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IMPACT OF 17-BETA ESTRADIOL AND MODERATE-INTENSITY EXERCISE ON MESENTERIC ARTERIAL FUNCTION OF UC DAVIS TYPE-2 DIABETES MELLITUS RATS

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DEDICATION

This dissertation is dedicated to my parents Md Shahjahan Ali, Ms. Rabeya Khatun, and my lovely wife, Dil Afroz Rownak, who loved and supported me unconditionally my whole life and during my Ph.D.

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Abstract

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The studies in this dissertation were designed to investigate the impacts of estrogen (17-β estradiol/E_2) and moderate-intensity exercise (MIE) on the mesenteric arterial (MA) function of the University of California Davis type-2 diabetes mellitus (UCD-T2DM) Rat model. Our recent report suggests that diabetes impairs MA vasorelaxation in both sexes of the UCD-T2DM model. Particularly, we reported that MA from prediabetic male rats showed a greater impairment compared to that in prediabetic females. However, when females become diabetic, they exhibit a greater vascular dysfunction than males. Therefore, the aim of the first study was to investigate whether female sex hormone, specifically E_2, preserves the MA vasorelaxation in female UCD-T2DM rats at the early prediabetic state. For this study, age-matched healthy Sprague Dawley (SD) and prediabetic female UCD-T2DM rats were ovariectomized and subcutaneously implanted with either a placebo or E_2 pellet for 45 days.

Regular aerobic exercise is a well-known therapeutic intervention for endothelial dysfunction, insulin resistance, and cardiovascular disease (CVD) risk in diabetes. However, there are still debates about the duration, intensity, and underlying mechanisms of benefits of exercise against deleterious metabolic consequences in diabetic patients. In the second study outlined in this dissertation, we examined the impact of exercise training on vascular function

and wall structure of the UCD-T2DM male rats. Age-matched male diabetic and SD control rats were randomly divided into sedentary and exercise-trained groups. The exercise-trained groups ran on a treadmill for eight weeks (1hr/day, 5days per week). For both studies (Studies I & II), metabolic parameters and MA responses to vasodilator and vasocontractile agents were determined. Furthermore, the expression of molecules associated with vascular signaling were also analyzed.

The specific aims of our studies were to investigate whether E_2 and moderate-intensity exercise (MIE) alter the 1) endothelium-dependent vasorelaxation (EDV) and vasoconstriction 2) relative contribution of endothelium-derived relaxing factors (EDRF) to vasorelaxation, and 3) expression of proteins associated with vascular signaling, in MA of UCD-T2DM rats.

In the first study, we demonstrated that acetylcholine (ACh)-induced vasorelaxation was impaired in MA of ovariectomized (OVX) prediabetic UCD-T2DM rats. Our data also showed that E_2 replacement improved MA relaxation in OVX prediabetic group to a similar level to that in control groups. Inhibition of cyclooxygenase (COX) by indomethacin (Indo) did not significantly affect the vascular responses in any groups, suggesting a minor role of COX metabolites in MA relaxation in the experimental groups. Inhibition of nitric oxide (NO) synthase (NOS) by L-NAME reduced vasorelaxation to ACh in control groups, but it did not completely abolish the vasorelaxation. We also showed that in control (healthy) groups, both NO and endothelium-derived hyperpolarizing factor (EDHF)-type relaxation were dominant in the MA relaxation of placebo and E_2 treated rats. However, in prediabetic groups, L-NAME completely abolished the vasorelaxation, regardless of E_2 treatment, suggesting a relative shift from EDHF-type relaxation to only NO-mediated relaxation in these groups. Furthermore, the sensitivity of MA to NO was significantly impaired in OVX prediabetic group, but E_2 treatment

enhanced the MA sensitivity to NO. Overall, our data suggest that a greater vasorelaxation in the E_2 treated OVX prediabetic group could be partly attributed to the elevated role of NO or improved sensitivity of MA to NO in this group.

The second study demonstrated that ACh-induced vasorelaxation of MA was significantly impaired in sedentary diabetic (DS) male rats. MIE significantly enhanced MA vasorelaxation in the exercise-trained diabetic (DE) group compared to the DS. However, no significant differences were observed between the vasorelaxation of control sedentary (CS) and control exercise-trained groups (CE). Inhibition of COX enhanced maximal vasorelaxation response (R_{max}) to ACh in DS arteries suggesting an elevated contractile COX contribution in MA of this group which could possibly be due to the observed increase in COX expression in the DS group. Unlike the DS group, inhibition of COX did not affect the vasorelaxation responses to ACh in the DE group. The addition of L-NAME resulted in a reduction in ACh-induced relaxation of MA from both DS and DE groups. However, the effect of L-NAME was more prominent in the DS group compared to the DE group, suggesting a major contribution of NO in DS arteries. On the other hand, a preserved role of NO with an enhanced EDHF-mediated relaxation was observed in the MA vasorelaxation of the DE group. Our data on the elevated small conductance calcium-activated potassium channel (SK_{Ca}) expression level in MA taken from the DE group compared to that in the DS group may suggest a role for SK_{Ca} in increased EDHF-type relaxation in the DE group. Furthermore, DS arteries exhibited a higher contractile response, myogenic tone, and wall thickness than those in MA of DE. Overall, our data suggest that MIE reduced myogenic tone (DE vs. DS) and improved EDV in mesenteric arteries of diabetic rats, possibly via a shift from contractile COX activity to both NO and EDHF-type relaxation.

In conclusion, the data generated in study-I suggest that estrogen may protect prediabetic female MA from early vascular dysfunction, possibly by elevating the contribution of NO to vasorelaxation as a compensatory mechanism to the loss of EDHF-type relaxation in this group. Although the results of the current study are in agreement with our previous report demonstrating a possible protective effect of female sex hormones in the MA function at prediabetic state, additional studies are needed to establish the specific role of E_2 in the progression of vascular dysfunction in the diabetic state.

Lastly, an intriguing observation of study-II was that MIE improved vasorelaxation and prevented the loss of EDHF-type relaxation in diabetic arteries. This was in addition to the changes induced to the wall thickness and myogenic tone in arteries of UCD-T2DM males. Given that sex differences play an important role in cardiovascular physiology, additional studies are needed to establish the specific role of MIE on vascular dysfunction in UCD-T2DM female rats and its underlying mechanisms.

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ACh	acetylcholine
Akt	protein kinase B
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CRC	concentration-response curve
E_2	17β-estradiol
EDCF	endothelium-derived contracting factors
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EDV	endothelium-dependent vasodilation
eNOS	endothelial nitric oxide synthase
ER	estrogen receptors
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	hydrogen peroxide
HbA1c	glycosylated hemoglobin A1c
IK_{Ca}	intermediate conductance calcium-activated potassium channels
Indo	indomethacin

IMT	intima medial thickness
iNOS	inducible nitric oxide synthase
K_{ir}	inward rectifier K+ channels
L-NAME	Nω-nitro-L-arginine methyl ester
mRNA	messenger ribonucleic acid
MA	mesenteric arteries
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NOX	nicotinamide-adenine-dinucleotide phosphate oxidase
$O_2^{\bullet-}$	superoxide anion radical
OVX	ovariectomized
PD	prediabetic
pD_2	-log [EC_{50}]
PE	phenylephrine
PGI_2	prostacyclin
R_{max}	maximum response
ROS	reactive oxygen species
SK_{Ca}	small conductance calcium-activated potassium channels
SKM	skeletal muscle
SMC	smooth muscle cell
SNP	sodium nitroprusside

TXA_2	thromboxane A_2
T1D	Type-1 diabetes mellitus
T2D	Type-2 diabetes mellitus
UCD-T2DM	University of the California Davis Type-2 Diabetes Mellitus

CHAPTER 1: INTRODUCTION

Cardiovascular diseases (CVD) are the leading causes of death for both men and women in the United States (CDC, 2020b). However, the risk of CVD in diabetic men and women is not similar. Although women suffer from fewer CVD events than men during premenopausal years due to the cardioprotective role of female sex hormones, this sex difference is abolished in menopause due to reduced estrogen level (Iorga et al., 2017). Premenopausal diabetic women not only lose the sex-based protective effect but also have a higher relative risk of CVD than diabetic men (Kannel & McGee, 1979). The transition of premenopausal women from a cardio-protective status to an increased CVD risk individual in diabetes is not well understood. Most type-2 diabetic patients are obese, and obesity is associated with a significantly increased risk of CVD in type-2 diabetes (T2D) (American College of Cardiology/American Heart Association Task Force on Practice Guidelines, Obesity Expert Panel, 2013, 2014; Daousi et al., 2006). Lifestyle modifications such as regular exercise and eating a balanced diet are at the forefront of preventing obesity and T2D. Although exercise training is a proven method to prevent CVD and diabetes, the type and intensity of exercise to improve vascular function in aging and diabetes remain debated (Craighead et al., 2019).

1.1 Diabetes Mellitus

Diabetes is a metabolic disorder characterized by high blood glucose level and occurs when pancreatic β-cells cannot produce enough insulin (type-1 diabetes) or when the body is not responsive to insulin despite having a proper blood insulin level (T2D). According to National Diabetes Statistics Report 2020, 34.2 million Americans have diabetes, and another 88 million Americans have prediabetes. CVD remains the primary cause of hospitalization of patients with

diabetes, and the total direct and indirect cost of diabetes was estimated at $327 billion in 2017 (Association, 2018).

1.1.1 Type-1 Diabetes (T1D)

T1D is also known as insulin-dependent diabetes mellitus. The initiation of type-1 diabetes occurs due to the destruction of insulin-producing pancreatic β-cells due to auto-immune disorder or environmental factors (such as viral infection). Therefore, the pancreas produces little to no insulin and thus resulting in high blood glucose level. Type-1 diabetes accounts for approximately 5-10% of the total diabetic patients in the USA.

1.1.2 Type-2 Diabetes (T2D)

T2D is the most common form of diabetes mellitus and accounts for 90-95% of the diabetic patients. In T2D, the tissues become unresponsive to insulin which is known as insulin resistance. Initially, the body counters insulin resistance by increasing insulin production from pancreatic β-cells. However, over time overproduction of insulin exhausts β-cells and results in diabetes mellitus. Genetic inheritance, together with lifestyle factors such as physical inactivity, high fat diet, smoking, consumption of alcohol, are critical factors that could lead to type-2 diabetes (Ripsin et al., 2009).

1.2 Prediabetes

Before an individual develops type-2 diabetes, they almost always go through a phase known as prediabetes. In prediabetes, the blood glucose level is elevated above the normal level but not high enough to be considered diabetes. According to CDC, fasting glucose between 100-125mg/dL or blood glucose level 140-199 mg/dL in glucose tolerance test or a HbA1c level between 5.7-6.4% is considered prediabetes. Prediabetes is prevalent in obese individuals with impaired glucose tolerance (Weiss et al., 2003). Approximately 88 million American adults have

prediabetes which is about 1 in every 3 American adults. Prediabetes increases the risk of developing type-2 diabetes and CVD (CDC, 2020a).

1.3 Diabetes and CVD

Cardiovascular risk is significantly higher in people with diabetes compared to non-diabetic individuals (Emerging Risk Factors Collaboration et al., 2010). Previous epidemiological report suggests that diabetes is an independent risk factor for CVD in both men and women and about 65% of the deaths in diabetic patients in due to some form of CVD (Grundy et al., 1999). Both type-1 and type-2 diabetic patients suffer from increased burden of CVD. However, since type-2-diabetes accounts for 90% cases of diabetes, most of the CVD deaths occur from type-2 diabetes. Due to the masking effect of diabetes against CVD, many of the symptoms remain undiagnosed and could be a reason for higher mortality in CVD in diabetes.

Clustering of metabolic risk factors is commonly referred as metabolic syndrome. Metabolic risk factors such as dyslipidemia, increased body weight, and abdominal obesity, hypertension, insulin resistance increase both risk of diabetes and CVD (Rochlani et al., 2017). Initiation of hyperglycemia in patients with metabolic syndrome accelerates the atherogenesis probably by the initiation of endothelial dysfunction and increasing advanced glycation end products. The injurious effect of hyperglycemia and metabolic syndrome on cardiovascular system can be observed in both macrovascular (coronary artery disease, stroke, peripheral artery disease) and microvascular (diabetic retinopathy, nephropathy, and neuropathy) levels (Fowler, 2008).

Microvasculatures with a relaxed diameter of less than 400μm are considered resistance arteries. Resistance arteries, although small, are a major controller of systemic vascular

resistance and blood pressure. For example, resistant mesenteric arteries (MA) and arterioles in times of cardiogenic shock are responsible for 40% of the increase in systemic vascular resistance (Ceppa et al., 2003). Thus, abnormalities in resistance arterial function have a significant role in the pathogenesis of hypertension and other vascular diseases in diabetes (Intengan & Schiffrin, 2000).

1.4 Endothelial Function

Vascular endothelium is the innermost layer of any vasculature that consists of a single-layered endothelial cells. Although it consists of only a single layer of endothelial cells, a healthy endothelium can respond to physical and chemical signals by producing a wide range of factors which can modulate vascular tone, cellular adhesion, smooth muscle cell (SMC) proliferation, and vessel wall inflammation. Therefore, impaired normal endothelial function, otherwise known as endothelial dysfunction, can lead to CVD and is often described in diabetes, hypertension, and atherosclerosis (Sun et al., 2019).

Endothelium-dependent vasorelaxation (EDV) is a reproducible parameter which can be investigated to check vascular health in pathophysiological conditions. Impaired EDV is the most common hallmark of endothelial dysfunction. To control the vascular tone, endothelium produces vasorelaxant and contractile factors known as endothelium-derived relaxing factors (EDRF) and endothelium-derived contractile factors (EDCF). EDRF is comprised of nitric oxide (NO), prostacyclin (PGI_2), and the elusive endothelium-derived hyperpolarization factor (EDHF). On the other hand, EDCF includes endothelin-1 (ET-1), angiotensin-II, thromboxane-A_2 (TXA_2), etc. Endothelial dysfunction or impaired vasodilation can occur when there is reduced production and sensitivity to EDRF, increased inactivation of EDRF, and/or increased production and sensitivity to EDCF.

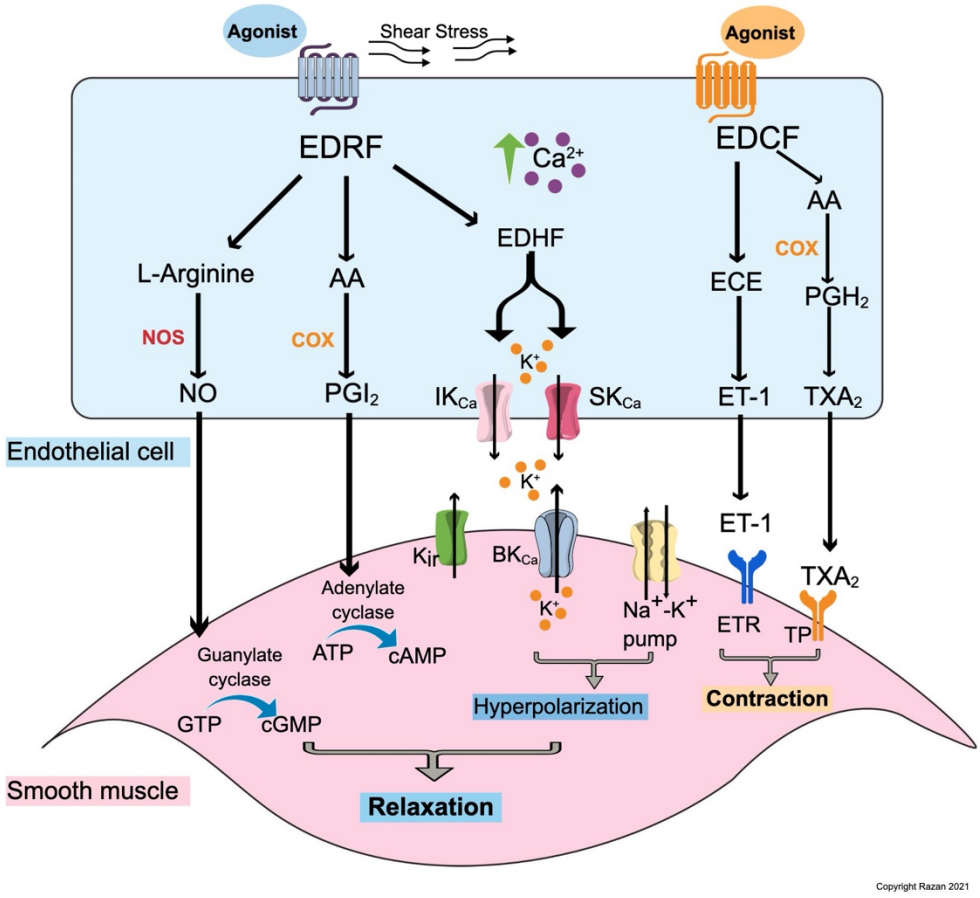

Figure 1.1 EDRF and EDCF. Nitric oxide (NO) activates soluble guanylate cyclase, yielding increased levels of cyclic GMP (cGMP). Prostacyclin (PGI_2) activates adenylate cyclase, leading to increased production of cyclic AMP (cAMP). The contribution of three mediators may differ based on vascular bed. NO may predominate in large arteries, whereas EDHF takes over in smaller blood vessels or in large arteries when the release of NO is curtailed. Depending on the species and vascular bed studied, the identity of EDHF has been narrowed down to include epoxyeicosatrienoic acids, K^+ ions, H_2O_2, and the myoendothelial junctions. AA, arachidonic acid; COX, cyclooxygenase; ECE, endothelin converting enzyme, NOS, NO synthase; L-Arg, L-arginine; ETR, endothelin receptor; TP, thromboxane receptor; SK_{Ca}, small conductance calcium-activated potassium channels; IK_{Ca}, intermediate conductance calcium-activated potassium channels.

1.4.1 Nitric Oxide (NO)

NO is the most well studied vasodilator to date. It is a soluble gas that is usually produced by nitric oxide synthase (NOS) from L-arginine in presence of oxygen (Tousoulis et al., 2012). So far three isoforms of nitric oxide synthase have been identified: a) nNOS (neuronal NOS; type I) b) iNOS (inducible NOS, type-II) c) eNOS (endothelial NOS, type-III) (Förstermann & Sessa, 2012). These isoforms of NOS differ in their structure and function. eNOS and nNOS are preferentially calcium-dependent enzymes, whereas iNOS is calcium-independent and stimulated in presence of inflammatory cytokines, bacterial lipopolysaccharide, and other agents. eNOS is the most prominent of the NOS enzymes in the cardiovascular system (Förstermann & Sessa, 2012).

Like all NOS, eNOS is a homo-dimer enzyme. Activation of eNOS occurs in presence of stimuli such as insulin, vascular endothelial growth factor (VEGF), estrogen, bradykinin, and fluid shear stress. Two important regulatory sites of eNOS phosphorylation are Serine 1177 (Ser1177) and Threonine 495 (Thr495), which are positive and negative regulatory sites respectively. Depending on the stimuli, eNOS activation can occur through different pathways. Bradykinin increases intracellular Ca^{2+} followed by phosphorylation of Ser1177 on eNOS by Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII) whereas phosphorylation of eNOS by estrogen happens through protein kinase B(Akt). Insulin can stimulate phosphorylation of eNOS by both 5' adenosine monophosphate-activated protein kinase (AMPK) and Akt (Förstermann & Sessa, 2012). Upon activation by phosphorylation, eNOS produces NO, which diffuses to the underlying smooth muscle of the vasculature and activates soluble guanylyl cyclase (sGC) and increases cGMP to relax vascular smooth muscle. Reduced activity of eNOS and/or increased elimination of NO can be associated with the vascular dysfunction in diabetes. Under oxidative

stress, NO is scavenged by superoxide to produce peroxynitrite and thus reduce the bioavailability of NO in the endothelium. Additionally, oxidative stress can cause eNOS uncoupling, which preferentially produces superoxide instead of NO and further deteriorates the oxidative stress in the vasculature (Förstermann et al., 2017; Radi, 2018).

Previous research has reported a wide range of biological activities of NO in the cardiovascular system, including vasorelaxant, anti-platelet aggregation agent, and anti-atherogenic, anti-inflammatory, angiogenic, anti-hypertrophic and other properties of NO (Strijdom et al., 2009).

1.4.2 Prostacyclin (PGI_2)

Prostaglandins (PG) were the first identified endothelium-derived vasoactive paracrine substances. The most common precursor of prostaglandin is arachidonic acid which is released from the cell membrane phospholipid by phospholipases. Cyclooxygenase (COX) enzymes metabolize arachidonic acid to produce prostaglandin H_2. Through the action of different prostaglandin synthases, different types of vasoactive prostanoids such as PGI_2, PGE_2 $PGF_{2\alpha}$ and thromboxane are synthesized. There are two major isoforms of COX: COX-1 and COX-2 (Mitchell & Warner, 2006). COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced in the presence of inflammatory stimuli. In a healthy blood vessel, both the endothelium and to a lesser extent smooth muscle express both the COXs, but COX-1 remains the predominant isoform. In most blood vessels, prostacyclin is the major metabolite of arachidonic acid and described as a potent anti-aggregating agent and vasodilator. Generally, PGI_2 through its Gs-coupled receptor activates adenylyl cyclase and increases intracellular cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA), which causes an inhibitory effect on myosin light chain kinase (MLCK) and relaxes smooth muscle to cause

vasorelaxation (Félétou et al., 2011; Raina et al., 2009). On the other hand, COX-derived EDCF contribute to vascular dysfunction and CVD through stimulation of thromboxane receptor (TP) (Félétou et al., 2011).

1.4.3 Endothelium Derived Hyperpolarization Factor (EDHF)

Besides NO and PGI_2, endothelium can also control vascular tone by hyperpolarizing the underlying smooth muscle. EDHF is more prominent in small resistance arteries compared to large arteries and also an important regulator of vascular tone (Tomioka et al., 1999). The contribution of EDHF on EDV and its identity could vary depending on the vascular bed under question (Leo et al., 2011). Although the exact identity of EDHF is still not clear, a lot of possible pathways have been suggested by previous research for the endothelium-dependent hyperpolarization of smooth muscle (Edwards et al., 1998, 2010; Sandow Shaun L. et al., 2002). In general, EDHF mediated response involves agonist-induced increase in endothelial cell Ca^{2+} concentration and subsequent opening of endothelial small conductance calcium-activated potassium channel (SK_{Ca}) and intermediate conductance calcium-activated potassium channel (IK_{Ca}). Two mechanisms have been proposed to explain SK_{Ca} and IK_{Ca} mediated smooth muscle hyperpolarization. The first mechanism involves the opening of SK_{Ca} and IK_{Ca} channels and a subsequent increase in potassium (K^+) in myoendothelial space. An increase in K^+ cloud in myoendothelial space causes the activation of inward rectifier (K_{ir}) and Na^+/K^+-ATPase pump in the smooth muscle causing hyperpolarization of smooth muscle (Edwards et al., 1998; Parkington et al., 2002; Sandow Shaun L. et al., 2002). The second mechanism involves the transmission of endothelial hyperpolarization by direct electrical coupling through myoendothelial gap junction (Edwards et al., 2010; Félétou & Vanhoutte, 2006). Another mechanism, independent of SK_{Ca} and IK_{Ca}, has also been described where Ca^{2+} dependent

release of epoxyeicosatrienoic acids (EET) and H_2O_2 from endothelium diffuse to smooth muscle and hyperpolarize it (Félétou & Vanhoutte, 2006).

1.5 Sex Difference in CVD in T2D

Sex difference in CVD is well accepted (Miller, 2010). The lower rate of CVD in premenopausal women compared to age-matched men has been attributed to the effect of female sex hormones, specifically estrogen. Although adverse cardiovascular events in premenopausal women are lower compared to men, women seem to lose their inherent sex-based protection against CVD in diabetes (Barrett-Connor, 1994; Wilson et al., 1998). Furthermore, women are at higher risk of CVD in diabetes compared to diabetic men. Framingham study showed that symptomatic heart failure is 2.4 times higher in diabetic men and 5 times higher in diabetic women compared to the non-diabetic cohort (Kannel et al., 1974). In a meta-analysis, Huxley et al. reported that the risk of fatal coronary heart diseases is 50% higher in diabetic women compared to diabetic men (Huxley et al., 2006). The underlying mechanism of higher CVD in diabetic women and the role of sex hormone on female vascular function in T2D is poorly understood.

1.6 Estrogen and Vascular Reactivity

Estrogen or Oestrogen is a category of female ovarian sex steroids. There are three major endogenous estrogens: a) estrone b) estradiol c) estriol. Estradiol is more potent compared to the other two major endogenous estrogens (Kuhl, 2005). Estrogen has many biological activities that render its cardiovascular benefits, including lipid-lowering, antioxidant, fibrinolytic, and a series of activities on the blood vessel. Vascular effect of estrogen occurs through both genomic and non-genomic pathway through estrogen receptor (ER) mediated signal transduction. The genomic regulation of estrogen has a delayed onset and prolonged duration of action, whereas

the non-genomic action has a rapid onset and short duration of action. Classical genomic action of estrogen occurs through interaction with intracellular ER-α and/or ER-β and is followed by increased eNOS messenger ribonucleic acid (mRNA) translated in protein in the endothelial cells. On the other hand, the non-genomic regulation of estrogen may be transduced through G-protein coupled estrogen receptor (GPER1/GPR30) and a sub-family of membrane bound ER-α and ER-β. Estrogen through the membrane ERs activates the phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway and leads to eNOS activation by phosphorylation on Ser1177 site (Duckles & Miller, 2010).

While many studies reported E_2 might increase COX-mediated production of PGI_2 in endothelial cells (Khalil, 2010), contradictory reports of the impact of estrogen on COX-mediated control of vascular tone are also present. Calkin et al. reported that E_2 mediated rapid cholinergic vasodilation in cutaneous vasculature is likely mediated by COX-2 pathway in postmenopausal women (Calkin et al., 2002). On the contrary, Jiang et al. reported that E_2 mediated vasorelaxation in rabbit coronary artery does not change in the presence of a non-selective COX inhibitor indomethacin, suggesting no role of prostanoids in E_2 mediated vasodilation in coronary artery (Jiang et al., 1991). Therefore, the effect of estrogen on COX-mediated production of PGI_2 may depend on vascular bed and species. It was also suggested, estrogen may modulate the crosstalk between NOS and COX pathways where increased NO-mediated vasorelaxation could decrease COX-mediated components of vasorelaxation (Case & Davison, 1999).

