Cellular and Vascular Changes in the Retina of Neonatal Rats after an Acute Exposure to Hypoxia

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PURPOSE. This study was undertaken to examine the effects of an acute hypoxic exposure on the retinal cells and production of vascular factors such as vascular endothelial growth factor (VEGF) and nitric oxide (NO), which may affect vascular permeability in the developing retina.

METHODS. Retinas of 1-day-old rats were examined at 3 hours to 14 days after hypoxic exposure. The mRNA and protein expression of hypoxia-inducible factor-1α (HIF-1α), VEGF, endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) were determined by real-time RT-PCR, Western blot analysis, and immunohistochemistry. Electron microscopy was used to examine the structural alterations in retinal cells, and rhodamine isothiocyanate (RhIC) or horseradish peroxidase (HRP) was administered intraperitoneally or intravenously to determine vascular permeability.

RESULTS. The mRNA and protein expression of HIF-1α, VEGF, eNOS, nNOS, and iNOS, along with VEGF concentration and NO production, were increased in response to hypoxia. Swollen Müller cell processes, apoptotic and necrotic cells in the inner nuclear layer, and changes in ganglion cells such as swollen and disrupted mitochondria were observed in hypoxic animals. Increased leakage of RhIC and HRP from retinal and hyaloid vessels was seen after hypoxic exposure.

CONCLUSIONS. The authors suggest that increased VEGF and NO production in hypoxia resulted in increased vascular permeability, leading to changes in Müller cells and degeneration of neural cells. Melatonin administration reduced VEGF and NO production, diminished leakage of RhIC and HRP, and promoted cell proliferation, suggesting this as a potential therapeutic agent in reducing hypoxia-associated damage in the developing retina. (Invest Ophthalmol Vis Sci. 2009;50: 5364–5374) DOI:10.1167/iovs.09-3552

Birth asphyxia or hypoxia-ischemia affects the normal development and maturation of many tissues including nervous tissue. Prenatal or fetal hypoxia can occur in association with many maternal causes, such as diabetes, asthma, anemia, smoking, and the use of alcohol or drugs. Reduced uteroplacental blood flow, premature onset of labor, and prolonged labor may also compromise fetal oxygenation. Significant cell death is known to occur after hypoxic-ischemic injuries in the developing brain.1–5 Based on morphologic, histochemical, and biochemical criteria, cell death has been classified as apoptotic or necrotic in the hypoxic-ischemic neonatal brain.4–14 Periventricular white matter damage, ventricular hemorrhages, and ventriculomegaly have also been reported to occur as a result of hypoxic-ischemic injury in premature infants.15–22 Retinopathy of prematurity (ROP) is known to result from relative retinal hypoxia, a period of hyperoxygenation causing regression of developing retinal blood vessels and a return to normal oxygen levels resulting in relative hypoxia in the now under-vascularized retina. Although premature birth and compromised pulmonary function have been considered important etiologic factors in the development of ROP,23,24 reports on cell death and other changes in the developing neural retina, which is an extension of the brain, are lacking. Literature on the effects of primary hypoxia on the structure of the developing neonatal retina is also scanty.

Hypoxia induces the expression of hypoxia inducible factor (HIF)-1α, a master regulator of cellular and developmental O2 homeostasis,25 which activates the production of vasoactive substances such as vascular endothelial growth factor (VEGF) and nitric oxide synthases (NOS) in the neonatal brain.22 We have reported earlier that VEGF and nitric oxide (NO) production is enhanced in the adult retina in response to hypoxia.26 We hypothesize that increased production of VEGF and NO, leading to increased vascular permeability and cellular toxicity in the developing retina, may result from hypoxic injury in the perinatal period.

