Hemodilutional anemia is associated with increased cerebral neuronal nitric oxide synthase gene expression

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Hare, Gregory M. T., C. David Mazer, William Mak, Reginald M. Gorczynski, Kathryn M. Hum, Steve Y. Kim, Leslie Wyard, Aiala Barr, Rong Qu, and Andrew J. Baker. Hemodilutional anemia is associated with increased cerebral neuronal nitric oxide synthase gene expression. J Appl Physiol 94: 2058–2067, 2003. First published January 17, 2003; 10.1152/japplphysiol.00931.2002.—Severe hemodilutional anemia may reduce cerebral oxygen delivery, resulting in cerebral tissue hypoxia. Increased nitric oxide synthase (NOS) expression has been identified following cerebral hypoxia and may contribute to the compensatory increase in cerebral blood flow (CBF) observed after hypoxia and anemia. However, changes in cerebral NOS gene expression have not been reported after acute anemia. This study tests the hypothesis that acute hemodilutional anemia causes cerebral tissue hypoxia, triggering changes in cerebral NOS gene expression. Anesthetized rats underwent hemodilution when 30 ml/kg of blood were exchanged with pentastarch, resulting in a final hemoglobin concentration of 51.0 ± 1.2 g/l (n = 7 rats). Caudate tissue oxygen tension (PbrO2) decreased transiently from 17.3 ± 4.1 to 14.4 ± 4.1 Torr (P < 0.05), before returning to baseline after ~20 min. An increase in CBF may have contributed to restoring PbrO2 by improving cerebral tissue oxygen delivery. An increase in neuronal NOS (nNOS) mRNA was detected by RT-PCR in the cerebral cortex of anemic rats after 3 h (P < 0.05, n = 5). A similar response was observed after exposure to hypoxia. By contrast, no increases in mRNA for endothelial (eNOS) or interleukin-1β were observed after anemia or hypoxia. Hemodilutional anemia caused an acute reduction in PbrO2 and an increase in cerebral cortical nNOS mRNA, supporting a role for nNOS in the physiological response to acute anemia. Cerebral blood flow; cerebral hypoxia; hemodilution

MAMMALS EXHIBIT HIGHLY REFINED MECHANISMS FOR OPTIMIZING TISSUE OXYGENATION IN RESPONSE TO HYPOXIC ENVIRONMENTAL CONDITIONS, INCLUDING INCREASES IN CIRCULATING BLOOD VOLUME AND HEMATOCRIT (14). CONVERSELY, SEVERE HEMODILUTIONAL ANEMIA MIGHT LEAD TO DECREASED TISSUE OXYGEN DELIVERY TO VITAL ORGANS, INCLUDING THE BRAIN, RESULTING IN ANEMIA-INDUCED TISSUE HYPOXIA. ACUTE ADAPTIVE MECHANISMS, INCLUDING A LUXURIANT INCREASE IN CEREBRAL BLOOD FLOW (CBF), ENSURE THAT CEREBRAL OXYGEN DELIVERY IS OPTIMIZED DURING ANEMIA (16, 25, 39, 44), POSSIBLY EXPLAINING WHY ANEMIA-INDUCED CEREBRAL HYPOXIA HAS BEEN DIFFICULT TO DEMONSTRATE (39).

RECENTLY, VAN BOMMEL ET AL. (48) DEMONSTRATED A REDUCTION IN CEREBRAL CORtical TISSUE OXYGEN TENSION AFTER SEVERE HEMODILUTIONAL ANEMIA BELOW AN AVERAGE CRITICAL HEMATOCRIT OF ~12% (48). THIS IS CONSISTENT WITH OTHER STUDIES THAT DEMONSTRATED THAT GLOBAL TISSUE OXYGEN CONSUMPTION BECOMES SUPPLY DEPENDENT BELOW A HEMOGLOBIN CONCENTRATION OF ~40 g/l (9), RESULTING IN ANAEROBIC METABOLISM AND DEMONSTRABLE TISSUE HYPOXIA (46). HOWEVER, ANEMIC HUMANS EXPERIENCE COGNITIVE IMPAIRMENT AT HEMOGLOBIN CONCENTRATIONS BETWEEN 50 AND 60 g/l AND ABOVE, SUGGESTING THAT REDUCED CEREBRAL OXYGEN DELIVERY AND CEREBRAL HYPOXIA MAY BE RESPONSIBLE FOR NEUROLOGICAL DYSFUNCTION AT HIGHER HEMOGLOBIN CONCENTRATIONS (33, 50). THE HIGH CEREBRAL METABOLIC REQUIREMENT FOR OXYGEN MAY EXPLAIN WHY FUNCTIONAL NEUROLOGICAL IMPAIRMENT OCCURS AT HEMOGLOBIN CONCENTRATIONS ABOVE THE LEVEL AT WHICH REDUCED CEREBRAL TISSUE OXYGEN TENSION IS OBSERVED IN EXPERIMENTAL STUDIES (48).