Estrogen may play a significant role in controlling smooth muscle hyperpolarization and vasorelaxation of small resistance arteries. It was reported that acetylcholine (ACh) induced hyperpolarization in rat MA is higher in intact females and E_2 replaced ovariectomized (OVX)

females. In E_2 deficiency, OVX female rat MA showed increased NO release but decreased EDHF relaxation due to a reduction in gap junction proteins (Nawate et al., 2005). Therefore, it was suggested that the contribution of EDRF may alter in female vasculature depending on menopausal status.

1.7 Aerobic Exercise and Vascular Adaptation

Vascular effect of aerobic exercise may include structural or functional adaptation. The physiological stimulus of aerobic exercise to cause these adaptations is repeated bout of increased shear stress on the vascular endothelium. Endothelial shear stress is the tangential force derived by the friction of flowing blood on the endothelium. The concept that shear stress is the stimulus behind vascular adaptation is supported by different cell culture, animal, and human studies (Chatzizisis et al., 2007; Green, 2009; Laughlin et al., 2004). Although evidence suggests exercise training has a systemic effect on the arterial tree and enhance both conduit and resistance arterial function and remodeling in human, however, no clear pattern emerges regarding the impact of exercise training on different vascular beds (Padilla et al., 2011).

1.7.1 Shear Stress and Endothelial Function

Enhanced laminar shear stress in exercise training may help to improve vascular endothelial function and prevent CVD by a number of possible mechanisms. Increased shear stress in aerobic exercise could induce the release of several anti-atherogenic factors such as NO and PGI_2 which are not only well known EDRFs but also known for their inhibitory effect on platelet aggregation, leukocyte migration, and SMC proliferation and inflammation (Traub & Berk, 1998). Shear stress was also reported as an important inducer of EDHF mediated vasodilation in rat mesenteric artery (Takamura et al., 1999).

Shear stress is the most potent physiological stimulus that causes continuous production NO from the endothelium. The shear stress mediated endothelial NO production could be attributed to the activation of both genomic and non-genomic pathways. Increased NO production by shear stress could occur through PI3K/Akt pathway mediated posttranslational activation of eNOS by phosphorylation on Ser1179 site. Alternatively, shear stress could increase NO production through enhancing eNOS gene expression in endothelial cells (Traub & Berk, 1998). In arterial branches where shear stress is low or in areas of disturbed flow, NO bioavailability may decrease due to reduced eNOS gene expression (Gambillara et al., 2006; Harrison et al., 2006).

Shear stress may also lead to the production of another important EDRF and anti-platelet aggregating agent, PGI_2 (Frangos et al., 1985). It is believed that COX-2 is responsible for PGI_2 synthesis in cardiovascular system, and thus selective inhibition of COX-2 explains the increased atherothrombotic events. However, Kirkby et al. reported under physiological condition, COX-1, but not COX-2, is responsible for PGI_2 synthesis in both human and mouse aortic endothelial cells using both static condition and under shear stress (Kirkby et al., 2012).

The shear stress could lead to activation of different mechanoreceptors present on the endothelial cells such as the potassium channels. SK_{Ca} and IK_{Ca} were suggested to be involved in shear stress mediated EDHF relaxation in rat MA (Takamura et al., 1999). Another important smooth muscle hyperpolarizing factor, EET, was reported to be released from endothelium under the influence of shear stress and hyperpolarize underlying smooth muscle in rodent gracilis muscle artery and MA (Huang et al., 2005).

1.7.2 Shear Stress and Vascular Wall Structure

Repeated bouts of shear stress by aerobic exercise can modulate vascular wall characteristics. Low shear stress could reduce the production of NO and PGI_2 and upregulate ET-1 production and thus could lead to vascular atherosclerosis (Chatzizisis et al., 2007). Low shear stress can induce both constrictive and expansive remodeling of coronary artery which could lead to coronary atherosclerosis. Furthermore, reduced shear stress was reported to be inversely related to intima medial thickness in carotid artery (Gnasso et al., 1996). Medial thickness could be increased due to increase in extracellular matrix protein (elastin and collagen) degradation by matrix metalloproteinases (MMPs) and SMC migration, differentiation, and proliferation.

1.8 Rodent Model of Obesity and T2D

Metabolic diseases such as obesity, dyslipidemia, T2D and hypertension often occur in combination. Many hereditary rodent models have been developed to study metabolic diseases such as ob/ob mouse, db/db mouse, Goto-Kakizaki rat, Zucker diabetic fatty (ZDF) rat, Otsuka Long-Evans-Tokushima-fatty (OLETF) rat, Spontaneously Diabetic Torii (SDT) rat, and spontaneously hypertensive rat (SHR). In addition, in recent decades genetically modified animals have also been added to the research of metabolic diseases. All the models added key insights to our understanding of T2D and metabolic disorder. However, limitation remains as no single individual model can potentially manifest all the key characteristics of T2D in human.

Although monogenic mutation is rarely the cause of human obesity and T2D, monogenic rodent models are often used in T2D research. Although these models answered some important questions in diabetes pathogenesis in human, they may not help us completely understand the full spectra of the disease. Therefore, polygenic models are more appropriate in the study of

obesity and diabetic condition in human. Several polygenic models of T2D are available such as Goto-Kakizaki, OLETF rat, New Zealand obese (NZO) mouse. However, the problem with majority of the polygenic rodent models is female animals do not develop diabetes or they develop diabetes under conditions such as high-fat diet or environmental stress. GotoKakizaki model, although resembles human T2D in several aspects, but unlike most human T2D patients does not get obese. Goto-Kakizaki rat is an excellent model for studying non-obese human T2D. OLETF rat model has a distinct mutation that causes little to no diabetes in female rats. NZO mice model on the other hand, have very early obesity and poor fertility. Therefore, there is increased urgency in developing a polygenic model that could manifest more of the human T2D characteristics. Another recently developed versatile polygenic model is University of the California Davis Type-2 Diabetes Mellitus (UCD-T2DM) Rat model. UCD-T2DM rat model were generated by selectively breeding insulin resistant obese Sprague Dawley rat with ZDF lean rat which had a defective β-cell function. UCD-T2DM shows adult onset-obesity, insulin resistance, has preserved leptin function, fertility and develops T2DM on a low-fat, low-sugar diet, and both sexes are affected (Kleinert et al., 2018). Hence UCD-T2DM represents an etiology which is very similar to human T2D as shown in Table 1.1. However, the male and female rats of UCD-T2DM model differ in their age of diabetic onset, with male being affected earlier (4-8months) than females (8-12 months).

Table 1.1

UCD-T2DM vs. ZDF Rat vs. Human T2D Comparison

Characteristics	UCD-T2DM	ZDF	Human T2D
Origin of obesity	Polygenic	Monogenic	Polygenic
Age of onset of T2DM	Males: ~183 days Females: ~286 days	Males: ~60-90 days Females: ~60 days on diet	Adolescent/Adult
Fertility	Preserved	Infertile	Preserved
Leptin function	Preserved	Deficient	Preserved
Plasma triglyceride	Moderate	Moderate	Severe

Adopted from (Cummings et al., 2008).

1.9 Statement of Research Problems, Hypotheses, Objectives, and Innovation

It is now well known that men and women are different in the age-dependent onset of CVD, severity, and symptoms. Premenopausal women exhibit lower incidence of CVD compared to age-matched men. The reason behind the reduced CVD events in premenopausal women compared to men is often attributed to the protective effect of the female sex hormone, estrogen. However, premenopausal diabetic women not only lose the sex-based advantage against CVD but also suffer from higher CVD-related events and mortality compared to men (Kannel & McGee, 1979). The underlying mechanism(s) of the loss of sex-based protection and the timeline for this loss in diabetes is not well understood.

T2D is often preceded by prediabetes. Prediabetes is characterized by a higher-than-normal blood glucose level but not too high enough to be classified as diabetes. Vehkavaara et al. reported elevated fasting glucose in patients, although not as high as diabetes, may still induce vascular dysfunction (Vehkavaara et al., 1999). Although several studies previously investigated the underlying mechanisms by which vascular dysfunction occurs in diabetes, too little is known about the vascular dysfunction at early prediabetic state, especially with regards to specific effects of female sex hormones. Furthermore, currently to our knowledge, no study has examined the impact of estrogen on vascular function in the prediabetic state, and whether estrogen alters the contribution of EDRF (PGI_2, NO, and EDHF) in vascular relaxation in this state.

Based on our recent report (Shaligram et al., 2020) on sex disparity in the development of MA dysfunction in prediabetic and diabetic UCD-T2DM rats, our central hypothesis for the first study in this dissertation is that estrogen protects MA from vascular dysfunction in prediabetic female rats. We further hypothesize that estrogen preserves EDV by enhancing the contribution

of EDRF and/or decreasing the contribution of EDCF in UCD-T2DM prediabetic female rats. Our aims were to test the hypotheses mentioned above by determining the effects of 17-β estradiol replacement on the 1) vasorelaxation and contractile responses, 2) contribution of EDRF to vasorelaxation, and 3) levels of expression of specific targets (i.e., eNOS, ER-α/β, NADPH oxidases) which are associated with vascular signaling, in MA from OVX Sprague Dawley (SD) and OVX prediabetic UCD-T2DM female rats.

Regular aerobic exercise is a proven method for intervening endothelial dysfunction, insulin resistance, and cardiovascular risk in T2D (Okada et al., 2010). In addition, the effects of exercise on vascularization, function, inflammation, and its metabolic consequences, including diabetes and CVD, are also well identified (Disanzo & You, 2014; Ertek & Cicero, 2012; Rodrigues et al., 2019). However, there is still debate about the intensity (moderate-intensity aerobic exercise vs. high-intensity interval training) and type of exercise (i.e., aerobic or resistant exercise) in preventing CVD in diabetes. Furthermore, underlying mechanisms of exercise-mediated changes in diabetic vascular function and wall properties are poorly understood.

Previous report from our group suggests that the UCD-T2DM model exhibited an impairment of MA vasorelaxation possibly, in part, due to a loss of EDHF-type relaxation. However, whether exercise improves the MA vasorelaxation in male UCD-T2DM rats has not been studied. Thus, our hypothesis (for the second study in this dissertation) was that moderate-intensity aerobic exercise (MIE) induces beneficial effects on arterial function and structure, possibly by altering the contribution of EDRF and/or EDCF on vasorelaxation and changing the mechanical properties of the wall in MA from male UCD-T2DM rats. To test this hypothesis, we aimed to investigate the effects of eight weeks of MIE on the 1) ACh-induced EDV and vascular responses to contractile agents, 2) contribution of EDRF in vasorelaxation, 3)

expression of proteins associated with NO-, COX-, and EDHF-mediated vasorelaxation and vasoconstriction, and 4) pressure-induced change of myogenic tone, wall thickness and elastin content in arteries (such as MA and aorta) isolated from male UCD-T2DM rats.

Since UCD-T2DM is an excellent model that imitates human T2D pathophysiology, investigation of vascular dysfunction of this model could lead to novel mechanistic insights of CVD in diabetes. Therefore, the innovation of this study lies in uncovering the contribution of estrogen and moderate-intensity aerobic exercise on resistant MA changes in EDRF (PGI_2, NO, EDHF), wall structure, and myogenic tone in a novel rodent model of T2D.

Eventually, a better understanding of the mechanism of action of estrogen and exercise on diabetic blood vessels could lead to sex-specific new therapeutic approaches to prevent higher CVD events in diabetic and menopausal women.

CHAPTER 2: STUDY I: 17β-ESTRADIOL (E_2) IMPROVES ACH-INDUCED RELAXATION OF MESENTERIC ARTERIES IN OVARIECTOMIZED UC DAVIS TYPE 2 DIABETES MELLITUS (UCD-T2DM) RATS IN PREDIABETIC STATE.

2.1 Introduction

The prevalence of type-2 diabetes (T2D) is increasing at an alarming rate worldwide (Zheng et al., 2018). Cardiovascular diseases (CVD) are the primary cause of death and disability in diabetic patients (Einarson et al., 2018). It is now well established that sex difference exists in CVD onset, severity, and outcomes (Miller, 2010). The prevalence of CVD in premenopausal women is less than age-matched men. The female sex hormone, estrogen in particular, plays a role in the sex-specific cardiovascular protection in women (Barrett-Connor, 1994; Iorga et al., 2017). Several studies including ours suggest that diabetes affects male and female vascular bed differently (Akther et al., 2021; Lum-Naihe et al., 2017; Shaligram et al., 2020; Witcher et al., 2010; Zhang et al., 2012). Diabetes not only abolishes the female-specific cardiovascular protection but also increases the relative events of CVD in premenopausal diabetic women compared to diabetic men (Castro', n.d.; Peters et al., 2015), suggesting that hyperglycemia may overcome some of the beneficial effects of female sex hormones. However, the timeline of the loss of female-specific cardiovascular protection in diabetic premenopausal women is not well understood.

An appropriate animal model may provide critical insights into the pathogenesis of cardiovascular dysfunction in T2D. The current study was performed in mesenteric arteries (MA) of a polygenic T2D rat model, the UC Davis type-2 diabetes mellitus (UCD-T2DM) rat. This model exhibits polygenic adult-onset obesity, insulin resistance, pancreatic beta-cell

decompensation, and preserved leptin signaling and fertility (Cummings et al., 2008). We recently reported sex difference exists in the MA function of UCD-T2DM as early as the prediabetic state (Shaligram et al., 2020). Specifically, vasorelaxation to acetylcholine (ACh) was impaired to a greater extent in MA from males than in females at the prediabetic state. In contrast, the arteries from diabetic females exhibited a greater impairment to ACh-induced vasorelaxation when compared with the diabetic males. Here, we hypothesized that estrogen might be responsible for delaying vascular endothelial dysfunction in prediabetic females. Thus, in the current study, we investigated the effect of 17-β estradiol (E_2) administration on the MA function of ovariectomized (OVX) rats both at healthy and prediabetic states.

Endothelial dysfunction is considered a critical early sign of CVD. Endothelium-dependent vasorelaxation (EDV) can be used as a reproducible parameter to examine endothelial function and vascular health. Reduced EDV was reported in both type-1 and type-2 diabetes (Han et al., 2014; Shaligram et al., 2020; Zhang et al., 2012). Altered EDV could result from reduced release and/or synthesis of endothelium-derived relaxing factors (EDRF) [nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarization factor (EDHF)] and/or increased production of endothelium-derived contractile factors (EDCF) [i.e., thromboxane A2 (TXA_2) and endothelin (ET-1)].

The contribution of EDRF to EDV may vary based on the vascular bed. While in large conduit arteries, NO is the major contributor for vasorelaxation, in small resistance arteries, EDHF was found to be the major contributor for EDV (Félétou, 2011; Zhang et al., 2012). Previous reports from our group suggest diabetes alters NO and EDHF contribution in both large and small resistance arteries and a reported sex difference in vascular function of UCD-T2DM (Akther et al., 2021; Shaligram et al., 2020; Zhang et al., 2012). However, whether the observed

sex difference in vascular function was due to the influence of sex hormones, was not investigated. Among male and female sex hormones, estrogen has a pronounced effect on vasculature. Receptor-mediated estrogen signaling could increase genomic transcription of endothelial nitric oxide synthase (eNOS) as well as increase eNOS activation via phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway (Duckles & Miller, 2010). Estrogen was also reported to increase EDHF-type relaxation in mesenteric and uterine resistance arteries (Burger et al., 2009). However, the specific role of estrogen on NO and EDHF contribution in MA relaxation at the prediabetic state was not investigated.

This study demonstrates that E_2 replacement improved MA vasorelaxation in ovariectomized rats at prediabetic state. Here, we provide the first evidence of the specific role of E_2 in protecting MA from early vascular dysfunction in female prediabetic UCD-TDM rats.

2.2 Materials and Methods

2.2.1 Chemicals

All chemicals were purchased from either Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO, USA) and dissolved in water unless otherwise stated.

2.2.2 Experimental Animals:

The UCD-T2DM rats were generated by selectively breeding obese Sprague Dawley (SD) rats with Zucker Diabetic Fatty (ZDF) lean rats at the animal facility in the Department of Nutrition at the UC Davis (Cummings et al., 2008). This model closely resembles human T2D and shows polygenic adult onset of diabetes, preserved fertility, and leptin function, and has inherent dysfunction of pancreatic β-cell (Cummings et al., 2008, 2011).

For this study, we selected 13-14-weeks-old female UCD rats that had not yet developed diabetes (prediabetic group) but had higher body weight than controls. Furthermore, they were

considered prediabetic when the blood glucose readings of animals were in the range of 150-200mg/dl (Guglielmino et al., 2012). These prediabetic female rats were then surgically ovariectomized (OVX) and implanted subcutaneously with either E2 (1.5mg 17β-estradiol/pellet, 60-days uniform release) or placebo pellet (Innovative Research of America, Sarasota, FL). Similarly, age-matched and OVX SD females implanted subcutaneously with E_2 or placebo pellets were purchased from Charles River Laboratories (Wilmington, MA). In general, animals were divided into four experimental groups: (1) OVX + placebo [OVX], (2) OVX + E_2, (3) prediabetic OVX + placebo [PD OVX], (4) prediabetic OVX + E_2 [PD OVX+E_2]. After pellet implantation, both prediabetic and SD rats stayed at their respective facility (UC Davis and Charles Rivers) for one week. The rats were later transferred to animal facility at the University of the Pacific.

All the animals at the University of the Pacific vivarium were kept in a humidity and temperature-controlled room with a 12hrs light/dark cycle and ad libitum access to water and standard rodent chow (Mazuri rodent chow). The animals were euthanized using carbon dioxide at 49±4 days after the implantation of the pellets. The euthanasia processes were completed following AVMA Guidelines for the Euthanasia of Animals: 2013 edition and the NIH Guidelines for the Care and Use of Laboratory Animals: Eighth Edition. In addition, all animal protocols were approved by the Institutional Animal Care and Use Committee of the University of the Pacific and UC Davis and complied with the Guidelines for the Care and Use of Laboratory Animals: Eighth Edition (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) and ARRIVE guidelines (Kilkenny et al., 2010).

2.2.3 Blood and Plasma Analysis

A drop of blood from the tail vein was used to measure random blood glucose and triglyceride level by hand-held point of care devices. Glucose levels were measured by a standard glucometer (OneTouch UltraMini) and, triglyceride levels were measured by Accutrend Plus System (Roche Farma, Barcelona, Spain) using specific strips for each device.

Blood samples were also obtained by intracardiac puncture after euthanizing the animals and collected in tubes containing heparin and sodium citrate. The tubes were then centrifuged at 10000xg for 5min at 4°C, and plasma was collected from the top and aliquoted into fresh tubes to be stored at -80°C for later analysis. Plasma insulin and E_2 level were measured using ELISA kits according to the manufacturer's protocol (Mercodia, Uppsala, Sweden; Abcam, Cambridge, MA). In addition, blood collected by intracardiac puncture was also used for glycated hemoglobin (HbA1c) level analysis using A1cNow kit (PTS diagnostics, Sunnyvale, CA) and following the manufacturer's instructions.

2.2.4 Measurement of MA Tension

Third-order MA branches were isolated and cleared from veins, fat, and other surrounding tissues and cut into 2mm rings. Each ring was then mounted in an organ bath, between the two jaws of a wire myograph (model 610M, Danish Myo Technology, Denmark) with the help of two tungsten wires (diameter 40μm). The organ bath contained Krebs solution of (in mM) 119 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.023 EDTA, and 6 glucose at 37°C, bubbled with 95% O_2–5% CO_2. A computer-based data acquisition system was used to monitor the arterial tension (LabChart version 7.3.8, Powerlab; ADInstruments, Colorado Springs, CO). The arterial rings were normalized to a resting tension of 13.3KPa and equilibrated for 30 mins to obtain a basal tone. 80mM KCl solution was then

used twice to stimulate the arterial segments. Subsequently, ACh (10μM) induced relaxation was recorded in phenylephrine (PE, 2μM) precontracted arteries to test the viability of the endothelium.

2.2.4.1 Relaxation Responses to Ach

MA rings were contracted with PE 2μM, which produced about 80% of the maximal contraction. Increasing doses of ACh (10^{-8} to 10^{-5} M) were added to PE precontracted MA rings to obtain the concentration response curve (CRC).

The vascular relaxation responses to ACh (10^{-8} to 10^{-5} M) in rat MA rings were then obtained after 20 mins pretreatment with indomethacin (Indo, 10 μM, a blocker of the cyclooxygenase (COX)), followed by the addition of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 200 μM, NO synthase (NOS) blocker) and then a combination of Apamin [1 μM, small conductance calcium-activated potassium channel (SK_{Ca}) inhibitor] and TRAM-34 [1 μM, intermediate conductance calcium-activated potassium channel (IK_{Ca}) inhibitor].

2.2.4.2 Relaxation Responses to Sodium Nitroprusside (SNP)

The CRC to SNP (10^{-9} to 10^{-5} M) was obtained in MA precontracted with PE (2μM).

2.2.4.3 Contractile Responses to PE

The CRC to PE was obtained by the addition of increasing concentrations of PE (10^{-8} to 10^{-5} M) to the organ bath. The concentration of drugs used to generate relaxation or contraction curves was based on the standard protocol used by our group (Han et al., 2014; Shaligram et al., 2020; Zhang et al., 2012).

2.2.5 Western Blot Analysis

All tissue samples harvested after euthanizing the animals were flash-frozen by liquid nitrogen and saved at -80°C for later analysis. MA and skeletal muscle (SKM) samples were

micronized either using gentleMACs tissue dissociator (Miltenyi Biotech, Bergisch, Germany) and following preset protocol for protein extraction or micronized by freezing with liquid nitrogen and grinding with a mortar and pestle as previously described (Baena et al., 2015). For both processes, commercial RIPA buffer supplemented with phosphatase and protease inhibitor cocktail (ThermoFisher Scientific, Waltham, MA) was used to obtain the total protein extract from the tissues. Briefly, for processing by gentleMACs tissue dissociator, tissues were placed in M-tubes (Miltenyi Biotech, Bergisch, Germany) containing RIPA buffer, phosphatase, and protease inhibitor cocktail. Protein extraction protocol was selected from the gentleMACs menu, and after 1 min, the blended tissue extract was centrifuged at 15000xg for 15 min at 4°C, and supernatants were collected. When the gentleMACs tissue dissociator was not available, MA samples were micronized under liquid nitrogen to obtain total protein extract. The powdered MA were then put in tubes containing RIPA buffer and phosphatase and protease cocktail and incubated for 1.5h at 4°C. Finally, the mixtures were centrifuged at 15000xg for 15 min at 4°C, and supernatants were collected. Total protein concentration of the extract was determined by BCA gold assay (ThermoFisher Scientific, Waltham, MA).

20-30µg of protein for each sample was subjected to sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE). Proteins were then transferred to a 0.45 µm nitrocellulose membranes (Bio Rad Laboratories Inc., Hercules, CA), blocked for 1 h at room temperature with 5% w/v BSA in 0.1% Tween 20-Tris-buffered saline, and incubated overnight at 4°C with primary antibodies similarly as described by us (Akther et al., 2021). Primary antibodies for eNOS, AMP-activated kinase-α (AMPK-α), phospho-AMPK-α (p-AMPK-α, Thr172), and insulin receptor substrate-1 (IRS1) were obtained from cell signaling technology (Danvers, MA) and antibodies against estrogen receptor-β (ER- β), NADPH oxidase-1 (NOX-1),

glucose transporter-4 (GLUT-4) were obtained from Abcam (Cambridge, MA). All primary antibodies were diluted to 1:1000 except IRS-1, which was diluted to 1:500. After incubation with primary antibody, the membranes were washed 4 times and incubated for 1hr at room temperature with IRDye 680 Donkey anti-Rabbit IgG secondary antibody (dilution 1:10000; LI-COR, Lincoln, NE). Finally, after removing the secondary antibody, membranes were washed 4 times with TBS containing 0.1% Tween-20, and bands were detected using the LICOR Odyssey imaging system. The bands were quantified by densitometry using LI-COR Image Studio Lite software. To confirm the uniformity of protein loading, blots were incubated with GAPDH antibodies, normalized to GAPDH level, and expressed as fold changes from the control E_2 treated group.

2.2.6 Statistical Analysis

Vasodilation to ACh and SNP were expressed as percent relaxation response from maximum PE (2μM) contraction at each concentration. The concentration that produces half of the maximum relaxation (EC_{50}) was calculated by a sigmoidal dose-response model with variable slope by GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) and expressed as sensitivity to the agonist; pD_2 values ($-\log EC_{50}$). Maximum relaxation response to agonist was expressed as $R_{max,}$ and maximum tension to contractile agent such as PE was expressed as $Tension_{max}$. One-way ANOVA was used to compare the means between different groups (i.e., EC_{50}, R_{max}, Blood glucose level). When one-way ANOVA returned $P<0.05$, Tukey's post hoc test was used to identify the groups which are different from each other. Comparison of CRCs between different groups were done by two-way ANOVA followed by Tukey's post hoc test. Two-way ANOVA with repeated measures followed by Bonferroni's post hoc analysis was used

to compare the CRCs before and after treatment with drugs within a group. Statistical analysis of protein expression was performed by one-way ANOVA and Tukey's post hoc analysis.

2.3 Result

2.3.1 Effects of E_2 Treatment on Metabolic Parameters

E_2 pellets significantly increased the concentrations of plasma estradiol (Figure 2.1A). E_2 concentrations were significantly lower in OVX, and PD OVX rats than those in respective E_2 treated OVX rats. In agreement with Ferrer et al. and our previous report (Ferrer et al., 1996; Rahimian et al., 1997), the body weights of E_2 treated rats were significantly lower than those of non-treated rats at the time of euthanizing the animals. The body weight was 348.0±9.0 g in OVX and 218.2±5.9 g in OVX + E_2, and 444.3±11.3 g in PD OVX, and 323.0±16.6 g in PD OVX + E_2. Similar to body weight, the percent of intra-abdominal adipose tissue (located around mesentery and omental) was significantly lower in E_2 treated OVX and PD OVX groups (Figure 2.1F). Both body weights and abdominal adiposity in E_2 treated OVX groups were comparable to their respective intact SD female and prediabetic female rats in our previous report (Shaligram et al., 2020).

