

The effect of hypoxia on nitric oxide and endothelial nitric oxide synthase in the whole heart and isolated cardiac cells: the role of the PI3-K / PKB pathway as a possible mediator.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part, submitted it at any university for a degree.

Signature

Name in full

_____/_____/_____

Date

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Dedication

This thesis is dedicated to the late Ms Florah Zanele Chamane, the superwoman behind this woman. If it were not for you, I would not have gotten this far. Ngiyabonga mawami!

Acknowledgements

- My Alpha and my Omega, God Almighty
- My supervisor Hans Strijdom, for exceptional support and guidance.
Thank you for this opportunity and for believing in me!
- My family. Without your love, support and trust I wouldn't have been able to fly so high. Thank you!
- My friends for keeping me sane 😊
- Members of ASF and the Anglican Communion at large for your love, prayers, support and motivation. Blessings in abundance be upon you!

Abstract

In the heart, endothelial nitric oxide synthase (eNOS) is regarded as the most important constitutively expressed enzymatic source of nitric oxide (NO), a major cardiac signalling molecule. On the whole, NO is regarded as a cardioprotective molecule. The role of eNOS during ischaemia / hypoxia is controversial; however, it is generally accepted that ischaemia / hypoxia results in increased cardiac NO production. Most studies focus either on the whole heart or isolated cell models. As yet, no study has compared findings with regard to NO metabolism in these two distinct models, in a single study. We hypothesise that observations in a whole heart model with regard to increased NO production and eNOS involvement in ischaemia are the result of events on cellular level and that the increase in NO production observed during hypoxia in cardiomyocytes and endothelial cells is at least in part due to the increase in expression and / or activation of eNOS. Furthermore, we hypothesize that these effects are mediated via the PI3-K / PKB pathway. We aimed to measure and compare NO-production and eNOS expression and activation in the whole heart and isolated cardiac cells and measure PKB expression and activation in the cells under normoxic and ischaemic / hypoxic conditions. We also aimed to determine the effects of PI3-K / PKB pathway inhibition on NO production and eNOS expression and activation in isolated cardiac cells under normoxic and hypoxic conditions. Adult rat hearts were

perfused and global ischaemia induced for 15 and 20 min. Tissue homogenates of perfused hearts were used for the measurement of nitrites and determination of expression and activation of eNOS. Expression of eNOS in the heart was also determined by immunohistochemical (IHC) analysis. Cardiomyocytes were isolated from adult rat hearts by collagenase-perfusion, and adult rat cardiac microvascular endothelial cells (CMEC) purchased commercially. In the cells, hypoxia was induced by covering cell pellets with mineral oil for 60 min. Cell viability was determined by trypan blue and propidium iodide (PI) staining and intracellular NO production measured by FACS analysis of the NO-specific probe, DAF-2/DA and by measurement of nitrite levels (Griess reagent). Results show that in ischaemic hearts, nitrite production increased by 12 % after 15 min ischaemia and 7 % after 20 min ischaemia. Total eNOS expression remained unchanged (Western Blot and IHC) and activated eNOS (phospho-eNOS Ser¹¹⁷⁷) increased by 38 % after 15 min ischaemia and decreased by 43% after 20 min ischaemia. In the cells, both viability techniques verified that the hypoxia-protocol induced significant damage. In isolated cardiomyocytes, NO-production increased 1.2-fold (by DAF-2/DA fluorescence), total eNOS expression increased 2-fold and activated eNOS increased 1.8-fold over control. In CMECs, NO-production increased 1.6-fold (by DAF-2/DA fluorescence), total eNOS increased by 1.8-fold and activated eNOS by 3-fold. With regards to our PI3-K / PKB investigations, results showed an increase of 84 % and 88 % in expression

and activation of PKB (phospho Ser⁴⁷³) in hypoxic cardiomyocytes, respectively. In hypoxic CMECs, there was no change in PKB expression but there was a 69 % increase in phosphorylated PKB. NO production in wortmannin-treated hypoxic cardiomyocytes decreased by 12 % as compared to untreated hypoxic cells. In treated hypoxic CMECs, NO production decreased by 58 % as compared to untreated hypoxic cells. Treatment with wortmannin did not change the expression of eNOS protein in the cardiomyocytes, however, activated eNOS decreased by 41 % and 23 % under baseline and hypoxic conditions in treated cells respectively. There was a significant increase in NO production after exposure to O₂ deficient conditions in all models investigated, a trend similar to what previous studies in literature found. However, the source of this NO is not fully understood although it has been discovered that NOS plays a role. Our data reveals similar trends in 15 min ischaemia in whole hearts and 60 min hypoxia in the cells; however, the trends observed at 20 min ischaemia are in conflict with our cell data (i.e. decrease in activated eNOS). This may be due to the severity of the ischaemic insult in whole hearts and/or the presence of other cell types and paracrine factors in the whole heart. Hypoxia increased the activation of PKB in the isolated cardiac cells. Inhibition of the PI3-K / PKB pathway reduced NO production and hypoxia-induced eNOS activation in cardiomyocytes. In conclusion, we have, for the first time, demonstrated that the increase in NO production during hypoxia is due (at least in part) to an

increase in eNOS phosphorylation at Ser¹¹⁷⁷ and that this is mediated via the PI3-K / PKB pathway.

Opsomming

Endoteel-afgeleide stikstofoksied sintetase (eNOS) word as die belangrikste konstitutief-uitgedrukte ensiematiese bron van stikstofoksied (NO) in die hart beskou. NO is 'n belangrike boodskapper in die hart, en word oor die algemeen as 'n kardio-beskerende molekule gesien. Die rol van eNOS tydens isemie / hipoksie is kontroversieel, hoewel dit algemeen aanvaar word dat isemie / hipoksie tot verhoogde kardiaale NO produksie lei. Die meeste studies fokus egter op óf heel-hart óf geïsoleerde sel modelle. Daar is geen aanduiding in die literatuur van enige studies wat bevindinge m.b.t. NO metabolisme in hierdie twee modelle, in 'n enkele studie, vergelyk het nie. Ons hipotese is dat waarnemings in 'n heel-hart model m.b.t. verhoogde NO produksie en eNOS betrokkenheid tydens isemie, die gevolg is van gebeure op sellulêre vlak, en dat die toename in NO produksie tydens hipoksie in kardiomyosiete en endoteelselle ten minste gedeeltelik die gevolg is van verhoogde uitdrukking en / of aktivering van eNOS. Ons hipotese lui verder dat lg. effekte via die PI3-K / PKB pad gemedieer word. Ons doel was dus om NO produksie en eNOS uitdrukking en aktivering in heel-harte en geïsoleerde selle te meet en vergelyk, asook om PKB uitdrukking en aktivering in die selle tydens normoksiese en isemiese / hipoksiese omstandighede te bepaal. Verder het die studie dit ten doel gehad om die effekte van PI3-K / PKB

inhibisie op NO produksie en eNOS uitdrukking en aktivering in geïsoleerde hartselle tydens normoksie en hipoksie te bepaal. Volwasse rotharte is geperfuseer en globale isgemie is vir 15 en 20 min toegepas. Weefsel homogenate van geperfuseerde harte is gebruik om nitriet-bepalings te doen, asook die bepaling van eNOS-uitdrukking en –aktivering. Proteïen uitdrukking van eNOS is ook immunohistochemies (IHC) bepaal. Kardiomiosiete is van volwasse rotharte m.b.v. kollagenase perfusie geïsoleer, en volwasse rot kardiaale mikrovaskulêre endoteelselle (CMEC) is kommersieel aangekoop. Hipoksie is in die selle geïnduseer deur sel-pellets met 'n lagie mineraalolie vir 60 min te bedek. Sellewensvatbaarheid was d.m.v. trypan blou en propodium jodied kleuring bepaal en intrasellulêre NO produksie is d.m.v. vloeisitometriese analise van die fluoresserende, NO-spesifieke detektor, DAF-2/DA gemeet, asook deur die bepaling van nitriet-vlakke (Griess Reagens). Die resultate toon dat in isgemiese harte, die nitriet-produksie met 12% toegeneem het na 15 min isgemie, en met 7% na 20 min isgemie. Totale eNOS proteïen uitdrukking het onveranderd gebly (Western blot en IHC), terwyl geaktiveerde eNOS (fosfo-eNOS serien 1177) met 38% na 15 min toegeneem het en met 43% afgeneem het na 20 min isgemie. In die selle het beide lewensvatbaarheidstudies bevestig dat die hipoksie protokol genoegsame selskade aangerig het. In die geïsoleerde kardiomiosiete het NO-produksie 1.2-voudig (d.m.v. DAF-2/DA fluoressensie) toegeneem, totale eNOS uitdrukking het 'n 2-voudige toename getoon, en geaktiveerde eNOS 'n

1.8-voudige toename in vergelyking met kontrole. In die CMEC groepe het NO-produksie 1.6-voudig (DAF-2/DA fluoressensie) toegeneem, totale eNOS uitdrukking 1.8-voudig, en geaktiveerde eNOS 3-voudig. Met betrekking tot die PI3-K / PKB ondersoek het ons resultate getoon dat daar 'n 84% en 88% toename in uitdrukking en aktivering (fosfo-PKB serien 473) onderskeidelik in die hipoksiese kardiomyosiete was. In hipoksiese CMEC het die totale PKB uitdrukking onveranderd gebly, terwyl daar 'n 69% toename in gefosforileerde PKB was. NO produksie in wortmannin-behandelde kardiomyosiete het met 12% afgeneem vergeleke met onbehandelde hipoksiese selle. In wortmannin-behandelde hipoksiese CMEC, het NO produksie met 58% afgeneem in vergelyking met onbehandelde hipoksiese selle. Behandeling met wortmannin het geen effek op die uitdrukking van eNOS proteïen in kardiomyosiete gehad nie, terwyl geaktiveerde eNOS met 41% en 23% in onderskeidelik basislyn en hipoksiese omstandighede afgeneem het in wortmannin-behandelde selle. Daar was 'n statisties beduidende toename in NO produksie na blootstelling aan verlaagde suurstof toestande in al ons modelle. Hierdie tendens bevestig wat deur ander studies in die literatuur gevind is. Die bron van hierdie NO is egter nog nie heeltemal bekend nie, hoewel dit bekend is dat NOS wel 'n rol speel. Ons data dui op soortgelyke tendense in die 15 min isemie groepe in die heel-hart model in vergelyking met 60 min hipoksie in die selmodelle; die 20 min isemie data in die heel-harte is egter teenstrydig hiermee (d.w.s verlaagde eNOS aktivering). Hierdie teenstrydigheid kan verklaar word deur

die felheid van die isgemiese besering na 20 min en / of die teenwoordigheid van ander seltipes en parakriene faktore in die heel-harte. Hipoksie het die aktivering van PKB laat toeneem in die geïsoleerde kardiaale selle. Inhibisie van die PI3-K / PKB pad het NO produksie en hipoksie-geïnduseerde eNOS aktivering in die kardiomyosiete laat afneem. Ter afsluiting: ons het vir die eerste keer aangetoon dat die toename in NO produksie tydens hipoksie (ten minste gedeeltelik) die gevolg is van 'n toename in eNOS fosforilering op serien¹¹⁷⁷ en dat dit 'n PI3-K / PKB gemedieerde meganisme volg.

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Abbreviations used

2, 3 BDM	2-3butanedione monoxime
6-PF2-K	6-phosphofructo-2-kinase
Akt	v-akt murine oncogene homologue1
AMPK	adenosine-5'-monophosphate-activated protein kinase
ATP	adenosine-5'-triphosphate
bFGF	basic fibroblast growth factor
BH ₄	tetrahydrobiopterin
BSA	bovine serum albumin
CaCl ₂	calcium chloride
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CCPA	2-chloro-N ⁶ -cyclopentyladenosine
cGMP	cyclic guanidine monophosphate
CMEC(s)	cardiac microvascular endothelial cell(s)
DAF-2	4, 5-diaminofluorescein
DAF-2/DA	4, 5-diaminofluorescein-2/diacetate
DAN-1 EE	ester derivative of 4-((3-amino-2-naphthyl)

	aminomethyl) benzoic acid
DETA/NO	diethylenetriamine
Dil-ac-LDL	Dil-labelled acetyl low-density lipoprotein
DNA	deoxyribonucleic acid
ECL	electrochemiluminescent
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetra-acetic acid
eNOS	endothelial nitric oxide synthase
FACS	fluorescence activated cell sorting
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FSC-H	forward scatter
g	gram
GSH	reduced glutathione
GSK 3	glycogen synthase kinase 3
GSNO	<i>S</i> -nitroglutathione
GTP	guanidine-5'-triphosphate
H ⁺	hydrogen ion

H ₂ O	water
H ₂ O ₂	hydrogen peroxide
haem	iron protoporphyrin IX
HDL	high-density lipoprotein
hEGF	human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFGF	human fibroblast growth factor
HIF-1	hypoxia-inducible factor 1
HM	hydrophobic motif
Hsp90	heat shock protein 90
IGF-I	insulin-like growth factor-I
IgG	immunoglobulin G
IHC	immunohistochemistry
IHD	ischaemic heart disease
ILK	integrin-linked kinase
iNOS	inducible nitric oxide synthase
IP	preconditioning
K _{ATP}	ATP-sensitive potassium channels
KCl	potassium chloride
kDa	kiloDalton(s)

kg	kilogram
KH_2PO_4	potassium dihydrogen phosphate
L-arg	L-arginine
L-NA	N^{ω} -nitro-L-arginine
L-NAME	N^{G} -nitro-L-arginine methyl ester
LSAB	labelled streptavidin biotin
MAPKAP-K1	mitogen-activated protein kinase activated-protein kinase 1
MAPKAP-K2	mitogen-activated protein kinase activated-protein kinase 2
mg	milligram
MgSO_4	magnesium sulphate
min	minute(s)
mM	millimolar
mPTP	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
mtNOS	mitochondrial nitric oxide synthase
n	sample size
Na_2HPO_4	sodium phosphate
Na_2SO_4	sodium sulphate

NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaH ₂ PO ₄	sodium dihydrogenphosphate
NaHCO ₃	sodium hydrogen carbonate
NaOH	sodium hydroxide
NF1	neurofibromatosis type 1
NGF	nerve growth factor
nm	nanometre
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO ₂ •	nitryl
NOS	nitric oxide synthase
NOS 1	neuronal nitric oxide synthase
NOS 2	inducible nitric oxide synthase
NOS 3	endothelial nitric oxide synthase
O ₂	oxygen
O ₂ •	superoxide
OH•	hydroxyl
OONO•	peroxynitrite
p	p-value

PBS	phosphate-buffered saline
PDE 3	phosphodiesterase 3
PDE 3B	phosphodiesterase 3B
PDGF	platelet-derived growth factor
PDGF-B	platelet-derived growth factor B
PDK1	PtdIns(3,4,5)P ₃ -dependent kinase 1
PDK2	PtdIns(3,4,5)P ₃ -dependent kinase 2
PED/PEA-15	phosphoprotein enriched diabetes / astrocytes-15
PH	pleckstrin-homology domain
phospho	phosphorylated
PI	propidium iodide
PI3-K	phosphatidylinositol-3-kinase
PIP ₃	phosphatidylinositol (3, 4, 5)-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKG	protein kinase G
PMSF	phenyl-methyl-sulphenyl-fluoride
PostC	postconditioning
PtdIns(3,4)P ₂	phosphatidylinositol (3, 4)-biphosphate

PtdIns(3,4,5)P ₃	phosphatidylinositol (3, 4, 5)-triphosphate
R ₃ IGF-1	83 amino acid analog of IGF-1
ROS	reactive oxygen species
rpm	revolutions per minute
Rsk	MAPKAP-K1
RyR	ryanodine receptors
SDS	sodium dodecylsulphate
Ser ¹¹⁴	eNOS serine 114 residue
sec	second(s)
SEM	standard error of mean
Ser	serine
Ser ¹¹⁷⁷	eNOS serine 1177 residue
Ser ⁴⁷³	PKB serine 473 residue
Ser ⁶¹⁵	eNOS serine 615 residue
Ser ⁶³³	eNOS serine 633 residue
sGC	soluble guanylate cyclase
SNAP	<i>S</i> -nitro- <i>N</i> -acetylpenicillamine
SOD	superoxide dismutase
SSC-H	side scatter
SWOP	second window of protection

t	time
TBE	trypan blue exclusion
Thr	threonine
Thr ³⁰⁸	PKB threonine 308 residue
Thr ⁴⁹⁵	eNOS threonine 495 residue
VEGF	vascular endothelium growth factor
VEGF-A	vascular endothelial growth factor A
vs	versus
WHO	World Health Organisation
XOR	xanthine oxidoreductase
α	alpha
β	beta
γ	gamma
°C	degrees Celcius
μm	micrometre
μM	micromolar

Chapter 1: Literature Overview

Chapter 1: Literature Overview

1.1 Introduction

1.1.1 Background

The incidence and prevalence of cardiovascular disease (including ischaemic heart disease (IHD), hypertension, rheumatic heart disease, heart failure, cerebrovascular disease, congenital heart disease and peripheral artery disease) in the 21st century are increasing dramatically with an estimated mortality of 20 million people by 2015 (WHO 2008). In 2005, 30% of all global deaths were due to cardiovascular disease (Figure 1.1), which is the highest cause of death worldwide (WHO 2008). Approximately 80% of these deaths occurred in low-middle income countries (WHO 2008). According to a study done by the Medical Research Council of South Africa in 2000, cardiovascular disease was the highest cause of death in the non-communicable disease category in South Africa (Figure 1.2) (South African National Burden of Disease Study 2000).

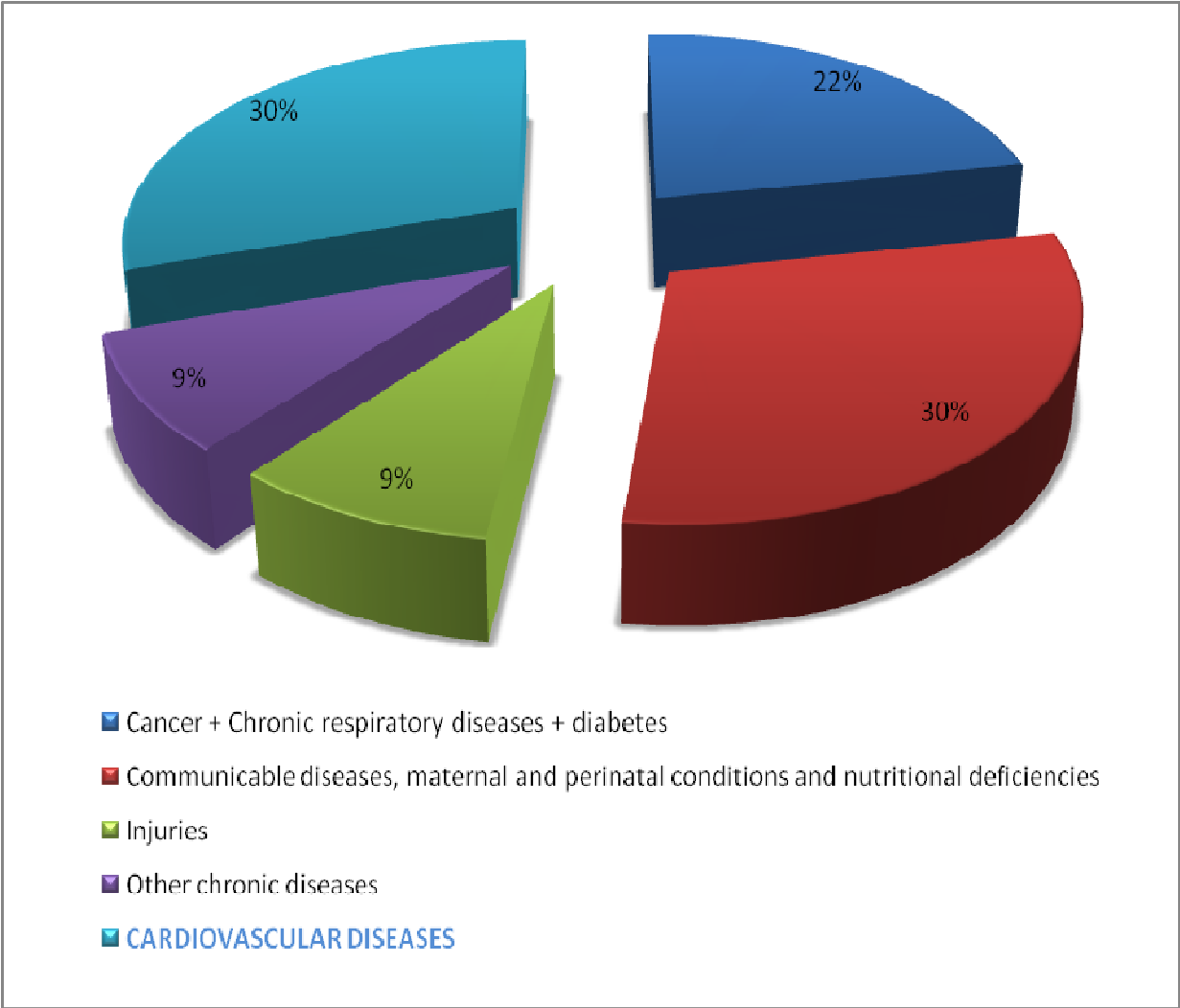


Figure 1.1: Global causes of death. (Adapted from WHO 2008).

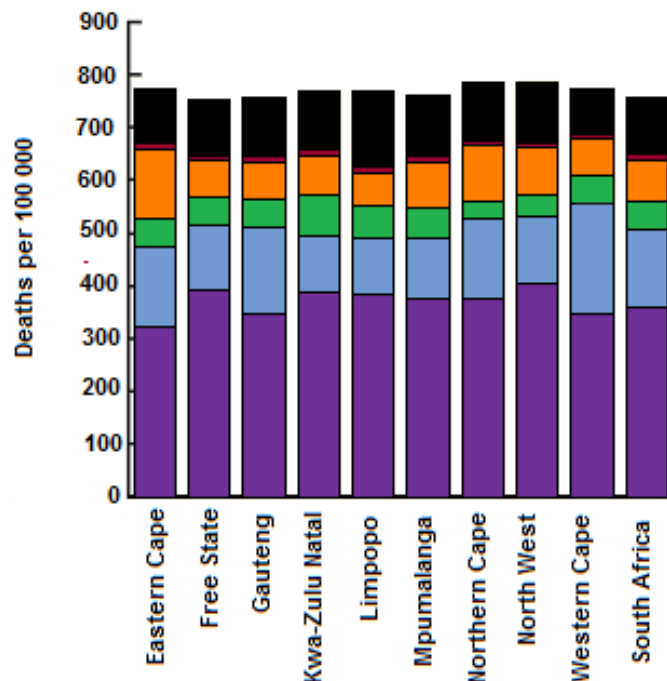


Figure 1.2: Mortality rates in non-communicable diseases in South Africa. (Adapted from the South African National Burden of Disease Study 2000)

IHD is fast becoming the number one cause of death in the world, a trend that seems entrenched in the developed countries and fast taking root in developing countries (WHO 2008). In South Africa, IHD is currently the fifth highest cause of mortality (Figure 1.3 A) (WHO 2008) and the highest cause of death in the Western Cape Province (Figure 1.3 B) (South African National Burden of Disease Study 2000).

