The Role of Neuronal Nitric Oxide Synthase (nNOS) in Ischaemia/Reoxygenation-induced injury and in protection of the Mammalian Myocardium

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Dedication

This thesis is dedicated to my late father Dr. Sitangshu Bikash Barua and my mother Mrs Romeli Barua, without their inspiration, guidance, unconditional love, I would not have the goals, I strive for today. I will always be grateful to them for their encouragement and for installing the self-belief that I can do anything if I approach it with confidence and optimism. I also would like to dedicate this thesis to my husband, Dr. Biplab Barua who supported me in all stages of thesis preparation.

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The study was carried out in the division of cardiac surgery, at the Clinical Science Wing in Glenfield Hospital and at the University of Leicester during the years 2006-2008.

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Abstract

Background: In physiological condition, NO is produced by two constitutive NOS isoform; eNOS and nNOS. Both isoforms have specific cellular locations and although the role of eNOS in myocardial ischaemic injury and in cardioprotection has been thoroughly addressed, but the role of nNOS remains unclear. Therefore, the aims of the thesis were to: (i) investigate the role of nNOS in ischaemia/reoxygenation-induced injury, (ii) determine whether its effect is species-dependent, (iii) elucidate the relationship of nNOS with mitoK_{ATP} channels and p38MAPK, two key components of IP and (iv) investigate whether modulation of the NO metabolism can overcome the unresponsiveness of the diabetic myocardium to IP.

Methods and Results: Ventricular myocardial slices from rats and mice, nNOS knockout mice, and also from human right atrial slices were subjected to 90min ischaemia and 120min reoxygenation (37^{o}C). Muscles were randomized to receive various treatments. Both the provision of exogenous NO and the inhibition of endogenous NO production significantly reduced tissue injury (creatine kinase release, cell necrosis and apoptosis), an effect that was species–independent. The protection seen with nNOS inhibition was as potent as that of IP, however, in nNOS-knocked out mice the cardioprotective effect of non-selective NOS (L-NAME) and selective nNOS inhibition (TRIM) and also that of IP was blocked while the benefit of exogenous NO remained intact. Additional studies revealed that the cardioprotection afforded by of exogenous NO and by inhibition of nNOS were unaffected by the mitoK_{ATP} channel blocker 5-HD although it was abrogated by p38MAPK blocker SB203580. Finally, in diabetic myocardium, IP did not decrease CK release neither reduced cell necrosis or apoptosis. In diabetic myocardium NO donor SNAP, inhibitor L-NAME and TRIM significantly reduced CK leakage, cell necrosis and apoptosis.

Conclusions: nNOS plays a dual role in ischaemia/reoxygenation on that its presence is necessary to afford cardioprotection by IP but its inhibition reduces myocardial ischaemic injury. The role of nNOS is species-independent and exerted downstream of the mitoK_{ATP} channels and upstream of p38MAPK. Moreover, both the provision of exogenous NO and the suppression of endogenous NO production resulted in potent protection of diabetic human myocardium, overcoming the unresponsiveness of these tissues to IP.

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The American Society for Cell Biology 48th annual meeting, San Francisco, USA, Dec 2008.

Neuronal Nitric Oxide Synthase Contributes to Myocardial Ischemic Injury Whilst Still Is Required for Cardioprotection.
The American Society for Cell Biology 48th annual meeting, San Francisco, USA, Dec 2008.

Can exogenous NO help to protect ischemic injury in diabetic myocardium?
Scandavian Society of Research in cardiothoracic surgery, Geilo, Norway, Feb 2009.

Modulation of the nitric oxide metabolism overcomes the unresponsiveness of the diabetic human myocardium to protection against ischemic injury
Heart Failure Congress, Nice, France. May 2009.

Publications

Dual Role of nNOS in ischemic injury and ischemic preconditioning.
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Modulation of the nitric oxide metabolism overcomes the unresponsiveness of the diabetic human myocardium to protection against ischemic injury.
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Abbreviations

ATP: Adenosine triphosphate

BH_4: Tetrahydrobiopterin

CaM: Calmodulin

cGMP: Cyclic guanosine 3'5' monophospate

CK: Creatine kinase

DAPI: 4',6-diamidino-2-phenylindole

DMSO: Dimethyl sulfoxide

eNOS: Endothelial nitric oxide synthase

FAD: Flavin adenine dinucleotide

FMN: Flavin mononucleotide

5-HD: 5-hydroxydecanoate

GTP: Guanosine 5' triphosphate

HIF1: Hypoxia inducible factor 1

HSP: Heat shock protein

IL-1: Interleukin -1

iNOS: Inducible nitric oxide synthase

IP: Ischaemic preconditioning

KHH: Krebs Henseleit Hepes buffer

L-NAME: Nω-Nitro-L-arginine methyl ester hydrochloride

MAPK: Mitogen activated protein kinase

MI: Myocardial infarction

MitoK_{ATP} Channels: Mitochondrial K_{ATP} channels

MPTP: Mitochondrial permeability transition pore

NADPH: Nicotinamide adenine dinucleotide phosphate hydrogenase

NO: Nitric oxide

NOS: Nitric oxide synthase

nNOS: Neuronal nitric oxide synthase

PBS: Phosphate buffered saline

PI: Propidium iodide

PKB: Protein kinase B

PKC: Protein kinase C

POC: Postconditioning

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

sCG: Soluble Guanylate Cyclase

SEM: Standard error of mean

SNAP: S-Nitroso-N-acetyl-DL-penicillamine

TRIM: 1-[2-(Trifluoromethyl)phenyl]imidazole(1)

TNF: Tumour necrosis factor

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

Chapter 1

Introduction

1.1. Ischaemia and reoxygenation induced myocardial injury

Ischaemic heart disease is the leading cause of death in the modern world. Myocardial ischaemia occurs when there is an imbalance between supply and demand for oxygenated blood in cardiomyocytes. Ischaemia compromises not only oxygen supply but also reduces availability of nutrient substances and inadequate removal of metabolites. Oxygen is therefore reduced for oxidative phosphorylation in the mitochondria. Cardiac muscle requires approximately 1.3ml of oxygen per 100gm of muscle per minute for cellular survival in comparison with approximately 8ml of oxygen per 100gm of muscle per minute in the contractility of the left ventricle. In reduced coronary blood flow, contractile activity decreased to the point that is sustained by oxygen availability, resulting in contractile dysfunction. These muscles can completely recover functionally by reperfusion. Myocardial ischaemia of less than 20 minutes duration, followed by reperfusion, results in full functional recovery without biochemical and functional evidence of tissue injury, whereas ischaemic periods of more than 45 minutes followed by reoxygenation, results in severe myocardial injury. There is increasing evidence that ischaemic reperfusion injury represents as a continuous phenomenon and occurs as a transition from reversible and irreversible cell injury.

1.2. Mechanism of ischaemia-reperfusion injury

Reoxygenation is essential to salvage the ischaemic tissue and to prevent the progressive pathological wave of ischaemia. Reperfusion re-supplies the oxygen and other metabolites to the myocardium whilst also washing out the toxic substrates produced by ischaemia. Reperfusion also has the potential to exacerbate lethal tissue injury by causing biochemical changes in the vicinity and by altering the morphology of cells already injured (1). Reperfusion induced microvascular injury causes haemorrhage and endothelial swelling that occludes the capillaries and may prevent local reperfusion to areas of critically injured myocardium. Myocardial ischaemia/reperfusion injury is clinically manifested by the development of contractile dysfunction, arrhythmias and/or myocardial cell death, that may be apoptotic and necrotic cell death, depending on the degree of injury. Opening of the mitochondrial permeability transition pore (MPTP) during reperfusion, after a sufficient duration of ischaemia, serves as a key mediator of cell death (2). During ischaemia there is increased depletion of adenosine triphosphate (ATP) and accumulation of Ca^{2+} (3). The reduction of ATP and the overload of Ca^{2+} do not directly cause immediate rupture of the plasma membrane at least in short periods of ischaemia. In addition, ischaemia induces metabolic acidosis and activates Na^{+}/ H^{+} exchange, resulting in transport of H^{+} in the extracellular space (4, 5, and 6). Accumulation of Ca^{2+} is augmented by ischaemia inducing membrane depolarisation and allows the opening of L-type Ca^{2+} channels. During reperfusion, oxygen re-enters the cells and is associated with a generation of reactive oxygen species (ROS), Ca^{2+} overload and recruitment of inflammatory cells (5-8). A diagram of ROS generation is shown in Figure 1.

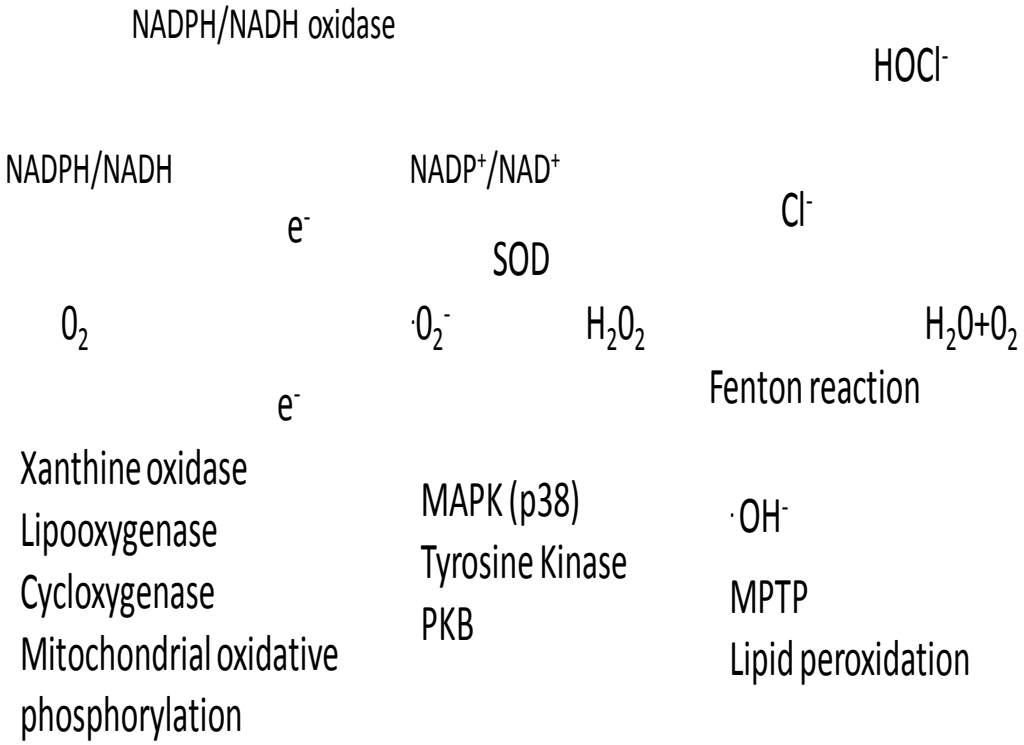


Fig 1: Generation of ROS: Many enzyme system stimulate the production of superoxide anion ($\cdot 0_2^-$) from 0_2. These include NADPH/NADP oxidase, xanthine oxidase, lipooxygenase, cycloxygenase, mitochondrial oxidative phosphorylation. Dismutation of $\cdot 0_2^-$ spontaneously or enzymatically by superoxide dismutase(SOD) produces hydrogen peroxide (H_2O_2) that can undergo further reaction to generate highly active hydroxyl radical ($\cdot OH^-$). Downstream target of H_2O_2 and superoxide anion are p38MAPK, tyrosine kinase, PKB.

The increase in ROS and the restoration of normal pH promote the opening of MPTP, which in turn causes ATP depletion and activation of caspases (9). Moreover, in addition to the loss of ATP and generation of ROS, the activation of proteolytic enzymes results in the loss of plasma membrane integrity. Ischaemia/reperfusion injury also triggers an inflammatory reaction, with production of cytokines and activation of the complement pathway (10). These events culminate in the recruitment of circulatory neutrophils resulting in additional injury (7, 8, and 10).

In addition to the molecular derangement discussed above, reperfusion causes marked microvascular and endothelial dysfunction resulting in vasoconstriction, platelet and leukocyte activation, along with increased fluid extravasation (11, 12). All these changes can cause contractile dysfunction. Thus, during the early phase of reperfusion, myocardium develops contracture and loses its compliance, a condition identified by cardiac surgeons as 'stone heart'. This contracture happens due to Ca^{2+} overload during ischaemia and reperfusion (13). During reperfusion, there is a rapid re-energisation of contractile cells with persistent Ca^{2+} overload which affects myofibrillar Ca^{2+} sensitivity, resulting in further contracture of the cardiomyocytes (13, 14). Therefore, the early phase of reperfusion is an important target for the application of cardioprotective strategies.

1.3. Cardioprotection

A large range of cardioprotective interventions have been proposed to counteract ischaemia/reperfusion injury, mainly addressed to overcome Ca^{2+} overload, inflammation and ROS. It is beyond the scope of this thesis to describe all the cardioprotection modalities of treatment studied. However, I will discuss the protective interventions that are more relevant for the present studies and for cardiac surgery such as cardioplegia and ischaemic preconditioning (IP).

1.3.1. Cardioplegia

During cardiac surgery, global ischaemia, rather than regional ischaemia occurs in myocardial tissues. In 1970, a high proportion of surgical patients died of transmural myocardial infarction (MI) perioperatively. Despite the presence of normal coronary arteries, transmural MI, scattered myocardial necrosis and confluent subendocardial necrosis were identified in post mortem autopsies. It is clear that postoperative low cardiac output syndrome is directly attributable to inadequate intraoperative myocardial protection. With this knowledge, multiple strategies have been applied to reduce the degree of ischaemic injury as well as to minimise the reperfusion damage. Among these, cardioplegia has been used as the gold standard for myocardial protection during open heart surgery for the last 30 years (15, 16). Whilst providing a bloodless, motionless operative field, cardioplegia also reduces myocardial oxygen demand to a level that would minimise the risk of intraoperative myocardial injury. Myocardial oxygen requirement, in the normothermic vented non-working heart, ranges between 6-

8ml O_2/min per 100gm of myocardium. Cardioplegia lowers myocardial energy requirement to a range of 1-1.5ml O_2/min per 100gm of myocardium, reducing energy requirement as much as 80-85%.

Various cardioplegic formulations, based on the intra or extracellular ionic compositions, have been utilised with and without the addition of agents aimed to correct specific alterations: (i) acidosis, (ii) ion imbalances, (iii) energy depletion, (iv) damage to cell membranes, (v) swelling, etc. However, it is also beyond the scope of this thesis to give a full account of the types of cardioplegic solutions and the mechanisms of protection involved.

1.3.2. Ischaemic preconditioning

Ischaemic preconditioning (IP), a phenomenon consisting of brief periods of ischaemia followed by reperfusion, induces tolerance to ischaemic injury and reduces the chance of sublethal damage to tissues. The exact mechanism of IP is still uncertain despite intensive research for the last two decades. Murry *et al*. (17) described this phenomenon in 1986 and demonstrated that one or more brief periods (>3min) of ischaemia followed by reperfusion affords remarkable reduction in infarct size. This phenomenon has also been demonstrated in the intestines, liver, kidneys and retina. It has been identified in all the studied animals as well, and therefore it appears to be a universal phenomenon by which organs adapt to recovery from ischaemic stress. In addition to a decrease in the infarct size, IP improves post-ischaemic recovery function and has an anti-arrhythmic effect. IP can reduce infract size by 50-75%, whereas medical

cardioprotection can achieve only a 10% reduction (18).

The classic or early IP referred to as 'the first window of protection' lasts from a few minutes to a few hours, whilst delayed IP or 'the second window of protection', is activated after 24 hours of the initial ischaemic insult, persisting up to 72 hours (19, 20). Different mechanisms are responsible for each type of IP; the early IP is induced by post translational modification of preexisting proteins whereas the delayed IP is caused by the synthesis of new proteins (21).

Preconditioning can also be induced by pharmacological agents activating a number of sarcolemmal receptors such adenosine A_1 and A_3 receptors, bradykinin and α_1 adrenergic receptors, endothelin and opioids receptors and also by other agents such as nitric oxide donors, phosphodiesterase inhibitors and various noxious agents (endotoxin, cytokines, reactive oxygen species). Pharmacological agents activate the signal transduction pathway of IP by stimulating or inhibiting any of the involved enzymes (22).

1.3.2.1. Mechanism of IP

A large body of research has been carried out to define the signal transduction pathway of IP, but the precise mechanism still remains unclear. During ischaemia, adenosine, bradykinin, prostaglandin, and opioids are released from the myocardium, which in turn activate protein kinase C (PKC), a key mediator for IP (23, 24). Moreover, mild oxidative stress can activate PKC. This is followed by translocation of an inactive

enzyme from the cytoplasm to the cell membrane where PKC exerts its activity (25). Yoshida *et al.* (26) reported that nitric oxide (NO) produced during the ischaemia/reoxygenation period induces PKC translocation and improves the recovery function in the rat heart. It is accepted that IP involves the activation (e.g. phosphorylation) of multiple protein kinases such as PKC and protein kinase B (PKB), which in turn open the putative mitochondrial K_{ATP} channels and activate the p38 mitogen-activated protein kinase (p38MAPK). p38MAPK may then act on the different targets (27, 28): (i) inhibition of cytocrome C release, (ii) alteration of Ca^{2+} concentration in the mitochondria, (iii) reduction in ROS production, (iv) increasing the expression and activity of nitric oxide synthase (NOS) in cytosol and mitochondria, and (v) phosphorylation of heat shock proteins (HSP).

It is well established that inhibition of the mitochondrial permeability transition pore (MPTP) is also a key factor for IP (29). Costa *et al.* described that intramitochondrial activity of several mediators such as PKC, ROS, MPTP and mitoK_{ATP} channels are vital for IP induced cardioprotection (30). Our own group has demonstrated that cardioprotection can be achieved by pharmacological manipulation of PKC, p38MAPK and mitoK_{ATP} channels in the human right atrial appendages and established that mitoK_{ATP} channels are upstream and p38MAPK are downstream of PKC (31, 32). Furthermore, it was shown that the PKCε isoforms activate the mitoK_{ATP} channels and then these in turn activate PKCα (31).

There is also increasing evidence that IP can be triggered by a non-receptor mechanism. Free radicals can act as a trigger for IP by directly activating PKC (23, 25). The dog

heart is abundant in xanthine oxidase, which is derived from purine catabolism. This contributes to non-receptor mediated IP. The perfusion of Ca^{2+} into the coronary circulation also induces cardioprotection by IP in the rat myocardium (33). Moreover, it has been suggested that transient hyperthermia induces cardioprotection by early and delayed IP (34), whereas stretching of myocardial fibers also provides cardioprotection to the ischaemic dog heart (35). A schematic diagram of ischemic preconditioning is shown in Figure 2.

There is a large body of evidence supporting the concept that IP consistently and reproducibly limits the infarct size in animal experimental models. What is required next is the availability of pharmacological mimetic of IP that could be ultimately used in the clinical setting.

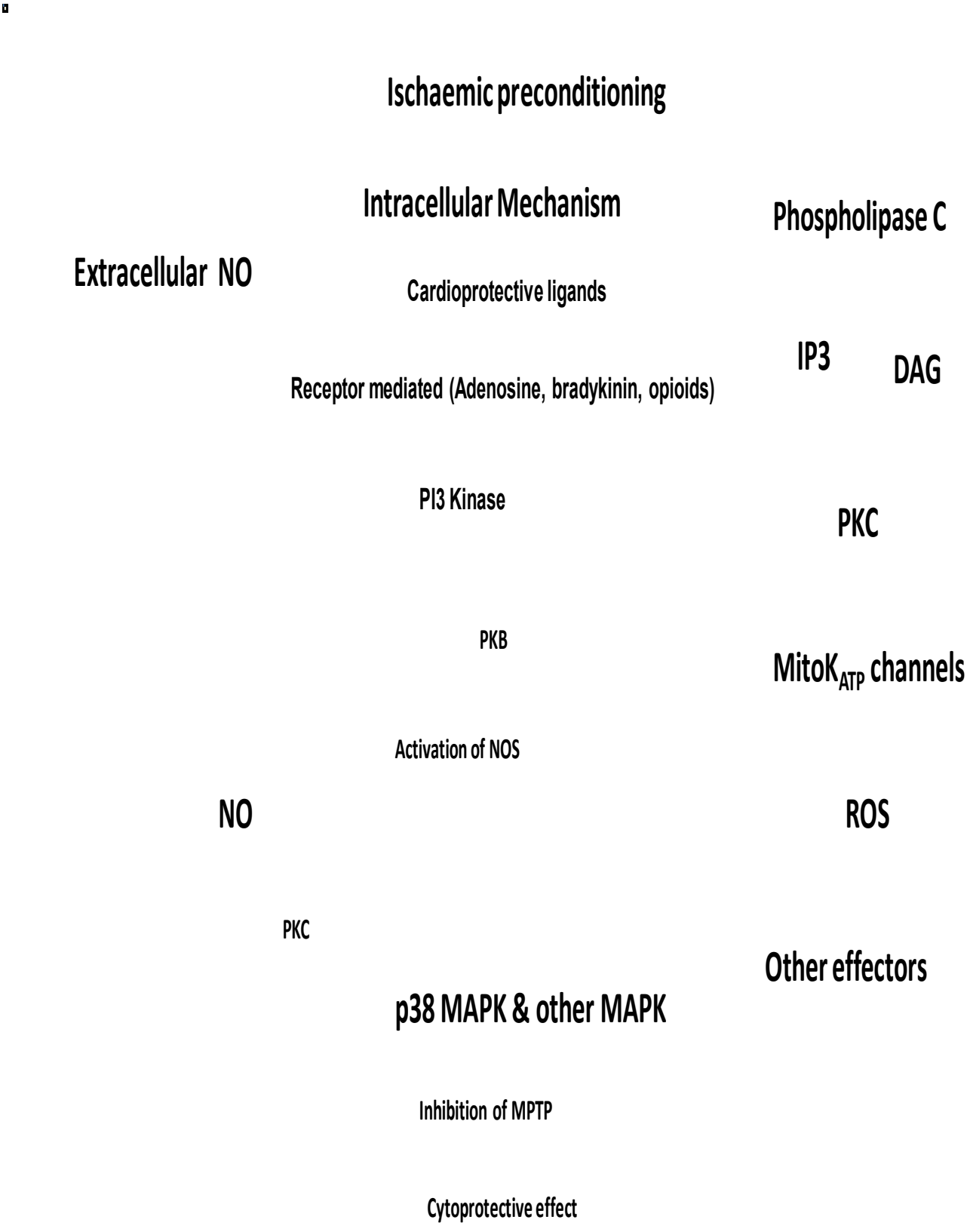


Fig 2. The schematic diagram of Ischaemic preconditioning.

1.3.3. Postconditioning

Postconditioning (POC) is a new concept of modifying ischaemia/ reoxygenation induced injury. A controlled brief repetitive cycle of ischaemia with intermittent reperfusion after prolonged ischaemia and at the beginning of reperfusion denotes postconditioning. Vinten-Johansen's group first reported POC phenomenon in canine heart (191). They have demonstrated that brief episodes of coronary occlusion and reperfusion at the onset of reperfusion after sustained ischaemic insult protects myocardium from ischaemia/ reoxygenation induced injury in dog. POC protocol was 30s reperfusion followed by 30s ischemia for 3 episodes at the very onset of reperfusion. Early moment is very crucial here as cardioprotection cannot be attained 1-3 min after the onset of reperfusion (192). It has been reported that ventricular premature beat- driven intermittent reperfusion of coronary vessels results in reduction of VF induced myocardial ischaemia/ reoxygenation induced injury (193). Several studies have suggested that POC is reproducible in *vivo* and *vitro* models of rodents, pigs, rabbits, canine and also documented in different organs (191, 194-199). POC reduces infract size ~20-70% depending on species and models (192, 2000, and 201). Evidences suggest that it reduces apoptosis, necrosis, endothelial dysfunction and activation, ROS production and inflammatory response (192).

An involvement of adenosine, kinin and opioids in early POC has been reported by many investigators (197, 202 and 203). Activation of intracellular survival kinase pathway such as ERK1/2, PI3, protein kinase B/Akt pathway are also associated with POC (194, 204-206). MitoK_{ATP} channels act as an end-effector in cardioprotection of

POC evidenced by several literatures (202, 207-209). It is suggested that MPTP is inhibited by increased NO production and decreased ROS and Ca^{++} in POC results in cardioprotection (210-215). Interestingly, some researchers suggest that similar pathway are involved in ischaemic preconditioning and post conditioning (191, 192, and 212). But there is difference as well; most obvious one is timing of initiation. Moreover, timing of activation of each and every component of both pathways may differ. The existing data suggest that IP and POC provide similar protection such as reduced infract size, reduced neutrophil and endothelial activation and interaction and decreased ROS generation (192).

A number of studies have been carried out to determine the co-treatment effect of IP and POC. Some reported that the degree of infract size was similar in IP and POC and no additive protective effect was ascertained by co-treatment (216-218). However, other researchers have shown additive benefits afforded by combination treatment (219, 220). It may be suspected that interchangeable end-effectors are activated by either intervention to a comparable degree.

Undoubtedly, the prediction of ischemic event is very limited, so the clinical implication of IP is not feasible. But POC can be applied in diverse clinical settings such as coronary artery bypass graft surgery, aortic cross clamp, and percutaneous coronary intervention where reperfusion can be initiated by mechanical or medical interventions. It has been demonstrate that brief periods of occlusion and perfusion during PCI for acute myocardial infarction reduce infarct size, improve coronary blood flow in distal site, improve heart function and decreased production of ROS (221-223).

Prior to removal of aortic cross clamp in cardiac surgery, POC can be induced effectively to improve the patient outcome (224, 225). Though little difference is surmised in pre and postconditioning, the two mechanisms may differ greatly in the compartmentalisation of the important trigger such as ROS and NO (226).

1.4. NO metabolism

Nitric oxide (NO), also known as endothelial derived relaxing factor (EDRF) was first described by Furchgott 30 years ago (36). NO is a relatively stable free radical and a signalling molecule which exerts both physiological and pathological actions. It is synthesised from L-arginine in the presence of O_2 and nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH). In low concentrations, NO maintains the normal homeostasis and mitigates cell injury. However, in high levels, NO is cytotoxic. Most of its toxic effects are triggered by the oxidation of molecules.

It is well accepted that NO plays an important role in modulating the heart's tolerance to ischaemia. Multiple factors jeopardise the elucidation of the specific role of NO in modulating ischaemia/reperfusion induced injury such as the short half-life of NO, diffusibility, existence in multiple redox state, the subcellular compartmentalisation of nitric oxide synthase (NOS) isoforms and the existence of multiple targets. On account of this, it is understandable that NO exerts a cardioprotective effect and contributes to myocardial injury simultaneously.

