

Research article

Pharmacological and ischemic preconditioning of the human myocardium: mitoK_{ATP} channels are upstream and p38MAPK is downstream of PKC

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Abstract

Background: These studies investigate the role of mitoK_{ATP} channels, protein kinase C (PKC) and Mitogen activated protein kinase (p38MAPK) on the cardioprotection of ischemic (IP) and pharmacological preconditioning (PP) of the human myocardium and their sequence of activation.

Results: Right atrial appendages from patients undergoing elective cardiac surgery were equilibrated for 30 min and then subjected to 90 min of simulated ischemia followed by 120 min reoxygenation. At the end of each protocol creatinine kinase leakage (CK U/g wet wt) and the reduction of MTT to formazan dye (mM/g wet wt) were measured. Similar protection was obtained with α_1 agonist phenylephrine, adenosine and IP and their combination did not afford additional cardioprotection. Blockade of mitoK_{ATP} channels with 5-hydroxydecanoate, PKC with chelerythrine, or p38MAPK with SB203580 abolished the protection of IP and of PP. In additional studies, the stimulation of mitoK_{ATP} channels with diazoxide or activation of PKC with PMA or p38MAPK with anisomycin induced identical protection to that of IP and PP. The protection induced by diazoxide was abolished by blockade of PKC and by blockade of p38MAPK. Furthermore, the protection induced by PMA was abolished by SB203580 but not by 5-hydroxydecanoate, whereas the protection induced by anisomycin was unaffected by either 5-hydroxydecanoate or chelerythrine.

Conclusions: Opening of mitoK_{ATP} channels and activation of PKC and p38MAPK are obligatory steps in the signal transduction cascade of IP and PP of the human myocardium with PKC activation being downstream of the opening of mitoK_{ATP} channels and upstream of p38MAPK activation.

Background

Ischemic preconditioning (IP) is a powerful protective endogenous adaptive response of the heart against a prolonged ischemic insult. [1,2] However, the application of IP requires a physical cut of the blood supply which can be difficult or impractical in many clinical situations. A

way to circumvent these potential problems associated with the clinical application of IP may be preconditioning by pharmacological (PP) means or manipulation of the signalling pathway involved in the protection. A number of membrane receptors are involved in the phenomenon of IP including α_1 [3–5] and β -adrenoceptors, [6] opioid

[7] and adenosine A1 and A3 receptors. [8,9] Other factors such as heat shock proteins, [10] bradykinin, [11] calcium [12] and nitric oxide synthase activity [13] have also been shown to participate in the protection of IP, however, whether the various forms of PP share the same molecular mechanism with IP is not fully elucidated.

The intracellular sequence of events that translate the binding of the various agonists to their membrane receptors into the protection of preconditioning remains under intense investigation. It has been reported that α_1 -adrenoceptors are coupled with protein kinase C (PKC) through phospholipase activity, [14] that in turn activate p38 Mitogen Activated Protein Kinase (p38MAPK) in some cardiac preparations. [15] ATP sensitive potassium channels have also been implicated in the signal transduction mechanism of IP, [16,17] and recent evidence from several investigators [18–20] including ourselves [21] has shown that the mitochondrial and not the sarcolemmal K_{ATP} channels are involved. The order of involvement of the above mediators remains controversial although recently it has been suggested that mitochondrial K_{ATP} channels are the triggers in the signal transduction mechanism rather than the end effectors. [18]

The aims of the present series of studies were to investigate the efficacy of pharmacological preconditioning of the human myocardium with α_1 -adrenoceptor and adenosine receptor agonists as compared to ischemic preconditioning and to elucidate the contribution and sequence of activation of PKC, p38MAPK and $mitoK_{ATP}$ channels.

Results

All samples entering the studies completed the applied protocol and were included in the analysis.

Ischemic versus pharmacological preconditioning (Study 1)

Figures 4A and 4B demonstrate that SI/R alone resulted in a significant increase in CK leakage and decrease in MTT reduction when compared to the aerobic controls. They also show that PP with phenylephrine or adenosine administered prior to SI/R is as protective as IP and that their use in combination does not result in additive protection.

Role of PKC, p38MAPK and $mitoK_{ATP}$ channels in IP and PP (Study 2)

Figures 5A and 5B show that both IP and PP with phenylephrine or adenosine are equally abolished by the PKC antagonist chelerythrine, the p38MAPK inhibitor SB203580 or the $mitoK_{ATP}$ blocker 5-hydroxydecanoate suggesting that these steps are necessary in the signal transduction cascade of preconditioning.

Sequence of activation of PKC, p38MAPK and $mitoK_{ATP}$ channels (Study 3)

The results on CK leakage and MTT reduction shown in Figures 6A and 6B demonstrate that identical protection is obtained with the $mitoK_{ATP}$ channel opener diazoxide, the PKC activator PMA and the p38MAPK activator anisomycin. Importantly, they also show that whilst the protection of diazoxide is abolished by the PKC antagonist chelerythrine and the p38MAPK antagonist SB203580, the protective effect of PMA is abolished by SB203580 but not by the $mitoK_{ATP}$ channel blocker 5-hydroxydecanoate, and the protective action of anisomycin is unaffected by chelerythrine and 5-hydroxydecanoate.

Discussion

The present studies have demonstrated that protection with PP by activation of α_1 -adrenoceptors or adenosine receptors is identical to that of IP in the human myocardium. In addition, they have shown that $mitoK_{ATP}$ channels, PKC and p38MAPK are an integral part of the cellular signal transduction involved in this cardioprotection in which $mitoK_{ATP}$ channels are placed upstream and p38MAPK placed downstream of PKC. These studies provide novel information to understand the underlying mechanism of protection by preconditioning of the human myocardium and the results have obvious clinical importance that warrants further discussion.

