ABSTRACT

Background & objectives: Diabetes is a stronger risk factor in the development of cardiovascular diseases in the female than the male gender. Diabetes-induced reactive oxygen species (ROS) alters the function of endogenous vasoconstrictors for which the antioxidant, quercetin, has been shown to restore in diabetic male rats, which has not been proven in the female. The female hormone, estradiol (a potent antioxidant), combined with quercetin may offer greater protection against diabetes/ROS-induced vascular reactivity. Therefore, the influence of gender on the response of normoglycemic/diabetic aorta to vasoconstrictors in the presence/absence of quercetin/estradiol, including mechanisms underlying any differences in tissue responses was examined.

Materials & methods: Isometric tension to cumulative concentrations of phenylephrine or angiotensin II were recorded in (age-and-sex-matched) thoracic aorta isolated from normoglycemic/streptozotocin-treated Wistar Kyoto rats. The role of ROS, 17β-estradiol, antioxidant enzymes, nitric oxide (NO) and prostaglandins (PG) in modulating the differences were explored.

Results & Discussion: Endothelium-intact normoglycemic male tissues contracted more to PE or Ang II than the female or the diabetic male. The normoglycemic (proestrus or diestrus) /diabetic female tissues contracted equally to PE. Ang II caused lesser contraction of normoglycemic (proestrus) /diabetic compared to normoglycemic female tissues in diestrus state. Endothelial-denudation or blockade of L-NAME/methylene blue (MB) pre-treatment reversed these differences, suggesting e-NOS-sGC-cGMP pathway regulated the differences. Endothelial-denudation, L-NAME/MB and acetylcholine produced lesser effect on the normoglycemic male and diabetic male/female tissues

compared to the normoglycemic female, which exhibited higher tissue vasorelaxants (NO/PGI_2). Therefore, the aorta of healthy female rat exists in a higher eNOS-sGC-cGMP (basal vasorelaxant) state. This feature was reversed by diabetes, supporting the hypothesis that the female vasculature succumbs more to diabetes-induced alterations than the male.

Contractile PG levels were higher in normoglycemic male/diabetic female tissues. Diabetes promoted relaxant PGI_2 synthesis in male/female tissues. This result is consistent with observed gender difference in tissue contraction (normoglycemic male >female and diabetic female>male). Higher diabetic female synthesis of contractile PGs consistently supports the greater negative impact of diabetes in the female. Enhanced diabetic-synthesis of vasodilators (PGI_2/EDNO) in male (/female) tissues perhaps represents a pathologic feature of short-term diabetes to counter increased diabetic state-stimulated contraction. These findings have implications for further understanding of the gender-related differences in the mechanism of diabetes-induced vascular disease.

The order of tissue oxidative stress and quercetin-induced reduction are diabetic (male >female)>normoglycemic (male >female) tissues, suggesting that quercetin effect is partially mediated by its action against oxidative stress. 17β-estradiol and/or quercetin-induced relaxation was greater in phenylephrine compared to angiotensin II-contracted diabetic male/female tissues, suggesting that quercetin/estradiol therapy appears more clinically relevant in managing phenylephrine than angiotensin II-mediated vasoreactivity during diabetes. L-NAME/MB reversed quercetin effect in normoglycemic male/female tissues (with/without endothelium) and male/female diabetic tissues (with but not without endothelium). L-NAME+indomethacin reduced quercetin effect in endothelium-intact

normoglycemic male and diabetic male/female tissues. Hence, quercetin action is partly mediated by endothelium-sensitive (eNO-sGC-cGMP/cyclooxygenase) and-insensitive (NO/cGMP) mechanisms, the latter of which appears inactive in male/female diabetic tissues. These findings have implication for the potential therapeutic usefulness of quercetin in the management of diabetes vascular disease.

ABSTRAK

Latar belakang & objektif: Diabetes adalah faktor risiko dalam perkembangan penyakit kardiovaskular serta lebih ketara pada golongan wanita berbanding dengan lelaki. Spesies oksigen reaktif (ROS) cetusan diabetes mampu mengubah fungsi vasokonstriktor endogen antioksidan, quercetin dan telah terbuktu berkesan pada tikus diabetik jantan tetapi belum terbukti dalam tikus betina. Hormon wanita, estradiol (antioksidan yang poten) jika berkombinasi dengan quercetin, boleh menawarkan perlindungan yang lebih berkesan terhadap kereaktifan vaskular yang diindusikan oleh diabetes/ROS. Maka, kajian ini bertujuan mengukur pengaruh jantina kepada respon aorta normoglisemik/diabetes kepada bahan vasokonstriktor dalam kehadiran/ketiadaan quercetin dan/atau estradiol. Mekanisme yang mendasari perbezaan dalam tindak balas tisu akan turut dikaji.

Bahan-bahan & Kaedah: Tindak balas ketegangan isometrik kepada kepekatan terkumpul phenylephrine atau angiotensin II (dengan atau tanpa bahan) telah direkodkan dalam aorta torasik (yang hampir sama umur-dan-seks) yang diasingkan daripada tikus Wistar Kyoto normoglisemik/streptozotocin dirawat. Peranan ROS, 17β-estradiol, enzim antioksidan, nitrit oksida (NO) dan prostaglandin (PG) yang boleh menyumbang terhadap perbezaan yang diperolehi akan turut diteroka.

Keputusan & Perbincangan: Tisu tikus jantan berendothelium-utuh normoglisemik, bertindak dengan lebih berpotensi daripada PE atau Ang II berbanding tikus betina atau jantan diabetik. Tisu normoglisemik /diabetes betina berkontraksi terhadap PE dengan lebih poten berbanding terhadap Ang II, yang kurang menghasilkan kontraksi pada aorta diabetes haiwan betina berbanding dengan tikus normoglisemik. Pemusnahan

endothelium , pra-rawatan L-NAME atau methylene blue (MB) melenyapkan perbezaan ini dan seterusnya mencadangkan bahawa laluan eNOS-sGC-cGMP menyumbangkan kepada perbezaan yang dilhat. Permusnahan endothelium, L-NAME/MB dan asetilkolin menghasilkan kesan yang kurang pada tikus jantan normoglisemik dan tisu diabetik jantan/betina berbanding dengan tikus betina yang sihat, yang mempamerkan vasorelaksan (NO/PGI_2) tisu yang lebih tinggi. Maka, aorta tikus betina yang sihat wujud dalam keadaan eNOS-SGC cGMP (basal vasorelaksan) yang lebih tinggi. Ciri ini telah dibalikkan oleh diabetes dan ini menyokong hipotesis bahawa vaskulatur haiwan betina lebih terpengaruh kepada perubahan-teraruh diabetes berbanding haiwan jantan.

Tahap penguncupan PG lebih tinggi dalam tisu normoglisemik jantan/diabetes betina. Diabetes mempromosikan sintesis relaksan PGI_2 dalam tisu jantan/betina. Keputusan ini adalah selaras dengan cerapan perbezaan gender dalam penguncupan tisu (normoglisemik jantan> betina dan betina diabetik> jantan). Sintesis penguncupan PG pada haiwan betina adalah lebih tinggi pada diabetik terus menyokong kesan negatif diabetes yang lebih ketara di kalangan haiwan betina. Sintesis vasodilator (PGI_2/EDNO) yang dipertingkatkan dalam tisu jantan(dan betina) mungkin mewakili ciri patologi diabetes jangka pendek untuk menentang peningkatan keadaan penguncupan diabetik dirangsang. Penemuan ini mempunyai implikasi untuk memahami dengan lebih lanjut perbezaan berkaitan gender dalam pada tisu vaskular akibat penyakit diabetes.

Urutan tekanan tisu oksidatif dan pengurangan kesan quercetin adalah seperti berikut: tisu jantan diabetik > betina diabetik > jantan normoglisemik > betina normoglisemik dan menyokong cadangan bahawa sebahagiannya kesan quercetin diperantarakan oleh tindakan terhadap tekanan oksidatif. 17β-estradiol dan/atau pengenduran akibat dorongan

quercetin adalah lebih ketara pada phenylephrine berbanding dengan angiotensin II-tisu diabetik jantan/betina yang dikuncupkan dengan angiotensin II, mencadangkan bahawa penggunaan quercetin/ estradiol mungkin lebih relevan secara klinikal dalam mempengaruhi reaktiviti terhadap phenylephrine berbanding dengan angiotensin II semasa diabetes. L-NAME/MB mebalikkan kesan quercetin dalam tisu jantan/betina normoglisemik (dengan atau tanpa endothelium) dan tisu diabetes jantan/betina (dengan tetapi tidak tanpa endothelium). L-NAME + Indomethacin mengurangkan kesannya dalam tikus jantan normoglisemik berendothelium-utuh dan tisu diabetes jantan/betina. Oleh itu, tindakan quercetin sebahagiannya diperantarakan oleh mekanisme yang endothelium sensitif (eNO-sGC-cGMP/cyclooxygenase) dan tidak sensitif (NO/cGMP), di mana kedua-duannya adalah tidak aktif dalam tisu diabetes jantan/betina. Penemuan ini mempunyai implikasi positif untuk kegunaan quercetin secara terapeutik dalam merawat ganguan pada sistem vaskular dalam pesakit penyakit diabetes.

LIST OF ORIGINAL COMMUNICATIONS

I. **Aloysius UI**, Achike FI, Mustafa MR. Gender difference in superoxide anion and hydrogen peroxide modulation of angiotensin II contraction in normal rat aorta, *Journal of Pharmacological Science, 2011, 115: supl. 1, pg. 209.*

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IV. **Aloysius UI**, Achike FI, Mustafa MR. Gender difference in superoxide anion and hydrogen peroxide modulation of angiotensin II-contraction in normal female rat aorta. *Journal of Endocrinology & Metabolism 2011, 2: supl. 1, pg. 2.*

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CONFERENCE PRESENTATIONS

I. **Aloysius UI**, Achike FI, Mustafa MR. Exploring Gender differences in the relaxant effect of quercetin on phenylephrine-contracted normal and diabetic rat aorta (2010). 16th (IUPHAR), World pharma, conference, Jul-2010, Copenhagen, Denmark.

II. **Aloysius UI**, Achike FI, Mustafa MR. Exploring the mechanism of quercetin gender-selective relaxant effect in phenylephrine contracted normal and diabetic rat aorta (2010). 4th Asian Society for Vascular Biology (ASVB), conference, Nov-2010, Hong Kong.

III. **Aloysius UI**, Achike FI, Mustafa MR. Gender difference in superoxide anion and hydrogen peroxide modulation of phenylephrine-induced contraction of rat aorta (2010). 24th scientific meeting of Malaysian Society of Pharmacology and Physiology (MSPP), 1st-3rd Jun-2010, SACC Convention Center, Shah Alam. Malaysia.

IV. **Aloysius UI**, Achike FI, Mustafa MR. Exploring the mechanism of quercetin in female rat aorta precontracted with phenylephrine (2010). Diabetology Asia Conference, 6th-10th, Oct-2010. Kuching Sarawak, Malaysia.

V. **Aloysius UI**, Achike FI, Mustafa MR. Quercetin exerts a gender-selective relaxant effect on phenylephrine contracted normal and diabetic rat aorta (2009). 10th International Symposium on mechanisms of vasodilatation, Jul-2009, Miyagi, Japan.

VI. **Aloysius UI**, Achike FI, Mustafa MR. Gender difference in quercetin attenuation of phenylephrine-induced vascular contraction of normal aorta (2008). 3rd scientific meeting of the Asian Society for vascular biology, Aug-2008, National University of Singapore.

VII. **Aloysius UI**, Achike FI, Mustafa MR. Effect of diabetes and gender on phenylephrine-induced vascular reactivity (2008). Diabetology Asia Conference, 26th-29th Oct-2008, Kuala Lumpur, Malaysia.

DEDICATION

Dedicated to my dearest wife Beatrice Sheila Susanne Aloysius

&

To my beloved wonderful children:

David, Ruth, Elisabeth and Hannah

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DECLARATION

No portion of the work contained in this thesis has been submitted in support of any application for any other degree or qualification in any other University or institute of learning.

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ALOYSIUS UMELO IGUEGBE

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LIST OF ABBREVIATIONS AND ACRONYMS

ACE	angiotensin-converting enzyme
ACh	acetylcholine
Ang II	angiotensin II
AT_1	angiotensin II type 1 receptor
AT_2	angiotensin II type 2 receptor
Ca^{2+}	calcium ion
$CaCl_2 \cdot 2H_2O$	calcium chloride dihydrate
CAT	catalase
cAMP	cyclic adenosine-mono-phosphate
cGMP	cyclic guanosine-mono-phosphate
COX	cyclooxygenase
CuSO4	copper II sulphate
CVD	cardiovascular disease
DG	diacylglycerol
DETCA	diethylthiocarbamic acid
DMSO	dimethyl sulfoxide
DPI	diphenylene iodonium
EDHF	endothelium-derived hyperpolarization factor
EDNO	endothelium-derived nitric oxide
EDTA	ethylenediaminetetra-acetic acid
eNOS (NOS III)	endothelial nitric oxide synthase
ET-1	endothelin-1
ET_A	endothelin receptor A
ET_B	endothelin receptor B

et al.	et alia (and others)
ER_a	estrogen receptor A
ER_b	estrogen receptor B
H_2O_2	hydrogen peroxide
g	gram
GTP	guanosine-tri-phosphate
ICAM-1	intercellular adhesion molecule-1
IDDM	insulin dependent diabetes mellitus
i.e.	*id est* (in other words)
i.p.	intraperitoneally
IP_2	inositol-di-phosphate
IP_3	inositolitri-phosphate
IL	interleukine
iNOS (NOS II)	inducible nitric oxide synthase
K^+	potassium ion
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen orthophosphate
KPSS	krebs physiological salt solution
LDL	low-density lipoprotein
L-NAME	N^{ω}-nitro-L-arginine methyl ester
µM	micromolar
M	molar
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
ml	millilitre

MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatise
mM	millimolar
mRNA	messenger ribonucleic acid
MB	methylene blue
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium hydrogen carbonate
NADH	nicotinamide adenine di-nucleotide
NA	noradrenaline
NADPH	β-nicotinamide adenine di-nucleotide phosphate
NaOH	sodium hydroxide
NIDDM	non-insulin dependent diabetes mellitus
NBT	nitro blue tetrazolium
NO	nitric oxide
nNOS (NOS I)	neuronal nitric oxide synthase
Lucigenin	N,N'dimethyl-9,9'-biacridinium dinitrate
O_2	molecular oxygen
$\bullet O_2^-$	superoxide anion
PE	phenylephrine-HCl
PG	prostaglandin
pEC_{50}	-log of median effective concentration
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2

PLC	phospholipase C
RAS	renin-angiotensin system
ROS	reactive oxygen species
SEM	standard error of mean
sGC	soluble guanyl cyclase
SNP	sodium nitroprusside
STZ	streptozotocin
SOD	superoxide dismutase
TNF	tumor necrosis factor
TXA_2	thromboxane A_2
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell
WKY	wistar-kyoto

CHAPTER 1

INTRODUCTION

1.1. Overview

Cardiovascular diseases (CVD) are the world's leading causes of premature death and permanent morbidity in both men and women (NCEP, 2002). Gender and diabetes are primary risk factors in the development of CVD. It has been shown that women on the average develop CVD 10 to 15 years later than men, and the female risk may increase after menopause due to declining effect of vasoprotective estrogens (Barrett-Conner and Bush, 1991). Under healthy physiological conditions, the vascular endothelium maintains blood flow and a balance between endothelium-derived contracting and relaxation factors (Pugley and Tabrizchi, 2000). In both hypertension and diabetes-induced vascular disease, dysfunction in renin angiotensin system (RAS) (which produces angiotensin II (a potent vasoconstrictor)) potentiates the synthesis and function of other endogenous vasoconstrictors and reactive oxygen species (ROS) (Touyz, 2004; Chu and Leung et al, 2009). Depending on the duration of vascular disease, increased ROS synthesis modifies and incapacitates the ability of the endothelium to secrete anti-atherosclerotic endothelium-derived relaxing factors (EDRF's) (such as nitric oxide (NO) and prostaglandin I_2 (PGI_2; prostacyclin) which ultimately results in endothelial dysfunction (Bayraktutan, 2002; Browne et al, 2007).

The mechanism involved in the short-term diabetes (4-12 weeks)-induced endothelial dysfunction (including altered vascular contraction) occurs earlier (and is more severe) in female than male subjects (Pinna et al, 2001), which suggests a role for gender in the pathogenesis of the disease. The mechanism of diabetes-induced endothelial dysfunction has been mostly demonstrated and not fully understood in the male gender (Ajay et al, 2006a,b; 2005; 2007; Chin et al, 2007) and may differ in the female. To

date, experimental studies on the effects of short-term diabetes on female models are sparse (Pinna et al, 2001). Since diabetes has been shown to be a stronger risk factor in the development of cardiovascular disease in the female gender than in the male (Barrett-Conner and Bush, 1991;Pinna et al, 2001), it is reasonable to suggest that more marked contractile dysfunction may occur in the vasculature of diabetic females than the male. Hence there is a need to investigate the effect of gender on short-term diabetes-induced alterations on vasocontractor function.

Furthermore, the endothelium of healthy male rats generates less endothelium-derived nitric oxide (EDNO) (Hayashi et al., 1992; Kauser and Rubanyi, 1994) and higher levels of oxidative stress factors compared to healthy females (Brandes and Mugge, 1997; Ide et al., 2002; Sartori-Valinotti et al., 2007). These gender differences are abolished by ovariectomy of the female (Barp et al, 2002), suggesting a higher protective role of the female endothelium and the female hormone, estradiol (17β-estradiol). Therefore, understanding the role of endothelium, ROS and estradiol in diabetes-induced vascular pathophysiology is important to developing correlations between experimental and clinical application.

Increasing evidence supports the use of dietary flavonoids to protect the endothelium from diabetes-induced vascular injury (Larson et al, 2010). The anti-oxidant, quercetin is ubiquitous in human diet and comprises about 60% of total dietary flavonoid consumption (Hertog et al. 1993; Larson et al, 2010). In the laboratory where the current study was undertaken, quercetin has been frequently shown to ameliorate vascular reactivity in diabetic animal models (Ajay et al, 2006a; 2007). These studies

however focused largely on male rats. Considering that the reproductive female has been identified as protective from cardiovascular disorders, and that female diabetics seem to lose much of this inherent protection (Pinna et al, 2001), there is a need to investigate possible gender differences in previously documented roles of ROS in the modulation of diabetic vasculopathy in rats. In addition, given that quercetin exerts its vasoprotective effects in diabetic male rat through the scavenging of free radicals and promotion of anti-oxidant enzyme function (Ajay et al, 2007; Sanchez et al, 2007), there is need to investigate if the vasodilator action of quercetin may differentiate between male and female tissues and if so, the possible mechanisms underlining any such difference in the action of quercetin against contractile dysfunction in the diabetic animal.

Furthermore, quercetin is the most potent anti-oxidant flavonoid, and it has been shown to preserve vascular endothelial function (Lakhanpal and Rai, 2007; Larson et al, 2010). On the other hand, compared to other sex hormones (progestins and androgens), estrogens (estriol, estrone, and estradiol) are more anti-oxidative and protective of vascular endothelial function (Czubryt et al. 2006). Given their individual vasoprotective potentials, a combined therapy of exogenous estradiol (17β-estradiol) and quercetin may hold a stronger promise in protecting the diabetic vasculature against oxidative stress and its excessive contractile effects. The findings in this study may have implications for further understanding of the gender-related differences in CVD events and the therapeutic usefulness of quercetin, estradiol or both in managing the outcomes.

1.2. Literature review

1.2.1. Vascular smooth muscle

The functionality of blood vessels is dependent on the maintenance of the structural integrity. The walls of blood vessels consist of three distinct layers of cells, namely, the intima, media, and adventitia. The intima consists of monolayer endothelial cells and connective tissue. The media layer is composed of elastin, collagen, and vascular smooth muscle (VSM) cells. The adventitia which surrounds the intima and the media is composed of strong fibrous tissue which maintains vessel structural integrity and shape (Pugley and Tabrizchi, 2000).

1.2.1.1. Vasocontraction

The contraction and relaxation of vascular smooth muscle (VSM) regulates vascular tone. Smooth muscle contraction is initiated and sustained by Ca^{2+} release from the sarcoplasmic reticulum stores, and from intracellular calcium influx from the extracellular space (Khalil and Breemen, 1995). The association of free intracellular Ca^{2+} ($[Ca^{2+}]i$) with calmodulin leads to the phosphorylation and activation of protein kinases (such as myosin light chain (MLC) kinase, Rho kinase, and mitogen activated protein kinase (MAPK)) coupled with the inhibition of myosin light chain kinase (MLC) phosphatase enzyme which degrades MLC kinase (Somlyo and Somlyo, 2000). Phosphorylated myosin cyclically binds to actin filaments resulting in force or the shortening of the smooth muscle (Webb, 2003) (Fig. 1.1). Also the interaction of vasoconstricting neurotransmitters and hormones with the G-coupled receptors on smooth muscle cell surface can initiate series of processes that promote vascular smooth muscle contraction. These receptors are activated by various types of guanosine 5-triphosphate (GTP) binding proteins (G-proteins), which are linked to different ion

channels and enzymes that modulate vasoconstriction. For example, activation of the G-coupled receptors enhances the degradation of plasma membrane phospholipids by phospholipase C (PLC), which converts inositol-di-phosphate (IP_2) to produce inositol triphosphate (IP_3) and diacylglycerol (DAG) (a promoter of protein kinase C (PKC) activity, release of $[Ca^{2+}]$i and contraction of smooth muscle (Fig. 1.1) (Webb, 2003, Orshal and Khalil, 2004). Activation of G-protein coupled receptors also promotes vasoconstriction via the inhibition of the activity of adenylate cyclase (which converts adenosine triphosphate (ATP) to cyclic adenosine-mono-phosphate (cAMP)), an inhibitor of MLCK contractile activation (Fig. 1.1). Further, stimulation of G-protein coupled receptors may also initiate contraction by promoting the depolarisation of smooth muscle cells leading to the activation of voltage-dependent Ca^{2+} channels and influx of extracellular Ca^{2+} into smooth muscle cells (Webb, 2003) (Fig. 1.1).

1.2.1.2. Vasorelaxation

On the other hand, the relaxation of smooth muscle occurs following the re-sequestering of Ca^{2+} into the sarcoplasmic reticulum by a plasma membrane Ca^{2+}pump (Hathaway et al. 1991) (and other Ca^{2+} pumps (Morel et al. 1981; Carafoli, 1991)) (Fig. 1.1). Several mechanisms are involved in the removal of $[Ca^{2+}]_{i.}$ For example, inhibition of the activities of sarcoplasmic reticulum Ca^{2+}-Mg^{2+}-adenosine 5'-triphosphate (ATP)ase activity, plasma membrane receptor-operated and voltage-operated Ca^{2+} channels reduce the mobilization of $[Ca^{2+}]_i$ and hence promotion of smooth muscle relaxation (Webb, 2003).

1.2.2. The endothelium

1.2.2.1. Physiology of vascular endothelium

The endothelium is a multifunctional organ comprising of simple monolayer of cells separating circulating blood from the vascular smooth muscle. Vascular endothelium plays an important role in the regulation of vascular tone, maintenance of blood fluidity and homeostasis. To maintain vascular homeostasis, the endothelium synthesizes several vasoactive substances, including the vasodilators, NO, PGI_2, endothelium-derived hyperpolarizing factors (EDHF's) and vasoconstrictors such as angiotensin II and endothelin-1 (Bayrantutan, 2002). A healthy endothelium maintains a balance in the function of vasodilator, vasoconstrictor, platelet aggregation, leukocyte adhesion and vascular smooth muscle growth factors (Cooke, 2000).

1.2.2.2. Pathophysiology of diabetes-induced endothelial dysfunction

Diabetes mellitus is a metabolic disorder characterized by high blood glucose levels resulting from defective insulin secretion and/or resistance to insulin action (Pazdro and Burgess, 2010). Diabetes mellitus is a crucial public health concern affecting approximately 100 million persons worldwide, and this number is expected to rise to 300 million by 2025 (Amos et al. 1997). Of the total diabetes population, 5-10% has insulin-dependent diabetes mellitus (IDDM, type 1 diabetes) and 90-95% has non–insulin-dependent diabetes mellitus (NIDDM, type 2). Type 1 or IDDM, which is caused by a combination of hereditary and environmental factors, is the most prevalent type of diabetes arising from the auto-immune mediated destruction of pancreatic β cells which reduce insulin bioavailability (Schwarz et al. 2009). NIDDM or type 2 diabetes which is characterized by insulin insensitivity coexists with hypertension and dyslipedimia (Schwarz et al. 2009). The incidence of type 2 diabetes is growing rapidly

due to increasing obesity, sedentary lifestyle patterns and unhealthy dietary habits (Fontana, 2009). In humans and experimental animals, both forms of diabetes are associated with endothelial dysfunction, which is characterized by the impairment of the vital functions of the endothelium, including the anti-inflammatory, anti-proliferative, anti-thrombic and vasodilatory properties (De Vriese et al. 2000; Bayrantutan, 2002) Further, increased synthesis of cyclooxygenase pathway-dependent vasoconstrictors, excessive synthesis or diminished destruction of ROS, and dysregulation in the gene encoding endothelial nitric oxide synthase (eNOS) (Zanetti, et al. 2000) have been proposed to contribute to the pathogenesis of diabetes endothelial dysfunction. This diabetes-induced derangement in vascular homeostasis leads to micro vascular (including nephropathy and retinopathy) and macro vascular (such as atherosclerotic cardiovascular disease: coronary artery disease, cerebrovascular disease and peripheral vascular disease) complications, the principal cause of death and disability in patients with diabetes (Cohen, 2005).

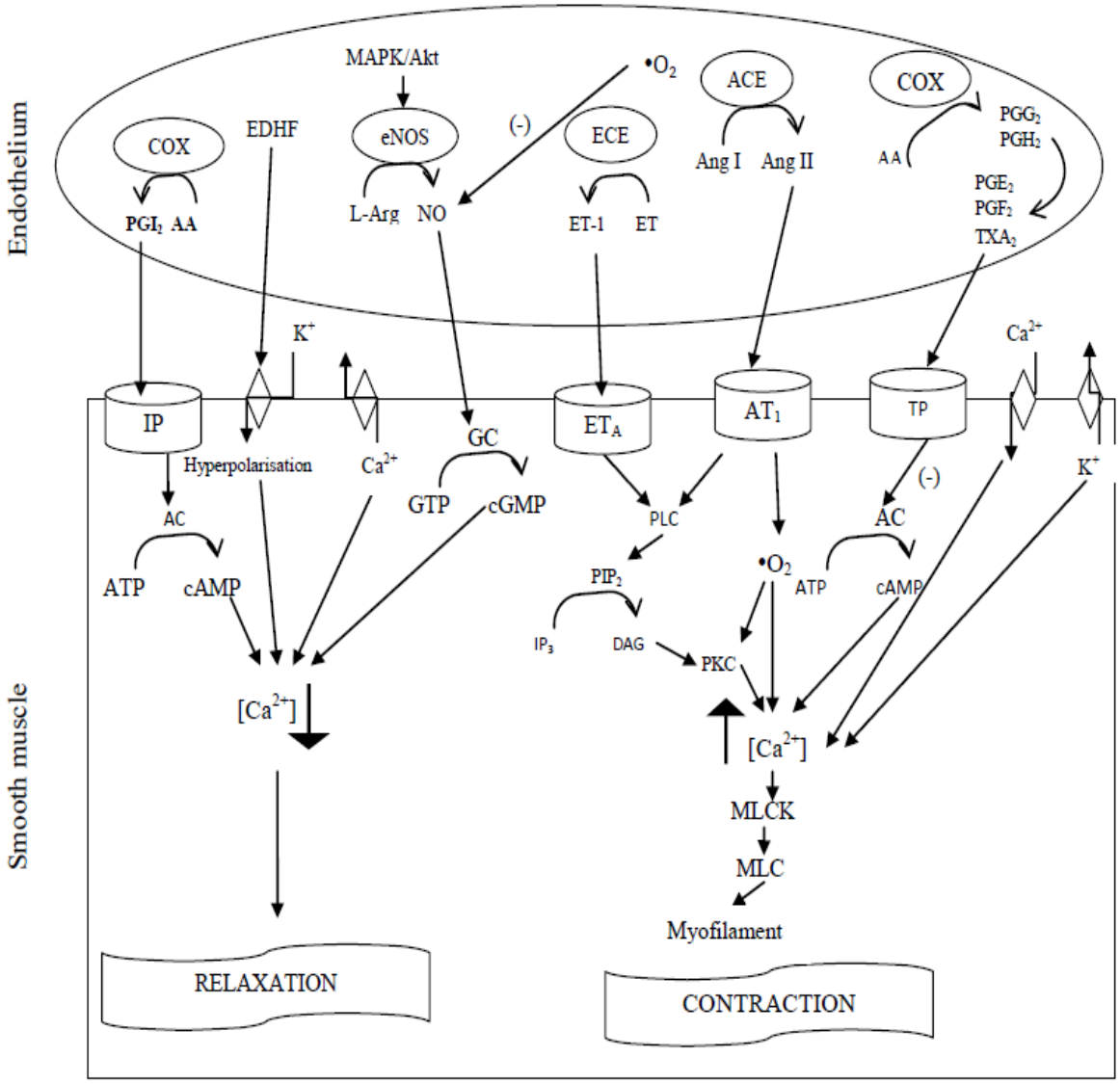

Figure1.1: Physiological and pathological stimuli involved in the mediation of vascular smooth muscle contraction or relaxation. Angiotensin converting enzyme (ACE), angiotensin I (Ang I), angiotensin II (Ang II), angiotensin II type I receptor (AT_1), adenosine triphosphate (ATP), cyclic adenosine 3',5'-monophosphate (cAMP), cyclic guanosine 3',5'-monophosphate (cGMP), cyclooxygenase (COX), endothelin-converting enzyme (ECE), endothelium-derived hyperpolarizing factor (EDHF), endothelin (ET), endothelin-1 (ET-1), endothelin-1 receptor A (ET_A), guanylate cyclase (GC), adenlate cyclase (AC), prostaglandin I_2 receptor (IP), inositol triphosphate (IP_3), mitogen-activated protein kinase (MAPK/Akt) pathway, L-arginine (L-Arg), nitric oxide (NO), endothelial nitric oxide synthase (eNOS), superoxide anion ($\bullet O_2^-$), phospholipase C (PLC), diacylglycerol (DAG), prostaglandin (PG), prostacyclin (PGI_2), thromboxane A_2 receptors (TP), thromboxane A_2 (TXA_2), cyclooxygenase (COX), channels, intracellular calcium $[Ca^{2+}]i$, myosin light chain kinase (MLCK), myosin light chain (MLC). Symbols: (-) (inhibition), ◊ (potassium (K^+) or Ca^{2+} ion channels). Arrows: ↑ **(i**ncrease), ↓ **(**decrease) in synthesis, → (activation).

1.2.3. Endogenous vasodilators

1.2.3.1 Endogenous vasodilator function in normal and diabetic states

A number of endothelium-derived factors play a vital role in endothelium-dependent vascular relaxation including, nitric oxide (NO), vasodilator prostaglandins (PGI_2), bradykinin and hyperpolarizing factors.

1.2.3.2 Acetylcholine

The neurotransmitter, acetylcholine (ACh) is produced endogenously at cholinergic synapses and neuroeffector junctions in the central and peripheral nervous system. It dilates vascular beds by stimulation of muscarinic receptor (M_3 subtype) located on the endothelial cells of the vessel wall despite poor cholinergic innervations of most arterial beds (Caulfield and Birdsall, 1998). Stimulation of the muscarinic receptors causes the endothelial cells to release several endothelium-derived factors, the most prominent of which is NO, PGI_2, and EDHF (Busse and Fleming, 1993). Clinical and animal models of diabetes are associated with impaired endothelium-dependent relaxation (i.e. endothelial dysfunction) as a result of diabetes-induced ROS-enhanced reduction in the synthesis and/or bioavailability of NO and deficiency of anti-oxidant enzymes (such as superoxide dismutase) (Gewaltig and Kojda, 2002). In early stage diabetes (4-12 weeks), endothelium-dependent ACh-induced vasodilation is enhanced, whereas at latter stages, vasodilatation is diminished (Pieper, 1999).

1.2.3.3. Nitric oxide

Nitric oxide (NO) is a highly reactive (free radical) gaseous compound which plays a critical role in the regulation of vascular tone. Its discovery as an endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980) and subsequent identification as NO (Ignarro et al. 1987), elicited enormous research interest. NO is synthesized from the endothelial cells by NO synthases (NOS) conversion of L-arginine to L-citrulline (Fig. 1.1) in response to physiological stimuli such as ACh, bradykinin, histamine, thrombin, ADP, ATP, substance P, oxidative stress and shear stress (Stuehr, 1999). NOS exists in three isoforms (neuronal nNOS (NOS I), inducible iNOS (NOS II), and endothelial eNOS (NOS III). iNOS which is Ca^{2+} independent is involved in the long-term regulation of vascular tone, whereas eNOS-Ca^{2+} dependent isoform plays a role in the short-term regulation of vascular tone (Orshal and Khalil, 2004). Once synthesized from the endothelium, the binding of NO to the heme moiety of soluble guanylate cyclase (sGC), increases the production of intracellular cyclic 3'-5'-guanosine monophosphate (cGMP) (Fig. 1.1). cGMP activates the cGMP-dependent protein kinase (PKG) which down regulates key pathways involved in Ca^{2+} homeostasis, resulting in reduced $[Ca^{2+}]i$ and sensitivity of contractile proteins to Ca^{2+}, thus promoting smooth muscle relaxation (Lincoln et al. 2001). Therefore, stimulation of NO counterbalances the direct endogenous vasoconstrictor effects of noradrenaline, serotonin, angiotensin II and endothelin on the vascular smooth muscle and its inhibition leads to significant peripheral vasoconstriction and elevation of blood pressure (Tresham et al. 1991). NO may also produce vasorelaxation by hyperpolarizing vascular smooth muscle which directly activates Ca^{2+}-activated K^+ channels (Kca) and Na^+-K^+ ATPase activity (Cohen and Vanhoutte, 1995).

Due to its anti-proliferative, anti-platelet aggregation and inhibitory effects on leukocyte adhesion, in blood vessels, NO plays an important role in the pathogenesis of diabetes (Bayrantutan, 2002). For example, in diabetes the gene encoding for eNOS (the main source of NO within the vascular endothelium) is impaired in experimental animals (Lund et al. 2000; Zanetti et al. 2000), hyperglycemic endothelial cells (Chakravarthy, 1998) and in patients with diabetes and hypertension (Endemann and Schiffrin, 2004).

1.2.3.4. Vasodilator prostaglandins

Prostaglandin (PGI_2) is the major vasodilatory PG produced by vascular endothelial cells. It is released from arachidonic acid by endothelium based COX-2 enzyme in response to shear stress, hypoxia, or to NO agonists (Fitz-Gerald and Patrono, 2001). PGI_2 induces relaxation of vascular smooth muscle by activating adenylate cyclase leading to increased production of cyclic adenosine monophosphate (cAMP) and hence reduction of $[Ca^{2+}]i$ and contraction (Fig. 1.1) (Cohen and Vanhuette 1995). PGI_2 may be involved in the pathogenesis of diabetic vascular disease. For example, diminished PGI_2 release in response to adrenaline has been observed in the aorta of STZ diabetic rats (Jeremy et al. 1993) and hence, the blockade of COX enzyme activity has been shown to potentiate endothelium-mediated vasodilatation in both hypertensive and diabetic rat arteries (Fitz-Gerald and Patrono, 2001). It has also been suggested that the diabetic state may cause an imbalance in endothelial production of eNOS and/or COX-derived PG factors and/or a defect in the eNOS-PG mediated cross-talk, all of which tends to make the vessel wall vasoconstrictive (Browne et al. 2007).

1.2.3.5. Bradykinin

The 9 amino acid peptide, bradykinin is synthesized from Kininogens from kallikrein-kinin system (KKS) located in both plasma and vascular tissues (Babe et al. 1996). Bradykinin primarily mediates the synthesis of blood clotting as well as vasodilator factors from the vascular endothelium. Bradykinin promotes vasodilatation by promoting the synthesis and the release of NO, PGI_2, EDHF and adenine nucleotides (ADP, ATP) via two of its kinin receptors, (B_1 and B_2). Stimulation of the same receptors also inhibits platelet adhesion and smooth muscle cell proliferation (Linz et al. 1999). There is growing evidence to suggest bradykinin-renin angiotensin cross talk may play a vital role in regulation of blood pressure and endothelial function (Hornig et al. 1997). For example, inhibition of angiotensin converting enzyme (ACE) is associated with increased levels of bradykinin, angoitensin (1-7), NO and decrease in levels of angiotensin II in normotensive and hypertensive blood vessels (Lima et al. 1997; Gainer et al 1998). Since diabetes promotes the activation of angiotensin II (Chu and leung, 2009), the bradykinin-renin angiotensin system may be involved in the pathogenesis of the disease.

1.2.3.6. Endothelium-derived hyperpolarizing factor

The role of endothelium-derived hyperpolarizing factor (EDHF) in modulating vascular tone remains unclear. EDHF may originate from cytochrome P450-derived arachidonic acid epoxides (Archer et al. 2003, Gauthier et al. 2005) and their contribution to endothelium-mediated relaxation is believed to be independent of NOS and COX and may vary with vascular beds (being more pronounced in smaller compared to larger arteries) (Feletou and Vanhoutte, 1999; McGuire et al. 2001). EDHF elicits vascular relaxation by opening of Ca^{2+}-regulated K^+ channels (BKCa) in the VSM cell

membranes which promote the sequestration and mopping up of free [Ca^{+2}]i (Feletou & Vanhoutte, 1999; Mc. Guire et al 2001). They may also promote vasodilatation via the activation of ATP-sensitive K^+ channels (K_{ATP}) and Na^+, K^+-ATPase, but Ca^{2+}-activated K^+ channels account more for its vasorelaxant effects (Mc. Guire et al 2001). EDHF-mediated vasodilatation becomes more pronounced in conditions of endothelial dysfunction (where NO bioavailability is impaired), an indication that EDHF may compensate for diminished endothelium-dependent vasorelaxation in the diabetic state (Taddei et al. 2001).

1.2.4. Endogenous vasoconstrictors

1.2.4.1. Endogenous vasoconstrictor function in normal and diabetic states

The diabetic blood vessel is not only characterized by altered vasodilator signaling but also by abnormal synthesis and function of endogenous vasoconstrictor factors, such as angiotensin II, noradrenaline, vasopressin, and 5-hydroxytryptamine, and contractile prostaglandins (De Vriese et al. 2000; Golovchenko et al. 2000). Diabetes-induced hyperglycemia coupled with excessive generation of ROS modifies the function of anti-atherosclerotic EDRFs such as NO. Depending on the duration of the disease, ROS ultimately impair vasoconstrictor function (Pieper, 1999). However, the extent to which vasoconstrictor function is modified by the diabetic state is inconsistent with some studies reporting attenuation (Myers and Messina, 1996; Misurski et al. 2001), enhancement (Abebe et al. 1990; Dresner et al. 1997) or unchanged contractile responses (Mulhern and Docherty, 1989; Chang and Stevens, 1992). Differences in variations in the mobilization of the L-arginine nitric oxide and/or cyclooxygenase pathway (Pieper, 1998; Browne et al. 2007) under the various experimental conditions (sex, age, and strain of the animal and type of vessels studied, diabetogenic agent

employed and duration of diabetes) have been suggested to contribute to these discrepancies (Bell and Hye, 1983; Pieper, 1999).

1.2.4.2. Angiotensin II

The renin-angiotensin system (RAS) is localized in vascular endothelial and smooth muscle cells. Angiotensin II (Ang II), the main effector hormone of the renin-angiotensin system (RAS), is an important mediator of cardiovascular disease including hypertension, myocardial infarction, stroke, renal failure, and diabetic vascular complications. Its physiological function is mediated mainly by four receptor subtypes AT_1 to AT_4 (Dzau et al. 2001). Its effects via AT_1 receptor include vasoconstriction, renal salt retention, and aldosterone and vasopressin release. It induces VSM cell contraction via the G-protein-coupled receptor signaling cascade (Fig. 1.1) and may augment the same by directly or indirectly potentiating the production and effect of other vasoconstrictors. It may also promote vascular remodeling (endothelial growth, apoptosis, inflammation, fibrosis and thrombosis) via the same receptor (Palatini, 2001). The AT_2 receptor stimulation (antagonizes/) blocks the effects of Ang II via AT_1 receptor (Unger, 2000). The function of AT_3 receptor is unclear but the AT_4 receptor may protect vascular integrity by stimulating endothelial release of plasminogen activator inhibitor-1 (the principal inhibitor of plasminogen and hence promote fibrinolysis (Unger, 2000; Palatini, 2001). In diabetes, Ang II- mediated effects via AT_1 receptor promotes excessive ROS generation, and increases the level and activation of other vasoconstrictors, thereby accelerating vascular remodeling of the endothelium (Touyz, 2004).

1.2.4.3. Endothelin-1

The peptides, endothelins (ETs), including ET-1, ET-2, and ET-3, are localized in VSM and endothelial cells and they possess potent and sustained vasoconstrictor properties (Schiffrin, 2001). Ang II and other physiological stimuli (including adrenaline, cytokines, free radical, and physical factors such as stretch, hypoxia, and low shear stress) stimulate the production and release of ET-1 (the predominant member of the ET family) from VSM and endothelial cells (Schiffrin, 2001). ET-1 contracts VSM cells and cardiomyocytes via ET_A G-protein-coupled receptor subtype resulting in sustained vasoconstriction. ET-1 may also potentiate the vasocontractile response of other vasoconstrictors such as norepinephrine (noradrenaline), and contractile prostaglandins. Its vascular effects via ET_B receptor oppose the effects of ET_A receptor. ET-1 is proinflammatory and promotes vascular smooth muscle cell growth, suggesting that it may be particularly relevant to the pathophysiology of vascular disease in diabetes (Creager et al. 2003). In support of this hypothesis, endogenous ET-1 mediated vasoconstriction is impaired in animal models of diabetes (Mcauley et al. 2000) and in patients with type II diabetes (Nugent et al. 1996).

