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Nitric Oxide System Alteration at Spinal Cord as a Result of Perinatal Asphyxia Is Involved in Behavioral Disabilities: Hypothermia as Preventive Treatment

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Perinatal asphyxia (PA) is able to induce sequelae such as spinal spasticity. Previously, we demonstrated hypothermia as a neuroprotective treatment against cell degeneration triggered by increased nitric oxide (NO) release. Because spinal motoneurons are implicated in spasticity, our aim was to analyze the involvement of NO system at cervical and lumbar motoneurons after PA as well as the application of hypothermia as treatment. PA was performed by immersion of both uterine horns containing full-term fetuses in a water bath at 37°C for 19 or 20 min (PA19 or PA20) or at 15°C for 20 min (hypothermia during PA-HYP). Some randomly chosen PA20 rats were immediately exposed for 5 min over grain ice (hypothermia after PA-HPA). Full-term vaginally delivered rats were used as control (CTL). We analyzed NO synthase (NOS) activity, expression and localization by nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) reactivity, inducible and neuronal NOS (iNOS and nNOS) by immunohistochemistry, and protein nitrotyrosilation state. We observed an increased NOS activity at cervical spinal cord of 60-day-old PA20 rats, with increased NADPH-d, iNOS, and nitrotyrosine expression in cervical motoneurons and increased NADPH-d in neurons of layer X. Lumbar neurons were not altered. Hypothermia was able to maintain CTL values. Also, we observed decreased forelimb motor potency in the PA20 group, which could be attributed to changes at cervical motoneurons. This study shows that PA can induce spasticity produced by alterations in the NO system of the cervical spinal cord. Moreover, this situation can be prevented by perinatal hypothermia.

Key words: NADPH-diaphorase; nitric oxide; inducible nitric oxide synthase; neuronal nitric oxide synthase; nitrotyrosine; immunohistochemistry; motoneurons; rats

Nitric oxide (NO) modulates central and peripheral neurotransmission, synaptic plasticity, blood flow, pain, memory, and other functions in the central nervous system (Garthwaite, 1991; Zorumski and Izumi, 1993; Moncada, 1994; Hobbs et al., 1999). Two constitutive isoforms of NO synthase (NOS), neuronal (nNOS) and endothelial (eNOS), and one inducible isoform (iNOS), catalyze the oxidation of the terminal guanidine nitrogen of L-arginine, yielding equimolar amounts of NO and citrulline (Moncada et al., 1997). The presence of nNOS (Ca^{2+} dependent) and iNOS (Ca^{2+} independent) has been described in the brain and spinal cord of mammals (Zhang et al., 1993; Saito et al., 1994; Uttenthal et al., 1998; Pullen and Humphreys, 1999; Rodrigo et al., 2001). In addition, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) reactivity (Saito et al., 1994) was localized in the brain and spinal cord of rats, monkeys, and cats (Zhang et al., 1993; Saito et al., 1994; Pullen and Humphreys, 1999; Dorfman et al., 2004) and has also been used as a marker of NOS activity in neurons (Young et al., 1997). NO is an intercellular messenger involved in neuroprotective processes (Bredt and Snyder, 1992); however, when it is increased, it may act in neurotoxic mechanisms (Moncada and Higgs, 1991; Moncada et al., 1991). Spinal motoneurons do not normally synthesize NO, but express it as a survival response after injury (Wu and Li, 1993). Asphystic situations induce overproduction of NO, which reacts

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with the free radical superoxide, exacerbating neurodegenerative processes through the formation of peroxynitrites, promoting protein nitration (Beckman, 1990, 1996; Koppal et al., 1999).

The use of hypothermia has been proposed as neuroprotective treatment against brain ischemia in humans (Katz et al., 2004; Gisselsson et al., 2005). Therapeutic hypothermia (33°C) was shown to diminish ischemia effects, decreasing metabolism of neurons (Gisselsson et al., 2005). Hypothermia reduces the production of reactive oxygen species (ROS) and inhibits toxic release of NO, protecting cells against damage (Lei et al., 1997; Loidl et al., 1998; Capani et al., 2003; Ekimova, 2003). Moreover, hypothermia has been shown to prolong the survival of rats dramatically (Herrera-Marschitz et al., 1993; Loidl et al., 2000).

Perinatal asphyxia (PA) is the most serious problem in perinatology around the world, because about one-third of asphystic neonates develop serious long-term neurological sequelae such as spasticity, dystonia, and motor abnormalities (Hill, 1991; Younkin, 1992; Palmer and Vannucci, 1993; Rivkin 1997). Spinal spasticity is partially caused by dysfunction of the spinal cord neural network (Hack and Fanaroff, 2000). Spastic motor abnormalities are attributed to concurrent spinal cord injury at motoneurons that are the target of brain systems (Harrison, 1988; Clancy et al., 1989). Large motoneurons subjected to ischemia suffer from apoptotic processes (Hayashi et al., 1998; de Louw et at, 2002).

Developmental studies have shown that corticospinal tract fibers reach the cervical region of the spinal cord at postnatal day (PND) 1 and the lumbar region at PND5 (Schreyer and Jones, 1982) in rats. The development of coordinated locomotor movements depends on maturation and functional integration of many subsystems (Lanuza et al., 2004). Newborn rats crawl using their forelimbs up to PND11, and then a rostrocaudal maturation of neuronal circuits changes the locomotion to a mature form around PND15. Motor skills could be affected by alterations at the neurons from spinal segments induced by situations of hypoxia-ischemia at birth (Schreyer and Jones, 1982).

Because function and survival of motoneurons that innervate forelimbs and hindlimbs muscles are influenced by NO, the aim of this work was to study the spinal cord NO system in rats that had suffered PA in order to analyze its relation to the spasticity. The nitric system was evaluated in the cervical and lumbar spinal cord, where motoneurons are localized. The use of hypothermia as a possible preventive treatment was studied. Moreover, a behavioral studied was assayed. An increased NOS activity was observed at cervical spinal cord of PA rats, whereas lumbar spinal cord was not altered. Hypothermia treatment was effective for preventing alterations. Also, a decreased forelimb motor potency with PA was observed. This study shows that the NO system changes at cervical motoneurons could be related to the behavioral alterations observed with spasticity.

