

**MYOCARDIAL ENDOGENOUS  
ADAPTATION FOLLOWING  
ISCHAEMIC PRECONDITIONING**

**Studying the second window of protection**

**Ph.D Thesis  
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## 1. INTRODUCTION

### 1.1 Classic ischaemic preconditioning:

Ischaemic preconditioning (IPC) describes the endogenous adaptation of the myocardium and was first described by Murry *et al.* as increased myocardial tolerance to a severe ischaemic insult that follows short bouts of non-lethal ischaemia-reperfusion cycles. Now termed classic preconditioning, it appears to be an acute and immediate response lasting not more than a few hours. The protection has been evoked by various preconditioning protocols and tested using different endpoints such as limitation in infarct size, reduced susceptibility to arrhythmia, better recovery from contractile dysfunction and cardiac enzyme release.

The intensive investigations that followed the original report by Murry have led to the emergence of several hypotheses regarding the mechanism behind this complex phenomenon. Likely triggers include adenosine, catecholamines, bradykinin, opioids, oxygen free radicals (OFR), and nitric oxide. Most act through 7 trans-membrane receptor, lead to phospholipase C or D (PLC or PLD) activation, which in turn generates diacylglycerol (DAG). DAG may then act on various isoenzymes of protein kinase C (PKC). It is believed that PKC activation and translocation is an important denominator in the preconditioning cascade in a number of species. Another kinase that may be acting either downstream of, or in concert to PKC, is protein tyrosine kinase (PTK). It is believed that PKC and PTK phosphorylate other kinase, including p38 mitogen activator protein kinase (p38 MAP kinase), and eventually leads to the opening of the mitochondria ATP-sensitive potassium (K<sub>ATP</sub>) channels. K<sub>ATP</sub> channel opening is believed to be the key effector in conferring early protection to the myocardium, although recent evidence suggests otherwise. Clearly further investigation is merited to define the complex mechanisms that confer early protection.

### 1.2 Delayed ischaemic preconditioning:

In 1993 it was observed that a second wave of protection appears 24 hours following the preconditioning protocol. This second wave of protection is now referred to as the second window of protection (SWOP). SWOP appears gradually, yet lasts as long as 72 hours or more. A fundamental difference between classic and delayed preconditioning may be in the means by which cardioprotection is conveyed. In the former, K<sub>ATP</sub> channels are suspected to be the end-effectors, in the latter newly synthesised cardio-protective proteins are thought to convey protection.

Literature data implies that both classic and delayed IPC share many common triggers and mediators. Adenosine, as with classic IPC, is thought to be a major contributor to the induction of SWOP. Apart from its importance in antiarrhythmic models, nitric oxide (NO) has also been shown to trigger delayed protection against both myocardial stunning and necrosis. However with respect to the role of oxygen free radicals in SWOP, comparatively little has been done.

It is interesting that in the late 80's Szekeres *et al.* published data on an extensive study describing the so called "late appearing and long lasting" protective effects of prostacyclin against electrophysiological changes. Perhaps an early observation of

what later became known as SWOP. Other possible avenues of conveying delayed protection exist or are yet to be explored, yet what remains a challenge is perhaps sustaining a preconditioned state through clinically applicable means. To that end a thorough understanding of its intricate inter-cellular pathways is needed.

Similar to classic preconditioning, PKC activation seems pivotal in delayed preconditioning. Protein tyrosine kinase has also been implicated, along with downstream activation of mitogen activator protein kinases. Overwhelming evidence supports the hypothesis that delayed protection is associated with acquisition of newly synthesised cytoprotective proteins or alterations in their activity. They include the heat shock protein family (such as HSP72), manganese-superoxide dismutase (Mn-SOD), and more recently nitric oxide synthase (NOS).

## 2. OVERALL AIM OF THE PROJECTS

Extensive research over the years in the field of ischaemic preconditioning has greatly extended our understanding of the underlying mechanism of cardiac adaptation. Although many questions are yet to be elucidated, especially with regard to the second window of protection, it remains one of the most powerful experimental tools in cytoprotection that may one day translate into a clinical reality.

