Hyperoxic sheep pulmonary microvascular endothelial cells generate free radicals via mitochondrial electron transport.

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J Clin Invest. 1993;91(1):46-52. https://doi.org/10.1172/JCI116198.

Research Article

Free radical generation by hyperoxic endothelial cells was studied using electron paramagnetic resonance (EPR) spectroscopy and the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Studies were performed to determine the radical species produced, whether mitochondrial electron transport was involved, and the effect of the radical generation on cell mortality. Sheep pulmonary microvascular endothelial cell suspensions exposed to 100% O2 for 30 min exhibited prominent DMPO-OH and, occasionally, additional smaller DMPO-R signals thought to arise from the trapping of superoxide anion (O2-.), hydroxyl (.OH), and alkyl (.R) radicals. Superoxide dismutase (SOD) quenched both signals suggesting that the observed radicals were derived from O2-.. Studies with deferoxamine suggested that the generation of .R occurred secondary to the formation of .OH from O2-. via an iron-mediated Fenton reaction. Blocking mitochondrial electron transport with rotenone (20 microM) markedly decreased radical generation. Cell mortality increased slightly in oxygen-exposed cells. This increase was not significantly altered by SOD or deferoxamine, nor was it different from the mortality observed in air-exposed cells. These results suggest that endothelial cells exposed to hyperoxia for 30 min produce free radicals via mitochondrial electron transport, but under the conditions of these experiments, this radical generation did not appear cause cell death.

Hyperoxic Sheep Pulmonary Microvascular Endothelial Cells Generate Free Radicals via Mitochondrial Electron Transport

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Abstract

Free radical generation by hyperoxic endothelial cells was studied using electron paramagnetic resonance (EPR) spectroscopy and the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Studies were performed to determine the radical species produced, whether mitochondrial electron transport was involved, and the effect of the radical generation on cell mortality. Sheep pulmonary microvascular endothelial cell suspensions exposed to 100% O_2 for 30 min exhibited prominent DMPO-OH and, occasionally, additional smaller DMPO-R signals thought to arise from the trapping of superoxide anion (O_2^-), hydroxyl ($\cdot OH$), and alkyl ($\cdot R$) radicals. Superoxide dismutase (SOD) quenched both signals suggesting that the observed radicals were derived from O_2^-. Studies with deferoxamine suggested that the generation of $\cdot R$ occurred secondary to the formation of $\cdot OH$ from O_2^- via an iron-mediated Fenton reaction. Blocking mitochondrial electron transport with rotenone (20 μM) markedly decreased radical generation. Cell mortality increased slightly in oxygen-exposed cells. This increase was not significantly altered by SOD or deferoxamine, nor was it different from the mortality observed in air-exposed cells. These results suggest that endothelial cells exposed to hyperoxia for 30 min produce free radicals via mitochondrial electron transport, but under the conditions of these experiments, this radical generation did not appear cause cell death. (*J. Clin. Invest.* 1993. 91:46–52.) Key words: antioxidant • electron paramagnetic resonance spectroscopy • endothelial cells • oxygen toxicity • spin trapping

Introduction

The pulmonary endothelial cell has been identified as a critical target of oxygen toxicity (1–3). Severe damage that includes swelling of mitochondria and rough endoplasmic reticulum, rupture of the plasma membrane, and decreased cell number has been observed in pulmonary endothelial cells of primates and rodents exposed to hyperoxia (1, 3, 4). Dependent upon duration of hyperoxic exposure, the changes in endothelial cell morphology become progressively more severe and appear to be related to the death of the animals (3). Cultured endothelial cells exposed to hyperoxia have been used to examine the mechanisms of this oxygen-induced injury. After 1–5 d of hyperoxia, monolayers of endothelial cells exhibit decreased DNA synthesis due to reductions in thymidine kinase activity, decreased protein synthesis due to defects in messenger RNA translation, decreased fluidity of the plasma membrane, and increased mortality indicated by elevations in cellular release of lactate dehydrogenase and ^{51}Cr (5–11). Antioxidants, including superoxide dismutase (SOD), glutathione peroxidase, and catalase, reduce many of the hyperoxia-associated changes, suggesting that reactive oxygen metabolites are key mediators of the injury (5, 8, 11).

The cellular mechanisms and biochemical consequences of free radical generation in oxygen toxicity remain uncertain. Previous studies using indirect methods of radical detection, have suggested that hyperoxia increased free radical production in lung tissue slices and mitochondria isolated from whole lung tissue (12, 13). To determine whether endothelial cells are a source of radicals in lung, we measured free radical generation from suspensions of intact sheep pulmonary microvascular endothelial cells exposed to 100% oxygen for 30 min. We used electron paramagnetic resonance (EPR)[1] spectroscopy and the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), to quantitate and characterize the radical species generated by the hyperoxic cells (14). Experiments were also performed to determine whether mitochondrial electron transport was an important source of radical generation in these cells and whether there was a correlation between free radical generation and cell mortality.

Methods

Sheep pulmonary microvascular endothelial cell cultures. Endothelial cells were isolated from sheep peripheral lung tissue (15). The tissue (1–2 cm^3) was shredded into small pieces with forceps, rinsed twice with Hanks' balanced salt solution (HBSS), and then incubated with 1 mg collagenase (C-9891, Sigma Chemical Co., St. Louis, MO)/ml HBSS for 20 min at 37°C. HBSS containing 20% fetal bovine serum (FBS) was added to the digested tissue and the tube was centrifuged at 150 *g* for 5 min. The pellet was resuspended in 48 ml of Medium 199 supplemented with 10% Nu-Serum IV (Collaborative Research, Inc., Bedford, MA), 10% FBS (Hyclone Laboratories, Inc., Logan, UT), 100 U of penicillin/ml, 100 μg of streptomycin/ml, 2 mM L-glutamine, and 0.25 μg of fungizone/ml (complete medium), and poured into tissue culture dishes. The dishes were then incubated at 37°C and monitored daily for growth of endothelial cells.

