Reactive Oxygen Species Released from Mitochondria during Brief Hypoxia Induce Preconditioning in Cardiomyocytes

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Reactive oxygen species (ROS) have been proposed to participate in the induction of cardiac preconditioning. However, their source and mechanism of induction are unclear. We tested whether brief hypoxia induces preconditioning by augmenting mitochondrial generation of ROS in chick cardiomyocytes. Cells were preconditioned with 10 min of hypoxia, followed by 1 h of simulated ischemia and 3 h of reperfusion. Preconditioning decreased cell death from 47% to 3% to 14% ± 2%. Return of contraction was observed in 3/3 preconditioned versus 0/6 non-preconditioned experiments. During induction, ROS oxidation of the probe dichlorofluorescin (sensitive to H$_2$O$_2$) increased ~ 2.5-fold. As a substitute for hypoxia, the addition of H$_2$O$_2$ (15 μmol/liter) during normoxia also induced preconditioning-like protection. Conversely, the ROS signal during hypoxia was attenuated with the thiol reductant 2-mercaptopyrrolylglycine, the cytosolic Cu,Zn-superoxide dismutase inhibitor diethylthiocarbamic acid, and the anion channel inhibitor 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate, all of which also abrogated protection. ROS generation during hypoxia was attenuated by myxothiazol, but not by diphenyleneiodonium or the nitric-oxide synthase inhibitor L-nitroarginine. We conclude that hypoxia increases mitochondrial superoxide generation which initiates preconditioning. Furthermore, mitochondrial anion channels and cytosolic dismutation to H$_2$O$_2$ may be important steps for oxidant induction of hypoxic preconditioning.

Myocardial preconditioning was initially described as an adaptive response of the heart to brief episodes of ischemia that decreased necrosis during subsequent prolonged ischemia (1). Reactive oxygen species (ROS; e.g. superoxide, H$_2$O$_2$, hydroxyl radicals) generated from brief ischemia/reperfusion have been recognized as possible “triggers” in the initiation of preconditioning (2). Evidence for this role includes intact heart studies where exposure to superoxide or H$_2$O$_2$ caused preconditioning-like protection (2, 3), and other studies demonstrating that antioxidants abolished the induction of preconditioning (4, 5). Few studies have directly measured ROS generation during brief hypoxia or ischemia induction (6). Such direct measures are needed to clarify important questions that remain regarding the role of ROS as inducing agents, including their source, where they are metabolized, and the relative contributions of different oxidant species to the induction of preconditioning protection.

Within the intact heart, possible sources of ROS include the cardiomyocytes, endothelial cells, neutrophils, or the auto-oxidation of catecholamines (7, 8). Within cardiomyocytes, sources of ROS could include superoxide generation from NAD(P)H or other oxidases such as cytochrome P450 (9–11), the mitochondrial electron transport chain (12), or even nitric-oxide synthase under conditions where arginine is depleted (13–15). Although it is likely that superoxide is the initial oxidant generated from these systems, the relative importance of superoxide, or its reduced products H$_2$O$_2$ or hydroxyl radical, in the signal transduction system involved in preconditioning is not known. Some evidence suggests that either superoxide or hydrogen peroxide can initiate preconditioning (2, 16, 17), so it is conceivable that H$_2$O$_2$ is the active signaling agent in this process.

The purpose of our study was to investigate the role of mitochondrial ROS in the induction of hypoxic preconditioning, and to clarify which ROS are required for the preconditioning response. For this study, we used chick cardiomyocytes, which have been shown to precondition with brief hypoxia (18, 19).

**Experimental Procedures**

**Cell Culture and Microscope Perfusion System**

Cardiac Culture Preparation—Embryonic ventricular cardiac myocytes were prepared as described previously (20). Heart ventricles from 10-day-old chick embryos were dissected, minced, enzymatically dispersed with 0.025% trypsin (Life Technologies, Inc.), and centrifuged differentially to yield 5–6 × 10$^6$ cells/embryo. Cells (0.7 × 10$^6$) were pipetted onto coverslips, incubated, and grown into contractile layers. Synchronous contractions were seen by the third day in culture. Cultures were checked for non-muscle cell contamination (greater than 95% of cells stain with anti-myosin heavy chain monoclonal antibodies, CCM-52). Experiments were performed with 3–5-day cardiac cell cultures, at which point viability exceeded 99%.

