Inducible NOS inhibitor 1400W reduces hypoxia/re-oxygenation injury in rat lung

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Nitric oxide (NO[•]) from inducible NO[•] synthase (iNOS) has been reported to either protect against, or contribute to, hypoxia/re-oxygenation lung injury. The present work aimed to clarify this double role in the hypoxic lung. With this objective, a follow-up study was made in Wistar rats submitted to hypoxia/re-oxygenation (hypoxia for 30 min; re-oxygenation of 0 h, 48 h, and 5 days), with or without prior treatment with the selective iNOS inhibitor 1400W (10 mg/kg). NO[•] levels (NO_x), lipid peroxidation, apoptosis, and protein nitration were analysed. This is the first time-course study which investigates the effects of 1400W during hypoxia/re-oxygenation in the rat lung. The results showed that the administration of 1400W lowered NO_x levels in all the experimental groups. In addition, lipid peroxidation, the percentage of apoptotic cells, and nitrated protein expression fell in the late post-hypoxia period (48 h and 5 days). Our results reveal that the inhibition of iNOS in the hypoxic lung reduced the damage observed before the treatment with 1400W, suggesting that iNOS-derived NO[•] may exert a negative effect on this organ during hypoxia/re-oxygenation. These findings are notable, since they indicate that any therapeutic strategy aimed at controlling excess generation of NO[•] from iNOS may be useful in alleviating NO[•]-mediated adverse effects in hypoxic lungs.

Keywords: 1400W, hypoxia, nitric oxide, inducible nitric oxide synthase, re-oxygenation period, apoptosis, lipid peroxidation, nitrotyrosine formation

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[•]) overproduction.¹ In the lungs, endogenous NO[•] plays an important role in regulating a number of physiological and pathological processes, including hypoxic situations. 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 calcium influx⁴ and its transcription can be activated in a wide variety of cell types by some stimuli, including inflammatory mediators (chemokines and cytokines)^{5,6} and exogenous factors (hypoxia).^{4,7} Two main sites of NO[•] production have been identified in the lung, the vasculature and the airways.^{8,9} The main isoform in vascular endothelium is eNOS. In the airways, all three isoforms have been detected in the bronchial epithelium,^{8,10} iNOS being the major airway epithelial isoform.¹¹ Also, iNOS has been found in vascular smooth-muscle cells⁹ and endothelial cells after hypoxia.¹²

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The re-oxygenation of the hypoxic tissues is characterized by the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in wide-spread lipid peroxidation, protein oxidative and nitrosative modifications, alterations in DNA,¹³ and cell apoptosis as well as necrosis.¹⁴ In particular, peroxynitrite (ONOO⁻), formed by the reaction of NO[•] and O₂^{-•} (superoxide), is a ROS and RNS that can alter protein function by nitrating phenolic rings, including tyrosine, to create nitrotyrosines.¹⁵

Recently, NO[•] derived from iNOS has been reported to act both as a destructive and a protective agent in the pathogenesis of the lung hypoxia/reoxygenation injuries. In this sense, while some studies have demonstrated that iNOS-derived NO[•] is involved in many pathological states related to hypoxia/reoxygenation in the lung,^{12,16,17} there is also evidence supporting the benefit of NO[•] generated by iNOS during such situations.^{18–20} All this controversy highlights the need to provide a clearer view of the role of iNOS-derived NO[•] during lung hypoxia/reoxygenation. The present work, therefore, aims to clarify this double role by analysing parameters of cell and tissue damage (lipid peroxidation, apoptosis, and levels of nitrated proteins) after treatment with the selective iNOS inhibitor *N*-(3-(aminomethyl)benzyl)acetamide or 1400W in the lung of rats submitted to hypoxia/reoxygenation (0 h, 48 h, and 5 days). The inhibitor 1400W is either an irreversible inhibitor or an extremely slow reversible inhibitor of iNOS that acts as a competitive inhibitor of L-arginine.²¹ It is worth mentioning the dearth of literature dealing with the inhibitor 1400W in hypoxic lungs, making its possible effects on this organ completely unknown.

