Hypoxia-Induced Cellular and Vascular Changes in the Nucleus Tractus Solitarius and Ventrolateral Medulla

Charanjit Kaur, PhD, Sivakumar Viswanathan, PhD, and Eng-Ang Ling, DSc, PhD, BSc

Abstract

Major changes in arterial pressure, autonomic, and respiratory activity occur in response to hypoxia. We analyzed structural damage and increased vascular permeability in the ventrolateral medulla and nucleus tractus solitarius, which control autonomic, respiratory, and cardiovascular functions in adult Wistar rats subjected to 2 hours of hypoxia (7% oxygen + 93% nitrogen) for up to 14 days after hypoxic exposure. Brainstem tissue levels of vascular endothelial growth factor (VEGF), nitric oxide (NO), and glutamate were significantly increased over control levels after hypoxic injury. By electron microscopy, swollen neurons and dendrites, degenerating axons, disrupted myelin sheaths, and swollen astrocyte processes were observed in the nucleus tractus solitarius and ventrolateral medulla. Leakage of intravenously administered horseradish peroxidase was observed through vascular walls in hypoxic rats. These results suggest that increased VEGF and NO production in hypoxia resulted in increased vascular permeability, which, along with increased levels of glutamate, may have induced structural alterations of the neurons, dendrites, and axons. Administration of the antioxidant neurohormone melatonin (10 mg/kg) before and after the hypoxia reduced VEGF, NO, and glutamate levels and improved ultrastructural abnormalities induced by hypoxia exposure, suggesting that it may have a therapeutic potential in reducing hypoxia-associated brainstem damage.

Key Words: Glutamate, Hypoxia, Melatonin, Nitric oxide, Nucleus tractus solitarius, Vascular endothelial growth factor, Ventrolateral medulla.

INTRODUCTION

Catecholaminergic neurons in the ventrolateral medulla (VLM) and nucleus tractus solitarius (NTS) regulate respiratory and cardiovascular function. The NTS receives afferent projections from arterial baroreceptors, carotid chemoreceptors, and cardiopulmonary receptors (1). Neurons in the VLM are involved in regulating the sympathetic outflow to the cardiovascular system (2) and thus are involved in the changes in arterial pressure (3–5), autonomic, and respiratory activity associated with hypoxia (6–9). In addition to being the sites of integration of chemosensory inputs, NTS neurons may also be direct targets for modulatory effects of hypoxia (10–12). Activation of neurons in the NTS and VLM, as indicated by c-fos (13) and tyrosine hydroxylase (14) expression, occurs after hypoxic injury. Some neurons excited by hypoxia in the NTS project directly to the rostral VLM (15), and neuron excitation in the VLM also occurs as a direct effect of hypoxia (16). Hypoxia may impair neuronal excitability and synaptic transmission (17).

Alterations in brain function due to neuronal death can occur within minutes after hypoxic/ischemic challenges (18). Hypoxia is associated with many pathologic conditions including brain infarct, cardiac arrest, partial drowning, suffocation, and respiratory arrest. Hypoxia as a result of acute heart failure (19, 20), respiratory failure (21), or near-drowning (22) causes brainstem injury.

Hypoxia causes depolarization of the resting membrane potential in neurons (23), which may lead to increased firing patterns resulting in excitotoxicity via excess glutamate release (24). Indeed, excitotoxicity has been considered to be a major mechanism of cell death in hypoxic injuries (23). Activation of glutamate receptors, particularly N-methyl-D-aspartate subtype 1 (NR1) and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) after ischemic or hypoxic insults may be a triggering mechanism for neuronal death (25–28). In addition to excitotoxicity, factors such as increased production of nitric oxide (NO) and enhanced vascular permeability may be pivotal in the pathophysiology of cellular injury. Such factors have not been analyzed in the brainstem in response to hypoxia.

