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Hydrogen Peroxide Potentiates the EDHF Phenomenon by Promoting Endothelial Ca\(^{2+}\) Mobilization

David H. Edwards, Yiwen Li, Tudor M. Griffith

Objective—The purpose of this study was to test the hypothesis that H\(_2\)O\(_2\) contributes to the EDHF phenomenon by mobilizing endothelial Ca\(^{2+}\) stores.

Methods and Results—Myograph studies with rabbit iliac arteries demonstrated that EDHF-type relaxations evoked by the SERCA inhibitor cyclopiazonic acid (CPA) required activation of K\(_{Ca}\) channels and were potentiated by exogenous H\(_2\)O\(_2\) and the thiol oxidant thimerosal. Preincubation with a submaximal concentration of CPA unmasked an ability of exogenous H\(_2\)O\(_2\) to stimulate an EDHF-type response that was sensitive to K\(_{Ca}\) channel blockade. Imaging of cytosolic and endoplasmic reticulum [Ca\(^{2+}\)] in rabbit aortic valve endothelial cells with Fura-2 and Mag-fluo-4 demonstrated that H\(_2\)O\(_2\) and thimerosal, which sensitizes the InsP\(_3\) receptor, both enhanced CPA-evoked Ca\(^{2+}\) release from stores, and that the potentiating effect of H\(_2\)O\(_2\) was suppressed by the cell-permeant thiol reductant glutathione monoethylester. CPA-evoked relaxations were attenuated by exogenous catalase and potentiated by the catalase inhibitor 3-aminotriazole, and were abolished by the connexin-mimetic peptide 43Gap26, which interrupts intercellular communication via gap junctions constructed from connexin 43.

Conclusions—H\(_2\)O\(_2\) can enhance EDHF-type relaxations by potentiating Ca\(^{2+}\) release from endothelial stores, probably via redox modification of the InsP\(_3\) receptor, leading to the opening of hyperpolarizing endothelial K\(_{Ca}\) channels and an electrotonically-mediated relaxant response. (Arterioscler Thromb Vasc Biol. 2008;28:1774-1781)

Key Words: hydrogen peroxide ■ thimerosal ■ SERCA pump ■ EDHF

The endothelium regulates arterial tone through the release of nitric oxide (NO) and vasodilator prostanoids and an NO/prostanoid-independent mechanism that involves smooth muscle hyperpolarization. Some workers have attributed this electrical response to a freely diffusible endothelium-derived hyperpolarizing factor (EDHF), whose identity has variously been proposed as H\(_2\)O\(_2\), K\(^+\) ions or epoxyeicosatrienoic acid metabolites of arachidonic acid.\(^1\)\(^2\) Definitive classification of underlying mechanisms has nevertheless been obscured by the existence of species- and vessel-specific differences in the contribution of such factors to relaxation. Indeed, it is now widely recognized that increases in endothelial [Ca\(^{2+}\)], underpin the EDHF phenomenon by promoting the opening of Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{Ca}\)).\(^2\) and there is evidence that the resulting hyperpolarization can then spread electrotonically through the vascular wall via myoendothelial and homocellular smooth muscle gap junctions to promote relaxant response.\(^3\) Agents that elevate endothelial [Ca\(^{2+}\)], by stimulating the generation of InsP\(_3\) or activate store-operated Ca\(^{2+}\) entry (SOCE) by blocking Ca\(^{2+}\) sequestration via the endoplasmic reticulum SERCA pump, may thus evoke hyperpolarization and “EDHF-type” relaxations that can be suppressed either by pharmacological blockade of K\(_{Ca}\) channels or gap junctional communication.\(^3\)\(^4\)