ASSESSMENT OF CHANGES IN CEREBRAL GENETIC EXPRESSION ASSOCIATED WITH HEMODILUTION MAY PROVIDE A MORE SENSITIVE METHOD OF CHARACTERIZING THE PHYSIOLOGICAL RESPONSE TO ACUTE ANEMIA AND MAY YIELD FURTHER EVIDENCE OF ANEMIA-INDUCED CEREBRAL HYPOXIA. USING THIS APPROACH, WE FOCUSED ON POTENTIAL MEDIATORS OF THE INCREASE IN CBF ASSOCIATED WITH BOTH HYPOXIA AND ANEMIA. LOCAL PRODUCTION OF NITRIC OXIDE (NO) BY ENDOTHELIAL (eNOS) (27, 38, 51) AND NEURONAL NITRIC OXIDE SYNTHASE (nNOS) (11, 15, 17, 29) MEDIATES CBF UNDER A NUMBER OF PHYSIOLOGICAL CONDITIONS, INCLUDING ANEMIA AND HYPOXIA (2, 15, 17). RELATIVELY SPECIFIC INHIBITION OF nNOS HAS BEEN DEMONSTRATED TO IMPAIR THE INCREASE IN...
CBF associated with acute anemia (17) and hypoxia (15), implicating nNOS as an important mediator of CBF in both cases. However, inhibitor studies do not fully differentiate between the effects of eNOS and nNOS activity because a completely selective inhibitor is not yet available. Molecular and immunohistochemical studies have demonstrated increased nNOS expression in rat and shark brain after exposure to hypoxia (12, 32, 37). Such upregulation of nNOS partially characterizes the cerebral response to hypoxia. Conversely, hypoxia has been demonstrated to reduce eNOS mRNA in blood vessels, suggesting a differential response (24, 45).

Changes in cerebral NOS gene expression have not yet been demonstrated in response to acute anemia. This study was designed to test the hypothesis that acute anemia causes cerebral hypoxia, which triggers changes in cerebral NOS gene expression. We measured both cerebral eNOS and nNOS mRNA after anemia to determine which enzyme might be regulated during anemia. Our results demonstrated that acute anemia caused a transient fall in caudate tissue oxygen tension (PbrO2) and an increase in cerebral cortical nNOS, but not eNOS, mRNA levels. The observed upregulation of nNOS gene expression characterizes the cerebral response to acute anemia and may provide further evidence of anemia-induced cerebral hypoxia.

MATERIALS AND METHODS

Animal model. All animal protocols were approved by the Animal Care and Use Committee at St. Michael’s Hospital in accordance with the requirements of Canadian Animal Care Committee and the American Physiological Society’s “Guide for the Use and Care of Laboratory Animals.” Male Sprague-Dawley rats (Charles River, St. Constant, PQ) were induced with ketamine-xylazine (100/7.5 mg/kg ip; Parke-Davis/Bayer, Toronto, ON) and maintained with 1–2% isoflurane (Abbott, St. Laurent, PQ) after intubation and ventilation with a pressure-controlled ventilator (Kent Scientific, Litchfield, CT). Ventilation was adjusted to achieve normocapnia and normoxia as determined by blood-gas analysis (Radiometer ALB 500, London Scientific, London, ON). Cannulation of the right jugular vein (PE-90) and tail artery (PE-50) were performed to achieve vascular access for direct measurement of mean arterial blood pressure (MAP) and central venous pressure (CVP) and to perform acute hemodilution. Animals were then placed in a stereotaxic frame (ADI Instruments, Harvard Apparatus, St. Laurent, PQ), and their scalps were incised sagitally. Bilateral 5-mm-diameter burr holes were trephined at the level of the bregma, 2–3 mm lateral to the sagittal sinus, exposing the intact dura.

A calibrated polarographic oxygen-sensing microelectrode, with a maximal diameter of 500 μm and a sensing aperture 1 mm in diameter (LICOX GMS, Harvard Apparatus) was then inserted ~6 mm past the dura into the region of the right caudate nucleus, by using stereotaxic coordinates. The caudate nucleus was chosen because it is a large and relatively homogenous area of gray matter with a metabolic rate similar to the cerebral cortex (40), making it suitable for placement of an invasive oxygen electrode. This location for probe placement was also chosen because the caudate nucleus represents a region of the brain with relatively limited collateral circulation (11, 35), which may be more susceptible to hypoxic damage (1). Placement of the probes within the caudate nucleus was confirmed by brain dissection at the end of experimental procedures. For each experiment, sensitivity and placement of each probe were confirmed by assessing responses to induced changes in arterial PO2 (Pao2) and arterial Pco2 (Paco2) levels (7, 13, 22, 28). A corresponding temperature probe was placed at the same coordinates as the oximetry probe. PbrO2 results are reported as both raw and normalized data. The oxygen-sensing microelectrodes were calibrated before each use by exposing the probe to 100% oxygen. Once the maximal plateau was reached, the probe was then placed in 100% nitrogen, and the time to reach a reading below 0.5 Torr was recorded. Probes were also zeroed in zero calibration solution (0.1 M sodium hydrosulfitie; Sigma S-1256, Oakville, ON). Probes were stored in clean distilled water at 4°C between experiments and reused up to eight times if the calibration parameters remained within 10% of initial values.

