ASCORBIC ACID AND TETRAHYDROBIOPTERIN
POTENTIATE THE EDHF PHENOMENON BY GENERATING
HYDROGEN PEROXIDE

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Running Title: Ascorbate, tetrahydrobiopterin and EDHF

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Time for primary review: 23 days

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ABSTRACT

Aim Our objective was to investigate whether pro-oxidant properties of ascorbic acid (AA) and tetrahydrobiopterin (BH₄) modulate endothelium-dependent, electrotonically mediated arterial relaxation.

Methods and Results In studies with rabbit iliac artery (RIA) rings, NO-independent, EDHF-type relaxations evoked by the sarcoplasmic endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor cyclopiazonic acid and the G protein-coupled agonist acetylcholine (ACh) were enhanced by AA (1 mM) and BH₄ (200 μM), which generated buffer concentrations of H₂O₂ in the range of 40-80 μM. Exogenous H₂O₂ potentiated CPA- and ACh-evoked relaxations with a threshold of 10-30 μM, and potentiation by AA and BH₄ was abolished by catalase, which destroyed H₂O₂ generated by oxidation of these agents in the organ chamber. Adventitial application of H₂O₂ also enhanced EDHF-type dilator responses evoked by CPA and ACh in RIA segments perfused intraluminally with H₂O₂-free buffer, albeit with reduced efficacy. In RIA rings both control relaxations and their potentiation by H₂O₂ were overcome by blockade of gap junctions by connexin-mimetic peptides (YDKSFPISHVR and SRPTEK) targeted to the 1st and 2nd extracellular loops of the dominant vascular connexins expressed in the RIA. Superoxide dismutase attenuated the potentiation of EDHF-type relaxations by BH₄, but not AA, consistent with findings demonstrating a differential role for superoxide anions in the generation of H₂O₂ by the two agents.

Conclusions Pro-oxidant effects of AA and BH₄ can enhance the EDHF phenomenon by generating H₂O₂, which has previously been shown to amplify electrotonic hyperpolarization-mediated relaxation by facilitating Ca²⁺ release from endothelial stores.

Key Words: connexin-mimetic peptides, gap junction, superoxide anion
INTRODUCTION

In many arteries NO-independent relaxations can be inhibited by synthetic connexin-mimetic peptides that interrupt intercellular communication via myoendothelial and homocellular smooth muscle gap junctions, suggesting that such responses are electrotonic in nature, rather than mediated by a freely diffusible endothelium-derived hyperpolarizing factor (EDHF).\textsuperscript{1-3} We have previously provided evidence that H\textsubscript{2}O\textsubscript{2} can potentiate such “EDHF-type” relaxations in rings of rabbit iliac artery (RIA) when these are evoked by cyclopiazonic acid (CPA), an agent that promotes store-operated endothelial Ca\textsuperscript{2+} entry (SOCE) by inhibiting the SERCA pump of the endoplasmic reticulum (ER) Ca\textsuperscript{2+} store.\textsuperscript{4} This novel action of H\textsubscript{2}O\textsubscript{2} may reflect enhanced Ca\textsuperscript{2+} store depletion secondary to sensitization of the InsP\textsubscript{3} receptor, with the resulting increase in Ca\textsuperscript{2+} mobilization promoting the opening of the hyperpolarizing endothelial K\textsubscript{Ca} channels that are widely recognized to underpin the EDHF phenomenon.\textsuperscript{4-6} We have also shown that the inhibitory effects of connexin-mimetic peptides against EDHF-type relaxations are attenuated by ascorbic acid (AA) and R-5,6,7,8-tetrahydrobiopterin (BH\textsubscript{4}),\textsuperscript{7,8} whose ability to improve endothelial function in patients with hypertension, hypercholesterolaemia, diabetes and heart failure has widely been attributed to their ability to prevent uncoupling of the constitutive eNOS and thereby reduce production of the superoxide anion (O\textsubscript{2}-) by the oxygenase component of the enzyme.\textsuperscript{9-13} It is well known, however, that AA and BH\textsubscript{4} can generate H\textsubscript{2}O\textsubscript{2} following oxidation by molecular oxygen in aqueous solution.\textsuperscript{13-15} To examine whether this pro-oxidant activity also modulates endothelium-dependent relaxation, we have correlated the ability of AA and BH\textsubscript{4} to generate H\textsubscript{2}O\textsubscript{2} with potentiation of EDHF-type relaxations evoked by CPA and acetylcholine (ACh) in the RIA. In this vessel H\textsubscript{2}O\textsubscript{2} cannot be regarded as an EDHF because H\textsubscript{2}O\textsubscript{2}-evoked changes in smooth muscle membrane potential are much smaller than those associated with endothelium-dependent smooth muscle hyperpolarization, and H\textsubscript{2}O\textsubscript{2}-evoked relaxations of endothelium-denuded preparations are unaffected by blockade of K\textsuperscript{+} channels.\textsuperscript{4,16} Most experiments were conducted with ring preparations in which the endothelium was directly exposed to H\textsubscript{2}O\textsubscript{2}, but H\textsubscript{2}O\textsubscript{2} was also applied adventitiously in perfused arterial segments to mimic the in vivo situation where systemic administration of pharmacological doses of AA have been shown to generate high concentrations of H\textsubscript{2}O\textsubscript{2} in interstitial fluid, but circulating H\textsubscript{2}O\textsubscript{2} is efficiently destroyed by red cell glutathione peroxidase and catalase.\textsuperscript{17,18}
METHODS
Experiments were performed with iliac arteries from male NZW rabbits (2-2.5 kg) sacrificed by injection of sodium pentobarbitone (120 mg/kg i.v.). Protocols conformed to UK Home Office regulations and the Guide for the Care and Use of Laboratory Animals issued by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Tissues were transferred to oxygenated (95% O₂, 5% CO₂) Holman's buffer containing (in mM): NaCl 120, KCl 5, NaH₂PO₄ 1.3, NaHCO₃ 25, CaCl₂ 2.5, glucose 11, and sucrose 10. Myograph experiments were conducted with oxygenated Holman's solution (95% O₂, 5% CO₂) at 37°C and pH 7.4. To evaluate EDHF-type responses, in all experiments the cyclooxygenase inhibitor indomethacin (10 µM) and N⁶-nitro-L-arginine methyl ester (L-NAME, 300 µM) were added to the buffer 40 min before tone was induced by phenylephrine (PE, 1 µM). All pharmacological agents were obtained from Sigma, UK.

