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Nitric oxide modulates hypoxia-inducible factor-1 and poly(ADP-ribose) polymerase-1 cross talk in response to hypobaric hypoxia

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Martínez-Romero R, Cañuelo A, Siles E, Oliver FJ, Martínez-Lara E. Nitric oxide modulates hypoxia-inducible factor-1 and poly-(ADP-ribose) polymerase-1 cross talk in response to hypobaric hypoxia. *J Appl Physiol* 112: 816–823, 2012. First published December 15, 2011; doi:10.1152/japplphysiol.00898.2011.—The physiological response to hypobaric hypoxia represents a complex network of biochemical pathways in which the nitrergic system plays an important role. Previous studies have provided evidence for an interplay between the hypoxia-inducible factor-1 (HIF-1) and poly(ADP-ribose) polymerase-1 (PARP-1) under hypoxia. Here, we evaluate the potential involvement of nitric oxide (NO) in the cross talk between these two proteins. With this aim, we studied comparatively the effect of pharmacological inhibitors of NO production or PARP activity in the response of the mouse cerebral cortex to 4 h of exposure to a simulated altitude of 31,000 ft. Particularly, we analyzed the NO and reactive oxygen species production, the expression of NO synthase (NOS) isoforms, PARP-1 activity, HIF-1α expression and HIF-1 transcriptional activity, the protein level of the factor inhibiting HIF, and, finally, beclin-1 and fractin expression, as markers of cellular damage. Our results demonstrate that the reduction of NO level did not affect reactive oxygen species production but significantly *1*) dampened the posthypoxic increase in neuronal NOS and inducible NOS expression without altering endothelial NOS protein level; *2*) prevented PARP activation; *3*) decreased HIF-1α response to hypoxia; *4*) achieved a higher long-term HIF-1 transcriptional activity by reducing factor inhibiting HIF expression; and *5*) reduced hypoxic damage. The pharmacological inhibition of PARP reproduced the NOS expression pattern and the HIF-1α response observed in NOS-inhibited mice, supporting its involvement in the NO-dependent regulation of hypoxia. As a whole, these results provide new data about the molecular mechanism underlying the beneficial effects of controlling NO production under hypobaric hypoxic conditions.

poly(ADP-ribose) polymerase-1 inhibition; nitrergic system; cerebral hypoxia; hypoxia-inducible factor-1 activity

HIGH-ALTITUDE ILLNESS IS CAUSED primarily by hypobaric hypoxia (HH), which is considered as an acute physiological stress that affects the central nervous system and results in several physiological alterations. In general, the severity and duration of the symptoms vary, depending on the altitude and rate of ascent, sometimes persisting after returning to lower altitudes (5, 40, 47). The development of these symptoms has been linked to hypoxia-induced oxidative and nitrosative stress (4, 6, 7, 49–54, 65–68).

Poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) is a zinc-finger, DNA-binding protein that specifically detects DNA-strand breaks caused by genotoxic agents, such as reactive oxygen species (ROS) and peroxynitrite (24). Once activated, this enzyme modulates the activity of different nuclear proteins, including itself, by catalyzing ADP-ribosylation. Previous studies have established that pharmacological inhibition or genetic inactivation of PARP-1 may be beneficial in the treatment of certain diseases, especially those involving a hypoxic situation (e.g., ischemia, cancer, inflammation, neurodegenerative diseases) and increased oxidative and nitrosative stress (8, 11, 26, 57, 77). Although the molecular mechanisms underlying this protection have not been fully elucidated, it has been associated to a decrease in the oxidative/nitrosative status (62). Moreover, in vivo studies have demonstrated that pharmacological neutralization of peroxynitrite formation is associated with a reduced degree of PARP activation (reviewed in Ref. 63).

The molecular response to hypoxia is largely governed by the hypoxia-inducible factor-1 (HIF-1), a transcription factor that binds to hypoxia-response elements and activates more than 100 genes involved in erythropoiesis, angiogenesis, energy metabolism, cell proliferation/survival, and apoptosis, among others (reviewed in Refs. 42, 44). HIF-1 is a heterodimeric DNA-binding complex composed of α- and β-subunits (73). Because HIF-1β is constitutively expressed, HIF-1 activity depends on the HIF-1α subunit level. In normoxia, HIF-1α is hydroxylated by HIF prolyl hydroxylases (PHDs). This posttranslational modification mediates HIF-1α ubiquitination and degradation by the proteosome (29, 39). However, under hypoxic conditions, the inhibition of PHDs allows the functional complex formation. HIF-1 transcriptional activity can also be regulated by hydroxylation. This modification, catalyzed by an asparaginyl hydroxylase known as factor inhibiting HIF (FIH), blocks HIF-1α interaction with the transcription coactivator p300/cAMP response element binding protein and hence its transcriptional activity (38, 46). The inhibition of FIH activity, which also occurs in hypoxia, contributes to the expression of HIF-1 target genes.

