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Electron paramagnetic resonance evidence that cellular oxygen toxicity is caused by the generation of superoxide and hydroxyl free radicals

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Cells require molecular oxygen for the generation of energy through mitochondrial oxidative phosphorylation; however, high concentrations of oxygen are toxic and can cause cell death. A number of different mechanisms have been proposed to cause cellular oxygen toxicity. One hypothesis is that reactive oxygen free radicals may be generated; however free radical generation in hyperoxic cells has never been directly measured and the mechanism of this radical generation is unknown. In order to determine if cellular oxygen toxicity is free radical mediated, we applied electron paramagnetic resonance, EPR, spectroscopy using the spin trap 5,5'-dimethyl-1-pyrroline-*N*-oxide, DMPO, to measure free radical generation in hyperoxic pulmonary endothelial cells. Cells in air did not give rise to any detectable signal. However, cells exposed to 100% O_2 for 30 min exhibited a prominent signal of trapped hydroxyl radical, DMPO-OH, while cell free buffer did not give rise to any detectable radical generation. This cellular radical generation was demonstrated to be derived from the superoxide radical since the observed signal was totally quenched by superoxide dismutase, but not by equal concentrations of the denatured enzyme. It was confirmed that the hydroxyl radical was generated since in the presence of ethanol the $CH_3 \cdot CH(OH)$ radical was formed. Loss of cell viability as measured by uptake of trypan blue dye was observed paralleling the measured free radical generation. Thus, superoxide and hydroxyl radicals are generated in hyperoxic pulmonary endothelial cells and this appears to be an important mechanism of cellular oxygen toxicity.

Oxygen toxicity; Free radical; Cell injury; Superoxide

1. INTRODUCTION

While mammalian cells require oxygen for life, oxygen can paradoxically be toxic causing cell injury and death. The 21% oxygen contained in air is required for normal aerobic metabolism; however, oxygen tensions of 60–100% are lethal [1–3]. Thus, all cellular life exists in a well defined range of oxygen concentration and tension with lower values limiting aerobic energy metabolism and higher values causing cellular oxygen toxicity. Therapy with increased partial pressures of inspired oxygen is routinely used for treatment of patients with lung disease, heart attack, and heart failure, as well as in healthy divers and aviators. In all these settings, however, it has been noted that prolonged hyperoxia can cause lung damage [1–6]. The endothelial cell has been shown to be an important target of injury. Cellular oxygen toxicity is associated with reduced high energy phosphate concentrations, inactivation of Krebs cycle enzymes, alterations in the electron transport chain, altered glutamate metabolism, depletion of reduced glutathione, and release of lysosomal enzymes. However, investigations have failed to demonstrate whether any of these abnormalities is a cause rather than an effect of oxygen toxicity [3,4]. It has been proposed that oxygen toxicity may be mediated by the formation of highly reactive reduced oxygen free radicals including the

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superoxide anion radical, $O_2^{\cdot -}$, and the even more reactive hydroxyl radical, $OH^{\cdot}$ [3–5]. This free radical generation could be the central mechanism causing the various metabolic abnormalities which lead to cell death. The actual mechanism of cellular oxygen toxicity, however, remains unclear since oxygen free radicals have never been directly measured in hyperoxic cells or shown to be the molecules mediating cellular injury. In this report we have applied electron paramagnetic resonance, EPR, spectroscopy to measure the presence of free radical generation in hyperoxic pulmonary endothelial cells and to determine if this free radical generation can actually result in cellular oxygen toxicity.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Pulmonary endothelial cells were cultured from sheep lungs using a procedure similar to that described previously and used at fifth to seventh passage [7]. Sheep peripheral lung tissue was shredded into small pieces with forceps. The tissue was rinsed twice with Hanks balanced salt solution (HBSS) containing 15 mM Hepes buffer, pH 7.4, then incubated with 1 mg collagenase (CLS II, Cooper Biomedical)/ml for 20 min at 37°C. HBSS containing 10% fetal bovine serum was added, the tube centrifuged at 150 × *g* for 5 min, and the pellet resuspended in 20 ml medium 199 supplemented with 10% Nu serum, 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.25 μg/ml fungizone (complete media), and poured into tissue culture dishes. The dishes were incubated at 37°C and monitored daily for growth of endothelial cells. Once identified endothelial cell colonies were isolated by a cloning ring (Belco) and exposed to 0.05% trypsin, 0.53 mM EDTA in calcium and magnesium free Dulbecco's phosphate buffered saline 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 trypsin (0.05%) and transferred to tissue culture flasks.