**Perfusion Chamber—** Coverslips with synchronously contracting cells were placed inside a Sykes-Moore chamber (1.2-ml volume, Belco Glass Inc., Vineland, NJ). The chamber and inflow tubing were maintained at 37 °C. Flow rate (0.25 ml/min), pH, and oxygen tension (PO$_2$) of the perfusate were controlled. Hypoxic conditions were verified with an optical method of phosphorescence quenching (Oxyspot, Medical Systems Inc, Greenvale, NY) (21). An extracellular Pd-porphine dye bound to albumin (1–10 μM) was added to the perfusate, and the PO$_2$-dependent phosphorescence decay was recorded in response to pulsed excitation light. Perfusion with hypoxic media resulted in measured PO$_2$ values of 3 torr within the chamber during steady state perfusion. Tubing supplying perfusate to the chamber was of low O$_2$ permeability, constructed of PharMed (Cole-Parmer Instrument Co., Chicago, IL) or stainless steel to minimize O$_2$ leaks.

**Perfusion Media Composition**—Standard perfusion media consisted...
of oxygenated balanced salt solution (BSS) with a PO$_2$ of 100 torr, PCO$_2$ of 40, pH of 7.4, [K$^+$] of 4.0 mM, and a glucose of 5.6 mM. Simulated ischemia consisted of BSS containing no glucose, with 2-deoxyglucose (20 mM) added to inhibit glycolysis and a [K$^+$] of 8.0 mM. This was bubbled with 80% N$_2$ gas and 20% CO$_2$ to produce a PO$_2$ of less than 3 torr, a PCO$_2$ of 144 torr, and a final pH of 6.8. Hypoxic media used for preconditioning consisted of BSS with no glucose, bubbled with 95% N$_2$ gas and 5% CO$_2$. Reperefuson was with standard media unless stated otherwise.

**Video/Fluorescent Microscopy**—Cells were imaged with an Olympus IMT-2 inverted phase/epifluorescence microscope equipped with Hoffman modulation optics to accentuate surface topology of the cells. This facilitated detection of contractile movement in the confluent layer of cells. Phase-contrast images were recorded for contraction analysis with a CCD camera. Fluorescence was measured using a cooled Hamamatsu slow-scanning PC-controlled camera (Hamamatsu, Hamamatsu City, Japan) coupled with Image-One software (Image Pro Plus) for quantification of changes in emission fluorescence. Measurements of propidium iodide (PI) fluorescence to assess membrane integrity were made using an excitation of 540 nm, with 580-nm long pass and 590-nm band pass filters. Dichlorofluorescin (DCF) fluorescence used to assess oxidant generation was measured using excitation light of 480 nm, with 510-nm long pass and 520-nm band pass filters. An additional marker of oxidant generation, dihydroethidium (DHE), which becomes oxidized and bound as the fluorescent complex ethidium-DNA, was measured using the same filter settings used to visualize PI. To prevent interference between PI and DHE oxidation measurements, separate studies were conducted with one or the other of these probes.

**Viability Assay**—Cell viability was quantified over time using the nuclear stain PI (5 µM, Molecular Probes, Eugene, OR), an exclusion fluorescent dye that binds to chromatin upon loss of membrane integrity. This method is similar in principle to trypan blue staining, and has been reported to predict the transition from reversible to irreversible cell injury in cultured cardiomyocytes (22). PI is not toxic to cells over a course of 8 h, permitting its addition to the perfusate throughout the experiment. At the end of each experiment using PI, all nuclei in a field of approximately 500 cells were stained by permeabilizing cells with digitonin (300 µM). Percent loss of viability (ie cell death) over time was expressed relative to the maximal value seen after digitonin exposure (100%).

**Measurement of Intracellular ROS Generation**—Intracellular oxidant stress was monitored by measuring changes in fluorescence resulting from intracellular probe oxidation. DHE (1–10 µM, Molecular Probes) enters the cell and can be oxidized by ROS including superoxide and/or hydroxyl radical to yield fluorescent ethidium (Eth). Eth binds to DNA (Eth-DNA), further amplifying its fluorescence (23). Eth-DNA fluorescence is generally stable, but can be decreased with severe hydroxyl radical attack (24). Thus, increases in DHE oxidation to Eth-DNA (ie increases in Eth-DNA fluorescence) are suggestive of superoxide generation (25).