Besides oxygen, regulators of HIF-1α include ROS and nitrogen species. Although the involvement of ROS in HIF-1α stabilization was controversial, it is increasingly being recognized that mitochondrial ROS can impair PHD activity, promoting HIF-1α accumulation and activation (18, 19, 25). Concerning the role of nitric oxide (NO) in HIF-1α regulation, it has been proposed that NO inhibition of mitochondrial respiration leads to intracellular O_2 redistribution, increasing PHD activity, and preventing HIF-1α stabilization (16, 35). More recently, it has been reported that NO elicits a regulatory feedback mechanism between HIF-1α and PHD2 activity that leads to HIF-1α accumulation, which, in turn, stimulates PHD2 expression and, consequently, reduces HIF-1α in a later phase (9, 10).

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Recent reports have begun to provide evidence for a connection between PARP-1 and HIF-1 in carcinogenesis (27, 57) using in vitro and in vivo hypoxic models (55, 56). In this sense, our group has demonstrated in a HH model that the absence or inhibition of PARP-1 promotes a lower but sustained posthypoxic HIF-1 transcriptional activity in the cerebral cortex. Besides, we have also observed that the inhibition of PARP-1 diminishes NO but not ROS production (55). We, therefore, hypothesize that NO plays a crucial role in the PARP-1-mediated HIF-1 response to HH. To test the proposed hypothesis, we have analyzed the effect of pharmacological inhibitors of NO production or PARP activity in the HIF-1 posthypoxic activity.

MATERIALS AND METHODS

Animals. This study was performed on male C57/BL6 mice (4–5 mo). The animals were maintained under standard conditions of light and temperature and allowed ad libitum access to food and water. All the procedures followed the European Union and Spanish guidelines on the use of animals for research (RD 1201/2005) and were approved by the institutional Committee for Ethics.

Experimental model. Acute HH was induced as previously described (55). Briefly, animals were placed in a hypobaric chamber in which the air pressure was controlled by means of a continuous vacuum pump and an adjustable inflow valve. The chamber was also provided with a manometer to check the experimental altitude during the process. Hypoxia was induced by downregulating the environmental pressure to a final barometric pressure of 225 mmHg, resulting in an O_2 partial pressure of 48 Torr (equivalent to ~6% normobaric oxygen). These conditions simulate an altitude of 31,000 ft (8,100 m) and were maintained for 4 h. Ascent and descent rates were kept below 1,000 ft/min. After the hypoxia period, a return to normobaric normoxic conditions (150 Torr O_2 partial pressure in our setting) was attained in ≈30 min. Animals were either killed immediately after the hypobaric chamber was opened (0-h reoxygenation group) or kept at atmospheric pressure for 2 h and then killed (2-h reoxygenation group). Animals kept in the chamber under normobaric normoxic conditions served as controls. No longer reoxygenation times were selected because time course experiments, previously performed by our group, indicated that NO level and HIF-1 expression and transcriptional activity returned to control values after 2 h of reoxygenation.

After the corresponding reoxygenation times, the mice were killed by cervical dislocation, and the brains were immediately removed. Cerebral cortexes were dissected, rinsed in saline solution, frozen in liquid nitrogen, and stored at −80°C until their analysis.

L-NAME and DPQ treatment. The NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) was dissolved in tap water at a concentration of 1 mg/ml and orally provided ad libitum for a period of 2 wk before the onset of hypoxia. The daily intake of the drug, which was recorded through the measurement of fluid intake, was ≈150 mg/kg. PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ; Alexis Biochemicals, San Diego, CA) was administered intraperitoneally (15 mg/kg) 1 h before hypoxia (32).

NO measurement. NO production was indirectly quantified by determining nitrate/nitrite and *S*-nitroso compounds (NOx), using an ozone chemiluminescence-based method.

The cerebral cortexes of animals from each experimental group were homogenized at 4°C in three volumes of PBS. Homogenates were then sonicated, centrifuged at 10,000 *g* for 40 min, and deproteinized with 0.8 N NaOH and 16% $ZnSO_4$ solutions (1:0.5:0.5 wt/vol/vol). After centrifugation at 10,000 *g* for 5 min, supernatants were removed for chemiluminescence analysis (13) in a NO analyzer (NOA 280i Sievers Instruments). The NOx concentration was calculated by comparison with standard sodium nitrate solutions. The final NOx values were referred to the total protein concentration in the initial extracts (12).

Determination of thiobarbituric acid reactive substances. Thiobarbituric acid reactive substances (TBARS), major indicators of oxidative stress, were determined in mice cerebral cortexes following the instructions of the Oxitek TBARS Assay Kit (ZeptoMetrix). Concentration of TBARS in the samples was calculated by interpolation from a standard curve with malondialdehyde, ranging from 0 to 100 nmol/ml (0–0.550 of absorbance at 530 nm, respectively). Final values were referred to the total protein concentration in the initial extracts (12).