Compared to E_2 treated groups, the PD OVX group had a significantly higher glucose level (Figure 2.1C). Accordingly, HbA1c, plasma insulin, and triglyceride levels were significantly higher in the PD OVX group compared to all other experimental groups (Figure 2.1D, E, G).

2.3.2 Effects of E_2 Treatment on IRS1 and GLUT-4 Expression in SKM

The SKM is the major site for insulin-mediated glucose uptake. Although β-cell failure is a common characteristic of T2D, SKM insulin resistance and reduced glucose clearance are considered the initiating factors leading to overt hyperglycemia and β-cell dysfunction

(DeFronzo & Tripathy, 2009; Warram et al., 1990). Higher glucose and HbA1c levels, together with higher insulin level in the PD OVX group (Figure 2.1C, D, E), prompted us to analyze the expression of the primary insulin signal transducer, IRS1, and GLUT-4 in SKM. As shown in Figure 2.2A, IRS1 expression in SKM was significantly lower in the PD OVX group compared to the OVX and OVX+E_2 groups. There was no difference in IRS1 expression in the SKM of OVX and OVX +E_2. E_2 treatment increased IRS1 in SKM of the PD OVX group, although the difference did not reach to a significant level. When GLUT-4 expression was investigated, both the PD OVX and OVX groups showed significantly lower expression compared to the respective E_2 replaced group, regardless of the disease state (Figure 2.2B).

A previous report also indicates that E_2 may activate AMPK leading to an increase in glucose uptake in SKM in C57 BL/6 mice (D'Eon et al., 2005). Next, we investigated whether AMPK and phospho-AMPK (p-AMPK) levels in SKM were altered in E_2 treated groups. E_2 treatment significantly increased AMPK and p-AMPK levels in SKM (0.8- and 0.7-fold respectively) only in the PD OVX group compared to the respective E_2 treated control group (Figure 2.3 A-B).

2.3.3 Effects of E_2 Treatment on Relaxation Responses to ACh

ACh (10^{-8} –10^{-5} M) concentration-dependently relaxed PE-precontracted MA rings. There was no significant difference in ACh-induced relaxation in MA rings from OVX rats compared with those from OVX + E_2 rats, as indicated by no significant differences between R_{max} and pD_2 values of the OVX and OVX+E_2 CRCs to ACh (Figure 2.4 Table 2.2). However, the ACh CRC was markedly impaired in MA of the PD OVX group compared to those in OVX and OVX+E_2 groups (Figure 2.4). Both the R_{max} and PD_2 values were significantly lower in the MA of the PD OVX group when compared to the OVX and OVX+E_2 groups (Table 2.2).

However, E_2 treatment significantly enhanced the R_{max} and PD_2 to ACh in the PD OVX rats to the same level as seen in MA of OVX or OVX + E_2 (Figure 2.4, Table 2.2).

2.3.4 Effects of E_2 Treatment on the Relative Contribution of EDRF to MA Relaxation

The relative contribution of PGI_2, NO, and EDHF to vasorelaxation induced by ACh were determined by sequentially blocking COX, NOS, and a combination of SK_{Ca} and IK_{Ca} channels. Specifically, EDV to ACh (10^{-8} to 10^{-5} M) in rat MA rings precontracted with PE (2 μM) was obtained before and after pretreatment with Indo (10 μM), followed by the addition of L-NAME (200 μM) and then a combination of apamin (1 μM) and TRAM-34 (1 μM). The administration of Indo did not significantly change the ACh CRC in any of the groups when compared to no drug ACh CRC (Figure 2.5A-D). The addition of L-NAME significantly reduced the ACh relaxation in OVX and OVX+E_2 groups but did not completely block the relaxation. After the addition of L-NAME, the R_{max} to ACh was 75.3±9.4% in OVX and 96.7±1.4% in OVX + E_2 rats (Table 2.3). The remaining area under the curve (AUC) after the addition of L-NAME in the OVX and OVX + E_2 groups suggests a role of PGI_2-NO-independent relaxation responses in these groups. Finally, pretreatment of MA with apamin and TRAM-34 in the presence of Indo and L-NAME completely abolished the remaining ACh-induced vasorelaxation in these groups (Figures 2.5A-B)

On the other hand, after the addition of L-NAME, the ACh-induced relaxation was completely abolished in prediabetic groups, regardless of E_2 treatment (Figure 2.5C-D). However, the effect of L-NAME was more prominent in the E_2-treated prediabetic group compared with their respective control (OVX+ E_2) group. Specifically, ΔAUC between Indo and Indo+L-NAME CRCs in the PD OVX + E_2 group was significantly different from the

OVX+ E_2 and PD OVX group, suggesting an elevated role of NO-dependent relaxation responses in this group (Table 2.3, Indo+L-NAME column; Figure 2.5D, Pink shaded area)

2.3.5 Effects of E_2 Treatment on SNP-induced Relaxation

The MA sensitivity to NO was assessed by measuring the relaxation responses to increasing concentration of SNP (10^{-9} to 10^{-5} M). Similar to the effects on ACh-induced endothelium-dependent relaxation, SNP-induced relaxation was significantly impaired in PD OVX compared to that in MA of the OVX group. The pD_2 values to SNP were 6.59 ± 0.2 in OVX, and 3.6 ± 0.2 in PD OVX animals. The R_{max} to SNP in OVX and PD OVX animals was 86.6±3.0% and 19.1±4.3%, respectively. E_2 treatment enhanced the pD_2 and R_{max} to SNP in both OVX and PD OVX rats (Figure 2.6, Table 2.4). However, there was still a significant, rightward shift of SNP CRC in MA from PD OVX+E_2 rats relative to OVX+E_2 (Figure 2.6), as assessed by both pD_2 values and R_{max}. The pD_2 values to SNP was 8.17±0.2 in OVX+E_2, and 5.49±0.6 in PD OVX+E_2 animals. The R_{max} to SNP in OVX+E_2 and PD OVX+E_2 animals was 101.7±0.6% and 57.68±7.5%, respectively.

2.3.6 Effects of E_2 Treatment on PE-induced Contraction

Next, we examined whether an elevated contractile response of the arteries was responsible for reduced ACh and SNP relaxation in the PD OVX group. We determined the specific effects of E_2 treatment on contractile responses. Therefore, the CRCs to PE (10^{-8} to 10^{-5} M) were compared in MA among experimental groups (Figure 2.7). As shown in table 2.5, the maximum contractile response to PE ($Tension_{max}$) but not the sensitivity to PE was higher in MA of PD OVX and PD OVX+E_2 when compared to that in OVX and OVX+E_2. Furthermore, our data shows that E_2 treatment did not affect vasoconstrictor responses to PE, irrespective of the

health or disease status. There were no significant statistical differences in MA PE sensitivity (pD_2) and $Tension_{max}$ between OVX and OVX+E_2 or PD OVX and PD OVX+E_2.

2.3.7 Effects of E_2 Treatment on ER, eNOS and NOX-1 Expression

Previous reports, including ours (Thor, Zhang, et al., 2010) have shown that the vascular effects of E_2 are mediated via ER subtypes, and in part by augmenting the function of eNOS. Thus, next we analyzed the expression of ER in MA of our experimental groups, using Western blot analysis. There was no difference in ER-α expression between OVX and PD OVX groups regardless of E_2 treatment (Figure 2.9). However, ER-β expression significantly (~0.5 fold) increased the in PD OVX+E_2 groups when compared to that in PD OVX group (Figure 2.8 B).

To investigate the possible mechanisms underlying the elevated contractile responses (or decreased ACh responses) in PD OVX model, the protein expression of eNOS as well as NADPH oxidase (NOX) subtypes NOX-1, major source of superoxide in the small vessel wall arteries (Touyz et al., 2002) in MA were measured. Western blot analysis revealed that the expression of eNOS showed no significant difference between the PD OVX group and the OVX group, regardless of E_2 treatment (Figure 2.8A). However, as shown in Figure 2.10, NOX-1 expression was significantly elevated in MA tissues from PD group, ~6 fold in PD OVX and ~3.5 fold in PD OVX + E_2 compared with those in OVX and OVX + E_2. Although, the NOX-1 expression tended to be lower in PD OVX+E_2 than in PD OVX, the difference was not statistically significant.

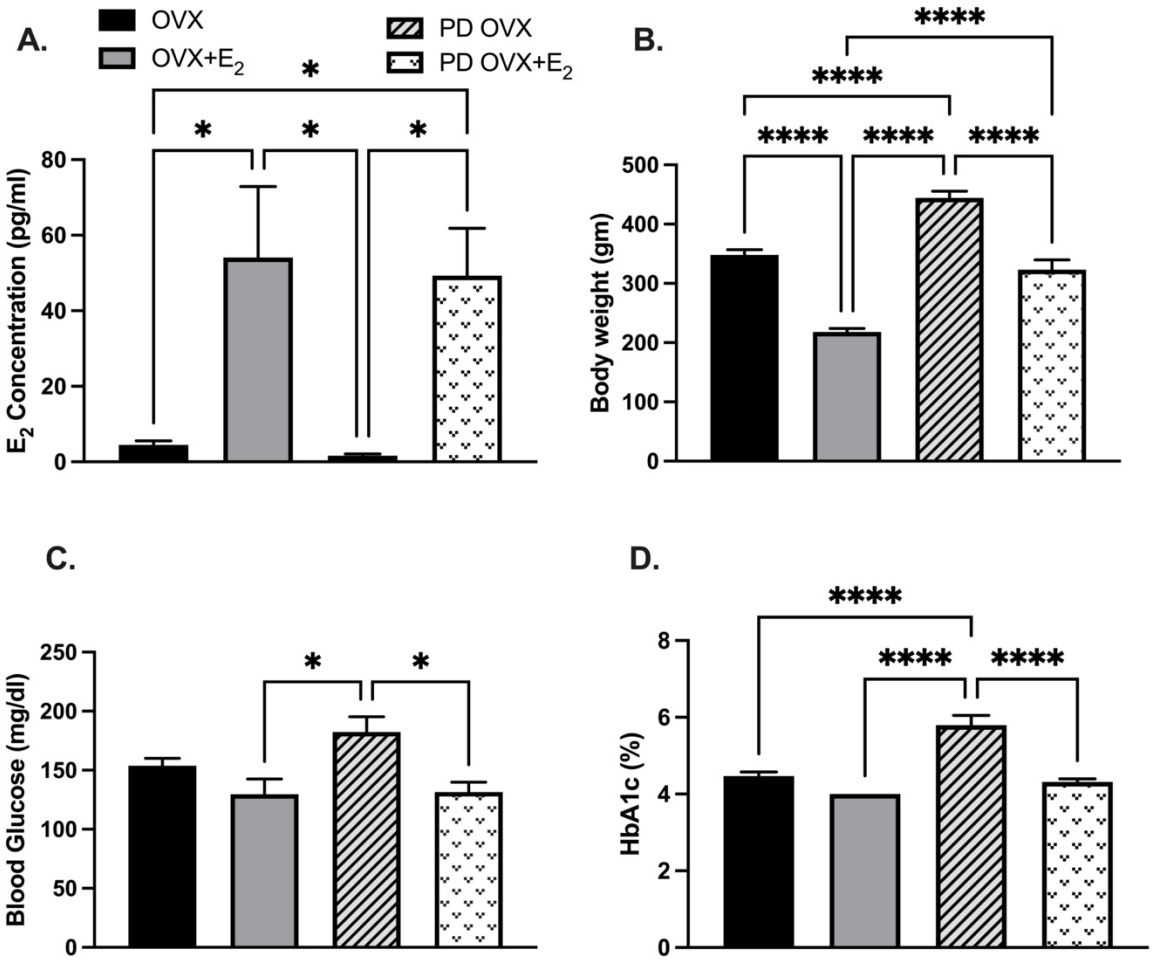

Figure 2.1 Metabolic parameters of ovariectomized and E_2 replaced SD control and prediabetic rats. (A) Plasma estradiol (E_2) concentration (B) Body weight (C) Blood glucose level (D) HbA1c (E) Plasma insulin level (F) Abdominal adipose tissue percentage to total body weight (G) Circulating triglyceride level (H) Plasma nitrate and nitrite level. Values are represented as mean ± SEM. Each bar represents the values obtained from n=5-6 animals per group. Capped lines indicate significant differences between two groups (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), analyzed by one-way ANOVA followed by Tukey's post hoc test.

(Figure 2.1 Continued)

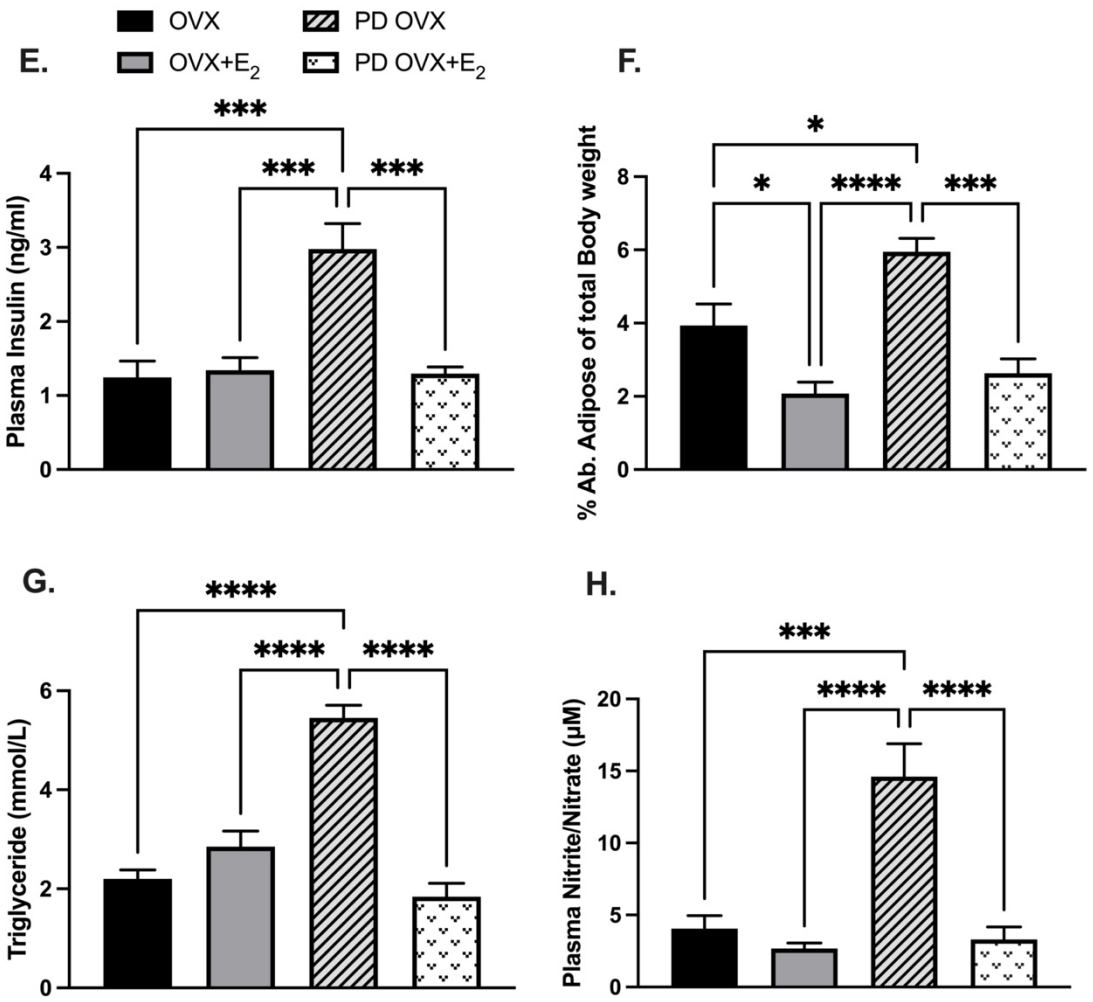

Table 2.1
Metabolic Parameters of Ovariectomized and E_2 Replaced SD Control and Prediabetic Rats

	OVX	OVX+E_2	PD OVX	PD OVX+E_2
Body Weight (g)	348.0±9.0	218.2±5.9#	444.3±11.3*#	323.0±16.6*$
Blood Glucose (mg/dl)	153.8±6.2	129.6±13.1	182.3±12.9*	131.5±8.3$
HbA1c (%)	4.47±0.1	4.0±0.0	5.80±0.2*#	4.31±0.1$
Abdominal Adipose Tissue (%)	3.94±0.6	2.08±0.3#	5.95±0.4*#	2.63±0.4$
Triglyceride (mmol/l)	2.2±0.2	2.85±0.3	5.45±0.3*#	1.93±0.3$
Plasma E_2 level (pg/ml)	4.47±1.1	58.08±18.8#$	1.60±0.5	49.27±12.6#$
Insulin (ng/ml)	1.25±0.2	1.34±0.2	2.98±0.3*#	1.3±0.1$
Plasma Nitrite+Nitrate (μM)	4.07±0.9	2.68±0.4	14.6±2.3*#	3.3±0.9$

Data are expressed as mean ± SEM; n=5-6 rats per group. #$p<0.05$ (vs. OVX), *$p<0.05$ (vs. OVX+E_2), \$$p<0.05$ (vs. PD OVX), analyzed using one-way ANOVA followed by Tukey's post hoc test.

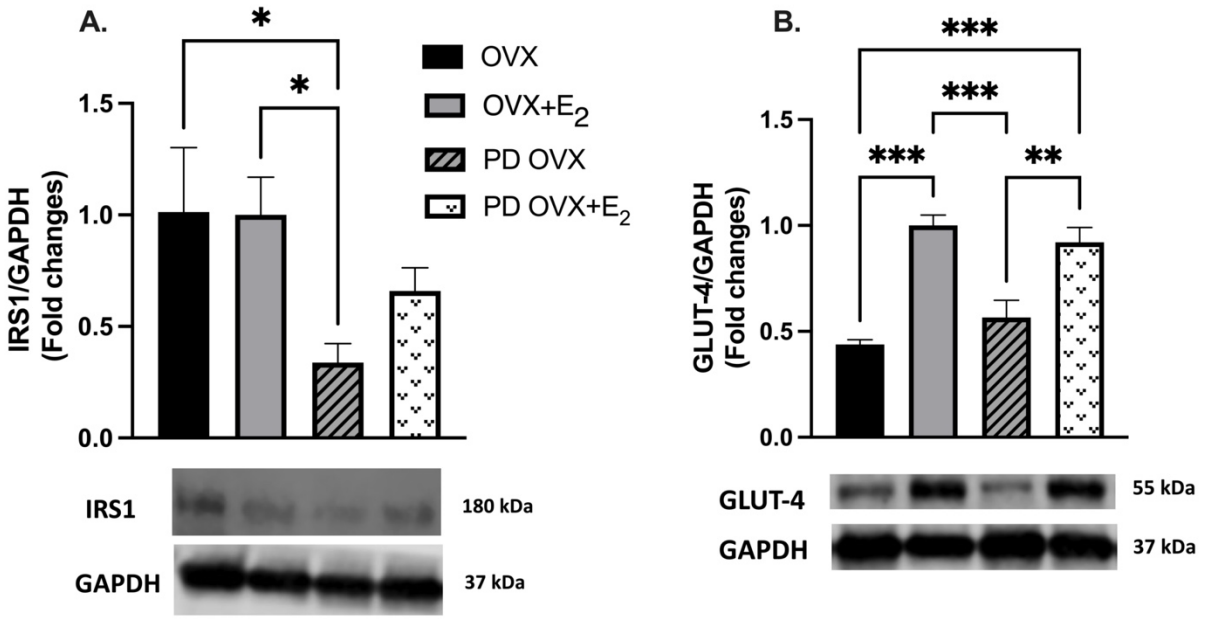

Figure 2.2 Western blot analysis of (A) IRS1 (B) GLUT-4 expression in skeletal muscle of ovariectomized and E_2 replaced SD control and prediabetic rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=4-5 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Capped lines indicate significant differences between two groups (*$p<0.05$, **$p<0.01$, ***$p<0.001$), analyzed using one-way ANOVA followed by Tukey's hoc test.

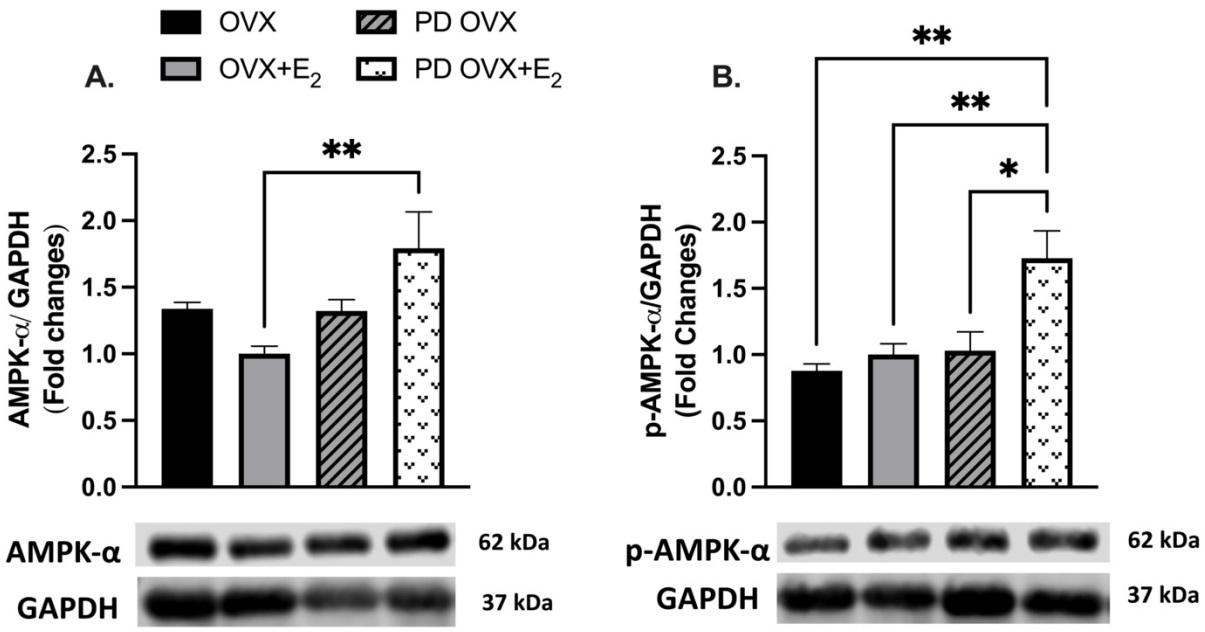

Figure 2.3 Western blot analysis of (A) AMPK-α (B) p-AMPK-α expression in skeletal muscle of ovariectomized and E_2 replaced SD control and prediabetic rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=4-5 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Capped lines indicate significant differences between two groups (*$p<0.05$, **$p<0.01$), analyzed using one-way ANOVA followed by Tukey's hoc test.

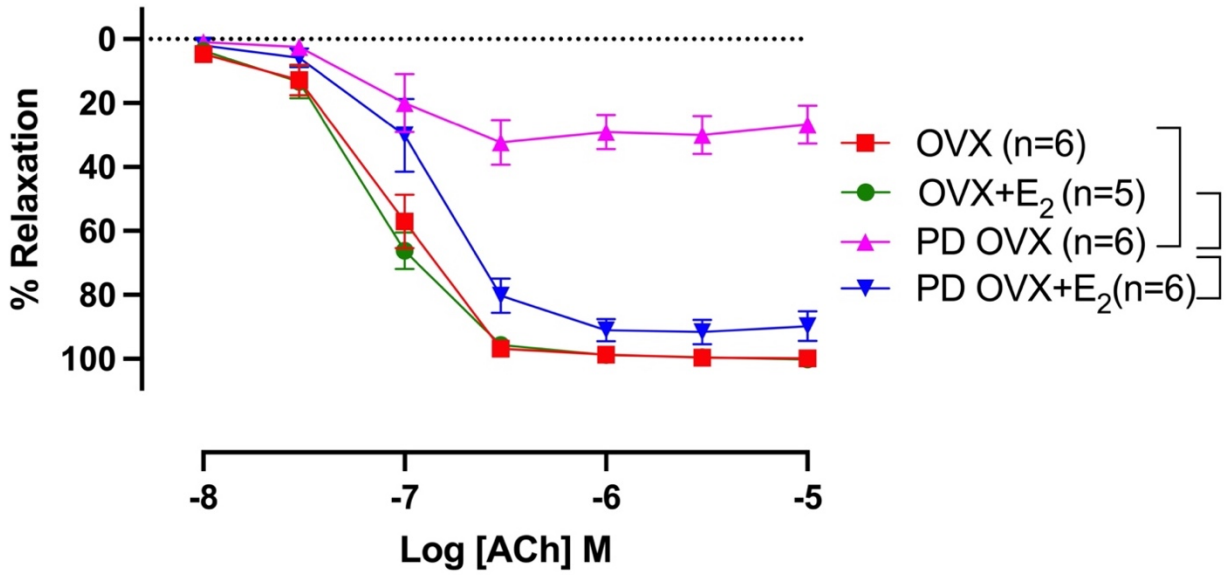

Figure 2.4 Relaxation responses to cumulative concentrations of acetylcholine (ACh, 10^{-8} to 10^{-5} M) in intact mesenteric arterial rings precontracted with phenylephrine (PE, 2µM) from ovariectomized and E_2 replaced SD control and prediabetic rats. Data are expressed as mean ± SEM. n=5-6 animals per group. Parenthesis indicates significant differences between groups (*p< 0.05) analyzed using 2-way ANOVA followed by Tukey's post hoc test.

