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# Detection of superoxide generated by endothelial cells

**(spin trapping/lipid peroxidation)**

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**ABSTRACT** **Superoxide and lipid free-radical generation in cultured endothelial cells treated with menadione or nitrazepam were measured using electron paramagnetic resonance spectroscopy. Superoxide was detected both intracellularly and extracellularly. Extracellular generation of superoxide and hydrogen peroxide was also measured, either by spectrophotometric measurement of succinoylated cytochrome *c* reduction or by polarography. Extracellular superoxide was generated due to reduced menadione diffusing across the plasma membrane and reacting with oxygen to generate superoxide in the medium. Increased intracellular oxygen tension favored intracellular oxidation of reduced menadione, thus decreasing diffusion of reduced menadione from the cells and, hence, decreasing extracellular superoxide production. The nitro anion free radical of reduced nitrazepam, which cannot cross the plasma membrane, did not generate detectable extracellular superoxide. Our results show that intracellular superoxide can be spin-trapped using 5,5-dimethyl-1-pyrroline-1-oxide and that secondary free-radical injury to membrane lipids, due to excess production of partially reduced species of oxygen by intact cells, can be detected by spin-trapping lipid free radicals with phenyl *N-tert*-butylnitrone.**

In 1968, McCord and Fridovich (1) showed that superoxide could be produced by the catalytic action of xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2). The superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) activity of a copper/zinc-containing protein, erythrocuprein, was subsequently described (2). The biological generation and reactions of superoxide are of considerable interest in studies of cellular metabolism and of the pathogenesis of diverse cytotoxic phenomena (3–6). The most difficult obstacle to the examination of biologically generated free radicals has been the identification and quantitation of these highly reactive species.

One method that has been used successfully to identify biologically generated free radicals is spin trapping. This technique consists of using a nitrone or a nitroso compound to "trap" the initial unstable free radical as a "long-lived" nitroxide free radical that can be observed at room temperature using conventional EPR spectrometric techniques. The hyperfine splitting of the spin-trapped adduct provides information that can aid in identification of the original free radical. Because the stable free radical accumulates, spin trapping is an integrative method of measurement and is inherently more sensitive than procedures that measure instantaneous or steady-state levels of free radicals.

We report that superoxide can be spin-trapped in suspensions of intact endothelial cells during the uncoupling of cellular reductases by menadione, a quinone capable of undergoing redox cycling, or by the aromatic nitro compound nitrazepam. Additionally, secondary free-radical injury to cell membranes was observed by spin-trapping lipid free radicals. Finally, no evidence was found for direct cellular production of hydroxyl radical under these conditions. The ability to detect superoxide produced by intact cells will facilitate investigations of the generation, metabolism, and mechanisms of superoxide-mediated cellular homeostasis and injury.

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## MATERIALS AND METHODS

**General Comments.** Menadione (2-methyl-1,4-naphthoquinone), *N,N*-bis(2-[bis(carboxymethyl)amino]ethyl)glycine (diethylenetriaminepentaacetic acid, DETAPAC), bovine erythrocyte Cu/Zn superoxide dismutase, glutathione, glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9), and NADPH were purchased from Sigma. Nitrazepam (1,3-dihydro-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one) was generously provided by Hoffmann–LaRoche. Chelex 100 ion-exchange resin was obtained from Bio-Rad. The spin traps 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and phenyl *N-tert*-butylnitrone (PBN) were synthesized according to the procedures of Bonnett *et al.* (7) and purchased from Eastman Kodak, respectively. Unless otherwise indicated, all buffers were passed through a Chelex 100 column to remove divalent metal ion impurities, as described by Poyer and McCay (8). DETAPAC (1 mM) was included in buffers to prevent hydroxyl radical formation; iron–DETAPAC is unable to catalyze hydroxyl radical generation from hydrogen peroxide plus superoxide (9, 10). EPR spectra were recorded on a Varian Associated Model E-9 spectrometer. NADPH-cytochrome *c* (P-450) reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) was purified from rat hepatic microsomes according to the procedure of Yasukochi and Masters (11), with minor modifications: Emulgen 913 (KAO-Atlas, Japan) at 30% higher concentration was substituted for Renex 690, and the purification was stopped after the affinity-column step. Porcine thoracic aorta endothelial cells were grown according to published procedures (12). These cells had the following enzyme specific activities (units/mg of cellular protein): Cu/Zn superoxide dismutase, 2.1 ± 0.2; Mn superoxide dismutase, 1.7 ± 0.2; catalase, 5.0 ± 0.5; glutathione peroxidase, 0.40 ± 0.05. For EPR studies, cells were harvested by trypsinization, washed, and suspended in 50 mM sodium phosphate/0.15 M NaCl/5 mM glucose/1 mM DETAPAC (pH 7.4). Cells were kept on ice after harvest and were studied at 25°C within 2 hr.

