Hydrogen Peroxide Promotes Endothelial Dysfunction by Stimulating Multiple Sources of Superoxide Anion Radical Production and Decreasing Nitric Oxide Bioavailability

Paul K. Witting,^{1,2} Benjamin S. Rayner,^{1,2} Beng-Jing Wu,^{1,3} Natasha A. Ellis² and Roland Stocker^{1,3}

¹Centre for Vascular Research, School of Medical Sciences, University of New South Wales and ²Vascular Biology Group, Concord Repatriation General Hospital, Sydney, ³Present address: Centre for Vascular Research, Faculty of Medicine, Bosch Institute, University of Sydney

Key Words

Vascular function • Inflammation • Nitric oxide • Superoxide anion radical • Oxidative stress

Abstract

Hydrogen peroxide (H_2O_2) is an oxidant implicated in cell signalling and various pathologies, yet relatively little is known about its impact on endothelial cell function. Herein we studied the functional and biochemical changes in aortic vessels and cultured porcine aortic endothelial cells (PAEC) exposed to H_2O_2 . Exposure of aortic rings to 25 or 50 μM , but not 10 μM , H_2O_2 for 60 min prior to constriction significantly decreased subsequent relaxation in response to acetylcholine (ACh), but not the nitric oxide ($\cdot NO$) donor sodium nitroprusside. Treatment of PAEC with 50 μM H_2O_2 significantly decreased ACh-induced accumulation of $\cdot NO$, as measured with a $\cdot NO$ -selective electrode, yet such treatment increased nitric oxide synthase activity ~3-fold, as assessed by conversion of L-arginine to L-citrulline. Decreased

$\cdot NO$ bioavailability was reflected in decreased cellular cGMP content, associated with increased superoxide anion radical ($O_2^{\cdot -}$), and overcome by addition of polyethylene glycol superoxide dismutase. Increased cellular $O_2^{\cdot -}$ production was inhibited by allopurinol, diphenyliodonium and rotenone in an additive manner. The results show that exposure of endothelial cells to H_2O_2 decreases the bioavailability of agonist-induced $\cdot NO$ as a result of increased production of $O_2^{\cdot -}$ likely derived from xanthine oxidase, NADPH-oxidase and mitochondria. These processes could contribute to H_2O_2 -induced vascular dysfunction that may be relevant under conditions of oxidative stress such as inflammation.

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Introduction

An intact and functional endothelium is vital for the maintenance of vascular homeostasis. The endothelium provides a physical barrier [1] excluding circulating blood components from the sub-endothelial space, and

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Dr Paul K. Witting
The ANZAC Research Institute, Concord Repatriation General Hospital
Sydney NSW 2139 (Australia)
Tel. +61-2-9767-9103, Fax +61-2-9767-9101
E-Mail pwitting@anzac.edu.au

synthesizes and releases a range of vasoactive factors. For example, endothelium-derived nitric oxide, produced through the action of nitric oxide synthase (eNOS) on L-arginine, regulates vascular tone [2], the interaction of platelets [3] and leukocytes [4] with the endothelium surface, and restricts vascular smooth muscle cell (VSMC) proliferation [5]. Furthermore, it is now recognized that endothelial dysfunction predicts the occurrence of vascular diseases such as atherosclerosis [6], diabetes mellitus [7] and essential hypertension [8].

In addition to NO, endothelial cells also produce endothelium-derived hyperpolarizing factor, another vasodilator of presently unknown identity. Candidates for endothelium-derived hyperpolarizing factor include hydrogen peroxide (H_2O_2) [9], arachidonate metabolites [10] and K^+ ions [11]. More recently, reactive oxygen species derived from VSMC mitochondria have been implicated in modulating vascular function [12]. Of these, H_2O_2 is increasingly viewed as important because its vessel dilating and hyperpolarizing properties are largely insensitive to inhibitors of eNOS and cyclooxygenase [13]. Notably, H_2O_2 produced via dismutation of superoxide radical anion ($\text{O}_2^{\cdot-}$) can enhance production of NO by cultured endothelial cells [14, 15], modulate Ca^{2+} [16] and K^+ ion [17] channel function, and inhibit VSMC proliferation [18].

A source of H_2O_2 in vascular cells is $\text{O}_2^{\cdot-}$ that itself can be generated by various processes including uncoupled eNOS [19], xanthine oxidase [20], mitochondria [21], cytochrome P-450 [22] and NAD(P)H oxidases [23, 24]. Interestingly, $\text{O}_2^{\cdot-}$ effectively impairs NO bioactivity via near diffusion-controlled bimolecular reaction of $\text{O}_2^{\cdot-}$ with NO [25]. This yields peroxynitrite that can inactivate eNOS directly [26] or indirectly [27], and this can result in further production of $\text{O}_2^{\cdot-}$ and hence H_2O_2 .

In addition to its potential direct function as a vasodilator, H_2O_2 is increasingly recognized as a cellular signalling molecule [28]. However, under certain pathophysiologic conditions, such as acute inflammation, activated phagocytes generate significantly higher concentrations of H_2O_2 locally. Indeed, cellular studies examining the potential pathophysiologic effects of H_2O_2 commonly employ the oxidant in the concentration range of 20–250 μM [14, 15].

Despite the potential for H_2O_2 to act as an endothelium-derived hyperpolarizing factor and the increasing appreciation that oxidative processes and inflammation contribute to endothelial dysfunction and cardiovascular disease [29], relatively little is known about the effect of pathophysiologic concentrations of H_2O_2 on

endothelial function. Three recent papers have focussed on the determinants of H_2O_2 -induced endothelial dysfunction in vitro. These studies suggest involvement of a labile iron-pool in enhancing oxidative stress [30]; a decrease in the eNOS co-factor tetrahydrobiopterin [31]; uncoupling of eNOS [32] and activation of NADPH oxidase [31, 32]. A common feature of the pathways proposed in these studies is increased cellular production of $\text{O}_2^{\cdot-}$, although the source(s) of this $\text{O}_2^{\cdot-}$ remains unclear. We therefore examined endothelium-dependent relaxation of isolated aortic vessel rings and NO production by endothelial cells exposed to H_2O_2 and explored likely sources of cellular $\text{O}_2^{\cdot-}$.

Materials and Methods

Vascular reactivity

Aorta were harvested from New Zealand White rabbits (2.5–3 kg), perfused, placed in modified Krebs-Henseleit solution (in mM: 11 D-glucose, 1.2 MgSO_4 , 12 KH_2PO_4 , 4.7 KCl, 120 NaCl, 25 NaHCO_3 , and 2.5 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and cut into 5 mm ring segments [33]. Where required, rings were incubated with 500 U/mL polyethylene glycol superoxide dismutase (PEG-SOD) or 50 mM phosphate buffered saline (vehicle control) at 4°C for 12h. This increased vascular SOD activity (see below) but did not affect constriction in response to phenylephrine (not shown). Rings were mounted in a myobath system (World Precision Instruments, Inc., Sarasota, FL) containing 20 mL of modified Krebs-Henseleit solution aerated at 37°C with 5% CO_2 and contracted by titrating with phenylephrine. A dose that caused half maximal contraction was selected for all further studies. A cumulative concentration-response curve to acetylcholine (ACh) or S-nitroso-penicillamine (SNP) (10^{-9} – 10^{-5} mol/L) was constructed, with relaxation expressed as percentage of initial contraction. Where indicated, mounted rings were pre-treated with H_2O_2 (10, 25 or 50 μM) or 1H-[1, 2, 4]oxadiazole[4, 3-a]quinoxalin-1-one (100 μM) for 60min followed by washing, pre-constriction and relaxation. After each concentration-response curve, the vessels were washed at least three times with, and allowed to equilibrate for 60min in modified Krebs-Henseleit buffer supplemented with 100 μM L-arginine before further exposure to ACh. Segments were used in three consecutive sequences of pre-constriction and relaxation then frozen for use in assessing SOD activity. Animal work was performed with local ethics approval.

