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Ascorbic acid and tetrahydrobiopterin potentiate the EDHF phenomenon by generating hydrogen peroxide

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Aims Our objective was to investigate whether pro-oxidant properties of ascorbic acid (AA) and tetrahydrobiopterin (BH_4) modulate endothelium-dependent, electrotonically mediated arterial relaxation.
Methods and results In studies with rabbit iliac artery (RIA) rings, NO-independent, endothelium-derived hyperpolarizing factor (EDHF)-type relaxations evoked by the sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase inhibitor cyclopiazonic acid and the G protein-coupled agonist acetylcholine (ACh) were enhanced by AA (1 mM) and BH_4 (200 μM), which generated buffer concentrations of H_2O_2 in the range of 40–80 μM. Exogenous H_2O_2 potentiated cyclopiazonic acid (CPA)- and ACh-evoked relaxations with a threshold of 10–30 μM, and potentiation by AA and BH_4 was abolished by catalase, which destroyed H_2O_2 generated by oxidation of these agents in the organ chamber. Adventitial application of H_2O_2 also enhanced EDHF-type dilator responses evoked by CPA and ACh in RIA segments perfused intraluminally with H_2O_2-free buffer, albeit with reduced efficacy. In RIA rings, both control relaxations and their potentiation by H_2O_2 were overcome by blockade of gap junctions by connexin-mimetic peptides (YDKSFPISHVR and SRPTEK) targeted to the first and second extracellular loops of the dominant vascular connexins expressed in the RIA. Superoxide dismutase attenuated the potentiation of EDHF-type relaxations by BH_4, but not AA, consistent with findings demonstrating a differential role for superoxide anions in the generation of H_2O_2 by the two agents.
Conclusion Pro-oxidant effects of AA and BH_4 can enhance the EDHF phenomenon by generating H_2O_2, which has previously been shown to amplify electrotonic hyperpolarization-mediated relaxation by facilitating Ca^{2+} release from endothelial stores.

1. 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).[1–3] We have previously provided evidence that H_2O_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^{2+} entry by inhibiting the sarcoplasmic endoplasmic reticulum (ER) Ca^{2+}-ATPase pump of the ER Ca^{2+} store.[4] This novel action of H_2O_2 may reflect enhanced Ca^{2+} store depletion secondary to sensitization of the $InsP_3$ receptor, with the resulting increase in Ca^{2+} mobilization promoting the opening of the hyperpolarizing endothelial K_{Ca} channels that are widely recognized to underpin the EDHF phenomenon.[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_4),[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 ($\cdot O_2^-$) by the oxygenase component of the enzyme.[9–13] It is well known, however, that AA and BH_4 can generate H_2O_2 following oxidation by molecular oxygen in aqueous solution.[13–15]

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To examine whether this pro-oxidant activity also modulates endothelium-dependent relaxation, we have correlated the ability of AA and BH_4 to generate H_2O_2 with potentiation of EDHF-type relaxations evoked by CPA and acetylcholine (ACh) in the RIA. In this vessel, H_2O_2 cannot be regarded as an EDHF because H_2O_2-evoked changes in smooth muscle membrane potential are much smaller than those associated with endothelium-dependent smooth muscle hyperpolarization, and H_2O_2-evoked relaxations of endothelium-denuded preparations are unaffected by blockade of K^+ channels.[4,16] Most experiments were conducted with ring preparations in which the endothelium was directly exposed to H_2O_2, but H_2O_2 was also applied adventitially 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_2O_2 in interstitial fluid, but circulating H_2O_2 is efficiently destroyed by red cell glutathione peroxidase and catalase.[17,18]

2. 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% O_2, 5% CO_2) Holman's buffer containing (in mM): NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11, and sucrose 10. Myograph experiments were conducted with oxygenated Holman's solution (95% O_2, 5% CO_2) at 37°C and pH 7.4. To evaluate EDHF-type responses, in all experiments, the cyclooxygenase inhibitor indomethacin (10 μM) and N^G-nitro-L-arginine methyl ester (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.

2.1 Tension myography

Rings (2–3 mm wide) were mounted in a myograph (model 610M; Danish MyoTechnology, Aarhus) placed under a resting tension of 1 mN and then allowed to equilibrate for ~30 min 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 BH_4 (200 μM) in the presence and absence of catalase (Cat No. C9322 derived from bovine liver) or superoxide dismutase (SOD) (Cat No. S7571 derived from bovine erythrocytes). Some rings were incubated with H_2O_2 (10, 30, or 100 μM) for 30 min before constriction. The role of gap junctions was investigated by pre-incubating for 20 min with ^{43}Gap26 (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), ^{43}Gap 26 and YDKSFPISHVR (dH_2O) and SRPTEK/srptek (10% acetic acid). Peptides were confirmed to be of greater than 85% purity by HPLC.

2.2 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 mmHg, respectively, giving a basal external arterial diameter ~1500 μm. PE (1 μM), CPA and ACh were administered intraluminally and in some experiments 100 μM H_2O_2 was added to the extraluminal Holman's 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).

2.3 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/BH_4 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 H_2O_2 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% O_2/5% CO_2 or exposed to air. In perfusion experiments, buffer was sampled from the myograph chamber and the effluent from the artery under study.

2.4 Mass spectra

Peptide mass spectra were recorded using either a Waters 1525 μ 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:deionized water. Formic acid (0.1% of eluent) was used as the proton source.

2.5 Statistics

In tension experiments, the maximal percentage reversal of PE-induced constriction (R_{max}) by CPA or ACh and concentrations giving 50% reversal of this constrictor response (IC_{50} for CPA) or 50% of maximal relaxation (EC_{50} for ACh) were determined for each experiment. The use of IC_{50} rather than EC_{50} 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 EC_{50} and D_{max}. All parameters were calculated as mean ± SEM 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.