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Received for publication 4 March 1992 and in revised form 30 June 1992.

J. Clin. Invest.

0021-9738/93/01/46/07 $2.00
Volume 91, January 1993, 46–52

1. *Abbreviations used in this paper:* DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; EPR, electron paramagnetic resonance.

Endothelial cell colonies were isolated by a cloning ring (Belco, Vineland, NJ) and exposed to 0.05% trypsin with 0.53 mM EDTA in calcium and magnesium-free Dulbecco's phosphate-buffered saline (hereafter referred to as PBS) for 4 min at 37°C. Complete medium was added and the cells were transferred to one well of a 24-well plate. When confluent, the cells were treated with 0.05% trypsin with 0.53 mM EDTA and transferred to tissue culture flasks. Endothelial cell cultures were positively identified by their cobblestone morphology and factor VIII staining (16). 47 different endothelial cell cultures were isolated and used for experiments at passages 5–7. Seven of the 47 cultures had been frozen at passage 4 or 5, thawed, placed back into culture, and studied at passage 6.

Protocol. Sheep pulmonary microvascular endothelial cells grown to confluence in a T150 flask were treated with 0.05% trypsin with 0.53 mM EDTA in PBS for 4 min at 37°C. Medium 199 with 5% FBS (13 ml) was added to the cells, which were then centrifuged at 150 *g* for 3 min, washed twice with 15 ml of PBS, and resuspended in PBS. Cell counts were performed with a hemocytometer. Cell viability was assessed by exclusion of 0.2% trypan blue dye. Two 7×10^6 cells in 0.8 ml of PBS were exposed to a vigorous stream of either 100% oxygen or air at 24°C for 30 min. In additional experiments, one or more of the following was added to the cells at the initiation of the exposure to oxygen: SOD (2–1,000 U/ml), deferoxamine (0.1 mM), rotenone (20 μM in ethanol), antimycin A (10–100 μM in ethanol), ethanol (2–4%), sodium cyanide (1–10 mM), or succinate (10 mM).

Purified DMPO (17) was added at a concentration of 50 mM for the final 5 min of exposure. One aliquot of the cell suspension (20 μl) was used to determine viability. The remaining cell suspension was transferred to an EPR flat cell which had been flushed with oxygen or air. EPR spectra were recorded at room temperature using an IBM-Bruker ER 300 spectrometer (Bruker Instruments, Inc., Billerica, MA) operating at X-band with a TM 110 cavity. The microwave frequency and magnetic field were precisely measured with a model 575 source locking microwave counter (EIP Microwave, Inc., San Jose, CA), and a Bruker ER 035M nuclear magnetic resonance gaussmeter, respectively. Serial 1-min EPR acquisitions were performed, blocked, and stored in the digital form at 10-min acquisitions. The Bruker digital data were transferred on line to personal computer (Premium Model 386, AST Research, Inc., Irvine, CA) for analysis. The components of the spectra were identified and simulated individually. The double integrals of the simulated spectra were then compared with that of a 0.2 μM aqueous solution of the free radical standard, 2,2,6,6-tetramethylpiperidine-*N*-oxide, measured under identical settings to calculate the concentration of the simulated components. The experimental spectra were then fitted with the appropriate simulated components using a least-squares fitting routine to determine the concentration of each component. During the EPR acquisition, 100% O_2 or air was delivered to the flat cell containing the cell sample via a small tube inserted into the top of the flat cell.

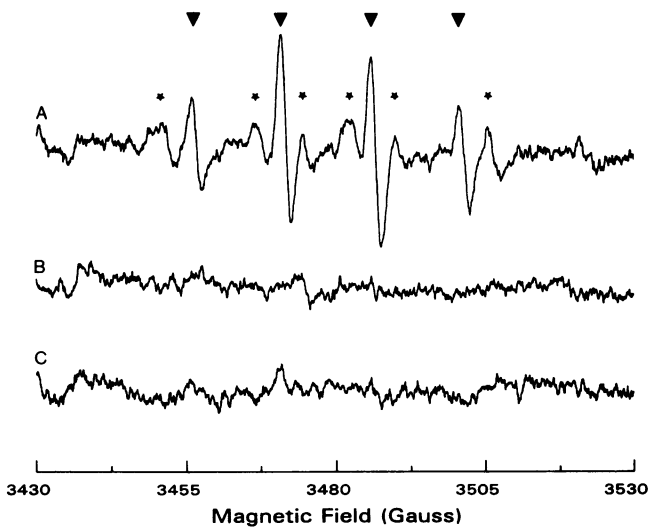

Figure 1. EPR spectra of DMPO adducts formed by endothelial cell suspensions in the presence of DMPO (50 mM) exposed for 30 min to (*A*) 100% O_2, (*B*) air, (*C*) 100% oxygen with 1,000 U SOD/ml. Arrows indicate the quartet generated by DMPO-OH and stars indicate the location of the six peaks generated by DMPO trapped alkyl radicals. Spectra are representative of one experiment and were obtained with microwave power of 20 mW and modulation amplitude of 0.5G.

