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Mitochondrial metabolism of a hydroperoxide to free radicals in human endothelial cells: an electron spin resonance spin-trapping investigation

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Oxidative damage to the vascular endothelium may be an important event in the promotion of atherosclerosis. Several lines of evidence suggest that lipid hydroperoxides may be responsible for the induction of such damage. Hydroperoxides cause loss of endothelial cell integrity, increase the permeability of the endothelium to macromolecules, and compromise its ability to control vascular tone via the secretion of vasoactive molecules in response to receptor stimulation. The molecular mechanisms responsible for these effects are, however, poorly understood. In this paper, we describe an e.s.r. spin-trapping investigation into the metabolism of the model hydroperoxide compound *tert*-butylhydroperoxide to reactive free radicals in intact human endothelial cells. The hydroperoxide is shown to undergo a single electron reduction to form free radicals. Experiments with metabolic poisons indicate that the mitochondrial electron-transport chain is the source of electrons for this reduction. The metal-ion-chelating agent desferrioxamine was found to prevent cell killing by *tert*-butylhydroperoxide, but did not affect free radical formation, suggesting that free metal ions may serve to promote free-radical chain reactions involved in cell killing following the initial conversion of the hydroperoxide to free radicals by mitochondria. These processes may well be responsible for many of the reported effects of hydroperoxides on endothelial cell integrity and function.

INTRODUCTION

Damage to the vascular endothelium may be an important event in the promotion of atherosclerosis [1,2]. Endothelial cells perform numerous physiological roles that are vital to the functioning of the vascular system. They provide a non-thrombogenic surface, act as a permeability barrier through which there is exchange of substances into the arterial wall, secrete growth regulatory molecules and cytokines, and maintain vascular tone via the release of small molecules such as nitric oxide, prostacyclin and endothelin [2,3]. Interference with one or more of these processes can lead to endothelial dysfunction, which may elicit further changes that culminate in atherosclerosis. Several lines of evidence indicate that oxidative stress, particularly that involving lipid peroxidation, may play an important role in endothelial cell damage during the development of atherosclerosis [4–6]. As well as affecting endothelial cell function, lipid peroxidation also appears to be involved in other processes associated with atherosclerosis, including the conversion of low-density lipoprotein particles into an oxidized form that is degraded by macrophages much more readily than the native form [7].

Oxidative stress occurs when the delicate redox balance of the cell is disturbed [8,9]. All aerobic cells generate potentially cytotoxic reactive oxygen species. For example, superoxide and hydrogen peroxide are generated in mitochondria by NADH–ubiquinone reductase and ubiquinol–cytochrome *c* reductase [10]. Under normal circumstances, reactive oxygen species are removed by a multi-level antioxidant defence system, consisting of both enzymes and free radical scavengers [9]. Consequently, in good health, the exposure of biomolecules to non-specific oxidation by 'free' reactive oxygen species (including the hydroxyl radical) appears to be minimal [8]. Levels of reactive oxygen species may, however, be enhanced under various conditions, resulting in oxidative stress. For example, activated neutrophils may contribute to endothelial injury by generating large amounts of superoxide during the respiratory burst [4,11,12]. Endothelial cells themselves have also been shown to generate both superoxide and hydrogen peroxide [13–17]. Conversely, oxidative stress may result from a relative deficiency in the cellular antioxidant defence system. For example, many drugs cause glutathione depletion, leaving the depleted cells more prone to oxidative damage. Glutathione deficiency is also believed to lead to oxidative stress in individuals infected with the human immunodeficiency virus [18].

The most important primary antioxidant defence mechanism in many cells, including endothelial cells, is the glutathione–glutathione peroxidase system [19]. Glutathione peroxidases remove hydroperoxides by linking their two-electron reduction to the oxidation of glutathione. The membrane-bound peroxidase reduces phospholipid hydroperoxides and the cytosolic form acts on fatty acid hydroperoxides and hydrogen peroxide [20]. Hydroperoxides are generated as intermediates during lipid peroxidation (which may be initiated by oxygen radicals), and are also formed by lipoxygenase [21,22]. Hydroperoxides are capable of promoting extensive biomolecular damage via the heterolytic cleavage of their peroxidic oxygen–oxygen bonds, which generates reactive alkoxyl and peroxyl free radicals [21,23], and have been implicated as causative agents of endothelial damage during oxidative stress. Linoleic acid hydroperoxide, for example, causes endothelial protein kinase C activation [24], increases the permeability of endothelial cell monolayers to macromolecules [25], and induces damage to the endothelium in laboratory animals [26,27].

The oxidant *tert*-butylhydroperoxide (TBHP), which is a substrate for glutathione peroxidase, has been used in many studies as a model hydroperoxide compound [28–30]. In endothelial cells, TBHP inhibits agonist-induced calcium signalling, causes a progressive increase in the resting cytosolic free Ca^{2+} concentration and induces lipid peroxidation [31–33]. The under-

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DFO, desferrioxamine; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; HFSC, hyperfine splitting constant; LDH, lactate dehydrogenase; TBHP, *tert*-butylhydroperoxide.

