Endothelial dysfunction in cardiac microvascular endothelial cells: an investigation into cellular mechanisms and putative role of oleanolic acid in reversing endothelial dysfunction.

Mashudu Mudau

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University of Stellenbosch

Supervisor: Prof Hans Strijdom

Faculty of Health Sciences

Division of Medical Physiology

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Declaration

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

Introduction: The discovery of the endothelium as a regulator of vascular tone, and the subsequent discovery of nitric oxide (NO) as the major endothelium-derived relaxing factor (EDRF), has opened up vast possibilities in the continued efforts to prevent and manage cardiovascular disease. Endothelial dysfunction (ED) is defined as reduced NO bioavailability and hence the reduced ability of the endothelium to maintain vascular homeostasis. ED represents the first, reversible step in the initiation of atherosclerotic disease and is thus regarded as a strong predictive tool of ischaemic heart disease (IHD). ED and its underlying mechanisms have been largely under-investigated in myocardial capillary-derived endothelial cells (cardiac microvascular endothelial cells, CMECs), and this study aimed to address this gap in the literature. Oleanolic acid (OA) is a bioactive triterpenoid derived from leaf extracts of African medicinal plants such as *Syzigium cordatum* (Water berry tree), and has been reported to elicit vasodilatory, hypoglycaemic and hypolipidaemic properties. However its effects particularly on CMECs and its putative role in reversing ED remain unclear, and this study aimed to investigate such effects.

Aims: The aims of this study were to: (1) Establish an *in vitro* model of ED in cultured myocardial capillary-derived CMECs by developing protocols for the induction of ED. (2) Asses ED induction by measurement of the following biomarkers: (i) intracellular NO production, (ii) superoxide (O_2^-) production, (iii) nitrotyrosine expression and (iv) NADPH oxidase expression. (3) Investigate underlying cellular mechanisms of our ED model by measuring and comparing eNOS and PKB/Akt expression and activation in control and dysfunctional CMECs.

(4) Investigate the effects of OA derived from leaf extracts obtained from *Syzigium cordatum* (Hochst.) [Myrtaceace], in both control and dysfunctional CMECs.

Methods: (1) To induce ED, hyperglycaemia and inflammation were simulated by incubation with 25 mM glucose (24 hours) and 1 ng/ml TNF-α (24 hours) or 5 ng/ml TNF-α (6 and 24 hours) respectively. Reduced intracellular NO production was used as the main indicator of ED. NO production and cell viability were quantified by FACS analysis of the fluorescent probes, DAF-2/DA and propidium iodide (PI) / Annexin V respectively. Cellular mechanisms were investigated by measurement of O_2^- levels via FACS analysis of DHE fluorescence, and measurement of total and activated PKB / Akt and eNOS, p22-phox, nitrotyrosine expression via Western blotting. **(2)** Effects of OA on CMECs were investigated by pre-treatment with 30 or 40 µM OA for 5 and 20 min followed by NO production and cell viability measurements. To investigate the effects of OA on ED, CMECs were pre-treated with 40 µM OA 1 hour prior ED induction followed by NO, cell viability, and eNOS expression / activation measurements.

Results: (1) 25 mM glucose (24hours), 1 ng/ml TNF-α (24 hours) and 5 ng/ml TNF-α (6 hours) failed to induce ED as verified by an increase in NO production in the treated cells. A model of ED was successfully achieved by incubating CMECs with 5 ng/ml TNF-α (24 hours), as verified by a significant decrease in NO production. Investigations into cellular mechanisms underlying our TNF-α-induced ED model, showed that activated eNOS and PKB / Akt levels were reduced. Furthermore, O_2^- levels remained unchanged, however p22-phox (NADPH) expression was

significantly increased suggesting oxidative stress. Nitrotyrosine levels (an oxidative / nitrosative stress marker and indirect measure of eNOS uncoupling) remained at control levels. **(2)** Investigations into the effects of OA on CMECs showed that 30 µM OA increased NO production after 5 and 20 min of incubation whereas 40 µM increased NO production after 20 min only. Pre-treatment with 40 µM OA significantly reversed ED by restoring NO production back to control levels. Data from cellular mechanism investigations showed that 40 µM OA significantly increased eNOS activation in both normal and dysfunctional CMECs. Cellular viability was not negatively affected by any of the above interventions.

Discussion and Conclusions: Based on our findings, reduced activation of the PKB / Akt-eNOS pathway appears to be the primary mechanistic pathway of the TNF-α-induced model of ED. Though O_2^- levels remained at control levels, the significant increase in p22-phox is indicative of increased expression of the O_2^- producing enzyme, NADPH oxidase, thus suggesting oxidative stress. However, based on our nitrotyrosine expression data, there was no strong evidence of eNOS uncoupling in our ED model. OA significantly stimulated NO production in our model of CMECs. Furthermore, our findings showed that OA is able to reverse ED. The NO production stimulatory effects of OA in our cells appear to be achieved via the increased activation of eNOS.

We have, for the first time as far as we are aware, developed a TNF-α-induced model of ED in myocardial capillary-derived endothelial cells. It appears that reduced activation of the PKB/Akt-eNOS pathway is the primary mechanism leading to decreased NO production in this model. However, we did find some evidence of

elevated oxidative stress, which led us to believe that eNOS uncoupling cannot be excluded as a mechanism of ED in our model. In this study, we report for the first time convincing evidence that OA has powerful NO-increasing properties in myocardial capillary-derived CMECs. Our study also show novel data, which suggest that OA is able to reverse ED in this model. Follow-up investigations could shed more light on the exact mechanisms underlying OA's effects in this model.

Abstrak

Inleiding: Die ontdekking dat endoteel ‘n reguleerder van vaskulêre tonus is, en die gevolglike ontdekking dat stikstofoksied (NO) die belangrikste endoteel-afgeleide verslappingsfaktor (EDRF) is, het verskeie moontlikhede in aangaande pogings om kardiovaskulêre siektes te voorkom en hanteer, ontsluit. Endoteel-disfunksie (ED), word gedefineer as verlaagde NO biobeskikbaarheid en dus ‘n ingekorte vermoë van die endoteel om vaskulêre homeostase te handhaaf. ED verteenwoordig die eerste, omkeerbare stap in die ontstaan van aterosklerotiese siekte en word dus beskou as ‘n sterk instrument waarmee isgemiese hartsiekte voorspel kan word. Studies oor ED en sy onderliggende meganismes, veral in miokardiale kapillêre-afgeleide endoteelselle (kardiale mikrovaskulêre endoteelselle, CMECs), word redelik afgeskeep in die literatuur, en hierdie studie het dit ten doel gehad om die gaping in die literatuur aan te spreek. Oleanoliese suur (OA) is ‘n bio-aktiewe triterpenoïede wat gevind word in blaar ekstrakte van inheemse medisinale plante soos bv. *Syzigium cordatum* (Waterbessie boom). OA het bewese vasodilatoriese, hipoglukemiese en hipolipidemiese eienskappe. OA se effekte op CMECs, en sy moontlike rol in die omkering van ED, is egter onbekend, en hierdie studie het dit ten doel gehad om sulke effekte te ondersoek.

Doelwitte: Die doelwitte van hierdie studie was: (1) Die vestiging van ‘n *in vitro* model van ED in gekultuurde CMECs afkomstig van miokardiale kapillêre deur protokolle vir die induksie van ED te ontwikkel. (2) Die evaluering van ED induksie deur die volgende bio-merkers te meet: (i) intrasellulêre NO produksie, (ii) superoksied (O_2^-) produksie, (iii) nitrotirosien uitdrukking en (iv) NADPH oksidase

uitdrukking. (3) Die ondersoek na onderliggende sellulêre meganismes van ED in ons model deur die meting en vergelyking van eNOS and PKB/Akt uitdrukking en aktivering in kontrole en disfunksionele CMECs. (4) Ondersoek na die effekte van OA afkomstig van blaar ekstrakte verkry van *Syzigium cordatum* (Hochst.) [Myrtaceace], in beide kontrole en disfunksionele CMECs.

Metodes: (1) Daar was gepoog om ED te induseer deur hiperglukemie en inflammasie te simuleer met onderskeidelik 25 mM glukose (24 uur) en 1 ng/ml TNF-α (24 uur) of 5 ng/ml (6 en 24 uur) inkubasie. Verlaagde intrasellulêre NO produksie was ingespan as die hoof indikator van ED. NO produksie en sellewensvatbaarheid was gekwantifiseer deur vloeisitometriese analises (FACS) van die fluoresserende agense, DAF-2/DA en propidium jodied (PI) / Annexin V onderskeidelik. Sellulêre meganismes was ondersoek deur O_2^- vlakke via FACS analise van DHE fluoressensie te meet, asook die meting van totale en geaktiveerde PKB / Akt en eNOS, p22-phox, nitrotirosien uitdrukking via Western blot tegnieke. **(2)** Effekte van OA op CMECs was ondersoek deur vooraf-behandeling met 30 of 40 µM OA vir 5 en 20 min gevolg deur NO produksie en sellewensvatbaarheid metings.

Resultate: (1) 25 mM glukose (24 uur), 1 ng/ml TNF-α (24 uur) and 5 ng/ml TNF-α (6 uur) kon nie daarin slaag om ED te induseer nie, soos blyk uit die verhoogde NO produksie waargeneem in die behandelde selle. ‘n Model van ED was suksesvol verkry deur CMECs met 5 ng/ml TNF-α (24 uur) te inkubeer, soos waargeneem deur verlaagde NO produksie. Ondersoek na sellulêre meganismes onderliggend tot ons TNF-α-geïnduseerde ED model, het getoon dat geaktiveerde eNOS en PKB / Akt vlakke verlaag was. Verder is gevind dat O_2^- vlakke onveranderd gebly het hoewel p22-phox (NADPH) uitdrukking betekenisvol toegeneem het, wat ’n aanduiding van

oksidatiewe skade is. Nitrotirosien vlakke (‘n oksidatiewe / nitrosatiewe stres merker en indirekte maatstaf van eNOS ontkoppeling) het onveranderd rondom kontrole vlakke gebly. **(2)** Ondersoek na die effekte van OA op CMECs het getoon dat 30 µM OA tot verhoogde NO produksie na 5 en 20 min inkubasie gelei het, terwyl 40 µM slegs na 20 min NO-verhogende effekte gehad het. Vooraf behandeling met 40 µM OA het ED betekenisvol omgekeer deur NO terug na kontrole vlakke te laat herstel. Ondersoek na sellulêre meganismes het getoon dat 40 µM OA eNOS aktivering betekenisvol verhoog het in beide normale en disfunksionele CMECs. Sellulêre lewensvatbaarheid was nie negatief geaffekteer deur enige van bogeneemde ingrepe nie.

Bespreking en afleidings: Gebaseer op ons bevindinge, blyk verlaagde aktivering van die PKB/Akt-eNOS pad die primêre meganistiese pad in ons TNF-α-geïnduseerde model van ED te wees. Alhoewel O_2^- vlakke rondom kontrole vlakke gebly het, was die betekenisvolle toename in p22-phox ‘n aanduiding van verhoogde uitdrukking van die O_2^- produserende ensiem, NADPH oksidase, wat dus suggererend van oksidatiewe stres was. Aan die ander kant was daar nie sterk bewyse van eNOS ontkoppeling in ons ED model nie, gebaseer op die nitrotirosien uitdrukking data. OA het duidelik NO produksie in ons model van CMECs gestimuleer. Verder wys ons resultate dat OA in staat is om ED om te keer. Die NO produksie-stimulerende effekte van OA in ons selle blyk die gevolg te wees van verhoogde aktivering van die PKB / Akt-eNOS pad.

Ons het hier vir die eerste keer, sover ons bewus is, ‘n TNF-α-geïnduseerde model van ED in CMECs afkomstig van miokardiale kapillêre gevestig. Dit blyk dat verlaagde aktivering van die PKB/Akt-eNOS pad die primêre meganisme was waardeur verlaagde NO produksie in ons model veroorsaak was. Ons het egter wel bewyse van verhoogde oksidatiewe stress gevind, wat ons laat glo dat eNOS ontkoppeling nie heeltemal as ‘n meganisme van ED in ons model uitgesluit kan word nie. In hierdie studie toon ons vir die eerste maal oortuigende bewyse dat OA kragtige NO-verhogende eienskappe in miokardiale kapillêre-afgeleide CMECs het. Ons studie bring ook nuwe data na vore, wat suggereer dat OA in staat is om ED in hierdie model om te keer. Opvolgstudies sal meer lig kan werp op die onderliggende meganismes van OA in hierdie model.

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List of abbreviations

ACE	angiotensin converting enzyme
ADMA	asymmetric dimethylarginine
AGEs	advanced glycation end-products
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BH_3^-	trihydrobiopterin radical
BH_4	(6R)-5,6,7,8-tetrahydrobiopterin
CaM	calmodulin
CaM kinase II	calcium / calmodulin-dependent kinase II
CECs	circulating endothelial cells
cGMP	guanosine 3':5'-cyclic monophosphate
CMECs	cardiac microvascular endothelial cells
CO_2	carbon dioxide
COX	cyclooxygenase
DAF-2/DA	4,5-diaminofluorescein-2/diacetate
DAF-2T	diaminofluorescein-triazol
DDH	dimethylaminohydrolases
DHE	dihydroethidium
DiI-ac-LDL	1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanineperchlorate-acetylated-low density lipoprotein
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAS	ethyl acetate-solubles
ED	endothelial dysfunction
EDCFs	endothelium derived contracting factors
EDHF	endothelial derived hyperpolarising factor
EDRF	endothelial derived relaxing factor
EECs	endocardial endothelial cells
EGM	endothelial growth medium
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FACS	flow activated cell sorter
FAD	flavin adenine dinucleotide
FBS	foetal bovine serum
FMN	flavin mononucleotide
GMP	guanosine monophosphate
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide

HDL	high density lipoprotein
Hsp90	Heat shock protein 90
HUVECs	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
IHD	ischaemic heart disease
IL-1	Interleukin-1
IL-6	Interleukin-6
iNOS	inducible nitric oxide synthase
LDL	low density lipoprotein
MAP	mean arterial pressure
MCP-1	monocyte chemoattractant protein-1
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2	nitrogen dioxide
NOS	nitric oxide synthase
O_2^-	superoxide anion
OA	oleanolic acid
$ONOO^-$	peroxynitrite
ox-LDL	oxidised low density lipoprotein
PBS	phosphate buffered saline
PDE	phosphodiesterase
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostracyclin
PI	propidium iodide
PI-3K	phosphatidyl-inositol-3-kinase
PKA	protein kinase A
PKB / Akt	protein kinase B / Akt
PKC	protein kinase C
PKG	protein kinase G
PRMTs	protein arginine methyltransferases
RAGE	receptor for advanced glycation end-products
RNS	reactive nitrogen species
ROS	reactive oxygen species
SDS	sodium dodecylsulfate
Ser 116	serine 116
Ser 617	serine 617
Ser 635	serine 635
Ser 1177	serine 1177
sGC	soluble guanylyl cyclase
SHR	spontaneously hypertensive rats

SOD	superoxide dismutase
Thr 495	Threonine 495
TNF-α	tumour necrosis factor-alpha
TXA_2	thromboxane A_2
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
vWF	von Willebrand factor
WHO	World Health Organisation
WPBs	Weibel-Palade bodies

Introduction

In 2004, the World Health Organisation (WHO) reported cardiovascular diseases / ischaemic heart disease (IHD) to be the leading cause of death worldwide and that cardiovascular deaths are envisaged to escalate to 23.4 million by the year 2030 (WHO 2009: http://www.who.int/healthinfo/global_burden_disease). In the year 2000, the Medical Research Council ranked IHD the number five cause of death in South Africa and the number one cause of death in the Western Cape Province under the non-communicable disease category (http://www.mrc.ac.za/bod/estimates.htm). Myocardial ischaemia is defined as reduced blood flow to the heart tissue, thus leading to oxygen and nutrient deprivation of the heart muscle and ultimately myocardial infarction (Choi et al 2009). Hypertension, tobacco use, hyperglycaemia, physical inactivity and obesity, all of which can potentially lead to atherosclerosis and eventually IHD, are considered to be the top risk factors for mortality worldwide (WHO 2009).

Endothelial dysfunction (ED), characterised by reduced nitric oxide (NO) bioavailability and hence diminished vasorelaxation in larger, muscular blood vessels, is considered to be the key step in initiation of atherosclerosis and is a reliable predictor of cardiovascular disease (Bonetti *et al* 2003). Atherosclerosis is a progressive disease commonly described as the narrowing and hardening of arteries due to build up of fatty streaks in the arterial wall, and underlies the pathogenesis of IHD (Szmitko *et al* 2003). Owing to its reversibility, ED represents a potentially key therapeutic target in the prevention of atherosclerosis and hence IHD. This study aims to explore cellular mechanisms involved in the pathogenesis of ED in

myocardial capillary-derived endothelial cells, and investigate possible ED reversing effects of an African medicinal plant extract-derived bioactive compound.

Chapter 1: Literature review

1.1 The endothelium

Wilhelm His devised the term endothelium in 1865, describing a cell layer that lined the vascular system, lymphatic system and mesothelial-lined cavities such as pleura, peritoneum and pericardium (Aird 2007). As research advanced the term was later confined to the cell layer lining the vascular and lymphatic systems (Aird 2007). Many years after its discovery, the endothelium was perceived as nothing more than a passive semi-permeable barrier between blood and underlying tissues (Mas 2009). Its role as a highly metabolically active organ, maintaining vascular homeostasis and blood flow, has only become apparent in recent years (Esper *et al* 2006).

In the larger, smooth muscle containing blood vessels, the endothelium is able to sense chemical or mechanical stimuli and elicit a response by producing a variety of vasoactive substances that can either dilate or constrict the blood vessel, depending on the type of the stimulus (Chhabra 2009). Under physiological conditions, the endothelium strives to maintain a balance between a vasoconstrictive state which is usually associated with pro-oxidant, pro-inflammatory and pro-thrombotic effects and a vasodilatory state which is usually associated with anti-oxidant, anti-inflammatory and anti-thrombotic effects [fig. 1.1] (Strijdom & Lochner 2009). A vasodilatory state is mediated by factors such as nitric oxide (NO), endothelium derived hyperpolarising factor (EDHF) and prostacyclins, while a vasoconstrictive state is mediated by factors such as endothelin-1 (ET-1), angiotensin II and thromboxane A2 (Strijdom & Lochner 2009; Chhabra 2009).

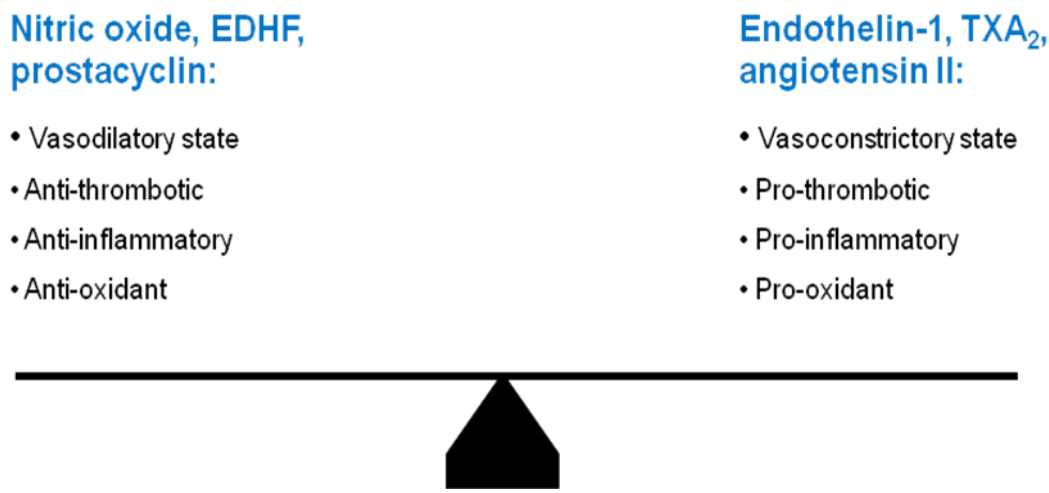


Figure 1.1: The endothelium maintains vascular homeostasis by regulating the intricate balance between a vasodilatory, anti-thrombotic, anti-inflammatory and an anti-oxidant state mediated by NO, prostacyclin and EDHF; and a vasoconstrictory, pro-thrombotic, pro-inflammatory and pro-oxidant state mediated by factors such as endothelin-1, angiotensin II and thromboxane. (Modified from Strijdom & Lochner 2009)

1.1.1 Anatomy and structure of the endothelium

The adult human endothelium is estimated to have a surface area of between 1 to 7000 m^2 (Limaye & Vadas 2006; Mas 2009), consisting of 1-6 × 10^{13} endothelial cells and approximated to account for about 1 kg of total body weight (Sumpio *et al* 2002; Limaye & Vadas 2006). An endothelial cell is flat with a large central nucleus, having a diameter of no more than 0.5 µm (Mas 2009) and shows distinct phenotypic characteristics from site to site in the vascular tree. They exhibit a circular shape in capillaries and venules whereas they are spindle shaped and arranged in the same direction as blood flow in arteries and arterioles (Mas 2009; Aird 2007). The plasma membranes of endothelial cells are profusely supplied with caveolae (Xu *et al* 2008). Caveolae are small lipid enriched invaginations of the plasma membrane which can be characterised by the presence of the protein caveolin (Dudzinski & Michel 2007).

Caveolae are generally thought to play a role in endocytosis, transcytosis and signal transduction (Xu *et al* 2008). They also control NO production through binding of endothelial nitric oxide synthase (eNOS; the most important enzyme responsible for NO production in endothelial cells) via protein caveolin, specifically caveolin-1 (Xu *et al* 2008).

Similar to most metabolically active cells, endothelial cells contain substantial numbers of mitochondria (Mas 2009). However, these cells preferentially derive their energy (ATP) anaerobically rather than from mitochondrial oxidative phosphorylation (Davidson & Duchen 2007). This led to a proposal that the principal role of mitochondria in endothelial cells may be to release nitric oxide (NO) and reactive oxygen species (ROS) as signalling molecules (Quintero *et al* 2006). Rod-shaped structures called Weibel-Palade bodies (WPBs) are uniquely expressed by endothelial cells (Weibel & Palade 1964) and are the major stores of the procoagulant von Willebrand factor (vWF) and some proinflammatory proteins including P-selectin (Mas 2009). Fusion of the WPBs with the cell membrane, followed by emptying of their contents into the blood is considered the first reaction to endothelial cell or vascular insult (Mas 2009).

Over the years, it has become apparent that the endothelium shows some structural heterogeneity across the vascular tree (Aird 2007). For example, tight junctions between endothelial cells of the larger arteries are better developed, which might reflect a structural adaptation to shear stress. In the microcirculation, the arterioles have tighter junctions than do capillaries whereas tight junctions at venules are loose

(Aird 2007). Not only do various endothelial cell subtypes differ structurally, but they have also been reported to show differences in antigenic properties and in their response to growth factors across the vascular tree (Ando *et al* 1999).

1.1.2 Endothelial cells in the heart

The endothelium has indeed emerged as an autocrine and paracrine signalling organ that has a far more complex and crucial role in maintaining cardiovascular health than merely serving as a barrier (Hsieh *et al* 2006). In the heart, endothelial cells can be categorised based on their proximity and interaction with the cardiomyocytes [fig. 1.2] (Strijdom & Lochner 2009). Endocardial endothelial cells, (EECs; endocardial endothelium) line the inner surface of the heart chamber walls, acting as barrier between circulating blood and the myocardium (Andries *et al* 1996). Owing to their close proximity with adjacent cardiomyocytes, EECs, together with the cardiac microvascular endothelial cells (CMECs; cells that line the myocardial capillaries), constitute the cardiac endothelium [Fig. 1.2] (Brutsaert 2003).

The cardiac endothelium has direct effects on the functioning and activity of cardiomyocytes. These effects include regulating metabolism, growth, contractility and rhythmicity and are mediated by autocrine and paracrine secretion of substances such as NO, ET-1, prostacyclins and a variety of growth factors (Brutsaert 2003; Hsieh *et al* 2006). On the other hand, endothelial cells from larger coronary arteries, which constitute the coronary vascular endothelium [Fig. 1.2], lie distant from cardiomyoctes (Strijdom & Lochner 2009) and indirectly affect the functioning and activity of cardiomyocytes by modulating blood supply to the myocardium (Brutsaert 2003).

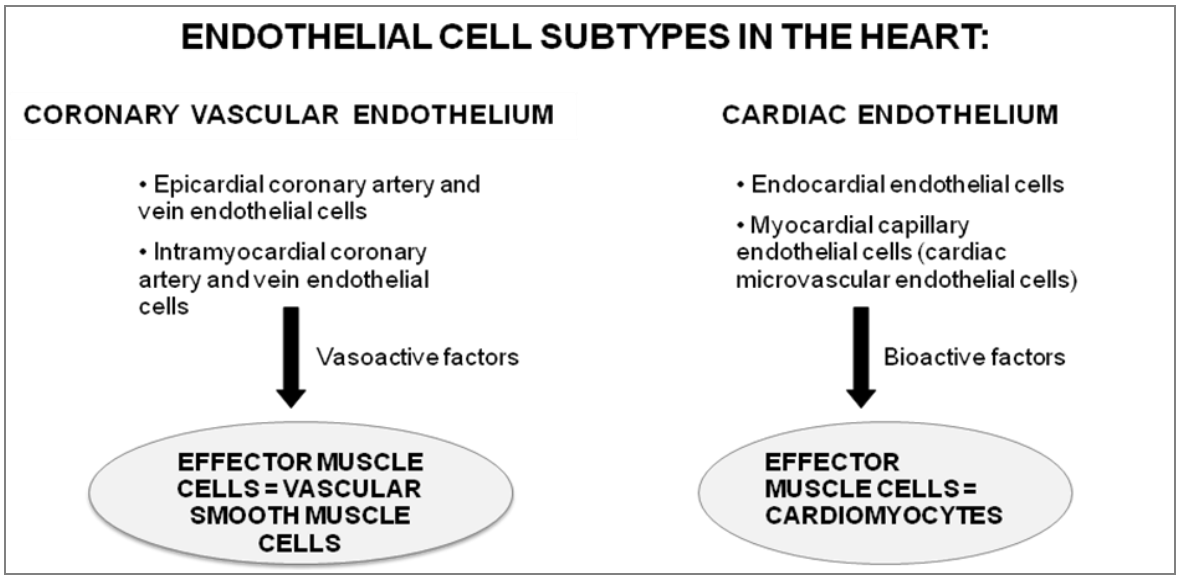


Figure 1.2: Classification of the endothelium in the heart. The coronary vascular endothelium is comprised of epicardial coronary artey and vein endothelial cells, and intramyocardial artery and vein endothelial cells and primarily target VSMCs. The cardiac endothelium is made up of EECs and CMECs which primarily targets cardiomyocytes. (Modified from Strijdom and Lochner 2009)

Owing to their location, EECs are exposed to 100 % of circulating blood unlike CMECs, which only receive about 3-5 % of the circulation (Brutsaert *et al* 1998). From this, Brutsaert *et al* (1998) deduced that aside from regulating the function and activity of the myocardium, EECs may also act as sensor of total blood derived factors. Furthermore, EECs have abundant microvilli and therefore a large contact surface area, contributing to the notion of a sensory function (Kuruvilla & Kartha 2003). The 3-5 % circulation that CMECs are exposed to would suggest that the concentration and partial pressure of blood derived factors, and not their total amount, would be of more importance in ensuring maximal diffusion between CMECs and cardiomyocytes (Brutsaert *et al* 1998). Since capillaries lack a vascular smooth muscle layer, the CMECs, unlike endothelial cells of the larger vessels do not exert vaso-active effects. Rather, it is likely that CMECs probably exclusively act

as regulators of myocardial function via autocrine and paracrine signalling (Brutsaert *et al* 1998).

It has been reported that for each cardiomyocyte there are at least 3 cardiac endothelial cells. Cardiomyocytes are estimated to vary between 10-100 µm in diameter and the intercapillary distance is approximated to vary between 15-50 µm [fig. 1.3]. The distance between each CMEC and the closest cardiomyocyte is approximately 1µm. This strategic arrangement facilitates endothelial cell-to-cardiomyocyte paracrine signalling (Brutsaert 2003).

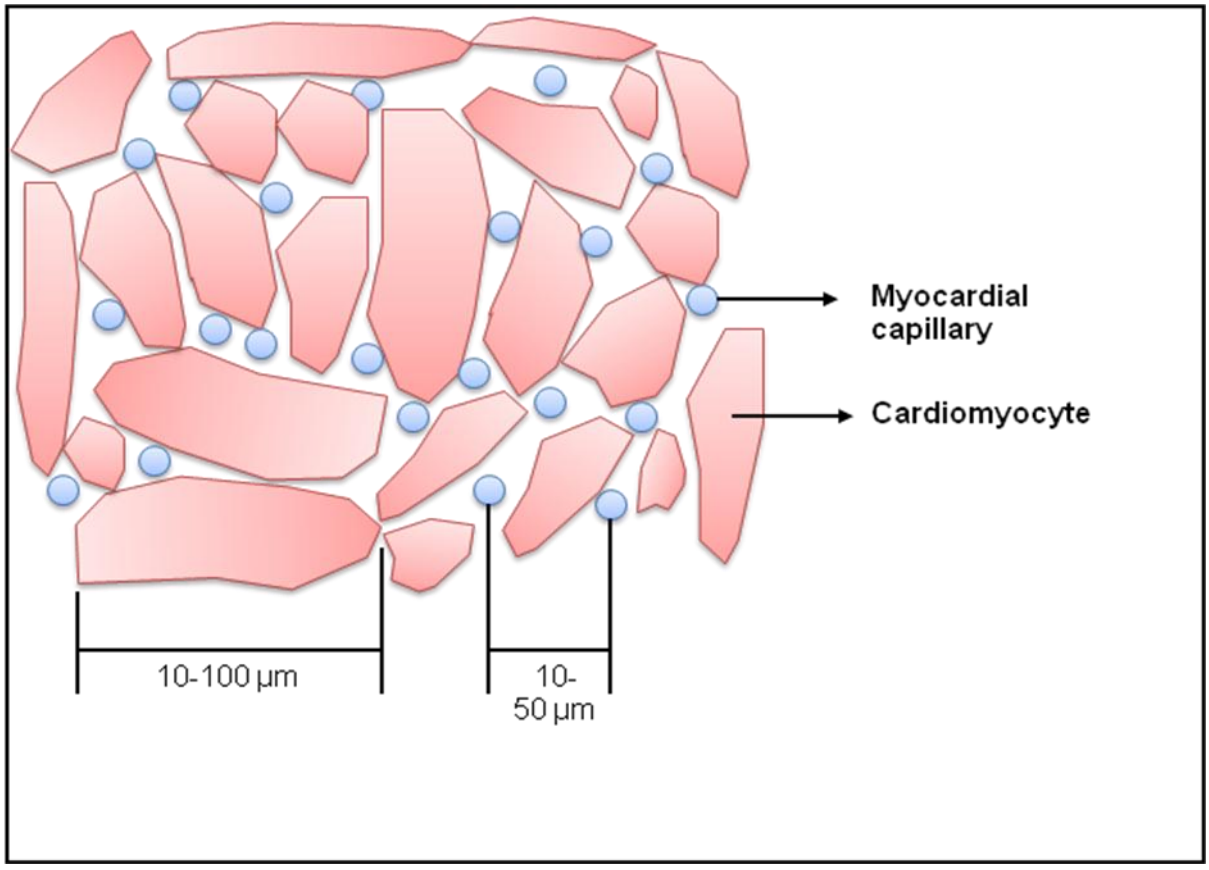


Figure 1.3: Myocardial capillary-to-cardiomyocyte distance. Each cardiomyocyte is about 10-100 µm in diameter and is surrounded by at least 3-4 myocardial capillaries. The intercapillary distance is about 10-50 µm. (Modified from Strijdom & Lochner 2009)

1.1.3 Endothelial cell-to-cardiomyocyte interaction

In the larger conduit vessels, endothelial cells act in a paracrine manner to regulate contraction/relaxation of the underlying vascular smooth muscle cells (VSMCs) via release of endothelium-derived factors such as NO, prostacyclin, ET-1 and angiotensin II. Since cardiac endothelial cells are in close proximity to cardiomyocytes, and not VSMCs, it would therefore be expected that they act in a paracrine manner to primarily influence the contractile activity of the underlying cardiomyocytes. Indeed there is evidence suggesting that cardiac endothelial derived factors act in both an autocrine and paracrine fashion to regulate function of cardiomyocytes [fig. 1.4] (Brutsaert *et al* 1998; Brutsaert 2003; Hsieh *et al* 2006; Shah & MacCarthy 2000).

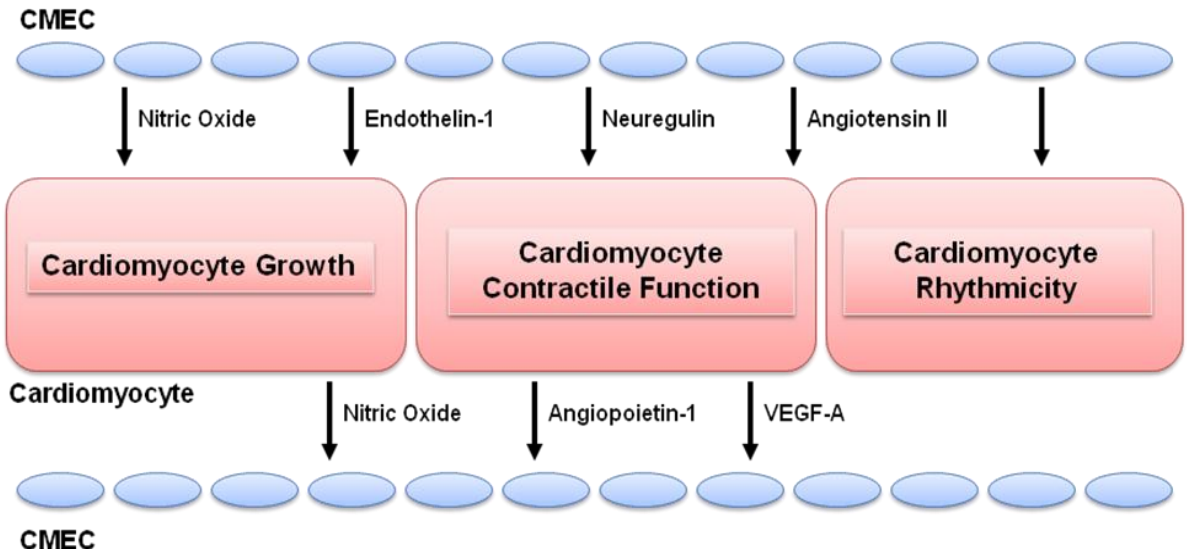


Figure 1.4: CMEC-to-cardiomyocyte interaction. CMECs regulate cardiomyocyte growth, contractile function and rhythmicity via paracrine release of factors such as NO, ET-1, angiotensin-II and neuregulin. Cardiomyocytes can in turn release factors such as NO, angiopoietin-1 and vascular endothelial growth factor-A (VEGF-A) that affect function and activity of CMECs. (Modified from Strijdom & Lochner 2009)

1.2. Normal endothelial function and endothelium-derived factors

1.2.1 Nitric oxide (NO)

The discovery that the endothelium-derived relaxing factor (EDRF) was in fact NO was rather astonishing, as NO had up till then been perceived as nothing more than a toxic environmental pollutant (Strijdom *et al* 2009 **(a)**) found in cigarette smoke, exhaust fumes of motor cars and industrial processes (Bruckdorfer 2005). In addition to its vasodilatory properties, NO was also found to inhibit platelet aggregation and their adhesion to VSMCs (Moncada *et al* 1988). This breakthrough led to an understanding of the mechanisms by which nitrovasodilators such as nitroglycerin and amyl nitrite brought about relief from angina pectoris (Moncada *et al* 1988; Strijdom *et al* 2009 **(a)**). To date, a vast number of studies have indeed proven that this molecule is endogenously synthesised in the body and plays a vital role in maintaining a healthy cardiovascular system (Strijdom *et al* 2009 **(a)**).