Pharmacological versus ischemic preconditioning

The demonstration that activation of α_1 -adrenoceptors with phenylephrine is as protective as IP of the human myocardium in these studies is supported by other results in the rat heart [3,5] but contrast with the results reported by Cleveland et al [4] in the human myocardium. The differing results may be due to the use of 50 μ M phenylephrine by Cleveland et al, [4] a concentration 500 times the one used in our studies. Using an identical preparation, we have recently shown [22] that phenylephrine exerts maximal protection at 0.1 μ M and that protection is lost at concentrations ≥ 10 μ M. It should also be pointed out that the human trabeculae preparation used by Cleveland et al [4] differed from our preparation in that muscles were electrically stimulated and subjected to only 45 min of simulated ischemia which may be another potential explanation.

The protection induced by adenosine was also identical to that of IP and the use of the two in combination did not result in additional benefit up and above that seen with each intervention alone. These results in the human myocardium are supported by studies in other animal species [23] but contrast with the results reported by Leesar et al [24] in the vivo human heart and by McCully et al [25] in the isolated rabbit heart. In the study by Leesar et al [24] patients undergoing percutaneous transluminal coronary

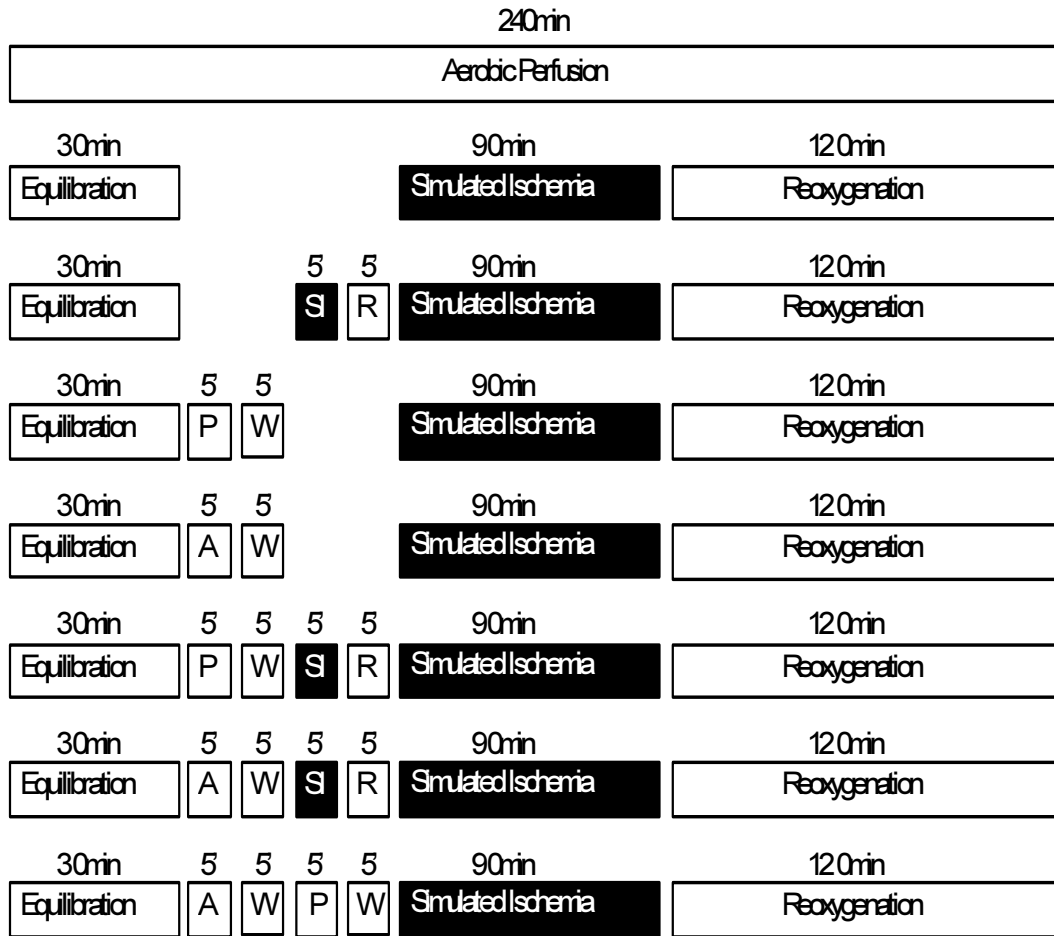


Figure 1

Protocol for Study I to investigate the efficacy of preconditioning via α_1 -adrenoceptors or adenosine (A) receptors alone and in combination with IP (n = 6 specimens/group): (i) time-matched aerobic control, (ii) SI/R alone, (iii) IP induced with 5 min of SI/ 5 min R before SI, (iv) phenylephrine (P) for 5 min and 5 min washout (W) before SI, (v) adenosine (A) for 5 min and 5 min washout (W) before SI, (vi) phenylephrine (P) for 5 min and 5 min washout (W) before IP, (vii) adenosine (A) for 5 min and 5 min washout (W) before IP and (viii) adenosine (A) for 5 min and 5 min washout (W) followed by phenylephrine (P) for 5 min and 5 min washout (W) prior to SI.

angioplasty were subjected to three 2-minute balloon inflations 5 minutes apart. Under these conditions the administration of adenosine was more effective in limiting ST-segment shift than the balloon inflation protocol. Although we have previously shown [2] that 4 to 5 min of ischemia are sufficient to precondition the human myocardium, independent of whether this time is attained with one or two cycles of ischemia, it is conceivable that

in their study [24] the balloon inflation protocol may not have been sufficient to induce enough ischemia to trigger preconditioning. This is a strong possibility under clinical conditions where collateral flow may lessen the severity of ischemia. Furthermore, in that study [24] changes in ST-segment, that are modulated by sarcolemmal K_{ATP} channels, [26] were used as the main end-point and it is now recognized that the protection of preconditioning may be