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In some artery types, there is evidence that exogenous H\(_2\)O\(_2\) can evoke smooth muscle relaxation by activating hyperpolarizing K\(_{Ca}\) channels and that EDHF-type relaxations and hyperpolarizations are associated with catalase-inhibitable endothelial H\(_2\)O\(_2\) production, leading to the hypothesis that endogenously-generated H\(_2\)O\(_2\) can serve as a freely diffusible EDHF.\(^1\) By contrast, in human mesenteric arterioles endothelium-dependent agonists promote H\(_2\)O\(_2\)-dependent relaxation, but exogenous H\(_2\)O\(_2\) causes constriction when such vessels are denuded of their endothelium, leading to the proposal that H\(_2\)O\(_2\) releases a chemically distinct EDHF.\(^5\) A further permutation, not previously described in the context of the EDHF phenomenon, is that H\(_2\)O\(_2\) promotes Ca\(^{2+}\) release from intracellular stores\(^6\)\(^7\) thereby elevating [Ca\(^{2+}\)], directly and enhancing SOCE, and contributing to a conducted hyperpolarizing response through the activation of endothelial K\(_{Ca}\) channels. To examine this scenario, we have therefore investigated the role of H\(_2\)O\(_2\) in EDHF-type relaxations of the rabbit iliac artery (RIA), a vessel in which H\(_2\)O\(_2\) cannot be regarded as a transferable EDHF because smooth muscle hyperpolarizations evoked by exogenous H\(_2\)O\(_2\) are much smaller than those associated with the authentic EDHF.
phenomenon.8 Because there is evidence that H2O2 depletes Ca2+ stores by sensitizing the InsP3 receptor, thereby mimicking the effects of agents that oxidize thiol groups such as the organic mercurial thimerosal,6,7,9–12 we compared the effects of H2O2 and thimerosal on EDHF-type relaxations and Ca2+ mobilization, using fura-2 and the low affinity Ca2+-probe Mag-fluo-4, which selectively loads the endoplasmic reticulum (ER), to monitor [Ca2+]i and [Ca2+]ER in the rabbit aortic valve (RAV) endothelium.13,14 This preparation was selected for Ca2+ imaging studies because H2O2 and thimerosal can both elevate vascular smooth muscle [Ca2+]i, and in intact vessels Ca2+ signals can be transmitted from the media to the endothelium via myoendothelial gap junctions.12,15,16 To avoid potentially confounding effects of H2O2 on receptor-coupled pathways mediated via phospholipase C (PLC),17 responses were evoked by the SERCA inhibitor cyclopiazonic acid (CPA). Although native arterial endothelial cells are widely recognized to express small- and intermediate-conductance SKCa and IKCa channels, also classified as SK1–3 and SK4 on the basis of structural and gating characteristics,7,18–21 in some artery types the endothelium also expresses functional large-conductance BKCa channels.22–26 The contributions of the 3 channel subtypes to EDHF-type relaxations were therefore investigated with selective SKCa, IKCa, and BKCa inhibitors (apamin, TRAM-34 and iberiotoxin, respectively), and the dominant role of electrotrophic signaling confirmed with a synthetic peptide (43Gap26) that blocks intercellular communication via gap junctions constructed from connexin 43.13,27,28

**Methods**

Full details are provided in the supplemental materials (available online at http://atvb.ahajournals.org). Mechanical responses were studied in myograph-mounted iliac artery rings obtained from male NZW rabbits and incubated in Holmans buffer. In some experiments the endothelium was removed by gentle abrasion. To study endothelial Ca2+ mobilization rabbit aortic valve leaflets were isolated and loaded either with Fura-2 AM to assess [Ca2+]i, or the low-affinity Ca2+ indicator mag-fluo-4 to assess [Ca2+]i in endothelial ER stores. Leaflets were imaged either with an inverted microscope to obtain background-corrected F43/38 ratios and calculate [Ca2+]i, or confocal microscopy to track the effects of interventions on Mag-fluo-4 fluorescence with changes in [Ca2+]i being assessed as fluorescence normalized to its value at the beginning of each experiment (ΔF/F0). All experiments were performed in the presence of L-NAME (300 μmol/L) and indomethacin (10 μmol/L) to inhibit the production of NO and prostanoids. Maximal percentage reversal of phenylephrine-induced constriction (Rmax) by CPA or H2O2 and concentrations giving 50% reversal of this constrictor response (IC50) in the case of CPA or 50% of maximal relaxation (EC50) in the case of H2O2 were determined. The use of IC50 was necessary to allow for a small initial constriction to CPA that was observed in many experiments. All data are presented as mean±SEM and were compared by the Student t test or ANOVA followed by an appropriate post test. *P<0.05 was considered significant; n denotes the number of animals studied for each data point.

**Results**

**Mechanical Responses to CPA**

In endothelium-intact rings constricted by phenylephrine (1 μmol/L), CPA-evoked relaxations were maximally equivalent to 85.6±1.4% of the constrictor response to phenylephrine (Rmax), although the threshold for relaxation was variable, being 10 or 30 μmol/L in different preparations, with an overall IC50 of 16 μmol/L (pIC50 4.79±0.02, n=54; data pooled from supplemental Table I). Relaxation was often preceded by small increases in tension (up to 10% at 3 μmol/L CPA), which are likely to reflect reduced buffering of Ca2+ influx following blockade of the smooth muscle SERCA pump.28

**Effects of H2O2 and Thimerosal**

Preincubation of endothelium-intact RIA rings with 100 μmol/L H2O2 markedly potentiated relaxations evoked by CPA at concentrations of 1 to 10 μmol/L, without alteration in overall Rmax (Figure 1A; supplemental Table I). Similarly, 100 μmol/L H2O2 potentiated the increases in [Ca2+]i, evoked by 1 to 10 μmol/L CPA in the endothelium of the RAV, but did not affect Ca2+ mobilization in response to 30 and 100 μmol/L CPA and minimally elevated basal [Ca2+]i (Figure 1B). In the presence of 100 μmol/L H2O2, the increase in [Ca2+]i, evoked by 10 μmol/L CPA attained the same level

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**Figure 1.** Effects of 100 μmol/L H2O2 on CPA-evoked responses. A, Potentiation of RIA relaxation (n=6); B, Synergistic elevation of RAV [Ca2+]i by H2O2 and 10 μmol/L CPA, whereas H2O2 was ineffective after 30 μmol/L CPA. Bar graphs confirm potentiation of RAV endothelial Ca2+ mobilization and its prevention by GSH-MEE (n=4 to 6). *P<0.05, **P<0.01 compared with corresponding CPA control.
observed with 30 µmol/L CPA and was not affected by the order in which these agents were administered (Figure 1B). Preincubation with GSH-MEE (1 mmol/L) blocked the ability of H2O2 to potentiate Ca2+ mobilization by 10 µmol/L CPA, but did not affect the control response to CPA (Figure 1B).