Cell culture

Porcine aortic endothelial cells (PAEC, Cell Applications Inc, CA) were cultured in M199 media (Sigma, Australia) supplemented with FBS (10% v/v), 50 $\mu\text{g/mL}$ heparin sulfate, 2 mM L-glutamine, 100 units/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C in an atmosphere containing 5% CO_2 as described previously [14]. Confluent PAEC, passage three-to-eight, were washed thoroughly, and overlaid with HEPES

buffered-physiologic salt solution (PSS) [14]. Cells were treated with vehicle (control) or H_2O_2 at the concentrations indicated in the legends to the Figures. In some experiments cells were pre-incubated with 200 μM N ω -nitro-L-arginine methyl ester (L-NAME) prior to the addition of H_2O_2 . Treated and control PAEC were further incubated at 37°C for 60min, washed with HEPES-buffered PSS supplemented with 100 μM L-arginine and exposed to 1 μM ACh. Cells were harvested, centrifuged and the cell pellet used for biochemical analyses.

Polymorphonuclear leukocytes (PMN) were obtained from fresh plasma using a Ficoll gradient (Sigma, Australia) combined with centrifugation as per the manufacturers' instruction. PMN were dispersed in phosphate buffer (50 mM, pH 7.4) and used within 1h of isolation. Isolated PMN were stimulated with phorbol ester (50 nM) and the generation of oxidants monitored by electron paramagnetic resonance (EPR) spectroscopy as detailed below.

'NO accumulation by PAEC

The accumulation of 'NO by cultured PAEC was monitored with a 'NO-selective electrode (ISO-NO MkII, World Precision Instruments Inc.) coupled to a DUO-18™ data recorder (v1.55). Cells were harvested, resuspended at $4-6 \times 10^6$ cells/mL in HEPES-buffered PSS containing 100 μM L-arginine. In some studies PAEC were pre-incubated with 500 U/mL PEG-SOD for 2h prior to use. The electrode was pre-equilibrated in the cell suspension at 20°C for 60min in the presence of vehicle (control), H_2O_2 (25 or 50 μM) or 200 μM L-NAME, before 1 μM ACh was added and the time-dependent increase in peak current response was monitored. Immediately after the current response reached a plateau, the area under the curve was estimated using the standard DUO-18™ software. The 'NO concentration was then determined using a standard curve generated from reaction of Cu(II) with serial dilutions of authentic S-nitroso-glutathione (standardized with $\epsilon_{336\text{ nm}} = 770\text{ M}^{-1}\text{cm}^{-1}$): the limit of 'NO detection was 9.8 nM [34]. Standard curves were generated after each series of analyses using identical buffer preparations as the electrode response varied from day-to-day and with different buffer preparations.

Assessment of $O_2^{\cdot -}$ production by PAEC and PMN

Production of $O_2^{\cdot -}$ by cells was assessed by monitoring oxidation of the cell-permeable spin label 3-isobutyl-1-methylxanthine-1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine.HCl (CMH) to the EPR-active CMH-nitroxide with EPR spectroscopy-CMH detects intra- and extra-cellular $O_2^{\cdot -}$ [35]. Briefly, confluent PAEC ($\sim 2-3 \times 10^6$ cells) were washed with HEPES-buffered PSS and then incubated at 37°C for 5min with the same buffer supplemented with L-arginine (100 μM) and CMH (250 μM), before exposure to 50 μM H_2O_2 for 60min and stimulation with 1 μM ACh or vehicle. Accumulation of CMH-nitroxide in the buffer over-laying the cells was monitored by EPR spectroscopy. Studies were also performed with isolated PMN (1×10^6 cells) treated with 50 nM phorbol-12-myristate-13-acetate (PMA) or vehicle (control) as controls. Samples were taken at 2min time intervals and CMH-nitroxide monitored in the absence or presence of PEG-SOD (500 units/mL).

In other studies, PAEC were grown to $\sim 95-100\%$ confluence, washed in HEPES buffered PSS and pre-incubated with the following either alone or in combination: 250 or 500 μM allopurinol (an inhibitor of xanthine oxidase); 10 μM diphenyliodonium (DPI, an inhibitor of NADPH-oxidase) or 20 μM rotenone (an inhibitor of mitochondrial complex I). After 15min of incubation at 37°C, cells were treated sequentially with 250 mM CMH and 50 μM H_2O_2 as described above. After 60min, cells were stimulated with 1 μM ACh and the supernatant collected and CMH-nitroxide monitored with EPR spectroscopy. The change in CMH-nitroxide was expressed as a percentage of that determined for cells treated with H_2O_2 alone. Inhibition determined in cells pre-treated with 500 μM allopurinol was not significantly different to that measured in the presence of 250 μM allopurinol (not shown). Subsequently studies with allopurinol were performed at the lower concentration.

EPR spectra were obtained as an average of 2 scans with modulation frequency 100 kHz and sweep time 84s, power 20 mW, modulation amplitude 0.1 mT. The sensitivity of the EPR assay for CMH-nitroxide was 5 nmol as determined by reaction of CMH with a serially diluted sample of authentic potassium superoxide (Sigma, Australia) or with the xanthine/xanthine oxidase system. Peak areas for the stable CMH-nitroxide radical were assessed by double integration of the nitroxide central peak with WINEPR software (Bruker, Germany), and standardized to cell protein. Data from the EPR studies were independently verified by determining PEG-SOD-inhibited reduction of ferricytochrome *c* [36] in separate experiments. Rates of ferricytochrome *c* reduction were normalized to total cell number to account for variation in cell density.

Western-blot analyses for endothelial nitric oxide synthase

To assess whether added H_2O_2 disrupted the eNOS dimer complex, PAEC were grown to near confluence and treated with 50 or 100 μM H_2O_2 or 400 μM reagent hypochlorous acid (positive control). The latter is known to completely dissociate the eNOS dimer to yield the corresponding monomer [38]. Cells were harvested after 15 (hypochlorous acid) or 60 (H_2O_2) min, lysed by three consecutive freeze thaw cycles, and eNOS precipitated and analyzed by SDS-PAGE with Western blotting as described [26].

Assessing eNOS gene regulation in PAEC treated with H_2O_2

A) Reverse Transcription. Cell RNA was extracted with a Total RNA Mini Kit (Sigma, Australia). Complementary DNA (cDNA) was constructed by reverse transcriptase-polymerase chain reaction (RT-PCR) using SuperScript II Reverse Transcriptase (Invitrogen, Australia) with a GeneAmp PCR Thermo-Cycler system (Applied Biosystems, Foster City, CA). Mixtures contained: 2 μL RNA, 1 μL oligodT (Invitrogen), and 9 μL of DEPC-treated MilliQ water, and were heated at 70°C for 5min to denature the RNA and then chilled to 4°C for 5min. The following was then added: 1 μL RnaseOut, 1 μL dNTPs, 4 μL of 5x reaction buffer, 1.75 μL DEPC-treated MilliQ water, and 0.25 μL Bioscript MMLV (Bioline, Australia) and the complete reaction mixtures heated to 42°C for 60min then 70°C for 10min

to terminate the RT-reaction. Transcribed cDNA were stored at -20°C for use in gene expression studies.

