Reactive oxygen species generated during myocardial ischemia enable energetic recovery during reperfusion

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ROS burst at the time of reperfusion is well studied, and cardiac preconditioning (3, 28). Whereas the role of the reactive oxygen species (ROS) seen on reperfusion is considered one of the key factors in the development of myocardial stunning after regional ischemia (6). Lower levels of ROS are also generated during ischemia (4, 18, 29) and are considered an important factor in the development of cardiac preconditioning (3, 28). Whereas the role of the ROS burst at the time of reperfusion is well studied, the role of ROS generated during the global ischemia experienced with cardiac arrest is less clear (6).

Previous studies have found that in a model of prolonged cardiac arrest, antioxidant (AOX) treatment either before (30) or after (1) ischemia promoted high-energy phosphate recovery during reperfusion and improved postischemic ventricular function. In these studies, the hearts were subjected to 30 min of ischemia, a duration that probably resulted in necrosis (3, 6). It is unknown whether such AOX treatment would influence energetic recovery or contractile function after shorter periods of ischemia that do not result in necrosis. It is likely that the pathophysiology leading to postischemic contractile dysfunction is not necessarily the same in stunned and infarcted myocardium (6).

In a study analogous to global ischemia in the heart, on hypoxic skeletal muscle (7) it was found that treatment with N-acetyl-L-cysteine (NAC), which increases intracellular glutathione concentration (27), or 1,2-dihydroxybenzene-3,5-disulfonate (Tiron), a superoxide scavenger, or N-acetyl-L-cysteine, was infused during either baseline or reperfusion. An additional group received deferoxamine as a bolus before ischemia. Hearts were freeze-clamped at baseline, at end of ischemia, and at end of reperfusion for analysis of high-energy phosphates. All AOX, when given before ischemia, inhibited recovery of ATP compared with controls. Both Tiron and deferoxamine also inhibited recovery of phosphocreatine. AOX given before ischemia decreased the efficiency of contraction during reperfusion compared with controls. All of the changes in energetics and efficiency brought on by preischemic AOX treatment could be blocked by a preconditioning stimulus. This suggests that reactive oxygen species, which are generated during ischemia, enhance bioenergetic recovery by increasing the efficiency of contraction.

N-acetyl-L-cysteine; Tiron; efficiency; myocardial stunning
Rats were anesthetized using an intraperitoneal injection of pentobarbital sodium (50–65 mg/kg). The right superficial jugular vein was isolated and heparin (1,000 U/kg) was administered. The trachea was cannulated and the rats were ventilated with room air with the use of a rodent ventilator. A midsternal thoracotomy was performed and a steel cannula (3–4 mm outside diameter) was placed through an aortotomy into the aorta and secured with a suture. While in situ, hearts were immediately perfused with Krebs-Henseleit bicarbonate buffer with the addition of 0.2 mM caprylic (octanoic) acid at a constant pressure of 85 mmHg. Buffer was bubbled with 95% O2-5% CO2 to attain a pH of 7.4. The heart was quickly excised from the chest and transferred to a Langendorff apparatus while continuously perfused. Perfusion pressure was maintained at 85 mmHg with 37°C Krebs-Henseleit buffer. A water-filled latex balloon that was attached to a pressure transducer by a stainless steel gavage needle was inserted through the left atrium into the left ventricle for measurement of left ventricular (LV) pressure. The heart was submerged in a jacketed, temperature-controlled glass chamber and allowed to equilibrate for 15 min. The balloon volume was set to maintain a LV end-diastolic pressure (EDP) of 5 mmHg. The signal from the pressure transducer was analyzed with the use of a heart performance analyzer (Digi-Med, Micro-Med; Louisville, KY). Developed pressure, ventricular EDP, and rate-pressure product (RPP; product of LV developed pressure and heart rate) were measured.

A blood gas analyzer (IL Critical Care Laboratory Synthesis 45) was used to measure Po2 of the perfusate at the level of the aortic cannula and the effluent from the pulmonary artery. The difference between the two measurements was used to calculate oxygen consumption (MVo2). The ratio of RPP to MVo2 was used as a measure of contractile efficiency (19, 23).

Perfusion Protocol

After a 15-min equilibration period, the hearts continued contracting during a baseline period of 10 min of perfusion with oxygenated buffer. We stopped the flow by turning a stopcock, and the hearts were then subjected to 20 min of warm ischemia. This was followed by 30 min of reperfusion at baseline perfusion pressures and Po2. This protocol does not result in myocardial infarction as measured by lack of creatine kinase release during reperfusion (M. G. Angelos, unpublished observations). The control hearts received only oxygenated buffer throughout the protocol (Fig. 1A). The experimental groups were perfused according to protocol with buffer containing 10 mM Tiron, a superoxide scavenger (14), or 4 mM NAC, a precursor of reduced glutathione (11) and a scavenger of hydroxyl radical (2). Each experimental group was further divided into two groups. One group received AOX treatment during baseline perfusion only (Fig. 1B) and the other experimental group received AOX during reperfusion only (Fig. 1C). A final group received a bolus infusion of 40 ml of 5 mM deferoxamine, an iron chelator, at the onset of ischemia (Fig. 1D) (n = 5–8 for all groups).

