Efficacy and Mechanism of Nicorandil in Perioperative Protection of Skeletal Muscle from Ischaemia and Reperfusion Injury in a Porcine Model

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ABSTRACT

Background: Prolonged ischaemia time during autogenous free muscle transfer, can lead to ischaemia reperfusion (I/R) injury and local necrosis of the muscle. It has been demonstrated that the phenomenon of ischaemic preconditioning (IPC) confers biphasic infarct protection in a porcine skeletal muscle flap model. Further to this, the hybrid nitrovasodilator and K_{ATP} channel opener Nicorandil, is known to induce 24h uninterrupted infarct protection in myocardial models. We therefore hypothesised that Nicorandil could pharmacologically confer late-phase infarct protection of skeletal muscle from I/R injury.

Methods: Yorkshire pigs (mean 17.9kg) with bilateral 8x13cm Latissimus Dorsi (LD) muscle flaps, received i.v. Nicorandil (3mg/kg) before being subjected to 4h ischaemia followed by 48h reperfusion.

Results: Nicorandil induced late-phase preconditioning appeared at 24h after Nicorandil injection and lasted for 72h before waning. LD infarction rates were reduced to 22+/-2, 25+/-2 and 28+/-2% at 24h, 48h and 72h respectively, compared to the ischaemic control of 40+/-2%. Further to this, Nicorandil preconditioning was associated with a reduction in mitochondrial free calcium content, preservation of muscle ATP content and attenuation of neutrophilic myeloperoxidase activity during the first hour of reperfusion. Injection with the specific sarcolemmal K_{ATP} (sK_{ATP}) inhibitor HMR-1098 or non-specific K_{ATP} inhibitor Glibenclamide before Nicorandil injection completely blocked the infarct-protective effects. Injection of the specific mitochondrial K_{ATP} (mK_{ATP}) inhibitor 5-HD or Glibenclamide before the onset of reperfusion also abolished Nicorandil preconditioning.

Conclusion: These findings support the hypothesis that a single dose of Nicorandil induces 48h of uninterrupted late-phase infarct protection in skeletal muscle. Further to this, sK_{ATP} and mK_{ATP} channels play a central role in the trigger and mediator mechanisms, respectively. Nicorandil is a potential new therapy to augment the ischaemic tolerance of skeletal muscle for patients undergoing autogenous free muscle transfer or composite tissue allotransplantation.

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The experiments and writings within this thesis are my own and relate to research carried out by myself at the Hospital for Sick Children, Toronto between June 2007 and July 2009. Other members of the research group who contributed to the laboratory and surgical work embodied in this thesis are specifically acknowledged above. The work has not been submitted for any other degree or other professional qualification.

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1.0 INTRODUCTION

1.1 History & The Clinical Problem

The specialty of Plastic and Reconstructive Surgery involves the use of tissue flaps to reconstruct form or function following trauma, oncological disease or congenital abnormality. The first half of the twentieth century saw the development of local and tubed-pedicled skin flaps in reconstructive challenges. These flaps were classed as 'random pattern flaps' and were therefore limited by their length to breadth ratio to prevent distal tip necrosis. At this time the vascular anatomy of the skin and fascia were not fully understood and therefore vascular patterns were not adhered to. The advent of 'axial pattern flaps' in the 1970s was in fact based on much earlier work by Salmon (Salmon et al. 1936). Their advantageous length-to-breadth ratios expanded the repertoire of plastic surgeons, enabling them to reconstruct more complex defects with fewer operations. It was not until Ger described the use of pedicled muscle flaps in lower leg trauma (Ger 1972) that advances in the knowledge of vascular muscle anatomy became apparent. It was soon realised that a skin paddle could be carried on a vascularised muscle pedicle, thereby improving the range of composite tissue used in reconstructive flap options. Mathes and Nahai provided the first definitive description of the muculocutaneous system of flaps and classified the different vascular anatomical axes (Mathes et al. 1981).

The 'free flap' was first introduced by Ian Taylor and colleagues in Melbourne, Australia 1973 (Daniel et al. 1973). They described the autogenous free transfer of a groin flap for wound coverage of a foot defect. The term was modified to

'free tissue transfer' to indicate that a wide range of tissues, such as skin, muscle and/or bone, could be isolated on their vascular supply and transplanted to distant sites in the body as a single-stage procedure. This discovery, spurred on by improvements in loupe magnification, micro-instruments and suture materials, became an important part of a plastic surgeon's armamentarium. Free tissue transfer is now used in the reconstruction and restoration of form, such as in breast reconstruction after mastectomy; and restoration of function, such as in facial paralysis surgery and in Volkmann's ischaemic contracture.

Along with this evolution in flap reconstruction, new issues have surfaced regarding the vascular supply to the flap. Free tissue transfer requires division of the vascular pedicle supplying, for example, a muscle / musculocutaneous flap, followed by an obligatory period of warm ischaemia prior to microsurgical anastomosis. There are circumstances when the restoration of the muscle flap circulation is delayed, such as a difficult and time-consuming anastomosis, perioperative vasospasm and thrombosis or external pedicle compression. This can result in a prolonged warm ischaemia time, which if left unmanaged, will eventually lead to lethal ischaemic insult and subsequent tissue necrosis. This culminates in failure of the free tissue transfer.

Further to this, prolonged tissue ischaemia and subsequent reperfusion injury can occur in other surgical scenarios, such as a protracted limb tourniquet time during orthopaedic / vascular procedures to provide a bloodless operative field; clamp control of major vessels during vascular bypass surgery; and limb replantation following traumatic amputation.

Despite the advances in surgical technology and the increased experience in specialist centres, the overall failure rate of free tissue transfer remains around 5% (Khouri 1992; Khouri et al. 1998). This is a significant clinical problem, as a flap failure constitutes a failure of the reconstruction of the patient's defect, necessitating further surgical procedures and exploration of alternative options. These additional procedures result in a prolonged hospital stay and add to the psychological morbidity of the patient.

The aetiology of free flap failure appears to be multifactorial, however it is believed that the incidence could be further reduced by the discovery of an effective treatment for the prevention of ischaemia reperfusion tissue injury, caused by a prolonged ischaemic insult followed by subsequent reperfusion of the tissues.

1.2 Ischaemia Reperfusion Injury in Musculoskeletal Reconstructive Surgery

Various tissues are commonly exposed to periods of warm ischaemia in both elective and traumatic surgical scenarios. The tolerance of a tissue for ischaemia depends on tissue type as well as its volume, metabolic activity and temperature. It is no surprise that skeletal muscle, with its high metabolic activity has the lowest tolerance to ischaemia (Blaisdell 2002) (Figure 1). This is due to the fact it comprises the vast majority of a limb's weight, blood-flow and therefore energy demands. However, these values are estimates as it is difficult to tell

when muscle has died. Macroscopic and even microscopic changes in the skeletal muscle cells do not become apparent for many hours following muscle death. Belkin et al went some way to quantifying this critical ischaemic interval by using spectrophotometric tetrazolium analysis of rat skeletal muscle to show that significant muscle damage occurred after 3 hours of ischaemia (Belkin et al. 1988). Eckert et al further reformed this level using nuclear magnetic resonance (NMR) spectroscopy, stating that in fact normo-thermic human skeletal muscle has a critical ischaemic tolerance of only 2 hours 15 minutes (Eckert et al. 1991). This critical ischaemia time was estimated by determining the point at which the Adenosine Triphosphate (ATP) pool was depleted; and is heavily dependent on muscle temperature (Eckert et al. 1991).

Tissue	Time
• Muscle	• 4 hours
• Nerve	• 8 hours
• Fat	• 13 hours
• Skin	• 24 hours
• Bone	• 4 days

Figure 1.1. *Critical tissue ischaemia time estimates based on assessment of absolute ischaemia induced prior to replantation (Blaisdell 2002).*

1.2.1 Skeletal Muscle Metabolism & Morphology

Normal physiological conditions in the skeletal myocyte involve oxidative phosphorylation of fatty acids, ketone bodies and phosphates to produce Adenosine Triphosphate (ATP), the cell's main energy supply. Phosphocreatine is an energy-rich phosphate compound found in high concentrations in the cell under these aerobic conditions. Phosphocreatine is tied to ATP by the enzyme creatine kinase and thus the ATP pool is consistently being replenished by the phosphocreatine reserve.

Under anaerobic conditions, as well as the phosphocreatine reserve, the cell continues to synthesize ATP by glycolysis and purine nucleotide deamination. With glycogen stores abundant in skeletal muscle, anaerobic glycolysis continues to produce ATP until it is inhibited by lactic acidosis (Walker 1986; Lindsay et al. 1990).

Much of the debate in the past has been regarding the point at which irreversible damage occurs in the myocyte, resulting in cell necrosis. Lindsay demonstrated a sustained level of muscle ATP content in up to 3 hours ischaemia in canine gracilis muscle (Lindsay et al. 1990) predominantly due to depletion of creatine phosphate stores. Only when the phosphocreatine pool has been exhausted, do we get a decline in the muscle ATP stores, known as Bendall's delay phase (Bendall 1973). This will eventually result in ATP depletion and cell death. During this interim phase, reperfusion of the muscle and restoration of oxidative phosphorylation can replenish ATP as long as the mitochondria are functioning

normally and there is availability of suitable substrates in the cell (Walker 1986). It is important to note that the rate of breakdown of ATP and phosphocreatine in the ischaemic myocyte is highly dependant on not only temperature, but species and fibre type as well.

Type 1 fibres are slow twitch muscle fibres more suited to endurance work and therefore more resistant to fatigue. They are the predominant fibre type in the postural muscles of the body. They have the highest oxidative capacity and the highest lipid stores, but in contrast, have the lowest glycogen stores and therefore a reduced amount of glycolytic enzyme capacity. Type 2 fibres, on the other hand, are fast twitch fibres found predominantly in limb musculature, like gastrocnemius and the hamstring muscles. They are sub-divided into 2a and 2b with increasing amounts of glycolytic capacity to provide energy by anaerobic metabolism. Intuitively, Type 1 fibres have predominantly oxidative metabolism of lipid stores as their energy source and are therefore much more vulnerable to hypoxia than Type 2 fibres (Lindsay et al. 1990; Blaisdell 2002).

1.2.2 Early Concepts of Reperfusion Injury

In autogenous free muscle transfer, a relatively small volume of skeletal muscle may be subject to reperfusion injury, causing a local inflammatory response with tissue hyperaemia and oedema related to the release of muscle breakdown products such as histamine, lactate, lysozomes, myoglobin and various proteolytic enzymes (Steinau 1988). These breakdown products not only trigger the inflammatory response but are also prothrombotic and therefore activate the

intrinsic clotting pathway, resulting in aggravation of pre-existing microvascular damage (Eckert et al. 1991; Blaisdell 2002).

If the volume of muscle involved is greater, for example, following extremity replantation or in a compartment syndrome, muscle necrosis with subsequent reperfusion can cause serious systemic complications affecting distant organs. This can result in life threatening metabolic consequences such as acidosis, hyperkalaemia and myoglobinuria (Walker 1986; Blaisdell 2002). This was first alluded to by the work of Bywaters in World War II, who noted the brown pigment of myoglobin in the urine of casualties that died of renal failure following crush injuries to the extremities (Bywaters et al. 1941). Haimovici et al clarified the association of these systemic metabolic changes with arterial reperfusion and later coined the term 'myonephropathic-metabolic syndrome' (Haimovici 1960). Further distant organ damage is apparent in the form of lung dysfunction, caused by the release of ROS and cytokines, resulting in diffuse capillary leakage and ultimately non-cardiogenic pulmonary oedema (Khalil et al. 2006). These catastrophic changes following replantation of a leg or arm, have resulted in re-amputation to avoid a potentially fatal outcome (McCutcheon et al. 2002).

By the late 1960s, the connection had been made between prolonged tissue ischaemia and subsequent reperfusion injury causing local necrosis and systemic metabolic sequelae. This trio of metabolic acidosis, hypotension and myoglobinuria had been termed 'de-clamping shock' (Vetto et al. 1968) referring to the flushing out of toxic metabolites following the release of vascular

clamps and reperfusion of a limb. However, little was known about the pathophysiology of ischaemia reperfusion injury.

1.2.3 Acute Ischaemia Reperfusion Injury

Reestablishment of blood flow to previously ischaemic tissues, resulting in local / systemic complications constitutes reperfusion injury. Ischaemia reperfusion injury was first described in a canine myocardial model by Cerra et al in 1975 (Cerra et al. 1975). Specifically, they observed that reperfusion after prolonged ischaemic anoxia, produced sub-endocardial haemorrhagic necrosis, the extent of which was proportional to the duration of the preceding ischaemic event. Further to this, a retrospective review of the mortality from valve replacement surgery, deduced that 30% of the deaths were due to post bypass haemorrhagic necrosis (Cerra et al. 1975). May and colleagues (May et al. 1978) first described the phenomenon of reperfusion injury in a free flap model, where they observed progressive obstruction of peripheral blood-flow in the flaps with increasing periods of ischaemia, up to a point where irreversible ischaemia and tissue necrosis occurred.

We know that an extended period of ischaemia damages cells, however paradoxically; reintroduction of oxygenated blood to ischaemic tissue results in further damage and can be lethal. Piper (Piper et al. 1998) defines reperfusion injury as “injury caused by restoration of blood flow after an ischaemic episode leading to death of cells that were only reversibly injured during that preceding ischaemic episode”. There has been much debate by investigators in the past

regarding the existence of reperfusion injury and the timing of lethal injury to cells during an ischaemic insult. In the past it was believed that all cellular injury developed during the ischaemic period whereas we now contend that reperfusion extends, and in fact contributes to the majority of tissue injury. Gute et al demonstrated that the characteristic myonecrosis of reperfusion injury will not occur in ischaemic skeletal muscle unless exposed to molecular oxygen by reperfusion (Gute et al. 1998). Tissue damage by reperfusion injury arises via a complex series of mechanisms. The earliest moments of I/R injury are characterised by a 'respiratory burst' with inflammatory cascades and release of reactive oxygen species (ROS), followed by mitochondrial dysfunction and eventually disruption of the microcirculation (no-reflow phenomenon) (Khalil et al. 2006; Gross et al. 2007).

1.2.3.1 Generation of Reactive Oxygen Species (ROS)

ROS are highly unstable oxygen molecules with unpaired electrons. They are produced in small quantities by normal cellular metabolism and are tightly regulated by ROS scavengers such as glutathione, superoxide dismutase and catalase (Granger et al. 1986). During ischaemia reperfusion injury sufficiently large quantities of ROS are produced which overwhelm the endogenous scavengers and therefore exert their effects on the cell.

Depending on the tissue in question, there are different sources of ROS. For example, following reperfusion injury in the lung the main source of ROS is cytochrome P450, whereas xanthine oxidase and NADPH oxidase have been

identified as important sources of ROS in skeletal muscle (Khalil et al. 2006; Zweier et al. 2006).

Xanthine oxidase (XO) is an enzyme located mainly within endothelial cells, in cardiac as well as skeletal muscle (Jarasch et al. 1986). During early reperfusion XO reacts with the anaerobic breakdown products of ATP, hypoxanthine and xanthine, to form hydrogen peroxide (Zweier et al. 2006). The localisation of XO to endothelial cells and their early exposure to oxygenated blood suggests their susceptibility to ROS-mediated reperfusion injury. The resultant endothelial cell damage may induce inflammatory cell chemotaxis, propagating the reperfusion injury.

Nicotinamide adenine dinucleotide phospate (NADPH) oxidase is another enzyme bound to phagocytic leucocytes. Neutrophil accumulation is a recognised sequelae of reperfusion injury, and therefore the increase in oxygen metabolism associated with the 'respiratory burst' results in oxidation of cytoplasmic NADPH to $NADP^+$. This produces highly unstable superoxide anion, which will further react with water and chloride ions to produce hydrogen peroxide and hypochlorous acid, respectively (Zweier et al. 2006).

During early reperfusion, the increased production of ROS have a direct toxic effect on the muscle cell by the process of lipid peroxidation resulting in breakdown of the cell membrane's phospholipid bilayer. This leads to a loss of membrane integrity, disruption of the osmotic gradient, cellular swelling and eventual cell death (Park et al. 1999; Zweier et al. 2006).

Due to the presence of ROS associated with the 'respiratory burst' in early reperfusion, several investigators have explored the potential of ROS scavengers to reduce reperfusion injury. There is evidence to show that lipid peroxidation can be inhibited by the administration of ROS scavengers, such as Vitamins A/E (Bilgin-Karabulut et al. 2001) and prostacyclin (Bozkurt 2002). However, other experiments looking at inhibition of NADPH oxidase (Dodd et al. 2000) and in NADPH oxidase-deficient mice (Hoffmeyer et al. 2000), have failed to show significant attenuation of reperfusion injury.

Despite xanthine oxidase (XO) being identified as a major pathway for ROS generation, the activity of XO is extremely variable, depending on the tissue or the species in question. For example, increased levels of the XO have been demonstrated in endothelial cells of ischaemic human skeletal muscle by Wilkins and colleagues (Wilkins et al. 1993), whereas very minute quantities of XO were observed in ischaemic canine skeletal muscle (Lindsay et al. 1990). This may explain the poor results obtained using XO inhibitors to ameliorate the effects of reperfusion injury. For example, Allopurinol, a xanthine oxidase inhibitor, failed to attenuate the extent of skeletal muscle necrosis in the pig, after 5 hours ischaemia and 48 hours reperfusion (Dorion et al. 1993).

It seems that ROS were initially thought to be major effectors of the reperfusion injury cascade but the failure to reach a consensus on the effects of ROS scavengers, have lead researchers to conclude that ROS are important mediators but only a minor contributor to reperfusion injury (Khalil et al. 2006; Zweier et al. 2006).

1.2.3.2 Mitochondrial Calcium Overload

There is now uniform consensus that calcium (Ca^{2+}) homeostasis is central to the development of ischaemia reperfusion injury. Cytosolic Ca^{2+} concentration is normally regulated by transport into and out of the endoplasmic reticulum or sarcoplasmic reticulum (ER/SR). It can also be transported out of cells via Na^{+}/Ca^{2+} exchangers (Berridge et al. 1998). During stress conditions, for example during ischaemia, Ca^{2+} transport can be inhibited or even reversed. In prolonged ischaemia the lack of ATP results in a failure to pump calcium out of the cytosol via the Ca^{2+} pump of the ER/SR, resulting in a relatively higher intracellular Ca^{2+} concentration. To compound this the burst of ROS during early reperfusion can cause the Na^{+}/Ca^{2+} exchanger to operate in reverse mode resulting in further Ca^{2+} influx into the cytosol (Ermak et al. 2002).

During ischaemic conditions, Ca^{2+} influx is essentially limited by intracellular acidosis, *i.e.*, anaerobic breakdown of ATP produces an excess of H^{+}, which results in a lowering of both the intracellular and extracellular pH. This ionic homeostasis inhibits the cation pumps, namely the Na^{+}/H^{+} exchanger which in turn inhibits the Na^{+}/Ca^{2+} exchanger, preventing further Ca^{2+} influx to the cytosol. The resupply of oxygen to the myocyte results in 3 changes within the cell: a) resupply of ATP to the cation pumps to initiate recovery of the cation balance; b) rapid normalisation of intracellular pH resulting in reactivation of Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchangers, which will aggravate cytosolic Ca^{2+} accumulation; c) too rapid normalisation of extracellular osmolality resulting in hyperosmolar intracellular fluid and cellular swelling. This is mainly due to the

reactivation of the Na^+/H^+ exchanger and the resultant cytosolic Na^+ overload (Piper et al. 1998).

It is well recognised that the final event in the reperfusion injury cascade is mitochondrial Ca^{2+} overload resulting in uncoupling of oxidative phosphorylation and the inability to synthesise ATP, leading to cell death (Ermak et al. 2002). Ca^{2+} concentrations in the mitochondria are dependent on those in the cytoplasm and therefore, under oxidative stress, mitochondrial Ca^{2+} accumulation can change from a physiological beneficial process to a cell death signal.

The inner membrane of the mitochondria is impermeable to almost all metabolites and ions under normal physiological conditions. Under certain pathological conditions, such as ischaemia, protein pores are formed within this membrane. They are collectively known as the mitochondrial permeability transition pore (mPTP) and play a vital role in both active and programmed cell death during reperfusion injury. mPTP can be induced by ROS or a high cytosolic Ca^{2+} concentration (Gateau-Roesch et al. 2006) and can be closed / blocked by acidosis and drugs such as Cyclosporin A (Halestrap et al. 1997). This illustrates that mPTP remain closed during ischaemia, but open during reperfusion. If the ischaemia reperfusion insult is severe and the mPTP remain open and disrupt the mitochondrial membrane potential. Mitochondria become uncoupled and hydrolyse ATP instead of synthesising it, leading to rapid ATP depletion, resulting in a loss of structural integrity and necrosis of the myocyte (Halestrap et al. 2000).

Programmed cell death or 'apoptosis' is also mediated via the mPTP. As apoptosis is an energy-dependent process, this may only occur if mPTP opening is less extensive or if they are able to reclose following a period of opening. Under stress conditions, the mPTP open to allow equilibration of matrix proteins. The osmotic force of these proteins may result in irrecoverable mitochondrial swelling and breach of the outer membrane with efflux of cytochrome C and other transcriptional factors that cause apoptotic cell death (Halestrap et al. 2000; Gateau-Roesch et al. 2006).

1.2.4 Late Ischaemia Reperfusion Injury

A later phase of reperfusion injury is thought to come about by the activation and invasion of neutrophils. The time-period of this response is variable, due to the multifactorial nature of the inflammatory response from myocyte necrosis. It is no surprise that polymorphonuclear (PMN) leucocytes accumulate gradually during prolonged ischaemia, as it is simply an inflammatory response like in any other tissue. During reperfusion, there are established interactions between neutrophils and vascular endothelium. Vascular endothelial cells provide an anchoring site for neutrophils by the increased expression of P / L selectin molecules (Lefer et al. 1994). This leads to activation of the inflammatory cascade, endothelial cell dysfunction and eventually cell death (Vinten-Johansen et al. 2005).

It is well known that granulocytic infiltration, predominantly neutrophilic, is a hallmark of myocardial infarction, appearing in the first 24 hours after vessel

occlusion (White et al. 1936). However, it is this intensive neutrophil accumulation during reperfusion, which has divided opinion on the role of neutrophils in reperfusion injury. Neutrophils are known to be a rich source of several ROS, such as superoxide, hypochlorous acid and lysosomal proteolytic enzymes (Baxter 2002). This has propagated the idea that neutrophil accumulation during reperfusion accounts for the burst of reactive oxygen species (ROS) resulting in early lethal reperfusion injury (Lucchesi et al. 1989; Reimer et al. 1989; Gumina et al. 1997). However, further research has failed to corroborate these findings and has not been able to robustly reproduce this phenomenon. As well as this the advent of isolated, buffer-perfused hearts, devoid of blood components demonstrates that reperfusion injury can be replicated in the absence of PMN (Xu et al. 2000; Vinten-Johansen 2004).

1.2.4.1 No reflow phenomenon

The "no reflow" phenomenon was first described by Ames in 1968 (Ames et al. 1968). He used this term to describe the total occlusion of ischaemically damaged capillary networks in the brain. The pathology of no reflow was first properly investigated by Kloner and colleagues in a canine coronary artery occlusion model, reporting that, following prolonged ischaemia, a distinct area of perfusion deficit was detected following restoration of coronary blood flow (Kloner et al. 1974). It is preceded by changes in the microcirculation that develop progressively during the ischaemic insult. Specifically, the formation of endothelial vesicles, differential endothelial swelling and development of gaps between cells, leads to an increase in vascular permeability. This results in

haemoconcentration and rouleau formation of red cells, leading to thrombosis (Hammersen et al. 1983). Neutrophil plugging is apparent in the capillaries of these areas of no reflow with some interstitial migration of these cells as well. Again, there is controversy as to whether no reflow occurs as a result of muscle infarction, or muscle necrosis occurs due to inadequate perfusion as a result of no reflow (Blaisdell 2002). We believe that these microvascular changes are occurring in areas of critically / irreversibly ischaemic muscle and are therefore a consequence of rather than a cause of this phenomenon.

1.2.4.2 Distant site injury

If a sufficiently large volume of muscle is involved in the ischaemic process the inflammatory response may become systemic. For example, in compartment syndrome or limb replantation, a large mass of skeletal muscle may be reperfused with the "washing out" of muscle breakdown products into the bloodstream.

Initially, this leads to a profound metabolic acidosis associated with hyperkalaemia, lactate acidosis and a systemic coagulopathy due to disseminated intravascular coagulation (DIC) (Walker 1986). The procoagulant breakdown products activate other inflammatory mediators in parallel, such as histamine, complement, thromboxane and bradykinin. These mediators create further damage to the vascular endothelium, leading to increased systemic vascular permeability (Blaisdell 2002; Khalil et al. 2006).

The clinical manifestations of this injury occur in the lung and kidney. Myoglobin is a direct renal tubular toxin that is released into the bloodstream during reperfusion and, in association with acidosis and oliguria, can ultimately lead to renal failure (Walker 1986). Elsewhere, activated neutrophils sequester in the lungs and, with concomitant ROS and cytokine damage, cause non-cardiogenic pulmonary oedema (Khalil et al. 2006). These sequelae can be fatal, even with modest ischaemia periods and young fit patients (McNeill et al. 1970). Blaisdell therefore advocates reperfusion of an ischaemic limb, only if the ischaemia time is less than 4-6 hours (Blaisdell 2002).

1.3 Protection of Skeletal Muscle Against Perioperative Ischaemia Reperfusion Injury

Ischaemia reperfusion injury can have devastating effects on different tissues and organs in the body, and this pathological process is evident in different medical / surgical specialties. For example, in cardiothoracic surgery, prolonged myocardial ischaemia during coronary bypass surgery can result in extended sub-endocardial necrosis upon reperfusion. Also, following lower limb trauma, patients that undergo limb revascularisation after prolonged ischaemia can suffer systemic dysfunction to distant organs, with reperfusion of a large mass of skeletal muscle. Conventional surgical wisdom is to minimise the ischaemic insult to the tissues with early reperfusion, however this is often beyond the surgeon's control. Several therapeutic strategies have been attempted to reduce the local and systemic effects of ischaemia reperfusion, such as limb cooling

(Sapega et al. 1988) and intra-arterial flushing (Rosen et al. 1987), producing mixed results in animal studies. Heparin anticoagulation, thrombolysis and angioplasty is the modern cornerstone of clinical intervention in the treatment of ischaemia reperfusion injury of the myocardium, however, although this restores blood flow to the ischaemic tissue to reduce the ultimate infarct size, it does not prevent the prior damaging effects of the ischaemic insult. There has therefore been a drive for other intervention strategies to try to prevent the effects of a prolonged ischaemic myocardial insult, which has translated to several treatment strategies targeting ischaemia reperfusion injury affecting skeletal muscle.

1.4 Acute Ischaemic Preconditioning

In 1986, a landmark study was published by Charles Murry, describing a potential treatment strategy, which he termed 'ischaemic preconditioning' (IPC) (Murry et al. 1986). In a canine myocardial model, the heart was exposed to four brief 5 min periods of alternating coronary artery occlusion and reperfusion, prior to an ischaemic insult of 40 min sustained coronary artery occlusion followed by 72 hours reperfusion. This resulted in a 75% reduction in myocardial infarct size, compared to ischaemic controls. The same group went on to show that this phenomenon was associated with reduction of myocardial energy demand during ischaemia and preservation of ATP (Murry et al. 1990).

