

**AN *IN VITRO* MODEL OF
ISCHAEMIA-REPERFUSION INJURY
IN MURINE MYOCARDIUM:
THE ROLE OF NITRIC OXIDE**

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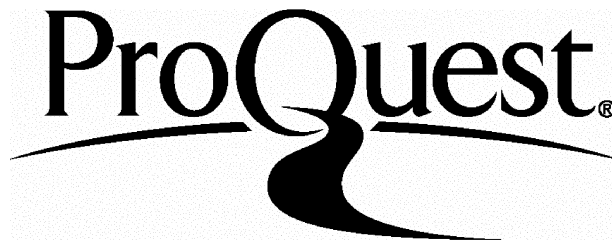
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This thesis is dedicated to my parents

ABSTRACT

Recent advances in understanding the mouse genome and genetic manipulative techniques have created a unique opportunity to explore the influence of single gene alterations on complex physiological systems. It is essential that physiologists respond to this challenge by careful design of appropriate, miniaturised systems to allow phenotypic changes to be explored. This thesis describes the stages in the establishment of a new model of ischaemia-reperfusion injury in murine myocardium. The first experimental chapter describes the detailed characterisation and validation of the model. Technical aspects of isolated mouse heart perfusion and construction of the Langendorff apparatus are discussed. The end-points of injury (infarct size, contractile recovery, and lactate dehydrogenase release) were co-correlated, and good dose-response relationships demonstrated against duration of global ischaemia. The second series of experiments showed that the model can be used to assess the efficacy of a well-known cardioprotective intervention (ischaemic preconditioning). Short episodes of global ischaemia were used to precondition against a subsequent 30 minute global ischaemic insult. Four periods of five minutes ischaemia were necessary to induce protection (a 42% reduction in infarct size). This was the first demonstration of this phenomenon in the isolated mouse heart using infarct size as the primary end-point.

Having established the model and demonstrated its capacity to assess the efficacy of a cardioprotective intervention, the final series of experiments investigated the role of nitric oxide in myocardial ischaemia-reperfusion injury using genetically-altered mice. 'Knockouts' for both constitutive isoforms of nitric oxide synthase (endothelial and neuronal) were used, together with wild-types, to assess the response to a protocol of global ischaemia-reperfusion injury. The results demonstrated, for the first time in murine myocardium, the protective effect of nitric oxide derived from endothelial nitric oxide synthase, and suggested a detrimental role for the neuronal isoform.

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CORRIGENDA

1. Section 1.3.4.2, page 28 (second paragraph, line 12): this sentence should be omitted.
2. Section 1.3.5, page 32: the first paragraph has been repeated from the introduction and should be omitted.
3. Section 3.1.5.2, page 61: the following sentence should be added to the end of the paragraph: 'Any hearts demonstrating abnormal coronary flow rate (<1.5 ml/min or >6 ml/min), bradycardia (heart rate <250 bpm), poor contractility (< 1.0 g developed force), or persistent arrhythmia (SVT, VT, or VF persisting after 20 min stabilisation) were excluded from further study.'
4. Table 3.1, page 62: a footnote should be added to this table as follows: 'Due to the chelating action of EDTA the level of free ionised Ca²⁺ in the buffer was 1.3 mM.'
5. Section 4.4.3.2, page 82 (first paragraph): the following sentence should be added at the end: 'In particular, the value of the LDH assay as a marker of myocardial necrosis in this model is in serious doubt for the reasons outlined above, and due to the considerable spread of the data it generates.'
6. Section 5.4.2.1, page 89 Figures 5.3 (a) and (b): the labelling of the column graphs is incorrect. Each control group should read (Group 5) and each preconditioned group should read, (Group 4).
7. Section 5.5 page 94 (second paragraph, line 6): the sentence starting 'Both species.....' should read as follows: 'Both species **have a relative** lack of the f_0/f_1 ATPase inhibitor.....'
8. Section 6.5.1, page 106: the following sentence should be inserted at the end of the first paragraph: 'The observed differences in coronary flow rates might also be related to the lower baseline heart rates (resulting in reduced metabolic rate) observed in both knockout groups.'

General points

1. In all experimental chapters, significance values (where relevant) are given in the footnotes for each table or figure.
2. Figure 4.4 and Figure 5.5 (a) are identical and are used to illustrate the characteristics of the contracture curve during global ischaemia.
3. It should be stressed that the mice used in the last experimental chapter (nitric oxide experiments) were a different strain from the NIH Swiss White mice used for characterisation and preconditioning studies.

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1. INTRODUCTION

1.1 INTRODUCTION TO MYOCARDIAL PROTECTION

The possibility that the heart may be rendered more resistant to the damaging effects of ischaemia-reperfusion injury is an attractive therapeutic goal. In the developed world, coronary heart disease now constitutes the most important independent cause of mortality. The treatment of myocardial infarction has been revolutionised by the use of thrombolytic agents which, if administered early, will frequently allow reperfusion and limit the size of the infarct. However, the benefit in terms of improved mortality diminishes rapidly if treatment is delayed. Endogenous mechanisms of myocardial protection provide a possible means by which the progress of myocardial necrosis might be slowed, increasing the time available for effective reperfusion. Similar considerations apply in patients with unstable angina and in those undergoing coronary artery surgery. Improved myocardial protection may also assist the preservation of explanted hearts prior to transplantation. A more detailed understanding of the cellular mechanisms involved in mediating cytoprotection may allow future development of pharmacological agents capable of invoking persistent protection against myocardial ischaemia.

The introduction to this thesis is split into three sections, reflecting the content and direction of the experimental chapters. The pathophysiology of ischaemia-reperfusion injury is discussed in some detail in the first section of the introduction since the cellular events are relevant to the interpretation of all the experimental data. The cardioprotective phenomenon of ischaemic preconditioning is discussed next to provide background information for the second experimental chapter in which its protective efficacy and the conditions necessary for its induction in the mouse heart were established. Finally the role of nitric oxide in modulating cardiac function and ischaemia-reperfusion injury is discussed, facilitating interpretation of the data from the last experimental chapter.

1.2 THE PATHOPHYSIOLOGY OF ISCHAEMIA-REPERFUSION INJURY

1.2.1 BACKGROUND

Myocardial injury during ischaemia and reperfusion is of considerable clinical relevance. Extensive laboratory and clinical research over recent years has concentrated on elucidating the pathophysiological events leading to irreversible cellular injury. Myocardial infarction is a dynamic process characterised by a transition from reversible to irreversible injury and culminating in fibrous scar tissue formation. The biochemical and structural changes that occur during ischaemia have been studied in detail, but the determinants of irreversible injury have not been identified. The area is further complicated by the concept of 'reperfusion injury' suggesting that restoration of blood flow to an ischaemic zone may cause further damage, notwithstanding the fact that it is a necessary prerequisite for tissue salvage. Furthermore, detailed characterisation of the events occurring during ischaemia and reperfusion involve the use of experimental models which do not accurately represent the clinical situation. Extrapolation of the findings from such studies must be considered with caution.

1.2.2 DEFINITION

A universally acceptable working definition of myocardial ischaemia that will facilitate appropriate design of experimental models has proved elusive. This is due to the continuous spectrum of severity and duration encountered in the complex pathophysiology of ischaemic heart disease. Coronary artery stenosis due to an atheromatous plaque may impede blood flow with metabolic insufficiency during exercise, but not at rest. An unstable plaque may rupture and complete vessel occlusion may occur due to the formation of platelet thrombus. This may (or may not) be followed by spontaneous lysis which could vary in extent. In the longer term, occluded vessels may undergo a process of recanalisation. Collateral vessels may open up during the evolution of coronary artery disease and the additional flow will frequently reduce the volume of myocardium 'at risk' from the consequences of reduced coronary blood flow. Changes in neurohumeral status, circadian rhythms, and alterations in substrate availability add further complexity.

Traditionally ischaemia has been defined in the relatively simple terms of 'supply and demand' with inadequate myocardial perfusion to meet the cellular metabolic requirements of the affected region. This leads to a state of metabolic failure (disequilibrium) and impaired electromechanical function. However, the lack of washout of metabolic waste products is also contributory. Ischaemia may be defined more generally as all the consequences for the cell of inadequate perfusion.

1.2.3 MYOCARDIAL INJURY DURING ISCHAEMIA

The extent of cellular damage resulting from an ischaemic insult will depend on its duration and severity, the degree of any collateral supply, cellular oxygen requirements, and the area of tissue 'at risk'. During the evolution of the process of necrosis, cells pass from a phase of reversible injury to one of inevitable cell death. Disruption of the sarcolemma is an early event in ischaemia, the presence of which indicates that the myocytes are already dead. Not all ischaemic myocytes become irreversibly injured simultaneously in experimental infarction, but myocytes die in a transmural wavefront of cell death proceeding from the subendocardial to the subepicardial myocardium (Jennings and Reimer, 1991). About six hours of ischaemia are required to complete the wave-front. During the reversible phase of ischaemic injury, reperfusion salvages all ischaemic myocytes in all layers, but once lethal injury begins to develop, reperfusion salvages reversibly injured myocytes that are located in the subepicardial and midmyocardial layers and thereby limits the transmural extent of infarction.

1.2.4 METABOLIC CHANGES DURING ISCHAEMIA

The metabolic changes associated with the sudden onset of ischaemia caused by occlusion of a major coronary artery include (a) cessation of aerobic metabolism, (b) depletion of creatine phosphate (c) onset of anaerobic glycolysis, and (d) accumulation of glycolytic products, such as lactate and alpha glycerol phosphate (α GP), and catabolites of the nucleotide pools in the tissue. These changes are associated with contractile failure and electrocardiographic alterations. Since the demand of the myocardium for high-energy phosphate exceeds the available supply, the net amount of adenosine triphosphate (ATP) in

tissue decreases. Eighty percent of the supply of high-energy phosphate utilised by severely ischaemic tissue comes from anaerobic glycolysis using glycogen as the principal substrate. Early in ischaemia, contractile activity utilises ATP, but much of the continuing utilisation of ATP by the ischaemic tissue is energy wasted via the mitochondrial ATPase. A lesser quantity of ATP is used by ion transport ATPases. Metabolic changes slow as the duration of ischaemia increases. Irreversibly injured myocytes exhibit (a) very low levels of ATP (less than 10% of control); (b) cessation of anaerobic glycolysis; (c) high levels of H⁺, adenosine monophosphate (AMP), inosine, lactate, and α GP; (d) a greatly increased osmolar load; (e) mitochondrial swelling and formation of amorphous matrix densities; and (f) disruption of the sarcolemma (Jennings and Reimer, 1991).

1.2.5 ION SHIFTS DURING ISCHAEMIA AND REPERFUSION

Within seconds of reperfusion of an irreversibly injured ischaemic zone a phenomenon known as 'contraction-band necrosis' occurs. It is characterised by (a) condensation of myofibrillar protein into irresolvable bands (Herdson *et al.*, 1965), (b) massive intracellular oedema (Jennings and Reimer, 1983), (c) the appearance of granular densities of calcium phosphate in the mitochondria (Shen and Jennings, 1972b), (d) focal disruption of the sarcolemma, (e) explosive myocyte swelling (Whalen *et al.*, 1974), and (f) massive increases in myocardial Ca²⁺, Pi, Na⁺, and Cl⁻, with decreased levels of Mg²⁺ and K⁺ (Whalen *et al.*, 1974). Of considerable importance is the finding that the source of the accumulated Ca²⁺ is the arterial blood reperfusing the tissue (Shen and Jennings, 1972a).

The appearance of contraction-band necrosis is thought to be due to the excessive influx of Ca²⁺ and precipitate rise in sarcoplasmic levels. Normal contraction is known to be triggered by a more modest rise and when the rise is excessive the result is uncontrolled contraction and the appearance of contraction bands. An almost identical lesion is known to occur experimentally in a model of calcium overload, 'the calcium paradox' (Ganote and Nayler, 1985)), and can be induced by catecholamine poisoning and prevented by use of Ca²⁺ channel blockers (Fleckenstein, 1977).

The ionic events preceding the catastrophic rise in sarcoplasmic Ca^{2+} have been extensively investigated. During ischaemia *in vivo*, myocytes undergo a process of swelling almost immediately (Jennings *et al.*, 1986). This is due to the increased osmotic load caused by ischaemic metabolites, and is associated with efflux of sarcoplasmic K^+ ('injury current'). During the phase of reversible injury the extracellular K^+ concentration doubles while the extracellular Na^+ falls (Hill and Gettes, 1980). With continuing ischaemia a phase of irreversibility is reached during which there is a characteristic rise in intracellular H^+ (Rouslin *et al.*, 1986) due to lactate production from anaerobic glycolysis. This, in turn, leads to a rise in intracellular Na^+ due to the activity of the Na^+/H^+ exchanger. The rise of intracellular Na^+ is further pronounced by the failing activity of the sodium pump (Na^+/K^+ ATPase) as a result of falling levels of ATP. Intracellular Na^+ is exchanged for Ca^{2+} via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Steenbergen *et al.*, 1990).

Upon reperfusion and the resumption of aerobic metabolism further Ca^{2+} loading occurs resulting in massive contraction of myofibrils. In addition it is likely that Ca^{2+} entry also occurs through holes in the sarcolemma of myocytes that had died before reperfusion (Jennings and Reimer, 1983).

1.2.6 REPERFUSION INJURY

It is now widely accepted that there is sufficient experimental evidence to support the concept of reperfusion-induced injury. It follows from this that cells may be reversibly injured during ischaemia but are lethally damaged during reperfusion. If the process of reperfusion could be modified (*eg* by altering the composition of the reperfusate) more tissue might be salvaged. Several studies have attempted to address this issue, but most have failed to provide convincing evidence of reperfusion injury. This has been partly due to inappropriate timing of the intervention *eg* before or during the ischaemic or hypoxic period (Opie, 1989), or as a result of failure to differentiate between an alteration in rate of recovery and an alteration in its extent.

There are several unfavourable consequences of reperfusion-induced injury and these are categorised below:

1.2.6.1 Reperfusion-induced arrhythmias

These characteristically occur within seconds of the onset of reflow (Manning and Hearse, 1984), and have been observed in all species including man (Goldberg *et al.*, 1983). The clinical relevance of this phenomenon has been debated in the past. Certainly it is clear that the human heart is more resistant to these arrhythmias than other animal species. It is possible that this discrepancy may be due to the more gradual (and often only partial) reperfusion seen in thrombolysis as opposed to the sudden reperfusion used in most experimental models. Additionally, reperfusion in man frequently occurs several hours after the onset of ischaemia at a time when the incidence of reperfusion arrhythmias in animal studies is low. However, with the advent of pre-hospital thrombolysis life-threatening ventricular tachyarrhythmias may become more common. Most of the experimental work in the area of reperfusion-induced arrhythmias has concentrated on two areas: generation of free radicals and disturbed calcium homeostasis. Support for an association between the generation of reactive oxygen intermediates and arrhythmogenesis has arisen from electrophysiological studies in several species (Pallandi *et al.*, 1987). Free radical generation was shown to be associated with changes in action potential characteristics, early and late after depolarisations, and increased automaticity. Subsequent studies suggest a molecular mechanism for these observations (Vandeplassche *et al.*, 1990). Oxidant stress may modify the activities of ion-translocating proteins in the sarcolemma and sarcoplasmic reticulum causing intracellular calcium overload. Subsequent oscillatory release from intracellular calcium stores is associated with afterdepolarisations and tachyarrhythmias.

1.2.6.2 Myocardial stunning

This is defined as post-ischaemic contractile dysfunction that occurs in the presence of normal coronary flow and is fully reversible on prolonged reperfusion (Braunwald and Kloner, 1982). The duration of the dysfunction greatly exceeds that of the preceding ischaemia (Bolli *et al.*, 1988), but interventions such as the use of inotropic drugs can override its effects. The causative cellular events underlying the phenomenon are thought to

be similar to those involved in the genesis of arrhythmias. In a series of experiments in the dog heart, Bolli and co-workers have been able to demonstrate enhanced postischaemic recovery following administration of free radical scavengers (superoxide dismutase, catalase, mercaptopropionyl glycine, and dimethylthiourea) as well as drugs that inhibit their formation (Bolli, 1988). Of significant interest are the findings in relation to timing of administration of these drugs. If administered before ischaemia or one minute before reperfusion they were equally effective. However, when given one minute after the onset of reperfusion they were ineffective, suggesting that the critical radical-mediated injury occurs in the first few moments of reperfusion. As mentioned in relation to arrhythmogenesis, calcium homeostasis is thought to be adversely affected by the generation of free radicals. An attractive hypothesis involves a free radical induced increase in calcium causing a transitory 'overload' early in reperfusion that damages the contractile elements and reduces their sensitivity to calcium. This would explain the prolonged ventricular dysfunction and gradual improvement as new contractile proteins are synthesised, and also the contractile reserve of stunned myocardium when treated with inotropic agents and exogenous calcium later in reperfusion.

1.2.6.3 Microvascular injury

This may take several forms including endothelial swelling, increased capillary permeability, altered vascular responsiveness, denudation and thrombosis. The most severe form of microvascular injury is the 'no reflow' phenomenon which is defined by the inability to reperfuse the tissue after removal of the obstruction to arterial flow. It was initially described in cerebral circulation and was first reported in the heart in 1966 (Krug *et al.*, 1966). Microvascular injury forms an important component of ischaemia-reperfusion injury. The mechanisms underlying this damage to endothelial cells have been divided into six broad categories: (a) physical obstruction of vessels with neutrophils, red blood cells and platelets; (b) neutrophil activation; (c) altered endothelial function particularly with reference to free radical production; (d) altered production and action of nitric oxide; (e) altered platelet activity; (f) action of endothelin. Although it is outside the scope of this

thesis to discuss in depth all the mechanisms mentioned above, the role of nitric oxide has particular relevance and a full examination of all the evidence is included in section 1.3.

1.2.6.4 Lethal reperfusion injury (LRI)

This is the most controversial area. Many experimentalists have held the view that LRI does not exist, and that cells that die after reperfusion were predestined to do so. Under these circumstances reperfusion would be responsible for a process of 'accelerated necrosis' (Jennings and Reimer, 1992). However, it is generally more widely held that reperfusion may contribute to a lethal injury in cells that may have been salvageable under modified conditions. The occurrence of LRI is likely to be due to a number of additive factors. Many of these underlie the other adverse consequences of reperfusion and include calcium overload, mitochondrial injury, accumulation and activation of leucocytes, osmotic stress and free radicals. Much of the experimental work in this area has concentrated on a role for free radical scavengers. Unfortunately the results have proved difficult to interpret with any confidence due to inappropriate study design (Reimer *et al.*, 1989).

1.3 ISCHAEMIC PRECONDITIONING

1.3.1 BACKGROUND

In 1986 a series of experiments was reported in the dog heart (Reimer *et al.*, 1986), designed to investigate the relative contributions of ATP depletion and catabolite accumulation in the genesis of lethal ischaemic injury. Their experimental model involved repetitive brief ischaemic episodes, working on the premise that each ischaemic episode would cause cumulative ATP depletion while the intermittent reperfusion would wash out ischaemic catabolites. To their surprise they found that following the initial ischaemic period, ATP levels were not depleted further by subsequent similar ischaemic challenges. They also noted that no infarction occurred in six of the seven dogs studied. This result was contrary to the previously accepted view that repetitive ischaemia would cumulatively lead to infarction. The observation led the same group (Murry *et al.*, 1986) to test the hypothesis that the preservation of high energy phosphates was due to a slowing of

consumption during ischaemia associated with a rapid and protective adaptation of the myocyte. They tested this hypothesis by subjecting the myocardium to four sets of five minute coronary occlusions, separated by five minutes' reperfusion, before a sustained 40 minute ischaemic insult. They found that the preceding brief periods of ischaemia and reperfusion were protective, reducing infarct size to 25% of that seen in the control group. This phenomenon was termed 'preconditioning with ischaemia'. Following these initial studies, the protection obtained has been further characterised both in terms of time course and various end-points of cellular injury.

1.3.2 GENERAL FEATURES OF ISCHAEMIC PRECONDITIONING

In the strictest sense, ischaemic preconditioning refers to the delay of infarct development induced by one or more preceding cycles of ischaemia and reperfusion. It is important to realise that the evolution of necrosis is delayed but not prevented. Preconditioning will limit infarct size during a temporary coronary occlusion but not during a prolonged or permanent occlusion.

1.3.2.1 Induction (protocols and species)

The conditions required for induction of preconditioning have been investigated in a variety of animal species. Following the initial observations in the dog using four, five minute coronary occlusions it was found that a single occlusion of 2.5, five, or 15 minutes is equally effective (Murry *et al.*, 1991b). In rabbits, either one, five minute occlusion (Cohen *et al.*, 1991) or two, two minute occlusions (Miura *et al.*, 1991) are adequate. In the rat, the threshold is probably a single five minute occlusion (Yellon *et al.*, 1992), although one study suggested that three, five minute occlusions were required (Liu and Downey, 1992). Studies in the pig have shown that two, ten minute periods of ischaemia are effective (Schott *et al.*, 1990). An important consideration, however, is the end-point chosen as evidence for the induction of protection. In the rat heart, for example, protection against ischaemia-induced arrhythmias exhibits a 'dose-dependent' effect proportional to the number of preconditioning cycles (Lawson *et al.*, 1993b).

Complete coronary occlusion, however, is not an absolute requirement as part of a preconditioning protocol. Cyclical variation in flow caused by the formation and dislodgement of thrombi may also induce protection (Ovize *et al.*, 1992a), as can flow restriction due to coronary stenosis (Ovize *et al.*, 1992c). In contrast, effective protection can also be induced using continuous high flow by simple hypoxia followed by reoxygenation (Shizukuda *et al.*, 1992).

There has been less work directed at ascertaining the minimum length of reperfusion required between brief ischaemic episodes. One study in the rat suggested that a minimum period of one minute was required and that 30 seconds was too short (Alkhulaifi *et al.*, 1993).

1.3.2.2 The time course of protection

Several important issues arise in relation to the time course of ischaemic preconditioning. If the time interval between the preconditioning protocol and subsequent sustained ischaemic insult is extended, a point must be reached beyond which the protective effect is lost. The decay of the protective effect as the intervening reperfusion period is extended appears to be gradual. Using a canine model, a delay of 120 minutes between preconditioning protocol and ischaemic insult results in a substantial fall off in protection with infarct size reduction decreasing from 92% (with five minutes of intervening reperfusion) to 54% (Murry *et al.*, 1991b). In rabbits the decay in protection appears to be more rapid. In one study infarct size reduction fell from 84% to 45% when the intervening reperfusion period was extended from ten to 60 minutes and disappeared completely by 120 minutes (Van Winkle *et al.*, 1991). In pigs protection disappears between 30 and 60 minutes of reperfusion (Schott *et al.*, 1990), and in rats protection has gone after one hour of intervening reperfusion (Li *et al.*, 1992).

Some investigators have explored the possibility of reinstating protection once it has waned. The situation is unclear, with studies in the rabbit (Yang *et al.*, 1993) indicating that reinstatement is possible (using five minutes of ischaemia after 120 minutes of reperfusion), but not in the pig (Sack *et al.*, 1993).

If the length of the ischaemic insult following preconditioning is extended it becomes clear that the protection conferred is not absolute. Eventually the size of the subsequent infarct matches that of control hearts. In the dog heart no protection is seen against 90 minutes of ischaemia, whereas with 60 minutes ischaemia there is still marked infarct size limitation (Nao *et al.*, 1990). This suggests that the onset of infarction is delayed during a prolonged ischaemic insult rather than prevented. It should be noted, however, that almost all the studies looking at this area have been performed using anaesthetised animals. The anaesthetic agents used will affect coronary dynamics as well as autonomic reflex pathways and may alter the protection observed following preconditioning. A recent study has suggested that in the conscious animal the window of protection may be extended further (Burckhardt *et al.*, 1995).

It also appears that there is a bimodal distribution of protection; the initial phase described by Murry, Reimer and Jennings lasts around one to three hours, depending on species and model, whilst a delayed preconditioning or 'second window of protection' (SWOP) originally identified and described in 1993 (Marber *et al.*, 1993), exists between 12 and 72 hours following the initial ischaemic insult. Since the underlying pathophysiology and mechanisms of these two phases of endogenous cardioprotection may be different, it is important to make critical distinctions between the two. Accordingly the early phase of protection is referred to as '*classic preconditioning*', and this thesis is limited to a discussion of this process.

1.3.2.3 Assessment of protection using other end points

Apart from infarction, ischaemia-reperfusion is associated with various other markers of myocardial injury. These include contractile dysfunction and reperfusion-induced arrhythmias.

The mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage has been termed 'stunning' (see section 1.2.6.2). It is clear that preconditioning of isolated hearts subjected to global ischaemia may result in greater recovery of functional parameters such left ventricular developed pressure (Cave and

Hearse, 1992). However the degree to which this effect is a manifestation of reduced necrosis rather than a reduction in myocardial stunning may be difficult to identify. A study designed to monitor regional wall motion using ultrasonic crystals implanted in the myocardium showed improved segment shortening on reperfusion in preconditioned hearts, but in addition these hearts had smaller infarcts (Cohen *et al.*, 1991). Some studies examining preconditioning during short regional ischaemic episodes which cause stunning without necrosis have failed to show any benefit (Ovize *et al.*, 1992b). Experiments using Doppler probes to monitor segment shortening are known to give conflicting results depending on the depth of the probe within the myocardium. This reflects the fact that the effects of ischaemia vary as one moves from epicardium to endocardium. Further studies in isolated hearts have shown that if functional recovery and infarct size are examined in the same hearts the improved recovery of preconditioned hearts correlates with infarct size limitation. It is likely, therefore, that most of the benefit is the result of a reduction in infarct size (Walker *et al.*, 1993).

Several investigators have examined the incidence of ischaemia- and reperfusion induced arrhythmias following preconditioning (Lawson *et al.*, 1993b). In a study using the rat heart a dose-dependent reduction (proportional to the number of preconditioning cycles) in the frequency of ischaemia-induced arrhythmias was demonstrated *in vitro* (Lawson *et al.*, 1993b). An earlier study, again in the rat, was able to show a protective effect of ischaemic preconditioning in terms of a reduced incidence of reperfusion-induced arrhythmias (Shiki and Hearse, 1987).

