Research article

Oxygen consumption and electron spin resonance studies of free radical production by alveolar cells exposed to anoxia: inhibiting effects of the antibiotic ceftazidime

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By EPR spectroscopy, we investigated free radical production by cultured human alveolar cells subjected to anoxia/re-oxygenation (A/R), and tested the effects of ceftazidime, an antibiotic previously demonstrated to possess antioxidant properties. Two A/R models were performed on type II pneumocytes (A549 cell line), either on cells attached to culture dishes (monolayer A/R model; 3.5 h of anoxia, 30 min of re-oxygenation) or after cell detachment (suspension A/R model; 1 h of anoxia, 10 min of re-oxygenation). Ceftazidime and selective inhibitors (SOD, Tiron, L-NMMA) were added before anoxia. Free radical production was assessed by the EPR spin trapping technique. Oxygen consumption was monitored, in parallel with EPR studies, in the suspension A/R model.

The production of free radical species was demonstrated by the generation of PBN-radical adducts: (a_N = 15.2 G) in the monolayer A/R model and a six-line EPR spectrum (a_N = 15.7 G and a_H = 2.7 G) in the suspension A/R model. A kinetic study performed by oximetry, in parallel with EPR spectroscopy, demonstrated marked alterations of the cell respiratory function and that the free radical production started during anoxia and increased during re-oxygenation. In the suspension A/R model, the amplitude of EPR spectra were decreased upon the addition of 200 U/ml SOD (37% inhibition), 0.1 mM Tiron (67% inhibition) and 1 mM L-NMMA (43% inhibition). Addition of 1 mM ceftazidime decreased the amplitude of EPR spectra (37% inhibition) in both A/R models. Complementary *in vitro* EPR studies demonstrated that CAZ scavenged the hydroxyl radical (produced by the Fenton reaction). The protective effect of ceftazidime in the cell model could thus be linked to its ability to scavenge superoxide anions, nitrogen-derived species and hydroxyl radicals.

Introduction

Pathological events that are subsequent to transient tissue hypoxia followed by oxygen reperfusion can account for numerous types of injury induced by surgery or environmental factors. Organ transplantation is a recognized cause of ischemia/reperfusion injury. Pathological changes consequent to reperfusion of an ischemic organ include oxidative stress due to the localized and short-lived generation of reactive oxygen species (ROS).[1–4] Subsequent to this, there is a rapid increase in the neutrophil adherence to endothelium, which is consistent with a neutrophil predominant inflammatory response. This leads to a secondary release of deleterious reactive species generated at the sites of damage.[5–8]

Several *in vivo* and *in vitro* models have been designed to simulate ischemia-reperfusion or anoxia-re-oxygenation

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(A/R), and to demonstrate the production of oxygen free radicals and reactive species, with subsequent lipid peroxidation, in organs[9–13] and cultured cells,[14–16] such as human umbilical vein endothelial cells. These endothelial cells have been demonstrated to be resistant to long periods of anoxia, maintaining 100% viability after 24 h at 0–1% oxygen, but showing cellular dysfunctions at re-oxygenation. This damage was clearly dependent upon the length of the period of anoxia and was attributed to oxygen radicals on the basis that the presence of free radical scavengers exerted a protective effect.[17] Endothelial cells subjected to anoxia were also found to increase the expression of adhesion molecules, eliciting increased adherence of neutrophils, via a pathway involving ROS production.[8] Aortic endothelial cells were more sensitive to anoxia with 5 h of anoxia resulting in a 50% loss of viability, cell dysfunctions and defects of lipid metabolism favouring peroxidation of membrane phospholipids.[18] Zweier *et al.*[15,16] demonstrated by the EPR spin trapping technique that, at re-oxygenation after 1 h of anoxia, human aortic endothelial cells produced superoxide free radicals that further reacted with iron to produce hydroxyl radicals. In this study, xanthine oxidase was implicated as the primary source of this radical generation. The NO pathway and peroxynitrite ($ONOO^-$), a potent oxidant agent derived from the reaction of nitric oxide ($NO^\bullet$) with superoxide anion ($O_2^{\bullet-}$), have also been recognized as being involved in the pathophysiological events of A/R.[19–21]

Ischemic conditions are present in the lungs during acute lung injury and acute respiratory distress syndrome, when alveoli are invaded by fluids and neutrophils.[22] The epithelial alveolar cells are then subjected to anoxic conditions with the risk of intracellular and extracellular oxidative damage. However, demonstration of free radical production by EPR spectroscopy at the cellular level remains difficult, and the kinetics of free radical release (during the ischemic/anoxic phase or at reperfusion/re-oxygenation) is still a matter of debate. In the presence of an exogenous toxic compound (paraquat), Horton *et al.*[23] detected free radical production by EPR spectroscopy during the oxidative process of rabbit alveolar cells exposed to anoxia. In isolated human alveolar cells, the detection of free radicals by EPR spectroscopy has never been demonstrated.

