Hydrogen peroxide relaxes porcine coronary arteries by stimulating BK_{Ca} channel activity

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Barlow, Robert S., and Richard E. White. Hydrogen peroxide relaxes porcine coronary arteries by stimulating BK_{Ca} channel activity. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1283–H1289, 1998.—It has been known for a number of years that neutrophils and macrophages secrete H_{2}O_{2} while fighting disease, and the levels obtained within the vasculature under these conditions can reach several hundred micromolar. Because the effect of H_{2}O_{2} on vascular smooth muscle is not fully understood, the present study examined the cellular effects of H_{2}O_{2} on coronary arteries. Under normal ionic conditions, H_{2}O_{2} relaxed arteries that were precontracted with prostaglandin F_{2a} or histamine (EC_{50} = 252 ± 22 μM). The effect of H_{2}O_{2} was concentration dependent and endothelium independent. In contrast, H_{2}O_{2} did not relax arteries contracted with 80 mM KCl, suggesting involvement of K\(^{+}\) channels. Single-channel patch-clamp recordings revealed that H_{2}O_{2} increased the activity of the large-conductance (119 pS), Ca\(^{2+}\)- and voltage-activated K\(^{+}\) (BK_{Ca}) channel. This response was mimicked by arachidonic acid and inhibited by eicosatriynoic acid, a lipoxygenase blocker, suggesting involvement of leukotrienes. Further studies on intact arteries demonstrated that eicosatriynoic acid not only blocked the vasodilatory response to H_{2}O_{2} but unmasked a vasoconstrictor effect that was reversed by blocking cyclooxygenase activity with indomethacin. These findings identify a novel effector molecule, the BK_{Ca} channel, which appears to mediate the vasodilatory effect of H_{2}O_{2}, and suggest that a single signaling pathway, arachidonic acid metabolism, can mediate the vasodilatory and vasoconstrictor effects of H_{2}O_{2} and possibly other reactive oxygen species.

There is increasing evidence that many cellular effects of reactive oxygen species are mediated by changes in membrane ionic conductance. Studies suggest that H_{2}O_{2} modulates currents carried via Na\(^{+}\) (36), Cl\(^{-}\) (27), or Ca\(^{2+}\) (14, 18) channels in various cell types. There is also substantial evidence that K\(^{+}\) channel activity is regulated by oxidation/reduction, and a majority of studies report that H_{2}O_{2} increases K\(^{+}\) conductance. For example, H_{2}O_{2} increases K\(^{+}\) currents in lung adenocarcinoma cells (17), renal epithelial cells (10), pyramidal neurons (28), and pancreatic β-cells (16). In addition, H_{2}O_{2} relaxes arterial smooth muscle by stimulating K\(^{+}\) conductance (30), but no single-channel studies have identified a specific K\(^{+}\) channel opened by H_{2}O_{2} in arterial myocytes. On the other hand, H_{2}O_{2} can also contract smooth muscle, including some blood vessels (21, 24); however, the signaling pathway(s) mediating the effects of H_{2}O_{2} on vascular smooth muscle has not been fully characterized.

H_{2}O_{2} is proposed to stimulate a variety of cellular transduction mechanisms, including protein kinase C (4, 11), phospholipase A_{2} (PLA_{2}) (5, 23), nitric oxide (NO)/L-arginine (6), hydroxytryptamine (33), arachidonic acid (AA) (22), cyclooxygenase (13, 25), and guanyl cyclase (3). Other studies have suggested that oxidation might directly affect ion channel proteins (26) or that a change in cellular redox status might modulate K\(^{+}\) channel gating (38); however, there is no general agreement on the mechanism of how H_{2}O_{2} may induce contraction or relaxation of smooth muscle (2, 13, 24, 31). In light of these apparently contradictory findings, the purpose of the present study was to identify a specific molecular effector of H_{2}O_{2} action in vascular smooth muscle and further characterize the signal transduction mechanism(s) stimulated by H_{2}O_{2} in these cells. We present direct evidence that H_{2}O_{2} opens the large-conductance, Ca\(^{2+}\)- and voltage-activated K\(^{+}\) (BK_{Ca}) channel in myocytes from porcine coronary arteries. Subsequent pharmacological characterization employing tissue and cellular studies suggested that this response is mediated via lipoxygenase metabolites of AA, whereas cyclooxygenase metabolites appeared to mediate H_{2}O_{2}-induced arterial contraction. These findings now identify a specific K\(^{+}\) channel and single transduction mechanism (AA metabolism) that could underlie many of the vascular effects of H_{2}O_{2} and other reactive oxygen species.