Because biochemical and morphologic alterations after hypoxic injury in the neurons in the NTS and VLM may be central to respiratory and cardiovascular alterations in hypoxic injury, we examined the expression of hypoxia-inducible factor-1α (HIF-1α), which plays a major role in a wide variety of responses to hypoxia (29) and regulates transcription of hypoxia-responsive genes, such as vascular endothelial growth factor (VEGF) (30). Because VEGF is upregulated in hypoxia and also increases vascular permeability, its expression was examined in the NTS and VLM. Increased vascular permeability was then assessed using intravenous horseradish peroxidase (HRP). We also examined the changes in NO and glutamate levels and the expression of NR1 and AMPA glutamate 2/3 (GluR2/3) receptors in these areas to determine whether they may be involved in the structural alterations. Because melatonin, a neurohormone and an antioxidant, reduces NO and VEGF production...
(31–33), its effect on these parameters was examined in the hypoxic NTS and VLM.

**MATERIALS AND METHODS**

**Animals**

Adult Wistar rats weighing 200 g each (n = 98) were exposed to hypoxia by placing them in a chamber (Model MCO 18M; Sanyo Biomedical Electrical Co, Ltd, Tokyo, Japan) filled with gas mixture of 7% oxygen and 93% nitrogen for 2 hours. They were then allowed to recover under normoxic conditions for 3 or 24 hours or 3, 7, and 14 days before death. Another group of 34 rats was kept outside the chamber as controls. An additional 20 rats subjected to hypoxia were used to assess effects of melatonin administration. The numbers of rats used for various procedures are given in Table 1. This study was approved by the Institutional Animal Care and Use Committee of National University of Singapore.

**Melatonin Administration**

To assess its effect on VEGF concentration, NO production, and glutamate release in the brainstem, melatonin (Sigma-Aldrich, St Louis, MO) was administered intraperitoneally as previously described (31). Each rat received 3 injections of melatonin: immediately before, immediately after, and at 1 hour after hypoxia exposure. Vascular endothelial growth factor concentration and NO production were determined at 24 hours and at 3 and 7 days (n = 5 rats at each time interval). The values between the hypoxic and hypoxic + melatonin administered rats were compared. Five hypoxia + melatonin administered rats were used for electron microscopy at 24 hours after the administration.

**Real-Time Reverse Transcription–Polymerase Chain Reaction**

Brainstems (extending from the obex to stria medullaris of the fourth ventricle) containing the NTS and VLM were removed from the hypoxia exposed rats at 3 and 24 hours and at 3, 7, and 14 days (n = 5 at each time) and the controls (n = 5). Total RNA was extracted using RNAeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol. The amount of total RNA was quantified with a BioPhotometer (Eppendorf, San Jose, CA).

For reverse transcription, 2 μg of total RNA was combined with 1 μM of Oligo (dT) 15 primer (Invitrogen, Carlsbad, CA). The mixture was heated at 70°C for 5 minutes and then placed on ice. Single-strand complementary DNA was synthesized from the RNA by adding the following reagents (final concentrations): 1 × first-strand buffer, 1 U/μL RNAsin, 25 μM of each dNTP, and 200U M-MLV reverse transcriptase (Promega, Madison, WI). The reaction mixture (20 μL) was incubated at 42°C for 50 minutes; heating the mixture to 95°C for 5 minutes terminated the reaction. The samples were stored at −20°C for polymerase chain reaction (PCR) analysis.

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) was carried out on a Light Cycler 2.0 instrument using a FastStart DNA Master plus SYBR Green I kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany), according to the manufacturer’s instructions. The amplified PCR products were separated on 1.5% agarose gel stained with ethidium bromide and photographed (SYNGENE; Chemi Genius Bio Imaging System, Cambridge, UK). The expression of target genes was measured in triplicate and was normalized to β-actin, as an internal control. Forward and reverse primer sequences for each gene and their corresponding amplicon size are listed in Table 2. Gene expression was quantified using a modification of the 2−ΔΔCt method, as previously described (34).

**Western Blotting**

The brainstem containing the NTS and VLM was removed from the hypoxia exposed rats at 3 and 24 hours and at 3, 7, and 14 days after hypoxia and the controls and was homogenized with tissue protein extraction reagent (Pierce Biotechnology, Inc, Rockford, IL) containing protease inhibitors. Samples of supernatants containing 20 μg of protein were heated to 95°C for 5 minutes and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% gels, in a Mini-Protean 3 apparatus (Bio-Rad, Hercules, CA). Protein bands were electroblotted onto 0.45 μm polyvinylidene difluoride membranes (Bio-Rad) and blocked with 5% (wt/vol) nonfat dried milk. The membranes were

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**TABLE 2. Sequence of Specific Primers**

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<th>Primer</th>
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eNOS, endothelial nitric oxide synthase; HIF-1α, hypoxia-inducible factor-1α; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; VEGF, vascular endothelial growth factor.

