ROS and NO trigger early preconditioning: relationship to mitochondrial K\textsubscript{ATP} channel

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Lebuffe, Gilles, Paul T. Schumacker, Zuohui Shao, Travis Anderson, Hirotoro Iwase, and Terry L. Vanden Hoek. ROS and NO trigger early preconditioning: relationship to mitochondrial K\textsubscript{ATP} channel. Am J Physiol Heart Circ Physiol 284: H299–H308, 2003. First published September 26, 2002; 10.1152/ajpheart.00706.2002.—Reactive oxygen species (ROS) and nitric oxide (NO) are implicated in induction of ischemic preconditioning. However, the relationship between these oxidant signals and opening of the mitochondrial ATP-dependent potassium (mitoK\textsubscript{ATP}) channel during early preconditioning is not fully understood. We observed preconditioning protection by hypoxia, exogenous H\textsubscript{2}O\textsubscript{2}, or PKC activator PMA in cardiomyocytes subjected to 1-h ischemia and 3-h reperfusion. Protection was abolished by K\textsubscript{ATP} channel blocker 5-hydroxydecanoate (5-HD) in each case, indicating that these triggers must act upstream from the K\textsubscript{ATP} channel. Inhibitors of NO synthase abolished protection in preconditioned cells, suggesting that NO is also required for protection. DAF-2 fluorescence (NO sensitive) increased during hypoxic triggering. This was amplified by pinacidil and inhibited by 5-HD, indicating that NO is generated subsequent to K\textsubscript{ATP} channel activation. Exogenous NO during the triggering phase conferred protection blocked by 5-HD. Exogenous NO also restored protection abolished by 5-HD or \textit{N}\textsuperscript{\textminus}nitro-\textit{l}\textsuperscript{\textminus}arginine methyl ester in preconditioned cells. Antioxidants given during pinacidil or NO triggering abolished protection, confirming that ROS are generated by K\textsubscript{ATP} channel activation. Coadministration of H\textsubscript{2}O\textsubscript{2} and NO restored protection in 5-HD-treated cells, indicating that ROS and NO are required downstream from the K\textsubscript{ATP} channel. We conclude that ROS can trigger preconditioning by causing activation of the K\textsubscript{ATP} channel, which then induces generation of ROS and NO that are both required for preconditioning protection.

hydrogen peroxide; nitric oxide; ischemia; cardiomyocytes

PRECONDITIONING CON有很大的影响 in the heart. After a brief triggering stimulus is applied, the "early window" of protection begins within minutes and lasts for several hours. The persistence of protection after removal of the trigger indicates that a signal transduction pathway has been activated. Numerous triggers of preconditioning have been identified, including brief hypoxia/ischemia, opioids, ACh, bradykinin, activators of PKC, and pharmacological agents that activate the mitochondrial ATP-dependent potassium (mitoK\textsubscript{ATP}) channel (10, 13, 15, 16, 26, 39, 50). However, the signal transduction sequence activated by these triggers is not fully understood.

Reactive oxygen species (ROS) have been implicated as participants in the signaling pathway involved in preconditioning triggering (1, 2, 4, 23, 41). We previously reported (42, 50) that exogenous H\textsubscript{2}O\textsubscript{2} induces preconditioning in cardiomyocytes and that mitochondrial ROS are involved in the triggering by hypoxia or by ACh administration. However, an interesting controversy has developed regarding the relationship between the mitoK\textsubscript{ATP} channel activation and the ROS signal during triggering. On one hand, Pain et al. (31) and Forbes et al. (6) showed that activation of the mitoK\textsubscript{ATP} channel elicits an increase in ROS generation that is required for preconditioning protection. Moreover, ROS generation during ACh triggering is abolished when the K\textsubscript{ATP} channel is inhibited, indicating that ROS signaling occurs as a consequence of channel activation (50). On the other hand, K\textsubscript{ATP} channel inhibitors given during hypoxic triggering abolished protection without attenuating the ROS signal (43). These findings suggest that oxidant signals may potentially participate at multiple steps during triggering by 1) contributing to the activation of the mitoK\textsubscript{ATP} channel during hypoxic triggering and 2) being generated as a consequence of mitoK\textsubscript{ATP} opening during all forms of triggering. In either case, although ROS appear to play a central role in the signaling associated with triggering, the relationship between the mitoK\textsubscript{ATP} channel and ROS generation during the triggering of early preconditioning is not fully understood.