Ischaemia is defined as a restriction in blood supply and hypoxia is defined as a reduction in oxygen supply. Ischaemia always leads to hypoxia but hypoxia can be present in the absence of ischaemia (Opie 2004). Myocardial ischaemia exists when the supply of oxygen to the myocardium fails to meet the oxygen demand of tissue due to a reduction in coronary flow (Opie 2004). Seconds after the onset of ischaemia, there is a reduction in cellular contraction which is further reduced by: (i) the energy imbalance created, which leads to an increase in inorganic phosphates and a decrease in phosphocreatine and subsequently adenosine-5'-triphosphate (ATP)- and; (ii) an increase in intracellular acidosis (Kloner & Jennings 2001). The energy imbalance further causes a decrease in fatty acid metabolism and an increase in anaerobic glycolysis (Opie 2004). As the ATP is depleted in the cell, the potassium / ATP channels open and potassium moves out of the cell. This shift of potassium from the cell contributes to the increase of intracellular sodium and cytosolic calcium levels (Allen et al. 1989). The acidosis that develops during ischaemia is biphasic: if mild (approximate decrease of 0.5 pH units), it is cardioprotective as it decreases contraction; if severe, irreversible cell damage occurs leading to apoptosis and necrosis (Bing et al. 1973; Cross et al. 1995).

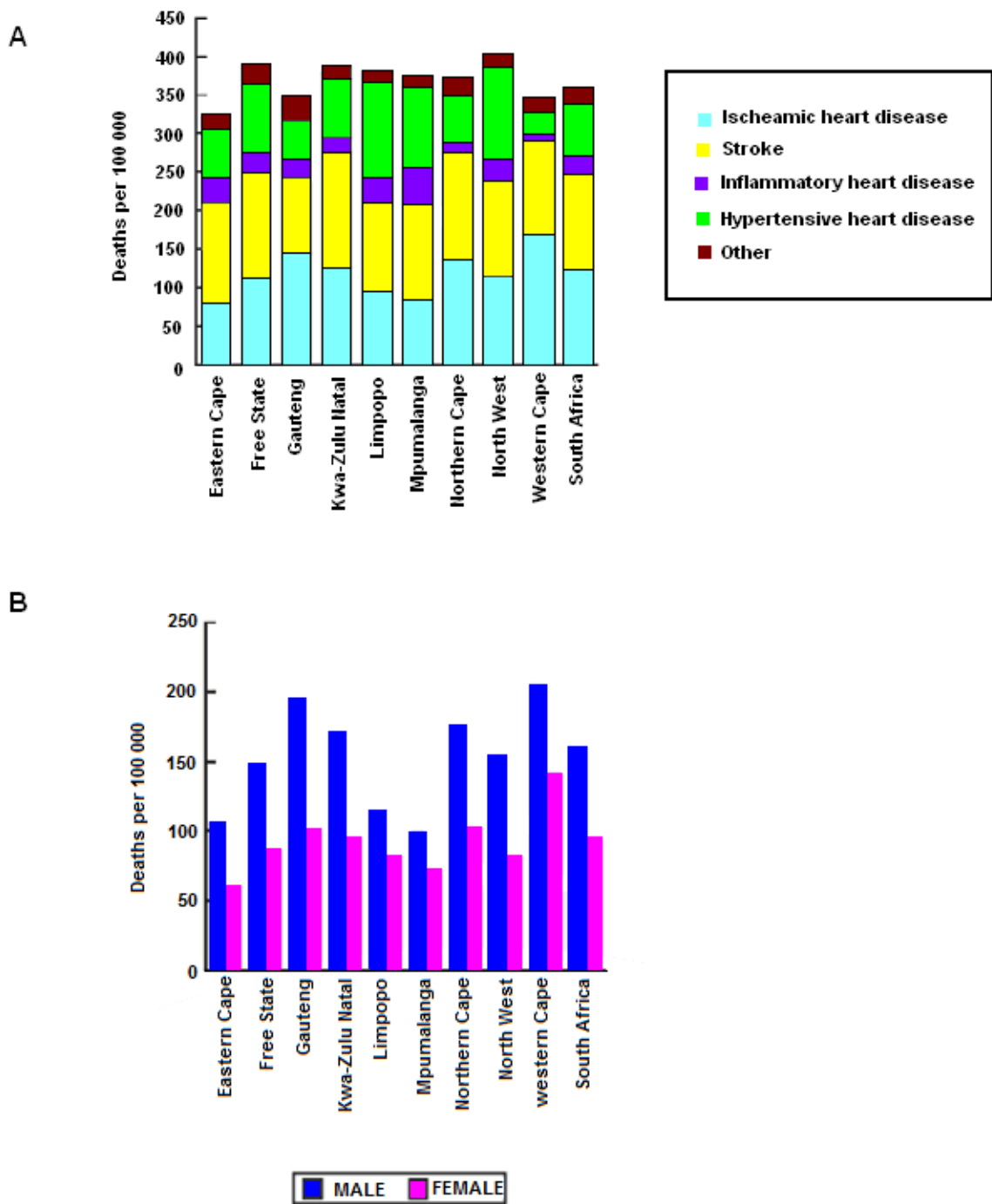


Figure 1.3: A. Mortality due to cardiovascular disease and (B) ischaemic heart disease in South Africa. Adapted from South African National Burden of Disease Study 2000)

Apoptosis is a genetically controlled process of cell death (McLellan & Schneider 1997). Apoptosis can be induced by ischaemia / reperfusion, reactive oxygen species (ROS) and myocardial remodelling (Kumar & Jugdutt 2003). Apoptosis is characterised by membrane blebbing, deoxyribonucleic acid (DNA) fragmentation and formation of apoptotic bodies (Table 1.1) (McLellan & Schneider 1997). As apoptosis is a controlled process, there is no inflammatory response (Kumar & Jugdutt 2003).

Cell death due to the loss of ATP is known as necrosis. A well known cause of necrosis is ischaemia (Kumar & Jugdutt 2003). Necrosis is characterised by loss of membrane integrity and spillage of cellular content into the interstitial space leading to an inflammatory response being elicited (Van Vuuren 2008 (MSc Thesis; Stellenbosch University); Kumar & Jugdutt 2003).

Table 1.1: Morphological and biochemical changes during apoptosis and necrosis (Adapted from Kumar & Jugdutt 2003)

APOPTOSIS	NECROSIS
<ul style="list-style-type: none"> • Membrane blebbing • DNA fragmentation • Cytoplasmic condensation • Caspase activation • No inflammatory immune response • Apoptotic bodies 	<ul style="list-style-type: none"> • Membrane rupture • DNA smear • Cell injury • Swelling • Inflammatory response • Cell debris

1.1.2 Cardioprotection against ischaemic / hypoxic injury

Clinically, cardioprotective therapies aim to reduce necrosis and acute myocardial infarct-associated complications (Kloner & Rezkalla 2004). Reperfusion is regarded as the most effective cardioprotective therapy against ischaemic injury (Zhao & Vinten-Johansen 2006). However, reperfusion also has a detrimental side, the so-called phenomenon of “reperfusion injury”, which includes myocardial stunning, reperfusion arrhythmias, calcium overflow and reactive oxygen species (ROS) formation, which can lead to microvascular damage and cell death (Opie 2004; Zhao & Vinten-Johansen 2006). There are other potent and well described forms of cardioprotection against ischaemic injury such as ischaemic preconditioning (IP) and postconditioning (PostC).

IP has been shown to protect the heart by limiting the infarct size during ischaemia (Murry et al. 1986). IP, considered the “gold standard” of experimental cardioprotection, switches on an intrinsic mechanism of protection elicited by exposing the myocardium to repeated brief ischaemic insults followed by reperfusion prior to the onset of prolonged (index) ischaemia (Gumina & Gross 1999). The clinically more relevant delayed phase of IP-protection, also called

second window of protection (SWOP), is triggered by ischaemia, heat stress, rapid ventricular pacing, exercise and pharmacological agents such as adenosine, bradykinin and nitric oxide (NO) (Marber et al. 1993; Yellon & Downey 2003).

The cardioprotective effects of bradykinin seem to be nitric oxide synthase (NOS) mediated (Ebrahim et al. 2001); a previous study on rat hearts showed that cardioprotection was lost if the hearts were treated with N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, prior to the administration of bradykinin (Ebrahim et al. 2001). NO has been shown to be an important cardioprotective role player during SWOP against MI and myocardial stunning (Takano et al. 1998). NO donors (diethylenetriamine [DETA/NO] and *S*-nitro-*N*-acetylpenicillamine [SNAP]) mimicked protection in the absence of ischaemia-induced SWOP (Takano et al. 1998; Guo et al. 1999). Inhibition of NOS at the onset of IP has been shown to abolish SWOP (Bolli et al. 1997; Takano et al. 1998; Xi et al. 1999). In a study on conscious rabbits, inhibition of NOS with N^ω-nitro-L-arginine (L-NA) at the onset of IP abolished SWOP (Bolli et al. 1997). In iNOS knockout mice treated with the adenosine A1-receptor agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA), SWOP was observed with a concomitant increase in endothelial NOS (eNOS) expression (Bell et al. 2002). Another study on

inducible NOS (iNOS) knockout mice showed no cardioprotection and no change in eNOS expression (Guo et al. 1999).

PostC is a recently discovered cardioprotective laboratory phenomenon. PostC is the rapid intermittent interruptions of bloodflow in the early phase of reperfusion following ischaemia. NO is involved in PostC via the cyclic guanosine monophosphate (cGMP) pathway- if L-NAME is infused before the onset of reperfusion, cardioprotection is lost (Pagliaro et al. 2004; Yang et al. 2005; Yang et al. 2004). Mechanisms of NO protection in PostC include anti-inflammatory actions, activation of ATP-sensitive potassium (K_{ATP}) channels and inhibition of the mitochondrial permeability transition pore (mPTP) (Pagliaro et al. 2004).

1.2 NO in the cardiovascular system: biochemistry, biological effects and synthesis

1.2.1 The discovery of NO as an endogenous molecule

The discovery of a potent vasodilatory molecule in the early 1980's, termed endothelium-derived relaxing factor (EDRF), introduced a paradigm shift in cardiovascular research (Cherry et al. 1982). The identity of this molecule remained elusive until 1987 when it was revealed that EDRF was in fact endogenously produced NO (Ignarro et al. 1987; Palmer et al. 1987; Furchgott 1988). This discovery came as a huge surprise as NO had until then been considered an air pollutant due to its presence in exhaust fumes and cigarette smoke (Nathan 1992). The establishment of a link between NO and vasodilatation finally shed light on the mechanism through which nitroglycerine, a widely prescribed drug since the early 1900's, was able to provide relief for angina pectoris (Chevigné et al. 1980; Marsh & Marsh 2000).

The discovery that NO was an endogenously produced signalling molecule in the cardiovascular system, resulted in the Nobel Prize for Physiology and Medicine being awarded to Murad, Ignarro and Furchgott in 1998 (Nobel Foundation). The importance of this discovery was further underlined when NO received the "Molecule of the Year" award in 1992 from the internationally acclaimed journal,

Science (Culotta & Koshland 1992; Rosselli et al. 1998). In the last decade or so it has become increasingly evident that NO possesses significant cardioprotective properties; therefore, the role of NO in conditions of low oxygen (O_2) supply (ischaemia and hypoxia) has become one of the fastest growing fields in basic cardiovascular research (Bolli 2001).

1.2.2 Biochemistry of NO

NO, in its natural state, exists as a gas and is uncharged (Lowenstein et al. 1996). On its own, NO is relatively unreactive, having a half life of 2 – 30 seconds, despite being a free radical (Lowenstein et al. 1996; Squadrito & Pryor 1998). However, in the presence of superoxide (O_2^\bullet), NO and O_2^\bullet combine in a chemical reaction to form peroxynitrite ($OONO^\bullet$) (Figure 1.4), which is a highly reactive and potentially harmful free radical (Squadrito & Pryor 1998). The reaction constant of NO and O_2^\bullet ($k = 6.7 \times 10^9$) is much higher than that of O_2^\bullet and its scavenger, superoxide dismutase (SOD) ($k = 2.0 \times 10^9$) (Squadrito & Pryor 1998), which explains why $OONO^\bullet$ forms so readily when both NO and O_2^\bullet are available to react. Further downstream, $OONO^\bullet$ undergoes additional reactions to form more harmful molecules, nitryl (NO_2^\bullet) and hydroxyl (OH^\bullet).

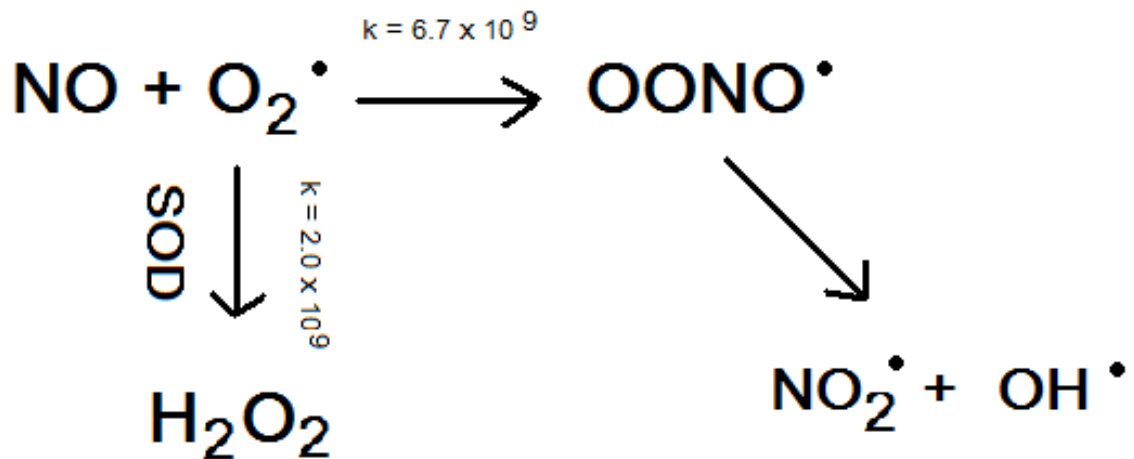


Figure 1.4: Reaction between nitric oxide and superoxide to form peroxynitrite. The rate constant of NO and superoxide is three-fold higher than that of superoxide and SOD. Peroxynitrite further breaks down into more harmful molecules, nitryl and hydroxyl. (Modified from Squadrito & Pyrro 1998)

NO is found in various tissues and organs in the body such as the gastrointestinal tract, reproductive system and cardiovascular system where it acts mostly as a signalling molecule and / or vasodilator (Tepavčević et al. 2007; Rosselli et al. 1998; Papapetropoulos et al. 1999). It is also present in the nervous system where it acts a neurotransmitter (Roy & Garthwaite 2006; Zhang & Snyder 1995).

The best described cellular receptor of NO is the enzyme, soluble guanylate cyclase (sGC) (Sarkar et al. 2001). When activated, sGC plays a pivotal role in the cardiovascular system as it is involved in the regulation of the vasculature, platelet adhesion and cardiomyocyte function (Sarkar et al. 2001). The binding of NO to sGC results in a 400-fold increase in the activity of the latter; activated sGC converts guanine-5'-triphosphate (GTP) to cGMP (Sarkar et al. 2001, Roy & Garthwaite 2006). The increase in sGC activity is proportional to the increase in cGMP. Further downstream, cGMP phosphorylates and activates target proteins such as cGMP-dependent protein kinases, cGMP-gated cation channels and cGMP-regulated phosphodiesterases through which the cellular effects of the NO-sGC-cGMP pathway are executed (Sarkar et al. 2001; Lepic et al. 2006; Roy & Garthwaite 2006).

NO may also act on mitochondria by inhibiting cytochrome oxidase thus inactivating the respiratory chain and ATP production (Sarkar et al. 2001). Low concentrations of NO induce a modest rise in cGMP accompanied by increased levels of cyclic adenosine monophosphate (cAMP), whilst high concentrations of NO are associated with an increase in cGMP and reduced levels of cAMP (Sarkar et al. 2001). This could be due to the inhibitory effect of cGMP on phosphodiesterase 3 (PDE 3) (Balligand et al. 1993).

1.2.3 Biological effects of NO

In the heart, NO has been found to be crucial during foetal and postnatal cardiac development (Lepic et al. 2006; Hammound et al. 2007). It also plays an important role in cardiomyocyte generation and proliferation, angiogenesis and cell survival (Hammound et al. 2007). NO has been shown to have anti-coagulatory and anti-inflammatory properties by regulating platelet function (Ülker et al. 2002; Schulz et al. 2004). Furthermore, NO inhibits neutrophil adhesion to the endothelium (Ülker et al. 2002) and the expression of cytokines (Schulz et al. 2004). On a functional level, NO is an important regulator of myocardial contractility and perfusion (Mount et al. 2007). Depending on its source (neuronal NOS [nNOS] - or eNOS-derived), NO can either enhance (nNOS) or depress contractility (eNOS) (Shah & McCarthy 2000; Barouch et al. 2002).

1.2.4. Synthesis of NO

NO is synthesised when L-arginine is converted to L-citrulline by the enzyme, NO synthase (NOS), in an oxygen- and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction (Kelly et al. 1996; Ülker et al. 2002; Fukuchi et al. 1998). NOS has three isoforms which are well documented: neuronal NOS

(nNOS, NOS 1) which was first described in the brain (Bredt et al. 1991), inducible NOS (iNOS, NOS2) first described in macrophages (Xie et al. 1992) and endothelial NOS (eNOS, NOS3) first described in endothelial cells (Lamas et al. 1992). The existence of a fourth isoform, mitochondrial NOS (mtNOS), has been proposed but there is considerable controversy in the literature as to whether mtNOS represents a distinct isoform of NOS (Lacza et al. 2006). All the major NOS isoforms are expressed in the heart, but eNOS seems to have the widest distribution being expressed in cardiomyocytes, endocardium and endothelium (Giraldez et al. 1997; Brutsaert 2003).

nNOS is a constitutively expressed and calcium-dependent enzyme (Kelly et al. 1996; Mount et al. 2007; Lepic et al. 2006). nNOS regulation mostly occurs at the post-transcriptional level (Alderton et al. 2001; Ferreiro et al. 2001). In the heart, nNOS is localised to the sarcoplasmic reticulum of cardiomyocytes where it is associated with ryanodine receptors (RyR) and it is also expressed in the intrinsic neurons that supply the heart (Ziolo & Bers 2003; Hassall et al. 1992). nNOS-derived NO stimulates contractility by increasing the release of calcium from the sarcoplasmic reticulum via RyR stimulation (Barouch et al. 2002).

iNOS is a high output calcium-independent enzyme (Alderton et al. 2001; Ferreiro et al. 2001). iNOS is not constitutively present, and NO-production via this isoform is therefore preceded by enzyme induction through factors such as: cytokines, bacterial endotoxins and other inflammatory processes (Ferreiro et al. 2001; Bloch 2001a). The amount of NO produced by iNOS is some 10^2 to 10^3 fold higher than by eNOS (Singh & Evans 1997; Ferreiro et al. 2001). The excessive amounts of NO generated by iNOS are often implicated in pathophysiological effects leading to myocardial dysfunction and tumour growth (Ferreiro et al. 2001; Sarkar et al. 2001). In mature cardiomyocytes, iNOS is localised along the T-tubules and sarcolemma and in the mitochondria (Kobzik et al. 1994; Ziolo & Bers 2003).

1.3 Endothelial NOS (eNOS)

The constitutively expressed, calcium-dependent eNOS isoform is responsible for physiological (baseline) production of NO in the cardiovascular system and is therefore generally regarded as a low output enzyme (Schulz et al. 2004). In fact, eNOS accounts for the majority of the NO produced in the myocardium during physiological conditions (Giraldez et al. 1997; Strijdom et al. 2006). Although the role of the eNOS isoform as the most important baseline generator of NO in the maintenance of physiological cardiac function is undisputed, its role during pathophysiological conditions such as low oxygen (O₂) supply is less clear (Ferreiro et al. 2001).

1.3.1 eNOS and caveolae

eNOS is anatomically closely associated with caveolae (Figure 1.5), which are surface area-increasing uncoated pits or “smooth” vesicles in plasma membranes (Yamada 1955). Caveolin, a 22 kDa protein, is expressed in caveolae and has three known isoforms: viz. caveolin-1, 2 and 3 (Razani et al. 2002). Caveolin-1 and -3 are associated with the formation of caveolae, and caveolin-3 (the isoform which is bound to eNOS) is the most predominant isoform present in skeletal and

cardiac tissue; furthermore, caveolin-3 is bound to eNOS (Sbaa et al. 2005). Caveolin-1 and -2 are expressed in endothelial cells and caveolin-3 is present in skeletal muscle tissue and cardiomyocytes (Rothberg et al. 1992).

Being membrane-bound, the association between eNOS and caveolin-3 provides an excellent location for eNOS-derived NO to exert paracrine effects (Sbaa et al. 2005). On the other hand, caveolin-3 acts as a regulator of eNOS: when bound to caveolin, eNOS is inhibited (Feron et al. 1996). Activation of eNOS happens in a calcium-dependent manner when calcium binds to calmodulin (CaM), leading to the displacement of caveolin and subsequent activation of eNOS (Sbaa et al. 2005; Bloch et al. 2001a; Kelly et al. 1996; Mount et al. 2007). No activation of eNOS can take place without the initial displacement of caveolin from eNOS by CaM.

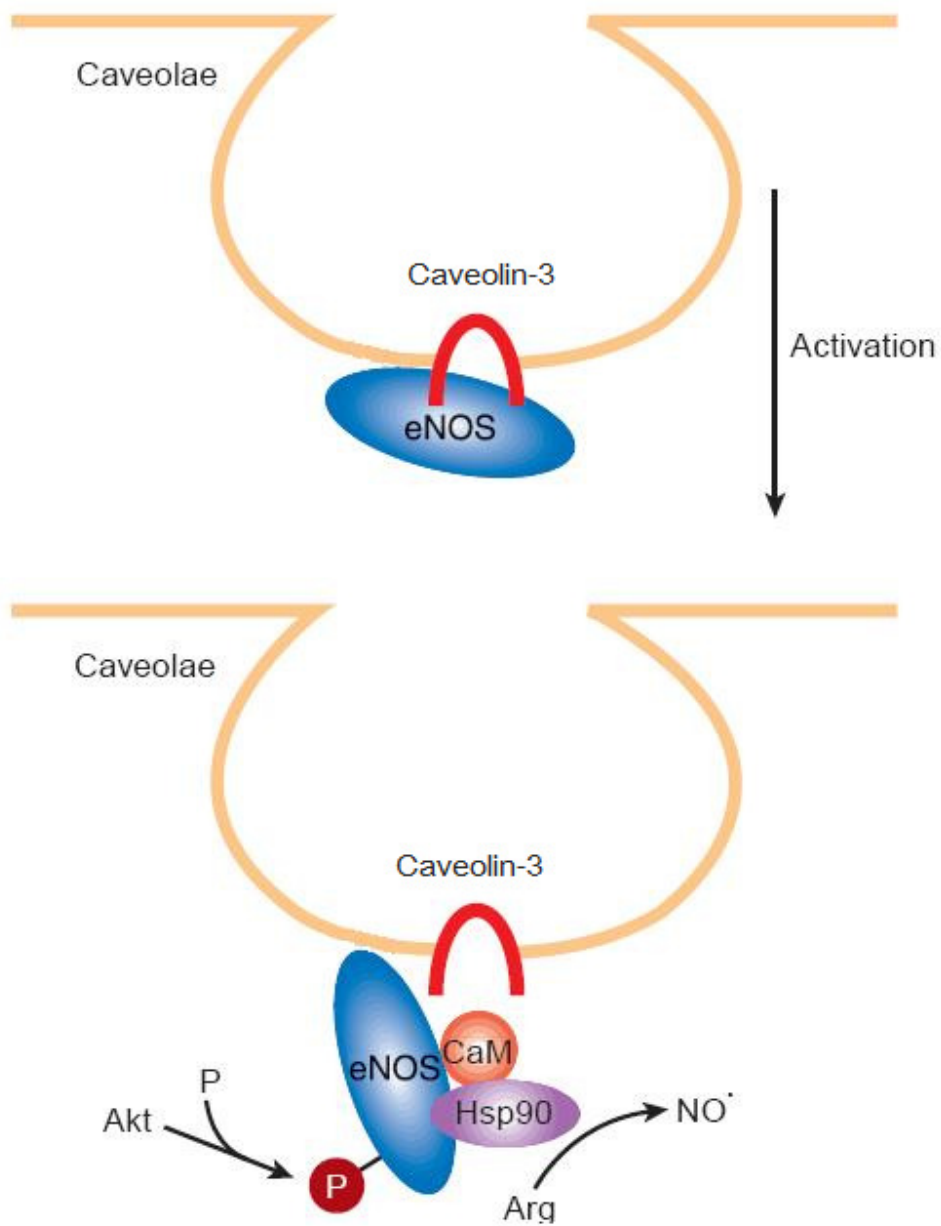


Figure 1.5: Association of eNOS to caveolae. Adapted from Brett 2003.