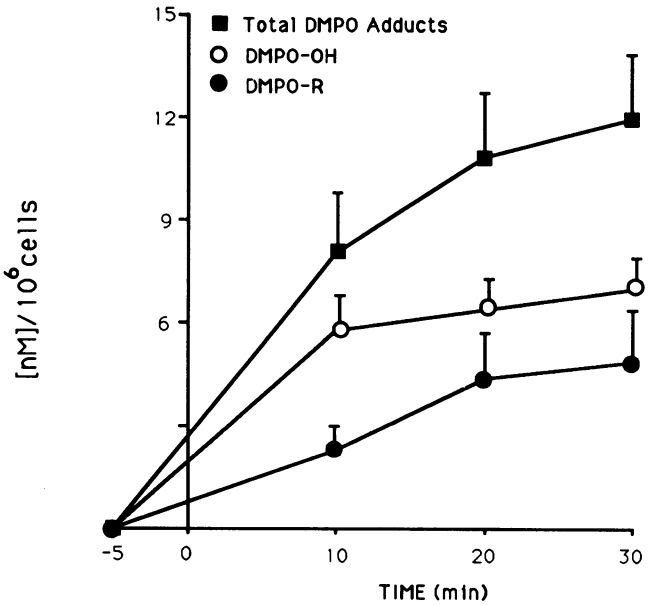

Figure 2. Generation of DMPO-R (●), DMPO-OH (○), or total DMPO adducts (■) by endothelial cell suspensions exposed to 100% oxygen for 30 min ($n = 12$). DMPO (50 mM) was added 5 min before transferring the cells to an EPR flat cell at time 0 when the EPR measurements were begun. Brackets indicate ±1 SE.

Purification of DMPO. DMPO was purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. and further purified by two sequential fractional vacuum distillations (18). The purified DMPO was stored under a nitrogen atmosphere at −20°C in amber vials to prevent light-induced degradation (14, 18). To define the possible background signals generated from DMPO alone, we routinely measured EPR spectra of solutions of purified DMPO (50 mM) in PBS, exposed to oxygen or air in the absence of cells.

Chemicals. The SOD used was the copper-zinc enzyme, either human recombinant from Biotechnology General (New York) or bovine from Sigma Chemical Co. Sodium cyanide was obtained from Aldrich Chemical Co. All other chemicals were obtained from Sigma Chemical Co. Tissue culture reagents were obtained from Gibco (Grand Island, NY).

Data analysis. Comparisons between experimental and control variables were made by analysis of variance. When significant variance ratios were obtained, pairwise comparisons of the means were performed with the least significant difference multiple-range test (19). Student's *t* test for dependent samples was used where appropriate. Differences were considered significant when $P < 0.05$.

Results

57 of 69 (83%) cell suspensions exposed to oxygen for 30 min exhibited free radical signals in the presence of DMPO. These

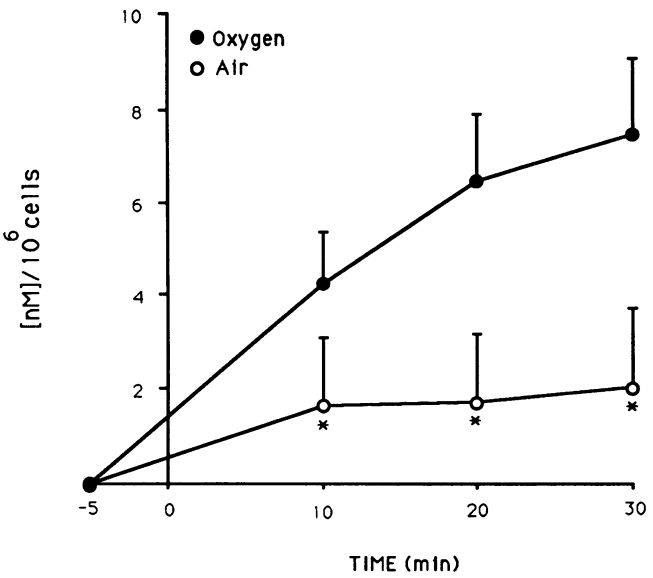

Figure 3. Generation of DMPO adducts by endothelial cell suspensions exposed to 100% oxygen (●, $n = 12$) or air (○, $n = 12$) for 30 min. DMPO (50 mM) was added 5 min before transferring the cells to an EPR flat cell at time 0 when the EPR measurements were begun. Brackets indicate ±1 SE; $^*P < 0.05$ air compared to oxygen.

EPR spectra always contained a 1:2:2:1 quartet pattern (Fig. 1 *A*) with hyperfine splitting constants ($a_N = a_H = 14.9$ G) indicative of the DMPO-OH adduct (20, 21). In 12 of the 57 (21%) preparations that exhibited the DMPO-OH signal, an additional small 1:1:1:1:1:1 sextet signal with hyperfine splitting constants ($a_N = 16.0$ G and $a_H = 22.8$ G) suggestive of trapped alkyl radicals, DMPO-R (20), was observed. As shown in Fig. 2, total DMPO adduct concentration detected from these cells increased from 0 to 20 min, but did not increase further at 30 min. Analysis of the individual components demonstrated that the DMPO-OH and DMPO-R concentrations reached maxima by 10 and 20 min, respectively. Similar time courses were observed for the hyperoxic cell preparations which exhibited only a DMPO-OH signal.