NO is a free radical gas with a half life of about 3-5 seconds (Rodeberg et al 1995). Owing to its gaseous and free radical nature, NO is able to diffuse easily between cells and tissues and react with a variety of molecules in the body (Strijdom *et al* 2009 **(a)**). Following its identification as the EDRF, it was reported that NO is synthesised from the amino acid L-arginine by a family of enzymes known as nitric oxide synthase (NOS) (Bruckdorfer 2005).

1.2.1.1 Nitric oxide synthase (NOS)

NOS enzyme occurs in three isoforms, namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Bruckdorfer 2005; Balligand & Cannon 1997; Bryan *et al* 2009). These isoforms were named according to where they were

first isolated. However, through advancement of research, it is now appreciated that cells can express more than one of these isoforms and that they are not exclusive to the area where they were first isolated (Bruckdorfer 2005; Bryan *et al* 2009). For example it has been found that the heart expresses all three isoforms (Balligand & Cannon 1997). iNOS was first discovered in macrophages and its expression as the name suggest, is induced by external stimuli such as cytokines (Balligand & Cannon 1997). Since these isoforms are not necessarily confined to the areas where they were initially isolated, they have also been named according to their order of isolation and cloning. nNOS, being the first isoform to be isolated, is also known as NOS I, iNOS (NOS II) was the second to be discovered and eNOS (NOS III) was the third to be isolated (Bruckdorfer 2005; Bryan *et al* 2009).

Physiologically, eNOS and nNOS are constitutive, calcium dependent enzymes and continuously produce low levels of NO. On the other hand, iNOS is calcium independent, its expression provoked by inflammatory cytokines, and producing large amounts of NO, about 1000-fold more than eNOS or nNOS (Strijdom *et al* 2009 **(a)**). This is potentially harmful as excess NO can react with the free radical superoxide anion (O_2^-) yielding another very harmful free radical known as peroxynitrite (Strijdom *et al* 2009 **(a)**). All NOS isoforms function optimally as homodimers (Balligand & Cannon1997; Bryan *et al* 2009) and require cofactors such as (6R)-5,6,7,8-tetrahydobiopterin (BH4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and iron protoporphyrin IX (haem) (Alderton *et al* 2001). Of the three isoforms, it has been proposed that eNOS is the major isoform responsible for NO production under physiological conditions in the cardiovascular system and

endothelial cells in particular (Dudzinski & Michel 2007) and thus, for the purpose of this study, eNOS will be the isoform of interest.

1.2.1.2 eNOS and biosynthesis of NO

eNOS, as all other NOS isoforms, is synthesised as two separate monomers which must form a dimer in order to catalyse formation of NO [fig. 1.5] (Forstermann & Munzel 2006). Each monomer contains a C-terminal reductase domain which binds cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), FAD, FMN and haem, and an N-terminal oxygenase domain that binds the cofactors BH4 and oxygen and the substrate L-arginine (Forstermann & Munzel 2006; Alderton *et al* 2001). These cofactors, in particular BH4, haem and L-arginine support and stabilise the dimeric structure of eNOS (Forstermann & Munzel 2006; Alderton *et al* 2001), in fact, haem is required for the dimerisation (Forstermann & Munzel 2006). At the interface of the dimer, a zinc ion is found tetrahedrally arranged to two cysteine residues from each monomer forming a zinc thiolate cluster. This site is crucial for the binding of BH4 and L-arginine (Forstermann & Munzel 2006; Alderton *et al* 2001).

During synthesis of NO, electrons flow from NADPH in the reductase domain via the flavins to the haem in the oxygenase domain (Steuhr *et al* 2001). Calmodulin, which is very important for eNOS activation, accelerates the rate of electron transfer across the reductase domain to the haem. Upon reaching the haem, these electrons reduce and activate oxygen (Steuhr *et al* 2001). eNOS catalyses conversion of L-arginine into NO in two steps. First L-arginine is hydroxylated to N-hydroxyl-L-arginine which is finally oxidised to NO and L-citrulline as a by-product (Steuhr *et al* 2001). Failure

of the enzyme to dimerise or absence of any of the cofactors mentioned above leads to the enzyme catalysing formation of O_2^- instead of NO, a mechanism referred to as eNOS uncoupling (Forstermann & Munzel 2006). A disruption in the flow of electrons can also lead to eNOS uncoupling (Forstermann & Munzel 2006).

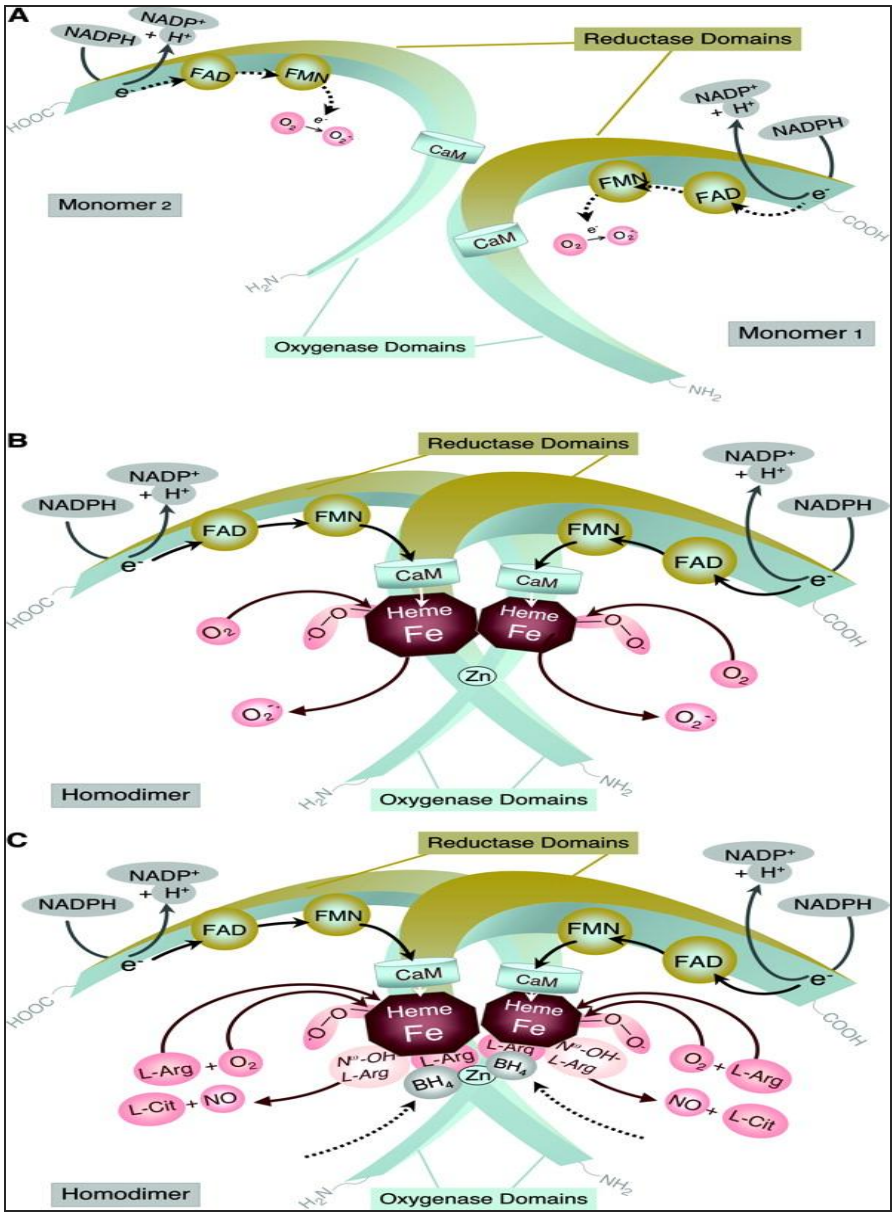


Figure 1.5: Structure and function of eNOS. **A**. eNOS is synthesised as monomers which must dimerize to catalyse production of NO. **B**. The two monomers come together and form a homodimer in the presence of the haem. However, without all the necessary cofactors and the substrate, eNOS catalyses formation of superoxide instead of NO. **C.** In the presence of all cofactors and the substrate L-arginine, eNOS can successfully catalyse conversion of L-arginine into NO and the by-product, L-citrulline. (Copied from Fostermann & Munzel 2006)

1.2.1.3 eNOS activation and phosphorylation

Activity of eNOS is regulated by intricate mechanisms such as subcellular localisation to caveolae, post-translational alterations such as phosphorylation, and association with regulatory proteins such as heat shock protein 90 (Hsp90) [fig. 1.6] (Takahashi & Mendelsohn 2003 **(a)**). Membrane-bound eNOS is localised to caveolae, where it interacts with the caveolae bound protein, caveolin-1 (Dudzinski & Michel 2007; Govers & Rabelink 2001). This interaction keeps eNOS inactive by attenuating binding of calmodulin to eNOS at low levels of calcium and thus hindering production of NO (Govers & Rabelink 2001). A surge in intracellular calcium mainly due to stimuli such as acetylcholine or bradykinin, prompts calmodulin to bind to eNOS resulting in displacement of eNOS from caveolin and activation of the enzyme (Bryan et al 2009, Dudzinski & Michel 2007, Govers and Rabelink 2001). This mechanism serves to regulate NO production under physiological conditions (Takahashi & Mendelsohn 2003 **(b)**).

Phosphorylation of eNOS by protein kinase B / Akt (PKB/Akt) at the serine 1177 (Ser 1177) site and interaction with Hsp90 are examples of complementary activation mechanisms of eNOS in addition to baseline, constitutive calcium-calmodulin activation (Dudzinski & Michel 2007). PKB/Akt is a serine/threonine kinase which activates eNOS by phosphorylating its Ser 1177 residue usually in response to stimuli such as shear stress, vascular endothelial growth factor (VEGF), insulin (Govers and Rabelink 2001) and hypoxia (Strijdom *et al* 2009 **(b)**). PKB/Akt stimulates activity of eNOS by either accelerating rate of electron transfer or diminishing dissociation of calmodulin from eNOS and may act independently of calcium (McCabe *et al* 2000; Dimmeler *et al* 1999). For example, Dimmeler *et al*

(1999) showed that PKB/Akt mediated eNOS phosphorylation in response to shear stress remained unaltered following the removal of calcium or antagonising of calmodulin by calmidazolium.

Other protein kinases such as protein kinase A (PKA), protein kinase C (PKC), AMP-activated protein kinase (AMPK), protein kinase G (PKG) and calcium/calmodulin-dependent protein kinase II (CaM kinase II) may also be involved in eNOS phosphorylation and depending on the phosphorylation site, may have a stimulatory or an inhibitory effect (Dudzinski & Michel 2007). Phosphorylation at threonine 495 (Thr 495) and Ser 116 inactivates eNOS, while phosphorylation sites such as Ser 635 and 617 in addition to the better characterised Ser 1177 site have an activating effect on eNOS (Li C *et al* 2007; Bauer *et al* 2003). Both PKA and PKB/Akt may mediate phosphorylation at Ser 617 leading to enhancement of binding of calmodulin to eNOS and thus eNOS activation (Michell *et al* 2002). Residue Ser 635 is phosphorylated by PKA while the inhibitory Thr 495 site is phosphorylated by PKC and AMPK, thereby inactivating eNOS by weakening eNOS-calmodulin interaction (Mount *et al* 2007, Dudzinski & Michel 2007). PKA, AMPK, PKG, and CaM kinase II can also phosphorylate eNOS at the Ser 1177 site (Dudzinski & Michel 2007; Mount *et al* 2007).

Hsp90, a molecular chaperone that participates in protein trafficking and folding, has also been implicated in eNOS activation (Michel & Vanhoutte 2010; Dudzinski & Michel 2007). Agonists such as VEGF, histamine, oestrogen, and shear stress induce binding of Hsp90 to eNOS (Takahashi & Mendelsohn 2003 **(b)**). Hsp90 increases eNOS activity by enhancing the binding of calmodulin to eNOS without a

rise in calcium or by stimulating flow of electrons in the reductase domain and thus augmenting eNOS activity (Takahashi & Mendelsohn 2003 **(b)**). According to Takahashi and Mendelsohn (2003) **(a)**, PKB/Akt and Hsp90 can act synergistically to increase activity of eNOS.

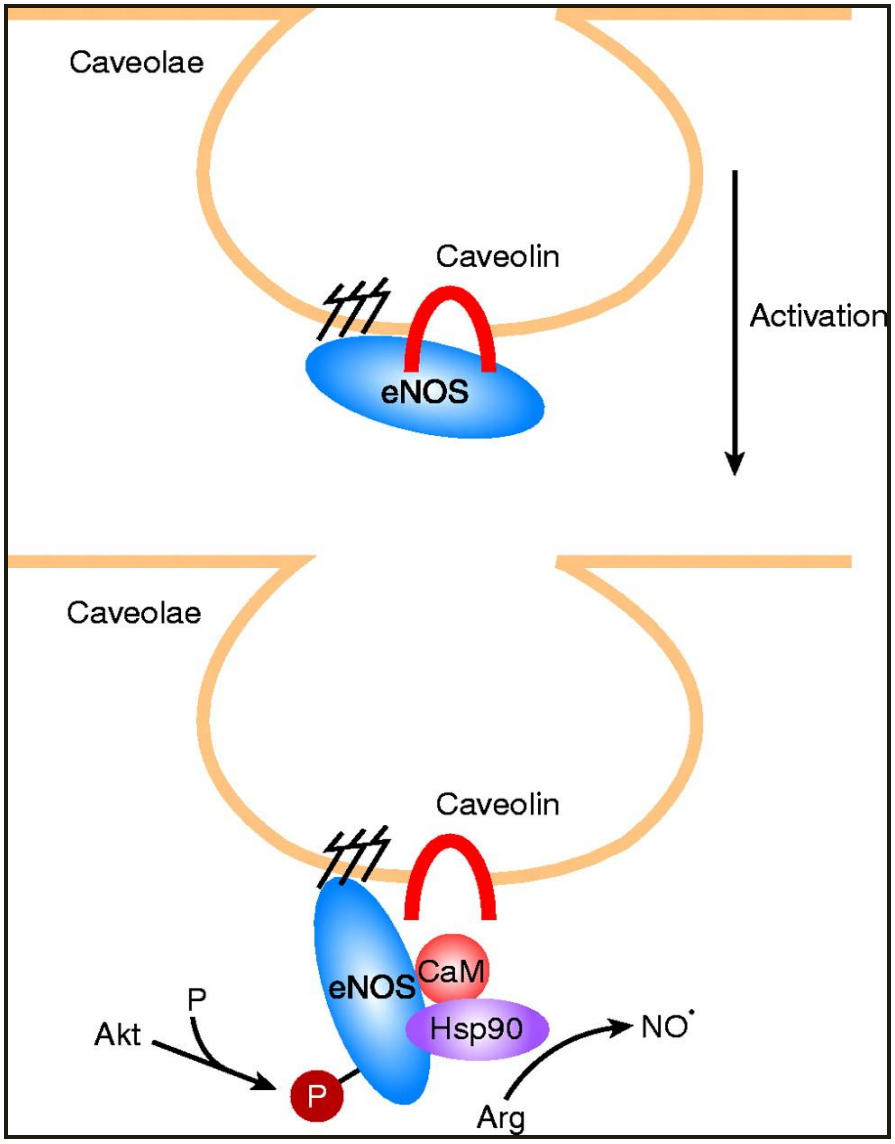


Figure 1.6: Regulation of eNOS activity. Inactive eNOS is targeted to the plasma membrane caveolae by interaction with caveolin-1. Upon stimulation or presence of an eNOS agonist such as shear stress and bradykinin, a rise in intracellular calcium, phosphorylation of eNOS, or binding of Hsp90, binding of eNOS to calmodulin (CaM) is enhanced. Binding of CaM disrupts the inhibitory interaction between eNOS and caveolin-1 leaving eNOS catalytically active to produce NO. (Copied from Bredt 2003)

1.2.1.4 Downstream mechanisms of NO

NO elicits its biological effects by binding to a haem-containing cytosolic enzyme, soluble guanylyl cyclase (sGC) [Fig. 1.7] (Bruckdorfer 2005). sGC is a heterodimeric enzyme composed of two subunits namely α and β. Each of these subunits occurs in two isoforms; α_1, α_2 and β_1, β_2 and the heterodimers are arranged as α_1/β_1 or α_2/β_1 respectively (Bruckdorfer 2005; Bryan *et al* 2009). The α_1/β_1 is the most predominant heterodimeric form of sGC and is widely expressed in tissues such as the heart, brain, lung, kidney, spleen and muscle (Bruckdorfer 2005; Bryan *et al* 2009). Binding of NO to the haem moiety of this heterodimeric enzyme activates it to catalyse conversion of guanosine triphosphate (GTP) into the second messenger guanosine 3':5'-cyclic monophosphate (cGMP) (Bruckdorfer 2005; Bryan *et al* 2009; Gewaltig & Kojda 2002). cGMP then in turn activates two cGMP-dependent protein kinases (PKG I and PKG II) (Gewaltig & Kojda 2002). Of these two kinases, PKG I is crucial for vasodilation and platelet aggregation inhibition. Activation of PKG I results in subsequent phosphorylation of a variety of membrane proteins, which in turn mediates the reduction of intracellular calcium levels and thus relaxation of the VSMCs [fig. 1.7]. Inhibition of platelet aggregation is also mediated via reduction of intra-platelet calcium concentration (Gewaltig & Kojda 2002).

The activity of cGMP is abolished by a family of hydrolytic enzymes called phosphodiesterases (PDE), particularly PDE 5, which hydrolyses cGMP into its inactive form GMP (Bryan *et al* 2009; Gewaltig & Kojda 2002).

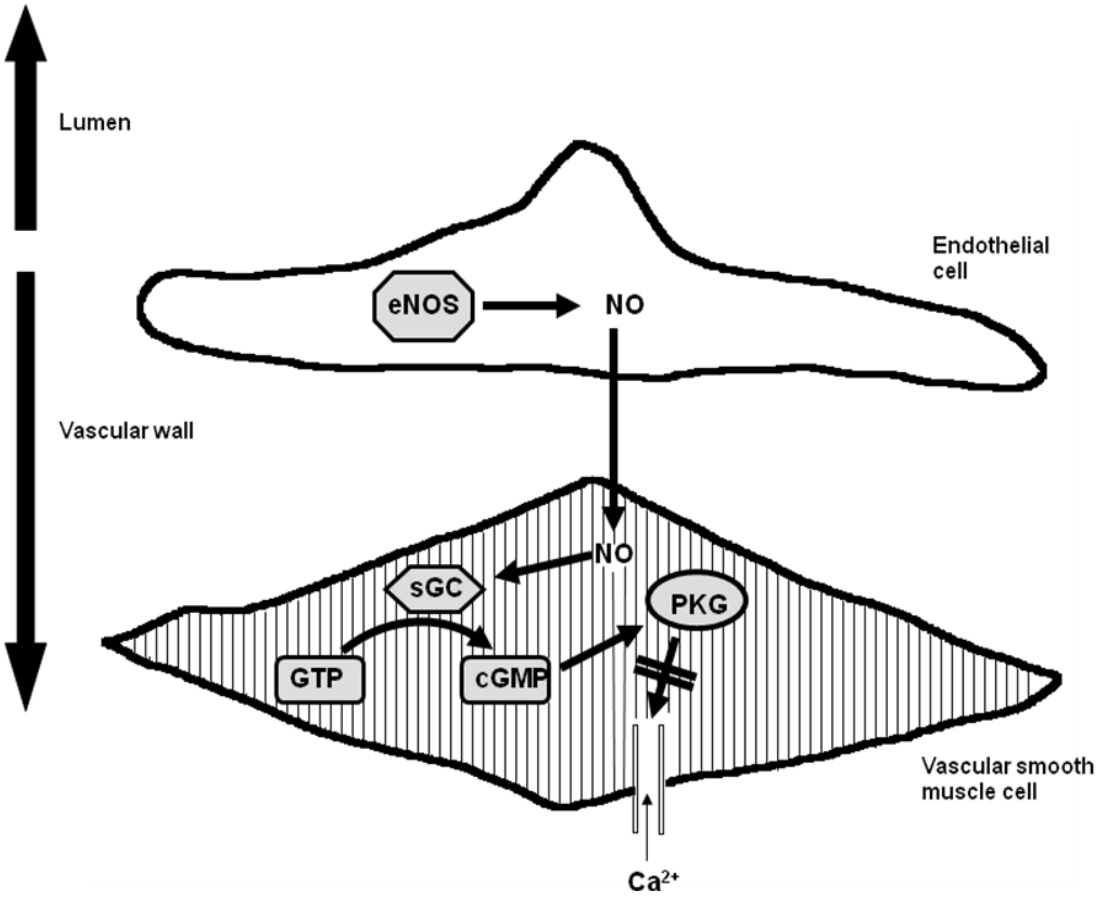


Figure 1.7: Downstream mechanisms of NO. After synthesis of NO in the endothelial cells, NO diffuses into the underlying VSMCs where it activates the sGC-cGMP-PKG pathway, which results in inhibition of the L-type calcium channels and the reduction of intracellular calcium levels. (Copied from Strijdom *et al* 2009 **(a)**)

1.2.1.5 Physiological roles of NO

Subsequent relaxation of the underlying vascular smooth muscle after NO production is the most well-known biological effect of NO in the vasculature (Gewaltig &Kojda 2002; Rastaldo *et al* 2007). However, the role that NO plays in maintaining vascular homeostasis extends beyond vasodilation in numerous ways including inhibition of detrimental factors such as smooth muscle cell proliferation and migration, platelet adhesion to endothelial cells and platelet aggregation and low density lipoprotein (LDL) oxidation (Yetik-Anacak & Catravas 2006; Balligand &

Cannon 1997). NO is also known to inhibit expression of adhesion molecules and white blood cell adhesion to endothelial cells (Yetik-Anacak & Catravas 2006; Balligand & Cannon 1997). Augmentation of these factors due to a reduction in NO bioavailability (i.e. ED) has been implicated in pathophysiological conditions such as hypercholesterolemia, hypertension and diabetes which are all considered risk factors of cardiovascular diseases (Yetik-Anacak & Catravas 2006).

1.2.1.6 NO in the heart

Cardiomyocytes have been reported to express eNOS and are thus capable of generating NO (Balligand & Cannon 1997). However, comparative studies have demonstrated that the cardiac endothelium expresses higher levels of eNOS than do cardiomyocytes and thus produce more NO (Shah & MacCarthy 2000; Brutsaert 2003; Hsieh *et al* 2006). This was recently confirmed by Strijdom *et al* (2006) via direct measurement of intracellular NO production in both cardiomyocytes and CMECs using a fluorescence-based technique. Their model of CMECs generated 26 times more NO per cell and exhibited a higher eNOS expression than cardiomyocytes, further highlighting the potential role of CMECs as putative paracrine regulators of cardiomyocytes. In the heart, the effects of NO involve more than regulation of vascular homeostasis. NO has been shown to play a role in regulation of contractile performance, heart rate, cardiac metabolism and modulation of cardiac growth (Brutsaert 2003), and the cardioprotective response of the heart to ischaemia-reperfusion injury (Bolli 2001).

Regulation of myocardial contractility is mediated by the NO-sGC-cGMP pathway. At low levels of NO, cGMP inactivates PDE 3, a phosphodiesterase which hydrolyses

3':5'-cyclic adenosine monophosphate (cAMP) to its inactive form AMP. NO can also act independently of cGMP by directly activating the β-adrenergic-adenylyl cyclase (AC) which catalyzes hydrolysis of ATP to cAMP (Rastaldo *et al* 2007; Ziolo *et al* 2008). Consequently cAMP accumulates in the cardiomyocytes and activates PKA which in turn mediates the opening of the voltage-gated calcium channels and subsequent intracellular influx of calcium resulting in a positive inotropic effect (myocardial contraction). Conversely, at high concentrations of NO, the cGMP-PKG pathway is activated resulting in a negative inotropic effect (myocardial relaxation) (Rastaldo *et al* 2007; Schulz *et al* 2004).

The growth-modulating role of NO on cardiomyocytes includes promoting growth and survival during cardiac development and suppressing growth in the adult heart in attempt to prevent cardiac remodelling (Brutsaert 2003). Mice lacking eNOS were shown to have a high incidence of cardiomyocyte apoptosis (Brutsaert 2003). Bradykinin (an eNOS agonist) has been reported to promote growth of cardiomyocytes in culture. However when adult cardiomyocytes are co-cultured with endothelial cells, bradykinin suppresses cardiomyocyte growth instead (Brutsaert 2003). Therefore the growth suppression effect in adult cells appears to be dependent on endothelium-derived NO and PGI_2, further highlighting the importance of cardiac endothelial-cardiomyocyte interaction (Brutsaert 2003). In addition to regulating growth, NO regulates cardiac metabolism by modulating cardiac oxygen consumption both in normal and disease conditions (Brutsaert 2003). For example, non-selective inhibition of NOS has been reported to increase myocardial oxygen consumption in both physiological and disease setting, thus posing a risk of oxygen toxicity (Chen *et al* 2002).

NO has been shown to play a role in ischaemia-reperfusion (Bolli 2001). However, the role NO plays in ischaemia-reperfusion is contradictory with some studies reporting beneficial effects and others reporting harmful effects in non-preconditioned hearts (Bolli 2001). The beneficial effects of NO include prevention of ischaemia-reperfusion injury (Flogel *et al* 1999). The harmful effects of NO during ischemia-reperfusion may be attributable to enhanced formation of reactive nitrogen species (RNS) e.g. peroxynitrite produced from the reaction of NO and O_2^- (Flogel *et al* 1999). Nonetheless, according to Bolli (2001), most studies demonstrate that NO is protective during ischaemia-reperfusion injury. eNOS transgenic mice demonstrated decreased infarct size after ischaemia-reperfusion suggesting a role of eNOS derived NO in cardioprotection during ischaemia-reperfusion injury (Jones *et al* 2004). NO has been shown to play a role in both early and late ischaemic preconditioning (Jones & Bolli 2006). However, its effects are more pronounced during late ischaemic preconditioning. Cardioprotection during late ischaemic preconditioning comprises an initial activation of eNOS and delayed subsequent activation of iNOS suggesting a biphasic role of both eNOS and iNOS derived NO (Jones & Bolli 2006). Recent advances in research demonstrate postconditioning as another possible cardioprotective mechanism (Zhao *et al* 2003). Postconditioning has been reported to prompt PKB / Akt and eNOS activation and inhibition of the upstream activator of PKB / Akt, phoshatidyl-inositol-3-kinase (PI-3K), has been shown to abolish the cardio-protective mechanism of postconditioning, indicating NO as a key role player (Tsang *et al* 2004; Yang *et al* 2005).

In summary:

• NO is one of the most important signalling molecules in the cardiovascular system;

• The main cellular source of NO is NOS (3 isoforms: nNOS, iNOS & eNOS, all expressed in the cardiovascular system);

• NO has variable effects depending on, inter alia, NOS isoform-specific production, amount of NO released, experimental considerations, etc.;

• In baseline, physiological conditions, NO is predominantly eNOS-derived with biological effects such as maintenance of vascular homeostasis, anti-clotting and anti-inflammatory actions, cardiogenesis and maintenance of contractile function;

• In hypoxia & ischaemia, the role of NO becomes more variable. Mostly associated with protection, effects can become harmful when NO reacts with O_2^- to form reactive and toxic radicals: $ONOO^-$, $NO_2^{\bullet}$, $OH^{\bullet}$;

• eNOS is an important source of hypoxia / ischaemia-induced ↑NO production via PI3-K / PKB-stimulated phosphorylation at the serine 1177 residue.

1.2.2 Prostacyclin and Thromboxane A_2

Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are other endothelium-derived factors which play a role in maintenance of the vascular homeostasis (Fetalvero *et al* 2007). The actions of these two factors appear to oppose each other, with PGI_2 having vasodilatory and anti-platelet aggregation properties, and TXA_2 having vasoconstrictory and platelet activating properties (Fetalvero *et al* 2007). PGI_2 and TXA_2 are prostanoids which belong to a family of prostaglandins derived from the fatty acid arachidonic acid (Fetalvero *et al* 2007; Mas 2009). The enzyme, cyclooxygenase (COX), which occurs in two isoforms, COX-1 for synthesis of TXA_2 and COX-2 for synthesis of PGI_2 [fig. 1.8], catalyses conversion of arachidonic acid to prostaglandin H_2 (PGH_2) which is further metabolised to TXA_2 by thromboxane synthase and PGI_2 by prostacyclin synthase respectively (Fetalvero *et al* 2007; Mas 2009). Maintaining a synthetic balance between these two prostanoids is critical for cardiovascular health (Fetalvero *et al* 2007). This was demonstrated by selective inhibition of COX-2, which lowered PGI_2 production without altering TXA_2 production shifting the balance in favour of TXA_2 leading to loss of vascular homeostasis and ultimately, initiation of vascular dysfunction (Fetalvero *et al* 2007).

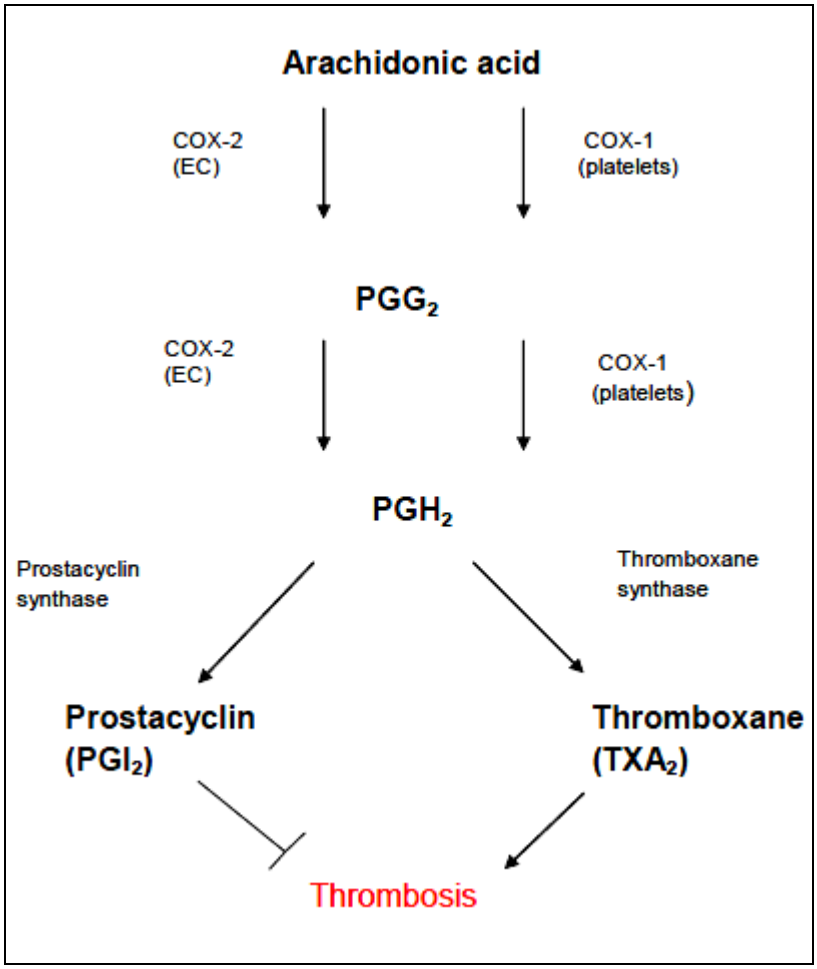


Figure 1.8: Production of prostacyclin (PGI_2) and thromboxane (TXA_2). COX metabolises arachidonic into prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2). PGH_2 is finally metabolised into PGI_2 by prostacyclin synthase in the endothelial cells (EC) or into TXA_2 by thromboxane synthase in the platelets. PGI_2 inhibits thrombosis whereas TXA_2 promotes thrombosis. (Modified from Fetalvero *et al* 2007)

1.2.3 Endothelium-derived hyperpolarising factor (EDHF)

EDHF is another known endothelium-derived vasodilator and causes relaxation of the VSMCs by means of membrane hyperpolarisation (Mas 2009) particularly in small arteries with a diameter of ≤ 300 µm (Luksha *et al* 2009). Its effects have been observed after pharmacological blocking of NO and PGI_2 synthesis (Mas 2009) suggesting that EDHF may act as supporting vasodilatory mechanism when NO

production is reduced (Luksha *et al* 2009; Deanfield *et al* 2007). The identity of EDHF is very controversial and several diffusible substances have been suggested such as epoxyeicosatrienoic acid (derived from arachidonic acid by cytochrome P450), potassium ions, hydrogen peroxide (H_2O_2), C type-natriuretic peptide and endocannabinoids (Mas 2009; Luksha *et al* 2009).

1.2.4 Endothelin-1 (ET-1)

ET-1 is one of the most potent vasoconstrictors released by endothelial cells due to stimuli such as hypoxia, angiotensin II (another potent vasoconstrictor), thrombin, platelet products, inflammatory cytokines, noradrenaline and vascular wall tension (Mas 2009). ET-1 elicits its effects via two receptors, namely ET_A and ET_B. Of these receptors, ET_B receptors are predominantly expressed in endothelial cells and ET_A on VSMCs. Activation of ET_A enhances vascular contraction whereas activation of ET_B stimulates eNOS activity leading to NO production and ultimately reduction in ET-1 production. ET_B receptor thus antagonises the vasoconstrictive action of ET-1 (Mas 2009).