1.4.1. Cellular and subcellular action of NO

NO interacts with the soluble guanylate cyclase (sGC) receptor which catalyses the conversion of guanosine 5' triphosphate (GTP) to cyclic guanosine 3' 5' monophosphate (cGMP) (37). Subsequently the increased cGMP activates cGMP-mediated protein kinase, cGMP regulated cation channels and cGMP mediated phosphorylation, all of them cause diverse and specific effects in different cells and tissues (38-40).

NO also acts independently of sGC via the S-nitrosylation of thiol. Thus, NO inhibits apoptosis by S-nitrosylation of caspase, a cystine protein involved in apoptosis (41). In addition, the NO-dependent S-nitrosylation modulates the activity of the sarcoplasmic ryanodine receptor that regulates Ca^{2+} channels (42). Furthermore, NO inhibits cytochrome oxide in the mitochondria and regulates the respiratory chain reaction and ATP production (43).

The chemical and pharmacological properties of NO were described by Louis J-Ignarro in 1989 (44) in a review paper as a chemically unstable molecule with a half-life of 3 seconds, that is spontaneously inactivated in the presence of oxygen and superoxide anion. NO is very lipophilic in nature, can be stabilised by superoxide dismutase and acidic pH, and readily permeates through biological membranes. It has a high binding affinity for and reactivity with haeme iron in haemoglobin, myoglobin and soluble guanyl cyclase to form corresponding nitrosyl-haeme product. Finally, the biological action is rapidly terminated by haemoglobin and myoglobin.

NO has been implicated in a wide range of physiological effects in the cardiovascular system. Endothelial cells continuously produce NO at a basal rate that regulates vascular tone, maintains integrity of vasculature and myocardial contractility via cGMP dependent mechanism. Belge *et al.* (45) showed that endogenous NO modulates rat cardiomyocyte response to adrenergic and cholinergic stimuli. Mellion *et al.* (46) demonstrated that NO inhibits ADP-induced platelet aggregation mediated by a cGMP dependent pathway. NO also inhibits leukocyte adhesions to endothelial cells as well as leukocyte emigration and aggregation by interacting with superoxide (47-49). NO maintains cell proliferation by impairing glycolysis and by inhibiting electron transport and stimulating ribonucleotide reductase in pathological condition (49).

1.4.2. Cytoprotective effect of NO

During ischaemia, accumulation of Ca^{2+} and NADPH provokes a small quantity of NO production. NO combines with superoxide anion to form peroxynitrate and other ROS (50). Several studies have suggested that ROS and NO trigger PKC activation, in turn eliciting IP (51, 52). One of these mechanisms may be the induction of heat shock protein (HSP 70).

NO modulates mitochondrial function through reversible and irreversible interactions with respiratory chain complexes. Thus, during ischaemia, NO helps in reducing oxygen consumption in the mitochondria without depletion of ATP. NO also induces the depolarisation of the mitochondrial membrane potential which limits Ca^{2+} overload in the mitochondria. In addition, NO also modulates inflammation by reducing polymorphonuclear mediated endothelial dysfunction in the myocardial ischaemia-

reoxygenation pathway. This process is probably due to its specific interaction with adhesion molecules. As discussed above, the cGMP produced from GTP by NO catalyses, reduces Ca^{2+} level by inhibition of L-type Ca^{2+} channels and also reduces concentration of cAMP. cAMP in turn reduces the myocardial contractility which ultimately decreases oxygen consumption and energy demand.

1.4.3. Toxic effect of NO

There are various toxic effects of NO. During ischaemia, hypoxanthine and xanthine accumulate while arginine and tetrahydrobiopterin (BH_4) are depleted, resulting in uncoupling of NOS and generation of more ROS (53-56). Moreover, ischaemia alters the NOS function affecting endothelial reactivity and switching the production of NO from superoxide.

Cytokines (e.g. interleukin IL-1, IL-2, tumour necrosis factor TNF, interferon γ) and endotoxins activate nitric oxide synthase (NOS) producing large amounts of NO with a potent vasodilatory effect. This attenuates the vasoconstrictor effects of other molecules such as endothelin, adenosine and vasopressin. Finkel *et al*. (57) described that cytokines are also a cause in myocardial stunning, mediated by NO. Release of cytokines from these cells stimulates NOS to produce more NO. The reaction of NO with superoxide produces peroxynitrite that causes membrane lipid peroxidation and protein oxidation (58, 59). Indeed, excess NO production causes sepsis-induced hypotension and myocardial depression.

1.4.4. Endogenous NO production by Nitric oxide synthase

As discussed earlier, NO is an important physiological mediator with the ability to promote both cell survival and cell death. This important mediator is enzymatically produced by the nitric oxide synthases (NOSs), converting L-arginine to L-citruline in the presence of NADPH, O_2 and other co-factors (60). Three NOS isoforms have been isolated, which are differentiated according to their expression and calcium dependence. All NOSs isoforms are widely distributed through most cells and tissues. The endothelial NOS (eNOS or NOS III, 2X134kDa) and neuronal NOS (nNOS or NOS I, 2X160kDa) are the constitutive and Ca^{2+} dependent NOSs. In comparison the expression of the inducible NOS (iNOS or NOS II, 2X130kDa) is Ca^{2+} independent and stress-induced. The other proposed NOS named as mitochondrial NOS, is considered to be nNOSα which is available in the inner membrane of mitochondria of cardiac myocytes (61). The tissue-specific expression and activity of NOS isoforms vary according to which cellular co-factor predominates. eNOS is located in chromosome 7, whilst nNOS is located in chromosome 12 and iNOS in chromosome 17. Figure 3 shows the structure of NOS isoforms.

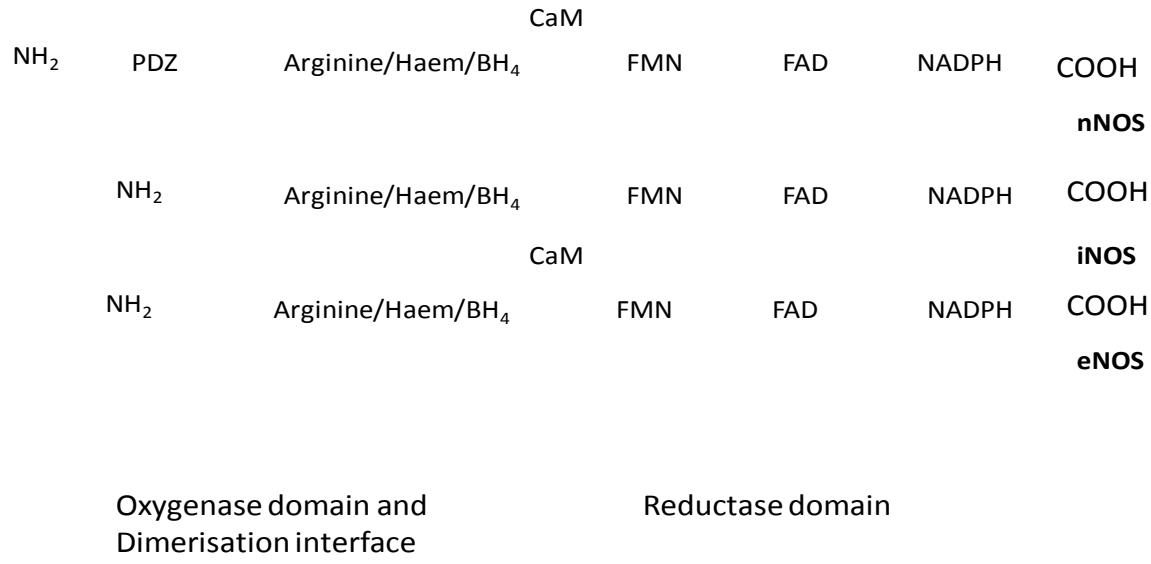


Fig 3. The general structure of NOS and the variation among the isoforms. The enzyme has both oxidation and reductive functions.

The eNOS is located in a special micro domain of the plasma membrane of almost all cells where is associated with the caveolins to regulate cellular function. Thus, the isoform caveolin 3, in association with eNOS, is responsible for cardiac contractility (62). Multiple factors contribute to the expression and induction of eNOS in cells. These include mechanical forces, hormones including catecholamines, vasopressin, bradykinin and histamine and even hypoxia and exercise (60). The exact subcellular localisation of eNOS is uncertain. It has been assigned to cytosol, plasma membrane, golgi apparatus. Majority of eNOS is localised in cell membrane and a small fraction is available in cytosol (227-230). eNOS in both endothelial cells and cardiomyocytes is localised to caveolae and detergent insoluble glycosphingolipid-rich microdomain in the plasmalemma (231, 232). Caveolae and these microdomains facilitate transport of

molecules across cells and the sites where GPCP and other molecules are available for triggering the signal transduction pathway (233, 234). Caveolin (principal structural protein of caveolae) may travel between plasma membrane and golgi appatatus (235-237). It has been suggested that eNOS co-localises in the golgi-specific marker mannosidase II (238, 239). eNOS is targeted to the membrane to dual acylation-palmitoylation/ depalmitoylation that is important in eNOS activity and its NO production (232 ,240). eNOS can be displaced from caveloin by altered lipid composition in the plasma membrane (241). This hypothesis is particularly important in the understanding of hypercholesterolemia-induced atherosclerosis and vascular diseases. Sullivan JC et al have hypothesized that in hypertension, only a portion of eNOS cycle from membrane to cytosol and produce lesser amount of NO, the remaining eNOS is not cycling properly and remains uncoupled and produce more ROS (242). New knowledge is required to determine the relationship between the intracellular compartmentalisation of eNOS and its enzyme activity in both endothelial cells and cardiomyocytes.

It has been documented that nNOS is located in the peripheral vagal nerves, sympathetic nerves and in the autonomic control region of the central nervous system. In cardiomyocytes, nNOS is expressed in sarcoplasmic reticulum, sarcolemma and mitochondria (63-65). nNOS expressed in neurons, cardiac myocytes and blood vessels (243, 244). Some studies have suggested that nNOS is anchored to plasma membrane by adaptor proteins (figure 3). Other studies have demonstrated that nNOS exist in particulate and soluble form in brain (245). It has been suggested that nNOS

immunoreactivity of neurons was mainly distributed in cytosol in a patch like forms (245). In cerebral astrocytes, nNOS immunoreactivity was high in cytoplasm for 6 days, then in nucleus and then again cytosols at day 7 (246). Some researchers have also found them in glial cells and neuronal cells (247). It can be surmised that different locations contribute the diverse function. Interestingly, nNOS activity is mediated by adapter proteins. Many investigators have suggested that variety of protein bearing PDZ domain can interact directly with PDZ domain of nNOS influencing the subcellular distribution and activity of nNOS in muscle cells and neurons (248, 249). Moreover, subcellular localisation of nNOS is changed when tissues are subjected to hypoxia or hypoglycaemia or increased glutamate concentration (250). It has been suggested that low cholesterol levels can redistribute nNOS from insoluble membrane to soluble fractions (251). It is probable that nNOS activity varied due to different subcellular location during ischemia and preconditioning (250). However, further studies are required for better perception of nNOS subcellular localisation during ischaemia/reoxygenation induced injury.

Disruption of the nNOS gene or inhibition of nNOS function, increases left ventricular ejection fraction and myocardial contractility (65). NO derived from nNOS influences the beat to beat regulation of basal cardiac function by control of Ca^{2+} fluxes (65, 66). In addition, it facilitates vagal stimulation and inhibits sympathetic stimulation by exerting its effect in central nervous system and peripheral nervous system.

As mentioned above, the activity of iNOS is stress induced. iNOS takes part in the inflammatory response and may have a role in myocardial protection in delayed IP (67).

iNOS derived NO can also reduce myocardial function by triggering apoptosis. It is well documented that iNOS induction and expression is significantly raised in many cardiac diseases such as cardiac failure, dilated cardiomyopathy, myocardial infarction, myocarditis and cardiac allograft rejection (68). Increased iNOS expression is evidenced by enhanced activity of other cytokines such as IL-1, TNFα. Large amounts of NO are formed from iNOS after exposure of the cells to the microorganism that interacts with superoxide to form ROS and kills the microorganisms by a non-specific immune defense mechanism.

eNOS and nNOS produce lesser amounts of NO in comparison to iNOS. After induction by bacterial lipopolysaccharide, iNOS produces 20 times more NO than constitutive NOS that, as discussed above, may have potentially damaging consequences. So, the difference in location, expression and regulation of NOS isoforms may play a major effect in the activity of NO in cardiac myocytes.

Catalytic active NOS is a homodimer of haemoprotein. Each NOS contains an oxidase domain at its amino terminal end and a reductase domain at its carboxyl-terminal end. The NOS reductase domain contains binding sites for the redox co-factors NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and calmodulin (CaM). Two reducing equivalents are transferred from NADPH via FMN and FAD to the oxidase domain under the control of the CaM/Ca^{2+} complex. The oxidase domain tightly binds with BH_4 and a cysteiinyl thiolate-ligated heme group, which are the reactive centres for oxidative reaction. BH_4 is acting as a redox co-factor and also has an allosteric action stabilising the structure of NOS (69).

The key elements of NOS regulation resides in the reductase and CaM binding domain. NOS is also subject to feedback inhibition by NO (70). Three kinetic parameters; rate of ferrous-haeme reduction, ferric-haeme-NO dissociation and ferrous-haeme-NO dissociation, regulate the distinct catalytic behaviour of the three NOS isoforms. eNOS exhibits the slowest rate of ferric to ferrous reaction and a relatively high ferric-NO dissociation rate. As a result, ferrous-NO complexes do not accumulate, which in turn prevents the generation of NO-oxidation products. Owing to a much faster ferrous-NO oxidation rate in iNOS, the production of NO in the steady state is greater with iNOS than with nNOS.

NO may also come from its breakdown of products such as nitrates or nitrites and from reaction of heme protein (71-73). The understanding that NOS can produce a range of reactive nitrogen species (RNS) depending on the isoforms and physiological circumstances is of crucial importance to fully elucidate the pathophysiological roles of NOS and its inhibition. This might explain why NOS inhibition is anti-inflammatory and cytoprotective as well as exogenous provision of NO is cardioprotective.

1.5. Nitric oxide synthase and ischaemic preconditioning

It is known that ischaemia increases NO production. eNOS is a reliable source of NO production during ischaemia/reperfusion (74) and its activity and expression are directly related to the duration of ischaemia (75). It has been reported that NO derived from eNOS is essential for eliciting IP (150).

A number of studies have suggested that iNOS is involved in delayed IP (67, 76-78). It has been argued that initial short episodes of ischaemia/reoxygenation activate the signal transduction pathway that induces iNOS expression (78). Increased expression of the iNOS gene and iNOS proteins mediated by the hypoxia inducible factor 1 (HIF1) have been observed in ischaemia (80).

However, there is still controversy about the role of nNOS. Thus, although nNOS expression is increased in the acute phase of myocardial infarction in both infarcted and non infarcted tissues in the rat heart (79), it has been reported that in the nNOS knockout mice there is no change in myocardial infarct size (81, 82). By contrast, it appears that delayed IP (72 hours) is dependent on nNOS in the rabbit (83), whereas a deficiency of nNOS reduces cerebral ischaemic injury in mice (84).

Nonetheless, the specific role of nNOS in early IP remains uncertain and their location with respect to other components of the signalling pathway such as mitoK_{ATP} channels, PKC and p38 MAPK, is unknown.

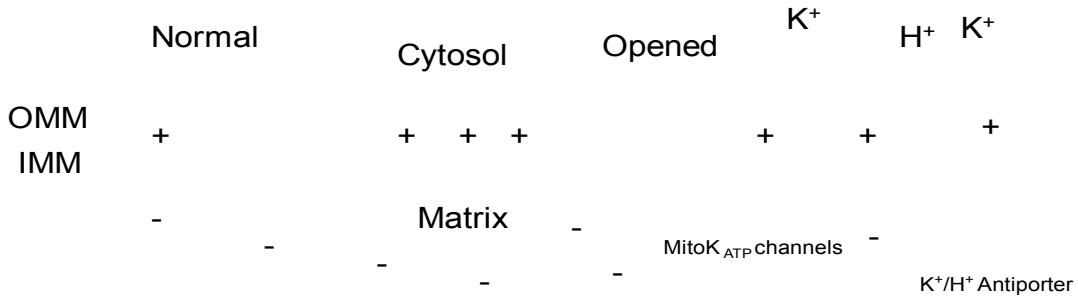
1.6. NO metabolism and mitoK_{ATP} channels

mitoK_{ATP} channels are located in the inner mitochondrial membrane, modulating the mitochondrial membrane potential and are an important trigger for IP (85, 86). Few studies have shown a link between mitoK_{ATP} and NO in IP induced cardioprotection. Sasaki *et al*. suggested that NO causes the opening of mitoK_{ATP} directly and indirectly (87) which in turn induces blunting of the Ca^{2+} overload in mitochondria, contributing

to the delayed IP. It has also been reported that the opening of the mitoK$_{ATP}$ channels by diazoxide induces cardioprotection and is NO-dependent (88-91). Furthermore, Rakhit *et al.* have demonstrated that the exogenous NO donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) induces early IP by a cGMP- dependent mechanism. It is also responsible for the delayed IP by cGMP-independent mechanism, mediated by activation of PKC and mitoK$_{ATP}$ channels (92). Nakano *et al.* suggested that SNAP induced cardioprotection cannot be reproduced by endogenous NO (93) implying that two separate pathways are working for the endogenous and exogenous NO-induced cardioprotection. The controversy in the results may be caused by the dose of SNAP used in the experiment. A reduced dose of SNAP (2μM) acts by a cGMP-independent mechanism whereas a higher dose of SNAP (100μM) acts by a cGMP-dependent mechanism (92-94).

Some investigators (95, 96) have reported that Diazoxide (mitoK$_{ATP}$ channel opener) mediated cardioprotection can be blocked by free radical scavengers and there is the belief that mitoK$_{ATP}$ channels lie upstream of the production of ROS (94, 95, 97). However, how mitoK$_{ATP}$ channels produce ROS and what the relationship is with NO metabolism remains unclear.

Figure 4 shows the relationship of mitoK$_{ATP}$ channels amd MPTP in ischemia reoxygenation injury and Figure 5 shows the consequence of opening of MPTP.



Reasons for opening of MPTP	Consequence of Mito K_{ATP} channels
Increased ROS	Increased threshold for opening of MPTP
Decreased ATP	Suppression of Ca^{++} overdose
Increased Ca^{++}	

Fig 4. The relationship of mitoK_{ATP} channels and MPTP during oxidative stress. During IP , PKC activation opens the mitoK_{ATP} channels which ultimately reduces ischemic and reperfusion injury.

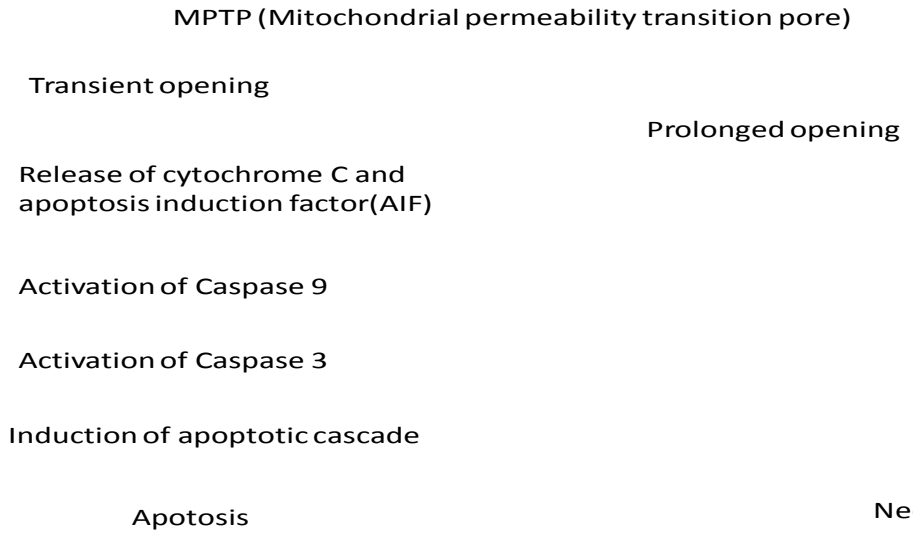


Fig 5. Consequence of opening of MPTP : Apoptosis requires ATP levels to be maintained whereas in necrosis ATP level falls. Transient opening of MPTP allows release of cytochromeC and mitochondrial swelling but resealing of pore restore ATO levels and favours apoptosis.

1.7. NO metabolism and p38MAPK

Our laboratory has previously demonstrated that the opening of p38MAPK is an obligatory step in IP that lies downstream of PKC activation (32). However, there is little information regarding the relationship of NO with p38MAPK in the myocardium. Thus, for example, Kim So et al showed that sodium nitroprusside activates p42/44 and p38MAPK in adult rat cardiomyocytes via both cGMP-dependent and cGMP-independent mechanisms (88). In another study, Wang *et al.* demonstrated that NO suppressed the angiotensin II induced activation of ERK in cardiac fibroblast (98). Other studies carried out in non-cardiac cells have shown that NO induced apoptosis is mediated directly by activation of MAPK in mitogenic cells (88, 98, and 99). Therefore, the relationship of p38MAPK with exogenous NO and endogenous NO produced by eNOS and nNOS remains largely unexplored and undefined.

1.8. nNOS and diabetic myocardium

Diabetes is a multiple organ disease. It increases the mortality and morbidity of coronary artery diseases (100). It also worsens the early and late outcome in acute coronary syndrome. Diabetes increases the risk of in-patient MI, complication of MI, and mortality after MI (101). Patients with diabetes also have an adverse long-term prognosis after MI including increased rate of reinfarction, congestive cardiac failure and death (102). In fact, 5 years mortality rate following MI may be as high as 50% more for diabetic patients than non-diabetic patients (103). During cardiac surgery

cardiopulmonary bypass induces greater oxidative stress in diabetic patients than in non diabetic patients that may be responsible, at least in part, for the increased postoperative mortality and morbidity among patients undergoing coronary artery bypass graft (104).

Diabetes represents a heterogeneous disorder and is characterised by endothelial dysfunction (105). All forms of clinical diabetes (IDDM, NIDDM), and also in animal models, exhibit reduced bioavailability of NO and altered expression and activity of NOS (106). The increased eNOS and decreased NO bioavailability seen in diabetic myocardium may be the result of high oxidative stress (107). There is also evidence that eNOS may be uncoupled in the diabetic state due to the lack of BH_4 (108). It has been suggested that iNOS is not expressed in the diabetic myocardium (107); however, it is still unknown about the expression and activity of nNOS in this clinical condition.

1.8.1. Diabetes and ischaemia-reperfusion injury

Diabetes alters neutrophil adhesion, platelet and fibrinolytic function, elicits endothelial and myocardial dysfunction and influences oxidative stress and myocardial energetic, each of them alone or in combination may be responsible for exaggerated reperfusion injury following a period of ischaemia (109). A number of studies have reported that diabetic myocardium is more sensitive to ischaemia/reperfusion injury, whereas some studies have shown contradictory results (110-113). The explanation for the differing results may be the use of different experimental conditions such as duration and severity

of diabetes, cardiac function, the duration of ischaemia and the type of metabolic substrate used. In addition, several intracellular mechanisms have been proposed to account for the variable responses of the diabetic heart to ischaemia/reperfusion injury: (i) alteration in the intracellular pH and Ca^{2+} (ii) increased production of ROS, (iii) alteration in the production of NO, and (iv) alteration in the regulation of mitoK_{ATP} channels.

The diabetic heart exhibits a decrease in the activities of Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchangers, and lower glycolytic rates during ischaemia may result in reduced accumulation of Na^{+} and Ca^{2+} with improvement of contractile function during reperfusion. However, during low flow of ischaemia, the degree of intracellular acidosis is less because of the wash out of lactate, and in addition to the low glycolytic rate, the diabetic heart may be more vulnerable to ischaemia/reperfusion injury than the non-diabetic heart (114).

Hyperglycaemia has been shown to acutely increase circulating inflammatory cytokines in human such as IL-6, TNFα. Over expression of inflammatory factors may exacerbate the ischaemia/reperfusion injury in the diabetic heart.

The enhanced oxidative stress seen in the diabetic heart may be responsible for more ischaemia/reperfusion injury in diabetic hearts than in non-diabetic hearts (115). Oxidative stress triggers the activation of caspase, the release of toxic metabolites into the cytoplasm and the alteration in mitochondrial membrane potential, which in turn results in the opening of the mitochondrial transitional pore.

At present, limited evidence exists that the upregulation or downregulation of NOS or any other modification of the NO metabolism will protect myocardium from ischaemia/

reperfusion injury under pathological conditions such as diabetes.

1.8.2. Diabetes and ischaemic preconditioning

The cardioprotective potential of IP in diabetes is also conflicting. Some studies reported that the cardioprotective effects are lost in diabetes whereas others have suggested that diabetic hearts are more protective than non diabetic hearts (116-119). The reported differences may be explained by the alteration in function of cardiac subcellular organelles including sarcolemma, sarcoplasmic reticulum and mitochondria (120). Diabetes is also associated with several abnormalities in energy metabolism, deranged Na^+/Ca^{2+} and Na^+/H^+ exchange activities, mitochondrial and sarcoplasmic Ca^{2+} pump, alteration of Na^+/K^+ pump and antioxidant defences that would contribute to the variable results (121). It is also known that diabetes alters the function and availability of vascular mitoK_{ATP} channels (122-125) and since these channels are recognised as major components of IP against infarction and stunning, it is logical to assume that IP-induced cardioprotection may be altered in diabetes. Our group has demonstrated that human diabetic myocardium cannot be preconditioned (126).

Both insulin and hyperglycaemia have also been shown to have detrimental and beneficial effects on cardiovascular diseases (127-131). Also, both have significant effect on mitoK_{ATP} channels that are central to the signal transduction mechanism of IP (132-137).

Another important factor that needs to be considered is the effect of anti-diabetic treatment on preconditioning. Some studies have reported that human myocardium from

patients with long-term exposure to oral sulfonylurea abolished cardioprotection by IP (133). As the induction of IP relies on mitoK_{ATP} channels, it is accepted that long term inhibition of these channels with oral hypoglycaemic agents would result in abrogation of cardioprotection, an effect that may explain the increased morbidity and mortality in these patients. Nonetheless other factors such as the impaired synthesis of stress-induced heat shock proteins could play a role in the loss of cardioprotective potential.

