Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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

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

Arterioscler Thromb Vasc Biol. 2008;28:1774-1781; originally published online July 31, 2008;
doi: 10.1161/ATVBAHA.108.172692
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

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_2O_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_2O_2 and the thiol oxidant thimerosal. Preincubation with a submaximal concentration of CPA unmasked an ability of exogenous H_2O_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_2O_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_2O_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 ^{43}Gap26, which interrupts intercellular communication via gap junctions constructed from connexin 43.

Conclusions—H_2O_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_2O_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 $[Ca^{2+}]_i$ 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 relaxation.[3] Agents that elevate endothelial $[Ca^{2+}]_i$ 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]

See accompanying article on page 1691

In some artery types, there is evidence that exogenous H_2O_2 can evoke smooth muscle relaxation by activating hyperpolarizing BK_{Ca} channels and that EDHF-type relaxations and hyperpolarizations are associated with catalase-inhibitable endothelial H_2O_2 production, leading to the hypothesis that endogenously-generated H_2O_2 can serve as a freely diffusible EDHF.[1] By contrast, in human mesenteric arterioles endothelium-dependent agonists promote H_2O_2-dependent relaxation, but exogenous H_2O_2 causes constriction when such vessels are denuded of their endothelium, leading to the proposal that H_2O_2 releases a chemically distinct EDHF.[5] A further permutation, not previously described in the context of the EDHF phenomenon, is that H_2O_2 promotes Ca^{2+} release from intracellular stores[6,7] thereby elevating $[Ca^{2+}]_i$ 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_2O_2 in EDHF-type relaxations of the rabbit iliac artery (RIA), a vessel in which H_2O_2 cannot be regarded as a transferable EDHF because smooth muscle hyperpolarizations evoked by exogenous H_2O_2 are much smaller than those associated with the authentic EDHF

Original received January 2, 2008; final version accepted July 23, 2008.

From the Wales Heart Research Institute, School of Medicine, Cardiff University, UK.

Correspondence to Tudor Griffith, Department of Diagnostic Radiology, Wales Heart Research Institute, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK. E-mail griffith@cardiff.ac.uk

Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.108.172692**

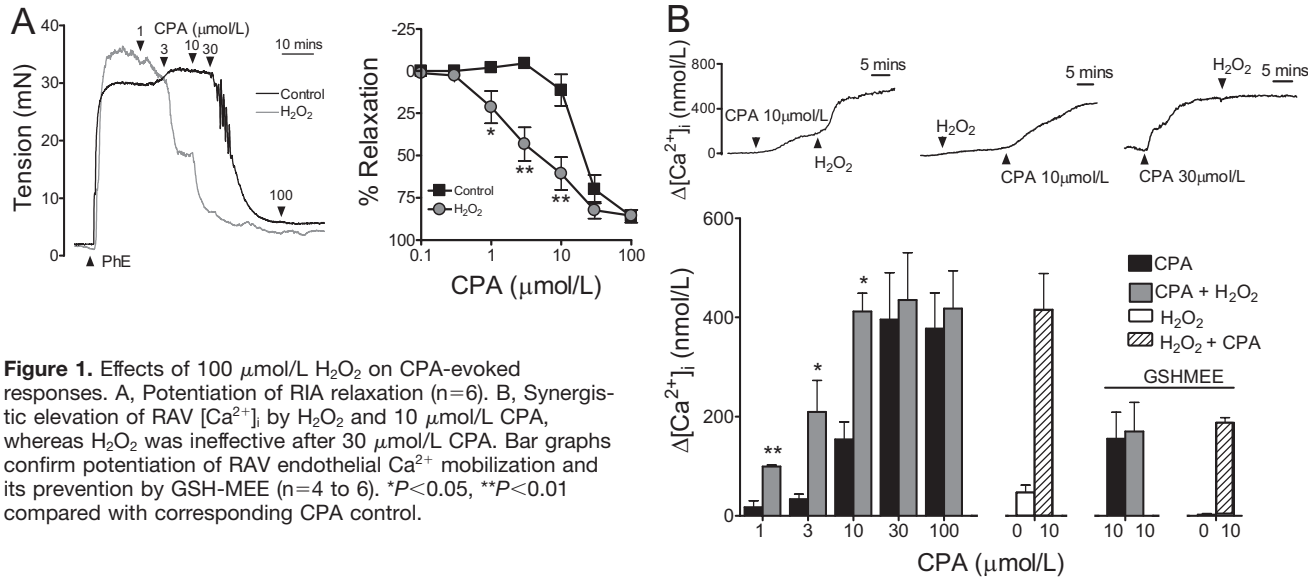


Figure 1. Effects of 100 μmol/L H_2O_2 on CPA-evoked responses. A, Potentiation of RIA relaxation (n=6). B, Synergistic elevation of RAV $[Ca^{2+}]_i$ by H_2O_2 and 10 μmol/L CPA, whereas H_2O_2 was ineffective after 30 μmol/L CPA. Bar graphs confirm potentiation of RAV endothelial Ca^{2+} mobilization and its prevention by GSH-MEE (n=4 to 6). *$P<0.05$, **$P<0.01$ compared with corresponding CPA control.