Identification as endothelial cells was accomplished by examination of cellular morphology at confluence and by the presence of factor VIII-related antigen as determined by immunofluorescence localization [8]. The cells were grown in T-150 flasks, harvested using 0.05% trypsin in Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium, centrifuged at 100 × *g* for 5 min, washed twice with 10 ml of PBS, and then suspended in the final desired volume of PBS. Cell counts were performed with a hemocytometer. Cell viability was assessed by exclusion of 0.02% trypan blue dye.

2.2. *Electron paramagnetic resonance*

Spin trapping studies were performed by using the spin trap 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) at a final concentration of 50 mM. Care was taken to keep the DMPO containing solutions covered to prevent any light-induced degradation. The DMPO was purchased from Aldrich Chemical Company and further purified by double distillation.

EPR spectra were recorded at room temperature using IBM-Bruker ER 300 spectrometer operating at X-band with a TM 110 cavity. The microwave frequency and magnetic field were precisely measured respectively with a EIP 575 source locking microwave counter and a Bruker ER 035M NMR Gaussmeter. The digitized Bruker spectral data (*x*-resolution: 1×2^{12}, *y*-resolution: 1×2^{32}) were transferred to an AST 286 or AST 386 personal computer (PC) for analysis. Spectral simulations were performed on the PC, using a program which assumes isotropic **g** and **A** tensors, and directly matched with the experimental data. Quantitation of the free radical signals was performed by comparing the double integral of the observed signal to that of a known concentration of the TEMPO free radical in aqueous solution [9].

3. RESULTS

Approximately 7×10^6 cells were suspended in 1.0 ml and exposed to a vigorous stream of either 100% oxygen gas or air at 24°C for 30 min. Double distilled DMPO was added to a final concentration of 50 mM for the final 5 min of exposure, after which the cells were transferred to the EPR flat cell which was prepurged with either oxygen or air and EPR measurements immediately started. Hyperoxic endothelial cells exhibited a prominent 1:2:2:1 quartet EPR spectrum (fig.1A). Measurement and computer simulation of this spectrum demonstrated that the hyperfine splittings were $a_N = a_H = 14.9$ G, indicative of trapped $OH^{\cdot}$,

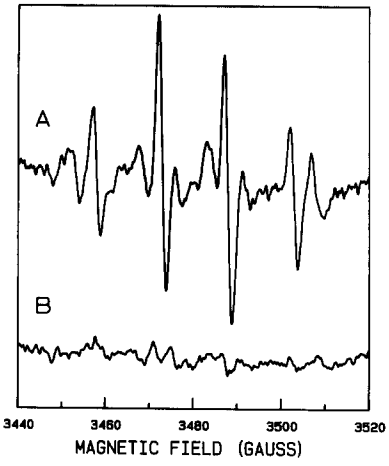

Fig.1. EPR spectra of endothelial cells subjected to 30 min of purging with O_2 (A) or with air (B). Spectra were recorded at room temperature with an IBM ER 300 spectrometer operating at X-band with 100 kHz modulation frequency, 20 mW microwave power and a TM 110 cavity.

DMPO-OH [10]. An additional small 1:1:1:1:1:1 sextet signal was observed with hyperfine splittings a_N = 16.0 G, a_H = 22.8 G suggestive of trapped alkyl radicals, DMPO-R [10]. Experiments were performed to measure the time course of the appearance of the DMPO-OH signal in the hyperoxic endothelial cell preparations. Serial 1 min EPR acquisitions were performed and blocked and stored as 5 or 10 acquisition spectral files. On examination of these spectra it was observed that the intensity of the DMPO-OH signal continued to increase for the first 20–30 min after the start of the EPR measurements (fig.2). In air exposed cells there was no detectable EPR signal (fig.1B). Even after 40 min the air equilibrated endothelial cells did not give rise to any signal (fig.2). In addition, no signal was observed when equal concentrations of DMPO in PBS were exposed to oxygen in the absence of cells. With varying concentrations of cells the intensity of the observed EPR signal appeared to be proportional to the number of cells. Therefore, the measured free radical generation appeared to be due to the effect of hyperoxia on the cells.

