Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria

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Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ. Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria. Am J Physiol Cell Physiol 294: C460–C466, 2008. First published December 12, 2007; doi:10.1152/ajpcell.00211.2007.—Cardiac ischemia decreases complex III activity, cytochrome c content, and respiration through cytochrome oxidase in subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM). The reversible blockade of electron transport with amobarbital during ischemia protects mitochondrial respiration and decreases myocardial injury during reperfusion. These findings support that mitochondrial damage occurs during ischemia and contributes to myocardial injury during reperfusion. The current study addressed whether ischemic damage to the electron transport chain (ETC) increased the net production of reactive oxygen species (ROS) from mitochondria. SSM and IFM were isolated from 6-mo-old Fisher 344 rat hearts following 25 min global ischemia or following 40 min of perfusion alone as controls. H2O2 release from SSM and IFM was measured using the amplex red assay. With glutamate as a complex I substrate, the net production of H2O2 was increased by 178 ± 14% and 179 ± 17% in SSM and IFM (n = 9), respectively, following ischemia compared with controls (n = 8). With succinate as substrate in the presence of rotenone, H2O2 increased by 272 ± 22% and 171 ± 21% in SSM and IFM, respectively, after ischemia. Inhibitors of electron transport were used to assess maximal ROS production. Inhibition of complex I with rotenone increased H2O2 production by 179 ± 24% and 155 ± 14% in SSM and IFM, respectively, following ischemia. Ischemia also increased the antimycin A-stimulated production of H2O2 from complex III. Thus ischemic damage to the ETC increased both the capacity and the net production of H2O2 from complex III and sets the stage for an increase in ROS production during reperfusion as a mechanism of cardiac injury.

Mitochondrial dysfunction contributes to myocardial injury during ischemia and reperfusion (11, 35). Cardiac ischemia results in damage to the mitochondrial electron transport chain (ETC) (33, 36, 49). In the isolated, buffer-perfused rat heart, 25 min of global ischemia damages the distal ETC, with decreases in complex III activity, cytochrome c content, and respiration through cytochrome oxidase in both subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) (12, 33). The blockade of mitochondrial respiration at complex I immediately before ischemia with the irreversible inhibitor rotenone (31) or the reversible inhibitor amobarbital (12) protects the distal ETC against ischemic damage. Protection of the ETC during ischemia by the reversible blockade of electron transport with amobarbital markedly decreases myocardial injury measured following reperfusion, supporting the premise that ischemic damage to mitochondria is a key factor in myocardial injury (1, 14). The current study addressed the question if ischemic damage to the ETC was sufficient to increase mitochondrial production and release of reactive oxygen species (ROS). If so, then ischemic damage to the ETC is likely to be a significant source of the ROS produced during reperfusion that can lead to opening of the mitochondrial permeability transition pore (58) and myocyte cell death (10).

The ETC is a major source of ROS, and complex III is the dominant site for production, in the baseline state (15, 22). Inhibition of complex I with rotenone or amobarbital decreases the ROS generation during ischemia in cultured myocytes (7) and in the intact isolated heart (1), suggesting that complex III remains the major site for ROS generation during ischemia. In contrast, inhibition of electron transport at cytochrome oxidase will lead to the accumulation of reducing equivalents at upstream complexes and enhance ROS generation from complex I (13). During ischemia, the internal myocyte milieu becomes altered with hypoxia (7), increased acidification (3), and calcium loading (27), all of which may increase ROS generation in situ in the absence of actual damage to the ETC. Thus, in the present study, we used mitochondria isolated from control and ischemic hearts assessed in the presence of oxygen, physiological pH, and low calcium concentration to test: 1) whether ischemic damage of the ETC alone is sufficient to increase ROS generation and 2) whether the sites of ROS generation in the ETC following ischemia correspond to the sites of ischemic damage.

