Is endothelial-nitric-oxide-synthase-derived nitric oxide involved in cardiac hypoxia/reoxygenation-related damage?

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Nitric oxide (NO) has been reported to act both as a destructive and a protective agent in the pathogenesis of the injuries that occur during hypoxia/reoxygenation (H/R). It has been suggested that this dual role of NO depends directly on the isoform of NO synthase (NOS) involved. In this work, we investigate the role that NO derived from endothelial NOS (eNOS) plays in cardiac H/R-induced injury. Wistar rats were submitted to H/R (hypoxia for 30 min; reoxygenation of 0 h, 12 h and 5 days), with or without prior treatment using the selective eNOS inhibitor L-NIO (20 mg/kg). Lipid peroxidation, apoptosis and protein nitration, as well as NO production (NOx), were analysed. The results showed that L-NIO administration lowered NOx levels in all the experimental groups. However, no change was found in the lipid peroxidation level, the percentage of apoptotic cells or nitrated protein expression, implying that eNOS-derived NO may not be involved in the injuries occurring during H/R in the heart. We conclude that L-NIO would not be useful in alleviating the adverse effects of cardiac H/R.

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1. Introduction

Hypoxia resulting from ischaemia, haemorrhage and other cardiovascular problems is common in clinical medicine and occurs in virtually all organ systems, making it vital to search for useful remedies. Although the underlying mechanisms of hypoxia-induced damage are still not fully understood, it has been shown that hypoxia induces nitric oxide (NO) overproduction (Kiang and Tsen 2006). In the heart, NO is known to play key roles in physiological and pathological conditions, including hypoxia. NO can be generated by haemoproteins NO synthases (NOS), which catalyse the conversion of L-arginine to NO and L-citrulline. Three NOS isoforms have been identified: endothelial NOS (eNOS, NOS 3), inducible NOS (iNOS, NOS 2) and neuronal NOS (nNOS, NOS 1) (Förstermann 1994). Whereas eNOS and nNOS are mostly calcium/calmodulin-dependent and usually express constitutively (Palmer *et al.* 1998), iNOS is typically independent of the intracellular calcium concentration and can be induced in a wide variety of cell types in response to some stimuli, such as hypoxia (Jung *et al.* 2000; Ricciardolo *et al.* 2004) or after stimulation with inflammatory mediators (Schulz *et al.* 1992; Kelly *et al.* 1996).

Most of the NOS activity in the heart corresponds to eNOS, which is located in the vascular endothelium within the myocardium as well as in cardiomyocytes (Kelly *et al.* 1996). Being localized in scattered nerves and ganglion cells (Ursell and Mayes 1995), nNOS is far less prominent. Finally, iNOS can be induced within the heart in macrophages, endothelial cells and cardiomyocytes (Ursell and Mayes 1995; Kelly *et al.* 1996; Grilli *et al.* 2003) by certain stimuli, including hypoxia. Under physiological conditions in the heart, NO, derived mainly from eNOS, maintains coronary

Keywords. Apoptosis; endothelial nitric oxide synthase; hypoxia; L-NIO; nitric oxide; reoxygenation

Abbreviations used: dUTP, desoxyuridinetriphosphate; eNOS, endothelial nitric oxide synthase; H/R, hypoxia/reoxygenation; NOS, nitric oxide synthase; PVDF, polyvinylidenefluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; TBA, 2 thiobarbituric acid; TBARS, thiobarbituric-acid-reactive substances; TCA, trichloroacetic acid; TdT, Terminal deoxynucleotidyl transferase; TUNEL, terminal-deoxynucleotidyl-transferase-mediated desoxyuridinetriphosphate nick end-labelling

vasodilatory tone (McGowan *et al.* 1994), inhibits platelet aggregation (Radomski *et al.* 1987a), and counteracts the adhesion of platelets (Radomski *et al.* 1987b; Groves *et al.* 1993) to the vascular endothelium.

The reoxygenation of the hypoxic cardiac tissue is one of the prime causes underlying cell and tissue damage upon reintroduction of molecular oxygen (O_2) under pathologic conditions, including hypoxic heart disease. Reoxygenation characteristically increases both reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Rouschop *et al.* 2009), resulting in widespread lipid peroxidation (one of the main mechanisms of oxidative stress), protein oxidative and nitrosative alterations, and cell apoptosis and necrosis, all of which can lead to profound cellular disturbances (McCord *et al.* 1985). In particular, 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 (Haddad *et al.* 1994). The oxidation of such residues forms the stable product 3-nitro-L-tyrosine (nitrotyrosine), which can be used as a marker of the potentially cytotoxic effect of NO (Beckman and Koppenol 1996).

