 202, 203] and progression of AD pathogenesis [130, 133], very limited information exists to explain a direct role of P2Y2 receptor-mediated cerebrovascular dysfunction in AD pathology.

ER stress is disturbances of the ER function by cellular stress and is triggered by various pathological factors, such as redox imbalance, Aβ toxicity and/or Aβ-induced calcium release, and hypoxia [12]. Increased accumulation of unfolded and misfolded proteins in ER lumen activates unfolded protein response (UPR) that is regulated by three ER stress-sensors; PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). These UPR signaling pathways modulate the restoration of ER homeostasis [176]. However, prolonged ER stress leads to CCAAT-enhancer-binding protein homologous protein (CHOP)-mediated apoptosis signaling that activates its downstream of anti-apoptotic Bcl-2 family and pro-apoptotic Bax/Bak [32]. Furthermore, it has been reported that ER stress is highly associated with endothelial dysfunction with reduction of NO bioavailability in cardiovascular disease [1, 176] and pathogenesis of AD [139, 145]. However, few investigations are currently available to determine the role of ER stress-associated cerebrovascular dysfunction in AD.

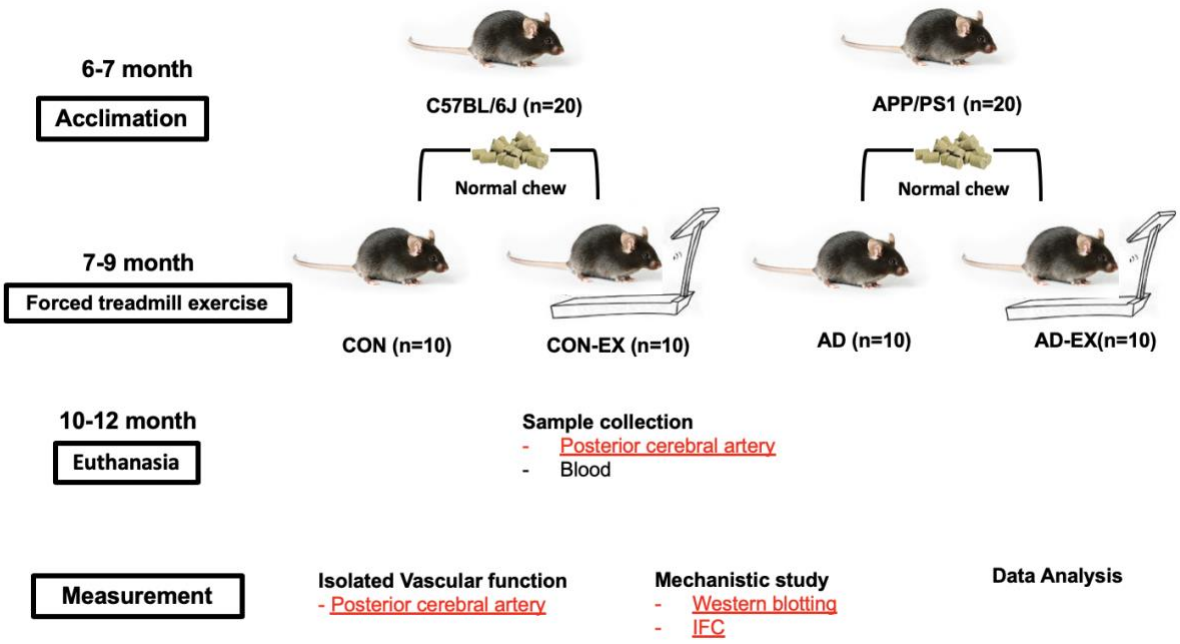
The protective effect of exercise on cardiovascular disease [192] and AD [204] has been widely studied. A large number of clinical studies have reported that exercise training increased CBF and reduced the accumulation of Aβ, neuron cell death, and cognitive decline in AD [44, 48, 205]. Also, elevated shear stress by exercise to the endothelial cells is one of the major mechanisms for the beneficial effect of exercise on cerebrovascular function via promoting angiogenesis, eNOS expression, and NO generation [192, 196]. However, no study has been identified the direct effect of exercise training on P2Y2 receptor-mediated cerebrovascular dysfunction and its downstream pathways in AD. Regular exercise training suppresses the elevated expression of ER stress markers including p-IRE1α, p-eIF2α, and CHOP-mediated apoptosis in AD brain [28], and ameliorates ER stress-mediated endothelial

dysfunction with the elevation of NO bioavailability in the diabetic aorta and mesenteric artery [150], as well as, in atherosclerosis [151]. However, there has been restricted evidence available to establish the linkage between the exercise training and ER stress-associated cerebrovascular dysfunction in AD and its underlying mechanisms.

Therefore, the main objective of this study is to determine the protective effect of exercise training on P2Y2 receptor-mediated and ER stress-associated cerebrovascular dysfunction in AD and its underlying mechanisms. To answer these questions, we evaluated 1) whether ATP-induced cerebrovascular function is impaired in cerebral artery in AD and exercise training reverses ATP-induced cerebrovascular dysfunction via P2Y2 receptor-dependent manner in cerebral artery in AD, and 2) whether ER stress and ER stress-associated apoptosis are elevated in AD brain, and exercise training ameliorates these signaling in AD brain.

## 4. 3. METHODS

**Figure 4. 1. Experimental design overview.**




### Animal models

APP/PS1 double transgenic mice that secrete human amyloid precursor protein (Mo/HuAPP695swe) and mutant human presenilin 1 (PS1-dE9), developing large amyloid deposits and CAA in the brain at 6 months of age. APP/PS1 males (The Jackson Laboratory, ME) were bred with C57BL/6J females (Stock No: 34835-JAX; The Jackson Laboratory, ME). Genotyping for transgenic screening in the offspring was performed using DNA obtained from the post-weaning tail snip at 2-3 weeks of age and confirmed by PCR. Male and female 7-9 month of age-matched wild type control (CON), APP/PS1 mice (AD), CON with exercise training (CON+EX), and APP/PS1 with exercise training (AD+EX) with were randomly assigned to one of four groups (at least10 mice per each group). All mice were housed in an

animal facility with controlled temperature (22-23°C) and 12 hours light/dark cycles, and allowed free access to water and chow. All experiments on animals were performed in accordance with the approved animal care, relevant guidelines, and regulation of the Institutional Animal Care and Use Committee at the University of Houston (14-009).

**Exercise training protocols**

At 7-9 months of age, CON+EX and AD+EX mice were performed exercise training for 10-12 wks on a motorized rodent treadmill (Columbus Instruments, Columbus, OH) and the EX groups of mice were acclimated to run on a treadmill prior to exercise training for 1 week. Each exercise training consisted of up of 1 hour of running on a motor-driven treadmill at 15m/min at a 5% grade, 5 days/week for 10-12 wks, as described previously (60-80% of $VO_2$max) [151]. The exercise protocol consisted up of 5 mins warm-up, 50 mins running, and 5 mins cool-down. Sedentary groups of mice were remained in under the same room and conditions throughout the experiment. At 10-12 months of age, mice were sacrificed within 24 hrs after the final training.

