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Preconditioning and Augmented Preconditioning via Manipulation of Metabolic and Signalling Pathways in the Rat Heart.

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TO MY PARENTS – Thanks for your support, encouragement, care, warmth and love that you gave me all these years, without you I would not have made it. TO MY BROTHERS – the late Xola Makaula, always in my heart big brother, Bagcine and Bonke, education is the key to success Bafowethu!!!!!

ABSTRACT

ABSTRACT

BACKGROUND – Cardiac ischaemic preconditioning (IPC) describes a biological phenomenon whereby a short ischaemic stimulus confers protection to the heart against subsequent prolonged ischaemia and reperfusion injury. Understanding this survival programme will enable us to augment tissue tolerance against cell death. Ischaemic preconditioning is poorly understood, however, certain metabolic events and activation intracellular signalling events are known to trigger this cardioprotection. The purpose of this study was to investigate the metabolic and intracellular signalling events which occur during ischaemic preconditioning and their effects on improvement of contractile recovery following an ischaemia / reperfusion insult.

Glucose deprivation substudy - we used glucose deprivation as a trigger of preconditioning to compare potential metabolic changes associated with glucose depletion, such as decreased glycolysis and enhanced fatty acid mobilisation, with conventional preconditioning signalling events such as activation of adenosine receptor and / or adrenergic receptor activation, protein kinase C activation and the subsequent opening of mitochondrial ATP sensitive potassium (mito K_{ATP}) channels. We therefore hypothesised that, transient glucose depletion preconditions the heart via an induction of adenosine / noradrenergic activation of protein kinase C (PKC) with resultant opening of mito K_{ATP} channels.

PKA inhibition substudy - Although preconditioning is cardioprotective against ischaemia / reperfusion injury, we proposed that the efficacy of ischaemic preconditioning on post ischaemic contractile function can be

augmented. We therefore suggested that augmentation of ischaemic preconditioning can be achieved by reducing the β-adrenergic / protein kinase A pathway induced accumulation of Ca^{2+} known to occur during ischaemia. We therefore hypothesised that activation of cyclic AMP dependent protein kinase A (PKA) could mediate deleterious effects during ischaemia and may limit post ischaemic contractile recovery afforded by single episode of preconditioning.

METHODS AND RESULTS – Isolated rat hearts were perfused with Krebs buffer containing 11mM glucose prior to 30 min global ischaemia and 30 min reperfusion (index I/R). Ischaemic preconditioning hearts had 10 min global ischaemia followed by 10 min reperfusion prior to index I/R.

Glucose deprivation substudy - Glucose deprivation preconditioned hearts were exposed to 15 min glucose free buffer perfusion followed by 10 min perfusion with glucose containing buffer prior to index I/R. Post ischaemic contractile recovery at the end of the experimental protocol was compared to the baseline and expressed as a percentage of basal contractile function. Ischaemic preconditioning and glucose deprivation preconditioning improved the functional recovery after 30 min reperfusion Control (28.5 ± 2.4 %) to ischaemic preconditioning (56.7 ± 3.6 %) and glucose deprivation preconditioning (59.8 ± 3.4 %). To evaluate the metabolic events occurring during the preconditioning stimulus; fatty acid mobilisation and glycolytic inhibition effects were investigated. This was achieved by perfusing a medium chain fatty acid, 11mM octanoate, glycolytic inhibitor, 11mM 2-Deoxyglucose (2DG) or an indirect inhibitor of glycolysis, 20 mM pyruvate

during the glucose deprivation period. Octanoate (50.2 ± 2.5 %) and a combination of octanoate and 2DG (49.3 ± 3.3 %) in the absence of glucose were not significantly different from GLU(-) (59.8 ± 4.0 %) were all significantly different compared to the control (32.1 ± 4.6 %). To assess the possible involvement of adenosine receptor and/or adrenergic receptor activation, adenosine antagonist 100μM SPT, adrenergic antagonist prazosin (α_1 blocker 10μM) and propranolol (β-blocker 10μM) were perfused. SPT (36.7 ± 3.3 %) blocked GLU(-) (59.8 ± 3.4 %) protection while prazosin (50.5 ± 3.1 %) and propranolol (55.5 ± 4.0 %) did not block glucose deprivation protection. To confirm the specificity of SPT, adenosine preconditioning ADO PC (60.9 ± 6.9 %) was abolished by SPT - ADO + SPT (28.4 ± 2.6 %). To investigate the role of PKC and mito K_{ATP} channel, inhibitors chelerythrine (PKC inhibitor 10μM) and 5-HD (K_{ATP} channel blocker 100μM) were perfused prior, during and after glucose depletion period prior to the index ischaemia. Chelerythrine (35.0 ± 4.8 %) or 5-HD (23.8 ± 3.4 %) also abolished glucose deprivation protection.

PKA inhibition substudy - It has been shown that the levels of cAMP decline with the number of ischaemia / reperfusion episodes[1, 2] hence ischaemic preconditioning protection improves with the number of cycles[3], therefore protein kinase A inhibition may be due to a stronger ischaemic preconditioning phenomenon. We therefore compared single episode to multiple episodes of ischaemia and further investigated whether β-adrenergic induced protein kinase A activation during single episode of ischaemic preconditioning may

limit this cardioprotection. We therefore evaluated whether we could augment the cardioprotection afforded by single episode of ischaemic preconditioning by attenuating the activation of protein kinase A.

Firstly, we compared the degree of improvement of post ischaemic contractile function with multiple cycles. Rat hearts preconditioned with single episode of 10 min global ischaemia improved post ischaemic contractile function, IPC 1 (45.0 ± 3.2 %) at the end of 30 min reperfusion. Moreover, rat hearts preconditioned with 3 x 5 min episodes of ischaemia, interspersed with 5 min reperfusion (IPC 3), showed significant improvement of post ischaemic contractile function, above that of single episode of ischaemic preconditioning, IPC 3 (62.8 ± 2.9 %). Prior to evaluating the role of protein kinase A activation on preconditioning its upstream mediator adenylate cyclase was activated. Hearts were pharmacologically preconditioned with the adenylate cyclase activator, forskolin 0.3μM (forskolin preconditioning). Forskolin used as a preconditioning mimetic showed a similar protection to ischaemic preconditioning, (forskolin preconditioning - 35.4 ± 0.9 % versus IPC 1 - 45 ± 3.2 %, NS). To investigate the role of protein kinase A activation during forskolin preconditioning stimulus, forskolin was concurrently perfused with protein kinase A inhibitor (2μM H89). H89 augmented forskolin preconditioning (forskolin preconditioning - 35.4 ± 0.9 % versus forskolin preconditioning + H89 - 50.9 ± 3.9 %). H89 was then perfused prior to trigger ischaemia of preconditioning. Again, H89 augmented the post ischaemic contractile function afforded by ischaemic preconditioning alone (IPC 1 - 45 ± 3.2 % versus IPC 1 + H89 - 58.0 ± 2.8 %, $p < 0.05$ vs. IPC 1). In collaboration with Professor Amanda Lochner, we measured protein kinase A activity

during the trigger ischaemia. In correlation with the above findings, we found that protein kinase A activity was significantly increased at the end of 10 min trigger ischaemia (baseline 1739 ± 190 pmol. min^{-1}. mg $protein^{-1}$ vs 10 min trigger ischaemia 3055 ± 255 pmol. min^{-1}. mg $protein^{-1}$, $p<0.001$). In H89 treated hearts protein kinase A activity was unaffected by 3 min H89 perfusion before trigger ischaemia (1440 ± 130 pmol. min^{-1}. mg $protein^{-1}$ vs baseline, NS). Moreover, H89 blocked the rise PKA activity following the end of 10 min trigger ischaemia (1120 ± 71 pmol. min^{-1}. mg $protein^{-1}$ vs baseline, NS).

CONCLUSION – Transient glucose deprivation preconditions the heart through adenosine receptor induced protein kinase C activation and mitochondrial K_{ATP} channel opening. However, protein kinase C activation during GLU(-) is not mediated via α_1 / β-adrenergic receptor activation. In addition, inhibition of protein kinase A activity further enhances the protective effect of subthreshold ischaemic preconditioning on post ischaemic contractile function.

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INTRODUCTION

1. INTRODUCTION

Reduced blood flow to the heart results in a reduction in oxygen and fuel substrate supply to the contractile high energy consuming myocardial tissue. This series of events is known as myocardial ischaemia. The damaging metabolic and functional consequences of myocardial ischaemia can lead to ischaemic heart disease (IHD) which induces cardiocytes cellular dysfunction (stunning / hibernation) or heart muscle cell death (myocardial infarction) and can lead to the development of ischaemic cardiomyopathy (heart failure)[4].

The spectrum of aetiologies resulting in ischaemic heart disease includes thrombus within the vessels, atherosclerotic plaque forming in the vessel wall and spasm of the coronary artery[5]. Ischaemic heart disease manifests as four clinical syndromes namely: angina pectoris, acute myocardial infarction (AMI), heart failure and sudden ischaemic cardiac death (SICD)[5]. Understanding the pathophysiology of these syndromes is crucial for preventative and therapeutic interventions for ischaemic heart disease. Although there may be ethnic and gender variabilities in the prevalence of ischaemic heart disease there are common risk factors that need to be taken into consideration. The risk factors can be divided into nonmodifiable and modifiable risk factors. The nonmodifiable risk factors include age, gender, and weight at birth, genetic predisposition and the penetrance of genetic defects. The modifiable risk factors include cholesterol levels, cigarette smoking, hypertension and diabetes and to a lesser extent fibrinogen level, stress and obesity[5].

As a practical consequence of the high incidence, prevalence, morbidity and mortality from ischaemic syndromes, multiple therapeutic approaches have

been developed in an attempt to decrease the adverse consequences of ischaemic heart disease. These include treatment that maintain oxygen supply by thrombolysis or mechanical opening of the artery by percutaneous transluminal-coronary angioplasty (PTCA) or coronary artery bypass grafting[5]. Another approach has been by reducing the energy demand of the heart e.g. by using beta blocking agents to lower the heart rate and reduce cardiac contraction[4].

An alternative approach would be to modify the cardiac cellular response to ischaemia thereby enabling the cardiac tissue to be more resistant / tolerant to ischaemic stress. In this regard, paradoxically, it has been found that short/transient episode(s) of ischaemia protect(s) against subsequent ischaemic insult. This biological phenomenon is termed ischaemic preconditioning (IPC)[6-8] and is currently regarded as one of the most powerful protectors of the heart. Of note, ischaemia has various individual components including low oxygen (hypoxia) and lack of cardiac fuel substrate, glucose, to mention but a few. Interestingly, as individual triggers these components of ischaemia have been shown to induce cardioprotection against ischaemia / reperfusion injury[9, 10]. Moreover, this ischaemic preconditioning phenomenon has been demonstrated in multiple tissue types including heart, skeletal muscle, brain, liver and kidney, and in numerous mammalian species[6-8] including human[11]. These observations suggest that preconditioning may be an evolutionary conserved cell survival programme, enabling many tissue types to have an augmented tolerance to ischaemic insults. Thus, understanding preconditioning may have widespread clinical application in enhancing tissue tolerance against cell death following for e.g.

myocardial infarction, stroke or multiple-organ failure following haemodynamic shock.

1.1 Cardiac Ischaemic Preconditioning

Two types of ischaemic preconditioning have been described. These include classic ischaemic preconditioning whereby an initial preconditioning insult results in enhanced tolerance against prolonged ischaemia. This protection lasts for 1-2 hours following the preconditioning stimulus[12, 13]. The delayed preconditioning phenomenon or second window of protection (SWOP) is where an initial preconditioning insult results in enhanced tolerance against prolonged ischaemia 24-72 hrs following the IPC insult / stimulus[14, 15]. In our study we have concentrated on the metabolic and signalling pathways during preconditioning insult.

To date, several endpoints of ischaemic preconditioning have been used in both classic preconditioning and SWOP but the classic end point is the delay of lethal damage by reducing infarct size[16]. Preconditioning has also been shown to attenuate the incidence of reperfusion induced arrhythmias[17], and to induce protection against post ischaemic contractile dysfunction with improved functional recovery[18].

1.2 Triggers, ligands and cell surface receptors of classic ischaemic preconditioning

The precise and elementary mechanisms underlying ischaemic preconditioning induced cardioprotection are currently under active investigation. To understand the mechanisms of preconditioning various individual components of ischaemia including hypoxia[9], mild increase in calcium[19] and deprivation of a major cardiac fuel substrate, glucose[10] have been investigated as the possible triggers in the preconditioning signalling cascade. As previously mentioned, all of these components of ischaemia induce ischaemic preconditioning like cardioprotection. Additionally, triggers known to be released during trigger ischaemia of preconditioning can independently induce cardioprotection. These triggers include adenosine[20] acetylcholine[21], bradykinin[10], noradrenaline[22], angiotensin II[23], endothelin[24] and opioids[25]. The significance of these ischaemic preconditioning triggers appears to be species dependent. Adenosine appears to be more important in rabbit[20], dog[26] and pig[25] while noradrenaline seems important in rats[22], however, other investigators have shown that adenosine plays an important role in the rat heart[27, 28]. Most ischaemic preconditioning triggers act on pertussis toxin sensitive inhibitory G-protein coupled cell surface receptors.

1.3 Post receptor signalling following ischaemic preconditioning

The stimulation of these G-protein coupled receptors, described above, lead to activation of either G stimulatory / inhibitory proteins. As an example, noradrenaline activates both α and β - adrenergic cell membrane receptors, with α receptor activation initiating the G inhibitory protein signalling pathway and β - receptor activation initiating G stimulatory signalling pathway.

The α - adrenergic, adenosine and opioids, to mention but a few, cell membrane receptors are G_i - protein coupled, and activate phospholipase C (PLC). Activated PLC converts phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5, -triphosphate (IP_3) and 1,2-diacyglycerol (DAG). The latter facilitates the translocation of protein kinase C (PKC) from the cytosol to sarcolemma[29]. The active PKC may phosphorylate the opening of the sarcolemmal and mitochondrial (K_{ATP}) channels[30,31] and activates other kinases like tyrosine kinase and MAP kinases[15]. The protein kinase C inhibitors or antagonists like chelerythrine have been shown to block protection afforded by ischaemic preconditioning therefore suggesting the importance of protein kinase C in the opening of these channels[29]. Protein kinase C agonists like phorbol myristate acetate or a diacylglycerol analogue were also shown to mimic ischaemic preconditioning[32]. Moreover, different isoforms of protein kinase C are involved in preconditioning cardioprotection[19, 30]. Recently, transgenic mice overexpressing PKC β were shown to be cardioprotective against post-ischaemic dysfunction[33].

On the other hand, the β - adrenergic cell membrane receptors are coupled to G-stimulatory protein in order to activate adenylate cyclase. This activation leads to rise in the cyclic AMP (cAMP) which in turn activates cAMP dependent protein kinase (PKA), resulting in the opening of L-type calcium channels, and eventually bringing about an increase in the cytosolic calcium. The trigger ischaemia of preconditioning is associated with the rise in cAMP with a concomitant increase in PKA activity[2, 34] leading to increase in Ca^{2+} influx[4].

1.4 The possible end effector of preconditioning

Mitochondrial ATP sensitive potassium channels

The activation of PKC and the resultant opening of the K_{ATP} channels has been considered as a likely end-effector of preconditioning. The consequences of mitochondrial ATP sensitive potassium (mito K_{ATP}) channel opening have been proposed to include mitochondrial swelling, membrane depolarisation, perturbation in ATP synthesis and in mitochondrial respiration[35, 36]. Interestingly, mitochondrial swelling is known to augment ATP production[37] and this may, in turn, be an adaptive mitochondrial response to cellular stress. Thus, these data suggesting that ischaemic preconditioning induced cellular resistance to ischaemia may be via the modulation of mitochondrial homeostasis. Moreover, it has been found that pharmacological agents that block the opening of mito K_{ATP} channels block ischaemic preconditioning[38] and pharmacological agents that open these channels likewise mimic ischaemic preconditioning[39].

Sarcolemmal ATP sensitive potassium channels

Although the involvement of mito K_{ATP} channels has been extensively studied in the context of preconditioning, the role of sarcolemmal ATP sensitive potassium channel (sarc K_{ATP} channels) in preconditioning remains unclear. Yao[40] suggested that sarc K_{ATP} channels are also involved in preconditioning protection. The consequences of the opening of sarc K_{ATP} channels have been suggested to be preservation of ATP, shortening phase 3 of the action potential and membrane hyperpolarisation leading to decreased calcium entry during ischaemia and subsequent cell survival[35]. Some investigators have shown that sarc K_{ATP} channel opening reduces infarct size following ischaemia / reperfusion insult[40-42]. However, some investigators have shown that a selective sarcolemmal channel blockers, HMR 1883 or HMR 1098, failed to abolish the protective effects of ischaemic preconditioning[43-45].

2

OBJECTIVES & HYPOTHESES

2. OBJECTIVES AND HYPOTHESES

Multiple therapeutic approaches have been developed in an attempt to decrease the adverse consequences of ischaemic heart disease. Moreover, ischaemic preconditioning (IPC)[6-8] is a biological phenomenon currently regarded the most powerful protector of the heart against ischaemic injury. However, the mechanism through which ischaemic preconditioning protects the heart is not clearly understood. As previously mentioned, ischaemia has various components e.g. hypoxia and glucose deprivation. As individual triggers these components can induce cardioprotection, therefore intensive investigation on how each of these components protect may lead to a much clearer understanding of ischaemic preconditioning. Of these components of ischaemia we decided to investigate the mechanism of glucose deprivation protection. We chose transient glucose deprivation as a preconditioning stimulus because it has been shown to protect against ischaemic injury, has not been extensively studied and it was considered as less severe preconditioning stimulus compared to ischaemic preconditioning.

2.1 Ischaemic preconditioning versus glucose deprivation preconditioning

These preconditioning stimuli are both known to elicit a powerful cardioprotection against ischaemia / reperfusion injury and they are both without glucose supply. However, ischaemic preconditioning has zero coronary flow, no oxygen supply, no cardiac function and cannot be directly stimulated or inhibited during preconditioning stimulus. As opposed to ischaemic preconditioning, glucose deprivation period has full coronary flow, normal oxygen supply, maintains cardiac function and can be directly

stimulated or inhibited before, during and after preconditioning stimulus. We were therefore in a better position to explore and investigate the mechanisms of glucose deprivation protection concerning contractile recovery following an ischaemic insult.

Of note, all the preconditioning triggers / stimuli are proposed to set in motion both metabolic events (decrease glycolysis and enhancement of fatty acid mobilisation) and cell signalling pathways (activation of adenosine and / or adrenergic receptors, protein kinase C activation and opening of ATP sensitive mitochondrial channels).

Thus the purposes of this section of our study was to compare and delineate potential metabolic changes with conventional preconditioning signalling events in the context of simulated ischaemia / reperfusion,

2.2 Delineation of metabolic and signalling pathways activated by glucose deprivation trigger of preconditioning

The exact mechanism through which glucose deprivation induces cardioprotection is unclear[10, 46]. As shown by Goto[10] the transient deprivation of glucose from the medium mimics ischaemic preconditioning. In this study we further investigate this phenomenon. The first objective of this study is to compare and delineate the mechanisms whereby transient deprivation of a major cardiac fuel substrate - glucose, can induce cardioprotection. It was hypothesised that transient glucose deprivation may induce the cardiac preconditioning programme either, by direct metabolic effects including inhibition of glycolysis and/or enhancement of fatty acid mobilisation / metabolism or, by directly mimicking classic ischaemic

preconditioning signalling pathways through the activation of PKC and mitochondrial K_{ATP} channel opening.

2.3 Is there a room for further protection of preconditioned hearts?

Although ischaemic preconditioning is regarded as most powerful protector of the heart against ischaemia / reperfusion injury, the post-ischaemic contractile function of the hearts subjected to a preconditioning stimulus does not revert completely back to pre-stimulus or baseline status following the index ischaemic insult.

One of many ways in which ischaemia can damage the heart is activation of PKA signalling pathway and subsequent accumulation of calcium. Within a short period of ischaemia, endogenous catecholamines[47] and noradrenaline release[48] lead to activation the β-adrenergic receptors, adenylate cyclase activation, rise in cAMP levels, activation of protein kinase A and Ca^{2+} accumulation. Excess accumulation of Ca^{2+} in acute myocardial ischaemia may lead to loss of Ca^{2+} handling and failing of Ca^{2+} ATPase leading to loss of Ca^{2+} responsiveness by contractile proteins i.e. actin and myosin[49], degradation of myofilament proteins, especially Troponin I[50]. McDonald[51] found that excess Ca^{2+} can result to decreased Ca^{2+} sensitivity of isometric tension and rate of crossbridge cycling in skinned myocytes isolated from pig myocardium. All this may account for the contractile dysfunction observed at reperfusion. We therefore hypothesised that activation of the β-adrenergic induced activation of cyclic AMP dependent protein kinase A (PKA) during the trigger of ischaemic preconditioning may limit this protection. Therefore, the question arising is whether the efficacy of ischaemic preconditioning on

contractile recovery can be further enhanced with the concurrent inhibition of the signalling pathway resulting in the augmentation of intracellular Ca^{2+} accumulation. If this could be achieved, we proposed that it could be possible to further enhance the protection afforded by the preconditioning stimulus.

Although we hypothesised that ischaemic preconditioning induced protection can be further augmented by blocking the PKA activation, it is important to mention that β-adrenergic signalling cascade is an essential trigger of ischaemic preconditioning since β-adrenergic blockade abolishes protective effect of preconditioning[2].

To test these hypotheses:

1) Firstly, we induced potential metabolic changes by inhibiting glycolysis with glycolytic inhibitor, 2-Deoxyglucose and a metabolic substrate known to indirectly inhibit glycolysis, pyruvate and enhancing fatty acid metabolism/mobilisation and indirectly inhibiting glycolysis with a medium chain fatty acid, octanoate. We also blocked the conventional preconditioning signalling events (activation of alpha and beta-adrenergic receptors, adenosine receptors, PKC and opening of K_{ATP} channels) with various pharmacological agents.
2) Secondly, we investigated a possible detrimental component of ischaemia by inhibiting PKA activation, a component of the β - adrenergic signalling pathway.

3

MATERIALS & METHODS

3. MATERIALS AND METHODS

In order to investigate the effects of glucose deprivation induced signalling and the effect of β-adrenergic pathway blockade on ischaemic preconditioning, we evaluated contractile recovery in isolated Langendorff perfused rat hearts following preconditioning triggers, global simulated ischaemia and reperfusion. The Langendorff perfusion apparatus is as shown in **figure 1(a)**. In this study, the improvement of post ischaemic contractile function was used as a marker of cardioprotection. **Figure 1(b),** a typical pressure trace obtained from our pilot studies, shows how would hearts perform when subjected to either ischaemia / reperfusion control (upper panel) compared to preconditioning ischaemia treatment (lower panel). Control hearts were subjected to a period of stabilisation followed by prolonged period of ischaemia, which they lost function, and reperfusion, in which they gained function. The value obtained at the end of the experiment was measured and compared to the baseline (value before any interruptions) and expressed as the percentage of baseline. The preconditioned hearts were subjected to 10 min of ischaemia followed by 10 min of reperfusion before being subjected to prolonged ischaemia and reperfusion. In comparison, the developed pressure in the ischaemic preconditioning group was significantly greater than that achieved by the control hearts at the end of the ischaemia / reperfusion insult (time point indicated by the vertical arrows).