Reactive nitrogen species (RNS) have also been suggested to participate in the intracellular signaling during preconditioning triggering (19, 29, 34, 49). However, the requirement for RNS in the triggering of preconditioning is controversial, as some groups find evidence for the involvement of nitric oxide (NO) during the early window of protection (21, 32) but others...
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**Materials and Methods**

Cardiac culture preparation. Embryonic chick ventricular myocytes were prepared as described previously (44). Spontaneously contracting cells on coverslips were studied at days 3–5 in a flow-through chamber under controlled \(O_2\) and \(CO_2\) conditions on an inverted epifluorescence microscope (43). The chamber and perfusate were maintained at 37°C; the flow rate was continuous (0.5 ml/min). Medium consisted of a balanced salt solution (BSS) with \(P_{O_2}\) of 100 mmHg, \(P_{CO_2}\) of 40 mmHg, pH 7.4, \(K^+\) concentration (\([K^+]\)) of 4.0 meq/l, and 5.6 mM glucose. Simulated ischemia consisted of BSS without glucose, with 2-deoxyglucose (20 mM) to inhibit glycolysis and \([K^+]\) of 8.0 meq/l. This solution was bubbled with 80% \(N_2\)-20% \(CO_2\) gas to produce \(P_{O_2}\) of 7 mmHg, \(P_{CO_2}\) of 144 mmHg, and pH 6.8. Hypoxic preconditioning medium consisted of BSS without glucose bubbled with 95% \(N_2\)-5% \(CO_2\). Reperfusion was with normal medium with glucose.

**Video fluorescent microscopy.** Fluorescent images were captured with a 12-bit digital camera (Metamorph, Universal Imaging). Cell viability was quantified with Sytox Green (100 \(\mu\)M; Molecular Probes), an exclusion fluorescent dye, to signal the loss of plasma membrane integrity associated with the loss of viability. This probe is not toxic to live cells, permitting its addition to the perfusate throughout. At the end of the experiment, cells were permeabilized with digitonin (300 \(\mu\)M) to obtain a measure of 100% cell death. Cell death during the experiment was calculated as the percentage of cells exhibiting nuclear staining relative to the value after digitonin.

**NO Measurements.** Endogenous NO production was assessed with the specific probe diaminofluorescein (DAF-2, 10 \(\mu\)M; Calbiochem; Ref. 14). After the cells are loaded with the diacetate form of the dye, covalent reaction with NO causes an increase in fluorescence. Fluorescence intensity was measured over time (excitation 480 nm, emission 520 nm) and expressed in arbitrary units.

**Experimental Design.** The sequence of experimental interventions is shown in Fig. 2. Cardiomyocytes were subjected to 60 min of simulated ischemia, followed by 180 min of reperfusion. Preconditioning was triggered by hypoxia (10 min), followed by 10 min of oxygenation before ischemia-reperfusion. Alternatively, preconditioning was triggered with \(H_2O_2\) (15 \(\mu\)M), PMA (200 nM), or pinacidil (10 \(\mu\)M) for 10 min in lieu of hypoxia. The following compounds were added to the perfusate at different times: \(L\)-NAME (200 \(\mu\)M), 5-hydroxydecanoate (5-HD, 500 \(\mu\)M), Go-6976 (100 \(\mu\)M), ebselen (5 \(\mu\)M), 2-mercaptopropionylglycine (2-MPG, 400 \(\mu\)M), trifluoperazine (50 \(\mu\)M). Exogenous NO was supplied from a tank containing 1% NO-99% \(N_2\). This gas was added to the \(O_2\)-\(CO_2\)-\(N_2\) mixture used to bubble the perfusate, at a rate determined to achieve a final concentration of 200 nM of NO in the headspace gas. NO and \(N_2\) concentrations were measured in the chamber every 10 min by chemiluminescence (Bedfont Scientific). Levels of \(NO_2\) generated from NO and \(O_2\) remained <2 ppm.
RESULTS