**Functional assessment of isolated posterior cerebral arteries of AD mice**

After euthanasia, the intact posterior cerebral artery (PCA), the main feed artery regulating blood flow to hippocampus [206], was rapidly excised from the brain and placed in a cold dissection chamber (4°C) with cold physiological saline solution (PSS) containing 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.2 mM $NaH_2PO_4$, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1% of BSA at pH 7.4. The isolated PCA was carefully removed the connective tissues from the surrounding artery under

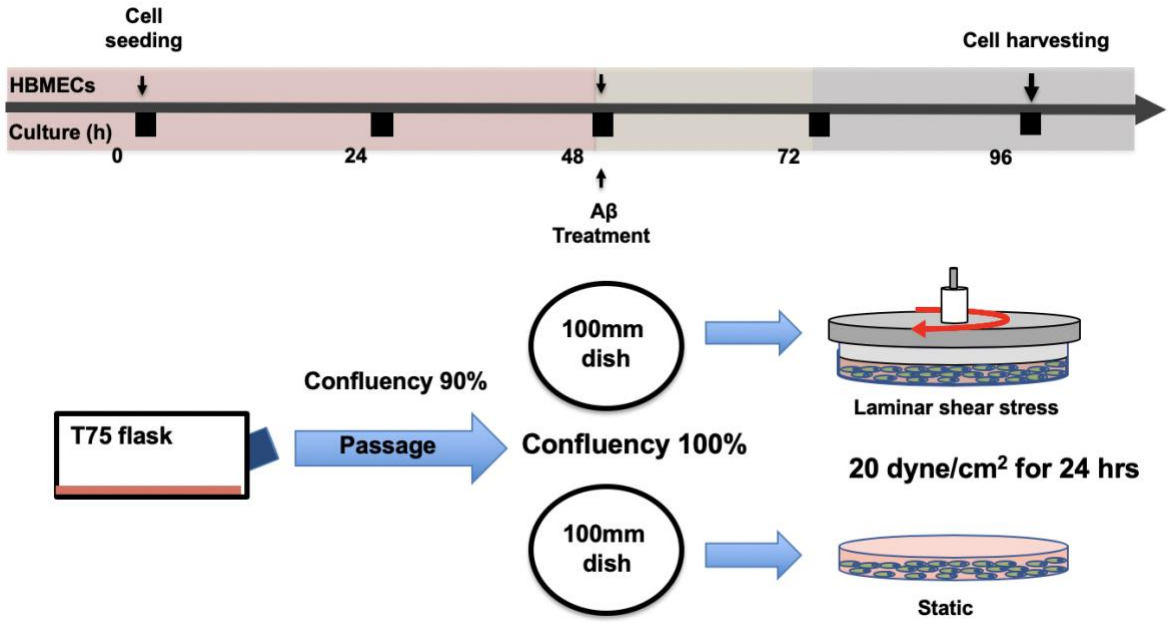
a dissecting microscope (Nikon SMZ1000). A single isolated PCA (100-130 μm in internal diameter and 0.5-1.0 mm in length) was transferred to a Lucite chamber containing PSS, cannulated with glass micropipettes filled with PSS-albumin solution, and secured with surgical nylon sutures. The chamber was transferred to a stage of an inverted microscope (Nikon Eclipse Ti-S), and the cannulated PCA was pressurized to 60cm$H_2O$ intraluminal pressure without altering the intraluminal flow. The temperature was maintained at 36.5°C. Vascular diameter changes were monitored by an inverted microscope with video caliper (Colorado Video, Boulder, CO, USA), as previously described by our laboratory [151]. After equilibrium (1 hr), the isolated PCA was pre-constricted with U46619 (thromboxane A2, 2 μmol/L) and the vasodilation function was established with endothelium-dependent vasodilator, 2-Methylthioadenosine-5'-O-triposphate (2-MeS-ATP; 1 nmol/L to 10 μmol/L) with a dose-dependent manner in PCA from four experiment groups.

**Cell culture and laminar shear stress (LSS) experiments**

Primary cultures of human brain microvascular endothelial cells (HBMECs) and CSC medium kits were purchased from Cell Systems, Inc (Kirkland, WA). The HBMECs were grown in CSC complete medium containing 10% serum, 2% CultureBoost™ (animal-derived growth factor), and 0.2% Bac-Off® antibiotic solution and cultured with 5% $CO_2$ at 37°C in dishes coated with 0.1% gelatin. For shear stress experiment, HBMECs, grown at 90-100% confluence in 100-mm tissue culture dishes, were exposed to unidirectional LSS at 20 dyne/$cm_2$ with various time point for 30 mins, 2hrs, and 24 hrs in shear medium by using cone-and-plate shear system (0.5° cone angle), which was placed inside of 5% $CO_2$ cell incubator. For some experiments, cells were treated with P2Y2 antagonist (AR-C 118925XX, 10μM)

before shear exposure. All experiments with HBMECs were conducted between passages 6-11.

**Figure 4. 2. Experimental overview for cultured HBMECs and LSS.**




## Immunoblotting

Brain tissue and HBMECs were homogenized and lysed in RIPA buffer with protease/phosphatase inhibitor cocktails, and then centrifuged 15,000 *g* for 20 mins at 4°C and protein concentration was measured by BCA assay. Proteins from brain and cell lysates (30µg per samples) were resolved by Tris-glycine SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membrane. Subsequently, the membrane was blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (0.05% TBST) for 30 mins at room temperature (RT) and incubated overnight with respective primary antibodies. The primary antibodies were purchased from the following

sources: p-eNOS (BD Biosciences; 612392), t-eNOS (BD Biosciences; 610296), p-Akt (Cell Signaling; 4060), t-Akt (Cell Signaling; 2920), P2Y2 (Alomone Labs; APR010), p-IRE1 (Abcam; ab48187), IRE1 (Abcam; ab37073), p-eIF2α (Invitrogen; 44728G), eIF2α (Cell Signaling; 9722), CHOP (Santa Cruz; sc575), APP (MillioreSigma; MAB348), Bax (Cell Signaling; 2772), Bcl-2 (Santa Cruz; sc7382), and B-actin (Santa Cruz; sc47778). The membrane was then washed three times in TBST and incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies for two hours at RT followed by washing three times with TBST. The blot bands were developed with enhanced chemiluminescence (Thermo Fisher Scientific Inc., MA, USA) for visualization, detected by ChemiDoc™ MP imager (Bio-Rad, CA), and quantified using NIH ImageJ software for densitometric analysis. Protein expression was normalized to the internal control, B-actin.

**Immunocytochemistry**

After 24 hrs LSS, HBMECs were rinsed three times with ice-cold PBS and fixed in 4% paraformaldehyde in PBS for 15 mins at RT. The fixed cells were washed three times with ice-cold PBS and were blocked with 5% goat serum in 0.03% Triton-X in PBS for 1hr in RT. The rabbit IgG P2Y2 receptor primary antibody, diluted in 5% of goat serum (APR010, 1:300), was incubated for overnight at 4°C. After washing the cells three times with PBS, secondary antibody (Alexa 594-conjugated, 1:500) was incubated for 1hr at RT and washed three times in PBS, and then mounted in mounting media with DAPI (VECTASHIELD antifade mounting medium with DAPI; VECTOR Lab) with cover slide in the darkroom. The images were obtained by 20X and 63X objectives using fluorescence microscopy.

**Microarray analysis**

To gain insight into global expression pattern of the purinergic receptor pathway related genes, microarray gene expression profiling was performed. Total RNA was purified using RNeasy kit (QIAGEN). Microarray analysis were performed from static (0 dynes/cm2, n=4) or laminar (20 dynes/cm2, 24 hrs, n=4) shear stress exposed HUVECs using Affymetrix whole-genome arrays containing 45,101 probe sets corresponding to ≈34,000 genes. Heat map was created with Gene-E ver. 3.0.214 (Broad Institute, Inc).

**Data analysis**

All data are presented as mean ± SEM. Statistical comparisons of vasoreactivity response between groups were performed with two-way analysis of variance (ANOVA) for repeated measure and intergroup differences were tested with Fisher's protected LSD test. The significance of intergroup differences observed in body weight, vessel diameters, and molecular studies were analyzed with one-way analysis of variance (one-way ANOVA) using software SPSS 22.0. Significance was accepted at $p < 0.05$.