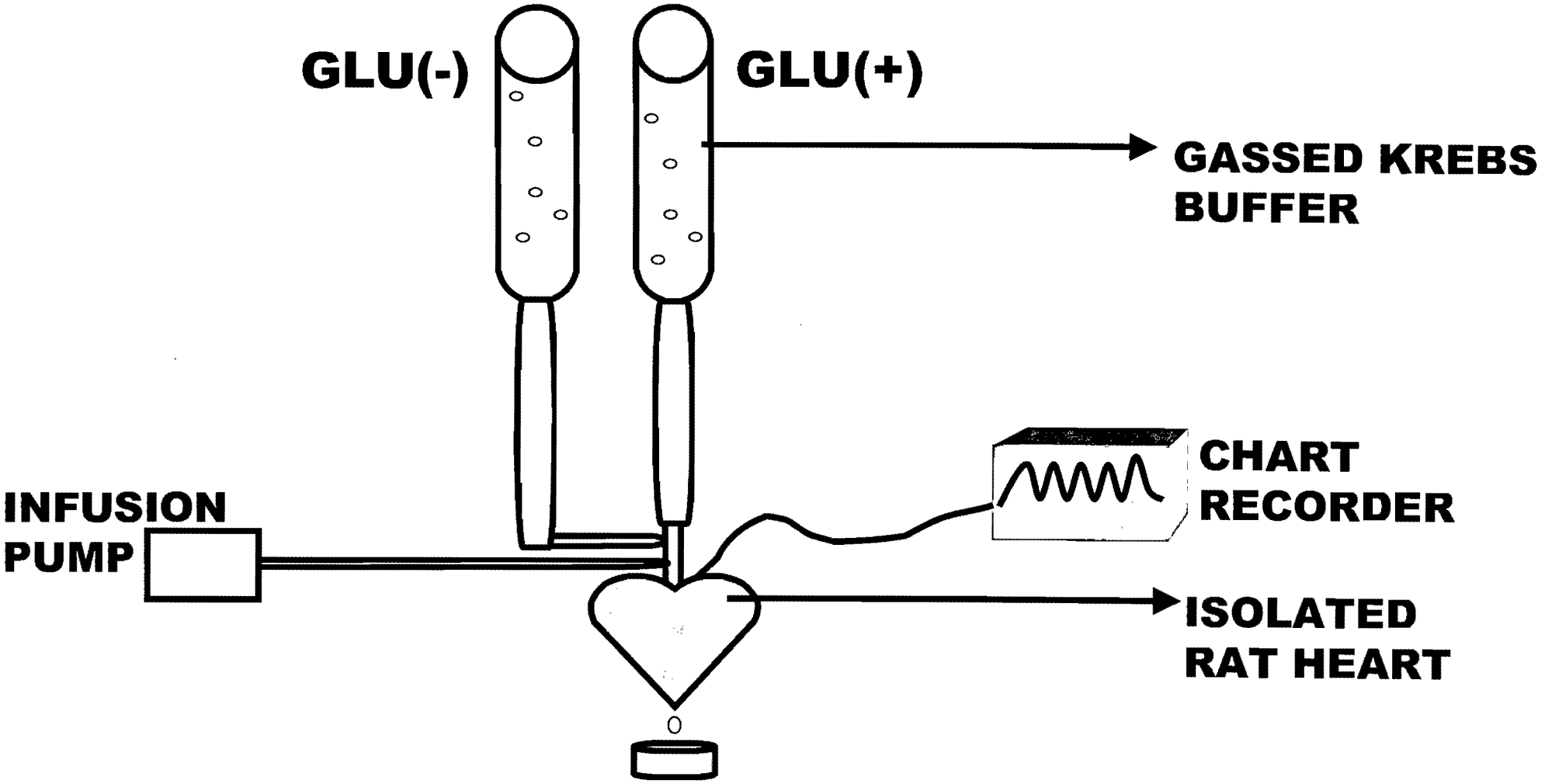


Figure 1(a) Schematic representation of Langendorff perfusion apparatus. The apparatus has two chambers, one with normal glucose containing perfusion medium GLU(+) and glucose deprived perfusion medium GLU(-). The perfusion medium was gassed with carbogen (95% O2 +5% CO2). Isolated rat hearts were mounted to the apparatus via the aorta and a water filled balloon was inserted into the left ventricle. The balloon was connected to a pressure transducer and the chart recorder. The pharmacological agents were administered via the infusion pump.

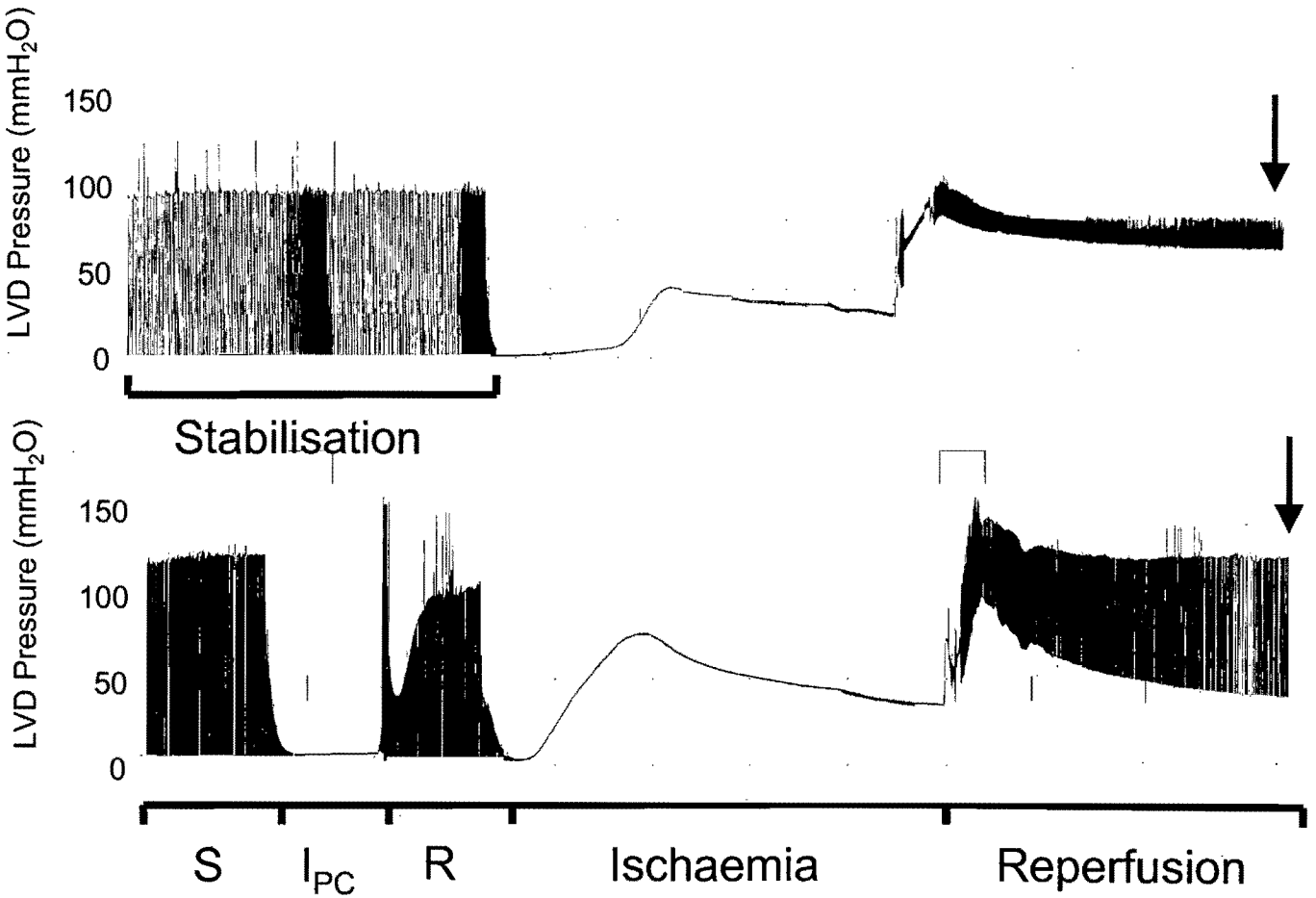


Figure 1(b) A typical pressure trace from the chart recorder. The top panel represents control pressure trace, after a period of uninterrupted perfusion (stabilisation) hearts were subjected to 30 min ischaemia, resulting in an initial period of zero developed pressure followed by the late onset of ischaemic contracture. Ischaemia was followed by re-establishment of the perfusion flow through the heart (reperfusion) and a marked reduction in the left ventricular developed pressure (LVDP) compared to stabilisation phase was observed. In ischaemic preconditioning (bottom panel) hearts were subjected to stabilisation phase(S) followed by 10 min ischaemia(IPC) / reperfusion(R) before subjected to 30 min ischaemia / reperfusion insult. IPC hearts had an early onset of ischaemic contracture and the developed pressure was significantly greater than that of the controls (vertical arrows).

3.1 Isolated rat heart perfusion

All experiments were conducted on adult male Long-Evans rats weighing 250-300g. All animals were *fed ad libitum* and cared for according to the *"Guide for the Care and Use of Laboratory animals"* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Rats were intraperitoneally anaesthetised with 0.35ml sodium pentorbabitone and then intravenously heparinised (200 IU) through the exposed femoral artery. The hearts were then rapidly excised, immersed in 4°C Krebs solution and mounted onto aortic cannula. Hearts were retrogradely perfused at a constant pressure (100cm H_2O) and temperature (37^0C) with a modified Krebs-Henseleit buffer containing (mM) NaCl 118.5, $NaHCO_3$ 25, KCl 4.75, $MgSO_4$ 1.19, KH_2PO4 1.18, $CaCl_2$ 1.36 and glucose 11. Oxygenation of the perfusion medium with 95%02 and 5%CO2 resulted in $PC0_2$ of approx. 40 mmHg, PO_2 of approx. 550 mmHg and pH 7.4. A compliant balloon was inserted into the left ventricle to measure left ventricular end diastolic, systolic pressure, heart rate and coronary flow. These functional parameters were recorded throughout the experiment using a computerised bridge amplifier/digitizer (PowerLab / 400, AD Instruments, Sydney, Australia), and continuously recorded using a Windows 95 operating system and PowerLab software. The criteria used for the exclusion of perfused rat hearts was a heart rate outside 260-410 beats/min, systolic pressure outside 70-120 mmHg, left ventricular end diastolic pressure of 4-10 mmHg and a rate pressure product outside 21.000-36.000 (mmHg beats/min) x 10^{-3}. Additionally, only hearts with a coronary flow 8-16 ml/min were used.

The formula below was used to calculate the rate pressure product (RPP) :

Rate pressure product:

$$= \frac{\text{Heart rate x (systolic pressure – diastolic pressure)}}{1000}$$

$$= \frac{\text{Heart rate x developed pressure}}{1000}$$

$$= \frac{\text{Heart function}}{1000}$$

$$RPP = (\text{beats/min x mmHg}) \times 10^{-3}$$

- Developed is the difference between systolic pressure and diastolic pressure.
- Heart function is the heart rate multiplied by developed pressure.
- Rate pressure product is the heart function divided by 1000.

3.1.1. Experimental Protocols

As a pilot study, to evaluate whether we could induce preconditioning via transient glucose deprivation (metabolic inhibition) we performed the following experimental protocols, **figure 2**.

In short, all hearts were excised into Krebs solution at 4°C and retrogradely perfused within 1 min of excision [52]. After stabilisation period, **Control** hearts (CONT) were subjected to 30 min global normothermic ischaemia and 30 min reperfusion (index I/R). During ischaemia hearts were immersed in an ungassed Krebs buffer solution at 37^0C and myocardial temperature monitored by a thermocouple indicator probe inserted into the pulmonary artery. **Ischaemic Preconditioning** (IPC), after stabilisation rat hearts were subjected to 10 min global normothermic ischaemia and 10 min reperfusion prior to index I/R.

Glucose deprivation preconditioning GLU(-), hearts were exposed to 15 min glucose free buffer perfusion followed by 10 min reperfusion with glucose containing buffer prior to index I/R.

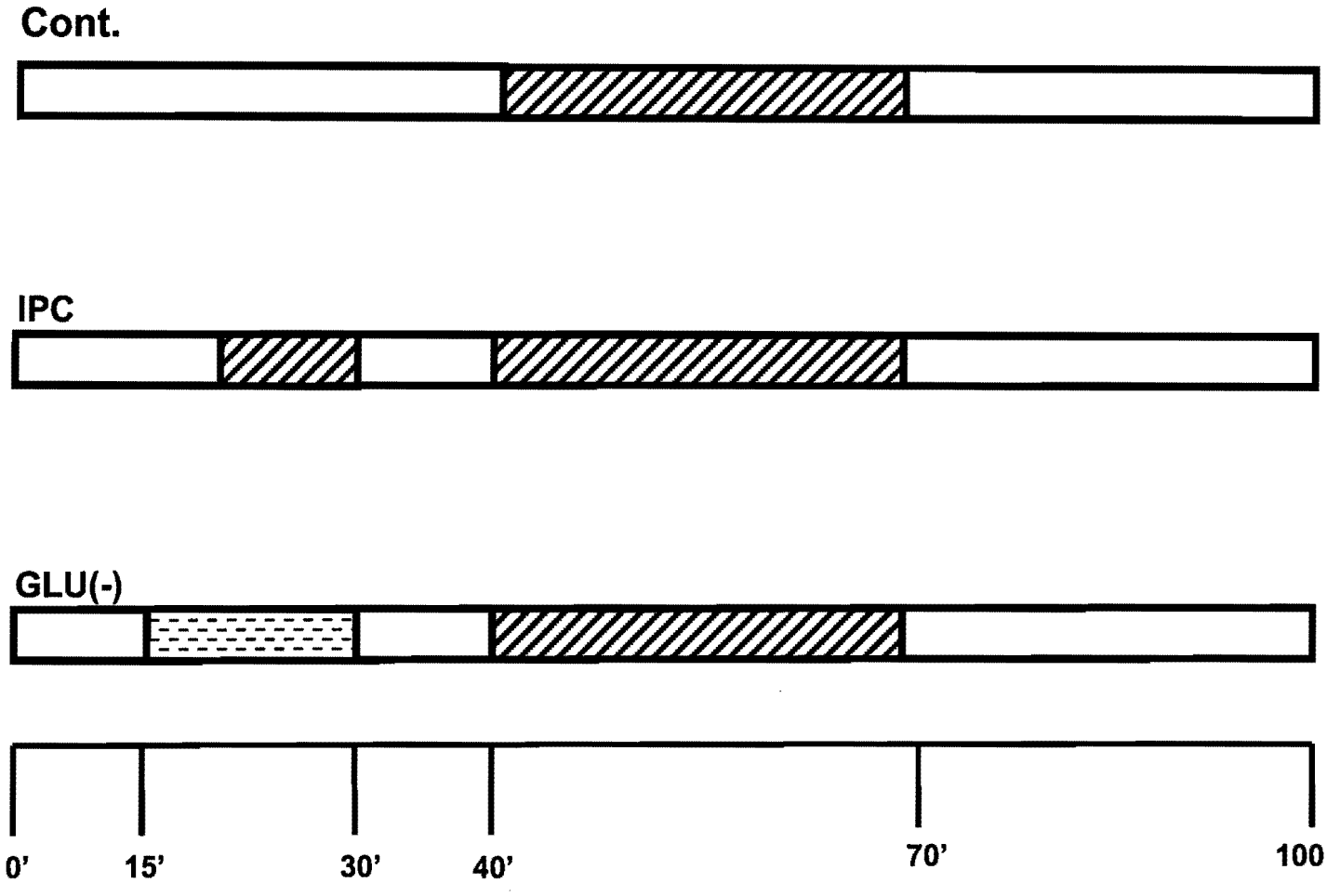


Figure 2: Experimental protocols depicting the timing of each intervention in isolated Langendorff perfused rat hearts. The white blocks represent normal aerobic perfusion, the hatched blocks represent global normothermic ischaemia and the black block represents 15 min glucose deprivation period. CONT = control, IPC = ischaemic preconditioning, GLU(-) = glucose deprivation preconditioning.

Glucose Deprivation Preconditioning and Cellular Metabolic Interventions

The potential alternate metabolic substrate hypothesis is discussed and illustrated in **figure 3(a).** In brief, glucose deprivation is a metabolic stress that may result in enhanced fatty acid mobilisation / metabolism and / or result in reduced rate of glycolysis. Moreover, fatty acid mobilisation may, in turn, inhibit glycolysis[53]. To test whether fatty acids as an alternate substrate or a direct effect of glycolytic inhibition was responsible for glucose deprivation protection, fatty acid supplementation and glycolytic inhibition were perfused in the presence of glucose. To enhance fatty acid mobilisation / metabolism, rat hearts were supplemented with a medium chain fatty acid, octanoate, also known to inhibit glycolysis[53]. An alternative approach was to inhibit glycolysis with 11mM 2-deoxyglucose (2DG) an agent which is known to inhibit glycolysis by binding to hexokinase. The protocols used to evaluate this hypothesis are as shown in **figure 3(b)**. Rat hearts were exposed to 15 min medium chain fatty acid, 11mM octanoate (OCT) or 11mM 2-deoxyglucose (2DG) in the presence of glucose containing buffer followed by 10 min washout period before being subjected to index I/R. Additionally, Goto et. al [10] showed that reduced glycolysis may effect glucose deprivation protection. To investigate this, a medium chain fatty acid, 11mM octanoate {GLU(-) OCT}, or a combination of 11mM octanoate and 11mM 2-deoxyglucose {GLU(-) OCT + 2DG} or 20 mM pyruvate {GLU(-) PYR}, a metabolic substrate also known to inhibit glycolysis [54], was added during 15 min glucose deprivation.

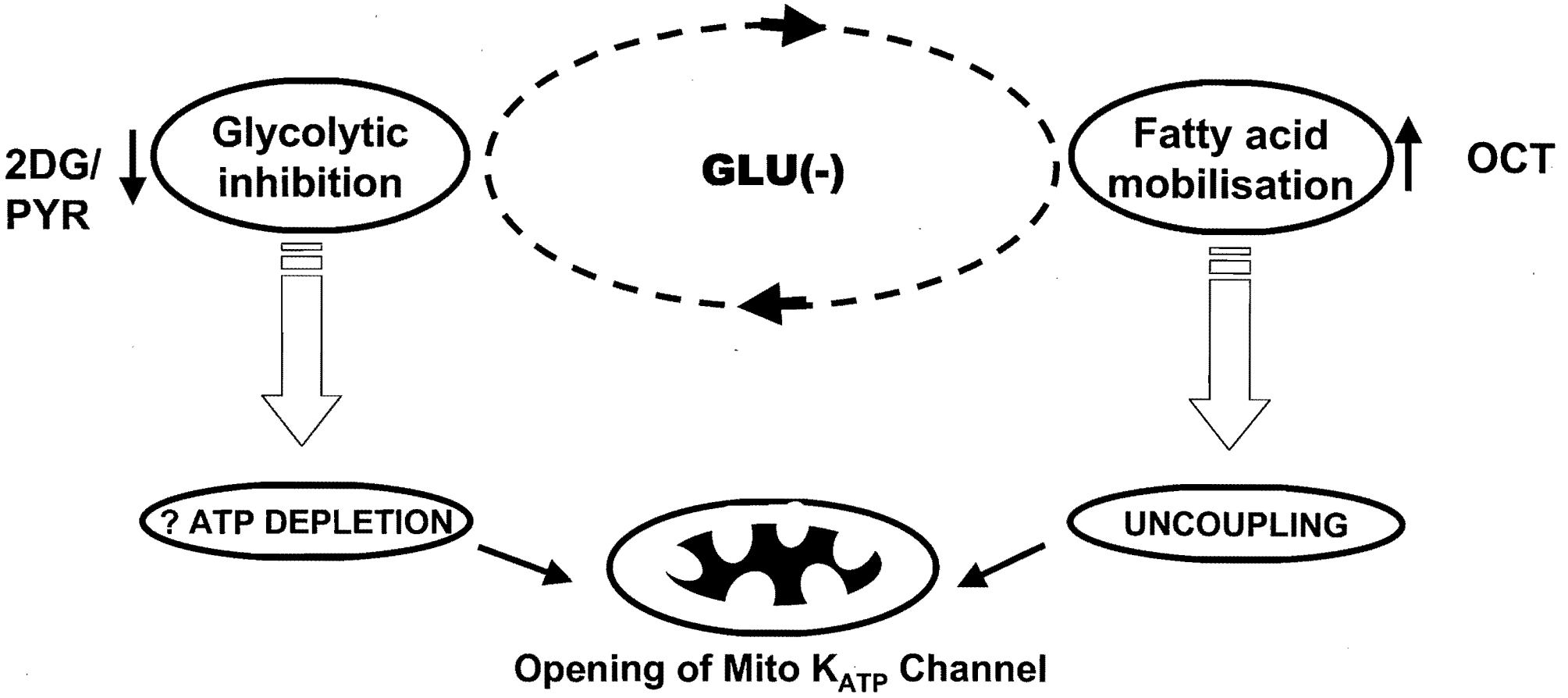


Figure 3(a) Schematic representation of the possible metabolic pertubations in the heart following glucose deprivation. Glucose deprivation is a metabolic stress leading to increased fatty acid mobilization and/or glycolytic inhibition. Both fatty acid mobilization, which may lead to uncoupling of the oxidative phosphorylation and glycolytic inhibition can result in ATP depletion. In turn, this hypothetical dpletion of ATP may lead to the opening of the mitochondrial ATP sensitive potassium channels. To test whether glucose deprivation protection acts via these metabolic pertubations a medium chain fatty acid, octanoate was used to enhance fatty acid mobilization and to inhibit glycolysis and a glycolytic inhibitor, 2-Deoxyglucose or pyruvate were perfused separately during glucose deprivation period.