Preincubation with 10 µmol/L thimerosal caused a pronounced leftward shift in the concentration-relaxation curve for CPA in endothelium-intact rings with potentiation again being evident over the range 1 to 10 µmol/L but Rmax unaltered (Figure 2A; supplemental Table I). Thimerosal did not itself evoke relaxation at concentrations ≥10 µmol/L, but at concentrations ≥30 µmol/L induced a triphasic response consisting of an endothelium-dependent relaxation superimposed on a biphasic direct smooth muscle response in which constriction preceded relaxation (Figure 2B). At the sub-threshold concentration of 10 µmol/L, thimerosal potentiated increases in [Ca2+]i, evoked by CPA over the range 1 to 10 µmol/L in the RAV endothelium, but not at 30 or 100 µmol/L CPA (Figure 2C). High concentrations of thimerosal themselves increased RAV [Ca2+]i, with an EC50 of ≈60 µmol/L (pEC50 4.25±0.12, n=4) with no elevation being evident at concentrations ≤10 µmol/L (Figure 2D).

Mag-fluo-4 fluorescence in the endoplasmic reticulum of the RAV was decreased by 10 and 30 µmol/L CPA in a concentration-dependent fashion (Figure 3A through 3C). In the presence of 100 µmol/L H2O2 or 10 µmol/L thimerosal, depletion of the ER Ca2+ store by 10 µmol/L CPA increased to a level that was statistically similar to that observed with 30 µmol/L CPA alone (Figure 3B and 3C). Neither 100 µmol/L H2O2 nor 10 µmol/L thimerosal affected ER fluorescence in the absence of CPA.

Mechanisms Contributing to CPA-Evoked Relaxation

CPA-evoked relaxations were unaffected by apamin (1 µmol/L) or TRAM-34 (10 µmol/L) individually, whereas...
Relaxation to Exogenous H₂O₂
Concentration-relaxation curves for H₂O₂ were similar in rings with and without endothelium and were unaffected by IbTX, apamin + TRAM-34 (data not shown) or apamin + TRAM-34 + IbTX in combination (Figure 5A; supplemental Table II). By contrast, in endothelium-intact rings preincubated with 10 μmol/L CPA, concentration-relaxation curves for H₂O₂ were shifted to the left with a small increase in Rₘₐₓ (Figure 5B; supplemental Table II), whereas no potentiation was seen in endothelium-denuded rings (data not shown). The potentiating effects of CPA in rings with endothelium were attenuated by IbTX, the combination of apamin + TRAM-34, and the triple combination apamin + TRAM-34 + IbTX (Figure 5B; supplemental Table II), thus confirming the participation of the three KCa channel subtypes.

Discussion
The principal finding of the present study is that H₂O₂ can promote EDHF-type relaxations of rabbit arteries via an endothelium-dependent mechanism that is distinct from its more widely-recognized smooth muscle action. The endothelial component of the response to H₂O₂ was shown to involve enhanced Ca²⁺ release from stores with secondary activation of KCa channels. H₂O₂ may thus contribute to relaxations mediated by the spread of endothelial hyperpolarization via gap junctions, rather than acting as a freely transferable EDHF, because its smooth muscle relaxing effects were insensitive to KCa channel blockade.

Preincubation of RIA rings with 100 μmol/L H₂O₂ markedly potentiated EDHF-type relaxations evoked by CPA when this SERCA inhibitor, which activates SOCE by depleting ER Ca²⁺ stores, was administered at concentrations ≥10 μmol/L. Correspondingly, preincubation of RAV leaflets with 100 μmol/L H₂O₂ amplified ER emptying and elevations in [Ca²⁺]ᵢ, evoked by CPA at concentrations ≥10 μmol/L, thus matching the range over which H₂O₂ potentiated relaxation. Because 100 μmol/L H₂O₂ did not itself induce changes in [Ca²⁺]ᵢ, this synergism suggests that H₂O₂ sensitizes the InsP₃ receptor. Indeed, heparin, an established antagonist of this receptor, abolishes H₂O₂-evoked relaxations were almost abolished by the connexin-mimetic peptide ^4^Gap26 (100 μmol/L; Figure 4B; supplemental Table I).