Many therapeutic strategies aim at reducing the alterations of the oxidant/antioxidant balance (redox balance) occurring during anoxia/re-oxygenation or ischemia/reperfusion by using exogenous molecules active against oxidizing agents and free radical species (hydrophilic or lipophilic antioxidants, polyphenols, anaesthetic agent).[18,20,21,24] Antibiotic molecules have been demonstrated to possess antioxidant-like properties, related to their chemical structures.[25–29] Ceftazidime, a third generation antibiotic of the cephalosporin family widely used in infectious diseases, is reported to inhibit lipid peroxidation and to act as an antioxidant against ROS produced by activated phagocytes. Ceftazidime is active against hypochlorous acid (HOCl) produced catalytically by neutrophil myeloperoxidase,[29,30] quenches singlet oxygen,[31] and protects endothelial cells subjected to oxidant stress.[30] The administration of ceftazidime by lung instillation is proposed for patients with nosocomial pneumonia linked to acute lung injury, a pathological situation leading to the hypoxia of alveolar cells.[32]

We were interested in studying the production of free radicals by cultured human alveolar epithelial cells subjected to anoxia/re-oxygenation and to evaluate the effects of ceftazidime on this free radical production. Two models of non-lethal A/R were designed, with cytotoxicity evaluated by the release of lactate dehydrogenase (LDH) into the supernatant. The production of free radicals was demonstrated by the EPR spin trapping technique and the dysfunction of respiratory function was evidenced by oximetry. The effects of ceftazidime were determined and compared to those of two superoxide anion scavengers (SOD and Tiron) and an inhibitor of NO synthase (L-NMMA).

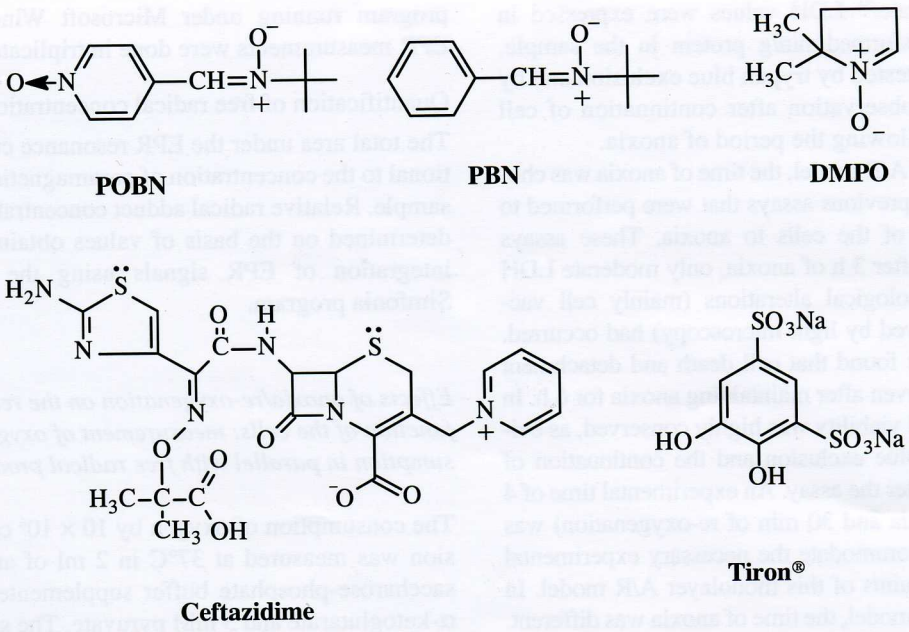
Materials and Methods

Reagents

Chemicals were dissolved in phosphate buffered saline (PBS: 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) or in Hanks' balanced salt solution added with glucose (HBSS-G: 138 mM NaCl, 5.4 mM KCl, 4 mM $NaHCO_3$, 34 mM Na_2HPO_4, 0.33 mM KH_2PO_4, 0.4 mM $MgSO_4.7H_2O$, 0.5 mM $MgCl_2.6H_2O$, 1.4 mM $CaCl_2.H_2O$, 5.6 mM glucose; pH 7.4). Analytical grade sodium phosphate, potassium, calcium and magnesium chloride, magnesium sulfate, sodium hydrogensulfate, $FeSO_4.7H_2O$, H_2O_2, glucose and Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) were purchased from Merck. Diethylenetriamine-pentaacetic acid (DETAPAC 98%) and the spin traps α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN), α-phenyl N-*tert*-butylnitrone (PBN) and 5,5-dimethyl pyrroline N-oxide (DMPO) were from Aldrich (Scheme 1). Ceftazidime was a gift from Glaxo-Welcome (Belgium). Analytical grade ethanol was from UCB Pharma, Belgium. Superoxide dismutase (SOD) was from Sigma and N^G-monomethyl L-arginine (L-NMMA) from Calbiochem. Minimal Essential Medium (MEM), L-glutamine, fetal calf serum, penicillin, streptomycin and trypsin solution were purchased from Gibco, Life Technologies. NADH, α-ketoglutarate and pyruvate were from Sigma.

Cell culture

Epithelial lung adenocarcinoma A549 cell line (ATCC, Rockville, MD, USA) was cultured in MEM supplemented



Scheme 1. Chemical formulae of some molecules used in the study: the 3 spin traps POBN, PBN and DMPO, the $O_2^{\cdot-}$ scavenger Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) and the antibiotic molecule ceftazidime.

with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin (0.1 µg/ml). This cell line has the characteristics of type II alveolar cells. Confluent cells were used after cell growth on plastic dishes in a humidified atmosphere (5% CO_2 and 95% air) at 37°C. For experimental assays of A/R, the confluent cells were treated directly on the culture dishes or used in suspension. The cell suspension was obtained by treatment of the confluent cells (5 min at 37°C) with a 0.05% trypsin solution in fetal calf serum/PBS. After centrifugation at 1000 *g* for 10 min, the cells were suspended in HBSS-G, counted and diluted to 8 or 10×10^6 cells/ml HBSS-G before use in A/R experiments.