3. Results

3.1 Effects of AA and BH_4 on CPA- and ACh-evoked relaxation

Pre-incubation of RIA rings with 1 mM AA or 200 μM BH_4 caused leftward shifts in the concentration–relaxation curves for CPA and ACh without affecting R_{max} and increased buffer [H_2O_2] to ~40 and ~60 μM after 60 min, respectively (*Figure 1A–D* and *Table 1*). Increases in myograph [H_2O_2] and the potentiating effects of AA and BH_4 on relaxation were abolished by 1000 U/mL catalase, which did not itself modulate control responses to either CPA or ACh (*Figure 1A–D* and *Table 1*).

3.2 Effects of H_2O_2 on CPA- and ACh-evoked relaxation/dilatation

Pre-incubation 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

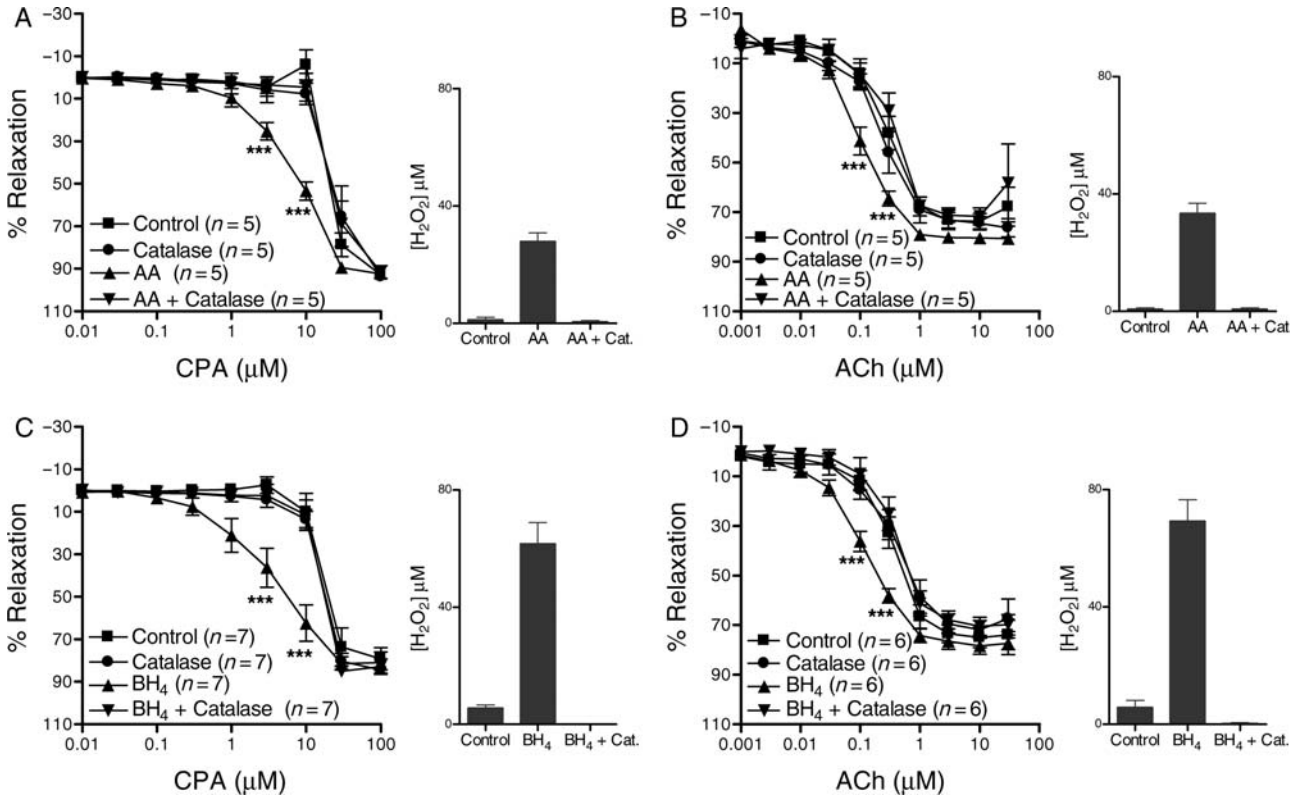

Figure 1 Effects of AA and BH_4 on EDHF-type relaxations to CPA and ACh in RIA rings. (*A* and *B*) 1 mM AA and (*C* and *D*) 200 μM BH_4 each elevated bath [H_2O_2] (bar graphs) and resulted in a catalase-sensitive potentiation of relaxation. [H_2O_2] was measured at the conclusion of each experiment, i.e. after 60 min incubation with AA or BH_4. ***$P < 0.001$ for specific concentrations of CPA or ACh compared with control.

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% of maximal 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* and *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*).

3.3 Role of superoxide anions

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 BH_4 (*Figures 3A* and *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 BH_4 was reduced by ∼50% after 30 min, but unaffected at 60 min (*Figures 3A* and *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 two- to three-fold greater in well-oxygenated buffer compared with buffer exposed to air (*Figure 3D*).

In analogous experiments with 200 μM BH_4, generation of H_2O_2 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*). The high concentrations of DTPA required to inhibit the accumulation of H_2O_2 in experiments with AA are likely to reflect competition between Ca^{2+} and other metal ions present in the buffer (see Supplementary material online).

Accumulation of H_2O_2 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 H_2O_2 was almost abolished by SOD at concentrations ≥300 U/mL after 30 min, whereas in well-oxygenated buffer H_2O_2 production was depressed only at SOD concentrations ≥900 U/mL, with 1200 U/mL SOD causing an overall reduction of ∼50% (*Figure 4D*).