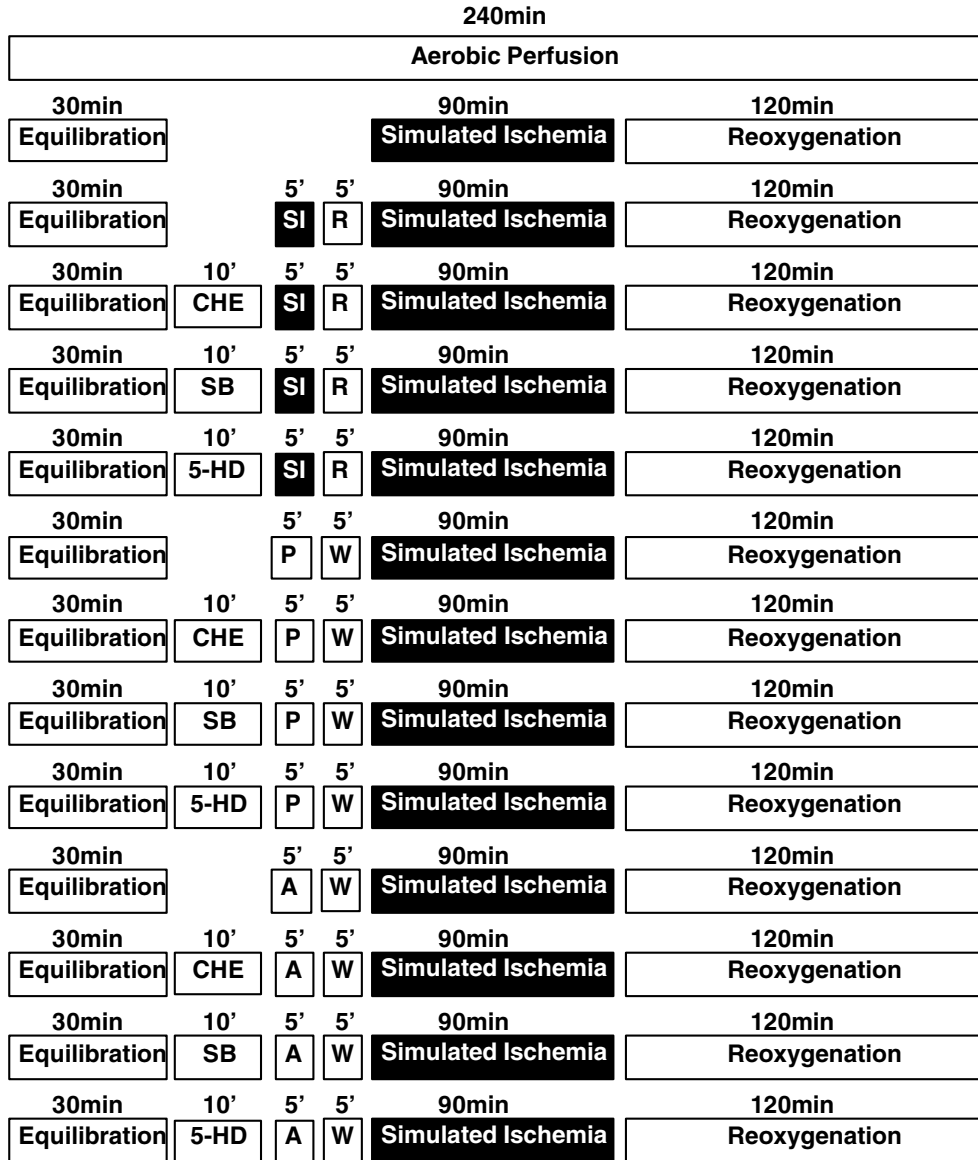


Figure 2

Protocol for Study 2 to investigate the role of PKC, p38MAPK and mitoK_{ATP} channels in the cardioprotective effect of IP and PP with phenylephrine (P) or adenosine (A) (n = 6 specimens/group): (i) time-matched aerobic control, (ii) SI/R alone, (iii) IP alone prior to SI, (iv) chelerythrine (CHE) for 10 min prior to IP, (v) SB203580 (SB) for 10 min prior to IP, (vi) 5-hydroxydecanoate (5-HD) for 10 min prior to IP, (vii) phenylephrine (P) for 5 min and 5 min washout (W) before SI, (viii) chelerythrine (CHE) for 10 min prior to phenylephrine (P) for 5 min and 5 min washout (W), (ix) SB203580 (SB) for 10 min prior to phenylephrine (P) for 5 min and 5 min washout (W), (x) 5-hydroxydecanoate (5-HD) for 10 min prior to phenylephrine (P) for 5 min and 5 min washout (W), (xi) adenosine (A) for 5 min and 5 min washout (W) before SI, (xii) chelerythrine (CHE) for 10 min prior to adenosine (A) for 5 min and 5 min washout (W), (xiii) SB203580 (SB) for 10 min prior to adenosine (A) for 5 min and 5 min washout (W) and (xiv) 5-hydroxydecanoate (5-HD) for 10 min prior to adenosine (A) for 5 min and 5 min washout (W).

