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Mitochondrial Damage and Metabolic Compensatory Mechanisms Induced by Hyperoxia in the U-937 Cell Line

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Experimental hyperoxia represents a suitable in vitro model to study some pathogenic mechanisms related to oxidative stress. Moreover, it allows the investigation of the molecular pathophysiology underlying oxygen therapy and toxicity. In this study, a modified experimental set up was adopted to accomplish a model of moderate hyperoxia (50% O_2, 96 h culture) to induce oxidative stress in the human leukemia cell line, U-937. Spectrophotometric measurements of mitochondrial respiratory enzyme activities, NMR spectroscopy of culture media, determination of antioxidant enzyme activities, and cell proliferation and differentiation assays were performed. The data showed that moderate hyperoxia in this myeloid cell line causes: i) intriguing alterations in the mitochondrial activities at the levels of succinate dehydrogenase and succinate-cytochrome *c* reductase; ii) induction of metabolic compensatory adaptations, with significant shift to glycolysis; iii) induction of different antioxidant enzyme activities; iv) significant cell growth inhibition and v) no significant apoptosis. This work will permit better characterization the mitochondrial damage induced by hyperoxia. In particular, the data showed a large increase in the succinate cytochrome *c* reductase activity, which could be a fundamental pathogenic mechanism at the basis of oxygen toxicity.

Keywords: Mitochondria, Oxidative stress, Oxygen toxicity, Reactive oxygen species (ROS)

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Introduction

Oxidative stress has been recognized as playing a role in the pathogenesis of different acute, chronic and, above all, degenerative diseases (Byung, 1994; Kazzaz *et al.*, 1996; McCord, 2000; Rahman, 2003). Normobaric hyperoxia is considered to elicit an oxidative stress similar to that occurring *in vivo* in types I and II epithelial alveolar cells, fibroblasts, endothelial cells and leukocytes, which are all forced to face atmospheric concentrations of O_2 (Gardner *et al.*, 1994; Lee *et al.*, 1996; Yan *et al.*, 1997). Some adopted experimental protocols seem to indicate mitochondria as the primary target of oxygen toxicity (Schoonen *et al.*, 1990), and one study has underlined the role of metabolic perturbations, i.e. glucose depletion in eliciting hyperoxia-related injuries (Allen *et al.*, 1988). Moreover, it has also been suggested that hyperoxia induces cell death via necrosis and not apoptosis (Li *et al.*, 1997; Lindsay *et al.*, 2000; Franek *et al.*, 2001), which stresses the need for further investigation on the interplay among oxidative stress, mitochondrial damage, metabolic perturbations and cell death.

This study mainly analyzed the metabolic modifications induced by moderate normobaric hyperoxia (50% O_2) in the U937 myeloid leukemia cell line. A moderate hyperoxia method was preferred over a full blown one (95-98% O_2) to provoke a condition of mild oxidative stress with the intent to point out the are most sensitive cell components and pathways and the corresponding adaptive mechanisms that are aroused for its counteraction. Some information could in fact be lost in a normobaric hyperoxia (95-98% O_2) set up, where the cells could be strongly damaged and rapidly wasted.

For these considerations, a better definition of the molecular mechanisms of cell injury, as determined by hyperoxia, adopting different methodological approaches could contribute to explaining some aspects of the complex pathophysiology of cellular oxidative stress and antioxidant

defenses, and the molecular aspects of O_2 clinical applications. Moreover, these studies could also shed some light on the oxidative metabolism modifications in mitochondria and their determination of cell death.

Materials and Methods

Materials and reagents Unless indicated, all chemicals and reagents (cell culture grade) were obtained from Sigma Chemical Co. (St. Louis, USA). The protein content was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, USA).

