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RESEARCH PAPER

Mitochondrial Antioxidants Alleviate Oxidative and Nitrosative Stress in a Cellular Model of Sepsis

Nadezda Apostolova • Remedios Garcia-Bou • Antonio Hernandez-Mijares • Raul Herance • Milagros Rocha • Victor M. Victor

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ABSTRACT

Purpose Mitochondrial dysfunction plays a key role in sepsis.

Methods We used a sepsis model of human endothelial cells (HUVEC) to study mitochondrial function during normoxic (21% O_2) and hypoxic (1% O_2) conditions.

Results When stimulated with a LPS cocktail, HUVEC displayed an increase of nitric oxide (NO) in normoxic and hipoxic conditions, being higher at 21% O_2. LPS-activation for 24 h at 1% O_2 increased ROS production, which was reversed with the mitochondrial antioxidant Mitoquinone (MQ) and Glutathione Ethyl Ester (GEE). Activated cells displayed diminished mitochondrial O_2 consumption with specific inhibition of Complex I, accompanied by increase in tyrosine nitration and Type II NOS protein expression, effects which were recovered by antioxidants and/or with L-NAME. These parameters varied with O_2 environment, namely inhibition of respiration observed in both O_2 environments at 24 h was very similar, whereas O_2 consumption rate fell earlier in 1% O_2-exposed cells. While no significant differences were detected at earlier time points, at 24 h tyrosine nitration was higher in normoxic *vs.* hypoxic cells.

Conclusions Mitochondria are heavily implicated in sepsis. Mitochondrial antioxidants provide a mechanistic model for the development of potential therapies.

KEY WORDS hypoxia · mitochondria · mitoquinone · reactive oxygen species · sepsis

ABBREVIATIONS

DAF-FM	4-amino-5-methylamino-2′,7′-difluorofluorescein
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
DHR123	dihydrorhodamine 123
GEE	glutathione ethyl ester
HBSS	Hank's balanced salt solution
HUVEC	human umbilical vein endothelial cells
L-NAME	L-N^G-nitroarginine methyl ester
MCB	monochlorobimane
MOF	multiple organ failure
MQ	mitoquinone
PBS	phosphate-buffered saline
ROS	reactive oxygen species

N. Apostolova • M. Rocha • V. M. Victor
Department of Pharmacology, University of Valencia
Valencia, Spain

N. Apostolova • M. Rocha • V. M. Victor
CIBERehd
Valencia, Spain

R. Garcia-Bou • M. Rocha • V. M. Victor (✉)
University Hospital Doctor Peset Foundation
Avda Gaspar Aguilar 90
46017, Valencia, Spain
e-mail: vmviktor@gmail.com

V. M. Victor
e-mail: victor.victor@uv.es

A. Hernandez-Mijares • M. Rocha • V. M. Victor
Endocrinology Service, University Hospital Doctor Peset
Valencia, Spain

A. Hernandez-Mijares
Department of Medicine, University of Valencia
Valencia, Spain

R. Herance
High Technology Institute-PRBB
Barcelona, Spain

V. M. Victor
Department of Physiology, University of Valencia
Valencia, Spain

TPP	triphenylphosphonium
Type II NOS	inducible nitric oxide synthase

INTRODUCTION

Severe sepsis is a systemic inflammatory response to infection characterized by septic shock and organ dysfunction and can progress into multiple organ failure (MOF), a condition defined as the presence of severely altered organ homeostasis in acutely ill patients (1,2). Although infections can be successfully managed with medication combined with other therapeutic approaches, such as intensive medical care, sepsis is still a major public health concern, as it represents the leading cause of morbidity and mortality among severely ill patients admitted to hospital intensive care units. It is estimated that there are 750,000 cases of severe sepsis each year in the USA, with a strikingly high mortality rate of around 30% for uncomplicated sepsis, which reaches 80% in cases of severe MOF (3).

There is substantial evidence to support the idea that MOF develops during sepsis mainly as a result of impaired utilization of cellular oxygen. Endothelial injury and malfunction are also crucial to sepsis pathogenesis (4). The complex function of the endothelium enables organ homeostasis by regulation of the vascular tone, maintenance of the selective vascular permeability and creation of an anticoagulant surface (5). In sepsis, the endothelium undergoes profound mechanical and functional alterations that contribute to pathogenesis of the inflammatory state.

The development of mitochondrial dysfunction in the endothelium during sepsis is extremely complex and still poorly understood (2). Patients under septic shock have been shown to display significant oxidative stress, manifested by increased levels of lipid peroxides, decreased antioxidant capacity and altered mitochondrial redox state (6–9). Moreover, post-mortem analyses of livers from patients with sepsis has revealed the presence of hypertrophic mitochondria with depressed Complex I and Complex IV activity (10). Similar results regarding mitochondrial dysfunction have also been obtained using cellular and animal models of sepsis (11,12). Several inflammatory mediators, including reactive oxygen species (ROS) and nitric oxide (NO), are overproduced during sepsis and have been shown to directly interfere with mitochondrial respiration. NO competes with molecular oxygen in binding to Complex IV of the electron transport chain (cytochrome *c* oxidase), which decreases the activity of the enzyme, blocks the electron transport and enhances the formation of superoxide. This radical further reacts with NO to produce peroxynitrite ($ONOO^-$) and other reactive nitrogen species (RNS). RNS have been reported, as modulators of a dozen other mitochondrial proteins, such as Complex I (13). Tissue hypoxia is frequently present in sepsis. This state may favour the competitive NO-mediated inhibition of cytochrome *c* oxidase, contributing to and/or enhancing the development of mitochondrial dysfunction (11). Moreover, hypoxia not only reduces mitochondrial respiration through reduced availability of oxygen but also enhances mitochondrial ROS production, an effect that has major implications in states of clinical shock (14). All this supports a pivotal role for NO, ROS and diminished oxygen concentration in the development of sepsis and also points the way for possible pharmacological modulation of mitochondrial function in the prevention and treatment of this inflammatory state.