MATERIALS AND METHODS

Hypoxic-Ischemic Injury

Severe PA was induced using a noninvasive model of hypoxia-ischemia as described previously (Loidl et al., 2000). All experimental protocols concerning animals were performed in agreement with international standards on animal treatment as well as with the approval of the Instituto de Biologia y Medicina Experimental Ethic Committee in accordance with minimal standards as defined by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health *Guide for the care and use of laboratory animals*. Appropriate proceedings were performed to minimize the number of animals used and their suffering. Animals were maintained under standard laboratory conditions at 24°C, with light/dark cycles of 12/12 hr, and food and water were given ad libitum. Timed pregnant Sprague-Dawley rats were sacrificed and hysterectomized after their first pup was delivered vaginally (CTL, n = 5). Then, asphyxia was performed by transient immersion of both uterine horns containing the full-term fetuses in a water bath at 37°C for 19 or 20 min (PA19 or PA20, respectively, n = 5 per group) or at 15°C during 20 min (HYP, n = 5). Asphyxia was assayed for 19 or 20 min according to previous studies in which 1 min of difference in the asphyctic period was enough to generate differences in striatal dopamine content and in adult behavior in rat (Herrera-Marschitz et al., 1993; Loidl et al., 1994, 2000). In addition, some PA20 rats were randomly chosen to be exposed over grain ice for 5 min previous to the recovery (HPA, n = 5). After asphyxia, the uterus horns were opened, and pups were removed and rapidly dried of delivery fluids and their umbilical cordons ligated. Pups were stimulated to breathe by clearing their fluids and performing tactile stimulation in the oral region. Then, they were placed for recovery under a heating lamp and given to a surrogate mother. Time of asphyxia was measured as the time elapsed from the hysterectomy up to the recovery from the water bath. The overall mortality rate was similar to that previously reported (Loidl et al., 2000), 40% for PA at 37°C and 0% for HYP. To avoid the influence of hormonal variations resulting from the female estrous cycle, only male rats were included in this study.

NOS Activity

Animals 21, 30, 60, and 90 days old were sacrificed by decapitation. Cervical and lumbar regions of the spinal cord were removed, frozen, and stored at –80°C until used. Ca^{2+}-dependent and -independent NOS activity was determined by measuring the amount of enzyme present by monitoring the conversion of L-(U-^{14}C)arginine into L-(U-^{14}C)citruline. Tissues were homogenized (1:3 w/v) at 4°C in HOSF buffer [20 mM HEPES, 0.2 M sucrose, 5 mM dithiothreitol (DTT), 1 mM EDTA, 10 μg/ml soyabean trypsin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4]. Homogenates were ultrasonicated and centrifuged for 30 min at 15,000 rpm (4°C) and supernatants collected. To use similar amount of proteins among samples, proteins concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. Then, samples of supernatants were incubated for 20 min at

37°C with 20 μM L-(U-^{14}C)arginine in incubation buffer (50 mM KH_2PO_4, 0.2 mM $CaCl_2$, 50 mM L-valina, 1 mM L-citrulina, 1.5 mM DTT, and 1 mM $MgCl_2$, pH 7.4, with 2 N KOH, plus 0.75 μg/ml NADPH, 2.15 μg/ml FMN, 3.74 μg/ml FAD, and 1.412 μg/ml BH_4). Reaction was stopped with Dowex-50 WX8-400 ionic interchange resin, and the resultant supernatant with L-(U-^{14}C)citruline was quantified. Upper samples described were considered as controls. To determine the activity of NOS Ca^{2+}-dependent enzyme isoform, we calculated the difference between the amount of (^{14}C)citruline produced in control samples and in samples processed in incubation buffer plus 2 mM ethylene glycol bis(β-aminoethyl ester)-N-N^1-tetraacetic acid (EGTA). To determine the activity of the NOS Ca^{2+}-independent isoform, the difference between the amount of (^{14}C)citruline produced in samples containing 2 mM EGTA and samples processed with incubation buffer plus 2 mM EGTA and 2 mM L-NAME was calculated.

Immunohistochemistry

PND60 animals were deeply anesthetized with pentobarbital (300 mg/kg body weight) and transcardially perfused with 0.9% saline solution followed by fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Spinal cords were removed after dorsal laminectomy and immersed in the fixative solution at 0–4°C for an additional 2 hr. After cryoprotection with 30% sucrose in phosphate-buffered saline (PBS) 0–4°C overnight, tissues were frozen in powered dry ice and stored at –80°C. Coronal cryostat sections of the cervical 5 and lumbar 5 region of the spinal cord (thickness 18 μm) were mounted onto gelatin-coated slides (2.5% gelatin, 1% Elmer's glue), air dried at room temperature, and stored at –80°C until use. For immunohistochemical assay, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Then, sections were incubated with blocking solution containing 10% normal serum goat in saline phosphate buffer, pH 7.4, for 1 hr, and nNOS, iNOS, and nitrotyrosine were detected using polyclonal rabbit antibodies (at dilutions of 1:1,000, 1:1,000, and 1:2,000, respectively) overnight at 0–4°C. Specificity of the three antibodies has been previously demonstrated (Uttenthal et al., 1998; Rodrigo et al., 2001; Alonso et al., 2002) and was corroborated here in adjacent sections by the omission of primary antibodies. Visualization of the immunoreactivity was performed with goat anti-rabbit IgG (1:100; Sigma, St. Louis, MO), developed with PAP assay (Sigma), and observed with 0.05% diaminobenzidine (DAB; Sigma) with 0.01% hydrogen peroxide in Tris buffer as a brown product. Sections were dehydrated with an increasing alcohol series, cleared in xylol, and coverslipped.

