Ischaemic preconditioning is not dependent on neutrophils or glycolytic substrate at reperfusion in rabbit heart

Guang Shung Liu, Alfred W H Stanley, and James M Downey

Objective: The aim was to determine whether reperfusion with either neutrophil free reperfusate or perfusate containing only pyruvate as the metabolic substrate would alter the protection against infarction afforded by preconditioning. Methods: Rabbit hearts (n=4-14 per group) underwent 30 minutes of regional ischaemia followed by 120 minutes of reperfusion. Blood reperfused groups experienced both ischaemia and reperfusion in situ while Krebs reperfused groups experienced regional ischaemia in situ but were reperfused with Krebs buffer in vitro. In another group, glucose in Krebs buffer was replaced by pyruvate. Results: Preconditioning with 5 minutes regional ischaemia caused smaller infarct size in the blood reperfused hearts: 43.0(SEM 5.4)% \(\pm\) 8.8(4.2)% in Krebs reperfused hearts, preconditioning caused a similar reduction of infarct size [49.9(2.5)% \(\pm\) 22.9(4.3)%] which was not different from that seen in the blood reperfused hearts. Replacing the glucose in the Krebs buffer by pyruvate also had no effect on infarct size in either the control or the preconditioned hearts [40.9(6.1)% \(\pm\) 11.8(5.2)%]. Histology of the ischaemic zones revealed 59.6(15.0) neutrophils per 10 high power fields in hearts reperfused in situ but only 2.6(0.6) in Krebs reperfused hearts, equal to the numbers in non-ischaemic blood perfused myocardium [2.8(0.9)]. Conclusions: The mechanism of preconditioning is not due to attenuation of neutrophil function during reperfusion. Furthermore, substituting pyruvate for glucose in the reperfusate did not prevent the protection against infarction afforded by ischaemic preconditioning.

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The in vitro reperfusion preparation also allows us to change the composition of the reperfusate. Two recent reports indicate that the protection afforded by preconditioning is lost when pyruvate, a mitochondrial substrate, is substituted for glucose in the reperfusate. 14 15 The pyruvate response would suggest that preconditioning may provide an increased glycolytic capability which allows the heart to recover in the presence of injured mitochondria. Those previous studies used the recovery of mechanical function and enzyme release as indicators of injury. In the present study we were able to repeat the experiment with infarct size as the end point.

Methods
Surgical preparation of animals
New Zealand White rabbits of either sex, weighing between 1.5 and 3.2 kg, were anaesthetised with intravenous sodium pentobarbitone (30 mg. kg\(^{-1}\)). Additional anaesthesia was given during the experiment as needed. The neck was opened with a ventral midline incision, and a tracheotomy was performed. The rabbits were mechanically ventilated with a positive pressure ventilator (MD Industries, Mobile, Alabama, USA) using 100% oxygen. Ventilation rate was 30-35 strokes min\(^{-1}\), and tidal volume was approximately 15 ml. The respiration rate was adjusted to keep the blood pH in the physiological range. Catheters, filled with heparinised saline (10 U/ml\(^{-1}\)), were placed in the left carotid artery and jugular vein for blood pressure monitoring and injecting drugs, respectively. A left thoracotomy was performed in the fourth intercostal space and the pericardium was opened to expose the heart. A 2-0 silk thread was passed around a branch of the left coronary artery with a taper needle and the ends of tie were threaded through a small vinyl tube to form a snare. The coronary branch was occluded by pulling the snare, which was then fixed by clamping the tube with a mosquito haemostat. Myocardial ischaemia was confirmed by regional