phenomenon.[8] Because there is evidence that H_2O_2 depletes Ca^{2+} stores by sensitizing the $InsP_3$ 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 H_2O_2 and thimerosal on EDHF-type relaxations and Ca^{2+} mobilization, using fura-2 and the low affinity Ca^{2+} probe Mag-fluo-4, which selectively loads the endoplasmic reticulum (ER), to monitor $[Ca^{2+}]_i$ and $[Ca^{2+}]_{ER}$ in the rabbit aortic valve (RAV) endothelium.[13,14] This preparation was selected for Ca^{2+} imaging studies because H_2O_2 and thimerosal can both elevate vascular smooth muscle $[Ca^{2+}]_i$, and in intact vessels Ca^{2+} signals can be transmitted from the media to the endothelium via myoendothelial gap junctions.[12,15,16] To avoid potentially confounding effects of H_2O_2 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 SK_{Ca} and IK_{Ca} channels, also classified as SK1–3 and SK4 on the basis of structural and gating characteristics,[2,18–21] in some artery types the endothelium also expresses functional large-conductance BK_{Ca} channels.[22–26] The contributions of the 3 channel subtypes to EDHF-type relaxations were therefore investigated with selective SK_{Ca}, IK_{Ca}, and BK_{Ca} inhibitors (apamin, TRAM-34 and iberiotoxin, respectively), and the dominant role of electrotonic signaling confirmed with a synthetic peptide (^{43}Gap26) 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 Ca^{2+} mobilization rabbit aortic valve leaflets were isolated and loaded either with Fura-2 AM to assess $[Ca^{2+}]_i$ or the low-affinity Ca^{2+} indicator mag-fluo-4 to assess $[Ca^{2+}]$ in endothelial ER stores. Leaflets were imaged either with an inverted microscope to obtain background-corrected $F_{340/380}$ ratios and calculate $[Ca^{2+}]_i$, or confocal microscopy to track the effects of interventions on Mag-fluo-4 fluorescence with changes in $[Ca^{2+}]_{ER}$ being assessed as fluorescence normalized to its value at the beginning of each experiment (F/F_0). 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 (R_{max}) by CPA or H_2O_2 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 H_2O_2) were determined. The use of IC_{50} 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 (R_{max}), although the threshold for relaxation was variable, being 10 or 30 μmol/L in different preparations, with an overall IC_{50} of ≈16 μmol/L (pIC_{50} 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 Ca^{2+} influx following blockade of the smooth muscle SERCA pump.[28]

Effects of H_2O_2 and Thimerosal

Preincubation of endothelium-intact RIA rings with 100 μmol/L H_2O_2 markedly potentiated relaxations evoked by CPA at concentrations of 1 to 10 μmol/L, without alteration in overall R_{max} (Figure 1A; supplemental Table I). Similarly, 100 μmol/L H_2O_2 potentiated the increases in $[Ca^{2+}]_i$ evoked by 1 to 10 μmol/L CPA in the endothelium of the RAV, but did not affect Ca^{2+} mobilization in response to 30 and 100 μmol/L CPA and minimally elevated basal $[Ca^{2+}]_i$ (Figure 1B). In the presence of 100 μmol/L H_2O_2 the increase in $[Ca^{2+}]_i$ evoked by 10 μmol/L CPA attained the same level

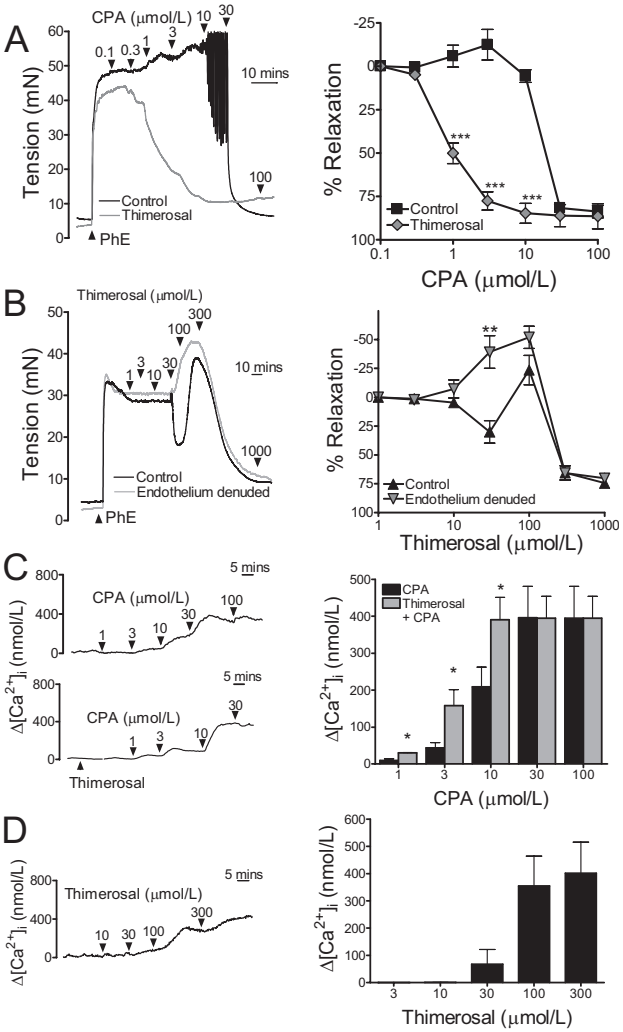


Figure 2. Effects of thimerosal. A, Potentiation of CPA-evoked RIA relaxation by 10 μmol/L thimerosal (n=8). B, Endothelium-dependent and -independent relaxant effects of thimerosal (n=9). C, Concentration-dependent elevation of RAV $[Ca^{2+}]_i$ by CPA in the presence and absence of 10 μmol/L thimerosal (n=7). D, Direct mobilization of Ca^{2+} by thimerosal in the RAV (n=4). *P<0.05, **P<0.01, and ***P<0.001 compared with corresponding control.