NADPH-d Histochemistry

The assay was performed as previously described (Dorfman et al., 2004). Briefly, coronal cryostat sections (thickness 18 μm) of the cervical 5 and lumbar 5 region of the spinal cord of PND60 trasncardially fixed rats were mounted onto gelatin-coated slides and incubated with 0.1% β-NADPH (Sigma) and 0.02% nitroblue tetrazolium (Sigma) diluted in 0.1 M phosphate buffer (PB), pH 7.4, with 1% Triton X-100, 1 hr at 37°C. For the negative control, β-NADPH was omitted in adjacent sections. NADPH-d reactivity was detected as a blue precipitate. Then, sections were washed in PB, dehydrated with 2-min washes in an increasing alcohol series, quickly cleared by xylol, and coverslipped.

SDS-PAGE and Western Blotting

PND60 rats were sacrificed by decapitation. Cervical and lumbar regions of the spinal cord were removed, frozen, and stored at –80°C until use. Tissues were homogenized (1:3, w/v) in HEPES buffer [20 mM *N*-(2-hydroxethyl)piperazine-*N*′-(2-ethanesulfonic acid)], pH 7.2, containing 0.2 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM DTT, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 2 μg/ml pepstatin, and 0.1 mM PMSF. All procedures were carried out at 4°C. Homogenates were centrifuged for 1 hr at 105,000*g*, and the supernatant was collected. To use similar amounts of proteins among samples (25 μg), proteins concentration was determined by the Bradford method (Bradford 1976), using bovine serum albumin as standard. Then, samples of supernatants were mixed 1:1 with sample buffer [10 ml Tris-HCl 0.5 M, pH 6.8, 16 ml sodium dodecyl sulfate (SDS) 10% (w/v), 8 ml glycerol, 2 ml 2-mercaptoethanol, and 0.2 ml bromophenol blue 0.1% (w/v)] and heated for 3 min at 95°C. Samples were run on an SDS-polyacrylamide gel electrophoresis (10% running gel with 3.5% stacking gel), with 0.25 M Tris-glycine, pH 8.3, as the electrolyte buffer, in a Bio-Rad Mini-Protein II (Bio-Rad, Madrid, Spain). Kaleidoscope Prestained Standards (Bio-Rad) were used as molecular weight markers. For Western blot analysis, proteins were transferred at 1.5 mA/cm^2 for 1 hr onto 0.2-mm polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA) by a semidry transfer (Bio-Rad). For nitrotyrosine protein species identification, membranes were incubated overnight at 4°C with antinitrotyrosine antibody (1:1,000). To normalize the results, monoclonal IgG antiactin (1:10,000; Sigma) was used in the same membranes. To reveal immunoreactivity, membranes were incubated with anti-rabbit chemiluminescence-labelled IgG (GE Biosciences, Miami, FL) and exposed to X-ray blue films (CEA, Strängnäs, Sweden). Revealed films were scanned with a computer-assisted densitometer (Bio-Rad GS-800), and immunoblot optical density was quantificated in Image-Quant 5.2 software (Molecular Dynamics, Sunnyvale, CA).

Image Analysis

Relative optical density (ROD) and cellular area (CA) were analyzed with an Olympus BH2 microscope attached to a video camera (CCD Sony-XC77) and coupled to a Macintosh computer equipped with a video card (Data Translation). Reactivity was analyzed in NIH Image (developed by Wayne Rasband, 1995, NIH, Research Services Branch, NIMH, Bethesda, MD). ROD was calculated using a gray scale of 255 gray levels. ROD levels of NADPH-d were considered as NADPH-d activity at NOS neurons according to Young et al. (1997). Because immunohistochemistry, NADPH-d histochemistry, and Western blotting are semiquantitative techniques, ROD values were expressed as percentage with respect to the CTL group, considering its ROD as 100%. All images

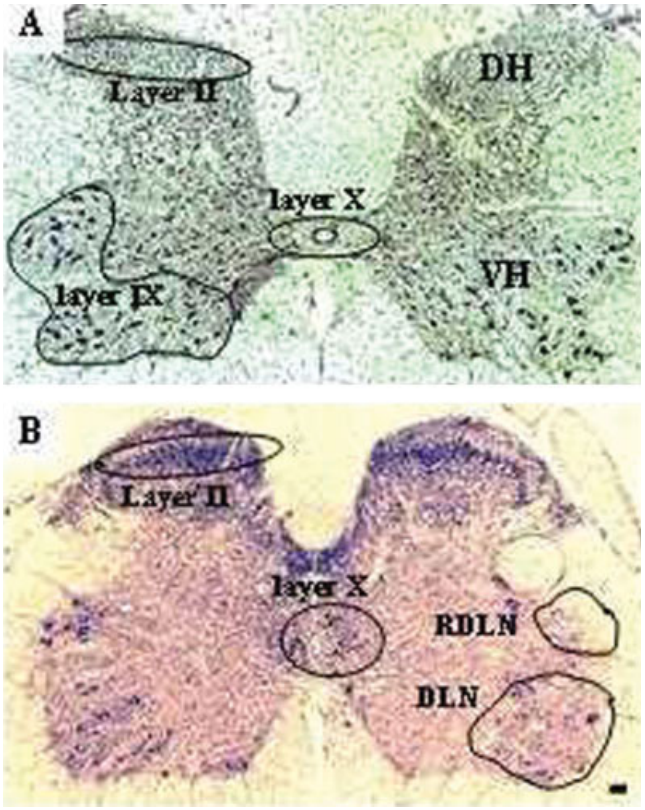

Fig. 1. Distribution of Rexed's layers at cervical and lumbar spinal cord. **A:** Rexed's layer II, layer IX (motoneurons localization), and layer X in a representative image of a CTL cervical 5 spinal cord stained with Nissl. **B:** Representative image of a CTL lumbar 5 spinal cord stained with NADPH-d reactivity. DH, dorsal horn; VH, ventral horn; RDLN, retrodorsal nucleus; DLN, dorsal nucleus. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were taken on the same day under the same light to avoid external variations. To enhance contrast and sharpness, post-processing of images in Adobe Photoshop CS was conducted using identical values for each image. Anatomical identification of the spinal cord regions was carried out according to Rexed's nomenclature using the Nissl staining technique performed with cresyl violet (0.5%; Sigma) according to standard protocols (Fig. 1). All sections were anatomically matched among animals before assays.

