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Sexual Dimorphism in Aortic Function of UC Davis Type 2 Diabetes Mellitus Rat Model: Estrogen Specific Responses

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SEXUAL DIMORPHISM IN AORTIC FUNCTION OF UC DAVIS TYPE 2 DIABETES MELLITUS RAT MODEL: ESTROGEN SPECIFIC RESPONSES

by

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DEDICATION

This dissertation is dedicated to my father Md. Nurul Alam, mother Bulbul Akther, loving husband Md. Zahir Uddin and our son Azan Zahir.

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Sexual Dimorphism in Aortic Function of UC Davis Type 2 Diabetes Mellitus Rat Model: Estrogen Specific Responses

Abstract

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Little is known about the interaction between diabetes and sex in vasculature. This study was designed to investigate the effects of estrogen as well as type 2 diabetes (T2D) on aortic function in rats with respect to sex. To test the effects of T2D and sex, UC Davis Type 2 Diabetes Mellitus (UCD-T2DM) rat model was used. To study the effects of estrogen, ovariectomized Sprague- Dawley female rats and UCD-T2DM rats at pre-diabetic stage were used and the rats were implanted subcutaneously either with placebo or 17 β-estradiol pellets (60 days release, 1.5mg/pellets). The plasma analytes for metabolic parameters and aortic responses to vasodilator and vasoconstrictor agents were determined. The expression of molecules associated with vascular response (e.g. endothelial nitric oxide (NO) synthase (eNOS), Nox1, Nox4, intermediate conductance calcium-dependent potassium channels (IK_{Ca}) and small conductance calcium-dependent potassium channels (SK_{Ca})) were also evaluated in aortic tissue.

The main objectives of the study were whether 1) sex differences exist in the development of abnormal vascular responses of UCD-T2DM rats, 2) there were changes in the relative contributions of endothelium-derived relaxing factors (EDRFs) in modulating vascular reactivity of aorta, and 3) estrogen replacement improves the aortic function of ovariectomized UCD-T2DM rats at pre-diabetic stage.

In the study of examining the effect of sex and T2D, diabetes significantly impaired relaxation responses to ACh and SNP in aortic rings from female UCD-T2DM rats, however, potentiated the relaxation in males. The responsiveness to PE was significantly enhanced in both diabetic groups regardless of sex. Accordingly, the basal nitric oxide (NO), as indicated by the potentiation of the response to PE after L-NAME, was reduced in aorta of both diabetic groups. Blocking of COX, sGC and NOS completely abolished the relaxation response in female diabetic group whereas male diabetic animals showed a significant remaining relaxation response to ACh. Further incubation of aortic rings of male animals with TEA or TRAM 34 blunted the relaxation responses to ACh in both control and diabetic groups. However, the inhibitory effects of TEA or TRAM 34 on the ACh-induced relaxation in male UCD-T2DM group was greater than their respective controls. By contrast, ACh responses were not affected following incubation with Apamin in either group of male rats. Moreover, protein expression of IKca were significantly higher in male diabetic group compared with the respective controls.

In the estrogen replacement study, treatment with E_2 markedly enhanced the ACh responses of aortic rings in both control and pre-diabetic groups compared to respective placebo treated group. Moreover, effect of E_2 in improving the ACh induced relaxation response was significantly higher in control group compared with pre-diabetic animals. The responsiveness to PE were significantly reduced in both E_2 treated groups. Basal NO level was significantly higher in both E_2 replaced groups but in control group the level was significantly higher than the pre-diabetic rats. Also, protein expression level of Nox1 were decreased in E_2 treated control and pre-diabetic group but eNOS were enhanced only in E_2 treated control groups.

In conclusion, this study suggests that the effects of type 2 diabetes on aortic ring are sex specific and we showed a differential contribution of EDRFs in male UCD-T2DM rats.

Furthermore, our data suggests that elevated eNOS and decreased Nox1protein level may contribute to the higher impact of estrogen in ovariectomized control groups compared to the pre-diabetic rats.

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[Ca2+]i	intracellular calcium concentration
ACh	acetylcholine
Akt	protein kinase B
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
BK_{Ca}	large conductance Ca2+-activated K+ channels
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CRC	concentration-response curve
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E_2	17β-estradiol
EC	endothelial cell
EDCF	endothelium-derived contracting factors
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EDV	endothelium-dependent vasodilation
eNOS	endothelial NOS
ER	estrogen receptors

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HbA1c	glycosylated haemoglobin A1c
HDL	high density lipoprotein
IKCa	intermediate conductance calcium-activated potassium channels
Indo	indomethacin
iNOS	inducible NOS
IP3	inositol-1,4,5-trisphosphate
ISI	Insulin sensitivity index
KIR	inwardly rectifying K+ channels
KV	voltage-gated K+ channels
LDL	low density lipoprotein
L-NAME	Nω-nitro-L-arginine methyl ester
mRNA	messenger ribonucleic acid
MA	mesenteric arteries
NADPH	nicotinamide-adenine-dinucleotide phosphate
NIDDM	noninsulin-dependent diabetes mellitus
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
Nox	nicotinamide-adenine-dinucleotide phosphate oxidase
$O_2^{\bullet-}$	superoxide anion radical
pD_2	-log [EC50]
PE	phenylephrine

PGI_2	prostacyclin
RNA	ribonucleic acid
ROS	reactive oxygen species
SK_{Ca}	small conductance calcium-activated potassium channels
SMC	smooth muscle cell
SNP	sodium nitroprusside
SOD	superoxide dismutase
STZ	streptozotocin
TXA_2	thromboxane A_2
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
WHO	World Health Organization

Chapter 1: Introduction

Cardiovascular diseases (CVD) are the major causes of morbidity and mortality in patients with diabetes. In healthy premenopausal women, the risk for CVD is lower compared to age-matched men (1-3). However, women after menopause experience higher incidence of CVD. This presumably related to the reduced level of estrogen (4-6). Multiple studies have shown that premenopausal women with diabetes not only lose the sex-based cardiovascular protection but also experience a higher relative risk of CVD compared to diabetic men (7-9). According to the "Female and CVD- 2019 Statistical Fact Sheet", CVD accounts for the highest percent (49%) of total death among female in United States, claiming over 412,244 deaths in 2016 (10).

1.1 Cardiovascular Diseases

CVDs are considered as a class of diseases of the heart and blood vessels which includes heart failure, coronary artery diseases, stroke, cardiomyopathy, peripheral artery disease, venous thrombosis, thromboembolic disease etc. (11). CVD are the number 1 cause of death globally (12). In 2016, approximated 17.9 million people died from CVD and 85% of these deaths occurred due to heart attack and stroke (12). Stroke and heart attacks mainly results from blockage of blood vessels that prevents the blood flow into the brain or heart. This blockage occurs mainly due to fatty deposits on the inner walls of the blood vessels (13). The common risk factors of developing CVDs include diabetes, hypertension, unhealthy diet, obesity, hyperlipidemia, tobacco use and physical inactivity. People with CVD or who are at high cardiovascular risk, has one or combination of risk factors.

1.2 Diabetes

In United States 30.3 million people have diabetes mellitus which is around 10% of the total U.S. population. 84.1 million U.S. adults have prediabetes. It is estimated that as many as 1 in 3 American adults will have diabetes in 2050 (data from American Diabetes Association and CDC).

According to WHO, diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively make use of the insulin produced (13). In patients with diabetes, high blood glucose can lead to several vascular diseases including micro-and macro-vascular consequences, like atherosclerosis, retinopathy and nephropathy.

1.2.1 Type 1 diabetes mellitus. Type 1 diabetes mellitus (T1DM) (previously known as insulin-dependent diabetes mellitus (IDDM)) is a form of diabetes mellitus in which pancreatic beta cells fail to produce enough insulin, leading to insulin deficiency. This type can be further classified as immune-mediated or idiopathic because T- cell mediated autoimmune attack destroy beta cells in the pancreas (14). In North America and Europe, T1DM accounts for around 5% of diabetes mellitus cases and there is no known preventive measure against it.

1.2.2 Type 2 diabetes mellitus. Type 2 diabetes mellitus (T2DM) is a type of metabolic disorder which is characterized by high blood glucose along with insulin resistance and relative insulin deficiency. Insulin resistance occurs primarily within the liver, muscles and adipose tissue. After developing insulin resistance cells lose the ability to respond adequately to normal levels of insulin. Although the specific defects are not known, insulin receptors are thought to be involve for the defective responsiveness of insulin to body tissues. T2DM accounts for 90% of

cases of diabetes. Several factors besides genetics are involved in the developments of T2DM such as lifestyle, age, obesity, diet, female sex hormones etc. Therefore, T2DM can be managed partly by lifestyle modification i.e. healthy diet and exercise. Diabetes ultimately leads to macrovascular and microvascular diseases due to the damage in large and small arteries respectively.

1.2.3 Gestational diabetes. Gestational diabetes is a form of diabetes which develops in some women during pregnancy. Majority of diabetes diagnosed during pregnancy is type 2 diabetes. Generally, after giving birth this type of diabetes does not exist. However, the person with gestational diabetes has a higher chance of developing T2DM in the later stage of life.

1.3 Diabetes and Cardiovascular Diseases

One of the major risk factors for CVD is diabetes. Both type 1 and type 2 diabetes are closely related to CVD. Diabetes causes cardiovascular damage in several ways and each may worsen or accelerate the effect of others. Thus, the heart and blood vessels are subjected to multiple attacks as diabetes progresses. It is more likely to develop CVD in individuals with diabetes than those without diabetes. Therefore, patients with diabetes are at high risk of morbidity and mortality due to the development of cardiovascular complications.

1.4 Endothelial Functioning

Endothelium is an inert anatomical barrier between blood and the vessel wall. It forms the inner lining of all vasculature. Under physiological and pathological conditions, endothelial cells act by releasing a variety of relaxing and contracting factors which can regulate the basal vascular tone by responding to neurohumoral mediators and mechanical forces (15). In the

pathogenesis of diabetic vascular disease, endothelial dysfunction plays a major role. Endothelial dysfunction refers to an impairment of the ability of endothelium to properly maintain the vascular homeostasis.

Endothelial cells regulate the tone of underlying vascular smooth muscle cells (VSMC) by producing several mediators of vasodilation. The endothelium-derived relaxing factors (EDRF) include nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factors (EDHF). In addition, endothelial cells also release several vasoconstrictor prostanoids (endothelium-derived contracting factors, EDCF). Endothelial dysfunction occurs when the release of EDRFs decreased and if the propensity of EDCF production is increased. This is the first step in the chain of events which ultimately leads to atherosclerosis and coronary disease (16). Endothelium-dependent vasodilation (EDV) is used as a reproducible parameter to probe endothelial function in different pathophysiological conditions (17) . It has been observed that EDV is impaired in various vascular beds of different diabetic animal models and in humans with type 1 and type 2 diabetes. The initiating factor in the development of diabetic vascular disease is the loss of modulatory role of endothelium. Although, the cellular basis of endothelial dysfunction in CVD with diabetes is widely studied but the interaction of sex with these conditions is not fully understood and demands detailed investigation. Impairment of EDV may occur through several mechanisms: decreased bioavailability of EDRFs, decreased responsiveness of the smooth muscle to EDRF and enhanced generation of EDCFs.

1.4.1 Nitric Oxide (NO). NO is produced through oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS). Different isoforms of NOS differ in structure and in function (18). There are three distinct isoforms of NOS : nNOS (neuronal NOS; type I), iNOS (inducible NOS, type II) and eNOS (endothelial NOS, type III). eNOS and nNOS are referred to as Ca^{2+}-

dependent enzymes and these enzymes are expressed constitutively. iNOS is Ca^{2+}- independent and induced by immunological stimuli such as cytokines, bacterial lipopolysaccharide and other agents. nNOS plays a role in cell communication and produces NO in skeletal muscle and nervous tissue. NO generated by eNOS plays a key role in the vascular tone regulation involving vasodilation, inhibition of leukocyte adhesion and vascular inflammation, inhibition of platelet aggregation and adhesion, regulation of vascular smooth muscle proliferation, angiogenesis stimulation, and endothelial progenitor cells activation (19).

eNOS is activated by agonists such as ACh which binds to G-protein-coupled receptors on the endothelial cell membrane, therefore increases the concentrations of intracellular Ca^{2+} and the increased Ca^{2+} binds to the calmodulin (20). NO mediates the formation of cyclic guanosine monophosphate (cGMP) by activating soluble guanylate cyclase and facilitates the vasodilation by diffusing into VSMCs (Figure 1). NO is metabolized rapidly to nitrite and then to nitrate before being excreted in the urine (21). The half-life of NO is less than 4s.

eNOS can also be activated by shear stress and the activation depends on ATP-gated Ca^{2+} channels that activates phosphatidylinositol 3-kinase (PI3K), leading to activation of protein kinase B (Akt), which directly phosphorylates eNOS on Ser1177, causing activation of eNOS (20, 22). A reduced activity and/or expression of eNOS could be responsible for a decrease in NO production. Notably, decreased NO production and/or increased NO elimination are closely associated with endothelial dysfunction.

In addition, accelerated degradation of NO by reactive oxygen species (ROS) is probably the major mechanism impairing NO bioavailability in diabetes. Uncoupling of eNOS due to oxidative stress can lead to the formation of superoxide ($O2^{\bullet -}$) which readily reacts with NO to

form peroxynitrite ($ONOO^-$), resulting decreased bioavailability of NO and impaired vasodilatory responses (23). Intracellular sources of ROS include peroxidases, xanthine oxidases, COX, lipoxygenases, the mitochondrial respiratory chain, heme-containing proteins, uncoupled NO synthases, and the NADPH oxidase (Nox) (24). Endothelial cell express five types of Nox subunits which includes Nox1, Nox2, Nox3, Nox4 and Nox5 (25). Nox1, Nox2 and Nox5 are considered as superoxide generating enzyme and promote endothelial dysfunction (25). On the other hand, Nox4 is hydrogen per oxide (H_2O_2) generating enzyme and has the vasoprotective effect (25-28). Vascular walls express high levels of Nox1, Nox2 and Nox4 (29). Nox1 is mainly expressed in large conduit vessels (30), whereas Nox2 is more highly expressed in resistance vessels (31).

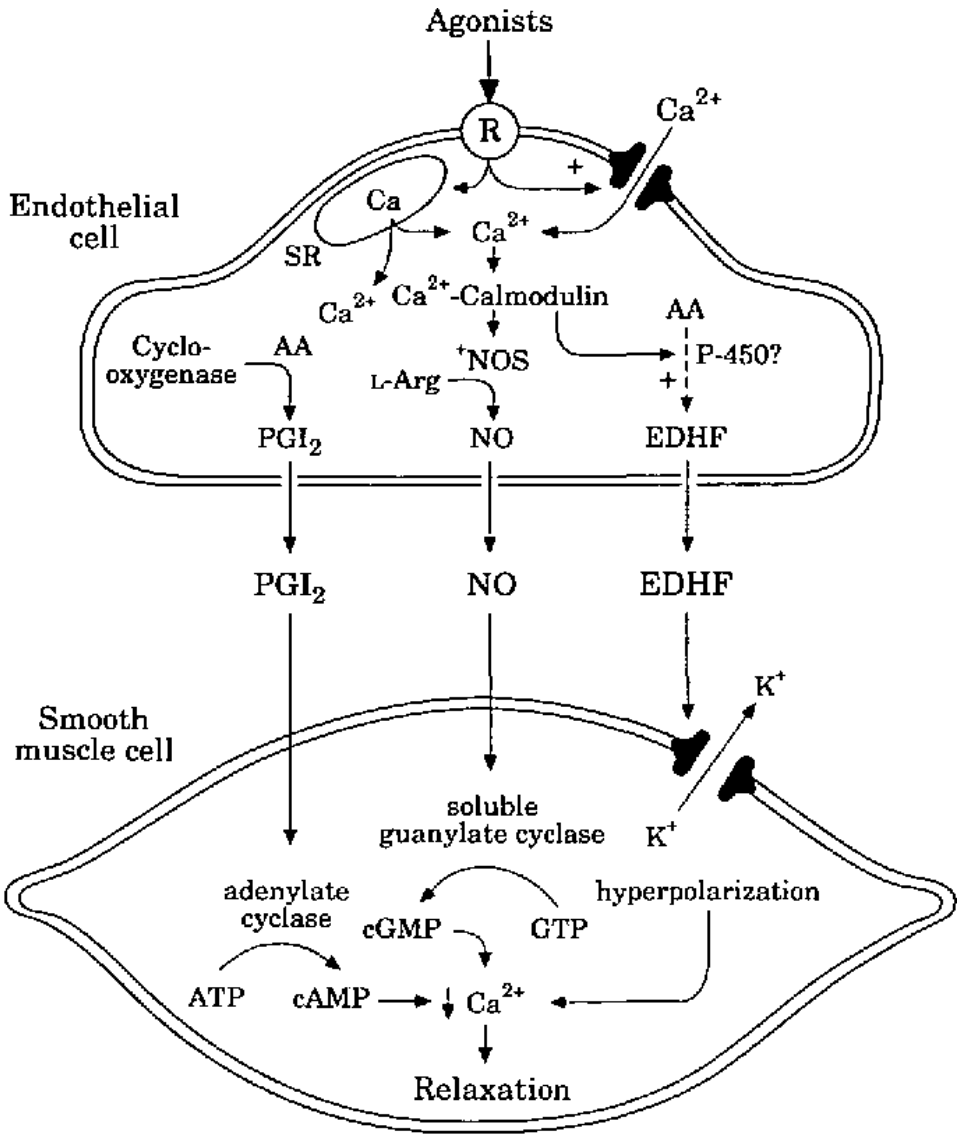

Figure 1: Release of endothelium-derived relaxation factors. Activation of endothelial receptor (R) induces Ca^{2+} influx into the cytoplasm of the endothelial cell. Upon binding with the agonists, inositol phosphate (IP3) increases which may contribute to the increased release of Ca^{2+} from sarcoplasmic reticulum (SR). Ca^{2+} causes the activation of nitric oxide synthase (NOS) and releases endothelium-derived hyperpolarizing factor (EDHF) after interacting with calmodulin. The elevated Ca^{2+} also leads to the formation of prostacyclin (PGI_2) from archidonic acid (AA) by cyclooxygenase. NO relaxes the smooth muscle by leading to the activation of cGMP from GTP. PGI_2 activates the formation of cAMP from ATP and causes the relaxation. EDHF causes relaxation by opening of K^{+} channels and hyperpolarization. Adapted from (32).

1.4.2 Prostacyclin (PGI_2). Endothelial prostanoids are produced by the phospholipases after releasing arachidonic acid from membrane-bound phospholipids. Cyclooxygenase (COX) metabolizes arachidonic acid and gives rise to prostaglandin H_2. Prostaglandin H_2 is then converted to several potent vasoactive prostanoids such as PGI_2, prostaglandins E and F and thromboxane A_2. Among the prostanoids, PGI_2 is the major prostanoid synthesized by endothelial cells. PGI_2 leads to the activation of adenylyl cyclase and an increase in the production of cyclic adenosine monophosphate (cAMP) by interacting with its GS-coupled PGI_2 receptor on the plasma membrane of vascular smooth muscle. cAMP then relaxes the vascular smooth muscle by activating protein kinase A. which phosphorylates selected target proteins and results in smooth muscle relaxation. Decreased secretion of PGI_2 are closely related to the clinical and experimental models of diabetes (33, 34).

1.4.3 Endothelium-derived hyperpolarizing factor (EDHF). EDHF, released from endothelium, is considered to be a substance and/or electrical signal that is produced or synthesized in the endothelium. EDHF relaxes the VSMCs by hyperpolarizing smooth muscle cells and this is associated with K^+ channels. The EDHF-signaling pathways is not fully understood. The contribution of EDHF in relaxation responses is dependent on the size of the vessel. It is well established that EDHF is more prominent in the smaller arteries than the larger ones and is also an important regulator of vascular tone. Two major pathways of EDHF are involved in vascular reactivity (35). In the classical pathway, intracellular Ca^{2+} increases by shear stress or by local hormones such as ACh, bradykinin, substance P or histamine. This elevated Ca^{2+} can activate two endothelial expressed potassium channels-small conductance calcium-activated potassium (SK_{Ca}) and intermediate conductance calcium-activated potassium channels (IK_{Ca}) and activates Na^+-K^+-ATPase and/or inwardly rectifying K^+ (K_{IR}) channels, then

subsequently hyperpolarize smooth muscle cells (36-38). The second pathway for EDHF does not involve the hyperpolarization of endothelial cells. It is associated with the release of eclectic variety of EDHFs followed by the activation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels or ATP sensitive-K^+ (K_{ATP}) channels, therefore hyperpolarizes the smooth muscle cells (39-41). The relative importance of the relaxation factors differs with vessel size (42). In large arteries and larger arterioles, NO is the major modulator of EDV. Decreased bioavailability of NO is considered as the main marker of endothelial dysfunction in aorta. NO, PGI_2 and EDHFs are thought to be involved in mediating vascular tone of resistance arteries (43). In diabetes, the contribution of EDHF to EDV were altered in small resistant arteries (44).

1.5 Diabetes, CVD and Sex Differences

Sex differences are the biological variances arise from variation in gene expression of sex chromosomes. This may be further transformed by changes in sex hormones, resulting in modified sex specific function (45). Earlier, majority of the clinical, epidemiological and experimental studies were conducted in male population and the outcomes were simply applied to females (46). The prevalence of CVD is higher in postmenopausal women compared to that of premenopausal women and the consequences are often worse compared with men. Even though, the risk of developing CVDs in healthy premenopausal women are much lower than that in age matched men, women lose their sex-based protection after becoming diabetic (47-49).