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**TABLE 1. Number of Hypoxic and Control Rats Killed at Various Time Points**

<table>
<thead>
<tr>
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EIA, enzyme immunoassay; NO, nitric oxide; RT-PCR, reverse transcription–polymerase chain reaction; VEGF, vascular endothelial growth factor.
then separately incubated with dilutions of antibodies to HIF-1α (Chemicon International, Temecula, CA; 1:500), VEGF (Santa Cruz Biotechnology, Inc, Santa Cruz, CA; 1:1000), neuronal NO synthase (nNOS; BD Transduction Laboratories, Rockville, MD; 1:500), endothelial NO synthase (eNOS; BD Transduction Laboratories; 1:2500), and inducible NO synthase (iNOS; BD Transduction Laboratories; 1:3000) in blocking solution overnight at 4°C. They were then incubated with the HRP-conjugated secondary antibodies (GE Healthcare, Amersham, Bucks, UK). Specific binding was revealed by an enhanced chemiluminescence kit (GE Healthcare) following the manufacturer’s instructions.

**Analysis of VEGF Concentration by Enzyme Immunoassay**

The amount of VEGF (ng/mL) released in the brainstem from control, hypoxic and hypoxia + melatonin rats (n = 5 each time interval) was determined with a Chemikine VEGF Enzyme Immunoassay (EIA) kit (Chemicon). Homogenates as described above for Western blotting were prepared, and

![Graphs showing fold change in expression of HIF-1α, VEGF, eNOS, iNOS, and nNOS](http://jnen.oxfordjournals.org/)

**FIGURE 1.** Reverse transcription–polymerase chain reaction analysis of hypoxia-inducible factor-1α (HIF-1α) (A), vascular endothelial growth factor (VEGF) (B), endothelial nitric oxide synthase (eNOS) (C), inducible nitric oxide synthase (iNOS) (D), and neuronal nitric oxide synthase (nNOS) (E) expression in the brainstem of rats at 3 and 24 hours and at 3, 7, and 14 days after hypoxia exposure and controls (C). Bar graphs represent fold changes quantified by normalization to β-actin as an internal control. Bar, mean ± SD. Differences in the mRNA levels are significant (*, p < 0.01) after hypoxia versus controls.
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(A) Optical density

(B) Optical density

(C) Optical density

(D) Optical density

(E) Optical density
FIGURE 2. Western blotting of hypoxia-inducible factor-1α (HIF-1α; 120 kDa), vascular endothelial growth factor (VEGF; 25 kDa), endothelial nitric oxide synthase (eNOS; 140 kDa), inducible nitric oxide synthase (iNOS; 130 kDa), and neuronal nitric oxide synthase (nNOS; 155 kDa) protein expression in the brainstem of rats at 3 and 24 hours and at 3, 7, and 14 days after hypoxic exposure and control rats (C). Immunoreactive bands are seen in the upper panel. Bar graphs (A, HIF-1α; B, VEGF; C, eNOS; D, iNOS; and E, nNOS) show significant changes in optical densities after hypoxic exposure (mean ± SD). Level of significance: *, p < 0.01, versus controls.

FIGURE 3. (A–C) Vascular endothelial growth factor (VEGF) (A), nitric oxide (NO) (B), and glutamate (C) concentrations in brainstem of control (C) rats and at 3 and 24 hours and at 3, 7, and 14 days after hypoxia exposure. Data represent mean ± SD. *, p < 0.01 for differences in levels between control and hypoxic rats. (D–F) Reductions in brainstem levels of VEGF (D), NO (E), and glutamate (F) at 24 hours and 3 days after melatonin administration in hypoxic rats. #, p < 0.01.
EIA measurements were performed according to the manufacturer’s protocol.