1.3.3 MYOCARDIAL METABOLISM AND PRECONDITIONING

As part of their original work, Murry and colleagues (Murry *et al.*, 1986) examined lactate release during ischaemia in preconditioned and control hearts. They found that less lactate was produced in the preconditioned group suggesting reduced anaerobic glycolysis during ischaemia. This suggested that the preservation in ATP levels observed was due to reduced utilisation rather than increased production. Since then there have been several reports confirming a slower decline in high energy phosphates in preconditioned tissue during the

early phase of sustained ischaemia in dogs and pigs (Jennings *et al.*, 1991a), although the effect is less dramatic in rats (Wolfe *et al.*, 1993). There is also evidence for faster recovery of ATP levels during reperfusion in preconditioned hearts (Flack *et al.*, 1991), but this is not significant in all studies (Schott *et al.*, 1990), and not apparent in some (Ovize *et al.*, 1992b).

Using the technique of NMR spectroscopy, intracellular pH can be estimated and any changes followed. Studies using this technique have shown that preconditioning attenuates acidosis during ischaemia (Wolfe *et al.*, 1993) and that this metabolic effect correlates better with functional recovery than preservation of ATP (Albuquerque *et al.*, 1994). However these studies have been conducted exclusively in the rat, and there is no available confirmatory data in any other species. It would seem safe to conclude, however, that any mechanistic explanation of ischaemic preconditioning must account for the apparent reduction in energy consumption observed during the subsequent ischaemic insult.

1.3.4 CELLULAR MECHANISMS

1.3.4.1 Discounted hypotheses

Several hypotheses concerning the likely mechanism of ischaemic preconditioning can be discounted immediately. The first concerns the possibility that the brief ischaemic episodes of a preconditioning protocol might open up collateral vessels, thus providing improved blood flow during the subsequent ischaemic insult. This is unlikely to be a contributing factor since protection has been demonstrated with varying degrees of collateral flow in the dog heart (Murry *et al.*, 1986), is present in species without significant collateral flow (Schott *et al.*, 1990), and is evident in isolated hearts subjected to global ischaemia (Asimakis *et al.*, 1992). The second hypothesis was based on the observation that brief regional ischaemia can lead to localised stunning (a reversible depression in contractility upon reperfusion after brief ischaemia). It was postulated that this would result in reduced metabolic demand and less necrosis during ischaemia. However preconditioning and stunning have different time courses (Murry *et al.*, 1991b), the degree of stunning induced

does not correlate with protection (Miura *et al.*, 1991), and reversing any stunning with an inotropic agent fails to prevent preconditioning (Matsuda *et al.*, 1993).

As discussed earlier the protection induced by ischaemic preconditioning appears to be due to reduced utilisation of high energy phosphates during ischaemia. One proposal for the reduction in energy demand involves the action of the mitochondrial ATPase inhibitor protein. During ischaemia mitochondrial ATPase functions in reverse and causes ATP to be 'wasted'. To counteract this tendency there is a reversible inhibitor of the ATPase which binds during ischaemia and is activated by a fall in intracellular pH (Rouslin and Pullman, 1987). It was suggested that this protein might mediate preconditioning either by binding more rapidly during a second ischaemic episode or by persistent binding during the brief reperfusion (Jennings *et al.*, 1991b). However, this explanation seems unlikely since binding of the inhibitor is very rapid (<90 seconds), reaching maximum inhibition within five minutes, with no persistence of inhibition on reperfusion (Vander Heide *et al.*, 1991). In addition, preconditioning occurs in the rat, a species known to have limited ATPase activity (Yellon *et al.*, 1992).

Glycolysis has been proposed as an alternative cellular mechanism for conservation of ATP and maintenance of cell viability during ischaemia (Opie, 1988). Inhibition of glycolysis during the preconditioning phase (by perfusing an isolated heart with pyruvate instead of glucose) has been shown to inhibit protection (Fralix *et al.*, 1991). Unfortunately the events occurring during the preconditioning ischaemia and subsequent ischaemic insult are somewhat confusing. A brief period of ischaemia increases glycolytic flux (Jennings *et al.*, 1990), but during the subsequent prolonged ischaemic insult glycolysis is decreased (Murry *et al.*, 1990). An alternative explanation involves the possibility that an increased capacity for glycolysis during the reperfusion period might preserve ATP levels. Support for this idea came from a study using the isolated rabbit heart in which subsequent protection was abolished by reperfusing with pyruvate after the long ischaemic insult (Omar *et al.*, 1991).

It is likely that the pathway involved in preconditioning involves at least three distinct levels.

1.3.4.2 Generation of the signal

There are several theories concerning the identity of the signal that triggers intracellular events leading to protection. One of the most popular involves the generation of adenosine by ischaemic tissue. Adenosine is rapidly released from cells under stress, including ischaemic myocytes (Bellardinelli *et al.*, 1989), and is thought to act as a local regulator of cell function via a feedback pathway (Stiles, 1991). During ischaemia adenosine levels reach the micro molar range (Van Wylen *et al.*, 1990), and its ability to induce cardioprotection against ischaemia-reperfusion injury has been demonstrated (Ely *et al.*, 1985). More direct evidence for its involvement in mediating the effects of preconditioning has been derived from several studies. This can be summarised as follows:

- (a) The reduction in the extent of necrosis following regional ischaemia and reperfusion in preconditioned rabbits is attenuated by drugs that antagonise the effects of adenosine at its specific receptors (Liu *et al.*, 1991).
- (b) Pre-treatment with adenosine or selective adenosine A₁ receptor agonists (but not A₂ receptor agonists) mimics the reduction in infarct size obtained with preconditioning (Downey *et al.*, 1993). In particular, the effect is still present when the marked systemic hypotension associated with these drugs is avoided (Tsuchida *et al.*, 1992).
- (c) Protection induced by preconditioning can be enhanced by inhibitors of nucleoside transport *eg* dilazep and draflazine, (Van Belle, 1995), and by acadesine, an adenosine regulating agent that preserves local levels by an unknown mechanism (Mullane, 1993).

However, the above studies have been carried out almost exclusively in the rabbit. There is some evidence suggesting that adenosine may be involved in the mechanism of preconditioning in the dog and the pig. Intracoronary infusion of R-phenyl-isopropyl-adenosine (R-PIA, an adenosine A₁ agonist) will limit infarct size *in vivo* in both these

species (Grover *et al.*, 1992), and several adenosine A₁ antagonists block the protective effect of preconditioning in dogs (Kitakaze *et al.*, 1993). The role for adenosine in the rat heart is unclear. In a study using recovery of post-ischaemic contractile function as the end point, preconditioning could be prevented by the use of an adenosine A₁ antagonist and induced by adenosine (Murphy *et al.*, 1991). However, several studies since have provided conflicting data, and it would seem unlikely that adenosine is an endogenous mediator of ischaemic preconditioning in the rat, although stimulation of adenosine receptors by addition of exogenous agonist does appear to confer protection. However, there is evidence supporting a role for endogenous adenosine in ischaemic preconditioning of human myocardium. Using isolated superfused strips of human atrial muscle, Yellon's group showed that the protection conferred using ischaemic preconditioning could be blocked by addition of an adenosine A₁ antagonist, 8-(p-sulphophenyl)theophylline (8-SPT), and mimicked by R-PIA (Walker *et al.*, 1995).

An alternative hypothesis for signal generation involves the observation that sympathetic neurotransmitter release and consequent α adrenoceptor activation can mimic the protective effects of ischaemic preconditioning. In a study using the rat heart levels of noradrenaline were found to increase following the period of transient ischaemia used for preconditioning (Banerjee *et al.*, 1993). Noradrenaline administered exogenously in place of the period of transient ischaemia-induced the same degree of protection against a subsequent global ischaemic insult. This effect was abolished in hearts from animals that were pre-treated with reserpine to deplete endogenous stores of noradrenaline. Noradrenaline induced preconditioning was simulated by phenylephrine and blocked by an α_1 adrenoceptor antagonist. These findings were then confirmed using an *in vivo* rabbit heart model (Bankwala *et al.*, 1994). However, a subsequent study, again using the isolated rat heart, found that sympathetic neurotransmission was not the dominant mechanism of preconditioning (Weselcouch *et al.*, 1995).

The potential role of mediators derived from the endothelium has been investigated. In anaesthetised mongrel dogs, the antiarrhythmic effect of preconditioning can be attenuated

or abolished by an inhibitor of the L-arginine nitric oxide (NO) pathway (Vegh *et al.*, 1992b) and by intracoronary administration of methylene blue, thereby preventing the effects of released NO on soluble guanylate cyclase (Vegh *et al.*, 1992a). Bradykinin is released early in ischaemia (Parratt, 1993) and has been postulated as a trigger for the release of NO and prostacyclin.

The possible involvement of bradykinin in ischaemic preconditioning has been investigated recently by Downey's group (Goto *et al.*, 1995). They used both *in vivo* and isolated rabbit heart models and examined the preconditioning effect by subjecting hearts to 30 minutes of regional ischaemia followed by reperfusion, using infarct size as the end-point. They found that bradykinin administered to isolated hearts mimics the effect of ischaemic preconditioning but that neither administration of a nitric oxide synthase inhibitor nor a prostaglandin synthase inhibitor affected the development of protection. However, polymixin B and staurosporin, both inhibitors of protein kinase C, abolished the effect. Protection induced by bradykinin was also inhibited by HOE 140, a bradykinin B₂ receptor inhibitor, but this inhibition could be overcome by amplifying the preconditioning stimulus using four cycles of five minute ischaemia with ten minutes of intervening reperfusion. HOE 140 did not abolish the protection induced by an ischaemic preconditioning protocol.

In contrast, in the *in situ* heart, HOE 140 will abolish the effect of ischaemic preconditioning if given prior to the stimulus. From these observations it seems likely that bradykinin formed *in vivo* from blood-borne kininogens is released during ischaemic preconditioning and is associated with the development of protection. It would also seem likely that this effect is mediated by activation of protein kinase C and does not involve production of nitric oxide or prostaglandins. However, when the action of bradykinin is blocked by a B₂ receptor blocker, protection can still be induced by amplifying the ischaemic preconditioning stimulus. This could be explained by the production of increased levels of other agonists (*eg* adenosine, noradrenaline) sufficient to reach threshold for induction of protection when bradykinin receptors are blocked. At the cellular level the metabolic adaptation induced is characteristic of an 'all or none' phenomenon, and the

situation would seem to be akin to that seen in synaptic transmission of neural impulses, with summation of different impulses until firing threshold is attained. This concept is well illustrated by a recent study using human atrial muscle (Morris and Yellon, 1997). Two individual stimuli (a sublethal hypoxic insult, and elevated bradykinin levels by angiotensin converting enzyme inhibition) were insufficient to induce a preconditioning response when administered separately, but, in combination, a full protective effect was observed.

1.3.4.3 Generation of the intracellular message

The mechanism by which the agonists mentioned above interact with the cell to cause generation of an intracellular message is thought to involve coupling of the agonist receptor to G-proteins that span the cell membrane. Evidence in support of this hypothesis has been derived from experiments in which pertussis toxin is used to inhibit the action of G-proteins. There is already considerable evidence that adenosine receptors are coupled to inhibitory G (G_i) proteins (Stiles, 1991), and that blockade using pertussis toxin attenuates protection induced by ischaemic preconditioning in the rabbit (Thornton *et al.*, 1993a). The ability of cholinergic receptors, which are also G_i -protein coupled, to mimic the protection of ischaemic preconditioning has been confirmed in the same study using carbachol to stimulate muscarinic (M_2) receptors (Thornton *et al.*, 1993a). In rats the situation is less clear, with reports both for (Piacentini *et al.*, 1993), and against (Lawson *et al.*, 1993a) involvement of pertussis-sensitive G-proteins in the anti-arrhythmic effects of preconditioning, and against involvement in infarct size limitation (Liu and Downey, 1993).

Following activation of G_i -protein-coupled receptors the activity of an enzyme (phospholipase C or D) leads to the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3) from membrane phospholipid. Another enzyme, protein kinase C (PKC), is activated by DAG (Fleming *et al.*, 1992). Activation of PKC is thought to play a key role in the intracellular signalling pathway and has been studied in several species. In a series of experiments using the isolated rabbit heart, preconditioning could be inhibited by administration of either of two PKC inhibitors (staurosporin or polymixin B), and was

mimicked by activators (phorbol myristate acetate or oleyl acetyl glycerol) given prior to the onset of ischaemia (Ytrehus *et al.*, 1994). The involvement of PKC has also been demonstrated in the rat (Speechly Dick *et al.*, 1994), a species in which adenosine does not appear to be a mediator. It would seem possible, therefore, that PKC may provide a unifying link in an effector pathway that may be activated by different agonists in several species. Activation of PKC is thought to involve translocation to the cell membrane, since inhibition of microtubular activity with colchicine prevents the development of protection following ischaemic preconditioning in the rabbit heart (Liu *et al.*, 1994). It should be stressed, however, that there is little direct evidence to confirm the physical presence of PKC in the cell membrane following preconditioning, although in a recent study using the isolated rat heart, immunocytochemical staining suggested that PKC is translocated to the sarcolemma following transient ischaemia (Mitchell *et al.*, 1995). Protein kinase C is thought to remain in the membrane for approximately one hour (Bogoyevitch *et al.*, 1993), thus providing a possible explanation for the persistence of protection ('memory effect') following preconditioning. However, phorbol ester induced translocation of PKC also leads to increases in intracellular cAMP and to cellular calcium overload, both of which would be detrimental to cardiac function. In addition, PKC aggravates hypoxic injury (Ikeda *et al.*, 1988) and is proarrhythmic (Black *et al.*, 1993). These inconsistent results suggest that the activation of PKC following ischaemic preconditioning may simply be an epiphenomenon. However, the use of poorly selective activators and antagonists of PKC may generate misleading data. A wide variety of isoenzymes of PKC may be activated by phorbol esters, whereas translocation and activation of one specific isoenzyme may follow an ischaemic preconditioning stimulus.

Recent work suggests that kinases other than PKC play a role in preconditioning. Experiments with the tyrosine kinase inhibitor genistein (Maulik *et al.*, 1996) suggest that tyrosine kinase activity is a crucial component of the signalling cascade. This inhibitor is reported to be relatively selective for tyrosine kinase when related to other kinases but ultimately some measure of tyrosine kinase activity is required to support the pharmacological data. The positions of tyrosine kinase, PKC and any other kinases in the

signalling cascade remain undetermined and add a further tier of complexity to the mechanisms of classic preconditioning. Nevertheless further work in this area may provide useful information towards more specific ways of manipulating the preconditioning phenomenon.

1.3.4.4 The effector mechanism

It is not known how the presence of activated PKC in the cell membrane leads to the changes in cell metabolism necessary to lead to the observed preservation in ATP levels associated with protection. The most popular theory, however, involves the ATP sensitive K^+ channel.

In 1983 the outward 'injury current' observed in myocardial ischaemia was identified (Noma, 1983). It was found to be due to K^+ efflux through channels opened by falling levels of ATP. It has been postulated that opening of the channel is responsible for the shortening of action potential duration observed during ischaemia and the consequent reduction in Ca^{2+} influx that may lead to reduced myocardial contractility and sparing of ATP. In the dog heart, a blocker (glibenclamide) of the ATP-sensitive K^+ channel has been shown to abolish the infarct size limitation of preconditioning (Gross and Auchampach, 1992). This effect has been repeated (Auchampach *et al.*, 1992) using a more specific blocking agent (5-hydroxydecanoate). However, these results could not be reproduced in the rabbit, and, furthermore, a K_{ATP} channel opener (pinacidil), did not confer protection (Thornton *et al.*, 1993b). If K_{ATP} channels are responsible for preconditioning then short episodes of ischaemia would have to increase channel opening during a subsequent, more prolonged, episode ('memory effect'). Experimentally, K_{ATP} channels are inhibited by much lower concentrations of ATP than those measured during early ischaemia (Nichols and Lederer, 1991), and although only minor increases in channel conductance (<1%) are necessary for a significant effect on action potential duration, the fall in ATP levels during ischaemia is insufficient to explain the observed reduction in action potential duration (Nichols and Lederer, 1990). It is unlikely, therefore, that a direct link exists between ATP

levels during ischaemia and K_{ATP} channel activity. One explanation might be provided by the action of an intermediary agent that is released during ischaemia causing activation of intracellular pathways leading to phosphorylation of the K_{ATP} channel. Adenosine has been proposed to fulfil this role and several investigators have attempted to demonstrate a convincing link by examining the cardioprotective effect of adenosine in the presence of K^+ channel blockers. In the pig heart, ischaemia, adenosine, and R-PIA have all been shown to induce protection (Van Winkle *et al.*, 1994). However, when R-PIA was given in the presence of 5-hydroxydecanoate (5-HD), a specific K_{ATP} channel blocker, the effect was lost. Similarly, in the dog heart, the protection conferred by administration of adenosine was inhibited by concomitant administration of K_{ATP} channel blockers (Yao and Gross, 1994a). Recent studies from our group (Speechly Dick *et al.*, 1995) using isolated human atrial trabeculae have provided encouraging evidence that the K_{ATP} channel is involved in the mediation of ischaemic preconditioning in man via activation of PKC. Protection could be abolished using chelerythrine (a PKC inhibitor), and mimicked using 1,2-dioctanoyl-sn-glycerol (DOG, a PKC agonist). Furthermore the protection induced by DOG could be abolished by glibenclamide.

The hypothesis that K_{ATP} channel openers exert their cardioprotective effect by accelerating sarcolemmal channel opening during ischaemia has been challenged by the observation that low dose bimakalim may induce cardioprotection without any effect on action potential duration (Yao and Gross, 1994b). Thus the involvement of K_{ATP} channels in other cell membranes, such as the mitochondrial plasma membrane, may be worthy of investigation. End-effectors other than the K_{ATP} have been suggested, including a critical step in energy expenditure (Murry *et al.*, 1991a) or alterations of the cytoskeleton (Ganote and Armstrong, 1993).

1.3.5 THE CLINICAL RELEVANCE OF ISCHAEMIC PRECONDITIONING

The possibility that the heart may be rendered more resistant to the damaging effects of ischaemia-reperfusion injury is an attractive therapeutic goal. In the developed world,

coronary heart disease now constitutes the most important independent cause of mortality. The treatment of myocardial infarction has been revolutionised by the use of thrombolytic agents which, if administered early, will frequently allow reperfusion and limit the size of the infarct. However, the benefit in terms of improved mortality diminishes rapidly if treatment is delayed. Endogenous mechanisms of myocardial protection provide a possible means by which the progress of myocardial necrosis might be slowed, increasing the time available for effective reperfusion. Similar considerations apply in patients with unstable angina and in those undergoing coronary artery surgery. Improved myocardial protection may also assist the preservation of explanted hearts prior to transplantation. A more detailed understanding of the cellular mechanisms involved in mediating cytoprotection may allow future development of pharmacological agents capable of invoking persistent protection against myocardial ischaemia.

Evidence is accumulating that it is possible to precondition the human myocardium. Isolated trabeculae from human atrium demonstrate enhanced resistance to hypoxia following preconditioning using a single episode of brief hypoxia. This effect is abolished in the presence of adenosine receptor antagonists (Walker *et al.*, 1995). The functional protection observed may also be inhibited using chelerythrine (a PKC antagonist), and glibenclamide, a K_{ATP} channel blocker, (Speechly Dick *et al.*, 1995). Isolated human ventricular myocytes demonstrate the same characteristics (Ikonomidis *et al.*, 1994). Further support for this phenomenon in man comes from two studies of percutaneous transluminal angioplasty. These show that following the initial balloon inflation, subsequent inflations are associated with less pain, less ST segment elevation and less lactate production than seen initially (Deutsch *et al.*, 1990). This attenuation in symptoms and signs is prevented by prior administration of glibenclamide (Tomai *et al.*, 1994). Of further interest is the observation that patients with a previous history of angina prior to an acute myocardial infarction sustained smaller infarcts as determined by reduced release of creatine kinase and the absence of Q waves on the electrocardiogram, as well as a lower incidence of cardiogenic shock and improved survival (Kloner *et al.*, 1995).

The possibility that improved collateralisation may play an important role in mediating this protection has made these observations difficult to interpret with confidence. However, the most direct evidence for preconditioning in man has emerged from a preliminary study in patients undergoing cardiac surgery in which resistance to global ischaemia was assessed (Yellon *et al.*, 1993). In this situation changes in collateral flow do not play a role. Intermittent application of the aortic cross clamp was used to deliver repeated episodes of global ischaemia to provide the preconditioning stimulus. Patients subjected to this protocol had better preservation of ATP levels in myocardial biopsies during a subsequent ten minute global ischaemic period. These metabolic changes were almost identical to those seen in dogs by Jennings' group (Reimer *et al.*, 1986). In a subsequent study using the same preconditioning protocol, serum levels of troponin-T were used as a marker of myocardial cell necrosis. Patients in the preconditioned group released significantly less troponin-T, demonstrating for the first time a potentially beneficial effect during the entire duration of the operation (Jenkins *et al.*, 1997).

It would appear from the evidence outlined above that human myocardium is amenable to preconditioning and that preconditioning may occur as a natural feature of some ischaemic syndromes. However, even with the development of pharmacological agents that can mimic or evoke the protection of ischaemic preconditioning, the timing of administration will be critical. Prompt reperfusion will always remain the most effective method of limiting ischaemic injury and is, therefore, the most important determinant of prognosis. However, there are certain situations in which the timing of treatment before the onset of ischaemia can be controlled to some extent.

Patients presenting with unstable angina are at high risk of myocardial infarction and would form a reasonably well-defined group for pre-emptive treatment. A therapy that stimulated or augmented the cellular preconditioning mechanisms over a period of several days or weeks could keep the myocardium protected. In the event of the patient suffering an acute myocardial infarction the treatment would enhance tissue tolerance and slow the rate of necrosis. Such a treatment would 'buy time' for the administration of revascularisation

therapies. A major theoretical hurdle is maintaining myocardium in a protected state by preconditioning. Experiments in Downey's laboratory suggest that continuous adenosine A₁ receptor activation with high dose chronic infusion of 2-chlorocyclopentyladenosine (CCPA) leads to down-regulation of the signalling mechanism and loss of protection (Tsuchida *et al.*, 1994). However, more encouraging data have been obtained recently using a different dosing schedule. Chlorocyclopentyladenosine was administered to rabbits by intermittent dosing over a 10 day period, and the persistence of myocardial protection assessed 48 hours after the final dose. The expected down-regulation of adenosine A₁ receptors was not observed (since the haemodynamic responses to administration of the agonist were preserved) and infarct size remained significantly reduced in the drug treated group (Dana *et al.*, 1998).

Preconditioning strategies might also be applied prior to a planned procedure involving a potentially injurious ischaemic insult. An example is coronary artery bypass graft (CABG) surgery. Highly effective strategies for myocardial preservation have already been developed including the use of various cardioplegic solutions, topical and systemic hypothermia, and intermittent aortic cross-clamping with ventricular fibrillation. In general, the rationale behind the use of cardioplegic techniques includes rapid diastolic arrest, membrane stabilisation, hyperosmolarity (to prevent intracellular oedema), acid buffering, and hypothermia. Additional strategies such as continuous coronary perfusion, warm instead of cold cardioplegia (to avoid cold injury), and the use of blood instead of crystalloid solutions (to improve oxygen delivery) have all added to the choices available to the cardiac surgeon.

However, although present cardioprotective measures are highly effective at minimising irreversible injury that might occur during these periods of imposed ischaemia, they are not without their limitations. Even with carefully controlled intra-operative ischaemic periods and hypothermia, sensitive markers of tissue injury such as troponin-T indicate that discrete necrosis occurs (Jenkins *et al.*, 1997). Moreover, as surgeons undertake more complex and higher risk operations, so the need for better preservation methods increases. In a situation

like CABG, the administration of an agent prior to surgery that could enhance myocardial defences would reduce susceptibility to focal necrosis during surgery and permit the extension of the intra-operative ischaemic period. High risk patients with poor pre-operative left ventricular function might certainly benefit if the degree of protection could be improved by invoking endogenous cellular adaptive mechanisms. The possibility that organ preservation prior to transplantation might be amenable to the same improved protection is also of significant interest.

Any clinical trial involving the use of a potential pharmacological agent designed to mimic the protection of ischaemic preconditioning will have to demonstrate its value in terms of relevant clinical end-points such as preservation of left ventricular function, attenuation of stunning, need for inotropic/balloon support, incidence of clinically detectable infarction, left ventricular failure, and post-operative death. However, studies so far have concentrated on low risk patients with good pre-operative status that would be expected to do well in any event. The benefit derived from ischaemic preconditioning in this group of patients is likely to be marginal. The end-points used presently are relatively insensitive; they provide us with indirect information on myocardial viability and are no substitute for direct measurement of infarct size. Measurement of total myocardial ATP content is not universally accepted as a sensitive marker of cell viability and the concept of a critical tissue concentration of ATP, below which cell death occurs, is now known to be incorrect (Opie, 1992). If it were possible to measure sub-cellular levels of ATP within different compartments (such as the mitochondrial fraction), and thereby assess local turnover, then more useful information might be available. End-points of clinical outcome are more likely to demonstrate a difference in studies conducted in a group of patients at higher risk, but these can only be performed once safety and tolerability have been established.

Exploitation of endogenous cardioprotective mechanisms may be possible in the context of carefully conducted clinical studies. There have been significant advances in our understanding of the mechanisms underlying ischaemia-reperfusion injury as a result of preconditioning research and potential pharmacological approaches to protection seem

feasible. However further development of pharmacological therapies should be based on sound experimental investigation and assessed in the context of other effective therapeutic strategies.

1.4 NITRIC OXIDE AND THE HEART

1.4.1 BACKGROUND

One of the greatest scientific discoveries in recent years has been the elucidation of the role of nitric oxide (NO) in biology and medicine. Nitric oxide is a unique, endogenous regulatory molecule involved in a variety of physiological processes within organ systems. These include a broad range of activities such as regulation of blood pressure, immunomodulation and neurotransmission. More specifically, its role in the heart relates to control of coronary tone and regulation of myocardial contractility as well as effects on platelet aggregation, neutrophil activation, and free radical production. Dysregulation of its mediated effects have been implicated in the pathogenesis of hypertension, atherosclerosis, allograft rejection, myocardial infarction, cardiomyopathy, and septic shock.