The probe 2,7′-dichlorofluorescin diacetate (DCFH-DA, 5 µM, Molecular Probes) enters the cell and the acetoxy group on DCFH-DA is cleaved by cellular esterases, trapping the nonfluorescent 2,7′-dichlorofluorescin (DCF) inside. Subsequent oxidation by ROS, particularly hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical, yields the fluorescent product DCF (23). Thus, increases in DCFH oxidation to DCF (ie increases in DCF fluorescence) are suggestive of H$_2$O$_2$ or hydroxyl generation (25).

The reported specificities of these two probes for different ROS have been verified in multiple cuvette and chick cardiomyocyte experiments, and have been described previously (25).

**Cell Contraction**—Cell contractions were observed as described previously (26). The criteria for a return of contraction was met if observable contractions were seen throughout the field of cells following the 3-hour period of reperfusion. A single field of cells was monitored for contractions throughout each experiment.

**Preconditioning Protocols**

In the ischemia/reperfusion protocol, cardiomyocytes were exposed to 1 h of simulated ischemia (simultaneous hypoxia, hypercarbic acidosis, hyperkalemia, and substrate deprivation) followed by 3 h of reperfusion. Previous work has shown that this yields significant cell death during reperfusion that appears to result from oxidant injury (20, 25). To induce preconditioning, cardiomyocytes were exposed to 10 min of hypoxia (PO$_2$ = 3 torr) without glucose, followed by 10 min of normoxic recovery in BSS prior to subsequent ischemia/reperfusion. Cell viability, contraction, and oxidant generation were measured during preconditioning induction and during subsequent ischemia and reperfusion. These results were compared with non-preconditioned cells studied under identical conditions.

**RESULTS**

**Cell Death and Contractile Function in Preconditioned Versus Non-preconditioned Cells**—As reported previously, cell death in this model of simulated ischemia/reperfusion occurred primarily during the reperfusion phase, whereas minimal cell death was seen during the ischemia phase (20). After 1 h of ischemia, cell death (PI uptake) in the present study was 1.2 ± 0.1% (n = 3) in hypoxia-preconditioned cells, which was not different from controls (1.6 ± 0.3% cell death, n = 6, p = 0.51) (Fig. 1). After 3 h of reperfusion, PI uptake in hypoxia-preconditioned cells averaged 14.4 ± 2.0% versus 47.4 ± 3.3% in non-preconditioned cells (p < 0.001). In the preconditioned studies, strong contractile activity returned (3 out of 3) after 3 h of reperfusion compared with 0 out of 6 in control experiments (data not shown). Thus, treatment with 10 min of hypoxia prior to simulated ischemia/reperfusion significantly reduced cell death and enhanced the return of contraction.

**Intracellular ROS Generation during the Induction of Preconditioning**—We next tested the role of ROS generation during hypoxic preconditioning. Fig. 2 shows DCF fluorescence during preconditioning with hypoxia. Brief hypoxia caused a rapid and significant increase in ROS generation compared with controls (p < 0.001). Of note, ROS generation occurred during preconditioning hypoxia, and decreased during recovery prior to ischemia. As seen in Fig. 3 the ROS generation during hypoxia was attenuated with the thiol-reducing agent 2-mercaptothiopropionyl glycine (2-MPG, 400 µM) (p = 0.003). Inhibition of nitric-oxide synthase (NOS, a potential nitric oxide and superoxide source during hypoxia) using N-nitro-l-arginine (100 µM) (reported to inhibit both nitric oxide and superoxide...
formation from NOS (27)) increased ROS generation during preconditioning hypoxia (Fig. 3).

Fig. 3. Effect of N-nitro-l-arginine or 2-MPG on DCFH oxidation during hypoxic preconditioning. ROS generation during 10 min of hypoxic preconditioning, suggested by increased DCFH oxidation, was attenuated by 2-MPG (400 μM), added during base-line conditions for 40 min and hypoxic preconditioning. However, the NOS inhibitor N-nitro-l-arginine (100 μM) had the opposite effect, increasing DCFH oxidation and DCF fluorescence.