We also sought to assess whether melatonin, an antioxidant and neuroprotective agent,27–28 has any protective effect on the hypoxic neonatal retina. Melatonin has been reported to reduce VEGF and NO production in the brain and adult retina and to reduce edema formation. Structural damage to the dendrites has also been reported to be mitigated by melatonin.29

MATERIALS AND METHODS

Animals

One-day-old Wistar rats were used in the present study. They were exposed to hypoxia by placing them in a chamber (MCO 18M; Sanyo Biomedical Electrical Co., Ltd., Tokyo, Japan) filled with a gas mixture of 5% oxygen/95% nitrogen for 2 hours. The rats were then allowed to recover under normoxic conditions for 3 hours, 24 hours, 3 days, 7 days, 14 days, or 21 days before being euthanatized. Another group of 1-day-old rats kept outside the chamber was used as age-matched controls. This study was approved by the Institutional Animal Care and Use Committee of National University of Singapore and was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Melatonin Administration

Melatonin (Sigma-Aldrich, St. Louis, MO) dissolved in normal saline (10 mg/kg body weight) was administered intraperitoneally, as described...
earlier, to 19 neonatal rats (subjected to hypoxia) to assess its effect on VEGF concentration, NO production, vascular permeability, and ultrastructure of the retina. VEGF concentration and NO production were determined at 3 and 24 hours (n = 5 rats at each time interval). Values between the hypoxia- and hypoxia + melatonin-administered rats were compared.

Real-time RT-PCR

Retinas were removed from the eyes of hypoxia-exposed rats at 3 hours, 24 hours, 3 days, 7 days, or 14 days (n = 5 at each time point) and their corresponding controls. Total RNA was extracted with a purification kit (RNasy easy mini kit; Qiagen, Valencia, CA) according to the manufacturer’s protocol. The amount of total RNA was quantified with a photometer (BioPhotometer; Eppendorf, San Jose, CA).

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

Quantitative RT-PCR was carried out on a PCR amplification system and detection system (Light Cycler 2.0; Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) using a hot start reaction (FastStart DNA Master plus SYBR Green I kit; Roche Diagnostics GmbH) according to the manufacturer’s instructions. 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 provided in Table 1.

Western Blot Analysis

Retinas were removed from the hypoxic rats at 3 hours, 24 hours, 3 days, 7 days, or 14 days (n = 5 at each time point) and their corresponding controls. They were homogenized with tissue protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL) containing protease inhibitors. Samples of supernatants containing 20 µg protein were heated to 95°C for 5 minutes and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% gels in an appropriate apparatus (Mini-Protean 3; Bio-Rad, Hercules, CA). Protein bands were electroblotted onto 0.45 µm polyvinylidene difluoride membranes (Bio-Rad) and were blocked with 5% (wt/vol) nonfat dried milk. The membranes were then separately incubated with dilutions of the polyclonal VEGF (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), nNOS (1:500; BD Transduction Laboratories, Lexington, KY), monoclonal HIF-1α (1:500; Chemicon International, Temecula, CA), eNOS (1:2500; BD Transduction Laboratories), and iNOS (1:3000; BD Transduction Laboratories) antibodies in blocking solution overnight at 4°C. They were then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare, Amersham, Bucks, UK). Specific binding was revealed by an enhanced-chemiluminescence kit (GE Healthcare) according to the manufacturer’s instructions.

Analysis of VEGF Concentration by Enzyme Immunoassay

The amount of VEGF (ng/mL) released in the retina from control and hypoxic rats (n = 5 at each time interval) was determined with a VEGF enzyme immunoassay (EIA) kit (Chemikine; Chemicon International Inc.). Homogenates were prepared as described for Western blot analysis, and EIA measurements were performed according to the manufacturer’s protocol.

Nitric Oxide Colorimetric Assay

The total amount of NO in the retina from control and hypoxic rats (n = 5 at each time interval) was assessed by the Griess reaction using a colorimetric assay kit (US Biological, Swampscott, MA) that detects nitrite (NO₂⁻), a stable reaction product of NO. Homogenates from the...
retina were prepared as for Western blot analysis, and nitrite colorimetric assays were performed according to the manufacturer’s protocol.

**Immunohistochemistry**

Rats exposed to hypoxia at 3 hours, 24 hours, 3 days, 7 days, or 14 days ($n = 3$ at each time point) and their corresponding controls ($n = 3$) were anesthetized with 6% pentobarbital and perfused with 2% paraformaldehyde. The eyes were removed, and 40-μm-thick frozen corneal sections were prepared and incubated with VEGF (1:200; Santa Cruz Biotechnology, Inc.), nNOS (1:500; BD Transduction Laboratories), eNOS (1:250; BD Transduction Laboratories), and iNOS (1:1000; BD Transduction Laboratories) antibodies diluted in phosphate buffered saline for 16 to 20 hours. Subsequent antibody detection was

