Preconditioning in Cardiomyocytes Protects by Attenuating Oxidant Stress at Reperfusion

Terry L. Vanden Hoek, Lance B. Becker, Zuo-Hui Shao, Chang-Qing Li, Paul T. Schumacker

Abstract—Cardiomyocyte death after ischemia/reperfusion correlates with oxidant stress, and antioxidants confer protection in that model. Preconditioning (PC) with hypoxia or adenosine also confers protection, leading us to hypothesize that PC protects by attenuating oxidant generation during subsequent ischemia/reperfusion. Chick cardiomyocytes were preconditioned with 10 minutes of hypoxia or adenosine (100 μmol/L), followed by 1 hour of simulated ischemia and 3 hours of reperfusion. Adenosine PC decreased cell death from 50±3% to 18±4% and enhanced the return of contractions during reperfusion, as observed previously with hypoxic PC. A transient burst of dichlorofluorescein (sensitive to H₂O₂) oxidation that was significantly attenuated by PC initiated by hypoxia or adenosine was seen at reperfusion. The protein kinase C (PKC) inhibitor Go-6976 and the mitochondrial ATP-sensitive K⁺ (KATP) channel inhibitor 5-hydroxydecanoate each abolished protection and abrogated the PC-induced attenuation of reperfusion oxidant stress. By contrast, when given only at reperfusion, the K⁺ channel opener pinacidil or the antioxidants 2-mercaptopropionylglycine and 1,10-phenanthroline decreased oxidant stress at reperfusion and improved survival and return of contractions. Thus, PC protection is associated with an attenuation of the oxidant burst at reperfusion, regardless of the method by which PC is triggered. Loss of PC protection associated with PKC inhibition or KATP channel inhibitors is associated with a restoration of that oxidant stress. These results suggest a mechanism for PC protection and reveal a functional link between PKC activation and KATP channel activation in that pathway. (Circ Res. 2000;86:541-548.)

Key Words: reperfusion ■ protein kinase C ■ hydrogen peroxide ■ KATP channels ■ reactive oxygen species

Myocardial preconditioning was first described as a phenomenon whereby brief episodes of nonlethal ischemia confer protection against subsequent ischemia/reperfusion (I/R) injury in the heart.1 Since then, work directed at understanding the factors that initiate and mediate preconditioning2 has identified multiple triggers, including transient hypoxia3,4 and adenosine.5,6 Despite the description of numerous preconditioning triggers, the process by which preconditioning protects the heart from subsequent ischemia and reperfusion injury is unclear. Activation of protein kinase C (PKC) and phosphorylation of ATP-sensitive K⁺ (KATP) channels have been implicated as common participants in this protective pathway, but the specific mechanism of protection is not known.7,8

Regarding the injury against which preconditioning protects, studies have linked I/R injury with the generation of reactive oxygen species (ROS), especially within the first few minutes of reoxygenation.9 Moreover, the development of cellular injury in these models correlates with the degree of oxidant stress (for review, see Reference 10). We hypothesized that preconditioning protects by attenuating the generation of oxidants at reperfusion. If so, then preconditioning should be associated with an attenuation of that oxidant stress regardless of the method used to trigger preconditioning protection. Furthermore, given that PKC inhibition blocks the protection associated with preconditioning, this should also abolish the attenuation of oxidant stress at reperfusion. Finally, given that activators of KATP channels confer preconditioning-like protection but that inhibitors abolish protection, we reasoned that KATP channel activation or inhibition should attenuate or augment oxidant generation at reoxygenation in accordance with this model.

Because it is technically difficult to detect transient changes in intracellular oxidant generation in intact organs,11 we studied cardiomyocytes that permit the use of intracellular dyes to assess oxidant generation during simulated ischemia and during reoxygenation.12 Previous studies demonstrated that cultured cardiomyocytes exhibit preconditioning13,14 and exhibit increased oxidant stress14 during I/R,15 rendering them suitable for addressing the hypothesis.