Continuing controversy, however, surrounds the nature of the involvement of NO in ischaemia-reperfusion injury. Given its anti-adhesion and vasodilatory properties, NO deficiency during reperfusion would be expected to contribute to the resultant injury. However, the formation of NO-derived free radical species, such as peroxynitrite, and the inactivation of iron-sulphur-centred enzymes involved in essential cellular activity, suggest that the presence of NO in large quantities (such as may occur during early reactive hyperaemia) may also be detrimental. Further complexity is added by the suggestion that NO may have a direct role to play as a cellular mediator of ischaemic preconditioning.

1.4.2 NITRIC OXIDE SYNTHESIS

Nitric oxide is synthesised within different cell types including the vascular endothelium (Palmer *et al.*, 1988), macrophages (Marletta *et al.*, 1988), and neuronal cells. The action of NO synthase (NOS) on the amino acid L-arginine cleaves the terminal guanidino nitrogen atom to combine with oxygen producing NO and L-citrulline. The three isoforms

of NOS have been characterised by cloning three different cDNA sequences. These isoforms differ with regard to their tissue distribution and sensitivity to calcium stimulation, and include an inducible NO synthase (iNOS) and two constitutively expressed isoforms (cNOS). The former isoenzyme (iNOS) is expressed in target tissue after stimulation with endotoxin or some cytokines, and is calcium independent (Moncada, 1992). The two constitutively expressed isoforms have been isolated from vascular endothelial cells (eNOS) and neuronal cells (nNOS), and are both calcium and calmodulin dependent.

Nitric oxide is a small, gaseous radical that is weakly soluble in water but readily soluble in lipid. These physical properties allow it to diffuse across biological membranes and also account for its rapid diffusion out of the circulation and into cells or circulating components of blood. The reported biological half-life of NO varies from approximately six to 30 seconds (Palmer *et al.*, 1987), but may be longer depending on local conditions. Nitric oxide is continuously released during baseline conditions, but synthesis is further enhanced by a variety of stimuli including fluid shear stress, pulsatile stretching of the vessel wall, and pharmacological agents such as acetylcholine and serotonin (Moncada *et al.*, 1991).

1.4.3 NITRIC OXIDE LOCALISATION WITHIN THE HEART

Nitric oxide synthase is a useful marker for the identification of areas subject to the actions of NO. Under basal conditions, eNOS and nNOS (*ie* constitutive isoforms) are present in the normal heart. All three isoforms may be present in the diseased heart or following a stressful stimulus. Until antibodies to NOS became available the enzyme was localised by use of a histochemical reaction involving the reduction of nitroblue tetrazolium to water-insoluble formazan by β -nicotinamide adenine dinucleotide phosphate (NADP). The nitric oxide synthases that catalyse the reaction are acting as NADPH-diaphorases (Hope *et al.*, 1991). Although the diaphorase reaction is not completely specific it is highly selective for NOS activity in the heart since there are no other diaphorases to confound localisation in this organ. In recent years, however, several laboratories have developed antibodies to the various isoforms of NOS (Dawson and Dawson, 1996). Standard immunofluorescence or

immunoperoxidase techniques may be employed with either polyclonal or monoclonal antibodies to yield excellent results.

Using both histochemical and antibody localisation techniques there is limited information available concerning the distribution of the different NOS isoforms within the hearts of different species. Endothelial NOS is found in endocardium and vascular endothelial cells along capillaries and arterioles with smaller quantities in the venules in both rat and pig heart (Klimaschewski *et al.*, 1992). Even less is known concerning the distribution of nNOS within the heart. This probably relates to the lack of understanding of the role of non-adrenergic/non-cholinergic nerves in cardiac innervation. However, in a survey of rat and guinea pig hearts, nNOS activity was demonstrated in nerve endings in the region of the sinu-atrial and atrio-ventricular nodes, in atrial and ventricular myocardium, and coronary arteries. The fact that up to 33% of rat and 28% of guinea pig coronary arteries are innervated by NOS-reactive nerves adds further weight to the evidence. In contrast, the pig heart contains very little nNOS, and eNOS predominates. The amount of nNOS in hearts of different species requires more precise quantification, but significant differences between species would appear to exist.

1.4.4 NITRIC OXIDE AND THE MICROCIRCULATION

In the absence of haemodynamically significant epicardial artery atherosclerosis, myocardial perfusion is regulated predominantly by microvessels less than 200 μm in diameter (Chilian *et al.*, 1986). In contrast to the large epicardial conductance vessels, the vascular wall of resistance vessels does not develop overt atheroma (Juergens, 1980). However, there is increasing evidence that abnormal reactivity of small diameter rather than large diameter vessels contributes significantly to myocardial ischaemia.

The endothelium, which covers the inner surface of all blood vessels like a tapestry, plays a major role in modulating vascular smooth muscle tone by the synthesis and metabolism of vasoactive substances including an endothelium-derived relaxing factor (EDRF), an endothelium-derived hyperpolarising factor, and prostacyclin (Bassenge and Busse, 1988). Endothelium-derived relaxing factor has been identified as NO or an NO carrier molecule

(Palmer *et al.*, 1987). Following synthesis by endothelial cells, NO traverses the subendothelial space and activates smooth muscle cell guanylate cyclase to increase cyclic guanosine monophosphate levels leading to smooth muscle cell relaxation. Nitric oxide is continuously synthesised and released under basal conditions. The most important mechanism underlying basal NO release is the shear stress due to blood flow upon the endothelial cell layer. The endothelial cells may be viewed as mechanotransducers, sensing increases in shear stress and converting this into vessel wall relaxation, thereby optimising tissue perfusion according to metabolic needs (Griffith *et al.*, 1987). Thus the continuous basal release of NO represents an important determinant of resting vascular resistance.

In addition to its central role as a vasodilator, NO inhibits the adherence of circulating blood cells (platelets and leucocytes) to the endothelium (Radomski *et al.*, 1987). This is likely to be due to an inhibitory effect of NO on the signalling and expression of adhesion molecules and chemokines (Tsao *et al.*, 1995). Furthermore, NO suppresses the proliferation of vascular smooth muscle cells (Garg and Hassid, 1989).

1.4.5 CROSS-TALK BETWEEN ENDOTHELIAL CELLS AND MYOCYTES

Endothelial cells line the endocardial cavities of the heart as well as the coronary vasculature. Although it has been established that denudation of the endocardial layers in isolated papillary muscle preparations produces characteristic changes in contractility (Brutsaert *et al.*, 1988), the relevance for NO release from endocardium in the whole heart is doubtful. This is due to the vastly greater thickness of myocardium relative to the endocardial monolayer, and the brief biological half life of NO. However, at the level of the coronary microvasculature, cardiac myocytes and endothelial cells are intimately related, so that NO released from the latter cell type could have global effects on cardiac function.

1.4.6 NITRIC OXIDE AND CARDIAC FUNCTION

Nitric oxide can influence cardiac function indirectly by virtue of its effects on peripheral and coronary vascular tone. These factors determine cardiac volume loading and coronary perfusion respectively. More recently it has been established that NO can exert direct effects on myocardial contractile function. Under resting conditions the primary mode of action

involves modulation of diastolic function (Grocott Mason *et al.*, 1994). However, in circumstances where there is β -adrenergic stimulation NO modulates inotropic and chronotropic responses (Balligand *et al.*, 1993). Nitric oxide appears to have paracrine actions when released from endothelial cells, and autocrine when released from cardiac myocytes themselves.

Available evidence suggests that NO produces its effects predominantly by activation of soluble guanylate cyclase (Waldman and Murad, 1987) and consequent elevation of the intracellular level of cyclic GMP (cGMP), although certain of its actions (*eg* nitrosylation of proteins) would appear to be cyclic GMP independent. There has been considerable disagreement in the scientific literature regarding the inotropic and chronotropic effects of NO. Exogenous administration of NO (using donor agents such as nitroprusside) has a significant negative inotropic effect *in vivo* (Paulus *et al.*, 1994) and a smaller effect *in vitro* (Balligand *et al.*, 1993). However, inhibition of basal NO synthesis has also been shown to have myocardial depressant actions in animals and in man (Klabunde *et al.*, 1991).

It would seem that the most likely explanation for these apparently disparate effects relates to the ambient concentration of NO. In a study using isolated cat papillary muscles, the concentration of NO donors used was found to influence contractility in a biphasic manner. Higher concentrations caused a decrease in active tension and duration of twitch. When the endocardial endothelium (a rich source of NO) was selectively damaged, the same concentration of NO donor had a positive inotropic effect (Mohan *et al.*, 1995). These changes would seem to have been mediated by changes in cGMP concentration since the effects were appropriately modified by zaprinast (a cGMP phosphodiesterase inhibitor) and mimicked by a cGMP analogue (8-bromo-cGMP). Thus preservation in myocardial function may be effected by subtle changes in basal NO release under physiological conditions.

However, pathological states such as septic shock could release large amounts of NO with consequent myocardial depression. There has been similar confusion with respect to the chronotropic effects of NO. Most studies to date have concentrated on the negative

chronotropic effect of exogenous NO on isolated sinu-atrial nodal cells (Han *et al.*, 1995). Nitric oxide would appear to be an important mediator of cholinergic heart rate control. However, in a recent study using guinea pig atria, the chronotropic effect of NO behaved in a biphasic manner (Musialek *et al.*, 1997). High concentrations caused a decrease in spontaneous beating rate and low concentrations caused an increase. The positive chronotropic effect was abolished following blockade of the hyperpolarisation-activated inward current I_h . No effect was observed when slow calcium channels were blocked with nifedipine.

1.4.7 SUBCELLULAR MECHANISMS

It seems likely that the cellular effects of NO are mediated via elevation of cGMP. The main target proteins for cGMP in myocardium are cGMP-dependent protein kinases (PKG) and cGMP-regulated cyclic nucleotide phosphodiesterases. Activation of these pathways has been reported to result in various effects on cell function. Probably the most well characterised of these relates to the relaxation and reduction in diastolic tone observed following exposure to NO or 8-bromo-cGMP. In a study using isolated rat cardiomyocytes (Shah *et al.*, 1994) the changes in contractility recorded following the administration of 50 μM 8-bromo-cGMP were not associated with any alteration in cytosolic calcium or action potential. The most probable explanation is an alteration in the sensitivity of the myofilaments to calcium, a conclusion which is supported by a previous study examining the effects of cGMP on myofilament calcium sensitivity in skinned cardiac fibres (Pfitzer *et al.*, 1982).

In addition to its effects on calcium sensitivity, NO is thought to influence sarcolemmal proteins. Activation of PKG could either directly (or indirectly via other protein kinases) lead to phosphorylation of proteins within the sarcolemma or sarcoplasmic reticulum. Recent work has suggested that the K_{ATP} channel is affected by NO in this way (Kubo *et al.*, 1994). Phosphorylation of these channels will increase the probability of any individual channel being in the open state. This would cause the transmembrane potential to move towards the equilibrium potential for K^+ resulting in hyperpolarisation. It is interesting to

note that the K_{ATP} channel is thought to be involved as an end-effector in ischaemic preconditioning (see section 1.3.4.4).

1.4.8 NITRIC OXIDE AND ISCHAEMIA-REPERFUSION INJURY DURING CARDIAC SURGERY

1.4.8.1 Surgical considerations

Cardiac surgery frequently requires a period of elective ischaemia in order to provide the surgeon with a quiescent and relatively bloodless operating field. Elective global ischaemia results from cross-clamping the aorta while on cardiopulmonary bypass and preventing blood flow down the coronary arteries. Each period of ischaemia and reperfusion carries with it the potential to produce injury. It was in the late 1960's that cardiac surgeons first recognised that myocardial necrosis was a possible complication of the operation itself, and was the cause of the low output syndrome seen in some patients dying early after cardiac procedures (Taber *et al.*, 1967). With the advent of coronary artery bypass grafting (CABG) in the 1970's it soon became apparent that transmural myocardial infarction occurred peri-operatively in a proportion of patients. Later *post mortem* studies confirmed that transmural infarction, scattered myocardial necrosis and confluent subendocardial necrosis could occur after cardiac surgery in patients with normal coronary arteries (Roberts *et al.*, 1973). Later studies showed that in patients surviving surgery there was an inverse correlation between the post-operative cardiac output and the extent of myocardial necrosis determined by creatine kinase MB levels in blood (Kirklin and Barratt-Boyes, 1993). It was also recognised that some patients went through a phase of depressed myocardial contractility in the early post-operative period, from which they later recovered - so called 'myocardial stunning' (Braunwald and Kloner, 1982). Today, with a better understanding of myocardial management during cardiac surgery and improved intensive care, cardiac surgery is much safer. However, with superior methods for detection of myocardial injury it is apparent that myocardial damage has not been eradicated and most published reports include some patients with electrocardiographic and/or enzymatic evidence of myocardial necrosis (Hake *et al.*, 1993).

1.4.8.2 Current methods of myocardial protection

Numerous protective strategies have been developed to minimise damage over the past twenty years. In the UK the most common techniques in use for CABG surgery are intermittent ischaemic arrest, and cold cardioplegic arrest with moderate whole body hypothermia. In the former, the surgeon operates on the heart for periods of approximately ten minutes while the aorta is cross clamped preventing coronary flow, and the heart is deliberately fibrillated to render it quiescent and to allow easier operating conditions. After this time the aortic cross-clamp is removed to allow reperfusion for at least five minutes with the heart beating. In this way the period of continuous ischaemia is limited. This method was used extensively in the 1960's and 1970's and is suitable for coronary artery surgery because the distal and proximal vein graft anastomoses can be alternated. The simplicity and speed of the technique make it popular with some surgeons for CABG and the clinical results are equivalent to those obtained with cardioplegic arrest (Pepper *et al.*, 1982).

In general, the rationale behind the use of cardioplegic techniques includes rapid diastolic arrest (preventing wasted energy consumption and reducing metabolite accumulation), membrane stabilisation, hyperosmolarity (to prevent intracellular oedema), acid buffering, and hypothermia. Additional strategies such as continuous coronary perfusion, warm instead of cold cardioplegia (to avoid cold injury), and the use of blood instead of crystalloid solutions (to improve oxygen delivery) have all added to the choices available to the cardiac surgeon.

These techniques, however, do not provide complete protection. During cardiopulmonary bypass (CPB), even with the heart perfused, the arterial pulse pressure is narrow and the intramyocardial tension is high, especially in the empty heart, and so the flow to the subendocardial layer is already reduced and further impaired by the addition of ventricular fibrillation. In patients undergoing cardiac surgery, the coronary arteries are likely to be diseased and not only is the myocardium more at risk from ischaemia, but the distribution of cardioplegia solutions may not be adequate to reach the most vulnerable areas (Becker *et*

al., 1981). The above effects are exaggerated in the hypertrophied ventricle. It has been well established that hearts protected with the various formulations of cardioplegic solution may exhibit systolic and diastolic dysfunction after reperfusion, a condition known as 'post-cardioplegic dysfunction' (Vinten-Johansen and Nakanishi, 1993). Cardioplegia solutions may also be washed out by the non-coronary collateral flow. Using the intermittent ischaemic technique the safe time period is limited and there is little time to allow for unexpected technical difficulties. There remains, therefore, a considerable need for improved methods of cardioprotection, especially as higher risk patients (often with poor pre-operative ventricular function) and more complex and time-consuming procedures become increasingly common.

1.4.8.3 Nitric oxide and cardioprotection

During ischaemia endothelial ATP levels fall, hypoxanthine levels increase, and xanthine dehydrogenase is converted to the oxidant-generating xanthine oxidase. On reperfusion there is an influx of molecular oxygen and calcium that precipitates a burst of oxygen-derived free radicals due to the action of xanthine oxidase and other endothelial oxidative enzymes (*eg* NADPH oxidase). This oxidative milieu generates lipid hydroperoxides that increase calcium permeability and activate phospholipase A₂. This, in turn, triggers endothelial expression of adhesion molecules, and the release of platelet activating factor, leukotrienes, thromboxane A₂ and other mediators of inflammation. Neutrophils are activated as they course through the inflammatory milieu and adhere to and infiltrate the reperfused tissue, generating more superoxide anion and inducing further tissue necrosis. In recent years it has been appreciated that the myocyte and its metabolism, morphology, and contractile capacity are not alone in bearing the brunt of surgical ischaemia-reperfusion, but that the vascular endothelium suffers considerable damage as well.

All of the above events are exacerbated by a drastic decline in NO activity (Johnson *et al.*, 1990). The mechanism by which NO activity is reduced with reperfusion injury is likely to be related to accelerated degradation by oxygen radicals (Huk *et al.*, 1997). Furthermore it seems that the situation is compounded by the generation of additional quantities of

superoxide anion by eNOS. Administration of L-arginine reverses this abnormality, possibly by competing with molecular oxygen as a substrate for electron transfer by eNOS, and resulting in beneficial NO synthesis.

Previous investigators (Johnson *et al.*, 1990) have shown that supplementing NO levels at the time of reperfusion by the use of NO donor agents leads to suppression of superoxide generation by neutrophils, inhibition of neutrophil infiltration and reduced tissue damage. It is likely, therefore, that the above mentioned decline in endogenous NO activity on reperfusion will contribute to the pathophysiology of reperfusion injury. There is, however, other evidence that supports an injurious role for NO if it is present in substantial excess. This could be especially relevant during early reactive hyperaemia that is mediated by an increase in NO release (Kostic and Schrader, 1992), or later when activated neutrophils capable of expressing iNOS have accumulated. Nitric oxide-dependent toxicity results from formation of NO-derived free radicals such as the peroxynitrite anion (Beckman *et al.*, 1990), and the inactivation of iron-sulphur-centred enzymes involved in essential cellular activity such as mitochondrial respiration. Evidence to support a noxious role for NO when overproduced comes from experimental models of myocardial injury after hypoxaemia (Matheis *et al.*, 1992), post-ischaemic cerebral reperfusion injury (Buisson *et al.*, 1992), and neutrophil-mediated tissue injury (Mulligan *et al.*, 1991).

Further complexity is added to the situation by the proposal that release of NO may be involved in the mechanism underlying the antiarrhythmic effect of classic ischaemic preconditioning. In anaesthetised mongrel dogs, this effect can be attenuated or abolished by an inhibitor of the L-arginine NO pathway (Vegh *et al.*, 1992b) and by intracoronary administration of methylene blue, thereby preventing the effects of released NO on soluble guanylate cyclase (Vegh *et al.*, 1992a). Bradykinin is released early in ischaemia (Parratt, 1993) and has been postulated as a trigger for the release of NO.

1.4.8.4 Conclusion

It would appear that NO plays an important role in modulating cardiac ischaemia-reperfusion injury in the myocyte and endothelial cell. The experimental evidence provides

somewhat conflicting data suggesting both beneficial and deleterious effects. It seems likely that these differences may be explained in part by the differing local concentrations of NO induced. Physiological levels of NO have a protective effect during reperfusion and a reduction below these levels is detrimental. An exaggerated rise in NO during early reperfusion is also associated with injury, and the use of NOS inhibitors under these conditions has been shown to reduce infarct size. As a result, the stage has not yet been reached when manipulation of NO synthesis or release can be incorporated into standard cardiac surgical cardioplegic techniques.

2. AIMS AND SCOPE

With the development of new techniques that allow controlled manipulation of specific genomic regions, the influence of individual gene products on cellular metabolism and homeostasis can be examined under physiological and pathological conditions. The aim of this the present study was the development and validation of a new model of ischaemia-reperfusion injury in murine myocardium that could be used in studies using genetically-altered mice. This objective had to be achieved in three stages:

1. Characterisation and validation of the model (Section 4).
2. Demonstration of the successful use of the model to assess a cardioprotective intervention, ischaemic preconditioning (Section 5).
3. Investigation of a putative cardioprotective modulator of ischaemia-reperfusion injury using genetically-altered mice (Section 6).

All the experiments were carried out using an *in vitro* model of Langendorff perfusion and the results have been interpreted with caution, avoiding direct extrapolation to the situation *in vivo*. It is anticipated that the results generated will allow further use of this model to examine in detail the role of nitric oxide (and the isoforms responsible for its synthesis) in modulating myocardial responses to ischaemic injury. In addition, genetically-altered mice with other genomic alterations thought to have relevance in ischaemia-reperfusion injury can be examined. It is hoped that the present study will stimulate the development of an *in vivo* model of regional ischaemia in the mouse heart so that similar experimental protocols can be examined in genetically-altered mice.

3. MATERIALS AND METHODS

3.1 GENERAL METHODOLOGY

All of the experiments comprising the present study were performed in the laboratory at the Hatter Institute, University College London Hospitals, in accordance with the United Kingdom Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by Her Majesty's Stationary Office, London.

3.1.1 REASONS FOR CHOICE OF ANIMAL MODEL

In vitro Langendorff perfusion of the isolated mouse heart had not been undertaken previously in the Hatter Institute. A handful of laboratories world-wide have developed the technology necessary to undertake this task. The size of the adult mouse heart renders its perfusion technically difficult and a considerable challenge to consistently obtain a stable, non-traumatised preparation that will generate data with physiological relevance. However, the reasons for constructing such a model are compelling. Traditionally there has been a considerable gulf between molecular biologists and physiologists in terms of the philosophy underlying their investigative techniques. However, recent advances in understanding the mouse genome and genetic manipulative techniques have created a unique opportunity to explore the influence of single gene alterations on complex physiological systems. It is essential that physiologists respond to this challenge by careful design of appropriate, miniaturised systems to allow phenotypic changes to be explored. In this way the contribution of individual gene products to cellular mechanisms underlying homeostasis, adaptation and disease can be examined.

In particular this experimental approach has several advantages over more traditional pharmacological techniques. The use of drugs to interact with cell surface receptors, ion channel proteins, intracellular signalling molecules and regulatory proteins, is fraught with difficulty. No agents can claim 100% specificity for their target, and other molecules with similar structure are frequently affected. Preferential binding is often dependent on local concentration, and this in turn will be affected by a variety of pharmacokinetic factors such

as route of administration, lipid solubility, presence of cofactors for absorption and rate of elimination. All of these may vary considerably between individual animals, and even within one animal such factors may be subject to diurnal variation and seasonal differences. In order to control for these effects it is often necessary to obtain additional tissue samples to assay for 'biological effect' of the agent in question. Such assays are not without their own limitations with respect to specificity and sensitivity.

Deliberate manipulation of the genome allows targeted deletion or overexpression of specific gene products. However, the results of such transgenic or 'knockout' experiments must also be interpreted with caution. The absence or increased abundance of certain substances within cells may lead to activation of local or more generalised homeostatic mechanisms that may affect the resultant phenotype. In fact, when such homeostasis fails, certain genetic alterations may result in death *in utero*.

3.1.2 ANIMAL MODEL

The mice used in the characterisation experiments and subsequently to investigate ischaemic preconditioning were all male NIH Swiss White. They were obtained pathogen-free from one supplier and housed under identical conditions. They were ordered by weight (approximately 25 g) in batches of 20. Each experiment was performed as a continuous series to minimise possible variation in ischaemic vulnerability with seasonal changes. Appropriate controls were included with each series of experiments, and historical controls were not utilised. The nitric oxide synthase 'knockout' mice used in the last series of experiments were a different strain and are described in more detail later.

3.1.3 CONSTRUCTION OF THE PERFUSION APPARATUS

Langendorff perfusion of the mouse heart necessitated design and manufacture of certain specialised items of equipment *de novo* (Figure 3.1).

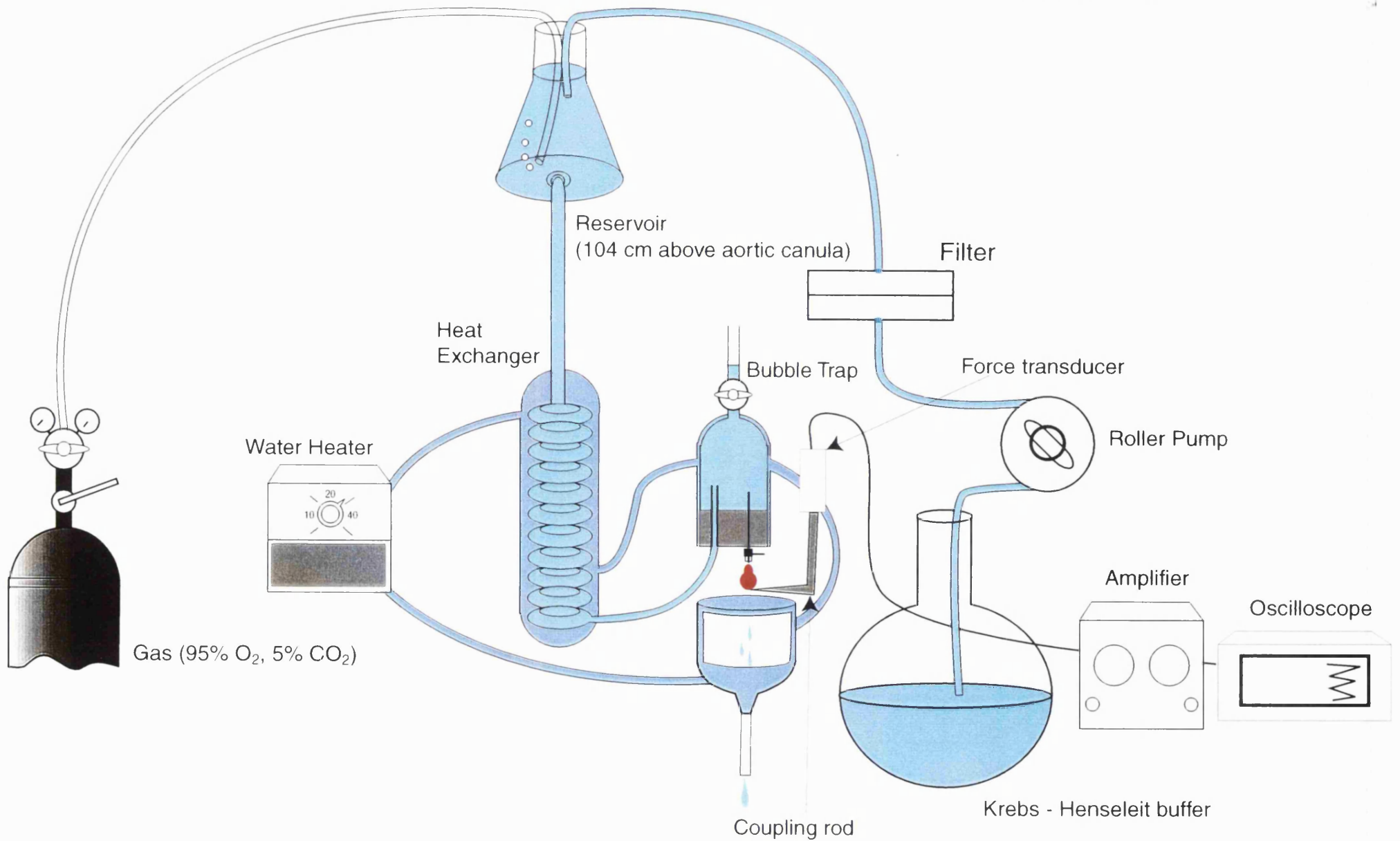


Figure 3.1 Diagrammatic representation of Langendorff perfusion circuit

3.1.3.1 Principle

The method for continuous perfusion of the isolated heart has changed little since its original description (Langendorff, 1895). An aortic cannula is positioned just above the sinuses of Valsalva. Perfusate (which may be crystalloid or blood) flows from the cannula and closes the aortic valve (as in diastole). A head of pressure, which may be generated either by gravity (a reservoir of buffer is positioned an appropriate height above the cannula) or by a pump, forces flow of perfusate down the coronary arteries. The coronary circulation returns the perfusate to the right atrium (mostly via the coronary sinus), and thence across the tricuspid valve into the right ventricle and out of the pulmonary conus. The perfusate runs down the epicardial surface of the heart and drips from the apex. In this way the isolated heart may be perfused by an oxygen-carrying nutrient buffer and maintained for several hours. It is important to distinguish this mode of perfusion from the 'working heart' mode which more closely mirrors the situation *in vivo*. During Langendorff perfusion the perfusate does not enter the left ventricular cavity, and the heart does not perform work as it does *in vivo*, with a pressure volume relationship.