In contrast to hyperoxic cells, air-exposed cells produced little or no EPR signal (Fig. 1 *B*). 4 of 12 cell preparations (33%) exposed to air produced small DMPO-OH signals and one had an additional small DMPO-R signal. As shown in Fig. 3, the mean concentrations of DMPO adducts from air-exposed cells were markedly less than those observed in oxygen-exposed cells studied simultaneously, and did not differ from zero or exhibit a significant time effect (Fig. 3).

To determine whether the observed radical signals were derived from superoxide anions, 14 cell suspensions were exposed to 100% oxygen in the presence of SOD (22). SOD (1,000 U/ml) completely quenched free radical adduct generation in 13 preparations and reduced it by 81% in 1 (Fig. 1 *C*), suggesting that the DMPO radicals were derived from superoxide anions. Inactive SOD, at the same protein concentration as the active enzyme, did not quench the DMPO signal generated by hyperoxic cells. The potency of SOD was assessed in a single experiment in which 500, 200, 20, or 2 U SOD/ml was added to cell suspensions immediately before exposure to 100% O_2. Radical signals were reduced by 93%, 100%, 100%, and 46%, respectively.

To address the possibility that hydroxyl radical contributed to DMPO-OH and DMPO-R formation, cells were exposed to 100% oxygen in the presence of deferoxamine (0.1 mM), a high-affinity iron chelator which blocks the hydroxyl radical-generating Fenton reaction (23). In one experiment in which the hyperoxic cells generated both DMPO-OH and DMPO-R signals, the addition of deferoxamine (0.1 mM) eliminated the DMPO-R signal, but did not significantly change the DMPO-OH concentration (Fig. 4, *A* and *B*). This suggested that the

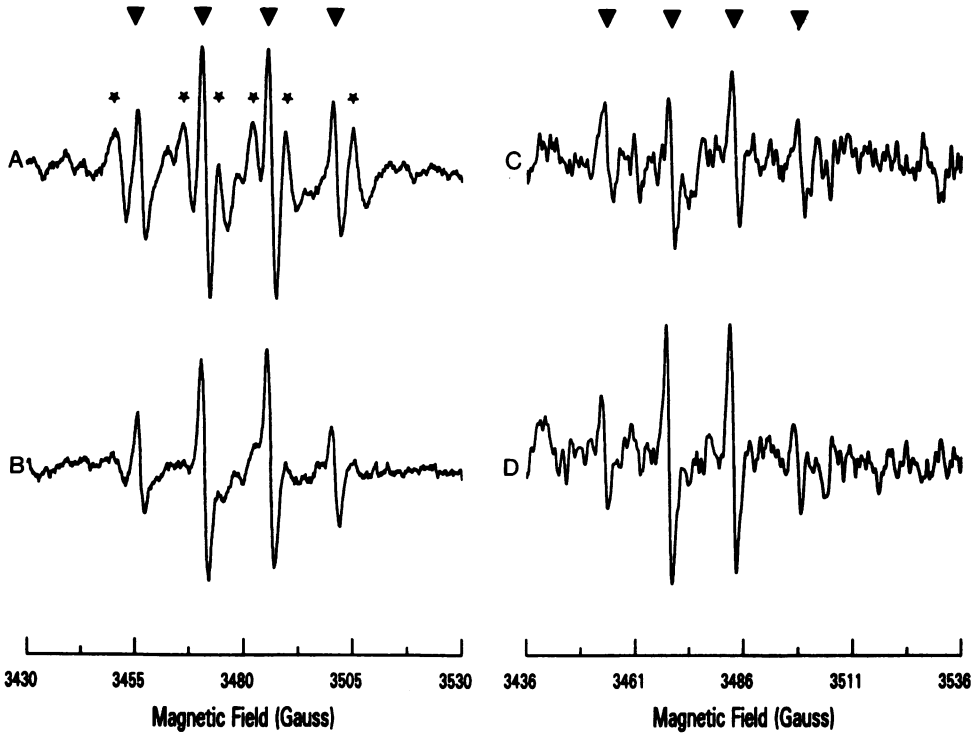

Figure 4. EPR spectra of DMPO adducts formed by endothelial cell suspensions in the presence of DMPO (50 mM) exposed for 30 min to 100% oxygen (*A* and *C*), or 100% oxygen with 0.1 mM deferoxamine (*B* and *D*). Arrows indicate the quartet generated by DMPO-OH and stars indicate the location of the six peaks generated by DMPO trapped alkyl radicals. Spectra were obtained from two different cell populations (*A*, *B* and *C*, *D*, respectively) with microwave power of 20 mW and modulation amplitude of 0.5G.

Fenton reaction was involved in the formation of DMPO-R. In three other experiments, in which DMPO-R was not detectable, deferoxamine (0.1 mM) caused a two-fold increase in the DMPO-OH concentration (Fig. 4, *C* and *D* and Fig. 5). This DMPO radical generation was significantly reduced by SOD (1,000 U/ml, Fig. 5), suggesting the DMPO-OH adduct was derived from superoxide anion.

We routinely assessed the purity of DMPO by measuring the EPR spectra of DMPO (50 mM) in PBS exposed to oxygen or air in the absence of cells. A small 1:2:2:1 quartet signal ($a_N = a_H = 14.9G$) and, in some solutions of DMPO, additional small 1:1:1:1:1:1 ($a_N = 16.0G$ and $a_H = 22.8G$) and 1:1:2:1:2:1:2:1:1 ($a_N = 16.2$ and $a_H(2) = 22.9G$) signals were detected. In that background signals were sometimes observed, we further investigated the effects of SOD and the addition of cells on the DMPO background signals. Addition of superoxide dismutase (1,000 U/ml) did not significantly reduce these radical signals. However, the addition of endothelial cells which had not been exposed to 100% oxygen eliminated the signal. Because these DMPO background signals were eliminated with the addition of cells and not quenched by SOD, they were unlikely to have been a source of the EPR spectra observed in the hyperoxic cell preparations.