**Nitric Oxide Colorimetric Assay**

Total amounts of NO in the brainstem from control, hypoxic and hypoxia + melatonin rats (n = 5 each time interval) were assessed by the Griess reaction using a colorimetric assay kit (US Biological, Swampscott, MA) that detects nitrite (NO$_2^-$), a stable reaction product of NO. Brainstem homogenates were prepared as for Western blotting, and nitrite colorimetric assays were performed according to the manufacturer’s protocol.

**FIGURE 4.** Vascular endothelial growth factor (VEGF) immunohistochemistry. (A, C) There is weak expression in branched cells (arrows) in close association with blood vessels (BV) in the nucleus tractus solitarius (NTS) (A) and ventrolateral medulla (VLM) (C) of a control rat. (B, D) The expression of VEGF is enhanced at 3 days after hypoxic exposure when compared with the controls. The stained branched cells are morphologically consistent with astrocytes. (E-G) The VEGF-immunopositive branched cells are identified as astrocytes (arrows) when doubly stained for glial fibrillary acidic protein (GFAP) (E, G; green) and VEGF (F; red) (arrows) in the VLM of a control rat. Colocalized expression of VEGF with GFAP-immunoreactive astrocytes can be seen in G. Scale bars = 10 µm (A-D), 50 µm (E-G).
FIGURE 5. Nitric oxide synthase immunohistochemistry. (A, B) Expression of endothelial nitric oxide synthase (eNOS) in blood vessels (arrows) in the nucleus tractus solitarius (NTS) is minimal in a control rat (A) but is markedly enhanced (arrows) at 3 hours after the hypoxic exposure (B). (C, D) Expression of nNOS in the NTS is very weak in neurons (arrows) in a control rat (C) but is enhanced at 24 hours after the hypoxic exposure (D). (E, F) Inducible nitric oxide synthase (iNOS) expression in the NTS (E) and ventrolateral medulla (F) at 24 hours after hypoxia exposure. Many neurons (arrows) show intense iNOS immunostaining. Scale bars = 50 μm (A–F).
Glutamate Assay

The glutamate concentration in the brainstem from control, hypoxic, and hypoxia + melatonin rats (n = 5 at each time point) was determined using an L-glutamate BioAssay kit (US Biological). Homogenates of the brainstem as described for Western blotting were prepared, and bioassay measurements were performed according to the manufacturer’s protocol.

Immunohistochemistry

Rats exposed to hypoxia at 3 and 24 hours and at 3, 7, and 14 days (n = 3 at each time) and controls (n = 3) and were

FIGURE 6. Nitric oxide synthase colocalization. (A–C) Confocal images showing the distribution of lectin-labeled (A, green) and endothelial nitric oxide synthase (eNOS)–labeled (B, red) blood vessels (arrows) in the nucleus tractus solitarius (NTS) at 24 hours after the hypoxic exposure. Colocalized expression (arrows) is shown in C. (D–G) Confocal images showing the distribution of tyrosine hydroxylase–labeled (D, G; green) and neuronal nitric oxide synthase (nNOS)/inducible nitric oxide synthase (iNOS)–labeled (E, H; red) neurons (arrows) in the NTS at 24 hours after hypoxia exposure. Colocalized labeling of tyrosine hydroxylase stained neurons with nNOS/iNOS (arrows) is detected in F and I. Scale bars = 20 μm (A–I).
FIGURE 7. Ionotropic AMPA GluR2/3 and NR1 immunohistochemistry. (A, B) Neurons (arrows) in the nucleus tractus solitarius (NTS) (A) and ventrolateral medulla (VLM) (B) in a control rat. (D, E) Enhanced expression of GluR2/3 is seen in the neurons (arrows) in the NTS (D) and VLM (E) at 24 hours after hypoxic exposure. (C, F) NR1 expression in the neurons (arrows) in the VLM of a control rat (C) is weak in comparison to its upregulation at 24 hours after hypoxic expression (F). Scale bars = 50 μm (A–F).
anesthetized with 6% pentobarbital and perfused with 2% paraformaldehyde. Brainstems were removed, and 40 μm thick frozen coronal sections were cut and incubated with the anti-VEGF (1:200), anti-nNOS (1:500), anti-eNOS (1:250), and anti-iNOS (1:1000), anti-GluR2/3 (recognizes both GluR2 and GluR3, 1:200; Chemicon), and anti-NR1 (1:200; Chemicon) antibodies diluted in phosphate-buffered saline (PBS) for 16 to 20 hours. Subsequent antibody detection was carried out as described (31). For negative controls, some sections from each group were incubated in a medium omitting the primary antibodies.