**Biochemical Analyses.** Endothelial cell protein quantitation and oxygen consumption measurement were described previously (12). Extracellular superoxide production was mea-

Abbreviations: DETAPAC, *N,N*-bis(2-[bis(carboxymethyl)amino]-ethyl)glycine; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO-OH, 5-hydroxy-2,2-dimethyl-1-pyrrolidinyloxyl radical; DMPO-OOH, 5-hydroperoxy-2,2-dimethyl-1-pyrrolidinyloxyl radical; DMPO-$CH_3$, 2,2,5-trimethyl-1-pyrrolidinyloxyl radical; PBN, phenyl *N-tert*-butylnitrone; $Me_2SO$, dimethyl sulfoxide.

sured by superoxide dismutase-inhibitable reduction of 30 $\mu$M succinoylated cytochrome *c*, monitored at 550 nm ($\varepsilon_{max}$ = 21 $mM^{-1}cm^{-1}$) according to the method of Kuthan *et al.* (13). The succinoylated cytochrome *c* had an isoelectric point of 4.4. The superoxide-mediated reduction of 30 $\mu$M succinoylated cytochrome *c* was corrected for the lower efficiency of the succinoylated protein in scavenging superoxide (10% compared with that of native cytochrome *c*). This calibration was performed as described (14), by measuring native and succinoylated cytochrome *c* reduction at 550 nm in 50 mM potassium phosphate/100 $\mu$M xanthine, pH 7.4, to which various amounts of xanthine oxidase (grade III, Sigma), were added to give rates of superoxide generation similar to those in our experiments.

**Spin Trapping.** In spin-trapping experiments designed to detect menadione- or nitrazepam-stimulated superoxide generation, reaction mixtures contained suspensions of cultured endothelial cells (≈400,000 cells per ml, equivalent to 0.4 mg of protein/ml), either menadione [0.01–1 mM in 0.14 M dimethyl sulfoxide ($Me_2SO$), final concentration] or nitrazepam (0.1–1.0 mM in 0.14 M $Me_2SO$, final concentration), the spin trap DMPO (0.1 M), and sufficient buffer (50 mM sodium phosphate/0.15 M NaCl/5 mM glucose/1 mM DETAPAC, pH 7.4) to bring the final volume to 0.5 ml. In all spin-trapping experiments, reaction mixtures were transferred to a flat quartz EPR cell and fitted into the cavity of the spectrometer.

Spin trapping of superoxide was also studied using purified NADPH-cytochrome (P-450) reductase. In a typical experiment, the reaction mixture contained the reductase (10 $\mu$g/ml), either menadione (0.1 mM in 0.14 M $Me_2SO$) or nitrazepam (0.1 mM in 0.14 M $Me_2SO$), DMPO (0.1 M), NADPH (250 $\mu$M), and sufficient buffer to bring the final volume to 0.5 ml. The effect of glutathione plus glutathione peroxidase on the EPR spectrum was investigated by adding glutathione peroxidase (10 units) and glutathione (0.1 mM) to the reaction mixture. Control experiments omitted either glutathione or glutathione peroxidase.