1.2.4.4. Noradrenaline

Noradrenaline (NA) or its analogue phenylephrine is a contractile agent of choice in the study of endothelial- dependent and -independent vascular relaxation responses. NA produces smooth muscle cell contraction by stimulating a_1-adrenoceptors, which results in the activation of PLC-G-protein-coupled receptor cascade (Fig. 1.1) and the promotion of vascular smooth muscle contraction. NA may additionally enhance vasoconstriction by depolarizing arterial smooth muscle cells consequently opening the

voltage-operated Ca^{2+}channels in the plasma membrane of the smooth muscle, leading to influx of [Ca^{2+}]i and sustained Ca^{2+}-dependent contraction (Nelson et al. 1990). Impaired vasocontractile response to noradrenaline or phenylephrine has been reported in experimental diabetes (Pinna et al. 2001; Sanz et al. 2003).

1.2.4.5. Serotonin (5-hydroxytryptamine)

Serotonin (5-hydroxytryptamine, 5HT) is a monoamine neurotransmitter primarily found in the gastrointestinal tract, platelets and in several parts of the central and peripheral nervous systems. Circulating serotonin originates in the gastrointestinal tract, where it overflows into blood and taken up by platelets. Serotonin released from activated platelets binds to its receptors on vascular smooth muscle cells and promotes G-protein- mediated contraction (Fig. 1.1) (Vanhoutte, 1990). In most blood vessels, this platelet (serotonin)-induced vasoconstriction is preventable with 5-HT_2-serotonergic antagonists such as ketanserin and naftidrofuryl (Vanhoutte, 1990). In healthy blood vessels, serotonin induces the endothelial cell synthesis of NO which may oppose platelet aggregation and its contractile stimulation (Vanhoutte, 1990). Serotonin is involved in the pathogenesis of coronary artery disease (Ichikawa et al. 1989), and diabetes- induced neuropathy (Sandrini et al. 1997), retinopathy (Pietraszek et al. 1992) and vasculaopathy (Hagen et al, 1985).

1.2.4.6. Potassium and calcium ions

Increase in extracellular potassium chloride (KCl) promotes vascular contraction by inhibiting Na^+, K^+-ATPase, which promotes Na^+ entry (into smooth muscle cells), membrane depolarization, influx of voltage-dependent Ca^{2+} ions and hence vasoconstriction (Karaki & Weiss, 1988). KCl-induced VSM cell depolarization liberates endogenous noradrenaline from vascular sympathetic nerve endings, which also augments contractile response (Fouda et al. 1991). Diabetes reduces Ca^{2+} and K^+-induced contractions of rat aorta suggesting diminished activity of Ca^{2+} channels and/or reduced levels of calmodulin in diabetic tissue (Ozturk, et al. 1994), although this was not corroborated by another study (Fulton et al. 1991).

1.2.4.7. Contractile prostaglandins

Endothelial cells produce contractile prostaglandins (PGs) from phosholipase A_2 – mediated metabolism of arachidonic acid. The enzyme cyclooxygenase (COX) converts arachidonic acid into PGG_2 and then into PGH_2. PGH_2 is then metabolized (by specific isomerases) into several contractile factors such as PGF_2, PGE_2, and TXA_2 (Fig. 1.1) (the most potent of contractile PGs) (Cohen and Vanhoutte, 1995; Feletou et al. 2010). Vasoconstrictor PGs endothelium-dependent contractions are mediated by thromboxane prostanoid (TP) receptors underlying vascular smooth muscle (Fig. 1.1). In addition, stimulation of TP receptors also promotes vascular remodeling (Feletou et al. 2010). Diabetes-induced hyperglycemia may promote the activity of COX-derived vasoconstrictor prostaglandins. For example, in cultured human aortic endothelial cells, hyperglycemic conditions was shown to up-regulate the mRNA expression/protein levels of COX-2 (enzyme involved in the synthesis of PGE_2 (Muscara et al. 2000) but not COX-1 (the enzyme involved in the synthesis of TXA_2) (Wallace et al. 1999). In

addition, diabetes induced an increase in levels of endothelium-derived vasoconstrictor prostaglandins which in turn augmented Ang II vascular effects (including vascular inflammation and thrombosis) in rabbit aorta (Tesfamariam et al. 1990). Hence, TP receptor antagonists may be useful in ameliorating contractile dysfunction, vascular inflammation and thrombosis (Feletou et al. 2010).

1.2.4.8. Influence of gender on vascular contraction in normal and diabetic states

Contractile effects of endogenous vasoconstrictors are regulated by gender. For example, it has been shown that VSM cell of males have more AT_1 receptors and ACE activity compared to those of reproductive females, an explanation for why the pressor effects of Ang II is more pronounced in intact male arteries than the female (Xue et al. 2007). Likewise, vascular contraction in response to catecholamines (nor noradrenaline and adrenalin) (Li and Duckles, 1994; Kneale et al. 2000) or to the sympathetic co-transmitter, neuropeptide Y (Zukowska-Grojec, 1998) is greater in male than the female gender. These sex-dependent differences in responses to contractile agonists have been associated with direct action of sex hormones (i.e. estradiol, progestins and androgens) on steroid receptors in endothelial and smooth muscle cells. For example, Stallone et al. 1991, and Crews & Khalil, 1999, demonstrated that the gender difference in the reactivity of isolated aortic ring to nor noradrenaline or adrenalin is abolished by ovarietomy of the female, suggesting a direct role for the female hormone, estradiol (17β-estradiol). On the other hand, the same authors found no difference in contraction between the castrated and intact male aorta but reported a significant elevation in the contraction of ovariectomized (OVX) females compared with intact females (Stallone et al. 1991), indicating that gender differences in vascular tone may be more likely related

to estrogens than to androgens. Also, intact females and OVX female animals implanted with estradiol exhibited reduced Ca^{2+} entry, $[Ca^{2+}]i$ and vascular contraction compared with males or OVX females without it (Crews and Khalil, 1999; Murphy and Khalil, 2000). Taken together, these findings suggest that sex differences in vascular tone are a direct consequence of the vascular effects of sex hormones. Since diabetes disrupts aromatase activity (Kim et al. 2006), the alterations in the function of these sex hormones may have further implications for understanding the role of gender in regulating vascular injury.

1.2.5. Steroid sex hormones

Sex hormones (androgens, progestins and estrogens) interact with cytosolic and nuclear receptors on vascular endothelial and smooth muscle cells to stimulate a host of genomic and non genomic effects that modulate vascular function. Growing research interest in the beneficial role of hormone replacement therapy (HRT) in reducing the incidence of cardiovascular disease and risk associated with it, has been the subject of many reviews (Gerhard and Ganz, et al. 1995; Palin et al. 2001).

1.2.5.1. Androgens

Androgen (testosterone, dihydrotestosterone and androstenedione) receptors are localized in endothelial and vascular smooth muscle cells in both genders but their levels are fourteen-fold higher in males than in females (Czubryt et al. 2006). The effects of androgens on vascular function are contradictory. For example, androgens (testosterone) may enhance vascular contraction (by inhibiting endothelium-dependent or independent relaxation) (Wynne and Khalil, 2003) as well as relaxation of arteries isolated from experimental animals (Costarella et al. 1996). However, the latter may not

be physiologically relevant, since it is achieved at supraphysiological concentrations; is endothelium-independent and more pronounced with structurally modified analogs (Honda et al. 1991).

1.2.5.2. Progestin

Progestin (progesterone) is produced more in females than males and is sixty-fold higher in premenopausal women than in men and post-menopausal women (Czubryt et al. 2006). Its effects on vascular reactivity are contradictory, ranging between no effect, inhibition or enhancement of relaxation (Thompson and Khalil, 2003). However, in laboratory animals, a number of studies support a vasodilatory role for progesterone via the promotion of NO and/or PGI_2 (Orshal and Khalil, 2004), but compared to estrogens, progesterone-induced vasorelaxation is smaller (Crews and Khalil, 1999). Further, progesterone may augment (Nickenig et al. 2000) or oppose the cardiovascular effects of estrogens (Wassmann et al. 2005)

1.2.5.3. Estrogens

Estrogens (17β-estradiol, estrone and estriol) are generated in both males and females, but are seven-fold higher in premenopausal women than in men and post-menopausal women (Czubryt et al. 2006). Compared to other gonadal sex hormones (progestins and androgens), the effects of estrogens on vascular function has been mostly studied owing to its beneficial potential in the treatment of cardiovascular complications in menopausal women (Gerhard and Ganz, et al. 1995; Palin et al. 2001). The vascular effects of estrogens are mediated via two estrogen receptors (ER) (ER_a and ER_β) located on endothelial and VSM cells. ER_β receptor is predominantly expressed in human VSM cells, particularly in women where it mediates direct vascular effects of estrogen. ER_a

receptor sub type plays an important role in regulating VSM cell differentiation and proliferation (Montague et al. 2006). The mechanisms by which estrogens exert their cardiovascular protective effects are not completely understood, but suggested mechanisms include their ability to modulate endothelial nitric oxide synthase (eNOS) expression and synthesis of NO via genomic and non genomic pathways. In the genomic pathway, estrogens interact with their receptors leading to a range of (delayed) effects involving the activation of MAPK/Akt-eNOS dependent transduction pathway (Geraldes et al. 2002) (Fig. 1.1). In the non-genomic (rapid onset) pathway, the binding of estrogens to endothelial surface membranes stimulate the phosphorylation of MARK/Akt pathway, thereby promoting the eNOS-mediated synthesis of vasorelaxant NO. In addition, estrogens induce direct and rapid non-genomic (endothelium-independent) effects via the VSM plasmalemmal receptors (Crews and Khalil, 1999; Orshal and Khalil, 2004) or estrogen receptors localized in the caveolae of endothelial cells (Wakeling et al. 2001).

In addition to stimulating NO synthesis, estrogens up-regulate COX-1 expression and promote the production of vasodilator COX products, such as PGI_2 (Geary et al. 2000; Sherman et al. 2002). The vasodilator effects of estrogens may also derive from direct inhibition of contractile COX-products, Ca^{2+} entry into VSM cells (Murphy and Khalil, 2000) and Ang II-induced vascular effects (including NADPH oxidase generation of $\bullet O_2^-$, VSM cell proliferation, migration, suppression and inflammation (Miller et al. 2007)). Due to the presence of a phenolic ring in their structure (which is absent in other sex hormones), estrogens are potent anti-oxidants (Ruiz-Larrea et al. 2000; Czubryt et al. 2006). For example, compared to intact female, OVX female rats are associated with lower anti-oxidant activity, reduced thiol groups, and increased plasma

lipoperoxides and vascular free radicals (Strehlow et al. 2003; Florian et al. 2004), all which are prevented by estrogen replacement (Hernandez et al. 2000; Strehlow et al. 2003).

1.2.5.4. Phases of the female reproductive cycle

Vascular tone may be influenced by hormonal changes occurring during the female reproductive cycle. For example, the vaginal epithelium in women and female rats are responsive to estrogens (17β-estradiol). Reproductive women and female rats undergo predictable phases of successive reproductive cycles (lasting between 21 - 35 days in women (Stenchever, 2001) and 4-5 days in rats (Marcondes et al. 2002). In women, the reproductive cycle consists of two (follicular and the luteal) phases (Stenchever, 2001)), and four (proestrus, estrus, metestrus, and diestrus) phases in rats (Marcondes et al. 2002). In the beginning of the reproductive cycle, both the plasma levels of estradiol and progesterone hormones are low. Estradiol rises in the middle of the follicular phase (in women or proestrus rats (Stenchever, 2001) leading to increased secretion of pituitary luteinizing hormone (LH) and follicular stimulating hormone (FSH) which results in ovulation / fall in estradiol levels. Progesterone levels start to rise in the beginning of the post ovulation phase (luteal phase in women (Stenchever, 2001) or early diestrus rats (Sportnitz et al. 1999), returning to baseline along with estradiol in late luteal in women or estrus rats (Stenchever, 2001; Marcondes et al. 2002).

1.2.5.5. Effect of reproductive cycle phases on vascular function in normal and diabetic state

Fluctuations in the plasma levels of female sex hormones during the different phases of reproductive cycle may or may not modulate vascular function. For example, aortic strips in estradiol rich proestrus rats are more hypo responsive to noradrenaline than estradiol deficient dietrus/metestrus rats (Zamorano et al. 1994). On the contrary, estradiol-induced vasodilatation of tail and mesenteric arteries of female rats were reported to be higher in proestrus compared to non proestrus rats (Kakucs et al. 2001). Further, arteries taken from cycling female rats (in either proestrus, estrus, metestrus, or diestrus) did not differ significantly in contractile response to either noradrenaline (Li et al. 1997) or to vasocostrictive prostanoids (Sanz et al. 2003) but differed in the effect and release of vasopressin (Stone and Crofton, 1989). These findings suggest that hormonal variation during the female reproductive cycle regulate vascular reactivity and hence should be taken into account in the assessment of the cardiovascular function in the female gender. Diabetes negatively modulates hypothalamus-pituitary axis and aromatase activity (Kim et al. 2006) leading to the disruption of reproductive cycle in women (Strotmeyer et al. 2003) and rats (Kim et al. 2006). This in turn may contribute to impairment of vascular function (Kim et al. 2006). Therefore, understanding the influence of diabetes on vascular function during different phases of the reproductive cycle may contribute further to the understanding of the mechanism of the disease in the female gender.

1.2.6. Reactive radicals

1.2.6.1. Reactive oxygen and nitrogen radicals

Under physiologic conditions, reactive oxygen species are formed as intermediates in redox processes involving the formation of water from oxygen (Brand, 2010). Molecular oxygen (O_2) undergoes a one electron reduction (by several different systems including NADPH oxidase, xanthine oxidase, cyclooxygenase, mitochondria electron transport chain and nitric oxide synthase) to yield superoxide anion ($\bullet O_2^-$). $\bullet O_2^-$ is then dismutated to hydrogen peroxide (H_2O_2) by the enzyme, superoxide dismutase (SOD) and to •OH by the Fe^{2+} thioredoxin or to H_2O and O_2 by catalase/ glutathione peroxidise (GPx) (Schafer and Buettner, 2001). When generated in excess, $\bullet O_2^-$ may interact with reactive nitrogen species such as nitric oxide (NO) to produce peroxinitrite ($ONOO^-$) (Darley-Usmar, et al, 1995). In a normal vasculature, a balance between the rate of production and elimination of these pro-oxidants ($\bullet O_2^-$, H_2O_2, •OH, NO, $ONOO^-$), is tightly regulated by anti-oxidant enzymes (such as SOD, catalase, thioredoxin, GPx), anti-oxidant vitamins, and other small molecules (Halliwell, 1999). Under pathological states, disequilibrium between the generation of these pro-oxidant molecules and anti-oxidant protection occurs resulting in the increased bioavailability of oxidant factors, which promotes a state of oxidative stress (a major cause of vascular injury in cardiovascular diseases) (Landmesser and Harrison, 2006).

1.2.6.2. The role of hyperglycemia-induced reactive free radicals

Increased oxidative stress as a result of hyperglycemia-induced oxidation of glucose, lipids and proteins is implicated in the onset and late stages of both insulin dependent and non-insulin independent diabetes (Etoh et al. 2003). In human cells and animal models of diabetes, both acute and chronic hyperglycemia promotes endothelial

dysfunction and hyper production of reactive oxygen species (ROS) (Johansen et al. 2005). The mechanisms through which diabetes-induced hyperglycemia increases the levels of oxygen radicals are not entirely clear but may involve a number of related biochemical pathways (including, glucose auto oxidation, activation of polyol (aldose-reductase)-sorbitol, protein kinase C (PKC) and hexamine pathways and the formation of advanced glycation end products, all of which aggravate vascular injury and dysregulation in vascular tone (Johansen et al. 2005).

1.2.6.3. Activation of polyol (sorbitol) pathway

In hyperglycemia, the oxidative metabolism of glucose via the polyol pathway results in excessive production of sorbitol and $\bullet O_2^-$ (Lorenzi, 2007). Increased polyol production of $\bullet O_2^-$ and other ROS, promotes the activation of NADPH oxidase (which accounts for most ROS generated in the vessel wall), xanthine oxidase, mitochondrial electron transport and NOS systems (Guzik et al. 2002; Aliciguzel et al. 2003). ROS from these free radical systems interact with •NO to generate the highly cytotoxic $ONOO^-$ which inhibits •NO function, blocks K^+ channels, promotes lipid peroxidation / •OH-induced apoptosis of myocytes, endothelial cells and fibroblasts (Maritim et al. 2003; Zou et al. 2004). All of these tend to make the vessel wall vasoconstrictive. NADPH oxidase-enhanced generation of ONOO- may also promote vasoconstriction by inducing a deficiency in arginine or tetrahydrobiopterin (BH_4) (an important cofactor for the synthesis of NO from L-arginine), which uncouples NOS (eNOS and /or iNOS) enzyme from preferentially causing it to produce $\bullet O_2^-$ instead of •NO (Zou et al, 2004). Furthermore, in diabetes, NADPH oxidase-generated ROS can augment vasomotor tone directly by activating $[Ca^{2+}]i$ and/or indirectly promoting it via the activation of the renin angiotensin (RAS) signaling cascade (Touyz et al. 2004) (Fig. 1.1). The

involvement of polyol pathway in the pathogenesis of diabetes is evidenced in the use of aldose reductase (which mediates sorbitol formation) inhibitors such as sorbinil to inhibit diabetes-induced lipid peroxidation in STZ-diabetic rats (Obrosova et al. 2002).

1.2.6.4. Depletion of anti-oxidant enzymes

Utilization of the polyol pathway for glucose metabolism during diabetes enhances $\bullet O_2^-$ synthesis at the expense of NADPH (a co-enzyme essential for the regeneration of anti-oxidant molecules such as, reduced glutathione (GSSG), ascorbate and tocopherol). Reductions in the levels of NADPH thus modifies the redox status of cells by decreasing NADPH/NADP+ and GSH/GSSG ratios thereby increasing susceptibility to intracellular oxidative stress (increased $\bullet O_2^-$ synthesis, increased inactivation coupled with decreased levels of anti-oxidant enzymes) (Michael, 2005). Hence, anti-oxidant therapy has been advocated for the management of oxidative stress-induced injury during diabetes. For example, diabetes-induced reductions in the levels of cytosolic Cu-Zn-SOD, extracellular (EC) SOD, and/or mitochondria Mn-SOD is normalized by treatment with exogenous SOD in humans (Atalay and Laaksonen, 2002) and/or with catalase in experimental animals (Kurzelewski et al. 2005). Also, the use of aldose reductase inhibitors to block the polyol synthesis of sorbitol and hence ROS, has been shown to restore reduced glutathione and ascorbate levels in experimental diabetes (Obrosova et al. 2002).

1.2.6.5. Activation of advanced glycation end products

Hyperglycemia-induced autoxidation of proteins generate advanced glycation end products (AGEs) which in turn produce $\bullet O_2^-$ which further quenches the bioavailability of NO, accelerates the oxidation of low density lipoproteins (LDL), the synthesis of inflammatory cytokines interleukin-1 (IL-1), tumor-necrosis factor-α (TNFα), adhesion molecules (VCAMs and ICAMs), as well as endothelial growth factors (Michael, 2005). Pharmacologic inhibition of AGEs prevents late structural changes in experimental diabetic retinopathy (Hammes et al. 1991).

1.2.6.6. Activation of protein kinase C

Hyperglycemia promotion of vascular oxidative stress also increases the synthesis and activation of the lipid second messenger DAG leading to the membrane translocation and activation of PKC (Nishikawa et al. 2000). Further, PKC quenches NO function by augmenting ROS levels and promoting the release of $[Ca^{2+}]i$ and hence the synthesis / activation of contractile factors (endothelin, contractile prostaglandins) (Fig. 1.1). Thus, the PKC-mediated aggravation of vascular oxidative stress may in turn promote endothelial cell growth, smooth muscle cell migration, proliferation and thrombosis (Michael, 2005). Several studies have shown that inhibition of PKC prevents early changes in the micro vascular beds of diabetic animals (Michael, 2005).

1.2.6.7. Activation of hexamine pathway

In hyperglycemia, most of the glucose metabolized via the glycolytic pathway leaks out as fructose-6 phosphate and is diverted into the hexamine signaling pathway, where the enzyme glutamine fructose-6 phosphate aminotransferase (GFAT) converts it to glucosamine-6 phosphate and finally to uridine diphosphate (UDP) N-acetyl

glucosamine. N-acetyl glucosamine adversely modifies transcription factors (such as factor Sp1) resulting in increased expression of transforming growth factor β-1 and plasminogen activator inhibitor-1, both of which promote diabetic vascular injury (Michael, 2005).

1.2.6.8. Influence of gender on the mechanism of diabetes vascular disease

The mechanisms involved in diabetes-induced vascular dysfunction has mostly been demonstrated in male but poorly studied and understood in the female (Pinna et al. 2001). In the male, increased oxidant burden (Hayashi et al. 1992; Brandes and Mugge, 1997; Sartori-Valinotti et al. 2007) and diminished anti-oxidant status (Faraci and Didion, 2004) contribute to the disease process. Since the female gender is said to lose its protection against development of cardiovascular disease (Sowers, 1998; Gaba et al. 1999), the gender gap in tissue content and effects of oxidant stress may be eliminated in diabetes. Further, healthy female subjects owe their protection from diabetes to estrogen-induced lowering of oxidant stress (Ruiz-Larrea et al. 2000; Strehlow et al. 2003; Florian et al. 2004). Since the synthesis, release, and bioactivity of endothelium-derived vasodilatory factors (NO, PGI_2, and EDHF's) and contracting factors (Ang II, ET-1, ROS and TXA_2) are regulated more by 17β-estradiol than other sex steroid hormones (Orshal and Khalil, 2004), the diabetes-induced modification in estrogen signaling may aggravate vascular injury more in the diabetic female than the male. Therefore, exploring the extent of this modification in the female, may offer insights into the pathogenesis and the mechanism of the disease in this gender. Also, given its anti-oxidant and endothelial protective role in cardiovascular disease (Mendelssohn and Karas, 1999), the female hormone, estradiol, has a strong promise for ameliorating the adverse contractile effects of diabetes-induced ROS in the male gender.

1.2.7. Flavonoids

Flavonoids are a group of plant-based polyphenolic compounds widely distributed in plants (vegetables, fruits, seeds, nuts, tea, and red wine) (Pietta, 2000). They constitute a large part of human diet with estimated daily intake of 23-500 mg day^{-1} (Hertog, 1993). More than 4000 varieties of flavonoids have been identified; each having unique structural characteristics that have been linkedd to health benefits (Nijveldt et al. 2001). Flavonoids are generally characterized by diphenylpropane skeletal structure consisting of fifteen carbon atoms arranged in three rings (C_6-C_3-C_6) with hydroxylated positions at 3, 5, 7, 2', 3', 4', 5'(Fig. 1.2) (Cao et al. 1997). Based on their hydroxyl and C=C contents, flavonoids are divided into sub groups, namely flavonols, flavones, flavanols, flavanones, isoflavonoids and anthocyanins (Lakhanpal and Rai, 2007) (Fig. 1.3). Compared to other flavonoids, flavonols show a wider range of biological activities and are specific in their action (Perez-Vizcaino and Duarte, 2010).

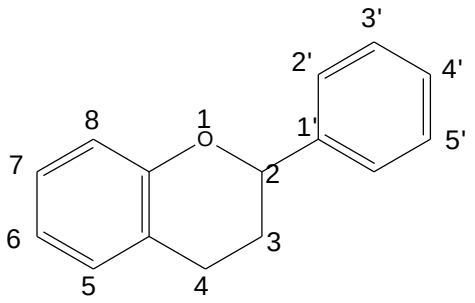

Figure 1.2: Basic structure of flavonoid (consisting of fifteen carbon atoms arranged in three rings) (Cao et al. 1997).

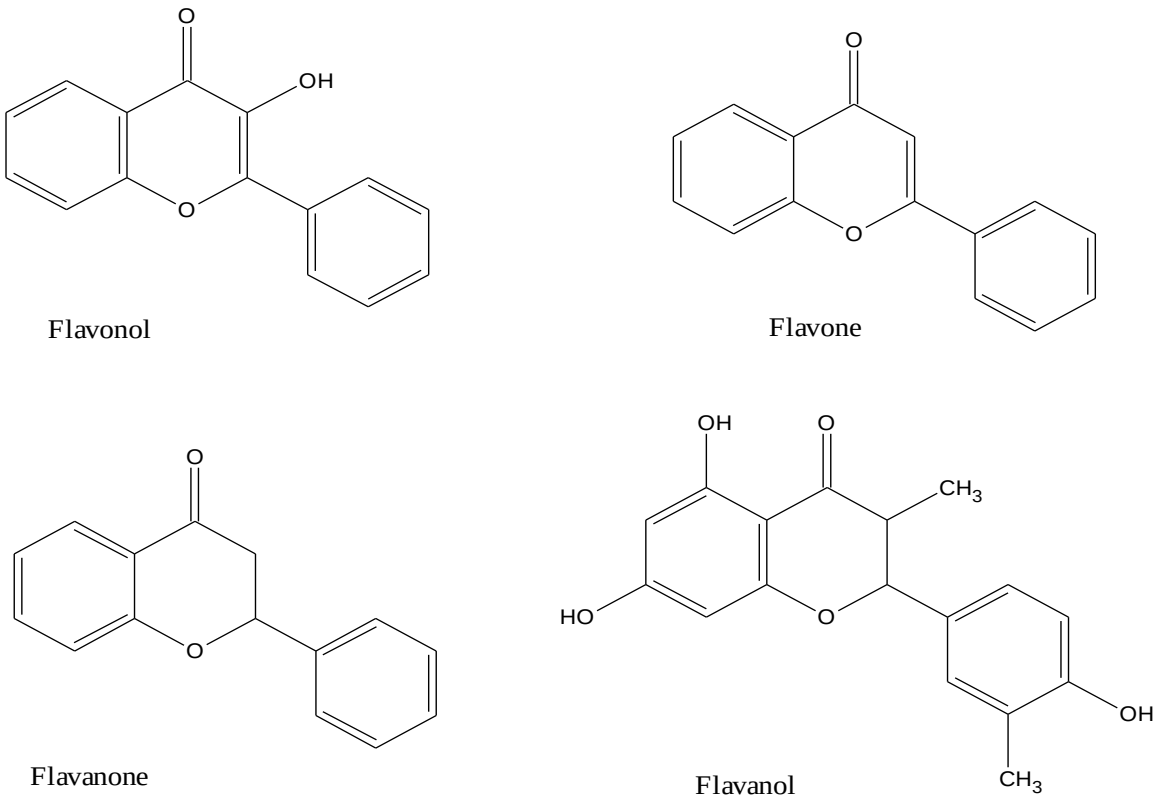

Figure 1.3: Structures of flavonoid; flavonol, flavones, flavanone, and flavanol (Lakhanpal and Rai, 2007).

In the last decade, flavonoids elicited profound research interest primarily because of the discovery of the 'French paradox' in which a large part of the French population who were associated with high consumption of red wine, exhibited relatively low cardiovascular mortality rate (Renaud and De Lorgeril, 1992). This observation has since been supported by animal studies (Hayek et al. 1997; Fuhrman et al. 2000) and further epidemiological evidence (Hertog et al. 1993; Geleijnse et al. 1999). The mechanisms behind the cardio protective properties of flavonoids are not fully clear. Flavonoids may protect against low density lipoprotein (LDL) oxidation via its scavenging of ROS, and decrease platelet promotion of thrombogenesis (Aviram et al. 2000). Its vascular effects may additionally derive from their anti-atherogenic actions against inflammatory and adhesion factors (Freedman et al. 2001).

1.2.7.1. Quercetin

Qquercetin (3, 3', 4', 5, 7-pentahydroxyflavone dehydrate or 2-(3,4-dihyroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one), (Fig. 1.4) is one of the most widely distributed flavonoid (flavonol) in human diet comprising about 60% of total dietary flavonoid consumption (Hertog et a1. 1993) (Table 1.1). Dietary levels of >33 mg/day has been linked with decreased risk of CVD (Knekt et al. 2002).

Table 1.1: Amount of quercetin in selected foods (USDA database, 2003)

Dietary source	Quercetin content (mg/100 g)
Capers	233.0
Raw onions	22.6
Cocoa powder	20.1
Cranberries	14.0
Lingo berries	7.4
Apples	4.6
Raw celery	3.5
Raw Brocoli	2.8
Green tea	2.7

1.2.7.1. 1. Bioavailability

The bioavailability of quercetin depends on the food matrix in which it is found, the type ingested, its absorption and metabolism. Pure quercetin (un-conjugated aglycone) and the form commonly found in food (quercetin glycosides) (Fig. 1.4) are readily bioavailable (Moon et al. 2008; Wiczkowski et al. 2008).

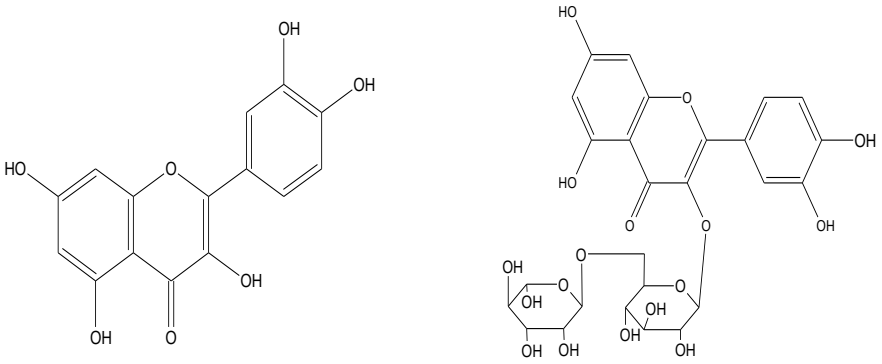

Figure 1.4: Un-conjugated quercetin aglycone and conjugated quercetin glycoside (Larson et al. 2010).

Following absorption into small intestine and colon, both forms of quercetin undergo glucuronate conjugation with metabolite (isorhamnetin, kaempferol and tamarixetin) formation in the intestinal epithelium (Larson et al. 2010). On absorption, levels of un-conjugated quercetin can reach up to 10 µM and levels up to 133 µM of conjugated derivatives and metabolites (isorhamnetin and kaempferol, tamarixetin) in human and animal circulation (Murota et al. 2003).

1.2.7.1.2. Cardiovascular effects

Although the use of antioxidant (flavonoid) therapy has been advocated in the management of cardiovascular disease (particularly, diabetes), clinical trials have produced conflicting evidence. For example, treatment with quercetin or its metabolites (isorhamnetin and kaempferol, tamarixetin) may or may not be useful in the management of high blood pressure in hypertensive patients (Johansen, 2005; Larson et al. 2010). On the other hand, in several animal models (including, high-sucrose, high-fat fed rats, NO deficient rats, Ang II or L-NAME infused rats, Dahls salt sensitive rats and in rats made hypertensive by chronic inhibition of NOS), quercetin was shown to consistently reduce blood pressure and/or the severity of hypertension (Ajay etal. 2005; 2006b; Edward et al. 2007; Egert et al. 2009). In diabetes, studies in the laboratory where the current research was conducted (Ajay et al. 2006a; 2007) and others (Roghani et al. 2005) have shown that quercetin decreases diabetes-induced hyperglycemia and endothelial dysfunction. The precise mechanism of the beneficial cardiovascular effects of quercetin is not fully understood. The literature evidence suggest that quercetin influences multiple targets via a combination of known and as yet unidentified mechanisms, including the inhibition of angiotensin converting enzyme (ACE), inflammatory factors, regulatory enzymes /proteins, such as NAD(P)H and xanthine oxidases, phospholipases and lipooxygenases, Ca^{2+}-dependent protein kinases (linked to smooth muscle contraction) (Perez-Vizcaino and Duarte, 2010; Larson et al. 2010). Quercetin-dependence on these mechanisms for its vascular action has been established in the male and requires to be investigated in the female.

1.2.7.1.3. Other effects

Other beneficial effects of quercetin has been documented in recent reviews (Lakhanpal and Rai, 2007; Perez-Vizcaino and Duarte, 2010; Larson et al. 2010). Quercetin inhibits the production and release of histamine and other allergic/inflammatory substances, and therefore, might be useful in the treatment of allergies, asthma, hay fever, and hives. Its inhibitory effects on both cyclooxygenase and lipooxygenase activities may promote its usefulness in treating inflammatory related conditions such as rheumatoid arthritis. Quercetin exerts antibacterial activity against all forms of bacteria-induced infections. Furthermore, in various animal and cell line studies, quercetin has been shown to inhibit the growth of cancer cells (breast, colon, prostate and lung tissues). Additionally, due to its potent anti-oxidant and angiogenic properties, quercetin inhibits oxidative stress-induced damage of DNA and suppression of PKC activity.This anti-oxidative action may also protect against oxidative-induced neurodegenerative disorders, such as Alzheimer's and Parkinson's disease.

1.2.7.1.4. Adverse effects of quercetin

Quercetin is neither mutagenic nor carcinogenic *in vivo* (Okamoto et al. 2005). At chronic concentrations up to 1,000 mg/day, both the short and long-term consumption of quercetin has been associated with few adverse effects, including nausea, headache, and tingling of the extremities (Harwood et al. 2007). However, regular consumption of foods (grapefruit juice, tea, onions) rich in quercetin may interfere with drug metabolizing enzymes, such as CYP_3A_4 (which is involved in the breakdown of commonly prescribed drugs such as, felodipine and digoxin). This interaction may enhance levels of these drugs (in circulation) and their potential to cause unwanted side

effects. Further, quercetin may also inhibit platelet aggregation, and hence, could increase risk of bleeding when taken along with anticoagulants (Vita, 2005).

1.2.7.1.5. Influence of gender on the quercetin vascular action

Since several biochemical pathways associated with diabetes-induced hyperglycemia (Section 1.2.6) (e.g. glucose autoxidation, polyol pathway, prostanoid synthesis, protein glycation) promote vascular injury by enhancing the synthesis of vascular ROS. Therefore, ameliorating oxidative stress through treatment with anti-oxidants has been proposed as an effective strategy for reducing diabetic vascular complications. For example, the laboratory where the current study was conducted (Ajay et al. 2005; 2006 a,b; 2007), quercetin corrected contractile dysfunction in aortic tissues isolated from WKY diabetic/SHR male rats via mechanisms that includes the scavenging ROS, enhancement of anti-oxidant capacity and the promotion of EDNO-sGC-cGMP activity. Quercetin is therefore a potential therapeutic agent in the management of diabetes vasculopathy. Very few studies have demonstrated the beneficial role of quercetin in the female diabetic model (Pinna et al. 2001). Compared to the male, female diabetic subjects have poorer outcomes following cardiovascular events (Barrett-Connor and Bush, 1991). Diabetic females lose the protective effects of the female hormone, estradiol, and hence may be more susceptible to vascular oxidative stress-induced damage (Sowers, 1998; Gaba et al. 1999, Pinna et al 2001). Therefore, it is possible that female diabetic rats may be more responsive to the vascular effects of flavonoids than the male.

1.2.7.1.6. Use of quercetin and/or estradiol in the management of diabetes-induced vasculopathy

Quercetin enhances estradiol levels *in vivo* (Weber et al 1996). For example, in ovariectomized women, blood estradiol levels were elevated significantly following consumption of estradiol and quercetin rich diet (grapefruit juice) than when estradiol was taken alone (Schubert et al. 1994). Given their exceptional anti-oxidant and vascular endothelial protective properties (estradiol (Section 1.2.5.3) and quercetin (1.2.7.3), respectively), a combined therapy of quercetin and exogenous estradiol may offer stronger promise in ameliorating diabetes-induced vascular oxidative stress, thus improving endothelial function. This may possibly offer new therapeutic window for the management of the disease outcome in both genders.

1.3. Experimental models

1.3.1. Choice of animals

Male and female Wistar Kyoto (WKY) rats were used in this study. WKY rats have common genetic background with spontaneously hypertensive (SHR) rats, and hence the former is widely employed as control strains for SHR model of essential hypertension (Okamoto and Aoki, 1963). The use of WKY rats in the current study was to facilitate a future extension of the work to hypertension studies. The choice of age 20-22 weeks for the animals was to match our current findings with earlier studies (Ajay et al. 2003; 2006 a, b; 2007) on the same theme. Also, this age allows for the rats to match the age of diabetic rats which had to be made diabetic for at least 8 weeks starting from age 12 weeks.

1.3.2. Animal model of diabetes

Streptozotocin (STZ) (2-Deoxy-2-([(methylnitrosoamino) carbonyl] amino)-D-glucopyranose) is a broad spectrum antibiotic with chemotherapeutic properties produced by Streptomyces achromogenes. Its diabetogenic property was first described in rodents and subsequently in dogs and monkeys. Since its discovery, it has been widely employed as the agent of choice for the induction of diabetes mellitus in animal models (Lenzen, 2008). Administration of STZ to laboratory animals results in symptoms similar to human insulin-dependent (Type 1) diabetes or to non-insulin-dependent (Type II) diabetes at later stages (Weir et al. 1981). Its mechanism of diabetes induction is based on the destruction of pancreatic β-cells. Due to its structural similarities with glucose, STZ is transported into liver hepatocytes and pancreatic β-cells (by the insulin-dependent glucose transporter GLUT-2), where it undergoes metabolism to yield glucose and methylnitrosourea. Methylnitrosourea adversely modifies DNA and other cellular macromolecules resulting in necrosis of β-cells and a state of insulin-dependent diabetes (Lenzen, 2008). STZ is inexpensive and readily available. It produces clear and reproducible end-organ effects of insulin deficiency in a relatively short time (within 2-4 days). Its draw back includes the liberation of minor levels NO and ROS, which do not contribute significantly to its mechanism of action (Lenzen, 2008).

1.4. Hypothesis

Gender differences exist in onset and progression of cardiovascular disorders. The mechanism involved in the diabetes-induced alteration of aortic contraction has been mostly demonstrated and understood in male animal diabetic model (Ajay et al. 2006a; 2007; Chin et al. 2007) which may differ from the female. Only few studies in this field have focused on the female gender (Pinna et al. 2001). Since diabetes has been shown to be a stronger risk factor in the development of cardiovascular disease in the female than in the male gender, it is reasonable to suggest that more marked adaptive changes may occur in the vasculature of females in the diabetic state. The current study therefore set out to investigate if the effect of short-term diabetes on aortic reactivity to endogenous vasoconstrictors were gender differentiated.

Diabetes elevates ROS and induces deficiency in anti-oxidant status, both of which are gender regulated (Brandes and Mugge, 1997; Ide et al., 2002; Sartori-Valinotti et al. 2007). ROS and other oxidative stressors may in turn promote abnormal tissue reactivity to endogenous vasoconstrictor molecules in a gender dependent manner. The current study therefore examined the influence of gender on the specific role of ROS in modulating phenylephrine (PE) or angiotensin II (Ang II)-induced tissue contraction in healthy and diabetic tissues. Given that quercetin exerts its vasorelaxant action through the scavenging of ROS, and considering that gender differences exist in the regulation and function of vascular tissue ROS and SOD (a key modulator of vascular ROS), the hypothesis is that the vasodilator action of the anti-oxidant quercetin may differentiate between male and female tissues. The study therefore, investigated possible gender differences and the underlining mechanism of any such difference in the effect of quercetin on PE or Ang II-contracted thoracic aortic rings. These differences may have

implications for further understanding of the gender-related differences in cardiovascular (diabetes) disease which may be explored for therapeutic intervention.

Further, the female hormone, estradiol is known to reduce the rate of cardiovascular events due to its ability to promote endothelial relaxant function and reduce vascular ROS. The current study examined the effect of exogenous estradiol or its combined administration with quercetin on diabetes-induced vascular oxidative stress and contractile outcome. The findings in this study may offer a new therapeutic window in the use of estradiol and/or quercetin in the management of diabetic vascular complications.

1.5. Objectives of the study

The study objectives in the current work are to investigate the following (in male versus female normoglycemic and diabetic WKY rat aorta):

1. The effect of gender on aortic tissue response to endogenous vasoconstrictors (PE and Ang II).
2. To investigate the influence of gender on the vascular effect of superoxide anion and anti-oxidant enzymes (SOD / CAT) during aortic tissue contraction.
3. To explore the possible role of gender in regulating the vasodilator effects of the anti-oxidant, quercetin and its mechanism of action.
4. To explore the possible role of 17β-estradiol or its combined therapy with quercetin in protecting against ROS-mediated contractile reactivity of normoglycemic and diabetic tissues.

1.6. Outline of the study

The hypothesis of this study is that gender differences exist in the vasodilator effect of the flavonoid quercetin. Therefore, *in vitro* studies were performed to assess the influence of gender on the contractile effects of PE or Ang II on aortic tissues isolated from normoglycemic and diabetic WKY rats in the presence or absence of various pharmacological probes. The present study was divided into two parts. In the first part, the influence of gender on PE-induced vascular contraction and the gender characteristic of quercetin action and/or estradiol in normoglycemic and diabetic tissues were assessed. Similarly, in the second part, the influence of gender on Ang II -induced contraction of normoglycemic and diabetic aortic tissues and the gender characteristic of quercetin and/or estradiol relaxant action was explored.

CHAPTER 2

MATERIALS AND METHODS

2.1. Experimental animal

All experiments involving animals were reviewed and approved by the University of Malaya and the International Medical University Animal Experimentation Ethics Committee. Male and female WKY rats were housed in groups of 3- 4 in a plastic cage, under standard laboratory room conditions (temperature: 22 ± 2 ^{o} C, humidity: 30 - 40%, 12/12-h light / dark cycle) with food (pelleted laboratory chow from Gold Coin Sdn. Bhd., Malaysia) and tap water available *ad libitum*. An adaptation period of 2-3 weeks was allowed before initiation of any of the following experimental protocols.

