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Endothelial NOS-derived nitric oxide prevents injury resulting from reoxygenation in the hypoxic lung

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Abstract

To date, the role that NO derived from endothelial NO synthase (eNOS) plays in the development of the injuries occurring under hypoxia/reoxygenation (H/R) in the lung remains unknown and thus constitutes the subject of the present work. A follow-up study was conducted in Wistar rats submitted to H/R (hypoxia for 30 min; reoxygenation of 0 h, 48 h and 5 days), with or without prior treatment using the eNOS inhibitor L-NIO (20 mg/kg). Lipid peroxidation, apoptosis, protein nitration and NO production (NOx) were analysed. The results showed that L-NIO administration lowered NOx levels in all the experimental groups. Contrarily, the lipid peroxidation level and the percentage of apoptotic cells rose, implying that eNOS-derived NO may have a protective effect against the injuries occurring during H/R in the lung. These findings could open the possibility of future studies to design new therapies for this type of hypoxia based on NO-pharmacology.

Keywords: *Hypoxia, nitric oxide, endothelial nitric oxide synthase, L-NIO, reoxygenation, lipid peroxidation, apoptosis*

Introduction

Hypoxia is one of the most frequently encountered forms of stress in health and disease. To find useful remedies capable of ameliorating its casualty is essential. Although the underlying mechanisms of the hypoxia-induced injury and cell death are still not fully understood, it has been shown that hypoxia induces nitric oxide (NO) over-production [1]. In the lungs, endogenous NO plays an important role in regulating a number of physiological and pathological processes, including hypoxia/reoxygenation (H/R). This molecule is formed during the conversion of L-arginine to citrulline by one of three different isoforms of NO synthase (NOS): endothelial (eNOS or NOS III), neuronal (nNOS or NOS I) and inducible (iNOS or NOS II) [2]. Whereas eNOS and nNOS are mostly calcium/calmodulin-dependent and are usually constitutively expressed [3], iNOS is typically independent of intracellular calcium concentration and can be induced in a wide variety of cell types in response to various stimuli, such as hypoxia [4,5]. Two main sites of NO production have been identified in the lung, the vasculature and the airways [6,7]. The main isoform in the vascular endothelium is eNOS, while in the airways all three NOS isoforms have been detected in the bronchial and bronchiolar epithelium [6,8].

The reoxygenation of the hypoxic tissues is characterized by the formation of both reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in widespread lipid peroxidation, protein oxidative and nitrosative modifications, alterations in DNA, apoptosis and necrosis [9–11]. Particularly peroxynitrite, formed by the reaction of NO and superoxide, is a ROS and RNS that can alter protein function by nitrating phenolic rings, including tyrosine, to create nitrotyrosine [12].

A great number of works in the literature have focused on the study of eNOS and the effects of NO under H/R in the lung. However, they have been addressed to analyse the role of eNOS and eNOS-derived NO on the physiological changes related to

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such situations in this organ [13–16], paying no attention to the study of the effects that this NO could exert in the development of the lung hypoxia-induced damage. To date, the role that eNOS-derived NO plays in the development of the lung injuries occurring during H/R remains completely unknown and thus constitutes the subject of the present work. Therefore, we seek to investigate the effects of this NO on parameters of cell and tissue damage in the lung submitted to H/R. For this, Wistar rats were submitted to hypoxia for 30 min and were studied during the subsequent reoxygenation period (0 h, 48 h and 5 days) as a novel approach to address the problems raised from assaults under such circumstances. We have analysed lipid peroxidation, apoptosis and nitrated protein expression before and after treatment with the selective eNOS inhibitor N5-(1-iminoethyl)-L-onornithine dihydrochloride (L-NIO) [17–19]. L-NIO is an L-arginine analogue that acts as an inhibitor of NO synthesis from L-arginine by the vascular endothelium. L-NIO is a competitive inhibitor of eNOS which has been reported to be ~ 5-times more potent as an inhibitor of this isoform than other L-arginine analogues [20,21]. It bears mentioning that the literature dealing with the inhibitor L-NIO in hypoxic lungs is very scant and thus its possible effects on this organ remain completely unknown. This is the first study that describes the time-course effects of this selective eNOS inhibitor in the adult rat lung submitted to H/R.

Methods

Animals

The study was performed on mature adult (4–5 months old) male albino Wistar rats kept under standard conditions of light and temperature and allowed *ad libitum* access to food and water. All the experiments were conducted according to E.U. guidelines on the use of animals for biochemical research (86/609/EU).