Lipid free radicals generated during the incubation of menadione with endothelial cells were detected using the spin trap PBN. In a typical experiment, the reaction mixture contained a suspension of cultured endothelial cells (≈400,000 cells per ml; 0.4 mg of protein/ml), menadione (0.1 mM in 0.1 M $Me_2SO$), 0.09 M PBN, and sufficient buffer to bring the final volume to 0.5 ml.

## RESULTS AND DISCUSSION

Incubation of cultured endothelial cell monolayers with menadione caused cell injury, indicated by a dose-dependent increase in release of $^{51}Cr$ (e.g., 20% at 0.1 mM menadione and 58% at 0.5 mM menadione) into the culture medium by prelabeled cells. In parallel experiments, polarographic measurement of endothelial cell oxygen consumption showed a 21-fold increase in oxygen utilization within seconds after menadione (0.1 mM) was added to cell suspensions (Table 1). This suggested a large increase in partial reduction of oxygen to superoxide, hydrogen peroxide, and other oxidant reaction products by endothelial cells. The decrease in apparent oxygen consumption upon addition of catalase confirms hydrogen peroxide production and accumulation in cell suspensions. If free radicals are generated when endothelial cells are incubated with menadione, spin-trapping techniques should illuminate the nature of these reactive intermediates.

Incubation of suspensions of cultured endothelial cells with menadione (0.1 mM) and the spin trap DMPO resulted in the formation of paramagnetic species (Fig. 1). In the absence of menadione, no EPR spectrum was observed. Scan B in Fig. 1, obtained 10 min after scan A was initiated, points to the existence of three distinct products: 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-$CH_3$), 5-hydroxy-2,2-dimethyl-1-pyrrolidinyloxyl (DMPO-OH), and 5-hydroperoxy-2,2-,dimethyl-1-pyrrolidinyloxyl (DMPO-OOH). Identification of these nitroxide radicals was based on a number of factors. First, it is known that quinones can be biologically reduced to semiquinones, which then autoxidize yielding superoxide (15). Second, previous studies document the reaction of hydroxyl radical with $Me_2SO$, giving a methyl radical (16–18). Finally, the EPR scans shown in Fig. 1 are identical to published spectra of these nitroxides (19, 20).

Table 1. Oxygen consumption by endothelial cell suspensions

| Addition(s) | nmol $O_2$ consumed · $min^{-1}$ · $mg^{-1}$ of protein |
|---|---|
| None | 0.6 |
| Menadione | 12.6 |
| Menadione + catalase | 5.7 |
| Dicoumarol | 0.9 |
| Menadione + dicoumarol | 9.1 |
| Menadione + dicoumarol + catalase | 5.0 |

Each value represents the average of triplicate experiments using the same cell suspension. Oxygen consumption was measured at 25°C in the same ambient air-saturated buffer used for EPR measurements. Cell suspension protein concentration was 0.12 mg/ml. Concentrations used: menadione, 0.1 mM; dicoumarol, 50 $\mu$M; catalase, 300 units/ml.