![Figure 2. Protein expression in the retina by Western blot analysis of HIF-1α, VEGF, eNOS, iNOS, and nNOS in rats at 3 hours, 24 hours, 3 days, 7 days, or 14 days after hypoxic exposure and their corresponding controls. Upper: HIF-1α (120 kDa), VEGF (25 kDa), eNOS (140 kDa), iNOS (130 kDa), and nNOS (155 kDa) immunoreactive bands. Bar graphs (lower) show significant changes in optical density after hypoxic exposure: (A) HIF-1α; (B) VEGF; (C) eNOS; (D) iNOS; (E) nNOS. Level of significance (*P < 0.05) compared with controls.](image-url)

**TABLE 1. Sequence of Specific Primers**

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</tr>
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<td>actccccggtctccctatg</td>
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*Level of significance (*P < 0.05) compared with controls.*
carried out as described earlier by us. For negative controls, some sections from each group were incubated in a medium omitting the primary antibodies.

**Double Immunofluorescence**

Three rats at 7 days after hypoxic exposure and their corresponding controls ($n = 3$) were used for double immunofluorescence. After deep anesthesia with 6% pentobarbital, the rats were killed by perfusion with 2% paraformaldehyde. Frozen coronal sections of the eyes at 40-μm thickness were cut with a cryotome (Frigocut; Leica, Wetzlar, Germany), divided into two sets, and rinsed in PBS. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes, and the sections were subsequently washed with PBS. The first set of sections was then incubated at room temperature with a cocktail mix of two primary antibodies, VEGF (1:200; Santa Cruz Biotechnology, Inc.) and glial fibrillary acidic protein (GFAP; 1:1000; Chemicon International, Inc.). GFAP is a specific marker of astrocytes. For the detection of apoptosis, the second set of sections was incubated at room temperature with a cocktail mix of two primary antibodies, caspase-3 (1:200; Cell Signaling Technology, Inc., Beverly, MA) and NeuN (1:100; Chemicon International, Inc.). Caspase-3 is a marker for cell apoptosis, and NeuN is a neuronal marker. Subsequent antibody detection was carried out with a cocktail mix of two secondary antibodies, Cy3-conjugated goat anti–rabbit IgG (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) and FITC-conjugated sheep anti–mouse IgG (1:100; Sigma-Aldrich). Mix of two secondary antibodies, Cy3-conjugated goat anti–rabbit IgG (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) and FITC-conjugated sheep anti–mouse IgG (1:100; Sigma-Aldrich). Co-localization of VEGF with GFAP and caspase-3 with NeuN was observed under a confocal microscope (FV 1000 Olympus Optical Co. Ltd., Tokyo, Japan). The number of caspase-3/NeuN-positive cells in the ganglion cell layer (GCL) was counted in six randomly selected microscopic fields from six sections of the retina in each rat at 60× magnification. The cells with NeuN labeling (green) overlapping with caspase-3 (red) were counted as NeuN-/caspase-3–positive cells, whereas those cells emitting only green fluorescence were counted as NeuN/caspase-3–negative cells. The percentages of cells with positive expression for caspase-3 were calculated and averaged.

**Electron Microscopy**

Rats exposed to hypoxia at 3 hours, 24 hours, 3 days, 7 days, 14 days, or 21 days ($n = 3$ at each time point), their corresponding controls ($n = 3$ at each time point), and those treated with melatonin ($n = 3$) were perfused with a mixed aldehyde fixative composed of 2% paraformaldehyde and 3% glutaraldehyde. The eyes were removed and processed as described earlier. Ultrathin sections were viewed under an electron microscope (CM 120; Philips, Eindhoven, The Netherlands).

**Analysis of Vascular Permeability with Tracers**

**Rhodamine Isothiocyanate.** Three control rats, three hypoxic rats at 24 hours, and three hypoxic rats administered melatonin were each given an intraperitoneal injection of rhodamine isothiocyanate (RhIC; 5 μL of 1% RhIC/g body weight; Sigma) dissolved in normal saline. The rats were killed by perfusion with 2% paraformaldehyde at 6 hours after RhIC injection in accordance with our earlier study. The eyes were removed, frozen coronal sections prepared and incubated with **Lycopersicon esculentum** lectin (1:100; Sigma-Aldrich), a marker for blood vessels and microglia, and were processed further, as described. Cellular colocalization was then studied by confocal microscopy (FV1000; Olympus).

