Preconditioning the human myocardium by simulated ischemia: studies on the early and delayed protection

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Abstract

Background: There are data supporting the existence of ischemic preconditioning in man. This study investigated the most effective preconditioning protocol for the human myocardium and whether the second window of ischemic preconditioning (24 h) is as protective as the first window (≤2 h). Methods and results: Right atrial appendages (n=6/group) obtained during coronary bypass surgery were prepared and superfused with normoxic and normothermic Krebs–Henseleit solution. After 30 min stabilisation, muscles were subjected to various preconditioning protocols followed by 90 min ischemia and 120 min reperfusion. At the end of each protocol, the leakage of creatinine kinase (CK, U/g wet wt) and the reduction of MTT to insoluble formazan dye (OD/mg wet wt), an index of cell viability, were measured. In study 1, preconditioning was induced by 2, 3, 5 and 10 min of ischemia followed by 5 min reperfusion. In study 2, 1–4 cycles of 2 or 5 min ischemia–5 min reperfusion were applied. In study 3, preconditioning was induced by 5 min ischemia–5 min reperfusion followed by 1, 2, 3 or 4 h reperfusion before the subsequent 90 min ischemia. In study 4, preconditioning with 5 min ischemia followed by 5 min reperfusion either immediately preceded 30 or 90 min ischemia/120 min reperfusion or was applied 24 h before. In study 1 and 2, optimal protection was achieved with 5 min or two cycles of 2 min preconditioning ischemia (CK=3.06±0.31 and 2.89±0.02; MTT=0.56±0.05 and 0.47±0.09, respectively vs. CK=5.56±0.52 and MTT=0.18±0.04 in ischemia alone group; P<0.05). In study 3, protection was observed 2 h after preconditioning (CK=3.43±0.22 and MTT=0.46±0.09; P<0.01 vs. ischemia alone group) but it was lost beyond 2 h (CK=6.30±0.56 and MTT=0.16±0.02 after 3 h; P=NS vs. ischemia alone group). In study 4, protection was observed 24 h following preconditioning when the atrial specimens were exposed to 30 min ischemia (CK=2.96±0.38 and MTT=0.61±0.01 vs. CK=4.56±0.26 and MTT=0.43±0.02 in ischemia alone group, P<0.05); however, when the period of ischemia was extended to 90 min the beneficial effect of preconditioning was lost (CK=10.28±0.5 and MTT=0.11±0.05 vs. CK=9.56±0.62 and MTT=0.10±0.05 in ischemia alone group, P=NS). Conclusions: In the isolated human myocardium maximal protection induced by preconditioning is achieved by a total 4–5 min ischemic stimulus, an effect that is lost beyond 2 h of its application. Two windows of protection were identified, the first (≤2 h) being more potent than the second (24 h). © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Ischemia; Preconditioning

1. Introduction

Brief periods of ischemia and reperfusion appear to protect the myocardium from a subsequent lethal ischemic injury. This phenomenon of ischemic preconditioning, originally described by Murry et al. [1], has been shown to exist in all animal species studied to date. There is now compelling evidence that it exists in humans. This evidence arises from in vitro experiments with human atrial trabeculae [2], ventricular trabeculae [3] and cultured ventricular myocytes [4], studies of patients undergoing planned procedures which invariably involve brief periods of ischemia such as percutaneous transluminal coronary angioplasty [5] and coronary bypass graft surgery [6]. Despite the wealth of information generated by these human studies, the most effective ischemic preconditioning protocol in man remains unknown.

In rats, rabbits, dogs and pigs, separation of the brief
preconditioning ischemic episodes from the long occlusion by 60 to 120 min results in complete or nearly complete loss of protection. However, if the duration of this separation is extended to 24 to 72 h, the infarct size will be reduced again. Hence there appears to be a distinct first (early) as well as a second (delayed) phase of protection. There is no evidence that this biphasic mode of protection exists in humans.

Although studies during angioplasty have given evidence for ischemic preconditioning in man, clearly there are practical and ethical limitations on the extent to which such situations can be used to investigate the characteristics of preconditioning in the human myocardium. In contrast, in vitro preparations allow a wide range of experimental manipulations. The aims of the present study were to investigate the most effective preconditioning protocol in human myocardium and also the existence and potency of a second window of protection. To achieve this, we subjected to simulated ischemia isolated, sliced and superfused right atrial trabeculae obtained from patients undergoing elective cardiac surgery. CK leakage and MTT reduction, an index of cell viability, were measured to assess myocardial injury.