In our first series of SWOP investigations, we aimed to define the role of oxygen free radicals as triggers of this adaptive process. Numerous studies suggest that OFR can trigger classic ischaemic preconditioning. Their role in delayed IPC though has been somewhat under-investigated. Since convincing evidence suggests that OFR may lead to activation of transcription factors thus leading to enhanced cytoprotective protein synthesis, which are likely to convey delayed protection, a role for OFR in SWOP is very plausible.

In the second series of investigations, we set upon examining SWOP in a swine model. Previous attempts at inducing delayed IPC in pig myocardium had thus far failed to yield meaningful protection against infarction. Nonetheless since swine heart closely resembles that of adult human heart, a successful demonstration of SWOP in pig myocardium would further fuel interest in delayed preconditioning and its potential clinical application. In addition we aimed to convey delayed protection employing a medically applicable technique (Percutaneous Transluminal Coronary Angiography, PTCA). We hypothesised that with the right protocol, pig myocardium, as with other species, may be amenable to delayed protection. Furthermore we aimed to examine if a subthreshold ischaemic stimulus (2x2 min) can be augmented pharmacologically to induce full protection against a severe ischaemic insult, which can perhaps be interpreted as a step closer towards realizing the clinical application of this powerful response.

### 3. DO OXYGEN FREE RADICALS INDUCE SWOP IN DOGS?

#### 3.1 Insight into the study:

Recently it has been suggested that at low concentrations oxygen free radicals can modulate functions within the cell. As previously mentioned Murry *et al* was the first to investigate their potential role as triggers of classic IPC. Their role in delayed preconditioning however remains ambiguous. At low concentrations, OFR are thought to directly activate PKC. They have also been shown to stimulate phospholipase D, which would indirectly lead to PKC activation. The aim of this study was to examine the contribution made by OFR to the induction of delayed protection against infarction in a large animal model. We tested the potential cardioprotection afforded by various numbers of brief cycles of ischaemia-reperfusion. We then investigated if the protection was lost with the administration of a potent free radical scavenger, N-2-mercapto-propionylglycine (MPG), given during the preconditioning protocols.

#### 3.2 Materials and methods:

Adult mongrel dogs of either sex were subjected to overnight fast prior to the experiments. Animals were pre-medicated with droperidol and atropine. Anaesthesia was induced by trapanal. Anaesthesia was maintained with narcofan in a nitrous oxide:oxygen gaseous mixture.

The experimental protocol stretched over two days. On day one, under sterile conditions, the right femoral artery was prepared and cannulated for systemic pressure monitoring and blood withdrawal respectively. Systemic blood pressures and ECG were continuously recorded. Arterial blood pH,  $PO_2$ ,  $PCO_2$  were monitored at selected intervals. The animal was given a muscle relaxant before a left thoracotomy to expose the heart. A 0.5 cm segment of the left anterior descending coronary artery (LAD) was then carefully dissected. A 3-0 silk suture was passed around the isolated portion of the LAD to form a snare. Following the completion of either sham or ischaemic preconditioning protocols (described below), the chest was closed, the animal allowed to recover.

On the following day, all animals were subjected to 60 min ischaemia followed by 180 min of reperfusion. Following conclusion of the reperfusion period, the heart was subjected to double staining using patent blue dye and 1% triphenyltetrazolium chloride (TTC). The percentage of area at risk and infarction within the area at risk was determined.

Transmural blood flow was also measured by fluorescent microspheres using the reference withdrawal technique. Transmural flow was measured before ligation and mid-way through the sustained occlusion on day 2. The values were expressed as ml/min/gram wet tissue. Results were excluded from the study if the subendocardial blood flow during ligation was more than 0.15 ml/min/g. In addition following a control measurement, ECG, heart rate and systemic blood pressures were registered and recorded at regular intervals. The occurrence of any ventricular tachycardia and fibrillation were recorded automatically.