Materials and Methods

Cardiac Cell Culture

Ventricular embryonic chick cardiomyocytes were prepared by using a method described previously.16 Cells were studied at days 3 to 5, when viability exceeded 99%. Cells were studied in a flow-through...
chamber as previously described. The perfusate consisted of balanced salt solution (with $P_{O_2}$ 149 mm Hg, $P_{CO_2}$ 36 mm Hg, pH 7.4, and $[K^+]_e$ 4.0 mEq/L) containing glucose (5.6 mmol/L). To simulate ischemia, the perfusate consisted of balanced salt solution without glucose containing 2-deoxyglucose (20 mmol/L) to inhibit glycolysis and $[K^+]_e$ of 8.0 mEq/L. This solution was bubbled with 80% $N_2$/20% $CO_2$ to produce a $P_{O_2}$ of $\approx$4 mm Hg, a $P_{CO_2}$ of 144 mm Hg, and a final pH of 6.8. Hypoxic media used for preconditioning consisted of balanced salt solution with no glucose bubbled with 95% $N_2$/5% $CO_2$. Reperfusion was with normal perfusate with glucose. Hypoxia ($P_{O_2}$ 15 mm Hg) and simulated ischemia (4 mm Hg) were verified by use of an optical phosphorescence quenching technique (Oxyspot, Medical Systems Inc) to confirm the $P_{O_2}$ in contact with the cells.

**Video/Fluorescent Microscopy**

Fluorescent images were obtained by using a cooled slow-scanning personal computer–controlled camera (Hamamatsu). Propidium iodide (PI, 5 µmol/L; Molecular Probes) was used to assess viability, as previously described. Cells were permeabilized with digitonin (300 µmol/L) at the end of the experiment to maximize PI staining. Vigorous cell contractile motion was always observed at the start of each study. Within minutes after the start of ischemia, visible contractile motion ceased in every experiment. During reperfusion, some contractile motion returned in some experiments. Return of contraction was assessed at 3 hours into reperfusion.

**Measurement of ROS Generation**

Intracellular oxidant stress was monitored as previously described by using the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA, 5 µmol/L; Molecular Probes). Present in the media throughout the experiment (5 µmol/L), this dye enters the cells and is cleaved by esterases, yielding nonfluorescent 2',7'-dichlorofluorescin (DCFH). Intracellular oxidants can lead to DCFH oxidation, yielding the fluorescent compound dichlorofluorescein (DCF).

**Preconditioning Protocols**

To simulate I/R, cardiomyocytes were exposed to 1 hour of simultaneous hypoxia, hypercarbic acidosis, hyperkalemia, and substrate deprivation, followed by 3 hours of reperfusion. This results in significant cell death, particularly during reperfusion, which appears related to oxidant damage. For preconditioning before I/R, cardiomyocytes were exposed to 10 minutes of hypoxia ($P_{O_2}$ 15 mm Hg) without glucose and then 10 minutes of normoxic recovery, followed by I/R. As a second preconditioning protocol, adenosine (100 µmol/L, Sigma Chemical Co) was used in lieu of hypoxia. Cell viability, contraction, and oxidant generation were measured during subsequent I/R.

**Data Analysis**

For each experiment, a single field of $\approx$500 cells was observed. Treatment and control groups were matched by using cells isolated on the same day. Additional coverslips were used for replicate experiments.

**Results**

**Effects of Preconditioning on Cell Death and Contraction**

In this model of simulated I/R, the majority of cell death develops during reperfusion. In cells preconditioned with adenosine (Figure 1), the pattern and extent of cell death were similar to those previously reported for hypoxia-preconditioned cells. After ischemia but before reperfusion, cell death was minimal and averaged $<2\%$ in all groups. Cell death after 3 hours of reperfusion in adenosine-preconditioned cells ($18\pm4\%$, $n=6$) was similar to that seen after preconditioning with hypoxia ($16\pm2\%$, $n=5$). In cells preconditioned by either hypoxia or adenosine, cell death was less than that found in nonpreconditioned control cells ($50\pm3\%$, $n=10$; $P<0.001$). After 3 hours of reperfusion, visible contractile activity returned in all preconditioned cells (11 of 11) compared with nonpreconditioned cells (0 of 10). Thus, preconditioning with adenosine or hypoxia conferred significant protection from cell death and contractile dysfunction.