Experimental Protocols

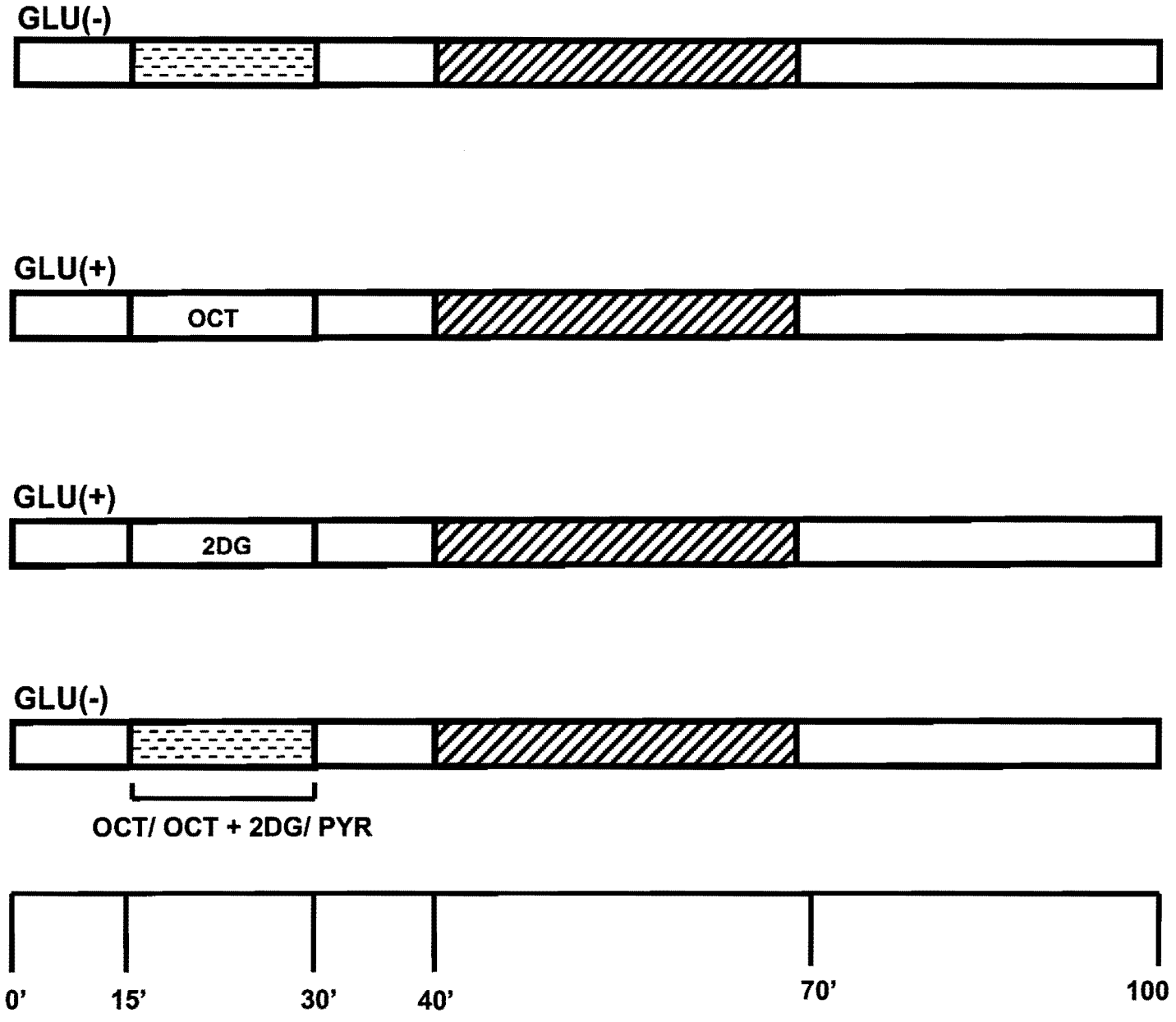
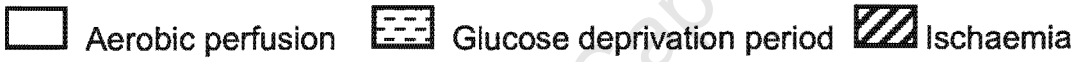


Figure 3(b): Experimental protocols depicting the timing of each intervention in isolated Langendorff perfused rat hearts. The white blocks represent normal perfusion, hatched blocks represent global normothermic ischaemia and the black block represents glucose deprivation period, GLU(-). The white blocks with OCT or 2DG represent a period when octanoate (OCT) or glycolytic inhibitor (2DG) was perfused together with normal glucose containing for 15 min. Black block with an arrow underneath means that octanoate (OCT) or a combination of octanoate and 2-Deoxyglucose (OCT and 2DG) or pyruvate (PYR) was perfused for 15 min during glucose deprivation period.



Glucose Deprivation Preconditioning and Cellular Signalling Interventions

As discussed previously, some of the classic signalling events known to induce ischaemic preconditioning, include adenosine and α, β - adrenergic receptor signalling cascades. To investigate whether metabolic preconditioning is via any of these classic signalling pathways, various antagonists were administered, as shown in **figure 4(a).** The protocols are as shown in **figure 4(b).** In brief, in control hearts antagonist infusion commenced 10 min prior to index I/R. In GLU(-) hearts, antagonist infusion was commenced 5 min prior to the GLU(-) period and stopped at the beginning of the 30 min I/R. To block adenosine activation 100μM 8-SPT (a nonselective adenosine receptor blocker – IC_{50} values for A1 and A2 are 4.3 and 6.2 respectively)[55] was used. α_1 and β-adrenergic receptor activation were blocked by the antagonists prazosin (α1-blocker-10μM)[56, 57] and propranolol (β-blocker-10μM)[58] respectively. As the adenosine antagonist studies implicated this nucleotide as a mediator during glucose deprivation, we performed an additional set of experiments to confirm the specificity of 100μM SPT, adenosine receptor blocker. In these experiments we preconditioned with 100μM adenosine as PC-mimetic. After a period of stabilisation, adenosine was administered for 5 min followed by 10 min washout prior to index I/R {ADO}. To evaluate the ability of SPT to block the effects of adenosine preconditioning, SPT was administered 2 min prior to, during and after the adenosine administration until the onset of index I/R {ADO + SPT}. Adenosine was also administered as a pretreatment for 5 min prior to index I/R {Pre ADO}. To investigate the role of PKC and the K_{ATP}

channel, inhibitors, chelerythrine (10μM)[59, 60] and 5-HD (100μM) [30, 61] were administered, respectively.

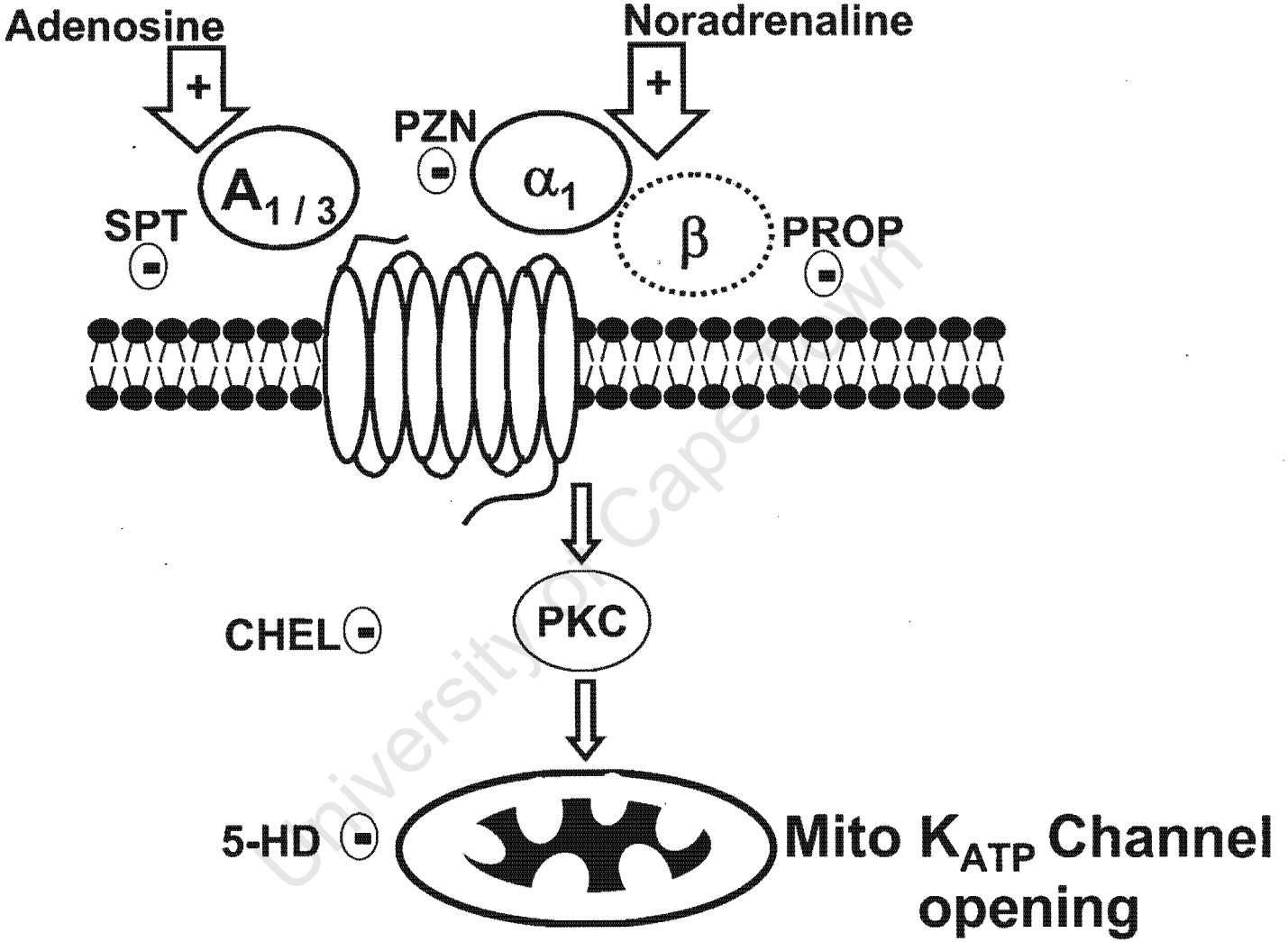


Figure 4(a) Schematic representation of the possible metabolic pathway through which glucose deprivation may confer protection. Glucose deprivation triggers the release of adenosine and activation of adenosine type 1 / 3 and/or noradrenaline release and activation of alpha1 and beta adrenergic receptors. Activation of these receptors may lead to activation of protein kinase C and subsequent opening of the mitochondrial ATP sensitive potassium channels. Various antagonists were used to block this pathway, namely: adenosine receptor blocker 8-(p-sulfophenyl) theophylline - SPT; alpha receptor blocker prazosin - PZN; beta blocker propanolol - PROP; protein kinase C inhibitor chelerythrine - CHEL; mitochondrial ATP sensitive potassium channel blocker 5-hydroxydecanoate - 5HD.

Cont.

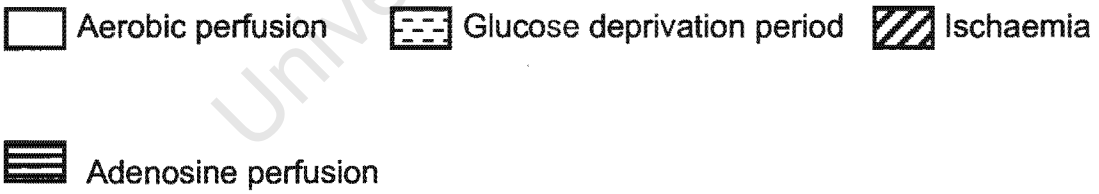
IPC

GLU(-)

$ADO_{PC\ mimetic}$

$ADO_{Pretreatment}$

Figure 4(b) Experimental protocols depicting the timing of each intervention in isolated Langendorff perfused rat hearts. The white blocks represent normal perfusion, hatched blocks represent global normothermic ischaemia and the black block represents glucose deprivation period, GLU(-). Arrows indicate the timing of pharmacological interventions. The following pharmacological agents were used in control and GLU(-) protocols: ADO, adenosine, SPT, 8-(p-sulfophenyl) theophylline)-adenosine receptor blocker; PZN, prazosin-α_1 adrenergic receptor blocker and PROP, propranolol-β adrenergic receptor blocker; CHEL, chelerythrine-PKC inhibitor; 5-HD, 5 hydroxydecanoate - Mito K_{ATP} channel blocker. Additionally, adenosine was used as PC mimetic (ADO) and SPT was used to block adenosine preconditioning like protection (ADO + SPT). In IPC group only CHEL and 5HD were used.



The role of protein kinase A inhibition in augmenting post ischaemic contractile function of single episode of preconditioning.

It has been shown that the levels of cAMP decline with the number of preconditioning ischaemia / reperfusion episodes[1, 2]. This increase in number of ischaemic preconditioning episodes correlates with much reduced infarct size and more improved cardioprotection compared to single episode of ischaemic preconditioning[3]. Thus, protein kinase A inhibition may be associated with greater ischaemic preconditioning protection. Moreover, it has been shown that ischaemic preconditioning is mediated by activation of β-adrenergic signalling pathway. The effects of an activated protein kinase A signalling pathway during the trigger ischaemia of preconditioning with pharmacological interventions is shown in **figure 5A**. To investigate the functional consequences of this correlation, we compared single episode to multiple episodes of ischaemia. Rat hearts were preconditioned with 3 x 5 min episodes of ischaemia, interspersed with 5 min reperfusion, before subjected to index I/R (IPC 3). To further investigate whether β-adrenergic induced protein kinase A activation during single episode of pharmacological or ischaemic preconditioning may limit this cardioprotection, hearts were initially preconditioned with an adenylate cyclase activator, 0.3μM forskolin. Forskolin was perfused as a PC mimetic for 5 min followed by a 10 min drug-free perfusion period before index of I/R (Forsk). In order to evaluate whether inhibition of PKA could further augment protection against ischaemia-induced contractile dysfunction, a specific PKA inhibitor, 2μM H89 (K_i for PKA = 0.048 μM)[62] was used. We subsequently evaluated the concurrent abrogation of

PKA activity with forskolin preconditioning. H89 was perfused 3 min prior and during 5 min forskolin perfusion followed by a 10 min drug-free perfusion period before index I/R (Forsk + H89).

To evaluate the effects of PKA inhibition with H89 against ischaemia / reperfusion and ischaemic preconditioning, four series of experiments were performed with H89. (1) H89 was perfused 3 min prior to the trigger ischaemia (IPC 1 + H89); (2) H89 was administered during the reperfusion period between preconditioning ischaemia and the index ischaemia (IPC 1 + $H89_{Rep}$); (3) H89 was administered as a ischaemic preconditioning mimetic for 5 min followed by 5 min washout ($H89_{PC\ mimetic}$); (4) H89 was perfused 3 min prior to the index I/R ($H89_{Pretreatment}$), **Figure 5B**.

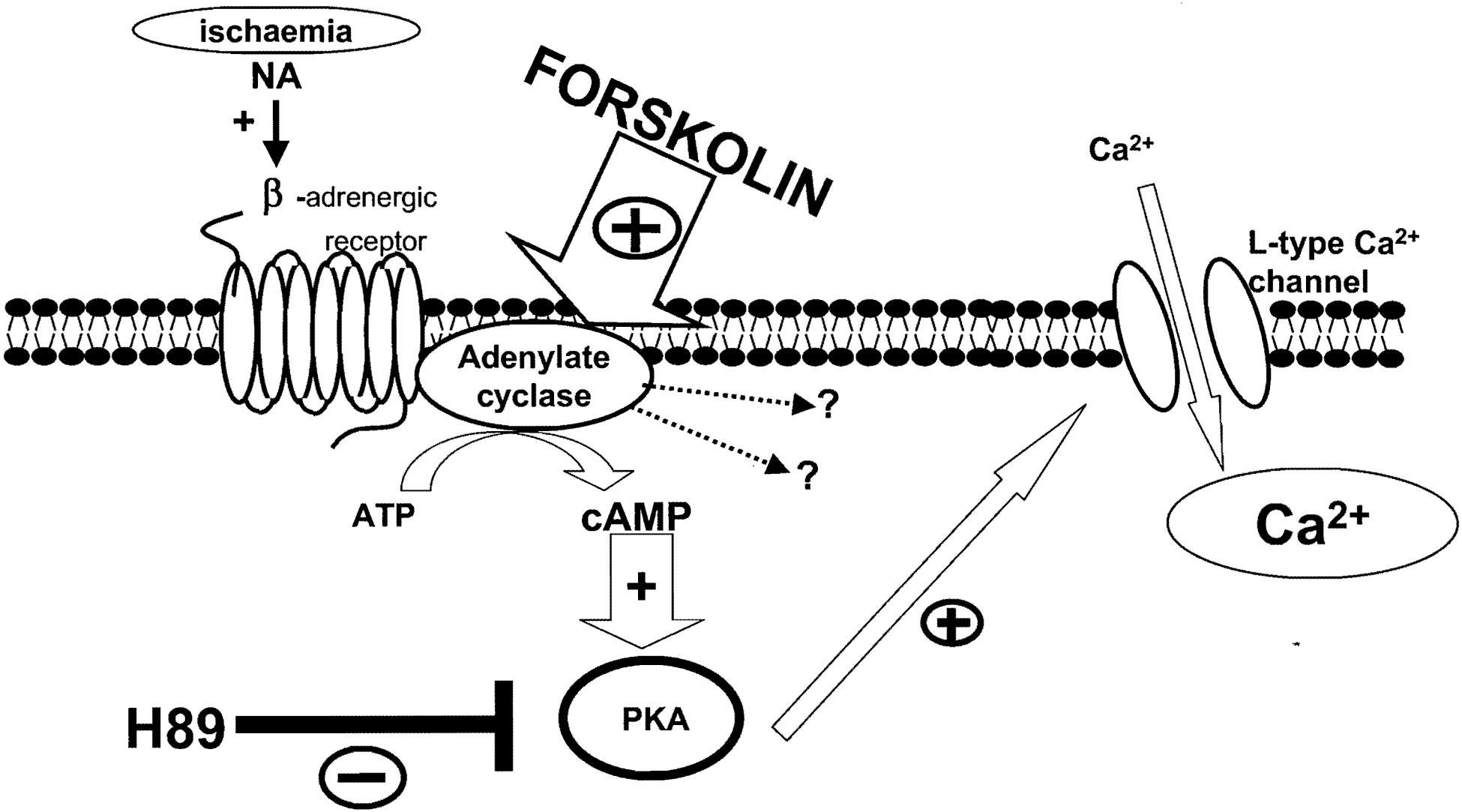


Figure 5 Schematic representation of β - adrenergic pathway and pharmacological interventions. Ischaemia leads to neuronal adrenaline (NA) release, activation of β - adrenergic receptors, activation of adenylate cyclase, amongst other things, it may lead to the rise in cAMP levels, activation of protein kinase A (PKA), opening of L-type calcium channels and subsequent calcium influx. Adenylate cyclase was activated by forskolin and PKA was inhibited by H89.

Experimental Protocols

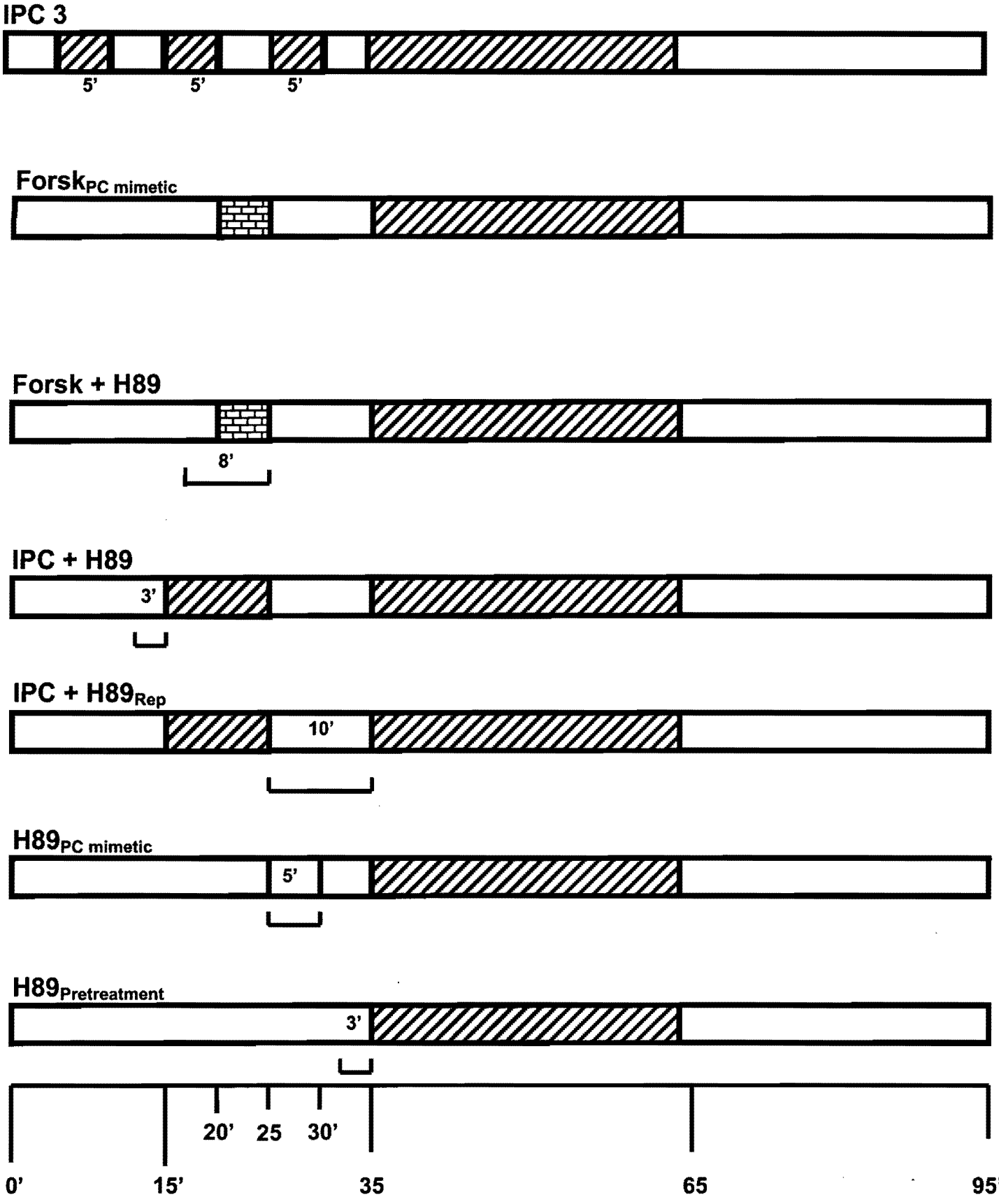


Figure 5B. Experimental protocols depicting the timing of each intervention in isolated Langendorff perfused rat hearts. The white blocks represent normal perfusion, hatched blocks represent global normothermic ischaemia. Lines indicate H89 perfusion period. The control hearts (CONT) were subjected to a period of 30 min ischaemia / reperfusion (index I/R) and preconditioning hearts were subjected to a single episode of 10 min global ischaemia followed by 10 min reperfusion before subjected to index I/R (IPC 1), figure 2. In another set of experiments, rat hearts were preconditioned with 3 x 5 min episodes of ischaemia, interspersed with 5 min reperfusion, before subjected to index I/R (IPC 3). Forskolin was perfused for 5 min followed by 10 min drug free perfusion (Forsk). H89 was perfused for 3min prior to and during 5 min forskolin perfusion (Forsk + H89) followed by 10 min drug free perfusion before being subjected to index I/R. H89 was also given 3 min prior trigger ischaemia (IPC 1 + H89) or 10 min during reperfusion period following 10 min trigger ischaemia before index ischaemia (IPC 1 + $H89_{Rep}$) or 5 min as preconditioning mimetic followed by 5 min washout ($H89_{PC\ mimetic}$) or 3 min prior index ischaemia ($H89_{Pretreatment}$).

 Aerobic perfusion  Ischaemia  Forskolin perfusion

3.2 Biochemical analysis of Protein Kinase A activity

To evaluate the degree of PKA inhibition we performed biochemical analysis of PKA activity with or without H89. Rat hearts were freeze-clamped at various time points during the experimental protocol with precooled Wollenberger tongs and immediately dipped into and stored in liquid nitrogen, **Figure 5C**. Tissue cAMP-dependent PKA activity was determined with an assay from Gibco BRL. Frozen tissue was homogenised in an extraction buffer (5mmol/L EDTA, 50mmol/L Tris, pH 7.5) and centrifuged for 10 min at 600g and the supernatant was quantitated and diluted to ± 5 μg protein / 10μL extract. Four assay conditions were used for each sample to measure the final percentage of activated PKA in the tissue. Two blank tubes with the tissue extract were set up, one with diluent and without inhibitor / activator and the other without diluent but with an inhibitor and an activator. Two assay tubes with an extract and diluent were set up, one with an inhibitor but without an activator and the other with an activator but without an inhibitor. Duplicates of each sample were made and tubes were incubated at room temperature for 15 minutes to allow inhibitor to bind. To evaluate the intrinsic tissue PKA activity, ^{32}P/Substrate solution was added (to each tube) and tubes were incubated at 30°C for 5 minutes. 20μM was removed (from each tube) and spotted onto the corresponding phosphocellulose disc. The phosphocellulose disc sheet was immersed in a tray containing about 200ml sulphuric acid and washed with rocking twice for 5 minutes, poured off (as radioactive waste. The phosphocellulose disc sheet was again washed twice for 5 minutes with distilled water. The phosphocellulose discs were punched out of the sheet with forceps and put into corresponding scintillation vials

containing 5ml scintillation fluid. To evaluate the intrinsic tissue PKA activity, ^{32}P incorporation with the PKA substrate was measured on a scintillation counter and the results were expressed as pmol activated PKA. min^{-1} . mg $protein^{-1}$. The pseudo-substrate was used as a negative control in order to inhibit ^{32}P incorporation into the PKA substrate. The protein content of samples was determined by either the Kaplan-Pedersen[63] or Lowry[64] technique.