**Double Immunofluorescence**

Brainstem sections of 6 rats at 24 hours after hypoxia and 6 controls were cut as above for immunohistochemistry, divided into 2 sets, and rinsed in PBS. Sections from the first set were incubated in a humidified chamber with a mixture of 2 primary antibodies (polyclonal anti-VEGF and monoclonal anti-glial fibrillary acidic protein [GFAP; Chemicon]) or monoclonal anti-nNOS/iNOS (BD Transduction Laboratories) or polyclonal anti-tyrosine hydroxylase (Chemicon International) diluted with PBS (VEGF, 1:200 and GFAP, 1:1000; nNOS/iNOS, 1:200 and tyrosine hydroxylase, 1:200). The sections were then incubated with a mix of Carbocyanine 3 (Cy3)-conjugated goat anti-rabbit immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (1:100; Sigma-Aldrich). After several washes with PBS, the sections were mounted with a fluorescent mounting medium (DAKO Cytomation, Glostrup, Denmark). The second set of sections was incubated at room temperature with primary antibody against polyclonal anti-eNOS (1:200) diluted in PBS. Subsequent antibody detection was carried out with Cy3-conjugated goat anti-rabbit IgG. After washing with PBS, the sections were incubated with FITC-conjugated lectin (Lycopersicon esculentum, 1:100; Sigma-Aldrich), a marker for vascular endothelial cells (35). The sections were then mounted with a fluorescent mounting medium. Colocalization was observed by confocal microscopy (FV 1000; Olympus Optical Co Ltd, Tokyo, Japan). Some sections were treated simultaneously without the primary antibodies to confirm the specificity of immunoreactivities.

**Analysis of Vascular Permeability Using HRP**

Control and hypoxic rats (24 hours after the hypoxic exposure, 12/group) were given an intravenous injection of HRP (type VI; Sigma) via the left external jugular vein (0.5 μL/g body weight; 7.2 mg of HRP dissolved in 50 μL of saline). The rats were killed by perfusion at 3 and 24 hours after the HRP injection using a fixative composed of 1.25% glutaraldehyde and 1% paraformaldehyde in 0.01 M phosphate buffer. The brainstem was removed, and the tissues processed for light and electron microscopy as previously described (36).

**Electron Microscopy**

After exposure to hypoxia, 15 rats were killed at 3 and 24 hours and at 3, 7, and 14 days (n = 3 at each time intervals) for electron microscopy along with hypoxia + melatonin (n = 5) and control rats (n = 3). The animals were anesthetized with 6% pentobarbital and perfused with a mixture of 2% paraformaldehyde and 3% glutaraldehyde. After perfusion, the brainstem was removed, and coronal slices (approximately 1 mm thick) containing the NTS and VLM were cut. Vibratome sections (100 μm thick) were prepared from these blocks and rinsed overnight in 0.1 M phosphate buffer. They were then postfixed for 2 hours in 1% osmium tetroxide, dehydrated, and embedded in Araldite mixture. Ultrathin sections stained in uranyl acetate and lead citrate were viewed in an electron microscope (CM 120; Philips, Worcester, MA).

**Statistical Analysis**

For RT-PCR, Western blots, ELISA, and NO colorimetric assay, data are reported as mean ± SD. Data were analyzed by 1-way analysis of variance followed by post hoc analysis using Dunnett test (SPSS version 15.0 software, SPSS Inc, Chicago, IL) to determine the statistical significance of differences between normal and hypoxic and between hypoxic and hypoxia + melatonin rats. A p value less than 0.01 was considered statistically significant.