Experimental protocol and endothelial NOS inhibitor administration

The acute hypobaric hypoxia was carried out as previously published by our group [22,23]. Briefly, animals were placed in a chamber in which the air pressure was controlled by means of a continuous vacuum pump and an adjustable inflow valve. Hypoxia was induced by down-regulating the environmental O_2 pressure to a final barometric pressure of 225 mm Hg, resulting in a 48 mm Hg O_2 partial pressure (pO_2). These conditions were maintained for 30 min. The ascent and descent speed were maintained below 1000 feet/min. After the hypoxia period, animals were kept under normobaric normoxic conditions for different reoxygenation times (0, 48 h and 5 days) and then were sacrificed. Control animals were maintained for 30 min in the chamber under normobaric normoxic conditions, before being sacrificed.

The selective eNOS inhibitor L-NIO or N5-(1-iminoethyl)-L-onornithine dihydrochloride (Tocris Biosciences; Ellisville, MO, USA) was injected intraperitoneally 20 min before hypoxia. L-NIO (20 mg/kg body weight) was dissolved in saline and the control was an equal volume of saline [24]. After the hypoxia period, animals were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 48 h and 5 days) and then were sacrificed. Control animals were maintained for 30 min in the chamber under normobaric normoxic conditions, before being sacrificed.

The following experimental groups ($n=5$ rats per group) were studied:

1) Control: rats maintained for 30 min in the chamber under normobaric normoxic conditions.
2) Hypoxia/reoxygenation: rats submitted to 30 min of hypoxia followed by 0 h, 48 h and 5 days of reoxygenation.
3) Control + L-NIO: control rats treated with L-NIO.
4) Hypoxia/reoxygenation + L- NIO: rats submitted to the same procedure as the second group but treated with L-NIO.

A total of 40 albino Wistar rats were used for the biochemical experiments (five animals per experimental group). The rats were killed by cervical dislocation and the lungs were immediately removed, rinsed in saline solution, and stored at −80°C until used. Another 40 rats were used for histochemistry and immunohistochemistry (five animals per experimental group). The rats were anaesthetized with Ketolar (Parke Davis, 1 mL/250 g weight; Madrid, Spain) by intraperitoneal injection and perfused in each reoxygenation time. Then, the lungs were removed, rinsed in saline solution and fixed.

NO measurement

The reaction of NO with ozone results in the light emission and this light (emitted in proportion to the NO concentration) is the basis for one of the most accurate NO assays available [25,26]. NO production was indirectly quantified by measuring nitrate/nitrite and S-nitrose compounds (NOx) using an ozone chemiluminescence-based method. For this technique, the rat lungs were homogenized in PBS with protease inhibitors. Homogenates were then sonicated, centrifuged and deproteinized with NaOH 0.8 N and $ZnSO_4$ 16% solutions. The total amount of NOx was determined by a modification [22] of the procedure

described by Braman and Hendrix [27] using a NO analyser (NOA^{TM} 280i Sievers Instruments; Boulder, CO, USA). A saturated solution of vanadium chloride (VCl_3) in 1 M HCl was added to the nitrogen-bubbled purge vessel fitted with a cold-water condenser and a water jacket to permit heating of the reagent to 90°C, using a circulating bath. HCl vapours were removed by a gas bubbler containing 1 M NaOH. The gas-flow rate into the detector was controlled by a needle valve adjusted to yield a constant pressure. Once the detector signal was stabilized, samples were injected into the purge vessel to react with the reagent, converting NOx to NO, which was then detected by ozone-induced chemiluminescence. NOx concentrations were calculated by comparison with standard solutions of sodium nitrate. Final NOx values were referred to the total protein concentration in the initial extracts.

Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined in lung homogenates as described by Buege and Aust [28]. Briefly, lungs were homogenized in PBS and then sonicated and centrifuged. In the supernatant, the amount of proteins was determined using the Bradford [29] assay. After 700 µL of thiobarbituric acid reagent (15% TCA or trichloroacetic acid, 0.38% TBA or 2 thiobarbituric acid and 2% HCl or chloride acid) was added to 300 µL of the supernatant, the solution was heated at 95°C for 15 min. After heating, the tubes were cooled in a water bath and centrifuged. The absorbance of the supernatant was read at 535 nm.

TUNEL assay for assessment of apoptotic cell death and image processing

Terminal deoxynucleotidyl transferase (TdT)-mediated desoxyuridinetriphosphate (dUTP) nick end-labelling (TUNEL) is a technique to estimate apoptosis in tissue sections. The protocol was performed in sections obtained from lungs embedded in paraffin according to the manufacturer's recommendations (TdT-FragELTM DNA Fragmentation Detection Kit, Calbiochem; Cambridge, MA, USA). Deionized water was replaced by TdT enzyme as a negative control. Apoptotic bodies were stained brown.

