Hypoxia and the heart: the role of nitric oxide in cardiac myocytes and endothelial cells

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DECLARATION:

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

Signature: ..……. **Date:** ……

ABSTRACT:

Nitric oxide (NO) is a major signaling molecule in the heart with various biological effects. The putative role of NO as a cardioprotective agent against ischaemia-reperfusion injury and in ischaemic preconditioning (IP) has made it one of the fastest growing fields in basic cardiovascular research. However, NO may also be associated with harmful effects, especially when released in excessive amounts. Little is known about the relative contributions to NO-production by the cardiac microvascular endothelial cells (CMECs) and the adjacent cardiomyocytes. Furthermore, the respective roles of endothelial NOS (eNOS) and inducible NOS (iNOS) are not well characterized in these cell types, particularly in hypoxia. In order to gain a better understanding of the role of NO in the hypoxic/ischaemic heart, the aims of this study were to: (1) develop an isolated cardiomyocyte model in which hypoxia and early IP can be induced and the role of NO assessed; (2) measure NO-production in cardiomyocytes and CMECs under baseline and hypoxic conditions; and (3) evaluate the expression, regulation and activation of eNOS and iNOS in cardiomyocytes and CMECs (baseline and hypoxia) and establish the relationship with NO-production under these conditions. Cardiomyocytes isolated from adult rat hearts and commercially purchased rat CMECs were used as cell models.

Results showed that: (1) Sustained hypoxia exerted significant cellular damage in isolated cardiomyocytes (viability of control cells: 100% vs. hypoxia: 46.2%). Although IP protected the cells against sustained hypoxia (viability of hypoxic cells: 46.2±1.8% vs. IP: 71.3±2.6%), a beneficial role for NO as trigger or mediator of protection could not be demonstrated. In view of these observations, the main focus

of further studies was aimed at the effects of hypoxia on the cardiomyocyte. (2) A novel method of direct intracellular NO-detection was developed (analysis of DAF-2/DA fluorescence by flow cytometry). Using this method, we demonstrated that CMECs produced ~26-fold more baseline NO per cell than cardiomyocytes and although hypoxia stimulated NO-generation in both cell types (increase in NO-production compared to control: cardiomyocytes: 1.6-fold; CMECs: 3.3-fold), CMECs were shown to produce ~52-fold more NO in hypoxia. Baseline peroxynitrite ($ONOO^-$) production was 2.2-fold higher in CMECs than cardiomyocytes; however there was a decrease in $ONOO^-$ production in both cell types during hypoxia. (3) Baseline eNOS expression was demonstrated in both cell types and CMECs expressed ~22-fold more baseline eNOS protein than cardiomyocytes; however, iNOS was detected in cardiomyocytes only. In hypoxic CMECs, eNOS was upregulated (18h $\downarrow PO_2$ hypoxia in cultured CMECs: 2.1-fold increase; 60min mineral oil hypoxia in trypsinized CMECs: 1.8-fold increase) and activated (phosphorylation at Ser1177) (18h $\downarrow PO_2$ hypoxia in cultured CMECs: 4.9-fold increase; 60min mineral oil hypoxia in trypsinized CMECs: 3-fold increase), which was closely linked to the hypoxia-induced NO-production. In the cardiomyocytes, eNOS regulation depended on the duration of hypoxia: exposure to longer periods of hypoxia and thus increased cellular injury caused a loss of eNOS protein; however, activated eNOS levels were unaffected and NO-production increased significantly; exposure to shorter hypoxia periods ($\downarrow$cellular injury), had no effect on eNOS expression but increased its activation. Thus, hypoxia-induced NO-generation in these cells was closely linked to eNOS activation. Preliminary data from mixed-cell investigations showed that intracellular NO levels in cardiomyocytes increased by 13-20% ($p < 0.05$ vs. myocytes only) when they were

co-incubated with CMECs under oxygenated conditions. This trend was also observed in hypoxia studies.

Summary and conclusions: Our findings show that IP exerted protection in a model of isolated cardiomyocytes, but that NO did not trigger or mediate protection. In fact, NO was harmful to the hypoxic myocyte. Direct NO-measurements showed that CMECs produced significantly more NO than cardiomyocytes during baseline and hypoxia. Hypoxia upregulated and activated eNOS in the CMECs, which seemed to be the predominant NOS-isoform in these cells. In cardiomyocytes, our data suggest that NO-production induced by longer hypoxic periods involved non-eNOS sources such as iNOS; however during shorter hypoxia, NO-production was closely linked to eNOS activation. Data from mixed-cell suspensions suggest that spillover diffusion of NO occurs from CMECs to the adjacent cardiomyocytes.

In conclusion, in this study cellular models of isolated cardiomyocytes and CMECs were successfully established. Furthermore, we developed and adapted several techniques for the evaluation of cell viability in both cell models. In view of a lack of direct NO-detection methods, a technique that directly measures intracellular NO generation in cardiomyocytes and CMECs was developed, viz. flow cytometric analysis of DAF-2/DA fluorescence. This detection technique allowed for new insights in the generation of NO by these cell types. Results suggest that eNOS was the main NOS isoform involved as source of the observed increases in hypoxia-induced NO levels, although there may be a role for iNOS in hypoxic myocytes. The ability of CMECs to produce more NO than the myocytes may have implications for

the in vivo scenario (e.g. possible spill-over diffusion into the myocytes), and future co-culture studies may shed more light on this possibility.

OPSOMMING:

Stikstofoksied (NO) is ‘n belangrike boodskapper in die hart met verskeie biologiese effekte. Die moontlikheid dat NO die hart teen isgemie-herperfusie skade kan beskerm (hetsy direk of indirek via isgemiese prekondisionering (IP)) het daartoe gelei dat dit ‘n snel ontwikkelende navorsingsveld in basiese kardiovaskulêre wetenskappe geword het. NO, wanneer in oormatige hoeveelhede afgeskei, kan egter skadelik wees. Onsekerheid bestaan oor die relatiewe bydraes van die kardiale mikrovaskulêre endoteelselle (CMECs) en die naburige kardiomiosiete tot NO-produksie. Verder is die relatiewe bydraes van endoteliale NOS (eNOS) en induseerbare NOS (iNOS) nie goed in hierdie seltipes gekarakteriseer nie, veral nie tydens hipoksie nie. Ten einde ‘n beter begrip van die rol van NO in die hipoksiese/isgemiese hart te verkry, het dié studie die volgende ten doel gehad: (1) ontwikkeling van ‘n geïsoleerde kardiomiosiet model waarin hipoksie en vroeë IP geïnduseer en die rol van NO-produksie evalueer kan word; (2) meting van NO-produksie in kardiomiosiete en CMECs tydens basislyn en hipoksiese omstandighede; en (3) evaluering van die uitdrukking, regulering en aktivering van eNOS en iNOS in kardiomiosiete en CMECs (basislyn en hipoksie) en bepaling van die verband met NO-produksie onder hierdie omstandighede. Kardiomiosiete, geïsoleer uit volwasse rotharte, en kommersiële rot CMEC kulture is as sel-modelle in die studie gebruik. Die uitslae het aangetoon dat: (1) Volgehoue hipoksie veroorsaak betekenisvolle sellulêre skade in geïsoleerde kardiomiosiete. Hoewel IP die selle teen volgehoue hipoksie beskerm het, kon ‘n voordelige rol vir NO as sneller en mediator van beskerming nie aangedui word nie. (2) ‘n Nuwe metode van direkte intrasellulêre NO-meting is ontwikkel (analise van DAF-2/DA fluoressensie m.b.v.

vloeisitometrie). Met hierdie tegniek kon gedemonstreer word dat CMECs ~26-voudig meer basislyn NO per sel as kardiomiosiete produseer en hoewel hipoksie NO-produksie in albei seltipes gestimuleer het, het die CMECs ~52-voudig meer NO tydens hipoksie gegenereer. Basislyn peroksinitriet ($ONOO^-$) produksie was hoër in CMECs as kardiomiosiete; daar was egter 'n daling in $ONOO^-$ produksie in beide seltipes tydens hipoksie. (3) Basislyn eNOS uitdrukking was teenwoordig in albei seltipes met 'n ~22-voudig hoër uitdrukking in die CMECs; iNOS kon egter slegs in kardiomiosiete aangetoon word. eNOS was opgereguleer en geaktiveer (fosforilering op Ser1177) in CMECs tydens hipoksie en dit was nou geassosieer met hipoksie-geïnduseerde NO-produksie. In die kardiomiosiete was eNOS-regulering van die duur van hipoksie afhanklik: blootstelling aan langer hipoksie periodes met gevolglike verhoogde sellulêre skade het tot eNOS proteïen verlies gelei terwyl die geaktiveerde eNOS vlakke onveranderd gebly en NO-produksie betekenisvol toegeneem het; blootstelling aan korter periodes van hipoksie (minder sellulêre skade) het egter geen effek op eNOS uitdrukking gehad nie, terwyl die aktivering wel verhoog is. Dus: hipoksie-geïnduseerde NO-produksie was nou met eNOS-aktivering in hierdie selle geassosieer. Opsommend wys ons uitslae daarop dat IP beskerming in 'n geïsoleerde kardiomiosiet-model uitgelok het, maar dat NO nie as sneller of mediator van beskerming opgetree het nie. NO was in der waarheid skadelik vir die hipoksiese kardiomiosiet. Direkte NO-bepalings het aangetoon dat CMECs betekenisvol meer NO as kardiomiosiete geproduseer het tydens basislyn en hipoksiese toestande. eNOS in die CMECs is deur hipoksie opgereguleer en geaktiveer en dit wil voorkom asof eNOS die oorheersende NOS-isoform in hierdie seltipe is. In die kardiomiosiete dui ons data daarop dat die verhoogde NO-produksie tydens langer hipoksie afkomstig van nie-eNOS bronne soos iNOS, is; tydens korter hipoksie is daar egter 'n

noue verband met eNOS aktivering. Uitslae van die gemengde seleksperimente dui daarop dat oorloop diffusie van NO van die CMECs na die naburige kardiomiosiete wel plaasvind. Ten slotte: ‘n beskermende rol vir NO kon nie in ons geïsoleerde kardiomiosiet-model aangetoon word nie, ten spyte van oortuigende bewyse tot die teendeel in die literatuur. Die ontwikkeling van die DAF-2/DA NO-bepaling tegniek het tot nuwe insigte in die produksie van NO deur kardiomiosiete en CMECs, en die verband daarvan met eNOS en iNOS, gelei.

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Ach	acetylcholine
ADMA	dimethylarginine
ADP	adenosine diphosphate
AMI	acute myocardial infarction
AMP	adenosine monophosphate
ANT	adenine nucleotide translocase
AT	angiotensin
ATP	adenosine triphosphate
2,3-BDM	2,3 butane dionemonoxime
BSA	bovine serum albumin
CABG	coronary artery bypass grafting
$CaCl_2$	calcium chloride
CaM	calmodulin
cAMP	3'-5'-cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CMECs	cardiac microvascular endothelial cells
CO_2	carbon dioxide
COX-2	cyclo-oxygenase 2
CsA	cyclosporine A
DAF-2/DA	diaminofluorescein 2/diacetate
DAF-2T	diaminofluorescein-triazol
DEA/NO	2-(N,N-Diethylamino)-diazenolate 2-oxide
DHR-123	dihydrorhodamine-123
DTT	dithiotreitol
EDRF	endothelium-derived relaxing factor
EGM	endothelial growth medium
EGTA	ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
eNOS	endothelium-derived nitric oxide synthase
ERK	extracellular-regulated kinase
ET	endothelin
ETC	electron transport chain
FACS	fluorescence-activated cell sorting
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FMN	flavin mononucleotide
Gi	inhibitory G-protein
GPCR	G-protein coupled receptor
GSH	reduced glutathione
GSNO	s-nitroglutathione
GTP	guanosine triphosphate
5-HD	5-hydroxy-decanoate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid
HIF	hypoxia inducible factor
His	histidine
H_2O_2	hydrogen peroxide
Hsp90	heat shock protein 90
HRP	horseradish peroxidase

IHD	ischaemic heart disease
iNOS	inducible nitric oxide synthase
IP	ischaemic preconditioning
JAK	Janus kinase
JNK	c-Jun NH_2-terminal protein kinase
K_{ATP} channel	ATP-sensitive potassium channel
KCl	potassium chloride
KCN	potassium cyanide
KHB	Krebs-Henseleit buffer
LCCA	left circumflex coronary artery
LDH	lactate dehydrogenase
LDL	low density lipoprotein
L-NA	N-nitro-L-arginine
L-NAME	N^W-nitro-L-arginine methyl ester
L-NMMA	NG-methyl-L-arginine
L-NNA	N-nitro-L-arginine
MAPK	mitogen-activated kinase
M-chol	muscarinic cholinergic receptor
$MgSO_4$	magnesium sulphate
MI	myocardial infarction
MPG	N-(2-mercapto-propionyl) glycine
MPTP	mitochondrial permeability transition pore
MtNOS	mitochondrial nitric oxide synthase
MTT	3-4,5-di-methylthiazol-2-yl-2,5-diphenyltetrazolium bromide
NAC	n-acetyl-cysteine
NaCl	sodium chloride
Na_2HPO_4	disodium phosphate
NaH_2PO_4	sodium dihydrogen phosphate
NCX	Na^+ / Ca^{2+} exchanger
NF-κB	nuclear factor κB
NHE	Na^+ / H^+ exchanger
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO^-	nitroxyl anion
NO^+	nitrosonium cation
NO_2^-	nitrite
NO_3^-	nitrous oxide
Non-IP	non ischaemic preconditioning
NOS	nitric oxide synthase
NOx	nitrates + nitrites
NTG	nitroglycerine
O_2	oxygen
$OH^{\cdot}$	hydroxyl radical
$ONOO^-$	peroxynitrite
P38 MAPK	p38 mitogen-activated protein kinase
PDE	phosphodiesterase
PI	propidium iodide
PIA	R(-)N6-(2-phenylisopropyl)-adenosine
PI3-K	phosphatidylinositol-3-kinase
PKA	protein kinase A

PKB	protein kinase B
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PMSF	phenyl methyl sulfonyl fluoride
PO_2	partial pressure of oxygen
PPi	inorganic pyrophosphate
PTK	protein tyrosine kinase
RNS	reactive nitrogen species
ROS	reactive oxygen species
RyR	ryanodine receptor
Ser	serine
SDS	sodium dodecylsulphate
sGC	soluble guanylate cyclase
SMT	S-methylisothiourea
SNAP	S-nitroso-N-acetylpenicillamine
SNO	s-nitrosothiols
SNP	sodium nitroprusside
SOD	superoxide dismutase
SPT	8-(p-sulfo-phenyl)theophylline
SR	sarcoplasmic reticulum
SWOP	second window of protection
TBE:	trypan blue exclusion
TCA	trichloroecetic acid
THB4	tetrahydrobiopterin
Thr	threonine
TNF-α	tumor necrosis factor alpha
TnT	troponin T
Tris	tris(hydroxymethyl)amino methane
VCAM-1	vascular cell adhesion molecule 1
VDAC	voltage-dependent anion channel
VDCC	voltage-dependent calcium channel
VEGF	vascular endothelial growth factor
VF	ventricular fibrillation
XO	xanthine oxidase

CHAPTER 1

LITERATURE REVIEW & HYPOTHESIS

A. Myocardial ischaemia, reperfusion and cardioprotection

A.1.1 Introduction to myocardial ischaemia, reperfusion and cardioprotection

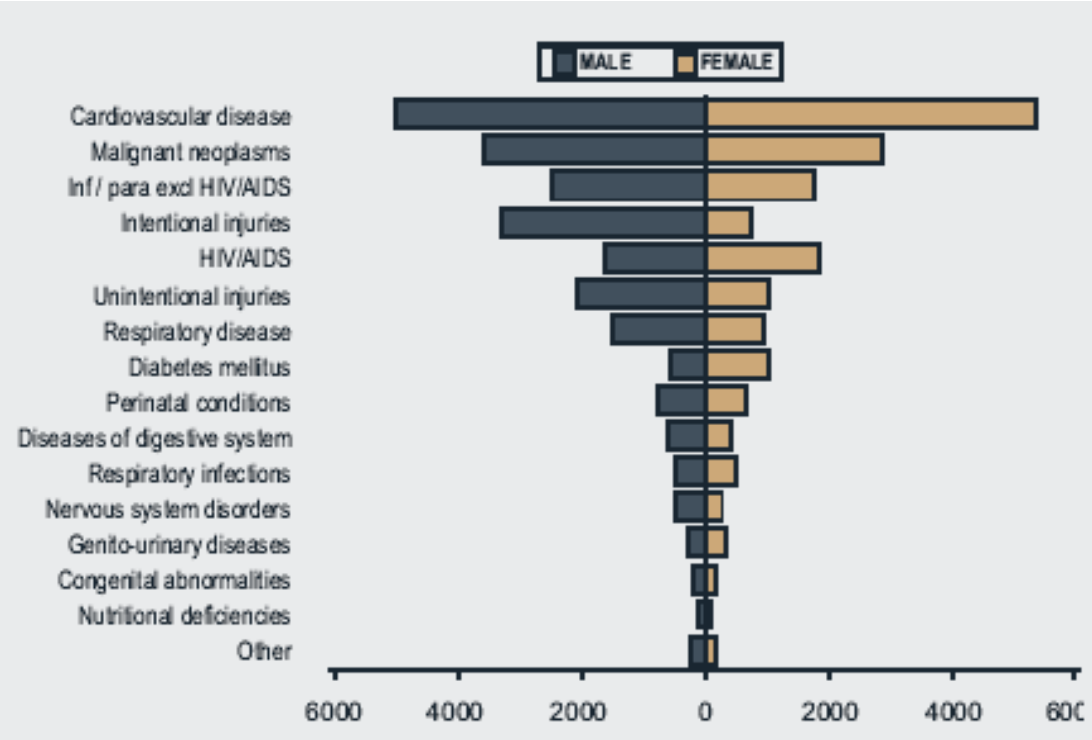
In order to gain a better understanding of the role of nitric oxide (NO) in the heart during ischaemia / hypoxia, it is necessary to give a brief introduction to the concepts of myocardial ischaemia, reperfusion and cardioprotection.

(i) Epidemiology

Ischaemic heart disease (IHD) is a major cause of death worldwide. This is also evident in South Africa, with latest statistics showing that IHD is the third most common overall cause of death, accounting for 5.6% of deaths. In the Western Cape, IHD is the leading cause of death accounting for 12% of the deaths (fig. 1.1). [South African National Burden of Disease Study 2000: Estimates of Provincial Mortality; MRC; South Africa; www.mrc.ac.za/bod/estimates.htm].

(ii) Myocardial ischaemia and infarction

Myocardial ischaemia is essentially an oxygen supply/demand imbalance that results from an impaired blood supply to the myocardium due to coronary artery occlusion typically triggered by atherosclerotic coronary artery disease [Opie 2004]. Short term effects of ischaemia are associated with the onset of tissue hypoxia, which induces adaptational changes in the myocardium aiming to decrease oxygen demand by reducing contractility and increasing glycolysis.



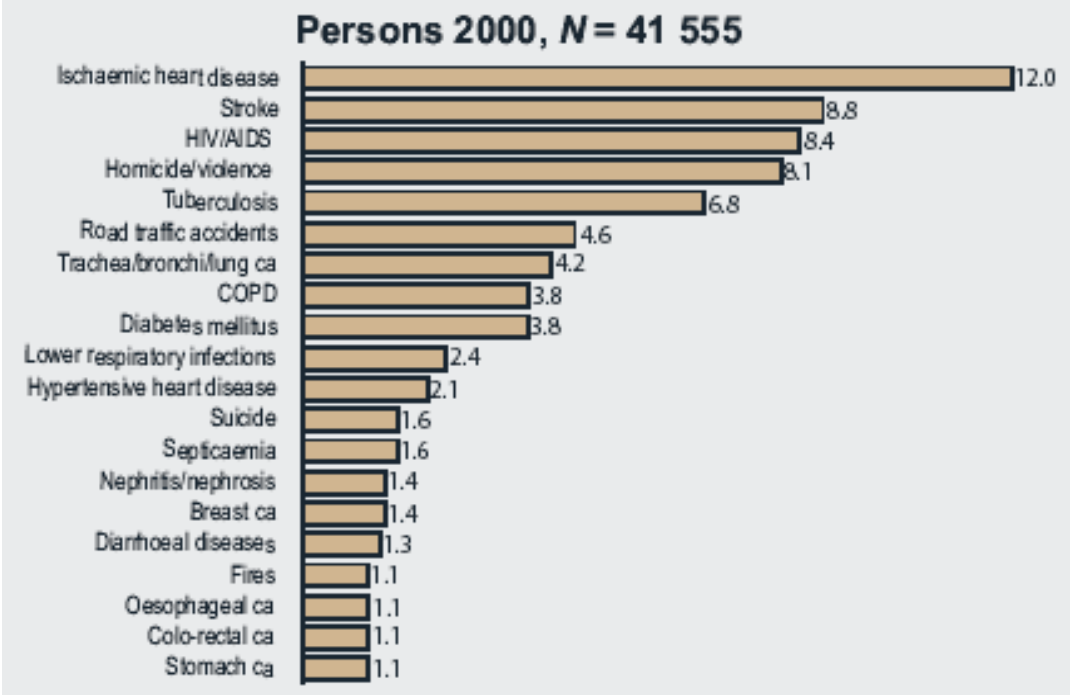


Fig. 1.1 Mortality statistics of the Western Cape Province, South Africa. **(A)** Causes of death in males and females ranked according to disease categories. **(B)** Single leading causes of death. (Source: South African Burden of Disease Study 2000: Estimates of Provincial Mortality; MRC of South Africa May 2005)

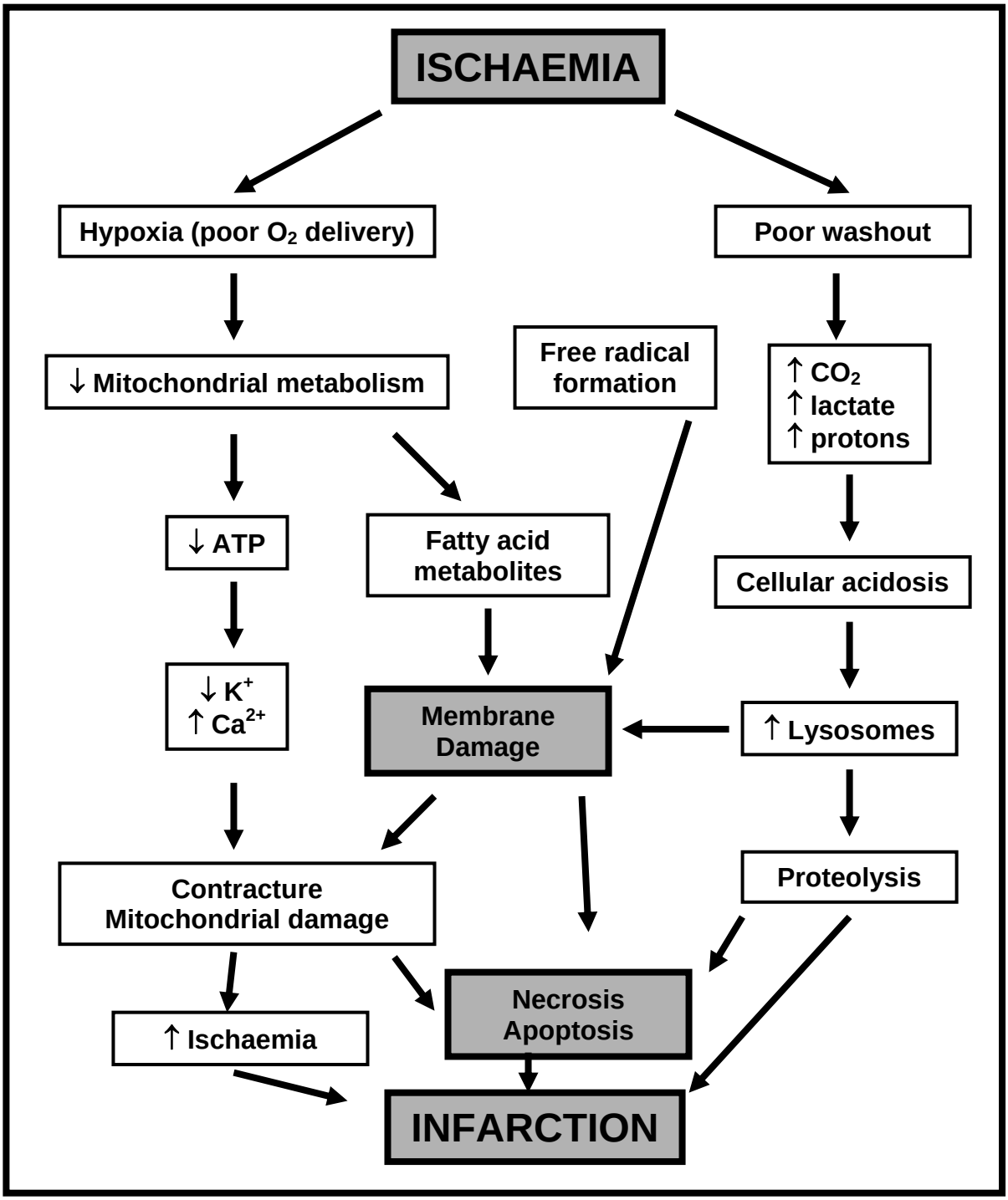


Fig. 1.2 Pathophysiological progress from myocardial ischaemia to infarction. (Modified from Opie 2004)

Severe ischaemia results in increased intracellular calcium, tissue acidosis and clinically a marked reduction in left ventricular performance. In irreversible ischaemic damage, cell death, which can be necrosis and/or apoptosis, and myocardial infarction, will follow (See fig. 1.2).

(iii) Cardioprotective therapy

The morbidity and mortality associated with acute myocardial infarction (AMI) has necessitated an increasing need for effective cardioprotective treatment. In the clinical setting, cardioprotection can be defined as the reduction of necrosis (i.e. myocardial infarct size), as well as AMI-associated complications such as heart failure and ventricular arrhythmias [Kloner & Rezkella 2004]. It is widely accepted that early reperfusion (before 3 h of coronary artery occlusion) of the infarcted myocardium has been the best strategy thus far to limit infarct size. Early reperfusion strategies include mechanical reversal of coronary artery occlusion (percutaneous transluminal coronary angioplasty; stents; urgent coronary bypass), and pharmacological reperfusion therapy with thrombolytic agents such as streptokinase and low-molecular-weight heparin [Opie 2004; Kloner & Rezkella 2004]. Despite the benefits associated with early reperfusion, harmful side effects are often observed when coronary blood flow is restituted, the so-called phenomenon of reperfusion injury (incl. stunning, reperfusion arrhythmias, microvascular damage, and accelerated death of severely damaged cells) [Opie 2004]. Myocardial stunning is a well described reperfusion injury event, and can be defined as the persistence of mechanical myocardial dysfunction after reperfusion, despite the absence of

irreversible damage and the return of normal or near-normal reperfusion [Kloner *et al* 1998].

Coronary artery bypass graft surgery (CABG) is a common treatment strategy used in patients with chronic IHD. However, the incidence of perioperative complications (mainly myocardial stunning and myocardial infarction [Ghosh 2003]) is relatively high, ranging from 3% - 30% [Kloner & Rezkella 2004]. In light of the complications associated with CABG, recent investigations have focused on new techniques and drugs that could achieve cardioprotection during surgery. One study in which high doses of adenosine were added to cold blood cardioplegia showed a reduction in the incidence of perioperative MI (fig. 1.3) [Mentzer *et al* 1999].

The quest for novel strategies in the treatment of surgical ischaemia-reperfusion injury has led clinicians to investigate a cardioprotective laboratory phenomenon first described in dog hearts, called ischaemic preconditioning (IP) [Murry *et al* 1986]. IP has been shown to be cardioprotective during ischaemia by prior conditioning of the heart with alternating pulses of ischaemia and reperfusion. In this regard, recent studies in human patients undergoing bypass surgery demonstrated that IP resulted in attenuated release of troponin T (an indicator of ischaemic damage) (Fig. 1.4) [Ghosh 2003; Yellon & Downey 2003]. These are two of a relatively small number of human studies that could successfully mimic IP-protection as observed in other animal models by direct application of the ischaemia-reperfusion protocol. IP will be discussed in more detail in the next chapter.

(iv) Summary

The incidence of cardiac ischaemia and myocardial infarction is increasing worldwide, and the morbidity and mortality associated with these conditions necessitate ongoing investigations in search of new and more effective modes of cardioprotective therapy. Currently, early reperfusion of the ischaemic / infarcted myocardium and cardiac bypass surgery are the two most effective cardioprotective therapies available to clinicians in the prevention and / or treatment of acute and chronic IHD respectively, supported by several adjunctive pharmacological agents. Unfortunately, both early reperfusion and CABG present with potentially harmful side effects. Early reperfusion (the best therapeutic option currently available to reduce AMI-derived necrosis) has been associated with myocardial stunning amongst others, whereas cardiac bypass surgery often manifests with complications such as perioperative MI and stunning. IP is a laboratory phenomenon with huge potential as a cardioprotective therapy, yet its direct application has had limited success in protecting the human heart in the clinical setting; most of the promising findings with IP have been observed in the context of CABG.

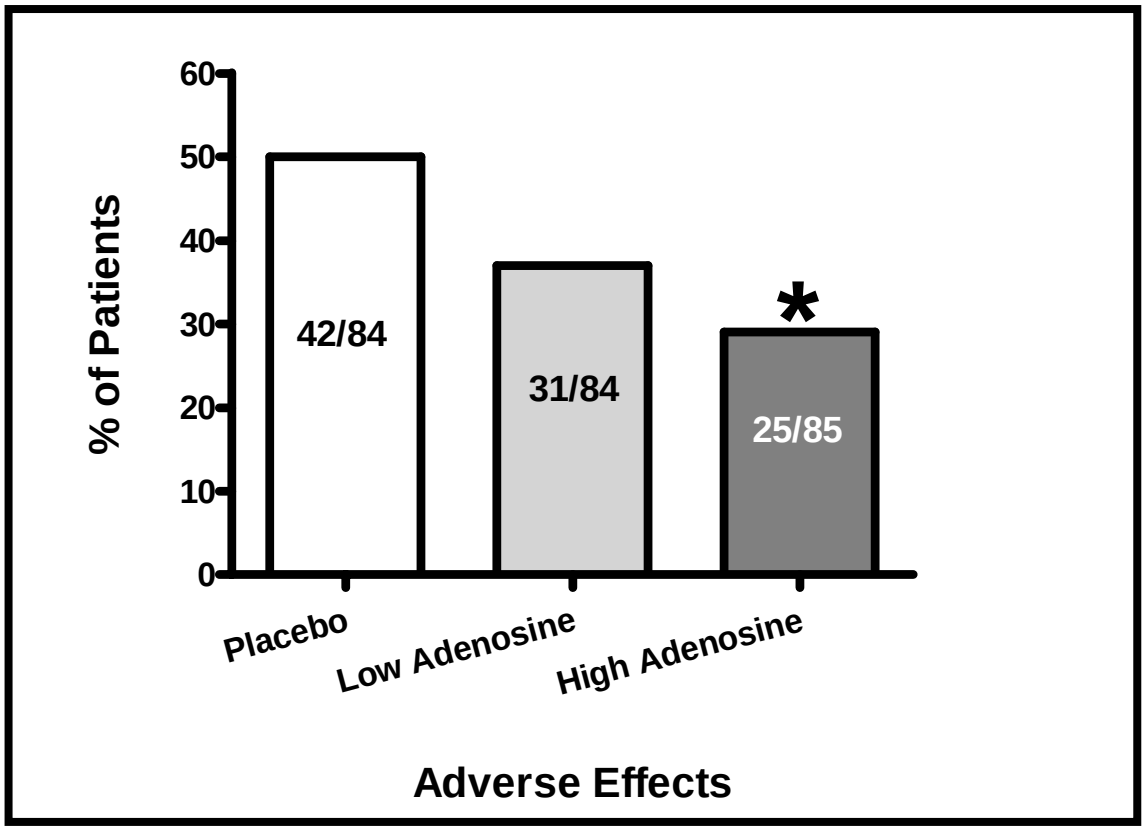


Fig. 1.3 Effects of adenosine treatment of patients undergoing coronary artery bypass grafting on postoperative complications. Patients received 500 μM (low adenosine) or 2 mM (high adenosine) adenosine administration intra-operatively. The high adenosine group was associated with significantly fewer adverse events (death, myocardial infarction, insertion of intra-aortic balloon, high-dose dopamine, or epinephrine use). *: P=0.006 vs. placebo. See text for further details. (Modified from: Mentzer *et al* 1999).

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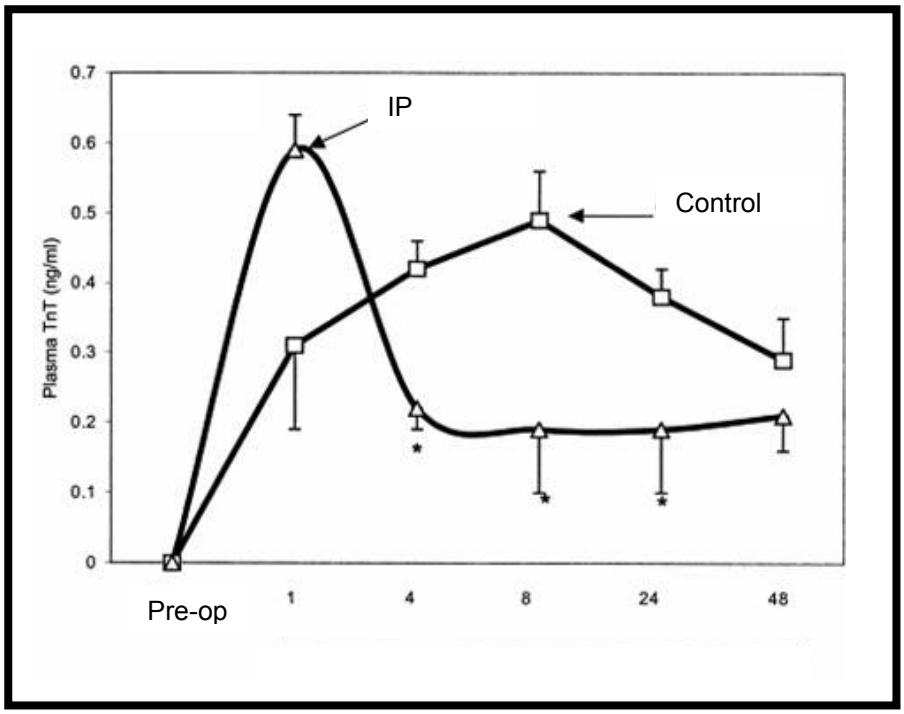

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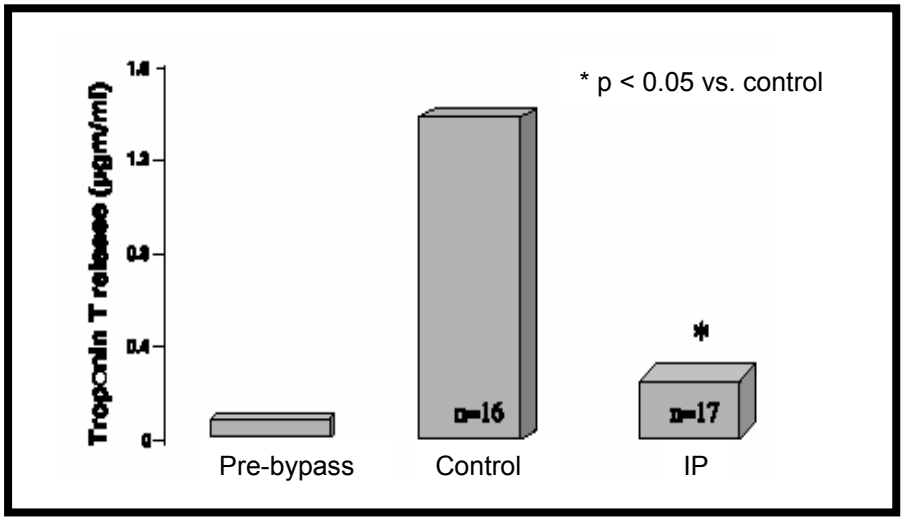

Fig. 1.4 Protective effect of ischaemic preconditioning in the human heart. **(A)** The time course of release of plasma cardiac troponin T (TnT) in preconditioned and control hearts in patients undergoing coronary artery bypass grafting without cardiopulmonary bypass. A reduction in cardiac TnT concentrations was observed in the preconditioned group. See text for details. (Modified from Ghosh 2003) **(B)** In another study, an IP-protocol of two 3min periods of aortic cross-clamping with 2min intervening reperfusion in patients undergoing CABG, exerted significant attenuation of serum TnT release in the IP group. (Modified from Yellon & Downey 2003).

A.1.2 Ischaemic preconditioning (IP)

(i) Background and context

Although reperfusion therapy has significantly reduced the mortality related to AMI, the functional recovery of reperfused hearts has been hampered by the harmful complications associated with the restitution of blood supply, resulting in an increase in the incidence of ischaemic heart failure [Sanada & Kitakaze 2004]. Clinicians are therefore constantly searching for novel and effective preventative or therapeutic strategies. In 1986, Murry and co-workers described an exciting, novel and very powerful form of cardioprotection in dog hearts, which they termed ischaemic preconditioning (IP) [Murry *et al* 1986]. In fact, its protective effect has proven to be so powerful, that IP has been referred to as *"the most potent form of protection against myocardial necrosis yet described"* [Lawson & Downey 1993]. At the time, the seemingly paradoxical contention that one could in effect exploit brief ischaemic insults to protect the heart from subsequent prolonged ischaemic injury was fascinating, and a promising proposition as a future clinical tool.

There has been a plethora of studies on IP since its discovery in the 1980's (a Pubmed search would typically produce between 4000 and 6000 hits). However, several excellent review articles have appeared covering all aspects of IP in detail [Dekker 1998; Cohen *et al* 2000; Bolli 2001; Yellon & Downey 2003; Sanada & Kitakaze 2004; Eisen *et al* 2004]. For the purposes of this dissertation therefore, only a few relevant aspects will be discussed. In order to understand the concept of IP, it is useful to revisit the protocol originally developed by Murry and co-workers (fig. 1.5A) [Murry *et al* 1986]. They opted for an open-chest canine model, in which the left

coronary circumflex artery (LCCA) was ligated for four 5 min periods, each separated by 5 min of reperfusion. Subsequently, the LCCA was occluded for a sustained period of 40 min. At the completion of the experiments, the ligation was removed to restore coronary blood flow, chest wounds were closed, and the animals allowed to survive for 4 days at which point they were sacrificed and their hearts removed for measurements. The results were astonishing, showing that IP reduced the infarct size (as a % of area at risk) from 29.4±4.4% (control hearts: 40 min ischaemia only) to 7.3±2.1% (fig. 1.5 B). Interestingly, the protection observed seemed to be time-dependent, since hearts subjected to 180 min of sustained ischaemia were not protected, leading the authors to believe that the protective properties of IP were to be found in its ability to delay the onset of, but not completely abolish, necrosis (fig. 1.5 C). This interpretation of the findings led King and Opie in a critical review of IP ten years later to suggest that IP "buys time, but does not cheat death". [King & Opie 1996]. In summary therefore, IP can be defined as an adaptation of the heart to brief sublethal ischaemia (or hypoxia), characterized by a shift to a preconditioned (defensive) phenotype [Stein *et al* 2004].

The study by Murry and co-workers was the first intervention other than revascularization that unequivocally limited MI. Consequently, in the two decades that followed, IP has been researched extensively, and has been shown to be a highly reproducible phenomenon across a wide spectrum of animal species (incl. rats, rabbits and pigs) and experimental models (*in vivo*, isolated hearts and isolated cells). However, a direct application of the IP protocol in humans has been hampered mainly by ethical

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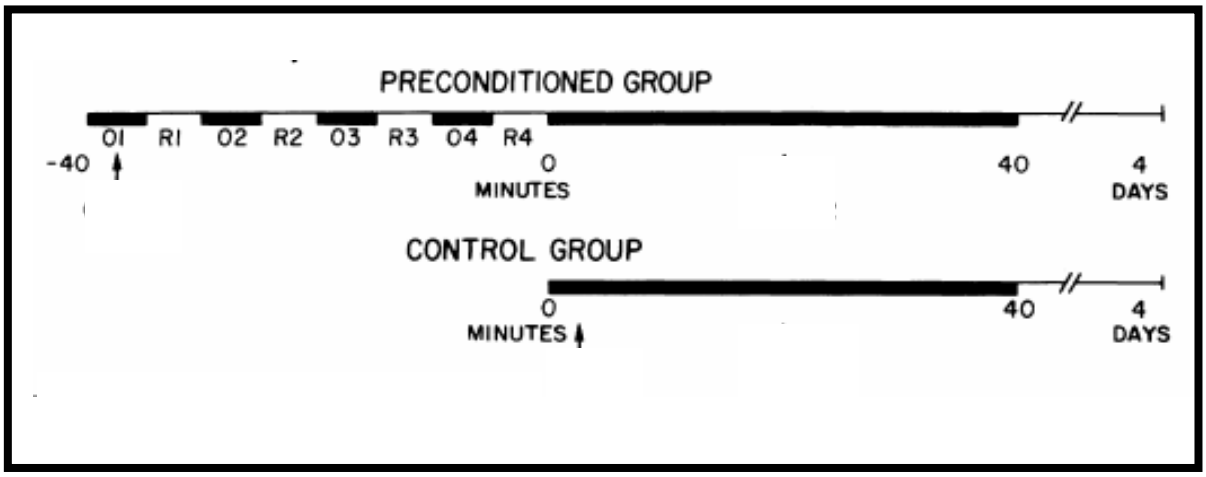

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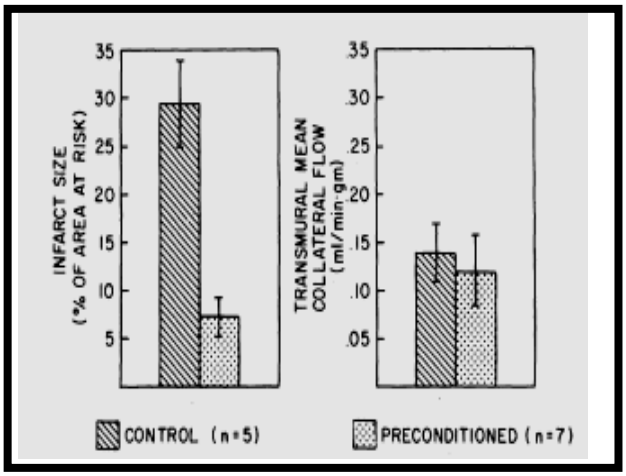

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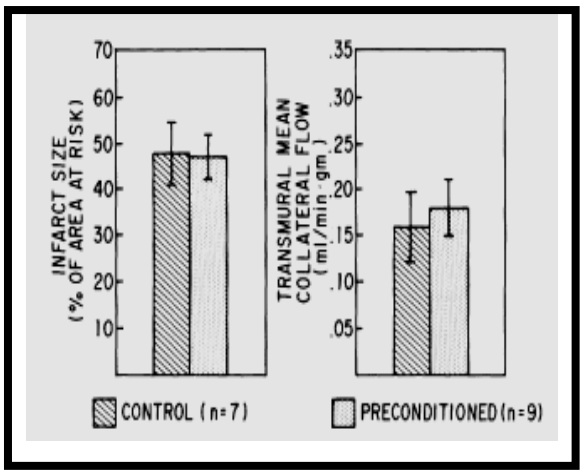


Fig. 1.5 Ischaemic preconditioning in *in situ* dog hearts by Murry *et al*. **(A)** The original IP protocol consisted of four 5 min periods of ischaemia each followed by 5 min of reperfusion prior to a sustained ischaemia period of 40 min. Coronary ligations were then removed and reperfusion allowed to continue for 4 days before measurements. **(B)** IP caused a significant reduction in infarct size in hearts subjected to 40 min of sustained ischaemia (bar chart left) with no difference in collateral blood flow between the groups (bar chart right). **(C)** IP had no effect on infarct size in hearts subjected to 180 min of sustained ischaemia (bar chart left) and no differences in collateral blood flow were observed (bar chart right). (Modified from Murry *et al* 1986)

and practical considerations [Cohen *et al* 2000], despite some attempts (fig. 1.3 and 1.4). Since the induction of myocardial ischaemia is not a feasible treatment option in human patients, researchers have rather shifted their focus on the cellular mechanisms of action of IP [Sanada & Kitakaze 2004] as a possible springboard for therapeutic design. Knowledge of the triggers, mediators and end-effectors of IP, could help researchers and clinicians to design other, more feasible cardioprotective therapies that mimic IP-protection.

(ii) Early (classical) IP-protection vs. second window (late) protection

The IP protocol and protective effects as described by Murry et al above, has been termed “early” or “classical” preconditioning, or “first window of IP-protection” [Yellon & Downey 2003]. In this first phase of IP-protection, the initial protection appears soon after the IP stimulus, and is robust but short-lived (1-2 hours) [Yellon & Downey 2003]. Subsequent to the early phase, a second, delayed phase of protection (late IP; second window of protection, “SWOP”) develops 12-24 hours after the initial stimulus; protection in this phase is less robust but lasts 3 to 4 days [Yellon & Downey 2003; Stein *et al* 2004] (fig. 1.6). For the purposes of this study, we will focus on early IP.

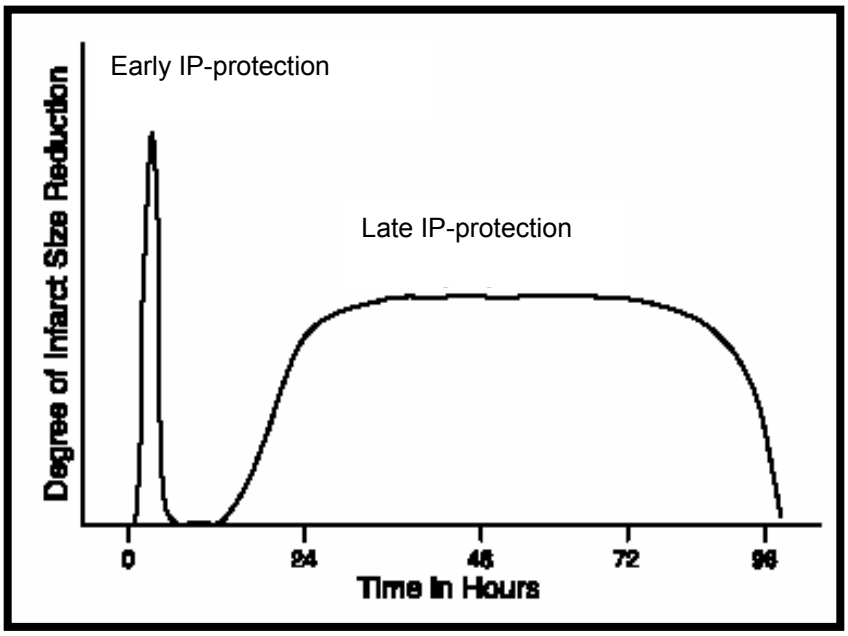


Fig. 1.6 Bi-phasic protection elicited by IP. Early protection within hours (“classical IP”) and late protection (“SWOP”, or second window of protection). (Modified from Yellon & Downey 2003)

(iii) The role of the adenine nucleotides and adenosine in early IP

In their seminal IP study, Murry *et al* attributed the protection by IP to, amongst others, reduced ATP depletion. This finding was supported by a separate study from the same laboratory on dog hearts [Reimer *et al* 1986]. Subsequently, the same group repeated their IP investigations in canine hearts, and measured myocardial ATP at different time-points during a 40 min sustained ischaemia period [Murry *et al* 1990]. Their results showed that IP slowed the rate of ATP-depletion after 10 min of sustained ischaemia compared to control. However, after 40 min of ischaemia, there was no difference in the ATP levels between the groups. They concluded that the ATP preservation observed in the early stages of sustained ischaemia in preconditioned hearts was due to reduced ATP consumption and not increased production. ATP preservation (and therefore reduced myocardial energy demand during ischaemia) as a putative cellular mechanism of IP protection was a plausible hypothesis, however, it subsequently proved not to be a ubiquitous finding.

In a separate study in rat hearts, sustained global ischaemia preceded by an IP-protocol did not reduce ATP depletion compared to control hearts [Headrick 1996]. The reduced ATP-depletion hypothesis could also not be demonstrated in another study on perfused rat hearts [Kolocassides *et al* 1996]. Despite the controversial findings surrounding relative ATP levels in preconditioned hearts, it is widely accepted that myocardial ischaemia *per se* causes ATP breakdown to ADP, AMP and eventually the final, bioactive metabolite, adenosine [Cohen *et al* 2000]. In fact, adenosine is released from the heart during any form of reduced oxygen supply or increased demand, including ischaemia and hypoxia [Hori & Kitakaze 1991]. The role

of adenosine as a major trigger of IP-induced protection has received much attention since 1991, when it was first discovered in rabbit hearts that pretreatment with A1 adenosine receptor antagonists abolished IP-protection as measured by infarct size [Liu *et al* 1991]. In addition, the group could also mimic IP-protection by substituting the brief IP ischaemia with intracoronary infusion of adenosine (fig. 1.7).

(iv) The Gi-coupled receptors as triggers of early IP-protection

Since the discovery of adenosine as an important trigger of IP-protection, it became clear that the Gi-coupled receptor activation is a common denominator in many of the protective pathways [Yellon & Downey 2003]. In fact, it is now accepted that any Gi-coupled receptor can trigger IP-protection via activation of Gi protein. Many triggers released during the brief IP ischaemia act in this way, *viz.* adenosine, norepinephrine [Banerjee *et al* 1993], bradykinin [Goto *et al* 1995] and the opioids [Schultz *et al* 1997]. Other triggers, whose release is not necessarily induced by ischaemia, can also act via the Gi-coupled receptor response, such as angiotensin (AT1 receptor), endothelin (ET1 receptor), and muscarinic receptor stimulation [Cohen *et al* 2000].

(v) Non-receptor triggered protection

Several triggers of IP-protection exist that do not act via a receptor-mediated process. Important examples of such triggers include free radicals and reactive oxygen species (ROS) [Tritto *et al* 1997; Altug *et al* 2000; Lebuffe *et al* 2003]; brief

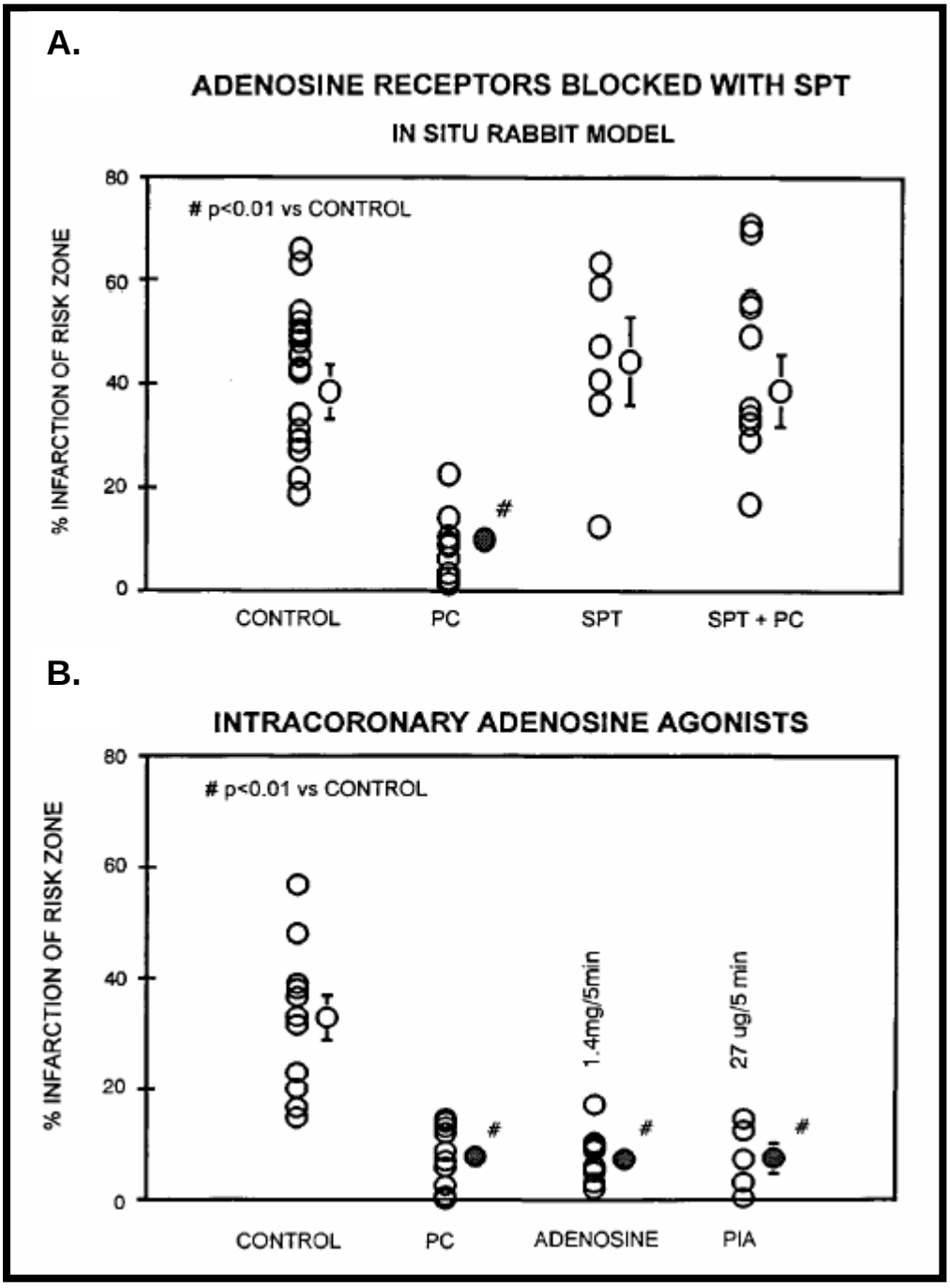


Fig. 1.7 Adenosine as a trigger of IP-protection. Pharmacological manipulation of the A1 adenosine receptor in the in situ rabbit heart demonstrates **(A)** the abolishment of IP-protection with A1 adenosine receptor antagonism; and **(B)** mimicking of protection by A1 adenosine receptor agonists. Abbreviations: SPT, 8-(p-sulfo-phenyl) theophylline; PIA, R(-)N6-(2-phenylisopropyl) adenosine. (Reproduced from Liu GS *et al* 1991)

periods of elevated coronary Ca^{2+} levels [Miyawaki *et al* 1996]; hyperthermia [Yamashita *et al* 1998]; ethanol [Krenz *et al* 2001], etc. One non-receptor-mediated trigger of IP that is of particular importance to the present study, is nitric oxide (NO) [Rakhit *et al* 2000; Lebuffe *et al* 2003; Lochner *et al* 2000]. The role of NO in IP will be discussed later in more detail.

(vi) Intracellular signal transduction in early IP

The intracellular signaling pathways through which IP exerts its protective actions are complex, multiple and crosstalk often occurs between the various pathways. For many years, the adenosine – protein kinase C (PKC) pathway has been considered to be the golden standard signaling pathway in early IP [Sanada & Kitakaze 2004; Yellon & Downey 2003]. It has since become clear that many other pathways are involved. Despite more than a decade of research into the mechanisms of IP-protection, the identification of a final effector pathway remains unresolved. The role of PKC as a mediator of early IP was suggested for the first time in 1994 when rabbits were treated with PKC antagonists prior to ischaemia - reperfusion in the presence or absence of a preceding IP protocol. Results demonstrated that the PKC inhibitors blocked IP-protection. In addition, they found that the administration of a PKC activator mimicked IP-protection [Ytrehus *et al* 1994]. Since these initial studies, several PKC isoforms have been described, but it is thought that the α [Wang & Ashraf 1998], δ [Zhao *et al* 1998] and ε [Liu GS *et al* 1999] isoforms are involved in IP-protection. Until now, the exact downstream intracellular targets of PKC have not been established [Yellon & Downey 2003].

Other protein kinase pathways have also been suggested to act as mediators of IP-protection. Our own laboratory investigated the role of the ß-adrenergic pathway in IP in isolated perfused rat hearts [Lochner *et al* 1999]. While activation of this pathway was regarded as trigger, attenuation of cAMP generation and subsequent PKA activation during sustained ischaemia was found to be essential for protection. A protective role for attenuation of PKA activation was later also demonstrated in a study on dog hearts, which additionally indicated that a third protein kinase cascade, namely the p38 mitogen-activated kinase (MAPK) family may be involved in IP [Sanada & Kitakaze 2001] (see fig. 1.8 for schematic diagram of the MAPK family). Our own investigations on isolated rat hearts demonstrated that IP-protection was associated with a transient increase in activated p38 levels during the brief ischaemic episodes and attenuation during sustained ischaemia [Marais *et al* 2001]. The findings also suggested that in the absence of IP, p38 activation was increased during sustained ischaemia, thereby suggestive of a harmful role for p38. These conclusions were supported by another study, this time in canine hearts, in which brief periods of ischaemia and reperfusion (IP) also resulted in strong activation of p38, whilst its activation was attenuated during sustained ischaemia [Sanada & Kitakaze 2001] (fig. 1.9). The role of protein kinase G (PKG) and its activation by NO will be discussed later.

Recent studies have also identified the phosphatidylinositol-3-kinase (PI3-K) – protein kinase B (PKB) pathway as an important mediator of IP-protection in isolated rat hearts, using contractile dysfunction [Tong *et al* 2000] and infarct size [Mocanu *et al* 2002] as end-points respectively. In both studies IP-protection was abolished in the presence of PI3-K inhibitors.

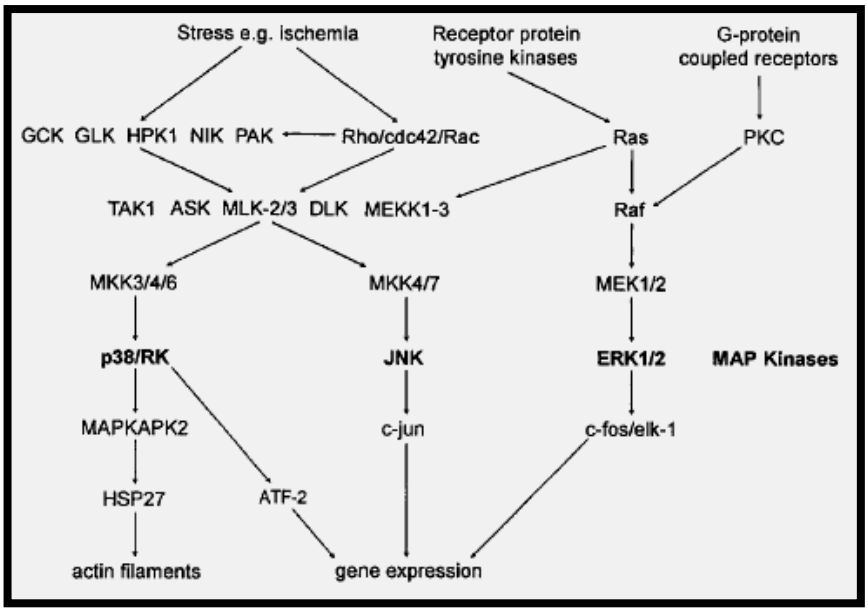


Fig. 1.8 The mitogen-activated protein kinase (MAPK) family. The two stress activated kinases (p38 and JNK) have been implicated in IP. (Reproduced from Cohen *et al* 2000)

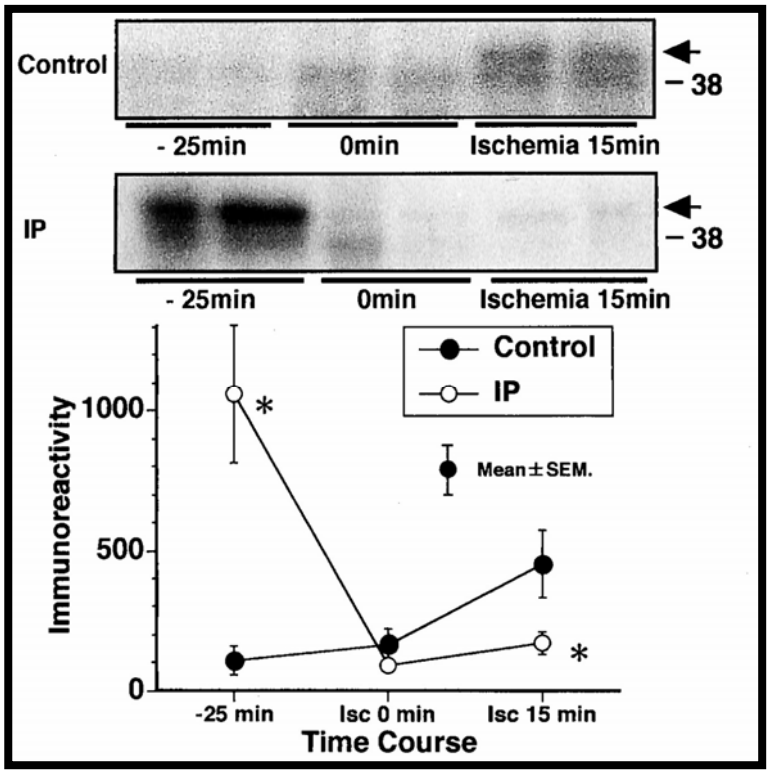


Fig. 1.9 Phasic activity of p38MAPK in control (non-IP) and IP hearts (Reproduced from Sanada & Kitakaze 2004).

(vii) Possible end-effectors of early IP

The nature of the end-effector (-s) ultimately responsible for the protection elicited by IP remains elusive [Cohen *et al* 2000]. For many years the cardiomyocyte K_{ATP} channel was the preferred candidate end-effector; initially the *sarcolemmal* K_{ATP} channel, and more recently the *mitochondrial* K_{ATP} channel, have been thought to be the final intracellular site onto which protective pathways converge causing the channels to open [Gross & Fryer 1999; Cohen *et al* 2000; Yellon & Downey 2003]. Mitochondrial K_{ATP} channel activation was shown to be cardioprotective in rat hearts exposed to ischaemia-reperfusion injury when the putative K_{ATP} channel opener, diazoxide, significantly improved heart function compared to untreated hearts [Garlid *et al* 1997]. The protection observed with diazoxide was subsequently completely abolished in the presence of the K_{ATP} channel blockers, glibenclamide and 5-hydroxy-decanoate (5-HD). Similar findings were obtained in rabbit cardiomyocytes [Liu Y *et al* 1998], and *in situ* rabbit hearts [Ockaili *et al* 1999]. See fig. 1.10 for a summary of the mechanisms thought to be involved in the activation of the mitochondrial K_{ATP} channel.

Opening of the mitochondrial K_{ATP} channel as a plausible end-effector and principal mediator of IP-protection is increasingly being questioned. One such concern is the bioenergetic effect of net K+ influx into the mitochondria when the channel opens, resulting in mitochondrial swelling [Garlid 2000]. Another problem regarding the investigation of mitochondrial K_{ATP} channels is the nature of their localization, which makes them difficult to study; in fact they have not yet been cloned in contrast to the sarcolemmal channel [Hanley & Daut 2005].

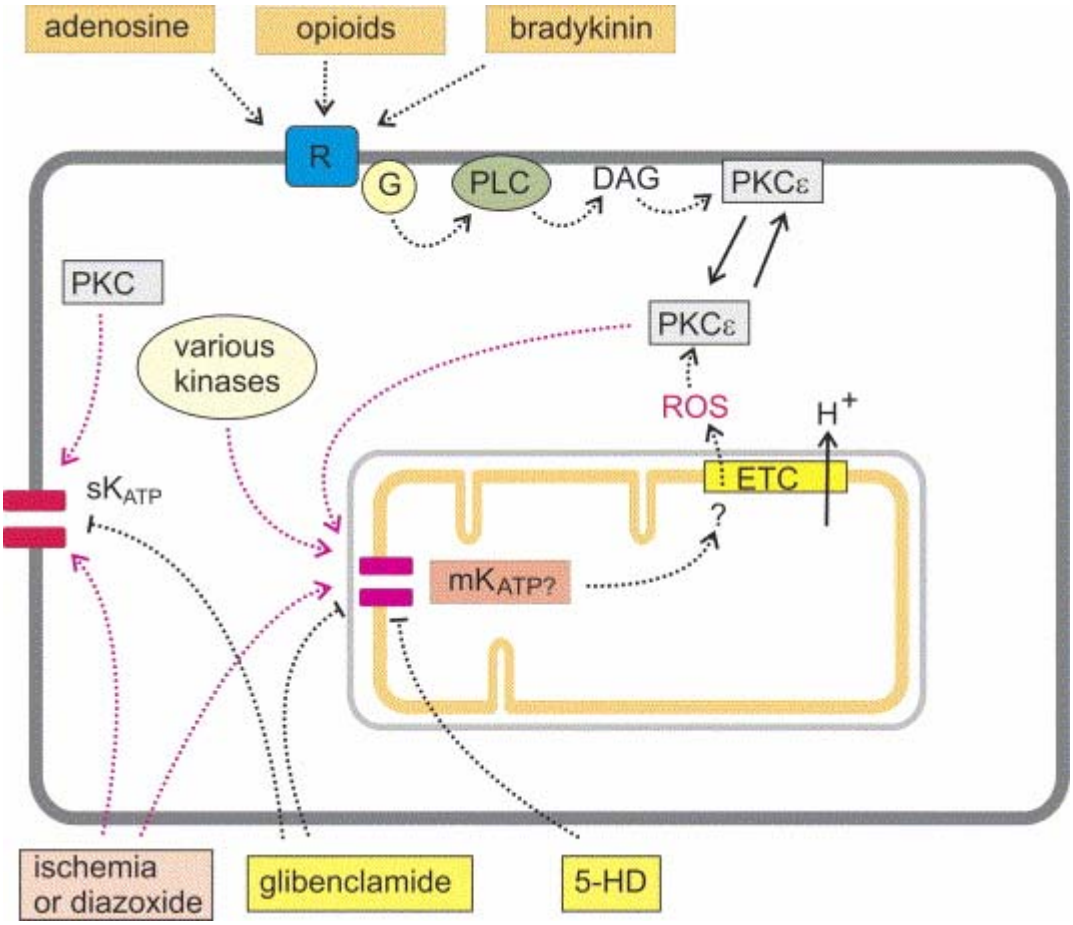


Fig. 1.10 The sarcolemmal and mitochondrial K_{ATP} channels. The most important activators and blockers of the K_{ATP} channels are shown here. Abbreviations: R, receptor; G, G-protein; PLC, phospholipase C, PKC, protein kinase C; ETC, electron transport chain. (Reproduced from Hanley & Daut 2005)

Questions surrounding the proposed mitochondrial K_{ATP} channel hypothesis of IP-protection have led experts in the field to explore alternative options as end-effectors, namely: ROS production [Hanley & Daut 2005; Oldenburg *et al* 2003]; changes in fatty acid metabolism [Hanley & Daut 2005] and the mitochondrial permeability transition pore (MPTP) [Hanley & Daut 2005; Hausenloy *et al* 2004]. The most promising current hypothesis implicates an IP-induced mechanism that ultimately leads to maintenance of the closed state of the MPTP [Hausenloy *et al* 2004] (fig. 1.11). From the results of this study, it is proposed that IP induces changes in mitochondrial function involving opening of the K_{ATP} channel resulting in attenuated matrix Ca^{2+} loading, improved energy production and decreased ROS release during reperfusion. As a result of the opening of the K_{ATP} channel and its sequelae, the opening probability of the MPTP is reduced, which in its turn prevents the release of the pro-apoptotic cytochrome C and uncontrolled influx of water and solutes into the mitochondria. Despite a plethora of investigations, we still do not know the exact nature of a final, common pathway through which IP-protection is exerted.

A summary of intracellular pathways and events elicited by IP based on current knowledge is shown in fig. 1.12 and 1.13.