Models of anoxia and re-oxygenation

Experiments with two models were performed: A/R of the adherent cells in monolayer (monolayer A/R) or A/R of cells in suspension (suspension A/R).

Monolayer A/R model

For the monolayer A/R model, adherent cells were subjected to anoxia (95% N_2, 5% CO_2) for 210 min at 37°C in the presence of 50 mM PBN followed by 30 min of re-oxygenation (95% air, 5% CO_2) at 37°C. The cells were then scraped and 30 ml of a chloroform:methanol (2:1) mixture added for PBN-adduct extraction.[33] After centrifugation, the chloroform phase was collected and evaporated to dryness under nitrogen. The residue was diluted with 0.5 ml $CHCl_3$ before EPR analysis. Controls (normoxia) were obtained in the same conditions with cells maintained in normal culture conditions for 240 min and extracted in the same manner.

Suspension A/R model

For the suspension A/R model, the cells were obtained as described above. To 0.8 ml of the cell suspension (8×10^6 cells/assay) was added 50 mM 4-POBN in the presence of 2% ethanol. The mixture was flushed for 15 min with 95% N_2, 5% CO_2 before sealing. A/R was maintained for 1 h at 37°C. The cell suspension was then flushed for 3 min with 100% O_2 (medical grade O_2, Air Liquide, Belgium) in order to quickly restore an oxygen concentration in the cell medium equivalent to that measured before anoxia (the oximetry assay was performed with a WTW-537 oximeter). The cells were further maintained for 7 min in air before EPR studies. Controls were obtained in the same conditions with cell suspensions maintained in normoxia at 37°C.

The antibiotic ceftazidime (1 mM), the superoxide anion scavengers SOD (200 U/ml) and Tiron (0.1 mM), or the NO-synthase inhibitor L-NMMA (1 mM) were added to the cells before starting A/R.

Cell viability and lactate dehydrogenase measurement

To evaluate the cytotoxic effects of A/R, the release of LDH into the medium was measured by a kinetic enzymatic spectrophotometric assay at 366 nm, using NADH and

pyruvate as substrate.[34] LDH values were expressed in µmol NADH transformed/min/g protein in the sample. Cell viability was tested by trypan blue exclusion and by light microscopy observation after continuation of cell culture for 48 h following the period of anoxia.

In the monolayer A/R model, the time of anoxia was chosen on the basis of previous assays that were performed to test the resistance of the cells to anoxia. These assays demonstrated that after 3 h of anoxia, only moderate LDH release and morphological alterations (mainly cell vacuolization as observed by light microscopy) had occurred. Additionally, it was found that cell death and detachment was not very great even after maintaining anoxia for 6 h. In the two models, cell viability was highly conserved, as evidenced by trypan blue exclusion and the continuation of cell development after the assay. An experimental time of 4 h (210 min of anoxia and 30 min of re-oxygenation) was thus selected to accommodate the necessary experimental and practical constraints of this monolayer A/R model. In the suspension A/R model, the time of anoxia was different. It was shortened taking into account that the trypsination slightly impaired the cells as demonstrated by the LDH release by control cells after 1 h in normoxia.

EPR spin trapping experiments

EPR on cells

For the suspension A/R model, the cells were transferred into the quartz flat cell in the cavity of the spectrometer and the presence of free radicals was evaluated by the generation of radical adducts in the presence of a spin trap agent (4-POBN, 20 mM) and 2% ethanol. For the monolayer A/R model, the residue of extraction was dissolved in 0.5 ml $CHCl_3$ and transferred into the quartz flat cell to monitor the presence of PBN-spin adducts.

In vitro EPR assays

The effects of ceftazidime (at concentrations in the range $4–8 \times 10^{-3}$ M) were tested on the EPR spectra of hydroxyl radicals ($HO^{\bullet}$) generated by the H_2O_2-Fe^{2+} system (Fenton reaction: 4×10^{-4} M $FeSO_4$, 4×10^{-4} M H_2O_2, 4×10^{-4} M DETAPAC) at room temperature in 1 ml PBS added with 100 mM DMPO (see above for EPR instrumental conditions). Recording of the EPR spectra was initiated 50 s after the mixture of all the reagents.

EPR measurements were carried out at room temperature with a Bruker spectrometer (Bruker, Karlsruhe, Germany) operating at X-band frequency (9.75 GHz) with non-saturating microwave power (20 mW). The instrumental settings were as follows: 100 kHz modulation frequency, 1.012 G modulation amplitude, 3480 ± 50 G magnetic field and receiver gain 2×10^4. The hyperfine splitting constants were measured directly from experimental spectra using a Bruker Win-Simfonia program running under Microsoft Windows. All the EPR measurements were done in triplicate.

Quantification of free radical concentrations

The total area under the EPR resonance curve is proportional to the concentration of paramagnetic species in the sample. Relative radical adduct concentrations were thus determined on the basis of values obtained by double-integration of EPR signals using the Bruker Win-Simfonia program.