Ten similar microphotographs per rat were digitally captured with a light microscope (Olympus, Hamburg, Germany). They were then analysed, after background subtraction (minimal particle size 10 pixels), in two different colour channels using ImageJ (an NIH image analysis and processing software downloaded free from http://rsbweb.nih.gov/ij/). The image derived from the green channel was used to determine the number of living cells while the image acquired from the red one was used to determine the apoptotic cells. The percentage of apoptotic cells in each microphotograph was quantified by computer-assisted image analysis using the same software.

Western blot analysis for nitrotyrosine expression

For Western blot analysis, equal amounts of denatured lung total-protein extracts were loaded and separated in 7.5% SDS-polyacrylamide gel. Proteins in the gel were transferred to a PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and then blocked. Polyclonal rabbit anti-3-nitro-L-tyrosine A4 antibody (1:3000, gift from Professor J. Rodrigo from Cajal Institute of Madrid) was used to detect nitrated proteins and monoclonal antibody to α-tubulin (Sigma, St. Louis, MO, USA) was used as the internal control. Antibody reaction was revealed with chemiluminescence-detection procedures according to the manufacturer's recommendations (ECL kit, Amersham Corp., Buckinghamshire, UK).

Nitrotyrosine immunohistochemistry

Lung samples were embedded in paraffin (Paraplast Extra, Tyco; Mansfield, MA, USA). Sections were incubated with 10% goat serum for 30 min. Afterwards, they were first incubated with diluted polyclonal rabbit anti-3-nitro-L-tyrosine A4 antibody (1:500, gift from Professor J. Rodrigo from Cajal Institute of Madrid), used to detect nitrated proteins, in PBS overnight at 4°C and later with a goat anti-rabbit biotinylated secondary antibody (Pierce; Rockford, IL, USA) followed by peroxidase-linked ABC. The peroxidase activity was demonstrated following the nickel-enhanced diamino-benzidine procedure [30]. Sections were mounted on slides, dehydrated and covered using DPX. Controls for background staining were performed by replacing the primary antibody with PBS.

Statistical analysis

Data were expressed as mean ± SD (standard deviation). The statistical treatment to evaluate significant differences between groups was performed with SPSS 15.0 software. The data followed a normal distribution (tested with Kolmogorov-Smirnov test) and the principle of homoscedasticity of variances (tested with Levene test) and were tested by a two-way ANOVA. The statistically significant differences regarding the control group in the untreated groups were expressed as $^{a}p<0.05$; $^{b}p<0.02$; $^{c}p<0.01$; and $^{d}p<0.001$. The statistically significant differences between the treated groups and the corresponding

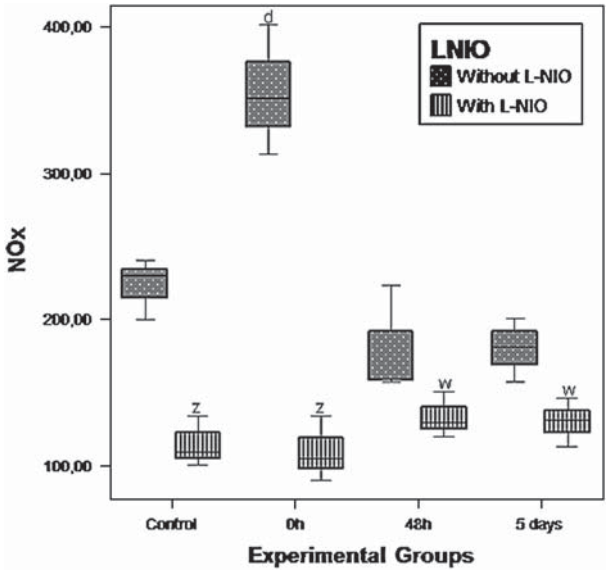

Figure 1. Influence of hypoxia/reoxygenation on nitrate, nitrite, and other nitrose compounds (NOx) in rat lung (μmol/mg protein). Experimental groups: Control and 0 h, 48 h, and 5 days post-hypoxia; Control L-NIO and 0 h L-NIO, 48 h L-NIO, and 5 days L-NIO post-hypoxia. Results are mean values of three independent experiments and five animals per group. The statistically significant differences regarding the control group in the untreated groups were expressed as $^{a}p<0.05$; $^{b}p<0.02$; $^{c}p<0.01$; $^{d}p<0.001$. The statistically significant differences between the treated groups and the corresponding untreated groups were expressed as $^{w}p<0.05$; $^{x}p<0.02$; $^{y}p<0.01$; $^{z}p<0.001$.