Materials and 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 experiments were conducted according to EU guidelines on the use of animals for biochemical research (86/609/EU).

Experimental protocol and inducible NOS inhibitor administration

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 O₂ pressure to a final barometric pressure of 225 mmHg, resulting in a 48 mmHg O₂ partial pressure (pO₂). These conditions were maintained for 30 min. The ascent and descent speed was maintained below 1000 ft/min. After the hypoxia period, animals were kept under normobaric, normoxic conditions for different re-oxygenation 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.

A total of 40 albino Wistar rats were used for the biochemical experiments (5 animals per experimental group). After the corresponding re-oxygenation times, 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 (5 animals per experimental group). The rats were anaesthetized, perfused for each re-oxygenation time, the lungs were removed, rinsed in saline solution and fixed.

The selective iNOS inhibitor *N*-(3-(aminomethyl)benzyl)acetamide or 1400W (Sigma) was injected intraperitoneally 20 min before hypoxia. The inhibitor 1400W (10 mg/kg body weight) was dissolved in saline, and the control was an equal volume of saline.²⁴ The following experimental groups (*n* = 5 rats per group) were studied:

1. Control – animals maintained for 30 min in the chamber under normobaric, normoxic conditions.
2. Hypoxia/re-oxygenation – animals submitted to 30 min of hypoxia followed by 0 h, 48 h and 5 days of re-oxygenation.
3. Control + 1400W – control animals treated with 1400W.
4. Hypoxia/re-oxygenation + 1400W – animals submitted to the same procedure as the second group but treated with 1400W.

NO[•] measurement

The reaction of NO[•] with ozone results in 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-nitroso compounds (NO_x) using an ozone chemiluminescence-based method. For this technique, lungs were homogenized in PBS with protease inhibitors. Homogenates were then sonicated, centrifuged, and deproteinized with 0.8 N NaOH and

16% ZnSO₄ solutions. The total amount of NO_x was determined by a modification²² of the procedure described by Braman and Hendrix²⁷ using a NO[•] analyser (NOATM 280i; Sievers Instruments). A saturated solution of vanadium chloride (VCl₃) in 1 M HCl was added to the nitrogen-bubbled purge vessel fitted with a cold water condenser and a water jacket to heat 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 NO_x to NO[•], which was then detected by ozone-induced chemiluminescence. NO_x concentrations were calculated by comparison with standard solutions of sodium nitrate. Final NO_x values were referred to the total protein concentration in the initial extracts.

Thiobarbituric acid reactive substances

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 assay.²⁹ After 700 µl of thiobarbituric acid reagent (15% trichloroacetic acid [TCA], 0.38% 2-thiobarbituric acid [TBA] and 2% HCl) was added to 300 µl of the supernatant and the solution 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 (A₅₃₅). Lung homogenate TBARS levels were expressed as A₅₃₅ per milligram of protein.³⁰

TUNEL assay for assessment of apoptotic cell death

Terminal deoxynucleotidyl transferase (TdT)-mediated desoxyuridine triphosphate (dUTP) nick end-labelling (TUNEL) is a reliable technique to estimate apoptosis in tissue sections.³¹ The protocol was performed in sections obtained from lungs embedded in paraffin (Paraplast Extra; Tyco) according to the manufacturer's recommendations (TdT-FragEL™ DNA Fragmentation Detection Kit; Calbiochem). Deionized water was used instead of TdT enzyme as a negative control. Apoptotic bodies were stained brown.

