Prolonged hypoxia increases vascular endothelial growth factor mRNA and protein in adult mouse brain

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Kuo, Ning-Tsu, David Benhayon, Ronald J. Przybylski, Richard J. Martin, and Joseph C. LaManna. Prolonged hypoxia increases vascular endothelial growth factor mRNA and protein in adult mouse brain. J. Appl. Physiol. 86(1): 260–264, 1999.—Brain hypoxia induces an increase in brain vasculosity, presumably mediated by vascular endothelial growth factor (VEGF), but it is unclear whether VEGF is required to maintain the increase. In these studies, brain VEGF mRNA and protein levels were measured in adult mice kept in hypobaric chambers at 0.5 atm for 0, 0.5, 1, 2, 4, 7, and 21 days. Hypoxia was accompanied by a transient increase of VEGF mRNA expression: twofold by 0.5 day and a maximum of fivefold by 2 days; these were followed by a decrease at 4 days and a return to basal levels by 7–21 days. VEGF protein expression induced by hypoxia was bimodal, initially paralleling VEGF mRNA. There was an initial small increase at 12 h that reached a maximum by day 2, and, after a transient decrease on day 4, the protein expression increased again on day 7 before it returned to normoxic levels after 21 days. Thus, despite continued hypoxia, both VEGF mRNA and protein levels returned to basal after 7 days. These data suggest a metabolic negative-feedback system for VEGF expression during prolonged hypoxia in the brain.

vascular growth factor; cerebral hypoxic adaptation; capillary angiogenesis; chronic hypoxia; messenger ribonucleic acid

PROLONGED MILD HYPOXIA is associated with polycythemia, increased brain capillary density, and increased cerebral glucose metabolism (13, 14, 25). These adaptive changes provide compensatory mechanisms to enhance tissue oxygenation by increasing blood oxygen-carrying capacity along with decreasing intercapillary distances. The molecular mechanisms of increased vascularity in the brain are not completely understood; however, recent reports demonstrated that vascular endothelial growth factor (VEGF), one of the hypoxia-inducible genes, plays a central role in the initiation of angiogenesis (2, 7, 15, 22, 26, 28). Furthermore, these results suggest that VEGF may play an important role in the hypoxia-induced increase in cerebral capillary vascularity in the early postnatal brain (23). Indeed, exposure of adult rats to prolonged periods of hypoxia has been shown to stimulate the expression of (VEGF) mRNA and protein (32), and exogenously administered VEGF induces brain angiogenesis (29). However, after vascular remodeling has been completed, it is unknown whether increased levels of VEGF are required to maintain the remodelled vasculature or whether VEGF levels can be regulated independently of continued hypoxic exposure.

The regulation of VEGF expression appears to be multifactorial. In addition to hypoxia, the expression of VEGF mRNA is upregulated by various vasoactive peptides and growth factors, such as tumor necrosis factor, endothelin-1, endothelin-3, basic fibroblast growth factor, growth hormone, and interleukin-1 (27, 30). Increased VEGF mRNA expression under hypoxic conditions can also be the result of an increase of VEGF mRNA stability (21). VEGF, a protein homodimer with a molecular mass of 45 kDa, is a potent mitogen that is transcriptionally induced through the activation of a transcription factor, hypoxia-inducible factor-1 (HIF-1) (6, 8). In the 3' untranslated region of the VEGF promoter, five hypoxia-inducible RNA protein binding sites were identified in human and rat genes. VEGF acts specifically on endothelial cells (20) through receptors present on their surface (2, 7). VEGF acts via two cell surface receptors, Flt and Flik. Flt was elevated in endothelial cells within a brain infarct (18), elevated expression of flk occurred in glioblastoma (24), and prolonged hypoxia enhanced the expression of flt-1 mRNA in rat brain (32), suggesting coincident regulation of VEGF and its receptor in the progression of angiogenesis.

Although it is clear that hypoxia increases cerebral microvessel density, and that VEGF plays an important role in mediating hypoxic angiogenesis, the role of VEGF in the continued maintenance of the brain microvessel population is unknown. Thus we studied the time course of the expression of VEGF mRNA and protein levels in the adult mouse brain subjected to 3 wk of continued hypoxia, i.e., longer than the time necessary for capillary bed remodeling.