Exogenous addition of antioxidants is a widely used approach to modulating increases in ROS and their consequences during the inflammatory process. However, there is a lack of conclusive evidence regarding the beneficial effect of antioxidants in critically ill patients (15), and this is believed to be due to the reduced capacity of these molecules to reach and/or accumulate within mitochondria. The most abundant cellular antioxidant is glutathione (GSH), which has multiple redox-related functions. Replacement therapy with antioxidants other than GSH is ineffective, as they do not exert all the important actions that GSH does *per se* (16). Since GSH alone is not effectively taken up by cells, several approaches to increase its intracellular levels have been developed, one of which is glutathione ethyl ester (GEE), a molecule that is converted intracellularly into GSH with a 1:1 molar stoichiometry and can efficiently reach mitochondria. Another group of antioxidants consists of selective mitochondria-targeted molecules with antioxidant properties, such as mitoquinone Q (MQ). This compound is composed of the lipophilic triphenylphosphonium cation (TPP) linked to the ubiquinone moiety of the endogenous coenzyme Q10 (17). Conjugation with TPP enables MQ to be readily transported into the cell and to concentrate several hundred fold within mitochondria attracted by the large mitochondrial inner membrane potential. This compound has been shown to have a beneficial effect in both cellular and animal models of sepsis (12).

In the present work, we addressed the temporal effect of endogenous NO and ROS production and diminished oxygen availability on LPS-activated human umbilical vein endothelial cells (HUVEC). We monitored NO release, peroxynitrite formation, nitrite levels, ROS production and GSH levels, mitochondrial respiration, Complex I activity and tyrosine nitration over an incubation period of 24 h. Additionally, through the use of MQ (18,19) and GEE (20), we confirmed the impairment of mitochondria in a model of sepsis.

MATERIALS AND METHODS

Cell Culture

All experiments were performed with HUVEC, a cellular model commonly employed for physiological and pharmacological studies of the endothelium. Umbilical cords were obtained from the Department of Gynaecology (Faculty of Medicine, University of Valencia). Cells were cultured in endothelial cell growth media-2 (EGM-2: Lonza, Walkersville, MD) as previously described (21) and were exposed to a humidified atmosphere composed of 94% N_2, 5% CO_2, and 1% O_2 for the hypoxia studies. All measurements were taken in a room air environment of 21% O_2, although additional studies involving washing, harvesting and lysis of cells carried out in a hypoxia tent produced similar results (data not shown). Cell activation was achieved by treatment with *E. coli* endotoxin LPS (10 μg/ml), IFN-γ (50 U/ml) and TNFα (20 ng/ml) (referred to herein as an "LPS cocktail") over a period of 24 h. In some experiments, the non-specific NO synthase (NOS) inhibitor L-N^G-nitroarginine methyl ester (L-NAME) (100 μM) was added immediately prior to cell treatment in order to evaluate whether subsequent changes were dependent on NOS activity. When necessary, cells were exposed to either MQ (1 μM) or GEE ester (0.1 mM) 1 h before treatment or/and hypoxia and were maintained in these conditions thereafter. In control experiments, the lipophilic cation linker TPP (1 μM), which is responsible for the targeting of MQ to mitochondria, produced no effect on any of the cellular responses observed. Cell viability was assessed at 24 and 48 h incubation by Trypan blue exclusion and by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diprenyl tetrazolium bromide) assay, a colorimetric assay based on the ability of cells to reduce a soluble yellow tetrazolium salt to blue formazan crystals. This reduction takes place only when mitochondrial reductase enzymes are active and is thus a marker of cell viability related to mitochondrial function. All experimental procedures were approved by the Ethics Committee of the University of Valencia and were performed in accordance with European Community guidelines.

Determination of Intracellular Nitric Oxide, Peroxynitrite and Nitrite

Intracellular NO and $ONOO^-$ were assessed by fluorimetry using the fluorescent probes 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) and dihydrorhodamine 123 (DHR123) respectively, both from Cambridge Biosciences, Cambridge, UK. HUVEC were seeded in a 96-well plate (5×10^5/well in 200 μl) and treated with LPS cocktail described previously in a 21% or 1% O_2 environment for 0, 6, 12 and 24 h. The culture medium was then replaced by HBSS solution, which was supplemented with 0.5 mM arginine, 20 mM glucose and the corresponding fluorochromes-either 10 μM DAF-FM ($\lambda_{exc}/\lambda_{em}$ of 495/515 nm) or 5 μM DHR123 ($\lambda_{exc}/\lambda_{em}$ of 500/530 nm). After 30 min incubation, fluorescence was detected using a Fluoroskan multiwell plate reader (Thermo Labsystems, Thermo Scientific, Rockford, IL).

The Griess method was employed to determine the presence of nitrite in the cell culture medium, which was considered an indicator of intracellular NO production (22).