Subanalysis showed that the ability of catalase to depress relaxation in individual rings correlated with their sensitivity to CPA, with inhibition being observed where the threshold was 10 μmol/L, but not in rings where the threshold for relaxation was 30 μmol/L (Figure 4C and 4D; supplemental Table I). In rings exhibiting a threshold at 30 μmol/L CPA, apamin + TRAM-34 or iberiotoxin effectively abolished relaxation, whereas in those with the lower threshold of 10 μmol/L, combined KCa channel blockade reduced Rₘₐₓ to ≈60% and this was further reduced by catalase to ≈40%. The catalase inhibitor 3-aminotriazole (ATZ, 50 mmol/L) selectively potentiated relaxations to 10 μmol/L CPA without affecting Rₘₐₓ (Figure 4E; supplemental Table I). Control CPA-evoked relaxations and their potentiation by 100 μmol/L H₂O₂ were unaffected by the PLC inhibitor U-73122 (10 μmol/L) (Figure 4F; supplemental Table I).

Figure 4. Mechanisms contributing to CPA-evoked relaxation. A, Combinatorial effects of apamin, TRAM-34 and iberiotoxin (n=4 to 25). Residual responses observed in the presence of apamin + TRAM-34 + IbTX were further attenuated by catalase (n=9). B, Responses were effectively abolished by ^4^Gap 26 (n=4). C and D, Differential effects of catalase and apamin + TRAM-34 + IbTX according to whether the threshold for relaxation was 10 or 30 μmol/L CPA (n=4 to 10). E, Relaxation was potentiated by 3-aminotriazole (n=6). F, U-73122 did not impair the potentiating effects of H₂O₂ on relaxation (n=5). *P<0.05, **P<0.01, ***P<0.001 compared with control (see also supplemental Table I).

Rₘₐₓ was reduced to ≈60% by preincubation either with the double combination apamin + TRAM-34 or iberiotoxin (IbTX, 100 mmol/L), and decreased to ≈35% in the presence of the triple combination apamin + TRAM-34 + IbTX (Figure 4A; supplemental Table I). This residual relaxation was further attenuated to ≈20% by catalase (2000 U/mL). Control relaxations were almost abolished by the connexin-mimetic peptide ^4^Gap26 (100 μmol/L; Figure 4B; supplemental Table I).
Figure 5. Interactive effects of CPA and K<sub>Ca</sub> inhibitors on H<sub>2</sub>O<sub>2</sub>-evoked relaxation. A, Relaxant effects of H<sub>2</sub>O<sub>2</sub> were unaffected by apamin+TRAM-34+iberiotoxin or endothelial denudation (n=5 to 8). B, Potentiation of H<sub>2</sub>O<sub>2</sub>-evoked relaxation by 10 μmol/L CPA in endothelium-intact rings was attenuated by iberiotoxin, apamin+TRAM-34, or apamin+TRAM-34+iberiotoxin (n=5 to 20). *P<0.05, **P<0.01, and ***P<0.001 compared with control (see also supplemental Table II).
Ca\textsuperscript{2+} release from ER stores in permeabilized endothelial cells,\textsuperscript{6} whereas the endothelium-specific SERCA3 pump isoform, which plays a key role in endothelium-dependent relaxation, is insensitive to H\textsubscript{2}O\textsubscript{2}, in contrast to the SERCA2b isoform found in smooth muscle.\textsuperscript{29,30} Whereas millimolar concentrations of H\textsubscript{2}O\textsubscript{2} can promote Ca\textsuperscript{2+} release from mitochondria or depress extrusion of cytosolic Ca\textsuperscript{2+} by the plasma membrane Ca\textsuperscript{2+} ATPase,\textsuperscript{6,31–33} the participation of these ER-independent mechanisms was excluded by observations that (1) 100 \textmu mol/L H\textsubscript{2}O\textsubscript{2} minimally affected [Ca\textsuperscript{2+}], and (2) relaxation and Ca\textsuperscript{2+} mobilization were maximal at CPA concentrations of 30 to 100 \textmu mol/L and not further increased by 100 \textmu mol/L H\textsubscript{2}O\textsubscript{2}. The possibility that the Ca\textsuperscript{2+} mobilizing effects of H\textsubscript{2}O\textsubscript{2} might involve activation of PLC and enhanced InsP\textsubscript{3} synthesis\textsuperscript{37} was excluded by the demonstration that the PLC inhibitor U-73122 did not affect the potentiation of CPA-evoked relaxation by H\textsubscript{2}O\textsubscript{2}. This is consistent with reports that H\textsubscript{2}O\textsubscript{2} does not increase endothelial InsP\textsubscript{3} synthesis, even at millimolar concentrations, and that U-73122 does not modulate H\textsubscript{2}O\textsubscript{2}-evoked Ca\textsuperscript{2+} mobilization in other cell types.\textsuperscript{6,7,31,32}