Tension Myography
Rings (2 to 3 mm wide) were mounted in a myograph (model 610M; Danish MyoTechnology, Aarhus) placed under a resting tension of 1mN and then allowed to equilibrate for ~30 minutes with readjustments of tension to compensate for stress relaxation. Cumulative concentration-response curves to CPA and ACh were constructed under control conditions and after 30 min incubation with AA (1 mM) or BH₄ (200 µM) in the presence and absence of catalase (Cat No C9322 derived from bovine liver) or superoxide dismutase (Cat No S7571 derived from bovine erythrocytes). Some rings were incubated with H₂O₂ (10, 30 or 100 µM) for 30 min before constriction. The role of gap junctions was investigated by preincubating for 20 min with 43Gap26 (VCYDKSFPISHVR; 100 µM), the truncated peptide YDKSFPISHVR, and the short peptides SRPTEK or its unnatural enantiomeric D-isomer srptek (each at 300 µM). Stock solutions were prepared in buffer with the exception of CPA (DMSO), indomethacin (5% ethanol), 43Gap 26 and YDKSFPISHVR (dH₂O) and SRPTEK/srptek (10% acetic acid). Peptides were confirmed to be of greater than 85% purity by HPLC.

Perfusion myography
Iliac artery segments (4-5 mm in length) were cannulated onto two glass micropipettes in a pressure myograph (Living Systems Instrumentation, USA). Flow and pressure were fixed at 0.5 ml/min and 75 mm Hg, respectively, giving a basal external arterial diameter ~1500 µm. PE (1 µM), CPA and ACh were administered intraluminally and in some experiments 100 µM H₂O₂ was added to the extraluminal Holmans solution in the myograph chamber 30 min before constriction. At the end of each experiment, arteries were perfused with
sodium nitroprusside (SNP, 100 µM) to induce full dilatation. Diameter changes were recorded on PowerLab 400 using Chart v4.1.2 software (AD Instruments, UK).

Hydrogen peroxide assay
Briefly, 300 µl samples of buffer were collected at the beginning and end of relaxation protocols in experiments with ACh or CPA (corresponding to AA/BH₄ incubation times of 30 and 60 min). These were added to Amplex Red (10 µM) and horseradish peroxidase (0.6 U/mL) in a 96-well plate and incubated in the dark at room temperature for 15 min. Absorbance was read at 560 nm using a Fluostar optima spectrophotometer (BMG Labtech) and H₂O₂ concentrations derived from a standard curve. Experiments were also performed in the absence of arterial rings with buffer maintained at 37°C and either oxygenated with 95% O₂/5% CO₂ or exposed to air. In perfusion experiments buffer was sampled from the myograph chamber and the effluent from the artery under study.

Mass Spectra
Peptide mass spectra were recorded using either a Waters 1525μ HPLC or UPLC Aquity autosamplers equipped with LCT Premier XE or Q-Tof micro mass sensitive detectors, respectively. Spectra were obtained in electrospray positive ion mode (ESI⁺) using a carrier solvent of 50:50 mixture of acetonitrile: deionised water. Formic acid (1% of eluent) was used as the proton source.