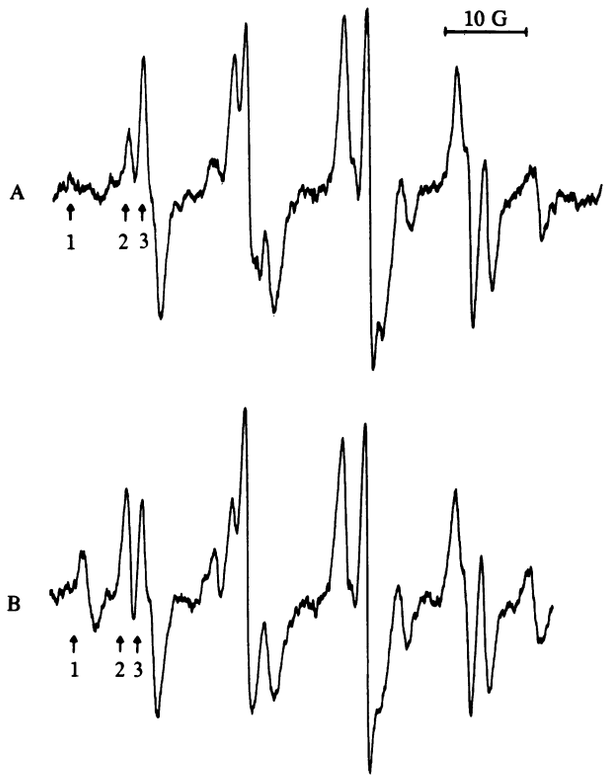


FIG. 1. EPR spectra obtained when endothelial cells were incubated with menadione (0.1 mM in 0.14 M $Me_2SO$) in the presence of the spin trap DMPO. Of interest is the observation that this spectrum is a composite of three different spin-trapped adducts: 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-$CH_3$, peak 1), 5-hydroxy-2,2-dimethyl-1-pyrrolidinyloxyl (DMPO-OH, peak 2), and 5-hydroperoxy-2,2-dimethyl-1-pyrrolidinyloxyl (DMPO-OOH, peak 3). Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 1.0 G. Sweep time was 6.25 G/min, and the receiver gain was $3.2 \times 10^4$ with a response time of 3.0 sec. The spectra were obtained under identical conditions except that scan B was initiated 10 min after the reaction commenced.

An EPR spectrum characteristic of DMPO-OH (Fig. 1) might suggest that hydroxyl radical was produced by menadione-treated endothelial cells. However, this is probably not the case, since DMPO-OOH rapidly decomposes into three species: DMPO-OH, an undefined nonradical, and hydroxyl radical (21, 22). Approximately 3% of DMPO-OH arises via this latter pathway (22). Confirmation that DMPO-OH results from the decomposition of DMPO-OOH and not menadione-stimulated production of hydroxyl radical is borne out by Fig. 1. First, consider the fate of a small quantity of hydroxyl radical generated in the presence of excess $Me_2SO$ (0.14 M, which is 40% greater than the DMPO concentration of reaction mixtures). Hydroxyl radical can react with $Me_2SO$, to give methyl radical, which can then be spin-trapped by DMPO (23). Because the rate constants for the reaction of either hydroxyl radical or methyl radical with DMPO are similar (24), and because the concentration of $Me_2SO$ is larger than that of either hydroxyl radical or DMPO, methyl radical, not hydroxyl radical, will be preferentially spin-trapped by DMPO.

The low-field peak of DMPO-$CH_3$ (whose rate of formation is almost diffusion-limited) was barely detected (scan A, Fig. 1), when DMPO-OOH (whose rate of formation is 10 $M^{-1}$ $sec^{-1}$; see ref. 24) is maximal. Furthermore, $t_{1/2}$ for DMPO-OOH is $\approx$8 min (19), whereas $t_{1/2}$ for DMPO-$CH_3$ is $\approx$1 hr (22). Thus, it appears that hydroxyl radical was not directly generated in cultured endothelial cells during the metabolism of menadione, but arose as the result of DMPO-OOH decomposition. Additional evidence supports this hypothesis. For hydroxyl radical to be generated biologically, a metal ion (e.g., $Fe^{+2}$) must reduce hydrogen peroxide to give hydroxyl radical. Depressing hydrogen peroxide concentration in the extracellular millieu by the addition of catalase should then decrease the amount of hydroxyl radical spin-trapped by DMPO (observed as DMPO-$CH_3$) by virtue of diminished hydrogen peroxide concentration in reaction mixtures. When catalase (300 units/ml) was added to endothelial cells in the presence of menadione (0.1 mM), menadione-stimulated oxygen consumption was decreased, confirming hydrogen peroxide accumulation (Table 1). When catalase was added to menadione-treated endothelial cells for EPR measurements, no change in the concentration of DMPO-$CH_3$ was observed, as compared to controls (minus catalase). Thus, under the conditions described herein, we did not find any compelling evidence for the production of hydroxyl radical during the metabolism of menadione by endothelial cells. The data show that DMPO-$CH_3$ probably results from the decomposition of DMPO-OOH to give small quantities of hydroxyl radical, which then reacts with $Me_2SO$ to produce methyl radicals.