MATERIALS AND METHODS

Arterial tension studies. Fresh porcine hearts were obtained from a local abattoir. The left anterior descending coronary artery was excised and placed into ice-cold dissociation medium of the following composition (mM): 110.0 NaCl, 5.0 KCl, 2.0 MgCl_{2}, 0.16 CaCl_{2}, 10.0 HEPES, 10.0 NaHCO_{3}, 0.5 KH_{2}PO_{4}, 0.5 NaH_{2}PO_{4}, 0.49 EDTA, 10.0 taurine, and 10.0 glucose (pH 6.9). Arteries were kept on ice during transport to the laboratory. Arterial rings (4–5 mm long, 2–4 mm diameter) were obtained from each left anterior descending coronary artery and prepared for isometric contractile force recordings as described previously (39). To control for possible indirect effects of endothelium-derived vasoactive factors, the endothelium was removed by rubbing the intimal surface. Rings were mounted between two triangular tissue supports, with one fixed to the bottom of the tissue bath and the other attached to a force-displacement transducer, and contractile force (grams) was recorded on a computer every 3 s. The tissue-bathing solution was a modified Krebs-Henseleit buffer of the following composition (mM): 118.0 NaCl, 4.8 KCl, 1.2 MgCl_{2}, 2.5 CaCl_{2}, 25.0 NaHCO_{3}, 1.2 KH_{2}PO_{4}, and 11.0 glucose (pH 7.4). This solution was continuously oxygenated (97% O_{2}/3% CO_{2}) and heated to 37°C. Coronary ring prepara-
tions were equilibrated for 90 min under an optimal resting tension of 2.0 g, and fresh solution was added every 30 min.

After equilibration, preparations were exposed to contractile agents [e.g., 10 µM histamine, 5 µM prostaglandin F2α (PGF2α)], which maximally contracted the arteries and ensured stabilization of the muscle. After agonist removal and reequilibration (30 min), the contractile agent was reapplied to the tissue bath, and when the tissue reached a stable maximum contraction, H2O2 was added to the bathing medium. All drug solutions were prepared fresh daily, and for solutions with high K+ concentration ([K+]o), NaCl was reduced to maintain normal osmolarity and Cl− concentration.

Cell isolation. Myocytes were isolated as described previously (39). Fat and connective tissue were removed, and the adventitia was carefully dissected away. Each artery was then cut into 1-mm strips and placed in test tubes containing dissociation medium as described above. Media strips were incubated at 37°C in 5 ml of the dissociation solution with 5.0 mg of papain, 2.3 mM dithiothreitol, and 0.2% BSA. After 30 min of shaking in a water bath at 37°C, the tissue was triturated and the enzyme activity was diluted by addition of excess enzyme-free solution. The solution was removed and centrifuged at 500 × g for 6 min at 4°C. The pellet was then resuspended in fresh medium and kept at 4°C. Experiments were performed within 6–8 h after cell dissociation.