**Effects of Preconditioning on Reperfusion Oxidant Generation via PKC**

If the mechanism of preconditioning protection involves an attenuation of oxidant stress at reperfusion, then induction of preconditioning by either hypoxia or adenosine should attenuate the oxidant burst at reperfusion. Figure 1 shows the effect of adenosine preconditioning on DCF fluorescence...
during baseline and I/R compared with no preconditioning. In the absence of preconditioning, control cells demonstrated an increase in DCFH oxidation to a peak value of 2.2 ± 0.1 at 10 minutes of reperfusion (n = 3), whereas adenosine-preconditioned cells increased to 1.6 ± 0.1 (n = 3, P = 0.02). Figure 2 shows the effect of hypoxic preconditioning on intracellular oxidant generation during I/R, as assessed by the oxidation of DCFH. As seen in adenosine preconditioning, a transient burst of DCF fluorescence was noted in nonpreconditioned cells at the start of reperfusion (1.4 ± 0.1 to 4.0 ± 0.3 by 10 minutes of reperfusion, n = 6). This was significantly attenuated in hypoxia-preconditioned cells (1.4 ± 0.1 to 2.6 ± 0.3, n = 9; P = 0.01).

PKC inhibition has been shown to block preconditioning protection in multiple studies. If preconditioning protects by attenuating oxidant stress at reperfusion, then PKC inhibition should abrogate this attenuation. In adenosine preconditioning, PKC inhibition with Go-6976, a selective PKC inhibitor,19 abrogated the attenuation of the reperfusion oxidant burst, causing DCFH oxidation to increase in preconditioned cells to 2.2 ± 0.1 by 10 minutes of reperfusion (n = 3), similar to nonpreconditioned cells (Figure 1). In hypoxic preconditioning, Go-6976 caused DCFH oxidation to increase from 1.5 ± 0.1 at the end of ischemia to 4.1 ± 0.3 at 10 minutes of reperfusion (n = 3) (P = NS versus non preconditioned controls, Figure 2). Go-6976 also abrogated preconditioning protection, with 46 ± 6% cell death at 0.01 to 0.1 μmol/L (n = 7) and no return of contractions compared with preconditioned cells (P < 0.001, Figure 2). Exposure to Go-6976 (0.1 μmol/L) for 4 hours under normoxia had no effect on viability or contraction (data not shown). In the absence of preconditioning, cells subjected to ischemia in the presence of Go-6976 (0.05 μmol/L) showed no exacerbation of cell death (n = 3, Figure 2). The less specific PKC inhibitors Ro-31-8220 (0.01 μmol/L, n = 3) and chelerythrine (2 μmol/L, n = 4) also abolished preconditioning protection in this system and were not associated with increased cell death during prolonged normoxia or during the standard I/R protocol (data not shown). These results confirm that preconditioning requires PKC activation20 and indicate that the attenuation of oxidant production during reperfusion involves PKC.

Role of the K<sub>ATP</sub> Channel in Reperfusion Oxidants

Studies have shown that K<sub>ATP</sub> channel openers can induce preconditioning-like protection, whereas inhibitors abolish protection in the heart. If preconditioning protects cells by attenuating the oxidant burst at reperfusion, then activators and inhibitors of that channel should modify the magnitude of the oxidant burst at reperfusion in accordance with our model. The compound 5-hydroxydecanoate (5-HD) inhibits mitochondrial K<sub>ATP</sub> channels20 and abolishes protection in chick cardiomyocytes.22 The attenuation of the reperfusion oxidant burst after hypoxic preconditioning was abolished by 5-HD (n = 3, Figure 3). Likewise, cell death after 3 hours reperfusion in hypoxia-preconditioned cells increased from 16 ± 2% to 50 ± 8% (P = 0.04) when 5-HD was added during the preconditioning (n = 4, Figure 3).