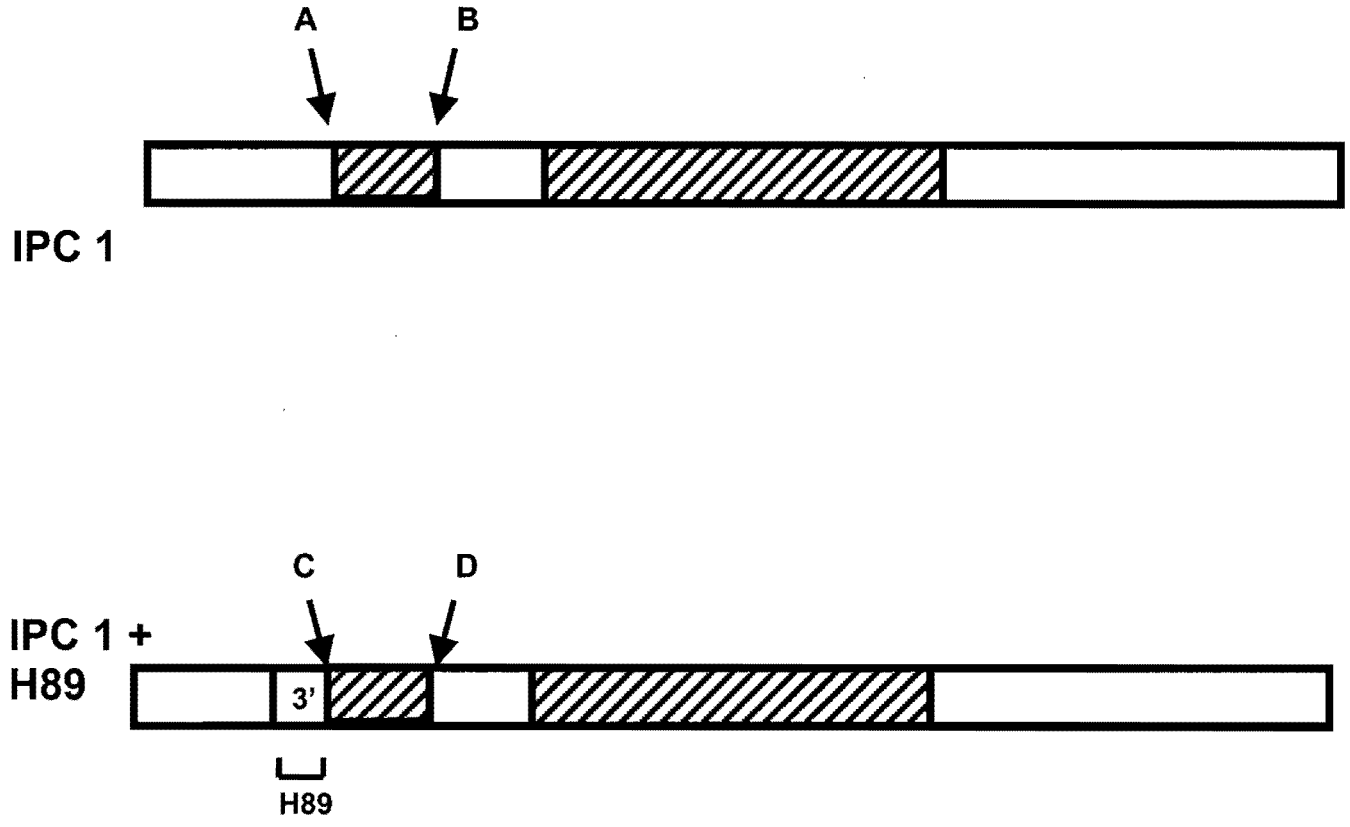


Figure 5C Experimental protocols depicting the timing of each PKA activity measurement in isolated Langendorff perfused rat hearts. The white blocks represent normal perfusion, hatched blocks represent global normothermic ischaemia. The line indicates the timing of the pharmacological interventions. Untreated hearts were freeze-clamped at the baseline (A) and the end of 10 min ischaemia (B). H89 treated hearts were freeze-clamped 3min after drug infusion (indicated by an arrow) before ischaemia (C), and at the end of 10 min ischaemia (D).

3.3 Materials

Forskolin hydrochloride, prazosin hydrochloride, 8-(p-sulfophenyl) theophylline, adenosine, chelerythrine chloride, 2-Deoxy-D-glucose, octanoic acid were all obtained from Sigma Chemical Corporation (St Louis, MO, USA) and dissolved in distilled water. 5-hydroxydecanoate was obtained from Research Biochemicals International (Natick, MA, USA). H89 was obtained as a gift from Hiroshi Hidaka, Nagoya University, Japan. Pyruvate was obtained from Roche molecular Biochemicals (South Africa). Chelerythrine, H89 and forskolin were stored as stock solutions of 200μM, 10mM and 10^{-3} M at -80^{0}C, respectively. All other stock solutions were made fresh daily.

3.4 Haemodynamic parameters

Heart rate, systolic pressure, diastolic pressure and the coronary flow were measured before index I/R (see tables 1-4) and at end of 30 min reperfusion for the glucose deprivation substudy and at 5min intervals until the end of 30 min reperfusion for PKA inhibition substudy. Developed pressure was calculated as the difference between systolic pressure and diastolic pressure. Baseline heart function (heart rate X developed pressure) was measured at the end of stabilisation phase and compared with the values at reperfusion. The heart function at reperfusion was compared to and expressed as the percentage the baseline heart function. During ischaemia the rise in the diastolic pressure was considered as an ischaemic contracture. The time to onset of contracture (TOC), i.e. the rise above the baseline diastolic pressure, was also recorded in all experiments.

3.5 Statistics

Data was expressed as mean ± SEM. The ANOVA one - way analysis of variance was used to determine significance between groups in the metabolic studies and signalling studies. One - way ANOVA with repeated measures was applied in reperfusion time course and haemodynamic measures. All post-hoc determinations (when ANOVA showed significant differences) were made by the Bonferroni test for multiple comparisons. A value of $P<0.05$ was used as the level of significance.

4

RESULTS

4. RESULTS

4.1 Exclusions

A total of 280 rats were used for this thesis. 248 rats were used for heart perfusion and 32 rat hearts were freeze clamped for protein kinase A activity measurements. Of 248 rats used for heart perfusion, a total of 25 rats did not fit the exclusion criteria. We report a data on 255 rats that successfully completed this study.

4.2 Transient glucose deprivation improves post ischaemic contractile function.

Haemodynamic variables prior to and during ischaemia

Ischaemic preconditioning hearts were subjected to 10 min global ischaemia (attenuated contractile function) and 10 min reperfusion (reversed the contractile function to the baseline level) prior to index ischaemia / reperfusion. To assess whether we could induce cardioprotection by transient glucose deprivation, hearts were subjected to 15 min glucose deprivation period, an effect which depressed the cardiac function, followed by glucose replenishment period, which returned the cardiac contractile function to baseline, before subjected to index I/R, (haemodynamic parameters prior to index I/R and time to onset of ischaemic contracture during ischaemia are shown in **table 1**).

	Baseline		Post treatment		Pre-ischaemia		TOC
	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	(min)
CONT	33.6 ± 0.9	11.3 ± 0.9	-	-	33.6 ± 0.9	11.3 ± 0.9	10.0 ± 1.3
IPC	33.5 ± 1.5	11.7 ± 0.8	-	-	30.1 ± 1.6	12.0 ± 1.1	4.70 ± 0.4^
GLU(-)	31.6 ± 1.7	11.5 ± 0.7	17.4 ± 1.4*	8.20 ± 0.9	25.8 ± 3.7	9.30 ± 0.7	6.7 ± 0.7
GLU(+)OCT	31.8 ± 2.2	11.6 ± 0.9	29.0 ± 2.0	10.4 ± 0.7	30.4 ± 1.6	10.3 ± 0.8	7.40 ± 0.9
GLU(+)2DG	37.4 ± 1.9	13.3 ± 1.0	17.6 ± 3.6*	10.5 ± 1.5	19.5 ± 1.3*	8.20 ± 0.7	8.80 ± 0.6
GLU(-)OCT	30.4 ± 2.2	12.2 ± 1.3	32.0 ± 1.7	12.8 ± 1.1	26.4 ± 3.3	10.4 ± 1.8	10.8 ± 1.4
GLU(-)OCT+2DG	34.4 ± 1.4	12.3 ± 0.6	26.9 ± 2.3	12.0 ± 1.0	26.1 ± 3.1	10.0 ± 1.4	6.30 ± 0.5
GLU(-)PYR	33.4 ± 0.7	14.3 ± 0.9	29.2 ± 2.6	13.6 ± 0.6	23.9 ± 2.7	11.9 ± 1.2	13.0 ± 1.0

Table 1 : Haemodynamic variables from the metabolism substudy

Baseline - at the end of stabilisation period; RPP - rate pressure product; CF - coronary flow; Post treatment - after 15 min GLU(-) and/or treatment with a pharmacological agent; Pre-ischaemia - before index ischaemia; TOC - time to onset of contracture during ischaemia. CONT - glucose control; IPC - ischaemic preconditioning; GLU(-) - glucose preconditionining; GLU(+) OCT / 2DG - octanoate / 2-deoxyglucose in the presence of glucose; GLU(-)OCT / OCT+2DG / PYR - octanoate/ a combination of octanoate and 2-deoxyglucose / pyruvate in the absence of glucose.

Values are mean ± SEM; $n \geq 6$ in all groups. *$p < 0.05$ vs baseline; ^$p < 0.05$ vs control

Post ischaemic contractile recovery

After a period of 30 min ischaemia / reperfusion, the control hearts (CONT) showed a significant reduction in post ischaemic contractile function compared to the baseline contractile function, CONT (28.5 ± 2.8 %). Ischaemic preconditioning (IPC) hearts were subjected to 10 min global ischaemia followed by 10 min reperfusion with a subsequent return of cardiac contractile function to the baseline level. Ischaemic preconditioning hearts showed significant improvement of the post ischaemic contractile recovery, IPC (56.0 ± 3.6 %). Thus, as predicted, in this pilot study we found that transient glucose deprivation improves post ischaemic contractile function, GLU(-) (59.8 ± 4.0 %). We therefore concluded that glucose deprivation preconditioning mimics ischaemic preconditioning and improves the post ischaemic contractile function of the control hearts, $p < 0.001$ vs control, **Figure 6**.

We therefore compared potential metabolic changes and consequences (decreased glycolysis and enhanced fatty acid mobilisation) with conventional ischaemic preconditioning signalling events (adenosine receptor and / or adrenergic receptor activation, protein kinase C activation and the subsequent opening of the ATP dependent potassium channels) as possible mechanisms involved in glucose deprivation protection.

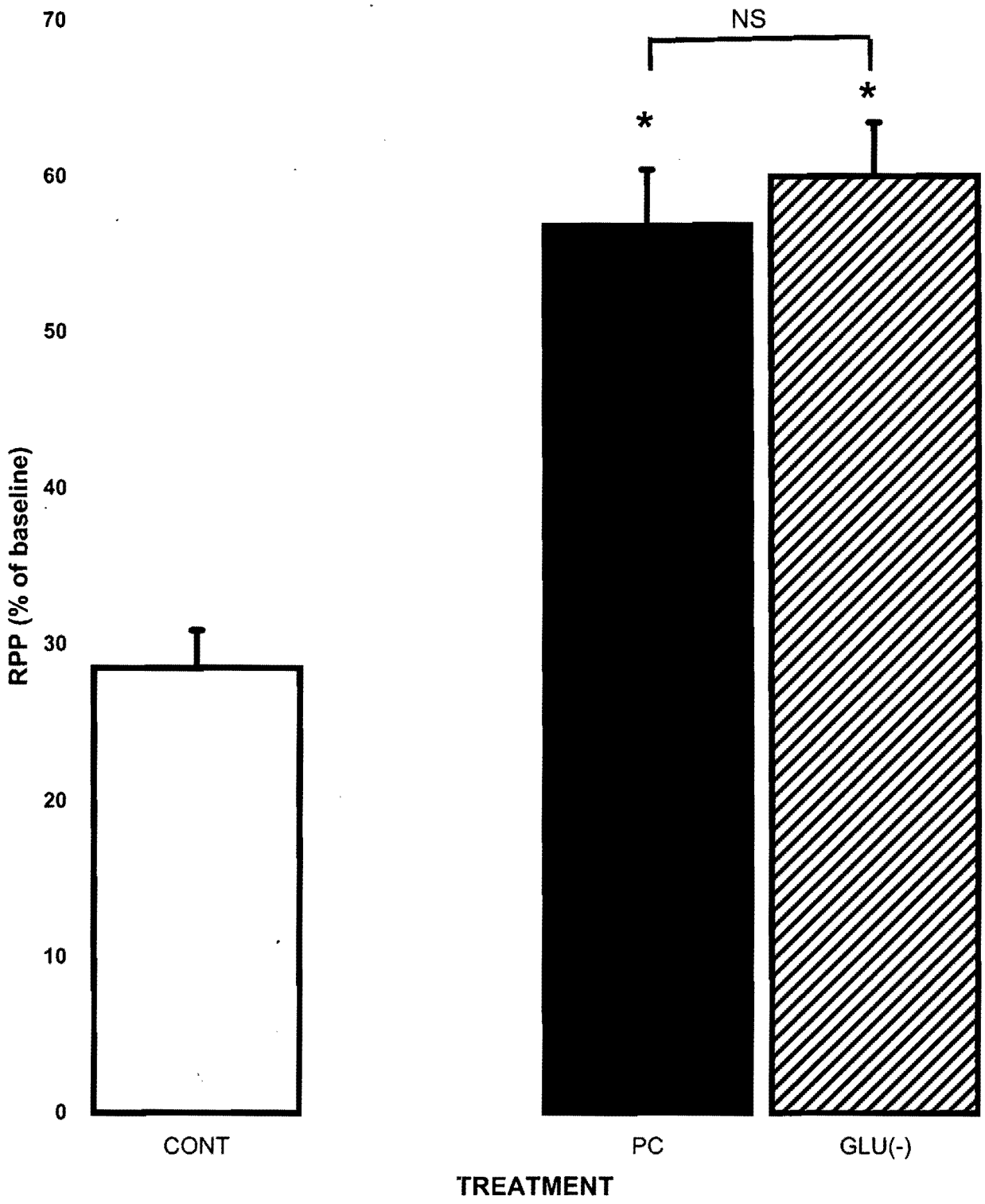


Figure 6 Transient glucose deprivation preconditioning mimicks ischaemic preconditioning and improve post ischaemic contractile function against ischaemia / reperfusion insult. RPP (expressed as percentage of baseline) = rate pressure product at 30 min reperfusion. $n \geq 6$ at the end of reperfusion. *$p < 0.001$ vs baseline.

4.3 Glucose Deprivation Preconditioning and Cellular Metabolic Pathway

Haemodynamic variables prior to and during ischaemia.

To investigate the possible metabolic pathways that may be active during glucose deprivation, a medium chain fatty acid, octanoate, and an inhibitor of glycolysis, 2-Deoxyglucose were used as substitutes for glucose deprivation period, GLU(-). Replacement of GLU(-) with octanoate (OCT hearts) did not have any effect on cardiac function prior to the index I/R, but replacement with 2-Deoxyglucose (2DG hearts) depressed the cardiac function, an effect that irreversibly depressed the cardiac function despite a glucose replenishment period, see **table 1.**

Post ischaemic contractile recovery

To study the role of fatty acid mobilisation or glycolytic inhibition we initially replaced 15 min glucose deprivation with octanoate or 2-deoxyglucose. The post ischaemic contractile recovery of octanoate, OCT (36.8 ± 3.5 %) or 2-deoxyglucose, 2DG (34.0 ± 4.3 %) when perfused for 15 min in the presence of glucose did not show any significant difference to the controls, CONT (32.1 ± 4.6 %) and were all significantly reduced compared to glucose deprivation protection, GLU(-) (59.8 ± 4.0 %), **figure7(a)**.

Octanoate was then perfused in the absence of glucose (for enhancement fatty acid metabolism / mobilisation and consequent inhibition of glycolysis) and was found to be cardioprotective against ischaemia / reperfusion insult, GLU(-)OCT (50.2 ± 2.5 %) compared to control (32.1 ± 4.6 %), slightly lower but not significantly different to glucose deprivation protection GLU(-) (59.8 ± 4.0 %). These data show that fatty acids as an alternate substrate did not abolish glucose deprivation mediated protection, we therefore conclude that glucose deprivation mediated protection does not directly act via enhanced fatty acid metabolism / mobilisation nor via possible octanoate-induced glycolytic blockade.

To further induce glycolytic blockade a combination of octanoate and 2-Deoxyglucose (glycolytic inhibitor) {GLU(-) OCT+2DG} was perfused in the absence of glucose. The use of alternate substrate (octanoate) probably contributed towards the maintenance of the cardiac function under these experimental conditions. This combination was found to be cardioprotective against ischaemia / reperfusion insult {GLU(-) OCT+2DG} - 49.3 ± 3.3 %. To further investigate the possible involvement of glycolytic blockade in glucose

deprivation protection we perfused with a different pharmacological agent known to indirectly inhibit glycolysis, pyruvate. Pyruvate abolished GLU(-) protection, GLU(-) PYR (26.6 ± 3.1%); NS vs control, **figure7(b)**

These results show that GLU(-) protection does not work via enhanced fatty acid metabolism and may partially act via an indirect inhibition of glycolysis by pyruvate. Our next step was to investigate possible cellular signalling pathways that may play a role in glucose deprivation mediated protection.

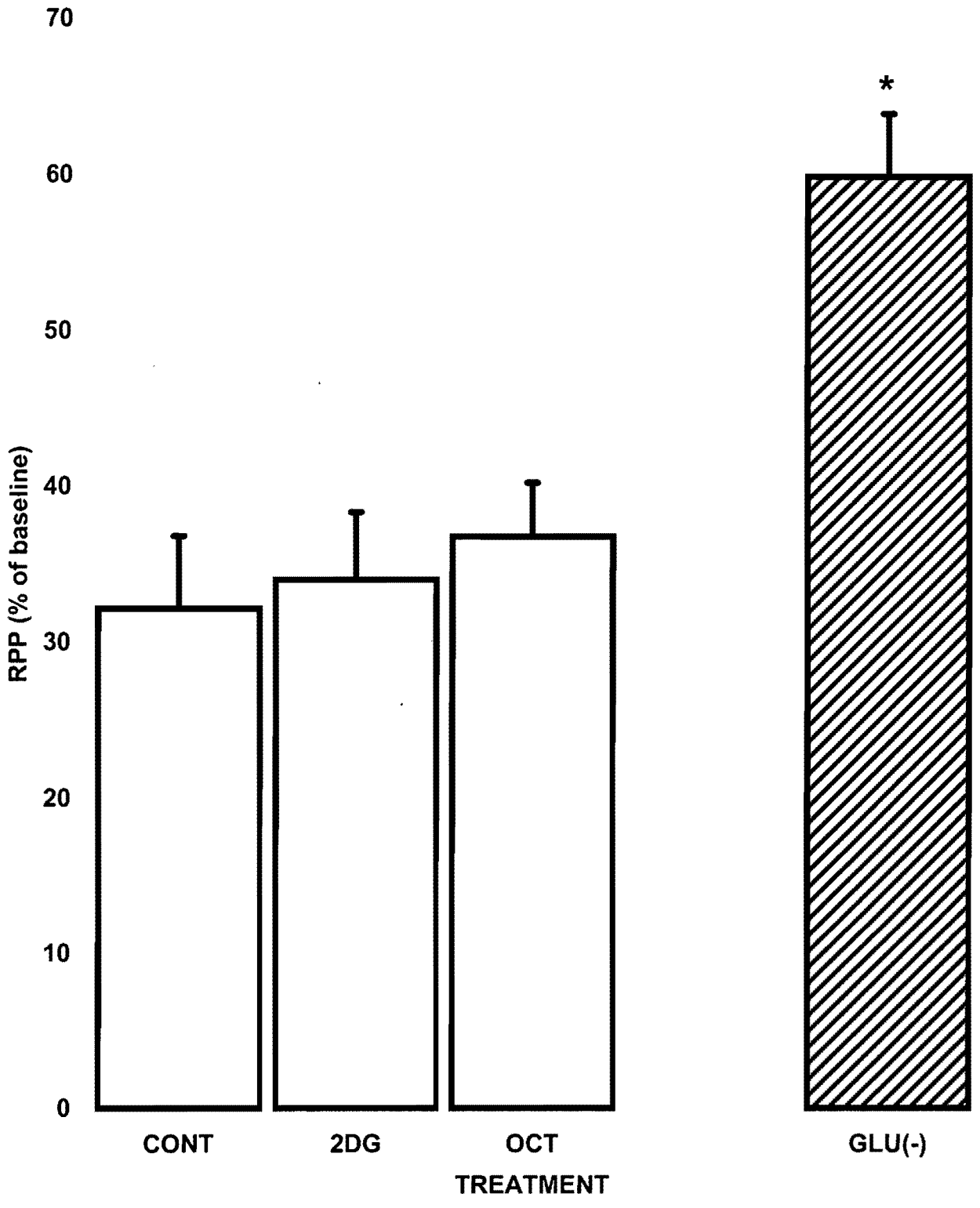


Figure 7 a The effects of enhanced fatty acid mobilization and glycolytic inhibition on post ischaemic contractile recovery when perfused as PC mimetic in the presence of glucose.
RPP (percentage of baseline) = rate pressure product at 30 min reperfusion, n > 6 at the end of reperfusion. All values mean + SEM. *p < 0.01 vs control / 2DG / OCT.