PARP activity assay. PARP activity was assayed using a colorimetric kit, according to the manufacturer's instructions (Universal Colorimetric PARP Assay Kit with Histone-Coated Strip Wells, Trevigen). PARP activity in the samples was calculated by interpo-

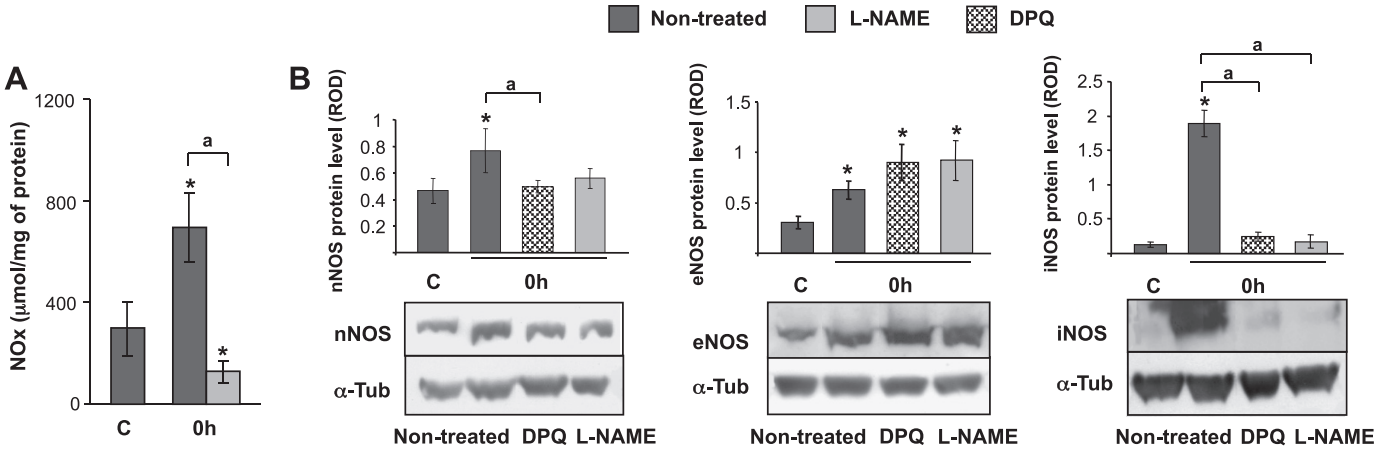

Fig. 1. Effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) treatment on posthypoxic nitrate/nitrite and *S*-nitroso compound (NOx) level and nitric oxide synthase (NOS) isoform expression. *A*: NOx levels (μmol/mg of protein) were determined in control (C), posthypoxic (0 h), and L-NAME-treated posthypoxic (0 h) mice. Values are means ± SD from three independent experiments ($n = 3$). *B*: densitometric quantifications of neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) expression in control, posthypoxic (0 h), and L-NAME or 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ)-treated posthypoxic (0 h) mice. Values are means ± SD relative to α-tubulin (Tub) from three independent experiments ($n = 3$). ROD, relative optical density. A representative immunoblot for nNOS, eNOS, and iNOS from a single experiment is shown. Levels significantly higher than the corresponding control group: $^{*}P < 0.05$. Statistically significant differences from the corresponding posthypoxic nontreated group: $^{a}P < 0.05$.

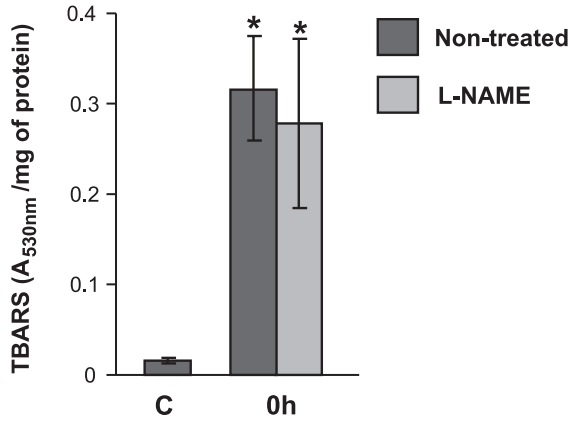

Fig. 2. Effect of L-NAME treatment on posthypoxic oxidative stress. Thiobarbituric acid reactive substances (TBARS) levels (535 nm absorbance/mg of protein) in control, posthypoxic (0 h), and L-NAME-treated posthypoxic (0 h) mice are shown. A_{530nm}, 530-nm absorbance. Values are means ± SD from three independent experiments ($n = 3$). Levels significantly higher than the corresponding control group: *$P < 0.05$.

lation from a standard curve with recombinant PARP enzyme ranging from 0 to 0.4 units. Final values were referred to the total protein concentration in the initial extracts (12).

