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 Stocker1,3

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$_2$O$_2$) 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$_2$O$_2$. Exposure of aortic rings to 25 or 50 µM, but not 10 µM, H$_2$O$_2$ for 60min prior to constriction significantly decreased subsequent relaxation in response to acetylcholine (ACh), but not the nitric oxide (•NO) donor sodium nitroprusside. Treatment of PAEC with 50 µM H$_2$O$_2$ significantly decreased ACh-induced accumulation of •NO, as measured with a •NO-selective electrode, yet such treatment increased nitric oxide synthase activity ~3-fold, as assessed by conversion of L-arginine to L-citrulline. Decreased •NO bioavailability was reflected in decreased cellular cGMP content, associated with increased superoxide anion radical (O$_2^•$−), and overcome by addition of polyethylene glycol superoxide dismutase. Increased cellular O$_2^•$− production was inhibited by allopurinol, diphenylidonium and rotenone in an additive manner. The results show that exposure of endothelial cells to H$_2$O$_2$ decreases the bioavailability of agonist-induced •NO as a result of increased production of O$_2^•$− likely derived from xanthine oxidase, NADPH-oxidase and mitochondria. These processes could contribute to H$_2$O$_2$-induced vascular dysfunction that may be relevant under conditions of oxidative stress such as inflammation.

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
endothelium-derived hyperpolarizing factor include a vasodilator of presently unknown identity. Candidates for endothelium-derived hyperpolarizing factor, another radical anion (O$_2^-$) and K$^+$ ions [11]. More recently, reactive oxygen species derived from VSMC mitochondria have been implicated in modulating vascular function [12]. Of these, H$_2$O$_2$ is increasingly viewed as important because its vessel dilating and hyperpolarizing properties are largely insensitive to inhibitors of eNOS and cyclooxygenase [13]. Notably, H$_2$O$_2$ produced via dismutation of superoxide radical anion (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$_2$O$_2$ in vascular cells is 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, O$_2$$^-$$^\cdot$ effectively impairs ‘NO bioactivity via near-diffusion-controlled bimolecular reaction of O$_2$$^-$$^\cdot$ with ‘NO [25]. This yields peroxynitrite that cannot inactivate eNOS directly [26] or indirectly [27], and this can result in further production of O$_2$$^-$$^\cdot$ and hence H$_2$O$_2$.

In addition to its potential direct function as a vasodilator, H$_2$O$_2$ 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$_2$O$_2$ locally. Indeed, cellular studies examining the potential pathophysiologic effects of H$_2$O$_2$ commonly employ the oxidant in the concentration range of 20-250 µM [14, 15].

Despite the potential for H$_2$O$_2$ 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$_2$O$_2$ on endothelial function. Three recent papers have focussed on the determinants of H$_2$O$_2$-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 O$_2^\cdot$-, although the source(s) of this 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$_2$O$_2$ and explored likely sources of cellular 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$_2$PO$_4$, 4.7 KCl, 120 NaCl, 25 NaHCO$_3$, and 2.5 CaCl$_2$$\cdot$2H$_2$O) and cut into 5 mm ring segments [33]. Where required, rings were incubated with 500 U/mL polyethylene glycol superoxide dismutase (PEG-SOD) or 50 mM phosphate buffered saline (vehicle control) at 4°C for 12h. This increased vascular SOD activity (see below) but did not affect constriction in response to phenylephrine (not shown). Rings were mounted in a myobath system (World Precision Instruments, Inc., Sarasota, FL) containing 20 mL of modified Krebs-Henseleit solution aerated at 37ºC with 5% CO$_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$^{-6}$-10$^{-5}$ mol/L) was constructed, with relaxation expressed as percentage of initial contraction. Where indicated, mounted rings were pre-treated with H$_2$O$_2$ (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 µg/mL heparin sulfate, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/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 overlayed with HEPES...
buffered-physiologic salt solution (PSS) [14]. Cells were treated with vehicle (control) or H2O2 at the concentrations indicated in the legends to the Figures. In some experiments cells were pre-incubated with 200 µM No-nitro-L-arginine methyl ester (L-NAME) prior to the addition of H2O2. 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 manufacturer’s 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 MKll, World Precision Instruments Inc.) coupled to a DUO-18™ data recorder (v1.55). Cells were harvested, resuspended at 4-6 x 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), H2O2 (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 cell number to account for variation in cell density.