**Horseradish Peroxidase.** Three control rats, three rats at 24 hours after hypoxic exposure, and three hypoxic rats treated with melatonin were each given an intravenous injection of HRP (type VI; Sigma) through the left external jugular vein (0.3 μL/g body weight; 7.2 mg HRP dissolved in 50 μL saline). They were killed by perfusion at 3 hours after HRP injection. The eyes were removed and processed according to the procedure described earlier.

**Statistical Analysis**

For RT-PCR, Western blot analysis, EIA, and NO colorimetric assay, data are reported as mean ± SD. Student’s $t$-test was used to determine the statistical significance of differences between normal and hypoxic and between hypoxic and hypoxia + melatonin rats. $P < 0.05$ was considered statistically significant.

![Figure 3](image-url)  
**Figure 3.** VEGF concentration (A) and NO production (B) in the retinas of control rats and at 3 hours, 24 hours, 3 days, 7 days, or 14 days after hypoxic exposure. Significant differences in VEGF concentration and NO production between control and hypoxic rats are indicated ($*P < 0.05$). Significant reductions ($#P < 0.05$) in levels of VEGF (C) and NO (D) at 3 and 24 hours after melatonin administration in hypoxic rats.
RESULTS

HIF-1α, VEGF, eNOS, nNOS, and iNOS mRNA Expression

Compared with controls, HIF-1α mRNA expression in the retina was increased significantly at 3 hours, 24 hours, and 3 days after hypoxic exposure but decreased below control levels at 7 and 14 days. VEGF and iNOS mRNA showed a significant increase up to 7 days after hypoxic exposure compared with control values. Increases in eNOS and nNOS mRNA were most marked at 3 hours to 3 days but subsided at 7 days (Fig. 1).

HIF-1α, VEGF, eNOS, iNOS, and nNOS Protein Expression by Western Blot Analysis

The immunoreactive band of HIF-1α protein, at approximately 120 kDa, was increased significantly up to 3 days after hypoxic exposure. VEGF and iNOS immunoreactive bands, approximately 25 kDa and 130 kDa, respectively, increased significantly at 3 hours to 7 days but declined at 14 days. The eNOS and nNOS protein bands, expressed approximately at 140 kDa and 155 kDa, respectively, increased significantly at 3 hours to 3 days after hypoxic exposure (Fig. 2).

VEGF Enzyme Immunoassay

VEGF concentration increased significantly in the retinas at 3 hours to 7 days in hypoxic rats compared with the controls (Fig. 3A). It was suppressed significantly in hypoxic rats given melatonin treatment (Fig. 3C).

Nitric Oxide Assay

NO levels in the retina were significantly increased at 3 hours to 3 days after hypoxic exposure compared with controls (Fig. 3B) but decreased at 7 to 14 days. With melatonin treatment, there was a significant reduction in NO levels compared with hypoxic rats not treated with melatonin (Fig. 3D).

Immunohistochemistry

eNOS. Weak eNOS expression was detected in some blood vessels in the nerve fiber layer (NFL) in control rats (Fig. 4A). The eNOS expression is enhanced drastically in retinal blood vessels (B, arrows) and in the hyaloid vessels (C, arrows) 24 hours after hypoxic exposure. Hyalocytes also express eNOS immunoreactivity (asterisks). Weak iNOS expression is seen in the hyaloid vessels (D, arrows) in a 2-day-old control rat. The expression of iNOS is enhanced in the hyaloid vessels (arrows) and in the INL (asterisks) 24 hours after hypoxic exposure (E, F). Many hyalocytes also show intense expression of iNOS (F, asterisks). Weak nNOS expression is seen in the ganglion cells (arrows) in the retina of a 2-day-old control rat (G). The expression in the ganglion cells (arrows) and in the INL (H, asterisk) is markedly upregulated 24 hours after hypoxic exposure. Scale bars: (A, C, D, F-H), 10 μm; (B, E) 50 μm.