MATERIALS AND METHODS

Animal preparation. Fifty-six adult mice were placed in hypobaric chambers in the Animal Resource Center at a pressure of 50.5 kPa (0.5 atm) except for ~1 h/day when the pressure was gradually returned over 10 min to atmospheric level for cage cleaning and for water and food replenishment. Room air flowing through the system under partial vacuum (~20 L/min, i.e., 10% of the chamber volume per minute) was sufficient to prevent accumulation of carbon dioxide and to maintain the ambient animal facility room temperature within the chamber. The duration of hypoxia was 0.5, 1, 2, 4, 7, and 21 days. The normoxic, control mice were housed in cages immediately adjacent to the hypobaric chambers and were fed and changed on the same schedule as were the hypoxic mice. The mice were weighed, and tail venous blood

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samples were obtained for hematocrit determination before the animals were killed. No mortality was noted over the 21 days of hypoxic exposure. Animals were killed by decapitation, and the brains were quickly removed, immediately frozen in liquid nitrogen, and stored at −80°C for RNA and protein isolation.

RNA isolation and Northern blot analysis. Total RNA from whole brains was isolated by the guanidine isothiocyanate method (4). Briefly, one-half of the brain was homogenized in a solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% N-laurylsarcosine, and 0.1 M β-mercaptoethanol. After sequential addition of sodium acetate, phenol, and chloroform-isomyl alcohol, the suspensions were vortexed and centrifuged at 14,000 g for 20 min. The RNA pellets were washed once with 70% ethanol, dissolved in diethyl pyrocarbonate-treated double-distilled water, and quantified with an ultraviolet (UV) spectrophotometer at 260 nm, and the ratio of light absorption at 260 nm to that at 280 nm was used as an indicator of the purity of the RNA. Total RNA samples (15 µg) were separated by electrophoresis through 1% agarose-formaldehyde gels followed by overnight capillary transfer onto a Nytran membrane. The membrane was UV cross-linked, baked at 80°C for 2 h and then hybridized for 2 h at 65°C in Church buffer (5), followed by an overnight hybridization at 65°C. The specific mRNA for VEGF was detected by using a VEGF cDNA probe (kindly provided by Dr. Eaton H. Agani, Johns Hopkins University, Baltimore, MD) (8) and was 32P radiolabeled by the random priming method. The membrane was washed twice for 20 min at room temperature with 2× sodium chloride-sodium citrate buffer (SSC)-0.1% SDS and twice for 20 min at 65°C with 0.2× SSC-0.1% SDS. After the final wash, the membranes were subjected to autoradiography at −80°C with Kodak film and intensifying screens. To correct for the slight variations in RNA in gel loading, the membranes were stripped by immersion in 0.1% SDS at 80°C for 20 min and reprobed with 18S ribosomal cDNA (16). The relative intensity was measured by densitometry (Sci-Scan, United States Biochemical). The ratio of relative intensities for VEGF to 18S ribosomal RNA was used for data analysis.

Protein preparation and Western blot analysis. The mouse brain was homogenized in a buffer containing 25 mM HEPES, 1% Triton X-100, 1 mM aprotinin, 0.1 mM leupeptin, 1 mM pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After being centrifuged at 14,000 g for 30 min, the Triton X-100-soluble fractions (100 µg protein) were electrophoresed in 10% SDS-polyacrylamide gels under nonreducing conditions. Two identical sets of gels were run simultaneously along with prestained molecular mass markers in each set of gels. A total of eight sets of gels were performed. After an overnight electrotransfer to polyvinyl difluoride membranes, the membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) (20 mM Tris, pH 7.6, and 150 mM NaCl) and incubated with polyclonal anti-VEGF antibody (Santa Cruz, CA) at 2 µg/ml dilution in TBS supplemented with 2% bovine serum albumin for 2 h at room temperature. This was followed by 1× 15-min and 2× 5-min washes with TBS plus 0.1% Tween 20. The membranes were then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase at 1:3,000 dilution in TBS plus 5% skim milk for 1 h at room temperature. After the final wash, the immunoreactive bands were detected by enhanced chemiluminescence with Kodak film, and the relative intensity was quantitated by densitometry (Sci-Scan, USB).