2.2. Drugs and chemicals

The following drugs were used: angiotensin II (Ang II), acetylcholine chloride (ACh), quercetin (3,3',4',5,7-pentahydroxyflavone dehydrate), indomethacin, N^{ω}-nitro-l-arginine methyl ester (L-NAME), methylene blue (MB), 17β-estradiol, phenylephrine-HCl (PE), superoxide dismutase (SOD), catalase (CAT), β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium hydrate (NADPH), N,N' dimethyl-9,9'-biacridinium dinitrate (lucigenin), diphenylene iodonium (DPI), diethylthiocarbamic acid (DETCA), Folin-Ciocalteu reagent, potassium sodium tartarate, disodium hydrogen phosphate, ethylene diamine tetraacetic acid (EDTA), riboflavin, L-methionine, Triton X-10, nitroblue tetrazolium, potasium hydrogen phosphate, sodium hydrogen phosphate, streptozotocin (STZ), phosphate buffered and sodium HEPES salts were purchased from Sigma-Aldrich (Co., St. Louis, Mo., USA). Sodium nitroprusside (SNP), sodium hydroxide (NaOH), copper II sulphate ($CuSO_4$) and Krebs salts were

purchased from BDH Limited (Poole, England), respectively. Ethanol was purchased from Fisher Scientific (Malaysia). Dimethyl sulfoxide (DMSO), sodium carbonate (Na_2CO_3) and hydrogen peroxide (H_2O_2) were purchased from MERCK (Germany). Hematoxylin and eosin Y alcohol staining solution were purchased from Richard-Allan Scientific (Kalamazoo, M. USA). Bovine serum albumin was purchased from Applichem (Germany). All the drugs were dissolved in distilled water with the exception of quercetin and 17β-estradiol (both of which were dissolved in DMSO) and indomethacin which was dissolved in Na_2CO_3 solution. Drug concentrations were expressed in the final molar concentration present in the organ bath.

2.3. Induction of (Type 1) diabetes mellitus (IDDM))

Type 1 diabetes was induced in (12-13 weeks old) male and female WKY rats by a single intraperitoneal injection (65 mg/kg of body weight) of STZ dissolved in cold normal saline. Plasma glucose levels and weight of the animals were measured 3 days following STZ-injection. These parameters were measured again 8 weeks after STZ injection, at sacrifice. Animals were considered diabetic only if their blood glucose levels were $\geq$ 17 mmol/l. Blood was collected from the rat tail vein with a 26 gauge needle and blood glucose levels ascertained with ACCU-CHEK Advantage-II test strip technique (Roche Diagnostics, Germany).

2.4. Collection of blood samples

At sacrifice, blood samples from normoglycemic or diabetic animals were collected by cardiac puncture, and on coagulation, serum was obtained by centrifugation (Allegra X-15, Beckman, USA) (10,000 rpm for 6 min at 4^oC) and stored in the refrigerator (Thermo Fisher scientific, USA) at -80 ^{o}C until analyzed.

2.5. Measurement of vascular function

The effects of quercetin on PE or Ang II contraction in the presence and absence of various pharmacological drug probes were examined in thoracic aortic rings isolated from age- (20-22 weeks) and sex-matched normoglycemic and diabetic groups.

2.5.1. Preparation of Krebs physiological salt solution

The Krebs physiological salt solution (KPSS) was prepared by dissolving weighed amounts of various salts (listed in Table 2.1) in distilled water. The solution was freshly prepared before each experiment.

Table 2.1: Composition of the Krebs physiological salt solution (KPSS)

Salt	**Concentration (mM)**
NaCl	118.2
$NaHCO_3$	25.0
KCl	4.7
KH_2PO_4	1.2
$MgSO_4.7H_2O$	1.2
Glucose	11.7
$CaCl_2 \cdot 2H_2O$	2.5
EDTA	0.026

2.5.2. Preparation and mounting of aortic ring.

The vascular function of aortic rings with or without endothelium was assessed using methods previously employed in our laboratory (Ajay et al. 2003; 2005; 2006 a,b; 2007). Following cervical dislocation, the chest cavity of rats (from each experimental group) was exposed and the descending thoracic aorta excised. Surrounding fat and connective tissue were carefully removed and the aorta was cut into small (3-4 mm) transverse rings. In some tissues, the endothelium was removed by gentle rotation of the rings on an appropriately-sized forceps. Following this, both the endothelium-intact and -denuded tissues were suspended between two L-shaped stainless steel hooks in a 5 ml organ bath containing normal KPSS (Table 2.1). The bath solution was maintained at 37 °C and gassed with a mixture of O_2 (95 %) and CO_2 (5 %) throughout the study. The rings were stretched to a preload tension of 1.0 -1.2 g and allowed to equilibrate for 35-40 min in KPSS, following which rings were contracted thrice (each for 5 min) with isotonic high potassium chloride solution (high K^+, 80 mM) with each successive addition accompanied by a washout. The integrity of the endothelium was assessed by exposing PE (1 μM)-contracted tissues to the endothelium-dependent relaxant, ACh (10 μM). The tissue was considered endothelium-denuded or endothelium-intact if the relaxation to ACh was < 5 % or ≥ 50 % of the peak PE-induced contraction, respectively. Some segments of aorta with or without quercetin treatment were either snap frozen in liquid nitrogen or were homogenized in chilled Krebs-HEPES (Table 2.2) or phosphate buffer solution (0.01 M phosphate buffer solution (pH 7.4) (containing 0.0027 M, KCl; 0.137 M, NaCl), following which the supernatant fraction was separated from cellular debris (using centrifugation (Allegra 64; 6,000 rpm for 5 min at 4^o C)) and stored at -80 ^{o} C for subsequent biochemical analysis.

2.5.3. Assessment of estrus cycle phases in female rats

The possibility exists that fluctuations in the levels of the female reproductive hormones during the estrus cycle may modulate vascular function (Zamorano et al. 1994; Kakucs et al. 2001). Therefore, all female rats were assessed for normal estrus cycling during a 2 week period and the stage of the estrus cycle on the day of sacrifice was determined (by vaginal cytology) (Diao et al. 2008) before vascular function assessment (Section 2.5.4). Only animals with normal cycles were included in the study. Vaginal smear samples were taken from rats using a cotton swab that was dipped with normal saline (NaCl 0.9%). Cotton swab was inserted into the vaginal and gently manipulated in a clockwise direction to avoid lesion of rat's vagina tissue. The swab was then smeared onto a glass slide, which was left to dry and then fixed with 70% ethanol and stained with hematoxylin (H) and eosin (E) stains. The phase of the estrus cycle was determined by microscopic (Leica, USA) identification of the morphological characteristics of the vaginal cells in the smear (using the objective lenses of magnifications: 10 x, 20 x and 40 x). The actual estrus phase of each animal remained unknown until the assessment of vascular function was complete.

2.5.4. Effect on contractile function

This part of the study was undertaken to examine the role of gender and the endothelium on the effects of quercetin and / or 17β-estradiol (estradiol) on agonist (PE or Ang II)-induced contraction of normoglycemic / diabetic tissues.

2.5.4.1. Preparation of quercetin and estradiol stock solution

Quercetin or estradiol stock solutions (10 mM) were prepared in 5% (v/v) DMSO. The final concentration of DMSO in quercetin or estradiol solution was adjusted to < 0.05 % (v/v) (concentration devoid of any observable effects on vascular tone (Duarte et al. 1993, Chen et al. 2009)).

2.5.4.2. Effect of quercetin and/ or estradiol on PE-induced contraction

Following preparation and mounting of aortic rings (Section 2.5.2), tissues were exposed to cumulative concentrations of PE (10^{-11}-10^{-5} M) at 4-5 min intervals after 20-25 min incubation in KPSS. This was preceded by a 25 - 30 min treatment of the tissues with DMSO (< 0.05 % (v/v)), quercetin (10^{-5} M) and /or estradiol (10^{-7} M). The use of 10^{-7} M estradiol concentration was based on the physiological levels of estradiol (Gilligan et al. 1994). The choice of 10^{-5} M of quercetin was based on the sub-maximal relaxant effect of quercetin on PE-induced contraction observed in previous studies (Roghani et al. 2005), including studies in the laboratory where current work was undertaken (Ajay et al. 2003; 2005; 2006 a,b; 2007).

2.5.4.3. Effect of quercetin and/or estradiol on angiotensin II-induced contraction

Following preparation and mounting of aortic rings (Section 2.5.2), endothelium-intact and -denuded aortic rings from respective experimental groups were exposed to cumulative concentrations of Ang II (10^{-11}-10^{-5} M) at 3 min intervals to minimize the vulnerability of Ang II to receptor desensitization (Meggs et al. 1985). This was preceded by a 20 - 25 min treatment of the tissues with or without DMSO (< 0.05 % (v/v), quercetin (10^{-5} M) and / or estradiol (10^{-7} M).

2.5.4.4. Mechanism underlining gender difference in agonist-induced contraction and the vasodilator action of quercetin

This part of the work was carried out to characterize the mechanism of quercetin action in PE- and Ang II-contracted endothelium-intact (or denuded) aorta from respective experimental groups. Following preparation and mounting of aortic rings (Section 2.5.2), tissues were exposed to various pharmacological probes for 4 - 5 min prior to and throughout incubation with quercetin (10^{-5} M) or its vehicle (DMSO (0.05 % (v/v)). To assess the role of the NO-cyclic GMP and / or cyclooxygenase pathways, the relaxant effects of quercetin on PE or Ang II (10^{-11}-10^{-5} M) concentration-response curves were recorded in aortic rings incubated with the sGC inhibitor- MB (10^{-5} M), nitric oxide synthase inhibitor- L-NAME (10^{-5} M), the cyclooxygenase inhibitor- indomethacin (10^{-5} M) or L-NAME + indomethacin. To examine the contribution of endogenous $\bullet O_2^-$ and/or H_2O_2 on the action of quercetin, PE or Ang II responses were examined in the presence of 150 U/ml of SOD, 236 U/ml of CAT or both. These concentrations of SOD and CAT have been shown to protect against free radical-induced endothelial dysfunction (Kurzelewski et al. 2005).

2.5.5. Characterization of the concentration-dependent vasorelaxant action of quercetin.

Following preparation and mounting (Section 2.5.2.), aortic rings from respective experimental groups were exposed to PE (10^{-6} M) and at the peak of the sustained contraction relaxation responses to cumulative concentrations of quercetin (10^{-8}-10^{-3} M) or its vehicle (DMSO (< 0.05 % (v/v)) were observed. The effect of ACh (10^{-14}-10^{-4} M) or SNP (10^{-14}-10^{-4} M) on PE (10^{-6} M)-induced contraction in the absence or presence of

quercetin (10^{-5} M) (or its vehicle (DMSO (< 0.05 % (v/v)) were also recorded at 4 min intervals.

2.5.6. Data presentation and statistical Analysis

Both the vasocontractile and vasorelaxant responses were recorded as mean ± standard error of the mean (S.E.M.) for the number of rats. The contractile responses of aortic rings to graded concentrations of PE or Ang II were expressed as percentages of the maximum contractile effect of high K^+ in respective tissues. Relaxation responses to cumulative concentrations of quercetin, ACh or SNP were calculated as percentage inhibition of PE-induced maximal contraction. The concentration–response curve for each experimental condition was plotted and from it were deduced the values of maximal agonist-induced response (Emax) and the concentration of the agonist (expressed as negative log molar concentration) producing 50% of Emax (pEC_{50}) (Prism version 5.0, Graph Pad Software, USA). Statistical evaluation of the data was performed using the Student's t-test for unpaired observations and by two-way analysis of variance (ANOVA) for multiple group comparison followed by the Bonferroni post-hoc test for selected pairs. In all cases 'p' value of less than 0.05 was considered statistically significant.

2.6. Biochemical measurements

2.6.1. Effect of gender on superoxide anion generation in aorta with or without quercetin

The *in vitro* (Ajay, 2006 a) and *in vivo* (Ajay, 2007) vasorelaxant function of the flavonoid, quercetin have been shown (in our laboratory) to be modulated by ROS in the male rat. In the current study, the possibility that gender influences the *in vitro* generation of superoxide anion (a key oxidant mediating endothelial dysfunction) (Richard et al. 2000; Kurzelewski et al. 2005) was explored in endothelium-intact aortic rings with or without quercetin (antioxidant) treatment.

2.6.1.1 Preparation of Krebs-HEPES buffer for $\bullet O_2^-$ assay

The Krebs-HEPES buffer solution was prepared by dissolving weighed amounts of various salts (listed in Table 2.2) in distilled water. The solution was freshly prepared before each experiment.

Table 2.2: Composition of the Krebs-HEPES buffer solution

Salt	Concentration (mM)
NaCl	99
$NaHCO_3$	25.0
KCl	4.7
KH_2PO_4	1.0
$MgSO_4.7H_2O$	1.2
Glucose	11.0
$CaCl_2 \cdot 2H_2O$	2.5
Na Hepes	20.0

2.6.1.2. Effect of gender on superoxide anion generation in aortic tissue supernatant samples

Following homogenisation and supernatant separation in phosphate buffer solution (Section 2.5.2.), generation of $\bullet O_2^-$ in tissue supernatant fraction was measured with a commercially available kit (Sigma-Aldrich). The kit uses a chemiluminescent method to measure the oxidation of luminol by xanthine/xanthine oxidase-induced superoxide generation. Briefly, test samples (aortic supernatant) (88 µL) were placed in duplicate

into appropriately assigned wells of a 96 well micro plate, containing luminol solution (5 µL), enhancer solution (5 µL) with or without SOD (1 µL). Reaction was initiated by adding xanthine oxidase working solution (2 µL). Luminescence intensity (photon emission) was measured within 5 minutes of the start of the reaction. $\bullet O_2^-$ production was expressed as average counts per mg/mL of homogenized tissue sample.

2.6.1.3. Effect of gender on superoxide anion generation in whole aortic tissue samples

Superoxide anion ($\bullet O_2^-$) was measured in whole tissue samples using lucigenin-enhanced chemiluminescence technique (Chan et al. 2003) with slight modification.

Following the preparation and mounting of aortic rings (Section 2.5.2), aortic rings were incubated for 45 min at 37 ^oC in a reaction mixture consisting of diethylthiocarbamic acid (DETCA) (1 mM) (20 µl) to inhibit SOD, NADPH (0.1 mM) (20 µl) (substrate for NADPH oxidase) and either DMSO (< 0.05 % (v/v) or quercetin (10^{-8}-10^{-3} M) or diphenylene iodonium (DPI) (5×10^{-6} M) (20 µl) (inhibitor of NADPH oxidase). Krebs-HEPES buffer (300 µl) (Table 2.2), lucigenin (5 µM), DMSO (< 0.05 % (v/v)) or DPI (5×10^{-6} M) were placed into separate wells of a 96-well optiplate and mounted into a (Top Count) single photon counter to count background photon emission over 15 min. A single ring segment of male or female aorta (3 mm) with or without quercetin was transferred to each appropriate well, and photon emission was re-counted for 15 min. Tissues were dried for 48 hours at 60 ^oC. $\bullet O_2^-$ production was expressed as average counts per mg of tissue dry weight.

2.6.2. Anti-oxidant measurement

Superoxide dismutase (SOD) and catalase (CAT) are major mediators of vascular oxidative stress and endothelial function (Richard et al. 2000; Kurzelewski et al. 2005). Since their levels may be gender differentiated (Brandes and Mugge, 1997; Ide et al., 2002; Kerr et al. 1999), the effect of gender on tissue concentrations of SOD and CAT were measured.

2.6.2.1. Superoxide dismutase activity

SOD activity was determined in aortic tissue homogenates using previously described method (Beyer and Fridovich, 1987). SOD quantitation was based on the generation of superoxide anion ($\bullet O_2^-$) by xanthine and xanthine oxidase, which react with nitro blue tetrazolium (NBT) to form a Formazan dye. The reaction mixture (containing 50 mM/L Potassium phosphate buffer (pH 7.8, 27 mL), 9.9 mM L-Methionine (1.5 mL), 0.025% Triton X- 100 (0.75 mL) and 57 μmol NBT (1 mL)) was placed into small glass tubes. This was followed by the addition of tissue supernatant samples (20 μL) and Riboflavin (10 μL) (4.4 mg/100 ml). A control (blank) glass tube containing 20 μL of buffer was run in parallel. The ensuing mixture in respective glass tubes were then illuminated for 7 min in an aluminum foil-lined box, containing two 20 W florescent tubes maintained at 25 °C (turning the color of mixture purple). The increase in absorbance due to Formazan formation was read spectrophotometerically at 560 nm. Under the described conditions, the increase in absorbance in controls was taken as 100 % and the SOD activity was calculated as follows:

$$\% \text{ of inhibition} = \frac{\text{Absorbance (blank-sample)}}{\text{Absorbance of blank}} \times 100$$

One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50 %.

SOD activity was normalized to the tissue protein content and expressed as:

$$\text{SOD specific activity (U/mg protein)} = \frac{\text{Enzyme unit}}{\text{Vs x [protein] x T}}$$

Where, Vs = volume of sample (ml)

[Protein] = protein concentration (mg/ml)

T = illumination time (7 min)

2.6.2.2. Catalase activity

CAT was measured in aortic tissue homogenates by a previously described method (Aebi, 1984). The assay is based on the decomposition of H_2O_2 by CAT present in the samples. 50 mM Potassium phosphate buffer (650 µl) and sample (50 µl) were added to a quartz cuvette. The reaction was started by adding 30 mM H_2O_2 (300 µl). The absorbance produced by the decomposition of H_2O_2 was measured spectrophotometrically at 240 nm at 25^{o}C for 30 seconds. CAT activity (U/s/mg protein) was normalized to the tissue protein content and expressed as:

$$\text{CAT specific activity (U/s/mg protein)} = \frac{2.3\ (-\log \text{net OD})}{\Delta T \times [\text{Protein}]}$$

Where, U = Unit of catalase required to consume 1 μmol of H_2O_2 per min/ mg of cellular protein.

Net OD = optical density (absorbance) of samples minus absorbance of blank standard

ΔT = time interval (10 s)

[Protein] = protein concentration (mg/ml) in samples.

2.6.2.3. Normalization of SOD or CAT levels to tissue protein concentration

The protein content of the tissue homogenates was measured using Bovine serum as a standard (Lowry et al. 1951). The assay relies on the reaction of the peptide bonds with the copper II sulphate solution (under alkaline conditions) resulting in the reduction of copper II ions (Cu^{2+}) to copper I (Cu^{+}) which reacts with Folin-Ciocalteu (Phosphomolybdic / Phosphotungstic acid) reagent to produce a colour change. The assay procedure was as follows: A stock solution (1 mg/mL) of Bovine serum albumin (BSA) powder was prepared in 1M NaOH solution and diluted serially in duplicates to achieve the concentrations (0, 10, 25, 50, 100, 200, 400 and 600 μg/mL), which was used to construct a BSA standard curve. Working standards (0.500 μl) and tissue supernatant samples (diluted 100 fold in NaOH) (0.500 μl) were placed into separate test tubes. Lowry reagent (5 mL) (containing 1% Copper II sulphate) (500 μl), 2% sodium potassium tartrate (500 μl) and 2% sodium carbonate Na_2CO_2 (50 ml) was added to each test tube and incubated at 27 ^{o}C for 15 min. The subsequent addition of 1N Folin- Ciocalteu's reagent (20 μl) into each test tube, followed by another incubation period (27 ^{o}C, 30 min), produced a colour change which absorbance was measured at

725 nm with a UV-spectrophotometer. The concentration of protein (mg/mL) was determined by comparing the average net optical density (OD) for each sample to the standard curve, which showed strong linearity (R^2= 0.93).

2.6.3. Tissue levels of prostaglandins and the effect of quercetin

Contractile (Tesfamariam et al. 1990; De Vriese et al. 2000) and vasodilator (Jeremy et al. 1993) prostaglandins (PG) have been implicated in the pathogenesis of diabetic vascular disease. In the male rat, the mechanism of quercetin vasorelaxant action may be PG mediated **(**Roghani et al. 2005). Therefore, the effect of gender and diabetes on the levels of PG products (vasoconstrictors: thromboxane (TXA_2) (as TXB_2; a stable metabolite of TXA_2); PGE_2 and the vasodilator: PGI_2 (as 6-keto-$PGF1_a$; a stable metabolite)) were measured in endothelium-intact tissues from respective experimental groups with or without quercetin treatment (10^{-5} M; 25 min). All assays were performed in homogenised tissue supernatant fractions using commercially procured enzyme immunoassay (EIA) kits (Cusabio Bioteck. Co. LTD) in accordance with the manufacturer's instructions. Levels of TXB_2, PGE_2 and PGI_2 were analysed as follows:

2.6.3.1. TXA_2 (TXB_2) concentration

Portions of working Standards (3.9, 7.80, 15.6, 31.2, 62.5, and 125.0 pg/ml) (100 µl) or tissue supernatant sample (100 µl) were added in duplicates to appropriate wells of a 96 well micro titer plate pre-coated with an antibody specific to TXB_2. TXB_2 specific Biotin-conjugated antibody preparation (100 µl) was added to each well and incubated for 1 hour at 37 °C. Following incubation, wells were aspirated and washed with wash buffer (200 µl) for a total of three washes. Avidin conjugated to Horseradish peroxidase (HRP) enzyme (50 µl) was added into each well and incubated for another 1 hour at

37°C. This was followed by the addition of 3, 3', 5, 5' tetramethyl-benzidine (TMB) substrate solution (90 µl) into each well to promote HRP enzyme-substrate reaction which produces a blue colour which absorbs spectrophotometrically 450 nm. The concentration of TXB_2 in the samples (pg/mL per mg of wet tissue weight) was determined by subtracting the average blank standard OD from the average OD for each working standard or sample. The average concentration of TXB_2 was determined by comparing the average net OD for each sample to the standard curve (linearity; R^2 = 0.99).

2.6.3.2. PGE_2 concentration

Portions of working Standards (0.4, 1.6, 6.3, 25.0, 100.0 pg/ml) or tissue supernatant samples (50 µl) were added in duplicates into appropriate wells of a 96 well microtiter plate pre-coated with PGE_2 specific Goat-anti-rabbit antibody. HRP-conjugate solution (50 µl) was added into each well (except for blank wells) and incubated for 1 hour at 37 °C following which each well was aspirated and washed with wash buffer (200 µl) for a total of three washes. Substrate solutions A (50 µl) and B (50 µl) were added into each well to promote HRP-substrate reaction. Sulphuric acid (stop solution) was added to terminate (yellow) colour development which absorbs spectrophotometrically at wavelength of 452 nm. The average net OD of PGE_2 in the samples was determined by subtracting the average blank standard OD from the average OD for each standard and sample. PGE_2 (pg/mL per mg of wet tissue weight) was determined by comparing the average net OD for each sample to the standard curve which showed strong linearity (R^2 = 0.94).

2.6.3.3. PGI_2 concentration

Portions of working Standards (7.8, 15.6, 31.2, 62.5, 125.0, 250.0, 500.0 pg/ml) or tissue supernatant sample (100 µl) was added in duplicates to appropriate wells of a 96 well microtiter plate pre-coated with PGI_2 (6-keto-PGF1a)- specific antibody and incubated for 2 hours at 37°C. Biotin-antibody solution (100 µl) was added into each well and incubated again for 1 hour at 37°C. Each well was aspirated and washed with wash buffer (200 µl) for a total of four washes. HRP-avidin working solution (100 µl) was introduced to each well and further incubated for another 1 hour at 37°C, at the end of which, well aspiration and washing was done for another 4 times. TMB substrate (developing) solution (90 µl) was added into each well and incubated again for 30 min at 37 °C to promote HRP enzyme-substrate reaction which was terminated by the addition of sulphuric acid following formation of yellow colour which absorbs spectrophotometrically at 450 nm. The average net OD for PGI_2 (6-keto-PGF1a) in the samples was determined by subtracting the average blank standard OD from the average OD for each standard and sample. The average concentration of PGI_2 (pg/mL per mg of wet tissue weight average) was determined by comparing the average net OD for each sample to the standard curve (linearity; $R^2 = 0.97$).

2.6.4. Total serum and tissue concentration of nitric oxide (as nitrite ion)

It has been suggested that the vascular endothelium of healthy females is more vasorelaxant than the male due to the formers higher basal release of endothelium-dependent NO (EDNO) (Hayashi et al. 1992; Kauser and Rubanyi, 1994). This enhanced EDNO release from the female is abolished following the development of diabetes (Pinna et al. 2001). Therefore, this part of the study compared the effect of gender and diabetes on total levels of nitric oxide (NO) (nitrite ion; a metabolite of NO)

production measured in serum (Section 2.4) and in endothelium-intact aortic tissue homogenate (Section 2.5.2) samples using commercially procured EIA kits (Assay Design, Stressgen). The assay was performed according to the manufacturer's instructions as follows: Zero Standard (reaction buffer) (50 µl), working Standards (3.13, 6.25, 12.5, 25, 50.0, and 100.0 µM/ml) (50 µl) or samples (50 µl) from each experimental group were placed into separate wells of a 96-well optiplate in duplicates. Reduced ß-Nicotinamide adenine dinucleotide (NADH) solution (25 µl), and Nitrate reductase enzyme solution (25 µl) were each added into wells containing the working Standard, zero Standard and samples and allowed to incubate at 37 °C, for 40 min. Under physiological conditions, NO is readily oxidized to nitrites and nitrates. During the incubation period, nitrate reductase enzyme converts all nitrates in the samples to nitrite ion which reacts with Griess reagent I (solution of Sulphanilamide in 2M HCl) (50 µl) and with Griess reagent II (solution of N-(1-Naphthyl) ethylenediamine in 2M HCl) (50 µl) resulting in colour development that absorbs strongly at 540 nm. The Griess method ability to specifically quantify cytotoxic peroxynitrites ($ONOO^-$) originally present in tissues is limited, given that $ONOO^-$ decomposes rapidly in physiological buffers with a half-life of 1 sec (Koppenol et al. 1992; Pfeiffer et al. 1997). Therefore, the total concentration of (stable) nitrite ion in each sample (µmol/L in serum or µmol/ml per mg of wet tissue weight) was determined by comparing the average net OD for each sample to the standard curve which showed strong linearity (R^2 = 0.99).

2.6.5. Serum and aortic tissues concentration of estradiol

This part of the experiment was assessed to determine the influence of diabetes on levels of estradiol and how this may modulate vascular function. Levels of estradiol in healthy (normoglycemic) and diabetic groups in diestrus phase of estrus cycle were determined in serum and in endothelium-intact tissue homogenate samples. Estradiol (17β-estradiol) measurement was performed using commercially procured EIA kits (Biocheck Inc. Foster City, CA). The assay was performed according to the manufacturer's instructions as follows: Estradiol antibody (Goat anti-rabbit IgG)-coated 96 well microtiter plate was incubated at 25 ^{o} C for 90 min with estradiol working Standards (0.0, 10.0, 30.0, 100.0, 300.0, and 1000.0 (pg/ml) (25 µl), blank controls (25 µl) or samples (25 µl) (serum or tissue supernatant fraction), Estradiol-HRP conjugate reagent (100 µl) and Rabbit anti-estradiol reagent (50 µl). During the incubation period, a fixed amount of HRP-labeled estradiol competes with the endogenous estradiol in the working standard, blank control or samples for a fixed number of binding sites on estradiol specific antibody. Following incubation, the microtiter wells were rinsed (with distilled water) and blotted dry (for a total of 5 washes) to remove unbound Estradiol-peroxidase conjugate. TMB (100 µl) reagent was added to each well and incubated for another 20 min at 25 ^{o} C (resulting in blue colour formation) at the end of which a stop solution (1 M HCl) (100 µl) was added to terminate colour development. The colour absorbance for each well was read spectrophotometrically at 450 nm as a measure of the amount of unlabelled estradiol bound to Estradiol-HRP in the samples or standards. The average concentration of estradiol (pg/mL of serum or pg/mL per mg of wet tissue weight) was determined by comparing the average net OD for each sample to the standard curve for estradiol (linearity; $R^2 = 0.77$).

2.6.6. Data presentation and statistical Analysis

For each experimental group, SOD or CAT activity and the concentration of $\bullet O_2^-$, TXA_2 (TXB_2), PGE_2, PGI_2 (6-keto-PGF1a), NO (nitrite ion), estradiol in serum or tissue were recorded as mean ± standard error of mean (SEM) of 4 or 8 determinations. The observed differences among the groups were tested for statistical significance using Student's t-test for unpaired observations and two-way analysis of variance (ANOVA) for multiple group comparison followed by the Bonferroni post-hoc test for selected pairs. In all cases 'p' value of less than 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1. Effect of gender and diabetes on body weight / plasma glucose levels

The mean ± SEM values for body weight and blood glucose levels in male and female normoglycemic /diabetic rats are summarized in Table 3.1. The normoglycemic female rats weighed significantly lesser than the normoglycemic male rats. Eight weeks after treatment with STZ, male and female diabetic animals attained body weight significantly lesser than their respective normoglycemic controls. Diabetic males showed a greater loss in body weight than the female (male = 16 % versus female = 9 %). Diabetic plasma glucose levels was similar in both genders, but was significantly higher than levels seen in age-matched normoglycemic controls.

Table 3.1: Body weight and plasma glucose levels in male and female normoglycemic and STZ-induced (8 weeks) diabetic WKY rats.

Groups	Weight (g)	Plasma glucose (mmol/L)	n
Males			
Normoglycemic	302.84 ± 6.04	5.94 ± 0.17	(34)
Diabetic	254.78 ± 7.69 #	26.52 ±1.16 #	(33)
Females			
Normoglycemic	205.90 ±3.24*	6.60 ± 0.19	(44)
Diabetic	188.35 ± 5.33#*	28.17 ± 1.29#	(32)

Values represent S.E.M ± mean. Statistics: # $p < 0.05$, diabetic compared to normoglycemic groups; *$p < 0.001$ female compared to male rats.

3. 2. Effect of diabetes on female reproductive (estrus) cycle and serum/aortic concentration of estradiol

3. 2.1. Effect of diabetes on reproductive (estrus) cycle of female rats

To assess the influence of the estrus cycle on vascular function in normoglycemic and diabetic female animals, the phase of the estrus cycle was determined by microscopic examination of vaginal smears from normoglycemic / diabetic rats (Section 2.5.3) collected on the day of sacrifice. Microscopic images of stained vaginal smears showing cell (type and their distribution) in each phase of the estrus cycle in a normal cycling female rat is shown in figure 3.1. Cells in proestrus phase consisted of mainly nucleated epithelial cells. Estrus phase samples were populated with mostly anucleated cornified cells. Metestrus phase samples exhibited the same proportion of conified, nucleated epithelial, and leukocyte cells, and the diestrus phase consisted of mainly leukocyte cells. Diabetes caused a cessation of normal estrus cycle resulting in a permanent metestrus / diestrus state (Fig. 3.1).

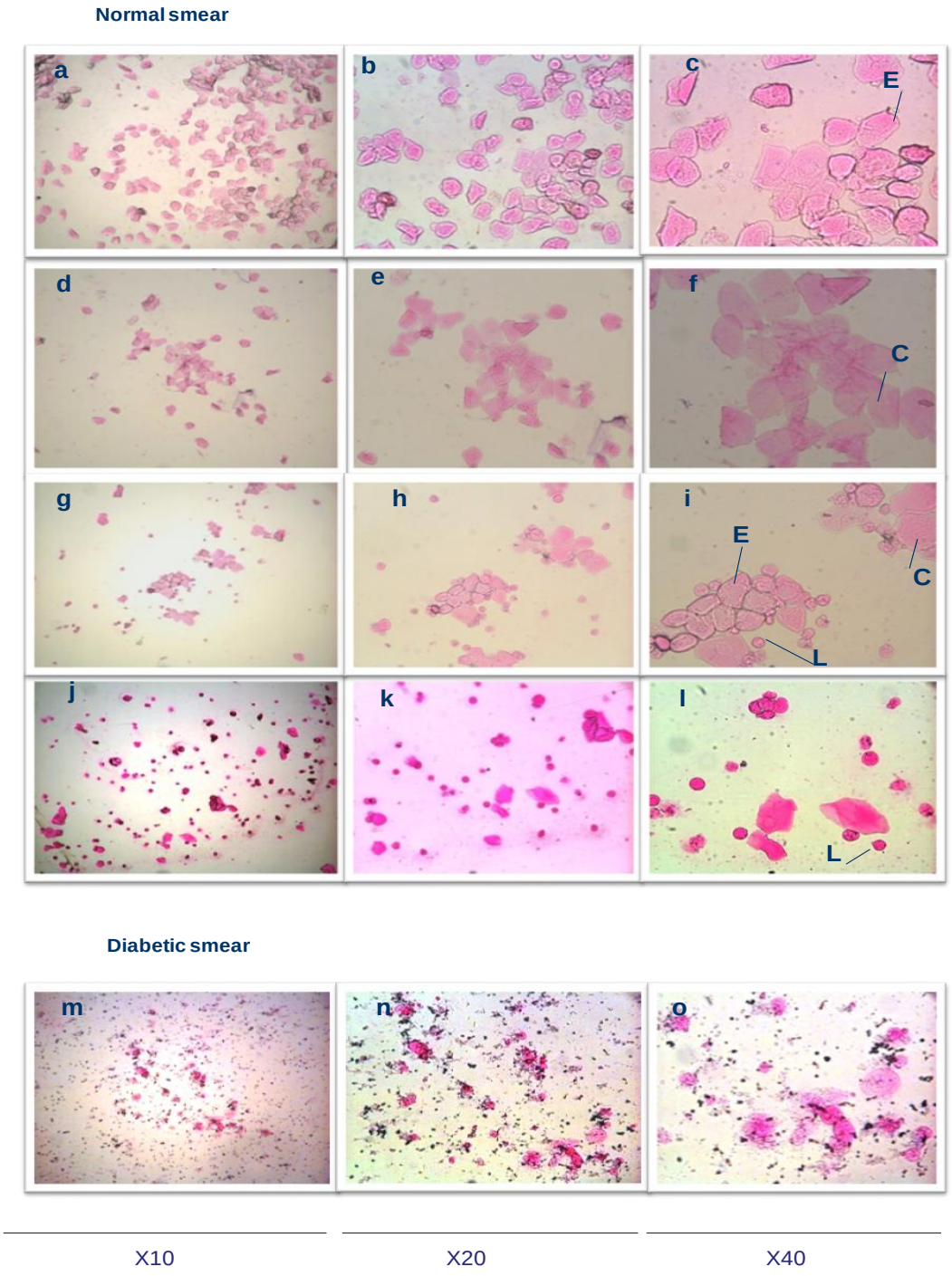

Figure 3.1: Shows (H+E) stained vagina smear images from normal cycling (upper panel) (a-l) and diabetic (lower panel) (m-o) female rats in various stages of estrus cycle displaying cells (magnified: X10 (left column), X20 (middle column) and X40 (right column)) in proestrus phase (a-c) (consisting of mainly nucleated epithelial cells (E)); estrus phase (d-f) (consisting of anucleated cornified cells (C)); metestrus phase (g-i) (howing conified cells (C), nucleated epithelial cells (E), and leukocytes (L)); and diestrus phase (j-l or m-o) predominantly exhibiting leukocyte cells (L) and fewer anucleated cornified cells. Slides represent vaginal smear samples from 22- 44 animals in each group.

3.2.2. Effects of gender and diabetes on serum and aortic tissue levels of estradiol

The effect of short-term diabetes on levels of estradiol (17β-estradiol) (Section 2.6.5) was measured in serum and (endothelium-intact) aortic tissue samples from the respective groups of rats. Normoglycemic female samples exhibited higher levels of estradiol (aorta = 1.0 ± 0.0; serum = 0.8 ± 0.2; ng/mL) compared to the male (aorta = 0.3 ± 0.4; serum = 0.4 ± 0.1; ng/mL) (Fig. 3.2). Compared to levels in normoglycemic samples, diabetes significantly reduced estradiol levels (by 50 %) in aortic tissue of female (0.5 ± 0.0 ng/mL) but failed to alter it in male aorta (0.5 ± 0.2 ng/mL). Diabetes enhanced serum estradiol levels (by about 30 %) in female (1.3 ± 0.2 ng/mL) and male (0.7 ± 0.2 ng/mL) samples (Fig. 3.2).

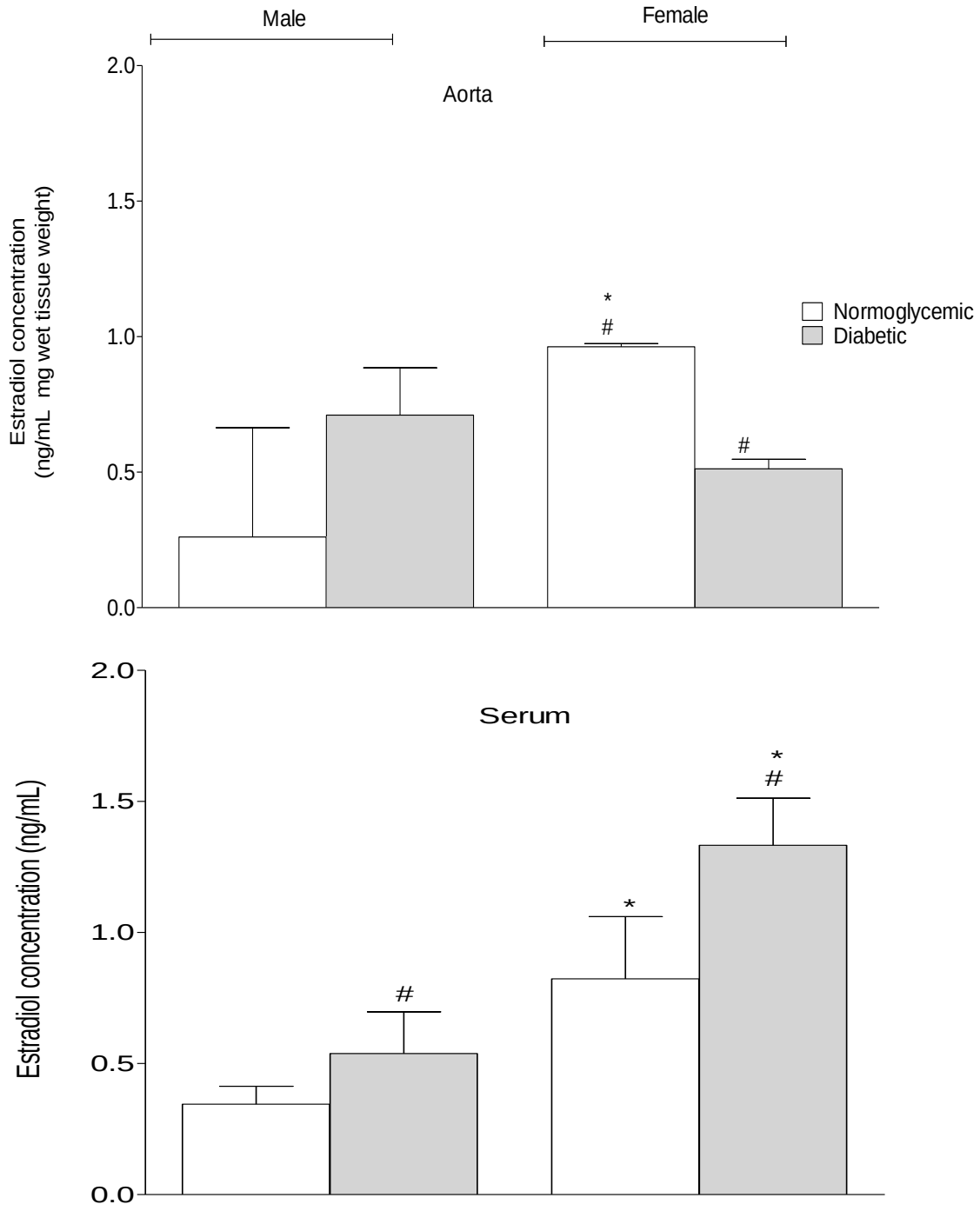

Figure 3.2: Levels of estradiol in (endothelium-intact) aortic tissues (upper panel) and serum (lower panel) from normoglycemic and diabetic male or female rats. Data represent mean ± SEM (n = 6-8 animals in each group). Statistics: $^{*}p < 0.05$: normoglycemic female compared to the male; $^{\#}p < 0.01$, diabetic compared to normoglycemic male or female samples.

3. 3. Effect of gender on agonist-induced vascular contraction

3.3.1. PE-induced contraction

Both normoglycemic and diabetic male and female tissues (with or without endothelium) produced concentration-dependent contractions to PE (Fig. 3.3).

In the normoglycemic group, the maximal contraction (Emax) to PE (% of high K+-induced contraction) was greater in endothelium-intact male (179.7 ± 7.1%) compared to the estradiol rich female tissues in proestrus phase (144.3 ± 8.6 %) (Fig. 3.3), which contracted similarly as estradiol-deficient female tissues in diestrus phase (145.1 ± 9.6 %). Endothelial denudation of normoglycemic tissues from both genders produced contractions of the same magnitude (male (217.7 ± 7.42 %) versus proestrus (215.1 ± 7.3 %) or diestrus (226.9 ± 7.4 %) female tissues). Endothelial denudation abolished or reversed the gender difference in the contraction of normoglycemic tissues. Since there was no difference in the effect of estrus cycle on the contraction to PE of endothelium-intact and -denuded normoglycemic female tissues (Fig. 3.3), results from proestrus or diestrus normoglycemic female tissues were pooled and used as controls for responses in corresponding diabetic female tissues which existed in permanent diestrus state (Fig. 3.1).

In the diabetic group, endothelium-intact female tissues contracted significantly more (Emax =159.9 ± 4.6 %) (Fig. 3.3) than the equivalent male tissues (Emax = 111.4 ± 5.6 %), which contracted lesser than its normoglycemic control (Emax = 179.7 ± 7.1 %). Contraction to PE in diabetic female tissues was similar in magnitude to its corresponding normoglycemic control (Emax = 145.0 ± 4.3 % (proestrus + diestrus)). Endothelial denudation of diabetic tissues enhanced contraction (Emax) more in male tissues (Emax = 238.4 ± 5.6 %) compared to the female (Emax = 179.8 ± 6.5 %). This intervention

eliminated or reversed the observed contractile differences between the following categories: normoglycemic versus diabetic male tissues, and between the diabetic male versus diabetic female tissues (Fig. 3.3).