3.1.3.2 Design of the aortic cannula

There are several important considerations in relation to mouse heart perfusion. The usual method to achieve cannulation in larger hearts involves lifting the excised heart and carefully placing the aorta around the cannula. The heart is usually held in place by a 'bull-dog' clip until secured by a silk suture. Retrograde perfusion usually starts simultaneously. This method, however, would not have been appropriate for the mouse heart, since the aortic diameter is so small that reliable placement on the cannula without causing excessive delay or aortic trauma before perfusion would have been impossible.

It was necessary, therefore, to achieve a design that would allow cannulation to take place 'on the bench', followed by transfer of the cannulated heart to the perfusion apparatus. In addition, a side-arm to permit gentle perfusion during transfer (and avoid air entering the cannula) had to be incorporated. The cannula was designed and constructed with the help of specialised technicians in the department of physiology, UCL. It was made from

stainless steel in two parts; the upper half was a simple cannula with a threaded expansion at its lower end, and the lower half had a screw thread and rubber 'O'-ring to allow a leak-proof seal between the two components when fitted together. The lower half also had a short metal side arm, which was machine pressed into a side hole in the cannula, and a half millimeter groove sited one millimeter from the end to accept a silk suture for securing the aorta. The original technical specifications are shown in Figure 3.2.

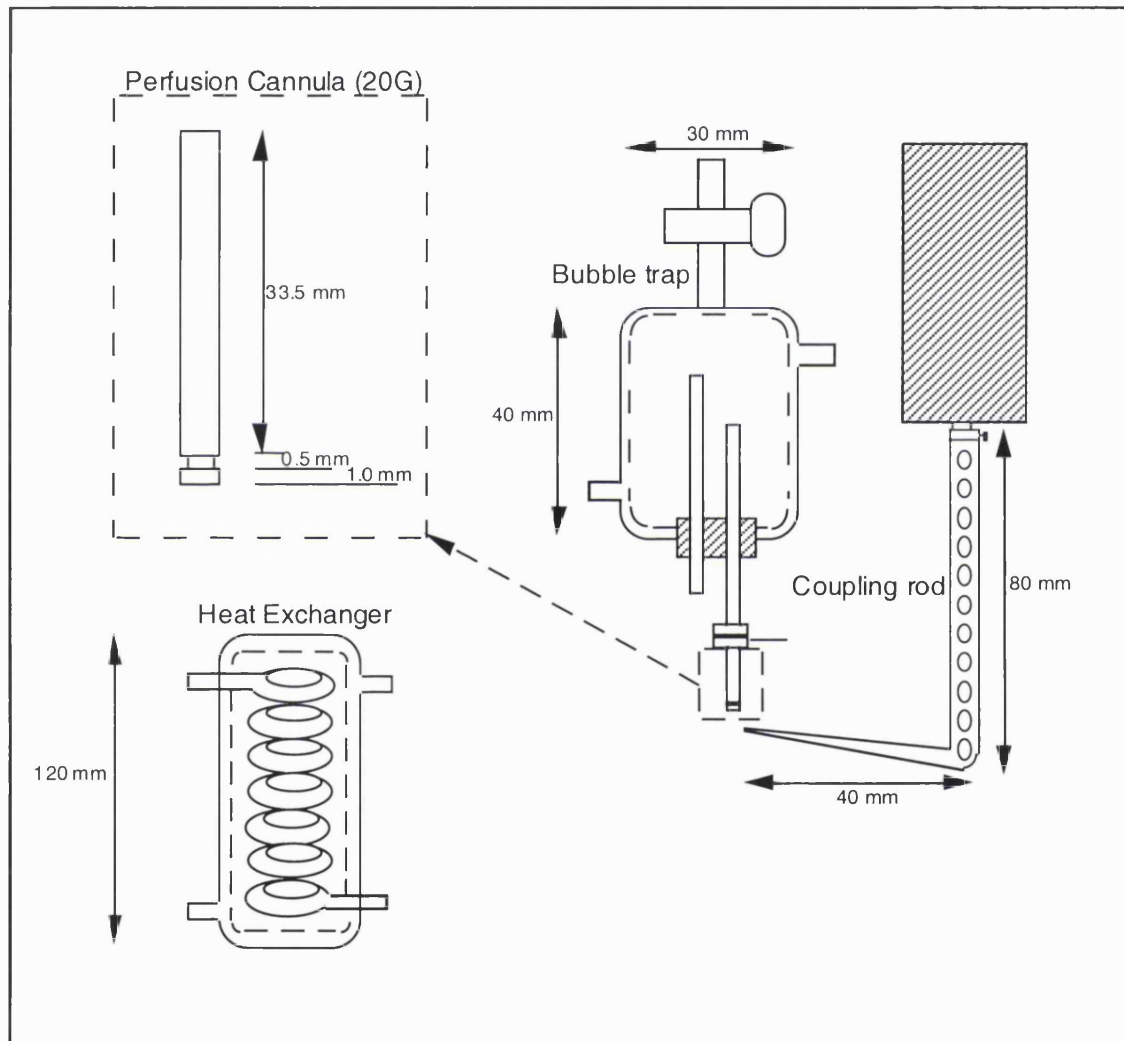


Figure 3.2 Technical specifications for perfusion cannula, bubble trap, coupling rod and heat exchanger

3.1.3.3 Design of glassware

Three items of glassware were made specifically for this model with the help of the glassblower in the department of chemistry, UCL. All the glass items were blown as a

double layer to allow warm water 'jacketing' and control of buffer temperature. A small bubble trap and a coiled heat exchanger were made to specification (Figure 3.2).

3.1.3.4 Thermocouple

Monitoring myocardial temperature forms an essential component of any Langendorff perfusion system. However, the placement of a wire into the right ventricle of such a small heart may have posed problems by displacement of its long axis and inaccurate measurement of developed force. As a result a very fine microthermocouple wire was purchased ('IT-18') which could be easily threaded retrogradely through the pulmonary conus, across the tricuspid valve and into the right ventricle. This was connected to a digital thermometer (Physitemp Bat-12; Sentsortek, Clifton, NJ) which displayed temperature continuously throughout an experiment.

3.1.3.5 Perfusion circuit, filter and pump

A constant pressure system was used to perfuse the coronary circulation. This was attained by elevating the reservoir of buffer a fixed height above the tip of the aortic cannula. A pressure head of 80 mmHg was used and accordingly the reservoir was placed 104 cm above the cannula. Any change in resistance to coronary flow, therefore, was reflected in a change in flow rate rather than perfusion pressure. The buffer used for perfusion (section 3.1.6.1) was filtered (2 μm glass microfibre filter, Whatman, Maidstone UK) to remove any contaminants or undissolved salts, thereby avoiding the danger of embolisation. The buffer was pumped (Watson Marlow Roller Pump, Nottingham, UK) from a conical flask through the filter to the reservoir. A bubble oxygenator was used to gas the buffer to the required pH (section 3.1.6.1). The buffer was heated to 37 °C by passage through the heat exchanger and any bubbles were removed in the bubble trap before the buffer flowed down the aortic cannula.

3.1.3.6 Construction of force transduction assembly

Accurate assessment of left ventricular contractility posed a considerable technical challenge. Commonly, left ventricular developed pressure is measured by placement of a

fluid-filled latex balloon into the cavity of the left ventricle with connection to a pressure transducer. Although this technique has been used successfully elsewhere, considerable difficulty was encountered eliminating tiny air bubbles from the fluid filled catheter between balloon and transducer. Due to the very small volume of fluid and fine catheter necessary, the smallest air bubble produced an unacceptable damping effect on the signal. In addition, following insertion and inflation of the balloon, considerable electrical instability resulting in troublesome arrhythmia led to the abandonment of this technique.

Transduction of developed force was adopted as an alternative measure of contractility. It was necessary to design the apparatus so that one axis of shortening could be transduced and amplified to an oscilloscope without imposing an unacceptable load on the heart or displacing it from the cannula. Furthermore the frequency response characteristics of the entire assembly had to accommodate a signal of 8 Hz (heart rate of 480 bpm) without loss of amplitude or resolution. Initially a hook and pulley system was tested (Figure 3.3).

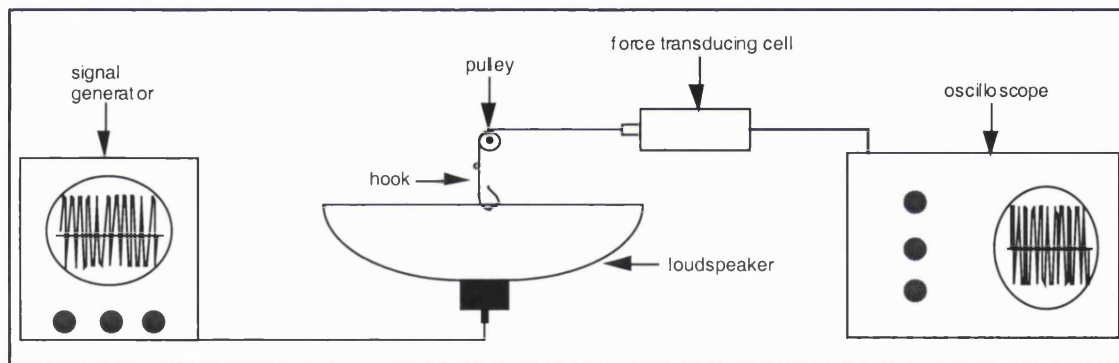


Figure 3.3 Diagrammatic representation of the testing assembly for assessment of the frequency-response characteristics of the hook and pulley system

The frequency response characteristics were assessed with the help of equipment from the department of physics, UCL. A signal generator was connected to a loud speaker and the frequency of oscillation of the drum varied up to 20 Hz, whilst the amplitude remained constant. The movement of the drum was transduced using the hook, pulley and force transducing cell (Statham, Gould Inc, Cleveland OH) and the signal prefiltered and amplified before display on the oscilloscope. The amplitude and resolution of the signal

remained constant over this frequency range and the assembly was incorporated into the perfusion apparatus.

During testing of the force transduction assembly, however, it soon became apparent that this method was not generating acceptable data. Considerable variation in signal was encountered during the course of a 20 minute stabilisation period. On close inspection of the apparatus it was clear that this was due to rotational instability of the heart and slippage of the suture material across the pulley.

An alternative method of force transduction had been assessed previously (Marber *et al.*, 1995), and used successfully in a similar model. A coupling rod was moulded from a light weight and rigid alloy with multiple air holes along the shaft to reduce its weight even further. A small hole drilled at the tip allowed the passage of 4-0 silk suture which had been passed through the apex of the left ventricle using a round-bodied needle. The suture was cut and tied so that the apex of the heart and the tip of the coupling rod were in close approximation using the shortest length of suture material possible. The upper end of the rod was attached to the force transducer. As the heart contracted, its long axis shortened pulling the coupling rod upwards. The frequency response characteristics of this assembly was flat to at least 50 Hz.

Adjustment of the distance between the heart and coupling rod enabled alteration in suture (diastolic) tension. However, fine control was needed to avoid pulling the heart off its attachment to the cannula. A hydraulic micromanipulator (designed for fine electrode puncture and single cell recording) was adapted for this purpose. A metal moulding attached the slave cylinder of the micromanipulator to the force transducer, and allowed very fine adjustments to the height of the coupling rod-force transducer assembly. The completed perfusion apparatus is shown in Figures 3.4 and 3.5.

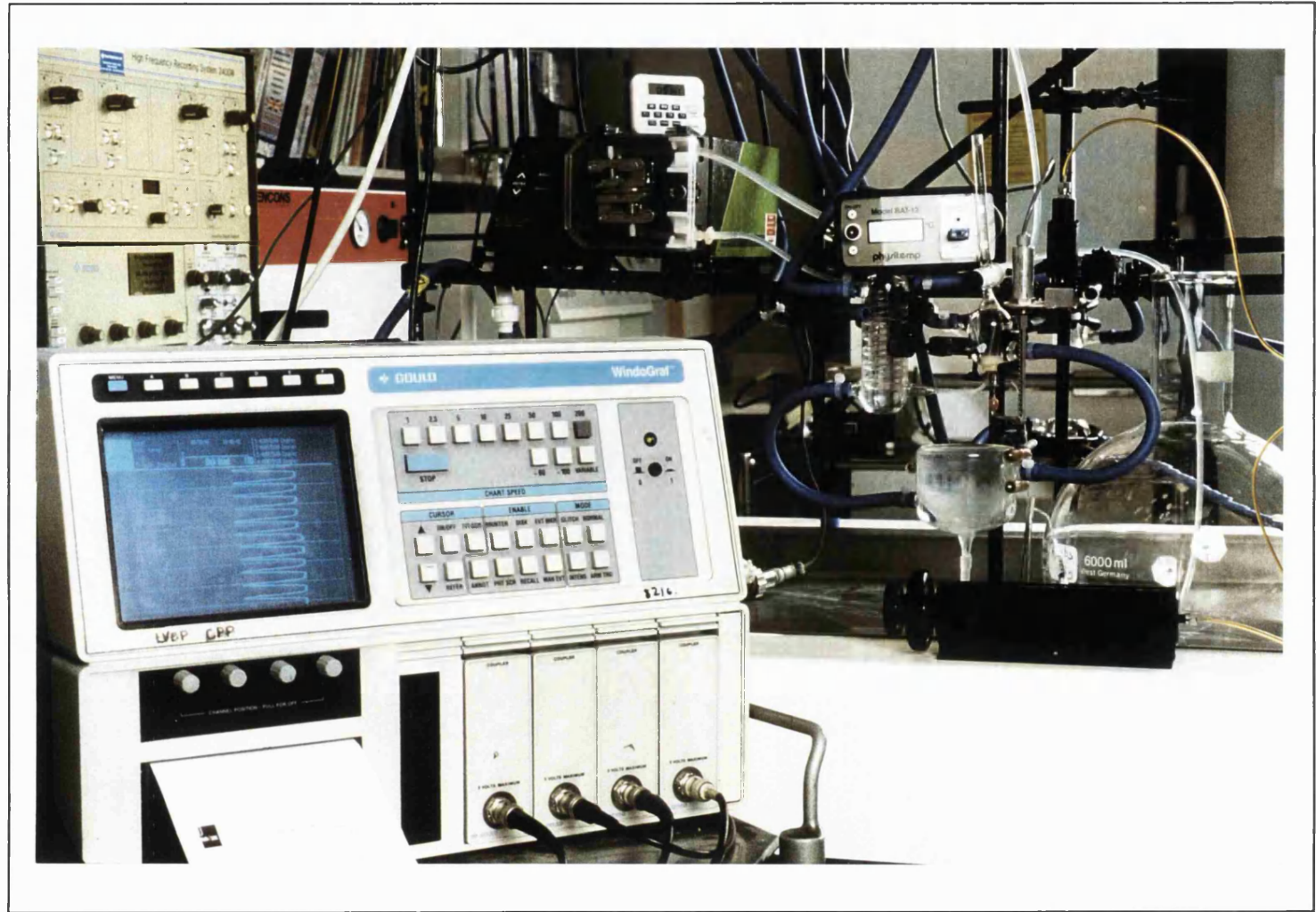


Figure 3.4 Perfusion apparatus and recording equipment

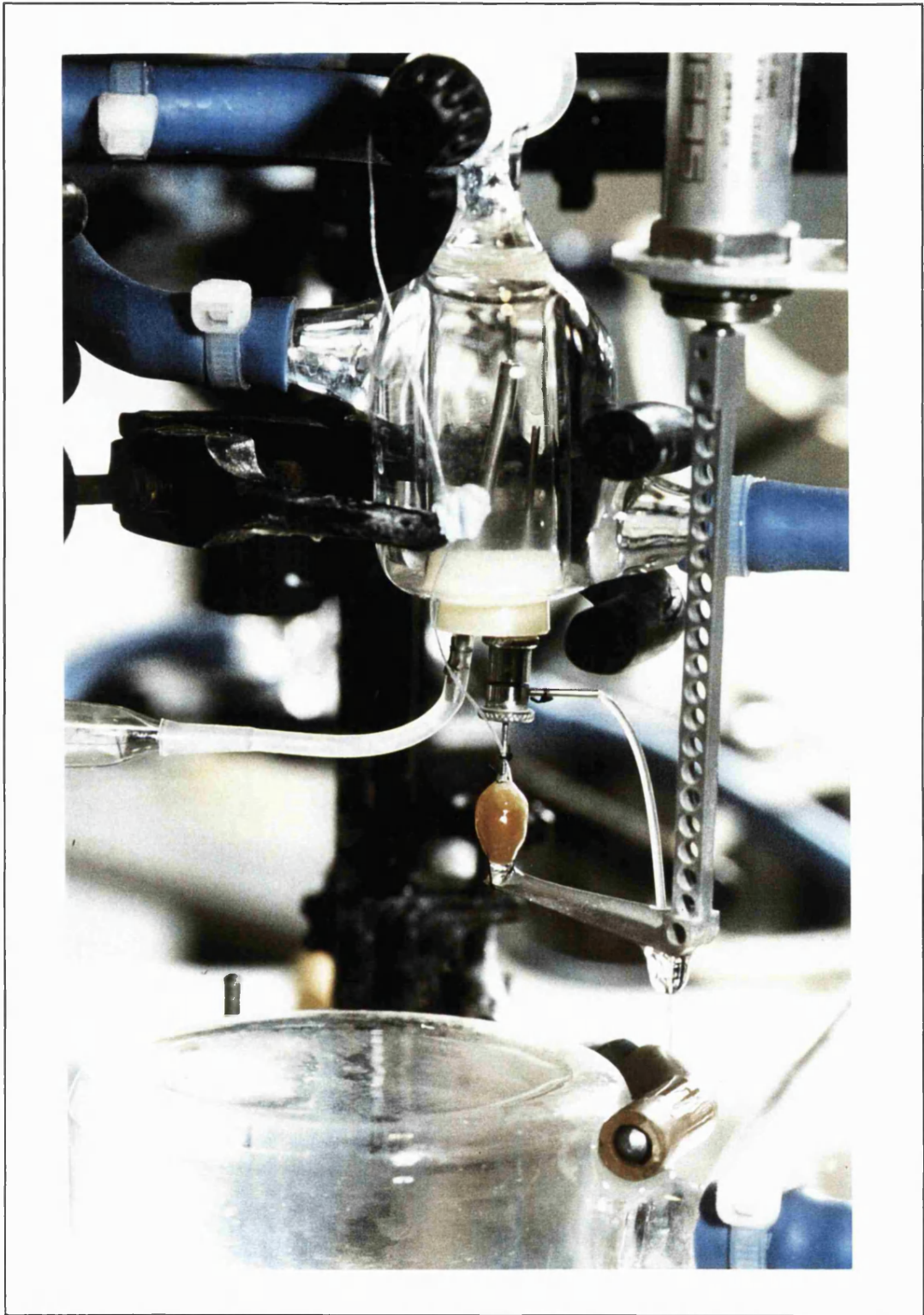


Figure 3.5 Close up view of perfused mouse heart attached to coupling rod

3.1.4 PARAMETERS MEASURED

3.1.4.1 Coronary flow rate

As detailed previously the perfusion method chosen maintained constant pressure by use of a reservoir at a fixed height above the aortic cannula. As a result, any change in resistance to coronary flow would be reflected by an alteration in flow rate. The main determinants of coronary resistance are impeding effects of contraction and arteriolar smooth muscle tone. Both of these alter the diameter of the coronary arterioles and effect a change in flow proportional to the fourth power of the vessel radius. Coronary flow rate was calculated simply by timed collections of effluent into a measuring cylinder.

3.1.4.2 Developed force

The principles employed have been described previously. At the beginning of every experiment it was essential to calibrate and balance the force transducer. Following cannulation and perfusion of the heart it was attached by a silk suture to the base of the coupling rod. The diastolic tension in the suture was adjusted using the micromanipulator to one gramme for every experiment. During the stabilisation period the silk suture gradually stretched and became saturated with perfusate. As a result the micromanipulator was used to gradually increase the distance between the heart and the coupling rod until the suture reached its 'elastic limit' and constant tension was obtained. As the perfusate dripped off the apex of the heart, it ran down the suture and along the coupling rod where it collected momentarily before falling off as a large droplet. This process caused a slight variation in the weight of the coupling rod and altered the baseline diastolic force transduced. This effect was small and did not cause difficulty interpreting the data. At fixed intervals during each experiment the force trace was recorded with the paper speed set to 100 mm/s. This allowed the shape of the force trace to be accurately assessed and its maximum point was clearly identified.

3.1.4.3 Temperature

The nature and position of the thermocouple wire have been described previously. Precise control of myocardial temperature for the entire duration of the experiment was essential,

especially during the period of global ischaemia. It has been shown that the cardiac temperature during ischaemia *in vivo*, even within a narrow 'normothermic' range, can alter the volume of infarction (Chien *et al.*, 1994). The high surface area to volume ratio of the mouse heart results in rapid heat loss when the continuous stream of warm perfusate no longer runs down the epicardial surface. During ischaemia it was necessary to immerse the entire heart (attached to coupling rod) in a water bath containing anoxic buffer at 37 °C. This proved to be an extremely effective method of maintaining myocardial temperature during this critical period. The temperature of the water circulating within the jacketing of the glassware was thermostatically controlled by a heated water circulator (Techne Circulator C-85, Cambridge, UK). Using these methods the myocardial temperature was maintained at 37 ± 0.2 °C for the entire duration of each experiment.

3.1.4.4 Heart rate

This parameter was simple to calculate from the force trace by counting the number of contractions within a measured distance and converting this to time with knowledge of the paper speed. The hearts were allowed to beat without electrical pacing for all the experiments described. This eliminated the possibility of splinting the heart by the presence of an additional wire attached to the right atrium and allowed the effects of any intervention on heart rate to be monitored. However, this approach necessitated a correction to the calculated developed force. During periods of bradycardia there is more time for intracellular accumulation of calcium between beats, resulting in increased contractility. To control for this effect the force-rate product was calculated. This was especially important where small differences between experimental groups might have been significant.

3.1.5 PREPARATION OF HEARTS FOR PERFUSION

3.1.5.1 Anaesthesia

All animals were deeply anaesthetised using sodium pentobarbitone (60 mg/kg) by intraperitoneal injection. Heparin (100 iu) was given concomitantly to prevent thrombus formation. Consciousness was usually lost within 60 seconds, and the animal was immediately weighed. It was then transferred to an operating board and the limbs secured.

An adequate depth of anaesthesia was confirmed by testing the hind-limb withdrawal reflex to pain.

3.1.5.2 Dissection

The chest was opened, excising the sternum and attached costal cartilages to give adequate access to the mediastinum. The heart was rapidly removed following transection of the pulmonary veins and great vessels, and placed in a culture dish containing iced buffer. Thymic and fatty tissue were carefully trimmed using fine dissecting scissors to reveal the ascending aorta. The cannula (primed with iced buffer to eliminate any air from the tubing attached to the side arm and the cannula) was held under the surface of the buffer by a clamp attached to a ball-jointed rod system. This allowed the position of the cannula to be adjusted, if necessary, beneath the buffer. A length of silk suture was looped around the tip of the cannula so that it could be tied to secure the aorta without wasting time. The ascending aorta was picked up between fine forceps and gently stretched around the tip of the cannula, where it was secured. Great care had to be taken to avoid tearing the aorta, or damaging the aortic valve and coronary ostia. During transfer to the perfusion apparatus the cannula was continuously perfused through a side arm with buffer under low pressure to prevent air entry. Once attached to the perfusion apparatus, retrograde perfusion at constant pressure was commenced. After considerable practice the time between excision of the heart and the commencement of perfusion never exceeded two minutes. It was particularly important to minimise this time and maintain consistency between hearts due to the theoretical risk of inadvertent preconditioning due to perfusion delay (Minhaz *et al.*, 1995).

3.1.6 TECHNICAL CONSIDERATIONS AND ANALYTICAL TECHNIQUES

Before each experiment the electrical circuit to the force transducing cell was switched on for at least 30 minutes. This was important since the flow of current had a significant warming effect on the delicate internal components of the cell and affected the mechanical transduction of force generated. This was in keeping with the recommendations of the manufacturer. The force transducing cell was held vertically and the coupling rod attached to its transduction arm. As a result, the effect of gravity and the weight of the coupling rod

displaced the position of the transduction arm from its neutral position. To simulate the effect of coronary perfusate, water was dripped along the coupling rod to add the relevant weight so that an appropriate neutral position was taken up. The wheatstone bridge of the transduction cell was then balanced using the preamplifier that provided the excitatory current. This ensured that the baseline did not shift when altering the gain on the amplifier. The force transducer was then calibrated using a series of weights (from 0.5 to 3 g). These steps were repeated every time the apparatus was used.