Data analysis. Data are presented as means ± SE, and statistical analysis was examined via unpaired t-test. Data were collected as sets of seven mice per group, with one mouse at each time interval in every set. The expression of VEGF mRNA was normalized to the expression of 18S ribosomal RNA (n = 4). The densitometry units obtained for the expression of VEGF protein (n = 7) were used to calculate the percent increase over the normoxic sample in the same gel. A P value < 0.05 was considered statistically significant.

RESULTS

Body weight and hematocrit measurements. After 7 days and 21 days of hypoxic exposure, body weights were significantly decreased (31.8 ± 1.0 and 31.9 ± 1.0 g, respectively) vs. the normoxic groups (37.3 ± 0.9 g; P < 0.01). The mice exposed to prolonged hypoxia developed polycythemia, with significant increases in hematocrit after 4, 7, and 21 days of exposure (59.8 ± 0.6, 61 ± 0.4, and 63 ± 1.3%, respectively) vs. the normoxic controls (51.3 ± 0.9%, P < 0.01). VEGF mRNA and protein expression. Northern blot analysis for VEGF mRNA and 18S ribosomal RNA in adult mouse brains from 0, 0.5, 1, 2, 4, 7, and 21 days of hypoxic exposure is shown as an image of a representative Northern blot (Fig. 1A) in comparison with the densitometric values obtained from all Northern blots (Fig. 1B). The expression of 18S ribosomal RNA was used to normalize for differences in the quantity of total RNA loaded in each lane, and the ratio of VEGF to 18S rRNA was calculated. The expression of VEGF mRNA
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DISCUSSION
In this study, our main finding is that the expression
VEGF mRNA and protein in the mouse brain stimulated
by mild hypoxia was elevated within 12 h of
exposure, and it remained elevated during continuous
hypoxia for at least 4 days but thereafter returned to
basal levels despite continued hypoxic exposure for 7
and 21 days. These data clearly indicate that continu-
eous, elevated expression of VEGF is not necessary to
maintain increased brain vascular density during pro-
longed mild hypoxia. The study by Xu and Severing-
haus (32) showed a similar rapid increase in VEGF
mRNA in rat brains exposed to 6–9% normobaric
hypoxia, with a peak threefold expression at 12–24 h.
This was followed by a decrease that continued toward,
but did not reach, control values on day 6, the end of
their study. In earlier studies in rats, we reported that
an increase in brain vascularity occurred after expo-
sure to 0.5 atm, which decreased the arterial oxygen
partial pressure by one-half to ~50 Torr (12, 19).
Significant increases of cerebral microvessel density in
adult rats occurred after 1 wk of hypoxia. The increases
reached a maximum after 2 wk and persisted for at
least 3 wk. These results lead us to speculate that brain
tissue responds to the hypoxic condition by producing
VEGF, which in turn stimulates endothelial cell prolif-
eration leading to an increase of cerebral vascularity.
Our present results on the expression of VEGF mRNA
and protein in mice and the results of Xu and Severing-
haus, who used rats (32), support this prediction.
However, it is obvious in our rat studies that brain
vascularity continues to increase for at least 1 wk after
mRNA levels return to control values but with VEGF
protein values exceeding the control values. Combining
these observations suggests that, when brain oxygen-
ation is improved because of an increase in vascularity,
a negative-feedback mechanism decreases the expres-
sion of VEGF, thereby returning expression to basal
levels to prevent an overgrowth in microvasculature.
Thus it appears that in rodent brains exposed to
prolonged hypoxia, VEGF is regulated by a mechanism
tive to tissue metabolic balance rather than to

VEGF protein expression (% change over control)