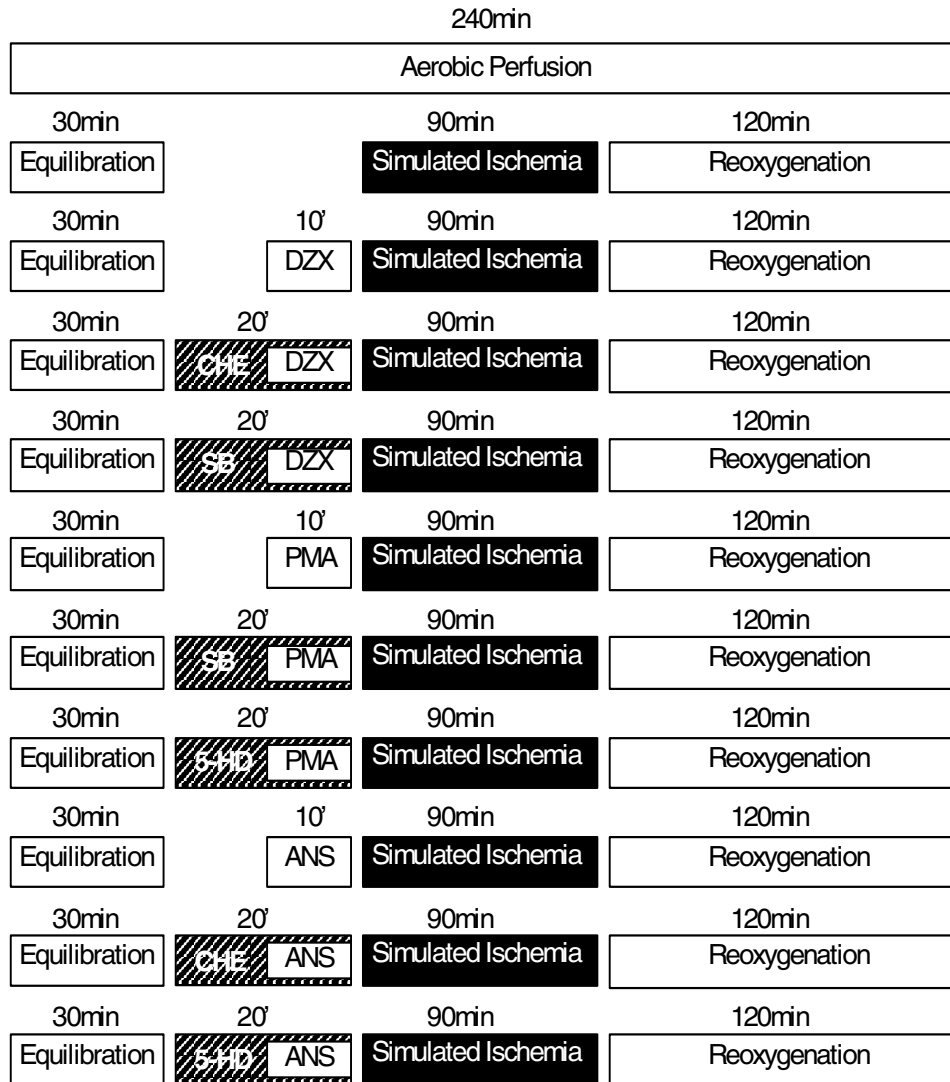


Figure 3

Study 3 protocol designed to elucidate the sequence of the participation of involvement of $\text{mitoK}_{\text{ATP}}$ channels, PKC and p38MAPK in the signal transduction cascade of cardioprotection. For this purpose, in addition to the (i) aerobic time-matched control and (ii) SI/R alone the following groups were studied (n = 6 specimens/group): (iii) diazoxide alone for 10 min prior to SI, (iv) chelerythrine (CHE) for 20 min with diazoxide added for the last 10 min prior to SI, (v) SB203580 (SB) for 20 min with diazoxide added for the last 10 min prior to SI, (vi) PMA alone for 10 min prior to SI, (vii) SB203580 (SB) for 20 min with PMA added for the last 10 min before SI, (viii) 5-hydroxydecanoate (5-HD) for 20 min with PMA added in the last 10 min before SI, (ix) anisomycin (ANS) alone for 10 min prior to SI, (x) chelerythrine (CHE) for 20 min with anisomycin (ANS) added for the last 10 min prior to SI and (xi) 5-hydroxydecanoate (5-HD) for 20 min with anisomycin (ANS) added for the last 10 min prior to SI.

mediated by mitochondrial rather than by sarcolemmal K_{ATP} channels. [18–21] The results reported by McCully et al [25] that adenosine is more potent than and extends the cardioprotection of IP in the rabbit heart are difficult to explain because they used a protocol of 5 min ischemia/5 min reperfusion for preconditioning which is identical to the one used in our studies and shown to afford optimal protection in other preparations. [5,9] However, since adenosine and IP use identical cellular signal transduction mechanism to induce protection, the most likely explanation of their results may be the presence of some unknown factor that may have influenced the severity of the IP insult and therefore its protective efficacy.

Mechanism of preconditioning

The elucidation of the factors involved in the signal transduction pathway of preconditioning has been the subject of intense investigation and although the participation of factors such as PKC [17,19,27] and $mitoK_{ATP}$ channels [16–21] is well established, their relevance and the sequence of activation remains controversial. The present studies are the first to demonstrate that PP and IP of the human myocardium share identical signal transduction cascade that involves $mitoK_{ATP}$ channels, PKC and p38MAPK in that order. Wang et al [28] showed that PKC inhibition with chelerythrine or calphostin C completely abolished the beneficial effects of diazoxide in the isolated rat heart, thus providing further support that $mitoK_{ATP}$ channels are upstream of PKC. However, Pain et al [18] and Miura et al [29] using chelerythrine and calphostin C to block PKC were unable to confirm this observation in the rabbit, suggesting that these differences could arise as a result of different animal species. In spite of this, it is interesting to note that Pain et al [18] reported that genistein, a tyrosine kinase antagonist, blocked the protection induced by diazoxide which indicates that activation of kinases also lies downstream of $mitoK_{ATP}$ channels in the rabbit heart.

Our demonstration that activation of any one of the components of the transduction cascade investigated in our studies ($mitoK_{ATP}$ channels, PKC, p38MAPK) can provide identical protection and that blockade of any of them individually completely abolishes protection indicates that in the human myocardium there is only one pathway of protection by preconditioning. The failure to obtain additional protection when more than one agent was used to induce PP or when these agents were used in combination with IP further support this thesis. But again, the mechanism of preconditioning the human myocardium may not be applicable to all species as suggested by the need to combine the inhibition of PKC and tyrosine kinase to abort the protection of preconditioning in pigs. [30]

The molecular interactions between the various components of the preconditioning pathway are not well understood. There is evidence that $mitoK_{ATP}$ channel opening increases radical oxygen species production which in turn may activate PKC, [18] but it is well known that $mitoK_{ATP}$ channels are also modulated by PKC [19]. The above and the realization of selective translocation of PKC with preconditioning, [27,28,31] suggest that PKC may play a dual role upstream and downstream of $mitoK_{ATP}$ channels. Our observation that blockade of PKC abolishes the protection by diazoxide does not eliminate that possibility.

Our results have shown that activation of p38MAPK is a crucial step in the transduction pathway of preconditioning in the human myocardium. Activation of p38MAPK has also been connected to preconditioning in the rabbit heart, [32] however the relationship could not be established in rat [33] and pig [34] hearts. Therefore, it seems that once more the components of the signal transduction pathway of preconditioning are species-dependent.