Statistics
In tension experiments the maximal percentage reversal of PE-induced constriction (Rmax) by CPA or ACh and concentrations giving 50% reversal of this constrictor response (IC₅₀ for CPA) or 50 % of maximal relaxation (EC₅₀ for ACh) were determined for each experiment. The use of IC₅₀ rather than EC₅₀ values was necessary to allow for a small initial CPA-induced constriction observed in ring experiments.⁴,⁸ In perfusion studies dilatations evoked by CPA and ACh were expressed as a function of the response to SNP, to calculate EC₅₀ and Dmax. All parameters were calculated as mean±S.E.M. and compared by the Student’s t-test or ANOVA followed by a Bonferroni post-test. P<0.05 was considered significant; n denotes the number of animals studied or assays performed for each data point.
RESULTS

Effects of AA and BH4 on CPA- and ACh-evoked relaxation
Preincubation of RIA rings with 1 mM AA or 200 µM BH4 caused leftward shifts in the concentration-relaxation curves for CPA and ACh without affecting R_max and increased buffer [H_2O_2] to ~40 µM and ~60 µM after 60 min, respectively (Figure 1A-D; Table 1). Increases in myograph [H_2O_2] and the potentiating effects of AA and BH4 on relaxation were abolished by 1000 U/ml catalase, which did not itself modulate control responses to either CPA or ACh (Figure 1A-D; Table 1).

Effects of H_2O_2 on CPA- and ACh-evoked relaxation/dilatation
Preincubation of endothelium-intact rings with increasing [H_2O_2] progressively lowered IC_{50}/EC_{50} values for CPA and ACh with a threshold for potentiation between 10 and 30 µM H_2O_2, but exerted no effect on R_max even at 100 µM H_2O_2 (Figure 2A; Table 1). Assay of buffer on completion of the relaxation protocols confirmed that intrinsic antioxidant mechanisms did not reduce applied myograph [H_2O_2] (Figure 2A). In perfused RIA segments concentrations of CPA and ACh causing 50% dilatation were similar to the IC_{50}/EC_{50} values observed in rings, and adventitial application of 100 µM H_2O_2 potentiated these responses to an extent intermediate between 30 and 100 µM H_2O_2 in rings, again without affecting D_max (Figure 2B; Tables 1,2). H_2O_2 was undetectable in buffer exiting from the lumen of segments exposed to 100 µM H_2O_2 on their adventitial surface (Figure 2B).

Role of superoxide anions
Superoxide dismutase (SOD, 1200 U/mL) did not affect the potentiation of CPA-evoked relaxation caused by 1 mM AA, but significantly attenuated the potentiation observed with 200 µM BH4 (Figures 3A,4A; Table 1). Corresponding assays of myograph [H_2O_2] demonstrated that generation of H_2O_2 from AA was unaffected by SOD at 30 or 60 min (i.e. the start and completion of the relaxation protocols), whereas H_2O_2 accumulation from BH4 was reduced by ~50% after 30 min, but unaffected at 60 min (Figures 3A,4A).

In experiments conducted in well-oxygenated buffer (95% O_2/5% CO_2) in the absence of arterial rings, generation of H_2O_2 from 1 mM AA rose to a plateau at 60-90 min (Figure 3B). The transition metal chelator DTPA caused a concentration-dependent reduction in H_2O_2 accumulation at 30 min, and no H_2O_2 was detectable in experiments employing oxygenated deionized water rather than buffer (Figure 3C). Generation of H_2O_2 from 1 mM AA was insensitive to the presence of SOD and was 2-3 fold greater in well-oxygenated buffer compared to buffer exposed to air (Figure 3D).
In analogous experiments with 200 µM BH₄, generation of H₂O₂ was rapid (~60 µM after 5 min) and rose to a peak at ~90 µM at 30 min before subsequently declining at 60 and 90 min (Figure 4B), but was unaffected by DTPA, in contrast to AA (Figure 4C). Accumulation of H₂O₂ after 30 min was similar at ~80 µM whether the buffer was well-oxygenated or exposed to air, but in buffer exposed to air accumulation of H₂O₂ was almost abolished by SOD at concentrations ≥ 300 U/mL after 30 min, whereas in well-oxygenated buffer H₂O₂ production was depressed only at SOD concentrations ≥ 900 U/mL, with 1200 U/mL SOD causing an overall reduction of ~50% (Figure 4D).