The discovery of IPC was a huge breakthrough, as previously there had been no effective therapeutic intervention to protect tissue from ischaemia reperfusion

injury. The clinical significance of an intrinsic intervention that produced a 75% infarct reduction rate was quickly realised, sparking a surge in research using myocardial animal models. This was a potential therapeutic strategy to reduce the extent of reperfusion-induced sub-endocardial necrosis in patients undergoing coronary angioplasty. Crucially, IPC is reliably reproducible and has been shown to limit infarct size in every species tested to date, such as in the rat (Yellon et al. 1992), rabbit (Liu et al. 1991), dog (Murry et al. 1986) and pig (Schott et al. 1990).

Acute IPC, also commonly referred to as 'classical' IPC, describes a preconditioning stimulus performed immediately before the onset of sustained ischaemia. IPC does not completely abolish infarction; rather, it delays the onset of cell death by inducing tolerance to ischaemia reperfusion injury.

The translational capability of the IPC phenomenon was quickly realised and in 1992, Mounsey introduced IPC as a technique to enhance survival in pedicled or free skeletal muscle flaps, often used in plastic and reconstructive surgery (Mounsey et al. 1992). Mounsey and colleagues also described a porcine model, using the latissimus dorsi skeletal muscle flap to study the preconditioning effect (Mounsey et al. 1992). The pig is a reliable model to study skeletal muscle IPC as its musculoskeletal and vascular anatomy closely resemble that of humans. In our lab, Pang and colleagues (Pang et al. 1995) reported that at least three cycles of 10 minutes ischaemia and 10 minutes reperfusion were required for preconditioning of both latissimus dorsi (LD) and gracilis muscles against a 4 hour ischaemic insult followed by 48 hours reperfusion. They reported a 44%

and 62% decrease in the extent of infarction in LD and gracilis flaps, respectively.

1.4.1 Efficacy

The number of preconditioning cycles as well as the duration, has importance in relation to the degree of infarct protection, and using too many cycles has been demonstrated to abolish the protective effects of IPC (Iliodromitis et al. 1997). There has been much debate about the optimum number of cycles of IPC required to exert a protective effect over myocardial / skeletal muscle from subsequent ischaemia reperfusion injury. In the canine myocardial model, Li et al (Li et al. 1990) demonstrated that a single cycle of 5 min IPC was as effective as six or twelve cycles in reducing the rate of infarction following an ischaemic insult of 60 mins. In the rabbit myocardial model, Downey's group were able to afford protection of myocardium with a single cycle of 5 min ischaemia / 10 min reperfusion (Liu et al. 1991; Thornton et al. 1993). On the other hand, two or more cycles of IPC are required to significantly protect porcine myocardium (Schott et al. 1990; Miyamae et al. 1993); and five cycles of 5 min ischaemia / 5 min reperfusion were required to effectively precondition in vivo rat hearts (Piot et al. 1997). When translating this to skeletal muscle preconditioning, Pang demonstrated that at least 3 cycles of 10 mins ischaemia / 10 mins reperfusion were required to effectively precondition porcine latissimus dorsi skeletal muscle flaps (Pang et al. 1995). Therefore, it appears that the threshold for IPC is both tissue and species-specific.

Investigators were initially looking at the myocardium as a recipient tissue of the IPC stimulus, instigated by occlusion and reperfusion of coronary vessels. However, in 1993 Przyklenk et al noted that IPC by cycles of occlusion / reperfusion of the circumflex artery in the dog, additionally protected the area of myocardium perfused by the left anterior descending artery (Przyklenk et al. 1993). This was termed 'intraorgan' remote IPC and lead to further investigation with the discovery of 'interorgan' remote IPC. Specifically, occlusion / reperfusion of the mesenteric artery in the rat (Gho et al. 1996) or the renal artery in the rabbit (Takaoka et al. 1999), protected the myocardium against subsequent ischaemia reperfusion. This form of IPC once again translated between species and was also effective in skeletal muscle, where Addison et al demonstrated that 3 cycles of 10 min occlusion / reperfusion of a hind-limb using a pneumatic tourniquet, significantly reduced infarct size in porcine LD, gracilis and rectus abdominis flaps, subjected to a 4 hr ischaemic insult followed by 48 hr reperfusion (Addison et al. 2003).

The common endpoint measured to demonstrate the efficacy of IPC in different species and tissues is the percentage of infarcted to viable muscle and then comparing this with the time-matched ischaemic control. In addition to infarct reduction, IPC is also associated with preservation of ATP content in myocardial (Murry et al. 1990) and skeletal muscle (Pang et al. 1995; Addison et al. 2003); as well as a reduction in neutrophil accumulation during reperfusion (Pang et al. 1997; Addison et al. 2003). Charles Murry's original paper reported a 75% reduction in the infarct size of preconditioned canine hearts (Murry et al. 1986) which is similar to the 78% reduction seen in ischaemic preconditioned porcine hearts by Schott (Schott et al. 1990). These 2 papers were seen as the gold

standard for IPC efficacy and other myocardial models have attempted to achieve this same degree of infarct reduction. Unfortunately, when translated to skeletal muscle infarct protection we see more modest reductions in infarction rate with Pang (Pang et al. 1995) reporting a 44% and 62% reduction in total infarct size in LD and gracilis muscles, respectively.

1.4.2 Mechanism

Ischaemia reperfusion injury has a multi-factorial pathway and as such, IPC can have more than one targeted therapeutic effect. The general consensus is that IPC is due to a signal transduction cascade, involving triggers during the preconditioning ischaemia (e.g. Adenosine A_1 receptor), a cascade of various mediators (e.g. Protein Kinase C) converging on end-effectors (e.g. ATP-sensitive Potassium channels). The overall effects are a reduction in the rate of ATP depletion in the cell as well as preservation of intracellular pH, resulting in stabilisation of mitochondrial membranes (Auchampach et al. 1993). The important triggers, mediators and end effectors are detailed below.

Adenosine receptors are found ubiquitously throughout the body in a variety of tissues. There are four subtypes with types 1 and 2 being found in myocardial and skeletal muscle. Stimulation of the A_1 receptor causes a profound reduction in heart rate via inhibition of conduction of electrical impulses via the Sino Atrial Node (SAN) (Auchampach et al. 1993). The activated A_1 receptors are also known to couple to a variety of inhibitory G-proteins (G_i proteins) which result in inhibition of ATP conversion to camp, *i.e.* ATP conservation. These G_i

proteins can also be bound and activated by opioids, noradrenaline and bradykinin. Another G_i protein coupled by A_1 receptors causes opening of the K_{ATP} channels, representing another avenue to conserve ATP. This illustrates that multiple receptors work in parallel to trigger preconditioning, which is important as pharmacological blockade of one receptor type does not completely block IPC-induced protection, but serves to increase the ischaemic threshold required to trigger protection (Yellon et al. 2003).

Acute adenosine treatment has been highlighted as a mimicker of IPC by initiation of signal transduction involving Protein Kinase C (PKC) and resulting in activation / opening of K_{ATP} channels. Adenosine is therefore responsible for the anti-infarctive effects in several myocardial models, such as the dog (Auchampach et al. 1993); rabbit (Liu et al. 1991) and pig (Van Winkle et al. 1994); but not in the rat heart model (Li et al. 1993). This triggering mechanism is similarly translatable to mammalian skeletal muscle, as Forrest (Forrest et al. 1997) demonstrated in the porcine LD muscle flap model.

Protein Kinase C (PKC) is one of the most important kinase mediators responsible for signal transduction and has been shown to be required for IPC to be effective in the rat and rabbit myocardial models (Ytrehus et al. 1994; Uchiyama et al. 2003). Our laboratory investigated PKC involvement in the pig skeletal muscle flap model, demonstrating that PKC-inhibitors blocked the infarct-protective effects of classical IPC and adenosine-induced IPC (Hopper et al. 2000). Other kinases involved in the signal transduction pathways include tyrosine kinase and the mitogen-activated protein kinases (MAPK) (Riksen et al. 2004).

The K^+ ion is the major cytosolic and mitochondrial cation, responsible for the regulation of energy production and maintenance of cellular Ca^{2+} homeostasis. Mitochondria have K^+- impermeable inner membranes and therefore require bidirectional K^+ cycling via specialised channels. The K^+/H^+ antiporter is responsible for K^+ efflux, with K^+ entry being regulated by the ATP-sensitive K^+ channels (K_{ATP} channel). These channels were originally described by Noma (Noma 1983) in the cardiac sarcolemma of isolated mammalian myocytes and were found to be an important regulator of cellular energy metabolism and membrane excitability. They have since been described in various other tissues, such as pancreatic B cells, vascular smooth muscle, human and animal skeletal muscle.

Further to Noma's description, there are 2 subtypes found in the sarcolemma (sK_{ATP}) and on the mitochondrial inner membrane (mK_{ATP}) (O'Rourke 2004). These channels are sensitive to changes in intracellular ATP concentration, i.e., the channel is activated (opened) in response to a fall in the intracellular concentration of ATP. Opening of K_{ATP} channels in cardiac myocytes during ischemia or hypoxia results in a shortening of the cardiac action potential (due in part to a prolonged phase 3 repolarisation), a reduction in Ca^{2+} influx and therefore ultimately prevention of cytosolic Ca^{2+} overload (Noma 1983; Gross 1995). These changes during an ischaemic insult result in a slowing of cellular ATP catabolism and effectively an increase in myocardial / skeletal ischaemic tolerance.

The critical role of K_{ATP} channel opening in the phenomenon of ischaemic preconditioning (IPC) was first demonstrated by Gross and Auchampach in 1992 in a canine myocardial model. Specifically, the selective K_{ATP} blocker Glibenclamide prevented myocardial preconditioning, whether given before or after the preconditioning ischaemia (Gross et al. 1992). This was corroborated by Geshi et al (Geshi et al. 1999) and again translated as a critical role in IPC of skeletal muscle where an infusion of the K_{ATP} channel opener Lemakalim mimicked the protective effect of preconditioning in porcine LD skeletal muscle flaps subjected to 4 hours ischaemia and 48 hours reperfusion. The pharmacological activation of K_{ATP} channels was also associated with preservation of mitochondrial ATP content and reduction in neutrophilic myeloperoxidase (MPO) activity (Pang et al. 1997). Further to this Moses et al demonstrated the central role that the mitochondrial K_{ATP} (mK_{ATP}) channel subtype has in both the trigger and mediator mechanisms of hind-limb remote IPC of skeletal muscle in the pig model (Moses et al. 2005).

Although sarcolemmal K_{ATP} channels are not specifically involved in the trigger and mediator mechanisms of acute or remote IPC, they have been shown to act as a trigger for delayed / late phase IPC in rat myocardium (Patel et al. 2005) and porcine skeletal muscle (Moses et al. 2005). They will be discussed in more detail in the chapter on late phase IPC.

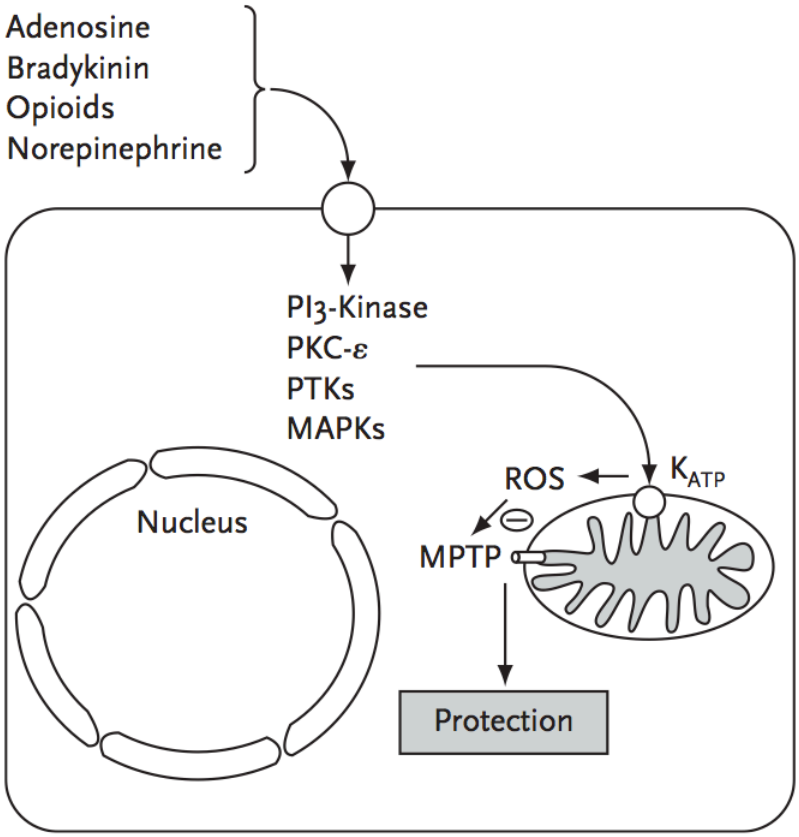

Figure 1.2. *The mechanism of acute / classical ischaemic preconditioning (IPC). During the IPC stimulus, several triggers are released (adenosine, bradykinin, opioids, noradrenaline). They activate a complex signaling cascade of protein kinases (PI_3-kinase, protein kinase c, tyrosine kinases, mitogen-activated-protein kinases (MAPKs)). This signaling cascade activates mitochondrial K_{ATP} channels, which in turn inhibit opening of the mitochondrial permeability transition pore (mPTP). Reproduced from Riksen et al (Riksen et al. 2004).*

1.5 Late Ischaemic Preconditioning

Acute or "classical" IPC describes an immediate adaptation of the cardiac / skeletal myocyte to sub-lethal ischaemic stresses. The acute IPC response develops immediately, however, wanes after 2-3 hours. It was therefore thought to be a transient phenomenon until in 1993 when Kuzuya and colleagues noted a similar reduction in infarction rate when the sustained ischaemic insult was induced 24 hours after the IPC stimulus (Kuzuya et al. 1993). They termed this "delayed" or "late phase" IPC, referring to a second window of preconditioning appearing 12-24 hours after the original IPC stimulus. Further to this, Baxter et al discovered that this late phase of protection lasted up to 72 hours, although the degree of infarct protection was not quite as robust as classical IPC, with infarct size reduction of 50% (Baxter et al. 1997).

Since Kuzuya's discovery, researchers have found that this biphasic pattern of IPC occurs in the myocardium of different species, such as the rabbit (Baxter et al. 1997); dog (Kuzuya et al. 1993) and pig (Tang et al. 1996). Following this, Moses et al then demonstrated late phase IPC in the porcine LD skeletal muscle flap model (Moses et al. 2005).

1.5.1 Efficacy

The therapeutic potentials of late IPC have been highlighted due to its prolonged window of preconditioning as well as its protective effects on muscle infarction and contractility. It has been noted by Roberto Bolli's group in the porcine

myocardial model (Tang et al. 1996) that late IPC protects against "myocardial stunning" (hypocontractility) as well as myocardial infarction. Further to this, Harralson et al (Harralson et al. 2005) found that late IPC protected rat LD muscle contractile function as well as infarct rate. Specifically, Harralson found that LD flaps were protected from infarction for a period of 72 hours, however the protection of contractile function lasted only 48 hours. Baxter and colleagues characterised the time course of late IPC in the myocardial rabbit model (Baxter et al. 1997). They noted a prolonged "second window" of infarct protection 24 – 72 hours following the IPC stimulus. This protective effect was then lost at 96 hours. Moses et al (Moses et al. 2005) confirmed this time-course in the porcine LD muscle flap model and noted that the degree of late-phase infarct reduction of 40-47% was similar to the 52% infarct reduction seen with acute IPC.

A further therapeutic potential that can be exploited with late-phase IPC, is the ability to repetitively precondition cardiac or skeletal muscle to convey a continuous period of infarct protection. This was illustrated by Derek Yellon's team in London, where they showed that intermittent pharmacological IPC, by activation of adenosine A_1 receptors, over a 10-day period maintained rabbit myocardium in a preconditioned state against ischaemia reperfusion injury (Dana et al. 1998). This could make late-phase IPC a powerful clinical tool in the realm of cardioprotection as well as augmentation of free flap ischaemic tolerance (Figure 1.3).

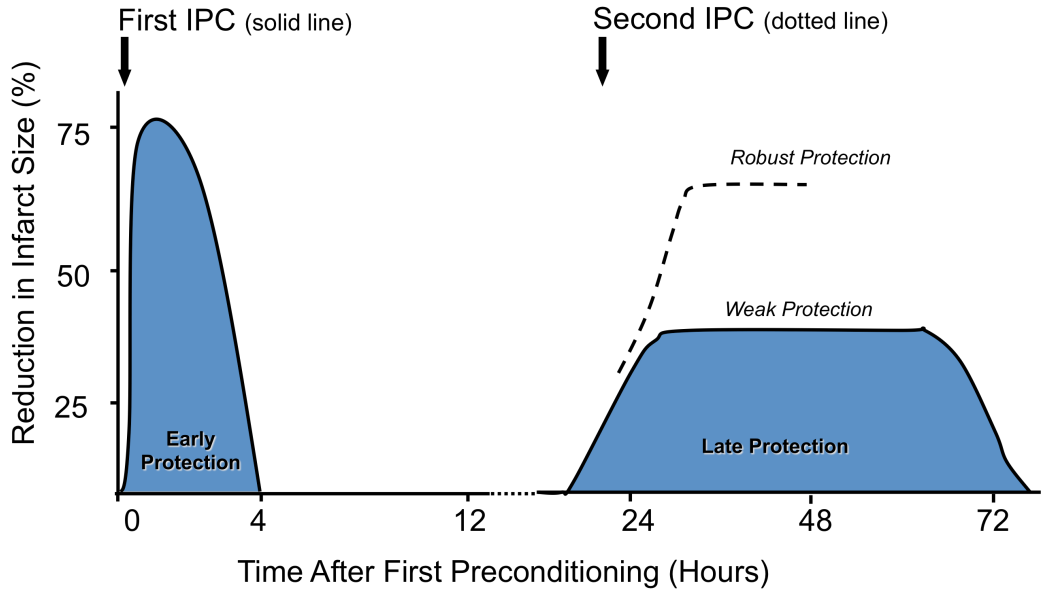

Figure 1.3. *The ischaemic preconditioning (IPC) stimulus can be repeated to potentially prolong the "preconditioned" state as well as providing a more robust protection of skeletal muscle from ischaemia reperfusion, during the second window of protection.*

1.5.2 Mechanism

Investigators have shown that classical and late-phase IPC mostly share the same signal transduction cascade involving various kinases, however the triggering stimuli and end effectors differ. The most striking difference between classical and delayed IPC is the activation of important transcriptional regulators, such as nuclear factor-$_k$B (Riksen et al. 2004), in the cell nucleus resulting in increased transcription of protective proteins and genetic modification of the myocyte to a more 'defensive' ischaemically tolerant phenotype. Several studies have looked at the triggering mechanism of this complex cascade. Baxter was the first to report that Adenosine, as well as in acute IPC, initiates late IPC (Baxter et al.

1994). The same group went on to classify the A_1 subtype responsible for triggering the delayed protection (Baxter et al. 1997). Bolli's group identified another important trigger, Nitric Oxide (NO) (Qiu et al. 1997). NO is an organic nitrovasodilator that can be formed endogenously by Nitric Oxide Synthase (NOS). There are three isoforms of this enzyme: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). NO is a cell-signaling molecule that can be best described as an endogenous modulator of vascular tone. Bolli has shown that myocardial adaptation to an ischaemic stress is due to an upregulation of eNOS, generating NO, which in turn triggers the kinase cascade leading to protein synthesis (Bolli et al. 1997). The involvement of NO as a trigger is further illustrated by the use of exogenous NO donors to mimic late IPC (Wang et al. 2000; Xuan et al. 2007).

The mediators of late IPC are described as the upregulated proteins that will enhance the tolerance of the cell to subsequent ischaemia. How they provide protection is still not fully understood. Interestingly NO has been identified as a mediator as well as trigger in the late IPC cascade. Takano et al (Takano et al. 1998) reported that iNOS was involved in the mediator mechanism of late IPC in the rabbit myocardial model. Other downstream mediators have been highlighted and are noted to be upregulated in preconditioned animals, such as Cyclooxygenase-2, superoxide dismutase and Aldose Reductase. There is a diversity of IPC initiators and mediators that converge on a common end-effector pathway resulting in K_{ATP} channel activation.

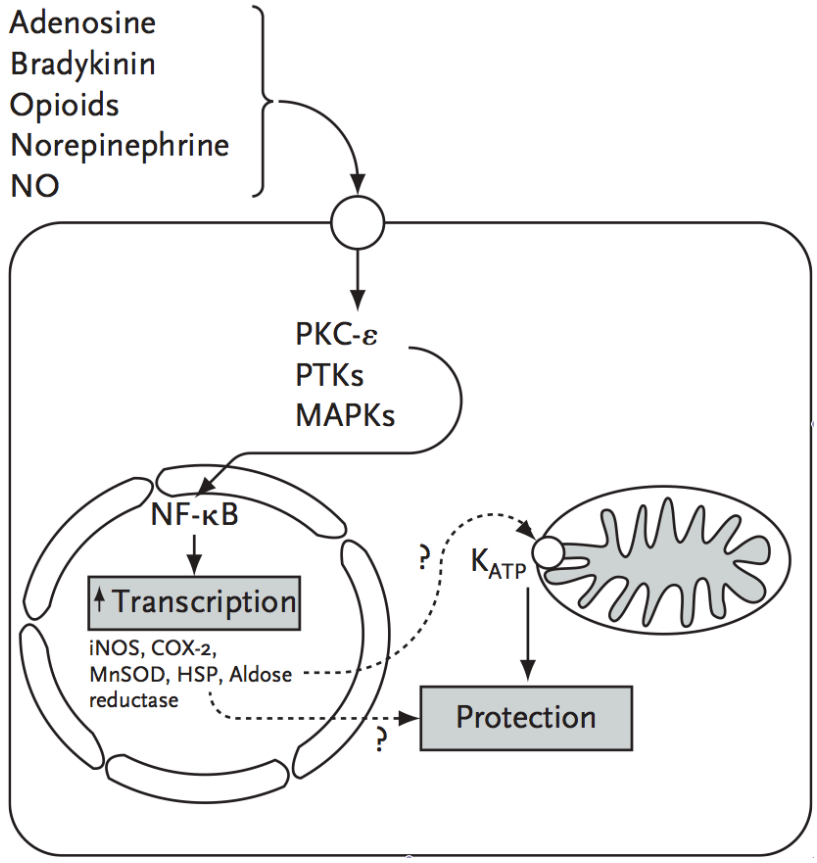

Figure 1.4. *The mechanism of delayed / late-phase ischaemic preconditioning (IPC). Nitric Oxide (NO) is an important additional trigger in delayed IPC. The kinase signal transduction cascade leads to activation of transcriptional regulator nuclear factors, which cause increased synthesis of protective proteins. Several mediators converge on a final common end-effector pathway resulting in mitochondrial K_{ATP} channel (mK_{ATP}) activation. Reproduced from Riksen et al (Riksen et al. 2004).*

It has been universally agreed that K_{ATP} channel opening is the final common pathway on which the signaling cascades converge, however, it remains to be determined whether its contribution is similar to that in acute IPC, where it has

been reported to inhibit ROS-induced mPTP opening during early reperfusion (Hausenloy et al. 2003).

Mei et al (Mei et al. 1996) were the first group to demonstrate that K_{ATP} channel activation was involved in the mediator pathway of late IPC. However, it was not until the more recently that mitochondrial K_{ATP} channels (mK_{ATP}) were highlighted as the likely mediator / end-effectors of late IPC (Bernardo et al. 1999; Takashi et al. 1999). This was facilitated by the development of highly selective pharmacological openers and inhibitors of mK_{ATP} channels, namely Diazoxide (Takashi et al. 1999) and 5-hydroxydecanoate (Takano et al. 2000). These mK_{ATP} channels, found on the inner membrane of the mitochondria, are postulated to act as a mechanism for osmotic regulation of mitochondrial volume and ultimately for mitochondrial Ca^{2+} homeostasis. Activation of these channels in mitochondria isolated from cardiac muscle, report membrane depolarisation produced by the K^+ entry, leading to reduced mitochondrial Ca^{2+} entry through the Ca^{2+} uniporter and further release of accumulated mitochondrial Ca^{2+} stores (Holmuhamedov et al. 1998; Holmuhamedov et al. 1999).

The translational skeletal muscle studies were carried out by Moses et al where they demonstrated, using the same pharmacological probes, that mitochondrial K_{ATP} channels play a central role in the mediator mechanism of late phase IPC in the protection of porcine skeletal muscle from infarction. Pharmacological activation of these channels was associated with muscle ATP preservation during sustained ischaemia and reperfusion, as well as a reduction in neutrophil accumulation during reperfusion (Moses et al. 2005).

The role of K_{ATP} channels in the triggering mechanism of delayed IPC was first examined by Garrett Gross' group in 2004. Specifically, they showed that administration of the selective sarcolemmal K_{ATP} (sK_{ATP}) channel blocker HMR-1098, but not 5-HD (mK_{ATP} blocker), abolished the IPC-induced infarct reduction 24 hours after the IPC stimulus (Patel et al. 2005). Using these same pharmacological probes, Moses et al further qualified the role of sK_{ATP} in the trigger mechanism of late phase IPC in the porcine LD skeletal muscle model (Moses et al. 2005).

1.6 Pharmacological Conditioning Against Perioperative Ischaemia Reperfusion Injury

Since the discovery of acute and late-phase IPC, researchers have elucidated a complex multi-factorial molecular mechanism associated with this phenomenon. These findings have provided us with several targets for pharmacological intervention and a huge clinical potential in the treatment of myocardial disease as well as the prevention of reperfusion-induced necrosis in musculoskeletal reconstructive surgery.

Acute IPC increases the ischaemic tolerance of a myocyte by subjecting it to sub-lethal periods of ischaemia prior to a sustained ischaemic insult. This is performed by invasively occluding and reperfusing the coronary vessels in a myocardial model, or the vascular pedicle in a skeletal muscle flap model. Performing this in a clinical human setting would be extremely invasive and

risks intimal damage to delicate vascular pedicles. Therefore the roles of various pharmacomimetics have been investigated in their capacity to pharmacologically precondition myocardial / skeletal muscle against I/R injury.

1.6.1 History

Despite the early interest in the role of oxygen-derived free radicals (now collectively known as "reactive oxygen species"), there has been little advance in pharmacological therapy to combat ROS-mediated I/R injury. For example, the roles of xanthine oxidase and NADPH oxidase are known as important sources of ROS, however, inhibition of these enzymes has failed to attenuate ischaemia reperfusion in different species (Dorion et al. 1993; Dodd et al. 2000; Hoffmeyer et al. 2000).