1.9. Hypothesis and aims of the thesis

The hypothesis of this thesis is that nNOS plays a key role in the myocardial injury induced by ischaemia and reoxygenation and in the cardioprotection of IP. Hence, the aims of the studies were to investigate:

1. the role of nNOS in ischaemic reoxygenation induced injury and cardioprotection by IP,
2. whether the role of nNOS in ischaemic reoxygenation induced injury is species dependent,
3. the relationship of nNOS with mito K_{ATP} channels,
4. the relationship of nNOS with p38MAPK, and
5. the role of diabetes in the effects mediated by nNOS.

Chapter 2

Methodology

In this chapter, the laboratory techniques applied in this thesis are presented.

2.1. Procurement of myocardial tissues

The right atrial appendage from patients undergoing elective, first time, coronary artery bypass graft or aortic valve replacement was obtained. This tissue is considered as a surgical surplus tissue, however, ethical approval was obtained from the local research ethics committees (Leicestershire Research Ethics Committee Ref No 7805) and written consent from each patient was sought. Patients with poor left ventricular function (ejection fraction <30%) and atrial fibrillation and also those on treatment with opioids, catecholamines and mitoK_{ATP} channels openers (e.g. nicorandil), were excluded. For some experiments the right atial appendage from diabetic patients was also taken for study. The hearts from rats and mice were obtained after culling the animals by cervical dislocation and followed by opening of the thorax.

2.2. Experimental preparation using myocardial tissues

The right atrial appendages from patients, and the ventricles from rats and both atrium and ventricles from mice, were mounted onto an ice cool, ground glass plate, with the epicardial surface face down. The tissues were sliced freehand with surgical skin graft blades (Shwann-Morton, United Kingdom). Sections weighing 30-40 mg and measuring

300-400 μm thick, were transferred to conical flasks containing 10ml of oxygenated Krebs Henseleit Hepes buffer (KHH). Sections were equilibrated for 30 minutes in KHH buffer solution which contained NaCl 118 mM, KCl 4.8 mM, $NaHCO_3$ 27.2 mM, KH_2PO_4 1 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.25 mM, glucose 10 mM, Hepes 20 mM. The KHH was bubbled with 95% oxygen (O_2) and 5% carbon dioxide (CO_2) at 37°C in a water bath. Simulated ischaemia was induced by bubbling the media with 95% nitrogen (N_2) and 5% CO_2 at 37°C and a pH of 6.8 and by removing glucose for 90 minutes. Reoxygenation was achieved by incubating tissues in 10ml oxygenated KHH medium with added glucose, at 37°C, for a period of 120 minutes. Some muscles were incubated in normoxic KHH buffer for the entire experimental period and served as time-matched aerobic controls. Other muscles were subjected to the IP protocol: this consisted of 5 minutes ischaemia followed by 5 minutes of reoxygenation, a protocol that has been shown to afford maximal protection in this model (183). The remaining muscles were randomized to receive various treatments, as described in the following chapters. A representative picture of oxygenation and ischemia/reoxygenation is shown in figure 6,7 and 8.

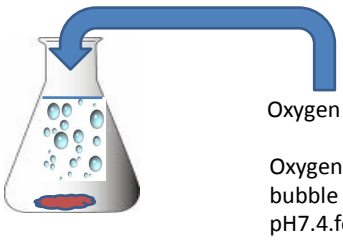


Figure 6: Aerobic control

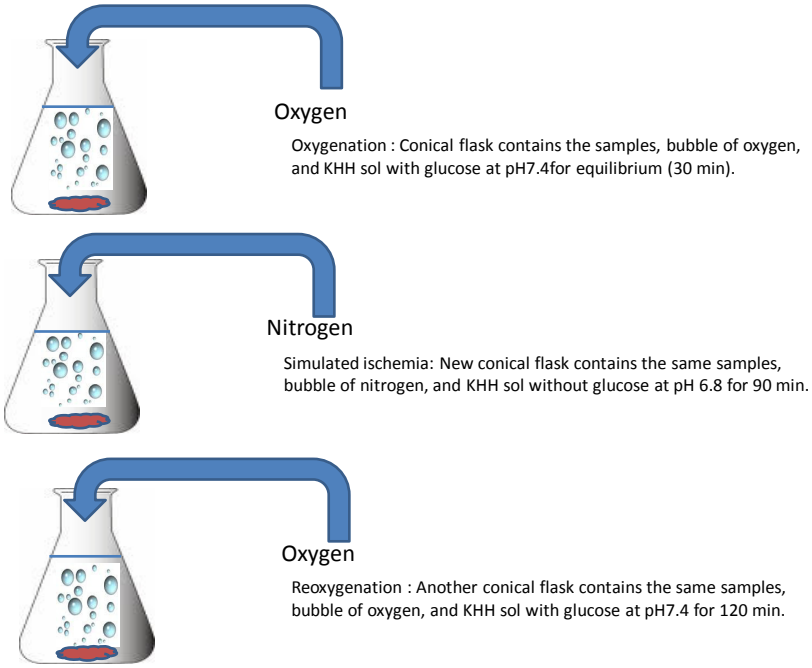


Figure 7: Ischaemia/reoxygenation

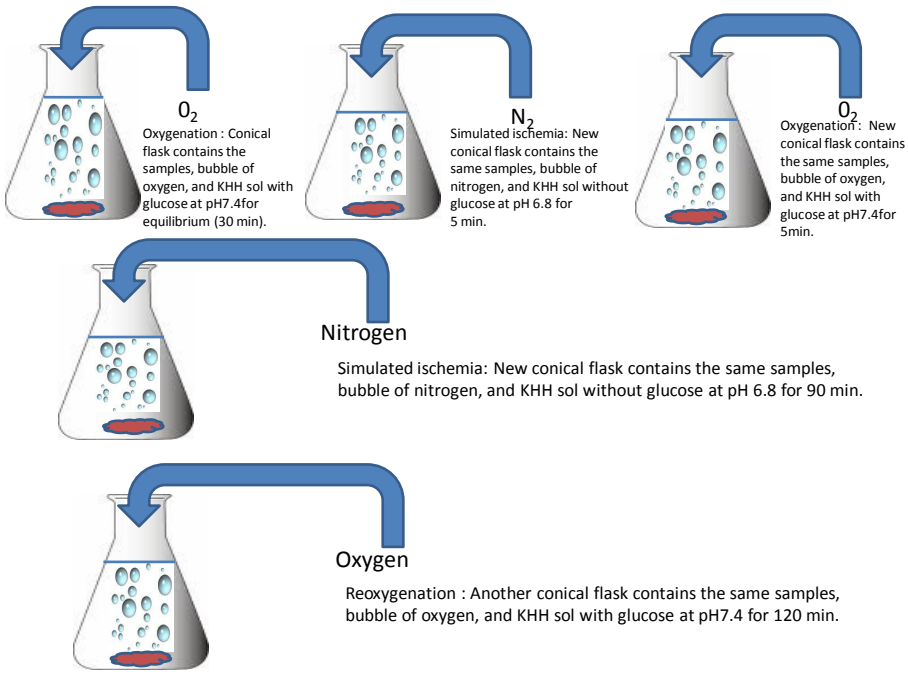


Figure 8: Ischaemic preconditioning

2.3. Solutions and Chemicals

Constituents for KHH buffer solution were purchased from Sigma, BDH and Fisher Scientific Int. Company. S-Nitroso-N-acetyl-DL-penicillamine (SNAP), Nω-Nitro-L-acetyl methyl ester hydrochloride (L-NAME), 1-[2-(Trifluoromethyl) phenyl] imidazole (1) (TRIM), 5-hydroxydecanoate (5-HD), SB203580 were obtained from Sigma. The KHH was prepared daily with deionized distilled water and contained NaCl 118 mM, KCl 4.8 mM, $NaHCO_3$ 27.2 mM, KH_2PO_4 1 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.25 mM, glucose 10 mM, Hepes 20 mM. 5-HD, TRIM and L-NAME were dissolved in deionised distilled water, and SNAP and SB203580 were dissolved in Dimethyl sulfoxide (DMSO). All the chemicals were of the highest purity. The concentrations of the agents were obtained from previous dose-response studies (139,140).

2.4. Assessment of tissue injury

Tissue injury was assessed by measurement of creatine kinase (CK) release into the media during the 120 minutes reoxygenation period. The enzyme activity was measured by an ultraviolet based method, on the formation of NADP. A commercial assay kit was used (30-3060/R2: Abbott Laboratories, Diagnostic Division, Kent, UK) with a 96 well, flat-bottom micro plate (Costar, Corning Life Sciences, Lowell, Massachusetts, USA). In this assay, NADP was reduced to NADPH, and light absorption at 340nm is measured using a spectrophotometer (Benchmark, Bio-Rad Laboratories, California, USA). Results were expressed as IU/mg wet weight after subtraction from aerobic control value.

2.5. Assessment of cell death

At the end of the experimental protocol, tissues were incubated for 15 minutes at room temperature, on the rocker, with 20μg/ml propidium iodide (PI, Sigma-Aldrich, Dorset, UK) in phosphate buffered saline (PBS) at pH 7.4 in order to identify the necrotic nuclei. Muscles were then washed twice with PBS for 5 minutes each time before fixation in 4% paraformaldehyde. They were kept overnight at 4-10°C and then transferred to 30% sucrose until the tissue sank. All the above steps were performed in the dark to avoid exposure to light. Following this, the muscles were embedded with Optical Cutting Temperature Embedding Matrix (Tissue-Tek®, Agar Scientific Ltd, Essex, UK). Frozen sections were then cut at a 7μm thickness in a Bright cryotome (model OTF) at –25°C, and sections were collected on SuperFrost Plus slides (Menzel Glasser, Braunschweig, Germany). The slides were then kept at -80°C until analysis.

To assess apoptosis, the slides were brought from -80°C to room temperature and washed with 20mM PBS. They were then permeabilised for 1 minute in a microwave oven (850 watts), in 200ml of 0.1% Triton X-100 in 0.1M Tri-sodium citrate buffer at pH 6.0. After this, the slides were rapidly cooled by adding 80ml distilled water and transferred to 20mM PBS solution. In addition, they were immersed in 3% bovine serum albumin (Sigma Aldrich, Dorset, UK) in 0.1M Tris-HCl buffer with 20% foetal bovine serum (Hyclone, Utah USA) at pH 7.5 for 30 minutes to block unspecific labeled activity. The terminal deoxynucleotidyl transferase (TdT) was used to incorporate fluorescein (FITC) labelled dUTP oligonucleotides to DNA strand breaks at the 3'-OH termini in a template- dependent manner (TUNEL technique). This took 90 minutes at 37°C in a humidity chamber using a commercially available kit (Roche Diagnostics, Penzberg, Germany). A negative control was performed each time. A negative control, obtained by not adding the enzyme solution, was performed every time.

To distinguish the total number of nuclei, sections were counter-stained with 1µg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, Oregon, USA) in PBS for 1 minute. Then the slides were washed 3 times for 5 minute each, in PBS. To reduce photobleaching, the sections were mounted with anti-fade solution (Prolong Antifade kit, Molecular Probes, Eugene, Oregon, USA) and covered with coverslips (Menzel Glasser, Braunschweig, Germany)

A fluorescent microscope (Axiovert 200M, Zeiss fluorescent microscope, Göttingen, Germany) at 40X magnification was use to assess necrosis and apoptosis. At least ten

fields per section, and one section per staining, were examined for each experiment. The fields were measured following the horizontal and vertical axes of the sections. Propidium iodide (PI) and FITC labelled nuclei were detected by the Cy3 and EGFP channels respectively, whilst DAPI labelling was detected by the DAPI channel. Only the necrotic or apoptotic signals coinciding with DAPI were considered true events. The NIH Image software (Scion Corp, Frederick, Maryland, USA) was used to determine the total events for each field. To avoid the inclusion of artefacts only fluorescent signals with areas greater than 16μm^2 were counted.

Representative picture of DAPI, DAPI and PI, DAPI and TUNEL are shown in figure 9, 10, and 11.

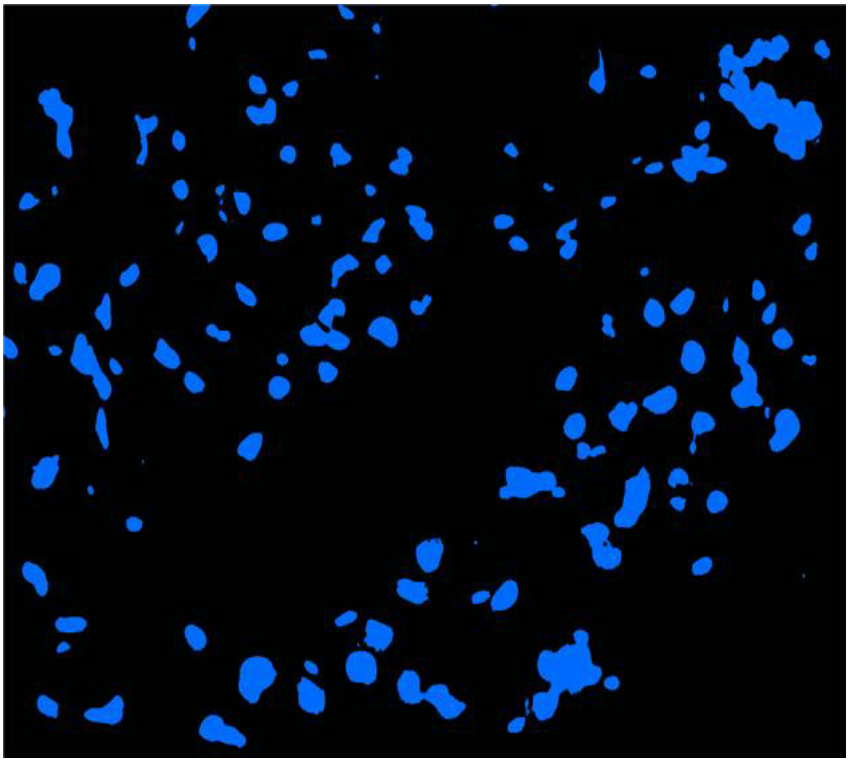


Figure 9: Representative picture of DAPI (blue-normal nuclei) at 40X magnification.

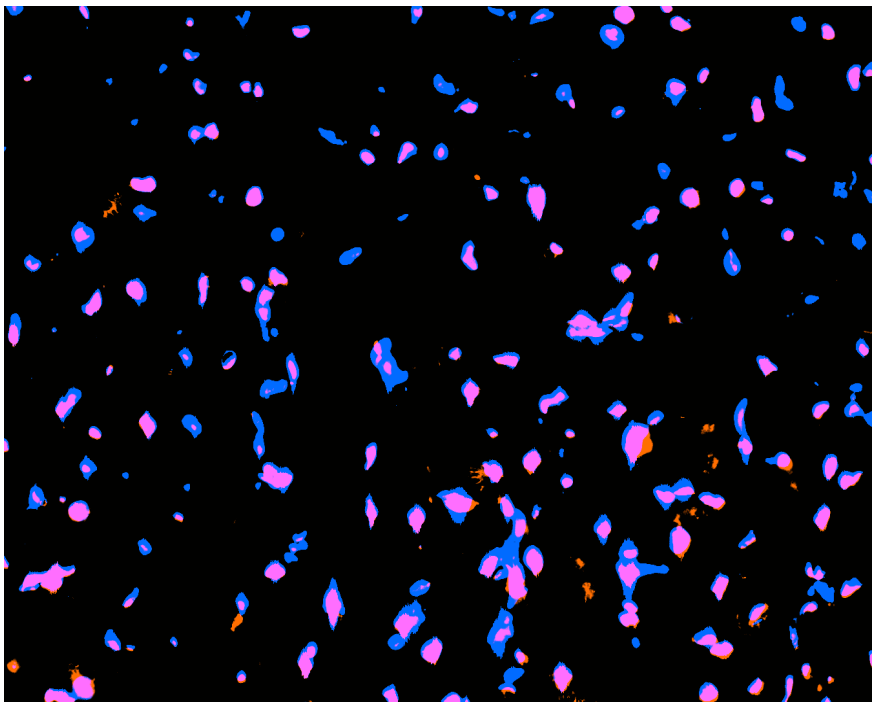


Figure 10: Representative picture of DAPI and PI staining (blue-normal nuclei, pink- necrotic nuclei) at 40X magnification.

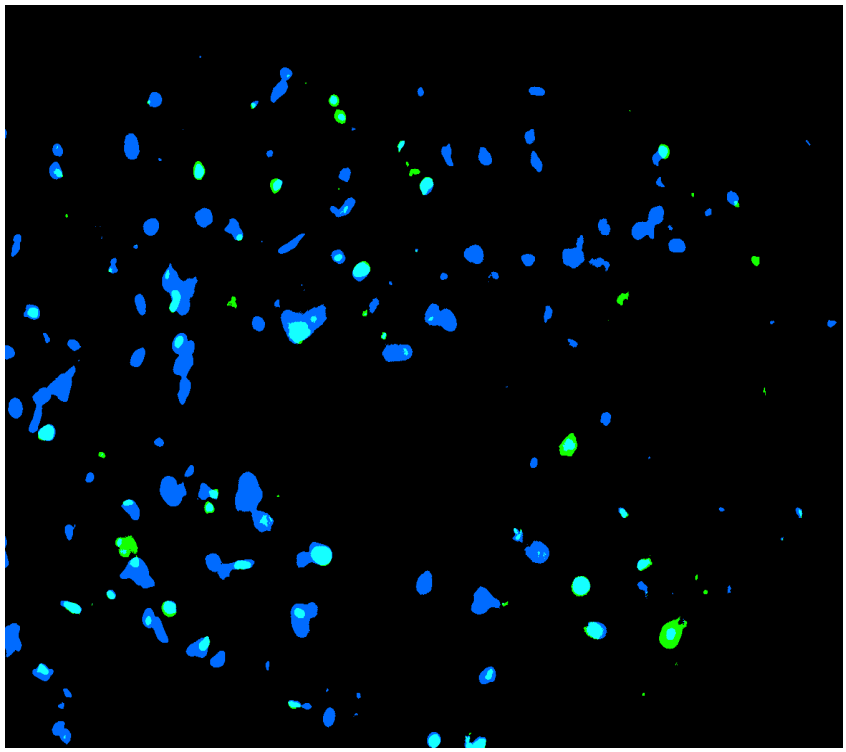


Figure 11: Representative picture of DAPI and TUNEL staining (blue-normal nuclei, green- apoptotic nuclei) at 40X magnification.

2.6. Discussion

The use of the right atrial appendage from patients is economic, simple to prepare and easily available. The myocardial sections remains stable for 24 hours under the described incubation conditions, and vascular integrity is not necessary for the metabolic function of the section (141). The thickness of the sections is 0.2-0.5mm and is appropriate for diffusion and metabolites. The degree of ischaemia/reoxygenation-induced injury is proportional to the severity of the ischaemia (183).

The use of *in vitro* experimental preparation in this thesis has advantages over *in vivo* preparations, as it is less complex and is not influenced by blood, neuroendocrine or hormonal factors. The prepared muscles sections are superfused and, moreover, the removal of the vasculature as the natural pathway for the provision of substrate may also be advantageous by eliminating the confounding factors such as the no-reflow phenomenon, and the variability of the collateral flow during ischaemia/reoxygenation.

Atrial and ventricular muscles were used in my studies and they may react differently to ischaemia/reoxygenation induced injury. For example, K_{ATP} channel and adenosine receptors, that play an important role in the degree of tissue damage, are present in both atrium and ventricles, but the density of these cellular components is different in the atrium and ventricles (142,143). Despite this concern, Walker *et al.* (144) demonstrated that the response to ischaemia/reoxygenation of ventricular and atrial muscles is similar. This is shown in the present studies when the right atrial appendages from human were compared to the ventricular myocardium from rats and mice. The results are consistent and reproducible.

Several investigators have demonstrated that the myocardium from patients with poor left ventricular function (ejection fraction <30%), and also from those on treatment with nicorandil cannot be cardioprotected by IP (145,146,185), therefore, these patients were not used as donors. Also, drugs such as opioids, sulphonylurea, the mitoK_{ATP} channels opener Diazoxide, and catecholamines have been shown to induce pharmacological preconditioning and therefore patients treated with any of these agents were not included as donors (133, 147,148).

The indexes of ischaemia/reoxygenation injury used in this thesis are widely utilized by many laboratories and are accepted by the scientific community as reliable markers of myocardial injury and because of this, they are not further discussed.

Chapter 3

Role of nNOS in ischaemia/reoxygenation-induced injury and protection by ischaemic preconditioning

3.1. Introduction

It is well established that enhanced bioavailability of endogenous NO affords cardioprotection against ischaemia and reoxygenation induced injury (149). As mentioned in Chapter 1, this important mediator is enzymatically produced by the three NOS isoforms (eNOS, nNOS and iNOS), widely distributed through most cells and tissues, by converting L-arginine to L-citrulline in the presence of NADPH, O_2 and other co-factors (60).

In cardiomyocytes nNOS is expressed in sarcoplasmic reticulum, sarcolemma and mitochondria (61, 63, and 64). Nitric oxide derived from nNOS influences the beat-to-beat regulation of basal cardiac function, serving negative feedback control of Ca^{2+} fluxes (65, 66). In addition, it facilitates vagal stimulation and inhibits sympathetic stimulation by exerting its effect in central nervous system (CNS) and peripheral nervous system (PNS).

NO derived from eNOS is essential for eliciting early ischemic preconditioning (150) however there is still controversy about the role of nNOS. The reason for these discrepancies may be related, at least in part, to the complexity of nNOS location and activation. Casadei *et al.* has reported in the review paper that the concentration and

activity of nNOS is enhanced in left ventricular myocardium of chronically infarcted animals and in failing human hearts, suggesting that nNOS attribution is essential in the myocardial response to stress (151). In this situation, nNOS seems to be located in the sarcolemmal membrane rather than in sarcoplasmic reticulum where it co-localises with RyR receptor (152, 153).

Bolli *et al.* hypothesised that NO derived from eNOS and nNOS participates in second window of ischaemic preconditioning in the rabbit model (83,154 and 155). In contrast, Jones *et al*. suggested that nNOS plays no role in early IP in mice and observed no change in infarct size when compared with wild type mice (81). But the same group has also demonstrated that NO generated from nNOS contributes to ischaemic brain necrosis whereas eNOS derived NO protects brain in nNOS knockout mice models (156). This investigation was done by occluding the middle cerebral artery in the context of ischaemia/reoxygenation.

Takimoto *et al.* has suggested that during acute MI, nNOS expression is increased in infarcted and non-infarcted regions. Nitric oxide synthesised from nNOS modulates autonomic activity and decreases heart rate in intact mammalian heart, and is associated with the development of ventricular fibrillation after MI (63, 79). Increased expression of eNOS, together with nNOS, may play a protective role by improving coronary blood flow by dilating coronary blood vessels in the setting of acute MI. Inducible NOS expression, however, is continuously increased in non-infarcted regions which is attributed to the gradual contractile dysfunction leading to heart failure after MI (63).

Even some investigators have suggested that in cardiomyocyte nNOS derived NO can be regarded as neutral bystanders without any influence on IP (93, 157). So, the precise role of nNOS in ischemia/reoxygenation and IP appears to vary between animal species and organs. There is little information in the literature relating to these processes in human.

A number of important issues remain to be addressed regarding the role of nNOS as a trigger of ischaemia/reoxygenation injury and IP. First to date, there is no evidence that nNOS is involved in early IP in myocardium and secondly there is controversy in the role of nNOS in different organs and species. To address these issues, I tried to provide evidence for the involvement of nNOS in ischemia and IP by using rat and mice ventricles and in addition, I investigated the role of nNOS human right atrial appendages.

Aims: The aims of the present studies were to investigate the role of nNOS in ischemia/reoxygenation injury and IP and to determine whether the role of nNOS is species dependent.

3.2. Materials and Methods

3.2.1. Study animals

Wister rats and C57BL/6J mice were purchased from Charles Rivers UK Ltd (Kent, UK). Animals were culled by cervical dislocation and the heart was rapidly dissected and sectioned, as described in chapter 2. These studies were undertaken in accordance

with the guidelines on the Operation of Animals (Scientific Procedure) Act 1986. All procedures are approved by the Animal Care and Use Committee of the University of Leicester.

3.2.2. Study patients

The right atrial appendage from patients undergoing elective heart surgery for coronary bypass surgery or aortic valve surgery was obtained prior to the initiation of cardiopulmonary bypass. Patients with atrial fibrillation, cancer, diabetes, poor LV function (EF<30%) were excluded from the study. In addition, patients undergoing concomitant surgical procedures or those being treated with opioids, catecholamine, K_{ATP} channel opener nicorandil were also excluded from the study. The study was conducted according to Declaration of Helsinki principles and approval was obtained from the Local Research Ethics Committee. All participants provided written consent.

3.2.3. Processing of samples and experimental preparation

The study was conducted using an established model described previously in Chapter 2 (Section 2.2).

3.2.4. Measurement of myocardial injury and viability

The index of myocardial injury, CK leakage and cell death by apoptosis and necrosis were used as described in section 2.4 and 2.5 in Chapter 2.

3.2.5. Study protocols

3.2.5.1. Study 1: The role of nNOS in myocardial ischaemia/reoxygenation injury and

in cardioprotection by IP

To investigate this, muscles from ventricles of rat hearts were randomized to receive the exogenous NO donor S-Nitroso-N-acetyl-DL-penicillamine SNAP (100μM), the non-selective NOS inhibitor Nω-Nitro-L-acetyl methyl ester hydrochloride L-NAME (100μM), or the selective nNOS inhibitor 1-[2-(Trifluoromethyl) phenyl] imidazole(1) TRIM (100μM), for 20 minutes prior to ischaemia. The concentrations of the reagents used were identified from the previous literature (139,140). Some preparations were subjected to IP (5 minutes ischaemia, 5 minutes reoxygenation).

3.2.5.2. Study 2: To elucidate whether the role of nNOS is species-dependent

This was achieved by applying a protocol identical to study 1 to ventricular myocardium from mouse and right atrial appendages myocardium from human.

3.2.6. Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Each reported value was obtained after subtracting the corresponding time-matched aerobic control value. A one way ANOVA followed by Bonferroni's test was used to compare the significance between groups. Analyses were performed using the SPSS program. Differences were considered to be statistically significant if $p<0.05$.

3.3. Results

Myocardial viability was assessed by 95% or more absence of necrosis and apoptosis by

the end of the experimental period in aerobically incubated myocardial slices.