Effects of anoxia/re-oxygenation on the respiratory function of the cells: measurement of oxygen consumption in parallel with free radical production

The consumption of oxygen by 10×10^6 cells in suspension was measured at 37°C in 2 ml of an air-saturated saccharose-phosphate buffer supplemented with 5 mM α-ketoglutarate and 5 mM pyruvate. The spin trap agent, POBN (50 mM, in the presence of 2% ethanol) was added at the beginning of the experiment. The oxygen concentration in the medium was measured by a Clark electrode, and the kinetics of O_2 consumption (slope of the curve) was recorded with an oxygraph (Physica Respirameter, Paar Physica, Austria). Anoxia was reached when the consumption of O_2 in the medium was complete, and was maintained for 10 min. Re-oxygenation was performed for 5 min by exposure of the stirred medium to air.[35] The samples for the EPR monitoring of free radical production were taken at the following points: (i) at the end of the anoxia period; (ii) upon initiating re-oxygenation; and (iii) after a 30 min re-oxygenation period.

RESULTS

Cytotoxic effects of anoxia: viability and LDH release

The short times of anoxia used in our study led to a low level of mortality: cell death was < 5% (compared to < 2% for controls) as demonstrated by trypan blue exclusion. In the monolayer A/R model, when the cells were maintained in culture for 48 h after the anoxia period, they remained viable and at confluence (light microscopy observations). However, some cell damage was demonstrated by the modest, albeit significant, release of LDH in the supernatant. Compared to the control assay (cells in normoxia), the concentration of LDH in the supernatant of the cells subjected to A/R was increased. For the monolayer A/R model, the LDH activity corresponded to the consumption of 1.75 ± 0.17 µmol NADH/min/g protein compared to 0.35 ± 0.02 µmol/min/g protein for control ($n = 9$; $P < 0.001$). In the second model (suspension A/R), the LDH activity was 3.35 ± 0.31 µmol

NADH/min/g protein versus 1.24 ± 0.15 µmol NADH/min/g protein in the control (n = 9; P < 0.001). In the suspension A/R model, the mean LDH activity found in the supernatant of control cells was higher compared to the monolayer model control, but the cells were detached by trypsination, a technique that can alter the cell membrane.

Free radical formation during anoxia: EPR spin trapping studies

The results of EPR experiments are shown in Figure 1 for the suspension A/R model. No free radicals were

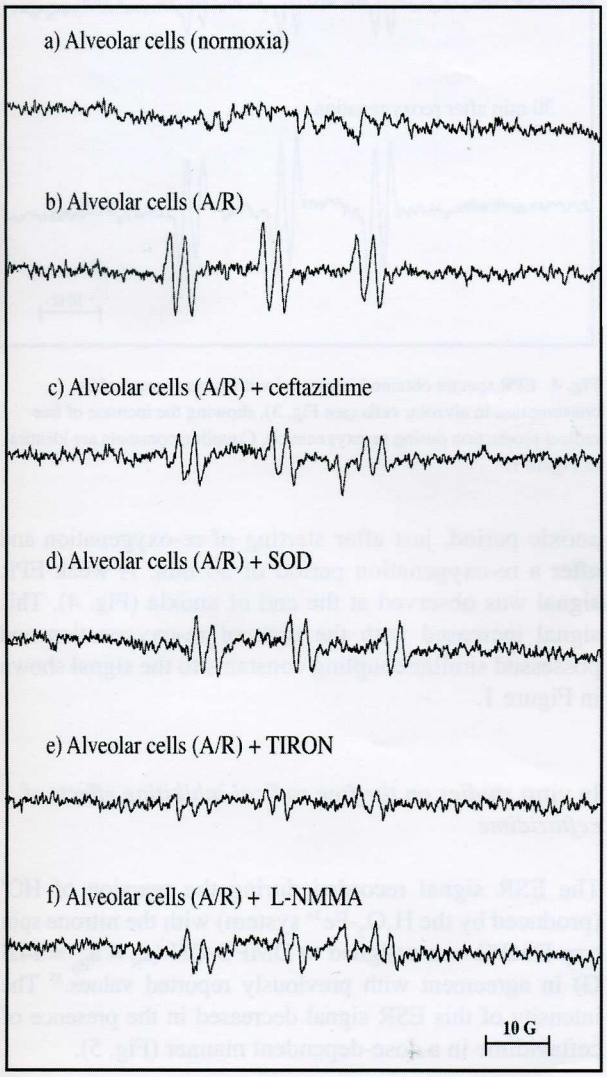


Fig. 1. Free radical production by 8×10^6 alveolar cells in suspension subjected to anoxia/re-oxygenation (suspension A/R model). The EPR spectra are characteristic of POBN/•CHCH$_3$OH adducts. EPR conditions and coupling constants are described in Materials and Methods; number of scans = 6. (a) Control – cells under normoxic conditions; (b) cells exposed to 1 h anoxia and 10 min re-oxygenation; (c–f) same as (b) in the presence of 1 mM ceftazidime (c), 200 U/ml SOD (d), 0.1 mM Tiron (e) or 1 mM L-NMMA (f). All the EPR measurements were done in triplicate.