For NO to be synthesised, various eNOS cofactors must be present, such as: tetrahydrobiopterin (BH₄), iron protoporphyrin IX (haem) and CaM, as well as the

electron transporters NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN); furthermore, the substrate L-arginine must be available in sufficient quantities (Figure 1.6) (Alderton et al. 2001; Mount et al. 2007). *In vitro* studies have shown that eNOS can become uncoupled in the absence of BH₄ and L-arginine, leading to the preferential synthesis of O₂[•] (Alderton et al. 2001).

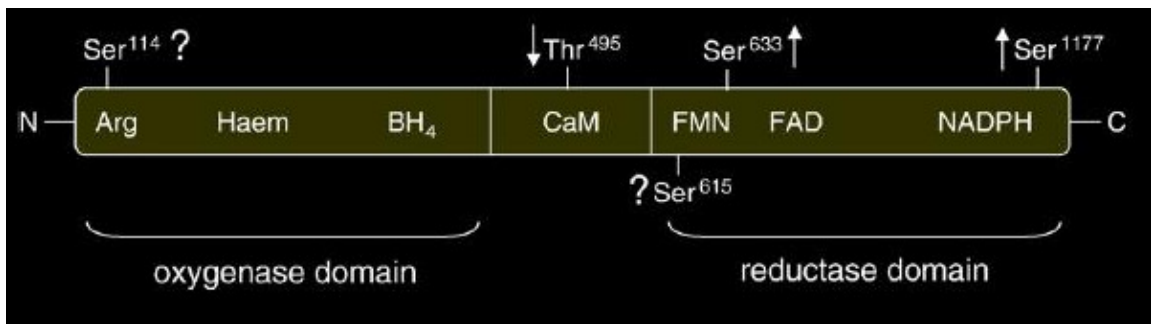


Figure 1.6: Phosphorylatable eNOS residues and cofactors. Adapted from Mount et al. 2007.

1.3.2 Regulation and activation of eNOS

When activated, eNOS exists as a dimer with an oxygenase domain (N-terminal) and a reductase domain (-COOH terminal) (Figures 1.5, 1.6 & 1.7) (Alderton et al. 2001; Mount et al. 2007). The domains are linked by CaM (Alderton et al. 2001; Mount et al. 2007). The reductase domain transfers electrons from NADPH via FAD and FMN to the haem in the oxygenase domain (Alderton et al. 2001).

The oxygenase domain catalyses the reaction between O₂ and L-arginine to NO and citrulline (Alderton et al. 2001).

The regulation and activation of eNOS are complex processes involving different molecules and pathways (Figure 1.7). Factors known to regulate eNOS expression and activation include bradykinin, vascular endothelium growth factor (VEGF), insulin, actin, shear stress, heat shock protein 90 (Hsp90), protein kinase B (PKB), protein kinase A (PKA), hypoxia and adenosine-5'-monophosphate-activated protein kinase (AMPK) (Mount et al. 2007; Lepic et al. 2006; Østergaard et al. 2007; Massion et al. 2003; Bloch et al. 2001a). Rho kinase is a negative regulator of eNOS messenger ribonucleic acid (mRNA) and protein (Østergaard et al. 2007).

The activation of eNOS is achieved by means of phosphorylation reactions at various potential sites on the enzyme. Currently, there are five possible known sites of phosphorylation on eNOS: Serine residues 114 (Ser¹¹⁴), 615 (Ser⁶¹⁵), 633 (Ser⁶³³), 1177 (Ser¹¹⁷⁷) and threonine residue 495 (Thr⁴⁹⁵) (Figure 1.6 & 1.7) (Mount et al. 2007). However, not all these sites activate the enzyme when they are phosphorylated; some are inhibitory, e.g. Thr⁴⁹⁵ (Mount et al. 2007). The effects of Ser¹¹⁴ and Ser⁶¹⁵ phosphorylation are controversial (Mount et al. 2007);

however, Ser¹¹⁷⁷ phosphorylation is the most important and best characterised activation mechanism of eNOS (Mount et al. 2007). Ser¹¹⁷⁷ is known to be phosphorylated by bradykinin, AMPK, shear stress, insulin (via the phosphatidylinositol-3-kinase (PI3-K) – PKB pathway) and statins (Mount et al. 2007). Phosphorylation of eNOS (especially Ser¹¹⁷⁷) plays an important role in the cardioprotective effects of various treatment regimes (such as statins) for acute myocardial infarction and chronic congestive cardiac failure (Mount et al. 2007). Statins increase eNOS expression by stabilising the eNOS-mRNA (Birnbaum et al. 2005 and references therein).

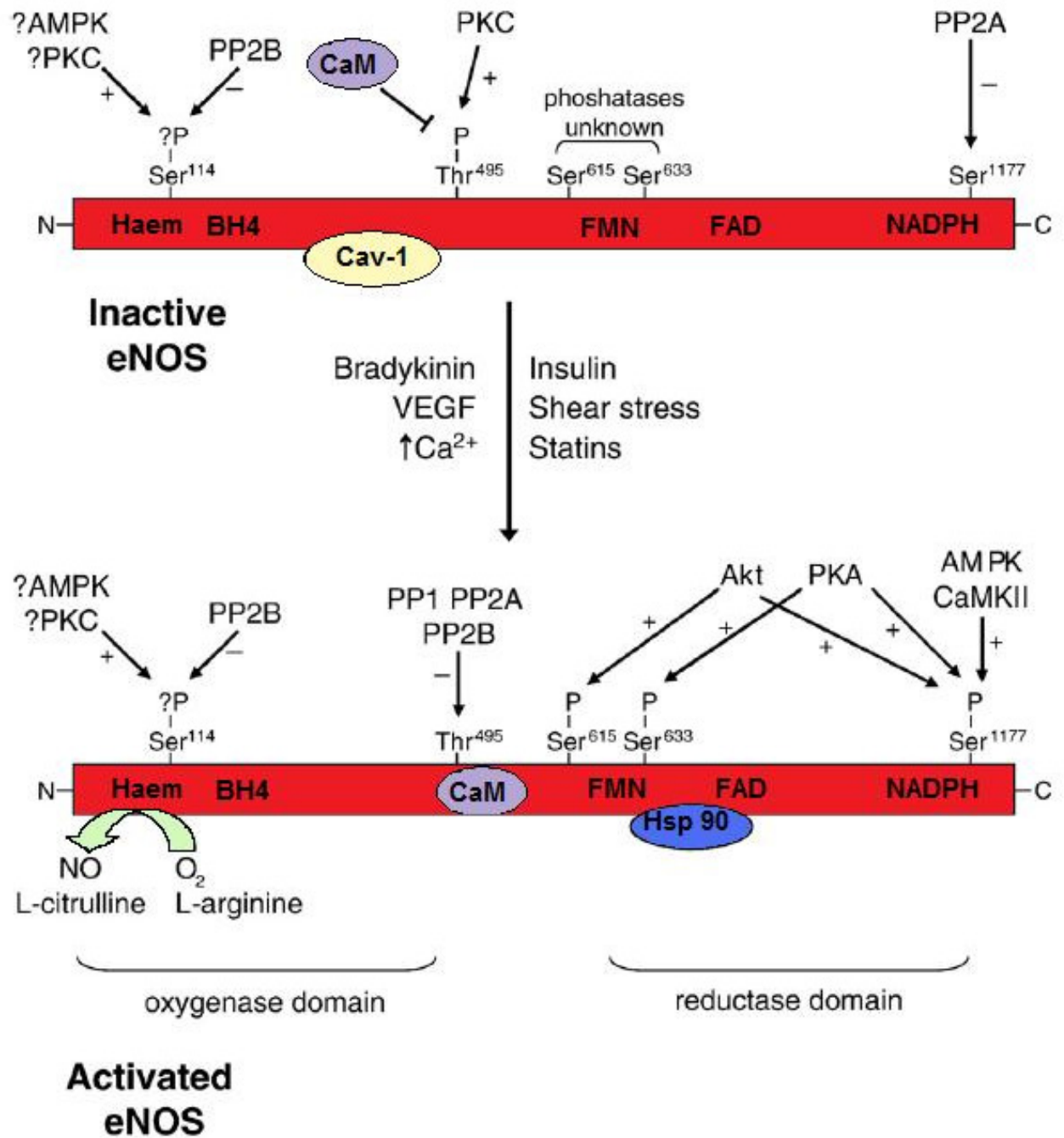


Figure 1.7: Diagram of eNOS in inactive and active states depicting location of cofactors, phosphorylatable sites and regulatory molecules. Adapted from Mount et al. 2007.

1.4 Cardiac NO and eNOS during low oxygen supply

Whereas the role of NO during baseline, physiological conditions is unquestionably beneficial of nature, its effects in the pathophysiological setting (hypoxia and ischaemia) are paradoxical (Table 1.2 & 1.3) (Culotta & Koshland 1992). In isolated hypoxic cardiac cells, we found the effects of hypoxia-induced NO to be harmful (Strijdom et al. 2004a). On the other hand, we have also shown data to the contrary, that NO is cardioprotective during hypoxia / ischaemia in isolated rat hearts (Du Toit et al. 1998; Lochner et al. 2000). Although these contradictory findings may be explained by differences in models and experimental protocols, underlying physiological mechanisms should not be discounted.

The harmful effects observed during situations where there is insufficient O₂ supply are not believed to be the result of NO *per se*, which is generally regarded as a ubiquitously protective molecule (Jones & Bolli 2006), but rather due to the reaction between NO and O₂[•] forming the significantly more reactive and harmful OONO[•] and its highly toxic catabolic products (pro-oxidants) which alter membrane integrity by oxidising membrane proteins and exert toxic effects on

lipids and nucleic acids (Figure 1.8) (Ferdinandy & Schulz 2003; Squadrito et al. 1998; Singh & Evans 1997).

Table 1.2: *In vivo* studies investigating the role of NO during ischaemia / reperfusion injury.

Adapted from Bolli 2001.

SPECIES	END-POINT	EFFECTS			COMMENTS
		Beneficial	No effect	Detrimental	
Rabbit Lamb	cardiac dysfunction mechanical function	■	■		L-NNA L-NAME L-arginine D-arginine
Rabbit	functional recovery			■	L-NMMA L-NAME SNP 8-bromo-cGMP
Rat Rabbit	mechanical function infarct size		■	■	L-NAME L-NAME SPT
Rat Rabbit	mechanical function mechanical function	■		■	L-NMMA L-NMMA L-NAME
Rat	pulse pressure vascular resistance	■			L-arginine Asprin
Rat	cGMP levels coronary flow	■			L-NAME L-NA Adenosine
Rat	functional recovery	■			L-arginine D-arginine L-NAME
Rat	contractile dysfunction pressure-rate product LVDP	■			L-NAME L-NAME PMN
Rat	infarct size	■			Bradykinin Chelerythrine HOE 140 p-benzamine SPT
Rat	contractile function coronary flow cGMP levels	■		■	L-NA L-arginine
Rat	aortic flow LDH		■		2% cholesterol diet L-NA cyclosporine A
Guinea pig	external heart work	■	■		L-NA bosentan VEGF
Rat	mechanical function vascular resistance	■	■		
Rat	functional recovery	■			L-arginine L-NA D-arginine
Rat	mechanical function generation of OONO			■	L-NMMA SNAP L-tyrosine
Rabbit	infarct size		■		cyclosporine A L-NAME FK-506
Mouse	contractile function LVDP			■	eNOS knockout L-NAME
Rabbit	infarct size		■		SNAP chelerythrine N-(2-mercaptopyronyl)-glycine
Mouse	infarct size	■			eNOS knockout nNOS knockout
Mouse	nitrite levels	■			eNOS knockout

Table 1.3: *In vitro* studies investigating the role of NO during ischaemia / reperfusion injury. Adapted from Bolli 2001.

SPECIES	END-POINT	EFFECTS			COMMENTS
		Beneficial	No Effect	Detrimental	
Piglets	contractility			■	
	lipid peroxidation				
Dogs	infarct size		■		
Rabbits	infarct size			■	L-NAME
Dogs	contractile function	■			L-NA
	myocardial bloodflow				
Rabbits	infarct size		■		L-NAME ramiprilat
Dogs	systolic wall thickening		■		L-NAME
Rabbits	infarct size	■			L-NAME
Rabbits	infarct size	■			L-NA
Rats	infarct size		■		L-NAME ramiprilat
Rabbits	infarct size	■			aminoguanidine
Rabbits	systolic wall thickening				L-NA
Dogs	infarct size	■			L-NAME
Pigs	contractile performance	■			L-NAME
					global ischaemia
Dogs	contractile function	■			L-NAME
Mice	LV function & morphology		■		eNOS knockout
Rabbits	infarct size	■			quinapril
Mice	infarct size	■			eNOS knockout
Mice	infarct size	■			MLA
Dogs	arrhythmias	■			
Mice	infarct size	■			MLA
Rat	iNOS mRNA			■	SMT aminoguanidine
Mice	infarct size	■			SMT CCPA DPCPX
Pigs	infarct size	■			SMT LU 135252
Pigs	infarct size		■		L-NMMA
Pigs	infarct size	■			L-NA L-NNA
Pigs	aortic constriction	■			LU 135252
	external work index				L-NNA

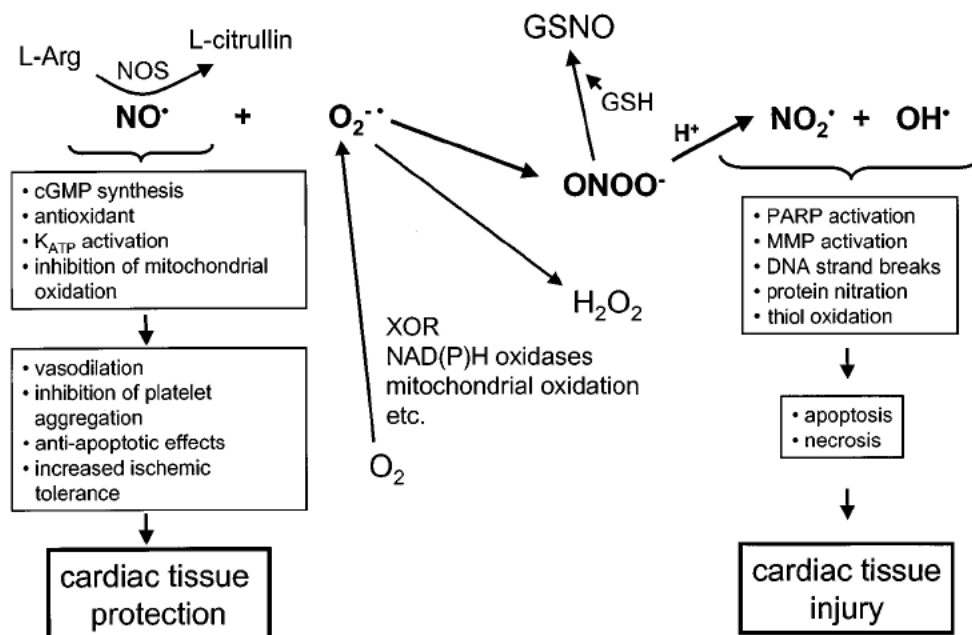


Figure 1.8: Cellular effects of NO, O₂^{•-} and ONOO[•] in the heart. GSH- reduced glutathione; GSNO- S-nitrosoglutathione; XOR- xanthine oxidoreductase. Adapted from Ferdinandy & Schulz 2003.

It is widely accepted that hypoxia and ischaemia induce increased NO production (Kitakaze et al. 1995; Depré et al. 1997; Csonka et al. 1999), and that this is (at least in part) NOS-derived (Shah & McCarthy 2000). Some studies have shown involvement of iNOS as a source of increased NO-production (Ding et al. 2005). iNOS mRNA and protein expression are increased after hypoxia / ischaemia (Jung et al. 2000; Takimoto et al. 2000) albeit with detrimental effects- In a study on swine cardiomyocytes, increased iNOS expression was associated with contractile dysfunction (Heinzel et al. 2008). Interestingly, another study showed

an increase in nNOS mRNA with no change in eNOS mRNA (Takimoto et al. 2000).

The role of eNOS during low O₂ supply is not well understood (Ferreiro et al. 2001). Some have shown eNOS expression and activation to be down-regulated during hypoxia / ischaemia (Giraldez et al. 1997; Faller et al. 1999; Phelan et al. 1996; Laufs et al. 1997); whereas others have shown eNOS activity to be up-regulated during hypoxia (Arnet et al. 1996; Shaul et al. 1992; Pohl & Busse 1989; Depré et al. 1997) and we were also able to show that eNOS plays a prominent role during hypoxia in isolated cardiac cells (Strijdom et al. 2006), although the latter study relied on indirect NOS-inhibition investigations.

1.5 The role of the PI3-K/ PKB pathway in the heart

The regulation and activation of eNOS during hypoxia and ischaemia is not well understood. Evidence in literature suggests that a putative mechanism in this regulation may be the PI3-K/ PKB pathway (Chen & Meyrich 2004).

PKB was discovered in 1991 and named as such based on its homology to PKA and protein kinase C (PKC) (Coffer & Woodgett 1991; Bellacosa et al. 1991; Jones et al. 1991a). Also known as Akt, PKB has four isoforms: PKB alpha (PKB α ; Akt1), PKB beta 1 and 2 (PKB β -1 / -2; Akt2) and PKB gamma (PKB γ ; Akt3) (Jones et al. 1991b; Konishi et al. 1995). All the isoforms have a serine / threonine domain, a carboxy-terminal domain containing a hydrophobic motif (HM) which is characteristic of the so-called AGC kinases (PKA, protein kinase G [PKG], PKC) and a pleckstrin-homology (PH) domain (Figure 1.9) (Marte & Downward 1999; Hanada et al. 2004).

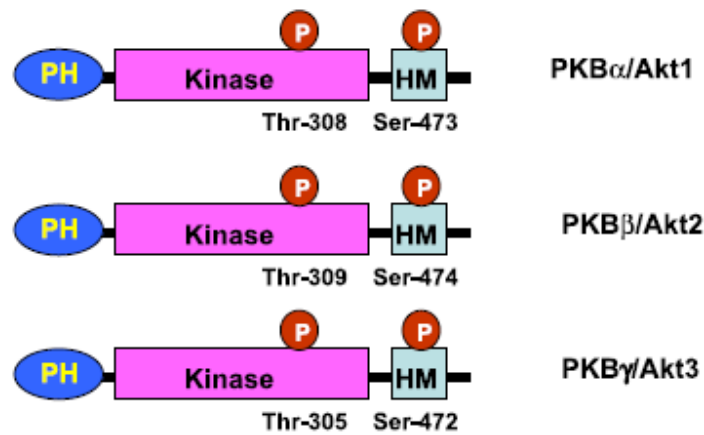


Figure 1.9: Domains and isoforms of PKB. Adapted from Hanada et al. 2004

PKB is activated by insulin and growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), thrombin, nerve growth factor (NGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I) (Marte & Downward 1999; Hanada et al. 2004). Various studies have shown that PKB is regulated by PI3-K: inhibition of PI3-K by wortmannin prevents activation of PKB (Franke et al. 1995; Burgering & Coffey 1995; Kohn et al. 1995) however; there are PI3-K-independent regulators of PKB such as heat shock and hyperosmolality which act through the p38 / HOG1 kinase cascade (Konishi et al. 1996).

PI3-K is thought to activate PKB in a two-step manner: (i) the production of phosphatidylinositol (3, 4)-biphosphate (PtdIns(3,4) P_2) and phosphatidylinositol (3, 4, 5)-triphosphate (PtdIns (3, 4, 5) P_3 ; PIP₃) by PI3-K which bind to the PH domain causing a conformational change and; (ii) translocation from the cytosol to the plasma membrane (Andjelkovic et al. 1997). PKB is also activated by PtdIns (3, 4, 5) P_3 -dependent kinase 1(PDK 1) at Thr³⁰⁸ which has no effect on Ser⁴⁷³ (Alessi et al. 1997; Stephens et al. 1998). The molecule which activates Ser⁴⁷³ remains elusive and has been termed PtdIns (3, 4, 5) P_3 -dependent kinase 2(PDK 2) (Hadjuch et al. 2001; Marte & Downward 1997).

Mitogen-activated protein kinase activated-protein kinase 2 (MAPKAP-K2), Rsk (also known as MAPKAP-K1) and intergrin-linked kinase (ILK) have been proposed as the elusive PDK2 molecule (Delcommenne et al. 1998; Vanhaesebroeck et al. 2001). However, studies have shown that MAPKAP-K2 is not activated by stimuli that activate PKB nor is it activated in a PI3-K-dependent manner thus ruling it out as a major kinase for Ser⁴⁷³ phosphorylation (Shaw et al. 1998). ILK has been shown not to be required for Ser⁴⁷³ phosphorylation: a study on ILK null mice showed phosphorylation of Ser⁴⁷³ to be the same as that of wild type mice (Sakai et al. 2003) and ILK dead mutants were able to induce Ser⁴⁷³ phosphorylation (Lynch et al. 1999).

With more than fifty targets identified to date, PKB has been implicated in glucose metabolism and cell survival amongst others (Figure 1.10). In glucose homeostasis, PKB activates a number of proteins in response to insulin stimulation: phosphorylation of glycogen synthase kinase 3 (GSK3) leads to the inactivation of glycogen synthase (Burgering & Coffey 1995); phosphodiesterase 3B (PDE3B) contributes to the regulation of intracellular levels of cAMP and cGMP (Kitamura et al. 1999) and phosphorylation of 6-phosphofructo-2-kinase (6-PF2-K) promotes glycolysis (Deprez et al. 1997).