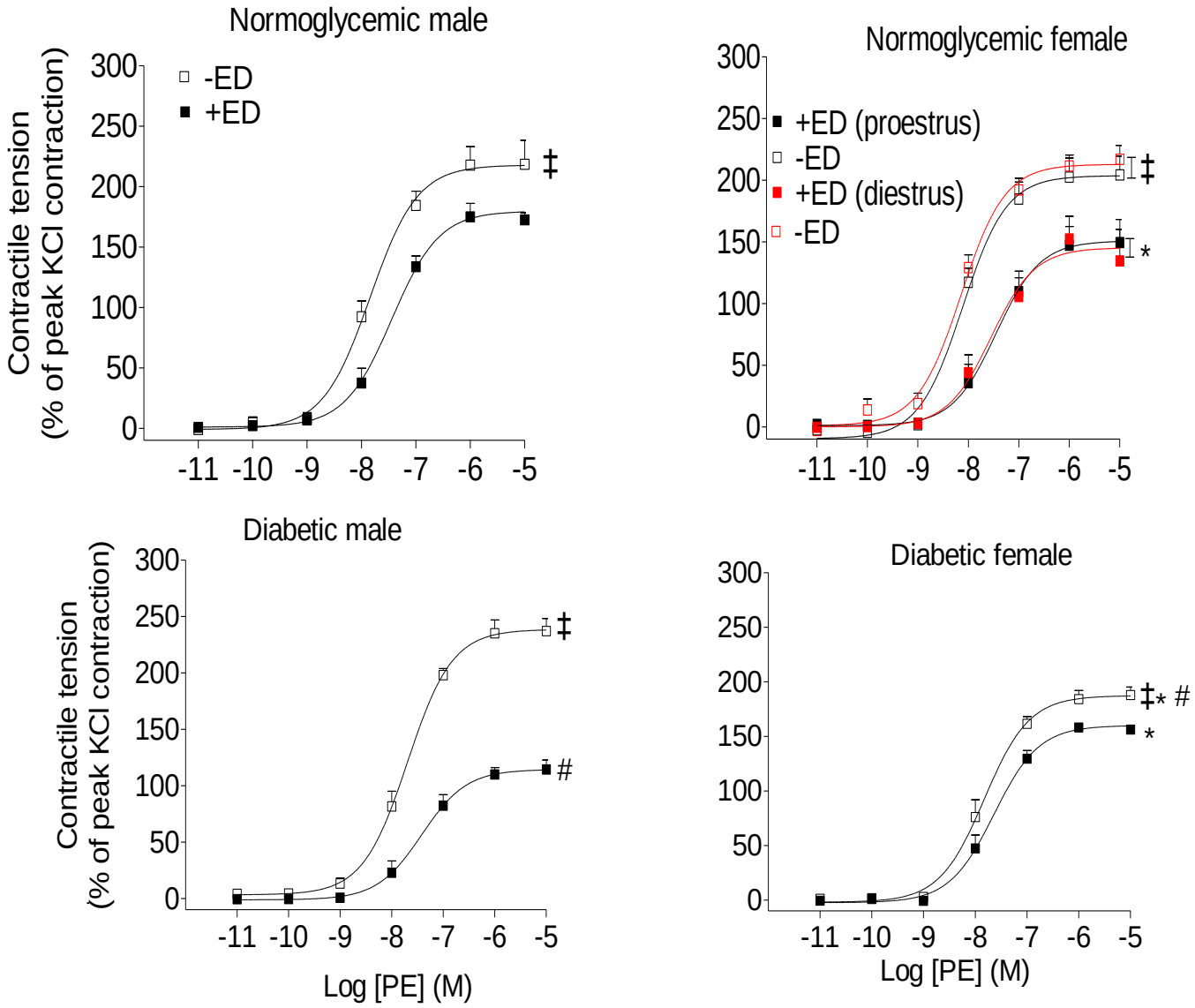

Figure 3.3: Cumulative phenylephrine (PE) (10^{-11}-10^{-5} M) concentration response curves of endothelium-intact (+ED) or –denuded (-ED) aortic rings from male/female normoglycemic (proestrus/diestrus) (upper panel) or diabetic (lower panel) WKY rats. Data represent mean ± SEM (n = 5 -11) in each group. Statistics: *$p < 0.01$, female compared with corresponding male tissues; $^{\#}p < 0.001$, compared with the corresponding male/female normoglycemic group, $^{‡}p < 0.01$, compared with endothelium-intact corresponding male/female normoglycemic or diabetic group.

3.3.2. Ang II-induced contraction

Both normoglycemic and diabetic male and female tissues (with or without endothelium) produced concentration-dependent contractions to Ang II (Fig. 3.4).

In the normoglycemic group, contraction to Ang II was greater in endothelium-intact normoglycemic male (Emax = 70.1 ± 3.0 %) compared to proestrus female (Emax = 40.7 ± 5.2 %) but not the diestrus female tissues (Emax = 128.6 ± 2.9 %) (Fig.3.4). Endothelium denudation similarly enhanced contraction in male (Emax = 105.0 ± 9.7 %) and proestrus female (Emax = 84.1 ± 4.0 %), but did not change it in diestrus female (Emax = 109.0 ± 11.9 %) tissues. This effectively reversed the difference in normoglycemic tissue contraction between the male and the proestrus female, and reduced it between the proestrus and diestrus female groups (Fig. 3.4).

Ang II-induced contraction was similar in endothelium-intact diabetic tissues from both genders (male Emax = 37.8 ± 5.6 % versus female Emax = 43.9 ± 3.5 %) (Fig. 3.4). Male tissue contraction was lesser than contraction seen in the corresponding normoglycemic controls, but normoglycemic (proestrus)/diabetic female tissue contracted similarly. Endothelial denudation equally enhanced contraction of diabetic male (Emax = 133.0 ± 9.0 %) and female (Emax = 113.1 ± 4.1 diestrus) tissues, thereby abolishing the contractile difference between the normoglycemic versus diabetic tissues from both genders (Fig. 3.4). Since diabetes caused a permanent diestrus state in the diabetic female, responses in normoglycemic (proestrus) tissues were used as controls for diabetic tissue responses in order to reflect estradiol-mediated difference.

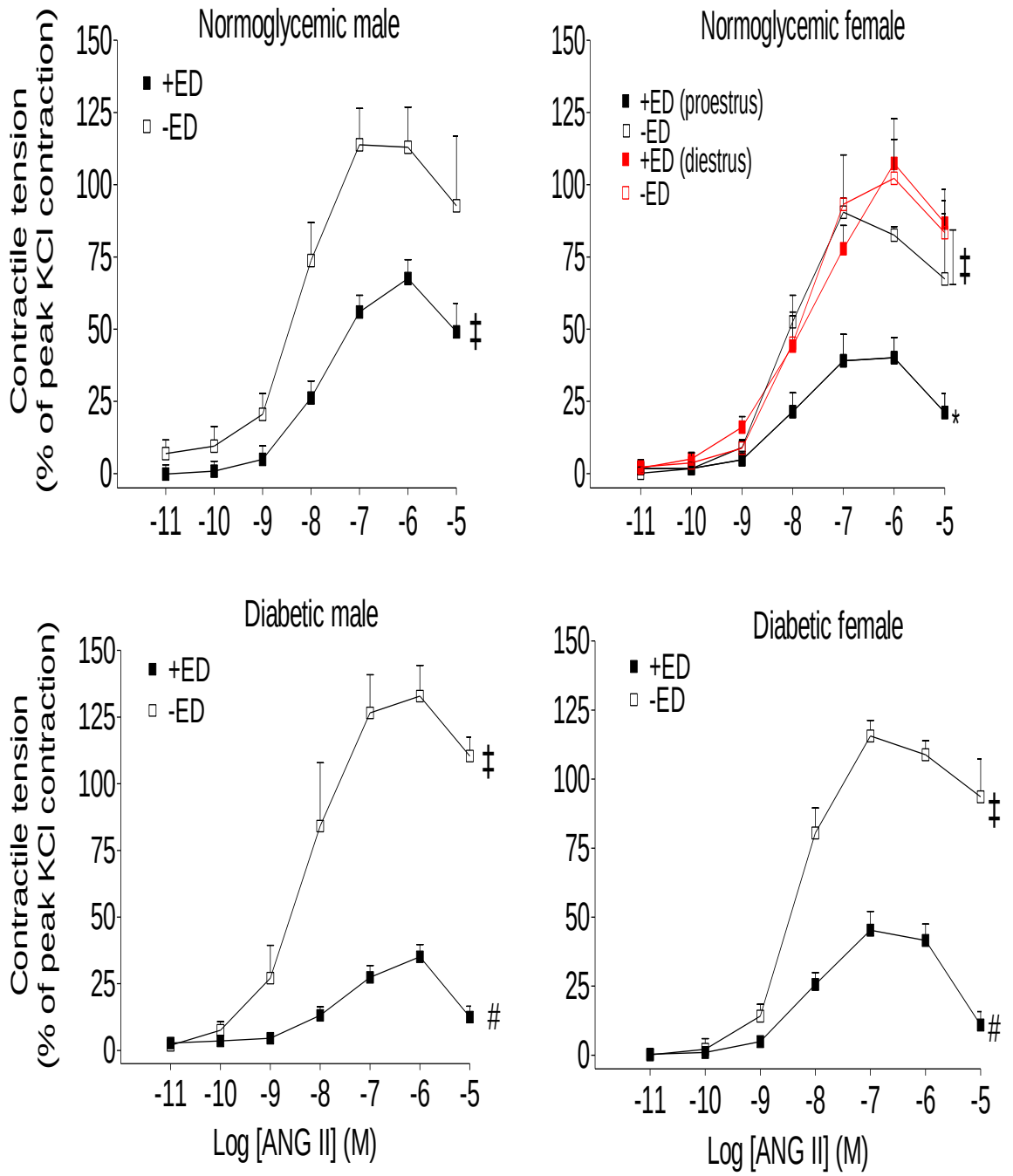

Figure 3.4: Cumulative angiotensin II (Ang II) (10^{-11}-10^{-5} M) concentration response curves of endothelium-intact (+ED) or –denuded (-ED) aortic rings from male / female normoglycemic (proestrus / diestrus) (upper panel) or diabetic (lower panel) WKY rats. Data represent mean ± SEM (n= 5-10) in each group. Statistics: *$p < 0.001$, normoglycemic (proestrus) or diabetic female compared to corresponding male; $^{\#}p < 0.001$, compared with the corresponding male/female normoglycemic group, $^{‡}p < 0.01$, compared with endothelium-intact corresponding normoglycemic or diabetic male/female group.

3.3.3. The effect of gender on quercetin vasodilator action

3.3.3.1. Concentration-dependent effects of quercetin (10^{-8}-10^{-3}M) on PE-induced contraction

The vasodilator activity of quercetin was tested in pre-contracted male and female aortic rings (Section 2.5.5). In both genders, quercetin (10^{-8}-10^{-3}M) concentration-dependently relaxed PE (10^{-6} M) contraction in endothelium-intact and -denuded normoglycemic and diabetic tissues (Fig. 3.5). Repeated exposure of tissues to DMSO (up to 0.05 % (v/v)) over the experiment time frame (time control) yielded no responses, and hence was removed from the graph.

At the maximum concentration tested (10^{-3} M), quercetin induced a higher percentage relaxation of PE-induced contraction in endothelium-intact normoglycemic male (Emax = 70 %) compared to the female (Emax = 32 %). In endothelium-denuded tissues, the quercetin-induced relaxation was comparable in both genders (male Emax = 53 % versus female Emax = 69 %) (Fig. 3.5 / Table 3.2).

In the diabetic group, the order of relaxation induced by quercetin is: endothelium-intact female (Emax = 85 %) > male (Emax = 66 %) and endothelium-denuded male (Emax =71 %) > female (Emax = 55 %) tissues (Fig. 3.5 / Table 3.2).

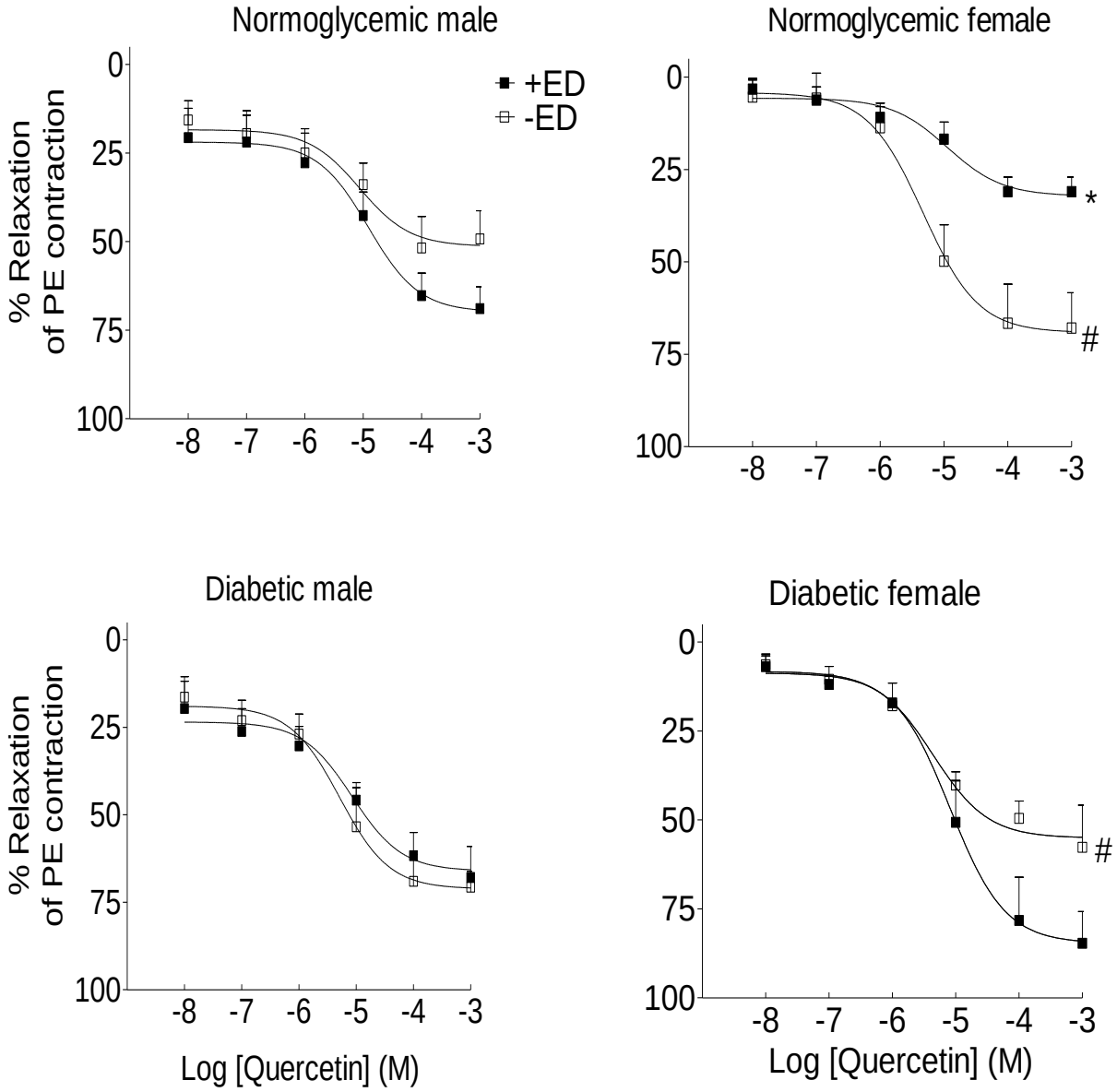

Figure 3.5: Relaxant responses to quercetin (10^{-8}-10^{-3} M) in endothelium-intact (+ED) or -denuded (-ED) aortic rings from male and female normoglycemic (upper panel) or diabetic (lower panel) WKY rats pre-contracted with phenylephrine (1 µM). Data are shown as mean ± SEM (n = 6 - 8). Statistics: $^{*}p < 0.01$, female compared with the male. $^{\#}p < 0.01$, endothelium-denuded versus -intact tissues. Repeated exposure of tissues to DMSO (up to 0.05 % (v/v)) over the experiment time frame (time control) yielded no responses, and hence was removed from the graph.

Table 3.2: Relaxant responses (Emax and pEC_{50} values) of quercetin (10^{-8}-10^{-3} M) in endothelium-intact or -denuded normoglycemic/diabetic male and female WKY aortic rings pre-contracted with phenylephrine (10^{-6} M).

Experimental groups	Male		Female	
	Emax ±SEM	**pEC_{50} ± SEM**	**Emax ±SEM**	**pEC_{50} ± SEM**
Normoglycemic				
Endothelium-intact	70.0 ± 6.5	4.9 ± 0.3	32.2 ± 3.2*	5.0 ± 0.3
Endothelium-denuded	52.6 ± 5.4	5.1 ± 0.4	69.1 ± 6.0$^{\#}$	5.3 ± 0.3$^{\#}$
Diabetic				
Endothelium-intact	66.1 ± 5.8	5.1 ± 0.3	84.7 ± 7.0‡	5.1 ± 0.3
Endothelium-denuded	71.1 ± 6.2	5.3 ± 0.4	55.0 ± 4.8$^{\#}$	5.4 ± 0.3$^{\#}$

3.3.3.2. Effects of sub-maximal concentrations of quercetin and/or 17β-estradiol on agonist-induced contraction

Endothelium-dependent and -independent responses to quercetin (10^{-5} M) and/or estradiol (17β-estradiol) (10^{-7} M) in aorta contracted with PE (Fig. 3.6a / 3.6b) or Ang II (Fig. 3.7) was obtained from normoglycemic and diabetic male/female groups. In tissues from both genders, DMSO (upto 0.05 % (v/v) which served as vehicle for quercetin or exogenous estradiol treatment) did not alter PE (Table 3.3) or Ang II (Table 3.4) contractions in endothelium-intact or –denuded tissues, hence responses to PE or Ang II (without DMSO) was used as controls for studies on these tissues.

3.3.3.2.1. PE-induced contraction

In the normoglycemic group, pre-treatment of tissues with quercetin attenuated the PE concentration-response curve in endothelium-intact male but not the female tissues (Fig. 3.6a / Table 3.3). Exogenous estradiol alone or its combination with quercetin attenuated contraction (Emax and tissue sensitivity (pEC_{50}) in male but neither of these treatments had any effect on the contraction in female tissues to PE (Fig. 3.6a / Table 3.3).

In male tissues, endothelium removal did not alter the prodilator effect of quercetin, but it enhanced the effects of estradiol or its combination with quercetin. These treatments produced relaxation of female tissues which originally were unresponsive to either of these treatments in the presence of endothelium (Fig. 3.6b / Table 3.3).

In the diabetic group, quercetin-induced effect was marginally greater in endothelium-intact female tissues (where quercetin caused 75 % inhibition of contraction (Emax)) compared to (51 % in) male. Estradiol significantly enhanced contraction (Emax) in endothelium-intact male but had no effect on female tissues (Fig. 3.6a / Table 3.3). In both genders, estradiol significantly reduced quercetin relaxation of endothelium-intact tissues (Fig. 3.6a / Table 3.3). Pre-treatment of endothelium-denuded tissues with estradiol and/or quercetin produced similar vasorelaxant effects in male/ female tissues (Fig. 3.6b / Table 3.3).

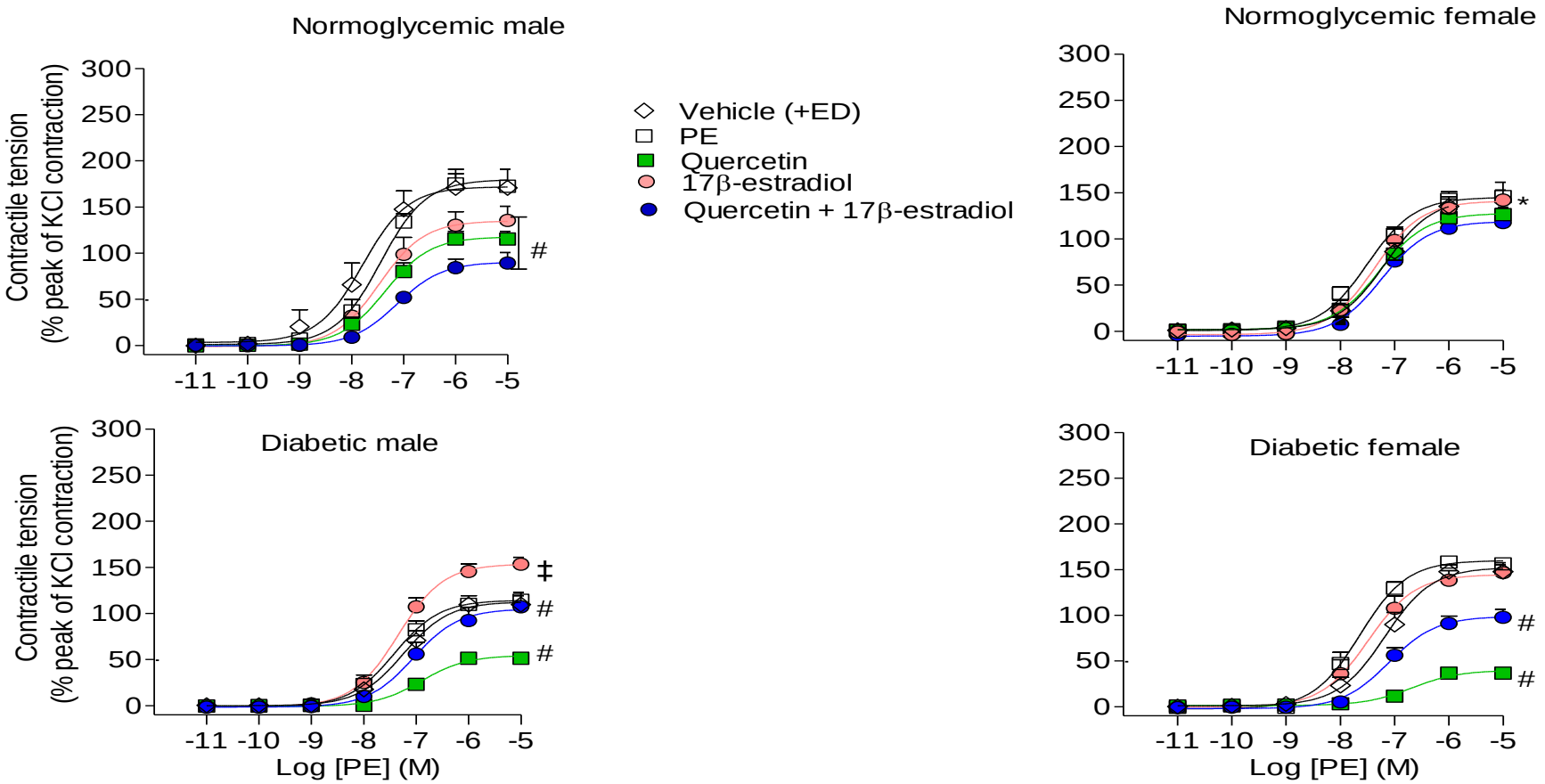

Figure 3.6a: Contractile responses of endothelium-intact normoglycemic/diabetic male and female tissues to phenylephrine (PE) in the presence of vehicle (DMSO < 0.05 % (v/v), 17 β-estradiol (10^{-7} M), quercetin (10^{-5} M) or 17 β-estradiol + quercetin. Data are shown as mean ± SEM (n= 5 -20 in each group). Statistics: *$p < 0.01$, female compared with the male; $^{\#}p < 0.001$ or ǂ$p < 0.001$, compared with corresponding male or female normoglycemic or diabetic controls.

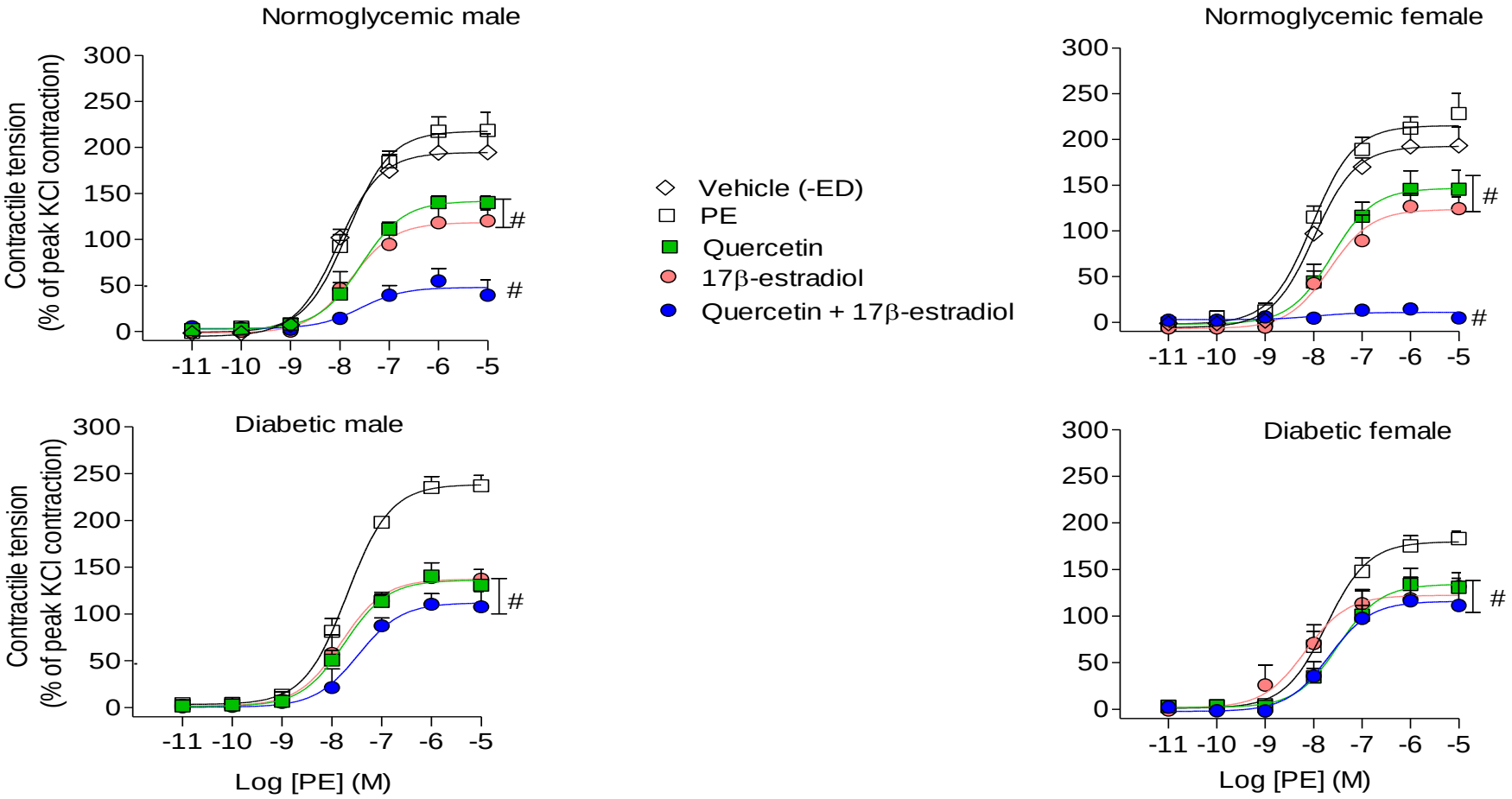

Figure 3.6b: Contractile responses of endothelium-denuded normoglycemic/diabetic male and female tissues to phenylephrine (PE) in the presence of vehicle (DMSO < 0.05 % (v/v)), 17 β-estradiol (10^{-7} M), quercetin (10^{-5} M) or 17 β-estradiol + quercetin. Data are shown as mean ± SEM (n= 4 -9 in each group).Statistics: *$p < 0.01$, female compared with the male; $^{\#}p < 0.001$, compared with corresponding male or female normoglycemic / diabetic controls.

Table 3.3: Emax and pEC_{50} values derived from PE (10^{-11}-10^{-5}M)-induced contraction of endothelium-intact /-denuded male / female normoglycemic /STZ-induced diabetic WKY aorta in absence or presence of vehicle (DMSO < 0.05 % (v/v)), quercetin and/or17 β-estradiol.

Rats	Treatment group	Male		Female	
Normoglycemic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (PE)	179.7 ± 7.1	7.4 ± 0.1	145.0 ± 4.3*	7.5 ± 0.1
	Vehicle	171.8 ± 11.1	7.8 ± 0.2#	135.6 ± 5.5*	7.4 ± 0.1*
	Quercetin	117.6 ± 4.6#	7.4 ± 0.1	127.7 ± 3.2	7.3 ± 0.1
	17β-estradiol	134.9 ± 8.8#	7.5 ± 0.2	141.1 ± 8.0	7.4 ± 0.2
	Quercetin+ 17β-estradiol	90.3 ± 4.6#	7.1 ± 0.1#	119.0 ± 4.5	7.2 ± 0.1
Endothelium-denuded	Control (PE)	217.7 ± 7.4ǂ	7.8 ± 0.1ǂ	215.1 ± 7.3ǂ	8.0 ± 0.1ǂ
	Vehicle	194.5 ± 8.0	8.0 ± 0.1	192.5 ± 7.3	8.0 ± 0.1
	Quercetin	141.7 ± 4.6#	7.8 ± 0.1	153.6 ± 8.2#	7.7 ± 0.2#
	17β-estradiol	118.4 ± 8.1#	7.8 ± 0.2	123.4 ± 10.5#	7.7 ± 0.2#
	Quercetin+ 17β-estradiol	47.8 ± 6.4#	7.6 ± 0.4	10.9 ± 2.4#	7.9 ± 0.9

Values represent mean ± SEM (n = 4 -20). Statistics: *p< 0.001, female compared to male tissues; #p < 0.01, compared with corresponding male/ female normoglycemic or diabetic controls; ǂp < 0.001, compared with corresponding endothelium-intact tissues.

Table 3.3 (continued): Emax and pEC_{50} values derived from PE (10^{-11}-10^{-5}M)-induced contraction of endothelium-intact /-denuded male / female normoglycemic /STZ-induced diabetic WKY aorta in presence or absence of vehicle (DMSO < 0.05 % (v/v)), quercetin and/or 17 β-estradiol.

Rats	Treatment group	Male		Female	
Diabetic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (PE)	111.4 ± 5.6#	7.4 ± 0.1	159.9 ± 4.6*	7.6 ± 0.1#
	Vehicle	112.6 ± 5.3	7.4 ± 0.1	152.3 ± 5.6	7.2 ± 0.1
	Quercetin	54.6 ± 3.0#	6.9 ± 0.1#	39.8 ± 3.5#	6.7 ± 0.2#
	17β-estradiol	153.6 ± 4.5#	7.5 ± 0.2	144.6 ± 6.9	7.5 ± 0.1
	Quercetin+ 17β-estradiol	105.0 ± 7.5#	7.1 ± 0.2#	98.8 ± 4.4#	7.1 ± 0.1#
Endothelium-denuded	Phenylephrine	238.4 ± 5.6ǂ	7.7 ± 0.1ǂ	179.8 ± 6.5	7.7 ± 0.1
	Vehicle	ND	ND	ND	ND
	Quercetin	136.1 ± 6.5#	7.8 ± 0.1	134.2 ± 5.1#	7.7 ± 0.2
	17β-estradiol	137.3 ± 5.6#	7.8 ± 0.1	122.3 ± 9.1#	8.2 ± 0.2#
	Quercetin+ 17β-estradiol	111.9 ± 8.9	7.5 ± 0.2#	115.9 ± 14.6	7.7 ± 0.4#

Values represent mean ± SEM (n = 4-20). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.01$, compared with corresponding male/ female normoglycemic or diabetic controls; ǂ$p < 0.001$, compared with corresponding endothelium-intact tissues.

3.3.3.2.2. Ang II-induced contraction

In the normoglycemic group, quercetin attenuated contraction in endothelium-intact male aorta but not the proestrus female. Exogenous estradiol alone or its combination with quercetin had no effect on the contraction of male tissues but it enhanced pEC_{50} in the female (Fig. 3.7 / Table 3.4). Endothelial denudation significantly enhanced contraction in both male and female groups. It failed to abolish quercetin vasorelaxant action in male, but unmasked it in female tissues (Table 3.4). Effect of estradiol and/ or quercetin on contraction of endothelium-denuded normoglycemic tissues was not determined.

In the diabetic group, quercetin significantly inhibited contraction in tissue from both genders with equal magnitude (Fig. 3.7 / Table 3.4). Estradiol had no effect on Emax value of both male/female tissues but it enhanced sensitivity (pEC_{50}) to Ang II in male tissues. Quercetin relaxation of Ang II contraction was inhibited by estradiol in diabetic tissues from both genders (Fig. 3.7 / Table 3.4). Endothelial denudation significantly enhanced contraction but failed to abolish quercetin relaxant effect in diabetic tissue from both genders (Table 3.4). Effect of estradiol and/ or quercetin on contraction of endothelial denuded diabetic tissues was not determined.

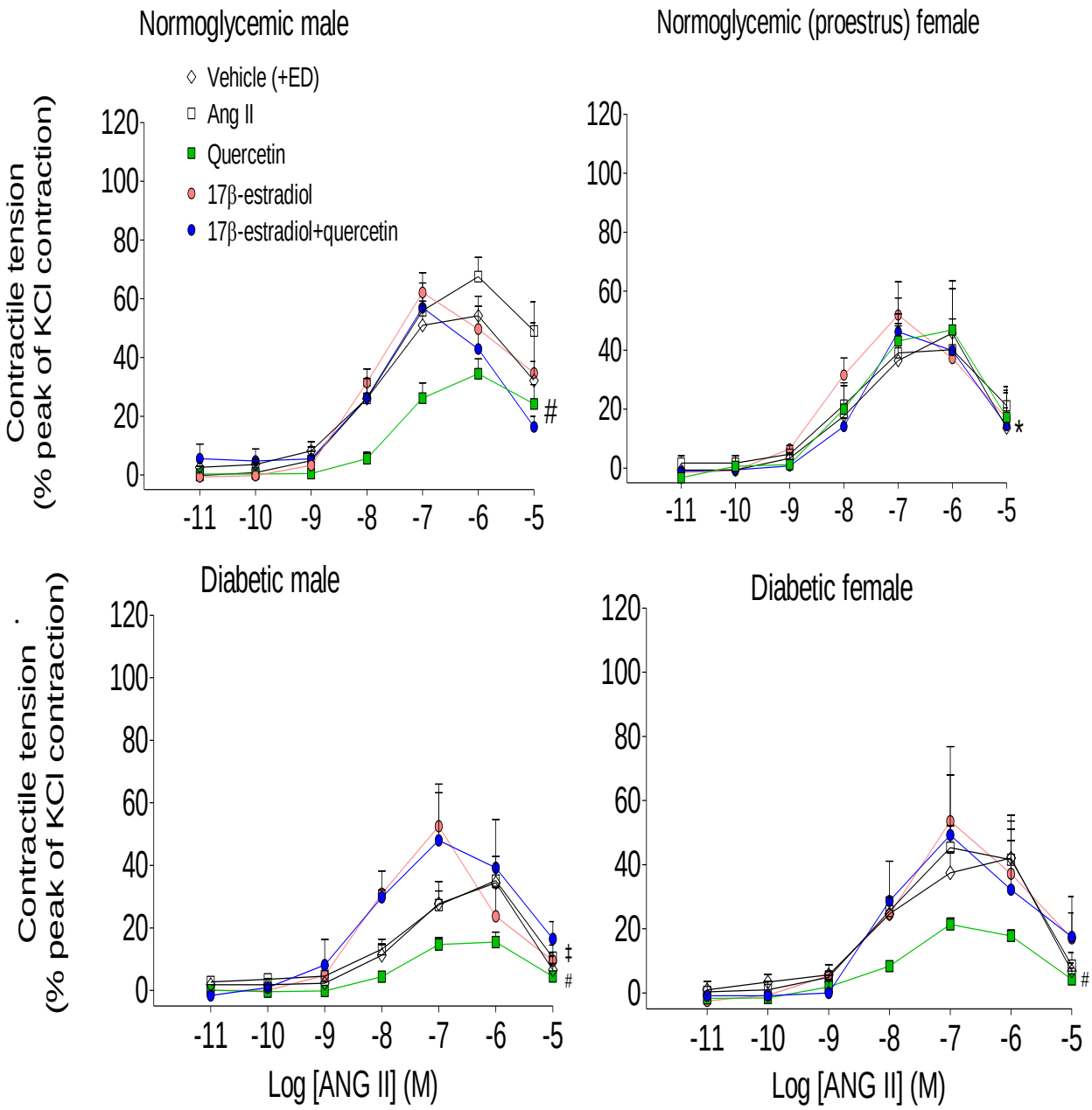

Figure 3.7: Contractile responses of endothelium-intact normoglycemic/diabetic male and female tissues to angiotensin II (Ang II) in the presence of vehicle (DMSO < 0.05 % (v/v %)), 17 β-estradiol (10^{-7} M), quercetin (10^{-5} M) or 17 β-estradiol + quercetin. Data are shown as mean ± SEM. Statistics: *p < 0.01, female compared with the male; $^{\#}$p < 0.001 or ǂp < 0.01, compared with corresponding male / female normoglycemic or diabetic controls.

Table 3.4: Emax and pEC_{50} values derived from Ang II (10^{-11}-10^{-5}M)-induced contraction of endothelium-intact /-denuded male / female normoglycemic /STZ-induced diabetic WKY aorta in absence or presence of quercetin and/or17 β-estradiol.

Rats	Treatment group	Male		Female	
Normoglycemic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (Ang II)	70.1 ± 3. 0	7.9 ± 0.1	40.7 ± 5.2*	8.0 ± 0.2
	Vehicle	55.4 ± 6. 0	7.9 ± 0.1	48.3 ± 14.8	7.7± 0.6
	Quercetin	35.2 ± 3.8#	7.4 ± 0.4#	47.3 ± 9.6	7.9 ± 0.4
	17β-estradiol	56.0 ± 3.7	8.0 ± 0.5	44.5 ± 6.0	8.4 ± 0.4 #
	Quercetin+ 17β-estradiol	50.0 ± 3.4	8.0 ± 0.3	43.1 ± 4.4	8.0 ± 0.9 #
Endothelium-denuded	Control (Ang II)	105.0 ± 9.7 ǂ	8.2 ± 0.2ǂ	84.1 ± 4.0 ǂ	8.1 ± 0.1
	Vehicle	ND	ND	ND	ND
	Quercetin	53.2 ± 9.7#	8.0 ± 0.3	53.0 ± 7.8#	8.0 ± 0.2
	17β-estradiol	ND	ND	ND	ND
	Quercetin+ 17β-estradiol	ND	ND	ND	ND

Values represent mean ± SEM (n = 4-7). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.01$, compared with corresponding male/ female controls; ǂ$p < 0.001$, compared with corresponding endothelium-intact tissues.

Table 3.4 (continued): Emax and pEC_{50} values derived from Ang II (10^{-11}-10^{-5}M)-induced contraction of endothelium-intact /-denuded male / female normoglycemic / STZ-induced diabetic WKY aorta in absence or presence of quercetin and/or17 β-estradiol.

Rats	Treatment group	Male		Female	
Diabetic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (Ang II)	37.8 ± 5.6#	7.5 ± 0.6#	43.9 ± 3.5	8.1 ± 0.2*
	Vehicle	35.4 ± 6.5	7.6 ± 0.4	41.7 ± 5.3	8.1 ± 0.3*
	Quercetin	15.5 ± 1.9#	7.8 ± 0.3#	19.5 ± 1.6#	8.0 ± 3.0
	17β-estradiol	37.8 ± 6.7	8.4 ± 0.5#	45.4 ± 7.7	8.0 ± 0.2*
	Quercetin+ 17β-estradiol	43.9 ± 7.8	8.4 ± 0.5	40.7 ± 10.0	8.1 ± 0.1
Endothelium-denuded	Control (Ang II)	133.0 ± 9.0ǂ	8.3 ± 0.2ǂ	113.1 ± 4.1ǂ	8.3 ± 0.1
	Vehicle	ND	ND	ND	ND
	Quercetin	63.4 ± 6.6#	8.3 ± 0.3	65.8 ± 8.5#	8.1 ± 2.2
	17β-estradiol	ND	ND	ND	ND
	Quercetin+ 17β-estradiol	ND	ND	ND	ND

Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.01$, compared with corresponding male/ female controls; ǂ$p < 0.001$, compared with corresponding endothelium-intact tissues.

3.4. Mechanism underlining gender differences in agonist-induced vasoconstriction and the vascular action of quercetin and/or 17β-estradiol.

3.4.1. Effect of ROS and antioxidant enzyme level

The hypothesis that levels of ROS ($\bullet O_2$-: a major modulator of the bioavailability of other endothelial-derived oxidants) and antioxidants enzymes (SOD and CAT) may be gender differentiated, and hence, may regulate normoglycemic / diabetic tissue contraction or the vasodilator action of quercetin was tested.

3.4.1.1. Effect of diabetes on superoxide anion levels

Superoxide anion ($\bullet O_2^-$) levels was significantly higher in normoglycemic male aorta (homogenized tissue fraction (105.4 ± 14.4 mg/mL) (Fig. 3.8) or whole tissue (106.9 ± 9.0 mg/mL)) (Fig. 3.9) compared to the female (homogenized tissue fraction (59.3 ± 20.7 mg/mL) or whole tissue (76.0 ± 4.8 mg/mL) sample. Diabetes significantly elevated $\bullet O_2^-$ content in male (homogenized tissue fraction (201.8 ± 45.7 mg/mL) (Fig. 3.8) or whole tissue (134.7 ± 39.4 mg/mL) Fig. 3.9) and female (homogenized tissue fraction (155.8 ± 45.7 mg/mL) or whole tissue (133.7 ± 23.1 mg/mL) samples (Fig. 3.8-3.9). Diabetes-induced enhancement of $\bullet O_2^-$ content was significantly higher in female (homogenized fraction = 164 % and whole tissue = 76 %) samples compared to the male (homogenized fraction = 92 % and whole tissue = 26 %).