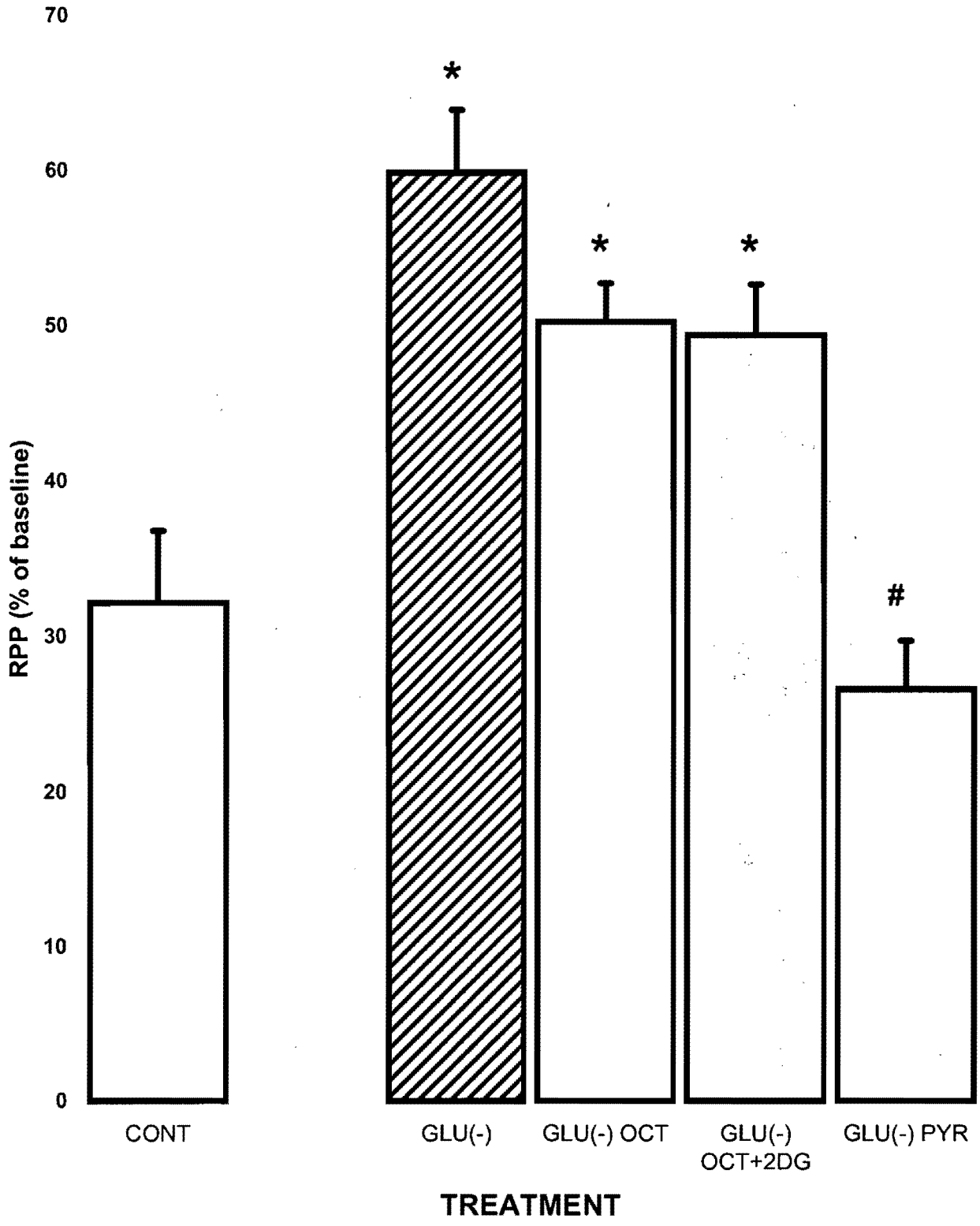


Figure 7 b The effects of enhanced fatty acid mobilization and glycolytic inhibition on post ischaemic contractile function. RPP (percentage of baseline) = rate pressure product at 30 min reperfusion n > 6 at the end of reperfusion. All values mean ± SEM. *p < 0.01 vs control; #p < 0.01 vs GLU(-)

4.4 Glucose Deprivation Preconditioning and Cellular Signalling Pathway

Cell surface receptor blockade and glucose deprivation reconditioning.

As previously discussed, some of the classic signalling events known to induce ischaemic preconditioning, include adenosine, α and β-adrenergic cell surface receptor signalling cascades. To investigate whether metabolic preconditioning acts via any of these classic signalling pathways, adenosine, α_1 and β-adrenergic receptor activation, SPT (nonselective adenosine blocker), prazosin (α1-blocker - 10μM) & propranolol (β-blocker - 10μM) were administered, in separate experiments with or without metabolic preconditioning.

Haemodynamic variables prior to and during ischaemia

In control hearts antagonists were given 10 min prior to the index I/R. As shown in **table 2**, propranolol depressed the cardiac function but PZN and SPT did not have any effect to the cardiac function of control hearts prior the index I/R. In GLU(-) hearts antagonists were administered before, during and after GLU(-) period. The antagonists further depressed the cardiac function of GLU(-) hearts, an effect that was not reversed by glucose replenishment period, except for SPT. Administration of adenosine significantly depressed the cardiac function except when adenosine was given with SPT. The coronary flow (CF) was not affected by GLU(-) or antagonists perfusion but adenosine perfusion significantly increasing coronary flow even after its administration.

	Baseline		Post treatment		Pre-ischaemia		TOC
	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	(min)
CONT.	33.7 ± 2.0	11.7 ± 1.2	-	-	33.7 ± 2.0	11.7 ± 1.2	10.0 ± 1.6
GLU(-)	36.1 ± 2.6	12.5 ± 1.1	25.6 ± 1.9*	12.0 ± 1.4	32.4 ± 2.2	10.8 ± 1.1	6.7 ± 0.7
CONT+PROP	36.2 ± 1.9	12.3 ± 0.8	-	-	21.7 ± 1.1*	11.2 ± 0.8	13.8 ± 2.1
GLU(-)PROP	30.9 ± 2.0	12.4 ± 0.4	14.3 ± 1.5*	9.6 ± 0.7	14.6 ± 2.9*	10.0 ± 0.6	13.7 ± 1.1
CONT+PZN	35.4 ± 0.9	13.0 ± 0.4	-	-	30.2 ± 2.7	9.8 ± 0.6	11.2 ± 1.1
GLU(-)PZN	34.3 ± 1.9	12.3 ± 0.7	17.0 ± 3.5*	7.2 ± 1.6	24.0 ± 3.6	9.3 ± 1.0	5.2 ± 0.6^
CONT+SPT	38.6 ± 2.0	14.0 ± 0.9	-	-	36.1 ± 2.4	14.0 ± 0.9	11.2 ± 1.4
GLU(-)SPT	38.3 ± 1.0	13.5 ± 0.6	23.8 ± 2.6*	9.3 ± 1.1	31.8 ± 3.2	11.2 ± 1.1	5.8 ± 0.5^
CONT+ADO	37.9 ± 1.5	14.1 ± 0.6	22.8 ± 4.6*	18.1 ± 0.9*	22.8 ± 4.6*	18.1 ± 0.9*	9.4 ± 0.7
ADO PC	32.7 ± 2.4	11.3 ± 1.1	19.1 ± 4.8*	17.3 ± 1.4*	26.5 ± 2.2	10.2 ± 0.8	8.3 ± 0.8
ADO + SPT	31.5 ± 1.6	9.6 ± 0.4	30.9 ± 2.8	13.8 ± 1.4*	29.5 ± 2.0	9.5 ± 0.7	7.5 ± 0.7

Table 2 : Haemodynamic variables from cell surface receptor signalling substudy

Baseline - at the end of stabilisation period; RPP - rate pressure product; CF - coronary flow; Post treatment - after 15 min GLU(-) and/or treatment with a pharmacological agent; Pre-ischaemia - before index ischaemia; TOC - time to onset of contracture during ischaemia. CONT - glucose control; GLU(-) - glucose deprivation preconditioning; CONT+ PROP/ PZN/ SPT/ ADO - pretreatment with propranolol (PROP)/ prazosin (PZN)/ 8-(-p-sulfophenyl) theophylline (SPT)/ adenosine (ADO); GLU(-) PROP/ PZN / SPT / ADO - pharmacological agents were given before, during and after GLU(-) before index ischaemia. ADO PC - adenosine was administered as a preconditioning mimetic; ADO + SPT - adenosine preconditioning with adenosine receptor blocker, SPT. Values are mean ± SEM; $n \geq 6$ in all groups. *$p < 0.05$ vs baseline; ^$p < 0.05$ vs control

Post ischaemic contractile recovery

When antagonists were administered as pretreatment, α and β - adrenergic receptor blockade significantly improved the post ischaemic contractile recovery of the control from (29.2 ± 3.4 %) to CONT+PZN (45.5 ± 5.4 %) and CONT+PROP (56.2 ± 4.4 %), $p < 0.05$ vs control. This may have been due to decreased energy demand prior to ischaemia. Adenosine receptor blockade did not have any effect on the cardiac function of the controls, CONT+SPT (22.3 ± 1.9 %), NS vs control. Of note, in these results antagonists were given for only 10 min instead of 30 min because in our pilot study the effect was the same, as to save pharmacological agents we only perfused for 10 min), **figure 8(a)**.

When antagonists were perfused with GLU(-), α and β -adrenergic receptor blockade did not have any additional protection on post ischaemic contractile function, GLU(-) (56.5 ± 1.0 %), GLU(-) PZN (50.2 ± 3.3 %) and GLU(-) PROP (55.5 ± 4.0 %). Adenosine blocker (SPT) significantly abolished GLU(-) protection from (56.5 ± 1.0 %) to GLU(-) SPT (36.7 ± 3.3 %), $p < 0.01$, figure **8(b)**. To test the specificity of SPT in blocking adenosine, in our system, we perfused adenosine as PC mimetic (ADO PC). ADO PC conferred protection on post ischaemic contractile recovery, ADO PC (60.0 ± 6.9 %).

The preconditioning like effect of adenosine was then blocked by SPT (28.4 ± 2.6 %). When adenosine was given as a pretreatment it did not have any effects against index I/R (34.3 ± 5.5 %), **figure 8(c)**. Collectively, these data suggest that GLU(-) protection protects through adenosine release.

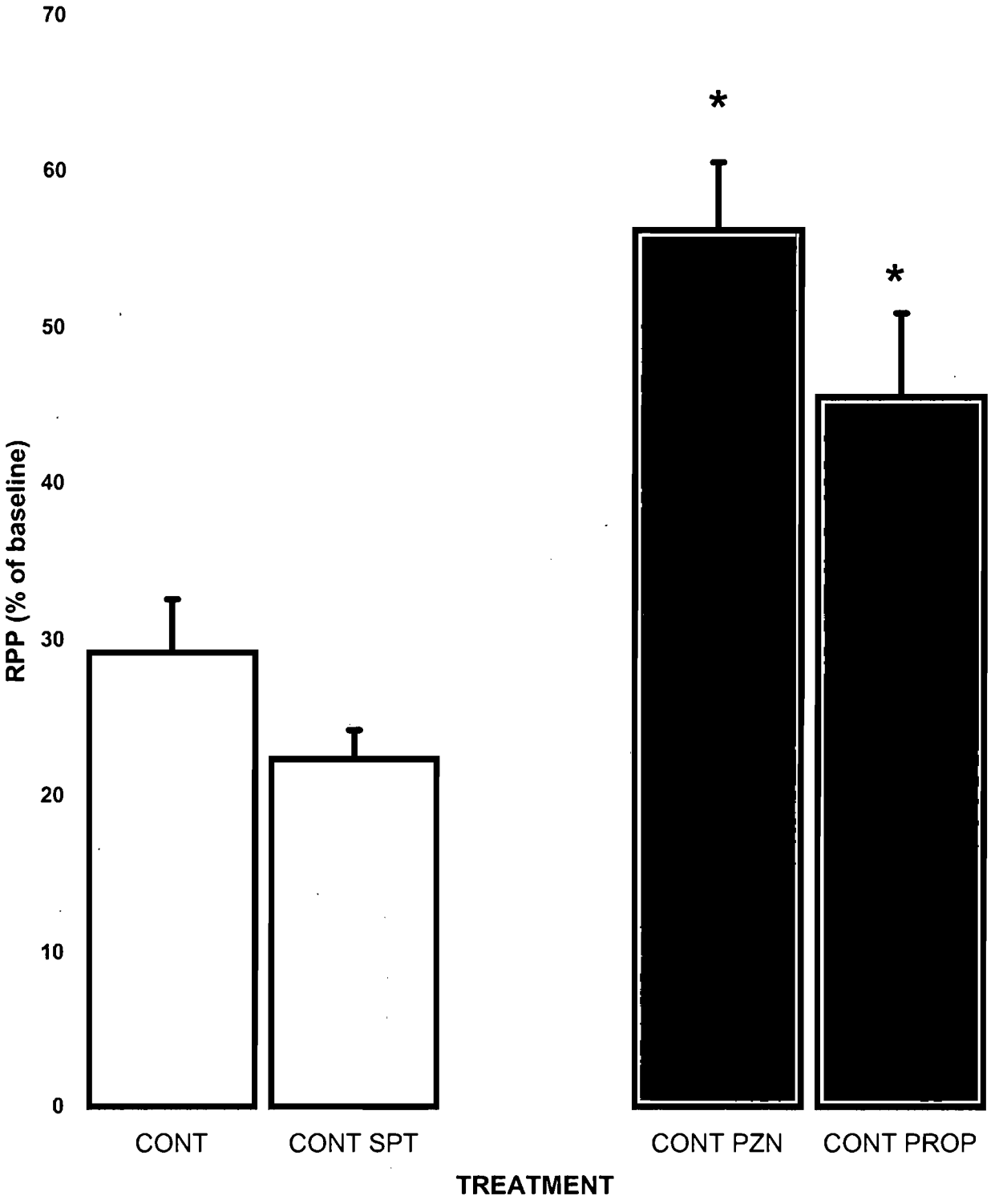


Figure 8a The effects of adenosine, alpha and beta receptor blockade on post ischaemic contractile function when perfused as a pretreatment. Alpha blockade using prazosin (PZN) and beta blockade using propanolol (PROP) improved the post ischaemic contractile function when administered as a pretreatment whilst adenosine blockade using SPT did not have any effects. RPP (% of baseline) = rate pressure product at 30 min reperfusion. $n \geq 6$ at the end of reperfusion. All values are mean + SEM. *$p < 0.001$ vs control.

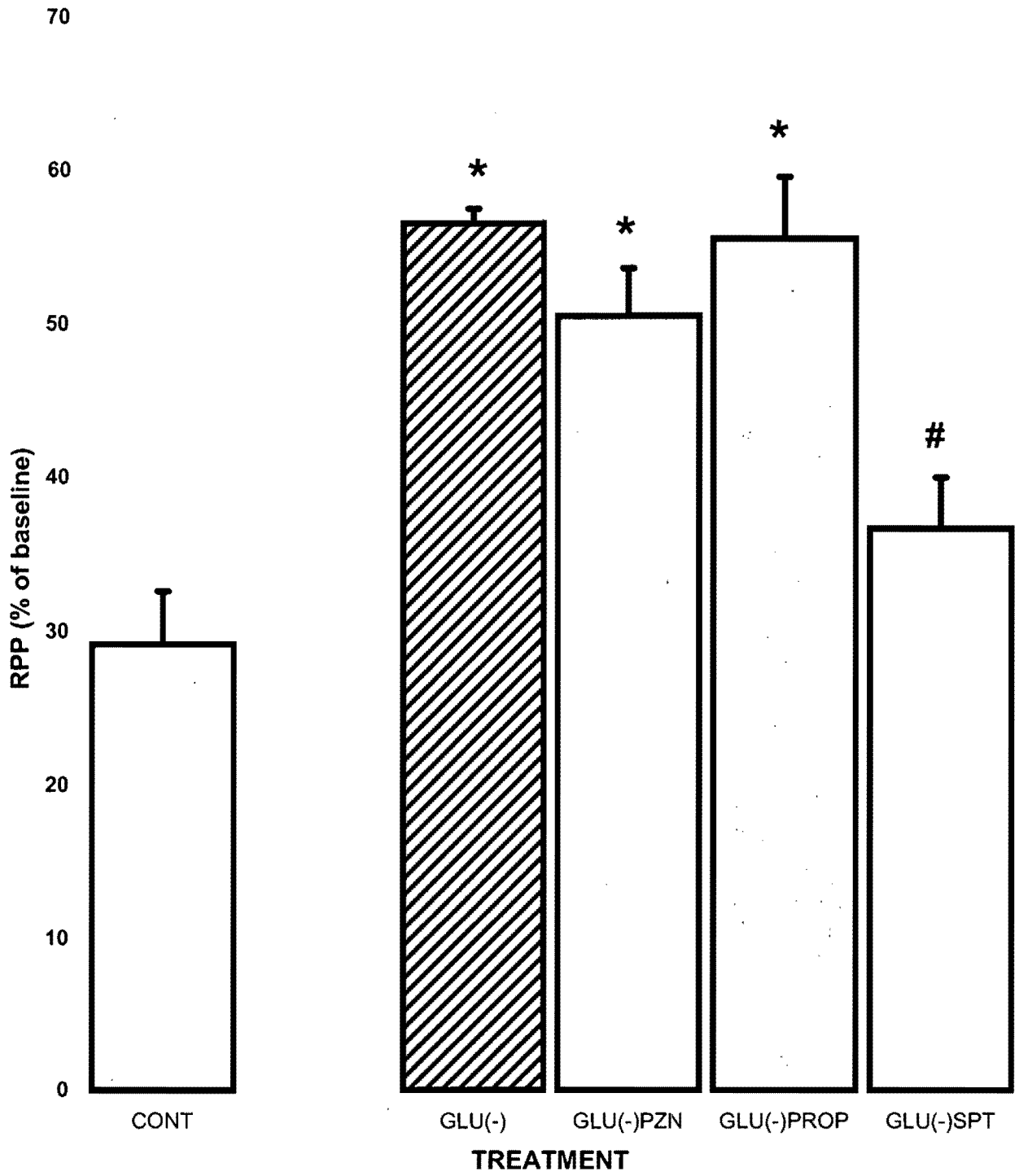


Figure 8b The effects of adenosine, alpha and beta receptor blockade on post ischaemic contractile function by SPT, prazosin and propanolol, respectively, in metabolically preconditioned hearts. RPP (% of baseline) = rate pressure product at 30 min reperfusion. $n \geq 6$ at the end of reperfusion. All values mean + SEM. *$p < 0.001$ vs control, #$p < 0.01$ vs GLU(-).

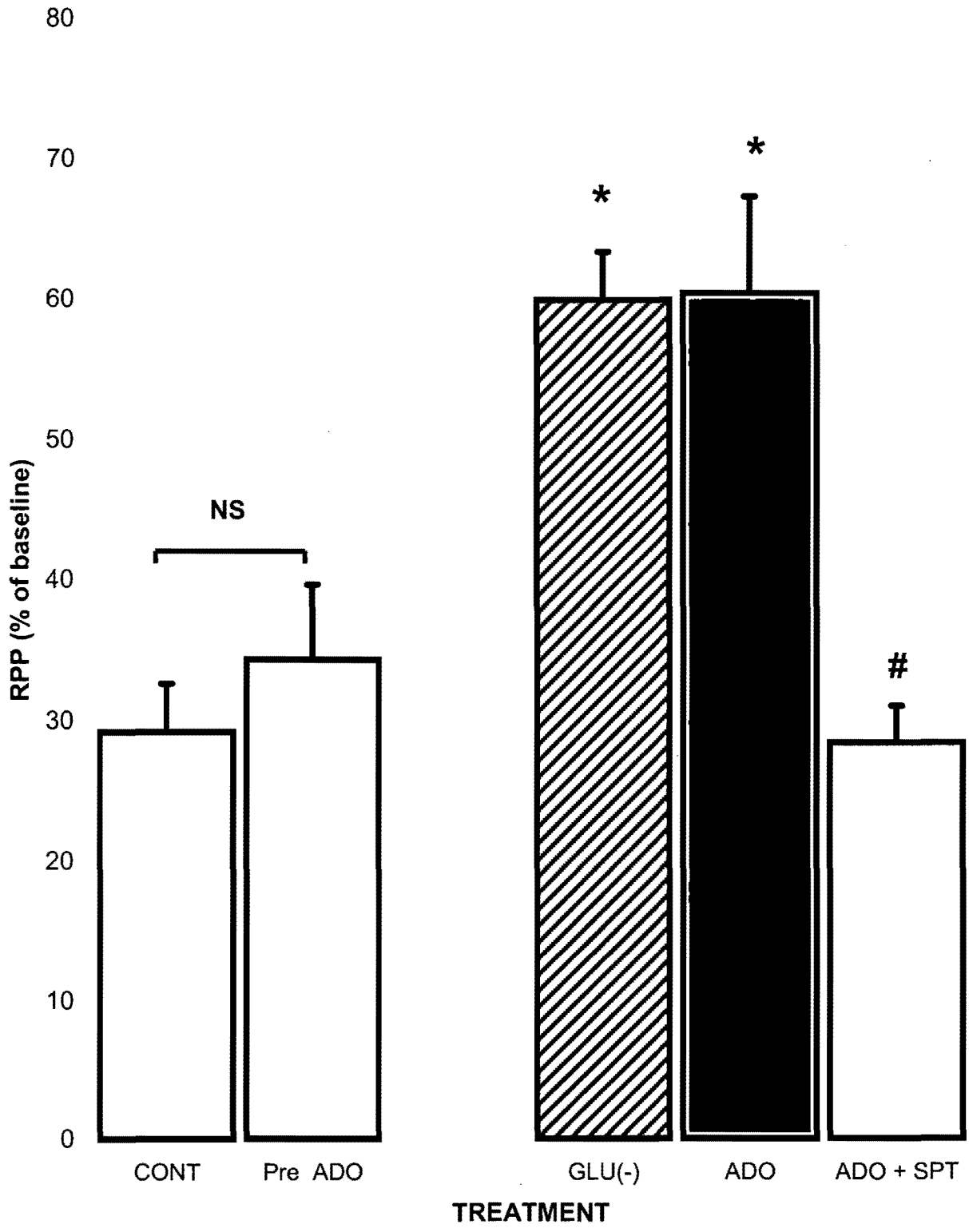


Figure 8c The effects of adenosine when administered as a pretreatment or PC mimetic. Adenosine (ADO) mimicks GLU(-) protection and adenosine protection was abolished by adenosine receptor blocker (ADO + SPT). Adenosine pretreatment (Pre ADO) had no effect on the control. RPP (% of baseline) = rate pressure product at 30 min reperfusion.
All values mean + SEM, $n \geq 6$. *$p < 0.05$ vs control; #$p < 0.05$ vs ADO; NS vs control

Post cell surface receptor blockade

The activation of cell surface receptors leads to the activation of the downstream signalling events. These include the activation of protein kinase C and the subsequent opening of the mitochondrial K_{ATP} channels. We therefore determined whether protein kinase C and mitochondrial K_{ATP} channel were activated in metabolic preconditioning by using various pharmacological inhibitors. Post cell surface receptor blockade included the inhibition of PKC and blockade of the mito K_{ATP} channels.

Haemodynamic variables prior to and during ischaemia.

The protein kinase C inhibitor (chelerythrine) and the K_{ATP} channel blocker (5-hydroxydecanoate) did not have any effects on the cardiac function and coronary flow of the control, IPC and GLU(-) hearts prior to the index I/R. During ischaemia, IPC and GLU(-) hearts, with or without antagonists, had an early onset of contracture and all the controls expectedly encountered a late onset. At 30rnin reperfusion all groups had a slight reduction in coronary flow (CF) compared to the baseline, **Table 3**.