3.4 Role of gap junctions

Pre-incubation of RIA rings with 100 μM ^{43}Gap26 attenuated relaxations to CPA, reducing R_{max} to ∼30%, as previously reported.[3,7,8] Potentiation of CPA-evoked relaxation by 100 μM H_2O_2 was lost in the presence of this peptide, which normalized relaxation (*Figure 5A*). Electrospray mass spectrometry demonstrated that samples of ^{43}Gap26 treated with H_2O_2 (both at 100 μM) at pH ∼7 that were allowed to stand for 60 min either in buffer or deionized water displayed substantial oxidation to cystine-bridged

Table 1 Effects of pharmacological interventions on EDHF-type relaxations evoked by CPA and ACh by in the presence and absence of H_2O_2 or catalase

CPA	*n*	pIC_{50}	R_{max} %	ACh	*n*	pEC_{50}	R_{max} %
Control	5	4.61 ± 0.01	92.8 ± 2.1	Control	5	6.55 ± 0.09	75.8 ± 2.7
Catalase	5	4.66 ± 0.07	93.8 ± 2.4	Catalase	5	6.64 ± 0.08	77.2 ± 3.3
AA	5	5.12 ± 0.05***	92.3 ± 2.3	AA	5	7.00 ± 0.06*	81.9 ± 1.2
AA+catalase	5	4.59 ± 0.11	91.5 ± 3.0	AA+catalase	5	6.48 ± 0.10	75.3 ± 2.2
Control	7	4.70 ± 0.10	79.6 ± 5.4	Control	6	6.43 ± 0.08	76.6 ± 3.8
Catalase	7	4.74 ± 0.04	85.8 ± 2.5	Catalase	6	6.39 ± 0.10	73.1 ± 3.6
BH_4	7	5.39 ± 0.15***	85.1 ± 2.1	BH_4	6	6.91 ± 0.04**	79.7 ± 3.1
BH_4+catalase	7	4.76 ± 0.05	83.9 ± 2.5	BH_4+catalase	6	6.42 ± 0.08	71.2 ± 3.9
Control	7	4.64 ± 0.04	84.3 ± 3.1	Control	8	6.50 ± 0.06	74.9 ± 4.4
H_2O_2 (10 μM)	7	4.80 ± 0.04	90.3 ± 1.6	H_2O_2 (10 μM)	7	6.55 ± 0.08	72.7 ± 5.2
H_2O_2 (30 μM)	7	4.96 ± 0.10	91.6 ± 2.5	H_2O_2 (30 μM)	6	6.87 ± 0.20	76.8 ± 5.5
H_2O_2 (100 μM)	7	5.36 ± 0.17***	86.0 ± 2.5	H_2O_2 (100 μM)	9	7.25 ± 0.15***	74.7 ± 4.1
Control	4	4.80 ± 0.03	92.6 ± 1.4	Control	7	6.46 ± 0.09	69.0 ± 3.0
SOD (1200 U)	4	4.71 ± 0.04	84.1 ± 2.0	SRPETK	7	–	26.5 ± 7.3***
AA	4	5.53 ± 0.19**	87.5 ± 3.6	srptek	5	6.29 ± 0.24	68.9 ± 5.6
AA+SOD	4	5.53 ± 0.15**	90.2 ± 2.0				
Control	4	4.67 ± 0.16	93.4 ± 3.2	Control	7	6.63 ± 0.10	77.9 ± 5.4
SOD (1200 U)	4	4.66 ± 0.06	80.0 ± 7.9	SRPTEK	7	–	34.2 ± 8.9**
BH_4	4	6.01 ± 0.20***	88.1 ± 7.1	SRPTEK+H_2O_2	5	–	39.0 ± 11.1*
BH_4+SOD	4	5.39 ± 0.21*	85.5 ± 9.1	H_2O_2 (100 μM)	5	7.16 ± 0.1**	74.6 ± 7.1
Control	6	4.92 ± 0.11	78.0 ± 7.6				
^{43}Gap26 (100 μM)	6	-	30.6 ± 6.9**				
^{43}Gap26+H_2O_2	4	5.03 ± 0.17	73.5 ± 10.4				
H_2O_2 (100 μM)	4	6.31 ± 0.15***	72.8 ± 7.9				
Control	6	4.73 ± 0.05	83.1 ± 3.8				
YDKSFPISHVR (300 μM)	4	-	35.2 ± 14.1***				
YDKSFPISHVR+H_2O_2	4	4.59 ± 0.01	80.2 ± 3.3				
H_2O_2 (100 μM)	6	5.30 ± 0.11***	84.3 ± 4.2				

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 ± SEM.

*$P < 0.05$ compared with control.

**$P < 0.01$ compared with control.

***$P < 0.001$ compared with control.

dimers as evidenced by the presence of the triply protonated species $[(VCYDKSFPISHVR)_2 - 2H]+3H^+$ (calculated *m/e* for $C_{140}H_{212}N_{38}O_{38}S_2+3H^+$ $\{M+3H^+\}$ 1033.52; found 1033.46), the triple sodium adduct $[(VCYDKSFPISHVR)_2 - 2H]+3Na^+$ (calculated *m/e* for $C_{140}H_{212}N_{38}O_{38}S_2+3Na^+$ $\{M+3Na^+\}$ 1055.50; found 1055.80), and mixed proton/sodium adducts at intermediate mass values.

Pre-incubation with a cysteine-free truncated form of ^{43}Gap26 (YDKSFPISHVR at 300 μM) closely mimicked the effects of ^{43}Gap26 at 100 μM (*Figure 5A*). Pre-incubation with the short peptide SRPTEK at 300 μM markedly attenuated relaxations to ACh, reducing R_{max} to less than 30%, whereas the corresponding D-isomer srptek was inactive; SRPTEK similarly abolished the potentiated ACh-evoked relaxations observed in the presence of 100 μM H_2O_2 (*Figure 5B*). Mass spectrometry confirmed the lack of dimerization of the YDKSFPISHVR or SRPTEK oligopeptides (data not shown).