PKB exerts its cell survival effects by inhibiting various pro-apoptotic proteins and activating anti-apoptotic proteins in a PI3-K-dependent manner. PKB phosphorylates BAD which prevents it from binding to the anti-apoptotic proteins, Bcl-2 and Bcl-X (del Peso et al. 1997; Datta et al. 1997). Cytochrome-c-induced cleavage of pro-caspase 9 is prevented by phosphorylation in a Ras-dependent manner (Donepudi & Grutter 2002). Phosphoprotein enriched diabetes / astrocytes-15 (PED / PEA-15), a cytosol protein thought to inhibit caspase 3, is protected from degradation and maintains its anti-apoptotic effect during serum deprivation when phosphorylated by PKB (Trencia et al. 2003).

The PI3-K/ PKB pathway has been shown to phosphorylate eNOS (Ser¹¹⁷⁷) during shear stress (Dimmeler et al. 1998; Mount et al. 2007). In fact, it is through this pathway that insulin is able to stimulate eNOS phosphorylation and thus activation (Montagnani et al. 2001). VEGF phosphorylates PKB and it has been shown to activate eNOS through PKB (Dimmeler et al. 1999). In order for PKB to phosphorylate eNOS, it needs to be bound to Hsp90, thus it seems Hsp90 acts as a scaffolding protein (Chen & Meyrich 2004; Fontana et al. 2002). In view of these interactions, it is possible that the PI3-K / PKB pathway may be involved in the phosphorylation and activation of eNOS during hypoxia.

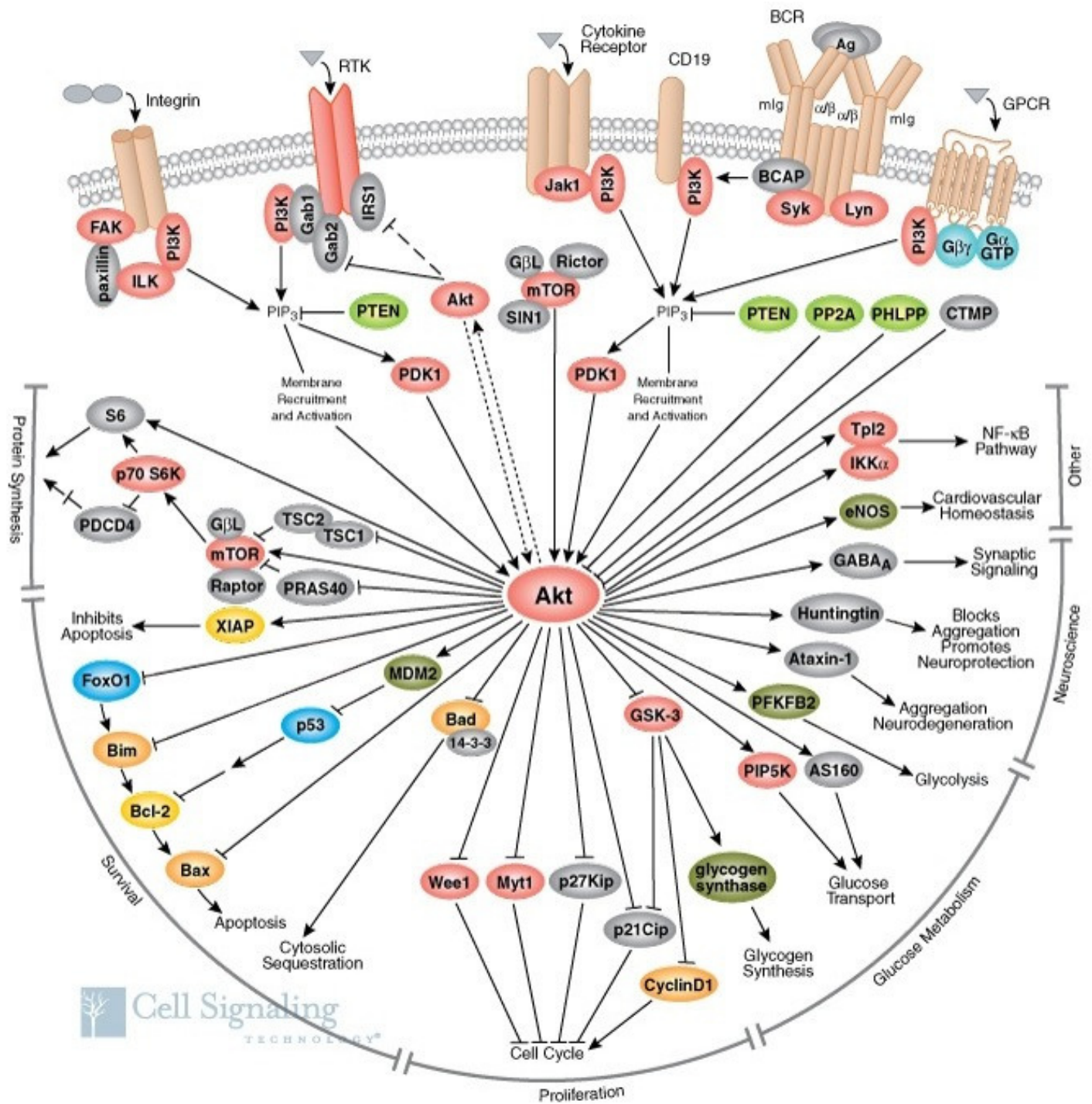


Figure 1.10. Downstream effects of PKB / Akt activation. Adapted from Cell Signaling Technology.

1.6 Endothelial cell – cardiomyocyte interactions in the heart

There is sufficient evidence to support the existence of various biological interactions and paracrine communication between endothelial cells and cardiomyocytes. However, little is known about the specific nature and mechanisms of such interactions (Narmoneva et al. 2004). In the myocardium there is at least one capillary for every cardiomyocyte, and endothelial cells outnumber cardiomyocytes at a ratio of 3:1 (Brutsaert 2003).

The importance of paracrine interactions by neuregulin, neurofibromatosis type 1 (NF1), platelet-derived growth factor B (PDGF-B), vascular endothelial growth factor A (VEGF-A), angiopoietin-1, endothelin-1 and NO between endothelial cells and cardiomyocytes has been highlighted in a number of studies that have shown that absence of certain molecules from one or the other of these cell types lead to the development of cardiac failure (Table 1.4 and Figure 1.11).

Both endothelial cells and cardiomyocytes express NOS and produce NO (Giraldez et al. 1997; Bloch et al. 2001b). Therefore, given the close proximity of these two NO-generating cell types as well as NO's gaseous nature and ability to

readily diffuse in and out of cells, NO is considered to be a major paracrine signalling factor in the myocardium (Hsieh et al. 2006). NO generated by cardiomyocytes plays an important role on neighbouring cells i.e. endothelial cells and sympathetic nerve fibres (Bloch et al. 2001a). eNOS-derived NO from the endothelium influences local cardiomyocyte function: a study on isolated muscle strips showed a decrease in twitch duration and force of contractility in the absence of the endothelium (Sarkar et al. 2001). Given the high probability of NO-mediated cardiac microvascular endothelial cell-cardiomyocyte paracrine communication, it is indeed possible that hypoxia-induced NO production could result in increased crosstalk between these cell types. This phenomenon has not received much attention and co-culture studies could shed more light.

Table 1.4: Signaling molecules with paracrine effects on the endothelium and cardiomyocytes.
Adapted from Hsieh et al. 2006.

MOLECULE	SOURCE	EFFECT
Neuregulin	endothelium	Formation of trabeculae and cardiac cushions Promote proliferation, survival and hypertrophic growth of neonatal cardiomyocytes
Neurofibromatosis type 1 (NF1)	endothelium	Downregulation of Ras activity
Platelet-derived growth factor B (PDGF-B)	endothelium	Cardiovascular development
Vascular endothelial growth factor A (VEGF-A)	cardiomyocytes	Angiogenesis during embryogenesis Inhibition of cardiac myocardial-to-mesenchymal transformation
Angiopoietin-1	cardiomyocytes	Prevents vascular oedema Regulates stabilisation and maturation of neovasculature
Endothelin-1	endothelium	Vasoconstriction
NO	cardiomyocytes endothelium	Ventricular relaxation Vascular smooth muscle relaxation

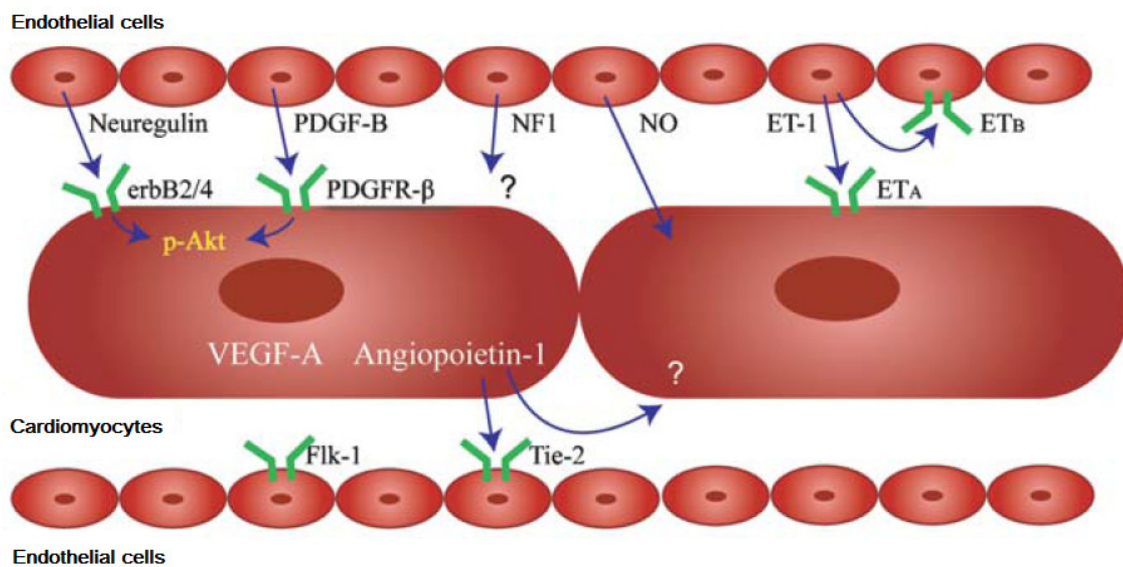


Figure 1.11: Paracrine interactions between endothelial cells and cardiomyocytes. Adapted from Hsieh et al. 2006.

1.7. Motivation and Aims

1.7.1. Motivation

NO is a major signalling molecule in the heart with a wide range of effects, both in health and in disease. On the whole, NO is regarded as a cardioprotective agent, particularly in the ischaemic myocardium in the context of ischaemic preconditioning, postconditioning, but also in ischaemia only.

However, many aspects of the role of NO in the heart still remain unclear, which makes continued research into the role of NO on a cellular and mechanistic level, imperative. Aspects of NO that need further research attention include: enzymatic sources of NO (especially in conditions of oxygen deprivation); contributions of distinct cardiac cell types (e.g. cardiomyocytes and endothelial cells) and; mechanisms of NO production.

It is now well known that both cardiomyocytes and endothelial cells express all three NOS isoforms and produce NO (Kelly et al. 1996; Lepic et al. 2006). We and others have shown that NO production increases significantly during hypoxia, and enzyme inhibition studies demonstrated that such increases were

NOS-derived (Kitakaze et al. 1995; Depré et al. 1996; Strijdom et al. 2006). However, it was not clear which NOS isoform (-s) was/ were responsible for the increased, hypoxia-induced NO production since inhibitors are not always NOS-isoform specific.

Of the three NOS isoforms, eNOS is the most widely distributed in the heart; being present in cardiomyocytes and endothelial cells. Although eNOS is the isoform that is mainly associated with physiological NO production, its regulation and possible activation in ischaemia / hypoxia is not clear. Could eNOS be an important source of NO production during ischaemia / reperfusion? Previous studies have shown the expression of eNOS to be increased during ischaemia / hypoxia (Arnet et al. 1996; Shaul et al. 1992; Pohl & Busse 1989). A study done on porcine coronary artery endothelial cells showed PKB to play a role in eNOS phosphorylation during hypoxia (Chen & Meyrich 2004), which strengthens this as a putative mechanism.

1.7.2. Problem statement

Few studies have investigated NO production by direct intracellular real-time detection techniques along with the expression and activation of eNOS.

Furthermore, most studies have been done either on isolated cells or whole hearts. To date, we are not aware of any study that has compared the effects of ischaemia / hypoxia in the whole heart with ischaemia / hypoxia effects at cellular level. The mechanism by which eNOS is regulated as a putative source of increased NO production in hypoxia is not well researched nor is it fully understood.

1.7.3. Hypothesis

We hypothesise that observations in a whole heart model with regard to increased NO production and eNOS involvement in ischaemia are the result of events on cellular level. We further hypothesise that the increase in NO production observed during hypoxia in cardiomyocytes and endothelial cells is at least in part due to the increase in expression and / or activation of eNOS and that these effects are mediated via the PI3-K / PKB pathway.

1.7.4. Aims

We aim to test our hypothesis by: (i) comparing changes in NO production and eNOS expression and / or activation observed in myocardial tissue sections from

perfused whole hearts subjected to oxygenated or ischaemic conditions with changes in isolated cells subjected to oxygenated or hypoxic conditions; (ii) investigating changes in PKB expression and activation in isolated cardiac cells under oxygenated and hypoxic conditions and; (iii) determining the effects of PI3-K / PKB inhibition on NO production in isolated cardiac cells and eNOS expression and / or activation in isolated cardiomyocytes subjected to oxygenated or hypoxic conditions.

Chapter 2: Methods and Materials

CHAPTER 2: Methods and Materials

2.1 Animals

Adult male Wistar rats (250 – 300 g) were used for isolated cardiomyocyte and whole heart studies. Before anaesthesia (160 mg / kg pentobarbital sodium intraperitoneally), rats were allowed free access to water and food. This investigation conforms to the “Guide for the Care and Use of Laboratory Animals” (US National Institutes of Health; NIH publication no 85 – 23, revised 1985). This project was approved by the Ethics Committee of the Faculty of Health Sciences of Stellenbosch University (Project No: P06/11/022).

2.2 Materials

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pyruvic acid, 2-3-butane dione monoxime (2,3-BDM), Griess Reagent, endothelial cell-specific trypsin, propidium iodide (PI) and wortmannin were obtained from Sigma (St Louis, MO); bovine serum albumin (BSA fraction V, fatty acid free) was obtained from Roche (Cape Town); collagenase type 2, class 2 from Worthington

(Lakewood, NJ); eNOS, phospho (Ser 1177) eNOS, PKB and phospho (Ser 473) PKB antibodies were obtained from Cell Signalling Technology (Beverly, MA); and 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) from Calbiochem (San Diego, CA). Trypan Blue was obtained from Merck (Cape Town). All other chemicals were of Analar grade from Merck.

2.3 Whole heart investigations

The perfusion technique was performed as previously described (Lochner et al. 2000). Briefly, after excision, the hearts were subjected to 15 min of Langendorff perfusion, 15 min of working heart perfusion followed by the experimental perfusion of 20 min. The hearts were perfused with Krebs-Henseleit buffer containing (in mM): sodium chloride (NaCl) 119, sodium hydrogen carbonate (NaHCO₃) 25, potassium chloride (KCl) 4.75, potassium dihydrogen phosphate (KH₂PO₄) 1.2, magnesium sulphate (MgSO₄.7H₂O) 0.6, sodium sulphate (Na₂SO₄) 0.6, calcium chloride (CaCl₂.H₂O) 1.2 and D-glucose 10.

2.3.1 Experimental groups and protocols

Whole hearts were divided in two groups: ischaemia and control (oxygenated). The control hearts were retrogradely perfused (Langendorff) in a time-matched fashion to the ischaemic hearts (Figure 2.1 A). In the ischaemic groups, hearts were subjected to 15 or 20 min low-flow global ischaemia post-working heart perfusion. Thereafter, hearts were freeze-clamped and stored at -80°C (for tissue nitrite measurements) or in liquid nitrogen, (for Western Blot analyses). Whole hearts generating an aortic output less than 30 ml / min during the initial stabilizing period and post-ischaemic hearts that did not have a sinus rhythm were discarded.

2.3.2 Nitrite measurements

NO production in the whole hearts was assessed by a colorimetric assay that determines nitrite levels (nitrite = breakdown product of NO metabolism) using the Griess reagent. Heart tissue was homogenised in 2.5 ml phosphate-buffered saline (PBS) followed by two cycles of centrifugation at 14 000 rpm for 20 min then 10 min at 4°C. Each time, the supernatant was retained and the pellet discarded. Samples were kept on ice as the protein content of the samples was

determined using the Lowry method as previously described (Lowry et al. 1951). Thereafter, a standard curve was calibrated from sodium nitrite standards and the tissue nitrite concentration of the samples was determined (in triplicate) against the standard curve. Values were determined by spectrophotometry at an absorbance of 540 nm.

2.3.3 Immunohistochemical analysis of total eNOS

Whole hearts were perfused as described above. At the end of the experimental intervention (ischaemia / control), hearts were perfusion-fixed with 4 % formalin for 5 min and embedded in paraffin. Histological processing of the heart tissue occurred within 12 hours of perfusion. 5 µm sections were mounted on slides, rehydrated and unmasked using citrate buffer. Endogenous peroxidase activity was then inhibited with 3% sodium hydroxide (NaOH). Thereafter, the slides were stained with eNOS antibody (Santa Cruz Biotechnology Inc, Santa Cruz, USA). The labelled streptavidin biotin (LSAB) kit (Dako, Glostrup, Denmark) was used to conjugate the eNOS and hematoxylin and eosin. To show that staining was due to eNOS antibody binding, negative control slides were prepared from the same tissue using the same protocol but omitting the primary antibody. Thereafter slides were dehydrated and cover slips were mounted. Slides were

then photographed with an AxioCam digital camera and analysed with AxioVision
4.6. using a Zeiss Axioskop 2 light microscope. Stain intensity was measured per
field.

2.4 Isolated, calcium-tolerant adult cardiomyocytes

2.4.1 Cardiomyocyte isolation procedure

Adult rat ventricular cardiomyocytes were isolated using a previously described method (Fischer et al. 1991) and subsequently modified in our laboratory (Strijdom et al. 2004). After excision, hearts were cannulated via the aorta and perfused retrogradely (37°C, gassed with 100 % O₂) in a calcium free, Krebs-Henseleit buffer ("Solution A", containing in mM: KCl 6, sodium phosphate (Na₂HPO₄) 1, sodium dihydrogenphosphate (NaH₂PO₄) 0.2, MgSO₄ 1.4, NaCl 128, HEPES 10, D-glucose 5.5, and pyruvic acid 2) for five minutes to rinse out the blood followed by perfusion, in a recirculating fashion, with a digestion buffer ("Solution B": solution A + 0.7 % BSA (fatty acid free) + 0.1 % collagenase + 15 mM 2,3- BDM) for 30 – 35 min. CaCl₂ was readministered at 20 and 25 min of total perfusion time to reach a total concentration of 200 µM. After digestion, hearts were removed from the perfusion apparatus and the ventricles carefully removed from atrial and vascular remnants. The ventricular tissue was then gently torn apart and incubated in a post-digestion buffer ("Solution C": 1 part Solution A + Solution B + 1 % fatty acid free BSA + 200 µM CaCl₂) for 15 min at 37°C in a shaking waterbath. A step-wise readministration of calcium followed

until the final concentration reached 1 mM. Thereafter, the tissue was filtered through a nylon mesh (200 x 200 µm) and gently centrifuged (100 rpm for 3 min). The cell pellet was re-suspended in an incubation buffer ("Solution D": Solution A + 1 mM CaCl₂ + 2 % fatty acid free BSA) and left to stabilise on a slow rotator at room temperature for at least an hour. Each heart typically yielded approximately 3-5 million cardiomyocytes.

2.4.2 Experimental groups and protocols

Cardiomyocyte samples (approximately 500 000 cells / sample) were selected from different heart preparations (n = 4 – 11). Oxygenated control conditions were simulated by incubating isolated cardiomyocytes suspended in solution D in 35 mm petri dishes in a standard tissue culture incubator under oxygenated conditions (40 – 60 % humidity; 37°C; 21 % O₂, 5 % CO₂) for the duration of the experiments (180 min). Hypoxic samples were also incubated in solution D, under identical incubation conditions as described for the cardiomyocytes, for the duration of the experiments. After a pre-incubation period of 120 min (from t = 0 min to t = 120 min), cells were subsequently subjected to hypoxia for 60 min (from t = 120 min to t = 180 min) (Figure 2.1 B).

2.4.3 Induction of hypoxia in the isolated cardiomyocytes

Hypoxia was induced as previously described (Armstrong et al. 1995) and modified in our laboratory (Strijdom et al. 2004). Briefly, cell samples were gently centrifuged (250 rpm for 30 sec) in microcentrifuge tubes followed by removal of 2/3 of the supernatant, and thereafter layering the pellet and supernatant with mineral oil (Figure 2.2).

2.4.4 Determination of cardiomyocyte viability

Cardiomyocytes were subjected to two independent indices of viability: (i) trypan blue exclusion test (TBE) and (ii) propidium iodide (PI) staining. TBE is an indicator of the ability of cell membranes to exclude the dye (i.e. trypan blue uptake = non-viable) (Figure 2.3). Sample viability was expressed as a percentage of the number of dye-excluding, viable cells over the total number of cells. Cell counting was performed under light microscopy using a haemocytometer. Samples with a time zero viability of <70 % were not considered for investigations. PI is a fluorescent probe that stains cell nuclei when cell membranes have lost their integrity and allow the probe to enter the cell. Such cells are regarded as non-viable. PI staining was evaluated by flow

cytometric analysis. Cardiomyocytes were incubated with 1 μ M PI for 15 min following the experiments (i.e. at t = 180 min). Thereafter, cells were rinsed and re-suspended in probe free media prior to fluorescence activated cell sorting (FACS) analysis.

2.4.5 Measurement of NO production in isolated cardiomyocytes

To determine intracellular NO production in the isolated cardiomyocytes, the NO-specific fluorescent probe, diaminofluorescein (DAF-2/DA), was used and fluorescence intensity determined by flow cytometric analysis (see later). This NO-detection technique has previously been developed and validated in our laboratory (Strijdom et al. 2004b; Strijdom et al. 2006). Cardiomyocytes in control samples were incubated in solution D containing 10 μ M DAF for 180 min from t = 0 min to t = 180 min. DAF-2/DA was present for the full duration of the experiments (180 min) in both control and hypoxia samples. At the end of the experiments, cells were re-suspended in probe-free media and analysed flow cytometrically.

Nitrite production was determined by mixing 500 μ l incubation media (from the cardiomyocytes) with 500 μ l Griess reagent. Absorbance was measured at 550

nm and nitrite levels were calculated using a standard curve calibrated with sodium nitrite.

For the PI3-K / PKB investigations, cardiomyocytes were incubated with a previously validated concentration of 100 nM wortmannin (PI3-K inhibitor) (Huisamen et al. 2002) 15 min prior to the induction of hypoxia and thereafter washed out at the end of 60 min hypoxia.

