Mitochondrial reactive oxygen species: which ROS signals cardioprotection?

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Garlid AO, Jaburek M, Jacobs JP, Garlid KD. Mitochondrial reactive oxygen species: which ROS signals cardioprotection? Am J Physiol Heart Circ Physiol 305: H960–H968, 2013. First published August 2, 2013; doi:10.1152/ajpheart.00858.2012.—Mitochondria are the major effectors of cardioprotection by procedures that open the mitochondrial ATP-sensitive potassium channel (mitoKATP), including ischemic and pharmacological preconditioning. MitoKATP opening leads to increased reactive oxygen species (ROS), which then activate a mitoKATP-associated PKCe, which phosphorylates mitoKATP and leaves it in a persistent open state (Costa AD, Garlid KD. Am J Physiol Heart Circ Physiol 295, H874–H882, 2008). The ROS responsible for this effect is not known. The present study focuses on superoxide (O2·−), hydrogen peroxide (H2O2), and hydroxyl radical (HO·), each of which has been proposed as the signaling ROS. Feedback activation of mitoKATP provides an ideal setting for studying endogenous ROS signaling. Respiring rat heart mitochondria were preincubated with ATP and diazoxide, together with an agent being tested for interference with this process, either by scavenging ROS or by blocking ROS transformations. The mitochondria were then assayed to determine whether or not the persistent phosphorylated open state was achieved. Dimethylsulfoxide (DMSO), dimethylformamide (DMF), deferoxamine, Trolox, and bromoeno lactone each interfered with formation of the ROS-dependent open state. Catalase did not interfere with this step. We also found that DMF blocked cardioprotection by both ischemic preconditioning and diazoxide. The lack of a catalase effect and the inhibitory effects of agents acting downstream of HO· excludes H2O2 as the endogenous signaling ROS. Taken together, the results support the conclusion that the ROS message is carried by a downstream product of HO· and that it is probably a product of phospholipid oxidation.

mitochondria; cardioprotection; reactive oxygen species; KATP channels; cardiac ischemia; ROS signaling

REACTIVE OXYGEN SPECIES (ROS) are second messengers of preconditioning (21) and have long been known to be required for cardioprotective signaling (3, 10, 18, 34, 43, 49). The mechanism of increased ROS is reasonably well understood: signaling from the plasma membrane leads to opening of the mitochondrial ATP-sensitive K+ channel (mitoKATP; Ref. 19), and the increased K+ influx into the matrix causes an increase in ROS, which derive downstream of the respiratory chain (1). The ROS transformations that take place in mitochondria are summarized in Figs. 1 and 2. The primary ROS produced by the mitochondrial respiratory chain is superoxide (O2·−), which is formed by single-electron reduction of oxygen. The majority of O2·− undergoes dismutation to hydrogen peroxide (H2O2) both spontaneously and through the action of superoxide dismutases located in the matrix and the intermembrane space (7, 26, 33). Much of the H2O2 generated is reduced by the glutathione and thioredoxin reductase antioxidant systems, which prevent the emission of excess H2O2 to the cytosol (42). H2O2 is also decomposed by catalase, which is present in the matrix of heart mitochondria. Some of the H2O2 leads to formation of hydroxyl radical (HO·) through the Fenton reaction of HO2 with transition metal ions (29). The highly reactive HO· oxidizes proteins and lipids in diffusion-limited reactions (27).

The consequences of ROS signaling for mitochondrial physiology are that two mitochondrial protein kinase-Cε (PKCe) are activated by oxidation of their thiol groups (28). Activation of PKCe1, which is associated with mitoKATP at the mitochondrial inner membrane, leads to opening of mitoKATP (13, 25). Activation of PKCe2 leads to inhibition of the mitochondrial permeability transition (MPT; Ref. 12). Progress is being made in these areas and in the molecular identification of mitoKATP (17). However, the ROS responsible for activating these PKCe in vitro or in vivo is still not known. Because the signaling ROS not only originates in mitochondria but also acts on mitochondria, it should be possible to narrow the search for the signaling ROS by studies on isolated rat heart mitochondria. The objective of these studies was to identify the point in the reaction sequence of ROS transformations at which the ROS signal is formed and, ultimately, to determine the identity of the ROS signal itself.