As stated above, it has been shown that hypoxia/reoxygenation (H/R) situations induce NO overproduction (Kiang and Tsen 2006). Moreover, NO reportedly acts as both a destructive and a protective agent in the pathogenesis of injuries occurring during such conditions (Naoki *et al.* 1999; Shirai *et al.* 2003; Baber *et al.* 2005; Barer *et al.* 2006). Some works have suggested that this dual role of NO depends directly on the isoform of NOS involved (Foster *et al.* 2003; Thippeswamy *et al.* 2006). Therefore, it would be critical to examine the role that NO derived from each NOS isoform plays in H/R-induced damage. To determine this, we have previously studied the role of iNOS-derived NO in such situations in the rat heart (Rus *et al.* 2011) and showed that this NOS isoform may have a cardioprotective effect against H/R injuries, challenging the conventional wisdom that iNOS is deleterious under these conditions. In this work, we investigated the role that eNOS-derived NO plays in cardiac H/R-induced injury. For this, *Wistar* rats were submitted to 30 min of hypoxia and were studied during the subsequent reoxygenation period (0 h, 12 h and 5 days) as a novel approach to address the events triggered by such circumstances. Lipid peroxidation, apoptosis and nitrated protein expression were analysed before and after treatment with the selective eNOS inhibitor N5-(1-iminoethyl)-L-onornithine dihydrochloride (L-NIO) (Vaupel *et al.* 1995; Benamar *et al.* 2003; Xu and Krukoff 2004). Inhibitors of NOS are invaluable tools for investigating physiological and/or pharmacological roles of NO, and extensive research in this field has identified selective inhibitors for each NOS isoform. In particular, L-NIO is an L-arginine analogue that acts as 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 approximately eight times more potent against eNOS than nNOS, four times more potent against eNOS than iNOS, and five times more potent as an inhibitor of eNOS than other L-arginine analogues (Rees *et al.* 1990). It bears mentioning that the literature dealing with the inhibitor L-NIO in hypoxic hearts is very scant, and so the possible effects it has on the heart remain completely unknown.

2. Methods

2.1 *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 EU guidelines on the use of animals for biochemical research (86/609/EU).

2.2 *Experimental protocol and eNOS inhibitor administration*

Acute hypobaric hypoxia was carried out as previously published by our group (Lopez-Ramos *et al.* 2005; Martínez-Romero *et al.* 2006). In brief, the 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 mmHg, resulting in a 48 mmHg O_2 partial pressure (pO_2). These conditions were maintained for 30 min. The ascent and descent speeds were kept at less than 1000 ft/min. After the hypoxia period, the animals were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 12 h and 5 days), and were then sacrificed. Control animals were sacrificed after being maintained for 30 min in the chamber under normobaric normoxic conditions.

The selective eNOS inhibitor L-NIO or N5-(1-iminoethyl)-L-onornithine dihydrochloride (Tocris Biosciences) 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 (Viñas *et al.* 2006). After the hypoxia period, the animals were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 12 h and 5 days) and then were sacrificed. Control animals were sacrificed after being maintained for 30 min in the chamber under normobaric normoxic conditions. 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, 12 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 ($n=5$ rats per experimental group). The rats were killed by cervical dislocation, and the hearts were immediately removed, rinsed in saline solution and stored at −80°C until use. Another 40 rats were used for histochemistry and immunohistochemistry ($n=5$ rats per experimental group). The rats were anaesthetized with Ketolar (Parke Davis, 1 ml/250 g weight) by intraperitoneal injection and perfused at each reoxygenation time. Then, the hearts were removed, rinsed in saline solution and fixed.

2.3 *NO measurement*

The reaction of NO with ozone results in the emission of light, and this light (emitted in proportion to the NO concentration) is the basis for one of the most accurate NO assays available (Laitinen *et al.* 1993; Fontijn *et al.* 1997). NO production was indirectly quantified by measuring nitrate/nitrite and *S*-nitrose compounds (NOx) with an ozone chemiluminescence-based method. For this technique, the hearts were homogenized in PBS with protease inhibitors. Homogenates were then sonicated, centrifuged, and deproteinized with NaOH 0.8N and $ZnSO_4$ 16% solutions. The total amount of NOx was determined by a modification (Lopez-Ramos *et al.* 2005) of the procedure described by Braman and Hendrix (1989) using a NO analyser (NOA™ 280i Sievers Instruments). 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 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 so they could react with the reagent, and NOx was converted 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.