Role of Cytosolic Superoxide Dismutase during the Induction of Preconditioning—Mitochondria have been shown to generate superoxide via univalent electron transfer to O₂, especially at the ubisemiquinone site (29). This superoxide may be converted to H₂O₂ by superoxide dismutase (SOD) in the mitochondria or in the cytosol (Fig. 5). To test whether hypoxia generates superoxide that is dismutated by Cu,Zn-superoxide dismutase (Cu,Zn-SOD) in the cytosol, we assessed ROS generation using two fluorescent probes (DHE, 10 μM; and DCFH, 5 μM) to measure superoxide and H₂O₂ generation. The Cu,Zn-SOD inhibitor diethyldithiocarbamic acid (10 mM, DDC) was used to inhibit the cytosolic conversion of superoxide to H₂O₂ (30). As seen in Fig. 6A, DDC abolished the increase in DCF fluorescence seen during hypoxic preconditioning (p < 0.001). By contrast, DDC augmented the extent of DHE oxidation during hypoxic preconditioning (p < 0.001) (Fig. 6B). These results suggest that superoxide generated by mitochondria during hypoxic preconditioning can enter the cytosol, where it is converted to H₂O₂, and a decrease in oxidation of H₂O₂-sensitive DCFH.

To further study the importance of cytosolic SOD for the induction of preconditioning, DDC was given during preconditioning hypoxia and the effect on subsequent preconditioning protection was measured. As shown above, this inhibition should increase the lifetime of superoxide while decreasing H₂O₂ formation. Thus, if superoxide radical was sufficient to activate preconditioning protection, SOD inhibition could augment this protection. By contrast, if H₂O₂ was the active signaling species, then Cu,Zn-SOD inhibition should abolish preconditioning protection.

As seen in Fig. 7, transient addition of DDC during hypoxic preconditioning abolished preconditioning protection. No difference in PI uptake was detected at the end of ischemia/reperfusion between preconditioned cells given DDC during preconditioning and non-preconditioned cells. Moreover, there was no return of contraction in any of the preconditioned cells treated with DDC. DDC by itself was not associated with directly toxic effects. In this regard, the same extent of cell death
was seen when DDC was given prior to ischemia/reperfusion (in non-preconditioned cells) and in cells exposed to ischemia/reperfusion without preexposure to DDC (46.6 ± 8.6% cell death after ischemia/reperfusion with pre-exposure to DDC, n = 3; versus 47.4 ± 3.3% in non-preconditioned cells). Finally, cardiomyocytes exposed continuously for 4 h to DDC showed no significant increase in PI uptake and continued to exhibit vigorous contractions (results not shown).

Effects of Exogenous H$_2$O$_2$ or the Thiol Reducing Agent 2-MPG—The results with DDC suggested that H$_2$O$_2$, rather than superoxide, was primarily responsible for the induction of preconditioning. We therefore tested whether low levels of exogenous H$_2$O$_2$ given prior to ischemia/reperfusion could elicit preconditioning-like protection. Cardiomyocytes were superfused with BSS containing H$_2$O$_2$ (15 μmol/liter) for 10 min followed by a 10-min washout prior to ischemia/reperfusion. Exposure to H$_2$O$_2$ for 10 min during normoxia resulted in significant protection against cell death during subsequent ischemia/reperfusion (p < 0.001) (Fig. 8). In addition, 3/3 H$_2$O$_2$-treated groups showed a return of contraction compared with 0/6 untreated control experiments.

We next attempted to prevent preconditioning using the thiol reductant 2-MPG at a concentration shown previously to attenuate the ROS signal generated during hypoxia (Fig. 3). By maintaining the cytosolic pool of reduced glutathione, 2-MPG is presumed to enhance the scavenging of H$_2$O$_2$ (Fig. 5). Addition of 2-MPG during the first 40 min of equilibration and 10 min of hypoxic preconditioning abolished preconditioning protection (43.3 ± 4.5% cell death in 2-MPG-treated hypoxic preconditioned cells, n = 3; versus 14.4 ± 2.0% in nontreated hypoxic preconditioned cells; p < 0.01) (Fig. 8). In addition, 0/3 2-MPG-treated hypoxic preconditioned studies showed a return of contraction compared with 3/3 nontreated hypoxic-preconditioned experiments. These results further support the role of H$_2$O$_2$ in the induction phase of preconditioning in cardiomyocytes.