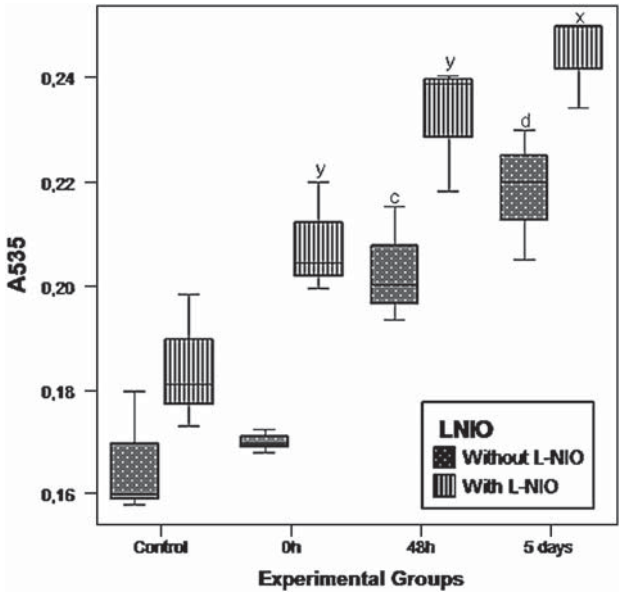

Figure 2. Influence of hypoxia/reoxygenation on lipid peroxidation in rat lung. Results were expressed as absorbance at 535 nm. Experimental groups: Control and 0 h, 48 h, and 5 days post-hypoxia; Control L-NIO and 0 h L-NIO, 48 h L-NIO, and 5 days L-NIO post-hypoxia. Results are mean values of three independent experiments with five animals per group. The statistically significant differences regarding the control group in the untreated groups were expressed as $^{a}p<0.05$; $^{b}p<0.02$; $^{c}p<0.01$; $^{d}p<0.001$. The statistically significant differences between the treated groups and the corresponding untreated groups were expressed as $^{w}p<0.05$; $^{x}p<0.02$; $^{y}p<0.01$; $^{z}p<0.001$.

Results

NO production

Figure 1 shows the determinations of nitrate/nitrite and other S-nitrose compounds (NOx) in the different experimental groups. NOx levels significantly rose early, at 0 h of reoxygenation ($p < 0.001$), in the untreated groups. However, the administration of the selective eNOS inhibitor L-NIO significantly lowered NOx level in control ($p < 0.001$) and throughout the reoxygenation periods: 0 h ($p < 0.001$), 48 h ($p < 0.05$) and 5 days ($p < 0.05$).

Thiobarbituric acid reactive substances (TBARS)

Malondialdehyde and other lipid peroxidation products, which react with thiobarbituric acid, are good indicators of oxidative stress [31]. Figure 2 shows that hypoxia/reoxygenation significantly raised the TBARS level at 48 h ($p < 0.01$) and 5 days ($p < 0.001$) of reoxygenation in the untreated groups. Moreover, in the groups treated with L-NIO, TBARS increased throughout the post-hypoxia period: 0 h ($p < 0.01$), 48 h ($p < 0.01$) and 5 days ($p < 0.02$).

TUNEL assay

The TUNEL assay, which identifies apoptotic cells, showed that hypoxia/reoxygenation significantly raised the percentage of area of apoptotic cells at the late

Table I. Quantitative data from image analysis of histological sections of rat lung stained for TUNEL assay.

Group		Percentage of area of apoptotic cells (%)
Untreated groups	Control	3.31 ± 1.10
	0 h	3.80 ± 1.00
	48 h	20.18 ± 2.52^{d}
	5 days	34.67 ± 2.47^{d}
L-NIO-treated groups	Control	5.79 ± 2.17
	0 h	18.18 ± 3.42^{z}
	48 h	27.80 ± 2.11^{y}
	5 days	41.46 ± 4.19^{y}

Experimental groups: Control and 0 h, 48 h and 5 days post-hypoxia; Control L-NIO and 0 h L-NIO, 48 h L-NIO and 5 days L-NIO post-hypoxia. Results are mean values of 50 microphotographs (10 microphotographs per animal and five animals per group) ± SD. The statistically significant differences regarding the control group in the untreated groups were expressed as $^{a}p < 0.05$; $^{b}p < 0.02$; $^{c}p < 0.01$; $^{d}p < 0.001$. The statistically significant differences between the treated groups and the corresponding untreated groups were expressed as $^{w}p < 0.05$; $^{x}p < 0.02$; $^{y}p < 0.01$; $^{z}p < 0.001$.