(i) The role of nNOS in ischaemic injury and IP

Figure 12A shows the experimental protocols for Study 1. Figures 12B-12D show that, in the rat myocardium, the selective inhibition of nNOS by TRIM resulted in a significant reduction in CK release, and in cell necrosis and apoptosis, when compared to the mean values in the ischaemia/reoxygenation alone group with no TRIM treatment. The degree of this protection was similar to the administration of exogenous NO, but greater than the non-selective NOS inhibitor L-NAME. Importantly, the cardioprotection afforded by the exogenous NO donor SNAP, and by the selective nNOS inhibitor TRIM was significantly greater than that of IP.

(ii) Is the role of nNOS species-dependent?

Figure 12E shows the experimental protocols for Study 2. As seen in Figures 12F-12K, and in comparison with the results observed in Figures 12B-12D, the order of magnitude of ischaemic injury for CK release, and cell necrosis and apoptosis varied slightly between species. However**,** the identical effects on NOS inhibition after the administration of exogenous NO to rat myocardium (Figures 12F- 12K) were observed for both mouse and human myocardium. Again, in both species, treatment with SNAP and TRIM afforded greater protection than L-NAME.

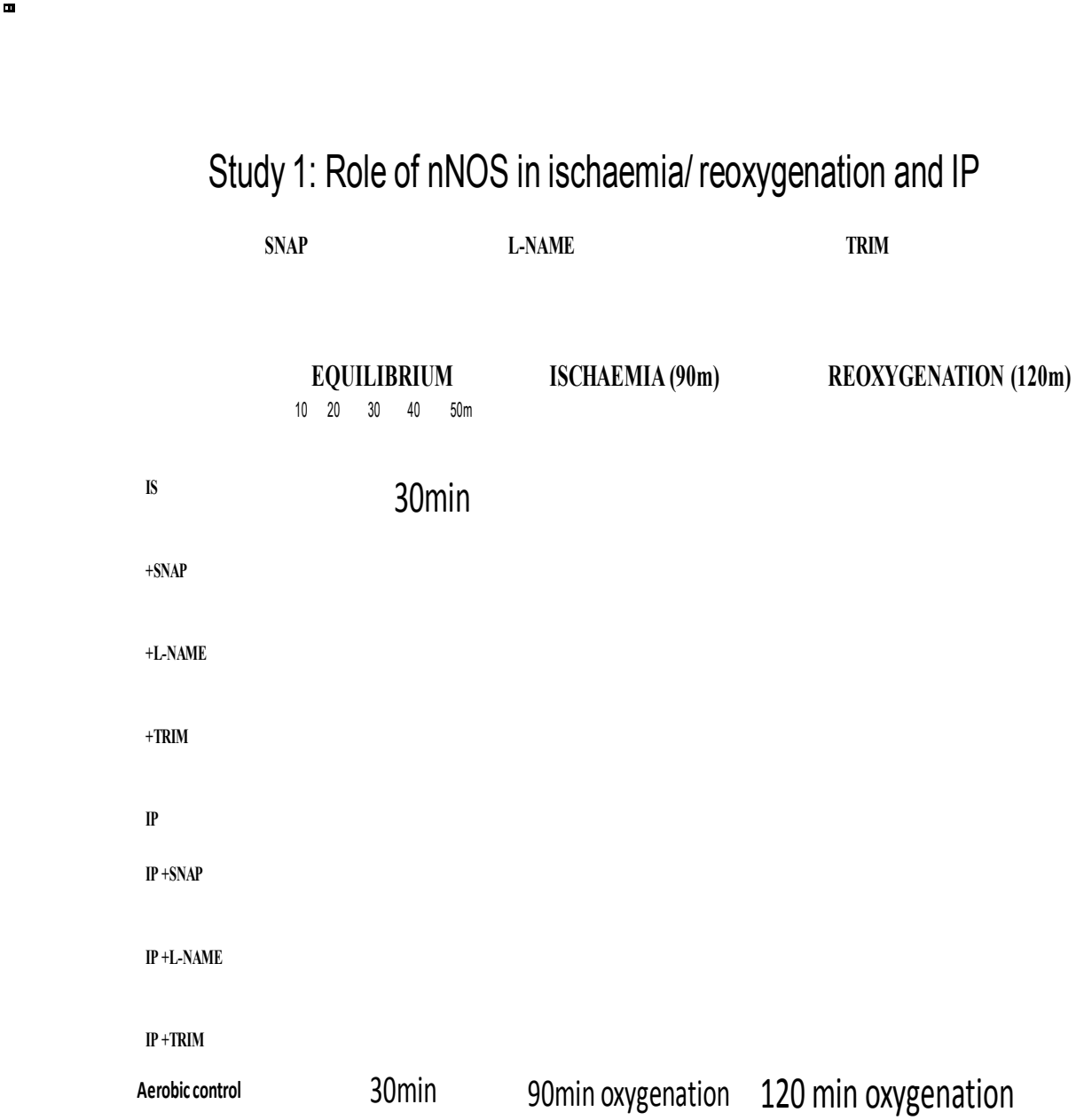


Figure 12 A. Experimental protocols for Study 1.

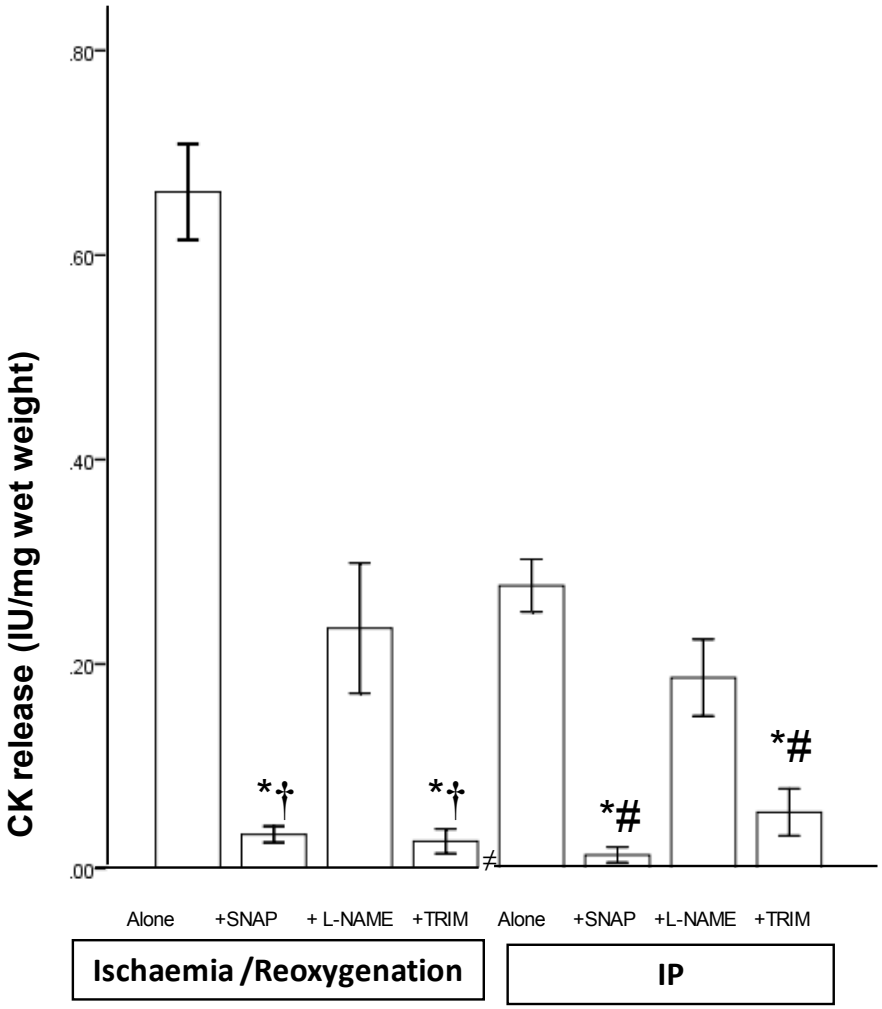


Figure 12B: CK release in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6 /groups means 6 organ). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia.

(*p value<0.05 vs. ischaemia,† p<0.05 vs. L-NAME and # p<0.05 vs. IP group).

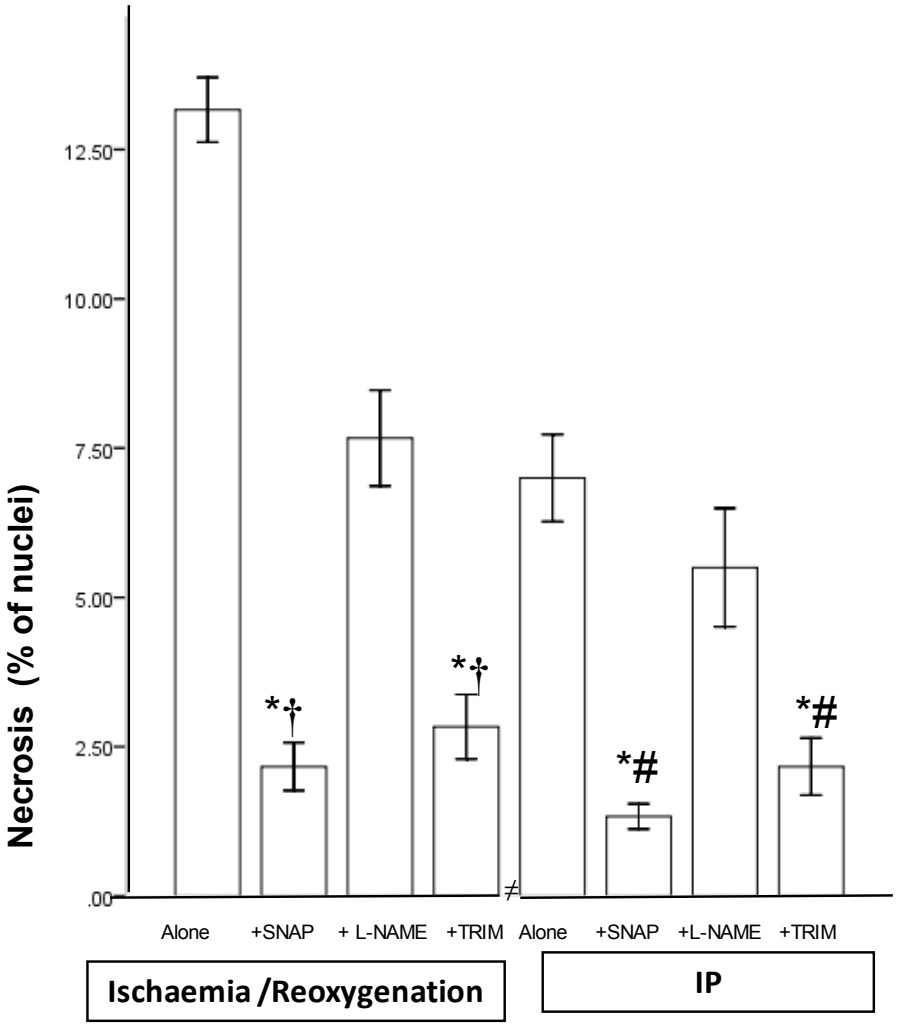


Figure 12C: Cell necrosis in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organ). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia. (*p value<0.05 vs. ischaemia,† p<0.05 vs. L-NAME and # p<0.05 vs. IP group).

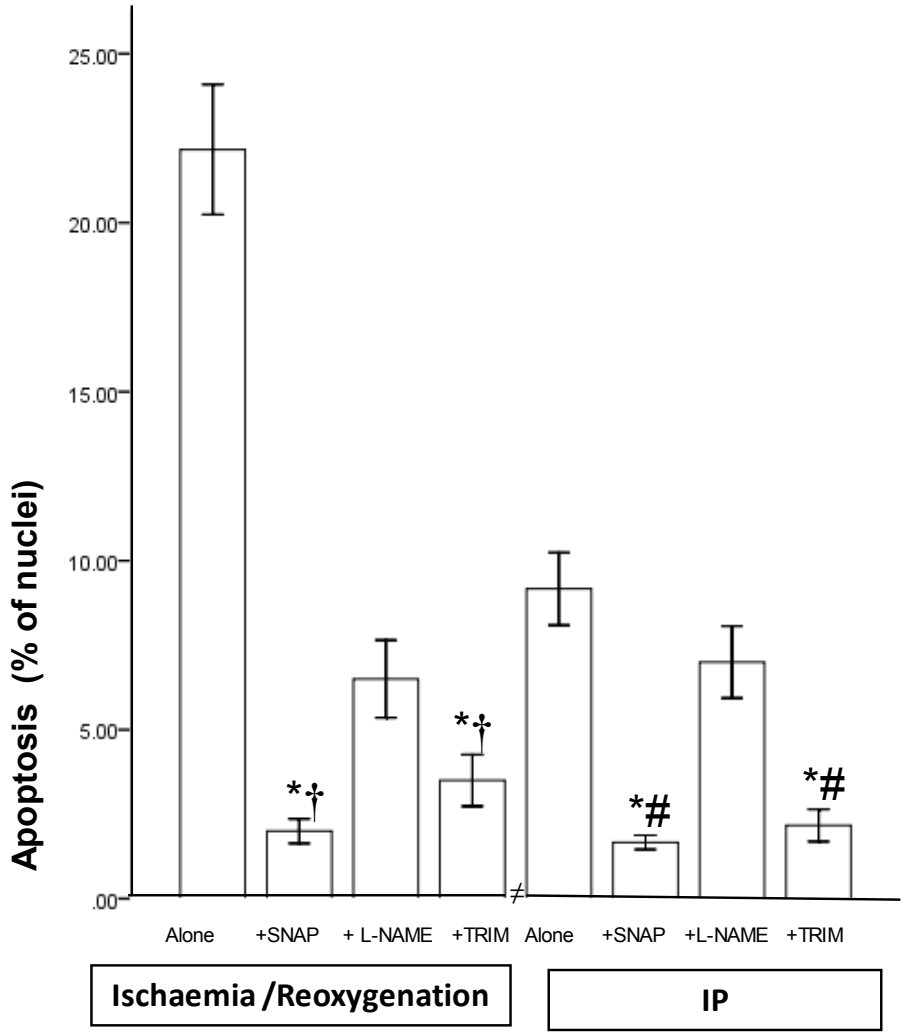


Figure 12D: Cell apoptosis in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organ). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia.

(*p value<0.05 vs. ischaemia, † p<0.05 vs. L-NAME and # p<0.05 vs. IP group).

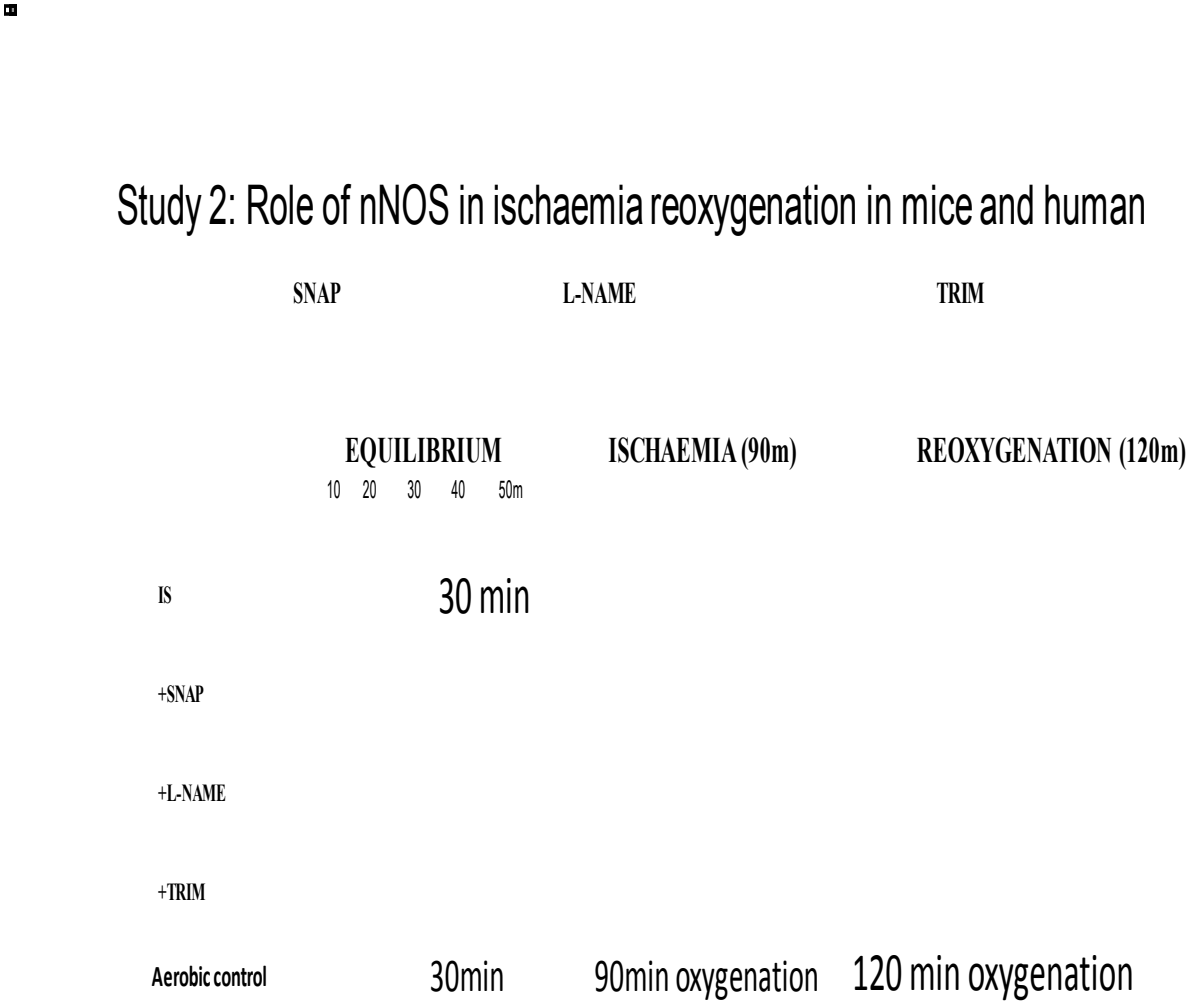


Figure 12E. Experimental protocols for Study 2.

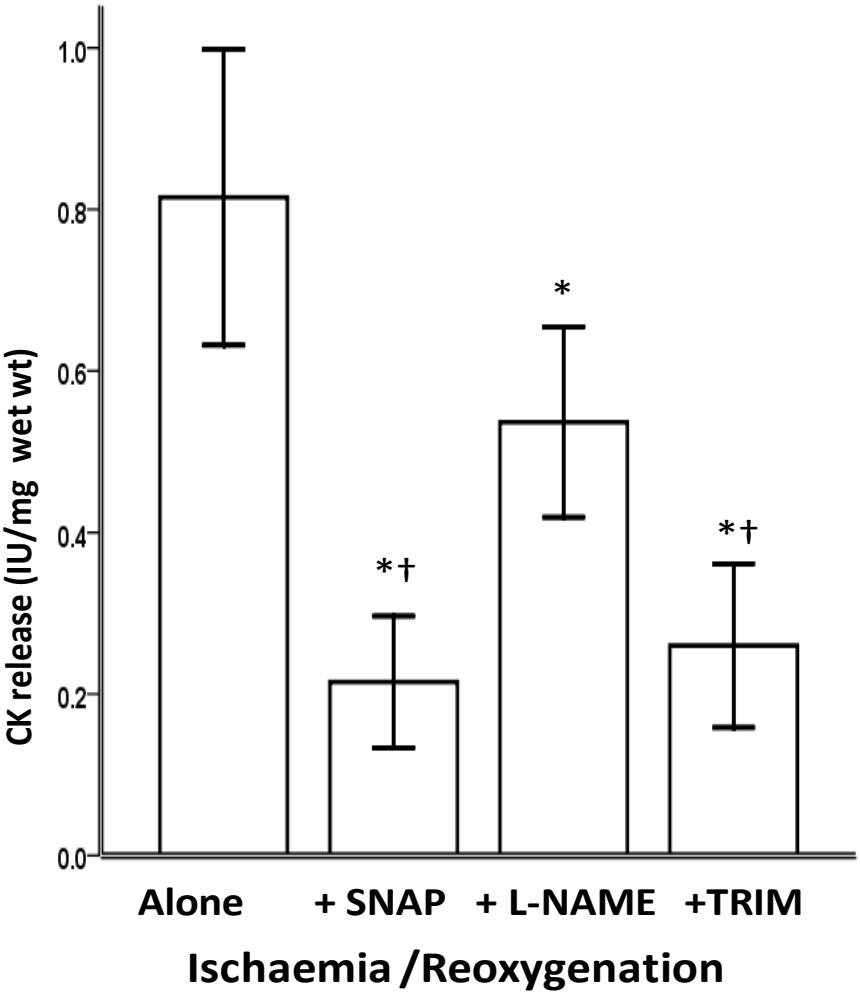


Figure 12F: CK release in mouse ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia.

*$P < 0.05$ vs. ischaemia**;** †P<0.05 vs. L-NAME treated groups.

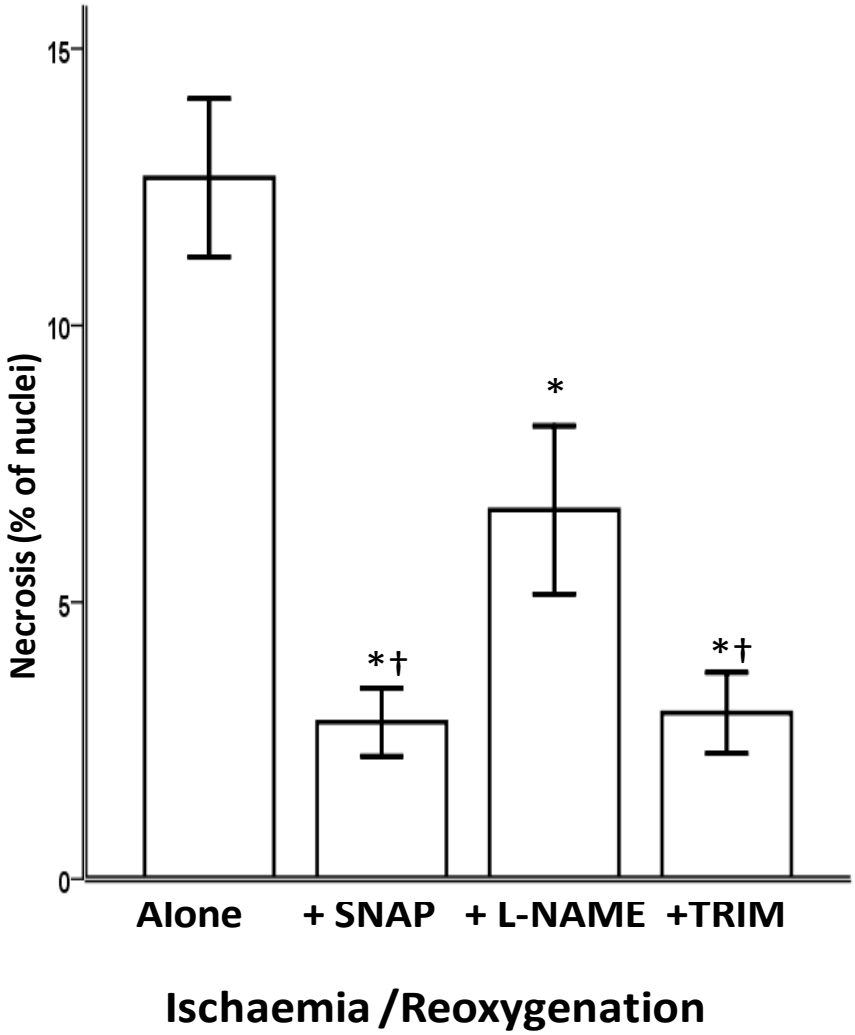


Figure 12G: Cell necrosis in mouse ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia.

*$P < 0.05$ vs. ischaemia**;** †P<0.05 vs. L-NAME treated groups.

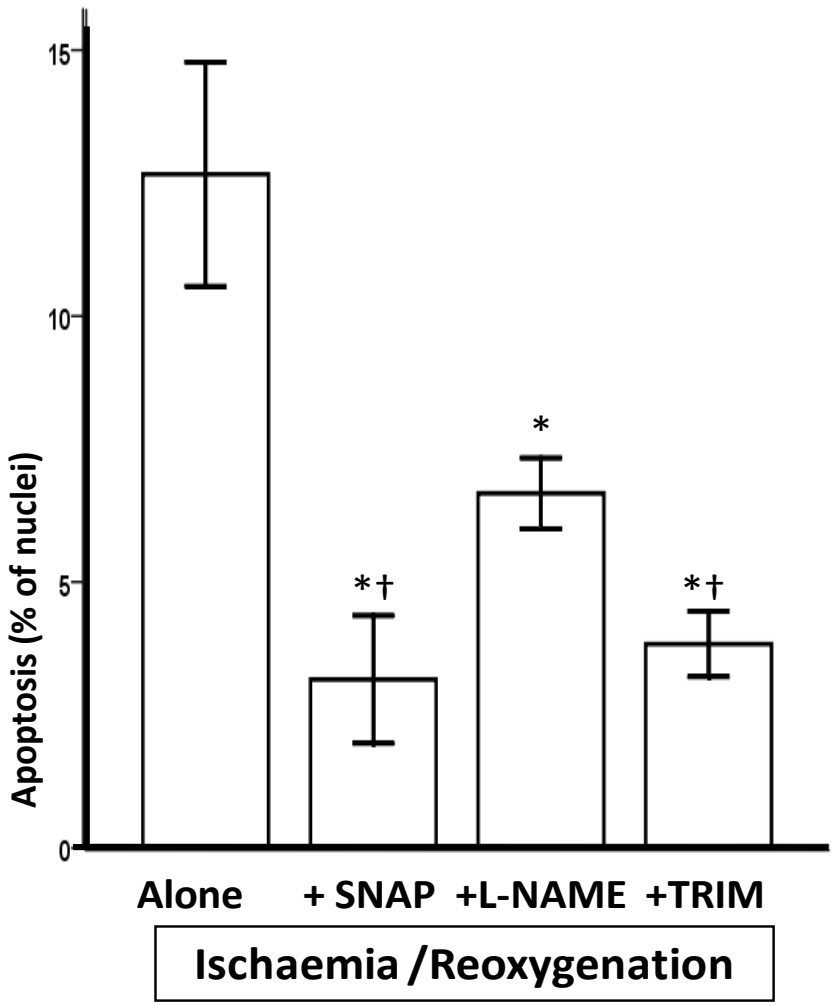


Figure 12H: Cell apoptosis in mouse ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia. *$P < 0.05$ vs. ischaemia; †P<0.05 vs. L-NAME treated groups.