Assessment of O2•− production by PAEC and PMN

Production of O2•− by cells was assessed by monitoring oxidation of the cell-permeable spin label 3-isobutyl-1-methylxanthine-1-hydroxy-3-methoxycarbonyl-2,5,5-tetramethyl-pyrroline.HCl (CMH) to the EPR-active CMH-nitroxide with EPR spectroscopy-CMH detects intra- and extra-cellular O2•− [35]. Briefly, confluent PAEC (~2-3 x 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 H2O2 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 x 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 ~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 diphenylidionium (DPI, an inhibitor of xanthine 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 H2O2 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 H2O2 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 H2O2 disrupted the eNOS dimer complex, PAEC were grown to near confluence and treated with 50 or 100 µM H2O2 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 (H2O2) 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 H2O2

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 Thermocycler system (Applied Biosystems, Foster City, CA). Mixtures contained: 2 µL RNA, 1 µL oligo(dT) (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 Bioline PCR Master Mix (Bioline, Australia) and the complete reaction mixtures heated to 42°C for 60min then 70°C for 10min.

Superoxide Anion Radical from Endothelial Cells Exposed to H2O2

Cell Physiol Biochem 2007;20:255-268

257
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, 5 min), followed by repeated denaturation (94°C, 30s), annealing (30s), elongation (72°C, 1 min) and a final elongation step (72°C, 10 min). Primers were synthesized by Prologi (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 µM H2O2 or vehicle (control) for 60 min, harvested and re-suspended at ~1x10⁶ cells/mL in 50 mM phosphate buffer, pH 7.4. Incubation of parallel samples with 200 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) served as a positive control. All samples were then incubated further with 2 µM 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl-benzimidoazolyl-carboxycyanine iodide for 20 min 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 (10⁶ cells/mL) exposed to 50 µM 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 ± H2O2 (in HEPES-buffered PSS with and without 200 µM L-NAME) was determined after stimulation with Ach (1 µM) in the presence of 100 µM L-arginine and 200 µM 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].
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 µM DTPA at 4°C prior to use.

**Statistical analysis**

Vessel function data are expressed as mean ± 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 ± 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 µM H₂O₂ had no significant effect on subsequent vessel relaxation elicited by ACh (Fig. 1A). Pre-treatment of the vessels with 25 or 50 µM H₂O₂ significantly decreased endothelium-dependent relaxation to ACh, as did pre-treatment with 100 µM 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 µM H₂O₂ had no effect on endothelium-independent relaxation induced by the •NO-donor SNP (Fig. 1B). Together, these data shown that H₂O₂ at = 25 µM promotes endothelial dysfunction.

**Effect of H₂O₂ on •NO bioactivity in cultured PAEC**

Endothelial cells contain soluble guanlyl 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 guanlyl 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 µM H₂O₂ for 60min (Fig. 2A). In contrast, pre-treatment of the cells with 50 µM H₂O₂ did not alter 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 x 10⁶ PAEC/well) at 25 or 50 µM final concentration was consumed rapidly: after

---

Superoxide Anion Radical from Endothelial Cells Exposed to H₂O₂

**Cell Physiol Biochem 2007;20:255-268**

---

![Fig. 2. Accumulation of cGMP by PAEC.](image-url)
20min ~70% of the added H₂O₂ was no longer detectable, and after 1h the residual H₂O₂ in the cell supernate was 11 ± 1.1 and 12.9 ± 3.5 % mol/mol (mean ± SD, n=3) for cells exposed to 25 and 50 µM, respectively. Additionally, at a fixed cell density (1 x 10^6 PAEC/well) the steady state extent of H₂O₂ consumption was not dependent on the volume of peroxide added but rather the rate of consumption (data not shown).