The generality of these conclusions was substantiated by experiments with thimerosal, which is known to sensitize the InsP\textsubscript{3} receptor to InsP\textsubscript{3} and Ca\textsuperscript{2+} via the oxidation of critical thiol groups,\textsuperscript{8–12,32} and whose ability to facilitate intracellular Ca\textsuperscript{2+} mobilization has been dissociated from alterations in InsP\textsubscript{3} synthesis,\textsuperscript{9,10} SERCA activity,\textsuperscript{11} mitochondrial Ca\textsuperscript{2+} release\textsuperscript{1,32} and blockade of the membrane extrusion Ca\textsuperscript{2+} ATPase.\textsuperscript{32} At concentrations \(\geq 30 \textmu mol/L\), thimerosal itself evoked EDHF-type relaxations in the RIA and elevated basal [Ca\textsuperscript{2+}], in the RAV endothelium. However, a subthreshold concentration (10 \textmu mol/L) that did not affect [Ca\textsuperscript{2+}], or [Ca\textsuperscript{2+}]\textsubscript{im} mimicked the effects of H\textsubscript{2}O\textsubscript{2} by potentiating relaxations and Ca\textsuperscript{2+} mobilization evoked by CPA at concentrations \(\leq 10 \textmu mol/L\), but not further elevating [Ca\textsuperscript{2+}], when stores were depleted by 30 or 100 \textmu mol/L CPA. Evidence that oxidation of thiol groups similarly underpins the response to H\textsubscript{2}O\textsubscript{2} was obtained in experiments showing that the cell permeant thiol reductant GSH-MEE abolished the synergy between H\textsubscript{2}O\textsubscript{2} and CPA in RAV leaflets without compromising the ability of CPA to elevate [Ca\textsuperscript{2+}]. While the precise molecular effects of H\textsubscript{2}O\textsubscript{2} on the InsP\textsubscript{3} receptor remain to be delineated, it possesses a number of accessible cysteine residues that are susceptible to oxidative modification.\textsuperscript{34} More specifically, thimerosal induces a conformational change in the N terminus of the receptor with the interaction between amino acids 1 to 225 (suppressor domain) and amino acids 226 to 604 (InsP\textsubscript{3}-binding core) being strengthened interactively by Ca\textsuperscript{2+} and thimerosal, leading to the formation of a highly InsP\textsubscript{3}-sensitive Ca\textsuperscript{2+}-release channel.\textsuperscript{12}

Thimerosal also promotes the formation of epoxyeicosatrienoic acid (EET) metabolites of CYP\textsubscript{450} epoxygenases by inhibiting acyl-coenzyme A (CoA)/lysolecithin acyltransferase, and in some species EETs may function as transferable EDHFs that activate smooth muscle BK\textsubscript{Ca} channels.\textsuperscript{2,35} In rabbit arteries, however, exogenously-administered EETs fail to relax endothelium-denuded preparations directly, thus excluding a role as an EDHF, whereas in preparations with endothelium they evoke indirect EDHF-type relaxations that involve signaling via gap junctions.\textsuperscript{21,23,36} Endogenous EET production is nevertheless unlikely to contribute to the ability of thimerosal or H\textsubscript{2}O\textsubscript{2} to potentiate the CPA-evoked Ca\textsuperscript{2+} mobilization demonstrated in the present study because (1) rabbit endothelial cells normally do not synthesize EETs,\textsuperscript{37} (2) EETs elevate endothelial [Ca\textsuperscript{2+}], by stimulating Ca\textsuperscript{2+} influx via TRP channels\textsuperscript{38} rather than promoting intracellular Ca\textsuperscript{2+} release, and (3) H\textsubscript{2}O\textsubscript{2} impairs EET synthesis by directly inhibiting CYP\textsubscript{450} epoxygenases.\textsuperscript{39}

The connexin-mimetic peptide \textsuperscript{49}Gap 26 effectively abolished CPA-evoked relaxations, thus confirming the primarily electrotonic nature of the EDHF phenomenon in the RIA.\textsuperscript{13,27,28} This peptide possesses homology with the first extracellular loop of Cx43, the dominant connexin expressed in the media of the RIA, and interrupts the spread of endothelial hyperpolarization via homocellular smooth muscle gap junctions without impairing CPA-evoked endothelial hyperpolarization.\textsuperscript{13,27} Experiments with selective inhibitors of SK\textsubscript{Ca}, IK\textsubscript{Ca}, and BK\textsubscript{Ca} channels provided evidence that all three subtypes contribute to this initiating hyperpolarization. Thus, apamin and TRAM-34, which block SK\textsubscript{Ca} and IK\textsubscript{Ca} channels, were individually ineffective against relaxation, but combined SK\textsubscript{Ca}/IK\textsubscript{Ca} blockade with apamin+TRAM-34 or BK\textsubscript{Ca} blockade with iberiotoxin significantly reduced relaxation, and further inhibition was evident with the triple combination apamin+TRAM-34+iberiotoxin, leaving a residual response equivalent to \(\approx 35\%\) of control. It should be noted that considerable heterogeneity in the endothelial expression of these \(K\textsubscript{Ca}\) subtypes and their functional contribution to the EDHF phenomenon across species and vessels is evident in the literature.\textsuperscript{2,3,18–26} In the rabbit mesenteric artery, for example, EDHF-type relaxations and hyperpolarizations are insensitive to iberiotoxin,\textsuperscript{20,21} whereas in the rabbit renal artery relaxation is partially inhibited by iberiotoxin and abolished by the combination of apamin and iberiotoxin, even though apamin alone is without effect.\textsuperscript{23} Iberiotoxin also attenuates relaxation in 1st order rat mesenteric arteries, but is inactive in 3rd order branch arteries.\textsuperscript{26} Indeed, in 3rd order arteries endothelium-dependent smooth muscle hyperpolarizations are abolished by apamin under resting conditions and therefore entirely attributable to the opening of SK\textsubscript{Ca} channels, but when depolarized by phenylephrine the combination of apamin+TRAM-34 is necessary to abolish relaxation, suggesting a specific role for IK\textsubscript{Ca} channels during repolarization.\textsuperscript{19,26}