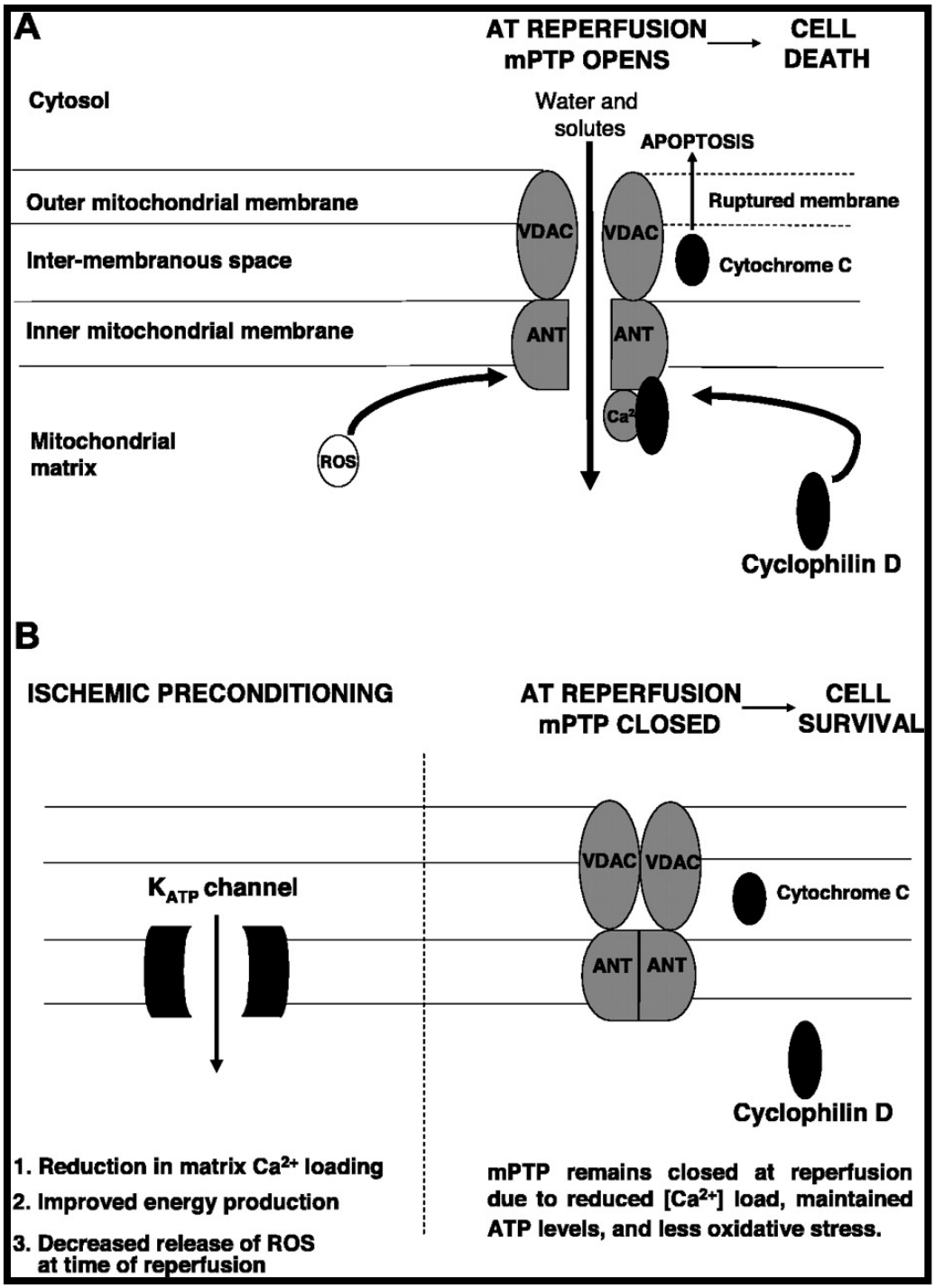


Fig. 1.11 Scheme illustrating proposed protective mechanism of MPTP in IP. **(A)** Events during ischaemia-reperfusion without IP: ROS and Ca^{2+} result in opening of MPTP and inflow of water. Rupture of the outer mitochondrial membrane and loss of cytochrome C to the cytosol follows. **(B)** Inhibition of MPTP opening in IP in response to K_{ATP} channel opening and reduced Ca^{2+} and ROS production. (Reproduced from Hausenloy *et al* 2004)

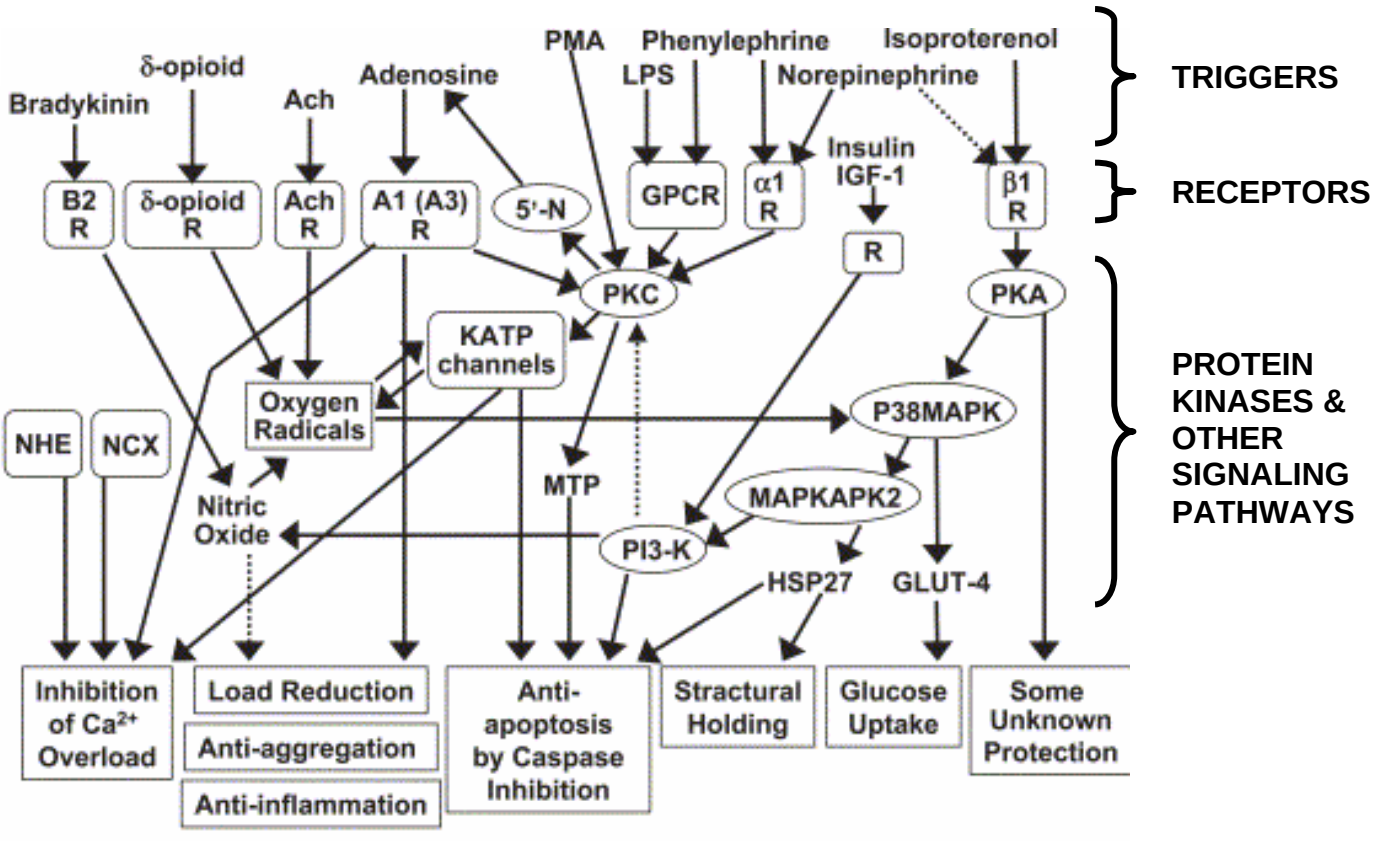


Fig. 1.12 Summary of the triggers, mediators, intracellular signaling pathways and proposed end-effectors of early IP-protection. Triggers such as bradykinin, the opioids, adenosine, norepinephrine and isoproterenol bind to receptors, activating several protein kinase pathways, including PKC, PKA, PI3-K and p38MAPK. Important examples of triggers and mediators that do not act via the conventional protein kinase pathways are ROS and NO. Putative end-effectors of protection are the mitochondrial K_{ATP} channels and the mitochondrial permeability transition pore ("MTP" on the diagram). Abbreviations: NHE, Na^+ / H^+ exchanger; NCX, Na^+ / Ca^{2+} exchanger; Ach, acetylcholine; GPCR, G-protein coupled receptors; MTP, mitochondrial transition pore. (Modified from Sanada & Kitakaze 2004)

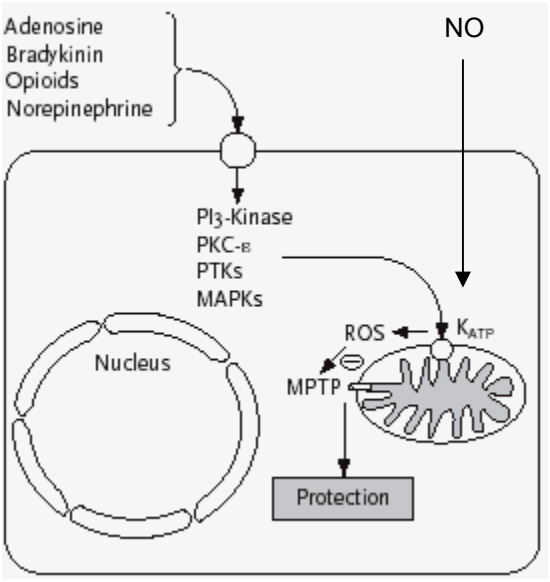


Fig. 1.13 Simplified scheme depicting the mechanism of early IP. (Modified from Riksen *et al* 2004)

(viii) Late preconditioning (second window of protection)

In contrast to early IP, the second phase of protection in IP lasts much longer (early IP: 1-2 hours vs. late IP: 3-4 days), and although less robust, protects against myocardial infarction as well as stunning [Stein *et al* 2004; Bolli 1996]. The stimuli, triggers, pathways and mediators of late IP-protection are summarized in fig. 1.14. Late IP typically involves activation of cardioprotective genes and synthesis of new proteins (as opposed to activation of existing proteins) that are cardioprotective. NO, and its generating enzyme NO synthase (NOS) play a crucial role in the mechanism of late IP [Bolli 2001]. Of particular importance is the de novo synthesis of the inducible isoform of NOS (iNOS) [Stein *et al* 2004]. It seems as if NO plays a dual role in the pathophysiology of late IP by initially acting as a trigger (eNOS-derived) and subsequently as a mediator (iNOS-derived) of late protection [Stein *et al* 2004; Jones & Bolli 2006] (see fig. 1.14). The role of NO in IP will be discussed in more detail later.

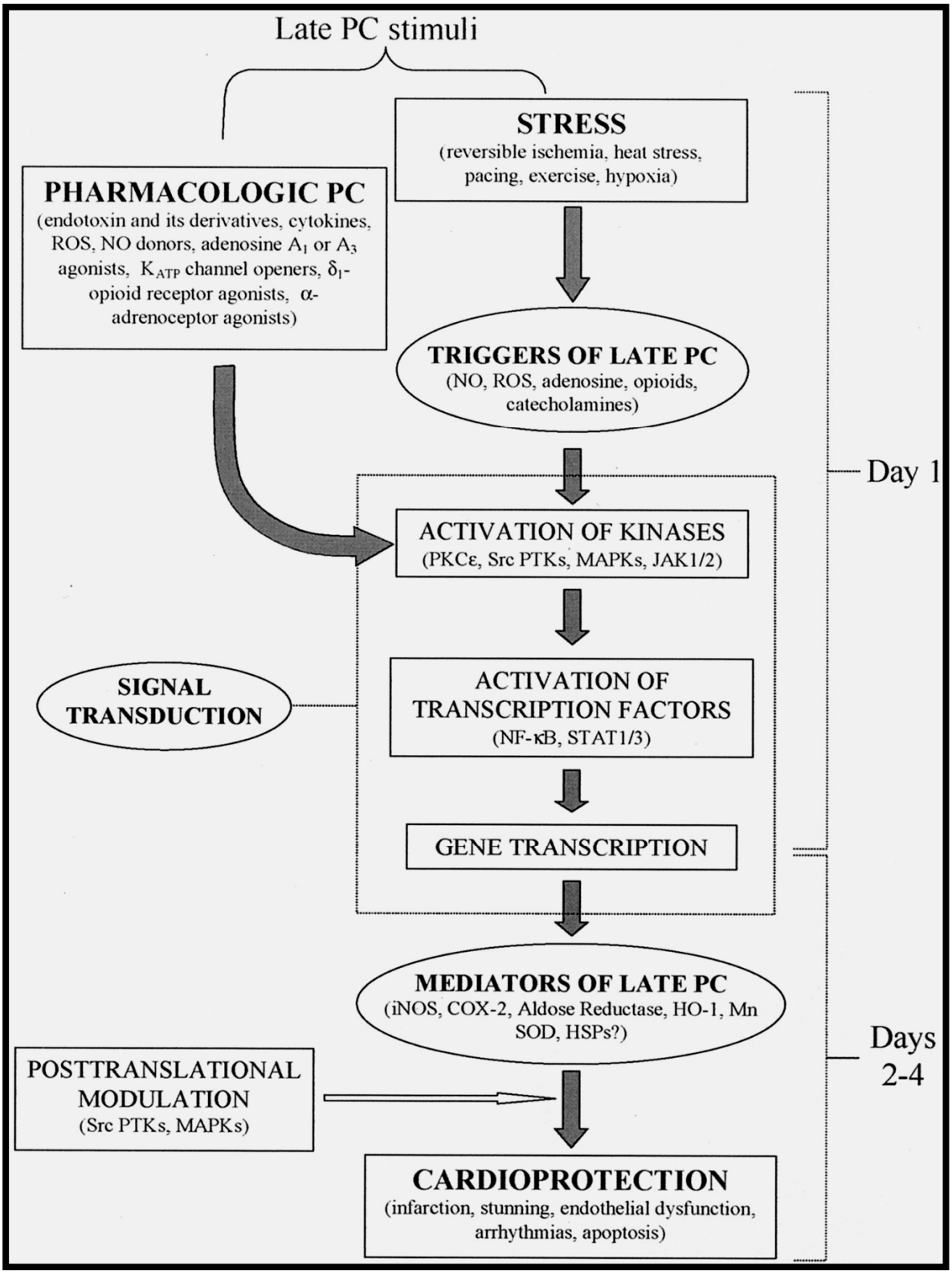


Fig. 1.14 Schematic diagram depicting the underlying cellular mechanisms of late IP. (Reproduced from Stein *et al* 2004)

B. Nitric oxide (NO) and its role in the heart

(i) The biochemistry of NO

NO (structural formula: N=O) is a simple, diatomic gas and free radical that was originally regarded only as an atmospheric pollutant present in exhaust fumes and cigarette smoke [Singh & Evans 1997]. The possibility that NO could also be endogenously produced in the body was not considered until the existence of so-called “nitrovasodilators” or guanylyl cyclase activators resulting in smooth muscle cell relaxation was proposed in the early 80’s [Furchgott & Zawadski 1980; Review by Murad 1998]. The ability of endothelial cells to produce a so-called endothelium-derived relaxant factor (EDRF) leading to arterial smooth muscle cell relaxation was also demonstrated [Furchgott & Zawadski 1980]. In 1987 it was discovered that, based on the significant similarity between their actions, EDRF was in fact NO [Ignarro *et al* 1987; Palmer RM *et al* 1987]. Since then, the progress in understanding the biological role of NO has been remarkable, culminating in the Nobel Prize for Medicine and Physiology awarded to Murad, Ignarro and Furchgott in 1998 for their discoveries concerning NO as a signaling molecule in the cardiovascular system [Official website of the Nobel Foundation: http://nobelprize.org/nobel_prizes/medicine/laureates/1998/].

The half-life of NO at physiological concentration is short (seconds) and it decomposes to nitrite (NO_2^-) and nitrous oxide (NO_3^-) in aqueous solutions, a reaction catalyzed by transition metals such as iron [Singh & Evans 1997].

It is therefore no wonder that NO is inactivated by haemoglobin in a reaction that forms methaemoglobin, NO_2^- and NO_3^-. Due to its distinct chemical properties, NO is

able to participate in a wide range of nitrogen-based biological reactions [Gow & Ischiropoulos 2001]. The nature of these reactions is mainly determined by the presence of an unpaired electron (i.e. NO as a free radical), or the existence of nitrogen in a variety of oxidation states (reminiscent of oxygen). Therefore, nitrogen can exist as a stable, fully reduced molecular nitrogen form, or fully oxidized as nitrate. However, nitrogen can also exist in several partially reduced states, viz. nitroxyl anion (NO^-); nitric oxide (NO); nitrosonium cation (NO^+); or as nitrite (NO_2^-). Each of the partially reduced forms of nitrogen, also referred to as reactive nitrogen species (RNS), has distinct reactivity properties. It is the existence of such a variety in reactivity that explains much of the biochemical behaviour of NO [Gow & Ischiropoulos 2001]. One of the most significant properties of NO with regards to its biological effects is its ability to react with a number of molecules in the body. Indeed, NO and other RNS have been shown to react with proteins, nucleic acids, lipids and sugars [Brune & Lapetina 1995; O'Donnell *et al* 1999; Yermilov *et al* 1995]. For the purposes of this study, we will focus on the reactions of NO with proteins, which can be divided into 3 broad categories, namely reaction with metal-containing proteins, thiol-containing proteins and oxides [Gow & Ischiropoulos 2001].

The discovery that NO reacts with, and activates, soluble guanylate cyclase (sGC) [Ignarro *et al* 1987; Murad 1994; Murad 1998] was the first known physiological interaction described for NO. In fact, the reaction between NO and the heme prosthetic group of sGC is the trigger of the signaling cascade that leads to smooth

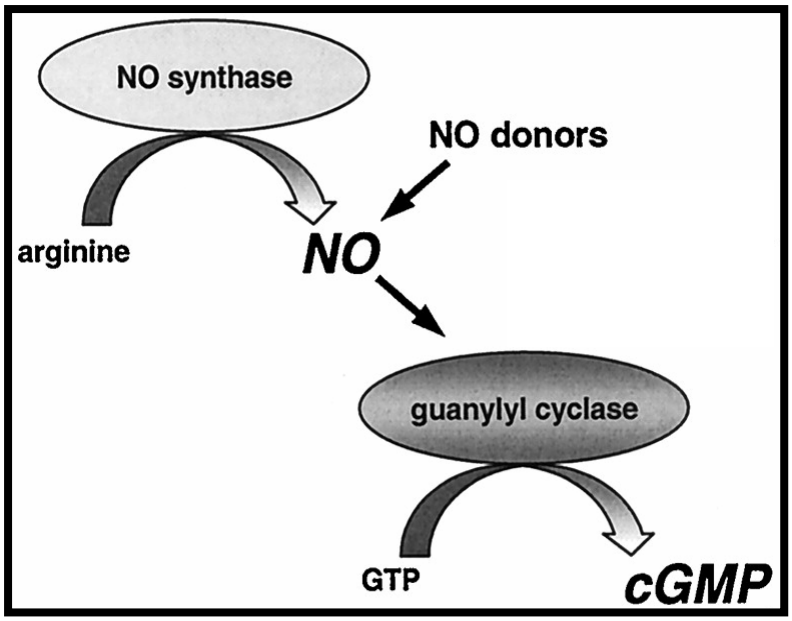


Fig. 1.15 The NO-sGC-cGMP pathway. (Modified from Friebe & Koesling 2003).

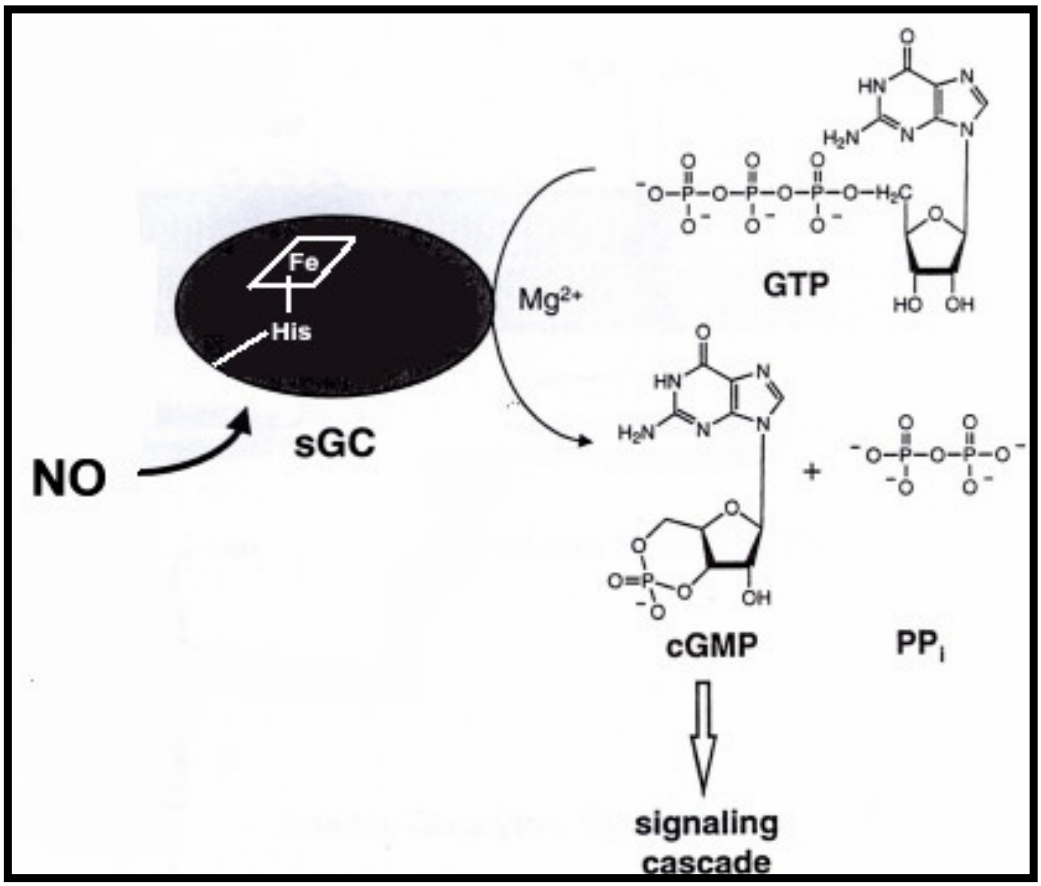


Fig 1.16 The NO-sGC reaction and activation of sGC. (Modified from Denninger & Marletta 1999)

muscle relaxation, and sGC is generally viewed as the most important receptor for NO [Friebe & Koesling 2003]. The NO-sGC interaction is an example of NO's ability to react with metals, since it binds to the iron within the heme group, which leads to conformational changes in the protein and ultimately enzyme activation [Murad 1994]. Stimulation of sGC by NO results in a profound 200-fold increase in the guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) conversion rate [Denninger & Marletta 1999; Friebe & Koesling 2003] (fig. 1.15 and 1.16). Soluble GC has a very high affinity for NO; in fact the EC_{50} value for sGC is as low as 2 nM NO; which explains why NO, released at relatively low physiological concentrations in cells, is able to function as a signaling molecule since most of its biological effects are via sGC activation [Friebe & Koesling 2003]. The mechanism of sGC activation by NO is thought to be a 2-step process: (a) NO-binding to heme results in formation of a NO-Fe_2^+-His-complex; (b) subsequently, breakage of the histidine-to-iron bond occurs, which initiates conformational changes and enzyme activation [Friebe & Koesling 2003]. In addition to sGC, NO also reacts with other metal-containing protein molecules including hemoglobin, myoglobin and cytochrome P450 [Gow & Ischiropoulos 2001].

A second class of NO-sensitive proteins is the thiol-containing proteins. NO's reaction with these proteins leads to the formation of so-called S-nitrosothiols (SNOs) [Gow & Ischiropoulos 2001]. Proteins that have been shown to be S-nitrosylated (leading to either activation or inhibition) by NO include p21 ras [Lander *et al* 1996], hemoglobin [Jia *et al* 1996] and caspase-3 [Kim *et al* 1997]. One of the most significant S-nitrosylation reactions is between NO and the signaling protein, p21 ras, which leads

to the activation of the latter [Gow & Ischiropoulos 2001], and as a result, activation of various intracellular signaling pathways.

Reaction of NO with oxides includes the well-known oxidation of NO by molecular oxygen [Gow & Ischiropoulos 2001] with formation of the ultimate final product, nitrite. In addition, due to NO's free radical nature, it also readily reacts with superoxide to form the highly reactive peroxynitrite, with a wide range of (often harmful) effects.

In summary, in view of NO's gaseous nature and its high degree of reactivity, it is clear that there is potentially a huge number of biological reactions in which NO can participate. Generally, the degree of exposure to NO, availability of target molecules and structure of target proteins determine the reaction route taken by NO in a cell. Exposure to NO is determined by a combination of intracellular production (via NOS) and external sources (from other cells or in plasma) of NO. Indeed, NO's reactions with thiol-containing proteins, superoxide and molecular oxygen are critically dependent on the flux of NO (from inside the cell and / or external sources) relative to the concentrations of these target molecules [Gow & Ischiropoulos 2001]. A good example of a protein that is structurally suited for reaction with NO, is sGC, which not only contains the heme-iron moiety for NO binding, but also cysteine residues making it susceptible to S-nitrosylation.

The reaction of NO with superoxide to form peroxynitrite ($ONOO^-$) deserves special mention. It is known to be the fastest biological reaction in which NO is involved [Gow & Ischiropoulos 2001]. In physiological conditions, superoxide generation is kept

within an acceptable range by its scavenger enzyme, superoxide dismutase (SOD) [Singh & Evans 1997]. The rate of reaction of superoxide with SOD is 2 x 10^9 M^{-1} sec^{-1}, whereas the rate of reaction of superoxide with NO is 6-10 x 10^9 M^{-1} sec^{-1} [Estevez & Jordan 2002]. As a result, NO combines at least 3 times faster with superoxide than SOD, which has important biological implications. Therefore, should a situation develop where SOD is ineffective in scavenging superoxide, or where there is excess NO generation, the reaction will be directed towards $ONOO^-$ formation [Singh & Evans 1997; Estevez & Jordan 2002; Ferdinandy & Schulz 2003]. Refer to Table 1.2 for a summary of the molecular mechanisms, targets and biological effects of NO.

Compared to $ONOO^-$, NO is a relatively stable and non-reactive free radical [Estevez & Jordan 2002]. However, $ONOO^-$ on the other hand is an unstable, pro-oxidant species that exerts toxic effects on many molecules, including nucleic acids, lipids and proteins [Singh & Evans 1997]. It is thought that many of NO's harmful effects are in fact mediated by $ONOO^-$ and not by NO itself [Ferdinandy & Schulz 2003], particularly when NO occurs in excess concentrations (such as generation by inducible NOS) [Singh & Evans 1997]. See figures 1.17 and 1.18 for schematic representations of the biologically important interaction between superoxide, NO and $ONOO^-$.

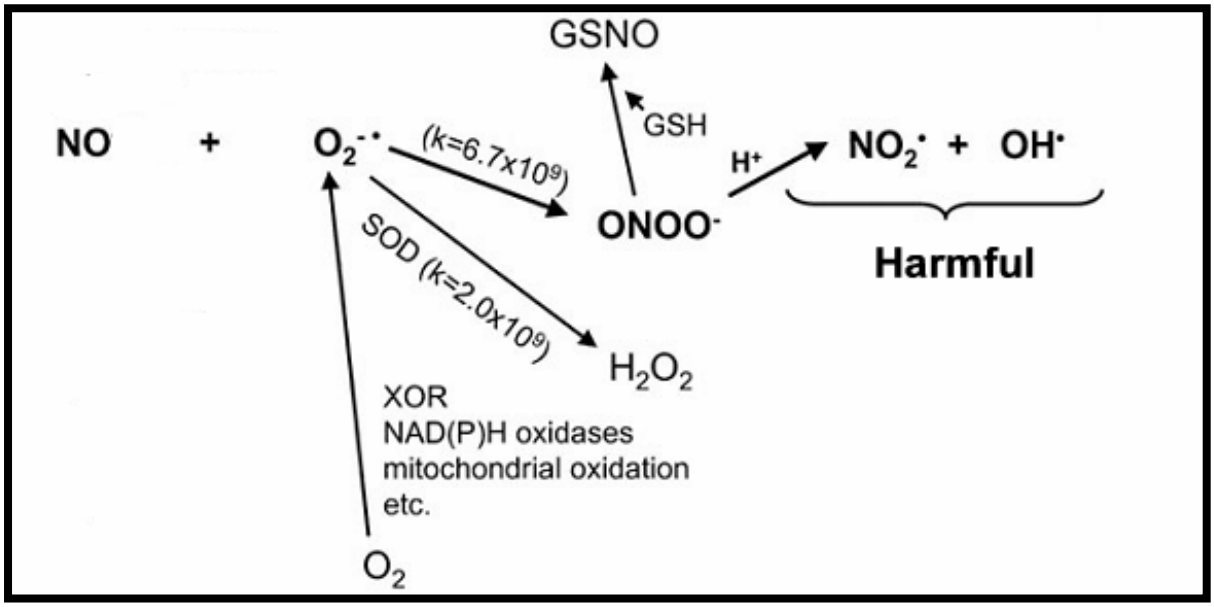


Fig. 1.17 The cellular interactions between superoxide, NO and $ONOO^-$. Although NO is not harmful by itself under physiological conditions, it becomes detrimental when the critical balance between cellular concentrations of NO, superoxide and SOD is disturbed leading to $ONOO^-$ generation (e.g. during ischaemia-reperfusion injury). Sources of superoxide in the body include NAD(P)H oxidases, xanthine oxidases (XOR) and mitochondrial electron transport activity. Detoxification of superoxide occurs when it is converted to H_2O_2 by its scavenger, SOD. $ONOO^-$ is detoxified when it combines with reduced glutathione (GSH) to form s-nitroglutathione (GSNO). $ONOO^-$ further decomposes to other highly reactive oxidants such as hydroxyl radical ($OH^{\bullet}$) leading to tissue damage. (Modified from Ferdinandy & Schulz 2003)

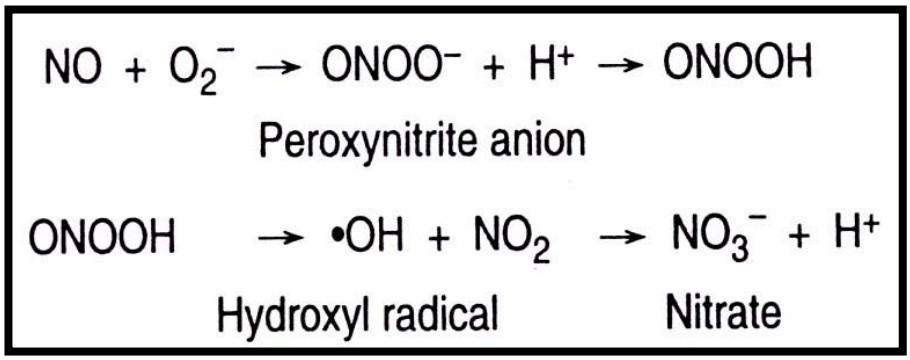


Fig. 1.18 Generation of harmful reactive nitrogen and oxygen species resulting from NO's reaction with superoxide. (Reproduced from Singh & Evans 1997)

(ii) Enzymatic generation of NO in the heart

The enzymes responsible for endogenous NO-generation in the body are called the NO synthases (NOS) [Schulz *et al* 2004]. NO is unique amongst the signaling molecules of the body, since it is a diffusable gas that can easily penetrate cell membranes [Bredt 2003]. Therefore, unlike conventional biological mediators, NO is not stored in vesicles, which means that NO release and signaling specificity must be controlled at the level of synthesis. Indeed, it has been suggested that the NOS enzymes are amongst the most tightly controlled in the body [Bredt 2003]. Currently, three main NOS isoforms have been described [Balligand & Cannon 1997]. Neuronal NOS (nNOS or NOS1) was originally described in the brain [Bredt *et al* 1991]; inducible NOS (iNOS or NOS2) in macrophages [Xie QW *et al* 1992] and endothelial NOS (eNOS or NOS3) in endothelial cells [Lamas *et al* 1992]. NOS is widely distributed throughout the body [Balligand & Cannon 1997]: neuronal NOS is expressed in neurons, cardiac conduction tissue, nerve terminals, epithelial cells, and skeletal muscle; iNOS in macrophages, endothelial cells, vascular smooth muscle cells, fibroblasts, and cardiomyocytes and eNOS in endothelial cells, kidney epithelial cells, hippocampal pyramidal neurons, skeletal myocytes, and cardiomyocytes [Balligand & Cannon 1997].

All three NOS isoforms share a common structure [Balligand & Cannon 1997] (fig. 1.19). The enzyme consists of two functionally complementary portions (connected by a calmodulin-binding domain in the middle): a carboxyl-terminal reductase domain and an amino-terminal oxygenase domain. The latter contains binding sites for heme, L-arginine and tetrahydrobiopterin (THB4). Upon activation of the enzyme, NADPH

releases electrons that are transferred from flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) (in the carboxyl-terminal) to heme iron, which is subsequently activated to bind oxygen. The electron transfer from the flavins to heme is calmodulin-dependent. The activated heme iron, in the presence of oxygen and the substrate L-arginine, finally catalyzes the synthesis of NO and L-citrulline [Balligand & Cannon 1997] (fig. 1.20). Neuronal NOS and eNOS require the presence of physiological concentrations of calcium for calmodulin-binding and the transfer of electrons to heme [Bredt & Snyder 1990], whereas in iNOS, calmodulin-binding seems to be calcium-independent [Balligand & Cannon 1997].

Neuronal NOS and eNOS are both constitutively expressed in cells with a result that they are generally associated with the generation of limited amounts of NO [Ziolo & Bers 2003]. In fact, eNOS is thought to be the source of continuous NO production under baseline, physiological conditions [Singh & Evans 1997]. Inducible NOS is normally not constitutively expressed, but has to be synthesized upon induction by factors such as cytokines [Balligand & Cannon 1997]. Inducible NOS is associated with high-output NO production and produces up to 1000-fold more NO than eNOS [Singh & Evans 1997].

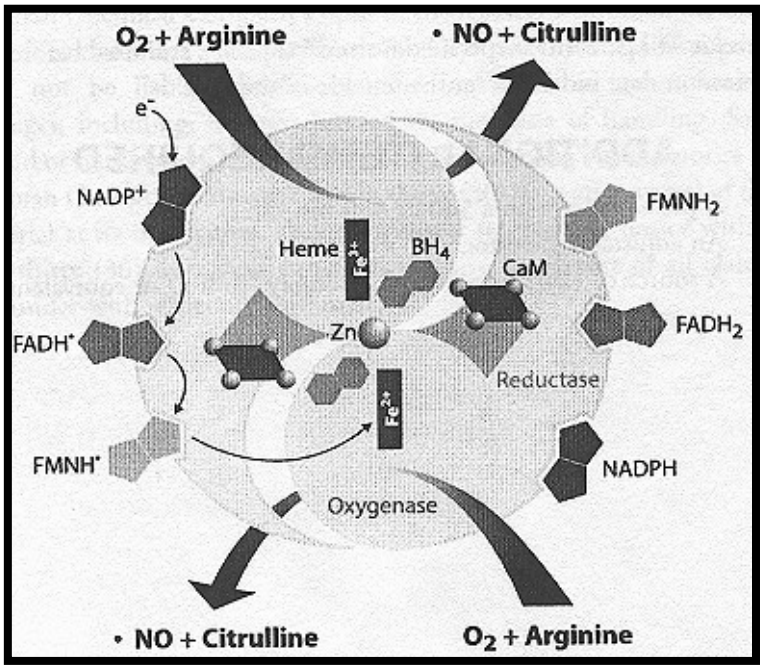


Fig. 1.19 Schematic representation of NOS, its co-factors, substrates and products (see text for details). Upon activation and calmodulin-binding, L-arginine is catalyzed to NO and citrulline in the presence of oxygen.

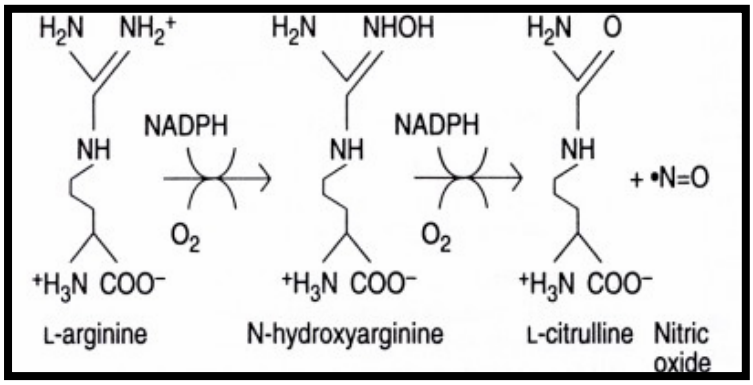


Fig. 1.20 Chemical reactions involved in the synthesis of NO. (Reproduced from Singh & Evans 1997)

The heart expresses all three NOS isoforms [Balligand & Cannon 1997]. Neuronal NOS is expressed in cardiac sympathetic nerve terminals [Balligand & Cannon 1997; Schwarz *et al* 1995], conduction tissue [Balligand & Cannon 1997] and cardiomyocytes [Danson *et al* 2005]; iNOS has been demonstrated in cardiomyocytes [Balligand *et al* 1994; Buchwalow *et al* 2001], vascular smooth muscle cells [Balligand & Cannon 1997], cardiac endothelial cells [Balligand *et al* 1995(a)], and cardiac fibroblasts [Balligand & Cannon 1997]; and eNOS in cardiomyocytes [Balligand *et al* 1995(b)] and cardiac endothelial cells [Balligand & Cannon 1997]. Recently, the expression of another calcium-dependent NOS enzyme has been described, viz. mitochondrial NOS (mtNOS) [Haynes *et al* 2004]. Transcripts of mtNOS mRNA have subsequently been identified in heart tissue [Gonzales *et al* 2005]. The exact nature of the mtNOS enzyme is still unclear; in the liver it has been identified as an isoform of nNOS [Haynes *et al* 2004], however this was disputed by another group who could not demonstrate the presence of any of the known NOS isoforms (nNOS, eNOS or iNOS) in liver mitochondria [Lacza *et al* 2003]. The strategic location of NO-generating enzymes within an organelle that plays such a crucial role in the mechanism of cardioprotection, makes further investigations into the biological effects of mtNOS imperative.

The location of NOS enzymes in specific subcellular domains plays an important role in their ability to be activated by calcium, to ensure proximity to the downstream targets of NO [Bredt 2003] and ultimately, in determining the cellular effects of NO [Ziolo & Bers 2003]. eNOS enzymes are specifically targeted to the Golgi complex and to flask-shape invaginations of the plasma membrane called caveolae [Bredt 2003; Schulz *et al* 2004; Gratton *et al* 2000]. Caveolae are localized hubs of signaling

activity within the cell and many signaling molecules are associated with caveolae, including G-protein-coupled receptors, ion channels and pumps (especially those involved in the regulation of intracellular calcium) [Bredt 2003]. In fact, in endothelial cells, caveolae have been described as the major plasmalemmal vesicle structure as opposed to clathrin-coated vesicles [Gratton *et al* 2000]. The distinct location of eNOS enables the enzyme to interact with ß-adrenoceptors and L-type calcium channels [Schulz *et al* 2004]. (See fig. 1.21)

Furthermore, the regulation of eNOS activity and exposure to external stimuli is the result of various protein interactions within the caveolae [Bredt 2003]. One of the major regulatory interactions with eNOS is with the most important protein in caveolae: caveolin-1 in endothelial cells and caveolin-3 in cardiomyocytes [Massion *et al* 2003], which results in eNOS inhibition [Bredt 2003; Ziolo & Bers 2003]. Inhibition of eNOS by caveolin is thought to be abolished by calmodulin since the latter causes displacement of caveolin from eNOS [Bredt 2003], a process that is further enhanced by binding of heat shock protein 90 (Hsp90) to eNOS [Gratton *et al* 2000].

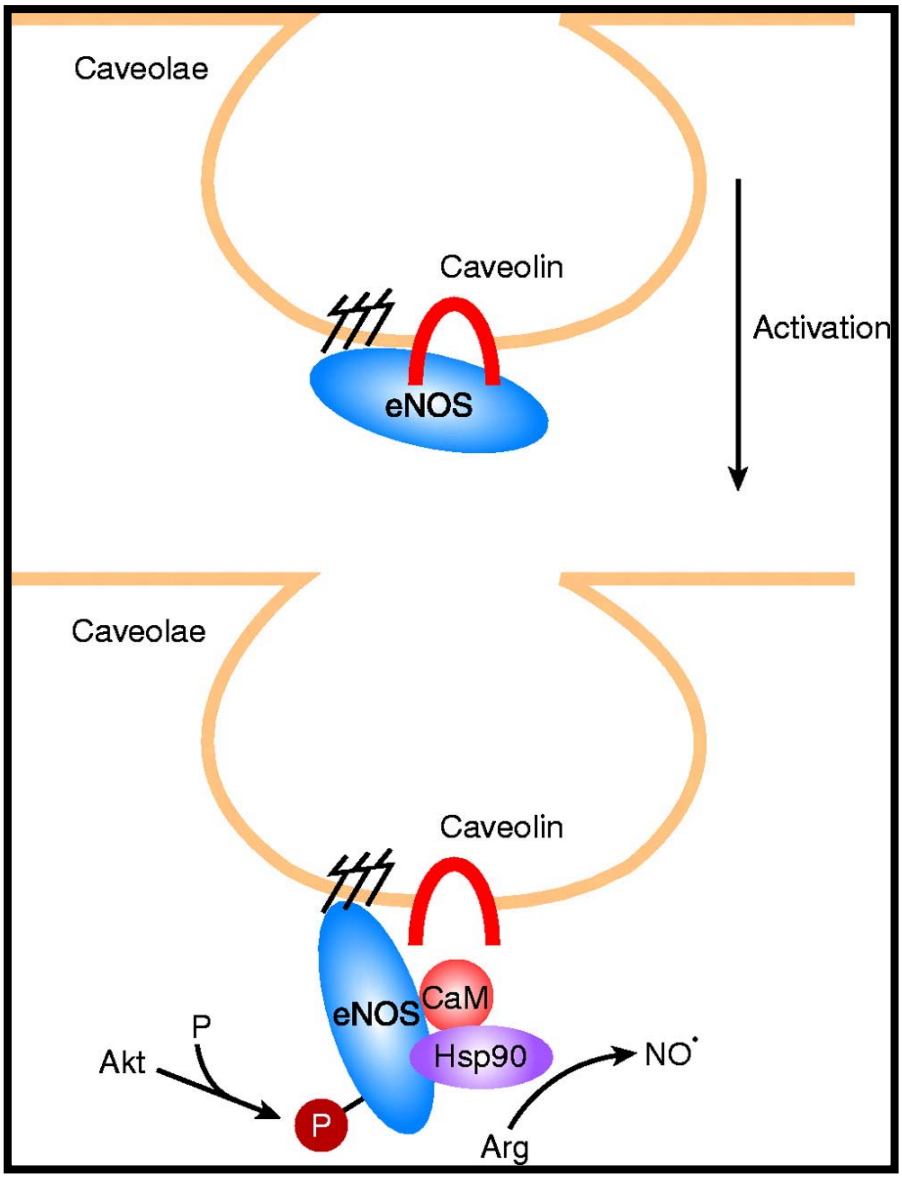


Fig 1.21 eNOS is localized in caveolae where it is regulated locally. The enzyme is targeted to the caveolus by myristoylation and palmitoylation (jagged lines). Upon an activating stimulus such as shear stress, Hsp90 is recruited, which in its turn recruits calmodulin, and activates eNOS. Additional activation of eNOS by Akt phosphorylation is also possible. (Reproduced from Bredt 2003)

Neuronal NOS is localized to the sarcoplasmic reticulum (SR) and is thought to associate with ryanodine receptors [Ziolo & Bers 2003], thereby increasing calcium release from the SR [Schulz *et al* 2004]. In the cardiomyocyte, the distinct subcellular localization of eNOS and nNOS has important consequences in the regulation of heart contraction [Bredt 2003]. In the caveolae, eNOS activation will lead to inhibition of the L-type voltage-dependent calcium channels, whereas nNOS (associated with the SR) will bind the ryanodine receptor, leading to calcium-release from the SR into the cytosol (see fig. 1.22). Inducible NOS, in contrast to eNOS and nNOS, is not believed to be localized or compartmentalized, and therefore occurs as a cytosolic enzyme [Ziolo & Bers 2003]. However, in a study that described the first immunocytochemical identification of iNOS in neonatal and adult cardiomyocytes, it was found that the enzyme was associated predominantly with the particulate component, i.e. the mitochondria, contractile fibres, plasma membrane and T-tubules [Buchwalow *et al* 2001]. They also demonstrated a constitutive expression of iNOS in the myocytes, which is in contrast to the commonly accepted view. It was speculated that based on the close association of iNOS with mitochondria and contractile fibers in their myocyte model, there may be a relationship between iNOS-derived NO, energy production, and contractile function in cardiac muscle.

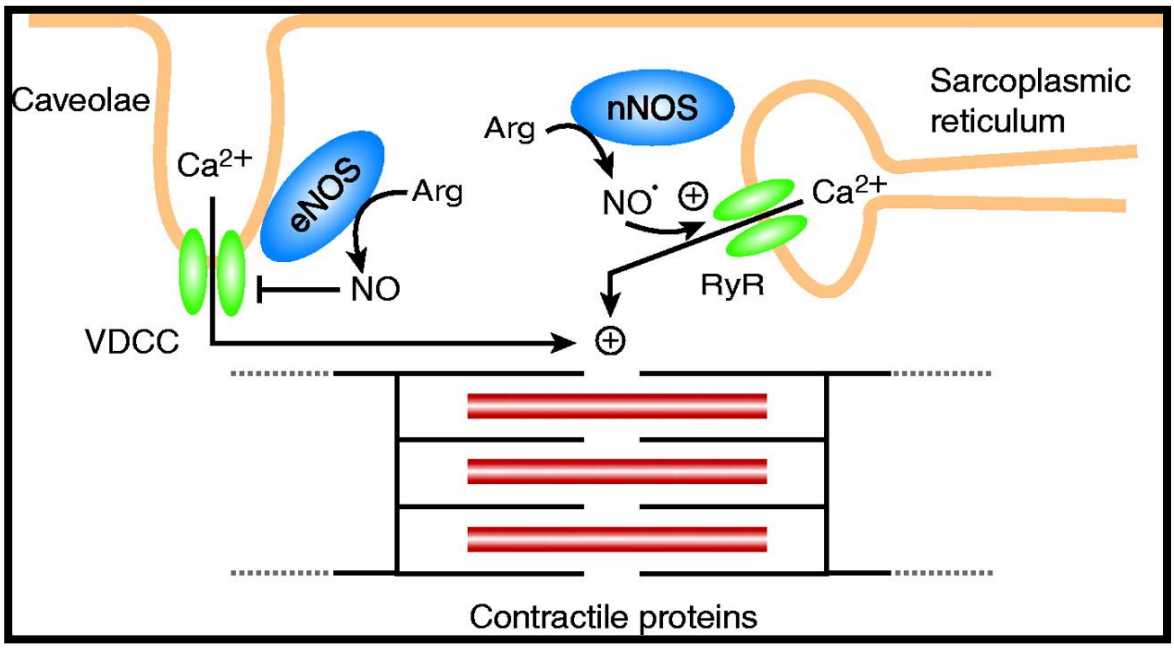


Fig 1.22 Opposing effects of eNOS and nNOS in the regulation of cardiomyocyte contraction. eNOS activation leads to an attenuation of calcium influx through the L-type voltage-dependent calcium channel (VDCC), whereas nNOS activation results in stimulation of the ryanodine receptor (RyR) and increased calcium release from the SR into the cytosol. Therefore, eNOS activation leads to negative inotropic effects and nNOS to positive inotropic effects. (Reproduced from Bredt 2003)

The precise nature and effects of the subcellular localization and distribution of nNOS and eNOS in cardiac cells are not well understood yet, and investigations often produce paradoxical findings. The proposed opposite effects exerted by nNOS and eNOS on calcium-levels are not necessarily a conclusion shared by all researchers [Ziolo & Bers 2003; Massion *et al* 2003]. A case in point relates to the localization of nNOS: in addition to being associated with the ryanodine receptors (RyR) as described above [Bredt 2003], nNOS also colocalizes with the L-type calcium channels, which could, upon nNOS activation, lead to subsequent inhibition of the channels and therefore *decreased* NO-levels [Ziolo & Bers 2003]. In such a case, nNOS would result in two opposite effects on myocyte contractility. The effects of endogenous NOS and NO in cardiac cells, and the different hypotheses will be discussed later.

(iii) Regulation of NOS in the heart

The primary regulatory mechanism of eNOS and nNOS activity is increased intracellular calcium, without which calmodulin cannot bind to the enzymes [Bredt 2003]. Furthermore, as discussed in the preceding section, the subcellular localization of eNOS and nNOS is a crucial component of NOS regulation. In the case of eNOS, caveolin (the primary coating protein of the eNOS-containing caveolae) acts as a negative regulator of the enzyme [Massion *et al* 2003], in addition to its role as a scaffolding protein. Caveolin's eNOS regulating role has been described in both cardiomyocytes [Feron *et al* 1998], and endothelium [Bucci *et al* 2000].

The eNOS-caveolin interaction can therefore be regarded two-fold:
(1) regulation of basal NO production, and (2) localization of eNOS in specific subcellular areas where it is available for agonist activation [Massion *et al* 2003].

Reference has previously been made of the role of Hsp90 as an activator of eNOS by promoting eNOS-caveolin dissociation. Furthermore, increased Hsp90-eNOS interaction is observed in the presence of vascular endothelial growth factor (VEGF) stimulation, histamine or shear stress [Brouet *et al* 2001]. In addition to this, Hsp90 promotes the recruitment of other eNOS-activating proteins, most notably that of the protein kinase PBK / Akt, which activates eNOS by phosphorylation on serine 1177 [Brouet *et al* 2001]. Indeed, serine 1177 is the best-characterized phosphorylation (and activation) residue of eNOS [Massion *et al* 2003; Dimmeler *et al* 1999], and PI3-K / Akt phosphorylation of serine 1177 results in a 15-20-fold increase in eNOS activity [Bredt 2003]. Other factors that are thought to activate eNOS by serine 1177 phosphorylation include shear stress, isometric vessel contraction, insulin [Bredt 2003] and cardiac muscle stretch [Massion *et al* 2003]. Ischaemia has also been shown to activate eNOS within minutes [Depre *et al* 1997]; however prolonged ischaemia associated with increased tissue acidosis attenuates eNOS activity [Giraldez *et al* 1997].

It is interesting to note that eNOS must be targeted to membranes (intracellular or peripheral) in order to be phosphorylated by Akt [Massion *et al* 2003]. The fate of eNOS after serine 1177 phosphorylation is still disputed, but is thought to be either translocated to the cytosol or moved within membrane structures [Massion *et al* 2003]. Four other phosphorylatable residues have been identified, namely Ser116,

Thr495, Ser615 and Ser633 [Massion *et al* 2003]. Phosphorylation of Thr495 by AMP-activated kinase or PKC has been shown to inactivate eNOS [Schulz *et al* 2004]. Although eNOS is a constitutive enzyme, its protein expression levels can be altered by various factors [Shah & MacCarthy 2000, Schulz *et al* 2004]. Angiotensin II (increased levels observed during myocardial ischaemia) has been shown to upregulate eNOS expression [Noda *et al* 1993], whereas TNF-α (also rapidly released in the ischaemic myocardium) decreases eNOS protein expression [de Frutos *et al* 2001]. In macrovascular endothelial cells, chronic fluid shear stress, exposure to transforming growth factor (TGF)-ß, cell proliferation, chronic exercise, and pregnancy have been shown to upregulate eNOS expression [Shah & MacCarthy 2000]. Treatment with the Gi-protein inhibitor, pertussis toxin, has been shown to both upregulate and activate eNOS in perfused rat hearts [Hare *et al* 1998]. In cardiomyocytes, 24 h elevation of cAMP levels have resulted in downregulation of eNOS, as did treatment of animals with the PDE inhibitor, milrinone [Shah & MacCarthy 2000].

The regulation of iNOS is predominantly driven by modulation on transcription level [Massion *et al* 2003], although it is generally believed that this enzyme does not require the fine regulation of the calcium-dependent NOS enzymes. However, recently at least four proteins have been described that interact with iNOS, including caveolin-3 and Rac2 [Massion *et al* 2003]. Regulation of nNOS has not been researched as extensively as the other NOS isoforms, however, it has been shown to be expressed in cardiomyocytes [Bredt 2003] where chronic hypoxia exposure is thought to reduce nNOS expression [Mohan RM *et al* 2001]. Please refer to Table 1.1 for a summary of the regulation of eNOS, nNOS and iNOS in the heart.

NOS protein expression		NOS activity	
nNOS			
+/0	Chronic angiotensin II	+	Hsp90
–	Chronic intermittent hypoxia	–	dystrophin deletion (mdx mice)
iNOS			
+	IL1β, INFγ LPS, TNFα+IL6 Phenylephrine (α-AR) Norepinephrine(α and β) Isoproterenol (β2) Hypoxia High glucose C-reactive protein Estrogen		
–	Corticoids , Cyclosporine A, FK506	–	Arginine deficiency
eNOS			
+	Shear stress Exercise Hypoxia (acute , chronic) Hormones and autacoids: Estrogens (ERβ) , Insulin Angiotensin II (AT2 and calcineurin) Drugs and toxins: Angiotensin converting enzyme inhibitors Angiotensin II type 1 receptor antagonists Some Ca^{++} channel blockers β adrenoceptors antagonists Statins Nicotin Nicorandil Pertussis toxin	+	Myristoylation Palmitoylation Serine 1177 phosphorylation: Stretching , AMPK , Insulin , Corticoids Hsp90 , as in chronic hypoxia Dynamin Shear stress Acute pacing Hormones and autacoids: Bradykinin Estradiol VEGF Acetylcholine Substance P Histamine Angiotensin II Drugs: β3-agonist Ca^{++}channel blockers ACE inhibitors Statins
–	Lipopolysaccharides LDL (native , glycosylated and oxidized) Hyperglycemia Cortisol Milrinone SNAP, 8-Br-cGMP and IBMX Erythropoietin	–	Caveolin-1 Caveolin-3 NOSTRIN Phosphorylation changes: hyperglycemia (Ser1177–) AMPK (Thr495+) BH4 deficiency ADMA ROS

Table 1.1: Regulation of NOS protein expression and activity in the heart. Abbreviations: **+**, stimulation; **-**, inhibition (Modified from Massion *et al* 2003)

(iv) The physiological effects of NO in the heart

NO-sGC-cGMP signaling

Originally, the effects of NO were mainly associated with its role in the regulation of the resting vascular tone, i.e. activation of the NO-sGC-cGMP-PKG pathway, which results in vasodilation via inhibition of the voltage-gated calcium channels [Schulz *et al* 2004]. In addition to the direct hemodynamic effects of endothelium-derived NO, it also exerts several other vascular actions. These include inhibition of platelet adhesion, activation and aggregation [Singh & Evans 1997], reduction of monocyte adhesion to the endothelium [Bath *et al* 1991], and induction of vascular cell adhesion molecule (VCAM)-1 [Marui *et al* 1993], thus making NO an important antithrombotic and anti-inflammatory role-player in the endothelium. However, the realization that all three NOS isoforms are expressed in the cardiomyocytes themselves, led researchers to believe that NO may indeed also have direct myocardial effects [Massion *et al* 2003].

The NO-sGC-cGMP pathway is regarded as the predominant molecular mechanism of NO actions [Balligand & Cannon 1997]. Activation of the NO-sensitive sGC results in the conversion of guanosine triphosphate (GTP) to the second messenger cGMP (fig. 1.23), which activates two cGMP-dependent protein kinases (PKG I and PKG II) [Schulz *et al* 2004]. A second class of molecular targets of cGMP is the phosphodiesterases (cGMP activates PDE II and inhibits PDE III) [Schulz *et al* 2004]. cGMP has also been shown to modulate the activity of cGMP-regulated ion channels

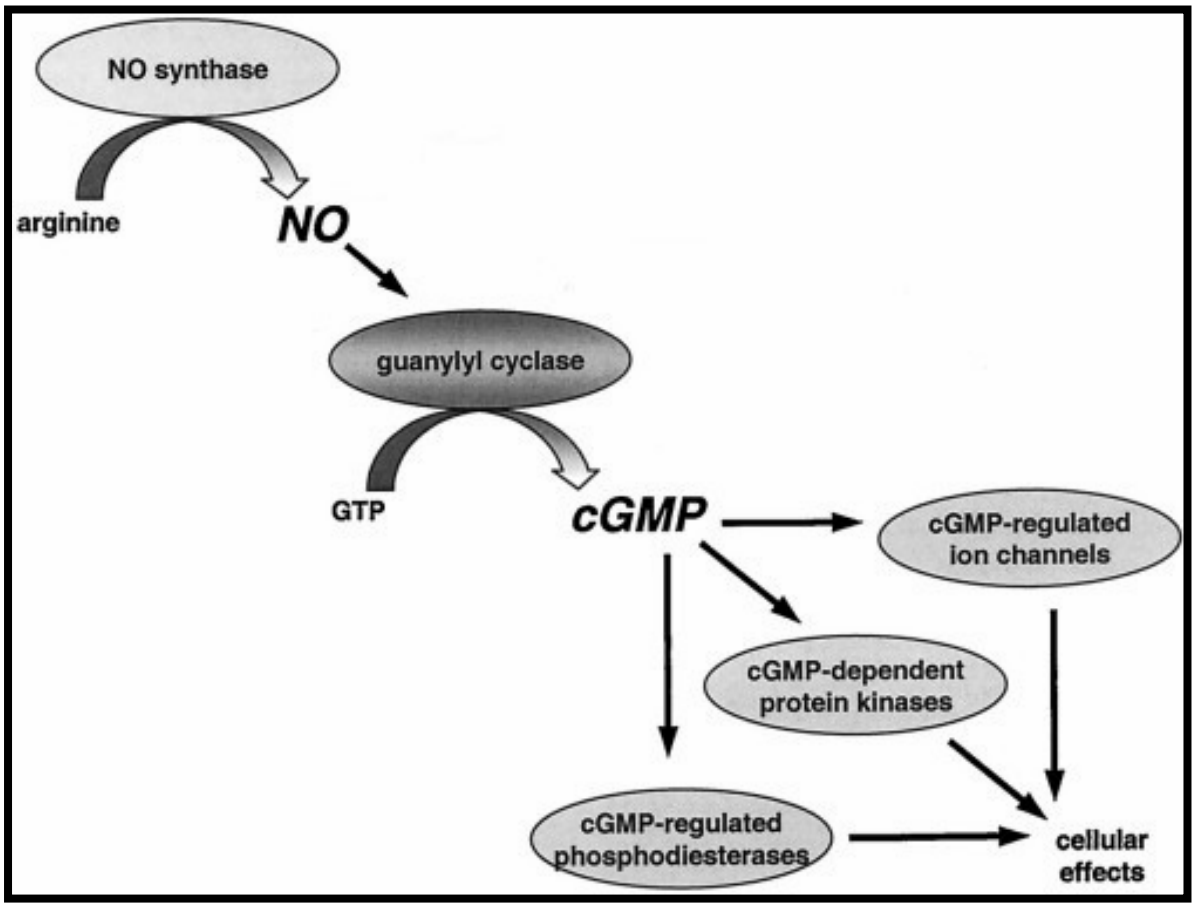


Fig 1.23 The NO-sGC-cGMP pathway (Reproduced from Friebe & Koesling 2003)

[Friebe & Koesling 2003]. Collectively, these effectors are involved in the regulation of several physiological functions in the cardiovascular system. In summary, cGMP-associated myocardial effects include (1) modulation of sarcolemmal calcium influx, (2) attenuated myofilament sensitivity for calcium, (3) altered SR function, (4) changes in the action potential, (5) cell volume modulation, and (6) reduction in oxygen consumption [Shah & MacCarthy 2000]. Several NO actions on the heart are mediated through cGMP-*independent* mechanisms [Shah & MacCarthy 2000; Balligand & Cannon 1997]. These include direct NO reactions with (1) proteins (amino, thiol, diazo and tyrosyl residues), (2) heme, (3) iron (Fe^{2+}) and (4) adenylyl cyclase (AC) in cardiomyocytes [Shah & MacCarthy 2000]. It is thought that cGMP-independent actions are particularly relevant when the high-output NO generating iNOS isoform is induced [Shah & MacCarthy 2000].

Effects on myocardial contractility: inotropic and lusitropic actions

In 1991, the first study appeared that demonstrated a direct link between NO and effects on myocardial contractile function [Smith *et al* 1991]. It has since become clear that NO has several, but often contradictory effects in the heart [Shah & MacCarthy 2000]. The role of the NOS isoforms and subcellular localization in the diversity of NO-actions has been discussed earlier. Other factors in the microenvironment that could account for the modulation of the biological effects of NO include: (1) the cellular source of NO (e.g. endothelial cells produce more NO than cardiomyocytes), (2) the amount of NO produced (e.g. high-output iNOS-derived NO vs. low-output eNOS), (3) the number and availability of molecular targets within diffusion distance from NOS, (4) prevailing redox balance and antioxidant status, (5) interactions with neurohormonal and other stimuli, (6) the presence of disease, and

(7) species and methodological differences in the experimental context [Balligand & Cannon 1997; Shah & MacCarthy 2000].

Modulation of the ß-adrenergic pathway in cardiomyocytes is an important mechanism underlying the effects of NO on myocardial contractility [Balligand & Cannon 1997; Balligand 1999] (see fig. 1.24). The ß-adrenergic-adenylyl cyclase (AC)-cAMP-PKA pathway results in phosphorylation (opening) of L-type calcium channels, increased intracellular calcium concentration, and ultimately the enhancement of myofibrillar contraction [Balligand & Cannon 1997]. Simultaneous increases in NO-production and cGMP levels may either activate phosphodiesterase (PDE) II or inhibit PDE III [Schulz *et al* 2004]. PDE II activation leads to increased cAMP breakdown and thus attenuated calcium-inflow and cardiomyocyte contraction. Conversely, cGMP-dependent PDE III inhibition potentiates ß-adrenergic-cAMP stimulatory effects [Balligand & Cannon 1997]. A third possible mechanism that can explain NO modulation of ß-adrenergic effects is direct L-type channel inhibition by PKG, thus potentiating the effects of the cGMP-PDE II attenuation of cAMP [Balligand & Cannon 1997].

Given the above opposing effects of NO on myocardial contraction, as well as the previously described differences in eNOS- and nNOS-derived NO actions, it is clear that the modulatory effects of NO on contractile function are complex and contradictory [Massion *et al* 2003]. The original discovery that the NO-cGMP pathway exerts effects of NO on myocardial contraction under basal (unstressed) conditions was regarded as a novel concept in cardiac function regulation at the time [Mohan *et al* 1995].

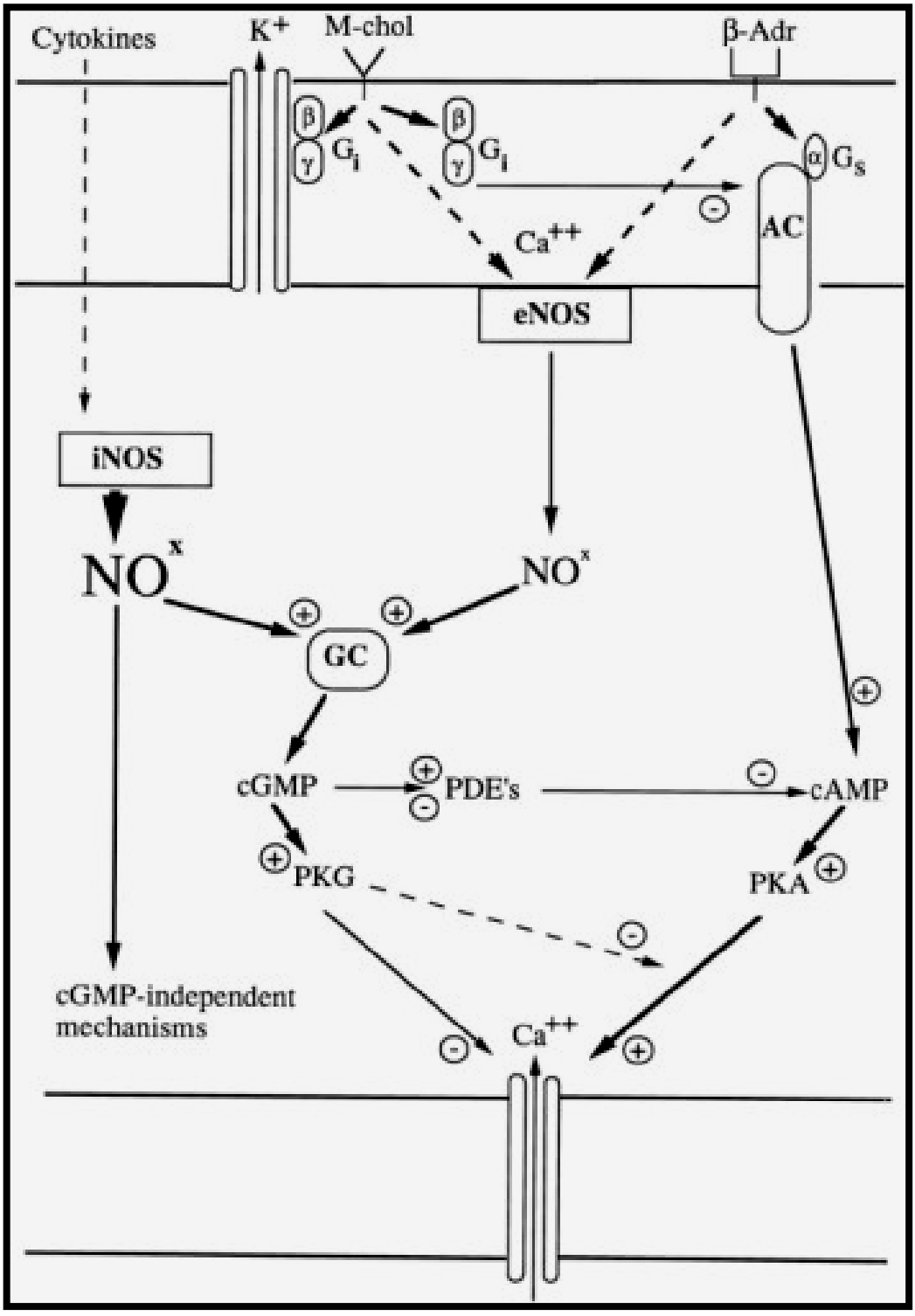


Fig. 1.24 Modulation of ß-adrenergic signaling in cardiomyocytes by NO. Refer to text for details. Abbreviation: M-chol, muscarinic cholinergic receptor. (Reproduced from Balligand & Cannon 1997)

From this study, it was suggested that basal, tonic release of endogenous NO under physiological conditions led to a preservation of myocardial function [Mohan *et al* 1995]. The NO effects followed bimodal pattern: at low (submicromolar NO doses), a small positive inotropic effect was observed, and at high (micromolar or above doses) a negative inotropic effect [Mohan *et al* 1995; Shah & MacCarthy 2000]. In a study on isolated rat cardiomyocytes, it was shown that NO donors (SNAP and DEA/NO) exerted a negative inotropic effect independent of cGMP-elevation [Sandirasegarane & Diamond 1999]. Another study on cultured rat cardiomyocytes showed that iNOS-derived-NO resulted in attenuated cAMP levels in ß-adrenergic stimulated cells, which was partly mediated by a cGMP-dependent mechanism [Joe *et al* 1998]. The conclusion from these findings was that NO generated by iNOS might participate in cardiac contractile depression in this manner, particularly in situations of iNOS induction such as cytokine exposure.

Although most studies have focused on the inotropic effects of NO, others have demonstrated a significant role for NO in *myocardial relaxation and diastolic properties* (lusitropic effects) [Shah *et al* 1994; Paulus *et al* 1994]. In these studies treatment with cGMP analogues (in isolated cardiomyocytes), endothelium-dependent agonists (in papillary muscle) and NO donors (in isolated hearts) resulted in the induction of earlier isotonic twitch relaxation, increased diastolic cell length and premature LV relaxation [Shah & MacCarthy 2000]. In isolated cardiomyocytes, NO was found to result in a negative chronotropic effect by decreasing the rate of spontaneous beating [Feron *et al* 1998]. Currently, it is thought that NO derived from nNOS (in cardiac ganglia) and eNOS (in cardiomyocytes) act to potentiate parasympathetic (vagal) inhibition of heart rate [Massion *et al* 2003].

Metabolic effects of NO

NO does not only affect myocardial contractile function. NO (endogenous and exogenous) has also been shown to exert metabolic effects on the myocardium [Brutsaert 2003]. These include decreased myocardial oxygen consumption (Brutsaert 2003; Loke *et al* 1999; Trochu *et al* 2000], the regulation of mitochondrial metabolism by direct inhibition of the respiratory chain [Stumpe *et al* 2001], as well as utilization of energy substrates by reducing myocardial glucose uptake [Tada *et al* 2000]. These effects are suggestive of a putative cardioprotective mechanism for NO [Brutsaert 2003]. NO has also been shown to inhibit electron transfer in the mitochondria [Cleeter *et al* 1994]. In addition, NO has recently been shown to directly activate the mitochondrial K_{ATP} channels [Sasaki *et al* 2000], which led researchers at the time to believe that NO might play a crucial role in what was then regarded as the candidate end-effector of IP-protection. Furthermore, a possible role for NO derived from iNOS in the induction of apoptosis has been shown in neonatal mouse cardiomyocytes treated with the cytokine TNF-α [Song *et al* 2000].

See Table 1.2 for a summary of molecular mechanisms and targets of NO, and fig. 1.25 for a schematic representation of the biological effects of low and high NO concentrations in the cardiovascular system.

MECHANISMS	TARGET MOLECULES	FUNCTIONAL EFFECTS
1. Interaction with Heme proteins	Guanylyl Cyclase	cGMP generation
	Hemoglobin, myoglobin	Inactivation of NO
	Cyclo-oxygenase 2	Activation of prostaglandin and thromboxane generation
	NOS	Inhibition of NO production
2. S-Nitrosation of thiols	NADPH oxidase	Enzyme inhibition
	Glutathione	Depletion of intracellular stores
	Hemoglobin	Exchange of NO to other acceptors
	Tissue plasminogen activator	Activates vasodilatation and anti-platelet effects
3. Binding to non-heme iron	Mitochondrial electron transport chain (complexes I, II and IV)	Inhibition of high-energy phosphate metabolism
	Ribonucleotide reductase	Inhibition of DNA synthesis
	Aconitases	In mitochondria: inhibition of Krebs Cycle; In Cytosol: regulation of iron metabolism
5. Oxidation	DNA strand breaks	Depletion of cell energy stores; contractile dysfunction
6. Tyrosine nitration	Cytoskeletal proteins, contractile filaments	Contractile dysfunction?

Table 1.2: Molecular mechanisms and targets and effects of NO relevant to cardiovascular biology. Please see text for detail. (Modified from Balligand & Cannon 1997)

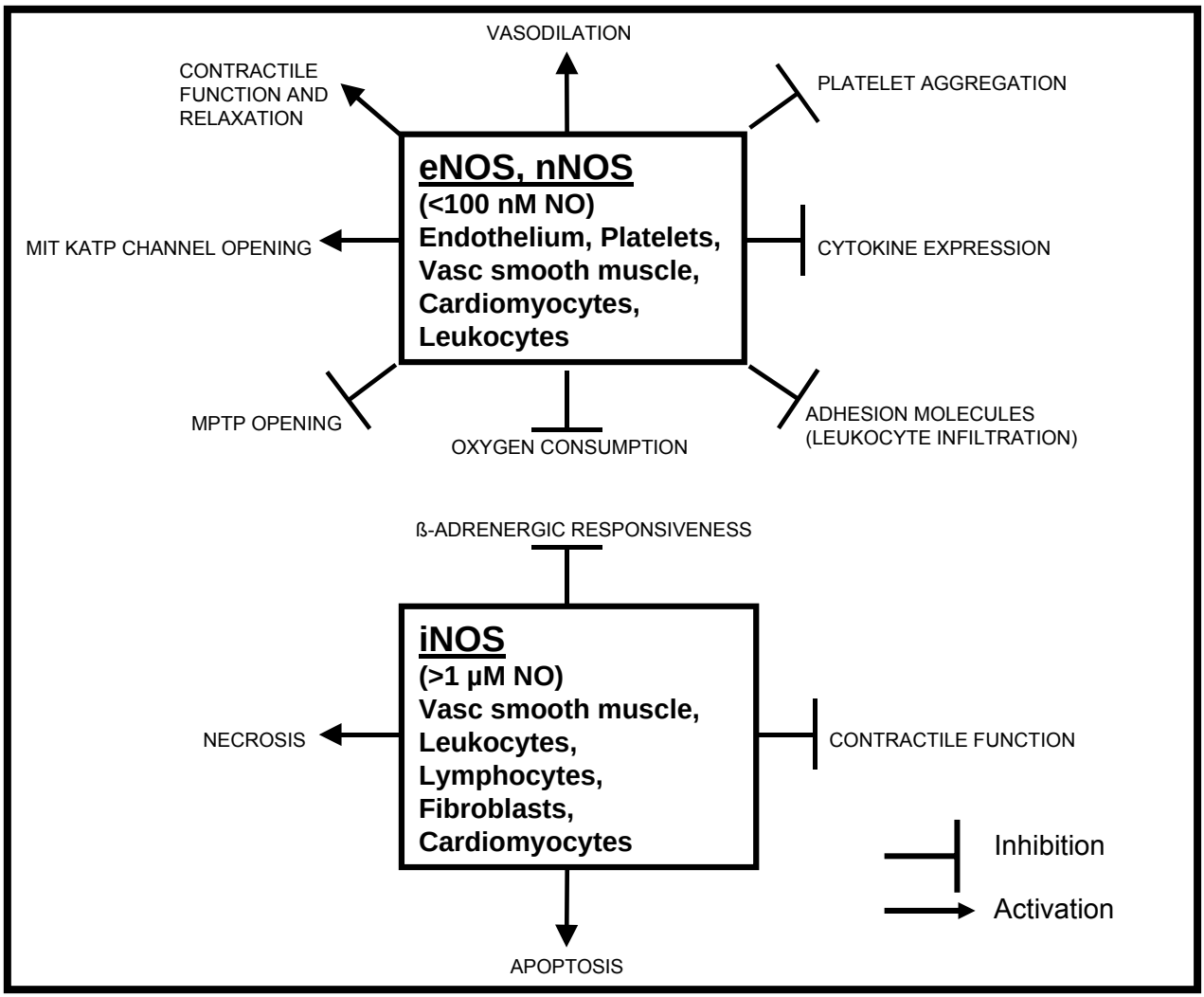


Fig 1.25 Effects of low (eNOS- or nNOS-derived) or high (iNOS-derived) NO concentrations in the cardiovascular system. (Modified from Schulz *et al* 2004)

(v) NO in myocardial hypoxia, ischaemia and ischaemia-reperfusion

Evidence for production of NO during hypoxia and ischaemia / reperfusion

NO (or the lack thereof) affects various aspects of hypoxia, ischaemia and ischaemia-reperfusion in the heart [Shah & MacCarthy 2000]. A role for NO under pathophysiological conditions in which oxygen availability is compromised has been demonstrated in a variety of experimental models and animals [Node *et al* 1995; Komarov *et al* 1997; Depré *et al* 1997; Pohl & Busse 1989; Kitakaze *et al* 1995]. It is generally accepted that ischaemia and hypoxia (in the absence of reperfusion or reoxygenation) result in increased levels of NO in the myocardium [Shah & MacCarthy 2000], however this seems to depend on the duration of ischaemia / hypoxia (within ~ 30 min) [Shah & MacCarthy 2000; Schulz *et al* 2004].