Table 2.2

Sensitivity (pD_2: $-logEC_{50}$), and Maximum Response (R_{max}) to ACh in Mesenteric Arteries from Ovariectomized and E_2 Treated SD Control and Prediabetic Rats

ACh	**n**	**pD_2($-logEC_{50}$)**	**R_{max} (%)**
OVX	6	7.1±0.05	99.75±0.27
OVX+E_2	5	7.1±0.03	100.1±0.1
PD OVX	6	2.5±0.9*#	41.77±5.66*#
PD OVX+ E_2	6	6.8±0.07$	91.06±3.93$

Data are expressed as mean ± SEM; n=5-6 rats per group. #p<0.05 (vs. OVX), *p<0.05 (vs. OVX+E_2), $p<0.05 (vs. PD OVX), analyzed using one-way ANOVA followed by Tukey's post hoc test.

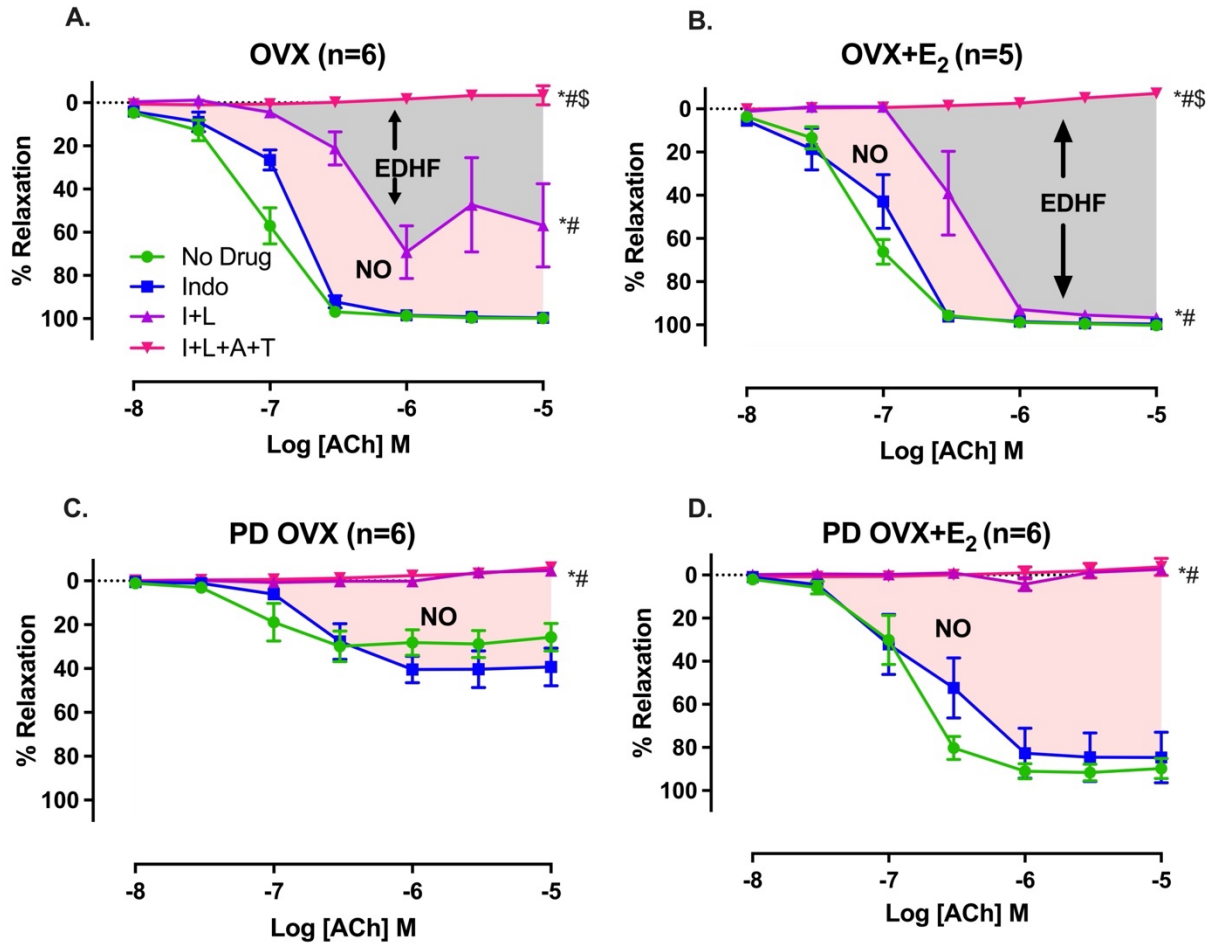

Figure 2.5 Effects of inhibiting cyclooxygenase, nitric oxide synthase, small conductance and intermediated conductance calcium-activated potassium channels (SK_{ca} and IK_{ca}) on acetylcholine (ACh)-induced vasorelaxation in mesenteric arteries taken from ovariectomized and E_2 replaced SD control and prediabetic rats; (A) OVX (B) OVX+E_2 (C) PD OVX (D) PD OVX+E_2.

Concentration response curve (CRC) to ACh (10^{-8} to 10^{-5}) was generated in the presence of no inhibitor (No drug) or in the presence of Indo, Indo+L-NAME (I+L), and Indo+L-NAME+Apamin+TRAM-34 (I+L+A+T) in mesenteric arterial rings. Indo (indomethacin; 10µM), L-NAME (N^{ω}-nitro-L-arginine methyl ester; 200µM), Apamin (1µM), TRAM-34; [1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole; 1µM]. * ($P<0.05$) vs. no drug; # ($P<0.05$) vs. Indo; $ ($P<0.05$) vs. Indo+L-NAME, analyzed using 2-way ANOVA with repeated measures followed by Bonferroni post hoc test.

Table 2.3

Sensitivity (pD_2: -logEC50), Maximum Response (R_{max}), and Area Under the Curve to ACh in Mesenteric Arteries from Ovariectomized and E_2 Replaced SD Control and Prediabetic Rats

Groups	No Drug			Indo			Indo+L-NAME			Indo+L-NAME+Apamin+TRAM-34		
	pD_2	R_{max},(%)	ΔAUC	pD_2	R_{max} (%)	ΔAUC	pD_2	R_{max} (%)	ΔAUC	pD_2	R_{max} (%)	ΔAUC
OVX	7.1±0.05	99.75±0.27	ND	6.88±0.1a	99.65±0.3	20.1±10.2	ND	75.3±9.4^{ab}	103.5±26.3	ND	1.73±3.7^{abc}	86.0±25.6
OVX+E_2	7.10±0.03	100.1±0.1	ND	7.06±0.1	99.56±0.2^{a}	8.8±13.7	ND	96.7±1.4	65.4±19.7	ND	-1.91±2.9^{abc}	139.6±15.3
PD OVX	2.5±0.9*#	41.77±5.66*#	ND	4.56±0.5*#	50.68±6.6*#	6.86±17.6	ND	1.88±1.1*#^{ab}	64.4±12.6	ND	-2.41±1.0^{abc}	3.7±1.7*#
PD OVX+E_2	6.8±0.07$	91.06±3.93$	ND	6.30±1.1$	85.0±11.3$	22.8±26.2	ND	4.23±2.9*#^{ab}	147.0±12.2*$	ND	-1.0±2.7^{ab}	3.5±3.2*#

A comparison of sensitivity (pD_2), maximum response (R_{max}) and ΔAUC to acetylcholine in the absence (no drug) or in the presence of Indo, Indo+L-NAME, and Indo+L-NAME+Apamin+TRAM-34in MA rings from mesenteric arteries taken from ovariectomized and E_2 replaced SD control and prediabetic rats. ΔAUC were measured by measuring the area between two curves. ΔAUC of Indo indicates COX contribution, ΔAUC of Indo+L-NAME indicates NO contribution and, ΔAUC of Indo+L-NAME+Apamin+TRAM-34 indicates EDHF-type contribution in ACh-induced vasorelaxation of MA.

Data are expressed as mean ± SEM; n=5-6 rats per group. Analysis between group: #$p<0.05$ (vs.OVX), *$p<0.05$ (vs. OVX+E_2), \$$p<0.05$ (vs. PD OVX), analyzed using one-way ANOVA followed by Tukey's post hoc test. Analysis within group: $^{a}p<0.05$ vs. no drug control within each group, $^{b}p<0.05$ vs. indo within each group (paired Student's t-test). ND; not determined.

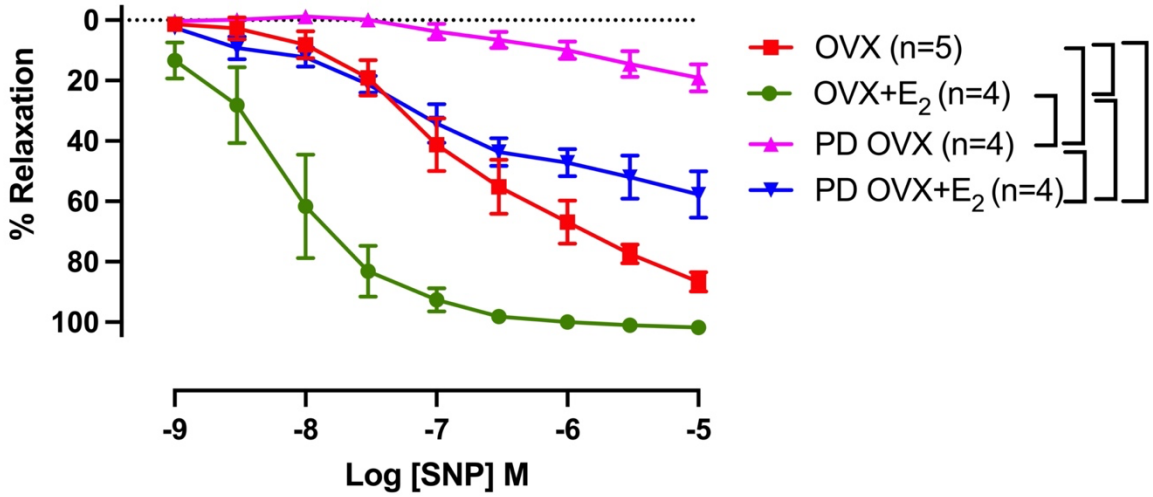

Figure 2.6 Relaxation responses to cumulative concentrations of sodium nitroprusside (SNP) (10^{-9} to 10^{-5} M) in intact mesenteric arterial rings pre-contracted with phenylephrine (PE, 2 μM) from ovariectomized and E_2 replaced SD control and prediabetic rats. Data are expressed as mean ± SEM. n= 4-5 per group. Parenthesis indicates significant difference ($p<0.05$) between groups analyzed using 2-way ANOVA followed by Tukey's post hoc test.

Table 2.4
Sensitivity (pD_2: -$logEC_{50}$), and Maximum Response (R_{max}) to Sodium Nitroprusside (SNP) in Mesenteric Arterial Rings from Ovariectomized and E_2 Replaced SD Control and Prediabetic Rats

SNP	**n**	**pD_2**	**R_{max} (%)**
OVX	5	6.59±0.2	86.6±3.0
OVX+E_2	4	8.17±0.2#	101.7±0.6
PD OVX	4	3.67±0.2*#	19.12±4.3*#
PD OVX+E_2	4	5.49±0.6*$	57.68±7.5*#$

Data are expressed as mean ± SEM; n=4-5 rats per group. #p<0.05 (vs. OVX), *p<0.05 (vs. OVX+E_2), $p<0.05 (vs. PD OVX), analyzed using one-way ANOVA followed by Tukey's post hoc test.

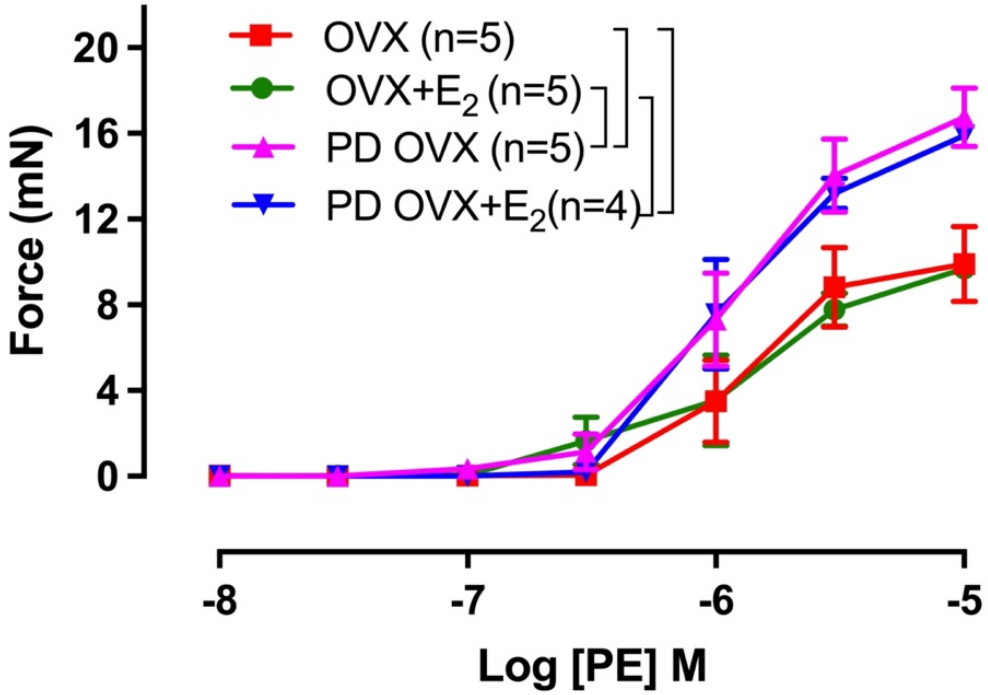

Figure 2.7 Concentration-response curves to phenylephrine (PE, 10^{-8} to 10^{-5} M) in intact mesenteric arterial (MA) rings of ovariectomized and E_2 replaced SD control and prediabetic rats. Data are expressed as mean ± SEM. n=4-5 per group. *$p<0.05$ between two groups analyzed using two-way ANOVA followed by Tukey's post hoc test.

Table 2.5

Sensitivity (pD_2: $-logEC_{50}$), and Maximum Tension ($Tension_{max}$) to Phenylephrine (PE) in Rat Mesenteric Arterial Rings from Ovariectomized and E_2 Replaced SD Control and Prediabetic Rats

PE	**n**	**pD_2**	**$Tension_{max}$ (mN)**
OVX	5	6.02±0.1	9.91±1.8
OVX+E_2	5	5.93±0.2	9.75±0.5
PD OVX	5	5.92±0.1	16.75±1.3*#
PD OVX+E_2	4	5.96±0.1	15.91±0.4*#

Data are expressed as mean ± SEM; n=4-5 rats per group. #$p<0.05$ (vs. OVX), *$p<0.05$ (vs. OVX+E_2), analyzed using one-way ANOVA followed by Tukey's post hoc test.

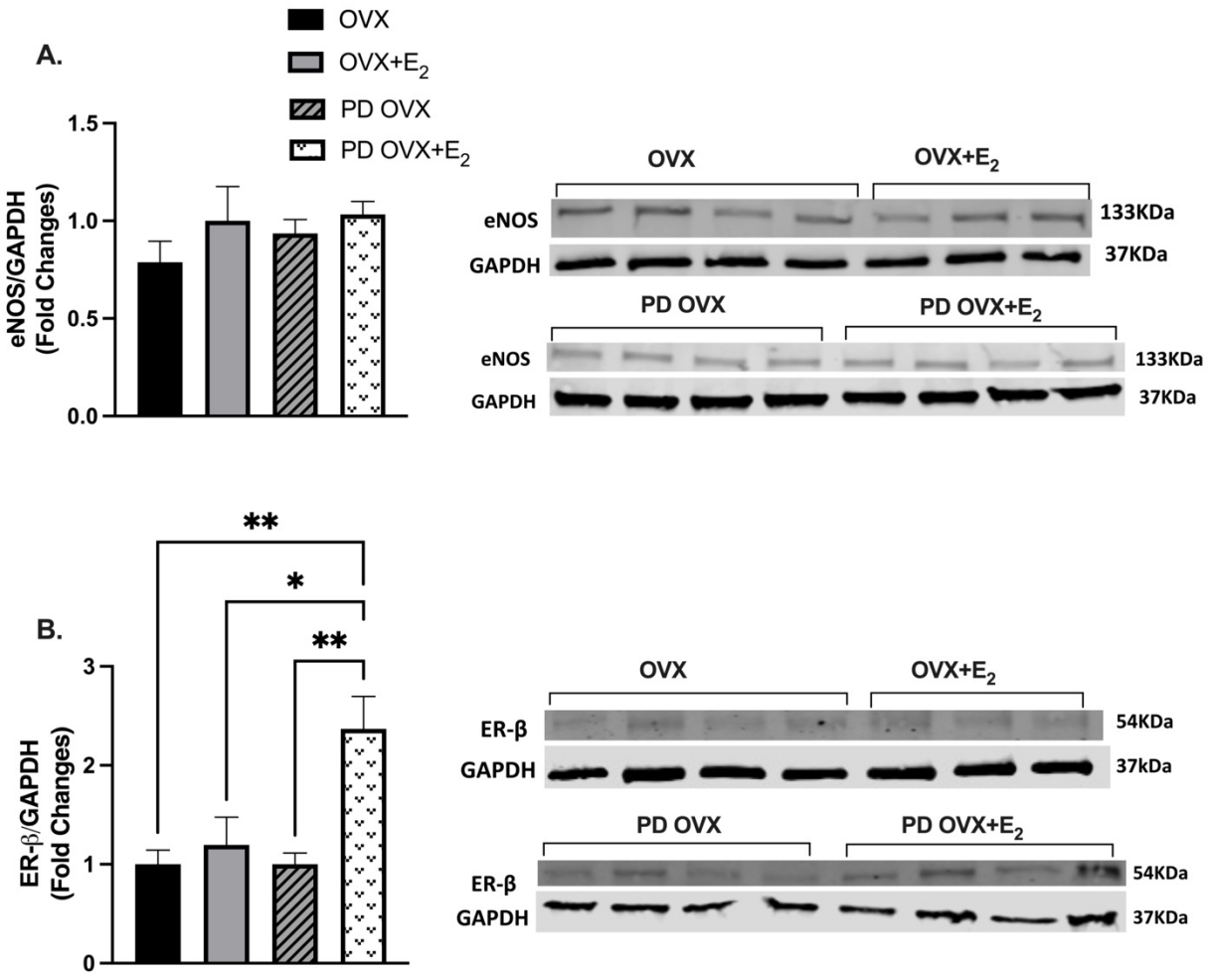

Figure 2.8 Western blot analysis of (A) eNOS (B) ER-β expression in mesenteric artery of ovariectomized and E_2 replaced SD control and prediabetic rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=3-4 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Capped lines indicate significant differences between two groups (*$p<0.05$, **$p<0.01$) as analyzed by one-way ANOVA followed by Tukey's post hoc test.

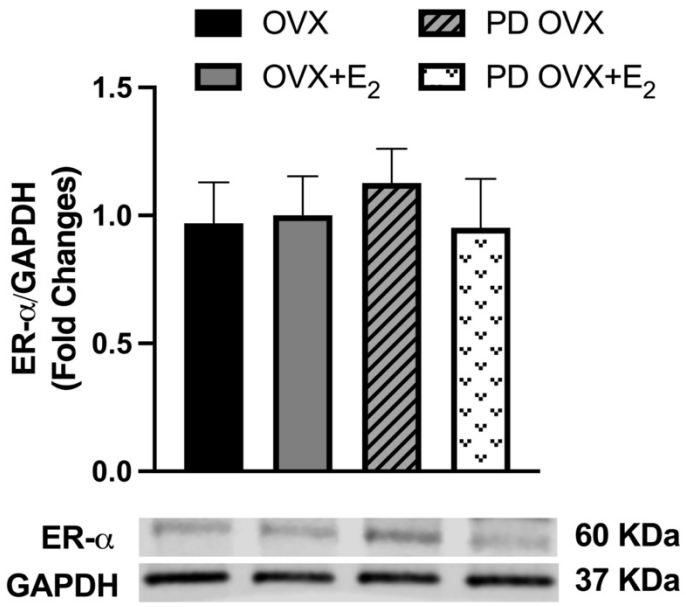

Figure 2.9 Western blot analysis of ER-α expression in mesenteric artery of ovariectomized and E_2 replaced SD control and prediabetic rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=3-4 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Data was analyzed by one-way ANOVA followed by Tukey's post hoc test.

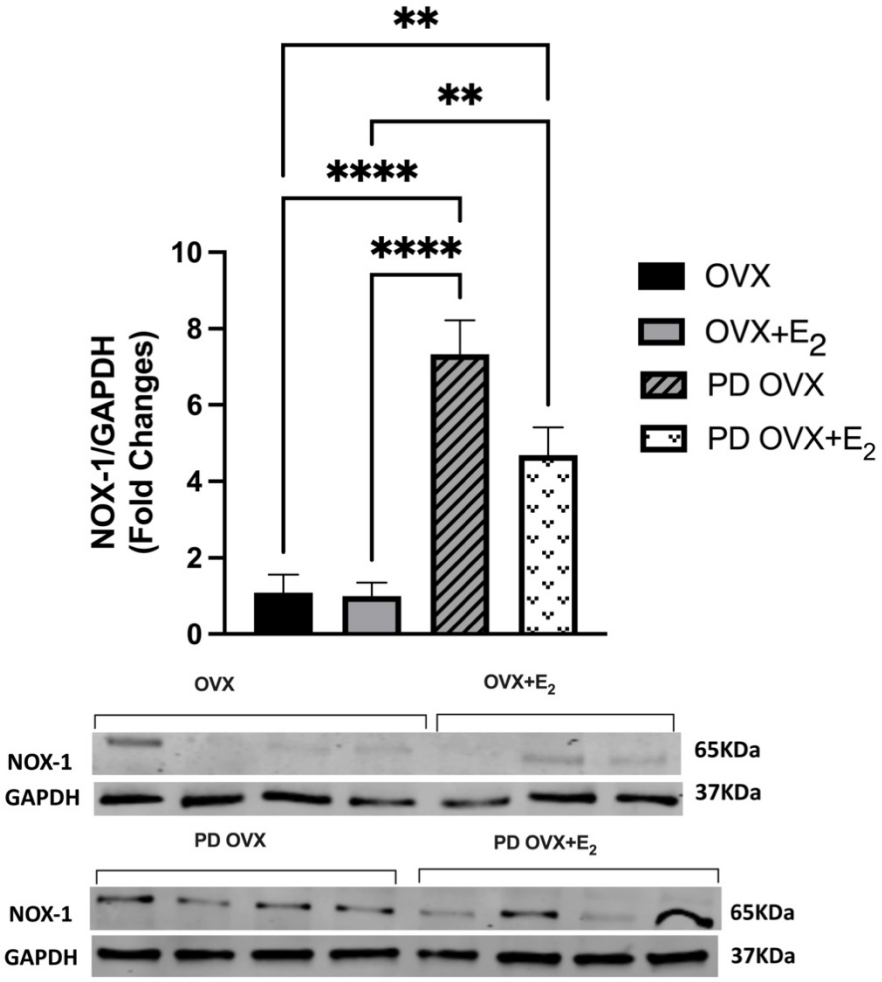

Figure 2.10 Western blot analysis of NOX-1 expression in mesenteric artery of ovariectomized and E_2 replaced SD control and prediabetic rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=3-4 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Capped lines indicate significant differences between two groups (**$p<0.01$, ****$p<0.0001$) as analyzed by one-way ANOVA followed by Tukey's post hoc test.

2.4 Discussion

Here, we set out to study the influence of E_2 replacement on MA function in OVX female UCD-T2DM rats at the prediabetic stage. We observed that ACh relaxation responses were impaired in MA of OVX prediabetic UCD-T2DM rats, and E_2 treatment improved MA relaxation in this group. Additionally, we showed that the improved vasorelaxation of MA in E_2 treated PD OVX rats could be in part due to enhanced contribution of NO to ACh responses.

Lack of estrogen in postmenopausal women results from loss of ovarian activity. Postmenopausal women are at higher risk for metabolic and cardiovascular abnormalities such as obesity and T2D (Chen et al., 2019; Mauvais-Jarvis et al., 2017; Sternfeld et al., 2005). Changes in lipid metabolism, and body fat composition and distribution with estrogen deficiency are thought to be the main factors contributing to an elevated risk of metabolic syndrome and CVD in postmenopausal women compared to premenopausal individuals (Lovejoy et al., 2008; Mauvais-Jarvis et al., 2017). In the present study, we used OVX female rats to evaluate the effects of ovarian hormone deficiency and E_2 replacement on the metabolic and vascular function, as well as the expression of specific markers involved in insulin signaling in healthy and prediabetic UCD-T2DM rats.

Both OVX and PD OVX groups exhibited significantly higher body weight compared to their respective E_2-treated groups. In addition, when the abdominal fat percentage to total body weight was compared, the PD OVX group showed significantly higher abdominal fat compared to all other experimental groups. E_2 treatment, however, significantly reduced abdominal obesity in both OVX and PD OVX rats, indicating a potential role of estrogen in reducing abdominal obesity. It was reported that an increased fat and body mass occur as a result of ovariectomy (Latour et al., 2001; Laudenslager et al., 1980; Rachoń et al., 2007; Richard et al., 1987).

Previous studies have shown that E_2 replacement in OVX rodents decreases abdominal fat accumulation, improves serum lipid profiles (Shinoda et al., 2002; Wu et al., 2004), and restores insulin action on muscle glucose transport (Kumagai et al., 1993; Puah & Bailey, 1985). Along similar lines, Ritland et al. reported in postmenopausal women, central obesity could be related to a decrease in adiponectin levels in serum (Ritland et al., 2008). However, in another report, Babaei et al. showed that ovarian hormone withdrawal leads to higher body weight and visceral adipose tissue in rats but surprisingly does not change adiponectin levels (Babaei et al., 2010). In Babaei's report, although a substantial decrease in body weight was achieved by estrogen replacement in OVX animals, the beneficial metabolic effects of weight loss seem to be related to a tendency in improving insulin sensitivity without elevating adiponectin production.

The underlying molecular and cellular mechanisms of the metabolic actions of estrogen are poorly understood. Much of our current view of the actions of estrogen on adiposity and metabolism derives from the ovariectomized rodent model of menopause. In the present study, we did not measure adiponectin level; however, when glucose and HbA1c levels were compared among the experimental groups, the PD OVX group showed significantly higher glucose and HBA1c levels compared to both E_2 treated groups. Although the OVX group showed a slight increase in glucose and HbA1C levels compared to those in the OVX + E_2 group, the difference didn't reach to a significant level.