2.5 Cardiac microvascular endothelial cell (CMEC) cultures

2.5.1 Cell preparation and validation of purity

Primary rat CMEC cultures were purchased from VEC Technologies (Rensselaer, NY). Cells were received in 25 ml or 75 ml fibronectin coated culture flasks and grown to confluency in an endothelial cell growth medium (Clonetics EGM-2 MV; Lonza, Walkersville, MD) as described previously (Strijdom et al. 2006). The media was supplemented with standard endothelial cell growth factors (hydrocortisone, human fibroblast growth factor (hFGF), VEGF, 83 amino acid analog of IGF-1 (R₃IGF-1), ascorbic acid, human epidermal growth factor (hEGF) and GA-1000) (Lonza) and 10 % foetal bovine serum (Highveld Biological, Johannesburg). Once confluent, cultures were passaged in a 1:3 ratio by trypsinisation followed by resuspension in fresh media and plating on sterile fibronectin-coated plates. Sub-cultures became confluent in approximately 5 days. Cells of the third and fourth passage were used for experiments. Purity of the CMEC cultures was verified by microscopic analysis. At confluency, endothelial cells displayed a typical “cobblestone” monolayer morphology, which is characteristic of endothelial cells in culture (Piper 1990).

Functional characterisation was determined by measuring the uptake of fluorescence labelled Dil-labelled acetyl low-density lipoprotein (Dil-ac-LDL) (Biomed Technologies, Stroughton, MA), specific for endothelial cells (Nishida et al. 1993; Piper 1990; Walsh et al. 1998; Fan & Walsh 1999), by flow cytometry.

2.5.2 Experimental groups and protocols, cell viability and NO measurements

For experimental purposes, CMECs were cultured in 35 mm petri dishes, containing approximately 750 000 cells / dish. Each petri dish represented an experimental sample. Cells were removed from culture by trypsinisation prior to experimentation. Control (oxygenated) and hypoxic CMEC samples were incubated and treated in an identical fashion to that described for the isolated cardiomyocytes (Figure 2.1 C).

Cell viability of CMECs was determined by flow cytometric analysis of PI staining. CMECs were incubated with 5 μ M PI for 15 min in a manner identical to that described for the cardiomyocytes.

For the detection of NO production, CMEC samples were loaded with DAF-2/DA (10 μ M) at $t = 0$ min for a total of 180 min as described for the cardiomyocytes.

After washing out the probe at the end of the experiments, samples were analysed by flow cytometer for their fluorescence intensity.

Nitrite production was determined by mixing 500 μ l incubation media (from the CMECs) with 500 μ l Griess reagent. Absorbance was measured at 550 nm and nitrite levels were calculated using a standard curve calibrated with sodium nitrite.

For the PI3-K / PKB investigations, CMECs were incubated with 100 nM wortmannin (Huisamen et al. 2002) 15 min prior to the induction of hypoxia and thereafter washed out at the end of 60 min hypoxia.

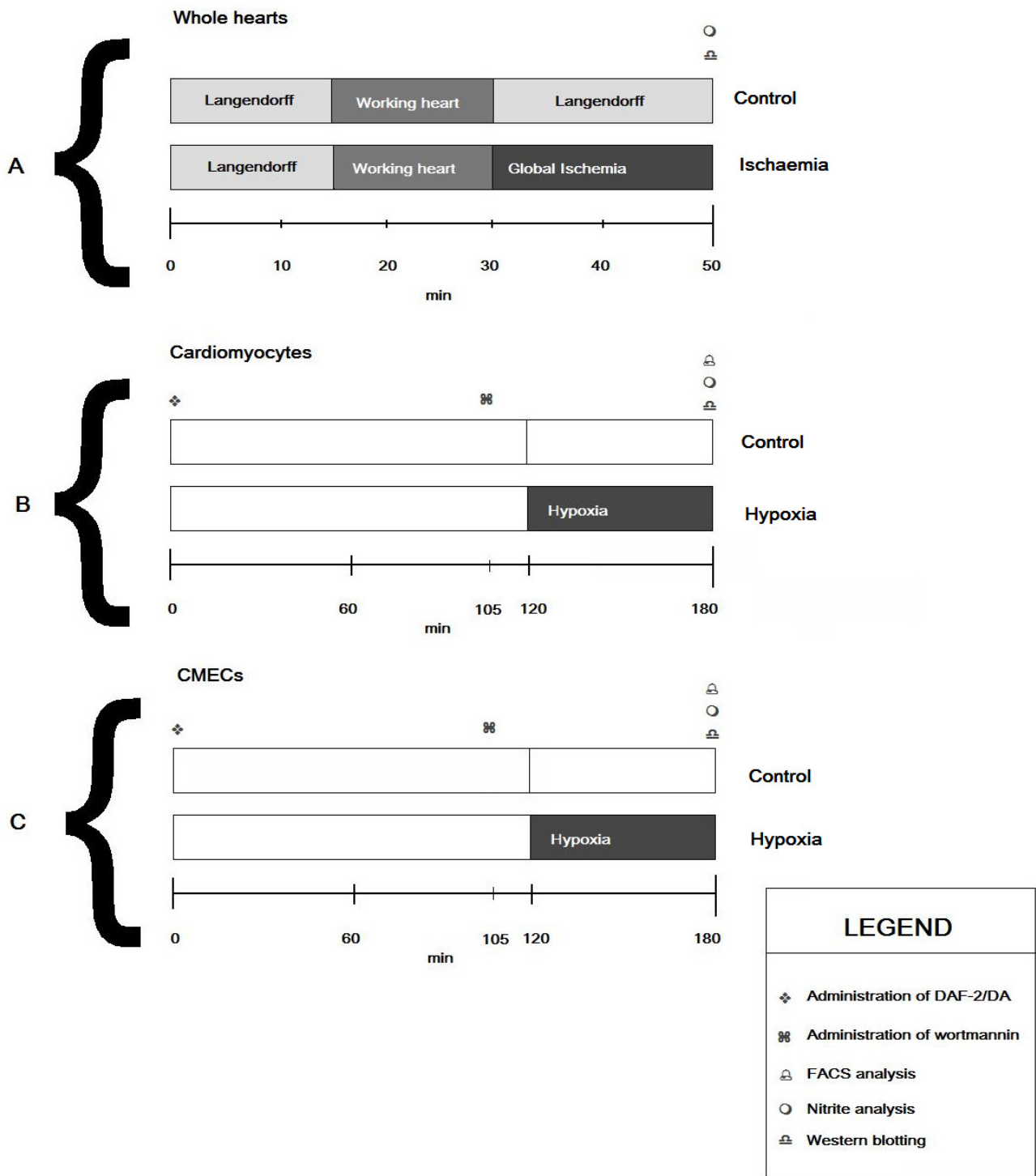


Figure 2.1: Experimental groups and protocols. A: Whole heart investigations B: Cardiomyocyte investigations C: CMEC investigations.

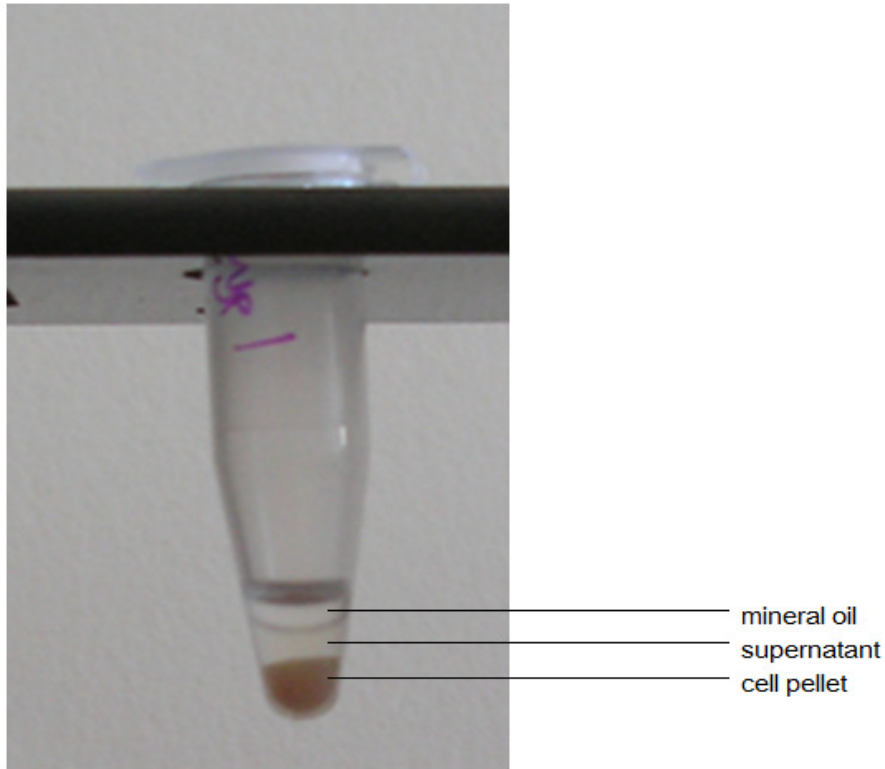


Figure 2.2: Hypoxia protocol for isolated cells

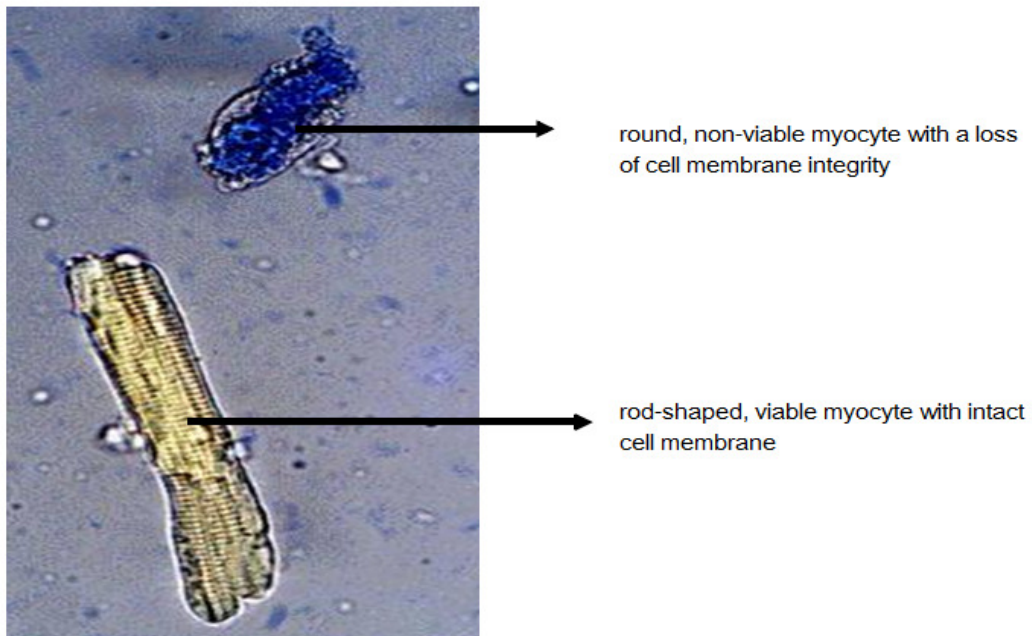


Figure 2.3: Cardiomyocytes incubated with trypan blue. Standard light microscope; 100x magnification.

2.6 Flow cytometric analysis

Flow cytometric analysis of DAF-2/DA and PI treated cells was performed using a method previously developed in our laboratory (Strijdom et al. 2004; Strijdom et al. 2006). At the end of the respective experiments, samples were resuspended in probe-free media followed by analysis on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). DAF-2/DA fluorescence was analysed in the FL1-H channel whilst PI fluorescence was analysed in the FL2-H channel. Analysis was done on a single cell level with the initial acquisition performed on 30 000 – 50 000 events from which 5 000 – 15 000 cells were selected for FACS analysis. Selection of the final sample was achieved by gating control samples according to their side scatter (cell granularity) and forward scatter (cell length) (Figure 2.4 A & B) to ensure that debris and non-cellular particles were excluded. Fluorescence signals were analysed using Cellquest® 3.3 software (Becton Dickinson and Co, San Jose, CA) and were expressed as mean fluorescence intensity (% of control, control adjusted to 100%).

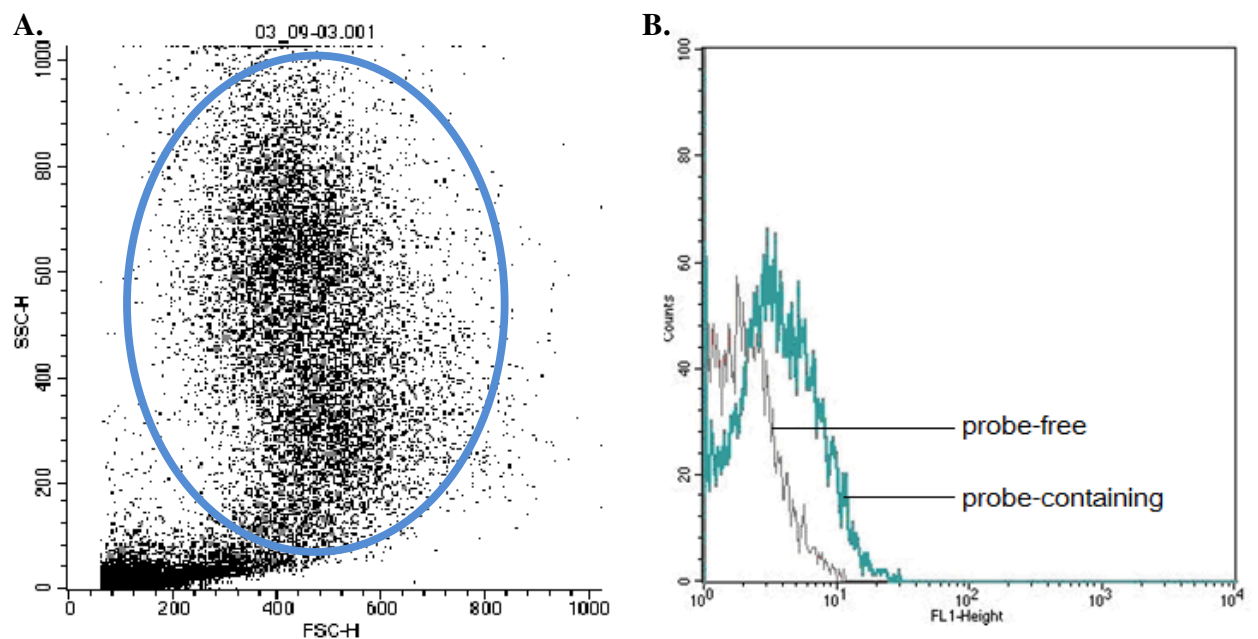


Figure 2.4: Flow cytometric analysis of isolated cardiomyocytes. (A) Representative scatterplot of a cardiomyocyte sample showing the distribution based on forward scatter (FSC-H) and side scatter (SSC-H) properties of the cells. The blue encircled region is the cell population of interest, which excludes debris and other particles. (B) A typical fluorescence histogram (FL1 channel) showing fluorescence of the probe-free cells (autofluorescence; grey curve) and DAF-2/DA-loaded control cells (green curve).

2.7 Western blot measurements

Western blotting was used to determine the amount of total and activated (phosphorylated (Ser 1177)) eNOS in both cell types and whole hearts. To determine eNOS expression and activation, samples were lysed in a buffer containing (in mM): Tris 20; ethylene glycol tetra-acetic acid (EGTA) 1;

ethylenediaminetetra-acetic acid (EDTA) 1; NaCl 150; β -glycerophosphate 1; sodium pyrophosphate 2.5; sodium orthovanadate 1; sodium dodecylsulphate (SDS) 10 %; Triton-X100 1 %; phenyl-methyl-sulphonyl-fluoride (PMSF) 50 μ g / mL; aprotinin 10 μ g / mL; leupeptin 10 μ g / mL. To measure PKB expression and activation, samples were lysed in a buffer containing (in mM): Tris 20; EGTA 1; EDTA 1; NaCl 150; β -Glycerophosphate 1; sodium orthovanadate 1; tetrasodium diphosphate 2.5; PMSF 1; 0.1% SDS; aprotinin 10 μ g / ml; leupeptin 10 μ g / ml, and 1% Triton-X100 was used. Protein levels were determined using the Bradford method (Bradford 1976). Lysate protein of equal amounts (100 -200 μ g for eNOS and 20 – 50 μ g for PKB) was loaded on 7.5 % (eNOS) and 10% (PKB) SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were probed with the respective rabbit polyclonal antibodies. The secondary antibody was horseradish peroxidase-linked anti-rabbit immunoglobulin G (IgG) (Amersham, Buckinghamshire, UK). The immunoreaction was visualised using the ECL™ system and film densitometry was analysed on Un-SCAN-IT (Silk Scientific, Orem, UT, USA). Data is presented as percentage of control, control adjusted to 100 %.

2.8 Statistical Analysis

All data is expressed as mean \pm SEM unless otherwise stated. For statistical analysis, Student's t-test (unpaired) or one-way ANOVA tests (with Bonferroni post-test if $p < 0.05$) were used. Results were considered significant if a p-value < 0.05 was achieved. Statistical analysis was performed on GraphPad Prism[®] 5.01.

Chapter 3: Results

Chapter 3: Results

3.1 Whole hearts

3.1.1 Nitrite production

When NO is broken down, it forms nitrite, therefore nitrite measurements can be used as an (indirect) indication of NO production, as described previously (Kleinborgard et al. 2002). After 15 min ischaemia nitrite production increased from control 75.21 ± 0.78 nmoles / mg protein to ischaemia 83.86 ± 1.08 nmoles / mg protein and; 20 min ischaemia increased nitrite production from control 70.51 ± 0.29 to ischaemia 75.17 ± 4.40 nmoles / mg protein ($p = 0.0021$; $n = 3$).

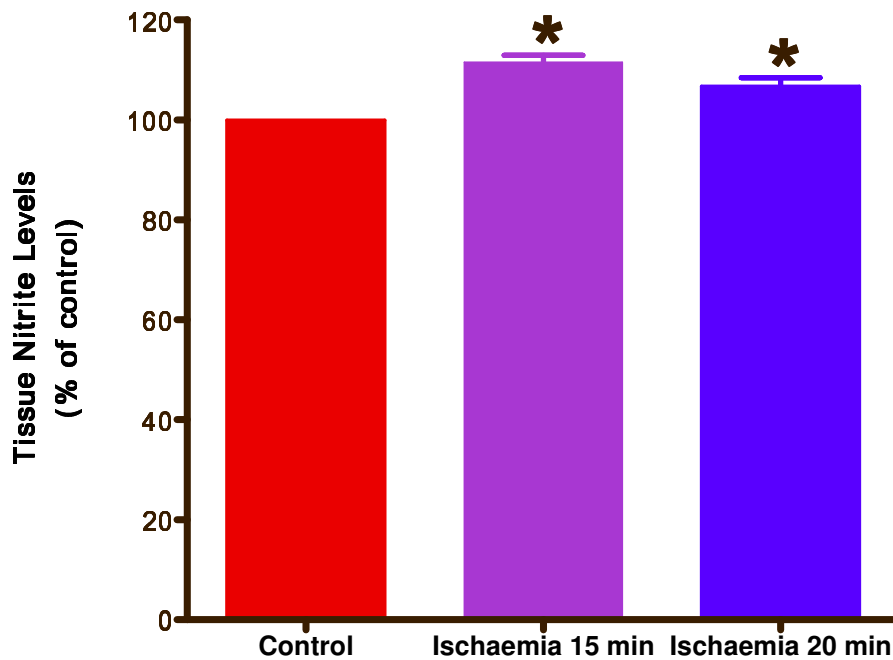


Figure 3.1: Nitrite production in whole hearts. Nitrite production increased significantly across all groups. Values are expressed as percentage of control (control adjusted to 100 %). *: $p = 0.0013$ at 15 min ischaemia and $p = 0.0139$ at 20 min ischaemia; $n = 3$.

3.1.2 eNOS expression and activation

3.1.2.1. Total eNOS protein

There was no change in total eNOS protein expression as measured by Western blotting (Figure 3.2) between the groups. These findings were validated by immunohistochemical (IHC) detection of eNOS which also showed no change in eNOS expression between control and ischaemic (20 min) hearts (Figure 3.3). To

validate that the IHC staining observed was due to the eNOS antibody, there was a significant difference between the means of the negative and positive controls ($p = 0.0151$) (Figure 3.4).

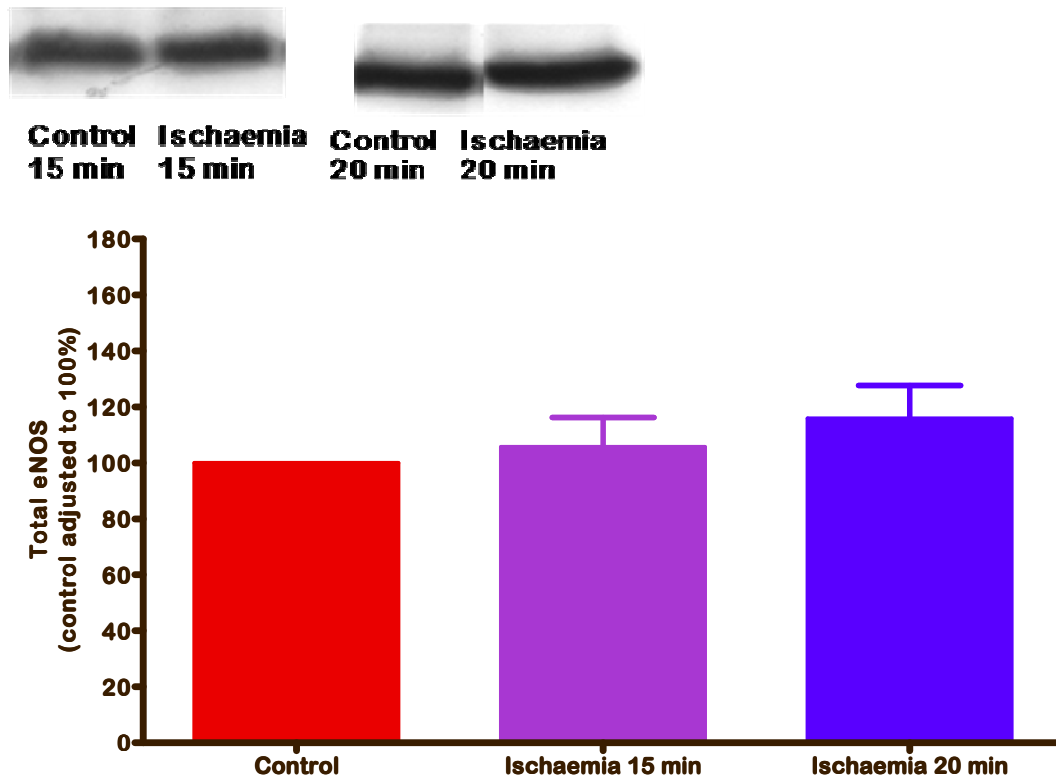
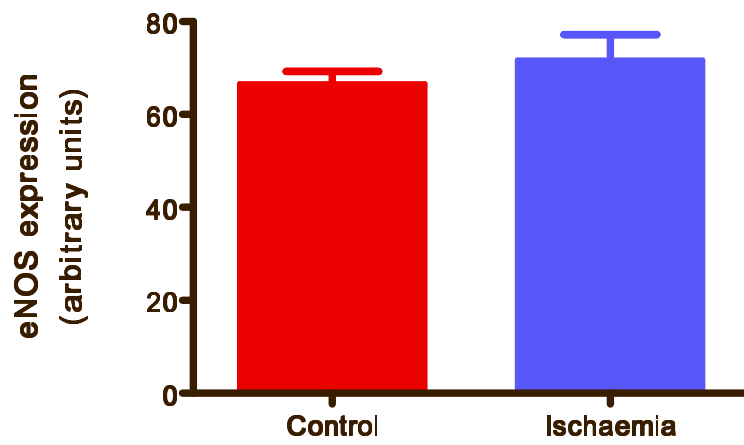


Figure 3.2: eNOS expression in whole hearts by Western blot. Expression of eNOS was unchanged between the groups. $n = 3-4$.