Measurement of ROS Production and Glutathione Content

Following 30 min incubation with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 5 μM), total ROS production was assessed using fluorescence microscopy (Leica, Heidelberg, Germany) as described elsewhere (23).

Intracellular GSH content was determined by fluorimetry, employing the fluorochrome monochlorobimane (MCB, 40 μM) (24). To summarise, cells seeded on 96-well plates were incubated with MCB diluted in PBS at 37°C for 15 min, after which fluorescence intensities were measured using excitation and emission wavelengths of 380 and 485 nm, respectively, in a Fluoroscan multiwell plate reader (Thermo Labsystems, Thermo Scientific, Rockford, IL).

Measurement of Oxygen Consumption

HUVEC were treated with LPS cocktail with or without L-NAME (100 μM), in 1% and 21% O_2 environments for 6, 12 and 24 h. The cells were then collected, centrifuged (3,000*g* for 5 min) and resuspended (5×10^6 cells/ml) in incubation medium (HBSS supplemented with L-arginine 0.3 mM and HEPES 25 mM). Oxygen consumption was monitored using a Clark-type oxygen electrode (Rank Brothers, Bottisham, UK) precalibrated with air-saturated incubation medium and maintained at 37°C throughout the measurement. Cellular respiration was assessed as the rate at which the partial pressure of oxygen (PO_2) dropped, assuming a steady-state oxygen concentration of 210 μM (atmospheric 21% O_2). Data were obtained using the data-acquisition device Duo.18 (WPI, Stevenage, UK). Sodium cyanide (1 mM) confirmed that O_2 consumption was mainly mitochondrial (data not shown). The Trypan blue exclusion test showed no significant changes in cell viability (data not shown).

Assessment of Complex I Activity

Mitochondrial Complex I activity was determined by assessing the rate of NADH oxidation by spectrophotometry (25) using Multiscan Plate Reader Spectrophotometer (Thermo Labsystems, Thermo Scientific, Rockford, IL). In short, 20 µl of cellular homogenate (0.3 mg of total protein) was mixed with 1 ml of (PBS) (10 mM), containing 0.1 mM NADH. Complex I activity was determined by measuring the decrease in absorbance at 340 nm and confirmed by inhibition with rotenone (40 µM). Basal absorbance was recorded at 37°C for 1 min, 5 µl of ubiquinone (10 µM) were then added and the rate of NADH oxidation was recorded over a 2-min period. Complex I activity was calculated from the time-dependent fall in the slope of the absorbance using an NADH extinction coefficient of 6.81 mM/cm at 340 nm.

Cellular Fractionation

The analysis of protein nitration was performed in HUVECs exposed to the LPS cocktail at 21% and 1% O_2 over 24 h. Cells were pelleted (3,000*g*, 10 min), and cellular fractionation was carried out using the method of Rickwood *et al.* (26). In brief, the cell pellet was resuspended in isolation buffer (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EGTA, 1 mM Na-EDTA, 10 µM aprotinin and 10 µM leupeptin, pH 7.4) in 250 ml of sucrose. The homogenate was centrifuged (2,500*g* for 30 min at 4°C) to pellet cell membrane and nuclei. The mitochondrial fraction (pellet) was obtained by centrifugation of the supernatant (12,000*g* for 30 min at 4°C). The three subcellular fractions (membrane, mitochondria and cytosol) were subsequently freeze-dried and stored at −80°C. Nitrotyrosine was determined with the Oxiselect nitrotyrosine ELISA kit (Cell Biolabs, San Diego, CA).

Western Blot

HUVEC seeded in 6-well plates (10^5 cells/ml) were incubated for 3 days (medium was refreshed daily) with 100 µM L-NAME, which was employed in order to study whether the basal level of nitration was dependent on NOS activity. Cells were lysed with 200 µl lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml aprotinin, 1 mg/ml leupeptin, and 150 mM NaCl). Lysates were centrifuged (12,000 g 10 min 4°C), and the resulting supernatant was stored at −70°C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using a 7.5% polyacrilamide gel. Samples containing 5 mg of total protein were diluted in 10 µl of Laemmli reducing sample buffer (58 mM Tris–HCl, 6% glycerol, 1.67% SDS, 0.002% bromophenol blue, and 1% 2-mercaptoethanol, pH 6.8) and boiled for 3 min. The nitrocellulose membrane was probed with anti-type II NOS polyclonal antibody (1:2000) (BD Biosciences, San Jose, CA). Goat anti-rabbit horseradish peroxidase conjugate (1:3000) was used as the secondary antibody (Vector Laboratories, Burlingame, CA), and detection was carried out with the enhanced chemiluminescent (ECL) detection system (Amersham, Little Chalfont, UK) and the digital luminescence image analyzer FUJIFILM LAS300 (Fujifilm). ImageQuant software version 4.0 was used for densitometric analysis.

The protein content in the samples for immunoblot analysis and for the other assays was quantified using a bicinchoninic acid (BCA)-based method (Pierce, Rockford, IL).

Drugs and Solutions

Medium 199 and HBSS were purchased from Cambrex (Verviers, Belgium), DAF-FM, DHR123 and DCFH-DA from Calbiochem (San Diego, CA), MCB from Molecular Probes (Eugene, OR) and MTT was from Roche Diagnostics (Mannheim, Germany). The remaining reagents were supplied by Sigma-Aldrich (St Louis, MO). MQ was synthesized following a reported method (27), and GEE was prepared as described elsewhere (28).