As seen previously with this model (43), cell death was minimal during ischemia but rose to 58.1 ± 2.9% (n = 8) after 3-h reperfusion in non preconditioned cells. Preconditioning conferred significant protection regardless of whether the triggering stimulus was hypoxia (33.0 ± 2.2%, n = 10; P < 0.0001), the KATP channel agonist pinacidil (29.9 ± 2.0%, n = 5; P < 0.0001), exogenous H2O2 (36.7 ± 3.5%, n = 6; P = 0.0005), or PMA (26.2 ± 1.3%, n = 3; P = 0.0001) (Fig. 3).

Role of ROS and PKC in opening of mitoKATP channel during triggering. Hypoxic preconditioning requires ROS generation from the mitochondrion electron transport chain, and exogenous H2O2 given during normoxia also confers protection (42). If these ROS cause activation of the mitoKATP channel, then inhibitors of that channel should abrogate the protection they confer. In the present study, the mitoKATP channel inhibitor 5-HD given throughout the experiment abolished protection induced either by hypoxia (55.5 ± 1.4%, n = 4) or by exogenous H2O2 (64.9 ± 7.2%, n = 6), thereby confirming the requirement for KATP channel opening in these responses (Fig. 3A). Protection was also abrogated when 5-HD was administered only during baseline and triggering associated with hypoxic (57.3 ± 5.5%, n = 3) or exogenous H2O2 (54.0 ± 1.6%, n = 3) preconditioning. These findings implicate ROS in the signaling that occurs upstream of the mitoKATP channel during triggering (Fig. 1).

Previous investigators have proposed that PKC promotes activation of the KATP channel during triggering (17, 36, 39). In this study, cells preconditioned with the PKC activator PMA exhibited significant protection compared with nonpreconditioned cells. Addition of 5-HD throughout the study abolished this protection (Fig. 3A). Furthermore, PKC inhibition by Go-6976, a selective PKC inhibitor, blocked the protection conferred by H2O2 (P = 0.01; Fig. 3B) but did not block protection induced by pinacidil. These results are consistent with the proposed activation of PKC by ROS at a site upstream from the mitoKATP channel (Fig. 1).

Role of NO in preconditioning triggering. To clarify whether NO participates in the signaling during triggering, L-NAME was given throughout the study to inhibit NO synthase. This compound abolished the protection conferred by hypoxic preconditioning (52.7 ± 1.5% cell death, n = 5; Fig. 4A), by pinacidil (54.5 ± 3.4%, n = 5; Fig. 4B), and by exogenous H2O2 (56.1 ± 1.0%, n = 3; not shown). Preliminary studies showed that lower concentrations (50–100 µM) of L-NAME did not abolish protection (data not shown). When given only during baseline and triggering, L-NAME also abolished protection by pinacidil (52.5 ± 1.6%, n = 3) and by PMA (60.7 ± 2.2%, n = 3). Protection was also abolished when NOS was inhibited by the calmodulin inhibitor trifluoperazine given only during baseline and triggering (52.7 ± 3.7%, n = 3). These findings are consistent with the proposed involvement of NO in the signaling associated with multiple triggers, at a site that must occur downstream from the opening of the KATP channel (Fig. 1).