## 4. 4. RESULTS

***Animal and vascular characteristics.***

Body weight was measured at 10-12 months of age. There was no difference in the body weight among the groups (Table 4. 1). Maximal intraluminal diameters of PCA were not significantly different in CON and AD vessels with or without exercise training (Table 4. 1). No significant difference was observed in the initial diameter and percentage constriction tone of PCA in all groups (Table 4. 1).

**Table 4. 1. Animal and vessel characteristics (PCA).**

| | Sedentary | | Exercise | |
|---|---|---|---|---|
| | **CON** | **AD** | **CON-EX** | **AD-EX** |
| **Animal Body weight (mg)** | 26.66±1.25 | 26.65±1.26 | 29.19±1.30 | 27.73±1.91 |
| **Posterior cerebral arteries constriction tone, %** | 21.14±4.51 | 14.57±2.75 | 15.07±1.38 | 12.78±2.48 |
| **Posterior cerebral arteries initial lumen diameter, μm** | 113.62±4.49 | 121.96±6.38 | 122.29±0.89 | 128.27±5.40 |
| **Posterior cerebral arteries maximal lumen diameter, μm** | 144.80±5.62 | 142.49±3.43 | 144.11±2.55 | 147.27±6.40 |

Values are mean ± SEM; CON, control mice; CON-EX, control mice with exercise training; AD, APP/PS1 double transgenic mice; AD-EX, APP/PS1 double transgenic mice with exercise training. The number of animals used for the body weight was 10-14 per group, and 4 mice per group was used for measuring the constriction tone, initial lumen, and maximal lumen of mesenteric arteries.

***Exercise training decreases amyloid peptide precursor (APP) expression in APP/PS1 double transgenic mice.***

To demonstrate the effectiveness of exercise training on AD pathogenesis in AD mice brain, we measured the expression of APP. The expression of APP was significantly higher in AD mice compared with CON ($p < 0.05$), but it was significantly reduced by exercise training in AD+EX ($p < 0.05$; Fig. 4. 3). This data suggests that exercise training prevents amyloidogenic processing of APP which associated with the formation of Aβ in AD brain.

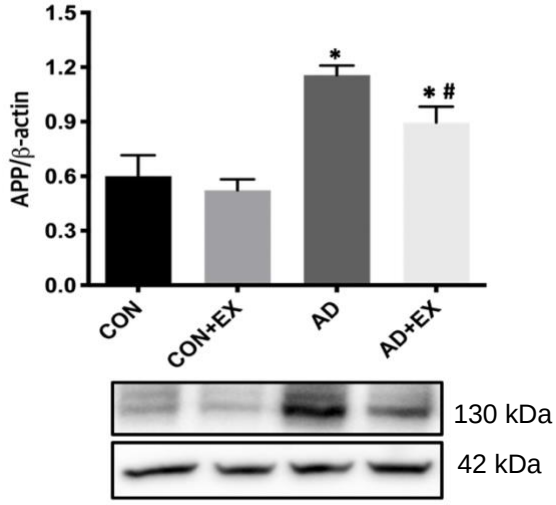



**Figure 4. 3. Effect of exercise training on the expression of APP in APP/PS1 double transgenic mice brain.**

The protein expression of APP was measured by immunoblot analysis. Bar graph presents densitometric analysis of APP protein expression in the brain from each group. Values are mean ± SEM (n=6/group). $^{*}p< 0.05$ vs CON, $^{\#}p<0.05$ vs AD.

***Exercise training reverses ATP-induced cerebrovascular dysfunction in PCA and improves P2Y2 receptor-mediated eNOS signaling pathways in APP/PS1 double transgenic mice.***

To evaluate the beneficial effects of exercise training on 2-MeS-ATP-induced cerebrovascular dysfunction in AD, we first measured 2-MeS-ATP-induced vascular reactivity in the isolated PCA. 2-MeS-ATP induced the vasodilation in PCA from CON mice, but it constricted in PCA from AD mice ($p < 0.05$; Fig. 4. 4A). Notably, 2-MeS-ATP induced the vasodilation in PCA from AD+EX mice showed the similar vasodilatory pattern in PCA from CON mice (Fig. 4. 4A). These results suggest that 2-MeS-ATP-mediated vasoconstriction was revered to vasodilation by exercise training in PCA from AD+EX. To investigate whether the cerebrovascular response of ATP was changed by the alteration of P2Y2 receptor expression, we measure the expression of P2Y2 receptor in the brain. Fig. 4. 4B showed that the expression of P2Y2 receptor was significantly decreased in AD mice brain compared to CON mice brain ($p < 0.05$), but exercise training significantly increased P2Y2 receptor expression in AD+EX mice brain compared to AD mice brain ($p < 0.05$). Taken together, these result findings imply that the alteration of ATP-induced vascular reactivity by AD and exercise training are P2Y2 receptor-dependent manner.

Activation of P2Y2 receptor by ATP produces NO, which is the key factor in regulating cerebrovascular reactivity via Akt/eNOS signaling [203, 207]. Therefore, we measured the protein expression of Akt and eNOS to confirm whether alteration of Akt and eNOS protein expression were associated with 2-MeS-ATP-induced P2Y2 receptor-mediated cerebrovascular dysfunction in AD. We found that phosphorylation of Akt (p-Akt) expression was significantly reduced in AD mice brain compared with CON mice brain ($p < 0.05$; Fig. 4. 5A), but exercise training significantly restored the p-Akt expression in AD+EX mice brain compared with AD mice brain ($p < 0.05$; Fig. 4. 5A). However, there was no statistical difference on the expression of total Akt (t-Akt) among the groups (Fig. 4. 5B). Furthermore,

p-eNOS was significantly decreased in AD mice brain compared with CON mice brain ($p < 0.05$; Fig. 4. 5C), but exercise training prevented the reduced p-eNOS expression in AD+EX mice brain ($p < 0.05$; Fig. 4. 5C). However, there was no statistical difference on t-eNOS expression among groups (Fig. 4. 5D). These results suggest that AD-pathology induces cerebrovascular dysfunction through the impairment of P2Y2/eNOS signaling pathways, but exercise training ameliorated cerebrovascular dysfunction via improvement of P2Y2/eNOS signaling pathways in AD.

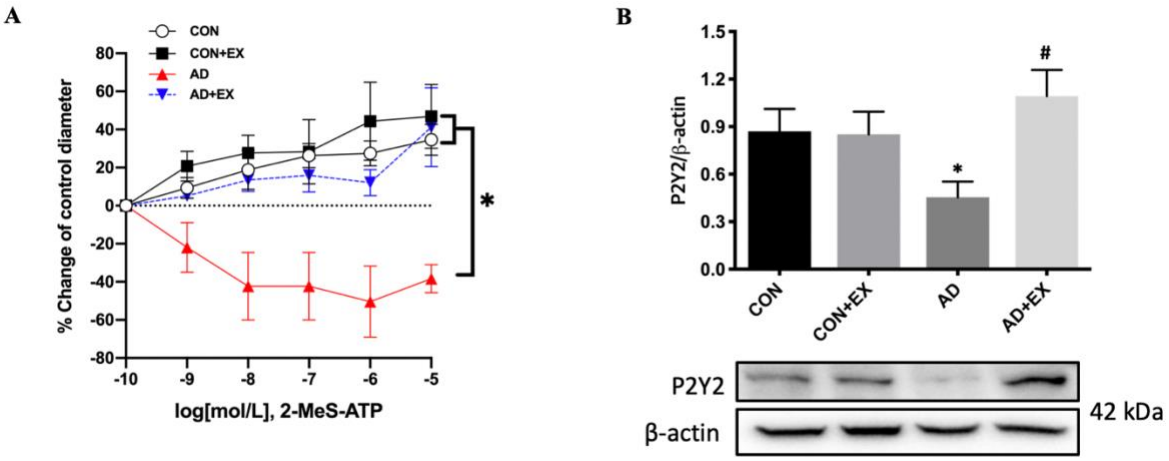



**Figure 4. 4. Effects of exercise training on 2-MeS-ATP-induced vasoreactivities in PCA and on the P2Y2 receptor expression in APP/PS1 double transgenic mice.**

**A**: Using isolated PCA from CON, CON+EX, AD, and AD+EX mice, 2-MeS-ATP-induced vasoreactivies were measured in a dose-dependent manner (n=4/group). **B**: The protein expression of P2Y2 receptor was analyzed by western blotting. Values are mean ± SEM (n=6/group). $*p< 0.05$ vs CON, $\#p<0.05$ vs AD.