The K<sub>ATP</sub> channel opener pinacidil induces preconditioning-like protection in the chick cardiomyocyte system.23 In the present study, pinacidil (10 μmol/L) that was added for 1 hour at the start of reperfusion abrogated oxidant generation at reperfusion and reduced cell death (Figure 4). In addition, this attenuation in oxidant generation and cell death could be reversed with the addition of 5-HD. Compared with cell death in nonpreconditioned control cells (50 ± 3%), cells treated with pinacidil exhibited 30 ± 2% cell death, with 3 of 4 groups exhibiting return of contraction during reperfusion (versus 0 of 10 in control cells). The addition of 5-HD before and during pinacidil treatment at reperfusion reversed this attenuation in cell death, resulting in 46 ± 8.4% cell death and 0 of 4 groups exhibiting any return of contraction.

Preconditioning and Antioxidant Protection

If protection conferred by preconditioning is mediated by an attenuation of oxidant stress at reperfusion, then similar
protection should be obtained when antioxidants are given only at reperfusion. The antioxidants 1,10-phenanthroline and 2-mercaptopropionylglycine were previously found to attenuate oxidant stress in this model.14 Figure 5 shows the percent reduction in cell death achieved with antioxidants compared with other interventions in the present study. When 1,10-phenanthroline (10 μmol/L) plus 2-mercaptopropionylglycine (400 μmol/L) was given only at reperfusion, cell death decreased to a similar extent as seen with preconditioning. Return of contractile motion was observed in 4 of 4 studies using antioxidants compared with 0 of 10 control studies. Addition of the PKC inhibitor Go-6976 or the KATP channel inhibitor 5-HD largely abrogated the protection conferred by hypoxic preconditioning. In non preconditioned cells, addition of the KATP channel opener pinacidil reduced cell death by 37 ± 6% only at reperfusion.

Discussion

Ischemic preconditioning was initially described in the heart,1 but isolated cardiomyocytes demonstrate similar protection from I/R after brief exposure to adenosine, hypoxia, or ischemia,3,4,13,23 suggesting that the same mechanisms act in both systems. In our model, preconditioning conferred a reproducible degree of protection against cell death, and it improved recovery of contractile function during reperfusion. Using DCFH to assess ROS generation, we detected a transient burst of oxidant stress at the start of reoxygenation, which was consistent with previous findings in the intact heart.9 Preconditioning induced by hypoxia, adenosine, or KATP channel activation attenuated this oxidant burst. By contrast, inhibition of PKC or inhibition of mitochondrial KATP channels abolished the protection and restored oxidant generation at reperfusion to a level indistinguishable from the control level. The level of protection seen with preconditioning was similar to that seen with antioxidants given only at reperfusion. These findings suggest that preconditioning can
Compared with nonpreconditioned cells subjected to I/R, cell death decreased to a similar extent in cells preconditioned with hypoxia or adenosine. Both the PKC inhibitor Go-6976 (0.05 μmol/L) and the K<sub>ATP</sub> channel inhibitor 5-HD (500 μmol/L), given during the entire experiment, abrogated this protection. By contrast, the K<sub>ATP</sub> channel opener pinacidil (10 μmol/L), given only during reperfusion, and antioxidants (10 μmol/L 1,10-phenanthroline plus 400 μmol/L 2-mercaptopropionylglycine, given only at reperfusion) conferred significant protection and produced return of contractile motion in nonpreconditioned cells (4 of 4 experiments). *P<0.05 vs untreated nonpreconditioned cells exposed to I/R. Values are calculated as percentage in cell death without intervention minus cell death with intervention relative to cell death without. Groups are as follows: I/R (no PC), n=10; adenosine PC, n=6; hypoxic PC, n=5; hypoxic PC plus Go-6976, n=7; hypoxic PC plus 5-HD, n=4; I/R with pinacidil, n=3; and I/R plus antioxidants, n=4.