Cells and treatments The human myeloid leukemia U-937 cell line was obtained from Interlab Cell Line Collection (CBA, Genoa, Italy). Cells were cultured (3×10^5 cell/ml, 40 ml total volume), in 75 cm^2 filter vent flasks (Costar, Boston, USA) for 96 h (Heraeus B5060EX/CO_2 incubator, Milan, Italy) at 37°C under a humidified atmosphere of 5% CO_2-21% O_2 and 5% CO_2-50% O_2 for normoxia and hyperoxia conditions, respectively. The culture medium, RPMI 1640, was supplemented with 2 mmol/l glutamine, 0.1 g/ml streptomycin, 100 U/ml penicillin and 10% fetal calf serum. In a set of experiments, the U-937 cell line was cultured in presence of N-acetyl cysteine (NAC-concentrations ranging from 1 to 4 mmol/l) dissolved in NaOH buffered Krebs-Ringer-Phosphate (KRP) solution (pH 7.4). The antioxidant agent was administered to cell cultures only once, on day 0.

Cell numbers were determined using a Neubauer hemocytometer, and the viability assessed by their ability to exclude trypan blue. The cytostatic activity, determined by microscopic assay, was confirmed in an alternative assay by measuring the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) reductase activity (Skehan, 1995). The latter assay was performed in quintuplicate in 96 well plates (Costar, Boston, USA). These two growth and viability assays essentially produced the same results. Apoptotic cells were identified by fluorescence microscopy using acridine orange, as previously described (Ramachandra and Studzinski, 1995).

Determination of medium glucose consumption The medium glucose and LDH activity were measured with a Hitachi 917 automated analyzer (Roche Diagnostics, AG, Rotkreuz, Switzerland) and appropriate reagent kits (LDH kit, Sigma).

To normalize the glucose consumption to different proliferation rates of the cells, data were expressed as AUC (area under curve) versus AUC of the cell growth during 96 h of culturing.

Determination of antioxidant enzyme activities The cell pellets obtained after centrifugation at 1,200 rpm for 5 min were washed in phosphate buffered saline solution (PBS), suspended in a lysis buffer at a density of a 2×10^7 cells/ml and sonicated twice for 30 s at 20 W with a sonicator, model VC50 Vibracell (Sonics & Materials Inc, Newtown, USA), according to the method of Brambilla *et al*. (1997). The catalases were analyzed by AP1 kits (Bioenzyme Laboratories, Milan, Italy); Superoxide-dismutase and Glutathione-peroxidase by Randox kit SD125 and RS505, respectively (Laboratories LTDI, London, UK).

Determination of antioxidant molecules The a-tocopherol and coenzyme Q (CoQ_{10}) were determined by HPLC methods (Vadhanavikit *et al*, 1984).

Mitochondrial respiratory chain enzyme assay The NADH-cytochrome *c* reductase (complex I-III), succinate-cytochrome *c* reductase (complex II-III) and cytochrome *c* oxidase (complex IV) were assayed in digitonin-permeabilized cells spectrophotometrically following the methods of O'Donnell *et al*., (1995) and the succinate dehydrogenase (complex II) activity was measured in isolated mitochondria, according to the same method. The NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase and cytochrome *c* oxidase activities were expressed as nmol of cytochrome *c* reduced/min/10^6 cells. Succinate dehydrogenase was expressed as nmol of 2,6-dichlorophenol-indophenol (DCPIP) reduced/min/10^6 cells.

NMR metabolite determinations The supernatant (3 ml) was mixed with an equal volume of cold 12% (v/v) $HClO_4$ solution. Denatured material was centrifuged for 10 min (2000 g), the upper solution neutralized with potassium carbonate, lyophilized and then dissolved in 0.7 ml D_2O, containing 0.75% sodium-3-(trimethylsilyl)-(2,2,3,3-$^2H_4$)-1-propionate (TSP, used as reference peak), for ^{1}H-NMR analysis. In ^{1}H-NMR spectra, registered at 25°C by a Gemini 300 spectrometer (Varian, Palo Alto, USA), signals for lactate, alanine and acetate methyl groups were evident (1.33, 1.42 and 1.92 ppm, respectively). The concentrations of the three metabolites were determined on the basis of the peak area of the corresponding methyl groups, normalized with respect to the area of the signal of a known concentration of TSP, according to the method of Florian *et al*., (1997).