All results are expressed as mean values  $\pm$  SEM. Infarct size data were analysed with 1-way analysis of variance (ANOVA) followed by unpaired t-test with Bonferroni's correction for multiple comparisons. Haemodynamic data were analysed using repeated measures ANOVA. The null hypothesis was rejected when  $P < 0.05$ .

#### 3.3 Experimental protocol:

The study was divided into two phases. In phase I, comprising of 4 groups, a model of delayed ischaemic preconditioning was designed with various cycles of IPC:

GI- Control (sham thoracotomy)  
GII- 4x5 IPC (4 x 5-min ischaemia, 10min reperfusion)  
GIII- 2x5 IPC (2 x 5-min reperfusion)  
GIV- 1x5 IPC (1 x 5-min ischaemia, 10min reperfusion)

In phase II we sought to block OFR by administering an antioxidant. On day 1, N-2-mercapto-propionylglycine (1.5 mg/kg/min) was universally administered as a continuous infusion, 30 min prior to any protocol. Four groups were designed, reciprocal to the four groups in phase I:

GV- MPG (drug control)  
GVI- 4x5 IPC + MPG  
GVII- 2x5 IPC + MPG  
GVIII- 1x5 IPC + MPG

On the second day all animals were subjected to a 60-min index ischaemia, followed by 180 min of reperfusion.

#### 3.4 Results:

Changes in heart rate, systolic and diastolic blood pressures, and rate-pressure products were comparable across the groups and it is unlikely that changes in infarct size could be attributed to haemodynamic variations. Similarly the size of area at risk (AAR) ranging from 22.9 to 25.1%, demonstrated that all groups were subjected to comparable areas at risk.

In phase I of the study the control group ( $n=6$ ), showed a mean infarct size of  $39.5 \pm 5.1\%$  of the AAR. Preconditioning with four cycles (4x5 IPC) reduced the infarction to  $15.8 \pm 1.3\%$  ( $n=6$ ,  $p<0.05$ ). Similarly the 2x5 IPC group ( $n=5$ ), demonstrated a significant limitation in infarct size ( $11.4 \pm 1.3\%$ ,  $p<0.05$ ). The 1x5 IPC also conferred some protection compared to controls, however this reduction was not statistically significant.

The mean infarction values of phase II of the study were as follows: drug control group (MPG), had a mean value comparable to that of the controls in Phase I ( $37.0 \pm 4.2\%$ ,  $n=5$ ). Addition of MPG had little effect on the protection afforded by 4x5 IPC ( $n=6$ ), as the reduction in infarction was still statistically significant ( $13.7 \pm 2.1\%$ ,  $p<0.05$ ). Adding MPG however abolished the previously observed protection with either 2x5 or 1x5 IPC ( $28.8 \pm 3.2\%$ ,  $36.6 \pm 3.4\%$  respectively).

Although we also monitored the incidence of arrhythmia and premature ventricular beats in every experiment, it can be said that no specific pattern, or significant

difference was noted between the groups. However a distinct pattern of reduced susceptibility to VF was seen in most preconditioned groups.

### **3.5 Conclusions from this study:**

The 4x5 and 2x5 IPC stimuli both conveyed significant cardioprotection. However this protection did not reach a significant level with a single cycle of IPC in the dog myocardium. In the second phase of the study the addition of MPPG did not reverse the cardioprotection afforded by 4x5 IPC. However the significant protection seen with 2x5 IPC and the partial infarct limitation afforded by 1x5 IPC were both abolished. Yet there seems to be dissociation in the MPPG+2x5 IPC group, between loss of protection against infarction as opposed to maintained protection against ventricular fibrillation. The reason for this observation is unclear. Nonetheless it can be assumed that in dogs, generation of oxygen free radicals during the brief cycles of ischaemia-reperfusion is an integral part of triggering delayed cardioprotection. However this role is only crucial with fewer bouts of IPC. As the number of cycles rise (rigorous stimuli), blocking OFR seems to have no adverse effect on the induction of SWOP. With multiple cycles other possible triggers (e.g. adenosine, nitric oxide etc.) might be released in sufficient amounts to confer cardioprotection, even in the absence of oxygen free radicals.