METHODS

Preparation of rat hearts for perfusion. The Animal Care and Use Committees of the Louis Stokes Cleveland Department of Veterans Affairs Medical Center and Case Western Reserve University approved the protocol. Male Fisher rats (6–8 mo of age) were anesthetized with pentobarbital sodium (100 mg/kg ip) and anticoagulated with heparin (1,000 IU/kg ip). Hearts were excised and perfused retrograde via the aorta with modified Krebs-Henseleit (K-H) buffer oxygenated with 95% O2-5% CO2 as previously described (12). Left ventricular developed pressure (LVEDP) was measured with a balloon inserted into the left ventricle. Isolated rat hearts initially were perfused for 15 min followed by 25 min global ischemia at 37°C. Hearts in the time control group were perfused for 40 min. The LVEDP

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at 40 min perfusion was maintained at 101 ± 8% of the LVDP at 15 min perfusion (n = 8). Ischemia resulted in cessation of contraction within 5 min and increased diastolic pressure [9 ± 1 mmHg at 15 min equilibration vs. 56 ± 4 mmHg at 25 min ischemia (n = 9) P < 0.01].

Isolation of SSM and IFM mitochondria. At the end of the experiment, hearts were removed from the cannula and placed into buffer A (in mM: 100 KCl, 50 MOPS, 1 EGTA, 5 MgSO4·7H2O, and 1 ATP; pH 7.4) at 4°C. Cardiac mitochondria were isolated using the procedure of Palmer et al. (43) except that trypsin was used as the protease (12, 38). Cardiac tissue was finely minced and placed in buffer A containing 0.2% bovine serum albumin and homogenized with a polytron tissue processor (Brinkman Instruments, Westbury, NY) for 2.5 s at a rotor setting of 6.0. The polytron homogenate was centrifuged at 500 g for 5 min and increased diastolic pressure [9

Mitochondria were isolated using the procedure of Palmer et al. (43) except that trypsin was used as the protease (12, 38). Cardiac tissue was finely minced and placed in buffer A containing 0.2% bovine serum albumin and homogenized with a polytron tissue processor (Brinkman Instruments, Westbury, NY) for 2.5 s at a rotor setting of 6.0. The polytron homogenate was centrifuged at 500 g, the supernatant was saved for isolation of SSM, and the pellet was washed. The combined supernatants were centrifuged at 3,000 g for 10 min at 4°C. SSM and IFM were washed twice and then suspended in 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA. Mitochondrial protein concentration was measured by the Lowry method by using bovine serum albumin as a standard.

Mitochondrial oxidative phosphorylation. Oxygen consumption by mitochondria was measured using a Clark-type oxygen electrode at 30°C (28, 36). Mitochondria were incubated in 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH2PO4, and 1 mg defatted, dialyzed BSA/ml at pH 7.4. Glutamate (complex I substrate, 20 mM) and succinate (20 mM) plus rotenone (5 μM) (complex II substrate) were used and state 3 (0.2 mM ADP-stimulated), state 4 (ADP-limited) respiration, respiratory control ratio, rate of uncoupled respiration (0.2 μM dinitrophenol), maximal rate of state 3 respiration (2 μM ADP), and the ADP/O ratio were determined. Mitochondria were used within 5 min perfusion (n = 8). Detecting of H2O2 production. H2O2 production from intact mitochondria was measured using the oxidation of the fluorogenic indicator amplex red in the presence of horseradish peroxidase (15). Glutamate and succinate were used as complex I and complex II substrates, and the presence of substrates is the same as that used to measure oxidative phosphorylation. Decylubiquinone could not be used as a substrate for complex III due to unacceptable background fluorescence. Cyclosporine A (1 μM) was added in select assays (45).

Measurement of cytochrome content. Cytochrome contents were determined in mitochondria solubilized in 2% deoxycholate in 10 mM sodium phosphate buffer using the difference of sodium dithionite reduced and air-oxidized spectra (34, 59). Cytochrome content was not determined in one heart in ischemia group due to use of the entire sample for other assays.

Statistical analysis. Data are expressed as means ± SE. Differences among groups were compared by two-tailed Student’s t-test (SigmaStat 3.5). A difference of P < 0.05 was considered significant.