Western blot analysis. The cerebral cortexes of mice brains were dissected on ice, flash-frozen with liquid nitrogen, and stored at −80°C. For nuclear extracts, brain-tissue preparations were homogenized, and the nuclear proteins were purified using the NE-PER Nuclear and Cytoplasmic Extraction Kit (PIERCE, Rockford, IL). A protease-inhibitor cocktail (Complete Mini, Roche) and reducing agents containing 1 mM dithiothreitol and 0.5 mM phenymethylsulfonyl fluoride were added to each reagent. For whole protein extracts, the cerebral cortexes were homogenized in 1:3 (wt/vol) of 30 mM Tris·HCl (pH 7.4) containing 0.01% Triton X-100. Protease inhibitors and reducing agents were also added as described above. The resulting homogenates were sonicated and centrifuged for 40 min at 10,000 *g*. All procedures were performed at 4°C. The protein concentration was determined by the Bradford method (12). Equal amounts of nuclear protein (HIF-1α: 25 μg) or of total protein extract [inducible NOS (iNOS), neuronal NOS (nNOS), endothelial NOS (eNOS): 40 μg; FIH, fractin: 20 μg] were loaded and separated on a 7.5% or 10% SDS-polyacrylamide gel (Mini Protean II; BioRad), respectively, as described by Laemmli (45). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). Membranes were blocked and incubated overnight at 4°C with diluted monoclonal anti-iNOS and eNOS (1:1,000; Transduction Laboratories), polyclonal anti-nNOS (gift from V. Riveros-Moreno of Welcome Research Laboratories, Beckenham, UK), anti-HIF-1α (1:500, Bethyl Laboratories), anti-FIH (1:500; Santa Cruz Biotechnology), or anti-fractin (1:500; Chemicon International, Temecula, CA) in blocking solution. Bound antibody was revealed by means of an enhanced chemiluminescence kit (ECL plus; Amersham). After immunodetection, membranes were probed with anti-α-tubulin (Sigma) as a loading control. The relative amount of the proteins in each sample was quantified by densitometric scanning.

Quantitative RT-PCR. Gene expression of different HIF-1 target genes [adrenomedullin (AM), vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1), erythropoietin (EPO), and lactate dehydrogenase (LDH)] and of the myosin-like BCL-2 interacting protein (beclin-1), as a marker of autophagy, was quantitatively assessed by real-time PCR using β-actin as the normalizing gene. Total RNA was isolated from cell extracts using Trizol reagent (Invitrogen), according to the manufacturer's instructions. After treatment with DNase, cDNA was synthesized from 1.5 μg total RNA using reverse transcriptase (Superscript III RT, Invitrogen) with oligo(dT)15 primers (Promega). Real-time PCR was performed on the Stratagene MxPro 3005P qPCR system using the Brilliant II SyBR Green QPCR Master Mix (STRATAGENE, La Jolla, CA). The following primer pairs were used: AM, 5′-CAGCAATCAGAGCGAAGC-3′ and 5′-ATGCCGTCCTTGTCTTTGTC-3′; VEGF, 5′-CAAGATCCGCAGACGTGTAA-3′ and 5′-CGCCTTGGCTTGTCACAT-3′; GLUT-1, 5′-TATTGCTGTGGCTGGCTTCT-3′ and 5′-GCCTTTGGTCTCAGGGACTT-3′; EPO, 5′-ACTCTCCTTGCTACTGATTCCT-3′ and 5′-ATCGTGACATTTTCTGCCTCC-3′; LDH, 5′-GTTACACATCCTGGGCCATT-3′ and 5′-TCACAACATCCGAGATTCCA-3′; beclin-1, 5′-GGTGGAAAAAGGAGAGACTCG-3′ and 5′-TCCACTGCTCCTCCGAGTTA-3′; β-actin, 5′-TGAGGAGCACCCTGTGCT-3′ and 5′-CCAGAGGCATACAGGGAC-3′.

Experiments were performed with triplicates, and the relative quantities of target genes corrected with the normalizing gene, β-actin, were calculated using the STRATAGENE MxPro QPCR Software.

Statistical analysis. Data are expressed as means ± SD from three independent experiments ($n = 3$). Statistical comparisons between reoxygenation groups and the corresponding control group were made by ANOVA with post hoc Bonferroni test, accepting $P < 0.05$ as the level of significance. Student's *t*-test was performed to evaluate the significance of the PARP-1-dependent differences and the effect of the reoxygenation time, accepting $P < 0.05$ as the level of significance.

RESULTS

NO system response to HH. First, we evaluated the cerebral cortex NO system response to 4 h of HH. As previously reported, hypoxia promoted a significant but transient increase in NO production (Fig. 1*A*). Treatment with L-NAME, a pan-NOS activity inhibitor, significantly decreased posthypoxic (0 h) NO to levels resembling those found in control mice, demonstrating the involvement of the NOS system in NO production.

We then analyzed the protein expression of constitutive NOS and iNOS isoforms immediately after hypoxia, experimental time in which NO level peaked (Fig. 1*B*). As shown in Fig. 1*B*, the rise in NO level was accompanied by the upregulation of nNOS, eNOS, and, more consistently, iNOS expres-

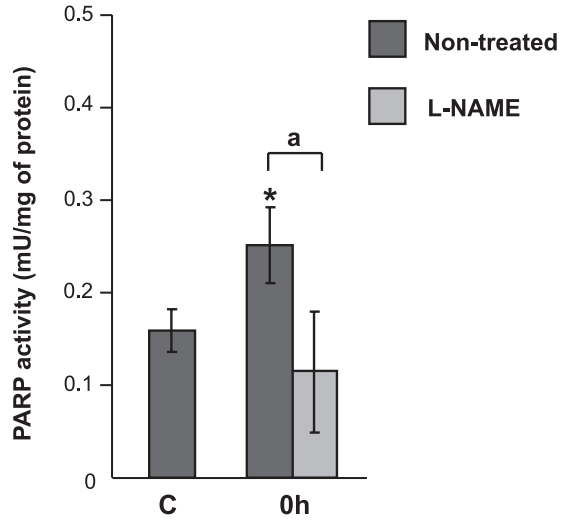

Fig. 3. Poly(ADP-ribose) polymerase (PARP) activity in control, posthypoxic (0 h), and L-NAME-treated posthypoxic (0 h) mice. Values are means ± SD from three independent experiments ($n = 3$). Unit definition: 1 unit PARP incorporates 100 pmol poly(ADP) from NAD^+ into acid-insoluble form in 1 min at 22°C. Statistically significant differences from the control group: *$P < 0.05$. Statistically significant differences from the corresponding posthypoxic nontreated group: [a] $P < 0.05$.