2.4 *Thiobarbituric-acid-reactive substances*

Thiobarbituric-acid-reactive substances (TBARS) were determined in heart homogenates as described by Buege and Aust (1978). In brief, the hearts were homogenized in PBS, and then sonicated and centrifuged. In the supernatant, the amount of protein was determined using the Bradford assay (1976). 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 recorded at 535 nm.

2.5 *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 of heart embedded in paraffin according to the manufacturer's recommendations (TdT-FragELTM DNA Fragmentation Detection Kit, Calbiochem). Deionized water was replaced by TdT enzyme as a negative control. Apoptotic bodies were stained brown.

Ten similar microphotographs per rat were digitally made 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 number of apoptotic cells. The percentage of apoptotic cells in each microphotograph was quantified by computer-assisted image analysis using the same software.

2.6 *Western blot analysis for nitrotyrosine expression*

For Western blot analysis, equal amounts of denatured heart total-protein extracts were loaded and separated in 7.5% SDS-polyacrylamide gel. Proteins in the gel were transferred to a polyvinylidenefluoride (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and then blocked. Polyclonal rabbit anti-3-nitro-L-tyrosine A4 antibody (1:3000, a gift from Professor J Rodrigo from CSIC Cajal Institute of Madrid, Spain) was used to detect nitrated proteins. After immunodetection, the membranes were probed with monoclonal antibody to β-actin (Sigma)

as a loading control. Antibody reaction was revealed with chemiluminescence detection procedures according to the manufacturer's recommendations (ECL kit, Amersham Corp., Buckinghamshire, UK). The relative amount of the proteins in each sample was quantified by densitometric scanning and expressed as arbitrary units (AU). These values were used to calculate the ratio of bulk-nitrated proteins to β-actin.

2.7 *Nitrotyrosine immunohistochemistry*

Sections of heart embedded in paraffin (Paraplast Extra, Tyco) 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, a gift from Professor J Rodrigo from CSIC Cajal Institute of Madrid, Spain), 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 avidin-biotin complex (ABC). The peroxidase activity was demonstrated following the nickel-enhanced diamino-benzidine procedure (Shu *et al.* 1988). Sections were mounted on slides, dehydrated, and covered using dextrene polystyrene in xylene (DPX) mounting media. Controls for background staining were performed by replacing the primary antibody with PBS.

2.8 *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 the 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 the differences between means. The statistically significant differences vs. 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 $^{w}P<0.05$; $^{x}P<0.02$; $^{y}P<0.01$; $^{z}P<0.001$.

3. Results

3.1 *NO production*

Figure 1 shows the values of NOx levels (nitrate/nitrite and other *S*-nitrose compounds) in the obtained in heart homogenates in the different experimental groups, as per the protocol described in Methods. NOx levels rose at 0 h of reoxygenation ($P<0.001$) vs. control in the untreated groups. On the other hand, the administration of the eNOS inhibitor L-NIO lowered NOx levels in all the experimental groups as compared with the corresponding untreated groups: control ($P<0.02$), 0 h ($P<0.001$), 12 h ($P<0.01$), and 5 days ($P<0.05$) post-hypoxia.

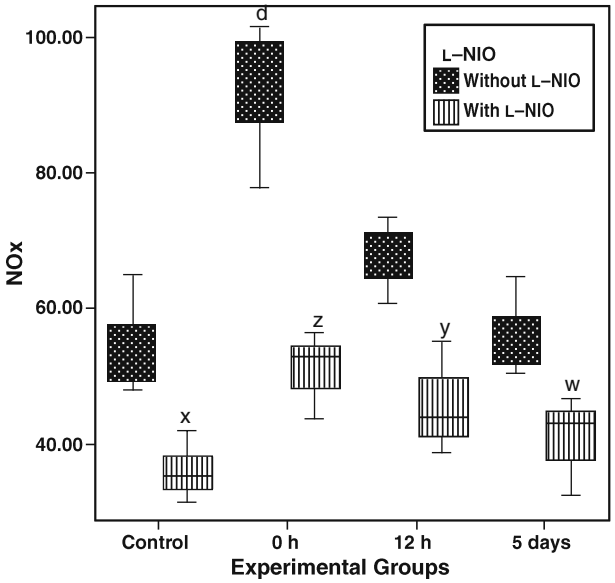

Figure 1. Influence of hypoxia/reoxygenation on nitrate, nitrite and other nitrose compounds (NOx) in rat heart (μmol/mg protein). Experimental groups: control and 0 h, 12 h and 5 days post-hypoxia; control L-NIO and 0 h L-NIO, 12 h L-NIO and 5 days L-NIO post-hypoxia. The results are the mean values of three independent experiments with five animals per group. The statistically significant differences vs. 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 $^{w}P<0.05$; $^{x}P<0.02$; $^{y}P<0.01$; $^{z}P<0.001$.