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cyanosis. At the end of the ischaemia, the hearts were reperfused for 2 h with either either blood in situ, or Krebs buffer in vitro. Hearts were reperfused with blood by simply removing the snare, and reperfusion was confirmed by the hyperaemic appearance of the region. Krebs reperfused hearts were randomly removed from the animal and mounted on a Langendorff apparatus. After 2 h with either blood in situ, or Krebs buffer in vitro. Hearts were reperfused with Krebs buffer containing (in mmol·litré⁻¹): NaCl 118.5, KCl 4.7, MgSO₄ 1.2, K₂HPO₄ 1.2, NaHCO₃ 24.3, CaCl₂ 2.5, and glucose 10. The Krebs buffer was gassed with 95% O₂ plus 5% CO₂ which results in a pH of 7.4±0.2, and a temperature of the Krebs buffer was maintained at 37°C. The perfusion pressure was maintained at 75 mm Hg. Pacing electrodes were placed in the right atrium and the isolated heart was paced at 180 beats·min⁻¹ with pulses of 5 V and 4 ms duration. A fluid filled latex balloon, which was connected to a transducer via PE240 tubing, was inserted into the left ventricle. Balloon volume was adjusted to maintain the left ventricular systolic pressure near 90 mm Hg. Total coronary artery flow was measured by timed collection of buffer from the chamber into a graduated cylinder. After 30 minutes of ischaemia the snare was released and the segment was reperfused with Krebs buffer along with the rest of the heart. All of the procedures involving the rabbits were approved by our institutional animal care and use committee.

Measurement of infarct and risk area

At the end of each in situ study the heart was quickly removed and mounted on a Langendorff apparatus. All hearts were flushed with room temperature saline for 30 min. The coronary branch was then reoccluded and the Krebs reperfused hearts along with the rest of the heart. All of the procedures involving the rabbits were approved by our institutional animal care and use committee.

Results

The data were derived from 64 rabbits. In the various groups shown in the table. There were six rabbits used for the histology studies. Haemodynamic data and infarct size for all groups are summarised in the table. There were no significant differences in heart rate, mean aortic pressure (measured at the onset of the 25 min coronary occlusion), and the size of the risk area among any of the eight groups. The infarct size, expressed as a percentage of the area at risk, averaged 43.6(SEM 5.4)% in the non-reperfused blood reperfused hearts. Preconditioning caused infarct size to be much smaller [8.8±4.2]% from the same ischaemic insult. These differences in infarct size were significant (p<0.05). The infarct size in the non-preconditioned Krebs reperfused hearts was 49.2±4.2%. Although the mean was larger than in the blood reperfused hearts, the difference was not significant. Preconditioning caused infarct sizes to be smaller in the Krebs reperfused hearts [22.9±4.1], p<0.05. There was no significant difference between infarct sizes in the preconditioned blood reperfused and Krebs reperfused hearts, or in the non-preconditioned hearts in the two groups. These data indicated that reperfusion with neutrophil free buffer did not alter infarct size nor did it block the protection afforded by preconditioning. The infarct sizes for the above four groups appear in fig 1.