**Accumulation of NO by PAEC**

Stimulation of PAEC with ACh in the presence of L-arginine resulted in NO accumulation over 20min (Fig. 3A). Pre-treatment of cells with L-NAME blocked (Fig. 3B) while H₂O₂ (50 µM) decreased NO accumulation (Fig. 3C). Exposure of PAEC to PEG-SOD (500 U/mL) prior to H₂O₂ and ACh reversed the H₂O₂-mediated decrease in NO accumulation (Fig. 3D). In contrast, PEG-SOD had no material effect on ACh-stimulated NO-accumulation by PAEC in the absence of H₂O₂ (not shown).

**H₂O₂ stimulates production of O₂•⁻ by PAEC**

The observed decrease in bioactivity and accumulation of NO induced by H₂O₂ 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 H₂O₂ in the absence of ACh (Fig. 2B) is indicative of unaltered eNOS activity, we speculated that decreased NO-availability was responsible for the H₂O₂-induced impaired vascular relaxation. In this context, O₂•⁻ was considered a likely candidate contributing to diminished NO bioactivity, as O₂•⁻ and NO react at near diffusion-controlled rates [25].

We therefore determined whether H₂O₂ caused PAEC to produce more O₂•⁻. In support of this notion, H₂O₂ 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 H₂O₂ (Figs. 4B & D). To validate accumulation of CMH-nitroxide as a measure of cellular O₂•⁻ production, we exposed PMA-
activated PMN, a well-known cellular source of $O_2^{•−}$, to CMH. This resulted in substantial formation of CMH-nitroxide, in a process that was inhibited 82 ± 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 $O_2^{•−}$, caused accumulation of CMH-nitroxide over 30 min, and this was inhibited 71 ± 6% by 500 U/mL PEG-SOD (not shown). In addition, and as an independent measure of cellular $O_2^{•−}$ 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 µM $H_2O_2$ increases ACh-elicited cellular production of $O_2^{•−}$.

Source(s) of $O_2^{•−}$ in PAEC treated with $H_2O_2$

Production of $O_2^{•−}$ 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 $O_2^{•−}$ 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 $O_2^{•−}$. To test this further, we monitored changes in the mitochondrial membrane
permeability transition potential. Similar to carbonyl cyanide 3-chloro-phenylhydrazone, H$_2$O$_2$ increased membrane depolarisation in PAEC (Fig. 6) indicating that H$_2$O$_2$ enhances mitochondrial dysfunction and that mitochondria are a source of O$_2^•^-$ in these cells. Our recent study indicated that 85-90% of PAEC remained viable 1 h after insult with 50 µM H$_2$O$_2$ [49]. Therefore, enhanced O$_2^•^-$ production in PAEC in response to sequential treatment with H$_2$O$_2$ 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 O$_2^•^-$ in endothelial cells. As such uncoupling is associated with conversion of eNOS dimer to monomers, we examined whether H$_2$O$_2$ caused eNOS monomerization. As can be seen in Fig. 7, even 100 µM H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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 µM H$_2$O$_2$. Also, H$_2$O$_2$ did not change total cellular SOD activity (not shown), suggesting that Cu,Zn-SOD does not contribute to O$_2^•^-$ production, e.g., as a result of reaction of its copper with H$_2$O$_2$ as suggested by others [50].
Next, we investigated whether removal of in situ produced O$_2^\cdot$ restored NO bioavailability in vessels treated with H$_2$O$_2$. Aortic rings were incubated without (control) or with PEG-SOD for 12h. This significantly increased aortic content of superoxide dismutase from 2.4 ± 0.6 to 5.6 ± 1.8 ng total SOD/mg protein (mean ± 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 µM H$_2$O$_2$ (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 H$_2$O$_2$-treated PAEC (Fig. 3D), and this was matched by a significant decrease in cellular O$_2^\cdot$ generation as judged by the extent of CMH oxidation (Fig. 4D).