sion. L-NAME treatment dampened the increase in nNOS and iNOS expression, but did not alter eNOS protein level (Fig. 1*B*). The pharmacological inhibition of PARP with DPQ (Fig. 1*B*) reproduced the NOS expression pattern observed after L-NAME treatment, suggesting the involvement of this protein in the nitrergic system response to hypoxia.

L-NAME effect on ROS response to hypoxia. Some authors have ascribed an antioxidant activity to L-NAME in different brain regions and nervous cells (31, 69, 71). However, under our experimental conditions, L-NAME administration did not significantly diminish the oxidative stress response to hypoxia observed in nontreated animals (Fig. 2).

Posthypoxic PARP activity under L-NAME treatment. PARP-1 activation is induced primarily by DNA-strand breaks, which can be triggered by oxidative and nitrosative stress, among other factors. Besides, PARP-1 activity is also known to induce NO production. Our results showed that, as expected, PARP activation in response to hypoxia was inhibited in L-NAME-treated mice (Fig. 3).

Modulation of HIF-1 response to hypoxia by NO production. As mentioned above, NO and ROS have been implicated in HIF-1α stability under hypoxia. Therefore, we analyzed the effect of L-NAME (a treatment that only diminishes NO level) in the HIF-1α expression level (Fig. 4*A*). Immediately after hypoxia (0 h), the results indicated that the treatment significantly decreased the HIF-1α response. To confirm the implication of PARP-1 in the modulation of HIF-1α by NO, we also evaluated the HIF-1α accumulation in pharmacologically PARP-inhibited mice. As shown in Fig. 4*A*, DPQ treatment significantly reduced HIF-1α expression (0 h). At 2 h of reoxygenation, HIF-1α protein level returned to control values in nontreated mice, and no additional effects were observed when animals were pretreated with L-NAME or DPQ.

In addition to HIF-1α stabilization, we also evaluated the effect of L-NAME on HIF-1 transcriptional activity in response to hypoxia by analyzing the transcription level of five HIF-1 target genes: AM, LDH, GLUT-1, VEGF, and EPO (Fig. 4*B*). According to the lower HIF-1α expression, immediately after hypoxia L-NAME treatment significantly decreased the mRNA level of three (AM, LDH, GLUT-1) out of the five genes analyzed. To check the duration of HIF-1 transcriptional activity, we performed the same analysis after 2 h of reoxygenation. Interestingly, although the transcription level of all of the target genes returned to control values in nontreated animals, L-NAME administration maintained the mRNA levels detected immediately after hypoxia (0 h).

The transcriptional activity of HIF-1 is also regulated by FIH, a protein that seems to be regulated by NO (58). As shown in Fig. 5, L-NAME treatment significantly diminished posthypoxic FIH expression to the levels found in control mice.