Role of Anion Channels during the Induction of Preconditioning—Previous reports suggest that membrane anion channels may be required for transit of superoxide across cell membranes (31), and that this transit can be inhibited by 4,4'-dithiobiocynato-stilbene-2,2'-disulfonate (DIDS) (32). As illustrated in Fig. 5, superoxide generated in the mitochondria may enter the cytosol, where it may be dismutated by Cu,Zn-SOD to H$_2$O$_2$, which then activates subsequent mediators of preconditioning. If mitochondrial anion channels are involved in superoxide movement into the cytosol, then inhibitors of those channels should attenuate H$_2$O$_2$ generation in the cytosol and prevent preconditioning protection. To test this, cardiomyocytes were superfused with BSS containing DIDS (200 μM) during 10 min of hypoxic preconditioning. As seen in Fig. 9A, DIDS during hypoxic preconditioning abolished ROS generation as measured by DCF fluorescence. DIDS given during hypoxic preconditioning also abolished preconditioning protection (Fig. 9B). DIDS exhibited no apparent toxicity, as evidenced by an absence of increased PI uptake after 4 h of superfusion under normoxic conditions (data not shown).

**DISCUSSION**

Our results show that 10 min of hypoxia in chick cardiomyocytes elicits a transient increase in ROS generation, predominantly H$_2$O$_2$. This ROS signal was attenuated by the mitochondrial site III electron transport inhibitor myxothiazol, but not NAD(P)H oxidase or nitric-oxide synthase inhibitors. These results suggest that the ROS generated during hypoxia originated from the mitochondria. Protection against subsequent ischemia and reperfusion was reversed by agents that attenuated this H$_2$O$_2$ signal. In that regard, the thiol-reducing agent 2-MPG, the cytosolic SOD inhibitor DDC, and the anion channel inhibitor DIDS all abolished preconditioning protection. Finally, transient exogenous H$_2$O$_2$ administration during normoxia induced preconditioning-like protection. We conclude that ROS participate in the signal transduction pathways involved in hypoxic preconditioning in this model. These ROS appear to originate as superoxide from the mitochondrial electron transport chain, which enter the cytosol via anion channels. There, dismutation by Cu,Zn-SOD appears to be necessary for the activation of subsequent steps involved in preconditioning protection.

**Oxidants as Signaling Molecules for the Induction of Preconditioning**

**Do Oxidants Induce Preconditioning?—**Our data are consistent with studies that have indirectly implicated ROS as signaling agents that elicit preconditioning. Most previous studies have been done in intact hearts and show that antioxidants given during ischemic preconditioning abrogate its protective effect against ischemia/reperfusion injury (4, 33). However, our study extends previous work by identifying mitochondria as the source of ROS responsible for induction, and by showing
that these ROS are generated during hypoxic preconditioning rather than at reoxygenation. Some previous studies were not able to abolish preconditioning with antioxidants (5, 34), raising the possibility that the type of antioxidant, its dose, or the timing of administration did not attenuate the oxidant signal responsible for induction. Our measurements of ROS generation indicated that the antioxidant compounds were acting as expected, and more directly support a role for ROS in the induction of hypoxic preconditioning.

Which Reactive Oxygen Species Participate in the Initiation of Preconditioning?—Our results show that hypoxic preconditioning is associated with significant oxidation of DCFH (sensitive to H₂O₂), and with significant DHE oxidation (sensitive to superoxide) when Cu,Zn-SOD is inhibited. Thus, a predominant ROS pathway during hypoxic preconditioning appears to involve H₂O₂ generated from superoxide metabolism. However, the specificity of these fluorescent probes for different reactive species is limited, so the precise delineation of each ROS is not possible. Nevertheless, the suggested role of H₂O₂ as the ROS trigger for preconditioning is supported by the observations that exogenous superoxide or H₂O₂ can produce preconditioning-like protection in the intact heart (2, 3, 35). Both superoxide and H₂O₂ have been shown to activate putative mediators of preconditioning.

FIG. 6. Effects of DDC on ROS generation during hypoxic preconditioning. DDC (1 mM) was added 20 min prior to and during 10 min hypoxic preconditioning. A, DCF fluorescence increases during hypoxic preconditioning were attenuated by DDC. B, ethidium fluorescence was increased by DDC. These results suggest that cytosolic SOD is involved in metabolizing superoxide generated by hypoxic preconditioning to H₂O₂.