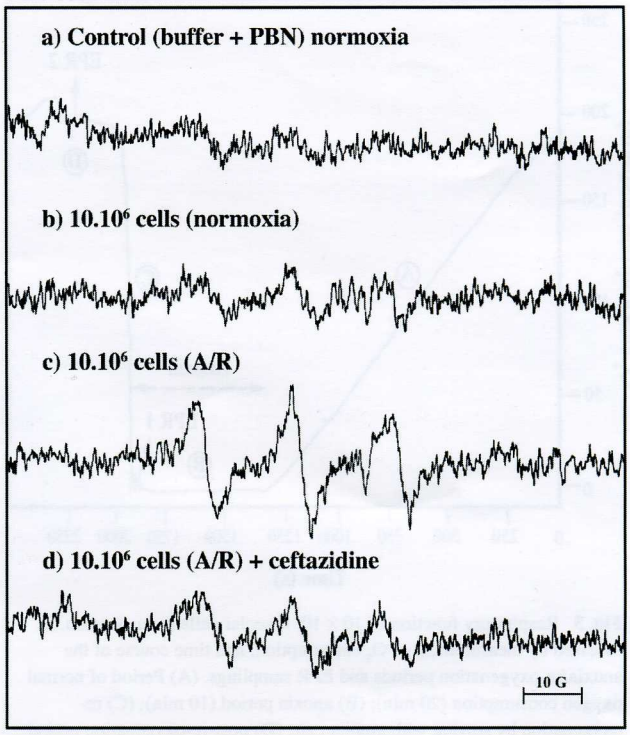


Fig. 2. EPR spectra of PBN spin adducts of 10×10^6 adherent alveolar cells subjected to anoxia/re-oxygenation (monolayer A/R model). EPR conditions and coupling constants are described in Materials and Methods; number of scans = 10. (a) Control of PBN maintained in buffer for 4 h in normoxia; (b) control – cells under normoxic conditions; (c) cells exposed to 3.5 h anoxia and 30 min re-oxygenation (95% air and 5% CO_2); (d) same as (c) + 1 mM ceftazidime.

produced by control cells in normoxia (Fig. 1a). After 1 h of anoxia at 37°C and 10 min of re-oxygenation in the presence of the 4-POBN/EtOH mixture, a six-line EPR spectrum was observed, characteristic of the POBN/•CH(OH)CH$_3$ adduct (a_N = 15.7 G and a_H = 2.7 G; Fig. 1b). The addition of 1 mM ceftazidime or 200 U/ml SOD decreased the EPR signal intensity (37% inhibition; Fig. 1c,d). The decrease in the EPR signal intensity was more pronounced with 0.1 mM Tiron, a superoxide anion scavenger (67% inhibition; Fig. 1e), while the use of heat-inactivated SOD, in similar conditions, did not inhibit the production of free radical species as evidenced by EPR spectroscopy (data not shown). The addition of 1 mM L-NMMA resulted in 43% inhibition and demonstrated the participation of NO• or NO•-derived radicals in the EPR signals (Fig. 1f).

In Figure 2, the results of the monolayer A/R model are shown. Cells exposed to an anoxia period of 210 min followed by 30 min re-oxygenation produced free radicals as indicated by the appearance of the EPR spectrum of PBN adduct (Fig. 2c). This spectrum has a coupling constant value of a_N = 15.2 G. This signal was assigned to a carbon-centered radical derived during lipid oxidation on the basis of comparison with data previously published by Arroyo *et*

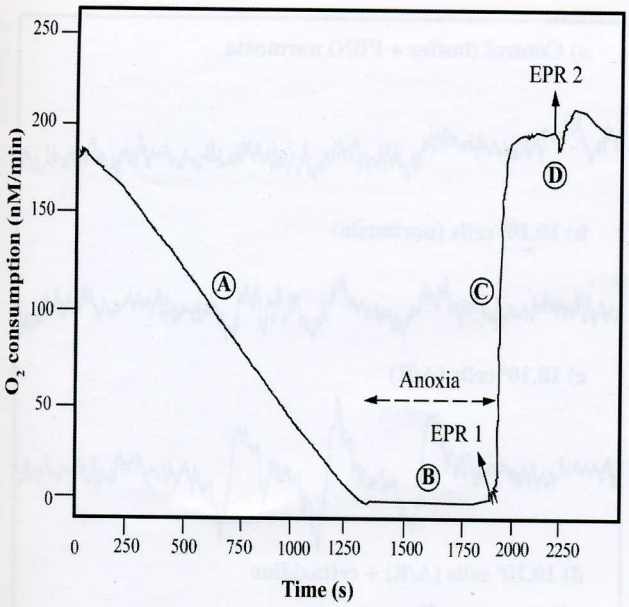


Fig. 3. Respiratory function of 10×10^6 alveolar cells in suspension, as assessed by measurement of O_2 consumption, and time course of the anoxia/re-oxygenation periods and EPR samplings. (A) Period of normal oxygen consumption (20 min); (B) anoxia period (10 min); (C) re-oxygenation by stirring with ambient air; (D) post-re-oxygenation period with altered respiratory function (no oxygen consumption). Arrows indicate the sampling times for EPR study: at the end of anoxia (EPR 1), after 3 min of re-oxygenation (EPR 2).