To clarify the requirement for NO during triggering, exogenous NO was added to the gas mixture used to bubble the media during baseline and triggering. The NO was begun before triggering to allow the NO concentration to reach the target value of 200 nM. That concentration was selected on the basis of the preliminary dose-response observation that intracellular sig-
naling was activated but that mitochondrial potential was maintained (not shown). In cells treated throughout the experiment with L-NAME, exogenous NO restored protection conferred by hypoxia (24.5 ± 4.4%, n = 4; Fig. 4A) or pinacidil (28.5 ± 2.4%, n = 4; Fig. 4B). Furthermore, this ability to restore protection was not abolished by concomitant administration of 5-HD in cells treated with L-NAME and preconditioned by hypoxia (33.1 ± 1.5%, n = 3; Fig. 4A). These findings are consistent with the involvement of NO in the triggering of preconditioning at a site downstream from the K<sub>ATP</sub> channel activation (Fig. 1).

Administration of exogenous NO to hypoxic preconditioned cells restored the protection (26.7 ± 3.1%, n = 4) that had been abrogated by 5-HD (55.5 ± 1.4%, n = 4; P = 0.0001; Fig. 4C). Similarly, protection triggered by exogenous H<sub>2</sub>O<sub>2</sub> (36.7 ± 3.5%) was abrogated by 5-HD (64.9 ± 7.2%) but was restored when exogenous NO was given only during baseline and triggering (25.1 ± 2.7%, n = 3; Fig. 4C). These findings are consistent with the proposed involvement of ROS upstream from the mitoK<sub>ATP</sub> channel and the requirement for NO downstream from that site during the triggering phase (Fig. 1).

If NO is required for the triggering of preconditioning, and if NO generation is regulated by the opening of the K<sub>ATP</sub> channel, then induction of preconditioning by either hypoxia or pinacidil should stimulate NO generation. Figure 5A shows the effects of repeated 10-min exposures to hypoxia on DAF-2, which becomes fluorescent on combination with NO. The increase in DAF-2 fluorescence was inhibited by L-NAME at 200 μM but not by lower concentrations (50 μM). The increase was also blocked by trifluoperazine, which attenuates NOS activity by inhibiting calmodulin (Fig. 5A). The increase in DAF-2 fluorescence during hypoxia was also attenuated by 5-HD (Fig. 5B). In the presence of pinacidil, this response was significantly amplified (Fig. 5C). Collectively, these observations confirm that DAF-2 is sensitive to NO production, and they support the proposed relationship between the activation of the mitoK<sub>ATP</sub> channel by hypoxia or pinacidil and the resulting stimulation of NOS activity during triggering (Fig. 1).
anism (31). To test this, exogenous NO was administered to nonpreconditioned cells during baseline and triggering. This conferred significant protection (Fig. 6A), which was abrogated by the antioxidant 2-MPG. Protection by NO was also abolished when 5-HD was given during baseline and triggering. This is consistent with the ability of exogenous NO to activate the mito-KATP channel (35).

Compared with nonpreconditioned cells (58.1 ± 2.9%, n = 10), exogenous NO only partially restored protection in PMA-preconditioned cells whose mito-KATP channels were inhibited by 5-HD (46.7 ± 0.8%, n = 3, P = 0.04; Fig. 6B). However, exogenous NO plus H₂O₂ during triggering completely restored protection conferred by PMA in 5-HD-treated cells (n = 3; Fig. 6B). These findings are consistent with the proposed requirement for both NO and ROS in signaling distal to the KATP channel (Fig. 1).

To confirm the requirement for ROS downstream from the KATP channel, ebselen, a glutathione peroxidase mimic, was administered only during 10-min pinacidil triggering. Ebselen accelerates conversion of H₂O₂ to H₂O (38), and it abolished the protection conferred by pinacidil (53.0 ± 2.0%, n = 3; Fig. 6C). To further confirm the requirement for ROS downstream from the mitoKATP channel, 2-MPG was administered with the same protocol. 2-MPG also abrogated protection in pinacidil-treated cells (n = 3; Fig. 6C). These findings are consistent with the proposed involvement of ROS in signaling downstream from the KATP channel (Fig. 1).