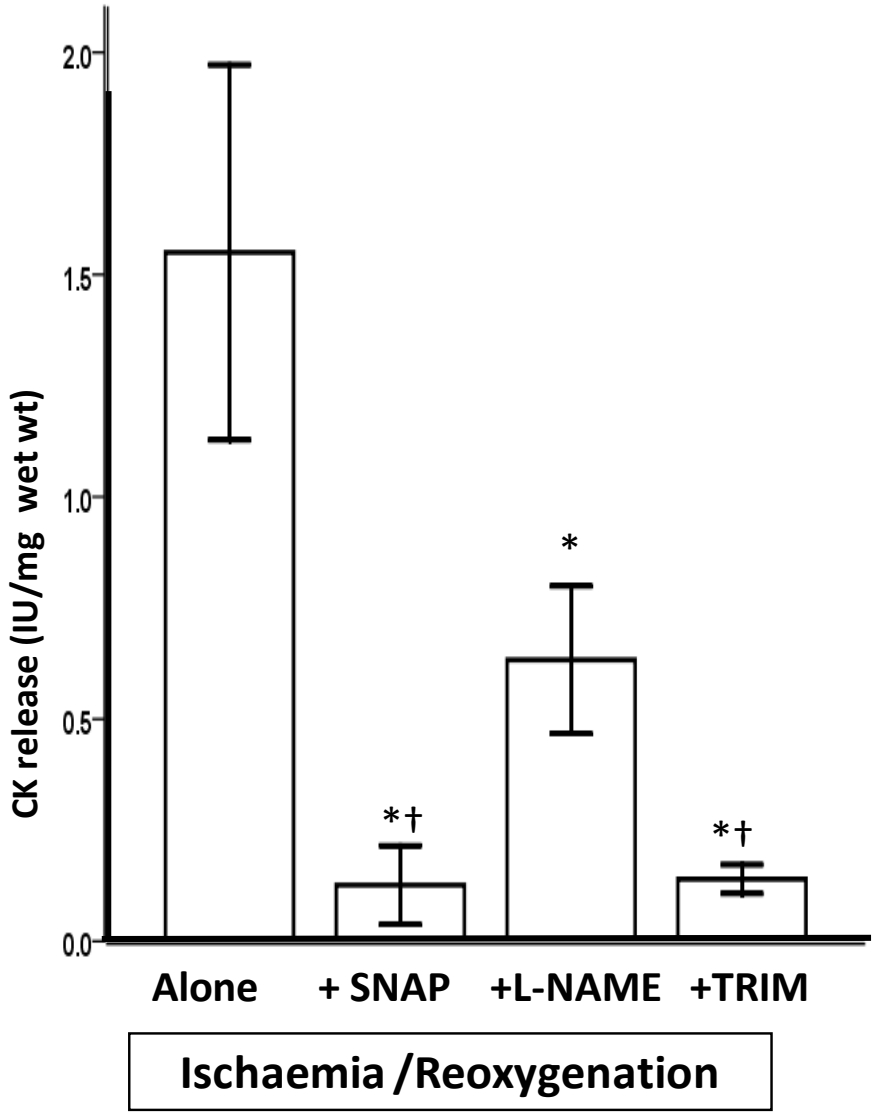


Figure 12I: CK release in human right atrial myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organs). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia.

*$P < 0.05$ vs. ischaemia**;** †P<0.05 vs. L-NAME treated groups.

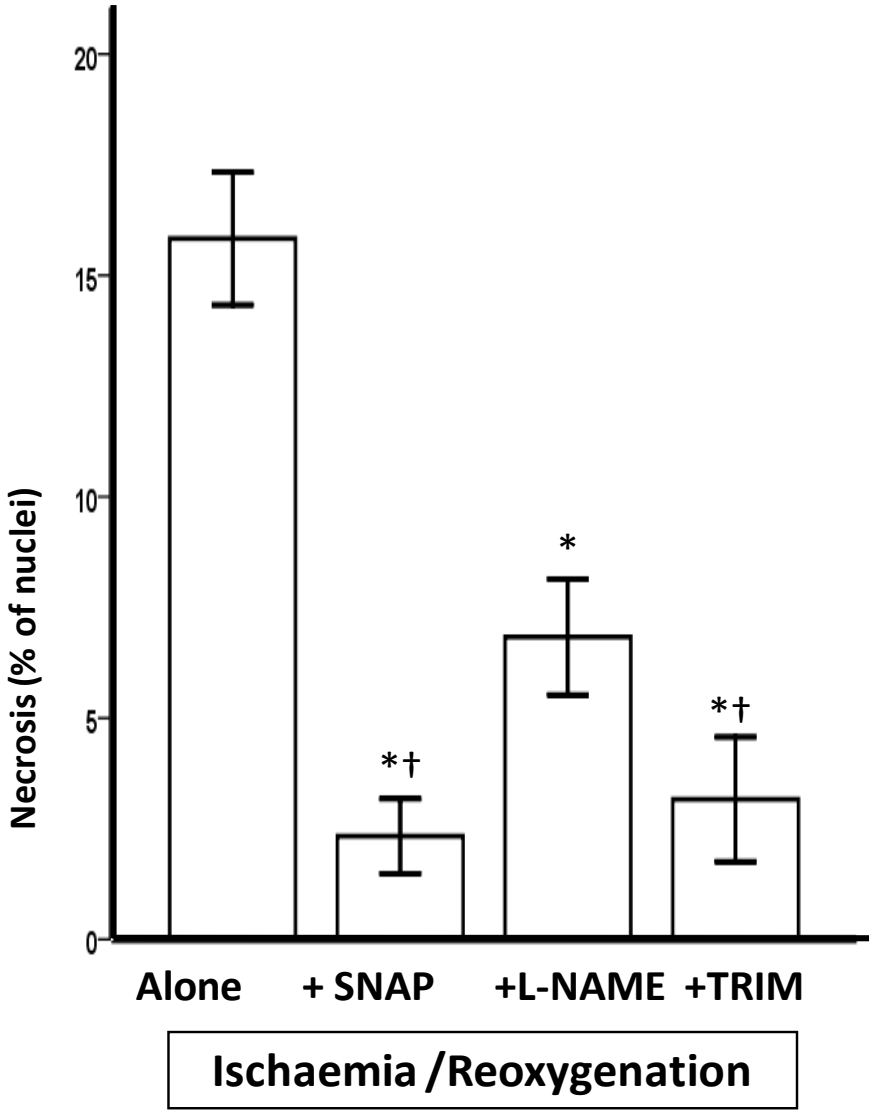


Figure 12J: Cell necrosis in human right atrial myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organ). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischeamia.

*$P < 0.05$ vs. ischaemia**;** †P<0.05 vs. L-NAME treated groups.

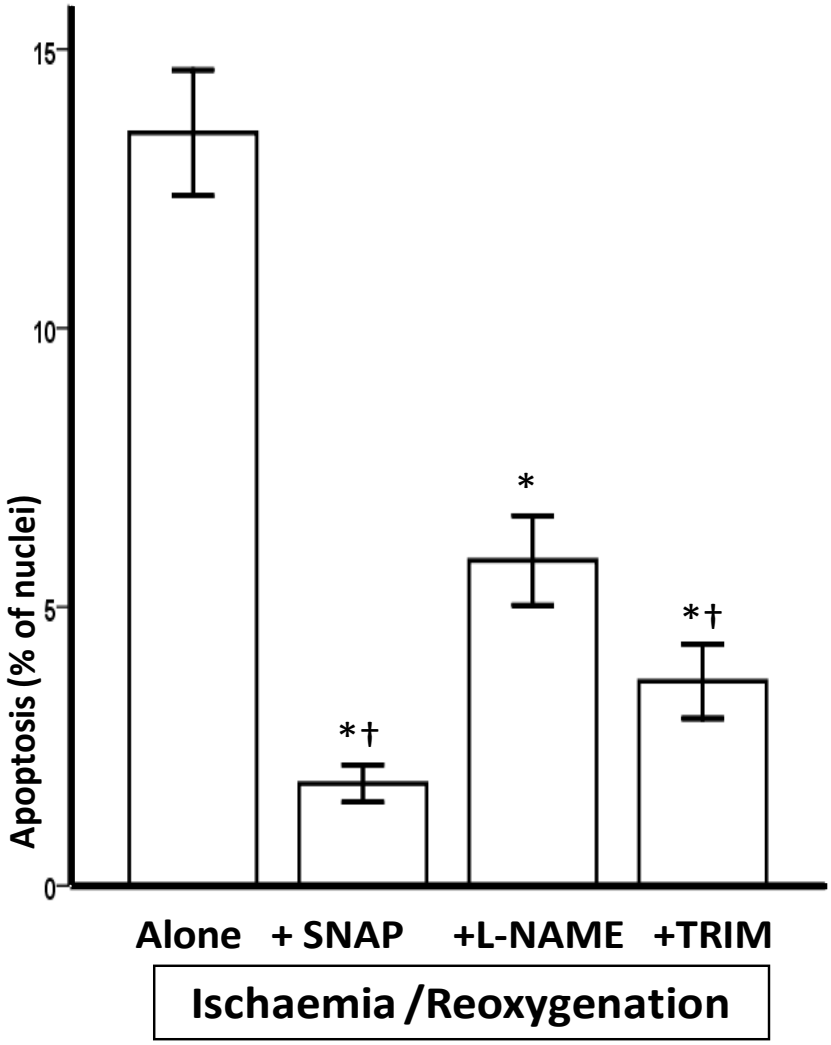


Figure 12K: Cell apoptosis in human right atrial myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organ). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia.

*$P < 0.05$ vs. ischaemia**;** †P<0.05 vs. L-NAME treated groups.

3.4. Discussion

The present studies have demonstrated that inhibition of nNOS reduces myocardial ischaemia/reoxygenation induced injury. In addition, they have shown that the protection obtained by the inhibition of nNOS is as potent as the exogenous administration of NO and that of IP, effects that are species-independent.

The duplicity of nNOS derived effects seen in our studies can be explained on one hand by a physiological production of NO, that triggers the cardioprotective intracellular mechanism of interventions such as IP, and on the other hand by a much greater production of NO induced by ischaemia/reoxygenation, that is detrimental. This augmentation of ischaemic injury by nNOS may be due to interaction of ROS and NO in the vicinity of infarction (84,158,159) as well as less availability of NO in the subcellular level. Iadecola postulated in his review paper that during the initial period of ischaemia, NO facilitates blood flow in the ischemic area by vasodilatation, decreased platelet aggregation, leukocytes adhesion and scavenging of ROS (160). Later stage of ischaemia, NO causes toxicity by oxidative damage, DNA damage leading to apoptotic cell death.

In the connection of IP, some investigators have reported that the protection of delayed IP in the rabbit heart is dependent on nNOS (83). It is also interesting to note that eNOS and nNOS fail to show any protection in early cerebral IP (161). But some investigators demonstrated that co-production of NO both constitutive NOS (eNOS and nNOS)

elaborate NO that is essential to induce IP tolerance in retina (162). The explanation of the hypothesis is that the critical level of cGMP and ROS balance is associated with tonic generation of NO by constitutive NOS is mandatory to permit full activity of other regulatory mediators molecule involved in IP. This supports my findings that the presence of nNOS is necessary to afford cardioprotection by IP.

During the past decade, all studies of nNOS in ischaemia/reoxygenation were done by using animal models such as mice, rat, rabbit, and chick cardiomyocytes. There is apparently no documentation of involvement of nNOS in IP in human myocardium. Moreover, there is controversy in different species. For the extrapolation of data obtained from experimental animals to the human situation, it is important to know the similarities and differences between animal species. To address all these issues, I have used mice ventricles and right atrial appendages from patients' undergoing cardiac surgery. An additional important finding is that the identical role is played by nNOS in the three species studied (rat, mouse and human), suggesting that the function of the enzyme has been preserved throughout evolution. These results may have clinical, logistic and economic implications since the findings observed from the animal studies could be extrapolated to human beings without the need for additional confirmatory investigations. It is also worth emphasizing that, despite the difference between atrial and ventricular myocardium, both tissues respond similarly to ischaemia/reoxygenation injury and to cardioprotection by manipulation of the NO metabolism.

Having demonstrated that nNOS plays a significant role in ischaemia/reoxygenation induced injury, so that its selective inhibition reduced myocardial damage, and the

effect is species-independent, my aim in the next chapter was to elucidate the role of nNOS in cardioprotection by IP using nNOS knocked out mice.

Chapter 4

The effect of nNOS deletion in myocardial ischaemia/reoxygenation induced injury and IP

4.1. Introduction

To further ascertain the role of nNOS in the injury sustained during ischaemia/reoxygenation and to elucidate its action in the cardioprotection induced by IP, I used nNOS knockout mice in this study. A number of inhibitors potentially selective for all NOS isoforms are available commercially but none is completely selective for one specific isoenzyme. Moreover, they may have additional pharmacological effects unrelated to NO pathway. Their effects may be jeopardised by variable bioavailability of NOS. To counteract these issues, several lines of knockout mice have been generated to describe the role of NO from each NOS isoform. The targeted disruption of gene offers a useful approach to examine the affects of their deletion in the pathogenesis of ischaemia/reoxygenation injury. Here in this study, we use nNOS knockout mice to elucidate the role of nNOS.

The nNOS knockout mouse was first developed by Fishman MC in 1993 (163) that exhibits a well defined phenotype consisting of enlarged stomach, pyloric stenosis, drug induced atherosclerosis and aggressive behaviour. nNOS plays a critical role in the regulation of multiple cardiac functions such as Ca^{2+} cycling , Na^{+} transport and maintenance of heart rate and, as expected, the nNOS deletion is associated with higher

L-type Ca^{2+} channel activity, systolic and diastolic Ca^{2+} elevation, high $[Ca^{2+}]i$ in myocytes leading to arrhythmia (65).

It has been reported that nNOS deletion is associated with increased ROS production, accelerated LV remodelling and apoptosis after acute MI in mice (164). This is also associated with impaired S-nitrosylation of ryanodine receptor (RyR2) leading to a proarrhythmic phenotype in isolated cardiomyocytes. However, the results of published studies using nNOS knocked out mice are conflicting. Thus for example whilst some investigators have reported that nNOS knockout mice are more resistant to focal and global cerebral ischaemia, others have shown that in nNOS knocked out mice, IP cannot be induced also in a model of cerebral ischaemia (161). The effect of deletion of nNOS in ischaemia/reoxygenation induced injury and IP in myocardium remains unclear.

Aims

In this study, I used nNOS knocked out mice to elucidate the role of nNOS in ischaemia/reoxygenation and IP. We tested the hypothesis that NO derived from nNOS plays a critical role in ischaemia/reoxygenation injury and IP.

4.2. Materials and Methods

4.2.1. Study animals

nNOS knockout mice (C57BL/6J) were also obtained from The Jackson Lab (Bar Harbor, Maine, USA). Animals were culled by cervical dislocation and the heart was rapidly dissected and sectioned as described in Chapter 2. These studies were undertaken in accordance with the guidelines on the Operation of Animals (Scientific

Procedure) Act 1986. All procedures are approved by the Animal Care and Use Committee of the University of Leicester.

4.2.2. Processing of samples and experimental preparation

The study was conducted using the model described previously in Chapter 2 (Section 2.2).

4.2.3. Measurement of tissue injury and viability

The index of myocardial injury, CK leakage and cell death by apoptosis and necrosis were used as described in Section 2.4 and 2.5 of Chapter 2.

4.2.4. Study protocols

In this study, myocardial slices from nNOS knockout mice were used subjected to 90 minutes ischaemia followed by 120 minutes of reoxygenation as described in Section 2.2. Muscles were randomised to be control or to receive SNAP (100μM), L-NAME (100μM) or TRIM (100μM) for the last 20 minutes of equilibration before ischaemia. In addition, IP was applied in a group of muscles.

4.2.5. Statistical analysis

Data are expressed as mean ± SEM. Each reported value was obtained after subtracting the corresponding time-matched aerobic control value. One way ANOVA followed by Bonferroni's test was used to compare the significance between groups. Analyses were performed using the SPSS program. Differences were considered to be statistically

significant if $p<0.05$.

4.3. Results

Figure 13A shows the experimental protocols for Study 3. Figures 4B-4D show that as opposed to wild type mice (Chapter 3), the myocardium of nNOS knockout mice could not be protected by the specific nNOS inhibitor TRIM as the mean values for CK release and cell necrosis and apoptosis were not significantly different from muscles subjected to control (ischemia/reoxygenation alone). The absence of benefit when L-NAME was added to the myocardium of knockout nNOS myocardium, supports the thesis that although the endogenously produced NO by eNOS may be beneficial, its deficit did not cause further ischaemic injury. As seen in wild type mice, again the NO donor SNAP almost abolished the CK release and the cell death induced by ischaemia and reoxygenation. Importantly, the myocardium of nNOS knocked out mice could not be protected by IP since the mean values for CK release and cell necrosis and apoptosis did not differ from the values in the control group.

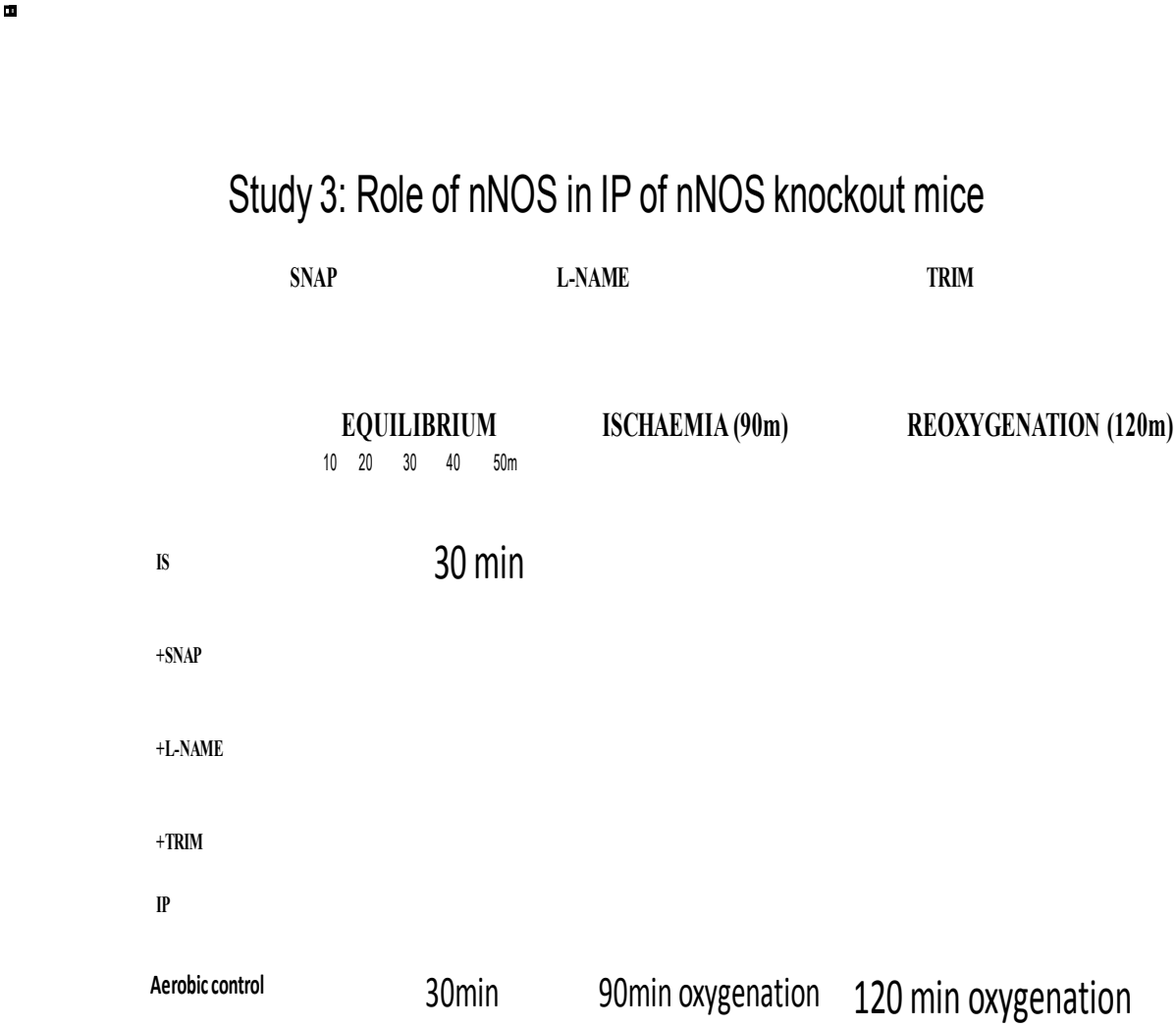


Figure 13A. Experimental protocols for Study 3.

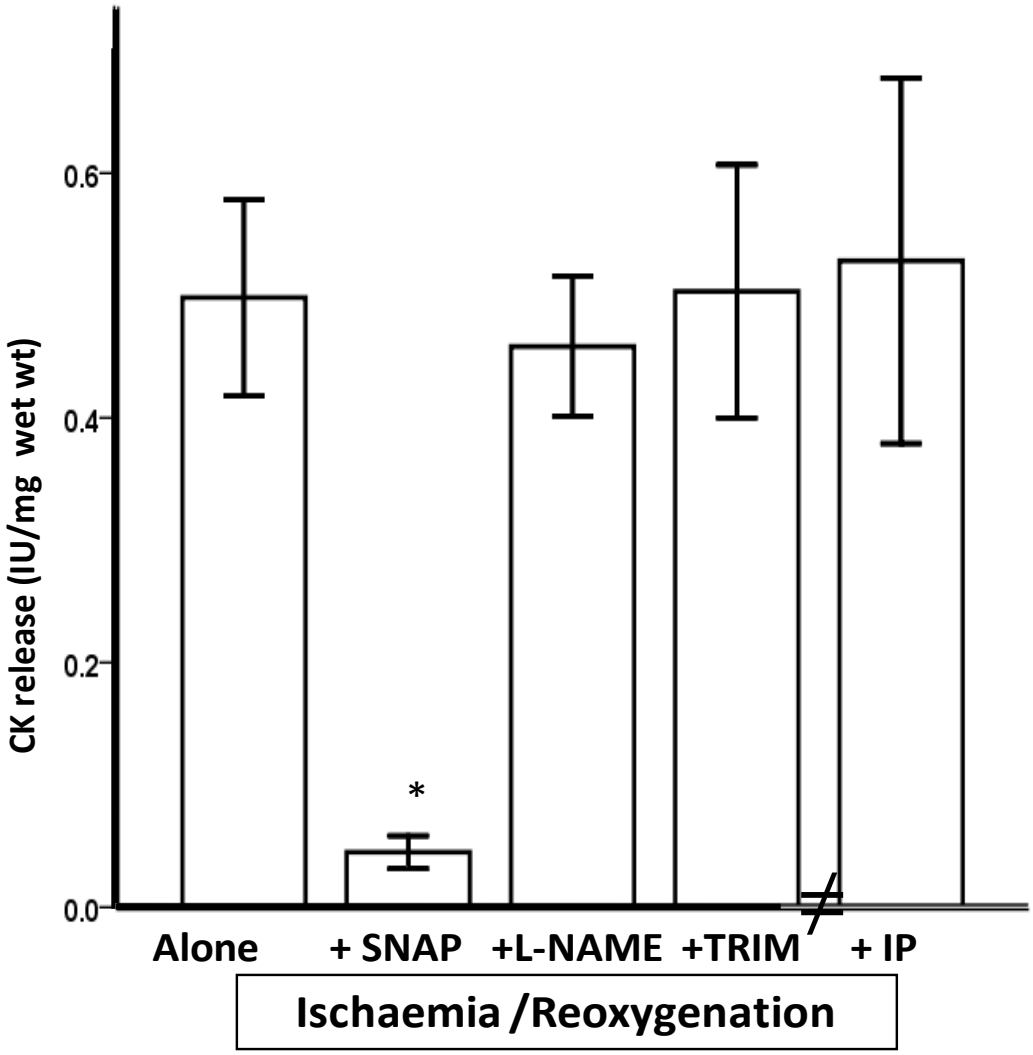


Figure 13B: CK release in nNOS knockout mice ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia. For comparison, some muscles were subjected to IP. *$P < 0.05$ vs. ischaemia.

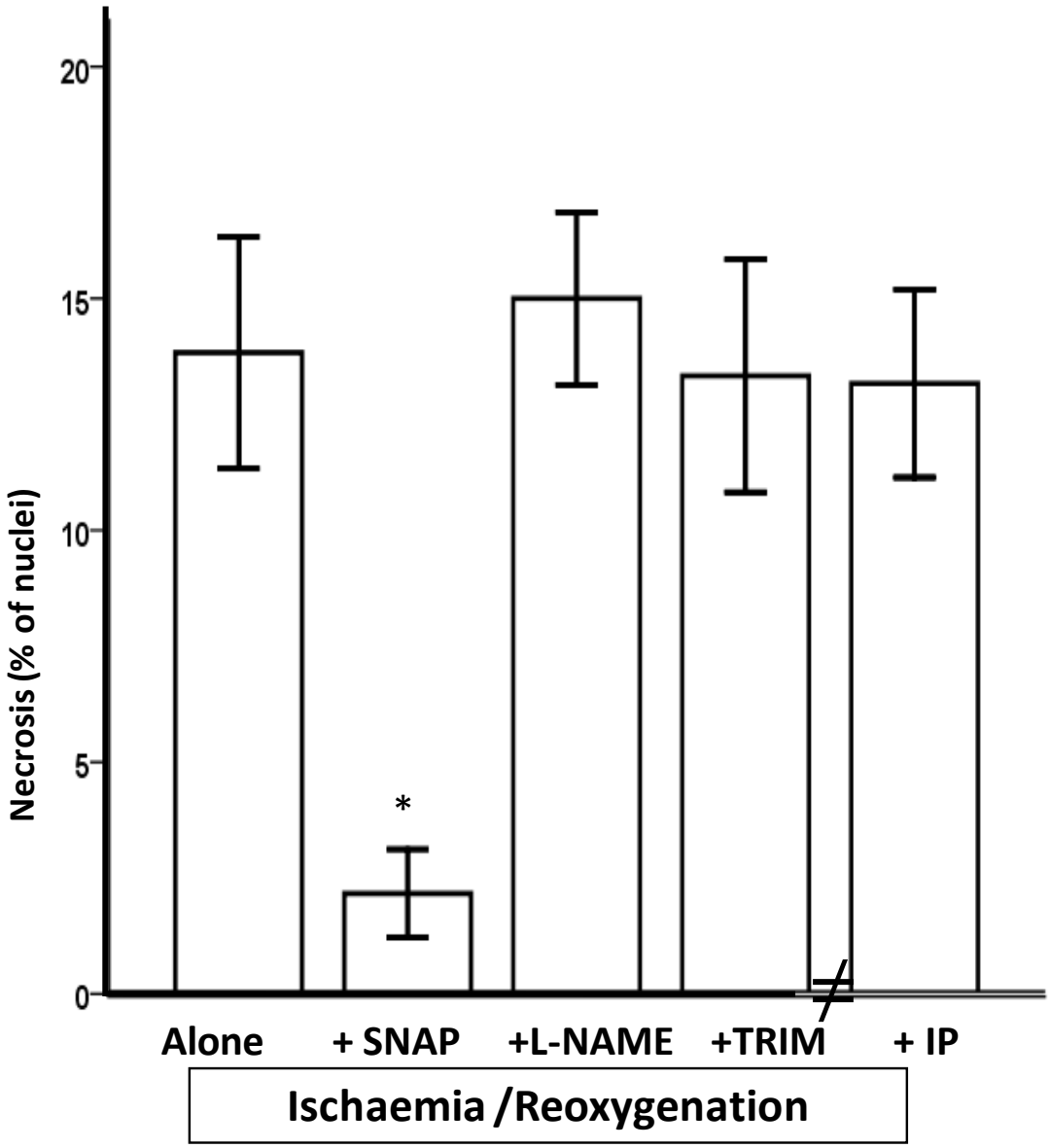


Figure 13C: Cell necrosis in nNOS knockout mice ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia. For comparison, some muscles were subjected to IP. *$P < 0.05$ vs. ischaemia.