Data Analysis

Data are represented as mean±SEM of at least five individual experiments. Statistical analysis was conducted using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). Data were submitted to a frequency distribution analysis by Kolmogorov-Smirnov's test, and as data presented normality, parametric test were used. One-way repeated measures analysis of variance (ANOVA) was performed for comparing within-group differences. Between-group differences were analysed using one-way ANOVA or unpaired Student's *t*-test. Student-Newman-Keuls (SNK) was used as post hoc. $P<0.05$ was considered statistically significant.

RESULTS

Activation of HUVEC with LPS cocktail (10 µg/ml LPS, 50 U/ml IFN-γ and 20 ng/ml TNFα) resulted in a progressive increase in the production of NO and NO-metabolites both in normoxic and hypoxic environments. However, production of NO (detected as DAF-FM fluorescence) and $ONOO^-$ (detected as DHR123 fluorescence) was significantly higher in cells exposed for 12 or 24 h to 21% compared to 1% O_2 (both $P<0.05$) (Fig. 1a and b),

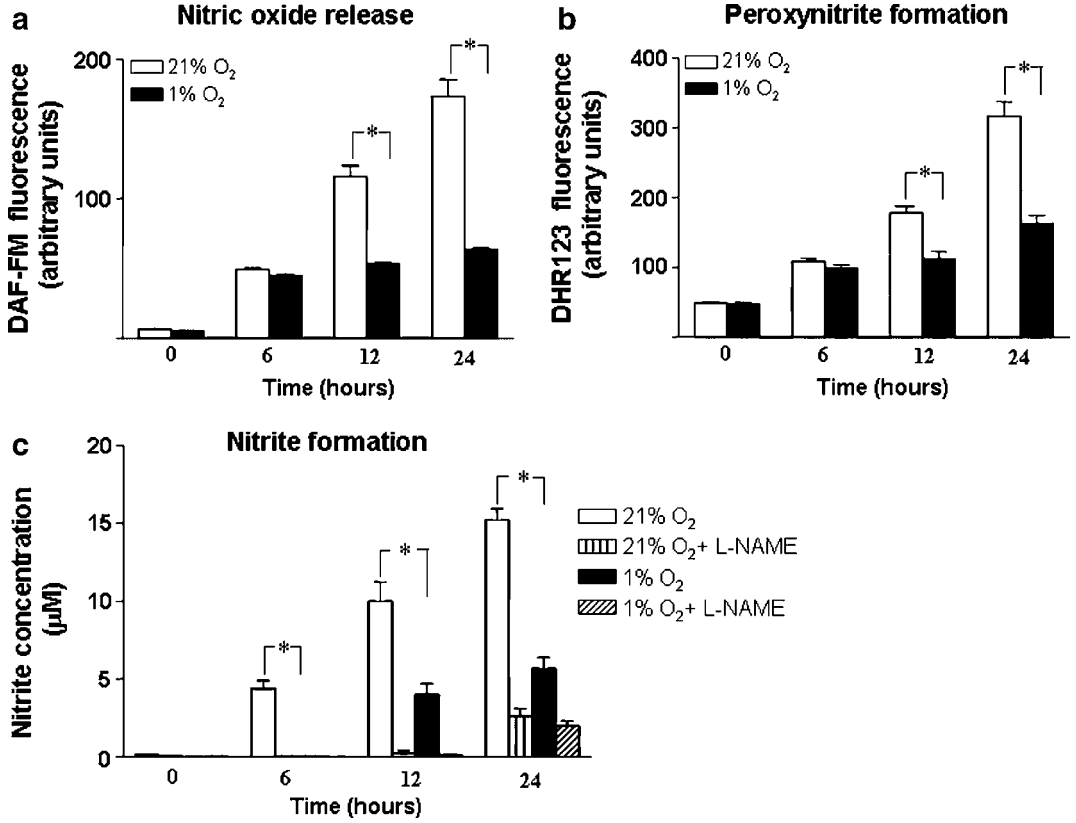

Fig. 1 Production of nitric oxide (**a**), peroxynitrite (**b**) and nitrite (**c**) in HUVEC activated with an LPS-cocktail at 21% O_2 (*white bars*) and 1% O_2 (*black bars*). Activated HUVEC were incubated at the indicated time points with either 10 μM DAF-FM (**a**) or 5 μM DHR123 (**b**) for 30 min, and fluorescence was detected as described in the text. C: nitrite (μM) was determined in the culture media with or without 100 μM L-NAME, after incubation at 21% O_2 (*white bars*) or 1% O_2 (*black bars*). Values are expressed as mean±SEM of 6 experiments. Data were statistically analysed by means of a Student's *t*-test; *$P<0.05$ between the indicated pairs.

whereas no difference was detected after 6 h exposure. The augmented NO release in LPS-activated cells was also reflected in the progressive increase of nitrite production measured over 24 h and similarly to NO and $ONOO^-$; activated cells subjected to normoxia showed significantly more pronounced nitrite formation than those in 1% O_2 (Fig. 1c, $P<0.05$). As expected, coincubation with L-NAME, a widely used NOS inhibitor, greatly reduced the accumulation of nitrite both in normoxia and hypoxia (Fig. 1c, $P<0.05$), thereby confirming the NOS-specificity of the response. At 24 h incubation, a Trypan blue exclusion test and MTT assay of cellular viability revealed the absence of significant alterations under all the experimental conditions (results not shown). Next, we aimed to assess ROS production and the redox state in activated HUVEC. LPS-activation for 24 h at 1% O_2 led to an increase of DCFH fluorescence, indicating an augmented production of ROS (Fig. 2a), an effect reversed by the antioxidant molecules MQ (1 μM) and GEE (0.1 mM). Oxidative stress is due to both an increase in ROS production and an undermining of the cellular antioxidant defences. As shown in Fig. 2b, activated HUVEC (24 h, 1% O_2) manifested a significantly decreased MCB fluorescence, pointing to a reduction in GSH levels, which was reversed with MQ, whereas, expectedly, GEE boosted the fluorescence signal.