A



B

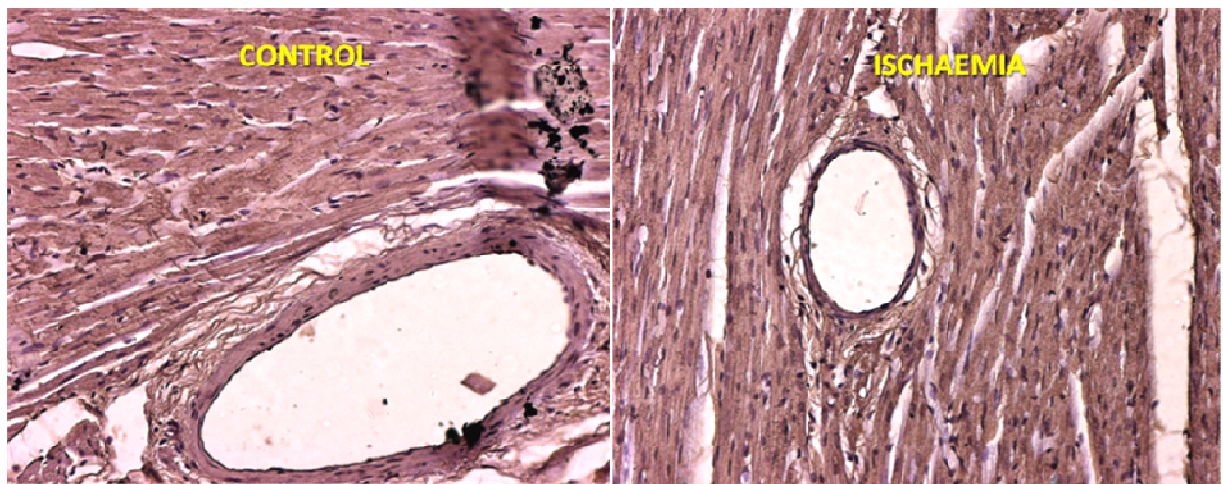
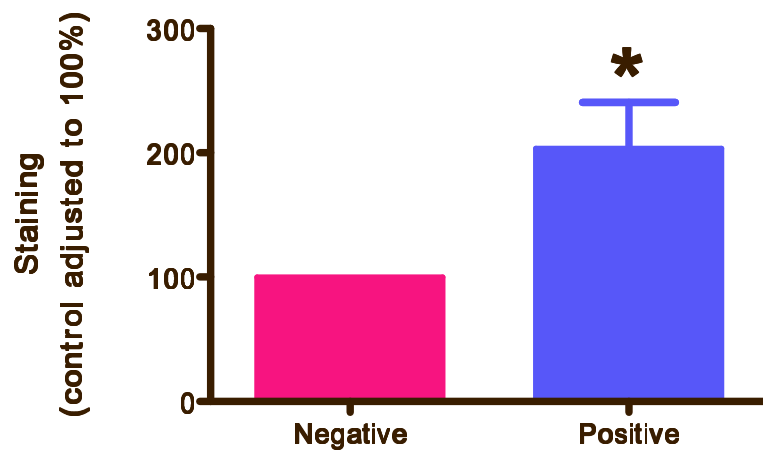


Figure 3.3: eNOS expression in whole hearts by IHC. Expression of eNOS in ischaemic hearts as detected by immunohistochemistry showed no difference between the groups. A: $p = 0.4632$. B: No difference in stain intensity was visually visible, magnification: 20x. $n = 4$.

A



B

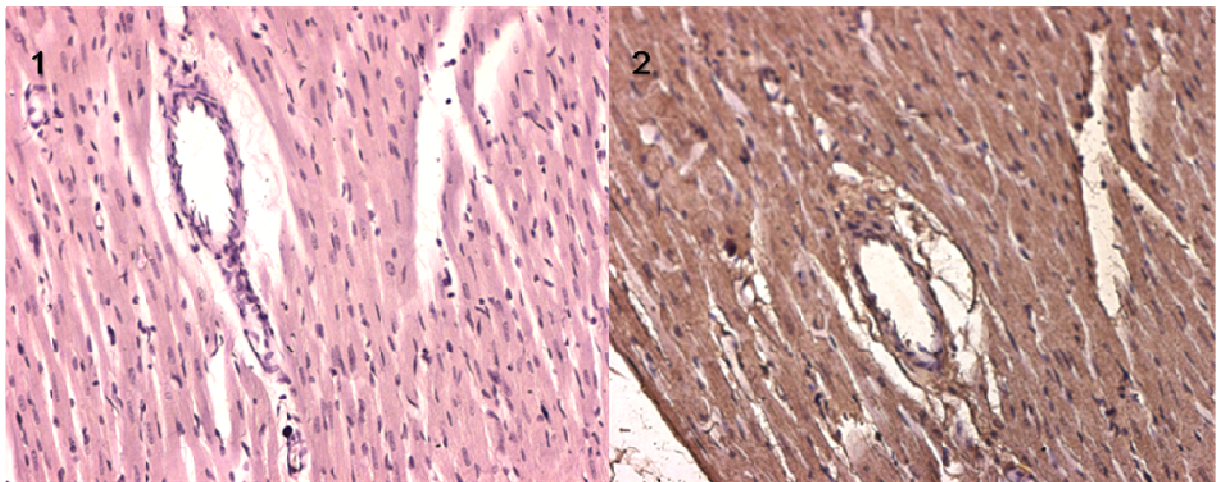


Figure 3.4: Negative control of Immunohistochemistry. A: $p = 0.0151$. B: 1 negative control; 2 positive control. Magnification: 20x, $n = 4$.

3.1.2.2 Activated (phosphorylated) eNOS- Ser¹¹⁷⁷

Phosphorylated eNOS (Figure 3.5) changed significantly in hearts subjected to ischaemia. Phosphorylated eNOS increased by 38 % after 15 min ischaemia (control: 100 % vs. ischaemia: 138.40 ± 7.82 %; n = 4). However, at 20 min ischaemia phosphorylated eNOS levels decreased by 48 % compared to control levels (control: 100 % vs ischaemia: 51.80 % ± 7.00 %; n = 4).

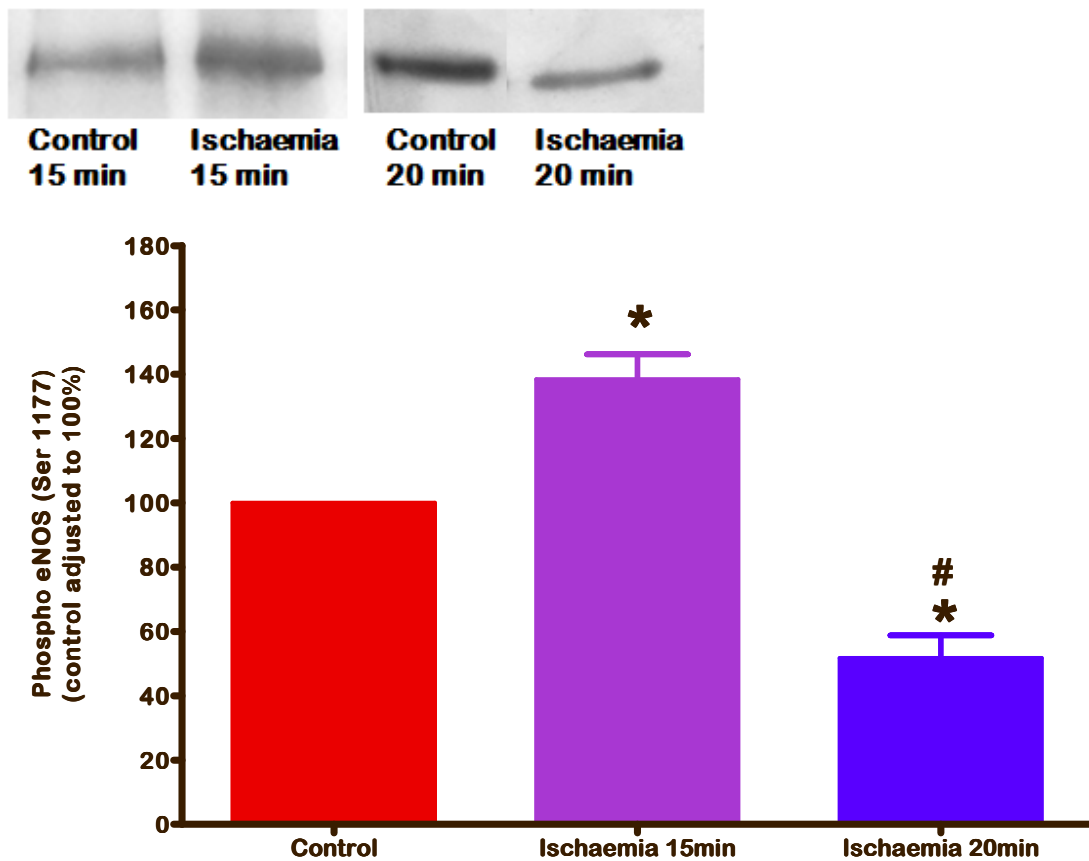


Figure 3.5: Phosphorylated eNOS in whole hearts. Phosphorylated (activated) eNOS increased after 15 min ischaemia but decreased after 20 min ischaemia. *: p < 0.0001 compared to control; #: p < 0.0001 compared to ischaemia 15 min.

3.2. Cardiomyocytes

3.2.1 Viability

To determine whether there was any hypoxia-induced injury to the cardiomyocytes we used the TBE and PI protocols. Both protocols (Figures 3.6 & 3.7) showed that hypoxia induced a significant degree of damage: TBE results show a 40 % reduction in viability (control: 100 % vs hypoxia: 58.71 ± 6.39 %; $n = 20$) whilst PI staining showed a 28 % reduction in viable cells (control: 100 % vs hypoxia: 72.11 ± 1.84 %; $n = 9$).

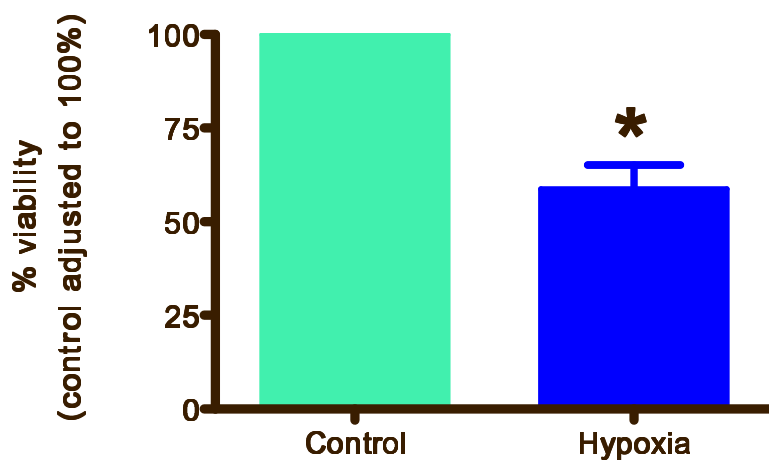


Figure 3.6: Cell viability as determined by TBE. Hypoxia-induced cell injury caused a decrease of 40 % in viability. *: $p = 0.0002$ vs control.

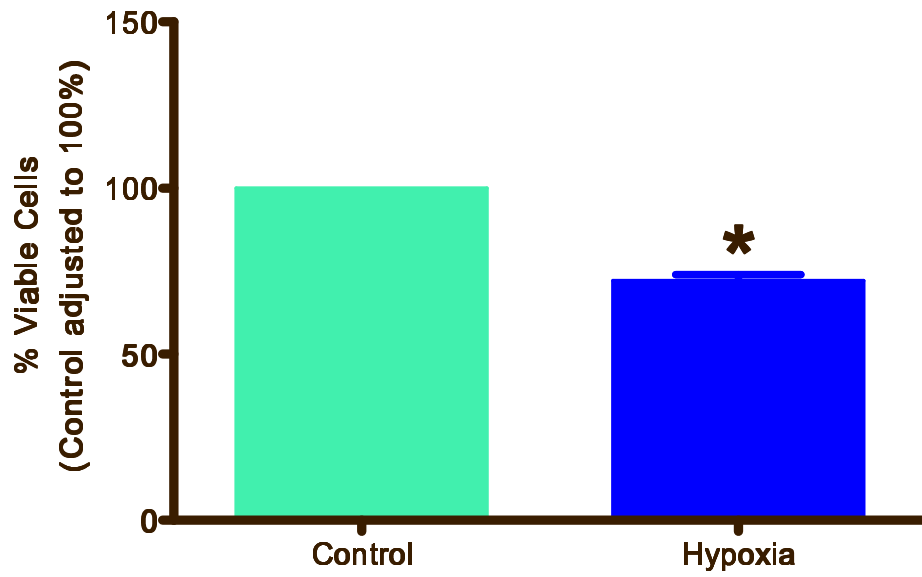


Figure 3.7: Cell viability as determined by PI uptake. Cell viability decreased by 28 % in hypoxic cells. *: $p < 0.0001$ vs control.

3.2.2 NO production

NO production was determined with the NO-specific fluorescent probe, DAF-2/DA. Results show a significant increase in mean DAF-2/DA fluorescence intensity in cardiomyocytes subjected to hypoxia (control: 100 % vs hypoxia: 124.10 ± 3.94 %; $n = 8$) (Figure 3.8). Although there seems to be a trend, there was no significant difference in nitrites between control and hypoxic cells (Figure 3.9).

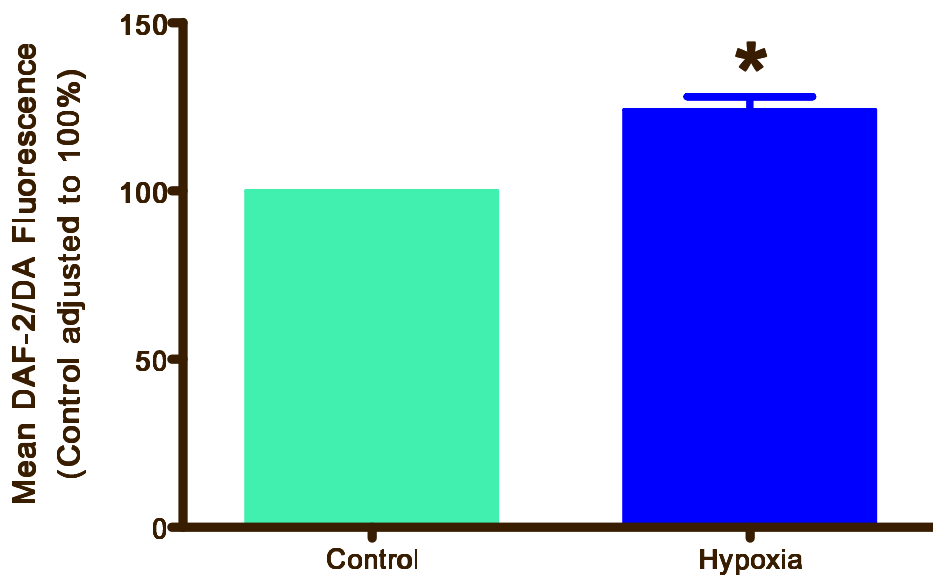


Figure 3.8: NO production in cardiomyocytes measured by DAF-2/DA fluorescence. *: $p < 0.0001$ vs control.

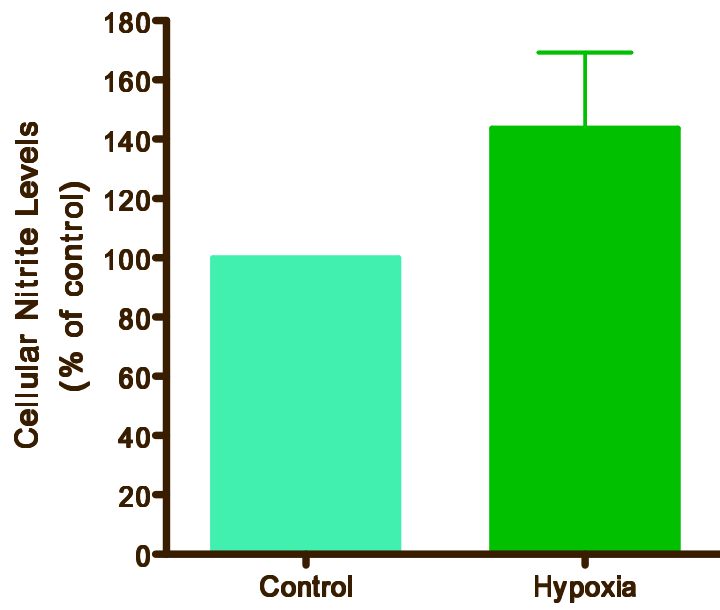


Figure 3.9: Nitrite production in cardiomyocytes. Nitrite levels were unchanged in both groups.

3.2.3 eNOS expression and activation

The expression and activation of eNOS were quantified using standard Western blotting techniques. The expression of eNOS protein in hypoxic cells increased to 165.20 ± 16.47 % compared to control (control: 100 %; n = 8) (Figure 3.10 A). Activated eNOS (phospho eNOS Ser¹¹⁷⁷) levels increased to 197.80 ± 24.98 % compared to control (control: 100 %; n = 7) (Figure 3.10 B).

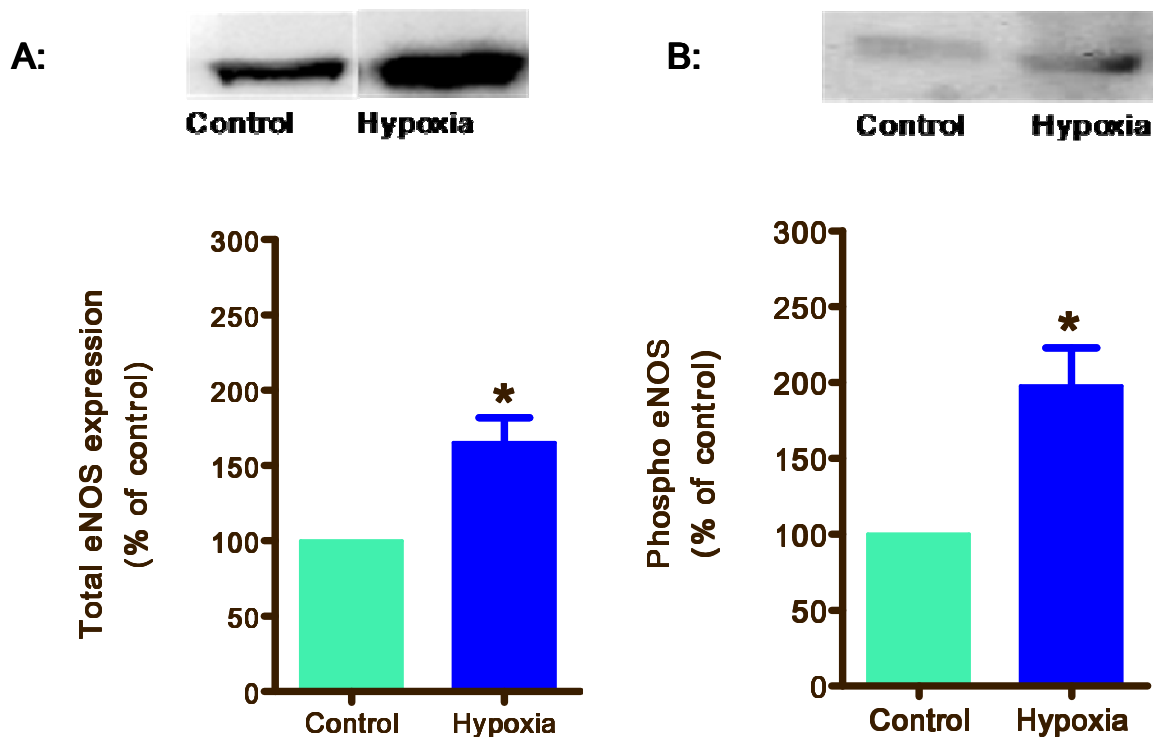


Figure 3.10: Expression and activation of eNOS in cardiomyocytes. **A:** Expression of eNOS protein increased by 65 % in hypoxic cells. *: p = 0.0105 compared to control. **B:** Phosphorylated eNOS increased by 98 % in hypoxic cells. *: p = 0.0384 compared to control. The relative phospho eNOS / total eNOS despite an increase in total eNOS.

3.3 CMECs

3.3.1 Viability

In hypoxic CMECs, cell viability decreased by 41 % compared to control (control: 100 % vs hypoxia: 58.73 ± 9.86 %; $n = 4$) (Figure 3.11). The viability data demonstrates that the hypoxia protocol was effective in causing cell injury.

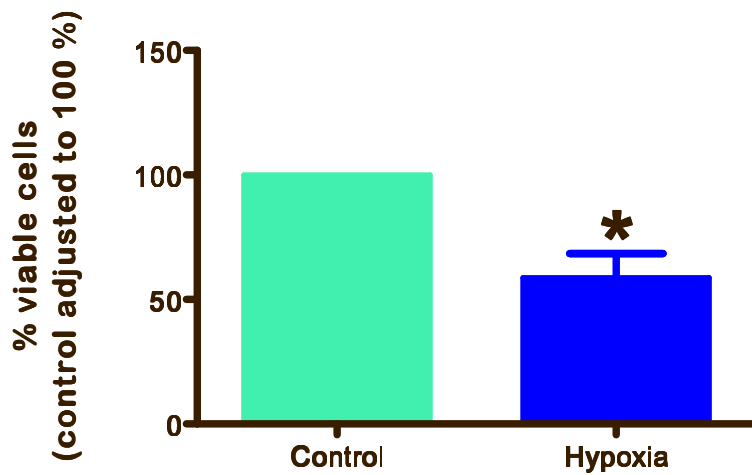


Figure 3.11: Cell viability as determined by PI uptake. Hypoxia-induced cell injury resulted in a 41 % reduction in viability. *: $p = 0.0057$ compared to control.

3.3.2 NO production

Cells were incubated in media containing 10 μ M DAF-2/DA to determine NO production. Mean DAF-2/DA fluorescence intensity increased by 55 % in hypoxic cells (control: 100 % vs hypoxia: 155.10 ± 7.00 %; $n = 10$) (Figure 3.12). Hypoxia did not have a significant effect on nitrite levels (Figure 3. 13).