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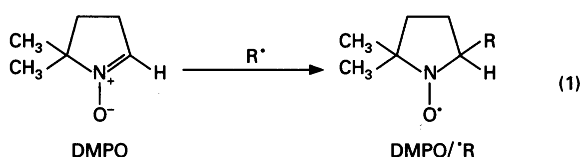
lying molecular mechanisms responsible for these effects are poorly understood. It is possible that many of the effects of hydroperoxides on endothelial cells are indirect, being caused by their free-radical metabolites. In order to address this problem, the present e.s.r. spectroscopy study was undertaken to investigate the possible formation of free radicals in human endothelial cells incubated with TBHP. Because of the extremely short half-lives of most free radicals (e.g. for an alkoxyl radical, $t_{1/2} = 10^{-6}$ s), it is usually necessary to employ spin-trapping compounds to ensure their detection by e.s.r. in biological systems [34,35]. Spin traps react with unstable free radicals to form nitroxide radical adducts, which may be stable for several hours. In this study, we employed the nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which has been used successfully for the detection of a variety of free radicals in biological systems:



From the e.s.r. spectrum of the DMPO radical adduct formed, DMPO/•R, it is usually possible to determine the identity of the parent radical, R• [34,35]. Using this approach, evidence was obtained for the one-electron reduction of TBHP to an alkoxyl radical, which undergoes a rapid β-scission reaction to form a carbon-centred radical. The formation of these free radicals from TBHP may be responsible for some of the pharmacological and toxicological effects of the compound towards endothelial cells.

MATERIALS AND METHODS

Human umbilical vein endothelial cells

Cells were isolated from umbilical cords essentially as described [36]. Veins were cannulated and flushed with PBS (170 mM NaCl, 10 mM Na_2HPO_4, 3.3 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) before being filled with 0.05% (v/v) collagenase in serum-free M-199 medium (GIBCO). Following a 10 min incubation at 37 °C (5% CO_2), the collagenase was removed by flushing with PBS and centrifugation for 10 min at 500 *g* to pellet the cells. The cells were then resuspended in growth medium [M-199 containing 20% (v/v) fetal calf serum, 2 mM L-glutamine, 200 i.u./ml penicillin and 100 i.u./ml streptomycin] and seeded into Nunc T25 cm^3 culture vessels. Confluent cells were then passaged once into T80 cm^3 vessels and grown to confluence in medium supplemented with 0.02 mg/ml endothelial cell growth factor supplement (Sigma) and 0.09 mg/ml heparin. The cells were identified as endothelial at day 2 of culture by immunofluorescent staining for factor VIII antigen [37]. Confluent monolayers were trypsinized and washed three times with PBS containing 5 mM glucose which had been treated with chelating resin to remove contaminating metal ions using the batch method [38]. Cells were then resuspended in this medium and kept on ice before incubation.

Endothelial cell incubations and e.s.r. spectroscopy

Cells (5×10^5) were pre-incubated, with gentle stirring, at 37 °C in PBS–glucose containing 80 mM DMPO (Sigma) (washed with activated charcoal) for 5 min before the addition of 200 μM TBHP (Sigma) to give a final volume of 0.5 ml. After a further 15 min, cells were transferred to a quartz flat-cell and e.s.r. spectra were recorded using a Varian E104 spectrometer equipped with a rectangular cavity and using the following instrument settings: modulation frequency, 100 kHz; sweep width, 100 G ($1G = 10^{-4}$ T); scan time, 8 min; time constant, 0.5 s; modulation amplitude, 2.5 G; gain, 5×10^4. Spectra which were to be used for computer simulation (Figure 3a) were recorded on a Bruker E106 spectrometer with the modified settings: scan time, 671 s; time constant, 163.84 ms; modulation amplitude, 2 G; gain, 2×10^6. As indicated, incubations were also carried out in which TBHP, cells, or both components were omitted. When the presence of either antimycin A or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (both from Sigma) is indicated, they were included in both the 5 min pre-incubation and the 15 min incubation. Spectra shown in each figure are from incubations carried out on the same day from the same batch of cells and are representative of at least three independent experiments.

Hyperfine splitting constant (HFSC) values were either measured directly from spectra or taken from computer simulations obtained using the SIMEPR program, written by D. R. Duling (Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, NC, U.S.A.). This program sequentially varies all parameters of each radical species until a minimum in the error surface is located. Goodness-of-fit was judged by both a minimum in the sum of the squared residuals and visual comparisons.

Chemical generation of radical adducts

The *tert*-butoxyl and methyl radical adducts of DMPO were generated from TBHP via the addition of 0.4 mM $FeSO_4$ (from a 100 mM stock solution in deoxygenated water) to 0.4 mM TBHP in 50 mM $KHPO_4$ buffer, pH 7, containing 80 mM DMPO, and the spectrum recorded as above, but with the following instrumental modifications: time constant, 0.25 s; scan time, 4 min; modulation amplitude, 1.25 G; gain 8×10^3. The DMPO methyl radical adduct was also generated independently of TBHP, via the addition of 2 mM $FeSO_4$ to 50 mM $KHPO_4$ buffer containing 2 mM H_2O_2, 1 M dimethyl sulphoxide and 80 mM DMPO, and the spectrum was recorded as above, but with a 1.25×10^3 gain setting.