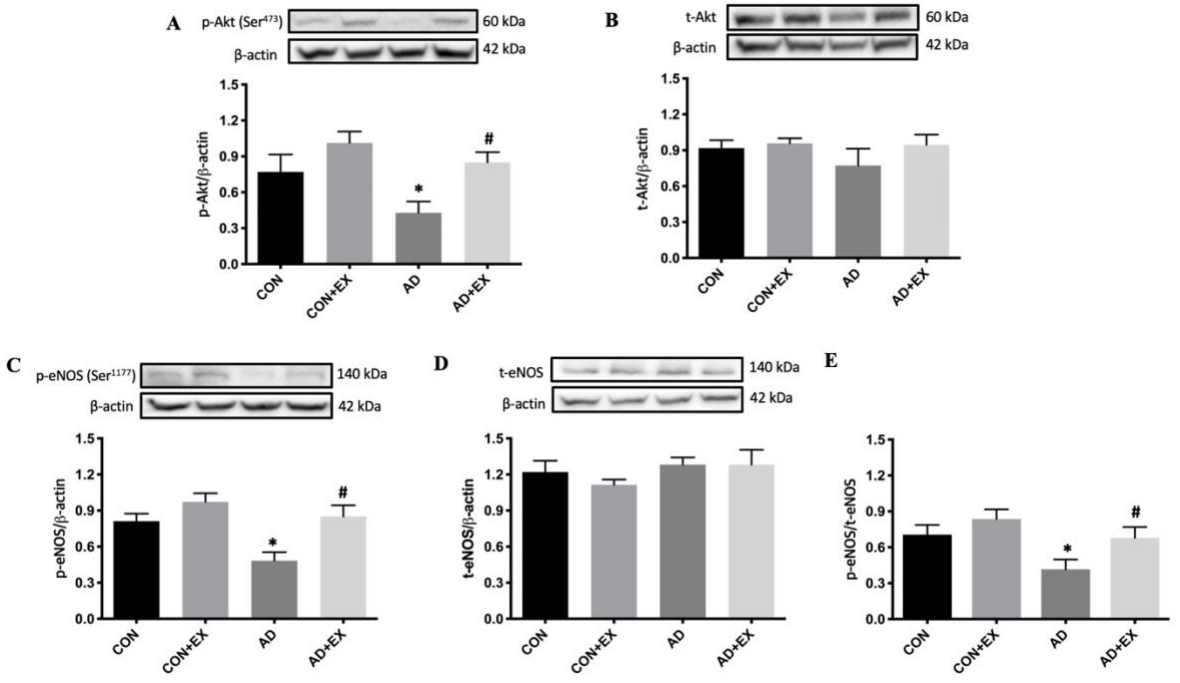



**Figure 4. 5. Effect of exercise training on eNOS signaling pathways in APP/PS1 double transgenic mice brain.**

**A-D**: Protein expression of p-Akt (A), t-Akt (B), p-eNOS (C), and t-eNOS (D) were analyzed by western blot (n=7-10/group). **E**: The ration of p-eNOS and t-eNOS proteins in the mice brain. Values are presented as mean ± SEM. $*p< 0.05$ vs CON, $\#p<0.05$ vs AD

***The role of P2Y2 receptor in cerebrovascular function in response to laminar shear stress (LSS) in vitro.***

To examine the effect of shear stress on the expression of P2Y2 receptor and eNOS signaling pathway, HBMECs were subjected to exercise-mimicking LSS (20 dyne/cm2) for different LSS time points (30 mins, 2 hrs, 24 hrs). The results obtained from the preliminary analysis of microarray data showed that P2Y2 receptor gene only was significantly elevated in response to the different intensity of LSS (5 & 20 dyne/cm2) among the nine genes of P2Y2

receptor in human umbilical vein cells (HUVECs) (Fig 4. 6A & B). The morphology changes of HBMECs by LSS presented an elongation shape after exposure to 24 hrs LSS (Fig. 4. 6C). The expression of P2Y2 receptor was significantly increased by 24 hrs LSS compared with static ($p < 0.05$; Fig. 4. 6D & E). Likewise, the critical factor of endothelial dysfunction, p-eNOS was also significantly increased by 2 hrs and 24 hrs LSS ($p < 0.05$; Fig. 4. 7A), respectively. Thus, t-eNOS was significantly elevated by 24 hrs LSS ($p < 0.05$; Fig. 4. 7B.

Then, we sought to examine whether P2Y2 receptor directly relevant to the regulation of eNOS signaling in HBMECs. The results showed that P2Y2 receptor inhibitor, AR-C, incubation in 24 hrs LSS-induced HBMECs abolished the increase of P2Y2 receptor, p-t/eNOS compared to 24 hrs LSS-induced HBMECs ($p < 0.05$; Fig. 4. 7C-E). Taken these findings together, an increase in laminar shear stress, as aerobic exercise mimetic effect, improves cerebral endothelial function via upregulation of P2Y2 receptor-dependent eNOS signaling pathway in HBMECs.