Evidence that endogenously-generated H\textsubscript{2}O\textsubscript{2} may contribute to EDHF-type relaxations was provided by experiments with catalase, which attenuated relaxation in RIA rings responsive to 10 \textmu mol/L CPA, but was without effect in preparations where the threshold for relaxation was 30 \textmu mol/L CPA. Correspondingly, 3-aminotriazole, which inhibits H\textsubscript{2}O\textsubscript{2} degradation by binding to the active site of catalase,\textsuperscript{40} significantly amplified EDHF-type relaxations evoked by CPA at 10 \textmu mol/L, but not at other concentrations. These results are consistent with the finding that exogenous H\textsubscript{2}O\textsubscript{2} potentiated EDHF-type relaxations evoked by low concentrations of CPA, but not at concentrations causing near-maximal depletion of the ER Ca\textsuperscript{2+} store (ie, 30 or 100 \textmu mol/L). They also imply that endothelial [H\textsubscript{2}O\textsubscript{2}], during
Application of 100 μmol/L H2O2 will be lower than that attained after application of 100 μmol/L H2O2, because the threshold for relaxation was then reduced to 1 μmol/L CPA, as compared to 10 or 30 μmol/L CPA under control conditions. We attempted to assess endogenous H2O2 formation in RAV preparations with dichlorofluorescein (DCF), a probe that has been widely used to image [H2O2], (see supplemental Figure I), but were unable to detect changes in DCF fluorescence after stimulation with 10 or 30 μmol/L CPA or application of 100 μmol/L H2O2, whereas increased fluorescence was consistently observed with 1 mmol/L H2O2, as reported in pancreatic cells. Although the findings with exogenous H2O2 at 100 μmol/L could in theory reflect an ability of intrinsic antioxidant mechanisms to establish an cytosolic/extracellular [H2O2] gradient of ≈1/10,41 DCF is resistant to oxidation by H2O2 in the absence of intracellular catalysts such as transition metal ions or haem-containing peroxidases, but is readily oxidized by species such as peroxy radicals and peroxynitrite, thus questioning both its sensitivity and specificity as a H2O2 probe.42

In preparations responding to 10 μmol/L CPA, residual relaxations observed in the presence of apamin+TRAM-34+iberiotoxin were attenuated by catalase, whereas in preparations with the higher threshold of 30 μmol/L, relaxation was effectively abolished by this triple combination of KCa channel inhibitors. These observations suggest that endogenously-generated H2O2 can modulate KCa channel activity. Further insights into this interaction were obtained in experiments with endothelium-intact rings preincubated with a submaximal concentration of CPA (10 μmol/L), which unmasked an endothelium-dependent relaxant response to exogenous H2O2 that was superimposed on its direct smooth muscle activity. This response exhibited characteristics similar to the relaxation evoked by CPA as it could be attenuated by combined SKCa/IKCa blockade or by BKCa blockade, thus providing additional evidence that concerted opening of endothelial BKCa, IKCa, and SKCa channels secondary to elevations in [Ca2+]i underpins the EDHF phenomenon in the RIA. Observations that control concentration-relaxation curves for H2O2 were identical in RIA rings with and without endothelium, and were unaffected by the combination of apamin+Tram-34+iberiotoxin, exclude the possibility that H2O2 activates endothelial KCa channels directly or functions as a freely transferable EDHF that activates smooth muscle KCa channels. Indeed, there is evidence that millimolar concentrations of H2O2 inhibit, rather than activate, BKCa and IKCa channels in porcine and bovine endothelial cells,25,43 and that 100 μmol/L H2O2 does not evoke significant smooth muscle hyperpolarization in the RIA (<3 mV).8 Because concentration-relaxation curves for H2O2 in denuded RIA rings are also unaffected by blockade of KATP or KCa channels or by inhibition of guanylyl and adenylcyclase,8 the precise nature of H2O2-evoked smooth muscle relaxation in the RIA remains unclear. One possibility is that H2O2 modulates the Ca2+ sensitivity of the contractile machinery. Indeed, in the rabbit aorta H2O2 can mediate relaxation while “paradoxically” elevating smooth muscle [Ca2+], rather than causing reductions in [Ca2+], secondary to the hyperpolarization that would expected if H2O2 functioned as an EDHF.16

In conclusion, we have provided evidence that H2O2 may participate in the EDHF phenomenon by enhancing ER Ca2+ release and promoting the activation of endothelial KCa channels. Further studies are required to define the subcellular mechanisms that generate H2O2 in rabbit endothelial cells in view of evidence that Ca2+ influx evoked by SERCA inhibitors promotes H2O2 production by mitochondria,44 whereas agonist-stimulated H2O2 production may be secondary to the generation of superoxide by NADPH oxidase.7 It also remains to be determined whether the increased endothelial oxidant stress that characterizes many vascular disease states enhances the EDHF phenomenon by potentiating intracellular Ca2+ release, thereby offsetting an associated reduction in NO bioavailability.