Reactive Oxygen Species (ROS) metabolism assay ROS metabolism was studied by a chemiluminescence (CL) assay, as previously described (De Baetselier and Scrham, 1986), using luminol as the chemiluminigenic probe. ROS production was measured by luminol amplified luminescence. Phorbol-12-Myristate-13-Acetate (PMA) and zymosan stimulated ROS metabolism was adopted as a marker of differentiation in the human myeloid cell line, U-937. Assays were performed in triplicate in an automatic luminometer (Autolumat LB 953, EG&G, Turku, Finland) at 25°C for 120 min, with cycles of five minutes each.

Statistical analysis All results are expressed as mean ± SEM. The group means were compared by Student-Newman-Keuls tests. When required, multiple comparison of means was achieved by analysis of variance (ANOVA) followed by a multiple comparison of means by the Dunnet test; $p < 0.05$ was considered significant.

Results

Mitochondrial respiratory enzymes The mitochondrial respiratory enzyme activities determined in the digitonin-permeabilized cells, using cytochrome *c* as an electron donor/acceptor (ODonnel *et al*., 1995), showed no significant modification of the NADH-cytochrome *c* reductase and

Table 1. Mitochondrial respiratory enzyme activities, antioxidant scavenging enzyme activities, NMR metabolite determinations and differentiation analysis of U-937 cells cultured for 96 h in normoxia and in hyperoxia (see "Materials and Methods")

Parameter		NORMOXIA	HYPEROXIA	
Mitochondrial respiratory enzyme activities	NADH cytochrome *c* reductase	3.90 ± 2.40	4.50 ± 2.80	NS
	Succinate dehydrogenase	7.10 ± 2.40	4.70 ± 2.00	P < 0.05
	Succinate cytochrome *c* reductase	8.90 ± 2.80	26.2 ± 4.70	P < 0.001
	Cytochrome oxidase	6.50 ± 2.40	6.90 ± 2.30	NS
Antioxidant enzyme activities	Superoxide dismutase	1.19 ± 0.07	1.65 ± 0.06	P < 0.05
	Glutathione peroxidase	120.4 ± 30.9	262.0 ± 50.5	P < 0.01
	Catalase	67.2 ± 3.2	89.4 ± 5.7	P < 0.05
Metabolite level	Lactate	5.8 ± 0.05	17.4 ± 2.1	P < 0.001
	Acetate	0.62 ± 0.14	0.65 ± 0.07	NS
	Alanine	0.47 ± 0.07	0.72 ± 0.16	P < 0.01
Differentiation marker by CL	Basal	3.73 ± 0.15	3.03 ± 0.21	P < 0.05
	PMA induced	3.40 ± 0.20	2.73 ± 0.36	NS
	Zymosan induced	4.30 ± 0.21	4.56 ± 0.06	NS

Results represent the mean ± S.E.M. calculated from four experiments, each performed in duplicate. *p < 0.05; **p < 0.01; ***p < 0.001.

cytochrome oxidase activities in hyperoxia-treated cell compared to those in the control cells (Table 1).

Conversely, the succinate dehydrogenase activity decreased significantly in the U-937 cells cultured in moderate hyperoxia, as has already been reported in Chinese hamster ovary cells (Schoonen *et al*., 1990).

Importantly, the succinate-cytochrome *c* reductase assay showed a dramatic increment of the malonate-inhibitable rate of cytochrome *c* reduction, which according to a previous report (Zhang *et al*., 1998) could depend on the generation of a superoxide anion, which is scavenged by exogenous cytochrome *c*, which gives an apparent increase of this mitochondrial enzymatic activity (Table 1).