We have previously reported a sex differences in aortic and mesenteric arterial function in STZ- induced diabetic model and hyperglycemic rats (50-52). In those studies, we reported a predisposition to vascular injury of female rat aorta and mesenteric arteries in these pathological conditions. Moreover, in epidemiological studies, it has been observed that the number of

women with impaired glucose tolerance (IGT) is 20% higher than in men and the total number of women with T2DM is 10% higher than in men (53) . Thus, further studies are required to understand the underlying mechanism for these sex difference reported. This may help in developing different therapeutic approaches and setting new therapeutic possibilities which may offer a route to improved care for women.

1.6 Estrogen and Cardiovascular System

Estrogens are a group of sex hormones that promote the development and maintenance of female characteristics in the human body (54). Estrogen has favorable effects on vasculature, antioxidant activity, enhanced fibrinolysis and lipid profile. Effects of estrogen in vascular function occurs through genomic and nongenomic mechanisms (55-57). The genomic vascular effects involve modifications in expression of vascular cell gene by two distinct nuclear ERs (ERα and ERβ) and ultimately leads to an improvement of endothelial function and the responses to injury of blood vessels and development of atherosclerosis (55-57). The nongenomic effect of estrogen occurs due to its interaction with plasmalemmal receptors in the endothelium and VSMCs. For example, estrogen results in vasodilation by increasing NO production through activating eNOS or by inhibiting vascular contraction (58, 59). Depending on the endothelial NO production and ion- channels, nongenomic vasodilator effects of sex hormones appear to have both endothelium-dependent and endothelium-independent mechanisms respectively (56, 57, 60). Sex differences in vascular reactivity partly related to the density of specific receptors as the expression of sex hormone receptors may vary depending on gonadal function, sex and degree of atherosclerosis. Female arteries have higher amount of estrogen receptor (ER) than those of men (61). Moreover, postmenopausal women and women with coronary artery disease have reduced number and activity of ERs in the vasculature (62, 63). In diabetic women,

estrogen level decreases than those of nondiabetic women which results in progressive decline in progressive decline in glucose-stimulated insulin secretion and increase insulin resistance by reducing insulin sensitivity (63, 64).

1.6.1 Estrogen and NO. Premenopausal women have elevated level of total NO than in men (65). The differences in endothelial NO production may explain the sex differences in modulating vascular tone but the cellular origin of the increased NO in women is not well understood (64, 65). It is well established that estrogen has a major role in endothelial NO release (66-70). One of the reasons for elevated eNOS may be due to the activation of genomic ERs (71). In addition, estrogen can interact with specific ERs in endothelial cell membrane and also activate nongenomic signaling pathways, thus regulate vascular tone by producing NO (68, 70). Intracellular calcium concentration ($[Ca^{2+}]i$) has a major role on the effect of estrogen in eNOS activation and NO production. In the vascular endothelium, sex differences in the endothelial $[Ca^{2+}]i$ regulation has been correlated to the estrogen effects on the Ca^{2+} handling mechanisms in the vascular endothelium (70, 72, 73). Evidence suggests that estrogen can stimulate NO release and increases in $[Ca^{2+}]_i$ by activating cell surface ERs in human endothelial cells. It has also been suggested that the sensitivity of eNOS to $[Ca^{2+}]i$ may differ in male and female vasculature. Estrogen can activate eNOS through the PI3K/Akt pathway by inducing phosphorylation, thus reduces the requirement of $[Ca^{2+}]i$ for activation (74). The antioxidant effects of estrogen may also have a role on NO pathway. In human umbilical vein endothelial cells, estrogen inhibited NADPH oxidase expression and the generation of reactive oxygen species (ROS) and peroxynitrate ($ONOO^-$) (75). The amount of superoxide ($O_2^{\bullet-}$) is higher in male aortic rings than in female rats (76). Moreover, estrogen increases NO bioactivity and decreases $ONOO^-$ release by decreasing the generation of $O_2^{\bullet-}$ in cultured bovine aortic

endothelial cells (77). Therefore, estrogen administration may improve endothelial function and prevent oxidative stress by decreasing vascular tone and arterial pressure.

1.6.2 Estrogen and PGI_2. The production of COX products such as PGI_2 may be augmented by estrogen (68) . At physiological concentration, estrogen can upregulate the PGI_2 synthesis bovine fetal pulmonary artery and human umbilical vein endothelial cells (78). Studies have shown that the COX-2 pathway plays a specific role in estrogen-induced potentiation of cholinergic vasodilation in postmenopausal women (79). However, it has been reported that estrogen-induced relaxation does not alter by the addition of indomethacin in coronary artery, suggesting that the release of prostanoids may not be involved in the estrogen-induced coronary relaxation (80). It has also been shown that estrogen may enhance the NO-mediated endothelium derived relaxation by modulating cross-talk between the NO synthase and COX pathways of vasodilation which is associated with a decrease in the COX component (81).

1.6.3 Estrogen and EDHF. The differences in the endothelium-dependent hyperpolarization of VSMCs may be associated with the higher endothelium- derived relaxation in females than in males (82). It has been suggested that ACh-induced vasorelaxation and hyperpolarization of mesenteric arteries are reduced in intact male and ovariectomized female rats compared with that in intact female rats. Interestingly blocking of the K^+ channels by apamin or charybdotoxin eliminated the differences in ACh-induced relaxation responses between male and female. Furthermore, treatment with estrogen improved the hyperpolarizing response to ACh in ovariectomized female rats. These data suggest that EDHF -mediated relaxation is attenuated in estrogen- deficient states (83, 84).

1.6.4 Estrogen and male vasculature. Estrogen also affects vascular function in males. The level of estrogen in males are dependent on androgen production. In males, almost 80% of estrogen in plasma derives from aromatization of androstenedione and testosterone into estrogen. Furthermore, estradiol can stimulate ERs of endothelial and VSMCs which is produced directly in the male vasculature. Interestingly, estrogen has been shown to improve endothelial function at low-dose in men with endothelial dysfunction, aromatase deficiency and diffuse atherosclerosis (85, 86). Although estrogen is considered to be the major factor in sex-based differences in CVDs, the effect of other sex hormones such as androgen, progesterone and testosterone cannot be ignored. It has been suggested that these sex hormones have role in regulating vascular tone, lipid profile and cell proliferation. For example, studies have reported that both progesterone and testosterone have antiatherosclerotic effect and influence the release of EDRF from endothelial cells, inhibit proliferation of smooth muscle cells. Furthermore, progesterone can increase high density lipoprotein and decrease LDL (87). Effect of these sex hormone are not clearly understood and warrants further investigation.

1.7 Study of Type 2 Diabetes and Type 2 Diabetic Rodent Model

Metabolic diseases and the risk factors associated with the disease frequently occurs in combination such as diabetes, obesity, dyslipidemia, and hypertension. Several hereditary animal models have been used to investigate these diseases which includes KK-Ay mouse, ob/ob mouse, db/db mouse, Zucker Diabetic Fatty (ZDF) rat, GotoKakizaki (GK) rat, Spontaneously Diabetic Torii (SDT) rat, OtsukaLong-Evans-Tokushima-fatty (OLETF) rat, and Spontaneously Hypertensive Rat (SHR). In addition, genetically modified animals,chemical (e.g. streptozotocin (STZ), alloxan) and diet-induced (e.g., high fat, high fructose, high sucrose, high cholesterol) fructose, high fat, high cholesterol) experimental animal models have also been used widely (88,

89). Type 2 diabetic rodent models have been available for many years. There are several reasons for using rodent models of human disease which includes smaller size, shorter breeding time and inexpensive housing. Due to the smaller size, rodent models can be used to design preventive strategies and treatments with minimal expense. For the study of human type 2 diabetes, variety of rodent models are commercially available. Several factors are associated with human type 2 diabetes including the degree of obesity at disease onset, age of onset, and the degree of deterioration in beta cell function observed over the course of the disease. All the potential characteristics of disease will not be manifested by a single individual. Likewise, no single rodent model represents all possible pathologies associated with type 2 diabetes. Furthermore, due to the inherent species difference and methodologies used to breed rodent models, the ability of rodents to mimic accurately human type 2 diabetes is challenging. Therefore, there is an ongoing demand for novel models which would better resemble the pathologies of human disease.

In human, obesity is primarily caused by polygenic mutation, but experimental studies of type 2 diabetes are conducted by using monogenic model of obesity. The most commonly used monogenic rodent models of obesity have leptin signaling deficiency. Major function of leptin is to induce satiety, therefore, deficiency in leptin signaling causes hyperphagia and subsequent obesity in these animals. These models include the Lep ob/ob mouse, the Lepr db/db mouse and Zucker Diabetic Fatty rat. These models are frequently used to test new therapeutic approaches for type 2 diabetes. However, lack of leptin signaling leads to early onset of diabetes, infertility, severe hyperlipidemia, lack of insulin sensitizing effect and lipotoxic effects which are disparate to those seen in human type 2 diabetes.

Rodent models with polygenic origin of obesity may represent more accurate model of human disease states. Although, there are different polygenic rodent models of diabetes, glucose intolerance and obesity exist, but unlike the monogenic models, there are no wild-type controls. In addition, biasness towards male sex is more extreme in these models (90).

The NZO mouse is generated by selective breeding, has a polygenic origin of obesity. It has deficiency in leptin transportation across the blood brain barrier, resulting in hyperphagia and obesity due to the lack of centrally mediated actions of leptin. In addition, NZO mice have early onset of obesity and poor fertility (89).

Another new model of type 2 diabetes is Spontaneously Diabetic Torii *Lepr f a* (SDT fatty) rats which are generated by introducing the fa allele of the ZDF rat into the SDT rat genome. Both sexes of SDT fatty rats became overtly obese and showed a significant hyperphagia (91).

In OLETF rat, diabetes is induced by two mutations which are the particular concern regarding their non-human-like characteristics. This model of rats become hyperphagic due to the mutation in CCK-A receptor subtype. Also, they have x-linked mutation (obd-1) which results in the diabetes phenotype in males but female rats do not develop diabetes. In addition, to manifest diabetes, OLETF rats required testosterone unlike humans.

It is very important to consider the lean animal model of type 2 diabetes in the study as not all the type 2 diabetic patients exhibit obesity. The Goto Kakizaki rats resembles several aspects of human type 2 diabetes but does not become obese unlike most of the human type 2 diabetic patients. This lean model of type 2 diabetes characterized by impaired insulin secretion and glucose intolerance. Aberrant beta cell function and/or mass results in defective glucose

metabolism and insulin resistance is not the main reason for initiating hyperglycemia in this model (92) . Finally, the major disadvantage with most of these models is the fact that typically female animals do not develop diabetes, or they develop diabetes under environmental stressed conditions. High fat and high sugar diet can induce hyperglycemia in ZDF female rats and in Wistar female rats required high sucrose diet to induce hyperglycemia. In contrast, rate of developing diabetes in human females is slightly higher than males (89).

1.7.1 Need for a novel model of type 2 diabetes. To compensate the deficiencies of existing models, it became very crucial to develop novel models of type 2 diabetes. An ideal model should have the combination of two primary defects observed in human type 2 diabetes, i.e. adult onset of polygenic origin of obesity with insulin resistance, and defect in beta-cell compensation for insulin resistance. An ideal model should develop diabetes in both sexes at approximately equal rates and would not require any gene mutations to induce obesity or diabetes.

1.8 Statement of Research Problem and Hypothesis

The prevalence of developing CVD is lower in premenopausal women compared to age matched man (1-3). This lower risk is related to the presumptive cardioprotective effects of female sex hormones, estrogen in particular. However, diabetic premenopausal women not only lose this sex-based cardiovascular protection, they also experience a higher risk of CVDs compared to diabetic men (7-9). Several lines of evidence suggest that endothelial dysfunction represents early steps in the development of vascular complications in diabetes, and hyperglycemia is the central initiating factor for those complications. Nonetheless, there is

insufficient evidence to establish the timeline of the loss of this female-specific protection in premenopausal patients with diabetes.

With the increasing prevalence of type 2 diabetes, creating an effective preclinical model of T2DM has become crucial for the study of disease prevention and treatment. Dr. Havel and colleagues at University of California (UC) Davis, developed a rat model of type diabetes that has been validated in several studies including all features of type 2 diabetic pancreatic, hepatic and gastrointestinal dysfunction (93-99). UCD-T2DM rat model more closely resembles the pathophysiology of human T2DM. Till now there is no data on aortic function of UCD-T2DM rats. Previously we reported sex differences in the vasodilation of rat or rabbit aorta after acute exposure to high glucose (100). We also observed sex-based differences in impaired EDV of aorta and mesenteric arteries in STZ- induced type 1 diabetic rats (52). However, the pathophysiology of T1DM differs from T2DM.

Our hypothesis is aortic endothelial function is altered in UCD-T2DM rats. Furthermore, we hypothesize that there is a sexual dimorphism in aortic endothelial function of UCD-T2DM rats. If our data show the loss of vascular protective effect in diabetic females; we will investigate the timeline of its loss by examining EDV in pre-diabetic UCD-T2DM rats. Particularly, we will examine the effect of female sex hormones (specifically estrogen) by replacing estrogen following removal of ovaries.

1.9 Specific Aims

1) To investigate whether the aortic reactivity is altered in a novel and validated model of type 2 diabetes, UC Davis-T2DM mellitus (UCD-T2DM) rats with respect to sex;

2) To determine whether sex and type 2 diabetes altered the relative contributions of PGI_2, NO and EDHF to aortic endothelial function in male and female UCD-T2DM rats;

3) To determine the underlying mechanisms of sex-based differences in the vascular effects by studying expressions of specific proteins that are associated with arterial function (eNOS, peNOS, Nox1- and Nox4-dependent NADPH oxidase, as well as IK_{Ca} and SK_{Ca} channels) and insulin signaling (Akt, pAkT, IRS-1 and IRS-2);

4) To investigate the effects of 17β-estradiol (E_2) on aortic function in ovariectomized UCD-T2DM rats at the prediabetic stage;

5) To determine the effects of 17β-estradiol (E_2) on the underlying mechanisms of vascular effects by studying expressions of specific proteins that are associated with aortic function (eNOS, Nox1- and Nox4 dependent NADPH oxidase).

Chapter 2: Study I: Sex Differences in Aortic Reactivity of UC Davis Type 2 Diabetes Mellitus (UCD- T2DM) Rats: A Shift in the Relative Importance of Endothelium Dependent Relaxation Factors (EDRF)

2.1 Introduction

The prevalence of T2DM is on the rise worldwide, thereby creating an effective preclinical model of T2DM has become crucial for the study of disease pathogenesis, prevention, and treatment. Among the existing rodent models of T2DM (as described in Section1.7), most commonly used rodent models are Zucker diabetic fatty (ZDF) rat, Otsuka Long Evans Tokushima fatty (OLETF) rat, and the db/db mouse. They all exhibit obesity-associated insulin resistance and impaired β- cell function which results from a monogenic mutation. Even though these established models have generated a great deal of useful data, but they have several disadvantages. Obesity phenotype is the common problem with these rodent models of T2DM. In humans, obesity and insulin resistance is adult onset and polygenic in origin, monogenic mutations are not very common to onset diabetes. Thus, effective translation of research to clinical type 2 diabetes can be challenging (63).

Owing to the difference in the etiology, our collaborators at UC Davis, Dr.Havel et.al have developed a new rat model of T2DM that more closely models the human T2DM. This model is developed by crossing obese insulin-resistant Sprague-Dawley (OSD) rats with ZDF-lean rats, both have only one of the primary defects associated with type 2 diabetes and each are nondiabetic. ZDF-lean rats have defect in pancreatic β-cell insulin production and do not have the leptin receptor mutation unlike ZDF rats (101), results in diabetes only in the presence of insulin resistance. On the other hand, OSD rats, which were originally obtained from Charles River Laboratories (Wilmington, MA) differ from healthy SD animals as the OSD rats gain

weight more rapidly than SD rats purchased from a different vendor. The OSD rats do not develop diabetes due to strong β-cell compensation, exhibit adult-onset polygenic obesity and hyperphagia and more metabolically efficient than normal weight animals (102). After using selective breeding, subsequent generations successfully resulted in diabetes in both male and female animals. UCD-T2DM rat exhibit adult-onset obesity, impaired glucose tolerance, insulin resistance, β-cell decompensation, and preserved fertility in both sexes. Therefore, this models more closely mimics human T2DM. There is no data on the aortic function of UCD-T2DM rats. Thus, our preliminary aim was to investigate whether the aortic function is impaired and sex differences exist in the development of abnormal vascular responses in male and female UCD-T2DM rats.

Endothelial dysfunction is an early sign of diabetic vascular diseases. Endothelial dysfunction can be defined as a reduced EDV to vasodilators, such as ACh and bradykinin, or flow-mediated vasodilation. EDV is considered as a reproducible parameter to investigate endothelial function under various pathological conditions (17). In diabetes, enhanced (103), impaired (104) and preserved (105) EDV have been reported. EDV is largely depends on EDRFs, such as NO, PGI_2 and EDHFs.

The relative contribution of different EDRFs on EDV varies between the size of arteries (106) and vascular beds (107). NO has been considered as a major contributor of EDV in large conduit arteries (108), whereas EDHF plays the predominant role in small resistant arteries (109).

Different pathological conditions can alter the relative contribution of various EDRFs to EDV. Therefore, another aim of our study was to investigate the relative contribution of EDRFs in EDV of UCD- T2DM male and female rats.

2.2 Material and Methods

2.2.1 Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in water, unless otherwise stated.

2.2.2 Animals and experimental design. Rats were housed and bred in the animal facility in the Department of Nutrition at the University of California, Davis. All animal protocols were approved by the Animal Care Committee of the University of the Pacific and University of California Davis Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (US National Institutes of Health 2011) and with ARRIVE guidelines. Rats were maintained with water and standard rodent chow food ad libitum at constant humidity and temperature, with a light/dark cycle of 12 h. After acclimation for 1 week, animals were euthanized for experiments using carbon dioxide as euthanasia agent, according to the recommendations from the 2013 AVMA Guidelines on Euthanasia (110) and the NIH Guidelines for the Care and Use of Laboratory Animals: Eighth Edition (US National Institutes of Health 2011).

In the present study, male and female UCD-T2DM rats were used. Diabetic phase was determined by measuring blood glucose level for three subsequent measurements using a standard glucose test meter (OneTouch, LifeScan, CA). Animals were considered diabetic when nonfasting blood glucose levels were higher than 300 mg/dl. Age-matched male and female non-obese and non-diabetic SD rats (Simonsen Laboratories, Gilroy, CA) were employed as

controls for UCD-T2DM rats. All animals were average 19-20 weeks old. The diabetic animals used in the study were diabetic for about 35± 2.7 (males) and 31±3.1 (females) days.

2.2.3 Measurement of metabolic parameters in the plasma. Blood glucose were measured in 12-h fasted rats using standard glucose test meter (OneTouch, LifeScan, CA) and triglycerides were measured by using an Accutrend Plus System (hand-held point-of-care device) and specific test strips (Roche Farma, Barcelona, Spain) with a drop of blood collected from the tail vein. Blood samples were collected from intracardiac puncture and obtained in tubes containing heparin as an anticoagulant. Plasma was obtained by centrifugation at 10,000xg for 5 min at 4°C and stored at -80°C until used. Insulin levels were determined in plasma samples by using ELISA kits according to the manufacturer's protocol (Spi Bio, Montigny Le Bretonneux, France). Insulin sensitivity index (ISI) was determined from fasting plasma glucose and insulin using the following formula:

ISI = [2/ (blood insulin (nM) x blood glucose (μM) + 1].

2.2.3.1 HbA1C measurement. HbA1c is glycated hemoglobin (A1c), which identifies average plasma glucose concentration. In Particular, the test measures the percentage of blood glucose attached to the hemoglobin. HbA1c can also be measured using point-of-care technology, such as the Bayer A1cNow test kit. A1cNow test kit consists of a monitor and a sample dilution kit. Blood sample was collected after sacrificing the animal to measure HbA1c. The diluted sample was mixed and added to the monitor. After adding the sample, the monitor begins the analyze and results will be displayed in the display window after 5 minutes.

General range of HbA1c :

• An A1C level < 5.6 percent is considered normal

• An A1C level between 5.6 and 6.4 percent is considered pre-diabetes

• An A1C level > 6.5 percent on two separate tests indicates type 2 diabetes

2.2.4 Measurement of arterial tension. The thoracic aortas were cut into 2mm rings after being excised and cleaned off adhering connective tissues. To measure isometric tension, the rings were suspended horizontally between two stainless steel hooks in individual organ baths containing 20 ml of Krebs buffer (in mM: 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.9 $NaHCO_3$, 0.023 EDTA, 1.6 $CaCl_2$, and 6.0 glucose) at 37°C bubbled with 95% O_2 and 5% CO_2. Isometric tension was continuously monitored with a computer-based data acquisition system (PowerLab; ADInstruments, Colorado Springs, CO). To develop a stable basal tone, aortic rings were equilibrated under 1g resting tension for 40 min. Rings were stimulated two times with 80mM KCl every 20 min until maximum contraction was achieved. The ability of acetylcholine (ACh, 10 μM) to induce relaxation of phenylephrine (PE, 2 μM) pre-contracted vessels was taken as evidence for the preservation of an intact endothelium. For the relaxation studies, we used an equal submaximal concentration of PE (2 μM) in both males and females.

2.2.4.1 Relaxation responses to ACh. Aortic rings were precontracted with PE (2 μM), which represented a concentration that produced 80% of the maximal effect (EC_{80}). The concentration response curves (CRC) were obtained by the addition of increasing concentrations of ACh (10^{-8} to 10^{-5} M).

The first CRC was obtained by the addition of increasing concentrations of ACh (10^{-8} to 10^{-5} M). Tissue was washed 2-3 times with Krebs solution to allow it to return to basal tone. After it reached the basal tone, indomethacin was added (Indo; 10 μM; dissolved in DMSO), a COX inhibitor for 20 min. The rings were again precontracted with PE and the second CRC to

ACh was generated. The third CRC to ACh in PE-precontracted rings was obtained after incubation with Indo and 1H- [1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ; 10 μM), an inhibitor of soluble guanylate cyclase (sGC) for 20 min. Finally, the fourth CRC to ACh was obtained after incubation with a combination of Indo, ODQ and N-nitro-L-arginine (L-NNA; 100 μM), a nonselective NO synthase (NOS) inhibitor for 20 min.