The first major advance was the discovery of the Adenosine A_1 receptor involvement in the trigger mechanism. Researchers were then able to mimic the IPC response by using both exogenous Adenosine (Forrest et al. 1997) and a specific Adenosine A_1 agonist (Auchampach et al. 1993; Tsuchida et al. 1993). However, due to the short elimination time of Adenosine, it is not really suitable for administration to elicit pharmacological IPC. Instead, the extracellular concentration of endogenous Adenosine can be pharmacologically raised by the nucleoside transport inhibitor Dipyridamole, which has shown promising results in a rabbit myocardial I/R model (Miura et al. 1992). Other IPC triggers prone to pharmacologically activation are: Bradykinin (elevated by the inhibited breakdown by ACE inhibitors (Jaberansari et al. 2001)); Morphine, as an opioid

agonist (Schultz et al. 1996); and Nitroglycerin as a NO donor (Leesar et al. 2001).

Downstream in the signal transduction pathway is the stimulation of Protein Kinase C (PKC), a series of iso-enzymes that play an important role as secondary messengers in the signal transduction cascade of IPC. Downey's group demonstrated that pharmacological activation of PKC mimicked IPC in rabbit myocardium (Ytrehus et al. 1994). Further to this, our lab demonstrated that pharmacological activation of PKC resulted in infarct protection of porcine LD muscle via a K_{ATP} channel-opening mechanism (Hopper et al. 2000). Despite the range of pharmacological 'triggers' of IPC that have been investigated, more recently the focus has been on studying pharmacological activation of the end-effectors of classical and delayed IPC.

One of the final events in the pathogenesis of I/R injury is mitochondrial Ca^{2+} overload, preceded by cytosolic Ca^{2+} overload (Ermak et al. 2002). The Na^{+}/H^{+} ion exchange protein (NHE) plays a central role, along with the Na^{+}/Ca^{2+} exchanger, in the maintenance of intracellular pH, Na^{+} and Ca^{2+} content. Pharmacological inhibition of the NHE before ischaemia results in protection of the myocardium from I/R injury in the pig model (Rohmann et al. 1995). Further to this, in our lab we demonstrated in our porcine skeletal muscle model, that the selective NHE inhibitor Cariporide protected LD muscle flaps from I/R injury when administered before ischaemia or reperfusion (McAllister et al. 2008).

As described earlier, the opening of mitochondrial K_{ATP} channels (mK_{ATP}) is essential for both acute and late-phase IPC to occur. Therefore recent effort has gone into the exploration of various pharmacological mK_{ATP} openers as well as inhibitors, which can arrogate or abolish the preconditioning stimulus. For example Glibenclamide, one of the sulphonyl ureas used in the treatment of Type 2 diabetes, is a potent non-selective K_{ATP} channel inhibitor and abolishes IPC in both myocardial (Gross et al. 1992; Schultz et al. 1996) and skeletal muscle (Pang et al. 1997; Moses et al. 2005; Moses et al. 2005) of numerous species. Diazoxide is a clinically licensed vasodilator has been shown to selectively activate mK_{ATP} channels (Liu et al. 1998). There is extensive research demonstrating pharmacological preconditioning by Diazoxide in myocardial animal models (Takashi et al. 1999; Takano et al. 2000; Rousou et al. 2004; Tsukamoto et al. 2005). The other clinically available K_{ATP} channel activator, which has had more recent clinical focus is Nicorandil and it will be discussed in more detail below.

1.6.2 Properties

The properties required for a clinically relevant pharmacomimetic are that the drug is preferably used in clinical practice already, has a relatively low side-effect profile and will be able to induce late IPC to augment the ischaemic tolerance of the myocardial / skeletal muscle tissue throughout a perioperative period, whether this be coronary angioplasty, heart transplantation, or free muscle transfer for reconstructive surgery.

Specific mK_{ATP} openers such as BMS-191095 (Moses et al. 2005) or the sK_{ATP} channel opener P-1075 (Moses et al. 2005) have shown favourable responses in producing infarct reduction comparable to invasive IPC, however, these are non-licensed drugs that have extensive side-effect profiles. Diazoxide has shown great promise as a pharmacological preconditioning agent for the myocardium (Takashi et al. 1999; Takano et al. 2000; Rousou et al. 2004; Tsukamoto et al. 2005), however, the drug can cause systemic hypotension and also has the significant side-effect of inhibiting insulin release from the pancreas, thereby raising blood glucose. Researchers therefore turned to other licensed clinical drugs to test in myocardial animal models, such as Nicorandil.

Nicorandil is a nicotinamide nitrate and is a clinically licensed drug used in the treatment of angina pectoris. Nicorandil was initially discovered by Japanese investigators and first reported internationally in 1979 as an experimental drug SG-75 with the potential as an anti-anginal medication due to its vasodilatory properties without calcium antagonism (Yanagisawa et al. 1979). Initially Nicorandil (SG-75) was seen as a new therapy in ischaemic heart disease. However, over time the cardioprotective potential has received much more attention and is now the main focus for investigators.

Nicorandil is a hybrid drug with both nitrate vasodilator and potassium-channel activating properties. The nitro-vasodilator effects of are thought to act by releasing nitric oxide, which in turn up-regulates intracellular cyclic guanine monophosphate (cGMP) levels and causes smooth muscle relaxation (Kukovetz et al. 1992). Nicorandil is also a potent ATP-sensitive potassium (K_{ATP}) channel opener, whose effect results in a shortening of the myocyte action potential

duration. This inhibits calcium influx, causing a reduction in intracellular calcium, leading to relaxation of vascular smooth muscle (Kukovetz et al. 1992). Clinically the nitrate properties dilate venous capacitance vessels, whereas the K_{ATP} channel activation leads to dilation of arterial resistance vessels. Nicorandil therefore acts as a balanced coronary artery and peripheral vasodilator, which reduces both preload and afterload, ultimately having a reductive effect on mean arterial pressure.

Nicorandil has a rapid rate of absorption; the therapeutic dosage range of 5 to 40 mg reaches a maximal plasma concentration after approximately 30 minutes, and this plasma concentration has a linear dose relationship (Frydman 1992). The absolute bioavailability of Nicorandil after both oral and intravenous administration in healthy human volunteers is in the region of 75-80%, implying there is no significant liver first-pass effect. Metabolism is by denitration to nicotinamide metabolites, which are mainly renally excreted (Frydman 1992). A single dose of the drug is eliminated from plasma within 8 hours, with an elimination half-life of 45 minutes after oral or intravenous administration (Markham et al. 2000). Verdouw et al studied the regional and systemic haemodynamics of Nicorandil in the anaesthetised and conscious pig. They reported an increase in cardiac output associated with a dose-dependent reduction of systemic vascular resistance and mean arterial pressure. Their investigation of the regional haemodynamics found that at the highest doses of Nicorandil, there were increases in blood flow to vital organs; however, blood flow to skeletal muscle was minimally affected (Verdouw et al. 1987).

The current dosage recommendations for human patients with stable angina pectoris are 10-30mg twice daily. The most commonly reported adverse effect is headache and dizziness, although there have been reports of patients developing aphthous or severe mouth ulceration (Markham et al. 2000). Another rare but well-reported complication of chronic Nicorandil treatment is idiopathic perianal ulceration (Katory et al. 2005).

The beneficial effects of Nicorandil in myocardial infarct protection were actually reported before the phenomenon of IPC was discovered by Charles Murry (Murry et al. 1986). Lamping et al studied the effects of Nicorandil on ischaemia reperfusion-induced myocardial infarct size in a canine model. They commented that animals infused with Nicorandil during the ischaemia and reperfusion period, had significantly lower infarct size compared to ischaemic controls. They also revealed that this beneficial effect was not related to any improvement in myocardial bloodflow (Lamping et al. 1984).

Following the discovery of IPC and the early success of pharmacomimetic triggers of IPC, Nicorandil has now emerged as an important new pharmacological preconditioning agent. It has been shown to have a cardioprotective effect in several species, such as dog (Mizumura et al. 1995; Geshi et al. 1999), rabbit (Ohno et al. 1997; Imagawa et al. 1998) and pig (Galie et al. 1995). In later experiments several of these investigators subsequently reported the involvement of K_{ATP} channels in the mediator mechanism of Nicorandil-induced IPC (Mizumura et al. 1996; Ohno et al. 1997; Imagawa et al. 1998). In in-vitro studies using isolated ventricular myocytes, Sato et al have in fact reported mK_{ATP} channel opening as the likely mediator of Nicorandil-

induced cardioprotection (Sato et al. 2000; Ishida et al. 2004). In a similar in-vitro model, Ishida et al (Ishida et al. 2004) demonstrated that Nicorandil attenuates mitochondrial Ca^{2+} overload via mK_{ATP} channel opening, which as we know, is a final event in ischaemia reperfusion-induced myocyte necrosis. Further therapeutic potential of Nicorandil was realised when Bolli's group demonstrated that Nicorandil could induce the second window of preconditioning against myocardial infarction when given 24 hours before the onset of sustained ischaemia (Tang et al. 2004).

1.7 Rationale

Elective autogenous muscle transfer is employed frequently in plastic and reconstructive surgery to reconstruct form and/or function. Since the 1970s, free tissue transfer has evolved and become one of the modern cornerstones of reconstructive surgery. Skeletal muscle flaps are useful as their bulk lends itself to reconstruction of large defects, as well as neurotisation of flaps to provide functional muscle reconstruction in cases such as facial palsy and Volkmann's contracture. With meticulous planning and technique, the obligatory ischaemic interval in free tissue transfer can be kept to a minimum and the overall failure rate has now improved in recent years to around 5% (Khouri et al. 1998). However, unforeseen complications can occur to prolong this ischaemic interval, such as vascular thrombosis. This is a more common complication of free tissue transfer with thrombosis rates of 8-10%, necessitating salvage surgery (Khouri et al. 1998; Selber et al. 2012). The vast majority of these complications occur in

the first 48 hours post operatively and this has been suggested as the 'golden period' for salvage of a free flap compromised by pedicle thrombosis.

In vascular, orthopaedic and plastic surgery, the use of pneumatic tourniquets is commonplace to provide a bloodless field during an operation. These tourniquets often remain inflated for a duration of 2-3 hours, seen as the acceptable upper limit of tourniquet time (Klenerman 1980), but resulting in prolonged warm ischaemia with accompanied metabolic changes in compartments of muscles in the lower / upper limb (Ostman et al. 2004). These changes occur due to the high metabolic demands of skeletal muscle, which is known to have a relatively short critical ischaemic period of 2.5 hours, compared to other tissues (Sjostrom et al. 1982).

Since the advent of free tissue transfer, plastic surgeons have been looking for ways to protect skeletal muscle and composite tissue flaps from ischaemia reperfusion injury. Except for the early restoration of blood-flow to the flap / muscle there has been little else available to be used as a direct therapeutic intervention. The significant breakthrough was the discovery of ischaemic preconditioning by Murry et al in 1986, whereby subjecting a tissue to brief sub-lethal cycles of ischaemia, results in robust infarct protection against a subsequent ischaemic insult (Murry et al. 1986). Following this discovery of an immediate adaptation in a tissue's ischaemic tolerance, Kuzuya (Kuzuya et al. 1993) revealed that the IPC phenomenon was biphasic, with an initial 'acute' preconditioning effect, waning after 4-6 hours, and returning at 12 – 24 hours following the preconditioning stimulus/treatment. The further discovery that this second window of protection lasts for up to 72 hours (Baxter et al. 1997; Moses

et al. 2005) makes late phase IPC more applicable for clinical practice, maintaining a continuous level of ischaemic tolerance within the tissue for at least 48 hours.

Acute / classical IPC, as described by Murry, requires a fairly invasive procedure to instigate the protection, involving repetitive clamping of coronary vessels or vascular pedicles. It would be difficult to convince a microsurgeon to perform repetitive clamping on the delicate vascular pedicles of free flaps! It was not until Przyklenk et al (Przyklenk et al. 1993) coined the term 'remote IPC', where a distant site to the preconditioned area is also protected, did a potential for practical clinical use become more accepted. This new finding was adapted by Addison et al (Addison et al. 2003), when remote IPC was induced using a hind-limb tourniquet in the porcine skeletal muscle flap model. Further to this, Moses et al combined these 2 evolutions in IPC, employing the hind-limb tourniquet technique to instigate late-phase infarct protection by remote IPC (Moses et al. 2005). This resulted in a robust form of infarct protection over a period of 48 hours, however, required preliminary animal anaesthesia to perform repetitive tourniquet inflations 24 hours before the flap surgery and subsequent ischaemic insult (Moses et al. 2005).

With the discovery of pharmacomimetic drugs such as Adenosine (Forrest et al. 1997) and Diazoxide (Takashi et al. 1999), the next logical step was to simulate the preconditioning effect without the need for invasive procedures such as vessel clamping or painful prolonged limb tourniquet inflation. Scientists have strived to find a suitable pharmacomimetic drug that will simulate the

preconditioning effect and provide a robust form of infarct protection over a prolonged period of time.

Nicorandil, a currently licensed anti-anginal medication, has been extensively studied in myocardial preconditioning models (Galie et al. 1995; Mizumura et al. 1996; Ohno et al. 1997; Imagawa et al. 1998; Geshi et al. 1999). The mechanism responsible for this pharmacological effect is via activation of ATP sensitive K^+ channels and Nicorandil has additionally been shown to produce a robust second window of infarct protection in a rabbit myocardial model (Tang et al. 2004). Incidentally, Moses et al demonstrated the central role of ATP sensitive K^+ channels in the mechanism of late-phase IPC of porcine skeletal muscle (Moses et al. 2005). As the efficacy and mechanism of acute and late-phase IPC translates from myocardial to skeletal muscle, it is feasible that Nicorandil would be able to induce a robust period of infarct protection in a skeletal muscle flap model. However, to date no studies have looked at Nicorandil-induced late phase preconditioning beyond 24 hours in cardiac or skeletal muscle.

1.8 Hypothesis

The well validated porcine latissimus dorsi skeletal muscle flap model will be used in order to examine the hypothesis that a single intravenous injection of Nicorandil will induce biphasic ischaemic preconditioning, with a second (late) phase that is robust enough to provide at least 48 hours continuous infarct protection of skeletal muscle from an ischaemic insult of 4 hours, followed by 48 hours of reperfusion.

It is believed that the mechanism of Nicorandil-induced preconditioning is triggered by sarcolemmal ATP-sensitive K^+ channels and the end-effector mechanism involves opening of mitochondrial ATP-sensitive K^+ channels. It is hypothesised that this infarct protection is associated with a reduction in mitochondrial calcium overload, a reduction in neutrophil accumulation and ultimately preservation of muscle ATP content during early reperfusion.

1.9 Specific Aims

1. To test the above hypotheses it is intended to use our lab's well-validated porcine LD muscle flap model. It is planned to validate the consistency of this model and to achieve a consistent reliable ischaemic control infarction rate with an ischaemic insult of 4 hours followed by 48 hours of reperfusion.

2. To test the hypothesis that a single i.v. dose of Nicorandil elicits late-phase IPC when administered 24 hours before the ischaemic insult. It is intended to demonstrate a linear dose-response relationship of Nicorandil with the % reduction in muscle infarction rate, compared to an ischaemic control.

3. To test the physiological response of the pig to an intravenous bolus injection of Nicorandil, with measurement of the mean arterial pressure.

4. To test the hypothesis that a single i.v. dose of Nicorandil elicits a biphasic IPC response, with an acute IPC phase and a late / delayed phase, which maintains infarct protection for a period of at least 48 hours in the porcine model.

5. To test the hypothesis that pharmacological preconditioning with Nicorandil involves prevention of mitochondrial free calcium ($m[Ca^{2+}]$) overload, attenuation of neutrophil accumulation and preservation of muscle ATP content.

6. To test the hypothesis that sarcolemmal ATP-sensitive K^+ channels (sK_{ATP}) have a role in the triggering mechanism, and mitochondrial ATP-sensitive K^+ channels (mK_{ATP}) have a role in the effector mechanism of Nicorandil-induced pharmacological preconditioning.

2.0 METHODS

2.1 Animal Model

2.1.1 Choice of Porcine Model

The pig as an animal model for muscle flap surgery is extremely useful as there is a close correlation between the musculoskeletal anatomy, origin/insertion, vascular territories and blood/nerve supply of porcine and human skeletal muscles (Patterson et al. 1967; Milton 1972; Meyer et al. 1978; Prather et al. 1979; Millican et al. 1985). Pigs have almost identical macroscopic tissue qualities of the skin, fascia and muscle, with the exception of the *panniculus carnosus*: a thin muscular layer adherent to the dermis along the flanks and neck of the animal that is useful for shivering and temperature control. Further justification of the porcine latissimus dorsi muscle flap model is discussed later in Chapter 4.

We used Yorkshire pigs for our experiments, as they were readily obtainable from a local farm in Ontario. Yorkshire piglets are born in litter sizes of 10-15 at around 1.5kg in weight and are weaned at around 35 days of age. We aimed to perform our muscle flap surgery on 18 – 20kg pigs, therefore giving an age range of 8 – 10 weeks, using standard growth rate charts (Bell 1964). The Yorkshire breed do not reach sexual maturity until 5 – 6 months of age and therefore the animals used in this experiment would be classed as juvenile / adolescent (Reiland 1978).

All animals were male and castrated to reduce animal aggression and hormonal influences, which may have interfered with results. All the animal surgical procedures were performed at the Research Institute of the Hospital for Sick Children in Toronto. The animal lab and staff have extensive training in dealing with pigs and have trusted animal suppliers providing healthy vaccinated animals. The facilities enabled initial apprentice-style training in the surgical and anaesthetic techniques required for our surgical protocol. An animal-handling course was also undertaken with the Research Institute prior to any surgical training. This enabled confident handling, without causing any undue stress to the animals.

2.1.2 Latissimus Dorsi (LD) Muscle Flap Model

The Latissimus Dorsi (LD) muscle flap model was chosen for this research project primarily due to its extensive validation from previous studies performed in our lab with the pig model (Mounsey et al. 1992; Pang et al. 1995; Hopper et al. 2000; Addison et al. 2003; Moses et al. 2005; McAllister et al. 2008). Other investigators have also reported the use of LD muscle / myocutaneous flaps to study critical ischaemia times in porcine skeletal muscle (Zelt et al. 1986; Picard-Ami et al. 1990; Thomson et al. 1991). The LD is a superficial muscle, lying just deep to the pannniculus carnosus with borders that are easily palpable *(Figure 2.1)*. The muscle can be easily raised and isolated on its principle thoracodorsal pedicle, which also lies superficial to the deep fascia (Getty 1975).

Using 18kg animals, one can harvest a reasonable size and thickness of muscle flap (13 x 8 cm) for simulating ischaemia/reperfusion injury as well as for multiple muscle biopsies. The muscles are readily accessible through bilateral flank incisions and, like human LD muscle, are not vital to general mobility and locomotion. The skin incisions are in such an area that they heal well without any undue tension or risk of infection.

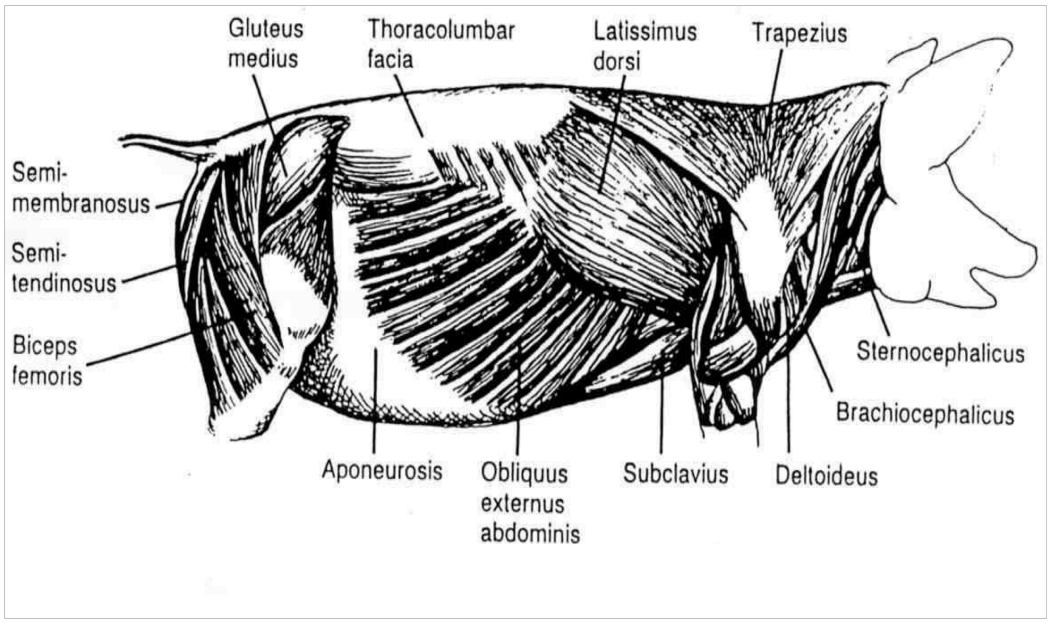

Figure 2.1. *The musculoskeletal anatomy of the pig is remarkably similar to that of the human. The Latissimus Dorsi (LD) muscle originates from the lower 4 ribs, inserting into the humerus. The principle vascular pedicle is the thoracodorsal vessels with secondary intercostal perforator supply. The muscle lies superficially on the flank deep to the pannniculus carnosus and is easy to palpate behind the forelimb shoulder (Getty 1975).*

2.2 Animal Management and Care

Pigs were ordered from a local supplier (M. Allin Farm) and were delivered to the animal care facility once per week. On arrival, the pigs were examined by the resident vetinarian and passed fit. Animals were housed in temperature-controlled (22^0C) and light-controlled (07:00 – 19:00) rooms with individual pens, for a period of at least 3 days prior to surgery to allow for acclimatisation and to alleviate stress from transportation. All pigs were offered the same commercial diet and tap water *ad libitum*; animals were fasted the night before surgery, but water was continued freely until the time of surgery. Animals were transported from their pens to the anaesthetic room in large mobile cages

The experimental surgery and animal management described in the following studies was approved by the Animal Care and User Committee of the Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada, and was in compliance with the guidelines of the Canadian Council of Animal Care (1980). Sacrificed animals were incinerated according to a protocol approved by the Animal Care and User Committee. The animal management and animal surgical procedures have been previously reported.

2.3 Animal Anaesthesia

2.3.1 Induction

On the day of surgery fasted animals were brought up to the induction room at 6:30 – 7:00 am. An intramuscular "premed" injection was given of Akmezine (1

ml contains: Ketamine Hydrochloride 58.82 mg, Acepromazine 1.18 mg, Atropine 0.09 mg; dose 1 ml/6 kg body weight) for animal sedation. The pig was then pre-oxygenated with O_2 and N_20 (1:1 volume). The posterior auricular surface was shaved and intravenous access was gained with a 20/22-gauge cannula. General anaesthesia was induced with intravenous pentobarbitone sodium (12-15 mg/kg; Ceva Sante Animal, Québec, Canada). After endotracheal intubation (6.0/6.5 Fr orotracheal tube) the pig was ventilated to a tidal volume of 15 ml/kg (Airshields 'Ventimeter' Ventilator, Pennsylvania, USA).

A preoperative dose of antibiotic prophylaxis was provided at this time with an intramuscular injection of 1ml Penicillin G (50,000 units/kg; Véloquinol, Lavaltrie, Quebec, Canada). The flanks were shaved and the animal washed with warm water and chlorhexidine soap before being transferred to the operating room.

2.3.2 Maintenance

Following arrival in the operating room, the animal was reconnected to the ventilator and ventilation pressure set to a 25cm water pressure (tidal volume of 15ml/kg). Ventilation rate was set to 14 breaths/min; however, rate was adjusted depending on the end-tidal CO_2 measurement. Fluid balance and anaesthesia was maintained by an intravenous infusion (Harvard Syringe Infusion Pump, Model 2720, Bard Medical System Division, Massachusetts, USA) of normal saline containing pentobarbitone sodium (0.5 – 1.0 mg/kg/hr).

Pentobarbitone sodium was selected for maintenance anaesthesia due to its common use in large animal surgery as well as our laboratory's extensive experience using the drug. Pentobarbitone is a short-acting barbiturate that produces steady-state anaesthesia, with minimal haemodynamic fluctuation. Traditional volatile anaesthesia was not used due to several reports that volatile anaesthetic agents mimic or enhance ischaemic preconditioning. Isoflurane, enflurane, halothane, sevoflurane and desflurane have all been implicated in various animal models, through their activation of ATP-sensitive potassium channels (K_{ATP}) (Zaugg et al. 2003). Perioperative opioid use was also restricted due to its indirect activation of Protein Kinase C, a known mediator in ischaemic preconditioning (Schultz et al. 1996; Zaugg et al. 2003).

Heart rate, oxygen saturation, respiratory rate, end-tidal CO_2 and rectal temperature were all monitored during surgery (Surgivet Monitor, Model V9212AR, Smiths Medical, Wisconsin, USA). Rectal temperature was kept within the normal range for the pig (38 – 39^0C). The animal was kept warm by ensuring the operating room was at a constant 22^0C, as well as by using a heated blanket (Aquamatic K Thermia Heating Blanket, Cincinnati, Ohio, USA) and infrared heat lamp.

2.3.3 Recovery

During the final hour of the ischaemic insult the animal was weaned from the pentobarbitone infusion by reducing the infusion dose by 50% and then stopping the infusion following closure of skin wounds. This facilitated an earlier recovery of the animal following the surgery. Following completion of surgery,

inhaled N_2O was stopped and the ventilatory rate reduced to allow end-tidal CO_2 to rise and stimulate spontaneous respiration. Mucous secretions were cleared from the oropharynx by suction catheter as required and the animal was extubated once appropriate oro-pharyngeal reflexes were present, the i.v. cannula was removed at this stage. The animal remained in the operating room until sufficient oxygen saturation was maintained on room air, and then was returned to a warm pen in the animal holding room with food and water. Animals were reviewed bi-daily and given analgesia as required.

2.3.4 Sacrifice

Following the completion of the surgical protocol and harvesting of bilateral LD muscle flaps under general anaesthesia, animals were euthanised with an overdose of sodium pentobarbitol (10ml (2.4g) Euthanyl; Bimeda Animal Health Ltd., Cambridge, ON, Canada). Animals were bagged and placed in a refrigerated locker until collection for incineration.

2.4 Surgical Protocol

2.4.1 Raising of LD Muscle Flaps

The animal was placed in the left or right lateral decubitus position and one of the flanks prepped with Betadine solution (10% povidone-iodine) and draped with sterile cloth drapes followed by skin markings for the incision. The skin incision was designed using the forelimb shoulder and scapular tips as markers

and a 15cm long trap-door incision drawn to ensure a 13x8cm flap could be raised under direct vision *(Figure 2.2)*. Skin incisions were made on each flank using a scapula and the trap-door skin flap was raised with the pannniculus carnosus *en-bloc,* exposing the LD muscle *(Figure 2.3)*. A 13x8cm flap was marked on the muscle, measuring 1cm from the ventral edge of the muscle. The LD muscle was then raised using knife and bipolar diathermy (Pfizer Valleylab Force 2 Electrosurgical Generator, New York, USA) from distal to proximal, with preservation of the thoracodorsal pedicle on the underside of the flap *(Figure 2.4)*.