Direct chemical interaction between ^{43}Gap26 and H_2O_2 was confirmed by the demonstration that there was ~50% consumption of 100 μM H_2O_2 30 min after addition to air-exposed Holman's buffer containing 100 μM ^{43}Gap26, whereas consumption of H_2O_2 was not evident in buffer containing 100 μM SRPTEK (*Figure 5C*).

4. Discussion

The major finding of the present study is that AA and BH_4 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 H_2O_2 generated by the oxidation of AA and BH_4 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 BH_4 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 $[H_2O_2]$ after 60 min incubation revealed conversion rates of ~4% and ~30% for AA and BH_4, respectively, consistent with evidence that BH_4 is more readily oxidized by molecular oxygen than AA in physiological buffer.[13] The potentiating effects of AA and BH_4, but not control responses, were inhibited by catalase and could be mimicked by exogenous H_2O_2, which itself enhanced EDHF-type relaxations at a threshold concentration of 10–30 μM. The observation that H_2O_2 amplifies relaxant responses to ACh, which mobilizes Ca^{2+}

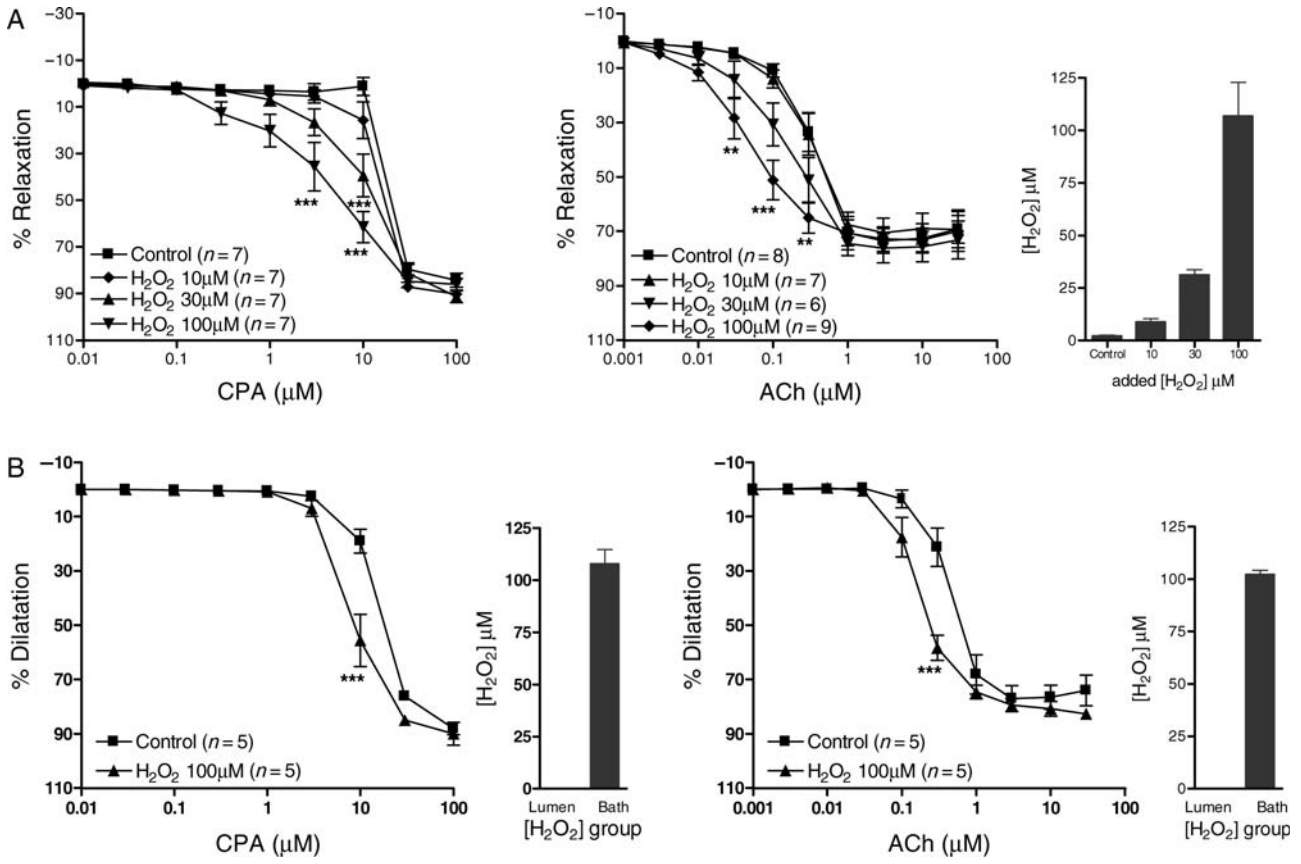

Figure 2 Concentration-dependent potentiation of EDHF-type relaxations/dilatations to CPA and ACh in the presence of exogenous H_2O_2. (*A*) In tension myograph experiments, buffer $[H_2O_2]$ was unchanged at the conclusion of the experiments (bar graph, illustrated for ACh). (*B*) In perfusion experiments, intraluminal and extraluminal $[H_2O_2]$ 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.