Behavioral Testing

One week prior to sacrifice, 60-day-old healthy rats (250–280 g corporal weight) underwent behavioral testing. According to Bures et al. (1976), muscular tone, motor skills, grasping reflex, and placing reactions were studied. 1) Forelimbs muscular tone was evaluated by inducing animals to hang from a metal grid (2 mm wire diameter, 20 × 20 mm holes). The time for which rats remained hanging from the grid by their forelimbs (elapsing between the moment when the grid was turned 180° and that when rats fell onto a smooth mattress) was scored. Each animal was assessed in five similar trials, with 15 min for recovery between trials. 2) Motor function was evaluated based on the skill of rats walking on a horizontally suspended wooden bar (2 cm diameter and 30 cm long) elevated 40 cm from the floor. The time for which animals were able to walk and remain standing on the bar was scored. 3) Grasping reflex as a measure of fine motor control was determined by the ability of the rats to grip wire bars separately with their four limbs. The palm was touched with a wire bar (1 mm diameter) and the toe flexion ability around the bar was evaluated. 4) To evaluate placement reactions, the examiner suspended the rat in the air, with the limbs separately approaching to a table in order to touch it with the back of the limb and to evaluate the intention to put the palm of the limb over the table. All tests were evaluated by two examiners (one of them blind to the treatment).

Statistical Analysis

Values are expressed as mean ± SD. At least two similar separate experiments were evaluated in all cases. Twelve sections of each animal were analyzed. Spinal cord analysis was bilaterally performed. Results were evaluated using one-way ANOVA, and comparisons among groups were made by Fisher, Scheffe, and Bonferroni-Dunn tests. Differences were considered significant at $P < 0.05$.

RESULTS

NOS Activity Induction by Perinatal Hypoxia

The time course of NOS activity at cervical and lumbar spinal cord of perinatal asphyctic animals at 21, 30, 60, and 90 days old was assayed. When the cervical region was studied, a significant increase of NOS Ca^{2+}-independent activity at PND60 and PND90 of PA20 rats was observed (78% ± 3% and 105% ± 7%, respectively) compared with the age-matched CTL group (Fig. 2A). NOS Ca^{2+}-independent activity at PND60 and PND90 in rats of the PA19 and HYP groups did not show significant alterations compared with the age-matched CTL group (Fig. 2A). In addition, in each experimental situation, animals showed a significant 81% ± 5% increment in the activity of NOS Ca^{2+}-independent isoform at PND60 and PND90 with respect to PND30 and PND21 (Fig. 2A). On the other hand, the NOS Ca^{2+}-dependent isoform showed pattern similar to that of the NOS Ca^{2+}-independent isoform, with 98% ± 8% significantly increased activity at PND60 and PND90 in PA20 rats with respect to younger groups and 103% ± 10% with respect to the age-matched CTL (Fig. 2B). At lumbar spinal cord, NOS Ca^{2+}-dependent and -independent isoforms did not show significantly altered activities according to the age and to perinatal treatments (not shown).

NOS Induction by Perinatal Hypoxia

Because we observed a significant NOS activity increment at PND60, we focused next on evaluating NADPH-d reactivity and nNOS and iNOS localization and expression in 60-day-old perinatally asphyctic rats.

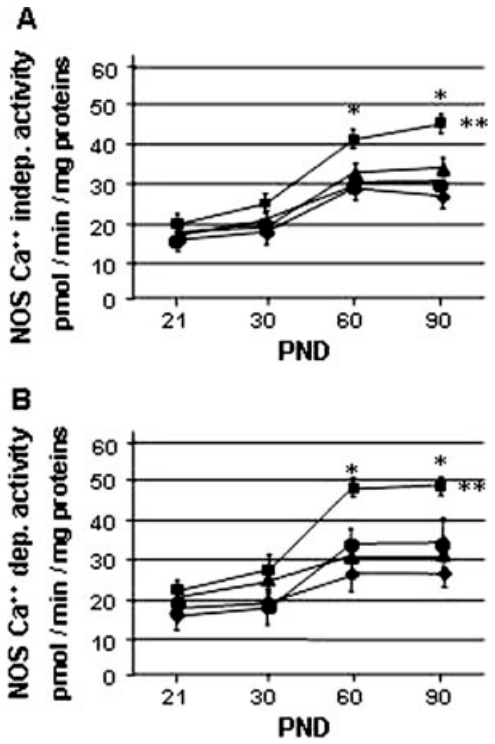

Fig. 2. Increased NOS activity by perinatal asphyxia. NOS Ca^{2+}-independent activity (**A**) and NOS Ca^{2+}-dependent activity (**B**) at cervical 5 spinal cord. Significant increment was observed in PND60 and PND90 rats with respect to younger rats (**$P < 0.05$). In addition, PND60 and PND90 PA20 rats showed a significant NOS activity increment with respect to age-matched CTL, PA19, and HYP rats (*$P < 0.05$). Every point indicates mean ± SD. Lozenges, CTL; squares, PA20; circles, PA19; triangles, HYP.