3.2 *Thiobarbituric-acid-reactive substances*

Malondialdehyde and other lipid peroxidation products, which react with thiobarbituric acid, are good indicators of oxidative stress (Drapper *et al.* 1993). Figure 2 shows that H/R raised the TBARS levels immediately after hypoxia (0 h; $P<0.01$) vs. the control group in the untreated groups. Nevertheless, TBARS level did not change in the groups treated with L-NIO vs. the corresponding untreated ones.

3.3 *TUNEL assay*

The TUNEL assay, which identifies apoptotic cells, is a good indicator of apoptosis. The quantification of apoptotic

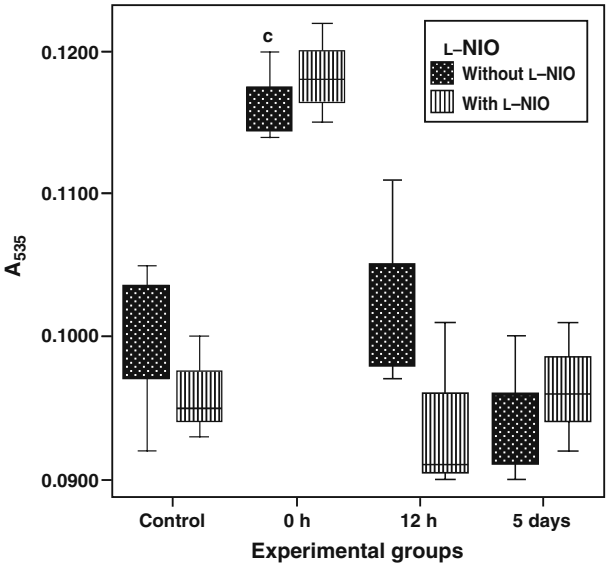

Figure 2. Influence of hypoxia/reoxygenation on lipid peroxidation in rat heart. The results are expressed as absorbance at 535 nm. Experimental groups: control and 0 h, 12 h and 5 days post-hypoxia; control L-NIO and 0 h L-NIO, 12 h L-NIO and 5 days L-NIO post-hypoxia. The results are the mean values of three independent experiments with five animals per group. The statistically significant differences vs. 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 $^{w}P<0.05$; $^{x}P<0.02$; $^{y}P<0.01$; $^{z}P<0.001$.

cells (table 1) showed a statistically significant increase at 0 h post-hypoxia vs. control in the untreated groups ($P<0.001$). On the other hand, the percentage of apoptotic cells did not change in the groups treated with the inhibitor L-NIO vs. the corresponding untreated groups (figure 3).

3.4 *Nitrotyrosine expression*

Three nitrotyrosine immunoreactive bands, corresponding to proteins of 80, 70 and 45 kDa, were detected in all the experimental groups (figure 4, right panel). The quantitative evaluation of bulk-nitrated proteins increased at 12 h post-hypoxia ($P<0.01$) vs. the control group in the untreated groups (figure 4, left panel). Nonetheless, the administration of the eNOS inhibitor L-NIO did not alter nitrated protein expression in the treated groups vs. the corresponding untreated ones.

3.5 *Nitrotyrosine immunohistochemistry*

In all the experimental groups, nitrotyrosine immunoreactivity was detected in cardiomyocytes and vascular endothelium (figure 5). The administration of L-NIO did not alter the nitrotyrosine-positive staining in the animals treated with this eNOS inhibitor vs. the corresponding untreated groups.

4. Discussion

Hypoxia-associated pathophysiology is intricate and complex but noticeably significant. Many adverse effects of hypoxia are commonly observed under conditions provoked by ischaemia and haemorrhage. As with reperfusion, reoxygenation does not completely reverse the hypoxia-induced changes. The complexity of the cellular response to hypoxia complicates the efforts to treat or prevent injury resulting from reoxygenation.