The contribution of K^+ to ACh-induced relaxation was assessed in aortic rings by obtaining the CRC to ACh before and after incubation for 30-min with tetraethylammonium (TEA; 2mM), a nonselective K^+ channel blocker; 1-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole (TRAM-34; 1 μM), a selective blocker of the intermediate-conductance Ca^{2+} activated K^+ (IK_{Ca}) channel; apamin (1 μM), a small-conductance Ca^{2+} activated K^+ (SK_{Ca}) channel inhibitor. Tissues were washed with Krebs buffer between each concentration response curves to allow the rings to return to basal tone.

2.2.4.2 Relaxation responses to Sodium Nitroprusside (SNP). Responses to SNP (10^{-9} to 10^{-5} μM), a NO-donor, were obtained in the endothelium denuded aortic rings pre-contracted with PE (2 μM) taken from all experimental groups.

2.2.4.3 Contractile responses to PE. The constrictor CRC to PE (10^{-8} to 10^{-5} M) were generated before and after incubation with Nω-Nitro-L-arginine methyl ester (L-NAME, 200 μM), a NOS inhibitor in the presence of indomethacin (Indo, 10 μM, dissolved in DMSO), a COX inhibitor. Between each CRC, tissues were washed with Krebs buffer to allow the rings to return to the basal tone. A vehicle-only (no drugs present) study was performed simultaneously in aortic rings from the same animal (Data not shown).

2.2.4.4 Western blot analysis.

2.2.4.4.1 Preparation of total protein extracts. Aortic tissue samples were micronized through freezing with liquid nitrogen and grinding with a mortar (111). To obtain total protein extract, samples were incubated with RIPA buffer (Sigma-Aldrich, St. Louis, MO) containing Protease Inhibitor Cocktail (UltraCruz, Santa Cruz Biotechnology, Dallas, TX) for 1.5 h at 4°C and centrifuged at 1500xg for 15 min at 4°C, and supernatants were collected. Protein concentrations were by the bicinchoninic acid assay (BCA Assay).

Protein (20–30 µg) was subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Proteins were then transferred to 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. Primary antibodies for p-V-akt murine thymoma viral oncogene homolog-2 (Akt) (Ser^{473}), total Akt, p-endothelial NO synthase (eNOS) (Ser^{1177}), endothelial NO synthase (eNOS), insulin receptor substrates IRS- 1, IRS-2 were supplied by Cell Signaling (Boston, MA). Antibodies against Nox1, Nox4, KCNN3 (SK_{Ca}) and KCNN4 (IK_{Ca}) were obtained from Abcam (Cambridge, MA). Incubation with secondary antibody (LI-COR donkey anti-Rabbit IgG IRDye 680 or anti-mouse IgG IRDye 800CW, 1: 10,000) was performed in the blocking buffer for 1 h at room temperature. Before analyzing, the membrane was washed four times with TBS containing 0.1% Tween-20. Detection was done by using a LI-COR Odyssey imaging system (Lincoln, NE). Uniformity of the protein loading was confirmed by incubating the blots with GAPDH and β- actin (Cell Signaling, Boston, MA) as a control.

2.2.4.5 Statistical analysis. All values were expressed as mean of n values ± standard error of the mean (SEM). Here, n denotes data from one rat. Relaxation responses to ACh- and SNP were calculated as the percentage of relaxation from maximum PE contraction at each concentration. Similarly, the recorded increase in the force of contraction was calculated as the percentage of maximum contraction obtained with PE at the highest dose. The concentration of the agonist, which produced half of the maximum effect (E_{max}) was expressed as EC_{50} and calculated by a sigmoidal dose-response model (for variable slope) using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). The sensitivity of the agonists was expressed as pD_2 values (-log [EC_{50}]), which were normally distributed. The area under the curve (AUC) was determined using GraphPad Prism 7 with trapezoidal technique. To compare the effect of different drugs such as TRAM-34 on the ACh response, the ACh results were expressed as differences of area under the concentration response curve (ΔAUC) in control (absence of TRAM-34) and experimental (presence of TRAM-34) condition. Comparison of CRCs between two groups was done using two-way ANOVA, with one factor being concentration and the other being groups (male vs female and control vs. diabetic). When the ANOVA test returned $P < 0.05$, post hoc analysis using Bonferroni's or Tukey's test was performed. Comparison of CRCs in a pre/post-test format within a group was done using two-way ANOVA with repeated measures. Student's unpaired t-test was used for comparisons of two group means. A probability value of less than 5% ($P<0.05$) was considered significant.

2.3 Results

2.3.1 Effects of sex and type 2 diabetes on metabolic parameters. Body weights of both male and female diabetic rats were significantly higher compared with the respective non-diabetic controls (Table 1). Accordingly, the adipose tissue weight, as well as their ratios to the

body weight were significantly higher in diabetic rats than those in non-diabetic control groups in both sexes. Moreover, male and female UCD-T2DM rats had higher triglyceride level in plasma than that of non- diabetic controls. However, when compared to male UCD-T2DM rats, female UCD-T2DM rats had significantly higher circulating triglyceride level. Also, diabetic groups showed increase in adiposity. Interestingly, female diabetic had higher adiposity compared to male diabetic rats, respectively.

The glycosylated haemoglobin A1c (HbA1c) test indicates average of blood glucose level for the past two to three months of study. Both glucose and HbA1c levels were higher in male and female diabetic rats compared to their respective non- diabetic controls. The plasma insulin concentration was significantly higher in female diabetic rats compared with those in non-diabetic female controls (Table 1). However, there was no difference in plasma insulin level in male diabetic rats when compared with their respective controls.

Table 1: Body weight and adipose weight, blood glucose levels, HbA1c, and other metabolic parameters of male and female control and diabetic rats

	n	Male Control	Male Diabetic	Female Control	Female Diabetic
Body Weight (g)	19-22	306.99±12.09	514.04±14.98*	211.77±3.80	402.79±5.85*
Adipose Tissue (g)	19-22	1.32±0.21	10.89±0.87*	1.56±0.22	14.80±1.68*#
Adipose Tissue/ Body Weight (g)	19-22	0.0045±0.0006	0.02±0.001*	0.007±0.001	0.037±0.004*#
Triglyceride (mmol/l)	19-20	1.64±0.12	2.15±0.18*	1.45±0.06	3.64±0.27*#
Blood Glucose (mg/dl)	19-20	143.33±6.26	361.6±29.81*	152.17±9.75	357.19±29.21*
HbA1c level	14-18	4.23±0.10	11.07±0.58*	4.40±0.06	10.09±0.58*
Insulin (ng/ml)	8-10	0.91±0.52	0.96±0.25	0.49±0.14	5.71±1.91*#
ISI	8-10	1.35±0.13	0.60±0.15*	0.89±0.23	0.10±0.03*#

Data are expressed as mean ± SEM. *$p<0.05$ (vs. control, same sex), #$p<0.05$ (vs. male, respective group), using Student's unpaired t-test. Insulin sensitivity index (ISI) = [2/ (blood insulin (nM) x blood glucose (μM) + 1]. HbA1c = glycated hemoglobin (A1c).

2.3.2 Effects of sex and type 2 diabetes on insulin signaling. Insulin sensitivity index (ISI), inversely indicative of insulin resistance, was significantly lower in both diabetic groups regardless of sex. However, when compared to male UCD-T2DM rats, female UCD-T2DM rats had lower ISI level (Table 1), suggesting that insulin signaling may be impaired in both diabetic groups. Therefore, we analyzed the expression of main insulin signal transducers, insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) in aortic tissue. Our results showed that IRS-1 expression was significantly reduced in both diabetic groups (by 0.5 fold in

male diabetic group and by 0.4 fold in female diabetic group), with a significantly lower expression in female diabetic than male diabetic animals (Figure 2A). On the other hand, the protein expression of IRS-2 was reduced by 0.5 fold only in female diabetic group compared to non-diabetic female control group. IRS-2 expression level was not altered in male by disease condition (Figure 2B). The expression of Akt, one of the main transducers of insulin signaling downstream of IRS was also analyzed in aortic tissue. The ratio of p-Akt/total Akt was significantly reduced in the aorta of both male and female diabetic groups (by 0.4 fold and by 0.5 fold respectively) compared to their respective control group, whereas total Akt protein remained unaltered in UCD- T2DM rats, irrespective of sex (Figure 3).

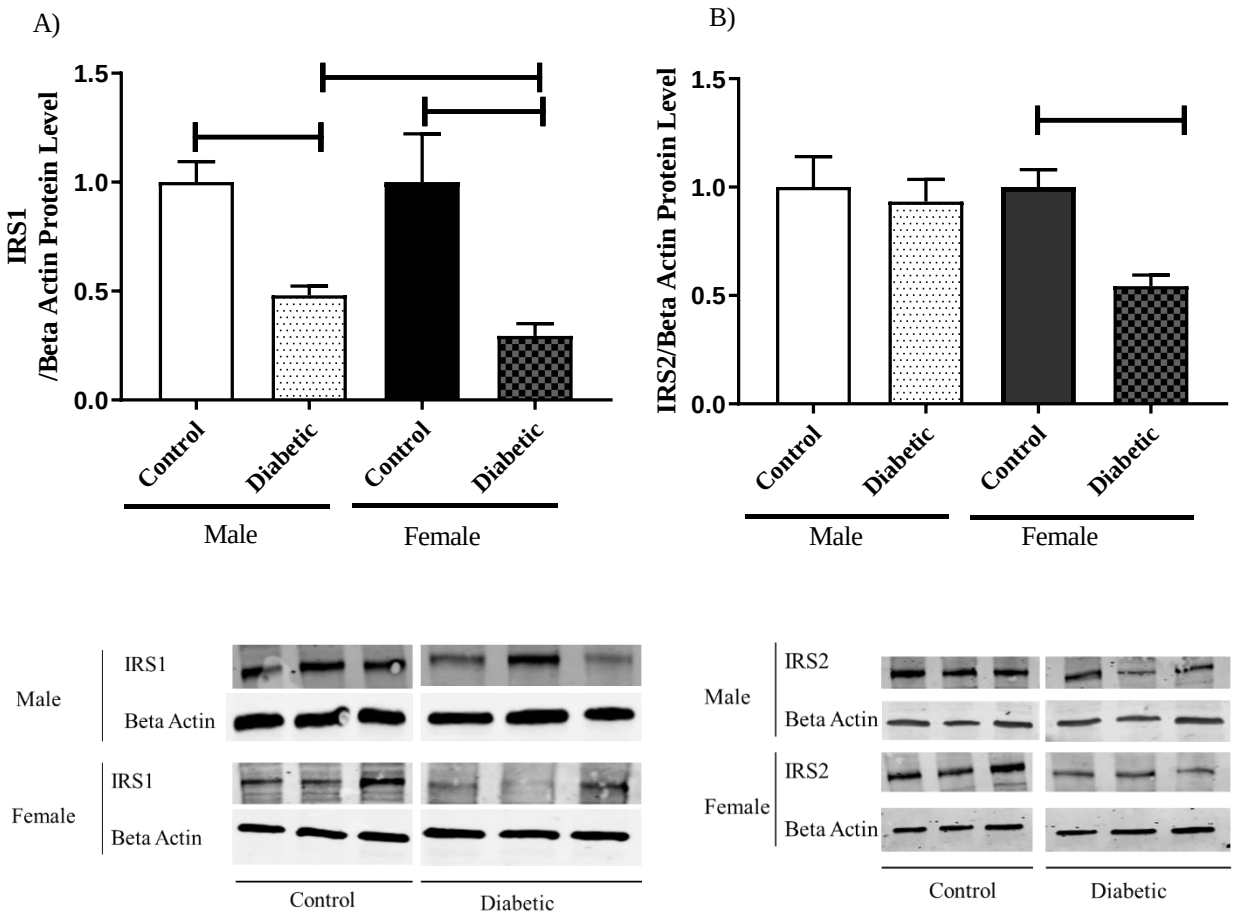

Figure 2: Protein levels of aortic insulin receptor substrate IRS-1 (A) and IRS-2 (B) from the samples of male and female control and diabetic rats. IRS-1 (A) and IRS-2 (B) were quantified by densitometry and normalized to corresponding beta actin. Values are represented as mean± SEM. Each bar represents the values obtained from n=5-6 animals per group. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($P<0.05$), as analyzed by Student's unpaired t-test.

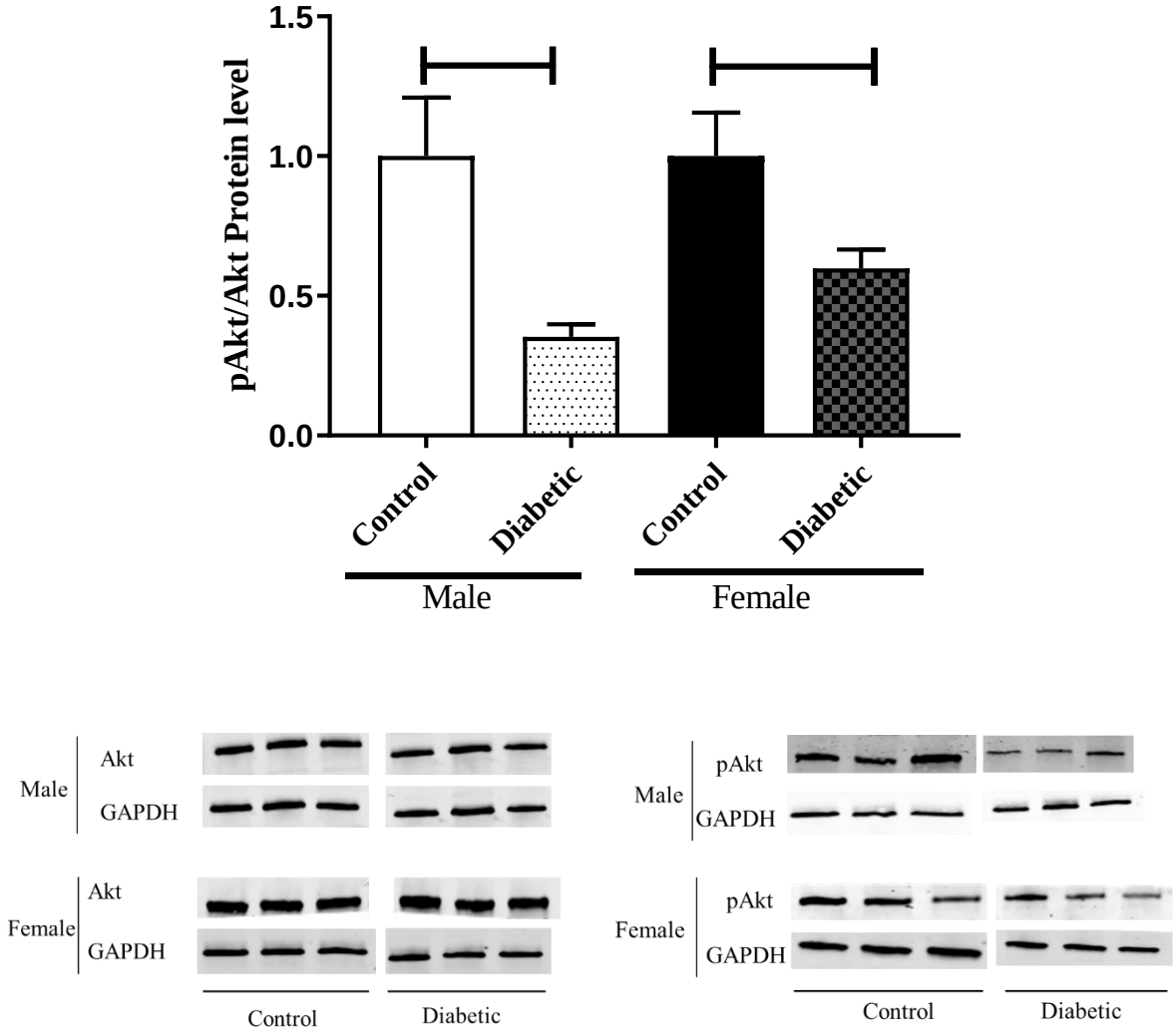

Figure 3: Protein levels of aortic phosphorylated/total V-akt murine thymoma viral oncogene homolog-2 (Akt) from the samples of male and female control and diabetic rats. pAkt/Akt were quantified by densitometry and normalized to corresponding Beta Actin. Values are represented as mean± SEM. Each bar represents the values obtained from n=5-6 animals per group. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($P<0.05$), as analyzed by Student's unpaired t-test.

2.3.3 Effects of sex and type 2 diabetes on relaxation responses to ACh. CRC to ACh were obtained to evaluate the effect of type 2 diabetes on the receptor-mediated endothelium-dependent release of NO or stimulated release of NO. Diabetes slightly but significantly impaired the relaxation responses to ACh in aortic rings from female diabetic group compared to non- diabetic female controls (Figure 4B). The E_{max} but not the sensitivity (as assessed by -log [EC_{50}] (pD_2)) of aortic rings to ACh were reduced in female diabetic group compared to their respective non- diabetic control group (Table 2). On the contrary, a potentiated relaxation response to ACh was observed in male diabetic group (Figure 4A). Both the E_{max} and pD_2 of aortic rings to ACh were significantly enhanced in the aortic rings of male diabetic group compared with their respective non-diabetic controls (Table 2).

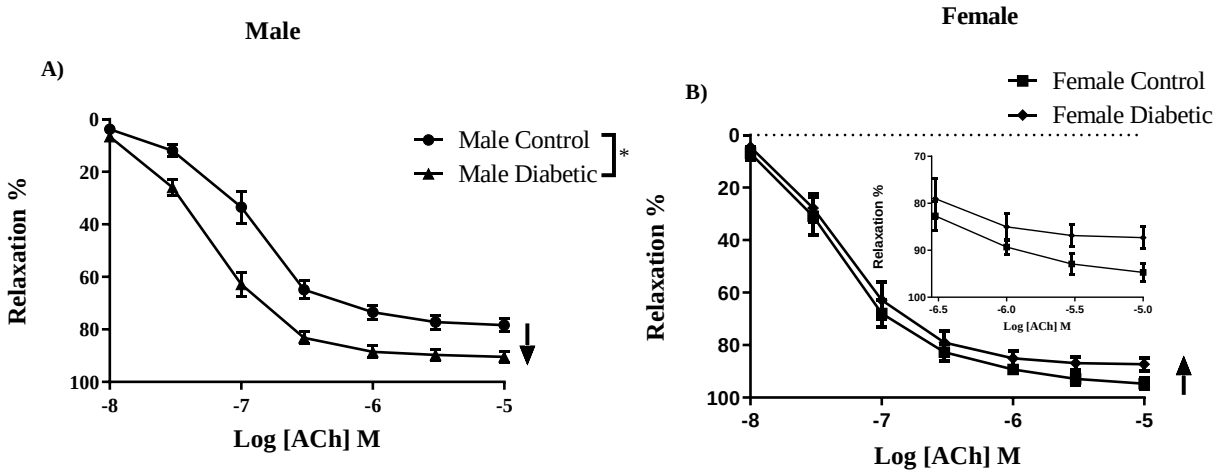

Figure 4: Relaxation responses to cumulative concentrations of acetylcholine (ACh, 10^{-8} to 10^{-5} M) in intact aortic rings precontracted with phenylephrine (PE, 2μM) from male (A) and female (B) control and diabetic rats. Data are expressed as means ±SEM. n=6-12 animals per group. *P < 0.05 between two groups analyzed using 2-way ANOVA followed by Bonferroni's post hoc test.

Table 2: pD_2 and E_{max} to acetylcholine (ACh) in aortic rings from male and female control and diabetic rats

ACh	n	pD_2	E_{max} %
Male Control	12	6.57± 0.10	81.14±1.63
Male Diabetic	11	7.14±0.057*	94.04±0.98*
Female Control	6	7.20±0.10#	94.72±1.90#
Female Diabetic	7	7.07±0.11	87.31±2.31*#

Data are expressed as mean ± SEM; n, number of rats per group. *p<0.05 (vs. control, same sex), #p<0.05 (vs. male, respective group), analyzed using Student's unpaired t-test.

2.3.4 Effects of sex and type 2 diabetes on relative contributions of EDRFs to ACh-induced relaxation. The relative contributions of PGI_2 and NO to vasorelaxation induced by ACh were estimated by sequentially inhibiting COX, sGC, and NOS. Specifically, EDV to ACh (10^{-8} to 10^{-5} M) in rat aortic rings precontracted with PE (2 µM) was obtained before and after pretreatment with Indo (10 µM), followed by addition of ODQ (10 µM) and L-NNA (100 µM). When ODQ was added, the EDV reduction is thought to represent the impact of C-GMP in EDV (112). Addition of L- NNA represent the impact of NO, and the remaining EDV to ACh is referred to as the L-NNA/Indo-insensitive component, or EDHF-type relaxation (113, 114).

The administration of Indo to block COX activity had no apparent effects on pD_2 and E_{max} to ACh, regardless of sex or diabetes. Moreover, the ΔAUC, defined as difference in the area under the curve between the ACh-CRC before and after Indo, were not different between UCD-T2DM groups and respective non- diabetic control groups, in both sexes (Figure 5, Table 3). In both males and females, the addition of ODQ substantially blocked the remaining

relaxation in all experimental groups. However, a significant relaxation response was remaining in male diabetic group compared with the non-diabetic control and female diabetic animals (Table 3). ΔAUC between the ACh-CRC before and after ODQ in the presence of Indo did not change in any of the diabetic group compared with their respective control. Finally, in females, addition of L-NNA to block NO synthesis completely abolished the remaining Indo- and ODQ resistant vasorelaxation in both control and diabetic animals (Figures 5C & 5D). In males, however diabetic group showed a significant remaining vasorelaxation compared with the non-diabetic control animals after blocking NO synthesis with L-NNA (Figures 5A & 5B). ΔAUC between the ACh-concentration response curve before and after L-NNA was significantly elevated, suggesting a role of NO independent (possibly EDHF) relaxation responses in male diabetic animals (Table 3). Therefore, from this point we continued our investigation using male groups to further study the contribution of EDHF in ACh- induced relaxation responses.