NADPH-d and iNOS Expression at Motoneurons

We observed NADPH-d reactivity and iNOS localization in cytoplasm of cervical and lumbar spinal motoneurons. Cervical motoneurons showed significantly increased NADPH-d ROD compared with CTL in all perinatal asphyctic groups (Fig. 3A,E,F); however, only PA20 rats showed significant increment in iNOS ROD with respect to the CTL group (Fig. 3B,G,H). Moreover, the cellular area of cervical motoneurons studied by NADPH-d staining showed a significant increment at PA20 in relation to CTL (Fig. 3C,E,F). Both motoneuron nuclei of the lumbar spinal cord (retrodorsolateral nucleus and dorsolateral nucleus), involved in hindlimb innervation, showed a similar significant decrease in NADPH-d ROD at HYP with respect to CTL, whereas PA20 and PA19 were unchanged (Fig. 3D). At lumbar motoneurons, iNOS immunoreactivity was not altered among groups.

NADPH-d and nNOS Expression in Layer X

NADPH-d reactivity and nNOS localization were detected in soma and dendrites of neurons from layer X at cervical and lumbar spinal cord in all analyzed groups.

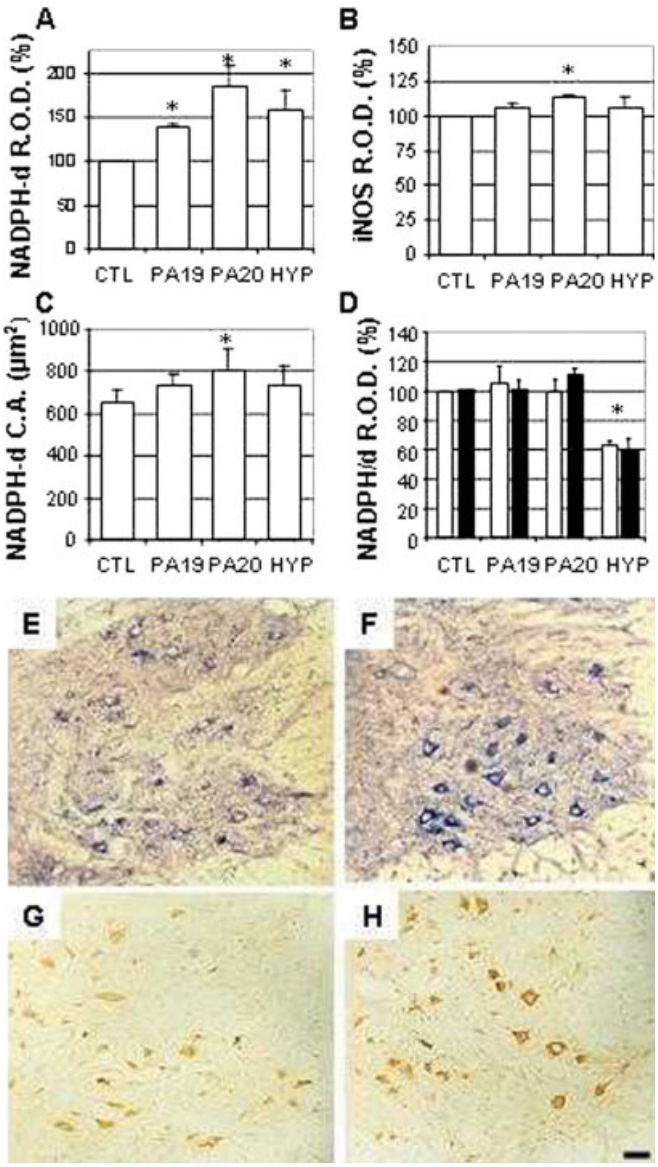

Fig. 3. Increment in NADPH-d reactivity and iNOS immunoreactivity at motoneurons induced by perinatal asphyxia. **A:** Significant increase in cervical NADPH-d ROD at PA19, PA20, and HYP with respect to CTL (PA19: 38% ± 5%, $P = 0.01$, PA20: 85% ± 22%, $P < 0.0001$, HYP: 60% ± 20%, $P = 0.005$). **B:** Significant increase in the iNOS ROD at PA20 with respect to CTL (PA20: 17% ± 1%, $P = 0.003$). **C:** Significant increase in NADPH-d C.A. at PA20 with respect to CTL (CTL: 647 ± 68 μm^2, PA19: 733 ± 40 μm^2, PA20: 804 ± 59 μm^2, $P = 0.0004$, HYP: 735 ± 69 μm^2). **D:** Significant decrease in lumbar NADPH-d ROD with HYP with respect to CTL in DLN (open bars; HYP: 61% ± 5%, $P = 0.01$) and RDLN (solid bars; HYP: 65% ± 2%, $P = 0.003$). *Significant with respect to CTL. Bars are mean ± SD. CA, cellular area; ROD, relative optical density. **E,F:** Representative images of NADPH-d staining in motoneurons of cervical spinal cord of CTL and PA20 respectively, showing ROD and CA increases in PA20 rats. **G,H:** Representative images of iNOS immunoreactivity at motoneurons of cervical spinal cord of CTL and PA20 animals, respectively. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