Dissection was performed under loupe vision into the axilla and then the flap was denervated by dissection of the thoracodorsal nerve from the pedicle and excising a section of it *(Figure 2.5)*. This denervation was performed to prevent painful muscle contractions during the 48-hour recovery period as well as acting as a surgical sympathectomy to reduced any neural influences on the flap. To support the flap and protect the thoracodorsal pedicle, the musculotendinous insertion was maintained and was ligated with a 1-0 silk tie to ensure the only blood supply to the flap was via the thoracodorsal pedicle. The muscle flap was then sutured back in position with 3-0 vicryl and the skin closed with 3-0 nylon, leaving an axillary opening for access to the pedicle.

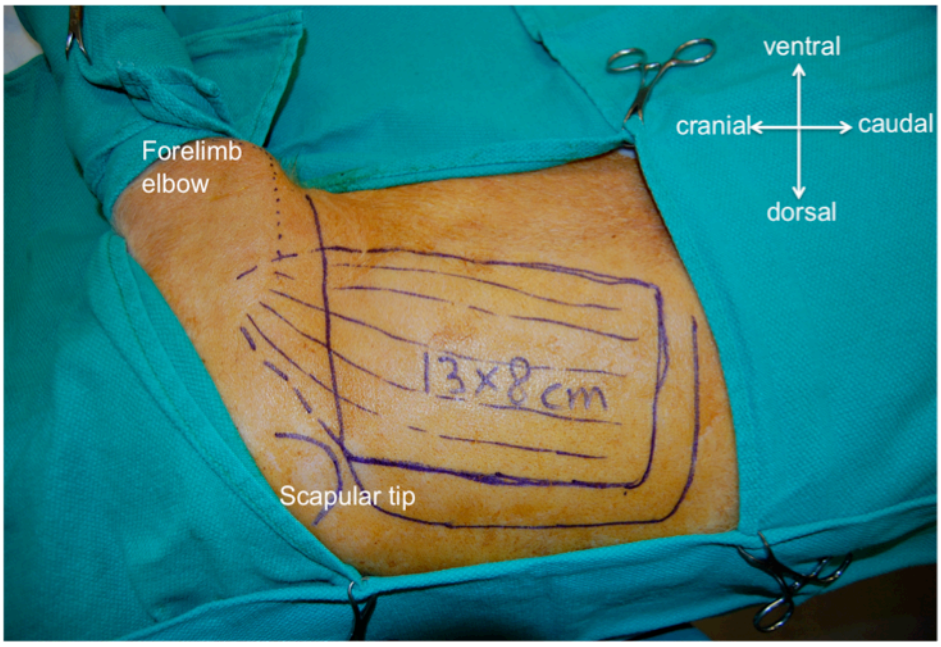

Figure 2.2. *The animal is draped with sterile green towels and the skin is prepped with 10% Betadine solution. The LD muscle is palpated and skin incision marked. Identical skin exposure was performed in every animal in the study.*

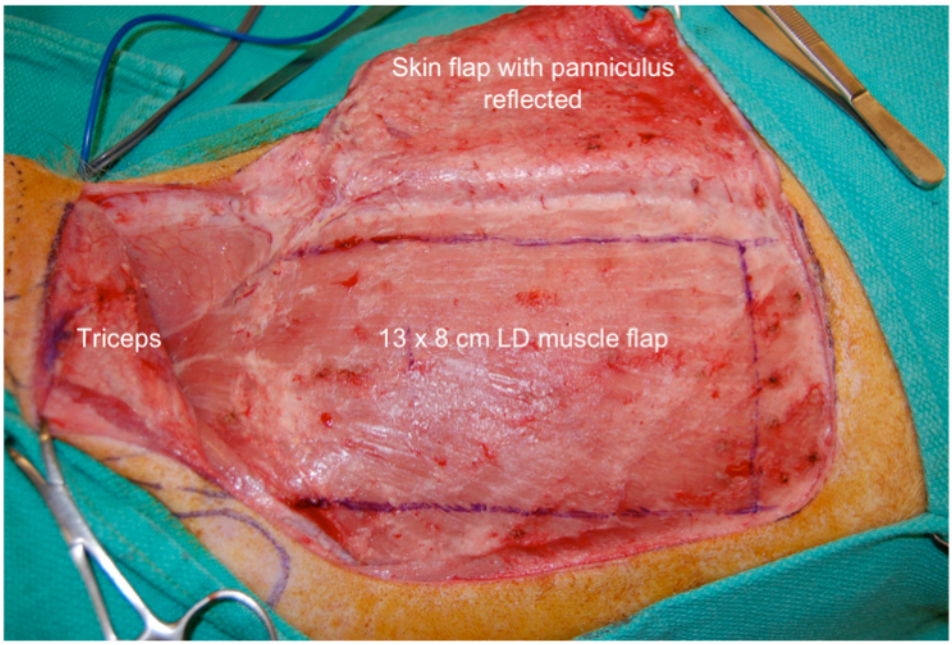

Figure 2.3. *Once the skin and pannniculus layer is reflected the dimensions of the 13 x 8cm LD muscle flap are marked and the flap raised with sharp dissection.*

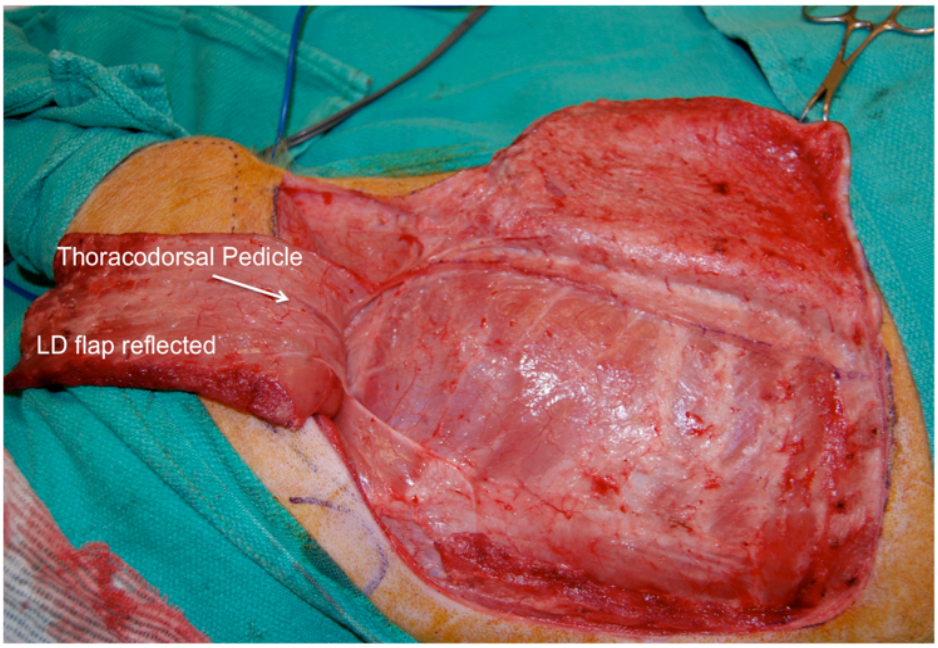

Figure 2.4. *The flap is mobilised onto the osseo-tendinous humeral insertion, exposing the thoracodorsal pedicle on the ventral surface of the muscle. Note the thickness of the LD flap in this picture (approx. 1cm thick).*

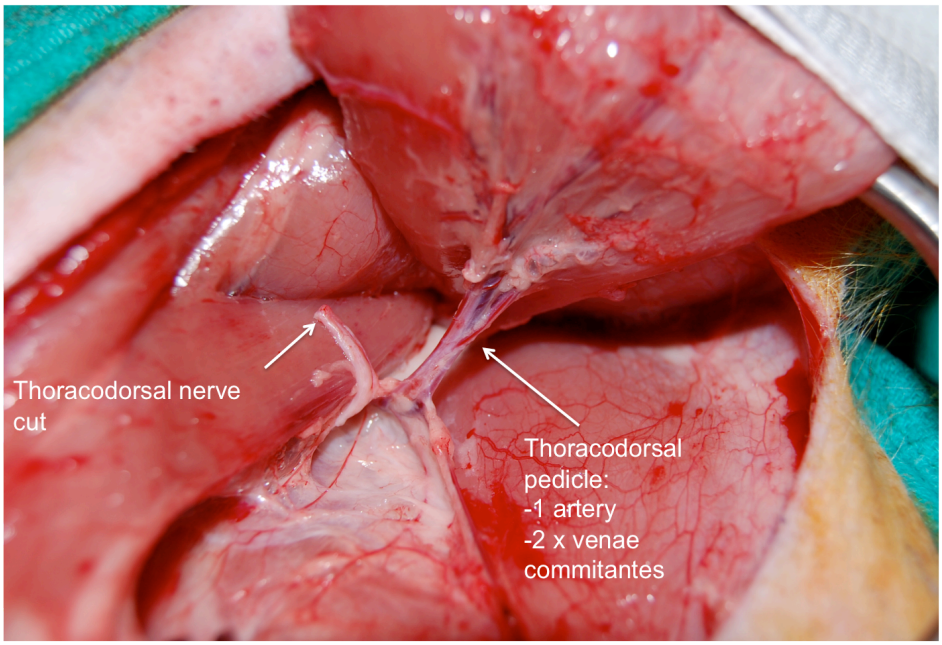

Figure 2.5. *The thoracodorsal pedicle is skeletised to allow for robust clamping of the artery and venae commitantes. The thoracodorsal nerve is divided to denervate the flap (sympathetic denervation). This also reduces painful contractions of the muscle for the animal postoperatively and mirrors the procedure of autogenous transfer in humans.*

2.4.2 Global Ischaemia of LD Muscle Flaps

Once bilateral LD flaps have been raised and sutured back in position, the pig was positioned prone on the operating table and the muscle flaps allowed to equilibrate for 15 minutes. Following this, two microvascular arterial clamps (2 x 8 mm; Weck) were applied to the pedicle to occlude circulation and render the flap globally ischaemic *(Figure 2.6)*. The animal was then placed prone again for the duration of the standard 4-hour ischaemic insult. Intravenous injection of Fluorescine dye (15 mg/kg; Sigma Chemical Co., Oakville, ON, Canada) was performed following placement of the vascular clamps. Global flap ischaemia was confirmed bilaterally by observation of a lack of green fluorescence *(Figure 2.7)* in the LD flaps under an ultraviolet Wood's lamp (Mineralight lamp, Model UVG – 11, Ultraviolet Products Inc., San Gabriel, CA, USA). Operating room temperature (22^0C) and rectal temperature (38 - 39^0C) were monitored during the 4-hour ischaemic insult.

Reperfusion of the LD muscles, after removal of vascular clamps, was confirmed by the hyperaemic response of the reperfused muscle flap and/or a second intravenous injection of Fluorescine and swift return of yellow fluorescence to the LD flaps. The remaining axillary skin incisions were then closed with 3-0 nylon and the pig allowed to recover from anaesthesia.

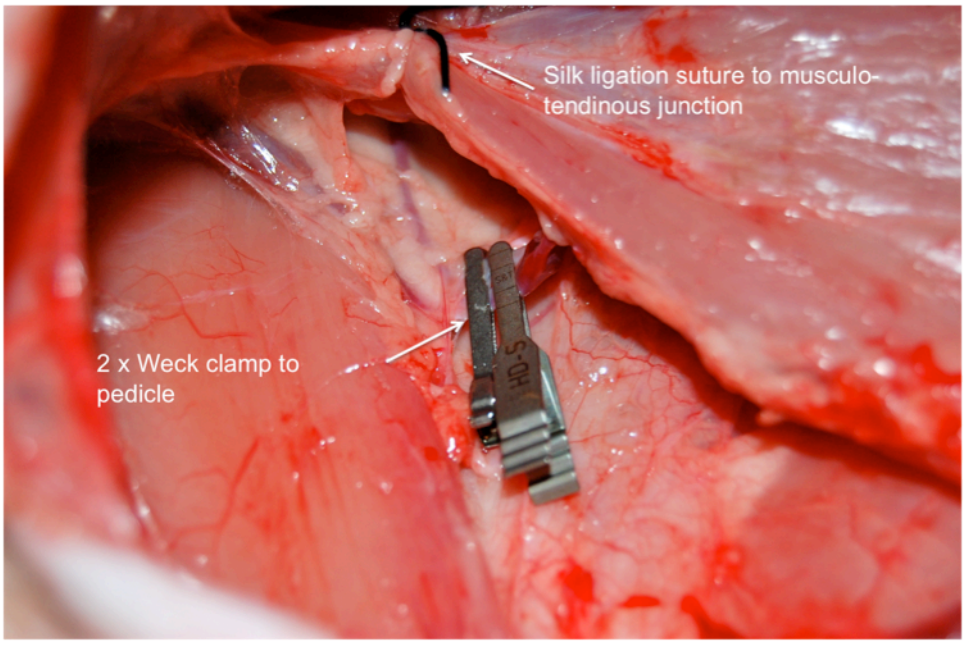

Figure 2.6. *Two 8mm Weck clamps were used to ensure cessation of blood-flow across the pedicle. A 1-0 silk ligation suture was tied around the musculo-tendinous insertion to insure that there was no collateral blood flow into the muscle. Clamps were left in place for a period of 4 hours to simulate a global ischaemic insult of the LD muscle flap.*

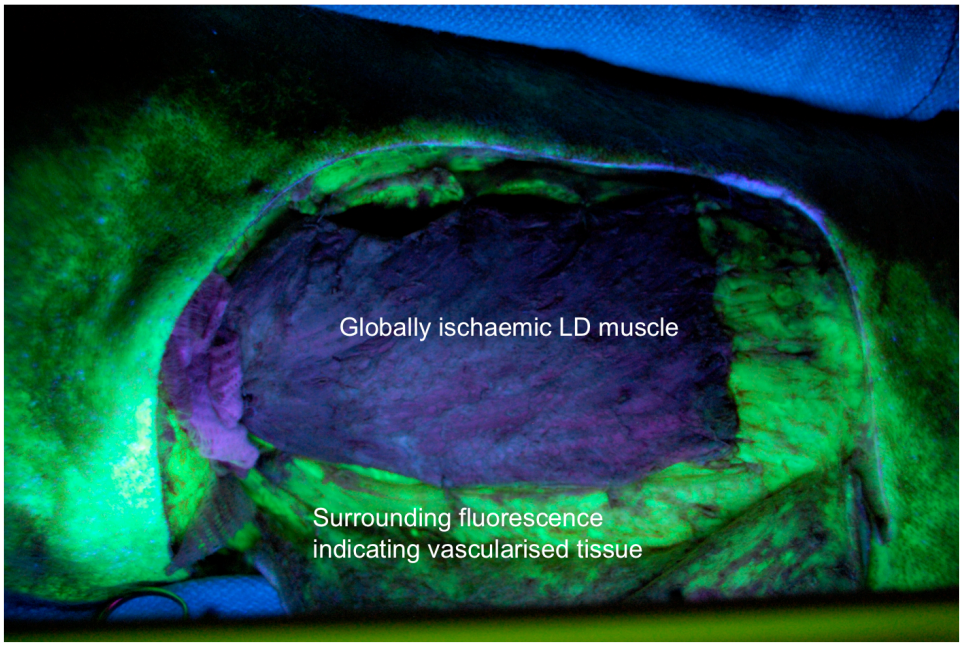

Figure 2.7. *Intravenous injection of Fluorescine dye (15 mg/kg) was performed following placement of the clamps. The bilaterally clamped LD flaps were then inspected under an ultraviolet Wood's lamp. Absence of fluorescence in the flap indicated global ischaemia.*

The ischaemic insult, induced by the temporary clamping of the thoracodorsal pedicle, was for a period of 4 hours. Previous work in this laboratory by Morris et al has looked at incremental time periods of ischaemic insult by this method of pedicle clamping in the porcine LD flap model (Morris et al. 1993). They found that when pedicles are clamped for greater than 4 hours there is risk of irreversible endothelial damage from clamp pressure resulting in thrombosis and immediate necrosis of the flap. Previous experiments using a 4-hour ischaemic insult have resulted in muscle infarction control rates of 40-50% (Pang et al. 1995; Hopper et al. 2000; Addison et al. 2003). In order to standardise our results in line with previous research on this model, we chose an ischaemic interval of 4 hours.

2.4.3 Nicorandil Injection

Nicorandil (Chugai Pharmaceutical, Tokyo, Japan) was stored in powder form and dissolved in normal saline prior to injection. The animal was brought to the anaesthetic room and given an intramuscular dose of Akmezine 1ml / 6kg animal weight (Ketamine, Acepromazine, Atropine) as described earlier. I.v. cannulation of an ear vein was performed with the animal under sedation and masked with O_2 and N_20 (1:1 volume). Intravenous injection of Nicorandil was carried out via injection of the dissolved drug into a 125ml bag of normal saline (100 μg/kg bolus injection, followed by 30 $\mu g.kg^{-1}.min^{-1}$). The bag was given over a period of 60 min resulting in a total of 3 mg/kg infused. The animal's heart rate and O_2 saturation was monitored throughout the infusion.

2.4.4 Measurement of Arterial Blood Pressure

Following the harvesting of LD muscle flaps and prior to animal euthanisation, invasive measurement of arterial blood pressure was performed over a 90 min period. The animal was placed in the supine position and the right groin explored. The right femoral artery was exposed by surgical dissection and cannulated with a polyethylene cannula (Becton Dickinson, Franklin Lakes, NJ, USA) under direct vision. A pressure transducer was connected and calibrated to give a Mean Arterial Pressure (MAP) trace on the monitor (Hewlett Packard 78534A, Andover, USA) and allowed to equilibrate for 5 min. Nicorandil was then given as before by i.v. injection via ear vein cannulation over a period of 60 minutes. The MAP was recorded at 10-minute intervals throughout this period, as well as for 30 minutes after the infusion. The animal was maintained under general anaesthesia as before on an infusion of normal saline (2ml/min) containing pentobarbitone sodium (0.5 – 1.0 ml/kg/min).

2.4.5 Assessment of LD Muscle Viability

Following 48 hours of reperfusion, animals were returned to the operating room and anaesthetised and intubated as before. Bilateral flank incisions were reopened and LD flaps harvested. The LD muscle flaps were then sectioned into thirteen 1x8 cm strips. These strips were then incubated in Nitroblue tetrazolium (Sigma Chemical Co., Oakville, ON, Canada) dye for 30 minutes at 22^0C. Nitroblue tetrazolium (NBT) is made up using 200 mg of tetrazolium in 400 ml of 0.2% Tris buffer at pH 7.4 and temperature 22 ˚C to give a final concentration

of 0.5 mg/ml. Tissue hydrogenases in viable muscle reduce NBT salts to an intense blue formazine stain. Therefore viable muscle in each strip stained blue while the necrotic areas remained pink in colour *(Figure 2.8)* (Morris et al. 1993).

The muscle strips were then removed from the NBT solution, blotted and laid out in cross-section on white card and a digital photograph taken (Sony Cybershot DSC-P9). All pictures were taken with the same camera under the same lighting conditions. Computer planimetry with digital imaging software (Adobe Photoshop CS) was used to map out the necrotic and viable areas and therefore calculate a percentage muscle infarction for each respective muscle flap. This method has been well established in our lab, (Addison et al. 2003; Moses et al. 2005; McAllister et al. 2008) and other labs (Labbe et al. 1988; Galie et al. 1995), and has been found to correlate closely with the previously published template method (Morris et al. 1993). Other studies have looked at the accuracy of NBT reduction in the assessment of skeletal muscle viability. Labbe et al (Labbe et al. 1988) demonstrated accuracy of NBT reduction to assess the necrosis rate of bilateral canine gracilis skeletal muscle flaps after 48 hours reperfusion. Electron microscopy was employed to confirm the validity of this method and the same method of computer planimetry was used to estimate the percentage necrosis.

Previous work in our lab (Pang et al. 1995) has demonstrated that 8x13 cm LD skeletal muscle flaps exhibit no necrosis when they were incubated in NBT,

prior to any ischaemic insult. Therefore a non-ischaemic control group was not planned for this thesis project to avoid unnecessary death of animals.

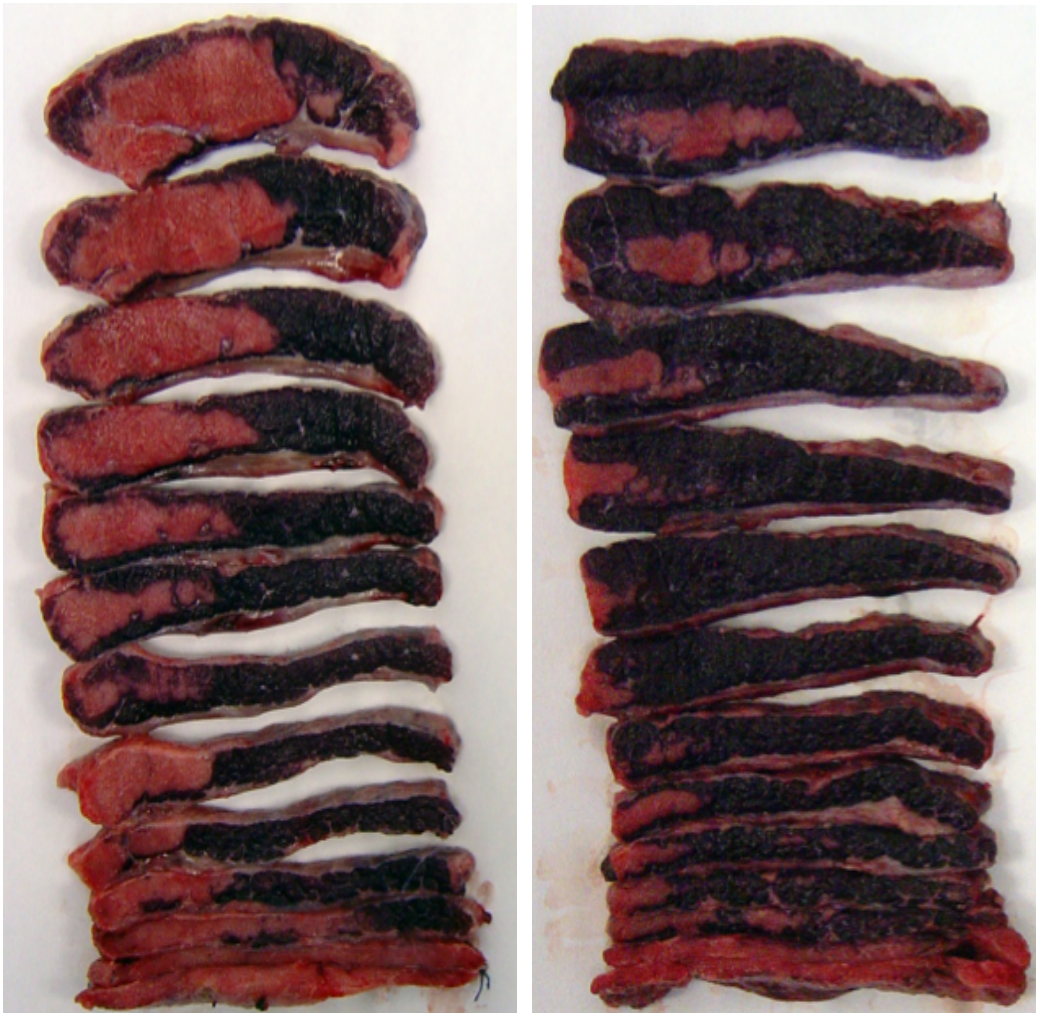

Figure 2.8. *Demonstration of the pattern of skeletal muscle infarction in the porcine latissimus dorsi (LD) muscle flaps subjected to 4hr ischaemia and 48hr reperfusion. Each muscle flap is cut transversely into thirteen 8 x 1cm segments, which were incubated in 0.5 mg/ml nitroblue tetrozolium dye solution at 22^0C for 30 mins. The viable areas are stained dark blue, and the non-viable (necrotic) areas remain red in colour. The flap on the left is from an ischaemic control animal and the flap on the right is from a Nicorandil pre-treated animal.*

2.5 Laboratory Analyses

2.5.1 Collection & Preparation of Muscle Biopsies

For the m[Ca^{2+}], ATP and MPO assays, a series of muscle biopsies were taken (1 x 1cm) with a scalpel, sequentially from the dorsal (thick) edge of each LD muscle, starting 1cm proximal to the free distal edge of the flap, and moving proximally, (i.e. taken at 7, 6, 5 and 4cm from the proximal flap edge. The decision to take biopsies from the thicker dorsal edge of the muscle flap is due to the fact that skeletal muscle in this area is subjected to maximal reperfusion induced muscle necrosis (see *Figure 2.8*). Biopsies were taken immediately before; at 2h and 4h ischaemia; and then finally at 1h reperfusion. Each muscle biopsy specimen was rinsed with cold (4^0C) isotonic saline. Part of the biopsy was then frozen in liquid nitrogen and stored at -80 ^{0}C in order to carry out ATP and MPO assays at a later stage. The remaining fresh tissue was processed immediately to assay the mitochondrial free Calcium content (m[Ca^{2+}]).

2.5.2 Measurement of Mitochondrial Free Calcium Content (m[Ca^{2+}])

The method of isolating mitochondria from fresh tissue by differential centrifugation, was adapted from that published by Rousou and colleagues (Rousou et al. 2004). Each muscle biopsy was cut into small pieces with a scalpel, washed with cold (4 ˚C) isotonic saline and placed in 7 ml of cold Buffer A (4 ˚C), containing 300 mM sucrose, 10 mM K^+-Hepes, (pH 7.2) and 1 mM K^+-

EGTA (pH 8.0). The tissue was then homogenised (Polytronic PT-10-35, Kinematic, ON, Canada) for 40 sec at 4 ˚C. Nagarse (0.2 mg/ml homogenate; Sigma Chemical Co., Oakville, ON, Canada) was added to the homogenate. This was then incubated on ice for 10 min, and subsequently centrifuged at 750 *g* for 6 min. The supernatant was saved, bovine serum albumin (BSA) was added (1 mg/ml), and this was re-centrifuged at 750 *g* at 4 ˚C for 6 min. The pellet was discarded, and the resulting supernatant was centrifuged at 9000 *g* at 4 ˚C for 10 min at ˚C. The mitochondrial pellet was saved and resuspended in 7 ml cold Buffer A (4 ˚C) containing 1 mg/ml BSA, then centrifuged twice at 9000 *g* at 4 ˚C for 10 min. The final pellet was suspended in 0.2 ml of respiration medium, containing 250 mM sucrose, 2 mM KH_2PO_4, 10 mM $MgCl_2$, 20 mM K^+-Hepes (pH 7.2) and 0.5 mM EGTA (pH 8.0) at 4 ˚C.