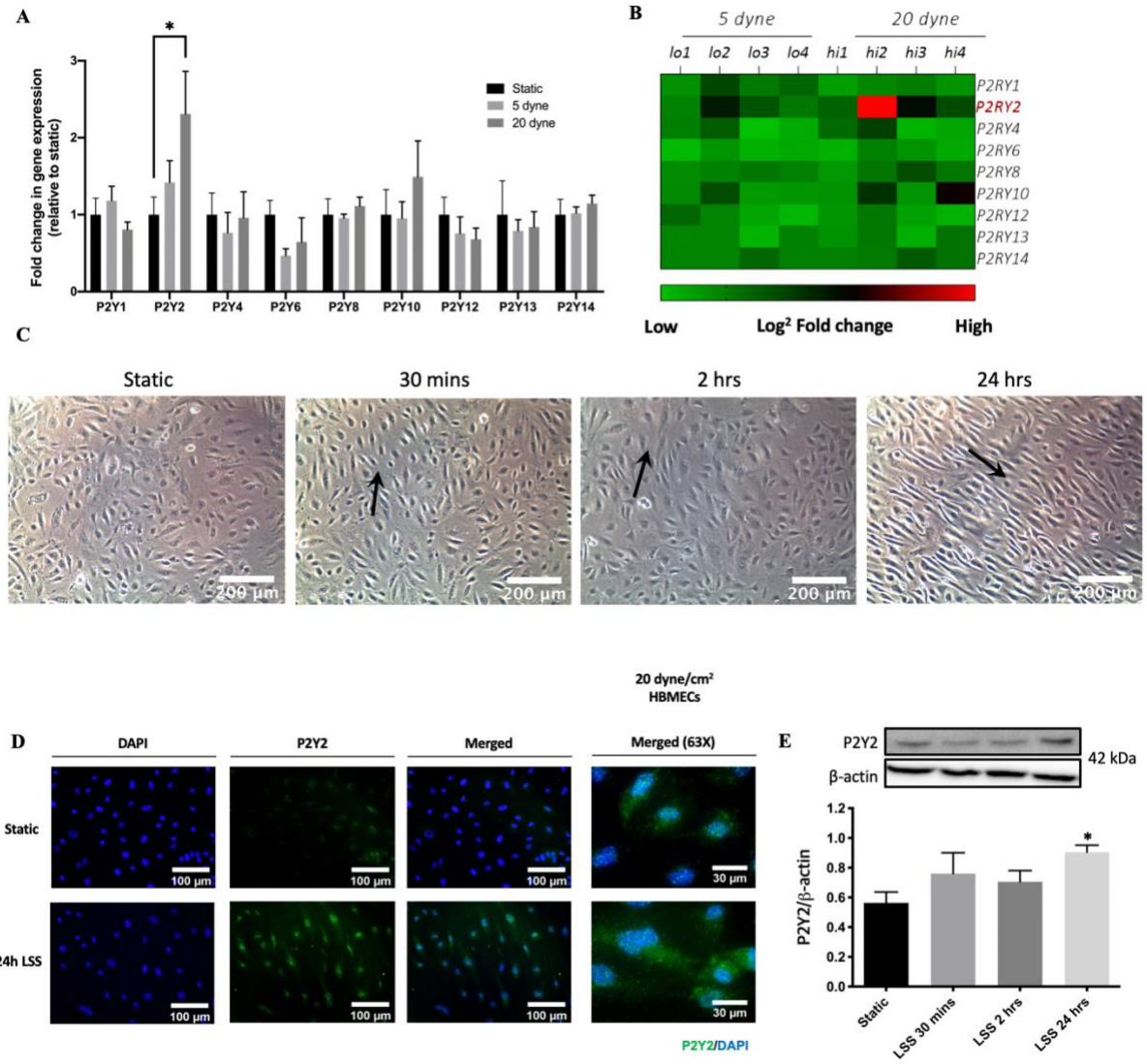



**Figure 4. 6. The expression of P2Y2 receptor in LSS-induced HUVECs and HBMECs.**

**A and B**: The gene expression of P2Y receptors in response to LSS in human umbilical vein cells (HUVECs). The P2Y2 gene only showed the highest expression among P2Y subtypes in response to the different intensity of LSS in HUVECs. Values are mean ± SEM (n=4). $^{*}p<$ 0.05 vs static. **C**: The morphology changes of HBMECs in response to LSS in a time-dependent manner; Phase-contrast images of HBMECs exposed to LSS at 20 dyne/cm2 for 30 mins, 2 hrs,

and 24 hrs. Black arrows indicate the direction of shear flow. Magnification: x10. Scare bar = 200μm. **D**: Fluorescence microscopic image of the expression of P2Y2 receptor in LSS-induced HBMECs. Magnification: x20 and x63. Scale bar= 30-100μm. **E**: The protein expression of P2Y2 receptor in response to LSS was analyzed by western blotting. Bar graph values are presented as mean ± SEM from 3 independent experiment. $*p < 0.05$ vs static.

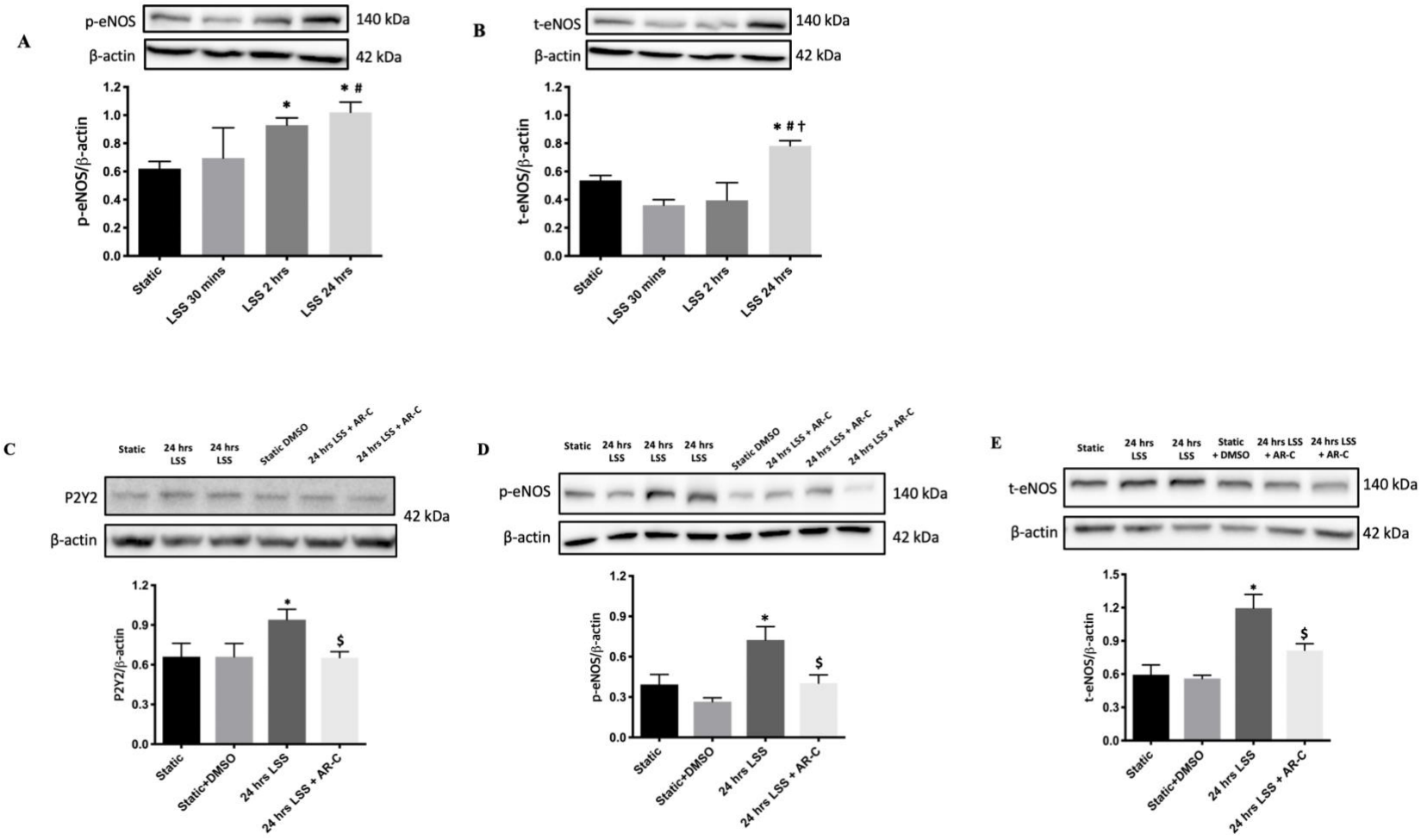

A
p-eNOS
β-actin
140 kDa
42 kDa
p-eNOS/β-actin
Static
LSS 30 mins
LSS 2 hrs
LSS 24 hrs
B
t-eNOS
β-actin
140 kDa
42 kDa
t-eNOS/β-actin
Static
LSS 30 mins
LSS 2 hrs
LSS 24 hrs
C
Static
24 hrs LSS
24 hrs LSS
Static DMSO
24 hrs LSS + AR-C
24 hrs LSS + AR-C
P2Y2
β-actin
42 kDa
P2Y2/β-actin
Static
Static+DMSO
24 hrs LSS
24 hrs LSS + AR-C
D
Static
24 hrs LSS
24 hrs LSS
24 hrs LSS
Static DMSO
24 hrs LSS + AR-C
24 hrs LSS + AR-C
24 hrs LSS + AR-C
p-eNOS
β-actin
140 kDa
42 kDa
p-eNOS/β-actin
Static
Static+DMSO
24 hrs LSS
24 hrs LSS + AR-C
E
Static
24 hrs LSS
24 hrs LSS
Static + DMSO
24 hrs LSS + AR-C
24 hrs LSS + AR-C
t-eNOS
β-actin
140 kDa
42 kDa
t-eNOS/β-actin
Static
Static+DMSO
24 hrs LSS
24 hrs LSS + AR-C

**Figure 4. 7. The role of P2Y2 receptor on eNOS signaling pathways in LSS-induced HBMECs.**

**A-B**: The effect of LSS on p-eNOS (A) and t-eNOS (B) expression. The protein expression of p-eNOS and t-eNOS were analyzed by western blotting. **C-E**: The effect of P2Y2 receptor inhibitor (AR-C,10μM) on the expression of P2Y2 receptor, p-eNOS, and t-eNOS in 24 hrs LSS-induced HBMECs. The protein expression of P2Y2 receptor (C), p-eNOS (D), and t-eNOS (E) were analyzed by western blotting. Bar graph values are presented as mean ± SEM from 3 independent experiment. $^{*}p< 0.05$ vs static, $^{\#}p<0.05$ vs 30 mins, $^{\dagger}p<0.05$ vs 2hrs, $^{\$}p<0.05$ vs 24 hrs LSS.