Over the years OFR have been shown, to either directly or indirectly stimulate the signalling cascades that culminate to IPC. One such cascade is via the activation and translocation of protein kinase C, which then leads to gene transcription, yielding proteins instrumental in delayed cardioprotection. More recently evidence is emerging on free radicals inducing a PTK-dependent pathway leading to cardioprotection. Downstream the signal transduction leads to enhanced phosphorylation and activation of other mediators. The nuclear transcription factor NFkB has also been recognized to play a crucial role in delayed IPC. There is strong evidence that oxidative stress can also lead to NFkB translocation and activation, further proof of the possible role of OFR in triggering the cellular cascades that lead to delayed ischaemic adaptation.

## **4. SWOP IN PIG MYOCARDIUM**

### **4.1 Insight into the study:**

After more than a decade, we are yet to reach the ultimate goal of pharmacological preconditioning. Bradykinin B<sub>2</sub> receptor stimulation has been proven to contribute to induction of classic IPC. Furthermore it has been shown that angiotensin converting enzyme (ACE) inhibitors confer myocardial cytoprotection, and seem to do so by augmenting bradykinin level. Thus in an *in vivo* swine model, we first aimed to establish delayed cytoprotection, using PTCA to precondition the animals. We then subjected the pigs to a subthreshold ischaemic stimulus, and examined if the addition of an ACE inhibitor, perindoprilat (0.06 mg/kg), during the IPC protocol would augment the protection afforded. If so, this would be the first study to establish pharmacological enhancement of a clinically relevant subthreshold stimulus in a model of delayed cardioprotection.

### **4.2 Materials and methods:**

Following an overnight fast, pigs were immobilized by administration of ketamine and diazepam. Anaesthesia was induced by intravenous administration of thiopentone sodium. Surgical anaesthesia and ventilation were maintained using a gaseous mixture of nitrous oxide:oxygen in addition to 1.0-1.5% isoflurane.

This study was designed to investigate delayed ischaemic preconditioning in pig myocardium, therefore each procedure again comprised of two days. On day 1, each animal underwent either sham or IPC procedure via employing percutaneous transluminal coronary angioplasty (PTCA). On day 2, following a mid-line thoracotomy, all animals were subjected to 40 min ligation of left anterior descending coronary artery (LAD) followed by 180 min of reperfusion. Infarct size was taken as the end point of the experiment and was determined (as described above) following completion of reperfusion.

To further elaborate on the induction of delayed adaptation in this model, we also investigated one of the proposed end-effectors of this process, namely Mn-SOD content in cardiomyocytes. In every group additional two pigs were subjected to relevant procedures on day 1. On day 2 following anaesthesia the chest was opened and the heart was extracted. Samples were taken and sent for immunohistochemical investigations.

Throughout the procedure standard limb ECG and systemic blood pressures (BP) were monitored continuously. Arterial pO<sub>2</sub> and pH were maintained within the physiologic range. In case of ventricular fibrillation (VF), if defibrillation could not be accomplished within 60 seconds, the experiment was terminated. Similar statistical evaluations, as employed in the first series of investigations, were applied in this study.

### **4.3 Experimental protocol:**

Five groups were designed to determine if pig myocardium can convey delayed cardioprotection, and furthermore whether a subthreshold ischaemic stimulus (2x2 IPC) can be augmented pharmacologically to convey significant protection; GI- control (sham PTCA)

GII- 4x5 IPC (4 x 5-min ischaemia, 10min reperfusion)  
GIII- 2x2 IPC (2 x 2-min ischaemia, 10min reperfusion)  
GIV - perindopril (drug control)  
GV- 2x2 IPC + Perindopril

All animals were subjected to the same protocol on day 2 as described above.