Western blot analysis

For Western blot analysis, equal amounts of denatured lung total-protein extracts were loaded and separated in 7.5% SDS-polyacrylamide gels. 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 the Cajal Institute, Madrid) was used to detect nitrated proteins and monoclonal antibody to α -tubulin (Sigma) was utilized as an 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). Sections were incubated with 10% goat serum for 30 min. Afterwards, they were incubated first with diluted polyclonal rabbit anti-3-nitro-L-tyrosine A4 antibody (1:500), used to detect nitrated proteins, in PBS overnight at 4°C, and later with a goat anti-rabbit biotinylated secondary antibody (Pierce) followed by peroxidase-linked ABC. The peroxidase activity was demonstrated following the nickel-enhanced diaminobenzidine procedure.³² Sections were mounted on slides, dehydrated, and covered using DPX. Controls for background staining were performed by replacing the primary antibody with PBS.

Image processing

The number of apoptotic cells and the nitrotyrosine-immunoreactive (N-Tyr-IR) area were quantified by computerized-assisted image analysis using ImageJ (an NIH image analysis and processing software downloaded free from <<http://rsbweb.nih.gov/ij/>>) connected to a light microscope (Olympus, Hamburg, Germany). Ten similar microphotographs per rat were digitally captured and analysed after background subtraction (minimal particle size 10 pixels). As a means of avoiding the usual biased segmentation of the stained structures, the immunoreactive area and the staining intensity, from black (0) to white (255), of each microphotograph was calculated as a function of the optical density following Sternberger's method.³³

For the quantification of the apoptotic cells in lung sections stained for TUNEL assay, the microphotographs were analysed in two different colour channels using Image J. The image resulting from the green channel for each microphotograph was used to determine the number of living cells, and the image from the red one was used to determine the number of apoptotic cells using the same software.

After the analysis of the lung sections stained for nitrotyrosine immunohistochemistry using Image J, the line segment corresponding to the nitrotyrosine-immunoreactive (N-Tyr-IR) area was mathematically

Figure 1 Influence of hypoxia/re-oxygenation on nitrate, nitrite, and other nitroso compounds (NOx) in rat lung ($\mu\text{mol}/\text{mg}$ protein). Experimental groups: control, and 0 h, 48 h, and 5 days post-hypoxia; control 1400W, and 0 h 1400W, 48 h 1400W, and 5 days 1400W post-hypoxia. Results are the mean values of three independent experiments and five animals in each 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 $P < 0.01$, and ^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 $P < 0.01$, and ^z $P < 0.001$

Figure 2 Influence of hypoxia/re-oxygenation on lipid peroxidation in rat lung. Results were expressed as absorbance at 535 nm (A_{535}) per milligram of protein. Experimental groups: control, and 0 h, 48 h, and 5 days post-hypoxia; control 1400W, and 0 h 1400W, 48 h 1400W, and 5 days 1400W post-hypoxia. Results are the 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 $P < 0.01$, and ^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 $P < 0.01$, and ^z $P < 0.001$

extrapolated using Origin v5.0, a data analysis and technical graphics software package.³⁴ The number of N-Tyr-IR cells was not quantified because most showed an immunoreactivity of little-defined contours for which visual counting is highly prone to error, even though Sternberger's quantitative method eliminates the need to discriminate immunostained structures for the background, while calculating only the percentage of immunoreactive area.³³

Statistical analysis

Data were expressed as mean $\pm$ SD. The statistical treatment to evaluate significant differences between groups was performed with SPSS v15.0 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 statistical significance was established by applying an unpaired Student's *t*-test to compare differences

Table 1 Quantitative data from image analysis of histological sections of rat lung stained for TUNEL assay

Group	Apoptotic cells (%)
Untreated	
Control	3.77 $\pm$ 1.48
0 h	4.10 $\pm$ 1.42
48 h	35.26 $\pm$ 4.35 ^d
5 days	41.63 $\pm$ 1.64 ^d
1400W-treated	
Control	3.32 $\pm$ 1.25
0 h	5.09 $\pm$ 0.79
48 h	18.67 $\pm$ 2.19 ^z
5 days	20.15 $\pm$ 2.21 ^z