We previously described feedback activation of mitoKATP, in which mitoKATP opening by a KATP channel opener leads to endogenous ROS formation and a persistent open state of mitoKATP. The phenomenon was shown to involve PKCe1 and an inner membrane phosphorylation event (13). This persistent open state of mitoKATP is thought to be responsible for “memory,” which is seen with all preconditioning (13).

We employed a preincubation protocol to probe the feedback activation system for the identity of the signaling ROS. Diazoxide was used to induce mitoKATP opening, which leads to endogenous ROS formation and activation of PKCe1. This process establishes the prolonged phosphorylation-dependent open state of mitoKATP (13). We then reisolated the mitochondria and probed them for this state by measuring the resulting steady-state mitochondrial matrix volume using the light-scattering technique (5, 11, 14, 20). Agents or conditions that interfered with any step in the generation of the signaling ROS would prevent formation of the phosphorylation dependent open state. Two additional assays were carried out to eliminate agents that interfered either with mitoKATP-dependent K+ uptake or with ROS activation of PKCe1. Together, these...
mitoKATP.

This conclusion excludes H2O2 as the signaling ROS. This conclusion was based on the observation that the HO• scavenger DMF blocked cardioprotection by ischemic preconditioning and diazoxide. Taken together, each of these results supports the conclusion that the ROS message is carried by a downstream product of hydroxyl radical (HO•). The actions of BEI suggest a product of phospholipid oxidation.

**RESEARCH DESIGN AND METHODS**

*Langendorff-perfused hearts.* Hearts from male Sprague-Dawley rats (200–240 g) were perfused as previously described (15, 22, 37) with Krebs-Henseleit buffer containing the following (in mM): 118 NaCl, 5.9 KCl, 1.75 CaCl2, 1.2 MgSO4, 0.5 EDTA, 25 NaHCO3, 16.7 glucose at 37°C and pH 7.4 and gassed with 95% O2-5% CO2. Treatment protocols are described in Fig. 3. Hearts were stabilized for 25 min with Krebs Henseleit buffer before treatment with buffer containing drugs or agents and then subjected to 25-min global ischemia followed by 2 h of reperfusion and measurement of infarct size. Ischemic preconditioning was established by two cycles of 5-min global ischemia followed by 5-min reperfusion before the index ischemia. Ischemic postconditioning was performed with six cycles of 10-s ischemia plus 10-s reperfusion (45). Hearts were not paced, and mechanical performance was evaluated as the product of heart rate and left ventricular developed pressure and reported as a percentage of rate pressure product at stabilization, t = 0. Infarct size was determined by the method of Ytrehus et al. (48) and reported as a percentage of slice cross section. Experimental protocols complied with the *Guiding Principles in the Use and Care of Animals* published by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Portland State University.

*Mitochondrial isolation.* Male Sprague-Dawley rats (200–240 g) were anesthetized with CO2 and immediately decapitated, and the heart was rapidly excised and perfused with buffer as described in **RESEARCH DESIGN AND METHODS.** After 45 min of stabilization, hearts were subjected to 25 min global ischemia (GI), followed by 2 h of reperfusion (R) and, finally, processing for infarct size estimation. Protocol for ischemic reperfusion (IR) without additional treatment is labeled “IR.” Ischemic preconditioning (IPC) was established by 5-min global ischemia followed by 10-min reperfusion before the index ischemia. Diazoxide (Dzx; 50 μM) was administered 5 min before the first IPC ischemia and before Dzx; it was continued for 5 min after these treatments and this was followed by 5-min reperfusion with buffer before the index ischemia.

We performed experiments on MPT in which diazoxide was used to induce inhibition of MPT via ROS activation of PKCe2 (12). This effect was blocked by DMSO, also implicating a ROS signal downstream from HO•. Finally, we found that the HO• scavenger DMF blocked cardioprotection by ischemic preconditioning and diazoxide. Taken together, each of these results supports the conclusion that the ROS message is carried by a downstream product of hydroxyl radical (HO•). The actions of BEI suggest a product of phospholipid oxidation.