The in-line filter was changed every day (glass microfibre 'paper') to ensure maximum efficiency. To further reduce the possibility of embolisation within the circuit the perfusion apparatus was thoroughly washed through with boiled purified and filtered water. This ensured that the risk of fungal contamination was minimised. As an extra precaution the perfusion circuit was flushed through with a biological detergent 'Decon 90' (Rhône-Poulenc) once weekly.

3.1.6.1 Perfusion buffer

Hearts were perfused with a modified Krebs-Henseleit buffer (Krebs and Henseleit, 1932) that had previously been used successfully for isolated perfusion of the mouse heart (Marber *et al.*, 1995), the composition of which is shown in Table 3.1.

Table 3.1 Composition of modified Krebs-Henseleit buffer

Reagent	Concentration
NaCl	118.0 mM
NaHCO ₃	24.0 mM
KCl	4.0 mM
NaH ₂ PO ₄	1.0 mM
CaCl ₂	2.5 mM
MgCl ₂	1.2 mM
di-sodium EDTA	0.5 mM
D-glucose	10 mM

Buffer was made with purified water (reverse osmosis, organic adsorption, deionisation) from an Elga UHP and Elgastat spectrum purifier (Elga Ltd, High Wycombe, UK) and

fresh buffer was made each day. The buffer was prefiltered as described previously and then gassed with a mixture of 95% O₂ and 5% CO₂. The flow rate of gas from the cylinder was adjusted to achieve a physiological pH (7.36 - 7.44).

3.1.6.2 Infarct size analysis

The primary end-point used for quantification of ischaemic injury in this model was infarct volume. Measurement of infarct size following global ischaemia in the isolated mouse heart had not been fully validated previously, although a preliminary report suggested it was feasible (Marber *et al.*, 1995). Many investigators have been reluctant to accept that an accurate assessment is possible in such a small heart, and the technique used would have to generate clearly delineated images of sufficient size to planimeter accurately. Traditionally the identification of necrotic tissue has been achieved using conventional histological techniques such as light microscopy. However the changes observed are only seen 48 hours or more following the ischaemic insult, which is clearly not a feasible time frame for an isolated heart preparation. Electron microscopy, however, may be used to detect changes as early as 20 minutes following ischaemia but tiny biopsies, however numerous, are liable to considerable sampling error and areas of necrosis may be missed. Fortunately, an acceptable alternative method for delineation of infarcted tissue, tetrazolium staining, has been well characterised (Fishbein *et al.*, 1981), and has been used extensively in other animal models following both *in vivo* and *in vitro* infarction protocols.

3.1.6.3 Principles of tetrazolium staining

Triphenyltetrazolium chloride (TTC) is a dye that crosses cell membranes and binds to intracellular dehydrogenase enzymes, resulting in its reduction and a colour change to deep red. Necrotic cells lose these enzymes during lysis and the process of reperfusion washes them out of the necrotic zone. As a result, only viable myocardial cells take up the dye and stain a 'brick-red' colour. An adequate process of reperfusion is essential, therefore, to allow delineation of infarcted tissue and some authorities state that periods in excess of two hours are mandatory (Ytrehus and Downey, 1993).

3.1.6.4 Staining of murine myocardium

Considerable time was spent assessing the use of TTC staining in this model. Particular consideration was given to the length of the reperfusion period and the method of dye delivery. Initially, a two hour reperfusion period was used before staining. However, a shorter reperfusion period had already been used in a similar model and found to generate identical data (Marber *et al.*, 1995). The explanation for the adequacy of a shorter reperfusion period may be related to the high coronary flow rate of the mouse heart per gramme heart weight. In comparison to the isolated rabbit heart (approximately 4 ml/g/minute) the mouse heart has a considerably greater rate of wash-out (20 ml/g/minute). Furthermore, if reperfusion is essential to wash dehydrogenases out of infarcted areas then the shorter the reperfusion period the more dead tissue should stain ('falsely') with TTC. Conversely, the longer the reperfusion period the greater the extension of the necrotic area. However, this assumption has not been borne out by experimental observation (Ganz *et al.*, 1990).

In order to address this issue, a direct comparison was made between a short reperfusion period (30 minutes) and a longer one (120 minutes) during the course of the characterisation experiments. The data are shown in section 4.4.1.2 and confirm that 30 minutes reperfusion were adequate to give consistently reliable infarct size determination.

Two methods of dye delivery were assessed during the preliminary stages of establishing the model. The first, which was subsequently abandoned, involved 'pan-staining' of the heart slices by incubating them in a 1% solution of TTC (Sigma, Loius, USA) in phosphate buffer (pH 7.4) at 37°C until deeply stained. Whilst this proved an effective method of delivery, the heart slices tended to curl up into rolls, and subsequent compression between plates for planimetry proved difficult. The second method (subsequently adopted) involved injection of the TTC down the side arm of the aortic cannula at the end of the reperfusion period whilst still 'on the rig'. The hearts were then removed once deeply stained and blotted dry before weighing. They were then frozen overnight at -80°C to facilitate slicing the next day. As the hearts were thawing they were sliced using a razor blade into one

millimeter sections in a direction parallel to the atrioventricular groove from the apex upwards. Approximately eight slices of ventricular tissue were obtained per heart. Slices were then incubated in 2% formaldehyde overnight to fix the stain.

3.1.6.5 Planimetry to calculate infarct volume

A magnified image of each heart slice (of known thickness) was obtained with use of a specially constructed camera assembly. A high resolution lens with appropriate focal length was incorporated into a camera system with adjustable height. The S-VHS signal output was connected to the appropriate port of an Apple Macintosh 7500 computer. The signal was digitised by the computer's video card and displayed on a high resolution colour monitor (Figure 3.6).

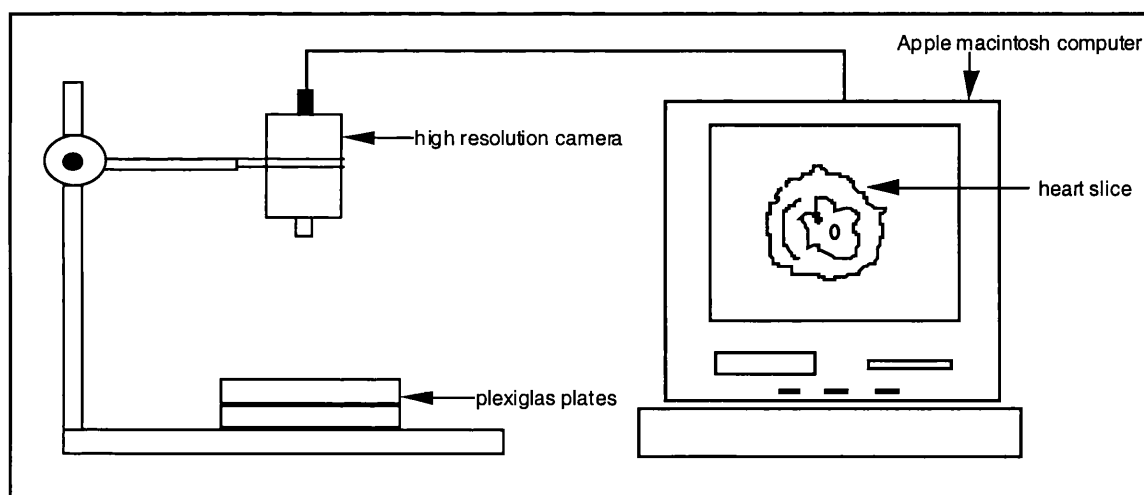


Figure 3.6 Diagrammatic representation of camera and computer assembly for planimetry of heart slices

The slices from each heart were placed between perspex plates that had been specifically made for this purpose, and were exactly 0.57 mm apart. A small piece of graph paper was placed next to the slices to act as a calibration grid. Using image analysis software (NIH Image version 1.61) the areas of infarcted tissue could be delineated using the computer's mouse. The number of pixels inside each area was displayed and recorded, and the calibration grid used to convert the values to mm^2 . The infarct volume was calculated by multiplying the area by the thickness of each slice (0.57 mm). This was then expressed as a percentage of the total volume of each slice (volume 'at risk' of necrosis). A typical

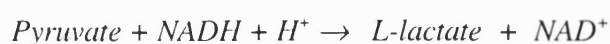
example is shown in Figure 3.7. All heart slices were planimetered blind to the experimental group from which they originated.



Figure 3.7 Digitised image of a typical mouse heart slice

3.1.6.6 Lactate dehydrogenase assay

Samples of coronary perfusate were collected and frozen at -80°C . Samples were later allowed to defrost and analysed for lactate dehydrogenase (LDH) activity. Lactate dehydrogenase catalyses the interconversion of lactate and pyruvate. The reaction equation is as follows:



During reduction of pyruvate an equimolar amount of NADH is oxidised to NAD. The oxidation of NADH results in a decrease in absorbance of the reaction mixture at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to the LDH activity in the sample.

A standard assay kit was purchased from Sigma Diagnostics (Dorset, England). The reagents necessary for the reaction were in powder form.

Reagent 'A' (0.194 mmol/L NADH; 54 mmol/L phosphate buffer, pH 7.5) was reconstituted with 20 ml of deionised water and reagent 'B' (16.2 mmol/L pyruvate) with 5 ml of deionised water. The reaction mixture was prepared by adding 0.8 ml of reagent 'B'

to 20 ml of reagent 'A'. Although the reaction mixture was stable for 24 hours when stored at 4 °C, fresh reagents were reconstituted and used immediately for each experiment.

Aliquots of coronary perfusate (0.1 ml) were added to the reaction mixture (2.5 ml), and gently mixed. Samples were analysed spectrophotometrically (Uvikon 810 spectrophotometer, Kontron, Germany) at 340 nm against deionised water as the control. An initial reading was taken at 30 seconds followed by three more at one minute intervals. The temperature of the cell within the spectrophotometer was allowed to drift from room temperature, but the temperature in each cuvette was measured using a thermocouple at the end of each assay. The mean change in absorbance per minute ($\Delta A/\text{min}$) over the three minute period was calculated and recorded.

The LDH activity per litre of perfusate was calculated from the following equation:

LDH activity (U/L)	=	$\frac{\Delta A/\text{min} \times 2.6 \times 1000}{6.22 \times 1.0 \times 0.1}$
LDH activity (U/L)	=	$\Delta A/\text{min} \times 4180$

Since the temperature of the reaction chamber was not controlled it was necessary to make a correction for the measured temperature using a conversion factor. The conversion factor was derived from a graph plotted using the data shown in Table 3.2

Table 3.2 Conversion factors for correction of assay temperature (measured in cuvette) to the standard temperature (37°C)

Assay temperature	Conversion factor (to 37°C)
25 °C	1.98
30 °C	1.47
37 °C	1.00

All the LDH data was corrected for myocardial mass by dividing the activity per litre of perfusate by the heart weight in grammes (iU/L/g).

3.1.7 STATISTICAL ANALYSIS

Results are presented as group means and standard error of the mean (SE) unless indicated otherwise. For comparison of two groups, differences within and between group means were analysed with Student's unpaired t-tests. For comparison of more than two groups, one way analysis of variance (ANOVA) was used and where a significant F value was obtained, Fishers protected least significance difference test was applied for between group comparisons. For comparison of more than two measurements recorded at different times, ANOVA for repeated measures was applied. Association between data was tested by Spearman rank correlation and linear regression was used to analyse the association for each group. Statistical significance was defined as a *P* value of ≤ 0.05 . Statistical analysis was performed on a Power Macintosh computer using commercially available software (Statview v. 4.5 , Abacus concepts).

4. CHARACTERISATION AND VALIDATION OF THE MODEL

4.1 INTRODUCTION

The advantages of setting up a model capable of reflecting the pathophysiology of ischaemia-reperfusion injury in the mouse heart have been discussed at some length previously. However, the temptation to assume that the measured end-points of injury accurately reflect the severity of ischaemic insult and the degree of necrosis should be avoided. Models that utilise single indicators of injury are disadvantaged because, even if a correlation can be demonstrated between severity of ischaemia and the end-point measured, other variables that may be influenced by ischaemia may underlie the effect observed. A good example of such a deficiency would be the assessment of contractile function following an ischaemic insult. Depressed contractility may reflect the underlying degree of necrosis, but can also be due to a 'stunning effect' which is fully reversible given adequate reperfusion for sufficient time. The use of several end-points should minimise the chances of making such misinterpretations. The extent to which these end-points co-correlate for any given severity of injury will reflect the degree to which they reflect the same pathology, and a graded increase in severity of insult should cause an associated rise in each parameter assessed. It is only after thorough characterisation that new models should be used in experiments designed to investigate various pathophysiological states.

4.2 AIMS

4.2.1 PRELIMINARY EXPERIMENTS

The aims of the preliminary experiments were as follows:

1. to assess the feasibility of isolated mouse heart perfusion using the newly-constructed apparatus
2. to demonstrate the stability of the preparation and the absence of injury following two hours of normal perfusion

3. to examine the adequacy of 30 minutes reperfusion following global ischaemia for accurate assessment of infarct size

4.2.2 VALIDATION EXPERIMENTS

The aims of the validation experiments were twofold:

1. to examine the responses to a graded increase in ischaemic injury in terms of the three end-points measured
2. to demonstrate that these three end-points co-correlated

4.3 METHODS

Male Swiss White mice (25 to 35 g body weight) were used for all experiments. Animals were deeply anaesthetised with sodium pentobarbitone (60 mg/kg *ip*). Hearts were isolated for perfusion as described in section 3.1.5.

4.3.1 EXPERIMENTAL PROTOCOL

4.3.1.1 Preliminary experiments

Twelve hearts were perfused of which two were excluded (an aortic tear, high flow rates, and ineffective coronary perfusion in one heart, and persistent bradycardia and poor contractility in the other). The remaining ten hearts were divided into one of two groups:

Group 1: hearts were perfused continuously for 120 minutes

Group 2: hearts were perfused for 30 minutes (stabilisation) before 25 minutes global ischaemia followed by either (a) 30 minutes reperfusion or (b) 120 minutes reperfusion

A summary of these protocols is represented diagrammatically in Figure 4.1.

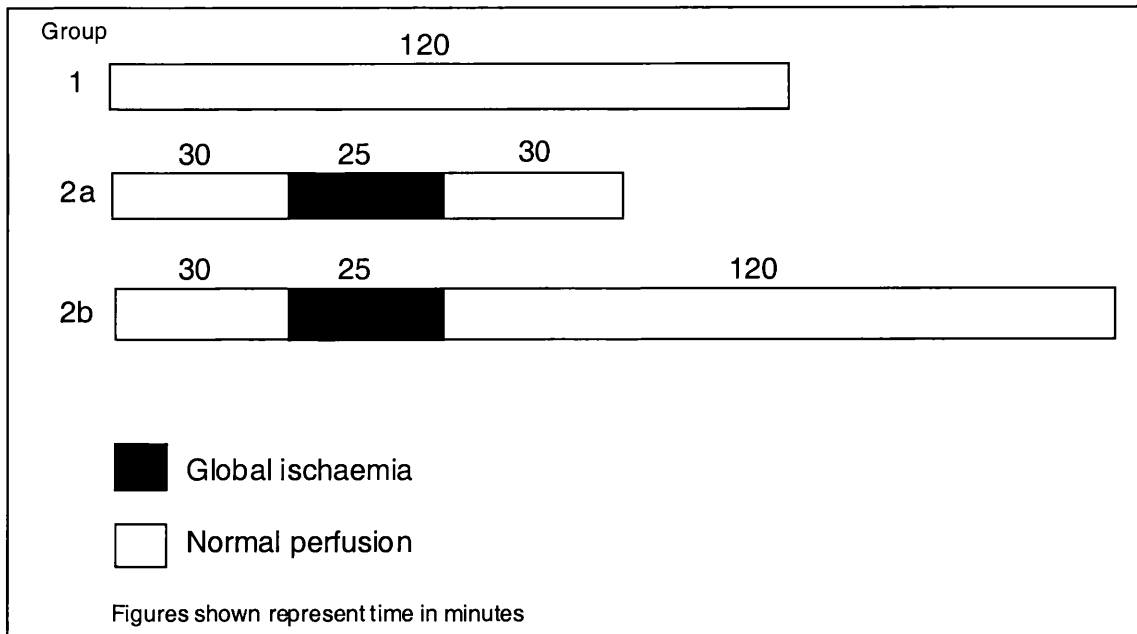


Figure 4.1 Diagrammatic representation of the three perfusion protocols used in the preliminary experiments

Throughout the perfusion period of Group 1, the following parameters were measured: myocardial temperature, developed force, heart rate, coronary flow rate and LDH release. After 120 minutes of perfusion had been completed, an assessment of infarct size was made as described in section 3.1.6.2.

Infarct size analysis only was carried out on hearts randomised to both Groups 2a and 2b.

4.3.1.2 Validation experiments

Forty-four hearts were entered into this study but eight were excluded during the stabilisation period and before any data had been recorded. This was due to aortic damage in five cases and persistent tachyarrhythmia in two cases. One heart was excluded due to severe bradycardia. Therefore a total of thirty six hearts were randomised into one of six groups.

The control group (Group 1) comprised six hearts that received continuous perfusion. The five experimental groups each consisted of six hearts receiving differing periods of global ischaemia: Group 2, 10 minutes; Group 3, 15 minutes; Group 4, 20 minutes; Group 5, 25 minutes, and Group 6, 30 minutes. Reperfusion was then carried out for 30 minutes following the ischaemic insult.

A summary of these perfusion protocols is shown in Figure 4.2.

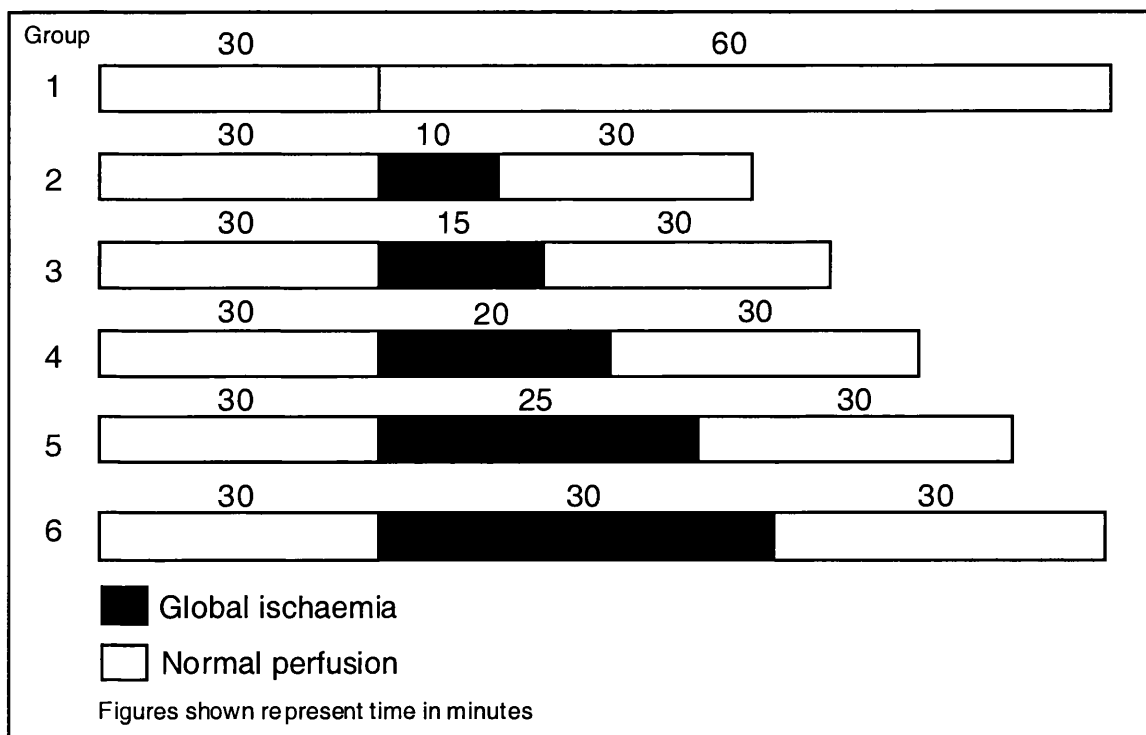


Figure 4.2 Diagrammatic representation of the six perfusion protocols used in the validation experiments

Baseline measurements were made in all six groups prior to the hearts receiving any ischaemia. The following parameters were assessed: body weight of the donor mouse, coronary flow rate, heart rate and developed force of the isolated hearts. Heart weight was measured at the end of each experiment.

Following the ischaemic insult and subsequent 30 minute reperfusion period of the isolated hearts, these parameters were measured in all groups: recovery of contractile force, coronary flow rate and heart rate. Myocardial temperature was monitored throughout the period of global ischaemia and during reperfusion. Infarct size analysis was carried out as described in section 3.1.6.2

4.4 RESULTS

4.4.1 PRELIMINARY EXPERIMENTS

4.4.1.1 Group 1

Hearts randomised to Group 1 underwent 120 minutes of continuous perfusion, and the results of these preliminary experiments are shown in Table 4.1.

Table 4.1 Mean values for temperature, developed force, heart rate, coronary flow rate and lactate dehydrogenase release during 120 minutes continuous perfusion of murine hearts (Group 1)

Time perfused (min)	Temperature (°C)	Developed force (% baseline)	Heart rate (bpm)	CFR (ml/min)	LDH (iU/L/g)
20	37.0 (0.2)	100.0 (0.0)	335 (08.2)	3.4 (0.2)	41.3 (05.3)
30	37.2 (0.0)	96.4 (3.6)	316 (12.8)	2.8 (0.3)	47.3 (16.0)
40	37.0 (0.1)	96.7 (3.3)	316 (17.4)	2.6 (0.2)	32.5 (10.2)
50	37.0 (0.1)	96.7 (3.3)	315 (08.1)	2.6 (0.2)	45.1 (08.6)
60	37.1 (0.1)	95.0 (3.4)	323 (12.0)	2.6 (0.2)	30.9 (04.8)
90	37.1 (0.1)	95.2 (3.1)	320 (15.2)	2.5 (0.3)	43.2 (09.4)
120	37.0 (0.2)	95.3 (3.2)	315 (09.7)	2.5 (0.3)	52.4 (10.1)

Standard error values shown in parentheses

Group 1 n = 4

bpm beats per minute

CFR coronary flow rate

LDH lactate dehydrogenase

4.4.1.1.1 Myocardial temperature

Within a few seconds following the initiation of perfusion the myocardial temperature rose rapidly to 37 °C. The temperature then remained stable throughout the perfusion period.

4.4.1.1.2 Developed force

This is expressed as a percentage of the force generated in grammes after 20 minutes perfusion. During this initial stabilisation period the resting tension was gradually increased to one gramme for every heart. The developed force remained constant after 30 minutes

perfusion with no evidence of deterioration. Heart rate and coronary flow also remained stable after 30 minutes perfusion.

4.4.1.1.3 Lactate dehydrogenase release

A constant low level of background LDH was released during perfusion.

4.4.1.1.4 Infarct size analysis

All four hearts stained uniformly deep red with no discernible evidence of infarction in any heart.

4.4.1.2 Group 2

Hearts in Group 2 underwent 25 minutes of global ischaemia followed by (a) 30 minutes or (b) 120 minutes reperfusion. The results are shown in Table 4.2.

Table 4.2 Mean infarct sizes in murine hearts after 25 minutes of global ischaemia followed by either 30 minutes (Group 2a) or 120 minutes reperfusion (Group 2b)

Group	Time of reperfusion (min)	Infarct size (I/R%)
2a	30	25.2 (1.6)
2b	120	23.8 (1.2)

Standard error values shown in parentheses

Group 2a: n = 3

Group 2b: n = 3

4.4.2 VALIDATION EXPERIMENTS

The key baseline characteristics of the 36 donor mice and isolated hearts subsequently randomised to one of six groups did not differ significantly prior to any period of ischaemia (Table 4.3).

Table 4.3 Mean values for body weight of 36 donor mice and the heart weight, coronary flow rate, heart rate, and developed force of their isolated hearts randomised to one of six groups prior to delivery of a varying ischaemic insult

Group	Body weight (g)	Heart weight (g)	CFR (ml/min)	Heart rate (bpm)	Developed force (g)
1	28.4 (0.8)	0.15 (0.01)	3.4 (0.2)	336 (08.2)	2.5 (0.4)
2	26.9 (0.3)	0.15 (0.01)	3.0 (0.4)	355 (11.8)	3.0 (0.2)
3	27.7 (0.8)	0.17 (0.01)	3.5 (0.6)	357 (15.6)	3.0 (0.6)
4	27.0 (0.9)	0.19 (0.03)	3.7 (0.8)	343 (16.7)	3.6 (0.5)
5	26.1 (1.3)	0.15 (0.01)	3.8 (0.9)	367 (16.7)	2.8 (0.2)
6	30.9 (0.9)	0.22 (0.02)	3.0 (0.4)	372 (16.8)	3.6 (0.3)

Standard error values shown in parentheses

Groups 1-6 n = 6

bpm beats per minute

CFR coronary flow rate

4.4.2.1 Infarct size

Volume of infarcted myocardium was expressed as a percentage of ventricular volume. Myocardium was stained with tetrazolium after 30 minutes of reperfusion. The results (Figure 4.3a) demonstrate a graded injury in response to increasing periods of global ischaemia. Ventricular volume did not differ significantly between groups (Figure 4.3b). It can be seen that there was minimal infarction until a 25 minute global ischaemic insult had been administered ($23.3\% \pm 1.5\%$). There was a significant increase in infarct size compared with controls at 25 and 30 minutes ($23.3\% \pm 1.5\%$, $59.8\% \pm 5.1\%$) indicating that tissue necrosis did not increase linearly with duration of ischaemia.