During the metabolism of menadione, superoxide is generated, some arising via the extracellular reaction of menadione with 2-methyl-1,4-dihydroxynaphthalene (menadiol), yielding an autoxidizable semiquinone (25). Dicoumarol inhibition of the two-electron reduction of menadione by DT-diaphorase [NAD(P)H:(quinone-acceptor)oxidoreductase (EC 1.6.99.2); see ref. 25] diminishes extracellular superoxide production. This occurs by decreasing 2-methyl-1,4-dihydroxynaphthalene available for extracellular diffusion and subsequent reduction of menadione to the semiquinone. Even though DMPO will diffuse across cell membranes (26), the EPR spectrum depicted in Fig. 1 could have resulted from intracellularly or extracellularly generated superoxide. Addition of superoxide dismutase (20 $\mu$g/ml) to endothelial cells in the presence of menadione and DMPO gave the EPR spectrum shown in Fig. 2. Only the EPR spectrum of DMPO-OH was observed. As discussed above, absence of the DMPO-OOH EPR signal does not negate the presence of superoxide, but merely defines the stability of DMPO-OOH under the experimental conditions. Since endothelial cells have an active glutathione peroxidase (0.4 unit/mg of protein) that can reduce hydroperoxides to alcohols (27), it may be that, under the experimental conditions described, DMPO-OOH was enzymatically reduced to DMPO-OH at such a rate that EPR-detectable concentrations of DMPO-OOH are never achieved. To test this hypothesis, we incubated purified rat hepatic NADPH-cytochrome *c* (P-450) reductase with menadione, NADPH, and DMPO, and we obtained the typical DMPO-OOH EPR spectrum (scan A, Fig. 3). When superoxide dismutase (10 $\mu$g/ml) was added to this reaction mixture, no EPR spectrum was observed. When glutathione and glutathione peroxidase were incubated with purified NADPH-cytochrome *c* (P-450) reductase, NADPH, menadione, and DMPO, the EPR spectrum was indicative of DMPO-OH (scan B, Fig. 3). In the absence of either glutathione or glutathione peroxidase, the EPR spectrum obtained was identical to that shown in scan A (Fig. 3). Although these *in vitro* experiments provide only circumstantial evidence for endothelial cellular glutathione peroxidase reduction of DMPO-OOH to DMPO-OH, the similarity of the EPR spectra in Fig. 2 and Fig. 3 (scan B) suggests that this reduction occurs in cellular preparations. EPR spectra of reaction mixtures containing endothelial cells, DMPO, superoxide dismutase (20 $\mu$g/ml), and menadione (0.01–1.0 mM in 0.14 M $Me_2SO$) showed that the spin-trapped superoxide adduct signals (observed as DMPO-OH) were dependent on menadione concentration.

Thor *et al.* (25) proposed that, in hepatocytes, intracellular reduction of menadione yields menadione semiquinone. Reaction with oxygen gives superoxide, which diffuses across the cell membrane into the surrounding medium. We tested whether superoxide diffused from cultured endothelial cells incubated with menadione by measuring succinoylated cytochrome *c* reduction by extracellular superoxide and by spin trapping of superoxide, in parallel experiments. Reduction of cytochrome *c* can be due to enzymatic processes, which are