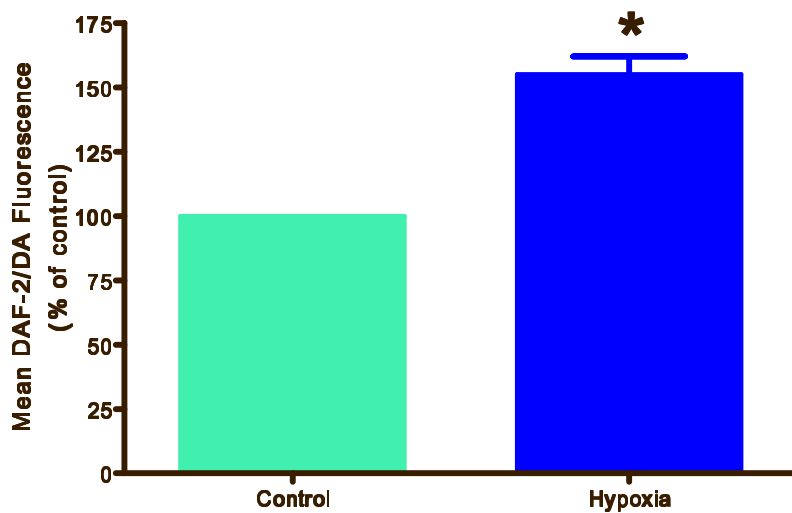


Figure 3.12: NO production in CMECs measured by DAF-2/DA fluorescence. In hypoxic CMECs, fluorescence increased by 55 %. *: $p < 0.0001$ vs control.

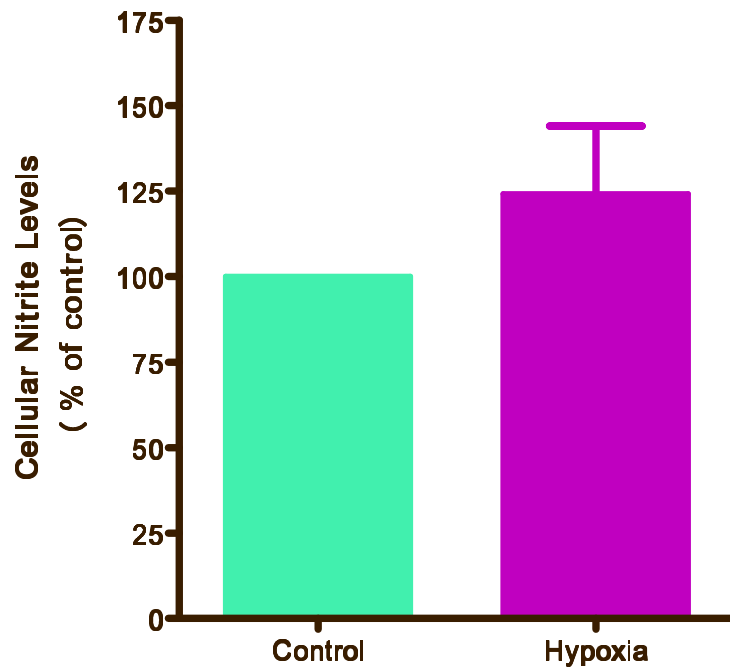


Figure 3.13: Nitrite production in CMECs. Hypoxia did not have an effect on nitrite levels.

3.3.3 eNOS expression and activation

Total eNOS protein expression increased to $179.50 \pm 34.23\%$ in hypoxic CMECs compared to control (control: 100 %; n = 5) (Figure 3.14 A). A concomitant increase in phosphorylated eNOS to $294.00 \pm 86.62\%$ was also observed in the hypoxic CMECs (control: 100 %; n = 6) (Figure 3.14 B).

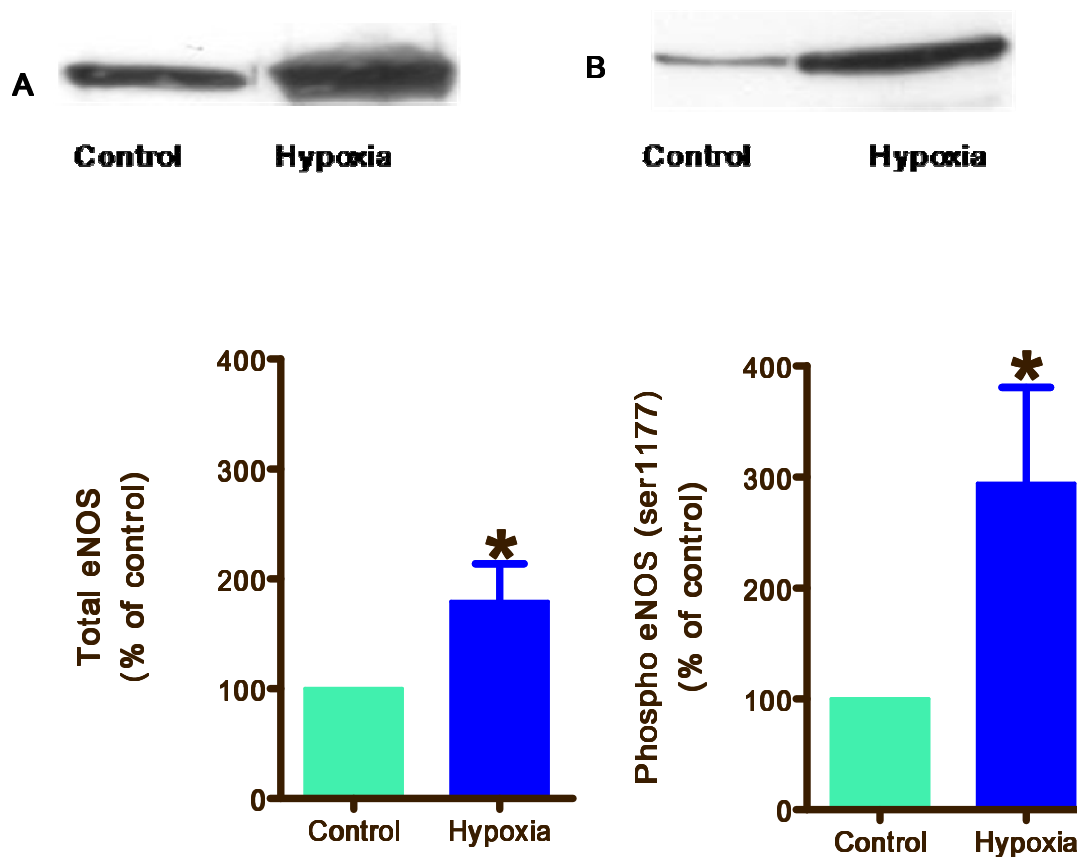


Figure 3.14: Expression and activation of eNOS in CMECs. A: expression of eNOS increased to $179.5 \pm 34.23\%$. *: p = 0.0487 vs control. B: Activated eNOS levels increased to $294.0 \pm 86.62\%$. *: p = 0.0490 vs control. The relative phospho eNOS / total eNOS ration increased despite an increase in total eNOS.

3.4 PI3-K / PKB investigations

3.4.1 Expression and activation of PKB

Expression and activation of PKB was determined using standard western blotting techniques. In hypoxic cardiomyocytes, expression and activation of PKB increased 84 % (control: 100 % vs hypoxia: 184.40 ± 23.13 %; n = 6) and 88 % (control: 100 % vs hypoxia: 188.40 ± 32.65 %; n = 6) compared to control, respectively (Figure 3.15). In hypoxic CMECs, there was no change in PKB expression but, a 69 % increase was observed in phosphorylated PKB, compared to control (control: 100 % vs hypoxia: 168.70 ± 15.06 %; n = 6) (Figure 3.16).

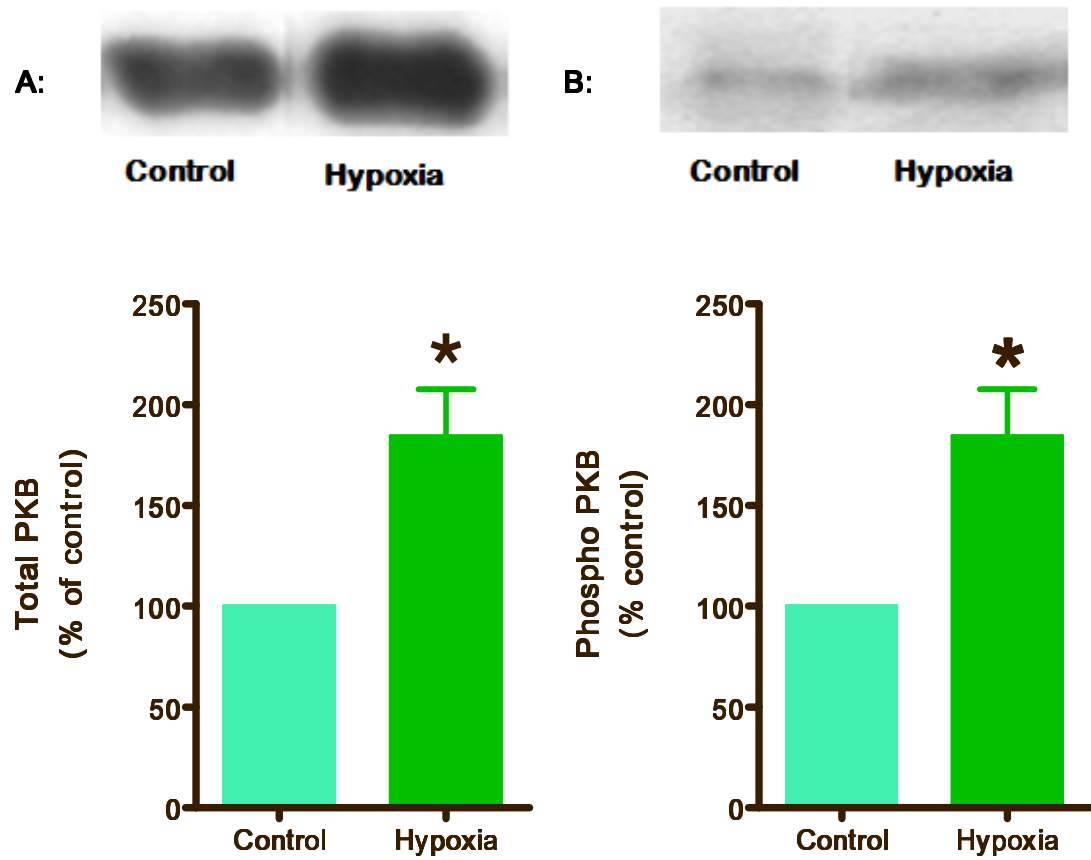


Figure 3.15: Expression and activation of PKB in cardiomyocytes. A: Expression of PKB increased by 84 % in hypoxic cells. *: $p = 0.0193$ compared to control. B: Activation of PKB increased by 88 % in hypoxic cells. *: $p = 0.0220$ compared to control.

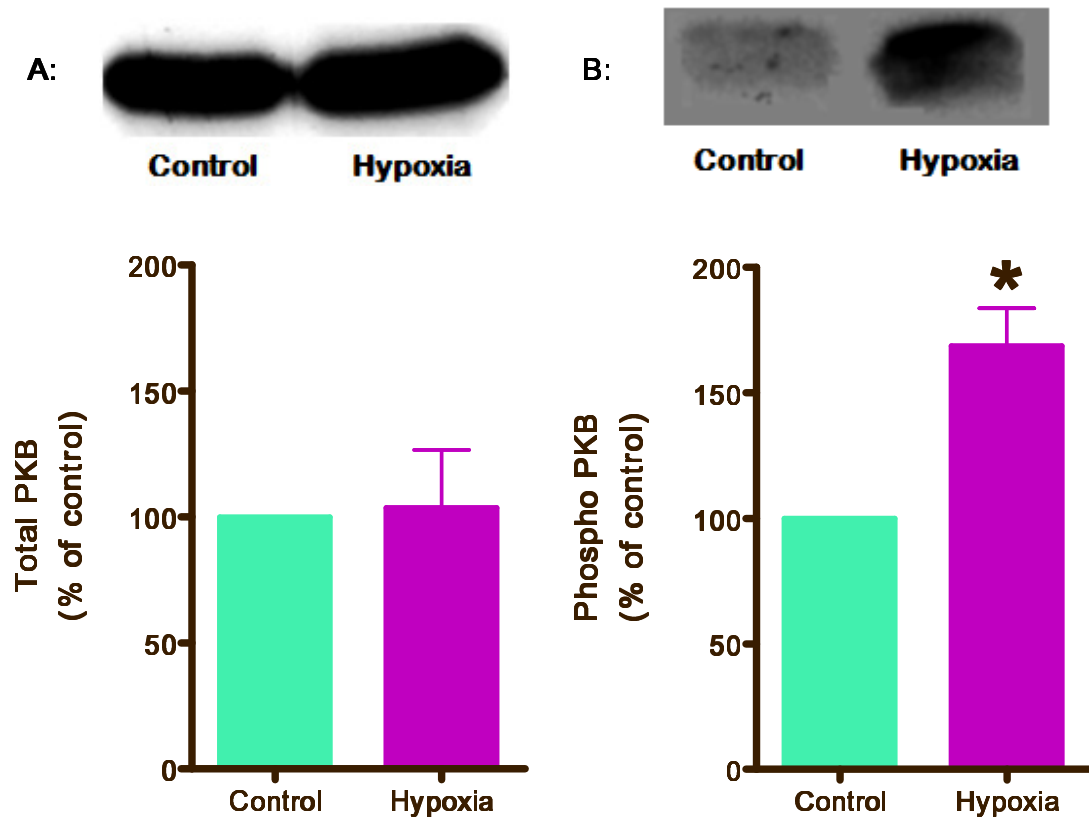
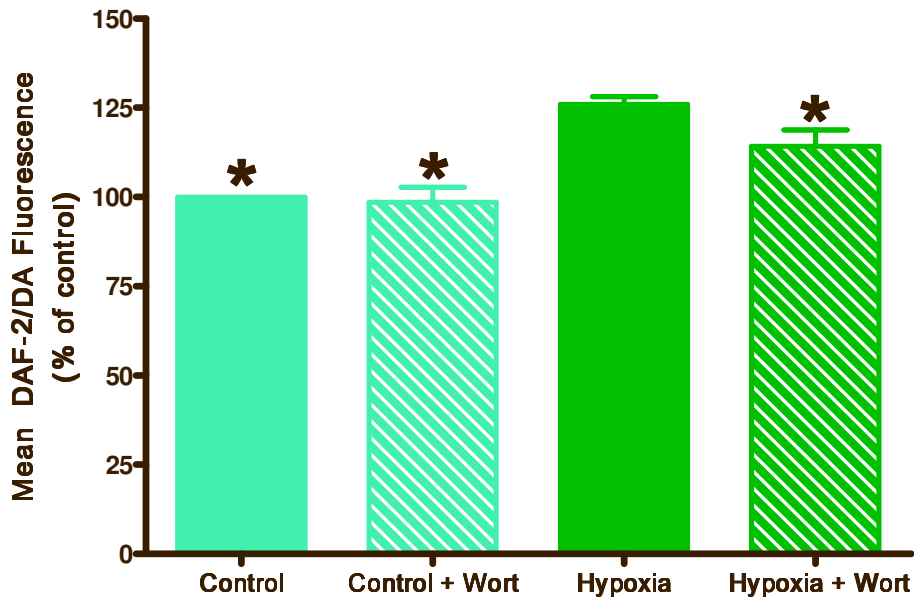


Figure 3.16: Expression and activation of PKB in CMECs. A: Expression of PKB did not change between the control and hypoxic cells. B: Activation of PKB increased by 69 % in hypoxic cells. *: $p = 0.0129$ vs control.

3.4.2 Inhibition of PI3-K / PKB pathway: effect on NO production

DAF-2/DA fluorescence was measured in isolated cardiac cells treated with 100 nM wortmannin (PI3-K inhibitor) to determine whether the PI3-K / PKB pathway plays a role in NO production during hypoxia. In cardiomyocytes, DAF-2/DA fluorescence decreased by 12 % in treated hypoxic cells compared to untreated hypoxic cells (untreated control: 100%; treated control 98.55 ± 4.25 %; untreated hypoxia: 126.00 ± 2.14 %; treated hypoxia: 114.20 ± 4.60 %; n = 5) (Figure 3.17 A). NO production was also significantly decreased in the CMECs: treated hypoxic cell fluorescence decreased by 58 % compared to untreated hypoxic cells (untreated control: 100 %; treated control: 93.64 ± 5.87 %; untreated hypoxia: 187.10 ± 20.95 %; treated hypoxia: 129.20 ± 12.03 %; n = 5) (Figure 3.17 B).

A:



B:

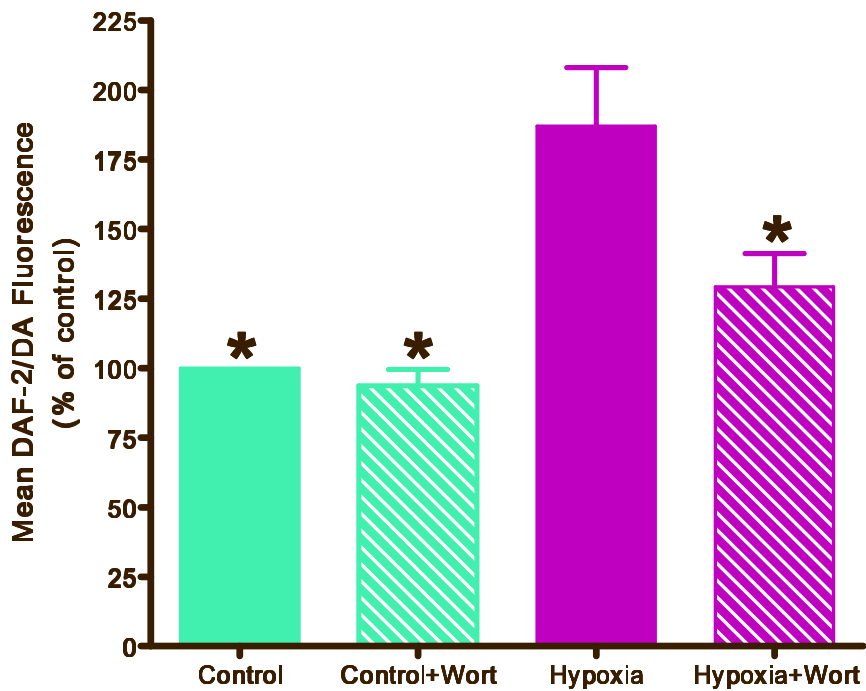


Figure 3.17: Effect of wortmannin on NO production in isolated cardiac cells. A: In cardiomyocytes, wortmannin significantly decreased DAF-2/DA fluorescence in treated hypoxic cells (114.2 ± 4.597 %) compared to untreated hypoxic cells. *: $p < 0.0001$ vs. untreated hypoxia. B: in CMECs, wortmannin significantly decreased DAF-2/DA fluorescence in treated hypoxic cells (129.2 ± 12.03 %) compared to untreated hypoxic cells. *: $p < 0.0001$ vs. untreated hypoxia.

3.4.3 eNOS expression and activation in the presence of PI3-K / PKB inhibition

To determine the effects of the PI3-K / PKB pathway on eNOS expression and activation during hypoxia, we incubated cardiomyocytes with 100 nM wortmannin (PI3-K inhibitor). eNOS expression and activation were determined by Western blotting. Wortmannin had no effect on the expression of eNOS during hypoxia (n = 6) (Figure 3.18).

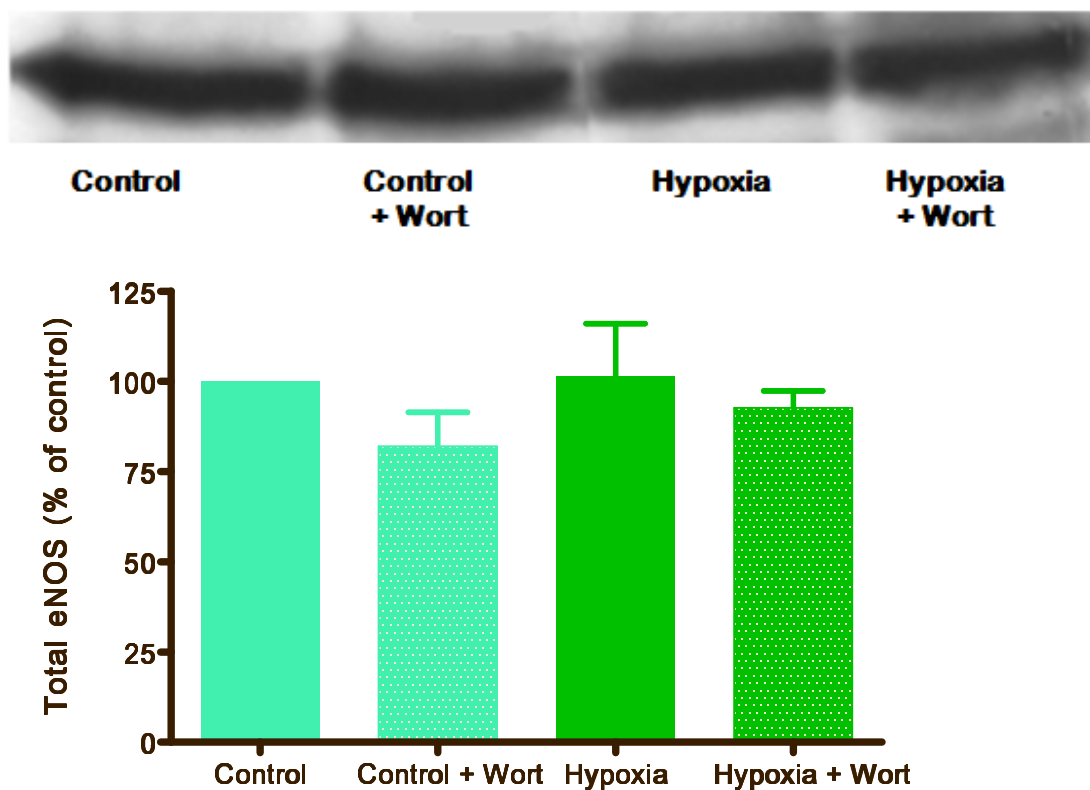


Figure 3.18: Effects of wortmannin on eNOS expression in cardiomyocytes. Wortmannin had no effect on eNOS expression.

Treatment with wortmannin decreased phosphorylated eNOS by 41 % under baseline conditions in treated cells (untreated control: 100 % vs treated control: 58.65 ± 6.63 %; n = 4) and in treated hypoxic cells it decreased by 23 % compared to untreated hypoxic cells (untreated hypoxia: 124.80 ± 3.22 % vs treated hypoxia: 101.80 ± 7.13 %; n = 6) (Figure 3.19).