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hearts were removed for mitochondrial isolation exactly as described previously (14). Hearts were washed and finely minced in ice-cold isolation buffer containing 250 mM sucrose, 10 mM HEPES at pH 7.2, and 0.5 mM K\(^+\)-EGTA. The suspension was diluted threefold with isolation buffer supplemented with 1% fatty acid-free BSA. The time between decapitation and completion of homogenization was kept as brief as possible and was completed within 2 min; mitoK\(_{ATP}\) activity shows a sharp dependence on the length of this period. The suspension was homogenized with a motorized Teflon pestle and centrifuged for 3 min at 1,500 \(g\). The supernatant was centrifuged for 5 min at 9,000 \(g\), and the resulting pellets were resuspended in isolation buffer lacking BSA and centrifuged for 3 min at 2,300 \(g\). This supernatant was centrifuged for 5 min at 9,000 \(g\). The final mitochondrial pellet was resuspended with isolation buffer to 35–40 mg protein/ml and kept on ice. Where indicated, rat liver mitochondria were isolated using a similar protocol, as previously described (5). Mitochondrial protein concentration was estimated using the Biuret reaction (23).

**Measurements of mitoK\(_{ATP}\) activity.** MitoK\(_{ATP}\) opening causes mitochondrial swelling due to respiration-driven uptake of K\(^+\) salts and water. These volume changes were followed by light scattering, as previously described (5, 11, 14, 20). Light-scattering changes of 0.1 mg/ml were monitored by light scattering in medium containing 200 \(\mu\)M ATP and lacking EGTA. MitoK\(_{ATP}\) opening was synchronized by sequential additions of CaCl\(_2\) (100 \(\mu\)M free Ca\(^{2+}\)), ruthenium red (0.1 \(\mu\)M, to block further Ca\(^{2+}\) uptake), and carbonyl cyanide m-chlorophenyl hydrazine (250 nM), at 20, 40, and 60 s, respectively (12). These experiments used mitochondria from livers taken from the animals supplying the heart.

**RESULTS**

**Endogenous ROS signaling and PKC\(\varepsilon\)-dependent feedback activation of mitoK\(_{ATP}\).** Feedback activation of mitoK\(_{ATP}\) can be divided into three steps as described in Fig. 5. In *step 1*, K\(^+\) channel openers such as diazoxide open mitoK\(_{ATP}\) (21), and the resulting K\(^+\) influx and matrix alkalinization leads to increased generation of superoxide (1). *Step 2* encompasses the ROS transformations that result in formation of the signaling ROS, i.e., the ROS that activates PKC\(\varepsilon\). In *step 3*, the activated PKC\(\varepsilon\) establishes the phosphorylation dependent open state of mitoK\(_{ATP}\) (13). The rationale for this division is that we can study the effects of agents on the overall cycle and, independently, on steps 1 and 3. In this way, we can identify agents that interfere specifically with formation of the signaling ROS (*step 2*).

Figure 6 contains results of control experiments using the preincubation protocol described in *Research Design and Methods*. When mitochondria were preincubated with diazoxide and ATP, they exhibited maximum volume change in the normal assay medium containing ATP and no further effect was seen with the addition of diazoxide (1st 2 bars of Fig. 6). This result is characteristic of conditions that allow mitoK\(_{ATP}\) to achieve the phosphorylation-dependent open state. When *step 1* was blocked by omission of diazoxide (2nd pair of bars) or inclusion of 5-HD (3rd pair of bars) in the preincubation, mitoK\(_{ATP}\)
Fig. 5. Reactive oxygen species (ROS)-dependent feedback activation of mitoK\textsubscript{ATP}. Step 1 of the process encompasses the sequence from opening to superoxide (O\textsubscript{2}\textsuperscript{-}) formation. MitoK\textsubscript{ATP} is opened by K\textsubscript{ATP} channel openers such as Dzx (21). Consequent uptake of K\textsuperscript{+} and anions leads to increased matrix volume (↑Vol), which is the basis of the light-scattering assay for mitoK\textsubscript{ATP} activity (14). The cytosolic concentration difference between [K\textsuperscript{+}] and [phosphate] means that more K\textsuperscript{+} than phosphate will be taken up, leading to matrix alkalinization (↑pH; Ref. 14). Matrix alkalinization, in turn, inhibits complex I, leading to increased production of superoxide (O\textsubscript{2}\textsuperscript{-}; Ref. 1). Step 2 encompasses the many ROS transformations that take place and lead to the signaling ROS, which activates protein kinase-C\textsubscript{ε}1 (PKC\textsubscript{ε}1). In step 3, the activated PKC\textsubscript{ε}1 phosphorylates a protein, possibly mitoK\textsubscript{ATP} itself, which leads to the phosphorylated open state of mitoK\textsubscript{ATP} (13). In parallel (not represented here), the ROS signal activates PKC\textsubscript{ε}2, leading to inhibition of mitochondrial permeability transition (MPT) opening (12).

behaved as if there were no preincubation; that is, mitoK\textsubscript{ATP} activity was inhibited by ATP and opened by diazoxide. When step 3 was blocked by inclusion of the specific PKC\textsubscript{ε} inhibitor εV\textsubscript{1-2} in the preincubation (last pair of bars), feedback activation was also prevented and mitoK\textsubscript{ATP} responded normally to ATP and diazoxide.