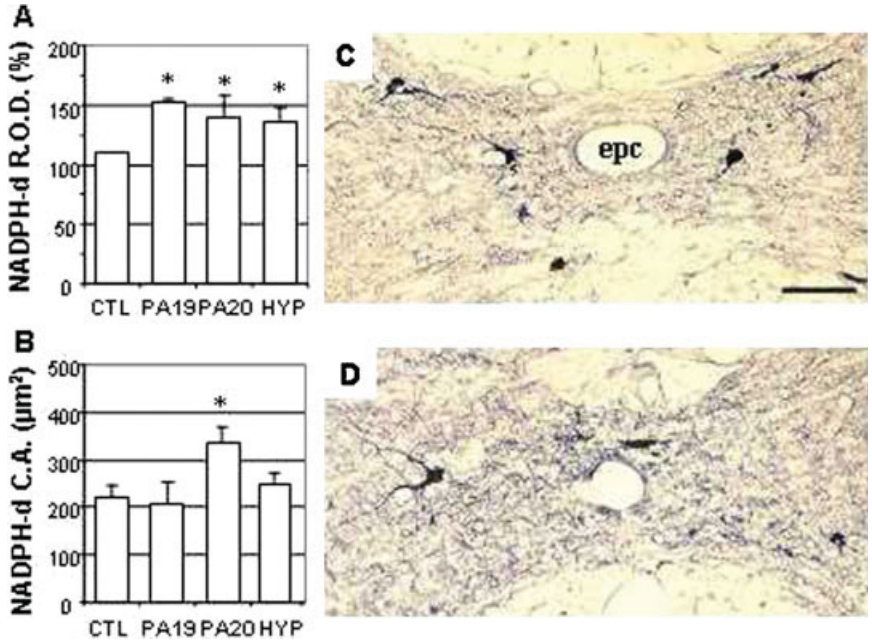

Fig. 4. Increment in NADPH-d reactivity in neurons from layer X of the cervical spinal cord. **A:** Significant increase in the NADPH-d ROD at PA19, PA20, and HYP with respect to CTL (PA19: 50% ± 2%, $P = 0.01$, PA20: 36% ± 13%, $P = 0.001$, HYP: 34% ± 8%, $P = 0.02$). **B:** Significant increase in the NADPH-d CA at PA20 with respect to CTL (CTL: 209 ± 31 μm^2, PA19: 253 ± 48 μm^2, PA20: 275 ± 21 μm^2, $P < 0.0001$, HYP: 253 ± 18 μm^2). CA, cellular area; ROD, relative optical density. *Significant with respect to CTL. Bars represent mean ± SD. **C,D:** Representative images of NADPH-d staining in layer X of the spinal cord of CTL and PA20, respectively, showing CA increase at PA20 rats. epc, Ependimal canal. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cervical cells showed a significant increase ($P < 0.01$) in the ROD of NADPH-d in PA19, PA20, and HYP groups (Fig. 4A); however, no changes were observed in the ROD of nNOS. Moreover, a significant increase in the cellular area ($P < 0.05$) was detected by NADPH-d in PA20 with respect to the CTL group (Fig. 4B–D), whereas no significant differences were observed in PA19 and HYP rats.

NADPH-d and nNOS Expression in Dorsal Horns

NADPH-d reactivity and nNOS immunoreactivity were observed in the interneurons localized in the dorsal horns (layer II) of cervical and lumbar spinal cord. NADPH-d histochemistry detected a decreased cellular area at cervical neurons of HYP with respect to CTL (CTL: 65.5 ± 3.6 μm^2, HYP: 53.0 ± 2.7 μm^2, PA19: 59.5 ± 4.3 μm^2, PA20: 59.1 ± 3.1 μm^2, $P < 0.01$), whereas nNOS immunoreactivity was not altered among groups. On the other hand, neither NADPH-d reactivity nor nNOS immunoreactivity of the cells localized at the lumbar dorsal horns was changed among the groups.

Induction of Protein Nitration by Perinatal Hypoxia

With the observed NOS alterations at PND60, we studied nitrotyrosine expression and localization at this age. Light-specific immunoreactivity was observed at motoneurons of the cervical and lumbar spinal cord of CTL rats. Granular deposits of reaction product were observed in the cytoplasmic, perinuclear, and nuclear areas of all analyzed groups (Fig. 5A,B). Cervical motoneurons of PA20 rats showed a significant increase with respect to CTL in cellular area and ROD (Fig. 5C,D, respectively). However, lumbar motoneurons did not show nitrotyrosine-immunodetectable alterations among the groups. Two nitrotyrosine protein species of 140 kDa and 75 kDa were detected in the immunoblot of cervical and lumbar spinal cord of all analyzed animals. The cervical region of PA20 and HYP rats showed a significant increase with respect to CTL in both proteins (Fig. 5E,F), whereas the lumbar nitrated proteins were not altered among the groups.

Neuroprotective Effect of Hypothermia

With assay after perinatal asphyxia, hypothermia was able to prevent alterations at almost all regions studied. HPA cervical motoneurons did not show alterations either at NADPH-d ROD and CA or at iNOS ROD with respect to CTL. In addition, nitrotyrosine ROD did not show differences between HPA and CTL. However, lumbar motoneurons showed significantly (29%) decreased NADPH-d ROD at HPA compared with CTL (Table I).

Neurons of the cervical spinal cord localized at layer X did not show ROD alterations between HPA and CTL studied by NADPH-d staining and nNOS