FIG. 7. Effects of DDC given during hypoxic preconditioning on cell death from ischemia/reperfusion. The protection conferred by hypoxic preconditioning was abolished when DDC was added during base-line and hypoxic preconditioning stages. These results suggest that the metabolism of superoxide to H₂O₂ by cytosolic SOD is an important step in the pathway from ROS generation to preconditioning protection.

FIG. 8. Preconditioning with exogenous H₂O₂ >2 and abolition of preconditioning with 2-MPG. Preconditioning-like protection was induced by exposure to H₂O₂ (15 μmol/liter) for 10 min during normoxia prior to ischemia/reperfusion. By contrast, the protection of hypoxic preconditioning was abolished when the thiol reductant 2-MPG (400 μM) was added during base-line and hypoxic preconditioning stages, resulting in the same amount of cell death after ischemia/reperfusion compared with non-preconditioned cells.
it is difficult to know whether superoxide itself or H\textsubscript{2}O\textsubscript{2} was ing exogenous superoxide did not employ an SOD inhibitor, so drial ROS generation to preconditioning protection. channels are an important component in the pathway from mitochon-
seen with hypoxic preconditioning. These results suggest that anion tion during brief anoxia can also activate signaling
moral inflammatory mediators localized in the microcirculation may release ROS in intact heart (7). However, the chick cardiomocyte cultures used in the present study do not contain inflammatory or endothelial cells, or significant amounts of xanthine oxidase (39), so the contribution from non-cardiomocyte systems to ROS generation is unlikely. NAD(P)H and mixed function oxidases also seem unlikely as a source of ROS because DPI, an inhibitor of these flavoproteins (11), did not attenuate ROS generation during hypoxia. Also, superoxide generation by NADH oxidase would be expected to decrease as [\text{O}_2\textsuperscript{-}] decreases (11), whereas our studies revealed an increase in ROS generation during hypoxia. Regarding NOS as a potential oxidant source during induction, our results showed an increase in ROS generation when NOS was inhibited. Thus, it is unlikely that nitric oxide is the oxidant signal responsible for inducing preconditioning. In addition, both N-nitro-l-arginine and DPI have been reported to abolish superoxide generation from NOS (15, 28). Neither agent attenuated the ROS generation during hypoxic preconditioning, making NOS an unlikely source of superoxide. Our data, along with the work of others, implicate the mitochondrial electron transport chain as an important source of free radicals in isolated cells (7, 40). The mitochondrial inhibitor myxothiazol decreased the ROS generation during hypoxic preconditioning, suggesting that these ROS originated from the cytochrome b-c\textsubscript{1} segment of complex III in the respiratory chain (41). This result is consistent with work by others who have found that two segments of the respiratory chain are primarily responsible for superoxide generation: the reduced flavin mononucleotide of NADH dehydrogenase in complex I (42) and the ubisemiquinone associated with the cytochrome b-c\textsubscript{1} segment of complex III (29). Because myxothiazol abolished the ROS generation during hypoxia, and DPI (expected to inhibit the flavoprotein NADH dehydrogenase) had no detectable effect, it is likely that complex III is the predominant source of superoxide.

The data also show that the ROS generation and protection associated with preconditioning is abolished with the anion channel inhibitor DIDS. Thus, superoxide would appear to enter the cytosol via mitochondrial anion channels. Anion channels are known to transport superoxide across membranes (32), and have been described on both the outer and inner mitochondrial membranes (43, 44). No studies to date have investigated the importance of such channels to the induction of preconditioning, but further studies are needed to clarify their importance.

Although previous studies have demonstrated the potentially destructive role of ROS generated during prolonged ischemia/reperfusion (12), a growing body of data suggests that signaling levels of ROS generated by mitochondria may activate intracellular signaling cascades involved in protective responses. In this regard, recent studies have shown that mitochondrial ROS generated during prolonged, moderate hypoxia appear to participate in the reversible suppression of ATP utilization and contraction in cardiomyocytes (45). The present study extends those findings by revealing that mitochondrial ROS generated during brief anoxia can also activate signaling cascades involved in protecting cardiomyocytes from subsequent ischemia/reperfusion injury.

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