***Exercise training alleviates ER stress and ER stress-associated apoptosis in APP/PS1 double transgenic mice.***

To determine the effect of exercise training on AD pathology-associated ER stress in AD brain, we investigated whether exercise training reduces the expression of ER stress markers, IRE1, eIF2α, and CHOP, in the AD brain. As shown in Fig. 4. 8A, the expression of p-IRE1 was significantly higher in AD mice brain compared with CON mice brain, but its expression was significantly reduced by exercise training in AD+EX mice brain compared with AD mice brain ($p < 0.05$; Fig. 4. 8A). However, the expression of t-IRE1 was not significantly changed among the groups (Fig. 4. 8B). Likewise, p/t-eIF2α were significantly elevated in AD mice brain compared with CON mice brain ($p < 0.05$; Fig. 4. 8C & D), but the expressions of p-eIF2α was significantly abated by exercise training in AD+EX mice brain compared with AD mice brain ($p < 0.05$; Fig. 4. 8C). These results imply that AD pathology

increases ER stress response, but exercise training mitigates ER stress signaling pathways in AD mice brain.

CHOP is the downstream of IRE1 and eIf2α, and the pro-apoptotic UPR transcription factor [137], as well as associated with endothelial dysfunction [32, 208]. Therefore, we determined whether exercise training positively regulates ER stress-associated apoptosis in AD brain. We detected that the expression of CHOP was significantly elevated in AD and CON+EX mice brain compared with CON mice brain ($p < 0.05$; Fig. 4. 8E), but CHOP expression was significantly decreased in AD+EX mice brain compared with AD mice brain. Then, we subsequently investigated whether exercise training regulates CHOP-mediated pro-apoptotic factor; Bax and anti-apoptotic factor; Bcl-2 in AD mice brain. Fig. 4. 8F showed that Bax was significantly increased in AD mice brain compared with CON mice brain ($p < 0.05$), but exercise training significantly suppressed the Bax expression in AD+EX mice brain compared with AD mice brain ($p < 0.05$). Also, anti-apoptotic factor, Bcl-2 was significantly diminished in AD mice brain compared with CON mice brain ($p < 0.05$; Fig. 4. 8G), but its expression was significantly augmented by exercise training in AD+EX mice brain compared with AD mice brain ($p < 0.05$). Taken together, these results indicate that ER stress-induced CHOP expression and its downstream of Bax and Bcl-2 are affected by AD pathology, but exercise training alleviates ER stress-induced CHOP and its apoptosis pathways in AD mice brain.

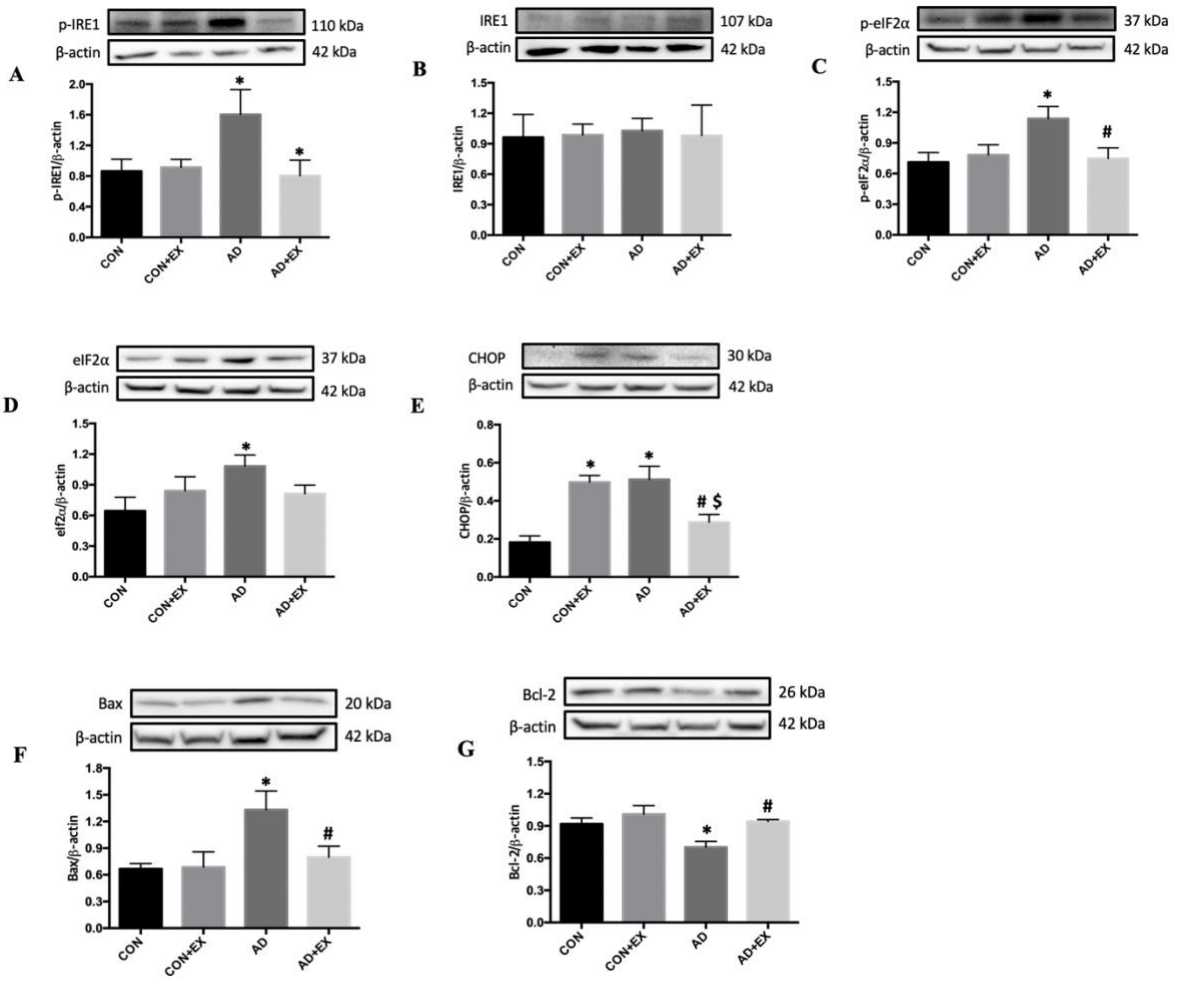



**Figure 4. 8. Effect of exercise training on ER stress and apoptosis in APP/PS1 double transgenic mice brain.**

**A-E**: The effect of exercise training on the protein expression in ER stress markers. The protein expression of p-IRE1 (A), IRE (B), p-eIF2α (C), eIF2α (D), and CHOP (E) were analyzied by western blotting. **F** and **G**: The effect of exercise training on the protein expression in Bax and Bcl-2. The protein expression of Bax (F) and Bcl-2 (G) were measured by immunoblot analysis from each group. Bar graphs present densitometic analysis of Bax and Bcl-2 in the brain from each group. Values are mean ± SEM (n=4-7/group). Values are mean ± SEM. $^{*}p< 0.05$ vs CON, $^{\#}p<0.05$ vs AD.