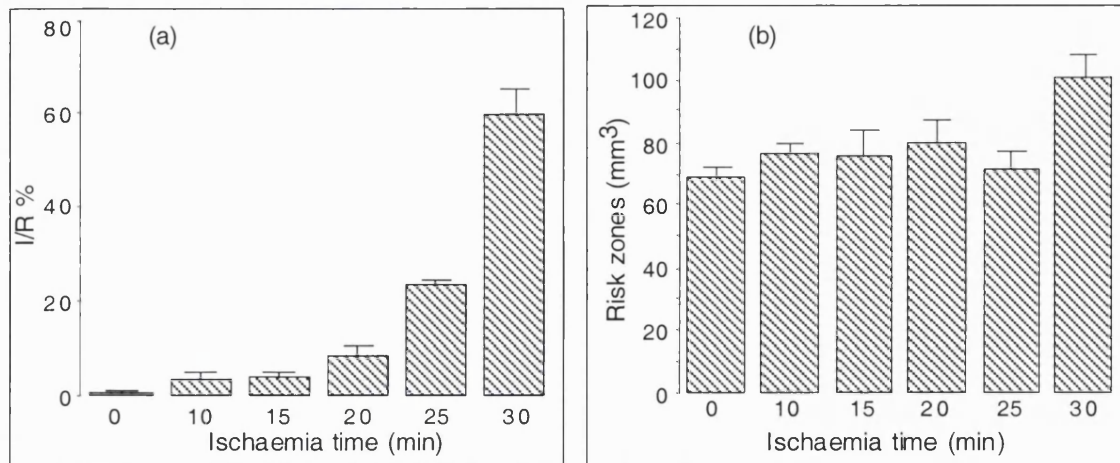


Figure 4.3 (a) Infarct size and (b) ventricular volume (risk zone) against duration of global ischaemia in 36 isolated murine hearts

4.4.2.1.1 Contractile recovery

During ischaemia, hearts demonstrated an initial reduction in diastolic tone followed by the onset of severe contracture and loss of contractile activity (Figure 4.4). The first five minutes of reperfusion show a further increase in resting diastolic tone and little recovery of contractile function.

Developed force during the reperfusion period was recorded at regular intervals and, following an initial hypercontractile phase during the first two minutes, maximum recovery had occurred by 30 minutes reperfusion.

A typical force tracing recorded during 30 minutes of global ischaemia is shown in Figure 4.4.

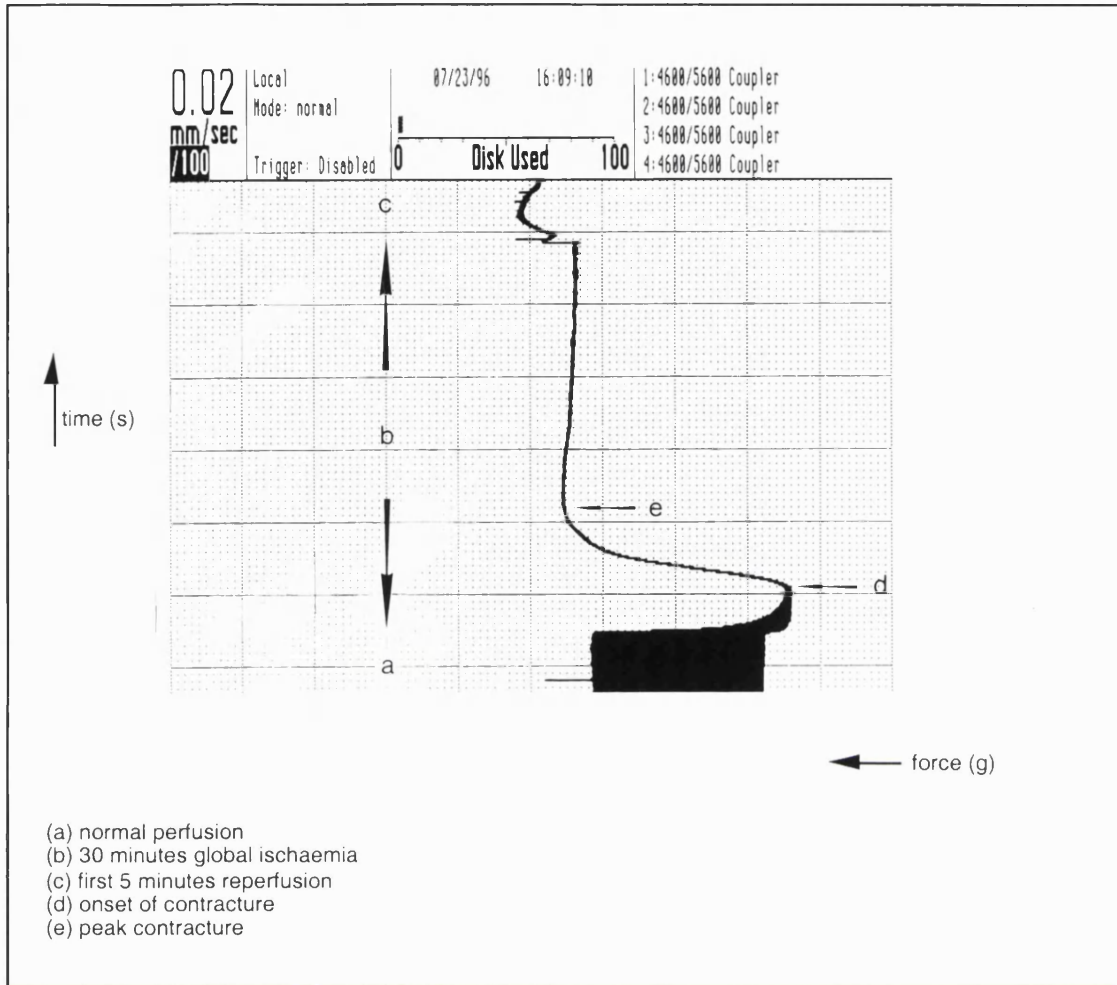


Figure 4.4 Force tracing of mouse heart during 30 minutes of global ischaemia followed by five minutes of reperfusion

Contractile force at the end of 30 minutes reperfusion was expressed as a percentage of baseline (Figure 4.5).

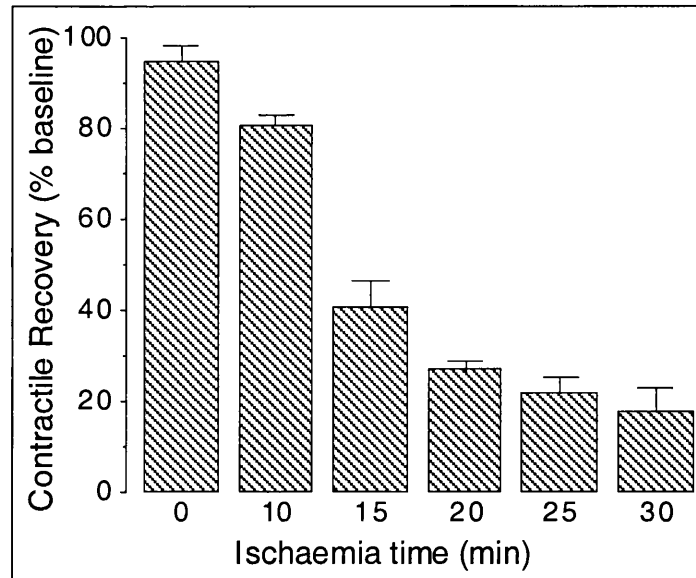


Figure 4.5 Contractile recovery of 36 hearts after 30 minutes reperfusion against duration of ischaemia

In contrast to tissue necrosis, contractile function was depressed considerably following a shorter duration of global ischaemia. All groups exhibited significantly reduced contractile function compared to controls, but hearts subjected to 25 and 30 minutes ischaemia did not differ significantly from those subjected to 20 minutes ischaemia. The component of contractile dysfunction that is clearly unrelated to myocardial necrosis probably reflects a significant 'stunning' effect.

4.4.2.1.2 Lactate dehydrogenase release

Samples of coronary perfusate were collected at regular intervals during reperfusion and analysed for LDH activity. Release peaked at 30 minutes reperfusion and Figure 4.6 shows values for each of the groups.

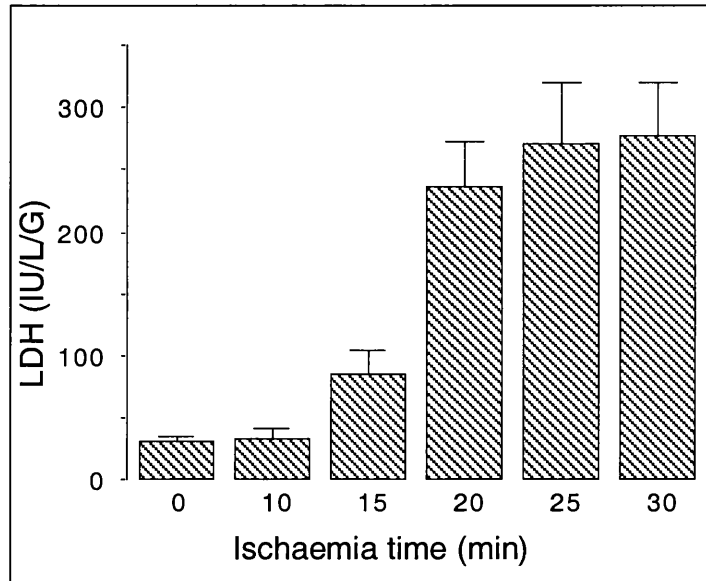


Figure 4.6 Lactate dehydrogenase (LDH) activity in coronary perfusate (collected after 30 minutes reperfusion) against duration of ischaemia in 36 hearts

Considerable variability was present and standard errors were large. Hearts subjected to periods of ischaemia in excess of 15 minutes released significant quantities of LDH compared to controls, but did not differ significantly from each other.

4.4.2.1.3 Comparison of infarct volume with contractile recovery and LDH release

Figures 4.7 (a) and (b) show that functional recovery and enzyme leakage correlated well with infarct size ($r = 0.77$, $P < 0.001$ and $r = 0.73$, $P < 0.001$ respectively).

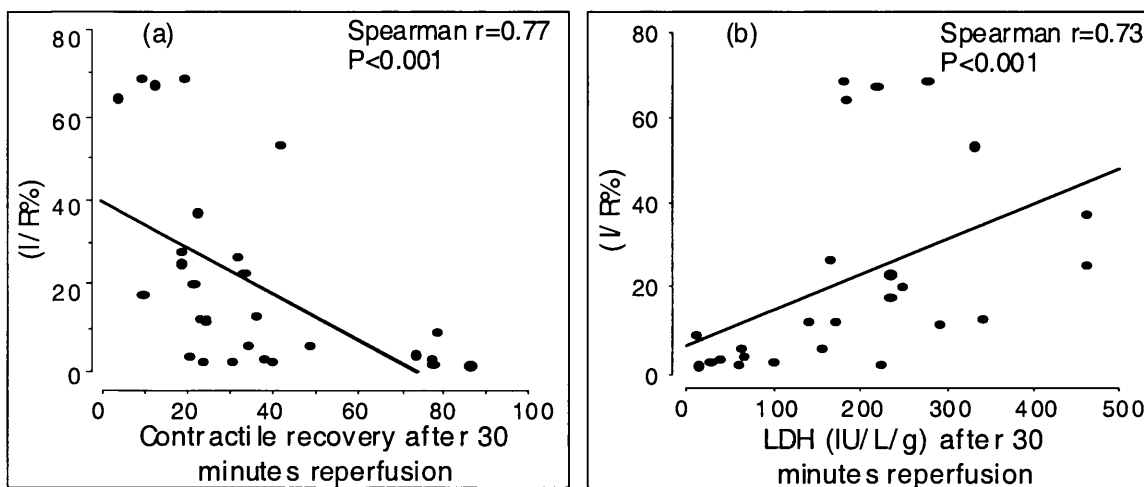


Figure 4.7 Correlation between infarct size and (a) contractile recovery and (b) LDH release in 36 hearts

Hearts in the group subjected to 30 minutes ischaemia displayed significantly greater necrosis without a corresponding effect on either contractile recovery or release of LDH (both of which plateaued after 20 minutes ischaemia). Different mechanisms of injury are likely to be responsible.

4.4.3 DISCUSSION

4.4.3.1 Preliminary experiments

These established the stability of the model during prolonged isolated perfusion of the mouse heart. The longevity of the preparation will have been affected by the choice of perfusion buffer. The decision to use Krebs' solution (modified for mouse heart perfusion) was based on a number of factors. The advantages of crystalloid solutions include consistency of formulation, simplicity, accepted reliability, ease of addition of drug treatments, and no necessity to re-circulate the perfusate. The disadvantages of blood perfusion include the need for an anaesthetised support animal with re-circulation of blood. This would have posed considerable technical problems given that the total blood volume of an adult mouse is less than two millilitres. It is recognised, however, that crystalloid perfusion is unphysiological due to the absence of cellular elements, low oxygen carrying capacity but high oxygen tension, and low oncotic pressure. These deficiencies result in myocardial oedema in the crystalloid perfused heart and necessitate a much higher coronary flow rate to maintain adequate oxygen delivery. For this reason a constant perfusion pressure of 80 mmHg was chosen, accepting that this is somewhat higher than murine aortic diastolic pressure *in vivo* (55 mmHg).

Several other important technical considerations were successfully addressed. Myocardial temperature was effectively maintained at a constant value throughout the perfusion period. There was a slight decline in contractility, heart rate and coronary flow over time, but after 30 minutes perfusion these values changed very little. It seemed logical, therefore to use a 30 minute stabilisation period for all subsequent experiments. The reperfusion protocol for assessing infarct size was also confirmed. As in previous studies (Marber *et al.*, 1995) a

30 minute period for reperfusion was adequate for wash-out of dehydrogenase enzymes from infarcted areas, since the infarct volume after two hours reperfusion was identical.

4.4.3.2 Validation experiments

These firmly established the reliability and reproducibility of the model in terms of the different end-points of injury used. The feasibility of measuring infarct size by tetrazolium staining in such a small heart was established despite the potential problems with this technique. A magnified video image can be digitised by computer video card allowing accurate planimetry using appropriate software.

This model was not designed to assess the incidence and nature of arrhythmias occurring during the reperfusion period, and electrocardiographic recordings were not made. However, certain observations were of interest. In general the incidence of arrhythmia increased with more prolonged periods of ischaemia. Eighty percent of hearts subjected to ten or 15 minutes of global ischaemia suffered extrasystoles (which may have been of ventricular or supraventricular in origin) occurring approximately ten minutes into the reperfusion period but disappearing after 20 minutes. One half of the hearts subjected to 20, 25 or 30 minutes of global ischaemia suffered ventricular fibrillation which began 15 minutes into the reperfusion period, and resolved by 30 minutes in approximately 80% of hearts.

Several other interesting areas have been highlighted. It would appear that there is probably a significant component of myocardial stunning in this model, although this hypothesis has not been tested directly. Following 15 minutes of ischaemia, contractile recovery was reduced to 40% of baseline, but infarct size was minimal (4%). It was only after 25 minutes of ischaemia that there was appreciable necrosis (I/R 23%), and there was a disproportionate increase to 60% after 30 minutes ischaemia. However, there was very little further increase in contractile dysfunction from 20 to 30 minutes ischaemia, indicating that contractility was likely to have been depressed by a mechanism independent of necrosis. It should be noted, however, that even after short periods of ischaemia, some necrosis had occurred and may have been responsible for the impaired contractility

observed at these time points. It is also interesting to note that release of LDH rose rapidly between 15 and 20 minutes ischaemia and then reached a plateau. This was in contrast with the time period of necrotic injury and indicates that LDH might be released from cells that are still viable. Alternatively, wash-out of LDH may be impaired following larger infarction due to lack of effective subendocardial reperfusion. It is possible that both these factors may have contributed to the effect observed. Taken together, all three end-points provided valuable information about the nature of the myocardial injury, but their differences emphasised the importance of avoiding the use of single end-points in models of ischaemia-reperfusion injury.

The isolated mouse heart has been perfused in Langendorff (Marber *et al.*, 1995) and working mode (Ng *et al.*, 1991) in previous studies. *In vivo* models, utilising regional ischaemia, have also been developed (Hutter *et al.*, 1996). The potential for well characterised physiological models to be used with new transgenic technology in the investigation of a number of myocardial pathological conditions has been recognised (Chien, 1995). It is essential that development of such models with their various end-points of injury should incorporate a thorough process of characterisation before meaningful conclusions can be drawn about the results they generate.

5. PRECONDITIONING MURINE MYOCARDIUM

5.1 INTRODUCTION

Since its recognition in 1986 (Murry *et al.*, 1986) the phenomenon of ischaemic preconditioning has been investigated in a variety of animal species. To date, experimental evidence suggests that this powerful form of endogenous cellular adaptation is universally conserved in mature cells. However, ischaemic preconditioning has not been demonstrated to reduce infarct size following global ischaemia in murine myocardium. Furthermore, it has been proposed that such protection would not be induced in animals with fast heart rates lacking mitochondrial f_1/f_0 ATPase inhibitor protein.

As discussed earlier, the protection induced by ischaemic preconditioning appears to be due to reduced utilisation of high energy phosphates during ischaemia. One proposal for the reduction in energy demand involves the action of the mitochondrial f_1/f_0 ATPase inhibitor protein. During ischaemia mitochondrial ATPase functions in reverse in an attempt to regenerate a depleted proton gradient so that supplies of reduced NADP can be replenished. This causes ATP to be 'wasted'. To counteract this tendency there is a reversible inhibitor of the ATPase which binds during ischaemia and is activated by a fall in intracellular pH (Rouslin and Pullman, 1987). It was suggested that this protein might mediate preconditioning either by binding more rapidly during a second ischaemic episode or by persistent binding during the brief reperfusion (Jennings *et al.*, 1991b). However, this explanation seems unlikely since binding of the inhibitor is very rapid (<90 seconds), reaching maximum inhibition within five minutes, with no persistence of inhibition on reperfusion (Vander Heide *et al.*, 1991). In addition, preconditioning occurs in the rat, a species known to have limited ATPase activity (Yellon *et al.*, 1992). As a result, if classic preconditioning could be demonstrated in the mouse heart this would further refute the involvement of this protein as a mediator of cytoprotection.

The mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage has been termed 'stunning'. It is clear that preconditioning of isolated hearts subjected to global ischaemia can result in greater recovery of functional parameters such as left ventricular developed pressure (Cave and Hearse, 1992). However the degree to which this effect is a manifestation of reduced necrosis rather than a reduction in myocardial stunning may be difficult to identify.

A study designed to monitor regional wall motion using ultrasonic crystals implanted in the myocardium showed improved segment shortening on reperfusion in preconditioned hearts, but in addition these hearts had smaller infarcts (Cohen *et al.*, 1991). Some studies examining preconditioning during short regional ischaemic episodes which cause stunning without necrosis have failed to show any benefit (Ovize *et al.*, 1992b). Experiments using Doppler probes to monitor segment shortening are known to give conflicting results depending on the depth of the probe within the myocardium. This reflects the fact that the effects of ischaemia increase as one moves from epicardium to endocardium. Further studies in isolated hearts have shown that if functional recovery and infarct size are examined in the same hearts, the improved recovery of preconditioned hearts correlates with infarct size limitation. It is likely, therefore, that most of the benefit is the result of a reduction in infarct size (Walker *et al.*, 1993).

The ability to assess recovery of contractile function and infarct size following an ischaemic insult in the isolated mouse heart allowed this issue to be addressed. It is already clear from the characterisation experiments (section 4) that there is a likely to be a significant component of stunning involved in this model after ischaemia-reperfusion injury. As a result, any protection seen against stunning could be demonstrated.

5.2 AIMS

The aims of the experiments in this section were:

1. to use the model in an assessment of classic ischaemic preconditioning as a method of cardioprotection against global ischaemic injury in the mouse heart

2. to establish the preconditioning protocol necessary to achieve protection
3. to investigate the nature of the protection provided (infarction vs. stunning)

5.3 METHODS

Male Swiss White mice (25 to 35 g body wt) were used for all experiments. Animals were deeply anaesthetised with sodium pentobarbitone (60 mg/kg *ip*). Hearts were isolated for perfusion as described in section 3.1.5.

Initially, neither an effective preconditioning protocol nor a suitable period of 'index' global ischaemia was known. A preliminary series of experiments (series 1) was conducted to establish these conditions, followed by a subsequent series (series 2) to confirm the efficacy of the preconditioning protocol.

5.3.1 EXPERIMENTAL PROTOCOL

5.3.1.1 Series 1

Nine hearts were randomised to one of three groups. In each group, all three hearts were allowed to stabilise for 30 minutes but then were subjected to differing periods of global ischaemia and reperfusion:

- Group 1** (Control group) hearts were perfused for a further 20 minutes before 25 minutes of global ischaemia followed by 30 minutes reperfusion
- Group 2** hearts were subjected to two, five minute periods of global ischaemia interspersed with two, five minute periods of reperfusion, before 25 minutes of global ischaemia followed by 30 minutes of reperfusion
- Group 3** hearts were subjected to two, three minute periods of global ischaemia interspersed with two, two minute periods of reperfusion, before 25 minutes of global ischaemia followed by 30 minutes reperfusion.

A summary of these perfusion protocols is represented diagrammatically in Figure 5.1

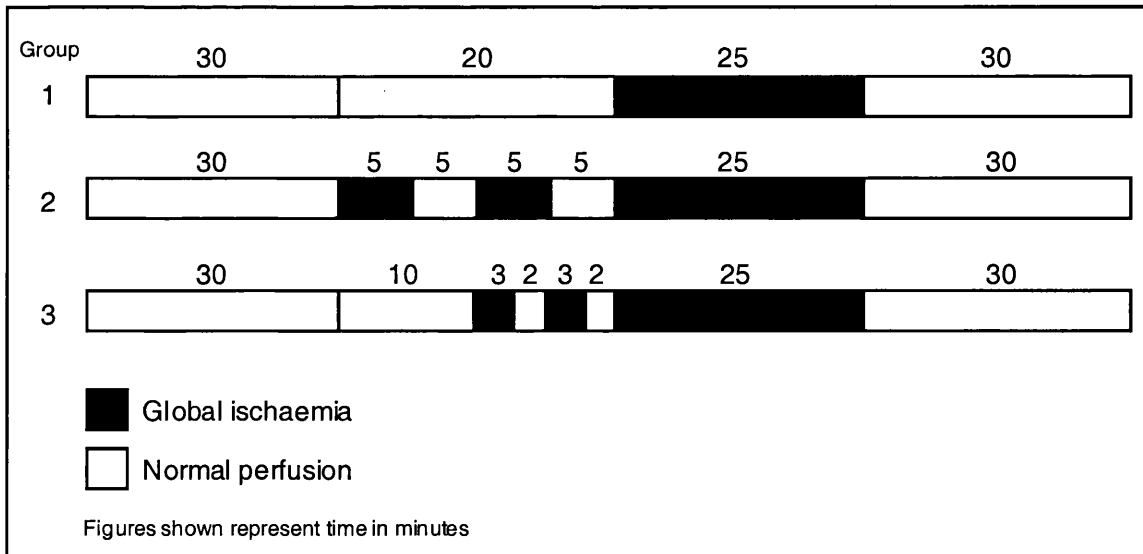


Figure 5.1 Diagrammatic representation of the three perfusion protocols used in series 1

Infarct size analyses were carried out on the nine hearts in this series to determine a suitable preconditioning protocol.

5.3.1.2 Series 2

A total of 26 hearts were perfused in this series of experiments. However, eight hearts were excluded during the stabilisation period due to persistent arrhythmia (5), severe bradycardia (1), and poor contractility (2). The remaining 18 hearts were randomised into one of two groups. In both groups, all nine hearts were allowed to stabilise for 30 minutes but then were subjected to differing periods of global ischaemia and reperfusion:

Group 4 hearts were subjected to four, five minute periods of global ischaemia interspersed with four, five minute periods of reperfusion before 30 minutes of global ischaemia followed by 30 minutes reperfusion

Group 5 (Control group) hearts were perfused for a further 40 minutes before 30 minutes of global ischaemia followed by 30 minutes reperfusion

A summary of these protocols is represented diagrammatically in Figure 5.2.

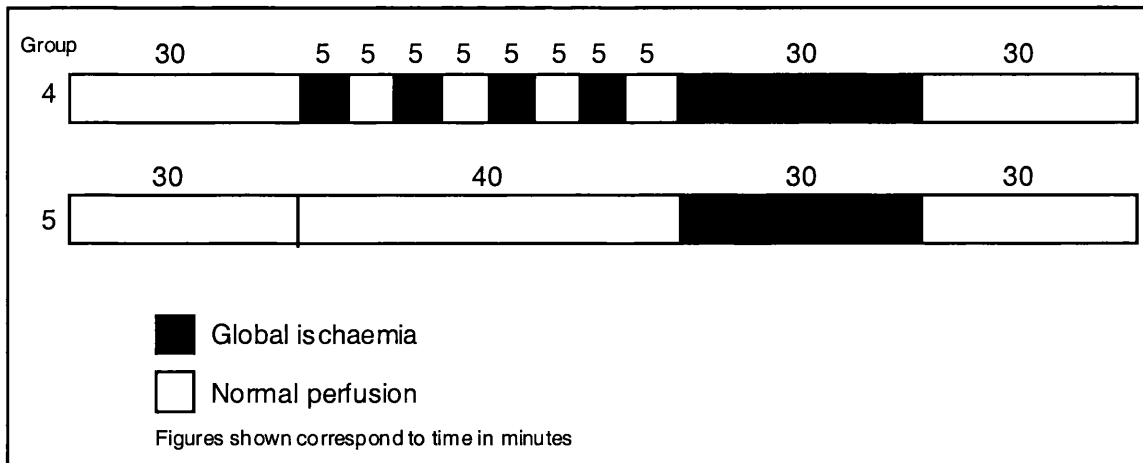


Figure 5.2 Diagrammatic representation of the two perfusion protocols used in series 2 experiments

Throughout the duration of each experiment, myocardial temperature, coronary flow rate, heart rate and developed force were measured and recorded at regular intervals. During global ischaemia the paper speed was set to 0.02 mm/s and the force trace later examined to calculate the time to onset and severity of contracture. The coronary perfusate was collected and frozen for subsequent assay of LDH activity. Following 30 minutes reperfusion, hearts were stained with tetrazolium. Planimetry was carried out later in a blinded fashion.

5.4 RESULTS

5.4.1 SERIES 1

The infarct sizes of the nine hearts included in series 1 are shown below (Table 5.1).

Table 5.1 Infarct sizes of nine murine hearts randomised to one of three different perfusion protocols

Group	Infarct size (I/R%)			Mean infarct size (I/R%)
1	25	27	23	25 (1.1)
2	25	30	32	29 (2.0)
3	25	25	20	23 (1.5)

Standard error values shown in parentheses

Group 1 (Control) n=3

Group 2 (Preconditioned) n=3

Group 3 (Preconditioned) n=3

The data from Table 5.1 indicated that there was no evidence of protection from either of the two preconditioning protocols tested (groups 2 and 3). Therefore a more intense preconditioning protocol was devised for the experiments in series 2. In addition, for series 2, the period of index ischaemia was extended from 25 to 30 minutes.