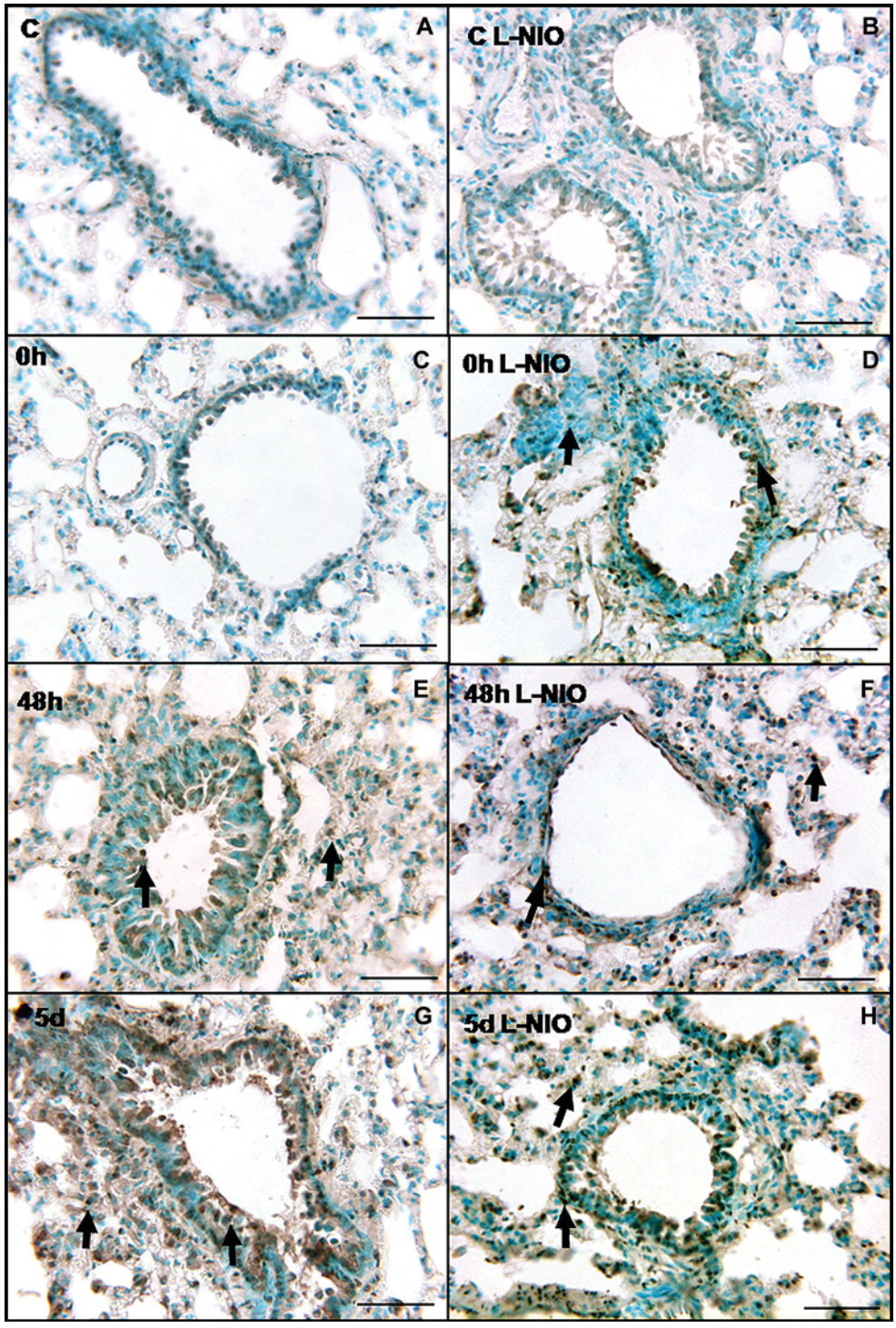

Figure 3. A-H: Micrographs showing histological sections of rat lung stained for TUNEL assay. Experimental groups: Control (C) and 0 h, 48 h, and 5 days (5d) post-hypoxia; Control (C) L-NIO and 0 h L-NIO, 48 h L-NIO, and 5 days (5d) L-NIO post-hypoxia. Apoptotic, TUNEL-positive cells are indicated by the brown nuclear staining (arrows). Micrographs E and G show higher apoptosis levels when compared with the control group in the untreated groups. Micrographs D, F, and H show higher apoptosis levels in relation to the corresponding untreated groups. Scale bars: 50 μm.

post-hypoxia times (48 h: $p < 0.001$, 5 days: $p < 0.001$) in the untreated groups (Table I). However, after the treatment with L-NIO, this percentage rose at 0 h ($p < 0.001$), 48 h ($p < 0.01$) and 5 days ($p < 0.01$) of reoxygenation when compared with the corresponding untreated groups (Figures 3A, C, E and G; taken from [32]).