Table 2 Effects of adventitially applied H_2O_2 on EDHF-type dilatations evoked by CPA and ACh

	n	pEC_{50}	D_{max} %
CPA			
Control	5	4.79 ± 0.04	88.0 ± 2.3
H_2O_2 (100 μM)	5	5.09 ± 0.05*	91.4 ± 2.9
Ach			
Control	5	6.30 ± 0.08	78.2 ± 4.7
H_2O_2 (100 μM)	5	6.75 ± 0.09*	82.6 ± 2.0

Potency (negative log EC_{50}) and maximal dilatation (D_{max}) expressed as a percentage of the dilator response to SNP are given as mean ± SEM.
*$P < 0.01$ compared with control.

from the ER store via the formation of $InsP_3$, is consistent with evidence that H_2O_2 enhances Ca^{2+} release by sensitizing the $InsP_3$ receptor and extends previous findings with CPA which elevates endothelial $[Ca^{2+}]_i$ by blocking ER Ca^{2+} uptake.[4–6] EDHF-type relaxations evoked by CPA and ACh can also be potentiated by the sulphydryl reagent thimerosal, which amplifies Ca^{2+} release from the ER by oxidizing critical thiol groups present in the $InsP_3$ receptor, thus raising the possibility of a molecular target common to H_2O_2 and thimerosal.[4,19–21] It should be appreciated that the concentrations of buffer H_2O_2 generated from AA and BH_4 in the present study are likely to correspond to intracellular levels within the suggested physiological range (1–10 μM), since glutathione peroxidase, catalase, and other mechanisms are thought to limit cytosolic $[H_2O_2]$ to 1–15% of that applied extracellularly.[22]

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 H_2O_2 and the ascorbyl radical (a marker of AA oxidation) at concentrations that correlate directly with plasma [AA], with interstitial fluid $[H_2O_2]$ rising to 20–150 μM but H_2O_2 remaining undetectable in the intravascular compartment.[17,18] To mimic this *in vivo* situation, 100 μM H_2O_2 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_2O_2. This reduction in potency is likely to reflect a concentration gradient of H_2O_2 across the wall of the segments because their intraluminal perfusate did not contain H_2O_2 and the RIA possesses ~10 layers of smooth muscle cells and is therefore relatively thick-walled.[3] It thus seems likely that putative H_2O_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_2O_2 would be smaller than in conduit arteries and (ii) gap junction-dependent mechanisms can dominate over NO-mediated vasodilatation in resistance arteries,[23] consistent with

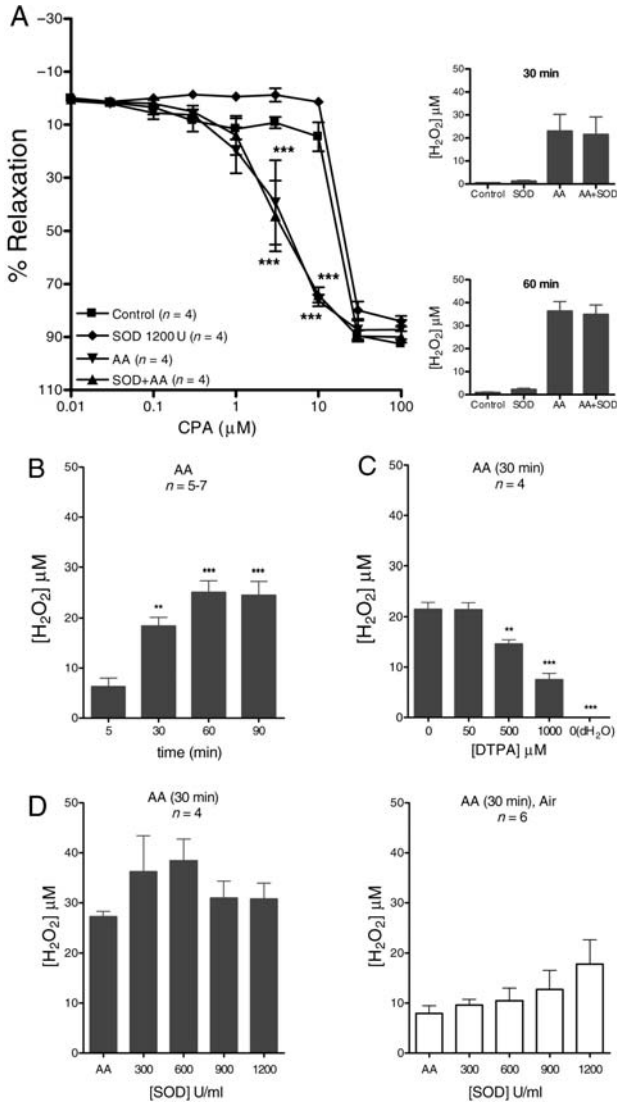

Figure 3 Effects of SOD (1200 U/mL) on concentration–response curves for CPA and generation of H_2O_2 from 1 mM AA. (*A*) SOD did not affect the potentiating effects of AA on relaxation or elevations in myograph $[H_2O_2]$ at 30 and 60 min (insets). (*B*) Time course of the generation of H_2O_2 in oxygenated buffer. (*C*) DTPA reduced the formation of H_2O_2 in well-oxygenated buffer and no generation of H_2O_2 was evident in comparative experiments performed in deionized water (dH_2O). (*D*) SOD did not affect the generation of H_2O_2 either in well-oxygenated buffer or air-exposed buffer (open columns). ** and *** denote $P < 0.01$ and 0.001 compared with the appropriate control.