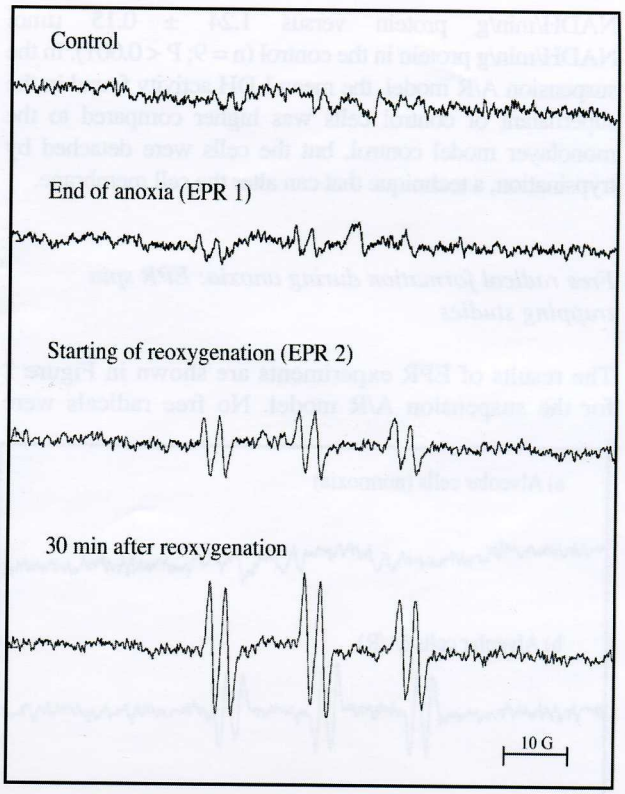


Fig. 4. EPR spectra obtained in parallel with measurement of O_2 consumption in alveolar cells (see Fig. 3), showing the increase of free radical production during re-oxygenation. Coupling constants are identical to Figure 1.

al.[36] In this study of ischemic canine heart tissue, a carbon-centered radical was observed with the following hyperfine coupling constant values: $a_N = 15.20$ G and $a_H = 3.85$ G (data from spin trap database of NIEHS at www.epr.niehs.nih.gov). These splitting constants were assigned to a carbon-centered radical (PBN/•CL). In control assays in buffer without cells, no EPR signals were observed (Fig. 2a). With control cells, maintained in normoxia, a weak EPR spectrum was observed (Fig. 2b). In this monolayer A/R model, 1 mM ceftazidime also inhibited free radical production (Fig. 2d).

Effects of anoxia on the respiratory function of alveolar cells in suspension

Figure 3 shows that the respiratory function of the alveolar cells in suspension was normal. Oxygen dissolved in the medium was consumed after a period of 20 min (Fig. 3A) and the medium was completely anoxic after this period. After 10 min of anoxia (Fig. 3B), re-oxygenation was performed in ambient air for 5 min (Fig. 3C) and the respiratory function of the cells was tested again (Fig. 3D). The dramatic change of the slope of the oxygen consumption curve indicated that the respiratory function of the cells was altered as a consequence of the A/R period. EPR assays were performed at the end of the anoxic period, just after starting of re-oxygenation and after a re-oxygenation period of 30 min. A weak EPR signal was observed at the end of anoxia (Fig. 4). This signal increased with the time of re-oxygenation and possessed similar coupling constants to the signal shown in Figure 1.

In vitro *studies on the free radical inhibiting effects of ceftazidime*

The ESR signal recorded during the reaction of HO• (produced by the H_2O_2-Fe^{2+} system) with the nitrone spin trap DMPO was assigned to DMPO-OH ($a_N = a_{H\beta} = 14.8$ G) in agreement with previously reported values.[35] The intensity of this ESR signal decreased in the presence of ceftazidime in a dose-dependent manner (Fig. 5).

Discussion

Epithelial alveolar cells are normally exposed to a 21% oxygen environment and appear resistant to modifications of the oxygen concentration, especially hyperoxia, although ROS have been shown to be produced in these

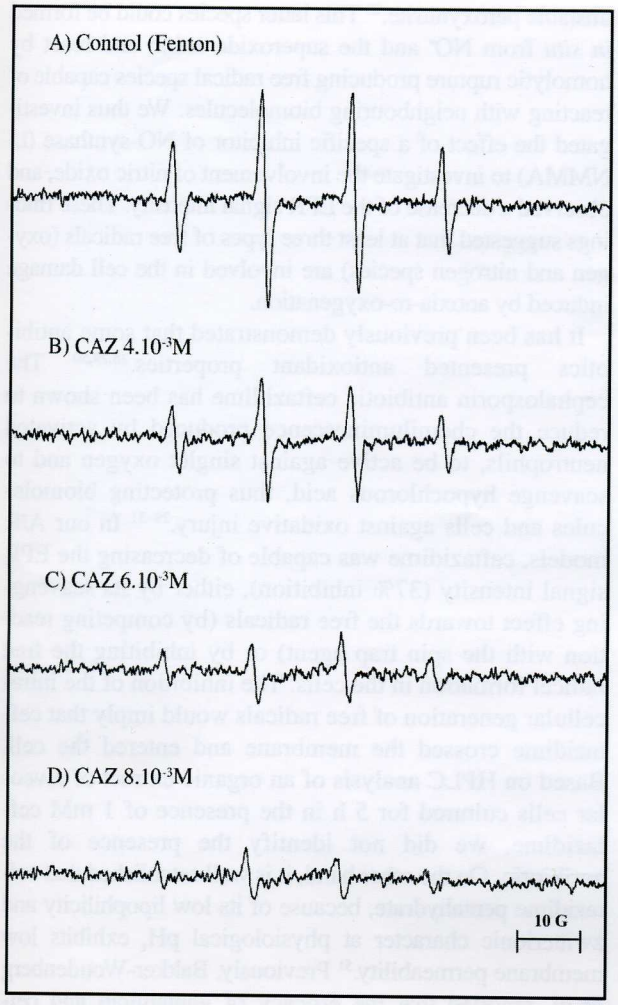


Fig. 5. EPR spectra of DMPO spin adducts formed with hydroxyl radicals produced by the H_2O_2-Fe^{2+} system (Fenton reaction), and inhibiting effects of increasing doses of ceftazidime.