The correlation between menopause and dysglycemia remains controversial. Kim et al. reported no association of natural menopause with T2D (Kim et al., 2011). However, Heianza et al. reported postmenopausal status is associated with dysglycemia independent of age (Heianza et al., 2013). Ovariectomy in rats has been reported to cause decreased (Kumagai et al., 1993; Rincon et al., 1996) or unchanged glucose tolerance tests (Latour et al., 2001). Decreased

insulin-stimulated glucose transport in SKM of OVX rodents has also been reported (Kumagai et al., 1993; Puah & Bailey, 1985).

Chronically elevated insulin without hypoglycemia is common in obesity and metabolic disorders, and it is referred to as hyperinsulinemia. Prior to overt metabolic disorder, abnormally elevated lipid level and hyperinsulinemia are reported (Erion & Corkey, 2017). In subjects with obesity but without diabetes, hyperinsulinemia and insulin hypersecretion are more prevalent than insulin resistance (Ferrannini et al., 1997; Thomas et al., 2019; Weyer et al., 2000) and hence may precede and contribute to insulin resistance. Furthermore, it has been shown that hyperinsulinemia is self-perpetuating and is more likely to be a primary defect rather than a compensation for insulin resistance in the general population (Mari et al., 2011; Tricò et al., 2018). Here, we observed a significant increase in triglyceride and insulin levels in the PD OVX group. Although the intact prediabetic groups were not included in the current study, our recent report on metabolic parameters in intact prediabetic male and female rats showed comparable metabolic values to those observed in PD OVX and PD OVX+E_2, respectively (Shaligram et al., 2020). Overall, these data would appear to support E_2 protection against weight gain, central adiposity, dyslipidemia, and hyperglycemia.

Insulin resistance is an important characteristic in the pathogenesis of T2D. SKM is the primary organ which under normoglycemic conditions is responsible for approximately 80% of insulin-mediated glucose uptake (DeFronzo & Tripathy, 2009; Klip & Pâquet, 1990). Therefore, SKM has been shown to play an important role in determining glycemic status. Consistent with the increases in circulating glucose and insulin levels in PD OVX, the expression of IRS1, a substrate of insulin receptor tyrosine kinase with a central role in the insulin signaling pathway and glucose uptake in SKM (Kovacs et al., 2003), was significantly low in SKM of this group.

Remarkably, we also observed a lower level of GLUT-4 expression in SKM of OVX groups compared to E_2 treated groups, regardless of disease status. Along similar lines, Gorres et al. recently reported estrogen treatment improved insulin signaling and enhanced SKM glucose uptake in OVX SD rats (Gorres et al., 2011).

An alternative pathway for GLUT-4 translocation to the membrane and increasing glucose transport, independent of insulin, is by phosphorylation of AMP-activated protein kinase (AMPK) (Gorres et al., 2011). AMPK is an energy-sensing enzyme that is activated when cellular energy levels are low, and it signals to stimulate glucose uptake in SKM, among other insulin-sensitizing effects. There is substantial evidence suggesting that AMPK is dysregulated in animals and humans with T2D and that AMPK activation (physiological or pharmacological or hormonal) could improve insulin sensitivity (Coughlan et al., 2014). E_2 treatment is shown to increase AMPK activation in C2C12 myotubes (D'Eon et al., 2008). However, in the current study, E_2 treatment only increased phosphorylated AMPK-α (p-AMPK-α) in the PD OVX group but not in the OVX. Although we did not attempt to elucidate the underlying mechanisms responsible for the enhancement of p-AMPK-α by E_2 treatment in the SKM of the PD OVX group, D'Eon et al. reported that E_2 promotes the partitioning of free fatty acids toward oxidation and away from triglyceride storage in SKM by up-regulating the expression of peroxisome proliferation activator receptor-δ (PPAR-δ) and its downstream targets and also by directly and rapidly activating AMP-activated protein kinase (D'Eon et al., 2005). Thus, the elevated SKM expression of p-AMPK-α observed in E_2-treated PD OVX may suggest that at the elevated glycemic condition, female sex hormone, in particular E_2, may have a beneficial effect in lowering glucose levels, in part, by AMPK activation.

In the current study, we observed a comparable trend between increased SKM GLUT-4 and p-AMPK expression and improved vascular function, as ACh relaxation was significantly enhanced in E_2 treated OVX during the prediabetic stage.

Endothelial dysfunction is considered the initiating factor for vascular complications in diabetes (Bakker et al., 2009; Eringa et al., 2013; Takenouchi et al., 2009). A commonly used technique to investigate endothelial dysfunction is by investigating EDV. Here we specifically demonstrated that E_2 replacement did not cause any changes in EDV in the ovariectomized rats. The ACh Emax were 99.7± 0.2% and 100.1 ± 0.1% in arteries of OVX and OVX + E_2, respectively. It is also important to note that there were no statistical differences in the ACh pD_2 values between OVX and OVX+E_2, and the values were in line with our recent report in normoglycemic male and female arteries (Shaligram et al., 2020). Therefore, our data suggest that ovariectomy for 7 weeks did not cause any EDV dysfunction in healthy controls. It has been reported that endothelial dysfunction gradually increases following ovariectomy, and the maximal effects of ovariectomy on vasculature could be overserved at 12 weeks in normoglycemic rats (Moien-Afshari et al., 2003). In the current study, the EDV was however significantly impaired in PD OVX groups. Accordingly, Vehkavaara et al. reported that a higher glucose level, but not as high as the diabetic state, could still lead to vascular dysfunction (Vehkavaara et al., 1999). We specifically showed that in absence of E_2, relaxation responses in MA of OVX prediabetic animals were significantly impaired. However, an intriguing observation of this study was that E_2 replacement restored the relaxation responses in the PD OVX group (Figure 2.4, Table 2.2). These observations are in line with our recent report showing that vascular relaxation to ACh was impaired to a greater extent in MA from males in the prediabetic stage than in their female counterparts (Shaligram et al., 2020).

Sex difference in vascular function is well studied (Miller, 2010; Shaligram et al., 2020). However, T2D abolishes the sex based protective effect against cardiovascular diseases in premenopausal women (Steinberg et al., 2000). Here, our data reinforces our hypothesis that female sex hormones may, in fact protect the effect of prediabetes on endothelial function. To the best of our knowledge, this study is the first to characterize the role of E_2 on vascular function as early as the prediabetic stage.

It has been shown that both NO-dependent and NO-independent mechanisms are involved in rat mesenteric arterial relaxation (Zygmunt et al., 1995). However, the specific role of E_2 on NO and NO-independent pathways in the prediabetic state is not clear. In the present study, we showed that the effects of Indo and L-NAME on mesenteric artery vasorelaxation in ovariectomized rats were dependent on disease as well as the presence of E_2. The inhibition of COX metabolites by Indo slightly but not significantly reduced sensitivity to ACh-mediated mesenteric relaxation in all four experimental groups, suggesting that metabolites in the COX pathway play only a minor role in EDV of mesenteric arteries. Consistent with this interpretation are data demonstrating that COX metabolites have a less important role in the relaxation of smaller arteries, such as mesenteric arteries (Shimokawa et al., 1996). When L-NAME is administrated after Indo, the further reduction in EDV is generally considered to represent the role of NO. Any remaining EDV to ACh after incubation with Indo and L-NAME is referred to as the L-NAME/Indo-insensitive component, or EDHF-type relaxation (Feletou & Vanhoutte, 1988; Komori et al., 1988). This remaining EDV can be subsequently blocked by inhibiting small and intermediate conductance calcium-activated potassium channels (SK_{Ca} and IK_{Ca}) (Doughty et al., 1999).

In all four experimental groups, the addition of L-NAME led to a further reduction of EDV (Figure 2.5A-D). However, the added effect of L-NAME in blunting ACh-mediated vasodilation was much more prominent and completely blocked the remaining relaxation responses to ACh in prediabetic arteries when compared with their respective control (Figure 2.5C-D). Overall, these data suggest that in OVX control groups, regardless of E_2 replacement, both NO and EDHF contributed to the MA relaxation responses (Figure 2.5A-B, Table 2.3). This observation is also in line with published reports by us and others who investigated ACh relaxation in mesenteric arteries of intact male and female rats (McCulloch & Randall, 1998; Shaligram et al., 2020; Zhang et al., 2012). Here, we show that in prediabetic animals, NO is the only contributor to mesenteric relaxation and EDHF type relaxation is absent, regardless of presence or absence of E_2. The alteration of EDHF contribution in the small arteries of diabetic animals has been shown by other investigators and us (Fukao et al., 1997; Oniki et al., 2006; Shi et al., 2006). Therefore, the impairment of relaxation responses to ACh observed in the OVX prediabetic rats may, in part, be due to loss of EDHF-mediated relaxation, which is one of the major vasodilatory mediators in arteries in these groups. However, an intriguing observation of this study was that E_2 replacement significantly increased the NO contribution to ACh vasorelaxation in prediabetic group (PD OVX+E_2) when compared to their respective controls (PD OVX or OVX+E_2). Therefore, it is possible that observed enhanced relaxation in the E_2 treated prediabetic models could be mediated by the elevated contribution of NO in vascular relaxation in this group.

Several investigators, including our group (Thor, Uchizono, et al., 2010; Lahm et al., 2008; Montgomery et al., 2003), have previously shown that the vascular effects of E_2 are mediated via ER-α and ER-β subtypes, and in part by augmenting the function of eNOS (Lantin-

Hermoso et al., 1997; Weiner et al., 1994). Here, we didn't directly measure eNOS activity, but our data show that the expression of ER-β (but not ER-α) was enhanced while the expression of eNOS was not changed in MA from E_2 treated OVX prediabetic rats compared to placebo-treated prediabetic rats. Additional studies will be needed to document the relative importance of NO to elevated relaxation responses in E_2 treated prediabetic rats.

In addition to a possible role for altered contributions of endothelial NO, other mechanisms that could explain the improved vascular function of prediabetes in the presence of E_2 may include factors such as enhanced sensitivity of MA to NO or decreased vasoconstrictor response to agents such as PE. Here, we observed that MA relaxation to SNP, an indicator of arterial sensitivity to NO, was clearly impaired in the prediabetic models. Therefore, the impaired responses to ACh in prediabetic models may in part occur at NO interaction with MA. This finding is in agreement with our recent report on impairment of SNP-induced relaxation in both prediabetic males and females (Shaligram et al., 2020). However, interestingly, E_2 replacement significantly increased the SNP-induced relaxation in both healthy and prediabetic groups when compared to their respective controls (OVX and PD OVX), suggesting that enhanced sensitivity of MA to NO in E_2 treated prediabetic arteries may in part be responsible for the enhanced vasorelaxation in this group.

We also examined the vasoconstrictor response to PE, an α-adrenoceptor agonist. We showed no changes in the sensitivity to PE in rat mesenteric arteries of prediabetic groups compared with non-prediabetic groups, irrespective of the presence or absence of E_2. However, maximal contractile responses were significantly higher in prediabetic groups compared with non-prediabetics, regardless of E_2 treatment.

Theoretically, the enhanced responses to PE observed in prediabetic models (Figure 2.7, Table 2.5) may partially result from a decreased release of relaxing factors (NO or EDHF), a reduced sensitivity of MA to NO, or an enhanced release of contracting factors.

Here, we did not measure endothelium-derived contractile factors. Still, consistent with our working hypothesis are data demonstrating that expression levels of NOX-1 catalytic subunit of NADPH oxidases, the major source of superoxide in the vessel wall, was significantly elevated in MA taken from prediabetic rats compared with healthy rats. Although the functional consequence of elevated NOX-1 expression in MA of prediabetic rats was not investigated, these data were in line with elevated PE responses in these groups. In diabetes, superoxide production has been shown to play an important role in activating endothelium-derived contracting factors-mediated responses (Shi et al., 2007; Shi & Vanhoutte, 2008; Vanhoutte & Tang, 2008; Zhang et al., 2012). Interestingly, in prediabetic groups, E_2 treatment resulted in a decrease in the NOX-1 level, although the difference was not statistically significant compared to the absence of E_2.

In summary, we have shown that mesenteric endothelial function in ovariectomized prediabetic rats was severely impaired. Furthermore, this study suggests that the predisposition of ovariectomized rat mesenteric arteries to vascular injury at the prediabetic state may be due to a shift away from a putative EDHF, initially the major vasodilatory factor, toward a reliance on NO. However, our data showed that the E_2 replacement preserved vasorelaxation in prediabetic rats. Thus, the basis for the protective effects of E_2 on the vascular relaxation may be partly attributed to the enhancement of NO sensitivity and its contribution to vascular relaxation as well as improved insulin signaling and elevated SKM glucose uptake in this group. Overall, the shift from EDHF-type relaxation towards a greater reliance on NO in estrogen replaced prediabetic

group could be a possible compensatory mechanism to protect female arteries from early vascular dysfunction.

CHAPTER 3: STUDY II: EFFECTS OF MODERATE-INTENSITY AEROBIC EXERCISE (MIE) ON MESENTERIC ARTERIAL FUNCTION AND WALL STRUCTURE IN MALE UC DAVIS TYPE 2 DIABETES MELLITUS (UCD-T2DM) RATS

3.1 Introduction

The prevalence of type-2 diabetes (T2D) is rising globally due to obesity and physical inactivity overlaid on a genetic predisposition, among other factors. It is generally believed that T2D and cardiovascular diseases (CVD) can be prevented and managed with healthy food choices and routine physical activity. Exercise prevents CVD by reducing cardiometabolic risk factors (i.e., insulin resistance, glucose intolerance, circulating lipid concentration) and improving cardiovascular function and adaptation (Padilla et al., 2011; Pinckard et al., 2019). Although regular aerobic exercise is a proven method for intervening endothelial dysfunction, insulin resistance, and cardiovascular risk in T2D (Okada et al., 2010), there is still debate about the intensity and type of exercise required to prevent CVD in diabetes.

Increased CVD risk in diabetes is associated with vascular structural, mechanical, and functional alteration. These alterations include increased wall to lumen ratio and stiffness, impaired endothelium dependent vasodilation (EDV), and enhanced responses to vasoconstricting agents (Bagi et al., 2005; De Vriese et al., 2000; Schofield et al., 2002; Souza-Smith et al., 2011). Endothelial dysfunction and impaired EDV, which precede overt CVD in diabetes, can occur through a myriad of pathways. The fundamental mechanisms include insulin resistance and impaired endothelial nitric oxide synthase (eNOS) activation through IRS-1/PI3K/Akt pathway, increased oxidative stress, and reduced nitric oxide (NO) bioavailability,

and pro-inflammatory activation of the endothelium (Tabit et al., 2010). Increased oxidative stress is linked with reduced bioavailability of NO, increased vascular inflammation, and adverse vascular remodeling (Touyz et al., 2002). Regular physical activity was correlated with reduced oxidative stress and inflammation in pathologic conditions such as hypertension (Briones & Touyz, 2009). Increased shear stress due to repeated bouts of aerobic exercise could also increase NO and endothelium-derived hyperpolarization factor (EDHF) production by stimulating endothelial cells (Traub & Berk, 1998; Takamura et al., 1999). Despite reported anti-inflammatory, antioxidant, and improved generation of endothelium-derived relaxing factors (EDRF) by exercise training, too little is known about the mechanism by which exercise mediates vascular benefits in diabetes, possibly due to the use of different intensity, type, and duration in rodent models and human.

EDRFs, mainly NO and prostacyclin (PGI_2), beside controlling vascular tone, also control other vital aspects that may determine vascular health and atherosclerosis. NO, and PGI_2 are well known for their inhibitory effect on platelet aggregation, leukocyte adhesion and migration, and vascular smooth muscle cell (VSMC) proliferation and inflammation (Traub & Berk, 1998). VSMC proliferation and/or hypertrophy, also described as an increase in intima medial thickness (IMT), is a marker of atherosclerosis (de Groot et al., 2004; McCloskey et al., 2014). Sedentary lifestyle reduces the shear stress on the arterial wall, and subsequently reduces the production of NO and PGI_2, and promotes thickening of intima-media (Gnasso et al., 1996). Besides IMT, increased collagen deposition and reduced elastin content have been reported in aged rat arteries which increase stiffness (Briones et al., 2007). Increasing evidence suggests, besides shear stress induced increase in production of EDRFs, the beneficial effect of exercise against CVD is obtained partly due to its direct impact on arterial wall properties such as elastin

and collagen content, wall thickness, wall to lumen ratio (Matsuda et al., 1993; Thijssen et al., 2012; Xiong et al., 2021). Despite these reports, the impact of exercise on diabetic arterial wall remodeling is not well understood.

The current study was designed to investigate the impact of moderate-intensity aerobic exercise (MIE) on vascular reactivity and structural remodeling of vessels in male (and female in future studies) diabetic UCD-T2DM rats. We hypothesize that MIE enhances mesenteric arterial (MA) relaxation by alteration of the relative contributions of EDRF and the expression of genes or activity of gene products associated with EDV. Furthermore, exercise training enhances EDV by altering the MA wall structure (collagen and elastin content, wall thickness) and myogenic tone in UCD-T2DM male rats. To test the hypotheses mentioned above, we investigated the effects of eight weeks of MIE on the 1) ACh-induced EDV and vascular responses to contractile agents, 2) contribution of EDRF in vasorelaxation, 3) expression of proteins associated with NO-, cyclooxygenase (COX)-, and EDHF-mediated vasorelaxation and vasoconstriction, and 4) pressure-induced change of myogenic tone, wall thickness, and elastin content in arteries (such as MA and aorta) isolated from male UCD-T2DM rats.

This study demonstrates that eight weeks of MIE improved vasorelaxation in MA from male UCD-T2DM rats. Here we provide the first report of the specific impact of MIE on the modulation of EDRF, myogenic tone, contractile response, and wall properties which may have improved EDV in the UCD-T2DM model.

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals were purchased from either Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO, USA) and dissolved in water unless otherwise stated.

3.2.2 Experimental Animals

For this study, we selected 19-20 weeks old male UCD-T2DM rats that developed overt diabetes. The mean diabetic age was (32±2) days at the time of exercise initiation. Age-matched Sprague Dawley (SD) rats were ordered from the Simonsen laboratory. Both diabetic and SD rats were randomly divided into either exercise-trained or sedentary groups. In total, four experimental groups were selected: 1) Control Sedentary (CS), 2) Control Exercise-trained (CE), 3) Diabetic Sedentary (DS), and 4) Diabetic Exercise-trained (DE).

The exercise-trained groups were acclimatized with the treadmill for one week (5 days). The acclimation protocol is elaborated in table 3.1 and adopted from (Teixeira-Coelho et al., 2017). After acclimation, the rats underwent MIE over the next eight weeks (1 hour/day, 5days/week). The exercise training was divided into two sessions/day with a 5 mins break to ensure the obese diabetic animal could complete the 1-hour session without exhaustion. The details of the exercise training protocol are elaborated in table 3.2.

Table 3.1
Five-days Acclimation Protocol for Running on a Treadmill

1st day	2nd day	3rd ,4th ,5th day
5 min (rest)	5 min (rest)	5 min (rest)
1 min (10 m/min)	1 min (12 m/min)	5 min (15 m/min)
1 min (12 m/min)	5 min (15 m/min)	
5 min (15 m/min)		

Table 3.2
Moderate-intensity Treadmill Running Protocol for SD Control and Diabetic Rats. The Protocol Was Followed for 5days/week for Eight Weeks

	1st session		5 mins break	2nd Session	
	5 mins warm up	25 mins MIE		25 mins MIE	5 mins cool down
Speed:	10m/min	15m/min		15m/min	10m/min

MIE is defined as the exercise intensity at which the oxygen consumption reaches 50-65% of the maximum oxygen consumption (VO_{2max}). Previous studies in rodents have used treadmill speed ranging from 12-18m/min with various inclinations for MIE (Chung & Diffee, 2012; Howarth et al., 2008; Yang et al., 2020). We selected the speed of 15m/min with 0% inclination on the treadmill for our protocol. The sedentary group is placed on the stationary treadmill three times a week to provide a matched environment without MIE.

During the study period, all the animals at the University of the Pacific vivarium were kept in a humidity and temperature-controlled room with a 12h light/dark cycle and *ad libitum* access to water and standard rodent chow (Mazuri rodent chow). The animals were euthanized using carbon dioxide after eight weeks of MIE. The euthanasia processes were completed by following AVMA Guidelines for the Euthanasia of Animals: 2013 edition and the NIH Guidelines for the Care and Use of Laboratory Animals: Eighth Edition. In addition, all animal protocols were approved by the Institutional Animal Care and Use Committee of the University of the Pacific. After the rats were sacrificed, the isolated MA function and wall characteristics were investigated using the wire and pressure myography technique.

3.2.3 Measurement of MA Tension by Wire Myography

Please see chapter 2, section 2.2.4, for a detailed experimental protocol.

3.2.3.1 Relaxation Responses to Sodium Nitroprusside (SNP)

Relaxation responses to SNP, a NO donor, were slightly modified from the first study to measure the smooth muscle sensitivity. MA segment was incubated for 20 mins with a combination of indomethacin (Indo, 10μM), N_{ω}-nitro-L-arginine methyl ester (L-NAME, 200μM), Apamin (1 μM) and 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34, 1μM) to prevent the contribution of EDRF from endothelial cells on the relaxation of smooth

muscle. Concentration response curves (CRC) to increasing dosage of SNP (10^{-9} to 10^{-5} M) were obtained in MA precontracted with phenylephrine (PE, 2μM).

3.2.4 Measurement of MA Myogenic Tone and Wall Thickness by Pressure Myograph

Pressure myograph is a strong tool to investigate myogenic tone, wall thickness, wall to lumen ratio, and change of inner diameter of artery at different pressures. The myogenic tone is a contractile response which is generated when the blood vessel is stretched radially due to increased intravascular pressure. Similar to study-I, the 3rd order MA was isolated by clearing from fat and other connective tissues and mounted on the glass cannula of a Danish Myo Technology (DMT) pressure myograph (DMT 114p). Both ends of the artery were secured on the cannulas by a thin nylon suture so that the artery and the cannulas together make a close system. The MA was then checked for any leak by pushing a small amount of the Krebs solution into the system. When the arteries maintained the bulbous formation, the system was considered a close system. Care was taken not to introduce any bubble in the system. The MA was then pressurized to 120mm Hg and checked for any bends. Any bend observed in the MA segment was corrected by stretching the artery using the horizontal movement screw. The MA segment was allowed to rest at 60mmHg for 30 mins, and then the viability of the arteries was determined by assessing ACh (10μM) mediated relaxation in PE (2μM) precontracted arteries. The artery segment was washed and allowed to rest for 20 mins before moving onto pressurized experiments.

MA inner and outer diameter, wall thickness, and myogenic tone were assessed over increasing intraluminal pressures in steps between 20-120 mmHg. The MA was allowed to develop a spontaneous tone by reaching a stable diameter (Active Diameter, D_a) at each pressure step, which was typically achieved over 5 min intervals. The pressure-response was repeated in

Ca^{2+}-free physiological salt solution (PSS) to determine the corresponding passive diameters (D_p). The myogenic tone is expressed as a percent of passive diameter (D_p) and calculated as ($D_p - D_a$)/ D_p x100. Myoview software, in combination with an inverted microscope (Zeiss Axio Vert.A1) connected with a camera, was used to record data for vascular wall characteristics (wall thickness, inner and outer diameter) at different pressures.

3.2.5 Western Blot Analysis

Please see chapter 2, section 2.2.5, for a detailed procedure. Additionally, the primary antibody for COX-1 was obtained from cell signaling technology (Danvers, MA), and an antibody against COX-2 was obtained from Abcam (Cambridge, MA). All primary antibodies were diluted to 1:1000 unless otherwise stated. The dilutions of secondary antibody were either 1:10000 or 1:15000.

3.2.6 Histological Studies of the Aortic Wall

Formalin-fixed paraffin-embedded aortic tissues were used for immunohistochemical analysis. Briefly, the aortic (2mm) segment was fixed in 10% formalin and dehydrated using increasing concentrations of ethanol. Aortic sections were then immersed in xylene and finally embedded in paraffin to make a block. The paraffin-embedded tissues are then sectioned at a thickness of 4-5µm using a microtome (MICROM HM 325, Thermo Fisher Scientific, Waltham, MA) and put on the warm water bath to stretch. Finally, the sections were fixed on a glass slide for further analysis.

3.2.6.1 Hematoxylin and Eosin Staining of the Aorta

To investigate the general histology of the aortic wall, aortic sections were stained by hematoxylin and eosin (H&E). Briefly, aortic sections on the glass slides were deparaffinized and immersed into water for rehydration. The hydrated sections were stained using hematoxylin

and eosin stain kit (Vector Lab, Burlingame, CA) following the manufacturer's protocol. The pictures of stained aortic sections were taken by a camera attached with a light microscope (Nikon Eclipse E200). The intima medial thickness of the aorta was measured by a line tool software provided by Nikon (NIS Element D). 4-5 pictures were taken from different areas of the same aorta, and measurements were taken from at least three different areas of each picture. All the readings were averaged, and the aortic mean medial thickness was determined for that animal.

3.2.6.2 Verhoeff Van Gieson Staining of the Aorta:

Aortic sections were also stained to investigate elastin content using the Verhoeff Van Gieson stain kit (StatLab, McKinney, TX) and following the manufacturer's protocol. Like H&E staining, 4-5 pictures were taken per aorta, and the area of elastin and the total area were analyzed using ImageJ software (NIH). Elastin content was expressed as a percentage of the total area.