Inhibitors of NOS are invaluable tools for investigating physiological and/or pharmacological roles of NO, and extensive research in this field has identified inhibitors with relative selectivity for each NOS isoform. In this work, we investigated the role that NO derived from eNOS plays in cardiac H/R-induced injury by using the selective eNOS inhibitor L-NIO. As mentioned in the introduction, remarkably few works deal with L-NIO in hearts submitted to H/R, making its possible effects on this organ completely unknown.

Alterations in the production of NO are critical in the injury that occurs during myocardial H/R situations. The results indicate that our H/R model significantly raised NO levels, indirectly quantified by nitrate/nitrite and *S*-nitrose compounds (NOx), at 0 h post-hypoxia in the untreated groups.

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

Group		Percentage of apoptotic cells (%)
Untreated groups	Control	3.94±1.80
	0 h	16.89±2.82^{d}
	12 h	2.29±0.99
	5 days	4.01±1.26
L-NIO-treated groups	Control	4.76±2.21
	0 h	14.65±1.29
	12 h	2.43±1.15
	5 days	5.56±1.49

Experimental groups: Control and 0 h, 12 h and 5 days post-hypoxia; Control L-NIO and 0 h L-NIO, 12 h L-NIO and 5 days L-NIO post-hypoxia. Results are mean values of 50 microphotographs (ten microphotographs per animal and five animals per group)±SD (standard deviation). 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 $^{w}P<0.05$; $^{x}P<0.02$; $^{y}P<0.01$; $^{z}P<0.001$.