A laser-Doppler flow probe (OxyFlo, Oxford Optronix, Oxford, UK) was positioned on the dura on the contralateral side, avoiding any visible large dural vessels. We measured regional cortical CBF (rCBF) using a probe at the surface of the cerebral cortex and not in the caudate nucleus to minimize local tissue trauma that might alter PbrO2 measurements.

A steady baseline was established within 1 h during which a heating pad and heating lamp were used to maintain the brain temperature of rats near 37°C. Brain oxygenation (PbrO2), temperature, CBF, MAP, CVP, and heart rate were recorded with a computerized data-acquisition system (DA-SYLab 5.6, Kent Scientific).

Experimental procedures. Rats were divided into three groups: those exposed to control conditions (negative control, n = 6), those exposed to hypoxia (positive control, n = 6), and those exposed to hemodilutional anemia (n = 7) for up to 3 h under general anesthesia. The hypoxic group was included to serve as a positive control for the measurement of reduced PbrO2 (7) and changes in cerebral nNOS expression (12, 32). Acute physiological measurements were performed during the first hour. After 20 min of baseline measurements, acute hemodilutional anemia was induced by simultaneously exchanging 30 ml/kg of arterial blood (50% of the estimated blood volume), withdrawn from the tail artery, with an equivalent volume of pentastarch (Pentaspan, DuPont Pharma, Mississauga, ON) infused via the jugular vein (n = 7 rats). We used a programmable “push-pull” pump (PHD 2000, Harvard Apparatus) for volume exchange (performed over a 10-min period). Tail artery cannulation was utilized for continuous blood pressure measurement, before and after the exchange-transfusion period. Both MAP and CVP measurements were interrupted during the volume-exchange period. After completion of volume exchange, all parameters were recorded for an additional 30 min before the animal was killed by anesthetic overdose (100 mg iv ketamine, Parke-Davis). Control animals were treated in a similar manner but did not undergo hemodilution (n = 6 rats). Animals in the hypoxic group were exposed to 15% oxygen in nitrogen, after a stable baseline was established (n = 6).

Brain harvesting for RNA extraction and RT-PCR reaction. Subgroups of control, anemic, and hypoxic animals were maintained under general anesthesia for 3 h after induction of acute hemodilutional anemia, hypoxia (15% oxygen), or control conditions prior to decapitation and rapid extraction of brains (n = 5 rats per group). Sections of cerebral cortex were selected from areas of the brain remote from probe site placement, flash frozen in liquid nitrogen within 4 min of decapitation, and then stored at −80°C until RNA extraction was performed.

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For total RNA extraction, ~50 mg of frozen cerebral cortex were mechanically disrupted and then RNA was extracted by using standard techniques (RNasy Mini Kit, Qiagen, Mississauga, ON). We determined the 260- to 280-nm optical density using 10 μl of sample, and the remaining volume was divided into 30-μl aliquots (~0.2 μg/μl) and frozen at ~80°C.

We performed RT-PCR using previously utilized primers for β-actin (19), interleukin (IL)-1β (19), nNOS (10, 12), and newly generated primers for eNOS based on the partial rat cDNA sequence (45) (GeneTool 1.0, BioTools, Edmonton, AB) (Table 1), using the One-Step RT-PCR kit (Qiagen). Serial dilutions of total RNA were performed to provide 0, 12.5, 25, and 50 ng of total RNA for the RT-PCR reaction. Reverse transcription was performed for 30 min at 50°C before inactivation of RT and activation of HotStar Taq DNA polymerase at 95°C for 15 min. Linear generation of PCR product was established for each set of primers. Samples then went through either 20 (β-actin) or 32 PCR cycles (all other primers). Each cycle consisted of denaturation (1 min at 94°C), annealing (1 min at 60°C for β-actin and IL-1β, 65°C for nNOS, and 62°C for eNOS), and extension (1 min at 72°C). Final extension was performed for 10 min at 72°C.

Quantitation of RT-PCR reaction. On each 1.5% agarose gel, RT-PCR product from a single control, anemic, and hypoxic brain sample were loaded for comparison. For each of these three experimental conditions, RT-PCR product generated from 0, 12.5, 25, and 50 ng of total RNA was included. These serial dilutions were used to determine whether the generation of RT-PCR product was linear with respect to the amount of total RNA used for reverse transcription. Gels were then read on a computerized digital imaging device (AlphaImager, 8-bit digital camera, Canberra Packard, Toronto, ON), and the band density was expressed in pixels. A linear regression was performed between the digitized band density and the total RNA loaded (ng). An r value of >0.9 was required for each curve before inclusion of the data for analysis. Data are presented as nanograms of PCR product per nanogram RNA for each sample. For each gel, the number of pixels associated with the 500-bp band (25 ng DNA) was used to standardize the slope, generating a number representing the amount of PCR product (ng) per total RNA loaded (ng). An r value of >0.9 was required for each curve before inclusion of the data for analysis. Data are presented as nanograms of PCR product per nanograms of total RNA. The individual ratios between anemia-control and hypoxia-control were also calculated for each gel, and the averages of these ratios are presented. Two different brain samples were assessed from each of five different animals to provide 10 gels for analysis.