**Role of gap junctions**

Preincubation of RIA rings with 100 µM 43Gap26 attenuated relaxations to CPA, reducing R_max to ~30%, as previously reported. 3,5,6 Potentiation of CPA-evoked relaxation by 100 µM H₂O₂ was lost in the presence of this peptide, which normalized relaxation (Figure 5A). Electrospray mass spectrometry demonstrated that samples of 43Gap26 treated with H₂O₂ (both at 100 µM) at pH ~7 that were allowed to stand for 60 min either in buffer or deionised water displayed substantial oxidation to cystine bridged dimers as evidenced by the presence of the triply protonated species [(VCYDKSFPISHVR)₂ - 2H] + 3H⁺ (calculated m/e for C₁₄₀H₂₁₂N₃₈O₃₈S₂ + 3H⁺ {M + 3H⁺} 1033.52; found 1033.46), the triple sodium adduct [(VCYDKSFPISHVR)₂ - 2H] + 3Na⁺ (calculated m/e for C₁₄₀H₂₁₂N₃₈O₃₈S₂ + 3Na⁺ {M + 3Na⁺} 1055.50; found 1055.80) and mixed proton/sodium adducts at intermediate mass values.

Preincubation with a cysteine-free truncated form of 43Gap26 (YDKSFPISHVR at 300 µM) closely mimicked the effects of 43Gap26 at 100 µM (Figure 5A). Preincubation with the short peptide SRPTEK at 300 µM markedly attenuated relaxations to ACh, reducing R_max to <30%, whereas the corresponding D-isomer srptek was inactive; SRPTEK similarly abolished the potentiated ACh-evoked relaxations observed in the presence of 100 µM H₂O₂ (Figure 5B). Mass spectrometry confirmed the lack of dimerization of the YDKSFPISHVR or SRPTEK oligopeptides (data not shown).

Direct chemical interaction between 43Gap26 and H₂O₂ was confirmed by the demonstration that there was ~50% consumption of 100 µM H₂O₂ 30 min after addition to air-exposed Holman’s buffer containing 100 µM 43Gap26, whereas consumption of H₂O₂ was not evident in buffer containing 100 µM SRPTEK (Figure 5C).
DISCUSSION

The major finding of the present study is that AA and BH₄ potentiate EDHF-type relaxations of rabbit arteries evoked by CPA and ACh through a mechanism that is sensitive to catalase. Control and potentiated responses were both inhibited by connexin-mimetic peptides. It follows that H₂O₂ generated by the oxidation of AA and BH₄ can amplify NO-independent arterial relaxations mediated by the spread of endothelial hyperpolarization via gap junctions.

Incubation of RIA rings with 1 mM AA or 200 μM BH₄ enhanced EDHF-type responses with the threshold for relaxation decreasing from 10-30 μM to ~1 μM with CPA and from ~0.1 μM to ~0.01 μM with ACh. Assay of myograph [H₂O₂] after 60 min incubation revealed conversion rates of ~4% and ~30% for AA and BH₄, respectively, consistent with evidence that BH₄ is more readily oxidized by molecular oxygen than AA in physiological buffer. The potentiating effects of AA and BH₄, but not control responses, were inhibited by catalase and could be mimicked by exogenous H₂O₂, which itself enhanced EDHF-type relaxations at a threshold concentration of 10-30 μM. The observation that H₂O₂ amplifies relaxant responses to ACh, which mobilizes Ca²⁺ from the ER store via the formation of InsP₃, is consistent with evidence that H₂O₂ enhances Ca²⁺ release by sensitizing the InsP₃ receptor, and extends previous findings with CPA which elevates endothelial [Ca²⁺]i by blocking ER Ca²⁺ uptake. EDHF-type relaxations evoked by CPA and ACh can also be potentiated by the sulphydryl reagent thimerosal, which amplifies Ca²⁺ release from the ER by oxidizing critical thiol groups present in the InsP₃ receptor, thus raising the possibility of a molecular target common to H₂O₂ and thimerosal. It should be appreciated that the concentrations of buffer H₂O₂ generated from AA and BH₄ in the present study are likely to correspond to intracellular levels within the suggested physiological range (1 to 10 μM), since glutathione peroxidase, catalase and other mechanisms are thought to limit cytosolic [H₂O₂] to 1-15% of that applied extracellularly.

In the rat, microdialysis techniques have shown that systemic administration of AA at pharmacological doses sufficient to achieve circulating AA levels of 1-10 mM causes extravascular accumulation of H₂O₂ and the ascorbyl radical (a marker of AA oxidation) at concentrations that correlate directly with plasma [AA], with interstitial fluid [H₂O₂] rising to 20-150 μM but H₂O₂ remaining undetectable in the intravascular compartment. To mimic this in vivo situation 100 μM H₂O₂ was selectively applied to the adventitia of perfused RIA segments and found to potentiate NO-independent CPA- and ACh-evoked...
dilatations, although less effectively than in ring preparations where the endothelium was directly exposed to the same concentration of H$_2$O$_2$. This reduction in potency is likely to reflect a concentration gradient of H$_2$O$_2$ across the wall of the segments because their intraluminal perfusate did not contain H$_2$O$_2$ and the RIA possesses ~10 layers of smooth muscle cells and is therefore relatively thick-walled. It thus seems likely that putative H$_2$O$_2$-dependent pro-oxidant effects of AA and BH$_4$ on endothelial function in vivo would be most pronounced in the microcirculation because (i) the adventitial-endothelial concentration gradient of H$_2$O$_2$ would be smaller than in conduit arteries, and (ii) gap junction-dependent mechanisms can dominate over NO-mediated vasodilatation in resistance arteries, consistent with evidence that the number of myoendothelial connections per endothelial cell is highest in small arteries.