RESULTS

Mitochondrial oxidative phosphorylation. Ischemia markedly decreased the rate of state 3 and increased the rate of state 4 respiration with glutamate substrate compared with mitochondria isolated from time controls (Table 1). Ischemia also decreased succinate-stimulated respiration in both SSM and IFM (Table 1). The ADP/O ratio was not changed by ischemia. Ischemia caused cytochrome c loss from both SSM and IFM. The contents of cytochrome c (in nmol/mg protein) in SSM and IFM after ischemia were 0.14 ± 0.01 (n = 8) and 0.19 ± 0.02 (n = 8), respectively, and were markedly decreased compared with time control SSM (0.27 ± 0.03, n = 8, P < 0.01) and IFM (0.36 ± 0.06, n = 8, P < 0.01). Ischemia did not change the contents of cytochrome c1, b, and a3 (data not shown). These findings are consistent with the results of previous studies using this model (12, 34).

ETC enzyme activities. Ischemia decreased complex I activity (NADH:ubiquinone reductase) in both SSM and IFM (Table 2). The decreased activity of NADH:cytochrome c reductase (NCR, rotenone sensitive) (Table 2) also largely reflects decreased complex I activity since complex I is the rate-limiting step in this assay (23, 24, 28). NFR reflects the

Table 1. Rate of oxidative phosphorylation in subsarcomeml mitochondria and interfibrillar mitochondria in the presence and absence of ischemia using glutamate (complex I) and succinate (complex II) as substrates

<table>
<thead>
<tr>
<th></th>
<th>Glutamate (20 mM)</th>
<th>Succinate (20 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC (n = 8)</td>
<td>ISc (n = 9)</td>
</tr>
<tr>
<td><strong>Subsarcomeml mitochondria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>210±16</td>
<td>95±10</td>
</tr>
<tr>
<td>State 4</td>
<td>32±4</td>
<td>47±4</td>
</tr>
<tr>
<td>RCR</td>
<td>7.2±0.6</td>
<td>2.0±0.2*</td>
</tr>
<tr>
<td>ADP/O</td>
<td>2.95±0.05</td>
<td>3.09±0.25</td>
</tr>
<tr>
<td>DNP</td>
<td>214±25</td>
<td>101±15*</td>
</tr>
<tr>
<td><strong>Interfibrillar mitochondria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>276±15</td>
<td>159±21*</td>
</tr>
<tr>
<td>State 4</td>
<td>34±3</td>
<td>56±7*</td>
</tr>
<tr>
<td>RCR</td>
<td>8.9±1.0</td>
<td>3.1±0.4*</td>
</tr>
<tr>
<td>ADP/O</td>
<td>3.12±0.05</td>
<td>3.06±0.25</td>
</tr>
<tr>
<td>DNP</td>
<td>282±18</td>
<td>160±27*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of experiments. ISC, ischemia; RCR, respiratory control ratio; DNP, dinitrophenol (0.2 mM). When succinate was used, rotenone (7.5 μM) was present to prevent reverse electron flow to complex I. Respiration with succinate as substrate was not measured in one heart in the time control group and two hearts in the ischemia group. *P < 0.05 vs. time control.
activity of NADH dehydrogenase, the first component of complex I (41, 49). Ischemia did not decrease NFR activity in either SSM or IFM, indicating that the site of ischemic damage to complex I is located distal to the NADH dehydrogenase (Table 2). Decreased complex I activity has been observed in SSM and IFM (14) and in mixed population of mitochondria following ischemia and reperfusion (44, 49, 57). The activity of citrate synthase, a mitochondrial matrix marker enzyme that cannot occur. In mammalian heart mitochondria, complex II appears not to be a site of ROS generation (5). During succinate oxidation in the presence of rotenone, H$_2$O$_2$ is generated from complex III (15, 21, 22). Oxidizing succinate in the presence of rotenone, the amount of H$_2$O$_2$ generated by SSM and IFM following ischemia was much higher compared with control SSM and IFM, indicating that ischemia increases H$_2$O$_2$ generation from complex III (Fig. 1). CSA did not affect the rate of H$_2$O$_2$ generation in SSM and IFM with or without ischemia (data not shown).