In order to determine if the observed DMPO-OH signal was derived from superoxide, similar experiments were performed in which cells were exposed to 100% oxygen in the presence of the superoxide scavenging enzyme, superoxide dismutase (SOD). Prior to exposure to hyperoxia 1000 U/ml of recombinant human copper-zinc superoxide dismutase or an equal concentration of the denatured enzyme, prepared by a modification of the procedure of Hodgson and Fridovich, was added to the cells [11,12]. In the presence of active SOD complete quenching of free radical generation was observed with no detectable EPR spectrum (fig.3A). Identical concentrations of the denatured enzyme did not quench free radical generation and a similar DMPO-OH signal was observed to that seen in the absence of the enzyme (fig.3B).

The DMPO-OH signal can arise either from the direct trapping of the hydroxyl radical, OH·, or from the breakdown of the superoxide adduct of DMPO, DMPO-OOH [10]. In order to determine if hyperoxic pulmonary endothelial cells generate OH·, experiments were performed subjecting the cells to hyperoxia in the presence of ethanol. Generation of the hydroxyethyl radical, CH_3·CH(OH), would be expected in the presence of OH· since OH· will extract a hydrogen atom from ethanol. In the presence of ethanol (5% volume), a 40% decrease in the DMPO-OH signal was observed with the appearance of a prominent 1:1:1:1:1:1 sextet signal a_N = 15.8 G, a_H = 22.8 G indicative of the trapped CH_3·CH(OH) radical, fig.3C [10]. Thus, these experiments confirm that the hyperoxic cells do generate OH·.

To determine if free radical generation by the hyperoxic endothelial cells causes cell injury and cell death, measurements of cell viability were performed in parallel with the EPR measurements on

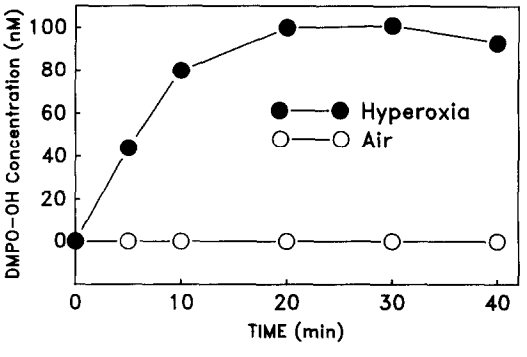

Fig.2. Time course of the appearance of the DMPO-OH signal in a preparation of 1.4 × 10^7 cells/ml exposed to 25 min of hyperoxia followed by addition of DMPO, 50 mM final concentration, and transfer to an oxygen purged EPR flat cell at time 0.

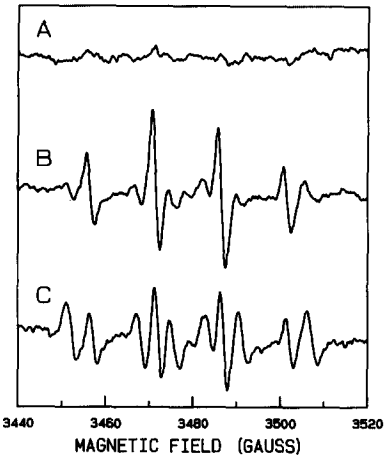

Fig.3. EPR spectra of endothelial cells subjected to 30 min of purging with O_2 in the presence of: (A) 1000 U/ml SOD; (B) an equal concentration of inactivated SOD; (C) ethanol 5% volume. Spectra were recorded as in fig.1.