2. Methods

2.1. Experimental preparation

Experiments were performed on trabeculae obtained from the right atrial appendage of patients undergoing elective coronary artery surgery or aortic valve replacement. Patients were excluded if they had large atriums, atrial arrhythmias, poor left ventricular function (ejection fractions <30%), right ventricular failure or were taking oral hypoglycaemic agents or opioid analgesia. Local ethical committee approval was obtained for the harvesting of specimens. The specimens were collected in oxygenated HEPES buffered solution at 4–5°C and immediately sectioned and prepared for study. Briefly, the appendage was mounted onto a ground glass plate with the epicardial surface faced down and then sliced using surgical skin graft blades (Shwann-Morton, UK) to a thickness of between 300 and 500 μm. The specimen and the slide were always kept moist throughout the procedure. The muscles (weight 30–50 mg) were then transferred to conical flasks (25 ml Erlenmeyer flasks, Duran, Astell Scientific, Kent, UK) containing 10 ml of oxygenated buffered solution. Following this, the flasks were placed in a shaking water bath maintained at 37°C. The oxygenation of the incubation medium was maintained by a continuous flow of 95% O₂–5% CO₂ gas mixture to obtain a P₀₂ between 25 and 30 kPa and a P₃CO₂ between 6 and 6.5 kPa. The P₀₂, P₃CO₂, and pH in the incubation medium were monitored by intermittent analyses of the effluent by using an automated blood gas analyser (model 855 Blood Gas System, Chiron Diagnostics) and the pH was kept between 7.36 and 7.45. For the induction of simulated ischemia, the medium was bubbled with 95% N₂–5% CO₂ (pH 6.80–7.00) and D-glucose removed (see below). In this preparation, tissue injury and viability were assessed (see below) but the atrium was not paced and the force developed was not measured.

2.2. Solutions

The incubation medium was prepared daily with deionized distilled water and contained (in mmol/l): NaCl (118), KCl (4.8), NaHCO₃ (27.2), KH₂PO₄ (1), MgCl₂(1.2), CaCl₂ (1.25), D-glucose (10) and HEPES (20). During simulated ischemia, to maintain a constant osmolality, D-glucose was removed and substituted with 2-deoxy glucose (10 mmol/l). All reagents were obtained from Sigma.

2.3. Experimental protocols

After sectioning the atrium, the preparations were allowed to stabilise for 30 min and then randomly allocated to various protocols. In most studies simulated ischemia was induced for a period of 90 min followed by 120 min of reperfusion.

2.3.1. Study 1

In this study, the effect of the duration of the preconditioning ischemic period was investigated. The preparations (n = 6/group) were preconditioned with 2, 3, 5 or 10 min of ischemia followed by 5 min of reperfusion before the 90 min long ischemic insult. Fig. 1 shows the time course for the six study groups.

2.3.2. Study 2

In this study, the effect of the number of cycles of ischemia–reperfusion for preconditioning was investigated. In study 2A, preconditioning was induced by 1 to 4 cycles of 2 min ischemia–5 min reperfusion (n = 6/group), whereas in study 2B, preconditioning was induced by 1 to 3 cycles of 5 min ischemia–5 min reperfusion (n = 6/group). Fig. 2 displays the time course for the two study groups.

2.3.3. Study 3

In this study, the duration of the initial protective effect of preconditioning (‘early protection’ or ‘first window of protection’) was investigated. The preparations (n = 6/group) were preconditioned with the protocol attaining the greatest protection in studies 1 and 2; this was one single cycle of 5 min ischemia. Then the tissues were reperfused for 1, 2, 3, or 4 h before the 90 min of ischemia. Fig. 3 shows the experimental time course for the six study groups.
Fig. 1. Experimental protocols for Study 1. All groups were equilibrated for 30 min in aerobic conditions (37°C). Following this, the right atrial slices (n = 6/group) were preconditioned (IP) with various periods of ischemia followed by 5 min reperfusion. The study groups were matched with an aerobic control and ischemia alone group.