Metabolite Analysis

Additional experiments, separate from those done to determine functional results, were performed with the use of an identical perfusion protocol. However, the hearts were freeze-clamped at the end of baseline, the end of ischemia, and the end of reperfusion and were freeze-dried and stored at −80°C for later analysis. The hearts were ground under liquid nitrogen, and metabolites extracted with 0.6 N perchloric acid and neutralized with NaHCO3. Neutralized extracts were analyzed spectrophotometrically for lactate, phosphocreatine (PCr), ATP, Cr, and Pi, using standard methods (5, 24) (n = 6–8 for all groups).

Preconditioning

A separate group of experiments was performed in which the hearts were subjected to a preconditioning stimulus of 5 min of global ischemia, followed by 5 min of reperfusion before the onset of the 20-min global ischemia insult. The ischemic period was followed by 30 min of reperfusion. There were four preconditioned groups. Three of the groups received one of the AOXs, Tiron, NAC, or deferoxamine (Fig. 1, A–G), given as described above, during the 5 min after preconditioning and before prolonged ischemia. The fourth group received no drug treatment (Fig. 1E). An additional group of untreated, nonpreconditioned controls, separate from those run for the earlier, nonpreconditioned experiments, was also run contemporaneously (Fig. 1A). Values were n = 4 for all groups except for NAC-treated animals, where n = 6. All hearts were freeze-clamped at the end of reperfusion. Functional and metabolic analyses were performed as described above.

Data Analysis

High-energy phosphates were expressed as micromoles per gram dry weight. All values are expressed as means ± SE. Significant differences between means were determined by ANOVA, with Tukey’s or Dunnett’s post hoc tests performed, as applicable, using the JMP version 3.2.2 (SAS Institute) statistical software package. Values of P < 0.05 were considered statistically different.
RESULTS

AOX Treatment before Ischemia

High-energy phosphates. Neither Tiron nor NAC had any effect on PCr or ATP during baseline perfusion (Figs. 2 and 3). The deferoxamine group was treated identically to the control group during baseline and received the AOX as a bolus immediately before ischemia and therefore baseline energetic measurements were not performed.

Treatment with any of the AOXs before ischemia had no significant effect on ATP levels at the end of ischemia compared with untreated hearts (Fig. 3). In contrast, the end of ischemia PCr was almost twice as high in the Tiron-treated hearts compared with either untreated hearts or NAC- or deferoxamine-treated hearts (Fig. 2).

An increase in glycolytic flux during ischemia is one possible explanation for the increase in PCr seen in hearts treated with Tiron before ischemia. Lactate, the product of anaerobic glycolysis, was quantified in the control and Tiron-treated hearts as an indirect measure of glycolysis. There was no significant difference between groups (data not shown).

The effect of AOX treatment on energetic status at the end of reperfusion was markedly different from that seen at the end of ischemia. Treatment with any of the AOXs before ischemia had no significant effect on ATP levels at the end of ischemia compared with untreated hearts (Fig. 3). In contrast, the end of ischemia PCr was almost twice as high in the Tiron-treated hearts compared with either untreated hearts or NAC- or deferoxamine-treated hearts (Fig. 2).

Functional results. Treatment with Tiron depressed baseline LV function, and this was reflected in significantly lower developed pressure in the Tiron group compared with all other groups (Table 1). There were no other baseline differences between groups (Table 1). There was no difference in developed pressure or EDP at the end of reperfusion compared with any groups (Table 1).

However, there was a decrease in efficiency of contraction (19, 23) during reperfusion in the groups treated with NAC and deferoxamine (Fig. 4). There was a trend toward lower efficiency in the Tiron-treated group, although this did not reach the level of statistical significance. This was also reflected in an increase in the mean O₂ consumption in the NAC and deferoxamine groups, although this change did not reach the level of significance (Table 2). None of the AOX treatments had an effect on baseline efficiency (data not shown).

Treatment With AOX Postischemia

A second set of experiments was performed in which hearts received either Tiron or NAC only during reperfusion. Neither Tiron nor NAC improved functional recovery during reperfusion (data not shown). Unlike treatment with AOX before ischemia, hearts that received AOX during reperfusion only showed no change in either end of reperfusion ATP or PCr (Fig. 3 and 4).