In the heart, ET-1 has been reported to exert autocrine effects by binding to ET_B receptors on cardiac endothelial cells resulting in release of NO and prostacyclin and ultimately vasodilation (Hsieh *et al* 2006). Its paracrine effects are achieved by binding to ET_A receptors expressed on underlying cardiomyocytes leading to myocardial contraction (Hsieh *et al* 2006).

1.2.5 Angiotensin II

Another potent vasoconstrictor released by endothelial cells is known as angiotensin II. Formation of angiotensin II results from conversion of angiotensin I to angiotensin II by the enzyme angiotensin converting enzyme (ACE), which is expressed in the cardiovascular system and is also found in the circulation (Dostal & Baker 1999; Mas 2009). ACE is expressed in CMECs and cardiomyocytes, suggesting the production of angiotensin II in both these cell types (Brutsaert 2003). Angiotensin II acts in an autocrine and paracrine manner to influence growth of cardiomyocytes (Brutsaert 2003).

Angiotensin II elicits its effects via two receptors, AT_1 and AT_2 (Dostal & Baker 1999; Schmieder *et al* 2007). Activation of AT_1 receptor is associated with effects such as vasoconstriction and cell proliferation whereas AT_2 receptors appear to oppose such effects (Schmieder *et al* 2007). Augmented binding of angiotensin II to AT_1 receptors has been associated with production of pro-inflammatory cytokines, enhanced activity of NADPH and xanthine oxidase leading to generation of oxygen free radicals, oxidation of LDL and hence ED (Schmieder *et al* 2007).

1.3 Endothelial dysfunction (ED)

ED is commonly described as reduced NO bioavailability or an inability of the endothelium to initiate vasodilation in response to stimuli such acetylcholine or shear stress and is considered a predictor of atherosclerosis and hence ischaemic heart disease (Chhabra 2009; Bonetti *et al* 2003). ED comprises a loss of balance between endothelial-derived vasodilatory and vasoconstrictive factors, where vasoconstrictive factors and effects become dominant leading to a pathophysiological state termed endothelial activation i.e. a pro-inflammatory, pro-oxidant, proliferative, pro-coagulatory and pro-adhesive state (Chhabra 2009; Bonetti *et al* 2003). Chronic exposure to cardiovascular risk factors such as hyperglycaemia, hyperlipidaemia, hypertension, tobacco smoking, etc., overwhelms the defensive mechanisms of the endothelium compromising the integrity of the endothelium and ultimately initiating ED (Deanfield *et al* 2007).

1.3.1 Cardiovascular risk factors associated with ED

1.3.1.1 Diabetes/ insulin resistance

Both type 1- and type 2 diabetes are independent risk factors of cardiovascular disease (Esper *et al* 2006). The endothelial damage or dysfunction that is observed in diabetic conditions may be attributable to upregulated expression and activity of NADPH oxidase, and increased formation of advanced glycation end-products (AGEs) (Guzik *et al* 2002; Avogaro *et al* 2008). NADPH oxidase is the main source of reactive oxygen species (ROS) in the vascular system, and thus its increased activity is associated with increased O_2^- production (Guzik *et al* 2002; Avogaro *et al* 2008). However, other enzymatic sources of ROS such as xanthine oxidase, COX

and uncoupled eNOS may also play a role (Guzik *et al* 2002). Not only does O_2^- scavenge NO leading to production of the potent oxidant peroxynitrite, but it also modifies activity and regulation of eNOS, and promotes VSMC proliferation and inflammation (Guzik *et al* 2002).

Excess blood sugar results in non-enzymatic glycation of intracellular and extracellular proteins and lipids forming AGEs, which accumulate in the vascular wall and reduce NO activity by quenching NO (Avogaro *et al* 2008; Soldatos *et al* 2005). The AGEs bind to specific surface receptors called receptor for AGE (RAGE) which are expressed on cells such as monocytes, macrophages and VSMCs resulting in amplification of an inflammatory response (Avogaro *et al* 2008; Soldatos *et al* 2005). Binding of AGEs to these receptors also results in increased vascular permeability, expression of vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM), and oxidative stress (Soldatos *et al* 2005).

Furthermore, hyperglycaemia activates PKC which decreases eNOS activity leading to reduced NO and increased ET-1 production (Avogaro *et al* 2008). In the setting of ED, ET_B receptor-mediated effects of ET-1 are blunted and thus the vasoconstrictory effects predominate (Versari *et al* 2009). PKC also enhances expression of adhesion molecules such as ICAM, VCAM and E-selectin (Avogaro *et al* 2008).

ED has been reported to occur early in insulin resistance (Hsueh *et al* 2004). Often insulin resistance is associated with central adiposity and hence the metabolic syndrome i.e. hypertriglyceridaemia, low high density lipoprotein (HDL) levels, high low density lipoprotein (LDL) levels and hypertension, all of which favour

development of ED and eventually atherogenesis (Hsueh *et al* 2004). It has therefore been suggested that advancement of insulin resistance to type 2 diabetes parallels advancement of ED to atherosclerosis [fig. 1.9] (Hsueh *et al* 2004).

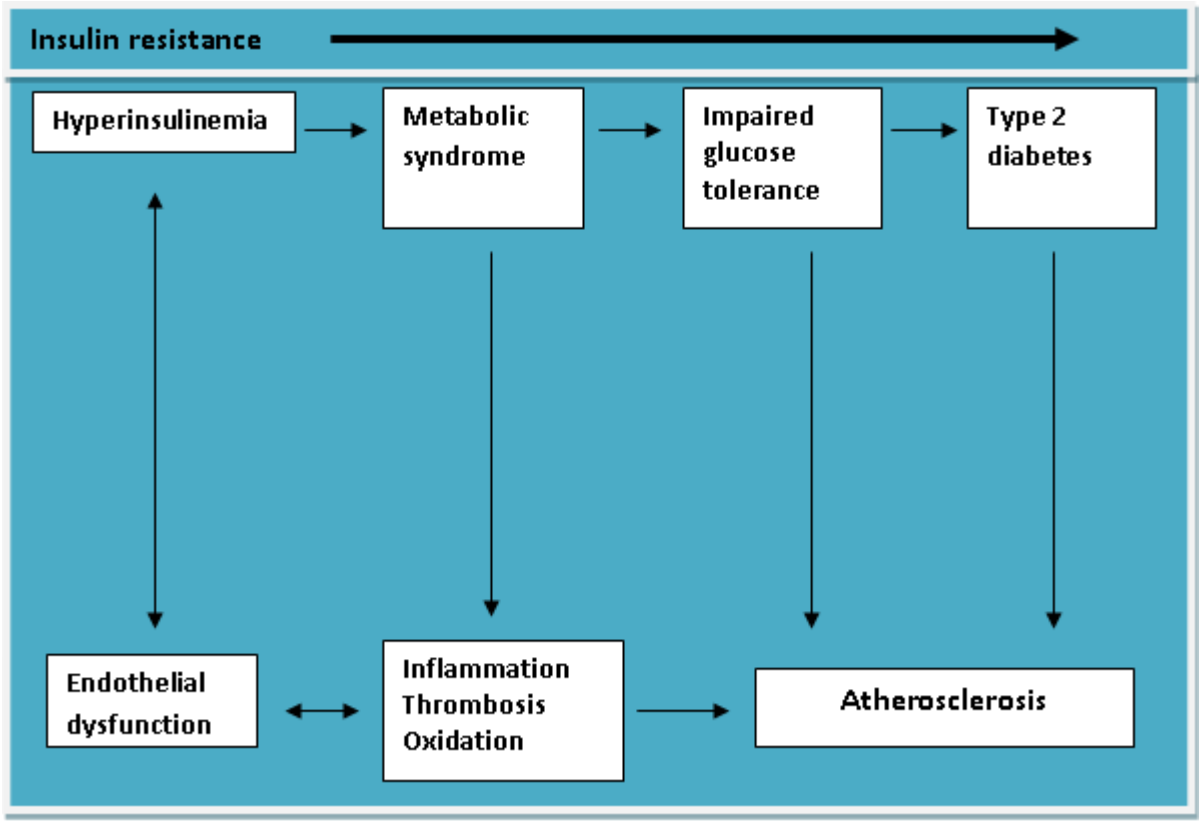


Figure 1.9: A parallel relationship is proposed to exist between progression of insulin resistance to type 2 diabetes and progression of endothelial dysfunction to atherosclerosis. (Modified from Hsueh *et al* 2004)

1.3.1.2 Hyperlipidaemia

Hyperlipidaemia constitutes increased circulating lipids including cholesterol and triglycerides, a state which predisposes to ED. Mechanisms underlying hyperlipidaemia-induced ED include upregulation of NADPH oxidase and thus oxidative stress, increased plasma levels of asymmetric dimethylarginine (ADMA) (Warnholtz *et al* 2001) and oxidation of LDL (Sawamura 2004). ADMA is an

endogenous inhibitor of eNOS. It competes with L-arginine for its binding site on eNOS, thus resulting in decreased NO production. Plasma concentrations of ADMA have been reported to be increased in hypercholesterolaemia (Böger *et al* 1998; Böger *et al* 2003) and ADMA is considered to be both a marker and risk factor of ED (Böger *et al* 1998).

In addition to scavenging NO, excess O_2^- modifies LDL cholesterol to oxidized LDL (ox-LDL) which plays a major role in atherogenesis. ox-LDL has been reported to promote ET-1 production (Boulanger *et al* 1992), expression of adhesion molecules and chemoattractants, and VSMC migration and proliferation (Sawamura 2004). Furthermore, ox-LDL is engulfed by macrophages forming foam cells which adhere to the vessel wall initiating an atherosclerotic plaque (Sawamura 2004). Both LDL and ox-LDL have been shown to increase the activity of S-adenosylmethionine-dependent methyltransferases, which lead to increased ADMA synthesis. Therefore, LDL and ox-LDL maybe be accountable for the increased plasma levels of ADMA in hypercholesterolaemia (Warnholtz *et al* 2001). LDL or ox-LDL may also upregulate caveolin-1 synthesis and thus inhibit eNOS activity (Davignon and Ganz 2004; Hamburg & Vita 2005).

1.3.1.3 Hypertension

Patients with hypertension demonstrate blunted forearm blood flow in response to stimuli such as acetylcholine and bradykinin (Tang & Vanhoutte 2010). Increased production of ROS and endothelial derived contracting factors (EDCFs) such as ET-1, angiotensin II, PGH_2 and TXA_2, and thus decreased NO bioavailability are all observed in hypertension (Versari et al 2009; Tang & Vanhoutte 2010). Shear stress

is known to be one of the important mechanisms of inducing NO-mediated vasodilation in both micro- and macrovasculature. However, this response is not observed in hypertension. Paniagua et al (2001) reported diminished vasodilation in response to shear stress in the microvasculature of hypertensive patients. In addition to this, Iaccarino et al (2004) found abolished PKB/Akt dependent-activation of eNOS due to mislocalisation of PKB/Akt to the plasma membranes in a model of spontaneously hypertensive rats (SHR).

1.3.1.4 Smoking

Tobacco smokers exhibit impaired flow mediated vasodilation, decreased NO bioavailability, and increased levels of ox-LDL (Puranik & Celermajer 2003). Passive smoking is also implicated in impairment of endothelial function (Barnoya & Glantz 2005; Puranik & Celermajer 2003). It appears that the harmful effects of smoking on the endothelial cells are dose-dependent and reversible upon smoking cessation (Puranik & Celermajer 2003). Oxidative stress appears to be the major link between smoking and ED (Puranik & Celermajer 2003; Barnoya & Glantz 2005; Burke & FitzGerald 2003). Cigarette smoke is rich in free radicals and directly delivers free radicals into the body. Besides being the supplier of free radicals, cigarette smoke facilitates endogenous release of ROS via activation of inflammatory cells (Burke & FitzGerald 2003; Antoniades *et al* 2008). Smokers have also been shown to have increased platelet activation and circulating levels of fibrinogen, factors that predispose to thrombogenesis (Burke & FitzGerald 2003). Furthermore, smoking has been reported to decrease the levels of high density lipoprotein (HDL) cholesterol, which is known to have anti-atherosclerotic properties (Puranik & Celermajer 2003).

1.3.1.5 Aging

Increasing age has been recognised as one of the factors that predisposes to ED (Katusic 2007; Herrera *et al* 2010). With aging, the ability of the endothelium to produce NO is reduced (Vanhoutte 2002). Furthermore, some studies have reported reduced expression and activity of eNOS as well decreased expression of sGC on VSMCs and its activity in older animals (Vanhoutte 2002). In addition to the decreased NO production, other EDRFs (prostacyclin and EDHF) are also reduced (Vanhoutte 2002), while EDCFs such as ET-1 and COX-derived prostanoids, and ROS production are increased (Herrera et al 2010; Vanhoutte 2002). Plasma levels of ADMA also rise with aging (Vanhoutte 2002).

One of the mechanisms contributing to reduced NO in aging may be the increased activity of arginase I (Katusic 2007; Herrera *et al* 2010). Arginase I is an enzyme that catalyses conversion of L-arginine to L-ornithine and urea, and it thus competes with eNOS for L-arginine (Katusic 2007). Thus the increased activity of this enzyme as observed with advancing age may result in ED (Katusic 2007; Herrera *et al* 2010). Clearly the balance between EDRFs and EDCFs is lost with advancing age, establishing aging as a risk factor for cardiovascular disease. Moreover, aging is often associated with conditions such as diabetes, hypertension, and hypercholesterolaemia further exacerbating the risk of developing atherosclerosis and thus cardiovascular diseases (Herrera *et al* 2010).

1.3.2 Proposed mechanisms of ED

Oxidative stress appears to be the common ED inducing pathway in all of the above discussed risk factors. According to the literature, cardiovascular risk factors are

associated with upregulation of sources of ROS, especially NADPH oxidase (Forstermann & Munzel 2006; Chhabra 2009). However other sources of ROS such as xanthine oxidase, COX and mitochondria are not completely sidelined (Guzik *et al* 2002). In fact, eNOS itself is a potential ROS generator when uncoupled (Forstermann & Munzel 2006). Damaging mechanisms of oxidative stress include increasing VSMC proliferation, endothelial cell apoptosis, and increased expression and activity of matrix metalloproteinases (Pennathur & Heinecke 2007).

Oxidative stress comprises increased rates of oxidant production and decreased levels of antioxidants such as superoxide dismutase (SOD), vitamin C and E, etc. (Pennathur & Heinecke 2007). Under physiological conditions, the enzyme SOD regulates the levels of O_2^- (Landmesser *et al* 2006). However, upregulated generation of O_2^- overwhelms the defensive mechanisms of this enzyme, leaving O_2^- free to react with other molecules such as NO (Landmesser *et al* 2006). O_2^- is implicated in direct induction of ED by scavenging of NO leading to production of potentially harmful peroxynitrite (Yokoyama 2004). In fact, the reaction between O_2^- and NO has been reported to occur much faster (rate constant=6.7 x 10^9 m/s^{-1}) than that of dismutation of O_2^- by SOD (rate constant=2.0 x 10^9 m/s^{-1}) (Huang 2003). High levels of peroxynitrite are injurious to the cells, oxidatively damaging DNA, lipids and proteins. In addition to being cytotoxic, peroxynitrite damages the intricate eNOS structure leading to eNOS uncoupling (Kuzkaya *et al* 2003).

Peroxynitrite has been reported to oxidise the essential cofactor of eNOS, BH_4 to its inactive form trihydrobiopterin radical (BH_3^-) which leads to uncoupling of eNOS [Fig.

1.10A] (Katusic 2001; Forstermann & Munzel 2006; Kuzkaya *et al* 2003). Furthermore, peroxynitrite may oxidize the zinc thiolate cluster resulting in the loss of the zinc ion and formation of disulfide bonds between the monomers, and thus disruption of the binding site for BH_4 and L-arginine [Fig. 1.10B] (Forstermann & Munzel 2006; Zou *et al* 2002 **(a)**). Some studies have reported that BH_4 is only oxidized at 10 to 100-fold higher levels of peroxynitrite than required to oxidize the zinc thiolate cluster. This suggests that oxidation of the zinc thiolate cluster may be the chief mechanism of uncoupling eNOS rather than oxidation of BH_4 (Forstermann & Munzel 2006; Zou *et al* 2002 **(a)**). Vitamin C is able to recycle BH_3^- to BH_4 (Kuzkaya *et al* 2003; Katusic 2001) and supplementation with BH_4 has been reported to restore endothelial function in conditions such as insulin resistance, hypercholesterolaemia (Katusic 2001), diabetes mellitus, and essential hypertension, as well as in chronic smokers (Forstermann & Munzel 2006).

In view of the decreased NO bioavailability observed in ED, one would expect that eNOS expression will also be decreased. However, in most cases, cardiovascular risk factors are associated with augmented eNOS expression which may be attributable to surplus hydrogen peroxide (H_2O_2) observed in oxidative stress (Li H *et al* 2002). The ability of H_2O_2 to increase eNOS expression via transcriptional and post-transcriptional mechanisms has been shown by several studies (Drummond *et al* 2000). However, despite increased eNOS expression, there is a paradoxical marked decrease in NO production, associated with an increase in O_2^- production due to eNOS uncoupling.

Zou *et al* (2002) **(b)** observed decreased phosphorylation and activity of PKB/Akt in bovine aortic endothelial cells following treatment with peroxynitrite. They further

found that, peroxynitrite increased AMPK-mediated phosphorylation of eNOS. However, the increased eNOS phosphorylation was not associated with NO but with O_2^- production.

Therefore, during conditions of oxidative stress, eNOS deviates from its role of being an essential regulator of the functioning of the cardiovascular system to being an O_2^- releasing enzyme. Peroxynitrite appears to play a major role in eNOS uncoupling. Uncoupled eNOS synthesises O_2^- at the expense of NO, further aggravating oxidative stress.

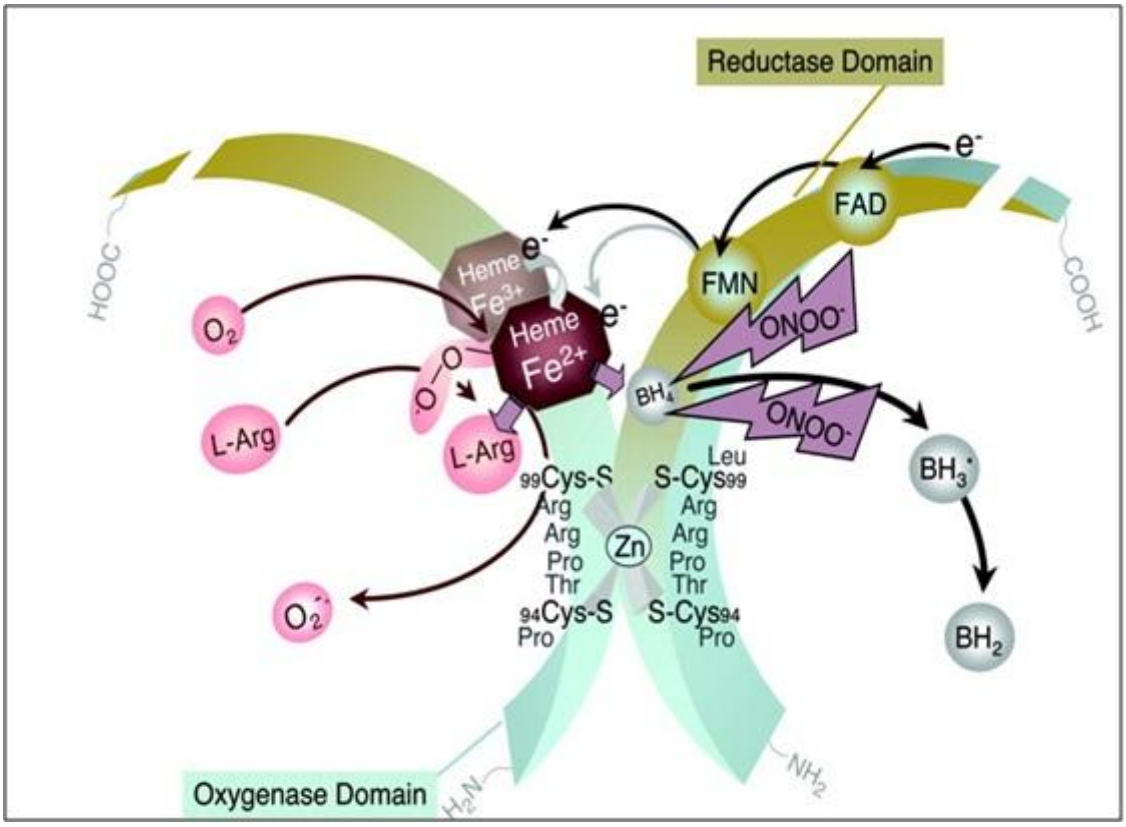


Figure 1.10A: Peroxynitrite and eNOS function. Peroxynitrite ($ONOO^-$) oxidises BH_4 into its inactive form BH_2 and therefore uncoupling eNOS. (Copied from Fostermann & Munzel 2006)

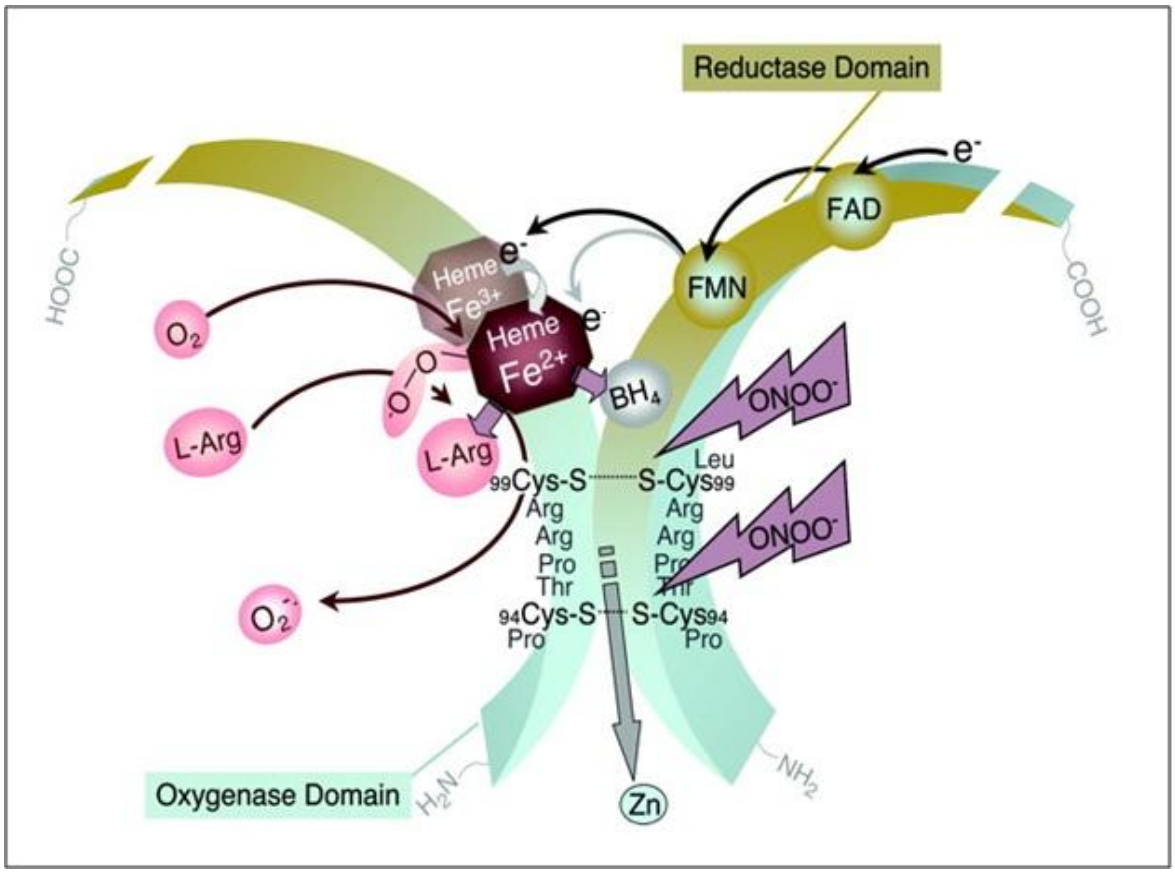


Figure 1.10B: Peroxynitrite and eNOS function. Alternatively, peroxynitrite oxidises the zinc thiolate cluster resulting in the loss of zinc and formation of disulfide bonds between monomers, and therefore disruption of the BH_4 and L-arginine binding site, and ultimately eNOS uncoupling. (Copied from Fostermann & Munzel 2006)

Inflammation is another common underlying mechanism of ED (Libby *et al* 2002). Under physiological conditions, the endothelium regulates vascular inflammation (including expression of adhesion molecules and leukocyte adhesion) via the release of NO (Osto & Cosentino 2010). It is therefore more likely that ED will promote sustained vascular inflammation which is detrimental to the vascular system. However, several studies have reported that inflammation also promotes ED and it is thus recognised as a novel risk factor for cardiovascular diseases (Szmitko *et al* 2003; Libby *et al* 2002). There seems to be a causal relationship between oxidative

stress and inflammation. Oxidative stress may amplify vascular inflammation signalling pathways (Madamanchi *et al* 2005) and inflammatory cells release O_2^-. Inflammation is often associated with overexpression of inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1). These inflammatory cytokines in turn prompt endothelial cells or macrophages to release adhesion molecules such as VCAM-1 and ICAM-1, MCP-1, interleukin-6 (IL-6) resulting in a state of endothelial activation (Blake & Ridker 2001).

The role of TNF-α in ED is now well appreciated. TNF- α has been associated with cardiovascular diseases such as acute myocardial infarction, chronic heart failure, atherosclerosis, myocarditis, etc. (Meldrum 1998). TNF-α levels are also significantly correlated with obesity which is an independent risk factor of ED (Fain *et al* 2004). This inflammatory cytokine has been reported to promote ROS formation via NADPH oxidase, xanthine oxidase, etc. (Zhang *et al* 2009). For example, Gao *et al* (2007) reported that TNF-α induces ED via increased NADPH oxidase activity in coronary arterioles of type 2 diabetic mice. In addition, TNF-α has been implicated in the downregulation of eNOS expression by accelerating eNOS mRNA degradation (Hamburg & Vita 2005, Stenvinkel 2001; Zhang *et al* 2009). According to Zhang *et al* (2006), ED observed in myocardial ischaemia-reperfusion injury may be attributable to increased TNF-α expression via enhancement of xanthine oxidase activity.

For a summary of proposed mechanisms of ED, please refer to figure 1.11.

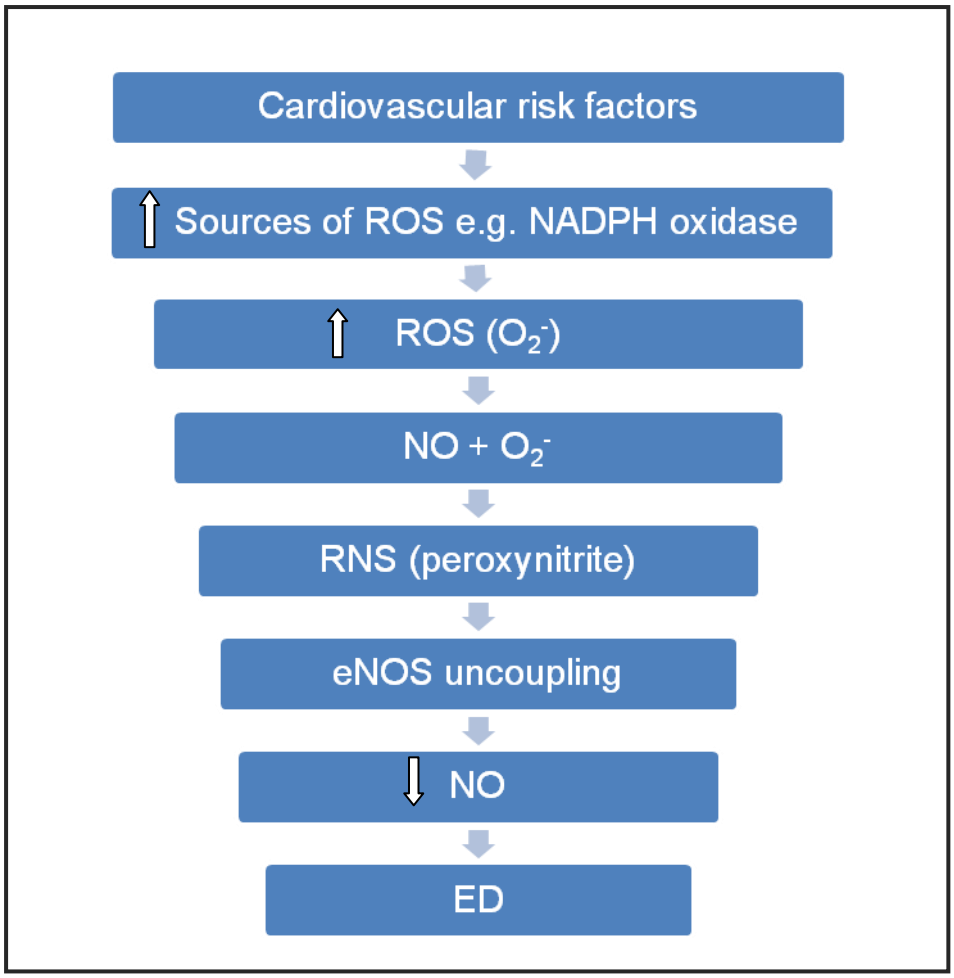


Figure 1.11: A summary of proposed mechanisms of ED.

1.3.3 Markers of ED

1.3.3.1 Reduction of NO bioavailability

Reduction in endothelial-derived NO production or bioavailability represents a key step in pathogenesis of cardiovascular diseases. Indeed, reduced NO bioavailability is the hallmark of ED (Davignon and Ganz 2004). The major NO reducing pathways include scavenging of NO by O_2^-, decreased eNOS expression or activation and eNOS uncoupling. Elevated TNF-α and ox-LDL have been reported to decrease eNOS expression by accelerating eNOS mRNA degradation (Hamburg & Vita 2005; Stenvinkel 2001). eNOS activation is another critical determinant of NO bioavailability. However, a risk factor such as hyperglycaemia is associated with

PKC activation which phosphorylates eNOS at its inhibitory site, Thr 495, thus inhibiting its activity (Avogaro *et al* 2008).

1.3.3.2 NADPH oxidase upregulation

Under physiological conditions, vascular NADPH oxidase slowly produces low physiological amounts of ROS that participate in cellular signalling (Griendling et al 2000). However, upregulated NADPH oxidase expression as observed in a variety of cardiovascular risk factors is implicated in atherogenesis. Vascular NADPH oxidase is characterized by two membrane bound subunits, p22phox and gp91phox, and two cytosolic subunits p67phox, p47phox and a protein rac1 or sometimes rac2 depending on cell type of expression (Griendling *et al* 2000; Brandes & Kreuzer 2005). Each of these subunits is employed in research to determine expression and activity of the NADPH oxidase enzymes, and thus an indicator of oxidative stress.

Prolonged treatment with angiotensin II in rats has been reported to increase expression of p22phox mRNA whereas TNF-α increased expression of p22phox mRNA in VSMCs (Griendling *et al* 2000). On the other hand, Li J-M et al (2002) reported increased p47phox-dependent ROS generation following treatment with TNF-α in coronary microvascular endothelial cells.

1.3.3.3 Nitrotyrosine upregulation

In addition to uncoupling the eNOS enzyme, peroxynitrite undergoes protonation to form peroxynitrous acid or combine with carbon dioxide (CO_2) to form nitroso-peroxocarboxylate, both of which yield tyrosine-nitrating compounds (Souza *et al* 2008; Hurst 2002). Via formation of these compounds, peroxynitrite leads to nitration

of tyrosine residues of proteins forming nitrotyrosine. Tyrosine nitration involves addition of a nitro group (NO_2) on the aromatic ring of tyrosine residues (Pacher *et al* 2007). Under normal conditions, low levels of free or protein-bound nitrotyrosine are detectable which may indicate low levels of oxidants and nitrating species produced during physiological processes. However, significant nitrotyrosine upregulation is observed in conditions that are associated with nitroxidative stress such as inflammation, cardiovascular disease, neurodegenerative disorders, etc (Souza *et al* 2008). Tyrosine nitration may modify the structure and function of proteins, leading to alterations in catalytic activity of enzymes, production of antigenic epitopes, and impaired cell signal transduction (Pacher *et al* 2007).

1.3.3.4 Circulating endothelial cells

Circulating endothelial cells (CECs), which are mature cells that have detached from the endothelium (Boos *et al* 2006), represents a new biomarker of endothelial injury (Erdbruegger *et al* 2006). In a healthy person, the endothelium is constantly refurbished at a replication rate of < 1 % and levels of CECs are very low (Erdbruegger *et al* 2006). Studies using a flow activated cell sorter (FACS) isolation technique reported CECs ranging from 50 - 7,900 cells/ml in healthy individuals and up to 39,100 cells/ml in individuals with vascular diseases. On the other hand, immunomagnetic studies revealed a range of only 0-10 CECs/ml in healthy individuals (Erdbruegger *et al* 2006). However, despite the methodological disparities in the overall cell number, both these techniques reveal lower levels of CECs in healthy individuals.

Increased levels of CECs are associated with ED, cardiovascular diseases and a variety of other diseases (Boos *et al* 2006; Erdbruegger *et al* 2006). Potential mechanisms underlying endothelial cell detachment may be mechanical injury, action of proteases and/or cytokines, defective endothelial cell adhesion to ECM, cellular apoptosis and actions of cardiovascular risk factors (Boos *et al* 2006; Erdbruegger *et al* 2006).

1.3.3.5 ADMA

ADMA has emerged as both a marker and a mediator of ED (Böger *et al* 2003). ADMA is endogenously synthesised via methylation of arginine residues in the nuclear proteins (Landim *et al* 2009). ADMA competitively inhibits eNOS resulting in decreased NO production, and may induce eNOS uncoupling. Synthesis of ADMA is catalysed by protein arginine methyltransferases (PRMTs) and its degradation is catalysed by dimethylarginine dimethylaminohydrolases (DDAH) (Landim *et al* 2009; Sydow & Munzel 2003). The latter is often decreased in a variety of cardiovascular disease and thus leading to upregulation of ADMA. For example, treatment of endothelial cells with TNF-α, ox-LDL and glucose has been reported to diminish the activity of DDAH (Landim *et al* 2009; Sydow & Munzel 2003). Furthermore, PRMTs and DDAH are ROS-sensitive and thus activity of the latter is impaired whereas activity of the former is increased in conditions of oxidative stress (Sydow & Munzel 2003).