Patch-clamp studies. For cell-attached patches, several drops of cell suspension were placed in a recording chamber (Warner Instruments) containing a solution of the following composition (mM): 140 KCl, 10 MgCl2, 0.1 CaCl2, 10 HEPES, and 30 glucose (pH 7.4, 22–25°C). Single K+ channels were measured in cell-attached patches by filling the patch pipette (2–5 MΩ) with Ringer solution composed of (mM) 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, and 10 HEPES and making a gigahm seal on a single myocyte. Voltage across the patch was controlled by clamping the cell at 0 mV with the high-[K+] extracellular solution. In experiments recording K+ channel activity of inside-out patches, the bathing solution exposed to the cytoplasmic surface of the membrane consisted of the following (in mM): 60 K2SO4, 30 KCl, 2 MgCl2, 0.16 CaCl2, 10 HEPES, 5 ATP, 1,1,2-bis(2-aminoophenacyl)ethane-N,N,N′,N′-tetraacetic acid, and 10 glucose (pH 7.4, 22–25°C). The pipette solution was the same Ringer solution described above. Currents were filtered at 2 kHz and digitized at 10 kHz. Average channel activity [open channel probability (NPo)] in patches with multiple BKCa channels was determined as described previously (39).

Drugs. Eicosatriynoic acid (ETI), papain, indomethacin, dithiothreitol, PGF2α, tetraethylammonium (TEA), and histamine were purchased from Sigma Chemical (St. Louis, MO). ODQ [1H-[1,2,4]oxadiazolo[4,3-a]quinolizin-1-one] and 1,2-bis(2-aminoophenacyl)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) were purchased from Calbiochem (La Jolla, CA). H2O2 was purchased from Fisher Scientific (Pittsburgh, PA).

Statistical analysis. Data from tissue studies are expressed as the percentage of maximum relaxation, and all other data are expressed as means ± SE. Statistical significance between two groups was evaluated by Student’s t-test for paired data. Comparison between multiple groups was carried out by one-way ANOVA, with a post hoc Tukey’s test to determine significant differences among the data groups. P < 0.05 was considered to indicate a significant difference.

RESULTS

H2O2 relaxes porcine coronary arteries. H2O2 produced concentration-dependent relaxation of endothelium-denuded coronary artery ring preparations precon-
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Fig. 2. H2O2-induced relaxation of coronary arteries requires physiological K+ gradients. A: H2O2 (300 µM) did not relax arteries precontracted with 80 mM KCl (n = 4). These same preparations had previously demonstrated H2O2-induced relaxation when precontracted with 10 µM histamine in physiological extracellular K+ concentration. Dashed line, baseline tension; w/o, washout of all drugs. B: comparison of 300 µM H2O2-induced relaxation responses in agonist-contracted (histamine) arteries and in arteries contracted with 80 mM KCl. *Significant depression of relaxation effect.

After a stimulatory effect of 300 µM H2O2 pipette solution on K+ channels was demonstrated in cell-attached experiments (NPo = 0.583 ± 1.09), the patch was excised into an inside-out configuration. K+ channel activity persisted (NPo = 0.254 ± 1.03), but subsequent addition of 1 mM TEA almost completely abolished channel activity (NPo = 0.017 ± 0.01; P = 0.0002, n = 5). At this concentration, TEA is a selective blocker of BKCa channels. Previous experiments from our laboratory measured the single-channel conductance of this same channel to be 119 ± 14 pS and also demonstrated its sensitivity to intracellular Ca2+, 1 mM TEA, and charybdotoxin (39). Therefore, this pharmacological and biophysical analysis clearly identifies the BKCa channel as an important target of H2O2 action in porcine coronary arteries. In contrast to our previous studies with estrogen and NO (7, 39), the stimulatory effect of H2O2 on BKCa channels did not involve production of cGMP, inasmuch as pretreating myocytes with 10 µM ODQ, a selective inhibitor of guanylyl cyclase activity (IC50 = 20 nM), had no effect on H2O2-induced channel activity but attenuated stimulation by 10 µM nitroprusside (NPo = 0.0, 0.112, and 0.975 for control, nitroprusside, and H2O2, respectively, n = 3; data not shown).