Figure 7 contains results of preincubation experiments in which we evaluated agents that may block ROS signaling (Step 2) in the feedback loop shown in Fig. 5. DMSO, DMF, and MPG have all been described as reactants with HO\textsuperscript{•-}, RO\textsuperscript{•-} (alkoxy radicals), and ROO\textsuperscript{•-} (alkylperoxyl radicals; Refs. 2, 32), each of which may play a role in endogenous ROS signaling. Trolox, a vitamin E analog, acts as a chain-breaking antioxidant and peroxyl radical scavenger (31). Figure 7A demonstrates that each of these agents prevented formation of the phosphorylation-dependent open state, suggesting that HO\textsuperscript{•-} is involved in the generation of the signaling ROS.

Deferoxamine is a chelator of iron (29, 41) and acts as a preventive antioxidant by reducing the rate of iron-dependent hydroxyl and peroxyl radical chain initiation. BEL (racemic bromoenol lactone) is an inhibitor of Ca\textsuperscript{2+}-independent phospholipase (iPLA\textsubscript{2}; Ref. 8), and it prevents the release of oxidized phospholipids such as hydperoxy fatty acids (FAOOH) from the bilayer, a possible downstream oxidation product in the reaction sequence. Figure 7B shows that both deferoxamine and BEL blocked feedback activation.

Fig. 6. Feedback activation of mitoK\textsubscript{ATP} is blocked by 5-HD and εV\textsubscript{1-2}. Shown are the effects of various agents on mitoK\textsubscript{ATP} activity after preincubation with ATP (200 μM) plus Dzx (30 μM). Mitochondria were preincubated with the agents indicated at step 1 and then assayed in K\textsuperscript{+} medium with ATP or ATP + Dzx as indicated below (described in RESEARCH DESIGN AND METHODS). With no further additions to the preincubation (−−−−), mitoK\textsubscript{ATP} remains in the phosphorylated open state with full activity, and diazoxide (ATP + Dzx) has no further effect in the subsequent light-scattering assay. When mitoK\textsubscript{ATP} opening during preincubation was prevented by omission of diazoxide (No Dzx) or inclusion of the mitoK\textsubscript{ATP} blocker 5-HD (300 μM; +5-HD), the phosphorylation-dependent open state was blocked. When feedback activation of PKC\textsubscript{ε}1 was prevented by inclusion of εV\textsubscript{1-2} (0.5 μM; +εV\textsubscript{1-2}), the open state was also blocked. Data are means of mitoK\textsubscript{ATP} activity ± SD of at least 3 independent experiments. *P < 0.05.

Fig. 7. Effects of agents that may block formation of the signaling ROS on feedback activation of mitoK\textsubscript{ATP}. A: effects of various ROS and radical scavenging agents and antioxidants on mitoK\textsubscript{ATP} activity after preincubation with ATP plus Dzx in the presence of DMSO (1% vol/vol), DMF (1% vol/vol), MPG (1 mM), and Trolox (100 μM). Each of these agents blocked formation of the phosphorylation-dependent open state of mitoK\textsubscript{ATP}. In B, deferoxamine (Dfo; 1 mM) and BEL (10 μM) also blocked this state, but uric acid (100 μM; +UA; peroxynitrite scavenger) and catalase (250 U/ml; H\textsubscript{2}O\textsubscript{2} scavenger) did not interfere with feedback activation of mitoK\textsubscript{ATP}. Mitochondria were preincubated with the agents indicated in the figure, then assayed in K\textsuperscript{+} medium, as described in RESEARCH DESIGN AND METHODS. Data are means of mitoK\textsubscript{ATP} activity ± SD of at least 3 independent experiments. *P < 0.05.
tion of mitoK\textsubscript{ATP}, further suggesting that hydroxyl radicals and their downstream oxidation products are involved in the generation of the signaling ROS. When uric acid, a per-oxynitrite scavenger (44), was included in the preincubation, the phosphorylation-dependent open state was preserved, indicating that peroxynitrite is not involved with any of the steps and, therefore, is not the signaling ROS. Catalase is an enzyme that catalyzes the decomposition of H\textsubscript{2}O\textsubscript{2}, and catalase was also unable to block this process, suggesting that H\textsubscript{2}O\textsubscript{2} is not the signaling ROS.

