Astrocyte responses to injury: VEGF simultaneously modulates cell death and proliferation

Nicole Schmid-Brunclik, Carole Bürgi-Taboada, Xanthi Antoniou, Max Gassmann, and Omolara O. Ogunshola

Institute of Veterinary Physiology, Vetsuisse Faculty and Zürich Centre for Integrative Human Physiology, University of Zürich, Zürich, Switzerland

Submitted 26 July 2007; accepted in final form 23 June 2008

Hypoxic/ischemic insults cause activation of adaptive mechanisms and alteration of gene expression within the injured areas to combat the progression of pathological events (1). Accordingly, at the cellular level an oxygen-sensing system is crucial to enable rapid adaptation to altered oxygen tensions. A well-characterized molecular pathway that mediates oxygen sensing occurs through the induction of the transcription factor hypoxia-inducible factor (HIF-1). HIF-1 consists of an oxygen-dependent α-subunit and a constitutively expressed β-subunit. Both subunits are basic-helix-loop-helix proteins of the PAS family, and complex binding enhances the transcription of target genes involved in glycolysis, erythropoiesis, and angiogenesis (16, 46). HIF-1α is mainly regulated at the protein level by oxygen. During normal oxygen supply, HIF-1α is hydroxylated by oxygen-dependent prolyl-4-hydroxylases, ubiquitinated, and rapidly degraded by the proteosomal system (11, 41). Hypoxia stabilizes HIF-1α and promotes its translocation to the nucleus; accumulation and dimerization with HIF-1β ultimately enhancing transcriptional activity of its target genes through binding to its hypoxia response element.

A major HIF-1 target gene known to have a cytoprotective role is the potent angiogenic molecule VEGF (10, 13). VEGF binds with high affinity to two receptor tyrosine kinases, flt-1 (VEGFR-1) and flk-1 (VEGFR-2), and activates downstream pathways (reviewed in Ref. 36) promoting angiogenesis during development, as well as (patho)physiological events, such as pregnancy, wound healing, rheumatoid arthritis, cardiovascular disease, and cancer (12, 34). Notably, astrocytes secrete basal levels of VEGF under normal physiological conditions, and hypoxia further induces both mRNA and protein levels (8, 23, 39). Although application of endogenous VEGF also causes increased vascular permeability and proliferation of glial cells (24, 49), the importance of endogenous VEGF production to survival of astrocytes has not been shown conclusively.

Since astrocytes play a crucial role in brain homeostasis, their response to hypoxic/ischemic insult deserves further investigation. The aim of the present study was to investigate and compare the astrocytic response to mild and severe hypoxic/ischemic insults. Specifically, we examined the influence of oxygen- and glucose-deprivation on HIF-1 stabilization and expression of VEGF in primary rat astrocytes. We show that both of these events can have significant detrimental effects on the brain, understanding how these complex cells respond to insult is important to limit vascular leakage and subsequent brain damage.

Address for reprint requests and other correspondence: O. O. Ogunshola, Institute of Veterinary Physiology, Vetsuisse Faculty, Univ. of Zurich, Winterthurerstrasse 260, Zurich CH 8057, Switzerland (e-mail: Larao@access.uzh.ch).
astrocytes induce HIF-1α only during prolonged severe insult but that this induction is not fully responsible for VEGF production, indicating other mechanisms of control. Importantly, endogenous VEGF expression constitutes an important astrocyte mitogen and survival factor.

**MATERIALS AND METHODS**

All experiments were performed in accordance with Swiss animal protection laws and Zürich University institutional guidelines. Our protocols were approved by the Kantonales Veterinaireamt, Zurich.

**Primary Culture of Astrocytes**

Primary astrocytes were prepared from newborn Wistar rat pups as previously described (8). Briefly neonatal rats were anesthetized by hypothermia, decapitated, and cerebral cortices removed. Meninges were removed, and cortices minced and placed in ice-cold buffer (Kreb solution 120 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 11 mM glucose and 0.6 g/l BSA). Homogenized tissue was digested with 7.5 mg trypsin for 15 min at 37°C and dissociated cells were cultured in DMEM containing 10% FBS, 150 mg/lL-glutamine, and 50 mg/ml gentamicin at 37°C. The culture medium was changed every 4 to 5 days. After 10 days, cultures displayed uniform (>96%) glial fibrillary acidic protein immunoreactivity. Astrocytes were passaged a maximum of three times and used at 80–90% confluency. In some experiments, a potent and selective inhibitor of Flk-1 kinase, SU1498 (Calbiochem), was used at a concentration of 0.1 and 10 μM to inhibit VEGF activity.