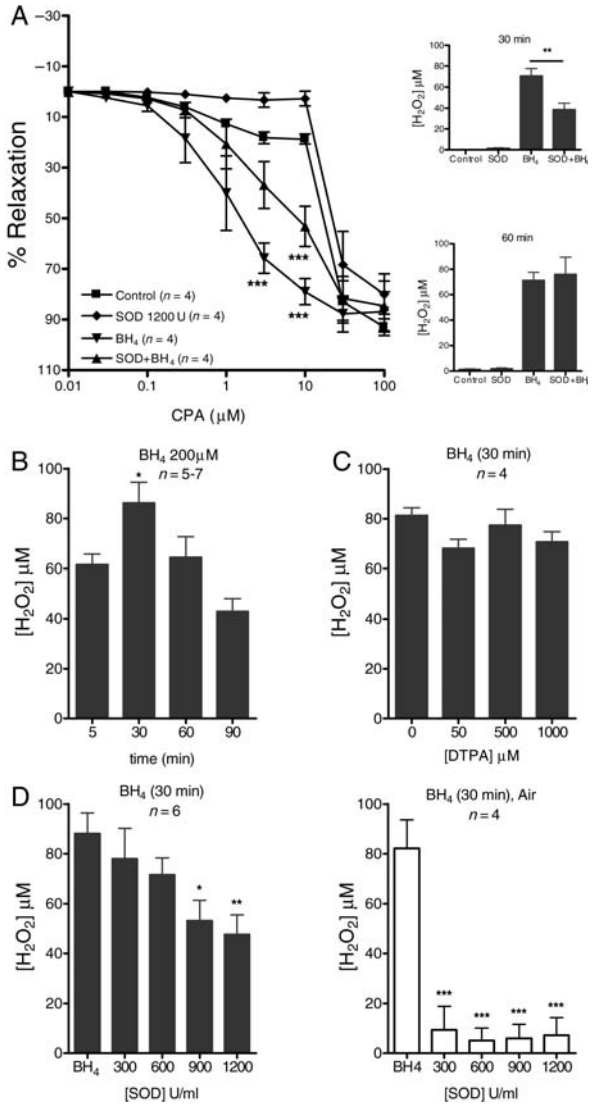

Figure 4 Effects of SOD (1200 U/mL) on concentration–response curves for CPA and generation of H_2O_2 from 200 μM BH_4. (*A*) SOD attenuated the potentiating effects of BH_4 on relaxation and the associated increase in myograph $[H_2O_2]$ after 30 but not 60 min (insets). (*B*) Time course of H_2O_2 generation in oxygenated buffer. (*C*) DTPA did not affect the formation of H_2O_2 in oxygenated buffer. (*D*) Inhibitory effects of SOD were much less evident in well-oxygenated buffer than air-exposed buffer (open columns). *, ** and *** denote $P < 0.05$, 0.01, and 0.001 compared with control.

evidence that the number of myoendothelial connections per endothelial cell is highest in small arteries.[24]

4.1 Mechanisms involved in the generation of H_2O_2

To gain insights into the pathways whereby AA and BH_4 generate H_2O_2 in physiological buffer, experiments were conducted to evaluate the contribution of the superoxide anion ($\cdot O_2^-$), which has been implicated in the autoxidation of BH_4, but whose role in the oxidation of AA is controversial.[14,15,25,26] In myograph studies with rings, SOD attenuated the potentiating effects of BH_4 on CPA-evoked relaxations and associated formation of H_2O_2, whereas the generation of H_2O_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 $\cdot O_2^-$, 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.[15] Loss of this chain reaction is likely to explain the ability of SOD to attenuate H_2O_2 formation and relaxation in the present study, since SOD causes a marked reduction the rate of BH_4 autoxidation.[15] In 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 catalyze its oxidation.[25,27] We were thus unable to detect formation of H_2O_2 from AA in deionized water and found that the generation of H_2O_2 from AA in buffer was substantially reduced in the presence of DTPA, a polydentate scavenger of Fe^{3+} and Cu^{2+} ions,[25,27] whereas formation of H_2O_2 from BH_4 was unaffected. Conversely, 1 μM Fe^{3+} or

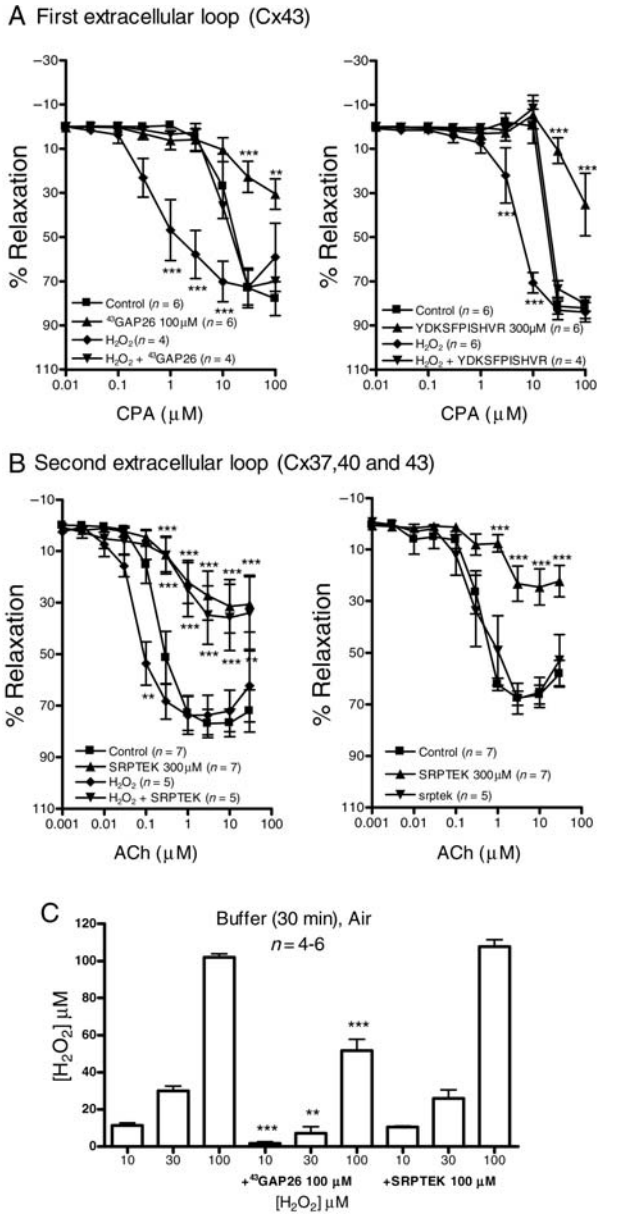

Figure 5 Effects of connexin-mimetic peptides on control EDHF-type relaxations and their potentiation by exogenous H_2O_2. (*A*) Attenuation of CPA-evoked relaxations by ^{43}Gap 26 and its truncated form YDKSFPISHVR were both reversed by 100 μM H_2O_2. (*B*) SRPTEK almost abolished relaxations to ACh, both in the presence and absence of 100 μM H_2O_2, whereas srptek was inactive. (*C*) ^{43}Gap 26 but not SRPTEK reduced applied [H_2O_2] in oxygenated buffer. ** and *** denote $P < 0.01$ and 0.001 compared with control.