B) RT-PCR. Gene amplification was optimised for each primer set to avoid saturation of the PCR reaction. Mixtures consisted of Biomix Red (Bioline, Australia), sense and anti-sense primers (final concentration 400 nM), and the cDNA template from reverse transcription (above). Cycling of the mixture consisted of activation (94°C, 5min), followed by repeated denaturation (94°C, 30s), annealing (30s), elongation (72°C, 1min) and a final elongation step (72°C, 10min). Primers were synthesized by Proligo (Lismore, NSW, Australia). Sense and anti-sense primers were 5'-TAA CCA GCA CCT TTG GGA AC-3' and 5'-GCG CTG TCT GTG TTA CTG GA-3' for eNOS; 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TGC TG TA-3' for GAPDH. Products were resolved on 1 % w/v agarose with ethidium bromide and visualized under short-wavelength UV light. Densitometry was performed with Image J software (v1.30, rsb.info.nih.gov/ij/download.html) and expressed as a fold-change relative to GAPDH expression. Images were downloaded to MS Power-Point for manipulation.

Assessment of mitochondrial membrane potential

Mitochondrial depolarization was measured with a commercial kit (MitoProbe Invitrogen, Australia) as described previously [38]. Briefly, PAEC were grown in 6-well plates, washed in HEPES-buffered PSS and exposed to 50 μ M H₂O₂ or vehicle (control) for 60min, harvested and re-suspended at $\sim 1 \times 10^6$ cells/mL in 50 mM phosphate buffer, pH 7.4. Incubation of parallel samples with 200 μ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP) served as a positive control. All samples were then incubated further with 2 μ M 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide for 20min at 37°C and analysed with flow cytometry (FACScalibur, BD Biosciences) at excitation = 488 nm.

Superoxide dismutase activity

Vessel rings (~ 0.3 g wet weight) that were pre-treated with PEG-SOD or vehicle (control), and employed in vascular function studies, or cultured PAEC (10^6 cells/mL) exposed to 50 μ M H₂O₂ or vehicle alone (control), were suspended in phosphate buffer (50 mM, pH 7.4) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and homogenised as described previously [39]. Next, SOD activity was determined by inhibition of pyrogallol oxidation, as described [40].

Electronic spectroscopy

Absorbance spectroscopy was performed using a multi-well plate reader (Victor III, Perkin Elmer Australia). The content of cGMP in vessels or cultured PAEC $\pm$ H₂O₂ (in HEPES-buffered PSS with and without 200 μ M L-NAME) was determined after stimulation with ACh (1 μ M) in the presence of 100 μ M L-arginine and 200 μ M 3-isobutyl-1-methylxanthine, using a commercial kit (Cayman Chemical, Ann Arbor, MI). Where required residual hydrogen peroxide in cell supernatants was determined as described previously [41].

Fig. 1. Aortic rings treated with H₂O₂ show inhibited endothelium-dependent but not endothelium-independent relaxation. Rabbit aortic rings were suspended in a myobath bio-assay system and pre-incubated with H₂O₂ at 10 (diamonds), 25 (filled triangles) or 50 μ M (circles); 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (positive control, open triangles) or vehicle alone (control, hatched squares) for 60min. Treated rings were washed thoroughly and treated further with phenylephrine to yield half-maximal constriction then dilated by addition of increasing dose of the endothelium-dependent (ACh) (A) or endothelium-independent (S-nitrosopenicillamine; SNP) (B) relaxant. Data is presented as percent of initial constriction (mean $\pm$ SEM, n = 5 except for vessels exposed to 25 μ M H₂O₂ where n = 4 vessels). *Significantly different (P < 0.05) from that obtained with control vessels except for vessels pre-treated with 10 μ M H₂O₂ which was not different from the control.

Chemicals

ACh, CMH, PMA, L-NAME, PEG-SOD, HEPES buffer, trypsin (type III), L-glutamine, L-arginine, diethylenetriaminepentaacetic acid, penicillin, streptomycin, FBS, SNP, allopurinol, DPI, rotenone, xanthine, xanthine oxidase and 30% w/v reagent H₂O₂ were from Sigma (Australia). 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one, an inhibitor of soluble guanylyl cyclase, was from Alexis Biochemicals (San Diego,

CA). S-nitroso-glutathione was from Cayman Chemicals (Ann Arbor, MA). Endothelial cell growth factor was from Starrate Inc (Sydney, Australia). Chemicals were of the highest possible grade. Buffers were prepared with Millipore Water, treated with Chelex100® and stored with 100 μ M DTPA at 4°C prior to use.

Statistical analysis

Vessel function data are expressed as mean $\pm$ SEM, and statistical analyses were performed using the Prism statistical program (GraphPad, San Diego, CA). Concentration-response curves were compared by two-way ANOVA for repeated measures. Other data are shown as mean $\pm$ SD and differences between paired data sets were determined with either the Mann-Whitney or Neuman-Keul's multiple comparison tests. Statistical significance was accepted at the 95% confidence interval ($P < 0.05$).

Results

H₂O₂ decreases endothelium-dependent relaxation of aortic vessels

The effect of H₂O₂ on agonist-induced vessel relaxation remains unclear with some [42, 43] though not all studies [44] reporting a diminished response to ACh. Pre-treatment of rabbit aortic rings with 10 μ M H₂O₂ had no significant effect on subsequent vessel relaxation elicited by ACh (Fig. 1A). Pre-treatment of the vessels with 25 or 50 μ M H₂O₂ significantly decreased endothelium-dependent relaxation to ACh, as did pre-treatment with 100 μ M 1H-[1, 2, 4]oxadiazole[4, 3-a]quinoxalin-1-one, an inhibitor of soluble guanylyl cyclase used as positive control (Fig. 1A). In contrast to agonist-induced vessel relaxation, 10, 25 or 50 μ M H₂O₂ had no effect on endothelium-independent relaxation induced by the 'NO-donor SNP (Fig. 1B). Together, these data shown that H₂O₂ at = 25 μ M promotes endothelial dysfunction.

Effect of H₂O₂ on 'NO bioactivity in cultured PAEC

Endothelial cells contain soluble guanylyl cyclase [45] and therefore can be used as a 'within cell' index of 'NO bioactivity that involves all facets of 'NO metabolism including 'NO production and inactivation, as well as soluble guanylyl cyclase stimulation [46]. Stimulation of PAEC with ACh increased the cellular content of cGMP (Fig. 2A). ACh-induced accumulation of cGMP was inhibited substantially by blocking eNOS or soluble guanylyl cyclase with L-NAME or 1H-[1, 2, 4]oxadiazole[4, 3-a]quinoxalin-1-one, respectively, or by pre-treating PAEC with 50 μ M H₂O₂ for 60min (Fig. 2A). In contrast, pre-treatment of the cells with 50 μ M H₂O₂ did not alter

Fig. 2. Accumulation of cGMP by PAEC. Confluent PAEC were pre-treated for 60 min in HEPES-buffered PSS containing vehicle (control) or L-NAME (200 μ M), 1H-[1, 2, 4]oxadiazole[4, 3-a]quinoxalin-1-one (100 μ M), H₂O₂ (50 μ M) or L-NAME (200 μ M) together with H₂O₂ (50 μ M) prior to exchanging to HEPES-buffered PSS containing 100 μ M L-arginine. Next, cells were stimulated with (A) ACh (1 μ M) or (B) 50 mM phosphate buffer saline pH 7.4 (vehicle control). Cellular cGMP was then determined as described in "Methods". Data represent mean $\pm$ SD of 5 independent analyses each performed in duplicate and using different PAEC preparations. Significantly different to the corresponding (*) control or (**) H₂O₂-treated cells ($P < 0.05$). Note the difference in the Y-axes scales for Panels A and B.

baseline cellular content of cGMP that was decreased by L-NAME (Fig. 2B). These results suggest that consistent with previous reports [13, 42], H₂O₂ does not decrease eNOS activity in PAEC under conditions where ACh-induced increase in cellular cGMP is impaired.