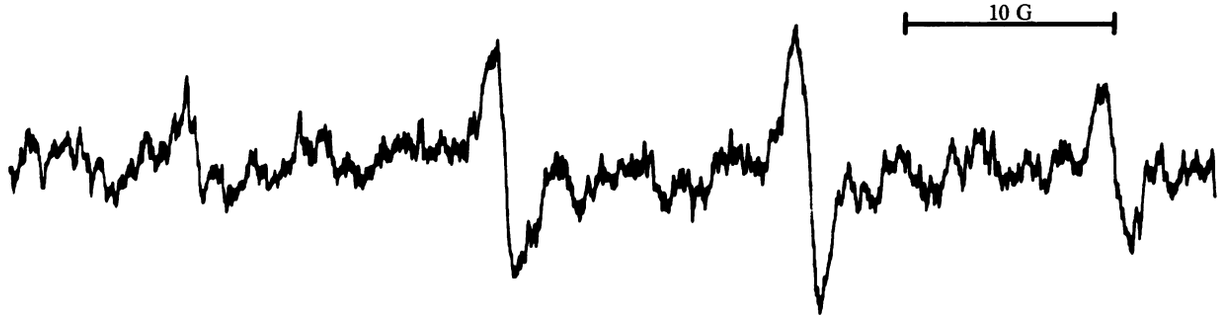


FIG. 2. EPR spectrum obtained when endothelial cells were incubated with menadione (0.1 mM in 0.14 M $Me_2SO$) in the presence of DMPO and superoxide dismutase (20 $\mu$g/ml). Scanning conditions were as described in the legend to Fig. 1, except that the receiver gain was $4.0 \times 10^4$. Hyperfine coupling constants: $A_N = A_H = 14.9$ G.

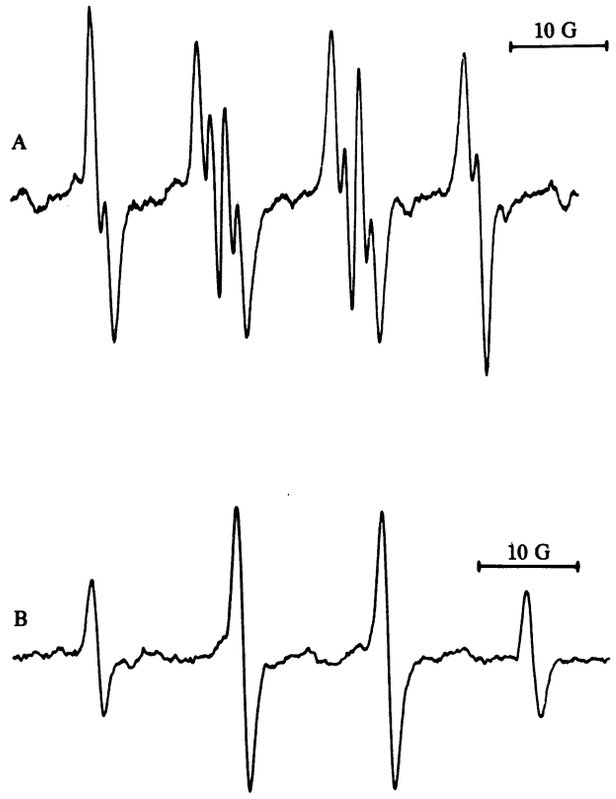


FIG. 3. Scan A: EPR spectrum obtained when purified NADPH-cytochrome *c* (P-450) reductase was incubated with NADPH, menadione (0.1 mM in 0.14 M $Me_2SO$), and the spin trap DMPO. Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 0.63 G. Sweep time was 12.5 G/min, and the receiver gain was $8 \times 10^3$ with a response time of 1.0 sec; $A_N$ = 14.3 G, $A_H^\beta$ = 11.7 G, and $A_H^\gamma$ = 1.25 G. Scan B: EPR spectrum was obtained under identical conditions except that glutathione and glutathione peroxidase were included in the reaction mixture. Sweep time was 25 G/min. $A_N = A_H$ = 14.9 G.

minimized by using the succinoylated protein (14), as well as by reaction with superoxide. Since cytochrome *c* does not diffuse across cell membranes, addition of superoxide dismutase to cell suspensions showed that 15–20% of menadione-stimulated succinoylated cytochrome *c* reduction was enzymatic. The remaining succinoylated cytochrome *c* reduction was a consequence of extracellular superoxide, but this did not prove intracellular to extracellular diffusion (Table 2). Maintenance of cells in 95% oxygen decreased menadione-stimulated superoxide reaction with succinoylated cytochrome *c*. Furthermore, incubation of endothelial cells