Increased plasma levels of ADMA have been documented in patients with conditions such as hyperlipidaemia, hypertension, coronary artery disease, stroke, end-stage renal disease, etc. (Sydow & Munzel 2003). Since ADMA induces eNOS uncoupling

and reduces NO production, it may contribute to the pathogenesis of cardiovascular diseases (Landim *et al* 2009; Sydow & Munzel 2003).

1.4 ED in atherosclerosis

ED has emerged as a useful prognostic tool for atherosclerosis and ultimately cardiovascular disease (Yang & Ming 2006). In fact, ED plays a major role in the development of atherosclerosis (Yang & Ming 2006). An intact endothelium is almost impermeable and does not promote leukocyte adherence and invasion, or platelet aggregation and adherence (Libby *et al* 2002). However, ED is often associated with enhanced endothelial permeability, upregulated pro-inflammatory cytokines and, expression of adhesion molecules such as VCAM-1 and ICAM-1 which facilitate leukocyte adhesion to the endothelium (Libby *et al* 2002). Leukocyte adhesion represents one of the first steps in the initiation of atherosclerosis. In fact, authors now define atherosclerosis as an inflammatory disease (Lind 2003; Hansson 2009). For example, a high fat diet has been associated with recruitment of inflammatory cells into the endothelium (Ruberg & Loscalzo 2005).

After adhering to the endothelium, leukocytes (monocytes and lymphocytes) cross the endothelium and migrate into the intima (Ruberg & Loscalzo 2005; Osto & Cosentino 2010). Upon reaching the intima, monocytes transform into macrophages and express receptors that facilitate uptake of lipids. Uptake and accumulation of lipids lead to transformation of macrophages into foam cells which initiate an atherosclerotic lesion and further enhance release of inflammatory cytokines leading to VSMC proliferation and migration into the vessel lumen (Ruberg & Loscalzo 2005; Osto & Cosentino 2010). Through these complex mechanisms, a cascade of events,

which begins with formation of an early atherosclerotic lesion leading to an advanced lesion characterised by a plaque formation, ensues (Ruberg & Loscalzo 2005). At this stage, the vessel diameter narrows and blood flow is decreased leading to ischaemic conditions (Ruberg & Loscalzo 2005). Progression of this disease is eventually associated with cardiac complications such as myocardial infarction.

1.5. ED in cardiac microvascular endothelial cells

As previously discussed, the endothelium in the heart is categorised into two subtypes, namely the coronary vascular endothelium and the cardiac endothelium [Fig. 1.2] (Strijdom & Lochner 2009). The coronary vascular endothelium constitutes the epicardial coronary arterial and venous endothelial cells, and the intramyocardial coronary arterial and venous endothelial cells, whereas the cardiac endothelium constitutes the CMECs (> 90 % myocardial capillary derived) and EECs (Strijdom & Lochner 2009). It is well known that ED in the coronary circulation is a predecessor of atherosclerosis and eventually ischaemic heart disease (IHD) which is a consequence of decreased blood flow and thus reduced oxygen supply to the heart tissues (Choi *et al* 2009). To date, most studies investigate models of larger coronary blood vessels, however, studies on myocardial capillaries and myocardial capillary-derived endothelial cells (CMECs) have been neglected and their role in the context of ED remains poorly understood.

Taking into consideration the structural and functional endothelial heterogeneity across the vascular tree (Aird 2007), endothelial cells in different blood vessels may not completely respond in the same manner to injury or insult. Furthermore, due to their lack of VSMCs, myocardial capillaries do not dilate nor constrict and therefore

the paracrine substances such as NO released by CMECs cannot be involved in regulation of vascular tone (Strijdom & Lochner 2009). As mentioned previously, CMECs release more NO than do cardiomyocytes (Strijdom *et al* 2006) in addition to other paracrine substances (Shah & MacCarthy 2000). Considering the strategic arrangement and likelihood of paracrine communication between CMECs and cardiomyocytes, the putative role of CMEC-derived substances such as NO may be to regulate the function and activity of underlying cardiomyocytes in addition to maintaining integrity of the cardiac endothelium. In view of this, it is likely that ED in CMECs could have a direct harmful impact on the underlying cardiomyocytes. For this reason, it is imperative that more studies are undertaken to investigate ED in myocardial capillary-derived CMECs, and to explore underlying cellular mechanisms.

1.6. Role of traditional medicinal plants in vascular function and possible relevance to ED

A number of traditional medicinal plants have been reported to demonstrate a wide variety of beneficial properties including anti-inflammatory, anti-hypertensive, hypoglycaemic, anti-tumorigenic and anti-viral effects (Musabayane *et al* 2005; Lin *et al* 2009). Plants with medicinal properties include *Syzigium cordatum* (Hochst.) [Myrtaceace] (water berry), *Cornus officinalis* (cornus), *Crataegus pinnatifida* (hawthorn), *Radix ginseng* and *Olea europaea* (olive tree) (Musabayane *et al* 2005, Hsu *et al* 2006, Lin *et al* 2009, Xi *et al* 2009, Sato *et al* 2007). The triterpenoid, oleanolic acid (OA), has recently been isolated from several medicinal plant extracts, including *Syzigium cordatum,* and is proposed to be the candidate bioactive compound accountable for many of the beneficial effects observed (Mapanga *et al* 2009; Li J *et al* 2002). OA has also been isolated from olive oil. Thus the low

incidence of cardiovascular diseases and cancer associated with high consumption of olive oil such as observed in the Mediterranean diet may in part be attributable to OA (Rodriguez-Rodriguez *et al* 2008).

Rodriguez-Rodriguez *et al* (2008) observed endothelium-dependent vasodilation in response to OA in both superior and small mesenteric arteries of rats. This response was abolished by incubation with the NOS inhibitor ADMA, suggesting a role for NO in OA-induced vasodilation. They further reported that OA treatment resulted in phosphorylation of PKB/Akt at residue Ser 473 (thus stimulating its activity) and phosphorylation of eNOS at Ser 1177 in human umbilical vein endothelial cells (HUVECs). From these observations it appears that OA stimulates eNOS activity via the PKB/Akt pathway. Another study reported a COX-2 dependent release of PGI_2 from human coronary smooth muscles following treatment with OA (Martinez-Gonzalez *et al* 2008). In addition to its vasoactive properties, OA has been shown to lower blood sugar levels in the setting of hyperglycaemia or diabetes mellitus. In a study of streptozotocin-induced diabetic rats, Mapanga *et al* (2009) reported reduced mean arterial pressure (MAP) and blood sugar levels, and improved renal function following a five week treatment with OA isolated from *Syzigium cordatum*. The reduced MAP was also observed in non-diabetic rats. Moreover, OA has been reported to have insulin secretory properties from pancreatic β-cells via stimulation of muscarinic M3 receptors by acetylcholine released from cholinergic nerve terminals (Hsu *et al* 2006).

From these observations, OA appears to have some potential in the management of diabetes and exert beneficial effects on vascular function. However, further studies

investigating cellular mechanisms involved in OA actions are required, particularly in the context of ED and myocardial capillary-derived endothelial cells.

1.7 Rationale, Motivation and Aim

1.7.1 Rationale and motivation

ED represents one of the first steps in the initiation of IHD and it is widely accepted as a very useful prognostic tool in predicting the development of atherosclerosis (Yang & Ming 2006). Given its reversibility, ED may signify an important therapeutic target in the prevention of atherosclerosis and hence cardiovascular disease. For this reason, ED has received much attention in research over the years and its role in the large coronary vessels is very well defined. However, despite a vast amount of publications on ED to date, its role and mechanisms in myocardial capillary-derived CMECs is not clear.

CMECs possess a special functional characteristic in that they line the myocardial capillaries, which due to their lack of VSMCs are unable to dilate or constrict. Each cardiomyocyte is surrounded by at least 3-4 capillaries and the intercapillary distance between each cardiomyocyte is estimated be around 15-50 µm. This strategic arrangement allows for optimal paracrine communication between CMECs and cardiomyocytes (Brutsaert 2003; Strijdom & Lochner 2009). CMEC-derived factors such as NO therefore principally regulate the function and activity of cardiomyocytes and are not involved in the regulation of vascular tone such as their endothelial cell counterparts in larger, muscular arteries. In view of this, cardiac microvascular ED could have significant effects on the function of cardiomyocytes. Interestingly, the role and mechanism of ED in CMECs have not been readily explored. As per

previous reports, ED may play a major role in potentiating IHD. According to Nishida *et al* (1993), hypertension and diabetes associated ED primarily targets the microcirculation of the heart. CMECs represent about 90 % of the total endothelial cells in the heart (Piper *et al* 1990). Furthermore, cultures of microvascular endothelial cells are compulsory for a comprehensive investigation of cellular metabolism and physiological function (Piper *et al* 1990). However, when exploring the mechanisms of ED, *in vitro* models of CMECs have been neglected and endothelial cells obtained from larger coronary vascular vessels have taken preference. This is probably due to the fact that they are easier to isolate and culture and to the (incorrect) notion that all endothelial cells exhibit the same cellular mechanisms thus overlooking their functional and structural heterogeneity (for review see Aird 2007). In this study, we attempt to address the lack of available data as described above by investigating ED in a model of cultured endothelial cells obtained from myocardial capillaries.

Anecdotal evidence supporting the cardioprotective, renoprotective and anti-diabetic effects of traditional medicinal plants is vast but scientific investigations to validate such assertions and explore underlying mechanisms are relatively rare. Musabayane and co-workers (2005) have recently isolated OA as the bioactive compound in leaf extracts from *Syzigium cordatum*, a widely used medicinal plant in Southern Africa. OA has been shown to have hypoglycaemic, hypotensive and vasodilatory effects, and insulin secretory properties (Mapanga *et al* 2009; Rodriguez-Rodriguez *et al* 2008; Hsu *et al* 2006). However, to our knowledge, the role of OA in ED, and CMECs in particular, has not been investigated. In an attempt to explore the putative

therapeutic properties of OA on ED, we aimed to investigate the role of OA in CMECs during physiological conditions and in the context of ED.

1.7.2 Aims

Given the lack of available data on ED in myocardial capillary-derived CMECs, this study is explorative and observational, and therefore not hypothesis-driven.

In this study we aim to:

- Establish an *in vitro* model of ED in cultured myocardial capillary-derived CMECs by developing protocols for the induction of ED.
- Asses ED induction by measurement of the following biomarkers: (i) intracellular NO production, (ii) O_2^- production, (iii) nitrotyrosine expression and (iv) NADPH oxidase expression.
- Investigate underlying cellular mechanisms by measuring and comparing eNOS and PKB/Akt expression and activation in control and dysfunctional CMECs.
- Investigate the effects of OA derived from traditional medicinal plant extracts, in particular *Syzigium cordatum* (Hochst.) [Myrtaceace], in both control and dysfunctional CMECs.

Chapter 2: Materials and methods

2.1 Materials

Endothelial cell-specific trypsin (500 BAEE units trypsin / 180 µg EDTA.4Na / ml in Dulbecco's phosphate buffered saline (PBS)), propidium iodide (PI) and human recombinant TNF-α were obtained from Sigma Chemical Co (St Louis, Mo, USA); 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) was purchased from Calbiochem (San Diego, CA, USA); alexa fluor® 647 annexin V was obtained from BioLegend (San Diego, CA, USA); dihydroethidium (DHE) was obtained from Invitrogen (Carlsbad, CA) and fibronectin from Roche (Roche Diagnostics, Randburg, RSA); eNOS, phospho-eNOS (Ser 1177), PKB/Akt, phospho PKB/Akt (Ser 473) antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA), and p22-phox and nitrotyrosine antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). All other chemicals were of Analar grade and were purchased from Merck (Cape Town, RSA).

2.2 Cell cultures and endothelial cell purity

Adult rat CMECs were purchased commercially from VEC technologies (Rensselaer, New York, USA). The microvascular endothelial cell harvesting technique followed by the company has previously been described (Nishida *et al* 1993; Piper *et al* 1990), and ensures the highest possible myocardial capillary-derived CMEC yield, precluding contamination by other endothelial cell types. Cells were received in 25 or 75 ml fibronectin coated tissue culture flasks and grown in microvascular endothelial growth medium [EGM] (Clonetics EGM-2MV; Lonza, Walkersville, MD) until they were fully confluent. The basal growth medium was supplemented with 10 % fetal

bovine serum [FBS] (Highveld Biological, RSA), standard endothelial growth factors, ascorbic acid and antibiotics. The cells were grown in a standard tissue culture incubator at an atmospheric composition of 21 % oxygen, 5 % carbon dioxide, 40-60 % humidity and temperature maintained at 37 ºC. Confluent cells (cells evenly distributed across flask and completely covering the flask) were removed from culture by addition of trypsin, resuspended in growth medium and subcultured to the next generation in a 1:2 ratio in 35 mm fibronectin coated petri dishes for experimental purposes.

Microscopic examination of cultured CMECs revealed characteristic “cobblestone” monolayer morphology, a distinct trait of endothelial cells in culture, thus confirming morphological purity (Piper *et al* 1990) [fig. 2.1]. Furthermore, functional purity tests were performed by measuring CMEC uptake of fluorescently labelled 1,1-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate-acetylated-low density lipoprotein [Dil-ac-LDL] (Biomed Technologies, Stoughton, MA), a probe which is specific for endothelial cells (Nishida *et al* 1993, Piper *et al* 1990). Briefly CMECs were incubated with 10 ug/ml Dil-ac-LDL at 37ºC for 4 hours. The probe was subsequently washed out, and cells were removed from culture by trypsinisation, resuspended in PBS and analysed by FACS in the FL2-H channel. A high fluorescence uptake (80-100%) indicates endothelial cell purity, and no experiments were conducted on CMEC subcultures with < 80 % purity [fig. 2.2].

Subcultures of the 4^{th} to 7^{th} generations demonstrated optimal endothelial cell purity and were thus used for experimental purposes. Upon confluency, the cell cycle is

arrested at the G_0 phase due to cell-to-cell contact and thus cell proliferation (mitotic activity) ceases, thereby minimising possible cell cycle variability when evaluating experimental results (Vinals & Pouyssegur 1999). Only confluent cultures were thus used for experimental purposes. Petri dishes were randomly assigned to respective control and experimental groups. Each experiment was repeated three times and the sample size varied from n = 6-12.

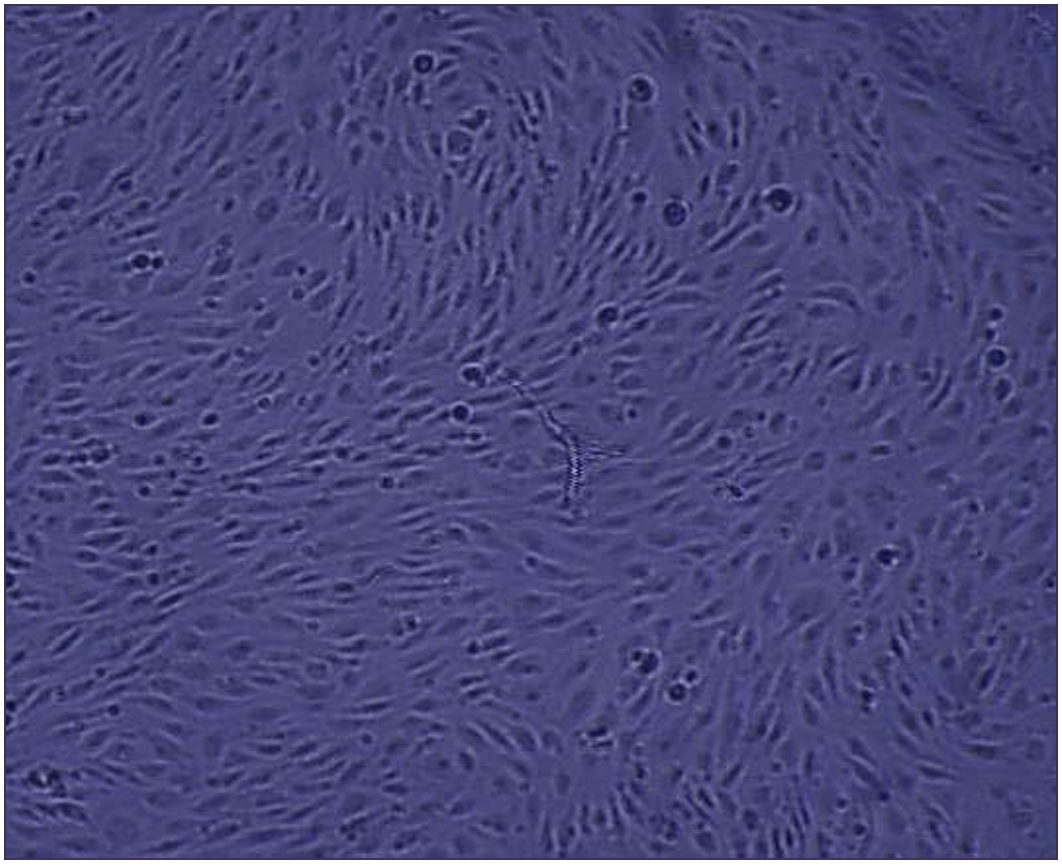


Figure 2.1: Micrograph of CMECs showing a characteristic “cobblestone” morphology in culture (10x magnification; Carl Zeiss inverted microscope, West Germany)

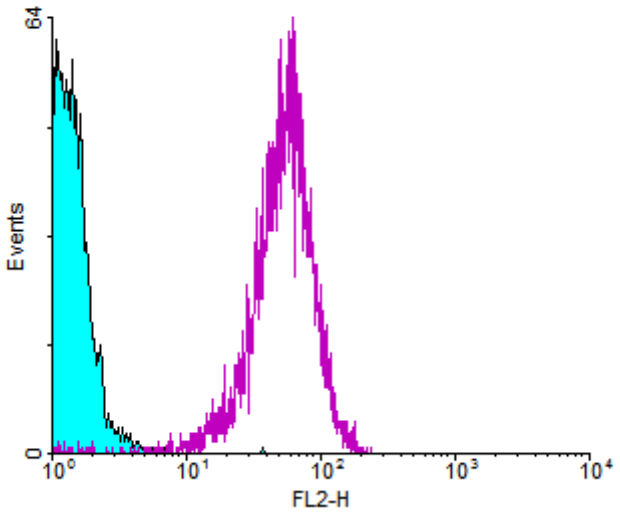


Figure 2.2: A typical histogram representation of autofluorescence (probe-free control) (blue) and DiI-ac-LDL fluorescence (purple).

2.3 Flow cytometry

Flow cytometric analysis of all fluorescent probes was performed by a flow activated cell sorter (FACS) [Becton-Dickinson FACSCalibur, Franklin Lakes, NJ], using protocols previously developed in our laboratory (Strijdom *et al* 2004 & 2006). A total of 5 000-10 000 events were routinely analysed per sample and a final cell population of interest was selected by placing a gate on control samples according to their side scatter (cell granularity) and forward scatter (cell size) [Fig. 2.3A & B], thereby excluding debris and non-cellular particles. Fluorescence data were analysed with Cellquest Pro® version 5.2.1 software (Becton-Dickson and Co, San Jose, CA). Unless stated otherwise, all fluorescence data are expressed as follows: (i) Mean fluorescence intensity expressed as % of control (control adjusted to 100 %), and (ii) % fluorescent cells of total gated population.

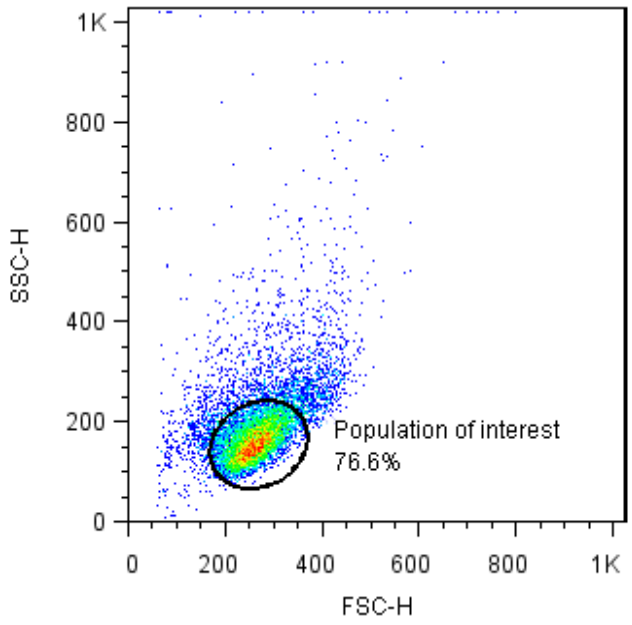


Figure 2.3A: A representative diagrammatic representation of a dot plot of a CMEC sample showing the side scatter (Y axis) which measures cell granularity and forward scatter (X axis) plot which measures cell size. The population of interest subjected to analysis is selected by placing a gate.

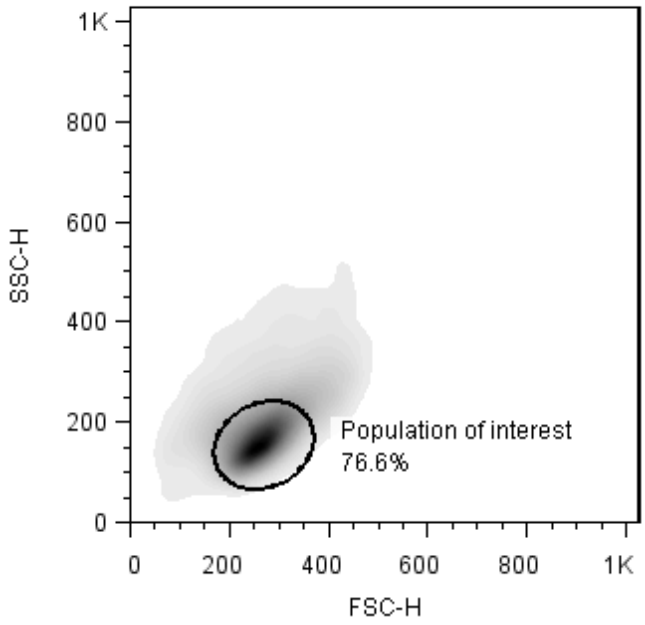


Figure 2.3B: A density plot of the same sample showing the side and forward scatter plot, and the gated population of interest.

2.4 Induction of endothelial dysfunction

In order to establish an *in vitro* model of ED, two different cardiovascular risk factors that are clinically associated with the development of ED, were simulated, namely hyperglycaemia and inflammation. Hyperglycaemia was achieved by incubating CMECs with 25 mM D-glucose (Merck chemicals, Gauteng, RSA) for 24 hours and inflammation simulated by incubating CMECs with the pro-inflammatory cytokine, TNF-α (1 ng/ml for 24 hours and 5 ng/ml for 6 or 24 hours). For diagrammatic representation of the experimental groups and protocols, please refer to fig. 2.4. The control samples were incubated with fresh, untreated medium for the same incubation period corresponding to the experimental groups.

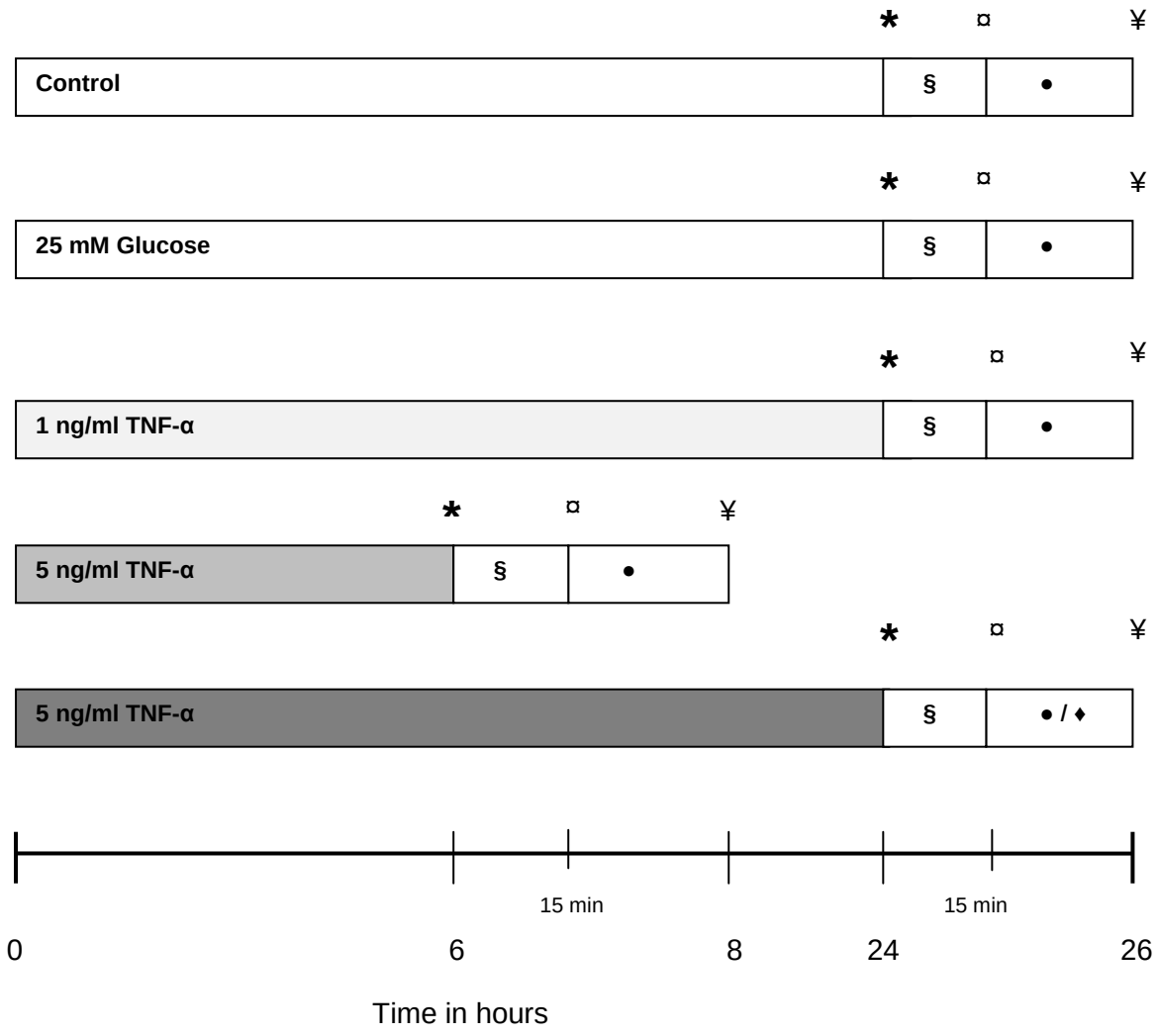


Figure 2.4: Experimental groups and protocols for ED induction.

Legend:

* Administration of fluorescent probes / sample preparation for Western blot analysis.

§ PI / annexin V incubation (15 min).

● DAF-2/DA incubation (2 hours).

♦ DHE incubation (2 hours).

¤ Sample preparation for FACS analysis of PI / annexin V fluorescence.

¥ Sample preparation for FACS analysis of DAF-2/DA fluorescence (or DHE fluorescence in the case of 5 ng/ml TNF-α 24 over hours samples).

2.4.1 Cell viability tests

At the end of the experimental incubation period, cell viability was confirmed, where indicated, by treatment of cells with 5 µM of PI and 5 µM annexin V (conjugated with Alexa Fluor® 647) for 15 minutes [fig. 2.4] prior to FACS analysis in the FL2-H and FL4-H channels respectively [fig. 2.5A & B]. Briefly, cells were removed from culture by trypisinisation, washed sequentially in staining and binding buffer (BioLegend) and subsequently treated with 5 µM of PI and annexin V, and incubated at room temperature in the dark for 15 min, followed by FACS analysis. Cell membrane permeability (loss of membrane integrity) grants the PI probe entrance into the cell which subsequently stains the nucleus. Therefore, cells demonstrating PI uptake represent a non-viable (necrotic) cell population. Apoptotic cells are identified by binding of annexin V to phosphatidylserine protein, which in healthy cells is normally located on the intracellular surface of the plasma membrane but translocates to the external surface of the plasma membrane during apoptosis. PI and Annexin V staining therefore distinguishes between cells undergoing necrosis and apoptosis respectively [fig. 2.6] (Wilkins *et al* 2002).

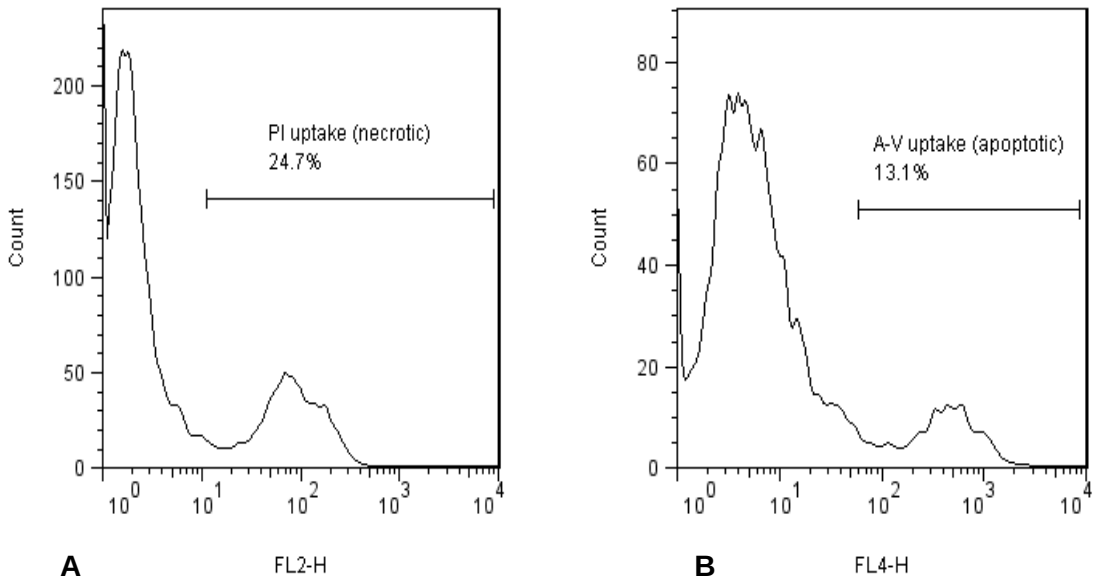


Figure 2.5: **A.** A histogram depicting flow cytometric analysis of PI fluorescence in channel FL2-H. The second peak represents cells that positively stained with the PI probe and hence regarded as non-viable (necrotic). **B.** Flow cytometric analysis of Annexin V fluorescence in channel FL4-H with the second peak representative of cells that positively stained with the Annexin V and are thus considered non-viable (apoptotic).

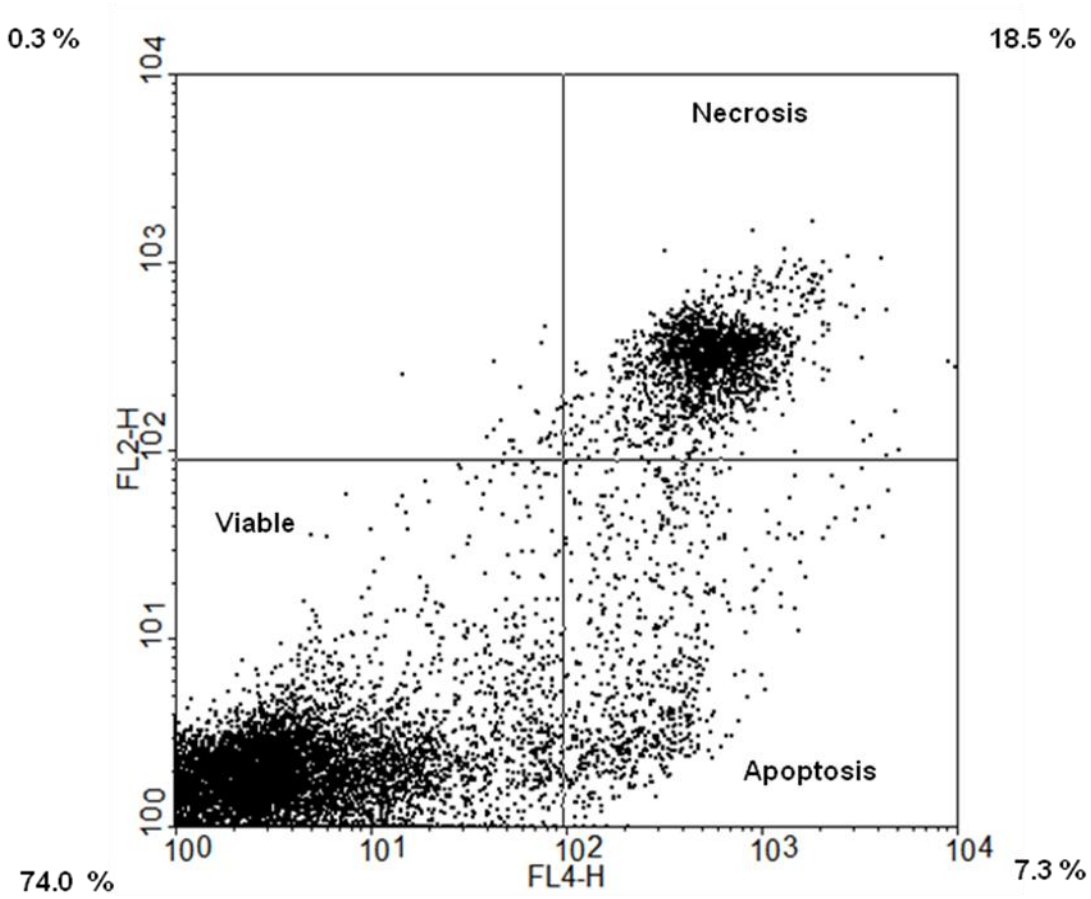


Figure 2.6: A dot plot illustrating PI fluorescence (FL2-H) and annexin V fluorescence (FL4-H), which allows the investigator to distinguish between apoptotic cells (lower right quadrant: annexin V staining positive, 7.3 % of the total gated population in the above sample) and necrotic cells (upper right quadrant: PI staining positive,18.5 % of the total gated population in the above sample). Viable cells (PI and annexin V negative, 74 % of the total gated population in the above sample) appear in the lower left quadrant.