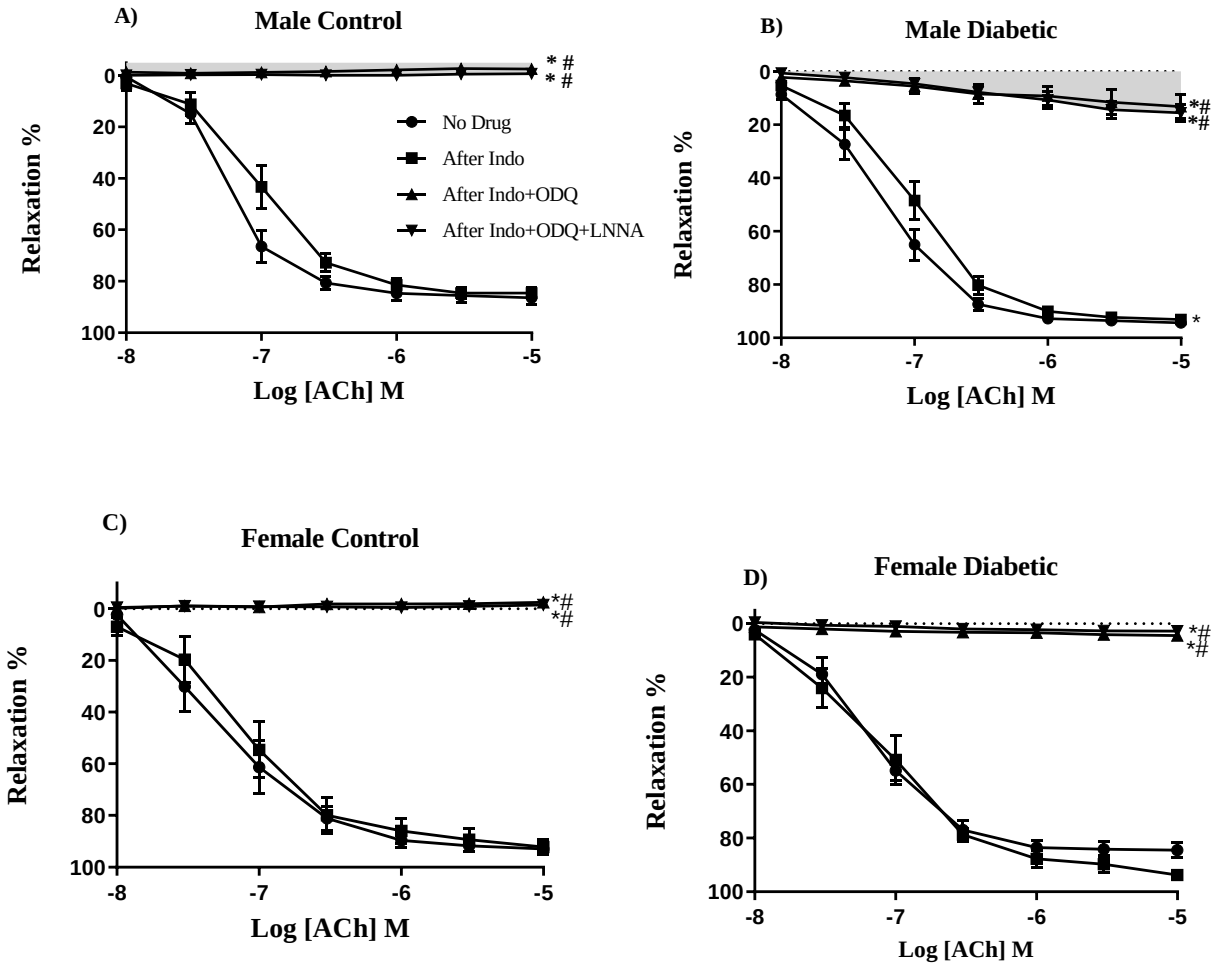

Figure 5: Effects of inhibiting cyclooxygenase, soluble guanylyl cyclase and nitric oxide synthase on acetylcholine (ACh)-induced vasorelaxation in aortic rings taken from male control (A), male diabetic (B), female control (C) and female diabetic (D) rats. ACh relaxation was measured in the presence of indomethacin (Indo ;10 µM), followed by addition of ODQ (10µM), and then with N-nitro-L-arginine (L-NNA;100µM). Data are expressed as means ± SEM. *P <0.05 vs. no drug; #P < 0.05 vs. Indo; analyzed using 2-way ANOVA with repeated measures followed by Bonferroni post hoc test (n=5-8 per group). Light grey shaded area: Contribution of EDHF to EDV.

Table 3: ΔAUC, pD_2 and E_{max} to acetylcholine (ACh) in rat aortic rings from male and female control and diabetic rats

Groups	No Drug			Indo			Indo+ODQ			Indo+ODQ+L-NNA		
	pD_2	E_{max}, %	ΔAUC	pD_2	E_{max}, %	ΔAUC	pD_2	E_{max}, %	ΔAUC	pD_2	E_{max}, %	ΔAUC
Male Control	6.83±0.04	86.54±4.30	ND	6.74±0.09	83.62±1.02	28.1±4.74	ND	2.18±0.25ab	163.99±9.28	ND	0.55±0.04ab	2.79±1.29
Male Diabetic	7.14±0.09*	94.16±1.11*	ND	6.95±0.12	93.97±0.33	23.7±0.92	ND	10.14±0.54*ab	166.05±5.18	ND	13.38±1.28*ab	11.15±1.63*
Female Control	6.79±0.08	92.84±0.76	ND	6.76±0.07	92.14±2.88	6.38±1.07	ND	2.48±1.13ab	185.60±16.98	ND	1.54±0.78ab	3.27±1.12
Female Diabetic	7.13±0.05*	84.50±2.70*#	ND	7.02±0.05	93.78±0.94	9.71±4.68	ND	4.39±0.63ab#	187.59±13.55	ND	2.72±0.72ab#	3.21±1.27

A comparison of the ΔAUC, sensitivity (pD_2) and maximum response (E_{max}) to acetylcholine in the absence (no drug) or in the presence of indo, indo+ODQ, and indo+ ODQ+L-NNA in aortic rings from male and female control and diabetic rats. Data are expressed as mean ± SEM. *P<0.05 (vs. control, same sex), #p<0.05 (vs. male in respective group) (Student's unpaired t-test); [a]P<0.05 vs. no drug control within each group, [b]P<0.05 vs. indo within each group (Student's paired t-test) , n= 5-8 per group. ND, not determined.

2.3.5 Effects of type 2 diabetes on the relative contributions of K^+ channels to ACh-induced relaxation responses in male rats. In order to investigate the contribution of K^+ channels in vasorelaxation in male groups, ACh responses were measured before and after incubation of aortic rings with TEA (a nonselective K^+ channel blocker). Incubation with TEA blunted the relaxation response to ACh in both male diabetic and control groups (Figures 6A & 6B). However, the ΔAUC between the ACh-CRC before and after TEA was higher in diabetic group suggesting that the inhibitory effect TEA on vasorelaxation was greater in diabetic group compared with their non-diabetic control group (Table 4).

Next the contribution of specific calcium-activated K^+ channels were evaluated by incubating the aortic rings with the selective blockers, TRAM 34 (IK_{Ca} blocker) and Apamin (SK_{Ca} blocker). Addition of TRAM 34 significantly reduced the pD_2 and E_{max} to ACh in male diabetic group compared with the nondiabetic control animals (Figures 7A & 7B). Similarly, ΔAUC between the ACh-concentration response curve before and after TRAM-34 was significantly higher in diabetic group compared with the control animals (Table 4). However, blocking of SK_{ca} channels by addition of Apamin had no apparent effect on pD_2 and E_{max} to ACh in male control and diabetic groups (Figures 8A and 8B). Consequently, the protein expression level of IK_{ca} and SK_{ca} channels were analyzed in the aortic tissues using western blot. Western blot analysis revealed that the expression of both SK_{ca} and IK_{ca} were significantly increased (by 10.0-fold and by 2.0-fold, respectively) only in the aortic tissue from male diabetic rats (Figures 9A & 9B).

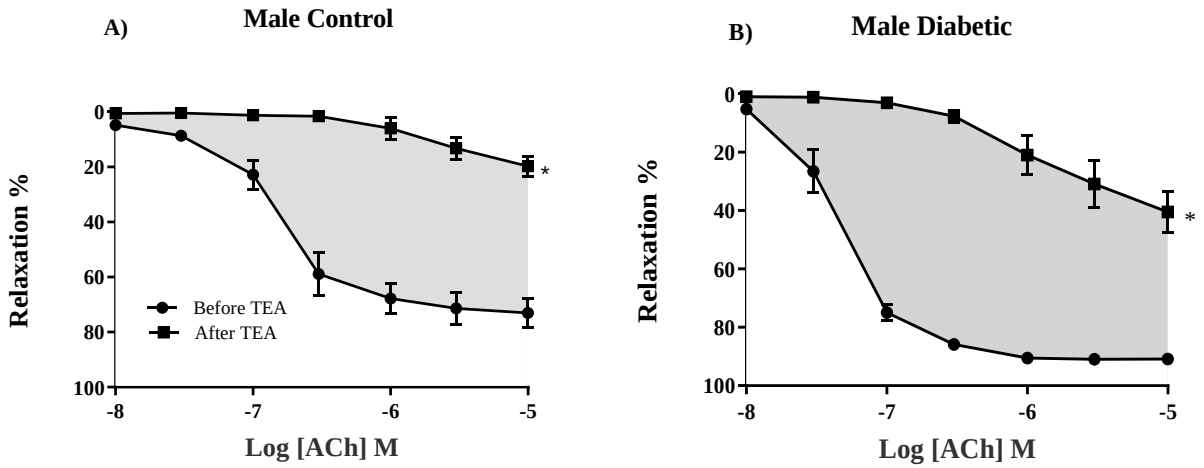

Figure 6: Effects of inhibiting calcium activated K^+ channels on ACh-induced vasorelaxation in aortic rings taken from male control (A) and diabetic (B) rats. Concentration response curves for ACh was measured before and after treatment with TEA (2mM). Data are expressed as mean ± SEM. **P* <0.05 vs. before TEA; analyzed using two-way ANOVA with repeated measures followed by Bonferroni post hoc test, n=6-8 per group. Light grey shaded area: Contribution of calcium activated K^+ channels to endothelium-dependent vasodilation (EDV).

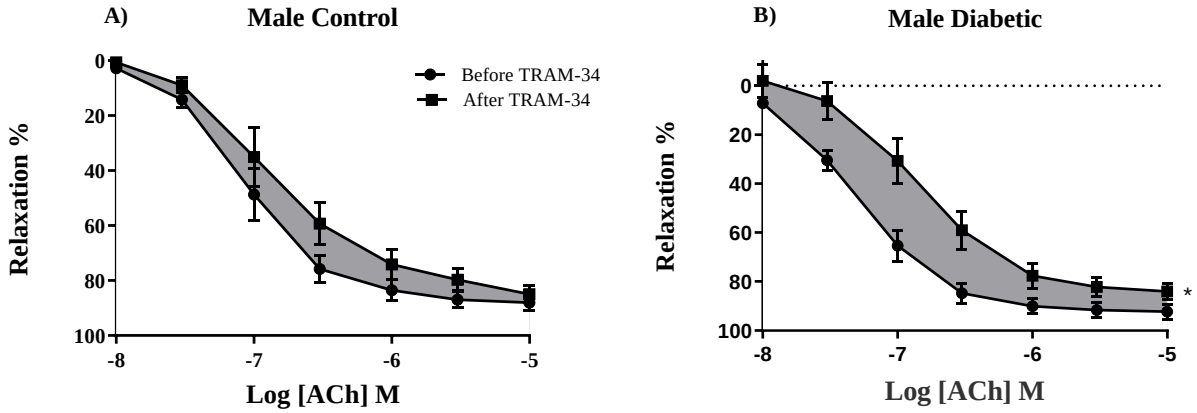

Figure 7: Effects of inhibiting intermediate-conductance Ca^{2+} activated K^+ (IK_{Ca}) channels on ACh-induced vasorelaxation in aortic rings taken male control (A) and diabetic (B) rats. Concentration response curves for ACh were measured before and after treatment with TRAM-34 (1uM). Data are expressed as mean ± SEM. *P <0.05 vs. before TRAM-34; analyzed using two-way ANOVA with repeated measures followed by Bonferroni post hoc test, n=8-9 per group. Dark grey shaded area: Contribution of intermediate-conductance Ca^{2+} activated K^+ (IK_{Ca}) channels to endothelium-dependent vasodilation.

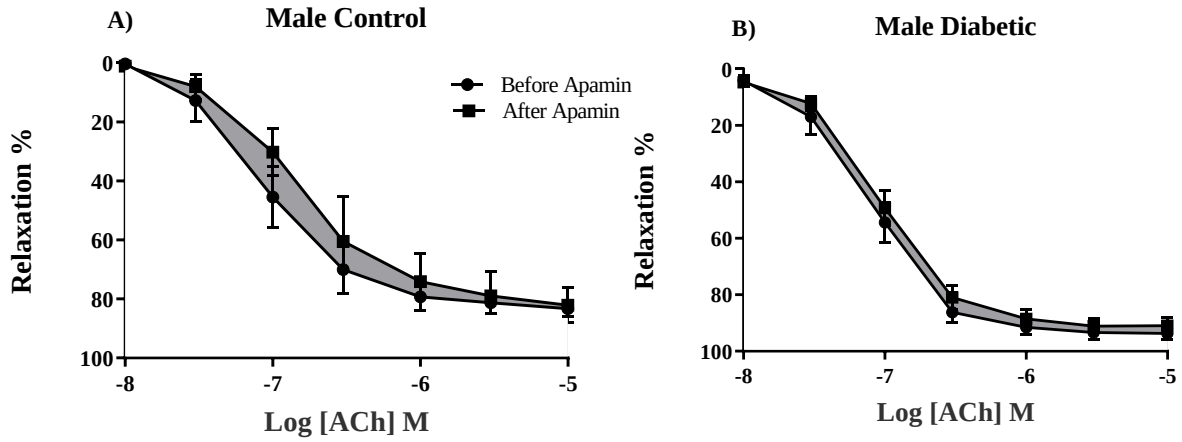

Figure 8: Effects of inhibiting small-conductance Ca^{2+} activated K^+ (SK_{Ca}) channels on ACh-induced vasorelaxation in aortic rings taken from male control (A) and diabetic (B) rats. Concentration response curves for ACh were measured before and after treatment with Apamin (1uM). Data are expressed as means ± SEM; analyzed using two-way ANOVA with repeated measures followed by Bonferroni post hoc test, n=8-9 per group. Dark grey shaded area: Contribution of small-conductance Ca^{2+} activated K^+ (SK_{Ca}) channels to endothelium-dependent vasodilation.

Table 4: ΔAUC, pD_2 and E_{max} to acetylcholine (ACh) in rat aortic rings from male control and diabetic rats

	Male Control			Male Diabetic		
	pD_2	**E_{max}, %**	**ΔAUC**	**pD_2**	**E_{max}, %**	**ΔAUC**
No Drug	6.32± 0.188	73.05±5.06	117.38±12.17	7.16±0.083*	92.25±1.01*	160.28±4.78*
TEA	4.14± 0.04#	19.68±3.42#		4.75±0.94#	39.53±6.69#	
No Drug	6.87±0.12	88.04±2.88	27.16±3.72	7.29±0.05*	96.07±1.08*	52.62±6.65*
TRAM-34	6.55±0.23#	84.93±3.02		6.77±0.12#	87±2.99#	
No Drug	6.77±0.20	83.27±2.68	24.7±9.39	7.04±0.099*	93.75±2.10*	10.76±2.27
Apamin	6.51±0.30	82.13±5.82		6.93±0.076	90.95±2.67	

A comparison of the ΔAUC, sensitivity (pD_2) and maximum response (E_{max}) to acetylcholine in the absence (no drug) or in the presence of TEA or TRAM-34 or Apamin in aortic rings from male control and diabetic rats. Data are expressed as mean ± SEM. *P <0.05 vs control, Student's unpaired t-test; # P <0.05 vs No Drug in respective treatment group, Student's paired t-test, n=8-9 per group. TEA, tetraethylammonium; TRAM-34, 1-[(2-chlorophenyl) (diphenyl)methyl]-1H-pyrazole.

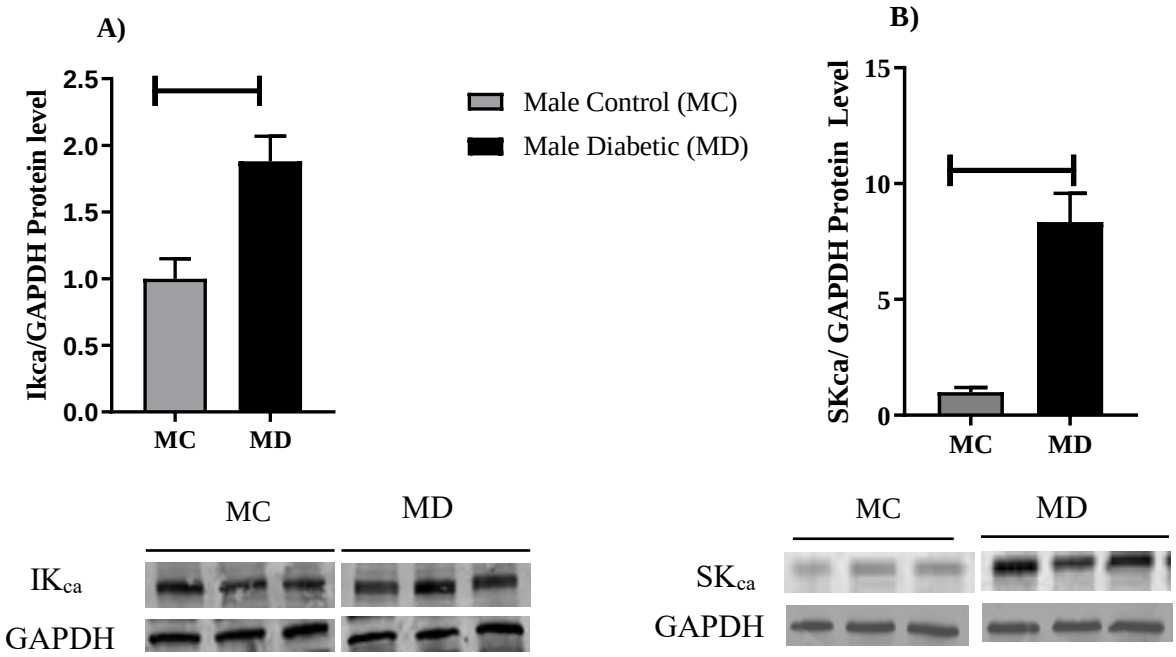

Figure 9: Protein levels of aortic intermediate conductance potassium channel (IK_{Ca}) (A) and small conductance potassium channels (SK_{Ca}) (B) were measured from the samples of male control and diabetic rats. Values are represented as mean± SEM. Each bar represents the values obtained from n=5-6 animals. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($P<0.05$), as analyzed by Student's unpaired t-test.

2.3.6 Effects of sex and type 2 diabetes on relaxation responses to SNP. The smooth muscle sensitivity to NO was investigated by performing CRC to SNP (10^{-9} to 10^{-5} M) in endothelium-denuded aortic rings. There was a significant rightward shift in the CRC to SNP in female diabetic rats compared with their controls (Figure 10B). Diabetes significantly reduced only the sensitivity as assessed by pD_2 values to SNP in aortic rings from female diabetic group compared with non-diabetic controls (Figure 10B and Table 5). However, no significant differences in CRC to SNP was observed in male diabetic and control animals (Figure 10A and Table 5).

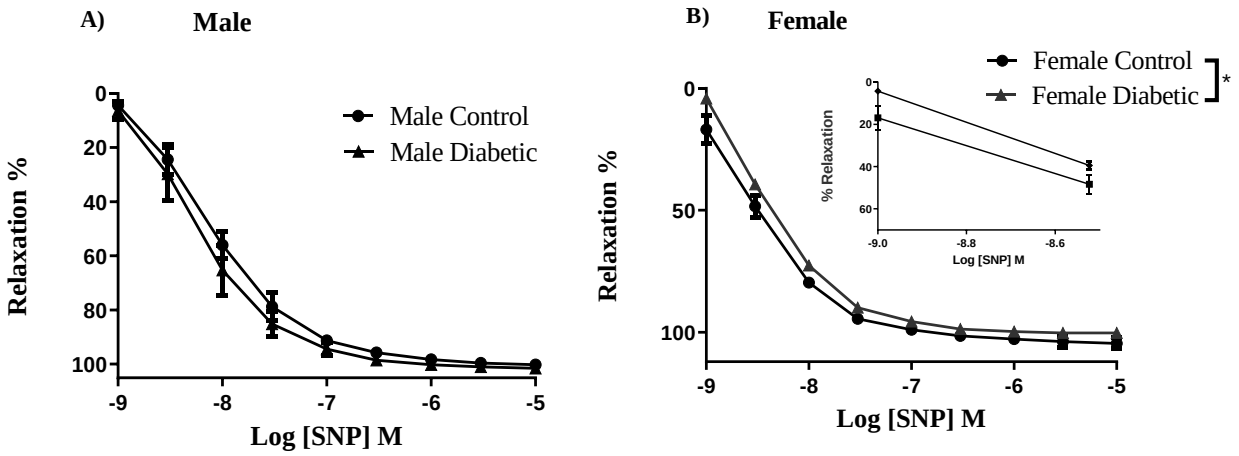

Figure 10: Relaxation responses to cumulative concentrations of sodium nitroprusside (SNP, 10^{-9} to 10^{-5} M) in endothelium denuded aortic rings pre-contracted with phenylephrine (PE, 2 μM) from male (A) and female (B) control and diabetic rats. Data are expressed as mean ± SEM. n= 5-7 per group.