Under the conditions employed, a bolus of H₂O₂ added to cultured PAEC (1 $\times$ 10⁶ PAEC/well) at 25 or 50 μ M final concentration was consumed rapidly: after

Fig. 3. Accumulation of $\cdot\text{NO}$ by PAEC. Confluent PAEC ($4\text{--}6 \times 10^6$ cells/mL) were pre-treated for 60 min in HEPES-buffered PSS (A) without (control) or with L-NAME ($200 \mu\text{M}$) (B) or H_2O_2 ($50 \mu\text{M}$) (C), washed thoroughly with HEPES-buffered PSS containing $100 \mu\text{M}$ L-arginine and stimulated with $1 \mu\text{M}$ ACh (arrow). The time-dependent change in peak current generated by the $\cdot\text{NO}$ -selective electrode was monitored continuously. In separate studies PAEC were equilibrated for 2 h with PEG-SOD (500 U/mL) or vehicle (control) prior to addition of $25 \mu\text{M}$ H_2O_2 (60 min) and $1 \mu\text{M}$ ACh (not shown). Peak responses were quantified (D) as described previously [34]. Data shown represents mean $\pm$ SD of 5 independent analyses using different PAEC preparations except for PEG-SOD data that was obtained from 4 independent samples. *Significantly different to the corresponding control in the absence of PEG-SOD ($P < 0.05$). **Significantly different to the corresponding sample in the presence of PEG-SOD ($P < 0.05$).

20 min $\sim 70\%$ of the added H_2O_2 was no longer detectable, and after 1 h the residual H_2O_2 in the cell supernate was 11 ± 1.1 and $12.9 \pm 3.5 \%$ mol/mol (mean $\pm$ SD, $n=3$) for cells exposed to 25 and $50 \mu\text{M}$, respectively. Additionally, at a fixed cell density (1×10^6 PAEC/well) the steady state extent of H_2O_2 consumption was not dependent on the volume of peroxide added but rather the rate of consumption (data not shown).

Accumulation of $\cdot\text{NO}$ by PAEC

Stimulation of PAEC with ACh in the presence of L-arginine resulted in $\cdot\text{NO}$ accumulation over 20 min (Fig. 3A). Pre-treatment of cells with L-NAME blocked (Fig. 3B), while H_2O_2 ($50 \mu\text{M}$) decreased this NO accumulation (Fig. 3C). Exposure of PAEC to PEG-SOD (500 U/mL) prior to H_2O_2 and ACh reversed the H_2O_2 -mediated decrease in $\cdot\text{NO}$ accumulation (Fig. 3D). In contrast, PEG-SOD had no material effect on ACh-stimulated $\cdot\text{NO}$ -accumulation by PAEC in the absence of H_2O_2 (not shown).

H_2O_2 stimulates production of $\text{O}_2^{\cdot-}$ by PAEC

The observed decrease in bioactivity and accumulation of $\cdot\text{NO}$ induced by H_2O_2 may result from decreased NO-production, its enhanced elimination, or a combination of the two processes. As the lack of change to cGMP levels in cells exposed to H_2O_2 in the absence of ACh (Fig. 2B) is indicative of unaltered eNOS activity, we speculated that decreased $\cdot\text{NO}$ -availability was responsible for the H_2O_2 -induced impaired vascular relaxation. In this context, $\text{O}_2^{\cdot-}$ was considered a likely candidate contributing to diminished NO bioactivity, as $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}$ react at near diffusion-controlled rates [25]. We therefore determined whether H_2O_2 caused PAEC to produce more $\text{O}_2^{\cdot-}$. In support of this notion, H_2O_2 increased the intensity of the steady state signal of CMH-nitroxide (Figs. 4A & D) compared to vehicle control (Figs. 4C & D), and this was prevented completely when PEG-SOD was added to the cells prior to H_2O_2 (Figs. 4B & D). To validate accumulation of CMH-nitroxide as a measure of cellular $\text{O}_2^{\cdot-}$ production, we exposed PMA-

Fig. 4. Generation of $O_2^{\cdot-}$ by PAEC. PAEC ($2-3 \times 10^6$ cells) were washed with HEPES-buffered PSS containing 100 μ M diethylenetriaminepentaacetic acid and 250 μ M CMH for 5min without (A) and with (B) 1000 units/mL PEG-SOD or phosphate buffered saline (50 mM, pH 7.4) vehicle alone (C). Cells were then incubated for a further 60min with or without H_2O_2 (50 μ M) and the extent of $O_2^{\cdot-}$ generation (D) determined by measuring CMH-nitroxide accumulation with EPR spectroscopy 30min after addition of 1 μ M ACh. Peak areas were quantified by double integration of the centre line of each nitroxide triplet signal and were normalized to total cell protein measured in the identical sample. Spectrometer parameters are described in “Methods” and spectral manipulations were performed using standard Bruker software. Data represent mean $\pm$ SD of 5 independent analyses using different PAEC preparations. *Significantly different to cells treated with ACh alone ($P < 0.05$). **Significantly different to the corresponding H_2O_2 -treated sample in the absence of PEG-SOD ($P < 0.05$). Commercial CMH contains detectable CMH-nitroxide as indicated by the sample treated with phosphate buffered saline alone.

activated PMN, a well-known cellular source of $O_2^{\cdot-}$, to CMH. This resulted in substantial formation of CMH-nitroxide, in a process that was inhibited $82 \pm 3\%$ (mean, $n=4$ independent experiments) by pre-treatment of the cells with 500 U/mL PEG-SOD (not shown). Similarly, incubation of CMH with xanthine (5 mM) plus xanthine oxidase (10 mU/mL), a known enzymatic source of $O_2^{\cdot-}$, caused accumulation of CMH-nitroxide over 30min, and this was inhibited $71 \pm 6\%$ by 500 U/mL PEG-SOD (not shown). In addition, and as an independent measure of cellular $O_2^{\cdot-}$ production [36], PMA-treated PMN (Fig. 5A) and H_2O_2 -treated PAEC stimulated with ACh reduced cytochrome *c* in a manner inhibited by SOD (Fig. 5B). At similar cell density, the extent of cytochrome *c* reduction by PAEC was significantly less than that seen with activated PMN. Cytochrome *c* reduction was not observed with PAEC treated with ACh alone. These data show that exposure of PAEC to 50 μ M H_2O_2 increases ACh-elicited cellular production of $O_2^{\cdot-}$.