2.4.2 NO production measurements

The measurement of intracellular NO levels was used as an end-point to verify whether ED induction was successfully achieved. Intracellular levels of NO were directly measured using the NO-specific DAF-2/DA fluorescent probe as previously described by Strijdom *et al* (2004 & 2006). Upon reacting with NO, DAF-2/DA is oxidised to diaminofluorescein-triazol (DAF-2T) which emits a green fluorescence analysed in the FL1-H channel by FACS [fig. 2.7]. At the end of the ED induction period, cells were washed with PBS and incubated with 10 µM DAF-2/DA at 37 ºC for 2 hours. After 2 hours, DAF-2/DA was washed out and cells removed from culture by trypisinisation and resuspended in probe-free PBS for FACS analysis [fig. 2.4]. All experiments included a probe-free absolute control sample (to determine autofluorescence) and probe-containing control samples [fig. 2.7]. A significant increase in baseline fluorescence in probe-containing control samples confirms proper DAF-2DA uptake by the cells, as demonstrated in fig. 2.7.

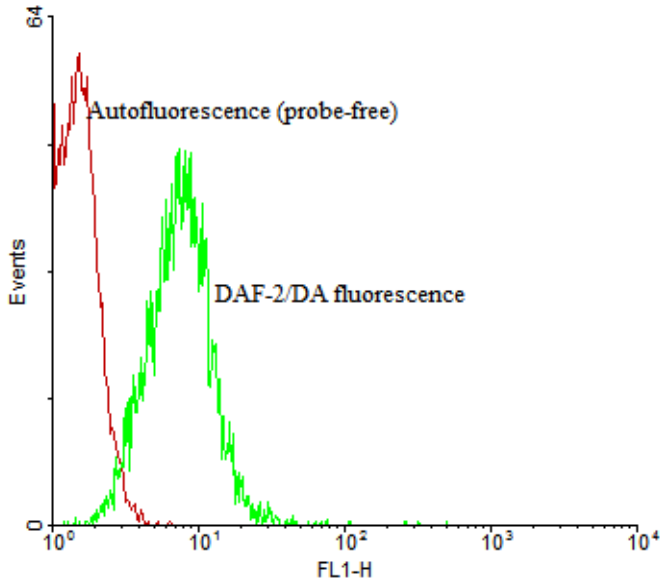


Figure 2.7: A histogram representation of autofluorescence of the cells (a probe-free control, used as an absolute control) and DAF-2/DA fluorescence (DAF-2/DA treated control) in the FL1-H channel.

2.4.3 Superoxide measurements

We measured O_2^- production in CMECs using a superoxide-sensitive probe, DHE. After 24 hours of ED induction, cells were washed with PBS and treated with 5 µM DHE in PBS and incubated at 37 ºC for 2 hours as per previous descriptions (Navarro-Antolin *et al* 2001) [fig. 2.4]. After 2 hours, probe-containing PBS was washed out and cells removed from culture by trypisinisation. Cells were resuspended in probe-free PBS and DHE fluorescence was analysed by FACS in channel FL2-H.

2.4.4 Protein expression and phosphorylation measurements

Total and phosphorylated (activated) eNOS and PKB / Akt, and nitrotyrosine and NADPH oxidase protein expression were measured by Western blot analysis. The membrane bound NADPH oxidase subunit, p22-phox, was used to quantify NADPH oxidase expression (Griendling *et al* 2000). β-tubulin was also measured to confirm equal protein loading in all samples. To extract protein, cells were lysed in a lysis buffer that comprised: 20 mM Tris; 1mM EGTA; 150 mM NaCl; 1mM β-glycerophosphate; 1 mM sodium orthovanadate; 2.5 mM tetra-sodium diphosphate; 1 mM PMSF; 0.1 % sodium dodecylsulfate (SDS); 10 µg/ml aprotinin; 10 µg/ml leupeptinin; 50 nM NaF and 1 % triton-X100. After lysis, cells were subjected to sonication, after which cell lysate protein of 50 µg / 10 µl was obtained. Cell lysate proteins of equal amounts were subsequently loaded onto a 7.5 % (for eNOS, PKB/Akt, and β-tubulin); 10 % (for nitrotyrosine) and 12 % (for p22-phox) SDS-polyacrylamide gel and transferred onto PVDF membrane (immobilon™-P, from Millipore). Non-specific binding sites on membranes were blocked with 5 % fat-free milk in Tris-buffered saline, 0.1 % tween-20.

Following Western blotting, membranes were probed with the specific rabbit polyclonal primary antibodies (anti-eNOS, anti-phospho eNOS (Ser 1177), anti-PKB/Akt, anti-phospho PKB/Akt (Ser 473), anti-β-tubulin) [Cell Signalling Technology, Beverly, MA, USA], and (anti-p22-phox and anti-nitrotyrosine) [Santa Cruz Biotechnologies, Santa Cruz, CA, USA]. The membranes were subsequently exposed to the secondary antibody, horseradish peroxidise-linked anti-rabbit IgG (Amersham, Buckinghamshire, UK). The ECL^{TM} system was employed to observe the immunoreactions and films were analysed by densitomentry (UN-SCAN-IT, Silk Scientific, Orem, UT, USA). All Western blot data were expressed as ratio of control with control adjusted to 1.

2.5 Plant extracts studies

2.5.1 OA isolation

OA powder extract was kindly supplied by Prof Cephas Musabayane (University of KwaZulu-Natal, South Africa). The leaves of *Syzigium cordatum* (Hochst.) [Myrtaceae] were obtained from the Durban region (KwaZulu-Natal). Professor H Baijnath, former chief taxonomist/ curator at the University of KwaZulu-Natal, verified the identity of the plant.

The leaves were air-dried and pulverised into fine powder. The powder was consecutively extracted three times at 24 hour intervals with three litres of hexane, dichloromethane, ethyl acetate and methanol as previously described (Mapanga *et al* 2009). The crude acetyl acetate extract (ethyl acetate-solubles; EAS) contained the bioactive compound as has been previously verified by Musabayane *et al* (2005)

and Samova *et al* (2003) and was further purified. The purified EAS subsequently underwent a series of open column chromatography steps, ultimately yielding OA as a white powder as described previously (Mapanga *et al* 2009). The structure of OA was ascertained by H and C-NMR (1D and 2D) spectroscopy. The purity of OA was 99 % when compared to spectral data available in existing literature based on the chemical and physicochemical properties (Mapanga *et al* 2009).

2.5.2 Pilot studies to assess the effects of OA on CMECs

Confluent CMECs were treated with either 30 µM or 40 µM OA dissolved in dimethyl sulfoxide (DMSO) for 5 and 20 min [fig. 2.8]. The vehicle control samples were treated with corresponding volumes of DMSO only and the controls received fresh medium only. At the end of each incubation period, NO production (DAF-2/DA) and cell viability (PI and Annexin V) were measured using methods described in previous sections.

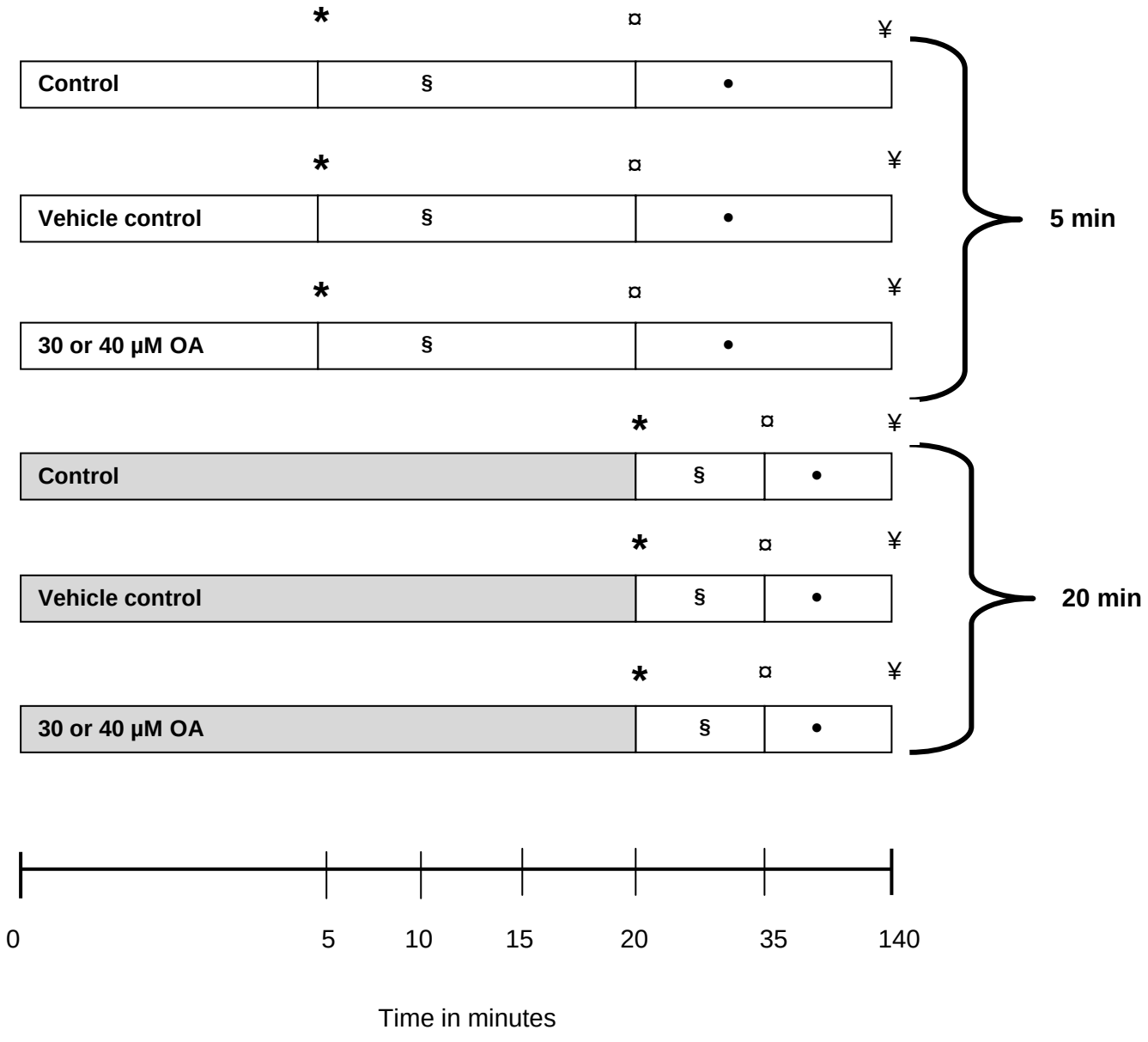


Figure 2.8: Experimental groups for pilot studies to asses effects OA on CMECs.

Legend:

* Administration of fluorescent probes

§ PI / annexin V incubation (15 min)

● DAF-2/DA incubation (2 hours)

¤ Sample preparation for FACS analysis PI / annexin V fluorescence

¥ Sample preparation for FACS analysis DAF-2/DA fluorescence

2.5.3 The effects of OA in ED

2.5.3.1 Cell viability and NO measurements

To determine the effects of OA in dysfunctional CMECs, confluent CMECs were pre-treated with 40 µM OA 1 hour prior to ED induction (5 ng/ml TNF-α for 24 hours). The experiments consisted of four groups, namely (i) untreated controls, (ii) OA treated cells, (iii) TNF-α treated cells (ED group), and (iv) TNF-α + OA treated cells (OA + ED group) [fig. 2.9]. Following the incubation period, NO production and cell viability were determined. Separate studies showed that administration of the vehicle for OA (DMSO) for 24 hours had no significant effect on NO production or cell viability (data not shown).

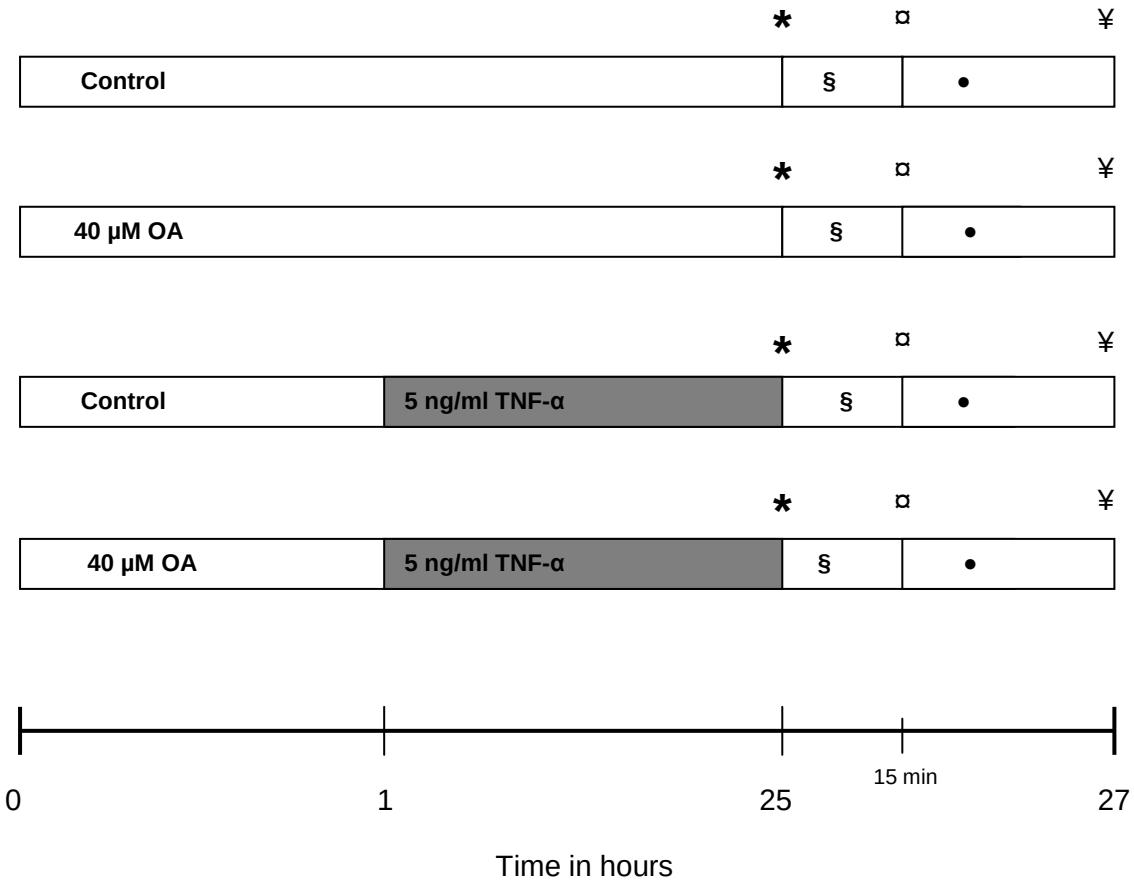


Figure 2.9: Experimental groups and protocols for OA investigations in normal and dysfunctional CMECs.

Legend:

* Administration of fluorescent probes / sample preparation for Western blot analysis.

§ PI / annexin V incubation (15 min)

● DAF-2/DA incubation (2 hours)

¤ Sampling for FACS analysis of PI / annexin V fluorescence

¥ Sampling for FACS analysis of DAF-2/DA fluorescence

2.5.3.2 Protein expression and phosphorylation measurements

Protein expression and activation were determined for OA investigations [fig. 2.9]. Total and activated eNOS expression was measured by Western blot analysis using protocols previously described on section 2.4.4.

Statistical analyses

All data are expressed as mean ± SEM. Statistical analyses were done with Student's t-test or one-way ANOVA (with Bonferroni post-hoc test if $p<0.05$). Data displaying a p-value of <0.05 were considered statistically significant. Graph Pad Prism ® version 5.01 software was used for all analyses.

Chapter 3: Results

3.1 ED induction (model establishment)

Nitric oxide levels are expressed as mean DAF-2/DA fluorescence intensity or % cells that stain positive with DAF-2/DA (% DAF-2/DA fluorescent cells).

3.1.1 Glucose incubation to induce hyperglycaemia

25 mM glucose incubation for 24 hours

Mean DAF-2/DA fluorescence intensity increased significantly in glucose treated cells when compared to untreated control (control: 100 % vs. glucose: 114.4 ± 5.9 %; $p = 0.03$, $n = 9$) [fig. 3.1A]. However, % DAF-2/DA fluorescent cells did not differ significantly between control and glucose treated samples (control: 100 % vs. glucose: 140.5 ± 19.37 %; $p = 0.053$, $n = 9$) [fig. 3.1B]. Cell viability was not affected by glucose treatment (control: 100 % vs. glucose: 101.1 ± 0.53 %; $p > 0.05$, $n = 9$) [fig. 3.1C].

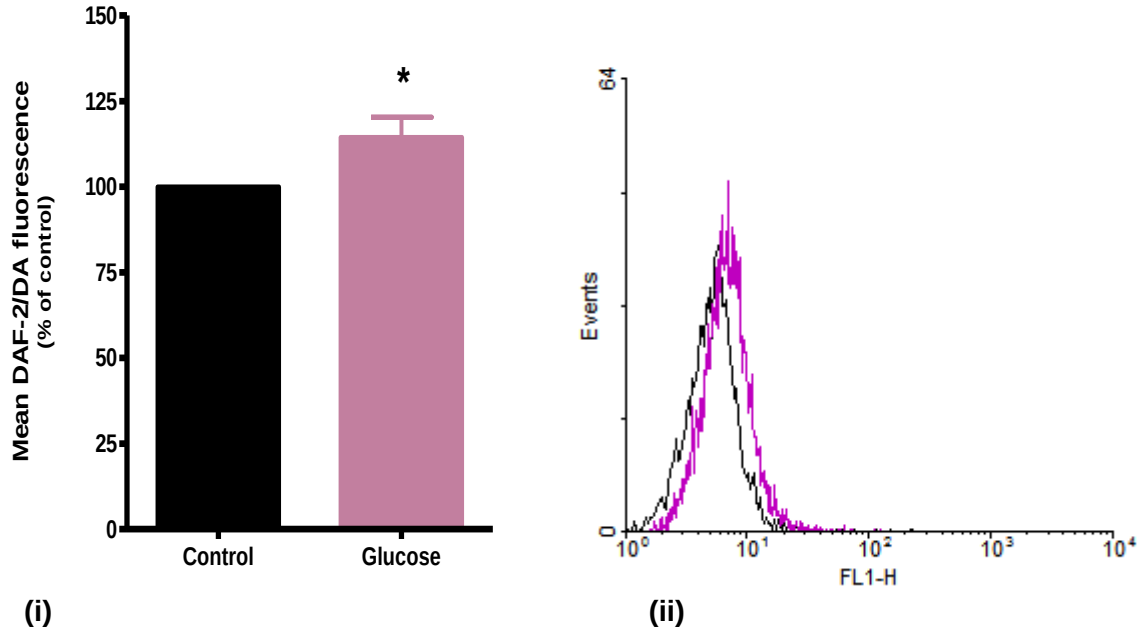


Figure 3.1A: **(i)** Bar graph depicting mean DAF-2/DA fluorescence intensity expressed as percentage of control. Glucose vs. control. *****: p = 0.03. **(ii)** A representative histogram showing DAF-2/DA fluorescence on the FL1-H axis in control (black) and glucose (pink) treated cells.

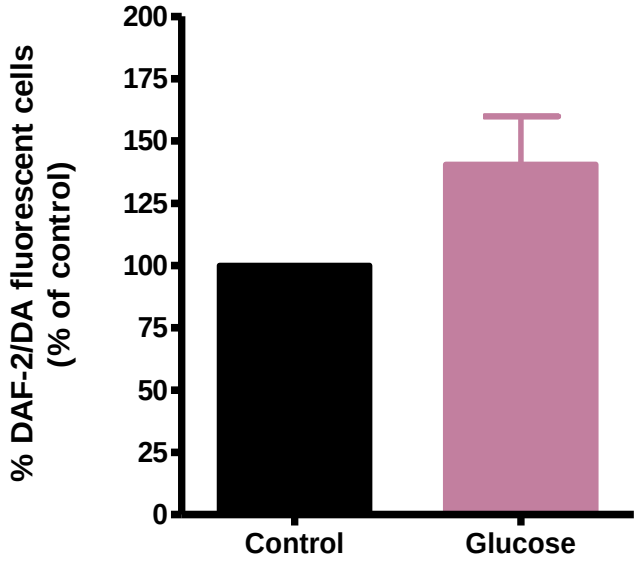


Figure 3.1B: % DAF-2/DA fluorescent cells expressed as percentage of control. Glucose vs. control. p > 0.05

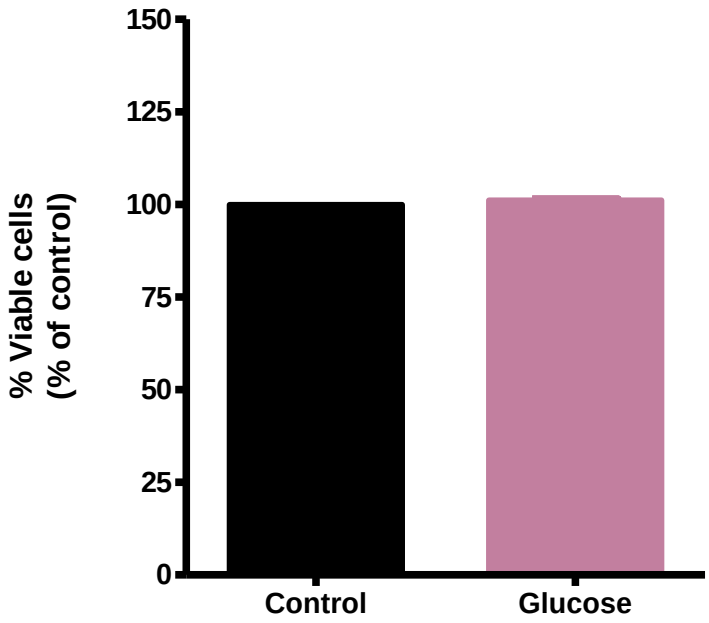


Figure 3.1C: Cell viability obtained from FACS analysis of cells excluding PI (i.e. viable cells) and expressed as percentage of control. Glucose vs. control, $p > 0.05$.

3.1.2 TNF-α incubation to simulate inflammation

To simulate inflammation, CMECs were treated with the pro-inflammatory cytokine, TNF-α, at 1 ng/ml for 24 hours, 5 ng/ml for 6 hours and 5 ng/ml for 24 hours.

1 ng/ml TNF-α for 24 hours

At 1 ng/ml (24 hours), TNF-α increased mean DAF-2/DA fluorescence intensity significantly by 24 % when compared to control (control: 100 % vs. TNF-α: 124.3 ± 6.8 %; p = 0.002, n = 10) [fig. 3.2A] and % DAF-2/DA fluorescent cells increased significantly by 53.5 % compared to control (control: 100 % vs. TNF-α: 153.5 ± 18.46 %; p = 0.009, n = 10) [fig. 3.2B]. No significant differences were observed in cell viability (control: 100 % vs. TNF-α: 102.3 ± 0.64; p = 0.07, n = 2) [fig. 3.2D].

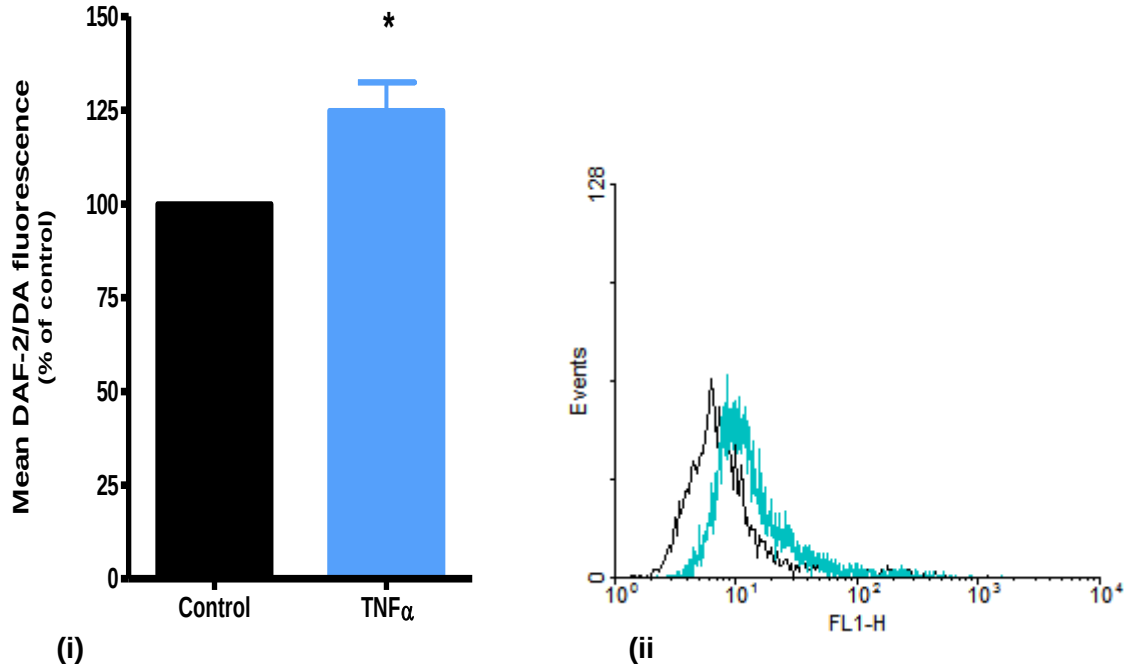


Figure 3.2A: **(i)** Mean DAF-2/DA fluorescence intensity expressed as % of control. 1 ng/ml TNF-α vs. control (24 hours), *****: p = 0.002. **(ii)** A representative histogram showing DAF-2/DA fluorescence on the FL1-H axis in control (black) and TNF-α (blue) treated cells.

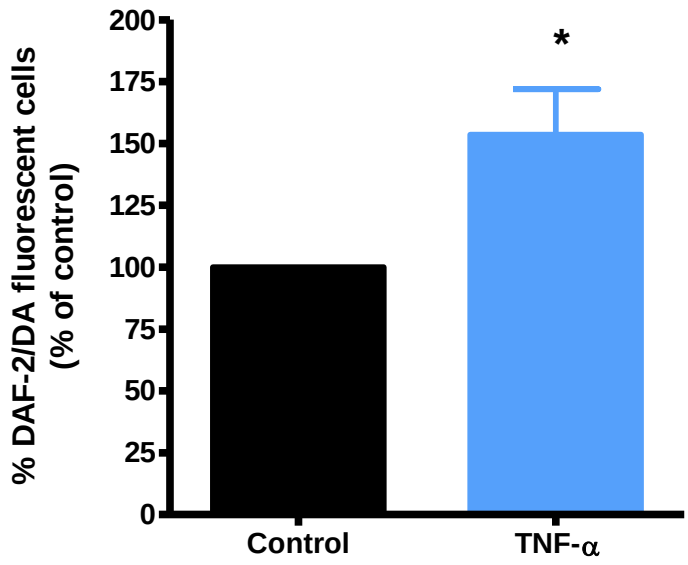


Figure 3.2B: % DAF-2/DA fluorescent cells expressed as percentage of control. 1 ng/ml TNF-α vs. control (24 hours) *****: p = 0.009

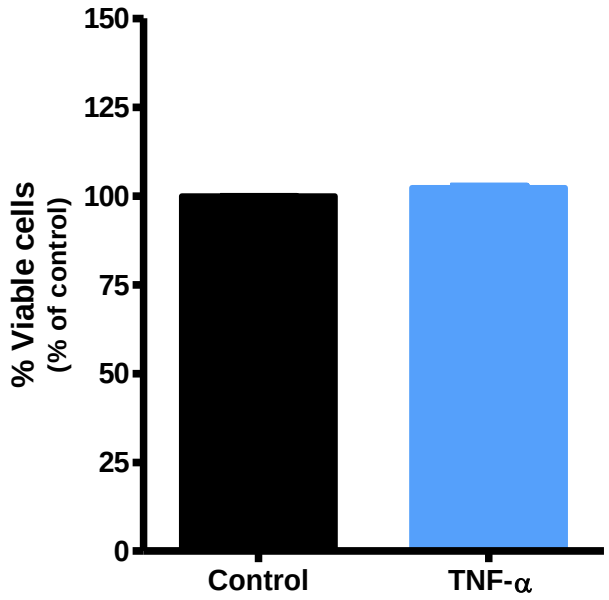


Figure 3.2C: Percentage of viable cells (expressed as % of control) as determined from FACS analysis of cells excluding PI. 1 ng/ml TNF-α vs. control (24 hours), p = 0.07.

5 ng/ml TNF-α for 6 hours

A 31 % increase in mean DAF-2/DA fluorescence intensity was observed when CMECs were treated with 5 ng/ml for 6 hours (control: 100 % vs. TNF-α: 130.7 ± 9.8 %; p = 0.04, n = 14) [fig. 3.3A]. % DAF-2/DA fluorescent cells remained unchanged (control: 100% vs. TNF-α: 100 ± 1.17 %; p = 0.97, n = 14) [fig. 3.3B]. Cell viability was not altered by this intervention (control 100 % vs. TNF- α: 99.5 ± 0.64 %; p = 0.48, n = 3) [fig. 3.3C].

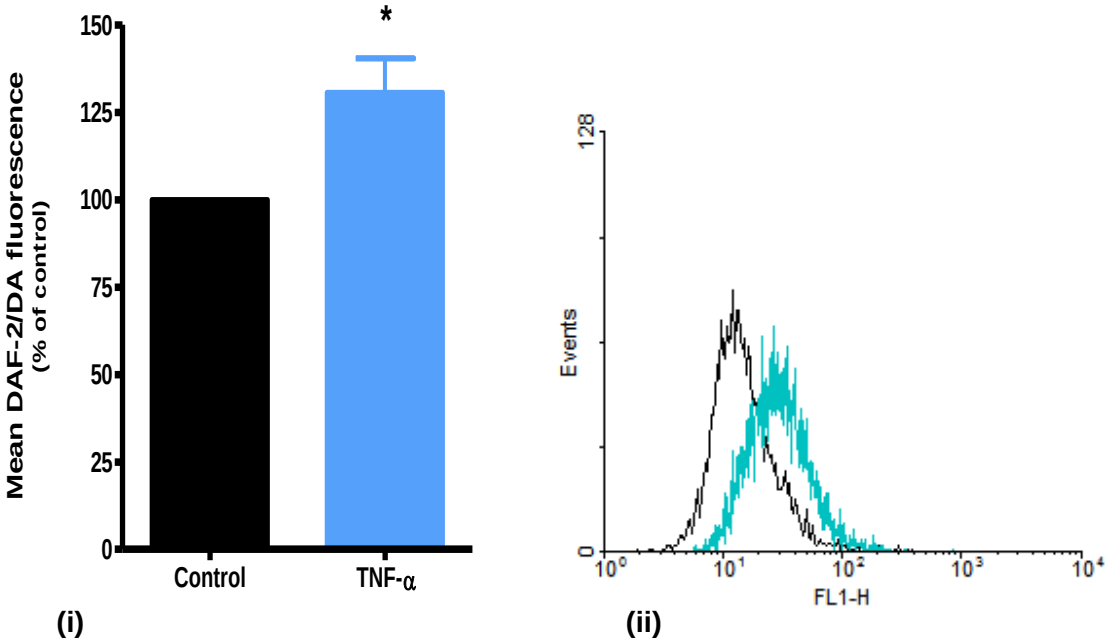


Figure 3.3A: **(i)** Mean DAF-2/DA fluorescence intensity expressed % of control. 5 ng/ml TNF-α vs. control (6 hours), *****: p = 0.04. **(ii)** A representative histogram showing DAF-2/DA fluorescence on the FL1-H axis in control (black) and TNF-α (blue) treated cells.

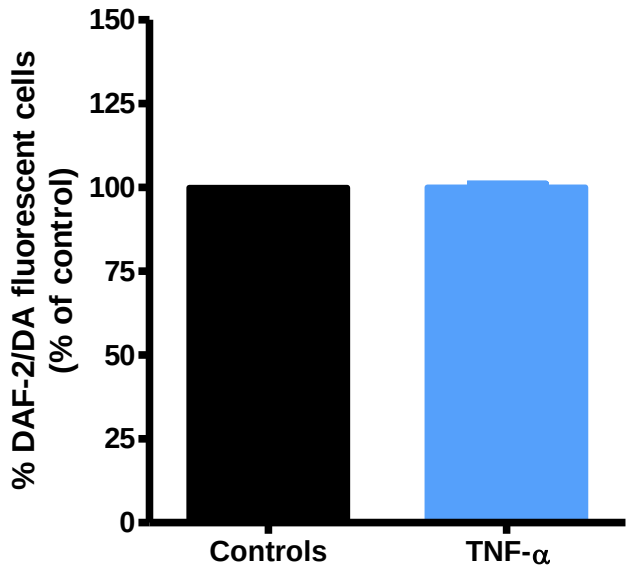


Figure 3.3B: % DAF-2/DA fluorescent cells expressed as percentage of control. 5 ng/ml TNF-α vs. control (6 hours), p = 0.97.

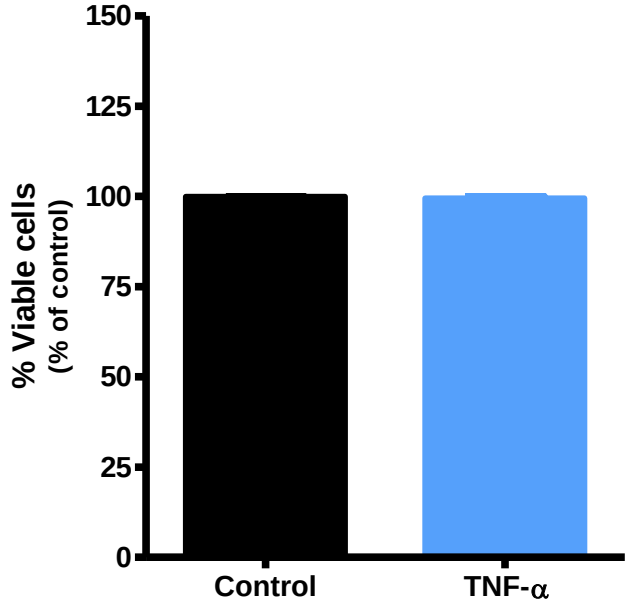


Figure 3.3C: Cell viability expressed as percentage of control as determined from cells excluding PI. 5 ng/ml TNF-α vs. control (6 hours), p = 0.48.