**P*<0.05 between two groups analyzed using two-way ANOVA followed by Bonferroni's post hoc test.

Table 5: pD_2 and E_{max} to sodium nitroprusside (SNP) in rat aortic rings from male and female control and diabetic rats

SNP	**n**	**pD_2**	**E_{max}, %**
Male Control	5	8.06± 0.09	100.15±0.35
Male Diabetic	6	8.19±0.13	101.46±1.41
Female Control	6	8.50±0.07	104.34 ±3.29
Female Diabetic	7	8.32±0.01*	100.18±0.45

Data are expressed as mean ± SEM; n, number of rats per group. *P<0.05 (vs. control, same sex), analyzed using Student's unpaired t-test.

2.3.7 Effects of sex and type 2 diabetes on contractile responses to PE. Contractile responses to α-adrenoceptor agonist (PE) were analyzed by measuring the CRC to PE (10^{-8} to 10^{-5} M). Both pD_2 and $Tension_{max}$ to PE were significantly enhanced in diabetic groups compared with the non- diabetic control rats, regardless of sex (Figures 11A & 11B and Table 6).

To indirectly measure the level of basal NO, CRC to PE (10^{-8} to 10^{-5} M) was performed in aortic rings before and after pretreatment with the NO synthase inhibitor, L-NAME (200 μM) in the presence of Indo (10 μM). The changes in the contractile level to PE after addition of L-NAME would reveal the effect of basal NO release on contraction (115, 116).

Incubation of the aortic rings with L-NAME resulted in a significant increase of the contractile responses to PE in all experimental groups (Figure 12). However, as indicated by ΔAUC (the difference in area under the curve between PE CRC before and after L-NAME) were lower in aortas of the diabetic rats compared with the control group, regardless of sex (Table 7). Furthermore, the basal NO level was significantly lower in male diabetic animals compared with female diabetic group as assessed by ΔAUC (Table 7).

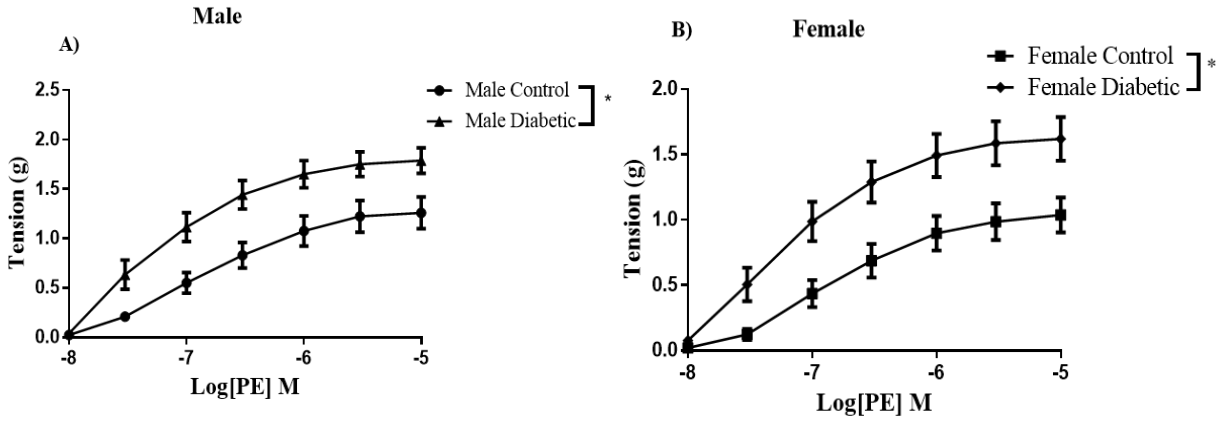

Figure 11: Concentration-response curves to phenylephrine (PE, 10^{-8} to 10^{-5} M) in intact aortic rings of male (A) and female (B) control and diabetic rats. Data are expressed as mean ± SEM. n=5-7 per group. *P<0.05 between two groups analyzed using two-way ANOVA followed by Bonferroni's post hoc test.

Table 6: pD_2 and $Tension_{max}$ to phenylephrine (PE) in aortic rings from male and female control and diabetic rats

PE	n	pD2	Tension max (g)
Male Control	5	6.81±0.047	1.26±0.16
Male Diabetic	5	7.19±0.09*	1.79±0.12*
Female Control	5	6.78±0.08	1.04±0.13
Female Diabetic	6	7.14±0.05*	1.53±0.16*

Data are expressed as mean ± SEM; n, number of rats per group. *P<0.05 (vs. control, same sex), analyzed using Student's unpaired t-test.

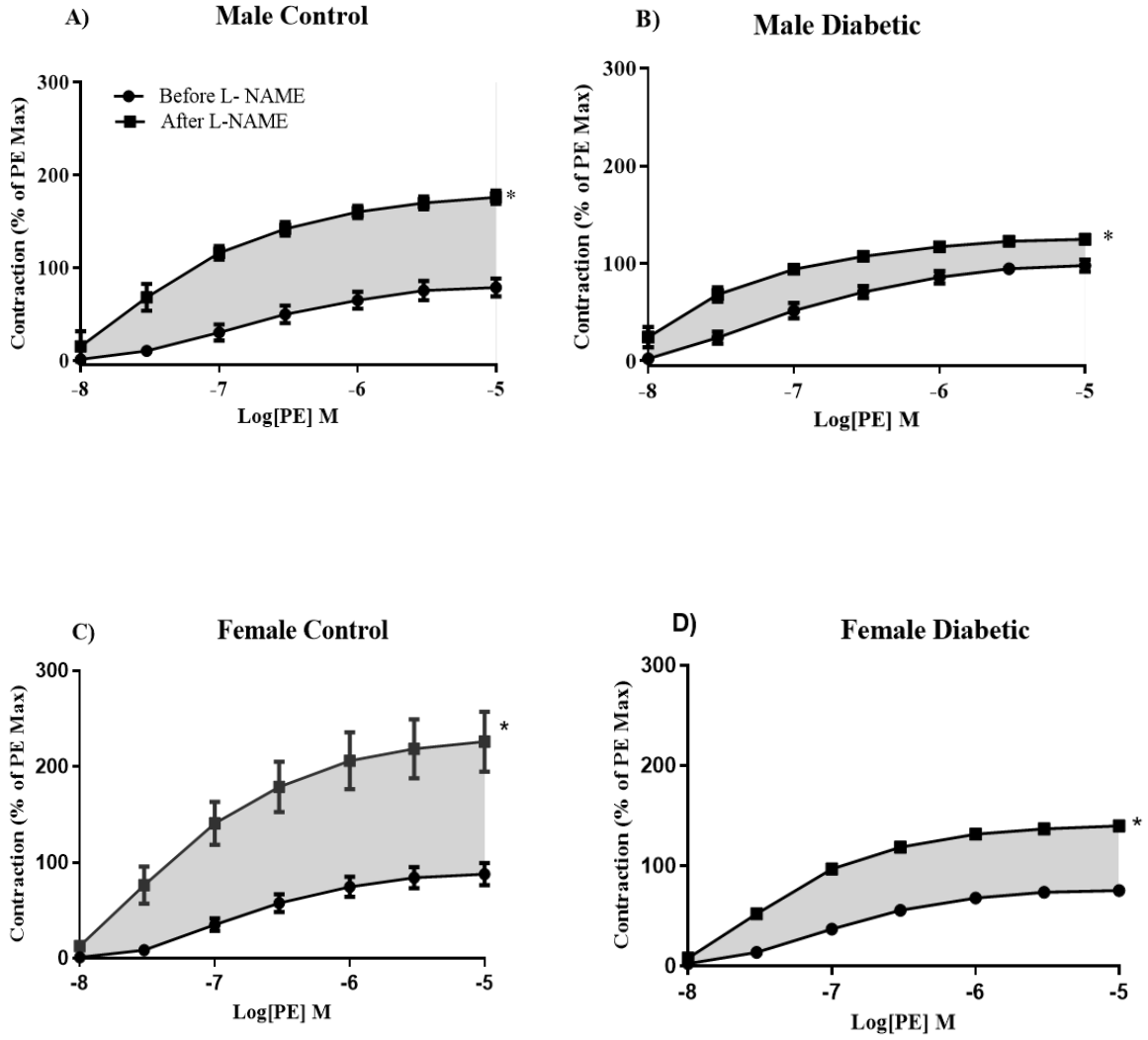

Figure 12: Contractile response to cumulative concentrations of phenylephrine (PE) in intact aortic rings from male control (A), male diabetic (B), female control (C) and female diabetic (D) rats. Contraction to PE was measured before and after incubation with Nω-Nitro-L-arginine methyl ester (L-NAME, 200 μM). Responses were performed in the presence of indomethacin (10 μM).
Data are expressed as mean±SEM, with *P<0.05 vs. before L-NAME in all groups as analyzed using two-way ANOVA with repeated measures, n=5-8 per group.

Table 7: ΔAUC, pD_2 and $Tension_{max}$ to phenylephrine (PE) in aortic rings from male and female control and diabetic rats

	$Tension_{max}$, g	**pD2**	**ΔAUC**
Male Control			
Before L-NAME	1.09±0.11	6.74±0.085	239.10±5.00
After L-NAME	2.47±0.29a	7.29±0.07a	
Male Diabetic			
Before L-NAME	1.66±0.17*	6.95±0.12	103.66±18.15*
After L-NAME	2.26±0.11a	7.46±0.099a	
Female Control			
Before L-NAME	0.93±0.17	6.76±0.077	319.44±59.81
After L-NAME	2.25±0.25a	7.21±0.11a	
Female Diabetic			
Before L-NAME	1.19±0.16	7.008±0.054*	167.42±6.87*#
After L-NAME	1.98±0.13a	7.35±0.059a	

Data are expressed as mean± SEM. L-NAME, *N*-nitro-L-arginine methyl ester. **P* <0.05 vs.control (Same sex), #*P*<0.05 vs male diabetic, Student's Unpaired t-Test; [a]*P*<0.05 vs before L- NAME, Student's paired *t*-test. n=6-8 per group.

2.3.8 Effects of sex and type 2 diabetes on eNOS and Nox expression. To investigate the possible mechanisms underlying the alterations of the ACh responses in male and female diabetic rats, the protein expression of eNOS and NADPH oxidase (Nox) subunits, Nox1 and Nox4 were measured. Moreover, eNOS activation was measured by determining the level of peNOS. Western Blot analysis revealed that the ratio of peNOS over total eNOS were significantly decreased in male diabetic group compared with the non- diabetic control rats. However, ratio of peNOS over total eNOS protein levels showed no significant difference between female control and diabetic groups (Figure 13).

The expressions of Nox1 was significantly elevated in aortic tissues taken from diabetic groups, regardless of sex (by 1.5-fold in male diabetic and by 1-fold in female diabetic, Figure 14A). However, protein expression of Nox4 showed no significant differences among all experimental groups (Figure 14B).

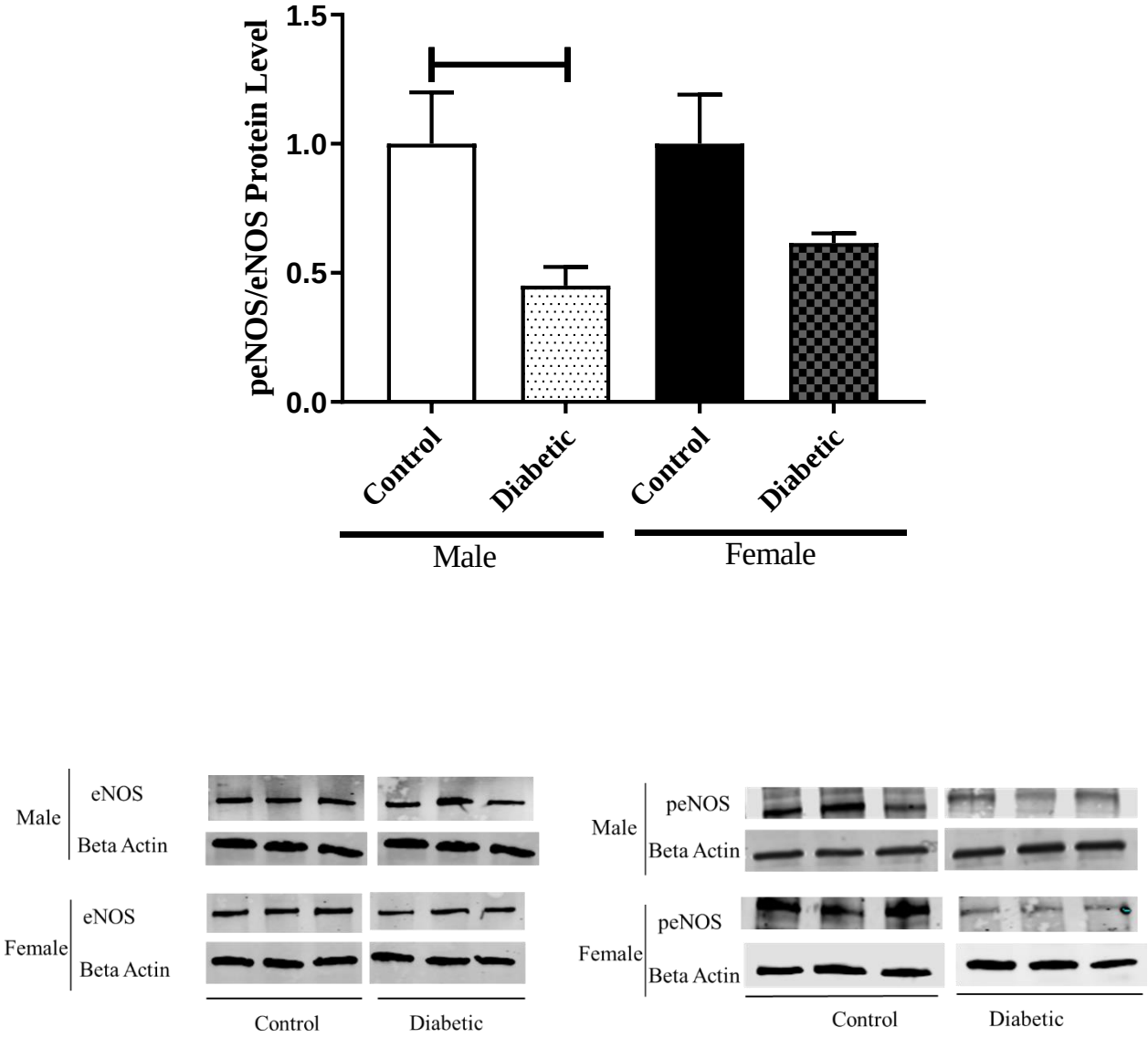

Figure 13: Protein levels of phosphorylated/ total eNOS in aortic samples from male and female control and diabetic rats. Each bar represents the mean ± SEM of values obtained from n=5 animals per group. To show representative bands images from different parts of the same gel have been juxtaposed, which is indicated by white dividing lines. Capped lines indicate significant differences between two groups ($P<0.05$), as analyzed by Student's unpaired t-test.

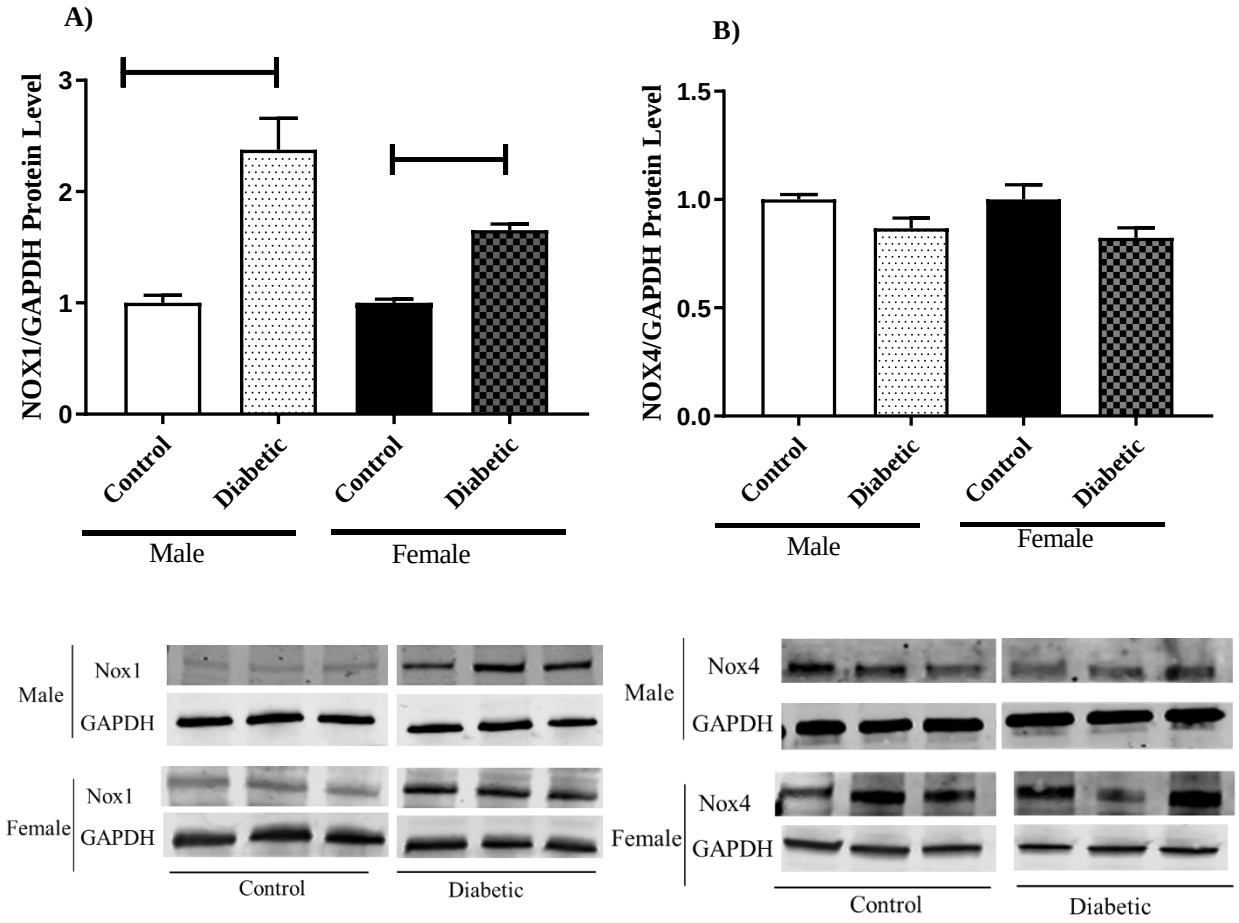

Figure 14: Protein levels of aortic Nox1 (A) and Nox4 (B*)* from the samples of male and female control and diabetic rats. Values are represented as mean± SEM. Each bar represents the values obtained from n=4-5 animals per group. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($P<0.05$), as analyzed by Student's unpaired t-test.

2.4 Discussion

This study is the first report on the aortic function of a well-characterized and validated model of T2D, the UCD-T2DM rat. Furthermore, it reveals a sex difference in the aortic reactivity of this model. Obesity and insulin resistance are some of the important features of T2D results from monogenic mutation in commonly used animal models. However, obesity is rarely initiated by monogenic mutations in humans, therefore UCD-T2DM rat model is a polygenic model of T2D which more closely resembles the pathophysiology of diabetes in humans.

Our data shows that both male and female diabetic rats had higher body weight and hyperglycemia compared with non-diabetic control rats. Moreover, female diabetic group exhibited higher adiposity, hyperinsulinemia and higher triglyceride level than non-diabetic control and male diabetic rats. Similarly, Ohta et al. reported an elevated blood insulin level in spontaneously diabetic torii (SDT) female rats compared with SDT male rats (91). In Ohta report, authors suggested that rising of blood glucose and declining of blood insulin level in male rats occurred earlier than in female rats. In the current study, insulin sensitivity index (ISI) was lowered in diabetic groups, irrespective of sex. However, when compared to male diabetic rats, female diabetic rats exhibited a lower ISI. Accordingly, we showed that insulin signaling was impaired in the aortic tissues in diabetic groups in both sexes. Particularly, aortic IRS-1 was reduced to a similar extent in both diabetic groups, but IRS-2 was reduced only in female diabetic group. Along similar line, it has been reported that the downregulation of IRS-2 level in endothelial cell is induced by hyperinsulinemia in obese subjects (117). Furthermore, in the current study, we showed that the ratio of p-Akt/Akt were significantly decreased in diabetic groups, regardless of sex, suggesting an impaired insulin signaling in these groups.

Endothelial dysfunction is considered to be an early step in the development of vascular complications in diabetes (17, 118, 119). In T2D, impaired (104), enhanced (120) or preserved (105) EDV have been reported. Similar with previous reports showing an impairment of EDV in aorta of T2D (104, 121), we demonstrated that the maximal relaxation to ACh was significantly reduced in aortic rings from female UCD-T2DM rats compared to its respective controls. However, an intriguing observation of this study was that, aortic rings from male diabetic animals exhibited a potentiation in EDV compared with that in male control. Similar observations were also made by our group using Zucker diabetic fatty (ZDF) male rats. Specifically, obesity-induced diabetes (ZDF model) significantly impaired relaxation responses to ACh in aortic rings taken from females, however, potentiated the relaxation in males (data not reported). In accordance with our data, Zhong et al. reported an elevated relaxation responses to ACh in aortic rings of GK male rats (122). On the other hand, Nemoto et al. and Kazuyama et al. reported an impaired EDV in aortic rings from GK male rats.