Figure 5. Decrease in cell death conferred by preconditioning (PC) with hypoxia or adenosine or by pinacidil or antioxidants. Compared with nonpreconditioned cells subjected to I/R, cell death decreased to a similar extent in cells preconditioned with hypoxia or adenosine. Both the PKC inhibitor Go-6976 (0.05 μmol/L) and the K<sub>ATP</sub> channel inhibitor 5-HD (500 μmol/L), given during the entire experiment, abrogated this protection. By contrast, the K<sub>ATP</sub> channel opener pinacidil (10 μmol/L), given only during reperfusion, and antioxidants (10 μmol/L 1,10-phenanthroline plus 400 μmol/L 2-mercaptopropionylglycine, given only at reperfusion) conferred significant protection and produced return of contractile motion in nonpreconditioned cells (4 of 4 experiments). *P<0.05 vs untreated nonpreconditioned cells exposed to I/R. Values are calculated as percentage in cell death without intervention minus cell death with intervention relative to cell death without. Groups are as follows: I/R (no PC), n=10; adenosine PC, n=6; hypoxic PC, n=5; hypoxic PC plus Go-6976, n=7; hypoxic PC plus 5-HD, n=4; I/R with pinacidil, n=3; and I/R plus antioxidants, n=4.

protect cells from I/R by attenuating oxidant generation at reperfusion, through a process involving activation of a PKC-dependent signaling pathway and K<sub>ATP</sub> channels.

Cell viability was assessed by using PI, which showed a progressive increase in staining during 3 hours of reperfusion yet only a minimal increase during ischemia. Previous studies in cardiomyocytes have shown minimal cell death during 1 hour of ischemia but accelerated cell death during 3 hours of reperfusion.13 Our present results link the transient oxidant burst at reperfusion to the loss in cell viability, yet PI staining continued to increase during reperfusion even though the transient oxidant burst had long since subsided. This may reflect a delayed response of PI, which provides a measure of sarcolemma permeability, to a lethal oxidant stress occurring at the start of reperfusion. Alternatively, a burst of oxidant stress at reperfusion could activate a sequence of events that later results in the increase in cell permeability. Could the PI measurements reflect a delayed response to a lethal insult incurred during ischemia? We argue against this, on the basis of the observation that cell death was minimal after 4 hours of continuous ischemia, when cells were studied without reperfusion.16

Preconditioning Protection and Attenuation of Oxidant Stress

Our findings are consistent with reports suggesting that preconditioning decreases oxidant stress, but our findings are novel in suggesting that this process occurs during the “early window” of protection. In rat cardiomyocytes, Zhai et al24 and Zhou et al25 found decreases in superoxide levels and increases in magnesium superoxide dismutase (magnesium SOD) activity by 24 hours after preconditioning. They suggested that preconditioning may activate existing SOD during the early window of protection and confer protection during the late phase by causing the expression of additional SOD. However, Turrens et al26 found no increase in the activities of SOD, catalase, or glutathione peroxidase during the early window of protection. Another report has suggested that mitochondria isolated from preconditioned hearts generates less superoxide than those isolated from nonpreconditioned hearts.27 Those results suggest that preconditioning could attenuate oxidant generation by mitochondria. However, controversy exists regarding the source of the ROS generated at reperfusion. Yabe et al28 found that preconditioning preserved mitochondrial function in rat hearts, raising the possibility that preconditioning may attenuate ROS generation and thereby lessen mitochondrial injury by oxidants produced elsewhere in the cell. Indeed, studies suggest that oxidants originate from a nonmitochondrial source at reperfusion.29,30 In the present study, antioxidants administered at reperfusion were as effective as preconditioning in protecting cells.

These findings suggest that ROS generation at reperfusion is responsible for the cell injury and that preconditioning protects by attenuating that oxidant stress. Because the cardiomyocyte system used in these studies does not contain neutrophils, endothelial cells, or other potential sources of oxidants, the present study also demonstrates that the cardiomyocytes themselves can act as a source of lethal oxidant stress.