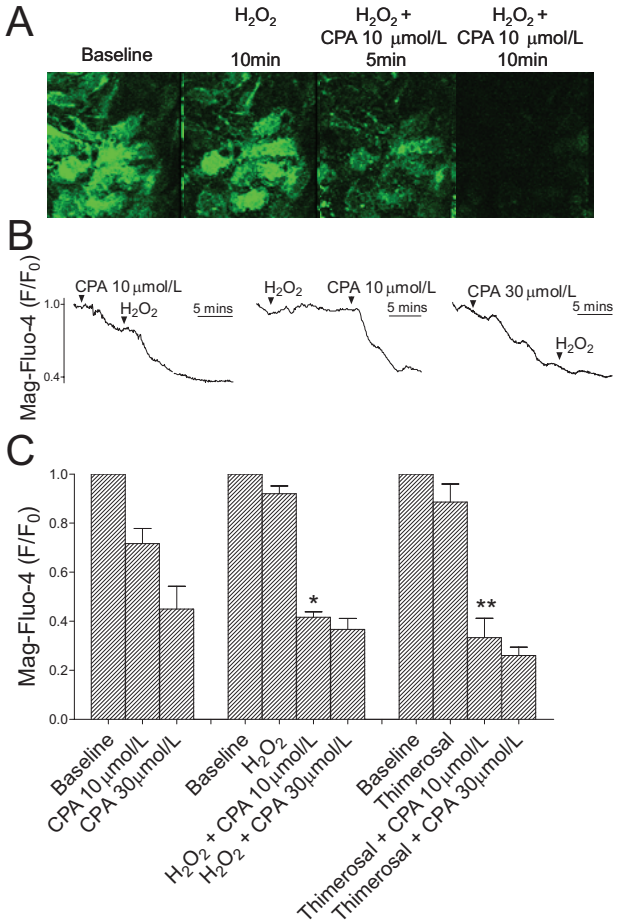


Figure 3. Effects of 100 μmol/L H_2O_2 and 10 μmol/L thimerosal on CPA-evoked depletion of ER Ca^{2+} stores loaded with Mag-fluo-4. A, Confocal images showing that H_2O_2 itself did not affect $[Ca^{2+}]_{ER}$ but facilitated store depletion in the presence of 10 μmol/L CPA. B, Traces illustrating that the effects of H_2O_2 and CPA were synergistic. Changes in $[Ca^{2+}]_{ER}$ are expressed as relative fluorescence ratio, F/F_o. C, Bar graphs showing that store emptying by CPA was potentiated to an equivalent extent by H_2O_2 and thimerosal (n=4 in each case). *P<0.05, **P<0.01 compared with 10 μmol/L CPA alone.

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 H_2O_2 to potentiate Ca^{2+} 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 R_{max} 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 subthreshold concentration of 10 μmol/L, thimerosal potentiated increases in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ with an EC_{50} of ≈60 μmol/L (pEC_{50} 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 H_2O_2 or 10 μmol/L thimerosal, depletion of the ER Ca^{2+} 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 H_2O_2 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

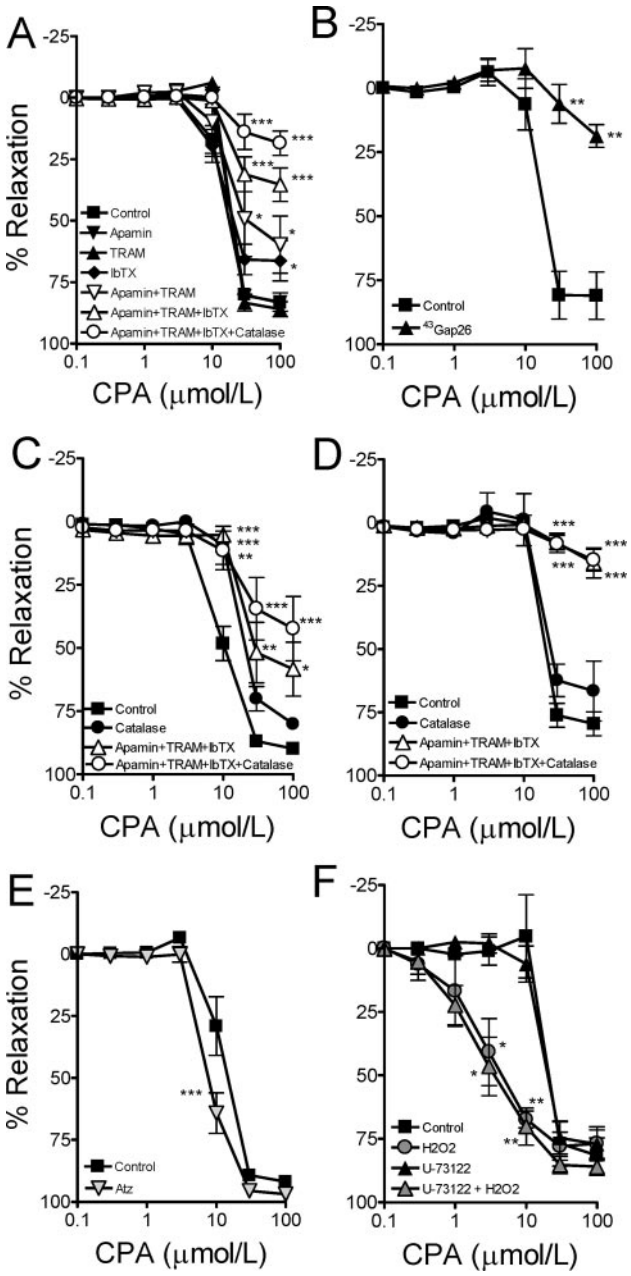


Figure 4. Mechanisms contributing to CPA-evoked relaxation. A, Combinatorial effects of apamin, TRAM-34 and iberiotoxin (n=4 to 25). Residual responses observed in the presence of apamin+TRAM-34+IbTX were further attenuated by catalase (n=9). B, Responses were effectively abolished by ^{43}Gap 26 (n=4). 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 H_2O_2 on relaxation (n=5). *P<0.05, **P<0.01, ***P<0.001 compared with control (see also supplemental Table I).

R_{max} was reduced to ≈60% by preincubation either with the double combination apamin+TRAM-34 or iberiotoxin (IbTX, 100 nmol/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 ^{43}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 K_{Ca} channel blockade reduced R_{max} 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 R_{max} (Figure 4E; supplemental Table I). Control CPA-evoked relaxations and their potentiation by 100 μmol/L H_2O_2 were unaffected by the PLC inhibitor U-73122 (10 μmol/L) (Figure 4F; supplemental Table I).

Relaxation to Exogenous H_2O_2

Concentration-relaxation curves for H_2O_2 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 H_2O_2 were shifted to the left with a small increase in R_{max} (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 K_{Ca} channel subtypes.

Discussion

The principal finding of the present study is that H_2O_2 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 H_2O_2 was shown to involve enhanced Ca^{2+} release from stores with secondary activation of K_{Ca} channels. H_2O_2 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 K_{Ca} channel blockade.