The suspension of mitochondrial pellet was used for assay of free (m[Ca^{2+}]), which was determined using a spectrofluoresence technique. The 0.2 ml of pellet suspension was added to 1.8 ml of loading buffer containing 140 mM NaCl, 5 mM KCl, 5.6 mM glucose and 5 mM HEPES (pH 7.4), and then loaded with 1 μM Fura-2-AM (Molecular Probes, Eugene, OR, USA) in DMSO (final concentration 0.1%) and 0.006% Pluronic F-127 (Molecular Probes, Eugene, OR, USA) and incubated in a shaking bath for 15 min at 30 ˚C in the dark. This was then centrifuged at 9000 *g* for 10 min at 30 ˚C to pellet the mitochondria, and the mitochondrial pellet was re-suspended in 0.2 ml loading buffer and added to a black 96-well microplate (BD Biosciences, Mississauga, Ontario, Canada). Sample blanks received 0.1% (final concentration) DMSO as a vehicle only. This was read in a microplate reader (Spectra Max Gemini EM, Molecular Devices Corporation, Downington, PA, USA) at 340 and 380 nm (excitation)

and 510 nm (emission). Calibration was performed using a calcium calibration kit and Fura-2 penta-potassium salt (Molecular Probes, Eugene, OR, USA). The amount of mitochondrial free Ca^{2+} was determined from the ratio of emission induced by the two excitation wavelengths.

Mitochondrial protein content was determined by the Bradford method (Bio-Rad Life Sciences, Hercules, CA, USA). The Bradford method for protein determination utilises a dye (Coumassie® Brilliant Blue G-250), which, under acidic conditions, exists predominantly in the red cationic form. When the dye binds to protein, it is converted to a stable blue form, with a shift in absorbance maximum from 465 nm to 595 nm.

The mitochondrial protein for assay was added to a 96-well microplate (BD Biosciences, Mississauga, Ontario, Canada). Sample blanks received 10 mg/ml (final concentration) bovine serum albumin as a vehicle only. This was read in a microplate reader (Spectra Max Gemini EM, Molecular Devices Corporation, Downington, PA, USA) at 595 nm (absorbance). Calibration was performed using bovine serum albumin standards (Bio-Rad Life Sciences, Hercules, CA, USA). The free m[Ca^{2+}] was expressed as nanomol/mg mitochondrial protein.

2.5.3 Measurement of Adenosine Triphosphate (ATP) Content

The muscle biopsies (~200 mg), previously stored at -80^{0}C, were homogenised in 2 ml of cold (4 ˚C) tricholoroacetic acid (2.5 % vol/vol) and the homogenate was centrifuged for 10 min at 1000 *g* at 4 ˚C. The supernatant was neutralised

with 1 M Tris base (120 μl/ml supernatant), and was then used for assay of ATP content using a FL-AA bioluminescent assay kit (Sigma, Oakville, ON, Canada).

The kit can be used for the quantitative analysis of ATP within the range 2 x 10^{-12} to 8 x 10^{-5} ml/litre. The following reactions occur:

$$\textbf{ATP + Luciferin} \xrightarrow{\text{Firefy Luciferase}} \textbf{Adenyl-luciferin + Ppi}$$

$$\textbf{Adenyl-luciferin + } O_2 \longrightarrow \textbf{Oxyluciferin + AMP + } CO_2 \textbf{ + Light}$$

The light emission from this reaction is determined using a luminometer and the ATP concentration is measured from standard curves prepared from solutions of known ATP concentration provided with the assay kit. The pellet was neutralised with 1 ml of 0.5 M NaOH and the protein content was determined by the Bradford method (Bio-Rad Life Sciences, Hercules, CA, USA), as described before. The muscle content of ATP was expressed as micromoles per gram of protein (McAllister et al. 2008).

2.5.4 Measurement of Myeloperoxidase (MPO) Activity

Myeloperoxidase is a lysosomal proteinase, prevalent in neutrophil granulocytes. It has been well validated as a marker of neutrophil accumulation in both

myocardial (Jordan et al. 1999; Wang et al. 1999) and skeletal muscle (Pang et al. 1997; Hopper et al. 2000).

Frozen muscle samples (~200 mg) were homogenised (Polytronic PT-10-35, Kinematic, ON, Canada) in 2 ml of cold Solution A (4 ˚C), containing 100 mM NaCl, 20 mM NaH_2PO_4 and 15 mM EDTA (pH 4.7) for 30 sec at 4 ˚C. This was then centrifuged at 12000 *g* for 20 min at 4 ˚C. The supernatant was discarded, and the pellet homogenised for 45 sec in 2 ml of ice-cold solution B, containing 50 mM KH_2PO_4 and 0.5% (by volume) cTAB (hexadecyltrimethylammonium bromide), at pH 5.4. The homogenate was sonicated for 10 sec on ice, and then underwent three freeze-thaw cycles in liquid nitrogen, before being centrifuged at 12 000 *g* for 20 min at ˚C. The resulting supernatant was collected and assayed for neutrophilic MPO activity using a spectrophotometric technique previously described by us (Pang et al. 1995; Pang et al. 1997; Pang et al. 1997; Hopper et al. 2000; Moses et al. 2005).

The enzyme activity was evaluated by measuring the H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB). A spectrophotometer (Beckman DU50, Beckman Coulter Canada Inc., Mississauga, ON, Canada) was used to measure the generation of oxidised TMB at 655 nm and 37 ˚C. The reaction mixture contained 10 μl of supernatant, 1.6 mM TMB, 0.3 mM H_2O_2, 80 mM sodium phosphate buffer (pH 5.4), 8% N,N-dimethyl formanide, and 40% Dulbecco's phosphate-buffer saline in a total volume of 550 μl. MPO activity was expressed as initial velocity of absorbance increase at 655 nm per minute per gram wet tissue. One unit of enzyme activity was defined as the amount of MPO that produced an absorbance change of 1.0 optical density unit·min^{-1}·g wet $muscle^{-1}$ at 37 ˚C.

2.5.5 Biochemicals

All chemical reagents and assay kits were purchased from Sigma Chemical Co. Ltd. (Oakville, ON, Canada), unless otherwise stated. Purified water from a Milli-Q Water System (Bedford, MA, USA) was used to make all solutions and standards. Nicorandil (N-(2-hydroxyethyl)-nicotinamide nitrate) was a gift from Chugai Pharmaceutical, Tokyo, Japan. 1-[[5-[2-(5-Chloro-0-anisamido)ethyl]-2-methoxyphenyl]sulphonyl]-3-methyl-thiourea sodium salt (HMR-1098, Aventis Pharma) was dissolved in 10 ml of saline (0.9%). 5-Hydroxydecanoate sodium (5-HD; 10mg/kg) was dissolved in 10 ml of saline (0.9%). We chose a dose of 6 mg/kg for HMR-1098 and 10 mg/kg for 5-HD, following the published information by Moses et al (Moses et al. 2005). All drug solutions were prepared not more than 30 min before intravenous injection.

2.6 Experimental Protocol

2.6.1 Study 1: To investigate the dose-response effect of Nicorandil-induced late phase IPC when given 24 hours before instigation of I/R injury in porcine LD muscle flaps.

Incremental concentrations of Nicorandil (0.1, 0.3, 1, 1.5, 2 and 3 mg/kg) were given intravenously 24 hours before the onset of 4 hours muscle ischaemia, followed by 48 hours reperfusion. After 48 hours reperfusion, muscle infarction was assessed by nitroblue tetrazolium reduction technique, as described earlier.

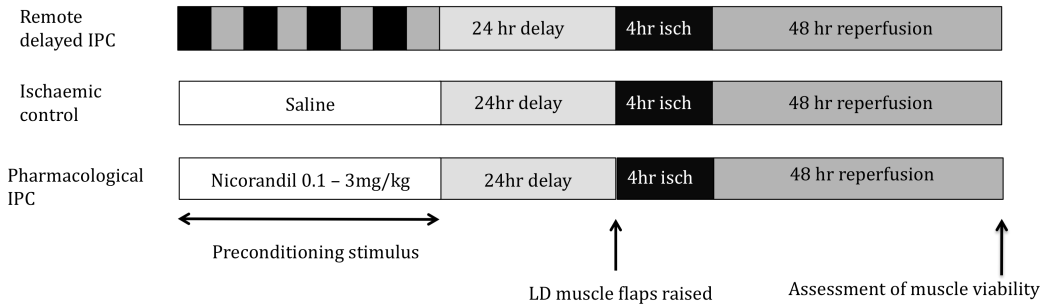

Figure 2.9. *Investigating the dose-response effect of Nicorandil (Study 1). Pigs received a saline infusion (ischaemic control) or saline containing 0.1 – 3 mg/kg Nicorandil (treatment group) over a 60-minute period, 24 hours prior to muscle flaps subjected to 4 hours sustained ischaemia and 48 hours reperfusion. The illustration of remote delayed IPC is for comparative purposes only.*

2.6.2 Study 2: To investigate the effect of intravenous Nicorandil on mean arterial blood pressure (MAP) in the porcine model.

The aim of this experiment was to investigate the effects of intravenous Nicorandil on the pig's MAP while under anaesthesia. From the results of Study 1, an optimal dose of Nicorandil (3 mg/kg) was chosen. While under anaesthesia, animals had their right femoral artery cannulated and MAP monitored for a 90 min period following the injection of saline alone or saline containing Nicorandil (3 mg/kg) over a period of 60 min.

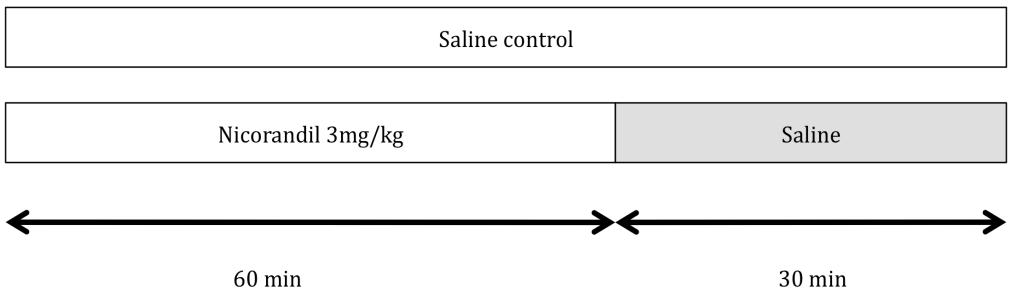

Figure 2.10. *The effect of intravenous Nicorandil pretreatment on mean arterial pressure (MAP). MAP was measured via cannulation of the right femoral artery under general anaesthesia, for a 90-min period. Saline or saline containing 3 mg/kg Nicorandil was infused over 60 min followed by 30 min saline infusion. MAP (mmHg) was recorded in 10-min intervals.*

2.6.3 Study 3: To investigate the efficacy of Nicorandil in induction of late IPC for uninterrupted perioperative protection of skeletal muscle against I/R injury in porcine LD muscle flaps.

This study was designed to investigate the efficacy of Nicorandil in inducing late IPC, when given at a series of time points before the onset of 4 hours ischaemia, followed by 48 hours reperfusion. Intravenous Nicorandil was prepared as described earlier and given to the animal under sedation, over a period of 60 min. Incremental time periods between Nicorandil injection and the onset of ischaemic insult were as follows: 0, 4, 8, 12, 24, 48, 72 and 96 hr. Following 4 hr ischaemia and 48 hr reperfusion, LD muscle infarction was assessed as before.

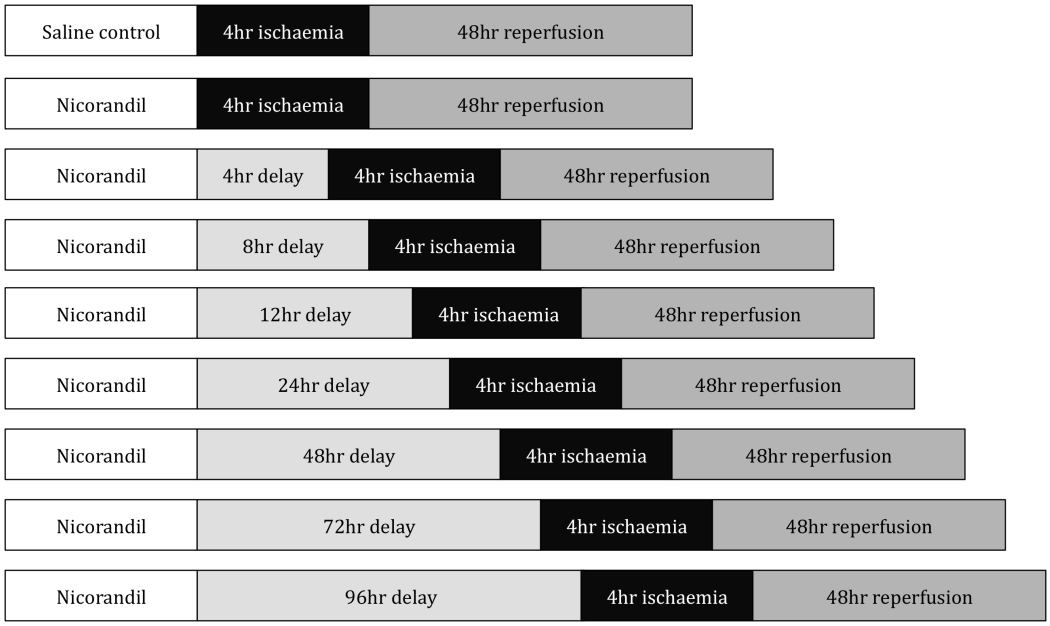

Figure 2.11. *The efficacy and time-course of Nicorandil-induced preconditioning. Nicorandil (3 mg/kg) was administered intravenously 0, 4, 8, 12, 24, 48, 72 and 96 hours before LD muscle flaps were subjected to 4 hours sustained ischaemia and 48 hours reperfusion. All muscle flaps were then harvested and assessed for viability.*

2.6.4 Study 4: To investigate the effect of Nicorandil-induced late IPC on muscle content of m[Ca^{2+}], ATP and MPO activity in porcine LD muscle flaps subjected to 4 hours ischaemia and 48 hours reperfusion.

In this study, animals were assigned to a control or treatment group. The control group received intravenous injection of saline and the treatment group, injection of Nicorandil (3mg/kg), 24 hr before the onset of 4 hr sustained ischaemia. Muscle biopsies (1x1 cm) were taken from both LD muscle flaps immediately

before sustained ischaemia; at 2 hr and 4 hr ischaemia; and at 1 hr reperfusion. Fresh muscle biopsies were processed for assay of m[Ca^{2+}]. Remaining tissue was frozen at -80˚C for assay of ATP content and MPO activity.

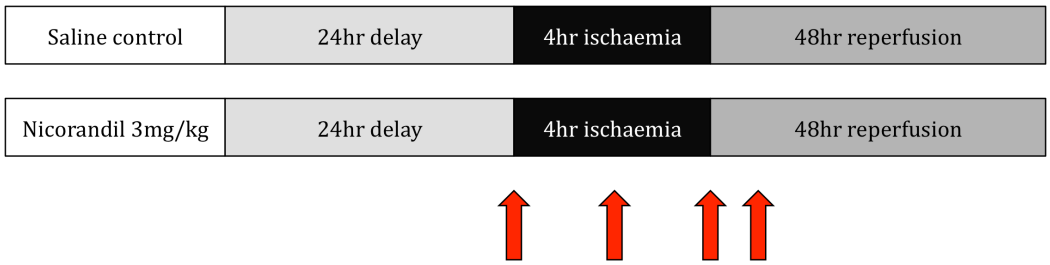

Figure 2.12. *Animals were assigned to saline ischaemic control or Nicorandil treatment group. After a 24-hour delay LD flaps were raised and subjected to 4 hours ischaemia and 48 hours reperfusion. Skeletal muscle biopsies were taken at 0hr, 2hr and 4hr of ischaemia; and at 1hr reperfusion (⇧). Biopsies were assayed for m[Ca^{2+}], ATP content and MPO activity.*

2.6.5 Study 5: To investigate the role of sarcolemmal K^+ (sK_{ATP}) channels in the trigger mechanism of Nicorandil-induced late IPC.

Study 5 was designed to demonstrate the involvement of sK_{ATP} channels in the trigger mechanism of Nicorandil-induced late IPC. Animals were assigned to an ischaemic control and seven treatment groups: 1) Saline; 2) Nicorandil; 3) HMR-1098 + Nicorandil; 4) 5-HD + Nicorandil; 5) Glibenclamide + Nicorandil; 6) HMR-1098 + Saline; 7) 5-HD + Saline and 8) Glibenclamide + Saline.

The sK_{ATP} channel inhibitor HMR-1098, the mK_{ATP} channel inhibitor 5-HD or the non-specific K_{ATP} inhibitor was injected intravenously 10min before administration of saline or saline containing Nicorandil (3 mg/kg), 24hr prior to subjecting LD muscle flaps to 4hr ischaemia and 48hr reperfusion.

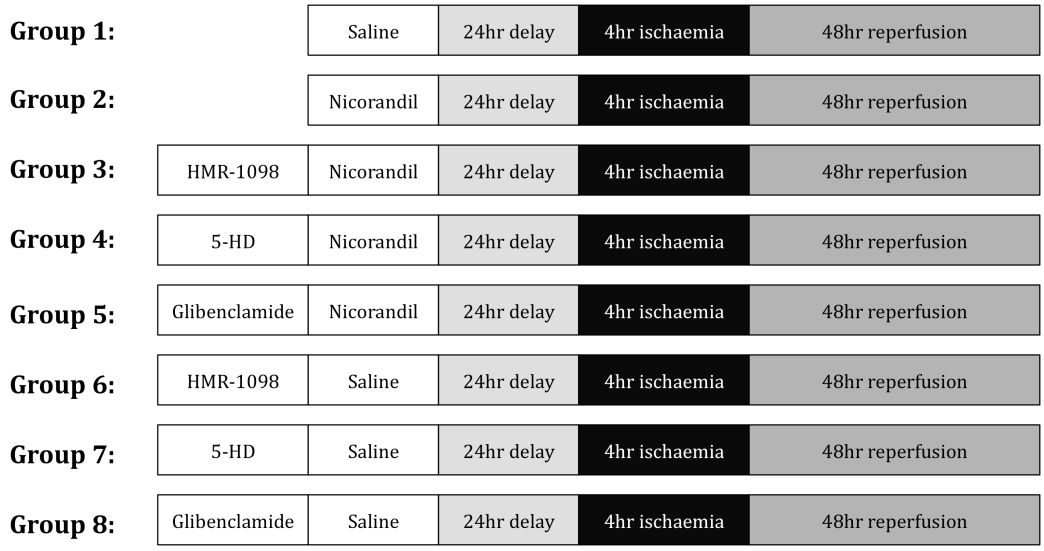

Figure 2.13. *Protocol for investigation of the trigger mechanism in Nicorandil-induced late protection of skeletal muscle against ischaemia / reperfusion injury. There were 4-5 pigs with bilateral LD muscle flaps in each group. All the LD muscle flaps in the control and treatment groups were subjected to 4h ischaemia and 48h reperfusion. Intravenous administration of saline or saline containing Nicorandil (3 mg/kg) was performed 24h before 4h ischaemia (i.e. late protection). The sarcolemmal K_{ATP} (sK_{ATP}) channel inhibitor HMR 1098 (HMR; 6 mg/kg), the mitochondrial K_{ATP} (mK_{ATP}) channel inhibitor 5-hydroxydecanate (5-HD; 10 mg/kg), and the nonspecific K_{ATP} channel inhibitor glibenclamide (Glib; 1 mg/kg) were injected intravenously at 10 min before intravenous injection of saline or saline containing Nicorandil. Muscle infarction was assessed after 48h reperfusion, using the nitroblue tetrazolium dye staining technique.*

2.6.6 Study 6: To investigate the role of mitochondrial K^+ (mK_{ATP}) channels in the effector mechanism of Nicorandil-induced late IPC.

Study 6 was designed to demonstrate the involvement of mK_{ATP} channels in the effector mechanism of Nicorandil-induced late IPC. Animals were assigned to an ischaemic control and seven treatment groups: 1) Saline; 2) Nicorandil; 3) Nicorandil + HMR-1098; 4) Nicorandil + 5-HD; 5) Nicorandil + Glibenclamide; 6) Saline + HMR-1098; 7) Saline + 5-HD and 8) Saline + Glibenclamide.

In Study 6, saline or saline containing Nicorandil (3 mg/kg) was administered, 24hr before subjecting LD muscle flaps to 4hr ischaemia and 48hr reperfusion. 10min before the onset of 4hr ischaemia, the sK_{ATP} channel inhibitor HMR-1098, the mK_{ATP} channel inhibitor 5-HD or the non-specific K_{ATP} channel inhibitor was injected intravenously as a bolus.

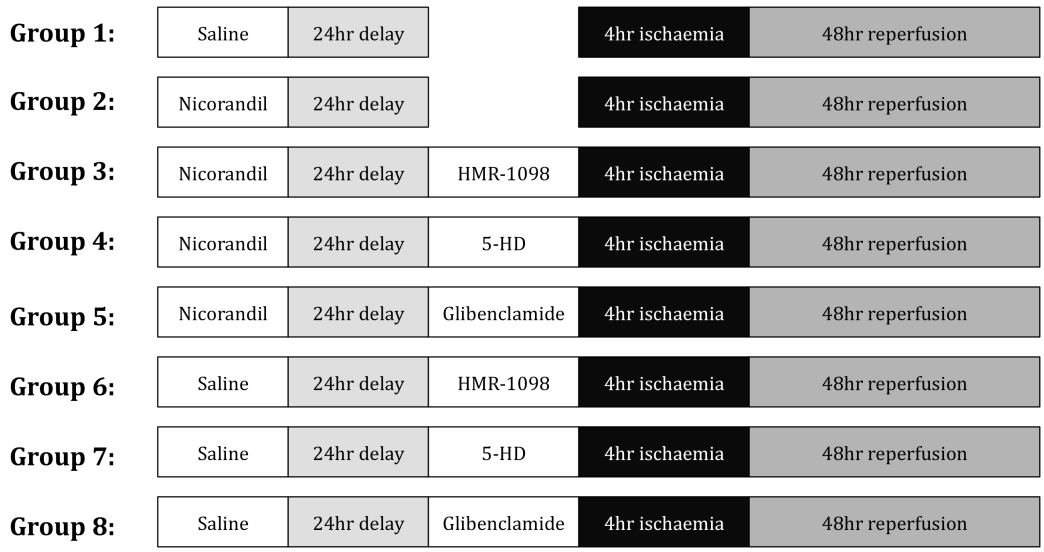

Figure 2.14. *Protocol for investigation of the mediator mechanism in Nicorandil-induced late protection of skeletal muscle against ischaemia / reperfusion injury. The protocol was the same as the protocol for investigation*

of the trigger mechanism in Figure 2.9 except that HMR-1098 (sK_{ATP} channel inhibitor), 5-HD (mK_{ATP} channel inhibitor) and Glibenclamide (non-specific K_{ATP} channel inhibitor) were injected intravenously at 24h after intravenous injection of saline or saline containing Nicorandil (3 mg/kg).

2.7 Statistical Analysis

All values are expressed as means +/- Standard Error of the Mean (SEM) unless otherwise stated. The number of observations in each study is described in the legends of the figures and tables. In studies 1-3, 5 and 6 mean values at each time point were compared using one-way Analysis of Variance (ANOVA) and Newman-Keuls multiple comparison test. For study 4 two-way ANOVA and students t-test were employed. Statistical significance was set at $P < 0.05$ for all tests. All statistical analyses were performed using the biostatistics software package SPSS (Chicago, Illinois 60606).

3.0 RESULTS

3.1 Animal Demographics

A total of 109 castrated male Yorkshire pigs were used in the following experiments, including 9 pilot controls used to gain consistency in the experimental model. The mean animal weight was 17.9kg (range 15 – 24kg; stdev 1.4kg). There were 4 premature animal deaths: 2 on-table intra-operative deaths; 1 animal required on-table euthanasia due to ongoing metabolic acidosis; 1 animal died on the 1st post-operative day of respiratory complications.

There were no significant differences between treatment groups when animal weight, duration of operation, or recovery time was taken into account.

3.2 Study 1: To investigate the dose-response effect of Nicorandil-induced late phase IPC when given 24 hours before instigation of I/R injury in porcine LD muscle flaps.

In this preliminary study we performed a series of dosage experiments to find the optimum dose of Nicorandil to effectively protect the LD muscle flap from I/R injury.

We looked at the dosages other investigators were using in large animal studies, such as Tang et al with rabbits (Tang et al. 2004); Mizumura et al with dogs (Mizumura et al. 1996) and Galie et al in pigs (Galie et al. 1995).

As described earlier, incremental concentrations of Nicorandil (0.1 mg/kg to 3 mg/kg) were injected intravenously with an initial bolus injection followed by a 60 min infusion. 24 hours later, the LD muscle flaps were subjected to 4 hours global ischaemia followed by 48 hours reperfusion. After 48 hours reperfusion, muscle infarction was assessed by nitroblue tetrazolium reduction technique.

No. of pigs	Control	0.1 - 0.3mg/kg	1-2mg/kg	3mg/kg
1	55	33	55	28
	41	-	43	15
2	40	36	27	33
	36	37	24	29
3	37	26	41	14
	45	22	30	10
4	36	21	27	34
	47	25	29	10
5	39	46	24	20
	41	39	17	22
6			27	
			17	
Mean	**41.7**	**31.7**	**30.1**	**21.5**
SEM	**1.9**	**2.9**	**3.2**	**2.9**

Table 3.1. *Incremental dosages of i.v. Nicorandil (mg/kg) given 24 hr before ischaemic insult. Flap necrosis expressed as % infarction, mean and standard error of the mean (SEM), n = 5-6 pigs.*

We observed a dose-dependent effect of i.v. Nicorandil, whereby increasing the concentration resulted in a reduction in percentage infarction of LD muscle flaps, i.e. an increase in infarct protection of the skeletal muscle (Figure 3.1).

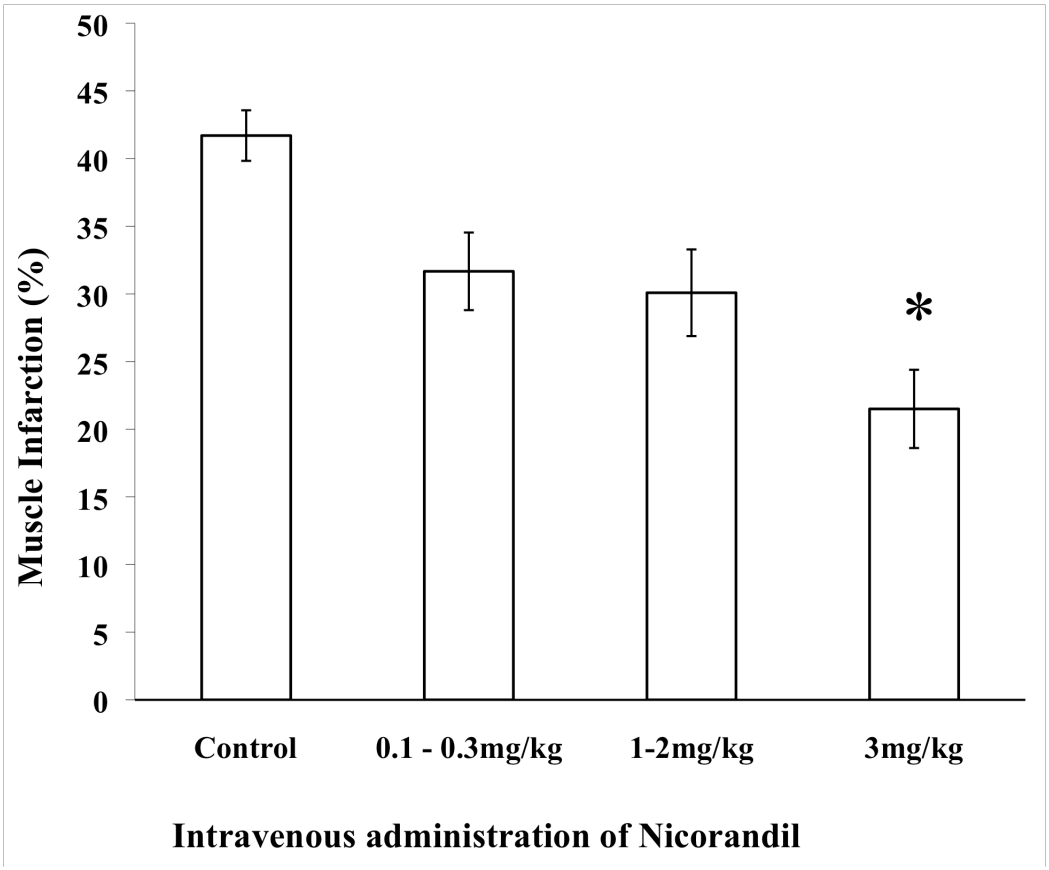

Figure 3.1. *Dose-response effect of intravenous administration of Nicorandil for induction of late phase infarct protection in skeletal muscle. There were between 5 and 6 pigs with bilateral LD muscle flaps in each group. All LD muscle flaps were subjected to 4h ischaemia starting at 24h after intravenous administration of saline (control) or saline containing Nicorandil. Muscle infarction was assessed after 48h reperfusion. Means with an asterisk are similar and are significantly different from the control (oneway ANOVA and Newman Keuls multiple range test; *p < 0.05).*

3.3 Study 2: To investigate the effect of intravenous Nicorandil on mean arterial blood pressure (MAP) in the porcine model.