It is still unknown whether the activation of p38MAPK is the last step of the transduction cascade that phosphorylates the end-effector and whether there is a simple or multiple effectors and their location. p38MAPK can phosphorylate a wide range of proteins, some of which may be potential candidates for end-effectors of preconditioning. Thus, for example, the low molecular weight heat shock protein HSP27 may be phosphorylated by p38MAPK via the intermediate MAPKAPK2 [35] and this may lead to polymerization of actin [36] and to increase tolerance of the cytoskeleton to stress. [37] Translocation of PKC isoforms to mitochondrial sites, intercalated discs and nucleus may suggest that p38MAPK activation in these places may activate enzymes involved with energy production, intercellular communication through cell junctions or gene transcriptions.

Our results suggest that $mitoK_{ATP}$ channels are not the end-effectors of cardioprotection by preconditioning of the human myocardium. The concept that $mitoK_{ATP}$ channels may be the end-effectors of preconditioning has been based on the efficacy of diazoxide, a highly selective $mitoK_{ATP}$ channel opener, to mimic cardioprotection by preconditioning [20,21] and the blockade of this protection by 5-hydroxydecanoate, [20,21] a specific $mitoK_{ATP}$ channel blocker; however, it was never clear whether the actions of these channels were limited to the mitochondria or were part of a more complex signal transduction cascade with effects on other cellular structures. The effect of opening the $mitoK_{ATP}$ channels remains controversial and whereas some investigators have reported large decreases in mitochondrial membrane potential affecting respiration and resulting in a reduction in Ca^{2+} uptake

into mitochondria, [38] others have observed little effect on membrane potential, bioenergetics or Ca^{2+} uptake but important changes on matrix and inter-membrane space volumes. [39] The reason given for these discrepancies was the use of high doses of mitoK_{ATP} channel openers in the former studies [39] but this does not clarify how changes in mitochondrial volume are connected to PKC activation, that is downstream in the signal transduction cascade.

The diagram in Figure 7 describes our proposal of the signal transduction cascade of preconditioning in the human myocardium. It is suggested that upon activation of sarcolemmal receptors (e.g. adenosine receptors, α_1 -adrenoreceptors) mitoK_{ATP} channels are opened via G_i proteins and PKC, possibly PKC- δ [28]. The opening of mitoK_{ATP} channels will activate PKC possibly via the production of radical oxygen species [18]. PKC may then translocate to various cellular sites including the mitochondria, sarcolemma and intercalated discs, and nucleus, a phenomena that may involve specific PKC isoforms, where p38MAPK will be activated. In turn, p38MAPK may activate a simple or multiple end-effectors directly or via MAPK intermediates. It is clear that more studies are required to fully elucidate the signal transduction pathway of preconditioning and the coupling between its components.

Clinical implications

The findings that the cardioprotection achieved by activation of α_1 -adrenoreceptors or adenosine receptors is as potent as the one obtained with IP and that their use in combination does not result in additional benefit have obvious major clinical implications because maximal protection can be attained without the need to occlude the coronary arteries to induce ischemia. The procurement of cardioprotection with stimuli of receptors as diverse as α_1 -adrenoreceptors and adenosine receptors can be clinically advantageous because some of these agents may be contraindicated in certain conditions (e.g. α_1 -adrenoreceptor agonists in hypertension, adenosine in the presence of alterations of the cardiac conduction system). These interventions can be useful to combat ischemic injury in different clinical conditions such as coronary angioplasty, cardiac surgery and heart transplantation; however, it is necessary to mention that our studies were performed in an *in-vitro* preparation and therefore any extrapolation to the clinical setting should be made with caution.

The realization that cardioprotection by PP and IP of the human myocardium is mediated by one obligatory signal transduction pathway also opens the therapeutical window for direct manipulation of its components. Recently, we have demonstrated that although the myocardium from patients with poor left ventricular function (ejection fraction < 30%) or with diabetes cannot be protected with

IP, the mitoK_{ATP} channel opener diazoxide elicited protection in the former but not in the latter [40]. This suggests that if part of the signal transduction cascade is affected by disease states, cardioprotection can still be obtained by bypassing the defective components. It is also of clinical relevance that blockade at any stage of the signal transduction involved in preconditioning does not seem to exacerbate injury suggesting that this pathway is solely used for cardioprotection and not in tissue injury.

Possible limitations of the study

A potential limitation of our studies was the use of atrial tissue as opposed to ventricular myocardium and the fact that this was a necrosis *in vitro* model, and therefore any extrapolation must be conducted with caution. Another limitation might be that right atrial appendages were obtained from patients subjected to various medical treatments such as nitrates and beta blockers, which may have influenced the simulated ischemia/reoxygenation injury and the protection induced by preconditioning. However this effect is unlikely to be significant as all the specimens responded similarly to ischemic injury and preconditioning.

Conclusions

The current studies demonstrate that protection with pharmacological preconditioning by activation of α_1 -adrenoreceptors or adenosine receptors is identical to that of ischemic preconditioning in the human myocardium. In addition, they show that mitoK_{ATP} channels, PKC and p38MAPK are an integral part of the cellular signal transduction involved in this cardioprotection in which mitoK_{ATP} channels are placed upstream and p38MAPK placed downstream of PKC.

Materials and methods

Experimental preparation

Experiments were performed on trabeculae obtained from the right atrial appendage of patients undergoing elective coronary artery bypass graft surgery or aortic valve replacement in a cell necrosis model that was developed and characterised in our laboratory and that has been described previously [41]. The investigation conforms with the principles outlined in the Declaration of Helsinki. Donor patients were excluded if they had enlarged atriums, atrial arrhythmias, poor left ventricular function (ejection fraction <30%), right ventricular failure or were taking oral hypoglycaemic agents, opioid analgesia, K_{ATP} channel openers or catecholamines. Local ethical committee approval was obtained for the harvesting technique. Temperature was maintained at 37°C throughout the experiments and simulated ischemia was induced by bubbling the medium with 95% N₂/5% CO₂ (pH 6.8–7.0) instead of 95% O₂/5% CO₂ (pH 7.36 and 7.45) and replacing D-glucose with 2-deoxy-D-glucose.