Preincubation of RIA rings with 100 μmol/L H_2O_2 markedly potentiated EDHF-type relaxations evoked by CPA when this SERCA inhibitor, which activates SOCE by depleting ER Ca^{2+} stores, was administered at concentrations ≤10 μmol/L. Correspondingly, preincubation of RAV leaflets with 100 μmol/L H_2O_2 amplified ER emptying and elevations in $[Ca^{2+}]_i$ evoked by CPA at concentrations ≤10 μmol/L, thus matching the range over which H_2O_2 potentiated relaxation. Because 100 μmol/L H_2O_2 did not itself induce changes in $[Ca^{2+}]_{ER}$, this synergism suggests that H_2O_2 sensitizes the $InsP_3$ receptor. Indeed, heparin, an established antagonist of this receptor, abolishes H_2O_2-evoked

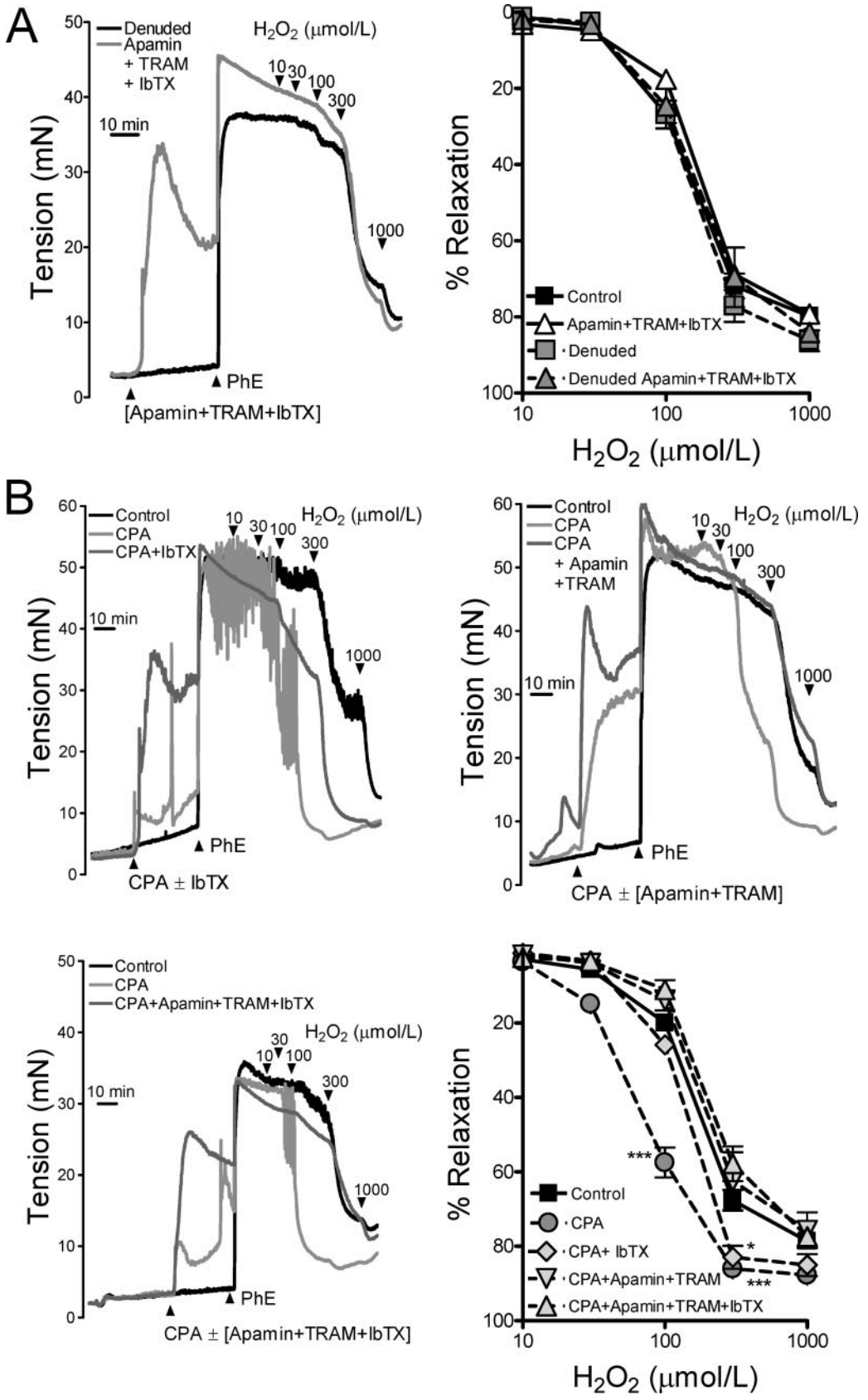


Figure 5. Interactive effects of CPA and K_{Ca} inhibitors on H_2O_2-evoked relaxation. A, Relaxant effects of H_2O_2 were unaffected by apamin+TRAM-34+iberiotoxin or endothelial denudation (n=5 to 8). B, Potentiation of H_2O_2-evoked relaxation by 10 μmol/L CPA in endothelium-intact rings was attenuated 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^{2+} release from ER stores in permeabilized endothelial cells,[6] whereas the endothelium-specific SERCA3 pump isoform, which plays a key role in endothelium-dependent relaxation, is insensitive to H_2O_2, in contrast to the SERCA2b isoform found in smooth muscle.[29,30] Whereas millimolar concentrations of H_2O_2 can promote Ca^{2+} release from mitochondria or depress extrusion of cytosolic Ca^{2+} by the plasma membrane Ca^{2+} ATPase,[6,31–33] the participation of these ER-independent mechanisms was excluded by observations that (1) 100 μmol/L H_2O_2 minimally affected $[Ca^{2+}]_i$ and (2) relaxation and Ca^{2+} mobilization were maximal at CPA concentrations of 30 to 100 μmol/L and not further increased by 100 μmol/L H_2O_2. The possibility that the Ca^{2+} mobilizing effects of H_2O_2 might involve activation of PLC and enhanced $InsP_3$ synthesis[17] was excluded by the demonstration that the PLC inhibitor U-73122 did not affect the potentiation of CPA-evoked relaxation by H_2O_2. This is consistent with reports that H_2O_2 does not increase endothelial $InsP_3$ synthesis, even at millimolar concentrations, and that U-73122 does not modulate H_2O_2-evoked Ca^{2+} mobilization in other cell types.[6,7,31,32]