Sex-based differences in vascular function are well studied (123), and there are also several reports including ours on the effects of sex hormones on the vascular endothelial function in diabetic rabbits, rats and mice (51, 52, 100, 124, 125). However, we are the first to characterize vascular function in the UCD-T2DM rat model with respect to sex. It has been well established that in conduit arteries, NO plays the major role in EDV (42, 126). The decreased ACh-induced relaxation observed in female diabetic arteries may in part result from decreased NO bioavailability or reduced in sensitivity of smooth muscle to NO in this group.

It has been reported that T2D reduce the synthesis of NO in aorta by phosphorylation of eNOS at Ser1177 (127) and our results show that ratio of eNOS phosphorylation (peNOS) at this position over total eNOS is decreased in aortic tissues from both diabetic groups (although it

didn't reach to significance level in female diabetic arteries) (Figure 13). There is an established negative regulatory effect of NO on EDHF synthesis (128). On the other hand, an augmented EDHF response was shown to compensate for the loss of NO-mediated vasorelaxation in arteries in diabetic rats (109, 129). In agreement with those studies that demonstrate compensatory interactions between pathways, the potentiation of ACh response in aortic rings from male diabetic group suggests that other molecules besides NO (e.g., EDHF or PGI_2) may be involved in ACh-relaxation in this group.

Here, we showed that the inhibition of COX metabolites by Indo didn't alter relaxation responses to ACh significantly in aortic rings of any of four experimental groups. Consistent with these results, Malakul et al. reported hypercholesterolemia and type 1 diabetes did not have any effect on COX- mediated EDV in rat aorta (129). In the current study, addition of ODQ or L-NNA completely abolished the EDV in aortic rings of both control groups and female diabetic group, but not in male diabetic rats. The remaining of a slight but significant relaxation response to ACh after addition of L-NNA in male diabetic group, indicating a contribution of EDHF-type relaxation (in addition to NO) in this group (Figure 5B). Along similar line, Malakul et al. reported a potential role of EDHF in EDV in aorta of type 1 diabetic male rat arteries (129). There are also reports of decreased NO-dependent relaxation response and increased EDHF activity in saphenous arteries (130) and carotid arteries (131) of high fat diet-induced obese male rats and STZ- induced type 1 diabetic male rats respectively.

The reasons for the shift in the role of NO to both NO and EDHF in vasorelaxation of aorta in male diabetic group, but not in female diabetic rats is not clear. However, this is consistent with our working hypothesis that the potentiated relaxation of aorta in this group may be partly attributed to the elevated response to EDHF.

Endothelial hyperpolarization-mediated by K_{Ca} channels has been suggested to play a critical role in initiating EDHF-type relaxation. K_{Ca} currents are mainly mediated by IK_{Ca} and SK_{Ca} channels (132) on responses in conduit and resistance-sized arteries in many species, including humans (133, 134). Next, we investigated whether inhibition of these channels influenced the EDV in the aortic rings from male diabetic group. We demonstrated that inhibition of IK_{Ca} channels with TRAM-34 abolished EDV in male diabetic group to a greater extent than that in their non- diabetic controls (Table 4). Accordingly, the protein expression level of both IK_{Ca} and SK_{Ca} channels were significantly greater in male diabetic group compared with the control rats (Figure 9). These results are in accordance with Scach et al. who reported that T2D increased the expression and contribution of IK_{Ca} in mesenteric arteries of ZDF rats (135). Regardless of higher SK_{Ca} channel expression, addition of Apamin had no effect on ACh-mediated relaxation responses in the aortic rings from either male diabetic or male control rats (Figure 6). Although IK_{Ca} and SK_{Ca} have the equal ability to generate K_{Ca} currents but it has been reported that these channels make distinct contributions to ACh-induced EDHF-mediated smooth muscle hyperpolarization (132). Brahler et al. reported that IK_{Ca} plays a key role in ACh- induced smooth muscle hyperpolarization in mice carotid arteries (132). Specifically, they showed that the loss of IK_{Ca} channels impaired the hyperpolarization of smooth muscle in a greater extent than the absence of SK_{Ca} channels (132). In the current study, the overexpression of SK_{Ca} channels was not associated with a larger EDHF-mediated relaxation response in aorta of male diabetic group. This maybe in line with Brahler et al. who reported that overexpression of SK_{Ca} channels in IK_{Ca} wild type mice did not produce a larger EDHF response (132). It has been reported that the distinct role of these channels may be due to the spatial separation in the endothelium (132, 136). SK_{Ca} channels are localized at endothelial cell junctions (132, 136, 137)

and possibly in caveolae (132, 138), and IKca channels are predominantly localized at endothelial projections through the holes in the elastic lamina (132, 136, 137). Therefore, those investigators concluded that IK_{Ca} channels can be activated by ACh-triggered Ca^{2+} release events (132, 136, 137).

Besides the possibility of altered contribution of EDRF, aortic dysfunction in UCD-T2DM model may be explained by changes of smooth muscle responsiveness to NO or contractile agents. We observed that SNP-induced relaxation of endothelium-denuded aortic rings was not altered male diabetic groups. Similarly, Nemoto et al. also observed no significant difference in SNP-induced relaxation in aortic rings of male GK rats (127). On the other hand, in the current study smooth muscle sensitivity NO was significantly reduced in female diabetic rats. These data suggest that decreased the vascular responsiveness to NO may in part be responsible for the impaired ACh-induced relaxation observed in aorta of diabetic female rats.

In the current study, both sensitivity and maximum tension to PE were enhanced significantly in aortic rings of UCD-T2DM groups compared with their respective non- diabetic control, regardless of sex (Figure 11). The elevated PE response may in part explain the decreased ACh-induced relaxation in aorta of female diabetic rats. It is important to note that regardless of increased PE-induced contraction, ACh response potentiated in male diabetic arteries. This, therefore, excludes the diminished PE contractile responsiveness as the cause of the increased ACh responses observed in male diabetic arteries. These data are in line with previous findings that type 1 diabetes results in increased vascular contraction in rat aorta (139) and mesenteric arteries (140).

The elevated contractile responses to PE observed in UCD-T2DM male and female rats may partially result from a decreased release of basal NO or an enhanced of contracting factors (52). We assessed endothelium-derived NO by measuring the differences in the degree of PE-induced contraction in the absence and presence of L-NAME (51, 141). Pre-treatment with L-NAME caused a significantly lower potentiation of the PE responses in aortic rings of UCD-T2DM rats, regardless of sex (Figure 12 B and D) compared with those in non- diabetic controls. This suggests that the decreased level of NO may in part be responsible for the elevated PE contractile responsiveness in UCD-T2DM rats.

It has been reported that in diabetes, superoxide production may play a crucial role in enhancing the contracting responses (142-144). The superoxide acts by scavenging NO, thus decreasing its bioavailability (145), will also elevate endothelium-dependent contractions. In the present study, we did not directly measure NO or superoxide production. However, we determined expression of Nox proteins in rat aorta. Among the Nox isoforms, Nox1, Nox2 and Nox5 are considered as superoxide generating enzyme and promote endothelial dysfunction (25). On the other hand, Nox4 is hydrogen per oxide (H_2O_2) generating enzyme and has the vasoprotective effect (25-28). Vascular walls express high levels of Nox1, Nox2 and Nox4 (29). Nox1 is mainly expressed in large conduit vessels (30), whereas Nox2 is more highly expressed in resistance vessels (31). Here, we observed an elevated expression of Nox1 in aorta taken from diabetic groups, irrespective of sex, whereas Nox4 expression was not changed (Figure 14A and B). Youn et al. reported that the activation of Nox1, but not Nox4 or Nox2, was associated with eNOS uncoupling and endothelial dysfunction in STZ-induced diabetic mice aorta (146). Along the similar line Gray et al. reported genetic deletion of Nox1 in diabetic mice led to reduced diabetes mellitus symptoms suggesting a key role of Nox1 derived reactive oxygen species in

diabetes (147). Therefore, our results on Nox1 overexpression in diabetic arteries suggest that the elevation of responses to PE observed in diabetic animals may be partially due to the elevated oxidative stress. Further studies required to examine the level of superoxide which may be linked to sex-specific vascular function in UCD-T2DM rats.

In conclusion, this study represents the first report showing that the aortic function in UCD-T2DM rats is altered in both sexes. We also showed that basis for sex differences in the development of aortic dysfunction in this model may be partly attributed to changes in the importance of NO and EDHF to the regulation of vascular reactivity. Specifically, potentiation of EDV in diabetic male arteries was associated to a shift from NO toward a reliance to both NO and EDHF. Our data also suggest that a lower sensitivity to NO and/or enhanced responsiveness to contractile agents may contribute to the impaired EDV in aortic rings of UCD-T2DM female rats.

Chapter 3: Study II: Effects of Estrogen Replacement in Aortic Function of Pre-diabetic Ovariectomized Rats

3.1 Introduction

Cardiovascular diseases (CVD) are the major causes of morbidity and mortality in patients with diabetes. It is well established that hyperglycemia and diabetes affect male and female vascular beds differently (51, 52, 100, 148). Clinically, premenopausal women have a lower incidence of CVDs compared with age-matched men. In premenopausal women the blood pressure and the prevalence of renal and heart diseases are much lower than age- matched men, but women loses this sex based protection with increasing age and decreasing level of estrogen after menopause (4-7, 149). Several studies reported that women are less likely to meet low density lipoprotein cholesterol level and blood pressure goals compared to men with type 2 diabetes and CVD (148, 150-152). This may be indicative of greater risk of CVD in association with diabetes in women than do men. Therefore, women with diabetes not only lose their sex based cardiovascular protection but have a higher risk of morbidity and mortality compared to the age matched men (153, 154).

It is well established that endothelial dysfunction represents early steps in the development of vascular complications in diabetes, and hyperglycemia is the central initiating factor for those complications. Several reports have suggested that early, repeated episodes of asymptomatic hyperglycemia increase the risk of CVD even in absence of overt clinical symptoms of diabetes (155, 156). Hyperglycemia can lead to several changes in vascular homeostasis, and endothelial dysfunction is one of the major hallmarks of hyperglycemia-induced vascular disease which is characterized by reduced NO dependent vasodilation (157). Furthermore, premenopausal women with hyperglycemic condition, lose their gender-based

cardiovascular protection (156-158). There is insufficient evidence to establish the timeline of the loss of female-specific protection in premenopausal patients with diabetes. Thus, our objective of this study was to investigate the effects of 17β-estradiol (E_2) replacement on aortic function in ovariectomized UC Davis type 2 diabetes mellitus (UCD-T2DM) rats at the prediabetic stage. We also evaluated the expressions of specific proteins that are associated with aortic reactivity including eNOS, Nox1- and Nox4 dependent NADPH oxidase.

3.2 Material and Methods

3.2.1 Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in water, unless otherwise stated.

3.2.2 Animals and experimental design. All animal protocols were approved by the Animal Care Committee of the University of the Pacific and complied with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (2011). Rats were euthanized using a carbon dioxide gas chamber according to the recommendations from the 2007 AVMA Guidelines on Euthanasia and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

3.2.2.1 Rat model. UCD rats that their blood glucose level is high but not high enough to be considered diabetic are called pre-diabetic. These animals have the potential to become diabetic at a later stage. The Sprague-Dawley (SD) rats were used as non-diabetic/control, as these animals do not have the potential to become diabetic at any stages without manipulation.

Ovariectomized female SD rats were purchased from Charles River (Wilmington, MA) and ovariectomized female pre-diabetic rats were purchased from University of California,

Davis. All animals (ovariectomized SD and pre-diabetic rats) were implanted subcutaneously either with placebo or 17 β-estradiol pellets (60 days release, 1.5mg/pellets). Rats were assigned to four different groups. Group 1 was Control ovariectomized, placebo treated (Control+Placebo); group 2 was control ovariectomized, 17 β-estradiol (1.5mg/pellet)- treated (Control+E_2); group 3 was pre-diabetic ovariectomized, placebo treated (Pre-diabetic+Placebo) and group 4 was pre-diabetic ovariectomized, 17 β-estradiol (1.5mg/pellet)- treated (Pre-diabetic +E_2). All animals were average 3.5-4 months old.

3.2.3 Measurement of metabolic parameters in plasma. Blood glucose were measured in 12-h fasted rats using standard glucose test meter (OneTouch, LifeScan, CA) and triglycerides were measured by using an Accutrend Plus System (hand-held point-of-care device) and specific test strips (Roche Farma, Barcelona, Spain) with a drop of blood collected from the tail vein. Blood samples were collected from intracardiac puncture and obtained in separate tubes containing heparin and sodium citrate as anticoagulants. Plasma was obtained by centrifugation at 10,000xg for 5 min at 4°C and stored at -80°C until used. HbA1c was measured by using Bayer A1cNow test kit. Blood sample was collected after sacrificing the animal to measure HbA1c. The diluted sample was mixed and added to the monitor. After adding the sample, the monitor begins the analyze and results will be displayed in the display window after 5 minutes.

3.2.4 Measurement of arterial tension. The thoracic aortas were cut into 2mm rings after being excised and cleaned off adhering connective tissues. To measure isometric tension, the rings were suspended horizontally between two stainless steel hooks in individual organ baths containing 20 ml of Krebs buffer (in mM: 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.9 $NaHCO_3$, 0.023 EDTA, 1.6 $CaCl_2$, and 6.0 glucose) at 37°C bubbled with 95% O_2 and 5% CO_2. Isometric tension was continuously monitored with a computer-based data acquisition

system (PowerLab; ADInstruments, Colorado Springs, CO). To develop a stable basal tone, aortic rings were equilibrated under 1g resting tension for 40 min. Rings were stimulated two times with 80mM KCl every 20 min until maximum contraction was achieved. The ability of acetylcholine (ACh, 10 μM) to induce relaxation of phenylephrine (PE, 2 μM) pre-contracted vessels was taken as evidence for the preservation of an intact endothelium. For the relaxation studies, we used an equal submaximal concentration of PE (2 μM) in all experimental groups.

3.2.4.1 Relaxation responses to ACh. Aortic rings were precontracted with PE (2 μM), which represented a concentration that produced 80% of the maximal effect (EC_{80}). The concentration response curves (CRC) were obtained by the addition of increasing concentrations of ACh (10^{-8} to 10^{-5} M).

3.2.4.2 Relaxation responses to Sodium Nitroprusside (SNP). Responses to SNP (10^{-9} to 10^{-5} μM), a NO-donor, were obtained in the endothelium denuded aortic rings pre-contracted with PE (2 μM) taken from all experimental groups.

3.2.4.3 Contractile responses to PE. The constrictor CRC to PE (10^{-8} to 10^{-5} M) were generated before and after incubation with Nω-Nitro-L-arginine methyl ester (L-NAME, 200 μM), a NOS inhibitor in the presence of indomethacin (Indo, 10 μM, dissolved in DMSO), a COX inhibitor. Between each CRC, tissues were washed with Krebs buffer to allow the rings to return to the basal tone. A vehicle-only (no drugs present) study was performed simultaneously in aortic rings from the same animal (Data not shown).

3.2.4.4 Western blot analysis.

3.2.4.4.1 Preparation of total protein extracts. Aortic tissue samples were micronized through freezing with liquid nitrogen and grinding with a mortar (111). To obtain total protein extract, samples were incubated with RIPA buffer (Sigma-Aldrich, St. Louis, MO) containing Protease Inhibitor Cocktail (UltraCruz, Santa Cruz Biotechnology, Dallas, TX) for 1.5 h at 4°C and centrifuged at 1500xg for 15 min at 4°C, and supernatants were collected. Protein concentrations were by the bicinchoninic acid assay (BCA assay).

Protein (20–30 µg) was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to 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. Primary antibodies forendothelial NO synthase (eNOS) were supplied by Cell Signaling (Boston, MA). Antibodies against Nox1 and Nox4 were obtained from Abcam (Cambridge, MA). Incubation with secondary antibody (LI-COR donkey anti-Rabbit IgG IRDye 680 or anti-mouse IgG IRDye 800CW, 1: 10,000) was performed in the blocking buffer for 1 h at room temperature. Before analyzing, the membrane was washed four times with TBS containing 0.1% Tween-20. Detection was done by using a LI-COR Odyssey imaging system (Lincoln, NE). Uniformity of the protein loading was confirmed by incubating the blots with GAPDH and β- actin (Cell Signaling, Boston, MA) as a control.

3.2.4.5 Measurement of plasma estradiol level. Plasma estradiol levels were assessed by using ELISA kit from Abcam (Cambridge, MA) according to manufacturer's instruction.

3.2.4.6 Statistical analysis. All values were expressed as mean of n values ± standard error of the mean (SEM). Here, n denotes data from one rat. Relaxation responses to ACh- and SNP were calculated as the percentage of relaxation from maximum PE contraction at each concentration. Similarly, the recorded increase in the force of contraction was calculated as the percentage of maximum contraction obtained with PE at the highest dose. The concentration of the agonist, which produced half of the maximum effect (E_{max}) was expressed as EC_{50} and calculated by a sigmoidal dose-response model (for variable slope) using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). The sensitivity of the agonists was expressed as pD_2 values (-log [EC_{50}]), which were normally distributed. The area under the curve (AUC) was determined using GraphPad Prism 7 with trapezoidal technique. To compare the effect of pharmacological agents such as L-NAME on the PE response, the PE results were expressed as differences of area under the concentration-response curve (ΔAUC) in control (absence of drug) and experimental (presence of drug) condition. Comparison of CRC between two groups was done using two-way ANOVA, with one factor being concentration and the other being groups (pre-diabetic vs control and placebo vs. E_2). When the ANOVA test returned $P < 0.05$, post hoc analysis using Bonferroni's or Tukey's test was performed. Comparison of CRC in a pre/post-test format within a group was done using two-way ANOVA with repeated measures. Student's unpaired t-test was used for comparisons of two group means. A probability value of less than 5% ($P<0.05$) was considered significant.

3.3 Results

3.3.1 Effects of 17β- estradiol (E_2) replacement on plasma estradiol level. E_2 treatment significantly increased the concentrations of plasma E_2 level in control and pre-diabetic rats compared to placebo treated animals (Figure 15). In the current study plasma E_2

concentrations were 4.46±1.10 pg/ml in placebo-treated control group, 45.24±17.05 pg/ml in E_2-treated control group, 1.60±0.58 pg/ml in placebo-treated pre-diabetic group and 49.27±12.54 pg/ml in E_2 treated pre-diabetic group.

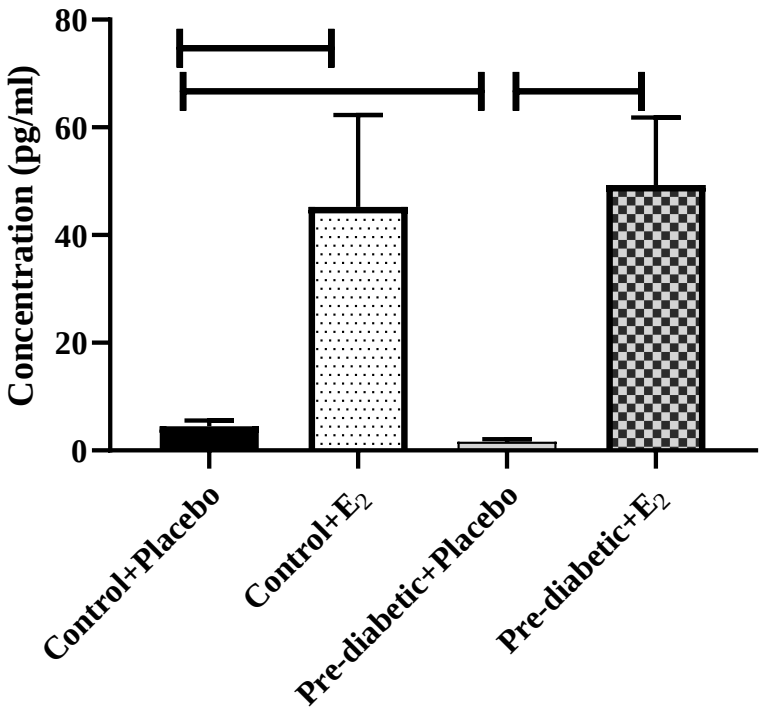

Figure 15: 17β-estradiol (E_2) level in plasma of placebo and E_2 -treated control and pre-diabetic rats. Data are expressed as mean±SEM. Capped lines indicate significant differences ($P < 0.05$) between 2 groups, analyzed using Student's unpaired t-test; n = 5 per group.

3.3.2 Effects of E_2 replacement on metabolic parameters. E_2 replacement significantly lowered the body weights of animals than those of placebo treated rats in both control and pre-diabetic groups. However, pre-diabetic animals had higher body weight compared to the control animals, regardless of treatment with E_2. (Table 8). Accordingly, replacement with E_2 lowered the adiposity in rats compared to the placebo treated groups of control and pre-diabetic rats. However, pre- diabetic animals had higher adiposity than control animals, irrespective of E_2 treatment. Moreover, blood glucose and HbA1c levels were significantly reduced after treatment with E_2 in both control and pre-diabetic rats.

Table 8: Body weight and adipose weight, blood glucose levels and HbA1c of placebo and E_2 treated control and pre-diabetic rats

	n	**Control+Placebo**	**Control+E_2**	**Pre-diabetic+Placebo**	**Pre-diabetic+E_2**
Body Weight (g)	7-8	355.14±10.43#	224.24±7.78*^	439.71±9.96	317.42±14.15*
Adipose Tissue (g)	6	15.61±2.13#	4.69±0.77*^	27.75±1.72	7.73±0.48*
Blood Glucose (mg/dl)	6	153.83±6.18#	129.6±0.11*	182.33±12.87	131.5±8.30*
HbA1c level	6	4.46±0.10#	4.00±0.00*	5.63±0.26	4.31±0.07*

Data are expressed as mean ± SEM. *$p<0.05$ (vs. placebo treated, respective group), #$p<0.05$ (vs. pre-diabetic+Placebo), ^$p<0.05$ (vs. pre-diabetic+E_2), analyzed using Student's unpaired t-test

3.3.3 Effects of E_2 replacement on relaxation responses to ACh. CRC to ACh were obtained to evaluate the effect of E_2 and prediabetes on the receptor-mediated endothelium-dependent release of NO. ACh- induced relaxation responses were higher in placebo-treated controls than placebo-treated pre-diabetic group (Figure 17A). In both control and pre-diabetic groups, treatment with E_2 markedly enhanced the ACh-responses of aortic rings compared to respective placebo treated group (Figures 16A and 16B). Both the E_{max} and sensitivity (as assessed by -log [EC_{50}] (pD_2)) to ACh were significantly enhanced in the aortic rings of E_2 -treated rats than those of placebo-treated animals regardless of being healthy or pre-diabetic (Table 9). However, when compared to E_2 -treated pre-diabetic animals, E_2- treated controls expressed higher ACh- induced relaxation responses (Figure 17B).