Mechanisms involved in the generation of H$_2$O$_2$

To gain insights into the pathways whereby AA and BH$_4$ generate H$_2$O$_2$ in physiological buffer, experiments were conducted to evaluate the contribution of the superoxide anion (‘O$_2^-$’), which has been implicated in the autoxidation of BH$_4$, but whose role in the oxidation of AA is controversial. In myograph studies with rings SOD attenuated the potentiating effects of BH$_4$ on CPA-evoked relaxations and associated formation of H$_2$O$_2$, whereas the generation of H$_2$O$_2$ from AA and its potentiating effects on relaxation were unaffected. Spin trap analysis has shown that BH$_4$ reacts with molecular oxygen at physiological pH to generate ‘O$_2^-$’, which then drives a chain reaction involving the reduction of molecular oxygen via an intermediary BH$_4$ radical that accelerates the rate of BH$_4$ oxidation by ~5-fold. Loss of this chain reaction is likely to explain the ability of SOD to attenuate H$_2$O$_2$ formation and relaxation in the present study, since SOD causes a marked reduction the rate of BH$_4$ autoxidation. By contrast, aqueous solutions of AA are stable at pH ~7 unless trace concentrations of Fe$^{3+}$ or Cu$^{2+}$ ions (normally present in commercially available salts) are present as contaminants that catalyse its oxidation. We were thus unable to detect formation of H$_2$O$_2$ from AA in deionized water and found that the generation of H$_2$O$_2$ from AA in buffer was substantially reduced in the presence of DTPA, a polydentate scavenger of Fe$^{3+}$ and Cu$^{2+}$ ions, whereas formation of H$_2$O$_2$ from BH$_4$ was unaffected. Conversely, 1 µM Fe$^{3+}$ or Cu$^{2+}$ catalysed the generation of H$_2$O$_2$ from 1 mM AA, with Cu$^{2+}$ being by far the more active cation (see Supplemental Materials). Although metal-catalysed oxidation of AA has been suggested to involve the interaction of ‘O$_2^-$’ with ascorbate ions and the ascorbyl radical, the effects of SOD on the rate of AA oxidation were modest (<2-fold decrease), and it has also been proposed that metal-catalysed oxidation (eg of 6-hydroxydopamine and 1,2,4-benzenetriol) can proceed via a 2-electron mechanism in which H$_2$O$_2$ is generated directly from molecular oxygen (rather
than \( \text{O}_2 \)) and is therefore insensitive to SOD.\(^{28,29}\) The impact of oxygenation on \( \text{H}_2\text{O}_2 \) accumulation was examined in experiments conducted with buffer exposed to air, because isolated arterial preparations are conventionally maintained in buffer gassed with \( \text{O}_2 \) at levels well above the physiological range. Such studies demonstrated a \( \sim \)50\% reduction in \( \text{H}_2\text{O}_2 \) generated from AA after 30 min compared to oxygenated buffer, whereas \( \text{H}_2\text{O}_2 \) formation from BH\(_4\) was unaltered. Major differences in the role of intermediary \( \text{O}_2 \) in the oxidation of AA and BH\(_4\) were nevertheless highlighted by observations that the generation of \( \text{H}_2\text{O}_2 \) from BH\(_4\) in buffer exposed to air was suppressed by SOD, whereas its formation from AA under the same conditions was unaffected.