Antioxidant scavenging enzymes Under these pro-oxidant culture conditions, some adaptive compensatory mechanisms were seen to develop. In particular, spectrophotometric determinations of the main antioxidant enzymes showed a significant increase of activity in the U-937 cells cultured in hyperoxia. The total cellular superoxide dismutase (SOD) and catalase activities (CAT) significantly increased in hyperoxia-cultured U-937 cells compared to the normoxia-cultured cells. Interestingly, the total glutathione peroxidase activity (GSH-PX) increased to a much greater extent than the other antioxidant enzymes in the hyperoxic cultures and in normoxia controls (Table 1). Moreover, the intracellular levels of CoQ_{10} and alpha tocopherol showed no difference in the hyperoxic cultures compared to the controls (data not shown).

Cell metabolism in hyperoxia The U-937 cells exposed to hyperoxia showed a time dependent increase in glucose consumption, but this was deliberately kept constant in the control cultures (Fig. 1). The demonstrated alteration in glucose consumption, added to the evidence of the

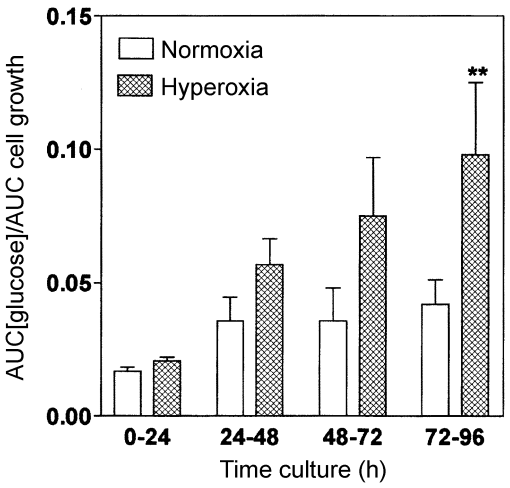

Fig. 1. Glucose consumption in U-937 cell cultures incubated for 96 h in normoxia and in hyperoxia (see "Materials and Methods"). Results expressed by AUC and normalized per single cell represent the mean ± S.E.M. calculated from three experiments, each performed in duplicate. **p < 0.01.

perturbation of succinate-cytochrome *c* reductase activity, and in particular to the decrease of succinate dehydrogenase (SDH) activity in hyperoxia-cultured U-937 cells, lead us to analyze the secondary metabolic alterations due to hyperoxic pro-oxidant culture conditions. NMR spectroscopy of culture media clearly showed different amounts of metabolites in the hyperoxic-treated cells compared to the control cells (Table 1). In particular, the former showed a remarkable increase of lactate, while the acetate levels showed no significant difference between the normoxic and hyperoxic cultured samples. These data clearly indicated that hyperoxic culture conditions in U-937 cells caused a dramatic drop in the

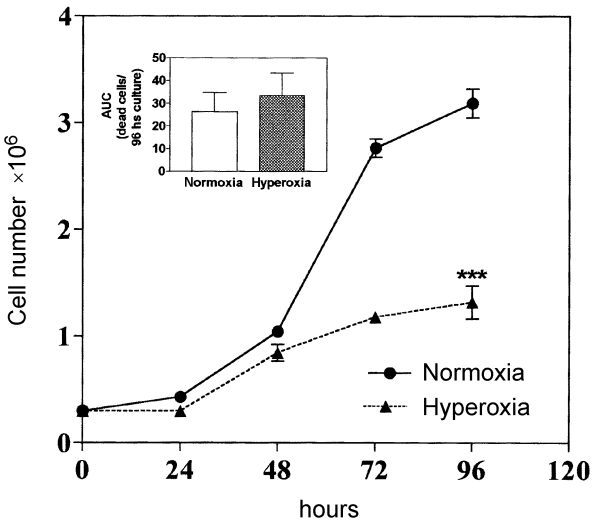

Fig. 2. Cell growth curve for U-937 cells cultured for 96 h in normoxia and in hyperoxia. Inside: cell death expressed as AUC during 96 h of culturing. Results represent the mean ± S.E.M. calculated from four experiments, each performed in duplicate. ***$p < 0.001$.