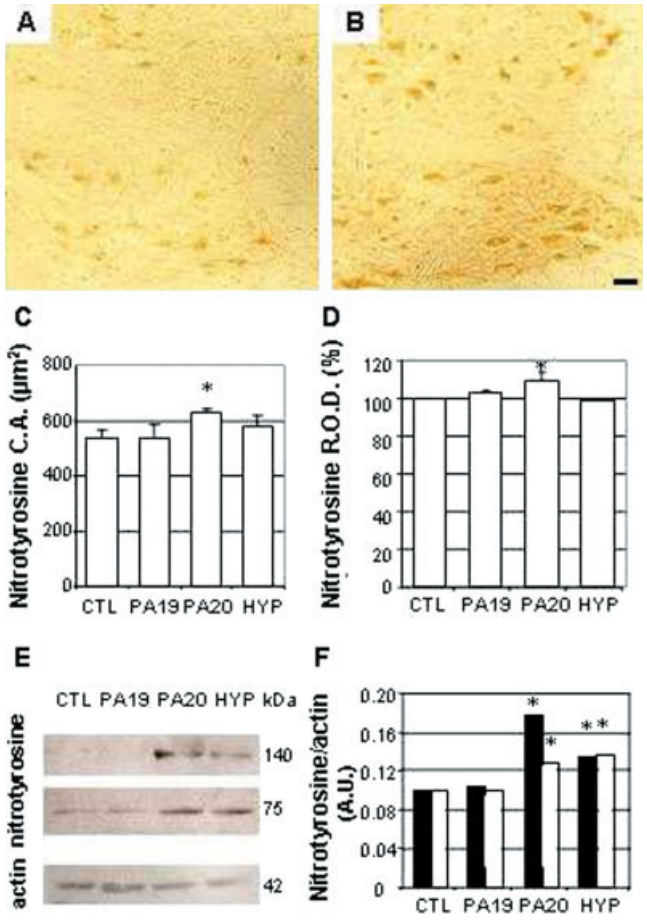

Fig. 5. Nitrotyrosine induction in cervical motoneurons by perinatal asphyxia. **A,B:** Representative images of nitrotyrosine immunoreactivity at motoneurons of CTL and PA20 animals, respectively. **C,D:** Significant increase in the nitrotyrosine-immunoreactive CA and ROD in motoneurons of PA20 with respect to CTL (CA = CTL: 541 ± 26 μm², PA19: 539 ± 48 μm², PA20: 626 ± 20 μm², $P = 0.0002$, HYP: 577 ± 41 μm²; ROD = CTL: 100%, PA19: 102 ± 2, PA20: 111 ± 4, HYP: 98 ± 1). *$P < 0.05$ with respect to CTL. Bars represent mean ± SD. CA, cellular area; ROD, relative optical density. **E:** Western blot of nitrotyrosine in cervical spinal cord showing two different species of nitrotyrosine proteins. Actin was probed on the same membrane with antiactin IgG. **F:** Western blot quantification shows induction of the two species of nitrotyrosine proteins levels by 20 min of perinatal asphyxia (normalized by actin). AU, arbitrary units. Solid bars, 140-kDa nitrotyrosine protein; open bars, 75-kDa nitrotyrosine protein. Bars indicate mean ± SD of three separate experiments. *$P < 0.05$ with respect to CTL. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

immunohistochemistry. However, cellular area studied by NADPH-d was significantly 40% increased in the HPA group with respect to CTL. On the other hand, lumbar HPA neurons of layer X showed no NADPH-d and nNOS ROD alterations related to CTL (Table I). Finally, hypothermia was also able to prevent alterations at neurons of the cervical and lumbar dorsal horns, showing similar values between HPA and CTL (Table I).

TABLE I. Neuroprotective Effect of Hypothermia[†]

	CTL	HPA
Cervical region		
Motoneurons		
NADPH-d ROD	100	98 ± 2
NADPH-d CA	650 ± 52	638 ± 45
iNOS ROD	100	101 ± 4
Nitrotyrosine ROD	100	103 ± 2
Layer X		
NADPH-d ROD	100	99 ± 3
NADPH-d CA	219 ± 20	309 ± 35★
nNOS ROD	100	97 ± 4
Dorsal horns		
NADPH-d ROD	100	102 ± 3
NADPH-d CA	66.3 ± 2.1	64.4 ± 3.6
nNOS ROD	100	103 ± 5
Lumbar region		
Motoneurons		
NADPH-d ROD	100	71 ± 4★★
NADPH-d CA	744 ± 39	728 ± 35
iNOS ROD	100	99 ± 2
Nitrotyrosine ROD	100	102 ± 3
Layer X		
NADPH-d ROD	100	102 ± 3
NADPH-d CA	207 ± 14	217 ± 23
nNOS ROD	100	98 ± 3
Dorsal horns		
NADPH-d ROD	100	99 ± 2
NADPH-d CA	69 ± 5.8	72 ± 6.3
nNOS ROD	100	101 ± 3

[†]HPA ROD (relative optical density) values are expressed as percentage with respect to the CTL group, considering CTL ROD as 100%. CA (cellular area) is expressed in μm².
★$P < 0.05$ with respect to CTL.
★★$P = 0.0032$ with respect to CTL.

Altered Muscular Tone by Hypoxic Condition

To evaluate whether any correlation exists between the observed cellular alteration and spastic symptoms, behavioral tests were performed. In the muscular tone testing, we observed that PA20 and HYP animals were able to remain hanging from the grid for a significantly shorter time than CTL, PA19, and HPA rats. (CTL: 4.3 ± 0.4 sec, PA19: 4.1 ± 0.3 sec, PA20: 1.7 ± 0.2 sec, $P < 0.0001$, HYP: 1.5 ± 0.3 sec, $P < 0.0001$, HPA: 3.9 ± 0.2 sec). No other behavioral abnormalities were observed among groups, including the studies of motor skills, grasping reflexes, and placing reactions.

DISCUSSION

Here we show that perinatal asphyxia produces alterations in both inducible and constitutive NOS isoforms, predominantly at the cervical region of the spinal cord. We describe a significant increase in NOS Ca^{2+}-dependent and -independent activity according to the age, whereas hypoxia-ischemia was shown to increase this function. In addition, this study provides evidence on the neuroprotective effect of hypothermia over the spinal cord. We observed that 20 min of PA at 37°C

significantly increased NOS activity, whereas 19 min of PA or 20 min of PA in hypothermia did not show alterations in this activity. In addition, low levels of NOS activity were detected at PND21 and PND30, with a significant increase at PND60. Then, we explored NOS localization and tissue expression in 60-day-old animals exposed to perinatal asphyxia. Colocalization of NADPH-d staining with areas of NOS reactivity has been well studied, indirectly showing NOS activity in the central nervous system (Dawson et al., 1991; Hope et al., 1991). Here, we observed that asphyxia during birth was able to increase NADPH-d ROD in cervical motoneurons and that this activity may correspond to an increase in iNOS isoform expression. Similar alterations were previously reported in the brain of animals subjected to oxygen deprivation, resulting in an increase of nNOS, iNOS, and nitrotyrosine expression (Moro et al., 1998; Fernández et al., 2003; van den Tweel et al., 2005a). We observed that 20 min of PA induced iNOS alterations, whereas 19 min of PA or PA under hypothermia were not sufficient to induce significant differences at cervical motoneurons. On the other hand, lumbar motoneurons of rats subjected to hypothermia during PA showed a pattern different from the cervical one. A decrease at NADPH-d ROD induced by 20 min of perinatal asphyxia, without altering iNOS, reflects a decrease in NOS activity without changes in its expression. As well as motoneurons, cervical neurons at layer X showed an increase of NADPH-d ROD by PA without altering nNOS expression, whereas lumbar neurons of this area were not affected.