**Role of Gap Junctions**

Studies with connexin-mimetic peptides have confirmed the underlying electrotonic nature of NO- and prostanoid-independent responses to CPA and ACh in rabbit arteries, and shown that such peptides do not attenuate direct NO-mediated relaxation or endothelial release of endogenous NO in sandwich bioassay experiments.\(^{3,7,8,30}\) As previously reported, CPA-evoked relaxations of the RIA were attenuated by the peptide VCYDKSFPISHVR (\(^{43}\text{Gap26}; 100 \ \mu\text{M})\), which possesses homology with the 1st extracellular loop of Cx43, the dominant connexin expressed in the media of the RIA and interrupts the spread of CPA-evoked endothelial hyperpolarization through the vessel wall.\(^{3,7,8}\) In the present study the inhibitory effects of 100 \( \mu\text{M} \) \(^{43}\text{Gap26}\) and the potentiating effects of 100 \( \mu\text{M} \) \( \text{H}_2\text{O}_2 \) on CPA-evoked relaxation cancelled when both agents were present simultaneously, with relaxation then being maintained at control levels. This may, at least in part, reflect oxidation of the thiol group of the cysteine residue of \(^{43}\text{Gap26}\) by \( \text{H}_2\text{O}_2 \) because dimerization via the formation of an intermolecular cystine bridge was confirmed by mass spectrometry and there was 50\% consumption of applied \( \text{H}_2\text{O}_2 \) in the presence of equimolar peptide concentrations, consistent with the reaction \( 2\text{R-SH} + \text{H}_2\text{O}_2 \rightarrow \text{R-S-S-R} + 2\text{H}_2\text{O} \). However, we also found that a truncated peptide YDKSFPISHVR mimicked the effects of \(^{43}\text{Gap26}\) when applied at 300 \( \mu\text{M} \), suggesting specific gap junction blockade because oxidation/dimerization of this peptide was prevented by deletion of the N-terminal valine and cysteine (VC) residues. Furthermore, the peptide SRPTEK, which corresponds to a highly conserved sequence in the 2nd extracellular loops of the principal endothelial connexins expressed in the RIA (Cx37 and Cx40), as well as Cx43,\(^3\) caused near-complete inhibition of ACh-evoked EDHF-type relaxations, even when these were potentiated by \( \text{H}_2\text{O}_2 \). This short peptide may therefore be considered a general, redox-insensitive inhibitor of gap junction signalling because (i) SRPTEK did not dimerize in the presence of \( \text{H}_2\text{O}_2 \), (ii) assay revealed no consumption of buffer \( \text{H}_2\text{O}_2 \), and (iii) the D-isoform (srptek) was
biologically inactive, suggesting that loss of relaxation driven by SRPTEK involves a specific molecular recognition event at the cell surface.

We have previously shown that 100 μM BH₄ opposes the ability of equimolar concentrations of 43Gap26 or the related peptide 37,40Gap26 (VCYDQAFPISHIR, which targets Cx37 and Cx40) to inhibit CPA-evoked EDHF-type relaxations and smooth muscle hyperpolarization in the RIA.⁷ At this concentration BH₄ generates myograph H₂O₂ concentrations of ~40 μM (data not shown), and could therefore contribute to the normalization of EDHF-type responses both via the potentiation of ER Ca²⁺ mobilization and peptide oxidation. However, at concentrations normally found in plasma (50 to 100 μM), which are lower than those employed in the present experiments, AA can also oppose the inhibition of CPA-induced relaxation and smooth muscle hyperpolarization by 43Gap26 and 37,40Gap26.⁸ It should also be noted that in non-vascular cells H₂O₂ has been variously shown to enhance or inhibit intercellular coupling via gap junctions constructed from Cx43, probably by altering the phosphorylation/oxidation status of residues present in the intracellular cytoplasmic tail of this connexin subtype.³¹-³⁵ Indeed, the hyper phosphorylation of Cx43 that follows administration of H₂O₂ or phorbol esters (which generate O₂⁻) can be prevented by a spectrum of antioxidants, including AA, with preservation of channel function.³⁶,³⁷ Further studies are therefore necessary to evaluate the effects of competing pro- and anti-oxidant mechanisms on gap junctional communication in the endothelial and smooth muscle layers of the vessel wall, and how their contributions might vary under different experimental conditions because AA and BH₄ are both capable of reducing H₂O₂ to H₂O as well as generating H₂O₂.¹⁴,¹⁵,³⁸,³⁹

Conclusions
Clinical studies have suggested that AA and BH₄ can both improve endothelial dysfunction in human conduit arteries by increasing the bioavailability of NO, provided that pharmacological doses are administered systemically.⁹,¹² The present findings raise the possibility that high concentrations of AA and BH₄ might also reverse endothelial dysfunction by amplifying the EDHF-type responses that have been postulated to compensate for loss of NO-dependent dilatation. It should thus be noted that the concentration of AA employed in the present in vitro studies (1 mM) is lower than the venous concentrations (1.5-3.2 mM) associated with restoration of endothelial responsiveness to ACh/methacholine in forearm plethysmographic studies in patients with hypertension or peripheral arterial disease following intra-brachial arterial administration of AA.¹⁰,¹¹ As noted above, such concentrations of circulating AA can elevate interstitial
fluid [H$_2$O$_2$] to levels that potentiated NO-independent dilatation when applied to the adventitia of RIA segments. Corresponding measurements of interstitial [H$_2$O$_2$] are not available for BH$_4$ although the infusion rates found necessary to prevent endothelial dysfunction following ischaemia-perfusion injury in the human forearm lead to total circulating biopterin concentrations of ~100 μM and appear to involve mechanisms distinct from the role of BH$_4$ as a co-factor for eNOS.$^{40}$ Further studies are therefore needed to assess the effects of extracellularly-generated H$_2$O$_2$ on endothelial function in vivo. Because H$_2$O$_2$ production from O$_2^-$ is normally attenuated by NO through an interaction that results in the formation of peroxynitrite, it also remains to be determined if the reduction in NO bioavailability that characterizes many vascular disease states upregulates the EDHF phenomenon directly.