Following the preliminary dosage study we chose an optimum concentration of 3mg/kg i.v. Nicorandil. We investigated the effect of this concentration on changes in mean arterial pressure (MAP) following i.v. infusion in the porcine model.

Time (min)	0	10	20	30	40	50	60	70	80	90
1	83	73	78	75	74	72	72	72	72	72
2	71	69	68	71	73	73	75	75	74	75
3	87	88	88	90	92	93	93	93	93	93
Mean	**80.3**	**76.7**	**78**	**78.7**	**79.7**	**79.3**	**80**	**80**	**79.7**	**80**
SEM	**4.8**	**5.8**	**5.8**	**5.8**	**6.2**	**6.8**	**6.6**	**6.6**	**6.7**	**6.6**

Table 3.2. *Mean Arterial Pressure (mmHg) for saline control group, n= 3 pigs*

Time (min)	0	10	20	30	40	50	60	70	80	90
1	58	40	43	42	22	19	26	84	62	67
2	92	42	41	44	68	65	62	58	80	90
3	59	17	17	33	55	54	53	42	49	56
4	105	89	60	70	52	47	42	49	-	-
5	90	60	58	60	51	53	53	-	-	-
6	99	87	63	57	66	65	59	57	74	-
Mean	**82.8**	**63.6**	**55**	**55.2**	**55.6**	**53.3**	**52.6**	**58**	**66.2**	**71**
SEM	**5.1**	**7.9**	**5.9**	**4.3**	**5.5**	**5.5**	**4.6**	**7.1**	**6.9**	**10.0**

Table 3.3. *Mean Arterial Pressure (mmHg) for Nicorandil (3mg/kg) treatment group, n= 6 pigs.*

As described earlier, MAP was measured for 90 mins in anaesthetised animals via a cannula placed in the right femoral artery. Nicorandil (3mg/kg) was

injected as an infusion over the first 60min with an infusion of normal saline (2ml/min), continuing for another 30min. During the first 60 minutes of i.v. Nicorandil infusion there was a significant reduction in MAP when compared to the saline-infused animals. However, the MAP normalised within 30 min of the end of the Nicorandil infusion (Figure 3.2).

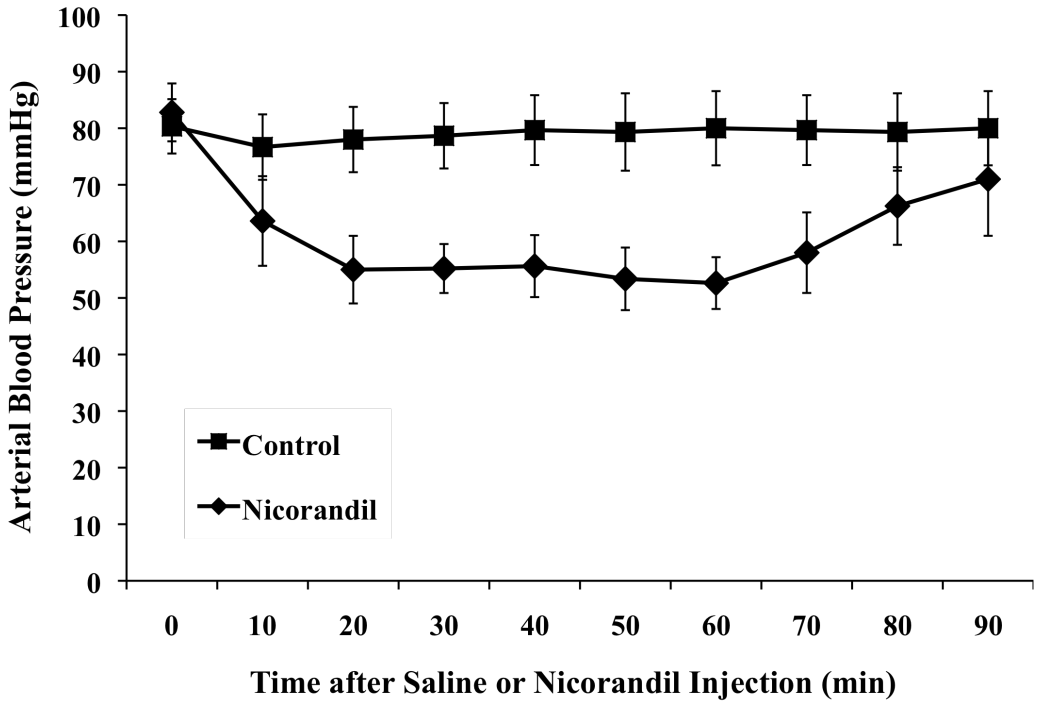

Figure 3.2. *Change in Mean Arterial Pressure (MAP) after intravenous administration of Nicorandil (3mg/kg). The control and Nicorandil treatment groups received intravenous infusion of saline and saline containing Nicorandil (3mg/kg), respectively. Values are mean +/- SEM; n = 5 pigs. Comparisons at each timepoint were calculated using one-way ANOVA and Newman Keuls multiple range test; $p < 0.05$.*

3.4 Study 3: To investigate the efficacy of Nicorandil in induction of late-phase IPC for uninterrupted perioperative protection of skeletal muscle against I/R injury in porcine LD muscle flaps.

Incremental time periods between Nicorandil injection (3mg/kg) and the onset of ischaemic insult were as follows: 0, 4, 8, 12, 24, 48, 72 and 96 hr. Following 4 hr ischaemia and 48 hr reperfusion, LD muscle infarction was assessed as before.

n =	control	0hr	4hr	8hr	12hr	24hr	48hr	72hr	96hr
1	34	33	13	39	26	23	33	27	57
	41	30	37	51	33	17	15	34	55
2	39	22	21	33	52	26	20	23	25
	55	29	21	28	61	16	29	18	38
3	41	27	31	39	30	27	24	33	34
	39	28	24	53	43	28	22	29	44
4	35	31	21	44	53	15	31	28	54
	36	19	24	-	61	18	27	-	17
5	37		24			14			
	45					33			
Mean	**40.2**	**27.4**	**24**	**41**	**44.9**	**22.4**	**25.1**	**28.5**	**43.9**
SEM	**1.9**	**1.6**	**2.2**	**3.4**	**4.9**	**2.0**	**2.1**	**2.1**	**4.6**

Table 3.4*. Timecourse of Nicorandil injection (3mg/kg) prior to ischaemic insult. Flap necrosis expressed as % infarction, mean and standard error of the mean (SEM), n = 4-5 pigs.*

There was 40 +/- 2% infarction rate in control LD muscle flaps subjected to 4hr ischaemia and 48hr reperfusion. Ischaemic preconditioning with i.v. Nicorandil (3mg/kg) given at 0hr and 4hr before instigation of ischaemic insult significantly reduced the infarct size to 27.3 +/- 1% and 24.0 +/- 2 % respectively (Figure 3). However this Nicorandil-induced infarct protective effect waned, resulting in the muscle infarction rate increasing to 41.0 +/- 3% at 8hr and 44.8 +/- 5% at 12hr. These are similar rates of muscle infarction to the ischaemic control. Importantly, this "late phase" Nicorandil-induced infarct protective effect returned at 24hr and continued for another 48hr with infarction rates of 22.3 +/- 2%, 25.1 +/- 2% and 28.5 +/- 2% at 24hr, 48hr and 72hr respectively. When Nicorandil was given 96hr before the onset of 4hr ischaemia, the infarct protective effect was lost with an infarction rate of 44 +/- 6%, similar to the ischaemic control (Figure 3.3).

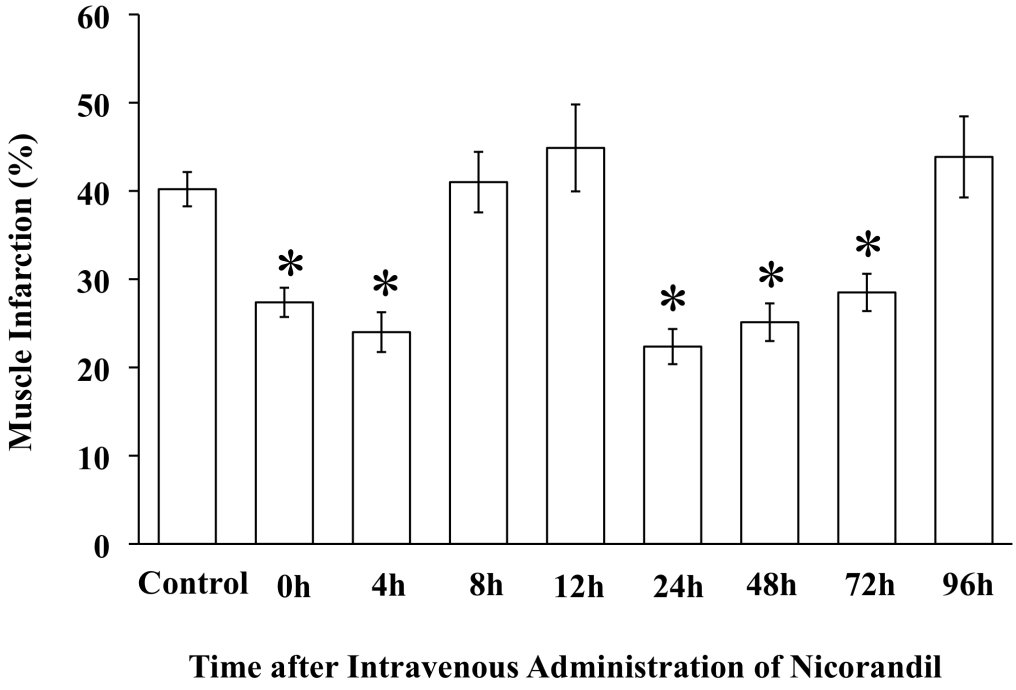

Figure 3.3. *Induction of biphasic infarct protection in skeletal muscle by intravenous administration of Nicorandil. There were 4 – 5 pigs with bilateral LD muscle flaps for each time point. All control and treatment LD muscle flaps were subjected to 4h of ischaemia and 48h of reperfusion. In Nicorandil treatment groups, 4h of sustained ischaemia started at 0, 4, 8, 12, 24, 48, 72, or 96 hr after intravenous administration of Nicorandil. Muscle infarction values are expressed as mean +/- SEM. Means with an asterisk are similar and are significantly different from the rest of the means without an asterisk (oneway ANOVA and Newman Keuls multiple range test; *p < 0.05).*

3.5 Study 4: To investigate the effect of Nicorandil-induced late IPC on muscle content of m[Ca^{2+}], ATP and MPO activity in porcine LD muscle flaps subjected to 4 hours ischaemia and 48 hours reperfusion.

The ischaemic control group received intravenous injection of saline and the Nicorandil pre-treatment group, injection of Nicorandil (3mg/kg), 24 hr before the onset of 4 hr sustained ischaemia. Muscle biopsies (1x1 cm) were taken from both LD muscle flaps immediately before sustained ischaemia; at 2 hr and 4 hr ischaemia; and at 1 hr reperfusion. Fresh muscle biopsies were processed for assay of mitochondrial free Ca^{2+} content (m[Ca^{2+}]). Remaining tissue was frozen at -80˚C for assay of ATP content and MPO activity.

Control	pre-ischaemia	2hr ischaemia	4hr ischaemia	1hr reperfusion
1	213.72	159.69	279.34	606.80
2	213.89	187.75	265.29	865.16
3	214.11	112.91	140.83	639.94
4	213.94	208.76	201.85	995.78
5	214.56	82.64	80.09	353.18
6	213.91	229.58	326.77	987.93
Mean	**214.02**	**163.56**	**215.70**	**741.47**
SEM	**0.12**	**23.18**	**37.87**	**103.27**

Table 3.5. *Ischaemic control muscle biopsies for mitochondrial free calcium content at various time-points during ischaemia / reperfusion; n = 6 pigs.*

Nicorandil	pre-ischaemia	2hr ischaemia	4hr ischaemia	1hr reperfusion
1	213.97	181.01	239.63	553.38
2	213.75	204.48	229.91	429.17
3	213.67	123.89	177.72	660.71
4	213.44	111.24	179.87	223.31
5	213.98	80.04	234.50	312.40
Mean	**213.76**	**140.13**	**212.33**	**435.79**
SEM	**0.10**	**22.94**	**13.78**	**79.00**

Table 3.6. *Nicorandil pre-treatment muscle biopsies for mitochondrial free calcium content at various time-points during ischaemia / reperfusion; n = 5 pigs.*

m[Ca^{2+}] remained stable during the ischaemic insult, both in the control and treatment group, when measured at 0hr (213.8 / 214.0 nmol/mg); 2hr (140.1 / 163.6 nmol/mg); and 4hr (212.3 / 215.7 nmol/mg). However, at 1 hr reperfusion there was a significant rise in m[Ca^{2+}] in both the ischaemic control and Nicorandil pre-treatment groups (741.5 and 435.8 nmol/mg respectively), when compared to ischaemic groups. Further to this, in the Nicorandil pre-treatment

group, the rise in m[Ca^{2+}] was significantly lower during the first hour of reperfusion, when compared to the control group, ($p < 0.05$) (Figure 3.4).

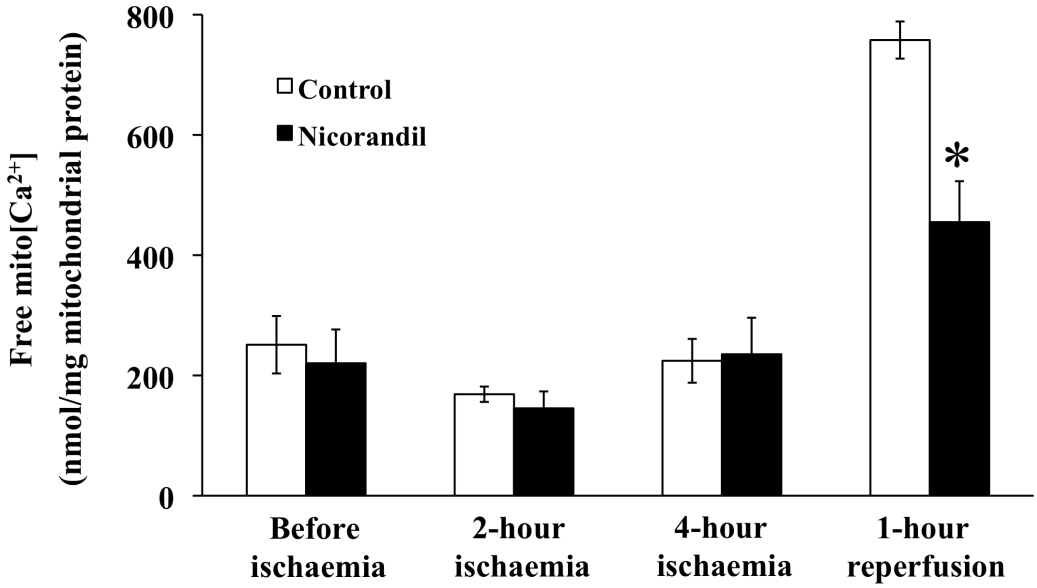

Figure 3.4. *Mitochondrial Free Calcium content (m[Ca^{2+}]) in porcine LD muscle flaps immediately before and at the end of 2hr and 4hr ischaemia and 1hr reperfusion. The control and treatment groups received intravenous infusion of saline and saline containing 3mg/kg Nicorandil, respectively. Infusions were given 24hr before the start of 4hr sustained ischaemia (late phase of protection). Values are expressed as mean +/- SEM; n = 5 pigs. Means with an asterisk are significantly different from the time-matched control (two-way ANOVA and t-test at each time-point; *p < 0.05).*

Adenosine Triphospate (ATP) content in LD muscle flaps was measured using a quantitative bioluminescent technique, as described earlier. ATP content in the muscle biopsies showed a gradual reduction during the ischaemic insult with no

significant differences between control and treatment groups (30.8 +/- 1.7 vs. 30.8 +/- 2.3 before ischaemia; 21.3 +/- 2.7 vs. 24.0 +/- 3.1 at 2hr ischaemia; 6.5 +/- 1.2 vs. 9.8 +/- 2.7 at 4hr ischaemia mmol/g prot). However, in the first hour of reperfusion there was a significant increase in muscle ATP content of the Nicorandil pre-treatment group, when compared to the control group (22.6 +/- 2.0 vs. 12.4 +/- 1.8 mmol/g prot; $p < 0.05$) (Figure 3.5).

Control	pre-ischaemia	2hr ischaemia	4hr ischaemia	1hr reperfusion
1	31.34	18.43	2.58	7.68
2	38.48	13.97	2.90	9.22
3	35.39	29.33	5.34	16.94
4	29.27	24.12	8.79	14.58
5	30.66	23.77	8.41	11.79
6	19.88	18.62	10.76	14.00
Mean	**30.84**	**21.37**	**6.46**	**12.37**
SEM	**1.68**	**2.73**	**1.17**	**1.79**

Table 3.7. *Ischaemic control muscle biopsies for muscle ATP content at various time-points during ischaemia / reperfusion; n = 6 pigs.*

Nicorandil	pre-ischaemia	2hr ischaemia	4hr ischaemia	1hr reperfusion
1	33.70	13.63	7.62	19.37
2	33.90	24.54	14.72	24.86
3	34.87	21.31	5.26	26.55
4	22.24	29.98	17.75	25.91
5	29.68	30.75	3.77	16.22
Mean	**30.88**	**24.04**	**9.82**	**22.58**
SEM	**2.34**	**3.13**	**2.73**	**2.04**

Table 3.8. *Nicorandil pre-treatment muscle biopsies for muscle ATP content at various time-points during ischaemia / reperfusion; n = 5 pigs.*

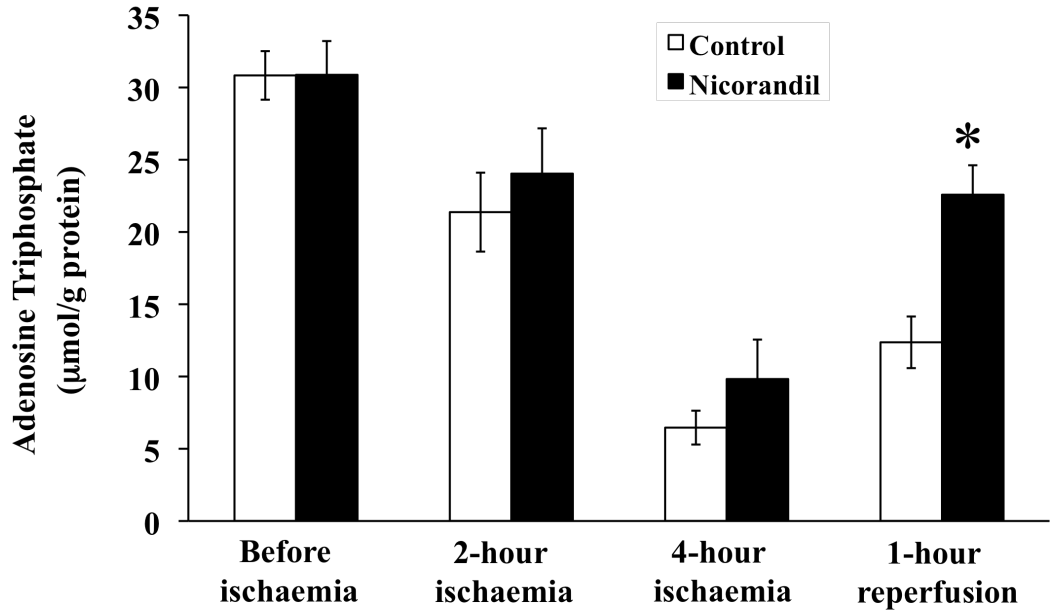

Figure 3.5. *Adenosine Triphosphate (ATP) content in porcine LD muscle flaps immediately before, and at the end of 2hr and 4hr ischaemia and at 1hr of reperfusion. The control and treatment groups received intravenous infusion of saline and saline containing 3mg/kg Nicorandil, respectively. Infusions were given 24hr before the start of 4hr sustained ischaemia (late phase of protection). Values are expressed as mean +/- SEM; n = 5-6 pigs. Means with an asterisk are significantly different from the time-matched control (two-way ANOVA and t-test at each time-point; *p < 0.05).*

Myeloperoxidase (MPO) activity is a well-validated marker of neutrophil accumulation in skeletal muscle. MPO was assayed in the skeletal muscle using a spectrophotometric technique. During 0, 2 and 4hr of the ischaemic insult MPO levels remained low and negligible in both control and treatment groups (0.12 +/- 0.02 vs. 0.13 +/- 0.02 before ischaemia; 0.15 +/- 0.02 vs. 0.14 +/- 0.02 at 2hr ischaemia; 0.19 +/- 0.04 vs. 0.13 +/- 0.02 at 4hr ischaemia). During the

first hour of reperfusion there were significant increases in MPO activity of the control group (0.95 +/- 0.07) and the Nicorandil pre-treatment group (0.38 +/- 0.08). Further to this, the rise in MPO activity of the Nicorandil pre-treatment group was significantly less than the rise in the control group (0.38 +/- 0.08 vs. 0.95 +/- 0.07 units/g wet muscle; $p < 0.05$) (Figure 3.6).

Control	pre-ischaemia	2hr ischaemia	4hr ischaemia	1hr reperfusion
1	0.14	0.08	0.07	1.02
2	0.11	0.15	0.24	1.04
3	0.11	0.15	0.32	0.64
4	-	0.20	0.20	1.01
5	0.17	0.16	0.14	1.09
Mean	**0.13**	**0.15**	**0.20**	**0.96**
SEM	**0.01**	**0.02**	**0.04**	**0.07**

Table 3.9. *Ischaemic control muscle biopsies for myeloperoxidase content at various time-points during ischaemia / reperfusion; n = 5 pigs.*

Nicorandil	pre-ischaemia	2hr ischaemia	4hr ischaemia	1hr reperfusion
1	0.10	0.15	0.19	0.62
2	0.11	0.09	0.12	0.25
3	0.11	0.18	0.10	0.42
4	0.20	0.18	0.09	0.45
5	0.12	0.11	0.14	0.13
Mean	**0.13**	**0.14**	**0.13**	**0.38**
SEM	**0.02**	**0.02**	**0.02**	**0.08**

Table 3.10. *Nicorandil pre-treatment muscle biopsies for myeloperoxidase content at various time-points during ischaemia / reperfusion; n = 5 pigs.*

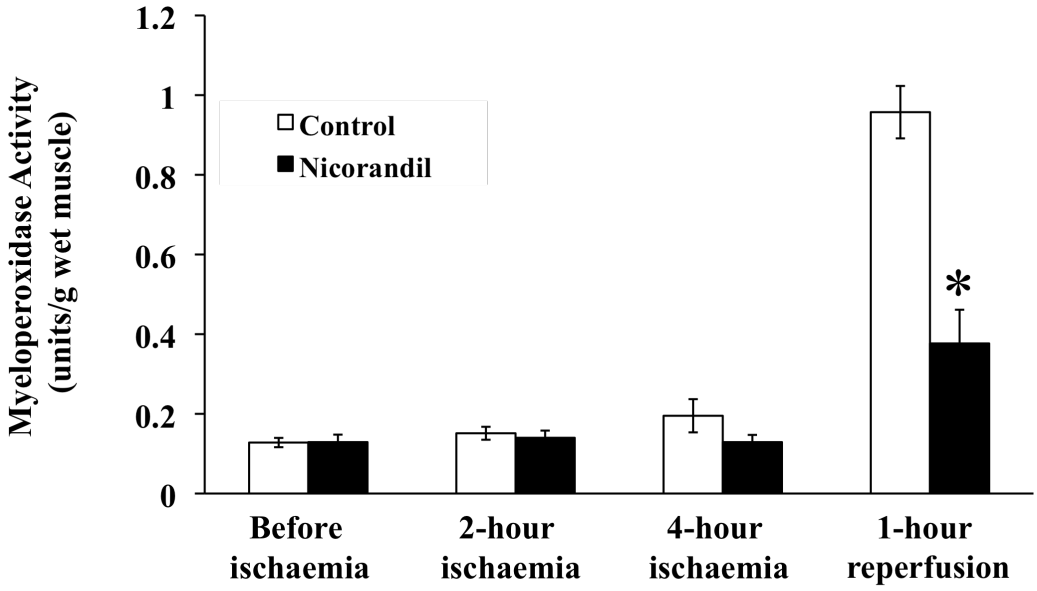

Figure 3.6. *Muscle neutrophilic myeloperoxidase (MPO) activity in porcine LD muscle flaps immediately before, and at the end of 2hr and 4hr ischaemia and at 1hr of reperfusion. The control and treatment groups received intravenous infusion of saline and saline containing 3mg/kg Nicorandil, respectively. Infusions were given 24hr before the start of 4hr sustained ischaemia (late phase of protection). Values are expressed as mean +/- SEM; n = 5 pigs. Means with an asterisk are significantly different from the time-matched control (two-way ANOVA and t-test at each time point; *p < 0.05).*

3.6 Study 5: To investigate the role of ATP-sensitive K^+ (K_{ATP}) channels in the trigger mechanism of Nicorandil-induced late IPC.