Cytotoxicity assays

Cells were seeded into 24-well plates in growth medium (as above). Two days later, cells were incubated in the presence or absence of 200 μM TBHP in Kreb's Ringer buffer (100 mM NaCl, 50 mM Hepes, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, pH 7.4). Either DMPO (0–80 mM) or desferrioxamine (Sigma) (0–2 mM) was added to control and TBHP-treated samples immediately before the addition of the peroxide. Following a 6 h incubation at 37 °C (5% CO_2), lactate dehydrogenase (LDH) activities in the incubation medium were determined as a measure of cytotoxicity [39]. Total LDH activity was determined for control samples following solubilization with 1% (v/v) Triton X-100.

RESULTS

Following the incubation of endothelial cells with 200 μM TBHP in the presence of DMPO, we obtained an e.s.r. spectrum consisting of signals from at least two trapped radicals (Figure 1a). Similar weaker signals were also obtained using 100 μM TBHP (results not shown). Omission of TBHP, cells, or both components resulted in the failure to detect signals (Figures 1b–1d). The very weak signal detected in the absence of cells is

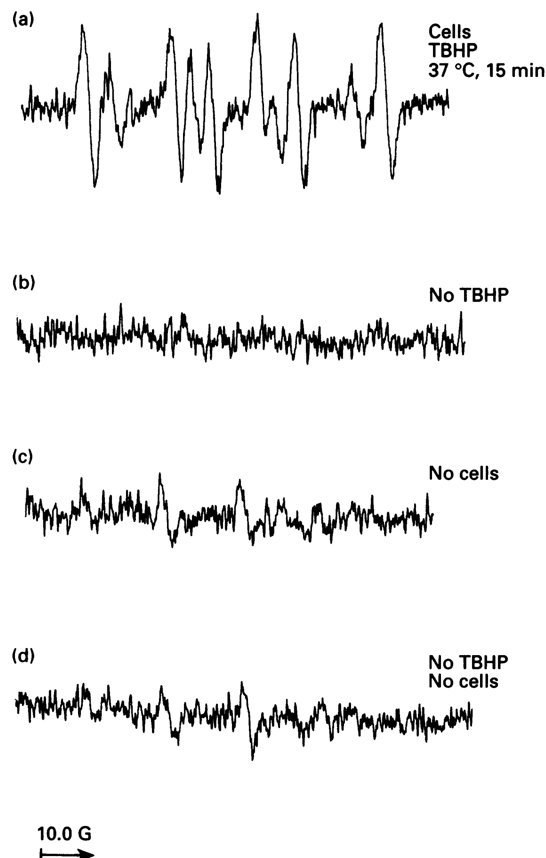


Figure 1 E.s.r. spectra of radical adducts detected following the incubation of endothelial cells with TBHP

(a) Cells (5×10^5) were pre-incubated, with gentle stirring, at 37 °C in PBS–glucose containing 80 mM DMPO for 5 min before the addition of 200 μM TBHP (0.5 ml final volume). After a further 15 min, cells were transferred to a quartz flat-cell and spectra were recorded as described in the Materials and methods section. As indicated, incubations were also carried out in which TBHP (b), cells (c), or both components (d) were omitted.

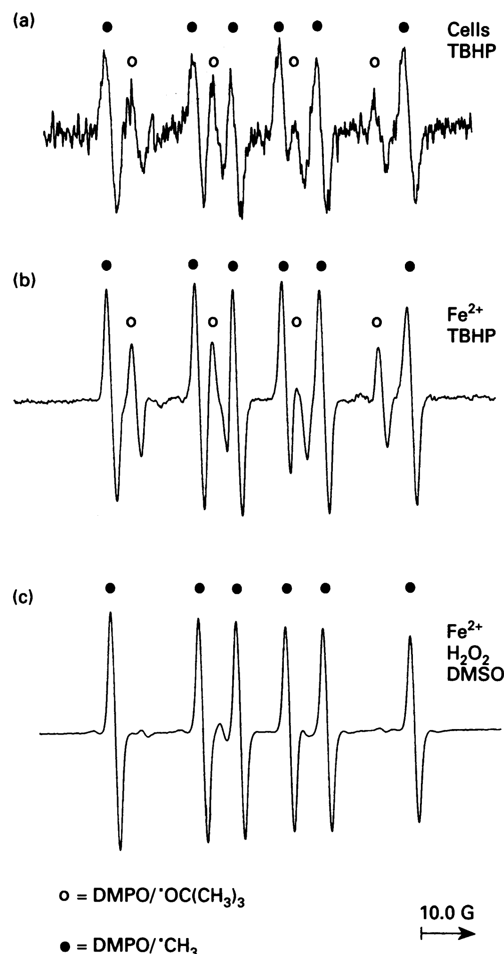


Figure 2 Independent chemical generation of radicals detected in endothelial cell incubations with TBHP

(a) Cells were incubated with TBHP in the presence of DMPO and the e.s.r. spectrum recorded, exactly as described in Figure 1(a) (spectrum reproduced for comparison). Radicals were also generated via the reaction of 0.4 mM $FeSO_4$ with 0.4 mM TBHP in 50 mM $KHPO_4$ buffer, pH 7, containing 80 mM DMPO (b). The DMPO methyl radical adduct was also generated independently of TBHP, using a reaction mixture containing 2 mM $FeSO_4$, 2 mM H_2O_2 and 1 M dimethyl sulphoxide in 50 mM $KHPO_4$ buffer, pH 7, containing 80 mM DMPO (c).

from an impurity in the DMPO, which may be formed via reactions involving contaminating metal ions from the glassware (Figures 1c and 1d). This signal is not present in spectra from samples containing cells alone (Figure 1b), indicating that the species is converted to a non-radical by the cells (see, for example, [40]). For this reason, the contribution of this contaminant signal to the spectrum in Figure 1(a) is also believed to be negligible.