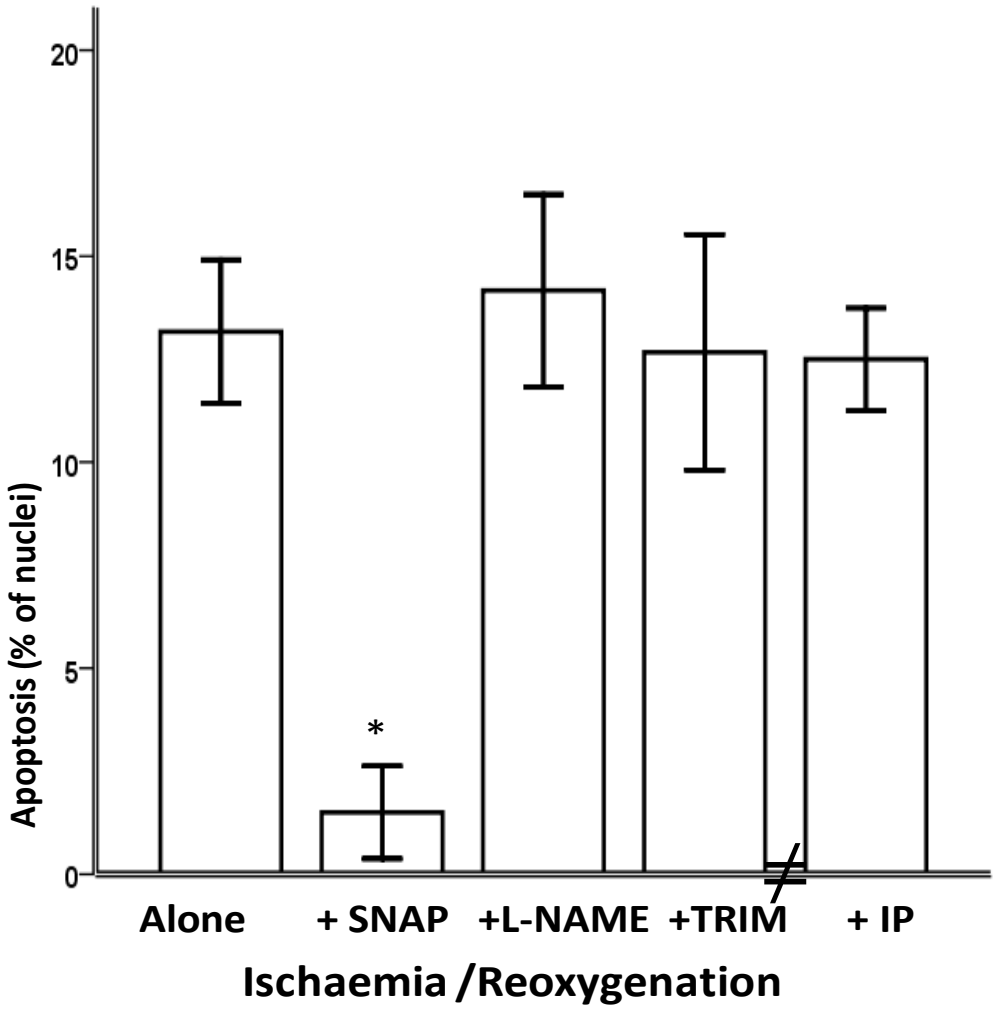


Figure 13D: Cell apoptosis in nNOS knockout mice ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP, L-NAME or TRIM for 20 min prior to ischaemia. For comparison, some muscles were subjected to IP. $*P < 0.05$ vs. ischaemia.

4.4. Discussion

The present results together with the results in Chapter 3 demonstrate that nNOS possesses a dual action and whilst its inhibition is cardioprotective, its presence is necessary to obtain the beneficial effect of interventions such as IP. Further reports of the key role of nNOS in IP by Bolli's laboratory showed that delayed IP or the second window of IP in the rabbit heart is dependent on nNOS (154,155).

It has been reported that nNOS knock out mice exhibit a larger amount of O_2- generation, increased myocardial oxygen consumption (MVO_2) and impaired sympathetic control, all of them predisposing to a greater ischaemic injury (165,166). However, it is worth noting that in this study myocardial injury (CK release and cell death) was not increased by ischaemia/reoxygenation in nNOS knockout mice, a finding supported by the absence of changes in infarct size in nNOS knockout mice (81). By contrast, a deficit in nNOS in mice reduced the degree of cerebral ischaemic injury (84), thus suggesting that the role of nNOS in ischaemia/reoxygenation could be organ-specific.

The only intervention protecting the myocardium of the nNOS knocked out mouse was the administration of exogenous NO (SNAP). While it would be interesting to fully elucidate the molecular mechanism of exogenous NO in signalling the cardioprotective pathway and whether the interaction is direct or mediated through other intermediary factors, it may be possible to speculate that NO quenches and removes other damaging ROS.

The complex biology of NOS and NO metabolism during ischaemia and reoxygenation is also reflected by the greater protection of the myocardium obtained with inhibition of nNOS than with the non-selective inhibition of NOS, a finding that was also observed in Chapter 3 with wild mice myocardium and also with the rat and human myocardium. In trying to find out an explanation for these results, it is possible to postulate the special confinement of the NOS isoforms within the cardiomyocytes; eNOS being located in the sarcolemmal caveolae (167) and nNOS localised in the sarcoplasmic reticulum, sarcolemma, cardiac mitochondria, and associated with ryanodine receptor (152). These co-localisations may be the reason for differing effects. Indeed, by regulating the proteins with which they are associated, the NOS isoforms may have distinctive functions and, therefore, it will not be surprising that the manipulation of their activity during ischaemic insults affords different degrees of protection. Moreover, it can be suggested that ischaemia/ reoxygenation and ischaemic preconditioning may activate in two separate pathways. A schematic diagram has been shown in figure 14. Nonetheless, two different phenomena may trigger NO production from multiple different sources. Certainly, this is an area of research that would require further investigation.

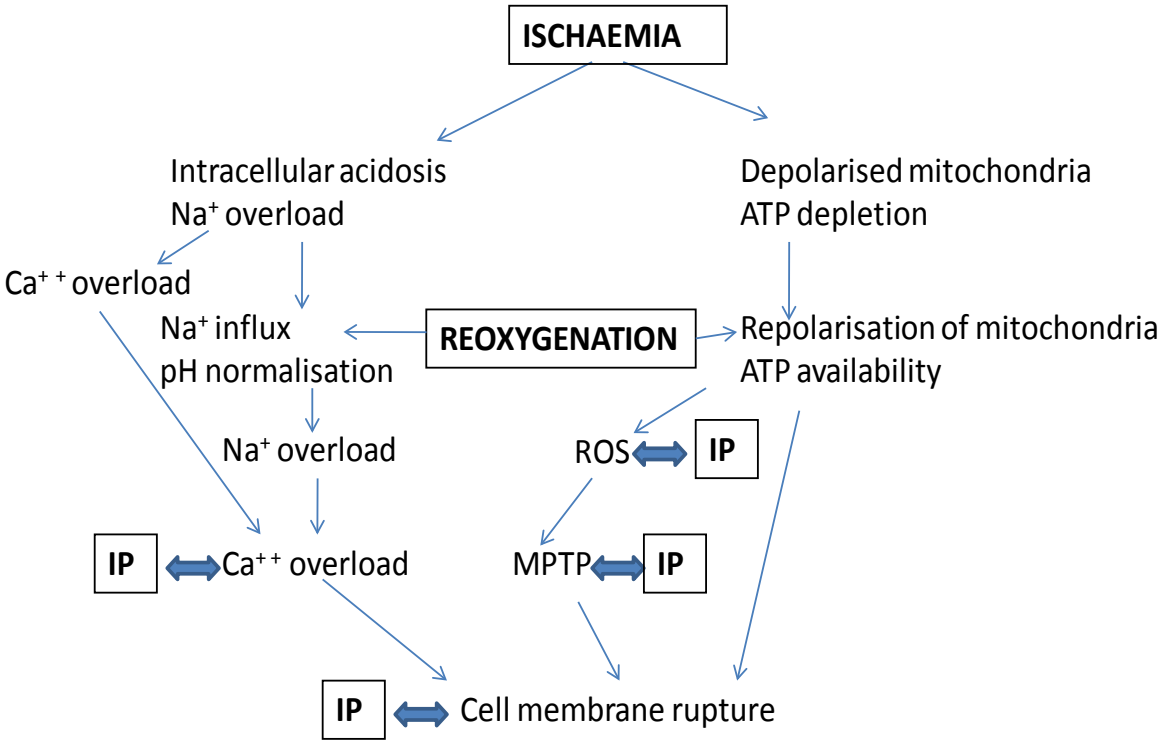


Fig 14. The schematic diagram of the separate pathway of ischemia/ reperfusion injury and IP.

Another explanation of this finding is increased expression of iNOS. Liang *et al.* demonstrated that iNOS expression was increased after ischaemia/reoxygenation and that was followed by increased nitrosotive stress (168). Moreover, iNOS may be over expressed in nNOS knocked out mice which may cause IP induced injury. It is well established that iNOS plays a negative effect in IP. Moreover, compensatory increased activity or expression of any of the NOS isoforms could occur in mutant mice. Further investigation is required to specify the expression, concentration and activation of each NOS isoform in preconditioned myocardium in nNOS knocked out mice.

Having delineated the role played by nNOS in ischaemia/reoxygenation injury and in IP, I turned my interest to how nNOS and the NO metabolism are related to key elements of the signal transduction of IP, namely the mitoK_{ATP} channels and p38MAPK.

Chapter 5

The relationship of nNOS and NO metabolism with the mitoK$_{ATP}$ channels and p38MAPK

5.1. Introduction

5.1.1. NOS and mitoK$_{ATP}$ channels

It is widely accepted that mitoK$_{ATP}$ channels, are an intrinsic component of the signal transduction pathway of IP (169). Diazoxide, a mitoK$_{ATP}$ channel opener, causes oxidation of mitochondrial flavoproteins isolated in cardiomyocytes consistent with activation of mitoK$_{ATP}$ channels. Opening of mitoK$_{ATP}$ channel results in K^+ influx that has two effects depending on the bioenergetics status of the cardiomyocytes (170):

(i) In resting cells, mitochondria membrane potential is high; mitoK$_{ATP}$ channel opener causes increased K^+ in the mitochondria resulting in the swelling of the mitochondria and alkalisation of the matrix. This leads to increased ROS production. ROS in turn activates other kinases participating in cardioprotection.

(ii) In ischaemia, mitochondria depolarise due to lack of oxygen and in reperfused cells, mitochondria also depolarise due to an increased rate of electron transport. When membrane potential is low, the mitoK$_{ATP}$ channel opens, adds a parallel K^+ conductance that counteracts the decrease in K^+ influx and matrix contraction.

Moreover, opening of the mitoK$_{ATP}$ channels protects the cardiac mitochondria from the

deleterious effects of Ca^{2+} overload (171).

It has been postulated that NO activates guanyl cyclase to produce cGMP resulting in stimulation of PKG which acts as an intermediate in the pathway leading to mitoK_{ATP} opening and ROS generation (172). Some investigators have reported that NO acts as a mitoK_{ATP} channel opener in delayed IP and independently of cGMP recruitment (87), while others have shown that the cardioprotection induced by SNAP in neonatal rat ventricular myocytes appears to be cGMP-dependent but independent of PKC, sarcolemmal K_{ATP} or mitoK_{ATP} channels (92). Therefore, the relationship between these important key modulators, NO and mitoK_{ATP} channels in the signal transduction pathway of IP is still controversial and the role played by nNOS is largely unexplored.

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5.1.2. NOS with p38MAPK

In cardiac tissues four types of p38MAPK have been identified; p38α, p38β, p38γ and p38δ. Among them, p38β is expressed in abundance in heart tissues. p38 can be activated by pacing, oxidative stress, nitroprusside, cGMP as well as weak activation of p38 being observed with receptor independent PKC activation by phorbol ester.

Numerous studies have been carried out to identify the role of MAPK in the context of IP. However, there is still controversy about the activity and potential role of p38 in IP. The reported inconsistent results may be due to species difference or related to the IP protocol used or to specific p38 isoform activity (173, 174). Thus, in 1996, Maulik *et al.* demonstrated that the increase in p38 phosphorylation and in MAPKAPK2 activity during IP is cardioprotective (175), a protection that is eliminated by the p38MAPK

blocker SB203580. These findings were later corroborated in the rat heart by the Mocanu *et al.* in 2000 (176). Our lab have shown that p38MAPK plays a critical role in IP of the human myocardium, being located downstream of PKC.

There is very little information regarding the relationship of NO with p38MAPK in the myocardium. Kim *et al.* showed that the ability of sodium nitroprusside to activate p42/44, and p38MAPK in the adult rat cardiomyocyte via both the cGMP-dependent and cGMP-independent mechanism (88). Ruusalepp *et al.* also suggested that NO mimics IP by phosphorylation of ERK1/2 and p38 MAPK (177). An indirect modulation of MAPK by NO was evidenced by the demonstration that NO suppresses the angiotensin I induced activation of ERK in cardiac fibroblast (98). By contrast, some investigators have demonstrated that sodium nitroprusside-induced p38MAPK activation does not modulate cardiomyocyte death (178); whereas other investigators have shown that SNAP induced apoptosis is blocked by SB203580 indicating that p38MAPK serves as mediator of NO induced apoptosis (179). As seen with mitoK_{ATP} channels, there is little and conflicting evidence on the relationship of NO metabolism with p38MAPK. Hence, there is no definitive evidence in the literature regarding the relationship of nNOS with mitoK_{ATP} and p38MAPK.

Aims

Therefore, the aim of the study in this chapter was to elucidate the relationship of nNOS and the NO metabolism with mitoK_{ATP} channels and p38MAPK in their mitigation of ischaemia/reoxygenation injury.

5.2. Materials and Methods

5.2.1. Study animals

Wister rats were purchased from Charles Rivers UK Ltd (Kent, UK). Animals were culled by cervical dislocation and the heart was rapidly dissected and sectioned as described below. These studies were undertaken in accordance with the guidelines on the Operation of Animals (Scientific Procedure) Act 1986. All procedures are approved by the Animal Care and Use Committee of the University of Leicester.

5.2.2. Processing of samples and experimental preparation

The study was conducted using the model described in Section 2.2.

5.2.3. Measurement of tissue injury and viability

The index of myocardial injury, CK leakage and cell death by apoptosis and necrosis were used as described in Section 2.4 and 2.5 of Chapter 2.

5.2.4. Study protocols

Rat ventricle muscles were randomized to receive the NO donor SNAP (100μM), or the selective nNOS inhibitor TRIM (100μM), for 20 minutes prior to ischaemia in the absence and presence of the mitoK_{ATP} channel blocker 5-HD (1mM) or the p38MAPK inhibitor SB203580 (10μm) for 30 minutes. In addition, some preparations were subjected to IP.

5.2.5. Statistical analysis

Data are expressed as mean ± SEM. Each reported value was obtained after subtracting the corresponding time-matched aerobic control value. One way ANOVA**,** followed by Bonferroni's test**,** was used to compare the significance between groups. Analyses were performed using the SPSS program. Differences were considered to be statistically significant if $p<0.05$.

5.3. Results

Figure 15A shows the experimental protocols for Study 4. Figures 15B-15D demonstrate that the reduction in CK release and in cell necrosis and apoptosis caused by the NO donor SNAP and the inhibition of nNOS with TRIM, was unaffected by the mitoK$_{ATP}$ channel blocker 5-HD, and that, as expected, it blocked the protection by IP.

Figure 16A shows the experimental protocols for Study 5. Figures 16B-16D show that the p38MAPK blocker SB203580 abolished the protective effect of exogenous NO and of nNOS inhibition, along with the benefit of IP; all indicating that the effect of both the exogenous NO and the endogenously produced NO by nNOS exert their effect downstream of mitoK$_{ATP}$ channels and upstream of p38MAPK.

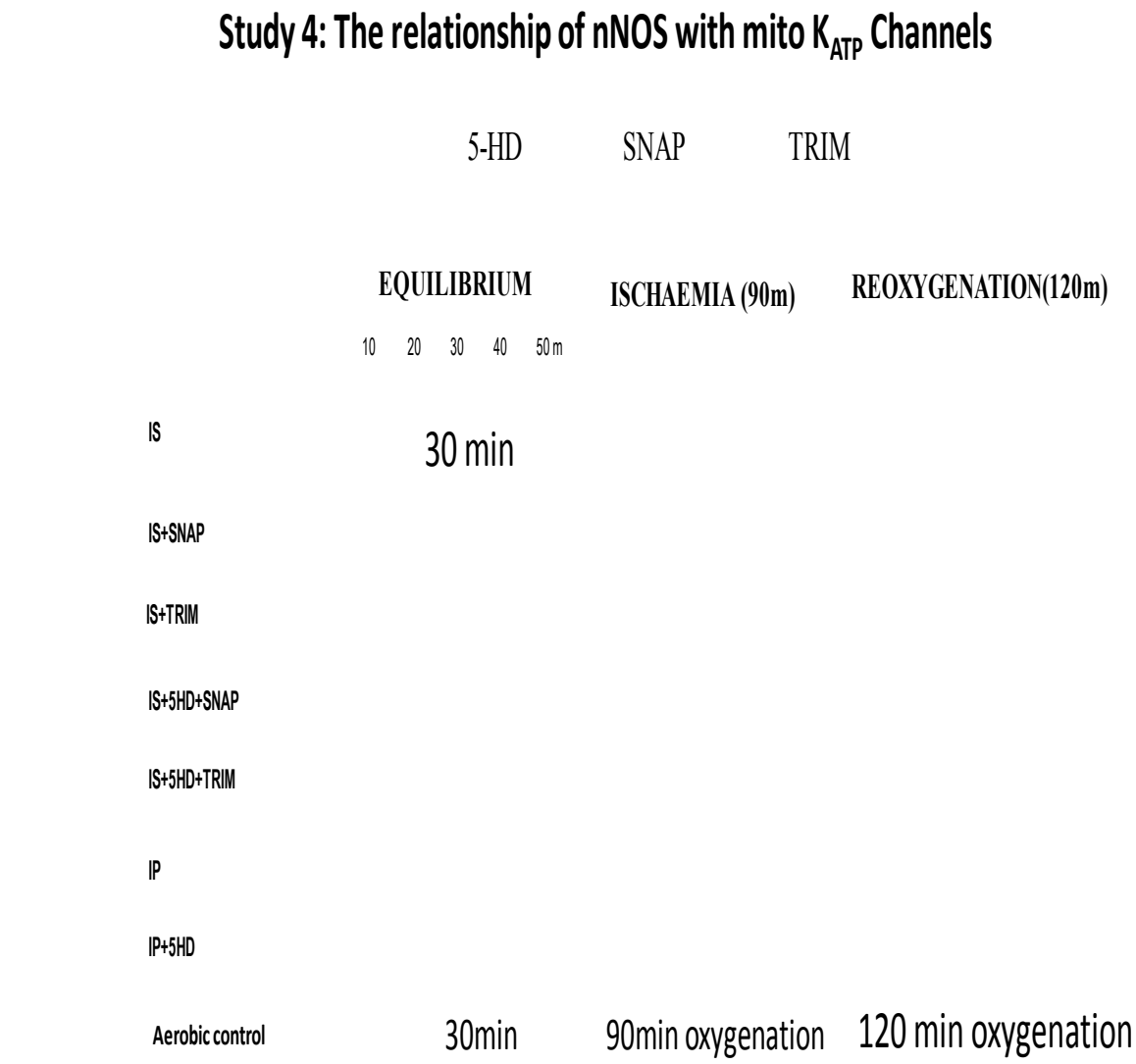

Figure 15A. Experimental protocols for Study 4.

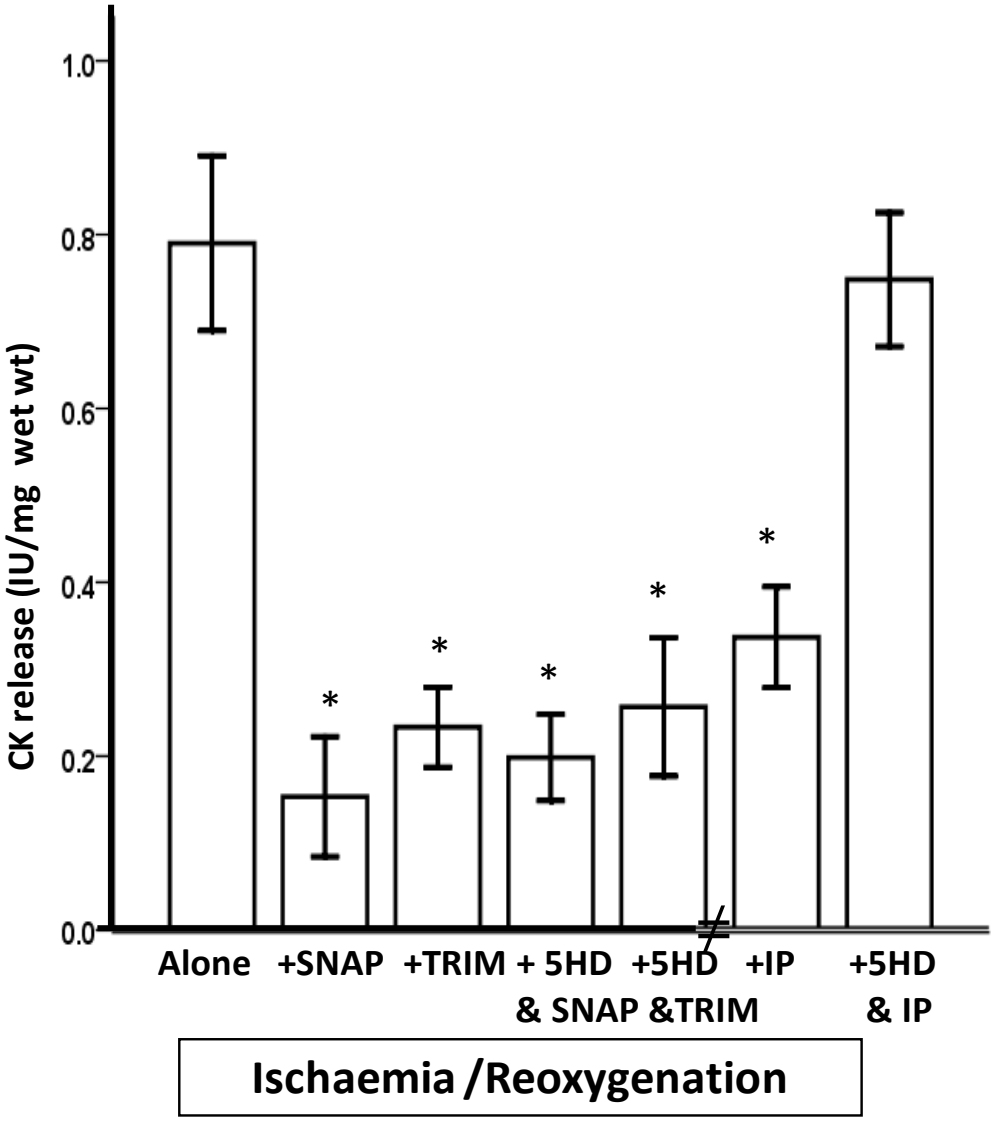

Figure 15B: CK release in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP or TRIM for 20 min prior to ischaemia in the absences and presence of 5-HD. For comparisons, some muscles were subjected to IP in the absences and presence of 5-HD. *P < 0.05 vs. ischaemia.