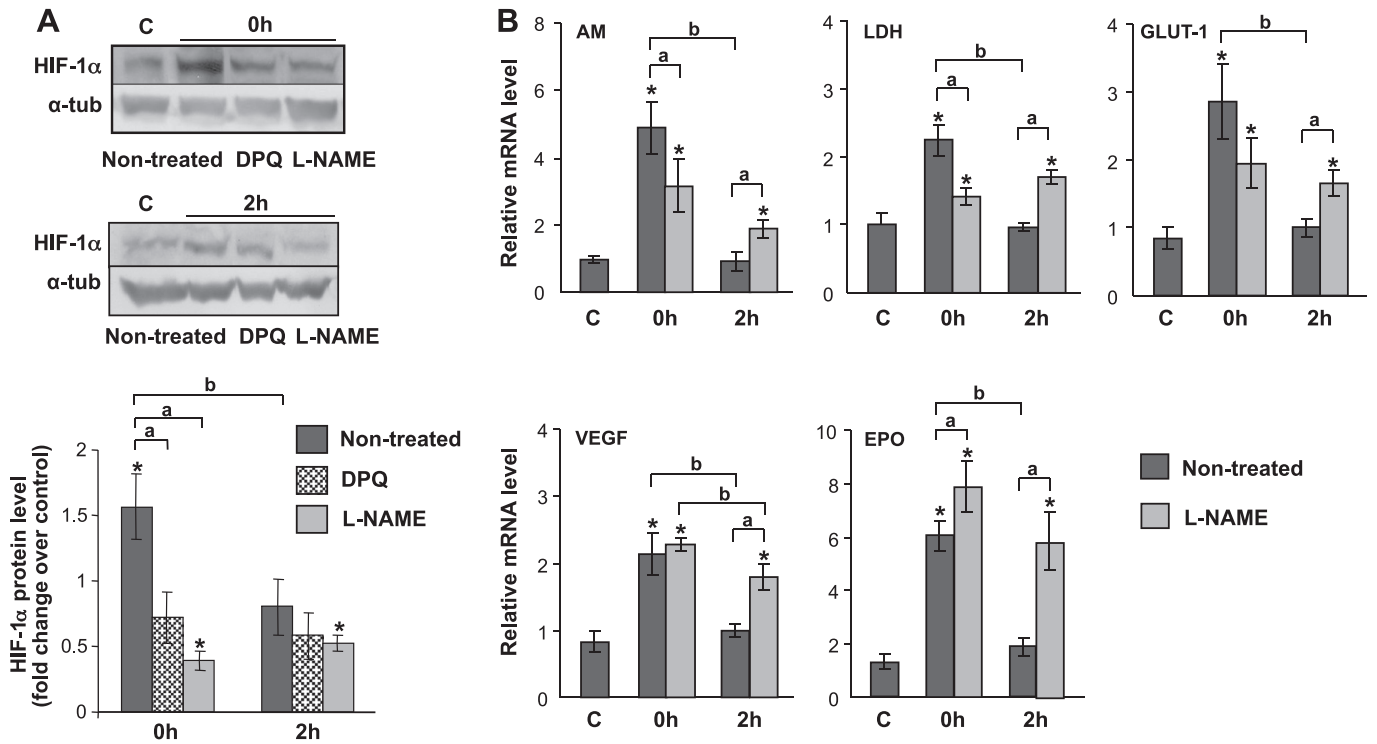

Fig. 4. *A*: Western blot analysis of the effect of L-NAME on hypoxia-induced hypoxia-inducible factor-1α (HIF-1α) stabilization. Semiquantitative densitometry measurements of HIF-1α immunoreactivity in control, posthypoxic (0 and 2 h), and L-NAME- or DPQ-treated posthypoxic (0 and 2 h) mice, expressed as fold change over the corresponding control value, are shown. α-Tubulin immunodetection was included as a protein-loading control. Values are means ± SD from three independent experiments ($n = 3$). A representative immunoblot for HIF-1α from a single experiment at 0 and 2 h is shown. *B*: mRNA expression of adrenomedullin (AM), lactate dehydrogenase (LDH), vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1), and erythropoietin (EPO) in control, posthypoxic (0 and 2 h), and L-NAME-treated posthypoxic (0 and 2 h) mice. The results are expressed as mRNA expression relative to control animals after normalization against β-actin. Each sample was analyzed in triplicate. The mean ± SD of three RNA extracts for each experimental group is represented. Statistically significant differences from the corresponding control group: $^{*}P < 0.05$. Statistically significant differences from the corresponding posthypoxic nontreated group: $^{a}P < 0.05$. Statistically significant differences between 0 h and its counterpart 2-h experimental group: $^{b}P < 0.05$.

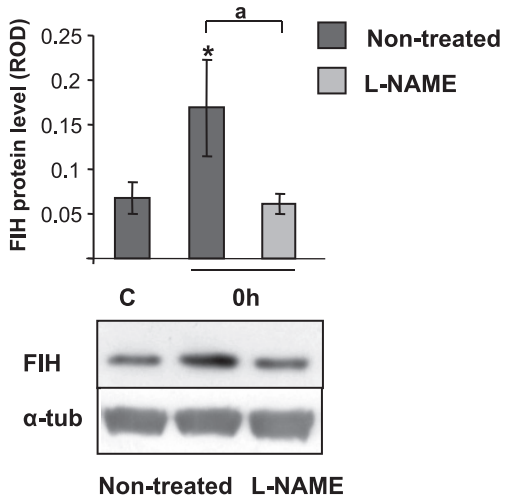

Fig. 5. Western-blot analysis of the effect of L-NAME on factor inhibiting HIF (FIH) levels. Semiquantitative densitometry measurements of FIH expression in control, posthypoxic (0 h), and L-NAME-treated posthypoxic (0 h) mice are shown. Values are means ± SD relative to α-tubulin from three independent experiments ($n = 3$). Statistically significant differences from the corresponding control group: *$P < 0.05$. Statistically significant differences from the corresponding posthypoxic nontreated group: [a] $P < 0.05$. A representative immunoblot for FIH from a single experiment is shown.

Inhibition of NO production and hypoxic damage. To corroborate the previously reported beneficial effect of reducing NO level in hypoxic disease (15, 67), we analyzed fractin and beclin-1, markers of apoptosis and autophagy, respectively, in our experimental conditions. As shown in Fig. 6*A*, the level of beclin-1 mRNA was significantly lower in L-NAME-treated animals (0 and 2 h). Moreover, the progressive and statistically significant increase in fractin expression (Fig. 6*B*) was also reduced with L-NAME treatment.