Data analysis. PbrO2 was normalized due to the known heterogeneity of cerebral tissue oxygenation levels between individual animals and within the brain (8). CBF measurements are presented as normalized data, recognizing that laser-Doppler flowmetry primarily reflects relative changes in red blood cell flux. We used the Statistical Analysis System software (SAS Institute Inc, Cary, NC) to initially assess data for any time and group effect using a two-way ANOVA. We performed comparisons between and within groups using Wilcoxon’s rank sum and signed-rank tests, respectively. Statistical significance was assigned at a P value <0.05. Data are presented as means ± SE.

RESULTS

Arterial blood-gas analysis and hemoglobin concentrations. In both control and anemic groups, the pH, PaCO2, and PaO2 remained unchanged throughout the experiment without any significant differences between groups (Table 2). After induction of acute hemodilutional anemia, the hemoglobin concentration decreased from a baseline value of 127 ± 7 g/l to a minimum of 51 ± 1 g/l (P < 0.05) and remained at a low level for the remainder of the experiment. This corresponded to a reduction in the blood oxygen content from 7.6 ± 0.4 to 3.1 ± 0.1 mmol/l (P < 0.05) (Table 2). After exposure to hypoxia, the PaO2 was reduced from 109.5 ± 7.7 to 48.9 ± 5.4 Torr, resulting in a corresponding decrease in arterial oxygen content from 6.8 ± 0.5 to a minimum of 4.6 ± 0.7 mmol/l (Table 2; n = 6, P < 0.05, for both). Hypoxia was also associated with a decrease in PaCO2 from 39.1 ± 2.6 to 28.4 ± 2.3 Torr (P < 0.05), whereas the pH remained unchanged.

Hemoglobin concentrations in the control and hypoxic groups remained unchanged throughout the experiment.

Physiological data, including PbrO2 and rCBF. Brain temperature remained near 37°C without any differences over time or between groups (Figs. 1A and 2A). Heart rate increased significantly over the duration of the experiment in both the control and anemic groups (P < 0.05) and tended to decrease in the hypoxic group. Despite these changes, there were no differences in heart rate between groups (Figs. 1C and 2C). CVP increased significantly in the anemic group, following exchange transfusion, from 3.5 ± 0.4 to 6.1 ± 0.5 mmHg (P < 0.05) but returned to baseline values by the end of the experiment (Fig. 1D). There were no

Table 1. Primers utilized for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence (5'–3')</th>
<th>Predicted Size, bp</th>
<th>Anneal Temperature, °C</th>
<th>Cycle No.</th>
<th>GenBank Accession</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>CTG GAG AAG AGC TAT GAG C</td>
<td>330</td>
<td>60</td>
<td>20</td>
<td>J00691</td>
<td>19</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AGG ATA GAG CCA CCA ATC C</td>
<td>331</td>
<td>60</td>
<td>20</td>
<td>J00691</td>
<td>19</td>
</tr>
<tr>
<td>nNOS</td>
<td>CCC GCA GAC GGA CCC AAG TTT</td>
<td>511</td>
<td>62</td>
<td>32</td>
<td>AF085195</td>
<td>GeneTool, 45</td>
</tr>
<tr>
<td>eNOS</td>
<td>GAG ACC ACC GTG ATG GCT GAA C</td>
<td>62</td>
<td>32</td>
<td>AF085195</td>
<td>GeneTool, 45</td>
<td></td>
</tr>
</tbody>
</table>

IL-1β, interleukin-1β; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.