cells under hyperoxic conditions.[37,38] Alveolar cells possess an important defense against ROS and are able to increase their defense mechanisms in stress conditions.[39–41] Indeed, breathing 100% oxygen is effectively toxic and lethal for animals after around 3–4 days, but toxic effects are only clearly apparent after 24 h. In humans, slight toxic effects (cough and some lack of comfort in breathing) were detected at 100% O_2, but only after 10 h and these effects were reversible.[42,43]

Alveolar cells can also be subjected to anoxia in pathological conditions such as those encountered in acute lung injury or acute respiratory distress syndrome, conditions in which reactive oxygen and nitrogen species are produced in the alveoli.[44,45] However, no direct evidence for free radical production in alveolar cells during A/R, such as the observation of radicals using EPR spectroscopy, has previously been obtained. An article by Horton *et al.* reported radical formation in freshly isolated rabbit alveolar cells, but after paraquat uptake.[23] Paraquat, a defoliant of the bipyridium class of pesticides, is a highly toxic compound because, within cells, it is reduced and oxidised in a cyclic manner to produce superoxide anions. Thus, in the study by Horton *et al.*, radical production in alveolar cells was initiated by radical generation from an added toxic compound. The present study contrasts with this model since the A/R conditions used were quite different; in particular, exogenous compounds known to initiate free radical production were absent.

We designed two models of A/R on alveolar cells in culture and demonstrated that sublethal A/R resulted in the generation of free radicals and cell damage, the latter assessed by LDH release and alterations to the respiratory function of the cells. The LDH release in the two A/R models was significant compared to control values (cells maintained in normoxia), but in the suspension A/R model, the LDH release data indicated that cell damage had occurred in the control cells. The trypsin treatment used to obtain the cell suspension explained the higher LDH values for control cells in suspension A/R model, compared to the monolayer A/R model. The poor medium (HBSS with added glucose) used for the A/R experiments could also be partially responsible for this cell damage, but the use of serum was excluded due to its capacity to scavenge free radicals. Nevertheless, the LDH release by the cells after A/R was significantly higher in cells exposed to anoxia, compared to controls.

By the EPR spin trapping technique, we demonstrated that A/R resulted in the generation of free radical species in alveolar cells, and that this free radical production increased with the time of re-oxygenation (Fig. 4). The EPR signal observed with the monolayer A/R model possessed coupling constants (a_N = 15.2 G) characteristic of a lipid-derived carbon-centered radical generated during lipid peroxidation, based on comparison with literature data. The observed coupling constants appeared to be quite close to those reported by Arroyo *et al.*[36] (a_N = 15.20 G and a_H = 3.85 G) for a species assigned as a lipid-derived carbon-centered radical PBN adduct (PBN/$^{\bullet}$CL). Controls were performed which showed that the observed EPR signals were not artefacts due to reaction of the extraction solvent with metal ions. On this basis, the observation of EPR signals was attributed to free radical production by the cells, occurring during the A/R sequence.

In the suspension A/R model, 1 h of anoxia followed by a 10 min period of re-oxygenation resulted in the appearance of a six-line EPR spectrum due to POBN/$^{\bullet}$CH(OH)CH_3 adducts, while in control cells (normoxia), EPR signals were not observed. The formation of POBN-radical adducts in the anoxic cells was favoured by the presence of EtOH (2% v/v) that rapidly reacted with ROS producing a radical that was trapped by POBN. These EPR signals were of higher intensity than in the monolayer A/R model, a difference that could

be attributed to the nature of the spin trap. POBN is the water soluble analog of PBN; it is a hydrophilic spin trap that enters cells and is used *in vivo* and *in vitro*.[46] Intracellular POBN concentrations may be expected to be much higher than intracellular PBN concentrations under the same conditions. Thus, trapping by POBN of the free radical species formed during the cascade of events that accompanied A/R may have been favourable. The free radicals produced by the cells could be due to lipid peroxidation. The alveolar cells produce a lipid-rich surfactant that could be a preferred target for lipid peroxidation. Indeed, although surfactant is relatively resistant to oxidation due to the predominance of saturated phospholipids, oxidizable cholesterol and unsaturated phospholipids represent ~35% of the weight of surfactant, and 15% of all surfactant phospholipids contain two or more double bonds.[47] More recently, Blanco and Catala[48] reported that the main polyunsaturated fatty acids (C18:2 ω6 and C20:4 ω6) were found in the lung surfactant and the amount of these lipid compounds decreased after a 180 min lipid peroxidation process induced by an ascorbate-Fe^{2+} system. However, POBN spin adducts were observed during A/R of isolated liver mitochondria and were attributed to the reaction of the POBN/ethanol couple with superoxide anion.[35,49] POBN could thus cross the mitochondrial membranes and reach the matrix compartment. The EPR signals that we reported here were similar to those obtained with isolated mitochondria, and suggested a mitochondrial origin.