The e.s.r. spectrum shown in Figure 1(a) is very similar to the spectra reported previously following the incubation of TBHP with isolated rat liver mitochondria [41], submitochondrial particles [42], rat liver cytosol [43] or, indeed, red blood cells [44]. In each of these previous studies, spectra were considered to consist of signals attributed to the DMPO adducts of both the *tert*-butoxyl radical [$^{\bullet}OC(CH_3)_3$] and the methyl radical ($^{\bullet}CH_3$). The *tert*-butoxyl radical is generated following the single-electron reduction of TBHP, and the methyl radical is formed via the subsequent β-scission of untrapped *tert*-butoxyl radicals:

$$(CH_3)_3COOH \xrightarrow{1e} (CH_3)_3CO^{\bullet} + OH^- \quad (2)$$

$$(CH_3)_3CO^{\bullet} \rightarrow CH_3(CO)CH_3 + {}^{\bullet}CH_3 \quad (3)$$

In order to confirm the possible presence of signals from these two radical adducts in the e.s.r. spectra reported here, reference spectra were obtained from adducts generated independently in chemical systems. The *tert*-butoxyl adduct [DMPO/$^{\bullet}OC(CH_3)_3$] was generated via the reaction of Fe^{2+} with TBHP (Figure 2b). The e.s.r. spectra of nitroxide radical adducts generally exhibit characteristic hyperfine structure, resulting from interaction of the unpaired electron with the magnetic nuclei of the nitroxide nitrogen and β-hydrogen moieties. The magnitudes of these interactions are reflected in the nitrogen and the β-hydrogen HFSC values, which can be measured directly from e.s.r. spectra and therefore used to identify radical adducts. The HFSC values, measured directly from the four-line spectrum of the chemically generated DMPO/$^{\bullet}OC(CH_3)_3$ adduct ($a^N = 15.12$ G, $a^H_\beta = 16.0$ G) (Figure 2b), are in good agreement with those reported elsewhere for this species [41–44], and a reasonable match was obtained when this signal was compared with the four-line signal present in the spectrum from the endothelial cell incubation (albeit limited by the poor signal-to-noise ratio) (Figure 2a). The stronger (six-line) signal, also present in the

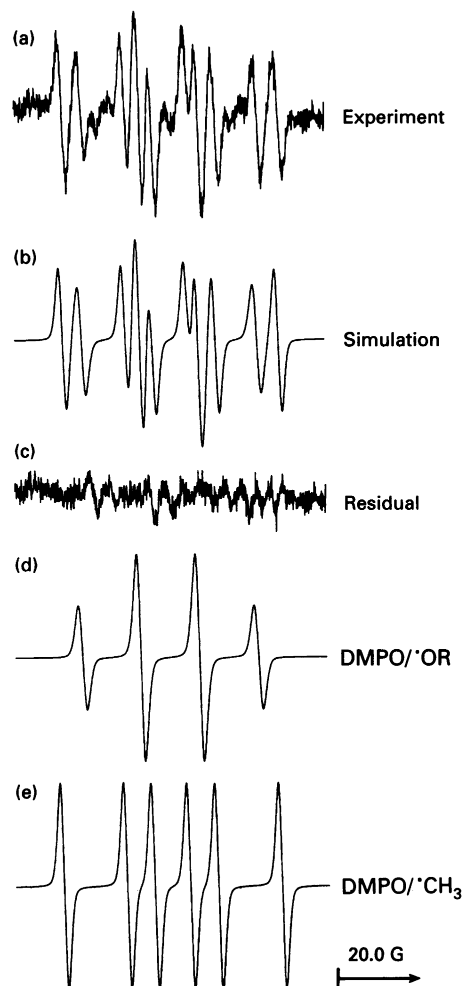


Figure 3 Experimental and computer simulated spectra of radical adducts detected following the incubation of endothelial cells with TBHP

(a) Cells were incubated for 15 min with 200 μM TBHP (as in Figure 1a) and the spectrum was recorded. (b) Simulated spectrum (see text for HFSC values obtained) ($r = 0.968$). (c) Residual spectrum obtained following the subtraction of (b) from (a). (d) Simulation of four-line component signal, in which R is either •OH or •OC(CH_3)$_3$. (e) Simulation of six-line component signal, assigned to DMPO/•CH_3.