The mechanisms of action of these agents suggests that they are acting on step 2, the formation of the ROS signal; however, it is necessary to demonstrate that they are not acting elsewhere in the loop, i.e., on step 1 or step 3. Accordingly, we tested each of the agents for their effect on mitoK\textsubscript{ATP} activity in the straight light-scattering assay with no preincubation and found that none of them interfered with diazoxide opening of mitoK\textsubscript{ATP} (data not shown). Note that this assay also detects respiratory inhibition or uncoupling, which inhibit K\textsuperscript{+} uptake by reducing the driving force.

To evaluate their effect on step 3 (induction of the phosphorylated open state by PKC\textsubscript{ε}1), we examined whether the agents were able to block mitoK\textsubscript{ATP} opening induced by H\textsubscript{2}O\textsubscript{2}, which works by activating PKC\textsubscript{ε}1 (13). Figure 8 contains representative light-scattering traces using mitochondria without preincubation. Here, we find that Trolox had no effect. MPG did block H\textsubscript{2}O\textsubscript{2}-induced mitoK\textsubscript{ATP} opening, which means that we cannot distinguish whether MPG acts on step 2 or step 3 from these experiments. The results of the step 3 assays are summarized in Fig. 9 and show that deferoxamine, DMSO, DMF, Trolox, and BEL did not inhibit H\textsubscript{2}O\textsubscript{2}-induced mitoK\textsubscript{ATP} opening (step 3). Because they also did not interfere with diazoxide opening of mitoK\textsubscript{ATP} (step 1), we infer that these agents are acting to prevent formation of the endogenous ROS signal that activates PKC\textsubscript{ε}1 (step 2).

These findings may be summarized as follows. 1) Deferox-amine, DMSO, DMF, Trolox, and BEL each prevent formation of the endogenous ROS signal. These results imply that the ROS signal arises downstream from OH\textsuperscript{•}. 2) H\textsubscript{2}O\textsubscript{2} is produced upstream of OH\textsuperscript{•}, and catalase, the H\textsubscript{2}O\textsubscript{2} scavenger, had no effect on the formation of the phosphorylation-dependent open state. Therefore, H\textsubscript{2}O\textsubscript{2} can be excluded as the signaling ROS. 3) The action of MPG is independent of ROS scavenging. Like all thiols, MPG can scavenge OH\textsuperscript{•}, but OH\textsuperscript{•} is not involved in the path away responsible for the results in Fig. 8. Moreover, MPG has little or no H\textsubscript{2}O\textsubscript{2} scavenging ability (6, 30). We propose that this action of MPG is due to its strong thiol reductant properties, holding PKC\textsubscript{ε}1 in a reduced state and thereby preventing its activation.

Cardioprotection by ischemic preconditioning and diazoxide is blocked by DMF. The implication of the previous mitoK\textsubscript{ATP} experiments is that intercepting the HO\textsuperscript{•}-dependent reactions will block all modes of ROS-dependent cardioprotection. To examine this question, we perfused the heart with 1% DMF, the HO\textsuperscript{•}-scavenger, following the protocols described in Fig. 3. As demonstrated in Fig. 10, DMF inhibited the improvement in postischemic functional recovery (Fig. 10A) and blocked the infarct-size reduction (Fig. 10B) normally observed with ischemic preconditioning and diazoxide. DMF alone had no effect on infarct size in these conditions (Fig. 10B). Again, the effect of DMF on cardioprotective signaling implicates a downstream oxidation product of HO\textsuperscript{•} as the signaling ROS.