3.3 Results

3.3.1 Effects of MIE on Metabolic Parameters

MIE significantly reduced the body weight in the diabetic group; however, it did not reach to the level of control groups. The body weight was 383.3±4.2 g in CS and 366.7±5.1 g in CE, and 559.3±30.4 g in DS and 469.3±6.2 g in DE (Figure 3.1A, Table 3.3). The normalized (to body weight) intra-abdominal adipose tissue level (located around mesentery and omental) was significantly higher in DS groups compared to the exercise-trained control and diabetic group (Figure 3.1B). Similarly, when circulating triglyceride levels were compared, the DS group showed significantly higher triglyceride level (3.52±0.3 mmol/L) compared to CS, CE, and DE (1.35±0.2, 1.36±0.1, and 1.54±0.2 mmol/L, respectively). However, MIE had no

significant effect on blood glucose, HbA1c, and plasma insulin level regardless of disease status. Blood glucose and HbA1c levels were higher, and plasma insulin levels were lower in diabetic groups than in the control group (Figure 3.1D-F).

3.3.2 Effects of MIE on Relaxation Responses to ACh

Both sensitivity (pD_2) and maximal response (R_{max}) to ACh were significantly reduced in MA from DS rats compared with those in CS. Interestingly, exercise improved the ACh response only in the MA of the diabetic group (DE); the R_{max} to ACh in MA rings in the DE group (90.74±2.4%) was markedly enhanced compared to DS (59.85±2.2%) (Figure 3.2 Table 3.4). Similarly, MA from DE animals showed improved sensitivity [$pD_2(-\log EC_{50})$] to ACh (6.72±0.1) compared to the respective sedentary group (5.80±0.2).

3.3.3 Effects of MIE on the Relative Contribution of EDRF to MA Relaxation

The relative contributions of COX metabolites, NO, and EDHF to vasorelaxation induced by ACh were estimated by sequentially inhibiting COX, NOS, and a combination of small and intermediate conductance calcium-activated potassium channel (SK_{Ca} and IK_{Ca}) inhibitors. Inhibition of COX by Indo enhanced ACh-induced R_{max} from 59.85±2.2% to 98.61±0.4% in DS arteries, suggesting the elevation of contractile COX metabolites in the DS group (Figure 3.3 C, Table 3.5). The addition of L-NAME resulted in a reduction in ACh-induced vasorelaxation of arteries from both control and diabetic sedentary rats. However, the effect was more prominent (and almost completely blocked ACh relaxation) in the DS group (DS vs. CS, Figure 3.3 A, C). The difference in area under the curve (ΔAUC) between ACh CRCs before and after an inhibitor was calculated as described in our previous study (Sangüesa et al., 2017). Particularly, an intriguing observation was that MIE prevented the loss of EDHF-mediated relaxation in male UCD-T2DM rats (DE vs. DS, Figure 3.3 C-D). The R_{max} of ACh-induced vasorelaxation after

Indo+L-NAME was 10.27±3.7% and 38.58±13.9% in DS and DE groups, respectively. Additionally, ΔAUC of Indo and L-NAME resistant relaxation was significantly lower in DS (6.24±1.83) compared to DE (48.38±9.1) (Figure 3.3C-D, gray shaded area; Table 3.5, column of Indo+L-NAME+Apamin+TRAM-34). Furthermore, unlike in the DS group, inhibition of COX with Indo did not affect ACh responses in MA of the DE group (DE vs. DS, Figure 3.3C-D).

3.3.4 Effects of MIE on the SNP-induced Relaxation

To investigate whether impaired smooth muscle sensitivity to NO led to reduced MA relaxation of DS rats, we examined the relaxation responses to SNP (NO donor). There was no significant difference observed between the CRCs to SNP regardless of MIE and/or disease status in any of the experimental groups (Figure 3.4, Table 3.6).

3.3.5 Effects of MIE on COX-1 and COX-2 Expression

COX metabolites are major regulators of vascular tone. Matsumoto et al. suggested that in T2D OLETF rats, impaired EDV of MA could be due to the overexpression of COX-1 and COX-2 as well as increased production of vasoconstrictive prostanoids (Matsumoto et al., 2007). In the current study, we observed an increase in MA relaxation in DS arteries by ACh in presence of Indo, suggesting an elevated contractile function of COXs in DS arteries. Hence, we have investigated both subtypes of COX (COX-1 and COX-2) expression in MA. Arteries taken from the DS group exhibited a greater level of both COX isoforms than those in exercise-trained groups (CE, DE) (Figure 3.5).

3.3.6 Effects of MIE on eNOS Expression

We observed a relative contribution of NO-mediated relaxation in diabetic groups compared to control groups. Zhang et al. reported an increase in eNOS gene expression in MA

of type-1 diabetic female rats, which was possibly correlated with the elevated NO contribution to EDV (Zhang et al., 2012). To investigate the origin of elevated NO-mediated relaxation, we determined the eNOS protein expression in the MA of experimental groups. Both DS and DE showed significantly higher eNOS expression than the CS and CE groups, approximately 4 and 3 folds, respectively (Figure 3.6).

3.3.7 Effects of MIE on IK_{Ca} and SK_{Ca} Expression

We observed a diminished EDHF-type relaxation in DS arteries, and MIE improved EDHF-type relaxation in the diabetic group. To determine whether EDHF-type relaxation was associated with the alteration of calcium-activated potassium channels, we investigated the expression of small and intermediate conductance calcium-activated potassium channels (SK_{Ca} and IK_{Ca}) in MA. There were no significant changes in the expression of IK_{Ca} in MA of any groups (Figure 3.7A). However, SK_{Ca} expression in DS was significantly reduced compared to CS and CE. When only diabetic groups were compared, SK_{Ca} level was significantly higher (0.3-fold) in DE than in the DS group (Figure 3.7B, top right section, DS vs. DE bar graph, analyzed by t-test).

3.3.8 Effects of MIE on the PE-induced Contraction of MA

A dysfunctional endothelium also becomes a source of other substances and mediators that are detrimental to the arterial wall, such as thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2)(Taddei et al., 2005). These factors have pro-atherosclerotic features and also cause vasoconstriction via activation of thromboxane prostanoids receptors (TP) on vascular smooth muscle (VSM). We, therefore, compared the CRCs to a contractile agent PE (10^{-8} to 10^{-5} M) in MA from the experimental groups. The MA from the DS group exhibited a higher maximal contractile response to PE than all other experimental groups (Figure 3.8, Table 3.7). MIE

significantly reduced the PE maximal tension in diabetic arteries to the same level as those in CS and CE.

3.3.9 Effects of MIE on the MA Myogenic Tone

The microvasculature regulates blood flow to organs by virtue of myogenic tone (constriction in response to intraluminal pressure) and therefore ensures smooth blood flow to organs despite changes in systemic hemodynamics. Increased myogenic tone could indicate an increase in vascular resistance and a subsequent rise in blood pressure. MA myogenic tone was assessed over increasing intraluminal pressure (20-120 mmHg). The myogenic tone in DS arteries was significantly higher compared to the other groups (Figure 3.9). Similar to the contractile response to PE, MIE significantly reduced the myogenic tone in the DE group, although it did not reach to the level of the control groups (CS and CE). There was no difference in the myogenic tone (%) between control groups (CS and CE).

3.3.10 Effects of MIE on NADPH oxidase-1 (NOX-1) Expression

To further investigate other possible mechanisms besides COX metabolites, that may have contributed to the elevated contractile responses (or decreased ACh responses) in the DS group, NOX-1 protein expression was measured in MA. NOX-1 expression was slightly elevated in MA from DS groups, but the difference between DS and the other experimental groups was not statistically significant (Figure 3.10).

3.3.11 Effects of MIE on the MA and Aortic Wall Thickness

Increased IMT could be an early marker for atherosclerosis (McCloskey et al., 2014). Adverse MA remodeling in the type-1 and type-2 diabetic models has been reported (Crijns et al., 1999; Souza-Smith et al., 2011). In the current study, wall thickness was determined to investigate the effect of diabetes and MIE on resistant MA remodeling. The MA taken from the

DS group exhibited a significantly higher wall thickness compared to all other groups. However, the wall thickness of the DE group was comparable to that of the control groups. There was no difference in the MA wall thickness among CS, CE, and DE groups (Figure 3.11).

To further investigate whether diabetes causes an adverse remodeling of conduit arteries (i.e., aorta) in the UCD-T2DM model and examine the impact of MIE, we analyzed aortic IMT by histological staining. Hematoxylin and eosin (H&E) stained aortic sections were investigated for IMT of the aorta (Figure 3.12**)**. DS aorta showed a significantly greater IMT (132.4±4.9μm) compared to all other groups. MIE reduced the thickness in the DE aorta to a level comparable to the control groups. There was no difference in the aortic IMT of CS (108.7±2.5μm), CE (109.8±2.0μm), and DE (109.9±5.3μm) groups.

3.3.12 Effects of MIE on Aortic Elastin Content

Vascular elastin and collagen content are passive indicators of its stiffness (Wagenseil & Mecham, 2012). Increased elastin degradation, together with collagen deposition in the intima, increases the stiffness of the artery. Aortic elastin content was measured by Verhoeff Van Gieson staining to investigate the impact of MIE on mechanical changes in the aortic wall structure in diabetes. MIE increased elastin (% to the total area) content in CE (32.0±3.2%) group compared to all other groups. Although there was no significant difference in CS (21.9±1.2%), DS (17.3.0±0.7%), and DE (21.8±1.8%) aortic elastin content, the DS group showed a decreasing trend in aortic elastin content among the groups.

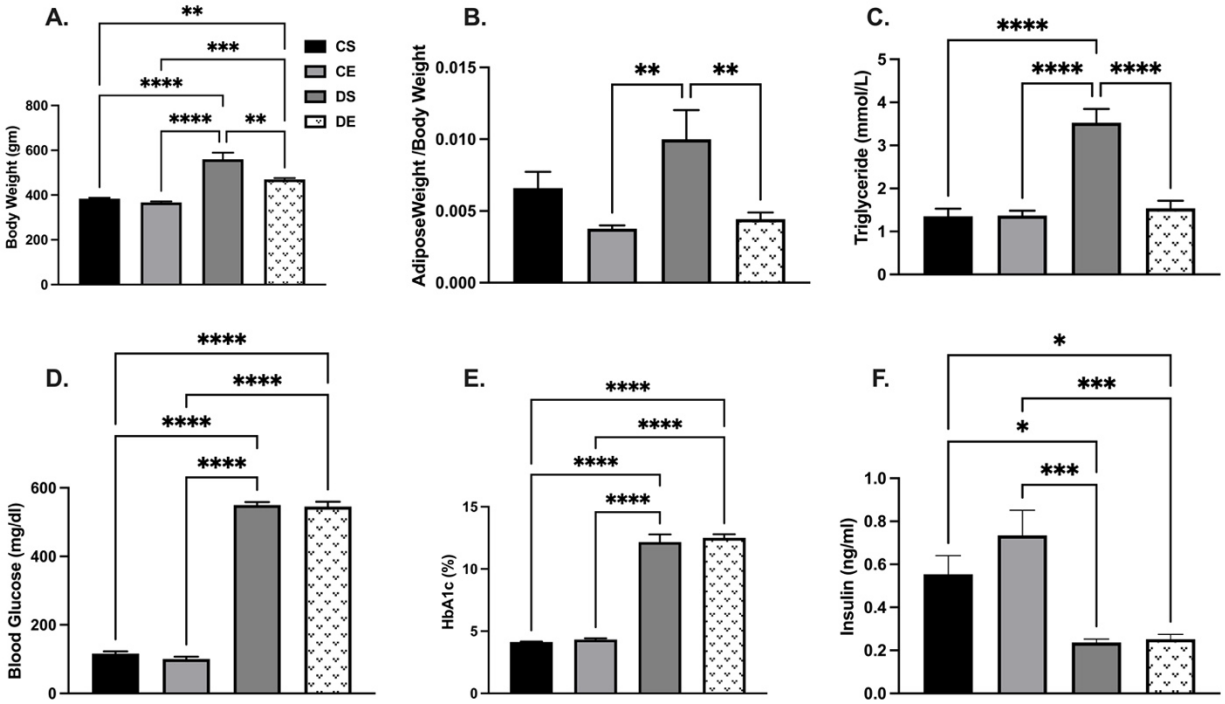

Figure 3.1 Effects of MIE on metabolic parameters of sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. (A) Body weight (B) Adipose tissue to Body weight ratio (C) Triglyceride level (D) Blood glucose level (E) HbA1c (%) (F) Insulin level. Values are represented as mean ± SEM. Each bar represents the values obtained from n=5-7 animals per group. Capped lines indicate significant differences between two groups (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), analyzed by one-way ANOVA followed by Tukey's post hoc test.

Table 3.3
Effects of MIE on Metabolic Parameters of Sedentary and Exercise-trained SD (CS, CE) and Diabetic (DS, DE) Rats

	CS	CE	DS	DE
Body Weight (g)	383.3±4.2	366.7±5.1	559.3±30.4*#$	469.3±6.2*#
Adipose Tissue (g)	2.53±0.4	1.37±0.1	5.89±1.4*#$	2.14±0.2
Adipose Tissue/ Body Weight (g)	0.006±0	0.003±0	0.009±0#$	0.004±0
Triglyceride (mmol/l)	1.35±0.2	1.36±0.1	3.52±0.3*#$	1.54±0.2
Blood Glucose (mg/dl)	111.0±6.4	105.0±6.4	518.0±32.6*#	545.0±14.5*#
HbA1c level	4.13±0	4.33±0.1	12.17±0.6*#	12.50±0.2*#
Insulin (ng/ml)	0.74±0.2	0.83±0.2	0.39±0.2*#	0.25±0.0*#

Data are expressed as mean ± SEM; n=5-6 rats per group. #$p<0.05$ (vs. CS), *$p<0.05$ (vs. CE), \$$p<0.05$ (vs. DE), analyzed using one-way ANOVA followed by Tukey's post hoc test.

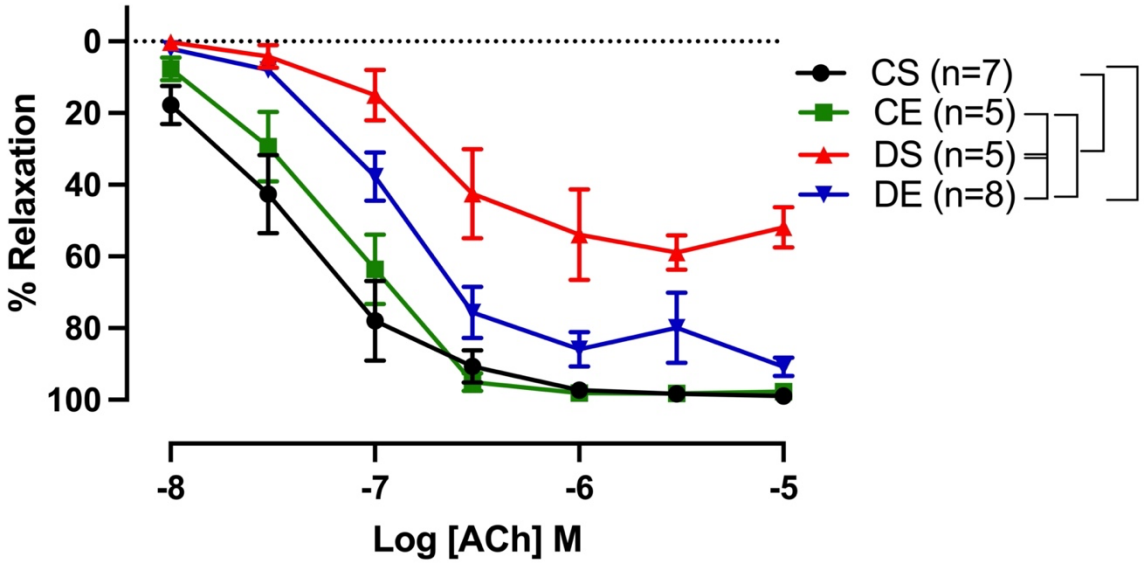

Figure 3.2 Relaxation responses to cumulative concentrations of acetylcholine (ACh, 10^{-8} to 10^{-5} M) in intact mesenteric arterial rings precontracted with phenylephrine (PE, 2μM) from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Data are expressed as mean ± SEM. n=5-8 animals per group. Parenthesis indicates significant differences between groups ($p< 0.05$) analyzed using 2-way ANOVA followed by Tukey's post hoc test.

Table 3.4

Sensitivity (pD_2: $-logEC_{50}$), and Maximum Response (R_{max}) to ACh in Mesenteric Arteries from Sedentary and Exercise-trained SD (CS, CE) and Diabetic (DS, DE) Rats

ACh	**n**	**pD_2($-logEC_{50}$)**	**R_{max} (%)**
CS	7	7.40±0.2	98.91±0.6
CE	5	7.22±0.1	98.60±1.0
DS	5	5.80±0.2*#$	59.85±2.2*#$
DE	8	6.72±0.1#	90.74±2.4*#

Data are expressed as mean ± SEM; n=5-8 rats per group. #p<0.05 (vs. CS), *p<0.05 (vs. CE), $p<0.05 (vs. DE), analyzed using one-way ANOVA followed by Tukey's post hoc test.

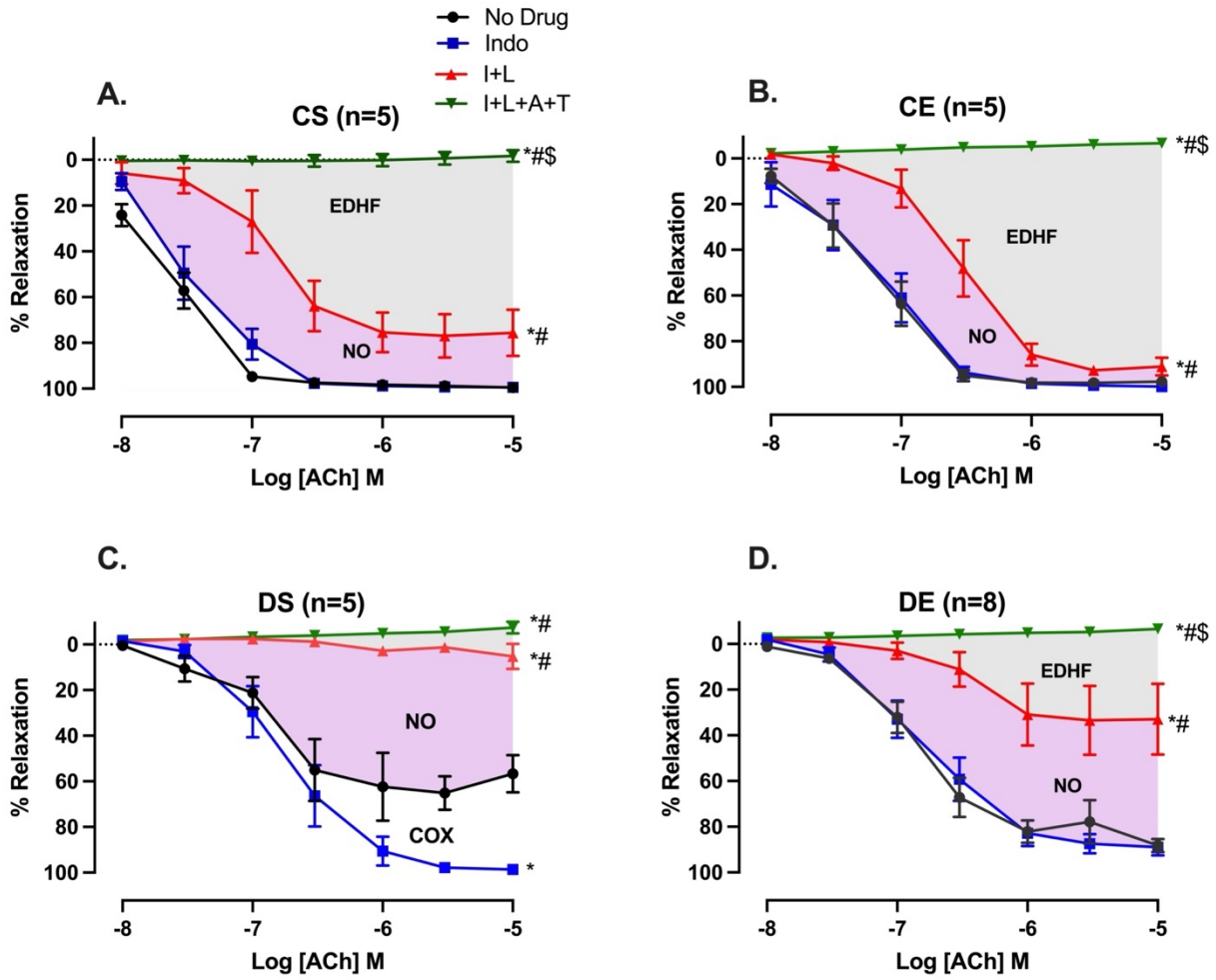

Figure 3.3 Effects of inhibiting cyclooxygenase (COX), nitric oxide synthase (NOS), small conductance and intermediated conductance calcium-activated potassium channels (SK_{ca} and IK_{ca}) on ACh-induced vasorelaxation in mesenteric arteries taken from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats; (A) CS, (B) CE, (C) DS, (D) DE.

CRC to ACh (10^{-8} to 10^{-5} M) was generated in the presence of no inhibitor (No drug) or in the presence of Indo, Indo+L-NAME (I+L), and Indo+L-NAME+Apamin+TRAM-34 (I+L+A+T) in mesenteric arterial rings. Indo (indomethacin; 10µM), L-NAME (N^{ω}-nitro-L-arginine methyl ester; 200µM), Apamin (1µM), TRAM-34; [1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole; 1µM]. * ($P<0.05$) vs. no drug; # ($P<0.05$) vs. Indo; $ ($P<0.05$) vs. Indo+L-NAME, analyzed using 2-way ANOVA with repeated measures followed by Bonferroni post hoc test.

Table 3.5

Sensitivity (pD_2: -logEC50), Maximum response (R_{max}), and Area Under the Curve to ACh in Mesenteric Arteries from Sedentary and Exercise-trained SD (CS, CE) and Diabetic (DS, DE) Rats

Groups	No Drug			Indo			Indo+L-NAME			Indo+L-NAME+ Apamin+TRAM-34		
	pD_2	R_{max} (%)	ΔAUC	pD_2	R_{max} (%)	ΔAUC	pD_2	R_{max} (%)	ΔAUC	pD_2	R_{max} (%)	ΔAUC
CS	7.63±0.1	99.57±0.1	ND	7.49±0.1	99.41±0.2	13.80±5.71	ND	80.54±7.3	93.90±9.62	ND	-1.71±2.5^{abc}	147.3±8.34
CE	7.22±0.12	98.60±0.9	ND	7.26±0.2	99.83±0.2	1.0±7.66	ND	93.88±1.9	75.20±8.13	ND	-6.67±1.4^{abc}	144.7±5.63
DS	5.80±0.2*#$	59.85±2.2*#$	ND	6.70±0.2#a	98.61±0.4^{a}$	47.20±4.77*#$	ND	10.27±3.7*#^{ab}	115.9±3.45*	ND	-7.36±2.5^{abc}	6.24±1.83*#$
DE	6.72±0.1#	90.74±2.4*#	ND	6.61±0.1*#	88.92±3.4*#	0.5±7.49	ND	38.58±13.9*#^{ab}	107.7±10.1	ND	-6.5±1.4^{abc}	48.38±9.1*#

A comparison of sensitivity (pD_2), maximum response (R_{max}) and ΔAUC to acetylcholine in the absence (no drug) or in the presence of Indo, Indo+L-NAME, and Indo+L-NAME+Apamin+TRAM-34in MA rings from sedentary and exercise-trained SD control (CS, CE) and diabetic (DS, DE) rats. ΔAUC were measured by measuring the area between two curves. ΔAUC of Indo indicates COX contribution, ΔAUC of Indo+L-NAME indicates NO contribution and, ΔAUC of Indo+L-NAME+Apamin+TRAM-34 indicates EDHF-type contribution in ACh-induced vasorelaxation of MA.

Data are expressed as mean ± SEM; n=5-8 rats per group. Analysis between the group: #p<0.05 (vs. CS), *p<0.05 (vs. CE), $p<0.05 (vs. DE), analyzed using one-way ANOVA followed by Tukey's post hoc test. Analysis within the group: ^{a}p<0.05 vs. no drug control within each group, ^{b}p<0.05 vs. Indo within each group, ^{c}p<0.05 vs. Indo+L-NAME, (paired Student's t-test). ND; not determined.

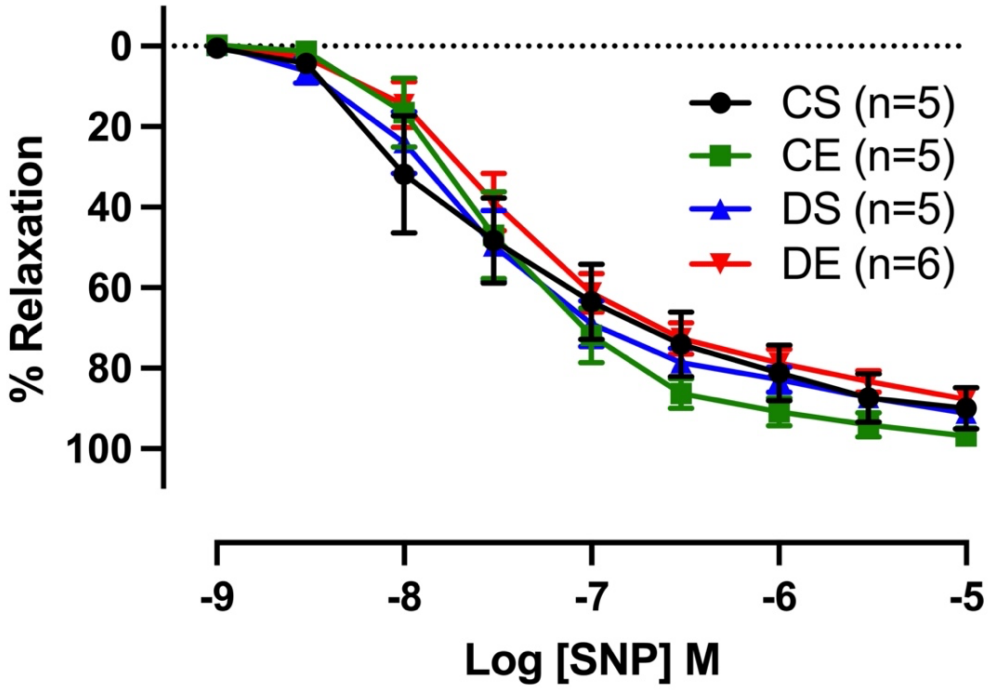

Figure 3.4 Relaxation responses to cumulative concentrations of SNP (10^{-9} to 10^{-5} M) in intact mesenteric arterial rings precontracted with phenylephrine (PE, 2μM) from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Data are expressed as mean ± SEM. n=5-6 animals per group, analyzed using 2-way ANOVA followed by Tukey's post hoc test.