**RESULTS**

**Hypoxia Increased HIF-1α, nNOS, eNOS, iNOS, and VEGF Gene Expression**

Significant differences were observed in messenger RNA (mRNA) expression of HIF-1α, nNOS, eNOS, iNOS, and VEGF between control and hypoxic groups (Fig. 1). Hypoxia-inducible factor 1α mRNA was significantly elevated up to 24 hours, which was followed by a significant decrease at 3 days in the brain stem after hypoxia exposure. Vascular endothelial growth factor, iNOS, and nNOS mRNAs were increased significantly at all time points versus controls. Endothelial NO synthase mRNA showed a significant increase up to 3 days and again at 14 days but was markedly depressed at 7 days after hypoxia exposure.

**Hypoxia Increased HIF-1α, nNOS, eNOS, iNOS, and VEGF Protein Expression**

Densitometry of the HIF-1α immunoreactive band (approximately 120 kDa) increased significantly from 3 to 24 hours after hypoxia versus control levels (Fig. 2). An immunoreactive band of approximately 25 kDa was detected.
with the VEGF antibody; its optical density was significantly increased at all time points after hypoxia exposure. Immunoreactive bands of eNOS, iNOS, and nNOS were detected at 140, 130, and 155 kDa, respectively. The eNOS band showed a significant increase at 3 and 24 hours and 3 and 14 days, with a decrease at 7 days after hypoxia, whereas iNOS and nNOS showed a significant increase at all time intervals.

Tissue Concentrations of VEGF, NO, and Glutamate

The concentration of VEGF in the brainstem increased significantly versus controls at all time points in hypoxic rats (Fig. 3A). Levels of NO were significantly increased from 3 hours to 7 days after hypoxic exposure (Fig. 3B). At 14 days, the difference between the control and the hypoxic group was not significant. The glutamate concentration in the brainstem was increased significantly in hypoxic rats at 24 hours and 3 days versus controls (Fig. 3C).

After melatonin administration in hypoxic rats, the VEGF (Fig. 3D), NO (Fig. 3E), and glutamate (Fig. 3F) levels were significantly increased up to 3 days compared with those in the hypoxic rats that did not receive melatonin.

Immunohistochemistry

Vascular Endothelial Growth Factor

In the NTS and VLM of control rats there were VEGF-immunoreactive branched cells (Figs. 4A, C). The processes of many VEGF-positive cells were closely associated with blood vessels throughout these areas. In rats subjected to hypoxia exposure, VEGF expression in the branched cells and their blood vessel-associated processes was markedly increased (Figs. 4B, D). The VEGF-positive cells were identified as astrocytes by colocalization with GFAP using double immunofluorescence labeling in both the hypoxic and control animals (Figs. 4, E–G).

Endothelial, Neuronal, and Inducible NO Synthases

Blood vessels in the NTS and VLM in the control rats expressed weak eNOS immunoreactivity in control rats (Fig. 5A). After hypoxia, many blood vessels appeared to be dilated and exhibited enhanced eNOS immunoreactivity for up to 3 days (Fig. 5B). The eNOS immunoreactivity was reduced at longer time intervals after the exposure compared with the earlier time points. There was weak nNOS immunoreactivity in the neurons in NTS (Fig. 5C) and VLM in control rats. The expression was enhanced at all time points after the hypoxic exposure (Fig. 5D).

In control rats, there was an extremely weak iNOS immunoreactivity in the NTS and VLM. The expression was markedly enhanced after the hypoxic exposure (Figs. 5E, F) from 3 hours to 14 days. Many neurons showed intense iNOS immunoreactivity, both at the soma and at the dendrites (Figs. 5E, F).

Double immunofluorescence labeling confirmed that the expression of eNOS was in endothelial cells (Figs. 6, A–C), whereas that of nNOS and iNOS was in tyrosine hydroxylase–positive catecholaminergic neurons (Figs. 6, D–I).

Glutamate Receptors

Weak expression of GluR2/3 was observed in the neurons in the NTS (Fig. 7A) and VLM (Fig. 7B) of control rats. The expression of NR1 receptors was also weak in the controls in the NTS (not shown) and VLM (Fig. 7C). The expression of GluR2/3 and NR1 was enhanced up to 3 days after hypoxia exposure (Figs. 7, D–F) but was comparable to the controls at later time intervals.