Ischemia also increased net H$_2$O$_2$ generation from SSM and IFM in the presence of rotenone (complex I inhibitor) when compared with that of time controls (Fig. 2), indicating that ischemic damage increases the capacity for the maximal net release of H$_2$O$_2$ from complex I. There was no difference in antimycin A-enhanced H$_2$O$_2$ generation between SSM and IFM from time control (3,025 ± 142 SSM, 3,277 ± 243 IFM, n = 8) and ischemic hearts (2,539 ± 221 SSM, 3,447 ± 235 IFM, n = 9) with glutamate as the substrate. Ischemia increased the antimycin A-stimulated H$_2$O$_2$ generation from SSM with succinate plus rotenone (Fig. 2), and a similar trend to increased antimycin A stimulated H$_2$O$_2$ production was evident in IFM (Fig. 2). Thus ischemic damage also increases capability of complex III to generate ROS.

The oxidation of succinate in the absence of rotenone can generate H$_2$O$_2$ from either complex I or complex III. With succinate as the substrate in the absence of rotenone, ischemia did not increase H$_2$O$_2$ generation [SSM: time control 1,023 ± 98 pmol·mg$^{-1}$·min$^{-1}$, mg protein$^{-1}$ (n = 8) vs. ischemia 900 ± 72 (n = 9), P = not significant (NS); IFM: time control 1,610 ± 184 (n = 8) vs. ischemia 1,111 ± 83 (n = 9), P < 0.05 for a decrease]. The rate of H$_2$O$_2$ generation at complex I by reverse flow can be estimated as the difference between production with succinate alone minus the rate of succinate with rotenone (37). Thus ischemia decreased the rate of H$_2$O$_2$ generation due to reverse flow into complex I (Fig. 3) while increasing the rate of production of H$_2$O$_2$ from complex III (Figs. 1 and 2).

Aconitase is an enzyme located in the mitochondrial matrix and is sensitive to inactivation by superoxide (25). Aconitase is used as an index of increased net superoxide generation directed into the mitochondrial matrix. Ischemia did not decrease

<table>
<thead>
<tr>
<th>Subsarcolemmal mitochondria</th>
<th>Complex I</th>
<th>NCR</th>
<th>NFR</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>604±64</td>
<td>4,346±142</td>
<td>2,914±184</td>
<td>2,244±93</td>
</tr>
<tr>
<td>ISC</td>
<td>376±48*</td>
<td>3,210±239*</td>
<td>2,672±351</td>
<td>2,100±60</td>
</tr>
<tr>
<td>Interfibrillar mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>795±51</td>
<td>6,066±362</td>
<td>3,640±218</td>
<td>2,693±125</td>
</tr>
<tr>
<td>ISC</td>
<td>442±53*</td>
<td>3,861±480*</td>
<td>3,404±302</td>
<td>2,813±170</td>
</tr>
</tbody>
</table>

Values are means ± SE (in pmol·min$^{-1}$·mg protein$^{-1}$); n = 8 experiments. Complex I, NADH:cytochrome c oxidoreductase; NCR, NADH:ferricyanide oxidoreductase; CS, citrate synthase. TC, time control. *P < 0.05 vs. TC.
the activity of aconitase in SSM and IFM following ischemia and rotenone inhibition increases net H$_2$O$_2$ generation from both SSM and IFM after ischemia with glutamate as substrate, indicating that ischemia increases the capacity for H$_2$O$_2$ generation from complex I (A). In the presence of succinate plus rotenone, antimycin A increases the production of H$_2$O$_2$ in SSM, indicating that ischemia increases the capacity for H$_2$O$_2$ generation from complex III (B). Data are means ± SE. *$P < 0.05$ ischemia ($n=9$) vs. time control ($n=8$). IFM in B also exhibits a trend for increased production, but it did not reach the statistical significance.