Table 2. Reduction of succinoylated cytochrome *c* by endothelial cell suspensions

| Addition(s) | nmol reduced · $min^{-1} \cdot mg^{-1}$ of protein |
|---|---|
| None | 0.42 |
| Superoxide dismutase | 0.42 |
| Menadione | 2.64 |
| Menadione + superoxide dismutase | 0.39 |
| Dicoumarol | 0.38 |
| Menadione + dicoumarol | 1.32 |
| Menadione + dicoumarol + superoxide dismutase | 0.46 |
| 95% oxygen | 0.39 |
| 95% oxygen + menadione | 1.72 |

Each value represents the average of triplicate experiments using the same cell suspension. Succinoylated cytochrome *c* reduction was measured at 25°C in ambient air-saturated buffer, unless otherwise specified. Endothelial cells were suspended (0.18 mg of protein/ml) in the same buffer used for EPR measurements, containing 100 μM succinoylated cytochrome *c* (oxidized form). Conditions, superoxide dismutase, 20 μg/ml; menadione, 0.1 mM; dicoumarol, 50 μM; 95% $O_2$, buffer plus cells were bubbled with 100% oxygen to adjust oxygen in solution to 1.075 mM (95% of saturation).

maintained in 95% oxygen with superoxide dismutase (20 μg/ml), DMPO, and menadione (0.1 mM in 0.14 M $Me_2SO$) led to a marked increase (≈40%) in intracellular spin trapping of superoxide. These results suggest that superoxide does not diffuse across the plasma membrane, but rather that superoxide is produced both intracellularly and extracellularly.

Intracellular endothelial cell reductases can univalently reduce menadione to menadione semiquinone, which can then transfer an electron to oxygen, yielding superoxide. Alternatively, cellular DT-diaphorase can divalently reduce menadione to 2-methyl-1,4-dihydroxynaphthalene, which can diffuse across cell membranes and reduce menadione, yielding menadione semiquinone by disproportionation. Reduction of oxygen by the semiquinone generates superoxide. When cells were maintained in 95% oxygen, increased intracellular oxygen concentration resulted in greater intracellular superoxide generation from oxygen reduction by menadione semiquinone, since the fate of the semiquinone depends on oxygen tension. The concomitant decrease in 2-methyl-1,4-dihydroxynaphthalene available to diffuse out of the cell decreased subsequent extracellular superoxide production (Table 2). Enhanced oxygen concentration has been utilized to demonstrate that superoxide does not diffuse across bacterial cell membranes (28). Addition of dicoumarol (50 μM) to menadione-treated cells also diminished extracellular superoxide, presumably by decreasing 2-methyl-1,4-dihy-