## 4. 5. DISCUSSION

The current study provides the first evidence that exercise training improves the cerebrovascular dysfunction in AD, possibly through P2Y2 receptors- and ER-stress-dependent endothelial function. Our findings demonstrate that; 1) 2-MeS-ATP-induced vasoconstriction in PCA from AD mice was reversed to vasodilation by exercise training; 2) reduced expression of P2Y2 receptor and eNOS signaling pathway in AD mice brain were restored by exercise training; 3) LSS increased the expression of P2Y2 receptor and eNOS signaling in HBMECs; 4) inhibition of P2Y2 receptor blunted the LSS-induced increase in eNOS expression in HBMECs; 5) abnormal expression of ER stress and ER stress-associated apoptosis in AD mice brain were normalized by exercise training.

ATP is the potent vasodilator and released by hypoxia and shear stress to regulate vascular reactivity acting on P2X and P2Y purinergic receptors in cerebral vessels [20, 209]. G protein-coupled P2Y receptors composed of eight sub-isoforms play an essential role in regulating cerebrovascular tone and CBF via the release of NO production [201, 210]. The previous study reported that ATP-induced endothelium-dependent vasodilation was decreased in Aβ-incubated cerebral penetrating arterioles in Tg2576 mice, which supports our findings in part that 2-MeS-ATP-induced vasoreactivity in PCA from AD mice was constricted rather than dilation (Fig. 4. 4A). Reduced CBF and impaired vasodilatory function in the cerebral artery are a potential risk factor of early onset of AD [211, 212], and our findings support the notion that reduced CBF by 2-MeS-ATP-induced vasoconstriction in PCA of AD mice (Fig. 4. 4A) possibly exacerbates the AD pathology (Fig. 4. 3), which ultimately induces cognitive impairment.

P2Y2 receptor is one of the isoforms of P2Y receptors, and is associated with AD pathology [131, 133], as well as regulate ATP-induced vasodilation through eNOS pathways in cerebral vessels [20, 213]. Our finding demonstrated that P2Y2 receptor expression was reduced in AD brain compared to CON mice brain (Fig. 4. 4B), which consisted with previous findings that the expression level of P2Y2 receptors was lower in AD patients compared to age-matched controls [131]. Also, NO plays an important factor for vascular homeostasis and therapeutic target in AD pathology [214]. APP and APP cleaving enzyme 1 (BACE1) protein levels are significantly increased in eNOS inhibitor-treated brain micro endothelial cells (BMECs) and eNOS knock-out mice brain [77, 215]. Our study results also reported that the downstream of P2Y2 receptor; p-Akt and p-eNOS protein expression were significantly reduced in AD mice brain compared to CON mice (Fig. 4. 5A & C) and APP expression was highly elevated in AD mice brain compared with CON mice (Fig. 4 .3). Collectively, these data suggest that reduced expression of P2Y2 receptor in AD brain might directly impair 2-MeS-ATP-induced vasodilatory response in NO-dependent manner in PCA and exacerbate AD pathology possibly through the decrease of NO bioavailability in AD mice.

The emerging number of clinical trials report the beneficial effect of exercise training on CBF[212] and cerebrovascular dysfunction [216] by enhanced eNOS signaling pathway. Notably, our finding showed that exercise training revered 2-MeS-ATP-induced vasoconstriction in PCA from the AD mice to vasodilation accompanied with the increase in P2Y2 receptor expression (Fig. 4. 4A & B). These findings suggest that exercise training ameliorated cerebrovascular dysfunction through the alteration of P2Y2 receptor in AD mice brain. One of the possible mechanisms for beneficial effect of exercise is the increased eNOS expression by enhanced LSS on endothelial cells of the vessel [44, 217]. The microarray

analysis data (Fig. 4A & B) demonstrated that P2Y2 was the most responsible gene to exercise-mimicking LSS in a dose-dependent manner among the sub-isoforms of P2Y receptors in HUVECs. It was critical preliminary evidence suggesting that exercise training improves cerebrovascular function via P2Y2 receptor-dependent mechanism, and the current study mainly focuses on P2Y2 receptor. Likewise, our data demonstrated that both exercise training and exercise-mimicking LSS increased the expression of P2Y2 receptor (Fig. 4. 4B, 4. 6D & E) with the elevation of eNOS signaling pathways in HBMEC (Fig. 4. 7A & B) and in the mice brain (Fig. 4. 5A & C). It's been well studied that long-term exercise training increases the expression of PI3K, p-Akt, and eNOS in hypertensive-induced rat brain [218] and in the basilar artery [219] and pial arterioles [220] in diabetic rats. Activation of eNOS signaling reduces the expression of APP and BACE-1 induced by blockage of eNOS signaling by eNOS inhibitor, L-NAME, in BMECs [215]. This study strongly supports our finding that elevated eNOS expression by exercise training (Fig. 4. 5A & C) improves 2-MeS-ATP-induced vasodilation (Fig. 4. 4A) and may delay AD pathology by reducing APP expression in AD mice brain (Fig. 4. 3). Furthermore, we directly report that exercise-induced overexpression of eNOS is regulated by P2Y2 receptor signaling pathways in brain endothelial cells. AR-C, P2Y2 receptor inhibitor, blunted the elevation of eNOS signaling by LSS in HBMECs (Fig. 4. 7D & E). Similar results were found in recent studies reporting inhibition of P2Y2 receptor by siRNA reduces LSS-induced activation of eNOS signaling in HUVECs and bovine aorta endothelial cells (BAECs) [221, 222]. The current study suggest that P2Y2 receptor is the central modulator to regulate cerebrovascular function through eNOS signaling pathway and prolonged exercise training ameliorates 2-MeS-ATP-induced cerebrovascular dysfunction possibly through upregulation of P2Y2-dependent eNOS signaling in AD mice.

The direct effect of ER stress on the etiology of AD has been well corroborated and is regarded as a possible therapeutic intervention to conquer AD [142]. GRP78, p-PERK, IRE1α, and eIF2α are markedly elevated in AD brain when compared to age-matched controls [143], and higher expression of CHOP-mediated pro-apoptotic makers are also found in AD mice brain [12, 142] and microvessels from AD mice brain [223]. The vascular functional studies reported that ER stress inducer, tunicamycin, impairs ACh-induced endothelial function with decreased expression of p-eNOS and total nitrate/nitrite level in mouse aorta [186], but inhibition of ER stress ameliorated endothelial dysfunction via increased eNOS expression and decreased oxidative stress/apoptosis, which eventually elevate NO bioavailability in mesenteric arteries in atherosclerosis [151] and aorta in diabetic mice [150]. Regular exercise training positively alters the expression of ER stress markers; GRP78, p-PERK, p-eIF2α, ATF6, and CHOP and apoptosis markers; caspase3/12, Bax, and Bcl-2 in AD mice brain [28, 224], and increase the NO bioavailability through up-regulation of eNOS signaling pathway in aorta from diabetic mice [150]. The current study also reports that ER stress (p-IRE1, p-eIf2α, and CHOP) was higher with a negative alteration of CHOP-mediated pro/anti-apoptosis protein expressions (increased Bax & decreased Bcl-2) (Fig. 4. 8A-G) and lower eNOS signaling in AD mice brain (Fig. 4. 5A & C). These findings imply that increased ER stress is associated with cerebrovascular dysfunction possibly through the reduced eNOS signaling pathway in AD mice. More importantly, however, exercise training significantly reversed the increased expression of ER stress markers and ER stress-associated apoptosis production (Fig. 4. 8A-G), and it elevated the reduced eNOS expression in AD mice brain (Fig. 4. 5A & C). Collectively, current findings suggest that exercise training ameliorates ER stress-associated

cerebrovascular dysfunction possibly through elevation of eNOS signaling by counteracting UPR demand and shifting cellular pro-apoptotic signals to pro-survival pathway in AD.