Late phase ischaemic preconditioning was instigated by Nicorandil injection (3 mg/kg) 24hr before subjecting LD muscle flaps to 4hr ischaemia and 48hr reperfusion. The selective sarcolemmal K_{ATP} (sK_{ATP}) channel inhibitor HMR-1098 (6 mg/kg) was injected as an intravenous bolus 10 minutes before Nicorandil injection. HMR-1098 effectively blocked the preconditioning effect of Nicorandil, resulting in an infarction rate of 40.8 +/- 5%, similar to the ischaemic control rate of 46.1 +/- 3% (Figure 3.7).

n =	Control	Nicorandil	5-HD + Nico.	HMR + Nico.	Gliben + Nico.	5-HD	HMR-1098	Gliben
1	55	17	16	25	39	40	27	46
	37	26	20	34	40	45	33	40
2	41	16	31	32	40	41	48	47
	58	27	25	22	43	46	51	40
3	41	28	25	64	41	43	49	40
	45	15	21	63	42	46	42	43
4	39	18	33	50	49	40	33	46
	58	14	26	40	-	45	50	34
5	47	33		27			49	
	40	29		35			55	
Mean	**46.1**	**22.3**	**25.9**	**40.8**	**42.4**	**43.1**	**43.6**	**42.1**
SEM	**2.6**	**2.2**	**2.0**	**4.8**	1.2	**1.3**	**4.2**	**2.2**

Table 3.11. *The addition of pharmacological probes 5-HD, HMR-1098 and Glibenclamide before Nicorandil pre-treatment. Flap necrosis expressed as % infarction, mean and standard error of the mean (SEM), n = 4-5 pigs.*

Further to this, the non-specific K_{ATP} channel inhibitor Glibenclamide (1 mg/kg) was injected as an intravenous bolus 10 minutes before Nicorandil injection. Glibenclamide also effectively blocked the preconditioning effect of Nicorandil, resulting in an infarction rate of 42.4 +/- 1% (Figure 3.7).

However, the selective mitochondrial K_{ATP} (mK_{ATP}) channel inhibitor 5-HD (10 mg/kg), when given 10min before Nicorandil injection, had no significant effect (25.9 +/- 2%) when compared to Nicorandil pre-treatment (22.3 +/- 2%). The equivalent doses of 5-HD, HMR-1098 or Glibenclamide were also injected 10 minutes before sham saline injection at 24hr before the onset of 4hr ischaemia and 48hr reperfusion. This resulted in infarction rates of 43.6 +/- 1%, 43.7 +/- 4% and 41.5 +/- 1% respectively, which are similar to the ischaemic control rate of 46.1 +/- 3% (Figure 3.7).

Taken together, these observations indicate that sK_{ATP}, but not mK_{ATP} channels, are involved in the trigger mechanism of Nicorandil-induced preconditioning for late-phase protection of skeletal muscle against infarction.

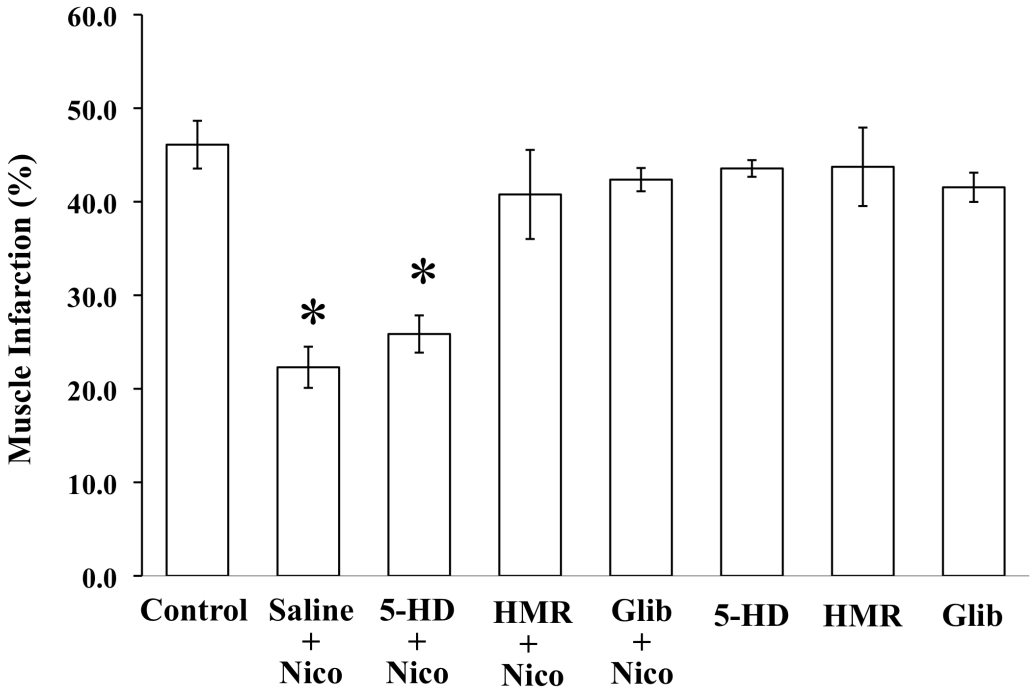

Figure 3.7. *Role of sarcolemmal K_{ATP} (sK_{ATP}) and mitochondrial K_{ATP} (mK_{ATP}) channels in the trigger mechanism of Nicorandil-induced late phase preconditioning against I/R injury in porcine skeletal muscle. There were 4-5 pigs with bilateral LD muscle flaps in each group. All LD muscle flaps were subjected to 4hr ischaemia and 48hr reperfusion. Intravenous administration of saline or saline containing Nicorandil (3 mg/kg) was performed 24hr before 4hr of sustained ischaemia (late phase protection). The sK_{ATP} channel inhibitor HMR-1098 (6 mg/kg), the mK_{ATP} channel inhibitor 5-HD (10 mg/kg) or the nonspecific K_{ATP} channel inhibitor Glib. (1 mg/kg) was injected intravenously at 10 min before intravenous administration of saline or saline containing Nicorandil. 5-HD was also co-infused intravenously (5mg/kg) for 30min after bolus injection. Muscle infarction was assessed after 48hr reperfusion. Values are expressed as mean +/- SEM; n = 4-5 pigs. Means with an asterisk are*

*similar and are significantly different from means without an asterisk (one-way ANOVA and Neuman-Keuls multiple range test; *p < 0.05).*

3.7 Study 6: To investigate the role of ATP-sensitive K^+ (K_{ATP}) channels in the effector mechanism of Nicorandil-induced late IPC.

Nicorandil (3mg/kg) was injected intravenously 24hr before subjecting LD muscle flaps to 4hr ischaemia and 48hr reperfusion. The selective mK_{ATP} channel inhibitor 5-HD was injected intravenously as a bolus 10min before the onset of the 4hr ischaemic insult. This effectively blocked the late-phase preconditioning effect of Nicorandil, resulting in an infarction rate of 35.7 +/- 3%, similar to the ischaemic control of 42.0 +/- 4% (Figure 3.8).

Further to this, the non-specific K_{ATP} channel inhibitor Glibenclamide (1 mg/kg) was also injected as an intravenous bolus 10 minutes before the onset of 4hr ischaemia. Glibenclamide also completely blocked the late-phase of infarct protection conferred by Nicorandil, resulting in an infarction rate of 42.8 +/- 1%, similar to the ischaemic control of 42.0 +/- 4% (Figure 3.8).

n =	Control	Nicorandil	Nico. + 5-HD	Nico. + HMR	Nico. + Gliben.	5-HD	HMR-1098	Gliben.
1	40	23	20	44	45	40	27	46
	35	17	31	49	46	45	33	40
2	48	26	31	35	41	41	48	47
	36	16	38	29	39	46	51	40
3	36	27	26	27	42	43	49	40
	29	28	32	30	43	46	42	43
4	62	15	10			40	33	46
	51	18	24			45	50	34
5							49	
							55	
Mean	**42.0**	**21.3**	**26.5**	**35.7**	**42.8**	**43.1**	**43.6**	**42.1**
SEM	3.8	1.9	**3.1**	**3.6**	**1.1**	**0.9**	**3.0**	**1.6**

Table 3.12. *The addition of pharmacological probes 5-HD, HMR-1098 and Glibenclamide 24 hr after Nicorandil pre-treatment, before ischaemia. Flap necrosis expressed as % infarction, mean and standard error of the mean (SEM), n = 3-5 pigs.*

On the other hand, the selective sK_{ATP} channel inhibitor HMR-1098, when given 10min before the 4hr ischaemic insult, had no significant effect (26.5 +/- 3%), when compared to Nicorandil-induced late phase preconditioning (21.3 +/- 2%). Equivalent doses of HMR-1098, 5-HD and Glibenclamide were also injected 24hr following sham saline injection and 10min before the onset of 4hr ischaemia and 48hr reperfusion. This resulted in infarction rates of 43.6 +/- 3%, 43.1 +/- 1% and 42.1 +/- 2% respectively, similar to the ischaemic control of 42.0 +/- 4% (Figure 3.8).

Taken together, these observations indicate that mK_{ATP}, but not sK_{ATP} channels, are involved in the mediator mechanism of Nicorandil-induced preconditioning for late phase protection of skeletal muscle against infarction.

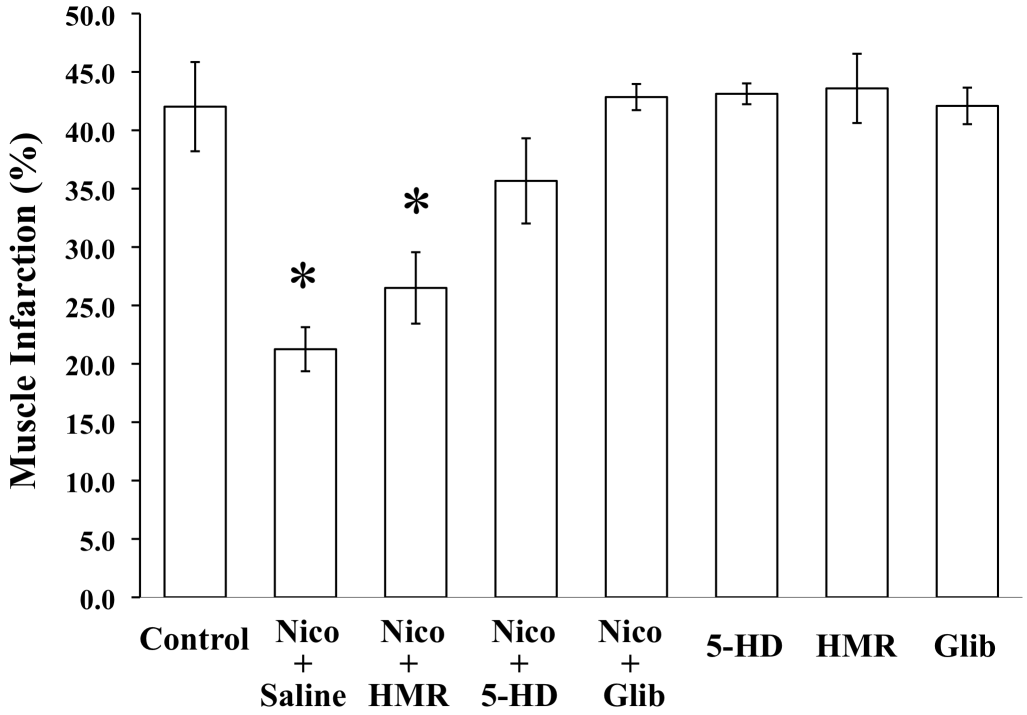

Figure 3.8. *Role of sarcolemmal K_{ATP} (sK_{ATP}) and mitochondrial K_{ATP} (mK_{ATP}) channels in the effector mechanism of Nicorandil-induced late phase preconditioning against I/R injury in porcine skeletal muscle. There were 3-5 pigs with bilateral LD muscle flaps in each group. All LD muscle flaps were subjected to 4hr ischaemia and 48hr reperfusion. Nicorandil was injected at 24hr prior to the onset of 4hr sustained ischaemia (late-phase preconditioning). The sK_{ATP} channel inhibitor HMR-1098 (6 mg/kg), the mK_{ATP} channel inhibitor 5-HD (10 mg/kg) or the nonspecific K_{ATP} channel inhibitor Glibenclamide (1 mg/kg) was injected intravenously 24hr after intravenous administration of saline or saline containing Nicorandil (3 mg/kg), 10 min before the onset of the 4hr ischaemic insult and 48hr of reperfusion. 5-HD was also co-infused*

*intravenously (5mg/kg) for 30min after bolus injection. Muscle infarction was assessed after 48hr of reperfusion. Values are expressed as mean +/- SEM; n = 3-5 pigs. Means with an asterisk are similar and are significantly different from means without an asterisk (one-way ANOVA and Neuman-Keuls multiple range test; *p < 0.05).*

4.0 DISCUSSION

4.1 Choice of Porcine Model

As stated earlier, the pig was chosen as the large animal model for our study due in part to the extensive research into its musculoskeletal anatomy and vascular territories of skin, fascia and muscle (Patterson et al. 1967; Milton 1972; Meyer et al. 1978; Prather et al. 1979; Millican et al. 1985). As well as this, our lab has prior experience in conducting experiments and in the perioperative care of these animals (Mounsey et al. 1992; Morris et al. 1993; Pang et al. 1995; Hopper et al. 2000).

The pig has been used as an experimental animal model in anatomical research for many years. When compared to other potential large animals such as a canine or rabbit model, the pig is a tight-skinned mammal with almost identical tissue qualities to human skin, with the exception of the *pannniculus carnosus*. In the late 60's Patterson et al (Patterson et al. 1967) demonstrated that despite the subtle differences in the anatomy of porcine skin, this thin muscular layer did not contribute to the blood supply of the skin. Our interest in this model was regarding its skeletal muscle anatomy. Millican and Poole accurately described the reliable and consistent anatomy of the Latissimus Dorsi (LD) muscle, raised as a pedicled myocutaneous flap (Millican et al. 1985). They further demonstrated a consistent thoracodorsal pedicle as the principle arterial supply with two accompanying venae comitantes and thoracodorsal nerve. They also reported that perforators from underlying intercostal vessels supplied the caudal part of the LD muscle, a feature present in humans.

Several large landmark studies in myocardial preconditioning have been performed using a canine model (Murry et al. 1986; Auchampach et al. 1993; Mizumura et al. 1995; Hausenloy et al. 2009). Further to this the canine LD muscle flap has been described for dynamic cardiomyoplasty in a canine chronic cardiac failure model (Soberman et al. 1990; Takemura et al. 1991). However, several investigators have noted distal ischaemia of the pedicled LD muscles after transfer, and have therefore resorted to vascular delay to improve LD viability and function for cardiomyoplasty (Carroll et al. 1997; Carroll et al. 1997). The reason for the distal necrosis in canine pedicled LD flaps is due to their different vascular territories in relation to human LD muscle. In particular, the pattern of branches between thoracodorsal and intercostal perforators differs between the dog and the human (Yang et al. 1999). Therefore pedicled canine LD muscle flaps are not a good predictor of their human counterpart.

We used castrated male Yorkshire pigs for our study with a mean animal weight of 17.9kg (range 15 – 24kg). We realised that this size of animal would be required to give us a consistent magnitude of 13 x 8cm LD muscle flaps. The 13 x 8cm flap allowed us to standardise our results with previous studies using the porcine LD flap model, resulting in further validation. In this weight range, animals were approximately 8 – 10 weeks of age and had not reached sexual maturity. Despite this, castrated animals were used to reduce animal aggression and to prevent any hormonal influences on the study results. It can be debated that as we have not used an 'adult' large animal model, there may be a potential effect on translational validity. When comparing our mean animal weight to other porcine studies in myocardial ischaemic preconditioning, Bolli's group

used pig models in two studies with mean weights of 25kg (Tang et al. 1996) and 28kg (Qiu et al. 1997). As well as this, Galie et al used 30 – 35kg pigs to study Nicorandil-induced myocardial protection (Galie et al. 1995). The findings from these myocardial protection models have translated to subsequent human trials such as CESAR 2 (Patel et al. 1999) and IONA (2002), demonstrating the validity of an adolescent porcine model.

It can be argued that a smaller animal model could have been used to simulate the ischaemia-reperfusion injury, such as the rat LD skeletal muscle flap model (Harralson et al. 2005). However, there are species differences in these smaller animal models with their vascular anatomy and muscle morphology. Firstly, there is a difference in the ratio of type 1 and 2 fibres in rat and pig skeletal muscle. Specifically, skeletal muscles of small animals like the mouse and rat have a more substantial proportion of type 2B fast twitch fibres. As body size increases, the 2B fibre population diminishes, producing a reversed ratio of type 1 to type 2 fibres, present in larger animals like the pig and human (Sutherland et al. 2006). Secondly, the LD muscle in humans and pigs has a Mathes & Nahai Type 5 blood supply (Mathes et al. 1981) where the muscle is supplied by a principle vascular pedicle and secondary segmental supply. In the rat model the principle thoracodorsal pedicle is not sufficient to maintain a vascularised flap and the intercostal supply must therefore remain intact (Carroll et al. 1997; Harralson et al. 2005). In the human and pig, the muscle can be reliably raised on the principle pedicle and our lab has shown the porcine LD muscle to remain viable on this pedicle for up to 48 hours in a sham control group (Pang et al. 1995). Further to this, the thoracodorsal pedicle in a 20kg animal is of a sufficient caliber to allow for repeated vascular clamping without fear of undue

shearing forces and intimal damage. Another concern in small animal models is the thickness / volume of the LD flap. A thin flap may be able to obtain sufficient oxygenation and nutrition by direct diffusion from adjacent tissues, thereby with partial muscle survival independent of the vascular pedicular supply.

A total of 9 animals were used in preliminary 'pilot' control studies, as well as a further 4 animals in model development for another study (McAllister et al. 2008). There is a learning curve of surgical technique required to raise bilateral muscle flaps and skeletise the thoracodorsal vascular pedicle consistently to allow for reliable vessel clamping. There were 4 premature animal deaths in our study. 1 animal died on the 1st postoperative day due to respiratory complications; 3 of the animals died or had to be euthanised intra-operatively due to metabolic acidosis / multi-organ failure. The most likely explanation for the intra-operative deaths is a syndrome called malignant hyperthermia, which can be triggered in various pig breeds by stress or several volatile anaesthetic agents such as Halothane, Isoflurane and Enflurane (Rosenberg et al. 1994). This syndrome, also known as Porcine Stress Syndrome (PSS), has been associated with a genetic mutation present in 19% of the Yorkshire breed of pigs (O'Brien et al. 1993).

4.2 The Bilateral LD Muscle Flap Model

As stated earlier, the anatomy of the LD muscle flap in the pig model is remarkably similar to the human LD muscle. Getty states that the LD muscle

takes origin from the lower 4 ribs and inserts together with teres major into the humerus. The muscle lies superficially deep to the pannniculus carnosus and can be easily isolated on its principal thoracodorsal vascular pedicle, which remains superficial to the deep fascia (Getty 1975).

There are a variety of muscles in the pig that could be used in a skeletal muscle flap model. The rectus abdominis is a good-sized muscle with reliable vascular anatomy, however is technically a more challenging dissection taking a longer period of time (Boyd et al. 1990). The gracilis muscle has been raised by Addison et al in a hindlimb remote IPC porcine model (Addison et al. 2003). However, due to the hind limb anatomy of the pig, this muscle is shorter and more triangular than in the human. Further to this, the resultant scar and postop swelling on the inner aspect of the hindlimb may interfere with animal mobility after surgery.

The LD muscle flap is a superficial flap with surface markings that are easy to palpate behind the forelimb shoulder. The skin incisions are in such a place that they heal well with low risk of infection or dehiscence. With an average 18kg animal, we were able to consistently raise bilateral 13 x 8cm LD muscle flaps, isolated on the thoracodorsal pedicle. The musculotendinous humeral insertion was left intact but ligated to insure only a single blood supply remained. The muscles can be elevated and denervated without affecting the animal's forelimb locomotion. All muscles were raised through standardised trap-door skin incisions on the flanks.

We decided not to involve a microvascular anastomosis of the thoracodorsal vessels in our muscle flap model. We realise that effectively, we are using a pedicled muscle flap model to validate ischaemic preconditioning in free muscle transfer. To ensure there were no neurogenic influences, the motor nerve to LD was transected, however it can be argued that there are microscopic sympathetic nerves adherent to the pedicular vessels which will not be divided. This was addressed by Addison et al who showed that intravenous injection of the ganglionic blocker Hexanthonium prior to preconditioning failed to block the preconditioning effect of remote IPC (Addison et al. 2003). Therefore, we believe that there are no neurogenic influences in our model and it is representative of a model for free tissue transfer.

The porcine LD muscle flap model has been extensively employed and validated in our lab (Mounsey et al. 1992; Pang et al. 1995; Pang et al. 1997; Hopper et al. 2000). We raised a standard 8 x 13cm LD flap, which has been used in the previous studies performed with this muscle flap model. We subjected our flaps to a standardised 4-hour ischaemic interval, which produced an infarct rate of 40 +/- 2%. This infarction rate compares to previously published results using a 4-hour ischaemic interval: 38 +/- 6% (McAllister et al. 2008); 46 +/- 2% (Moses et al. 2005); 44 +/- 4% (Addison et al. 2003).

4.3 The dose-response effect of Nicorandil-induced late phase infarct protection

Nicorandil was given as an intravenous bolus + 60 minute infusion 24 hours before the onset of the 4 hour ischaemic insult followed by 48 hours of reperfusion. When deciding on which incremental doses to use, we were guided by previous large animal studies. Tang et al (Tang et al. 2004) gave a similar i.v. bolus + 60 minute infusion to conscious rabbits, with a total concentration of 1.9 mg/kg. Mizumuru et al, in a canine myocardial infarct model, used a dose of 0.85 mg/kg given over 75 minutes and a dose of 2 mg/kg given over 190 minutes (Mizumura et al. 1995; 1996). Galie et al used an intravenous infusion of Nicorandil with a range of dosage from 1.6 to 3.6 mg/kg in a porcine myocardial infarct model (Galie et al. 1995). Verdouw et al also employed a range of dosage from 0.15 to 1.5 mg/kg over an infusion period of 10 minutes in a porcine myocardial model (Verdouw et al. 1987). Finally, Lamping et al (Lamping et al. 1984) used an i.v. bolus + 140 minute infusion in a canine myocardial model, giving a total Nicorandil concentration of 3.6 mg/kg.

We therefore chose a range of incremental concentrations of Nicorandil (0.1 mg/kg to 3 mg/kg), delivered by an initial i.v. bolus followed by a 60 minute infusion. 24 hours later, the LD muscle flaps were subjected to 4 hours global ischaemia followed by 48 hours reperfusion. After 48 hours reperfusion, muscle infarction was assessed by the nitroblue tetrazolium reduction technique.

At dosages of 0.1 – 0.3 mg/kg Nicorandil, there was only partial protection conferred against I / R injury with a reduction in percentage muscle infarction

from 42% to 32%. Further increasing the dose to a range of 1 - 2 mg/kg resulted in a minimal further reduction in muscle infarction to 27%. Once the Nicorandil concentration was increased to 3 mg/kg, we achieved a more stable response rate with a reliable reduction in muscle infarction with a mean of 22%. Despite the significant reduction in infarction achieved with a Nicorandil concentration of 1-2 mg/kg, we found a rather inconsistent range of results with these dosage parameters. It was therefore decided to use the optimal concentration of 3 mg/kg for our experiments.

Taken together these results demonstrate that with incremental i.v. Nicorandil concentration, increasing reduction in the percentage muscle infarction was seen, when compared to the ischaemic control. Several animals were injected with Nicorandil concentrations of 4-5 mg/kg, however no further reductions in the infarction were seen and not enough numbers were performed to include in our charted results.

4.4 The effect of intravenous Nicorandil on mean arterial blood pressure (MAP) in the porcine model

The measurement of mean arterial pressure (MAP) was performed over a period of 90 minutes on anaesthetised animals. Our results have shown that a dose of 3 mg/kg i.v. Nicorandil resulted in a significant reduction in MAP over a period of 60 minutes, but the MAP normalised once the Nicorandil infusion has ceased (Figure 3.2).

This larger dose of Nicorandil was similar to that used by Lamping et al in their canine myocardial model (Lamping et al. 1984). The infarct reduction induced by Nicorandil was also accompanied by a 25 – 30 mmHg reduction in MAP in Lamping's study. Mizumura et al surmised that the beneficial effect on infarct size could be due to its peripheral haemodynamic effect as opposed to a direct action on the K_{ATP} channels (Mizumura et al. 1995). That may be true in the case of acute phase ischaemic preconditioning, as in Lamping's study. However, in our study, a hypotensive dose of i.v. Nicorandil was given 24 hours before the onset of our ischaemic insult. Markham et al (Markham et al. 2000) reviewed the pharmacokinetics of the drug and stated that "a single dose of intravenous Nicorandil is almost entirely eliminated from plasma within 8 hours". They further reported that the half-life of this rapid phase of elimination was approximately 45 minutes. These findings correspond to our blood pressure data showing a rapid normalisation of MAP after completing the i.v. infusion. Further to this, Verdouw et al demonstrated in a porcine model, that Nicorandil exerted a pronounced effect on preload (venous dilatation) and afterload (systemic arterial dilatation) even at lower concentrations. They also performed regional haemodynamic studies using the radioactive microsphere technique and demonstrated that even at the higher concentrations of Nicorandil, there was no significant increase in regional blood flow to skeletal muscle (Verdouw et al. 1987).

One potential limitation of our methodology, which may have influenced the MAP data, is regarding the device used to measure blood pressure. The polyethylene cannula (Becton Dickinson, Franklin Lakes, NJ, USA) that was used, was too wide bore to cannulate any peripheral arteries, therefore the

femoral artery was used. This meant an exploratory incision and dissection of the vessel to allow ligation and placement of the cannula. This required induction of general anaesthesia and intubation of the animal prior to MAP measurement. A pentobarbital infusion was used, as described earlier in the methodology. Pentobarbital sodium was used as maintenance anaesthesia during the flap-raising surgery due to it's non-hypotensive effects, however this preservation of blood pressure during anaesthesia is due to an increase in systemic vascular resistance as shown by Thomson et al (Thomson et al. 1991). In the presence of a potent nitrate vasodilator like Nicorandil, inhibiting systemic vascular resistance reduces afterload. Therefore pentobarbital anaesthesia may in fact contribute to the hypotensive effects seen during Nicorandil infusion. To combat this issue the lab has recently upgraded the apparatus to allow dorsal ear arterial cannulation and monitoring under ketamine sedation. Unpublished findings more recently have demonstrated higher resting / control MAP, as well as a less pronounced reduction in MAP. Further work is required to substantiate these observations.

4.5 The Efficacy of Nicorandil in Induction of Late Phase IPC of Porcine Skeletal Muscle

This is the first report on the efficacy and time course of pharmacological late phase IPC to protect skeletal muscle from I/R injury. Intravenous Nicorandil (3 mg/kg) given 24 hr before the onset of a 4 hr ischaemic insult followed by 48 hr reperfusion, results in a significant reduction in skeletal muscle infarction from

40 +/- 2% (ischaemic control) to 22 +/- 2%. This equates to a 45% reduction in muscle necrosis. This is comparable to the efficacy of remote ischaemic preconditioning, where instigation of 3 cycles of 10 minute occlusion / reperfusion in the hind limb of a pig induced a late phase of protection with a 40% reduction in the muscle necrosis (Moses et al. 2005). Further to this, the efficacy of Nicorandil-induced late IPC is comparable to acute / classical IPC. Pang et al demonstrated a 44% reduction in infarct size after acute preconditioning of porcine LD muscles with 3 cycles of 10 minutes occlusion / reperfusion (Pang et al. 1995).