**DISCUSSION**

This study examined the relationships among ROS, NO, PKC, and the mitoKATP channel during the triggering of preconditioning. Preconditioning by hypoxia, H₂O₂, or PMA conferred protection that was blocked by mitoKATP channel inhibition (Fig. 1). Therefore, the signals activated by these triggers must precede opening of the KATP channel. The data also reveal that NO is required during triggering, based on the observations that 1) hypoxia-, H₂O₂-, or PMA-induced protection was blocked by NOS inhibitors, 2) exogenous NO restored protection that had been abrogated by NOS inhibition, and 3) fluorescence of the NO-sensitive probe DAF-2 increased during triggering. The stimulation of NO production during triggering must have been a consequence of KATP channel activation because 1) inhibitors of that channel attenuated the DAF-2 response, 2) protection induced by pinacidil was abolished by NOS inhibition, 3) pinacidil was associated with an augmentation of DAF-2 fluorescence, and 4)
exogenous NO restored protection that had been abolished by 5-HD treatment in preconditioned cells.

These conclusions are based on the assumption that the inhibitors used in the study were specific for their intended targets. Several pharmacological agents used in the study of ischemic preconditioning have been shown to act at nonspecific sites, which could confound interpretation of this study. For example, Hanley et al. (11) recently reported that 5-HD acts as a substrate for acyl-CoA synthetase, that pinacidil selectively inhibits NADH oxidation, and that diazoxide can inhibit succinate dehydrogenase. Although it is not clear how these nonspecific targets could explain the effects of these drugs on preconditioning protection, it is clear is that future confirmation of the findings of our study will require the use of more definitive tools.

ROS signaling upstream of KATP channel. We (42) and others (1, 2, 41) have found that oxidant signals contribute to hypoxia-induced preconditioning. The ROS signal during hypoxic triggering was abolished by complex III inhibitors, which implicates the mitochondria in the oxidant generation (42). ROS signals appear to contribute to the activation of the KATP channel during certain types of preconditioning. Recently it was shown that superoxide can activate reconstituted mitoKATP channels in a planar lipid bilayer (51). In our study, hypoxia- or H2O2-induced protection was abolished by 5-HD given either throughout the study or only during the triggering phase. However, KATP channel inhibition did not abolish the ROS signal during hypoxic triggering (42). Therefore, at least some of the oxidant signaling during hypoxic triggering does not require opening of the KATP channel (Fig. 1).

Requirement for ROS downstream from mitoKATP channel. Data also suggest that KATP channel opening causes ROS generation. Pain et al. (31) found that antioxidants blocked protection induced by diazoxide, whereas Forbes et al. (6) found direct evidence of ROS generation in response to that KATP channel opener. Our finding that antioxidants abolished pinacidil-induced protection is consistent with their conclusions. During ACh-induced preconditioning, Yao et al. (50) found that KATP channel inhibition abolished both protection and the oxidant signal during triggering, suggesting that ROS must have been generated downstream from that channel. In cells preconditioned with opioid agonists, 5-HD also abolished the ROS signals and protection during triggering (23). Therefore, preconditioning by KATP channel agonists or certain receptor-mediated triggers causes KATP channel opening and subsequent ROS signaling. Hence, with certain triggers ROS do not appear to be involved until after the KATP channel opens (Fig. 1).

Relationship between ROS and PKC activation. The targets of PKC during the induction of preconditioning are not known. The notion that oxidants precede PKC activation is consistent with the observation that ROS can activate PKC (8, 37). Moreover, PKC inhibitors block protection without altering the ROS signal during hypoxic triggering (43), indicating that PKC acts upstream from the KATP channel but downstream from