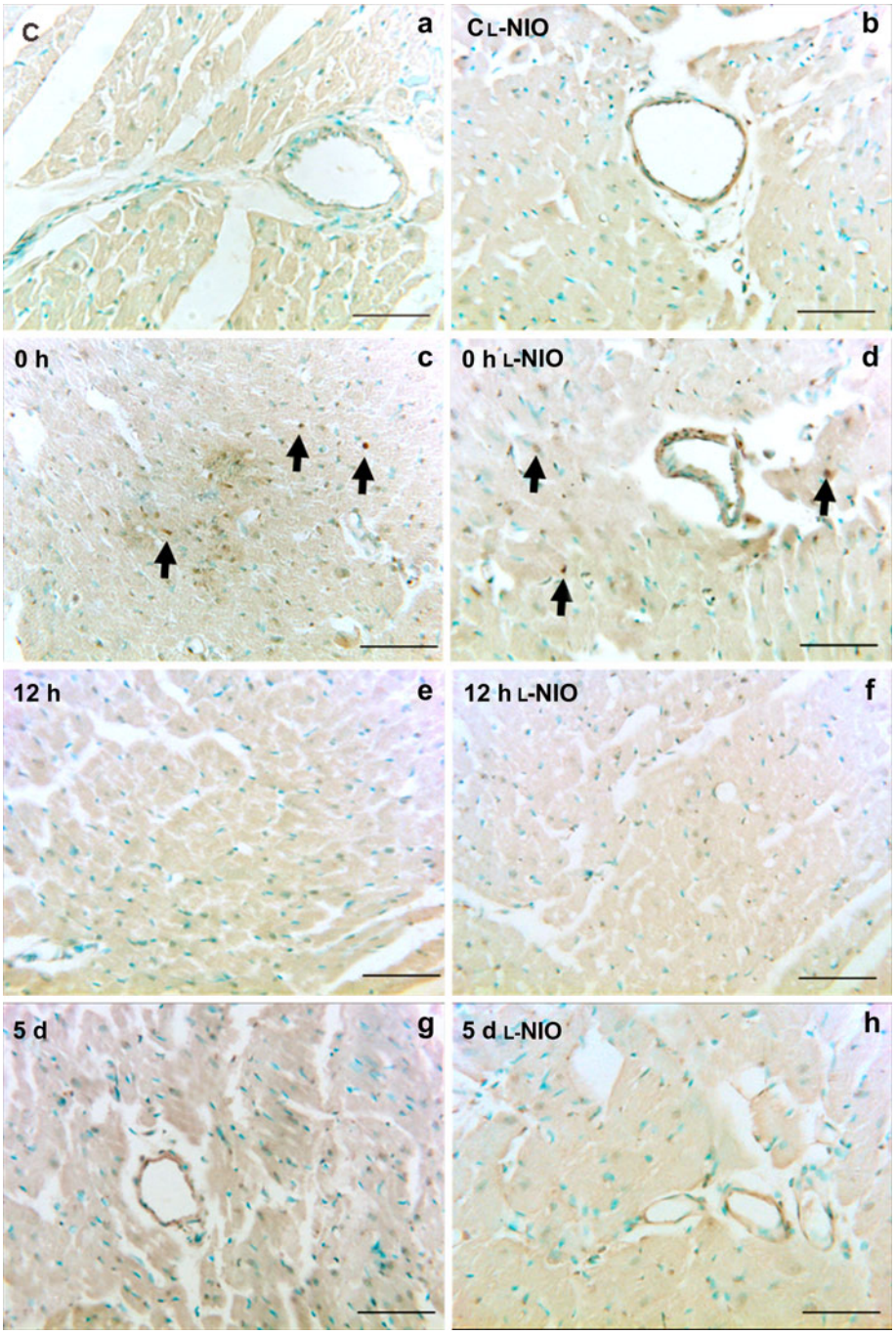

Figure 3 (**a**–**h**). Micrographs showing histological sections of rat heart stained for TUNEL assay. Experimental groups: Control (C) and 0 h, 12 h and 5 days (5 d) post-hypoxia; control (C) L-NIO and 0 h L-NIO, 12 h L-NIO and 5 days (5 d) L-NIO post-hypoxia. Apoptotic, TUNEL-positive cells are indicated by the brown nuclear staining (*arrows*). Micrograph **c** shows higher apoptosis levels when compared with the control group in the untreated groups. Micrographs **b**, **d**, **f** and **h** show no changes in apoptosis levels in relation to the corresponding untreated groups. Scale bars: 50 μm.

Nevertheless, after the administration of the inhibitor L-NIO, NOx levels significantly fell in control and throughout the reoxygenation periods (0 h, 12 h and 5 days). Given that it has been reported that L-NIO is a selective eNOS inhibitor (Viñas *et al.* 2006; Tsai *et al.* 2007; Chaitoff *et al.* 2008; Mehta *et al.* 2008; Sosroseno *et al.* 2008), the lower NOx levels found after L-NIO administration can be attributed to eNOS inhibition.

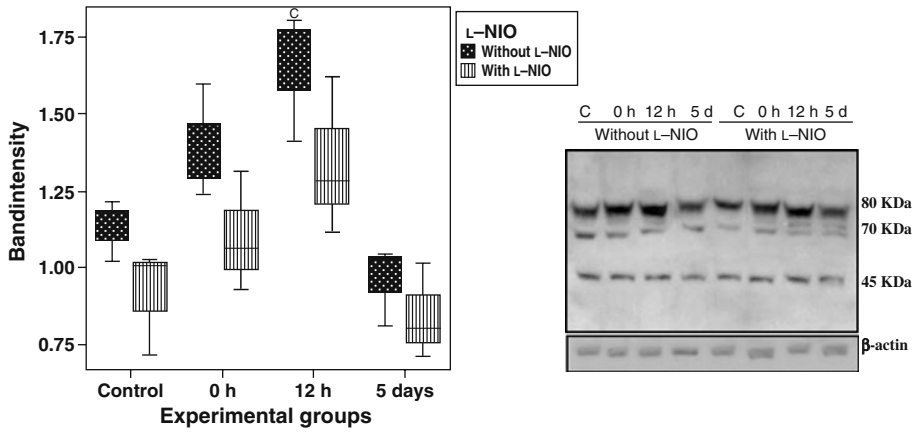

Figure 4. Influence of hypoxia/reoxygenation on nitrotyrosine-modified protein expression in rat heart. Left panel: densitometric quantification of bulk-nitrated proteins in the experimental groups: control (C) and 0 h, 12 h and 5 days (5 d) post-hypoxia; control (C) L-NIO and 0 h L-NIO, 12 h L-NIO and 5 days (5 d) L-NIO post-hypoxia. The results are expressed as arbitrary units (AU). The results are the mean values of three independent experiments with five animals per group. Right panel: representative autoradiography of the nitrotyrosine-modified protein bands; β-actin immunodetection was also included as a protein-loading control. The statistically significant differences vs. 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 $^{w}P<0.05$; $^{x}P<0.02$; $^{y}P<0.01$; $^{z}P<0.001$.