The generality of these conclusions was substantiated by experiments with thimerosal, which is known to sensitize the $InsP_3$ receptor to $InsP_3$ and Ca^{2+} via the oxidation of critical thiol groups,[9–12,32] and whose ability to facilitate intracellular Ca^{2+} mobilization has been dissociated from alterations in $InsP_3$ synthesis,[9,10] SERCA activity,[11] mitochondrial Ca^{2+} release[11,32] and blockade of the membrane extrusion Ca^{2+} ATPase.[32] At concentrations $\geq$30 μmol/L, thimerosal itself evoked EDHF-type relaxations in the RIA and elevated basal $[Ca^{2+}]_i$ in the RAV endothelium. However, a subthreshold concentration (10 μmol/L) that did not affect $[Ca^{2+}]_i$ or $[Ca^{2+}]_{ER}$ mimicked the effects of H_2O_2 by potentiating relaxations and Ca^{2+} mobilization evoked by CPA at concentrations $\leq$10 μmol/L, but not further elevating $[Ca^{2+}]_i$ when stores were depleted by 30 or 100 μmol/L CPA. Evidence that oxidation of thiol groups similarly underpins the response to H_2O_2 was obtained in experiments showing that the cell permeant thiol reductant GSH-MEE abolished the synergy between H_2O_2 and CPA in RAV leaflets without compromising the ability of CPA to elevate $[Ca^{2+}]_i$. While the precise molecular effects of H_2O_2 on the $InsP_3$ receptor remain to be delineated, it possesses a number of accessible cysteine residues that are susceptible to oxidative modification.[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 ($InsP_3$-binding core) being strengthened interactively by Ca^{2+} and thimerosal, leading to the formation of a highly $InsP_3$-sensitive Ca^{2+}-release channel.[12]

Thimerosal also promotes the formation of epoxyeicosatrienoic acid (EET) metabolites of CYP_{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_{Ca} channels.[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.[21,23,36] Endogenous EET production is nevertheless unlikely to contribute to the ability of thimerosal or H_2O_2 to potentiate the CPA-evoked Ca^{2+} mobilization demonstrated in the present study because (1) rabbit endothelial cells normally do not synthesize EETs,[37] (2) EETs elevate endothelial $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx via TRP channels[38] rather than promoting intracellular Ca^{2+} release, and (3) H_2O_2 impairs EET synthesis by directly inhibiting CYP_{450} epoxygenases.[39]

The connexin-mimetic peptide ^{43}Gap 26 effectively abolished CPA-evoked relaxations, thus confirming the primarily electrotonic nature of the EDHF phenomenon in the RIA.[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.[13,27] Experiments with selective inhibitors of SK_{Ca}, IK_{Ca}, and BK_{Ca} channels provided evidence that all three subtypes contribute to this initiating hyperpolarization. Thus, apamin and TRAM-34, which block SK_{Ca} and IK_{Ca} channels, were individually ineffective against relaxation, but combined SK_{Ca}/IK_{Ca} blockade with apamin+TRAM-34 or BK_{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_{Ca} subtypes and their functional contribution to the EDHF phenomenon across species and vessels is evident in the literature.[2,3,18–26] In the rabbit mesenteric artery, for example, EDHF-type relaxations and hyperpolarizations are insensitive to iberiotoxin,[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.[23] Iberiotoxin also attenuates relaxation in 1st order rat mesenteric arteries, but is inactive in 3rd order branch arteries.[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_{Ca} channels, but when depolarized by phenylephrine the combination of apamin+TRAM-34 is necessary to abolish relaxation, suggesting a specific role for IK_{Ca} channels during repolarization.[19,26]

Evidence that endogenously-generated H_2O_2 may contribute to EDHF-type relaxations was provided by experiments with catalase, which attenuated relaxation in RIA rings responsive to 10 μmol/L CPA, but was without effect in preparations where the threshold for relaxation was 30 μmol/L CPA. Correspondingly, 3-aminotriazole, which inhibits H_2O_2 degradation by binding to the active site of catalase,[40] significantly amplified EDHF-type relaxations evoked by CPA at 10 μmol/L, but not at other concentrations. These results are consistent with the finding that exogenous H_2O_2 potentiated EDHF-type relaxations evoked by low concentrations of CPA, but not at concentrations causing near-maximal depletion of the ER Ca^{2+} store (ie, 30 or 100 μmol/L). They also imply that endothelial $[H_2O_2]_i$ during

application of CPA will be lower than that attained after application of 100 μmol/L H_2O_2, 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 H_2O_2 formation in RAV preparations with dichlorofluorescein (DCF), a probe that has been widely used to image $[H_2O_2]_i$ (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 H_2O_2, whereas increased fluorescence was consistently observed with 1 mmol/L H_2O_2, as reported in pancreatic cells.[33] Although the findings with exogenous H_2O_2 at 100 μmol/L could in theory reflect an ability of intrinsic antioxidant mechanisms to establish an cytosolic/extracellular $[H_2O_2]$ gradient of ≈1/10,[41] DCF is resistant to oxidization by H_2O_2 in the absence of intracellular catalysts such as transition metal ions or haem-containing peroxidases, but is readily oxidized by species such as peroxyl radicals and peroxynitrite, thus questioning both its sensitivity and specificity as a H_2O_2 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 K_{Ca} channel inhibitors. These observations suggest that endogenously-generated H_2O_2 can modulate K_{Ca} 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 H_2O_2 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 SK_{Ca}/IK_{Ca} blockade or by BK_{Ca} blockade, thus providing additional evidence that concerted opening of endothelial BK_{Ca}, IK_{Ca}, and SK_{Ca} channels secondary to elevations in $[Ca^{2+}]_i$ underpins the EDHF phenomenon in the RIA. Observations that control concentration-relaxation curves for H_2O_2 were identical in RIA rings with and without endothelium, and were unaffected by the combination of apamin+Tram-34+iberiotoxin, exclude the possibility that H_2O_2 activates endothelial K_{Ca} channels directly or functions as a freely transferable EDHF that activates smooth muscle K_{Ca} channels. Indeed, there is evidence that millimolar concentrations of H_2O_2 inhibit, rather than activate, BK_{Ca} and IK_{Ca} channels in porcine and bovine endothelial cells,[25,43] and that 100 μmol/L H_2O_2 does not evoke significant smooth muscle hyperpolarization in the RIA (<3 mV).[8] Because concentration-relaxation curves for H_2O_2 in denuded RIA rings are also unaffected by blockade of K_{ATP} or K_v channels or by inhibition of guanylyl and adenylyl cyclases,[8] the precise nature of H_2O_2-evoked smooth muscle relaxation in the RIA remains unclear. One possibility is that H_2O_2 modulates the Ca^{2+} sensitivity of the contractile machinery.[16] Indeed, in the rabbit aorta H_2O_2 can mediate relaxation while "paradoxically" elevating smooth muscle $[Ca^{2+}]_i$, rather than causing reductions in $[Ca^{2+}]_i$ secondary to the hyperpolarization that would expected if H_2O_2 functioned as an EDHF.[16]

In conclusion, we have provided evidence that H_2O_2 may participate in the EDHF phenomenon by enhancing ER Ca^{2+} release and promoting the activation of endothelial K_{Ca} channels. Further studies are required to define the subcellular mechanisms that generate H_2O_2 in rabbit endothelial cells in view of evidence that Ca^{2+} influx evoked by SERCA inhibitors promotes H_2O_2 production by mitochondria,[44] whereas agonist-stimulated H_2O_2 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 Ca^{2+} release, thereby offsetting an associated reduction in NO bioavailability.