Endogenous ROS signaling and mitoK\textsubscript{ATP}-dependent MPT inhibition. MPT synchronization, described in research design and methods, was employed to determine the role of HO\textsuperscript{•}, RO\textsuperscript{•}, and/or ROO\textsuperscript{•} in diazoxide induced MPT inhibition, which depends on ROS activation of a second mitochondrial PKC\textsubscript{ε}, PKC\textsubscript{ε}1 (12). Results of a representative experiment are contained in Fig. 11. CsA completely blocked MPT activity, and diazoxide inhibited MPT opening by ~60%, which is the customary extent observed in these experiments (13). This inhibition was reversed by 5-HD, MPG, DMSO, and DMF (not shown) also reversed MPT inhibition by diazoxide. These results also imply that the signaling ROS that activates PKC\textsubscript{ε}1 must be a downstream oxidation product of HO\textsuperscript{•}.
Our objective is to identify the mitoK<sub>ATP</sub>-dependent endogenous ROS that activates PKCe1 to leave mitoK<sub>ATP</sub> in a phosphorylated open state. Superoxide is the first ROS that increases due to increased K<sup>+</sup> uptake into the matrix (see Fig. 1) and has been proposed to be the ROS signal (38). Xanthine/xanthine oxidase (X/XO) induced mitoK<sub>ATP</sub> opening in isolated mitochondria; however, superoxide rapidly dismutates to H<sub>2</sub>O<sub>2</sub> in aqueous solution, and Costa et al. (13) showed that X/XO did not open mitoK<sub>ATP</sub> in the presence of catalase. Therefore, the observed effect of X/XO was mediated by H<sub>2</sub>O<sub>2</sub>, and superoxide can be excluded as a PKCe activator or mitoK<sub>ATP</sub> opener. Queliconi et al. (38) repeated the X/XO experiment and obtained the same results; however, they failed to control for H<sub>2</sub>O<sub>2</sub> production and erroneously concluded that superoxide opens mitoK<sub>ATP</sub>. PKCe is activated by nitric oxide (NO; Ref. 4) and H<sub>2</sub>O<sub>2</sub> (28), which oxidize the thiols in the zinc finger of PKCe, leading to its activation. Consistent with this biochemistry, H<sub>2</sub>O<sub>2</sub> and NO cause mitoK<sub>ATP</sub> opening (13, 25) that is blocked by the PKCe peptide inhibitor εV<sub>1,2</sub>. The absence of an effect of H<sub>2</sub>O<sub>2</sub> and NO in the presence of εV<sub>1,2</sub> demonstrates that these agents have no direct effect on mitoK<sub>ATP</sub>. Again, Queliconi et al. (38) repeated these experiments and obtained the same result; however, they failed to control for PKCe involvement and erroneously concluded that H<sub>2</sub>O<sub>2</sub> and NO open mitoK<sub>ATP</sub> directly.

Liu et al. (30) concluded that hydroxyl radical (HO·) is responsible for ROS signaling in the heart, but this conclusion was based on experiments showing that MPG blocks ROS signaling and the assumption that this block was due to HO· scavenging. This assumption may be incorrect. The data in Fig. 7 show that MPG blocks H<sub>2</sub>O<sub>2</sub>-induced mitoK<sub>ATP</sub> opening. HO· is not involved in this process, and MPG has little or no H<sub>2</sub>O<sub>2</sub> scavenging ability (6, 30). Moreover, eliminating HO· requires very high concentrations of scavenger due to the transitory nature of HO·, and it is unlikely that submillimolar concentrations of MPG would be effective. Thus most scavengers “will never be present at levels remotely approaching those of endogenous molecules that react at diffusion-controlled rates with HO·” (24). Therefore, we infer that MPG prevents signaling, not by scavenging ROS, but rather by virtue of controlling rates with HO·/RO· reactant. Data presented are representative of 3 independent experiments with P < 0.05.
of its powerful thiol reduction properties, keeping PKCε in a reduced, inactive state. Conclusions drawn from experiments in which MPG blocks ROS signaling, including those of Liu et al. (30), should be reevaluated in view of this new finding related to the mechanism of action of MPG.