H2O2 activation of BKCa channels involves the lipoxygenase pathway. Previous studies suggested that some of the vascular effects of H2O2 might be mediated by metabolites of the PLA2/AA cascade (5, 11), and the importance of this mechanism was tested on cell-attached patches. Addition of 10 µM AA to the bath solution stimulated BKCa channel activity by ~200-fold (NPo from 0.0017 to 0.2857, +40 mV, n = 3, P = 0.003) in cell-attached patches (Fig. 4). This increase was very similar to the ~200-fold increase in NP0 produced by H2O2. Interestingly, AA-stimulated channel activity was reversed ~80% by 5 µM ETI (30 min, NPo = 0.0657), an inhibitor of 5-, 12-, and 15-lipoxygenase activity. The inhibitory effect of ETI was subsequently reversed by 10 µM indomethacin, an inhibitor of cyclooxygenase activity. Because AA mimicked the stimulatory effect of H2O2 and appeared to enhance lipoxygenase metabolism, the next experiments tested whether these metabolites might also mediate the effects of H2O2 on BKCa channels. Once again, H2O2 enhanced single-channel activity within 30 min of exposure (NPo from 0.00 to 0.825; Fig. 5A), but subsequent addition of 5 µM ETI (30 min) almost completely reversed H2O2-induced BKCa channel activity (NPo = 0.067). On average, 5 µM ETI inhibited the response to H2O2 by 84 ± 13% (P = 0.005, n = 4). This reversal was similar to the inhibitor effect of ETI on AA-induced channel activity (80%) and suggested that lipoxygenase metabo-

Fig. 3. H2O2 stimulates large-conductance Ca2+- and voltage-activated K+ (BKCa) channel activity in myocytes from porcine coronary arteries. A: consecutive recordings from a cell-attached patch (-40 mV) before and 30 min after administration of 300 µM H2O2. Upward deflections, channel openings; dashed line, channel closed state. B: summary of single-channel studies identifying BKCa channel as a target of H2O2. H2O2 (300 µM, within the patch pipette) stimulated channel activity in cell-attached (C-A) patches (+40 mV). Patches were then excised into inside-out (I-O) configuration, and effects of 1 mM tetraethylammonium (TEA) on channel activity were determined (n = 5). *Significant increase in BKCa channel open probability compared with control (C-A and I-O) or significant inhibition of channel activity produced by TEA.

Fig. 4. Arachidonic acid (AA) stimulates BKCa channel activity via lipoxygenase metabolites. Cumulative data from 3 cell-attached patch experiments (+40 mV) are shown. AA (10 µM, 30 min) stimulated BKCa channel activity, and this effect was reversed by 5 µM eicosatriynoic acid (ETI; 30 min). Subsequent addition of indomethacin (Indo; 10 µM) restored AA-stimulated channel activity. All drugs were added cumulatively. *Significant increase in channel open probability vs. control.
lites were involved in the vascular response to H2O2. A complete time-course plot of channel activity before and after H2O2, with subsequent inhibition by ETI, is illustrated in Fig. 5B.

H2O2 induces vasoconstriction when lipoxygenase activity is inhibited. To further characterize the potential involvement of AA metabolites, tension studies were performed to test whether lipoxygenase metabolites were also involved in the relaxation effect of H2O2. Arteries were initially contracted with histamine or PGF2α, and exposure to 300 µM H2O2 induced the expected relaxation. After washout, preparations were incubated with 5 µM ETI for 30 min, and after preconstriction, subsequent addition of H2O2 (5 min) never induced significant relaxation (Fig. 6A; n = 5). In contrast, when arteries were preincubated with ETI and then exposed to 300 µM H2O2 without precontraction, the oxidant contracted the arteries (Fig. 6B). This vasoconstrictor effect was observed in 11 arteries pretreated with ETI (P = 0.0003), whereas only a vasodilator effect was observed in these same arteries in the absence of lipoxygenase inhibition. Interestingly, this contractile response to H2O2 was reversed by further treatment with 10 µM indomethacin (Fig. 6C). Indomethacin reversed H2O2-induced contraction by an average of 85 ± 4% (n = 4). These findings demonstrated that H2O2 can act as a vasodilator or a vasoconstrictor, depending on the disposition of AA metabolism. Additional control experiments demonstrated that the inhibitory effect of ETI did not involve inhibition of guanylyl cyclase activity. Pretreating arteries with 5 µM ETI did not inhibit relaxation produced by 10 µM sodium nitroprusside, which induced 100% relaxation in the absence or presence of ETI (n = 4; data not shown).