Ultrastructural Changes

In the NTS and VLM of control rats, the neurons had pale nuclei and abundant cytoplasm that contained widely distributed cisternae of rough endoplasmic reticulum, mitochondria, and Golgi apparatus (Fig. 8A). At 3 hours to 3 days after hypoxia exposure, the neuron cytoplasm appeared watery and, in some areas, was devoid of cytoplasmic organelles or showed clumping of the rough endoplasmic reticulum (Fig. 8B). These abnormalities were less evident at 7 and 14 days. Instead, at these time points, the cytoplasm of some cells was laden with dense bodies and vacuoles (Fig. 8C). Many dendritic profiles in the NTS (Fig. 8D) and VLM (Fig. 8E) were swollen and contained variable numbers of vacuoles. At 3 hours to 3 days after hypoxia exposure, the axons in some sectional profiles appeared detached from the surrounding myelin sheaths, which appeared distorted or disorganized (Fig. 8F). Some axons had vacuoles or complete disappearance of organelles (Fig. 8G). Another degenerative feature evident at longer time intervals was the presence of closely packed dense bodies or fibrillar material in the axoplasm (Fig. 8H). In addition to these neuronal changes, edema was evidenced by the presence of swollen astrocyte processes (Fig. 9A), notably those associated with blood vessels (Fig. 9B). Another salient feature in the hypoxic brainstem was the occurrence of many perivascular cells containing fibrillar material (Figs. 9C, D). After melatonin administration, the swelling of neurons (Fig. 9E) and astrocyte processes (Figs. 9F) was diminished. Melatonin also reduced disruption of myelin sheaths of the axons (Fig. 9G) and swelling of dendrites (Fig. 9H).

Horseradish Peroxidase

Horseradish peroxidase–labeled cells or processes were not observed in the neuropil of NTS and VLM in the control rat.
rats (i.e. those not subjected to hypoxia), although some endothelial or perivascular cells showed weak labeling (Figs. 10A, B). At 3 hours after HRP administration in hypoxic rats, perivascular cells associated with the blood vessels in the NTS (Fig. 10C) and VLM were labeled with HRP reaction product. At 24 hours after the injection, HRP reaction products inundated the NTS (Fig. 10D) and VLM.

By electron microscopy, in addition to the perivascular cells, HRP reaction products were observed in the cytoplasm of microglial cells at both time points in hypoxic rats (Fig. 10E). At 24 hours after hypoxia exposure, HRP was additionally localized in the pale hypertrophic processes of astrocytes (Fig. 10F).

**DISCUSSION**

Central nervous system neurons are extremely vulnerable to oxygen deprivation; their impaired functions can occur within minutes of severe hypoxia in conditions such as stroke and apnea (37). Several factors, such as excitotoxicity and excess NO production, may contribute to neuronal loss. Disruption of the blood-brain barrier may also lead to neuron injury (38). We found that neurons, axons, and dendrites in the NTS and VLM show structural alterations such as swelling and degeneration with hypoxia exposure. These alterations may be linked to their activation and altered functions including reduction in ventilatory response to hypoxia (14, 39, 40).

Brainstem HIF-1α mRNA and protein expression peaked at 24 hours after hypoxic exposure. The drastic decline at 3 days suggests that the response was rapid in onset but was not sustained. Hypoxia-inducible factor 1α is upregulated in various tissues in hypoxic conditions, and it activates the expression of many genes such as VEGF at the transcriptional level (41). Indeed, we observed increased VEGF mRNA and protein expression in the brainstem as early as 3 hours after hypoxic exposure. Vascular endothelial growth factor is an inducer of vascular leakage, and its upregulation promotes leakage of plasma proteins from blood vessels in the brain (31, 42). Double immunofluorescence labeling with GFAP and VEGF showed complete colocalization, indicating that VEGF is produced by astrocytes under normal conditions. Moreover, the intensity of VEGF staining was greatly enhanced in astrocytes and their blood vessel-associated processes after hypoxia. The elevated VEGF expression was associated with leakage of HRP, which inundated the tissue and was taken up by perivascular cells. Dendrite and astrocyte process swelling appeared soon after hypoxic exposure, suggesting that the blood-brain barrier breakdown affected both neurons and glial cells.