**FUNDING**

The study was supported by the British Heart Foundation (Grant PG/05/133/19892) and the Cardiff Institute of Tissue Engineering (CITER).

Funding to pay the Open Access charge was provided by the British Heart Foundation

**DISCLOSURES**

None
REFERENCES


FIGURE LEGENDS

Figure 1 Effects of AA and BH4 on EDHF-type relaxations to CPA and ACh in RIA rings. (A,B) 1 mM AA and (C,D) 200 μM BH4 each elevated bath [H2O2] (bar graphs) and resulted in a catalase-sensitive potentiation of relaxation. [H2O2] was measured at the conclusion of each experiment i.e. after 60 min incubation with AA or BH4. *** denotes \( P < 0.001 \) for specific concentrations of CPA or ACh compared with control.

Figure 2 Concentration-dependent potentiation of EDHF-type relaxations/dilatations to CPA and ACh in the presence of exogenous H2O2. (A) In tension myograph experiments buffer [H2O2] was unchanged at the conclusion of the experiments (bar graph, illustrated for ACh). (B) In perfusion experiments intraluminal and extraluminal [H2O2] at the conclusion of each experiment were similarly unchanged (bar graphs). ** and *** denote \( P < 0.01 \) and 0.001 for specific concentrations of CPA or ACh compared with control.

Figure 3 Effects of SOD (1200 U/mL) on concentration-response curves for CPA and generation of H2O2 from 1 mM AA. (A) SOD did not affect the potentiating effects of AA on relaxation or elevations in myograph [H2O2] at 30 and 60 min (insets). (B) Time course of the generation of H2O2 in oxygenated buffer. (C) DTPA reduced the formation of H2O2 in well-oxygenated buffer and no generation of H2O2 was evident in comparative experiments performed in deionised water (dH2O). (D) SOD did not affect the generation of H2O2 either in well-oxygenated or air-exposed buffer (open columns). ** and *** denote \( P < 0.01 \) and 0.001 compared with the appropriate control.

Figure 4 Effects of SOD (1200 U/mL) on concentration-response curves for CPA and generation of H2O2 from 200 μM BH4. (A) SOD attenuated the potentiating effects of BH4 on relaxation and the associated increase in myograph [H2O2] after 30 but not 60 min (insets). (B) Time course of H2O2 generation in oxygenated buffer. (C) DTPA did not affect the formation of H2O2 in oxygenated buffer. (D) Inhibitory effects of SOD were much less evident in well-oxygenated than air-exposed buffer (open columns). *, ** and *** denote \( P < 0.05, 0.01 \) and 0.001 compared with control.

Figure 5 Effects of connexin-mimetic peptides on control EDHF-type relaxations and their potentiation by exogenous H2O2. (A) Attenuation of CPA-evoked relaxations by \(^{43}\text{Gap 26}\) and its truncated form YDKSFPISHVR were both reversed by 100 μM H2O2. (B) SRPTEK almost abolished relaxations to ACh, both in the presence and absence of 100 μM H2O2.
whereas srptek was inactive. (C) GAP 26 but not SRPTEK reduced applied [H₂O₂] in oxygenated buffer. ** and *** denote $P<0.01$ and 0.001 compared with control.
Table 1. Effects of pharmacological interventions on EDHF-type relaxations evoked by CPA and ACh by in the presence and absence of H$_2$O$_2$ or catalase.