5.4.2 SERIES 2

The baseline characteristics of the 18 donor mice and their corresponding isolated hearts randomised to one of two perfusion protocols did not differ significantly prior to any period of ischaemia (Table 5.2).

Table 5.2 Mean values for body weight of 18 donor mice and the heart weight, coronary flow rate, heart rate, and developed force of their isolated murine hearts randomised to either a preconditioned or control perfusion protocol

Group	Body weight (g)	Heart weight (g)	CFR (ml/min)	Heart rate (bpm)	Developed force (g)
4	32.1 (1.1)	0.19 (0.01)	3.3 (0.4)	399 (11.0)	3.44 (0.32)
5	30.4 (0.8)	0.20 (0.01)	3.5 (0.4)	399 (8.6)	3.36 (0.21)

Standard error values shown in parentheses

Group 4 (Preconditioned) n=9

Group 5 (Control) n=9

bpm beats per minute

CFR coronary flow rate

5.4.2.1 Infarct size

The volume of infarcted myocardium was expressed as a percentage of ventricular volume (Figure 5.3).

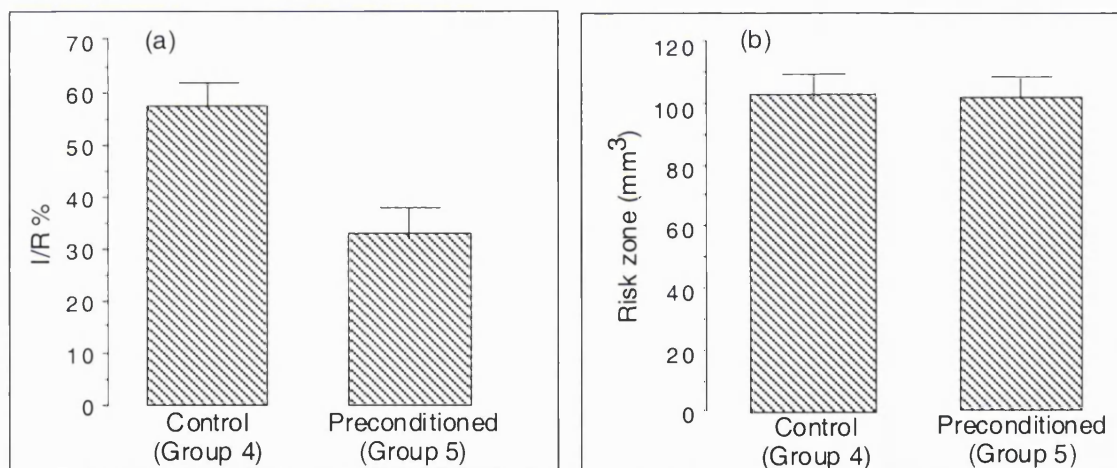


Figure 5.3 Infarct size (a) and ventricular volume (b) in control and preconditioned murine hearts

The results in Figure 5.3 (a), demonstrate a significant reduction in infarct size seen in the preconditioned group ($P=0.003$). Figure 5.3 (b) shows that ventricular volume (risk zone) did not differ significantly between groups.

5.4.2.2 Contractile recovery

The extent of contractile recovery in hearts from both experimental groups following 30 minutes global ischaemia is shown in Figure 5.4.

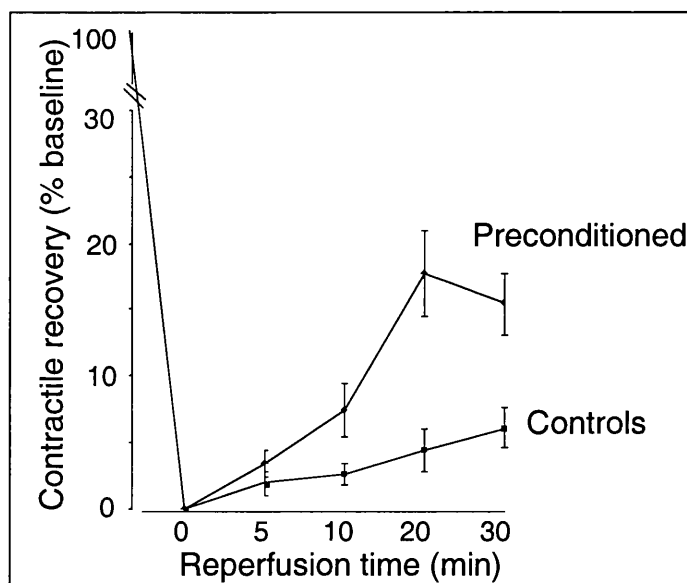


Figure 5.4 Percentage recovery of contractile function following 30 minutes global ischaemia in control and preconditioned murine hearts, during 30 minutes reperfusion

Hearts were not paced during these experiments and both groups suffered from persistent bradycardia. As a result, a correction for heart rate was incorporated to allow a more meaningful comparison between the two groups. Thus the force-rate product was calculated by multiplying the developed force (grammes) by the heart rate (bpm). The contractility data during reperfusion were then expressed as the percentage of baseline force-rate product. Once this correction had been made, a significant improvement in recovery was observed in the preconditioned group using a repeated measures ANOVA ($P=0.004$). These data, together with the uncorrected force data, are shown in Table 5.3.

Table 5.3 Infarct size and contractile data for murine hearts randomised to either preconditioned and control perfusion protocols

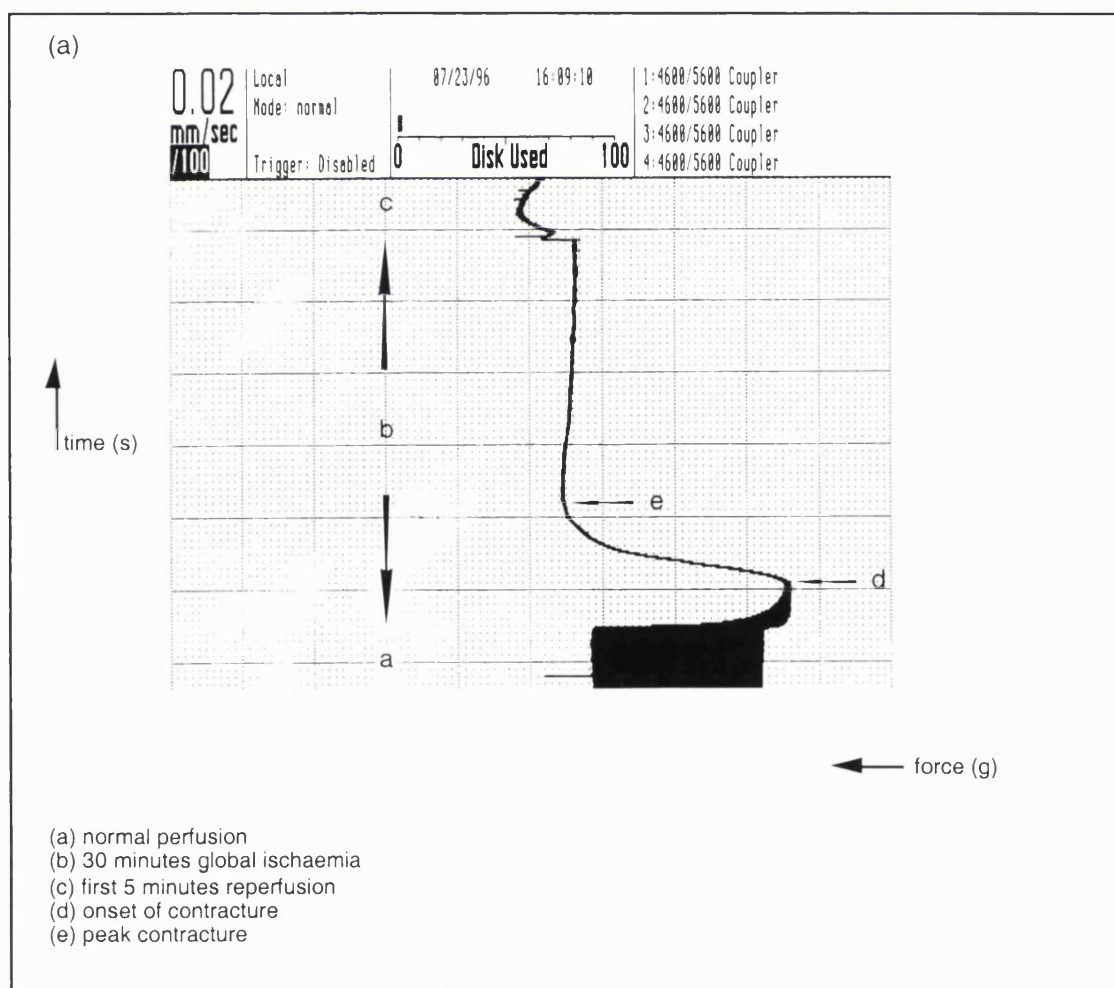
Group	I/R (%)	Risk zone (mm ³)	Developed Force (% baseline)	Force-Rate (% baseline)
4	33 (5.0)	102.2 (6.7)	17.0 (3.0)	15.5 (2.4)
5	57 (4.9)	103.1 (6.5)	9.3 (2.6)	6.3 (1.4)

Standard error values shown in parentheses

Group 4 (Preconditioned) n=9;

Group 5 (Control) n=9

The force data were also examined to allow a comparison of diastolic function between the two groups. A sample tracing (one from each group) is shown in Figure 5.5.



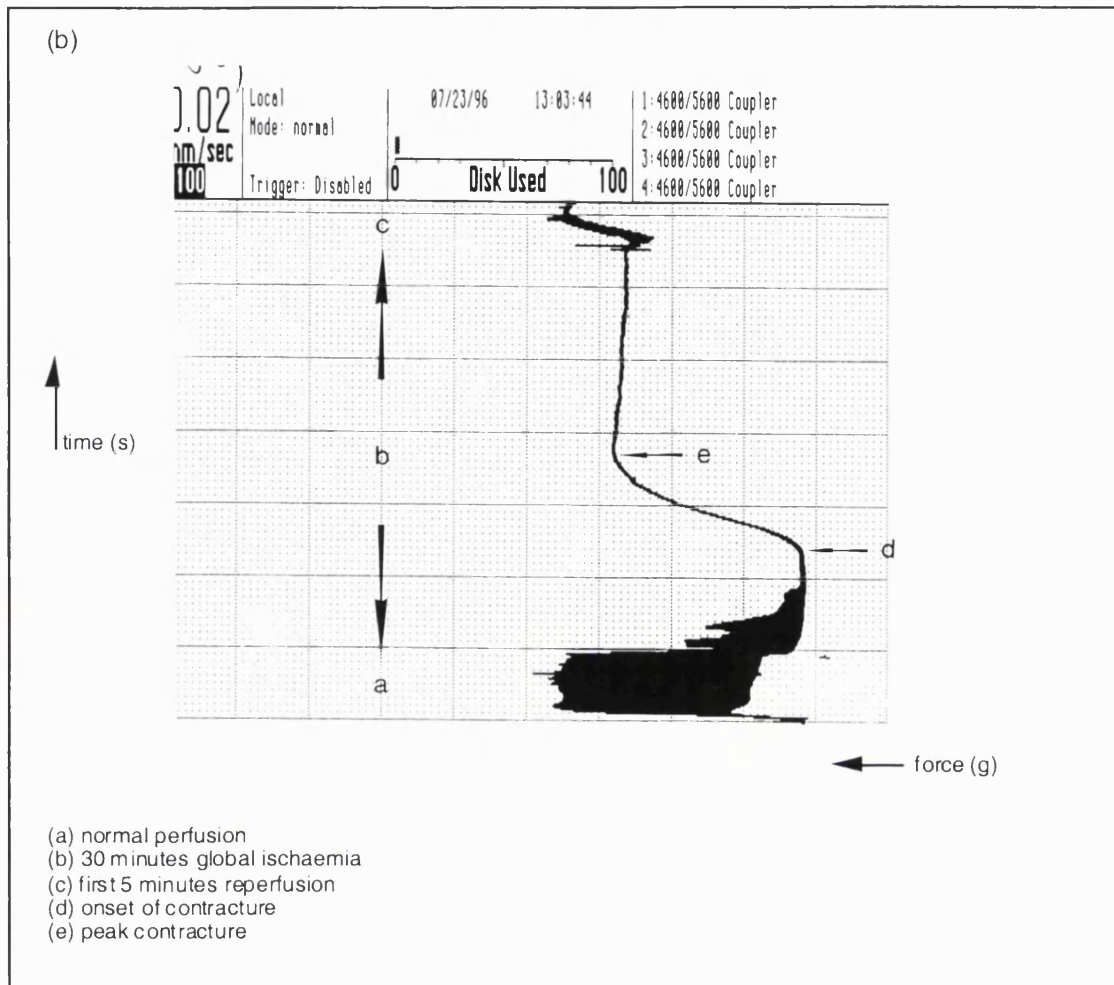


Figure 5.5 Force tracings during 30 minutes of global ischaemia followed by five minutes reperfusion in (a) control and (b) preconditioned murine hearts

Immediately following the onset of global ischaemia, the resting tension decreased (usually about 0.5 g) and rapidly stabilised. After a variable period of time, resting tension started to rise as contracture developed. Although there was a trend towards an earlier time to onset of contracture in the control group (mean 10.6 ± 2.7 minutes) compared with the preconditioned group (mean 12.2 ± 1.2 minutes), this was not significant. The peak contracture showed a stronger trend in the same direction (control group mean 4.6 ± 1.1 g; preconditioned group mean 2.7 ± 0.19 g), but this just failed to reach statistical significance ($P=0.08$).

5.4.2.3 Lactate dehydrogenase release

Hearts in both the control and preconditioned groups released large quantities of LDH into the coronary perfusate, with peak release occurring at 20 minutes reperfusion (Figure 5.6).

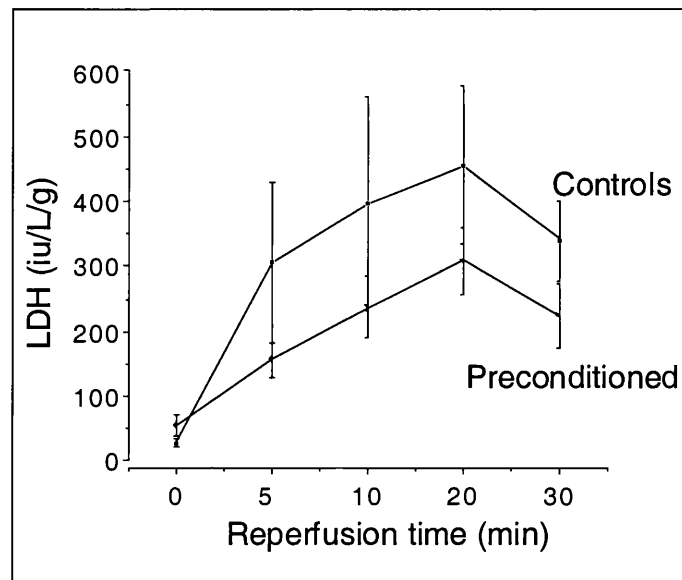


Figure 5.6 Release of LDH into coronary perfusate by control and preconditioned murine hearts during 30 minutes reperfusion

A consistently higher level was seen in the control group at all time points but, due to the considerable spread of the data and large standard deviation, the difference between the groups failed to reach significance ($P=0.43$ repeated measures ANOVA).

5.4.2.4 Coronary flow rate

Since the perfusion system was constant pressure, changes in coronary vascular resistance were reflected by changes in the flow rate of coronary perfusate. This was monitored during the 30 minute reperfusion period and the mean values expressed as a percentage of baseline flow for each of the two groups. The results are shown in Table 5.4 and demonstrate a significant reduction in flow rate in the control group ($P=0.0001$ repeated measures ANOVA).

Table 5.4 Coronary flow rate in murine hearts in both control and preconditioned groups during 30 minutes reperfusion

Group	Reperfusion time (minutes)				
	Baseline	5	10	20	30
4	100 (0)	86.6 (4.9)	84.5 (5.0)	81.7 (5.0)	79.8 (5.0)
5	100 (0)	56.4 (9.5)	55.1 (9.7)	56.9 (9.4)	55.7 (9.7)

Standard error values shown in parentheses
 Group 4 (Preconditioned) n=9;
 Group 5 (Control) n=9

5.4.2.5 Arrhythmias

As discussed in section 4, electrocardiographic recordings were not available. However, it was possible to identify periods of ventricular fibrillation (VF) with reasonable confidence from the force tracing. Supraventricular and ventricular tachycardias could not be distinguished, however. The incidence of reperfusion VF was similar in both groups (six hearts in the control group; seven hearts in the preconditioned group).

5.5 DISCUSSION

The protective effect of ischaemic preconditioning against a global ischaemic insult had not been demonstrated previously in the isolated mouse heart, and, as a result, the most effective protocol for preconditioning ischaemia was unknown. Previous studies have not examined infarct size reduction as the primary indicator of protection (Gabel *et al.*, 1996), however, no animal species examined to date has failed to demonstrate this phenomenon in the myocardium.

The preliminary experiments described in this chapter (series 1) suggested that a moderate quantity of global ischaemia (two, five minute periods, or two, three minute periods) were ineffective preconditioning stimuli to reduce infarct size after a 25 minute period of global ischaemia. It was possible that these protocols may have been either too severe, or insufficient in duration. The rat heart is another 'fast heart rate' animal, and has similar myocardial energetics to the mouse. Both species lack the f_0/f_1 ATPase inhibitor protein, and have a high rate of turn-over of adenine nucleotides. Previous work in the rat heart has

suggested that a fairly severe preconditioning protocol may be necessary to induce protection. One study just achieved a protective effect with one cycle of five minutes regional ischaemia (Yellon *et al.*, 1992), but another study found that three cycles of five minutes regional ischaemia were necessary (Liu and Downey, 1992). On this basis, the preconditioning protocol was modified to include four periods of five minutes global ischaemia (series 2).

Preconditioning is known to *delay* the onset of infarction, so that infarct size may be the same after 30 minutes ischaemia in a preconditioned heart, as after 25 minutes ischaemia in an untreated heart. The amount of time that would be 'bought' was also hard to predict in the mouse heart. However, from the characterisation data (section 4), after 30 minutes global ischaemia, infarct size would be approximately 60% of the total ventricular volume. If the predicted benefit from preconditioning was in the region of three to five minutes, infarct size should be reduced to 25 to 30% of ventricular volume. If the same benefit was conferred against 25 minutes global ischaemia, the predicted infarct size would be a reduction from 25% to 9-15% of ventricular volume. Since the mouse heart is so small, the technique used to assess infarct size is more accurate when the infarcted volume of myocardium is larger. For this reason the period of index ischaemia was extended from 25 to 30 minutes.

Using the revised protocol (series 2) a significant reduction in infarct size was seen from 57% in control hearts to 33% in the preconditioned hearts following 30 minutes global ischaemia ($P=0.003$). Using the data from the characterisation experiments (section 4) this equates to a saving of 3.5 minutes of ischaemic time, and represents a reduction of 42% in infarct size after the same duration of ischaemia.

Systolic contractile function was also improved in the preconditioned group. When developed force after 30 minutes reperfusion (expressed as a percentage of baseline) for the two groups was compared, the difference just failed to reach significance ($P=0.07$). However, once the data had been corrected for heart rate to give the 'force-rate product', preconditioned hearts had significantly better preserved systolic function ($P=0.004$). This

improvement in function may be due to a reduction in the amount of infarcted myocardium present in the preconditioned group. From the characterisation experiments (section 4), the predicted improvement in contractility expected when 3.5 minutes of ischaemic time are 'saved' by a protective intervention is 15%. However, systolic function was 1.5 times better in the preconditioned group than controls. This suggests, but does not prove, that a large element of the improvement observed in systolic function was due to a reduction in myocardial stunning.

Diastolic function was not significantly improved by the preconditioning protocol although there was a trend in that direction. In addition, recovery of baseline coronary flow rate was significantly better in preconditioned hearts suggesting a lesser degree of contracture. Traditionally the appearance of contracture has always been regarded as an indicator of severe ischaemic injury, since the underlying mechanism reflects depletion of ATP and permanent 'rigor bonds' between myofilaments. However, a study in the isolated rat heart has provided interesting evidence that the presence and extent of contracture may not necessarily predict the likely recovery on reperfusion (Kolocassides *et al.*, 1996). Preconditioned hearts in this study demonstrated accelerated contracture, which was paralleled by a more rapid decline in ATP levels than control hearts during ischaemia. However, on reperfusion, preconditioned hearts demonstrated improved contractile recovery. Not only does this cast serious doubt over the value of the presence of contracture as a predictor of post-ischaemic recovery, but it also suggests that events on reperfusion may be far more important determinants of recovery from an ischaemic insult.

Release of LDH into the coronary perfusate was consistently lower in the preconditioned group. However, due to the considerable spread of the data, the difference failed to reach significance ($P=0.43$). This is consistent with data from previous experiments, and probably reflects the variation in effective reflow in the subendocardial region.

The incidence of reperfusion VF was high in both groups with no evidence of any difference between them. There has been no experimental work to date examining reperfusion arrhythmias in the isolated mouse heart, however certain characteristics appear

to distinguish this species. Unlike the rat heart, in which arrhythmias occur early (within one minute) during reperfusion, the mouse heart suffered arrhythmia after ten to 15 minutes of reperfusion. Episodes were usually multiple and self-limiting, sometimes lasting a few seconds, but occasionally several minutes. After 30 minutes reperfusion, episodes of VF were infrequent. It would appear that ischaemic preconditioning does not confer protection against the occurrence of reperfusion VF in this species, but this observation requires confirmation using a model specifically designed to assess electrocardiographic changes.

In conclusion, this model of ischaemia-reperfusion injury in the isolated mouse heart has been used to demonstrate the protective efficacy of a protocol of ischaemic preconditioning. Both infarct size and contractile recovery were improved in the preconditioned hearts. Further evidence has been provided to refute the involvement of the mitochondrial f_0/f_1 ATPase inhibitor protein in the mechanism of ischaemic preconditioning.

6. NITRIC OXIDE AND MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY

6.1 INTRODUCTION

The presence of NO in different cell types and its implication in many diverse biological processes has been extensively discussed (section 1.4). Nitric oxide would certainly appear to play an important role in modulating myocardial ischaemia-reperfusion injury, but its precise effects vary according to experimental model and individual protocol. As a result continuing controversy surrounds the nature of its involvement in ischaemia-reperfusion injury and the balance between deleterious and beneficial effects.

In the past, the use of pharmacological blockade to eliminate NO production by its synthetic enzymes has been complicated by lack of specificity for the three isoforms of NOS. No agent exists that exhibits complete specificity. However, targeted disruption of the genes for the various isoforms offers a useful genetic approach to study the roles of each isoform and examine the effects of their deletion on the pathophysiology of ischaemia-reperfusion injury.

Investigators examining the effects of NO on brain injury following ischaemia have been the first to appreciate the potential of this approach. Animal models of focal ischaemia have employed occlusion of nutrient cerebral vessels, most commonly the middle cerebral artery. In addition, primary neuronal cortical cultures have been exposed to excitotoxic or ischaemic conditions and the activities of NOS isoforms or NO production evaluated. Genetically-altered mice lacking expression of one of the individual NOS isoforms have been examined in models of excitotoxic and ischaemic injury (Huang *et al.*, 1994). The observation that eNOS appears to reduce infarct size (probably by maintenance of cerebral blood flow) and that nNOS would appear to exacerbate ischaemic injury, is of considerable interest. This experimental approach has not been applied to myocardial ischaemia-reperfusion injury, however.

The existence of such mutant mice offers the possibility of investigation using both *in vivo* and *in vitro* models. The isolated mouse heart perfused with crystalloid buffer in Langendorff mode clearly represents an 'unphysiological' situation. The heart is denervated, does not perform work with the usual relationship between ventricular preload and afterload, and is perfused with crystalloid buffer which lacks cellular elements, has low oxygen carrying capacity but high oxygen tension, and low oncotic pressure. These deficiencies result in myocardial oedema and necessitate a much higher coronary flow rate to maintain adequate oxygen delivery. Despite these limitations, however, there are certain advantages to the use of the *in vitro* mouse heart. The lack of blood-borne cellular elements allows their contribution to the evolution of ischaemia-reperfusion injury to be isolated from the model, and the potential still exists to perfuse with leucocyte-enriched buffer as part of a separate experiment. The use of global (rather than regional) ischaemia more closely mirrors the surgical situation.

The use of mutant mice with absent expression of the individual isoforms of NOS allow their individual contributions to be examined. The mechanisms underlying the contribution of NO to ischaemia-reperfusion injury involve both blood-mediated and non blood-mediated effects. Its effects on leucocyte activation and platelet adherence will be absent in any investigation using the *in vitro* crystalloid-perfused mouse heart. However, the effects of NO on endothelial cell function including vasomotor tone and free radical generation persist. Furthermore, any direct myocardial inotropic or chronotropic effects of NO will also be apparent. These considerations allow certain inferences to be drawn about the role of NO derived from individual isoforms in the absence of its effects on blood-borne cellular elements.

6.2 AIMS

The aims of this set of experiments were twofold:

1. to demonstrate that genetically-altered mice can be used successfully to investigate the interaction between their phenotype and the pathophysiology of myocardial ischaemia-reperfusion injury
2. to investigate the role of the individual constitutive isoforms of NOS in modulating myocardial ischaemia-reperfusion injury

6.3 METHODS

Nitric oxide synthase knockout (NOS KO) and wild-type mice (WT) were used for these experiments. Targeted deletions of the mouse genome had generated eNOS and nNOS knockout mice. In 1993 Huang *et al.* reported the results of their experiments in which they had generated homozygous mutant mice lacking the nNOS gene. They cloned and mapped the nNOS gene prior to disrupting it using a targeting vector (the neomycin resistance gene) in an embryonic stem cell line (Huang *et al.*, 1993). Identical techniques were used to generate mutant mice lacking the eNOS gene (Huang *et al.*, 1995). Homozygous mutants for each gene were obtained from this source and bred at University College, London.

The three experimental groups comprised eNOS KO, nNOS KO and wild type mice. Animals were deeply anaesthetised with sodium pentobarbitone (60 mg/kg *ip*). Hearts were isolated for perfusion as described in section 3.1.5.