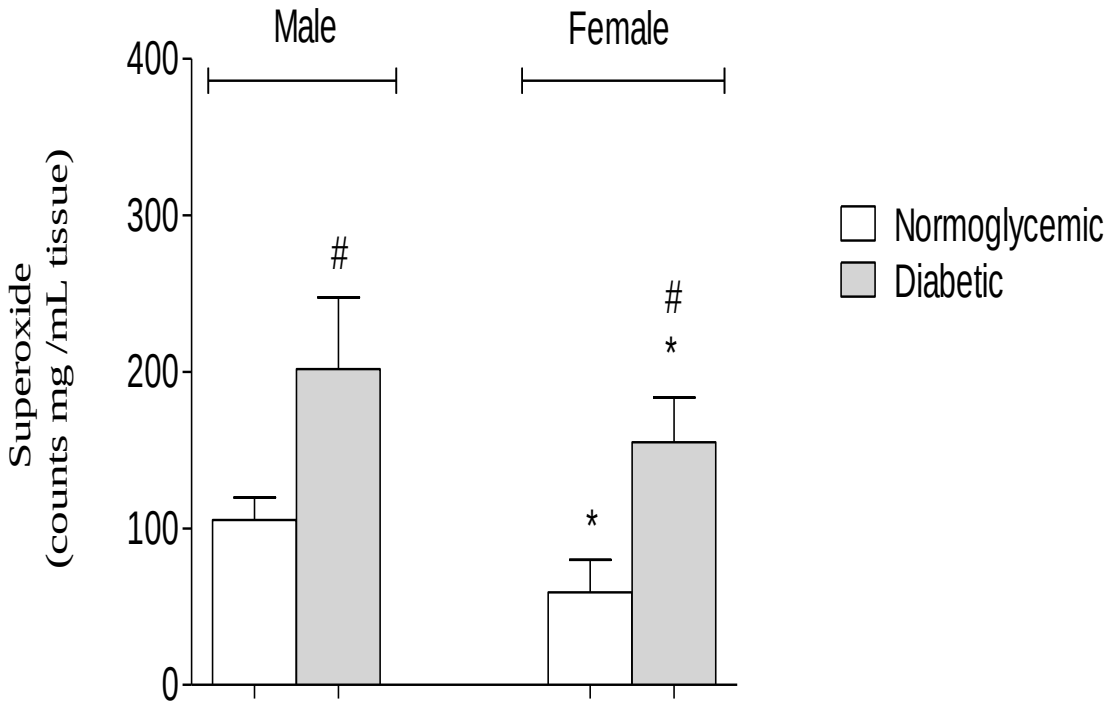

Figure 3.8: Superoxide anion concentration in homogenized aortic tissue supernatant samples from normoglycemic/diabetic male and female animals. Data are shown as mean ± SEM (n = 6 - 8 in each group). Statistics: $^{\#}p < 0.001$, diabetic compared with normoglycemic tissues; $^{*}p < 0.01$, female compared with male.

3.4.1.1.1. Concentration-dependent inhibitory effects of quercetin on superoxide anion levels

Quercetin reduced $\bullet O_2^-$ generation in a concentration-dependent manner in male (normoglycemic/diabetic) and female (diabetic) aorta (Fig. 3.9). In normoglycemic tissues, quercetin caused a much higher percentage lowering of $\bullet O_2^-$ generation in male than female tissues with significant difference between male and female tissues for all except the lowest concentration (10^{-8} M) (which significantly elevated $\bullet O_2^-$ in the female).

Quercetin inhibition of $\bullet O_2^-$ levels was comparable in diabetic tissues from both genders (Fig. 3.9). At the highest concentration tested (10^{-4} M), the order (%) of quercetin inhibition of $\cdot O_2^-$ in aortic tissues is: diabetic male (98 %) > diabetic female (96 %) > normoglycemic male (94 %) > normoglycemic female (52 %). The % reduction of $\bullet O_2^-$ generation by the NADPH oxidase inhibitor, DPI, was significantly greater in diabetic compared to normoglycemic tissues (Fig. 3.9). The order of DPI-induced lowering of $\bullet O_2^-$ production is: diabetic male (98 %) > diabetic female (95 %) > normoglycemic male (81 %) > normoglycemic female (49 %).

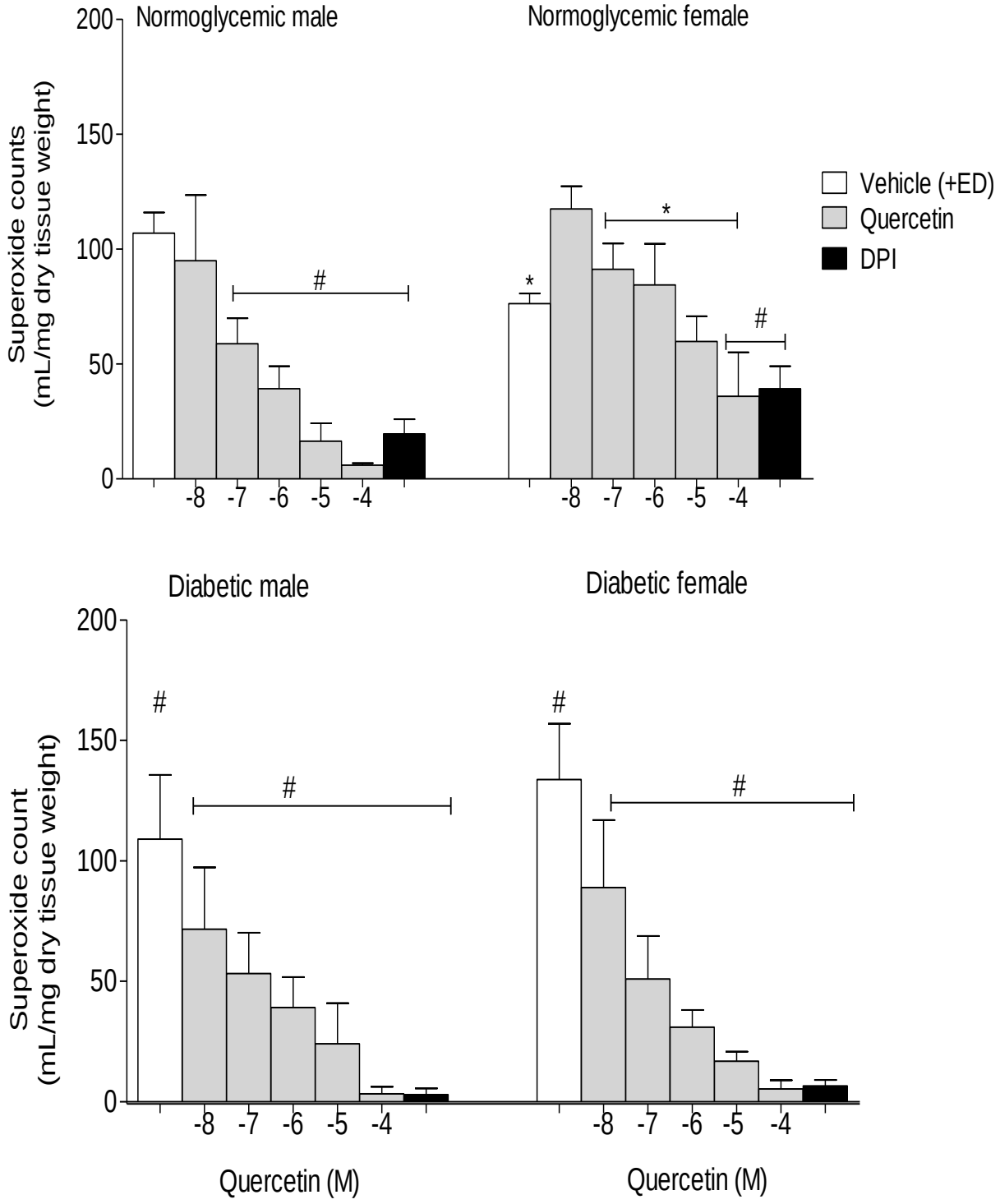

Figure 3.9: Levels of superoxide anion generated by endothelium-intact (+ED) normoglycemic (upper row) and diabetic (lower row) male/ female aortic tissue samples in the absence (vehicle, DMSO < 0.05 % (v/v)) or presence of quercetin (10^{-8} – 10^{-4} M) or DPI (NADPH oxidase inhibitor) (5 x 10^{-6} M), measured using lucigenin-enhanced chemiluminescence. Data are shown as mean ± SEM (n = 4 - 9). Statistics: $^{\#}p < 0.01$, compared with corresponding male or female control (vehicle-treated) tissues; $^{*}p < 0.05$, male compared with the female.

3.4.1.2. Effect of gender and diabetes on antioxidant enzyme levels

In the normoglycemic group, tissue SOD activity was lower in male (2.1 ± 0.2 U/mg) compared to the female (2.9 ± 0.1 U/mg) (Fig. 3.10), but CAT activity were similar in male (19.2 ± 0.7 U/s/mg) and female (19.5 ± 0.4 U/s/mg) tissues (Fig. 3.10).

Diabetes significantly reduced SOD activity more in female tissues (1.4 ± 0.1 U/mg) (52 % reduction) than the male (1.6 ± 0.2 U/mg) (24 % reduction). CAT activity was also reduced in tissues from both genders (female (14.9 ± 0.7 U/s/mg) (24 % reduction) versus male (15.3 ± 1.2 U/s/mg) (20 % reduction)) (Fig. 3.10).

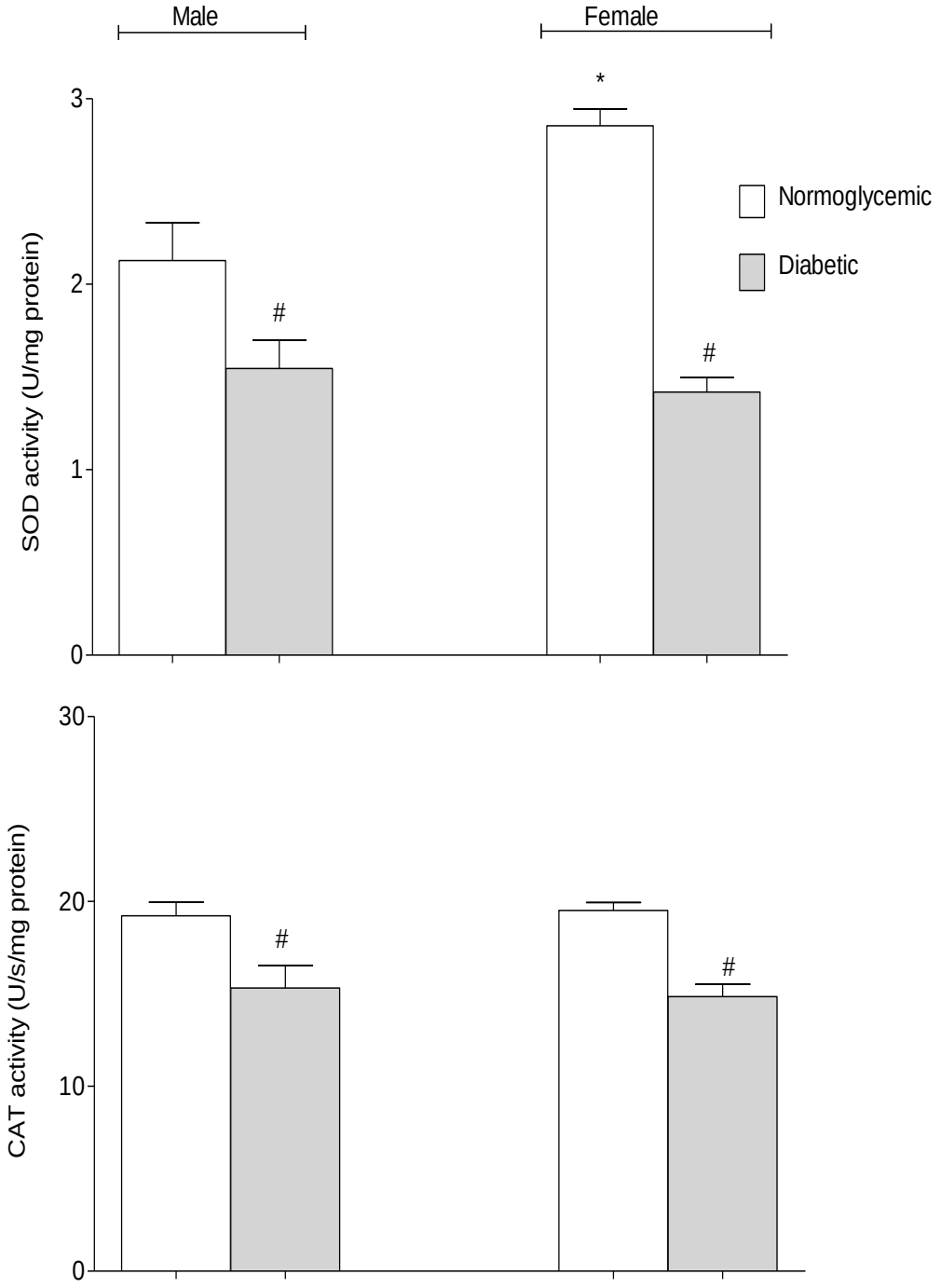

Figure 3.10: Superoxide dismutase (SOD) (upper row) and catalase (CAT) (lower row) activity in aortic tissue homogenate samples from normoglycemic/diabetic male and female rats. Data are shown as mean ± SEM (n= 6 - 8 in each group). Statistic: $^{\#}p < 0.05$, diabetic compared with normoglycemic tissues; $^{*}p < 0.001$, female compared with male.

3.4.1.3. Effect of the inhibition of ROS on agonist-induced contraction

The direct role of reactive oxygen species (ROS) ($\bullet O_2^-$ and/or H_2O_2) in modulating gender differences in PE or Ang II–induced contraction and quercetin action was measured. Endothelium-intact male and female tissues from respective groups were incubated with SOD and/or CAT, following which concentration responses to PE or Ang II (10^{-11}-10^{-5} M) were assessed (Section 2.5.4.4).

3.4.1.3.1. PE-induced contraction

In normoglycemic male, PE contraction was significantly reduced in the presence of SOD and/or CAT, in contrast to the female, where only CAT treatment attenuated contraction at the highest dose tested (Table 3.5). In the male, quercetin attenuation of PE contraction was significantly enhanced with SOD or SOD + CAT but not CAT treatment. In the female, SOD, CAT or both unmasked a quercetin relaxant effect (Table 3.5).

In diabetic male, SOD and/or CAT enhanced contraction in contrast to their effects in corresponding normoglycemic control. In the diabetic female, PE-induced contraction was reduced with SOD + CAT but was unchanged with SOD or CAT treatment. In diabetic tissues from both genders, SOD and/or CAT treatment significantly reduced quercetin relaxant effect on PE contraction (Table 3.5).

Table 3.5: Emax and pEC_{50} values derived from phenylephrine (10^{-11}-10^{-5}M)-induced contraction of endothelium-intact male / female normoglycemic /STZ-induced diabetic WKY aorta in absence or presence of quercetin, SOD, CAT, SOD + quercetin, CAT + quercetin or SOD + CAT + quercetin.

Rats	Treatment group	Male		Female	
	Normoglycemic	Emax ± SEM	pEC_{50} ± SEM	Emax ± SEM	pEC_{50} ± SEM
Endothelium intact	Control (PE)	179.7 ± 7.1	7.4 ± 0.1	145.0 ± 4.3*	7.5 ± 0.1
	Quercetin	117.6 ± 4.6#	7.4 ± 0.1	127.7 ± 3.2	7.3 ± 0.1
	SOD	114.5 ± 5.6#	7.6 ± 0.1	121.7 ± 7.9	7.2 ± 0.1*
	SOD + Quercetin	84.4 ± 6.9#	7.2 ± 0.2#	77.1 ± 8.7#	7.1 ± 0.2
	CAT	129.0 ± 4.7#	7.6 ± 0.1	117.5 ± 7.7#	7.5 ± 0.1
	CAT+ Quercetin	106.2 ± 7.7	7.4 ± 0.2	82.4 ± 7.1#	7.0 ± 0.1#
	SOD + CAT	121.4 ± 12.5#	7.2 ± 0.2	123.1 ± 5.3	7.2 ± 0.1
	SOD + CAT+ Quercetin	92.5 ± 5.5 #	7.2 ± 0.1	70.5 ± 11.0#	7.4 ± 0.3

Values represent mean ± SEM (n = 6 -20). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.001$, compared with corresponding male/ female control group.

Table 3.5 (continued): Emax and pEC_{50} values derived from phenylephrine (PE) (10^{-11}-10^{-5}M)-induced contraction of endothelium-intact male / female normoglycemic / STZ-induced diabetic WKY in absence or presence of quercetin, SOD, CAT, SOD + quercetin, CAT + quercetin or SOD + CAT + quercetin.

Rats	Treatment group	Male		Female	
	Diabetic	Emax ± SEM	pEC_{50} ± SEM	Emax ± SEM	pEC_{50} ± SEM
Endothelium intact	Control (PE)	112.6 ± 5.3	7.4 ± 0.1	159.9 ± 4.6*	7.6 ± 0.1
	Quercetin	54.6 ± 3.0$^{\#}$	6.9 ± 0.1$^{\#}$	39.8 ± 3.4$^{\#}$	7.2 ± 0.2$^{\#}$
	SOD	154.0 ± 3.6$^{\#}$	7.6 ± 0.1	137.7± 2.9	7.4 ± 0.1
	SOD + Quercetin	104.0 ± 7.9$^{\#}$	7.2 ± 0.1$^{\#}$	94.7 ± 5.8$^{\#}$	7.1 ± 0.2$^{\#}$
	CAT	154.0 ± 4.6$^{\#}$	7.6 ± 0.1	142.5 ± 5.2	7.6 ± 0.1
	CAT+ Quercetin	108.6 ± 6.7$^{\#}$	7.3 ± 0.2$^{\#}$	94.5 ± 3.5$^{\#}$	7.2 ± 0.1$^{\#}$
	SOD + CAT	144.5 ± 6.6$^{\#}$	7.5 ± 0.1	118.1 ± 2.7$^{\#}$	7.3 ± 0.1$^{\#}$
	SOD + CAT+ Quercetin	109.4± 8.4$^{\#}$	7.3 ± 0.2	90.3 ± 4.1$^{\#}$	7.1 ± 0.1

Values represent mean ± SEM (n = 6 - 20). Statistics: *$p < 0.001$, female compared to male tissues; $^{\#}p < 0.001$, compared with corresponding male/ female control group.

3.4.1.3.2. Ang II-induced contraction

In normoglycemic male tissue, Ang II contraction was unchanged by SOD or CAT (SOD + CAT: not determined), in contrast to female tissues, where either of these treatments augmented contraction (Emax) (Table 3.6). In male tissues, quercetin relaxant effect (Emax) was unchanged with SOD or CAT treatment. In the female tissues, where SOD or CAT enhanced contraction, quercetin relaxant effect was unmasked compared to its lack of effect in untreated tissues (Table 3.6).

In the diabetic group, Ang II contraction was augmented by CAT (Emax / pEC50) or SOD (pEC_{50}) in male tissues (SOD + CAT: not determined) (Table3.6). SOD or CAT treatment did not alter female tissue response to Ang II. In male tissues, quercetin relaxation of Ang II contraction was unchanged by SOD, but CAT significantly inhibited it in diabetic female tissues. SOD enhanced quercetin relaxant action (Emax) but CAT attenuated it.

Table 3.6: Emax and pEC_{50} values derived from Ang II (10^{-11}-10^{-5}M)-induced contraction of endothelium-intact male / female normoglycemic /STZ-induced diabetic WKY aorta in absence or presence of quercetin, SOD, CAT, SOD + quercetin, CAT + quercetin or SOD + CAT + quercetin.

Rats	Treatment group	Male		Female	
		Emax ± SEM	pEC_{50} ± SEM	Emax ± SEM	pEC_{50} ± SEM
	Normoglycemic				
Endothelium intact	Control (Ang II)	70.1 ± 3.0	7.9 ± 0.1	40.7 ± 5.2*	8.0 ± 0.2
	Quercetin	35.2 ± 3.8$^{\#}$	7.4 ± 0.2$^{\#}$	47.3 ± 9.6	7.9 ± 0.4
	SOD	67.3 ± 13.0	8.0 ± 0.3	66.0 ± 7.4$^{\#}$	8.0 ± 0.4
	SOD + Quercetin	23.4 ± 4.0$^{\#}$	8.0 ± 0.3	36.5 ± 7.2$^{\#}$	8.0 ± 0.4
	CAT	61.1 ± 8.2	8.0 ± 0.3	77.7 ± 6.2$^{\#}$	8.2 ± 0.2
	CAT+ Quercetin	26.8 ± 9.6$^{\#}$	8.6 ± 0.9$^{\#}$	41.1 ± 6.0$^{\#}$	8.2 ± 0.3
	Diabetic				
Endothelium intact	Control (Ang II)	37.8 ± 5.6	7.5 ± 0.6	43.9 ± 3.5	8.1 ± 0.2*
	Quercetin	15.5 ± 1.9$^{\#}$	7.8 ± 0.3$^{\#}$	19.5 ± 1.6$^{\#}$	8.0 ± 0.3
	SOD	44.2 ± 10.5	8.0 ± 0.6$^{\#}$	37.2 ± 9.1	8.1 ± 0.1
	SOD + Quercetin	22.1± 2.5$^{\#}$	7.9 ± 0.4	4.7 ± 1.8$^{\#*}$	9.4 ± 0.2$^{\#}$
	CAT	75.8 ± 5.4$^{\#}$	7.8 ± 0.2$^{\#}$	45.9 ± 4.2*	8.2 ± 0.5*
	CAT+ Quercetin	49.1 ± 5.0$^{\#}$	7.8 ± 0.3	39.6 ± 7.5	8.0 ± 0.3

Values represent mean ± SEM (n = 6- 10). Statistics: *$p < 0.001$, female compared to male tissues; $^{\#}p < 0.05$, compared with corresponding male/ female control group.

3.4.2. Effect of nitric oxide pathway

3.4.2.1. PE-induced contraction

Both male and female endothelium-intact/-denuded normoglycemic and diabetic aorta showed concentration-dependent contractions to PE in the presence of L-NAME or MB (Fig. 3.11).

In the normoglycemic group, L-NAME or MB treatment significantly enhanced contraction in male (pEC_{50}) and female (Emax / pEC_{50}) tissues. This was more so in endothelium-intact female, where L-NAME or MB enhanced contraction by 22 % or 30 % compared to the male (L-NAME = 4 %; MB = <0 %) (Fig. 3.11 / Table 3.7). Inhibition of eNOS or sGC activity with L-NAME or MB respectively, significantly reduced quercetin action in male tissues, with quercetin causing approximately 18 % or 3 % reduction of contraction (Emax) in L-NAME or MB-treated tissues as against 34 % in the untreated tissues (Table 3.7). In the female, % quercetin-induced effect in L-NAME (15 %) or MB (19 %)-treated tissues was comparable with its effect (13 % reduction of contraction) in untreated female controls (Fig. 3.11 / Table 3.7). In endothelium-denuded tissues from both genders, MB treatment attenuated contraction, while L-NAME did not alter it (Fig. 3.11/ Table 3.7). Quercetin relaxant action was abolished by L-NAME or MB in endothelium-denuded tissues from both genders (Table 3.7).

In the diabetic group, L-NAME or MB significantly enhanced contraction more in endothelium-intact male tissues (L-NAME = 39 %; MB = 31 %) compared to the female (L-NAME = 11 %; MB = <0 %) (Fig. 3.11 / Table 3.7). In effect, pre-treatment of endothelium-intact tissues with either L-NAME or MB abolished the observed

gender differences in contraction between normoglycemic male versus the female / the diabetic male, and between diabetic male versus the female. L-NAME or MB significantly reduced quercetin action in diabetic tissues from both genders. In the diabetic male, quercetin caused a 5 % or 1 % reduction of contraction (Emax) in L-NAME or MB-treated tissues as against 51 % in the untreated male tissues. In the diabetic female, quercetin produced a 17 % or 4 % reduction of contraction in L-NAME or MB-treated tissues as against 75 % in the untreated female tissues (Fig. 3.11 / Table 3.7). Further, in endothelium-denuded tissues from both genders, MB (L-NAME = not determined) significantly attenuated PE-induced contraction but failed to alter quercetin relaxant effect in these tissues (Table 3.7).

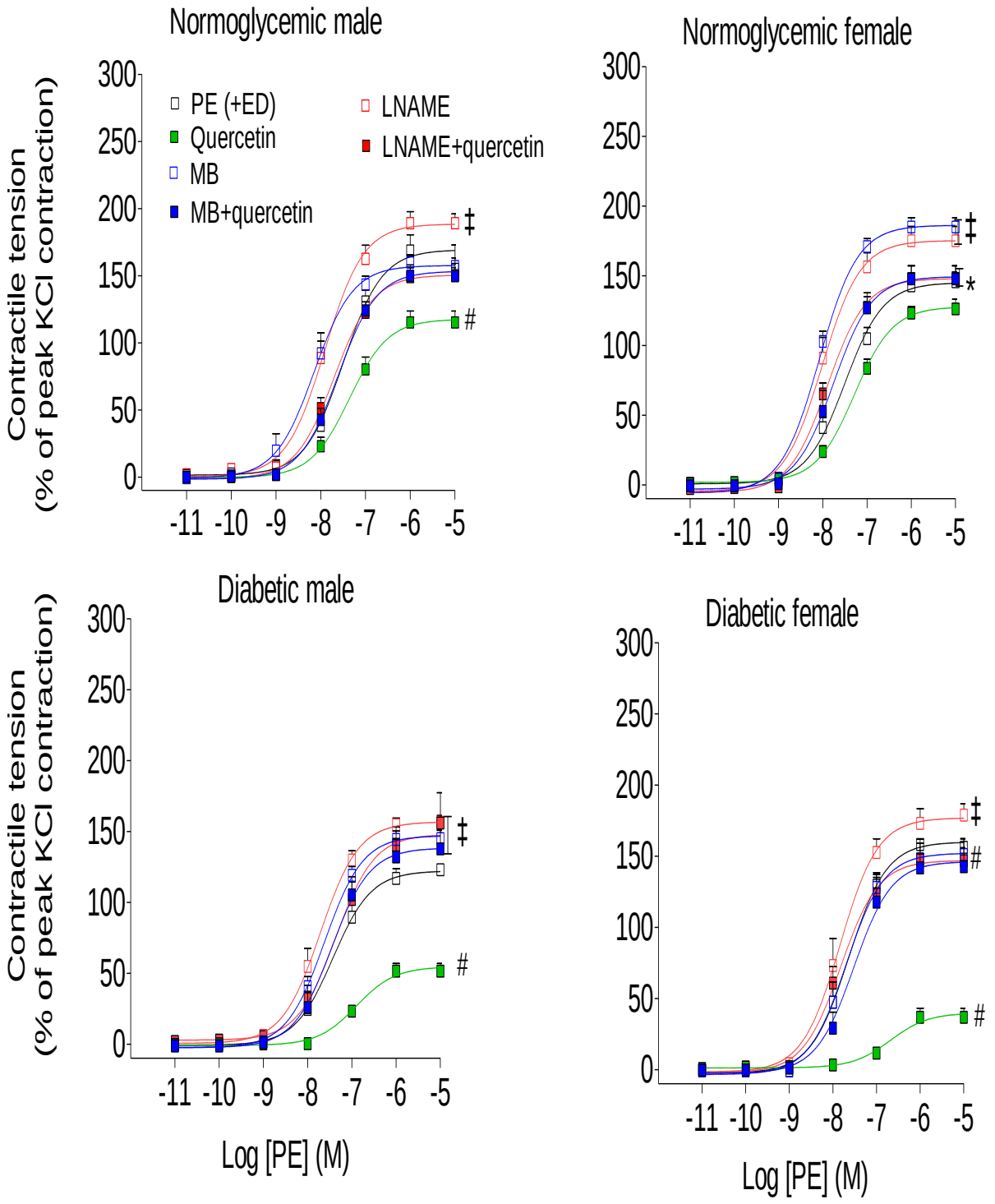

Figure 3.11: Contractile responses of endothelium-intact normoglycemic (upper panel) and diabetic (lower panel) male / female tissues to phenylephrine (PE) in control, with quercetin (10^{-5} M), methylene blue (10^{-5} M) (MB), MB + quercetin, L-NAME (10^{-5} M) or L-NAME + quercetin. Data are shown as mean ± SEM (n = 6 - 20). Statistics: $^{*}p <$ 0.01, female compared with the male; $^{\#}p < 0.001$ or $^{\ddagger}p < 0.01$, compared with corresponding male or female normoglycemic or diabetic controls.

Table 3.7: Emax and pEC_{50} values derived from phenylephrine (10^{-11}-10^{-5} M) concentration-response curves of endothelium-intact or -denuded normoglycemic/ diabetic male and female WKY aortic rings in the absence or presence of MB, L-NAME , MB + quercetin or L-NAME + quercetin.

Rats	Treatment group	Male		Female	
Normoglycemic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (PE)	179.7 ± 7.1	7.4 ± 0.1	145.0 ± 4.3*	7.5 ± 0.1
	Quercetin	117.6 ± 4.6#	7.4 ± 0.1	127.7 ± 3.2	7.3 ± 0.1
	MB	159.7 ± 5.7	8.0 ± 0.1#	187.0 ± 4.1#*	8.1 ± 0.1#
	MB + quercetin	154.5 ± 4.1	7.6 ± 0.1#	150.6 ± 7.0#	7.7 ± 0.1#
	L-NAME	187.3 ± 6.2	8.0 ± 0.1#	175.3 ± 6.4#	8.0 ± 0.1#
	L-NAME + quercetin	154.2 ± 4.7#	7.8 ± 0.1	147.9 ± 4.7#	8.0 ± 0.1
Endothelium-denuded	Control (PE)	217.7 ± 7.4	7.8 ± 0.1	215.1 ± 7.3	8.0 ± 0.1
	Quercetin	141.7 ± 4.6#	7.8 ± 0.1	153.6 ± 8.2#	7.7 ± 0.2#
	MB	108.5 ±19.2#	7.9 ± 0.5	166.4 ± 10.2#*	7.7 ± 0.2#
	MB+ quercetin	193.6 ± 2.54	7.9 ± 0.1	189.0 ± 11.7	8.3 ± 0.2
	L-NAME	195.0 ± 10.1	7.9 ± 0.2	207.5 ± 12.1	8.1 ± 0.2
	L-NAME + quercetin	216.4 ±11.4	7.9 ± 0.2	202.0 ± 7.2	8.0 ± 0.1

Values represent mean ± SEM (n = 6-20). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.01$, compared with corresponding male/ female controls. ND = not determined.

Table 3.7 (continued): Emax and pEC_{50} values derived from concentration-response curves of endothelium-intact or -denuded normoglycemic/ STZ-induced diabetic male / female WKY aortic rings to phenylephrine (10^{-11}-10^{-5} M) in the absence or presence of MB, L-NAME , MB + quercetin or L-NAME + quercetin.

Rats	Treatment group	Male		Female	
Diabetic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (PE)	112.6 ± 5.3	7.4 ± 0.1	159.9 ± 4.6*	7.6 ± 0.1
	Quercetin	54.6 ± 3.0#	6.9 ± 0.1#	39.8 ± 3.4#	7.1 ± 0.2#
	MB	147.0 ± 3.3#	7.6 ± 0.1	152.1 ± 5.8	7.7 ± 0.1
	MB + quercetin	138.3 ± 6.7	7.5 ± 0.1	146.1 ± 6.0	7.5 ± 0.1
	L-NAME	156.7 ± 4.0#	7.7 ± 0.1#	176.8 ± 6.0	7.8 ± 0.1#
	L-NAME + quercetin	147.9 ± 7.8	7.4 ± 0.1#	146.9 ± 6.4#	7.8 ± 0.1
Endothelium-denuded	Control (PE)	238.4 ± 5.6	7.7 ± 0.1	179.8 ± 6.5	7.7 ± 0.1
	Quercetin	136.1 ± 6.5#	7.5 ± 0.1	134.2 ± 5.1#	7.7 ± 0.2
	MB	149.3 ± 4.8#	7.8 ± 0.1	120.1 ± 7.9#*	7.6 ± 0.2
	MB+ quercetin	147.3± 4.4	7.8 ± 0.1	107.5 ± 9.3	7.6 ± 0.2
	L-NAME	ND	ND	ND	ND
	L-NAME + quercetin	ND	ND	ND	ND

Values represent mean ± SEM (n = 6-20). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.01$, compared with corresponding male/ female normoglycemic or diabetic controls. ND = not determined.

3.4.2.1.1. The effect of ACh on PE-induced contraction

Both male and female endothelium-intact normoglycemic and diabetic aorta pre-contracted with PE showed concentration-dependent relaxation to ACh (Fig. 3.12).

In the normoglycemic group, ACh induced a higher % relaxation (Emax) in normoglycemic female (88.0 ± 5.5 %) compared to the male tissues (60.0 ± 3.9 %) with no significant change to pEC_{50} (female = 7.7 ± 0.3 versus male = 7.7 ± 0.2) (Fig.3.12). Quercetin pre-treatment did not alter response (Emax) of female (102.4 ± 3.6 %) or male (74.3 ± 4.2 %) tissues to ACh, but it enhanced pEC_{50} values in both (male = 8.2 ± 0.3; female = 8.3 ± 0.2) tissues (Fig. 3.12).

In the diabetic group, responses to ACh was marginally more in diabetic female (Emax = 100.7 ± 4.6 %; pEC_{50} = 8.1 ± 0.2) compared to the male tissues (Emax = 82.3 ± 5.5 %; pEC_{50} = 7.9 ± 0.3) (Fig. 3.12). In the presence of ACh, quercetin did not alter Emax value in male (82.9 ± 2.1 %) or female (78.6 ± 4.2 %) tissues, but it significantly enhanced the pEC_{50} of male (8.9 ± 0.1), but not the female tissues (7.9 ± 0.3) (Fig. 3.12).

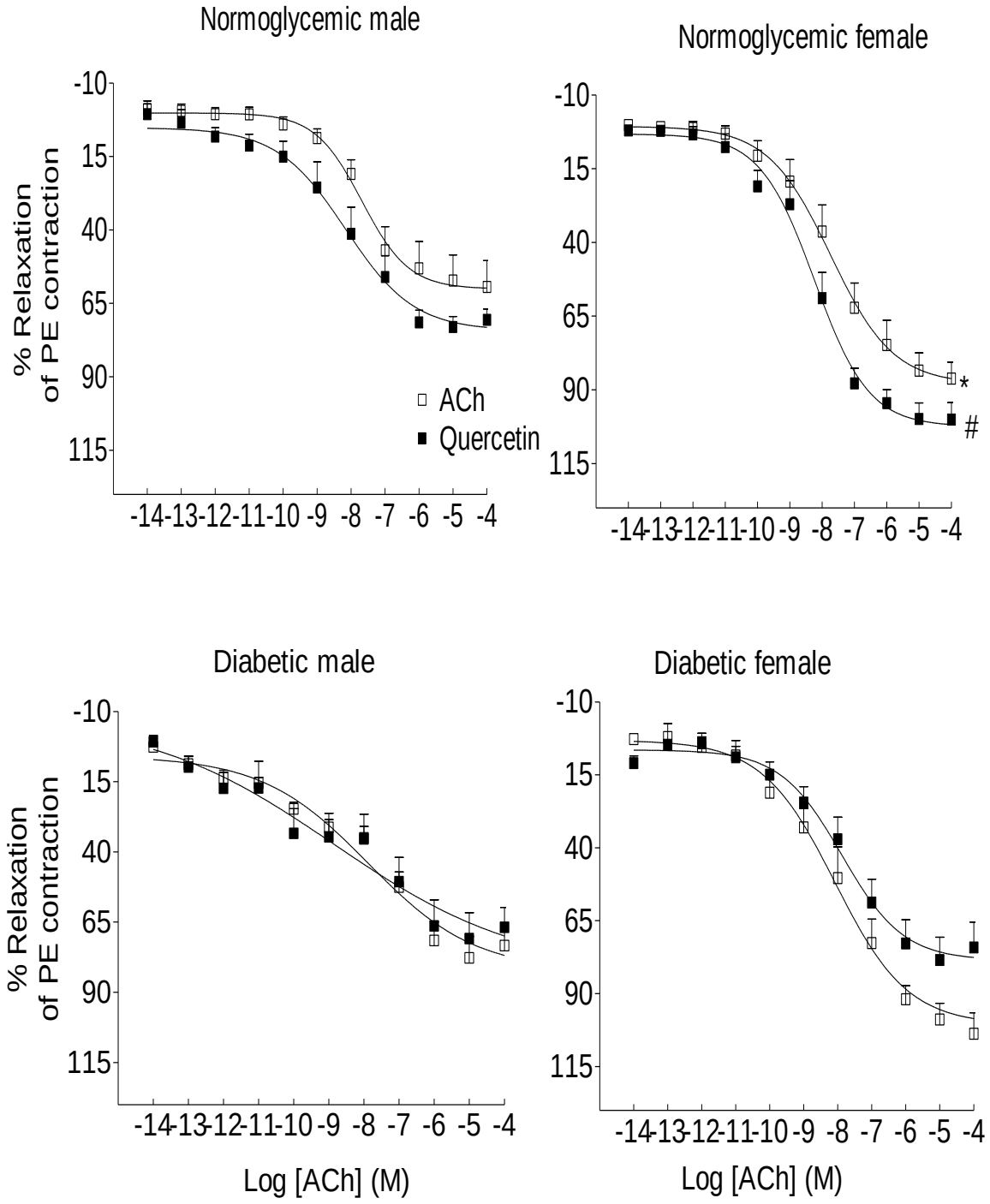

Figure 3.12: Cumulative acetylcholine (ACh) (10^{-14}- 10^{-4} M) concentration response curves of aortic rings from male (left column) and female (right column) normoglycemic (upper row) /diabetic (lower row) WKY rats pre-contracted with phenylephrine (PE) in the presence of quercetin (10^{-5} M) or its vehicle (DMSO< 0.05 % (v/v)). Data are shown as mean ± SEM (n = 5 - 9). Statistics: $^{\#}p < 0.05$, compared with the corresponding male or female vehicle-treated controls. Repeated exposure of tissues to DMSO (up to 0.05 % (v/v)) over the experiment time frame (time control) yielded no responses, and hence was removed from the graph.

3.4.2.1.2. The effect of SNP on PE-induced contraction

Both male and female endothelium-intact normoglycemic and diabetic aorta pre-contracted with PE showed concentration-dependent relaxation to SNP (Fig. 3.13).

In both genders, normoglycemic tissues relaxed (Emax) similarly to the exogenous NO releasing compound, SNP (male =104.1 ± 1.2 % and female =114.4 ± 9.7 %), but tissue sensitivity (pEC_{50}) to SNP was greater in male (10.6 ± 0.1) compared to female tissues (9.7 ± 0.5) (Fig. 3.13). Quercetin failed to alter Emax value of SNP-treated male (100.4 ± 1.5 %) or female (114.9 ± 8.7 %) tissues, but it reduced the pEC_{50} in male (10.3 ± 0.1) but not the female (pEC_{50} = 9.6 ± 0.4) tissues (Fig. 3.13).

In the diabetic tissues from both genders, relaxant response (Emax) to SNP, was also comparable (male = 108.9 ± 3.8 % versus female = 114.0 ± 4.0 %), with male exhibiting greater pEC_{50} (10.1 ± 0.3) compared to female (9.3 ± 0.2) tissues (Fig. 3.13). In both genders, quercetin equally failed to alter SNP-induced Emax of diabetic (male = 103.6 ± 2.6 %; female = 122.0 ± 8.2 %) tissues. However, diabetes increased the pEC_{50} value in quercetin + SNP-treated female (10.2 ± 0.7) but not male (10.0 ± 0.2) tissues (Fig. 3.13).

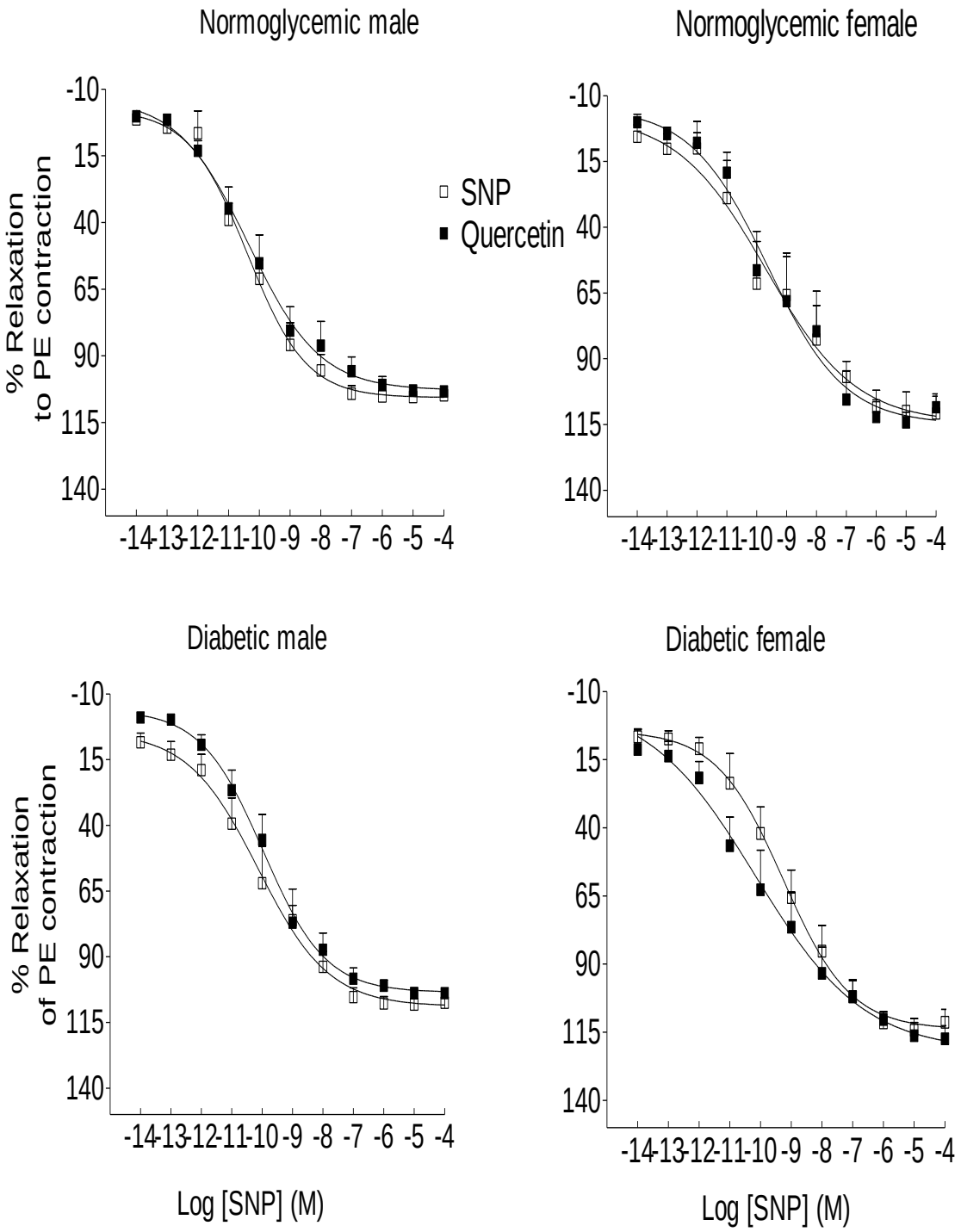

Figure 3.13: Cumulative sodium nitroprusside (SNP) (10^{-14}- 10^{-4} M) concentration response curves of aortic rings from male (left column) and female (right column) normoglycemic (upper row) /diabetic (lower row) WKY rats pre-contracted with phenylephrine (PE) in the presence of quercetin (10^{-5} M) or its vehicle (DMSO) (< 0.05 % (v/v)). Data are shown as mean ± SEM (n = 5 - 8). Statistics: $^{\#}p < 0.05$, compared with the corresponding male or female vehicle-treated controls. Repeated exposure of tissues to DMSO (up to 0.05 % (v/v)) over the experiment time frame (time control) yielded no responses, and hence was removed from the graph.