In canine hearts, increased levels of nitrates + nitrites (NOx; metabolic end products of NO) were measured in the coronary blood during ischaemia [Node *et al* 1995]. In another study on isolated rat hearts, 40 min of global ischaemia also resulted in increased NO-production [Komarov *et al* 1997]. The same trend was observed in isolated perfused rabbit hearts subjected to different periods of low-flow ischaemia [Depré *et al* 1997]. In this study, using the L-citrulline assay as an indicator of NOS activity, increased activity was observed, which disappeared after the onset of reperfusion (fig. 1.26). In 1989, hypoxia (as opposed to ischaemia) was also shown to be an important stimulus for increased NO-production in a study on femoral artery segments [Pohl & Busse 1989]. Subsequently, it was demonstrated that hypoxia

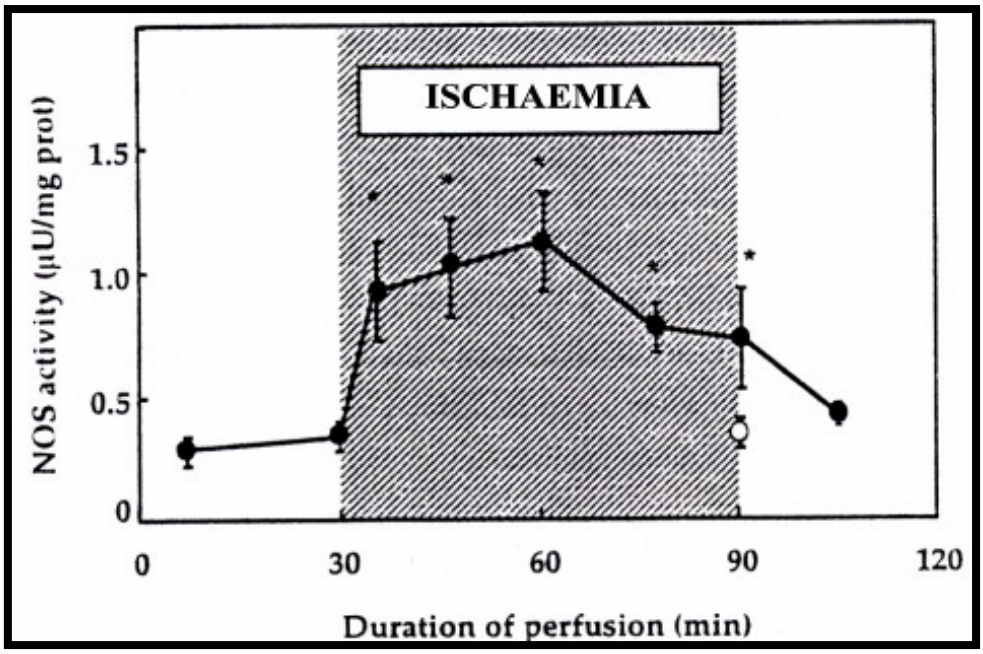


Fig. 1.26 NOS activity in isolated rabbit hearts during ischaemia (30 min – 90 min) and reperfusion (90 min – 105 min). An increase in NOS activity, as measured by the L-citrulline assay, was observed rapidly after the onset of low-flow ischaemia and started decreasing again after 30 min of ischaemia and during reperfusion. (Modified from Depré *et al* 1997)

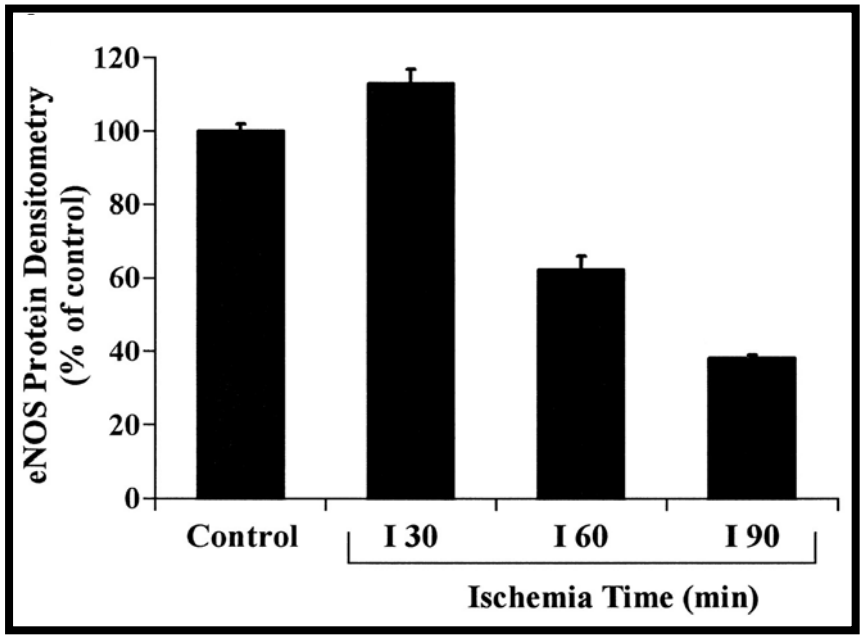


Fig. 1.27 Total eNOS protein expression in heart tissue at different time-points of exposure to ischaemia. At 60 min ischaemia a ~ 40% reduction in eNOS was observed compared to control levels. (Reproduced from Giraldez *et al* 1997)

induced NO-production in coronary endothelial cells [Park *et al* 1992], as well as isolated adult rat cardiomyocytes [Kitakaze *et al* 1995].The same group in a subsequent study demonstrated on open-chest dogs that treatment with a NOS-inhibitor (L-NMMA) resulted in a reduction in blood flow during regional low-flow ischaemia, which was associated with a worsening of contractile function and metabolic function [Kitakaze *et al* 1996]. This study was one of the first to suggest a putative protective role for endogenously produced NO during ischaemia [Shah & MacCarthy 2000]. Ischaemia-induced NO-production seems to change over time since it has been shown that prolonged myocardial ischaemia is associated with a decline in NO levels [Shah & MacCarthy 2000; Schulz *et al* 2004]. Prolonged ischaemia of more than 60 min in isolated perfused rat hearts resulted in a loss of total eNOS protein expression as well as NOS activity, which was ascribed to the increasing levels of tissue acidosis [Giraldez *et al* 1997] (fig. 1.27). This trend was also observed in a model of isolated cardiomyocytes subjected to 2 h of simulated ischaemia, where it was shown that acidosis attenuated guanylyl cyclase-induced cGMP synthesis [Agullo *et al* 2003].

Despite several investigations examining the role of NOS and NO during hypoxia and ischaemia, more research is necessary to establish the significance of NOS regulation in cardiomyocytes during hypoxia / ischaemia [Jung *et al* 2000]. Furthermore, most of the data available focus on the role of eNOS, whereas the role of the high-output NO generating iNOS is comparatively under-investigated. It is unlikely that the contribution of nNOS is important in this regard since its expression seems to be downregulated during hypoxia [Mohan RM *et al* 2001]. A role for iNOS was demonstrated in a study on rat hearts in which it was shown that hypoxia

induced iNOS expression in cardiomyocytes, and that the iNOS gene was regulated by hypoxia inducible factor (HIF)-1 [Jung *et al* 2000].

Detrimental effects of NO during ischaemia and hypoxia

Although it is thought that most of the detrimental effects of NO during ischaemia-reperfusion occur during the reperfusion phase (see later), some studies have demonstrated a harmful role for NO released during ischaemia and hypoxia in the absence of reperfusion or reoxygenation. In perfused rabbit hearts the addition of a NOS inhibitor prior to and during ischaemia, but not reperfusion, protected the hearts against ischaemic damage (improved functional recovery and attenuated enzyme release) [Depré *et al* 1995] (See fig. 1.28). Another study on isolated working rabbit hearts also demonstrated similar trends [Schulz & Wambolt 1995], where the administration of L-NAME and L-NMMA (NOS inhibitors) prior to the onset of ischaemia, but not present at the beginning of reperfusion, resulted in beneficial effects. In fact, in the case of the L-NAME treated hearts, an almost complete protection against myocardial mechanical dysfunction was observed, suggesting a damaging role for ischaemia-induced NO-release [Schulz & Wambolt 1995]. In a more recent study on endothelial cells, it was demonstrated that hypoxia potentiated NO-mediated apoptosis, probably via the formation of peroxynitrite [Walford *et al* 2004]. In another study that investigated a role for *endogenous* NO during ischaemia, isolated rat hearts were subjected to cardioplegic arrest +/- a NOS inhibitor (L-NMMA). Results showed a significant improvement in post-ischaemic mechanical function in the L-NMMA treated group [Amrani *et al* 1995]. See fig. 1.29 for a

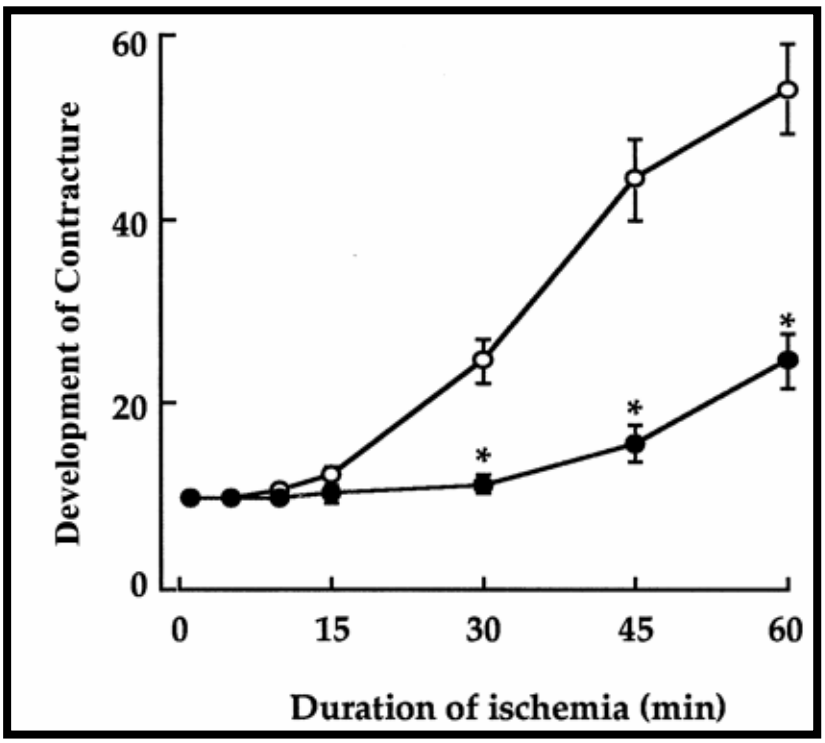


Fig. 1.28 Development of contracture in perfused rabbit heart exposed to 60 min ischaemia and 30 min reperfusion. Treatment with a NOS inhibitor (L-NMMA) prior and during ischaemia, but not reperfusion, delayed the onset and reduced degree of contracture (filled circles on graph), compared to untreated controls (open circles). These results suggested that NO produced during ischaemia was harmful and contributed to the ischaemic damage. (Reproduced from Depré *et al* 1995)

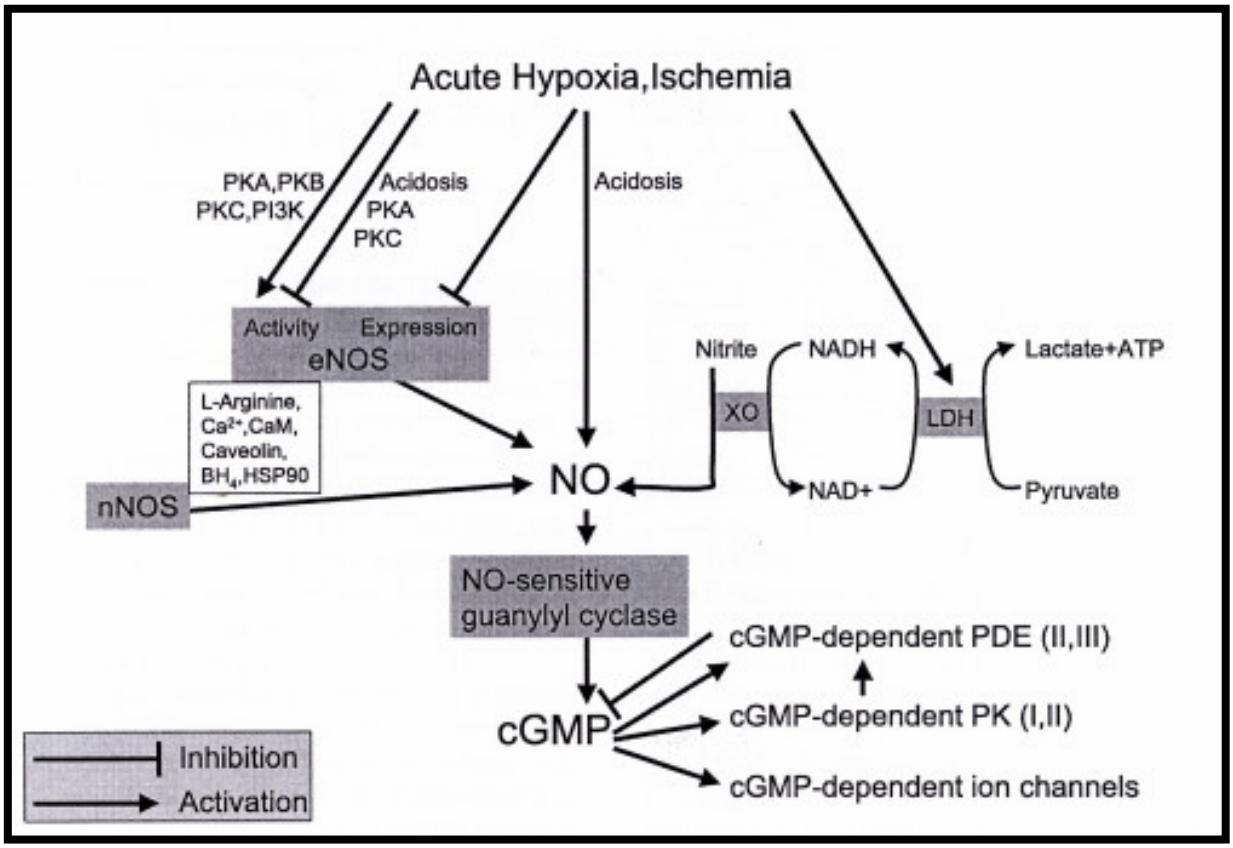


Fig. 1.29 Signaling events involving NO production during hypoxia and ischaemia. Hypoxia / ischaemia activates PI3-kinase and PKB (protein kinase B or Akt) which results in activation of eNOS via phosphorylation of Serine1177. PKA and PKC can either activate or inhibit eNOS. Hypoxia- / ischaemia-associated tissue acidosis inhibits the activity and downregulates the expression of eNOS, however it has been shown to increase NO-production in an eNOS-independent manner. Hypoxia / ischaemia can also increase NO production by LDH activation and nitrite-to-NO conversion catalyzed by xanthine oxidase (XO). (Reproduced from Schulz *et al* 2004)

schematic representation of the signaling events leading to increased NO production during hypoxia and ischaemia.

Detrimental effects of NO during ischaemia-reperfusion

Studies demonstrating a harmful role for NO are more abundant in the context of ischaemia *and reperfusion* injury [Shah & MacCarthy 2000]. In a study on piglets subjected to hypoxia-reoxygenation on cardiopulmonary bypass, it was shown that treatment with a NOS inhibitor resulted in nearly complete protection against myocardial reoxygenation injury [Matheis *et al* 1992]. When the NO substrate, L-arginine was co-administered with the NOS inhibitor, the protective effects of NOS inhibition were abolished. In another study on isolated rat hearts subjected to hypoxia – reoxygenation, it was shown that addition of SNP (a NO donor) during reoxygenation was detrimental, as opposed to when it was added during the hypoxia period [Draper & Shah 1997]. Another group [Csonka *et al* 1999] also demonstrated harmful accumulation of NO in isolated perfused rat hearts during ischaemia-reperfusion. They reported a marked increase in tissue NO levels after 30 min ischaemia by a spin trapping method, which was reduced in hearts pretreated with the NOS inhibitor, LNA. Reduction in NO levels was accompanied by improved postischaemic cardiac performance, decreased LDH release and reduced incidence of ventricular fibrillation (VF), which led the authors to conclude that the accumulation of NO during ischaemia and reperfusion contributed to the injury. Others have also demonstrated a harmful role for NO in ischaemia-reperfusion by reporting a reduction in infarct size in the presence of NOS inhibition [Woolfson *et al* 1995; Patel *et al* 1993].

A harmful role for iNOS-derived NO was described in a study on rabbits in which myocardial infarction (MI) was induced [Wildhirt *et al* 1995]. In this study, MI was induced by coronary occlusion, and iNOS activity measured 72 h post-MI. Findings showed that iNOS activity increased significantly (in the absence of changes in eNOS activity compared to baseline), which contributed to the observed left ventricular contractile depression and reduced myocardial bloodflow. The harmful effects of iNOS-derived NO were significantly reversed in the presence of the iNOS-specific inhibitor, SMT.

A plausible explanation for the detrimental effects of NO in ischaemia-reperfusion may be the formation of its highly reactive derivative, peroxynitrite [Ferdinandy & Schulz 2003]. In a study on isolated rat hearts exposed to global ischaemia and reperfusion, greatly increased NO levels were observed during the early post-ischaemic phase of reperfusion, which was associated with myocardial injury and a concurrent burst of superoxide and peroxynitrite release [Wang & Zweier 1996]. Pretreatment with L-NAME (NOS inhibitor) or SOD (superoxide dismutase; superoxide scavenger) was associated with attenuated reperfusion injury, suggesting that the harmful effects of NO during ischaemia-reperfusion may in fact be mediated by the actions of peroxynitrite, rather than by NO itself (see section 1.3 (i)). In another study on rat papillary muscle, it was found that exposure to hypoxia and reoxygenation resulted in reduction of contraction and O_2-uptake (respiration), which was significantly reversed by a NOS inhibitor, a ROS scavenger, and a peroxynitrite scavenger [Xie *et al* 1998]. When endogenous peroxynitrite release was mimicked (administering a combination of a NO-donor, SNAP, and pyrogallol, a superoxide-

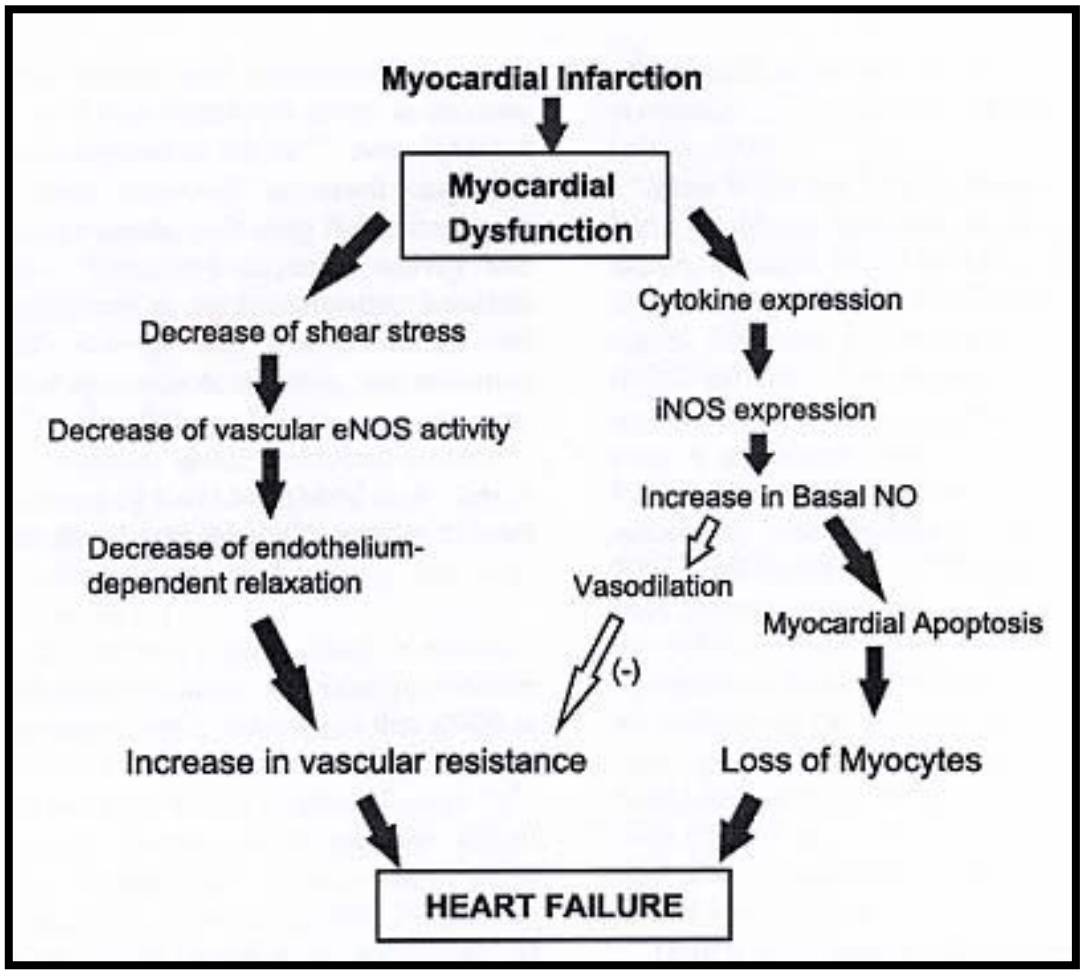


Fig. 1.30 Schematic representation of the proposed roles for NO in post-MI heart failure. See text for details. (Reproduced from Razavi *et al* 2005)

releasing agent), similar effects on contraction and respiration were observed than in hypoxia-reoxygenation injury.

A dual role for NO has also been proposed in the development of heart failure following myocardial infarction (MI) [Razavi *et al* 2005]. Two major pathways have been suggested: (1) decreased vascular eNOS activity and NO-dependent vasodilatation, which contributes to increased vascular resistance, and (2) increased cytokine expression (e.g. TNF-α) leading to iNOS induction and production of large amounts of NO. High levels of NO may lead to cardiomyocyte apoptosis. However, it can also result in vasodilatation, which is beneficial. See fig. 1.30 for a schematic representation of the proposed role for NO in post-MI heart failure.

(vi) The role of NO in protection against ischaemia –reperfusion injury

In the previous section, attention was mostly given to the harmful role of NO and its reactive derivative, peroxynitrite, in hypoxia, ischaemia and ischaemia-reperfusion. However, NO has also been shown to be beneficial in these conditions [For reviews see: Bolli 2001; Schulz *et al* 2004; Shah & MacCarthy 2000; Ferdinandy & Schulz 2003]. It is important to distinguish the protective role of NO in the ischaemia-reperfused myocardium from the role of NO in the *preconditioned* myocardium [Bolli 2001]. In this section, focus will be on the former. The modulation of the severity of ischaemia-reperfusion injury by NO has been widely studied. Most of the studies have used a pharmacological approach (either inhibition of NOS or enhancement of NO availability with NO-donors) prior to, during or after ischaemia, whereas some have utilized NOS-deficient animals [Bolli 2001]. In an extensive review on the role of

NO in cardioprotection, a meta-analysis of 92 studies during the preceding 10 years indicated that 73% had demonstrated a protective effect for NO in myocardial ischaemia (either endogenous or exogenous NO) [Bolli 2001]. In this review, it was suggested that the beneficial effects of NO were independent of the type of ischaemia applied (regional or global), the type of animal species, the experimental model (in vivo, isolated heart or isolated cell) or the measured endpoint. In a more recent review, the same authors argue that the role of NO in ischaemia should be regarded as ubiquitously cardioprotective [Jones & Bolli 2006].

The proposed mechanisms of NO protection in ischaemia are summarized in fig. 1.31. The classical NO-sGC-cGMP-PKG signaling pathway has been discussed earlier, and this pathway is regarded as a mechanism through which NO may exert protection by reducing intracellular calcium [Ferdinandy & Schulz 2003]. NO can also protect by the termination of chain propagating lipid radical reactions due to oxidant stress [Rubbo *et al* 1994]; prevention of platelet adhesion to the endothelial surface by inhibiting the activity of platelets and neutrophils [Kubes *et al* 1991; Radomski *et al* 1987]; counteraction of the toxic effects of peroxynitrite [Villa *et al* 1994]; mitochondrial K_{ATP} channel activation [Sasaki *et al* 2000] and anti-apoptotic effects [Weiland *et al* 2000].

Beneficial effects of NO during ischaemia have been observed in *ex vivo* (isolated heart and isolated cell) studies as well as *in vivo* [Ferdinandy & Schulz 2003]. In isolated perfused heart investigations, protective effects of NO have generally been reported based on improved post-ischaemic contractile function, reduction in infarct

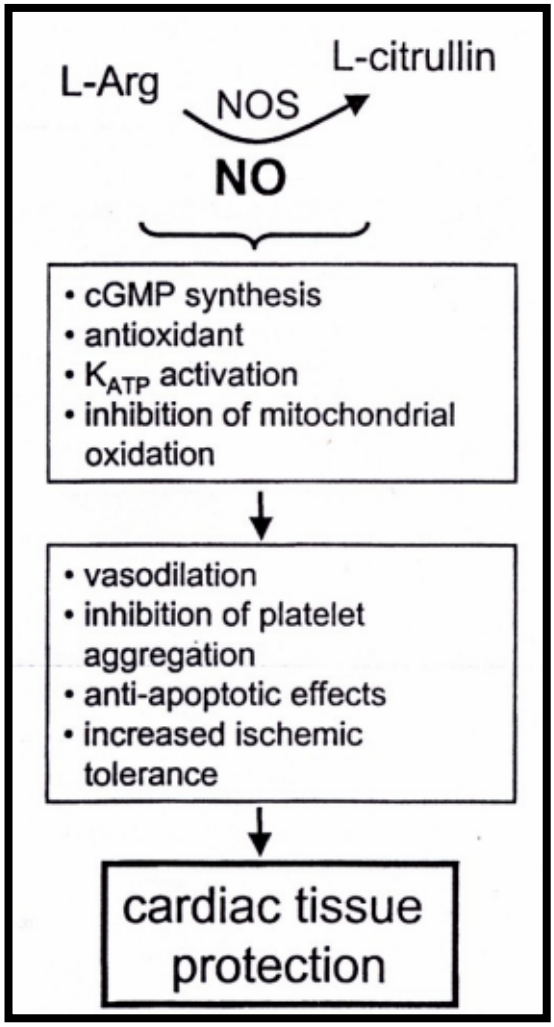


Fig. 1.31 Proposed mechanisms of protection of NO in ischaemia (Reproduced from Ferdinandy & Schulz 2003)

size and in arrhythmia's [Shah & MacCarthy 2000]. Given the multitude of studies, only a few will be highlighted here. A protective role for *endogenous* NO against ischaemia-reperfusion injury was shown in a study on isolated mouse hearts in which eNOS was knocked out [Sumeray *et al* 2000]. Results showed that the eNOS knock-out hearts suffered significantly larger infarcts than wildtypes. A protective role for *exogenous NO* was demonstrated in a study on isolated rabbit hearts [Horimoto *et al* 2000]. Hearts were pretreated with the NO precursor L-arginine and subsequently subjected to an ischaemia-reperfusion protocol; results indicated that the treated hearts developed significantly smaller infarct sizes than control groups. In another study on isolated rat hearts, the NO-donor nitroprusside was administered to hearts subjected to ischaemia-reperfusion to examine the effect of exogenous NO. They found that nitroprusside significantly improved post-ischaemic mechanical function compared to untreated hearts [Du Toit *et al* 1998].

Several *in vivo* studies have also demonstrated a beneficial role for NO during ischaemia [for review, see: Bolli 2001]. In a study on rabbits, hearts were subjected to coronary occlusion followed by reperfusion and the effect of *endogenous* NO inhibition was investigated. Results showed a significant increase in infarct size compared to control in the NOS inhibitor treated group [Williams *et al* 1995]. The role of *exogenous* NO during ischaemia-reperfusion was investigated in an open-chest working pig heart by administering the NO-precursor, L-arginine. Findings indicated that L-arginine reduced myocardial stunning and arrhythmias compared to untreated hearts [Engelman *et al* 1995].

(vii) Summary of the role of NO in ischaemia

From the above it is clear that the literature contains conflicting data with regards to the effects of NO during hypoxia, ischaemia and ischaemia-reperfusion. Although it is Bolli's contention that NO is fundamentally cardioprotective and that its beneficial properties are not influenced by factors such as experimental models or protocols (See Section 1.3 (vi)) [Bolli 2001; Jones & Bolli 2006], others are of the opinion that several factors other than NO itself can influence the outcome (beneficial or harmful) [Shah & MacCarthy 2000]. These include (1) the experimental preparation (*in vivo* vs. *ex vivo*; isolated heart vs. isolated cell; different animal species), (2) the experimental protocol (perfusion buffer composition; type and duration of ischaemia; ischaemia alone or followed by reperfusion), and (3) the end-points measured (functional recovery vs. infarct size). A factor that has become increasingly evident in the interpretation of data recently, is the role of peroxynitrite formation, especially when it is formed in high concentrations [Shah & MacCarthy 2000]. Peroxynitrite is generated in situations of high NO production such as during reperfusion of the ischaemic myocardium or iNOS induction [Ferdinandy & Schulz 2003]. Under these conditions, NO will predominate over SOD and react with superoxide to form peroxynitrite [Ferdinandy & Schulz 2003]. The deleterious effects of peroxynitrite are further influenced by the existing antioxidant status of the microenvironment. In hindsight, it is clear that many investigators did not consider the effect of peroxynitrite simply because its potential role as a harmful by-product of NO metabolism was not yet fully understood at the time.

A further factor that should be considered in the interpretation of data emanating from NO studies, is the use of NOS inhibitors [Ferdinandy & Schulz 2003]. As described earlier, several studies that employed the use of NOS inhibitors during ischaemia observed cardioprotection [Schulz & Wambolt 1995; Depre *et al* 1995; Woolfson *et al* 1995]. In the interpretation of these findings, one should also consider other factors that are not necessarily related to decreased endogenous NO-production. The dosage of NOS inhibitors could influence results, as well as the NOS isoform-specificity of the inhibitor. Drug effects unrelated to NOS inhibition may also play a role. An example of the latter is the discovery that L-NAME at high concentrations (>100 μM) has been shown to be a muscarinic receptor antagonist [Buxton *et al* 1993].

(viii) The role of NO in early (classical) preconditioning

Early (classical) preconditioning has been explained earlier. Protection is observed soon (within minutes) after the preconditioning stimulus (either a brief cycle (-s) of ischaemia and reperfusion, or mimicked pharmacologically), it is robust, but disappears within 1-2 hours after the initial stimulus [Yellon & Downey 2003]. For the purposes of this study, a “trigger” of preconditioning refers to an event or release of a factor during the initial stimulus, whereas a “mediator” of preconditioning is considered a factor or event that occurs during the sustained ischaemic period. One should also distinguish between ischaemic preconditioning (“IP”), where the initial stimulus is brief ischaemia, and pharmacological preconditioning, in which case IP is mimicked or simulated by substituting the brief ischaemia with a drug or other chemical substance.

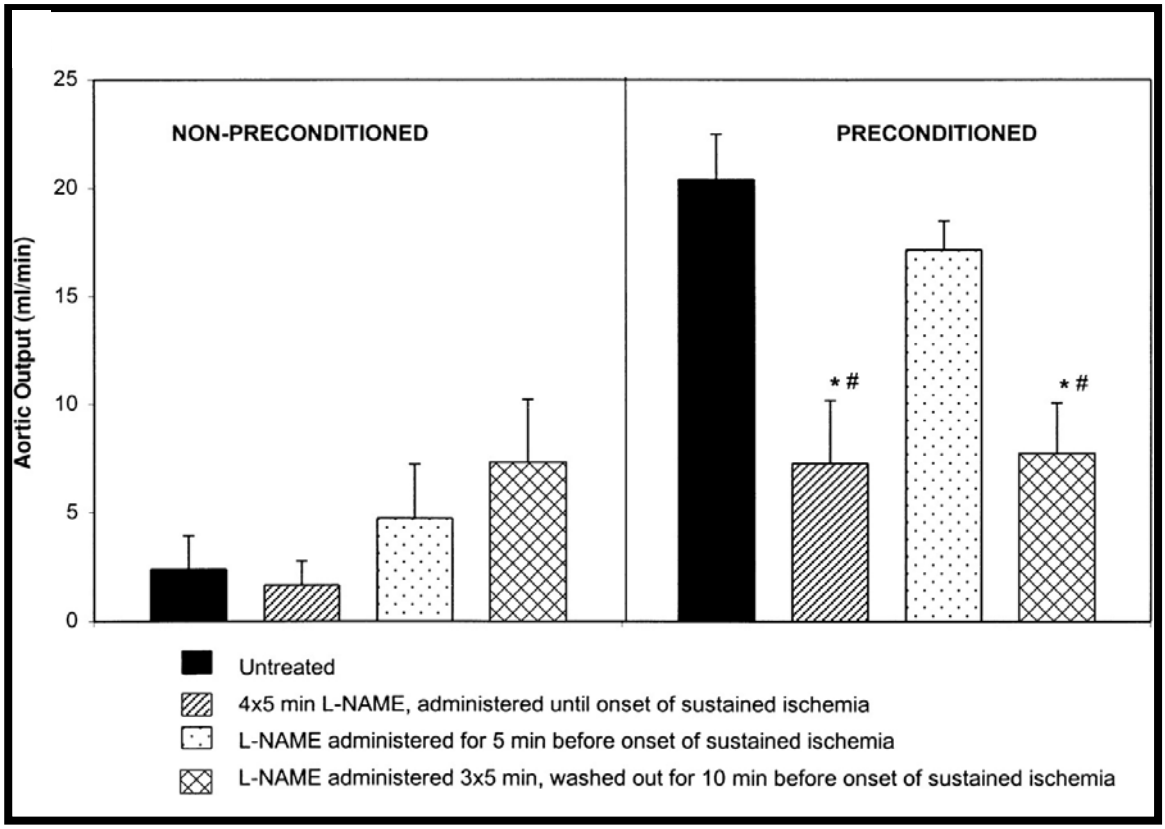


Fig 1.32 Demonstration of NO as a trigger of IP-protection. From the preconditioned results (bar chart on the right), it is clear that administration of the NOS inhibitor, L-NAME, prior and during the IP protocol resulted in a partial reversal of the protective effects (end-point: aortic output). (Reproduced from Lochner e*t al* 2000)

NO as trigger

A triggering role for NO in early IP was shown for the first time in 1992 in an *in vivo* study on dogs [Vegh *et al* 1992]. In this study, IP was induced by a two-cycle ischaemic protocol followed by a sustained ischaemic period. Protection was confirmed by a significant attenuation in the severity of ischaemia-induced arrhythmias (ventricular premature beats, ventricular fibrillation and ventricular tachycardia). When the NOS inhibitor L-NAME was administered before the first ischaemic episode of the IP protocol, some of the marked protective effects of IP were lost. Other studies also demonstrated a triggering role for NO in IP. An investigation conducted in our own laboratory demonstrated similar effects on isolated perfused rat hearts [Lochner *et al* 2000] (fig. 1.32). When L-NAME was added before and during the IP protocol, functional recovery of the heart as observed during IP alone was partially attenuated. In addition, pharmacological preconditioning by pre-administering the NO-donors SNAP and SNP before sustained ischaemia conferred protection, similar to that observed with the IP protocol. In another study, on cultured neonatal rat cardiomyocytes, an IP protocol of simulated ischaemia-reperfusion conferred protection as measured by a cell viability test and LDH-release [Rakhit *et al* 2000]. Administration of the NOS inhibitor, L-NMMA during the IP protocol blocked the protection observed in untreated IP groups.

Pharmacological preconditioning with SNAP also protected the cells in a fashion similar to that of IP. An investigation into the role of NO as a trigger of IP-protection in embryonic chick cardiomyocytes reported results similar to the above; however, their findings suggested that the stimulation of NO production during the triggering period

was a consequence of mitochondrial K_{ATP} channel opening [Lebuffe *et al* 2003]. In addition, this study provided evidence of a significant role for ROS as a trigger of IP, upstream of the mitochondrial K_{ATP} channel (see fig. 1.33).

Endogenous vs. exogenous NO

Despite the evidence supporting a possible triggering role for *endogenous* NO in early IP by utilizing NOS inhibition, other studies failed to demonstrate protection in this way [Bolli 2001; Weselcouch *et al* 1995; Lu *et al* 1995; Woolfson *et al* 1995; Post *et al* 2000; Nakano *et al* 2000]. Therefore, the role of endogenous NO as a trigger of early IP does not seem to be fully established. In fact, there seems to be disagreement in the literature as to the role of endogenous NO production as a requirement for the triggering mechanism of IP. Bolli in his review stated that endogenous biosynthesis of NO was not required for the development of the early phase of IP [Bolli 2001], conversely, Ferdinandy & Schulz stated in their review that intact NO biosynthesis was indeed required [Ferdinandy & Schulz 2003]. Despite the conflicting data and opinions, it does seem as if most authors agree that *exogenous* NO in the form of pharmacological preconditioning does elicit protection. Several studies utilized NO-donors to induce protection: SNAP [Lochner *et al* 2000; Rakhit *et al* 2000; Nakano *et al* 2000], SNP [Lochner *et al* 2000], NTG [Bilinska *et al* 1996; Hill *et al* 2001], SIN-1 [Bilinska *et al* 1996], and L-arginine [Horimoto *et al* 2000].

ROS and peroxynitrite

Closely associated with the role of NO in IP are reactive oxygen species (ROS) and peroxynitrite [Ferdinandy & Schulz 2003]. A triggering role for mitochondria-derived ROS in IP-protection was demonstrated in a study on isolated cardiomyocytes [Vanden Hoek TL *et al* 1998]. Results also showed that ROS, generated by K_{ATP} channel activation, performed a triggering role in IP [Lebuffe *et al* 2003]. See fig. 1.33 for a schematic representation of the proposed interaction between ROS, NO and the mitochondrial K_{ATP} channel in the triggering of early IP.

Given the evidence that both NO and ROS are involved as triggers of IP, it became plausible to hypothesize a triggering role for peroxynitrite, a product of NO and superoxide [Ferdinandy & Schulz 2003]. Indeed, in a study on isolated rat hearts, brief administration of peroxynitrite prior to sustained ischaemia exerted protection against arrhythmias [Altug *et al* 2000]. Furthermore, administration of the ROS scavenger, MPG, prior to peroxynitrite treatment or the IP protocol, significantly reversed the protection, thereby confirming a triggering role for peroxynitrite. A protective role for peroxynitrite was confirmed in subsequent studies [Altug *et al* 2001; Soylemez *et al* 2003].

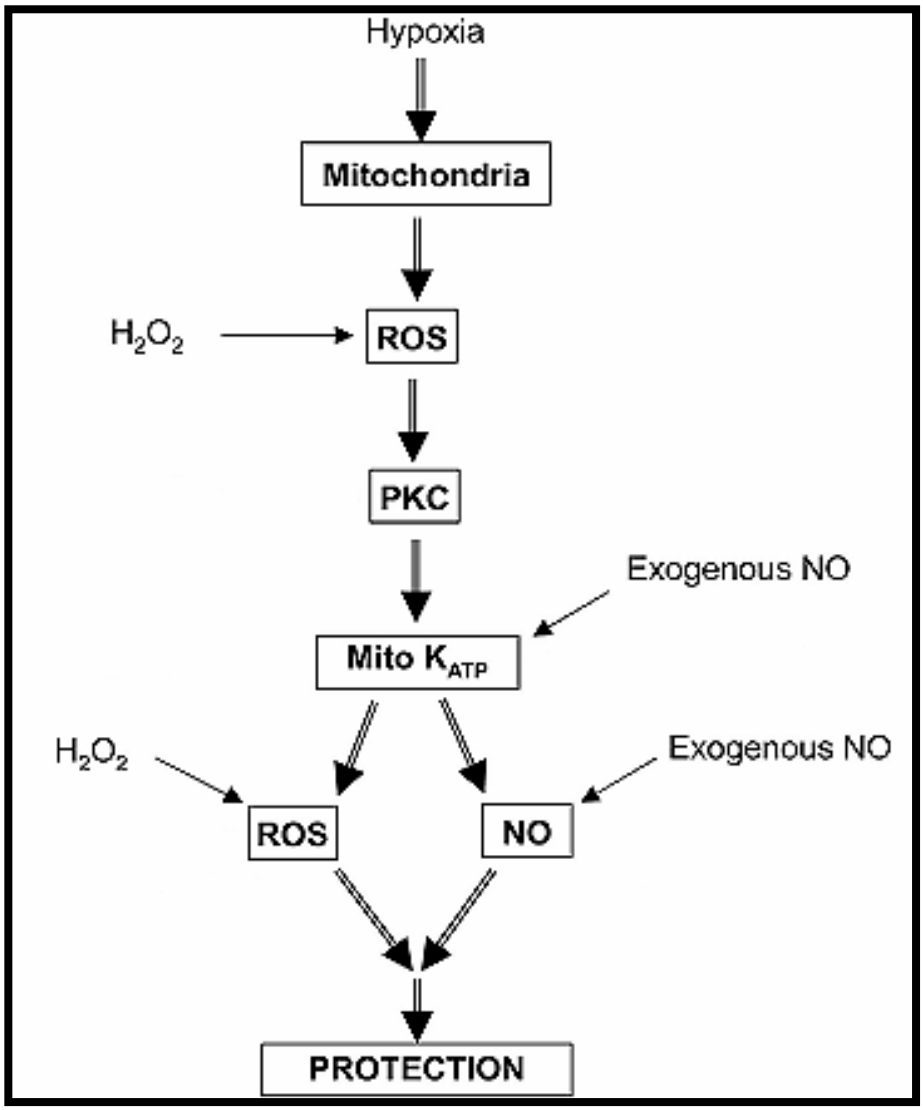


Fig. 1.33 Proposed signal transduction relationships between ROS, NO and the mitochondrial K_{ATP} channel in the triggering of early IP. (Reproduced from Lebuffe *et al* 2003)

(ix) The role of NO in late preconditioning

The late phase of IP occurs approximately 12 – 24 h after the initial IP stimulus, and although it lasts for up to 72 h, the protection elicited is not as robust as that of early IP [Bolli 2001]. However in addition to the fact that the protection lasts longer than early IP-protection, late IP has been shown to protect against both infarction and stunning [Bolli *et al* 1997; Bolli 2000]. These qualities have prompted some researchers to attribute greater clinical relevance to late IP than early IP [Bolli 2001]. The triggering mechanism of late IP is very similar to that of early IP, however, there seems to be little dispute over the role of NO as a trigger of late IP. A triggering role for endogenous as well as exogenous NO in late IP has been demonstrated mainly in the in vivo setting [Bolli 2001; Qiu *et al* 1997; Takano *et al* 1998; Banerjee *et al* 1993; Guo *et al* 1999; Ping *et al* 1999; Hill *et al* 2001].

Generally, the main mechanistic difference between early and late IP is the fact that late IP involves genetic reprogramming of the heart , which leads to the *de novo* synthesis of protective proteins [Bolli 2000], whereas early IP seems to involve rapid post-translational modification of *existing* proteins. An intriguing aspect of the role of NO in late IP is its dual role as both a trigger and mediator, involving two different NOS isoforms [Bolli 2001]. It is believed that during the triggering phase, brief ischaemia activates eNOS to release NO, which then subsequently leads to the induction of iNOS protein expression and a second wave of NO release [Bolli 2001] (see fig. 1.14). The mechanisms for the iNOS-derived NO protection are listed in table 1.3.

PROBABLE MECHANISMS
Inhibition of calcium influx
Antagonism of ß-adrenergic stimulation
Reduced contractility
Reduced myocardial oxygen consumption
Opening of KATP channels
Antioxidant actions (superoxide and peroxynitrite)
Activation of cyclo-oxygenase 2
POSSIBLE MECHANISMS
Preserved endothelium-dependent vasodilatation
Reduced “no reflow”
Reduced leukocyte infiltration
Reduced release of cytokines
Reduced expression of VCAM-1

Table 1.3: Mechanisms of protection of iNOS-derived NO release during late IP (Modified from Bolli 2001)

(x) The non-uniform distribution of NOS and NO-production in cardiac cells

From the previous sections, it is clear that the heart is able to endogenously generate NO. It has also been established that all the major cell types of the heart, viz. endothelial cells, cardiomyocytes, fibroblasts and vascular smooth muscle cells express NOS and produce NO. Although the physiological role of NO is relatively well established and uncontroversial, it seems that the actions of NO in pathophysiological conditions, when oxygen supply is compromised (hypoxia, ischaemia, ischaemia-reperfusion), are not well established. Similar conclusions can be drawn from studies investigating the role of NO in early IP. On the whole, NO seems to exert ambivalent actions under such conditions, ranging from harmful to protective, or of no consequence at all. Many explanations for the conflicting data have been proposed (see Section 1.3 (vii)): the nature of the experimental preparation and protocol, variability in end-points used, the role of oxidant generation (superoxide and peroxynitrite), and the existing redox status in the cellular microenvironment. Another (relatively poorly investigated) explanation for the contradictory effects of NO in the heart could be the differences in its production by the various cardiac cell types due to a non-uniform distribution of NOS and NO-producing capacity.

The amount and ultimate action of NO produced by the different cell types could vary depending on NOS isoenzyme predominance (e.g. high-output iNOS vs. low-output eNOS/nNOS), as well as the size of the respective cell type populations [Brutsaert 2003; Shah & MacCarthy 2000]. Evidence points to the fact that the cardiac endothelial cells and ventricular cardiomyocytes collectively form the majority of NO-

producing cells in the heart [Brutsaert 2003; Shah & MacCarthy 2000]. However, the relative importance of endothelium- and cardiomyocyte-derived NO remains to be established [Shah & MacCarthy 2000]. A non-uniform distribution of eNOS expression exists among the different cell subpopulations of cardiac endothelium (*macrovascular*: epicardial, endocardial and coronary, vs. *microvascular*: myocardial capillaries) [Brutsaert 2003; Brutsaert *et al* 1998]. It is thought that the cardiac microvascular endothelial cells (CMECs) express relatively lower levels of eNOS than the other endothelial cell types [Brutsaert 2003; Brutsaert *et al* 1998].

Although most cell types in the heart demonstrate eNOS expression and activity, there seems to be a predominance of eNOS expression in cardiac endothelium compared to considerably lower expression in the cardiomyocytes [Brutsaert 2003]. It has to be noted, though, that this conclusion was derived from eNOS-labeling and staining studies, and not via direct measurements of eNOS protein or NO-production. The ratio of all cardiac endothelial cells to cardiomyocytes is ~ 3:1 [Brutsaert 2003], and it is thought that CMEC comprise ~ 33% of the cells in the myocardium [Nishida *et al* 1993]. Ventricular cardiomyocytes seem to be present in similar numbers to CMECs, since it has been reported that they constitute <40% of the total myocardial cell number [Gödecke *et al* 2001]. It is therefore fair to assume that the CMEC: cardiomyocyte cell number ratio in the myocardium is at least 1:1. Despite their suggested low eNOS expression relative to other endothelial cell types, it is likely that CMECs are of greater *functional* relevance with regard to interaction with the underlying ventricular cardiomyocytes, since no single CMEC is more than 2-3 μm from a cardiomyocyte [Shah & MacCarthy 2000]. The proximity within which these cell types are located from each other in the myocardium, suggests they are likely to

participate in paracrine crosstalk involving the highly diffusable NO [Shah & MacCarthy 2000; Brutsaert 2003; Brutsaert *et al* 1998; Andries 1998]. It is unclear, however, whether such a NO paracrine messenger pathway exists, and if it does, in which direction the net diffusion gradient would be. It is also unclear what the effects of external NO would be on the recipient cell particularly during pathophysiological conditions when NO-production is thought to increase.

C. Motivation and Aims

(i) Problem identification, rationale and motivation

NO is a free radical produced throughout the body by enzymes called NO synthases (NOS), and occupies a unique position amongst the body's signalling molecules due to its gaseous nature and ability to rapidly exert paracrine effects by simple diffusion across cell membranes. In the heart, NO has been recognized as a major regulatory factor in physiological conditions [Brutsaert 2003]. However, the discovery that NO is a mediator in myocardial hypoxia / ischaemia has made it one of the fastest growing fields in basic cardiovascular research in recent years [Bolli 2001]. The interest in the role of NO has been fuelled by mounting evidence that it may act as a potent cardioprotective mediator during myocardial ischaemia and particularly in the context of late ischaemic preconditioning [Bolli 2001]. In fact, in a recent review, Jones & Bolli argue that the effects of NO should be regarded as fundamentally cardioprotective except when present in pharmacological doses [Jones & Bolli 2006].

Indeed, NO has been shown to exert a harmful role during myocardial ischaemia / hypoxia, especially when produced in excessive amounts, or when its generating enzyme, NOS, experiences a lack of substrate or cofactors, in which case the generation of harmful radicals is favoured [Ferdinandy & Schulz 2003]. The exact mechanism that causes a switch from NO-induced protection to damage is not well understood. Some suggest that it may be due to a shift away from endothelial NOS (eNOS) activity (thought to be responsible for basal, low-output NO production) towards induction of the inducible NOS isoform, iNOS [Klein 2002]. There is evidence to suggest that iNOS is responsible for high-output NO generation during stress

conditions such as hypoxia / ischaemia, resulting in the generation of harmful reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) [Ferdinandy & Schulz 2003]. Interestingly, eNOS *per se* has also been shown to be activated during myocardial ischaemia leading to increased NO levels [Schulz *et al* 2004]. eNOS can therefore not be disregarded as a possible source of harmful NO levels during hypoxia; however, this aspect needs further investigation. Regulation and activation of eNOS in cardiac endothelial microvascular cells (CMECs) and cardiomyocytes is an area that needs more research [Shah & MacCarthy 2000]. Although eNOS has traditionally been regarded as a constitutive protein, it is now recognized that its expression levels can be altered by several stimuli [Shah & MacCarthy 2000], however, the role of hypoxia in the induction of increased eNOS levels in cardiac cells is not well established. Although nNOS is also expressed in cardiac tissue, the function and regulation of nNOS-derived NO are not well characterized [Danson *et al* 2005]; in addition, the expression of nNOS in cardiomyocytes and its physiological role is still largely under investigation [Brutsaert 2003; Mohan *et al* 2001].

The non-uniform cellular distribution of eNOS (and therefore NO production) in the myocardium, and the cell-to-cell variation in NOS isoform expression, suggest that the production and effects of NO in the heart are unpredictable and likely to be characterized by many paracrine crosstalk pathways, e.g. between CMECs and ventricular cardiomyocytes. In view of the assumption that CMECs produce larger amounts of NO than cardiomyocytes, and their proximity to the cardiomyocytes in the myocardium, spill over diffusion into the underlying cardiomyocytes may occur.

A further question that is not yet fully answered is the role of NO as a protective agent in early IP. In isolated, perfused rat hearts, findings from our laboratory have demonstrated a clear role for NO as a trigger of ischaemic preconditioning (IP) [Lochner *et al* 2000]. However, according to a recent meta-analysis study [Bolli 2001], the majority of studies could not find a triggering role for NO in early IP-protection. Clearly, this matter needs more research. Many studies investigating the role of NO in ischaemia / hypoxia and IP relied on indirect measures to assess NO (e.g. the use of NO-donors, NOS inhibitors, nitrate+nitrite measurements, etc.) and did not determine actual NO generation or eNOS expression / activation. This shortcoming in the methodology complicated the interpretation of data (particularly in the case of negative or controversial findings) [Ferdinandy & Schulz 2003]. Therefore, more studies utilizing direct NO measurement methods are required.

In summary, from the above, the following salient conclusions were made: (1) NO may play an important role in myocardial hypoxia; (2) Studies utilizing direct NO measuring methods are lacking, thereby contributing to the confusion about the role of NO in myocardial ischaemia / hypoxia and early IP; (3) There is insufficient data available to establish the exact role of eNOS in the heart during hypoxia, and to what extent eNOS is regulated / activated by hypoxia; (4) Although cardiomyocytes and endothelial cells collectively produce the bulk of NO in the heart, the relative contribution to NO-production by these respective cell types is not well established; (5) CMECs and cardiomyocytes are located in close proximity to each other, and therefore likely to participate in paracrine crosstalk involving many messengers, including NO; (6) CMECs express more eNOS than cardiomyocytes, which makes the existence of CMEC-to-cardiomyocyte spill over diffusion of NO conceivable;

however this has not been shown yet; (7) The role of NO as a trigger of early IP is still unclear and needs further investigation, particularly in the isolated cardiomyocyte model where the influence of nonmyocyte sources of NO are excluded

(ii) Hypothesis

In view of the above, we hypothesize that:

(1) Hypoxia-induced NO generation by cardiomyocytes acts as a trigger in eliciting IP protection against subsequent sustained hypoxia in these cells.
(2) Exposure to hypoxia induces higher production of NO compared to baseline conditions in both cardiomyocytes and CMECs.
(3) CMECs produce more baseline and hypoxia-induced NO than cardiomyocytes; this is associated with higher eNOS expression levels in the CMECs; in addition, eNOS expression and activation and iNOS induction play an important role in hypoxia-induced NO production in both CMECs and cardiomyocytes.

(iii) Specific aims:

In order to test the above hypotheses, the development of suitable experimental models was a prerequisite. Specific aims were therefore:

(1) Establishment of isolated adult rat ventricular cardiomyocyte and CMEC models.
(2) Establishment of techniques for the evaluation of cell viability in isolated cardiomyocytes and CMECs.

(3) Development of a technique for direct measurement of intracellular NO generation in cardiomyocytes and CMECs.

(4) Design and development of protocols for the induction of hypoxia and early IP in isolated cardiomyocytes, and hypoxia in CMECs.

(5) Determination of the role of NO and ROS in hypoxia and early IP in cardiomyocytes; determination of the role of NO and ROS in hypoxia in CMECs.

(6) Quantification of total and activated eNOS, and total iNOS, in cardiomyocytes and CMECs under baseline and hypoxia conditions. These two NOS isoforms were chosen for the purposes of this study, since more is known about their role in the heart, and their expression and activation have been more extensively reported in the literature compared to nNOS. The role of nNOS, and its regulation in cardiac tissue have only recently become more evident. In addition, in the only study to investigate the effect of hypoxia on nNOS expression in cardiomyocytes, it was found that the enzyme was downregulated, which would make it an unlikely candidate source of increased NO-production during hypoxia.

CHAPTER 2

MATERIALS AND METHODS

2.1 The isolated cardiomyocyte model

(i) General

In this study, all investigations involving cardiomyocytes were performed on ventricular cardiomyocytes isolated from the hearts of adult male Wistar rats (250 – 300g). Rats were allowed free access to water and food prior to anaesthesia (30mg of pentobarbital sodium intraperitoneally). The aspects of the project pertaining to animal studies were approved by the Ethics Committee of the Faculty of Health Sciences, Stellenbosch University. Furthermore, investigations conformed to the "Guide for the care and use of laboratory animals" (US National Institutes of Health; NIH publication no 85-23, revised 1985).

(ii) Isolation of adult rat ventricular cardiomyocytes

Chemicals: HEPES, pyruvic acid, and 2,3 butane dionemonoxime (2,3-BDM) were obtained from Sigma Chemical Co. Bovine serum albumin (BSA) (fraction V, fatty acid free) was obtained from Roche, and collagenase (Type 2 Class 2) from Worthington. All other chemicals were of Analar grade and obtained from Merck.

The myocyte isolation technique was based on a previously described method [Fischer *et al* 1991]. After removal, hearts were perfused retrogradely (at a pressure of 100 cm water) with a nominally calcium-free Krebs-Henseleit buffer to rinse blood from the coronary vessels. The rinsing solution (solution A) contained in mM: KCl 6; Na_2HPO_4 1; NaH_2PO_4 0.2; $MgSO_4$ 1.4; NaCl 128; HEPES 10; D-glucose 5.5 and pyruvic acid 2 (37°C, pH 7.4, gassed with 100% O_2). After 5 minutes, perfusion was

switched to a digestion buffer (solution B) consisting of 0.7% BSA, 0.1% collagenase and 15 mM BDM added to solution A. Perfusion with the digestion buffer was done in a recirculating fashion and continued for a further 25 – 30 minutes until the tissue was digested. Calcium (100 μM) was added at 15 minutes and 20 minutes of digestion respectively.

At the end of the perfusion, ventricles were carefully separated from the atria and remnants of the large vessels, gently torn apart and placed in a post-perfusion digestion buffer (solution C = 1 part solution A + 1 part solution B) containing 200 μM $CaCl_2$, 2% BSA, 0.05% collagenase and 7.5 mM BDM. The cell suspension was then incubated in a shaking waterbath (under an O_2 atmosphere, 37°C) for 15 minutes, followed by a step-wise calcium readministration period of 5 minutes to render calcium-tolerant cells (final concentration: 1 mM). The digested cell suspension was then filtered through a nylon net (mesh size 200 x 200 μm) and gently centrifuged at 100 r.p.m. for 3 minutes (room temperature), after which the supernatant (containing non-viable myocytes, other cells and debris) was removed and the remaining pellet resuspended in a final incubation buffer consisting of solution A, containing 1 mM $CaCl_2$ and 2% BSA (solution D). Finally, cells were allowed to sediment for 5 minutes at room temperature, after which the supernatant was removed and the final pellet resuspended in solution D. The final suspension was stabilized for 1 hr by slow rotation under an O_2 atmosphere at room temperature. After the stabilization period, the purity of the pellet was further enhanced by filtering it through 2% BSA-containing solution D. See fig. 2.1 for a microphotograph of typical viable rod-shaped cardiomyocytes isolated by the collagenase-perfusion method in our laboratory.

(iii) Assessment of cardiomyocyte viability

During the course of this study, we investigated four different methods of cell viability assessment in the isolated cardiomyocytes, viz. (1) trypan blue exclusion test [Armstrong & Ganote 1991; Cheung *et al* 1985; Marber 2000], (2) % rod-shaped myocytes [Armstrong & Ganote 1991; Cheung et al 1985; Marber 2000], (3) propidium iodide (PI) nuclear staining [Marber 2000; Vanden Hoek 1998; Yao 1999], and (4) MTT- (3-4,5-di-methylthiazol-2-yl-2,5-diphenyltetrazolium bromide) staining [Gomez *et al* 1997].

The *trypan blue exclusion test* was a modification of the one originally described by Armstrong & Ganote [Armstrong & Ganote 1991]. Sampling for time zero baseline viability was done on the initial cell pellets before subdivision into experimental groups. The pellets of the respective groups were again sampled at the end of the experiments. Sampling was done by removing 12.5 µl (representing approximately 80 000 - 100 000 myocytes) directly from pellets and suspending them in 100µl of hypotonic solution D (diluted 1:1 with deionized water, containing 5mM KCN) for 3 minutes. Subsequently, 25 µl was removed from the KCN-Sol D-cell suspension and mixed with 25 µl counting medium consisting of hypotonic solution D, 5mM KCN, 0.5% glutaraldehyde (Merck) and 1% trypan blue (Merck) for 30 seconds. Samples (10 µl each) of the final counting suspension were placed on a haemocytometer and examined light microscopically (100x magnification) to evaluate the absorption (non-viable) or exclusion (viable) of trypan blue dye. In this way, cells were osmotically challenged by placing them in hypotonic counting solutions thereby allowing assessment of osmotic fragility.

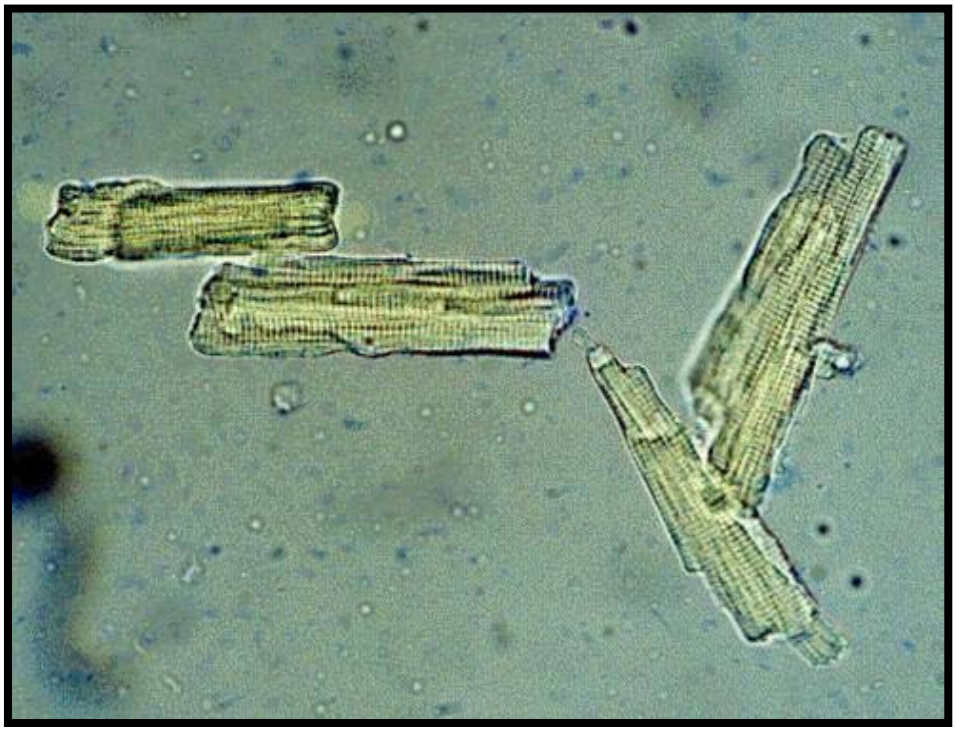
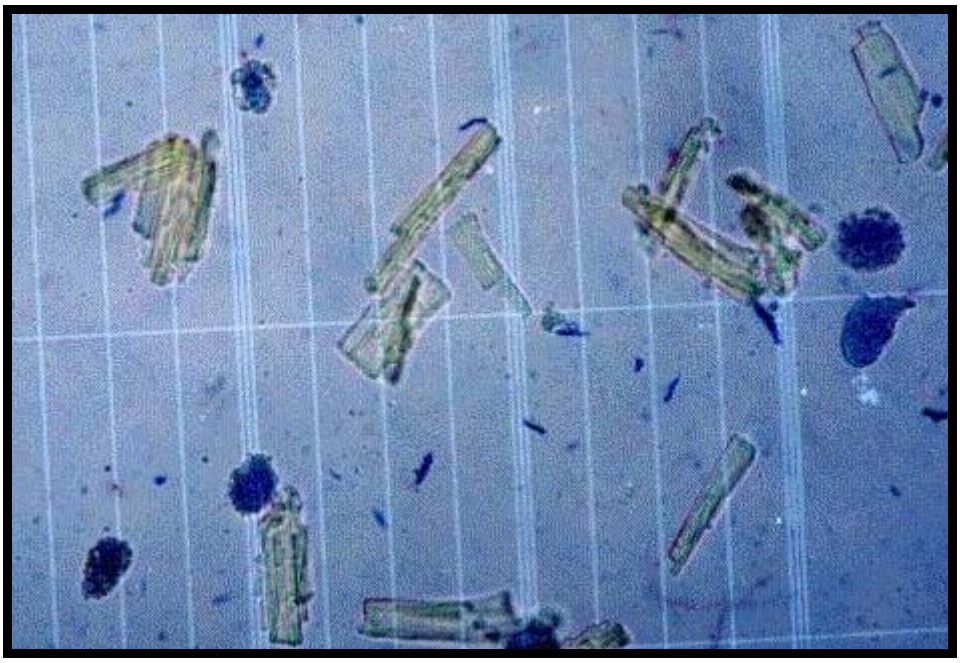


Fig. 2.1 Rod-shaped cardiomyocytes photographed (light microscope; 100x magnification; no trypan blue added) after isolation in our laboratory. Myocytes are considered viable when they are rod-shaped and exhibit a length:width ratio of ≥ 3:1 as the shown in the microphotograph.

A.



B.

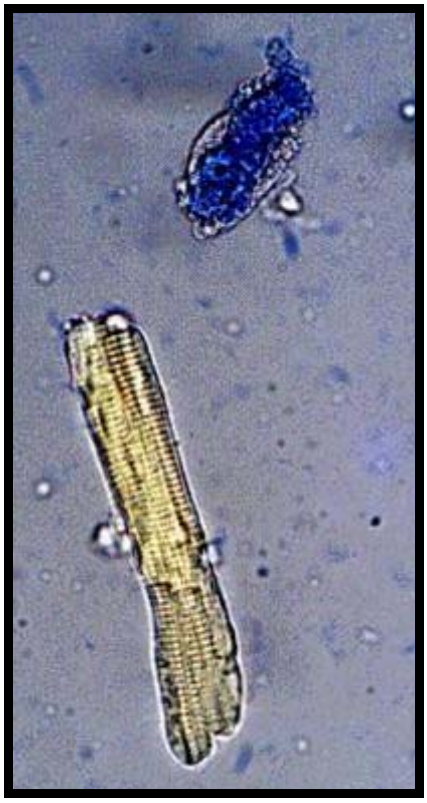


Fig. 2.2 Microphotographs of isolated cardiomyocytes stained with 1% trypan blue. **(A)** 10x magnification shows a population of mainly trypan blue-excluding, rod-shaped viable myocytes, with a few blue-stained round non-viable cells. **(B)** 100x magnification demonstrating a viable rod with a non-viable blue round myocyte.

Time zero viability (viable cells calculated as a percentage of the total cells in a sample) varied between 70% and 80% and all cell isolates of less than 70% viability were discarded. For experimental purposes, Trypan blue excluding cells were expressed as a percentage of the total number of viable cells (%TBE) as determined before experimentation. See fig. 2.2 for microphotographs of viable and non-viable cardiomyocytes when stained with trypan blue.

The use of myocyte morphology (*% rod-shaped myocytes*) as an index of viability was also modified from a technique described by the same group [Armstrong & Ganote 1991]. More or less the same procedure was followed as described for the trypan blue exclusion test, with the exception that cells were suspended in an *isotonic* solution D and counting medium. Rod-shaped cells (length:width ratio $\geq$ 3:1; see fig. 2.1) were considered viable, whereas square-shaped (length:width ratio $\leq$ 3:1) and round cells were considered non-viable. Cell viability was expressed as the percentage rod-shaped cells present. See fig. 2.1 & 2.2 for microphotographs demonstrating morphological properties of cardiomyocytes.

We also employed *propidium iodide (PI) nuclear staining* to assess myocyte viability. A modification of a technique previously described for endothelial cells was used [Navarro-Antolin *et al* 2001(b)]. Accordingly, cell membrane permeability to, and subsequent nuclear staining by PI (Sigma Chemical Co.) was evaluated by FACS analysis (Becton-Dickinson FACSCalibur analyzer, Franklin Lakes, NJ). Cardiomyocytes were incubated with 1 μM PI in solution D for 15 min followed by flow cytometric analysis of fluorescence in the FL-2 channel; data were expressed as

mean fluorescence intensity (% of control signal). Increased PI fluorescence indicates reduced viability.

The *MTT assay* was based on a modification of a technique described in isolated cardiomyocytes [Gomez *et al* 1997]. This assay is based on the reduction of the water-soluble yellow coloured MTT (Sigma Chemical Co.) to its water-insoluble purple coloured formazan product. Cells were suspended in 35 mm Petri dishes to which 1% MTT in solution D was added, followed by 120 min incubation at 37°C. Following removal of the supernatants, cells were lysed in a mixture containing 1% HCL in isopropanol and 0.1% Triton in distilled water for 5 minutes on a rotator after which cells were centrifuged and the supernatant analyzed spectrophotometrically at 540 nm. High optical density readings indicate increased viability.

(iv) Induction of hypoxia in cardiomyocytes

Unless stated otherwise, ischaemia in the myocytes was simulated by employing a technique referred to as “ischaemic pelleting”, which is essentially a form of hypoxia. The technique was a modification of a method described in isolated rabbit cardiomyocytes [Armstrong *et al* 1994(a); Armstrong & Ganote 1994(b); Armstrong & Ganote 1994(c); Armstrong *et al* 1995]. Cells were gently compacted into a pellet by centrifugation (250 rpm; 40 sec) in microcentrifuge tubes. Subsequently, most of the supernatant was removed, leaving behind a layer of approximately ⅓ of the pellet thickness. The supernatant was then covered with a layer of mineral oil to exclude air for the required duration (See fig. 2.3).

(v) Experimental groups

On average the digested heart yielded 3 – 6 million ventricular cardiomyocytes, sufficient for at least 6-12 myocyte fractions (experimental groups), containing 500 000 myocytes each. Investigations were repeated on myocyte fractions from different hearts, with sample sizes varying from n = 5 - 15. All experimental groups were incubated at 37°C in a standard tissue culture incubator (21% O_2, 5% CO_2, 40-60% humidity). Cells of all experimental groups were suspended in solution D for the indicated time durations, unless stated otherwise. Generally, all non-hypoxia samples were incubated as suspension cultures in 1 ml solution D in 35 mm tissue culture dishes, whereas samples subjected to hypoxia were incubated in microcentrifuge tubes as stated above. Please refer to the chapters that follow for more specific descriptions of the various experimental groups and protocols.

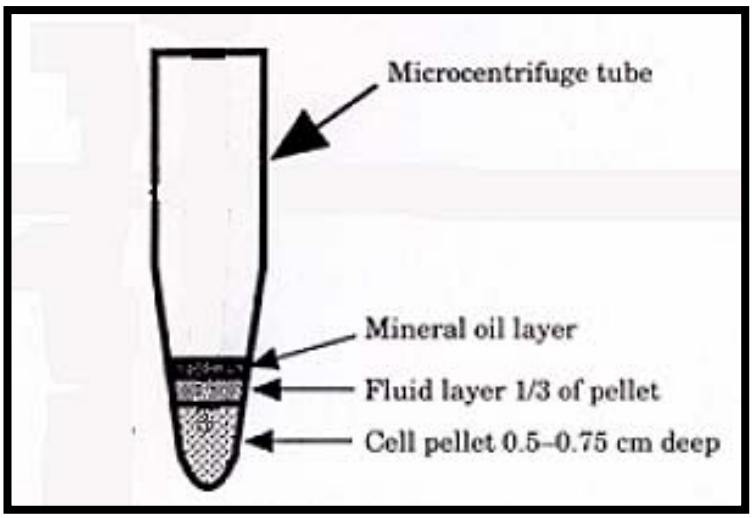


Fig. 2.3 Schematic representation of the ischaemic pelleting technique of hypoxia induction. Myocyte suspensions are placed into microcentrifuge tubes, gently centrifuged thereby compacting the cells to form a pellet. The supernatant is then aspirated leaving behind a layer of about a third of the pellet thickness. Finally, the supernatant is covered with a mineral oil layer and the sample incubated for the required duration in a standard tissue culture incubator at 37°C. (Modified from Armstrong *et al* 1995)

2.2 Cardiac microvascular endothelial cell (CMEC) cultures

(i) Primary CMEC cultures purchased from VEC Technologies ™, USA

Primary rat CMEC cultures were purchased from VEC Technologies (Rensselaer, NY, USA). The isolation procedure followed by the manufacturer was designed to ensure the highest possible yield of microvascular endothelial cells, and steps were taken to avoid contamination with large-vessel epicardial and endocardial endothelial cells based on a previously described technique [Nishida *et al* 1993]. Cells were received in 75 ml fibronectin-coated tissue culture flasks and grown to confluency in a microvessel endothelial cell growth medium, EGM (Clonetics EGM-2MV; Cambrex BioScience). The medium was supplemented with 10% fetal bovine serum (FBS; Highveld Biological), 0.1% gentamicin/amphotericin B, and standard endothelial cell culture growth factors (Clonetics). Cultures for experimental purposes were prepared from confluent primary cultures by exposing cells to trypsin 500 BAEE U/ml and EDTA 180 µg/ml in Dulbecco's phosphate-buffered saline (Sigma Chemical Co.), followed by resuspension of detached cells in growth medium and subculture in a 1:3 ratio. Subcultures, plated in fibronectin-coated dishes, needed ~5 days to become confluent. Cultures from the third or fourth passage were used for the experiments.