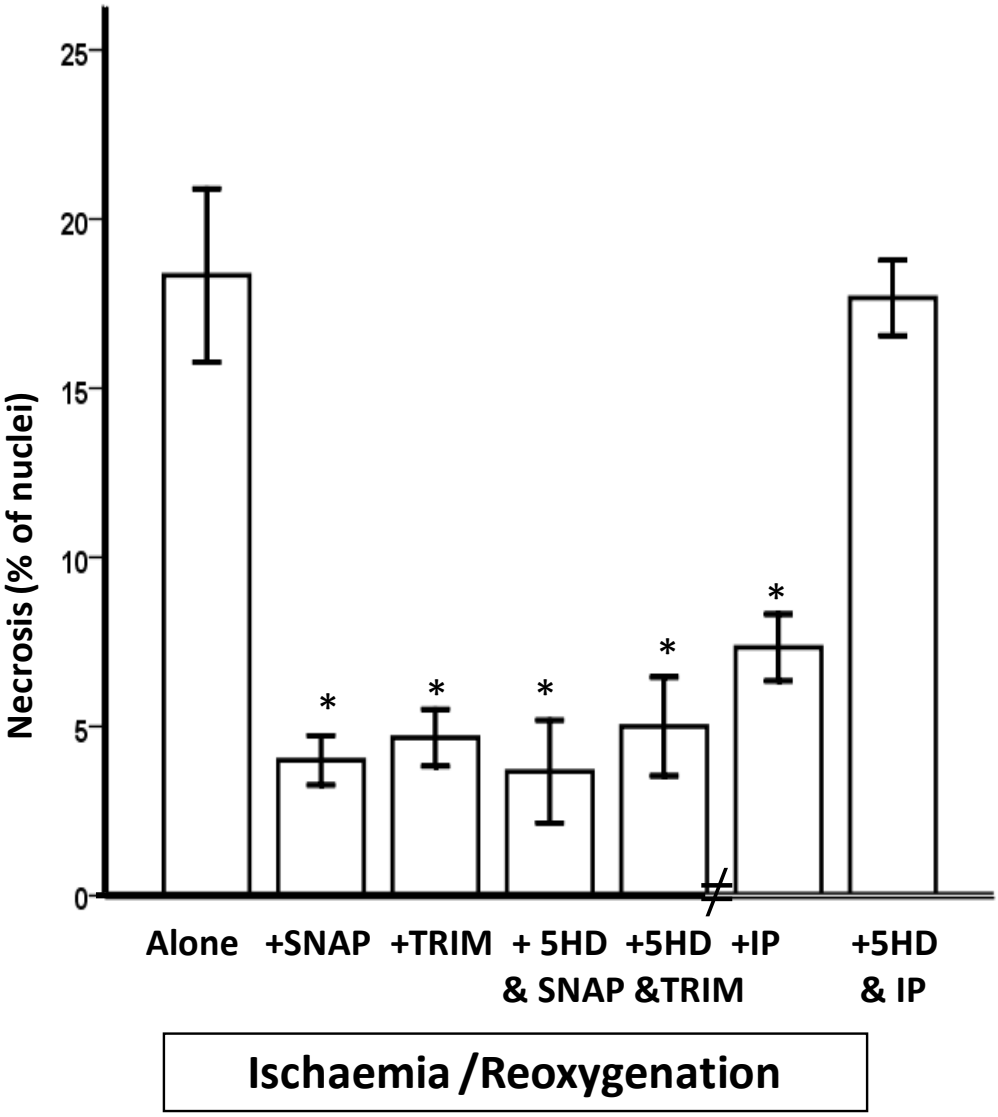

Figure 15C: Cell necrosis in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP or TRIM for 20 min prior to ischaemia in the absences and presence of 5-HD. For comparisons, some muscles were subjected to IP in the absences and presence of 5-HD. *$P < 0.05$ vs. ischaemia.

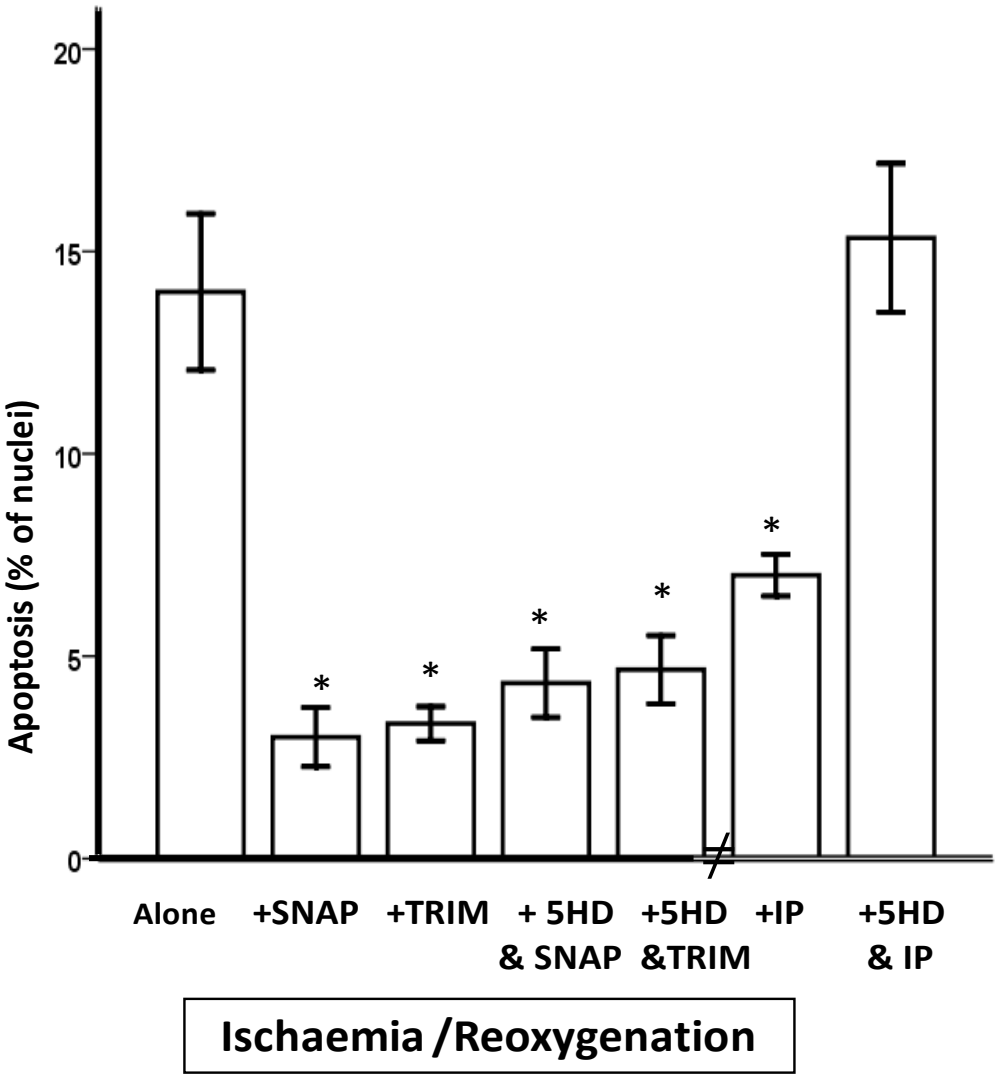

Figure 15D: Cell apoptosis in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP or TRIM for 20 min prior to ischaemia in the absences and presence of 5-HD. For comparisons, some muscles were subjected to IP in the absences and presence of 5-HD. *P < 0.05 vs. ischaemia.

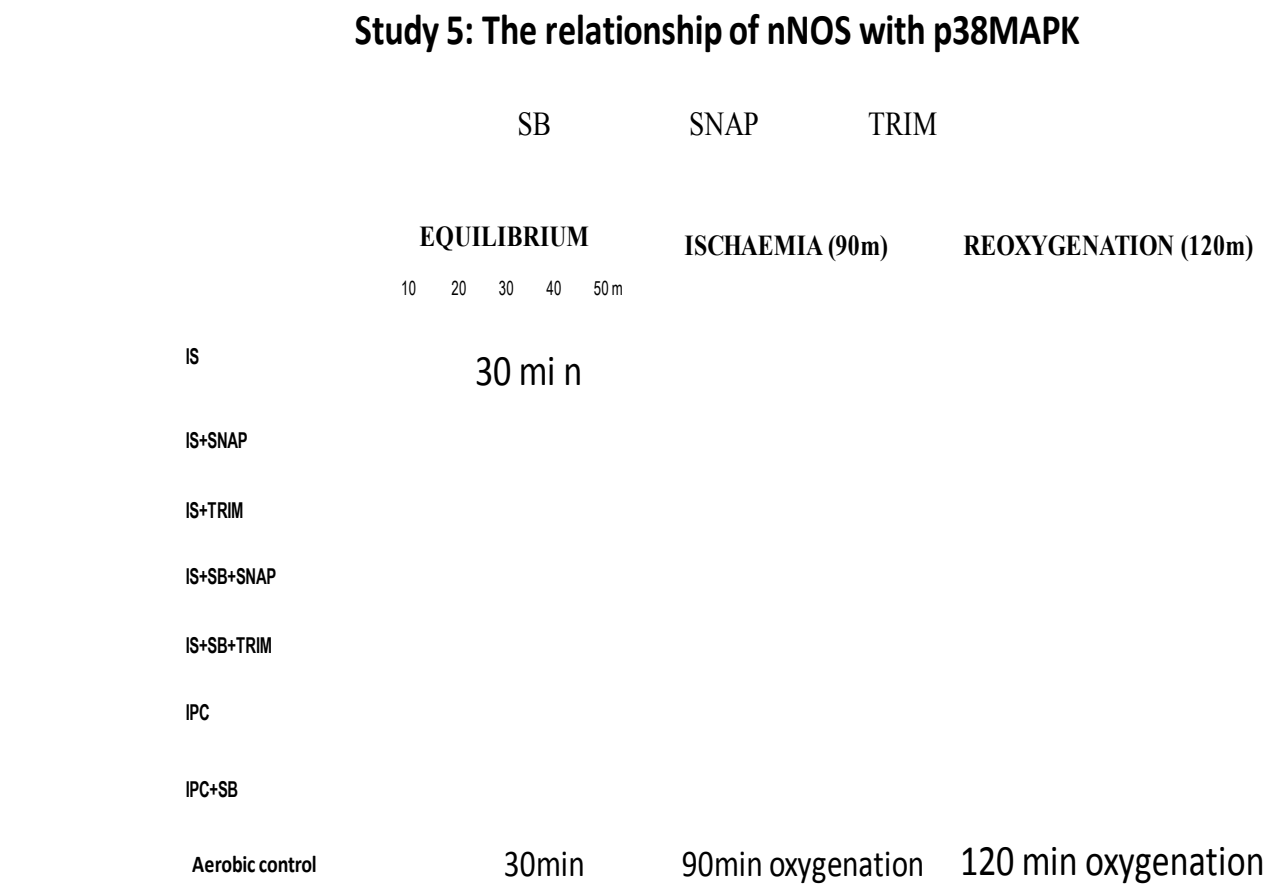

Figure 16A. Experimental protocols for Study 5.

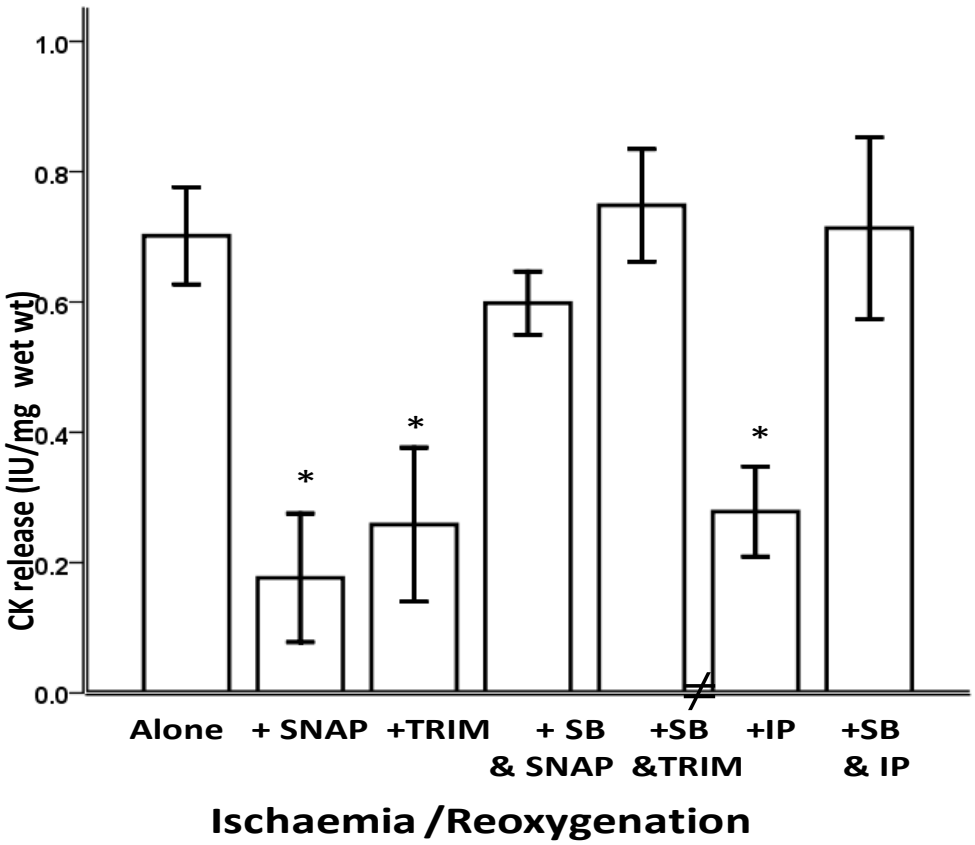

Figure 16B: CK release in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP or TRIM for 20 min prior to ischaemia in the absences and presence of SB203580 (SB). For comparisons, some muscles were subjected to IP in the absences and presence of SB203580.*$P < 0.05$ vs. ischaemia.

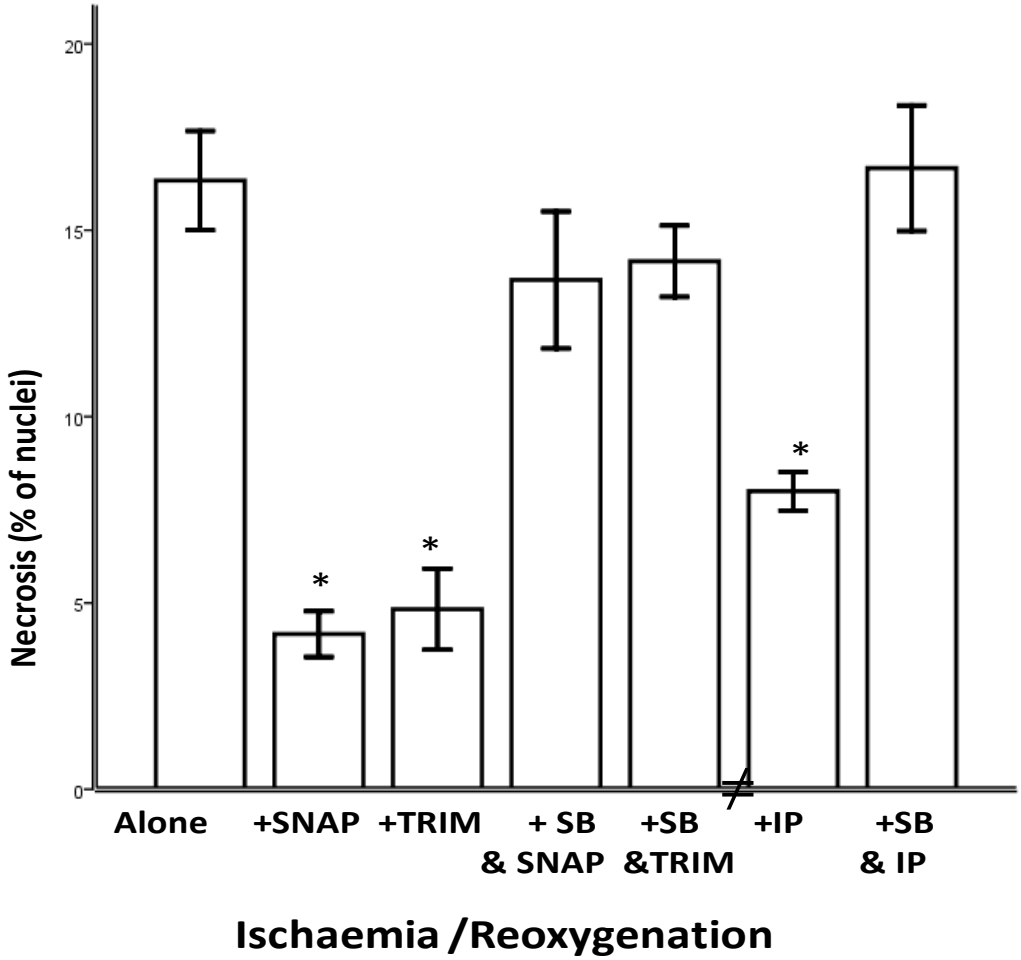

Figure 16C: Cell necrosis in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group > 6organs). Muscles are incubated with SNAP or TRIM for 20 min prior to ischaemia in the absences and presence of SB203580 (SB). For comparisons, some muscles were subjected to IP in the absences and presence of SB203580. *$P < 0.05$ vs. ischaemia.

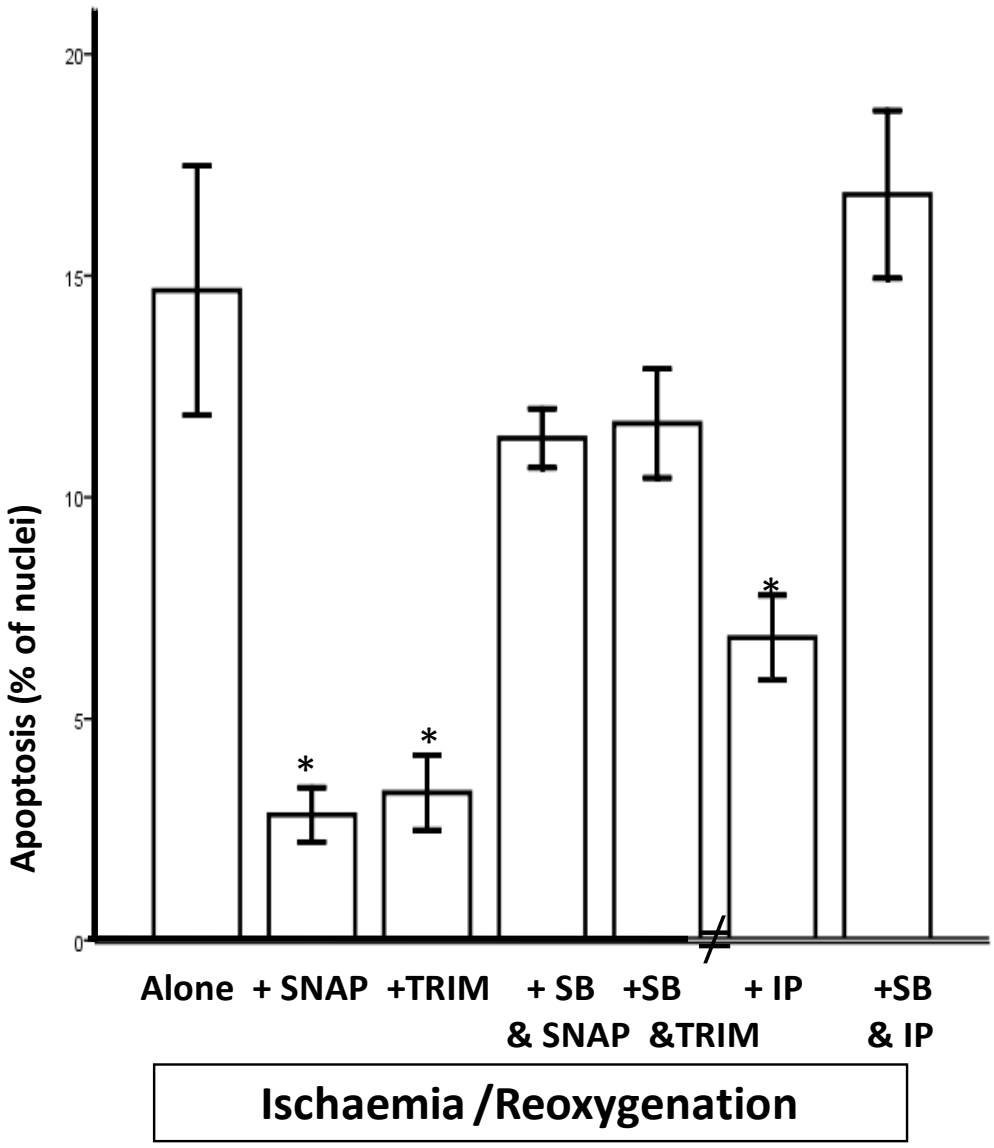

Figure 16D: Cell apoptosis in rat ventricular myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group> 6organs). Muscles are incubated with SNAP or TRIM for 20 min prior to ischaemia in the absences and presence of SB203580 (SB). For comparisons, some muscles were subjected to IP in the absences and presence of SB203580.

*$P < 0.05$ vs. ischaemia.

5.4. Discussion

The present studies locate the action of nNOS downstream of the mitoK$_{ATP}$ channels since the protection induced by the nNOS-specific inhibitor TRIM was blocked by 5-HD, a mitoK$_{ATP}$ channel inhibitor. The finding that exogenous NO is also acting at the same point reinforces the view that the production of endogenous NO by nNOS is the mechanism of protection. Indeed, other laboratories have shown that the protection induced by exogenous NO is not affected by blockade of mitoK$_{ATP}$ channels with 5HD or glibenclamide (30) and that the protection induced by the mitoK$_{ATP}$ channel opener diazoxide is NO-dependent, further supporting the thesis that NO metabolism is a critical component of the cell survival mechanism. The different location of the two constitutive forms of NOS, nNOS and eNOS, may also suggest that NO plays a distinctive role depending on the cellular site where it is produced. This thesis gets support from the finding that the specific inhibition of nNOS was more protective than the non-specific inhibition of NOS. Certainly, this is an important question that would require further investigation.

In contrast to the above, Wang *et al.* suggested that during ischaemia the increase in endogenous production of NO triggers the opening of mitoK$_{ATP}$ channels resulting in a reduction in apoptosis of cardiomyocytes (89). Other investigators reported that exogenous NO selectively opens mitoK$_{ATP}$ channels, an action that is potentiated by the presence of the selective mitoK$_{ATP}$ channels opener diazoxide (87). It is not clear what is the reason for the variable results, the differences in the experimental models used

could play a role. The diffusion and availability of NO may vary from single cell preparations to more complex *in vivo* models.

To further our understanding on the mechanism of NO-induced cardioprotection, it is important to realise that in intact cells, cytosolic protein kinase (PKG and/or PKC) are too large to cross the outer mitochondrial membrane (OMM) and regulate the mitoK_{ATP} channels embedded in the inner mitochondrial membrane. NO generated from NOS can diffuse easily across OMM and may then serve as a more efficient transducer of preconditioning signals to targets inside the mitochondria than cytosolic kinases. On the other hand, NO may exert its effect on myocardial protection by regulating protein translocation between the OMM and the IMM, via direct effect or through activation of cGMP-PKG or other unknown mediators. Certainly, the elucidation of this mechanism would require further investigation.

It is still unknown whether the activation of p38MAPK is the last step of the ischaemic preconditioning pathway that phosphorylates the end-effectors or whether there are multiple effectors. p38MAPK can phosphorylate a wide range of proteins, some of which may be potential candidates for end-effectors for preconditioning. Low molecular heat shock protein HSP27 may be phophorylated by p38MAPK via a intermediate MAPKK2 and this may lead to polymerisation of actin and to increase the tolerance of cytoskeleton to stress. Translocation of PKC isoforms to mitochondrial sites, intercalated discs and nucleus may suggest that p38MAPK activation in these places may activate enzymes involved with energy production, intercellular communication through cell junctions and gene transcription. During ischaemia/reoxygenation,

exogenous NO or NO donor activates MAPK especially ERK1/2 and p38 MAPK. In the present study, administration of NO donor SNAP or inhibition of nNOS by TRIM in the presence of p38MAPK inhibitors SB203580, demonstrates no cardioprotection from ischaemia/reoxygenation induced injury. This result suggested that NO activity or nNOS activity is underlying in the upstream of p38 MAPK.

Previously, our laboratory has shown that p38MAPK is a key factor of IP-induced cardioprotection located beyond the mitoK$_{ATP}$ channels (32). Here I have also shown that the effect of NO metabolism in the intracellular signalling pathways of cardioprotection is exerted upstream of p38MAPK, as the beneficial effects of exogenous NO donation and nNOS inhibition were abolished by the p38MAPK blocker SB203580. A schematic diagram is shown in Figure 17.

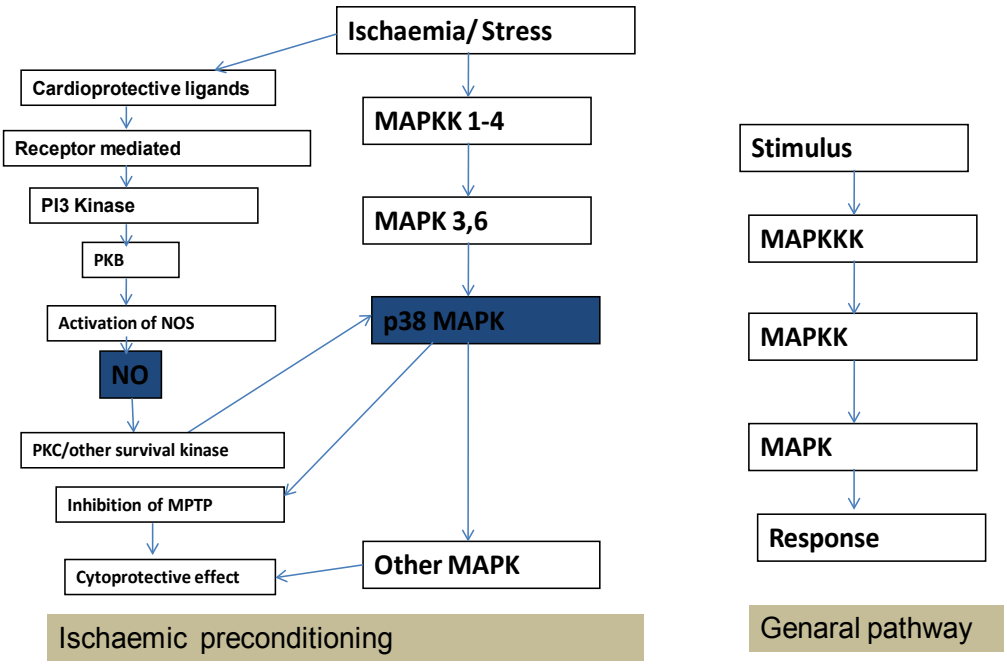

Fig 17. The schematic diagram of MAPK pathway in IP and general.
MAPK Mitogen activated protein kinase , MAPKK Kinase of MAPK , MAPKKK Kinase of MAPKK

Having demonstrated that nNOS and NO metabolism are downstream of mitoK_{ATP} channels and knowing that a defective mitochondrion is responsible for the deficit of cardioprotection in the diabetic myocardium, my next aim was to investigate whether the unresponsiveness of cardiac tissues from diabetic subjects can be overcome by manipulation of nNOS and the NO metabolism.