3.4.2.1.3. Nitric oxide (nitrite ion) levels

Serum nitrite levels was significantly higher in normoglycemic female serum (283.0 ± 43.0 μMol/L) compared to the male (167.9 ± 4.8 μMol/L), but aortic tissue nitrite levels were similar in both genders (female: 5.6 ± 1.1 versus male: 4.7 ± 0.7 μMol/L/mg) (Fig. 3.14).

Diabetes significantly elevated nitrite levels more in serum samples from male (381.8 ± 37.4 μMol/L; 127 % increase) compared to the female (576.4 ± 14.5 μMol/L; 104 % increase). Diabetes significantly enhanced nitrite concentration in female diabetic aorta (21.5 ± 0.7 (284 % increase) μMol/L/mg) but did not significantly alter it in the male (3.9 ± 0.6) (< 1 % reduction) μMol/L/mg)) (Fig. 3.14).

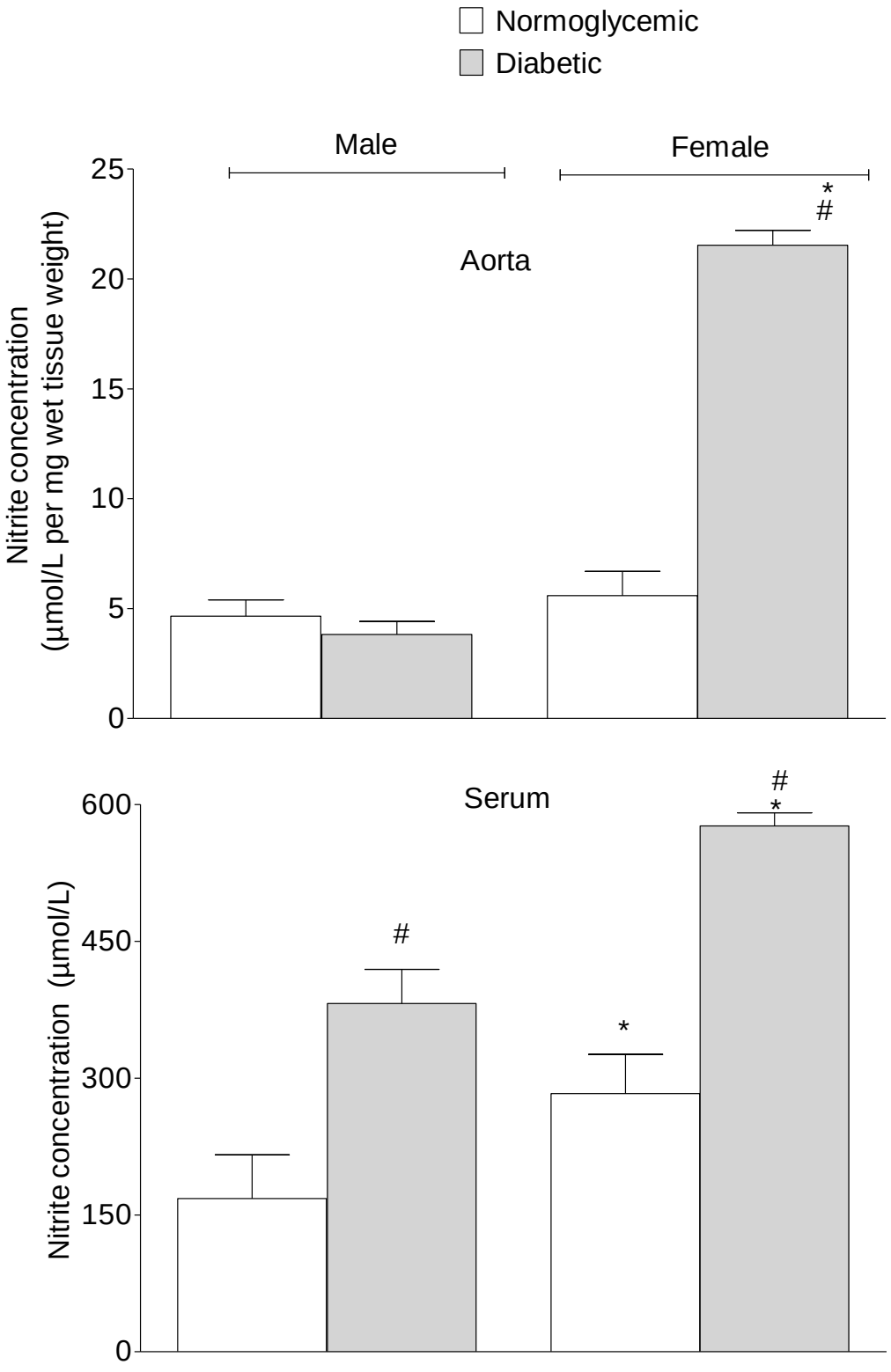

Figure 3.14. Nitrite concentration in serum (upper row) or aortic tissue (lower row) from normoglycemic/diabetic male and female animals. Data are shown as mean ± SEM (n= 6 - 7 in each group). Statistics: $^{\#}p < 0.001$, diabetic compared with normoglycemic tissues; $^{*}p < 0.001$, female compared with male.

3.4.2.2. Ang II-induced contraction

Both male and female endothelium-intact /-denuded normoglycemic and diabetic aorta showed concentration-dependent contractions to Ang II in the presence of L-NAME or MB (Fig. 3.15 / Table 3.8).

In the normoglycemic group, L-NAME or MB-enhanced contraction in tissues from both genders with L-NAME or MB producing (143 % or 145 %) greater increase in Emax value in female compared to male (L-NAME = 31 %; MB = 29 %) tissues. These interventions abolished the difference in contraction between the normoglycemic male versus the female (Fig. 3.15 / Table 3.8). L-NAME or MB pre-treatment significantly reduced quercetin action in endothelium-intact male tissues with quercetin causing approximately 4 % or 11 % reduction of contraction (Emax) in L-NAME or MB-treated tissues as against 50 % in the untreated tissues (Table 3.8). In female tissues, where quercetin originally exerted no effect, quercetin caused a 31 % or 26 % relaxation of contraction (Emax) in L-NAME or MB-treated tissues (Table 3.8). In endothelium-denuded tissues from both genders, Ang II-induced contraction in MB (L-NAME = not determined)-treated tissues was similar to responses in respective untreated controls. MB significantly attenuated quercetin relaxant action (Table 3.8).

In the diabetic group, L-NAME or MB treatment of endothelium-intact tissues resulted in greater % contraction (Emax) in male (L-NAME =163 %; MB = 146 %) compared to the female (L-NAME =125 %; MB = 93 %). In effect, either of the treatments abolished the contractile difference between the normoglycemic versus diabetic tissues (Table 3.8). Pre-treatment with L-NAME or MB reduced quercetin relaxant action significantly more in male (by 18 % or 15 % versus 58 % induced effect in control tissues) compared

to (46 % or 36 % versus 55 % induced effect in untreated) female tissues (Fig. 3.15 / Table 3.8). In endothelium-denuded tissues from both genders, MB pre-treatment similarly attenuated contraction. MB treatment did not alter quercetin action in endothelial-denuded diabetic aorta from both genders (Table 3.8).

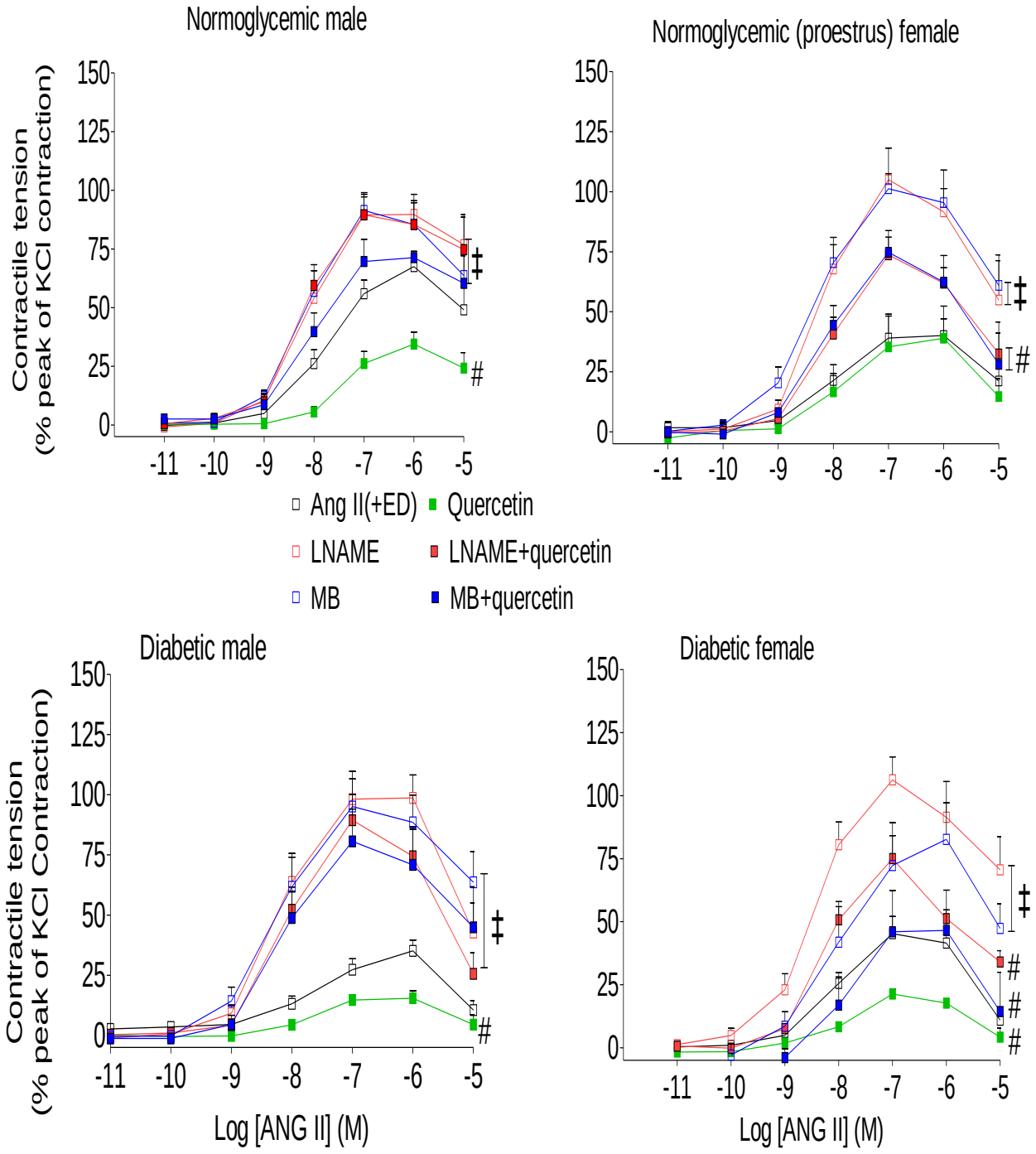

Figure 3.15: Contractile responses of endothelium-intact normoglycemic (upper panel) and diabetic (lower panel) male / female tissues to angiotensin II in control (Ang II), with Quercetin (10^{-5} M), methylene blue (10^{-5} M) (MB), MB + quercetin, L-NAME (10^{-5} M) or L-NAME + quercetin. Data are shown as mean ± SEM (n = 5 – 10 in each group). Statistics: $^{*}p < 0.01$, female compared with the male; $^{\#}p < 0.001$ or $\ddagger p < 0.001$, compared with corresponding male or female normoglycemic or diabetic controls.

Table 3.8: Emax and pEC_{50} values derived from concentration-response curves of endothelium-intact or -denuded normoglycemic/STZ-induced diabetic male and female WKY aortic rings to Ang II (10^{-11}-10^{-5} M) in the absence or presence of MB, L-NAME , MB +quercetin or L-NAME + quercetin.

Rats	Treatment group	Male		Female	
Normoglycemic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (Ang II)	70.1 ± 3. 0	7.9 ± 0.1	40.7 ± 5.2*	8.0 ± 0.2
	Quercetin	35.2 ± 3.8$^{\#}$	7.4 ± 0.4$^{\#}$	47.3 ± 9.6	7.9 ± 0.4*
	MB	89.6 ± 5.1	8.2 ± 0.1$^{\#}$	99.7 ± 4.5$^{\#}$	8.4± 0.1$^{\#}$
	MB + quercetin	79.9 ± 4.5	8.2 ± 0.1	68.9 ± 5.4$^{\#}$	8.2 ± 0.2
	L-NAME	91.9 ± 5.1	8.2 ± 0.1$^{\#}$	98.7 ± 7.8$^{\#}$	8.2± 0.2
	L-NAME + quercetin	88.3 ± 5.1	8.2 ± 0.1	68.0 ± 4.0$^{\#}$	8.1 ± 0.1
Endothelium-denuded	Control (Ang II)	105.0 ± 9.7	8.2 ± 0.2	84.1 ± 4.0	8.1 ± 0.1
	Quercetin	53.2 ± 9.7$^{\#}$	8.0 ± 0.3	53.0 ± 7.8$^{\#}$	8.0 ± 0.2
	MB	100.3 ± 9.5	8.2 ± 0.3	84.2 ± 8.4	7.8 ± 0.2$^{\#}$
	MB+ quercetin	88.2 ± 10.4	7.9 ± 0.1$^{\#}$	86.3 ± 11.7	8.3 ± 0.2$^{\#}$
	L-NAME	ND	ND	ND	ND
	L-NAME + quercetin	ND	ND	ND	ND

Values represent mean ± SEM (n = 5-10). Statistics: *$p < 0.001$, female compared to male tissues; $^{\#}p < 0.01$, compared with corresponding male/ female controls. ND = not determined.

Table 3.8 (continued): Emax and pEC_{50} values derived from concentration-response curves of endothelium-intact or -denuded normoglycemic/STZ-induced diabetic male and female WKY aortic rings to Ang II (10^{-11}-10^{-5} M) in the absence or presence of MB, L-NAME, MB + quercetin or L-NAME + quercetin.

Rats	Treatment group	Male		Female	
Diabetic		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Endothelium-intact	Control (Ang II)	37.8 ± 5.6	7.5 ± 0.6	43.9 ± 3.5	8.1 ± 0.2*
	Quercetin	15.5 ± 1.9#	7.8 ± 0.3#	19.5 ± 1.6#	8.0 ± 3.0
	MB	93.0 ± 6.7#	8.3 ± 0.2#	85.3 ± 15.0#	8.1 ± 0.4
	MB + quercetin	76.1 ± 6.4	8.2 ± 0.2	46.8 ± 9.9#	7.9 ± 0.3#
	L-NAME	100.0 ± 5.6#	8.2 ± 0.1#	99.0 ± 6.4#	8.5 ± 0.2#
	L-NAME + quercetin	82.0 ± 5.2	8.1± 0.4	63.0 ± 4.3#	8.4 ± 0.2
Endothelium-denuded	Control (Ang II)	133.0 ± 9.0	8.3 ± 0.2	113.1 ± 4.1	8.3 ± 0.1
	Quercetin	63.4 ± 6.6#	8.3 ± 0.3	65.8 ± 8.5#	8.1 ± 2.2
	MB	46.7 ± 12.2#	8.6 ± 0.1#	65.9 ± 7.5#	8.3 ± 0.3*
	MB+ quercetin	25.6 ± 5.2#	8.2 ± 0.2#	65.1 ± 11.7	8.1 ± 0.4
	L-NAME	ND	ND	ND	ND
	L-NAME + quercetin	ND	ND	ND	ND

Values represent mean ± SEM (n = 5 -10). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.01$, compared with corresponding male/ female controls. ND = not determined.

3.4.3. Effect of cyclooxygenase and/or eNO pathways

3.4.3.1. PE-induced contraction

In the normoglycemic group, contractile response (Emax) to PE in endothelium-intact tissues was significantly reduced by indomethacin in both genders (Table 3.9). L-NAME significantly attenuated or reversed the relaxant action of indomethacin in tissues from both genders (Table 3.9). In the male, quercetin attenuated contraction with or without indomethacin, with a magnitude similar to indomethacin treatment alone. In the female tissues, quercetin attenuated contraction with but not without indomethacin (Table 3.9). In quercetin treated tissues from genders, L-NAME + indomethacin intervention, significantly shifted the PE response curve leftwards effectively attenuating quercetin relaxant effect (Table 3.9).

In the diabetic group, contractile response to PE was significantly enhanced (Emax) by indomethacin treatment in endothelium-intact male in contrast to the female, where, indomethacin reduced contraction (Table 3.9). L-NAME significantly reversed the relaxant action of indomethacin in female but failed to alter its contractile effects in male tissues (Table 3.9). In both genders, quercetin effect was significantly reduced with indomethacin or indomethacin + L-NAME pre-treatment (Table 3.9).

Table 3.9: Emax and pEC_{50} values derived from concentration-response curves of endothelium-intact normoglycemic/STZ-induced diabetic male and female WKY aortic rings to phenylephrine (PE) (10^{-11}-10^{-5} M) in absence or presence of indomethacin and/or L-NAME, indomethacin and/or SOD with or without quercetin treatment.

Rats	Treatment group	Male		Female	
Endothelium-intact		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Normoglycemic	Control (PE)	179.7 ± 7.1	7.4 ± 0.1	145.0 ± 4.3*	7.5 ± 0.1
	Quercetin	117.6 ± 4.6#	7.4 ± 0.1	127.7 ± 3.2	7.3 ± 0.1
	Indomethacin	122.2 ± 7.8#	7.4 ± 0.2	109.2 ± 5.2#	7.6 ± 0.1#
	Indomethacin+ quercetin	117.8 ± 7.2	7.1 ± 0.2#	101.9 ± 5.6	7.4 ± 0.2
	L-NAME	187.3 ± 6.2	8.0 ± 0.1#	175.3 ± 6.4#	8.0 ± 0.1#
	L-NAME + quercetin	154.2 ± 4.7#	7.8 ± 0.1	147.9 ± 4.7#	8.0 ± 0.1
	L-NAME + indomethacin	173.0 ± 4.0	7.8 ± 0.1	178.1 ± 4.5	7.7 ± 0.1#
	L-NAME+ indomethacin + quercetin	147.4 ± 3.0#	7.6 ± 0.1	152.0 ± 3.7#	7.6 ± 0.1
	SOD	114.5 ± 5.6#	7.6 ± 0.1	121.7 ± 7.9	7.2 ± 0.1*
	SOD + quercetin	84.4 ± 6.9#	7.2 ± 0.2#	77.1 ± 8.7#	7.1 ± 0.2
	SOD + indomethacin	159.4 ± 6.3#	7.4 ± 0.1	128.3 ± 8.7*	7.7 ± 0.2#*
	SOD + indomethacin + quercetin	141.3 ± 6.8	7.3 ± 0.2	69.1 ± 6.8#	7.2 ± 0.3#

Values represent mean ± SEM (n = 6-20). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.001$, compared with corresponding male/ female control group.

Table 3.9 (continued): Emax and pEC_{50} values derived from concentration-response curves of endothelium-intact normoglycemic /STZ-induced diabetic male and female WKY aortic rings to PE (10^{-11}-10^{-5} M) in absence or presence of indomethacin and/or L-NAME, indomethacin and/or SOD with or without quercetin treatment.

Rats	Treatment group	Male		Female	
Endothelium-intact		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Diabetic	Control (PE)	112.6 ± 5.3	7.4 ± 0.1	159.9 ± 4.6*	7.6 ± 0.1
	Quercetin	54.6 ± 3.0#	6.9 ± 0.1#	39.8 ± 3.4#	7.2 ± 0.2#*
	Indomethacin	159.4 ± 6.3#	7.5 ± 0.1	113.7 ± 5.9#*	7.3 ± 0.1#
	Indomethacin+ quercetin	102.2 ± 7.6#	7.3 ± 0.2	85.0 ± 5.9#	7.1 ± 0.2
	L-NAME	156.7 ± 4.0#	7.7 ± 0.1#	176.8 ± 6.0	7.8 ± 0.1#
	L-NAME + quercetin	147.9 ± 7.8	7.4 ± 0.1#	146.9 ± 6.4#	7.8 ± 0.1*
	L-NAME + indomethacin	149.2 ± 3.8	7.6 ± 0.1	182.5 ± 4.9	7.6 ± 0.1
	L-NAME+ indomethacin + quercetin	141.4 ± 4.6	7.3 ± 0.1#	138.7 ± 6.2#	7.6 ± 0.1
	SOD	154.0 ± 3.6#	7.6 ± 0.1	137.7 ± 2.9	7.4 ± 0.1
	SOD + quercetin	104.0 ± 7.9#	7.2 ± 0.1#	94.7 ± 5.8#	7.1 ± 0.2#
	SOD + indomethacin	150.2 ± 6.7	7.6 ± 0.1	108.6 ± 8.9#*	7.4 ± 0.2
	SOD + indomethacin + quercetin	92.1 ± 9.0#	7.0 ± 0.2#	73.5 ± 4.3#	7.0 ± 0.1#

Values represent mean ± SEM (n = 6-20). Statistics: *$p < 0.001$, female compared to male tissues; #$p < 0.001$, compared with corresponding male/ female control group.

3.4.3.1.1. Effect of gender and quercetin on synthesis of cyclooxygenase products

The contribution of prostaglandins (PG) (TXA_2, PGE_2 and PGI_2) in modulating observed gender-dependent contractile responses and the action of quercetin on these endothelium-intact tissues were explored by measuring the levels of prostaglandins in endothelium-intact tissues from normoglycemic / diabetic rats treated with or without quercetin (Section 2.6.3).

In the normoglycemic group, male tissues exhibited higher levels of TXA_2 (TXB_2) (11.4 ± 1.6 pg/mL/mg) and PGE_2 (1.8 ± 0.2 pg/mL/mg) compared to the corresponding female tissues (TXB_2 = 5.7 ± 1.5; PGE_2 =1.3 ± 0.1 pg/mL/mg) (Fig. 3.16). Female tissues exhibited higher PGI_2 (6-keto-$PGF1_a$) (0.9 ± 0.1 pg/mL/mg) content compared to the male (0.3 ± 0.1 pg/mL/mg) (Fig. 3.16).

Acute pre-treatment with quercetin (10^{-5} M, 25 min) significantly reduced TXB_2 (male: = 5.4 ± 1.1 versus female: = 1.1± 0.2 pg/mL/mg) but did not alter PGE_2 levels in tissues from both genders (male: PGE_2 = 1.6 ± 0.3 versus female: PGE_2 =1.3 ± 0.7) (Fig. 3.16). The % reductions of TXB_2 was higher in female (80 %) compared to male (53 %) tissues. Quercetin enhanced PGI_2 levels in tissues from both genders (male: 0.8 ± 0.2 pg/mL/mg) (female: 1.2 ± 0.2 pg/mL/mg) (Fig. 3.16). This enhancement was more in male (175 %) compared to female (31 %) tissues.

In the diabetic group, tissue concentrations of TXA_2 (2.0 ± 0.3 pg/mL/mg) and PGE_2 (0.9 ± 0.1 pg/mL/mg) were significantly reduced in male compared to its normoglycemic control. In contrast, diabetes enhanced TXA_2 (16.3 ± 6.7 pg/mL/mg) but not PGE_2 (1.5 ± 0.0 pg/mL/mg) content in the female in comparison to the female

normoglycemic tissues. The levels of both prostanoids were significantly elevated in the female compared to the male tissues (Fig. 3.16). Diabetes induced an increase in PGI_2 levels in tissue from both genders (male = 0.6 ± 0.1 pg/mL/mg versus female = 1.5 ± 0.2 pg/mL/mg) and this was higher (100 %) in diabetic male compared to female (67 %) tissues (Fig. 3.16).

Quercetin reduced TXB_2 (male = 0.5 ± 0.1 versus female = 3.2 ± 0.7 pg/mL/mg) but not PGE_2 levels (male: =1.0 ± 0.3 versus female: 1.4 ± 0.0 pg/mL/mg) (Fig. 3.16) in tissue from both genders. It significantly reduced PGI_2 levels in diabetic tissues from both genders (male = 0.4 ± 0.0 and female = 0.6 ± 0.1 pg/mL/mg). This reduction of PGI_2 levels was significantly more in diabetic female (60 % reduction) compared to the male (33 % reduction) tissues (Fig. 3.16).

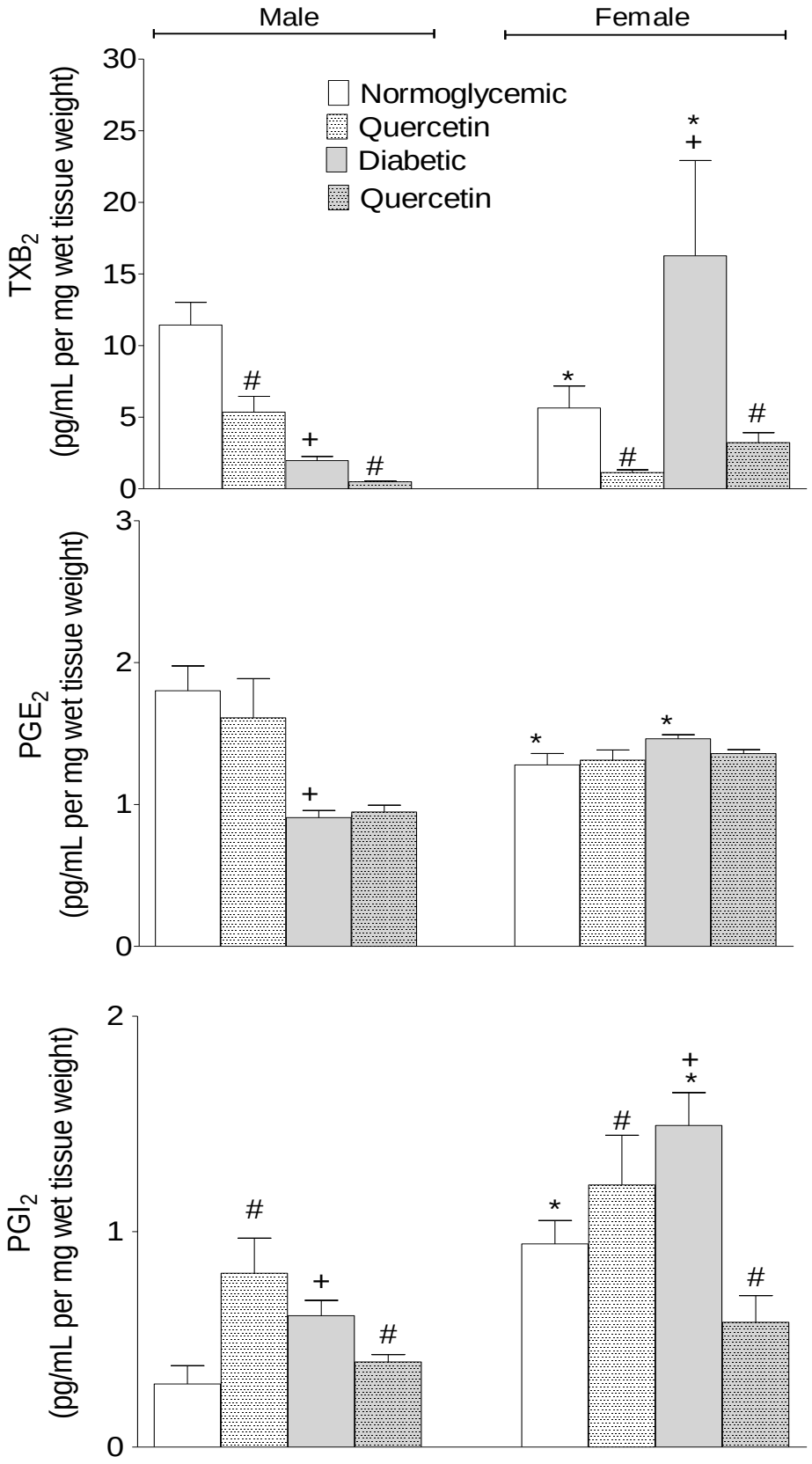

Figure 3.16: Levels of thromboxane (TXB_2) (upper row), prostaglandin E_2 (PGE_2) (middle row) and prostaglandin I_2 (PGI_2; prostacyclin) (lower row), in aortic tissue from normoglycemic and diabetic male/female animals. Data are shown as mean ± SEM (n= 5-7 in each group). Statistic: $^{\#}p < 0.001$ or $^{+}p < 0.001$, compared with respective male or female control; $^{*}p< 0.01$, female compared with male.

3.4.3.1.2. Effect of indomethacin and/or SOD on PE-induced contraction

The effect of $\bullet O_2^-$ interaction with the cyclooxygenase pathway on PE-induced contraction of endothelium-intact normoglycemic / diabetic male and female aorta in the presence or absence of quercetin was investigated.

In normoglycemic male aorta, SOD or indomethacin (but not indomethacin + SOD) significantly relaxed contraction (Emax). In these tissues, quercetin relaxant effect was enhanced by SOD, while its combination with indomethacin + SOD produced lesser relaxation compared to quercetin treatment alone (Table 3.9). In the female, indomethacin attenuated contraction but not SOD, quercetin or indomethacin + SOD treatments. Quercetin combined with SOD or indomethacin + SOD to inhibit contraction (Table 3.9).

In diabetic male tissues, contractile response (Emax) to PE was significantly enhanced by SOD, indomethacin or indomethacin + SOD in contrast to responses produced by these treatments in corresponding normoglycemic tissues. Quercetin in combination with SOD or indomethacin + SOD significantly attenuated the contractile effects of SOD or indomethacin + SOD (Table 3.9). In the female tissues, PE-induced contraction (Emax) was significantly reduced by indomethacin or indomethacin + SOD (but not with SOD) treatment. Quercetin significantly relaxed contraction in SOD- or indomethacin + SOD –treated tissues, although this was lesser than relaxation produced by quercetin treatment alone (Table 3.9).

3.4.3.2. Ang II-induced contraction

In normoglycemic tissues, Ang II contraction (Emax) in endothelium-intact male tissues was significantly attenuated by indomethacin, which failed to alter it in the female tissues (Table 3.10). L-NAME significantly attenuated or reversed the relaxant action of indomethacin in the male but did not significantly alter its effect in female tissues (Table 3.10). In the male tissues, quercetin relaxant effect was unchanged with indomethacin treatment (Table 3.10). In female tissues, indomethacin had no effect on quercetin action. Quercetin relaxant effect was present in L-NAME + indomethacin-treated male and also in female tissues, where quercetin action was originally absent (Table 3.10).

In the diabetic group, indomethacin enhanced contraction in male (Emax) tissues, but elicited minimal effect in female tissues (Table 3.10). L-NAME enhanced the contractile effects of indomethacin in diabetic tissues from both genders (Table 3.10). Quercetin relaxation of male diabetic tissues was significantly inhibited by indomethacin, but this was not the case with female tissues, where it was marginally enhanced (Table 3.10). Indomethacin + L-NAME induced reduction of quercetin effect was significantly more in diabetic male than female aorta (Table 3.10).

Table 3.10: Emax and pEC_{50} values derived from concentration-response curves of endothelium-intact normoglycemic /STZ-induced diabetic male and female WKY aortic rings to Ang II (10^{-11}-10^{-5} M) in presence or absence of indomethacin and/or L-NAME with or without quercetin treatment.

Rats	**Treatment group**	**Male**		**Female**	
Endothelium-intact		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Normoglycemic	Control (Ang II)	70.1 ± 3. 0	7.9 ± 0.1	40.7 ± 5.2*	8.0 ± 0.2
	Quercetin	35.2 ± 3.8$^{\#}$	7.4 ± 0.4$^{\#}$	47.3 ± 9.6	7.9 ± 0.4*
	L-NAME	91.9 ± 5.1	8.2 ± 0.1$^{\#}$	98.7 ± 7.8$^{\#}$	8.2 ± 0.2
	L-NAME + quercetin	88.3 ± 5.1	8.2 ± 0.1	68.0 ± 4.0$^{\#}$	8.1 ± 0.1
	Indomethacin	26.6 ± 2.8$^{\#}$	8.0 ± 0.1	55.7 ± 5.5	8.3 ± 0.4$^{\#}$
	Indomethacin + quercetin	26.8 ± 3.8	8.0 ± 0.4	52.5 ± 6.0	8.0 ± 0.1
	L-NAME + indomethacin	102.6 ± 3.3	8.2 ± 0.1	69.0 ± 6.2*	8.3 ± 0.3
	L-NAME+ indomethacin + quercetin	43.7 ± 3.4$^{\#}$	8.0 ± 0.6	30.4 ± 3.5$^{\#}$	8.0 ± 0.3$^{\#}$

Values represent mean ± SEM (n = 6- 10). Statistics: *$p < 0.001$, female compared to male tissues; $^{\#}p < 0.001$, compared with corresponding male/ female control group.

Table 3.10 (continued) : Emax and pEC_{50} values derived from concentration-response curves of endothelium-intact male and female WKY aortic rings to Ang II (10^{-11}-10^{-5} M) in presence or absence of indomethacin and/or L-NAME with or without quercetin treatment.

Rats	**Treatment group**	**Male**		**Female**	
Endothelium-intact		**Emax ± SEM**	**pEC50 ± SEM**	**Emax ± SEM**	**pEC50 ± SEM**
Diabetic	Control (Ang II)	37.8 ± 5.6	7.5 ± 0.6	43.9 ± 3.5	8.1 ± 0.2*
	Quercetin	15.5 ± 1.9$^{\#}$	7.8 ± 0.3$^{\#}$	19.5 ± 1.6$^{\#}$	8.0 ± 3.0
	L-NAME	100.0 ± 5.6$^{\#}$	8.2 ± 0.1$^{\#}$	99.0 ± 6.4$^{\#}$	8.5 ± 0.2$^{\#}$*
	L-NAME + quercetin	82.0 ± 5.2	8.1± 0.4	63.0 ± 4.3$^{\#}$	8.4 ± 0.2*
	Indomethacin	60.2 ± 5.9$^{\#}$	8.0 ± 0.9$^{\#}$	51.7 ± 5.1	8.4 ± 0.3*$^{\#}$
	Indomethacin + quercetin	52.1 ± 5.2	8.0 ± 0.4	12.1 ± 2.6$^{\#}$*	8.0 ± 0.9$^{\#}$
	L-NAME + indomethacin	93.4 ± 3.9	8.4 ± 0.1	97.4 ± 6.3	8.3 ± 0.2
	L-NAME+ indomethacin + quercetin	72.8 ± 5.5	8.2 ± 0.4	53.6 ± 7.0$^{\#}$	8.0 ± 0.3$^{\#}$

Values represent mean ± SEM (n = 6-10). Statistics: *$p < 0.001$, female compared to male tissues; $^{\#}p < 0.001$, compared with corresponding male/ female control group.

CHAPTER 4

DISCUSSION

This study explored possible gender differences in agonist (phenylephrine (PE) or angiotensin II (Ang II))-contracted rat aorta, and in the effects of the antioxidant flavonoid-quercetin and/or 17β-estradiol (estradiol) on contractile function. The roles of oxidative stress, nitric oxide-cGMP and / or cyclooxygenase pathway in regulating the observed gender differences were also examined. Our data provide evidence to suggest that normoglycemic and diabetic aortic tissue contraction to PE or Ang II, and the vasorelaxant function of quercetin or estradiol is influenced by gender-dependent, endothelium-mediated factors and the diabetic state.

4.1. Influence of gender and the endothelium on agonist-induced contraction

Eight weeks following STZ -induced diabetes, the endothelium-intact normoglycemic male tissues contracted more (to PE or Ang II) compared to the normoglycemic female aorta or the diabetic male. The normoglycemic and diabetic female contracted equally to PE regardless of estrus cycle phase. Ang II induced lesser response in normoglycemic (proestrus)/diabetic female compared to corresponding normoglycemic tissues in diestrus phase (Fig. 3.3 / 3.4). This result is consistent with earlier studies (Tostes et al. 2000; Wangensteen et al. 2004; Robert et al. 2005), which show that normoglycemic male tissues respond significantly more to agonist-induced vasoconstriction than the female. It also supports previous studies (Pinna et al. 2001) showing that the female blood vessel may become more reactive following the development of diabetes. Removal of the endothelium (Fig. 3.3 / 3.4) or pre-treatment with L-NAME or MB (Fig. 3.11 / 3.15) abolished the gender differences in (PE or Ang II) contraction between the normoglycemic male versus the diabetic male /

normoglycemic female, and between the diabetic male versus the diabetic female aorta. Taken together, these results suggest that gender-related, endothelium-dependent factors mediated the observed tissue differences in contraction.

In figure 3.3 & 3.4, the diabetes-induced attenuation of contraction in male (PE or Ang II-contracted) and female (Ang II- contracted) tissues, supports previous observations (Myers and Messina, 1996; Misurski et al. 2001), including studies from our laboratory (Chin et al. 2007), showing that vascular hypo reactivity to endogenous vasoconstrictors exist in (short-term) diabetes. This outcome perhaps represents an early attempt by the diabetic tissue endothelium to counter the increased contractility subsequently seen in this condition (Myers and Messina, 1996; Misurski et al. 2001). Furthermore, endothelium removal resulted in a greater percentage increase in Emax of normoglycemic female (PE = 49 %; Ang II = 107 %) compared to male (PE = 21 %; Ang II = 50 %) tissues (Table 3.3 / 3.4), indicating that the endothelium of healthy (normoglycemic) female is in a higher vasorelaxant state compared to the male in agreement with previous studies (Hayashi et al. 1992; Kauser and Rubanyi, 1994). This female 'advantage' was evidently diminished in female diabetic tissues, where removal of the endothelium resulted in lesser % contraction (PE = 12.5 %; Ang II = 158 %) compared to contraction in equivalent male tissues (PE =114 %; Ang II = 250 %) (Table 3.3/3.4). This reduced contractile response in endothelium-denuded diabetic female tissues perhaps supports the hypothesis that female vascular endothelium succumb more to diabetes-induced damage compared to the male (Pinna et al. 2001).

4.1.1. The role of estrus cycle on agonist-induced contraction of female aorta

The development of diabetes caused a cessation of the normal reproductive (estrus) cycle in the female rats resulting in a permanent diestrus state and reduced levels of estradiol in line with earlier finding (Kim et al. 2006) (Fig. 3.1). However, this negative effect of diabetes on female estrus cycle did not alter PE-induced contraction in agreement with earlier studies (Li et al. 1997; Sanz et al. 2003). On the other hand, endothelium-intact normoglycemic female tissues in diestrus phase produced greater reactivity to Ang II in comparison with corresponding proestrus tissues (Fig. 3.4). This suggests that estradiol-deficiency (a feature of tissues in diestrus phase (Walmer et al. 1992)) augments female vascular reactivity to Ang II as has been demonstrated (Xue et al. 2007). The attenuated response of diestrus diabetic tissues to Ang II stimulation was perhaps a consequence of the opposing effects of diabetic (endothelium)-activation of vasodilator factors (Table 3.5 / 3.6 & fig. 3.16)- a pathophysiological mechanism to compensate for increased tissue contraction associated with this condition (Myers and Messina, 1996; Misurski et al. 2001).

4.2. The influence of quercetin and/or estradiol on tissue contraction

4.2.1. The effect of quercetin on tissue contraction

PE (Bleeke et al. 2004; Tsai and Jiangi, 2010) and Ang II (Touyz, 2004; Chu and Leung et al. 2009) are well known triggers for increased vascular oxidative stress. On the other hand, the vasoprotective effects of antioxidant flavonoids are greater in the presence of oxidative stress (Nascimento et al. 2003, Lopez-Lopez et al. 2004). It is therefore no surprise that quercetin (10^{-8}-10^{-3} M or 10^{-5} M) vasodilator action was predominant in PE- or Ang II- treated tissues (normoglycemic /diabetic male and the diabetic female) (Fig. 3.6 a, b / 3.7 / Table 3.3 / 3.4), where oxidative stress (Fig. 3.8-3.9) was higher

compared to its lesser effect in endothelium-intact female tissues with reduced oxidative stress). Taken together, in PE-contracted tissues, these results confirm earlier studies (Duarte et al. 1993; Roghani et al. 2005) including those from our laboratory (Ajay, 2003; 2006 a,b; 2007), which show that quercetin exerts its vasorelaxant action via endothelium-dependent and -independent mechanisms in male rats. The current data also supports earlier findings in male rats (Ajay et al. 2007; Sanchez et al. 2007) showing that the endothelium-mediated mechanism for quercetin action is partly regulated by the presence of oxidative factors. It goes further to propose for the first time, that the same factors influence quercetin action in female rat aorta. Furthermore, compared to diabetic female aorta (with lesser estradiol levels (Fig. 3.2), the absence of quercetin vasorelaxant action in endothelium-intact normoglycemic female tissues may probably be the result of (physiological) endothelium-mediated suppressive effects of (the female hormone) estradiol against oxidative stress (Strehlow et al. 2003; Florian et al. 2004). This led to the investigation of the direct role of estradiol and / or quercetin on vascular contraction.