The efficacy of Nicorandil-induced late phase IPC has been demonstrated in other species. Iliodromitis et al demonstrated that an oral dose of Nicorandil recaptured the waning effect of acute IPC in a rabbit myocardial infarct model, however they were unable to show that Nicorandil initiated IPC by itself (Iliodromitis et al. 2003). Bolli's group, in their rabbit myocardial infarct model, also demonstrated the efficacy of Nicorandil-induced late phase IPC. Using an i.v. bolus + 60 minute infusion of Nicorandil 24 hours before the ischaemic insult, they showed a 54% reduction in myocardial infarct size compared to the ischaemic control (Tang et al. 2004).

Since the advent of Nicorandil as a potent pharmacological preconditioning agent against myocardial ischaemia, researchers have investigated its potential to protect other solid organs from ischaemia reperfusion injury. More recently Suto et al demonstrated Nicorandil-induced protection of small intestine from I/R injury (Suto et al. 2011). Shimizu and colleagues reported that I/R injury of rat kidney was ameliorated by Nicorandil (Shimizu et al. 2011). Further to this,

Yamazaki et al demonstrated the effectiveness of i.v. Nicorandil in providing protection of hepatic tissue from subsequent ischaemic injury (Yamazaki et al. 2011).

4.6 The Time Course of Nicorandil in Induction of Late Phase IPC of Porcine Skeletal Muscle

We clearly demonstrated a biphasic response in infarct reduction instigated by Nicorandil injection. Nicorandil induced an acute phase of IPC, which lasted for 4-6 hours before waning. A second window of protection appeared 12-24 hours following Nicorandil injection, which was as robust as the acute phase and was maintained for a period of 48 hours before disappearing (Figure 3). This is the first study to report the entire biphasic time course of Nicorandil-induced IPC, showing the initial waning period at 4-6 hours, followed by the disappearance of the preconditioning effect after 72 hours post injection. This is also the first report of Nicorandil-induced late IPC in skeletal muscle.

Nicorandil-induced late IPC has been studied before in Bolli's rabbit myocardial infarct model. They reported a significant reduction in myocardial infarct size with a single i.v. dose of Nicorandil, given 24 hours before the ischaemic insult. However, they only reported on a single time-point of 24 hours for late phase IPC (Tang et al. 2004). There have been several earlier studies published on pharmacological late phase IPC of skeletal muscle. Interestingly, Wang et al triggered late-phase IPC in rat cremaster muscle with sodium nitroprusside (a donor of nitric oxide) (Wang et al. 2000). Maldonado's lab published two

studies demonstrating the efficacy and time-course of late-phase IPC in rat LD muscle models (Quan et al. 2004; Harralson et al. 2005). They corroborate our evidence that the duration of late-phase protection lasts from 24 to 72 hours before ischaemia, but disappears at 96 hours (Harralson et al. 2005). However, this late IPC stimulus was elicited mechanically and is demonstrated in a devolved animal model with inherent differences in skeletal muscle anatomy and morphology as has been discussed earlier.

4.7 Mechanism of Nicorandil-Induced Late Phase IPC of Porcine Skeletal Muscle

Late-phase preconditioning with a single intravenous dose of Nicorandil 24 hours before the onset of ischaemia resulted in a significant reduction of mitochondrial free calcium (m[Ca^{2+}]), preservation of muscle ATP content and a reduction in myeloperoxidase activity during the 1st hour of reperfusion, when compared with the ischaemic control levels. This is the first in-vivo study to demonstrate a reduction in m[Ca^{2+}] secondary to Nicorandil-induced IPC.

These findings agree with previous work in our lab by Moses et al. They induced late-phase IPC by noninvasive remote IPC using a hindlimb tourniquet. Muscle biopsies were taken at similar ischaemic and reperfusion time-points from the dorsal edge of the LD muscle flap. They also reported that late-phase IPC is associated with preservation of muscle ATP content and a significant reduction of neutrophilic myeloperoxidase activity in the first 90 min of

reperfusion (Moses et al. 2005; Moses et al. 2005). However, they did not measure m[Ca^{2+}].

4.7.1 Mitochondrial Free Calcium Content

We chose to measure the mitochondrial free calcium content (m[Ca^{2+}]) because of its central role in the mechanism of mitochondrial calcium overload leading to cell necrosis. Ca^{2+} is primarily transported into mitochondria via uniporters, once the cytosolic Ca^{2+} concentration rises to a certain threshold. Ca^{2+} equilibration in the mitochondria is brought about by mitochondrial Na^{+} / Ca^{2+} exchangers that pump Ca^{2+} back into the cytoplasm. During oxidative stress conditions, i.e. reperfusion injury, these cation exchangers can reverse resulting in Ca^{2+} transport back into the mitochondria (Ermak et al. 2002).

The potassium ion (K^{+}) is the most important cation regulator of mitochondrial activity. As stated earlier, mitochondria have K^{+} impermeable membranes and therefore rely on bidirectional K^{+} cycling via K^{+} / H^{+} antiporters (K^{+} efflux) and ATP-sensitive K^{+} channels (K^{+} entry). Opening of K_{ATP} channels in skeletal muscle cells during an ischaemic insult, results in a shortening of the myocyte action potential, which leads to a reduction of cellular Ca^{2+} influx and ultimately prevention of cytosolic Ca^{2+} overload.

Nicorandil is a potent K_{ATP} channel opener, which has been shown in our study to induce a late phase of IPC resulting in infarct protection associated with a significant reduction in m[Ca^{2+}] during the first hour of reperfusion. Nicorandil-

induced activation of K_{ATP} channels leading to prevention of m[Ca^{2+}] overload has been demonstrated in several models of isolated cardio-myocytes (Holmuhamedov et al. 1999; Ishida et al. 2004). However to date there are no in-vivo animal studies that have demonstrated this link.

4.7.2 Muscle ATP Content

We have reported here that preconditioning with i.v. Nicorandil, results in a significant preservation of skeletal muscle ATP content during the first hour of reperfusion. This is in keeping with previous studies in our lab demonstrating an increase in ATP content during reperfusion, following late remote IPC (Moses et al. 2005), early remote IPC (Addison et al. 2003) and early local IPC (Pang et al. 1995).

Other investigators have demonstrated a similar preservation of muscle ATP content in the myocardium of the dog (Murry et al. 1990), rabbit (Janier et al. 1994; Takaoka et al. 1999) and rat (Kobara et al. 1996). In contrast, there is a scarcity of papers demonstrating this finding in Nicorandil preconditioning. Early investigations of Nicorandil by Garrett Gross' lab reported preservation of endocardial ATP and total adenine nucleotides in Nicorandil-induced acute preconditioning in a canine myocardial model (Pieper et al. 1987). This finding was later corroborated by Murakami et al in a pig I/R model, where Nicorandil pretreatment preserved energy metabolism in the sub-endocardium by increasing ATP (Murakami et al. 2002).

There is still debate regarding the mechanism by which IPC preserves total muscle ATP content. Murry and colleagues propose that this increase in ATP content is due to a reduction in myocardial energy demand during ischaemia, as well as a reduced rate of ATP consumption (Murry et al. 1990). Janier et al concluded that IPC enhanced glucose uptake and anaerobic glycolytic production of ATP (Janier et al. 1994). Vuorinen et al (Vuorinen et al. 1995) indicated that it was in fact the inhibition of the enzyme mitochondrial ATP synthase activity that leads to sparing of ATP in the reperfusion period. This hypothesis has since been disproved by Vander Heide et al (Vander Heide et al. 1996) and Bosetti et al (Bosetti et al. 2000), who state that there is no difference in mitochondrial ATP synthase levels between IPC and ischaemic controls.

4.7.3 Neutrophilic Myeloperoxidase Activity

We have demonstrated in this study that levels of neutrophilic myeloperoxidase (MPO) activity did not change during the 4-hour ischaemic insult in both ischaemic control and Nicorandil pre-treated animals. However, during the first hour of reperfusion there was a significant reduction in the rise of MPO activity in the Nicorandil pre-treated group compared to the control group. This is in keeping with previous studies in our lab where late remote IPC (Moses et al. 2005), early remote IPC (Addison et al. 2003) and early local IPC (Pang et al. 1997) significantly attenuated MPO activity during early reperfusion of porcine skeletal muscle.

It has been well documented that neutrophil accumulation occurs during early reperfusion. Several myocardial models have demonstrated that reduction of myocardial I/R injury by classical IPC is associated with a reduction in neutrophilic myeloperoxidase accumulation during early reperfusion (Mizumura et al. 1995; Wang et al. 1999). Further to this, Nicorandil-induced preconditioning of the myocardium in the dog (Mizumura et al. 1995) and pig (Galie et al. 1995) model has reported that the infarct protection is associated with a reduction in myeloperoxidase accumulation during reperfusion.

Despite these findings there is still much controversy regarding the role of neutrophils in I/R injury. Neutrophils are known to generate Reactive Oxygen Species (ROS), which can result in tissue injury, and so are seen causally in the development of I/R injury. On the other hand, neutrophil accumulation is seen simply as an inflammatory response to myocyte necrosis. This is supported by evidence indicating that I/R injury occurs in neutrophil-free systems, such as buffer-perfused rabbit hearts (Liu et al. 1992) and isolated ventricular myocytes (Maddaford et al. 1999). Further to this Vanden Hoek et al (Vanden Hoek et al. 1997) were able to generate ROS during the ischaemic insult to isolated cardiomyocytes.

4.8 Pathways of Nicorandil-Induced Late Phase IPC of Porcine Skeletal Muscle

The phenomenon of IPC is a complex multifactorial pathway, occurring in various species, in a number of tissue types. Researchers have targeted several

of the upstream 'triggers' of IPC, such as the Adenosine receptor (Auchampach et al. 1993; Baxter et al. 1994; Addison et al. 2003), opioid receptor (Addison et al. 2003), as well as the signal transduction pathway involving Protein Kinase C (Hopper et al. 2000; Uchiyama et al. 2003). These different triggering and mediator mechanisms converge on a downstream end-effector pathway resulting in activation of ATP-sensitive potassium (K_{ATP}) channels, prevention of mitochondrial calcium overload and preservation of muscle ATP content.

We decided to investigate this end-effector pathway of K_{ATP} activation and its involvement in Nicorandil-induced late-phase IPC of skeletal muscle. The involvement of K_{ATP} channels in late-phase IPC was identified by Mei et al in a canine myocardial model (Mei et al. 1996), and by Bernardo et al (Bernardo et al. 1999) in a rabbit model. This involvement was also discovered to translate to skeletal muscle by Moses et al (Moses et al. 2005) in the porcine LD muscle flap model.

4.8.1 Role of the sarcolemmal K_{ATP} channel (sK_{ATP})

We have demonstrated, with the results of Study 5, that sK_{ATP}, but not mK_{ATP} channels, are involved in the trigger mechanism of Nicorandil-induced late phase IPC of porcine LD muscle flaps. Specifically, the selective sK_{ATP} channel inhibitor HMR-1098 abolished the preconditioning effect of Nicorandil, when given 24 hours before the onset of a 4-hour ischaemic insult followed by 48 hours reperfusion. The non-specific K_{ATP} channel inhibitor Glibenclamide was also effective in blocking the preconditioning effect of Nicorandil, when given

24 hours before the onset of the 4-hour ischaemic insult. Further to this, the selective mK_{ATP} channel inhibitor 5-HD was ineffective when given before Nicorandil injection, 24 hours before the onset of the ischaemic insult.

This result compares with previous findings in the porcine skeletal muscle model, where it was observed that sK_{ATP} channels were involved in the trigger mechanism of hindlimb remote IPC for late-phase protection of skeletal muscle against infarction (Moses et al. 2005). Our results in skeletal muscle corroborate those in the myocardium where Patel et al demonstrated that the sK_{ATP} channel was required as a trigger but not a mediator for delayed cardioprotection in the rat heart, elicited by mechanical late IPC (Patel et al. 2005) and opioid-induced late IPC (Patel et al. 2002). Further to this Gross et al found that the sK_{ATP} channel blocker HMR-1098 was able to block delayed cardioprotection elicited by Diazoxide (K_{ATP} agonist) (Gross et al. 2003). However, this goes against the findings of Takashi et al (Takashi et al. 1999), who reported in a rat myocardial infarct model, that activation of mK_{ATP} channels triggered late-phase preconditioning, although they did not look at sK_{ATP} channel function in this regard.

The activation of sK_{ATP} channels and their role in acute IPC is unclear. In Nicorandil-induced acute IPC, Sato demonstrated involvement of mK_{ATP} but not sK_{ATP} channels (Sato et al. 2000), whereas it was confirmed that both mK_{ATP} and sK_{ATP} are involved in the acute-phase cardioprotection afforded by Diazoxide (Tanno et al. 2001) and Nicorandil (Tsuchida et al. 2002). These complex findings can be summarised by stating that there is evidence in several species and tissues to implicate the involvement of sarcolemmal K_{ATP} channels in the

trigger mechanism of delayed IPC. The role of sK_{ATP} channels in acute IPC is less clear and probably involves both sK_{ATP} and mK_{ATP} channels.

4.8.2 Role of the mitochondrial K_{ATP} channel (mK_{ATP})

In study 6 we reported that mK_{ATP} channels, but not sK_{ATP} channels, are involved in the mediator mechanism of Nicorandil-induced late phase IPC of porcine skeletal muscle flaps. Intravenous injection of the selective mK_{ATP} inhibitor 5-HD or the non-selective mK_{ATP} inhibitor Glibenclamide 10min before the onset of 4 hours ischaemia abolished the late-phase preconditioning effect of Nicorandil. In contrast, the selective sK_{ATP} inhibitor HMR-1098 failed to block the late preconditioning effect of Nicorandil when given directly before the 4-hour ischaemic insult.

This again compares to the results by Moses et al, where it was reported that mK_{ATP} channels were involved in the effector mechanism of hindlimb remote IPC for late-phase protection of skeletal muscle against infarction (Moses et al. 2005).

The evidence for mK_{ATP} channels as a mediator / end-effector in late-phase IPC is fairly unanimous. Bernardo (Bernardo et al. 1999) and Mullenheim (Mullenheim et al. 2001) both reported, in the rabbit model, that late-phase cardioprotection was abolished by the non-selective K_{ATP} blocker Glibenclamide as well as the selective K_{ATP} blocker 5-HD, thereby implicating the mK_{ATP} channel as a mediator in late IPC. This was further corroborated by Mei and

colleagues, who reported that both Glibenclamide and 5-HD inhibited pharmacologically induced late-phase IPC in the canine myocardial model (Mei et al. 1996).

As stated earlier, Nicorandil is a hybrid clinically licensed drug, acting as a nitric oxide donor and K_{ATP} channel opener. There is certainly some controversy regarding the role of Nitric Oxide (NO) in the late phase of preconditioning. Bolli's group have stated in several papers that NO is pivotal in the initiation and mediator pathways of late phase of IPC in the conscious rabbit myocardial model (Qiu et al. 1997; Takano et al. 1998). Further to this, Wang et al reported that NO initiated late-phase infarct protection in rat skeletal muscle (Wang et al. 2000) and murine myocardium (Wang et al. 2005).

On the other hand, when Nicorandil elicits its infarct reduction effect, Mizumura et al reported mediation by K_{ATP} channels and not by NO (Mizumura et al. 1996). Furthermore, Post et al confirmed that NO is not involved in classical (acute) IPC of porcine myocardium (Post et al. 2000). However, to date there are no studies examining the role of K_{ATP} channels in Nicorandil-induced late IPC. Tang et al demonstrated Nicorandil-induced late phase IPC in conscious rabbits, however they did not look at the role of K_{ATP} channels (Tang et al. 2004). In isolated myocyte preparations, Nicorandil acts directly to open mK_{ATP} channels (Sato et al. 2000) which in turn attenuates mitochondrial Ca^{2+} overload (Ishida et al. 2004). In addition to this, we have shown that the non-selective K_{ATP} channel inhibitor Glibenclamide completely abolished the Nicorandil-induced infarct protective effect, when given before Nicorandil or before ischaemia, returning the infarction rate to a level comparable with the ischaemic

control. One hypothesis is that the nitrate-type effects of Nicorandil may serve to potentiate the K_{ATP} channel activation, rather than operate independently, as eluded to in the ventricular myocyte model of Sasaki et al (Sasaki et al. 2000). This would suggest Nicorandil's main mechanism of action in late-phase IPC is by activation of K_{ATP} channels.

4.9 Conclusion

In surgical specialties such as Plastic, Orthopaedic and Vascular surgery, a muscle or groups of muscles are placed under ischaemic conditions, either through tourniquet or vascular clamp control. Further to this, autogenous free muscle transfer is used routinely in plastic surgery to reconstruct form and/or function. Human skeletal muscle is known to have a critical ischaemia time of approximately 2.5 hours, however functionality of muscle contraction may be affected well before this time. Protracted operations with long tourniquet times, as well as unpredictable complications such as thrombosis and vasospasm can prolong this ischaemic interval leading to irreversible muscle necrosis.

The phenomenon of IPC has evolved from the localised invasive cyclical clamping of blood vessels, to remote IPC with a hind-limb tourniquet. With the advent of pharmacomimetic drugs, we are now able to elicit infarct protection with a systemic single dose in myocardial as well as skeletal muscle.

This is the first study to demonstrate the efficacy of late-phase IPC with intravenous Nicorandil, to provide 48 hours of uninterrupted infarct protection from ischaemia / reperfusion injury in skeletal muscle. Specifically, pharmacological preconditioning by Nicorandil was biphasic with an acute phase that appeared when intravenous injection of Nicorandil was given immediately and up to 4-6 hours before the ischaemic insult. This acute phase of preconditioning waned; and then a second / late phase of protection appeared at 12-24 hours following Nicorandil injection and lasted for a period of 48 hours before disappearing. This late phase of protection was as robust as the acute phase, producing a 45% reduction in the amount of muscle necrosis, compared to ischaemic controls.

As well as producing an equally robust reduction in muscle necrosis, we demonstrated that late phase pharmacological preconditioning by Nicorandil reduced the amount of mitochondrial Ca^{2+} overload during early reperfusion. Nicorandil pre-treatment was also responsible for attenuating neutrophil infiltration during early reperfusion, as seen by a significant reduction in myeloperoxidase activity. We further demonstrated that Nicorandil preconditioning reduced the rate of ATP depletion in the myocyte during early reperfusion injury, with a significant preservation of muscle ATP content, compared to the ischaemic control.

Pharmacological probes were employed to delineate the molecular mechanism of Nicorandil preconditioning. The selective sarcolemmal K_{ATP} (sK_{ATP}) channel inhibitor HMR-1098 abolished the preconditioning effect when given before Nicorandil injection, but had no effect when given 24 hours later, before the

ischaemic insult. In addition, the selective mitochondrial K_{ATP} channel inhibitor 5-HD abolished the preconditioning effect when given before the 4-hour ischaemic insult but had no effect when given 24 hours earlier, before Nicorandil injection. Taken together these findings demonstrate that sarcolemmal K_{ATP} channels are involved in the trigger mechanism, and mitochondrial K_{ATP} channels in the mediator mechanism of Nicorandil-induced late phase IPC of skeletal muscle.

The additional use of the non-selective K_{ATP} inhibitor Glibenclamide abolished the preconditioning effect when given before Nicorandil pretreatment or before ischaemia. These findings infer that the late-phase preconditioning effect is primarily by the K_{ATP} channel-opening effect of Nicorandil and not by its nitrate donor properties. However, to confirm these findings, further study is required using pharmacological probes to block the nitrate effects of Nicorandil during the triggering mechanism.

With the establishment of the molecular mechanism of early and late phase pharmacological preconditioning by Nicorandil, it is hoped that further studies can be designed to look at the reliability of Nicorandil as a pharmacological preconditioning agent for patients undergoing elective free muscle transfer in reconstructive surgery.

5.0 PERSPECTIVES & LIMITATIONS

With the discovery of Nicorandil as a potent K_{ATP} channel opener, we have demonstrated the potential use of a clinical drug in elective global preconditioning of skeletal muscle prior to elective reconstructive, vascular or musculoskeletal surgery. Not only can Nicorandil trigger an immediate infarct-protective effect, it can transmute skeletal myocytes to an ischaemia-tolerant phenotype. This infarct protection can last for a period of 48 hours, which is the crucial post-operative window in autogenous free muscle transfer, with regard to anastomotic complications such as vasospasm and pedicle thrombosis.

Since the advent of the first human hand transplant in 1998, the field of composite tissue allotransplantation (CTA) has been advancing exponentially. Ischaemia Reperfusion (I/R) injury has been shown to contribute to acute rejection in animal allotransplant models (Pradka et al. 2009). Further to this, a clear link has also been demonstrated between prolonged ischaemia time and long-term graft survival in solid organ transplantation (Opelz et al. 1994). Several strategies have been investigated to prevent I/R injury in solid organ transplantation, such as the induction of heat shock proteins and the use of cooled preservation solutions like 'University of Wisconsin' solution (Caterson et al. 2013). However there is very little evidence of successful intervention strategies to reduce I/R injury in CTA.

CTA is more technically and logistically challenging than autogenous free tissue transfer and this is reflected by the prolonged ischaemia times. Hand

transplantation cases average 6 hour ischaemia times (Petruzzo et al. 2011) and face transplantation approximately 4 hours (Pomahac et al. 2012).

Baumeister et al used the intervention of heat stress preconditioning to upregulate heat shock protein 72 (HSP-72), demonstrating a reduction in I/R injury of hind limb allotransplants in a rat model (Baumeister et al. 2004). Further to this, Villamaria et al recently validated a porcine CTA model using allogeneic gracilis myocutaneous flaps (Villamaria et al. 2012). It would therefore be feasible to examine the impact of Nicorandil preconditioning in a porcine microvascular CTA model. This could lead to the discovery of an exciting new therapeutic intervention against I/R injury in solid organ and composite tissue allotransplantation.

A perceived limitation of this study was the use of Nicorandil in the intravenous form. The majority of studies have also looked at the efficacy of intravenous Nicorandil, given by a bolus + infusion. This has the risk of exposing the animal to the vasoactive effects of the drug, resulting in reduced vascular resistance and ultimately a fall in mean arterial pressure. Interestingly, Iliodromitis et al studied the efficacy of oral Nicorandil in a rabbit myocardial model. They gave an oral dose of 5 mg/kg for 5 consecutive days prior to ischaemic insult, reporting a preservation of the IPC effect, which waned after 4-6 hr in the ischaemic control. However, this cannot be concluded as true late-phase IPC as the interval was only 90 minutes, and the oral Nicorandil by itself was insufficient to initiate IPC (Iliodromitis et al. 2003).

Despite this, the efficacy of oral Nicorandil in human clinical trials, such as the 'Impact of Nicorandil in Angina' (IONA) study (2002) and the CESAR 2 trial (Patel et al. 1999), has demonstrated the myocardial protective effect of this drug when chronic oral dosing is employed. Further to this, Qi et al demonstrated that oral dosing with up to 30 mg/kg Nicorandil enhanced the survival of ischaemic skin flaps in a rat model (Qi et al. 2006). This effect was probably due, in part, to a direct nitrate vasodilator effect on the cutaneous vascular plexuses. Nicorandil's effect may have also been due to its anti-apoptotic action on endothelial cells. This has been confirmed in-vitro by Date et al where it was found that Nicorandil could inhibit apoptosis of endothelial cells by activation of mitochondrial K_{ATP} channels (Date et al. 2005).

The bioavailability of Nicorandil in the oral format is >75%. Peak plasma concentrations of the drug occur 0.5 to 1 hour after administration and steady state dosing can be achieved within 4 days (Markham et al. 2000). This suggests a novel application for this drug, where a repeated oral dose could be administered to provide a chronic level of infarct protection for a dedicated post-operative period. Oral Nicorandil could provide infarct protection for autogenous free muscle, myocutaneous and fasciocutaneous flap transfer. As well as this, our findings may lead to further work looking at Nicorandil-induced late-phase preconditioning of solid organs against I/R injury. They could have huge therapeutic potential in transplant surgery. There is already evidence that Nicorandil acutely protects intestine (Suto et al. 2011), kidney (Shimizu et al. 2011) and liver (Yamazaki et al. 2011) from I/R injury. More studies need to be designed to look at the effects of chronic oral Nicorandil dosing in providing a

robust continuous period of infarct protection during the prolonged ischaemia of organ transplant surgery.

Another limitation of this project was the measured endpoint of myocyte necrosis in the LD muscle flaps of the pig. The amount of cell necrosis was quantified using the well-validated Nitroblue tetrazolium reduction technique, followed by computer planimetry to map out the areas of necrosis and give an overall percentage muscle infarction rate. This method has been well validated in our lab with the porcine LD flap model (Addison et al. 2003; Moses et al. 2005; Moses et al. 2005; McAllister et al. 2008), as well as in other animal models (Labbe et al. 1988; Qiu et al. 1997). However, is there a more sensitive measure of permanent myocyte dysfunction, rather than eventual cell necrosis?

We know that the critical ischaemic limit of skeletal muscle is approximately 2.5 hours, after which point reperfusion-induced irreversible tissue necrosis occurs (Sjostrom et al. 1982). However, less is known about the effect reperfusion injury has on contractility. Gurke et al was able to demonstrate that 3 cycles of ischaemic preconditioning resulted in an improvement in muscle endurance and contractility of post-ischaemic extensor digitorum longus muscle in a rodent model (Gurke et al. 1996; Gurke et al. 2000). Further to this, McLaughlin et al employed a similar method using the rat cremaster skeletal muscle model to show that treatment with the endogenous amino acid taurine, protected electrophysiological function in skeletal muscle against ischaemia reperfusion injury (McLaughlin et al. 2000).

'Myocardial stunning' is the term given to abnormalities in systolic or diastolic contractile function of cardiomyocytes exposed to a period of ischaemia. Investigators have targeted this phenomenon, demonstrating the involvement of reactive oxygen species (ROS) and transient cytosolic calcium overload during the early phase of reperfusion (Bolli 1990; 1991). As we have seen throughout this discussion, the mechanisms of acute and late ischaemic preconditioning translate from myocardial to skeletal muscle animal models. Interestingly, late phase preconditioning has been shown to protect against myocardial stunning in the rabbit model (Bolli et al. 1997) but not in the pig (Qiu et al. 1997).

Although K_{ATP} channels are central to infarct protection, they do not appear to be involved in amelioration of myocardial stunning (Takano et al. 2000). Paradoxically, it appears that Nitric Oxide is more central to the reduction of myocardial stunning (Bolli et al. 1997; Bolli et al. 1997; Takano et al. 1998). However, there are few studies looking at the impact of Nicorandil on myocardial stunning. Iwamoto et al looked at Nicorandil's K_{ATP} opening and nitrate properties in the rabbit myocardial model and concluded that K_{ATP} channel opening was central to the protection of the myocardium from stunning but could not exclude a role for NO during reperfusion (Iwamoto et al. 1993).

The effects of Nicorandil on skeletal muscle contractile function have never been looked at and could highlight a therapeutic potential in optimising free muscle flaps for functional muscle transfer in the treatment of disorders such as facial palsy and Volkmann's ischaemic contracture.

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