6.3.1 EXPERIMENTAL PROTOCOL

A total of 41 hearts were perfused on the Langendorff apparatus. Five hearts were excluded during the stabilisation period; three hearts demonstrated poor contractility and severe bradycardia, one suffered frequent and refractory ventricular ectopics, and one sustained an aortic tear during cannulation. Mice from each of the three groups were selected at random. A final total of 36 hearts (12 from each group) were subjected to ischaemia-reperfusion injury as indicated below (Figure 6.1).

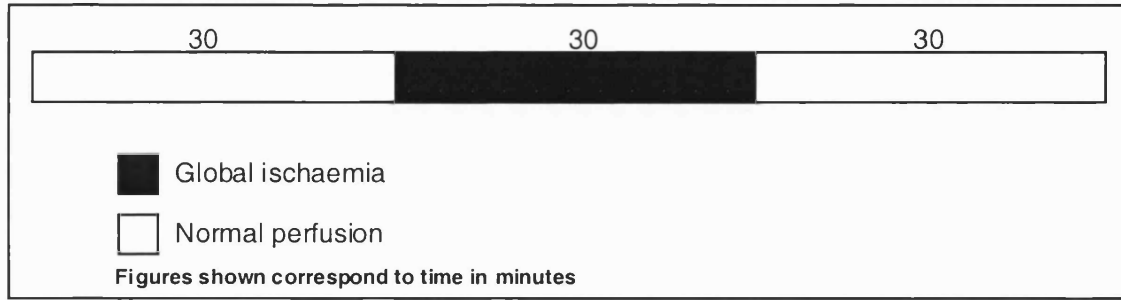


Figure 6.1 Experimental perfusion protocol for eNOS Knockout, nNOS Knockout, and Wild Type groups

Throughout the duration of each experiment myocardial temperature, coronary flow rate, heart rate and developed force were measured and recorded at regular intervals. Planimetry was carried out later in a blinded fashion.

6.4 RESULTS

The characteristics at baseline are shown for all three groups in Table 6.1.

Table 6.1 Mean values for body weight of 36 donor mice, and the heart weight, coronary flow rate, heart rate, and developed force of their isolated hearts prior to 30 minutes of global ischaemia

Group	Body weight (g)	Heart weight (g)	CFR (ml/min)	Heart rate (bpm)	Developed force (g)
WT	31.7 (0.4)	0.21 (0.01)	3.6 (0.3)	368 (18)	3.96 (0.27)
eNOS KO	33.2 (1.1)	0.22 (0.01)	2.9 (0.1)	318 (10)	4.29 (0.25)
nNOS KO	25.2 (1.0)	0.15 (0.01)	2.7 (0.2)	331 (13)	3.72 (0.28)

Standard error values shown in parentheses

Group WT (wild type) n=12

Group eNOS KO (endothelial nitric oxide synthase knockout) n=12

Group nNOSKO (neuronal nitric oxide synthase knockout) n=12

bpm beats per minute

CFR coronary flow rate

Certain differences were immediately obvious. Coronary flow rate was significantly lower in both eNOS and nNOS knockouts compared to wild-type (eNOS KO vs. WT P=0.02; nNOS KO vs. WT P=0.003). Heart rate was lower in both knockout groups but the difference only reached significance in the eNOS KO group (P=0.02 vs. WT). The other differences related to the size of the animals. Mean body weight and heart weight were

significantly lower in the nNOS group compared with both eNOS KO and WT mice ($P < 0.0001$). Developed force did not differ significantly between any of the groups.

6.4.1 INFARCT SIZE

The volume of infarcted myocardium was expressed as a percentage of ventricular volume (Figure 6.2).

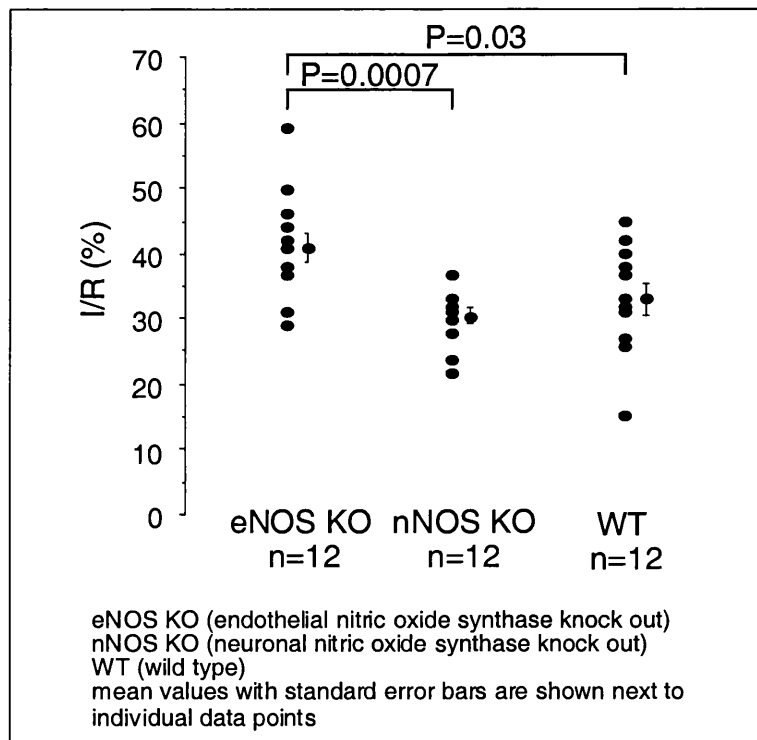


Figure 6.2 Infarct sizes in 36 murine hearts derived from eNOS Knockout, nNOS Knockout, and Wild Type groups

Infarct size in the eNOS KO group was significantly greater than WT ($P=0.03$) and greater than nNOS KO ($P=0.0007$). Infarct size in the nNOS KO group appeared slightly smaller than WT and the data appeared to exhibit far less spread. However, this difference was not significant ($P=0.3$). Figure 6.3 shows absolute values for (a) the mean infarct volume and (b) the ventricular volume of each group. Although hearts in the nNOS KO group had smaller infarct volumes than the other two groups, the hearts were smaller in this group.

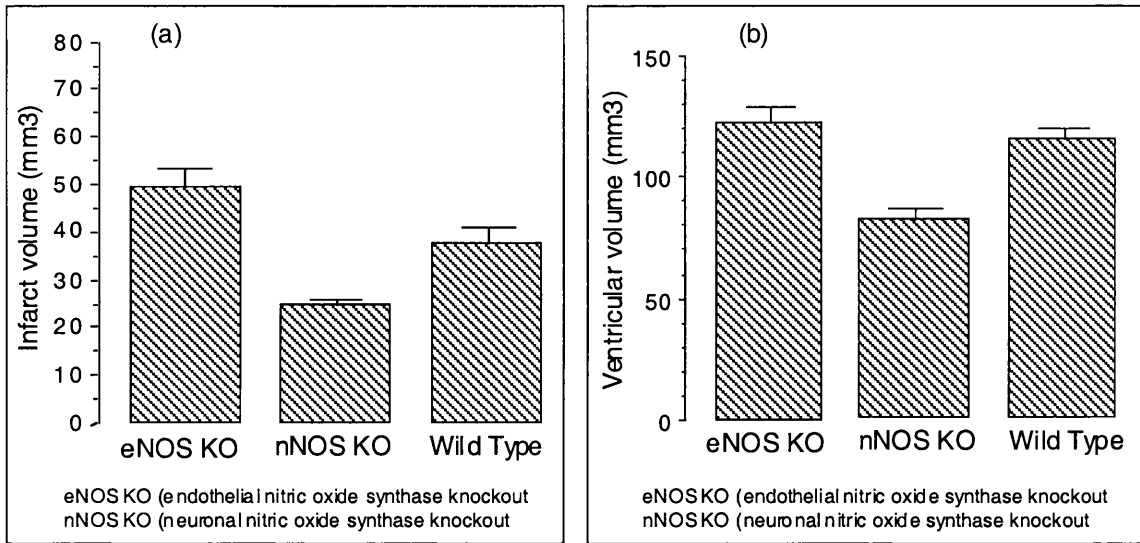


Figure 6.3 Mean values for (a) infarct volume and (b) ventricular volume for 36 murine hearts derived from eNOS Knockout, nNOS Knockout, and Wild Type groups

6.4.2 CONTRACTILE RECOVERY

The force-rate product was calculated for all hearts by multiplying the developed force (grammes) by the heart rate (bpm). The data was then expressed as a percentage of baseline force-rate product. These data are represented graphically in Figure 6.4.

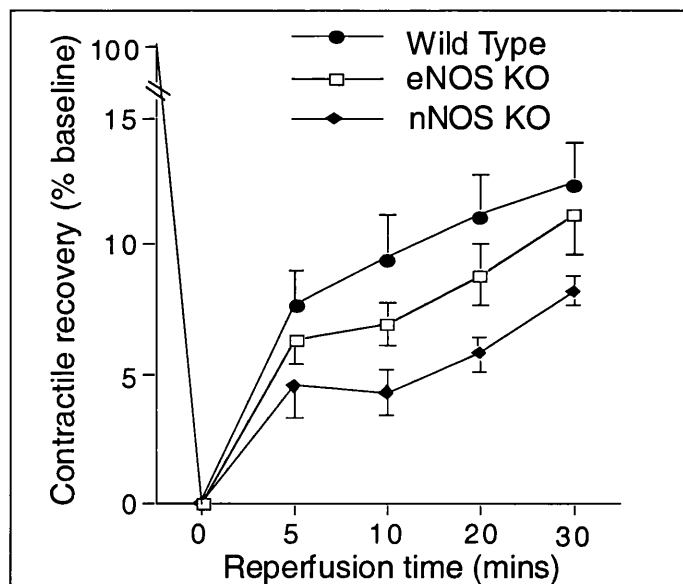


Figure 6.4 Recovery of contractile function in 36 murine hearts derived from eNOS Knockout, nNOS Knockout, and Wild Type groups during 30 minutes reperfusion

There were no significant differences in contractile recovery between groups (repeated measures ANOVA).

6.4.3 CORONARY FLOW

Coronary flow rate was calculated at baseline and then during reperfusion. The data was then expressed as percentage change from baseline and is shown in Table 6.2.

Table 6.2 Coronary flow rate (% baseline) in 36 murine hearts derived from eNOS Knockout, nNOS Knockout, and Wild Type groups during 30 minutes reperfusion

Group	Reperfusion time (minutes)				
	Baseline	5	10	20	30
WT	100	66.7 (4.5)	62.9 (4.5)	62.6 (4.4)	59.4 (4.0)
eNOS KO	100	63.9 (4.1)	61.1 (3.8)	55.4 (3.4)	51.8 (3.5)
nNOS KO	100	58.2 (3.3)	57.9 (4.0)	56.3 (4.9)	53.7 (3.6)

Standard error values shown in parentheses

Group WT (wild type) n=12

Group eNOS KO (endothelial nitric oxide synthase knockout) n=12

Group nNOSKO (neuronal nitric oxide synthase knockout) n=12

The reduction in coronary flow observed following ischaemia did not differ significantly between the groups. In absolute terms, coronary flow rate was significantly lower in both knockout groups for the entire duration of reperfusion, but flow rates in these groups were lower at baseline.

6.4.4 HEART RATE

Heart rate was recorded during reperfusion and the results expressed as a percentage of baseline values. No significant differences were observed between the three groups (Table 6.3).

Table 6.3 Heart rate (% baseline) in 36 murine hearts derived from eNOS Knockout, nNOS Knockout, and Wild Type groups during 30 minutes reperfusion

Group	Reperfusion time (minutes)				
	Baseline	5	10	20	30
WT	100	58 (6.0)	69 (5.0)	85 (3.0)	80 (2.0)
eNOS KO	100	62 (6.0)	74 (7.0)	90 (3.0)	97 (2.0)
nNOS KO	100	58 (6.0)	69 (3.0)	87 (6.0)	99 (1.0)

Standard error values shown in parentheses

Group WT (wild type) n=12

Group eNOS KO (endothelial nitric oxide synthase knockout) n=12

Group nNOSKO (neuronal nitric oxide synthase knockout) n=12

6.5 DISCUSSION

6.5.1 BASELINE CHARACTERISTICS

During the 30 minute stabilisation period prior to the ischaemic insult, certain important characteristics separated the three groups of mouse hearts. Baseline coronary flow rate was significantly lower in the eNOS and nNOS knockouts than in the wild types. This reflects an increase in coronary vascular resistance since the hearts are perfused at constant pressure. It is well recognised that basal release of NO is an important determinant of resting coronary tone (Griffith *et al.*, 1987). Furthermore, inhibition of NO synthesis in the coronary vasculature by L-arginine analogues leads to vasoconstriction of the coronary microcirculation (Amezcuca *et al.*, 1989), and can cause myocardial ischaemia (Patel *et al.*, 1993). This is consistent with the lower coronary flow rates observed in the knockout mice in these experiments. It is surprising, perhaps, that increased synthesis and release of other autocooids (*eg* prostacyclin, adenosine, or endothelium-derived hyperpolarising factor) does not occur during development and maturation, leading to compensatory coronary vasodilation. This apparent absence of homeostasis may be due to the fact that the increase in resting coronary tone is relatively mild and insufficient to cause a reduction in tissue oxygen content, accumulation of metabolites, or changes in intraluminal pressure. Furthermore, denervation by *in vitro* perfusion may have removed an important influence of altered autonomic tone. It is of note, however, that eNOS and nNOS knockouts seemed

similarly affected, implying that nNOS may also have a significant role in the basal synthesis of NO and regulation of coronary vascular resistance.

Heart rate was also noted to be significantly lower in the eNOS KO group compared to wild types. This trend was also present in the nNOS group although the effect failed to reach statistical significance. The direct chronotropic effects of NO would appear to behave in a concentration-dependent fashion. When present in large quantities (as may be encountered in pathological states such as septic shock) NO has a negative chronotropic (and inotropic effect). This has been confirmed by observation of the effect of exogenous NO on isolated sinu-atrial nodal cells (Han *et al.*, 1995). However, in a recent study using guinea pig atria, the chronotropic effect of NO behaved in a biphasic manner (Musialek *et al.*, 1997). High concentrations caused a decrease in spontaneous beating rate and low concentrations caused an increase.

At physiological concentrations, therefore, NO may act to accelerate heart rate. In eNOS KO mice resting NO levels will be lower still, and this positive chronotropic effect may be lost. It is also interesting to note that these observations are mirrored in male eNOS KO mice *in vivo* (Rees, DD unpublished data). However, resting blood pressure is higher in these animals providing a stimulus for a reflex bradycardia.

The last baseline characteristic that distinguished the groups relates to animal and heart weight. The nNOS KO group were significantly lighter than eNOS KOs and wild types both in terms of body weight and heart weight. The differences observed were not large, however, and, although the nNOS KO mice would have been capable of further growth (in terms of muscle mass rather than body fat), they were within the normal adult range. The differences probably arose due to the difficulty breeding this particular genetically-altered animal, with smaller numbers of younger mice available as a consequence.

6.5.2 INFARCT SIZE

The primary finding of this study concerns the effect of targeted deletion of individual constitutive isoforms of NOS on infarct size following an ischaemia-reperfusion protocol. Infarct size in mice lacking eNOS was significantly greater than wild types, and nNOS

KOs (41% vs. 33% and 30% respectively). This finding is consistent with the view that NO release during reperfusion attenuates the resultant injury. The endothelial isoform of NOS is likely to play an important role in maintaining coronary vasodilation and improving coronary flow following an ischaemic insult. Although the precise anatomical distribution of nNOS in the mouse heart is unknown, it is likely that it is present in smaller quantities and localised to innervated areas such as nodal and conducting tissue. Its contribution, therefore, to maintenance of coronary flow, is likely to be unimportant.

The other beneficial effects of NO that relate to its anti-adhesion and anti-inflammatory properties can be discounted in a crystalloid-perfused Langendorff preparation. It is interesting to note that these results mirror those obtained from recent studies examining cerebral ischaemia-reperfusion injury. Using an *in vivo* model of temporary middle cerebral artery occlusion, eNOS KO mice suffered larger infarcts (Huang *et al.*, 1996). Of further interest is the observation that nNOS KO mice suffer smaller infarcts than wild types (Huang *et al.*, 1994). Although this was not confirmed in the current study, there was a trend in this direction. Clearly the magnitude of any detrimental effect of NO synthesised by nNOS must be smaller in murine myocardium than neuronal tissue in these models. However, the current study was performed *in vitro*, and the damaging influences of adherent platelets and activated neutrophils will be absent. Production of free radicals will also be reduced since circulating leucocytes are absent, leaving endothelial cells and myocytes as the only source. It is certainly possible that any deleterious effects of nNOS might be apparent if the current experiments were repeated *in vivo* using a regional ischaemic insult.

6.5.3 CONTRACTILE RECOVERY

Recovery of contractile function was uniformly poor in all groups. In addition to the effects of necrosis (dead muscle will not contract), there is likely to be a significant contribution from stunned myocardium. This makes interpretation of contractility data less straightforward. On the basis of the infarct size data, the greatest recovery would have been expected in the nNOS KO group. It would appear, however, that despite smaller infarcts,

hearts in this group appeared to suffer more contractile dysfunction, presumably due to stunning. The direct effects of NO on myocardial contractility appear to be concentration-dependent, in a biphasic manner similar to the chronotropic effects. When present in large amounts, NO has a depressant effect on systolic function, whereas in smaller quantities a positive inotropic effect is observed (Mohan *et al.*, 1995). It is possible that NO is released in significant quantities by eNOS following ischaemia resulting in protection against infarction but depression of myocardial contractility. This might explain the poor contractile recovery in the nNOS KO group (eNOS is still present). The eNOS KO group might have demonstrated better contractile recovery than the other two groups, but this will have been obscured by the increase in infarct size.

6.5.4 CORONARY FLOW RATE

As discussed previously, both knockout groups demonstrated reduced baseline coronary flow rates compared to wild types. During reperfusion, the three groups did not differ significantly in the extent of recovery of baseline flow. Coronary flow rate during reperfusion will depend on the degree of myocardial contracture as well as coronary vascular resistance. The areas of necrosis apparent on tetrazolium staining were discrete and well-circumscribed (spreading mainly from the subendocardium) and reduction in flow in these areas might be accompanied by an increase in flow in other regions, leading to little overall change in flow rate. As a result, inferences drawn from examination of this data might be misleading, especially when comparisons involve small differences in ischaemia-reperfusion injury.

6.5.5 HEART RATE

Both knockout groups demonstrated almost complete recovery of baseline heart rate after 30 minutes reperfusion. Although not statistically significant, wild types did not exhibit such a complete recovery. Nitric oxide release following ischaemia would have greatest in this group and this may have contributed to the bradycardia observed (see section 6.5.1).

6.5.6 CONCLUSION

In conclusion, this study is the first to demonstrate the protective effect of eNOS against myocardial ischaemia-reperfusion injury in the isolated mouse heart. The findings are in keeping with a cardioprotective role for NO in myocardium which, in this model, is probably via a locally-mediated effect on coronary flow. The endothelial isoform of NOS is responsible for maintenance of coronary vasodilation and its absence in the knockout mice is likely to be responsible for the increase in necrosis observed. The neuronal isoform would not appear to be involved in this protection but may be injurious in its own right. *In vivo* experiments using regional myocardial ischaemia may confirm that nNOS exacerbates myocardial ischaemic injury in keeping with the observations following cerebral ischaemia.

7. CONCLUSION

7.1 SUMMARY

During the course of the present study several important issues relating to the investigation of the pathophysiology of ischaemia-reperfusion injury have been addressed. The principle underlying this study involved the possibility of constructing a valid physiological model to allow phenotypic investigation of genetically-altered mice. A large part of this work involved the construction of a suitable Langendorff apparatus for effective perfusion of the isolated mouse heart. A miniaturised assembly was designed to allow *in vitro* cannulation of the mouse aorta and then rapid transfer of the cannulated heart to the perfusion apparatus. During perfusion, myocardial temperature and contractility were monitored without interfering with effective coronary perfusion or causing trauma to the isolated heart. Infarct size was measured using tetrazolium staining, but due to the size of the heart, a dedicated camera system was constructed to generate a magnified image of each heart slice that could be digitised by computer video card. Since all of these techniques were new to the laboratory, detailed characterisation experiments were carried out to demonstrate the stability of the model and to confirm that the techniques used generated consistent data. It was essential to demonstrate that the end-points of injury co-correlated, and that appropriate dose-response relationships existed between each individual end-point and a gradation in severity of ischaemic insult.

Having established these criteria successfully the model had been fully characterised, and the next series of experiments involved the assessment of a well-known cardioprotective intervention 'ischaemic preconditioning'. This intervention was chosen to assess the model for several reasons. Ischaemic preconditioning had not been demonstrated previously in the isolated mouse heart using global ischaemia, with infarct size as the primary end-point. Since its existence had been successfully established in all animal species investigated so far, it seemed probable that the mouse heart would be similarly consistent. Furthermore, since the mouse heart does not contain any inhibitor protein for the mitochondrial f_0/f_1

ATPase, its involvement in the mechanism underlying the metabolic adaptation of ischaemic preconditioning could be discounted with the successful demonstration of this protective intervention in murine myocardium. A suitable preconditioning protocol was established after several unsuccessful attempts. With reference to the data from the characterisation experiments it was possible to predict the expected improvement in contractile recovery of a given reduction in infarct size. Ischaemic preconditioning provided a 42% reduction in infarct size after 30 minutes of global ischaemia but the contractile recovery was greater in the preconditioned group than would have been expected from the effect on extent of infarction. It is likely, therefore, that myocardial stunning may have been attenuated by ischaemic preconditioning in this model.

The model was fully characterised, and its ability to assess the efficacy of a cardioprotective intervention was demonstrated successfully before proceeding with the final series of experiments. These involved the use of genetically-altered mice with targeted deletion of the genes encoding specific isoforms of nitric oxide synthase (NOS). Wild type mice were compared with mice demonstrating absent expression of either endothelial NOS (eNOS) or neuronal NOS (nNOS). Both of these isoforms are normally expressed constitutively in myocardium. This allowed the contribution of NO to the development of ischaemia-reperfusion injury to be assessed with particular reference to the enzyme responsible for its synthesis. This approach did not suffer from the disadvantages of pharmacological inhibition studies. Of considerable interest was the finding that eNOS knockout mice suffered larger infarcts than wild types implying a protective role for NO synthesised by this enzyme during ischaemia-reperfusion injury. This is in agreement with studies carried out examining cerebral infarction following occlusion of the middle cerebral artery *in vivo*, using the same genetically-altered mice. The neuronal isoform was not found to be protective, with some suggestion that it may have a deleterious influence. This was also consistent with cerebral infarction studies.

Thus the role of NO in the modulation of ischaemia-reperfusion injury *in vitro* was investigated for the first time using genetically-altered mice. The suggestion that eNOS may have beneficial effects and nNOS deleterious effects on the evolution of myocardial

infarction following an ischaemic insult has implications for studies of cardioprotective interventions utilising pharmacological manipulation of NO levels.

7.2 FUTURE WORK

There is considerable scope for future research directly arising from the findings of the present study. With the establishment of a new model capable of assessing the pathophysiology of ischaemia-reperfusion injury in the isolated mouse heart, a number of possible approaches are available to improve our understanding of cellular events during myocardial ischaemia-reperfusion injury and to guide potential future therapy. For convenience and clarity, suggestions for future directions have been split into two broad categories:

7.2.1 NITRIC OXIDE AND ISCHAEMIA-REPERFUSION INJURY

The involvement of NO in modulating myocardial injury during ischaemia and reperfusion could be further elucidated. There is evidence implicating NO as an intermediate step in the protection against reperfusion arrhythmias induced by ischaemic preconditioning in the dog heart (Vegh *et al.*, 1992b). However, there have been no studies directly examining a role for NO as an intermediary in the mechanism underlying the protection of classic ischaemic preconditioning against infarct size. The role of eNOS and nNOS could be examined very easily using the model described in this study. Furthermore, the breeding of 'double knockouts' for both constitutive isoforms of NOS would provide an additional experimental group for comparison to quantify the total effect of NO in this phenomenon. This might provide useful clarification of situations in which NOS isoforms might substitute for one another. The presence of compensatory mechanisms in these genetically-altered mice is also an important consideration. Knockout animals may reveal pathways that act in parallel with NO signalling that may be explored with the aim of restoring responses in human conditions where NO production is diminished.

The role of the inducible isoform of NOS (iNOS) has not been discussed in this study. This is because, under basal conditions, there is negligible activity of this enzyme.

However, in certain disease states, and following stresses such as ischaemia, endotoxaemia, and certain pharmacological agents, the expression of iNOS is upregulated. This may have relevance to delayed myocardial protection (the 'second window') and it has been suggested that the reduction in infarct size seen in rabbits 24 hours after administration of the neutralised endotoxin 'MLA' may be effected by the K_{ATP} channel (Elliott *et al.*, 1996) with a possible role for iNOS in mediating this effect. It would be straightforward to establish the existence of delayed myocardial protection in the mouse by administration of endotoxin and measurement of infarct size following an ischaemia-reperfusion protocol 24 hours later. The involvement of the K_{ATP} channel could then be assessed using a blocking agent such as glibenclamide. Transgenic mice lacking iNOS expression are already available and could be incorporated in these studies to confirm or refute the involvement of iNOS in this form of delayed adaptation to ischaemia. If protection was not possible in iNOS knockout mice the possibility that it might be re-established by administration of a K_{ATP} channel opener could be explored. Other stresses such as heat shock, or certain pharmacological agents (*eg* adenosine) could be examined in a similar fashion. In addition, the possibility that iNOS may be involved in activating promoter sequences for heat shock protein expression could be examined in wild types and iNOS knockouts, and the presence and degree of any protection related to the quantity or phosphorylation state of particular heat shock proteins.

7.2.2 OTHER TRANSGENIC STUDIES

The same principles may be applied to mice with other genetic manipulations of interest. In time the number of such animals will increase greatly but several are already available. The role of membrane proteins (which may be receptors, ion channels, co-transporters *etc*), intracellular signalling molecules, and other gene products thought to be involved in cellular homeostasis and metabolism, could be examined in genetically-altered mice. Examples might include mice lacking expression of manganese superoxide dismutase (Mn-SOD), the sodium-hydrogen ion exchanger, specific isoforms of protein kinase C, certain heat shock proteins thought to be cytoprotective, the receptor sub-unit of tyrosine kinase, and many others. The recent advances in molecular biology and genetic manipulative techniques will

undoubtedly be utilised in the future by physiologists to examine phenotypic changes using appropriately characterised models of animal pathophysiology.

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