Sources of Funding

This work was supported by the British Heart Foundation.

Disclosures

None.

References

1. Shimokawa H, Morikawa K. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. *J Mol Cell Cardiol*. 2005;39:725–732.
2. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. *Trends Pharmacol Sci*. 2002;23:374–380.
3. Griffith TM. Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis? *Br J Pharmacol*. 2004;141:881–903.
4. Tomioka H, Hattori Y, Fukao M, Watanabe H, Akaishi Y, Sato A, Kim TQ, Sakuma I, Kitabatake A, Kanno M. Role of endothelial Ni^{2+}-sensitive Ca^{2+} entry pathway in regulation of EDHF in porcine coronary artery. *Am J Physiol*. 2001;280:H730–H737.
5. Hatoum OA, Binion DG, Miura H, Telford G, Otterson MF, Gutterman DD. Role of hydrogen peroxide in ACh-induced dilation of human submucosal intestinal microvessels. *Am J Physiol*. 2005;288:H48–H54.
6. Zheng Y, Shen X. H_2O_2 directly activates inositol 1,4,5-trisphosphate receptors in endothelial cells. *Redox Rep*. 2005;10:29–36.
7. Hu Q, Yu ZX, Ferrans VJ, Takeda K, Irani K, Ziegelstein RC. Critical role of NADPH oxidase-derived reactive oxygen species in generating Ca^{2+} oscillations in human aortic endothelial cells stimulated by histamine. *J Biol Chem*. 2002;277:32546–32551.
8. Chaytor AT, Edwards DH, Bakker LM, Griffith TM. Distinct hyperpolarizing and relaxant roles for gap junctions and endothelium-derived H_2O_2 in NO-independent relaxations of rabbit arteries. *Proc Natl Acad Sci U S A*. 2003;100:15212–15217.
9. Bootman MD, Taylor CW, Berridge MJ. The thiol reagent, thimerosal, evokes Ca^{2+} spikes in HeLa cells by sensitizing the inositol 1,4,5-trisphosphate receptor. *J Biol Chem*. 1992;267:25113–25119.
10. Parekh AB, Penner R. Activation of store-operated calcium influx at resting $InsP_3$ levels by sensitization of the $InsP_3$ receptor in rat basophilic leukaemia cells. *J Physiol*. 1995;489:377–382.
11. Montero M, Barrero MJ, Torrecilla F, Lobaton CD, Moreno A, Alvarez J. Stimulation by thimerosal of histamine-induced Ca^{2+} release in intact HeLa cells seen with aequorin targeted to the endoplasmic reticulum. *Cell Calcium*. 2001;30:181–190.
12. Bultynck G, Szlufcik K, Kasri NN, Assefa Z, Callewaert G, Missiaen L, Parys JB, De Smedt H. Thimerosal stimulates Ca^{2+} flux through inositol 1,4,5-trisphosphate receptor type 1, but not type 3, via modulation of an isoform-specific Ca^{2+}-dependent intramolecular interaction. *Biochem J*. 2004;381:87–96.
13. Griffith TM, Chaytor AT, Bakker LM, Edwards DH. 5-Methyltetrahydrofolate and tetrahydrobiopterin can modulate electrotonically mediated endothelium-dependent vascular relaxation. *Proc Natl Acad Sci U S A*. 2005;102:7008–7013.
14. Shmygol A, Wray S. Modulation of agonist-induced Ca^{2+} release by SR Ca^{2+} load: direct SR and cytosolic Ca^{2+} measurements in rat uterine myocytes. *Cell Calcium*. 2005;37:215–223.