FIGURE 4. Weak eNOS expression is detected in some blood vessels (arrows) in the retina and hyaloid vessels (asterisks) in a 2-day-old control rat (A). The eNOS expression is enhanced drastically in retinal blood vessels (B, arrows) and hyaloid vessels (C, arrows) 24 hours after hypoxic exposure. Hyalocytes also express eNOS immunoreactivity (asterisks). Weak iNOS expression is seen in the hyaloid vessels (D, arrows) in a 2-day-old control rat. The expression of iNOS is enhanced in the hyaloid vessels (arrows) and retinal (arrowheads) vessels 24 hours after hypoxic exposure (E, F). Many hyalocytes also show intense expression of iNOS (F, asterisks). Weak nNOS expression is seen in the ganglion cells (arrows) in the retina of a 2-day-old control rat (G). The expression in the ganglion cells (arrows) and in the INL (H, asterisk) is markedly upregulated 24 hours after hypoxic exposure. Scale bars: (A, C, D, F-H), 10 μm; (B, E) 50 μm.

FIGURE 5. Weak expression of VEGF is detected in astrocytes (arrow) in the retina of a 4-day-old control rat (A). VEGF expression is enhanced at 3 days (B) and 7 days (C) after hypoxic exposure. (B) VEGF-positive processes (arrows) of astrocytes can be seen associated with a blood vessel in the NFL. A large number of hyalocytes associated with the hyaloid vessels also express VEGF (D). Confocal images showing the distribution of GFAP (E, green) and VEGF (F, red) immunoreactive cells (arrows) in the retina at 7 days after hypoxic exposure. Colocalized expression of VEGF and GFAP (G). Scale bars: (A-D) 10 μm; (E-G) 20 μm.
which were in close association with the NFL. The eNOS immunoreactivity in the blood vessels in the NFL appeared to reduce at 7 and 14 days (data not shown) after hypoxic exposure compared with the earlier time intervals.

iNOS. Weak iNOS immunoreactivity was observed in the GCL and retinal and hyaloid vessels in the control rats of different age groups (Fig. 4D). Expression was markedly enhanced after hypoxic exposure at 24 hours to 7 days (Fig. 4E). Expression of iNOS was also observed in some blood vessels in the NFL. Hyaloid vessels and hyalocytes (Fig. 4F) showed intense iNOS immunoreaction. Many iNOS-positive hyalocytes were in close association with the NFL. At 14 days, iNOS expression was attenuated in the GCL but that in the hyaloid vessels and hyalocytes had disappeared (data not shown).

nNOS. Weak nNOS immunoexpression was observed in the GCL (Fig. 4G) in control rats. Expression was enhanced at 3 hours to 7 days after hypoxic exposure (Fig. 4H) but was comparable to that in controls in longer surviving rats. The hyaloid vessels and the hyalocytes did not express nNOS immunoreactivity in the control or hypoxic rats.

VEGF. Weak VEGF expression was observed in the retinas of control rats (Fig. 5A). After hypoxic exposure, immunoexpression of VEGF was increased up to 7 days in branched cells in the NFL (Figs. 5B, 5C). VEGF expression was not observed in other layers of the retina. Some blood vessels in the NFL were surrounded by VEGF-positive cell processes (Fig. 5B). Hyaloid vessels and hyalocytes in the 1-day to 8-day control rats also showed weak VEGF immunoreactivity. After hypoxic exposure, VEGF expression was markedly increased in the hyaloid vessels and hyalocytes (Fig. 5D). Branched cells expressing VEGF were identified as astrocytes in the hypoxic rats because they were completely colocalized with GFAP-positive cells (Figs. 5E–G).