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None.

References


44. Bogeski I, Bozem M, Sternfeld L, Hofer HW, Schulz I. Inhibition of protein tyrosine phosphatase 1B by reactive oxygen species leads to maintenance of Ca<sup>2+</sup> influx following store depletion in HEK 293 cells. Cell Calcium. 2006;40:1–10.

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METHODS

Mechanical responses
Male NZW rabbits (2-2.5 kg) were killed with sodium pentobarbitone (120 mg/kg; i.v.) according to University guidelines. Iliac artery rings 2-3 mm wide were obtained and mounted in a myograph containing oxygenated (95% O₂, 5% CO₂) Holmans buffer at 37°C, and maintained at a resting tension of 2 mN during a 1 h equilibration period followed by 40 min incubation with N⁶-nitro-L-arginine methyl ester (L-NAME, 300 µmol/L) and indomethacin (10 µmol/L). Agents under study were then added for a further 40 min, as required, before tone was induced by phenylephrine (1 µmol/L) and relaxation evoked by CPA or H₂O₂. Cumulative concentration-response curves for CPA were constructed under control conditions and in the presence of H₂O₂ (100 µmol/L), 3-aminotriazole (Atz; 50 mmol/L), mercury((o-carboxyphenyl)thio)ethyl sodium salt (thimerosal, 1 µmol/L), the PLC inhibitor 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)-hexyl)-1H-pyrrole-2,5-dione (U-73122, 100 µmol/L), 43Gap26 (VCYDKSFISHVR, 100 µmol/L) and the specific SKCa, IKCa and BKCa channel inhibitors apamin (1 µmol/L), TRAM-34 (10 µmol/L) and iberiotoxin (100 nmol/L), individually and in combination. Relaxations evoked by H₂O₂ were compared in endothelium-intact and -denuded rings in the presence and absence of 10 µmol/L CPA and/or combinations of apamin, TRAM-34 and iberiotoxin.

Calcium imaging of the rabbit aortic valve endothelium
Aortic valve leaflets were removed and incubated in oxygenated Holmans buffer containing Fura-2 AM (5 µmol/L) for 2 h at room temperature followed by washout for 30 min and mounted on an inverted microscope as previously described.¹ The preparations were alternately excited at 340/380 nm, and images were acquired at 2 s intervals with an exposure time of 100 ms at each wavelength. Background-corrected F₃₄₀/₃₈₀ ratios were calculated and [Ca²⁺], determined according to Grynkiewicz et al.³ and presented as the change from baseline following intervention. CPA, H₂O₂, glutathione monoethyl ester (GSH-MEE, 1 mmol/L), or thimerosal were added as required. While exogenous GSH and GSH-MEE both increase intracellular endothelial GSH concentrations, the action of GSH-MEE involves transmembrane transport followed by intracellular hydrolysis, whereas the effects of GSH require extracellular breakdown and subsequent intracellular resynthesis.² All such experiments were performed in the presence of L-NAME (300 µmol/L) and indomethacin (10 µmol/L).

To assess [Ca²⁺] in endothelial ER stores (|[Ca²⁺]ER), aortic valve leaflets were incubated with the low-affinity Ca²⁺ indicator mag-fluo-4 (2 µM) in buffer at room temperature for 60 min, followed by washing with indicator-free buffer for a further 90 min to unload mag-fluo-4 located in the cytosol, as described.⁴ The tissue was imaged on a Leica SP5 confocal microscope. Changes in mag-fluo-4 fluorescence following experimental interventions were normalized to the value at the beginning of each experiment (F₀). All such experiments were performed in the presence of L-NAME (300 µmol/L) and indomethacin (10 µmol/L).
Materials
All pharmacological agents were obtained from Sigma, UK. Stock solutions were prepared in buffer with the exception of CPA and U-73122 (DMSO), indomethacin (5% bicarbonate) and [3H]Gap 26 (dH2O). Fluorescent probes were obtained from Invitrogen, UK.

Statistics
In mechanical experiments the maximal percentage reversal of phenylephrine-induced constriction (R_{max}) by CPA or H2O2 and concentrations giving 50% reversal of this constrictor response (IC_{50}; in the case of CPA) or 50% of maximal relaxation (EC_{50}; in the case of H2O2) were determined for each experiment. The use of IC_{50} rather than EC_{50} values was necessary to allow for the small initial constriction to CPA that was observed in many experiments. R_{max}, pIC_{50} and pEC_{50} values and changes in cytosolic and ER [Ca^{2+}] were calculated as mean ± sem and compared by the Student's t-test or ANOVA followed by a post-test as appropriate. P<0.05 was considered significant; n denotes the number of animals studied for each data point.