Nitrotyrosine expression

Three nitrotyrosine immunoreactive bands, corresponding to proteins of 126, 112 and 72-kDa, were detected in all the experimental groups (Figure 4; right panel). The quantitative evaluation of the bulk-nitrated proteins increased significantly from 48 h to

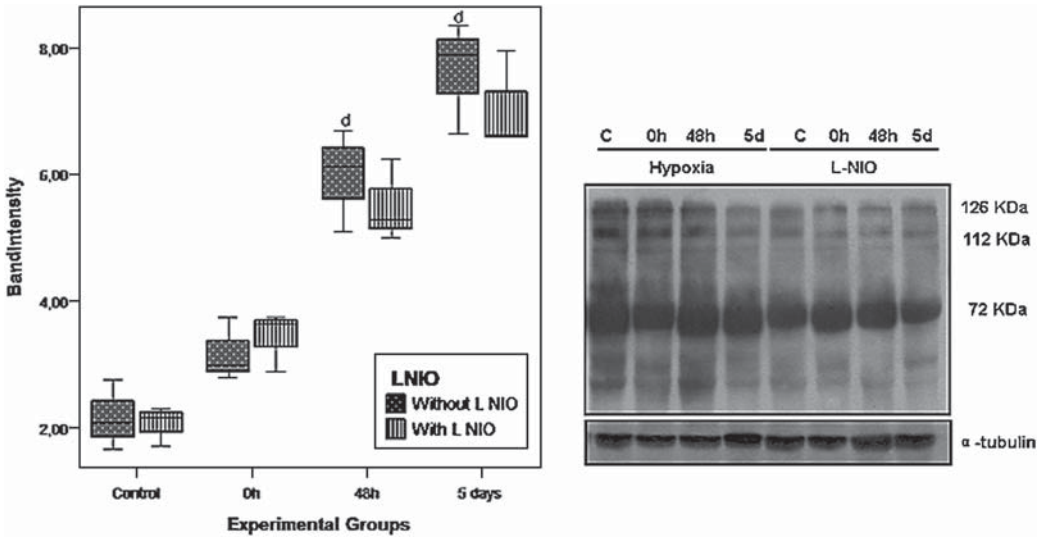

Figure 4. Influence of hypoxia/reoxygenation on nitrotyrosine-modified protein expression in rat lung. Left panel: densitometric quantification of nitrotyrosine-modified proteins in the experimental groups: Control (C) and 0 h, 48 h, and 5 days (5d) post-hypoxia; Control (C) L-NIO and 0 h L-NIO, 48 h L-NIO, and 5 days (5d) L-NIO post-hypoxia. Results were expressed as arbitrary units (A.U.). Results are mean values of three independent experiments with five animals per group. Right panel: representative autoradiography of the nitrotyrosine-modified protein bands; α-tubulin immunodetection was also included as a protein-loading control.
The statistically significant differences regarding the control group in the untreated groups were expressed as $^{a}p<0.05$; $^{b}p<0.02$; $^{c}p<0.01$; $^{d}p<0.001$. The statistically significant differences between the treated groups and the corresponding untreated groups were expressed as $^{w}p<0.05$; $^{x}p<0.02$; $^{y}p<0.01$; $^{z}p<0.001$.

5 days post-hypoxia ($p < 0.001$) in the untreated groups (Figure 4; left panel). However, the administration of the inhibitor L-NIO did not modify the nitrated protein expression when compared with the corresponding untreated groups.

Nitrotyrosine immunohistochemistry

In all the experimental groups, the nitrotyrosine immunoreactivity was detected in bronchiolar epithelial cells and vascular endothelial cells (Figures 5A, C, E and G; taken from [32]). Corroborating the previous results, the nitrotyrosine-positive staining was more intense from 48 h to 5 days post-hypoxia in these cell types in the untreated groups. The administration of L-NIO did not alter the nitrotyrosine-positive staining in the animals treated with this eNOS inhibitor.

Discussion

Hypoxia-associated pathophysiology is complex and elusive, but notably significant. Many adverse effects of hypoxia are normally observed under conditions generated by ischemia. Like reperfusion, reoxygenation eventually does not completely reverse the hypoxia-induced changes. The complexity of the cellular response to hypoxia complicates efforts to design approaches to treat or prevent injury resulting from reoxygenation.