To investigate the location giving rise to the endogenous ROS signal, we employed the preincubation protocol described in RESEARCH DESIGN AND METHODS and RESULTS. This protocol leads to feedback activation of mitoKATP, which depends on endogenous ROS signaling to PKCε1 (13). The results of Figs. 4–7 provide important clues to the identity of the ROS messenger. They are best understood in the context of Figs. 1 and 2. The antioxidants defereroxamine and Trolox each prevented mitoKATP opening. Deferoxamine is an iron chelator, and Trolox, a water soluble derivative of vitamin E, is a peroxyl radical scavenger and lipid peroxidation chain-breaking antioxidant. These data indicate that the redox-sensitive opening of mitoKATP is dependent on both iron and peroxyl radicals and are consistent with transition metal-catalyzed ROO-dependent signaling. These steps occur downstream of HO• production and strongly suggest that HO• is the precursor of the signaling molecule. This is supported by the finding that inclusion of DMSO or DMF, which react with HO•, RO•, and/or ROO• (2), in the preincubation prevented mitoKATP opening.

Many workers have proposed that H2O2 is the messenger of ROS-dependent mitoKATP opening (1, 46). H2O2 would appear to be a good candidate, not the least because it is effective in activating mitochondrial PKCε (11, 13, 25, 50) and in protecting ischemic heart (47, 49) and hypoxic cardiomyocytes (16). However, a major finding of the present study is that H2O2 can be excluded as the signaling ROS in mitochondria and that the signaling ROS originates downstream of hydroxyl radical. The evidence is 1) the ROS signal arises downstream from OH• radical, whereas H2O2 is produced upstream of OH radical (Fig. 1); and 2) catalase, the H2O2 scavenger, had no effect on the formation of the phosphorylation-dependent open state, whereas DMF and DMSO, which have neither H2O2 scavenging nor thiol reductant properties, do block PKCε-dependent mitoKATP activation. Circumstantial evidence also argues against H2O2 as the signaling ROS: H2O2 is produced in the matrix, whereas PKCε1 faces the intermembrane space. The matrix of heart mitochondria contains glutathione, thioredoxin reductase, and catalase, which may prevent sufficient H2O2 from reaching its target (42).

The conclusion that the ROS message is formed downstream from HO• is further supported by the effects of DMSO and DMF on endogenous ROS-dependent MPT inhibition (Fig. 10). Finally, the finding that DMF prevents cardioprotection by both ischemic preconditioning and diazoxide (Fig. 11) supports extension of the conclusion to the ex vivo and in vivo heart. That HO• is necessary for cardioprotective signaling should not be taken to mean that HO• itself is the signaling ROS. HO• is too reactive for signaling, with a half-life that is diffusion limited at ~1 ns (24). HO• will react with its nearest neighbors almost immediately after it is formed, allowing for the formation of downstream oxidation products that are most likely responsible for signaling.

Finally, the finding that BEL interrupted the ROS signal (Fig. 7) implicates hydroperoxy fatty acids (FAOOH) as the ROS signal. A full investigation of the effects of fatty acids and hydroperoxy fatty acids on mitoKATP and on ischemia-reperfusion injury will be presented in a subsequent communication.

Limitations. 1) The conclusions are based largely on in vitro experiments carried out on isolated mitochondria. The rationale for this approach is that the signaling ROS not only originates in mitochondria but also acts on mitochondria. We have no reason to believe that the ROS transformation reaction sequence is any different in vivo. 2) We stress that the conclusions relate specifically to ROS signaling secondary to mitoKATP opening. The reverse sequence, mitoKATP opening caused by ROS, may also be physiologically important. For example, it is possible that the increased ROS that occurs during ischemia opens mitoKATP, and this ROS may arise from a different location, such as complex III. The identity of the ROS signal in this setting has not yet been addressed. 3) BEL has been shown to inhibit activation of store-activated Ca2+ channels; however, this is mediated by inhibition of iPLA2 and is therefore a pharmacological effect (40). It has recently been shown that BEL inhibits voltage-gated Ca2+ and transient receptor potential canonical channels independently of iPLA2 (9). We consider it unlikely that these effects play a role in the effect of BEL on isolated mitochondria.

Conclusions and potential conceptual/pragmatic values of these findings. We conclude that the signaling ROS responsible for activating mitochondrial PKCεs is a downstream oxidation product of hydroxyl radical and that superoxide and H2O2 are not the signaling ROS. We further conclude that the main action of submillimolar MPG on mitochondria and perfused heart is not ROS scavenging but rather thiol reduction, causing inactivation of PKCε and other ROS-dependent kinases. MitoKATP-dependent ROS signaling is a central process in cardioprotection and cellular signaling generally. Determining the identity of the mitochondrial signaling ROS is important for future research in these areas.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