Our H/R model significantly increased the lipid peroxidation level, determined by TBARS, at 0 h post-hypoxia in the untreated groups, indicating that changes consistent with oxidative processes occur in the heart in response to hypoxia. Because this increase in TBARS coincides with higher NOx levels, it might at first be assumed that NO could be involved in the oxidative stress that occurs during cardiac H/R. In fact, a close relationship between NO and oxidative stress has long been proposed (Saugstad 2000). Peroxidative processes, including lipid peroxidation, depend on $HO^{\cdot}$ production and on the formation of oxidizing iron species such as ferryl ion, kinetically equivalent to $HO^{\cdot}$ (Yamazaki and Piette 1990). In this sense, NO may exert pro-oxidant effects by reducing ferric iron complexes, thereby inducing a release of bound iron and indirectly substituting for other reductants in the Haber–Weiss reaction-mediated production of $HO^{\cdot}$ from H_2O_2 (Reif and Simmons 1990). In addition, NO-derived peroxynitrite can induce lipid peroxidation in model systems without iron participation (Radi *et al.* 1991). Nonetheless, our results also show that after eNOS inhibition using L-NIO, the TBARS level did not change in the treated groups in comparison with the corresponding untreated ones, indicating no direct involvement of eNOS-derived NO in the oxidative processes occurring in the heart during such conditions.

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, a significant increase in the percentage of apoptotic cells was detected at 0 h post-hypoxia in the untreated groups, suggesting that apoptotic events occur in the heart in response to hypoxia. As in the previous case, this increase in apoptosis coincides with a rise in NOx levels, implying that NO could be responsible for the programmed cell death that occurs during cardiac H/R. Actually, a relation between NO, apoptosis and hypoxia has previously been reported. Hypoxia raises NO production and increased NO reacts with superoxide to form peroxynitrite. Then, peroxynitrite causes the mitochondria to swell up and release cytochrome C, which is subsequently caged by Apaf-1 and caspase-9 to form apoptosome. The apoptosome, therefore, activates caspase-3, thereby leading to apoptosis (Kiang and Tsen 2006). Nevertheless, after L-NIO administration, it was noticeable that the number of apoptotic cells did not vary in the hypoxic rat heart, implying that eNOS-derived NO may not mediate the programmed cell death that occurs in this organ during H/R. There are, however, controversial results in the literature concerning the effects of the eNOS inhibitor L-NIO on apoptosis in other experimental models and organs. While some authors have reported that L-NIO does not modify the pattern of DNA fragmentation in rat peritoneal neutrophils (Fierro *et al.* 1995), proposing that eNOS-derived NO is not involved in cell apoptosis, others have demonstrated that the number of TUNEL-positive cells decreases with L-NIO in the ischaemic rat kidney, suggesting that eNOS contributes to the development of the apoptotic events during renal ischaemia/reperfusion (Viñas *et al.* 2006).

Finally, our results also show that our H/R model significantly augmented bulk-nitrotyrosine-modified protein expression, found in vascular endothelial cells and cardiomyocytes, at 12 h post-hypoxia in the untreated groups. Nitration of tyrosine residues could affect tyrosine phosphorylation and thus interfere with impor-