Source(s) of $O_2^{\cdot-}$ in PAEC treated with H_2O_2

Production of $O_2^{\cdot-}$ in endothelial cells has been assigned previously to xanthine oxidase [47] and NADPH oxidase [48]. The contribution of these enzymes, as well as mitochondria, to cellular $O_2^{\cdot-}$ production was assessed using CMH oxidation and a pharmacological approach. DPI (an inhibitor of flavin-dependent enzymes), PEG-

SOD, rotenone and allopurinol all inhibited CMH-nitroxide accumulation by H_2O_2 -treated PAEC (Table 1). Combinations of allopurinol plus DPI, or allopurinol, DPI plus rotenone resulted in additive inhibition. Control experiments with the inhibitors in the absence of H_2O_2 showed no measurable change in CMH-nitroxide. The decrease in CMH-oxidation by added rotenone suggests a mitochondrial origin of $O_2^{\cdot-}$. To test this further, we monitored changes in the mitochondrial membrane

Fig. 5. Reduction of ferri-cytochrome *c* by PMN and PAEC. (A) PMN ($4\text{--}5 \times 10^6$ cells/mL) or (B) PAEC ($2\text{--}3 \times 10^6$ cells) pre-treated with H_2O_2 ($50 \mu\text{M}$) were stimulated with PMA (50 nM) or ACh ($1 \mu\text{M}$), respectively, and the production of $\text{O}_2^{\cdot-}$ determined as PEG-SOD-inhibited reduction of ferricytochrome *c* measuring absorbance at 550 nm as described in “Methods”, using an extinction coefficient $\epsilon_{550 \text{ nm}} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [43]. Note the difference in the Y-axes scales for Panels A and B. *Significantly different to the corresponding non-activated cells ($P < 0.05$).

Fig. 6. H_2O_2 induces mitochondrial dysfunction in cultured PAEC. PAEC cultured to near confluence were washed, treated with $50 \mu\text{M}$ H_2O_2 or with CCCP (positive control) or vehicle and then treated with CCCP as detailed in the Methods. Cells were harvested and mitochondrial membrane depolarisation determined with flow cytometry. Data represent mean $\pm$ SD derived from $n = 3$ independent experiments. *Significantly different to the control ($P < 0.05$).

permeability transition potential. Similar to carbonyl cyanide 3-chloro-phenylhydrazone, H_2O_2 increased membrane depolarisation in PAEC (Fig. 6) indicating that H_2O_2 enhances mitochondrial dysfunction and that mitochondria are a source of $\text{O}_2^{\cdot-}$ in these cells. Our recent study indicated that 85–90% of PAEC remained viable 1 h after insult with $50 \mu\text{M}$ H_2O_2 [49]. Therefore, enhanced $\text{O}_2^{\cdot-}$ production in PAEC in response to sequential treatment with H_2O_2 and ACh is not a general response to toxicity.

Uncoupling of eNOS is known to transform the enzyme into an oxidase [19] and hence represents another potential source of $\text{O}_2^{\cdot-}$ in endothelial cells. As such uncoupling is associated with conversion of eNOS dimer to monomers, we examined whether H_2O_2 caused eNOS monomerization. As can be seen in Fig. 7, even $100 \mu\text{M}$

H_2O_2 failed to induce eNOS monomerization. In contrast, treatment of PAEC with hypochlorous acid caused eNOS monomerization (Fig. 7), as has been reported previously [37]. Also, H_2O_2 had no material effect on the expression of the eNOS gene (Fig. 7). These results are consistent with NOS activity measurements (Table 2) that showed H_2O_2 pre-treatment of PAEC to increase conversion of [^3H]L-arginine to [^3H]L-citrulline in a manner inhibited by L-NAME. Together, these data suggest that eNOS activity is retained, and uncoupling of eNOS does not occur when PAEC are exposed to $50 \mu\text{M}$ H_2O_2 . Also, H_2O_2 did not change total cellular SOD activity (not shown), suggesting that Cu,Zn-SOD does not contribute to $\text{O}_2^{\cdot-}$ production, e.g., as a result of reaction of its copper with H_2O_2 as suggested by others [50].

Table 1. Cellular superoxide radical anion trapping with CMH. Confluent PAEC (3-4 x 10⁶ cells) were washed with HEPES-buffered PSS and then pre-treated sequentially with the indicated inhibitor, 250 mM CMH and 50 μ M H₂O₂ or PSS (control) and incubated at 37°C for 60min. Next, acetylcholine (1 μ M) was added, after which CMH-nitroxide was measured in the supernatant with EPR spectroscopy. Where added, PEG-SOD was

Treatment	Peak area/mg protein (SD)	% Inhibition	(n)
Control (no cells)	1.5 (0.1)		4
PAEC (vehicle alone)	1.6 (0.1)		8
PAEC + H ₂ O ₂	5.0 (0.2)*		8
PAEC + PEG-SOD + H ₂ O ₂	1.8 (0.1)	94	4
PAEC + allopurinol	1.6 (0.2)		3
PAEC + DPI	1.7 (0.1)		3
PAEC + rotenone	1.9 (0.1)*		3
PAEC + allopurinol + H ₂ O ₂	4.0 (0.2)*,**	29	4
PAEC + DPI + H ₂ O ₂	3.2 (0.2)*,**	53	4
PAEC + rotenone + H ₂ O ₂	3.1 (0.2)*,**	56	4
PAEC + allopurinol + DPI + H ₂ O ₂	2.3 (0.1)*,**	79	4
PAEC + allopurinol + DPI + rotenone + H ₂ O ₂	2.0 (0.2)*,**	97	3

present at 500 U/mL, and allopurinol, DPI and rotenone at 250, 10 and 20 μ M, respectively. Inhibitor studies were performed in batches, with each batch including a sample of each PAEC + vehicle and PAEC + 50 μ M H₂O₂ as controls. Percent inhibition represents the decrease in superoxide radical anion trapping by CMH as calculated by comparing the decrease in CMH-nitroxide peak area with the maximum change in CMH-oxidation measured in the presence of 50 μ M H₂O₂. Data represents the mean $\pm$ (SD) from (n) independent experiments. Differences between paired samples were assessed using the Newman-Keul's Multiple Comparison test. *Significantly different to vehicle control; P < 0.05. **Significantly different to PAEC + H₂O₂; P < 0.05.

Table 2. Endothelial nitric oxide activity in porcine aortic endothelial cells. PAEC (1-2 x 10⁶ cells) were cultured to near confluence, washed with HEPES-buffered PSS and then pre-treated with 50 μ M H₂O₂ in the presence of absence of L-NAME or PSS (control) and then incubated at 37°C. After 60min, cells were

Treatment	Fold-change in yield of [³ H] L-citrulline (SD)	(n)
PAEC (vehicle alone)	1	9
PAEC + H ₂ O ₂	2.8 (0.2)*	9
PAEC + L-NAME + H ₂ O ₂	0.1 (0.2)*,**	6

harvested, incubated in lysis buffer and eNOS activity measured using a commercial kit (Alexis Biochemicals, CA) as per manufacturer's instructions. Data is expressed as the fold-change in the yield of L-citrulline relative to the respective vehicle control (arbitrarily set to 1). Data represents the mean $\pm$ (SD) from (n) independent experiments. Differences between paired samples were assessed using the Newman-Keul's Multiple Comparison test. *Significantly different to PAEC + vehicle; P < 0.05. **Significantly different to PAEC + H₂O₂; P < 0.05.