DISCUSSION

It is clear that H2O2 is a vasoactive substance; however, our understanding of how this oxidant affects vascular smooth muscle cells is far from complete. For example, H2O2 may act as a vasoconstrictor (24) or a vasodilator (6, 33), depending on the specific artery, species, or experimental conditions. There is even less certainty regarding the signal transduction and/or effector mechanism(s) that may be involved in the vascular response to H2O2, e.g., protein kinase C (4, 11), PLA2 (5, 23), guanylyl cyclase (3), or NO (6). The present study helps resolve some of this controversy by providing direct evidence for an effector molecule that can medi-
H2O2-induced vasodilation, the BKca channel, and also proposes that a single signaling pathway, AA metabolism, can mediate the vasodilatory and vasocostricter effects of H2O2 on porcine coronary arteries.

Twenty years ago, Olson and Boerth (19) found that H2O2 exerted beneficial effects on the coronary circulation. More recent studies have demonstrated that H2O2 increases coronary blood flow (29) and relaxes coronary arteries in vitro by a mechanism that is specific to H2O2 and does not involve superoxide anion or hydroxyl radical (2). In the present study, physiological concentrations of H2O2 relaxed porcine coronary arteries precontracted with agonists. In contrast, H2O2 failed to relax arteries precontracted by elevating extracellular [K+] to 80 mM. Raising external [K+] reduces the driving force for K+ efflux, thereby functionally limiting the influence of K+ channels on vascular reactivity. The inability of H2O2 to relax arteries precontracted with high extracellular [K+] suggested that the relaxation response to H2O2 involved stimulation of K+ conductance, a mechanism that has also been suggested from pharmacological studies of arterial smooth muscle (30, 36) and microelectrode/whole cell studies employing other cell types (10, 28). In contrast, the present study has measured the effects of H2O2 on single-channel activity directly in cell-attached patches. H2O2 increased the open probability of a large-conductance (> 100 pS), Ca2+- and 1 mM TEA-sensitive K+ channel, which we and others previously identified as the BKca channel in these cells (34, 39). These channels are expressed in high density in myocytes from porcine or human (12) coronary arteries and are important effectors that mediate vasodilatation produced by a number of agents by inducing membrane repolarization. Further support for BKca channel involvement is gained from previous studies indicating that H2O2 hyperpolarizes myocytes from rat carotid arteries by a mechanism that was partially sensitive to charybdotoxin (15). Moreover, H2O2-induced relaxation of cerebral arteries is inhibited by 1 mM TEA, further implicating a role for BKca channels (30).

Although there is increasing evidence that ion channels mediate the effects of H2O2 and other oxidants on cell excitability, we have found no studies examining the effects of H2O2 on single ion channels expressed in their native cells. Microelectrode and/or whole cell studies revealed that H2O2 increases a Ca2+-activated Cl− current in oocytes (27) and slows inactivation of tetrodotoxin-sensitive Na+ currents in rat ventricular myocytes (36). In addition, H2O2 opens or closes Ca2+ channels incorporated into artificial lipid bilayers, depending on the concentration employed (14, 18). Several studies also indicate that H2O2 increases K+ currents. For example, H2O2 induces a K+ conductance in CA1 pyramidal neurons (28), activates a gliobalamide-sensitive K+ conductance in LLC-PK1 cells (10), and inhibits inactivation of K+ currents in oocytes expressing cloned K+ channels (35). Studies utilizing oxidizing agents other than H2O2 have also demonstrated stimulation of BKca channel activity in cell-attached patches on myocytes from pulmonary or ear artery (20). In contrast, H2O2 decreases open probability of cloned hskoCa2+-activated K+ channels expressed in oocytes or HEK-293 cells (8). The rationale for the variance between this finding and those of the present and other studies (20) is not apparent but may be related to differences in cell or channel properties. For example, studying the regulation of cloned channels expressed in transfected cells can be limited by the fact that signaling systems and/or regulatory proteins that influence channel activity under normal conditions may not be present in transfected cells.