2.3.4. Study 4

In this study, the ‘delayed protection’ or ‘second window of protection’ was investigated. We have previously demonstrated in our laboratory [7] that the human right atrial preparation used in the present studies remains viable for at least 24 h but is more sensitive to ischemia following 24 h aerobic incubation. For this reason, two periods of ischemia, 30 min (Study 4A) and 90 min (Study 4B) were studied (n = 6/group). Again the preconditioning protocol consisted of a single cycle of 5 min ischemia–5 min reperfusion. Fig. 4 shows the experimental time course.

2.4. Assessment of tissue injury and viability

At the end of each experimental protocol, tissue injury was determined by measuring the leakage of creatinine kinase (CK) into the incubation medium and tissue viability by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to blue formazan product.

2.4.1. CK leakage

The activity of CK leakage into the media during the reperfusion period (U/g wet wt) was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma Catalogue No. 1340-K).

2.4.2. MTT reduction

At the end of the experimental time, the tissue was loaded into a Falcon conical tube (15 ml, Becton Dickinson, New Jersey, USA) and 2 ml of phosphate buffer solution (0.05 M) containing MTT (1.25 mg/ml, 3 mM at final concentration) was added, incubated for 30 min at 37°C and then homogenized in 2 ml dimethyl sulfoxide (Homogenizer Ultra-Turrax T25, dispersing tool G8, IKA-Labortechnic, Staufen, Germany) at 9500 rpm for 1 min. The homogenate was then centrifuged at 1000 g for 18 min and 0.2 ml of the supernatant was dispensed into a 96-well flat-bottom microtiter plate (Nunc Brand Products, Denmark). After this, the absorbance was measured on a plate reader (Benchmark, Bio-Rad, CA, USA) at 550 nm and the results expressed as OD/mg wet wt.

2.5. Statistical analysis

All data are presented as mean±S.E.M.. All values were compared by ANOVA with application of a post hoc Tukey’s test. Statistical significance was assumed at the P<0.05 level.

3. Results

Samples were obtained from patients with stable ischemic heart disease or aortic valve disease undergoing elective coronary bypass grafting or aortic valve replacement. All samples entering the studies completed the applied experimental protocol and were included in the analysis.

3.1. Study 1 — effect of the duration of the preconditioning ischemic stimulus

As shown in Fig. 5, 90 min of ischemia resulted in a
A. Study 2A

<table>
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<th>Protocol</th>
<th>Equilibration</th>
<th>Ischemic Preconditioning</th>
<th>Ischemia</th>
<th>Reperfusion</th>
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<td>Aerobic Control</td>
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<tr>
<td>Ischemia Alone</td>
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Fig. 2. Experimental protocols for Study 2A and 2B. Right atrial slices in all groups (n=6/group) were equilibrated for 30 min. In Study 2A, the slices were then preconditioned (IP) with 1–4 cycles of 2 min ischemia – 5 min reperfusion before being subjected to 90 min of ischemia – 120 min reperfusion. In Study 2B, after equilibration, the slices were subjected to 1–3 cycles of 5 min ischemia – 5 min reperfusion preconditioning before the 90-min ischemic period.

B. Study 2B

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<th>Protocol</th>
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<th>Ischemia</th>
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significant increase in CK leakage and a decrease in MTT reduction. An inverted bell shape curve was observed for CK leakage when various periods of ischemic preconditioning were applied. 2 min of ischemia was not protective; 3 min of ischemia was the minimum period required to achieve a significant reduction in CK leakage but maximal protection was obtained with 5 min ischemic preconditioning with mean CK leakage values not significantly different from those in the aerobic control group. Surprisingly, protection was lost when ischemic preconditioning was extended to 10 min.

A mirror image to that seen for CK leakage was observed for MTT reduction. Thus, 5 min of ischemic preconditioning afforded maximal protection such that MTT reduction values were similar to those seen in the aerobic control group, and again, protection was lost when the duration of ischemia was less than 3 min or increased to 10 min.