Purity of CMEC cultures was verified by microscopic identification of a typical "cobblestone" monolayer morphology, a distinct characteristic of cultured endothelial cells [Nishida *et al* 1993; Piper 1990] (See fig. 2.4). Contaminating cells such as fibroblasts and cardiomyocytes were absent. In addition, functional characterization was done by measuring the uptake of fluorescently labeled Dil-ac-LDL (Biomed Technologies, Stoughton, MA), a marker specific for endothelial cells [Nishida *et al*

1993; Piper 1990; Walsh *et al* 1998; Fan *et al* 1999] by FACS analysis in the FL-2 channel. Tests were performed on cells of randomly selected culture dishes, and a positive staining rate of 93–95% was recorded throughout. See fig. 2.5 for a fluorescence microphotograph of LDL-staining CMECs in culture.

(ii) Assessment of CMEC viability

In CMEC investigations, two cell viability tests were used, *viz.* trypan blue exclusion and PI staining. For trypan blue staining, CMECs were removed from culture by trypsinization (as described above) and subsequent suspension of the isolated cells in a 1% Trypan Blue-solution D mixture in a procedure similar to that described for the isolated cardiomyocytes. The only modification to the staining method used for the myocytes was that the counting solution remained isotonic. CMECs were subsequently assessed by light microscopy and the number of trypan blue-excluding cells (i.e. viable cells) calculated as a % of the total cell number. CMEC viability was also assessed by staining trypsinized cells with 5 μM PI for 15 minutes at 37°C, similar to a method previously described for endothelial cells [Navarro-Antolin *et al* 2001(b)]. Stained cells (non-viable) were subsequently quantified by FACS analysis of the mean fluorescence intensity as measured in the FL-2 channel, similar to the procedure described for myocytes.

(iii) Induction of hypoxia in CMECs

CMECs were subjected to two different hypoxia protocols: (1) overnight hypoxic incubation of cultured CMECs, or (2) ischaemic pelleting of isolated, trypsinized CMECs in a method similar to the one described for isolated cardiomyocytes.

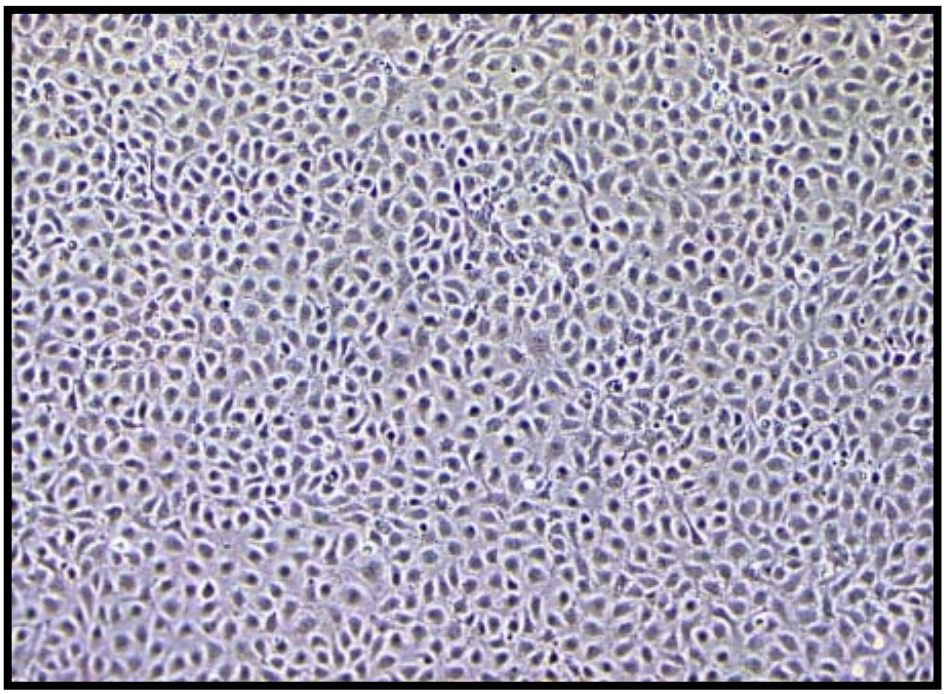


Fig. 2.4 Microphotograph of confluent CMEC culture (10x magnification) demonstrating the typical cobblestone appearance.

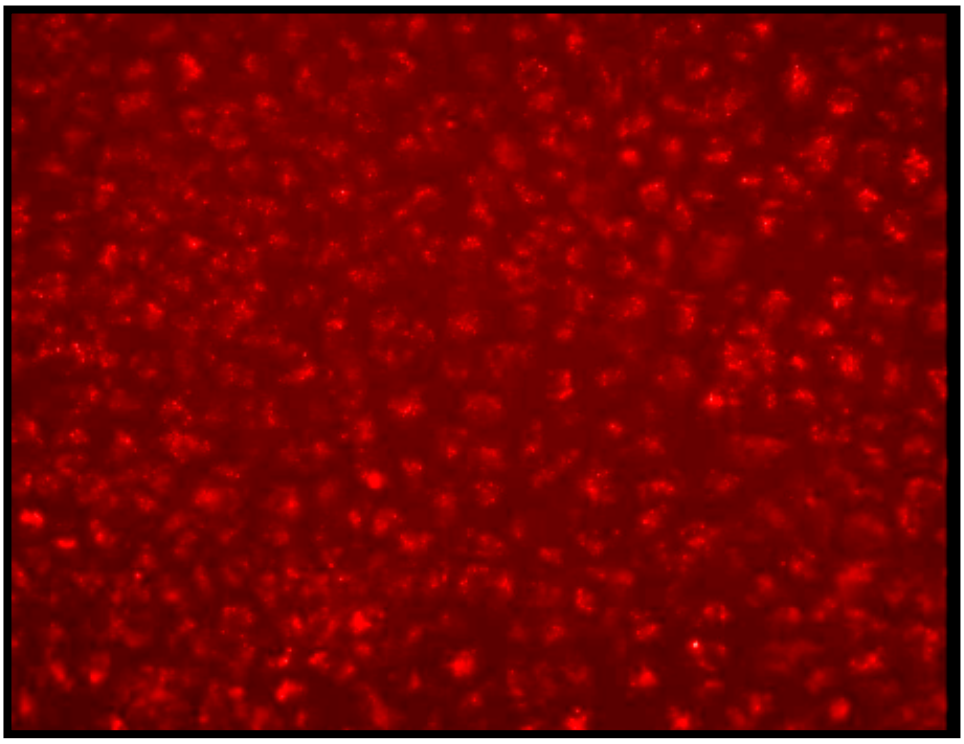


Fig. 2.5 Fluorescence microphotograph of LDL-staining CMECs in culture (20x magnification). On average CMEC cultures demonstrated >93% positive staining.

In the *hypoxic incubation* protocol, CMECs were retained in their original culture (35 mm fibronectin-coated petri dish). Before induction of hypoxia, the 10% FBS-containing endothelial growth medium (10% EGM) was removed and substituted with serum-reduced 5% EGM. Hypoxia was induced by lowering the oxygen tension in a standard multi-gas tissue incubator (↓PO_2 incubation) (1% O_2, 5% CO_2, 40–60% humidity, 37°C) for 18 h. The second hypoxia protocol (*ischaemic pelleting*) was designed to match the technique described for the fresh isolated cardiomyocytes. Cultured CMEC were isolated by detachment from their fibronectin-coated dishes with trypsin. After trypsin was washed out, isolated CMEC were resuspended in fresh solution D in 35 mm petri dishes. Hypoxia was induced by compacting isolated CMECs into a pellet in microcentrifuge tubes (centrifugation @ 1000 rpm), followed by removal of most of the supernatant and finally layering with mineral oil. Hypoxic samples were incubated in a standard tissue culture incubator (37°C).

(iv) Experimental groups

For experimental purposes, CMECs of the third or fourth generation were either incubated in culture or as trypsinized isolated cells, depending on the protocol (see later for detailed description) in 35 mm dishes at a density of 0.5×10^6 cells/dish. Experimental groups (varying sample sizes, n = 4–12) consisted of cells obtained from different culture dishes, equally representative of culture passages 3 and 4. All experimental groups were incubated at 37°C in a standard tissue culture incubator (21% O_2, 5% CO_2, 40-60% humidity). Incubation media were either 1 ml 5% EGM for the cultured CMEC experiments or 1 ml solution D for the isolated trypsinized CMEC experiments.

[For all other methods, protocols and experimental designs please refer to the specific chapters.]

2.3 Statistical analyses

Unless stated otherwise, all data are expressed as percentages (mean ± SEM). For comparative studies, Student's t test (unpaired) or one-way ANOVA (with Bonferroni post-hoc test if $p<0.05$) was used for statistical analyses. Differences were considered statistically significant if a p-value of <0.05 was achieved. Analyses were performed by Graph Pad Prism® Version 4.01 software.

CHAPTER 3

HYPOXIA AND EARLY ISCHAEMIC PRECONDITIONING IN ISOLATED CARDIOMYOCYTES: THE ROLE OF NO AND ROS

3.1 Introduction

The aim of the investigations in this chapter was to examine the role of NO (endogenous and exogenous), ROS and cellular cGMP in a model of simulated, early hypoxic preconditioning and hypoxia using isolated adult rat cardiomyocytes. The isolated cell model is sufficiently free of any interference by non-myocytes, thus providing the opportunity to focus on these factors in myocytes only.

3.2 The isolated cardiomyocyte model

In 1972, a method that successfully isolated calcium-tolerant ventricular cardiomyocytes from an adult rat heart was described for the first time [Gould & Powell 1972]. In this seminal study in the field of cardiac cellular research the observation was made that a straightforward coronary perfusion with buffer containing bacterial collagenase was sufficient to provide a good yield of cells able to survive in solutions containing physiological levels of calcium, thus the term “calcium-tolerant” myocytes [Powell 1985]. The biggest advantage of the perfusion-based isolation technique as opposed to incubating tissue fragments with the enzyme is that the heart is continuously supplied with essential substrates during the stressful period of tissue dispersal; in addition, the perfusion method has shown to provide higher cell yields [Powell 1985].

In the years following, many studies aimed to relate isolated cardiomyocyte responses to those observed in whole tissue in an attempt to establish whether cell isolation compromises cellular behaviour [Powell 1985]. There is general appreciation of the fact that isolated cell preparations are not a substitute for the *in*

vivo situation or even isolated heart models [Hearse & Sutherland 2000; Marber 2000; Stowe & Riess 2004]. Essentially, the advantages of the isolated cell preparation occur at the expense of a cell phenotype that differs from the intact heart [Marber 2000]. See Table 3.1 for a summary of the advantages and disadvantages of the isolated cardiomyocyte model compared to the intact heart. However, despite the shortcomings of the isolated myocyte preparation, it does provide an ideal opportunity for the investigation of a particular cell type in isolation from external influences (excluding complicating effects of vascular, neuronal or humoral factors) [Cave *et al* 1996]. Furthermore, it allows the contribution of cellular heterogeneity to be assessed and provides a relatively small volume of distribution, which makes the use of drug manipulation easier and more cost-effective [Marber 2000].

The use of suspensions of freshly isolated adult cardiomyocytes (as in the present study) compared to other myocyte-based models also deserves some comment. Generally, researchers either make use of immature cardiomyocytes (embryonic, neonatal or dedifferentiated adult cells) or mature cardiomyocytes (adult cells) [Marber 2000]. As a rule, immature myocytes are studied in culture. The biggest disadvantage of studying embryonic and neonatal cardiomyocytes relate to their immature genotype and phenotype, particularly with regards the expression of ion channels and contractile protein isoforms that differ significantly from adult cells [Mitcheson *et al* 1998]. Adult cardiomyocytes can also be studied in culture [Mitcheson *et al* 1998], however placing them in culture is complicated by their adaptation to the culture environment and therefore the fact that they cannot be considered to be in a stable steady-state [Mitcheson *et al* 1998]. Furthermore, cultured adult myocytes undergo reorganization of their cytoskeletal and contractile

proteins as the cells adapt from a 3-dimensional to the 2-dimensional environment of the tissue culture dish. It is also thought that adult myocytes dedifferentiate to the fetal phenotype [Mitcheson *et al* 1998], or even die [Marber 2000] in long-term cultures. Regardless of whether freshly isolated or cultured adult cardiomyocytes are used, it is generally agreed that the single most important factor for obtaining a high and viable yield is the quality of the isolation procedure [Powell 1985; Mitcheson *et al* 1998].

We and others [Mitcheson *et al* 1998] have observed that freshly isolated myocytes remain viable for at least 8 –12 hours. Therefore, given the temporal nature of our investigations, and the other considerations discussed above we have decided to opt for the freshly isolated adult cardiomyocyte model.

ADVANTAGES	DISADVANTAGES
Homogenous cell type; absence of external (nonmyocyte) factors	Revelance to intact heart uncertain
Study of interactions between cells (co-culture models)	Compromised function due to absence of adjoining or distant cells
Genetic manipulation	Lack of humoral or neuronal influence
Low volume of distribution (cost-effective when using drugs)	Uncertain maintenance conditions
Separation of apoptosis from necrosis	Results influenced by maintenance conditions
Extracellular environment can be manipulated	Isolation procedure shock
Channel activity and redox can be measured	Interventions such as ischaemia have to be simulated

Table 3.1: Relative advantages and disadvantages of the isolated cardiomyocte model compared to the intact heart. (Modified from Marber 2000).

3.3 Experimental groups, protocols and drug treatment (see fig. 3.1)

(i) Oxygenated controls

Oxygenated control cells were incubated in suspension in solution D under an O_2 atmosphere for the full duration of the experiment (2½ h).

(ii) Ischaemic preconditioned cells

An IP protocol previously described for isolated cardiomyocytes [Armstrong *et al* 1995] was modified and applied in this study. Briefly, myocytes were subjected to a single cycle preconditioning protocol of 10 min hypoxia (ischaemic pelleting; see section 2.1 (iv)) followed by 20 min reoxygenation. Reoxygenation was achieved by removing the hypoxic cells beneath the oil layer and resuspending them in fresh solution D (gassed with 100% O_2, 37°C). After the IP protocol, cells were once again pelleted in a microcentrifuge tube and covered with mineral oil to induce 2 h sustained hypoxia.

(iii) Non-preconditioned (non-IP; hypoxic) cells

The non-IP group was subjected to an initial intervention-free, oxygenated incubation period of 30 min (to correspond with the IP protocol), followed by sustained hypoxia of 2 h.

(iv) NOS inhibition

The role of NO was investigated by the administration of L-NAME (50 μM; Sigma), a non-specific NOS inhibitor under two different conditions. L-NAME was dissolved in a stock solution (distilled water) from which an appropriate volume was directly added to the cell suspensions. (i) L-NAME was present before (10 min) and during the *short* hypoxia of the IP protocol in order to prevent activation of NOS and NO generation during this time period. At the end of the short hypoxia, L-NAME was washed out twice before the reoxygenation period. In this way, a possible *trigger* effect of NOS and NO could be investigated. (ii) To investigate a possible *mediator* effect for NOS and NO, L-NAME was present during *sustained* hypoxia, with a 10 min pre-administration period to ensure proper absorption into the cells at the start of hypoxia. Non-preconditioned groups were exposed to L-NAME at corresponding time-periods to serve as appropriate controls. The above investigations were repeated with L-NAME at a higher concentration (200 μM) and L-NNA (50 μM), an alternative NOS inhibitor.

(v) iNOS inhibition

In order to investigate whether the inducible form of NOS, iNOS, was involved as a trigger of IP, the iNOS-specific inhibitor SMT (10 μM; Sigma) was administered in a similar fashion as described for L-NAME above, 10 min before the onset of the brief hypoxia of the IP protocol and was also present during the brief hypoxia. After the brief hypoxia, the drug was washed out twice and cells were resuspended in fresh, oxygenated buffer (reoxygenation), followed by 2 h sustained hypoxia. Untreated IP

myocytes were used as controls. In a second group, SMT was present during the sustained hypoxia period only (with a 10 min pre-administration period), to investigate a possible mediator role of iNOS. Non-preconditioned groups were exposed to SMT at corresponding time-periods to serve as appropriate controls.

(vi) NO donor studies

This protocol was designed to establish whether exogenous NO could act as a trigger of protection against hypoxia. Briefly, cells were pre-treated with the NO donor, SNP (100 μM; Sigma) for 10 min before a wash-out period of 20 min in fresh buffer followed by 2 h sustained hypoxia. Myocytes undergoing hypoxic preconditioning were used as appropriate controls for the SNP groups.

(vii) H_2O_2 pretreatment

In order to assess whether ROS could mimic IP in our model, cells were pretreated with 100 μM H_2O_2 for 10 min, followed by a wash-out period of 20 min, and sustained hypoxia of 2 h. Viability was measured and compared to non-IP and hypoxic IP groups.

(viii) Inhibition of reactive oxygen species (ROS)

The significance of the generation of ROS (particularly NO-derived ROS) during the IP protocol was investigated using the ROS scavenger, N-(2-mercapto-propionyl) glycine (MPG; 300 μM) (Aldrich, Germany), which was present 10 min prior to and

during the entire preconditioning protocol, i.e. during the brief hypoxia and subsequent reoxygenation. MPG is known to react avidly with oxidant species such as peroxynitrite and hydroxyl radical due to its thiol group [Xuan *et al* 2000]. MPG was washed out prior to sustained hypoxia. Untreated IP myocytes served as controls. In another series, MPG was also administered 10 min before and during the sustained hypoxia period, to investigate whether ROS-generation during this period had an effect on IP. Non-IP groups were exposed to MPG at corresponding time periods to serve as appropriate controls. Investigations were repeated with 0.5 mM N-acetyl-cysteine (NAC; Sigma), an alternative, non-specific ROS scavenger.

3.4. Measurement of cardiomyocyte cGMP content

Cardiomyocyte cyclic GMP extraction was done with 5% trichloro-acetic acid (TCA), after which samples were washed 4 times with ether. Measurement of cellular cyclic GMP levels was done with a radio-immuno assay kit (Amersham, UK). Cellular protein content was measured using the Lowry [Lowry *et al* 1951] and Bradford [Bradford 1976] techniques, and results were expressed as pmol cGMP / mg protein. Sampling was done as shown in fig. 3.1.

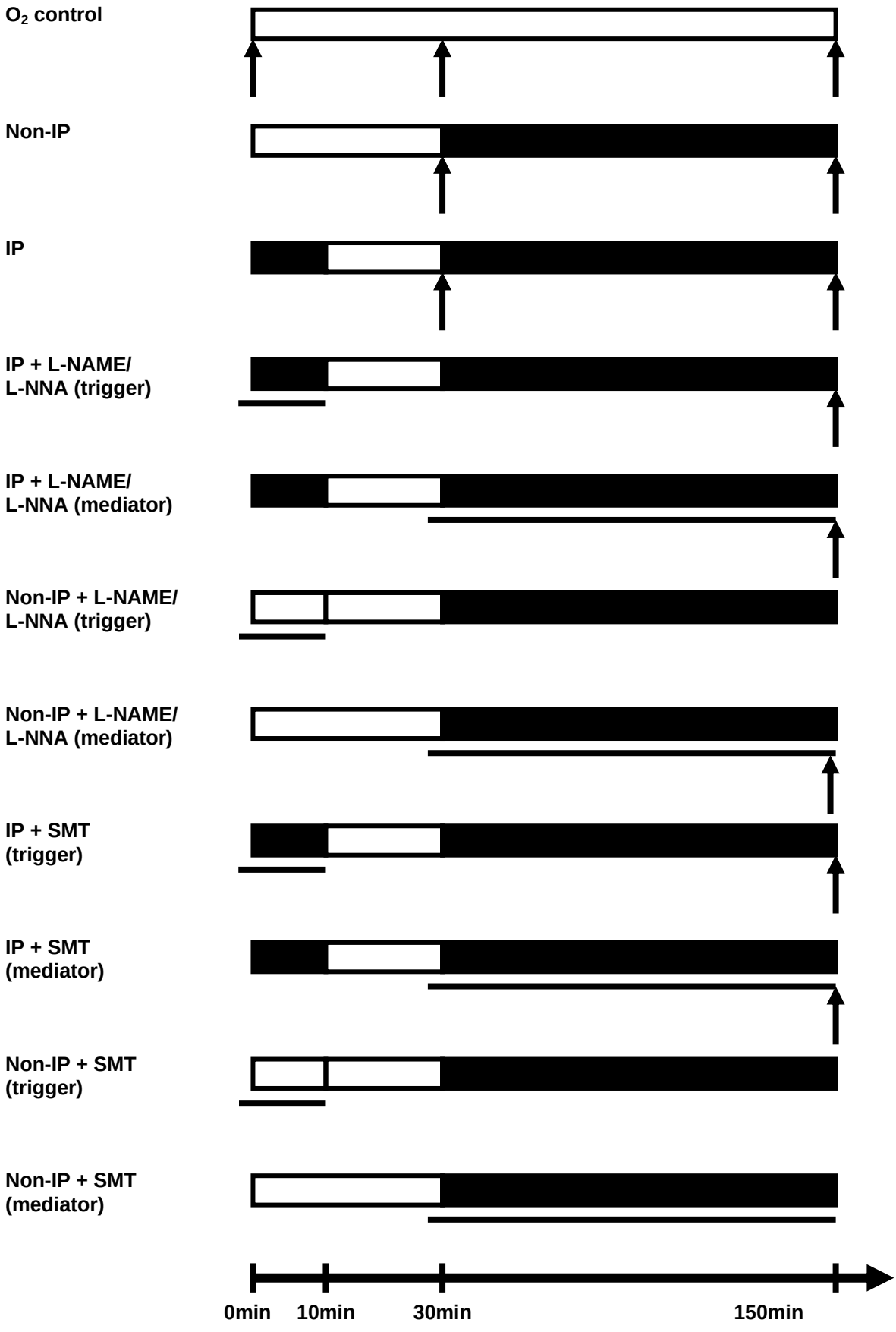


Fig. 3.1 Experimental groups and protocols. See next page for legend.

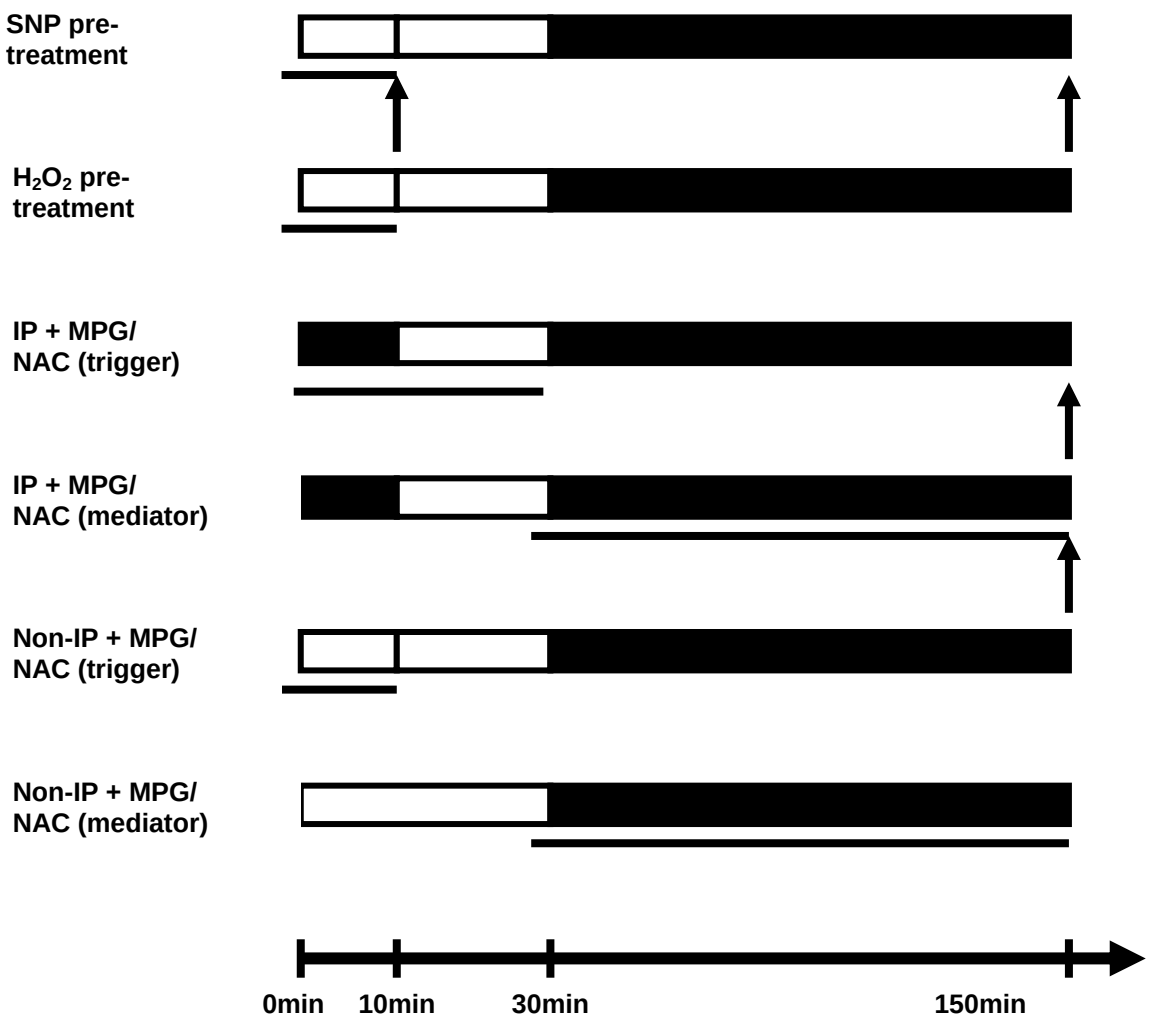


Fig. 3.1 Experimental groups and protocols. Unshaded bars represent control oxygenated incubation, and black shaded bars hypoxic conditions. Black lines below the bars indicate drug administrations. L-NAME, L-NNA, SMT, MPG and NAC were all pre-administered for 10 min. All pharmacological agents were removed by washing twice with fresh buffer. Sampling points for myocyte viability determinations were done at the end of 150 min in all groups, and cellular cGMP sampling is indicated by the arrows.

3.5 Results

(i) Simulated ischaemia and preconditioning protocol (fig. 3.2 A & B)

Exposure to 2 h sustained hypoxia lead to a significant reduction in viability compared to cells subjected to oxygenated control conditions. When the sustained hypoxia period was preceded by the IP protocol, cell viability improved significantly. The protective effects of the IP protocol were demonstrated by two independent end-points of viability, namely the percentage of cells excluding trypan blue (viable cells increased by 54% with IP, $p<0.05$), and myocyte morphology (30% increase in rod-shaped myocytes, $p<0.05$). As a third end-point of viability, the MTT reduction test was utilized. In this case, 2 h sustained hypoxia reduced viability significantly by ~90% ($p < 0.05$), which was partially reversed in the IP-groups (non-IP: 7.08 ± 2.50% vs. IP: 54.2 ± 15.2%; $p > 0.05$).

(ii) Inhibition of NOS with L-NAME (fig. 3.3 A & B)

Inhibition of NO generation with 50 μM L-NAME during 10 min hypoxic IP (investigating the *trigger* effect of NOS and NO) had no effect on cell viability (44% and 46% viable cells in treated and untreated IP cells respectively). The administration of L-NAME to inhibit NOS activity during the sustained hypoxia period (*mediator* effect) similarly had no effect on the viability of preconditioned myocytes (51% and 46% viable cells in treated and untreated IP myocytes respectively). Administration of L-NAME for 10 min, followed by washout, to non-preconditioned cells, was also without effect. However, when *non-preconditioned* cells were treated with L-NAME during the sustained hypoxia period, cell viability increased significantly

from 36% to 51% trypan blue excluding myocytes ($p<0.05$, fig. 3.3 A) in one series, and from 39.5% to 54.9% in a separate series ($p < 0.05$, fig. 3.3 B) respectively. Morphological evaluation revealed a similar significant pattern (13% increase in rod-shaped cells, $p<0.05$; fig. 3.3 B). Treatment of oxygenated control cells with L-NAME for 2½ h had no effect on viability, thereby excluding any possible drug-effects (data not shown).

In order to determine whether the results obtained with 50 μM L-NAME were dose-dependent, the above investigations were repeated using 200 μM L-NAME. Results with the increased drug concentration followed a similar trend as observed before: While L-NAME had no effect on the viability of IP cells, the protective effect exerted by NOS inhibition on non-preconditioned cells during sustained hypoxia could be successfully reproduced by 200 μM L-NAME (%TBE myocytes increased from 41.9 ± 1.8% to 55.7 ± 1.9%, $p < 0.05$), as well as by the administration of an alternative NOS inhibitor, 50 μM L-NNA (42.2 ± 1.6% to 66.03 ± 6.7% increase in TBE myocytes, $p < 0.05$). Co-administration of the NOS substrate, L-arginine (10 mM), with L-NAME during sustained hypoxia did not alter L-NAME induced protection (non-IP+ L-NAME: 54.87 ± 2% TBE; non-IP + L-NAME + L-arginine: 53.2 ± 3.3% TBE, $p>0.05$).

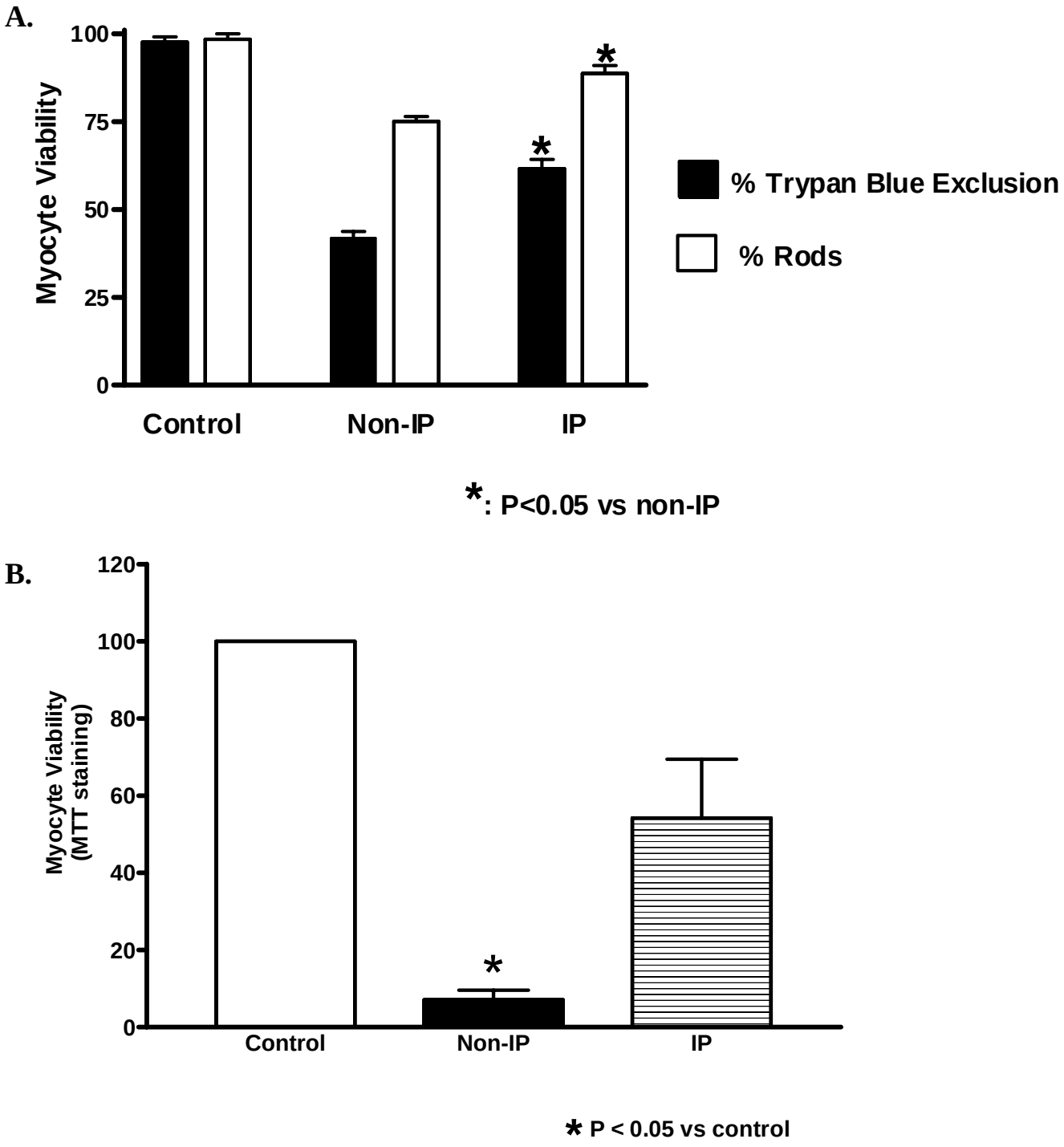


Fig. 3.2 Demonstration of IP protection. **(A)** Using trypan blue exclusion (TBE) and myocyte morphology assessment (the ability of myocytes to retain their viable rod-shaped structure - %rods) as end-points. IP increased the viable, trypan blue excluding myocytes from 46,2% to 71,3%, and the number of rod-shaped cells from 74% to 96% (n = 4-8 / group). **(B)** When MTT reduction was used as end-point, similar patterns were observed (viability reduced from 100% to 7 ± 2.5% in non-IP cells, which improved to 54 ± 15.2% in IP groups (n = 4 / group).

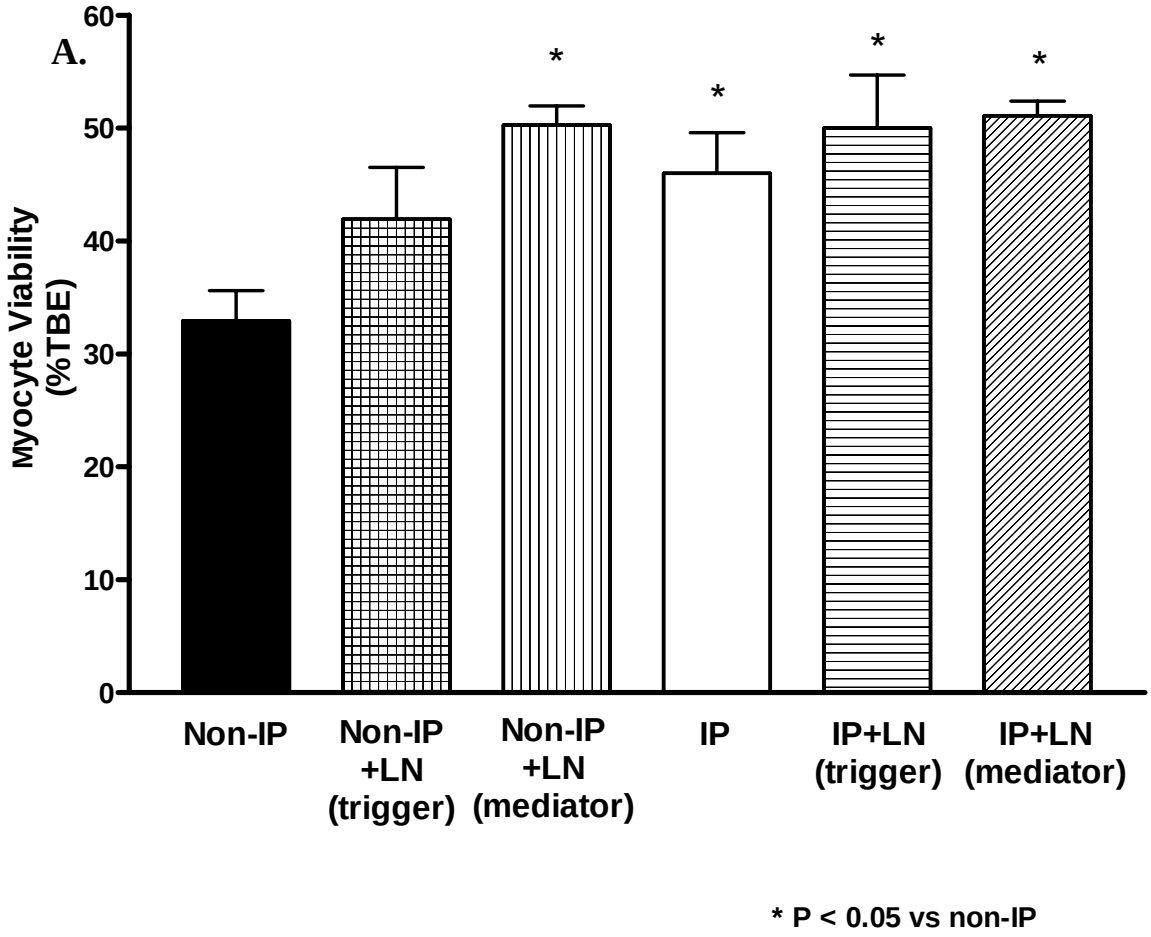


Fig. 3.3 (A) Effect of NOS inhibition with L-NAME (50 μM) in non-IP and IP groups. The drug was administered to preconditioned cells during either the short hypoxia period of IP ("trigger effect") or the sustained hypoxia ("mediator" effect) period. Non-preconditioned (non-IP) cells were treated with L-NAME in the same manner. NOS inhibition had no effect on the viability of preconditioned cells, but significantly increased viable myocytes in non-preconditioned groups, when administered during sustained hypoxia; (n = 5-15 / group).

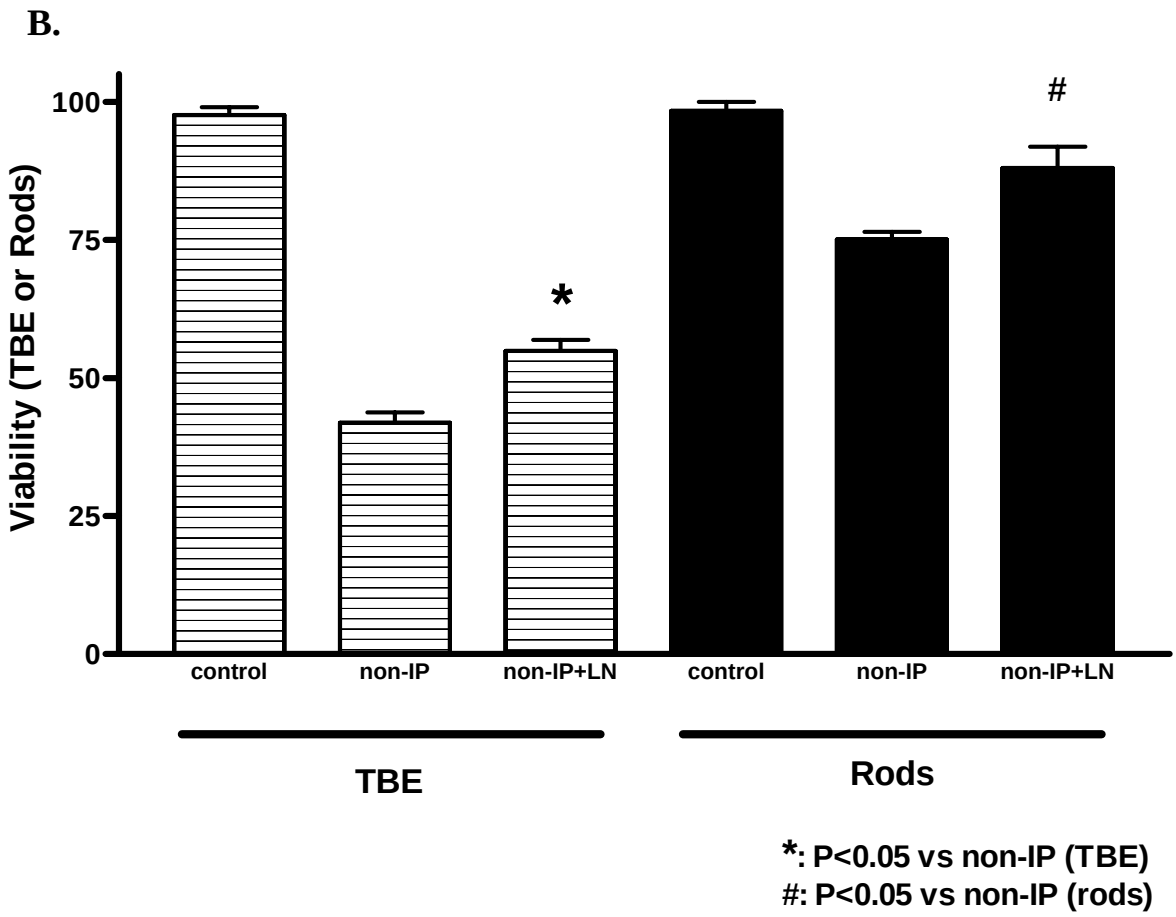


Fig. 3.3 (B) Effect of NOS-inhibition with L-NAME (50 μM) on non-IP groups (i.e. investigating the role of NOS during sustained hypoxia in the absence of IP). A significant improvement in viability was observed when NOS was inhibited; viable trypan blue excluding cells increased from 39.5 ± 0.9% in non-IP to 54.9 ± 2% in IP (n = 6 / group), and % rods increased from 75.1 ± 1.4% to 88.1 ± 3.9% respectively (n = 6 / group).

(iii) Inhibition of iNOS with 10 μM SMT (fig 3.4)

The administration of SMT before and during hypoxic preconditioning had no effect on the percentage viable myocytes after 2 h of sustained hypoxia and values similar to those of untreated preconditioned groups were obtained (fig. 3.4 A). Similarly, the percentage viable cells was unchanged in both IP and non-IP groups when SMT was administered during sustained hypoxia (fig. 3.4 B). No drug-effects were observed with SMT when administered to oxygenated control myocytes (data not shown).

(iv) Pre-treatment with NO donor, 100 μM SNP (fig. 3.5)

The viability of myocytes treated with the NO donor, SNP, for 10 min followed by 20 min wash-out remained unchanged compared to untreated non-IP myocytes after 2 h sustained hypoxia, indicating that exogenous NO did not act as a trigger of protection.

(v) Reactive oxygen species studies (figs. 3.6 A & B)

Treatment of myocytes with the ROS scavenger, MPG (300 μM), during the full IP protocol (short hypoxia and reoxygenation) had no effect on viability after 2 h sustained hypoxia compared to untreated preconditioned myocytes (fig. 3.6 A). Similarly, administration of MPG during sustained hypoxia had no effect on the viability of either preconditioned or non-preconditioned cells (fig. 3.6 B). MPG-treatment did not alter the viability of oxygenated control cells, thereby excluding drug-effects (results not shown).

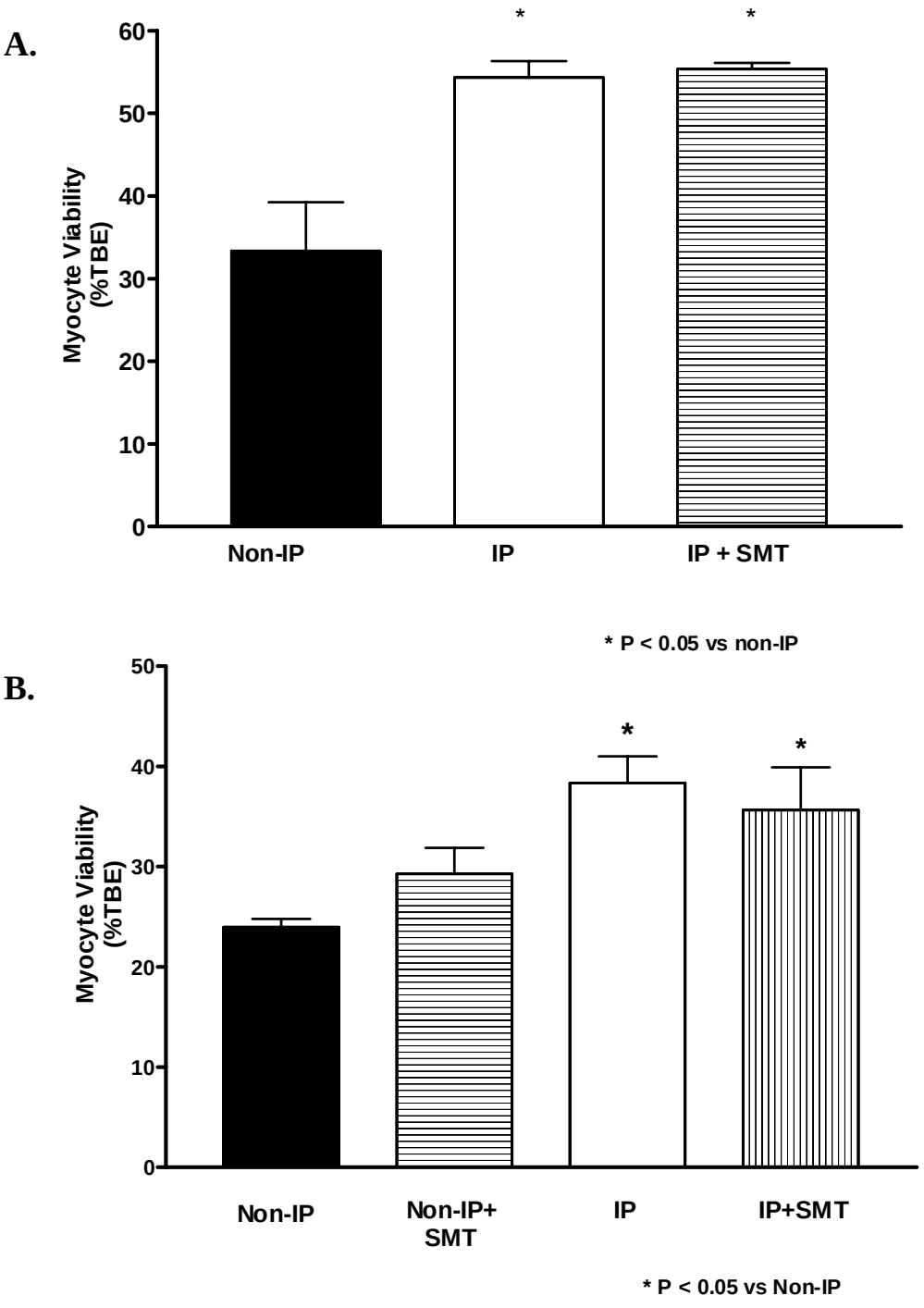


Fig. 3.4 The effect of iNOS inhibition in IP and sustained hypoxia. **(A)** Administration of the specific iNOS antagonist, SMT (10 μM), prior to and during the brief hypoxia period of the IP protocol, had no effect on the myocyte viability (n = 6 / group). **(B)** Similarly, SMT treatment during sustained hypoxia also had no effect on viability in either IP or non-IP cardiomyocytes compared to their respective untreated controls (n = 4 / group).

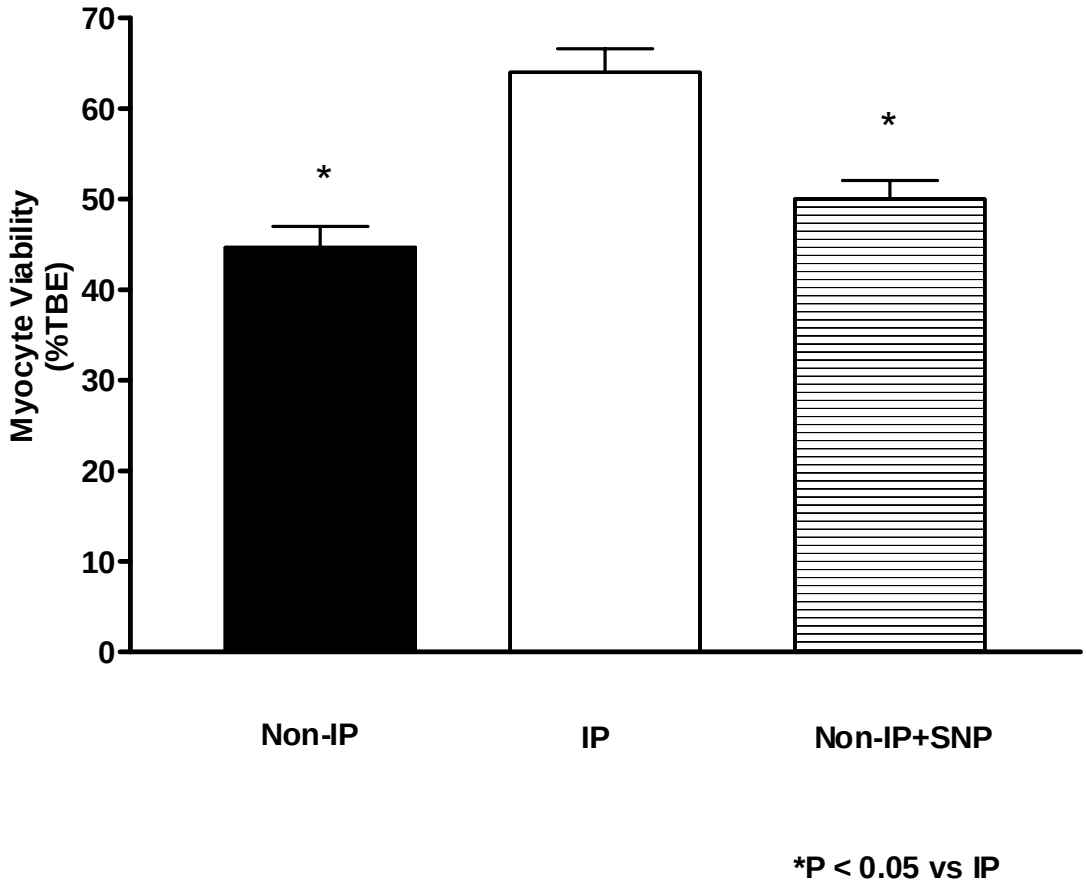


Fig. 3.5 Pre-treatment with NO donor, SNP (100 μM), followed by wash-out (20 min) and 2 h sustained hypoxia could not mimic IP protection; n = 9-13 / group.

In addition, an alternative ROS scavenger, N-acetyl-cysteine (NAC, 0.5 mM), was administered in similar fashion to MPG as described above, and the findings remained unchanged from those observed in MPG-treated IP and non-IP groups (data not shown). Pretreatment of the myocytes with H_2O_2 (100 μM), a ROS generator, failed to protect against sustained hypoxia, and viability remained at levels observed in non-IP cells (non-IP: 42.9 ± 1.4%; IP: 63.3 ± 2.6% and H_2O_2 treated cells: 42.8 ± 3.1%; $p > 0.05$).

(vi) cGMP determinations (fig. 3.7 A & B)

A transient elevation in cGMP levels was observed in preconditioned cells at the end of the IP protocol, compared to levels in oxygenated control and non-IP cells (IP: 63.2 ± 8.6 pmoles / mg prot vs control and non-IP: 41.6 ± 3.4, $p < 0.05$). Cyclic GMP levels of non-IP cells were significantly elevated after 2 h hypoxia when compared to controls ($p < 0.05$) and IP cells ($p < 0.05$, fig. 3.7 A). L-NAME administration had no additional lowering effect on the cGMP levels of both non-IP and IP myocytes compared to their untreated counterparts, after 2 h hypoxia (fig. 3.7 A). The iNOS inhibitor SMT had no effect on the cGMP levels of either IP (fig. 3.7 A) or non-IP groups (data not shown). MPG administration during the triggering phase of IP cells did not alter cGMP levels, however, when administered during the sustained hypoxia period, levels were significantly increased compared to untreated IP cells (IP+MPG: 62.3 ± 5.3 pmoles / mg prot vs untreated IP: 41.5 ± 4, $p < 0.05$.). SNP administration for 10 min followed by 20 min wash-out and 2 h sustained hypoxia caused a gradual, significant increase in cGMP levels over time, when compared to untreated groups (fig. 3.7 B).

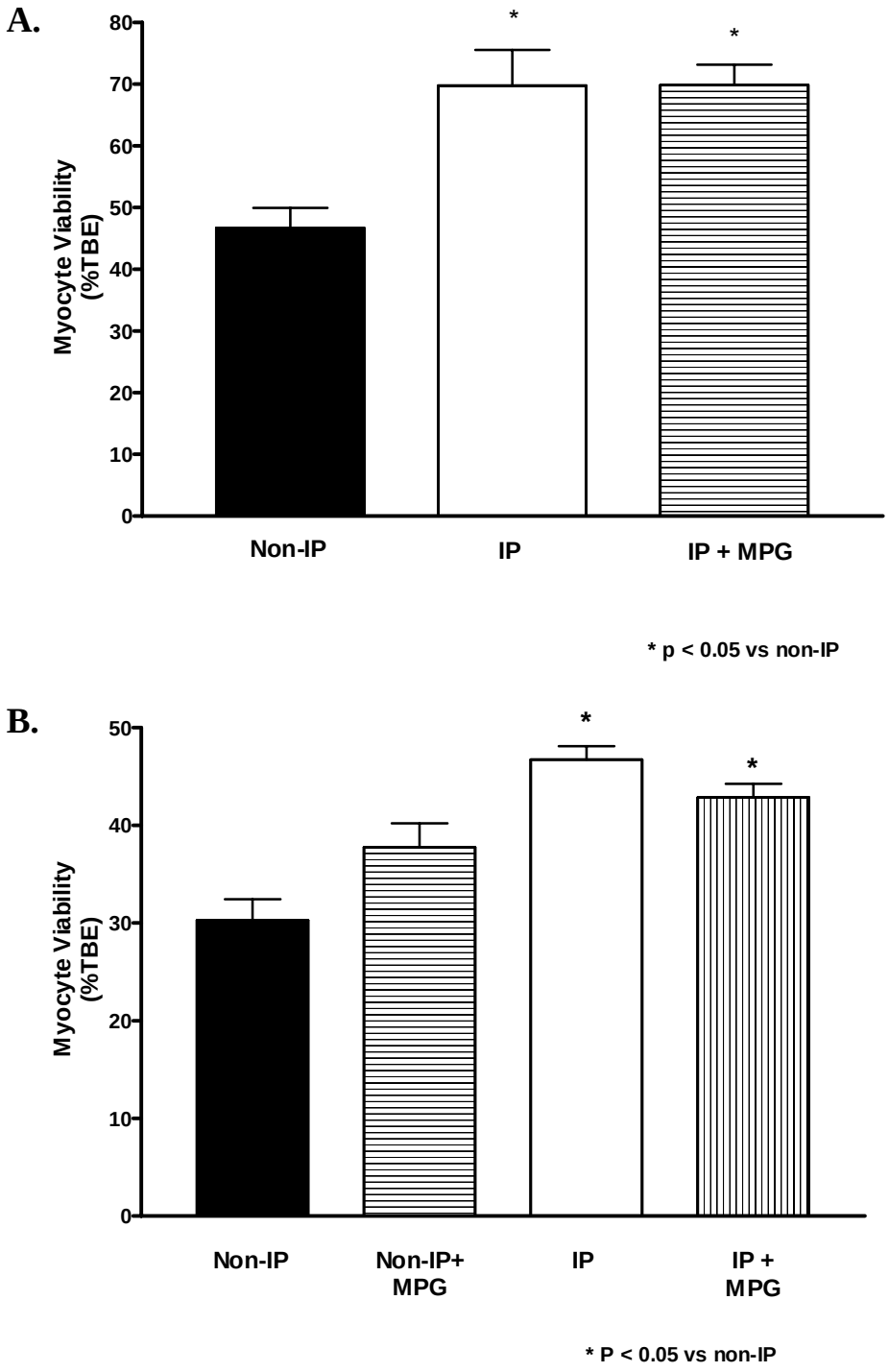


Fig. 3.6 Treatment with the ROS scavenger, MPG (300 μM). **(A)** Administration of MPG during the IP protocol (i.e. brief hypoxia and subsequent reoxygenation) had no significant effect on the cell viability of preconditioned myocytes. **(B)** MPG treatment during sustained hypoxia did not alter viability in either IP or non-IP cells compared to their respective untreated controls; n = 6 / group.

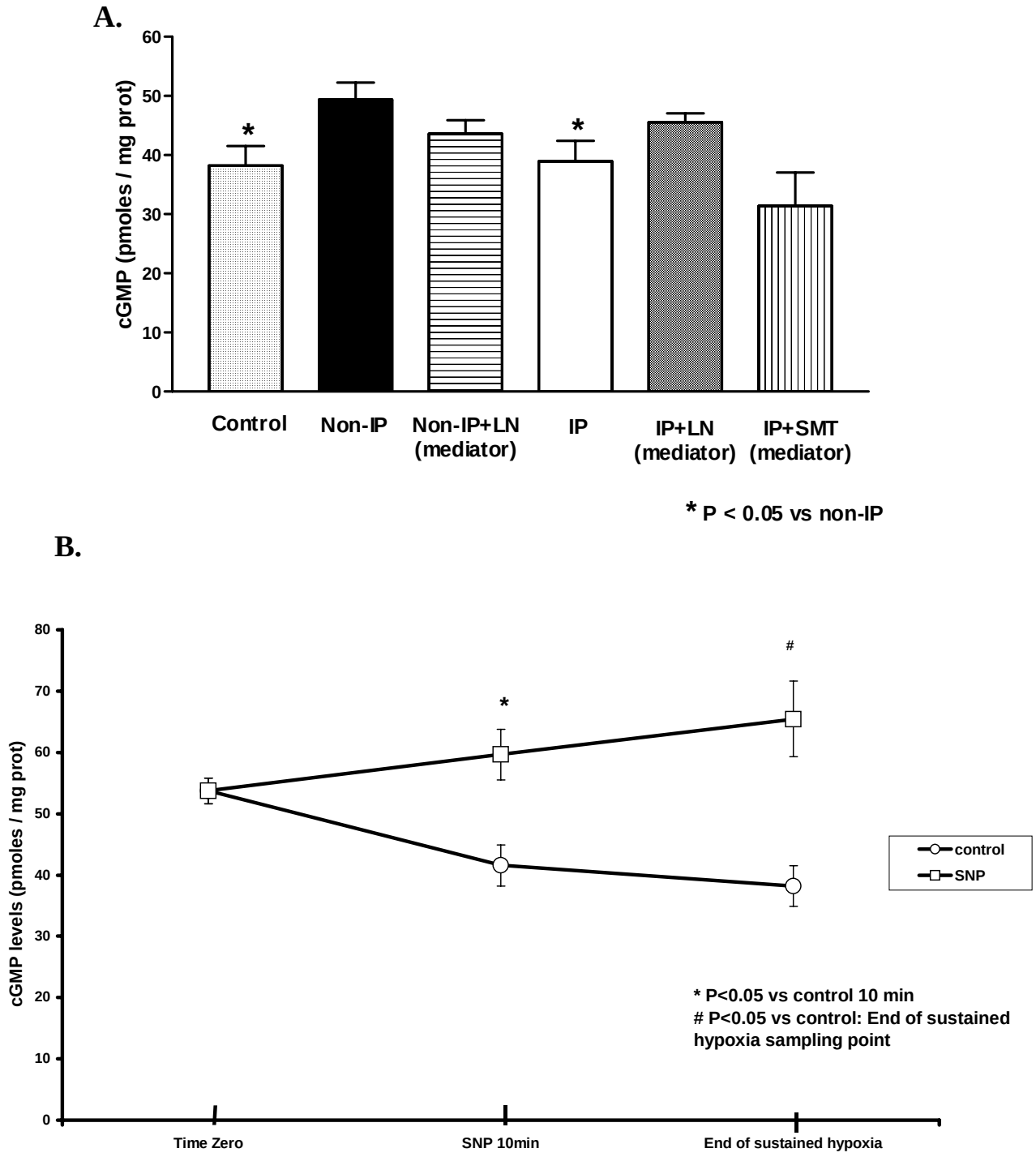


Fig. 3.7 Cyclic GMP levels in cardiomyocytes. **(A)** Cellular cGMP levels after 2 h hypoxia. Effects of L-NAME and SMT. See text for details (n = 4 – 8 / group). **(B)** The effect of 10 min SNP pretreatment on cGMP levels in non-IP cells over time. SNP-treated cells showed elevated cGMP levels compared to corresponding O_2 control groups at the end of the 10 min treatment period. Cyclic GMP levels continued to rise in SNP-treated cells until the end of sustained hypoxia.

3.6 Discussion

(i) The isolated adult cardiomyocyte model

The isolation procedure used in this study is a standard technique widely used and accepted to yield a very pure myocyte preparation. The final suspension does not contain contaminating cell types that could influence findings in any significant way.
Of particular concern in this study would be the presence of endothelial cells, since they possess NOS, and could therefore contribute to the overall NO production. Although small clusters of stripped endothelium were occasionally noted in random samples, the results obtained from such samples did not differ significantly from the others, and it is doubtful whether they would contribute to NO production in any significant way. The isolated myocyte model therefore represents an ideal opportunity to establish whether NO generated in cardiomyocytes plays a role (beneficial or harmful) in IP protection.

(ii) The IP protocol

The current study showed that the IP stimulus (10 min hypoxia, 20 min reoxygenation) could trigger inherent, anti-hypoxia cardioprotection in freshly isolated adult cardiomyocytes, manifesting within 2½ h. Protection was demonstrated by two independent end-points (%TBE cells and morphological studies), which are independent markers of viability: trypan blue exclusion measures osmotic fragility of the cell membrane, whereas the morphology test evaluates cell appearance, independent of trypan blue uptake. Although the margin of increase in viability

differed between the two end-points, both demonstrated a clear protective effect induced by prior hypoxic preconditioning.

The findings observed with trypan blue are in line with previous results of a study upon which our IP protocol was based [Armstrong *et al* 1995]. The latter study, on isolated adult rabbit myocytes, demonstrated ~ 50% increase in viable TBE cells after 2 hr sustained hypoxia, compared to 54% in the present study. These observations confirm that cardiomyocytes per se are able to release triggers of protection in response to the IP stimulus [Cohen *et al* 2000; Miura 1996; Schultz *et al* 1997], and this may help explain why preconditioned isolated myocytes show some degree of protection. In our model, protection was observed despite the fact that we were unable to demonstrate a triggering role for NO, which indicates that other, non-NO triggers were released. Furthermore, the rather stressful isolation procedure may in itself serve as a still unknown preconditioning stimulus [Piper & Ladilov 1997].

(iii) NOS and NO as a possible trigger of protection

NOS inhibition studies

It has previously been established that a short period of ischaemia (e.g. 5 min) can rapidly stimulate NOS activity in the heart [Depré *et al* 1997]. We therefore studied whether the 10 min simulated ischaemia of the IP protocol could trigger protection via activation of the NOS - NO pathway. Results showed that NOS inhibition with L-NAME during the triggering phase did not influence IP protection (fig. 3.3 A), suggesting that NOS activation was not necessary to trigger protection. To our

knowledge this is the first demonstration that NOS inhibition does not affect the protection elicited by IP in a fresh, isolated adult rat cardiomyocyte preparation.

Although it has recently been suggested that NO could also act independently (such as direct K_{ATP} channel activation, inherent antioxidant properties and COX-2 stimulation) [Bolli 2001], its cellular actions are usually mediated through its second messenger, cGMP [Balligand & Cannon 1997; Denninger & Marletta 1999; Villa-Petroff *et al* 1999]. Therefore, we also measured the effect of NOS inhibition during the triggering phase on cGMP generation at the end of sustained hypoxia. Results show that L-NAME did not attenuate overall cGMP generation during this period, also suggesting that NOS-NO activation during the triggering phase does not play an important role in preconditioning (results not shown).

A number of studies that applied NOS inhibition just before or during the IP protocol showed a similar trend, albeit with different experimental models and protocols. In a study on isolated, perfused rabbit hearts, it was found that addition of L-NAME 10 min before the IP protocol conferred no additional benefit compared to IP alone (end-point: infarct-to-risk ratio) [Woolfson *et al* 1995]. Similar results with L-NAME + IP treatment were obtained in rabbit hearts with infarct / risk zone as end-point [Patel *et al* 1992], and arrhythmias as end-point [Lu *et al* 1995], and in isolated rat hearts with contractile function and LDH release as end-points [Weselcouch *et al* 1995]. Similar findings were obtained in swine hearts (no alteration in the reduction of IP-induced infarct size development) using a different NOS inhibitor, L-NA [Post *et al* 2000]. In another study on isolated rabbit hearts, it was demonstrated that L-NAME + IP treatment did not alter cardioprotection and it was suggested that endogenous production of NO during the IP protocol was insufficient and therefore ineffective to

trigger protection [Nakano *et al* 2000]. Perhaps the best evidence was an observation made in isolated working rat hearts that IP did not increase basal cardiac NO content as measured by electron spin resonance [Csonka *et al* 1999].

In contrast, other studies found that inhibition of IP-induced NO synthesis did cause partial attenuation of IP protection. A study from our own laboratory demonstrated that L-NAME administration before and during the IP protocol partially attenuated functional recovery in isolated rat hearts [Lochner *et al* 2000]. Similarly, a study on anaesthetized, open-chest dogs, showed a partial attenuation of IP protection with L-NAME pretreatment (end-point: arrhythmias) [Vegh *et al* 1992]. Differences in results between the present study and that of Lochner *et al* and Vegh e*t al* could be explained by the presence of an intact coronary circulation, thereby allowing for an additional major source of NO (endothelium-derived NOS - NO). It is possible that the additional NO release and administration of NOS inhibitors may have had profound effects on the coronary vascular tone that could account for some of the findings in the IP protocols used. Furthermore, both the mentioned groups used multi-cycle IP protocols, compared to the single-cycle IP protocol of the present study.

There are considerable variations in the concentrations of L-NAME used in the above studies; ranging from 30 μM, which failed to block protection [Weselcouch *et al* 1995], to a concentration of 50 μM that did succeed in attenuating IP protection [Lochner *et al* 2000]. Recently, another group could only achieve inhibition of IP protection at a concentration of 200 μM in embryonic chick myocytes [Lebuffe *et al* 2003]. In order to verify whether our results with 50 μM were dose-dependent, we repeated the investigations with 200 μM, which produced results identical to the

50 μM series, indicating that our model did not follow the trend observed by Lebuffe *et al*. A possible explanation for this discrepancy could be the difference in model used (cultured, embryonic chick myocytes vs isolated adult rat myocytes).

L-NAME has been reported to be pharmacologically non-specific [Curtis & Pabla 1997]. It is therefore possible that the drug may cause inhibition of both the eNOS *and* iNOS isoforms. Despite some controversy regarding the presence of NOS in isolated myocytes, it has been shown quite convincingly that myocytes express both iNOS and eNOS isozymes [Balligand *et al* 1995(b); Balligand *et al* 1994]. More recently, it has also been demonstrated that cardiomyocytes express the nNOS isoform [Danson et al 2005]; however, more investigations are necessary to establish its role [Brutsaert 2003]. Most studies that investigated IP, however, did not distinguish between the isoforms. Contrary to the commonly accepted view, a study conducted on rat hearts demonstrated that iNOS was constitutively expressed both in vitro and in vivo [Buchwalow *et al* 2001]. It is therefore possible that iNOS could be activated in a similar fashion to the constitutively present eNOS. In order to investigate a possible trigger role for iNOS - NO in the present study, SMT, a known iNOS-specific inhibitor [Wang YP *et al* 2001; Wildhirt *et al* 1997], was administered during the brief hypoxia period of the IP protocol. Results indicated that iNOS inhibition during the triggering phase did not affect IP protection or cGMP generation, similar to the observations with L-NAME. Furthermore, iNOS inhibition during sustained hypoxia had no effect on either cell viability or cGMP levels at the end of the experiments, negating a role for iNOS activation in IP. In summary, observations with L-NAME and SMT treatment suggest that the protection conferred by IP in our model was not dependent on eNOS or iNOS activation during the brief hypoxia

period. In addition, these findings suggest that activity of the NOS-NO-cGMP pathway at the end of the experiments was not affected by NOS inhibition during the triggering phase.

NO donor studies

Another technique widely used to investigate the potential role of NO as a trigger is to mimic IP by the administration of NO. SNP pretreatment could not induce protection against sustained hypoxia in our model (fig. 3.5). In one of the few studies to show a similar finding, albeit in an entirely different experimental model, it was demonstrated that pre-treatment of anaesthetized rats with a NO-donor, C87-3754, did not influence the incidence or severity of ventricular arrhythmias induced by sustained ischaemia [Sun & Wainwright 1997].

In one of the few studies investigating NO in early IP in an isolated cell model (cultured neonatal rat myocytes), IP-protection (90 min simulated ischaemia – 30 min reoxygenation, followed by 6 h sustained ischaemia) was successfully mimicked by pretreating the cells with SNAP (S-nitroso-N-acetylpenicillamine; NO-donor) for 90 min [Rakhit *et al* 2000]. Our own investigations showed that administration of the NO donors SNAP or SNP could successfully mimic classic IP in isolated perfused rat hearts [Lochner *et al* 2000]. Another study on conscious rabbits found that pretreatment with the NO-donor nitroglycerine (NTG) attenuated myocardial stunning [Shinmura *et al* 1999]. Interestingly, it also appears that exogenous, rather than endogenous, NO could trigger IP protection. It was demonstrated in eNOS knockout (KO) mice that IP significantly attenuated infarct size, indicating that eNOS-NO was

not pivotal in the protection of IP [Bell & Yellon 2001]. However, the administration of SNAP to both KO and wildtype mice successfully mimicked IP. Similarly, SNAP treatment was shown to be protective against ischaemia-reperfusion in isolated rabbit hearts [Nakano *et al* 2000].

However, despite other observations made to the contrary, it is evident from our results that neither endogenous NOS - NO, nor exogenous NO, act as triggers of IP protection in our model of freshly isolated rat cardiomyocytes. It remains unclear why exogenous NO administration could not elicit protection in our model, despite the observation that SNP treatment caused a significant, transient increase in cGMP levels compared to untreated control myocytes at corresponding time points (fig. 3.7 B). These increases suggest that the failure to protect was not due to possible ineffective SNP concentrations, or desensitization of the downstream enzymes and effectors of the NOS – NO pathway (i.e. guanylate cyclase and cGMP), occurring as a possible result of the robust isolation procedure or potential pH changes.

The role of ROS as triggers and mediators of IP

An increasing number of studies are focusing on the role of ROS and reactive nitrogen species (RNS) as possible triggers of IP, most of which showed a protective role for ROS and RNS. For example a trigger role for peroxynitrite ($ONOO^-$) in IP-protection was demonstrated in isolated rat hearts, and administration of the ROS scavenger MPG increased the incidence of arrhythmias in both the IP and peroxynitrite treated cells [Altug *et al* 2000]. Similarly, in a study on isolated rabbit hearts, MPG blocked SNAP's cardioprotection [Nakano et al 2000]; and in guinea pig

hearts MnTBAP, a O_2^- scavenger, also abrogated protection [Kevin *et al* 2003]. A triggering role for ROS was demonstrated in a model of isolated embryonic chick ventricular myocytes [Lebuffe *et al* 2003; Vanden Hoek *et al* 1998]. However, ROS generation does not appear to trigger protection in our model of adult rat cardiomyocytes: (i) pretreatment of cells with H_2O_2, a ROS generator, did not confer protection, and (ii) administration of MPG or NAC before and during our IP protocol did not abolish protection. The administration of MPG and NAC during the sustained hypoxia period had no effect on the viability of IP and non-IP groups. However, it is interesting to note that MPG treatment during this period induced a significant elevation in cGMP generation at the end of sustained hypoxia. It is not clear what caused this observation since MPG is not known to be a direct cGMP activator. However, one explanation may be that certain ROS (e.g. superoxide) act as "NO scavengers" [Estevez & Jordan 2002], and their removal with a relatively non-specific ROS scavenger could therefore lead to increased NO levels, with a resultant upregulation of the NO-cGMP pathway.