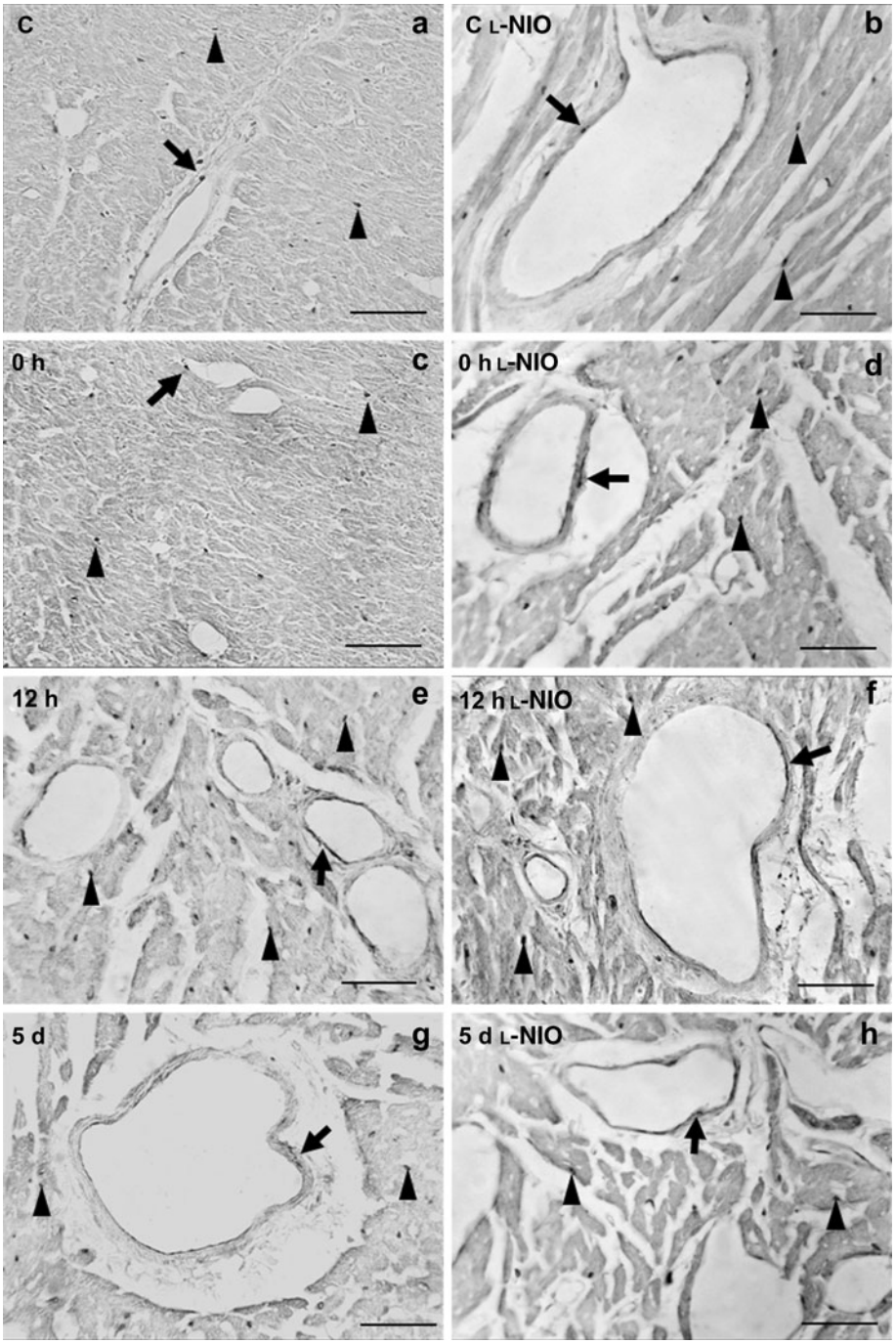

Figure 5 (**a**–**h**). Micrographs showing nitrotyrosine immunoreactivity in rat heart sections in endothelial cells (*arrows*) and cardiomyocytes (*arrow heads*). Experimental groups: control (C) and 0 h, 12 h and 5 days (5 d) post-hypoxia; control (C) L-NIO and 0 h L-NIO, 12 h L-NIO and 5 days (5 d) L-NIO post-hypoxia. Micrographs **b**, **d**, **f** and **h** show no changes in nitrotyrosine-staining intensity in relation to the corresponding untreated groups. Scale bars: 50 μm.

tant signalling pathways (Van der Vliet *et al.* 1994), including the nitration of mitochondrial respiratory-chain enzymes, which could lead to cell death (Bolaños *et al.* 1994; Alonso *et al.* 2002). As in the previous cases, the treatment with the inhibitor L-NIO did not alter bulk-nitrated protein expression, suggesting that eNOS-derived NO may not be involved in the formation of nitrotyrosine in the hypoxic rat heart. Contrary to these results, it has been reported that

the up-regulation of eNOS during chronic hypoxia leads to the formation of nitrotyrosine in the endothelium of the pulmonary arteries (Demiryürek *et al.* 2000).

In short, the results of this study indicate that treatment with the selective eNOS inhibitor L-NIO does not affect parameters of cell and tissue damage (lipid peroxidation, apoptosis and nitrated protein expression) in the rat heart submitted to H/R. These findings imply that eNOS-derived NO may not be involved in the damage that occurs during such situations in the heart. From these results, we propose that L-NIO would not be useful in alleviating the adverse effects of H/R in this organ. Moreover, this work confirms that the dual role (protective/destructive) played by NO in the pathogenesis of the injuries occurring during cardiac H/R depends directly on the NOS isoform involved. In this sense, while iNOS-derived NO may have a cardioprotective effect against the damage that occurs under such conditions (Rus *et al.* 2011), eNOS-derived NO appears not to be related to cardiac H/R-induced injury. Furthermore, we have previously investigated the role that eNOS-derived NO plays during H/R in the rat lung (Rus *et al.* 2010), and contrary to what happens in the heart, this NOS isoform may exert a protective effect against lung H/R-related damage. Taking these results together, we conclude that the dual role of NO during H/R depends not only on the producer NOS isoform but also on the organ involved.

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