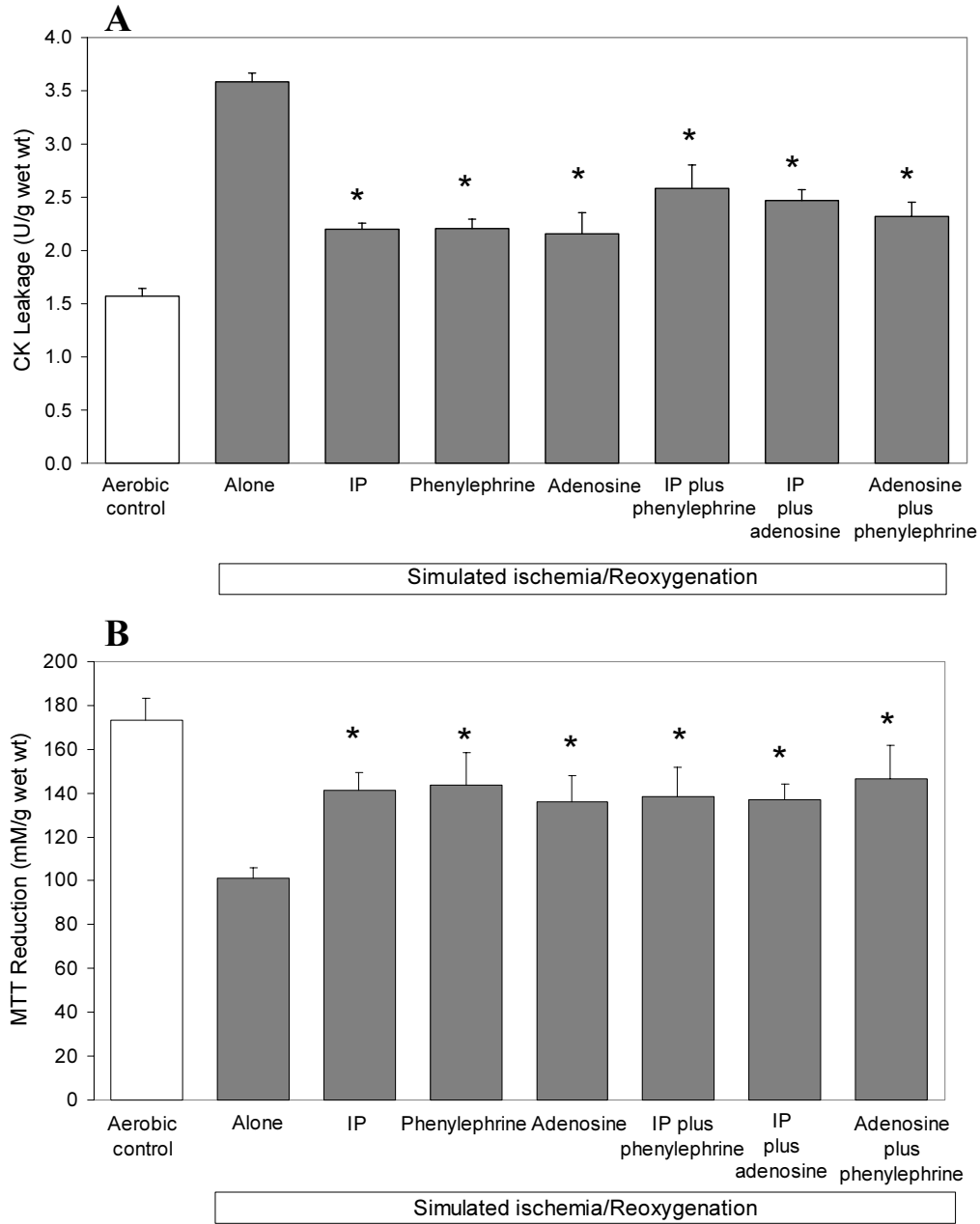


Figure 4

Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see Figure 1) to investigate the efficacy of preconditioning via α_1 -adrenoreceptors or adenosine receptors alone and in combination with IP (Study I). Data are expressed as mean \pm SEM of six experiments. * $p < 0.05$ vs SI/R alone group.