Reperfusion with Krebs buffer virtually eliminated neutrophils from the ischaemic zone. Histology of the ischaemic zone revealed 59.6±15 neutrophils per 10 high power fields in blood reperfused hearts but only 2.8±1.6 in Krebs reperfused hearts. Figure 2 shows that the neutrophil count in non-ischaemic blood perfused myocardium was 2.8±2.6 per 10 high power fields. An unexpected finding in this study was the observation that removing neutrophils did not alter infarct size in non-preconditioned hearts. We were concerned that the Ca²⁺ in the Krebs buffer might be contributing to calcium loading and thus mask any protective effect derived from removing the neutrophils. To test this hypothesis we examined two groups in which Ca²⁺ was either increased or decreased. Halving reperfusion Ca²⁺ to 1.25 mM (group 5) or doubling it to 5.00 mM (group 6) had no effect on infarct size in non-preconditioning hearts.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Preischämie</th>
<th>HR (beats·min⁻¹)</th>
<th>AOP (mm Hg)</th>
<th>25 min occlusion</th>
<th>AOP (mm Hg)</th>
<th>AOP (mm Hg)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON-B</td>
<td>5</td>
<td>266(17)</td>
<td>87(15)</td>
<td>244(22)</td>
<td>77(6)</td>
<td>0.42(0.09)</td>
<td>0.18(0.05)</td>
<td>43(5.4)</td>
</tr>
<tr>
<td>CON-K</td>
<td>5</td>
<td>229(12)</td>
<td>72(8)</td>
<td>234(8)</td>
<td>63(4)</td>
<td>0.49(0.06)</td>
<td>0.30(0.05)</td>
<td>43(5.4)</td>
</tr>
<tr>
<td>PC-K</td>
<td>14</td>
<td>257(8)</td>
<td>79(2)</td>
<td>244(9)</td>
<td>65(3)</td>
<td>0.50(0.04)</td>
<td>0.12(0.05)</td>
<td>22(9.2)</td>
</tr>
<tr>
<td>PC-Y</td>
<td>5</td>
<td>295(15)</td>
<td>84(4)</td>
<td>249(17)</td>
<td>71(5)</td>
<td>0.59(0.03)</td>
<td>0.27(0.03)</td>
<td>46(14.4)</td>
</tr>
<tr>
<td>DCa-K</td>
<td>5</td>
<td>259(14)</td>
<td>81(5)</td>
<td>253(8)</td>
<td>78(3)</td>
<td>0.75(0.06)</td>
<td>0.32(0.06)</td>
<td>40(6.1)</td>
</tr>
<tr>
<td>DCa-Y</td>
<td>6</td>
<td>269(9)</td>
<td>83(4)</td>
<td>262(9)</td>
<td>76(4)</td>
<td>0.77(0.14)</td>
<td>0.11(0.06)</td>
<td>11.8±5.2</td>
</tr>
</tbody>
</table>

HR=heart rate; AOP=aortic pressure; I/R=infarct size to area at risk; CON=control; PC=preconditioning; B=reperfused with blood; K=reperfused with Krebs; HCa=replacing Ca²⁺; DCa=doubling Ca²⁺; PP=pyrovanadate; *p<0.01 v CON-B; tp<0.05 v CON-K; t*p<0.05 v CON-PY.
Lack of dependence of ischaemic preconditioning on neutrophils or glycolytic substrate

Figure 1 Ischaemic hearts reperfused with blood in situ are shown on the left and those reperfused ex vivo with Krebs buffer are shown on the right. Infarct size, expressed as a percent of the risk zone infarcted, is plotted. Open symbols indicate individual experiments while the mean and SEM for each group is shown by the filled symbol. PC=preconditioning.

Figure 2 Neutrophils in the ischaemic and normal flow zones of the hearts reperfused with either blood or Krebs buffer. Neutrophils were counted manually in PAS stained histological sections. HPF=high power field. *p<0.05 v control.

Figure 3 Infarct size in the hearts reperfused with Krebs buffer over a broad range of Ca²⁺ concentration. There is no significant difference in infarct size among the 1.25 mM, 2.5 mM, and 5.0 mM Ca²⁺ groups.

Figure 4 Infarct size in the hearts reperfused with Krebs buffer containing either pyruvate or glucose. Substituting pyruvate for glucose in Krebs buffer had no effect on infarct size in non-preconditioned hearts and did not block protection in preconditioned hearts. Con=control; PC=preconditioning; GLU=glucose; PY=pyruvate. *p<0.05 v control.

Discussion

Our data do not support the hypothesis that preconditioning protects the myocardium from ischaemia by attenuating neutrophil function during reperfusion. We found that reperfusing the hearts with Krebs buffer, which effectively eliminated the accumulation of neutrophils in the ischaemic zone, did not alter the protection conferred by preconditioning. Recent data indicate that preconditioning may be mediated by adenosine. Because adenosine has been shown to inhibit neutrophil function, one of the aims in this study was to determine whether neutrophils might play a role in the protection afforded by preconditioning. Because the protective effect of preconditioning was not altered by elimination of neutrophils, we must conclude that preconditioning does not protect by attenuating neutrophil function.