Excitotoxicity due to increased release of glutamate has been considered a major mechanism of neuronal loss in hypoxic conditions. Increased tissue levels of glutamate and enhanced expression of NR1 and GluR2/3 receptors in the present study suggest that neuronal damage may be triggered through this mechanism, as reported previously in other parts of the brain (25–28). Overactivation of NMDA and AMPA glutamate receptors in the neurons results in accumulation of toxic concentrations of calcium, which triggers a range of downstream neurotoxic cascades including activation of NO synthase (43). Activation of nNOS mediated by excitatory amino acids increases production of NO (44), which is believed to mediate neuronal injury (45, 46). It is possible that the neuronal changes we observed occurred partly through increased activation of NR1 and GluR2/3 and nNOS expression in the NTS and VLM neurons.

Endothelial NO synthase mRNA was also increased up to 3 days after the hypoxic exposure, and increased eNOS immunoreactivity in the blood vessels in the NTS and VLM was also evident. The expression of eNOS in astrocytes has been reported earlier in the NTS (47) but was not observed in our study. Nitric oxide produced through eNOS may be responsible for the vasodilatation that occurs in hypoxic tissue (44). A subtle regulatory mechanism may be involved in vasodilation by NO that might explain the rebound of eNOS at 14 days after its decline at 7 days. In addition to being a potent vasodilator, NO has also been implicated in blood-brain barrier permeability (48), and the serum leakage through the dilated blood vessels may have contributed to the neuronal and axonal damage in the NTS and VLM.

We also observed enhanced iNOS expression at all time points after hypoxia. Hypoxia induces iNOS expression (32), and expansion of brain damage in hypoxic/ischemic conditions occurs as a result of production of toxic levels of NO by iNOS (45). Nitric oxide produced from both iNOS and nNOS contributes to cell death and axonal damage through inhibition of mitochondrial respiration and rapid glutamate release from both glia and neurons (48–50).

Melatonin reduces hypoxia-associated oxidative stress (51, 52), is neuroprotective in asphyxia (53), and has been reported to be beneficial in the treatment of hypoxic brain damage (54). It decreases NO levels in many tissues by inhibiting eNOS and iNOS production (55). In the present study, NO and VEGF levels in the brainstem declined after melatonin administration, and this may have resulted in decreased vascular leakage and edema. Melatonin treatment also reduced glutamate levels in the brainstem, suggesting reduced excitotoxicity and hence its role in neuroprotection. It also appeared to preserve the structure of neurons, axons, and dendrites that may have been due to reduced vascular leakage and glutamate levels.

In conclusion, we have shown that a hypoxic insult results in damage to the neurons, axons, and dendrites in the

**FIGURE 10.** Horseradish peroxidase (HRP) labeling studies. (A, B) HRP is localized in endothelial cells (arrow) of blood vessels (BV) (A) and occasional perivascular cells (B) (arrow) in the nucleus tractus solitarius (NTS) of a control rat at 3 hours (A) and 24 hours (B) after HRP administration. (C, D) HRP leakage from a blood vessel (BV) in the NTS of a hypoxic rat at 3 hours after HRP injection is evidenced by the labeling of perivascular cells (arrows) with HRP reaction product. Some HRP reaction products are also seen in the neuropil (C). At 24 hours after the injection (D), the leaked HRP (arrows) is present throughout the NTS. (E, F) Electron micrographs showing that HRP (arrows) is internalized in the microglial cell (M) in E and the pale profiles identified as hypertrophic astrocyte processes in F. Scale bars = 10 µm (A–D), 1 µm (E), 2 µm (F).
brainstem. Increased release of glutamate in the brainstem may result in excitotoxicity through enhanced expression of NR1 and GluR2/3 receptors. An increase in VEGF and NO production augments the permeability of the blood vessels evidenced by HRP leakage. Melatonin may be beneficial in protecting the neurons and axons in the brainstem because it reduces glutamate, VEGF, and NO levels and hence mitigates neuronal damage that results from excitotoxicity and increased vascular permeability.

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REFERENCES


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52. Ng KM, Lau CF, Fung ML. Melatonin reduces hippocampal beta-amyloid generation in rats exposed to chronic intermittent hypoxia. Brain Res 2010;1354:163–71