Experimental groups: control, and 0 h, 48 h, and 5 days post-hypoxia; control 1400W, and 0 h 1400W, 48 h 1400W, and 5 days 1400W post-hypoxia. Results are the mean values of 50 microphotographs (10 microphotographs per animal and five animals per group) $\pm$ 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 $P < 0.01$, and ^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 $P < 0.01$, and ^z $P < 0.001$

Figure 3 (A–H) Micrographs showing histological sections of rat lung stained for the TUNEL assay. Experimental groups: Control (C), and 0 h, 48 h, and 5 days (5d) post-hypoxia; Control (C) 1400W, 0 h 1400W, 48 h 1400W, and 5 days (5d) 1400W post-hypoxia. Apoptotic, TUNEL-positive cells are indicated by the brown nuclear staining (arrows). Micrographs E and G showed increased apoptosis levels when compared with the control group in the untreated groups. Micrographs F and H showed lower apoptosis levels in relation to the corresponding untreated groups. Scale bars: 50 μ m

between means. 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 untreated groups were expressed as ^w $P < 0.05$, ^x $P < 0.02$, ^y $P < 0.01$, and ^z $P < 0.001$.

Results

NO[•] production

Figure 1 shows the determinations of nitrate/nitrite and other S-nitroso compounds (NOx) in the different experimental groups. NOx levels rose early, at 0 h of re-oxygenation ($P < 0.001$), in the untreated groups.

Figure 4 Influence of hypoxia/re-oxygenation 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) 1400W, and 0 h 1400W, 48 h 1400W, and 5 days (5d) 1400W post-hypoxia. Results were expressed as arbitrary units (AU). Results are the 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 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 $P < 0.01$, and ^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 $P < 0.01$, and ^z $P < 0.001$

However, the administration of the iNOS inhibitor 1400W significantly lowered NOx levels in all the experimental groups: control ($P < 0.02$), 0 h ($P < 0.001$), 48 h ($P < 0.001$), and 5 days post-hypoxia ($P < 0.001$).

Lipid peroxidation

Malondialdehyde and other lipid peroxidation products, which react with thiobarbituric acid, are good indicators of oxidative stress.³⁵ Figure 2 shows that hypoxia/re-oxygenation raised the TBARS level at 48 h ($P < 0.01$), and 5 days ($P < 0.001$) post-hypoxia in the untreated groups. Nevertheless, the administration of 1400W lowered the TBARS at 48 h ($P < 0.05$), and 5 days ($P < 0.001$) of re-oxygenation.

TUNEL assay

The TUNEL assay, which identifies apoptotic cells, showed that hypoxia/re-oxygenation significantly raised the percentage of apoptotic cells at the late post-hypoxia times (48 h and 5 days: $P < 0.001$) in the untreated groups (Table 1). After treatment with 1400W, this percentage decreased at 48 h ($P < 0.001$), and 5 days ($P < 0.001$) of re-oxygenation when compared with the corresponding untreated groups (Fig. 3).

Nitrotyrosine expression

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

Table 2 Quantitative data from image analysis of histological sections of rat lung stained for nitrotyrosine immunohistochemistry

Group	Nitrotyrosine-immunoreactive area (AU)
Untreated	
Control	0.50 ± 0.05
0 h	0.56 ± 0.05
48 h	0.94 ± 0.05 ^d
5 days	1.02 ± 0.08 ^d
1400W-treated	
Control	0.58 ± 0.08
0 h	0.62 ± 0.06
48 h	0.66 ± 0.05 ^z
5 days	0.68 ± 0.04 ^z

Experimental groups: control, and 0 h, 48 h, and 5 days post-hypoxia; control 1400W, and 0 h 1400W, 48 h 1400W, and 5 days 1400W post-hypoxia. Results were expressed as arbitrary units (AU). Results are the 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 $P < 0.01$, and ^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 $P < 0.01$, and ^z $P < 0.001$

48 h to 5 days post-hypoxia ($P < 0.001$) in the untreated groups (Fig. 4, left panel). However, the administration of the inhibitor 1400W decreased nitrated protein expression in the late re-oxygenation period (48 h, and 5 days; $P < 0.01$) in the treated groups.