It has been reported in several recent studies that isolated Krebs perfused hearts could be preconditioned, with protection reflected in the recovery of mechanical function and enzyme release following a standard ischaemic insult. It was also observed that protection would be lost if the hearts were reperfused with a buffer containing pyruvate in place of the glucose. These data suggest that ischaemia causes a defect in the mitochondria. The preconditioned hearts appeared to be able to provide additional metabolic support through glycolysis when glucose was present, which allowed the myocytes to survive a critical convalescent period. The proposed explanation was that hearts reperfused with the mitochondrial substrate, pyruvate, could not benefit from the added glycolytic support present in the preconditioned hearts. The present study tested that hypothesis in an infarct size model. Unfortunately, the present results did not support the theory, as preconditioning was just as protective with pyruvate as with glucose in the buffer. We have no explanation for the discrepant data except to caution that the recovery of function in the reperfused hearts reflects a combination of both necrosis and stunning.

Substituting pyruvate for glucose did not alter infarct size in either the non-preconditioned [40.9(6.1)%, respectively]. The calcium data appear in fig 3.

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The relative contributions are always unknown and factors which affect stunning may be quite different from those affecting necrosis. It would appear that pyruvate may tended to be larger in the Krebs reperfused hearts, although which affect stunning may be quite different from those both preconditioned and non-preconditioned hearts which infarct sizes to hearts reperfused in situ. In fact the infarcts component might be is not apparent but it does not appear to be calcium as changes in the calcium concentration of the reperfusate had little effect on infarct size. The alternative explanation, of course, is that neutrophils do not contribute to necrosis in the reperfused rabbit heart.

It has been proposed that activated neutrophils contribute to reperfusion injury by either plugging the microcirculation or attacking the myocytes in a process termed phagocytosis. Chemotactic substances are generated in the tissue during myocardial ischaemia. At reperfusion, neutrophils respond to these chemotactic signals and enter the myocardium in quantity. While the severity of injury is usually in proportion to the quantity of neutrophils present, a cause and effect relationship has been difficult to prove. While activated neutrophils clearly can injure myocytes in vitro, the key question has been whether activated neutrophils attack otherwise recoverable tissue to kill a significant quantity of myocardium during reperfusion. Unfortunately, it has been impossible to make an animal completely neutropenic or to eliminate neutrophils entirely from the infarct and for this reason none of the evidence supporting the neutrophil hypothesis has offered clear proof of the theory. Therefore we cannot exclude the possibility that neutrophils may contribute to cell death in the reperfused rabbit heart.

We used tetrazolium staining after only two hours of reperfusion to visualise the infarcts in the present study. It is our experience that tetrazolium as used here is an accurate indicator of infarct size in the untreated heart but that certain interventions such as superoxide dismutase may alter the ability of the method to distinguish between living and dead tissue. The salvage induced with preconditioning in the rabbit does seem to be accurately indicated by tetrazolium, however. When infarct sizes were measured in control and preconditioned hearts by histology after three days of reperfusion, infarct sizes in both groups were virtually identical to those seen here.

In conclusion, we find that 30 minutes of in situ ischaemia followed by two hours of in vitro reperfusion with Krebs buffer virtually eliminates neutrophils from the ischaemic zone but has no effect on infarct size in either non-preconditioned or preconditioned hearts. This argues against a modulation of neutrophil mediated injury as a mechanism of preconditioning. Substituting pyruvate for glucose in the reperfusate also failed to alter infarct size in either non-preconditioned or preconditioned hearts.

For invited correspondence on this paper, see p 1226.

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Key terms: preconditioning; neutrophils; metabolism; pyruvate; myocardial infarction.

18 Cave AC, Downey DM, Hearn DJ. Adenosine fails to substitute for preconditioning in the globally ischemic isolated rat heart. (Abstract) J Mol Cell Cardiol 1991;23(supp III):s-76.