Although some studies in the intact heart have shown a beneficial effect of antioxidants given only at reperfusion,31 other studies have failed to detect a beneficial effect unless they are given before reperfusion (as reviewed by Opie32). To the extent that a rapid burst of ROS generation at reperfusion is an important determinant of myocardial injury, antioxidants administered at reperfusion will be protective only if they reach their targets before significant oxidant stress has occurred. Any delay in the delivery of the drug to the site of oxidant generation will likely degrade the extent of protection by allowing unchecked oxidant stress to begin. The consistent protection afforded by antioxidants in our cardiomyocyte model may reflect the ability of such a system to delivery the drugs simultaneously with oxygen at the start of reperfusion.

Role of K<sub>ATP</sub> Channels in Reducing Reperfusion Oxidant Injury

The K<sub>ATP</sub> channel may function as an effector of preconditioning, although the mechanism by which it confers protection is unknown.33–35 Antagonists of the channel, such as glibenclamide and 5-HD, block ischemic preconditioning protection in the heart, and K<sub>ATP</sub> channel openers mimic the beneficial effect of preconditioning.36,37 Some studies indicate that K<sub>ATP</sub> channel activators are protective only when given during ischemia (for review, see References 38 and 39), whereas other studies suggest that it can be effective when given just before reperfusion.40 Because reperfusion oxidant stress occurs rapidly after reperfusion begins, any delay in intracellular access could degrade the extent of protection. We found that pinacidil was protective when administered at the start of reperfusion, which may be explained by a more...
rapid access of the drug to the cells in a flow-through system. The ability of pinacidil to confer protection when given at the start of reperfusion points to a possible connection between that channel and the system responsible for ROS generation. To the extent that the $K_{\text{ATP}}$ channel is the effector of preconditioning, our data suggest that the channel modulates oxidant generation at the start of reperfusion. The inhibitor 5-HD is selective for the mitochondrial $K_{\text{ATP}}$ channel, and mitochondria have the potential to generate ROS. This might suggest that $K_{\text{ATP}}$ channels function by modulating mitochondrial superoxide generation, yet other evidence points away from mitochondria as the source of oxidants generated at reperfusion. We speculate that the mitochondrial $K_{\text{ATP}}$ channel may influence oxidant generation by affecting the supply of NAD(P)H from the mitochondrial matrix dehydrogenases to a nonmitochondrial oxidase system. However, additional work is required to test this hypothesis.

Interestingly, in the present study, the extent of protection conferred by pinacidil was somewhat less than that conferred by preconditioning, yet it appeared to be as effective as preconditioning at attenuating the reoxygenation ROS burst. One explanation is that preconditioning protects cells by a second mechanism that is independent of the $K_{\text{ATP}}$ channel activation. Alternatively, it is possible that pinacidil exhibits nonspecific detrimental effects that undermine the extent of protection otherwise afforded by $K_{\text{ATP}}$ channel activation. However, the finding that 5-HD abolished preconditioning strongly implicates the $K_{\text{ATP}}$ channel as a significant effector for preconditioning protection with pinacidil in this system.

### Role of PKC Signaling

PKC has been implicated as a mediator of preconditioning in different models. However, the specific targets of PKC in preconditioning have not been fully established. In ventricular myocytes, Hu et al. found that PKC activation reduced the sensitivity of sarcoplasmic $K_{\text{ATP}}$ channels to ATP, an effect that would tend to promote channel opening at a given [ATP]. Light et al. showed that PKC activation increased the open probability of the $K_{\text{ATP}}$ channel in cardiomyocyte membrane patches. Speechly-Dick et al. found that preconditioning of human trabeculae induced by PKC activation could be blocked by inhibition of the $K_{\text{ATP}}$ channel. Sato et al. have suggested that activated PKC acts on mitochondrial $K_{\text{ATP}}$ channels. Collectively, these findings support a model in which the PKC targets include the $K_{\text{ATP}}$ channel(s). Our findings confirm the linkage between PKC activation and $K_{\text{ATP}}$ channel activation in preconditioning and extend previous work by linking this pathway to a modulation of oxidant generation at reoxygenation. This is based on the observation that the effects of preconditioning on the reoxygenation oxidant burst were ablated when either PKC activation or $K_{\text{ATP}}$ channel activation was inhibited.