spectrum from the chemical reaction, is from the DMPO methyl radical adduct (DMPO/•CH_3) ($a^N = 16.3$ G, $a^H_\beta = 23.4$ G) (Figure 2b). This signal is present in the spectrum obtained from the cell incubation (Figure 2a). The DMPO/•CH_3 adduct was also generated independently of the DMPO/•OC(CH_3)$_3$ adduct, via reaction of the hydroxyl radical (from Fenton's reagent, Fe^{2+}–H_2O_2) with dimethyl sulphoxide [45]. The six-line signal obtained from the DMPO/•CH_3 adduct (Figure 2c) is identical to the signal obtained from both the Fe^{2+}–TBHP reaction (Figure 3b) and the endothelial cell incubation with TBHP (Figure 2a). Although our assignment of the strong six-line signal present in the spectrum from the endothelial cell incubations to the DMPO/•CH_3 adduct is unambiguous, the poor signal-to-noise ratio of this spectrum makes the assignment of the weaker four-line signal to the DMPO/•OC(CH_3)$_3$ less conclusive. A more accurate determination of HFSC values can be obtained from poorly resolved spectra, containing more than one species, using computer-simulation techniques. A simulation of the multi-component signal obtained following the incubation of TBHP with endothelial cells in shown in Figure 3(b), along with simulated spectra of the two individual component signals (Figures 3d and 3e). The residual spectrum (Figure 3c) was obtained by subtraction of the simulated spectrum (Figure 3b) from the experimental spectrum (Figure 3a). The HFSC used by the computer program to reproduce the six-line component signal of the spectrum ($a^N = 16.14$ G, $a^H_\beta = 23.17$ G) confirm that this signal is from the DMPO/•CH_3 adduct [41–44,46]. However, the HFSC used to simulate the four-line component signal of the spectrum ($a^N = 15.04$ G, $a^H_\beta = 15.02$ G) does not confirm unambiguously that the signal is from the DMPO/•OC(CH_3)$_3$ adduct. Indeed, the small a^H_β splitting (cf. the 16.0 G value obtained from the chemically generated *tert*-butoxyl adduct) brings the HFSC values for this signal closer to those of the DMPO hydroxyl radical adduct (DMPO/•OH) [47]. Because the *tert*-butoxyl radical is the precursor of the methyl radical (reactions 1 and 2), the detection of the DMPO/•CH_3 adduct confirms that TBHP has undergone a single-electron reduction by the endothelial cells and that the *tert*-butoxyl radical has been formed. However, at the poor signal-to-noise ratio achieved, even with computer simulation, it is not possible to assign unambiguously the four-line signal to DMPO/•OC(CH_3)$_3$, DMPO/•OH or possibly a mixture of both (the computer-simulated spectrum obtained when two trapped oxygen-centred-radicals were included was not as satisfactory as that obtained using a single trapped oxygen-centred radical).

We next sought to identify the reductant necessary for the formation of these radicals from TBHP. Measurements of LDH from cells at the concentrations of TBHP employed in these experiments failed to detect significant cell killing during the short incubation periods (results not shown). This suggests that the radicals are not generated as a consequence of cell death involving, for example, the interaction of TBHP with decompartmentalized metal ions and reducing agents. Similarly, incubation of cells with TBHP at 4 °C, rather than 37 °C, resulted in the failure to detect signals, indicating the involvement of metabolic, rather than purely chemical, processes (results not shown). It has been reported previously that isolated rat liver mitochondria can bring about the reduction of TBHP to the same free radicals as detected in our endothelial cell incubations [41]. In order to identify a possible role for mitochondria in the reduction of TBHP by intact endothelial cells, incubations were carried out in the presence of the respiratory-chain poison antimycin A and the uncoupling agent CCCP. No e.s.r. signals were detected in the presence of antimycin A (Figure 4b), indicating that electron transfer from the mitochondrial respiratory chain to TBHP is responsible for free radical formation. Radical formation was also inhibited by CCCP, which again indicates a role for the mitochondrial electron-transport chain in TBHP reduction to free radicals (Figure 4c).

Isolated rat liver microsomes have also been shown to metabolize TBHP to free radicals, largely via cytochrome-*P*-450-dependent mechanisms [48]. The cytochrome-*P*-450 inhibitor metyrapone was found to have no effect on radical generation in our system (results not shown). Similarly, the iron-chelating agent desferrioxamine (DFO) has been reported to protect hepatocytes from killing by TBHP [29]. The presence of DFO in endothelial cell incubations had no effect on radical generation (results not shown), suggesting that reactions involving free iron are not responsible for the conversion of TBHP to free radicals. Iron could, however, participate in reactions necessary for cell killing that occur following radical generation. In order to examine this possibility, we measured cell killing by TBHP, as LDH leakage, in both the absence and presence of DFO. In

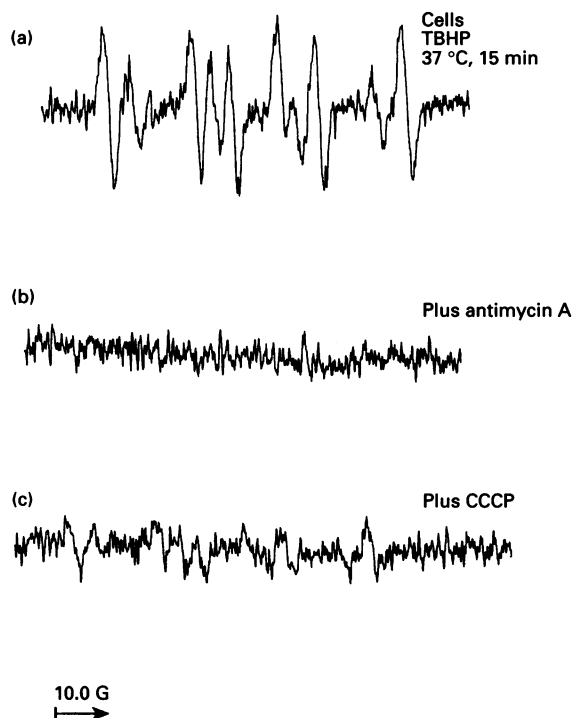


Figure 4 Effects of antimycin A and CCCP on radical generation from TBHP by endothelial cells