4.2.2. The effect of estradiol on tissue contraction

The effect of exogenous estradiol on vascular responses to contractile agonists is controversial. Attenuation (Thomas et al. 1995), enhancement (Miller and Vanhoutte, 1990) or no effect (Nadarali et al. 2001; Tep-Areenan et al. 2003) have all been reported. In the current study, estradiol attenuated PE contraction or marginally reduced it in Ang II contracted endothelium-intact normoglycemic male aorta compared to its lack of effect in female tissues (Fig. 3.6a / 3.7) in agreement with earlier findings (Thomas et al. 1995; Nadarali et al. 2001; Tep-Areenan et al. 2003). In the current study, these results suggest that the lower contraction of normoglycemic endothelium-

intact female compared to the male aorta was partly attributable to estradiol hormone-modulated relaxation and inhibition of oxidant stress (Fig. 3.2) (Binko and Majewski, 1998; Tep-Areenan et al. 2003). This reasoning is supported by the observation that normoglycemic female tissues exhibited greater response to estradiol (in PE-contracted tissues) following exposure to conditions (endothelial denudation (Table 3.3 / 3.4) which promotes oxidative stress (Brandes and Mugge, 1997).

In endothelium-intact diabetic male tissues (contracted with PE or Ang II), estradiol tended to enhance contraction rather than relax it (Miller and Vanhoutte, 1990), while eliciting no apparent effect on female tissues (Fig. 3.6a / 3.7). These data suggest that estradiol relaxant action is reversed during early-stage diabetes (at least in the male) in line with previous findings (Maggi et al. 2003). It also supports the view that estradiol protective (vasorelaxant) function wanes once cardiovascular disease develops (Barrett-Corner et al. 1991; Bolego et al. 1999). In the female, the apparent lack of estradiol effect in (PE or Ang II-contracted) endothelium-intact normoglycemic / diabetic tissues may be reflective of a number of reasons: 1) the normoglycemic female tissues were already fully primed with estradiol (Fig. 3.2) and hence, were insensitive to the physiological concentration (10^{-7} M) of estradiol tested; 2) the vasorelaxant action of estradiol may have been masked by the diabetic state reduction in estradiol levels (Fig. 3.2) and synthesis of vasoconstrictor prostaglandins (Fig. 3.16); 3) It is also possible that the effects of estradiol against diabetic tissue contraction only become more observable in the female tissues in latter stages of the disease when endothelial function deteriorates even more as evidenced in figure 3.6b and Table 3.3 / 3.4. These are interesting speculations warranting further studies.

Furthermore, estradiol is known to exert vascular action via genomic and non genomic mechanisms (Orshal and Khalil, 2004). The classic genomic pathway (producing (delayed) chronic vascular effects of estradiol) requires receptor–specific transcription and protein synthesis, while in the non genomic (rapid onset) pathway; estradiol promotes vasodilatation by directly regulating Ca^{2+} entry mechanisms. Since $\geq$ 1 hour (vascular tissue exposure to estradiol) is required to trigger genomic effects (Binko and Majeswski, 1998) and 5-25 min for non genomic events (Teoh et al. 2000)), the 20-25 min exposure period employed in the present study probably support a non genomic mechanism for the vasodilator effect of estradiol in normoglycemic (with or without endothelium) / diabetic male tissues (without endothelium), and endothelium-denuded normoglycemic / diabetic female tissues.

4.2.3. The effect of estradiol on quercetin vasodilator action

Quercetin is thought to enhance estradiol levels *in vivo* (Schubert et al. 1994; Weber et al 1996). Since estradiol and quercetin are potent antioxidants (Strehlow et al. 2003; Florian et al. 2004), it could be predicted that estradiol would promote and/or augment quercetin vasorelaxant action. In agreement, this was the case in PE-contracted endothelium-intact/-denuded male and endothelium-denuded normoglycemic female tissues (Fig. 3.6a / b). However, although quercetin + estradiol- induced relaxation was present in PE-contracted endothelium-intact diabetic tissues from both genders, it was lesser in magnitude compared to relaxation caused by quercetin alone treatment (Fig. 3.6a / b). This is contrary to current study hypothesis and may be related to the negative effects of diabetes on estradiol signaling in tissues from both genders (Fig. 3.6a), which probably caused a reduction in the anticipated synergy in quercetin + estradiol-induced vasorelaxation. Equally, in Ang II-contracted male / female normoglycemic / diabetic

tissues, estradiol tended to oppose rather than enhance quercetin effect (Fig. 3.7). Taken together, these data indicate that in early-stages of diabetes, a combined administration of quercetin + estradiol (17β-estradiol) may not offer greater protection than quercetin alone treatment against diabetic tissue reactivity (to PE or Ang II), where this combination appears to have no added clinical benefit.

4.3. Mechanisms underlining gender differences in normoglycemic / diabetic tissue contraction and quercetin action

4.3.1. Role of oxidative stress

In healthy and diseased conditions, endothelial cells release $\bullet O_2^-$ and H_2O_2 both of which modulate vascular reactivity differently (Jakus, 2000; Chin et al., 2007). On the other hand, the vascular action of quercetin (Cogolludo et al., 2007) and SOD (Jakus, 2000) against $\bullet O_2^-$ produces H_2O_2, a potent vasodilator (Fujimoto et al., 2001). We therefore explored the specific role of $\bullet O_2^-$ and / or H_2O_2 in regulating the observed gender-different responses to PE or Ang II in the absence or presence of quercetin.

In line with previous studies (Brandes and Mugge, 1997; Kerr et al. 1999), our data show that healthy (normoglycemic (endothelium-intact)) male aorta exists in a state of greater oxidative stress (increased $\bullet O_2^-$ / reduced SOD/CAT activity) compared to female aorta (with reduced $\bullet O_2^-$ / higher SOD/CAT concentrations) (Fig. 3.8 - 3.9). Consequently, blockade of $\bullet O_2^-$ (with SOD), H_2O_2 (with CAT) or both (with SOD + CAT) significantly inhibited PE (but not Ang II) contraction in male tissues (Table 3.5 / 3.6). In contrast, in female tissues, blockade of $\bullet O_2^-$ (but not H_2O_2) failed to alter PE contraction, but removal of both factors significantly enhanced Ang II response.

These results suggest that the higher normoglycemic male tissue contraction (to PE) was promoted by enhanced synthesis of $\bullet O_2^-$ / H_2O_2 compared to the female tissues, where both factors was minimal and exerted negligible effect on contraction. In contrast, although, these ROS factors ($\bullet O_2^-$ and/or H_2O_2) were minimally present in normoglycemic female tissues, they appear to promote vasorelaxation (Table 3.5 / 3.6). In summary, these results support a role for $\bullet O_2^-$ and/or H_2O_2 in modulating vascular contraction in male (Ongil et al. 2001; Hilgers and Stumpf, 2002; Chin et al. 2007) and female rat aorta under physiological conditions.

Quercetin concentration-dependent reduction in $\bullet O_2^-$ levels was greater in normoglycemic male compared to female with lesser oxdant ($\bullet O_2^-$) burden. Also, quercetin vasodilator action was greater in the endothelium-intact normoglycemic male than female tissues (contracted with PE or Ang II), suggesting that the vasodilator action of the antioxidant quercetin appears to be more active in tissues (normoglycemic male) with increased endothelial-derived oxidative stress but not the female (Fig. 3.8 - 3.10 & Table 3.5 / 3.6). However, quercetin induced enhancement in female tissue $\bullet O_2^-$ at low concentration (10^{-8} M) probably supports the observation that antioxidants may be pro-oxidants in healthy tissues at certain conditions (Chen et al. 2007; 2008). Further, quercetin combined with blockers (17 β-estradiol (Table 3.3 / 3.4), SOD and/or CAT (Table 3.5 / 3.6)) of $\bullet O_2^-$ and/or H_2O_2 to further relax (PE or Ang II) contraction in normoglycemic male, further supporting the notion that quercetin exerts its relaxant effect in the male rat aorta by removing these oxidant factors. Since quercetin antioxidative action yields the vasodilator H_2O_2 (Congolludo et al. 2007; Khoo et al. 2010), it was not surprising that pre-treatment of male tissues with SOD or SOD + CAT enhanced quercetin effect in these tissues more than CAT. In the normoglycemic

female, pre-treatment with anti-oxidants (17β-estradiol, SOD / CAT) alone had no effect on contraction, but combined with quercetin, they unmasked a quercetin relaxant effect (Table 3.5 / 3.6). We propose that the presence of ROS ($\bullet O_2^-$ / H_2O_2) factors significantly inhibited quercetin vasorelaxant action in PE-treated female, but enhanced it in male tissues. In Ang II –treated tissues from both genders, removal of $\bullet O_2^-$ and/or H_2O_2 (which tended to promote vasoconstriction) enhanced quercetin effect in male and unmasked it in the female. These data are in agreement with the current study hypothesis suggesting that the vascular action of quercetin may select between male and female tissues partly in response to gender-related differences in the bioavailability and vascular function of ROS.

Compared to levels seen in normoglycemic controls, oxidative stress (increased $\bullet O_2^-$ versus lesser SOD/CAT activity) was significantly higher in diabetic tissues from both genders (Fig. 3.8-3.9). $\bullet O_2^-/H_2O_2$ appear to have promoted vasorelaxation in the diabetic male but contraction in the female (Table 3.5). The failure of $\bullet O_2^-$ to enhance diabetic male tissue contraction is surprising. It is probably a (gender specific) pathophysiologic feature of early-stage diabetes in the male. In the current study, the data support a stronger role for $\bullet O_2^-$ and/or H_2O_2 in modulating vascular tone in male compared to female aorta during early-stage diabetes. These results are in agreement with several studies (Tsuneo and Katsuo, 2002; Shastri et al. 2002; Chin et al. 2007), showing that ROS factors regulate vascular tone under pathological conditions (i.e. diabetes).

In keeping with its selectivity for oxidative stress (Lopez-Lopez et al. 2004; Sanchez et al. 2007), quercetin action was understandably more profound in diabetic than normoglycemic tissues contracted with PE (Table 3.3, / 3.5) or Ang II (Table 3.4 / 3.6).

Therefore, in endothelium-intact diabetic tissues from both genders, the blockade of (vasorelaxant) $\bullet O_2$ with antioxidants (17β-estradiol, SOD and/or CAT (Table 3.3-3.6) accordingly reduced quercetin vasodilator effect or augmented it in SOD (H_2O_2) - treated diabetic male and female tissues. These results confirm an earlier finding in (normoglycemic (Ajay et al. 2003; 2006a) / diabetic (Ajay et al. 2006 a, b; 2007; Sanchez et al. 2007)) male aorta, and supports the current hypothesis that the relaxant effect of quercetin is partly mediated by oxidative stress factors. Additionally, the finding (Fig 3.9) that, the NADPH oxidase inhibitor, DPI, significantly reduced $\bullet O_2^-$ generation to very low levels in both endothelium-intact normoglycemic / diabetic tissues from both genders, implies that NADPH oxidase enzyme is a key source of $\bullet O_2^-$ inhibited or neutralized by quercetin in these tissues as has been demonstrated (Sanchez et al. 2007; Romero et al. 2009).

4.3.2. Role of nitric oxide-cGMP pathway

Pre-treatment of tissues with L-NAME or MB abolished or reversed the contractile difference between the endothelium-intact normoglycemic male versus female tissues contracted with PE (Fig. 3.11) or Ang II (Fig. 3.15). In other words, the observed differences in agonist-induced normoglycemic tissue contraction were eNO-sGC-cGMP mediated. Since the inhibition of eNO or sGC (with L-NAME or MB) resulted in greater female than male tissue contraction to PE (Table 3.7) or Ang II (Table 3.8), endothelium-based eNO- sGC-cGMP function is greater in the aorta of healthy (normoglycemic) female than male rats. This is in agreement with earlier studies by Hayashi et al. (1992) and Kauser & Rubanyi (1994), demonstrating that endothelium of female rat produces a higher basal vasorelaxation compared to the male. Consistent with this finding, in figure 3.12, normoglycemic female aorta exhibited higher percentage

relaxation (than the male) to the endothelium-dependent vasodilator (ACh). This was not the case with the responses to the endothelium-independent NO donor (SNP) (Fig. 3.13), where male and female tissue responses were similar. Equally, in figure 3.14, normoglycemic female tissues exhibited higher nitrite ion content (a biomarker of NO function) than the male. In the male, higher tissue oxidative stress was probably responsible for diminished ACh-induced relaxation or nitrite levels in agreement with findings by Brandes and Mugge (1997) and Kerr et al. (1999). Taken together, these results suggest that the endothelium of the female rat exists in a higher basal (eNO-cGMP-induced) vasorelaxant state compared to the male. This higher activation of eNO-cGMP pathway in normoglycemic female likely accounts for why its tissues were more hypo responsive to vasoconstrictor stimulation (Fig 3.3 / 3.4) compared to the hyper reactive male tissues, where eNO-cGMP function was lesser.

In normoglycemic male tissues, inhibition of EDNO (with L-NAME) or sGC-cGMP (with MB) in endothelium-intact normoglycemic tissues attenuated quercetin action in PE (Fig. 3.11) or Ang II (Fig. 3.15)-contracted male tissues, suggesting that eNO-sGC-cGMP pathway mediates the quercetin relaxant effects in normoglycemic male aorta in line with previous findings (Ajay et al. 2003; Rogahni 2005). In the female, we suggest quercetin effect was reduced or masked given the higher activation of this pathway in female tissues. Further, since the concentration of L-NAME (10 µM) or MB (10 µM) used in this study was sufficient to block all the effects of endothelium and non-endothelium generated NO, the presence of quercetin action in these tissues was probably in response to enhanced ROS ($\bullet O_2^-$) generation seen in these conditions (Brend et al. 1989; Sekiguchi et al. 2004). These data therefore support the consistent

observation that quercetin action is selective of tissues undergoing oxidant stress (Ajay et al. 2007; Sanchez et al. 2007).

In figures 3.12 / 3.13, quercetin enhanced the % (endothelium-dependent) relaxation to ACh more in the male compared to female tissues, but did not alter the (endothelium-independent) SNP response which was similar in both genders. Since quercetin promotes endothelial relaxant function more in conditions of oxidative stress, the lack of (profound) quercetin enhancement of ACh (or SNP)-relaxation in male tissues was probably because ACh or SNP provided full NO-facilitated relaxation which reduced or masked quercetin effect. Taken together, in normoglycemic male aorta, quercetin action was perhaps largely mediated by the blockade of oxidant factors (deleterious to EDNO) which appear to play a minimal role in the female tissues, where estrogen-induced reduction of $\bullet O_2^-$ probably resulted in a lesser quercetin effect. In this regard, direct measurement of the effect of quercetin on NO, sGC and/or cGMP in the presence or absence of $\bullet O_2^-$, would be useful in a future study to further clarify these speculations.

In endothelium-denuded normoglycemic tissues, the current data further reveal that quercetin relaxant effect was completely abolished by L-NAME or MB in tissues from both genders (Table 3.7 / 3.8). These results suggest the existence of an endothelium-independent, L-NAME (or MB) - sensitive NO (Joly et al. 1994) or non-NO (Das et al. 1999) mechanism through which quercetin exerts its effect in both male and female endothelium-denuded tissues.

Short-term diabetes may result in reduced vascular contraction (Myers and Messina, 1996; Misurski *et al.* 2001) as observed in the diabetic male tissues contracted with PE (Fig. 3.3 / Table 3.3) or Ang II (Fig. 3.4 / Table 3.4). Diabetes may equally produce no change in contraction (Mulhern and Docherty, 1989; Chang and Stevens, 1992) as observed in the diabetic female tissues contracted with PE (Fig. 3.3 / Table 3.3) or Ang II (Fig 3.4/Table 3.4). These inconsistencies in the reactivity of diabetic tissue is explained partly by alterations in eNO-cGMP transduction pathway (Pieper, 1998; Browne et al. 2007) which appear to be attenuated more in diabetic female than male tissues (Table 3.7 / 3.8). Despite high NO release in the diabetic female aorta (Fig. 3.14), L-NAME or MB pre-treatment failed to produce much higher contraction to PE or Ang II (Fig. 3.11 / 3.15) in contrast to male tissues, where any of these treatments profoundly enhanced contractions (Table 3.7 / 3.8). These data support a greater attenuation of eNO or cGMP component of the eNO-cGMP relaxant cascade in diabetic female than male aorta. Impairment in vascular (eNO-cGMP) smooth muscle function is part of the pathophysiological mechanism of diabetes (Suzuki et al. 2001). This result is supportive of the view that diabetes-induced impairment in the function of vascular eNO-cGMP pathway may be more severe in the female than male gender (Bolego et al. 1999; Pinna et al. 2001).

Furthermore, nitrite ion content was significantly enhanced in diabetic tissues from both genders (more in female than male) (Fig. 3.14), suggesting that, during the early stages of diabetes, the endothelium promotes the synthesis of NO to compensate for increased diabetic tissue contractile stimulation (Myers and Messina, 1996; Misurski et al. 2001; Chin et al. 2007). This enhanced endothelium-release of NO accounts for the lesser diabetic male contraction (to PE / Ang II) (Fig. 3.3 / 3.4). Although this compensatory

synthesis of EDNO (Fig. 3.14) seems to occur more in diabetic female than male aorta, its effect in attenuating contraction appears less apparent in PE/Ang II-contracted female compared to the male tissues (Fig. 3.3). This discrepancy probably suggests that in addition to EDNO, other endothelium-based factors (such as cyclooxygenase) mediated the observed difference in diabetic tissue contraction.

In endothelium-intact diabetic tissues from both genders, quercetin relaxant action is mediated by EDNO-sGC-cGMP pathway, since the inhibition of this pathway (with L-NAME / MB) significantly attenuated quercetin action in PE- or Ang II-contracted tissues from both genders (Fig. 3.11 / 3.15). This result confirms an earlier study in our laboratory (Ajay et al. 2003; 2006 a, b; 2007), showing that EDNO-sGC-cGMP pathway mediates quercetin vasodilator action in diabetic male tissues. In the female, the current data describes for the first time, the existence of the same pathway in the diabetic female, which was not observed in corresponding normoglycemic control tissues. Furthermore, since the diabetic state promoted the synthesis of vasodilator factors (EDNO / PGI_2) (Fig. 3.14 / 3.16), it is understandable that ACh or SNP pre-treatment elicited no effect on quercetin action in diabetic tissues, given that these tissues were fully saturated with vasodilator factors which probably masked quercetin effect. It is also plausible that the diabetes-induced alterations in eNOS-cGMP cascade (Table 3.7 / 3.8) may have desensitized diabetic tissues to quercetin + ACh effect (Romero et al. 2009).

In endothelium-denuded diabetic tissues, the inhibition of EDNO (with L-NAME) or sGC-cGMP (with MB) failed to inhibit quercetin action in contrast to its effect in endothelium-denuded normoglycemic tissues. Since NO-sGC-cGMP pathway mediates quercetin vasodilator effect in normoglycemic tissues (Joly et al. 1994), the diabetic-induced attenuation of the function NO-sGC-cGMP pathway in diabetic aorta from both genders desensitized these tissues to quercetin effect, hence the failure of MB to block quercetin effects. Therefore, quercetin effect in these tissues is independent of NO-sGC-cGMP pathway. In these tissues, quercetin effect may have been in response to ROS occasioned by endothelial injury (endothelial removal) (Brandes and Mugge, 1997).

4.3.3. Role of cyclooxygenase and EDNO pathways

Inhibition of prostaglandin (PG) synthesis with indomethacin attenuated PE (Table 3.9) or Ang II (Table 3.10) contraction more in endothelium-intact normoglycemic male than female tissues, suggesting that contractile COX product(s) played a greater role in normoglycemic male than female tissue contraction. This interpretation is supported by the observation that treatment with L-NAME reversed the indomethacin-induced relaxation in these tissues, indicating that indomethacin inhibited the contractile COX product opposing EDNO release in these tissues (Table 3.9 / 3.10). In support of this view, tissue levels of TXA_2 (the main endothelium-derived vasoconstrictor released in response to COX-I activity (Rolland et al. 1984; Muscara et al. 2000) and PGE_2 (a disease-induced vasoconstrictor product of COX-II activation (Wallace et al. 1999; Yamamoto et al. 1993)) were higher in endothelium-intact aorta of normoglycemic male compared to the female. Taken together, enhanced synthesis of contractile factors ($\bullet O_2^-$, TXB_2 / PGE_2) and/or lower synthesis of vasodilator factors (EDNO or PGI_2) is the basis for greater PE contraction of normoglycemic male aorta than the female.

Consequently, the blockade of PG products and EDNO (with L-NAME + indomethacin) effectively removed the gender difference in normoglycemic tissue contraction. These data further support the consistent observation (Fig. 3.11 / 3.12/ 3.14 / 3.15) that the female aorta exists in a higher state of basal endothelium-mediated vasorelaxation compared to the male.

The mechanisms involved in the endothelium-mediated relaxant effects of flavonoids have been shown to be concentration dependent. At concentrations > 30 µM (Chan et al. 2000), flavonoids tend to promote endothelium (eNO-cGMP-cyclooxygenase)-dependent vasodilation through the inhibition of contractile proteins such as protein kinase C (Duarte et al. 1993), cAMP-phosphodiesterase (Beretz et al. 1980), and Ca^{2+} release from intracellular compartments (Chan et al. 2000). Therefore, at the concentration (10 µM) used in the current study, the L-NAME + indomethacin-induced inhibition of quercetin action in normoglycemic tissues (Table 3.9 / 3.10), suggests quercetin action is mediated in these tissues by eNO-cGMP-cyclooxygenase cross talk in aorta of male rats, consistent with earlier studies in our laboratory (Ajay et al. 2003; 2006 a). In the female, the apparent lack of quercetin action observed in untreated tissues may be attributed to the inhibitory effects of the cyclooxygenase (contractile PG) component of this eNO-cGMP-cyclooxygenase pathway (Table 3.9 / 3.10).

In the diabetic group, indomethacin pre-treatment enhanced PE (Table 3.9) or Ang II (Table 3.10) contraction in male, but reduced it in female tissues contracted with PE, but not Ang II. These results indicate that diabetes promoted a PG-mediated vasodilation in the male but contraction in the female. This is corroborated by the reduced synthesis of contractile PGs (TXA_2 / PGE_2) in diabetic male compared to

enhancement of the same in diabetic female tissues. Given the higher synthesis of vasodilator factors (EDNO and PGI_2) (Fig. 3.14 / 3.16) in the diabetic female, it is no surprise that the anticipated contractile synergy between increased levels of contractile PGs (TXB_2 / PGE_2) and PE or Ang II treatment was masked or suppressed.

Furthermore, the speculation was that the NO-sGC-cGMP relaxant cascade was down regulated by diabetes in female compared to male tissues. In agreement, the reactivity of diabetic female aorta to L-NAME was enhanced with indomethacin (figures 3.11 and 3.15), suggesting that diabetes exerted a greater negative effect on the female sGC-cGMP component of this pathway compared to NO. Defects in NO-sGC-cGPM signal transduction pathway during endothelial dysfunction has been demonstrated in hypertension (Morawietz et al. 2001) and may also exist in diabetes (Suzuki et al. 2001). Furthermore, consistent with earlier observations, increased synthesis of diabetic endothelium-derived vasodilator factors (EDNO (Fig. 3.14), PGI_2 (3.16)) are probably part of the pathophysiological response (in diabetic aorta of male and female WKY rats) to oppose excessive contractile stimulation (Browne et al. 2007; Csanyi et al. 2007)

In diabetic tissues, indomethacin reduced quercetin relaxation of male tissues contracted with PE or Ang II, and female tissues contracted with PE but not Ang II (Table 3.9 / 3.10), suggesting that indomethacin attenuation of PGI_2 activity in these tissues reduced quercetin vasodilator function. Consequently, in these tissues, L-NAME + indomethacin pre-treatment almost entirely abolished quercetin effect, indicating (as in normoglycemic male / female WKY rat aorta) that it's vascular effect in male / female diabetic tissues is mediated by NO-cGMP-cyclooxygenase coupled cross talk. This finding confirms an earlier observation in the male in which Roghani et al. (2005)

reported a quercetin induced vasodilatory effects that was NO-cGMP-cyclooxygenase mediated. The data also demonstrates for the first time, the existence of this mechanism for quercetin action in the diabetic female WKY rats.

4.3.4. Role of cyclooxygenase and $\bullet O_2^-$ pathways

The metabolism of arachidonic acid by cyclooxygenases in the absence of lipooxygenase activity may be influenced by $\bullet O_2^-$ (Oltman et al. 2003). In Table 3.9, it is interesting that treatment with indomethacin inhibited PE-induced contraction and reversed the relaxant action of SOD in male tissues but had minimal or no effect on any of these responses in the female (Ang II was not determined). Since normoglycemic male tissues produced more contractile factors ($\bullet O_2^-$, TXA_2, PGE_2 (Fig. 3.8, 3.9 / 3.16)) than the female, the lack of SOD or SOD + indomethacin effect in the female (Table 3.9) is understandable, given that $\bullet O_2^-$, TXA_2 & PGE_2 were minimally present for SOD / indomethacin to act upon in the female. Also, since SOD dismutation of $\bullet O_2^-$ results in the vasodilator, H_2O_2 (Khoo et al. 2010), it is also possible that indomethacin reversed SOD-mediated (via H_2O_2-PG) vasodilatation in normoglycemic male tissues. These results are in agreement with evidences suggesting that $\bullet O_2^-$ regulates vascular tone in healthy animals via a PG-mediated mechanism. (Tsuneo and Katsuo, 2002; Shastri et al. 2002; Chin et al. 2007). In the current study, we suggest that H_2O_2-PG mediated vasorelaxation appears more active in normoglycemic male than female WKY aorta.

Indomethacin pre-treatment significantly reduced SOD + quercetin, but not quercetin effects in normoglycemic male. This is in contrast to female tissues, where indomethacin unmasked a quercetin (but did not alter SOD + quercetin) relaxant effect (Table 3.9). In male tissues, it could be argued that indomethacin blocked the synthesis of contractile COX-product which reduced the generation of $^{\cdot}O_2^-$ resulting in the unchanged / diminished effects of quercetin or quercetin + SOD, respectively. In the female, where $^{\cdot}O_2^-$ is minimally present, indomethacin removed a contractile PG-product opposing quercetin relaxant action. In tissues from both genders, it is also possible that H_2O_2 produced by SOD and quercetin-induced removal of $^{\cdot}O_2^-$ exerted a relaxant effect via a PG-mediated mechanism (Thengchaisri et al. 2003) that is indomethacin-sensitive or –insensitive in male or female tissue, respectively. These are interesting speculations needing further studies.

SOD and/or indomethacin similarly enhanced PE contractions in diabetic male tissues, in contrast to the female, where these treatments tended to cause relaxation (Table 3.9). This result implies that diabetes activated vasodilator factors ($\bullet O_2^-$ and/or PGI_2) in the male tissues in contrast to the female, where it promoted contractile factors (TXB_2 / PGE_2). This data is consistent with literature evidences suggesting that $\bullet O_2^-$ mediates vascular tone in early-stage diabetes via a PG-mediated mechanism in rats (Thengchaisri et al. 2003; Chin et al. 2007). Furthermore, as earlier deduced (Table 3.5 / 3.6) and consistent with current data (Table 3.9), $\bullet O_2^-$ (or its dismutated product, H_2O_2) and PGI_2 (Fig. 3.16) are factors generated in the endothelium of diabetic rats to compensate for diminished NO-sGC-cGMP or oppose the increased contractile stimulation in the diabetic state (Csanyi et al. 2007; Chin et al. 2007).

In diabetic tissues of the male rat, quercetin relaxant action was significantly reduced by the removal of $\bullet O_2^-$ (with SOD), vasodilator PG (with indomethacin) or both (with SOD + indomethacin) (Table 3.9). This is expected, given that these vasodilator factors ($\bullet O_2^-$ / PGI_2) were activated in these tissues. Similarly in diabetic female tissues, where vasorelaxant (EDNO (Fig. 3.12) / PGI_2 (3.16)) factors were also activated, removal of contractile factors (TXA_2 / PGE_2 (Fig. 3.16)) expectedly reduced, but failed to abolish quercetin effect. These results confirm the finding that quercetin vasodilator effect is greater in diabetic tissues with higher ROS synthesis. The current data supports a quercetin vasorelaxant action that is largely mediated in male and female diabetic tissues by a $\bullet O_2^-$-H_2O_2-PG signal transduction mechanism, the blockade of which (with SOD + indomethacin) reduces its action in these tissues, regardless of gender.

4.4. Future areas of investigations

The present study has established, to a large extent, the mechanistic basis of the gender differences in contractile responses (to PE or Ang II) between normoglycemic and diabetic aorta. However, the possibility exists for other contributing mechanisms. For instance, at the age diabetes studied, the α_1-adrenoceptors was probably altered by diabetes in male but not female animals, whereas Ang II receptor could have been down regulated in aorta from both genders. It is possible that several other endothelium-derived hyperpolarisation factors (EDHF) contributed to the observed contractile differences in normoglycemic or diabetic aorta (Browne et al. 2007; Csanyi et al. 2007). Future studies are therefore, required to address this issue. $\bullet O_2^-$ (which is normally vasoconstrictive) appears to be vasorelaxant in early-stage diabetic (male) tissues. Further work is required to confirm this novel finding. The hyperglycemia state is a major contributor to the vascular pathophysiology in diabetes. The levels of

hyperglycemia achieved in this study, although similar to levels in other studies (Pinna et al. 2001; Sanz et al. 2003), were uncontrolled. Therefore the possibility exists that controlled (e.g. with insulin) levels of hyperglycemia could yield different patterns of data, which is worth exploring for its clinical relevance. There is a need to repeat the current investigation in resistance vessels for correlation with the micro vascular angiopathy of clinical diabetes. Furthermore, the mechanisms involved in quercetin action in both endothelium-intact and -denuded tissues needs to be investigated further to include the influence of quercetin on estrogen receptors during contractile stimulation, the expression of genes that modulate oxidant, antioxidant and cyclooxygenase proteins. These are just some of the potential areas for further exploration which we hope to pursue in the near future.

CHAPTER 5

SUMMARY AND CONCLUSION

The present study was designed to explore possible gender differences in agonist (phenylephrine (PE) or angiotensin II (Ang II))-contracted normoglycemic /diabetic rat aorta, and the effects of the antioxidant flavonoid-quercetin and/or 17β-estradiol (estradiol) on contractile outcome. The mechanisms underlining any observed gender differences were subsequently evaluated. The following are the major summaries of the findings in this study:

SUMMARY

1. There are gender differences in PE or Ang II-induced contraction of normoglycemic and diabetic aorta from WKY rats. Endothelium-intact normoglycemic male tissues contract more (to PE or Ang II) than the normoglycemic female or the diabetic male. The normoglycemic /diabetic female tissues contracted equally to PE regardless of phases of estrus cycle. Ang II caused lesser contraction of normoglycemic (proestrus) /diabetic compared to normoglycemic female tissues in diestrus state.

2. These differences in tissue contraction were reversed or abolished by the removal of the endothelium or by the development of diabetes-induced endothelial defects, suggesting that the differences are endothelium mediated and reflect the pathophysiology of the diabetic state in this study.

3. The lower female normoglycemic tissue contraction is attributed to higher female tissue synthesis and content of vasodilator factors, including estradiol, EDNO and PGI_2, coupled with reduced oxidative stress (decreased $\bullet O_2^-$ synthesis and increased antioxidant (SOD/CAT) status). The higher normoglycemic male tissue contraction is

attributable to an increase in contractile factors (enhanced synthesis of oxidative stress factors ($\bullet O_2^-$, TXA_2, and PGE_2 levels) coupled with reduced antioxidant (SOD/CAT) status).

4. Compared to normoglycemic controls, the lower diabetic male tissue contraction (to PE) is attributable to reduction in contractile PGs (TXA_2 and PGE_2) and/or the enhancement of vasodilator factors (NO, PGI_2 & $\bullet O_2^-$). The greater reactivity of diabetic female tissues (to PE) is attributable to enhanced tissue synthesis/activation of contractile PGs (TXA_2 and PGE_2), which appears to counteract the effect of diabetic tissue release of vasodilator factors (NO and/or PGI_2).

5. In the diabetic female rat, enhancement of contractile PGs (TXA_2 and PGE_2), attenuation of the cGMP component of the NO-cGMP relaxant cascade are observed pathological features of early-stage diabetes. This observation is in strong agreement with the well-documented reversal of the female cardiovascular protective effect once cardiovascular disease (e.g. diabetes) develops.

6. Contraction of aorta from male/female diabetic WKY rats is associated with endothelial promotion of the synthesis / activation of vasoconstrictor antagonists (such as EDNO and/or PGI_2). Compared to the male, diabetic female tissues produced higher levels of $\bullet O_2^-$ /(H_2O_2) which appears to have contributed to the greater female tissue contraction but appears to be a vasorelaxant in diabetic male tissues in early-stage diabetes. Enhanced diabetic synthesis of vasorelaxant factors (EDNO and/or PGI_2) are probably part of the pathophysiological mechanism in diabetic tissues to compensate for the increased tissue contraction associated with this condition.

7. The vasorelaxant action of quercetin is tissue-selective, and is more prominent in oxidatively stressed tissues (i.e. normoglycemic / diabetic male (with or without endothelium) and diabetic female (with or without endothelium) compared to tissues with minimal stress (i.e. normoglycemic female (without than with endothelium)). These results support the hypothesis that quercetin vasorelaxant action is partly mediated by its action against oxidative stress in aorta of male / female WKY rats.

8. In addition to anti-oxidative stress mechanisms, quercetin vascular protective effect is mediated by endothelium-dependent (eNO-sGC-cGMP and/or cyclooxygenase) and -independent (NO or cGMP) mechanisms. The former mechanism appears to be partly mediated by the $\bullet O_2^-/H_2O_2$-cyclooxygenase signal transduction pathway and is more predominant in male than female normoglycemic / diabetic WKY rat aorta. The latter mechanism is more prominent in normoglycemic than diabetic tissues from both genders. The current result in normoglycemic / diabetic female rat is the first of its kind to describe the vasodilator activity and mechanism of quercetin action in this gender.

9. Given that estradiol and/ or quercetin intervention reduced contraction in PE (but not Ang II)-contracted diabetic tissue from both genders, the combination of quercetin / estradiol may be more clinically relevant in managing PE than Ang II-induced reactivity. This may be particularly so, in the latter stages of diabetes disease when endothelial function deteriorates even more.

In conclusion, the present study suggests that: 1) gender differences exist in PE or Ang II-induced contraction of normoglycemic and diabetic aorta from WKY rats, 2) the aorta of the normoglycemic female WKY rats exists in a higher state of basal vasorelaxation and this female 'advantage' is attenuated by the development of diabetes,

3) the vascular protective action of the antioxidant quercetin is tissue- specific being more profound in tissues with higher oxidative stress factors, 4) in addition to oxidative stress, quercetin vasodilator action in normoglycemic /diabetic male / female aortic tissues are mediated by endothelium-dependent (eNO-sGC-cGMP and/or cyclooxygenase) and -independent (NO or cGMP) mechanisms, the latter of which is more prominent in normoglycemic than diabetic tissues from both genders, 5). Pharmacological intervention with the antioxidant flavonoid, quercetin, with or without estradiol may have clinical relevance in managing aortic tissue pathophysiology related to α_1-adrenoceptor stimulation (by PE) than those related to Ang II stimulation. The current findings have implications for further understanding of the gender-related differences in the mechanism of diabetes-induced vascular disease and the potential therapeutic usefulness of quercetin, estradiol or both in managing this major pathophysiology of a global disease-diabetes mellitus.

REFERENCES

Abebe, W., Harris, K.H., MacLeod, K.M. (1990). Enhanced contractile responses of arteries from diabetic rats to alpha α_1-adrenoceptor stimulation in the absence and presence of extracellular calcium. *J. Cardiovasc. Pharmacol.* **16(2),** 239-248.

Aebi, H. (1984). Catalase in vitro. *Enzymol.* **105,**121-126.

Ajay, M., Gilani, A.U., Mustafa, M.R. (2003). Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. *Life Sci.* **74,** 603–612.

Ajay, M., Achike, F.I., Mustafa, A.M., Mustafa, M.R. (2006 a). Effect of quercetin on altered vascular reactivity in aortas isolated from STZ-induced diabetic rats. *Diab. Res. Clin. Pract.* **3,** 1–7.

Ajay, M., Achike, F.I., Mustafa, A.M., Mustafa, M.R. (2006 b). Direct effects of quercetin on impaired reactivity of spontaneously hypertensive rat aortas: comparative study with ascorbic acid. *Clin. Exp. Pharmacol. Physiol.* **33(4),** 345–350.

Ajay, M., Mustafa, M.R. (2005). Chronic treatment with flavonoids prevents endothelial dysfunction in spontaneously hypertensive rat aorta. *J. Cardiovasc. Pharmacol.* **46,** 36-40.

Ajay, M., Achike, F.I., Mustafa, A.M., Mustafa, M.R. (2007). Quercetin, a flavonoid antioxidant, modulates endothelium-derived nitric oxide bioavailability in diabetic rat aortas. *Nitric Oxide* **16(4),** 4042–4447.

Aliciguzel, Y., Ozen, I., Aslan, M., Karayalcin, U. (2003). Activities of xanthine oxidoreductase and antioxidant enzymes in different tissues of diabetic rats. *J. Lab. Clin. Med.* **142(3)**, 172-177.

Amos, A.F., McCarty, D.J., Zimmet, P. (1997). The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabet. Med.* **14(5),** S1–S85.

Archer, S.L., Gragasin, F.S., Wu, X.C., Wang, S., McMurtry, M.S., Kim, D.K., Platonov, M., Koshal, A., Hashimoto, K., Campbell, W.B., Falck, J.R., Michelakis, E.D. (2003). Endothelial-derived hyperpolarizing factor in human internal mammary artery is 11, 12-epoxyeicosatrienoic acid and causes relaxation by activating smooth muscle BKca channels. *Circulation* **107(5),** 769-776.

Atalay, M., Laaksonen, D.E. (2002). Diabetes, oxidative stress and physical exercise. *J. Sports Sci. Med.* **1,** 1–14.

Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Coleman, R., Hayek, T., Presser, D., Fuhrman, B. (2000). Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am. J. Clin. Nutr.* **71(5),** 1062-1076.

Babe, K.S. Jr., Serafin, W.E. (1996). Histamine, bradykinin, and their antagonists. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG, eds. Goodman and Gilman's The Pharmacological Basis of Therapeutics. 9th ed. New York, NY: McGraw-Hill, 581-600.

Barp, J., Araújo, A.S.R., Fernandes, T.R.G., Rigatto, K.V., Llesuy, S., Belló-Klein A, et al. (2002). Myocardial antioxidant and oxidative stress changes due to sex hormones. *Braz. J. Med. Biol. Res.* **35,** 1075-81.

Barrett-Conner, E., Bush, T.L. (1991). Estrogen and coronary heart disease in women. *J. Am Med. Assoc.* **265,** 1861-1867.

Barrett-Conner, E.L., Cohn, B.A., Wingard, D.L., Edelstein, S.L. (1991). Why is diabetes mellitus a stronger risk factor for a fatal ischemic heart disease in women than in men? *J. Am. Med. Asso.* **265,** 627-631.

Bayraktutan, U. (2002). Free radicals, diabetes and endothelial dysfunction. *Diab. Obes. Met.* **4,** 224–238.

Bell, R.H., Jr Hye, R.J. (1983). Animal models of diabetes mellitus: physiology and pathology. *J. Surg. Res.* **35**, 433-460.

Beretz, A., Stoclet, J., Anton, R. (1980). Inhibition of isolated rat aorta contraction by flavonoids. Possible correlation with cAMP-phosphodiesterase inhibition. *Planta Medica,* **39,** 236.

Beyer, W., Fridovich, I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* **161,** 559–566.

Binko, J., Majweski, H. (1998). 17β-estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS. *Am. J. Physiol. Heart Circ. Physiol.* **274**, 853-859.

Bleeke, T., Zhang, H., Madamanchi, N., Patterson, C., Faber, J.E. (2004). Catecholamine-induced vascular wall growth is dependent on generation of reactive oxygen species. *Circ. Res.* **94,** 37-45.

Bolego, C., Cignarella, A., Zancan, V., Pinna, C., Zanardo. R., Puglisi, L. (1999). Diabetes abolishes the vascular protective effects of estrogen in female rats. *Life Sci.* **64,** 741–749.

Brand, M.D. (2010). The sites and topology of mitochondrial superoxide production. *Exp. Gerontol.* **45(7-8),** 466-472.

Brandes, R.P., Mugge, A. (1997). Sex differences in the generation of superoxide anions in the rat aorta. *Life Sci.* **60,** 391–396.

Brend, E., Jutta, H., Dieter, W. (1989). Singlet oxygen as an ultimately reactive species in Salmonella typhimurium DNA damage induced by methylene blue and visible light. *Carcinogenesis* **10,** 2019-2024.

Browne, D.L., Meekings, D.R., Allard, S., Munday, L.J., Shaw, K.M., Cumming, M.H. (2007). Vasodilator prostanoids compensate for attenuated nitric oxide mediated vasodilatation in type 1 diabetes. *Br. J. Diab.Vasc. Dis.* **7,** 288.

Busse, R., Fleming, I. (1993). The endothelial organ. *Curr. Opin. Cardiol.* **8,** 719-727.

Cao, G., Sofic, E., Prior, R.L. (1997). Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic. Biol. Med.* **22(5),** 749-760.

Carafoli E. (1991). Calcium Pump of the Plasma Membrane. *Physiol. Rev.* **71(1),** 129-153.

Caulfield, M.P., Birdsall, N.J. (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* **50,** 279-290.

Chakravarthy, U., Hayes, R.G., Stitt, A.W., McAuley, E., Archer, D.B. (1998). Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes* **47,** 945-952.

Chan, E.C.H., Drummond, G.R.,Woodman, O.L. (2003). 3′, 4′-dihydroxyflavonol enhances nitric oxide bioavailability and improves vascular function after ischemia and reperfusion injury in the rat. *J. Cardiovasc. Pharmacol.* **42,** 727–735.