acetate/lactate ratio. Interestingly, in the hyperoxia U-937 cells a significant increase in alanine content was determined (Table 1). Both lactate and alanine can be produced from the increased availability of pyruvate, whose utilization in the Krebs cycle is reduced by perturbation of the mitochondrial respiratory chain. This alteration of the mitochondrial metabolism could also justify the decrease of succinate dehydrogenase activity observed in the hyperoxic cultures.

These data, therefore, confirmed a metabolic shift to anaerobic glycolysis in hyperoxic cell cultures (Shoonen *et al.*, 1990).

Cell growth and cytotoxicity Dye-binding assays on U-937 cells cultured for 96 hours in normobaric hyperoxia ($pO_2 \approx 50\%$) showed a strong inhibition of cell growth compared to cells cultured in normoxia. This cell growth inhibition, however, did not appear to be related to the remarkable cytotoxicity (cell dead expressed as AUC/96 h: normoxia 26.3 ± 3.4; hyperoxia 33.3 ± 4.0, p = NS), as evaluated by the light microscopy assay (trypan blue exclusion) (Fig. 2).

These data were confirmed by the LDH release into the medium and by the MTT reductase activity. It is worth noting that no morphological (fluorescence microscopy) and biochemical criteria (DNA electrophoresis) of apoptosis were observed (data not shown).

These results indicated that moderate hyperoxia causes a significant alteration of cell growth, which was marked after 48 h of culturing.

Differentiation markers To evaluate the possible induction of differentiation by hyperoxia-pro-oxidant culture conditions, a set of functional differentiation assays were performed. In particular, it is well known that in human myeloid tumor cell lines restoration of the so-called 'respiratory burst' can be considered a fundamental functional marker of differentiation (Ehinger *et al.*, 1996). Chemiluminescence analyses clearly indicated that U-937 cells, cultured for 96 h in moderate hyperoxia, showed no recovery of the oxidative burst function (Table 1). These data confirmed that the metabolic shift related to the hyperoxic condition did not depend on cell metabolism modifications resulting from cell differentiation. Interestingly, the basal production of ROS was lower in samples containing cells cultured for 96 h in hyperoxia compared to the controls. This data, as evaluated by the chemiluminescence method under normoxic conditions, seem to indicate that the inhibition of the mitochondrial metabolism associated with the induction of antioxidant enzymes buffers the increased ROS generation induced by moderate hyperoxia.

Discussion

The results from the spectrophotometric measurements for the mitochondrial respiratory enzymes activities in digitonin permeabilized U-937 cells, coupled with the NMR spectroscopy analysis of the culture medium, have demonstrated that moderate hyperoxia (50% O_2) induced an alteration in the mitochondrial respiratory chain and a series of metabolic compensatory mechanisms in a myeloid leukemia cell line.

These data seem to confirm the studies of Shoonen *et al.* (1990) and Allen *et al.* (1988), in that they show a respiratory failure and stimulation of glycolysis in different cell lines exposed to normobaric hyperoxia (95-98% O_2).

Our experimental model of hyperoxia permits a better definition of the mitochondrial alteration, which consists of an intriguing increase in the succinate cytochrome *c* reductase activity that could be one of the pathogenic mechanisms by which hyperoxia causes oxidative cell damage. In facts, in our opinion, this increase represents the result of an aspecific scavenging action, by the exogenous cytochrome *c*, of the superoxide anions produced by hyperoxia-damaged succinate cytochrome c reductase, as hypothesized by Zhang *et al.*, (1998).