To examine the possibility that mitochondrial electron transport was a source of the free radicals generated by hyperoxic cells, experiments were performed with several inhibitors of electron transport (Fig. 6). Since two of these inhibitors (rotenone and antimycin A) were dissolved in ethanol as a vehicle, experiments were also performed to determine the effects of this vehicle. Ethanol (2% or 4%) did not alter the EPR signals produced by hyperoxic cells. Rotenone blocks mitochondrial electron transport from NADH dehydrogenase to coenzyme Q (24). As shown in Fig. 7, rotenone (20 μM) significantly decreased the radical adduct concentration. This decrease was reversed by 10 mM succinate, a substrate which transfers electrons to coenzyme Q via flavin adenine dinucleotide, distal to the site at which rotenone inhibits electron transport (Fig. 6). Preliminary studies have demonstrated the ability of endothelial cell suspensions to utilize exogenous substrate by a 48% increase in oxygen consumption in the presence of 10 mM succinate (83.3±11.5 nl of oxygen/min per 10^6 cells without succinate to 123.7±25.7 nl of oxygen/min per 10^6 cells with succinate, $n = 4$, $P < 0.04$). The radical adduct generation by hyperoxic cells in the presence of rotenone and succinate was completely blocked by SOD (Fig. 7). In isolated mitochondria, antimycin A blocks electron transport from cytochrome *b* to *c* (25). As shown in Fig. 8, 10 μM antimycin A decreased DMPO adduct concentration, whereas 100 μM caused a marked increase. This increase was totally blocked by SOD (1,000 U/ml). Cyanide blocks electron transport from cytochrome aa_3 to oxygen (25). As shown in Fig. 9, 1 mM cyanide did not significantly change the DMPO adduct con-

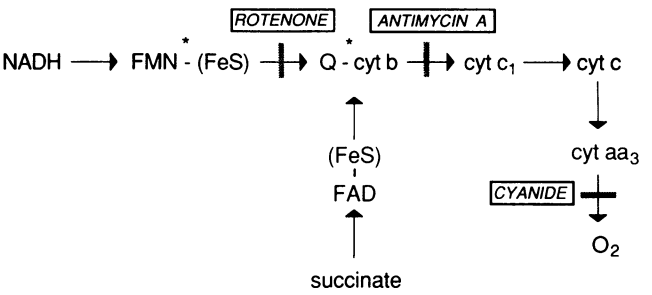

Figure 6. Schematic diagram of the mitochondrial electron transport chain which demonstrates the site of entry for the substrate succinate and the locations of inhibition by rotenone, antimycin A, and cyanide. Asterisks indicate possible sites of superoxide anion production (31, 32).

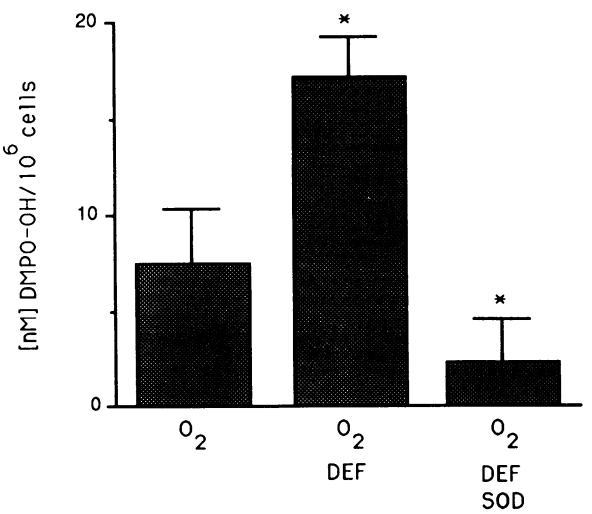

Figure 5. Concentration of DMPO adducts at 25 min after the addition of DMPO to endothelial cell suspensions exposed for 30 min to 100% oxygen in the absence ($n = 3$) and presence of 0.1 mM deferoxamine (*DEF*, $n = 3$) or 0.1 mM deferoxamine and 1,000 U SOD/ml ($n = 3$). Results are expressed as nanomolar DMPO-OH/10^6 cells. Brackets indicate ±1 SE; $^*P < 0.05$ for comparisons with cells exposed to oxygen in the absence of inhibitor.