	Baseline		Post treatment		Pre-index ischaemia		TOC
	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	(min)
CONT	32.9 ± 1.0	11.0 ± 0.9			32.9 ± 1.0	11.0 ± 0.9	11.7 ± 0.5
IPC	33.5 ± 1.5	11.7 ± 0.8			30.1 ± 1.6	12.0 ± 1.1	4.7 ± 0.4^
IPC + CHEL	33.3 ± 1.3	12.0 ± 0.8			32.0 ± 0.9	13.5 ± 1.0	3.9 ± 0.1^
IPC + 5HD	34.1 ± 1.9	11.6 ± 0.7			30.5 ± 2.6	12.7 ± 0.4	5.3 ± 0.4^
GLU(-)	31.6 ± 1.7	11.5 ± 0.7	17.4 ± 1.4*	8.2 ± 0.9	25.8 ± 3.7	9.3 ± 0.7	6.8 ± 0.7^
CONT+CHEL	36.9 ± 1.5	12.7 ± 1.3			35.0 ± 1.6	13.0 ± 1.5	10.1 ± 1.2
GLU(-)CHEL	35.7 ± 1.1	12.0 ± 0.2	26.2 ± 1.0	12.4 ± 0.3	31.5 ± 1.8	11.9 ± 0.6	5.0 ± 0.3^
CONT+5HD	36.9 ± 1.3	12.0 ± 0.5			35.5 ± 2.3	12.0 ± 0.7	13.3 ± 1.0
GLU(-)5HD	34.6 ± 2.3	11.7 ± 1.0	24.4 ± 3.1	12.2 ± 1.7	31.2 ± 2.5	11.2 ± 1.5	5.8 ± 0.3^

Table 3 : Haemodynamic variables from the post receptor signalling substudy.

Baseline - at the end of stabilisation period; RPP - rate pressure product; CF - coronary flow; Post treatment - after 15 min GLU(-) and/or treatment with a pharmacological agent; Pre-ischaemia - before index ischaemia; TOC - time to onset of contracture during ischaemia. CONT - glucose control; GLU(-) - glucose deprivation preconditioning; IPC - ischaemic preconditioning; CONT+ CHEL/ 5HD pretreatment with chelerythrine (CHEL)/ 5-hydroxydecanoate (5HD); IPC CHEL / 5HD - pharmacological agents were administered before ischaemic preconditioning trigger IPC or GLU(-) CHEL / 5HD - during and after GLU(-) before index ischaemia.
Values are mean ± SEM; $n \geq 6$ in all groups. *$p < 0.05$ vs baseline; ^$p < 0.05$ vs CONT.

Post ischaemic contractile recovery

Protein kinase C antagonist, chelerythrine and mito K_{ATP} channel blocker, 5-hydroxydecanoate did not have any significant effect on the post ischaemic contractile recovery of the controls, CONT (28.5 ± 2.8 %), CONT+CHEL (23.7 ± 3.8 %) and CONT+5HD (29.7 ± 2.6 %). As shown in **figure 9**, glucose deprivation protection, GLU(-) (59.8 ± 4.0 %), was abolished by PKC inhibitor GLU(-) CHEL (35.0 ± 4.8 %) and K_{ATP} channel blocker GLU(-) 5HD (23.8 ± 3.4 %), $p < 0.001$. PKC inhibitor and K_{ATP} channel blocker also abolished protection afforded by ischaemic preconditioning, from IPC (56.7 ± 3.6 %) to IPC + CHEL (37.4 ± 1.1 %); IPC + 5HD (36.8 ± 3.8 %), $p < 0.05$. These results suggest that transient glucose deprivation protects against post ischaemic contractile dysfunction by activation of protein kinase C and opening of the mitochondrial ATP dependent potassium channels.

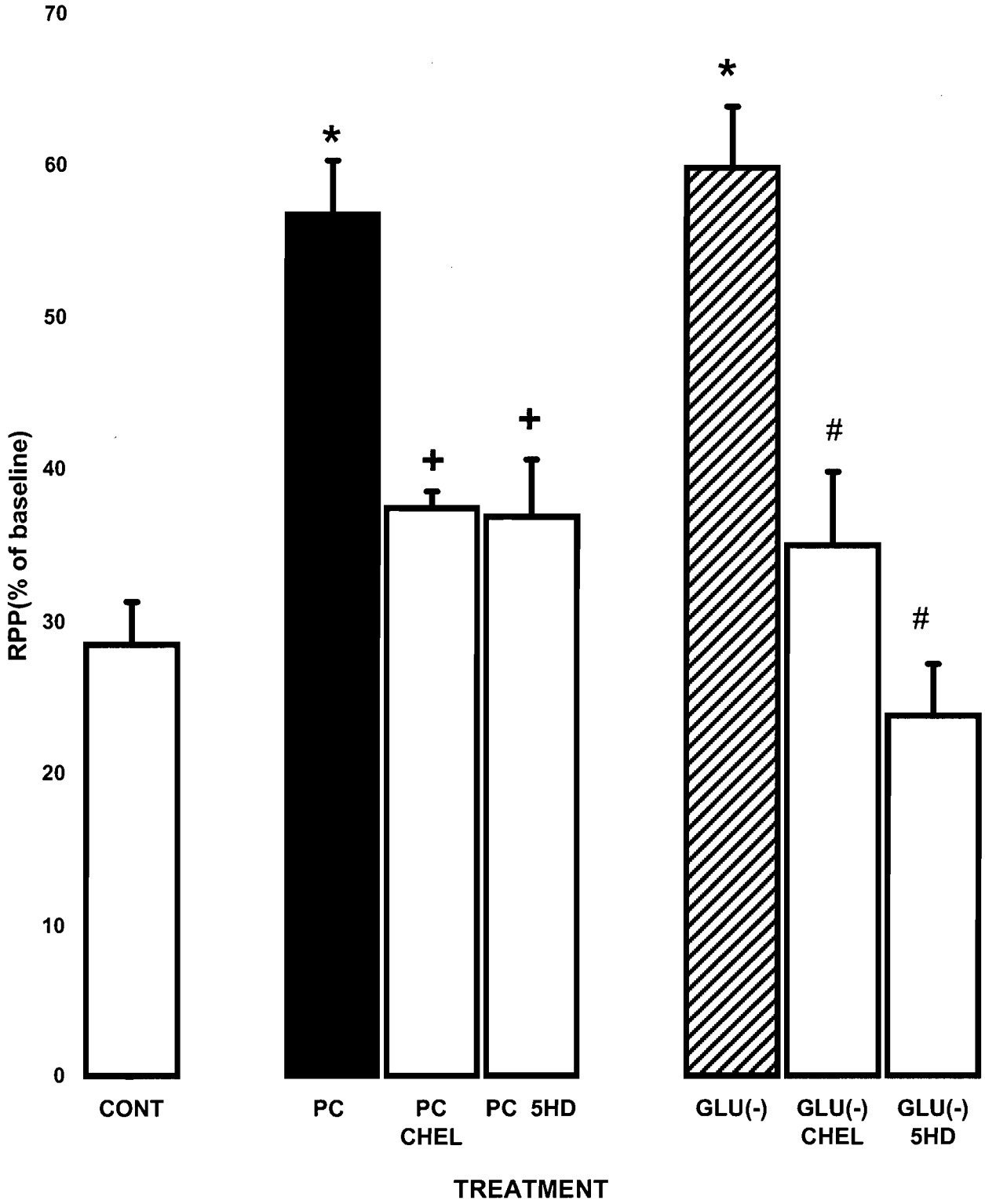


Figure 9 The effects of protein kinase C and mitochondrial ATP sensitive potassium channel blockade on post ichaemic contractile function. PKC inhibitor, chelerythrine (CHEL) and potassium channel blocker (5HD) abolished ichaemic preconditioning and glucose deprivation preconditioning. RPP (% of baseline) = rate pressure product at 30 min reperfusion. n > 6 at the end of reperfusion. All values mean + SEM. *p < 0.01 vs control, +p < 0.01 vs PC, #p < 0.01 vs GLU(-).

4.5 The role of protein kinase A inhibition in augmenting single episode of ischaemic preconditioning

It has been shown that the levels of cAMP decline with the number of ischaemia / reperfusion episodes[1, 2] and ischaemic preconditioning protection improves with the number of cycles[3]. Thus protein kinase A inhibition may contribute to improved post ischaemic contractile recovery after multiple episodes. We therefore compared single episode to multiple episodes of ischaemia and further investigated whether β-adrenergic induced protein kinase A activation during single episode of ischaemic preconditioning may limit this cardioprotection. In addition, we evaluated whether we could augment the cardioprotection afforded by single episode of ischaemic preconditioning by attenuating the activation of protein kinase A.

Single episode versus multiple episodes of ischaemia

After a period of 30 min ischaemia / reperfusion (index I/R), the control hearts (CONT) showed a significant reduction in post ischaemic contractile function compared to the baseline contractile function, CONT (14.8 ± 3.0 %). Rat hearts preconditioned with single episode of 10 min global ischaemia improved post ischaemic contractile function, IPC 1 (45.0 ± 3.2 %) at the end of 30 min reperfusion. Rat hearts preconditioned with 3 x 5 min episodes of ischaemia, interspersed with 5 min reperfusion, before subjected to index I/R showed additive improvement of post ischaemic contractile function, above

that of single episode of ischaemic preconditioning, IPC 3 (62.8 ± 2.9 %) at the end of 30 min reperfusion, **figure 10(a).**

This suggests that preconditioning with three episodes of ischaemia is better that one episode, but the exact mechanism underlying this phenomenon remains unclear. We therefore investigated whether a single episode of pharmacological preconditioning with an adenylate cyclase activator, forskolin and single episode of ischaemic preconditioning may be augmented by inhibition of PKA activity.

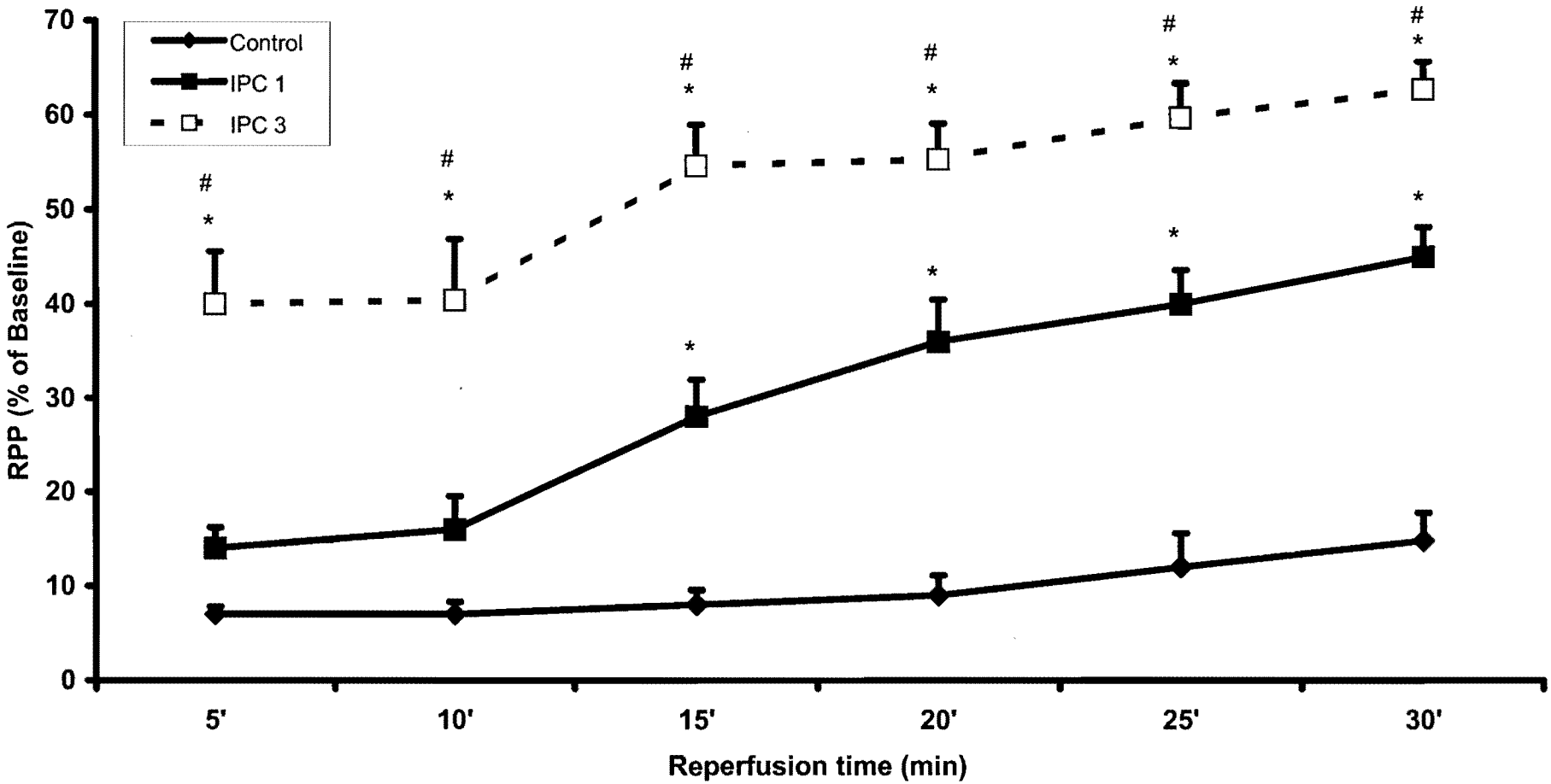


Figure 10 (a) Three cycles of ischaemic preconditioning further improves post ischaemic contractile function compared to single episode of preconditioning. RPP (% of baseline) = rate pressure product at 5 min intervals during 30 min reperfusion. All values mean ± SEM; $n \geq 6$ throughout. *$p < 0.01$ vs control; #$p < 0.05$ vs IPC 1.

PKA inhibition on pharmacological preconditioning

After a period of 30 min ischaemia / reperfusion, the control hearts (CONT) showed a significant reduction in post ischaemic contractile function compared to the baseline contractile function, CONT (17.3 ± 1.5 %). Ischaemic preconditioning (IPC 1) hearts were subjected to 10 min global ischaemia followed by 10 min reperfusion with a subsequent return of cardiac contractile function to the baseline level. Ischaemic preconditioning hearts showed significant improvement of the post ischaemic contractile recovery, IPC 1 (45.0 ± 3.2 %). To assess whether we could induce cardioprotection by transient activation of adenylate cyclase, an adenylate cyclase activator forskolin was used. Hearts were subjected to 5 min forskolin perfusion, an effect which significantly increased the cardiac function, followed by a 10 min drug-free perfusion with normal buffer, which returned the cardiac contractile function to baseline, before subjected to index ischaemia / reperfusion, (haemodynamic parameters prior to and during ischaemia) are shown in **table 4**). We found that when forskolin was given as a preconditioning mimetic, it improved post ischaemic contractile function, FORSK (35.4 ± 0.9 %), NS vs IPC 1. Thus, forskolin preconditioning had a similar effect to a single episode of preconditioning, $p<0.001$ vs control. To determine protein kinase A's effect on forskolin preconditioning, we concurrently abrogated PKA activation with PKA inhibitor, H89 3min before and during 5 min forskolin period (see protocol figures). H89 significantly augmented the post ischaemic contractile recovery of forskolin preconditioning, FORSK+H89 (50.9 ± 3.9 %) $p < 0.01$ vs forskolin preconditioning, **Figure 10(b)**. These data suggest that deleterious effect of protein kinase A activation during adenylate cyclase mediated

preconditioning; we therefore looked at the effect of abrogation of protein kinase A activity during ischaemic preconditioning.

	Baseline		Post Treatment		Pre - ischaemia		TOC
	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	RPP (10^{-3})	CF (ml/min)	(min)
CONT	33.8 ± 2.9	11.6 ± 0.7	-	-	33.8 ± 2.9	11.6 ± 0.7	10.3 ± 0.7
IPC	34.4 ± 2.1	12.4 ± 0.8	-	-	33.2 ± 2.9	14.7 ± 0.7	4.80 ± 0.3^
$FORSK_{PC\ mimetic}$	34.7 ± 1.4	12.4 ± 0.6	44.4 ± 2.1*	15.7 ± 0.4*	32.0 ± 2.3	10.8 ± 0.6	7.5 ± 0.4
FORSK + H89	37.2 ± 2.7	12.0 ± 0.5	46.2 ± 3.9	15.9 ± 0.2*	30.4 ± 1.9	10.7 ± 0.3	7.7 ± 0.5
IPC + H89	34.0 ± 1.7	12.6 ± 0.7	35.0 ± 1.4	14.0 ± 0.6	41.0 ± 4.8	13.2 ± 0.6	6.10 ± 1.1^
$IPC + H89_{Rep.}$	33.9 ± 2.1	12.7 ± 0.6	-	-	29.5 ± 1.9	12.0 ± 0.5	5.30 ± 0.8^
$H89_{PC\ mimetic}$	37.2 + 2.0	12.2 + 0.5	37.4 + 2.1	11.9 + 0.3	35.2 + 2.3	11.7 + 0.7	9.3 + 0.4
$H89_{Pretreatment}$	32.6 ± 3.0	13.2 ± 1.5	32.0 ± 2.0	14.0 ± 1.9	32.0 ± 2.0	14.0 ± 1.9	11.0 ± 1.1

Table 4 : Heamodynamic variables from protein kinase A inhibition substudy

Baseline - at the end of stabilisation period; RPP - rate pressure product; CF - coronary flow; Post treatment - after treatment with a pharmacological agent; Pre-ischaemia - before index ischaemia; TOC - time to onset of contracture during ischaemia. CONT - control; IPC - ischaemic preconditioning; $FORSK_{PC\ mimetic}$ - forskolin given as preconditioning mimetic; FORSK + H89 - forskolin PC with H89 given before and during forskolin period; H89 was administered as a PC mimetic (H89 PC mimetic) / 3min prior to trigger ischaemia (IPC + H89) / 3min before index ischaemia ($H89_{pretreatment}$) / post trigger ischaemia before index ischaemia ($IPC + H89_{Rep}$)
Values are mean ± SEM; $n \geq 6$ in all groups. *$p < 0.05$ vs baseline; ^$p < 0.05$ vs control.

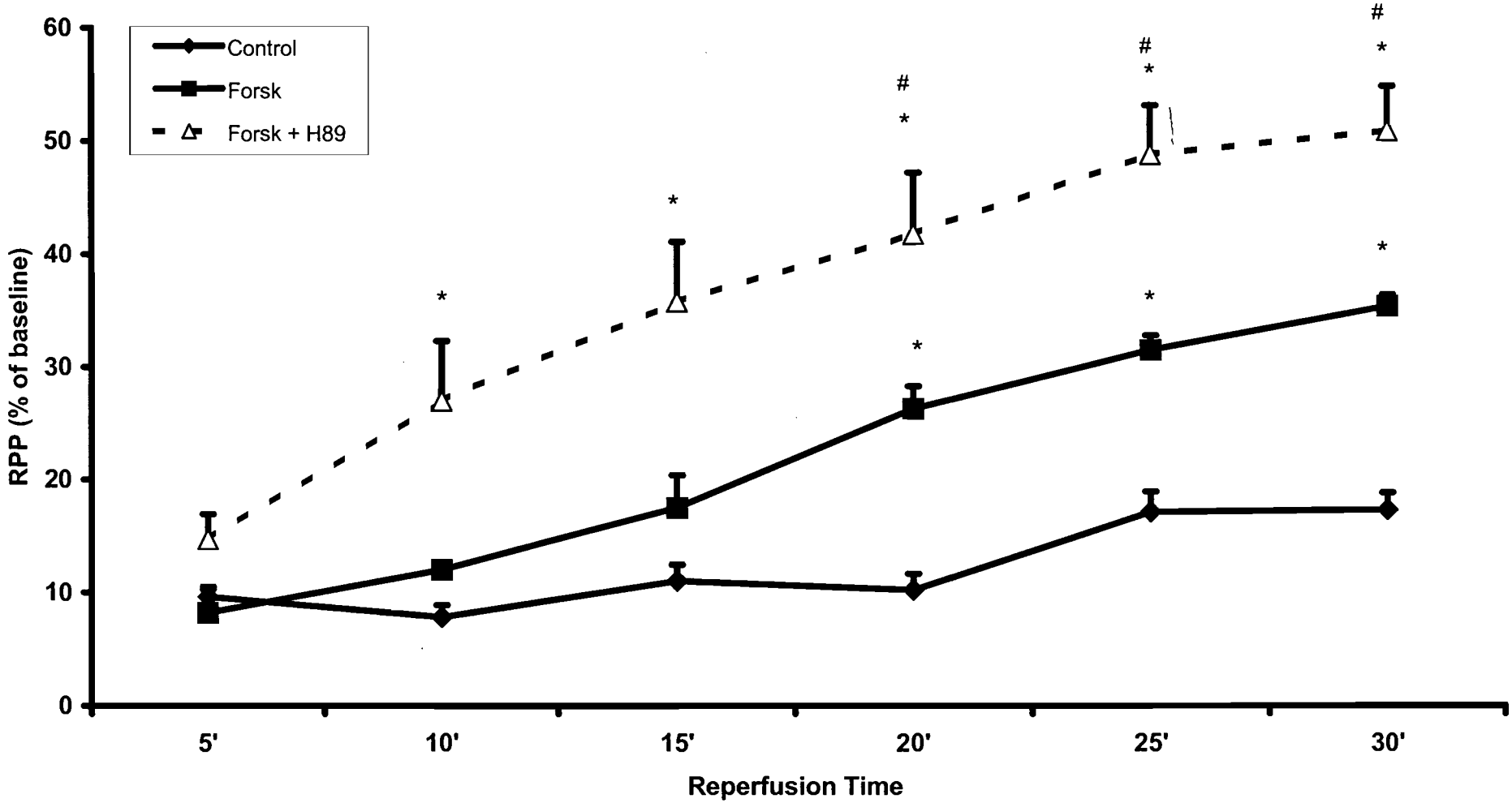


Figure 10 (b) Adenylate cyclase activator, forskolin, confers a preconditining like protection and PKA inhibitor(H89) further improves post ischaemic contractile function afforded by a single episode of forskolin preconditioning. RPP (% of baseline) = rate pressure product at 5 min intervals during 30 min reperfusion. All values mean ± SEM; $n \geq 6$ throughout. *$p < 0.01$ vs control; #$p < 0.05$ vs Forsk.

Protein kinase A inhibition on ischaemic preconditioning

When H89 was perfused prior to the single episode of trigger ischaemia (IPC 1 + H89) significant augmentation of post ischaemic contractile function was evident from the 5 min of reperfusion up to the end of the experiment. IPC 1 (45.0 ± 4.3 %) vs IPC 1 + H89 (58.0 ± 2.8 %), $p < 0.05$ at the end of the experiment, **figure10(c)**.

When H89 was given as a pretreatment to index ischaemia, post ischaemic contractile function was also significantly improved from 20min reperfusion up to the end of the experiment - $H89_{Pretreatment}$ (37.0 ± 2.1 %), $p<0.001$ vs control. IPC 1 and CONT+H89 were not significantly different at all time points IPC 1(45.0 ± 4.3 %) vs $H89_{Pretreatment}$ (37.0 ± 2.1 %), NS. When H89 was administered as a PC mimetic the post ischaemic contractile recovery was not significantly different from the control - $H89_{PC\ mimetic}$ (15.4 ± 1.5 %) vs control (14.8 ± 3.0 %). This suggests that H89 itself does not precondition, **figure10(d)**.

When H89 was administered following the trigger ischaemia before index ischaemia - IPC 1 + $H89_{Rep}$ (36.6 ± 4.4 %), did not have any effect on the ischaemic preconditioning induced cardioprotection (47.6 ± 5.0 %), **figure10(e)**. These data suggest that protein kinase A inhibition must be given prior to ischaemic preconditioning trigger to augment subthreshold ischaemic preconditioning effect or as a pretreatment against ischaemia / reperfusion insult to induce protection.

Taken together, these data suggest that single episode of ischaemic preconditioning protects against prolonged ischaemia induced contractile dysfunction. Moreover, inhibition of protein kinase A activity further enhances

the protective effects of single episode of ischaemic preconditioning and that its inhibition prior to prolonged ischaemic insult improves post ischaemic contractile function.