As mentioned in the Introduction, there is a remarkable dearth of literature concerning with the inhibitor L-NIO in lungs submitted to hypoxia/reoxygenation (H/R), making its possible effects on this organ completely unknown. This is the first available study to describe the time-course effects of this endothelial NOS inhibitor in the hypoxic adult rat lung.

Alterations in the production of NO are critical in the injury that occurs during H/R situations in the lung. The results indicate that our model of H/R significantly increased NO levels, indirectly quantified by nitrate/nitrite and S-nitrose compounds (NOx), at 0 h post-hypoxia in the untreated groups. However, once L-NIO was administered before the hypoxic episode, NOx levels significantly lowered in all the experimental groups. Because it has been long reported that L-NIO is a selective eNOS inhibitor [24,33–36], the lower NOx levels found here should be attributed to eNOS inhibition.

Our H/R model significantly raised the lipid peroxidation level, determined by thiobarbituric acid reactive substances (TBARS), at 48 h and 5 days post-hypoxia in the untreated groups, proposing that changes consistent with oxidative stress occur in the lung in response to H/R. Nonetheless, once L-NIO was administrated, the TBARS level rose throughout the reoxygenation period, indicating that eNOS-derived NO could have a beneficial role against the oxidative processes that occur in the lung during H/R situations. In fact, several mechanisms may explain the protective effects of NO against oxidative stress. First, NO as a weak free radical acts as a scavenger of lipid radicals *in vitro* [37] and may thus terminate the cascade leading to lipid peroxidation. On the other hand, peroxidative processes, including lipid peroxidation, depend on HO**.** production and on the