It is difficult to explain why our model did not follow the cardioprotective trend of ROS observed in other studies, and more investigations on the isolated rat myocyte model are required to elucidate this. Certainly, differences with the embryonic chick myocyte studies [Lebuffe *et al* 2003; Vanden Hoek *et al* 1998] may be attributed to differences in species, experimental models or, in the case of MPG, different dosages (300 μM vs 400 μM). Limitations of ROS scavengers should also be considered, particularly the fact that some are likely to be consumed by ROS in the absence of continued delivery, which limits their ability to scavenge fully [Kevin *et al* 2003], but this fails to explain why H_2O_2 could not trigger protection in our model.

NOS as a possible mediator of protection

We assessed the role of NOS-derived NO-release during sustained hypoxia in non-preconditioned and preconditioned cells. NO is known to be a major activator of the guanylate cyclase-cGMP pathway [Balligand & Cannon 1997; Csonka *et al* 1999; Takano *et al* 1998], therefore, in the present study, the cardiomyocyte cGMP levels were used as an indicator of the activity of this pathway. Results showed that cGMP levels were elevated in non-IP cells, suggestive of NO and cGMP generation during hypoxia (fig. 3.7), a finding similar to previous observations [Depré & Hue 1994; Lochner *et al* 1998]. It is well established that NOS activity is rapidly stimulated by ischaemia [Depré *et al* 1997], while the activation of cGMP - phosphodiesterase is attenuated [Lochner *et al* 1998], both processes which could contribute to the increase in cGMP observed (fig. 3.7). It has recently been shown that cellular acidosis and subsequent activation of the Na^+ / H^+ exchanger modulate production of endogenous NO [Kitakaze *et al* 2001]. Addition of exogenous arginine had no further deleterious effect on cell viability, indicating that NOS activation was probably maximal under the experimental conditions used.

The inhibition of NO generation during sustained hypoxia did not affect IP protection (fig. 3.3). In fact, it appears that IP decreases the accumulation of NO during sustained hypoxia, since significantly lower cGMP levels were found in preconditioned cells compared to non-IP cells at the end of sustained hypoxia (fig. 3.7). The decreased cGMP levels in IP cells could be indicative of an endogenous down-regulation of NOS - NO by the IP protocol, the mechanism of which remains to be established. It is also evident from our cGMP studies that the administration of

L-NAME, or SMT, to IP myocytes caused no further decrease in cGMP, suggesting that NOS / NO activity had already been attenuated maximally in IP groups.

Although most studies investigated the mediator role of NO in the context of delayed IP [Imagawa *et al* 1999; Takano *et al* 1998; Xuan *et al* 2000], a few authors did investigate NO as a mediator of classic IP. In isolated perfused rat hearts it was shown that the administration of L-NA before sustained ischaemia – reperfusion had no effect on either IP protection or NO generation during this time period [Csonka *et al* 1999]. A similar reduction of ischaemia-reperfusion induced NO accumulation was seen in the untreated IP hearts, suggesting that IP seemed to decrease the harmful accumulation of NO during sustained ischaemia - reperfusion, with the result that additional NOS inhibition had no effect. They argued that the decreased rate of NO production in preconditioned cells during ischaemia / reperfusion could be due to altered metabolic conditions such as changes in pH, availability of cofactors and/or substrates for enzyme synthesis. These results are in agreement with our observations. Another possibility was the activation of endogenous NOS inhibitors such as asymmetrical dimethylarginine (ADMA) [Ueno *et al* 1992; Usui *et al* 1998]. The distribution of ADMA correlates well with that of NOS [Ueno *et al* 1992], although no evidence could be found in the literature to suggest that ADMA is present in cardiomyocytes.

In summary, the results of the investigations on preconditioned myocytes suggest that: (i) either cardiomyocyte NOS was not activated by the IP protocol used, or that NO was not generated in sufficient quantities during the IP protocol to act as a trigger, and (ii) the NO generated during sustained hypoxia in preconditioned cells

was reduced (as indicated by the cGMP levels) so that pharmacological inhibition of the enzyme had no additional effects. These results suggest therefore that in our model of isolated cells, the cardiomyocyte NOS - NO can be regarded as a neutral bystander with no influence on the IP mechanism, similar to findings by Liu and co-workers [Liu *et al* 1999].

NOS inhibition in non-preconditioned, hypoxic myocytes

Interestingly, the current study showed that inhibition of NO synthesis (observed with both 50 µM and 200 µM L-NAME) during sustained hypoxia in non-preconditioned cells resulted in significant protection, the degree of which was comparable to the protection conferred by the IP stimulus (fig. 3.3 A & B). In separate experiments, L-NNA (50 µM), an alternative NOS inhibitor, showed similar, if not slightly enhanced, protective effects compared to L-NAME, excluding possible drug-specific reactions with L-NAME. The observation that the inclusion of L-arginine (NOS substrate essential for NO generation) did not alter the protection conferred by L-NAME indicates that the drug decreased NOS activity, even in the presence of excess substrate. However, it is surprising that arginine did not reverse the effects of L-NAME, the latter being an arginine based inhibitor. Obviously, this phenomenon needs to be further investigated.

Reasons for the observed protection of non-preconditioned myocytes by L-NAME are not clear. L-NAME treatment during sustained hypoxia did not attenuate cGMP levels, suggesting that the protection observed with NOS inhibition was not likely to be via a downregulation of the NOS-NO-cGMP pathway. The protective effects of

L-NAME could not be reproduced by SMT administration, which indicates that iNOS inhibition is not involved in this particular observation (fig. 3.4 B). It is possible that NOS inhibition prevents the formation of potentially harmful NO-derived reactive oxygen species (ROS) such as hydroxyl radicals (OH^-) and peroxynitrite ($ONOO^-$) during sustained hypoxia. However, results obtained when the ROS scavengers, MPG and NAC, were administered during sustained hypoxia, suggest that ROS formation was not harmful to non-IP cells in our model. The bimodal action of NO (i.e. physiological effects at low, submicromolar concentrations and harmful effects at higher, micromolar or above concentrations) should also be considered; it is possible that sustained hypoxia in our model resulted in the release of high (harmful) concentrations of NO and that subsequent NOS inhibition during this period conferred protection.

In summary, one has to consider the possibility that the protective effects of NOS-inhibition may occur by other, NO-independent mechanisms [Curtis & Pabla 1997; Nakano *et al* 2000; Sun & Wainwright 1997]. The fact that cGMP levels remained unchanged despite NOS inhibition and the observed cytoprotection could also suggest that sustained hypoxia induced NOS-NO to exert harmful effects, independent of cGMP.

3.7 Conclusion

The present study was originally designed to specifically exclude any significant contribution of endothelial cell eNOS – NO, a major source of overall NO, in the mechanism of cardiomyocyte preconditioning. The results of this chapter indicate that the activation of cardiomyocyte NOS (eNOS or iNOS) during the IP stimulus and

sustained hypoxia is not required for protection in this model of simulated ischaemic preconditioning. Similar results were obtained in other models [Bolli 2001; Post *et al* 2000; Weselcouch *et al* 1995; Woolfson *et al* 1995]. We also demonstrated that IP could not be mimicked by the administration of exogenous NO donors, despite transient increases in cGMP levels, which is indicative of increased NO-cGMP activity. Based on these results, one can speculate that NOS - NO in this isolated rat cardiomyocyte model may be an insignificant contributor to the overall pool of released NO, with no effect on IP on its own. One possibility is that adult rat myocytes are more dependent on non-myocyte NO-sources for the maintenance of their intracellular NO levels than chick embryonic myocytes or neonatal myocytes. In such a scenario, the absence of other NO-generating cell types in the experimental model could explain why NOS inhibition did not reverse IP. Future co-culture studies could establish a role for endothelial cell NOS-NO in IP and myocyte-endothelial interactions in NO release, thereby showing the relative roles of each of the cellular sources of NO in IP. It is also possible that adult rat myocyte-NOS generates superoxide in preference to NO during hypoxic stress, however, this would not explain why the ROS scavengers failed to reverse IP protection. The fact that isolated cardiomyocytes were nevertheless successfully preconditioned in the present study suggests that other triggers such as TNF-α must be involved [Lecour *et al* 2002].

The findings of this chapter suggest that fresh, isolated adult rat cardiomyocytes seem to have a different phenotype with regards to NOS-NO physiology than their neonatal counterparts and myocytes of other species. Follow-up studies using NO-

sensitive detection methods could help to clarify whether these isolated rat heart myocytes produce significant quantities of NO.

Finally, the results of our experiments on NOS activation during sustained hypoxia in non-preconditioned cells were suggestive of a harmful role for NOS and NO, but that this effect was seemingly cGMP-independent. In view of the fact that we were unable to show a role for NO in IP protection, together with the unexpected findings demonstrating a harmful role for NO during sustained hypoxia, it was decided that, for the purposes of the rest of this thesis, focus would be directed at the role of NO in hypoxia.

[The aims, methods, data and conclusions presented in this chapter appeared in: *Strijdom H, Genade S, Lochner A. Nitric oxide synthase (NOS) does not contribute to simulated ischaemic preconditioning in an isolated rat cardiomyocyte model. Cardiovasc Drugs Therapy 2004; 18: 99-112.* This publication received a dedicated editorial article written by two leading researchers in the field and appears as an addendum to this dissertation.]

CHAPTER 4

THE NEED FOR DIRECT INTRACELLULAR DETECTION OF NITRIC OXIDE IN ISOLATED CARDIOMYOCYTES: DEVELOPMENT OF A NOVEL TECHNIQUE

4.1 Introduction

Findings from the previous chapter suggested that there was no role for NO or ROS in IP-protection in our model of isolated cardiomyocytes. However, the results showed a seemingly harmful role for NO when released during sustained hypoxia. In view of this, it was decided to further investigate the role of NO as an important biological mediator during hypoxia / ischaemia. In contrast to our own findings, others have demonstrated a triggering and mediating role of NO in IP. These properties have made NO one of the fastest growing fields of interest in heart research [Bolli 2001]. However, despite extensive research, the exact underlying cellular mechanisms of NO are complex and remain poorly understood [Ferdinandy & Schulz 2003]. An important way by which more insight into NO's mechanisms of action could be obtained, is direct measurement of intracellular NO production. However, many studies (including the study from our own laboratory on which we reported in Chapter 3; Strijdom *et al* 2004(a)] depend on *indirect* methods to predict changes in intracellular NO levels, such as nitric oxide synthase (NOS) activity (citrulline assay and cyclic GMP measurements) [Depré *et al* 1997], NO_x (nitrate/nitrite) level determinations [Depré *et al* 1997; Kelm 1999], NOS protein [Rakhit *et al* 2000; Failli *et al* 2002], and mRNA expression [Failli *et al* 2002].

In addition, methods for *direct,* single-cell NO-detection (e.g. chemiluminescence assays, electron paramagnetic resonance spectroscopy and electrochemical electrode methods) are often complicated, insensitive and non-specific, and not readily available to the average equipped laboratory [Leikert *et al* 2001]. This is particularly true for low output NO-generating cells such as cardiomyocytes and endothelial cells. [Leikert *et al* 2001]. Diaminofluorescein –2/diacetate (DAF-2/DA), a

membrane-permeable, fluorescent, real-time indicator for NO [Kojima *et al* 1998], has recently been used to detect NO in cells of cardiac origin via flow cytometric analysis, *viz.* endothelial cells [Failli *et al* 2002; Leikert *et al* 2001; Navarro-Antolin *et al* 1991(a)] and embryonal rat heart-derived cell lines [Chen *et al* 2002]. DAF-2/DA was also used to qualitatively assess NO in cultured embryonic chick ventricular myocytes [Lebuffe *et al* 2003] and isolated cardiomyocytes [Zorov *et al* 2000], by means of video fluorescent microscopy and confocal microscopy respectively.

However, there is no evidence of studies using DAF-2/DA fluorescence to detect NO levels in *adult cardiomyocytes with flow cytometry* (fluorescence activated cell sorting, FACS). FACS has major advantages over other fluorescence detection techniques such as spectrofluorimetry and fluorescence microscopy [Vergne *et al* 1998]. The former requires high labeling intensity and does not distinguish between intracellular and extracellular fluorescence, while the latter is time-consuming and analyzes a small number of cells at a time. In contrast, FACS rapidly measures and analyzes thousands of cells, distinguishes between cell subpopulations and analyzes intracellular events on a single-cell level [Vergne *et al* 1998].

Therefore, we aimed to design a protocol for the FACS analysis of freshly isolated adult rat cardiomyocytes to assess whether baseline intracellular NO release in these cells could be detected with DAF-2/DA (Calbiochem). The NO donor 2-(N,N-Diethylamino)-diazenolate 2-oxide (DEA/NO) (Sigma) was administered to verify the probe's reported NO-sensitivity. Furthermore, since ischaemia stimulates cardiac NOS activity [Depré *et al* 1997], we investigated whether hypoxia could enhance

DAF-2/DA fluorescence in cardiomyocytes. L-NAME, a NOS inhibitor, was administered to establish whether the observed effects were due to NOS-induced NO-release. Levels of NO_x (nitrate/nitrite), major oxidative metabolites of NO [Vergne *et al* 1998] were determined and compared with DAF-2/DA data in order to validate and quantitate results obtained with FACS analysis.

4.2 Experimental groups and protocols (fig. 4.1)

The probe-incubation protocols were modifications of previously described methods in endothelial cells [Navarro-Antolin *et al* 2001(a); Navarro-Antolin *et al* 2001(b)]. Suspensions of ~$0.5x10^6$ myocytes / ml were incubated with a non-limiting dose of DAF-2/DA (10 μM) for 180 min (37ºC). Exposure to light was avoided as far as possible throughout experimentation. Experimental interventions (fig. 4.1) were introduced at different time-points during incubation: (i) DEA/NO at t = 60 min, (ii) the NOS activator, CsA, at t = 60 min, (iii) hypoxia at t = 60 min, and (iv) L-NAME at t = 30 min. Control groups were incubated in suspension under an O_2 atmosphere (21% O_2, 5% CO_2, 40-60% humidity) for the full duration of the experiment (180 min). Simulated ischaemia was achieved by ischaemic pelleting as previously described [Ch. 3; Strijdom *et al* 2004(a)].

4.3 Flow cytometry (fig. 4.2 & 4.3)

At t = 180 min, DAF-2/DA was washed out by centrifugation of the samples after removal of supernatants and mineral oil (in the hypoxia samples), subsequently, cells were resuspended in probe-free buffer followed by immediate FACS analysis.

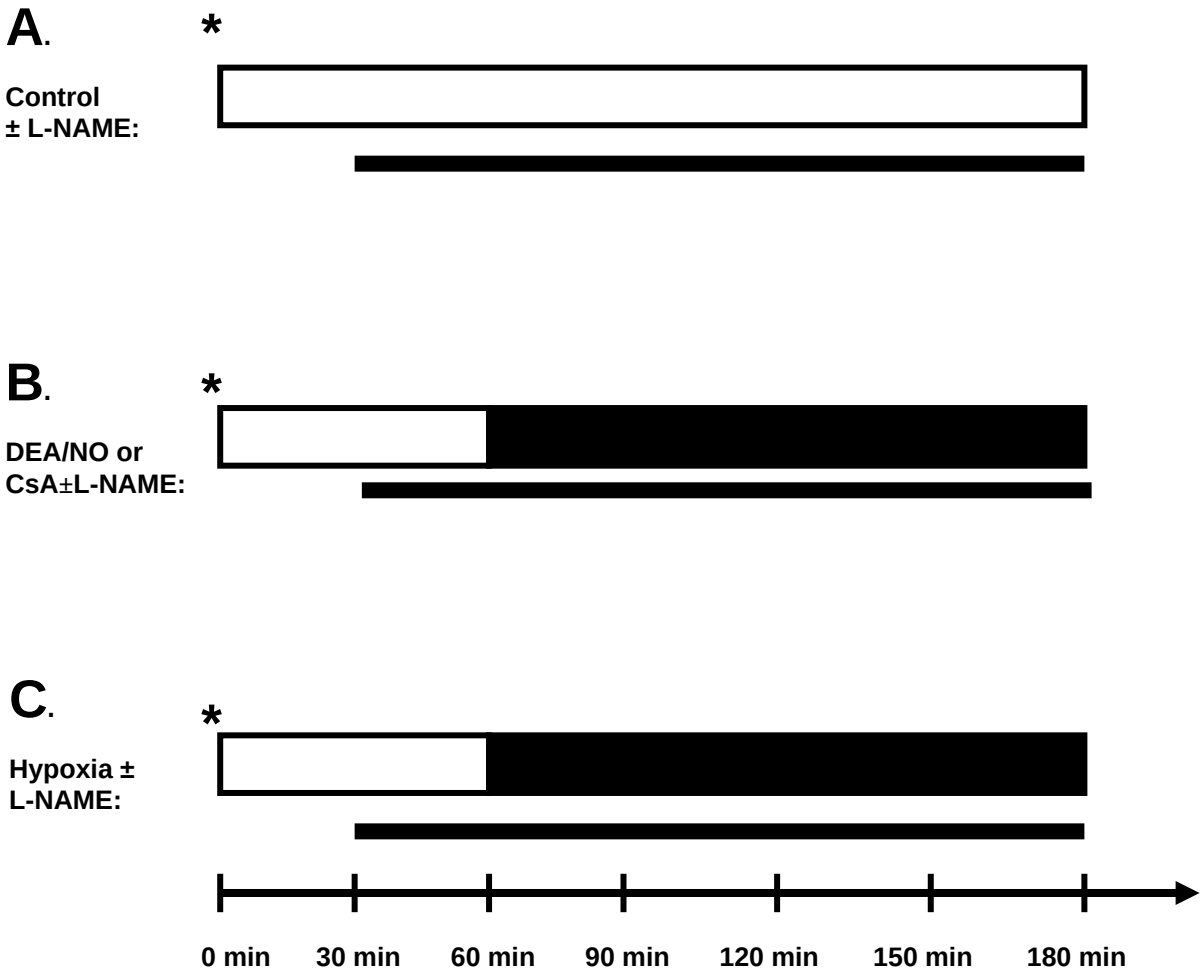


Fig. 4.1 Experimental protocols. Isolated myocytes were divided into sample fractions of ~ 0.5 x 10^6 cells each on which the respective experimental interventions were performed. Experimental groups consisted of samples from different hearts (*n*: 5 – 15 per group).
(A) Control samples were kept in suspension in 1 ml of solution D for 180 min at 37° C in a tissue culture incubator. **(B)** The NO-donor, DEA/NO was administered at different concentrations (100, 500 and 1000 µM respectively) for 120 min at t = 60 min to validate the NO-detection properties of DAF-2/DA. CsA (10 µM; NOS activator) was also added at t = 60 min ± NOS inhibition (L-NAME). **(C)** Ischaemia was simulated by ischaeming pelleting starting at t = 60 min and lasting for 120 min. The unshaded portions of the bars represent untreated, oxygenated control conditions. L-NAME (50 µM) was administered to control and hypoxia groups at t = 30 min, and incubation lasted until t = 180 min (represented by black lines under the bars). Asterisks indicate the start of DAF-2/DA incubation for FACS analysis at t = 0min. Samples were collected at t = 180 min for subsequent FACS analyses and viability assessment.

A Becton Dickinson FACSCalibur ® analyzer was used to quantify fluorescence (excitation wavelength: 488 nm and emission wavelength: 530 nm) at the single-cell level, and data was analyzed using Cellquest ® version 3.3 (Becton Dickinson) software. In each sample viable cell populations were determined by gating forward light scatter (cell size) and side light scatter (cellular granularity) signals as recorded on a dot plot (fig. 4.2), which resulted in the exclusion of non-cellular particles and debris (located on the bottom left corner of the dot plot). In this way, undesired effects on overall fluorescence were limited.

Pre-gated acquisition populations were limited to ~ 50,000 events (viable cells + non-viable cells and debris), and final, gated cell populations usually contained 10 000 – 15 000 cardiomyocytes (for analysis). To standardize selection of the analyzed cell populations between different experimental samples, we used the gating coordinates of control samples for all subsequent analyses in a particular FACS session. Caution was taken to ensure that selected samples contained equal cell numbers. Fluorescence was produced by oxidation of DAF-2/DA to its highly green-fluorescent diaminofluorescein-triazol (DAF-2T) form, and signals were recorded on a frequency histogram (fig. 4.3) in the FL-1 channel by logarithmic amplifiers. Fluorescence data were expressed as mean fluorescence intensity (% of control).

4.4 NO_X (nitrates + nitrites) measurements

Samples collected at t = 180 min were stored in liquid nitrogen until a nitrate/nitrite colorimetric assay (Cayman Chemical) was performed on homogenized cell suspensions. Photometric measurements of absorbance (540 nm) determine nitrate + nitrite concentrations, expressed as pmoles / 10^6 cells.

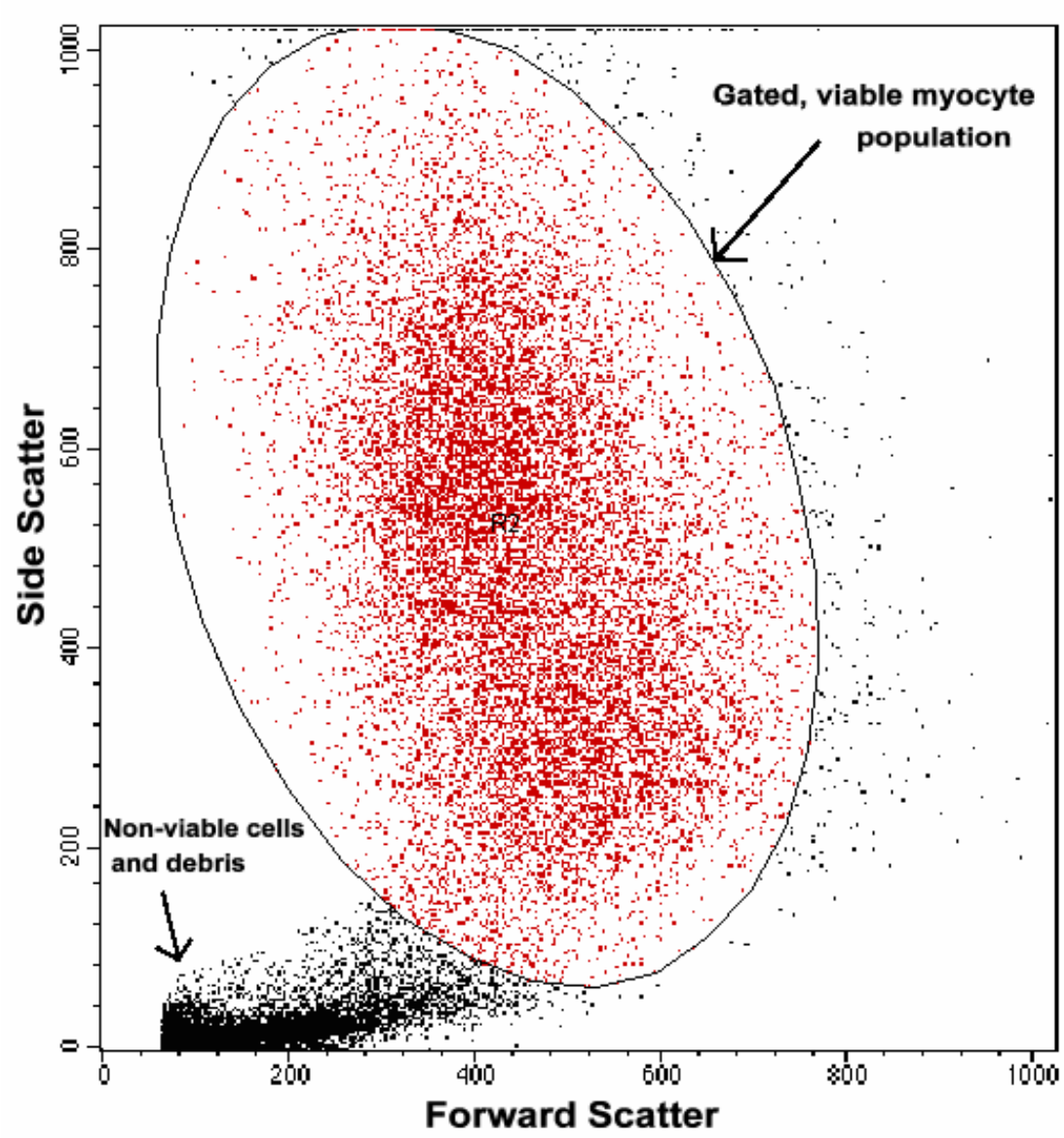


Fig. 4.2 Representative flow cytometry dot plot of a myocyte suspension showing the spread of the total recorded "events" (cells, particles and debris) calculated by their forward and side light scatter. The red eclipse-shaped area represents the gated cell population, which is ultimately analyzed and excludes the black zone in the left bottom corner representing cellular debris and other dissolved particles that may influence overall fluorescence. In total, 50 000 "events" were counted per sample, and gated cell populations usually contained 10 000 – 15 000 myocytes. Analysis of intracellular DAF-2/DA fluorescence was performed on the gated cell populations, and recorded on a frequency histogram.

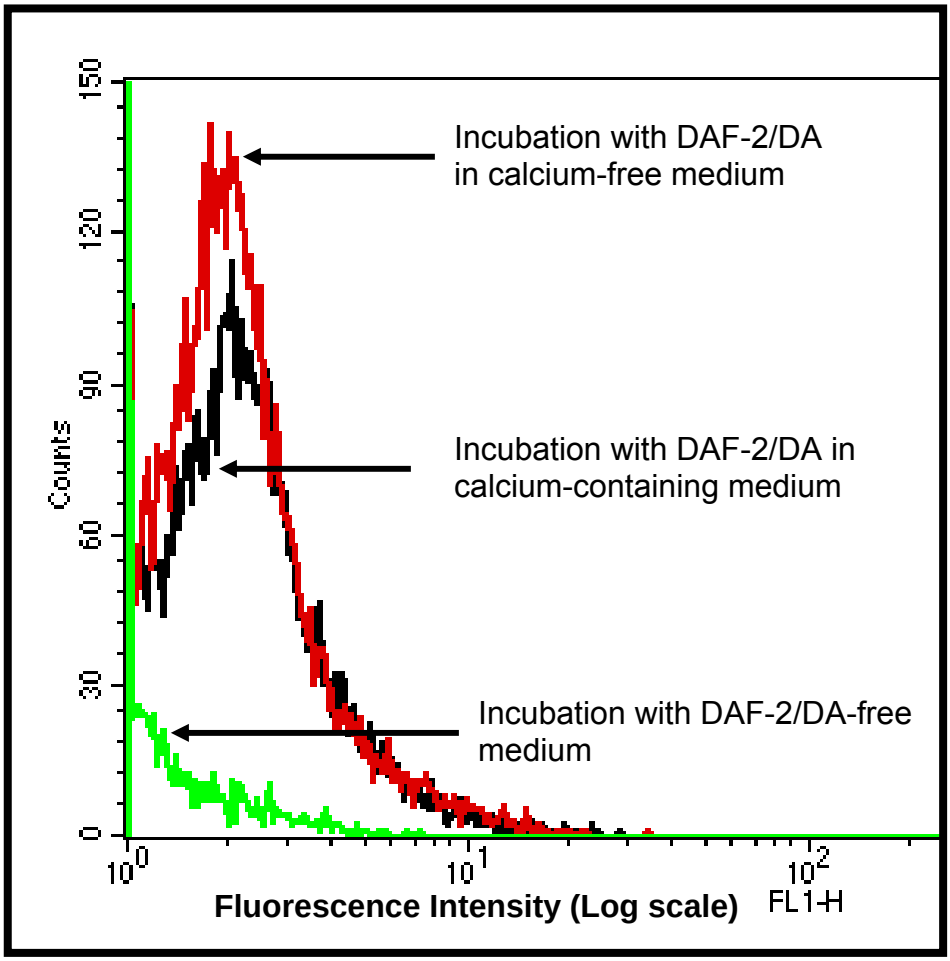


Fig. 4.3 A representative frequency histogram depicting the fluorescence intensity (log) on the x-axis (FLH-1: fluorescence channel 1 height detecting green fluorescence) and cell count on the y-axis. The black graph represents control (incubated with 10 μM DAF-2/DA in normal Ca^{2+} containing medium), the green graph cells incubated in DAF-2/DA-free buffer and the red graph cells incubated in a Ca^{2+} -free medium. Fluorescence data indicated that no significant autofluorescence was present in cells incubated without DAF-2/DA (green), and that incubating cells in Ca^{2+} -free medium had no effect on fluorescence compared to control cells that contained Ca^{2+} at physiological concentrations.

4.5 Results

(i) DAF-2/DA-fluorescence and FACS analysis (fig. 4.2 & 4.3)

FACS analysis of DAF-2/DA (10 μM) produced detectable mean baseline fluorescence in control cardiomyocytes after 180 min. Fig. 4.2 is a typical dot plot of a myocyte suspension showing the spread of the total recorded events. Cells incubated in DAF-2/DA-free buffer showed a 5-fold attenuation in fluorescence compared to control cells in the presence of the marker. Incubation of control myocytes in calcium-free buffer had no effect on fluorescence (fig. 4.3).

(ii) NO-specificity of DAF-2/DA (figs. 4.4, 4.5 & 4.6)

A dose-dependent increase in mean fluorescence was detected in DEA/NO-treated myocytes (100, 500 and 1000 μM: 129.4 ± 11, 282.5 ± 10.5* and 343.7 ± 61.6*% of control respectively, * $P<0.05$ vs control) (fig. 4.4 & 4.5). Cyclosporine A (CsA) is known to activate NOS in cells [Navarro-Antolin *et al* 2001(a)], and our results demonstrate that baseline DAF-2/DA fluorescence was increased by 50% in the presence of 10 μM CsA (control: 100% vs CsA 10 μM: 149.9 ± 9%; $P < 0.05$; $n = 5$ / group) (fig. 4.6). Co-administration of the NOS inhibitor L-NAME (50 μM) significantly attenuated the increased fluorescence in 10μM CsA-treated cells (CsA: 149.9 ± 8.2% vs. CsA+L-NAME: 111.1 ± 7.1% of control; $P<0.05$; $n = 5$ / group) (fig. 4.6).

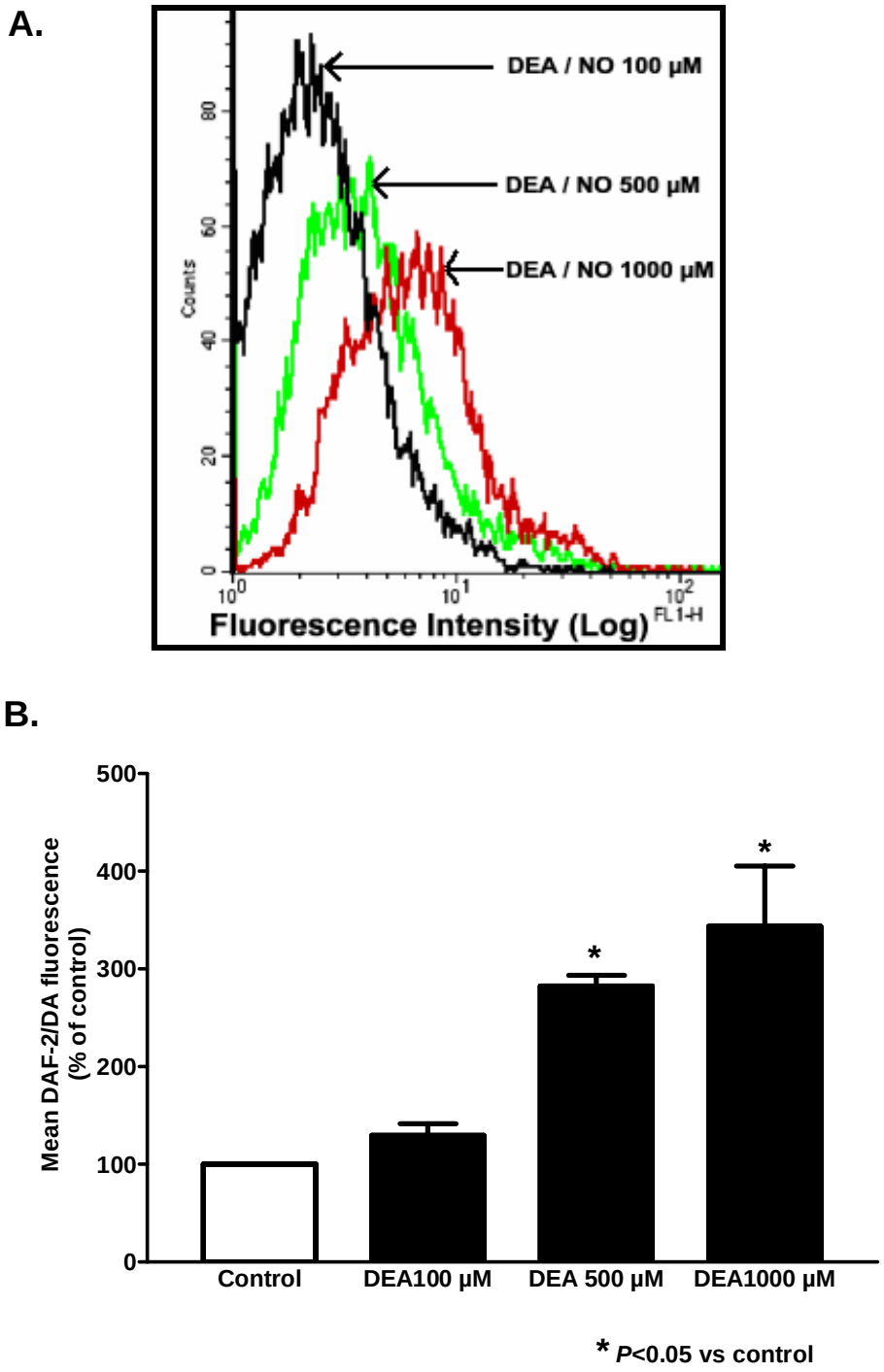


Fig. 4.4 Dose-dependent enhancement of DAF-2/DA fluorescence by the NO-donor, DEA/NO. Cells were incubated with DEA/NO for 120 min starting at t = 60 min. **(A)** A representative frequency histogram of the fluorescence intensity resulting from the administration of 100 µM (black graph), 500µM (green graph) and 1000 µM (red graph) DEA/NO respectively. **(B)** Bar chart of control and DEA/NO 100, 500 and 1000 µM fluorescence. Results are mean fluorescence expressed as % of control. DEA/NO 500 and 1000 µM enhanced mean fluorescence significantly compared to control (283% and 344% of control respectively, $P<0.05$ vs control, $n = 5$ / group).

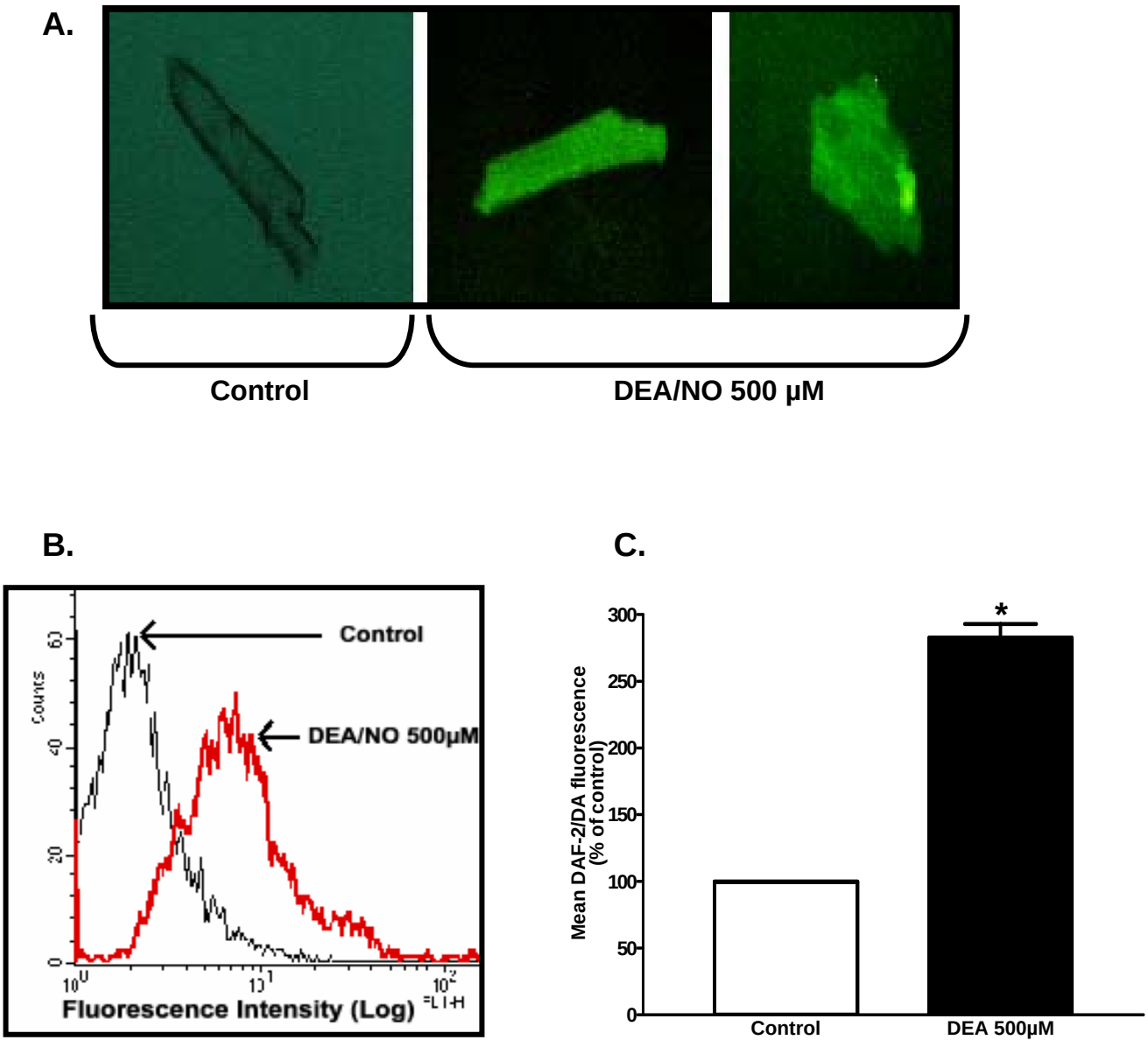


Fig. 4.5 (A) Fluorescence microphotographs of individual cardiomyocytes loaded with DAF-2/DA. The increase in the DAF-2/DA fluorescence signal is clearly visible in the myocytes treated with the NO-donor DEA / NO (500 µM) compared to the untreated control cell on the left. **(B)** Frequency histogram and **(C)** bar chart quantify the increased fluorescence observed with DEA / NO; n = 5 / group.

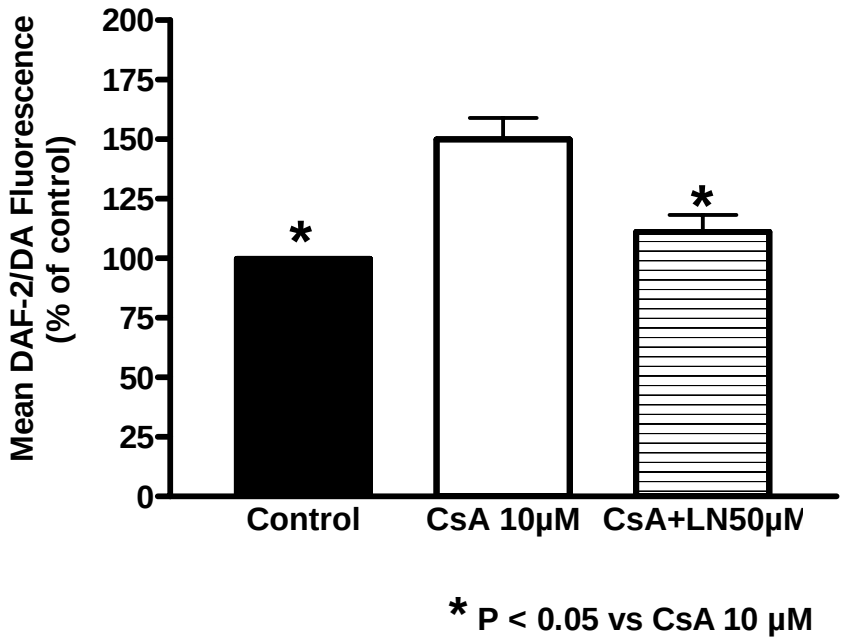


Fig. 4.6 Effect of treatment with CsA (10 μM), a known activator of NOS, and subsequent inhibition of NOS (50 μM L-NAME) in cardiomyocytes. Results demonstrate a significant increase in DAF-2/DA fluorescence in CsA-treated cells, partially reversed by NOS inhibition. (Abbreviation: LN = L-NAME) *: P<0.05 vs CsA; n = 5 / group.

(iii) Effects of hypoxia on viability and DAF-2/DA fluorescence

Myocytes subjected to 120 min hypoxia showed a 57% and 25% reduction in the percentage TBE cells and rods respectively ($P<0.05$ for both parameters; n = 10 / group). A 56.3% increase in mean DAF-2/DA fluorescence was observed in hypoxic cardiomyocytes (control: 100% vs hypoxia: 156.3 ± 5.6%; $P<0.05$, n = 15 / group) (fig. 4.7 A & C). Hypoxic myocytes incubated in DAF-2/DA-free buffer recorded a mean fluorescence of 77% of control compared to 163% in DAF-2/DA-incubated hypoxic myocytes ($P<0.05$; not shown).

(iv) Effects of NOS inhibition on DAF-2/DA fluorescence

Administration of the NOS inhibitor, L-NAME (50 μM), to control myocytes, resulted in a 16% attenuation of mean baseline fluorescence ($P<0.05$; not shown). Addition of 50 μM L-NAME to hypoxic cells caused a reduction of 30% in mean fluorescence compared to untreated hypoxic cells (untreated hypoxia: 156.3 ± 5.6% vs L-NAME-treated hypoxia: 126.4 ± 7.8%; $P<0.05$, n = 5 / group) (fig. 4.7 B & C).

(v) NO_x measurements (fig. 4.8)

Hypoxia increased NOx levels by 60% compared to control (control: 482.6 ± 42 vs hypoxia: 773.4± 107 pmoles / 10^6 myocytes; $P<0.05$) (fig. 4.8). Addition of L-NAME to hypoxic cells resulted in an attenuation of NOx to levels observed in control cells (469 ± 42 pmoles / 10^6 myocytes; $P<0.05$ vs hypoxia) (fig. 4.8). DEA/NO (500 μM) caused a 140-fold increase in NOx levels compared to control ($P<0.05$; not shown).

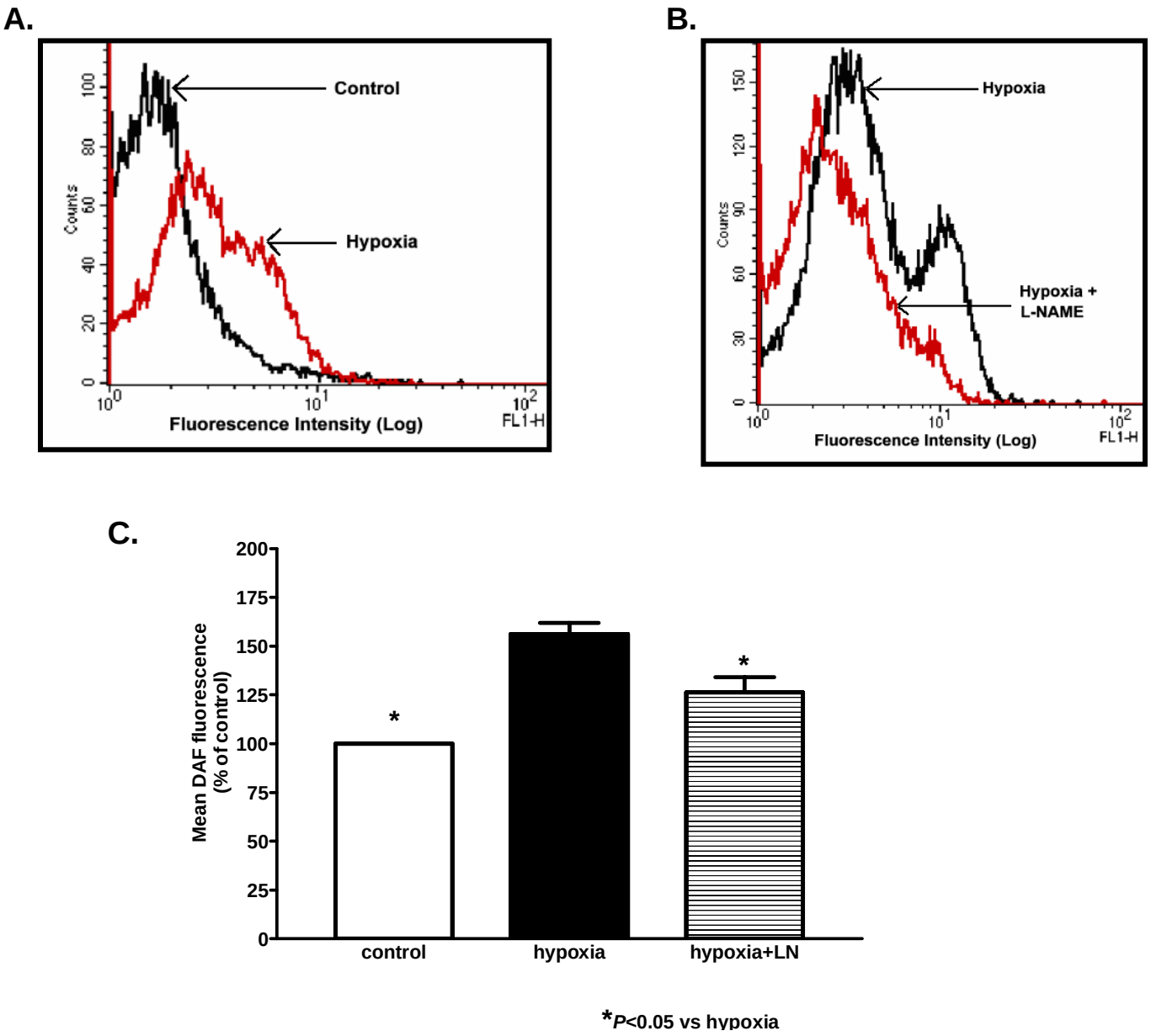


Fig. 4.7 (A) Representative frequency histogram of DAF-2/DA fluorescence observed in control (black graph) and hypoxia (red graph) myocytes. It is clear that 120 min of hypoxia resulted in enhanced fluorescence intensity compared to control. **(B)** Representative frequency histogram of hypoxia myocytes treated with 50 μM L-NAME (red graph) compared with untreated hypoxia cells (black graph), showing a clear attenuation in fluorescence intensity in the former. **(C)** Bar chart depicting the effects of hypoxia ± L-NAME compared with control. Results are expressed as mean fluorescence as a % of control. ("LN" = 50 μM L-NAME). Results show that 120 min of hypoxia enhanced DAF-2/DA fluorescence significantly, and L-NAME attenuated this effect by 30%; n = 5 – 15 / group.

4.6 Discussion

To the best of our knowledge, we have shown for the first time that FACS analysis detects NO levels in *isolated adult cardiomyocytes* incubated with the fluorescent NO-probe, DAF-2/DA. The NO-specificity of the probe in cardiomyocytes was demonstrated by the dose-dependent increase in fluorescence observed with the NO-donor, DEA/NO (fig. 4.4 & 4.5). The NO-specificity of DAF-2/DA has previously been questioned due to possible susceptibility to Ca^{2+} and light [Broillet *et al* 2001], however the authors who originally developed the probe, subsequently showed that the reaction between DAF-2 and NO was independent of Ca^{2+} and Mg^{2+}, and that the role of Ca^{2+} was rather to release NO from NO-donors [Suzuki *et al* 2002]. Our own investigations into a potential role for Ca^{2+} in DAF-2/DA fluorescence showed that Ca^{2+} had no effect (fig. 4.3). All possible precautions were taken to avoid light exposure during incubation and experimentation, however, the effect of incidental light cannot be completely excluded. In such an event, all samples would be affected equally. Freshly isolated adult cardiomyocytes, despite their shortcomings as described in Chapter 2, are physiologically superior preparations to cultured neonatal/embryonic myocytes, since the latter express an immature heart cell genotype and phenotype [Mitcheson *et al* 1998]. Furthermore, fresh cardiomyocytes are known to survive *in vitro* for a sufficient period of time (8-10 hours) [Mitcheson *et al* 1998], allowing the investigator sufficient time to study eNOS-NO metabolism.

Ischaemia has been shown to activate cardiac NOS [Depré *et al* 1997]. We investigated whether 120 min hypoxia could activate endogenous cardiomyocyte NO-production. Our FACS results indicate a significant increase in NO production

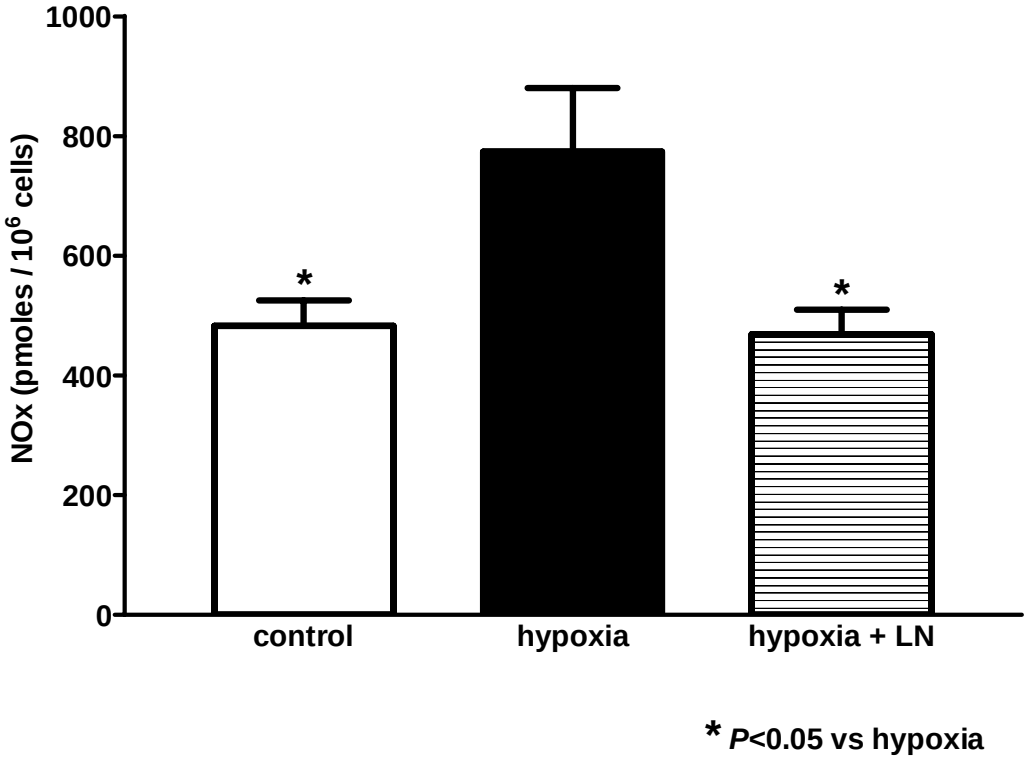


Fig. 4.8 Effects of hypoxia ± NOS inhibition on NOx (nitrates + nitrites) levels. Results show increased NOx levels after 120 min of hypoxia, reversed by the addition of L-NAME (*n* = 6 / group).

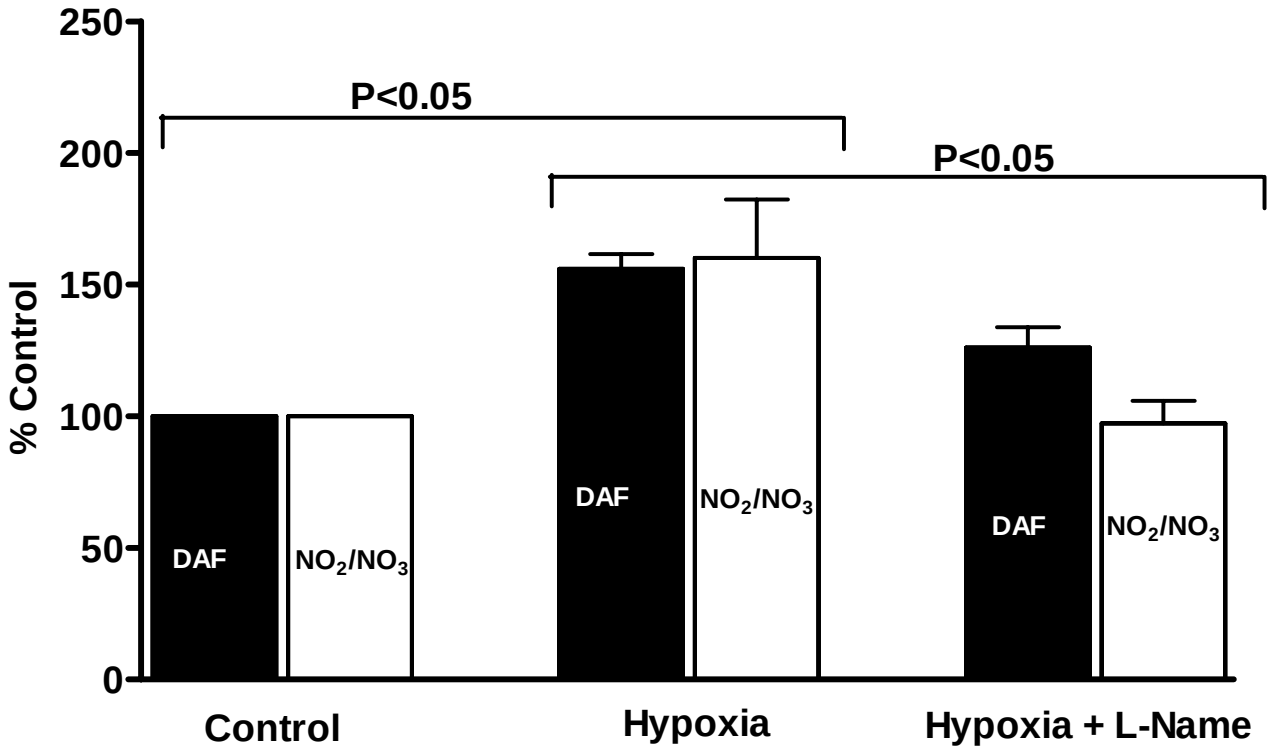


Fig. 4.9 Bar chart combining the DAF-2/DA FACS analysis and NOx data. Results of both methods are expressed as a percentage of control (control = 100%). Hypoxia results for both parameters were significantly greater than control and hypoxia + L-NAME values (DAF hypoxia and NOx hypoxia: 156 and 160% respectively; DAF hypoxia + L-NAME and NOx hypoxia + L-NAME: 126 and 97% respectively); n = 6-15 / group.

(fig. 4.7 A & C), associated by a pronounced reduction in cell viability confirming the efficacy of the protocol. Interestingly, the cardiomyocytes exhibit a bimodal pattern of mean DAF-2/DA fluorescence intensity (fig. 4.7 A & B), particularly the hypoxic cells. This is difficult to explain, but may be due to a change in the morphology of the myocytes, or that hypoxia induces a portion of the cells to develop greater fluorescence intensity. Possible autofluorescence induced by hypoxia was excluded by incubating hypoxic cells in a DAF-2/DA-free buffer. Increases in NO levels were significantly reversed by L-NAME (fig. 4.7 B & C), suggesting that the increase was due to activated cardiomyocyte NOS and therefore an endogenous mechanism. The ability of the probe to detect endogenous NO-production was further supported by the CsA data (fig. 4.6) CsA has been shown to be a potent activator of eNOS in endothelial cells [Navarro-Antolin *et al* 2001(a)]. In the present study, CsA significantly increased the DAF-2/DA fluorescence signal, and when NOS was inhibited by L-NAME, the increase was almost completely reversed, indicative of NOS activation, and therefore the probe's ability to detect changes in endogenous NO. We have previously demonstrated significant increases in cardiomyocyte cGMP (the most important second messenger of NO) induced by 120 min hypoxia [Ch. 3; Strijdom *et al* 2004(a)], supporting the present DAF-2/DA findings.

In a further attempt to validate this technique, we assayed cellular NOx levels, an acknowledged end-point for NO production [Kelm 1999], on all samples (fig. 4.8). The % changes in NOx between control, hypoxia and hypoxia + L-NAME groups was similar to observations with DAF-2/DA (fig. 4.9). This suggests that the FACS technique is at least as sensitive as NOx measurements, particularly in the case of *intracellular* control and hypoxia-induced, NOS-activated NO generation. It can

therefore be concluded that the 60% increase in hypoxia-induced DAF-2/DA fluorescence represents an intracellular release of ~ 300 pmoles NOx / 10^6 myocytes. A similar conclusion can be drawn from the respective hypoxia + L-NAME findings.

Attempts to use known concentrations of NO-donors and measurements of the NOx they release, as possible standards to calibrate fluorescence readings were unsuccessful. Increases in DEA/NO and SNP-induced (not shown) NOx generation over controls were 139- and 35.4-fold respectively, compared to 2.4 and 1.6 in FACS studies. Discrepancies between the results of the two detection methods can be explained by the fact that the NOx assay does not distinguish between intracellular and extracellular NOx, whereas FACS analyzes intracellular NO only. The investigator has limited control over the extent to which donors release NO in the extracellular compartment, particularly in the case of DEA/NO, known to spontaneously release NO on dissolution in aqueous media [Keefer 1998].

In summary, our findings suggest that the DAF-2/DA FACS analysis method can be regarded as an independent and validated technique that detects and measures ***intracellular*** NOS-activated (i.e. endogenous) NO-production in isolated cardiomyocytes. The application of this technique in isolated myocytes can help to elucidate the complex nature of intracellular NO actions, both in physiological and pathophysiological (i.e. hypoxia / ischaemia) conditions.

[The aims, methods, data and conclusions presented in this chapter appeared in: *Strijdom H, Muller C, Lochner A. Direct intracellular nitric oxide detection in isolated adult cardiomyocytes: flow cytometric analysis using the fluorescent probe, diaminofluorescein. J Mol Cell Cardiol 2004; 37(4): 897-902*.]

CHAPTER 5

NO-PRODUCTION AND NOS REGULATION IN CARDIOMYOCYTES AND CMECs: A COMPARATIVE STUDY

5.1 Introduction

It is important at this stage to reflect in a summarized fashion on the findings of our investigations in Chapters 3 & 4 before the next series of investigations are presented. The most important findings and milestones are as follows:

A viable freshly isolated adult rat ventricular cardiomyocyte model has been established. We have also developed a method of hypoxia induction, which has been validated by 3 independent viability indices. In addition, three independent NO-detection methods (direct and indirect) have been established, *viz.* intracellular cGMP level determination, NOx measurements, and FACS analysis of DAF-2/DA fluorescence. The development of the DAF-2/DA technique in freshly isolated cardiomyocytes signifies a novel NO-detection method in the field of basic cardiovascular research. We have established that a sustained period of hypoxia increases NO-production in the cardiomyocytes as measured by the three detection techniques described above and that the observed increase in NO-production is likely to be derived from endogenous NOS-activation. However, the evidence for this conclusion is mostly indirect, and the NOS isoforms involved remain unknown.

Although we were successful in the establishment of an early IP protocol that elicits protection against sustained hypoxia, our findings suggest that in our model of isolated cardiomyocytes, NO does not seem to be involved as a trigger or mediator of early IP-protection. We could also not demonstrate a triggering or mediating role for ROS in IP-protection. In addition, it seems as if NO released during sustained hypoxia was harmful in non-preconditioned cells, while its release had no effect in

preconditioned myocytes. In view of these observations, the rest of the thesis will focus on the role of NO during hypoxia.

In this chapter, we will attempt to further characterize the phenomenon of hypoxia-induced NO-production in cardiomyocytes. The NOS isoform (-s) involved needs to be established and its regulation and activation demonstrated. Furthermore, the role of NO-derived reactive species needs more research. NO-production in cardiac microvascular endothelial cells (CMECs), the most likely nonmyocyte cellular source of NO to the cardiomyocytes, needs to be investigated in baseline and hypoxic conditions, as well as the NOS enzyme (-s) involved. The relative contributions to NO production of each cell type has not been described yet, and we will attempt to establish models and protocols in which cell-to-cell comparisons can be investigated.

As discussed in Ch. 1, studies investigating the role of NO in the heart often report contradictory effects ranging from harmful to protective [Shah & MacCarthy 2000]. An important factor that could contribute to the contradictory effects is the relative contributions of NO-producing cardiac cells to overall NO actions [Shah & MacCarthy 2000]. Furthermore, protection may be dependent on the presence of nonmyocyte (e.g. endothelial cells) sources of NO. The amount and ultimate action of NO produced by the different cardiac cell types could vary depending on NOS isoenzyme predominance, as well as the size of the respective cell type populations, for example, cardiac endothelial cells and ventricular cardiomyocytes, which collectively form the majority of NO-producing cells in the heart [Ch. 1; Brutsaert 2003; Shah & MacCarthy 2000]. However, despite extensive research, the relative importance of endothelium- and myocyte-derived NO remains to be established [Shah & MacCarthy

2000]. Cardiac endothelial cells and ventricular cardiomyocytes both express the constitutive NO-generating enzyme, endothelial nitric oxide synthase (eNOS), albeit to a relatively low degree [Brutsaert *et al* 1998; Andries *et al* 1998; Balligand *et al* 1995(b)]. Compared with other cardiac endothelial cell types, the cardiac microvessel endothelial cells (CMECs) are of greater functional relevance with regards to endothelial cell-cardiomyocyte interaction, since they are in close proximity to the myocytes, facilitating rapid passage of signaling molecules such as NO [Ch. 1; Brutsaert 2003; Shah & MacCarthy 2000; Brutsaert *et al* 1998; Andries *et al* 1998].

Studies investigating production of NO in hypoxic endothelial cells show contradictory trends [Kerkhof *et al* 2002]: whereas some have shown hypoxia-induced increases in NO production and eNOS expression [Justice *et al* 2000; Xu *et al* 1995], others have shown decreased eNOS mRNA levels and cGMP production [McQuillan *et al* 1994]. In hypoxic cardiomyocytes, data seem to be more consistent. We have previously shown that hypoxic ventricular cardiomyocytes produce more intracellular NO than oxygenated control cells [Ch. 3 & 4; Strijdom *et al* 1994(a); Strijdom *et al* 1994(b)], a trend also observed by others [Depré *et al* 1997; Kitakaze *et al* 1995].

The contention that cardiac endothelial cells produce larger physiological (baseline) amounts of NO than cardiomyocytes is largely based on (indirect) eNOS-labeling and -expression studies [Brutsaert 2003; Shah & MacCarthy 2000; Brutsaert *et al* 1998; Andries *et al* 1998]. However, to the best of our knowledge, no evidence exists of studies that have compared actual physiological (basal) NO production in cardiac endothelial cells and cardiomyocytes by *direct measurements of intracellular NO* concentrations. Furthermore, studies investigating cardiomyocyte and CMEC NOS

(eNOS and iNOS) regulation during hypoxia are lacking [Jung *et al* 2000; Shah & MacCarthy 2000]. Hypoxia as a putative activator of eNOS is a case in point, and needs further investigation since there is no evidence of studies that directly compare NO production and the role of eNOS in these cell types during hypoxia. CMECs are thought to demonstrate the lowest eNOS expression of all cardiac endothelial cell types [Brutsaert 2003; Shah & MacCarthy 2000; Brutsaert *et al* 1998], and it would be interesting to compare their NO production with that of their ventricular cardiomyocyte neighbours, also thought to express low levels of eNOS. Direct eNOS expression studies in CMEC are lacking, which may be the result of possible eNOS down-regulation during culture *in vitro*, as reported by some authors [Lang *et al* 1999; Balligand *et al* 1995(a)].

The inducible isoform of NOS, iNOS, has also been described in both endothelial cells [Balligand *et al* 1995(a)] and cardiomyocytes [Balligand *et al* 1994], and its contribution to NO-production in the heart has been discussed earlier [Ch. 1]. Although several studies demonstrated increased iNOS expression under hypoxic conditions in cell types such as macrophages [Melillo *et al* 1995] and pulmonary endothelial cells [Palmer *et al* 1998], few have investigated the effects of hypoxia on iNOS in cardiomyocytes and CMECs. In a study on neonatal rat cardiomyocytes increased iNOS expression was observed after exposure to hypoxia, and data suggested that hypoxia inducible factor-1 (HIF-1) seemed to be the regulating factor for iNOS gene expression [Jung *et al* 2000].

Therefore, in this chapter, we aimed to test the hypothesis that CMECs produce more intracellular NO than cardiomyocytes during baseline and hypoxic conditions. A

protocol was designed that directly measured and compared intracellular NO production in isolated ventricular cardiomyocytes and CMECs using flow cytometric analysis of diaminofluorescein (DAF-2/DA), a NO-specific fluorescent probe [Kojima *et al* 1998]. In addition, we aimed to assess the subsequent intracellular production of NO's most reactive and potentially harmful metabolite, peroxynitrite ($ONOO^-$) under the same conditions. $ONOO^-$ is biologically important since it has both deleterious (many of the harmful actions of NO are mediated via $ONOO^-$ [Ferdinandy & Schulz 2003; Beckman & Koppenol 1996; Murphy *et al* 1998] and cardioprotective [Ferdinandy & Schulz 2003; Stowe & Riess 2004] effects. These findings could help explain the relative contributions of CMECs and cardiomyocytes to $ONOO^-$ production, and to what extent NO is metabolized to $ONOO^-$ in each cell type.

We furthermore aimed to measure and compare baseline and hypoxia-induced regulation of eNOS and iNOS to ascertain whether their content reflects possible differences observed with direct NO measurements in these cell types.

5.2 Experimental groups and protocols (fig. 5.1)

(i) NO-measurements in cardiomyocytes

Freshly isolated cardiomyocytes; hypoxia induced by ischaemic pelleting (fig. 5.1 A)

Oxygenated control conditions were simulated by normoxic incubation of isolated cardiomyocytes in solution D (500,000 cells / 35mm petri dish) in a standard tissue culture incubator (21% O_2, 5% CO_2, 40-60% humidity, 37°C) for 180 min. Hypoxia was simulated by the ischaemic pelleting method previously described [Ch. 2;

Strijdom *et al* 2004(a); Strijdom *et al* 2004(b)]. The protocol for the detection of NO was employed as previously described for endothelial cells [Navarro-Antolin *et al* 2001(a); Navarro-Antolin *et al* 2001(b)] and isolated cardiomyocytes [Ch. 4; Strijdom *et al* 2004(b)], with minor modifications (fig. 5.1 A). At the beginning of experiments (t = 0 min), control and hypoxic samples were loaded with non-limiting concentrations of the cell-permeable fluorescent probe diaminofluorescein-diacetate (DAF-2/DA, 10 μM in 1 ml solution D) for detection of intracellular production of NO. The probe was present throughout the experiments. Hypoxia was induced for a duration of either 120 min (at t = 60 min), or 60 min (at t = 120 min), while control samples were incubated under normoxic conditions for the same time periods. To assess the role of NOS in the 120 min hypoxia groups, L-NAME (non-selective NOS inhibitor, 50 μM), or SMT (iNOS-specific inhibitor, 100 μM and 1 mM), were administered at t = 30 min and were present for the remainder of the experiments (fig. 5.1 A). At t = 180 min, probes were washed out from all samples and cells resuspended in probe-free solution D followed by immediate FACS analysis.

NO-production in a cultured cardiomyocyte model (fig. 5.1 B)

To test whether subjecting cardiomyocytes to culture conditions (as opposed to the freshly isolated state) would influence NO-production, isolated cells were cultured in 35mm fibronectin-coated dishes in solution D under normoxic conditions for 24 h, after which the medium was removed, and cells loaded with DAF-2/DA-containing solution D for 60 min (fig. 5.1B). Subsequently, the probe was washed out, cells resuspended in probe-free medium, and analyzed by FACS. DAF-2/DA was administered at the end of the experiments, since separate investigations showed

that the incubation time in this model (24 h) was too long for sustainable production of fluorescence by the probe when administered at t = 0 min (data not shown).

Cardiomyocytes in suspension cultures; hypoxia by ↓PO_2 incubation (fig. 5.1 C)

To establish whether the NO-production observed in hypoxic cells was dependent on the nature of the hypoxia protocol, cardiomyocytes were suspended in substrate-free solution D in 35mm culture dishes with oxygenated control samples incubated under normoxic conditions in a standard tissue culture incubator, and hypoxic samples subjected to ↓PO_2 incubation (1% O_2, 5% CO_2, 40-60% humidity; 37°C) in a multi-gas tissue culture incubator (fig. 5.1 C). After 120 min hypoxic incubation, samples were removed and loaded with solution D containing DAF-2/DA for 60 min, before the probe was washed out in preparation for FACS analysis.

(ii) Peroxynitrite ($ONOO^-$) measurements in cardiomyocytes (fig. 5.1 A)

The protocol for the detection of $ONOO^-$ was employed as previously described for endothelial cells [Navarro-Antolin *et al* 2001(a); Navarro-Antolin *et al* 2001(b)] and isolated cardiomyocytes [Strijdom *et al* 2004(b)], with minor modifications (fig. 5.1 A). At the beginning of experiments (t = 0 min), control and hypoxic samples (120 min hypoxia groups) were loaded with non-limiting concentrations of the cell-permeable fluorescent probe dihydrorhodamine-123 (DHR-123, 2 μM in 1 ml solution D) for detection of intracellular $ONOO^-$ production. The probe was present throughout the experiments. At t = 180 min, the probe was washed out from all samples and cells resuspended in probe-free solution D followed by immediate FACS analysis.

(iii) NO-measurements in CMECs

CMECs isolated by trypsinization, hypoxia induced by mineral oil layering (fig. 5.1 A)

In trypsinized CMECs, the experiments were designed to match those described for the fresh isolated cardiomyocytes (Fig. 5.1 A). Briefly, for oxygenated control samples, cultured CMECs were isolated by detachment from their fibronectin-coated dishes with trypsin, as discussed in Ch. 2. Isolated CMECs were incubated in fibronectin-free petri dishes in fresh solution D (0.5×10^6 cells / 35mm petri dish) under an O_2 atmosphere as described for cardiomyocytes. Hypoxia was induced by gentle centrifugation of isolated CMECs (1000 r.p.m.) in microcentrifuge tubes, followed by removal of most of the supernatant and finally layering with mineral oil. Hypoxic samples were incubated in a tissue culture incubator (37°C).

For the detection of NO samples were loaded with 10 µM DAF-2/DA at t = 0 min. Control groups were subjected to normoxia for 180 min. In the hypoxic samples, a normoxic preincubation period was followed by the induction of hypoxia for a duration of 60 min or 120 min. NOS inhibitors were administered in an identical fashion to that described in isolated myocytes in the 120 min hypoxia group. At t = 180 min, all samples were washed and analyzed by FACS. To test whether incubation of CMECs isolated by prior trypsinization was harmful to the cells, we measured the viability and LDL-uptake in cells isolated and then resuspended in solution D for 180 min, and compared findings with those in cells cultured on fibronectin for the same time period.