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HEMODILUTIONAL ANEMIA INCREASES CEREBRAL CORTICAL nNOS mRNA

Table 2. Blood-gas analysis for control, anemic, and hypoxic rats

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Sample</th>
<th>pH</th>
<th>PaCO2, Torr</th>
<th>PaO2, Torr</th>
<th>Hemoglobin Concentration, g/l</th>
<th>O2 Content, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Baseline 1</td>
<td>7.38 ± 0.03</td>
<td>33.0 ± 3.2</td>
<td>124.7 ± 8.9</td>
<td>116 ± 10</td>
<td>6.9 ± 0.6</td>
</tr>
<tr>
<td>20</td>
<td>Baseline 2</td>
<td>7.31 ± 0.04</td>
<td>39.2 ± 3.5</td>
<td>131.4 ± 7.5</td>
<td>116 ± 9</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>35</td>
<td>Hemodilution 1</td>
<td>7.34 ± 0.02</td>
<td>35.1 ± 1.9</td>
<td>134.5 ± 16.6</td>
<td>116 ± 7</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>50</td>
<td>Hemodilution 2</td>
<td>7.35 ± 0.02</td>
<td>37.6 ± 2.1</td>
<td>115.0 ± 8.9</td>
<td>117 ± 7</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>70</td>
<td>Hemodilution 3</td>
<td>7.37 ± 0.03</td>
<td>35.3 ± 2.5</td>
<td>126.5 ± 11.5</td>
<td>119 ± 5</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>Hemodilution anemia (n = 7)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>Baseline 1</td>
<td>7.40 ± 0.03</td>
<td>34.5 ± 1.8</td>
<td>125.5 ± 10.4</td>
<td>127 ± 7</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td>20</td>
<td>Baseline 2</td>
<td>7.38 ± 0.02</td>
<td>34.7 ± 1.6</td>
<td>134.5 ± 16.1</td>
<td>123 ± 5</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>35</td>
<td>Hemodilution 1</td>
<td>7.34 ± 0.02</td>
<td>39.7 ± 1.5</td>
<td>138.0 ± 16.5</td>
<td>51 ± 1*</td>
<td>3.1 ± 0.1*</td>
</tr>
<tr>
<td>50</td>
<td>Hemodilution 2</td>
<td>7.38 ± 0.02</td>
<td>36.7 ± 1.8</td>
<td>124.9 ± 10.6</td>
<td>55 ± 2*</td>
<td>3.3 ± 0.1*</td>
</tr>
<tr>
<td>70</td>
<td>Hemodilution 3</td>
<td>7.38 ± 0.02</td>
<td>37.2 ± 2.2</td>
<td>124.2 ± 11.7</td>
<td>60 ± 3*</td>
<td>3.6 ± 0.2*</td>
</tr>
<tr>
<td>Hypoxia (n = 6)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Baseline 1</td>
<td>7.36 ± 0.03</td>
<td>39.1 ± 2.6</td>
<td>109.5 ± 7.7</td>
<td>125 ± 3</td>
<td>6.8 ± 0.5</td>
</tr>
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<td>Hypoxia 1</td>
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<td>52.3 ± 5.9*</td>
<td>125 ± 6</td>
<td>5.2 ± 0.8*</td>
</tr>
<tr>
<td>70</td>
<td>Hypoxia 3</td>
<td>7.37 ± 0.04</td>
<td>28.4 ± 2.3*</td>
<td>53.6 ± 4.6*</td>
<td>119 ± 12</td>
<td>4.9 ± 0.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of rats, PaCO2, arterial PCO2; PaO2, arterial PO2. *P < 0.05 compared with baseline.

statistically significant changes in MAP between or within anemic and control groups at baseline or after completion of hemodilution (Fig. 1B). In the hypoxic group, MAP decreased from a baseline of 64.1 ± 3.8 to 49.7 ± 4.0 mmHg after hypoxia exposure (Fig. 2B, P < 0.05).

In the control group, PbrO2 (19.5 ± 3.7 Torr), normalized PbrO2, and rCBF did not change significantly over the duration of the experiment (Fig. 1, E, F, and G). In the anemic group, there was a significant reduction in PbrO2 from a baseline value of 17.3 ± 4.1 to a minimum of 14.4 ± 4.1 Torr, during the 10-min period of exchange transfusion (Fig. 1E, P < 0.05). The PbrO2 remained reduced for a total of ~20 min (P < 0.05). Brain oxygenation then returned to a level that was not significantly different from baseline values, where it remained for the duration of the experiment. Normalized PbrO2 followed a similar pattern (Fig. 1F). By contrast, exposure to hypoxia resulted in a significant sustained reduction in PbrO2 from 22.8 ± 4.0 to 8.2 ± 0.8 Torr (Fig. 2, E and F, n = 6, P < 0.05).

After induction of acute anemia, normalized CBF increased from 0.97 ± 0.05 to a maximum of 1.58 ± 0.40 and remained elevated above baseline to the end of the experiment (Fig. 1G, P < 0.05). This increase in CBF began within 4 min of initiation of hemodilution, reaching its maximum 10 min after completion of hemodilution. Achievement of maximal CBF corresponded to the approximate time at which the PbrO2 recovered to baseline values. After exposure to hypoxia, cortical rCBF increased from a baseline value of 0.95 ± 0.03 to a maximum of 2.98 ± 0.68. This increase was sustained for the duration of the experiment (Fig. 2G, P < 0.05).

Determination of cerebral cortical mRNA levels using RT-PCR. Measurements utilizing serial dilutions of total RNA (0, 12.5, 25, and 50 ng) demonstrated that RT-PCR product was generated in proportion to the amount of total RNA utilized for reverse transcription (Fig. 3). Linear generation of PCR product with increasing PCR cycle number was confirmed for each primer (Fig. 4A).