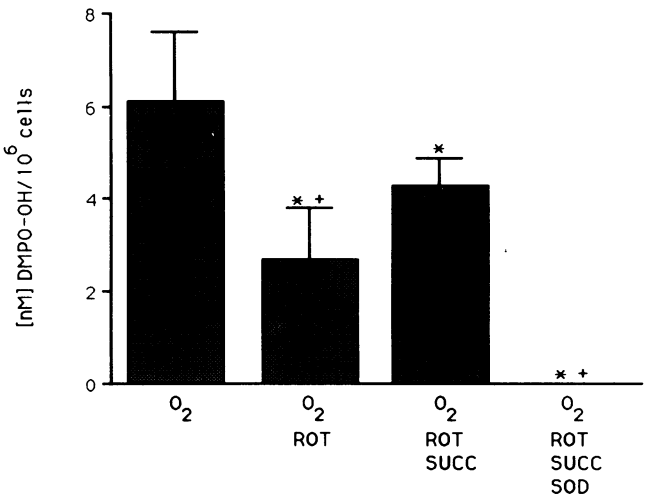

Figure 7. Concentration of DMPO adducts at 25 min after the addition of DMPO to endothelial cell suspensions exposed for 30 min to 100% oxygen in the absence ($n = 5$) and presence of 20 μM rotenone (*ROT*, $n = 5$), or 20 μM rotenone with 10 mM succinate (*ROT + SUCC*, $n = 5$), or 20 μM rotenone with 10 mM succinate and with 1,000 U SOD/ml ($n = 3$). Results are expressed as nanomolar DMPO-OH/10^6 cells. Brackets indicate ±1 SE, $^*P < 0.05$ for comparisons with cells exposed to oxygen in the absence of inhibitor, and $^+P < 0.05$ for comparison with cells exposed to 100% oxygen in the presence of rotenone and succinate.

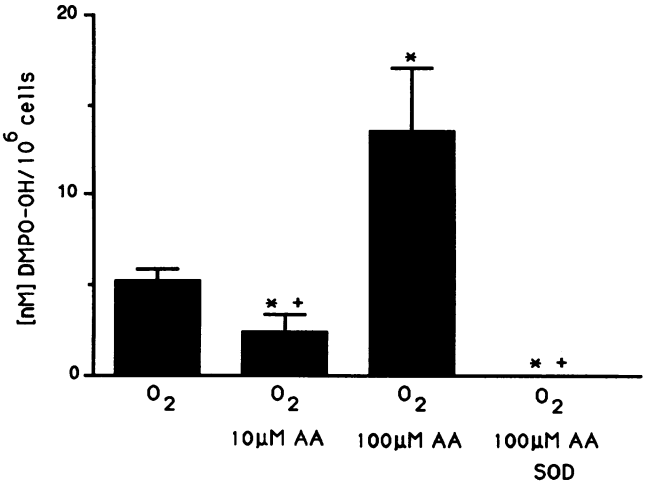

Figure 8. Concentration of DMPO adducts at 25 min after the addition of DMPO to endothelial cell suspensions exposed for 30 min to 100% oxygen in the absence ($n = 7$) and presence of 10 μM antimycin A (*AA*, $n = 4$), 100 μM antimycin A ($n = 3$), and 100 μM antimycin A with 1,000 U SOD/ml ($n = 3$). Results are expressed as nanomolar DMPO-OH/10^6 cells. Brackets indicate ±1 SE; $^*P < 0.05$ for comparisons with cells exposed to oxygen in the absence of inhibitor, and $^+P < 0.05$ for comparison with cells exposed to 100% oxygen in the presence of 100 μM antimycin A.

centration, whereas 10 mM cyanide caused an eight-fold increase. This increase was significantly reduced by the addition of SOD (1,000 U/ml).

Immediately following treatment with trypsin, cell mortality was 13.0±4.5% and was unaffected by the addition of the spin trap DMPO (50 mM). On the average, the 30-min exposure to 100% O_2 resulted in an additional mortality of 4.5±0.9% ($n = 64$). Cells exposed to air for 30 min had a mortality that was not significantly different from oxygen-exposed cells studied simultaneously (1.0±1.8% vs. 5.8±3.3%, $n = 9$, $P = 0.086$). As shown in Table I, the addition of cyanide (10 mM) or cyanide (10 mM) and SOD (1,000 U/ml) during the hyperoxic exposure significantly increased the mortality. No other significant differences were obtained.

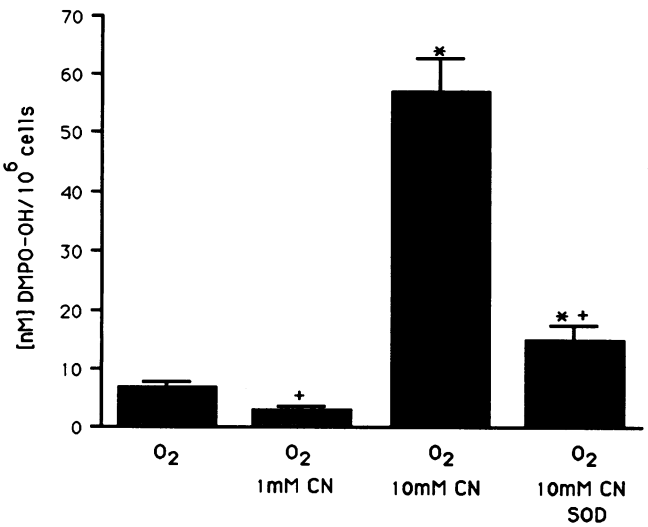

Figure 9. Concentration of DMPO adducts at 25 min after the addition of DMPO to endothelial cell suspensions exposed for 30 min to 100% oxygen in the absence ($n = 7$) and presence of 1 mM cyanide (*CN*, $n = 3$), or 10 mM cyanide ($n = 4$), or 10 mM cyanide with 1,000 U SOD/ml ($n = 3$). Results are expressed as nanomolar DMPO-OH/10^6 cells. Brackets indicate ±1 SE; $^*P < 0.05$ for comparisons with cells exposed to oxygen in the absence of inhibitor, and $^+P < 0.05$ for comparison with cells exposed to 100% oxygen in the presence of 10 mM cyanide.