Non-activated cells were found to consume significantly less O_2 in the 1% O_2 environment than in normoxic conditions over the 24 h period ($P<0.05$) at all the time points studied (6, 12 and 24 h) (Fig. 3a and b). However, Complex I activity studied over the 24 h incubation period showed no difference in control (non-activated) cells subjected to 1% O_2 *versus* those exposed to 21% O_2 (Fig. 3c and d). When activated cells were compared with control cells in normoxic conditions there was no detectable change in overall oxygen consumption or Complex I activity over the first 6 h, though there was a progressive ~75% fall ($P<0.05$) in both parameters after this time (Fig. 3a and c). A similar decrease was detected in the 1% O_2 group, but while the degree of inhibition observed in both oxygen environments at 24 h was very similar, the O_2 consumption rate fell earlier in 1% O_2-exposed cells (6 h, $P<0.05$ *vs.* 21% O_2). Coincubation with L-NAME reversed the drop in both oxygen consumption and Complex I activity by 75–80% ($P<0.05$) in

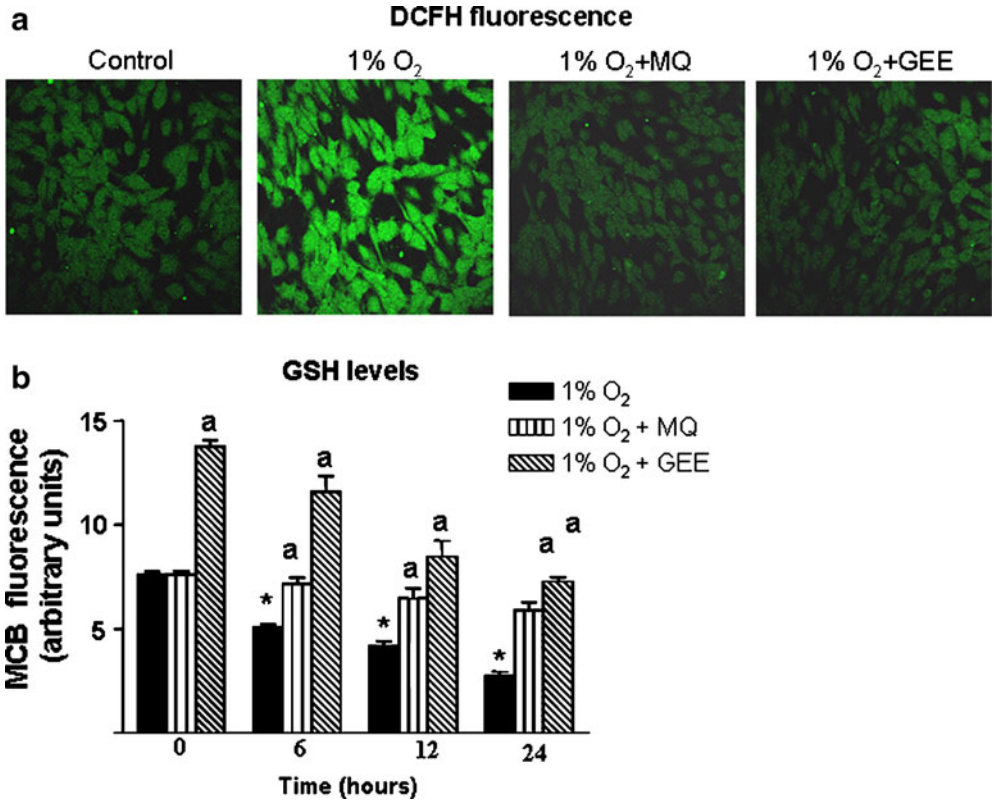

Fig. 2 ROS production and redox status in HUVEC stimulated with LPS cocktail. (**a**) Representative fluorescence microscopy images showing DCFH fluorescence after 24 h incubation with an LPS cocktail. Cells were incubated with DCFH-DA 5 μM for 30 min. (**b**) GSH levels in activated HUVEC. At the indicated time points, MCB 40 μM was added for 30 min incubation, and fluorescence was recorded as described in "Materials and Methods." Data were statistically analyzed by a one-way ANOVA multiple comparison test followed by a Student-Neuman-Keuls test, $^{*}P < 0.05$ *vs.* the control of 0 h incubation, and by a Student's *t*-test, $^{a}P < 0.05$ *vs.* the corresponding control value at each time point.