**DISCUSSION**

In the present study, we found that ischemic damage to the ETC increased the net production of H$_2$O$_2$ from complex I and complex III. Ischemia decreased complex I activity in both SSM and IFM, supporting that damage occurs to both the proximal ETC (current study) as well as the distal ETC (14, 33). Ischemia also increased the capacity for ROS production from both complex I and complex III. Since mitochondrial damage mainly occurs during ischemia (14, 32), the increased production and release of ROS following ischemic damage to the ETC observed in the current study sets the stage for the increased ROS generation during the reoxygenation of early reperfusion (6, 11, 40).

Ischemia decreased complex I activity without alternation of NFR. Ferricyanide accepts electrons from the flavin mononucleotide (FMN) cofactor of complex I (Fig. 4) (41). Preserved NADH dehydrogenase activity during ischemia localizes the site of damage distal to FMN (Fig. 4). However, in blocks of canine myocardium incubated in plastic bags at 37°C, both complex I activity and NFR decreased (49). The inconsistency between our study and this previous study may be due to the different model of ischemia used in that study, as well as the different species used.

Complex I is one of the sites for ROS generation from the ETC, but the exact site for ROS generation within complex I remains uncertain (5, 13, 15, 20, 41). FMN in NADH dehydrogenase (29, 30), iron sulfur cluster N$_2$, and the two tightly bound ubiquinone located distal in the path of electron flow through complex I (20, 41) are all potential sites for ROS generation. In the baseline state, the inhibition of NADH dehydrogenase decreases ROS generation from complex I, indicating that the site for ROS generation in complex I is distal to FMN center (41, 50), though other investigators previously assigned the production of ROS to NADH dehydrogenase (42, 56). In the present study, ischemia increased ROS generation from complex I with unaltered NADH dehydrogenase activity, localizing the site for ROS generation distal to FMN (20, 41, 50). Rotenone blocks electron transport at the ubiquinone acceptor site (9). Rotenone inhibition increased ROS generation from complex I in the mitochondria from ischemic hearts, bracketing the site for ROS generation proximal to the ubiquinone binding site (41) (Figs. 2 and 4). These observations suggest that the N$_2$ iron-sulfur site or the tightly bound quinones within complex I are the loci for production of ROS following ischemia. In contrast, when succinate was the substrate, ischemia decreased ROS generated by reverse electron

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**Fig. 2. Chemical inhibition of complex I and complex III increases H$_2$O$_2$ generation from SSM and IFM following ischemia. Rotenone inhibition increases net H$_2$O$_2$ generation from both SSM and IFM after ischemia with glutamate as substrate, indicating that ischemia increases the capacity for H$_2$O$_2$ generation from complex I (A). In the presence of succinate plus rotenone, antimycin A increases the production of H$_2$O$_2$ in SSM, indicating that ischemia increases the capacity for H$_2$O$_2$ generation from complex III (B). Data are means ± SE. *$P < 0.05$ ischemia ($n=9$) vs. time control ($n=8$). IFM in B also exhibits a trend for increased production, but it did not reach the statistical significance.**

**Fig. 3. Ischemia decreases the production of reactive oxygen species due to reverse electron flow into complex I from complex II. The difference of net H$_2$O$_2$ production with succinate as the substrate in the presence of rotenone inhibition of complex I (Fig. 1) minus the rate of net H$_2$O$_2$ production with succinate in the absence of rotenone (text) is shown. Ischemia decreased the component of H$_2$O$_2$ production attributed to reverse electron flow from complex II into complex I. Data are means ± SE. *$P < 0.05$ ischemia ($n=9$) vs. time control ($n=8$).**
flow (Figs. 3 and 4). This finding suggests that the site of ischemic damage is close to the distal portion of complex I because it likely limits reverse electron flow into complex I. Taken together, ischemia appears to have decreased complex I activity most likely by altering the portion of the complex that contains the two tightly bound quinones (Fig. 4). There are two tightly bound quinones in complex I, Qof (fast relaxing ubisemiquinone) and Qos (slow relaxing ubisemiquinone)(41). Based on the use of forward and reverse electron flow to localize the defect within complex I (41), the site of net H2O2 production from complex I following ischemia appears likely to lie within the iron-sulfur clusters of the complex, immediately proximal to the likely site of ischemic damage at the tightly bound quinones. Further experimental studies are needed to challenge this proposal.