Moreover, Brambilla *et al.* (1997) also showed that mitochondrial respiratory chain damage, induced by chloramphenicol and ethidium bromide, leads to the overexpression of antioxidant enzymes, like glutathione peroxidase and heme oxygenase, while the catalase activity shows no appreciable change. These modifications probably represent an adaptive response to an H_2O_2- mediated cytotoxic event secondary to mitochondrial stress.

In contrast, our results showed a large increase of glutathione peroxidase activity associated with significant increments in the catalase and total superoxide dismutase activities. These data point out the mechanisms of damage, as determined by superoxide leakage and repair, unlike other

types of oxidative stress.

It is also generally accepted that hyperoxia injures cells by virtue of the unbalanced production of ROS (including H_2O_2 and superoxide anion), which overcome the endogenous antioxidant defenses (Kazzaz *et al.*, 1996). These oxidants are cytotoxic and kill cells via apoptosis. However, hyperoxia induced cell death via necrosis, despite the activation of nuclear factor-kB (Li *et al.*, 1997; Franek *et al.*, 2001; Shishodia and Aggarwal, 2002).

Yet, the experimental model of moderated hyperoxia (50% instead of 98% of O_2) adopted in this study did not cause significant apoptosis in cell cultures, as shown by light and fluorescence microscopy. This could depend on the strong induction of glutathione peroxidase, which could partially protect mitochondrial membranes from oxidative damage and prevent cytochrome *c* release (Nomura *et al.*, 1999).

In conclusion, this study has shown that the following pathogenic mechanisms and adaptive responses represent a protective strategy against oxygen toxicity, namely:

- moderate hyperoxia mainly hampers mitochondrial respiration, which is the main source of ROS, and in an attempt to circumvent this metabolic perturbation, cells rapidly shift to glycolysis so that the availability of glucose becomes the limiting factor for cell growth progression;
- moderate hyperoxia seems to induce an oxidative stress, as demonstrated by the dramatic increase of the malonate-inhibitable rate of cytochrome *c* reduction at the level of succinate cytochrome *c* reductase, which could be related to an aspecific superoxide-scavenging action of the exogenous cytochrome *c* and the induction of antioxidant enzymes.
- hyperoxia-damaged mitochondria at the level of the electron respiratory chain showed a decrease in succinate dehydrogenase activity, an apparent increase in succinate-cytochrome *c* reductase activity, with no significant modification of NADH cytochrome *c* reductase activity, cytochrome oxidase activity and cellular concentrations of coenzyme Q_{10}. In relation to previous studies (Simon *et al.*, 1978; Gardner *et al.*, 1994; Yan *et al.*, 1997), our data leave, as a possible source of ROS generation, a derangement at the level of $FADH_2$ oxidation (i.e. at the level of glycerol catabolism by mitochondrial FAD-dependent glycerol 3 phosphate dehydrogenase and/or fatty acids beta oxidation via Electron Transferring Flavoprotein).

Intriguingly, a set of experiments were performed with the addition of n-acetyl-cysteine (1-4 mmol/l) to the culture medium, and showed no modification of the considered parameters (data not shown). The inefficacy of n-acetyl-cysteine could be related to the lack of entry into the cells or mitochondria, or to the fact that this antioxidant molecule fails to scavenge all the ROS (for example the superoxide anion) (Zafarullah *et al.*, 2003).

Moreover, the low level of ROS in cells cultured for 96 h in hyperoxia, determined by chemiluminescence in basal conditions (see again Table 1), clearly indicate that both the decrease of mitochondrial respiratory activity and the activation of the antioxidant defense systems seem to have a role in reducing the unbalance in the oxidative metabolism induced under these particular experimental conditions.

Normobaric hyperoxia has been shown to represent a useful experimental model to evaluate cellular defense mechanisms to oxidative stress and to the cellular toxicity determined by oxygen therapy.

The results of this study, while confirming several other reports, provide some novel information (i.e. the alteration of succinate cytochrome *c* reductase), which may be of value in the further understanding of the molecular mechanisms at the basis of oxygen toxicity.

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