12 h 1 day 2 day 4 day 7 day 21 day

![Enhanced chemiluminescence signals from a Western blot of brain samples used to quantitate amount of VEGF protein present in brain tissue with use of a polyclonal anti-VEGF antibody. Mice were exposed to hypobaric hypoxia for 0–21 days, and VEGF protein increased by day 1. B: average densitometric scans of the Western blots (n = 7) comparing VEGF in hypoxic brains with that in normoxic brains. It clearly shows a bimodal expression of VEGF protein, which increased significantly by day 1 of hypoxia and then returned to levels present in normoxic mouse brains by day 21. **Significantly different from control, P < 0.05.](image)
arterial oxygen partial pressure, which remains about one-half that of baseline throughout hypoxic exposure. In other studies (10) in which cultured myocardial vascular smooth muscle cells were used, it was shown that hypoxia stimulated the expression of VEGF protein and mRNA by fivefold after 24 h of hypoxia and returned to baseline within 24 h of normoxia. Electrical stimulation of skeletal muscle induces expression of VEGF mRNA, where hypoxia is thought to trigger vessel growth. Under chronic stimulation, the increase in VEGF mRNA was most pronounced after 4 days; the levels decreased gradually over the next several days but still were elevated threefold at 21 days (11). Thus evidence is accumulating to strongly support the hypothesis of a bidirectional homeostatic response that exists between the perfusion capabilities of blood vessels and the metabolic requirements of tissues and cells, which in turn leads to a modification of the vasculature to satisfy tissue and oxygen needs (1). Our results are compatible with this model of metabolic-feedback regulation of vascular beds.

Similar to VEGF, the glucose transporter protein GLUT-1 is also regulated by hypoxia by increasing its half-life and its transcription rate through the activation of HIF-1 (9, 31). However, in contrast to the transient increases in VEGF mRNA and protein discussed above, a sustained induction of cerebral capillary GLUT-1 protein was found even after 3 wk of continuous hypoxia in rats (12, 14), highlighting the existence of different regulatory mechanisms for VEGF and GLUT-1 expression by hypoxia through HIF-1.

From the extant reports (18, 23, 32), it appears that hypoxic exposure results in upregulation of brain VEGF mRNA within ~3 h and VEGF protein expression beginning at 12 h, with a maximum reached within a few days and returning to basal levels by 1 wk. However, there are some minor discrepancies among these reports. Xu and Severinghaus (32) identified two species of VEGF mRNA with a molecular size of 4.7 and 3.9 kb. In contrast, we only detected a single band in mouse brains below 285 rRNA corresponding to 4.4 kb. The discrepancy could be due to the difference in animal species or assay conditions. In the same report, Western blot analysis showed a single band of VEGF protein with a molecular mass of 23 kDa under reducing conditions. In the present report, we found two bands on the immunoblot at 27 and 45 kDa under nonreducing conditions. The 45-kDa band can be identified as the secreted homodimer of VEGF (105) [the murine form is VEGF 164 (7)]. The 23-kDa band obviously is the monomer obtained under reducing conditions. The 27-kDa protein could be a proteolytic product, because a 38-kDa band has been reported under nonreducing conditions after cerebral ischemia (18), or it could result from alternative RNA splicing and enzymatic cleavage (17).

We showed that a biphasic expression of VEGF protein occurred during hypoxic exposure. VEGF protein increased after 12 h, 1 day, and 2 days, but it decreased at 4 days, increased at 7 days, and decreased at 21 days. Although not discussed, a similar transient decrease at 3 days of hypoxic exposure is apparent in the data of Xu and Severinghaus (32). We speculate that the initial induction of VEGF could be due to an enhanced VEGF stability and half-life and that the later induction could be controlled by other transcription factors. Future studies on the kinetics of induction of hypoxia-inducible factors related to VEGF will be necessary to address this biphasic expression of VEGF during hypoxic exposure. Serum erythropoietin levels exhibited a similar transient decrease after 4–6 days of hypobaric hypoxia in rats, although they rebounded to elevated levels at 20 days (3).

In conclusion, our results indicate that hypoxia transiently induces VEGF mRNA expression for up to 7 days of exposure in the adult mouse brain. This elevation of VEGF occurs during the time of initial adaptation when capillary density increases through hypertrophy rather than hyperplasia (14). VEGF mRNA levels then subsequently fall to baseline even though hypoxia persists, whereas increases in VEGF protein are a little longer lasting, suggesting that VEGF expression is tightly regulated by tissue metabolic balance rather than arterial oxygen partial pressure and that VEGF is necessary for initiation of angiogenesis but not for maintenance of increased capillary density.

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