**Table 1.** Cellular superoxide radical anion trapping with CMH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak area/mg protein (SD)</th>
<th>% Inhibition</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no cells)</td>
<td>1.5 (0.1)</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>PAEC (vehicle alone)</td>
<td>1.6 (0.1)</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>PAEC + H$_2$O$_2$</td>
<td>5.0 (0.2)*</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>PAEC + PEG-SOD + H$_2$O$_2$</td>
<td>1.8 (0.1)</td>
<td>94</td>
<td>4</td>
</tr>
<tr>
<td>PAEC + allopurinol</td>
<td>1.6 (0.2)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>PAEC + DPI</td>
<td>1.7 (0.1)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>PAEC + rotenone</td>
<td>1.9 (0.1)*</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>PAEC + allopurinol + H$_2$O$_2$</td>
<td>4.0 (0.2)**</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>PAEC + DPI + H$_2$O$_2$</td>
<td>3.2 (0.2)**</td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>PAEC + rotenone + H$_2$O$_2$</td>
<td>3.1 (0.2)**</td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>PAEC + allopurinol + DPI + H$_2$O$_2$</td>
<td>2.3 (0.1)**</td>
<td></td>
<td>79</td>
</tr>
<tr>
<td>PAEC + allopurinol + DPI + rotenone + H$_2$O$_2$</td>
<td>2.0 (0.2)****</td>
<td></td>
<td>97</td>
</tr>
</tbody>
</table>

**Table 2.** Endothelial nitric oxide activity in porcine aortic endothelial cells. PAEC (1-2 x 10^6 cells) were cultured to near confluence, washed with HEPES-buffered PSS and then pre-treated with 50 µM H$_2$O$_2$ in the presence of absence of L-NAME or PSS (control) and then incubated at 37°C. After 60min, cells were 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 ± (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 + H$_2$O$_2$; P < 0.05.

PEG-SOD restores NO bioavailability

Table 1. Cellular superoxide radical anion trapping with CMH. Confluent PAEC (3-4 x 10^6 cells) were washed with HEPES-buffered PSS and then pre-treated sequentially with the indicated inhibitor, 250 mM CMH and 50 µM H$_2$O$_2$ or PSS (control) and incubated at 37°C for 60min. Next, acetylcholine (1 µM) was added, after which CMH-nitroxide was measured in the supernatant with EPR spectroscopy. Where added, PEG-SOD was present at 500 U/mL, and allopurinol, DPI and rotenone at 250, 10 and 20 µM, respectively. Inhibitor studies were performed in batches, with each batch including a sample of each PAEC + vehicle and PAEC + 50 µM H$_2$O$_2$ 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 µM H$_2$O$_2$. Data represents the mean ± (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.

**Table 2.** Endothelial nitric oxide activity in porcine aortic endothelial cells. PAEC (1-2 x 10^6 cells) were cultured to near confluence, washed with HEPES-buffered PSS and then pre-treated with 50 µM H$_2$O$_2$ in the presence of absence of L-NAME or PSS (control) and then incubated at 37°C. After 60min, cells were 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 ± (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 + H$_2$O$_2$; P < 0.05.
Discussion

Activated phagocytes produce large quantities of H$_2$O$_2$, yet relatively little is known about the impact of H$_2$O$_2$ on vascular function. Here we show that pre-treatment of rabbit aortic vessels with non-toxic concentrations of H$_2$O$_2$ 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$_2$O$_2$ exposure reversed the impaired endothelium-dependent relaxation. Studies with isolated PAEC revealed that H$_2$O$_2$ induced both a concentration-dependent increase in cellular O$_2^-$ production, and a decrease in ACh-induced ‘NO accumulation. Both processes were reversed by PEG-SOD. Our inhibitor studies with DPI implicate flavin-dependent enzymes, such as xanthine oxidase and NADPH oxidase, as a source of O$_2^-$, consistent with previous reports by others [31, 32]. Studies with allopurinol confirmed a role for xanthine oxidase.
oxidase. Our results further implicate mitochondria but not eNOS as a significant additional cellular source of O$_2^-$; and they exclude changes to NOS activity and NO production in PAEC exposed to H$_2$O$_2$. Together, these data suggest that at $\geq$ 50 µM, H$_2$O$_2$ causes endothelial dysfunction by increasing cellular O$_2^-$ production through multiple sources, which collectively decrease NO bioavailability.