Three hours after induction of hypoxia or anemia, an increase in nNOS mRNA level was observed (Figs. 4B and 5). For nNOS, the digitized band density increased from a baseline of 1.2 ± 0.1 × 10^-3 in control animals to values of 2.6 ± 0.6 × 10^-1 and 2.0 ± 0.4 × 10^-1 ng RT-PCR product/ng total RNA in anemic and hypoxic rats, respectively (Fig. 5B, P < 0.05 for both). When expressed as a ratio above control values, nNOS mRNA increased to a ratio of 2.4 ± 0.6 and 1.7 ± 0.2 for anemic and hypoxic brain samples, respectively (Fig. 5C, P < 0.05). Conversely, 3 h after initiation of hypoxia or acute anemia, mRNA levels for β-actin, IL-1β, and eNOS in cerebral cortex were not increased relative to controls (Fig. 5, B and C). Band density values for β-actin in the control, anemic, and hypoxic groups were 3.4 ± 0.4 × 10^-2, 3.5 ± 0.2 × 10^-2, and 2.7 ± 0.2 × 10^-2 ng RT-PCR product/ng total RNA, respectively.

DISCUSSION

Increased nNOS gene expression has been demonstrated in response to a diverse number of stimuli, including cerebral trauma (30), cerebral hypoxia (12, 32, 37), seizure disorders (41), and cerebral ischemia (38). Demonstration of increased cerebral cortical nNOS mRNA after acute hemodilutional anemia has not been previously reported and represents the major novel finding in this study. The increase in nNOS mRNA occurred after a transient reduction in cerebral oxygenation, suggesting that anemia-induced cerebral hypoxia may have been the stimulus for increased nNOS transcription (12).
editor nNOS gene promoter sequences supports the possibility that transcriptional regulation may play a significant role in modulating nNOS activity in response to acute anemia, as it does for other physiological stimuli (49). Hypoxia also initiated an increase in cerebral cortical nNOS mRNA levels in this study. This finding is consistent with previous reports in which hypoxia increased the number of NADPH-diaphorase-positive perivascular neurons in shark brain (37), increased rat cerebellar nNOS mRNA levels, and increased nNOS protein levels in rat cerebral cortex and cerebellum (12, 32). The elevation in cerebral nNOS mRNA observed after acute anemia may provide further evidence of anemia-induced cerebral hypoxia and characterizes the cerebral response to acute hemodilutional anemia.

By contrast, acute anemia and hypoxia did not cause any measurable increases in either IL-1β or eNOS mRNA levels. Hypoxia is known to stimulate production of IL-1β from astrocytes in vitro (52). The lack of an increase in cortical IL-1β mRNA in this study suggests that the limited period of hypoxia was insufficient to initiate increased IL-1β gene expression in vivo. Increased cerebral eNOS expression might also be expected after acute hemodilutional anemia due to changes in shear forces associated with the increase in CBF (4). However, increased eNOS mRNA was not detected after anemia in this study. This may reflect the effect of local tissue hypoxia, which has been demonstrated to cause a downregulation of eNOS expression (24, 45). Alternately, increased eNOS mRNA expression may have occurred at a slower rate, preventing its detection at 3 h.

Fig. 1. Rats undergoing hemodilutional anemia (solid horizontal bars, 10 min, •, n = 7) demonstrated stable brain temperature (Temp) (A), mean arterial blood pressure (B), and heart rate (C), which were not different from control values (○, n = 6). Central venous pressure (D) increased immediately after hemodilution but returned to baseline by the end of the experiment. Absolute (E) and normalized (F) caudate tissue oxygen tension decreased transiently during hemodilution, returning to baseline values within 20 min. Regional cerebral blood flow (rCBF; G) began to increase shortly after initiation of hemodilution, reaching its maximal increase of ~50% above baseline. The achievement of maximal cortical rCBF corresponded to the approximate time at which caudate tissue oxygen tension returned to baseline. *P < 0.05 relative to baseline.

Fig. 2. Rats exposed to hypoxia [15% oxygen (arrow), •, n = 6] demonstrated stable brain temperature (A), heart rate (C), and central venous pressure (D), whereas mean arterial blood pressure (B) decreased significantly compared with controls (○, n = 6). Absolute (E) and normalized (F) caudate tissue oxygen tension decreased with initiation of hypoxia (arrow), reaching a stable level of ~50% of the control value. Normalized cortical rCBF (G) increased with initiation of hypoxia (arrow) to a level more than double the control values. Cerebral hypoxia stimulated an increase in rCBF despite the reduced systemic blood pressure. *P < 0.05 relative to baseline.
nNOS influences a number of physiological functions within the brain, including synaptic transmission (5), neuronal development (42), and regulation of CBF (3, 11, 15, 17, 43). Studies utilizing the relatively specific nNOS inhibitor, 7-nitroindazole, suggest that nNOS activity is important in the regulation of CBF under basal conditions (11, 17, 20) and actively supports regulation of CBF in response to hypercapnia (29, 51), hypoxia (15), and acute hemodilution (17). These effects may be mediated by NO released from perivascular "nitrooxidergic" neurons capable of initiating cerebrovascular dilation in vitro (3, 18, 36, 43). Although a causal relationship between the anemia-induced increase in nNOS mRNA and increased rCBF was not established in this study, upregulated nNOS expression may be required to support the sustained eleva-