Go-6976 has been characterized as selective for Ca$^{2+}$-dependent isoforms of PKC, yet other studies have implicated Ca$^{2+}$-independent isoforms in the preconditioning of adult hearts. The particular isoforms involved in preconditioning of chick cardiomyocytes have not been characterized, so it is possible that this difference is due to the different source of our cardiomyocytes.

### Role of Oxidants in the Induction of Preconditioning

ROS have been implicated as second messengers in the activation of preconditioning. Exogenous oxidants induce preconditioning in intact hearts, and antioxidants block the beneficial effects of preconditioning. We previously reported that increased ROS are generated by mitochondria during hypoxic preconditioning. According to that model, low levels of mitochondrial ROS function as signaling agents required for the activation of protection. Antioxidants administered only during hypoxic preconditioning attenuated the transient ROS signal and abolished the protective effect. In the present study, evidence of an oxidant signal was again observed during hypoxic preconditioning (see Figure 2A, minute 40). However, compared with magnitude of the ROS burst at reperfusion, the magnitude of this signal is small.

Regarding the induction phase, no oxidant signal was observed during adenosine preconditioning, suggesting the existence of alternative pathways that do not involve oxidants. It is also possible that adenosine triggers preconditioning by acting downstream from the oxidant-dependent step. We speculate that mitochondrial oxidants generated during hypoxic preconditioning lead to the activation of PKC, which subsequently acts on the $K_{\text{ATP}}$ channel. Evidence suggests that oxidants can activate PKC, whereas adenosine may activate PKC by a receptor-mediated pathway, possibly involving diacylglycerol, that bypasses mitochondria. In either case, $K_{\text{ATP}}$ channel activation appears to attenuate the oxidant burst seen in reperfusion, which appears to be responsible for the cell death later in reperfusion. These findings present a dual role for ROS in preconditioning: during induction (when low levels of oxidants generated by mitochondria activate PKC and open $K_{\text{ATP}}$ channels) and during the protection phase (when the subsequent oxidant burst at reperfusion is attenuated).

Regarding the mechanism of reversal of preconditioning protection by 5-HD, it is noteworthy that 5-HD had no effect on the oxidant generation during hypoxia (see Figure 3A, minute 40). Thus, abrogation was not due to attenuation of mitochondrial oxidant generation during hypoxia, an intervention previously demonstrated with antioxidants to abrogate preconditioning protection. The result also suggests that oxidants generated during hypoxic preconditioning may originate from a source different from the oxidant burst after I/R, because interventions affecting the $K_{\text{ATP}}$ channel appear to alter the oxidants generated at reperfusion but not during preconditioning. Further work is needed to identify the oxidant source at reperfusion.

### Timing of Preconditioning Protection: Ischemia Versus Reperfusion

The shorter the duration of myocardial ischemia, the less is the degree of ischemic injury. Classically, it has been thought that most injury occurs during ischemia, yet a growing number of studies have shown that interventions at the start of reperfusion may improve recovery. It is difficult to know...
whether the protection conferred by preconditioning of intact hearts is mediated by its effects on events occurring during ischemia or whether preconditioning affects events during the first minutes of reperfusion. Because reperfusion injury may occur within seconds and because some amount of reperfusion of ischemic tissue is necessary to quantify the extent of necrosis in intact hearts when dyes are used, this issue has been difficult to address in the intact heart. In vitro models have the potential to shed light on this controversy by providing a continuous assessment of viability in the same cells during ischemia and reperfusion phases. To the extent that our findings in cardiomyocytes apply to the intact heart, these data suggest that interventions such as a K+ channel agonists or antioxidants applied at reperfusion have the potential to protect against the consequences of ischemia but that the rapid generation of oxidants on reperfusion leads to a narrow window of opportunity for therapy.

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