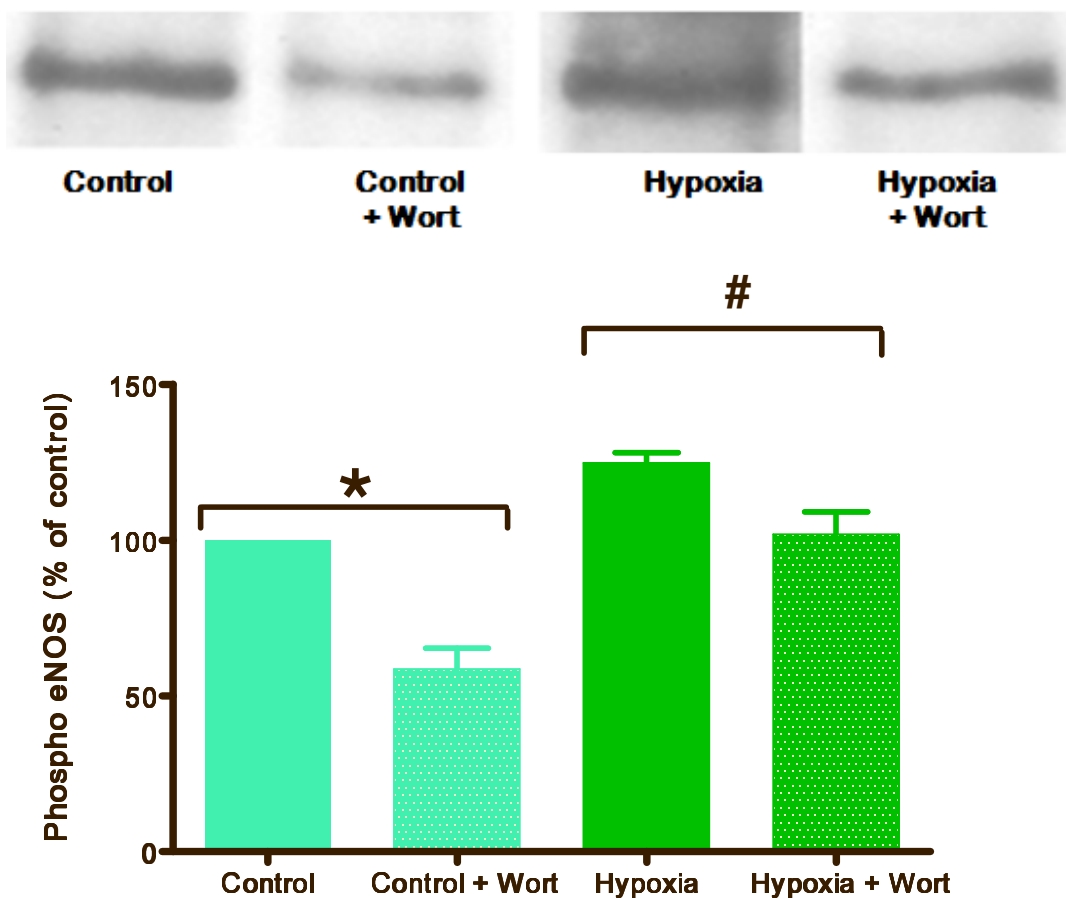


Figure 3.19: Effect of wortmannin on eNOS activation in cardiomyocytes. Wortmannin decreased baseline activation of eNOS to 58.65 ± 6.625 % compared to untreated control. In hypoxic cardiomyocytes, wortmannin significantly reduced phospho eNOS levels by 23 % compared to untreated hypoxic groups (untreated hypoxia: 124.8 ± 3.216 % vs treated hypoxia: 101.8 ± 7.125 %; n = 6). *: $p < 0.0001$; #: $p = 0.0230$.

Chapter 4: Discussion

Chapter 4: Discussion

4.1. Summary of results

4.1.1. NO production and eNOS regulation / activation in whole heart and cardiac cell models

Our whole heart data show that 15 min and 20 min of global ischaemia induced increased production of NO as measured by tissue nitrite levels (Figure 3.1); this trend was also observed in our cardiac cell models, viz. cardiomyocytes (Figure 3.8) and CMECs (Figure 3.12) exposed to 60 min hypoxia, as measured by a NO-specific fluorescent probe (DAF-2/DA). We repeated the studies using extracellular nitrite release as endpoint and similar changes were observed, albeit not significant (Figure 3.9 and Figure 3.13). Expression of total eNOS protein in the ischaemic whole hearts remained at control levels at both 15 min and 20 min as demonstrated by both IHC measurements (Figure 3.4) and Western blotting (Figure 3.2), whereas activated eNOS levels (Western blot analysis) increased at 15 min ischaemia, but were significantly reduced at 20 min ischaemia (Figure 3.5). In hypoxic cardiomyocytes, total eNOS expression

increased (Figure 3.10 A), which was accompanied by increased activated eNOS levels (Figure 3.10 B). These trends were also observed in CMECs (Figure 3.14 A and Figure 3.14 B).

4.1.2. The role of PI3-K / PKB in NO production and eNOS regulation / activation

Our investigations into a possible role for the PI3-K / PKB pathway in hypoxia-associated NO production indicate that inhibition of this pathway (by wortmannin, PI3-K inhibitor) resulted in reduced NO production in both cardiomyocytes (Figure 3.15 A) and CMECs (Figure 3.15 B), as measured by DAF-2/DA fluorescence analysis. Further exploration of possible PI3-K / PKB involvement demonstrated increased expression of total PKB protein (Western blot analysis) in cardiomyocytes at 60 min hypoxia (Figure 3.16 A) accompanied by increased activated PKB levels (Figure 3.16 B). In hypoxic CMECs, total PKB expression remained at control levels (Figure 3.17 A), but activated PKB increased (Figure 3.17 B). Finally, we looked at the effects of PI3-K / PKB pathway inhibition (wortmannin administration) on eNOS regulation / activation in hypoxic cardiomyocytes. Results show that although total eNOS expression was not affected (Figure 3.18), activated eNOS levels decreased significantly (Figure 3.19).

4.2. NO Production

4.2.1. Whole heart model vs. isolated cell models

It is now generally accepted that oxygen deprivation, whether it be ischaemia or hypoxia, results in increased NO production in the heart (Ferdinandy & Schulz 2003). This phenomenon has previously been demonstrated separately in whole heart models (Bolli 2001; Depré et al. 1997), and isolated cell models (Kitakaze et al. 1995; Strijdom et al. 2004). As far as we are aware, however, there is no evidence of studies that investigated and compared NO production under conditions of low oxygen supply in whole hearts and the important NO generating cell types that make up the myocardium *in a single* study.

Ventricular cardiomyocytes and cardiac microvascular endothelial cells are major sources of overall cardiac NO production, and gaining more knowledge on their distinct behaviour under hypoxic conditions could help explain whether whole heart findings are a reflection of events on cellular level. Our study shows an increase in NO production in all ischaemic / hypoxic models investigated, viz. whole hearts (11.5% increase after 15 min ischaemia and 6.6% after 20 min ischaemia), isolated cardiomyocytes (24% increase after 60 min hypoxia) and isolated CMECs (55% increase after 60 min hypoxia). Our data suggest that the

percentage increase in the whole heart model is not identical to the cell models; this could be due to differences in experimental models and protocols, or a lower sensitivity of the nitrite assay used in the whole heart experiments. However, the importance of the *similarity* in the observed trends (i.e. increased production) cannot be ignored, and our data suggest that the elevated NO levels in the ischaemic myocardium are at least in part due to the contributions of cardiomyocytes and CMECs.

4.2.2. Real-time, direct intracellular NO measurements

Our studies on NO production in isolated cells using extracellular nitrite levels, an indirect method of NO production detection, were not significant even though there was a trend suggestive of increased production. Therefore, we used a more sensitive and direct method to determine NO production in the isolated cells. Several fluorescent probe-based methods have been developed that aim to detect direct, real-time NO in live cells such as the ester derivative of 4-((3-amino-2-naphthyl) aminomethyl) benzoic acid (DAN-1 EE) and 4, 5-diaminofluorescein (DAF-2) (Kojima et al. 1997; Kojima et al. 1998). DAN-1 EE

was not considered for this study due to its cytotoxicity and strong autofluorescence (Kojima et al. 1998).

In order for DAF-2 to be able to enter the cell, it has to be acetylated (DAF-2/DA) and thereafter it is hydrolysed by intracellular esterases to DAF-2 (Kojima et al. 1998). DAF-2 is specific for NO and only fluoresces after reacting with NO (Kojima et al. 1998). Various studies have used DAF-2 to measure NO in vitro in different cell types, including smooth muscle cells (Kojima et al. 1998) and endothelial cells (Leikert et al. 2001) using microscopy techniques. However, the use of DAF-2/DA to determine intracellular NO production in adult cardiomyocytes by flow cytometry was demonstrated for the first time in our laboratory (Strijdom et al. 2004b).

4.2.3. Increased NO production: the next step

Our NO measurement data confirms previous findings in literature that low oxygen supply leads to increased NO production in the heart. The fact that NO production increases under these circumstances has important implications, since most authors agree that NO released during hypoxia and ischaemia is beneficial and protective (Jones & Bolli 2006; Du Toit et al. 1998; Lochner et al.

2000). As explained in the literature review chapter, IHD is associated with high morbidity and mortality and every research effort should be employed to gain better understanding of NOs protective properties, and therefore as a potential future therapeutic tool.

In order for us to gain more understanding of the underlying mechanisms of increased NO generation, exploration of the possible subcellular sources is necessary. We know that the increased NO generation is at least in part due to increased NOS activity (Schulz et al. 2004). An important NOS isoform candidate in this regard is eNOS, therefore, as a next step, we investigated eNOS expression and activation in our models.

4.3. Expression and activation of eNOS

4.3.1. The importance of investigating eNOS as a putative source of increased NO production

Of the three NOS isoforms, eNOS is the most abundant in the heart, being expressed in cardiomyocytes, endothelium, connective tissue and fibroblasts (Fan et al. 2007; Keil et al. 2002; Bloch et al. 2001b; Dudzinski et al. 2006; Shaul 2002; Jiang et al. 2008). Under physiological conditions, eNOS is responsible for NO production associated with the maintenance of baseline cardiac function (Sarkar et al. 2001; Giraldez et al. 1997; Strijdom et al. 2006). The role of eNOS during physiological conditions is relatively well understood, however, its role and regulation during ischaemia / hypoxia is under-researched and not well understood (Shah & McCarthy 2000; Jung et al. 2000; Ferreiro et al. 2001). In this study we attempted to address this shortcoming by investigating the role of eNOS in three distinct models, viz the perfused whole heart, isolated cardiomyocytes and CMECs.

4.3.2. Activation of eNOS on Ser¹¹⁷⁷

There are five possible phosphorylation sites on eNOS (see Figure 1.5), namely serine residues 114 (Ser¹¹⁴), 615 (Ser⁶¹⁵), 633 (Ser⁶³³), 1177 (Ser¹¹⁷⁷) and threonine residue 495 (Thr⁴⁹⁵) (Mount 2007). Of these five sites, Ser¹¹⁷⁷ has been the most widely researched. Phosphorylation of Ser¹¹⁷⁷ activates eNOS (see Figure 1.7) and a variety of molecules, i.e. shear stress, insulin, bradykinin and statins, have been shown to phosphorylate eNOS at this site (Boo et al. 2002; Montagnani et al. 2001; Harris et al. 2001; Harris et al. 2004). However, very few authors have investigated a link between hypoxia and phosphorylation of the eNOS Ser¹¹⁷⁷ residue, even though it is viewed as the most common activation site of eNOS. To our knowledge, this is the first study to demonstrate that hypoxia leads to eNOS activation via phosphorylation at Ser¹¹⁷⁷ in adult cardiomyocytes and CMECs.

4.3.3. Whole heart model vs. isolated cell models

Various studies have shown conflicting results with regards to the expression and activation of eNOS in ischaemic / hypoxic conditions either in whole hearts (Depré et al. 1997) or isolated cells (Faller 1999; Arnet et al. 1996; Giraldez et al.

1997; Laufs et al. 1997;). In literature, changes in eNOS expression are variable: some studies observed an increase (Bell et al. 2002), whilst some observe no change (Guo et al. 1999) and others still observed a decrease in whole hearts (Takimoto et al. 2000). We have shown that total eNOS protein expression remains unchanged in ischaemic whole hearts (confirmed by two independent eNOS protein detection techniques) whilst it increased in isolated hypoxic cells. To the best of our knowledge, this is the first study to compare eNOS expression and activation in a whole heart model with isolated cell models, in a single study, the implication of which will be discussed later.

In both the cardiac cell models, we observed an increase in eNOS protein expression. This upregulation in eNOS protein could have contributed partially to the increase in NO production observed in the cell models but not in the whole hearts. From the cell data, it appears as if hypoxia has direct effects on eNOS gene expression, leading to increased protein synthesis.

In the whole hearts, after 15 min ischaemia, activated eNOS increased; however, at 20 min ischaemia, there was a significant reduction in activated eNOS levels. Therefore, the 15 min global ischaemia data in the whole heart model followed similar trends observed on the cell models with regard to eNOS activation. The

reduction in activated eNOS after 20 min ischaemia was observed despite increased NO production. From this observation, it appears that eNOS cannot be considered as a likely source of NO at 20 min of ischaemia. Our findings are similar to those of a recent study on rabbit hearts in which hypoxia caused a decrease in activated eNOS levels, despite elevated levels of NO being maintained. This was attributed to an increased association of hsp90 with eNOS which is thought to play a role in increasing coupled eNOS activity (Shi et al. 2002). It is also possible that other (eNOS-independent) sources of NO were activated in the 20 min ischaemia group such as iNOS, nNOS and the reduction of nitrate and nitrite to NO due to ischaemia-induced acidosis (Ding et al. 2005; Millar et al. 1998; Zweier et al. 1999).

Furthermore, in the whole heart, one needs to keep in mind that there are many cell types present and their influence on eNOS regulation has not been explored even though it is known that eNOS is also expressed in fibroblasts, connective tissue, atrial cardiomyocytes and other sub-types of endothelial cells (Fan et al. 2007; Keil et al. 2002; Bloch et al. 2001b; Shaul 2002).

There is a greater possibility of paracrine communication between different cell types in the whole heart compared to isolated cell models and, given its ability to easily diffuse across membranes, NO is a candidate paracrine messenger.

NO has been shown to have a negative feedback regulatory role on eNOS (Grisvage et al. 1995). VEGF has also been shown to have a dual role: it can activate and inactivate eNOS depending on the kinase involved. If VEGF leads to activation of PKB / Akt, eNOS Ser¹¹⁷⁷ is phosphorylated and Thr⁴⁹⁵ is dephosphorylated, leading to activation of eNOS; on the other hand, if PKC is activated, it in turn, phosphorylates Thr⁴⁹⁵ and dephosphorylates Ser¹¹⁷⁷ leading to inactivation of eNOS (Chen et al. 1999; Michell et al. 2001). In summary, many factors are at play in the multicellular, whole heart model which could explain the contradictory findings observed at 20 min global ischaemia.

In the isolated cell models, findings suggest that in cardiomyocytes and CMECs, increased NO generation was due to a combination of protein expression and increased activation of eNOS. Therefore, we can conclude that an increase in the expression and activation of eNOS is an important source of increased NO production in the isolated cardiac cells during hypoxia.

By and large, it seems as if eNOS protein expression is to some extent (up-) regulated by hypoxia, at least in the isolated cell models, which could partly explain the increase in NO generation. Ischaemia and hypoxia also seem to activate eNOS in all models, with the exception of longer (more severe) duration of ischaemia in the whole hearts (20 min global ischaemia) as discussed earlier.

The upstream mechanism by which hypoxia could increase the activation of eNOS is not well understood; thus we next investigated a putative role for the PI3-K / PKB pathway.

4.4. PI3-K / PKB pathway

To date, the best known mechanism of eNOS Ser¹¹⁷⁷ phosphorylation is through activated PKB (Dimmeler et al. 1999). Various molecules, i.e. insulin and high density lipoproteins (HDL), have been shown to activate eNOS through the PI3-K / PKB pathway (Gao et al. 2002; Mineo et al. 2003). A fairly recent study on porcine coronary endothelial cells showed that hypoxia activated eNOS in a PI3-K / PKB –dependent mechanism (Chen & Meyrich 2004).

In our study, there was an increase in the expression of PKB protein after hypoxia in the cardiomyocytes although protein expression remained unchanged in the endothelial cells. Our observations in cardiomyocytes are contradictory to observations from studies in literature which show no change in PKB expression (Chen et al. 2001; Engelbrecht et al. 2004). This inconsistency could be due to the difference in age (adult vs. neonatal), animal species of the cardiomyocytes and, difference in protocols used.

We were able to show an increase in PKB phosphorylation (Ser⁴⁷³) in our isolated cardiac cell models after exposure to 60 min of hypoxia. Our results are in line with other studies in literature which show an increase in PKB activity after

hypoxia in human prostate cancer cells (Zhong et al. 2000), retinal epithelial cells (Treins et al. 2002) and porcine coronary artery endothelial cells (Chen & Meyrich 2004). The increase in activated PKB was temporally associated with the increase in eNOS phosphorylation on Ser¹¹⁷⁷ (at 60 min hypoxia). Nonetheless, this does not necessarily prove that hypoxia activates eNOS in a PI3-K / PKB-dependent mechanism.

Consequently, we looked at the effect of PI3-K inhibition on NO production and eNOS expression and activation. PI3-K inhibition by wortmannin reduced NO production in both hypoxic cardiomyocytes and endothelial cells. This was associated with a decrease in activated eNOS in the cardiomyocytes which seemed to indicate that wortmannin suppressed hypoxia-induced eNOS activation (Figure 3.19). These results are in agreement with previous studies on rat lung vascular endothelial cells (Hisamoto et al. 2001) and Chinese hamster ovary cells (Sampaio et al. 2007) which found that inhibition of the PI3-K / PKB pathway decreased eNOS phosphorylation. To the best of our knowledge, this is the first time that hypoxia has been shown to activate eNOS phosphorylation through a PI3-K / PKB-dependent mechanism: in adult cardiomyocytes and CMECs. Our data confirm the findings of a previous study on porcine coronary

endothelial cells, in which PKB was shown to be the mediator for eNOS activation by hypoxia (Chen & Meyrich 2004).

In summary, our results show that hypoxia increases the activation of PKB (phospho Ser⁴⁷³) in isolated cardiac cells. Inhibition of the PI3-K / PKB pathway reduces NO production in both cardiomyocytes and CMECs. Furthermore, hypoxia activates eNOS phosphorylation in cardiomyocytes by PKB activation as inhibition of the PI3-K / PKB pathway attenuated the hypoxia-induced increase in eNOS phosphorylation and subsequent NO production. These findings suggest that the PI3-K / PKB pathway can be considered as a likely upstream mechanism of eNOS derived-NO production in hypoxia. Further investigations are necessary to elucidate the signalling mechanisms through which hypoxia activates the PI3-K / PKB pathway.

Chapter 5: Conclusions

Chapter 5: Conclusions

5.1. Conclusions

To the best of our knowledge, this is the first study to investigate and compare the effects of ischaemia / hypoxia on NO production and eNOS expression in the whole heart and isolated cells in a single study. Our study demonstrates that increased NO production in the whole heart is indeed a result of increases observed in cardiomyocytes and CMECs as similar trends were observed. In addition, we have shown that at 15 min ischaemia the observations in the whole heart are similar to events at cellular level with regard to eNOS activation, but not at 20 min ischaemia. In both cell models hypoxia has a direct effect on eNOS protein expression. Inhibition of the PI3-K / PKB pathway reduced hypoxia-induced NO production in both cell models and hypoxia-induced eNOS activation in cardiomyocytes. Therefore, we have, for the first time, demonstrated that the increase in NO production during hypoxia is due (at least in part) to an increase in eNOS phosphorylation and this increase is mediated via the PI3-K / PKB pathway. A proposed diagram of the underlying mechanisms is depicted in figure 5.1.

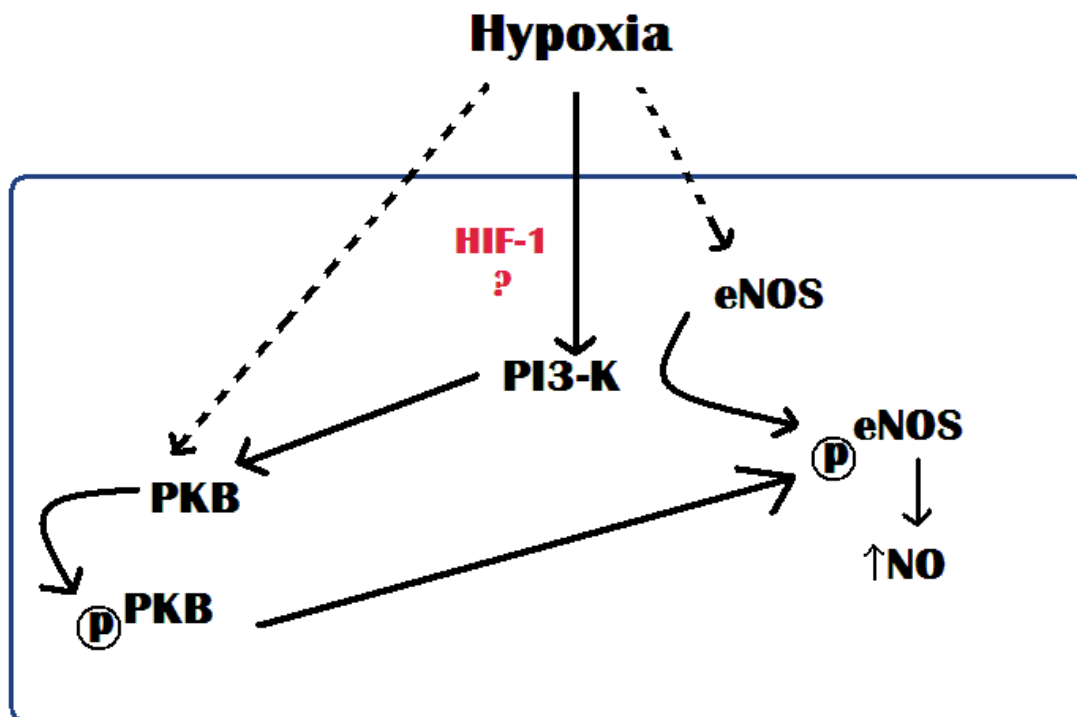


Figure 5.1: Proposed diagram of the underlying mechanisms of hypoxia-induced NO production via eNOS in cardiomyocytes. Solid lines depict actions as demonstrated in the present study and dotted lines depict increased protein expression. "p" depicts phosphorylated enzyme.

5.2. Shortcomings of the study

It is a well known fact that there are three NOS isoforms and they are all expressed in the heart. In this study we decided to focus on eNOS, however, one should acknowledge that iNOS and nNOS could also contribute to NO production in ischaemia / hypoxia. Of the five phosphorylatable sites of eNOS we focused on

phosphorylation of Ser¹¹⁷⁷. It would be interesting to investigate phosphorylation of the other known activation sites of eNOS, namely Thr⁴⁶⁵. Looking back at this study we realise we could have also done the PI3-K / PKB inhibition on the CMECs to determine their effect on NO production and eNOS expression and activation.

5.3. Future direction

The effect of hypoxia on iNOS and nNOS needs to be studied in whole hearts and isolated cells to provide a more complete picture on the effects of NOS in the heart. It is known that hypoxia-inducible factor 1 (HIF-1) is able to activate iNOS (Palmer et al. 1998). HIF-1 needs to be further investigated especially as another possible mechanism of eNOS activation in ischaemia / hypoxia and a possible link in the hypoxia-PI3-K / PKB pathway (see Figure 5.1).

It is known that microvascular endothelial cells express more eNOS per cell than cardiomyocytes; hence, they have the potential to produce more NO (Strijdom et al. 2006). The effect of NO as a paracrine molecule between cardiomyocytes and endothelial cells needs to be investigated. Future co-culture studies could help elucidate this possibility.

ADDENDUM 1

List of publications resulting directly from this study

- ✿ Strijdom H, Friedrich SO, Hattingh S, **Chamane N**, Lochner A. Hypoxia-induced regulation of nitric oxide synthase in cardiac endothelial cells and myocytes. Mol Cell Biochem 2008; DOI10.1007/s11010-008-9906-2

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

- ✿ Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B. *Curr Biol* 1997; 7: 261 – 269.

- ✿ Allen DG Lee JA, Smith GL. The effects of simulated ischaemia on intracellular calcium and tension in isolated ferret ventricular muscle. *J Physiol* 1989; 410: 297 - 323.

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