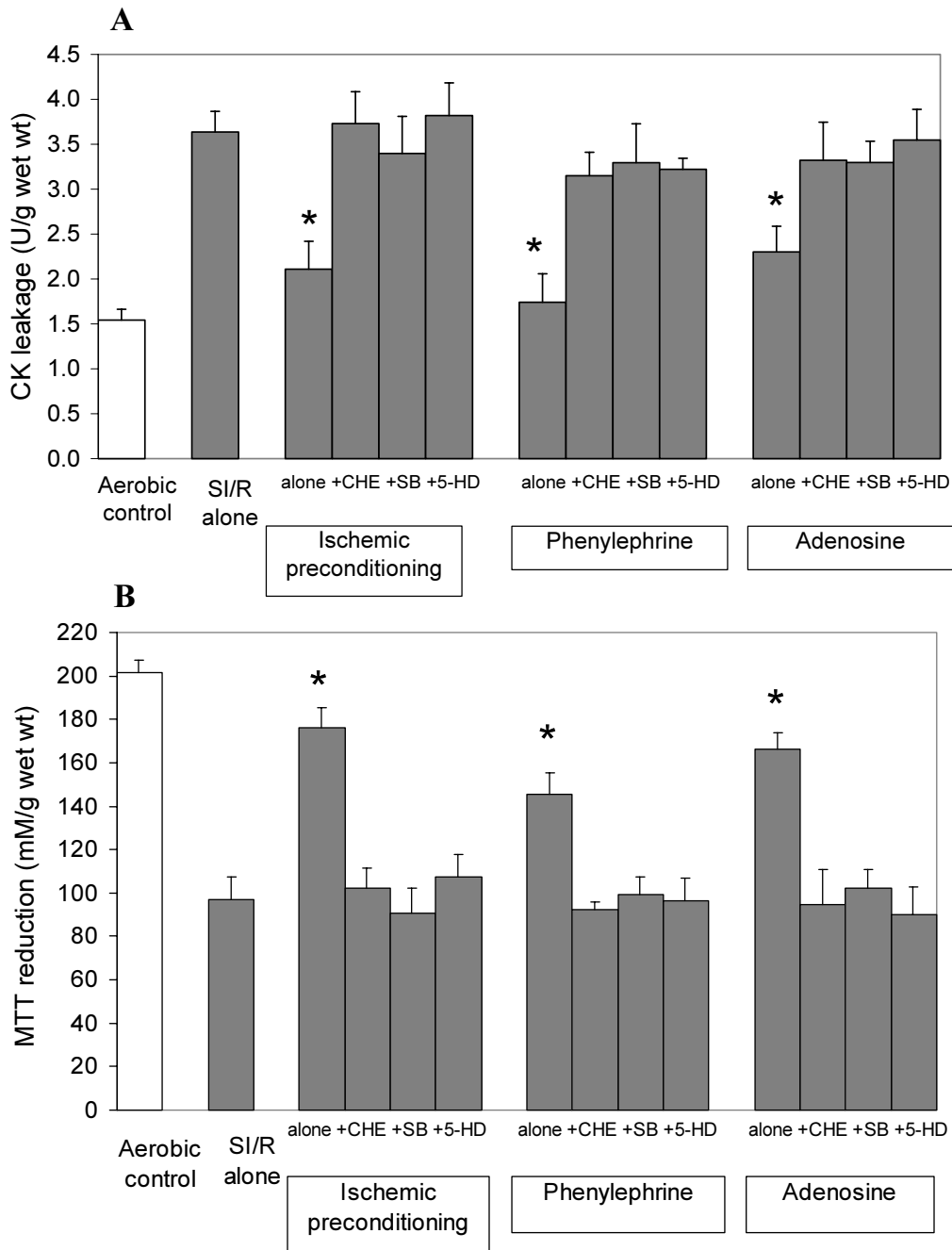


Figure 5
 Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see Figure 2) to investigate the role of PKC, p38MAPK and mitoK_{ATP} channels in ischemic and pharmacological preconditioning with phenylephrine (P) or adenosine (Study 2). Data are expressed as mean ± SEM of six experiments. *p < 0.05 vs. SI/R alone group. (5-HD: 5-hydroxydecanoate, SB: SB203580).

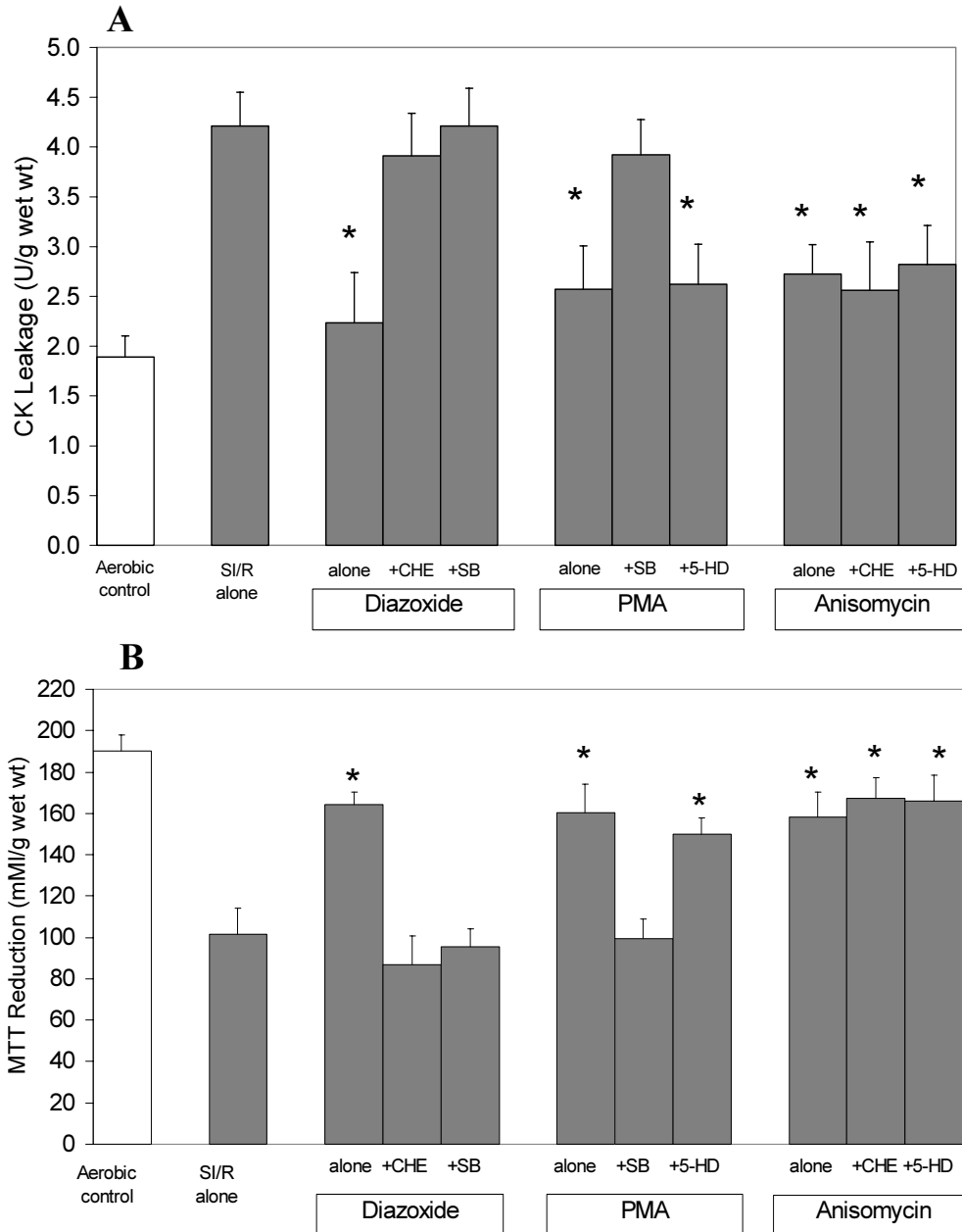


Figure 6
 Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see Figure 3) to investigate the sequence of activation of the mediators of pharmacological and ischemic preconditioning (Study 3). Data are expressed as mean \pm SEM of six experiments. * $p < 0.05$ vs SI/R alone group. (CHE: chelerythrine, 5-HD: 5-hydroxydecanoate, SB: SB203580, PMA: phorbol-12-myristate-13-acetate).