**Hypoxic and Glucose Deprivation Experiments**

For all experiments, primary astrocytes were incubated under normoxic (21% O2), hypoxic (1% O2), or near-anoxic (<0.1% O2) conditions in a humidified glove box incubator (In vivo 400; Ruskinn Technologies). Glucose deprivation experiments were carried out with 0.1% O2 and selective inhibitor of Flk-1 kinase, SU1498 (Calbiochem), was used at a concentration of 0.1 and 10 μM to inhibit VEGF activity.

**Western Blot Analysis**

Cells were scraped into lysis buffer [0.27 M sucrose, 2 mM EDTA (pH 8.0), 0.1% NP-40, in 0.6 M KCl, 150 mM NaCl, 150 mM HEPES (pH 7.5)] and centrifuged for 10 min at 16,000 g. Cytoplasmic fractions were frozen, and nuclear pellets resuspended in nuclear extraction buffer [20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA (pH 8.0)] and left 15 min on ice. Nuclear extracts were obtained after centrifugation (16,000 g) for 10 min and separated by denaturing SDS-PAGE. After transfer to nitrocellulose, membranes were incubated in 4% milk in PBS for 1 h at room temperature and then were incubated overnight at 4°C with antibodies against HIF-1α IgY (6) (diluted 1:100 in PBS). Following washes, membranes were incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Membranes were exposed to X-ray film after luminescence detection (25 μl of 90 mM coumaric acid in DMSO, 50 μl of 250 mM luminol in DMSO in 10 ml of 100 mM Tris, pH 8.5). For loading, controls blots were stripped and reprobed for the nuclear protein Sp-1. Results were quantified by densitometry using Quantity One software (BioRad Laboratories).

**VEGF ELISA**

Quantitation of VEGF was performed using a VEGF ELISA kit (R&D Systems) according to the manufacturer’s instructions. Optical density was measured using a microplate reader (Multiskan RC; Thermo Labsystems) at 450 nm with wavelength correction at 570 nm.

**EMSA**

EMSA was performed radioactively with nuclear extracts as previously described (22). Oligonucleotide probes derived from the EPO enhancer gene 5’-GCC CTA CGT GTC TCA-3’ (3’EPO enh) and 5’-TG A GAC AGC ACG TAG GGC-3’ (5’EPO enh.rev) were synthesized by Microsynth (Balchag, Switzerland). End labeling was performed with T4 PNK (Fermentas, LabForce, Nunningen, Switzerland) at 37°C for 1 h. Antisense oligonucleotides were added in twofold molar excess and annealed at 95°C for 3 min. Samples were loaded on a 4% nondenaturing gel and electrophoresis was performed at 200 V in 1× TBE buffer at 4°C. After drying, the gel was visualized using BioRad Molecular Imager FX.

**In Situ Labeling of Fragmented DNA**

Isolated terminal deoxyribonucleotid transferase-mediated dUTP nick-end labeling (TUNEL) was carried out according to the manufacturer’s instructions (Roche Biochemicals, Penzberg, Germany). Astrocytes grown on coverslips and subjected to different conditions were fixed for 30 min in 4% paraformaldehyde. Staining was carried out in a humidified atmosphere for 1 h, and nuclei were counterstained with DAPI for 3 min. Samples were analyzed by fluorescence microscopy.

**Proliferation Assays**

AlamarBlue assay (Biosource, Camarillo, CA) was used to quantitatively measure astrocyte proliferation. Experiments were performed according to the manufacturer’s instructions. In brief, primary astrocytes were seeded on 96-well plates at a density of 2,000 cells per well and incubated overnight. Four hours prior to measurement, 20 μl of AlamarBlue dye was added to each well, and plates were incubated under normoxic, hypoxic, or anoxic conditions with or without glucose as described above. Absorbance was measured with a plate reader (Multiskan RC; Thermo Labsystems) at 540 nm, subtracting the background absorbance at 630 nm.