Table 1

Correlation of radical generation and cell injury

Cell preparation	[Free radical][a] (nM)	Cell death[b] (%)
Air purged	0	0
O_2 purged	100	17
O_2 purged + 1000 U/ml SOD	0	0

[a] Quantitation of [free radical] performed by comparing the double integral of the observed signal to that of a known concentration nitroxide standard in aqueous solution in the same flat cell using nonsaturating power

[b] % cells taking up trypan blue

aliquots of cells removed just prior to the start of the EPR measurements. In repeat experiments with cells exposed to 100% O_2 for 30 min there was a 17–20% loss of cell viability as measured by trypan blue uptake. Cells exposed to air for 30 min, however, showed no loss of viability. It was observed that free radical generation was associated with cell death (table 1). Concentrations of SOD which abolished free radical generation also totally prevented cell death as measured by trypan blue uptake.

4. DISCUSSION

The generation of oxygen free radicals in hyperoxic cells has been hypothesised to be an important mechanism of oxygen toxicity for many years, however, all previous evidence of radical generation has been of an indirect nature. While EPR studies have demonstrated that activated leukocytes are potent radical generators, no solid tissue cell type had been similarly shown to endogenously generate these radicals [14]. It is known that activated endothelial cells phagocytose and mount a respiratory burst. Indirect studies have suggested that this respiratory burst may be accompanied by the generation of $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ [15,16]. Recently, spin trapping EPR studies of exogenous radical generation in endothelial cells have been performed which suggested the feasibility of measuring radicals in these cells using the spin trap DMPO [17]. Subsequently the first EPR measurements of endogenous free radical generation were performed in vascular endothelial cells subjected to an ischemic equivalent of anoxia and reoxygenation; however, no similar measurements of radical generation in hyperoxic cells have been performed [18]. Therefore, in this study we have applied EPR spin trapping techniques in an effort to provide more direct evidence for the formation of these radicals and their importance in the mechanism of hyperoxic cellular injury. Because oxygen toxicity in the lung is an important clinical problem which can occur in patients receiving oxygen and this toxicity is associated with endothelial cell damage we chose to perform these studies in isolated cultured pulmonary endothelial cells.

We observed that cultured pulmonary endothelial cells exposed to high oxygen tensions generate free radicals which are detectable by electron paramagnetic resonance spin trapping techniques. Prominent DMPO-OH signals and smaller DMPO-R signals were observed which totally disappeared in the presence of superoxide dismutase suggesting that $O_2^{\cdot -}$ and $O_2^{\cdot -}$-derived radicals were generated. The generation of the trapped $R^{\cdot}$ radicals implicated the presence of $OH^{\cdot}$ since $R^{\cdot}$ could only be formed by $OH^{\cdot}$ mediated hydrogen atom abstraction and not by $O_2^{\cdot -}$ alone. In the presence of the $OH^{\cdot}$ scavenger ethanol trapping of ethyl radicals was observed demonstrating that $OH^{\cdot}$ was generated. Since copper-zinc superoxide, a relatively large enzyme of 32 kDa which would not passively diffuse across the cell membrane, blocked the formation of DMPO-OH and DMPO-R radical adducts it may be that this $OH^{\cdot}$ generation occurs outside the cell via a metal catalyzed reaction of $O_2^{\cdot -}$ and H_2O_2. It has previously been demonstrated that $O_2^{\cdot -}$ can leave cells via membrane anion channels while H_2O_2 can passively diffuse across the cell membrane [19]. Alternatively, it is possible that some portion of the extracellular superoxide dismutase may have been endocytosed into the cell or entered via cell membrane breakdown. It was observed that the measured free radical generation paralleled cell injury and cell death. Superoxide dismutase concentrations which totally abolished this radical generation also prevented cell death.

Thus, pulmonary endothelial cells exposed to high oxygen tensions generate superoxide and hydroxyl free radicals which in turn cause cell injury or death. These results provide direct evidence for the validity of the free radical hypothesis of cellular oxygen toxicity and suggest that this toxicity can be decreased or prevented by superoxide

dismutase or free radical scavengers. Electron paramagnetic resonance spectroscopy is demonstrated to be a powerful technique for measuring and characterizing free radical generation in hyperoxic cells.

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