Cells were incubated for 15 min with 200 μM TBHP (as in Figure 1a), either with no additions (a) (spectrum reproduced from Figure 1a for comparison), or with the addition of either 100 μM antimycin A (b) or 50 μM CCCP (c). For incubations (b) and (c), cells were pre-incubated in medium containing either antimycin A (b) or CCCP (c) at 37 °C for 5 min before the addition of TBHP.

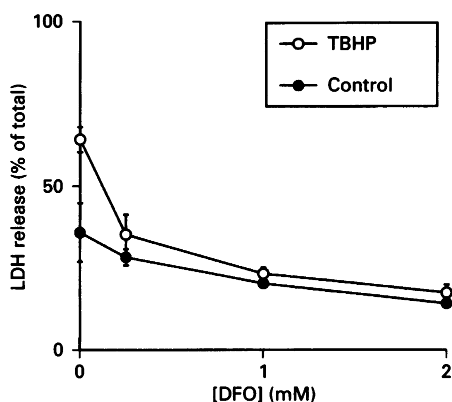


Figure 5 Effects of TBHP and desferrioxamine on LDH leakage from endothelial cells

Cells were incubated in the presence or absence of 200 μM TBHP in Kreb's Ringer buffer (see text for details). As indicated, desferrioxamine (0–2 mM) was added to control and TBHP-treated samples immediately before the addition of the hydroperoxide. Following a 6 h incubation at 37 °C (5% CO_2), LDH levels in the incubation medium were determined. Data represent means ± S.D. ($n = 4$).

order to induce a high level of cell killing by TBHP in cells not treated with DFO, 6 h incubations were carried out. As shown in Figure 5, after incubation for 6 h, almost twice as much LDH was released from cells incubated with 200 μM TBHP compared with cells not given the peroxide. DFO was found to reduce the toxicity of TBHP (Figure 5), indicating that iron may be involved in killing reactions (the high levels of LDH released from cells not given TBHP is believed to reflect cell killing caused by incubation in the absence of serum). Further support for the proposal that free radical formation may be responsible for the cytotoxicity of TBHP towards endothelial cells is provided by the finding that the spin-trap DMPO also prevents cell killing by the reagent (Figure 6).

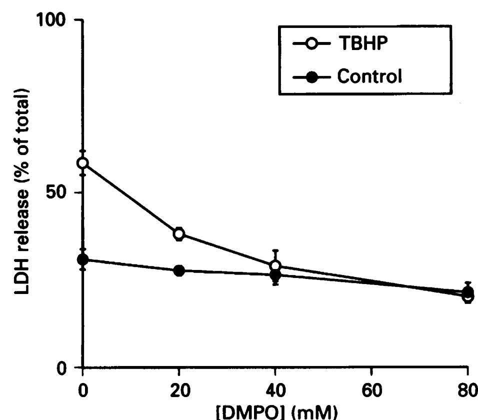


Figure 6 Effects of TBHP and DMPO on LDH leakage from endothelial cells

Cells were incubated in the presence or absence of 200 μM TBHP in Kreb's Ringer buffer (see text for details). As indicated, DMPO (0–80 mM) was added to control and TBHP-treated samples immediately before the addition of the hydroperoxide. Following a 6 h incubation at 37 °C (5% CO_2), LDH levels in the incubation medium were determined. Data represent means ± S.D. ($n = 4$).

DISCUSSION

The findings presented here provide direct e.s.r. evidence for free radical formation from a hydroperoxide by human endothelial cells. Due to the poor signal-to-noise ratios obtained from cell incubations containing TBHP, it is not possible to assign unambiguously the four-line component signal present in spectra to the *tert*-butoxyl adduct of DMPO: the small β-hydrogen coupling for this signal indicates that it may arise from the DMPO hydroxyl radical adduct [47]. Although the DMPO/$^{\bullet}$OH adduct has been detected in systems containing hydroperoxides [49,50], it is not an obvious metabolite of TBHP and may be formed as an artefact of the spin-trapping technique. For example, the DMPO hydroxyl radical adduct can be formed via nucleophilic substitution reactions in which the attacking radical is not trapped but is replaced with water [51]. Metal ions can also stimulate formation of the DMPO/$^{\bullet}$OH adduct via the non-radical nucleophilic addition of water to DMPO, even in the presence of a hydroperoxide [50,52]. Therefore, although it appears (from the HFSC values) that the four-line signal detected in our system is probably from the DMPO/$^{\bullet}$OH adduct, it is considered unlikely that this reflects the trapping of free hydroxyl radicals. The detection of the DMPO/$^{\bullet}CH_3$ adduct in endothelial cell incubations, however, confirms that TBHP has undergone a single-electron reduction and proves, indirectly, that *tert*-butoxyl radicals have been generated, regardless as to whether or not they have been trapped and contribute to the four-line signal.