DISCUSSION

There is an intricate interplay of biochemical pathways in response to HH that cause changes at the physiological, cellular, and molecular levels. In this sense, it has been extensively reported that the activation of the nitrergic system is involved in the cerebral cortex response to hypoxia. The results we present provide new data supporting the advantages of controlling the NO production under hypobaric hypoxic conditions, since reduction of NO level *1*) prevents PARP-1 activation as well as iNOS and nNOS response; *2*) maintains eNOS function; and *3*) prolongs HIF-1 activity, minimizing hypoxic cellular damage.

PARP-1 is a DNA binding protein that detects DNA-strand breaks, triggered by a variety of oxidants and free radical species, particularly peroxynitrite (1, 61, 63, 72). In this sense, our results in the cerebral cortex show that HH promotes an increase in NO production, which correlates with PARP activation. This augmented NO level was parallel to an enhanced expression of NOS isoforms, pointing to them as a potential source of the observed NO production. NOS-independent NO generation has also been reported in hypoxic conditions (33, 48, 78). In our HH model, an access to the animals is not feasible until the normobaric normoxic conditions are newly achieved and the chamber is opened. Thus it is not possible to analyze the NO sources during the hypoxic period. However, our results showing that treatment with L-NAME (a specific NOS inhibitor) before the onset of hypoxia abolishes PARP activation suggest that NOS-dependent NO should be, at least in part, involved in this effect. In this sense, NOS expression and activity have been analyzed in different central nervous system areas after acute HH models, showing an enhanced NO production and a main implication of the constitutive NOS isoforms as a result of an increase in *N*-methyl-D-aspartate receptor-mediated intracellular Ca^{2+} (43, 49, 50, 53, 65–67). In addition, a decreased PARP activity after brain ischemia has been reported in nNOS-knockout mice, suggesting that ischemia activates nNOS to produce peroxynitrite, which, in turn, activates PARP (28).

On the other hand, PARP-1 activity is also known to induce NO production. Indeed, activation of PARP plays an important role in the upregulation of inflammatory cascades via functional association with different proteins, including NF-kB, a key transactivator of the iNOS and nNOS gene (36, 60). In fact, PARP-1-deficient mice have reduced iNOS expression (21, 62). In our experimental conditions, the immediate posthypoxic increase in both NO production and NOS isoform expression, predominantly iNOS, could also reflect this effect.

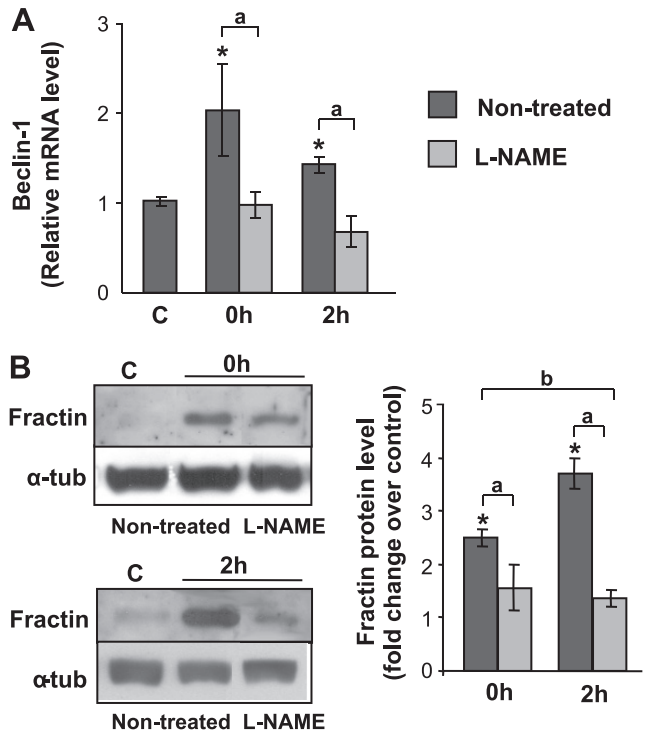

Fig. 6. Effect of L-NAME treatment on hypoxic cell death. *A*: mRNA expression of beclin-1 in control, posthypoxic (0 and 2 h), and L-NAME-treated posthypoxic (0 and 2 h) mice. The results are expressed as mRNA expression relative to control animals after normalization against β-actin. Each sample was analyzed in triplicate. The mean ± SD of three RNA extracts for each experimental group is represented. *B*: representative immunoblot of fractin level in control, posthypoxic (0 and 2 h), and L-NAME-treated posthypoxic (0 and 2 h) mice. α-Tubulin immunodetection was included as a protein-loading control. Semiquantitative densitometry measurements are expressed as fold change over control values. Values are means ± SD from three independent experiments ($n = 3$). Statistically significant differences from the corresponding control group: *$P < 0.05$. Statistically significant differences from the corresponding posthypoxic nontreated group: [a] $P < 0.05$. Statistically significant differences between 0 h and its counterpart 2-h experimental group: [b] $P < 0.05$.

Our study, analyzing for the first time the involvement of PARP-1 in the hypoxic response of the three NOS isoforms, demonstrates that pharmacological inhibition of PARP significantly decreased iNOS and nNOS expression, but did not affect eNOS protein level. iNOS and nNOS overactivation is involved in neurotoxicity, whereas eNOS induction is beneficial for the endothelial functionality in hypoxic situations. Consequently, our results would support and explain the beneficial effect of controlling NO production through the use of PARP inhibitors in hypoxic treatments.