PEG-SOD restores [•]NO bioavailability

Next, we investigated whether removal of in situ produced O₂^{•-} restored [•]NO bioavailability in vessels treated with H₂O₂. Aortic rings were incubated without (control) or with PEG-SOD for 12h. This significantly increased aortic content of superoxide dismutase from 2.4 $\pm$ 0.6 to 5.6 $\pm$ 1.8 ng total SOD/mg protein (mean $\pm$ SD, n = 4 vessels; P = 0.03), corresponding to a 2.3-fold increase in total SOD activity. Pre-treatment of aortic

rings with PEG-SOD partially restored endothelial dysfunction induced by 25 (Fig. 8A) and 50 μ M H₂O₂ (Fig. 8B), yet PEG-SOD had no effect on vessel relaxation induced by SNP (not shown). Pre-incubation with PEG-SOD also restored NO accumulation induced by ACh in H₂O₂-treated PAEC (Fig. 3D), and this was matched by a significant decrease in cellular O₂^{•-} generation as judged by the extent of CMH oxidation (Fig. 4D).

Fig. 7. eNOS mRNA expression and stability in PAEC exposed to H_2O_2 . (A) Near confluent PAEC were treated with H_2O_2 (50 μM) or vehicle alone (control) for 60min, cells harvested, total RNA isolated and the corresponding cDNA obtained as described in the Methods. Next, the level of eNOS mRNA and the house keeping gene GAPDH were determined in the control and H_2O_2 -treated samples with RT-PCR and visualised with ethidium bromide staining. (B) PAEC ($2-3 \times 10^6$ cells) were pre-treated with H_2O_2 (50 or 100 μM) or vehicle alone (control) for 60min, cells harvested and lysed and eNOS protein was immuno-precipitated and immuno-blotted as described in the Methods. Western-blot shows eNOS dimer complex or monomer from Lane 1: control cells; Lane 2: PAEC treated with 50 μM or Lane 3: 100 μM H_2O_2 ; Lane 4: eNOS dimer complex dissociates in cells treated with 400 μM hypochlorous acid. Data shown is representative of three independent experiments.

Discussion

Activated phagocytes produce large quantities of H_2O_2 , yet relatively little is known about the impact of H_2O_2 on vascular function. Here we show that pre-treatment of rabbit aortic vessels with non-toxic concentrations of H_2O_2 as low as 25 μM impaired endothelium-dependent vessel relaxation induced by ACh, although it had no material effect on relaxation induced by an NO -donor. Increasing arterial SOD activity prior to H_2O_2 exposure reversed the impaired endothelium-dependent relaxation. Studies with isolated PAEC revealed that H_2O_2 induced both a concentration-dependent increase in cellular $\text{O}_2^{\cdot-}$ production, and a decrease in ACh-induced NO accumulation. Both processes were reversed by PEG-SOD. Our inhibitor studies with DPI

Fig. 8. Partial reversal of H_2O_2 -induced endothelial dysfunction by added PEG-SOD. (A) Aortic rings were suspended in Krebs buffer and incubated with vehicle (filled triangles and hatched squares) or 500 U/mL PEG-SOD (open triangles) for 12h prior to pre-treatment with vehicle (hatched squares) or 25 μM H_2O_2 (triangles), followed by relaxation in response to increasing concentrations of ACh as described in the Legend to Fig. 1. (B) Rings were treated as in (A) using the H_2O_2 concentration indicated, except that maximal relaxation in response to 1 μM ACh was determined using vessels with (filled columns) or without exposure to 500 U/mL PEG-SOD (open columns). Data represent mean $\pm$ SEM of 4 separate studies with rings from different animals. *Significantly different ($P < 0.05$) to (A) the dose response curve generated with H_2O_2 -treated vessels and (B) corresponding vessels without PEG-SOD incubation.

implicate flavin-dependent enzymes, such as xanthine oxidase and NADPH oxidase, as a source of $\text{O}_2^{\cdot-}$, consistent with previous reports by others [31, 32]. Studies with allopurinol confirmed a role for xanthine

oxidase. Our results further implicate mitochondria but not eNOS as a significant additional cellular source of $O_2^{\cdot-}$, and they exclude changes to NOS activity and $\cdot NO$ production in PAEC exposed to H_2O_2 . Together, these data suggest that at $\geq 50 \mu M$, H_2O_2 causes endothelial dysfunction by increasing cellular $O_2^{\cdot-}$ production through multiple sources, which collectively decrease $\cdot NO$ bioavailability.

A decrease in the bioactivity of endothelium-derived $\cdot NO$ is an important manifestation of endothelial dysfunction, itself an independent predictor of cardiovascular events [51]. While there are many potential reasons for impaired $\cdot NO$ bioactivity caused by H_2O_2 , our results indicate that eNOS uncoupling is unlikely involved. Thus, eNOS activity increased (Table 2) rather than decreased in PAEC treated with H_2O_2 , and enzyme monomerization was not detected (Fig. 7). The latter observation contrast the situation reported for other oxidants, such as peroxynitrite [26, 27] or hypochlorous acid [38]. An oxidative mechanism thought to be important for endothelial dysfunction is reaction of $\cdot NO$ with $O_2^{\cdot-}$ because the resulting peroxynitrite activates soluble guanylyl cyclase less effectively than $\cdot NO$ itself [52]. There is strong support for the notion that $O_2^{\cdot-}$ is also implicated in H_2O_2 -induced endothelial dysfunction. Foremost, increasing SOD activity in PAEC and aortic vessels substantially reversed the adverse effects of H_2O_2 (Figs. 3 & 8). Also, exposure of PAEC to H_2O_2 increased cellular production of $O_2^{\cdot-}$ (Figs. 4 & 5), as indicated by CMH oxidation and cytochrome *c* reduction. The use of two different detection methods strengthens our conclusion of $O_2^{\cdot-}$ production, as it eliminates potential other interpretations. For example, while in addition to $O_2^{\cdot-}$ CMH oxidation can also be achieved by peroxynitrite [53], the latter oxidant does not engage in cytochrome *c* reduction. Therefore, our data support the notion that increases in endothelial cell production of $O_2^{\cdot-}$ is the likely cause for the H_2O_2 -induced endothelial dysfunction and decrease in $\cdot NO$ bioactivity. Whether the decreased $\cdot NO$ bioactivity was due to direct reaction of $O_2^{\cdot-}$ with $\cdot NO$ [25] appears uncertain, as in this case we would have expected H_2O_2 to also decrease vessel relaxation in response to the $\cdot NO$ donor SNP, which was not the case (Fig. 1). Thus, the precise mechanism underlying the H_2O_2 -induced and $O_2^{\cdot-}$ mediated impairment of endothelial function requires further elucidation.

Several cellular sources of $O_2^{\cdot-}$ have been identified in endothelial cells exposed to H_2O_2 [30–32], peroxynitrite [26] and hypochlorous acid [37]. In each case, the oxidant-stimulated production of $O_2^{\cdot-}$ was linked to vascular

dysfunction observed for endothelium-dependent, but not endothelium independent, relaxants. However, unlike peroxynitrite and hypochlorous acid, H_2O_2 does not cause appreciable eNOS uncoupling (Fig. 7B), indicating that eNOS did not contribute to the cellular $O_2^{\cdot-}$ production. Rather, our inhibitor studies implicate activation of xanthine oxidase and NADPH oxidase, and also mitochondria as sources of $O_2^{\cdot-}$ (Table 1). Additional studies are required to elucidate the relative contribution of the different pathways, as well as their cellular compartmentalization, to the observed endothelial dysfunction.