Ultrastructural Changes

In hypoxic rats, swelling of Müller cell processes at the inner limiting membrane and those closely associated with the ganglion cells in the GCL was observed as early as 3 hours after hypoxic exposure and remained so until 14 days thereafter (Fig. 6C). Some of the processes appeared to contain vacuoles. Such features were not observed in the Müller cell processes in control retinas (Fig. 6A). In control retinas, retinal ganglion cells (RGCs) had rounded nuclei with evenly dispersed chromatin and abundant cytoplasm containing mitochondria, rough endoplasmic reticulum, and a Golgi complex (Fig. 6B). After hypoxic exposure, RGCs had swollen mitochondria with dis-
ruptured cristae and large vacuoles at 3 to 21 days (Fig. 6D). At 14 to 21 days, clumping of nuclear chromatin and disintegration of mitochondria were observed in the RGCs. Another prominent feature was the occurrence of many apoptotic cells at 7 to 14 days (Fig. 7B) and necrotic cells at 21 days (Fig. 7C) in the inner nuclear layer (INL) after hypoxic exposure compared with controls (Fig. 7A). In necrotic cells, swelling of cytoplasm with loss of organelles, nuclei with dark chromatin clumps and separation of the two layers of the nuclear envelope were observed (Fig. 7C). A few apoptotic or necrotic cells were observed in the INL of the retinas in 4-day-old control rats but not in older rats. Retinal pigment epithelial (RPE) cells contained swollen mitochondria and vacuoles in their cytoplasm (Fig. 7D) after hypoxic exposure. The intercellular spaces between RPE cells were dilated. With melatonin administration, RGCs (Fig. 6F) and RPE cells were structurally comparable to corresponding cells in controls, whereas the occurrence in the INL (Fig. 7E) of apoptotic or necrotic cells and swelling of Müller cell processes was noticeably reduced (Fig. 6E). In addition, after melatonin administration, an increase in incidence of mitotic cells was observed in the outer nuclear layer (ONL; Fig. 7F) and in the GCL (Figs. 7G, 7H).

**Apoptosis Analysis**

Immunoreactive NeuN-labeled cells in the GCL and INL were identified in the retinas of control rats (Figs. 8A–C); few of them were labeled by caspase-3 (Fig. 8B). After hypoxic exposure, however, NeuN immunoreactive cells showing caspase-3 labeling were increased significantly (Figs. 8D–F). The percentage of NeuN/caspase-3–positive RGC 7 days after hypoxic exposure was significantly higher than that in the corresponding controls (Fig. 8G).

**Vascular Permeability**

**Rhodamine Isothiocyanate.** Lectin labeling of the blood vessels in the NFL, hyaloid vessels, and hyalocytes was observed in control, hypoxic and hypoxia + melatonin rats (Figs. 9 and 10). Retinas in hypoxic rats appeared to be inundated with extravasated RhIC (Figs. 9E, 9F) but not in the controls (Figs. 9B, 9C). There was evidence of some leakage of RhIC into the vitreous in control rats as the hyalocytes associated with the hyaloidal vessels emitted weak RhIC immunofluorescence (Fig. 10B). Increased RhIC leakage into the vitreous was observed in hypoxic rats (Fig. 10E). Leaking RhIC appeared to be internalized by the lectin-labeled microglia (Figs. 9E, 9F) and hyalocytes (Figs. 10B, 10E). Melatonin administration reduced RhIC leakage that was still slightly greater than in controls but less than in untreated hypoxic rats (Figs. 9H, 10H).

**Horseradish Peroxidase.** In control rats, the HRP reaction product blood was observed in vessels in the NFL and GCL (Fig. 11A), but no evidence was observed of HRP leakage from these blood vessels. Hyaloid vessels also showed the presence of HRP within their lumen as well some leakage into the vitreous, as evidenced by HRP-labeled hyalocytes. In hypoxic rats, leakage of HRP from blood vessels in the NFL was evidenced by the presence of granular deposits in the vicinity of the blood vessels (Fig. 11B). Hyaloid vessels also showed increased leakage of HRP, as evidenced by the presence of...
intensely labeled hyalocytes. After melatonin administration, the leakage of HRP from blood vessels in the NFL (Fig. 11C) and hyaloid vessels was greatly reduced.

**DISCUSSION**

The developing eye and the visual system have been reported to be susceptible to hypoxic-ischemic damage. Premature birth, compromised pulmonary function, and cyanotic heart disease that result in hypoxia have been reported as important etiologic factors in the development of retinopathy. Retinal hypoxia may enhance or induce the death of RGCs and other neurons in the developing retina, resulting in loss of vision. In the present study, hypoxic damage to the developing retina was evidenced by the widespread presence of apoptotic and necrotic cells, swelling of Müller cell processes, changes in the RPE, and increased vascular leakage. Although the precise timing of the retinal cell death process has not yet been completely characterized during normal development of the retina, RGC death has been reported to occur between postnatal days 1 to 6 in the rat animal model. In view of this, the degenerative changes observed in the RGCs, such as swollen and disrupted mitochondria in 7-day-old and older rats, appeared to be related to hypoxia. This was further supported by the occurrence of increased caspase-3 immunoreactivity in RGCs 7 days after hypoxic exposure, suggesting that these cells may die by apoptosis. Enhanced caspase-3 activity was also observed in the INL. Cell death in the INL during normal development has been reported to peak between postnatal days 7 and 10. In the present study, however, necrotic cells were observed up to 21 days in the INL after hypoxic exposure.