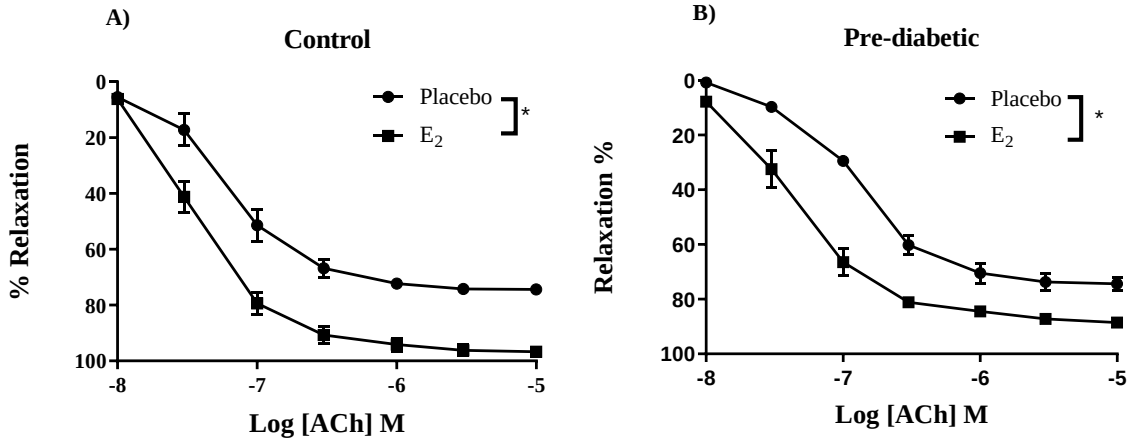

Figure 16: Relaxation response to cumulative concentrations of ACh (10^{-8} to 10^{-5} M) in intact aortic rings precontracted with phenylephrine (2 μM) from placebo and E_2 treated control (A) or pre-diabetic (B) rats. Data are expressed as means ±SEM. *P < 0.05 between two groups analyzed using 2-way ANOVA followed by Bonferroni's post hoc test.

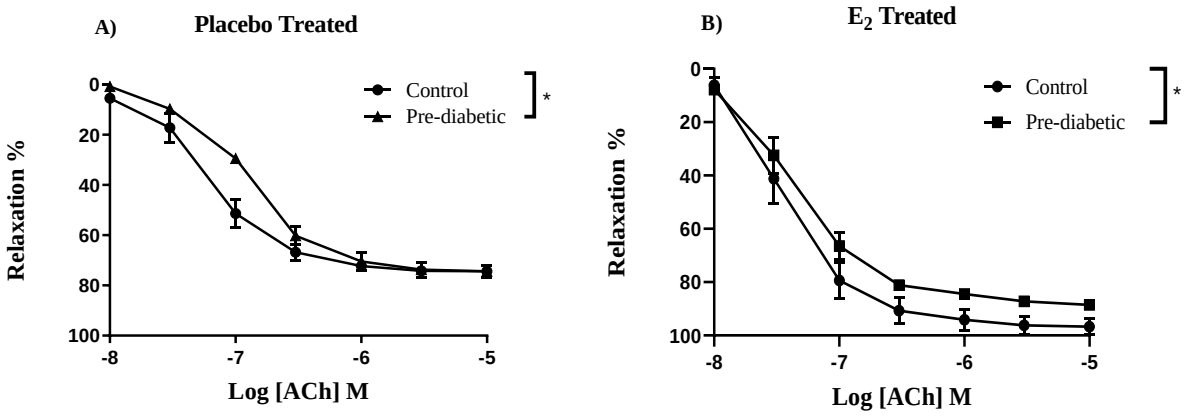

Figure 17: Relaxation response to ACh (10^{-8} to 10^{-5} M) in intact aortic rings precontracted with phenylephrine (2 μM) from placebo (A) and E_2 (B) treated control and pre-diabetic rats. Data are expressed as means ± SEM. *P < 0.05 between two groups analyzed using 2-way ANOVA followed by Bonferroni's post hoc test.

Figure 17 is a different representation of same data shown in Figure 16.

Table 9: pD_2 and E_{max} to acetylcholine (ACh) in aortic rings from placebo and E_2 treated control and pre-diabetic rats

ACh	n	pD_2	E_{max} %
Control+ Placebo	5	6.70± 0.13	74.36±1.42
Control+ E_2	5	7.45±0.03*	96.69±1.66*
Pre-diabetic+ Placebo	5	6.44±0.05	74.38±1.31
Pre-diabetic+ E_2	5	7.19±0.04*#	88.51±1.70*#

Data are expressed as mean ± SEM; n, number of rats per group. *$p<0.05$ (vs. placebo in respective group), #$p<0.05$ (vs. control+E_2), analyzed using Student's unpaired t-test.

3.3.4 Effects of E_2 replacement on relaxation responses to SNP. The smooth muscle sensitivity to NO was investigated by performing CRC to SNP (10^{-9} to 10^{-5} M) in endothelium - denuded aortic rings. In aortic rings from pre-diabetic groups, E_2 treatment improved the sensitivity as assessed by pD_2 values to SNP compared with placebo treated group (Figure 18B and Table 10). However, no significant differences in CRC to SNP was observed in control groups, regardless of E_2 treatment (Figure 18A and Table 10).

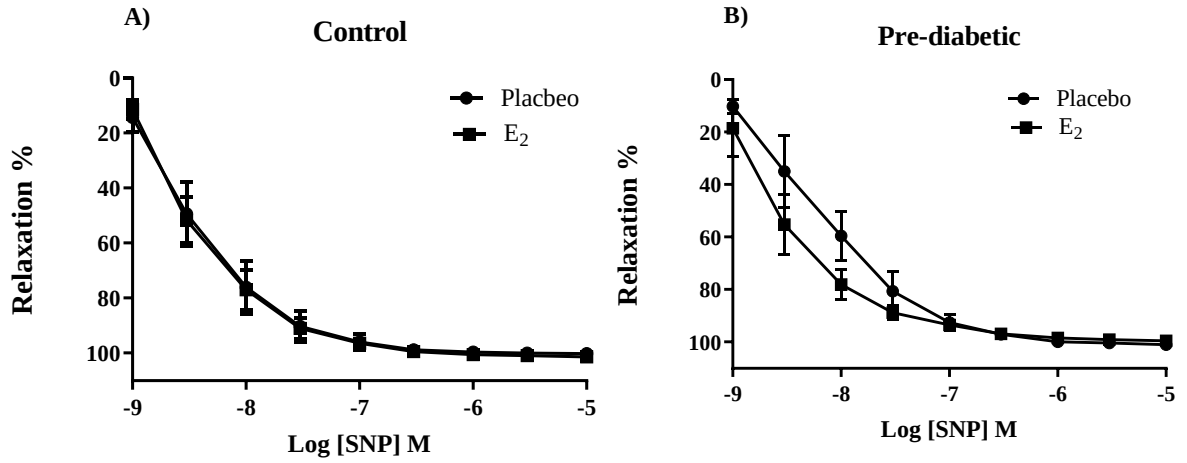

Figure 18: Relaxation responses to cumulative concentrations of sodium nitroprusside (SNP, 10-9 to 10-5 M) in endothelium denuded aortic rings pre-contracted with phenylephrine (PE, 2 μM) from placebo and E2 treated control (A) and pre-diabetic (B) rats. Data are expressed as mean ± SEM.

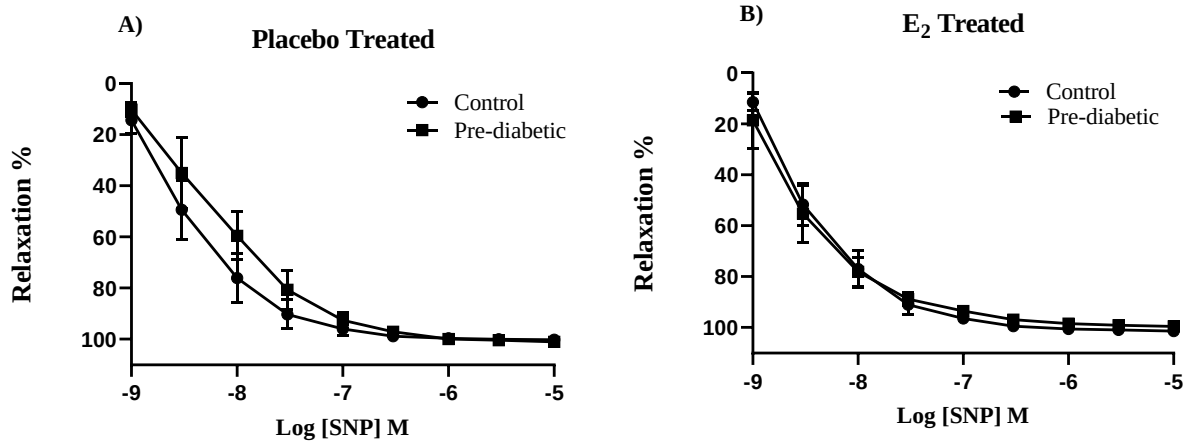

Figure 19: Relaxation responses to cumulative concentrations of sodium nitroprusside (SNP, 10^{-9} to 10^{-5} M) in endothelium denuded aortic rings pre-contracted with phenylephrine (PE, 2 μM) from placebo (A) and E_2 (B) treated control and pre-diabetic rats. Data are expressed as mean ± SEM.

Figure 19 is a different representation of same data shown in Figure 18.

Table 10: pD_2 and E_{max} to sodium nitroprusside (SNP) in rat aortic rings from placebo and E_2 treated control and pre-diabetic rats

SNP	**n**	**pD_2**	**E_{max}, %**
Control+ Placebo	5	8.43± 0.17	100.11±0.38
Control+ E_2	5	8.45±0.12	101.31±0.66
Pre-diabetic+ Placebo	5	8.15±0.18	100.98±0.34
Pre-diabetic+ E_2	5	8.76±0.08*	99.54±0.80

Data are expressed as mean ± SEM; n, number of rats per group. *P<0.05 (vs. placebo in respective group), analyzed using Student's unpaired t-test.

3.3.5 Effects of E_2 replacement on contractile responses to PE. Contractile responses to α-adrenoceptors were analyzed by measuring the CRC to PE (10^{-8} to 10^{-5} M). The $tension_{max}$ but not the sensitivity (as assessed by pD_2) to PE were significantly decreased in E_2 -treated pre-diabetic and control groups compared with their respective placebo- treated groups (Figures 20A & 20B and Table 11).

To indirectly measure the level of basal NO, CRC to PE (10^{-8} to 10^{-5} M) were performed in aortic rings before and after pretreatment with the NO synthase inhibitor, L-NAME (200 μM) in the presence of Indo (10 μM). The changes in the contractile level to PE after addition of L-NAME would reveal the effect of basal NO release on contraction (115, 116).

Incubation of the aortic rings with L-NAME resulted in a significant increase of the contractile responses to PE in all experimental groups (Figure 22). However, as indicated by ΔAUC (the difference in area under the curve between PE CRC before and after L-NAME) were higher in aortas of E_2 -treated pre-diabetic and control groups compared with their respective placebo-treated groups (Table 12). However, when compared to pre-diabetic group, E_2 -treated controls had a higher level of basal NO than E_2-treated pre-diabetic rats as assessed by ΔAUC (Table12).

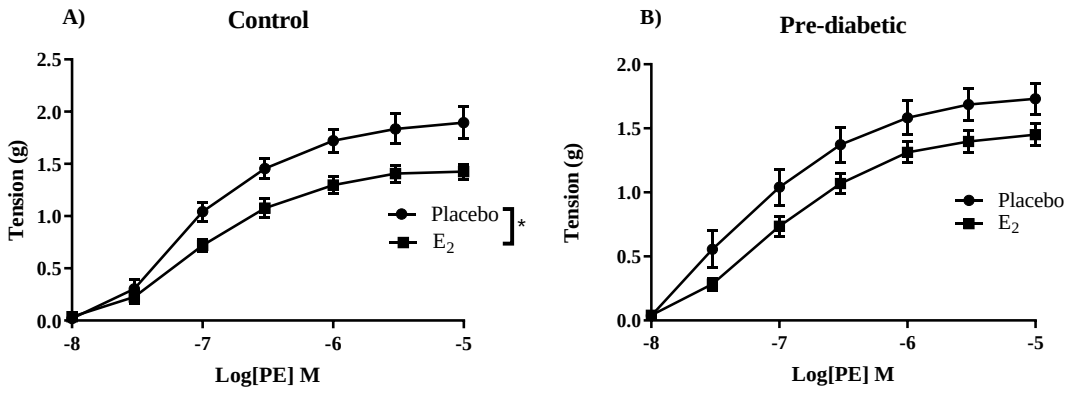

Figure 20: Concentration-response curves to phenylephrine (PE, 10^{-8} to 10^{-5} M) in intact aortic rings of placebo and E_2 -treated control (A) and pre-diabetic (B) rats. Data are expressed as mean ± SEM. *P<0.05 between two groups analyzed using two-way ANOVA followed by Bonferroni's post hoc test, n=5per group.

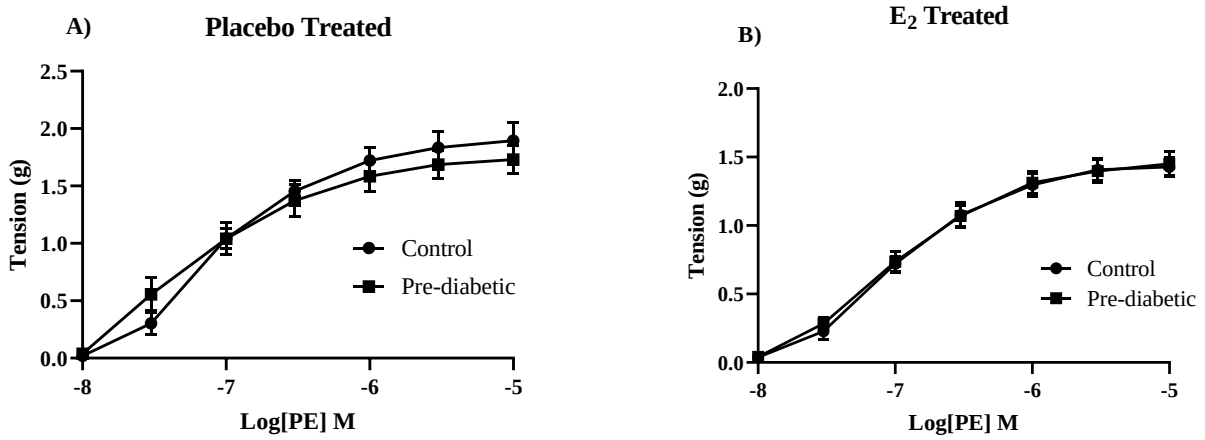

Figure 21: Concentration-response curves to phenylephrine (PE, 10^{-8} to 10^{-5} M) in intact aortic rings of placebo (A) and E_2 (B) treated control and pre-diabetic rats. Data are expressed as mean ± SEM.

Figure 21 is a different representation of same data shown in Figure 20.

Table 11: pD_2 and $Tension_{max}$ to phenylephrine (PE) in aortic rings from placebo and E_2 treated control and pre-diabetic rats

PE	n	pD2	Tension max (g)
Control+ Placebo	5	7.04±0.03	1.99±0.17
Control+ E_2	5	6.98±0.06	1.43±0.08*
Pre-diabetic+ Placebo	5	7.08±0.07	1.78±0.13
Pre-diabetic+ E_2	5	7.02±0.05	1.39±0.07*

Data are expressed as mean ± SEM; n, number of rats per group. *P<0.05 (vs. placebo in respective group), analyzed using Student's unpaired t-test.

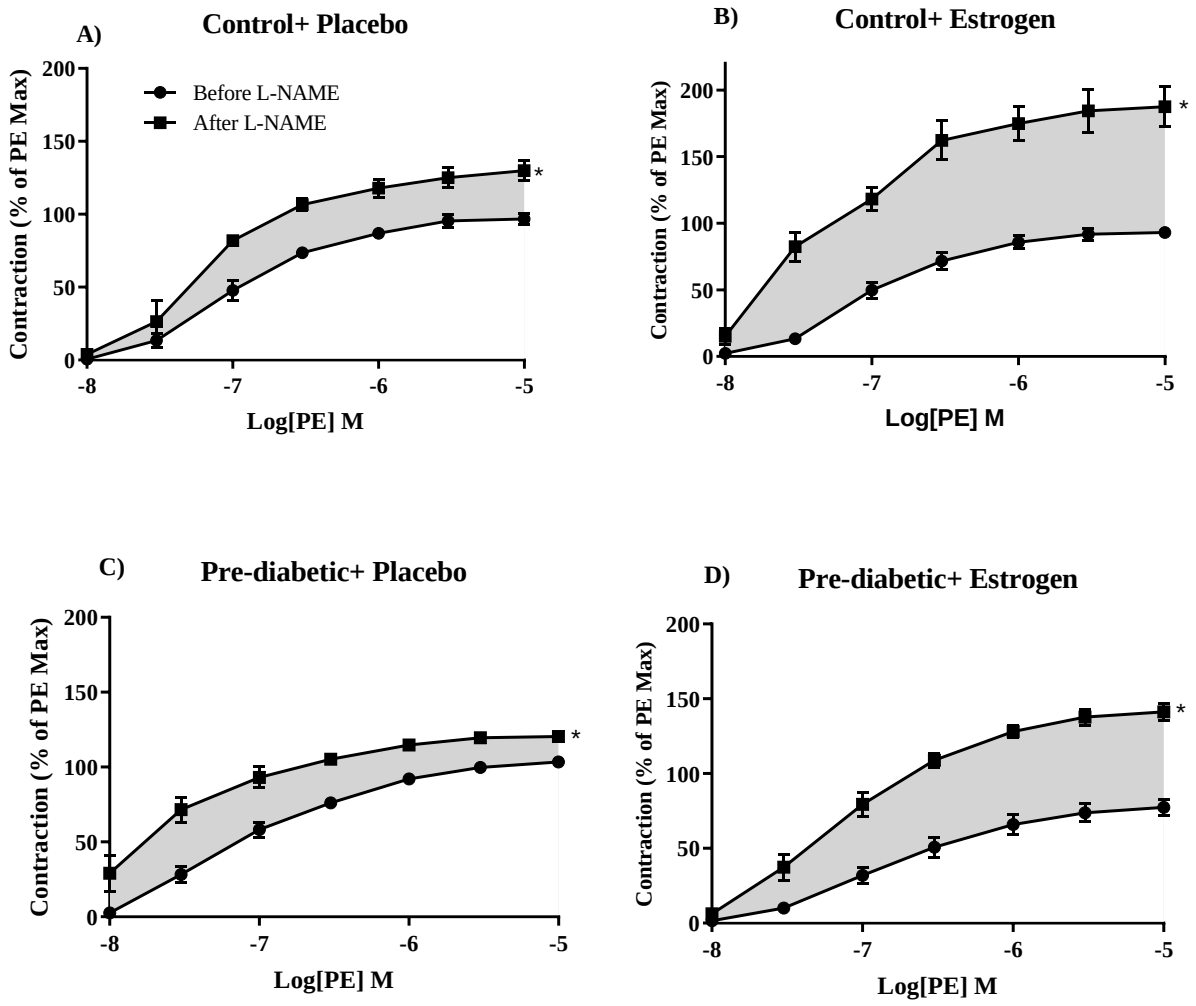

Figure 22: Contractile response to cumulative concentrations of phenylephrine (PE, 10^{-8} to 10^{-5} M) in intact aortic rings from control+ placebo (A), control+ E_2 (B), pre-diabetic+ placebo (C) and pre-diabetic+ E_2 (D) rats. Contraction to PE was measured before and after incubation with Nω-Nitro-L-arginine methyl ester (L-NAME, 200 μM). Responses were performed in the presence of indomethacin (10 μM). Data are expressed as mean±SEM, with *P<0.05 vs. before L-NAME in all groups as analyzed using two-way ANOVA with repeated measures, n=5 per group.

Table 12: ΔAUC**,** pD_2 and $Tension_{max}$ to phenylephrine (PE) in aortic rings from placebo and E_2 treated control and pre-diabetic rats

	$Tension_{max}$, g	**pD2**	**ΔAUC**
Control+ Placebo			
Before L-NAME	1.69±0.60	6.97±0.07	80.10±1.51
After L-NAME	2.27±0.94a	7.16±0.08a	
Control+ E_2			
Before L-NAME	1.56±0.21	7.02±0.05	232.57±21.63*
After L-NAME	2.26±0.19a	7.41±0.09a	
Pre-diabetic+ Placebo			
Before L-NAME	1.78±0.16	6.94±0.12	85.68±2.81
After L-NAME	2.30±0.13a	7.45±0.09a	
Pre-diabetic+ E_2			
Before L-NAME	1.22±0.10	6.75±0.79	139.58±10.49*#
After L-NAME	2.19±0.07a	7.09±0.10a	

Data are expressed as mean± SEM. L-NAME, *N*-nitro-L-arginine methyl ester. **P* <0.05 vs. placebo in respective group (Same sex), #*P*<0.05 vs E_2 treated control, Student's Unpaired t-Test; [a]*P*<0.05 vs before L- NAME, Student's paired *t*-test. n=5 per group.