#### 4.4 Results:

Heart rate and systemic blood pressures were monitored continuously and recorded at regular intervals, however there were no significant differences between the values at any given interval between the groups. Similarly mean AAR values were comparable across the groups.

The results of the infarct size study demonstrate that in contrast to two previous studies, pig myocardium is also amenable to delayed protection against infarction following IPC. There was a significant reduction in infarct size from  $42.8 \pm 3.2\%$  in the control group (n=9), to  $19.5 \pm 3.9\%$  ( $p < 0.05$ ) in the 4x5 IPC group (n=8). The subthreshold stimulus or perindopril alone could not confer significant delayed protection ( $33.4 \pm 3.9\%$ , n=7 &  $31.2 \pm 2.3\%$ , n=8 respectively). However when combined (2x2 IPC + Pr.), they conferred significant protection against infarction ( $18.4 \pm 3.1\%$ ,  $p < 0.05$ , n=7). This protection was comparable with that observed in the fully preconditioned group (4x5 IPC).

We did not observe any significant differences in reperfusion arrhythmias and fibrillation, between preconditioned and non-preconditioned groups, thus concluding that there was no protection against rhythm disturbances in this swine model of delayed IPC.

Results from Mn-SOD immunohistochemistry further confirmed the preconditioned state. The preconditioned groups showed far stronger positivity than non-preconditioned groups.

#### 4.5 Conclusions from this study:

The second window of protection may also be evoked in porcine myocardium, as evident from the protection against infarction. This observation is further substantiated with increased Mn-SOD in preconditioned hearts. Secondly and perhaps medically more significant, we were able to pharmacologically augment a clinically relevant (subthreshold) ischaemic stimulus in a model of delayed adaptation. The 2x2 min IPC chosen as our subthreshold stimulus is realistic and closely resembles clinical interventions performed on patients with ischaemic heart disease (e.g. PTCA). There are two reasons as to why we chose an ACE inhibitor in our model. First, since their introduction, they have become a cornerstone in treatment of hypertension. ACE inhibitors have also emerged as a crucial part of therapy in patients suffering from heart failure. They have been shown to prevent left ventricular remodeling as well as reduce myocardial ischaemic events. Thus they have become an indispensable tool in

treating patients with heart disease. To learn more of their potential role in delayed cardioprotection can be beneficial to millions of people worldwide. On the other hand ACE inhibitors have been shown to induce early protection in the setting of classic IPC. These considerations were the impetus behind our choice of pharmacological preconditioning in this model. Pharmacological preconditioning in humans may be a future option for adjunct therapy, and as demonstrated by this study, it is not an unattainable goal. On the other hand on a daily basis patients with ischaemic heart disease may be subjected to brief bouts of ischaemia (electively in the form of PTCA or non-electively in the form of angina pectoris). These short ischaemic episodes may simulate subthreshold preconditioning stimuli that along with the right measures could be enhanced pharmacologically to the benefit of the patient.

## 5. NOVEL FINDINGS

- In our first series of experiments we were able to demonstrate that oxygen free radicals play a crucial role in the induction of delayed cardioprotection against infarction in an *in vivo* dog model. This protection was seen as tolerance against both infarction as well as ventricular fibrillation 24 hours following ischaemic preconditioning. However as demonstrated, the role played by free radicals is overwhelmed by other triggers during multiple cycles of IPC.
- In the second series of experiments, delayed cardioprotection was demonstrated for the first time in pig myocardium. Although there was a clear limitation of the infarct size in preconditioned pigs, we did not observe an increased tolerance against reperfusion arrhythmias or fibrillations.
- Also novel to this work was the demonstration of pharmacological enhancement of subthreshold ischaemic stimuli in a model of delayed cardiac adaptation. The use of an ACE inhibitor to achieve this augmentation adds to the clinical importance of this observation.
- Based on previous results regarding blunted breakdown of bradykinin following pretreatment with ACE inhibitors, this study for the first time hints at the possible involvement of bradykinin in the induction of the second window of protection.

## 6. PUBLICATIONS

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