Hypoxia-dependent events in cells activate HIF-1α, which regulates cellular and developmental O2 homeostasis. Enhanced expression of HIF-1α and increased production of VEGF and NO was observed after hypoxic exposure. HIF-1α activates the expression of many genes at the transcriptional level, including VEGF, which has been suggested as a key mediator of hypoxia-induced neovascularization and an inducer of vascular leakage. Intravenous administration of VEGF in newborn mice increased blood-brain barrier permeability within 2 hours. In the present study, vascular permeability was increased in the blood vessels of the inner retina and in hyaloid vessels in hypoxic rats, as shown by the leakage of RhIC and HRP. The extravagated tracers were phagocytosed by the numerous hyalocytes in the vitreous or by the microglia in the NFL and GCL.

Although VEGF and NO levels were significantly increased in hypoxic retina, as shown by Western blot and colorimetric assay, respectively, it appears that the quantum of increase was small. One possible explanation for this was that total retinal tissue containing a mixed population of cells was removed for this analysis, possibly limiting the extent of actual changes that were more evident by immunostaining or RhIC labeling.

VEGF was expressed in the NFL by branched cells, previously identified as astrocytes in the developing retina. We...
confirmed that the cells were indeed the astrocytes. In addition, hyalocytes may be another cellular source of VEGF because they expressed VEGF immunoreactivity. Although Müller cells are present in the retina at birth in many species, VEGF expression was not observed in Müller cells in the present study.

VEGF has been reported to increase vascular permeability by the synthesis or release of NO predominantly derived from eNOS. Blood vessels in the NFL and the hyaloid vessels showed intense expression of eNOS immunoreactivity in hypoxic rats. The resultant increased production of NO was followed by vasodilatation and increased blood flow and by an increase in vascular permeability in the retina. In addition to eNOS, VEGF has been reported to induce the expression of iNOS. NO derived from iNOS is known to aggravate damage in ischemic conditions in many tissues, including the brain, and NO derived from nNOS in hypoxic-ischemic injury correlated with regions of selective vulnerability in the developing brain. Increased expression of iNOS and nNOS in the hypoxic retinas in the present study might have been responsible for the apoptotic and necrotic cell death of neurons in the INL and the degenerative changes observed in the RGCs. It appears that multiple factors, such as enhanced production of VEGF, NO, and increased permeability of blood vessels in the developing retina, may be responsible for damage to retinal neurons. Increased permeability of hyaloid vessels and increased expression of VEGF, eNOS, and iNOS by the hyalocytes, many of which were in close association to the retina, might have abetted the neuronal damage. VEGF expression in macrophages is known to be strongly upregulated by hypoxia and is thought to contribute to angiogenic activity. Recent in vitro studies have shown that hypoxia increases VEGF expression in hyalocytes.

A protective effect of melatonin was evidenced by the reduction in VEGF concentration and NO production and the concomitant decrease in vascular permeability in hypoxic rats. Our previous study also showed that melatonin was beneficial in abating VEGF and NO production in the developing hippocampus after hypoxic insult. In the present study, the swelling of Müller cell processes and cell death were reversed, and increased proliferation of cells in the ganglion cell and outer nuclear layers of the retina was induced by melatonin.

**CONCLUSIONS**

We have shown that hypoxia results in cell death and in swelling of Müller cells in the developing retina. Increased production of VEGF and NO lead to increased permeability of retinal and hyaloid blood vessels, evidenced by leakage of RhIC and HRP, and this may be one of the underlying causes of cell death. Melatonin may be beneficial in protecting the neurons and other elements in the developing retina because it reduces VEGF concentration, NO production, and, hence, vascular permeability.
FIGURE 11. Blood vessels (arrows) containing HRP are seen in the NFL of a control 2-day-old rat (A). Free HRP indicative of its leakage is evident in the vicinity of the blood vessels at 24 hours after hypoxic exposure (B). Leakage is reduced after melatonin administration (C). (A, arrowheads) HRP-labeled hyalocytes. Scale bars, (A–C) 10 μm.

Acknowledgments

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References