Thymidine incorporation assay was used as an alternative method to AlamarBlue. Cells were grown to 90% confluency on 24-well plates overnight. At time 0, individual plates were exposed to normoxic, hypoxic, or anoxic conditions in the presence or absence of glucose after the addition of 1 Ci [3H]thymidine. After 6 h, the astrocytes were washed with ice-cold PBS, trypsinized, and harvested onto glass fiber filters (Wallac, Hänenberg). The amount of incorporated radioactivity was counted using a Wallac counter.

In all proliferation assays, individual experiments, including those with inhibitor application, were performed 5 times in quadruplicate, and data were converted to fold change compared with normoxic cells grown in glucose containing media.

**ATP Measurements**

ATP was measured with an ATP bioluminescent assay kit (Sigma). Cells were lysed in buffer (105 mM NaCl, 1% Triton X-100, 1% NP-40, 50 mM Tris) for 10 min before centrifugation (10 min at 16,000 g). The supernatant was mixed with TCA 5% (1:1 vol/vol) to block residual ATPase activity, centrifuged for 1 min at 16,000 g and neutralized with saturated Tris solution to pH 7.8. Sample was added to diluted ATP assay mix (1:1), and luminescence was measured using Berthold luminometer (Detection Systems, Pforzheim, Germany). Cellular ATP levels were determined from a standard curve and normalized to total protein concentrations.

**Hif-1α siRNA Experiments**

RNA interference was performed using a pool of short interfering RNAs (siRNAs) targeting rat Hif-1α (as well as a nontargeting control siRNA) purchased from Dharmacon Research (ON-TARGET plus siRNA). Then 60% confluent rat astrocytes were transfected with 100 nM siRNA using oligofectamine (Invitrogen) according to manufacturer’s instructions. Briefly, cells were washed with PBS and astrocyte medium was replaced with DMEM without antibiotic, and then siRNA duplexes were added in Opti-MEM.
(GIBCO). After 4-h transfection, the medium was resupplemented with serum and antibiotics. Cells were exposed to hypoxia and/or glucose deprivation for the times indicated, and proteins were extracted for analysis 48 h posttransfection.

Statistical Analyses

Graphics and statistical analyses were performed using Microsoft Excel software. All results are expressed as fold change over normoxic values. Statistical significance (P < 0.05%) was calculated using Student’s t-test.

RESULTS

Increased Severity of Insult Stimulates Astrocytic Proliferation

Since injury induces increased proliferation of glial cells and formation of a glial scar in vivo (24, 49), we investigated whether the severity of insult is instrumental in modulating the proliferative rates of astrocytes. AlamarBlue and thymidine incorporation assays (Fig. 1, A and B, respectively) were used to determine the proliferative capacity of astrocytes in the presence and absence of glucose for 6 h. As expected, different severities presented distinct proliferation patterns (Fig. 1). No significant difference was observed between normoxic and hypoxic glucose-containing cells, but anoxia stimulated increased proliferation significantly (~30%). Surprisingly, glucose withdrawal further induced proliferation. Normoxic glucose deprivation caused a 40% increase in proliferation over normoxic glucose-containing cells, whereas near-anoxia combined with glucose deprivation increased proliferation a further twofold using both techniques. In conclusion, increased severity of insult augmented proliferation of astrocytes in vitro.

Combined Oxygen and Glucose Withdrawal Induces Significant Cell Death of Astrocytes

To get a handle on how astrocytes survive oxygen and/or glucose deprivation for prolonged periods of time, we measured cellular ATP levels (Fig. 1, C and D). In glucose-containing media (Fig. 1C), normoxic ATP levels were virtually constant for the duration of the experiment. Hypoxia and anoxia reduced ATP levels by 50–70% within the first 6–24 h but were subsequently maintained. Glucose deprivation (Fig. 1D) elicited a different response. A 50% decrease in normoxic ATP levels occurred within 6 h of glucose withdrawal before recovering to 70% at 48 h, and hypoxia had no additional detrimental effect on ATP levels in the absence of glucose. Anoxia combined with glucose deprivation resulted in a dra-
matic 70% decrease in ATP levels already at 6 h that were virtually zero at 24 h. Thus, although astrocytes are able to tolerate oxygen and glucose deprivation, simultaneous long-term withdrawal is ultimately lethal after 6 h. In agreement, in the presence of glucose, only few TUNEL-positive astrocytes were detected (data not shown), indicating that the cells survive oxygen deprivation well. Increased cell death processes in hypoxic glucose-deprived astrocytes were clearly identified after 24 h of exposure (Fig. 1E, top), and anoxia combined with glucose deprivation caused earlier (6 h) TUNEL-positive staining and nuclear condensation (Fig. 1E, middle). By 24 h, virtually all astrocyte nuclei (90%) were TUNEL positive (Fig. 1E, bottom) with obvious nuclear condensation and cell shape disruption.