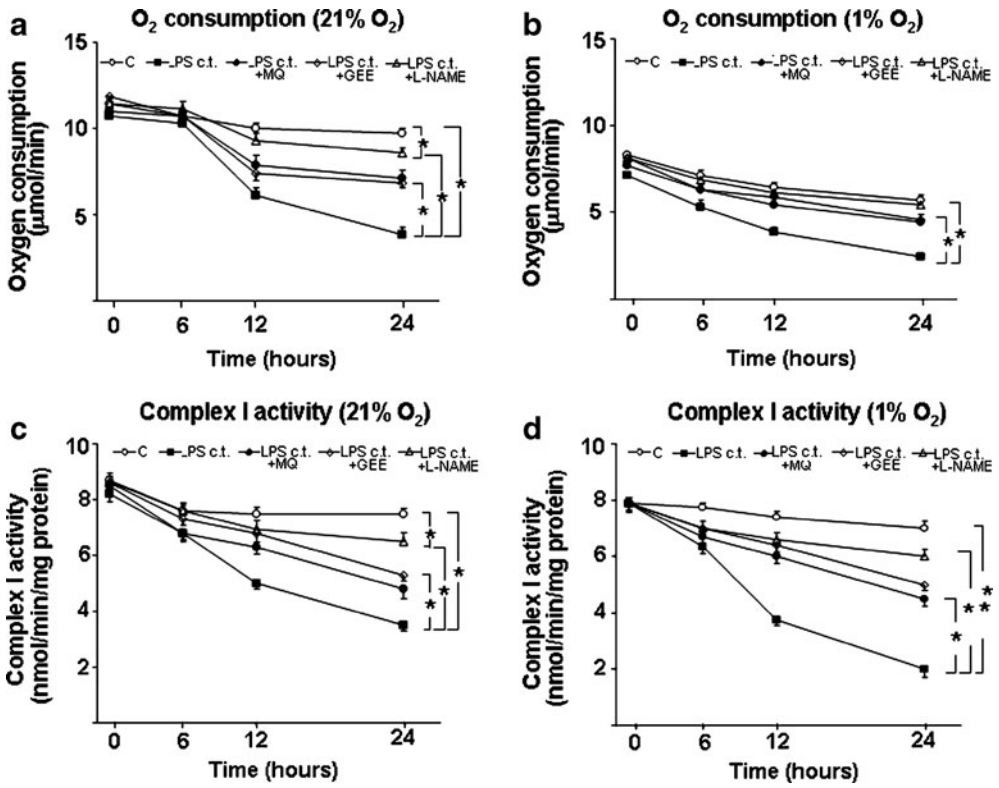

Fig. 3 O_2 consumption and mitochondrial Complex I activity in HUVEC stimulated with an LPS cocktail. After incubation for the indicated periods of time (0, 6, 12 and 24 h) O_2 consumption rate was measured in intact cells in normoxic (**A**) and hypoxic conditions (**B**), and Complex I activity was determined in normoxic (**C**) and hypoxic conditions (**D**). Data were statistically analysed by means of a one-way ANOVA multiple comparison test followed by a Student-Neuman-Keuls test, $^{*}P < 0.05$ between the indicated pairs.

both oxygen environments. Incubation of activated HUVEC for 1 h with GEE or MQ in LPS or cytokine-free medium resulted in a partial recovery of both oxygen consumption and Complex I activity ($P<0.05$), which was more effective at earlier time points in all the conditions assayed.

We also set out to study tyrosine nitration, one of the most common NO-mediated protein modifications. No differences were observed between normoxic and hypoxic conditions with respect to the concentration (0.2 ± 0.1 ng/mg protein) of total cell tyrosine nitration over a 24 h period (Fig. 4). Moreover, this low (basal) concentration was not affected by L-NAME (data not shown), suggesting it was independent of NOS activity. However, in activated cells, there was a progressive increase of tyrosine nitration in both oxygen environments, particularly evident at 24 h incubation (both $P<0.05$) (Fig. 4a and b). Of note, while no significant differences were detected at earlier time points, tyrosine nitration was higher in normoxic than in hypoxic cells at 24 h ($P<0.05$). Coincubation with L-NAME significantly reduced tyrosine nitration in both 21% and 1% O_2 environments. In addition, we assessed the nitrotyrosine content specifically in the mitochondrial and cytosolic cellular fraction (Fig. 4c and d). Similarly to the results obtained in whole-cell extracts, a significant increase in nitrotyrosine content was observed at 24 h incubation in 21% O_2 with respect to hypoxic conditions. This difference was more pronounced in the cytosolic fraction, but the increase of cytosolic nitrotyrosine was most obvious at 24 h incubation (Fig. 4d). Interestingly, at 6 h we detected a higher nitrotyrosine concentration in hypoxic than in normoxic conditions, and this was particularly evident in the mitochondrial fraction. Thus, the early (6 h) increase in nitrotyrosine concentration in the total cell extracts under hypoxia was due to the increased nitration in the mitochondrion, as shown in Fig. 4c. Interestingly, we detected no increase in tyrosine nitration in either the membrane or the nuclear fraction (data not shown).