Chan, E.C.H., Pannangpetch, P., Woodman, O.L. (2000). Relaxation of flavones and flavonols in rat isolated thoracic aorta: mechanism of action and structure-activity relationships. *J. Cardiovasc. Pharmacol.* **35,** 326–333

Chang, K.S., Stevens, W.C. (1992). Endothelium-dependent increase in vascular sensitivity to phenylephrine in long-term Streptozotocin diabetic rat aorta. *Br. J. Pharmacol.* **107,** 983-990.

Chen, G., Ye, Y.**,** Li, L.**,** Yang, Y., Qian, A., Hu, S. (2009). Endothelium-independent vasorelaxant effect of sodium ferulate on rat thoracic aorta. *Life Sci.* **84** (3-4), 81-88.

Chen, Q., Espey, M.G., Sun, A.Y., Pooput, C., Kirk, K.L., Krishna, M.C., et al. (2008) Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. *Proc. Natl. Acad. Sci. U S A.* **105,** 11105-11109.

Chen, Q., Espey, M.G., Sun, A.Y., Lee, J.H., Krishna, M.C., Shacter, E., et al. (2007). Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc. Natl. Acad. Sci. U S A,* **104 (21)**, 8749-8754.

Chin, L.C., Achike, F.I., Mustafa, M.R. (2007). Hydrogen peroxide modulates angiotensin II-induced contraction of mesenteric arteries from streptozotocin-induced diabetic rats. *Vasc. Pharmacol.* **46,** 223–228.

Chu, K.Y., Leung, P.S. (2009). Angiotensin II in Type 2 Diabetes Mellitus. *Curr. Protein Pept.* Sci. **10,** 75-84.

Cogolludo, A., Frazziano, G., Briones, A.M., Cobeno, L., Moreno, L., Lodi, F et al. (2007). The dietary flavonoid quercetin activates BKCa currents in coronary arteries via production of H_2O_2. Role in vasodilatation. *Cardiovasc. Res.* **73,** 424–431.

Cohen, R.A., (2005). Role of nitric oxide in diabetic complications. *Am. J. Ther.* **12,** 499–502.

Cohen, R.A., Vanhoutte, P.M. (1995). Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. *Circulation* **92,** 3337-3349.

Cooke, J.P. (2000). The endothelium: a new target for therapy. *Vasc. Med.* **5,** 49-53.

Costarella, C.E., Stallone, J.N., Rutecki, G.W., Whittier, F.C. (1996).Testosterone causes direct relaxation of rat thoracic aorta. *J. Pharmacol. Exp. Ther.* **277,** 34–39.

Creager, M.A., Lüscher, T.F., Cosentino, F., Beckman, J.A. (2003). Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Circulation* **108**, 1527-1532

Crews, J.K., Khalil, R.A. (1999). Antagonistic effects of 17β-estradiol, progesterone and testosterone on Ca^{2+} entry mechanisms of coronary vasoconstriction. *Arterioscler. Thromb. Vasc. Biol.* **19,** 1034–1040.

Csanyi, G., Lepran, L., Flesch, T., Telegdy, G., Gyula, S., Mezei, Z. (2007). Lack of endothelium-derived hyperpolarizing factor (EDHF) up-regulation in endothelial dysfunction in aorta in diabetic rats *Pharmacological Reports* **59,** 447-455.

Czubryt, M.P., Espira, L., Lamoureux, L., Abrenica, B. (2006). The role of sex in cardiac function and disease. *Can. J. Physiol. Pharmacol.* **84,** 93–109.

Darley-Usmar, V., Wiseman, H., Halliwell, B. (1995). Nitric oxide and oxygen radicals, a question of balance. *FEBS Letters*, **369,** 13-15.

Das, R., Kravtsov, G.M., Ballard, H.J., Kwan, C.Y. (1999). L-NAME inhibits Mg^{2+}-induced rat aortic relaxation in the absence of endothelium. *Br. J. Pharmacol.* **128 (2),** 493–499.

De Vriese, A.S., Verbeuren, T.J., van de Voorde, J., Lameire, N.H., Vanhoutte, P.M. (2000). Endothelial dysfunction in diabetes. *Br. J. Pharmacol.* **130,** 963-974.

Diao, W.F., Chen, W.Q., Hoger, H., Pollak, A., Lubec, G. (2008). Fluctuations of hippocampal neuronal protein levels over the estrus cycle in the rat. *Neurochem. Int.* **52,** 1002–1011.

Dresner, L.S., Wang, S.P., West, M.W., Ponomarenko, I.N., Mueller, C.M., Wait, R.B. (1997). Nitric oxide inhibition simulates the enhancement of α_1-agonist-induced vasoconstriction in diabetes. *J. Surg. Res.* **70,** 119–123.

Duarte, J., Perez-Vizcaino, F., Utrilla, P., Jimenez, J., Tamargo, J., Zarzuelo, A. (1993). Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure-activity relationships. *Gen. Pharmacol.* **24,** 857–862.

Dzau, V.J., Bernstein, K., Celermajer, D., Cohen, J., Dahlof, B., Deanfield, J., et al. (2001). The relevance of tissue Angiotensin-converting enzyme: manifestations in mechanistic and endpoint data. *Am. J. Cardiol.* **88 (9),** 1L-20L.

Edwards, R.L., Lyon, T., Litwin, S.E., Rabovsky, A., Symons, J.D., Jalili, T. (2007). Quercetin reduces blood pressure in hypertensive subjects. *J. Nutr.* **137,** 2405–2411.

Egert, S., Bosy-Westphal, A., Seiberl, J., Kurbitz, C.,Settler, U., Plachta-Danielzik, S. et al. (2009). Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: A double-blinded, placebo-controlled cross-over study. *Br. J. Nutr.* **102,** 1–10.

Endemann, D.H., Schiffrin, E.L. (2004). Endothelial dysfunction. *J. Am. Soc. Nephrol.* **15 (8),**1983-1992.

Etoh, T., Inoguchi, T., Kakimoto, M., Sonoda, N., Kobayashi, K., Kuroda, J., Sumimoto, H., Nawata, H. (2003). Increased expression of NAD(P)H oxidase subunits, NOX_4 and p22phox, in the kidney of streptozotocin-induced diabetic rats and its reversibility by interventive insulin treatment. *Diabetologia* **46,** 1428-1437.

Faraci, F.M., Didion, S.P. (2004). Vascular protection: Superoxide dismutase isoforms in the vessel wall. *Arterioscler. Thromb. Vasc. Biol.* **24,** 1367–73.

Feletou, M., Huang, Y., Vanhoutte, P.M. (2010). Vasoconstrictor prostanoids. *Pflugers Arch- Eur. J. Physiol.* **459**, 941–950.

Feletou, M., Vanhoutte, P.M. (1999). The alternative: EDHF. *J. Mol. Cell. Cardiol.* **31,** 15-22.

FitzGerald, G.A., Austin, S., Egan, K., Cheng, Y., Pratico, D. (2001). Cyclooxygenase products and atherothrombosis. *Ann. Med.* **32 (1),** 21-26.

Florian, M., Freiman, A., Magder, S. (2004). Treatment with 17β-estradiol reduces superoxide production in aorta of ovariectomized rats. *Steroids* **69,** 779–787.

Fontana L., (2009). Modulating human aging and age-associated diseases. *Biochim. Biophys. Acta***. 1790**, 1133–1138.

Fouda, A.K., Kaufmann, A., Thorin, E., Henrion, D., Capdeville-Atkinson, C. Atkinson, J. (1991). The role of endogenous nor epinephrine release in potassium-evoked vasoconstriction of the rat tail artery. *Eur. J. Pharmacol.* **205,** 63-72.

Freedman, J.E., Parker, C., Li, L. et al. (2001). Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. *Circulation* **103 (23),** 2792-2798.

Fuhrman, B., Rosenblat, M., Hayek, T., Coleman, R., Aviram, M. (2000). Ginger extract consumption reduces plasma cholesterol, inhibits LDL oxidation and attenuates development of atherosclerosis in atherosclerotic, apolipoprotein E-deficient mice. *J. Nutr.* **130 (5),** 1124-1131.

Fujimoto, S., Asano, T., Sakai, M., Sakurai, K., Takagi, D., Yoshimoto, N., Itoh, T. (2001). Mechanisms of hydrogen peroxide-induced relaxation in rabbit mesenteric small artery. *Eur. J. Pharmacol*. **412 (3),** 291–300.

Fulton, D.J.R., Hodgson, W.W.C., Sikorski, B.W., King, R.G. (1991). Attenuated responses to endothelin-1, KCI and $CaCl_2$, but not noradrenalin of aorta from rats with streptozotocin-induced diabetes mellitus. *Br. J. Pharmacol.* **104,** 928-932.

Furchgott, R.F., Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature (Lond.)* **288,** 373–376.

Gaba, M.K., Gaba, S., Clark, L.T. (1999). Cardiovascular disease in patients with diabetes: clinical considerations. *J. Assoc. Acad. Minor Phys.* **10,** 15–22.

Gainer, J.V., Morrow, J.D., Loveland. A., King, D.J., Brown, N.J. (1998). Effect of bradykinin receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects. *N. Engl. J. Med.* **339,** 1285-1292.

Gauthier, K.M., Edwards, E.M., Falck, J.R., Reddy, D.S., Campbell, W.B. (2005). 14, 15-epoxyeicosatrienoic acid represents a transferable endothelium-dependent relaxing factor in bovine coronary arteries. *Hypertension* **45 (4),** 666-671.

Geary, G.G., Krause, D.N., Duckles, S.P. (2000). Estrogen reduces mouse cerebral artery tone through endothelial NOS- and cyclooxygenase dependent mechanisms. *Am. J. Physiol. Heart. Circ. Physiol.* **279**. H511–H519.

Geleijnse, J.M., Launer, L.J., Hofman, A., Pols, H.A., Witteman, J.C. (1999). Tea flavonoids may protect against atherosclerosis: The Rotterdam Study. *Arch. Intern. Med.* **159 (18),** 2170-2174.

Geraldes, P., Sirois, M.G., Bernatchez, P.N., Tanguay, J.F. (2002). Estrogen regulation of endothelial and smooth muscle cell migration and proliferation: role of p38 and p42/44 mitogen-activated protein kinase. *Arterioscler. Thromb. Vasc. Biol.* **22,** 1585–1590.

Gerhard, M., Ganz, P. (1995). How do we explain the clinical benefits of estrogen from bedside to bench. *Circulation* **92**, 5–8.

Gewaltig, M.T., Kojda, G. (2002). Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovasc. Res.* **55 (2),** 250-260.

Gilligan, D. M., Quyyumi, A. A., Cannon, R. 0. (1994). Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. *Circulation* **89,** 2545-2551

Golovchenko, I., Goalstone, M.L, Watson, P., et al. (2000). Hyperinsulinemia angiotensin II, hyperglycemia, and advanced glycosylation end products in vascular smooth muscle cells. *Circ. Res.* **87**,746–752.

Guzik. T.J., Mussa, S., Gastaldi, D., Sadowski. J., Ratnatunga, C., Pillai, R., Channon, K.M. (2002). Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* **105 (14),** 1656-1662.

Hagen, A.A., Shinsava, Y., White, R.P. (1985). Experimental diabetes: Reduction of serotonin- induced vasoconstriction by meclofenamic acid in vitro. *Pharmacology* **30,** 197-204.

Halliwell, B. (1999). Antioxidant defence mechanisms: From the beginning to the end (of the beginning). *Free Radic. Res.* **31**, 261-272.

Hammes, H.P., Martin, S., Federlin, K., Geisen, K., Brownlee, M. (1991). Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc. Natl. Acad. Sci. USA* **88,** 11555–11558.

Harwood, M., Danielewska-Nikiel, B., Borzellaca, J.F., Flamm, G.W., Williams, G.M., Lines, T.C. (2007). A Critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem. Toxicol.* **45,** 2178-2205.

Hathaway, D.R.**,** March, K.L., Lash. J.A., Adam, L.P., Wilensky, R.L. (1991). Vascular smooth muscle: A review of the molecular basis of contractility. *Circulation* **83,** 382-390

Hayashi, T., Fukuto, J.M., Ignarro, L.J., Chaudhuri, G. (1992). Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: Implications for atherosclerosis. *Proc. Natl. Acad. Sci.* **89,** 11259–11263.

Hayek, T., Fuhrman, B., Vaya J., Rosenblat, M., Belinky, P., Coleman, R., Elis, A., Aviram, M. (1997). Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler. Thromb. Vasc. Biol.* **17 (11),** 2744-2752.

Hernandez, I., Delgado, J.L., Diaz, J., Quesada, T., Teruel, M.J., Llanos, M.C., Carbonell, L.F. (2000). 17β-estradiol prevents oxidative stress and decreases blood pressure in ovariectomized rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **279,** R1599–R1605.

Hertog, M.G.L., Feskens, E.J.M, Hollman, P.C.H., Katan, M.B., Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen elderly study. *Lancet.* ***342,*** 1007–1011.

Hilgers, K.F., Stumpf, C. (2002). Angiotensin II, the endothelium and superoxide anions. *J. Hypertens.* **20,** 1271–1273.

Honda, H., Unemoto, T., Kogo, H. (1999). Different mechanisms for testosterone-induced relaxation of aorta between normotensive and spontaneously hypertensive rats. *Hypertension* **34,** 1232–1236.

Hornig, B., Kohler, C., Drexler, H. (1997). Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. *Circulation* **95,** 1115-1118.

Ichikawa, Y., Yokoyama, M., Akita. H., Fukuzaki, H. (1989). Constriction of a large coronary artery contributes to serotonin-induced myocardial ischemia in the dog with pliable coronary stenosis. *J. Am. Coll. Cardiol.* **14 (2),** 449-459.

Ide, T., Tsutsui, H., Ohashi, N., Hayashidani, S., Suematsu, N., Tsuchihashi, M., Tamai, H., Takeshita, A. (2002). Greater oxidative stress in healthy young men compared with premenopausal women. *Arterioscler. Thromb. Vasc. Biol.* **22**, 438–442**.**

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci.* **84,** 9265-9269.

Jakus, V. (2000). The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease. *Bratisl. Lek. Listy* **101 (10),** 541–551.

Jeremy, J.Y., Thompson, C.S., Mikhailidis, D.P. (1993). Differential changes of adrenoceptor-and muscarinic receptor-linked prostacyclin synthesis by the aorta and urinary bladder of the diabetic rat. *Br. J. Pharmacol.* **108,** 1131-1136.

Johansen, J.S., Harris, A.K., Rychly, D.J., Ergul, A. (2005). Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice. *Cardiovasc. Diabetol.* **4,** 5.

Joly, G.A., Ayres, M., Chelly, F., Kilbourn, R.G. (1994). Effects of N^G-methyl-L -arginine, N^Gnitro-L-arginine, and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem. Biophys. Res. Commun.* **199,** 147-154.

Kakucs R, Varbiro S, Nadasy GL, Monos E, and Szekacs B. (2001). Acute, nongenomic vasodilatory action of estradiol is attenuated by chronic estradiol treatment. *Exp. Biol. Med. (Maywood)* **226,** 538–542.

Karaki, H., Weiss, G.B. (1988). Calcium release in smooth muscle. *Life Sci.* **42,** 111-122.

Kauser, K., Rubanyi, G.M. (1994). Gender difference in bioassayable endothelium-derived nitric oxide from isolated rat aorta. *M. J. Physiol.* **267,** H2311-2317.

Kerr, S., Brosnan, M.J., McIntyre, M., Reid, J.L., Dominiczak, A.F., Hamilton, C.A. (1999). Superoxide anion production is increased in a model of genetic hypertension: Role of the endothelium. *Hypertension* **33,** 1353-1358.

Khalil, R.A., van Breemen. C. (1995). Mechanisms of calcium mobilization and homeostasis in vascular smooth muscle and their relevance to hypertension. In: Hypertension: Pathophysiology, Diagnosis, and Management, edited by Laragh JH and Brenner BM. New York: Raven, p. 523–540.

Khoo, N.K., White, C.R., Pozzo-Miller, L., Zhou, F., Constance, C., Inoue, T., Patel, R.P., Parks, D.A. (2010). Dietary flavonoid quercetin stimulates vasorelaxation in aortic vessels. *Free Radic. Biol. Med.* **49,** 339–347.

Kim, N.N., Stankovic, M., Cushman, T.T., Goldstein, I., Munarriz, R., Traish, A. M. (2006). Streptozotocin-induced diabetes in the rat is associated with changes in vaginal hemodynamics, morphology and biochemical markers *BMC Physiology* **6**, 4.

Kneale, B.J., Chowienczyk, P.J., Brett, S.E., Coltart, D.J., Ritter, J.M. (2000). Gender differences in sensitivity to adrenergic agonists of forearm resistance vasculature. *J. Am. Coll. Cardiol.* **36,** 1233–1238.

Knekt, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliovaara, M., Reunanen, A., Hakulinen, T., Aromaa, A. (2002). Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* **76,** 560–568.

Koppenol, W.H., Moreno, J.J., Pryor, W.A., Ischiropoulos, H., Beckman, J.S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5 (6),** 834–842.

Kurzelewski, M., Czarnowska, E., Beresewicz, A. (2005). Superoxide-and nitric oxide-derived species mediate endothelial dysfunction, endothelial glycocalyx disruption, and enhanced neutrophil adhesion in the post-ischemic guinea-pig heart. *J. Physiol. Pharmacol.* **56,** 163–178.

Lakhanpal, P., Rai, D.K. (2007) Quercetin: a versatile flavonoid. *Internet Journal of Medical Update* Jul-Dec **2,** 22-37.

Landmesser, U., Harrison, D.G., Drexler, H. (2006). Oxidant stress: a major cause of reduced endothelial nitric oxide availability in cardiovascular disease. *Eur. J. Clin. Pharmacol.* **62 (1),** 13–19.

Larson, A.J., David, Symons J.D., Jalili, T. (2010). Quercetin: A Treatment for Hypertension? A Review of Efficacy and Mechanisms. *Pharmaceuticals* **3,** 237-250.

Lenzen S. (2008). The mechanisms of alloxan- and streptozotocin-induced diabetes *Diabetologia* **51,** 216–226.

Li, Z., Duckles, S.P. (1994). Influence of gender on vascular reactivity in the rat. *J. Pharmacol. Exp. Ther.* **268,** 1426-1431.

Li, Z., Krause, D.N., Doolen, S., Duckles, S.P. (1997). Ovariectomy eliminates sex differences in rat tail artery response to adrenergic nerve stimulation. *Am. J. Physiol. Heart. Circ. Physiol.* **272,** 1819-1825.

Lima, C.V., Paula, R.D., Resende, F.L., Khosla, M.C., Santos, R.A.S. (1997). Potentiation of the hypotensive effect of bradykinin by short-term infusion of angiotensin-(1-7) in normotensive and hypertensive rats. *Hypertension* **30,**542-548.

Lincoln, T.M., Dey, N., Sellak, H. (2001). cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J. Appl. Physiol.* **91,** 1421-1430.

Linz, W., Wohlfart, P., Schölkens, B,A. Malinski, T., Wiemer, G. (1999). Interactions among ACE,bradykinins and NO. *Cardiovasc. Res.* **43,** 549-561.

Lopez-Lopez, G., Moreno, L., Cogolludo, A., Galisteo, M., Ibarra, M., Duarte, J. et al. (2004). Nitric oxide (NO) scavenging and NO protecting effects of quercetin and their biological significance in vascular smooth muscle. *Mol. Pharmacol.* **65**, 851-859.

Lorenzi, M. (2007). The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient. *Exp. Diabetes Res.* **2007,** 61038.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin-Phenol reagents. *J. Biol. Chem.* **193,** 265-275.

Lund, D.D., Faraci, F.M., Miller, F.J., Heistad, D.D. (2000). Gene transfer of eNOS improves relaxation of carotid arteries from diabetic rabbits. *Circulation* **101,** 1027-1033.

Maggi, A., Cignarella, A., Brusadelli, A., Bolego, C., Pinna, C., Puglisi, L. (2003). Diabetes undermines estrogen control of inducible nitric oxide synthase function in rat aortic smooth muscle cells through overexpression of estrogen receptor- β. *Circulation* **108,** 211–217.

Marcondes, F. K., Bianchi, F. J., Tanno, A. P. (2002). Determination of the estrus cycle phases of rats: Some helpful considerations. *Braz. J. Biol.* **62,** 609-614.

Maritim, A.C., Sanders, R.A., Watkins, J.B. (2003). Diabetes, oxidative stress, and antioxidants: A review. *J. Biochem. Mol. Toxicol.* **17 (1),** 24-38.

McAuley, D. F., Nugent, A. G., McGurk, C., Maguire, S., Hayes, J. R. and Johnston, G. D. (2000). Vasoconstriction to endogenous endothelin-1 is impaired in patients with Type II diabetes mellitus. *Clin. Sci.* **99**, 175-179.

McGuire, J., Ding, H., Triggle, C. (2001). Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factors. *Can. J. Pharmacol.* **79**, 443-470.

Meggs, L.G. (1985). Specific desensitization of the canine renal vasculature to angiotensin II despite cyclo-oxygenase inhibition. *Yale J. Biol. Med.* **58,** 453.

Mendelssohn, M.E., Karas, R.H. (1999). Mechanisms of disease: The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med.* **340,** 1801–1811.

Michael, B. (2005). The Pathobiology of diabetic complications a unifying mechanism. *Diabetes* **54 (6),** 1615-1625.

Miller, A.A., Drummond, G.R., Mast, A.E., Schmidt, H.H.H.W., Sobey, C.G. (2007). Effect of gender on NADPH-oxidase activity, expression and function in the cerebral circulation: Role of estrogen. *Stroke* **38.** 2142–2149.

Miller, V.M., Vanhoutte, P.M. (1990). 17β-Estradiol augments endothelium-dependent contractions to arachidonic acid in rabbit aorta. *Am. J. Physiol.* **258** *(Regul. Int. Comp. Physiol.)* R1502–R1507.

Misurski, D.A., Hopfner, R.L., Gopalakrishnan, V. (2001). Attenuated agonist evoked vasoconstrictor responses in the perfused mesenteric vascular bed of Streptozotocin diabetic rats. *Exp. Biol. Med.* **226,** 940–946.

Montague, C.R., Hunter, M.G., Gavrilin, M.A., Phillips, G.S., Goldschmidt-Clermont, P.J., Marsh, C.B. (2006). Activation of estrogen receptor-alpha reduces aortic smooth muscle differentiation. *Circ. Res.* **99,** 477–84.

Moon, Y.J., Wang, L., Dicenzo, R., Morris, M.E. (2008). Quercetin pharmacokinetics in humans. *Biopharm. Drug Dispos.* **29,** 205–217.

Morawietz, H., Weber, M., Rueckschloss, U., Lauer, N., Hacker, A., Kojda, G. (2001). Upregulation of vascular NAD(P)H oxidase subunit gp91phox and impairment of the nitric oxide signal transduction pathway in hypertension. *Biochem. Biophys. Res. Commun.* **285**, 1130–1135.

Morel, N., Wibo, N., Godfraind, T. (1981). A calmodulin stimulated Ca^{2+} Pump in rat aorta plasma membranes. *Biochim. Biophys.* **644,** 82-88.

Mulhern, M., Docherty, J.R. (1989). Effects of experimental diabetes on the responsiveness of rat aorta. *Br. J. Pharmacol.* **97,** 1007–1012.

Murota, K., Terao, J. (2003). Antioxidative flavonoid quercetin: Implication of its intestinal absorption and metabolism. *Arch. Biochem. Biophys.* **417,**12-27.

Murphy, J.G., Khalil, R.A. (2000). Gender-specific reduction in contractility and [Ca^{2+}]i in vascular smooth muscle cells of female rat. *Am. J. Physiol. Cell. Physiol.* **278,** C834–C844.

Muscara, M.N., McKnight, W., Asfaha, S., Wallace, J.L. (2000). Wound collagen deposition in rats: Effects of an NO-NSAID and a selective COX-2 inhibitor. *Br. J. Pharmacol.* **129,** 681–686.

Myers, T.O., Messina, E.J. (1996). Depressed arteriolar responsiveness to norepinephrine in Streptozotocin-induced diabetes in the rat. *Prostaglandins* **52,** 415–430.

Nadarali, E.K., Smith, S.L., Dolye, P.J., Williams, G. (2001). Vasorelaxant effects of estradiols on guinea pigs: A role for gender differences. *Eur. J. Clin. Investig.* **31,** 215–220.

Nascimento, N.R.F., Costa-e-Forti, A., Peter, A.A., Fonteles, M.C. (2003). Free radical scavengers improve the impaired endothelium-dependent responses in aorta and kidneys of diabetic rabbits. *Diabetes Res. Clin. Pract.* **61,** 145–153

National cholesterol education program (NCEP). Third report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report (2002). *Circulation* **106 (25),** 3143-3421.

Nelson, M.T., Patlak, J.B., Worley, J.F, Standen, N.B. (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol.* **259,** C3-C18.

Nickenig, G., Strehlow, K., Wassmann, S., Baumer, A.T., Albory, K., Sauer, H., Bohm, M. (2000). Differential effects of estrogen and progesterone on AT1 receptor gene expression in vascular smooth muscle cells. *Circulation* **102,** 1828–1833.

Nijveldt, R.J., van Nood, E., van Hoorn, D.E., Boelens, P.G., van Norren, K., van Leeuwen, P. A. (2001). Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **74,** 418–425.

Nishikawa, T., Edelstein, D., Du, X.L., et al. (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycemia damage. *Nature* **404,** 787–790.

Nugent, A. G., McGurk, C., Hayes, J. R. and Johnston, G. D. (1996). Impaired vasoconstriction to endothelin 1 in patients with NIDDM. *Diabetes* 45, 105–107.

Obrosova, I.G., Van Huysen, C., Fathallah, L., Cao, X.C., Greene, D.A., Stevens, M.J. (2002). An aldose reductase inhibitor reverses early diabetes-induced changes in peripheral nerve function, metabolism, and antioxidative defense. *FASEB Journal* **16,** 123-125.

Okamoto, K., Aoki, K. (1963). Development of a strain of spontaneously hypertensive rats. *Jpn. Circ. J.* **27,** 282-293.

Okamoto, T. (2005). Safety of quercetin for clinical application (Review). *Int. J. Mol. Med.* **16,** 275-278.

Oltman, C.L., Kane, N.L., Miller Jr, F.J., Spector, A.A., Weintraub, N.L., Dellsperger, K.C. (2003). Reactive oxygen species mediate arachidonic acid-induced dilation in porcine coronary microvessels, *Am. J. Physiol. Heart Circ. Physiol.* **285**, H2309–H2315.

Ongil, S., Arilla, E., Rodriguez-Puyol, M., Rodriguez-Puyol, D. (2001). The role of hydrogen peroxide in the contractile response to angiotensin II. *Mol. Pharmacol.* **59 (1),** 104–112.

Orshal, J. M., Khalil, R.A. (2004). Gender, sex hormones, and vascular tone. *Am. J. Physiol. (Regul. Integr. Comp. Physiol.)* **286,** R233–R249.

Ozturk, Y., Altan, V.M., Aydın, S. (1994). Effect of short and long term streptozotocin diabetes on smooth muscle calmodulin levels in the rat. *Cell Calcium* **16 (2),** 81-86.

Palatini, P. (2001). Sympathetic overactivity in hypertension: a risk factor for cardiovascular disease. *Curr. Hypertens. Rep.* **3 (1),** S3-S9.

Palin, S.L., Kumar, S., Sturdee, D.W., Barnett, A.H. (2001). HRT in women with diabetes – review of the effects on glucose and lipid metabolism. *Diabetes Res. Clin. Pract.* **54,** 67-77.

Pazdro**,** R., Burgess, J.R. (2010). The role of vitamin E and oxidative stress in diabetes complications. *Mech. Ageing Dev.***131,** 276-286.

Perez-Vizcaino, F., Duarte, F. (2010). Flavonols and cardiovascular disease. *Mol. Aspects Med.* **31,** 478-94.

Pfeiffer, S., Gorren, A.C., Schmidt, K., Werner, E.R., Hansert, B., Bohle, D.S., Mayer, B. (1997). Metabolic fate of peroxynitrite in aqueous solution. *J. Biol. Chem.* **272 (6)**, 3465-3470.

Pieper, G.M., (1998). Review of alterations in endothelial nitric oxide production in diabetes: Protective role of arginine on endothelial dysfunction. *Hypertension* **31,** 1047–1069.

Pieper. G.M. (1999). Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. *Diabetologia* **42,** 204–213.

Pietraszek, M.H., Takada, Y., Takada, A., Fujita, M., Watanabe, I., Taminato, A., et al. (1992). Blood serotonergic mechanisms in type 2 (non-insulin-dependent) diabetes mellitus. *Thromb. Res.* **66 (6),** 765-774.

Pietta, P.G. (2000). Flavonoids as antioxidants. *J. Nat. Prod.* **63,** 1035–1042.

Pinna, C., Cignarella, A., Zanardo, R., Bolego, C., Puglisi, L. (2001). Gender differences and antioxidant treatment affect aortic reactivity in short-term diabetic rats. *Eur. J. Pharmacol.* **431,** 71–79.

Pugsley, M.K., Tabrizchi, R. (2000). The vascular system. An overview of structure and function. *J. Pharmacol. Toxicol. Methods.* **44 (2),** 333-340.

Renaud, S., de Lorgeril, M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet*. **339 (8808),** 1523-1526.

Richard F Ammar, Jr. R.F., Gutterman, D.D., Brooks, L.A., Dellsperger, K.C. (2000). Free radicals mediate endothelial dysfunction of coronary arterioles in diabetes. *Cardiovasc. Res.* **47(3),** 595-601.

Robert, R., Chagneau-Derrode, C., Carretier, M., Mauco, G., Silvain, C. (2005). Gender differences in vascular reactivity of aortas from rats with and without portal hypertension. *J. Gastroenterol. Hepatol.* **20 (6),** 890-894.

Roghani, M., Baluchnejadmojarad, T., Vaez-Mahdavi, M.R., Roghani-Dehkordi, F. (2005). Mechanisms underlying quercetin-induced vasorelaxation in aorta of sub chronic diabetic rats: An in vitro study, *Vasc. Pharmacol.* **42,** 31–35.

Rolland, P.H, Jouve, R., Pellegrin, E., Mercier, C., Serradimigni, A. (1984). Alteration in prostacyclin and prostaglandin E2 production: Correlation with changes in human aortic atherosclerotic disease. *Arteriosclerosis* **4,** 70–78.

Romero M, Jimenez R, Sanchez M, Lopez-Sepulveda R, Zarzuelo MJ, O'Valle F, et al. (2009). Quercetin inhibits vascular superoxide production induced by endothelin-1: Role of NADPH oxidase, uncoupled eNOS and PKC. *Atherosclerosis* **202 (1),** 58-67.

Ruiz-Larrea, M.B., Martin, C., Martinez, R., Navarro, R., Lacort, M., Miller, N.J. (2000). Antioxidant activities of estrogen against aqueous and lipophilic radicals; Differences between phenol and catechol estrogens. *Chem. Phys. Lip.* **105,** 179–188.

Sanchez, M., Lodi, F., Vera, R., Villar, I.C., Cogolludo, A., Jimenez, R., et al. (2007). Quercetin and isorhamnetin prevent endothelial dysfunction, superoxide production, and over expression of $p47^{phox}$ Induced by angiotensin II in rat aorta. *J. Nutr.* **137,** 910-915.

Sandrini, M., Vitale, G., Vergoni, A.V., Ottani, A., Bertolini, A. (1997). Streptozotocin-induced diabetes provokes changes in serotonin concentration and on $5\text{-}HT_{1a}$ and $5\text{-}HT_2$ receptors in the rat brain. *Life Sci.* **60 (16),** 1393-1397.

Sanz, E., Fernandez, N., Monge, L., Martınez, M.A., Climent, B., Dieguez, G., Villalon, A.L. (2003). Effects of diabetes on the vascular response to nitric oxide and constrictor prostanoids: Gender and regional differences. *Life Sci.* **72,** 1537–1547.

Sartori-Valinotti, J.C., Iliescu, R., Fortepiani, L.A., Yanes, L.L., Reckelhoff, J.F. (2007). Sex differences in oxidative stress and the impact on blood pressure control and cardiovascular disease. *Clin. Exp. Pharmacol. Physiol.* **34,** 938-945.

Schafer, F.Q., Buettner, G.R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30,** 1191-1212.

Schiffrin, E.L. (2001). Role of endothelin-1 in hypertension and vascular disease. *Am. J. Hypertens.* **14,** 83S-89S.

Schubert, W., Cullberg, G., Edgar, B., Hedner, T. (1994). Inhibition of 17 beta-estradiol metabolism by grapefruit juice in ovariectomized women. *Maturitas.* **20,** 155-163.

Schwarz, P.E., Li, J., Lindstrom, J., Tuomilehto, J. (2009). Tools for predicting the risk of type 2 diabetes in daily practice. *Horm. Metab. Res.* **41**, 86–97.

Sekiguchi, F., Yanamoto, A., Sunamo, S. (2004). Superoxide dismutase reduces the impairment of endothelium-dependent relaxation in the spontaneously hypertensive rat aorta. *J. Smooth Muscle Res.* **40,** 65–74.

Shastri, S., Gopalakrishnan, V., Poduri, R., Wang, H.D. (2002). Tempol selectively attenuates angiotensin II evoked vasoconstrictor responses in spontaneously hypertensive rats. *J. Hypertens.* **20 (7),** 1381–1391.

Sherman, T.S., Chambliss, K.L., Gibson, L.L., Pace, M.C., Mendelsohn, M.E., Pfister, S.L., Shaul, P.W. (2002). Estrogen acutely activates prostacyclin synthesis in ovine fetal pulmonary artery endothelium. *Am. J. Respir. Cell. Mol. Biol.* **26,** 610–616.

Somlyo, A,P. Somlyo, A.V. (2000). Signal transduction by G-proteins, rhokinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol.* **522**, 177–185.

Sowers, J.R. (1998). Diabetes mellitus and cardiovascular disease in women. *Arch. Intern. Med.* **158,** 617–621.

Sportnitz, U.M., Socin, C.D., David, A.A. (1999). Estrus stage determination in rats by means of scanning electron microscopic images of uterine surface epithelium. *The Anat. Rec*. **254,** 116-126.

Stallone, J.N., Crofton, J.T., Share, L. (1991). Sexual dimorphism in vasopressin-induced contraction of rat aorta. *Am. J. Physiol. Heart Circ. Physiol.* **260,** 453–458.

Stenchever, M.A., Droegemueller, W., Herbst A.L., et al. (2001). Reproductive endocrinology. In: Stenchever M.A., ed. Comprehensive Gynecology, 4th ed. St. Louis: Mosby, 71-124.

Stone, J.D., Crofton, J.T., Share, L. (1989). Sex differences in central adrenergic control of vasopressin release. *Am. J. Physiol.* **257**, R1040-R1045.

Strehlow, K., Rotter, S., Wassmann, S et al. (2003). Modulation of antioxidant enzyme expression and function by estrogen. *Circ. Res.* **93,** 170–177.

Strotmeyer, E.S., Steenkiste, A.R., Foley, T.P., Berga, S.L., Dorman, J.S. (2003). Menstrual cycle differences between women with type 1 diabetes and women without diabetes. *Diabet. Care* **26,** 1016 –1021.

Stuehr, D.J. (1999). Mammalian nitric oxide synthases. *Biochim. Biophys. Acta.* **1411,** 217-230.

Suzuki, L.A., Poot, M., Gerrity, R.G., et al. (2001). Diabetes accelerates smooth muscle accumulation in lesions of atherosclerosis: Lack of direct growth-promoting effects of high glucose levels. *Diabetes* **50,** 851–860.

Taddei, S., Virdis, A., Ghiadoni, L., Sudano, I., Salvetti, A. (2001). Endothelial dysfunction in hypertension. *J. Cardiovasc. Pharmacol.* **38 (2),** S11-S14.

Teoh, H., Quan, A., Leung, S.W.S., Man, R.Y.K. (2000). Differential effects of 17β-estradiol and testosterone on the contractile responses of porcine coronary arteries. *Br. J. Pharmacol.* **129,** 1301– 1308.

Tep-Areenan, P., Kendall, D.A., Randall, M.D. (2003). Mechanism of vasorelaxation to 17ß-estradiol in rat arteries. *Eur. J. Pharmacol.* **476,** 139-149

Tesfamariam, B., Brown, M.L., Deykin, D., et al. (1990). Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta. *J. Clin. Invest*. **85,** 929–932.

Thengchaisri, N., Kuo, L. (2003). Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: Role of cyclooxygenase and potassium channels. *Am. J. Physiol. Heart Circ. Physiol.* **285,** H2255-H2263.

Thomas, G., Ito, K., Zikic, E., Bhatti, T., Han, C., Ramwell, P. (1995). Specific inhibition of the contraction of the rat aorta by 17β-estradiol. *J. Pharmacol. Exp. Ther.* **273,** 1544–1550.

Thompson, J., Khalil, R.A. (2003). Gender differences in the regulation of vascular tone. *Clin. Exp. Pharmacol. Physiol.* **30,** 90–105.

Tostes, R.C., David, F.L., Carvalho, M.H., Nigro, D., Scivoletto, R., Fortes, Z.B. (2000). Gender differences in vascular reactivity to endothelin-1 in deoxycorticosterone-salt hypertensive rats. *J. Cardiovasc. Pharmacol.* **36 (1),** S99– S101.

Touyz, R.M. (2004). Reactive oxygen species and angiotensin II signaling in vascular cells - implications in cardiovascular disease. *Braz. J. Med. Biol. Res.* **37,** 1263-1273.

Tresham, J.J., Dusting, G.J., Coghlan, J.P., Whitworth, J.A. (1991). Haemodynamic and hormonal effects of N-nitro-L-arginine, an inhibitor of NO biosynthesis, in sheep. *Clin. Exp. Pharmacol. Physiol*. **18,** 327-330.

Tsai, M., Jiang, M.J. (2010). Reactive oxygen species are involved in regulating a1-adrenoceptor-activated vascular smooth muscle contraction. *J. Biomed. Sci.* **17,** 67

Tsuneo, K., Katsuo, K. (2002). Modulation by hydrogen peroxide of noradrenaline-induced contraction in aorta from streptozotocin-induced diabetic rat. *Eur. J. Pharmacol.* **441,** 83–89.

Unger, T. (2000). Neurohormonal modulation in cardiovascular disease. Cardiac Protection. *Am. Heart J.* **139**, S2-S8.

United States Department of Agriculture (USDA) database for the flavonoid content of selected foods. (2003) .Beltsville, http://www.nal.usda.gov/fnic/foodcomp /Data/ Flav /flav. pdf/.

Vanhoutte, P.M. (1990). Vascular effects of serotonin and ischemia. J. *Cardiovasc. Pharmacol.* **16 (3)**, S15-9.

Vita, J.A. (2005). Polyphenols and cardiovascular disease: Effects on endothelial and platelet function. *Am. J. Clin. Nutr.* **81,** 292–297.

Wakeling, A.E., Dukes, M., Bowler, J.A. (2001). Potent specific pure antiestrogen with clinical potential. *Cancer Res.* **51,** 3867–3873.

Wallace, J.L., Chapman, K., McKnight, W. (1999). Limited anti-inflammatory efficacy of cyclooxygenase-2 inhibition in carrageenan-airpouch inflammation. *Br. J. Pharmacol.* **126,** 1200–1204.

Walmer, D.K., Wrona, M.A., Hughes, C.A., Nelson, K.G. (1992). Lactoferrin expression in the mouse reproductive tract during the natural estrus cycle: correlation with circulating estradiol and progesterone. *Endocrinology* **131,** 1458–1466.

Wangensteen, R., Moreno, J.M., Sainz, J., Rodríguez-Gómez, l., Chamorro, V., Luna, J.D, et al. (2004). Gender difference in the role of endothelium-derived relaxing factors modulating renal vascular reactivity. *Eur. J. Pharmacol.* **486,** 281-288.

Wassmann, K., Wassmann, S., Nickenig, G. (2005). Progesterone antagonizes the vasoprotective effect of estrogen on antioxidant enzyme expression and function. *Circ. Res.* **97,** 1046–54.

Webb, R.C. (2003). Smooth muscle contraction and relaxation. *Adv. Physiol. Educ.* **27 (4),** 201-206.

Weber, A., Jager, R., Borner, A., et al. (1996). Can grapefruit juice influence ethinylestradiol bioavailability? *Contraception.* **53,** 41-47.

Weir, G.C., Clore, E.T., Zmachinski, C.J., Bonner-Weir, S. (1981). Islet secretion in a new experimental model for non-insulin dependent diabetes. *Diabetes* **30,** 590-595.

Wiczkowski, W., Romaszko, J., Bucinski, A., Szawara-Nowak, D., Honke, J., Zielinski, H., Piskula, M.K. (2008). Quercetin from shallots (*Allium cepa L. var. aggregatum*) is more bioavailable than its glucosides. *J. Nutr.* **138,** 885–888.

Wynne, F.L., Khalil, R.A. (2003). Testosterone and coronary vascular tone:implications in coronary artery disease. *J. Endocrinol. Invest.* **26,** 181–186.

Xue. B., Johnson, A.K., Hay, M. (2007). Sex differences in angiotensin II induced *Braz. J. Med. Biol. Res.* **40,** 727-734.

Yamamoto, Y., Kamiya, K., Terao, S. (1993). Modeling of human Thromboxane A2 receptor and analysis of the receptor-ligand interaction. *J. Med. Chem.* **36,** 820–825.

Zamorano, B., Bruzzone, M.E., Martinez, J.L. **(**1994). Influence of the estrus cycle on the norepinephrine-induced contraction of rat aorta: relationship to vascular prostanoids biosynthesis. *Biol. Res.* **27 (3-4),** 209-215.

Zanetti, M., Sato, J., Katusic, Z.S., O'Brien, T. (2000). Gene transfer of eNOS alters endotheliumdependent relaxations in aortas from diabetic rabbits. *Diabetologia* **43,** 340-347.

Zou, M.H., Cohen, R., Ullrich, V. (2004). Peroxynitrite and vascular endothelial dysfunction in diabetes mellitus. *Endothelium* **11 (2),** 89–97.

Zukowska-Grojec Z. (1998). Neuropeptide Y: An adrenergic cotransmitter, vasoconstrictor, and a nerve-derived vascular growth factor. *Adv. Pharmacol.* **42,** 125-128.

APPENDIX