Previous studies have proposed a variety of molecular pathways that can be stimulated by H2O2. For example, activation of guanylyl cyclase may underlie H2O2 relaxation of pulmonary arteries (3). Because we showed previously that stimulation of the NO/cGMP signaling cascade opened BKca channels in myocytes from porcine coronary arteries (7, 39), it was possible that H2O2 was also acting via this mechanism; however, inhibition of guanylyl cyclase did not affect the response to H2O2. Furthermore, ETI did not inhibit relaxation induced by nitroprusside, nor does cGMP induce vascular contraction as H2O2 does when lipoxigenase is blocked. These findings make it unlikely that the effect of H2O2 on BKca channels in porcine coronary arteries involves cGMP. On the other hand, H2O2 increases protein kinase C production in A7r5 smooth muscle cells (11) and pulmonary arterial smooth muscle, with subsequent increases in PLA2 activity and AA release (5). Because other studies had demonstrated a link between AA and K+ channels in neurons (1) and neuroendocrine cells (9), we considered that AA metabolites might mediate the stimulatory effect of H2O2 on BKca channels in coronary smooth muscle. Addition of exogenous AA stimulated BKca channel activity in cell-attached patches dramatically, and this increased activity was significantly reversed by ETI, an inhibitor of 5-, 12-, and 15-lipoxigenase activity. Moreover, the inhibitory effect of ETI was completely reversed by indomethacin, probably because of shunting of AA metabolism from cyclooxygenase toward the lipoxigenase pathway. These experiments suggested that lipoxigenase metabolites of AA stimulate the activity of BKca channels, and subsequent experiments further implicated AA metabolism as an important signaling mechanism in the response to H2O2. Not only did AA mimic the effect of H2O2 on BKca channels, but ETI also inhibited the effect of H2O2 on intact tissues and single myocytes. In fact, ETI unmasked a contractile effect of H2O2 (Fig. 6B). As in single-channel recordings, indomethacin reversed the effect of ETI by inhibiting H2O2-induced contraction. The ability of ETI and indomethacin to inhibit and disinhibit responses to H2O2, respectively, casts doubt on the possibility that H2O2 is acting directly on BKca channel proteins. Instead, these findings provide evidence that AA metabolism may account for H2O2-induced contraction and relaxation of coronary arteries.
We propose that under normal conditions $H_2O_2$ increases AA metabolism, probably via stimulation of PLA2 activity (11), and lipoxygenase derivatives of AA then promote vasorelation because of their powerful stimulation of BKCa channel activity; however, if lipoxygenase activity is inhibited, then AA metabolism is shunted toward cyclooxygenase, and vasoconstriction ensues because of production of contractile prostanoids (e.g., thromboxane). If this hypothesis is correct, then inhibition of cyclooxygenase should reverse the contractile effect of $H_2O_2$. Experiments with indomethacin revealed that this was indeed the case (Fig. 6C). Therefore, $H_2O_2$ appears to stimulate PLA2/AA metabolism, and, depending on which pathway is dominant, lipoxygenase or cyclooxygenase, H2O2 could act as a vasodilator or a vasoconstrictor. This mechanism is consistent with studies demonstrating that H2O2 stimulates PLA2 activity in arterial smooth muscle cells (23) and others demonstrating that indomethacin attenuates H2O2-induced contraction of tracheal smooth muscle (25, 33). Furthermore, Duerser et al. (9) demonstrated that the neurohormone somatostatin inhibits excitability of pituitary cells by opening BKCa channels via lipoxygenase metabolites. In this and the present study, ETI reversed agonist-induced stimulation of BKCa channel activity but had no direct inhibitory effect on channel proteins. Therefore, stimulation of BKCa channel activity by lipoxygenase metabolites may be a ubiquitous mechanism to depress activity of excitable cells; however, further studies are needed to identify the specific leukotrienes and/or AA metabolites involved. Identification of these products, coupled with a more complete understanding of their effects on cellular physiology, should help shed more light on our understanding of diseases such as ischemia-reperfusion injury, myocardial stunning, and obstructive airway diseases, where oxidative damage has been implicated.

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