The pathophysiologic relevance of the present study depends on both the likelihood of endothelial cells being exposed to the H_2O_2 concentrations used here and the relevance of ACh as a suitable agonist to mimic vessel relaxation in vivo. In humans circulating PMN are present at $1.5\text{--}6 \times 10^6$ cells/mL, and at this density activated cells generate 0.08 to 0.48 mM H_2O_2 per hour [54]. Another potentially relevant source of H_2O_2 are activated monocytes [55]. Notably, the enhanced rates of H_2O_2 production by cells are generally matched by increased rates of consumption, so that the steady state level remains relatively low. Whether this balance of production and degradation is affected by disease is not clear. Nevertheless, H_2O_2 is freely diffusible and relatively non-reactive therefore, it is plausible that the vascular endothelium becomes exposed (locally) to micromolar concentrations of H_2O_2 in the setting of inflammation. Additional studies are necessary to establish, or otherwise, the relevance of ACh as a vessel relaxing agent, although many substances and processes involved in vessel relaxation (shear stress, bradykinin, histamine, substance P, vasopressin and α -adrenergic agonists) stimulate endothelium-dependent relaxation via cGMP-dependent processes, which are likely affected by differences in $O_2^{\cdot-}$ in the vessel wall. It is also important to examine whether our results obtained with rabbit aorta extend to other arterial vessels, as the relative importance of $\cdot NO$ to vessel relaxation differs between species and vascular beds (reviewed in ref [56]).

The clinical implications of H_2O_2 -induced vascular dysfunction are widespread and may include chronic and acute disease states. Increased vascular concentrations of H_2O_2 are evident in animal models of diabetes, and there is evidence that links this to endothelial dysfunction. For example, treatment of isolated aortas from chronically diabetic animals with 3-amino-1, 2, 4-triazole that inhibits catalase, markedly inhibited relaxation induced by ACh, and this was enhanced by co-treatment with SOD [57]. Increased vascular production of H_2O_2 is implicated in

organ failure [58] and remodelling of the vessel wall [59], a characteristic of essential hypertension. The notion that biologically relevant concentrations of H₂O₂ can cause endothelial dysfunction by decreasing [•]NO bioavailability has important implications. Identifying its underlying mechanism may lead to the development of novel pharmacological strategies that inhibit this type of endothelial dysfunction.

Abbreviations

ACh (acetylcholine); cGMP (guanosine 3',5'-cyclic monophosphate); DPI (diphenyliodonium); eNOS (endothelial nitric oxide synthase); EPR (electron paramagnetic resonance spectroscopy); L-NAME (N ω -nitro-L-arginine methyl ester); [•]NO (nitric oxide);

O₂^{•-} (superoxide anion radical); PAEC (porcine aortic endothelial cells); PEG-SOD (polyethylene glycol superoxide dismutase); PMA (phorbol-12-myristate-13-acetate); PMN (polymorphonuclear leukocytes); PSS (physiological salt solution); SNP (S-nitroso-penicillamine); VSMC (vascular smooth muscle cells).

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References

- 1 Utoguchi N, Ikeda K, Saeki K, Oka N, Mizuguchi H, Kubo K, Nakagawa S, Mayumi T: Ascorbic acid stimulates barrier function of cultured endothelial cell monolayer. *J Cell Physiol* 1995;163:393-99.
- 2 Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G: Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987;84:9265-9269.
- 3 Azuma H, Ishikawa M, Sekizaki S: Endothelium-dependent inhibition of platelet aggregation. *Br J Pharmacol*. 1986;88:411-415.
- 4 Kubes P, Suzuki M, Granger DN: Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* 1991;88:4651-4655.
- 5 Tanner FC, Meier P, Greutert H, Champion C, Nabel EG, Luscher TF: Nitric oxide modulates expression of cell cycle regulatory proteins: a cytostatic strategy for inhibition of human vascular smooth muscle cell proliferation. *Circulation* 2000;101:1982-1989.
- 6 Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P: Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* 1986;315:1046-1051.
- 7 Matsumoto T, Yoshiyama S, Wakabayashi K, Kobayashi T, Kamata K: Effects of chronic insulin on endothelial dysfunction of basilar arteries from established streptozotocin-diabetic rats. *Eur J Pharmacol* 2004;504:119-127.
- 8 Panza JA, Garcia CE, Kilcoyne CM, Quyyumi AA, Cannon RO 3rd: Impaired endothelium-dependent vasodilation in patients with essential hypertension. Evidence that nitric oxide abnormality is not localized to a single signal transduction pathway. *Circulation* 1995;91:1732-1738.
- 9 Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A: Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* 2000;106:1521-1530.
- 10 Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, Busse R: Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 1999; 01:493-497.
- 11 Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH: K⁺ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* 1998;396:269-272.
- 12 Xi Q, Cheranov Y, Jaggar JH: Mitochondria-derived reactive oxygen species dilate cerebral arteries by activating Ca²⁺ sparks. *Circ Res* 2005;97:354-362.
- 13 Matoba T, Shimokawa H, Kubota H, Kunihiro I, Urakami-Harasawa L, Mukai Y, Hirakawa Y, Akaike T, Takeshita A: Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler Thromb Vasc Biol* 2003;23:1224-1230.
- 14 Thomas SR, Chen K, Keaney JF Jr: Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem* 2002;277:6017-6024.
- 15 Cai H, Li Z, Dikalov S, Holland SM, Hwang J, Jo H, Dudley SC Jr, Harrison DG: NAD(P)H oxidase-derived hydrogen peroxide mediates endothelial nitric oxide production in response to angiotensin II. *J Biol Chem* 2002;277:48311-48317.
- 16 Hu Q, Corda S, Zweier JL, Capogrossi MC, Ziegelstein RC: Hydrogen peroxide induces intracellular calcium oscillations in human aortic endothelial cells. *Circulation* 1998;97:268-275.