HIF-1α, a master regulator of the cellular oxygen homeostasis, is stabilized and activated by hypoxia and modulates the expression of several target genes (2, 22). Although the role of NO levels in controlling HIF-1 activity under hypoxic conditions offers conflicting data, recent studies center the regulation of HIF-1 by NO on the control of its degradation. In this sense, it has been proposed that NO directly inhibits PHDs, through its competitive binding with the Fe^{2+} at the catalytic site, promoting HIF-1α accumulation (10, 14, 58, 70). The present in vivo study supports this idea, since the decreased NO production, caused by the L-NAME treatment, could be responsible for the reduced posthypoxic HIF-1α levels observed. Besides, considering that DPQ reproduces this effect, we demonstrate the crucial role of PARP-1 in this mechanism. Finally, although an involvement of ROS in HIF-1α accumulation has also been reported (17–19), our results do not reflect this effect, since L-NAME treatment did not affect the increased posthypoxic ROS level (Fig. 1*B*).

In addition to HIF-1α abundance, the transcriptional activity of HIF-1 is also regulated under hypoxia. Our mRNA expression studies show that, parallel to the HIF-1α level, the initial response of some of the target genes analyzed (AM, LDH, GLUT-1) was higher in the presence of active PARP-1. However, after 2 h of reoxygenation, the expression of all of the analyzed genes returned to basal values only in nontreated mice, while it remained steady in L-NAME-treated animals (when NO levels were downregulated). The second major mechanism controlling HIF-1 activity is the modulation of its interaction with transcriptional coactivators by FIH (3, 41), a dioxygenase dependent on oxygen for enzymatic activity (46), but not for transcription (58). As previously reported by our group (55), PARP-1 seems to upregulate FIH expression, explaining the lower expression level of HIF-1 target genes in nontreated mice after 2 h of reoxygenation. However, L-NAME treatment downregulates FIH expression, promoting a longer HIF-1 transcriptional activity and supporting the implication of NO in this effect. In this sense, an inhibitor role of FIH activity has been attributed to NO (58, 64), although, to our knowledge, this effect has not been demonstrated in vivo. Our data do not seem to reflect this effect, since the higher posthypoxic NO level observed in non-L-NAME-treated mice correlates with a lower HIF-1 transcriptional activity (2 h), which cannot be explained by the inhibition of FIH. Therefore, we presume that NO could exert an indirect effect on FIH and, consequently, on HIF-1-dependent gene expression by modulating PARP-1 activity. Previous reports (27, 57) have proposed a role of PARP-1 as transcriptional coactivator of HIF-1 and suggest that HIF-1 transcriptional activity is dependent on the enzymatic activity of PARP-1. According to our results, the inhibition of NO production by L-NAME precludes PARP activation. In this situation, the inhibition of PARP activity could compromise its interaction with HIF-1, favoring the lower transcriptional activity detected in our study. Although this mechanism has not been demonstrated in vivo, it may cooperate with FIH in the regulation of HIF-1-dependent gene expression.

Strikingly, the mRNA level of EPO was not reduced by L-NAME treatment. In vitro and in vivo studies have involved either HIF-1 or both HIF-1 and HIF-2 isoforms in EPO expression (20, 30, 74, 76). Similarly, and as mentioned above, eNOS expression was not decreased by L-NAME. It has been demonstrated that, among other mechanisms, eNOS expression is transcriptionally activated by HIF-2 (23). Although the role of NO in regulating HIF-1 signaling has been intensively studied, to our knowledge, the involvement of NO in HIF-2 regulation has been hardly analyzed. The particular expression pattern of EPO and eNOS mentioned above seems to suggest that NO may not affect HIF-1 and HIF-2 in exactly the same way.

Finally, different studies have shown that a decreased level of HIF-1α can be neuroprotective under an ischemic/hypoxic stimulus through downregulation of proapoptotic genes (34, 37, 59, 75). In the present study, L-NAME treatment, which reduces HIF-1α expression, decreases the level of fractin and beclin-1. Thus, in addition to the benefit of decreasing the nitrosative environment, these results represent new data supporting the neuroprotective effect of reducing NO production in diseases involving a hypoxic situation.

Although further experiments demonstrating the in vivo interaction between PARP-1 and HIF-1 and the involvement of HIF-2 need to be performed, our present results demonstrate a crucial role of the nitrergic system, particularly of iNOS and nNOS, in the cross talk between PARP-1 and HIF-1 in the cerebral response to HH. These data should be taken into account when using PARP inhibitors in hypoxic treatments.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.M.-R., E.S., and E.M.-L. conception and design of research; R.M.-R., A.C., E.S., and E.M.-L. performed experiments; R.M.-R., A.C., E.S., and E.M.-L. analyzed data; R.M.-R., A.C., E.S., F.J.O., and E.M.-L. interpreted results of experiments; R.M.-R. and E.M.-L. prepared figures; R.M.-R., A.C., E.S., F.J.O., and E.M.-L. approved final version of manuscript; A.C., E.S., and E.M.-L. edited and revised manuscript; E.S. and E.M.-L. drafted manuscript.

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