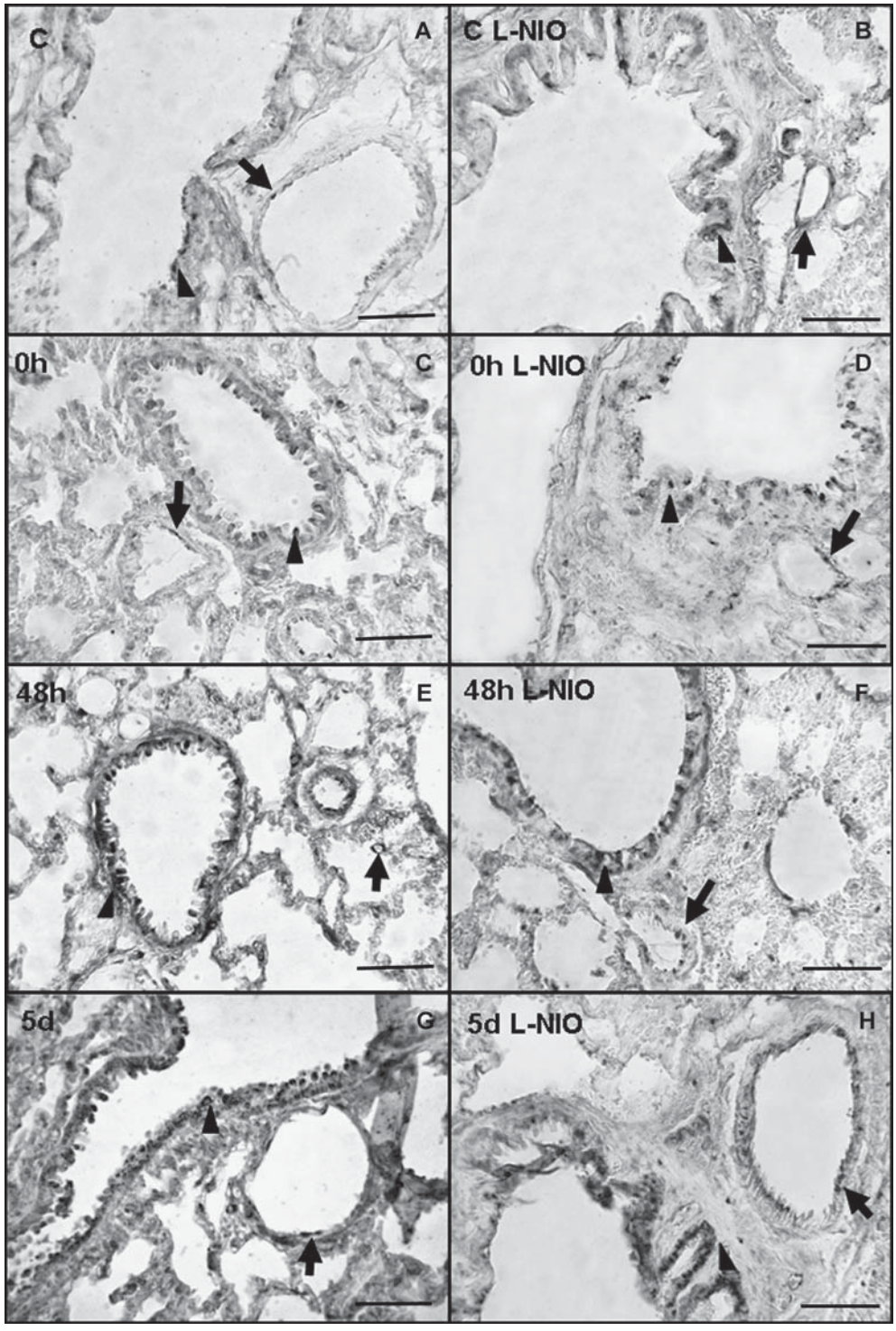

Figure 5. A-H: Micrographs showing nitrotyrosine immunoreactivity in rat lung sections in endothelial cells (arrows) and bronchiolar epithelial cells (arrow heads). Experimental groups: Control (C) and 0 h, 48 h, and 5 days (5d) post-hypoxia; Control (C) L-NIO and 0 h L-NIO, 48 h L-NIO, and 5 days (5d) L-NIO post-hypoxia. Micrographs E and G show higher nitrotyrosine-staining intensity when compared with the control group in the untreated groups. Micrographs B, D, F, and H show no changes in the nitrotyrosine-staining intensity in relation to the corresponding untreated groups. Scale bars: 50 μm.

formation of oxidizing iron species such as ferryl ion, kinetically equivalent to HO**.** [38]. In this sense, NO may exert anti-oxidant effects by inhibiting the function of ferrous iron as a transient metal [39].

The results have also shown that our model of H/R provokes cell damage, indicated by the use of the TUNEL assay, which measures DNA damage and is an indicator of apoptosis. In this sense, an increased percentage of apoptotic cells was detected at 48 h and 5 days of reoxygenation in the untreated groups. Nevertheless, after L-NIO administration, it is noticeable that the percentage of apoptotic cells significantly

rose throughout the post-hypoxia period, suggesting that eNOS-derived NO may exert a protective effect against the programmed cell death that occurs under these conditions in the rat lung. In this sense, it has been reported that NO can inhibit apoptosis by nitrosylating active sites of cysteine residues in caspases, essential enzymes for the apoptotic process [40]. Nevertheless, the effects of this inhibitor differ substantially in other experimental situations, models and organs. It has been reported that L-NIO administration reduced the number of TUNEL-positive cells, increased by ischemia/reperfusion, in the rat kidney [24], suggesting that eNOS contributes to the development of the apoptotic events during renal ischemia/reperfusion. Contrarily, other authors showed that this eNOS inhibitor did not alter the pattern of DNA fragmentation in rat peritoneal neutrophils [41].

From these results, we conclude that the peroxidative and apoptotic damage detected before the treatment with L-NIO in the rat lung submitted to H/R may not be related to eNOS-derived NO. Moreover, this NO could exert a beneficial role against the lung hypoxia-induced damage. In this sense, it has been reported that in situations of increased free-radicals production, as in the case of H/R, NO may operate as a scavenger of the ROS generated during such conditions, preventing them from causing damage [37,42,43].

Finally, the results have also shown that our H/R model significantly augmented nitrotyrosine-modified protein expression at the later reoxygenation times (48 h and 5 days) in the untreated groups, implying that changes consistent with nitrosative stress occur in the lung in response to H/R. Nitration of tyrosine residues could affect tyrosine phosphorylation and thus interface with important signalling pathways [44], including the nitration of mitochondrial respiratory-chain enzymes, which could lead to cell death [45,46]. However, the treatment with the inhibitor L-NIO did not modify nitrated protein expression, proposing that eNOS-derived NO could not be involved in the formation of nitrotyrosine in the hypoxic rat lung. These results are corroborated by the nitrated-proteins location data found in vascular endothelial cells and bronchiolar epithelial cells. Contrary to these results, it has been reported that the upregulation of eNOS during chronic hypoxia leads to the formation of nitrotyrosine in the endothelium of the pulmonary arteries [47].

In short, the results of this study show that a treatment with the selective eNOS inhibitor L-NIO raises the peroxidative and apoptotic damage in the hypoxic rat lung, suggesting that eNOS-derived NO may exert a protective effect against the injuries that occur during the H/R situations in the lung. These findings might provide an insight for a therapeutic design to prevent the hypoxia-induced injury and cell death.

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