Complex III is the dominant site for the net production of ROS from intact mitochondria in the baseline state (15, 21, 22, 39, 51). The increased production of H2O2 in mitochondria following ischemia with succinate as the substrate in the presence of rotenone indicates that complex III remains a key site for ROS generation during ischemia. Most of the ROS presence of rotenone indicates that complex III remains a key following ischemia with succinate as the substrate in the mitochondrial membrane) (Fig. 4), whereas the Qo site of complex III release reactive oxygen species directed toward mitochondrial matrix. In contrast, the Qo site of complex III, the quinol binding site located on the outer aspect (cytosol side) of the inner mitochondrial membrane, releases reactive oxygen species toward the intermembrane space.

An increase in ROS production from complex I also can occur due to blockade of the distal ETC in the absence of complex I damage (13, 15, 55) due to increased relative reduction and electron leak from complex I (15, 41, 55). The decrease in complex III activity and cytochrome c loss with ischemia will increase ROS production from upstream complex I (13), augmenting the impact of direct ischemic damage to complex I.

In intact mitochondria, complexes I and III produce superoxide and release it to both matrix and intermembrane space aspects of the inner membrane (15, 51, 55). In the baseline state, chemical inhibition of complex I with rotenone did not increase net ROS generation with a complex I substrate, supporting superoxide production directed toward the matrix (15, 47). In the current study, rotenone inhibition increased net ROS release from complex I after ischemia, suggesting a shift in the site for ROS generation to the N2/quinol site that now favors release toward the intermembrane space. The lack of a decrease in aconitase activity during in situ ischemia also favors ROS production oriented away from the mitochondrial matrix.

Superoxide generated in the matrix cannot cross the inner membrane unless an ion channel is opened (22, 41). The mitochondrial permeability transition pore (MPTP) opening is a well-known mechanism to induce leakage across both the inner and outer membranes (17, 18). Ischemia and reperfusion...
increase the probability of MPTP opening (58). However, in the isolated mitochondria studies, results do not support MPTP as a mechanism for superoxide egress since mitochondria are isolated in the presence of EGTA and ATP, which favor MPTP closure, and cyclosporin A did not block the increased H$_2$O$_2$ production from mitochondria after ischemia. Superoxide may traverse the inner membrane via the inner membrane anion channel (IMAC) (4, 8). Unfortunately 4,4’-diisothiocyanato-stilbene-2,2’-disulfonic acid disodium salt hydrate (DIDS), which blocks the IMAC channel (4), quenched fluorescence in our system and could not be used to evaluate the contribution of IMAC. Ischemia does not substantially inactivate glutaredoxin or glutathione peroxidase in isolated SSM and IFM (53). The content of manganese superoxide dismutase in the mitochondria is increased in postischemic reperfusion (26). Thus, changes in matrix antioxidant capacity during ischemia are an unlikely explanation for the observed increases in the net production of H$_2$O$_2$ after ischemia.

In the isolated heart model, superoxide production occurs during ischemia (1, 27). Perfusion of amobarbital, a complex I inhibitor that blocks electron flux into complex III (12, 14), decreases superoxide generation during ischemia (1), suggesting that ROS were generated by the ETC and that complex III is the dominant site. Blockade of electron transport during ischemia protects mitochondria against damage, supporting that the ETC itself contributes to the genesis of ischemic mitochondrial damage (12, 31). Ischemic damage increases ROS generation from both complex I and complex III as shown in the present study, in turn providing a mechanism for increased ROS production with the reoxygenation of early reperfusion. In support of this mechanism, reperfusion of myocardium when mitochondria are protected from ischemic damage to the ETC (12) markedly decreased H$_2$O$_2$ production and substantially limited infarct size (14). Approaches to modulate mitochondrial metabolism during reperfusion that include postconditioning (19, 52, 60), direct chemical inhibition of respiration (2), and hypoxic reperfusion (46) minimize myocardial injury following ischemia and reperfusion, likely by decreasing ROS production from mitochondria that were damaged by the antecedent ischemia.

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