5 ng/ml TNF-α for 24 hours

The model of ED in CMECs was established by incubation with 5 ng/ml TNF-α over a period of 24 hours. Mean DAF-2/DA fluorescence intensity decreased by 14.2 % in TNF-α treated CMECs when compared to control (control: 100 % vs. TNF-α: 85.80 ± 2.9 %; p = 0.0001, n = 16) [fig. 3.4A]. % DAF-2/DA fluorescent cells also showed a decrease in TNF-α treated cells compared to control (control: 100 % vs. TNF-α: 88.61 ± 4.42 %; p = 0.02, n = 16) [fig. 3.4B] No statistical significance was observed in cell viability between control and TNF-α treated CMECs (control 100 % vs. TNF-α: 99.31 ± 0.40; p = 0.10, n = 13) [fig. 3.4C]

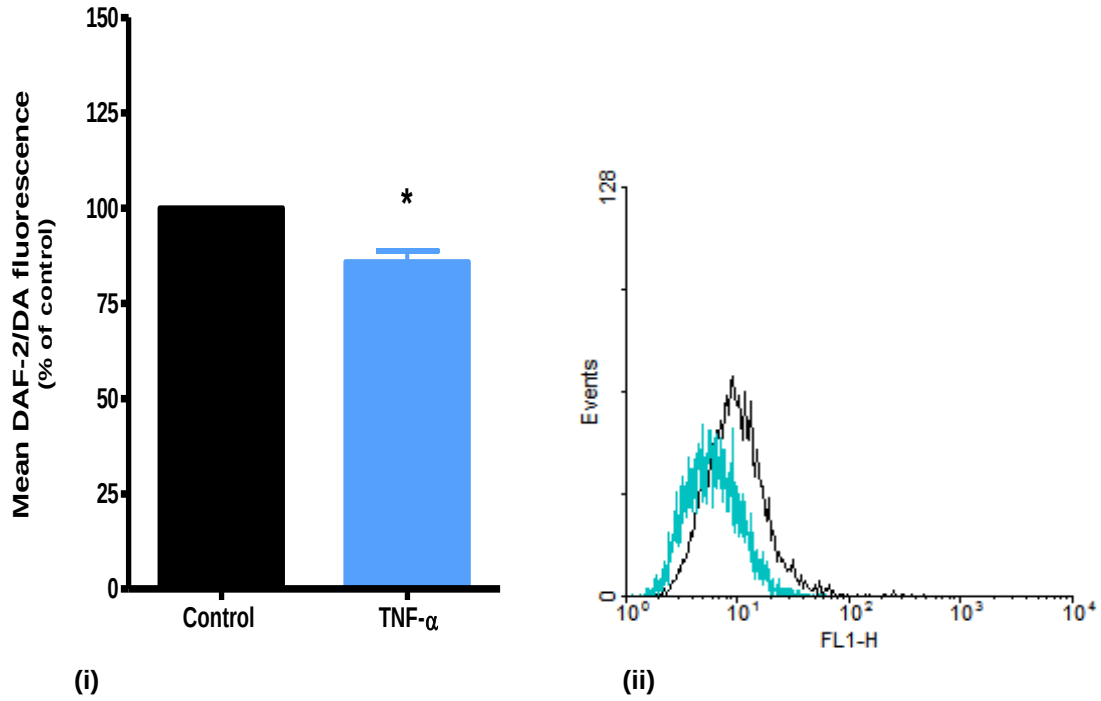


Figure 3.4A: **(i)** Mean DAF-2/DA fluorescence intensity expressed % of control. 5 ng/ml TNF-α vs. controls (24 hours), *****: p = 0.0001. **(ii)** A representative histogram showing DAF-2/DA fluorescence on the FL1-H axis in control (black) and TNF-α (blue) treated cells.

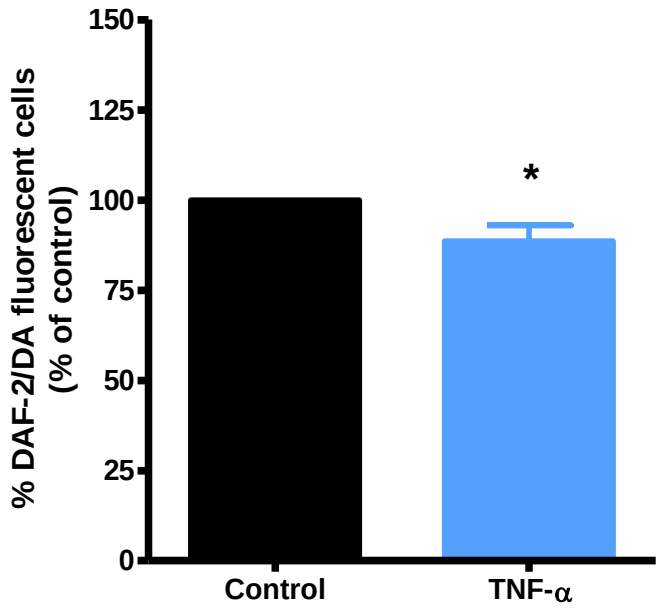


Figure 3.4B: % DAF-2/DA fluorescence cells expressed as percentage of control. 5 ng/ml TNF-α vs. controls (24 hours), *: p = 0.02.

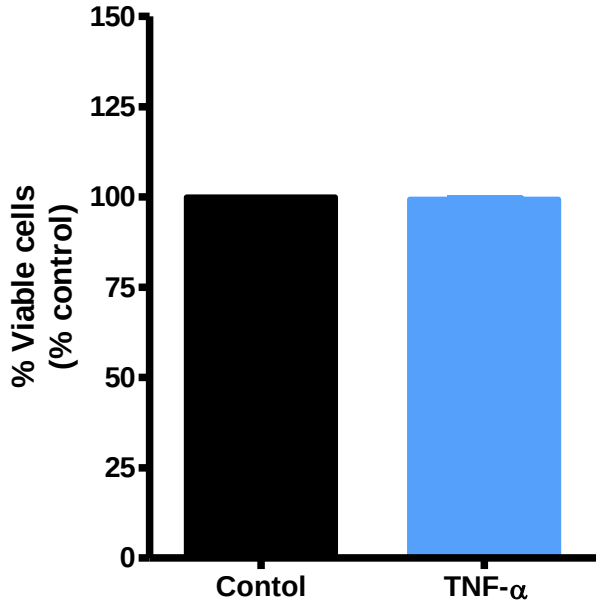


Figure 3.4C: Cell viability expressed as percentage of control obtained from FACS analysis of cell excluding PI. 5 ng/ml TNF-α vs. controls (24 hours), p = 0.10.

3.2 Superoxide measurements

We measured O_2^- levels in our model of ED (5 ng/ml TNF-α treated CMECs for 24 hours) by FACS analysis of DHE fluorescence. Levels of O_2^- are expressed as mean DHE fluorescence intensity or % cells that stain positive with DHE (% DHE fluorescent cells). There were no significant differences in both mean DHE fluorescence intensity (control: 100 % vs. TNF-α: 132 ± 26.6 %; p = 0.24, n = 12) [fig 3.5A] and % DHE fluorescent cells (control: 100 % vs. TNF-α: 97.7 ± 1.2 %; p = 0.07, n = 12) [fig 3.5B] when compared to control.

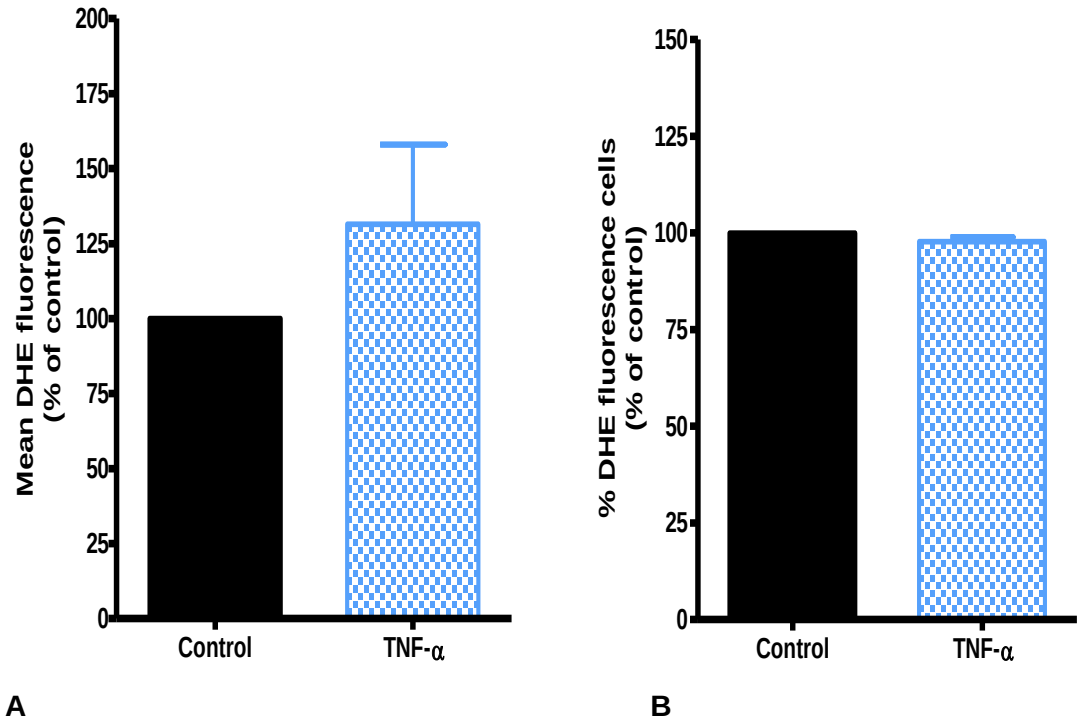


Figure 3.5: **A**: Mean DHE fluorescence intensity expressed as percentage of control. TNF-α vs. control, p = 0.24. **B:** % DHE fluorescent cells expressed as percentage of control. TNF-α vs. control, p = 0.07

3.3 Protein expression measurements

All protein expression and phosphorylation measurements were done on our ED model (5 ng/ml TNF-α treated CMECs for 24 hours) by Western blotting analysis.

3.3.1 Total eNOS expression and activation

Total eNOS

The increases observed in total eNOS expression in TNF-α treated samples did not differ significantly compared to control (control: 1 vs TNF-α: 1.70 ± 0.40; p = 0.99, n = 8) [fig. 3.6A].

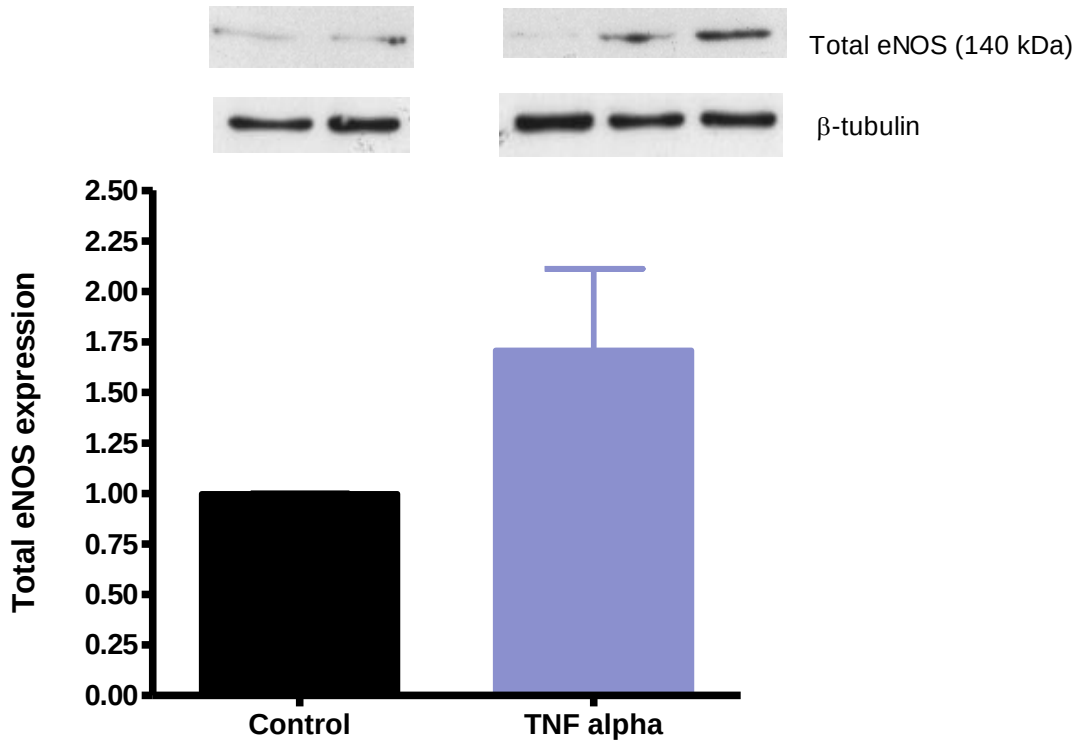


Figure 3.6A: Total eNOS expression in TNF-α vs. control, p = 0.99. β-tubulin shown for verification of equal protein loading.

Phosphorylated (activated) eNOS Ser 1177

eNOS phosphorylation decreased significantly by 40 % in TNF-α treated cells when compared to control (control: 1 vs TNF-α: 0.60 ± 0.095; p = 0.001, n = 8) [fig. 3.6B]

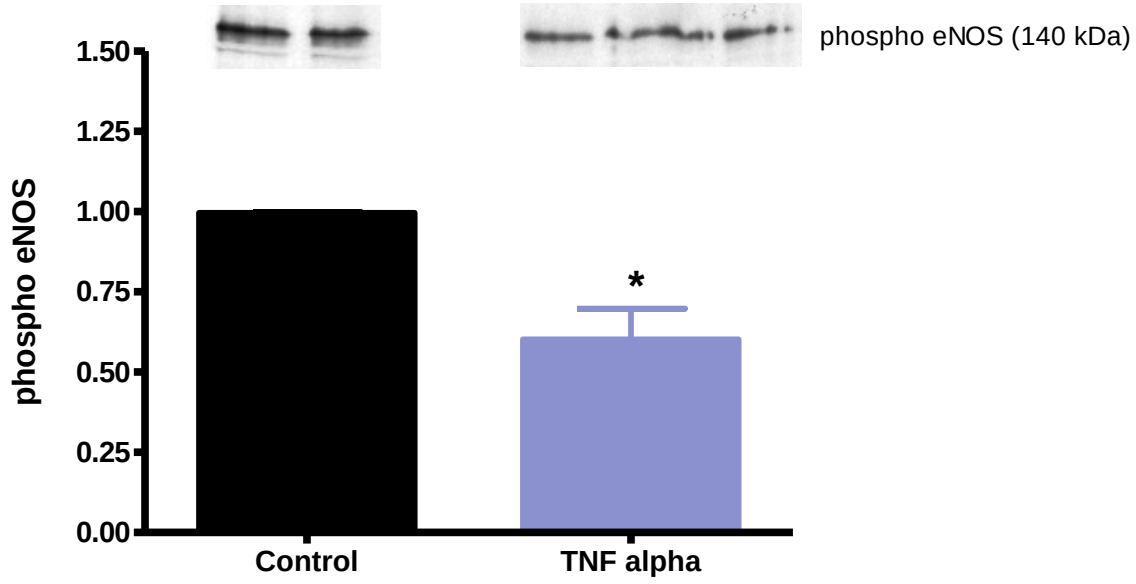


Figure 3.6B: Phosphorylated eNOS levels in TNF-α vs. control, *: p = 0.001.

Phospho / total eNOS ratios

When phosphorylated eNOS was expressed as a ratio of total eNOS, a significant decrease of 45 % was observed in TNF-α treated cells (control: 1 vs TNF-α: 0.55 ± 0.150; p = 0.009, n = 8) [fig. 3.6C].

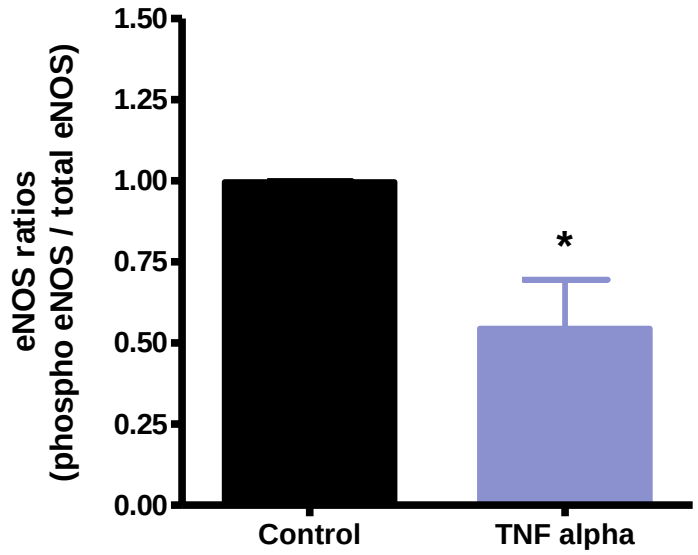


Figure 3.6C: Phosphorylated eNOS expressed as ratio of total eNOS in TNF-α vs. control, *****: p = 0.009.

3.3.2 Total PKB / Akt expression and activation

Total PKB / Akt expression did not differ significantly between control and TNF-α treated cells (control: 1 vs. TNF-α: 1.07 ± 0.058; p =0.198, n = 10) [fig. 3.7A]. However, phosphorylated PKB / Akt (Ser 473) levels decreased significantly by 42 % in TNF-α treated when compared to control (control: 1 vs. TNF-α: 0.52 ± 0.139; p = 0.003, n = 10) [fig. 3.7B].

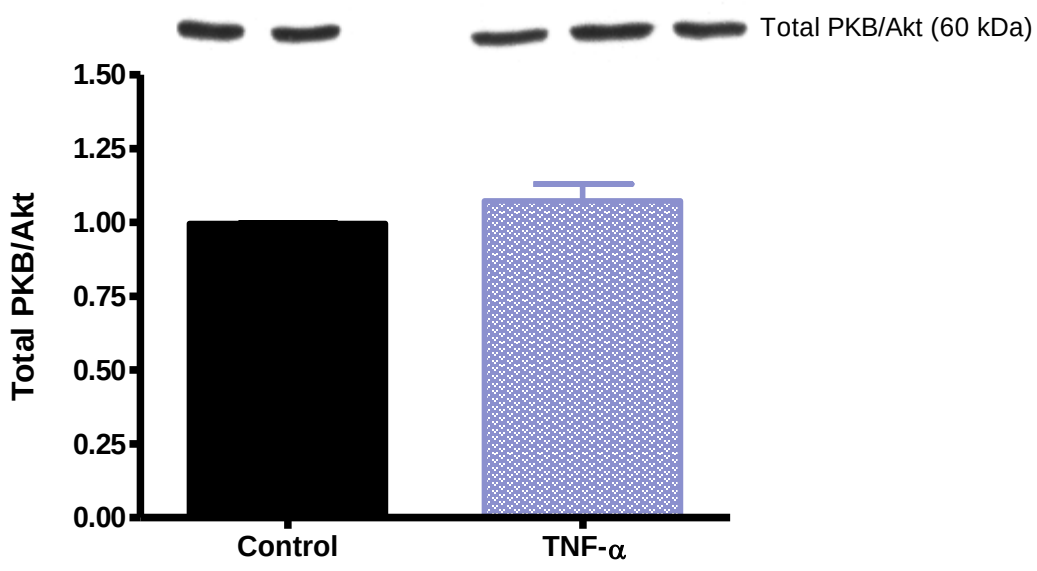


Figure 3.7A: Total PKB / Akt expression in TNF-α vs. control, p = 0.198.

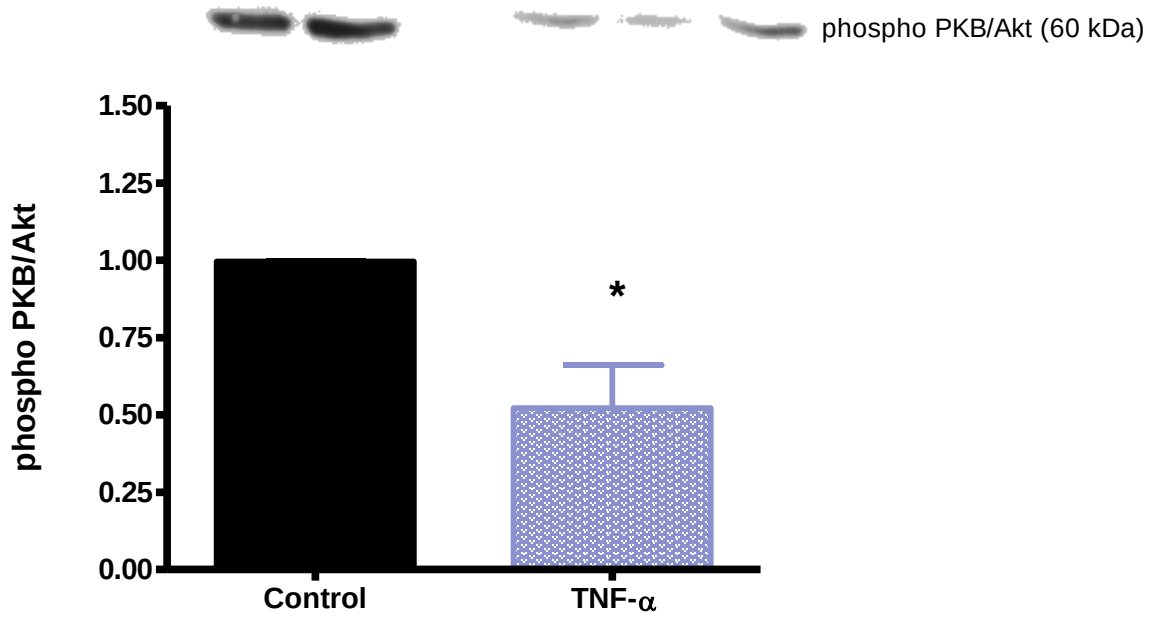


Figure 3.7B: Phosphorylated (activated) PKB / Akt Ser 473 in TNF-α vs. control, *****: p = 0.003.

3.3.3 p22-phox expression

p22-phox, a membrane-bound subunit of NADPH oxidase was used to quantify NADPH oxidase expression. p22-phox increased significantly by 3.2-fold in TNF-α treated when compared to control (control: 1 vs. TNF-α: 3.23 ± 0.353; p = 0.0001, n = 6) [fig. 3.8].

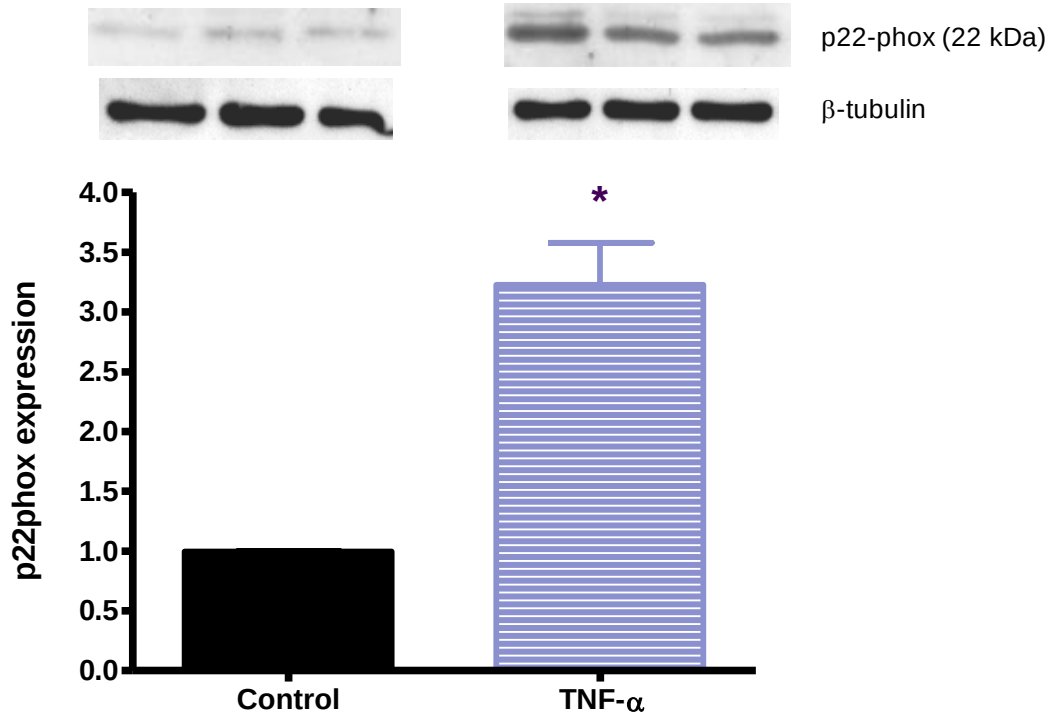


Figure 3.8: p22-phox expression in TNF-α vs. control, *: p = 0.0001. β-tubulin shown for verification of equal protein loading.

3.3.4 Nitrotyrosine expression

Protein nitration in our model of ED was quantified by Western blot analysis of nitrotyrosine expression and regarded as a marker for peroxynitrite induced oxidative stress. However, there were no significance differences in nitrotyrosine expression between control and TNF-α treated cells (control: 1 vs. TNF-α: 0.96 ± 0.041; p = 0.38, n = 10) [fig. 3.9].

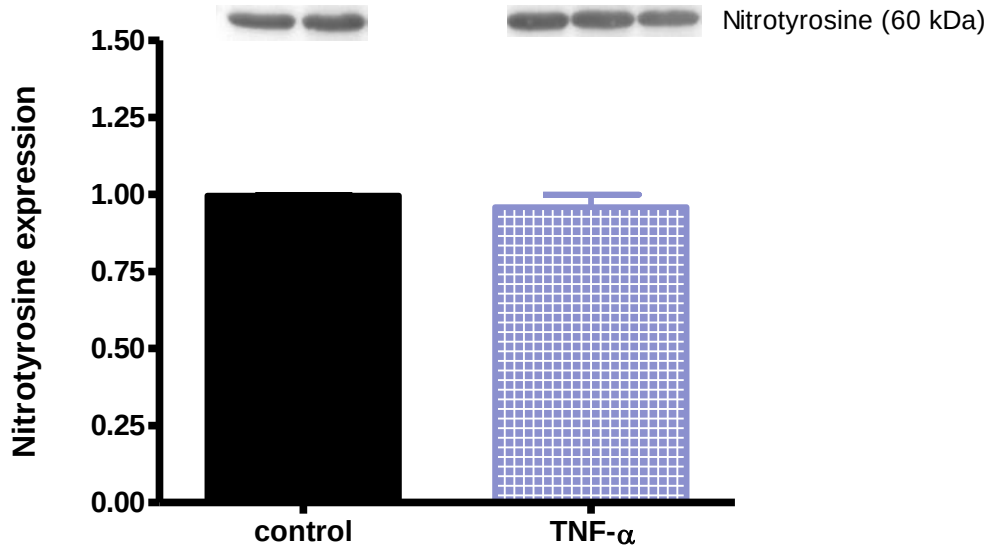


Figure 3.9: Nitrotyrosine expression in TNF-α vs. control, p = 0.38.

3.4 Plant extracts studies

3.4.1 Pilot studies to assess effects of OA on normal CMECs

30 µM OA incubation for 5 and 20 min

30 µM OA increased mean DAF-2/DA fluorescence intensity significantly by 44 % after 5 min of incubation (control: 100 % vs. OA: 144.3 ± 13.0; $p < 0.0001$, $n = 9$) and by 83 % after 20 min of incubation (control 100 % vs. OA: 183.0 ± 14.9; $p < 0.0001$, $n = 9$) [fig. 3.10A]. The vehicle controls treated with DMSO showed unaltered mean DAF-2/DA fluorescence intensity (control: 100 % vs. DMSO: 97.27 ± 7.4 %; $p > 0.05$, $n = 17$) [fig. 3.10A & 3.10B] and cell viability [fig. 3.10C], and OA had no significant effect on cell viability [fig. 3.10C].

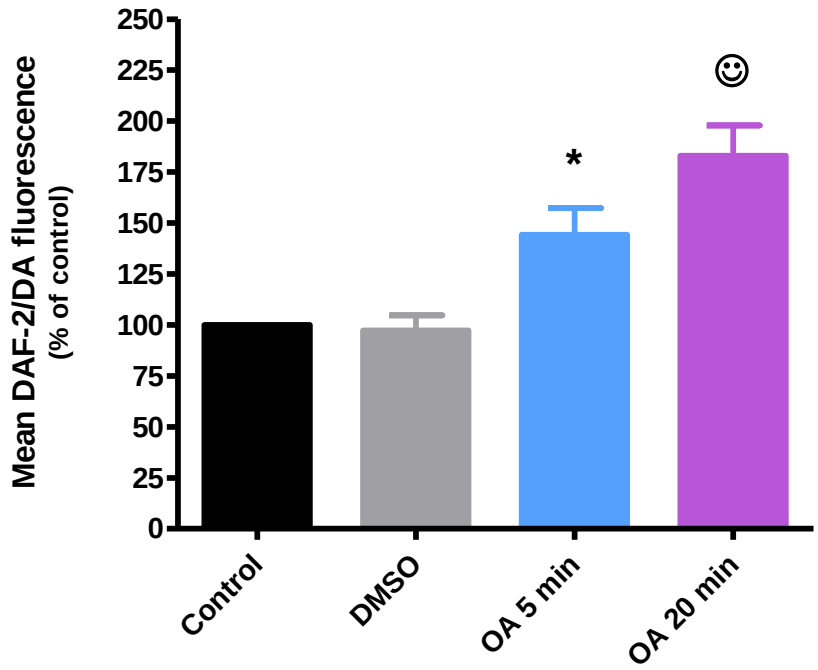


Figure 3.10A: Mean DAF-2/DA fluorescence intensity expressed as percentage control. 30 µM OA 5 min vs. control, *****: $p < 0.0001$ and 30 µM OA 20 min vs. control, ☺: $p < 0.0001$.

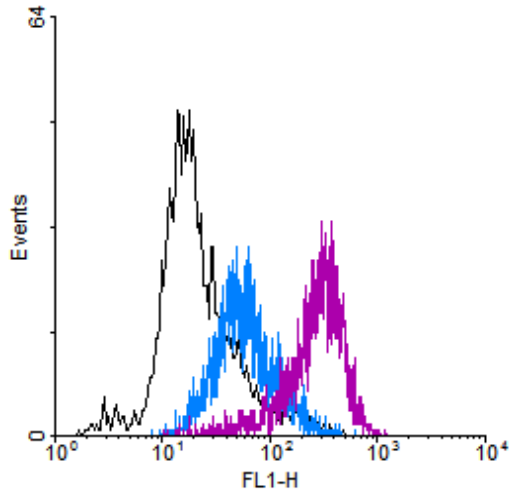


Figure 3.10B: A representative histogram showing DAF-2/DA fluorescence in control (black) and OA 5 min (blue) and OA 20 min (purple) with 30 µM treatment.

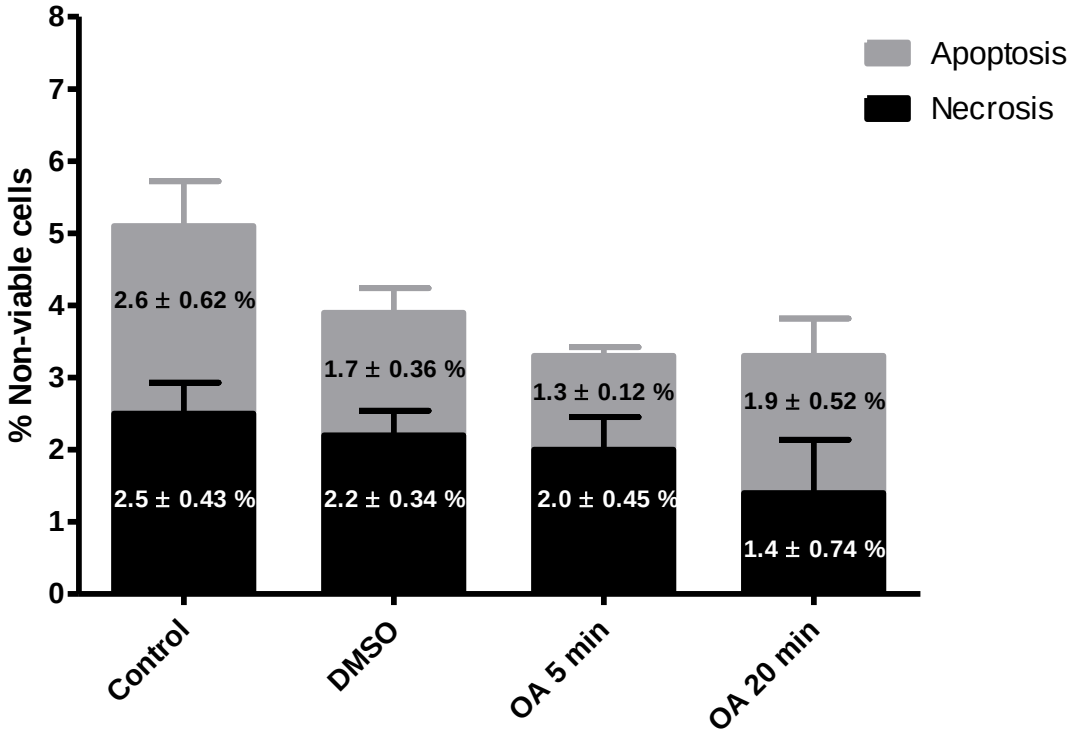


Figure 3.10C: Stacked bar-chart depicting changes in percentage of non-viable cells (apoptotic and necrotic cells) at 30 µM OA incubation for 5 and 20 min. Data expressed as mean ± SEM. $p > 0.05$ in all groups.

40 µM OA incubation for 5 and 20 min

At 40 µM OA, mean DAF-2/DA fluorescence intensity did not increase significantly after 5 min incubation (control: 100 % vs. OA: 125.2 ± 24.6 %; $p > 0.05$, $n = 9$). However, a significant 2.5-fold increase was observed in NO production after 20 min OA incubation at this dosage (control: 100 % vs. OA: 246 ± 34.4%, $p < 0.0001$, $n = 9$) [fig. 3.11A & 3.11B]. OA had no significant effect on cell viability [fig. 3.11C], and vehicle controls had no significant effect on either NO production (control: 100 % vs. DMSO: 102.8 ± 6.8 %; $p > 0.05$, $n = 17$) [fig. 3.11A] or cell viability [fig. 3.11C]

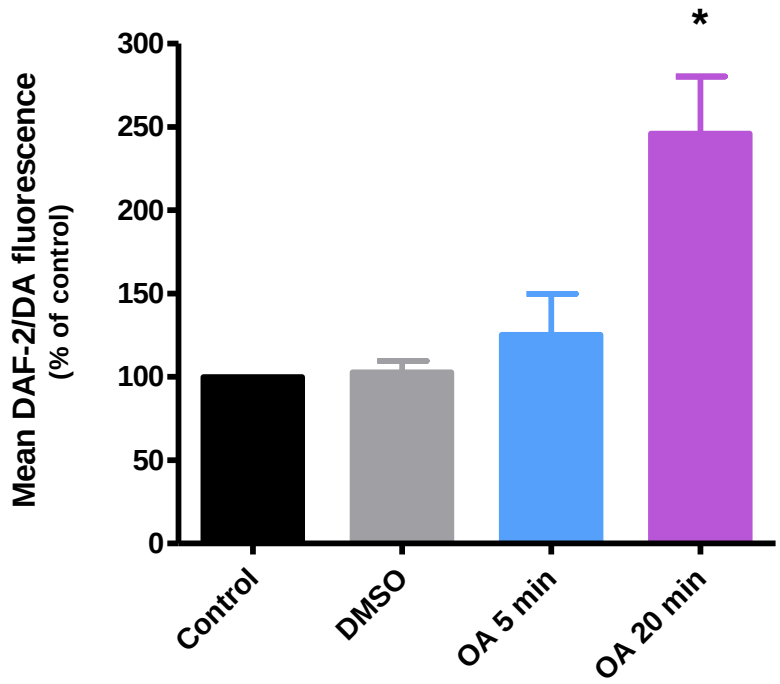


Figure 3.11A: Mean DAF-2/DA fluorescence intensity expressed as percentage of control. 40 µM OA 20 min vs. control, *: $p < 0.0001$.