15. Dora KA, Hinton JM, Walker SD, Garland CJ. An indirect influence of phenylephrine on the release of endothelium-derived vasodilators in rat small mesenteric artery. *Br J Pharmacol*. 2000;129:381–387.
16. Iesaki T, Okada T, Shimada I, Yamaguchi H, Ochi R. Decrease in Ca^{2+} sensitivity as a mechanism of hydrogen peroxide-induced relaxation of rabbit aorta. *Cardiovasc Res*. 1996;31:820–825.
17. Hong JH, Moon SJ, Byun HM, Kim MS, Jo H, Bae YS, Lee SI, Bootman MD, Roderick HL, Shin DM, Seo JT. Critical role of phospholipase Cγ1 in the generation of H_2O_2-evoked $[Ca^{2+}]_i$ oscillations in cultured rat cortical astrocytes. *J Biol Chem*. 2006;281:13057–13067.
18. Ledoux J, Werner ME, Brayden JE, Nelson MT. Calcium-activated potassium channels and the regulation of vascular tone. *Physiology*. 2006;21:69–78.
19. Crane GJ, Gallagher N, Dora KA, Garland CJ. Small- and intermediate-conductance calcium-activated K^+ channels provide different facets of endothelium-dependent hyperpolarization in rat mesenteric artery. *J Physiol*. 2003;553:183–189.
20. Murphy ME, Brayden JE. Apamin-sensitive K^+ channels mediate an endothelium-dependent hyperpolarization in rabbit mesenteric arteries. *J Physiol*. 1995;489:723–734.
21. Fujimoto S, Ikegami Y, Isaka M, Kato T, Nishimura K, Itoh T. K^+ channel blockers and cytochrome P450 inhibitors on acetylcholine-induced, endothelium-dependent relaxation in rabbit mesenteric artery. *Eur J Pharmacol*. 1999;384:7–15.
22. Rusko J, Tanzi F, van Breemen C, Adams DJ. Calcium-activated potassium channels in native endothelial cells from rabbit aorta: conductance, Ca^{2+} sensitivity and block. *J Physiol*. 1992;455:601–621.
23. Kagota S, Yamaguchi Y, Nakamura K, Kunitomo M. Characterization of nitric oxide- and prostaglandin-independent relaxation in response to acetylcholine in rabbit renal artery. *Clin Exp Pharmacol Physiol*. 1999; 26:790–796.
24. Ungvari Z, Csiszar A, Koller A. Increases in endothelial Ca^{2+} activate K_{Ca} channels and elicit EDHF-type arteriolar dilation via gap junctions. *Am J Physiol*. 2002;282:H1760–H1767.
25. Brakemeier S, Eichler I, Knorr A, Fassheber T, Kohler R, Hoyer J. Modulation of Ca^{2+}-activated K^+ channel in renal artery endothelium in situ by nitric oxide and reactive oxygen species. *Kidney Int*. 2003;64: 199–207.
26. Hilgers RH, Todd J Jr., Webb RC. Regional heterogeneity in acetylcholine-induced relaxation in rat vascular bed: role of calcium-activated K^+ channels. *Am J Physiol*. 2006;291:H216–H222.
27. Chaytor AT, Bakker LM, Edwards DH, Griffith TM. Connexin-mimetic peptides dissociate electrotonic EDHF-type signalling via myoendothelial and smooth muscle gap junctions in the rabbit iliac artery. *Br J Pharmacol*. 2004;144:108–114.
28. Edwards DH, Chaytor AT, Bakker LM, Griffith TM. Modulation of gap junction-dependent arterial relaxation by ascorbic acid. *J Vasc Res*. 2007; 44:410–422.
29. Liu LH, Paul RJ, Sutliff RL, Miller ML, Lorenz JN, Pun RY, Duffy JJ, Doetschman T, Kimura Y, MacLennan DH, Hoying JB, Shull GE. Defective endothelium-dependent relaxation of vascular smooth muscle and endothelial cell Ca^{2+} signaling in mice lacking sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform 3. *J Biol Chem*. 1997;272:30538–30545.
30. Grover AK, Samson SE, Misquitta CM. Sarco(endo)plasmic reticulum Ca^{2+} pump isoform SERCA3 is more resistant than SERCA2b to peroxide. *Am J Physiol*. 1997;273:C420–C425.
31. Granados MP, Salido GM, Gonzalez A, Pariente JA. Dose-dependent effect of hydrogen peroxide on calcium mobilization in mouse pancreatic acinar cells. *Biochem Cell Biol*. 2006;84:39–48.
32. Redondo PC, Salido GM, Rosado JA, Pariente JA. Effect of hydrogen peroxide on Ca^{2+} mobilisation in human platelets through sulphydryl oxidation-dependent and independent mechanisms. *Biochem Pharmacol*. 2004;67:491–502.
33. Bruce JI, Elliott AC. Oxidant-impaired intracellular Ca^{2+} signaling in pancreatic acinar cells: role of the plasma membrane Ca^{2+}-ATPase. *Am J Physiol*. 2007;293:C938–C950.
34. Joseph SK, Nakao SK, Sukumvanich S. Reactivity of free thiol groups in type-I inositol trisphosphate receptors. *Biochem J*. 2006;393: 575–582.
35. Chen YJ, Jiang H, Quilley J. The nitric oxide- and prostaglandin-independent component of the renal vasodilator effect of thimerosal is mediated by epoxyeicosatrienoic acids. *J Pharmacol Exp Ther*. 2003;304: 1292–1298.
36. Hutcheson IR, Chaytor AT, Evans WH, Griffith TM. Nitric oxide-independent relaxations to acetylcholine and A23187 involve different routes of heterocellular communication. Role of gap junctions and phospholipase A2. *Circ Res*. 1999;84:53–63.
37. Pfister SL, Falck JR, Campbell WB. Enhanced synthesis of epoxyeicosatrienoic acids by cholesterol-fed rabbit aorta. *Am J Physiol*. 1991;261: H843–H852.
38. Vriens J, Owsianik G, Fisslthaler B, Suzuki M, Janssens A, Voets T, Morisseau C, Hammock BD, Fleming I, Busse R, Nilius B. Modulation of the Ca^{2+} permeable cation channel TRPV4 by cytochrome P_{450} epoxygenases in vascular endothelium. *Circ Res*. 2005;97:908–915.
39. Larsen BT, Gutterman DD, Sato A, Toyama K, Campbell WB, Zeldin DC, Manthati VL, Falck JR, Miura H. Hydrogen peroxide inhibits cytochrome P_{450} epoxygenases. *Circ Res*. 2008;102:59–67.
40. Darr D, Fridovich I. Inhibition of catalase by 3,3′-diaminobenzidine. *Biochem J*. 1985;226:781–787.
41. Antunes F, Cadenas E. Estimation of H_2O_2 gradients across biomembranes. *FEBS Lett*. 2000;475:121–126.
42. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol*. 2004;142:231–255.
43. Cai S, Sauvé R. Effects of thiol-modifying agents on a K(Ca^{2+}) channel of intermediate conductance in bovine aortic endothelial cells. *J Membr Biol*. 1997;158:147–158.
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^{2+} influx following store depletion in HEK 293 cells. *Cell Calcium*. 2006;40:1–10.