3.3.6 Effects of E_2 replacement on eNOS and Nox expression. To investigate the possible mechanisms underlying the elevated of the ACh responses in ovariectomized E_2 -treated rats, the protein expression of eNOS and NADPH oxidase (Nox) subunits, Nox1 and Nox4 were measured. Western blot analysis revealed that the expression of eNOS were significantly increased after treatment with E_2 in control group (by 2.0 fold, Figure 23). Although eNOS protein levels tended to be higher in E_2 -treated pre-diabetic rats compared with placebo-treated pre-diabetic group, the difference did not reach to significance level (Figure 23).

The expressions of Nox1 was significantly reduced in aortic tissues taken from E_2 -treated control and pre-diabetic groups (by 0.8 fold in control and by 0.5 fold in prediabetic, Figure 24). However, the protein expression of Nox4 showed no significant differences among all experimental groups (Figure 25).

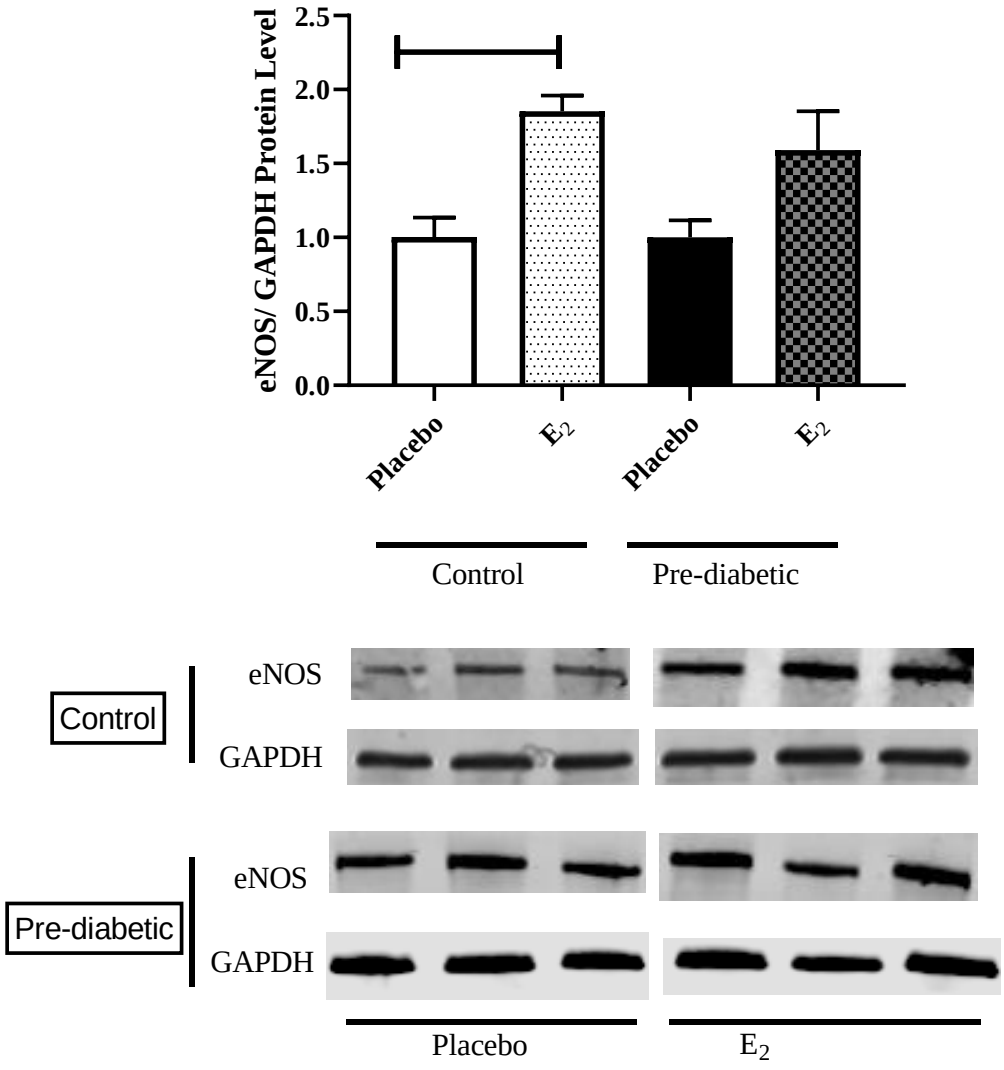

Figure 23: Western blots of eNOS normalized to GAPDH in aortic samples from placebo and E_2 -treated control and pre-diabetic rats. Each bar represents the mean ± SEM of values obtained from n=5 animals. To show representative bands images from different parts of the same gel have been juxtaposed, which is indicated by white dividing lines. Capped lines indicate significant differences between two groups ($P<0.05$), as analyzed by Student's unpaired t-test.

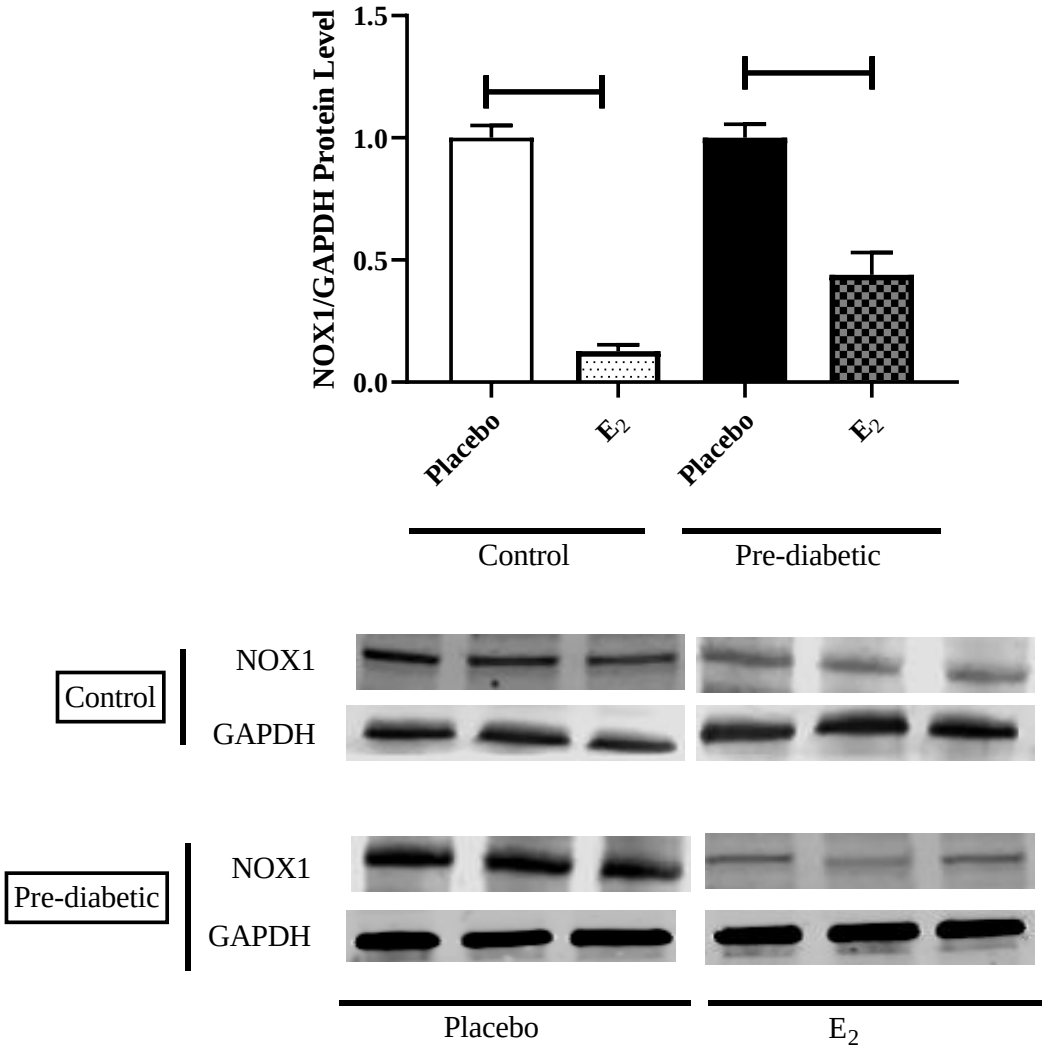

Figure 24: Western blots of Nox1 normalized to GAPDH in aortic samples from placebo and E_2 - treated control and pre-diabetic rats. Values are represented as mean± SEM. Each bar represents the values obtained from n=4-5 animals. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines. Capped lines indicate significant differences between two groups ($P<0.05$), as analyzed by Student's unpaired t-test.

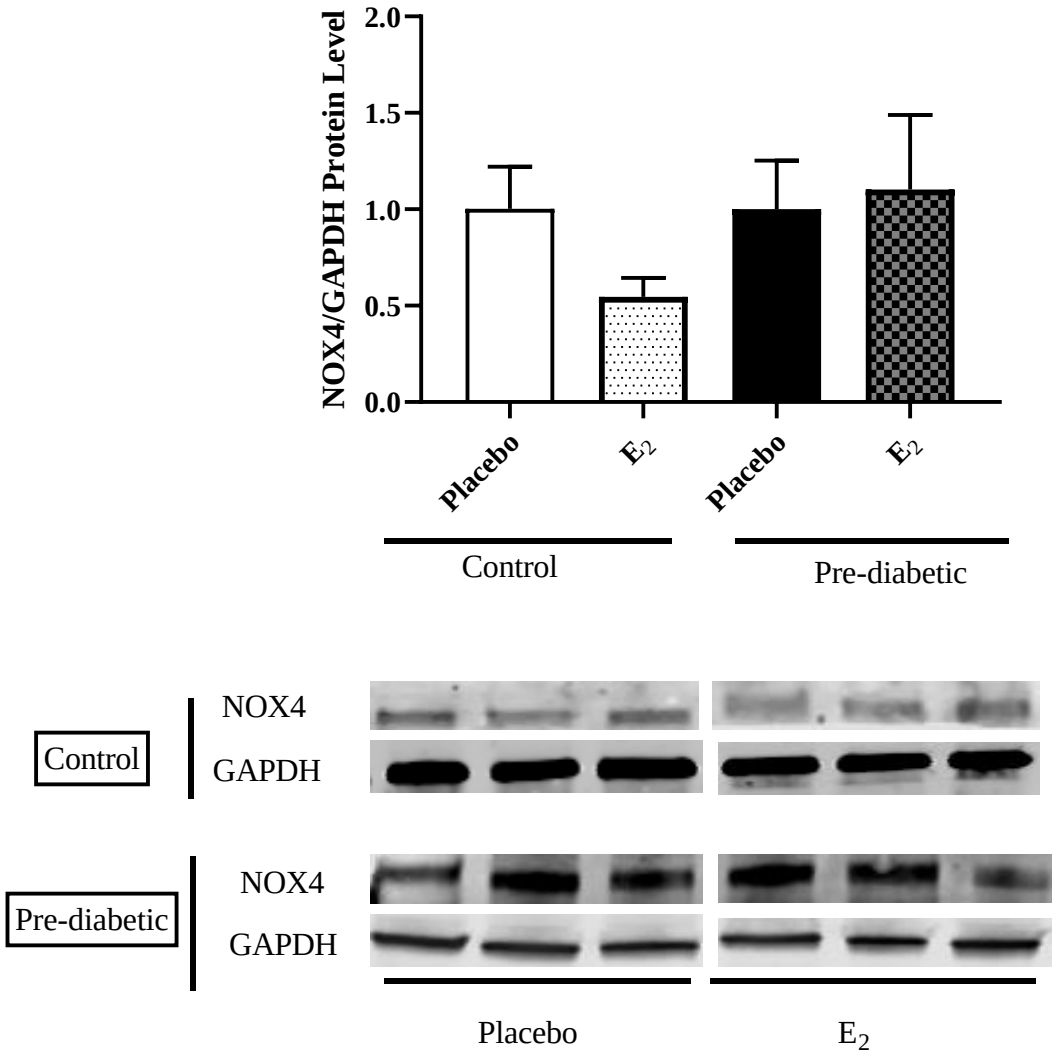

Figure 25: Western blots of Nox4 normalized to GAPDH in aortic samples from placebo and E_2 -treated control and pre-diabetic rats. Values are represented as mean± SEM. Each bar represents the values obtained from n=4-5 animals. To show representative bands, images from different parts of the same gel have been juxtaposed, indicated by white dividing lines.

3.4 Discussion

This study is the first report on the effects of 17β-estradiol (E_2) on aortic function in UCD-T2DM rats at the prediabetic stage. Using pre-diabetic model enabled us to investigate the timeline of loss of endothelial function. Furthermore, using ovariectomized animals with or without E_2 replacement allowed us to examine the role of estrogen while investigating endothelial function at pre-diabetic stage. The main findings of our investigation were that 1) the E_2 replacement improved the endothelial function in both control and pre-diabetic ovariectomized groups but the impact was significantly higher in control group compared with the pre-diabetic animals, and 2) E_2 replacement elevated the basal NO level in both control and pre-diabetic group, but the effect of E_2 was significantly higher in control group than that of pre-diabetic group. Accordingly, the protein expression level of eNOS were enhanced in both E_2-treated control group and pre-diabetic group but the effect was more prominent in control groups. Furthermore, E_2 treatment reduced the Nox1 level in control and pre-diabetic animals.

In the current study, treatment with E_2 increased the plasma estradiol levels in control and pre-diabetic rats. Our data shows that, E_2 replacement reduced the blood glucose level, HbA1c level, body weight and adipose tissue weight in control and pre-diabetic rats. In accordance with previous studies decreased body weight (159-162), adiposity (159, 163), blood glucose level (160, 163) and HbA1c (164) have been reported with E_2 -treated ovariectomized rats.

Endothelium-dependent vasodilatation (EDV) is used as a reproducible parameter to probe endothelial function in different pathophysiological conditions. Several lines of evidence suggest that the endothelial dysfunction represents early steps in the development of vascular complications in diabetes, and hyperglycemia is the central initiating factor for many types of vascular complications in diabetes (165-170). It has been reported that E_2 replacement improves

ACh- mediated relaxation responses in aortic rings of ovariectomized ZDF rats (160) and SD rats (160, 171, 172). Similar with previous reports, showing an improved EDV to ACh after E_2 replacement in ovariectomized SD rat aorta (160, 171, 172), we demonstrated that treatment with E_2 significantly enhanced the relaxation responses to ACh in control group compared to placebo treated controls. However, the impact of E_2 in improving the relaxation response is significantly lower in pre-diabetic stage compared with the control animals. To our knowledge, no data has yet been published on the effect of E_2 in ovariectomized pre-diabetic rats. In the current study, we observed that maximal relaxation to ACh was significantly higher in aortic rings from ovariectomized UCD-T2DM rats at pre-diabetic stage after E_2 replacement compared with that in placebo-treated group.

It has been well established that in conduit arteries, NO plays the major role in EDV (42, 126). The lower ACh-induced relaxation observed in placebo treated control and pre-diabetic arteries may in part result from decreased NO bioavailability or reduced in sensitivity of smooth muscle to NO in this groups. NO is produced in vascular endothelial cells by the enzyme endothelial nitric oxide synthase (eNOS) (173). It has been reported that E_2 enhanced eNOS expression in rat aorta (174, 175) and rat cerebral microvessels (176). Han et al. reported that E_2 replacement improved endothelial dysfunction in aortic rings of ovariectomized ZDF rats by improving the eNOS expression and signaling network (160). Accordingly, we demonstrated that protein expression level of eNOS were higher in E_2 treated groups compared with the placebo-treated groups (although it didn't reach to significance level in pre-diabetic group). By contrast, Barbacanne et al. found no effect of E_2 on eNOS expression in rat aorta (172).

The basis for discrepancy with Barbacanne et al. study, might be due to the difference in the duration of E_2 administration.

Besides the possibility of increased contribution of NO production, improved aortic endothelial function observed in E_2 treated groups may be explained by changes of smooth muscle responsiveness to NO or contractile agents. We observed that SNP-induced relaxation of endothelium-denuded aortic rings was not altered in control groups. Similarly, Barbacanne et al. also observed no significant difference in SNP-induced relaxation in aortic rings of ovariectomized SD rats (127, 172). On the other hand, in the current study smooth muscle sensitivity NO was significantly enhanced in pre-diabetic E_2 -treated group. These data suggest that increased vascular responsiveness to NO may in part be responsible for the improved ACh-induced relaxation observed in aorta of this group.

In the current study, maximum tension to PE were decreased significantly in aortic rings of E_2 -treated groups compared with their respective placebo treated groups regardless of control and pre-diabetic condition. The decreased PE response may in part explain the elevated ACh-induced relaxation in aorta of E_2 -treated rats. These data are in line with Ping et al. who reported that E_2 treatment attenuates the vascular contraction in hypertensive mice aorta (177).

The attenuated contractile responses to PE observed in E_2 -treated control and pre-diabetic rats may partially result from the elevated release of basal NO or an enhanced of contracting factors (52, 178). We assessed endothelium-derived NO indirectly by measuring the differences in the degree of PE-induced contraction in the absence and presence of L-NAME (51, 141). It has been reported that treatment with E_2 increases basal NO release in ovariectomized SD rat aorta (171). Here, we observed that pre-treatment with L-NAME caused a

significantly higher potentiation of the PE responses in aortic rings of E_2 -treated rats not only in control but also in pre-diabetic group (Figure 20 B and D) compared with those in respective placebo treated rats. This suggests that the increased level of NO may in part be responsible for the attenuated PE contractile responsiveness in E_2 treated rats. Moreover, when compared to E2-treated pre-diabetics, the basal NO level was significantly higher in E_2 -treated controls.

It has been reported that in diabetes and hyperglycemia, superoxide production may play a crucial role in enhancing the contracting responses (50, 142-144). The superoxide acts by scavenging NO, thus decreasing its bioavailability (145) leading to endothelium-dependent contractions. In the present study, we did not directly measure NO or superoxide production. However, we determined expression of Nox proteins in rat aorta. Among the Nox isoforms, Nox1, Nox2 and Nox5 are considered as superoxide generating enzyme and promote endothelial dysfunction (25). On the other hand, Nox4 is hydrogen per oxide (H_2O_2) generating enzyme and has the vasoprotective effect (25-28). Vascular walls express high levels of Nox1, Nox2 and Nox4 (29). In large conduit arteries, Nox1 is mainly expressed (30). Here, we observed a decreased expression of Nox1 in aorta taken from E_2 treated groups of control and pre-diabetic rats, whereas Nox4 expression was not changed (Figures 22 and 23). It has been reported that E_2 treatment significantly reduced the Nox1 expression level in Wistar rat aorta (179). Youn et al. reported that the activation of Nox1, but not Nox4 or Nox2, was associated with eNOS uncoupling and endothelial dysfunction in STZ-induced diabetic mice aorta (146). Along the similar line Gray et al. reported genetic deletion of Nox1 in diabetic mice led to reduced diabetes mellitus symptoms suggesting a key role of Nox1 derived reactive oxygen species in diabetes (147). Therefore, our results on attenuated expression of Nox1 in E_2 -treated arteries of control and pre-diabetic groups suggest that the decreased contractile responses to PE or enhanced ACh-

induced relaxation in E_2 -treated control or pre-diabetic groups may be partially due to the reduced oxidative stress. Further studies required to directly measure the level of superoxide and NO in the experimental groups.

In conclusion, we showed that EDV was impaired in ovariectomized rats in both control and prediabetic groups. E_2 -replacement improved the endothelial function in both control and pre-diabetic groups possibly due to the elevated NO resulted from higher eNOS or lower Nox1 expression in E_2-treated groups. When compared to prediabetic groups, E_2 -replacement had a higher impact on aortic function of control group. This might be due to higher eNOS leading to higher basal NO in this group.

Chapter 4: Overall Conclusion

We have shown that the aortic function are altered in UCD-T2DM rats and ovariectomized rats either in control or pre-diabetic groups. Specifically, the important findings of study I are, aortic function is altered in a novel and validated model of type 2 diabetes (UCD-T2DM rats) and sex differences exist in the development of abnormal vascular response. Our data shows that the lower sensitivity to NO and/or enhanced responsiveness to contractile agents contributed to the impaired EDV in aortic rings of UCD-T2DM female rats. Particularly, potentiation of EDV in diabetic male arteries was associated to a shift from NO toward a reliance to both NO and EDHF. In study II we showed that, E_2 -replacement improved the endothelial function in both ovariectomized control and pre-diabetic groups. The impact of E_2 was significantly higher in control group which may be due to the higher NO resulted from lower Nox1 in this group.

Chapter 5: Limitation

The effect of sex and diabetes on vascular activity varies in different vascular beds and species. Clearly for investigation on specific diabetic macro-vascular complications, the use of coronary artery and cerebral artery is desired. However, rat aorta was used in the current study. In addition, it is uncertain to what extent the rat model of diabetes can mimic human vascular disease in diabetes. But, due to the relative difficulty in obtaining vascular samples from human, the rat models of diabetes are still widely used to unmask the mechanisms of diabetic vascular complications.

Western blot analysis was done using whole aortic tissue, which contains both endothelial cells (ECs) and smooth muscle cells (SMCs). Hence the increase or decrease in expression of proteins cannot be precisely attributed to ECs or SMCs.

Here, we used UCD-T2DM rats. Although this is model is validated model of type 2 diabetes and it closely resembles the human T2DM, it is still under study and studies in certain areas such as hypertension remains to be determined.

In study II, we used ovariectomized rats as preclinical model of menopause. Although ovary secretes a multitude of hormones, only E_2 was replaced in this study. Therefore, the effects of progesterone and other ovarian hormones were not investigated. Further studies required to examine the effects of sex hormones in ovariectomized UCD-T2DM rats in diabetic stage.

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