A decrease in the bioactivity of endothelial-derived NO is an important manifestation of endothelial dysfunction, itself an independent predictor of cardiovascular events [51]. While there are many potential reasons for impaired NO bioactivity caused by H$_2$O$_2$, our results indicate that eNOS uncoupling is unlikely involved. Thus, eNOS activity increased (Table 2) rather than decreased in PAEC treated with H$_2$O$_2$, 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 NO with O$_2^-$ because the resulting peroxynitrite activates soluble guanylyl cyclase less effectively than NO itself [52]. There is strong support for the notion that O$_2^-$ is also implicated in H$_2$O$_2$-induced endothelial dysfunction. Foremost, increasing SOD activity in PAEC and aortic vessels substantially reversed the adverse effects of H$_2$O$_2$ (Figs. 3 & 8). Also, exposure of PAEC to H$_2$O$_2$ increased cellular production of O$_2^-$ (Figs. 4 & 5), as indicated by CMH oxidation and cytochrome c reduction. The use of two different detection methods strengthens our conclusion of O$_2^-$ production, as it eliminates potential other interpretations. For example, while in addition to O$_2^-$ 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$_2^-$ is the likely cause for the H$_2$O$_2$-induced endothelial dysfunction and decrease in NO bioactivity. Whether the decreased NO bioactivity was due to direct reaction of O$_2^-$ with NO [25] appears uncertain, as in this case we would have expected H$_2$O$_2$ to also decrease vessel relaxation in response to the NO donor SNP, which was not the case (Fig. 1). Thus, the precise mechanism underlying the H$_2$O$_2$-induced and O$_2^-$ mediated impairment of endothelial function requires further elucidation.

Several cellular sources of O$_2^-$ have been identified in endothelial cells exposed to H$_2$O$_2$ [30-32], peroxynitrite [26] and hypochlorous acid [37]. In each case, the oxidant-stimulated production of O$_2^-$ was linked to vascular dysfunction observed for endothelium-dependent, but not endothelium independent, relaxants. However, unlike peroxynitrite and hypochlorous acid, H$_2$O$_2$ does not cause appreciable eNOS uncoupling (Fig. 7B), indicating that eNOS did not contribute to the cellular O$_2^-$ production. Rather, our inhibitor studies implicate activation of xanthine oxidase and NADPH oxidase, and also mitochondria as sources of O$_2^-$ (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$_2$O$_2$ 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 1.5-6 x 10$^6$ cells/mL, and at this density activated cells generate 0.08 to 0.48 mM H$_2$O$_2$ per hour [54]. Another potentially relevant source of H$_2$O$_2$ are activated monocytes [55]. Notably, the enhanced rates of H$_2$O$_2$ 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$_2$O$_2$ is freely diffusible and relatively non-reactive therefore, it is plausible that the vascular endothelium becomes exposed (locally) to micromolar concentrations of H$_2$O$_2$ 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 $\alpha$-adrenergic agonists) stimulate endothelium-dependent relaxation via cGMP-dependent processes, which are likely affected by differences in O$_2^-$ 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 NO to vessel relaxation differs between species and vascular beds (reviewed in ref [56]).

The clinical implications of H$_2$O$_2$-induced vascular dysfunction are widespread and may include chronic and acute disease states. Increased vascular concentrations of H$_2$O$_2$ 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$_2$O$_2$ 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 $\text{H}_2\text{O}_2$ can cause endothelial dysfunction by decreasing $\cdot\text{NO}$ 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)
- $\cdot\text{NO}$ (nitric oxide)
- $\text{O}_2^-\cdot$ (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)

**References**


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.


Witting PK, Upston JM, Stocker R: Formation of F(-)Tocopherol Radical Initiates Lipid Peroxidation in LDL Exposed to Horse Radish Peroxidase. Biochemistry 1997;36;1251-1258

Mian KB, Martin W: Hydrogen peroxide-induced impairment of reactivity in rat isolated aorta: potentiation by 3-amino-1,2,4-triazole. J Pharmacol 1997;121:813-819.