Table 3.6

Sensitivity (pD_2: $-logEC_{50}$), and Maximum Response (R_{max}) to SNP in Mesenteric Arteries from Sedentary and Exercise-trained SD (CS, CE) and Diabetic (DS, DE) Rats

SNP	**n**	**$pD_2(-logEC_{50})$**	**R_{max} (%)**
CS	5	7.23±0.3	89.89±4.9
CE	5	7.38±0.2	96.88±2.1
DS	5	7.36±0.2	91.16±1.2
DE	6	7.08±0.1	87.78±2.0

Data are expressed as mean ± SEM; n=5-6 rats per group. Analyzed by one-way ANOVA followed by Tukey's post hoc test.

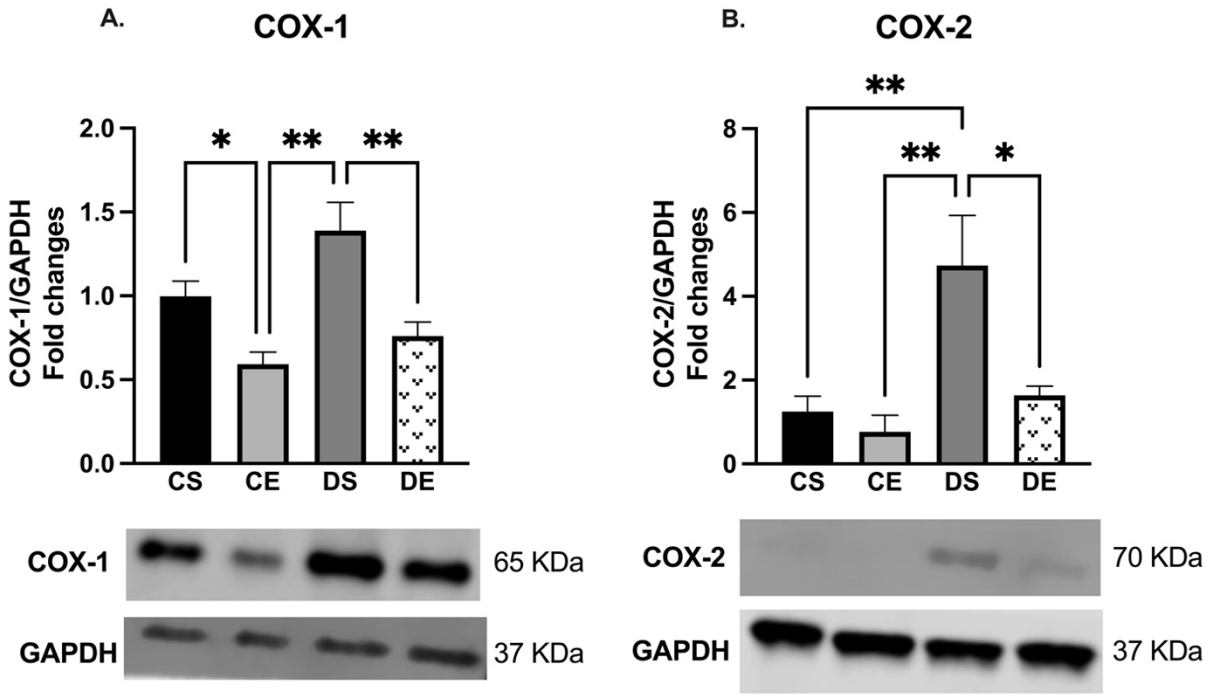

Figure 3.5 Western blot analysis of (A) COX-1 (B) COX-2 expression in mesenteric artery (MA) of sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=3-4 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Capped lines indicate significant differences between two groups (*$p<0.05$, **$p<0.01$), as analyzed by one-way ANOVA followed by Tukey's post hoc test.

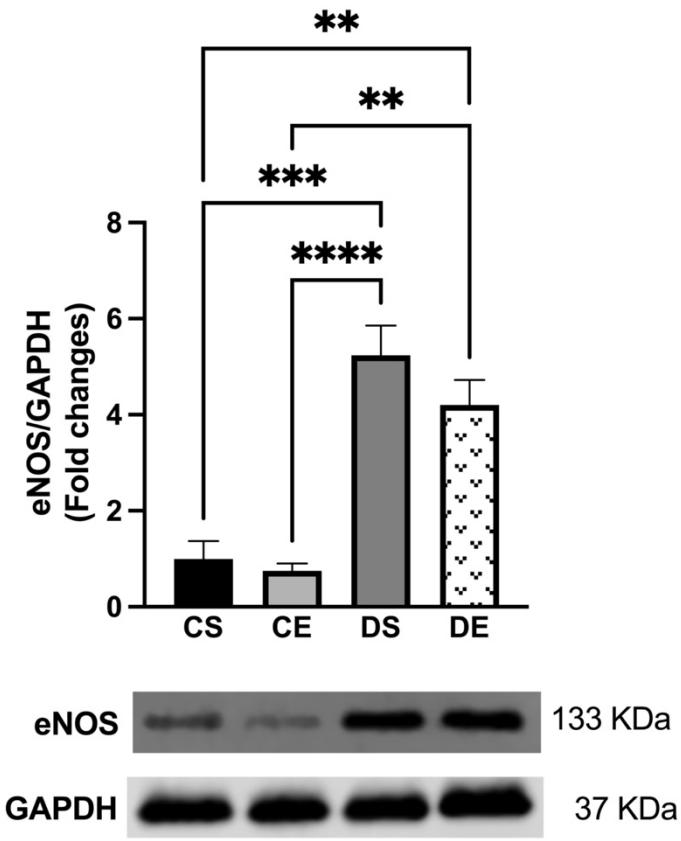

Figure 3.6 Western blot analysis of eNOS expression in mesenteric artery (MA) of sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=4-5 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Capped lines indicate significant differences between two groups (**p<0.01, ***p<0.001, ****p<0.0001), as analyzed by one-way ANOVA followed by Tukey's post hoc test.

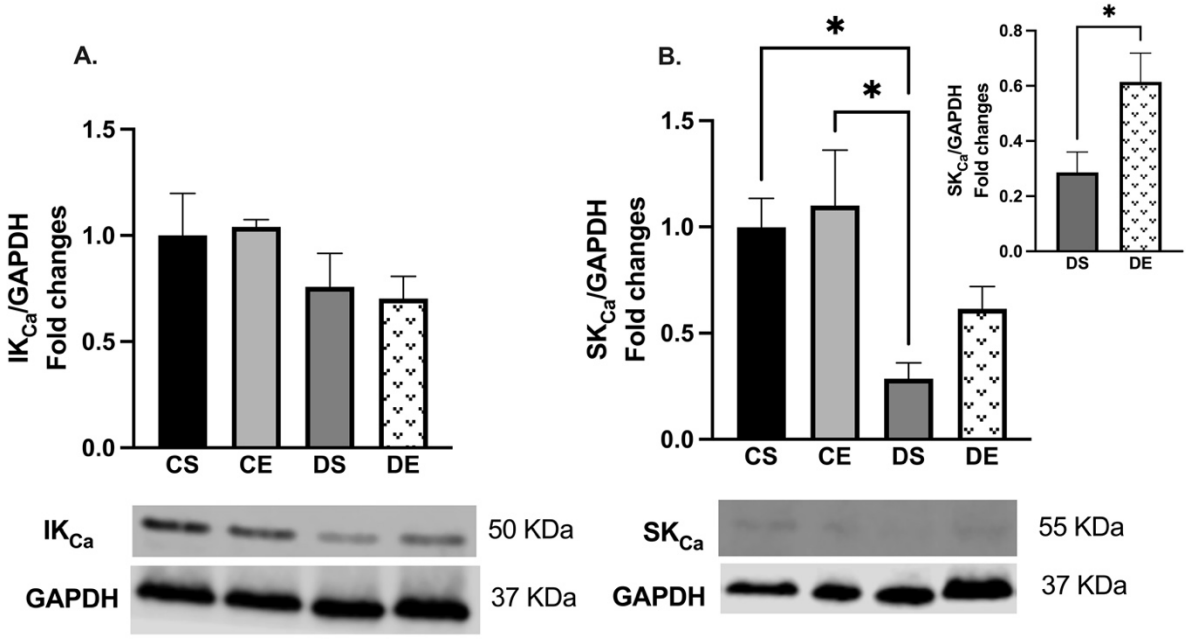

Figure 3.7 Western blot analysis of (A) IK_{Ca} ($K_{Ca}3.1$) (B) SK_{Ca} ($K_{Ca}2.3$) expression in mesenteric artery (MA) of sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Protein level were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=4-5 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes. Capped lines indicate significant differences between two groups (*$p<0.05$), as analyzed by one-way ANOVA followed by Tukey's post hoc test. Figure B) top right section is showing comparison of only diabetic groups (DS vs. DE) which was analyzed by Student's unpaired t-test.

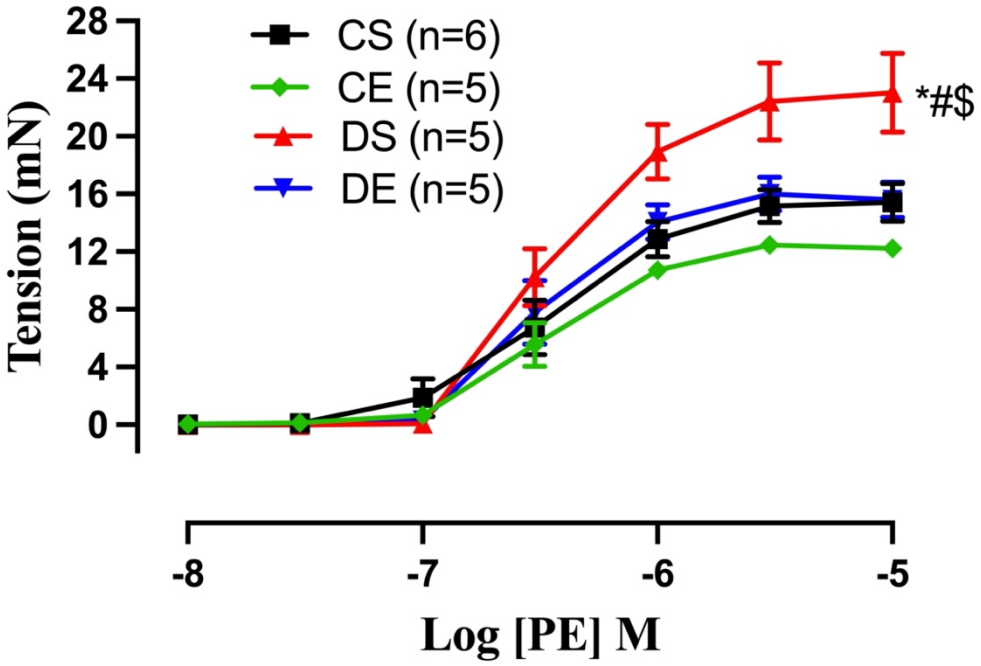

Figure 3.8 Concentration response curve to cumulative concentrations of phenylephrine (PE, 10^{-8} to 10^{-5} M) in intact mesenteric arterial rings from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Data are expressed as mean ± SEM. n=5-6 animals per group. #$p<0.05$ (vs. CS), *$p<0.05$ (vs. CE), \$$p<0.05$ (vs. DE), analyzed by using 2-way ANOVA followed by Tukey's post hoc test.

Table 3.7

Sensitivity (pD_2: $-logEC_{50}$), and Maximum Tension ($Tension_{max}$) to PE in Mesenteric Arteries from Sedentary and Exercise-trained SD (CS, CE) and Diabetic (DS, DE) Rats

PE	**n**	**$pD_2(-logEC_{50})$**	**$Tension_{max}$**
CS	6	6.49±0.16	15.45±1.24
CE	5	6.46±0.12	12.23±0.54
DS	5	6.46±0.18	23.03±2.74*#$
DE	5	6.41±0.11	16.20±1.18

Data are expressed as mean ± SEM; n=5-6 rats per group. #p<0.05 (vs. CS), *p<0.05 (vs. CE), $p<0.05 (vs. DE), analyzed using one-way ANOVA followed by Tukey's post hoc test.

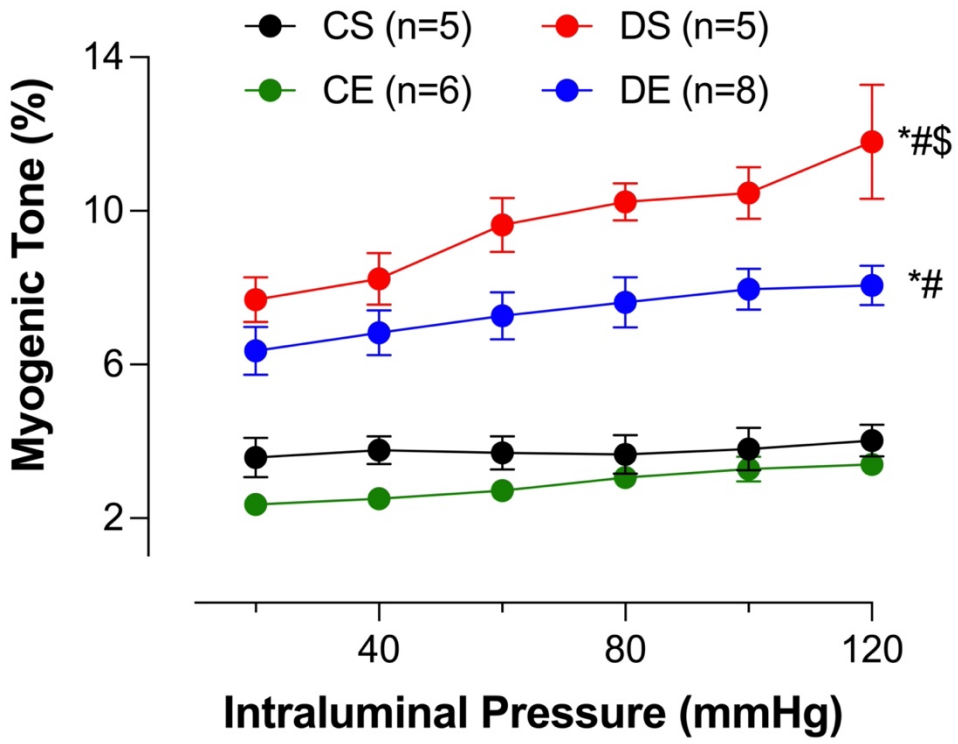

Figure 3.9 Myogenic tone (%) at different intraluminal pressure in intact mesenteric arterial rings from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Data are expressed as mean ± SEM. n=5-8 animals per group. #p<0.05 (vs. CS), *p<0.05 (vs. CE), $p<0.05 (vs. DE), analyzed using 2-way ANOVA followed by Tukey's post hoc test.

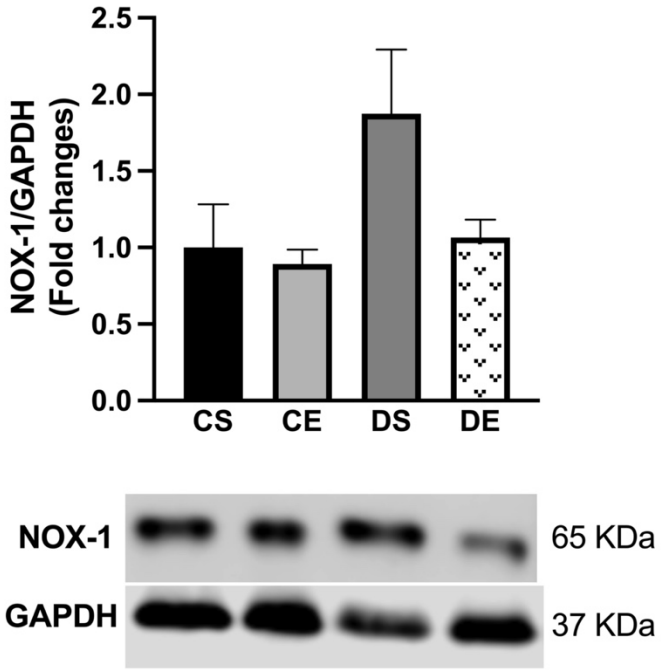

Figure 3.10 Western blot analysis of NOX-1 expression in mesenteric artery (MA) of sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Protein levels were quantified by densitometry and normalized to corresponding GAPDH. Values are represented as mean ± SEM. Each bar represents the values obtained from n=3-4 animals per group. Representative bands of target and housekeeping protein (GAPDH) were shown from the same membranes and analyzed by one-way ANOVA followed by Tukey's post hoc test.

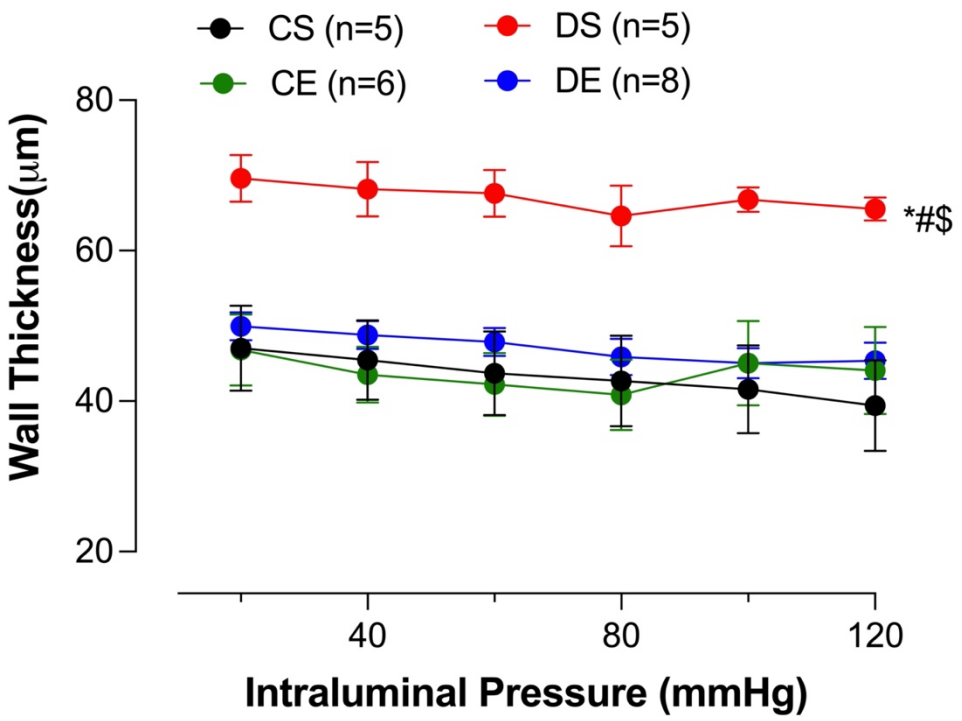

Figure 3.11 Wall thickness at different intraluminal pressure in intact mesenteric arterial rings from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Data are expressed as mean ± SEM. n=5-8 animals per group. #$p<0.05$ (vs. CS), *$p<0.05$ (vs. CE), \$$p<0.05$ (vs. DE), analyzed using 2-way ANOVA followed by Tukey's post hoc test.

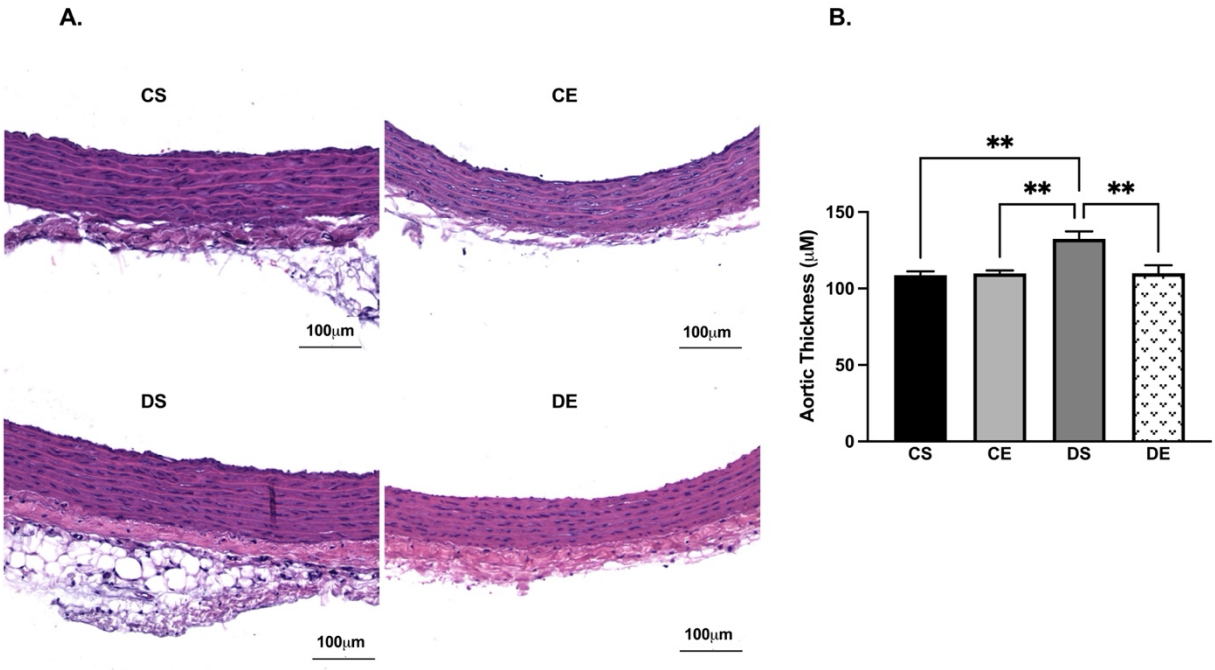

Figure 3.12 Effects MIE on aortic intima medial thickness. A) Hematoxylin and eosin (H&E) stained sections of aorta from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. B) Comparison of aortic intima medial thickness. Each bar represents the values obtained from n=4 animals per group. Capped lines indicate significant differences between two groups (**p<0.01,), analyzed by one-way ANOVA followed by Tukey's post hoc test.

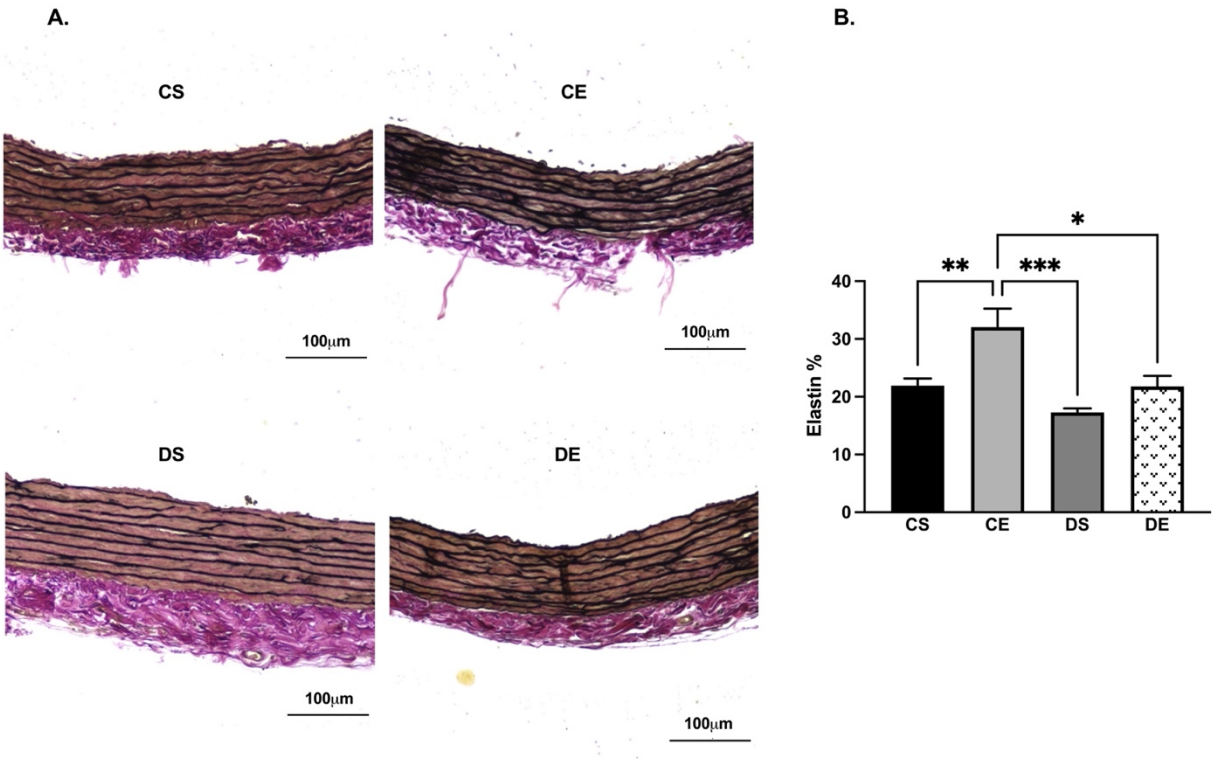

Figure 3.13 Effects MIE on aortic elastin content. A) Verhoeff and Van Gieson-stained sections of aorta from sedentary and exercise-trained SD (CS, CE) and diabetic (DS, DE) rats. Elastin fibers are stained as black lines in the intima. B) Comparison of aortic elastin content (% to total area). Each bar represents the values obtained from n=4 animals per group. Capped lines indicate significant differences between two groups (*$p<0.05$, **$p<0.01$, ***$p<0.001$), analyzed by one-way ANOVA followed by Tukey's post hoc test.