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**Fig. 6.** Cell death and the effects of preconditioning. A: exogenous NO conferred significant cardioprotection (n = 3) that was blocked by 5-HD (n = 4) or by 2-MPG (400 μM, n = 3) *P < 0.001 vs. control. B: 5-HD given throughout the study abolished PMA-induced protection (200 nM, n = 3). Protection was partially restored by exogenous NO (200 nM, n = 3) and was fully restored by the coinadministration of exogenous NO and H2O2 (15 μM) during triggering (n = 3). *P < 0.05 vs. control. C: pinacidil given only during triggering conferred significant protection that was abolished by the ROS scavengers eb-selen (5 μM) and 2-MPG (400 μM) (n = 3 for each group). *P < 0.0001 vs. control.
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Hyponxia-activated mitochondrial ROS generation (Fig. 1). Protection by exogenous H2O2 was abolished by PKC inhibition, indicating that ROS can potentially trigger preconditioning by promoting the activation of PKC. However, PKC inhibition did not block protection by pinacidil, indicating that PKC must be acting upstream from the KATP channel. Furthermore, protection induced by PKC activation was abolished by 5-HD, indicating that protection by PKC required the opening of the KATP channel. Collectively these observations indicate that ROS signals generated during hypoxia or H2O2 triggering are proximal to PKC activation (40). Furthermore, PKC induces protection through a mechanism requiring KATP channel activation (Fig. 1).

Liu et al. (17) reported that PKC activation can potentiate the KATP current induced by pinacidil, and Sato et al. (36) demonstrated that PKC primes the KATP channel to open earlier and more intensively during prolonged ischemia. Wang et al. (45) showed that PKC downregulation renders the KATP channel ineffective, thereby augmenting ischemic injury. These findings are consistent with a model in which PKC and the KATP channel are interrelated, with PKC acting as a regulator of that channel (Fig. 1). It is possible that ROS generated in response to KATP channel opening could augment PKC activation, which in turn could feedback to facilitate or prolong channel activity. Our findings confirm the linkage between PKC and activation of the KATP channel and extend previous work by linking this pathway to the early ROS and RNS bursts produced by various triggering agents.

Requirement for NO in early preconditioning. The role of NO in early preconditioning is controversial. In some studies, NOS inhibitors failed to abolish preconditioning-induced protection in rats (24, 46), rabbits (27, 47), and pigs (33). In the rat heart, Weselcouch et al. (46) found that protection was not blocked by L-NAME at 30 μM, yet Lochner et al. (19) were able to block protection with 50 μM. Nakano et al. (27) found that NO donors induced preconditioning in the rabbit heart but were unable to abolish protection with L-NAME at 100 μM. We found that L-NAME inhibited protection at 200 μM, but preliminary studies failed to show any effect at 50 or 100 μM. Thus dose-dependent effects of NOS inhibitors may explain, at least in part, the negative results observed in some previous reports.

Our data indicate that NO is generated during triggering, and the resulting signal is required for protection. Endothelial type NOS is constitutively expressed in cardiomyocytes (3, 25), and an isoform of NOS similar to the endothelial isoform is expressed in cardiac mitochondria (7, 20). This places a potential source of NO in close proximity to the mitoKATP channel. In conscious rabbits, Xuan et al. (48) observed a rapid activation of calcium-dependent NOS after repeated cycles of hypoxia were used to trigger late preconditioning. Early preconditioning can be induced by the same triggering protocol (12), raising the possibility that NO generation during preconditioning involves Ca2+/calmodulin-dependent NOS activation. Our finding that protection was abrogated when NOS was blocked with the calmodulin inhibitor trifluoperazine is consistent with that possibility.

ROS and NO signals during triggering: relationship to mitoKATP channel. Our data suggest that KATP channel activation triggers NO production. First, pinacidil-induced protection was abolished by L-NAME, and protection was restored by exogenous NO during triggering. Second, hypoxia-simulated NO production was abolished by KATP channel inhibition and was amplified by KATP channel openers. These findings indicate that NO generation is regulated by the KATP channel (Fig. 1). In rabbits, Ockaili et al. (29) reported that diazoxide mimicked both early and delayed preconditioning and that L-NAME applied before the sustained ischemia blocked protection. Our conclusion that NO is a mediator of protection regulated by KATP channel activation is therefore consistent with their data.