Cu^{2+} catalyzed the generation of H_2O_2 from 1 mM AA, with Cu^{2+} being by far the more active cation (see Supplementary material online). Although metal-catalyzed oxidation of AA has been suggested to involve the interaction of $\cdot O_2^-$ with ascorbate ions and the ascorbyl radical, the effects of SOD on the rate of AA oxidation were modest (less than 2-fold decrease),[14] and it has also been proposed that metal-catalyzed oxidation (e.g. of 6-hydroxydopamine and 1,2,4-benzenetriol) can proceed via a 2-electron mechanism in which H_2O_2 is generated directly from molecular oxygen (rather than $\cdot O_2^-$) and is therefore insensitive to SOD.[28,29] The impact of oxygenation on H_2O_2 accumulation was examined in experiments conducted with buffer exposed to air, because isolated arterial preparations are conventionally maintained in buffer gassed with O_2 at levels well above the physiological range. Such studies demonstrated a ~50% reduction in H_2O_2 generated from AA after 30 min compared with oxygenated buffer, whereas H_2O_2 formation from BH_4 was unaltered. Major differences in the role of intermediary $\cdot O_2^-$ in the oxidation of AA and BH_4 were nevertheless highlighted by observations that the generation of H_2O_2 from BH_4 in buffer exposed to air was suppressed by SOD, whereas its formation from AA under the same conditions was unaffected.

4.2 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}Gap26; 100 μM), which possesses homology with the first 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 μM ^{43}Gap 26 and the potentiating effects of 100 μM H_2O_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}Gap26 by H_2O_2, because dimerization via the formation of an intermolecular cystine bridge was confirmed by mass spectrometry and there was 50% consumption of applied H_2O_2 in the presence of equimolar peptide concentrations, consistent with the reaction $2R\text{-}SH + H_2O_2 \rightarrow R\text{-}S\text{-}S\text{-}R + 2H_2O$. However, we also found that a truncated peptide YDKSFPISHVR mimicked the effects of ^{43}Gap26 when applied at 300 μM, suggesting specific gap junction blockade because oxidation/dimerization of this peptide was prevented by deletion of the N-terminal valine and cysteine residues. Furthermore, the peptide SRPTEK, which corresponds to a highly conserved sequence in the second 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 H_2O_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 H_2O_2, (ii) assay revealed no consumption of buffer H_2O_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 BH_4 opposes the ability of equimolar concentrations of ^{43}Gap26 or the related peptide ^{37,40}Gap26 (VCYDQAFPISHIR, which targets Cx37 and Cx40) to inhibit CPA-evoked EDHF-type relaxations and smooth muscle hyperpolarization in the RIA.[7] At this concentration, BH_4 generates myograph H_2O_2 concentrations of ~40 μM (data not shown) and could therefore contribute to the normalization of EDHF-type responses both via the potentiation of ER Ca^{2+} mobilization and

peptide oxidation. However, at concentrations normally found in plasma (50–100 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 ^{43}Gap26 and ^{37,40}Gap26.[8] It should also be noted that in non-vascular cells H_2O_2 has been variously shown to enhance or inhibit intercellular coupling via gap junctions constructed from Cx43, probably by alternating the phosphorylation/oxidation status of residues present in the intracellular cytoplasmic tail of this connexin subtype.[31–35] Indeed, the hyperphosphorylation of Cx43 that follows administration of H_2O_2 or phorbol esters (which generate $\cdot O_2^-$) 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 antioxidant 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 BH_4 are both capable of reducing H_2O_2 to H_2O as well as generating H_2O_2.[14,15,38,39]

4.3 Conclusions

Clinical studies have suggested that AA and BH_4 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 BH_4 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_2O_2] to levels that potentiated NO-independent dilatation when applied to the adventitia of RIA segments. Corresponding measurements of interstitial [H_2O_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_2O_2 on endothelial function *in vivo*. Because H_2O_2 production from $\cdot O_2^-$ 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.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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References