The oximetry of alveolar cells in suspension indicated that the respiratory function of the cells was altered by a short period of A/R, that free radicals were produced, and that this free radical production was higher after re-oxygenation (Fig. 4). These observations confirm previous data reported for other anoxia models, suggesting that the cell alterations started during anoxia and increased during re-oxygenation.[1,2,4,15,21] The simultaneous demonstration of an altered respiratory function and free radical production confirmed our hypothesis that the mitochondrial respiratory chain was damaged and responsible, at least partially, for the production of free radicals that could be primarily superoxide anions. However, we can not exclude a cytosol production of superoxide anion by hypoxia-activated xanthine-oxidase, as previously suggested.[15,16]

In the presence of the two superoxide anion scavengers, SOD and Tiron, the intensity of EPR signals was partially reduced. Tiron was more potent than SOD; this could be explained by a higher capacity of Tiron to cross the cell membrane and reach the site (membranes, cytosol or mitochondria) where superoxide anions are produced. The incomplete inhibition observed with Tiron and SOD suggests that other free radicals were also produced. They could originate from lipid peroxidation, from an *in situ* Fenton reaction producing hydroxyl radicals or from superoxide-derived species such as the unstable peroxynitrite.[20] This latter species could be formed *in situ* from $NO^{\bullet}$ and the superoxide anion and react by homolytic rupture producing free radical species capable of reacting with neighbouring biomolecules. We thus investigated the effect of a specific inhibitor of NO-synthase (L-NMMA) to investigate the involvement of nitric oxide, and observed a decrease of the EPR signal intensity. These findings suggested that at least three types of free radicals (oxygen and nitrogen species) are involved in the cell damage induced by anoxia-re-oxygenation.

It has been previously demonstrated that some antibiotics presented antioxidant properties.[28,29,50] The cephalosporin antibiotic ceftazidime has been shown to reduce the chemiluminescence produced by activated neutrophils, to be active against singlet oxygen and to scavenge hypochlorous acid, thus protecting biomolecules and cells against oxidative injury.[29–31] In our A/R models, ceftazidime was capable of decreasing the EPR signal intensity (37% inhibition), either by its scavenging effect towards the free radicals (by competing reaction with the spin trap agent) or by inhibiting the free radical formation in the cells. The inhibition of the intracellular generation of free radicals would imply that ceftazidime crossed the membrane and entered the cell. Based on HPLC analysis of an organic extract of alveolar cells cultured for 5 h in the presence of 1 mM ceftazidime, we did not identify the presence of the antibiotic. On the other hand, it is well established that ceftazidime pentahydrate, because of its low lipophilicity and zwitterionic character at physiological pH, exhibits low membrane permeability.[51] Previously, Bakker-Woudenberg *et al.* reported that the efficacy of gentamicin and ceftazidime was increased when they were entrapped in liposomes.[52] Therefore, the extracellular scavenging effect seems more probable, which can also explain why inhibition due to ceftazidime was only partial. ROS-scavenging by ceftazidime was further confirmed by its inhibiting effect on the EPR signal intensity of the DMPO-hydroxyl radical spin adduct. This scavenging effect of ceftazidime raises the question of a radical intermediate of the drug. Indeed, several reports demonstrated that the radiosterilization of some antibiotics, including ceftazidime, resulted in the formation of free radical species. For instance, in the case of ceftazidime, three main species were produced: the first showed septet lines in an EPR spectrum and decayed at 230 K – it was assigned as a $^{\bullet}C(CH_3)_2COOH$ radical. The second showed triplet lines and decayed at 293 K – it was assigned as iminoxyl radicals ($>C=N-O^{\bullet}$). The third showed a broad singlet line and survived even at 295 K.[53] Although our experimental conditions to produce free radical species from ceftazidime are very different from irradiation conditions, we may not completely exclude the formation of radical intermediates from the drug, though we did not demonstrate it. The thioether bonds and the heterocyclic rings would be, at least in part, responsible for the antioxidant

properties of these drugs, as has been suggested especially for the scavenging of HOCl.[28,50] It may be extrapolated that the mechanism of radical scavenging by ceftazidime involves the reaction of free radicals with this molecule, resulting in rupture of the C–O bond leading to carbon-centered and iminoxyl radicals as described above. The ROS may also react with the heterocyclic ring or the thioether bond of this drug producing, for examples short-lived thiyl radicals that were undetectable in our experimental conditions.

Therefore, the interaction of ceftazidime with ROS may result in the protection of biomolecules, cells and tissues during A/R or in pathological states accompanied by an excessive phagocyte activation. The administration of ceftazidime in humans will combine an antioxidant protection with an antibacterial activity. Ceftazidime does not significantly enter into the neutrophils, and would thus not be able to inhibit the intraphagosomal lysis of bacteria by HOCl. It remains to be determined whether the reaction of ceftazidime with free radicals *in vivo* could produce toxic metabolites or induce a reduced antibacterial activity of the drug, since it has been demonstrated for other cephalosporins that their interactions with HOCl resulted in a depression of their antibacterial activity.[28]

Conclusions

The results demonstrate that anoxia-re-oxygenation of epithelial alveolar cells leads to the production of free radicals and to respiratory dysfunction. The free radical production was present in the anoxia phase but increased at re-oxygenation and seemed to originate, at least partially, from damaged mitochondria. Our results indicate that a number of activated species are produced by cells exposed to anoxia, specifically, the superoxide anion (or its derivatives) and NO-derived species, at least the NO-derived intermediate $ONOO^-$. Indeed, SOD, Tiron and L-NMMA reduced the appearance of the POBN/$^{\bullet}CH(OH)CH_3$ radical adduct. Ceftazidime, an antibiotic of the cephalosporin family, widely used in clinical practice and previously reported to possess an antioxidant activity towards ROS, was found to have significant efficacy in the trapping of free radicals.

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