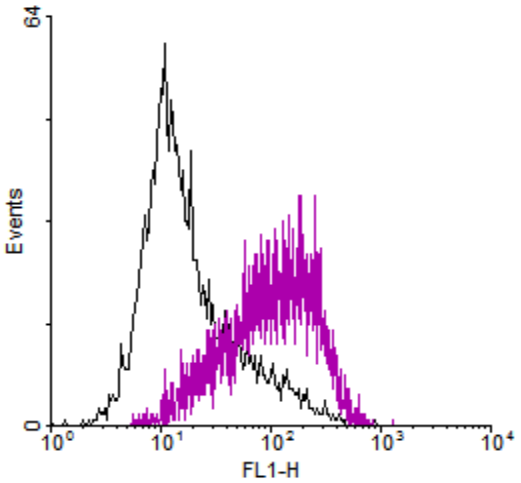


Figure 3.11B: A representative histogram showing DAF-2/DA fluorescence in control (black) and OA 20 min (purple) at 40 µM OA treatment.

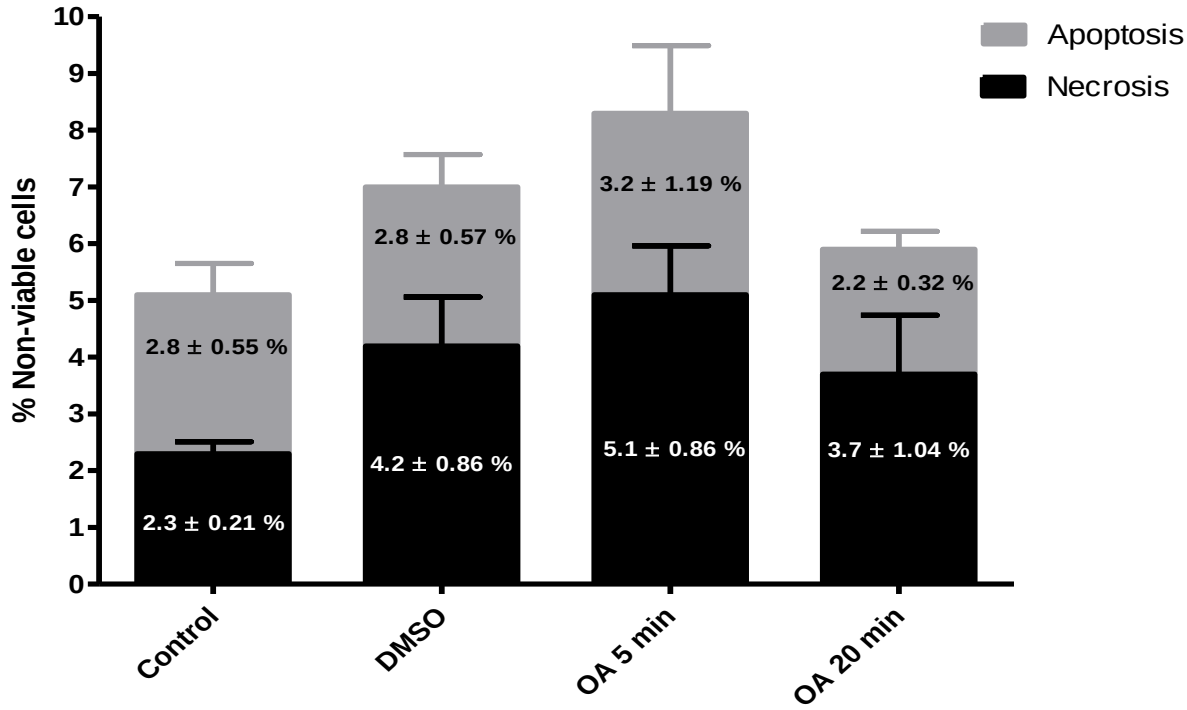


Figure 3.11C: A stacked bar-chart showing changes in percentage of non-viable (apoptotic and necrotic) cells at 40 µM incubation for 5 and 20 min. Data expressed as mean ± SEM. $p > 0.05$ in all groups.

3.4.2 The effects OA in ED

3.4.2.1 NO production and viability measurements

40 µM OA and 5 ng/ml TNF-α for 24 hours

To investigate the effects of OA in our model of ED, four groups were investigated: control, TNF-α treated group, OA group and TNF-α + OA group [fig. 2.9 under methods (chapter 2)]. TNF-α (5 ng/ml over 24 hours) induced ED by significantly decreasing mean DAF-2/DA fluorescence intensity by ~ 27 % (control: 100 % vs. TNF-α: 72.61 ± 5.3 %; $p < 0.0001$, $n = 15$) [fig. 3.12A]. No significant differences in mean DAF-2/DA fluorescence intensity were observed in the OA group when compared to control (control: 100 % vs. OA: 83.88 ± 8.1; $p > 0.05$, $n = 9$). However, pre-treatment of CMECs with OA before ED induction (TNF-α + OA group) significantly increased mean DAF-2/DA fluorescence intensity back to control levels when compared to the TNF-α only group (TNF-α: 72.61 ± 5.3 % vs. TNF-α + OA: 98.45 ± 10.9; $p < 0.0001$, $n = 9$) [fig. 3.12A & 3.12B]. Cell viability did not differ significantly in TNF-α, OA and TNF-α + OA groups when compared to control. However, pre-treatment with 40 µM OA significantly reversed the necrotic and apoptotic effects of TNF-α (**PI**: TNF-α 2.2 ± 0.4 % vs. TNF-α + OA: 0.6 ± 0.1 %; $p < 0.05$, $n = 6$ and **annexin V**: TNF-α: 6.0 ± 1.3 % vs. TNF-α + OA: 0.8 ± 0.1 %; $p < 0.05$, $n = 6$) respectively [fig. 3.12C & 3.13]. Separate investigations showed that DMSO alone (vehicle control for OA) had no effect on either DAF-2/DA fluorescence or cell viability (data not shown).

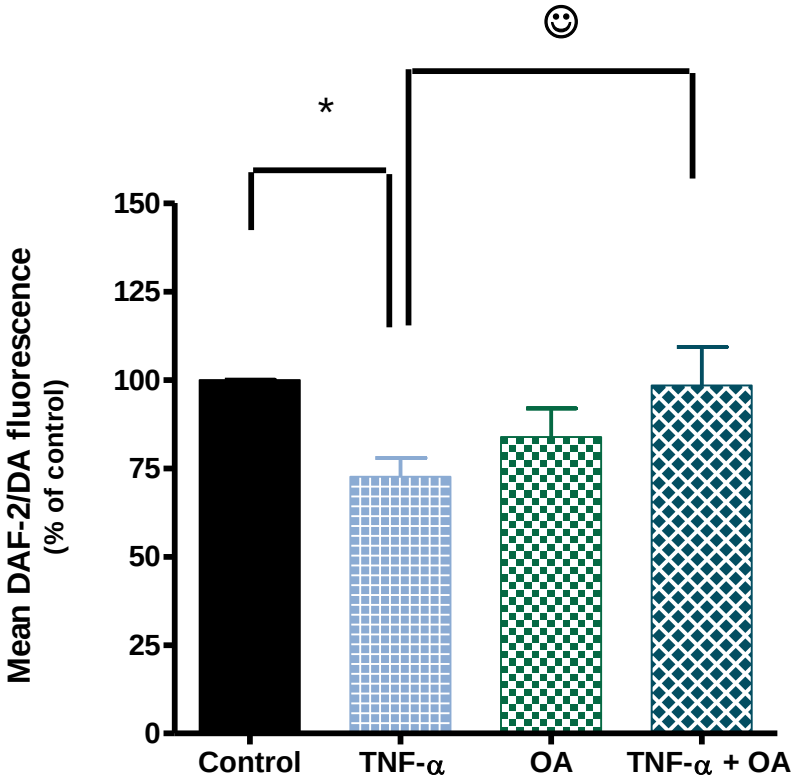


Figure 3.12A: Mean DAF-2/DA fluorescence intensity expressed as percentage of control. TNF-α vs. control, *****: $p < 0.0001$ and TNF-α + 40 μM OA vs. TNF-α, ☺: $p < 0.0001$

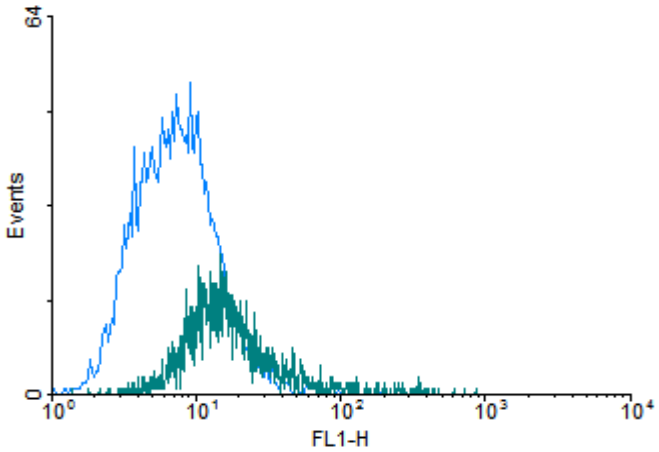


Figure 3.12B: A representative histogram showing DAF-2/DA fluorescence on the FL1-H axis in TNF-α (blue) and TNF-α + 40 μM OA (green).

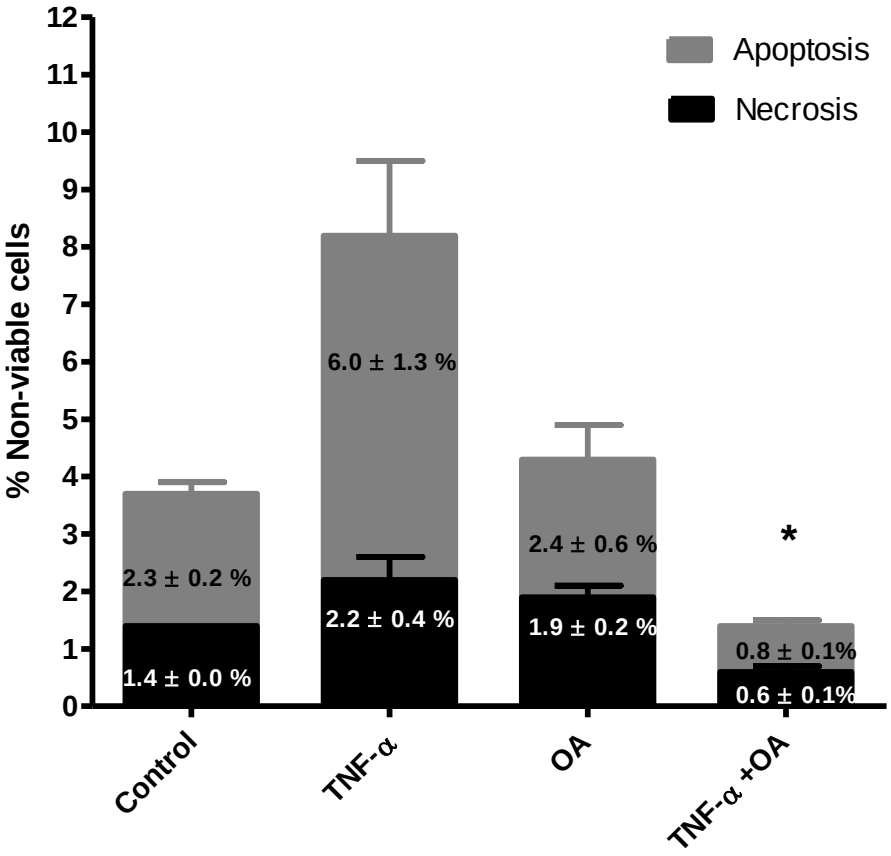


Figure 3.12C: A stacked bar chart showing the percentage of non-viable (apoptotic and necrotic) cells treated with 5 ng/ml TNF-α and / or 40 μM OA (24 hours). *****: $p < 0.05$ TNF-α vs. TNF-α + OA.

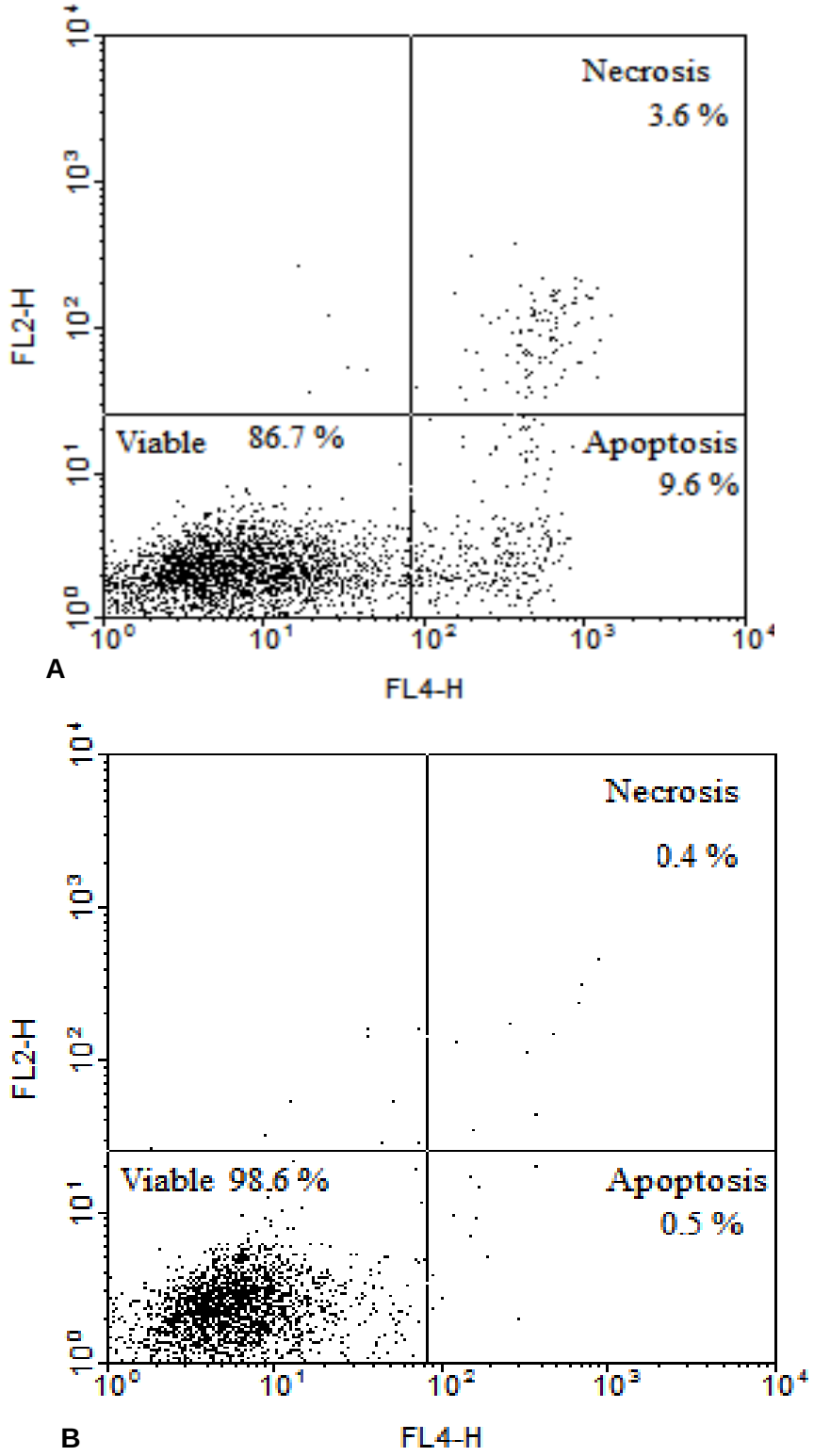


Figure 3.13: Representative dot plots showing PI and annexin V fluorescence in cells treated with 5 ng/ml TNF-α with or without 40 µM OA pre-treament (24 hours). **A**: Treatment of CMECs with TNF-α resulted in a 9.6 % incidence of apoptosis (annexin V positive cells) 3.6 % necrosis (PI positive cells). **B**: Pre-treatment with 40 µM OA significantly reversed the apoptotic and necrotic effects of TNF-α to 0.5 and 0.4 % respectively. Treatment with OA therefore increased % viable cells from 86.7 to 98.6 %.

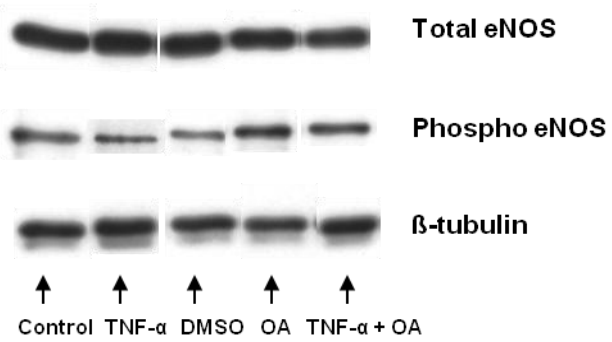
3.4.2.2 Cellular mechanisms of OA on CMECs and ED model

Total eNOS expression and phosphorylated (activated) eNOS

40 µM OA and 5 ng/ml TNF-α for 24 hours

Following our investigations into the effects of OA on NO production in normal and dysfunctional CMECs, we investigated the cellular mechanisms involved using the 40 µM dosage of OA. Our groups consisted of the non-treated control, vehicle control treated with DMSO only, ED group (5 ng/ml TNF-α over 24 hours), OA group and TNF-α + OA treated group. We found no significant differences in total eNOS expression and phosphorylated eNOS when comparing all our experimental groups. However, when phosphorylated eNOS was expressed as the ratio of total eNOS and equal protein loading was adjusted for, the following significant changes were observed [fig. 3.14]:

- eNOS phosphorylation / total ratio increased significantly in the OA treated group when compared to control (control: 1 vs. OA: 1.90 ± 0.13; $p < 0.05$, $n = 3$).
- eNOS phosphorylation / total ratio increased significantly in the TNF-α + OA treated group when compared to TNF-α (ED) group (TNF-α + OA: 1.4 ± 0.29 vs. TNF-α: 0.54 ± 0.15; $p < 0.05$, $n = 3$).



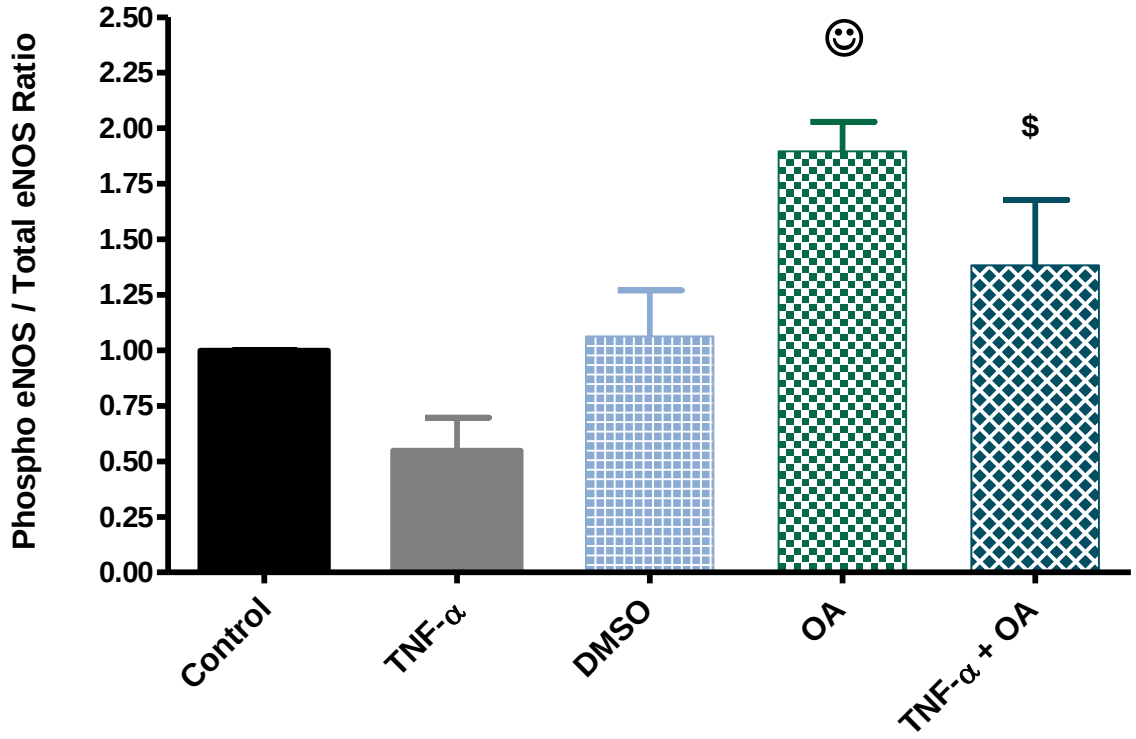


Figure 3.14: Phosphorylated eNOS expressed as a ratio of total eNOS expression. ☺: $p < 0.05$ OA vs. Control and **$**: $p < 0.05$ TNF-α + OA vs. TNF-α.

Chapter 4: Discussion

4.1 Summary of results

4.1.1 The development and establishment of an *in vitro* model of ED

In this study, we regarded reduced intracellular NO production as the primary end point to identify the development of ED in our model of CMECs. The following known inducers of ED were tested:

- Hyperglycaemia: 25 mM glucose (24 hours) resulted in an increase in NO production [fig. 3.1A].
- Inflammation: TNF-α 1 ng/ml (24 hours) increased NO production [fig. 3.2A].
  TNF-α 5 ng/ml (6 hours) increased NO production [fig. 3.3A].
  TNF-α 5 ng/ml (24 hours) decreased NO production [fig.3.4A].

Viability data showed that none of the above interventions affected cell viability. Based on the above, a model of ED was thus established (i.e. reduced intracellular NO production) when CMECs were incubated with 5 ng/ml TNF-α for 24 hours.

4.1.2 Exploration of cellular mechanisms in the TNF-α induced model of ED

1. eNOS (main source of NO in endothelial cells):

- Total eNOS expression remained at control levels [fig. 3.6A].
- Phosphorylated eNOS was reduced [fig. 3.6B].
- Total / phospho eNOS ratios were decreased [fig. 3.6C].

2. PKB / Akt (main upstream activator of eNOS by phosphorylation of residue Ser 1177):

- Total PKB / Akt expression remained at control levels [fig. 3.7A].

- Phosphorylated PKB / Akt was reduced [fig. 3.7B].

3. O_2^- production (marker of cellular oxidative stress) remained unchanged [fig. 3.5].

4. p22-phox expression (subunit of O_2^- producing NADPH oxidase) was increased [fig. 3.8].

5. Nitrotyrosine expression (marker of peroxynitrite induced protein nitration) remained at control levels [fig. 3.9].

4.1.3 Effects of OA on CMECs and ED

1. Our investigations into the effects of OA in control CMECs showed the following:

- 30 µM OA increased NO levels after 5 and 20 minutes of incubation [fig. 3.10A].
- 40 µM OA only increased after 20 minutes incubation [fig. 3.11A].
- Cell viability was not affected with either 30 µM or 40 µM treatments.

2. Investigations into the effects of OA in ED (TNF-α induced model) showed that pre-treatment with 40 µM OA reversed ED [fig. 3.12A]. Furthermore 40 µM OA reversed the pro-apoptotic and necrotic effects of TNF-α in our ED model [fig. 3.12C].

3. OA significantly increased eNOS phosphorylation in normal CMECs and pretreatment with 40 µM OA significantly restored eNOS phosphorylation back to control levels in TNF-α treated cells [fig. 3.14]

4.2 Discussion of results

4.2.1 ED model

In the first part of this study, we primarily aimed to (i) establish a model of ED in CMECs obtained from myocardial capillaries, and (ii) to investigate cellular mechanism associated with ED in CMECs. Unlike other endothelial cells, CMECs derived from myocardial capillaries possess a special attribute in that they are situated in blood vessels that do not dilate or constrict due the absence of smooth muscle layer (Strijdom & Lochner 2009). In view of this, ED in the myocardial capillaries will not result in a deficient NO-dependent vasodilatory response as observed in larger, smooth muscle containing vessels. Rather, due to their uniquely positioned setting in close proximity to cardiomyocytes, dysfunction of CMECs would potentially have implications on myocardial contractile function. Very few studies have investigated ED in endothelial cells derived from myocardial capillaries, and the present study aims to address this gap in the literature.

The association between diabetes and cardiovascular disease is indisputable (Soldatos *et al* 2005), and hyperglycaemia is a common feature of diabetes amongst other metabolic disorders (Potenza *et al* 2009). Both acute and chronic hyperglycaemia is implicated in endothelial damage; in fact, ED triggers micro- and macrovascular complications such as renal failure, blindness, and atherosclerosis associated with diabetes (Potenza *et al* 2009). Mechanisms of hyperglycaemia induced-ED include oxidative stress, increased formation AGEs and increased activity of PKC, all of which result in decreased NO bioavailability (Guzik et al 2002; Avogaro *et al* 2008). Inflammation is now recognised as both an independent risk factor and an underlying mechanism of ED (Libby *et al* 2002). Inflammation is often

associated with increased circulating levels of inflammatory cytokines such as TNF-α, which directly cause ED via enhancing oxidative stress and downregulation of eNOS expression (Zhang *et al* 2009; Valerio *et al* 2006). In order to establish a model of ED in CMECs, we therefore mimicked a hyperglycaemic and pro-inflammatory state by treatment with glucose (high concentration) and TNF-α respectively.

Our experimental model of ED was successfully achieved with treatment of CMECs with the pro-inflammatory cytokine TNF-α (5 ng/ml for 24 hours). Previous studies on endothelial cells derived from larger vessels have implicated TNF-α in the induction of ED. Possible mechanisms of TNF-α induced ED include increased O_2^- generation by NADPH or xanthine oxidase (Gao *et al* 2007; Picchi *et al* 2006) or downregulation of eNOS mRNA expression (Zhang *et al* 2009; Valerio *et al* 2006; Lai *et al* 2003). In this study, ED induction with TNF-α appeared to be dose and time dependent. Our data showed that 1 ng/ml (lower dosage) incubation for 24 hours and 5 ng/ml for 6 hours (shorter incubation period) did not induce ED. Gupta and Khandelwal (2004) showed that 1 ng/ml TNF-α over 24 hours favoured an increase in PKB / Akt phosphorylation in HepG2 cells. It is a known fact that PKB / Akt is an upstream activator of eNOS by phosphorylation of Ser 1177 residue thus leading to increased NO production. This may explain the rise in NO levels observed in our study with 1 ng/ml TNF-α treatment. De Palma *et al* (2006) demonstrated a time dependent increase in eNOS activation in human endothelial cells, peaking at 6 hours following TNF-α treatment. This may explain the rise in NO levels observed with 5 ng/ml TNF-α treatment over the shorter incubation time of 6 hours in our model.

Treatment with a high dosage of glucose also did not induce ED in our model of CMECs; in fact we observed a significant increase in NO production. These findings agree with those of Cosentino *et al* (1997), who also reported an increase in eNOS gene expression and NO production paralleled by an increase in O_2^- in human aortic endothelial cells following treatment high dosage (22.2 mM) of glucose over 5 days. We did not measure O_2^- in our glucose treated CMECs and follow-up studies could verify whether our model also exhibited oxidative stress. On the other hand, others showed a downregulation of eNOS activity in bovine aortic endothelial cells treated with 30 mM glucose for 48 hours (Du *et al* 2001). Endothelial cell heterogeneity, different dosages and incubation times may explain the discrepancies observed in these variable findings with glucose.

4.2.2 Cellular mechanisms of TNF-α induced ED in our model: eNOS and PKB / Akt expression and activation

In our attempts to explore cellular mechanisms of decreased NO production in TNF-α treated CMECs, we investigated the expression and activity of the main source of NO in endothelial cells. Activity of eNOS is dependent on factors such as dimerisation, displacement from caveolin-1 and phosphorylation at Ser 1177 (Forstermann & Munzel 2006; Dudzinski & Michel 2007). According to the literature, TNF-α can induce ED by upregulating NADPH or xanthine oxidase leading to oxidative stress and hence NO scavenging (Gao *et al* 2007; Picchi *et al* 2006), and/ or by accelerating eNOS mRNA degradation thus leading to a decrease in total eNOS protein expression (Zhang *et al* 2009; Valerio *et al* 2006; Lai *et al* 2003). Contrary to the latter findings, our data showed that total eNOS expression remained

unchanged. Phosphorylated (activated) eNOS as well as phospho eNOS / total eNOS ratios were however, significantly decreased in our ED model, which could explain the lower NO production observed.

PKB / Akt is a known upstream activator of eNOS by phosphorylation of Ser 1177, however, activity of PKB / Akt is dependent on phosphorylation of its residue Ser 473 by PI-3K (Strijdom *et al* 2009 **(a)**). Our data showed that total PKB / Akt expression remained at control levels, but phosphorylated (activated) PKB / Akt decreased significantly in our ED model. These findings suggest that reduced activation of the PKB / Akt-eNOS pathway may be the primary mechanistic pathway of ED in our model of CMECs. eNOS uncoupling and decreased eNOS expression are often the proposed mechanisms of ED in the literature. Though the activity of the PKB / Akt – eNOS pathway is a critical determinant of NO production, it is often overlooked in the context of ED. We have therefore, for the first time as far as we are aware, demonstrated reduced activation of the PKB / Akt-eNOS as the primary mechanistic pathway of ED in an *in vitro* model of ED in myocardial capillary-derived CMECs.

4.2.3 Cellular mechanisms of TNF-α induced ED in our model: Superoxide levels, p22-phox and nitrotyrosine expression (oxidative stress)

The data from most studies on ED seem to demonstrate that cellular oxidative stress is an important contributing factor (and a consequence) of ED (Gao *et al* 2007). Although the first part of the current study clearly showed reduced activation of the PKB / Akt-eNOS pathway as a principal mechanism of ED in our model, we went further to explore whether oxidative stress was also involved. To this end, we

measured three independent markers of oxidative stress, namely (i) p22-phox expression, (ii) intracellular O_2^- levels, and (iii) nitrotyrosine expression.

Overproduction of O_2^- is a common link between cardiovascular risk factors (such as diabetes mellitus, smoking, and hypertension) and ED. Furthermore, over-expression of the pro-inflammatory cytokine, TNF-α, has been associated with oxidative stress (Gao *et al* 2007; Li J-M *et al* 2002). We therefore measured O_2^- production in our ED model by FACS analysis of the O_2^- sensitive probe, DHE. We however, did not observe any significant changes in O_2^- levels with this probe in our ED model. Contrary to this finding, protein expression studies demonstrated a significantly higher p22-phox expression in our ED model, suggesting an over-expression of NADPH oxidase enzyme, the major source of O_2^- in the vascular system (Griendling *et al* 2000). Indeed, TNF-α has been reported to promote oxidative stress via over expression of NADPH oxidase (Picchi *et al* 2006; Gao *et al* 2007). Consistent with our finding, De Keulenaer *et al* (1998) reported an increase in p22-phox expression in VSMCs in response to TNF-α treatment. According to Djordjevic *et al* (2005), an increase in p22-phox expression was associated with a *delayed* increase in ROS generation in endothelial cells. This may explain the contradiction observed in O_2^- levels and p22-phox expression in our study. Despite not observing an increase in O_2^- levels as measured by DHE, the convincing increase in p22-phox expression does serve as strong indication that our ED model is, at least in part, associated with oxidative stress. The failure of the probe to demonstrate increased O_2^- levels could also be ascribed to the fact an initial burst of O_2^- might have rapidly been cancelled by the scavenging actions of molecules such as SOD and NO by the time the probe was added.

Nitrotyrosine expression is a useful indirect marker of increased peroxynitrite production (Ceriello *et al* 2001) and high levels of peroxynitrite are associated with the induction of eNOS uncoupling (Zou *et al* 2002 **(a)**; Kuzkaya *et al* 2003). Nitrotyrosine expression remained at control levels in our ED model. High levels of peroxynitrite are said to be a product of increased rates between NO and O_2^-. Though there was an increase in NADPH oxidase expression, NO production was decreased in our ED model. In view of the decreased PKB / Akt-eNOS activation pathway in our ED model, we speculate that the amounts of available NO were presumably not sufficient to result in formation of peroxynitrite levels high enough to trigger enhanced protein nitration and hence increased nitrotyrosine expression in our ED model. Furthermore, according to Pacher *et al* (2007), peroxynitrite reacts at a slow rate with biological compounds and thus our 24 hour incubation may have been too short for enhanced protein nitration to occur.

4.2.4 Effects of OA on CMECs

In the second part of the study, we aimed to investigate the effects of *Syzigium Cordatum* leaf extract-derived OA on our normal and dysfunctional CMECs. OA is a bioactive triterpenoid that has also been isolated from plants such as *Olea europaea* (olive oil) and *Radix ginseng,* known to have beneficial properties (Xi *et al* 2009; Sato *et al* 2007). Literature demonstrates OA to have anti-inflammatory, anti-hypertensive, and hypoglycaemic properties (Musabayane *et al* 2005; Mapanga *et al* 2009). Furthermore, OA has previously been reported to induce NO-dependent vasodilation in macrovascular endothelial cells (Rodriguez-Rodriguez *et al* 2008).