Having shown that PKA inhibitor, H89, was cardioprotective against ischaemia / reperfusion and augmented ischaemic preconditioning, to confirm this we measured the protein kinase A activity with or without H89.

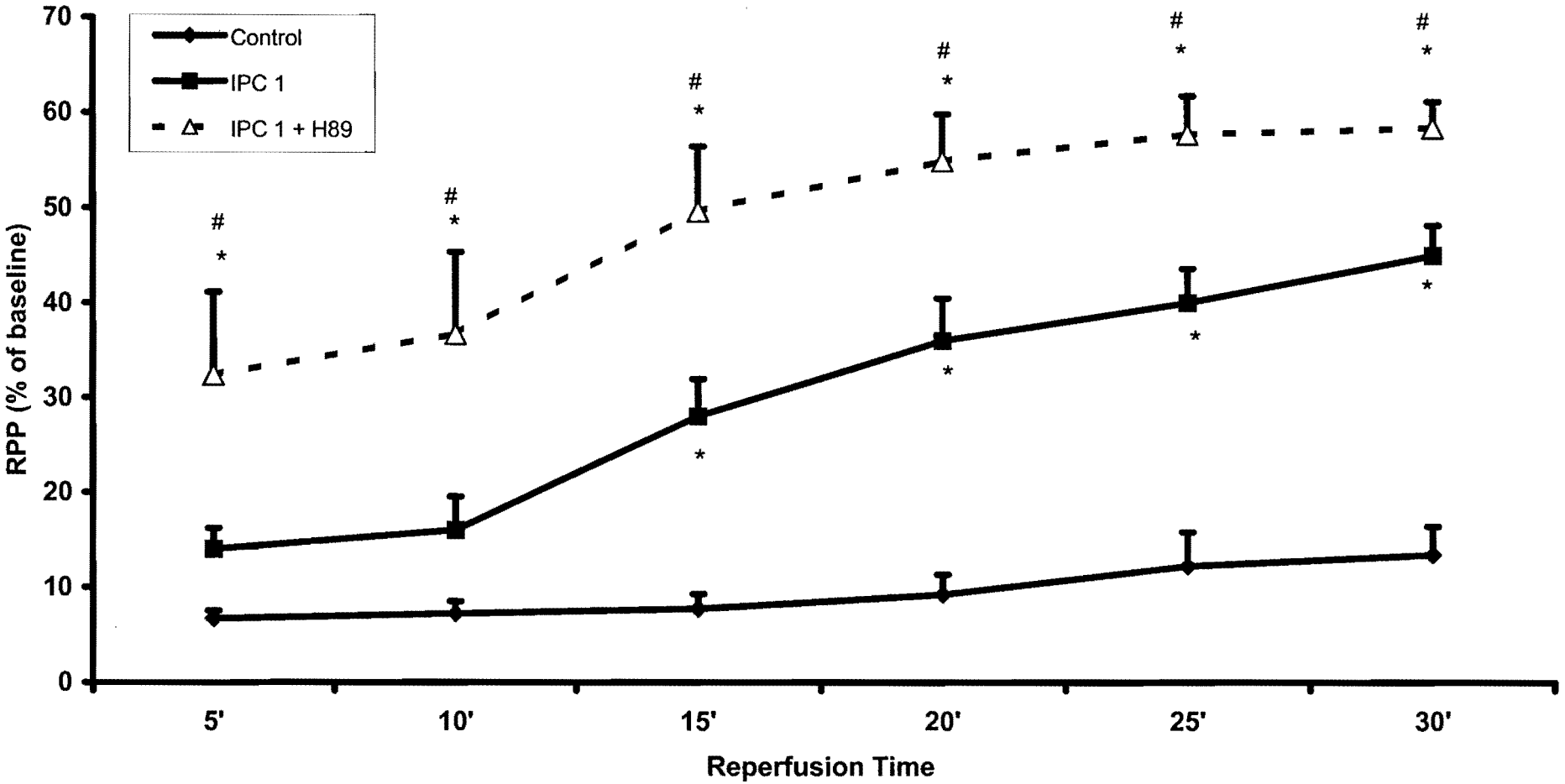


Figure 10 (c) A single episode of ischaemic preconditioning confers protection against ischaemia / reperfusion and PKA inhibitor(H89) further improves post ischaemic contractile function afforded by a single episode of ischaemic preconditioning. RPP (% of baseline) = rate pressure product at 5 min intervals during 30 min reperfusion. All values mean ± SEM; $n \geq 6$ throughout. *$p < 0.01$ vs control; #$p < 0.05$ vs IPC 1.

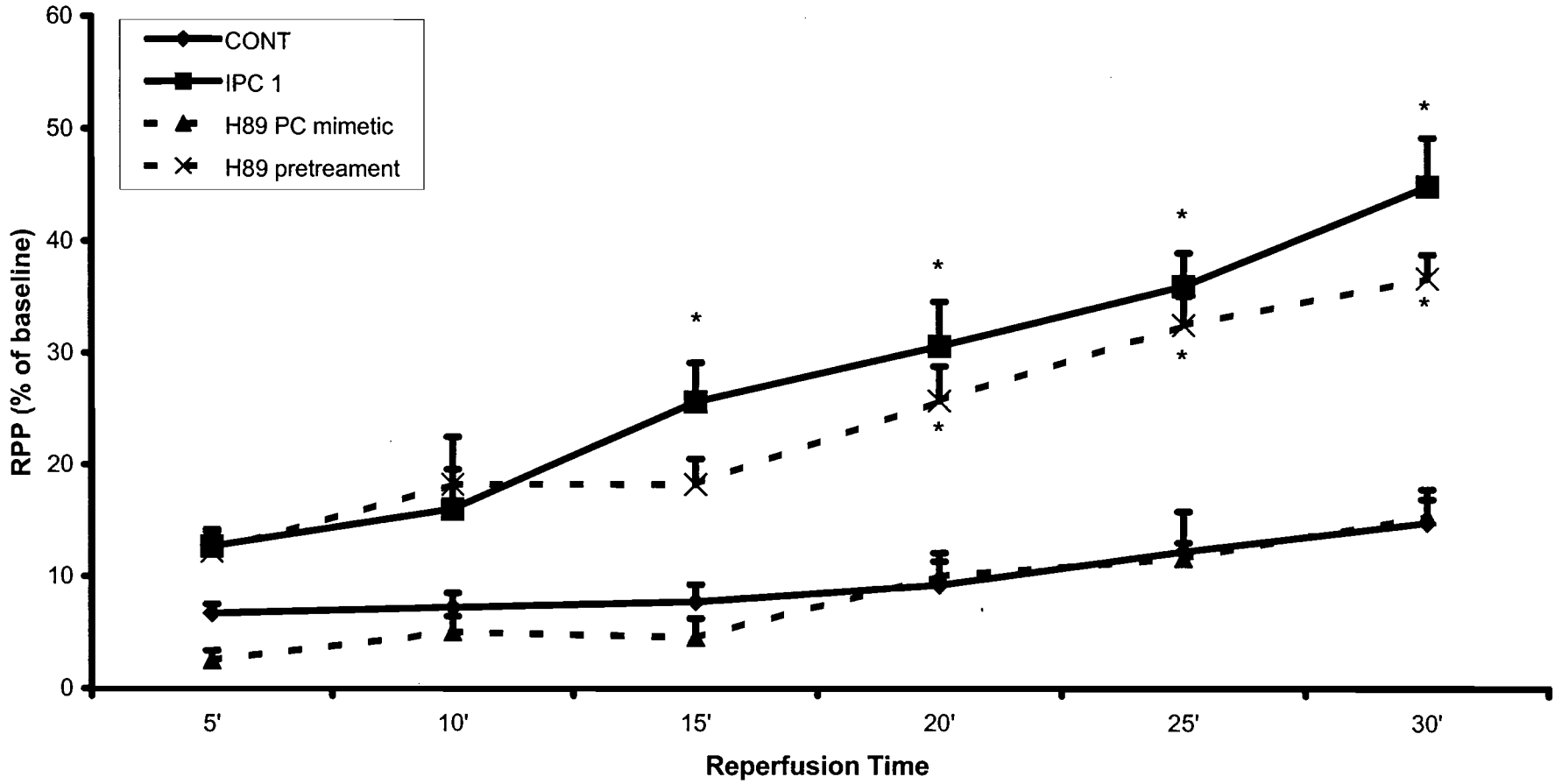


Figure 10 (d) PKA inhibitor(H89) perfused as a pretreatment improves post ischaemic contractile function and did not any significant effects on the controls when given as a PC mimetic. RPP (% of baseline) = rate pressure product at 5 min intervals during 30 min reperfusion. All values mean ± SEM; $n \geq 6$ throughout. *$p < 0.01$ vs control; IPC vs H89pretreatment, NS.

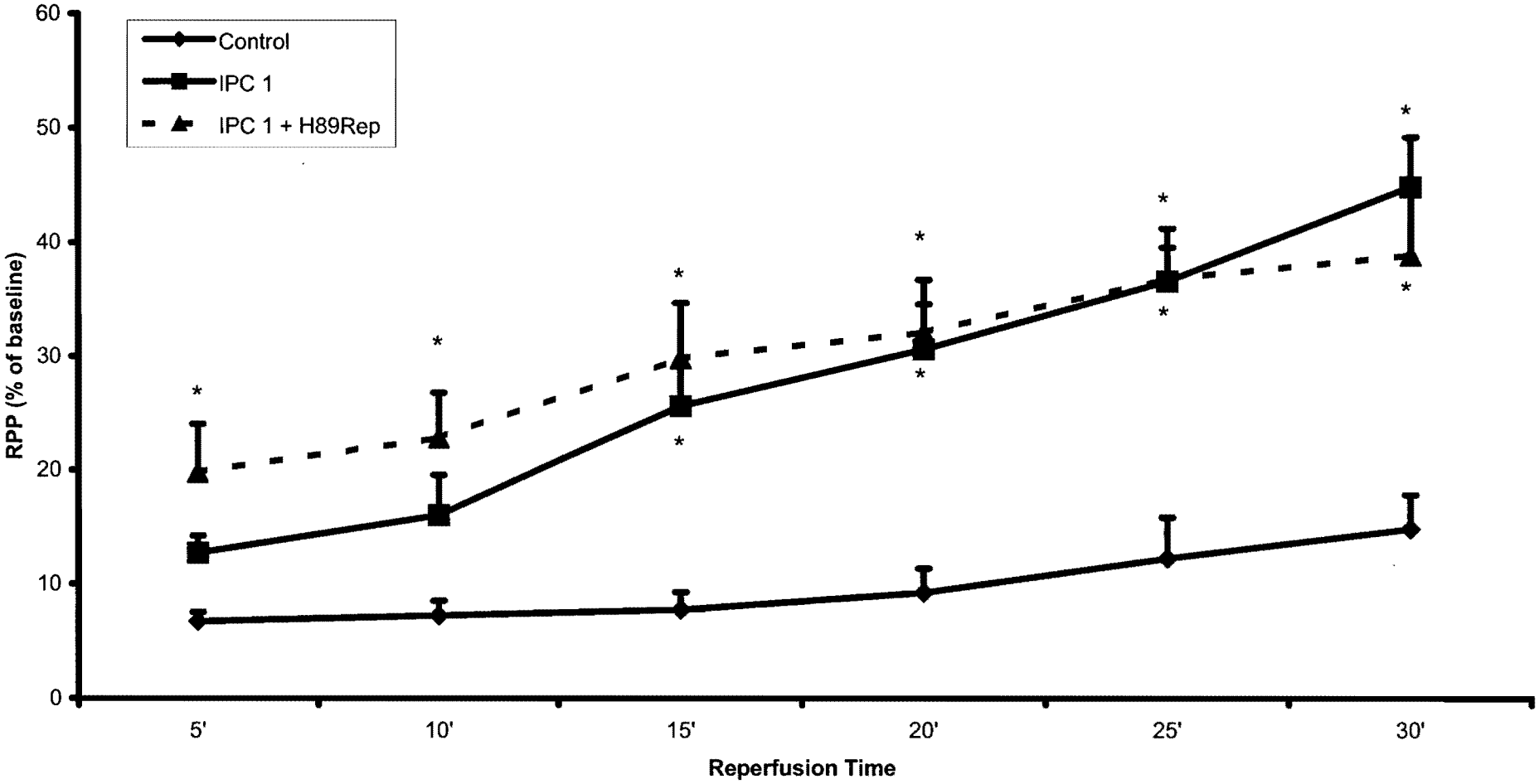


Figure 10 (e) A single episode of ischaemic preconditioning confers protection against ischaemia / reperfusion and PKA inhibitor(H89) perfusion following trigger ischaemia did not have any significant effect on post ischaemic contractile function afforded by a single episode of ischaemic preconditioning. RPP (% of baseline) = rate pressure product at 5 min intervals during 30 min reperfusion. All values mean ± SEM; $n \geq 6$ throughout.
*$p < 0.05$ vs control; IPC 1 vs IPC 1 + H89Rep; NS.

Protein kinase A activity during trigger ischaemia of preconditioning

In perfused hearts we have shown that protein kinase A inhibition protects against ischaemia-induced contractile dysfunction. Protein kinase A activity during 10 min acute myocardial ischaemia was therefore measured to correlate the inhibition of protein kinase A activity with augmentation of ischaemic preconditioning. In untreated hearts, the protein kinase A activity was significantly increased, baseline (1739 ± 190 pmol. min^{-1}. mg $protein^{-1}$) to end of 10 min trigger ischaemia (3055 ± 255 pmol. min^{-1}. mg $protein^{-1}$), $p<0.001$. Protein kinase A activity was unaffected during 3 min perfusion before ischaemia (1440 ± 255 pmol. min^{-1}. mg $protein^{-1}$ vs baseline, NS). When H89 was perfused 3 min prior to the ischaemic stimulus protein kinase A activity remained near to the baseline levels after 10 min ischaemia (1120 ± 71 pmol. min^{-1}. mg $protein^{-1}$ vs baseline, NS), **figure 10(f)**. The hearts that were subjected to ischaemic preconditioning protocol did not revert completely back to pre-IPC status following a significant ischaemic insult, however protein kinase A inhibition augmented ischaemic preconditioning induced cardioprotection suggesting that the trigger ischaemia of preconditioning comprises of two components, a protective component and a damaging component. The latter may be due to an increase in protein kinase A activity and subsequent Ca^{2+} influx during the trigger ischaemia of preconditioning. This may lead to limited ischaemic preconditioning cardioprotection. We therefore concluded that improved post ischaemic contractile function may have been due protein kinase A inhibition since we have shown that PKA inhibitor (H89) blocked the ischaemia-induced rise in PKA activity.

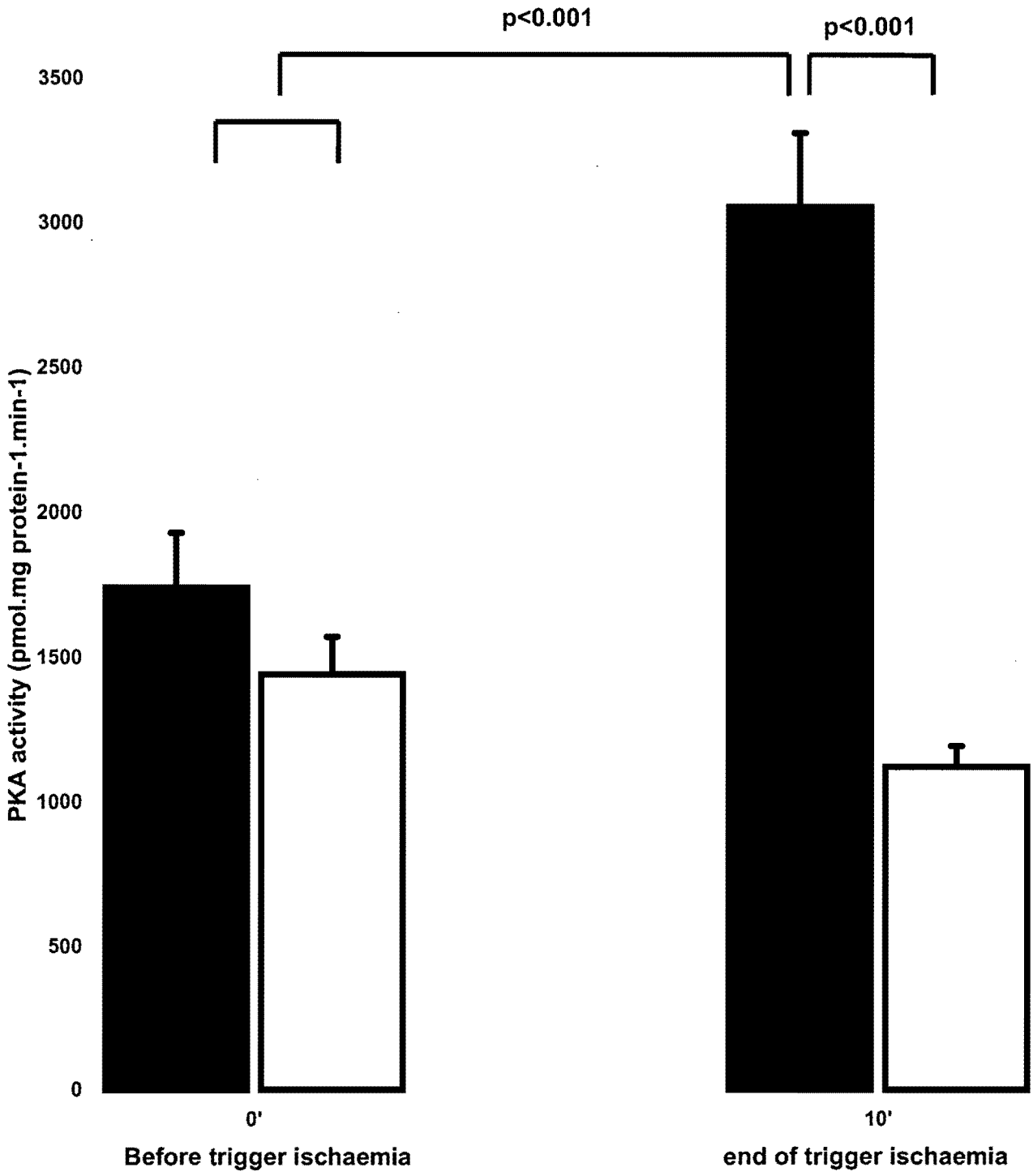


Figure 10(f) Total activated cAMP dependent protein kinase (PKA) activity. In untreated hearts (black bars), PKA activity was significantly increased at the end of 10 min trigger ischaemia. In H89 treated hearts (white bars), PKA activity was significantly reduced to the baseline levels at the end of 10 min trigger ischaemia. $p < 0.001$ vs baseline.

5

DISCUSSION

5. DISCUSSION

The most important findings of this study are: 1) transient glucose deprivation induced protection on post ischaemic cardiac function appears to be through adenosine receptor activation, stimulation of the downstream signal protein kinase C (PKC) and subsequent opening of the mitochondrial ATP sensitive potassium (mito K_{ATP}) channels. 2) ischaemic preconditioning can be augmented by inhibiting protein kinase A activity during the trigger of preconditioning.

5.1 Metabolic considerations and cardiac preconditioning

Glucose serves as an important substrate for generation of energy in the heart. The heart utilises energy in the form of adenosine triphosphate (ATP). Under normal aerobic conditions the heart generates ATP from fatty acid and glucose metabolism and under ischaemic conditions glucose serves as the most important supply of energy. When oxygen is abundant, fatty acid metabolism produces more ATP than glucose, however, when oxygen is rate limiting more ATP is generated, mole per mole by glucose compared to fatty acids. Glycolysis is a catabolic process that converts glucose into pyruvate. Each molecule of glucose converted to pyruvate produces a net of 2 ATP[4]. When glucose is limiting, intracellular stores of glycogen serve as the source of glucose[65], glycogen is then converted to glucose in a process called glycogenolysis.

In the context of preconditioning the potential role that may be played by these 3 substrates (glucose, glycogen and fatty acids) is discussed below.

Inhibition of glycolysis and cardiac preconditioning

Glycolysis is known to be inhibited by ischaemia, pyruvate or acetate[54, 66, 67], 2-Deoxyglucose (nonmetabolisable analogue of glucose)[68], octanoate[65, 69, 70] and glucose depletion[10]. A brief exposure to some of the above metabolic perturbations has been shown to confer a preconditioning-like cardioprotection. The mechanism for this preconditioning-like cardioprotection is hypothesised to be through inhibition of glycolysis.

In our study, transient inhibition of glycolysis alone by a glycolytic inhibitor, 2-Deoxyglucose, in the presence of glucose containing perfusate, produced no improvement of post ischaemic contractile function although glycolytic blockade appeared evident as demonstrated by a non-reversible depression of cardiac contractile function during and after administration of 2DG. Although we did not perfuse 2DG in the absence of glucose, a study by Wu[71] demonstrated that glucose depletion in the presence of 2DG still conferred cardioprotection in rat ventricular myocytes. We, therefore, perfused with pyruvate, a metabolic substrate known to indirectly inhibit glycolysis, in the absence of glucose. Pyruvate abolished glucose deprivation mediated protection. This may have been due to the ability of pyruvate to increase the cytosolic ATP above that of fatty acid fuels[53] thereby increasing mitochondrial ATP levels[72]. This increase in mitochondrial ATP levels is may block mitochondrial ATP sensitive potassium channels hence the blockade of glucose deprivation preconditioning. Taken together, these data do not support a direct effect of glycolytic inhibition in the preconditioning-like cardioprotection programme afforded by transient glucose deprivation.

Conversely, de Jonge[28] found that short ischaemic episodes afford protection through enhanced glycolysis. ATP generated during anaerobic glycolysis protects sodium pump and the calcium uptake pump of the sarcoplasmic reticulum in ischaemic cells[65]. In addition, it has been suggested that glycolysis shutdown in ischaemic cells may have deleterious effects including acidosis[73], ATP breakdown[4], excess influx of calcium[4, 49], accumulation of fatty acids[74] and proton accumulation. However, the inhibited glycolysis during a preconditioning stimulus and during prolonged ischaemia may have different effects.

The role of glycogen in cardiac preconditioning

A role for glycogen in preconditioning is controversial. Some investigators have shown that glycogen depletion can be induced by brief periods of ischaemia[73, 75] by brief periods of anoxia[73], following epinephrine infusion[76], pyruvate perfusion[77] and by glucose deprivation[10] confer ischaemic preconditioning-like cardioprotection. This cardioprotection through glycogen depletion is thought to be due to resultant reduction in lactate production and proton accumulation[78-80], via activation of Na^+/H^+ and Na^+/Ca^{2+} exchange and by decreasing acidosis[81]. In contrast, others have shown that protection against ischaemia requires glycogen availability[73, 82-88], whilst others suggest that glycogen depletion by itself is not effective enough in protecting against ischaemic injury[89].

In this study, we have shown that hearts subjected to transient glucose deprivation developed an earlier ischaemic contracture compared to glucose

perfused hearts. As previously shown by our laboratory, earlier onset of ischaemia contracture is associated with glycogen depletion[82]. Thus as could be expected, our data suggest that the transient glucose depletion used prior to the index ischaemic insult accelerated glycogen depletion. However, as we did not measure glycogen levels in our study, we cannot directly evaluate or comment on what potential role glycogen depletion played in the glucose deprivation cardioprotection.