3.2. Study 2 — effect of the number of cycles of preconditioning

Fig. 6 shows the results of preconditioning with increasing cycles of 2 min ischemia – 5 min reperfusion. The results from CK leakage and MTT reduction show that maximal protection was obtained with two cycles of 2 min ischemia. Interestingly, in this study preconditioning with one cycle of 2 min ischemia resulted in a small but statistically significant decrease in CK leakage. This result contrasts with that observed in study 1 where the enzyme leakage resulting from preconditioning with 2 min ischemia was similar to the mean values of the ischemia
alone group. This apparent contradictory result may be attributed, at least in part, to the greater CK leakage seen in the ischemia alone group in study 2, since the CK leakage mean values in the 2 min ischemic preconditioning groups were similar in both studies. Alternatively, it may suggest that CK leakage is more sensitive than MTT reduction in assessing tissue injury and that 2 min ischemia is in the threshold of protection by ischemic preconditioning, a possibility that would be supported by the absence of protection in terms of MTT reduction with 2 min of ischemic preconditioning in the two studies. As shown above, increasing the number of cycles and the total ischemic preconditioning period beyond 5 min reduced or abolished the protection seen in the results with CK leakage and MTT reduction.

Fig. 7 shows the results on CK leakage and MTT reduction of preconditioning with increasing numbers of cycles of 5 min ischemia–5 min reperfusion. Both CK leakage and MTT reduction demonstrate clearly that protection is lost beyond 5 min of ischemic preconditioning. Overall these studies show that to precondition the human atrial myocardium the most important factor is the total ischemic stimulus and not the number of cycles.

3.3. Study 3 — first window of protection

Fig. 8 shows the results on CK leakage and MTT reduction when ischemic preconditioning of atrial myocardium is followed by various reperfusion periods before the 90 min ischemia and 120 min reperfusion. The results show that the protection induced by preconditioning with only 5 min of reperfusion is maintained when the interval between the preconditioning and ischemia is within 2 h and that the beneficial effect is lost when that interval is extended to 3 or more hours.

3.4. Study 4 — second window of protection

We have shown in previous studies that the right atrial preparation used in the present experiments is viable for at least 24 h; however, after this time the preparation is more susceptible to ischemia–reperfusion injury than when incubated for shorter periods (unpublished data). For this reason, in this study two different periods of ischemia, 30 (moderate ischemia) and 90 min (severe ischemia), were used to investigate the late or second window of protection of ischemic preconditioning.

Fig. 9 shows the results with 30 min ischemia. Ischemia alone caused a significant increase in CK leakage and decrease in MTT reduction when compared with the aerobic control group. Both the first and second window of protection gave a similar decrease in CK leakage and amelioration of MTT reduction.

As shown in Fig. 10, extension of the period of ischemia to 90 min resulted in greater CK leakage and lower MTT reduction than with 30 min ischemia. As expected, the first window of preconditioning significantly improved CK leakage and MTT reduction, however, this beneficial effect was not seen with the second window of preconditioning and values were similar to those observed in the ischemia alone group.

4. Discussion

The present studies have characterised the ischemic preconditioning phenomenon in the human myocardium and have disclosed the following important results: (i) there is a graded narrow window of protection by preconditioning with 4–5 min of ischemia being the most effective period, (ii) the number of preconditioning cycles in itself does not influence protection, and (iii) as shown
A. Study 4A (with 30min ischemia)

![Diagram of Study 4A protocols]

B. Study 4B (with 90min ischemia)

![Diagram of Study 4B protocols]

Fig. 4. Experimental protocols for Study 4. Right atrial slices in all groups (n=6/group) were equilibrated for 30 min. In study 4A, after equilibration, the slices were aerobically incubated for 24 h. The ‘first window’ group were then preconditioned (IP) with 5 min ischemia–5 min reperfusion before being subjected to 30 min ischemia–120 min reperfusion. The ‘second window’ group were preconditioned initially with 5 min ischemia–5 min reperfusion before the 24 h incubation and then immediately subjected to 30 min ischemia–120 min reperfusion. In study 4B, identical protocols were applied except the ischemic time was extended to 90 min.

in other animal species, there are two windows of protection, the first (≤2 h) being more protective than the second (24 h). These results have significant clinical implications and warrant further discussion.