The inhibition of free-radical formation by the mitochondrial poison antimycin A suggests that the mitochondrial electron-transport chain is the subcellular site at which the single electron reduction of TBHP occurs. This conclusion is consistent with the mechanism proposed by Kennedy et al. [53] who, employing isolated rat liver mitochondria, showed that TBHP is reduced by a cytochrome component of the respiratory chain [53]. Inhibition of radical formation by the uncoupling agent CCCP is also consistent with this mechanism: when mitochondria are uncoupled, the respiratory-chain components are largely in the oxidized state, and therefore electron transfer to TBHP is prevented [53]. Uncouplers have a similar effect on mitochondrial superoxide radical formation, which also depends on electron leakage from the respiratory chain [54].

Because it is widely recognized that mitochondria are an important target for TBHP toxicity [28,30], the above findings suggest that free radical formation may play a role in the toxicity of TBHP. Moreover, the observation that the spin trap DMPO protects endothelial cells from TBHP (determined by LDH release) suggests that free radical formation may be directly involved in cell killing by the hydroperoxide. The iron-chelating agent DFO also protects endothelial cells from TBHP. DFO does not, however, prevent formation of the free radicals detected in the system described here. Although this would indicate that iron participates in reactions other than the initial activation of the hydroperoxide to free radicals by mitochondria, it is possible that the cellular uptake of DFO during the shorter incubation periods used in the e.s.r. experiments is insufficient for the chelator to influence radical formation. Indeed, in their studies employing isolated rat liver mitochondria, Kennedy et al. [53] found that chelating agents cause a slight attenuation of the signal from the DMPO/$^{\bullet}CH_3$ adduct, suggesting that TBHP interacts with a tightly bound metal to form free radicals. Similarly, using submitochondrial particles, Massa and Giulivi [42], have provided evidence for the participation of a tightly bound copper pool in the reduction of TBHP to free radicals. Iron is also expected, however, to promote the chain reactions of lipid peroxidation, which may be ultimately responsible for cytotoxicity [29], following initiation by TBHP-derived radicals. In support of this, Masaki et al. [29] found that pre-treatment with DFO markedly inhibited lipid peroxidation in hepatocytes exposed to TBHP.

As well as the induction of lipid peroxidation, free radicals generated from TBHP are also expected to cause oxidation of other critical target molecules, including nucleic acids and proteins (particularly at thiol moieties), which may be responsible for many of the reported injurious effects of the hydroperoxide on endothelial cells. For example, the inhibition of agonist-induced calcium signalling in endothelial cells caused by TBHP has been dissociated from lipid peroxidation and may be due to damage to an ion-conducting protein [33]. Clearly, free radicals are expected to cause oxidative damage to a wide variety of cellular targets, and may well be responsible for some of the numerous effects of TBHP on endothelial cell integrity and function.