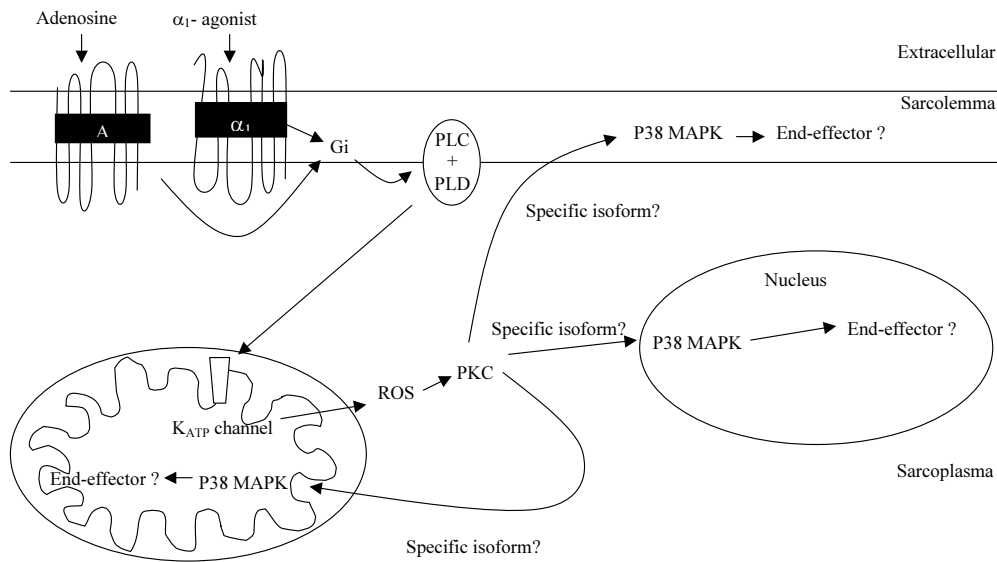


Figure 7

Proposed schematic representation of the signal transduction mechanism leading to cardioprotection by pharmacological and ischemic preconditioning of the human myocardium. Upon activation of sarcolemmal receptors $\text{mitoK}_{\text{ATP}}$ channels are opened via G proteins and possibly PKC- δ . The opening of $\text{mitoK}_{\text{ATP}}$ channels will activate PKC possibly via the production of radical oxygen species (ROS). PKC may then translocate to various cellular sites including the mitochondria, sarcolemma and intercalated discs, and nucleus, a phenomenon that may involve specific PKC isoforms, where p38MAPK will be activated. In turn, p38MAPK may activate a single or multiple end-effectors directly or via MAPK intermediates. PLC: phospholipase C, PLD: phospholipase D.

Solutions and chemicals

The incubation medium was prepared daily with de-ionized distilled water and contained (in mM): NaCl_2 (118), KCl (4.8), NaHCO_3 (27.2), MgCl_2 (1.2), KH_2PO_4 (1.0) CaCl_2 (1.25), HEPES (20) and D-glucose (10) or 2-deoxy-D-glucose (10). All the chemicals were purchased from Sigma Chemicals.

Experimental time course

All the muscles were equilibrated for a 30 min period before being randomly assigned to serve as time-matched aerobic controls or subjected to a 90 min period of simulated ischemia (SI). The muscles were then reoxygenated (R) for another 120 min by incubation in 10 ml of oxygenated medium with added glucose. At the end of the ex-

perimental protocols, samples from the incubation media used during the reoxygenation period were collected for the assessment of creative kinase (CK) leakage and the tissue was taken for the assessment of viability (reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)). All agents tested were added for 5, 10 or 20 min at the end of the equilibration period and before the induction of SI. The doses of the agent used in the present studies (phenylephrine at 0.1 μM , adenosine at 100 μM , chelerythrine at 10 μM , 5-hydroxydecanoate at 1 mM, SB203580 at 10 μM , PMA at 1 μM diazoxide at 100 μM anisomycin at 1 nM) were selected following preliminary dose-response studies for each of the drugs.

Study groups

In Study 1, the efficacy of preconditioning via α_1 -adrenoceptors or adenosine receptors alone and in combination with IP was investigated following the protocol described in Figure 1.

Study 2 investigated the role of PKC, p38MAPK and mitoK_{ATP} channels in the cardioprotective effect of IP and of PP with phenylephrine and adenosine following the protocol described in Figure 2.

Study 3 was designed as described in Figure 3 to elucidate the sequence of the participation of involvement of mitoK_{ATP} channels, PKC and p38MAPK in the signal transduction cascade of cardioprotection.

In the three studies, n = 6 specimens from different subjects/group were used.

Assessment of tissue injury and viability

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120 min reoxygenation period. This was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma Catalogue No. 1340-K) and the results expressed as U/g wet wt.

Tissue viability was assessed by the reduction of MTT to a blue formazan product at the end of the experimental time. The absorbance of the blue formazan formed was measured on a plate reader (Benchmark, Bio-Rad Laboratories, California, USA) at 550 nm and the results expressed as mM/g wet wt [41].

Statistical analysis

All data are presented as mean \pm SEM. All values were compared by ANOVA with application of a post hoc Tukey's test. Statistical significance was taken as $p < 0.05$.

Authors' Contribution

ML participated in the design of the study, carried out all the studies, collated and analyzed the data and drafted the manuscript. MG participated in the design of the study, analysis and presentation of the data and revision of the manuscript. Both authors read and approved the final manuscript.

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