Discussion

Suspensions of cultured sheep pulmonary microvascular endothelial cells exposed to 100% O_2 generated free radicals which were detected by election paramagnetic resonance spectroscopy using the spin trap, DMPO. The EPR signal generated from the hyperoxic endothelial cells in these experiments had one or two components which were identified by their characteristic hyperfine splitting constants. The predominant component was the DMPO-OH signal, which can arise from either trapped · OH or the decomposition of the O_2^- adduct DMPO-OOH (18, 21). The second component, which was only observed in ~ 20% of the hyperoxic cell preparations, was a smaller DMPO-R signal. This signal suggested generation of alkyl radicals or hydroxyl radicals, since · R can be formed by · OH-mediated hydrogen abstraction (18, 26).

Addition of SOD to cell suspensions exhibiting both DMPO-OH and DMPO-R adducts quenched both signals (Fig. 1), suggesting that both radicals were derived from superoxide anions. This result, which confirms our previous observation (14), can be explained by the generation of · OH and · R from superoxide anion via the Fenton-Haber-Weiss reactions (27):

Table I. Comparison of Cell Death in Endothelial Cell Suspensions Exposed to 100% Oxygen in the Presence and Absence of Inhibitors of Radical Generation

		Cell death*	
Intervention	*n*	100% O_2	100% O_2 + intervention
SOD (1,000 U/ml)	14	6.0±2.7	4.1±1.2
Deferoxamine (0.1 mM)	3	8.7±1.2	12.0±4.4
Rotenone (20 μM)	5	2.0±3.2	1.0±1.5
Rotenone (20 μM) + succinate (10 mM)	5	2.0±3.2	4.4±1.9
Rotenone (20 μM) + succinate (10 mM) + SOD (1,000 U/ml)	3	2.3±1.8	1.7±0.9
Antimycin A (10 μM)	4	0.5±0.9	6.0±4.4
Antimycin A (100 μM)	3	9.0±5.0	25.7±5.5
Antimycin A (100 μM) + SOD (1,000 U/ml)	3	9.0±5.0	18.0±1.2
Cyanide (1 mM)	3	7.0±2.3	5.3±5.0
Cyanide (10 mM)	4	4.5±3.8	82.3±4.2‡
Cyanide (10 mM) + SOD (1,000 U/ml)	3	3.3±5.3	65.7±18.9‡

* Percentage of cells taking up trypan blue dye±SE.
‡ $P < 0.05$ for comparison of cells exposed to 100% O_2 in the presence and absence of intervention.

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$2\,O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$

$$R\text{-}H + \cdot OH \rightarrow H_2O + \cdot R$$

In the presence of the iron chelator deferoxamine, the DMPO-R signal from hyperoxic cells was greatly diminished, suggesting that O_2^- was reacting via these iron-dependent reactions to form $\cdot OH$, which in turn reacted with alkyl groups to form $\cdot R$. In some experiments, deferoxamine increased the DMPO-OH signal. This observation suggests that chelating the iron decreased conversion of O_2^- to $\cdot OH$ thereby increasing the pool of O_2^- available to be trapped by DMPO. Increased trapping of O_2^- may have enhanced the production of DMPO-OOH, that subsequently decomposed to the DMPO-OH adduct (18, 26).

Small concentrations of DMPO adducts with hyperfine coupling constants similar to the adducts derived from hyperoxic cells, were also observed in PBS solutions of DMPO, which had been purified by fractional distillation. Addition of cells to the DMPO solutions, quenched these signals, suggesting that cells may metabolize the adducts to compounds not detectable by EPR (28). Furthermore, in contrast to the DMPO adducts derived from hyperoxic endothelial cells, adducts observed in PBS in the absence of cells were not quenched by SOD. These data suggest that the DMPO adducts that arise from the hyperoxic cells were derived from the cellular generation of superoxide anion, whereas those observed in PBS without cells were not. Finally, little or no signal was detectable from air-exposed cells, suggesting that the radical adduct generation was not only cellular in origin, but was due to the exposure to elevated oxygen tension.

The DMPO-R signal was observed in only a fraction of our cell preparations. Furthermore, 12 of 69 cell suspensions exposed to hyperoxia did not exhibit any EPR signals at all. These results suggest that only certain cell populations could produce O_2^-, $\cdot OH$ and $\cdot R$, or, more likely, that radicals were produced and reacted with cell components more readily than DMPO. In that measurement of radical adduct generation from hyperoxic cells is a dynamic process, dependent upon both production as well as degradation processes, it may be expected that there would be variable responses among cell cultures. For example, it has been demonstrated that variabilities in culture media protein and iron content, culture age, or antioxidant enzyme levels can alter the response of cells to oxidant stress (11, 29, 30). We have not yet examined the role of these or other factors in our system.

Previous studies using isolated lung mitochondria or submitochondrial particles have suggested that the mitochondrial electron transport chain, in particular the ubiquinone-cytochrome *b* region and the NADH dehydrogenase complex (Fig. 6), can generate superoxide anion by a one electron transfer to oxygen (1, 13, 25, 31). The predominant source of mitochondrial superoxide anion production has been attributed to the autooxidation of the ubisemiquinone molecule in the coenzyme Q cycle (25, 31). In our experiments, we used agents which inhibit mitochondrial electron transport at different loci to determine whether the mitochondria were an important source of superoxide anion generation in hyperoxic endothelial cells and to localize the site of this generation.