<table>
<thead>
<tr>
<th></th>
<th>CPA n</th>
<th>pIC$_{50}$</th>
<th>R$_{max}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>4.61±0.01</td>
<td>92.8±2.1</td>
</tr>
<tr>
<td>Catalase</td>
<td>5</td>
<td>4.66±0.07</td>
<td>93.8±2.4</td>
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<tr>
<td>AA</td>
<td>5</td>
<td>5.12±0.05***</td>
<td>92.3±2.3</td>
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<td>AA + Catalase</td>
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<td>4.59±0.11</td>
<td>91.5±3.0</td>
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<td>Control</td>
<td>7</td>
<td>4.70±0.10</td>
<td>79.6±5.4</td>
</tr>
<tr>
<td>Catalase</td>
<td>7</td>
<td>4.74±0.04</td>
<td>85.8±2.5</td>
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<td>BH$_4$</td>
<td>7</td>
<td>5.39±0.15***</td>
<td>85.1±2.1</td>
</tr>
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<td>BH$_4$ + Catalase</td>
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<td>4.76±0.05</td>
<td>83.9±2.5</td>
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<tr>
<td>Control</td>
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<td>4.64±0.04</td>
<td>84.3±3.1</td>
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<tr>
<td>H$_2$O$_2$ (10µM)</td>
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<td>4.80±0.04</td>
<td>90.3±1.6</td>
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<tr>
<td>H$_2$O$_2$ (30µM)</td>
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<td>4.96±0.10</td>
<td>91.6±2.5</td>
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<tr>
<td>H$_2$O$_2$ (100µM)</td>
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<td>5.36±0.17***</td>
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<td>4.80±0.03</td>
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<td>4.71±0.04</td>
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<td>5.53±0.19**</td>
<td>87.5±3.6</td>
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<td>AA + SOD</td>
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<td>5.53±0.15**</td>
<td>90.2±2.0</td>
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<td>Control</td>
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<tr>
<td>SOD (1200 U)</td>
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<td>4.66±0.06</td>
<td>80.0±7.9</td>
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<tr>
<td>BH$_4$</td>
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<td>6.01±0.20***</td>
<td>88.1±7.1</td>
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<td>BH$_4$ + SOD</td>
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<td>5.39±0.21*</td>
<td>85.5±9.1</td>
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<td>6</td>
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<td>43Gap26 (100 µM)</td>
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<td>H$_2$O$_2$ (100 µM)</td>
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<tr>
<td>YDKSFPIHSVHR (300 µM)</td>
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<td>35.2±14.1***</td>
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<tr>
<td>H$_2$O$_2$ (100 µM)</td>
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<td>5.30±0.11***</td>
<td>84.3±4.2</td>
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<th>ACh n</th>
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<th>R$_{max}$ %</th>
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<td>Control</td>
<td>5</td>
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<td>75.8±2.7</td>
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<tr>
<td>Catalase</td>
<td>5</td>
<td>6.64±0.08</td>
<td>77.2±3.3</td>
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<tr>
<td>AA</td>
<td>5</td>
<td>7.00±0.06*</td>
<td>81.9±1.2</td>
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<td>AA + Catalase</td>
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<td>6.48±0.10</td>
<td>75.3±2.2</td>
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<tr>
<td>Control</td>
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<td>76.6±3.8</td>
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<tr>
<td>Catalase</td>
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<td>6.39±0.10</td>
<td>73.1±3.6</td>
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<td>BH$_4$</td>
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<td>6.91±0.04**</td>
<td>79.7±3.1</td>
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<tr>
<td>BH$_4$ + Catalase</td>
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<td>71.2±3.9</td>
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<tr>
<td>Control</td>
<td>8</td>
<td>6.50±0.06</td>
<td>74.9±4.4</td>
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<td>H$_2$O$_2$ (10 µM)</td>
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<td>6.55±0.08</td>
<td>72.7±5.2</td>
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<td>H$_2$O$_2$ (30 µM)</td>
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<td>6.87±0.20</td>
<td>76.8±5.5</td>
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<td>H$_2$O$_2$ (100 µM)</td>
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<td>7.25±0.15***</td>
<td>74.7±4.1</td>
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<tr>
<td>Control</td>
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<td>69.0±3.0</td>
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<td>SRPETK</td>
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<td>SRPETK</td>
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<td>-</td>
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<td>H$_2$O$_2$ (100 µM)</td>
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<td>7.16±0.1**</td>
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---

Potency (negative log IC$_{50}$ or EC$_{50}$) and maximal relaxation (R$_{max}$) expressed as a percentage 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.
**Table 2.** Effects of adventitiously applied H$_2$O$_2$ on EDHF-type dilatations evoked by CPA and ACh.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>pEC$_{50}$</th>
<th>D$_{max}$%</th>
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<tr>
<td>CPA</td>
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<td></td>
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<tr>
<td>Control</td>
<td>5</td>
<td>4.79±0.04</td>
<td>88.0±2.3</td>
</tr>
<tr>
<td>H$_2$O$_2$ (100µM)</td>
<td>5</td>
<td>5.09±0.05**</td>
<td>91.4±2.9</td>
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</tbody>
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<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>pEC$_{50}$</th>
<th>D$_{max}$%</th>
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<tr>
<td>ACh</td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>5</td>
<td>6.3±0.08</td>
<td>78.2±4.7</td>
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<tr>
<td>H$_2$O$_2$ (100 µM)</td>
<td>5</td>
<td>6.75±0.09**</td>
<td>82.6±2.0</td>
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</table>

Potency (negative log EC$_{50}$) and maximal dilatation (D$_{max}$) expressed as a percentage of the dilator response to SNP are given as mean ± S.E.M. ** denotes P<0.01 compared with control.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A First extracellular loop (Cx43)

B Second extracellular loop (Cx37,40 and 43)

C Buffer (30 min), Air
n=4-6