![Image of agarose gels demonstrating RT-PCR products for different dilutions of total RNA extracted from rat cerebral cortex.](http://example.com/figure3)

Fig. 3. The agarose gels demonstrate RT-PCR products generated from different dilutions of total RNA (0, 12.5, 25, and 50 ng) extracted from rat cerebral cortex. Primers for rat β-actin (A), interleukin (IL)-1β (B), neuronal nitric oxide synthase (nNOS; C), and endothelial NOS (eNOS; D) were utilized. Serial dilutions demonstrate decreasing band density of RT-PCR product, which were normalized to the 500-bp DNA marker (asterisk).

![Image of agarose gels and graphs demonstrating linear generation of RT-PCR product for eNOS and nNOS between 30 and 34 PCR cycles, using 50 ng of total extracted RNA. Similar data were obtained for β-actin (20 cycles) and IL-1β (32 cycles).](http://example.com/figure4)

Fig. 4. A: agarose gels (top) and graphs (bottom) demonstrate linear generation of RT-PCR product for eNOS and nNOS between 30 and 34 PCR cycles, using 50 ng of total extracted RNA. Similar data were obtained for β-actin (20 cycles) and IL-1β (32 cycles). B: representative gels demonstrate RT-PCR product from cerebral cortical RNA (50 ng), using specific primers for eNOS and nNOS in control, anemic, and hypoxic rats. Anemia and hypoxia resulted in an increase in nNOS RT-PCR product relative to the control.
tion in CBF observed during acute and chronic anemia (17, 25).

Confllicting reports exist regarding the measurement of reduced cerebral tissue oxygen tension following hemodilution. Shen et al. (39) measured a slight increase in cerebral cortical tissue oxygen tension after the establishment of hemodilutional anemia using invasive oxygen-sensitive electrodes, when methoxamine was utilized to maintain a stable blood pressure in rats. They suggested that this increase was due to anemia-induced cerebral hyperemia and may reflect the relatively abundant collateral circulation within the cerebral cortex. Conversely, van Bommel et al. (48) measured a significant reduction in cortical tissue oxygen tension below a critical hematocrit of ~12% using a noninvasive, intravascular florescent dye in pigs. Although increases in carotid artery blood flow and cerebral oxygen extraction were measured, these mechanisms were overwhelmed at extremely low hematocrits, and tissue hypoxia ensued (48).

The present study demonstrated a transient reduction in PbrO2 for ~20 min after induction of hemodilution, lending support to the hypothesis that physiologically relevant anemia-induced cerebral hypoxia had occurred. We elected to measure cerebral tissue oxygenation in the caudate nucleus because it provided a relatively large homogenous gray matter structure for placement of our oxygen electrode and because of the similar metabolic requirements for oxygen as the cerebral cortex (40), although a potentially more limited end-arterial blood supply exists (11, 35). As such, the caudate nucleus may represent a region of the brain thought to be more susceptible to hypoxic injury (1).

The reduction in PbrO2 occurred in the absence of systemic hypotension, with a reduction in hemoglobin concentration to a minimum of 51.0 g/l. As in other studies, volume exchange with synthetic starch colloid was utilized to maintain adequate preload and prevent any significant reduction in blood pressure (26, 34, 44, 46). Although this resulted in a transient increase in CVP, maintenance of MAP was achieved without the use of sympathomimetic drugs. Maintenance of MAP in the anemic group was critical in determining the effect of acute anemia on cerebral oxygenation, since previous studies have clearly demonstrated that systemic hypotension reduced cerebral
tissue oxygen tension in hogs and rabbits after hemorrhagic anemia and acute hemodilution (13, 22, 26). In the present study, the postdilutional blood pressure did not decrease significantly and was not statistically different from control values at any point. Therefore, hypotension should not have influenced the \( PbrO_2 \) measurements after completion of hemodilution.

Our baseline measurements of \( PbrO_2 \) for anemic and control rats (17.3 ± 4.1 and 19.5 ± 3.7 Torr, respectively) were within the range reported for rat cerebral cortex (15.5 ± 1.5 to 29.6 ± 6 Torr) (7, 8, 39) and closer to the reported values for rat hippocampus (20 ± 3 and 22 ± 3 Torr) (8, 31). These published values were achieved with the use of different invasive oxygen electrodes with diameters ranging from 50 to 300 μm. They reflect the finding that measurements of cerebral tissue oxygen tension are ~10–15 Torr lower than corresponding sagittal sinus venous \( PO_2 \) values (7, 28). Therefore, the electrodes utilized in this study reflect published values for rat cerebral tissue oxygen tensions and should be adequately sensitive to detect the relative decreases in \( PbrO_2 \) observed after hemodilutional anemia, assuming that both groups experienced similar degrees of tissue trauma after placement of the oxygen electrode. Furthermore, studies that used oxygen microelectrodes, similar to those used in this study, have demonstrated cerebral cortical tissue oxygen tensions between 25.7 ± 8.3 and 33.3 ± 13.3 Torr in human patients (6), suggesting that our data and techniques may have potential clinical relevance.