Cultured CMEC model, hypoxia induced by ↓PO_2 incubation (fig. 5.1 D)

We tested whether performing the experiments in cultured CMEC would influence results by repeating the NO-detection investigations in CMECs that were not isolated by prior trypsinization (fig. 5.1 D), but retained in culture (35mm fibronectin-coated petri dish). Prior to experimentation, the 10% FBS-containing endothelial growth medium (10% EGM) was removed and substituted with serum-reduced 5% EGM in control and hypoxic samples. In oxygenated control groups, samples were incubated for 18 h under an O_2 atmosphere. Hypoxia was induced by ↓PO_2 incubation of cultured cells (1% O_2, 5% CO_2, 40-60% humidity, 37°C) for 18 h. At the end of 18 h incubation, EGM was removed and cells were loaded with 10 µM DAF-2/DA for 60 min. After the loading period, probe-containing medium was washed out and replaced with probe-free solution D before immediate FACS analysis. DAF-2/DA was administered at the end of the experiments, since separate investigations indicated that the incubation time in this model (18 h) was too long for sustainable production of fluorescence by the probe when administered at t = 0 min (data not shown).

(iv) $ONOO^-$ measurements in trypsinized CMECs (fig. 5.1 A)

At the beginning of experiments (t = 0 min), control and hypoxic samples (120 min hypoxia groups) were loaded with non-limiting concentrations of the cell-permeable fluorescent probe dihydrorhodamine-123 (DHR-123, 2 µM in 1 ml solution D) for detection of intracellular $ONOO^-$ production. The probe was present throughout the experiments. At t = 180 min, the probe was washed out from all samples and cells resuspended in probe-free solution D followed by immediate FACS analysis.

(v) NO-production in mixed-cell suspensions (fig. 5.1 E)

Isolated cardiomyocytes and trypsinized CMECs were co-suspended in solution D at a CMEC-to-cardiomyocyte cell number ratio of 1:1 (500 000 CMECs and 500 000 cardiomyocytes respectively). Prior to the co-suspension of the cells, isolated cardiomyocytes and trypsinized CMECs were incubated separately in solution D from t = 0 min to t = 120 min. In the control oxygenated groups, CMECs and myocytes were subsequently co-suspended in solution D at t = 120 min and incubated in 35 mm dishes for a further 60 min in a standard tissue culture incubator under oxygenated conditions (21% O_2; 5% CO_2; 40-60% humidity; 37°C). Hypoxia in the mixed-cell samples was induced at t = 120 min by pelleting the CMECs (centrifugation @ 1000 r.p.m.) in microcentrifuge tubes, followed by removal of the supernatant, addition of myocyte suspensions on top of the CMEC pellets, and a final centrifugation (@ 250 r.p.m.) in order to pellet the myocytes. As a last step, most of the supernatant covering the two pellets was removed and layered with mineral oil for 60 min until t = 180 min. In separate experiments, cardiomyocytes were co-incubated with *CMECs in culture* under control conditions by layering the myocyte suspension onto the cultured CMEC monolayer for 60 min after separately pre-incubating the myocytes and CMECs in solution D for 120 min.

5.3 Probe specificity

Specificity of DAF-2/DA was tested as described earlier [Ch. 4; Strijdom *et al* 2004(b)] by administration of the NO-donor, DEA/NO (see fig. 5.1 F for protocol). Incidental sensitivity of DAF-2/DA for $ONOO^-$ was evaluated by incubating DAF-2/DA-loaded myocyte suspensions with 100 µM authentic $ONOO^-$ and analyzing

fluorescence by FACS. Specificity of DHR-123 for $ONOO^-$ was tested by FACS analysis of myocyte samples loaded with 2 μM DHR-123 and subsequently incubated with increasing concentrations of authentic $ONOO^-$ (100 μM, 500 μM and 1 mM) for 120 min (fig. 5.1 F). Incidental sensitivity of the probe for NO was evaluated by incubating DHR-123-loaded myocyte samples with 500 μM DEA/NO (NO donor) and subsequently analyzing the fluorescence by FACS.

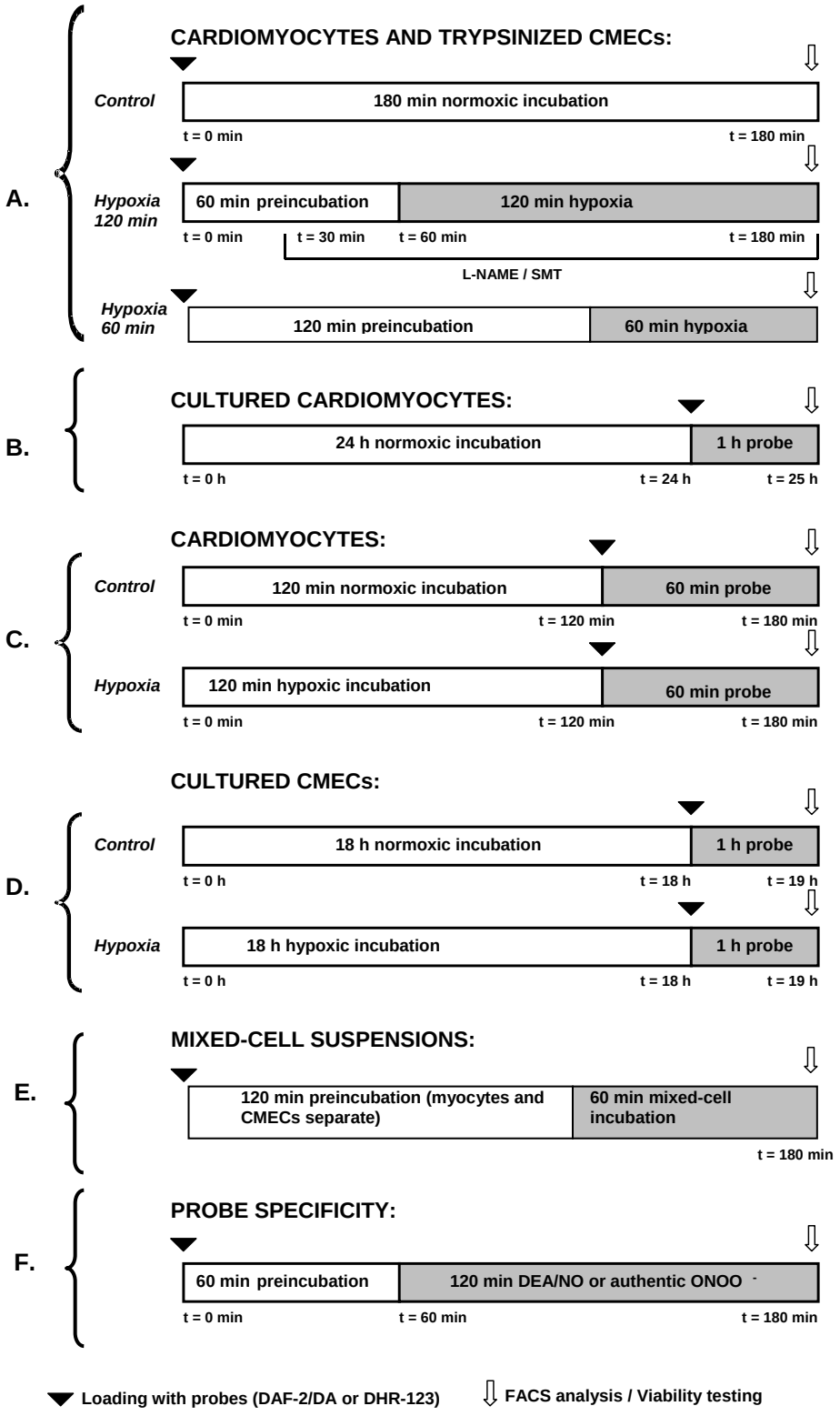


Fig. 5.1 Experimental groups and protocols for NO and $ONOO^-$ detection and viability testing (see next page for detailed legend)

Fig. 5.1 (A) Cardiomyocytes and CMECs (isolated by prior trypsinization) were incubated in substrate-containing solution D for 180 min. Control samples were incubated under a normoxic atmosphere, and hypoxia (ischaemic pelleting) was induced at *t* = 60 min or t = 120 min. Probes, 10 μM DAF-2/DA and 2 μM DHR-123, were loaded at *t* = 0 min and remained present throughout the experiments. At *t* = 180 min, samples were randomly collected for FACS analysis or viability assessment (propidium iodide or trypan blue). NOS inhibitors, 50 μM L-NAME or 100 μM and 1 mM SMT, were administered to 120 min hypoxia samples at *t* = 30 min and remained present until the end of experiments. **(B)** Cardiomyocytes were cultured in fibronectin-coated 35 mm dishes for 24 h followed by loading with DAF-2/DA for 60 min and FACS analysis. **(C)** Cardiomyocytes were incubated in substrate-free solution D for 120 min, loaded with DAF-2/DA for 60 min, followed by FACS analysis, and hypoxia induced by $\downarrow PO_2$ incubation for 2 h. **(D)** CMECs were incubated in culture for 18 h. Control samples were subjected to normoxia, and hypoxic samples by reduction of O_2 concentration to 1%. At *t* = 18 h, samples were loaded with DAF-2/DA for 1 h, followed by FACS analysis. **(E)** Cardiomyocytes and trypsinized CMECs (loaded with DAF-2/DA at t = 0 min) were preincubated separately under normoxic conditions for 120 min, followed by co-incubation (normoxic or hypoxic) for 60 min in a 1: 1 cell number ratio. **(F)** Probe specificity of DAF-2/DA and DHR-123 was assessed in myocytes by the administration of DEA/NO (100 μM, 500 μM, and 1 mM), and authentic $ONOO^-$ (100 μM, 500 μM, and 1 mM) at *t* = 60 min and present until *t* = 180 min, followed by FACS analysis.

5.4 Flow cytometry

FACS analysis of DAF-2/DA- and DHR 123-treated isolated cardiomyocytes and CMEC was performed as described previously [Ch. 4; Strijdom *et al* 2004(b); Navarro-Antolin *et al* 2001(b)]. Both the DAF-2/DA and DHR-123 fluorescence signals were recorded in the FL-1 channel. For PI-uptake viability tests [Ch. 2; Navarro-Antolin *et al* 2001(b)], cells were analyzed by FACS in the FL-2 channel. Unless stated otherwise, all FACS data are expressed as mean fluorescence intensity (percentage of control).

5.5 Cell viability tests

In both cardiomyocytes and CMECs, hypoxia-induced cell damage was evaluated by FACS analysis of the % increase in mean fluorescence of cells absorbing propidium iodide, based on the method previously described [Ch.2]. In the cardiomyocytes, the trypan blue exclusion test was used as a second indicator of viability based on a method previously described for myocytes [Ch.2-4].

5.6 Western Blot analyses of eNOS and iNOS (fig. 5.2)

The amount of total and phosphorylated (Ser 1177) eNOS in both cell types (respective sample collection time points shown in Fig. 5.2) was determined by Western blotting. In samples exposed to 120 min hypoxia and the cultured CMEC groups (fig. 5.2 A & B), cells were lysed in a buffer containing in (mM): Tris 20; p-nitrophenyl phosphate 20; EGTA 1; NaF 50; sodium orthovanadate 0.1; phenyl-methyl sulphonyl fluoride (PMSF) 1; dithiotreitol (DTT) 1; aprotinin 10 µg/ml; leupeptin 10 µg/ml and 1% Triton-X100. For the Western blots of the isolated

cardiomyocyte and trypsinized CMEC groups exposed to 60 min hypoxia (fig. 5.2 A & C), a lysis buffer containing (in mM): Tris 20; EGTA 1; EDTA 1; NaCl 150; ß-Glycerophosphate 1; sodium orthovanadate 1; tetra-sodium diphosphate 2.5; PMSF 1; 0.1% Sodium dodecylsulfate (SDS); aprotinin 10 µg / ml; leupeptin 10 µg / ml, and 1% Triton-X100 was used. After sonication, cell lysate protein (40 µg) was loaded on 7.5 or 8% SDS-polyacrylamide gel and transferred onto nitrocellulose. After Western blotting, membranes were probed with the respective rabbit polyclonal antibodies (Cell Signaling Technology). The secondary antibody was horseradish peroxidase-linked anti-rabbit IgG (Amersham). INOS determinations in isolated cardiomyocytes and trypsinized CMECs were done by using the cell lysis procedure as described above. Primary anti-iNOS rabbit polyclonal antibodies (BD Transduction Laboratories) were used, and the secondary antibody was anti-mouse HRP-linked (Upstate Cell Signalling). The immunoreaction for all samples was visualized using the ECL™ system, and films were densitometrically analyzed (UN-SCAN-IT, Silk Scientific, Orem, UT, USA).

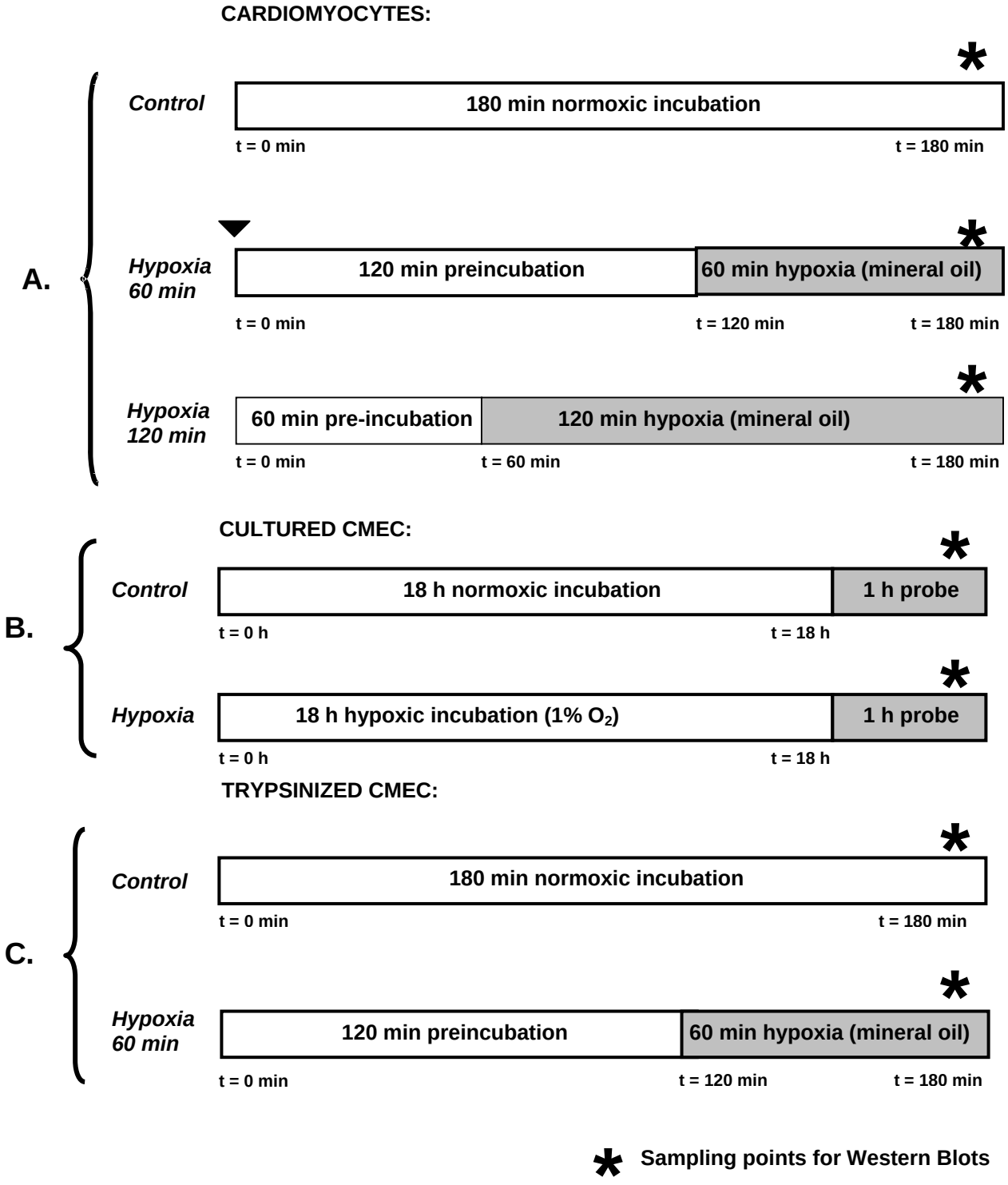


Fig. 5.2 Experimental groups and protocols for eNOS and iNOS determinations. See text for detailed description

5.7 Results

(i) Cell viability (fig. 5.3)

Hypoxia-induced cell injury was tested by evaluating changes in cell viability. Two viability tests were used, viz. trypan blue exclusion and propidium iodide staining [Ch. 2]. Unless stated otherwise, all data are given as the percentage of control (control adjusted to 100%). In isolated cardiomyocytes the % viable TBE cells decreased from 54% to 46.2±2% (P<0.05; n=10) after 120 min of mineral oil-induced hypoxia. Similarly, 120 min hypoxia increased the nonviable PI-staining cardiomyocytes by 59.3% (increased mean PI fluorescence to 159.3±8.8% of control, P<0.05; n=11). In cardiomyocytes exposed to *60 min* hypoxia a loss of 20% viable, trypan blue excluding cardiomyocytes was observed (control: 100% vs. hypoxia: 80.5 $\pm$ 1.3; p < 0.05; n = 4 / group) and a 30% increase in non-viable PI-fluorescence intensity was observed (100% vs. hypoxia: 129.8 $\pm$ 6.3%; p < 0.05; n = 8 / group) (Fig. 5.3 A & B). When the hypoxia protocol was changed to 120 min $\downarrow PO_2$ incubation (1% O_2 atmosphere), a 26% reduction of % viable TBE cells was observed (73.6±5% compared with control, P<0.05; n=3).

In CMECs isolated by prior trypsinization, 120 min hypoxia induced by ischaemic pelleting reduced % viable TBE cells to 84.2 ± 3.8% of control (P<0.05; n=6). Trypsinized CMECs exposed to 60 min hypoxia induced by ischaemic pelleting demonstrated 18% increase in PI fluorescence (control: 100% vs. hypoxia: 117.7 $\pm$ 3.7; p < 0.05; n = 5 / group) (fig. 5.3 F). When the hypoxia protocol was changed to 18 h of $\downarrow PO_2$ incubation of CMECs in culture, the percentage of non-viable PI-

staining cells increased to 121.5 ± 7.2% of control (P<0.05; n=5) (fig. 5.3 E). These data indicate that 60 min hypoxia (ischaemic pelleting) of trypsinized CMECs achieved the same degree of injury within a much shorter time than ↓ PO_2 incubation of cultured CMECs (18 h). These results also suggest that CMECs were less vulnerable to hypoxic injury than the cardiomyocytes. Finally, we tested whether isolation of CMECs by prior trypsinization had any effect on CMEC viability compared with CMECs in culture (both groups under control, oxygenated conditions). The viability as measured by actual PI fluorescence readings remained unchanged in the two groups (cells isolated by trypsinization 1.24±0.08, n= 5, and cultured cells: 1.22±0.14, P>0.05, n= 8). The ability of CMECs to take up fluorescence-labeled LDL was also not affected by the isolation procedure compared with cultured CMECs (data not shown).

(ii) Probe specificity (fig. 5.4)

The NO donor, DEA/NO, was administered in increasing concentrations to isolated myocytes preloaded with 10 μM DAF-2/DA as described earlier [Ch. 4]. The dose-response curve of DEA/NO vs. mean fluorescence intensity measurements by FACS analysis is shown in fig. 5.4 A. A step-wise increase in fluorescence was observed: DEA/NO 100 μM, 1.4-fold increase (142±5% of probe only); 500 μM, 2.6-fold increase (259±19%); and 1 mM, 3.2-fold increase (316±38%) (P<0.05 vs. probe only). These results are in agreement with the findings presented in Ch. 4. Incidental sensitivity of DAF-2/DA for $ONOO^-$ was tested by incubating probe-containing samples with 100 μM authentic $ONOO^-$ and comparing the fluorescence with that obtained with 100 μM DEA/NO (see graph inset fig. 5.4 A).

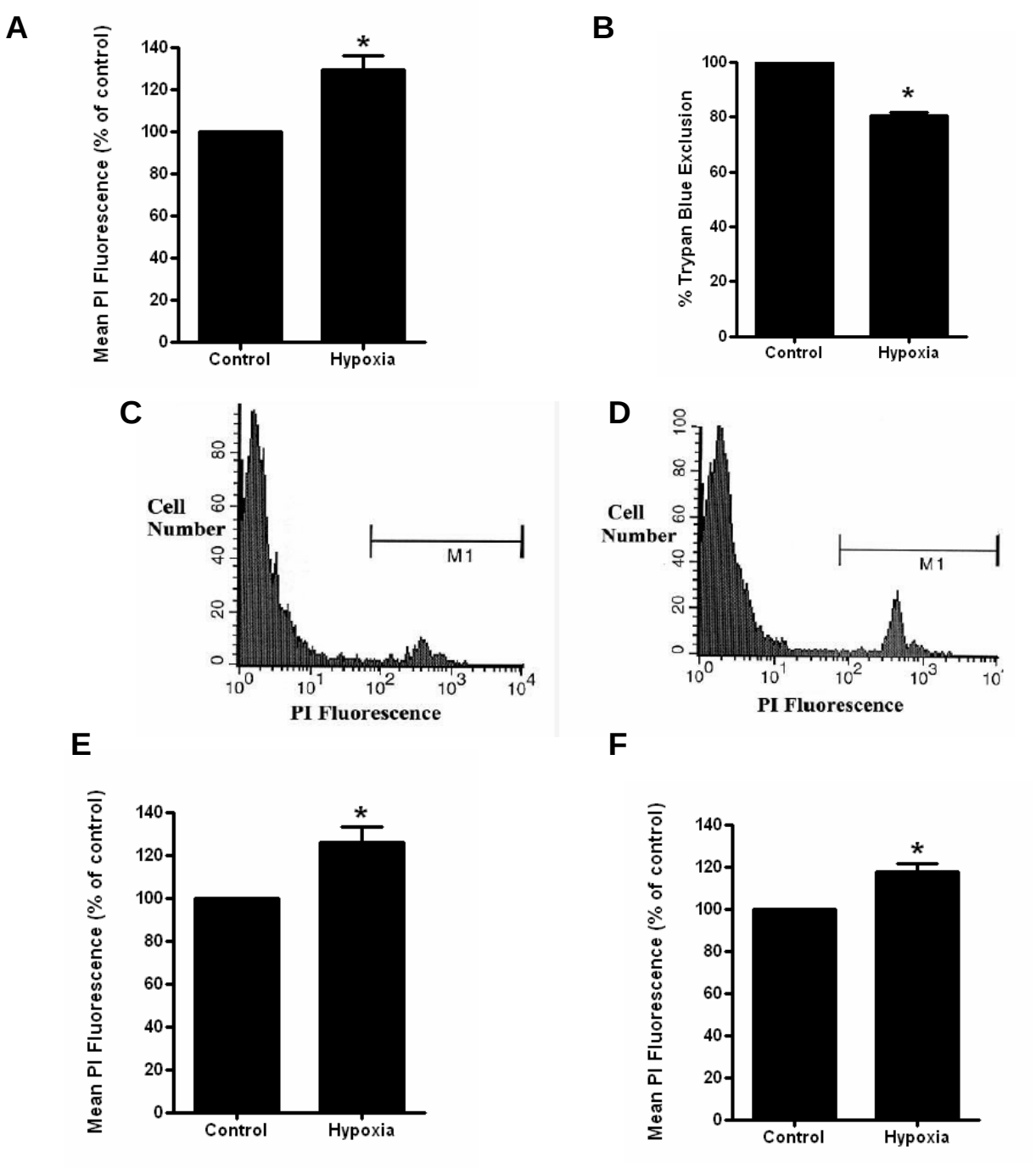


Fig 5.3 Cell viability results. **(A)** Bar chart showing mean PI fluorescence in control and hypoxic cardiomyocytes exposed to 60 min hypoxia induced by ischaemic pelleting. *:$p < 0.05$; n = 8 / group. **(B)** Trypan Blue Exclusion (% of control) in control and 60 min hypoxic cardiomyocytes. * $p < 0.05$; n = 4 / group. **(C)** Representative histogram depicting PI fluorescence intensity in control CMECs. The fluorescence peak on the right, gated by M1, represents cells that have absorbed PI (non-viable population). **(D)** Histogram of PI fluorescence in hypoxic CMECs showing increased population of PI-positive cells. **(E)** PI fluorescence in cultured CMECs (18 h control and ↓ PO_2 hypoxia). * $p < 0.05$; n = 4 / group. **(F)** PI fluorescence in trypsinized CMEC (control and 60 min mineral oil hypoxia). * $p < 0.05$; n = 5 / group.

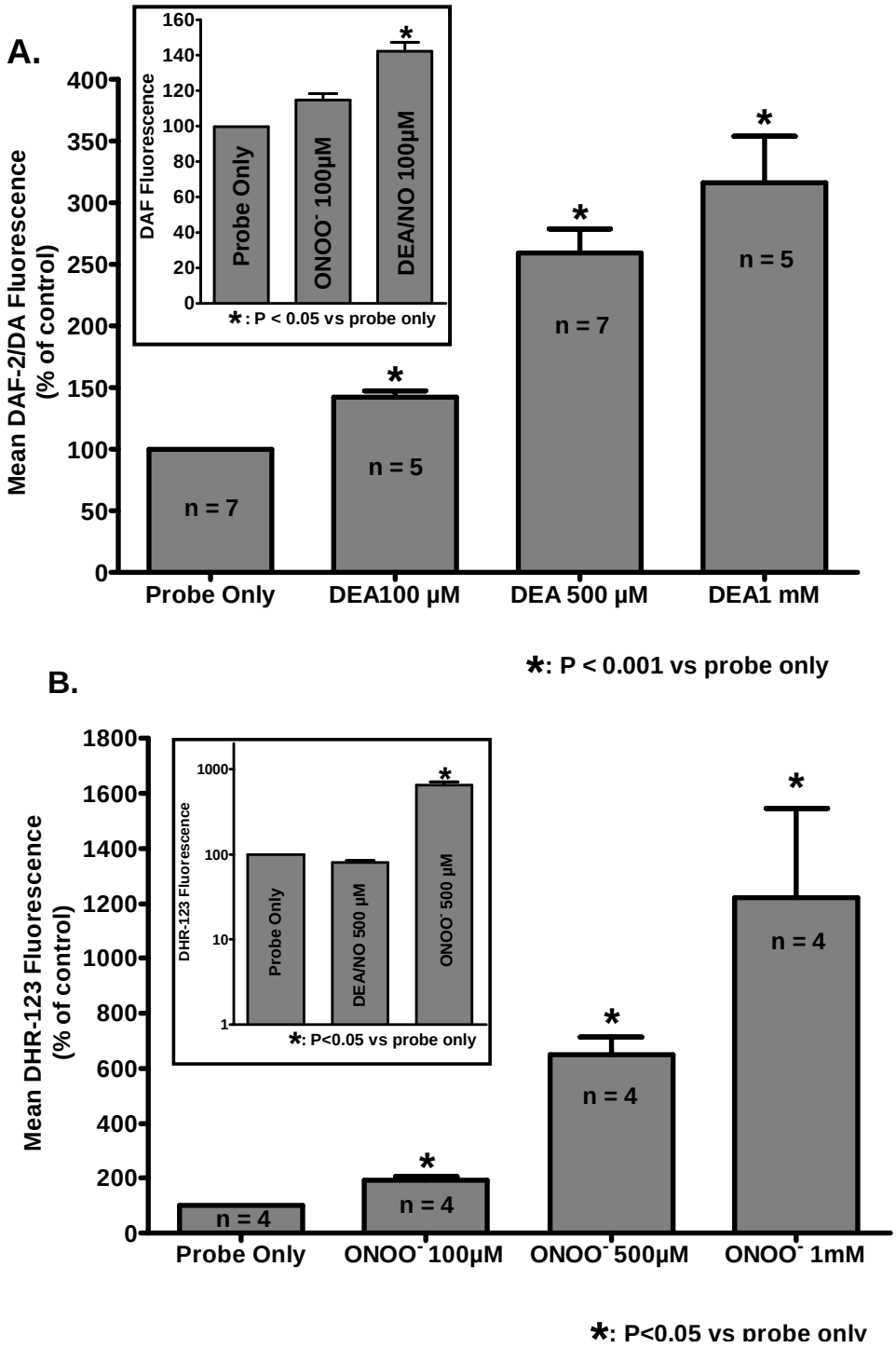


Fig. 5.4 (A) Specificity of DAF-2/DA for NO tested by incubating cardiomyocytes with increasing concentrations of the NO donor, DEA/NO (100 µM, 500 µM, and 1 mM) for 120 min. In the inset, incidental sensitivity of the probe for authentic $ONOO^-$ (100 µM) was tested, and fluorescence was compared with 100 µM DEA/NO. **(B)** Dose-response effect tested in cardiomyocytes preloaded with DHR-123 followed by 120 min incubation with authentic $ONOO^-$ (100 µM, 500 µM, and 1 mM). In the inset, incidental sensitivity of DHR-123 for 500 µM DEA/NO was tested, and fluorescence was compared with 500 µM authentic $ONOO^-$.

No significant enhancement of fluorescence was observed with authentic $ONOO^-$. To test probe specificity of DHR-123 for $ONOO^-$, we incubated cardiomyocyte samples preloaded with the probe with increasing concentrations of authentic $ONOO^-$ (fig. 5.4 B). A step-wise enhancement of mean DHR-123 fluorescence intensity was observed (compared with probe-only control samples adjusted to 100%): $ONOO^-$ 100 μM, 1.9-fold increase (192.5±12% of probe only); 500 μM, 6.5-fold increase (652±60%); and 1 mM, 12.2-fold increase (1223±321%) (P<0.05 vs. probe only). We also tested whether incubation with 500 μM DEA/NO would result in enhanced DHR-123 fluorescence. The graph inset (Fig. 5.4 B) shows that DHR-123 fluorescence was unaffected by the NO donor.

(iii) DAF-2/DA and DHR-123 fluorescence in cardiomyocytes (figs 5.5 & 5.6)

In cardiomyocytes, the induction of hypoxia by ischaemic pelleting for 120 min resulted in a 1.6-fold increase in DAF-2/DA fluorescence compared with control (155.6±4.6%; P<0.05; n=11; fig. 5.5 A). Co-incubation of hypoxic samples with the NOS inhibitors partially reversed fluorescence intensity: 50 μM L-NAME to 114 ± 6.1%* (n=8), 100 μM SMT to 136.1 ± 4.3%* (n=4), and 1 mM SMT to 126.4 ± 3.8%* (n=4) respectively (*:P<0.05 vs. hypoxia) (fig. 5.5 A). Changing hypoxia to 60 min ischaemic pelleting resulted in a 1.4-fold increase in DAF-2/DA fluorescence (control: 100% vs. hypoxia: 140.3 ± 4.6%; $p < 0.05$; $n = 8$ / group) (fig. 5.5 B). We tested whether the ability of cardiomyocytes to produce NO under baseline conditions would be affected by placing the cells in culture, as opposed to analyzing cells in the freshly isolated state. Cardiomyocytes were cultured for 24 h in fibronectin-coated dishes under normoxic conditions, after which DAF-2/DA fluorescence was measured.

Results showed that there was no difference in actual baseline DAF-2/DA fluorescence readings from those obtained in fresh cells in suspension (data not shown). Furthermore, to test whether the enhancement of fluorescence by hypoxia was a result of hypoxia *per se*, rather than the specific protocol used, studies were repeated in an alternative hypoxia protocol in which the PO_2 of the incubator was lowered to 1% O_2 ($\downarrow PO_2$ hypoxia) for 120 min. This model of hypoxia resulted in increased DAF-2/DA fluorescence to 109 ± 1.3% of control ($P<0.05$; $n=7$; fig. 5.5 C). These findings indicate that mineral oil-induced hypoxia was more effective in increasing NO production compared with control than hypoxia induced by $\downarrow PO_2$. In the last set of cardiomyocyte fluorescence studies, we measured DHR-123 fluorescence in oxygenated control and mineral oil-induced hypoxic cells. Results showed that 2 h of hypoxia resulted in a significant reduction in fluorescence (61.2±2.5%; $P<0.05$; $n=9$) compared with control cells (fig. 5.6).

In these studies, the focus was on the ability of the drugs to alter the production of NO and the possible effects of the inhibitors on cell viability were regarded not relevant.

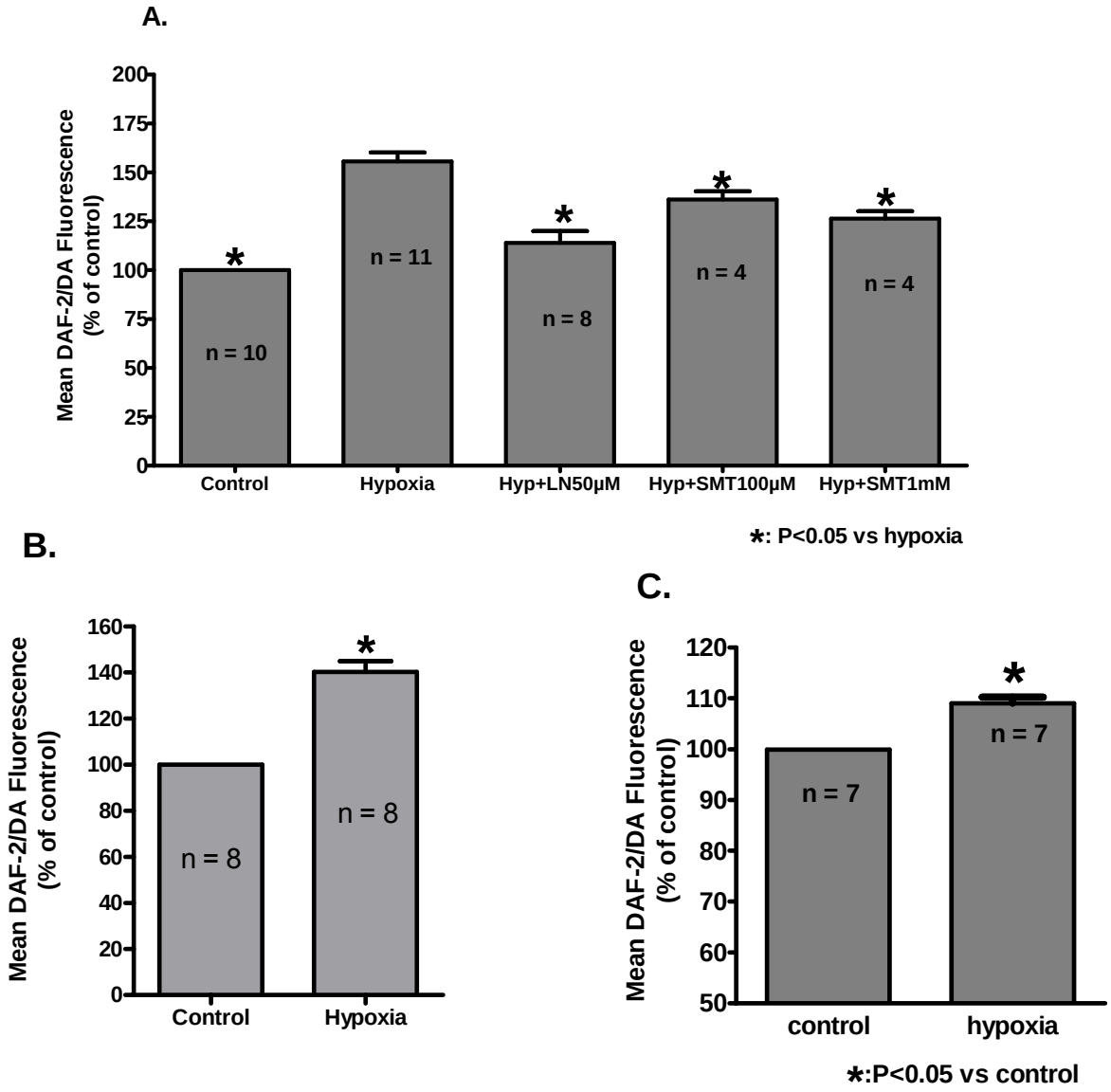


Fig. 5.5 DAF-2/DA fluorescence in cardiomyocytes. **(A)** Subjecting cardiomyocytes to 120 min hypoxia induced with ischaemic pelleting resulted in enhanced mean DAF-2/DA fluorescence intensity. NOS inhibition with 50 μM L-NAME, or 100 μM and 1 mM SMT during hypoxia reversed these effects, indicative of NOS activation by hypoxia in myocytes. **(B)** 60 min ischaemic pelleting hypoxia also resulted in increased DAF-2/DA fluorescence. **(C)** Induction of a different model of hypoxia in cardiomyocytes also resulted in enhanced DAF-2/DA fluorescence, albeit not to the same extent as that observed in mineral oil-treated cells. In this model of hypoxia, cardiomyocytes were suspended in substrate-free solution D for 120 min under a hypoxic atmosphere (1% O_2 concentration). Abbreviations: Hyp, hypoxia; LN, L-NAME.

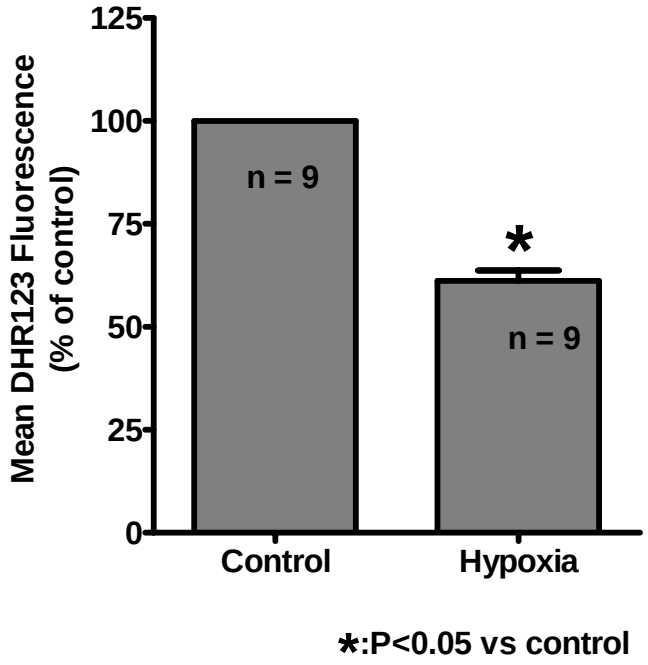


Fig. 5.6 DHR-123 fluorescence in cardiomyocytes. Induction of hypoxia by 120 min ischaemic pelleting resulted in attenuated DHR-123 fluorescence in cardiomyocytes compared with oxygenated control.

(iv) DAF-2/DA and DHR-123 fluorescence in CMECs (figs. 5.7 & 5.8)

Ischaemic pelleting-induced hypoxia (120 min) in CMECs isolated by prior trypsinization increased DAF-2/DA fluorescence 3.3-fold (331.5±43% of control; $P<0.05$; $n=12$; fig. 5.7 A). Pre-administration of 50 μM L-NAME, but not SMT, to hypoxic cells attenuated fluorescence from 331% to 223.2±17.6% of control ($P<0.05$ vs. hypoxia) (fig. 5.7 A). Exposure of trypsinized CMECs to 60 min of ischaemic pelleting hypoxia resulted in 1.55-fold increase in the DAF-2/DA signal (control: 100% vs. hypoxia: 155.1 ± 7%; $p < 0.05$; $n = 10$ / group) (fig. 5.7 B). To test whether the increased fluorescence observed in hypoxic isolated CMECs was due to the hypoxic insult *per se*, and not methodological considerations, we measured DAF-2/DA fluorescence in a separate series in which cultured CMECs were subjected to $\downarrow PO_2$ hypoxia incubation for 18 h. Results indicate that in this model of hypoxia, mean fluorescence intensity increased 1.4-fold over control (141±5%; $P<0.05$; $n=6$; Fig. 5.7 C). These findings suggest that hypoxia by ischaemic pelleting in trypsinized, isolated CMECs induced relatively more NO production over baseline than hypoxic incubation of cultured CMECs after 18 h. The effect of 120 min hypoxia (induced by ischaemic pelleting in isolated CMECs) on DHR-123 fluorescence is shown in fig. 5.8. Hypoxia attenuated mean DHR-123 fluorescence intensity to 78.7 ± 3% of control ($P<0.05$; $n=7$).

In these studies, the focus was on the ability of the drugs to alter the production of NO and the possible effects of the inhibitors on cell viability were regarded as not relevant.

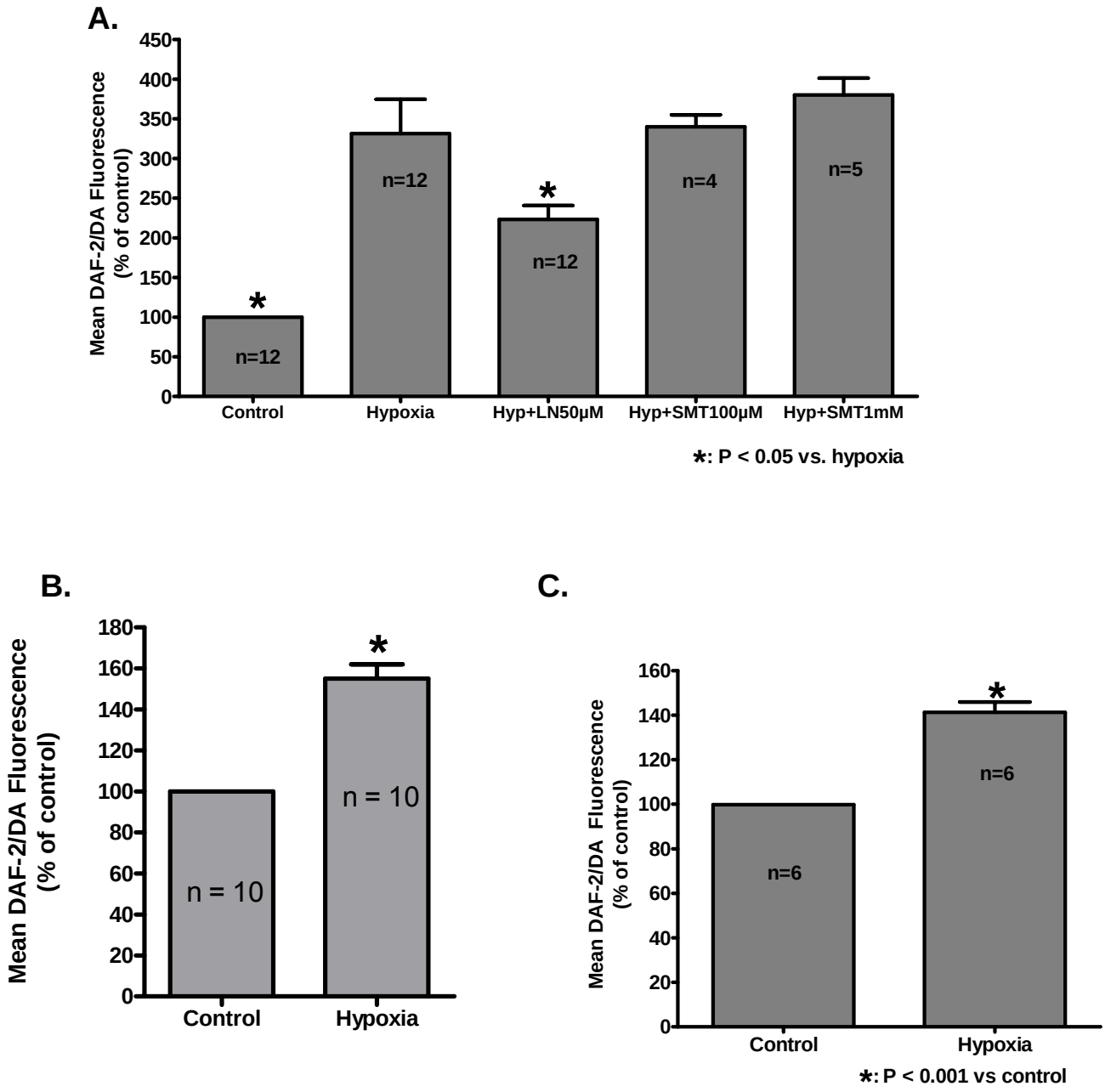


Fig. 5.7 DAF-2/DA fluorescence in CMECs. **(A)** Subjecting CMEC isolated by prior trypsinization to 120 min hypoxia induced with ischaemic pelleting resulted in enhanced mean DAF-2/DA fluorescence intensity. NOS inhibition with 50 μM L-NAME, but not 100 μM or 1 mM SMT, during hypoxia reversed these effects, indicative of a degree of NOS activation by hypoxia in CMECs. **(B)** Trypsinized CMECs exposed to 60 min of ischaemic pelleting hypoxia also increased the DAF-2/DA fluorescence signal. (**C)** Induction of a different model of hypoxia in CMECs also resulted in enhanced DAF-2/DA fluorescence, albeit not to the same extent as that observed in mineral oil-treated cells. In this model of hypoxia, cultured CMECs were incubated in serum-poor EGM for 18 h under a hypoxic atmosphere ($\downarrow PO_2$ hypoxia). Abbreviations: Hyp, hypoxia; LN, L-NAME.

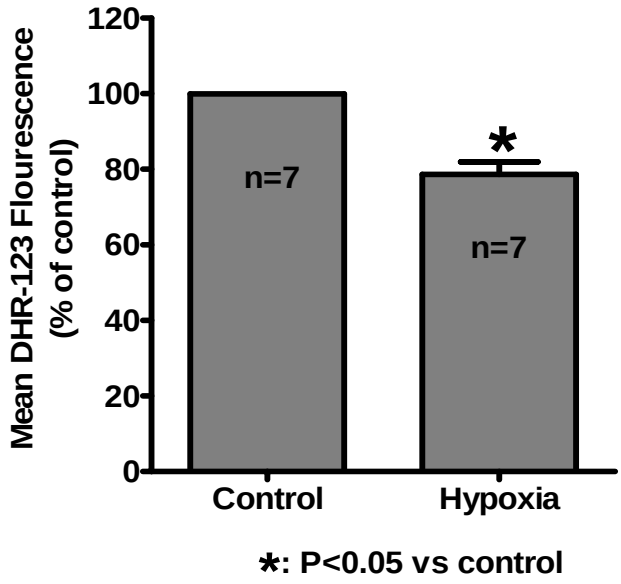


Fig. 5.8 DHR-123 fluorescence in CMECs. Induction of hypoxia by mineral oil in CMECs isolated by prior trypsinization resulted in attenuated DHR-123 fluorescence compared with oxygenated control.

(v) Direct myocyte-CMEC comparison of fluorescence data (fig. 5.9 - 5.11)

Direct comparisons of fluorescence measurements in cardiomyocytes and CMECs were made possible by selecting similar number of cells per cell type for FACS analysis and correcting for autofluorescence by subtracting background fluorescence produced by each cell type during probe-free incubation. Typical scatterplots and gated populations for subsequent FACS analysis are shown in fig. 5.9 A & B, and a histogram showing baseline mean DAF-2/DA fluorescence of myocytes and trypsinized CMECs is shown in fig. 5.9 C. In the 180 min groups (see fig. 5.1 A for the protocols), comparisons of actual DAF-2/DA mean fluorescence intensity readings showed that fluorescence signals in CMECs were 26 times stronger under control (baseline) conditions compared with cardiomyocytes (64.3±3.8 vs. 2.49±0.1; $P<0.05$). After 120 min ischaemic pelleting hypoxia, the signal was 52-fold stronger (185.1±24.1 vs. 3.55±0.3; $P<0.05$) (fig. 5.10). In the cultured cell models, CMECs produced 7 times more NO per cell than cardiomyocytes ($P<0.05$; data not shown) under baseline conditions. Similarly, when subjecting cells to the $\downarrow PO_2$ hypoxia protocol, CMEC also produced 7 times more NO per cell ($P<0.05$; data not shown). In these investigations, DAF-2/DA was administered for 1 h at the end of the experiments due to technical considerations (DAF-2/DA fluorescence is diminished with long incubation times). Data from separate investigations in which we compared 1 h probe exposure at the end of experiments to 3 h exposure demonstrated that there was no difference in the fluorescence signal (data not shown). Finally, oxygenated control DHR-123 fluorescence was 2.2 times greater in CMECs compared with cardiomyocytes (117.5±23 vs. 53.3±5.7 respectively; $P<0.05$) (fig. 5.11).

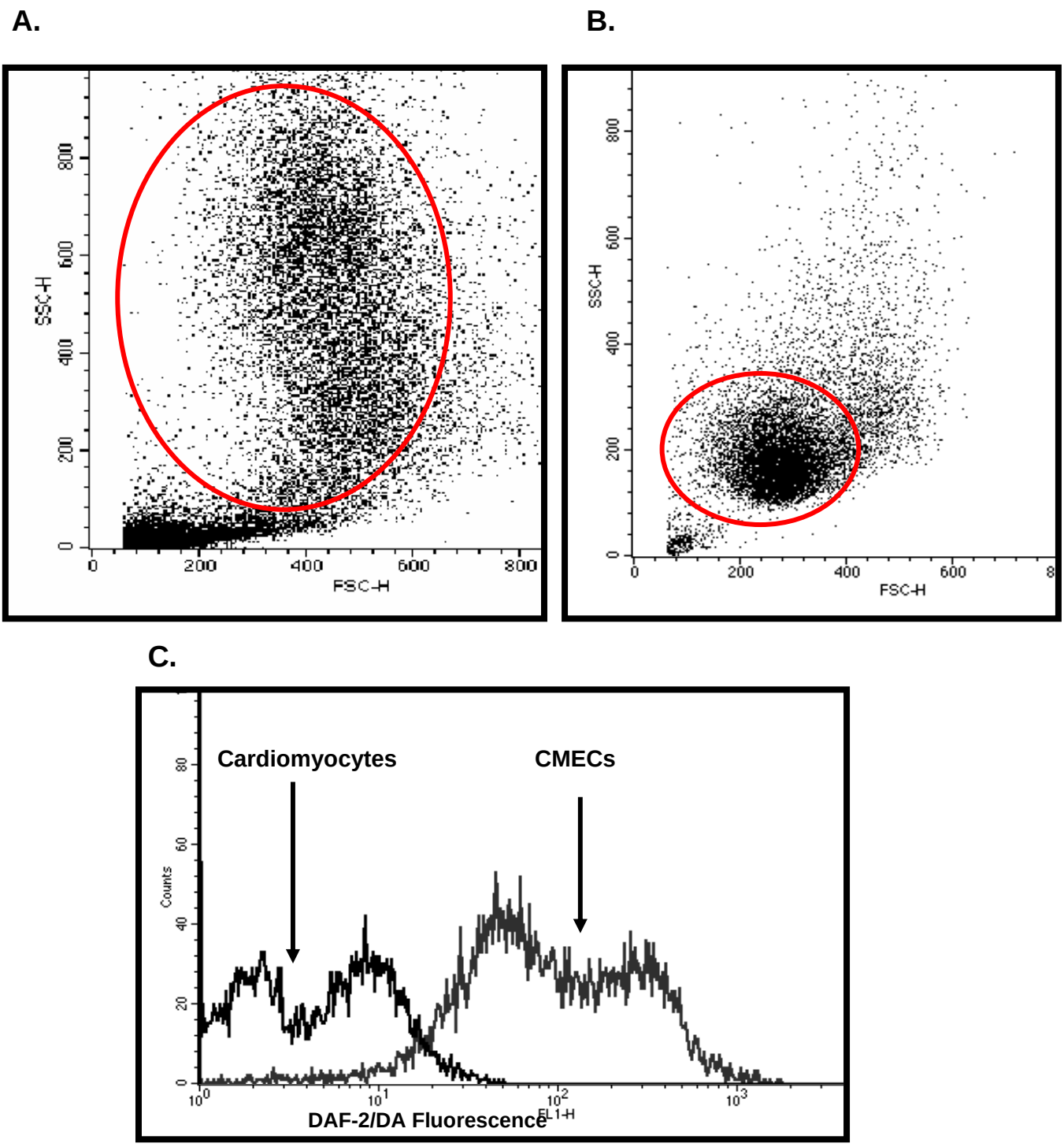


Fig. 5.9 FACS analysis data of cardiomyocytes and CMECs. (**A)** Typical scatterplot of cardiomyocytes and gated population indicated by red circle. **(B)** Scatterplot of CMECs with gated population indicated by red circle. **(C)** Combined frequency histogram depicting baseline DAF-2/DA fluorescence (x-axis) in the FL-1 channel of cardiomyocytes (left: lower fluorescence signal) and CMECs (right: higher fluorescence signal). The bi-modal appearance of the fluorescence in (C) may be a result of variable cell morphology or differential fluorescence intensity within the respective populations.

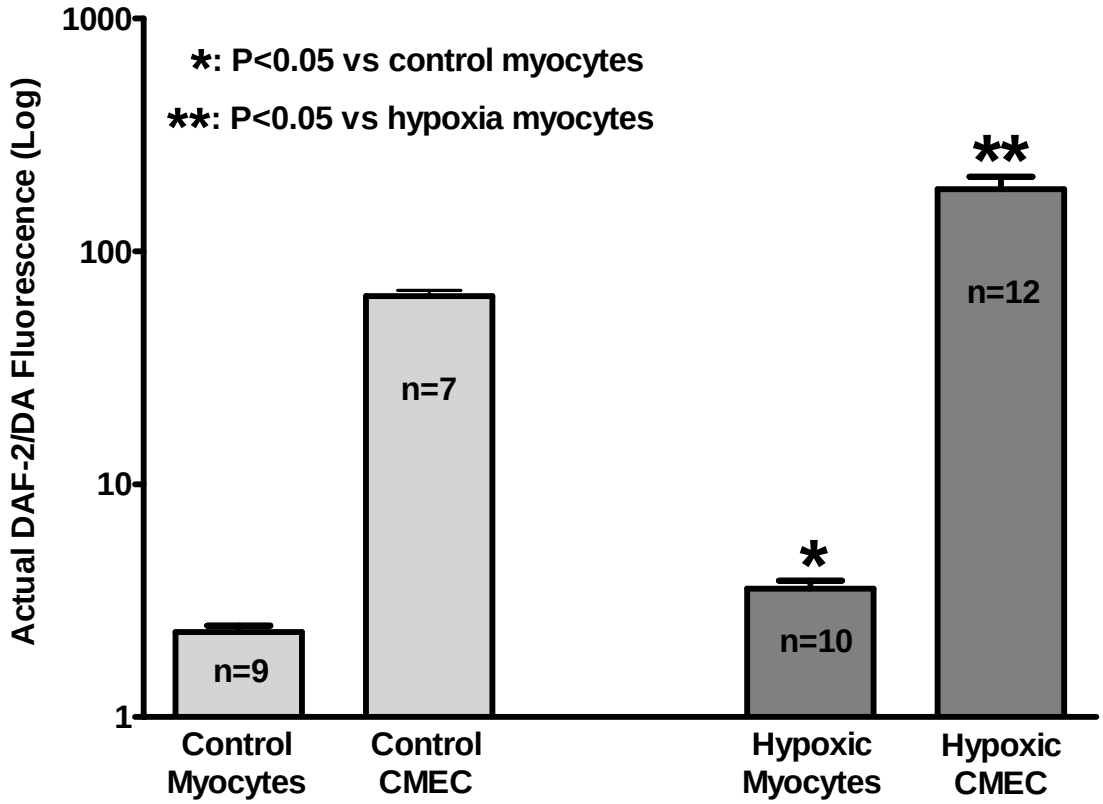


Fig. 5.10 Combined bar chart demonstrating actual DAF-2/DA fluorescence intensity in control (baseline) and 120 min hypoxia (ischaemic pelleting protocol) (see fig. 6.1 A for experimental design) as measured in the same number of cells per cell type. Control fluorescence signals were 26-fold stronger in trypsinized CMECs than in cardiomyocytes and in hypoxia 52-fold stronger.

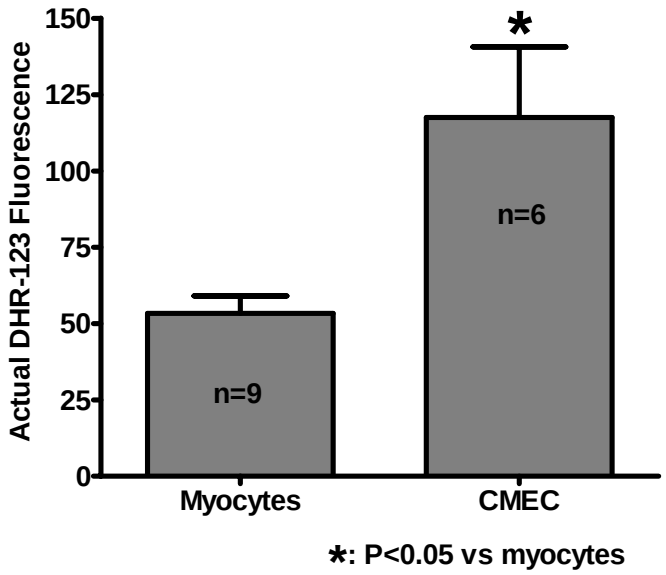


Fig 5.11 Bar chart depicting actual baseline DHR-123 fluorescence intensity readings measured in myocytes and trypsinized CMECs with 2.2-fold stronger fluorescence signals observed in CMECs. (117.5±23 vs. 53.3±5.7 respectively).

(vi) NO-production in mixed-cell suspensions (fig. 5.12)

Mean DAF-2/DA fluorescence increased significantly in cardiomyocytes that were co-suspended with trypsinized CMECs for 60 min under control conditions (myocytes only: 100% vs. myocytes+trypsinized CMECs: 126.8 ± 5.3%; $p < 0.05$; $n = 5$) (Fig. 5.12 A). In separate experiments, isolated cardiomyocytes were co-incubated with cultured CMECs under control conditions by placing the myocyte suspension onto the monolayer CMECs in culture; similar results were obtained (myocytes only: 100% vs. myocytes+cultured CMECs: 113.4 ± 1.1%; $p < 0.05$; $n = 4$) (Fig. 5.12 A). When the mixed-cell suspensions were exposed to 60 min hypoxia, the cardiomyocytes showed a 24% increase in DAF-2/DA fluorescence intensity compared to mixed-cell suspension control levels (mixed-cell control: 126.8 ± 5.3% vs. mixed-cell hypoxia: 150.7 ± 5.7%; $p < 0.05$; $n = 5$). Mixed-cell suspension subjected to 60 min hypoxia also showed a significant increase in DAF-2/DA fluorescence compared to myocyte-only hypoxia groups (hypoxia myocyte-only: 131.6 ± 4.7% vs. hypoxia mixed-cell: 150.7 ± 5.7%; $p < 0.05$; $n = 4$) (Fig. 5.12 B).

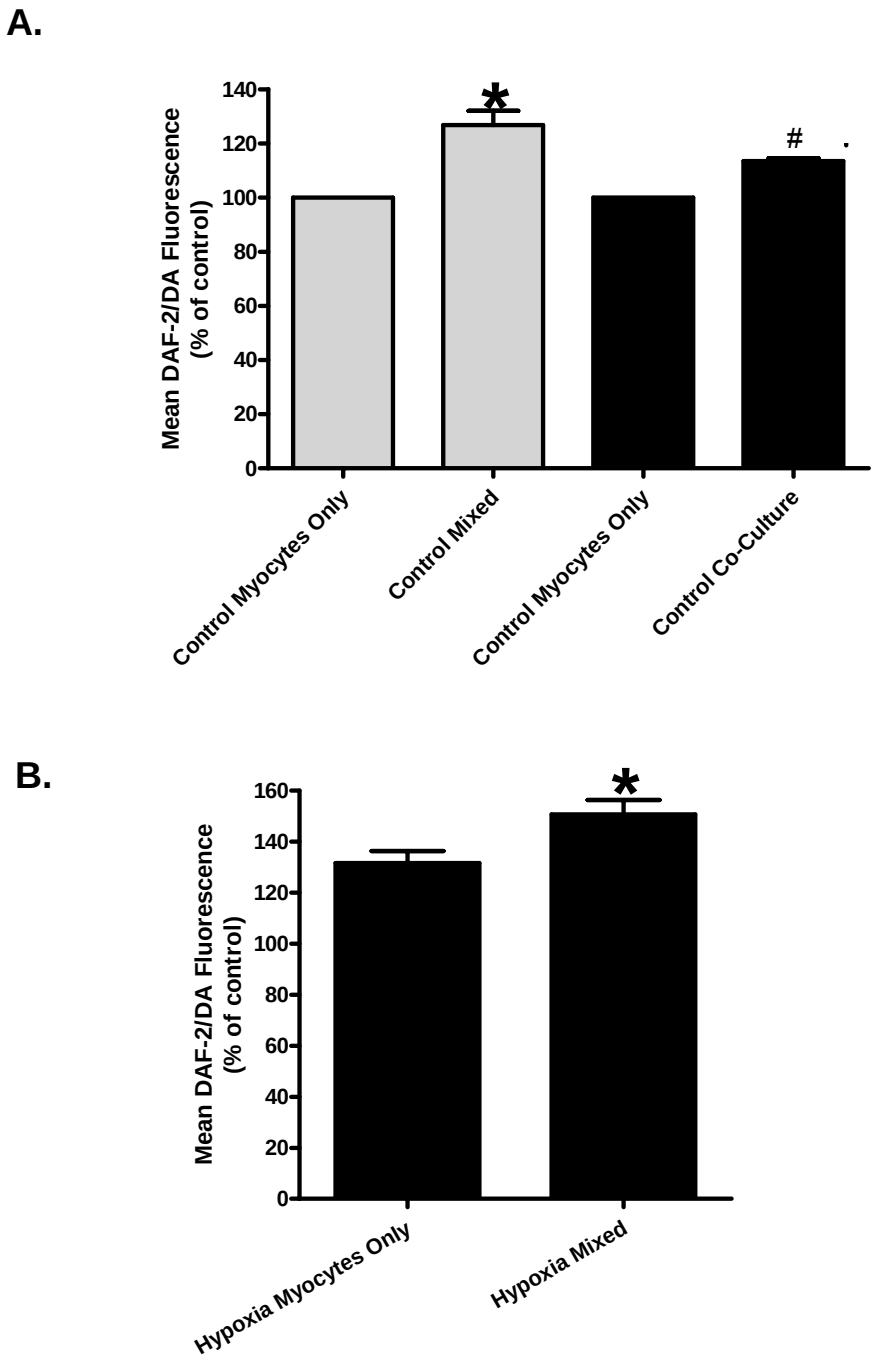


Fig. 5.12 Effects of co-incubation with CMECs on cardiomyocyte DAF-2/DA fluorescence. **(A)** DAF-2/DA fluorescence measured under control conditions in cardiomyocytes only and cardiomyocytes co-incubated with trypsinized CMECs ("Control Mixed") or suspended on cultured CMECs ("Control Co-culture") (*: $p < 0.05$ vs myocytes only; #: $p < 0.05$ vs. myocytes only). **(B)** 60 min ischaemic pelleting hypoxia in cardiomyocytes only or co-incubated with trypsinized CMECs ("Hypoxia Mixed") (*: $p < 0.05$ vs. myocytes only).

(vii) Total baseline eNOS and iNOS content in cardiomyocytes and CMEC (figs. 5.13 & 5.14)

Total eNOS protein content in isolated myocytes and CMECs (isolated by prior trypsinization) subjected to 180 min normoxic incubation in solution D was measured by Western blotting. Differences in cell size and number per sample were corrected for by loading identical amounts of total protein (50 µg per sample). In fig. 5.13, a representative blot of control cardiomyocyte and CMEC total eNOS (*n*=2; samples obtained from different preparations) shows increased eNOS in CMECs. Quantitation by densitometry indicates a 22-fold greater amount of eNOS / 50 µg cell protein in CMEC compared with cardiomyocytes. iNOS in both cell types was also measured by Western blotting and demonstrated a strong baseline signal in the cardiomyocytes, but no expression in CMECs (fig. 5.14).

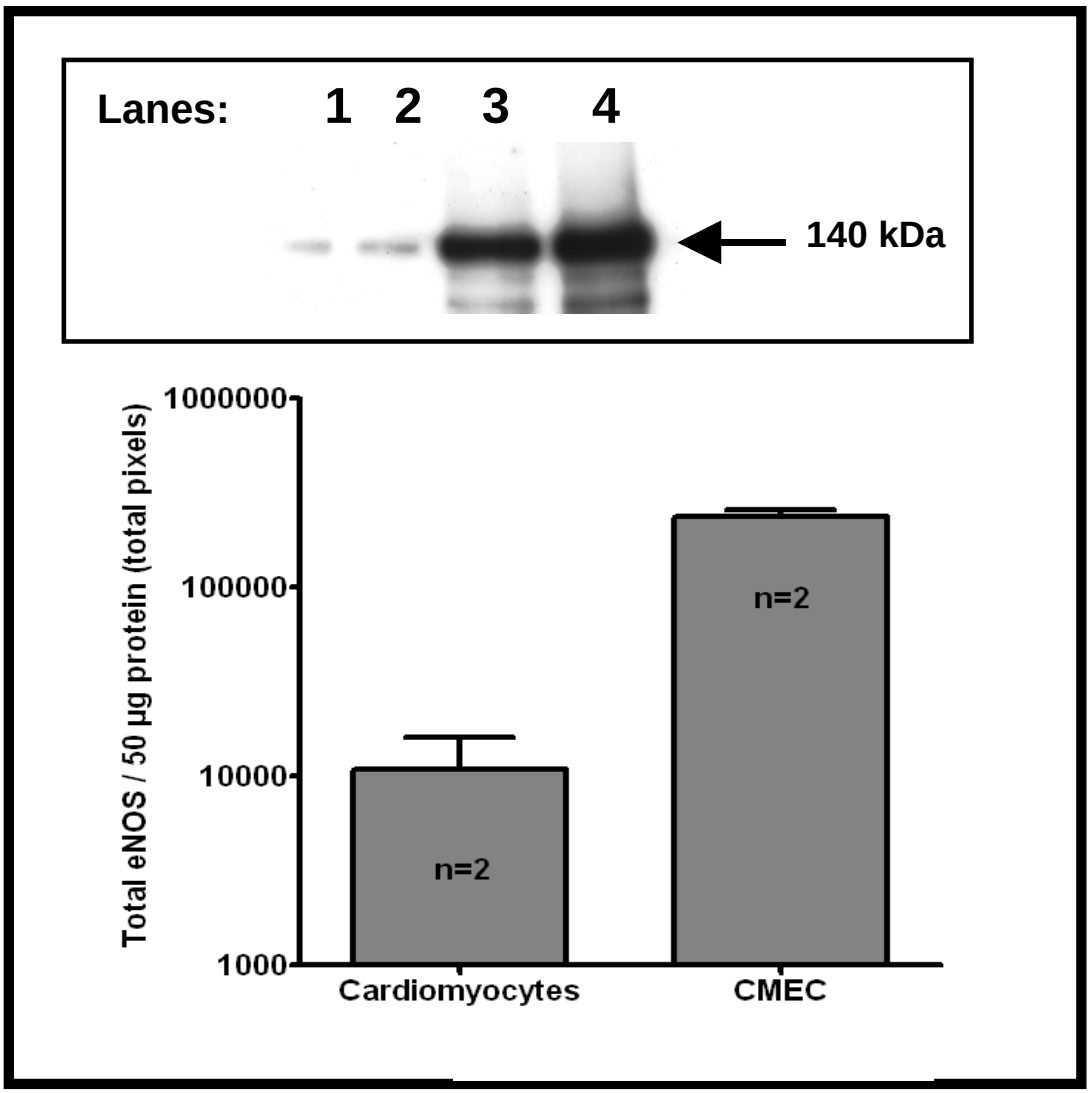


Fig. 5.13 Western blot analysis of total eNOS expression in cardiomyocytes and CMECs incubated for 180 min under normoxic conditions (isolated cell models; see fig. 5.1 for protocols). Equal amounts of total protein per cell type (50 µg) were loaded and analyzed on the same blot to enable direct comparisons (*n*=2, samples obtained from different preparations). **Upper panel:** Western blot depicting total eNOS expression in control cardiomyocytes (lanes 1 and 2) and control CMECs (lanes 3 and 4). **Lower panel:** Bar chart of total pixels in oxygenated control samples of each cell type as measured by densitometry. Total eNOS protein content was 22-fold higher in CMECs than in cardiomyocytes, which corresponds to the 28-fold increase observed in DAF-2/DA fluorescence measurements.

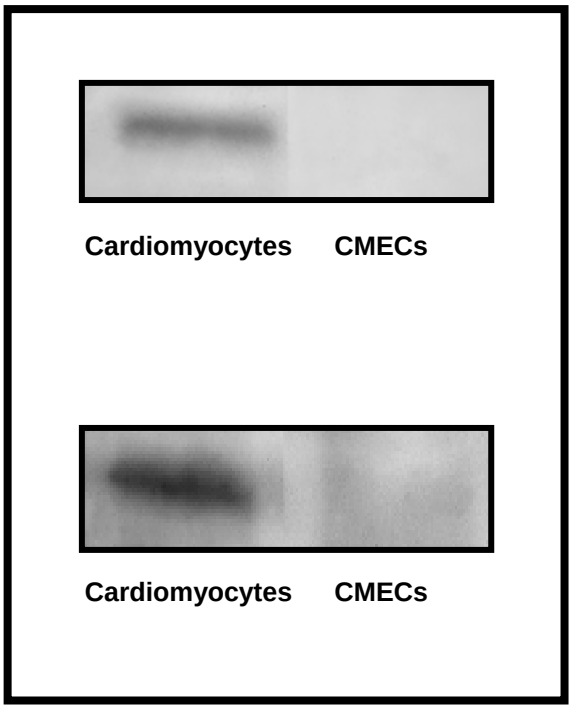


Fig. 5.14 Representative Western blots of inducible NOS (iNOS) expression under baseline control conditions in cardiomyocytes and CMECs. Both panels represent analyses done on cells from different hearts and CMEC cultures. On both occasions, the cardiomyocyte samples expressed iNOS whereas the CMECs showed no expression.

(viii) Total and phosphorylated (Ser1177) eNOS in hypoxia (figs. 5.15 & 5.16)

Total eNOS expression in cardiomyocytes exposed to *120 min* hypoxia (ischaemic pelleting) (see fig. 5.2 A for protocol) was significantly attenuated compared to control groups (control: 100% vs. hypoxia: 32.5 ± 11.8%; $P < 0.05$; n = 4) (fig. 5.15 A), however, the expression of phosphorylated eNOS remained constant compared to control conditions (control: 100% vs. hypoxia: 103.7 ± 12.05; $P > 0.05$; n = 4) (fig. 5.15 B). We subsequently exposed cardiomyocytes to a shorter hypoxia duration of 60 min. Results showed that 60 min hypoxia had no effect on total eNOS expression (control: 100% vs. hypoxia: 112.0 ± 5.5; $p > 0.05$; $n = 4$) (Fig. 5.15 C), but resulted in a 1.5-fold increase in phosphorylated eNOS (control: 100% vs. hypoxia: 152.6 ± 16.3%; $p < 0.05$; $n = 5$) (Fig. 5.15 D).

In cultured CMECs, 18 h of $\downarrow PO_2$ hypoxia resulted in a 2.1-fold increase in total eNOS expression (control: 100% vs. hypoxia: 210.3 ± 15%; $p < 0.05$; $n = 6$) (fig. 5.16 A), and this was associated with a 4.9-fold increase in phosphorylated eNOS levels (control: 100% vs. hypoxia: 493.9 ± 130.9%; $p < 0.05$; $n = 4$) (fig. 5.16 B). Trypsinized CMECs exposed to 60 min mineral oil hypoxia showed a 1.8-fold increase in total eNOS expression (control: 100% vs. hypoxia: 179.5 ± 34.2%; p < 0.05; n = 5) (Fig. 5.16 C), whereas phosphorylated eNOS levels increased almost 3-fold (control: 100% vs. hypoxia: 294 ± 86.6%; p < 0.05; n = 6) (Fig. 5.16 D).