Effects of 100 µmol/L \( \text{H}_2\text{O}_2 \) and 30 µmol/L CPA in rabbit aortic valves loaded with 10 µmol/L 2',7'-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCFDA) for 30 min at room temperature. Confocal imaging demonstrated that supraphysiological \( \text{H}_2\text{O}_2 \) concentrations (1 mmol/L) were required to increase fluorescence.
**Supplemental Table I** Effects of pharmacological interventions on EDHF-type relaxations evoked by CPA

<table>
<thead>
<tr>
<th>Intervention</th>
<th>n</th>
<th>pIC$_{50}$</th>
<th>$R_{\text{max}}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>4.72±0.06</td>
<td>88.3±5.2</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>6</td>
<td>5.31±0.20*</td>
<td>91.4±3.2</td>
</tr>
</tbody>
</table>

| Control                 | 8  | 4.78±0.04   | 83.5±3.5           |
| Thimerosal              | 8  | 5.96±0.10***| 85.4±6.5           |

| Control                 | 4  | 4.78±0.09   | 81.0±3.1           |
| 43 Gap26               | 4  | -           | 18.7±2.3**         |

| Control                 | 25 | 4.80±0.04   | 83.6±1.9           |
| Apamin                  | 4  | 4.76±0.04   | 83.8±3.3           |
| TRAM                    | 4  | 4.73±0.01   | 85.1±2.9           |
| IbTX                    | 7  | 4.80±0.05   | 66.3±4.5*          |
| Apamin+TRAM             | 4  | 4.78±0.15   | 60.1±6.7*          |
| Apamin+TRAM+IbTX        | 14 | -           | 37.2±4.1**         |
| Apamin+TRAM+IbTX + Catalase | 9  | -           | 20.7±6.8**         |

| Control                 | 10 | 5.02±0.04   | 89.9±1.6           |
| Catalase (threshold 10 µmol/L) | 10 | 4.73±0.06***| 80.7±2.3           |
| Apamin+TRAM+IbTX        | 5  | -           | 58.4±10.7**        |
| Apamin+TRAM+IbTX + Catalase | 5  | -           | 43.5±13.2**        |

| Control                 | 5  | 4.65±0.03   | 79.5±4.7           |
| Catalase (threshold 30 µmol/L) | 5  | 4.60±0.14   | 67.3±8.7           |
| Apamin+TRAM+IbTX        | 4  | -           | 16.0±5.9**         |
| Apamin+TRAM+IbTX + Catalase | 4  | -           | 14.8±4.4**         |

| Control                 | 6  | 4.93±0.05   | 91.2±2.0           |
| Atz                     | 6  | 5.09±0.06*  | 92.3±4.3           |

| Control                 | 5  | 4.64±0.04   | 81.9±7.2           |
| H$_2$O$_2$              | 5  | 5.52±0.31*  | 79.5±5.6           |
| U-73122                 | 5  | 4.70±0.05   | 77.4±5.7           |
| U-73122+H$_2$O$_2$      | 5  | 5.55±0.27**†| 86.9±2.8           |

Potency (negative log IC$_{50}$) and maximal relaxation ($R_{\text{max}}$) expressed as a function of the constrictor response to phenylephrine are given as mean ± s.e.m. *, ** and *** denote $P<0.05$, 0.01 and 0.001 compared with control; † denotes $P<0.05$ compared with U-73122 alone.
**Supplemental Table II** Effects of K$_{Ca}$ channel blockers on relaxations evoked by H$_2$O$_2$ in the presence and absence of CPA.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>n</th>
<th>pEC$_{50}$</th>
<th>$R_{\text{max}}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>3.88±0.05</td>
<td>80.6±2.2</td>
</tr>
<tr>
<td>Apamin+TRAM+IbTX</td>
<td>5</td>
<td>3.79±0.02</td>
<td>79.9±2.4</td>
</tr>
<tr>
<td>Denuded</td>
<td>7</td>
<td>3.87±0.03</td>
<td>86.9±2.6</td>
</tr>
<tr>
<td>Denuded+Apamin+TRAM+IbTX</td>
<td>8</td>
<td>3.88±0.04</td>
<td>84.8±1.6</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>3.80±0.03</td>
<td>79.0±1.4</td>
</tr>
<tr>
<td>CPA</td>
<td>16</td>
<td>4.14±0.04***</td>
<td>86.7±1.1**</td>
</tr>
<tr>
<td>CPA+IbTX</td>
<td>6</td>
<td>3.88±0.03</td>
<td>83.7±2.8</td>
</tr>
<tr>
<td>CPA+Apamin+TRAM</td>
<td>5</td>
<td>3.74±0.06</td>
<td>75.7±4.8</td>
</tr>
<tr>
<td>CPA+Apamin+TRAM+IbTX</td>
<td>15</td>
<td>3.68±0.04</td>
<td>78.3±2.5</td>
</tr>
</tbody>
</table>

Potency (negative log EC$_{50}$) and maximal relaxation ($R_{\text{max}}$) expressed as a function of the constrictor response to phenylephrine are given as mean ± s.e.m. ** and *** denote $P<0.01$ and 0.001 compared with control.