To our knowledge the effects of OA on NO production have not previously been investigated in myocardial capillary-derived CMECs. In a study by Rodriguez-Rodriguez *et al* (2008), OA resulted in a dose dependent increase in NO production in rat mesenteric arteries, with the highest increase in NO obtained at 30 µM OA concentration. Our investigations revealed that 30 µM of OA led to significantly increased NO levels (43 % after 5 min and 83 % after 20 min of incubation), whereas 40 µM OA increased NO production by 2.5-fold after 20 min, but had no significant effect after 5 min. In view of the considerable increase in NO production evoked by the 40 µM OA in our model of CMECs after 20 min, this dosage was used for further investigations.

4.2.5 Effects of OA on ED

Following the promising data observed with OA in normal CMECs, we undertook investigations to explore whether OA could reverse NO-lowering effects of TNF-α in our ED model. Several studies have reported OA to have anti-hypertensive, hypoglycaemic (Mapanga *et al* 2009) and hypolipidaemic properties (Liu *et al* 2007). Furthermore, OA is a component of the food substances such as olive oil that has been reported to have beneficial effects on the cardiovascular system (Rodriguez-Rodriguez *et al* 2008). For example, long-term intake of pomace olive oil supplemented with or without OA has been reported to enhance endothelial function in spontaneously hypertensive rats by enhancing eNOS expression and pomace olive oil is said to naturally have a high content of OA (Rodriguez-Rodriguez *et al* 2007). Though its putative vasorelaxant properties by enhancing either PGI_2 or NO production have been shown in several studies (Martinez-Gonzalez *et al* 2008; Rodriguez-Rodriguez *et al* 2008), the effects of OA in ED remain unclear. In this

study, we investigated the effects of OA using the 40 µM dosage, and our data showed that OA significantly reversed ED by restoring NO production back to control levels.

TNF-α has previously been associated with endothelial cell apoptosis (Deshpande *et al* 2000). Furthermore, TNF-α has also been shown to initiate ROS-dependent necrotic cell death (Morgan *et al* 2008). Albeit not significant, treatment with TNF-α induced some degree of apoptosis and necrosis in our model of CMECs. However, OA significantly reversed the pro-necrotic and pro-apoptotic effects of TNF-α thus restoring cell viability to control levels.

4.2.6 Cellular mechanisms of OA in CMECs and ED model

To determine the cellular mechanistic pathway of OA, we measured the expression and activation of the main source of NO in the endothelial cells, eNOS. When expressed as a ratio of total eNOS and following correction of equal protein loading, eNOS phosphorylation (activation) increased significantly in the OA group, when compared to the control group. Furthermore pretreatment with 40 µM OA restored eNOS activity in the TNF-α treated samples. Rodriguez-Rodriguez *et al* (2008) reported a rapid increase in eNOS phosphorylation Ser 1177 in response to OA treatment in human umblical vein endothelial cells. Phosphorylation was inhibited by PI3-K inhibitors suggesting PKB / Akt as the main key player of OA-induced eNOS phosphorylation in their study. From our observations and that of Rodriguez-Rodriguez *et al* 2008, it appears that OA stimulates the activity of the PKB / Akt-eNOS pathway thus leading to an increase in NO production. Others have also

reported OA to increase the expression of eNOS (Rodriguez-Rodriguez *et al* 2007). We however, found no changes in expression of eNOS.

Chapter 5: Conclusion

5.1 Conclusions

To date, there is very little information on ED in myocardial capillary derived endothelial cells / CMECs. The importance of the endothelium is now well appreciated; however, its structural and functional heterogeneity is often overlooked especially in the context of ED. For the first time, as far as we are aware, we have established an experimental model of ED in this functionally unique endothelial cell type. We have also demonstrated a novel, though surprisingly obvious, cellular mechanistic pathway of ED (reduced PKB / Akt-eNOS activation). The marked increase in p22-phox expression suggests that a degree of eNOS uncoupling might have been present, which could serve as an additional mechanism of ED in our TNF-α induced ED model. However, in view of the unchanged nitrotyrosine expression in our model and inability to demonstrate increased O_2^- levels, we propose that the decrease in the activity of PKB / Akt-eNOS pathway (thus leading to reduced NO production) was the primary mechanistic pathway of ED in our model (Fig. 5.1). For a summary of our findings, refer to table 5.1

Consistent with previous findings on macrovascular endothelial cells, OA appears to have NO production stimulatory effects in our model of CMECs. We also demonstrate the ability of OA to reverse ED by restoring NO production. OA appears to confer protective effects on our model of CMECs, as demonstrated by the ability of OA to reverse the pro-apoptotic and pro-necrotic effects of TNF-α. From our observations, the NO production stimulatory effects of OA are most likely mediated by the activation of the PKB / Akt-eNOS pathway. Our data therefore adds further

validation to the notion that OA represents a novel therapeutic tool in enhancing endothelial function and hence cardiovascular health.

5.2 Shortcomings of the study

Superoxide levels in ED cells: Our DHE fluorescence data were inconclusive and did not support the p22-phox results. We believe this could probably be due to technical considerations related to probe and time of incubation. We plan to expand our superoxide measurement studies with DHE (using at different time points) and to incorporate other possible superoxide measurement methods.

PKB / Akt, p22-phox and nitrotyrosine in OA treated samples: Due to time constraints and technical glitches, we were unable to complete Western blot analyses of these proteins in the OA experiments. Results from these studies would have given valuable insight into the mechanisms of OA, follow-up investigations to adress this will be undertaken shortly.

5.3 Future direction

Investigations on CMECs-to-cardiomyocyte interaction in the setting of ED and thus establishment of an in *vitro* coculture model of dysfunctional CMEC-cardiomyocyte are necessary to provide more insight on relevance of ED in myocardial derived CMECs.

Parameter	Decreased	Increased	No change
NO production	√		
eNOS activation	√		
PKB / Akt activation	√		
Superoxide production			√
P22-phox expression		√	
Nitrotyrosine expression			√

Table 5.1: A summary of our findings during establishment of an experimental model of ED (5 ng/ml TNF-α for 24 hours).

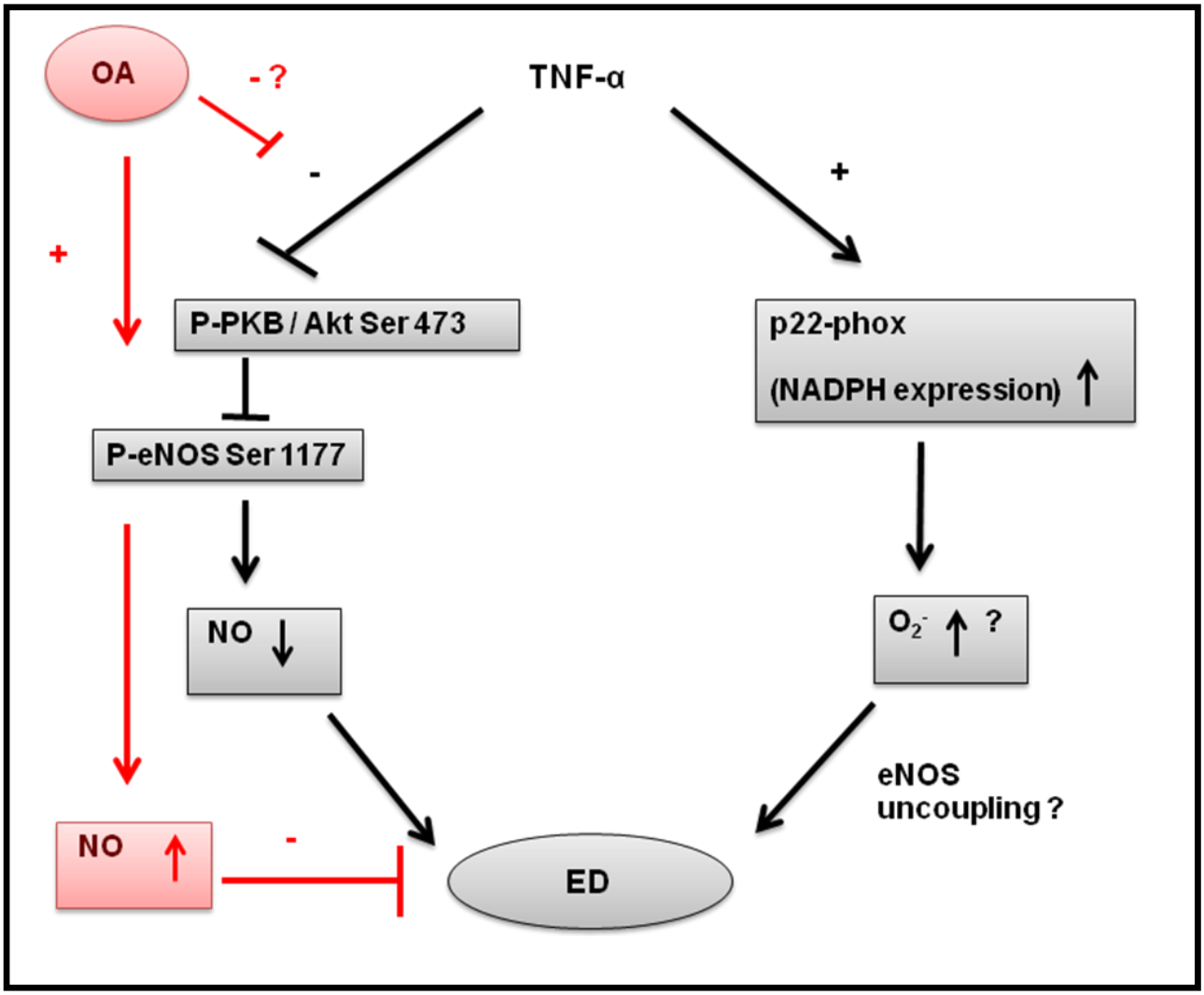


Figure 5.1: Proposed mechanisms of ED and OA in our model of CMECs.

References

- Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function and mechanisms. Circ Res 2007; 100:158-173.

- Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001;357: 593-615.

- Ando H, Kubin T, Schaper W, Schaper J. Cardiac microvascular endothelial cells express α-smooth muscle actin and show low NOS III activity. Am J Physiol 1999;276: H1755-H1768.

- Andries LJ, Kaluza G, De Keulenaer GW, Mebazaa A, Brutsaert DL, Sys SU. Endocardial endothelial dysfunction and heart failure. J Cardiac Failure 1996;2:S195-S202.

- Antoniades C, Tousoulis D, Stefanadis C. Smoking in Asians: it doesn't stop at vascular endothelium. Int J Cardiol 2008;128:151-153.

- Avogaro A, De Kreutzenberg S, Fadini G. Endothelial dysfunction: causes and consequences in patients with diabetes mellitus. Diabetes Res Clin Pract 2008;82:s94-s101.

- Balligand J-L, Cannon PJ. Nitric oxide synthases and cardiac muscle: autocrine and paracrine influences. Arterioscler Thromb Vasc Biol 1997;17:1846-1858.

- Barnoya J, Glantz SA. Cardiovascular effects of secondhand smoke: nearly as large as smoking. Circulation 2005;111:2684-2698.

- Bauer PM, Fulton D, Boo YC, Sorescu GP, Kemp BE, Jo H, Sessa WC. Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine

phosphorylation sites in endothelial nitric-oxide synthase. J Biol Chem 2003;278:14841-14849

- Blake GJ, Ridker PM. Novel clinical markers of vascular wall inflammation. Circ Res 2001;89:763-771.

- Böger RH, Bode- Böger S, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, Cooke JP. Asymmetric dimethlyarginine (ADMA): a novel risk factor for endothelial dysfunction, its role in hypercholesterolemia. Circulation 1998;98:1842-1847.

- Böger RH, Vallance P, Cooke JP. Asymmetric dimethlyarginine (ADMA): a key regulator of nitric oxide synthase. Atherosclerosis Suppl 2003;4:1-3.

- Bolli R. Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. J Mol Cell Cardiol 2001;33:1897-1978.

- Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 2003;23:168-175.

- Boos CJ, Lip GYH, Blann AD. Circulating endothelial cells in cardiovascular disease. J Am Coll Cardiol 2006;48:1538-1547.

- Boulanger CM, Tanner FC, Bea ML, Hahn AW, Werner S, Lüscher TF. Circ Res 1992;70:1191-1197.

- Brandes RP, Kreuzer J. Vascular NADPH oxidases: molecular mechanisms of activation. Cardiovasc Res 2005;65:16-27.

- Bredt DS. Nitric oxide signaling specificity – the heart of the problem. J cell Sci 2003;116:9-15.

- Bruckdorfer R. The basics about nitric oxide. Mol Aspect Med 2005;26:3-31.

- Brutsaert DL, Fransen P, Andries LJ, De Keulenaer GW, Sys SU. Cardiac endothelium and myocardial function. Cardiovasc Res 1998;38:281-290.

- Brutsaert DL. Cardiac endothelial-myocardial signaling: Its role in cardiac growth, contractile performance, and rhythmicity. Physiol Rev 2003;83:59-115.

- Bryan NS, Bian K, Murad F. Discovery of the nitric oxide signaling pathway and targets for drug development. Front Biosci 2009;14:1-18.

- Burke A, FitzGerald GA. Oxidative stress and smoking-induced vascular injury. Progr Cardiovasc Dis 2003;46:79-90.

- Ceriello A, Mercuri F, Quagliaro L, Assaloni R, Motz, Tonutti L, Taboga C. Detection of nitrityrosine in the diabetic plasma: evidence of oxidative stress. Diabetologia 2001;44:834-838.

- Chen Y, Traverse JH, Du R, Hou M, Bache RJ. Nitric oxide modulates myocardial oxygen consumption in the failing heart. Circulation 2002;106:273-279.

- Chhabra N. Endothelial dysfunction- A predictor of atherosclerosis. Internet J Med Update 2009; 4(1): 33-41.

- Choi D, Hwang K-C, Lee K-Y, Kim Y-H. Ischemic heart disease: current treatments and future. J Contr Release 2009;140:194-202.

- Cosentino F, Hishikawa K, Katusic ZS, Luscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 1997;96:25-28.

- Davidson SM, Duchen MR. Endothelial mitochondria: Contributing to vascular function and disease. Circ Res 2007;100:1128-1141.

- Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation 2004;109:27-32.

- De Keulenaer GW, Alexander RW, Ushio-Fukai M, Ishizaka N, Griendling KK. Tumour necrosis factor α activates a p22phox-based NADH oxidase in vascular smooth muscle. Biochem J 1998;329:653-657.

- De Palma C, Meacci E, Perrotta C, Bruni P, Clementi E. Endothelial nitric oxide synthase activation by tumor necrosis factor α through neutral sphingomyelinase 2, sphingosine kinase 1 and sphingosine 1 phosphate receptors. A novel pathway relevant to the pathophysiology of endothelium. Arterioscler Thromb Vasc Biol 2006;26:99-105.

- Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: Testing and clinical relevance. Circulation 2007;115:1285-1295.

- Deshpande SS, Angkeow P, Huang J, Ozaki M, Irani K. Rac 1 inhibits TNF-α-induced endothelial cell apoptosis: dual regulation by reactive oxygen species. FASEB J 2000;14:1705-1714.

- Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601-605.

- Djordjevic T, Pogrebniak A, Belaiba RS, Bonello S, Wotslaw C, Acker H, Hess J, Gorlach A. The expression of the NADPH oxidase subunit p22phox is regulated by a redox sensitive pathway in endothelial cells. Free Radic Biol Med 2005;38:616-630.

- Dostal DE, Baker KM. The cardiac rennin-angiotensin system: conceptual, or a regulator of cardiac function? Circ Res 1999;85:643-650.

- Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. Circ Res 2000;86:347-354.

- Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. J Clin Invest 2001;108:1341-1348.

- Dudzinski D, Michel T. Life history of eNOS: partners and pathways. Cardiovasc Res 2007;75(2):247-260.

- Erdbruegger U, Haubitz M, Woywodt A. Circulating endothelial cells: a novel marker of endothelial damage. Clin Chim Acta 2006;373:17-26.

- Esper RJ, Nordaby RA, Vilarino JO, Paragano A, Cacharon JL, Machado RA. Endothelial dysfunction: a comprehensive appraisal. Cardiovasc Diabetol 2006; 5:4.

- Fain JN, Bahouth SW, Madan AK. TNF-α release by the nonfat cells of human adipose tissue. Int J Obes 2004;28:616-622.

- Fetalvero KM, Martin KA, Hwa J. Cardioprotective prostacyclin signaling in vascular smooth muscle. Prostag Other Lipid Mediat 2007;82:109-118.

- Flogel U, Decking UK, Godecke A, Schrader J. Contribution of NO to ischemia-reperfusion injury in the saline-perfused heart: a study in endothelial NO synthase knockout mice. J Mol Cell Cardiol 1999;31:827-836.

- Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 2006;113:1708-1714.

- Gao X, Belmadani S, Picchi A, Xu X, Potter BJ, Tewari-Singh N, Capobianco S, Chilian WM, Zhang C. Tumor necrosis factor-α induces endothelial dysfunction in Lepr^{db} mice. Circulation 2007;115:245-254.

- Gewaltig MT, Kojda G. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. Cardiovasc Res 2002;55:250-260.

- Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. Am J Physiol Renal Physiol 2001;280:F193-F206.

- Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase, role in cardiovascular biology and disease. Circ Res 2000;86:494-501.

- Gupta D, Khandelwal RL. Modulation of insulin effects on phosphorylation of protein kinase B and glycogen synthesis by tumor necrosis factor-alpha in HepG2 cells. Biochim Biophys Acta 2004;1671:51-58.

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

- Hamburg NM, Vita JA. Endothelial dysfunction in atherosclerosis: mechanisms of impaired nitric oxide bioactivity. In: Loscalzo J (editor). Molecular mechanisms of atherosclerosis. Taylor and Francis 2005:95-110.

- Hansson GK. Atherosclerosis- an autoimmune disease: The anitschkov lecture 2007. Atherosclerosis 2009;202:2-10.

- Herrera MD, Mingorance C, Rodríguez- Rodríguez R, De Sotomayor MA. Endothelial dysfunction and aging: an update. Ageing Res Rev 2010;9:142-152.

- Hsieh PCH, Davis ME, Lisowski LK, Lee RT. Endothelial-cardiomyocyte interactions in cardiac development and repair. Annu Rev Physiol 2006;68:51-66.

- Hsu J-H, Wu Y-C, Liu I-M, Cheng J-T. Release of acetycholine to raise insulin secretion in Wistar rats by oleanolic acid, one of the active principles contained in *Cornus officinalis*. Neurosci Lett 2006;404:112-116.

- Hsueh WA, Lyon CJ, Quinones MJ. Insulin resistance and the endothelium. Am J Med 2004;117:109-117.

- Huang PL. Endothelial nitric oxide synthase and endothelial dysfunction. Curr Hypertens Rep 2003;5:473-480.

- Hurst JK. Whence nitrotyrosine? J Clin Invest 2002;109:1287-1289.

- Iaccarino G, Ciccarelli M, Sorriento D, Cipolletta E, Cerullo V, Iovino GL, Paudice A, Elia A, Santulli G, Campanile A, Arcucci O, Pastore L, Salvatore F, Condorelli G, Trimarco B. AKT participates in endothelial dysfunction in hypertension. Circulation 2004;109:2587-2593.

- Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol 2006;40:16-23.

- Jones SP, Greer JJ, Kakkar AK, Ware PD, Turnage RH, Hicks M, Van Haperen R, De Crom R, Kawashima S, Yokoyama M, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury. Am J Physiol Heart Circ Physiol 2004;286:H276-H282.

- Katusic ZS. Vascular endothelial dysfunction: does tetrahydrobiopterin play a role? Am J Physiol Heart Circ Physiol 2001;281:981-986.

- Katusic ZS. Mechanisms of endothelial dysfunction induced by aging, role of aeginase I. Circ Res 2007;101:640-641.

- Kuruvilla L, Kartha CC. Molecular mechanisms in endothelial regulation of cardiac function. Mol Cell Biochem 2003;253:113-123.

- Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interaction of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols. J Biol Chem 2003;278:22546-22554.

- Lai PFH, Mohamed F, Monge J-C, Stewart DJ. Downregulation of eNOS mRNA expression by TNF α: Identification and functional characterization of RNA-protein interactions in the 3'UTR. Cardiovasc Res 2003;59:160-168.

- Landim MBP, Filho AC, Chagas ACP. Asymmetric dimethylarginine (ADMA) and endothelial dysfunction: implications for atherogenesis. Clinics 2009;64:471-8.

- Landmesser U, Harrison DG, Drexler H. Oxidant stress- a major cause of reduced endothelial nitric oxide availability in cardiovascular disease. Eur J Clin Pharmacol 2006;63:13-19.

- Li C, Ruan L, Sood SG, Papapetropoulos A, Fulton D, Venema RC. Role of eNOS phosphorylation at Ser 116 in regulation of eNOS activity in endothelial cells. Vasc Pharmacol 2007;47:257-264.

- Li H, Wallerath T, Munzel T, Fostermann U. Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. Nitric Oxide Biol Chem 2002;7:149-164.

- Li J, Guo W-J, Yang Q-Y. Effects of ursolic acid and oleanolic on human colon cell line HCT15. World J Gastroenterol 2002;8:493-495.

- Li J-M, Mullen AM, Yun S, Wientjes F, Brouns GY, Thrasher AJ, Shah AM. Essential role of NADPH oxidase subunit $p47^{phox}$ in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-α. Circ Res 2002;90:143-150.

- Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;150:1135-1143.

- Limaye V, Vadas M. The vascular endothelium: structure and function. In Fitridge R and Thompson M, editors. Mechanisms of Vascular Disease: A Textbook for vascular Surgeons. Cambridge University Press 2006:1-13.

- Lin Y, Vermeer MA, Trautwein EA. Triterpenic acids present in hawthorn lower plasma cholesterol by inhibiting intestinal ACAT activity in hamsters. eCAM 2009;doi:10.1093/ecam/nep007.

- Lind L. Circulating markers of inflammation and atherosclerosis. Atherosclerosis 2003;169:203-214.

- Liu J, Sun H, Wang X, Mu D, Liao H, Zhang L. Effects of oleanolic acid and maslinic acid on hyperlipidemia. Drug Dev Res 2007; 68:261-266.

- Luksha L, Agewall S, Kublickiene K. Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. Atherosclerosis 2009;202:330-344.

- Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 2005;25:29-38.

- Mapanga RF, Tufts MA, Shode FO, Musabayane CT. Renal effects of plant-derived oleanolic acid in streptozotocin-induced diabetic rats. Ren Fail 2009;31:481-491.

- Martinez-Gonzalez J, Rodriguez-Rodriguez R, Gonzalez-Diez M, Rodriguez C, Herrera MD, Ruiz-Gutierrez V, Badiman L. Oleanolic acid induces prostacyclin release in human vascular smooth muscle cells through a cyclooxygenase-2-dependent mechanism. J Nutr 2008;138:443-448.

- Mas M. A closer look at the endothelium: its role in the regulation of vasomotor tone. Eur Urol Suppl 2009; 8:48-57.

- McCabe TJ, Fulton D, Roman LJ, Sessa WC. Enhanced electron flux and reduced calmodulin dissociation may explain “calcium-independent” eNOS activation by phosphorylation. J Biol Chem 2000;275:6123-6128.

- Meldrum DR. Tumor necrosis factor in the heart. Am J Physiol Regulatory Intergrative Comp Physiol 1998;274:577-595.

- Michel T, Vanhoutte PM. Cellular signaling and NO production. Pflugers Arch-Eur J Physiol 2010;459:807-816.

- Michell BJ, Harris MB, Chen Z-P, Ju H, Venema VJ, Blackstone MA, Huang W, Venema RC, Kemp BE. Identification of regulatory sites of phosphorylation of the bovine endothelial nitric oxide synthase at Serine 617 and Serine 635. J Biol Chem 2002;277:42844-42851.

- Moncada S, Palmer RM, Higgs EA. The discovery of nitric oxide as the endogenous nitrovasodilator. Hypertension 1988;12:365-372.

- Morgan MJ, Kim Y-S, Liu Z-G. TNF-α and reactive oxygen species in cell death. Cell Res 2008;18:343-349.

- Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J Mol Cell Cardiol 2007;42:271-279.

- Musabayane CT, Mahlalela N, Shode FO, Ojewole JAO. Effects of *syzigium cordatum* (Hochst.) [Myrtaceace] leaf extract on plasma glucose and hepatic glycogen in streptozotocin-induced diabetic rats. J Ethnopharmacol. 2005;97:480-495.

- Navarro-Antolin J, Lopez-Munoz MJ, Klatt P, Soria J, Michel T, Lamas S. Formation of peroxynitrite in vascular endothelial cells exposed to cyclosporine A. FASEB J 2001;15:1291-1293.

- Nishida M, Carley WW, Gerritsen M, Ellingsen O, Kelly RA, Smith TW. Isolation and characterization of human and rat cardiac microvascular endothelial cells. Am J Physiol Heart Circ Physiol 1993;264: 639-652.

- Osto E, Cosentino F. The role of oxidative stress in endothelial dysfunction and vascular inflammation. In: Ignarro LJ (editor). Nitric oxide: biology and pathobiology 2nd edition. Academic Press 2010:705-754.

- Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315-424.

- Paniagua OA, Bryant MB, Panza JA. Role of endothelial nitric oxide in shear stress-induced vasodilation of human microvasculature: diminished activity in hypertensive and hypercholesterolemic patients. Circulation 2001;103:1752-1758.

- Pennathur S, Heinecke JW. Oxidative stress and endothelial dysfunction in vascular disease. Curr Diabetes Rep 2007;7:257-264.

- Picchi A, Gao X, Belmadani S, Potter BJ, Focardi M, Chilian WM, Zhang C. Tumor necrosis factor α induces endothelial dysfunction in the prediabetic metabolic syndrome. Circ Res 2006;99:96-77.

- Piper HM, Spahr R, Mertens S, Krutzfeldt A, Watanabe H. Microvascular endothelial cells from heart. In: Piper HM (editor). Cell culture techniques in heart vessel and research. Springer-Verlag 1990:158-173.

- Potenza MA, Gagliardi S, Nacci C, Carratu MR, Montagnani M. Endothelial dysfunction in diabetes: From mechanisms to therapeutic targets. Curr Med Chem 2009; 16:94-112.

- Puranik R, Celermajer DS. Smoking and endothelial function. Progr Cardiovasc Dis 2003;45:443-458.

- Quintero M, Colombo SL, Godfrey A, Moncada S. Mitochondria as signalling organelles in the vascular endothelium. PNAS 2006;103(14):5379-5384.

- Rastaldo R, Pagliaro P, Capello S, Penna C, Mancardi D, Westerhof N, Losano G. Nitric oxide and cardiac fuction. Life Sciences 2007;81:779-793.

- Rodeberg DA, Chaet MS, Bass RC, Arkovitz MS, Garcia VF. Nitric oxide: An overiew. Am J Surg 1995;170:292-303.

- Rodriguez-Rodriguez R, Herrera MD, De Sotomayor MA, Ruiz-Gutierrez V. Pomace olive oil improves endothelial dysfunction in spontaneously hypertensive rats by increasing endothelial nitric oxide synthase expression. Am J Hypertens 2007;20:728-734.

- Rodriguez-Rodriguez R, Stankevicius E, Herrera MD, Ostergaard L, Andersen MR, Ruiz-Gutierrez V, Simonsen U. Oleanolic acid induces relaxation and calcium-independent release of endothelium-derived nitric oxide. Br J Pharmacol 2008;155:535-546.

- Ruberg FL, Loscalzo J. Inflammation and atherothrombosis. In: Loscalzo J (editor). Molecular mechanisms of atherosclerosis. Taylor and Francis 2005:45-60.

- Samova LI, Shode FO, Nadar A, Ramnanan P. Antihypertensive, antiatherosclerotic snd antioxidant activity of triterpernoids isolated from *Olea europaea*, subspecies Africana leaves. J Ethnopharmacol 2003;84:299-305.

- Sato H, Genet C, Strehle A, Thomas C, Lobstein A, Wagner A, Mioskowski C, Auwerx J, Saladin R. Anti-hyperglycemic activity of TGR5 agonist isolated from *Olea europaea*. Biochem Biophys Res Comm 2007;362:793-798.

- Sawamura T. LOX-1, a lectin-like oxidized LDL receptor identified form endothelial cells, in endothelial dysfunction. Int Congr 2004;1262:531-534.

- Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BMW. Renin-angiotensin system and cardiovascular risk. Lancet 2007;369:1208-19.

- Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischemia/reperfusion injury. Cardiovasc Res 2004;61:402-413.

- Shah AM, MacCarthy PA. Paracrine and autocrine effects of nitric oxide on myocardial function. Pharmacol Ther 2000;86:49-86.

- Soldatos G, Cooper ME, Jandeleit-Dahm KAM. Advanced-glycation end products in insulin-resistant states. Curr Hypertens Rep 2005;7:96-102.

- Souza JM, Peluffo G, Radi R. Protein tyrosine nitration-functional alteration or just a biomarker? Free Radic Biol Med 2008;45:357-366.

- Stenvinkel P. Endothelial dysfunction and inflammation-is there a link? Nephrol Dial Transplant 2001;16:1968-1971.

- Steuhr D, Pou S, Rosen GM. Oxygen reduction by nitric oxide synthases. J Biol Chem 2001;276:14533-14536.

- Strijdom H, Muller C, Lochner A. Direct intracellular nitric oxide detection in isolated adult cardiomyocytes: flow cytometric analysis using the fluorescent probe, diaminofluorescein. J Mol Cell Cardiol 2004;37:897-902.

- Strijdom H, Jacobs S, Suzel H, Page C, Lochner A. Nitric oxide production is higher in rat cardiac microvessel endothelial cells than ventricular cardiomyocytes in baseline and hypoxic conditions: a comparative study. FASEB J 2006;20:14-316.

- Strijdom H, Chamane N, Lochner A. Nitric oxide in the cardiovascular system: a simple molecule with complex actions. Cardiovasc J Afr 2009;20:303-310 **(a)**.

- Strijdom H, Friedrich SO, Hattingh S, Chamane N, Lochner A. Hypoxia-induced regulation of nitric oxide synthase in cardiac endothelial cells and myocytes and the role of the PI3-K/PKB pathway. Mol Cell Biochem 2009;321:23-55 **(b)**.

- Strijdom H, Lochner A. Cardiac endothelium: More than just a barrier! SA Heart 2009; 6(3):174-185.

- Sumpio BE, Riley JT, Dardik A. Cells in focus: endothelial cell. Int J Biochem Cell Biol 2002; 34:1508-1512.

- Sydow K, Munzel T. ADMA and oxidative stress. Atherosclerosis Suppl 2003;4:41-51.

- Szmitko PE, Wang C-H, Weisel RD, De Almeida JR, Anderson TJ, Verma S. New markers of inflammation and endothelial cell activation part 1. Circulation 2003;108:1917-1923.

- Takahashi S, Mendelsohn ME. Synergistic activation of endothelial nitric oxide synthase (eNOS) by HSP90 and Akt. J Biol Chem 2003;278:30821-30827 **(a)**.

- Takahashi S, Mendelsohn ME. Calmodulin-dependent and –independent activation of endothelial nitric-oxide synthase by heay shock protein 90. J Biol Chem 2003;278:9339-9344 **(b)**.

- Tang EHC, Vanhoutte PM. Endothelial dysfunction: a strategic target in the treatment of hypertension? Pflugers Arch-Eur J Physiol 2010;DOI 10.1007/s00424-010-0786-4.

- Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 2004;95:230-2.

- Valerio A, Cardile A, Cozzi V, Bracale R, Tedesco L, Pisconti A, Palomba L, Cantoni O, Clementi E, Moncada S, Carruba MO, Nisoli E. TNF-α downregulates eNOS expression and mitochondrial biogenesis in fat and muscle of obese rodents. J Clin Invest 2006;116:2791-2798.

- Vanhoutte PM. Ageing and endothelial dysfunction. Eur Heart J Suppl 2002;4:A8-A17.

- Versari D, Daghini E, Virdis A, Ghiadoni L, Taddei S. Endothelium-dependent contractions and endothelial dysfunction in human hypertension. Br J Pharmacol 2009;157:527-536.

- Vinals F, Pouyssegur J. Confluence of vascular endothelial cells induces cell cycle exit by inhibiting p42/p44 mitogen-activated protein kinase activity. Mol Cell Biol 1999;19:2763-2772.

- Warnholtz A, Mollnau H, Oelze M, Wendt M, Münzel T. Antioxidants and endothelial dysfunction in hyperlipidemia. Curr Hypertens Rep 2001;3:53-60.

- Weibel ER, Palade GE. New cytoplasmic components in arterial endothelia. J Cell Biol 1964;23:101-112.

- Wilkins RC, Kutzner BC, Truong M, Sanchez-Dardon J, McLean JRN. Analysis of radiation induced apoptosis in human lymphocytes: Flow cytometry using annexin V and propidium iodide versus neutral comet assay. Cytometry 2002;48:14-19.

- Xi J, Chang Q, Chan CK, Meng ZY, Wang GN, Sun JB, Wang YT, Tong HHY, Zheng Y. Formulation development and bioavailability evaluation of a self-nanoemulsified drug delivery system of oleanolic acid. AAPS Pharm Sci Tech 2009;10:172-182.

- Xu Y, Buikema H, van Gislt WH, Henning RB. Caveolae and endothelial dysfunction: filling the caves in cardiovascular disease. Eur J Pharmacol 2008;585:256-260.

- Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol 2005;100:57-63.

- Yang Z, Ming X-Z. Recent advances in understanding endothelial dysfunction in atherosclerosis. Clin Med Res 2006;4:53-65.

- Yetik-Anacak G, Catravas JD. Nitric oxide and the endothelium: history and impact on cardiovascular disease. Vasc Pharmacol 2006;45:268-276.

- Yokoyama M. Oxidant stress and atherosclerosis. Curr Opin Pharmacol 2004;4:110-115.

- Zhang C, Xu X, Potter BJ, Wang W, Kuo L, Michael L, Bagby GJ, Chilian WM. TNF-α contributes to endothelial dysfunction in ischemia/reperfusion injury. Arterioscler Thromb Vasc Biol 2006;26:475-480.

- Zhang H, Park Y, Wu J, Chen XP, Lee S, Yang J, Dellsperger KC, Zhang C. Role of TNF-α in vascular dysfunction. Clin Sci 2009;116:219-230.

- Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic precondition. Am J Physiol Heart Circ Physiol 2003;285:H579-H588.

- Ziolo MT, Kohr MJ, Wang H. Nitric oxide signaling and the regulation of myocardial function. J Mol Cell Cardiol 2008;45:625-632.

- Zou M-H, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. J Clin Invest 2002;109:817-826 **(a)**.

- Zou M-H, Hou X-Y, Shi C-M, Nagata D, Walsh K, Cohen RA. Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser^{1179} phosphorylation of endothelial nitric oxide synthase. J Biol Chem 2002;277:32552-32557 **(b)**.