Treatment With Deferoxamine Before Prolonged Ischemia

Because we were unable to show improvement in postischemic contractile function with deferoxamine, as Williams et al. (30) did, we attempted to more closely replicate their study by duplicating their prolonged ischemic time in an additional group of animals. As was seen in the groups subjected to a shorter ischemic time, treatment with deferoxamine before ischemia resulted in no improvement in postischemic function compared with control hearts when both groups were subjected to 30 min of ischemia (data not shown).

Preconditioning

Additional experiments were performed to determine whether a preconditioning stimulus would alter the response of the myocardium to AOXs given before reperfusion.
ischemia. In these experiments, the AOXs were given before ischemia but the administration of each particular drug was preceded by a brief period of ischemia, as outlined in METHODS.

Both AOX-treated and -untreated hearts that were subjected to preconditioning demonstrated improvements in functional recovery compared with untreated, nonpreconditioned hearts (Table 3). There was also an increase in ATP recovery after ischemia in all preconditioned hearts, except those treated with NAC, compared with nonpreconditioned, untreated hearts (Table 3). There was also a trend toward increased PCr recovery in preconditioned hearts, except those treated with NAC, compared with nonpreconditioned, untreated hearts (Table 3). During ischemia but not during reperfusion. All of the AOXs studied, when given before ischemia, prevented bioenergetic recovery during reperfusion. The uniformity of the results in all AOX groups treated before ischemia suggests an oxidant-mediated mechanism and not a drug effect. Antioxidant treatment given only during reperfusion had no effect on bioenergetics.

Ischemic preconditioning resulted in increased contractile efficiency as well as improvements in functional and energetic recovery compared with nonpreconditioned hearts. When comparing only preconditioned hearts, the presence of AOX before ischemia resulted in no changes in contractile efficiency or energetic recovery compared with untreated hearts.

**DISCUSSION**

In this study, none of the AOXs given either before or after ischemia attenuated postischemic contractile dysfunction. When given before ischemia, Tiron increased the PCr available to the myocardium only during ischemia but not during reperfusion. All of the AOXs studied, when given before ischemia, prevented bioenergetic recovery during reperfusion. The uniformity of the results in all AOX groups treated before ischemia suggests an oxidant-mediated mechanism and not a drug effect. Antioxidant treatment given only during reperfusion had no effect on bioenergetics.

**Functional Results**

The lack of functional improvement with AOX treatment was unexpected. After initial experiments with Tiron, NAC, and deferroxamine showed no functional improvement, additional experiments using a bolus of deferroxamine before 30 min of ischemia were performed. This protocol was previously shown to result in improved functional and bioenergetic recovery in perfused rabbit hearts (1). Similar to the results with AOX treatment before 20-min ischemia, this provided no attenuation of contractile dysfunction in our model, leading to the conclusion that the functional response to AOX is not ubiquitous and may be species dependent.

It is currently accepted that AOX treatment attenuates myocardial stunning (6). However, review of the literature reveals several other examples of the failure of AOXs to preserve postischemic contractile function (22, 26, 27). The buffer-perfused rat heart has also been shown to have a variable response to AOXs as protective agents against myocardial stunning (9, 25). It has been suggested that these variable results are secondary to differences in experimental protocol (10).

**Table 1. Developed pressure and end-diastolic pressure measured at the end of reperfusion in hearts that received antioxidant before ischemia**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Developed Pressure, mmHg</th>
<th>EDP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Reperfusion</td>
<td>Baseline</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>110 ± 7</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>Tiron</td>
<td>5</td>
<td>79 ± 10*</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>NAC</td>
<td>10</td>
<td>118 ± 6</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>Deferoxamine</td>
<td>6</td>
<td>107 ± 10</td>
<td>69 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. EDP, end-diastolic pressure; NAC, N-acetyl-L-cysteine. *P < 0.05, ANOVA.

**Table 2. Oxygen consumption at the end of reperfusion in hearts given the indicated treatment before onset of ischemia**

<table>
<thead>
<tr>
<th>Group</th>
<th>O$_2$ Consumption, ml · min$^{-1} ·$ wet wt$^{-1}$</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>0.143 ± 0.023</td>
</tr>
<tr>
<td>NAC</td>
<td>0.197 ± 0.020</td>
</tr>
<tr>
<td>Tiron</td>
<td>0.110 ± 0.030</td>
</tr>
<tr>
<td>Deferoxamine</td>
<td>0.192 ± 0.025</td>
</tr>
</tbody>
</table>

Values are means ± SE. P = 0.08, ANOVA.
For example, whereas we observed no protective functional effect of NAC, Dhalla et al. (10) were able to demonstrate improvements in postischemic function with NAC. However, whereas we administered the AOX either before or after ischemia, Dhalla et al. (10) perfused both before and after ischemia and used a substantially decreased dose of NAC.