4.1. Intensity of the preconditioning stimulus

Our studies are the first to demonstrate that maximal protection of the human myocardium by ischemic preconditioning is obtained with an ischemic stimulus of 4–5 min. It was not surprising that shorter periods of ischemia resulted in a decrease or loss protection since studies in animals have shown that the ischemic period should be greater than 2 min to achieve protection [8,9]. However, the loss of protection with ischemic periods beyond 6 min was unexpected since several investigators have reported that 10 min of ischemia preconditions the heart of a number of animal species [10–12]. The findings that 4–5 min of ischemia is the optimal time for preconditioning is supported by studies performed in the course of percutaneous transluminal coronary angioplasty (PTCA) [13,14]. In these studies, coronary arteries are typically occluded for 2 min by balloon inflation with 5 min apart. Consistently in all studies the severity of myocardial ischemia, assessed by changes in S-T segment shifts and angina symptoms, are less during the second and third balloon inflation than during the first inflation. This suggests that a total of 4–6 min of ischemic
preconditioning also confers maximal protection in the clinical setting.

The present studies have also shown a dose–response effect in preconditioning the human myocardium so that the phenomenon should be identified as a graded rather than an all-or-nothing event. It is worth noting that the time window of protection was confined to a limited period, between 3 and 6 min of ischemia, and therefore studies involving few ischemic times may give the false impression that preconditioning is an all-or-nothing phenomenon. This thesis is supported by studies on anesthetized pigs [15] and rabbits [16] where graded ischemia determined the extent of infarct size reduction. It is also worth noting that our findings are in agreement with those of Downey et al. [17] that preconditioning ischemia <2 min did not confer protection, indicating that preconditioning has a threshold somewhere between 2 and 5 min. Their explanation was that the threshold of protection reflects the duration of ischemia required to build up adenosine levels to the point where adenosine receptors are adequately populated. It should be mentioned however that preconditioning by repeated short periods of ischemia and reperfusion may have resulted in wash-out of tissue adenosine and that in fact adenosine may not have been raised sufficiently to reach the threshold of protection. If this is the case then the mechanism of protection induced by repeated short ischemic cycles should involve the stimulation of receptors other than or in addition to adenosine receptors. Indeed Goto et al. [11] has previously suggested that the threshold of protection by preconditioning can be obtained by the additive effect of the stimulation of several membrane receptors (i.e. adenosine receptors, α1-adrenoreceptors, bradykinin and opioid receptors).

Another finding of our study that may have clinical implications is the loss of protection when the preconditioning ischemic stimulus was extended to 10 min, a time that has been reported to elicit protection in several animal species. Thus, if our results are extrapolated to clinical situations it is possible that repeated occlusions of a coronary artery during PTCA or of the ascending aorta...
results argue the conventional wisdom that preconditioning can be made more effective by increasing the number of ischemic cycles. It should be emphasised however that this argument may apply to our model of ischemia–reperfusion and that it may not necessarily be valid for shorter or longer periods of ischemia or different degrees of severity of tissue injury.

4.3. First window of protection

To the best of our knowledge, our studies are also the first to demonstrate that classical preconditioning of the human myocardium, also known as the early or first window of preconditioning, is restricted to the initial 2 h following its application, and this has obvious clinical implications. A similar response has been reported in a variety of animal species [9,20,21] supporting the view that the underlying mechanisms of the first window of preconditioning may be identical in all species. Certainly, the stimulation of membrane receptors such as A₁/A₂ and α₁-adrenoreceptors and the activation of protein kinase C and K ATP channels have been shown to be involved in the majority of the animal species studied [11,22–25] and in man [3,26]. Our finding that preconditioning is a graded phenomenon is also compatible with the notion that a number of triggers could be activated to achieve protection.

The realization that preconditioning is a phenomenon probably shared by all mammalian species studied including man and the evidence that it may be elicited through Fig. 7. Study of the number of preconditioning cycles (5 min ischemia–5 min reperfusion) on the the (A) leakage of CK and (B) MTT reduction of right atrial slices (n = 6/group). Data are expressed as mean ± standard error of mean of six experiments. *P < 0.05 vs. ischemia alone group.

during cardiac surgery totalling 10 or more min of ischemia may result inadvertently in loss of protection.