The protective or neurotoxic character of NO depends on its synthesized amount and on its oxidative state. During postnatal development, NO shows predominantly a constitutive origin, which turns into an inducible origin with aging (Uttenthal et al., 1998). When synthesized at low concentration, NO appears to play a protective role in oxidative stress, acting as an antioxidant scavenger (Chiueh and Ranhala, 1999). Under pathological conditions such as acute global hypoxia-ischemia, NO is overproduced as an effort to reestablish normal blood flow, but it may also trigger free radical production resulting in a protein nitration system that alters brain function (Bartosz, 1996; Moro et al., 1998; Alonso et al., 2002). In our model of hypoxia-ischemia, overactivation of the nitrergic system is triggered long after asphyxia (60 days later); however, a long-term plasticity also results in protein nitration. These findings are supported by the fact that KO mice lacking nNOS and iNOS ($nNOS^{-/-}$ and $iNOS^{-/-}$) are protected from brain damage induced by neonatal hypoxia-ischemia (Ferriero et al., 1996). In addition, pharmacological inhibition of nNOS and iNOS activity also prevents brain damage induced by neonatal hypoxia-ischemia (van den Tweel et al., 2002, 2005b). Consequently, perinatal asphyxia inducing long-term NOS alterations is involved in the increase of oxidative stress.

Here we observed a significant increase in nitrotyrosine immunoreactivity at cervical motoneurons colocalizing with iNOS and NADPH-d expression. In accordance, we observed a significant increase in two species of nitrated proteins (140 and 75 kDa) at cervical spinal cord of PA20 rats. These observations are consistent with previous reports describing an increase in other species of nitrated proteins (30, 38, 45, and 52 kDa) in brains of young rats that suffered hypoxia during delivery (Fernández et al., 2003; van der Tweel et al., 2005a). This evidence makes it reasonable to think about regional specificities for protein nitration induced by hypoxia. It is also important to note that lumbar motoneurons that did not show NOS alterations by PA did not present nitrotyrosine changes.

Because significant alterations in the nitric system of spinal cord induced by PA were observed, we tried to prevent or reduce this injury with a drastic hypothermic treatment. We observed that placing PA20 pups on ice for 5 min (HPA group) prevented alterations at cervical motoneurons and at neurons around the ependyma. In accordance, it has been described that hypothermic treatment is able to mitigate spasticity symptoms (Katz et al., 2004; Gisselsson et al., 2005) by decreasing the production of hydroxyl radical (Hashimoto et al., 2003), neuronal metabolic activity (Ekimova, 2003), and neuronal actin polymerization rate (Gisselsson et al., 2005). Core body temperature of mammals decreases during hibernation to 15°C, with 10% of normal oxygen requirements and a natural state of reduced metabolism (Carey et al., 2003). Probably, the decreased temperature in our model that is representative of clinical situations of PA triggers a response similar to hibernation, limiting the cellular oxygen requirements and giving cells better resistance to a hypoxic environment. Insofar as lumbar motoneurons of HPA showed a loss of NOS activity, the induction of hypothermia by pharmacologic agents such as hydrogen sulfide (Blackstone et al., 2005) or N-acetylcysteine (Cakir et al., 2003) directly in the ischemic region could be a promising strategy for resolving anoxic injury.

To evaluate whether lifetime hypertonic motor deficits that are generally observed at spasticity (Strata et al., 2004) could be related to the alterations induced by PA at cervical motoneurons, behavioral tests to study the influence of hypoxia-ischemia on motor function were used. PA20 animals showed normal walking but showed altered capacities when motor potency was evaluated. This group was shown to resist for a shorter period of time than CTL in hanging from their forelimbs. However, although hypothermia has been able to reduce almost all pathological changes in immunocytochemical and biochemical assays, it was not sufficient to ameliorate alteration of muscle tone in the HYP group. Nitrergic system homeostasis is necessary to the maintenance of correct motor function; however, other pathways, such as the GABAergic and glutamatergic systems, are involved in the development of this skill (Ma et al., 1992; Deply et al., 2008). Although both of them are altered by the asphyctic process (Zhang et al., 2008), their action is also influenced by temperature (Nishi et al., 2007; Zhang et al., 2008), so hypothermia during asphyxia could be affecting muscle

tone in a way different from normothermia. Hypothermia during asphyxia could be enough to reduce nitrergic system changes; however, the reversion of motor function should be more complex. Given that motoneurons at cervical spinal cord innervate forelimb muscles (Ito et al., 2008), the observed motor behavioral abnormality could be partially attributed to the altered cervical motoneurons. On the other hand, PA19 rats did not show significant behavioral differences compared with CTL. The 1 min of difference at PA19 with respect to PA20 could represent the time window during which hypoxia is able to generate spastic motor sequelae in rats. In accordance with these findings, the work of Strata et al. (2004) showed similar motor abnormalities in perinatally asphyctic rats. Spastic motor sequelae were reported in humans with perinatal asphyxia, but no correlation was found between the severity of the perinatal complication and the grade of the sequelae (Michaelis et al., 1980; Krageloh-Mann et al., 2002).

The present data support the idea that the cervical spinal cord is highly sensitive to hypoxia-ischemia resulting from nitric system alteration contributing to neuronal dysfunction and motor sequelae such as spasticity produced by perinatal asphyxia. The iNOS, nNOS, and nitrotyrosine increments observed at PA20 suggest that oxidative stress may be involved in the neurodegenerative mechanisms of spasticity caused by hypoxia-ischemia in a time-course-dependent manner. In addition, hypothermia seems to be a promising treatment for preventinbg or attenuating sequelae produced in the spinal cord by severe PA. Better knowledge of the physiological mechanisms involved in hypothermia could allow development of new neuroprotective therapeutic strategies to ameliorate or prevent spasticity.

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