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REFERENCES

1 Ross, R. and Glomset, J. A. (1976) N. Engl. J. Med. **295**, 420–425
2 Ross, R. (1993) Nature (London) **362**, 801–809
3 Gimbrone, M. A., Jr. (1976) Prog. Hemostasis Thromb. **3**, 1–28
4 Warren, J. S. and Ward, P. A. (1986) Am. J. Med. Sci. **292**, 97–103
5 Hennig, B. and Chow, C. K. (1988) Free Rad. Biol. Med. **4**, 99–106
6 Halliwell, B. (1993) Br. Med. J. **307**, 885
7 Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3883–3887
8 Halliwell, B. (1993) Haemostasis **23**, Suppl. **1**, 118–126
9 Sies, H. (1993) Eur. J. Biochem. **215**, 213–219
10 Cadenas, E., Boveris, A., Ragan, C. I. and Stoppani, A. O. M. (1977) Arch. Biochem. Biophys. **180**, 248–257
11 Sacks, T., Moldow, C. F., Craddock, P. R., Bowes, T. K. and Jacob, H. S. (1978) J. Clin. Invest. **61**, 1161–1167
12 Siflinger-Birnboim, A. and Malik, A. B. (1993) J. Cell. Physiol. **155**, 234–239
13 Arroyo, C. M., Carmichael, A. J., Bouscarel, B., Liang, J. H. and Weglicki, W. B. (1990) Free Rad. Res. Commun. **9**, 287–296
14 Babbs, C. F., Cregor, M. D., Turek, J. J. and Badylak, S. F. (1991) Lab. Invest. **65**, 484–496
15 Terada, L. S., Willingham, I. R., Rosandich, M. E., Leff, J. A., Kindt, G. W. and Repine, J. E. (1991) J. Cell. Physiol. **148**, 191–196
16 Sundqvist, T. (1991) J. Cell. Physiol. **148**, 152–156
17 Panus, P. C., Radi, R., Chumley, P. H., Lillard, R. H. and Freeman, B. (1993) Free Rad. Biol. Med. **14**, 217–223
18 Staal, F. J. T., Ela, S. W., Roederer, M., Anderson, M. T., Herzenberg, L. A. and Herzenberg, L. A. (1992) Lancet **339**, 909–912
19 Vercellotti, G. M., Dobson, M., Schorer, A. E. and Moldow, C. F. (1988) Proc. Soc. Exp. Biol. Med. **187**, 181–189
20 Maiorino, M., Thomas, J., Girotti, A. W. and Ursini, F. (1991) Free Rad. Res. Commun. **12–13**, 131–135
21 Esterbauer, H. (1993) Am. J. Clin. Nutr. **57**, Suppl., 779S–786S
22 de Groot, J. J. M. C., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., Wever, R. and van Gelder, B. F. (1975) Biochim. Biophys. Acta **377**, 71–79
23 Davies, M. J. (1988) Biochim. Biophys. Acta **964**, 28–35
24 Taher, M. M., Garcia, J. G. N. and Natarajan, V. (1993) Arch. Biochem. Biophys. **303**, 260–266
25 Hennig, B., Enoch, C. and Chow, C. K. (1986) Arch. Biochem. Biophys. **248**, 353–357
26 Yagi, K., Ohkawa, H., Ohishi, N., Yamashita and Nakashima, T. (1981) J. Appl. Biochem. **3**, 58–65
27 Cutler, M. G. and Schneider, R. (1974) Atherosclerosis **20**, 383–394
28 Bellomo, G., Martino, A., Richelmi, P., Moore, G. A., Jewell, S. A. and Orrenius, S. (1984) Eur. J. Biochem. **140**, 1–6
29 Masaki, N., Kyle, M. E. and Farber, J. L. (1989) Arch. Biochem. Biophys. **269**, 390–399
30 Imberti, R., Nieminen, A.-L., Herman, B. and Lemasters, J. J. (1993) J. Pharmacol. Exp. Ther. **265**, 392–400
31 Elliot, S. J., Meszaros, J. G. and Schilling, W. P. (1992) Free Rad. Biol. Med. **13**, 635–650
32 Elliot, S. J. and Doan, T. N. (1993) Biochem. J. **292**, 385–393
33 Elliot, S. J., Doan, T. N. and Schilling, W. P. (1993) J. Pharmacol. Exp. Ther. **264**, 1063–1070
34 Mason, R. P. and Morehouse, K. (1989) in Oxygen Radicals in Biology and Medicine, eds. Simic, M. G., Taylor, K. A., Ward, J. F. and von Sonntag, C. (Plenum, New York), pp. 21–27
35 Mason, R. P., Hanna, P. M., Burkitt, M. J. and Kadiiska, M. B. (1994) Environmental Health Perspectives, in press.
36 Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. (1973) J. Clin. Invest. **52**, 2745–2756
37 Jaffe, E. C., Hoyer, L. W. and Nachman, R. L. (1973) J. Clin. Invest. **52**, 2757–2764
38 Buettner, G. R. (1988) J. Biochem. Biophys. Methods **16**, 27–40
39 Anuforo, D. C., Acosta, D. and Smith, R. V. (1978) In Vitro **14**, 981–988
40 Samuni, A., Carmichael, A., J., Russo, A., Mitchell, J. B. and Riesz, P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7593–7597
41 Kennedy, C. H., Pryor, W. A., Winston, G. W. and Church, D. F. (1986) Biochem. Biophys. Res. Commun. **141**, 1123–1129
42 Massa, E. M. and Giulivi, C. (1993) Free Rad. Biol. Med. **14**, 559–565
43 Davies, M. J. (1990) Free Rad. Res. Commun. **9**, 251–258
44 Maples, K. R., Kennedy, C. H., Jordan, S. J. and Mason, R. P. (1990) Arch. Biochem. Biophys. **277**, 402–409
45 Dixon, W. T., Norman, R. O. C. and Buley, A. L. (1964) J. Chem. Soc. **4**, 3625–3634
46 Timmins, G. S. and Davies, M. J. (1993) Carcinogenesis **14**, 1615–1620
47 Buettner, G. R. (1987) Free Rad. Biol. Med. **3**, 259–303

48 Greenley, T. L. and Davies, M. J. (1993) Biochim. Biophys. Acta **1157**, 23–31
49 Yagi, K., Ishida, N., Komura, S., Oshishi, N., Kusai, M. and Kohno, M. (1992) Biochem. Biophys. Res. Commun. **183**, 945–951
50 Chamulitrat, W., Iwahashi, H., Kelman, D. J. and Mason, R. P. (1992) Arch. Biochem. Biophys. **296**, 645–649
51 Davies, M. J., Gilbert, B. C., Stell, J. K. and Whitwood, A. C. (1992) J. Chem. Soc. Perkin Trans. **II**, 333–335
52 Hanna, P. M., Chamulitrat, W. and Mason, R. P. (1992) Arch. Biochem. Biophys. **296**, 640–644
53 Kennedy, C. H., Church, D. F., Winston, G. W. and Pryor, W. A. (1992) Free Rad. Biol. Med. **12**, 381–387
54 Boveris, A. and Cadenas, E. (1982) in Superoxide Dismutase, vol. II (Oberley, L., ed.), pp. 15–30, CRC Press, Boca Raton, FL
55 Duling, D. R. (1994) J. Magn. Reson. **104**, 105–110

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