Type II NOS protein was not detected in non-activated cells incubated at 21% O_2 or 1% O_2 for 24 h (data not shown). However, there was an increase in Type II NOS protein expression in the activated cells at 6 and 12 h in

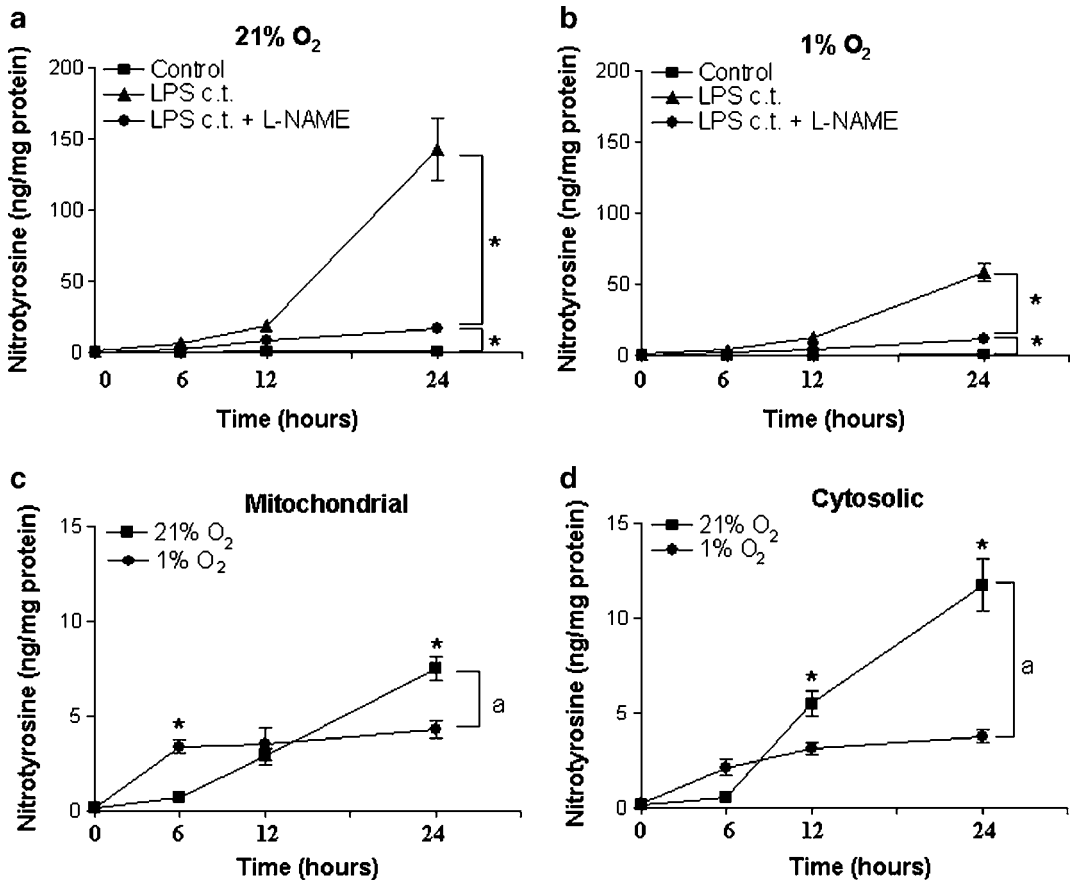

Fig. 4 Nitrotyrosine formation in HUVEC activated with an LPS cocktail for the indicated periods of time (0, 6, 12 and 24 h). Nitrotyrosine concentrations in whole-cell extracts of control cells and cells activated in the absence or presence of L-NAME 100 μM and exposed to normoxia (**a**) and hypoxia (**b**). Nitrotyrosine concentrations in specific subcellular fractions of cells exposed to normoxia and hypoxia: mitochondrial fraction (**c**) and cytosolic fraction (**d**). Data were statistically analyzed by means of a one-way ANOVA multiple comparison test followed by a Student-Neuman-Keuls test or Student's *t*-test, $^{*}P<0.05$ between the indicated pairs. $^{a}P<0.05$ *vs.* the corresponding control value at each time point.

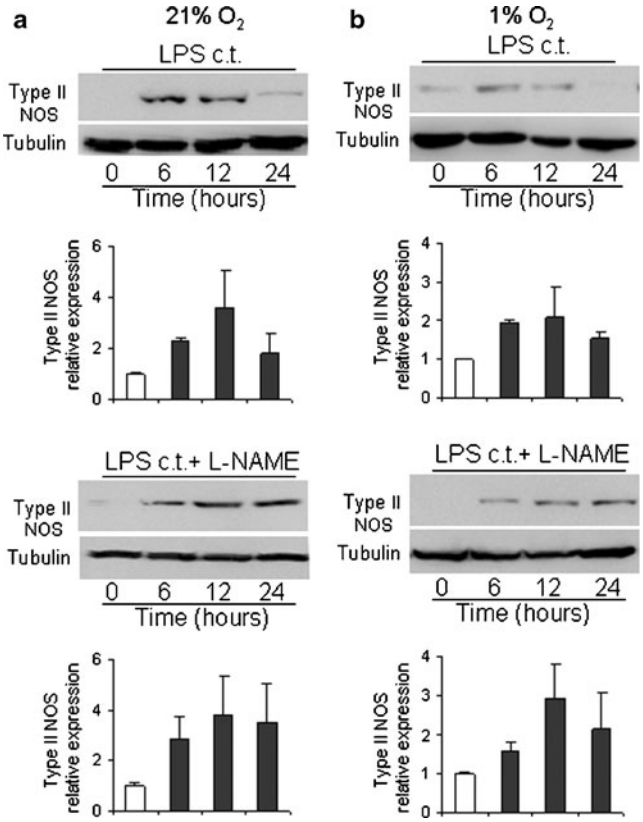

Fig. 5 Type II NOS expression in activated HUVEC exposed to normoxia (**A**) and hypoxia (**B**) for 0, 6, 12 and 24 h, in the presence and absence of L-NAME 100 μM. Representative western blot images are shown as well as bar charts expressing quantification of the expression of protein. Tubulin expression was used as a protein loading control.

both oxygen environments (Fig. 5a and b). In fact, Type II NOS expression was more marked in activated cells exposed to 21% *vs.* 1% O_2. Expression of Type II NOS seemed to return to nearly basal levels at 24 h in both oxygen conditions. In addition, L-NAME markedly attenuated the decrease in Type II NOS expression registered at 24 h and had no major effect at the other time points measured.