Rotenone is thought to inhibit mitochondrial electron transport from NADH dehydrogenase to coenzyme Q (24). When electron transport is blocked at this site, the redox state of distal sites which produce radicals may be more oxidized, thereby decreasing the availability of electrons for O_2^- generation. As shown in Fig. 7, rotenone decreased radical concentrations produced by hyperoxic endothelial cells by ~ 60% suggesting that a site distal to that blocked by rotenone, possibly the previously reported ubiquinone-cytochrome *b* site, was responsible for free radical generation in these cells. This possibility is further supported by the observation that the decrease in free radical signal in rotenone-treated cells was partially reversed by succinate, a substrate which provides electrons to coenzyme Q via flavin adenine dinucleotide, thereby bypassing the site of rotenone blockade (Fig. 6).

Antimycin A has been reported to block electron transport from cytochrome *b* to *c* in isolated mitochondria (25). When electron transport is blocked at this site, the redox state of proximal sites, including the ubiquinone-cytochrome *b* region and the NADH dehydrogenase complex, would be expected to become reduced, thereby increasing the availability of electrons for O_2^- generation. Studies using isolated lung mitochondria demonstrated that the addition of antimycin A (2 μM) led to an increased production of O_2^-, as would be predicted (25). In our studies using intact cells, however, antimycin A (10 μM), decreased the concentration of measured radical adducts. With the addition of higher concentrations of antimycin A (100 μM) to intact cells an increase in SOD-inhibitable DMPO radical adduct generation was observed. These results suggested that there may have been incomplete inhibition of mitochondrial electron transport in the hyperoxic cells in the presence of the inhibitor at the lower concentration. Furthermore, the increase was consistent with that observed in isolated mitochondria, supporting the idea that O_2^- generation occurs predominately proximal to the site of inhibition by antimycin A.

Cyanide has been reported to block electron transport from cytochromes aa_3 to oxygen (25). Blocking electron transport at this loci, should shift the redox state of proximal sites toward the reduced state and consequently increase O_2^- generation. Addition of 1 mM cyanide to intact cells did not significantly alter the production of radicals, whereas 10 mM cyanide markedly increased the SOD-inhibitable DMPO radical adduct generation. These results suggest that the lower concentration of cyanide may have been insufficient to completely inhibit electron transport, but at the higher concentration, electron transport was fully inhibited. The resultant increase, once again, suggests that O_2^- generation occurs at the proposed proximal sites.

The experiments with inhibitors of mitochondrial electron transport implicated the mitochondria as an important source of superoxide anion generation. Although this mechanism dictates that hyperoxic endothelial cells generated superoxide anions intracellularly, the locations of the DMPO adducts which were detected by EPR spectroscopy have not been determined. It is possible that DMPO, which is capable of diffusing across cell membranes, reacted intracellularly with the radicals produced by the mitochondria. This possibility implies that the added superoxide dismutase, a relatively large molecule which quenched the formation of DMPO adducts, entered cells via endocytosis or diffusion across breaks in the cell membranes (32, 33). Alternatively, superoxide anions generated within mitochondria may have diffused into the extracellular space through the cell membrane to generate extracellular DMPO adducts where they were quenched by extracellular SOD. Previous studies have suggested that intracellular superoxide anion can traverse the cell membrane via anion channels (34).

Endothelial cells exposed to 100% O_2 exhibited a small increase in cell mortality. This loss of viability was not different from that observed in air-exposed cells and was not significantly altered by the addition of antioxidants. These results suggest that the changes in mortality were a result of nonspecific effects of exposing the cells to a vigorous stream of gas. The absence of a correlation between radical generation and cell mortality may reflect the ability of the cell to protect itself against hyperoxic injury for 30 min. During such exposures, free radical generation may be detectable, but not yet lethal. It is probable that with prolonged exposure to hyperoxia and the associated free radical generation, mortality would increase, as reported for endothelial cell monolayers exposed to hyperoxia for many hours (5–11).

As shown in Table I, the only significant changes in mortality we observed were in cells exposed to a high concentration of cyanide. These cells exhibited marked increases in radical generation which may suggest that enhanced radical generation is associated with greater cell death. The change in mortality, however, is more likely to be a consequence of the effect of cyanide on the cell, in that SOD was able to inhibit the marked increase in radical generation, but did not prevent the cell mortality. Because of the variability of the data and number of experiments performed, we cannot rule out the possibility that antimycin A and/or rotenone may also increase cell mortality.

In summary, pulmonary endothelial cells have the potential to produce superoxide anion, and in some cases, hydroxyl and alkyl radicals. The antioxidant SOD quenched this radical generation, demonstrating that the observed radical signals were derived from superoxide anions. In some cells, the generation of $\cdot R$ occurred secondary to the formation of $\cdot OH$ from O_2^- via an iron-mediated Fenton reaction. Blocking mitochondrial electron transport with rotenone, antimycin A, and cyanide resulted in altered radical generation which implicated the mitochondria, possibly the ubiquinone-cytochrome *b* and NADH dehydrogenase sites of electron transfer, as an important source of superoxide anion generation. Under the conditions of our experiments, 30 min of hyperoxia-induced radical generation did not appear to cause cell death.

Acknowledgments

We express our gratitude to Dr. Richard G. Hansford for helpful discussions and Brenda L. Jordan for preparation of the manuscript.

This work was supported by National Institutes of Health grants HL-17655-13, HL-38324, HL-41970, and HL-34674, Squibb American Heart Association Clinician-Scientist Award, American Lung Association Award, and American Heart Association Award.

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