3.4 Discussion

To the best of our knowledge, this is the first study that measured the effects of MIE on vascular function and wall structure of the UCD-T2DM model. Here, we demonstrated an impairment of ACh-induced vasorelaxation in MA from DS rats. Our data also showed that the impaired vasorelaxation of DS arteries could be associated with the enhanced vasocontractile contribution of COX and/or loss of EDHF-type relaxation. Intriguingly, MIE improved vasorelaxation in MA, possibly due to a shift from contractile COX to NO- and EDHF-type relaxation in the diabetic group. Furthermore, MA from the DS group exhibited a higher contractile response and myogenic tone than in arteries from control rats. Our data also showed that exercise training reduced the PE-induced contractile response and myogenic tone of MA in diabetic rats. Additionally, we demonstrated that wall thickness in both small and large arteries were significantly greater in the DS group, and MIE reduced the wall thickness of diabetic arteries to the same level as controls.

Lack of physical activity was reported to be correlated with obesity (Ladabaum et al., 2014). On the other hand, according to the American College of Sports Medicine and American Diabetes Association, the recommended physical activity (150 mins/week) may help to lose weight. However, 60mins/day of physical activity may be required when relying on exercise alone to lose weight (Colberg et al., 2010). In the current study, eight weeks (1hr/day, 5days/week) of MIE reduced body weight, abdominal adiposity, and triglyceride level in the diabetic group (DE) compared to their sedentary counterpart (DS). Previously, changes in triglyceride levels were observed in both Zucker Diabetic Fatty (ZDF) rats and patients of coronary heart diseases after eight weeks of aerobic exercise training (D. Ito et al., 2015; Y. Wang et al., 2019). MIE did not alter the plasma insulin, blood glucose, and HbA1c level in

either control or diabetic groups. In agreement with our observation, a previous report suggested insulin secretion of T2D patients did not change after a single bout of MIE (Knudsen et al., 2015). Additionally, clinical trial from the US diabetes prevention program indicated MIE with caloric restriction could reduce the incidence of diabetes in impaired fasting glucose individuals up to 58% compared to 31% of metformin treated group (Knowler et al., 2002). We did not implement caloric restriction in our experimental groups since the focus of our study was to investigate the mechanism of MIE-induced changes in MA structure and function in diabetes. Still, we observed a reduction in metabolic risk factors such as body weight, abdominal adiposity, and triglyceride level in the exercise-trained diabetic group. However, MIE did not significantly improve the glycemic or insulin status of the diabetic group.

Endothelial dysfunction and impaired EDV is a hallmark of CVD in diabetes. Several studies, including ours, have reported endothelial dysfunction and impaired EDV in diabetes (De Vriese et al., 2000; Moien-Afshari et al., 2008; Shaligram et al., 2020; Takenouchi et al., 2010; Zhang et al., 2012). In addition, the protective effects of exercise against vascular dysfunction in diabetes are also reported in a number of studies (Qiu et al., 2018). In the current study, the sedentary UCD-T2DM rats showed a significantly impaired vasorelaxation of MA compared to control groups. MIE improved the vasorelaxation without changing the glycemic status, suggesting vascular dysfunction in diabetes could be prevented by MIE, independent of hyperglycemic status. Similarly, previous report has shown that MIE could improve the aortic function of db/db mice without changing body weight or hyperglycemic condition (Moien-Afshari et al., 2008). Furthermore, Sakamoto et al. reported exercise training but not food restriction prevents endothelial dysfunction in type-2 diabetic OLETF rat aorta, indicating

exercise as the major physiological stimulus required for improving vascular function in diabetes (Sakamoto et al., 1998).

Impaired EDV in diabetes may arise from several mechanisms: decreased production of EDRFs and increased production of endothelium-derived contractile factors (EDCF), enhanced inactivation of EDRFs, impaired diffusion of EDRF to underlying smooth muscle, and reduced sensitivity of the smooth muscle to EDRFs (De Vriese et al., 2000). The contribution of EDRFs to EDV may change depending on the vascular beds. Although NO is considered the major EDRF in conduit arteries, in small resistant arteries, EDHF plays a significant role in EDV (Félétou et al., 2011). Previous reports from our laboratory using type-1 and type-2 diabetic rats demonstrated that EDHF-type relaxation was diminished, whereas the dependency on NO-mediated relaxation was enhanced in diabetic MA (Shaligram et al., 2020; Zhang et al., 2012). Similarly, in the current study, we observed a loss of EDHF-type relaxation with an increased dependency on NO-mediated relaxation in the MA of the DS group. Increased NO contribution may be explained by elevated eNOS expression (Figure 3.6) observed in DS and DE arteries. The elevated NO contribution in diabetic MA could be a compensatory mechanism for loss of EDHF-type relaxation. Previous research suggests a negative feedback relationship exists between NO and EDHF. NO attenuated release of EDHF in rabbit carotid and porcine coronary arteries (Bauersachs et al., 1996; Brandes et al., 2000). On the other hand, an augmented EDHF response was reported to compensate for reduced NO-mediated relaxation in diabetic arteries (Garland et al., 1995; Malakul et al., 2008). Additionally, our study was in agreement with previous reports which described increased eNOS expression in the heart and aorta of type-2 diabetic Goto- Kakizaki rat model (Bitar et al., 2005; Desrois et al., 2010). Despite the higher relative importance of NO, vascular relaxation was significantly impaired in the sedentary

diabetic model, possibly due to of loss of EDHF. Furthermore, any increase in NO has the potential for free radical-mediated damage, particularly under conditions of oxidative stress in diabetes, where peroxynitrite is formed more easily (Zhang et al., 2012).

Although NO is generally considered the principal mediator of EDV, it has become clear that EDHF is also an important contributor to EDV, especially in small arteries like MA. The contribution of EDHF on vasorelaxation and its identity could vary based on vascular beds under question (Leo et al., 2011). Tomioka et al. reported EDHF contribution on EDV is inversely proportional to the vessel diameter (Tomioka et al., 1999). On the other hand, the chemical identity of EDHF while remains debated, but increasing report suggests the involvement of SK_{Ca} and IK_{Ca} channels in the endothelium-derived hyperpolarization of different arteries (Brähler et al., 2009; Doughty et al., 1999). The classical EDHF pathway involves an agonist-induced increase in cytosolic Ca^{2+} concentration, followed by the opening of SK_{Ca} and IK_{Ca}. Activation of these K_{Ca} channels subsequently hyperpolarize VSMC via activation of Na^+/K^+-ATPase, $K_{ir,}$ and BK_{Ca} channel or through myoendothelial gap junctions (Edwards et al., 1998; Parkington et al., 2002; Sandow Shaun L. et al., 2002).

Altered EDHF-mediated relaxation was reported in pathological conditions such as hypertension, atherosclerosis, and diabetes (Félétou & Vanhoutte, 2004). Specifically, in diabetic animals, both increased and decreased EDHF-mediated responses were reported (Gao et al., 2011). Nevertheless, previous studies, including ours, reported attenuated EDHF mediated responses in mesenteric arteries from both type-1 and type-2 diabetic rats (Makino et al., 2000; Shaligram et al., 2020; Zhang et al., 2012). In agreement with the previous reports showing impaired EDHF-relaxation, our current study has demonstrated a diminished EDHF-type relaxation in MA of DS rats. Intriguingly, MIE prevented the loss of EDHF-mediated relaxation

in male UCD-T2DM rats (DE vs. DS, Figure 3.3 C-D). Similarly, Minami et al. reported nine weeks of exercise training improved EDHF contribution in MA of type-2 diabetic OLETF rats (Minami et al., 2002). Next, we investigated whether altered EDHF relaxation response was related to a change in the K_{Ca} (IK_{Ca} and SK_{Ca}) level of MA. In the current study, when IK_{Ca} expression level was investigated, both diabetic groups, regardless of exercise training, showed a decreasing trend of IK_{Ca} protein level in MA compared to their respective controls. However, the difference did not reach to the significant level (Figure 3.7A). On the other hand, when SK_{Ca} expression was measured in MA, the DS group showed a significantly decreased in SK_{Ca} level compared to CS and CE (0.7 and 0.8-fold decreased respectively). When only diabetic SK_{Ca} protein level was compared (DS vs. DE, t-test), the DE group showed an enhanced SK_{Ca} level (0.3-fold increase) in MA compared to DS (Figure 3.7B). This data may suggest that the decrease of EDHF-type relaxation observed in the DS group, in part, could be due to lower expression of SK_{Ca} channel in MA of DS group. Furthermore, the pattern of K_{Ca} expression observed in the current study was in a similar line with our recent report on the K_{Ca} expression in UCD-T2DM animals (Shaligram et al., 2020).

In addition to a possible role of altered EDRF, other mechanisms that may explain MA dysfunction in diabetes could also result from a decreased sensitivity of smooth muscle to NO or an enhanced contractile response to contractile agents such as PE or endothelin-1 (ET-1). Previous studies have reported impaired EDV in the presence of preserved endothelium-independent relaxation (Leo et al., 2010; J. C. Li et al., 2021; Takenouchi et al., 2010). Similarly, in the current study, we did not observe a difference in SNP (a NO donor) induced relaxation in any of the groups after blocking the EDRF contribution with a combination of Indo+L-NAME+Apamin+TRAM-34. This may further emphasize that endothelial dysfunction,

but not reduced smooth muscle sensitivity to NO in the DS group may be responsible for reduced EDV in this group. In contrast, reports of impaired SNP-induced relaxation in diabetes are also available (Brown et al., 2001; Csanyi et al., 2007). Clearly, further studies are required to elucidate the relationship between diabetes and vascular smooth muscle sensitivity to NO.

To further investigate whether an enhanced contractile response was responsible for the impaired vascular function in the DS group, we measured PE-induced contractile response in MA. Conflicting data about diabetes and its effects on the contractile response of the vasculature are available with reports showing decreased, no change, and increased contractile response (Park et al., 2011; Matsumoto et al., 2008; Guo et al., 2005). Previous studies which reported an increase in contractile response of vessels from diabetic rats, mentioned the contribution of COX-1&2 and oxidative stress leading to hypersensitivity of smooth muscle (Shi & Vanhoutte, 2008; Guo et al., 2005; Santiago et al., 2016). In the current study, we investigated vasoconstriction responses to an α_1 adrenoceptor agonist (PE) in MA from control and diabetic rats. PE-induced vasoconstriction response was enhanced in the MA of DS groups compared to DE and control groups, which could be due to an increase in contractile metabolites in DS arteries (Figure 3.8, Table 3.7). Furthermore, unlike in the DS group, inhibition of COX with Indo had no effect on the ACh response of the DE group, suggesting that MIE may reduce the contractile COX activity in MA of male UCD-T2DM rats (DE vs. DS, Figure 3.3 C-D). Both COX-1 and COX-2 may produce metabolites that regulate vascular tone. While COX-1 is expressed constitutively in almost all tissues, COX-2 is often the inducible isoform and expressed in inflammatory sites. Prostacyclin (PGI_2), the major vasodilatory product of the COX pathway, was reported to be generated by COX-1 but not COX-2 (Kirkby et al., 2012). However, paradoxically PGI_2 can also cause contraction of arteries due to dysfunction of its

preferential PGI_2 receptor (IP). In spontaneously hypertensive rat aorta, PGI_2 acts as an EDCF due to dysfunction of IP receptors (Gomez et al., 2008). Despite reports of both COX isoforms in endothelium-dependent contraction, increasing reports suggest in pathological conditions such as obesity, oxidative stress and hypertension, COX-2 could be a major source for EDCF. It was reported that COX-2 contributes to cardiac oxidative stress and coronary vasoconstriction in obese Zucker rats (Santiago et al., 2016). In hypertension, up-regulation of COX-2 expression and NOX-mediated generation of reactive oxygen species (ROS) and the subsequent activation of p38 MAPK favors the emergence of the amplified endothelium-dependent contractions (Félétou et al., 2011). In the current study, we did not measure the COX-derived prostanoids or measure vasoconstriction to PE in the presence of selective COX inhibitors. Therefore, we cannot define the role of specific COX isoform on vasoconstriction of MA. Nevertheless, we observed an increase in both COX-1&2 protein expression in DS arteries compared to DE, suggesting an enhanced COX derived contractile prostanoids, at least in part, could be a mediator of higher contractile response in the DS group.

Pressure-induced constriction, also known as myogenic tone, is a major regulator of blood flow and an inherent property of resistant arteries. In response to physiological pressures, myogenic arteries constrict, and at lower physiological pressure, the arteries dilate, thereby autoregulating the blood flow to organs and tissues (Johnson, 1986). Myogenic tone is also thought to be a critical contributor of vascular resistance and hence blood pressure (Meininger & Davis, 1992). Enhanced myogenic tone, therefore, could lead to elevation of systemic blood pressure in T2D (Bagi et al., 2005). In the current study, we demonstrated that pressure-induced myogenic constriction in the MA of the DS group was enhanced, and MIE significantly decreased the tone in DE, although it did not reach to the level of control groups (Figure 3.9).

Similar reports of enhanced myogenic tone in mesenteric, cerebral, and skeletal muscle arteries were reported in T2D rodent models (Lagaud et al., 2001; Jarajapu et al., 2008; Frisbee et al., 2002). In contrast, myogenic tone was also reported to decrease in the ophthalmic artery of type-2 diabetic BBZDR/Wor rat (I. Ito et al., 2006), suggesting pressure-dependent autoregulation of vascular tone could be affected differently in various resistant vascular beds in T2D. In the current study, increased myogenic tone in DS could be due to enhanced contractile prostanoids generated by COXs. In agreement with our results, previous reports suggest increase in one or more vasoconstricting prostanoids could lead to an increase in myogenic tone in diabetes, and COX-2 could be a major player contributing to this increased arterial tone (Bagi et al., 2005; Lagaud et al., 2001).

Superoxide generation in diabetes also may play an important role in activating endothelium-derived contractile factors (Shi et al., 2007; Shi & Vanhoutte, 2008; Vanhoutte & Tang, 2008). NO scavenging by superoxide anion could decrease NO bioavailability and favor endothelium-dependent contraction (Rubanyi et al., 1986). NOXs are the major sources of reactive oxygen species in vasculature. NOX-1 and NOX-2 are important sources of superoxide in MA (Y. Li & Pagano, 2017). When we measured NOX-1 expression in the MA, although DS arteries showed a greater expression compared to DE and control group, it did not reach to the significance level (possibly due to the lower n number). However, NOX-1 is not the only isoform in MA, and the presence of NOX-1, NOX-2, and NOX-4 was reported in MA (Y. Li & Pagano, 2017). In the current study, we tried to measure NOX-4 beside NOX-1. However, no detectable bands were observed, possibly due to our experimental limitation (unable to load higher protein amount and/or lower sensitivity of the antibody). Although we did not measure the other isoforms, previous reports, including ours suggest, increased expression of NOX-1,

NOX-2, and NOX-4 in the diabetic aorta and mesenteric arteries (Akther et al., 2021; Galán et al., 2012; Shaligram et al., 2020). In addition, in our recent report we have demonstrated that ROS generation was enhanced in primary aortic endothelial cell from UCD-T2DM rats (Akther et al., 2021). Therefore, NOX isoforms could increase oxidative stress in diabetic MA. Clearly, further studies are required to elucidate the role of individual NOX isoform on MA function in diabetes.

The diabetes-associated vascular diseases manifest endothelial dysfunction followed by structural changes of large and small arteries (Spinetti et al., 2008). IMT is considered a surrogate marker of atherosclerosis in pathogenic conditions (de Groot et al., 2004). In small resistant MA from db/db mice, increased outward remodeling was characterized by an enhanced cross-sectional area and thus suggesting an increase in wall thickness in diabetic arteries (Souza-Smith et al., 2011). Increased wall thickness, together with increased collagen deposition and change in the elastic lamina, were associated with increased stiffness in MA from aged rats (Briones et al., 2007). In the current study, we demonstrated that diabetes significantly increased both the MA and aortic wall thickness in the DS group. However, the exercise training prevented the increase in wall thickness in diabetic group and the MA and aortic wall thickness in DE group was similar to control groups (CS and CE) (Figure 3.11 and 3.12). In agreement with our observation, aerobic exercise reduced MA wall thickness (media/lumen ratio) in the hypertensive SHR model, (Xiong et al., 2021). Additionally, a previous report also suggests exercise decreases arterial wall thickening in human subjects with existing CVD and cardiovascular risk factors (Thijssen et al., 2012).

Arterial stiffness is a critical functional and structural property of the vessel wall in diabetes. Stiffening predominantly affects the aorta and, to a lesser degree, the peripheral

muscular arteries. A host of indices have been introduced to quantify arterial stiffness. Elastin and collagen are two key elements in vascular remodeling, and they can determine the stiffness passively (Wagenseil & Mecham, 2012). Arterial elastin degradation is often accompanied by increased deposition of collagen in the intima, leading to arterial stiffening and thickening of intima-media (Adeva-Andany et al., 2021). In the current study, the CE group had significantly higher aortic elastin content compared to all other groups, which may indicate a possibility of improved elasticity and reduced stiffness of the aorta in this group. In agreement with our observation, exercise training increased elastin content in the aorta of Wister Kyoto and SD rats (Matsuda et al., 1993; Souza et al., 2017). On the other hand, although DS aortas showed a lower level of elastin compared to DE, the difference did not reach to the significant level. In the present study, we did not measure aortic stiffness by pulse wave velocity and/or measure collagen content. Therefore, we are unable to explain whether aortic stiffness is increased in UCD-T2DM rats. However, previous reports suggest diabetes may enhance collagen content in the aorta of T2D rodents and humans and, therefore, increase aortic stiffness (Katz et al., 2011; X. Wang et al., 2006). In agreement with this theory, previous studies which alternatively measured aortic stiffness by pulse wave velocity in T2D patients, reported increased aortic stiffness (Dec-Gilowska et al., 2020; Mansour et al., 2013).

In conclusion, this study represents the first report showing MIE improves MA function possibly by causing a shift from contractile COX to NO-and EDHF- type relaxation in UCD-T2DM male rats. Furthermore, our data suggest MIE may improve EDV by decreasing COX expression and possibly reducing vasocontractile prostanoids activity in diabetic MA. An intriguing observation was that MIE reduced the loss of EDHF-type relaxation in diabetic MA, possibly by enhancing SK_{Ca} contribution on EDHF-type relaxation. Additionally, MIE reduced

myogenic tone, and the contractile response of MA arteries of the diabetic group, which could have in part contributed to the enhanced vasorelaxation observed in exercise-trained diabetic group. Furthermore, we demonstrated that MIE prevented the increase in wall thickness/IMT in large and small arteries, suggesting that MIE possibly also reduces adverse remodeling of arteries in UCD-T2DM animals. Clearly, additional studies will be required to understand the mechanisms of exercise-mediated changes in vascular endothelial function and wall properties in diabetes.

CHAPTER 4: CONCLUSION, FUTURE DIRECTIONS AND LIMITATIONS

Cardiovascular diseases (CVD) remain the leading cause of death in diabetic patients, regardless of the recent advances in cardiovascular medicine and technology. Despite the existence of a number of studies on the underlying mechanisms of vascular dysfunction in diabetes, the effect of sex hormones and exercise on the diabetic vasculature remained poorly understood. The main objectives of the studies in this dissertation were to investigate the impacts of 17-β estradiol (E_2) and moderate-intensity exercise (MIE) on mesenteric arterial (MA) function in the University of California Davis type-2 diabetes mellitus (UCD-T2DM) Rats.

In the first study outlined in this dissertation, we demonstrated that acetylcholine (ACh)-induced vasorelaxation was impaired in MA of ovariectomized (OVX) prediabetic UCD-T2DM Rats. E_2 replacement improved MA relaxation in OVX prediabetic group to a similar level to that in control groups. Inhibition of cyclooxygenase (COX) by indomethacin did not significantly affect the vascular responses in any groups, suggesting a minor role of COX metabolites in MA relaxation in the experimental groups. Inhibition of nitric oxide (NO) synthase (NOS) by L-NAME reduced vasorelaxation to ACh in control groups, but it did not completely abolish the vasorelaxation. We also showed that in control (healthy) groups, both NO and endothelium-derived hyperpolarizing factor (EDHF)-type relaxation were dominant in the MA relaxation of placebo and E_2 treated rats. However, in prediabetic groups, L-NAME completely abolished the vasorelaxation, regardless of E_2 treatment, suggesting a relative shift from EDHF-type relaxation to only NO-mediated relaxation in these groups. Furthermore, the sensitivity of MA to NO was significantly impaired in OVX prediabetic group, but E_2 treatment enhanced the MA sensitivity to NO. Lastly, E_2 treated OVX prediabetic rats exhibited improved

insulin signaling and glucose uptake in skeletal muscle. Overall, our data suggest that a greater vasorelaxation in the E_2 treated OVX prediabetic group could be, in part, attributed to the elevated role of NO or improved sensitivity of MA to NO in this group.

We previously reported that MA from diabetic female rats showed a greater extent of impairment compared to that in diabetic males despite having a relatively preserved vascular function at the early prediabetic state. However, when females become diabetic, they exhibit a greater endothelial dysfunction than males (Shaligram et al., 2020). Although the results of the current study are in agreement with our previous report demonstrating a possible protective effect of female sex hormones in the MA function at prediabetic state, additional studies are needed to establish the specific role of E_2 in the progression of vascular dysfunction in the diabetic state.

In the second study of this dissertation, we have demonstrated that ACh-induced MA vasorelaxation was impaired in the diabetic sedentary (DS) group. However, MIE improved the vasorelaxation of UCD-T2DM male rats, possibly by causing a shift from the contractile COX action to NO and EDHF-type relaxation. Furthermore, our data suggested that MIE may decrease vasocontractile COX activity and elevate EDHF-type relaxation, possibly by reducing the expression of COX isoforms and increasing expression of small conductance calcium-activated potassium channel (SK_{Ca}) in diabetic MA. Additionally, MIE reduced contractile response and myogenic tone in diabetic MA, and it decreased the wall thickness of both large and small arteries. Although these results suggest exercise training alleviates vascular dysfunction in UCD-T2DM male rats, we did not measure the MIE effects in female diabetic rats. Given that sex differences play an important role in cardiovascular physiology, additional

studies are needed to establish the specific role of MIE on vascular dysfunction in UCD-T2DM female rats and its underlying mechanisms.

A number of reports, including ours, suggest that EDHF, a significant contributor of endothelium-dependent vasorelaxation (EDV) in MA, is lost in diabetes (Leo et al., 2011; Shaligram et al., 2020; Zhang et al., 2012). Accordingly, our study suggests that EDHF-type relaxation is significantly higher in healthy rat arteries, but it is lost in both prediabetic and diabetic states. Loss of EDHF-mediated vasorelaxation in diabetes may result from reduced expression and/or function of potassium channels (i.e., small and intermediate conductance calcium-activated potassium channels; SK_{Ca} and IK_{Ca}) that control hyperpolarization of endothelial cells and subsequently underlying smooth muscle. Other factors include irregularities of myoendothelial gap junction function and reduced synthesis of hyperpolarization factors such as epoxyeicosatrienoic acids (EET). However, there is a common acceptance of SK_{Ca} and IK_{Ca} as the major contributors to the classical EDHF pathway. In our second study, we observed an elevated contribution of EDHF-type relaxation in exercise-trained diabetic MA compared to the sedentary diabetic group. Interestingly, we observed an increase in SK_{Ca} channel expression in MA of DE compared to that in DS group. These data suggest that the observed elevated EDHF-type relaxation in MA of DE may be attributed to the increased SK_{Ca} channel expression in this group. Further studies are required to elucidate the role of these channels in controlling EDHF-mediated vasorelaxation in health and diabetes. Understanding the impact of exercise on these channels may bring forward new therapeutic strategies to treat diabetes-induced microvascular disease.

Limitations

1) Third-order resistant MA with a diameter ranging from 200-350μm were used in our studies. Although this artery represent resistance arteries, it cannot completely explain complications of other resistance arteries such as renal arteries. Overall, the effects of 17-β estradiol or MIE could vary depending on the vascular bed (Padilla et al., 2011; Tostes et al., 2003).

2) In addition to estrogen, ovariectomy may also alter the plasma concentration of other hormones and other factors such as progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (Yen & Tsai, 1971). Such changes can also affect arterial response to vasodilator. Our first study does not rule out this possibility. However, 'E_2 replacement in ovariectomized rats mimics a 'female' response' and is, therefore, an argument against a significant role for other factors involved in the modulation of female response. In addition, previous studies, including ours, have also used E_2 replacement to investigate the female-specific responses of the vasculature (Rahimian et al., 1997; Stice et al., 2009). Therefore, we believe our interpretation and conclusion drawn from the E_2 replacement study to record female-specific vascular function is reasonable.

3) Whole mesenteric arteries containing endothelial and smooth muscle cells were subjected to Western blot analysis. Therefore, the expression of COX, $SK_{Ca,}$ and IK_{Ca} in the mesenteric wall could not be attributed explicitly to either smooth muscle or the endothelial layer.

4) It is important to note that we did not directly measure the hyperpolarization of smooth muscle in the current study. However, the sensitivity of Indo+L-NAME resistant relaxation of control groups to a combination of specific potassium channel inhibitors (apamin and TRAM-34) are characteristic for EDHF-mediated responses.

5) The aerobic exercise intensity was not set based on the peak oxygen consumption (VO_{2max}) in individual rats. Exercise at which a subject uses 50-65% of VO_{2max} may be considered moderate-intensity exercise. Our treadmill setup was not capable of measuring $VO_{2max,}$ and thus we set up the intensity/treadmill speed following a thorough review of the literature. In our study, the obese diabetic rats likely had a different VO_{2max} due to their increased weight. Therefore, we cannot rule out some slight variation in exercise stimulus gained by diabetic and control groups. However, the variation was possibly negligible as both diabetic and control could complete the 1-hour session with little stimulus.

6) The exercise-trained groups were subjected to forced exercise training with a low electrical stimulus when they stopped running. The number of electrical stimuli was greater in the acclimation period and gradually became less frequent over the 1st week of training. Eventually, the electric shock was turned off from 2nd-3rd week to reduce the effect of inflammatory stimulus. Rats were given a gentle tap on their back when they stopped running. Although we cannot rule out that the exercise-trained groups received a higher inflammatory stimulus from the electric shock at the early stage of training than the sedentary group, the effect might have been negligible due to the reduction of electrical stimuli to a minimum.

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