- 17 Liu Y, Gutterman DD: Oxidative stress and potassium channel function. *Clin Exp Pharmacol Physiol* 2002;29:305-311.
- 18 Deshpande NN, Sorescu D, Seshiah P, Ushio-Fukai M, Akers M, Yin Q, Griendling KK: Mechanism of hydrogen peroxide-induced cell cycle arrest in vascular smooth muscle. *Antioxid Redox Signal* 2002;4:845-854.
- 19 Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr: Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 1998;95:9220-9225.
- 20 White CR, Darley-Usmar V, Berrington WR, McAdams M, Gore JZ, Thompson JA, Parks DA, Tarpey MM, Freeman BA: 1996 Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc Natl Acad Sci USA* 1996;93:8745-8749.
- 21 Lui Y, Zhao H, Li H, Kalyanaraman B, Nicolosi AC, Gutterman DD: Mitochondrial sources of H_2O_2 generation play a key role in flow-mediated dilation in human coronary resistance arteries. *Circ Res* 2003;93:573-580.
- 22 Fleming I, Michaelis UR, Bredenkotter D, Fisslthaler B, Dehghani F, Brandes RP, Busse R: Endothelium-derived hyperpolarizing factor synthase Cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. *Circ Res* 2001;88:44-451.
- 23 McNally JS, Davis ME, Giddens DP, Saha A, Hwang J, Dikalov S, Jo H, Harrison DG: Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress. *Am J Physiol Heart Circ Physiol* 2003;285:H2290-2297.
- 24 Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG: Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996;97:1916-23.
- 25 Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Nat Acad Sci USA* 1990;87:1620-1624.
- 26 Zou MH, Shi C, Cohen RA: Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 2002;109:817-826.
- 27 Kuzkaya N, Weissmann N, Harrison DG, Dikalov S: Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem* 2003;278:22546-22554.
- 28 Stone JR: An assessment of proposed mechanisms for sensing hydrogen peroxide in mammalian systems. *Arch Biochem Biophys* 2004;422:119-124.
- 29 Stocker R, Keaney JF Jr: Role of oxidative modifications in atherosclerosis. *Physiol Rev* 2004;84:1381-1478.
- 30 Thomas SR, Schulz E, Keaney JF Jr: Hydrogen peroxide restrains endothelium-derived nitric oxide bioactivity: role for iron-dependent oxidative stress. *Free Radic Biol Med* 2006;41:681-688.
- 31 Boulden BM, Widder JD, Allen JC, Smith DA, Al-Baldawi RN, Harrison DG, Dikalov SI, Jo H, Dudley SC Jr: Early determinants of H_2O_2 -induced endothelial dysfunction. *Free Radic Biol Med* 2006;41:810-817.
- 32 Coyle CH, Martinez LJ, Coleman MC, Spitz DR, Weintraub NL, Kader KN: Mechanisms of H_2O_2 -induced oxidative stress in endothelial cells. *Free Radic Biol Med* 2006;40:2206-2213.
- 33 Lau AK, Leichtweis SB, Hume P, Mashima R, Hou JY, Chaufour X, Wilkinson B, Hunt NH, Celermajer DS, Stocker R: Probucol promotes functional reendothelialization in balloon-injured rabbit aortas. *Circulation* 2003;107:2031-2036.
- 34 Rayner BS, Wu, B-J, Raftery M, Stocker R, Witting PK: Human S-nitroso-myoglobin is a store of vasoactive nitric oxide. *J Biol Chem* 2005;280:9985-9993.
- 35 Kuzkaya N, Weissmann N, Harrison DG, Dikalov SI: Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem* 2003;278:22546-54.
- 36 Schnur RA, Newman SL: The respiratory burst response to *Histoplasma capsulatum* by human neutrophils. Evidence for intracellular trapping of superoxide anion. *J Immunol* 1990;144:4765-4772.
- 37 Stocker R, Huang A, Jeranian E, Hou JY, Wu TT, Thomas SR, Keaney JF Jr: Hypochlorous acid impairs endothelium-derived nitric oxide bioactivity through a superoxide-dependent mechanism. *Arterioscler Thromb Vasc Biol* 2004;24:2028-2033.
- 38 Witting PK, Liao W Q, Harris MJ, Neuzil J: Expression of human myoglobin in H9c2 cells enhances toxicity to added hydrogen peroxide. *Biochem Biophys Res Commun* 2006;348:485-493.
- 39 Suarna C, Dean RT, May J, Stocker R: Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol* 1995;15:1616-1624.
- 40 Ahmed H, Schott EJ, Gauthier JD, Vasta GR: Superoxide dismutases from the oyster parasite *Perkinsus marinus*: purification, biochemical characterization, and development of a plate microassay for activity. *Anal Biochem* 2003;318:132-141.
- 41 Witting PK, Upston JM, Stocker R: Formation of α -Tocopheryl Radical Initiates Lipid Peroxidation in LDL Exposed to Horse Radish Peroxidase. *Biochemistry* 1997;36:1251-1258.
- 42 Mian KB, Martin W: Hydrogen peroxide-induced impairment of reactivity in rat isolated aorta: potentiation by 3-amino-1,2,4-triazole. *Br J Pharmacol* 1997;121:813-819.
- 43 Harrison GJ, Jordan LR, Willis RJ: Deleterious effects of hydrogen peroxide on the function and ultrastructure of cardiac muscle and the coronary vasculature of perfused rat hearts. *Can J Cardiol* 1994;10:843-849.
- 44 Kobayashi T, Kamata K: Mechanisms underlying the chronic pravastatin treatment-induced improvement in the impaired endothelium-dependent aortic relaxation seen in streptozotocin-induced diabetic rats. *Eur J Pharmacol* 2002;441:83-89.
- 45 Schmidt K, Schrammel A, Koesling D, Mayer B: Molecular mechanisms involved in the synergistic activation of soluble guanylyl cyclase by YC-1 and nitric oxide in endothelial cells. *Mol Pharmacol* 2001;59:220-224.
- 46 Moro MA, Russel RJ, Celtek S, Lizasoain I, Su Y, Darley-Usmar VM, Radomski M W, Moncada S: cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc Natl Acad Sci USA* 1996;93:1480-1485.
- 47 Erdei N, Toth A, Pasztor ET, Papp Z, Edes I, Koller A, Bagi Z: High Fat Diet-Induced Reduction in Nitric Oxide-Dependent Arteriolar Dilation in Rats. Role of Xanthine Oxidase-Derived Superoxide Anion. *Am J Physiol Heart Circ Physiol* 2006;291:H2107-H2115.
- 48 Johnson D K, Schillinger KJ, Kwiat DM, Hughes CV, McNamara EJ, Ishmael F, O'Donnell RW, Chang MM, Hogg MG, Dordick JS, Santhanam L, Ziegler LM, Holland JA: Inhibition of NADPH oxidase activation in endothelial cells by ortho-methoxy-substituted catechols. *Endothelium* 2002;9:191-203.

- 49 Witting PK, Harris HH, Rayner BS, Aitken JB, Dillon CT, Stocker R, Lai B, Cai Z, Lay PA: The Endothelium-derived Hyperpolarizing Factor, H₂O₂, Promotes Metal-ion Efflux in Aortic Endothelial Cells: Elemental Mapping by a Hard X-ray Microprobe. *Biochemistry* 2006;45:12500-12509.
- 50 Goto JJ, Gralla EB, Valentine JS, Cabelli DE: Reactions of hydrogen peroxide with familial amyotrophic lateral sclerosis mutant human copper-zinc superoxide dismutases studied by pulse radiolysis. *J Biol Chem* 1998;273:30104-30109.
- 51 Gokce N, Keaney JF Jr, Hunter LM, Watkins MT, Menzoian JO, Vita, JA: Risk stratification for postoperative cardiovascular events via non-invasive assessment of endothelial function: a prospective study. *Circulation* 2002;105:1567-72.
- 52 Tarpey MM, Beckman JS, Ischiropoulos H, Gore JZ, Brock TA. Peroxynitrite stimulates vascular smooth muscle cell cyclic GMP synthesis. *FEBS Lett* 1995;364:314-318.
- 53 Dikalov S, Skatchkov M, Fink B, Bassenge E. Quantification of superoxide radicals and peroxynitrite in vascular cells using oxidation of sterically hindered hydroxylamines and electron spin resonance. *Nitric Oxide* 1997;1:423-431.
- 54 Liu X, Zweier J L. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: evaluation in human polymorphonuclear leukocytes. *Free Radic Biol Med* 2001;31:894-901.
- 55 Rollins BJ, Walz A, Baggiolini M. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. *Blood* 1991;78:1112-1116.
- 56 Hill CE, Phillips JK, Sandow SL. Heterogeneous control of blood flow amongst different vascular beds. *Med Res Rev* 2001;21:1-60.
- 57 Karasu C. Time course of changes in endothelium-dependent and -independent relaxation of chronically diabetic aorta: role of reactive oxygen species. *Eur J Pharmacol* 2000;392:163-173.
- 58 Lacy F, Kailasam MT, O'Connor DT, Schmid-Schonbein GW, Parmer RJ. Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity. *Hypertension* 2000;36:878-884.
- 59 Zhang Y, Griendling KK, Dikalova A, Owens GK, Taylor WR. Vascular hypertrophy in angiotensin II-induced hypertension is mediated by vascular smooth muscle cell-derived H₂O₂. *Hypertension* 2005;46:732-737.