METHODS

Mechanical responses

Male NZW rabbits (2-2.5kg) were killed with sodium pentobarbitone (120mg/kg; i.v.) according to University guidelines. Iliac artery rings 2-3 mm wide were obtained and mounted in a myograph containing oxygenated (95% O_2, 5% CO_2) Holmans buffer at 37°C, and maintained at a resting tension of 2 mN during a 1h equilibration period followed by 40 min incubation with N^G-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 H_2O_2. Cumulative concentration-response curves for CPA were constructed under control conditions and in the presence of H_2O_2 (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)-1*H*-pyrrole-2,5-dione (U-73122, 100 μmol/L), ^{43}Gap26 (VCYDKSFPISHVR, 100 μmol/L) and the specific SK_{Ca}, IK_{Ca} and BK_{Ca} channel inhibitors apamin (1 μmol/L), TRAM-34 (10 μmol/L) and iberiotoxin (100 nmol/L), individually and in combination. Relaxations evoked by H_2O_2 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 2s intervals with an exposure time of 100 ms at each wavelength. Background-corrected $F_{340/380}$ ratios were calculated and $[Ca^{2+}]_i$ determined according to Grynkiewicz et al.[3] and presented as the change from baseline following intervention. CPA, H_2O_2, 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 $[Ca^{2+}]$ in endothelial ER stores ($[Ca^{2+}]_{ER}$), aortic valve leaflets were incubated with the low-affinity Ca^{2+} 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 (F_0). All such experiments were performed in the presence of L-NAME (300 μmol/L) and indomethacin (10 μmol/L).

Materials

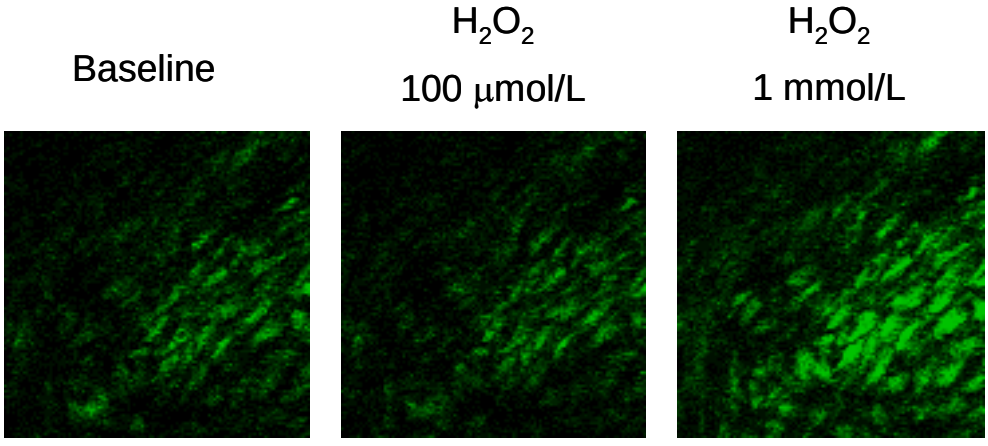
All pharmacological agents were obtained from Sigma, UK. Stock solutions were prepared in buffer with the exception of CPA and U-73122 (DMSO), indomethacin (5% bicarbonate) and ^{43}Gap 26 (dH_2O). Fluorescent probes were obtained from Invitrogen, UK.

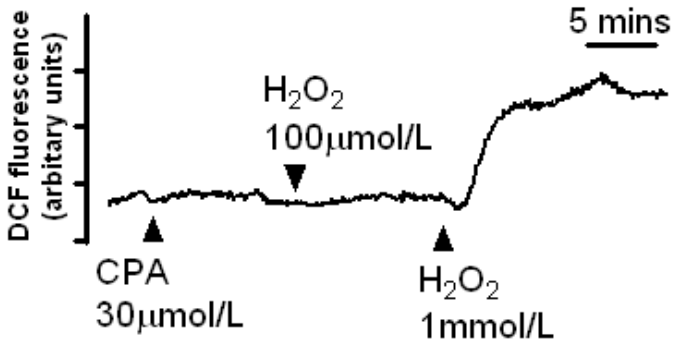
Statistics

In mechanical experiments the maximal percentage reversal of phenylephrine-induced constriction (R_{max}) by CPA or H_2O_2 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 H_2O_2) 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.

1. Griffith TM, Chaytor AT, Bakker LM, Edwards DH. 5-Methyltetrahydrofolate and tetrahydrobiopterin can modulate electrotonically mediated endothelium-dependent vascular relaxation. *Proc Natl Acad Sci U S A.* 2005;102:7008-7013.
2. Tsan MF, White JE, Rosano CL. Modulation of endothelial GSH concentrations: effect of exogenous GSH and GSH monoethyl ester. *J Appl Physiol.* 1989;66:1029-34.
3. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985;260:3440-50.
4. Shmygol A, Wray S. Modulation of agonist-induced Ca^{2+} release by SR Ca^{2+} load: direct SR and cytosolic Ca^{2+} measurements in rat uterine myocytes. *Cell Calcium*. 2005;37:215-23.

Supplemental Figure I





Effects of 100 μmol/L H_2O_2 and 30 μmol/L CPA in rabbit aortic valves loaded with 10 μmol/L 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 30 min at room temperature. Confocal imaging demonstrated that supraphysiological H_2O_2 concentrations (1 mmol/L) were required to increase fluorescence.

Supplemental Table I Effects of pharmacological interventions on EDHF-type relaxations evoked by CPA

Intervention	n	pIC_{50}	R_{max} %
Control	6	4.72±0.06	88.3±5.2
H_2O_2	6	5.31±0.20*	91.4±3.2
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±8.1**
Apamin+TRAM+IbTX + Catalase	9	-	20.7±6.8**
Control (threshold 10 μmol/L)	10	5.02±0.04	89.9±1.6
Catalase	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 (threshold 30 μmol/L)	5	4.65±0.03	79.5±4.7
Catalase	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_2O_2	5	5.52±0.31*	79.5±5.6
U-73122	5	4.70±0.05	77.4±5.7
U-73122+H_2O_2	5	5.55±0.27*‡	86.9±2.8

Potency (negative log IC_{50}) and maximal relaxation (R_{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_2O_2 in the presence and absence of CPA.

Intervention	n	pEC_{50}	R_{max} %
Control	6	3.88±0.05	80.6±2.2
Apamin+TRAM+IbTX	5	3.79±0.02	79.9±2.4
Denuded	7	3.87±0.03	86.9±2.6
Denuded+ Apamin+TRAM+IbTX	8	3.88±0.04	84.8±1.6
Control	20	3.80±0.03	79.0±1.4
CPA	16	4.14±0.04***	86.7±1.1**
CPA+IbTX	6	3.88±0.03	83.7±2.8
CPA+Apamin+TRAM	5	3.74±0.06	75.7±4.8
CPA+Apamin+TRAM +IbTX	15	3.68±0.04	78.3±2.5

Potency (negative log EC_{50}) and maximal relaxation (R_{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.