DISCUSSION

Mitochondria are reported to play a crucial role in the development of sepsis. In the present study, we have investigated the implication of mitochondria and the role of oxygen availability in LPS-induced HUVEC, which were used as an endothelial model of sepsis. In particular, we have discovered that stimulation with LPS leads to a decrease in mitochondrial oxygen consumption that is related to a specific inhibition at Complex I, enhanced ROS production and an altered redox state (decreased GSH content). These results support and expand previously published evidence regarding the presence of mitochondrial dysfunction, oxidative stress and/or GSH depletion in LPS-treated HUVEC (12) and HUVEC stimulated with plasma from patients with sepsis (29,30).

In view of the available evidence, we decided to assess the effect of mitochondria-specific antioxidants. We employed MQ, mitochondria-targeted ubiquinone and GEE, a molecule of ubiquitous intracellular distribution which also reaches the mitochondrion. Importantly, GSH, the most abundant cellular redox-active and antioxidant molecule, is not synthesized in the mitochondrion, and its presence in this compartment is fully dependent on its synthesis in the cytosol by the ATP-requiring enzymes γ-glutamylcysteine ligase and GSH synthetase, upon which it is transported into mitochondria (31). Bearing this is mind, it is logical to postulate that early oxidative stress in the mitochondria can affect intramitochondrial GSH levels prior to those in the other cellular compartments. This is why we chose a GSH-releasing molecule (GEE) that can reach mitochondria. In both *in vitro* and *in vivo* studies GEE has been shown to successfully reverse a fall in GSH levels and, therefore, to ameliorate the inflammatory cellular injury associated with several pathological conditions in the endothelium. For example, GEE was reported to attenuate endotoxin-induced injury in bovine pulmonary artery endothelial cells (BPAEC) (32) and to diminish exogenous $ONOO^-$-induced inhibition of mitochondrial respiration and nitrotyrosine generation in HUVEC (33). Efficient mitochondrial accumulation and ROS removal have been demonstrated for MQ in both cellular and animal models (34,35). In our cells, both MQ and GEE had a beneficial effect on all the parameters studied. Thus, the reduction in oxygen consumption and Complex I activity in activated cells under both normoxic and hypoxic conditions was almost fully reversed by co-treatment with MQ or GEE, which points to a major role of mitochondrial ROS in this effect. The antioxidant properties of these molecules were confirmed when we assessed ROS production and evaluated the redox state (GSH levels) in activated HUVEC. The increase in ROS generation and diminished GSH content were reversed by both MQ and GEE co-treatment, which also confirms the potential of these compounds as antioxidant and redox-active agents.

When stimulated with the LPS cocktail, HUVEC not only displayed redox changes and increased ROS generation but also manifested changes in NO production, such as increased Type II NOS expression and a subsequently enhanced release of NO. Type II NOS expression was enhanced both in normoxia and hypoxia at 6 and 12 h of incubation, whereas it returned almost to basal levels after 24 h incubation, independently of the oxygen environment. This latter effect was abolished with the application of the general NOS inhibitor L-NAME, probably due to a loss of the negative effect that

NO exerts on NOS activity. Activated HUVEC also displayed markers of nitrosative stress, such as enhanced $ONOO^-$ formation and an augmented tyrosine nitration. Importantly, $ONOO^-$, a radical formed by the reaction of NO with superoxide, impairs Complex I by S-nitrosylation and nitration. While S-nytrosylation of Complex I can be reversed by exposure to thiol-active compounds such as GEE (36), tyrosine nitration is thought to be the major mechanism responsible for the delayed and irreversible Complex I inhibition provoked by adding exogenous NO (37). Our results point to a major increase in nitrotyrosine formation upon treatment with the LPS cocktail, which is particularly evident at 24 h of incubation. This increase is less pronounced in HUVEC subjected to hypoxia, which is probably due to the lower level of NO production in these conditions. We detected lower NO production and, consequently, lower $ONOO^-$ generation at 1% O_2, which is in accordance with previously published results (11) and could be related, among other effects, to the fact that under diminished oxygen availability, the K_m for oxygen of NOS is elevated. Importantly, when NO production was inhibited by exposure to L-NAME, the generation of nitrotyrosine was almost completely reversed in both 21% O_2 and 1% O_2 environments, thus suggesting a fundamental role of NO in tyrosine nitration of proteins.

CONCLUSION

We describe the presence of mitochondrial dysfunction with specific inhibition of Complex I, and oxidative and nitrosative stress in a human endothelial cellular model of sepsis. We also show that oxygen concentration plays an important role during this inflammatory condition. We believe that the evidence reported here can contribute to a better general understanding of the cellular mechanisms involved in pathogenesis of sepsis. In addition, we provide evidence that mitochondrial antioxidants are of benefit in managing mitochondrial dysfunction and oxidative damage during sepsis. Also, we consider our data to be of major clinical importance, as they provide insights for the potential therapeutic use of mitochondrial antioxidants, such as GEE and MQ, and redox-active compounds during conditions of septic shock.

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