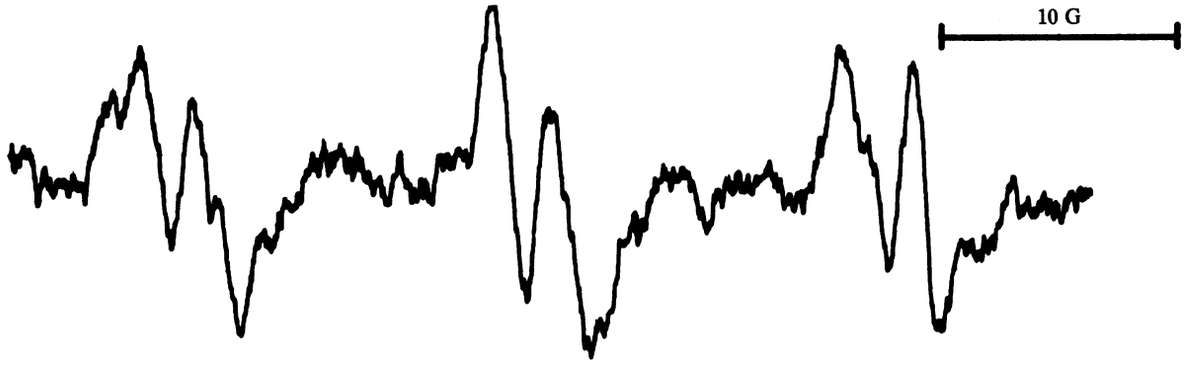


FIG. 4. EPR spectrum obtained when endothelial cells were incubated with menadione (0.1 mM in 0.14 M $Me_2SO$) and the spin trap PBN (0.09 *M*). Scanning conditions were as described in the legend to Fig. 2. $A_N$ = 14.8 G and $A_H$ = 2.5 G.

droxynapthalene available for extracellular diffusion (Table 2). In further support of this concept, the menadione-stimulated oxygen consumption of endothelial cells was depressed 25% (from 12.6 to 9.1 nmol $O_2$ consumed $min^{-1}mg^{-1}$ of protein) upon addition of 50 $\mu$M dicoumarol. This decrease was probably due to inhibition of generation (and, hence, extracellular diffusion) of 2-methyl-1,4-dihydroxynapthalene, which would yield superoxide and then hydrogen peroxide, by dismutation of the superoxide, thus inhibiting apparent oxygen consumption by decreasing hydrogen peroxide concentration. These data support our hypothesis that superoxide does not diffuse across the endothelial cell membrane.

Many aromatic nitro-containing compounds can be reduced by cellular reductases to corresponding nitro anion free radicals (29), to which the cell membrane is impermeable. This permits the study of intracellularly generated superoxide in endothelial cells. We have found that nitrazepam, a nitro-containing benzodiazepine, is reduced by NADPH-cytochrome *c* (P-450) reductase to a nitro anion free radical and that, in the presence of oxygen, superoxide is generated (30). Incubation of endothelial cells with nitrazepam (0.1 mM in 0.14 M $Me_2SO$) and DMPO (0.1 M) resulted in an EPR spectrum identical to that observed when endothelial cells are incubated with menadione, DMPO, and extracellular superoxide dismutase (Fig. 2). When superoxide dismutase (20 $\mu$g/ml) was added to the nitrazepam reaction mixture, no change in the EPR signal was seen. In the absence of nitrazepam, no EPR spectrum was observed. These results, in combination with those obtained using menadione, demonstrate that spin-trapping techniques can detect intracellularly generated superoxide. We examined cellular integrity by studying $^{51}Cr$ and vital dye exclusion and found that, under our conditions, DMPO, $Me_2SO$, menadione, and nitrazepam did not induce significant cell lysis.

Secondary radical damage to cell membranes (e.g., lipid peroxidation) consequent to menadione-induced superoxide generation by endothelial cells was studied using spin-trapping methods. The acyclic nitrone PBN has been used to monitor lipid free-radical formation both *in vitro* and *in vivo* (31, 32). Incubation of endothelial cells with menadione and PBN resulted in the EPR spectrum shown in Fig. 4. Addition of superoxide dismutase (20 $\mu$g/ml) resulted in an EPR spectrum whose line shape was similar to that shown in Fig. 4. However, the height of the signal (and thus the amount of free radicals spin-trapped) was only 10% of that observed in the absence of the dismutase, suggesting that significant lipid peroxidation was caused by extracellularly generated superoxide. No EPR spectrum was observed in the absence of menadione. From the similarity of the hyperfine-splitting constants previously reported (31, 32) to those calculated for the spectrum in Fig. 4, it is apparent that, after endothelial cells are exposed to menadione, lipid free radicals are generated and spin-trapped by PBN. This indicates that superoxide contributes to generation of cellular lipid free radicals.

In conclusion, we have shown that oxygen- and carbon-centered free radicals can be detected in whole cell suspensions by uncoupling electron transport. Thus, it is possible to study the mechanism(s) by which free radicals impinge upon cell integrity and function.

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