Chapter 6

Role of nNOS and NO metabolism in protection of the Diabetic Myocardium

6.1. Introduction

There is conflicting evidence in the literature regarding the ability to precondition the diabetic myocardium. Some studies have demonstrated a failure to cardioprotect diabetic tissues whilst others have reported a benefit of being preconditioned (117, 119, 137,180,181), arguing that diabetes reduces the intracellular acidosis leading to a lesser accumulation of lactate and H^+ during an ischaemic insult (113, 182). However, our laboratory has convincingly demonstrated that the diabetic human myocardium cannot be protected by IP (180).

Several factors could contribute to the failure of preconditioning the diabetic myocardium, including an impaired function of mitoK_{ATP} and sarcolemmel K_{ATP} channels, and also the presence of diabetic medication such as sulphonylureas. It is known that hyperglycaemia increases the risk of myocardial infarction in diabetes (100) and that its severity is a potential risk factor for irreversible ischaemic injury. However, there is still controversy on the role of hyperinsulinaemia in ischaemic injury.

The NO metabolism can also be influenced by diabetes. High glucose levels increase

the expression of constitutive NOS and high insulin also increases NO production as well as NOS expression. This altered NO metabolism may be related to the pathogenesis of diabetic myocardial damage. It is known that hyperglycaemia increases superoxide anion production via activation of multiple pathways including xanthine and NADPH oxidases, cycloxygenase, mitochondrial respiratory chain and advance glycation end products. The increased superoxide production favours the NOS expression and a greater generation of NO. Then the reaction of NO with superoxide forms the potent cytotoxic oxidant peroxynitrate that ultimately causes cell damage by lipid peroxidation and protein nitration and oxidation. Peroxynitrite also acts on the mitochondria decreasing mitochondrial membrane potential and releasing cytochome C and the apoptotic factor leading to caspase-dependent and independent apoptotic cell death. By now it is well accepted that peroxynitrite and nitrosative stress play a major role in the pathogenesis of diabetic cardiac diseases.

Aims

The aim in this chapter was to define the role of nNOS and NO metabolism in ischaemia/reoxygenation-induced injury of the diabetic human myocardium and to investigate whether their manipulation can overcome the unresponsiveness of these tissues to protection by IP.

6.2. Materials and Methods

6.2.1. Study subjects

The right atrial appendage from diabetic patients undergoing elective heart surgery for coronary bypass surgery or aortic valve surgery was obtained prior to the initiation of cardiopulmonary bypass. Patients with atrial fibrillation, cancer, poor LV function (EF<30%) or with additional surgical procedures or those being treated with opioid, catecholemines, K_{ATP} channel opener nicorandil were excluded from the study. The study was conducted according to Declaration of Helsinki principles and approval was obtained from the Local Ethics Committee. All participants provided written consent.

6.2.2. Processing of sample and Experimental preparation

The study was conducted using the experimental model described previously in Section 2.2.

6.2.3. Measurement of tissue injury and viability

The index of myocardial injury, CK leakage and cell death by apoptosis and necrosis were used as described in Section 2.4 and 2.5 of Chapter 2.

6.2.4. Study protocols

Muscles from the right atrial appendages were subjected to the protocol described in Section 2.2 (90 minutes ischaemia/120 minutes reoxygenation) were randomly allocated to the following groups (n=6/group): (i) ischaemia/reoxygenation alone (control), or (ii) exogenous NO donor SNAP (100μM), (iii) the non selective NOS inhibitor L-NAME

(100μM), or (iv) the selective nNOS inhibitor TRIM (100μM) incubated with myocardial slices for 20 minutes prior to ischaemia. The selected concentrations of the reagents used were found to be optimally effective in previous studies (139,140). For comparison, IP was induced in some myocardial sections.

6.2.5. Statistical analysis

Data are expressed as mean ± SEM. Each reported value was obtained after subtracting the corresponding time-matched aerobic control value. One way ANOVA followed by Bonferroni's test was used to compare the significance between groups. Analyses were performed using the SPSS program. Differences were considered to be statistically significant if $p<0.05$.

6.3. Results

Figure 18A shows the experimental protocols for Study 6. Figures 18B-18D show that, as shown before in our laboratory (180), the diabetic myocardium cannot be protected by IP since CK release and cell necrosis and apoptosis were similar to control. However, the provision of exogenous NO and also the inhibition of endogenous production of NO, both with the non-selective NOS inhibitor L-NAME and the selective nNOS inhibitors TRIM, resulted in a statistically significant reduction in CK release, cell necrosis and apoptosis as compared to the mean values in the control group.

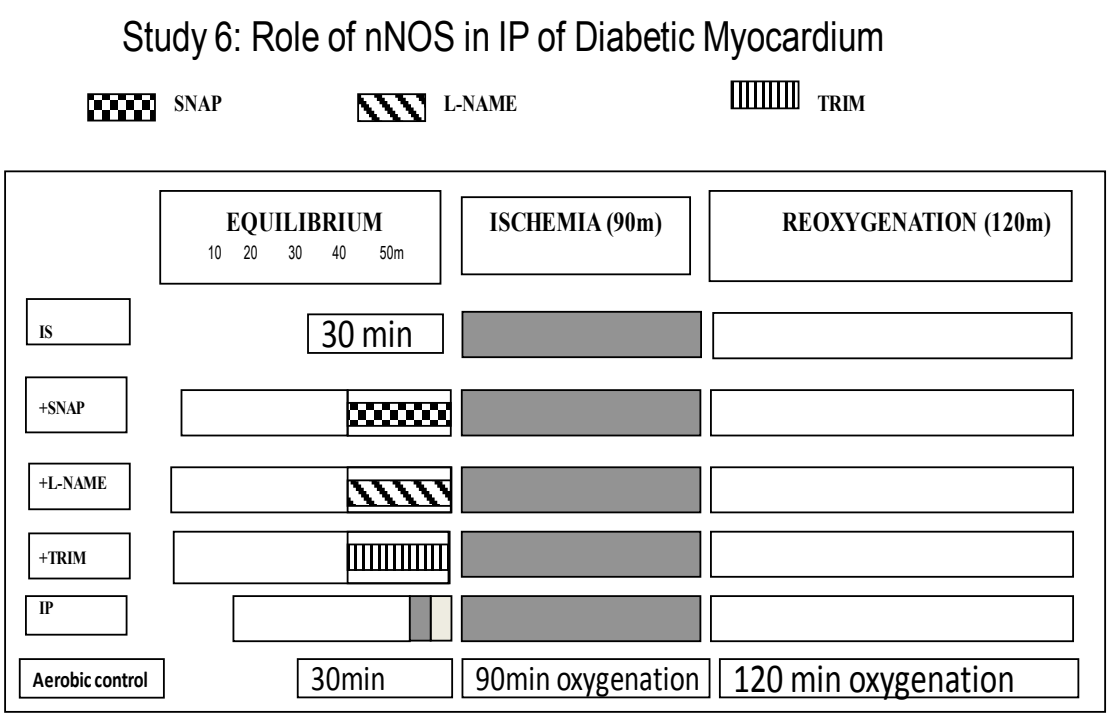

Figure 18A. Experimental protocols for Study 6.

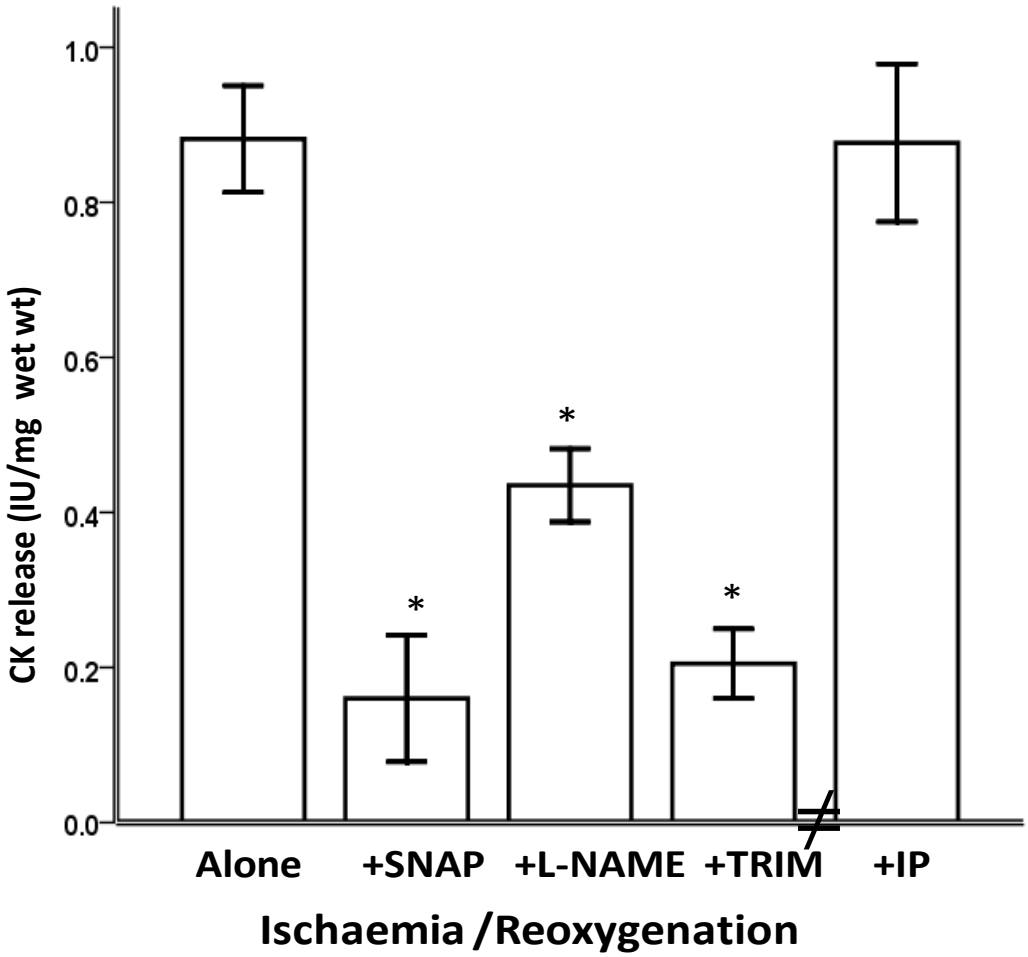

Figure 18B: CK release in right atrial myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organ). Muscles were incubated with SNAP (100μM), L-NAME(100μM) or TRIM(100μM) for 20 min prior to ischemia.

*$P < 0.05$ vs. ischaemia.

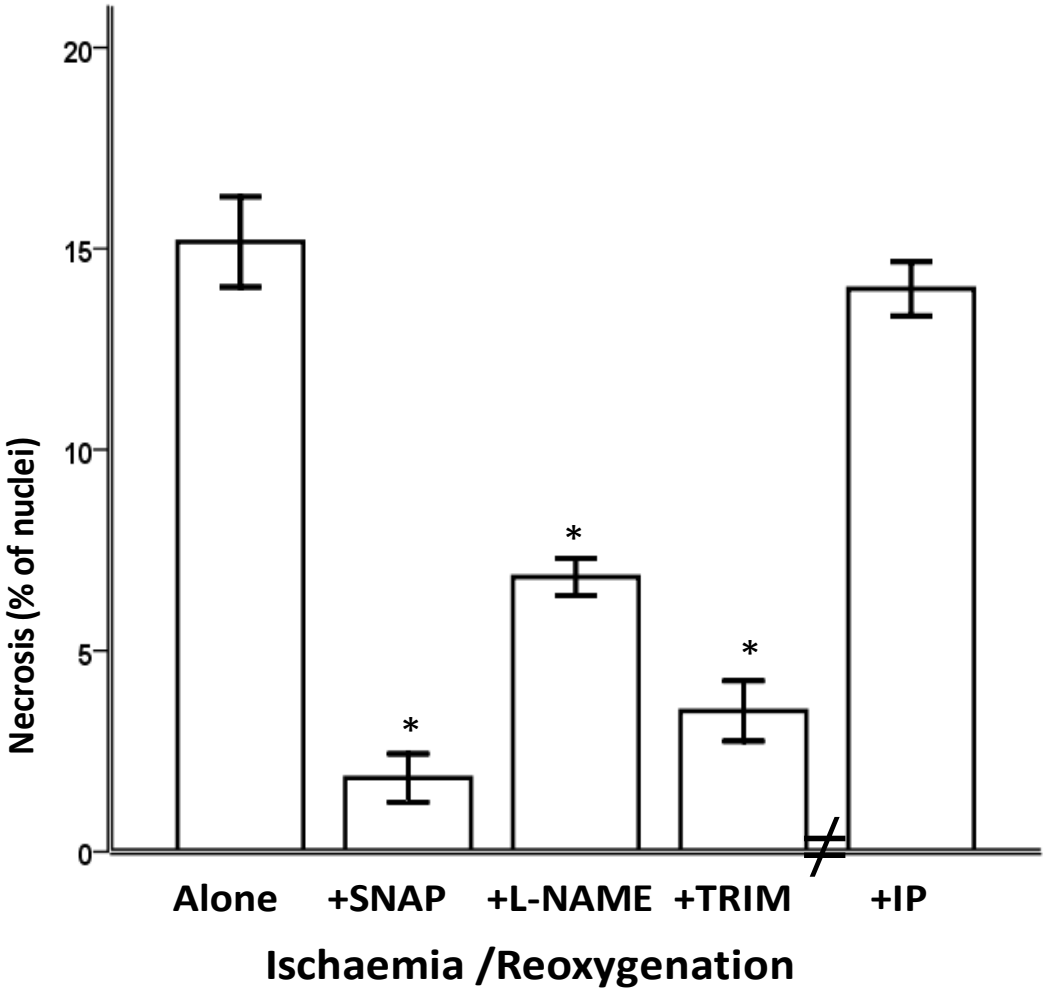

Figure 18C: Cell necrosis in right atrial myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organ). Muscles were incubated with SNAP (100µM), L-NAME(100µM) or TRIM(100µM) for 20 min prior to ischaemia.

*$P < 0.05$ vs. ischaemia.

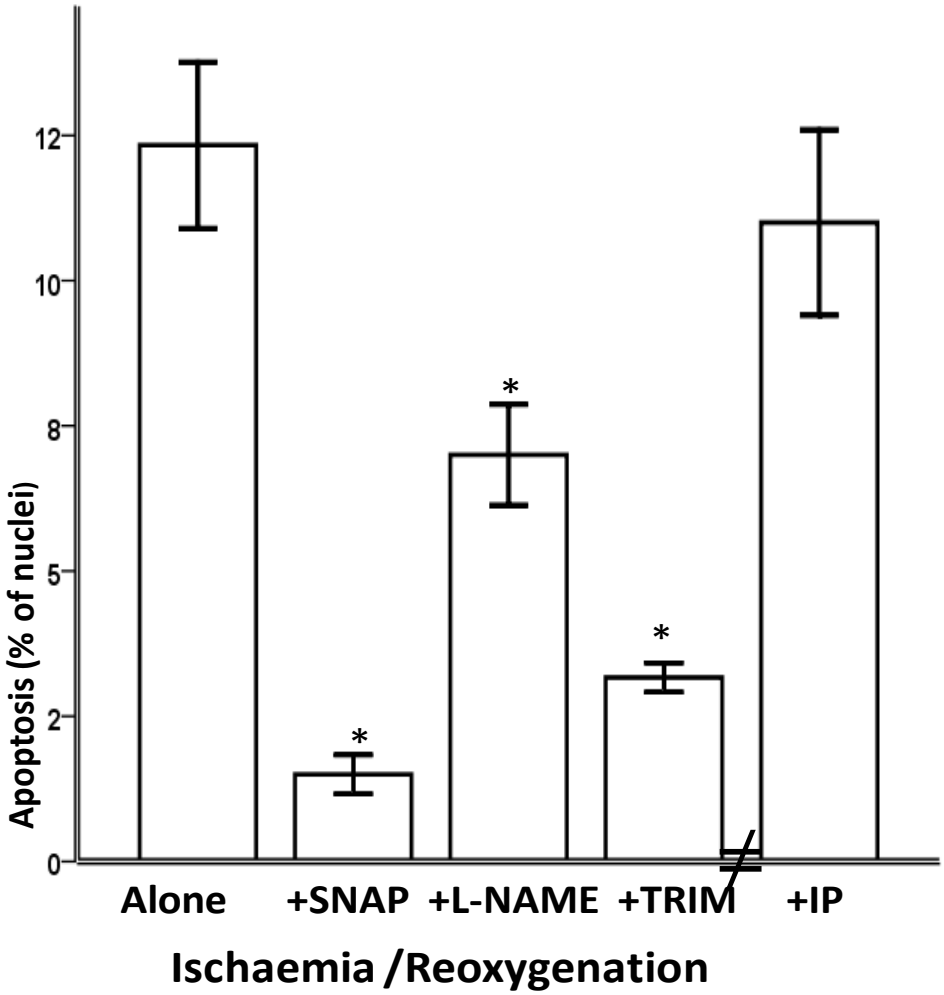

Figure 18D: Cell apoptosis in right atrial myocardium subjected to 90 min ischaemia followed by 120 min of reoxygenation (n=6/group means 6 organ). Muscles were incubated with SNAP (100μM), L-NAME(100μM) or TRIM(100μM) for 20 min prior to ischaemia.

*$P < 0.05$ vs. ischaemia.

6.4. Discussion

The present study has demonstrated for the first time that modulation of the NO metabolism by either provision of exogenous NO or suppression of the production of endogenous NO with the non-selective NOS inhibitor L-NAME or the selective nNOS inhibitor TRIM affords strong protection of the diabetic human myocardium that is unresponsive to protective interventions such as IP. These findings are of marked clinical relevance because a reduction in the myocardial damage sustained by the diabetic heart during an ischaemic insult, as occurs in cardiac surgery, may result in improvement of the clinical outcome.

Diabetes is a recognised risk factor for cardiovascular diseases and its presence carries greater mortality and morbidity in this group of patients (100,184). There is strong evidence, although no general agreement, that diabetes renders the myocardium more susceptible to ischaemic injury (104) and also that the diabetic heart cannot be protected by interventions such as IP (180). The higher oxidative and nitrosative stress seen in diabetes suggests that NO metabolism could play a major role in the abnormal response of the diabetic myocardium to ischaemia and IP. Hence, one could expect that in the presence of an elevated endogenous NO production in addition of exogenous NO would exacerbate ischaemic injury. However, our findings demonstrate that, in fact, the administration of the NO donor SNAP significantly reduces ischaemic injury of the diabetic myocardium, as seen by the reduction in CK release and cell necrosis and apoptosis, all suggesting that the provision of exogenous NO, rather than to add to nitrosative stress and tissue injury, mitigates them. These results in the diabetic myocardium are consistent with the findings in the non-diabetic myocardium in Chapter

3 and can be explained by the reported reduction in NOS activity by exogenous NO. Thus, a decrease in the production of endogenous NO would generate less oxidative and nitrosative compounds and therefore a reduction in nitrosative stress (186, 187). Another possible explanation is the known ability of the exogenous NO to quench other free radicals (188, 189).

The reduction in ischaemic injury seen in the diabetic myocardium by both L-NAME, a non-selective inhibitor of NOS, and TRIM, a specific nNOS inhibitor, is also consistent with our previous finding in the non-diabetic myocardium. The results support the concept that the endogenously produced NO is detrimental during ischaemia and reoxygenation, possibly due to an increased generation of nitrosative radicals. By contrast, we have demonstrated that the nNOS is necessary for the myocardium to be protected by IP and other investigators have found that overexpression of eNOS is cardioprotective. The complex biology of NOS and NO metabolism during ischaemia and reoxygenation is also reflected by the greater protection of the diabetic myocardium obtained with inhibition of nNOS than the non-selective inhibition of NOS, a finding that was also observed in the non-diabetic myocardium. In trying to find out an explanation for these results, it is possible to argue that the spatial confinement of the NOS isoforms within the cardiomyocytes, eNOS being located in the sarcolemmal caveolae and associated to protein caveolin 3 whilst nNOS is localised in the sarcoplasmic reticulum and associated with the ryanodine receptor (152), may play a role. By regulating the proteins with which they are associated, the NOS isoforms may have distinctive functions and, therefore, it will not be surprising that the manipulation of their activity during ischaemic insults affords different degrees of protection.

Certainly, this is an area of research that would require further investigations.

Previously, our laboratory has demonstrated that the cause of the failure to precondition the diabetic myocardium is a dysfunctional mitochondrion, probably the putative mitoK_{ATP} channels. The most important finding of the present study is that this deficit in cardioprotection can be overcome by manipulation of NO metabolism, an action that should be exerted beyond the mitochondrial defect. In chapter 5 it was shown that cardioprotection induced by the administration of exogenous NO and by selective inhibition of nNOS activity can be abrogated by the p38MAPK blocker ,SB203580, but is unaffected by the mitoK_{ATP} channel blocker, 5-HD, thus suggesting that the NO, whether exogenous or endogenous, is acting downstream of the mitoK_{ATP} and upstream of p38MAPK. Indeed, it would be interesting to fully elucidate the molecular mechanism of this signalling cascade and whether their interaction is direct or is mediated through other intermediary factors.

The clinical therapeutic exploitation of a molecule such as NO has important advantages because, in addition of being physiologically produced, it is highly diffusible (190) (150-300μm for a time of 4-15 seconds), it has short half-life (190) (<15 seconds) and potentially it may have less side effects than agents targeted to other elements of the cardioprotective intracellular signalling mechanism (e.g. protein kinase activators). However, an excessive production of NO may lead to hypotension and, hence, it would be advantageous to direct its action to the selected organ to achieve the most appropriate local concentration or production of NO. Nonetheless, it is necessary to emphasise that, although human myocardium was used, the present study was

performed in an *in vitro* experimental model and thus extrapolation to the clinical set up should be taken with caution.

In conclusion, here I have shown that the modulation of the NO metabolism is a potential therapeutic target to reduce ischaemic myocardial injury of the diabetic heart with potential to improve the clinical outcome of cardiac surgery and other conditions where ischaemic injury may occur.

Chapter 7

Conclusions and future directions

In this thesis, I have demonstrated that nNOS is a key factor, playing a dual role by contributing to myocardial ischaemia/reoxygenation injury and at the same time by being necessary to elicit myocardial protection by IP.

In the first studies (Chapter 3), I demonstrated that exogenous NO can elicit cardioprotection of a similar degree to that of IP and also that inhibition of nNOS is effectively cardioprotective. These studies expand the body of knowledge by demonstrating that the selective inhibition of nNOS prevents the development of ischaemia/reoxygenation induced myocardial injury. In addition, I showed that these effects are species-independent as the responses seen in rats were reproduced in mice and human myocardium.

Using nNOS knocked out mice in the next study (Chapter 4), I was able to determine that the inducing of IP requires the presence of nNOS. It provides evidence for the first time that nNOS is a necessary mediator for the cardioprotection elicited by the early or first window IP. This study provides new insight into the mechanism of IP.

In additional studies (Chapter 5) and using pharmacological tools, I was able to dissect the relationship of nNOS and the NO metabolism with other key factors of IP, namely, mitoK_{ATP} channels and p38MAPK. The results demonstrated that the effect of NO

metabolism and the action of nNOS in the intracellular signalling pathways of cardioprotection is exerted downstream of the mitoK_{ATP} channels and upstream of p38MAPK.

Following the interesting findings of the previous studies, I demonstrated in the last study of this thesis (Chapter 6) that the unresponsiveness of the human diabetic myocardium to cardioprotection can be overcome by the specific inhibition of nNOS and by the provision of exogenous NO with similar efficacy.

Collectively, the present studies provide noble understanding on the role of nNOS in myocardial ischaemia/reoxygenation induced injury and cardioprotection.

Clinical implications

At present, most research of early IP has shown to reduce ischaemia/ reoxygenation injury in laboratory. Only a handful of clinical evidence of ischaemic preconditioning is available for clinical implication. This may be due to complexity of ischaemia/ reperfusion and difficulties in application of effectors/ mediators in patients. In recent years, remote ischemic preconditioning (RIPC) represents a novel method of reducing ischemia/ reoxygenation injury. It was initially discovered by Przyklenk K *et al.* in 1993 (252). They have demonstrated that brief occlusion of circumflex artery preconditioned the myocardium not only in this territory but also in the territory of left anterior descending artery suggesting that factors released in the vicinity of the occlusion are transported throughout the heart . Subsequent studies have shown that remote ischemic preconditioning protects kidneys, lungs, liver, stomach and muscle flap from ischaemia/

reoxygenation induced injury (253-258).

Remote ischaemic preconditioning is a powerful innate mechanism of multiorgan protection that can be induced by transient occlusion of blood flow to a limb with a blood pressure cuff (259). This liberates blood borne effectors that circulate through the system to have multiple protective effectors (260). In RIPC, information is transferred not only by humoral mediators but also by neurogenic mediators (261). This new information triggers the signal transduction pathway directly or through receptor that results in cytoprotection (260, 261). Evidence suggested that NO has important effect in RIPC (262-266). The role of NO and NOS in RIPC in heart is controversial (268). These conflicting evidences suggest that the mechanism of protection through NO in RIPC may be organ specific (267, 268). However, it is also beyond the scope of this thesis to give a full account of the mechanisms of protection involved in remote ischemic preconditoning.

Preconditioning can be translated from experimental discovery to acceptable clinical technique as RIPC is easy to deliver, readily applicable and non-invasive. Nonetheless, more clinical studies probably multicentre studies are needed to determine the intra-organ mediators and effectors responsible for the beneficial effects of RIPC.

The present findings demonstrating significant cardioprotection by modulation of the activity of nNOS and the NO metabolism are of clinical relevance. This approach can be more advantageous, and associated with less unspecific effects, than the use of agents targeted to other elements of the cardioprotective intracellular signalling pathway such as protein kinase activators. In particular, it may be useful for eliciting

cardioprotection in the diabetic heart that can not be preconditioned by IP, since evidence from this study has shown that nNOS is downstream of the mitoK_{ATP} channels that are defective in this clinical condition.

Future directions

Nitric Oxide is central to injury and protection during ischaemia and reoxygenation and it is imperative that the role of NO produced by each NOS isoform, requiring different cellular locations and protein association, is fully elucidated, including the molecular interactions involved. In particular, the NO derived from nNOS is likely to be critically involved in both short and long term response of the myocardium to ischaemia and reperfusion injury and possibly IP, by bringing about changes in the phosphorylation of regulatory proteins in the signal transduction pathway involved. So the appropriate concentration of Nitric Oxide, exact location of three NOS isoforms and the precise sequence of reaction of NO with other mediators and effectors in IP pathway needs to be ascertained. I think this is the area where future research for preconditioning should be directed. These will decide whether classical or remote preconditioning can be used in a clinical setting for cardioprotection.

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