In conclusion, the current study provides valuable insight into the therapeutic strategy of exercise training on cerebrovascular dysfunction in AD by enhancing P2Y2 receptor-mediated eNOS signaling and reducing ER stress-associated pathways in AD. Furthermore, P2Y2 receptor is a critical modulator of both cerebrovascular dysfunction and the exercise-induced beneficial effect on cerebrovascular dysfunction in AD. Further investigation to find the direct pathogenic chain of ER stress and P2Y2 receptor-mediated vascular function in a cerebral artery in AD would discover the missing clues for developing novel therapeutic implications to conquer AD pathogenesis.

# Chapter 5: Conclusion

Here we show for the first time several key cell signaling proteins and pathways that are responsible for ameliorating endothelial dysfunction in response to exercise. We found that exercise training improves a) impaired ACh-induced vasodilation in both atherosclerotic mesenteric arteries and coronary arterioles and b) reduced 2-MeS-ATP-induced vasorelaxation in PCA from AD via a NO-dependent mechanism. Further, we found that exercise training mitigates ER stress-associated endothelial dysfunction in both atherosclerotic mesenteric arteries and coronary arterioles, as well as in the PCA from AD. Exercise inhibits the abnormal expressions of ER stress markers, the TXNIP/NLRP3 inflammasome, apoptosis and oxidative stress, as well as, reduces eNOS expression. Exercise training also promotes the conversion of 2-MeS-ATP-induced vasoconstriction to vasodilation in PCA from AD in a P2Y2 receptor-dependent manner. Lastly, we found that LSS, a function of increased hemodynamic flow and force in response to exercise, upregulates the expression of the P2Y2 receptor, which leads to increased eNOS signaling in HBMECs. Inhibition of the P2Y2 receptor, through pharmacological blockade, blunts these changes in LSS-induced HBMECs.

Atherosclerosis and Alzheimer's dementia (AD), as components of cardiovascular and neurovascular disease, are major public health concerns and are among the leading causes of death worldwide. These disorders are characterized by plaque formations that act as both physical and physiological barriers, and which lead to vascular dysfunction. Ultimately, the vascular dysfunction, evident in both of these disorders, results in significant reductions in perfusion to target organs, namely the heart and the brain, leading to reduced lifespan and reduced quality of life for those afflicted. Vascular dysfunction has been attributed to the

dysfunction of the endothelial cells that line the vasculature. Endothelial dysfunction is the early response to atherosclerosis and is involved in the etiology of AD. Prolonged ER stress has been identified as the possible underlying mechanism regulating endothelial dysfunction through increases in oxidative stress, inflammation and apoptosis, which leads to cell death. Alleviation of ER stress in the vascular system can be an important therapeutic strategy for the retardation and treatment of the pathophysiology stemming from atherosclerosis and AD.

Exercise is known to mitigate the consequences of both these disorders, though the specific cellular mechanisms responsible for these improvements in response to exercise have not been fully delineated. However, exercise training remains the premier therapy for the prevention and treatment of vascular dysfunction due to its role in the mitigation of oxidative stress, reductions in the inflammatory and apoptotic responses, as well as its capacity to increase NO bioavailability. However, the specific role of exercise training in ER stress-associated vascular dysfunction across different vascular beds, especially as it relates to atherosclerosis and AD is still largely unknown. Therefore, through this dissertation, we aimed to address these knowledge gaps by determining the underlying mechanisms responsible for the exercise-induced amelioration of ER stress-associated vascular dysfunction across different vascular beds and for different pathologies. Ultimately, the goal for our research is to determine the specific cellular proteins or pathways responsible for reducing or eliminating ER stress-associated endothelial dysfunction in response to exercise. In doing so, we potentially identify novel therapeutic targets and strategies for the treatment of afflicted patients who might otherwise be unable or unwilling to exercise.

Our results from studies one and two provide direct evidence that exercise training is an effective treatment to ameliorate ER stress-associated endothelial dysfunction in

atherosclerosis. Exercise increases NO bioavailability, through the down-regulation of ER stress, ER stress-associated TXNIP/NLRP3 inflammasome and ER stress-associated apoptosis signaling pathways in atherosclerotic mesentery artery and coronary arterioles. Additionally, we found that exercise training improved UCP-2 mediated endothelial dysfunction through the reduction of UCP-2 mediated oxidative stress in atherosclerotic mesentery arteries and coronary arterioles. A limitation of the current study is the absence of cellular and molecular data to confirm the direct effect of ER stress on UCP-2-mediated endothelial dysfunction in atherosclerosis. This is mainly due to the limited number of mesentery arteries and coronary arterioles available for study, as well as practical constraints. Further studies would be required to identify the direct effect of exercise on ER stress-induced UCP-2-mediated endothelial dysfunction using atherosclerotic resistance vessels or atherosclerosis-induced cultured cells. Taken together, our data suggest that exercise training is a useful countermeasure for patients with atherosclerosis and may help reduce atherosclerosis-related CVD risk factors such hypertension and myocardial infarction. Additionally, exercise would improve the health outcomes of atherosclerosis afflicted people and reduce their morbidity and mortality rates.

Our study three results demonstrate that exercise training is an optimal preemptive therapy for the prevention of AD pathology and a positive intervention for ER stress-purinergic receptor-associated cerebrovascular dysfunction in AD. Exercise training promotes the conversion of 2-MeS-ATP-induced vasoconstriction to vasodilation in PCA from AD via the elevation of P2Y2 receptor-dependent eNOS signaling pathway. Thus, the expression of the P2Y2 receptor and eNOS signaling are highly elevated in LSS-induced HBMECs, and inhibition of P2Y2 receptor blunts LSS-induced increase in eNOS signaling in HBMECs. Furthermore, exercise training mitigates ER stress-associated cerebrovascular dysfunction

possibly through reduction of ER stress and ER stress-associated apoptosis signaling. A potential limitation of this study is the absence of data to provide the causal effect between ER stress and P2Y2 receptor in cerebrovascular dysfunction in AD. Another limitation of this study is the limited number of experiments defining the beneficial effect of LSS on P2Y2 receptor-mediated eNOS signaling in AD pathology-induced HBMECs. Therefore, further experiments are necessary in order to determine the connection between ER stress and the P2Y2 receptor in cerebral arteries in AD and AD pathology-induced HBMECs. In addition, it is necessary to identify the specific cellular mechanisms responsible for the changes in P2Y2 receptor-mediated eNOS signaling in response to the exercise-mimicking LSS in AD pathology-induced HBMECs. Together, these data suggest that exercise training could serve as an important preventive intervention for people with AD. Exercise could slow or reverse cognitive decline and memory loss, and this would reduce the mortality of this disease and improve quality of life for AD patients and their caregivers.

In conclusion, ER stress is highly implicated in vascular dysfunction in different vascular beds and pathologies with alteration of its downstream pathways including inflammation, apoptosis and oxidative stress, as well as the NO signaling cascade. However, exercise training is an effective intervention and frontline therapy for regulating ER stress- and purinergic receptor-associated vascular dysfunction in different vascular beds and pathologies through the positive alteration of these molecular functions.

Future work

Further investigations are necessary to identify the most effective exercise protocol for maintaining and regulating cardiovascular health and cardiovascular risk

factors for patients with atherosclerosis and AD. Various modes of exercise training or duration/intensity of exercise training might obtain different treatment outcomes or cardiometabolic benefits for these patients. It is vital for exercise researchers to determine the adequate exercise strategy to prevent or reverse cardiovascular disease.

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