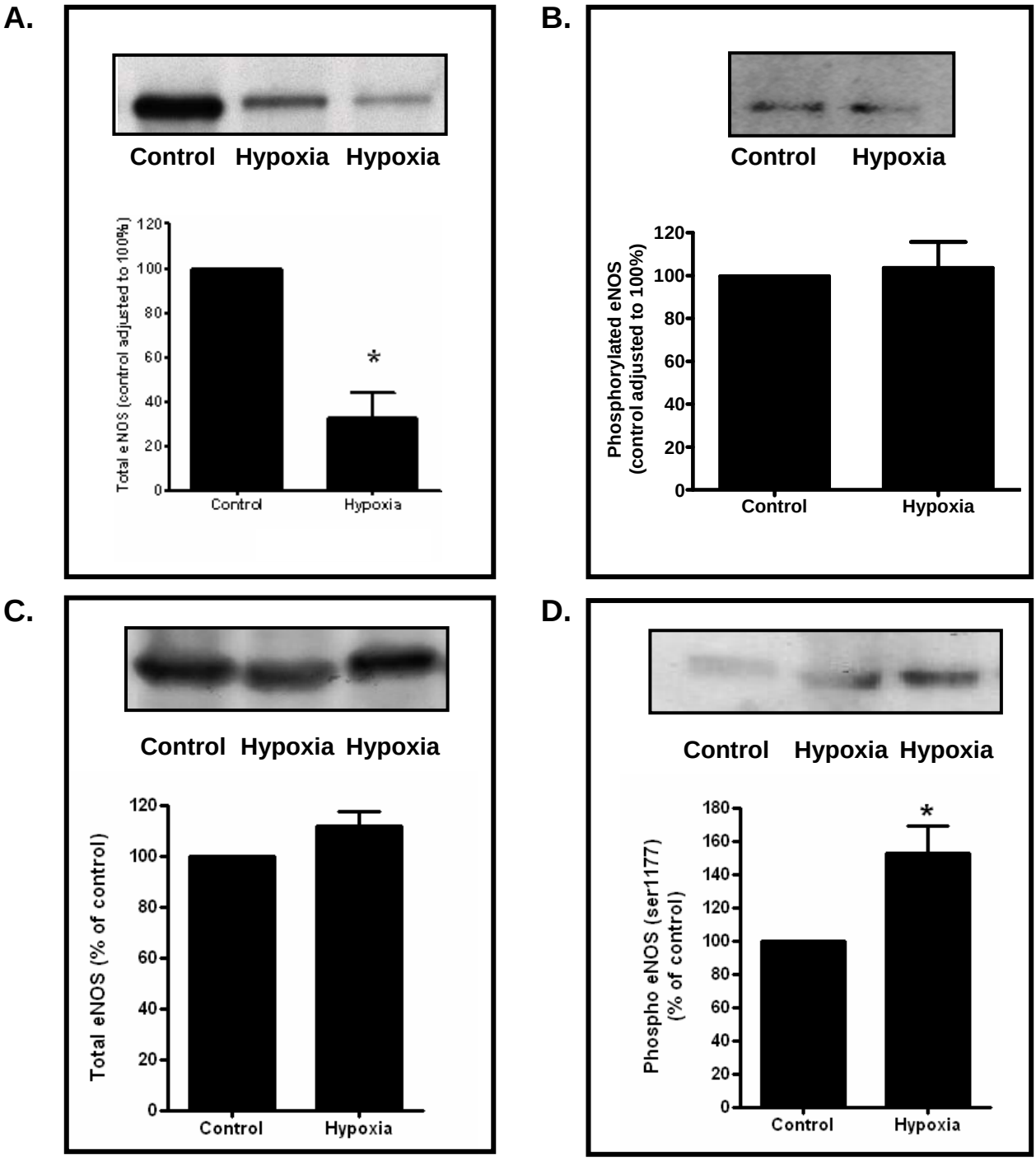


Fig. 5.15 Total and phosphorylated (Ser1177) eNOS in cardiomyocytes. **(A)** Total eNOS expression in cardiomyocytes exposed to 120 min ischaemic pelleting hypoxia showed a significant reduction compared to control. *: $P < 0.05$ vs. control **(B)** Phospho eNOS levels remained constant during 120 min hypoxia. **(C)** Myocytes subjected to 60 min hypoxia retained baseline total eNOS expression. **(D)** Phospho eNOS levels increased in cardiomyocytes after 60 min hypoxia. *: $P < 0.05$ vs. control.

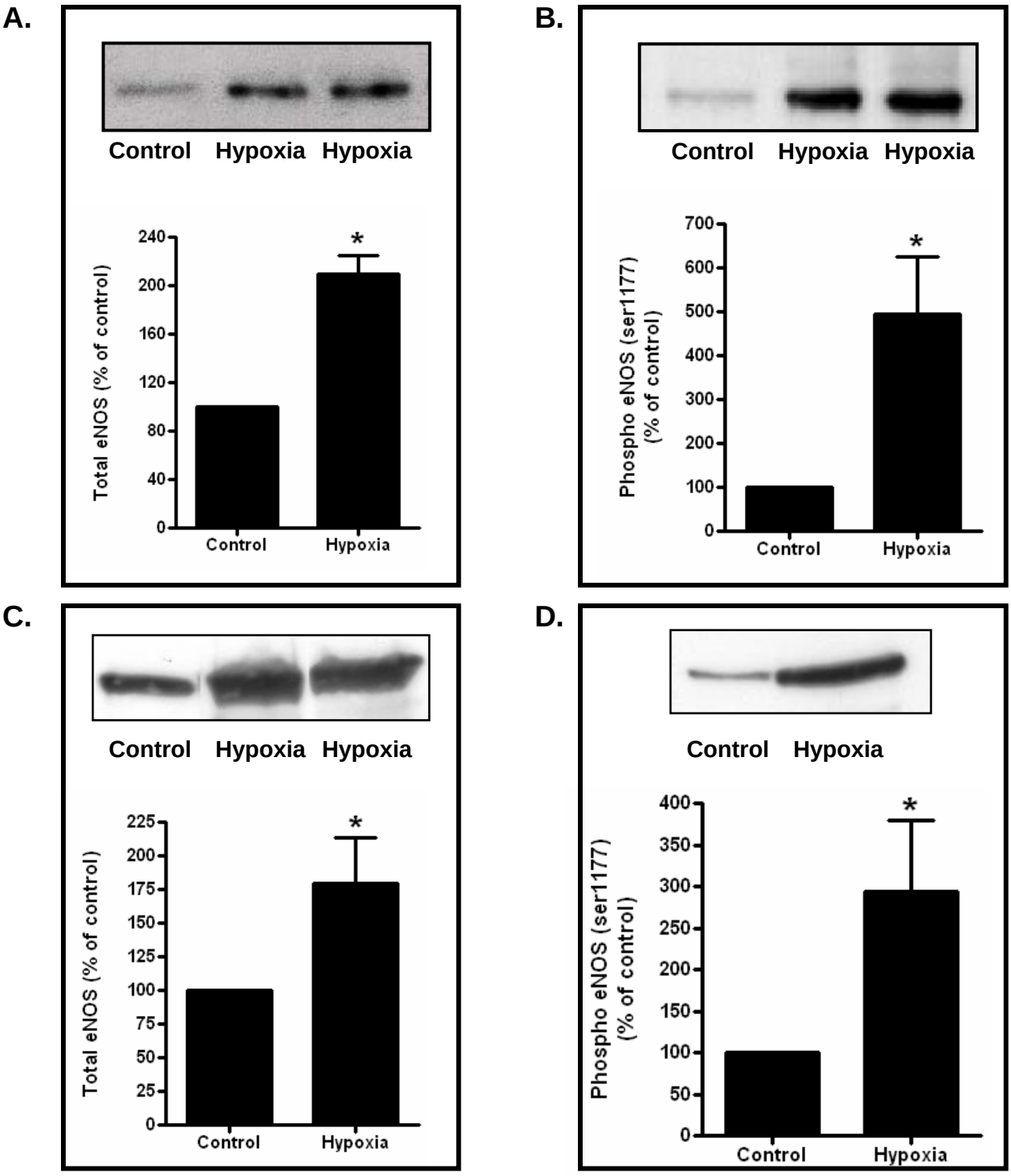


Fig. 5.16 Total and phosphorylated (Ser1177) eNOS in CMECs. **(A)** Total eNOS expression in cultured CMECs exposed to 18 hr hypoxic incubation showed a significant increase compared to control (*: $P < 0.05$). **(B)** Phospho eNOS levels increased after 18 hr hypoxia (*:$P < 0.05$). **(C)** Trypsinized CMECs subjected to 60 min hypoxia increased total eNOS expression compared to control (*:$P < 0.05$). **(D)** Phospho eNOS levels increased in trypsinized CMECs after 60 min hypoxia. *: $P < 0.05$ vs. control.

(ix) iNOS expression in cardiomyocytes and CMECs during hypoxia (fig. 5.17)

In cardiomyocytes exposed to 120 min ischaemic pelleting hypoxia, iNOS expression remained unchanged (control: 100% vs. hypoxia: 91.78 $\pm$ 34.3; $P > 0.05$; $n = 2$) (fig. 5.17), whereas no iNOS expression was observed in either control or hypoxic CMEC samples (not shown).

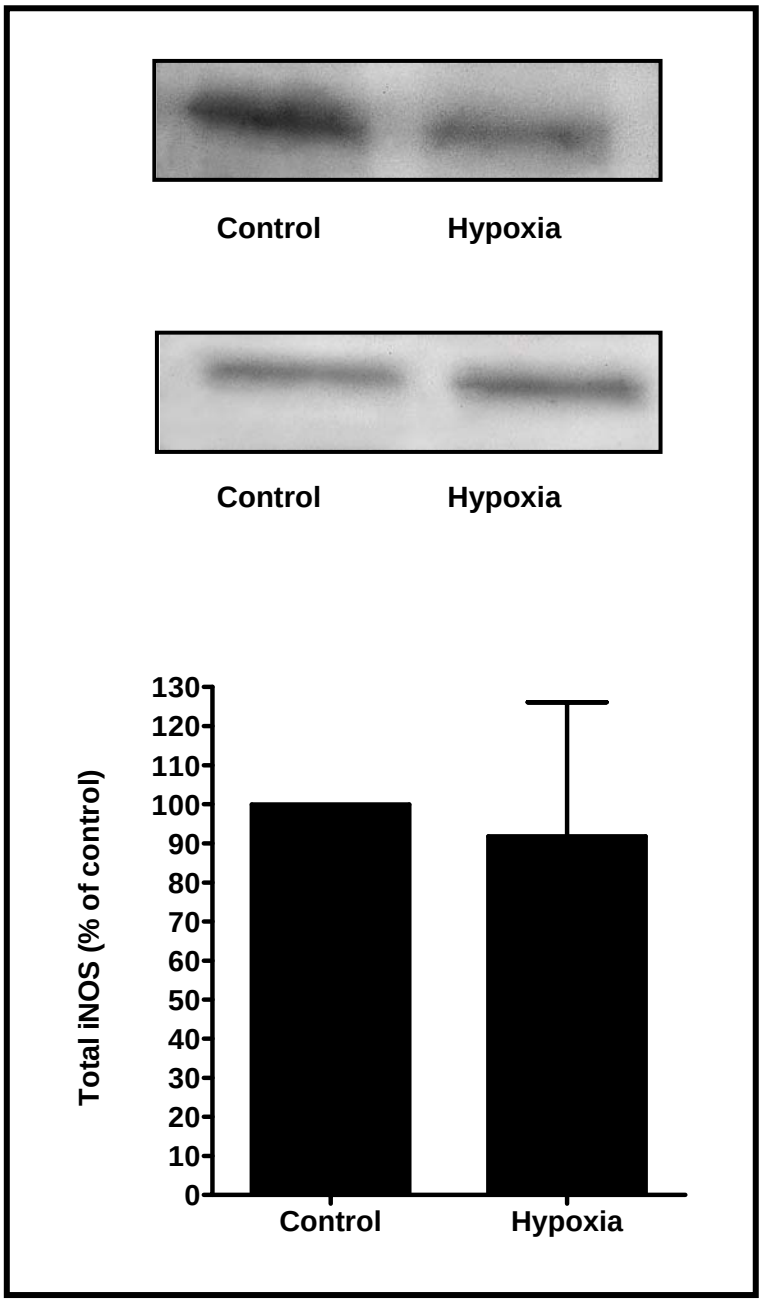


Fig. 5.17 iNOS expression in cardiomyocytes. Exposure to 120 min hypoxia did not affect control iNOS expression. Each Western blot panel represents separate myocyte samples obtained from different hearts.

5.8 Discussion

The relative importance of endothelium- and myocyte-derived NO remains to be established [Brutsaert 2003; Shah & MacCarthy 2000]. In our opinion, this hiatus in current knowledge is to a large extent due to a lack of studies that directly measured and compared *actual intracellular concentrations of NO* in these cell types. One way of addressing this is by designing studies that make use of direct cellular models. It is now widely accepted that cardiac endothelial cells produce more NO in baseline, physiological conditions than cardiomyocytes, but this conclusion is largely based on NOS-staining and -expression studies, and not direct NO measurements [Brutsaert 2003; Shah & MacCarthy 2000; Brutsaert *et al* 1998; Andries *et al* 1998].

There is a particular shortage of direct studies on the endothelial cell subtype that line the coronary microvessels (CMECs) [Shah & MacCarthy 2000]. CMECs are exposed and in closest proximity to the largest portion of myocardial muscle cells compared with any of the other endothelial cell subtypes. In fact, CMECs comprise about one-third of the total cell number in the ventricular wall [Shah & MacCarthy 2000; Nishida *et al* 1993]. CMECs are therefore ideal candidate external sources of NO in physiological and pathophysiological conditions for the cardiomyocytes. Compared with other cardiac endothelial cell subtypes, CMECs are thought to produce the lowest concentrations of NO [Brutsaert 2003; Shah & MacCarthy 2000; Andries *et al* 1998], yet it has been strongly suggested that a significant reciprocal NO-crosstalk mechanism, with major local physiological and pathophysiological effects, exists between the low-output NO-generating CMECs and cardiomyocytes [Brutsaert 2003; Shah & MacCarthy 2000; Brutsaert *et al* 1998]. For this reason, more knowledge about the relative amounts of NO produced by each cell type is

necessary to gain a better understanding of their relative roles in paracrine NO communication.

To achieve this, we measured intracellular NO production by flow cytometric analysis of the highly fluorescent oxidized product of DAF-2/DA, DAF-2T, a method previously developed for endothelial cells [Navarro-Antolin 2001(b)] and later modified for isolated ventricular cardiomyocytes [Ch. 4; Strijdom *et al* 2004(b)]. The advantage of flow cytometric measurement of changes in DAF-2/DA fluorescence above other fluorescence detection techniques is that it allows rapid real-time analysis of intracellular fluorescence on a single-cell level in thousands of cells at a time.

DAF-2/DA was developed as a NO-detection probe [Kojima *et al* 1998], and its specificity for NO has since been confirmed in a variety of cell types [Leikert *et al* 2001; Zorov *et al* 2000; Chen *et al* 2002; Lebuffe *et al* 2003] by validating results with other widely used indicators of NO production [Ch. 4; Strijdom *et al* 2004(b); Failli *et al* 2002; Havenga *et al* 2001]. In this chapter, DAF-2/DA specificity for NO was again demonstrated by observing a step-wise enhancement of fluorescence when increasing concentrations of the NO donor, DEA/NO, were administered [see Ch. 4]. Additionally, we excluded the possibility that $ONOO^-$ was a possible cause of changes in DAF-2/DA fluorescence (fig. 5.4 A). In a further attempt to validate probe specificity, we investigated whether DAF-2/DA could detect *endogenous* NO by administering 50 μM L-NAME to control cardiomyocytes; results showed an attenuation of the baseline fluorescence signal (control: 100% vs. control+L-NAME: 87%; $P<0.05$; $n=6$), in agreement with the findings in Ch. 4.

(i) NO and NOS in oxygenated control (baseline) investigations

Results demonstrate that CMECs generated significantly more NO than cardiomyocytes under baseline conditions. On a cell-to-cell basis, in the 180 min isolated cell incubation protocol (fig. 5.1 A), NO production was 26-fold higher in CMECs (fig. 5.10). Investigations were repeated in cells cultured overnight (see figs. 5.1 B & D for the protocols), and similar, albeit smaller, patterns were observed. We subsequently investigated whether eNOS was present in both cell types, and if so, whether there were differences in the expression of the enzyme in the isolated cell models (fig. 5.2 A for protocol). Results demonstrate the existence of baseline eNOS expression in both cell types (fig. 5.13), confirming previous observations [Brutsaert 2003; Shah & MacCarthy 2000; Brutsaert *et al* 1998; Balligand *et al* 1995(b)]. A comparison of the cell-to-cell expression of total eNOS content showed that expression was 22 times greater in CMECs (fig. 5.13), similar to the differences observed in NO production between the cell types.

We also investigated baseline iNOS expression in cardiomyocytes and CMECs, and found that there was expression in the myocytes, but not the CMECs, in which there was no measurable signal (fig. 5.14). However, one has to consider the possibility that endotoxin present in the collagenase used during the isolation procedure could have up-regulated iNOS in the myocytes [Tirmenstein *et al* 2000]. In separate investigations, we administered the iNOS inhibitor SMT (1 mM) to baseline (control) cardiomyocytes and found a modest, but significant attenuation in the DAF-2/DA signal (control: 100% vs. control+SMT: 88.79 $\pm$ 4.5%; $P < 0.05$; n =4), further indicative of baseline iNOS activity and contribution to NO-production in myocytes.

Similar investigations with SMT in control CMECs could not demonstrate reduction in DAF-2/DA fluorescence (data not shown), thereby supporting the Western blot findings in these cells.

Therefore, based on the NO and NOS data under baseline conditions, it seems that CMECs intrinsically produce more NO than myocytes on a cell-to-cell basis; however, the *amount* of NO produced by CMECs was affected by the cell model (CMEC produced 26-fold more NO in the isolated cell model vs. 7-fold in the cultured cell model). We furthermore established that the baseline eNOS expression in the isolated cell models was 22-fold higher in CMECs than cardiomyocytes, which was closely associated with the differences in NO-production; in addition our results suggest a possible contribution by iNOS to baseline NO production in the cardiomyocytes, but not in CMECs. To the best of our knowledge, there is no evidence of in vitro studies that have directly measured and compared cellular NO production and eNOS / iNOS expression between CMECs and cardiomyocytes. Results also demonstrate that the CMECs derived from cultures in this study (passage 3–4) retained their expression of eNOS, contrary to previous reports that this endothelial cell subtype loses eNOS expression in culture [Lang *et al* 1999; Balligand *et al* 1995(a)]. Indeed, given the possibility that earlier passages may have a higher expression of eNOS, the current data may well be an underestimation of the role of eNOS in CMECs.

(ii) NO and NOS during hypoxia: Isolated cardiomyocytes

The effects of NO in cardiac hypoxia / ischaemia can be beneficial or harmful [Ch. 1 for overview; Shah & MacCarthy 2000; Bolli 2001; Lochner *et al* 2000; Strijdom *et al* 2004(a)], which may, among others, be explained by the fact that its production and release by different cellular sources are variable and nonuniform [Shah & MacCarthy 2000]. In addition, the production of NO, on a cell-to-cell basis, during hypoxia in CMEC and cardiomyocytes is not known.

Hypoxia induced by ischaemic pelleting

We have shown that both 60 and 120 min of hypoxia induced by ischaemic pelleting significantly reduced cardiomyocyte viability as measured by two independent markers (Fig. 5.3). However, the injury exerted by the 120 min hypoxia protocol was more severe. In the 120 min hypoxia group, there was a 54% reduction in viable, trypan blue excluding cells compared to 20% in the 60 min hypoxia myocytes; similarly, cardiomyocytes exposed to 120 min hypoxia demonstrated an increase of 59% in non-viable PI-staining fluorescence compared to only 30% in 60 min hypoxia.

Despite the severity of the 120 min ischaemic pelleting hypoxia protocol and ensuing loss of viable cells, we observed a 1.56-fold increase in NO production compared to control (fig. 5.5 A). The increase in NO-production was observed in spite of a 68% loss of total eNOS protein (fig. 5.15 A). The *activated* (phospho Ser1177) eNOS levels remained unchanged, however, which effectively resulted in a 3.3-fold increase in the activated / total eNOS ratio (fig. 5.15 A & B). Another study also

reported a loss of total eNOS in isolated rat hearts subjected to prolonged ischaemia [Giraldez *et al* 1997]. After an initial increased expression at 30 min ischaemia, total eNOS levels started declining at 60 min ischaemia (40% reduction) and 90 min ischaemia (60% reduction). It is therefore evident that the total eNOS protein expression in cardiomyocytes is influenced by the duration and severity of the ischaemia / hypoxia insult.

In our study, pharmacological evaluation of NOS activity in cardiomyocytes during the 120 min hypoxia protocol demonstrated that nonselective NOS inhibition significantly attenuated NO production (fig. 5.5 A), which suggests a possible role for eNOS activity during the hypoxia protocol, despite the observed loss in total expression. However, other non-eNOS sources of NO are more likely to have been induced by hypoxia given the size of the loss of total eNOS. It is known that ischemia-induced acidosis can lead to NO-production that is not related to NOS activation [Schulz *et al* 2004; Zweier *et al* 1999]. Another possible source of non-eNOS NO-production may be the induction of iNOS [Jung *et al* 2000]. In fact, our findings suggest a role for iNOS in the cardiomyocytes, since the administration of the iNOS-specific inhibitor, SMT, during hypoxia significantly decreased NO production by 13 – 20% (fig. 5.5 A). In addition, we demonstrated iNOS expression in baseline and hypoxic cardiomyocytes (figs. 5.14 & 5.17). Although iNOS levels did not increase during hypoxia, we cannot exclude a contribution by iNOS to the observed increases in NO-production. More research is necessary to elucidate the role of iNOS in hypoxia in the cardiomyocytes.

In view of the loss of eNOS observed in the 120 min hypoxia cardiomyocytes, we decided to shorten the hypoxic insult to 60 min (see fig. 5.1 for the protocol). Under these conditions, there was no loss in total eNOS protein, in fact expression remained at control levels (Fig 5.15 C). Activated (phospho-eNOS ser1177) eNOS levels increased 1.5-fold (Fig. 5.15 D), which was associated with a 1.4-fold increase in NO production (Fig 5.5 B). From the 60 min hypoxia results, it is clear that a relatively close relationship existed between hypoxia-induced eNOS activation and NO production. This suggests that eNOS was a prominent source of increased cardiomyocyte NO levels in the shorter, less severe hypoxia protocol. Findings therefore indicate that the contribution of non-eNOS sources of NO was less in the 60 min hypoxia group than the 120 min group.

Isolated cardiomyocytes, hypoxia induced by ↓PO_2

The lowering of atmospheric O_2 to 1% and 2 h incubation in a substrate-free medium also significantly increased NO production in cardiomyocytes (see fig. 5.1 C for protocols), although the relative increase over baseline was less than observed in mineral oil-treated freshly isolated cells (fig. 5.5 A & B). This suggests that the *amount* of NO produced in cardiomyocytes was dependent on the hypoxia protocol used, and possibly a result of the greater degree of hypoxia-induced injury observed in cells subjected to the ischaemic pelleting protocol.

(iii) NO and NOS during hypoxia: CMECs

In the CMEC studies, we chose to use two different hypoxia protocols (fig. 5.1). The motivation for using two distinct CMEC cell models and protocols was to ensure that the trends and effects observed were not model-dependent. Furthermore, the trypsinized CMEC model allowed us to apply an incubation and hypoxia protocol that was identical to that used for the isolated cardiomyocytes, which facilitated comparisons of data between the cell types. The viability results confirm that the degree of cellular injury induced by the hypoxia protocols was similar in the two CMEC models and the cardiomyocytes (fig. 5.3 E & F).

CMECs in culture, hypoxia induced by ↓PO_2

In the cultured model of CMECs, 18 h ↓PO_2 hypoxia increased PI fluorescence by 22% (fig. 5.3 E), and induced a 2.1-fold increase in total eNOS protein compared to control cells, which indicates that there was hypoxia-induced upregulation of eNOS in CMEC (fig. 5.16 A). This was associated with significant activation of eNOS (5-fold increase in **absolute** phospho eNOS, and 2-fold **relative** increase in phospho eNOS: total eNOS ratio) (fig. 5.16 B) and a 1.4-fold increase in NO production (fig. 5.7 C). The close relationship between hypoxia, and eNOS activation and NO production in the cultured CMECs, suggests that the NO generated during hypoxia is predominantly eNOS-derived. There seems to be little participation from iNOS in our model of hypoxic CMECs, since we showed in the same model that the iNOS-specific inhibitor, SMT, had no effect on NO production in CMECs during hypoxia, in contrast to observations made in the cardiomyocytes. In addition, subsequent Western

blotting could not demonstrate iNOS expression in either control or hypoxic CMEC obtained from similar passages.

Trypsinized CMECs; hypoxia by ischaemic pelleting

Hypoxia by 120 min ischaemic pelleting (see fig. 5.1 for protocols) resulted in a 3.3-fold increase in NO production over control (fig. 5.7 A). Although nonselective NOS inhibition significantly reversed the hypoxia-induced increase, selective iNOS inhibition had no effect on NO production (fig. 5.7 A), contrary to observations made in cardiomyocytes. These trends were further substantiated by the absence of iNOS expression in CMECs (fig. 5.14). Ischaemic pelleting hypoxia of *60 min* in the trypsinized CMECs resulted in a 1.55-fold increase in NO production (Fig 5.7 B). This was associated with a 1.8-fold increase in total eNOS (fig. 5.16 C), and ~ 3-fold increase in activated eNOS (fig. 5.16 D), demonstrating a similar pattern to that observed in the cultured CMEC investigations. Therefore, our findings showed that, in two distinct models of hypoxia, eNOS is upregulated and activated in CMECs leading to increased NO production.

(iv) Cell models and hypoxia protocols used in the study

It is evident that many of the observations made in the NO and eNOS investigations were dependent on the cell model and/or hypoxia protocol used. This phenomenon may partly be explained by the greater degree of hypoxic injury observed in the cells subjected to the ischaemic pelleting protocol compared with their controls. Another explanation could be that isolation of cultured CMECs by trypsinization before

experiments might have induced additional cellular stress, resulting in the relatively high NO production, although in separate experiments we showed that trypsinized CMECs maintained their baseline viability and LDL-uptake ability compared with cultured CMECs. Follow-up studies measuring and comparing total and activated NOS in isolated and cultured CMECs may give more clarity.

A larger increase in NO production was also observed in cardiomyocytes when exposed to ischaemic pelleting hypoxia compared with $\downarrow PO_2$ hypoxia (fig. 5.5). In the myocyte experiments, however, there was no difference in the cell *models* (fresh isolated myocytes in suspension were used in both hypoxia protocols; see fig. 5.1 A & C); therefore, it is likely that the higher NO production in cells subjected to ischaemic pelleting was directly related to the greater cellular injury induced by this form of hypoxia. Finally, viability results suggest that CMECs were less vulnerable to hypoxic injury than cardiomyocytes, consistent with findings from other studies [Piper 1990; Buderus *et al* 1989].

(v) Peroxynitrite

In a separate series of experiments, we measured baseline $ONOO^-$ production in CMECs (isolated by trypsin) and cardiomyocytes (freshly isolated), and investigated how this compared with NO production. Little is known about the relative contributions of CMECs and cardiomyocytes to $ONOO^-$ production. DHR-123 was used to detect $ONOO^-$, based on findings of previous studies that showed a relative selectivity of the probe for $ONOO^-$ over other oxidants such as O_2^-, H_2O_2, or NO in cellular systems [Navarro-Antolin *et al* 2001(b); Murphy *et al* 1998; Kooy *et al* 1994;

Ischiropoulos *et al* 1999]. In the present study, selectivity for $ONOO^-$ was confirmed by enhancement of DHR-123 fluorescence when authentic $ONOO^-$ was administered (fig. 5.4 B) in a protocol adopted from a previous study [Navarro-Antolin *et al* 2001(b)]. It was interesting to note that the probe generated a detectable fluorescence signal, despite the relatively long incubation time of authentic $ONOO^-$, which is known for its short half-life.

The probe's ability to detect changes in *endogenous* $ONOO^-$ production was assessed by administering a ROS scavenger N-acetyl-cisteine (NAC) to control cells, but no attenuation of the fluorescence signal was observed (data not shown). However, the attenuation of DHR-123 fluorescence during hypoxia provides indirect evidence for this, since it is widely believed that endogenous ROS production is decreased during hypoxia in the absence of reoxygenation. Results showed that the baseline $ONOO^-$ production in CMECs was 2.2-fold higher than in cardiomyocytes (fig. 5.11). Therefore, in our model, CMECs was the greater cellular source of $ONOO^-$ in baseline conditions, although the ratio of increase was lower than that observed with NO production. There was a significant attenuation of DHR-123 fluorescence after exposure to hypoxia in both cell types (figs. 5.6 & 5.8), which is not unexpected since $ONOO^-$ is known to be generated during the reperfusion phase of ischaemia-reperfusion [Ferdinandy & Schulz 2003; Beckman & Koppenol 1996], and our model of hypoxia did not include reperfusion.

(vi) NO-production in mixed-cell studies

In order to investigate whether intracellular NO-levels in cardiomyocytes are affected by co-incubation with CMECs, we developed a mixed-cell model in which cardiomyocytes and trypsinized CMECs were co-incubated at a 1:1 ratio (fig. 5.1 E for protocol). Results showed that intracellular NO levels in the cardiomyocytes increased by 27% compared to myocyte-only groups in control oxygenated conditions (Fig 5.12 A). This observation was repeated when cardiomyocytes were suspended on a monolayer of cultured CMECs (13% increase compared to myocytes only) (Fig 5.12 B) under oxygenated conditions. When the mixed-cell cardiomyocyte+CMEC suspensions were exposed to 60 min hypoxia, a significant increase in NO production compared to myocyte-only hypoxia was observed (Fig 5.12 B).

The increases in intracellular cardiomyocyte NO levels during control oxygenated and hypoxic conditions when exposed to CMECs suggest a possible spillover effect from the higher NO-producing CMECs. However, it is possible that the CMECs released factors other than NO into the medium that could have been indirectly responsible for the increased NO levels observed in the cardiomyocytes (e.g. eNOS-activating factors). In order to exclude this possibility, we incubated cultured CMECs in Krebs-Henseleit Buffer (KHB) for 60 min in separate experiments, after which the medium was removed and transferred to the myocytes with subsequent DAF-2/DA loading and FACS analysis. The medium-transfer method was chosen to investigate this question, since it is very unlikely that, once the medium was removed from the CMECs, any significant amounts of the highly reactive and short-lived NO would be

present in the absence of a sustained CMEC-derived supply. Results indicated that there was no effect on intracellular myocyte NO (DAF-2/DA fluorescence in control myocytes: 100% vs. myocytes+medium: 99.6 ± 2.9%; $p > 0.05$; $n = 7$). These findings suggest that the CMECs were unlikely to release factors, other than NO itself, that could explain the increases observed in the myocytes. It should be borne in mind that these investigations are preliminary; however, findings thusfar are encouraging. More studies are necessary, including co-culture experiments in which live-cell microscopic analyses could be used to demonstrate real-time cell-to-cell spillover diffusion.

5.9 Conclusion

We have demonstrated that in baseline and hypoxia, rat CMECs produce significantly more NO than rat ventricular cardiomyocytes in different cellular models (isolated cells and cultured cells) and different hypoxia protocols (ischaemic pelleting and $\downarrow PO_2$). The total eNOS measurements under baseline conditions showed similar patterns, suggesting that differences in baseline NO production may be explained by the relatively greater eNOS expression observed in CMECs. Conversely, we observed baseline iNOS expression in the cardiomyocytes, but not in the CMECs, suggestive of a possible contribution to baseline NO-production in the myocytes. We have shown that hypoxia resulted in the generation of increased NO levels in both cell types compared with control, which was partially reversed by nonselective inhibition of NOS. In the cardiomyocytes, but not CMECs, iNOS seemed to play a role as a source of NO production in hypoxia, but this needs to be investigated further. Although nNOS was not measured in this study, it is unlikely that it could have been a non-eNOS source of NO during hypoxia, since the only study to date

that investigated nNOS expression in cardiomyocytes, demonstrated a reduction in nNOS expression [Mohan *et al* 2001]. Hypoxia in our study activated eNOS in both cardiomyocytes and CMECs by phosphorylation of eNOS (Ser 1177), resulting in increased NO production in both cell types. In the CMECs, eNOS regulation was further characterized by increased total eNOS protein expression, whereas in myocytes eNOS levels were either reduced (120 min hypoxia) or maintained at control levels (60 min hypoxia protocol).

Extrapolation to the *in vivo* situation is difficult since the exact CMEC-to-cardiomyocyte cell number ratio in the adult heart is unknown. However, it is thought that CMECs comprise ~33% of the total cell number in the ventricular wall [Nishida *et al* 1993], and the ratio of total cardiac endothelial cells (CMECs + endocardial endothelial cells) to cardiomyocytes is ~3:1 [Brutsaert 2003]. Therefore, collectively, CMECs are likely to produce more NO than ventricular cardiomyocytes under baseline conditions (derived mainly from the constitutive activity of eNOS), and after exposure to hypoxia. The fate of the relatively greater amounts of NO released by CMECs is unknown, but spillover diffusion into the underlying cardiomyocytes is possible. Preliminary mixed-cell suspension experiments in this chapter demonstrate encouraging trends, with elevated NO-production observed in both baseline and hypoxic cardiomyocytes when exposed to CMECs. Follow-up studies with co-culture cell models could demonstrate how the increased NO production in CMECs in baseline and hypoxia affects NO crosstalk between these cell types. Future studies may also be able to show whether the greater intracellular production of $NO/ONOO^{-}$ in CMECs could lead to spillover effects in cardiomyocytes, particularly in hypoxia.

[The aims, methods, data and conclusions presented in this chapter appeared in: *Strijdom H, Jacobs S, Hattingh S, Page C, Lochner A. Nitric oxide production is higher in rat cardiac microvessel endothelial cells than ventricular cardiomyocytes in baseline and hypoxic conditions: a comparative study. FASEB J 2006; 20(2): 314 – 316*.]

CHAPTER 6

CONCLUSIONS

The original purpose of this study was to investigate the possible cardioprotective properties of NO in the context of IP. As the findings in Chapter 3 clearly show, we were unable to prove that NO was either a trigger or mediator of hypoxia-induced early IP. The non-involvement of NO in IP protection in our model was extensively investigated using a variety of interventions. In addition, NO accumulation during sustained hypoxia was shown to be harmful to the adult cardiomyocyte, which is contrary to the overwhelmingly beneficial effects attributed to NO by Bolli and Jones [Bolli & Jones 2006]. In view of these rather unexpected findings, the focus of this PhD thesis was accordingly directed towards the role of NO in the context of hypoxia *per se*.

In order to achieve the aims of this study, a number of methods and protocols were developed in our laboratory. Viable cardiomyocytes were successfully isolated from adult rats, and an endothelial cell culture model (CMECs) was established. Both cell types were investigated in their isolated state as well as in cultured conditions, and appropriate models were designed for this purpose. Furthermore, a variety of cell viability techniques were developed or adapted from previous studies. This was necessary mainly to ensure that our experiments were conducted on cells of the highest possible viability, but also to serve as end-point for several interventions such as hypoxia and IP. The investigations conducted in Chapter 3 were mainly dependent on *indirect* NO assessment tools such as NOS inhibition and cGMP level determinations. This prompted us to develop the DAF-2/DA-FACS analysis technique of direct intracellular NO-detection, which was novel in the field of isolated cardiomyocyte research at the time. It provided us with a unique opportunity to measure real-time NO generation in thousands of cells simultaneously. Furthermore,

the DAF-2/DA-technique enabled us to conduct the first comparative study of its kind in cardiomyocytes and CMECs in which baseline and hypoxia-induced NO production could be measured separately.

The role of NO and ROS was investigated in early IP and hypoxia. To achieve this aim, different hypoxia protocols were developed. For the isolated cell models, hypoxia was induced by layering the cell pellet with mineral oil, and for cell cultures, a tissue culture incubator in which the partial pressure of oxygen was lowered, was used. IP in the cardiomyocytes was induced by a single-cycle brief hypoxia period followed by reoxygenation prior to sustained hypoxia. We have earlier referred to the findings with regards to the non-involvement of NO in IP in our model of isolated cardiomyocytes. We could also not demonstrate contributions from ROS to IP protection in this model. These findings led to a shift in emphasis from investigating the role of NO in IP to examining its role in hypoxia *per se*.

The development of the FACS-based detection method, enabled us to investigate NO and peroxynitrite (ONOO-) generation in hypoxia in both cardiomyocytes and CMECs. Results indicated that exposure to hypoxia induced both cell types to increase NO generation compared to baseline production. We could furthermore demonstrate that CMECs generate substantially more NO per cell than cardiomyocytes in both conditions. This was the first study of its kind in which NO-production in these cell types was directly compared in the context of hypoxia. We were able to demonstrate this phenomenon in different cell models *viz.* isolated cells and cultured cells. Furthermore, the $ONOO^-$ data demonstrated that CMECs

generated higher amounts of this radical than cardiomyocytes, although hypoxia attenuated its generation significantly in both cell types.

Having established that both cardiomyocytes and CMECs produce NO under hypoxic conditions, the next aim was to measure the expression and activation of two of the most important NOS isoforms in cardiac tissue, eNOS and iNOS. At the time, the role of nNOS was still under investigation, and its role and function in the heart were unclear. Furthermore, the only study to investigate the role of nNOS in hypoxia in cardiomyocytes demonstrated a reduction in its expression [Mohan et al 2001], making it an unlikely candidate for NO production in this particular scenario. It was therefore decided to focus on the role of eNOS and iNOS. Using Western blotting, it was shown that CMECs and cardiomyocytes both expressed baseline eNOS, but that iNOS was detected in the cardiomyocytes only. In the CMECs, hypoxia induced both eNOS expression and activation. The close relationship observed between hypoxia-induced NO generation and eNOS activation in the CMECs, suggested that eNOS was the predominant source of NO in hypoxia in these cells. In the myocytes, hypoxia of longer duration (120 min) caused a loss of eNOS although activated eNOS remained constant. Despite the loss of eNOS, NO production increased, which led us to believe that non-eNOS sources of NO may have been induced such as iNOS, as was indeed suggested by the Western blot and iNOS inhibition data (see Ch. 5). When cardiomyocytes were exposed to shorter hypoxia (60 min), total eNOS expression levels remained unchanged, and the increased activation of eNOS was closely linked to the hypoxia-induced NO generation.

From our results, it therefore seems that eNOS played a significant role in NO generation in both cell types leading us to believe that it is the predominant NOS isoform involved during baseline and hypoxic conditions. The relatively greater degree of NO-production capacity, eNOS-expression, and –activity observed in CMECs has important implications for our understanding of possible interactions between CMECs and cardiomyocytes in the myocardium. We believe that a spillover diffusion effect is likely to exist from CMECs to cardiomyocytes in both the resting and ischaemically-stressed myocardium. Preliminary mixed-cell studies from our laboratory show encouraging results pointing towards possible spillover diffusion, however, the existence and exact role of such a phenomenon have not been demonstrated yet and future studies using co-culture models could help elucidate this matter.

Below is a schematic representation of the possible implications of our findings, in which the close proximity of the CMECs and cardiomyocytes in the myocardium is shown (fig. 6 B). Due to the short diffusion distance between these cell types, it is likely that a mechanism of NO-crosstalk exists; however, this phenomenon and the relative importance of each cell type to such a crosstalk remains unknown. Our data show that, in the *in vitro* situation, CMECs produce 7-26 times more NO per cell than cardiomyocytes under baseline conditions (fig. 6 C). The NO-data correspond well with the relative eNOS expression observed. In addition, our findings demonstrated a possible role for iNOS in the cardiomyocytes, however no iNOS expression was detected in the CMECs. In the *in vivo* situation, it is therefore possible that the relatively greater amounts of NO released by the CMECs could create a spillover diffusion effect into the underlying cardiomyocytes. The additional, non-myocyte

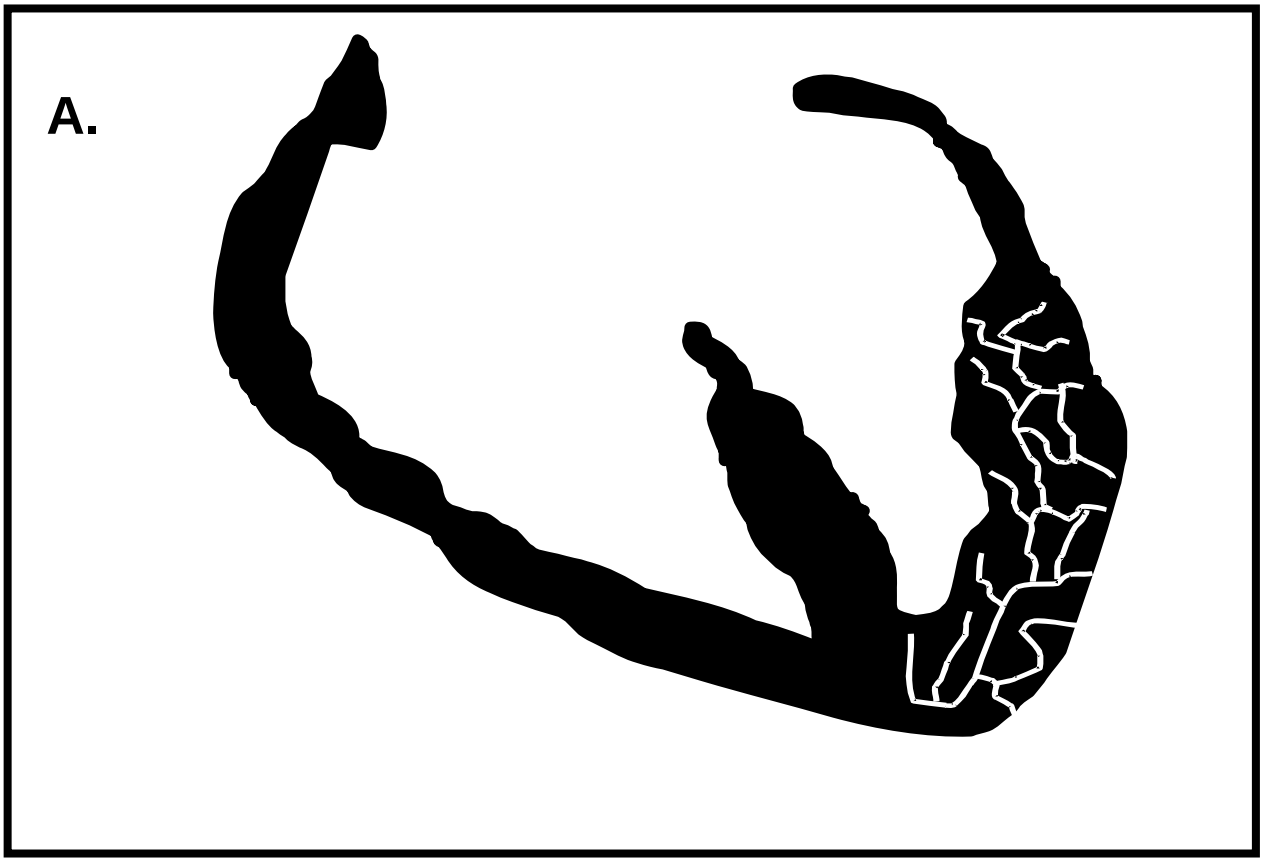
derived NO could contribute to baseline cardiomyocyte regulation and function. Future co-culture studies could help elucidate whether this phenomenon exists, and what the effects on the recipient cells would be.

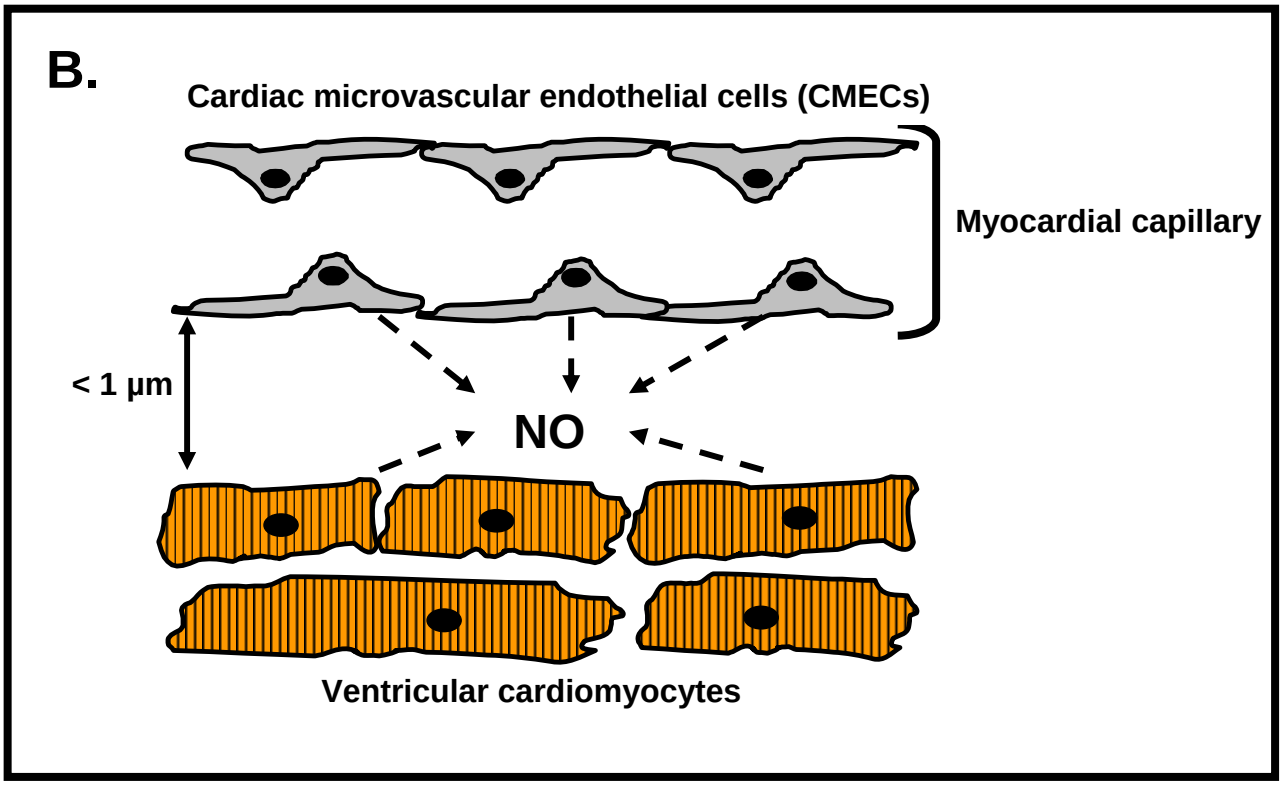
During hypoxia (fig. 6 D), eNOS was both upregulated and activated in the CMECs, which was associated with an increase in NO-production. In the cardiomyocytes, similar findings were observed, with the exception that there may be an additional role for iNOS in NO-generation. From these findings, it is possible that an even greater diffusion gradient may develop during hypoxia from the CMECs to the underlying cardiomyocytes. Future co-culture studies could indicate whether this does in fact occur, and if so, whether it is harmful or beneficial to the cardiomyocytes.

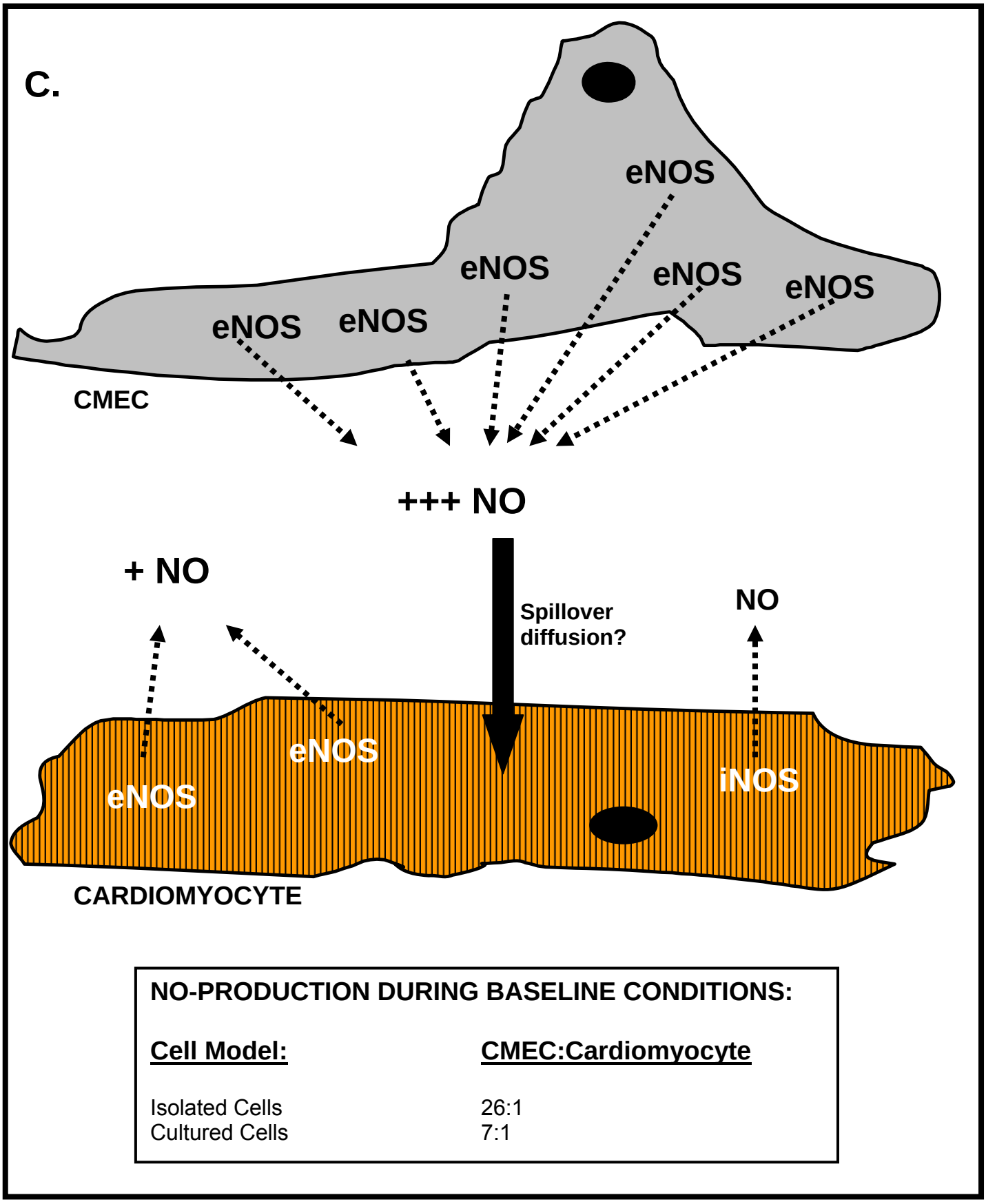
Topics for future consideration:

There is an overwhelming amount of data available in the literature on NO in the heart. Despite this, much confusion still exists as to the exact role of NO, particularly in the context of hypoxia / ischaemia. In view of this, and the findings of the current thesis, further investigations of the following topics may be worthwhile:

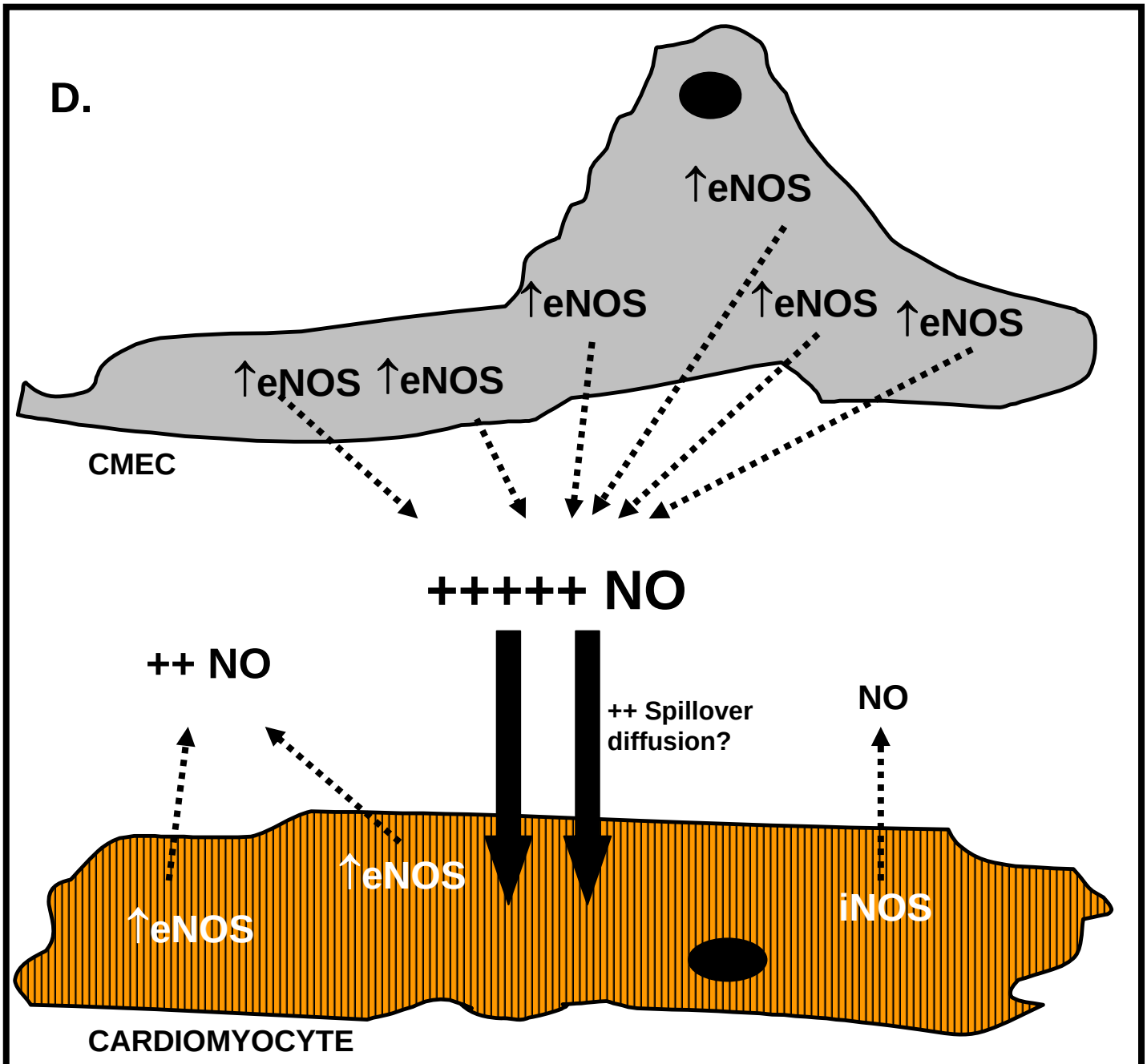
1. To investigate the role of nNOS expression and activation in baseline and hypoxic conditions in cardiomyocytes and CMECs;
2. Development of co-culture studies (cardiomyocytes + CMECs) in which IP is investigated in order to assess whether endothelial NO could act as a trigger and / or mediator of protection in cardiomyocytes;
3. Further investigations on isolated cardiomyocytes to explore other, non-NO, triggers and mediators of the IP protection observed in these cells.


A.


B.
Cardiac microvascular endothelial cells (CMECs)
Myocardial capillary
< 1 µm
NO
Ventricular cardiomyocytes



NO-PRODUCTION DURING BASELINE CONDITIONS:	
Cell Model:	**CMEC:Cardiomyocyte**
Isolated Cells	26:1
Cultured Cells	7:1



NO-PRODUCTION DURING HYPOXIC CONDITIONS:

Hypoxia Protocol:	**CMECs**	**Cardiomyocytes**
Ischaemic pelleting	200%↑	40-60%↑
↓PO_2 incubation	41%↑	9%↑

Fig. 6 (A) Sagittal section of the myocardium shows the network of myocardial capillaries in the left ventricular wall. **(B)** The proximity of the two NO-producing cell types investigated in this study, CMECs (lining the myocardial capillaries) and the underlying ventricular cardiomyocytes, is shown here. Due to the short diffusion distance between these cell types (<1μm), it is likely that a NO-crosstalk mechanism exists, however, the relative importance of CMEC- and cardiomyocyte-derived NO remains unknown. **(C)** In the *in vitro* situation, results of this study showed that CMECs produced 7 to 26 times more NO per cell than cardiomyocytes under baseline conditions, which corresponded with the relative eNOS expression observed (22x more total eNOS in CMECs compared to myocytes). Our results demonstrated a possible NO-producing role for iNOS in the cardiomyocytes, but not the CMECs. Therefore, collectively, CMECs are likely to produce significantly more NO than ventricular cardiomyocytes under baseline conditions in the heart, derived predominantly from eNOS in both cell types. The fate of the relatively greater amounts of NO released by CMECs is unknown, but spillover diffusion into the cardiomyocytes is possible where it may contribute to baseline regulation and function of the myocytes. **(D)** After exposure to hypoxia, eNOS was both upregulated (ischaemic pelleting protocol: 1.8-fold increase; $\downarrow PO_2$ incubation protocol: 2.1-fold increase) and activated (ischaemic pelleting: 4.9-fold increase; $\downarrow PO_2$ incubation: 3-fold increase) in CMECs. Upregulation and activation of eNOS in the CMECs were associated with 1.45 – 1.55-fold increase in NO-production. In the myocytes, 60 min ischaemic pelleting hypoxia caused eNOS activation (1.5-fold increase), which was associated with a 1.4-fold increase in NO-production. Inducible NOS (iNOS) was expressed in the myocytes, but no changes were observed in hypoxia. It is possible that the increases in NO-production observed over baseline will create an even bigger diffusion gradient from the CMECs to cardiomyoctes during hypoxia. This could lead to the diffusion of a significant portion of the CMEC-released NO into the underlying cardiomyocytes, however, the nature of the spill-over effects in this case is unknown, and could be either harmful or beneficial.

ADDENDUM 1: List of publications resulting directly from this study:

Strijdom H, Genade S, Lochner A. Nitric oxide synthase (NOS) does not contribute to simulated ischaemic preconditioning in an isolated rat cardiomyocyte model. Cardiovasc Drugs Ther 2004; 18: 99-112.

Strijdom H, Muller C, Lochner A. Direct intracellular nitric oxide (NO) detection in isolated adult cardiomyocytes: Flow cytometric analysis using the fluorescent probe, diaminofluorescein (DAF). J Mol Cell Cardiol 2004; 37: 897 – 902.

Strijdom H, Jacobs S, Hattingh S, Page C, Lochner A. Nitric oxide production is higher in rat cardiac microvessel endothelial cells than ventricular cardiomyocytes in baseline and hypoxic conditions: a comparative study. FASEB J 2006; 20(2): 14 – 316.

ADDENDUM 2: List of publications resulting indirectly from this study:

Marais E, Genade S, **Strijdom J**, Moolman J, Lochner A. Activation of p38 MAPK induced by a multi-cycle ischaemia preconditioning protocol associated with attenuated p38 MAPK activity during sustained ischaemia and reperfusion. J Mol Cell Cardiol 2001; 33: 769 – 778.

Marais E, Genade S, **Strijdom H**, Moolman J, Lochner A. P38 MAPK activation triggers pharmacologically-induced beta-adrenergic preconditioning, but not ischaemic preconditioning. J Mol Cell Cardiol 2001; 33: 2157 – 2177.

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Lampiao F, **Strijdom H**, Du Plessis S. Direct nitric oxide measurement in human spermatozoa: flow cytometric analysis using the fluorescent probe, diaminofluorescein. International Journal of Andrology 2006; In Press.

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ENDOKRINOLOGIE 3
ENDOCRINOLOGY 3

MBChB II

2007

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DIE VETOPLOSBARE HORMONE... Hierdie hormone behoort aan 'n klas molekules wat geredelik in vette kan oplos, en dus as gevolg hiervan oor selmembrane kan beweeg sonder die hulp van 'n RESEPTOR-SEINTRANSDUKSIE pad. Steroïed hormone is seker die bekendste voorbeelde van vetoplosbare hormone, maar daar is ander...

EIENSKAPPE IN PLASMA	TEIKEN-SEL GEBEURE	FINALE EFFEKTE
1. Vervoer via draerproteïene (kan nie in plasma oplos nie!)	1. Bind aan intrasellulêre reseptore (sitoplasma of nuklêer)	1. Lei tot die induksie (sintese) van nuwe proteïene of die staking van die sintese van proteïene
2. Plasma konsentrasies is baie stabiel en voorspelbaar	2. Finale effekte word bereik via hormone se werking in die selkern	2. Die proteïene wat deur vetoplosbare hormone beïnvloed word, is: intrasellulêre ensieme of hormoon / neurotransmitter reseptore
3. Neem redelik lank om uit plasma verwyder te word	3. Veroorsaak veranderinge op die vlak van DNA (transkripsie / proteïen-sintese); d.w.s. aktivering of onderdukking van gene	3. Lei gewoonlik tot 'n nuwe funksie of voorkoms van 'n sel (metamorfose / differensiasie)
4. Afskeiding in plasma word ALTYD gereguleer deur die sg. relay proteïen-hormone		4. Effekte op teikenselle is gewoonlik onomkeerbaar
		3. Vanweë die feit dat die effekte op transkripsie / proteïensintese vlak waargeneem word, is hulle werking redelik stadig

KLASSIFIKASIE VAN VETOPLOSBARE HORMONE...

Onthou asb dat nie alle vetoplosbare hormone NOODWENDIG steroïed hormone is nie!!

A. DIE HORMONE VAN DIE BYNIER KORTEKS

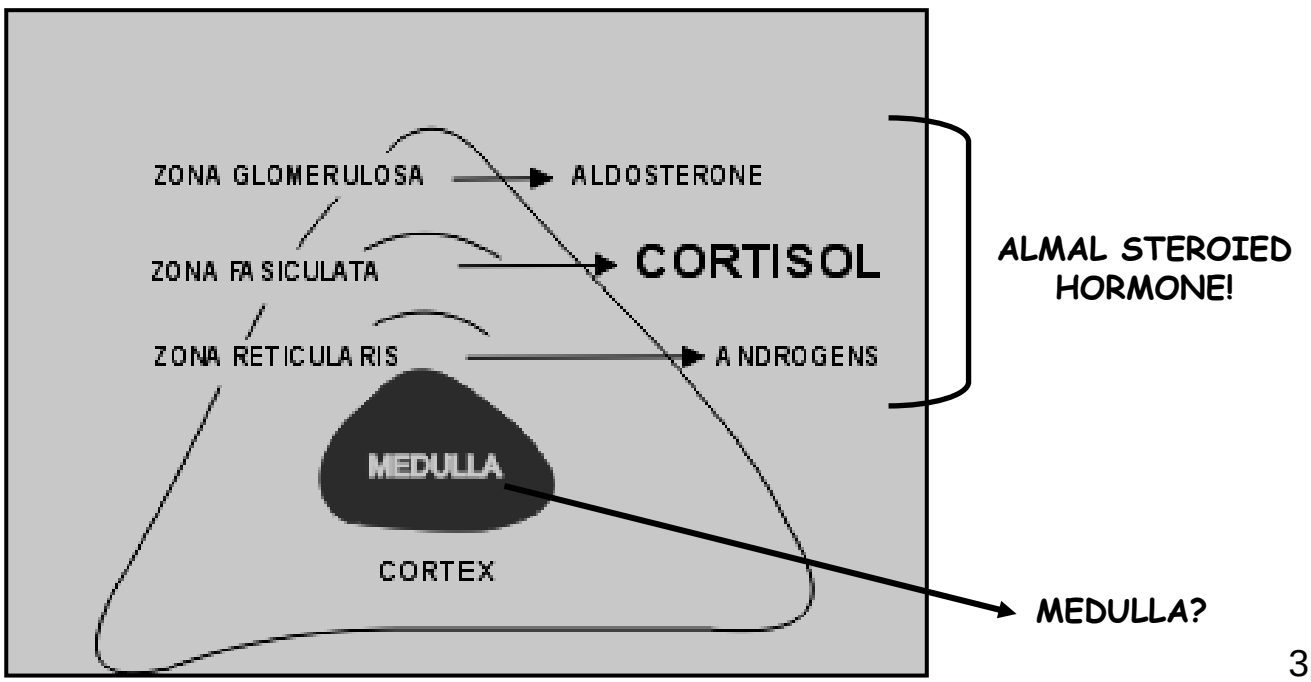
- Aldosteroon (vorm deel van 'n teuel beheer paar wat die interne omgewing reguleer!!)
- Glukokortikoiede (Kortisol en Kortikosteroon)
- Androgene (Dehidro-epi-androsteroon: DHEA)

B. DIE TIROIED HORMONE

C. DIE GESLAGSHORMONE

D. GROEI HORMOON: TREE SOOS VETOPLOSBAAR OP

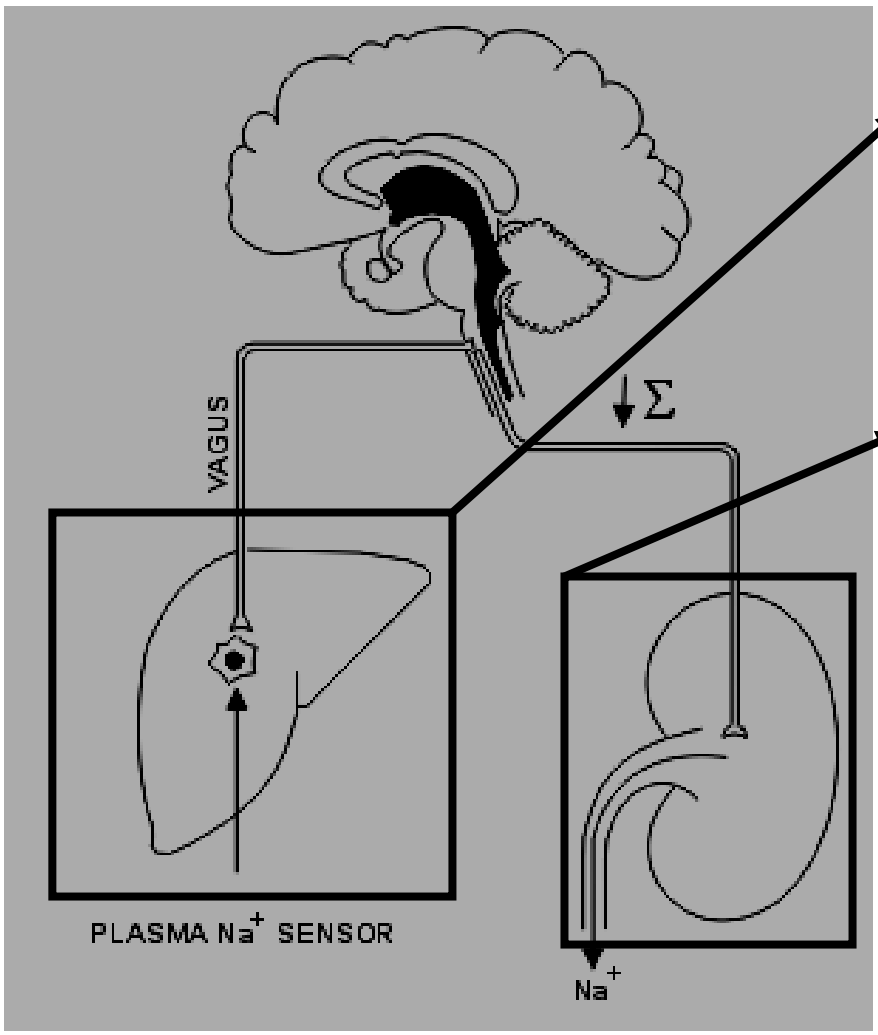
A. DIE BYNIERKORTEKS HORMONE



Die bynierkorteks hormone

DIE ALDOSTEROON - HEPATO-RENALE REFLEKS TEUEL BEHEER PAAR...

Hierdie voorbeeld van 'n teuel-beheer paar is in twee opsigte uniek: (i) Aldosteroon is nie 'n wateroplosbare hormoon nie, en (ii) die hepato-renale refleks is nie 'n hormoon nie. In alle ander opsigte voldoen hierdie paar aan die vereistes van interne omgewing reguleerders wat hul effekte via teuel-beheer uitoefen.



HEPATO = LEWER:

• Lewer selle bevat Na^+ sensitiewe reseptore wat deur ↑ Na^+ vlakke in die plasma gestimuleer word

RENALE = NIER:

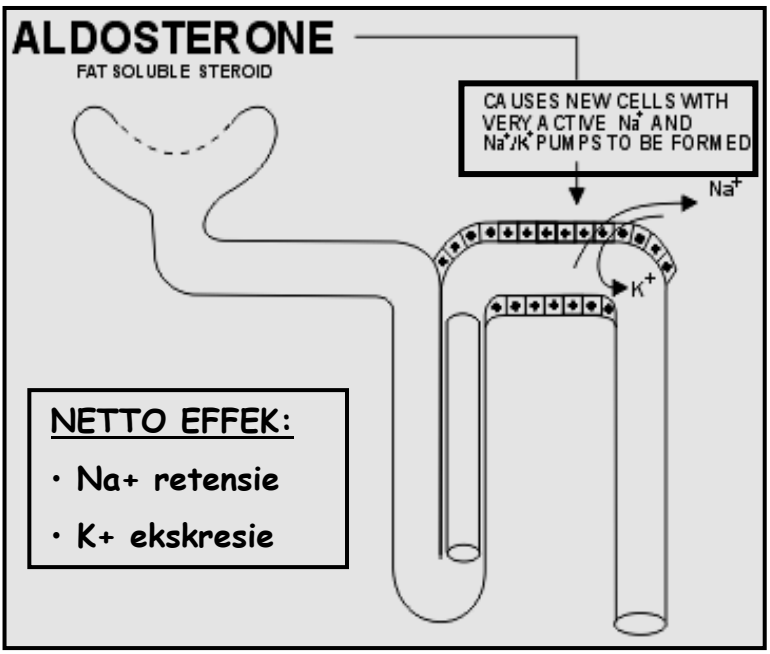
• Veranderinge in die renale gloeruli en tubules lei tot Na^+ ekskresie in die urine

REFLEKS = VAGUS:

• Na^+ sensitiewe selle in die lewer stimuleer Vagus wat tot die onderdukking van die simpatiese senuweesisteem en dus toename in glomerulêre filtrasie en Na^+ ekskresie lei.

Die Hepato-Renale Refleks

ALDOSTEROON... Steroïed hormoon; sy afskeiding word deur die relay hormoon ANG II beheer; Lei tot die sintese van Na^+ en Na^+/K^+ pompe in die distale nierbuisies.