Nitrotyrosine immunohistochemistry

The nitrotyrosine-immunoreactivity was detected in bronchiolar epithelial cells and vascular endothelial

cells in all the experimental groups (Fig. 5). Corroborating the previous results, the nitrotyrosine-immunoreactive area significantly increased from 48 h to 5 days post-hypoxia ($P < 0.001$) in these cell types in the untreated groups but decreased during those times (48 h and 5 days: $P < 0.001$) in the groups treated with the iNOS inhibitor 1400W (Table 2).

Discussion

Hypoxia-associated pathophysiology is complex and elusive but notably significant. Many adverse effects of hypoxia are commonly observed under conditions generated by ischaemia. Like reperfusion, re-oxygenation eventually does not completely reverse

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) 1400W, and 0 h 1400W, 48 h 1400W, and 5 days (5d) 1400W post-hypoxia. Micrographs E and G showed higher nitrotyrosine-immunoreactivity intensity when compared with the control group in the untreated groups. Micrographs F and H showed less nitrotyrosine-immunoreactivity intensity in relation to the corresponding untreated groups. Scale bars: 50 μ m

the hypoxia-induced changes. The complexity of the cellular response to hypoxia complicates efforts to design approaches to treat or prevent injury resulting from re-oxygenation.

As mentioned in the introduction, there is a remarkable lack of literature dealing with the inhibitor 1400W in lungs submitted to hypoxia/re-oxygenation, so that its possible effects on this organ remain completely unknown. This is the first available study to describe the time-course effects of this inducible NOS inhibitor in the hypoxic adult rat lung.

Alterations in the production of iNOS-derived NO[•] are critical in the damage that occurs in lung hypoxia/re-oxygenation situations. The results indicate that hypoxia/re-oxygenation significantly raised NO[•] levels, indirectly quantified as nitrate/nitrite and S-nitroso compounds (NOx), at 0 h of re-oxygenation in the untreated groups compared with the normobaric, normoxic control. On the other hand, the administration of the inhibitor 1400W before the hypoxic episode significantly lowered NOx levels in all the experimental groups. Because it is well known that 1400W is a highly selective iNOS inhibitor,^{24,36} the lower NOx levels reported here should be attributed to the iNOS inhibition.

Our hypoxia/re-oxygenation model significantly raised lipid peroxidation, determined by thiobarbituric acid reactive substances (TBARS), at 48 h and 5 days post-hypoxia in the untreated groups, suggesting that changes consistent with oxidative processes occur in the lung in response to hypoxia. However, once 1400W was administered, the TBARS levels significantly fell at the same times when compared with the corresponding untreated groups. These results indicate that iNOS-derived NO[•] may be involved in lipid peroxidation processes in the lung submitted to hypoxia/re-oxygenation, contributing to the oxidative stress that takes place under these conditions. In fact, a close relationship between NO[•] and oxidative stress has long been proposed.³⁷ Peroxidative processes, including lipid peroxidation, depend on HO[•] production and on the formation of oxidizing iron species such as ferryl ion, kinetically equivalent to HO[•].³⁸ In this sense, NO[•] may exert pro-oxidant effects by reducing ferric iron complexes, thus inducing a release of bound iron and indirectly substituting for other reductants in the Haber–Weiss reaction-mediated production of HO[•] from H₂O₂.³⁹ In addition, NO[•]-derived peroxynitrite can induce lipid peroxidation in model systems without iron participation.⁴⁰ In agreement with our results, other authors found similar effects of this iNOS inhibitor in other

experimental situations, models, and organs. In this sense, it has been reported that 1400W administration reduced lipid peroxidation in the diabetic liver and kidney,⁴¹ and in rats treated with lipopolysaccharide and lead.⁴²