1. Griffith TM. Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis? *Br J Pharmacol* 2004; **141**:881–903.
2. Sandow SL. Factors, fiction and endothelium-derived hyperpolarizing factor. *Clin Exp Pharmacol Physiol* 2004;**31**:563–570.
3. 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* 2005;**144**:108–114.
4. Edwards DH, Li Y, Griffith TM. Hydrogen peroxide potentiates the EDHF phenomenon by promoting endothelial Ca^{2+} mobilization. *Arterioscler Thromb Vasc Biol* 2008;**28**:1774–1781.
5. Hu Q, Zheng G, Zweier JL, Deshpande S, Irani K, Ziegelstein RC. NADPH oxidase activation increases the sensitivity of intracellular Ca^{2+} stores to inositol 1,4,5-trisphosphate in human endothelial cells. *J Biol Chem* 2000;**275**:15749–15757.
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. Griffith TM, Chaytor AT, Bakker LM, Edwards DH. 5-Methyltetrahydrofolate and tetrahydrobiopterin can modulate electrotonically mediated endothelium-dependent vascular relaxation. *Proc Natl Acad Sci USA* 2005;**102**:7008–7013.
8. 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.
9. May JM. How does ascorbic acid prevent endothelial dysfunction? *Free Radic Biol Med* 2000;**28**:1421–1429.
10. Sherman DL, Keaney JF Jr, Biegelsen ES, Duffy SJ, Coffman JD, Vita JA. Pharmacological concentrations of ascorbic acid are required for the beneficial effect on endothelial vasomotor function in hypertension. *Hypertension* 2000;**35**:936–941.
11. Pleiner J, Schaller G, Mittermayer F, Marsik C, MacAllister RJ, Kapiotis S *et al*. Intra-arterial vitamin C prevents endothelial dysfunction caused by ischemia–reperfusion. *Atherosclerosis* 2008;**197**:383–391.
12. Katusic ZS, d'Uscio LV, Nath KA. Vascular protection by tetrahydrobiopterin: progress and therapeutic prospects. *Trends Pharmacol Sci* 2009; **30**:48–54.
13. Tóth M, Kukor Z, Valent S. Chemical stabilization of tetrahydrobiopterin by L-ascorbic acid: contribution to placental endothelial nitric oxide synthase activity. *Mol Hum Reprod* 2002;**8**:271–280.
14. Scarpa M, Stevanato R, Viglino P, Rigo A. Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. Effect of superoxide dismutase. *J Biol Chem* 1983;**258**:6695–6697.
15. Kirsch M, Korth HG, Stenert V, Sustmann R, de Groot H. The autoxidation of tetrahydrobiopterin revisited. Proof of superoxide formation from reaction of tetrahydrobiopterin with molecular oxygen. *J Biol Chem* 2003;**278**:24481–24490.
16. 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 USA* 2003;**100**:15212–15217.
17. Chen Q, Espey MG, Sun AY, Lee JH, Krishna MC, Shacter E *et al*. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc Natl Acad Sci USA* 2007;**104**:8749–8754.
18. Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, Krishna MC *et al*. Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. *Proc Natl Acad Sci USA* 2008;**105**: 11105–11109.
19. 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.
20. Bultynck G, Szlufcik K, Kasri NN, Assefa Z, Callewaert G, Missiaen L *et al*. 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.
21. 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.
22. Schröder E, Eaton P. Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations. *Curr Opin Pharmacol* 2008;**8**:153–159.
23. Berman RS, Martin PE, Evans WH, Griffith TM. Relative contributions of NO and gap junctional communication to endothelium-dependent relaxations of rabbit resistance arteries vary with vessel size. *Microvasc Res* 2002;**63**:115–128.
24. Sandow SL, Hill CE. Incidence of myoendothelial gap junctions in the proximal and distal mesenteric arteries of the rat is suggestive of a role in endothelium-derived hyperpolarizing factor-mediated responses. *Circ Res* 2000;**86**:341–346.
25. Buettner GR. In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *J Biochem Biophys Methods* 1988;**16**:27–40.
26. Wee LM, Long LH, Whiteman M, Halliwell B. Factors affecting the ascorbate- and phenolic-dependent generation of hydrogen peroxide in Dulbecco's Modified Eagles Medium. *Free Radic Res* 2003;**37**:1123–1130.
27. Buettner GR. Ascorbate autoxidation in the presence of iron and copper chelates. *Free Radic Res Commun* 1986;**1**:349–353.
28. Zhang L, Bandy B, Davison AJ. Effects of metals, ligands and antioxidants on the reaction of oxygen with 1,2,4-benzenetriol. *Free Radic Biol Med* 1996;**20**:495–505.
29. Bandy B, Davison AJ. Interactions between metals, ligands, and oxygen in the autoxidation of 6-hydroxydopamine: mechanisms by which metal chelation enhances inhibition by superoxide dismutase. *Arch Biochem Biophys* 1987;**259**:305–315.
30. Chaytor AT, Evans WH, Griffith TM. Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J Physiol* 1998;**508**:561–573.
31. Rouach N, Calvo CF, Duquennoy H, Glowinski J, Giaume C. Hydrogen peroxide increases gap junctional communication and induces astrocyte toxicity: regulation by brain macrophages. *Glia* 2004;**45**:28–38.
32. Upham BL, Kang KS, Cho HY, Trosko JE. Hydrogen peroxide inhibits gap junctional intercellular communication in glutathione sufficient but not glutathione deficient cells. *Carcinogenesis* 1997;**18**:37–42.
33. Saez JC, Retamal MA, Basilio D, Bukauskas FF, Bennett MV. Connexin-based gap junction hemichannels: gating mechanisms. *Biochim Biophys Acta* 2005;**1711**:215–224.
34. Cho JH, Cho SD, Hu H, Kim SH, Lee SK, Lee YS *et al*. The roles of ERK1/2 and p38 MAP kinases in the preventive mechanisms of mushroom Phellinus linteus against the inhibition of gap junctional intercellular communication by hydrogen peroxide. *Carcinogenesis* 2002;**23**:1163–1169.
35. Hu J, Cotgreave IA. Glutathione depletion potentiates 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced inhibition of gap junctional intercellular communication in WB-F344 rat liver epithelial cells: relationship to intracellular oxidative stress. *Chem Biol Interact* 1995;**95**:291–307.
36. Hu J, Speisky H, Cotgreave IA. The inhibitory effects of boldine, glaucine, and probucol on TPA-induced down regulation of gap junction function. Relationships to intracellular peroxides, protein kinase C translocation, and connexin 43 phosphorylation. *Biochem Pharmacol* 1995;**50**:1635–1643.
37. Lee KW, Lee HJ, Kang KS, Lee CY. Preventive effects of vitamin C on carcinogenesis. *Lancet* 2002;**359**:172.
38. Deutsch JC. Ascorbic acid oxidation by hydrogen peroxide. *Anal Biochem* 1998;**255**:1–7.
39. Moore J, Wood J, Schallreuter K. H_2O_2-mediated oxidation of tetrahydrobiopterin: Fourier transform Raman investigations provide mechanistic implications for the enzymatic utilization and recycling of this essential cofactor. *J Raman Spect* 2002;**33**:610–617.
40. Mayahi L, Heales S, Owen D, Casas JP, Harris J, MacAllister RJ *et al*. (6R)-5,6,7,8-tetrahydro-L-biopterin and its stereoisomer prevent ischemia reperfusion injury in human forearm. *Arterioscler Thromb Vasc Biol* 2007;**27**:1334–1339.