**Energetic Results**

Treatment with Tiron before ischemia led to an increase in available PCr at the end of the ischemic period. Glycolysis can be inhibited by ROS (16, 17) and therefore the increase seen could be secondary to increased energy production. If so, it would have been expected that lactate levels would increase with Tiron treatment by preserving glycolysis, but this was not observed. We therefore feel it is unlikely that the changes in energetics at the end of ischemia are secondary to increases in glycolysis, although a direct measurement of glycolytic flux has not been made.

An alternative explanation is that the hearts treated with Tiron consumed less energy during the ischemic period. For example, if ROS impaired the integrity of the cell membrane, more energy would be consumed maintaining ion gradients. If attenuation of ROS by Tiron prevented this breach in membrane integrity, there would be less demand for energy consumption by active ion transport.

**End of Reperfusion Energetics**

Contrary to what was seen at the end of ischemia, all AOX treatments, when given before ischemia, showed an inhibition of high-energy phosphate recovery during reperfusion. There was no effect on energetic recovery during reperfusion when the AOX was given only during reperfusion.

This suggests that the low levels of ROS generated during ischemia (4, 29), as opposed to the large burst seen at reperfusion (12), may play a signaling role that promotes energetic recovery during reperfusion. One possibility is that, during ischemia, the low levels of ROS lead to an increased rate of energy production during reperfusion. For example, although (as mentioned earlier) in some systems high levels of ROS inhibit glycolysis, exposure to low levels of oxidants is known to stimulate glycolysis (13, 15, 20).

An alternative explanation is that ROS generated during ischemia led to a decreased energetic cost of contraction during reperfusion. This is supported by our data, which demonstrate a decreased efficiency of contraction during reperfusion in hearts treated with AOX. One possible explanation for changes in efficiency is suggested by work done in the area of cardiac preconditioning. It has been shown that ROS generated during an initial ischemic insult are necessary for the induction of the preconditioned state (3, 28). It is thought that the mechanism by which ROS stimulate preconditioning is through the activation of protein kinase C (PKC) (3, 8). It has also been demonstrated that activation of PKC increases contractile efficiency (21). It is possible that ROS generated during ischemia increase efficiency during reperfusion and that this was blocked by the administration of AOX before the onset of ischemia.

This hypothesis is supported by the data from the preconditioned hearts. Applying a preconditioning stimulus abrogated any effect of AOX given before 20 min of ischemia. This implies that a pathway involved in contractile efficiency and energetic recovery was activated by preconditioning and therefore was not able to be blocked by the presence of AOX during ischemia. Activation of PKC is one attractive candidate in that it fulfills all necessary requirements. Both preconditioning and ROS (3, 8) activate PKC, and its activation leads to increased efficiency (21). However, at this time we have no direct evidence to support this mechanism over any other.

The energetic results are in conflict with prior studies, which showed an improvement in postischemic energetic recovery when AOXs were given either before ischemia (30) or on reperfusion (1). However, in these studies, rabbits were used as opposed to rats, and an ischemia time of 30 min was used. This duration of ischemia is probably associated with myocardial necrosis (3, 6) and may reflect different pathophysiological mechanisms than myocardial stunning (6).

**Preconditioning**

The ischemic preconditioning stimulus resulted in an attenuation of postischemic ventricular dysfunction and improvements in energetic recovery. One possible explanation is an attenuation of necrosis in the preconditioned hearts. However, the length of ischemia stud-
ied (20 min) is not typically considered to be associated with ischemia (6), and studies in our laboratory demonstrate no increase in creatine kinase release after 20 min of ischemia (unpublished observation). At this time we have no data to support any particular explanation for these results.

In conclusion, we have shown that AOX treatment either before ischemia or on reperfusion has no effect on postischemic contractile function in this model of global ischemia. Treatment with Tiron before ischemia increased PCR concentration at the end of ischemia. This does not appear to be through an oxidant-mediated inhibition of glycolysis. However, treatment with any of the AOX before ischemia prevented energetic recovery during reperfusion. The data suggest that the mechanism by which ROS, generated during ischemia, promote energetic recovery is through an increase in contractile efficiency. Furthermore, it appears that the ROS-mediated mechanism that led to changes in efficiency is associated with mechanisms important in ischemic preconditioning. In contrast, ROS generated during reperfusion did not appear to play a role in energetic regulation. These findings suggest a physiological, rather than pathological, role for ROS generated not only during preconditioning but also during more prolonged ischemia.

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