Baseline \( PbrO_2 \) values measured in this study may be lower than some published reports due to mild hyperventilation of rats, which resulted in baseline \( PaCO_2 \) values of 33.0 ± 3.2 and 34.5 ± 1.8 Torr in control and anemic animals, respectively. Hypocapnia is known to reduce cerebral tissue oxygen tension due to cerebral vasoconstriction (13, 22, 28) and likely contributed to lowering the baseline \( PbrO_2 \) measurements. However, this effect was similar in control and anemic groups and does not explain the relative reduction in \( PbrO_2 \) following hemodilution.

The cerebral hyperemic response to anemia (16, 25, 39, 44) and hypoxia (44, 47) has been well documented. The increase in CBF associated with anemia has been attributed to both passive changes in blood rheology (16, 34, 39) and actively regulated cerebral vasodilation (9, 17, 34). NO is one of many factors known to influence vascular smooth muscle tone and may mediate the increase in CBF observed with anemia (17). The increase in rCBF associated with hemodilutional anemia augments cerebral oxygen delivery and may compensate for the reduction in blood oxygen-carrying capacity (39, 44). In this study, laser-Doppler flowmetry was used to determine the temporal relationship between changes in rCBF and \( PbrO_2 \). The observed increase in rCBF occurred within 4 min of initiation of hemodilution and reached is maximum value ~20 min after completion of hemodilution, coinciding with the approximate time that \( PbrO_2 \) returned to baseline. This suggests that increased cerebral oxygen delivery may help to maintain cerebral oxygenation after hemodilution.

Laser-Doppler flowmetry estimates rCBF on the basis of changes in red blood cell flux, which is affected by red cell velocity and hematocrit. The reduction in hematocrit observed after hemodilution would be expected to result in an underestimation of the true changes in CBF. Therefore, the absolute magnitude of the increase in rCBF cannot be determined in this study. However, this limitation would not have prevented us from detecting the expected relative increase in rCBF following hemodilution, as has been demonstrated by others (21, 39). The rationale for utilizing laser-Doppler flowmetry in this study is further supported by studies that have correlated changes in CBF and tissue blood flow detected by laser-Doppler flowmetry following hemodilution with other quantitative measurements of tissue blood flow, including labeled microsphere and hydrogen clearance methodologies (21, 23). In the present study, laser-Doppler probes were placed on the surface of the cerebral cortex instead of into the caudate nucleus, in an attempt to minimize local tissue damage at the site of the oxygen electrode. This prevented the measurement of flow and tissue oxygen tension at the same location. However, Rebel et al. (35) have measured comparable changes in cortical and caudate tissue blood flow following hemodilution, suggesting that our measure of rCBF in the cortex may reflect changes in CBF within the caudate nucleus. Further experimental studies would be required to establish this relationship.

In this study, the hypoxic group was designed to serve as a positive control, confirming our ability to measure reduced \( PbrO_2 \) (7) and increased rCBF (47) and to detect changes in cerebral NOS gene expression (12). For this reason, blood pressure and relative changes in blood oxygen content were not controlled for, as no direct comparisons between the hypoxic and anemic data were made. After hypoxia, blood pressure dropped significantly, contributing to the reduction in \( PbrO_2 \) to 8.2 ± 0.8 Torr. Similar values for cortical tissue oxygen tension were measured by Dunn et al. (7) at a comparable degree of hypoxemia. Hypoxic brain samples were used as a positive control for changes in NOS mRNA levels because published reports demonstrate changes in cerebral nNOS expression following hypoxia (12, 32, 37). Hypoxia also resulted in a reduction in the \( PaCO_2 \), likely secondary to hyperventilation of rats above the level of controlled ventilation and a reduction in CO2 production due to reduced oxygen metabolism in the hypoxic animals.

In summary, acute normotensive, hemodilutional anemia was associated with an increase in nNOS mRNA within the cerebral cortex in anesthetized rats. This increase occurred in association with transient cerebral hypoxia, suggesting that tissue hypoxia may have been the stimulus for the observed increase in gene expression. Recovery of cerebral tissue oxygenation occurred in association with maximally increased rCBF, suggesting that increased cerebral oxygen delivery contributed to maintaining \( PbrO_2 \). Increased nNOS
gene expression may play a role in sustaining the increase in CBF observed with anemia, but this remains to be confirmed. The mechanism by which anemia stimulates an increase in cerebral nNOS gene expression requires further elucidation.

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