The results also indicate that our hypoxia/re-oxygenation model provokes cell damage, according to the TUNEL assay, which measures DNA damage and is a reliable indicator of apoptosis.³¹ In this sense, a higher percentage of apoptotic cells was detected at 48 h, and 5 days post-hypoxia in the untreated groups. Nevertheless, this percentage decreased in the rat lung at the same time intervals after 1400W administration, suggesting involvement of iNOS-derived NO[•] in the programmed cell death that occurs during hypoxia/re-oxygenation in the lung. Similar results were found in the literature in other models, organs and species. In this way, it has been demonstrated that treatment with 1400W significantly reduced apoptosis during hypoxia in pancreatic β-cells,⁴³ and during ischaemia/reperfusion in rat kidney²⁴ and mouse heart.⁴⁴ Actually, a relationship has been reported between iNOS-derived NO[•], apoptosis, and hypoxia.¹ Hypoxia increases KLF6 and NF-κB and decreases KLF4, resulting in greater expression of iNOS. This increase in iNOS, in turn, boosts NO[•] production that reacts with O₂^{•-} to form peroxynitrite. Then, peroxynitrite causes mitochondria to swell and release cytochrome-c, which subsequently is caged by Apaf-1 and caspase-9 to form apoptosome. The apoptosome, therefore, activates caspase-3, thereby leading to apoptosis.¹

Our results show that hypoxia/re-oxygenation significantly augmented nitrotyrosine-modified protein expression from 48 h to 5 days post-hypoxia in the untreated groups. However, the treatment with 1400W significantly decreased nitrated protein expression at those times, suggesting that the increased expression of these proteins at 48 h and 5 days in the untreated groups would be due to iNOS-derived NO[•]. These findings indicate that this NO[•] may be related to the nitrosative stress that occurs in the lung during hypoxia/re-oxygenation. Nitration of tyrosine residues could affect tyrosine phosphorylation and thus interface with important signalling pathways,⁴⁵ including the nitration of mitochondrial respiratory-chain enzymes, which could lead to cell death.^{46,47} These results are corroborated by the nitrated-modified protein location data, found in vascular endothelial cells and bronchiolar epithelial cells. Agreeing with our results, some studies have demonstrated that treatment with 1400W abrogated nitrotyrosine expression under ischaemia/reperfusion

conditions in hamster heart,⁴⁸ and in rabbit lung.⁴⁹ A mechanism that relates iNOS, nitrotyrosine formation and hypoxia could be described. It has been reported that depletion of L-arginine resulted in a fall in NO[•] and a rise in O₂^{•-} production, accompanied by peroxynitrite and nitrotyrosine formation.⁵⁰ In this sense, experimental conditions that induce iNOS, including hypoxia, can increase arginase activity^{51,52} and could contribute to lowering the cytosolic level of L-arginine. Moreover, when iNOS is induced and the high output pathway of NO[•] synthesis is activated, L-arginine concentrations may become rate-limiting, favouring O₂^{•-} synthesis rather than NO[•].⁵³

Conclusions

The results of this study show that treatment with the selective iNOS inhibitor 1400W decreases lipid peroxidation, apoptosis, and nitrated protein expression in the rat lung submitted to hypoxia/re-oxygenation. These findings suggest that iNOS-derived NO[•] may exert a deleterious role by causing, or at least contributing to, the injuries related to lung hypoxia/re-oxygenation situations. Our results are noteworthy, since they indicate that any therapeutic strategy aimed at controlling excess generation of NO[•] from iNOS may be useful in alleviating NO[•]-mediated adverse effects in hypoxic lungs. Therefore, the administration of 1400W would be beneficial in treatment against lung hypoxia/re-oxygenation dysfunctions.

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