Fatty acid metabolism / mobilisation and cardiac preconditioning

Brief myocardial ischaemia stimulates moderate accumulation in fatty acids and severe cases of ischaemia lead to further increased accumulation of fatty acids[74]. Increased intracellular fatty acids during ischaemia may lead to imbalance of substrates and accumulation of unoxidised products of fatty acids[90]. High concentrations of fatty acids are said to disrupt cellular processes by causing non-specific detergent action on biomembranes[74], inhibition of enzymes[91], uncoupling of oxidative phosphorylation in mitochondria[92], stimulation of mitochondrial ATPase[93] and development of ventricular arrhythmias in dogs[94].

Although fatty acid accumulation has detrimental effects on the heart, our laboratory has shown that uncoupling of the oxidative phosphorylation in mitochondria is one of the mechanisms by which ischaemic preconditioning manifests its cardioprotective effects[95]. In addition, fatty acid accumulation inhibits glycolysis[65], which, as previously mentioned, has been found to be cardioprotective. Hence, we hypothesised that enhanced fatty acid

mobilisation may be cardioprotective during transient period of glucose deprivation. To investigate the role of enhanced fatty acid mobilisation glucose perfused hearts were subjected to 15 min of excess medium chain fatty acid, octanoate, perfusion followed by fatty acid free period before subjected to index I/R. There was no loss of cardiac function during octanoate perfusion and there was no improved recovery. A combination of octanoate and glucose deprivation period had no effect or additional cardioprotective effect compared to glucose deprivation protection alone. This suggests that enhanced fatty acid mobilisation is not involved in the glucose deprivation protection.

Following all the metabolic perturbation experiments, we concluded that the protective effect of glucose deprivation is not likely to be due to inhibition of glycolysis alone nor via fatty acid mobilisation. We therefore began to evaluate the possible cell signalling pathways that may be activated by transient glucose deprivation. We postulated that one or more of these signalling pathways might activate a preconditioning-like cardioprotective programme in response to glucose deprivation.

5.2 Cellular signalling and cardiac preconditioning

Neuronal noradrenaline release and cardiac preconditioning

Glucose deprivation is a metabolic stress and it may trigger the release of noradrenaline. Periods of ischaemia or anoxia are believed to result in the release of endogenous noradrenaline through nonexocytotic process from the neurons[48,96]. Noradrenaline release acts via α-adrenergic[97] or β-adrenergic pathways[2, 97]. Transient activation of any of these two pathways has been shown to mimic ischaemic preconditioning[2, 22, 47, 58, 97]. In our model, glucose deprivation protection was unchanged by the administration of α-adrenergic or β-adrenergic antagonists.

In line with our findings, α-adrenergic blockade with prazosin[59, 98] phenoxybenzamine[99] or bunazosin[100], when administered up to the onset of index I/R failed to prevent the protective effects of ischaemic preconditioning or transient β-adrenergic stimulation mediated preconditioning. Prazosin also failed to abolish ischaemic preconditioning effects[98] even when washed out before index I/R[59]. In contrast with our findings prazosin attenuated the cardioprotective effects of preconditioning in dog[101], this may have been due to differences in species used. Additionally, pretreatment with α-adrenergic antagonists conferred cardioprotection in the ischaemia / reperfusion control hearts in our studies. In line with this, Nayler[57] found that pretreatment with different concentrations of prazosin (1, 5 or 10μM respectively) was protective against ischaemia / reperfusion insult. However, bunazosin or phenoxybenzamine had no protective effect when given as a pretreatment to index I/R.

On the other hand, glucose deprivation preconditioning was not affected by β-adrenergic blockade with propranolol, likewise, Yabe[102] found that β-adrenergic blockade with timolol failed to abolish ischaemic preconditioning when perfused up to the onset of index I/R but abolished isoproterenol preconditioning. When propranolol was administered as a pretreatment it was cardioprotective against ischaemia / reperfusion insult. Reimer[103] also found that preatreatment with propranolol followed by coronary occlusion reduced necrosis in dogs. Contrary to this, pretreatment with β-adrenergic blocker, timolol, did not have any cardioprotective effect against ischaemia / reperfusion insult.

The cardioprotective effects of α-adrenergic or β-adrenergic antagonists pretreatment may have been due to decreased energy demand or attenuated beta-adrenergic response[59] or reduction in myocardial ischaemia / reperfusion induced Ca^{2+} overload prior to and / or during sustained ischaemia and during reperfusion[104].

Despite the controversies that appear to hinder the unravelling of the exact mechanisms of α-adrenergic or β-adrenergic signalling in preconditioning, we do not think that the failure of α-adrenergic or β-adrenergic blockade to abolish glucose deprivation preconditioning was due to administration of the antagonists up to the onset of index I/R. Moreover, due to the cardioprotective effect of α-adrenergic and β-adrenergic antagonists observed in ischaemia / reperfusion control hearts, we cannot exclude a protective role of activated adrenergic receptor pathway during glucose deprivation protection.

Adenosine production and cardiac preconditioning

Adenosine is an important nucleoside known to have homeostatic and protective effects on the heart[27]. Ischaemia, anoxia or metabolic inhibition lead to the production of adenosine from ATP breakdown[27, 105, 106]. Transient adenosine administration has been shown to mimic ischaemic preconditioning through activation of adenosine (A_1 and/or A_3) receptor types[105]. Although both receptor types are involved in preconditioning A_1 receptor has been thoroughly investigated. Adenosine A_1 receptor agonists, given as a trigger of ischaemia, confer cardioprotection against sustained ischaemia[106, 107].

This adenosine-induced cardioprotection may act in various ways. These include decreased catecholamine-induced metabolic and contractile responses[108], inhibition of norepinephrine release and/or reduction of intracellular calcium concentrations during ischaemia[27, 109], activation of PKC and subsequent opening of mito K_{ATP} channels via Gi protein activation[27, 105, 110-112].

In our study, we used glucose deprivation as a trigger of cardioprotection. Parallel to other studies, a non-selective adenosine receptor antagonist (SPT), abolished protection afforded by glucose deprivation. Although SPT is non-selective and cannot permeate the cell membrane we used sufficient dose (100μM) to block receptor activation (IC_{50} values for A_1 and A_2 are 4.3μM and 6.2μM, respectively)[55]. To confirm this we preconditioned with adenosine and abolished the protective effects of adenosine preconditioning with SPT. In line with our findings, ischaemic

preconditioning has been abolished by adenosine receptor blockade[98], suggesting that metabolic and ischaemic preconditioning involves adenosine receptor activation. Contrary to our findings, Goto[10] did not abolish protection induced by glucose deprivation with SPT. This finding may have been due to the different animal model and / or end-point used. In addition, Miura[105] argue that the extent of cardioprotection is mainly determined by interstitial adenosine levels achieved during ischaemic preconditioning and not by the level during sustained ischaemia. We therefore concluded that transient glucose deprivation leads to adenosine production, which as shown previously[27], plays an important role in preconditioning-like cardioprotection.

Role of protein kinase C in cardiac preconditioning

One of the important downstream signalling events that has been found to be involved in cardioprotection against ischaemic injury is the activation of protein kinase C. Various cell surface G_i - protein coupled receptors, including the α - adrenergic, adenosine and opioids facilitate the translocation of protein kinase C (PKC) from the cytosol to the sarcolemma[29, 105]. Translocation of protein kinase C results in the opening of the sarcolemmal and mitochondrial (K_{ATP}) channels[60, 105] and activation of other kinases like tyrosine kinase and MAP kinases[15]. Protein kinase C agonists like phorbol myristate acetate or a diacylglycerol analogue have been shown to mimic ischaemic preconditioning[32, 113]. Although ischaemic preconditioning seems to be dependent on PKC translocation, not all isoforms of PKC elicit cardioprotection when activated. The key role players appear to be PKC α, δ

or ε isoforms[114-117]. PKC α and ε are translocated to the plasma membrane and PKC δ is translocated to the mitochondria[30, 115] but some investigators[118, 119] believe that all three isoforms are translocated to the plasma membrane. Although, all of the three PKC isoforms are translocated by ischaemic preconditioning, PKC α rapidly dissociates from plasma membrane while PKC δ and ε are retained for a long time[116]. This evidence shows that PKC translocation as an important mediator of ischaemic preconditioning, we therefore looked at the role of PKC activation in the metabolic preconditioning model.

In our study we have shown that a selective PKC antagonist, chelerythrine, (shown to inhibit all PKC isoforms) abolished glucose deprivation protection, suggesting that transient period of glucose inhibition lead to activation of PKC. Thus, in line with other PKC studies PKC activation has shown to be pivotal to the glucose deprivation cardioprotection[10, 71] and in ischaemic preconditioning[29, 99]. We therefore concluded that glucose deprivation protection acts, in part, via PKC activation. In our model, we did not delineate the putative isoforms activated, however we postulated that the PKC δ and ε isoforms are involved.

The likely end effector of cardiac preconditioning

The activation of PKC and the resultant opening of the K_{ATP} channels has been considered as an important end-effector of ischaemic preconditioning. Moreover, it has been found that, pharmacological agents that block the opening of mito K_{ATP} channels block ischaemic preconditioning[38]. Although

there are still uncertainty about the mechanism by which the opening of mito K_{ATP} channels confer cardioprotection, some consequences of mito K_{ATP} channel opening have been suggested. These consequences include mitochondrial swelling, membrane depolarisation, perturbations in ATP synthesis, and in mitochondrial respiration[35, 36]. Interestingly, mitochondrial swelling is known to augment ATP production[37], this may be an adaptive mitochondrial response to cellular stress and/or to prevent Ca^{2+} overload[36]. These may be the mechanisms by ischaemic preconditioning confer cardioprotection against ischaemic insult.

In this study we looked at the possible role of mito K_{ATP} channel in glucose deprivation protection. We found that glucose deprivation protection was abolished by mito K_{ATP} channel inhibitor, 5-hydroxydecanoate. This is parallel to the findings that opening of mito K_{ATP} channels is involved in the mechanism by which preconditioning affords cardioprotection[25, 38, 52, 120]. According to our knowledge there is no work that has linked glucose deprivation preconditioning to the opening of mito K_{ATP} channels.

Other studies have linked ischaemic preconditioning cardioprotection to adenosine receptor activation, protein kinase C activation and subsequent opening of mito K_{ATP} channels. We therefore conclude that transient glucose deprivation mimics ischaemic preconditioning through adenosine receptor activation, protein kinase C activation and the subsequent opening of mito K_{ATP} channels.

5.3 Augmented preconditioning by inhibition of protein kinase A activity.

Involvement of β-adrenergic receptor pathway in cardiac preconditioning

The trigger ischaemia of preconditioning has been shown to protect the isolated rat heart against post-ischaemic contractile dysfunction[18, 52]. As previously shown in this study, one mechanism of ischaemic preconditioning involve the activation of protein kinase C and opening of the mitochondrial ATP-sensitive potassium channels. On the other hand, it is believed that periods of ischaemia or anoxia lead to stimulation of β-adrenergic pathways[2, 97, 121] and subsequent accumulation of Ca^{2+} [122]. Other studies have shown that transient β-adrenergic stimulation protects against prolonged ischaemia[2, 97, 100]. Other studies showed that transient β-adrenergic stimulation cardioprotection induces protein kinase C activation via an increase in the intracellular Ca^{2+} concentration[19, 102]. Moreover, Strasser[56] showed that independent activation of protein kinase C during short ischaemic episode stimulates adenylate cyclase, this activation occurred rapidly after the onset of ischaemia (5-15 minutes), therefore suggesting possible involvement of adenylate cyclase activation in cardioprotection. In this study we have shown that transient activation of adenylate cyclase, shown to increase the cAMP levels[2, 56], conferred a preconditioning-like cardioprotection. Suggesting a possible involvement of β-adrenergic receptor induced activation of adenylate cyclase during ischaemic preconditioning trigger as a mechanism whereby ischaemic preconditioning affords cardioprotection. However, Sandhu[1]

showed that decrease in adenylate cyclase activation and subsequent increase in cAMP levels during sustained ischaemic insult following three ischaemic episodes did not account for protection against myocardial infarction.

In contrast, other studies have shown that transient attenuation of β-adrenergic receptor activation preconditions the heart against sustained ischaemia[59]. In support of this concept, it is believed that adenosine released[27, 109], activation of G-inhibitory protein by opioids[25, 71, 123, 124] during ischaemic trigger of preconditioning protects the heart by attenuating the adverse effects of β-adrenergic receptor stimulation.

Although there is a controversy about the possible involvement β-adrenergic pathway stimulation, we have found that transient activation of adenylate cyclase by forskolin protects the isolated rat hearts against ischaemia / reperfusion insult. This controversy does not bring into the picture the possible role of protein kinase A activation during ischaemic preconditioning. Secondly we observed that the mechanical function of the hearts subjected to ischaemic preconditioning did not revert completely back to baseline mechanical function following the index ischaemic insult, hence there is still room for further protection of these hearts.

The possible role of protein kinase A in cardiac preconditioning

Myocardial ischaemia and pharmacological activation of adenylate cyclase lead to an increase in the cAMP levels[56] and the activation the cAMP dependent protein kinase (PKA)[4]. However, the levels of cAMP decline with the number of short ischaemia / reperfusion episodes[1, 2] and ischaemic

preconditioning protection improves with the number of cycles[3]. Thus protein kinase A inhibition may contribute to improved post ischaemic contractile recovery after multiple episodes. In this study, we looked at the effects of concurrent inhibition of protein kinase A activation in both ischaemic and pharmacologically induced preconditioning. In this study, when H89 (PKA inhibitor) was perfused concurrently with adenylate cyclase activator (forskolin preconditioning) it augmented the functional recovery achieved with forskolin preconditioning. We therefore perfused H89 prior to the trigger of ischaemic preconditioning. Again, H89 augmented ischaemic preconditioning and when H89 was perfused as a pre-treatment it protected the same way as ischaemic preconditioning. From the protein kinase A activity data, this was found to be due to reduced protein kinase A activity during ischaemic trigger of preconditioning. Although the mechanisms through which inhibition of protein kinase A activity protects is not known exactly, we hypothesised that cardioprotection may be due to an inactivation of L-type Ca^{2+} channels and subsequent decrease in ischaemia-induced cytosolic free Ca^{2+} accumulation. In line with our findings, Steenbergen[122] showed that there is an elevation of cytosolic free calcium concentration early in myocardial ischaemia, moreover, Miyawaki[19] showed that transient Ca^{2+} depletion followed by Ca^{2+} replenishment protects against ischaemic injury, this may therefore account for the damaging component of preconditioning ischaemia.

We demonstrated that three episodes of ischaemic preconditioning elicited greater post ischaemic contractile recovery than one cycle and that the protective effects of sublethal ischaemic and/or pharmacological preconditioning on post-ischaemic contractile function can be further

augmented by protein kinase A inhibition. We therefore conclude that increased PKA activity during the sublethal episode of preconditioning may limit the post ischaemic contractile recovery. Although we conclude that ischaemic preconditioning induced protection can be further augmented by blocking the PKA activation, it is important to mention that β-adrenergic signalling cascade is an essential trigger of ischaemic preconditioning since we have shown that adenylate cyclase activator, forskolin, preconditions and that β-adrenergic blockade abolishes protective effects of preconditioning [2].

6

CONCLUSION

6. CONCLUSION

We have found that transient glucose deprivation preconditions the heart through adenosine receptor induced protein kinase C activation and subsequent opening of the mitochondrial ATP sensitive potassium channel. However, protein kinase C activation during transient glucose deprivation is unlikely to be mediated via α_1 / β - adrenergic receptor activation. In addition, inhibition of protein kinase A activity further enhances the protective effects of subthreshold episode of preconditioning on post ischaemic contractile function. Suggesting that the trigger ischaemia of preconditioning may be limited by activation of protein kinase A.

7

IMPLICATIONS & UNANSWERED QUESTIONS

7. IMPLICATIONS AND UNANSWERED QUESTIONS

In the first part of this study we looked at the effect of transient glucose deprivation on post ischaemic contractile function. During glucose deprivation period and during administration of α_1 and β blockers (prazosin and propranolol, respectively) the contractile function dropped significantly (see tables 1-3). Decrease in cardiac function may lead to decrease in energy demands leading to protection against myocardial infarction[125], amelioration of left ventricular dysfunction[126-128] and protection against ischaemia induced arrhythmias and fibrillation[129]. We do not know if this decrease in contractile function played any significant role in cardioprotection.

In GLU(-) hearts prazosin and propranolol was given up to the onset of index I/R because some investigators predict that the protective effects of preconditioning stimulus/ stimuli like activation of PKC[116] and subsequent activation of MAP kinases[130] and tyrosine kinase[131, 132] remain activated after preconditioning stimuli and probably carried up to the index ischaemia where they effect protection. Kawamura[116] showed that PKC δ and ε (PKC isoforms regarded as key role players in preconditioning) are still highly activated 10 min later after preconditioning stimulus hence it is important to block PKC after preconditioning stimulus[32]. To prevent any possible alpha and/or beta adrenergic induced activation of PKC, MAP kinases and / or tyrosine kinase during the washout period we administered antagonists up to the onset of index I/R {Figures 8(a) and 8(b); Pgs. 61 and 62}.

Concerning the concentration of prazosin: Nayler[57], showed that different concentrations of prazosin (1, 5 & 10 μM) were protective against index I/R and found 1μM prazosin to be optimal but the same concentration failed to abolish ischaemic preconditioning protection[59]. Additionally, they showed that 90 min perfusion with different concentrations of prazosin (1, 5 & 10 μM) did not have any significant differences on ATP, CP and Ca^{2+} levels. We therefore think that concentration used in this study was probably sufficient to block alpha receptor activation.

Possible activation by β-adrenergic receptor activation and its effectcs on cardioprotection - it has been shown that β-adrenergic receptor activation may lead to activation of PKC via transient elevation of Ca^{2+}. Transient β-adrenergic receptor activation by isoproterenol led to translocation of PKC δ from the cytosol to membrane fraction (marker of PKC activation)[102]. Moreover, Miyawaki[133] showed that isoproterenol preconditioning led to transient elevation of Ca^{2+} and subsequent activation of PKC. Additionally, they showed that β-adrenergic receptor blocker, propranolol, and PKC inhibitor, chelerythrine, completely abolished cardioprotective effects of isoproterenol preconditioning. Therefore the aim of the study was to block any possible β-adrenergic receptor-induced activation of PKC, that may have occurred during GLU(-) {Figure, 4(a); Pg. 35}

PKA inhibition substudy - In the second part of our study we found that pharmacological preconditioning with adenylate cyclase activator, forskolin improves left ventricular contractile dysfunction. During forskolin perfusion there was a significant increase in the contractile function (both heart rate and the developed pressure). Some investigators have shown that a transient increase in cardiac function confers cardioprotection. Cell stretching or acute volume overload[134] and rapid cardiac pacing[135, 136], bypassing β adrenergic pathway stimulation, have been shown to protect the heart against ischaemic injury and myocardial infarction. We do not know whether forskolin preconditioning was due to β adrenergic pathway stimulation alone or merely due to increased heart rate and / or developed pressure.

Specificity of protein kinase A inhibitor, H89

The evidence for protein kinase A inhibition by H89 was provided by direct measurements of protein kinase A activity {Figure 10(d)}. However, the concentration of 2μM H89 used in this study may also inhibit cGMP protein kinase G (PKG)[62]. The possible involvement of PKG in this study is unlikely due to the fact that cyclic GMP and cGMP kinase have been shown to elicit protective effects[137, 138]. Inhibition of PKG would hypothetically lessen rather than augmenting ischaemic preconditioning protection. In addition, a closely related protein kinase A inhibitor, H-8 which is 30 times less potent than H89, has been shown to reduce the incidence of torsdes de pointes in rabbits[139], yet H89 inhibits PKG 10 times less[62]. We therefore conclude that our data relate to predominant inhibition of protein kinase A.

8

FUTURE DIRECTIONS

8. FUTURE DIRECTIONS

Glucose deprivation and metabolic pathways

In the first part of our study we looked at metabolic versus signalling events happening during glucose deprivation. In metabolism study we did not measure glycogen levels, rates of glycolysis nor rates of fatty acid oxidation. All these metabolic events may have played a significant role in glucose deprivation protection. We therefore aim to measure glycogen levels, rates of glycolysis and rates of fatty acid oxidation to get insight into these metabolic consequences.

Endpoint of ischaemic preconditioning

The end-point of ischaemic preconditioning used in this study is post ischaemic contractile dysfunction. Although post ischaemic contractile function is now considered as an alternate end point (because it is likely be a mixture of stunning, hibernation and apoptosis / necrosis). Investigators do, however, use ischaemic preconditioning to improve post ischaemic contractile dysfunction[2, 18, 52]. Several end-points are accepted, yet the measurement of infarct size remains the gold standard in both in vitro and in vivo models[140]. Additionally, as opposed to contractile dysfunction β adrenergic pathway may not play role in infarct size reduction[1]. As cell survival is the paramount endpoint we plan to use molecular manipulations in vitro to investigate the regulation of protein kinase A in cardiomyocytes or skeletal muscles (C_2C_{12}) cells in the context of preconditioning. Further studies are necessary to investigate the role of protein kinase A inhibition in reducing stunning, hibernation, apoptosis and / or necrosis. We do not know if protein

kinase A inhibition augmented ischaemic preconditioning by further reducing stunning / hibernation and decreasing apoptosis / necrosis.

Cyclic AMP / protein kinase A measurements

We have already shown that H89 inhibits protein kinase A activity during ischaemic preconditioning trigger and that augments preconditioning. To further investigate the harmful effect of index ischaemia that limits preconditioning, we plan to maximally activate cAMP / PKA with adenylate cyclase activator, forskolin and then measure the levels of cAMP during ischaemic trigger of preconditioning. Additionally, we are going to measure both cAMP levels and protein kinase A activity at the end of index ischaemia and compare untreated hearts and H89 treated hearts. This will allow us to further explore the newly discovered concept of ischaemic preconditioning augmentation.

9

PUBLICATIONS

9. PUBLICATIONS

ABSTRACTS

M.M. Awan, Siyanda Makaula, Sara Foresti, L.H. Opie; Metabolic Preconditioning: Substrates and Signalling, Circulation (1999) **100**, No. 18 (Supplement 1), p 717.

M.M. Awan, Siyanda Makaula, D.M. Yellon, L.H. Opie; Acute myocardial ischaemia lessens the potential protection afforded by ischaemic preconditioning, European Heart Journal (1999) **20**, (Abstract Supplement 1), p 167.

Siyanda Makaula, M.M. Awan, M.N. Sack, L.H. Opie; H-89, an inhibitor of PKA activity, enhances protective effect of ischaemic preconditioning of recovery of contractile function in isolated rat hearts. American Heart Association (2000), New Orleans, USA.

Siyanda Makaula, M.M. Awan, M.N. Sack, L.H. Opie; H-89, an inhibitor of PKA activity, enhances protective effect of ischaemic preconditioning of recovery of contractile function in isolated rat hearts. European Society of Cardiology (2000), Amsterdam, Netherlands.

MANUSCRIPTS

M. Moneeb Awan, Siyanda Makaula, Sara Foresti, Michael Sack and Lionel H. Opie; Mechanisms whereby glucose deprivation triggers metabolic preconditioning in the isolated rat heart, Molecular and Cellular Biochemistry Journal, ***in press***.

Siyanda Makaula, M. Moneeb Awan, Amanda Lochner, Michael Sack and Lionel H. Opie; H89, an inhibitor of protein kinase A activity, enhances protective effect of ischaemic preconditioning on recovery of contractile function in isolated rat hearts, in preparation for publication in Circulation Journal.

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