4.2. Number of preconditioning cycles

The results in Figs. 6 and 7 clearly demonstrate that the number of preconditioning cycles per se do not influence the outcome and that in fact protection is determined by the intensity of the ischemic stimulus. Clinical studies on preconditioning in the course of PTCA where changes in S-T segment shift and the severity of angina are reduced during the second and third coronary occlusion [5] may be the reflection of fulfilling the optimal ischemic stimulus (i.e., 4–6 min) rather than an effect directly promoted by the increasing number of ischemic cycles. Animal studies in which preconditioning was elicited by increasing the number of cycles but using cycles of one ischemic duration time only [1,18,19] cannot separate the effects of the number of preconditioning cycles from those corresponding to the total duration of ischemia and hence, do not support or refute the above suggestion. Therefore, our

5. Second window of protection

Our studies are again the first to demonstrate the existence of a second window of protection in the human myocardium. However, we showed that the second window is not as protective as the first window, a result that is consistent with other reports in anesthetized rabbits and dogs [27–29]. The issue is not without controversy and some investigators [30,31] have reported that in anesthetized rabbits preconditioning does not result in infarct size reduction if the ischemic insult is applied in the following 24 or 48 h. The reasons for these discrepancies are not entirely clear, but differences in the experimental preparations and protocols should be taken into account. Preliminary studies conducted in our laboratory showed that the extent of protection obtained in the second window of preconditioning was similar with one cycle and with repeated cycles of ischemia as long as the total ischemic stimulus was between 4 and 5 min (data not shown).

The contrast between the universal presence of the first
Fig. 8. Study of the duration of the initial protective effect of preconditioning (first window) on the (A) leakage of CK and (B) MTT reduction of right atrial slices (n=6/group). Data are expressed as mean±standard error of mean of six experiments. *P<0.05 vs. ischemia alone group.

window of protection and the controversial second window may suggest that the mechanism underlying the two windows are different. The cellular mechanisms underlying the second window are not fully understood at present. Some experimental evidence for the involvement of adenosine receptor stimulation and activation of protein kinase C (PKC) in the development of delayed protection has been reported in rabbits [32,33]. However, PKC could influence a host of other signal transduction pathways and it is possible that other protein kinase events play a role in the mechanism. Certainly, other end-effectors have been implicated in the second window. These include the intracellular antioxidant superoxide dismutase (SOD) [34], heat shock proteins [35] and nitric oxide synthetase [36]. At present, there is limited evidence to suggest that $K_{\text{ATP}}$ channels are involved in the delayed phase of protection [37]. It is quite clear that further research is needed in this area.

5.1. Limitations of the study and clinical implications

The present work has several limitations. First, in our preparation ischemia was induced by removing O$_2$ and nutrient substrate but toxic metabolites, usually accumulated during ischemia, freely diffused into the incubation media (simulated ischemia). We accept that there are important differences between this model and true ischemia, particularly in respect to the washout of ischemic metabolites and pH changes. Second, we used atrial tissue and any extrapolation to ventricular myocardium must be conducted with caution; however, Walker et al. [2] have suggested that identical protection can be obtained by preconditioning in both tissues. Third, right atrial specimens were obtained from patients subjected to medical treatments (e.g. nitrates, β-blockers, calcium antagonists) that potentially may themselves influence ischemia–re-
perfusion injury and the protection induced by preconditioning. Fourth, our model is an in vitro preparation and the results may not completely apply to the clinical setting, although the findings during coronary artery occlusion in the course of PTCA may suggest that protection by preconditioning can be achieved with similar protocols in both situations.

Our results have important clinical implications by revealing that the duration of the ischemic stimulus rather than the number of cycles is the most important element influencing myocardial protection by preconditioning. Furthermore, this protection is a graded phenomenon with maximal benefit with 4–5 min ischemic duration and reduction or loss of protection if the ischemic stimulus is extended beyond 5 min. We also have shown that the second window of protection by preconditioning is not as effective as the first window and that this may lessen its relevance as a potential therapeutical intervention. However, further studies may be required to confirm this latter finding.

Acknowledgements

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Fig. 10. Study of the effect of the ‘first and second window’ of preconditioning on the (A) leakage of CK and (B) MTT reduction of right atrial slices (n = 6/group) when subjected to 90 min of ischemia. Data are expressed as mean ± standard error of mean of six experiments. *P < 0.05 vs. aerobic control group; †P < 0.05 vs. ischemia alone group.

References