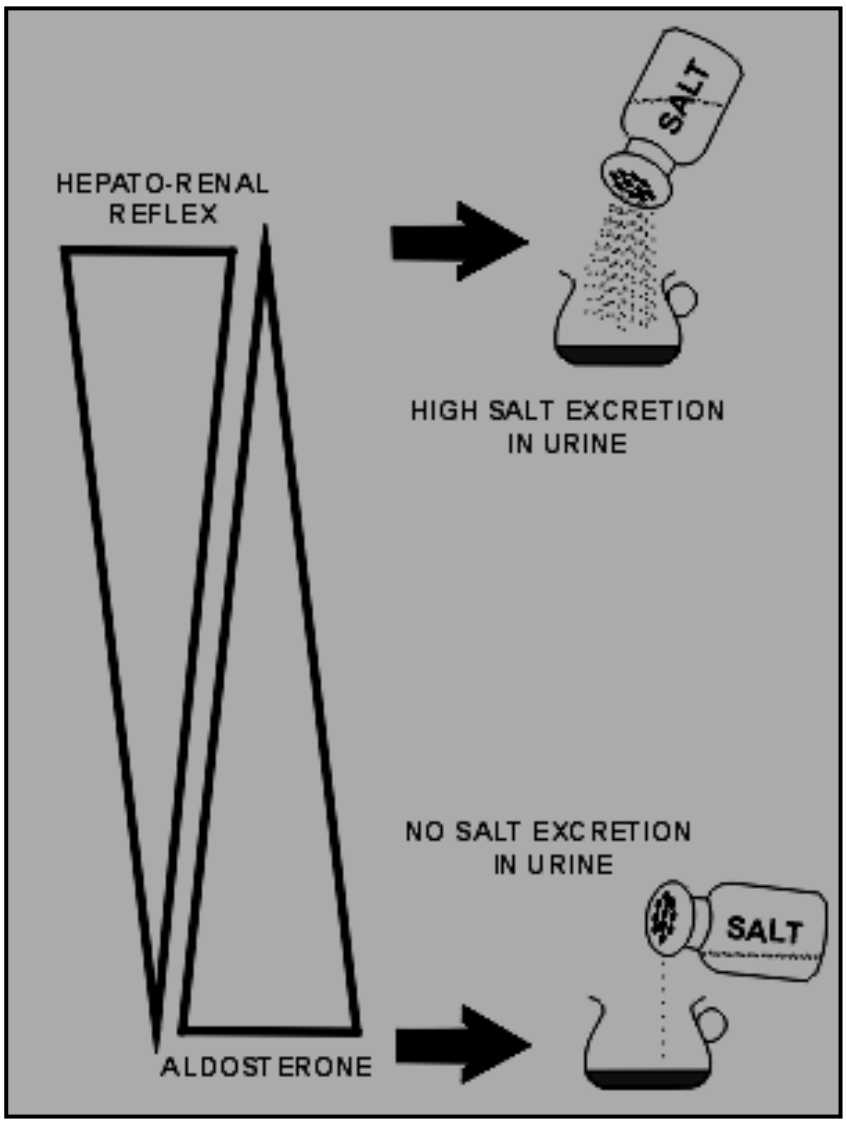
Effekte van Aldosteroon

ALDOSTEROON:

• Dit is 'n vetoplosbare hormoon wat as 'n INTERNE OMGEWING REGULEERDER dien;

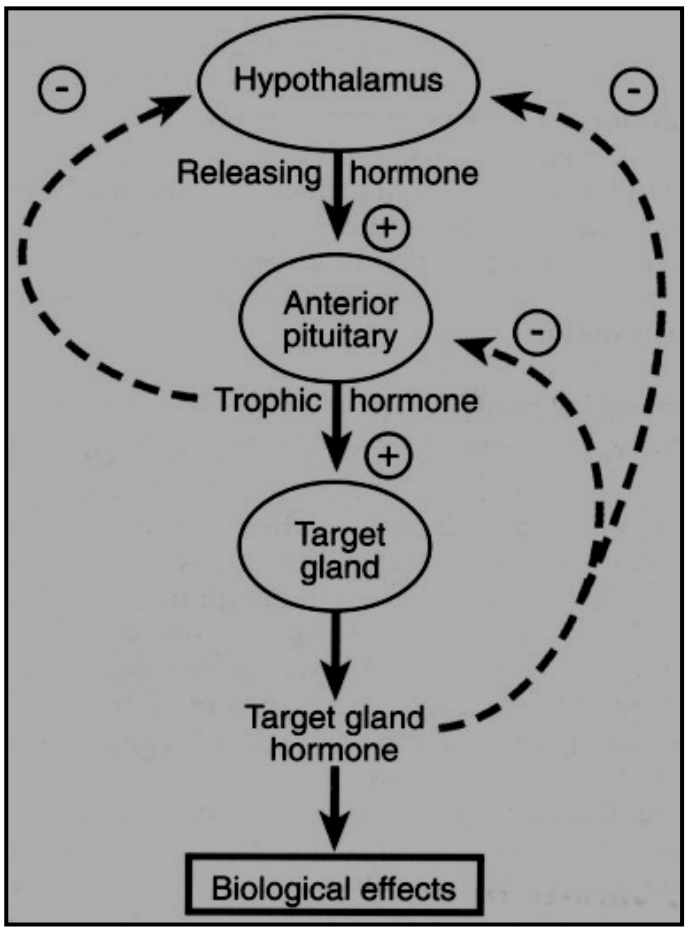
• Sy afskeiding vanuit die bynierkorteks word deur 3 FAKTORE gestimuleer: (i) ANG II, (ii) ↑ K^+ in die plasma, en (iii) ↓ plasma osmolaliteit

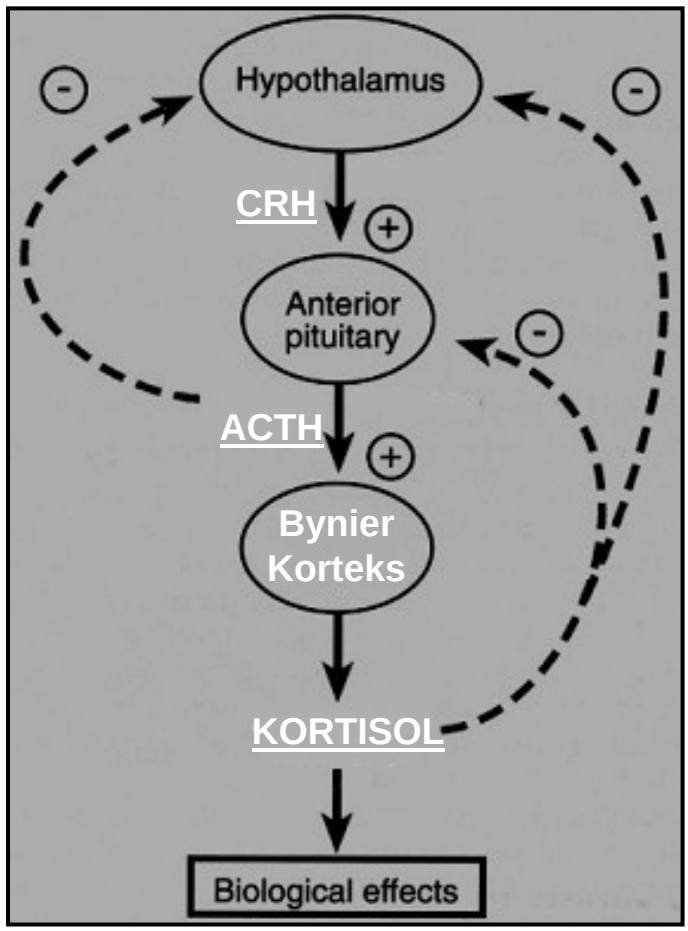
• Vorm ook 'n TEUEL-BEHEER paar saam met die hepato-renale refleks in die beheer van plasma Na^+ vlakke



Aldosteroon-HRR teuel-beheer

KORTISOL... Steroïed-hormoon. Afskeiding word deur die hipotalamiese-ant. hipofisêre as beheer (Hipotalamus: CRH = corticotropin releasing hormone; Ant. Hipofise: ACTH = adrenocorticotrophic hormone, of corticotropin)



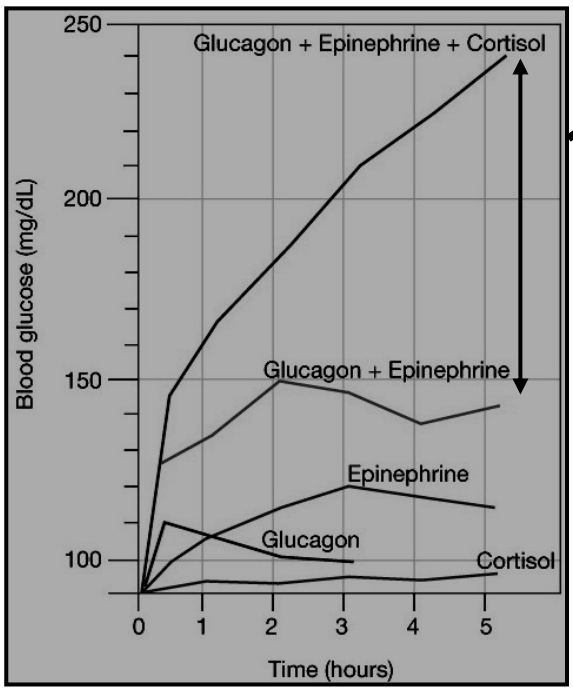


Kortisol se HH-as

BIOLOGIESE EFFEKTE VAN KORTISOL...

NB: "CORTISOL IS ESSENTIAL FOR LIFE"

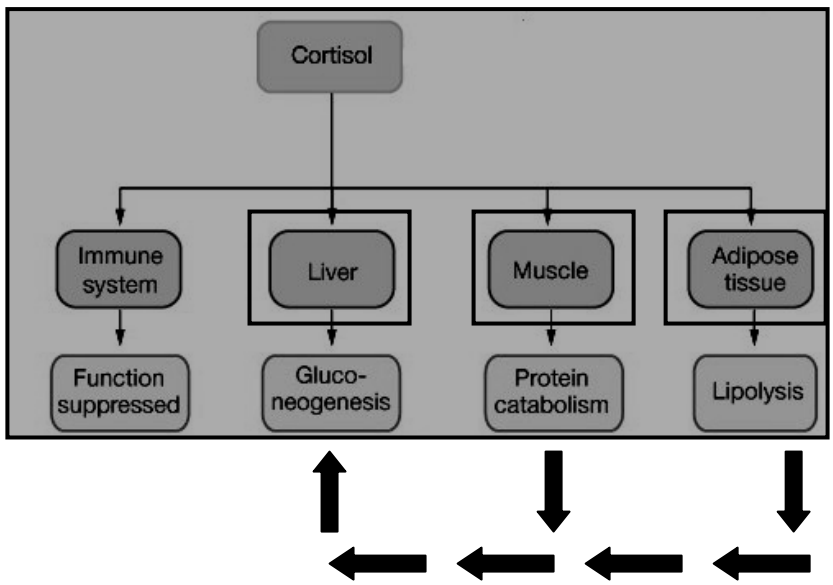
• Word algemeen as een van die liggaam se belangrikste stres-hormone geklassifiseer (Adrenalien: kort-werkend; Kortisol: lang-werkend). Die meting van kortisol-vlakke kan as aanduiding van emosionele stres gebruik word.
• Kortisol is basies 'n hormoon met METABOLIESE effekte, en die belangrikste uitwerking van kortisol is om HIPOGLUKEMIE teen te werk. Kortisol is dus, saam met Adrenalien en Glukagon, 'n diabetogene hormoon, want dit lei tot ↑ plasma glukose vlakke.



Kortisol se effek op Glukagon en Adrenalien se diabetogene effekte

• Kortisol is essensiëel vir Glukagon en Adrenalien om tydens hipoglukemie EFFEKTIEWE ↑ glukose-vlakke te bewerkstellig

Kortisol se biologiese effekte

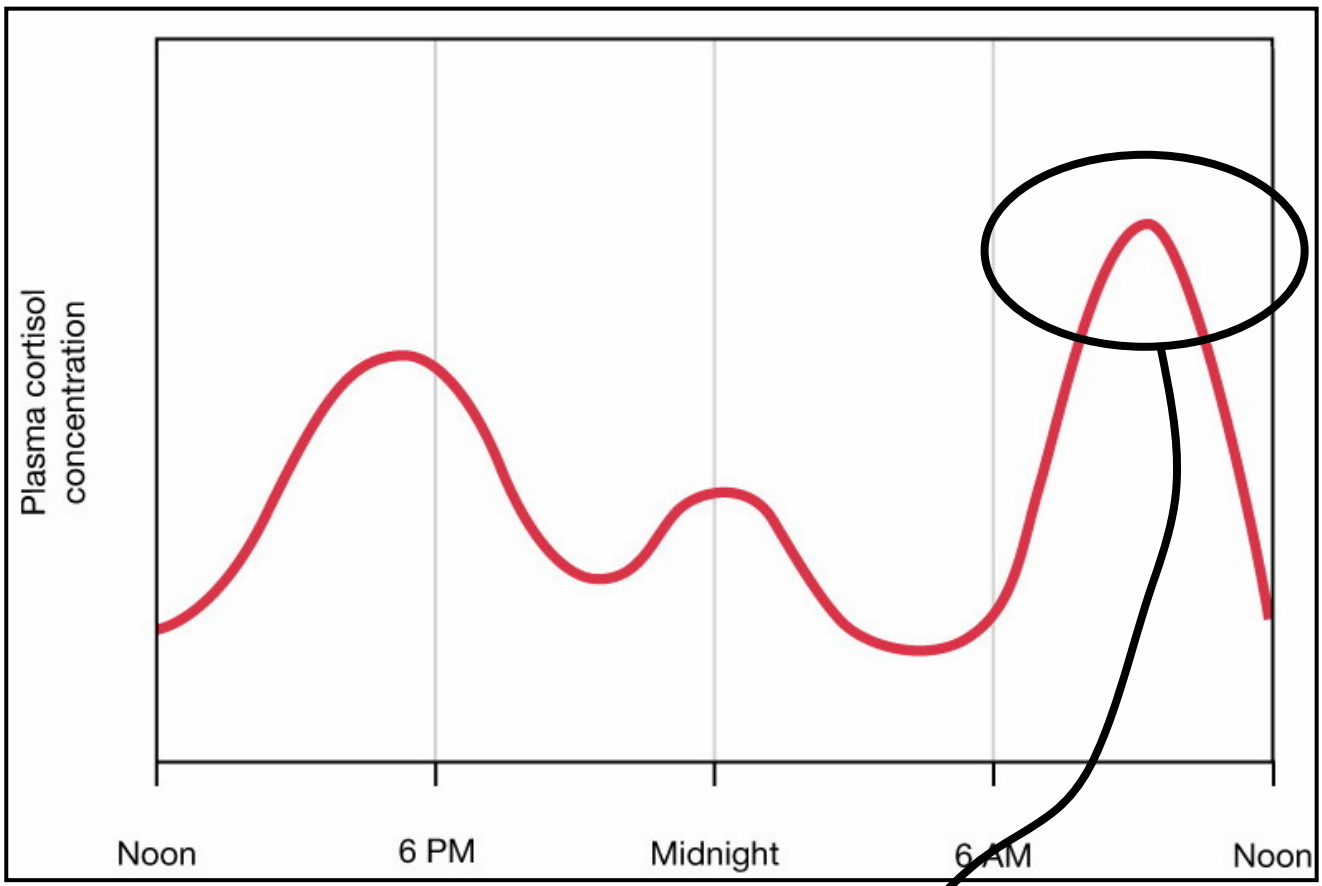


GLUKONEOGENESE!!

• Kortisol is ook 'n hoogs effektiewe immuun-onderdrukker. Dit word veroorsaak deur: (i) Inhibisie van sitokien-vrystelling en antiliggaam produksie deur witbloedselle; (ii) Onderdukking van die inflammatoriese reaksie deur leukosiet-beweging te inhibeer

POTENSIAAL AS 'N MEDIKASIE? Kortikosteroïede...

KORTISOL WORD RITMIES AFGESKEI... Vergelyk Melatonien en Groeihormoon!!



Kortisol se sirkadiese ritme

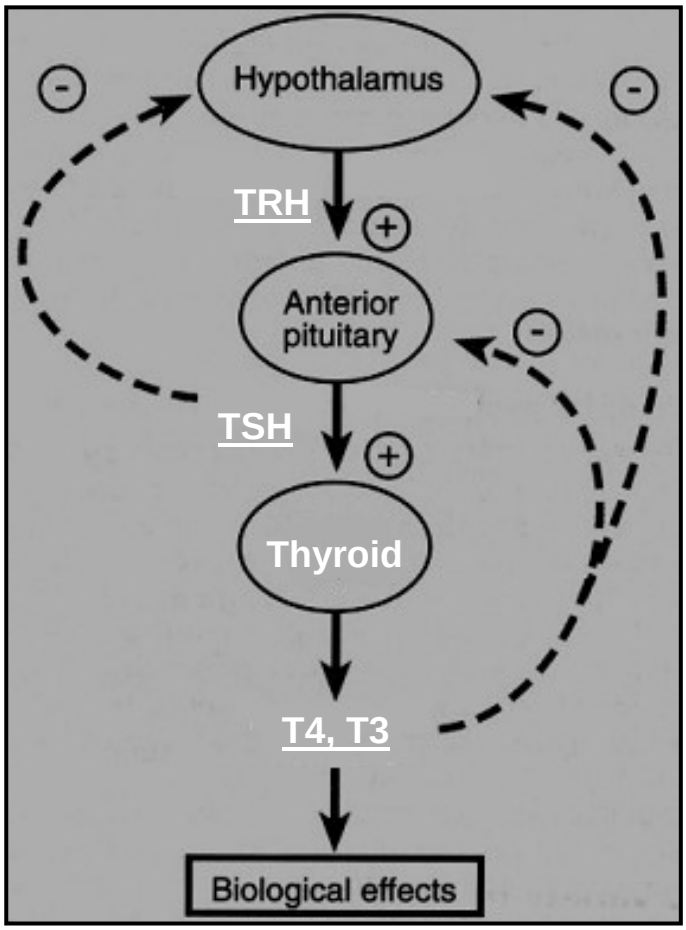
WATTER IMPLIKASIE HET DIT OP DIE TYDSTIP WANNEER 'N DOKTER BLOEDVLAKKE WIL BEPAAL?

SELFSTUDIE:

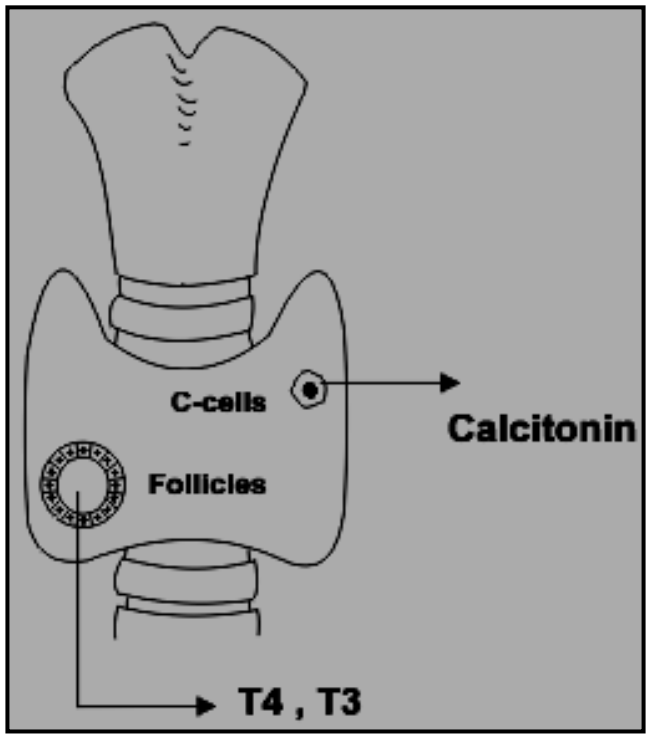
- KORTISOL TEKORT: "Addison se Siekte"
- KORTISOL OORMAAT: "Cushing se Siekte"
- http://academic.sun.ac.za/medphys/endo4.htm#i1

DHEA... Word in baie klein hoeveelhede in beide mans en vrouens afgeskei. In vrouens, is die bynierkorteks die belangrikste bron van androgene, terwyl dit 'n sekondêre rol in mans speel, veral na puberteit wanneer die androgene van die testis (veral testosteroon) oorheers.

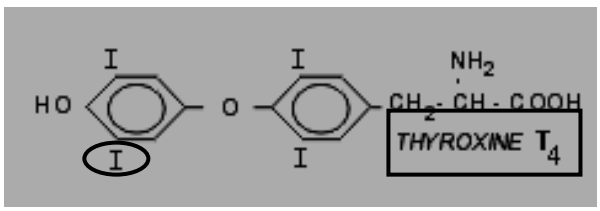
B. DIE TIROÏED HORMONE... Die tiroïed hormone is aminosuur-derivate (van tirosien), maar is nogtans vetoplosbaar. Die afskeiding van tiroïed hormone word beheer deur die HH-as (Hipotalamus = TRH: thyrotropin releasing hormone en Ant. Hipofise = TSH: thyroid stimulating hormone, of tirotropien). Die tiroïed hormone word mbv 'n draerproteïen (TBG) in bloed vervoer

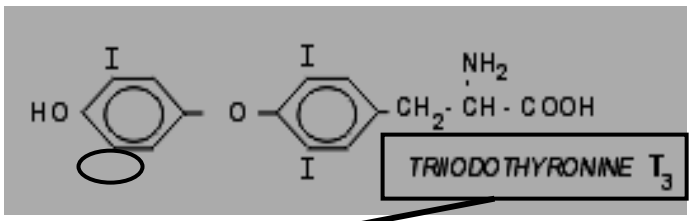


Die HH-as van T4 en T3



T4 en T3 afskeiding





3 - 5x meer aktief as T4!!

SELFSTUDIE:

Die sintese van tiroïed-hormone in die follikulêre selle, en die rol van kolloïed, jodium, en tiroglobulien. Silverthorn bl. 733, 744

BIOLOGIESE EFFEKTE VAN TIROÏED HORMOON:

- Essensieel vir normale groei en ontwikkeling in kinders
- ↑ suurstof verbruik in meeste weefsels
- ß-reseptor sintese
- Interaksie met ander hormone tydens metaboliese reaksies (bv. verhoog adrenalien se vermoë om vry vetsure van vetselle te sekreteer)

DIE EFFEKTE VAN TIROÏED HORMOON WORD DUIDELIKER AS DAAR 'N OORPRODUKSIE OF ONDER-PRODUKSIE PLAASVIND...

A. ONDER-PRODUKSIE TYDENS KINDERJARE: "Kretinisme"

sien: http://academic.sun.ac.za/medphys/endo3.htm#g1 vir meer inligting

B. ONDER-PRODUKSIE IN VOLWASSENES: "Hipotiroïedisme"

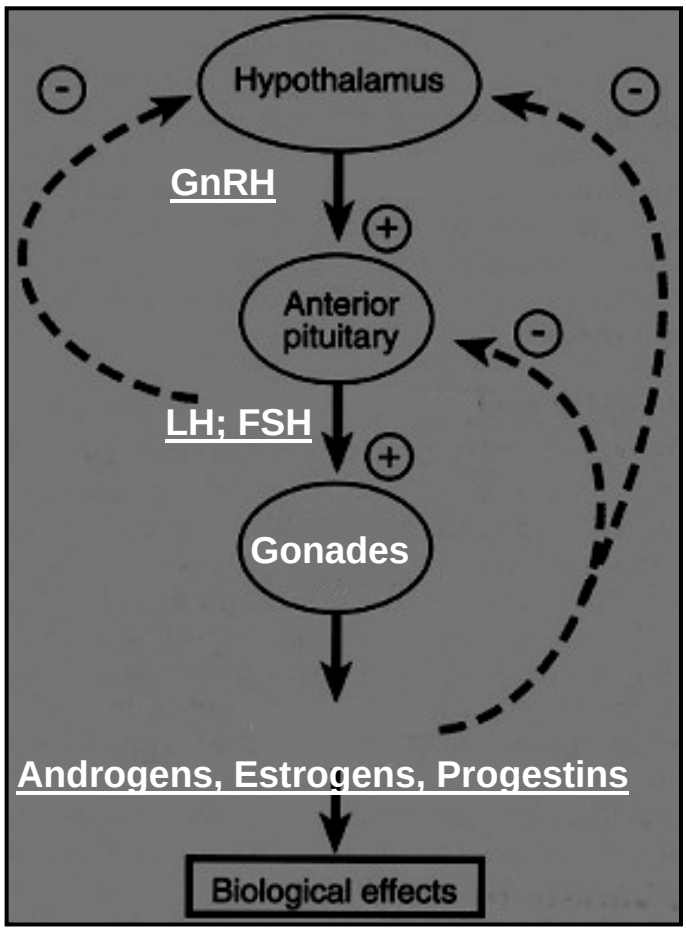
(i) ↓ metaboliese tempo en suurstof verbruik(d.w.s. minder interne hitte opgewek = koue intolerant); (ii) ↓ proteïensintese (swak naels, dun hare, droeë, dun vel); (iii) depressie; (iv) bradikardie

C. OOR-PRODUKSIE: "Hipertiroïedisme"

(i) ↑ metaboliese tempo en suurstof verbruik(d.w.s. meer interne hitte opgewek = hitte intolerant; klam, sweterige hande); (ii) ↑ prikkelbaarheid; irriteerbaar; slapeloosheid; (iii) tagikardie

C. DIE GESLAGS-HORMONE...

Die klassieke geslagshormone is steroïed-hormone wat in die gonades (man: testis; vrou: ovarium) gesintetiseer en afgeskei word. Daar is 'n derde groep geslags-hormone wat in die bynier korteks afgeskei word (androgeen: DHEA) – reeds behandel. Die geslagshormone staan onder beheer van die HH-as (Hipotalamus: GnRH = gonadotropin releasing hormone; Ant. Hipofise: LH = luteinizing hormone en FSH = follicle stimulating hormone).



Die HH-as van die geslagshormone

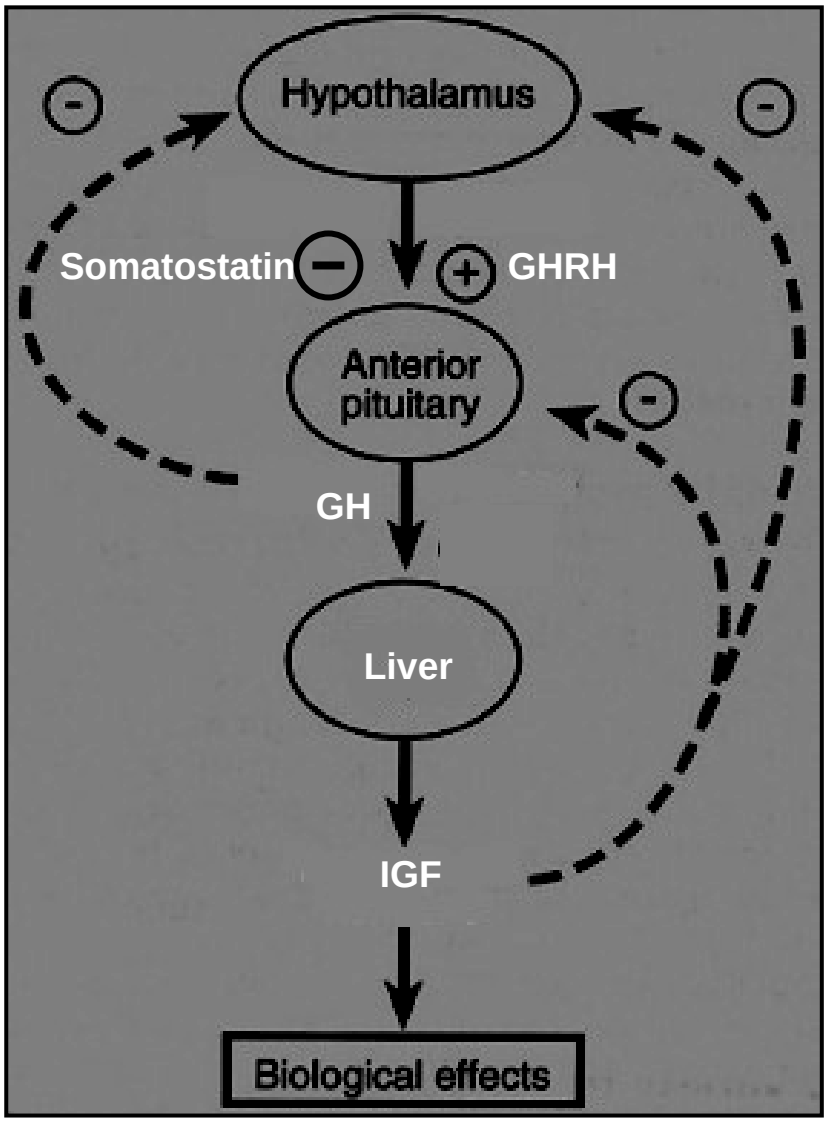
KLASSIFIKASIE:

- Androgene: Testosteroon (testis) en DHEA (bynierorteks)
- Estrogene: Estradiol
- Progestiene: Progesteroone

FUNKSIES:

- Testosteroon: Ontwikkeling van spermselle; ontwikkeling en maturasie van manlik geslagsorgane, insluitende sekondêre manlike geslagskenmerke.
- Estradiol: Ontwikkeling van die eierselle (oösiete); beheer van menstruasie (veral follikulêre fase); ontwikkeling en maturasie van die vroulike geslagsorgane, insluitende sekondêre geslagskenmerke.
- Progesteroon: Die hormoon van swnagerskap; voorbereiding en handhawing van swangerskap.

GROEIHORMOON... 'n Proteïen-hormoon wat vanaf die Ant. Hipofise afgeskei word; vorm saam met tiroïed hormoon, insulien en geslagshormone die klassieke groep hormone wat vir normale groei en ontwikkeling verantwoordelik is. 'n Tekort aan enige van hierdie hormone sal normale groei op een of ander manier beïnvloed. Alhoewel GH 'n proteïen-hormoon is, besit dit baie eienskappe wat eerder met vetoplosbare hormone verbind word: (i) GH se afskeiding word deur relay hormone vanaf die hipotalamus gereguleer, (ii) 'n belangrike meganisme van werking op sellulêre vlak is geen-aktivering en proteïensintese, en (iii) minstens 50% van die plasma GH worddmv 'n draerproteïen vervoer.



Groeihormoon afskeiding

GH FUNKSIES:

- GH reguleer groei en ontwikkeling in kinders (been en weefsel groei via die induksie van proteïen sintese)
- GH stimuleer verhoogde glukose vrystelling van die lewer, en dus verhoogde plasma glukose.
- GH die as 'n relay hormoon wat die sekresie van IGF (Insulin-like Growth Factor) reguleer. IGF is primêr verantwoordelik vir kraakbeengroei, hoewel dit ook bg funksies met GH deel.

AFWYKINGS VAN GH AFSKEIDING:

1. ONDER-PRODUKSIE IN KINDERJARE: Dwerg Groei
   (vergelyk 'n GH-tekort dwerg met 'n tiroïed-hormoon tekort dwerg)

2. OOR-PRODUKSIE IN KINDERJARE: Reusegroei
   ("Gigantism")

3. OOR-PRODUKSIE IN VOLWASSENES: Akromegalie

Meer info oor bg: http://academic.sun.ac.za/medphys/endo3.htm#h1

SELFSTUDIE:

Die verterings hormone ... Silverthorn: Table 21-2; p. 672-673

BELANGRIKE ONDERWERPE VAN TEMA 6, SESSIES 1-5:

KONSEPTE:

- Die Vetoplosbare Hormoon
- Hepato-Renale Refleks
- Die Glukokortikoïede
- Stres-hormoon
- Sirkadiese Ritme
- Die Hipotalamiese-Hipofisêre As
- Kretinisme, Hipotiroïedisme, Hipertiroïedisme
- Hipotiroïed-Dwerg vs. Groeihormoon-tekort dwerge
- Gigantisme vs. Akromegalie

UITKOMSTE IN STUDIEGIDS:

- Bl. 16, Uitkomste 1-6
- Bl. 17, Sessie 1 Selfstudie vrae 1,2,4
- Bl. 17 Sessies 2-6 uitkomste 1,2,4-6.

THE FAT SOLUBLE HORMONES... These hormones belong to a class of molecules that readily dissolve in fats, and are therefore able to move across cell membranes without the help of a RECEPTOR-SIGNALING PATHWAY. Steroid hormones are probably the most well known examples of fat soluble hormones, but there are others…

PROPERTIES IN PLASMA	**TARGET CELL EVENTS**	**FINAL EFFECTS**
1. Transported via carrier proteins (can't dissolve in plasma!)	1. Bind to intracellular receptors (cytoplasma or nuclear)	1. Leads to the induction (synthesis) of new proteins or the cessation of protein synthesis
2. Plasma concentrations are very stable and predictable	2. Final effects are achieved via actions of the hormones in the cell nucleus.	2. Proteins that are affected by fat soluble hormones are: intracellular enzymes or hormone/neurotransmitter receptors
3. Removal from plasma is relatively slow	3. Cause changes on the level of DNA (transcription / protein synthesis); thus activation or suppression of genes	3. Usually leads to a new function or appearance of a cell (metamorphosis / differentiation)
4. Secretion in plasma is ALWAYS regulated by the so-called relay protein hormones		4. Effects on target cells are usually irreversible
		5. Due to its effects happening on transcription / protein synthesis level, it follows that the action is relatively slow.

CLASSIFICATION OF FAT SOLUBLE HORMONES...

Please remember that not all fat soluble hormones are NECESSARILY steroid hormones!!

A. THE HORMONES OF THE ADRENAL CORTEX

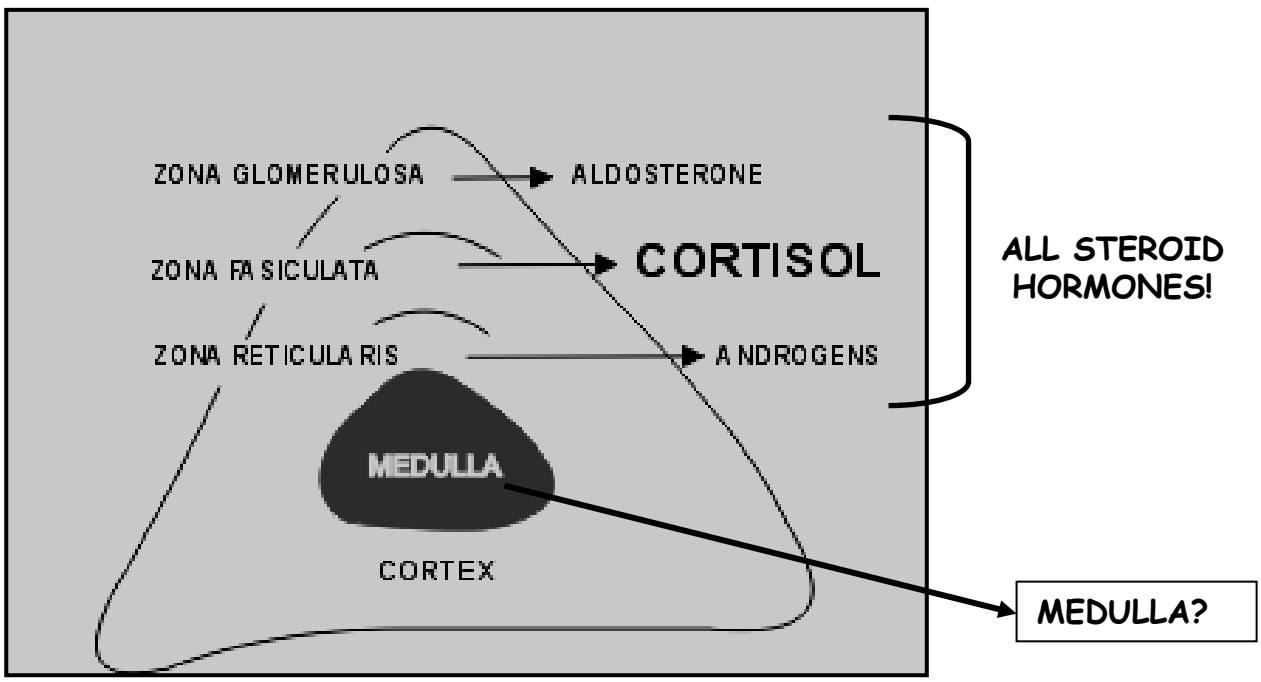
- Aldosterone (forms part of a rein control pair that regulates the internal environment!!)
- Glucocorticoids (Cortisol en Corticosterone)
- Androgens (Dehydro-epi-androsterone: DHEA)

B. THE THYROID HORMONES

C. THE SEX HORMONES

D. GROWTH HORMONE: *BEHAVES LIKE FAT SOLUBLE*

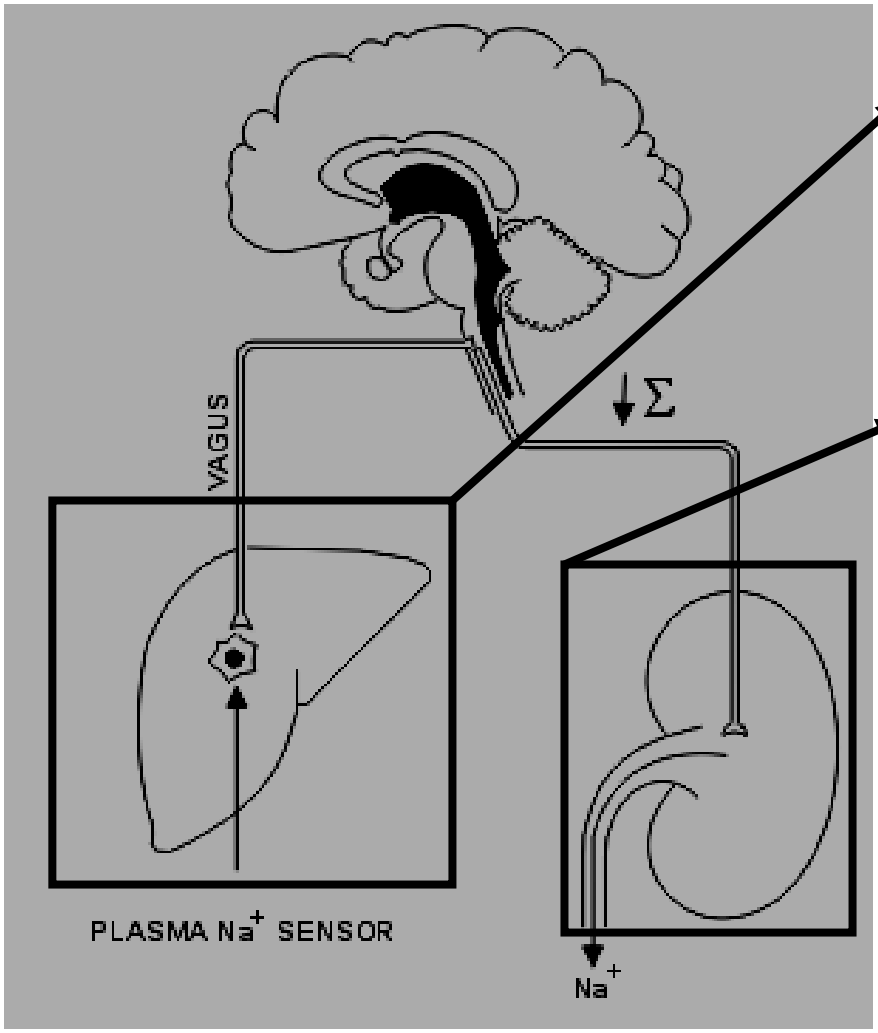
A. THE ADRENAL CORTEX HORMONES



The hormones of the adrenal cortex

THE ALDOSTERONE - HEPATO-RENALE REFLEX REIN CONTROL PAIR...

This example of a rein control pair is unique in two ways: (i) Aldosterone is not water soluble, and (ii) the hepato-renal reflex is not a hormone. In all other respects, this pair does fulfill the requirements of internal environment regulators that exert their effects via rein control mechanisms.



HEPATO = LIVER:

• Liver cells contain Na^+ sensitive receptors that are stimulated by ↑ Na^+ levels in the plasma

RENAL = KIDNEY:

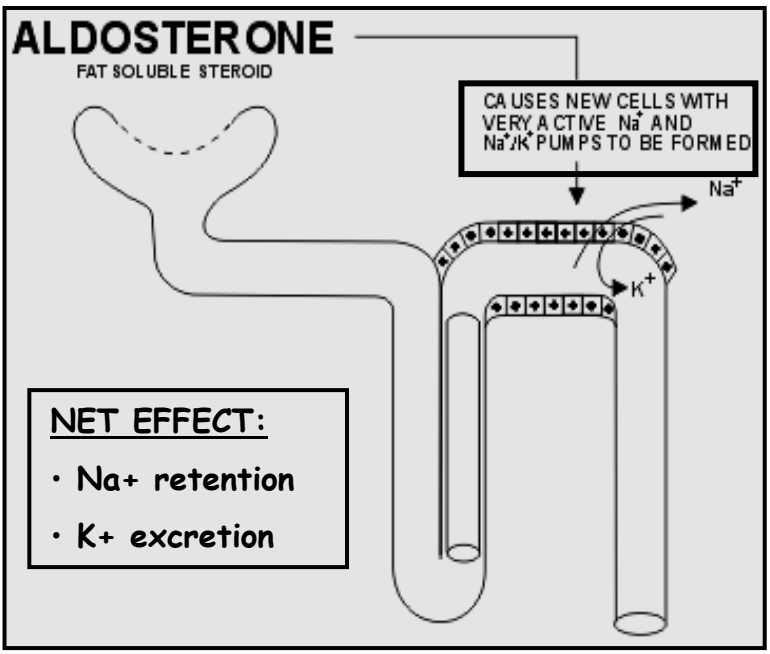
• Changes in the renal gloeruli and tubules lead to Na^+ excretion in the urine

REFLEX = VAGUS:

• Na^+ sensitive cells in the liver stimulate Vagus which leads to the suppression of the sympathetic nervous system and therefore increased glomerular filtration and Na^+ excretion.

The Hepato-Renal Reflex

ALDOSTERONE... Steroid hormone; its secretion is regulated by the relay hormone ANG II; Leads to the synthesis of Na^+ and Na^+/K^+ pumps in the distal renal tubules.



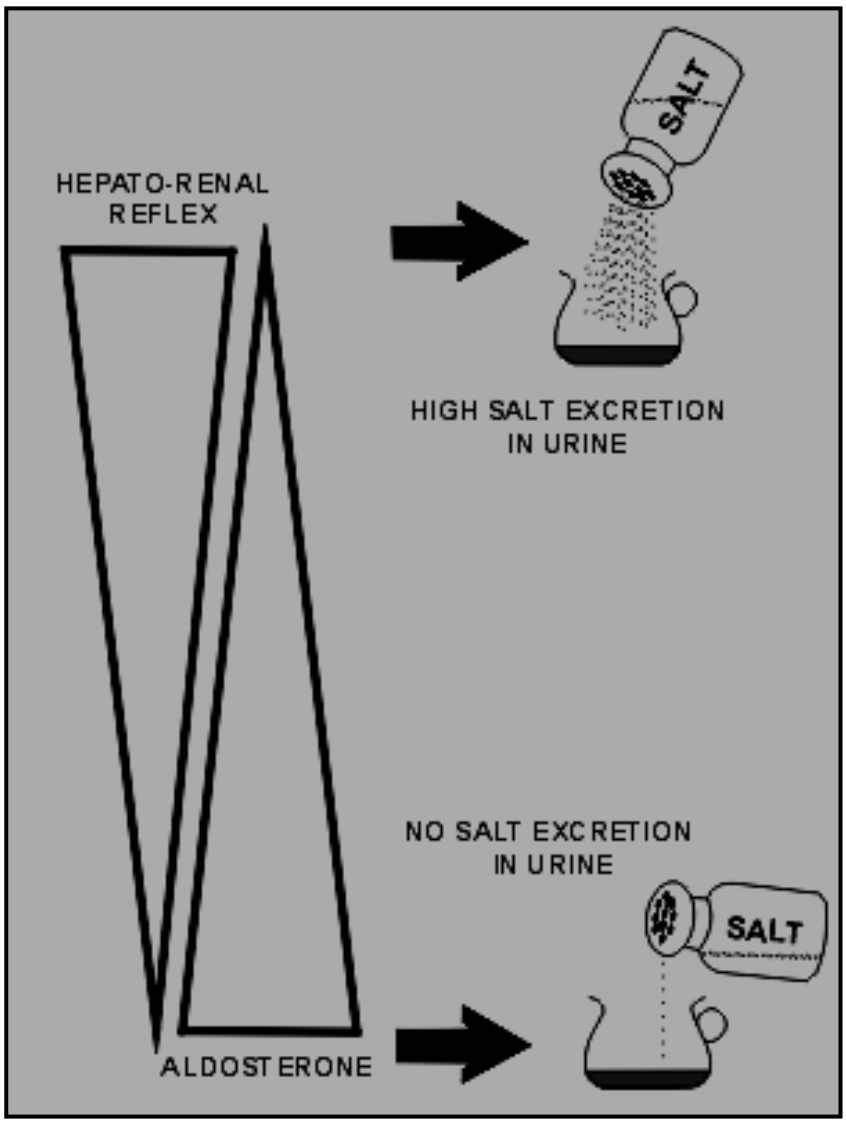
ALDOSTERONE:

• Is a fat soluble hormone that serves as an INTERNAL ENVIRONMENT REGULATOR;

• Its secretion from the adrenal cortex is stimulated by 3 FACTORS: (i) ANG II, (ii) ↑ K^+ in the plasma, and (iii) ↑ plasma osmolality

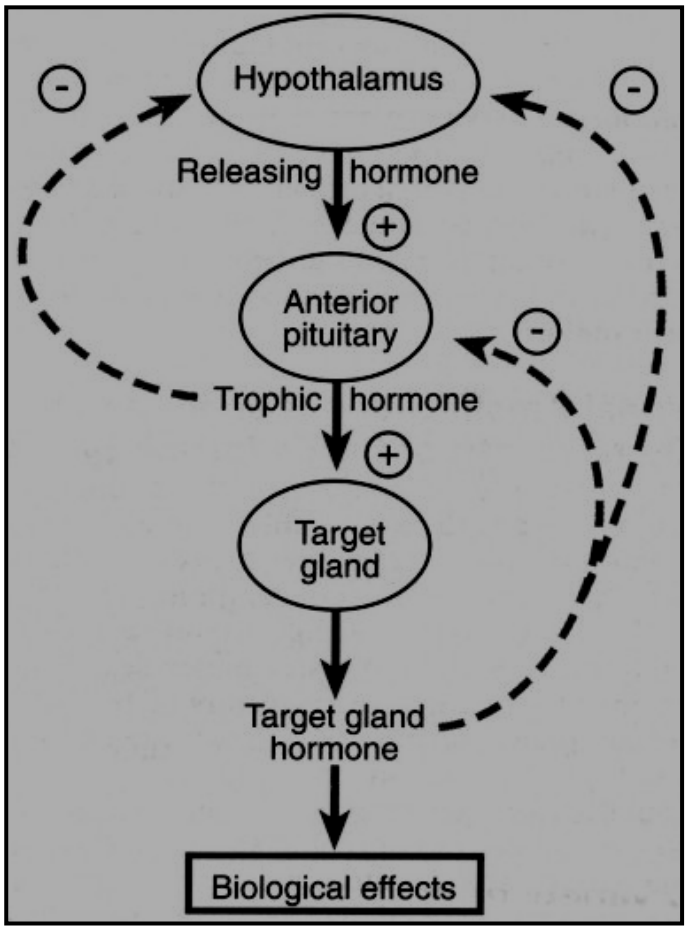
• Forms a REIN CONTROL pair with the hepato-renal reflex in the control of plasma Na^+ levels

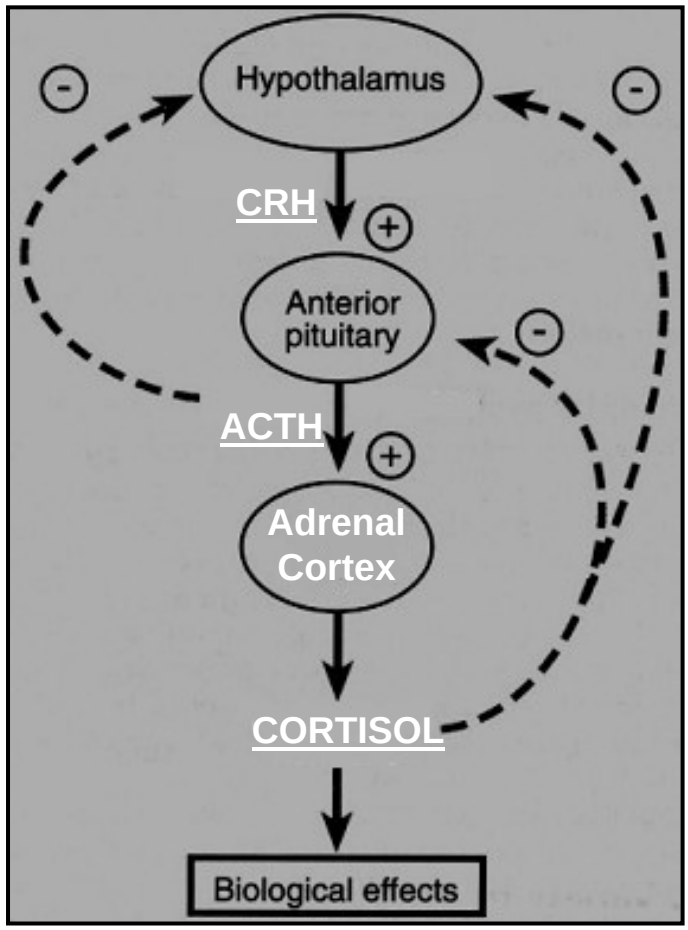
Effects of Aldosterone



Aldosterone-HRR rein control

CORTISOL... Steroid-hormone. Secretion is controlled by the hypothalamic-ant. pituitary axis (Hypothalamus: CRH = corticotropin releasing hormone; Ant. Pituitary: ACTH = adrenocorticotrophic hormone, or corticotropin)





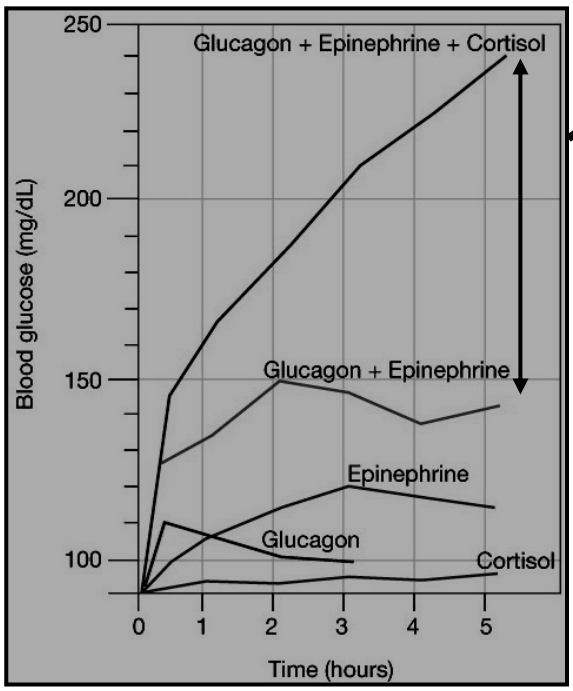
The HH-axis of cortisol

BIOLOGICAL EFFECTS OF CORTISOL...

NB: "CORTISOL IS ESSENTIAL FOR LIFE"

• Classified as one of the body's most important stress hormones (Adrenaline: short-working; Cortisol: long-working). The measurement of cortisol levels can be used as an indicator of emotional stress.

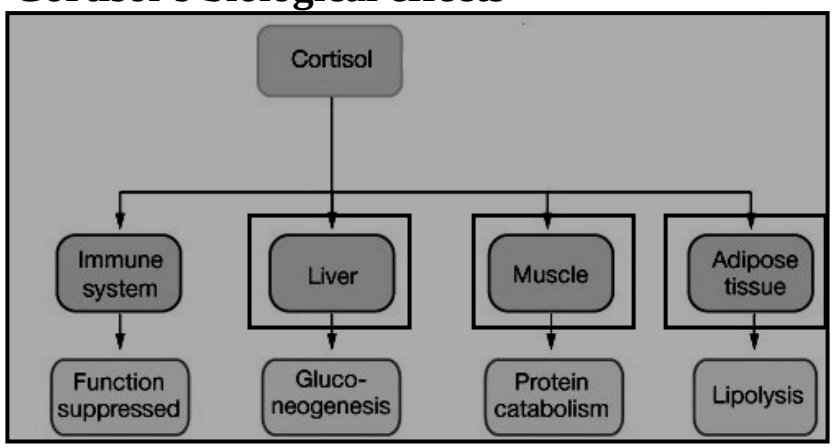
• Cortisol is basically a hormone with METABOLIC effects, and the most important effect of cortisol is to counter HYPOGLYCAEMIA. Cortisol is therefore, together with Adrenaline and Glucagon, a diabetogenic hormone, since it leads to ↑ plasma glucose levels.



Cortisol's effect on Glucagon and Adrenaline's diabetogenic effects

• Cortisol is essential for Glucagon and Adrenaline to achieve EFFECTIVE ↑ glucose-levels during hypoglycaemia

Cortisol's biological effects

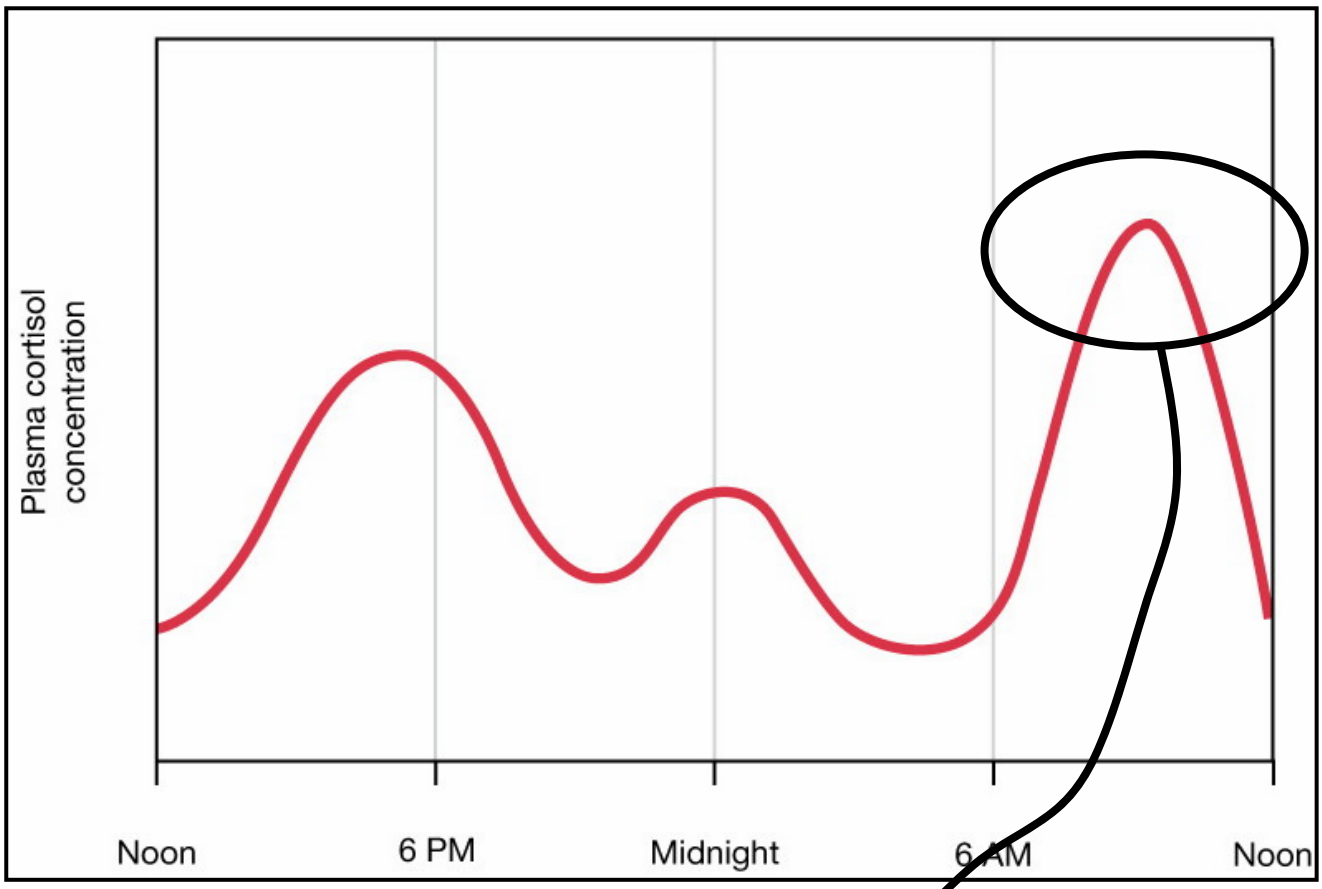


GLUCONEOGENESIS!!

• Cortisol is also a very efficient immune suppressant. It achieves this by: (i) Inhibition of cytokine-release and antibody production by white blood cells; (ii) Suppression of the inflammatory reaction by inhibiting leucocyte movement

POTENTIAL AS A MEDICATION? Corticosteroids...

CORTISOL-RELEASE IS RHYTHMIC... See also melatonin and Growth Hormone!!



Cortisol's circadian rhythm

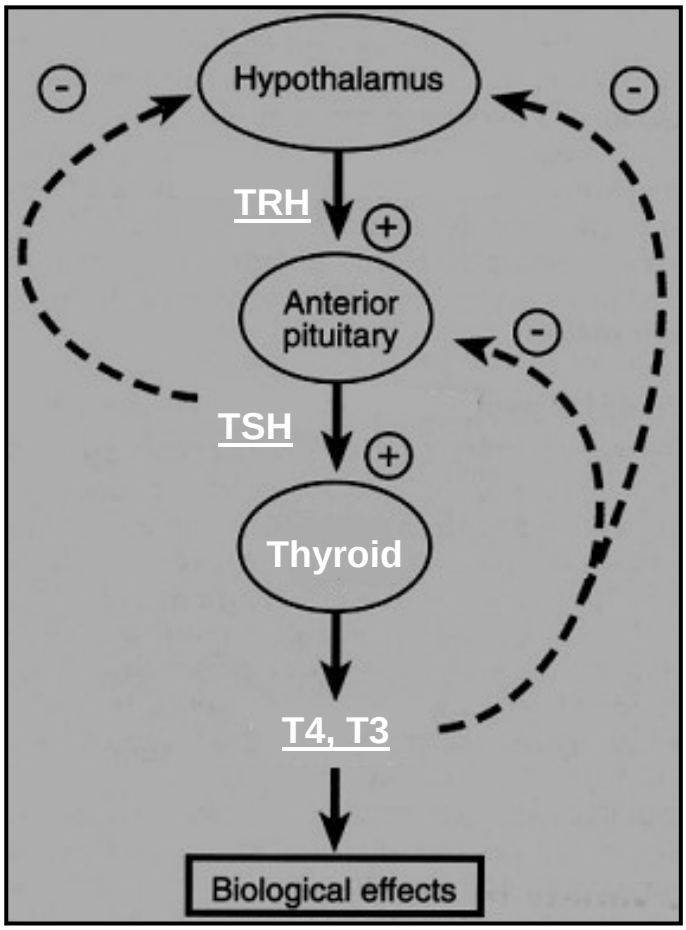
WHAT IMPLICATIONS DOES THIS HAVE ON THE TIMING OF BLOOD LEVEL MEASUREMENTS BY THE DOCTOR?

SELF STUDY:

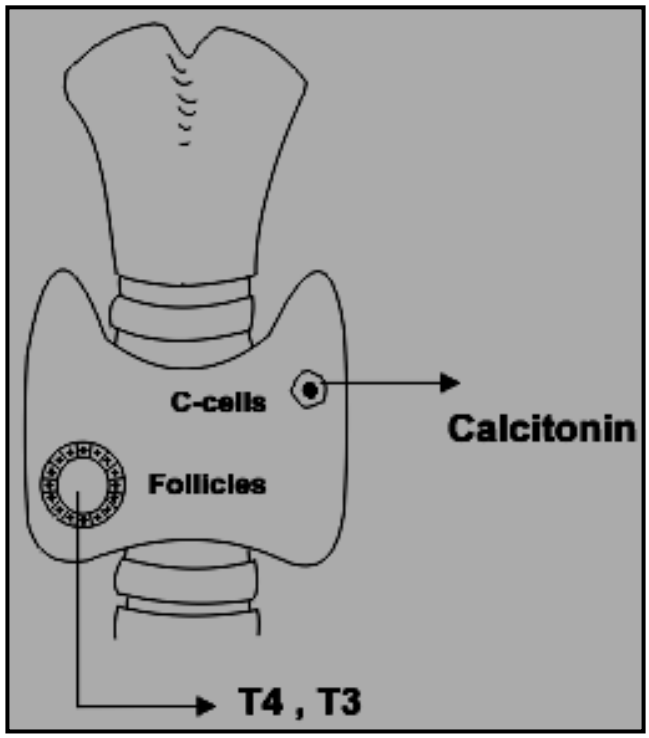
- CORTISOL DEFICIENCY: "Addison's Disease"
- CORTISOL EXCESS: "Cushing's Disease"
- http://academic.sun.ac.za/medphys/endo4.htm#i1

DHEA... Secreted in small quantities in both males and females. In females, the adrenal cortex is the most important source of androgens, whilst in males it plays a secondary role, especially after puberty when the androgens of the testis (testosterone) dominate.

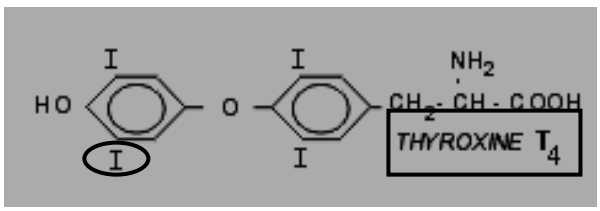
B. THE THYROID HORMONES... The thyroid hormones are amino acid derivatives (tyrosine), but are nevertheless fat soluble. The secretion of thyroid hormones is regulated by the HH axis (Hypothalamus = TRH: thyrotropin releasing hormone and Ant. Pituitary = TSH: thyroid stimulating hormone, or thyrotropin). The thyroid hormones are transported in blood by a carrier protein (TBG).

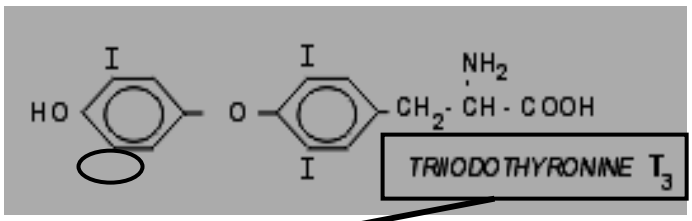


The HH-axis of T4 and T3



T4 and T3 secretion





3 - 5x more active than T4!!

SELF STUDY:

The synthesis of thyroid hormones in the follicular cells, and the role of colloid, iodide and thyroglobulin. Silverthorn p. 733, 744

BIOLOGICAL EFFECTS OF THYROID HORMONE:

- Essential for normal growth and development in children
- ↑ oxygen consumption in most tissues
- ß-receptor synthesis
- Interaction with other hormones during metabolic reactions (e.g. increases adrenaline's ability to release free fatty acids from adipocytes.)

THE EFFECTS OF THYROID HORMONES BECOME MORE APPARENT IN CASE OF INCREASED OR DECREASED PRODUCTION...

A. DECREASED PRODUCTION DURING CHILDHOOD: "Cretinism"

see: http://academic.sun.ac.za/medphys/endo3.htm#g1 for more info

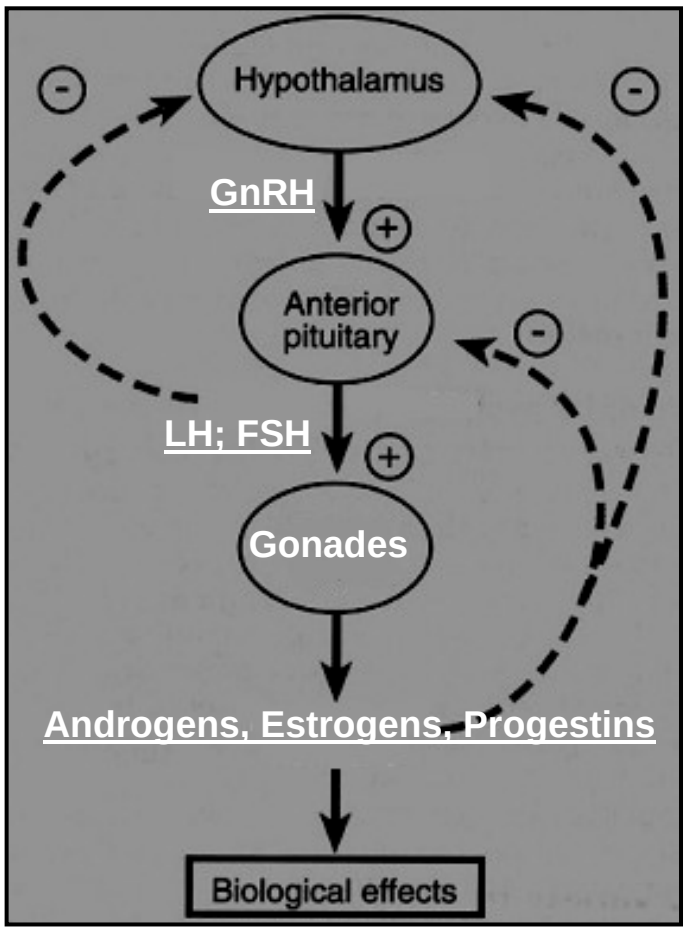
B. DECREASED PRODUCTION IN ADULTS: "Hypothyroidism"

(i) ↓ metabolic rate and oxygen consumption (i.e.. less internal heat generation = cold intolerant); (ii) ↓ protein synthesis (brittle nails, thin hair, dry, thin skin); (iii) depression; (iv) bradycardia

C. OVER-PRODUCTION: "Hyperthyroidism"

(i) ↑ metabolic rate and oxygen consumption (I.e. more internal heat generation - heat intolerant; clammy, sweaty hands); (ii) ↑ excitability; irritability; insomnia; (iii) tachycardia

C. THE SEX HORMONES... The classical sex hormones are steroid hormones that are synthesized and secreted in the gonades (male: testis; female: ovarium). There is a third group of sex hormones that are secreted in the adrenal cortex (androgen: DHEA) – see earlier. The sex hormones stand under control of the the HH-axis (Hypothalamus: GnRH = gonadotropin releasing hormone; Ant. Pituitary: LH = luteinizing hormone and FSH = follicle stimulating hormone).



The HH-axis of the sex hormones

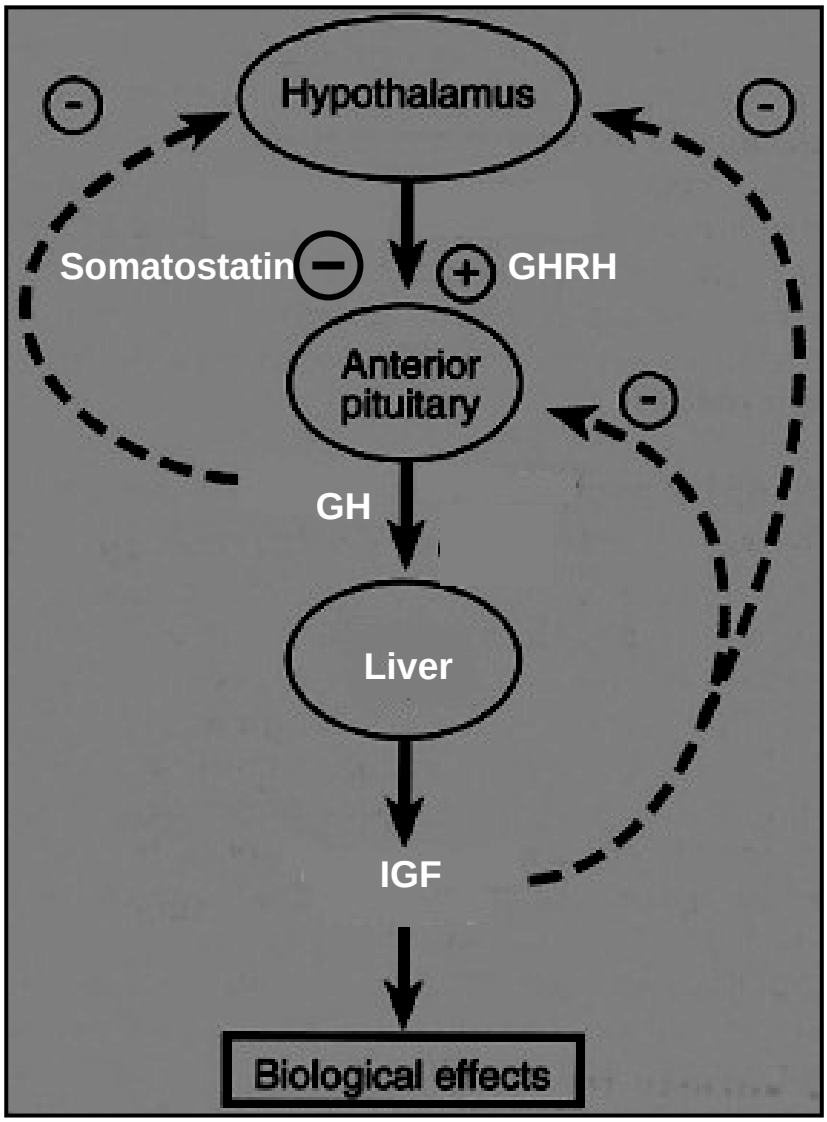
CLASSIFICATION:

- Androgens: Testosterone (testis) and DHEA (adrenal corteks)
- Estrogens: Estradiol
- Progestins: Progesterone

FUNCTIONS:

- Testosterone: Development of sperm cells; development and maturation of male sex organs, including secondary male sex features.
- Estradiol: Development of the egg cells (oocytes); control of menstruation (especially follicular phase); development and maturation of the female sex organs, including secondary female sex features.
- Progesterone: The hormone of pregancy; preparation and maintenance of pregnancy

GROWTH HORMONE... A protein-hormone that is secreted from the Ant. Pituitary; together with the thyroid hormone, insulin and the sex hormones, it forms a classical group hormones that are responsible for normal growth and development. A shortage of any of these hormones will affect normal growth in one way or the other. Although GH is a protein hormone, it does exhibit some features which rather resembles fat soluble hormones: (i) GH's secretion is regulated by relay hormones from the hypothalamus, (ii) an important mechanism of action on cellular levels is gene-activation and protein synthesis, and (iii) at least 50% of plasma GH is transported by carrier proteins.



Growth Hormone secretion

GH FUNCTIONS:

- GH regulates growth and development in children (bone and tissue growth via the induction of protein synthesis)
- GH stimulates increased glucose release from the liver, and thereby increases plasma glucose
- GH serves as a relay hormone that regulates the secretion of IGF (Insulin-like Growth Factor). IGF is primarily responsible for cartilage growth, although it shares the above functions of GH as well.

ABNORMAL GH RELEASE:

1. UNDER-PRODUCTION DURING CHILDHOOD: Dwarfism
   (compare a GH-deficient dwarf with a thyroid-hormone shortage dwarf)

2. OVER-PRODUCTION IN CHILDHOOD: Gigantism

3. OVER-PRODUCTION IN ADULTS: Acromegaly

More info: http://academic.sun.ac.za/medphys/endo3.htm#h1

SELFSTUDY:

The Digestive Hormones... Silverthorn: Table 21-2; p. 672-673

IMPORTANT TOPICS OF THEME 6, SESSIONS 1-5:

CONCEPTS:

- The fat soluble Hormone
- Hepato-Renal Reflex
- The Glucocorticoids
- Stress-hormone
- Circadian Rhythm
- The Hypothalamic-Hypophysis Axis
- Cretinism, Hypothyroidism, Hyperthyroidism
- Hypothyroid-Midget vs. Growth hormone-deficiency Midget
- Gigantism vs. Acromegaly

OUTCOMES IN STUDYGUIDE:

- p. 16, Outcomes 1-6
- p. 17, Session 1 Selfstudy questions 1,2,4
- p. 17 Sessions 2-6 outcomes 1,2,4-6.