Other studies show that exogenous NO facilitates KATP channel activation (35), which suggests that the channel can be a target of endogenous NO production. In our study, exogenous NO was protective and this effect was blocked by 5-HD, suggesting that NO protected by opening that channel (35). Activation of the mitoKATP channel also triggers ROS production (6, 31), so 5-HD may have blocked protection because it prevented the ROS generation normally triggered by mitoKATP opening. In support of this interpretation, we found that protection induced by pinacidil was reversed by the antioxidants 2-MPG and ebselen, confirming that ROS are required downstream from mitoKATP channel. Also, exogenous H2O2 given with NO conferred protection when the mitoKATP was blocked. Finally, the protective effect of exogenous NO was abolished by the antioxidant 2-MPG. Collectively these results indicate that exogenous NO can lead to opening of the mitoKATP channel. The opening of that channel then leads to the generation of ROS and NO, both of which are required for preconditioning. During hypoxic triggering, NO and ROS produced by mitoKATP channel opening could feedback on the channel itself, thereby prolonging its activation and facilitating the downstream effects, including the further generation of ROS and NO.

Requirement for ROS and RNS downstream from mitoKATP channel. Further evidence supporting the need for both ROS and NO downstream from the KATP channel came from cells preconditioned with PMA. In these cells, 5-HD blocked protection but a small degree of preconditioning was restored when exogenous NO was also given (Fig. 6B). If PMA protects by activating the KATP channel via PKC, then 5-HD should abolish protection (Fig. 1). In that situation, exogenous NO should fail to restore significant protection because of the lack of a simultaneous ROS signal. However, when both H2O2 and exogenous NO were administered, full protection was restored because both the ROS and NO arms were present. By the same reasoning, in cells preconditioned with H2O2 or hypoxia and blocked with 5-HD, exogenous NO was able to restore protection because the triggers were able to supply the necessary ROS component of signaling (Fig. 4C). Therefore, ex-
logenous H₂O₂ and NO given together confer protection even if the K<sub>ATP</sub> channel is blocked, because these molecules appear to act downstream of the channel in the hypoxic preconditioning process. We conclude that preconditioning triggers such as hypoxia or exogenous H₂O₂ can induce protection by causing activation of the mitoK<sub>ATP</sub> channel via oxidant signaling. Protection ensues when NO and additional ROS are generated as a result of K<sub>ATP</sub> channel opening. Triggers such as PMA or pinacidil can trigger K<sub>ATP</sub> opening without the need for ROS upstream from the channel. Once opened, the K<sub>ATP</sub> channel stimulates downstream production of ROS and NO, both of which are required for protection. Scavenging of either the NO or ROS signals generated downstream from the K<sub>ATP</sub> channel was sufficient to abolish protection.

Oxidant signaling during triggering versus oxidant stress at reperfusion. Our data implicate both NO and ROS in the redox signaling involved in the induction of preconditioning. We previously observed (43) a large burst of oxidative stress at the start of reperfusion. This burst was smaller in preconditioned cells, which showed an improvement in survival. This illustrates the paradoxical involvement of ROS and NO in ischemia-reperfusion injury, with small redox-dependent signals involved in the induction phase and large bursts of oxidant stress contributing to cell death during ischemia and reperfusion.

The mechanism responsible for protection in preconditioning is not known. We speculate that ROS and NO are protective because they inhibit the process of cell injury during ischemia that leads to the uncontrollable oxidant burst generated at reperfusion. Although some protection is achieved if an antioxidant cocktail is administered only during reperfusion (43), the full significance of the oxidant burst at reperfusion is not clear. Although the burst is attenuated in preconditioned cells, it is not known whether the burst represents the cause of cell death or whether it is merely a marker of irreversible injury. To the extent that cell death in this model occurs through an apoptotic pathway, it is possible that NO and ROS signals act to inhibit the release of apoptosis-inducing proteins from the intermembrane space of mitochondria or that they inhibit the activation of caspases. In either case, the degree of protection appears to be greater in the intact heart, where 60–80% reduction in cell death can be achieved with preconditioning compared with the 40–50% seen in our cardiomyocyte model. Regardless of the model used, future progress in revealing the mechanisms of preconditioning protection will require a clearer understanding of the sequence of events that commit cells to a death pathway in ischemia and reperfusion.

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REFERENCES