**HIF-1α Protein Stabilization in Astrocytes Occurs Only After Severe Oxygen Deprivation**

HIF-1 is the master regulator of the hypoxic response and modulates survival in many cell types. Many reports demonstrate that HIF-1α protein stabilization occurs immediately after hypoxic exposure (1% O₂) in various cell lines (19, 46). To observe time-dependent HIF-α accumulation in primary cultured astrocytes, nuclear protein extracts were prepared from normoxic (21% O₂), hypoxic (1% O₂), or near-anoxic (<0.1% O₂) cells. Surprisingly, HIF-1α protein was hardly detected by Western blot analysis under hypoxic conditions (Fig. 2A) even after 48 h of exposure. Only near-anoxic incubation induced the accumulation of HIF-1α protein in a time-dependent manner (Fig. 2B). Subsequent reoxygenation (21% O₂) for 5 h (Fig. 2B, 5R) abrogated HIF-1α protein stabilization. As expected, HIF-1β levels remained constant at all time points investigated (data not shown). To investigate whether the induced HIF-1α was active and capable of binding target hypoxia responsive element sequences, we performed EMSA analyses. Under normoxic and hypoxic conditions no HIF-DNA binding complex was detected (Fig. 2C); however, a weak binding signal was identified after 2 h of anoxia (Fig. 2D). The intensity of this band increased with extended incubations corresponding to Western blot data and confirmed that astrocytes stabilize HIF-1α only after severe oxygen deprivation.

**VEGF Protein Is Induced in the Absence of HIF-1α Stabilization**

VEGF, a major HIF-1α target gene, is strongly induced by hypoxia (5, 39). To determine whether VEGF is upregulated in astrocytes in accordance with HIF-1α stabilization, VEGF protein levels in conditioned media and cell lysates were measured by ELISA. Figure 2E shows increased VEGF expression in cytoplasmic fractions following oxygen deprivation. Notably, hypoxic incubation induced VEGF protein levels, immediately reaching a maximum after 6 h (4.5-fold increase over normoxic levels, **P = 0.001), although HIF-1α was not detected under these conditions. Interestingly, cyto-

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**Fig. 1.** Continued. Significant cell death occurs only during prolonged oxygen glucose deprivation (E). Isolated terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive nuclei were detected after 24 h of hypoxic incubation (Hx 24 h), whereas near-anoxic exposure for only 6 h resulted in visible nuclear condensation and positive staining of isolated cells as indicated by arrow (Ax 6 h). After 24 h of anoxia (Ax 24 h) most cells were TUNEL-positive. Right, colocalization of TUNEL-positive nuclei (green) with DAPI nuclear stain (blue) is seen. Scale bar = 20 μM.
plasmic VEGF induction after near-anoxia was similar until 6 h, but by 24 h a more than fivefold increase was observed (Fig. 2E), indicating that only prolonged anoxia (>24 h) significantly induces VEGF over hypoxic conditions. Time-dependent secretion of VEGF into the culture media was also monitored (Fig. 2F), but again no alterations between hypoxia and anoxia were observed until 24 h. Thus, during oxygen withdrawal, astrocytes produce similar amounts of VEGF, regardless of the insult severity during acute insult, but chronic exposure significantly elevates VEGF production and secretion.

Immediate HIF-1α Protein Stabilization Following an Ischemic Insult in Vitro

An ischemic event such as stroke deprives cells of both oxygen and glucose. To mimic in vivo models of ischemia, astrocytes were placed in glucose-free media immediately prior to anoxic exposure. Glucose deprivation alone caused immediate stabilization of basal HIF-1α protein levels (Fig. 3A, 0 h), and additional hypoxia further induced nuclear HIF-1α protein accumulation in a time-dependent manner. Near anoxic glucose-deprived astrocytes stabilized HIF-1α even more rapidly with a significant increase observed after just 2 h and threefold induction compared with hypoxic samples after 6 h (Fig. 3B). Note that after 24 h of anoxic incubation only residual protein levels were extracted implying major cell death at that time.

Although glucose withdrawal alone resulted in immediate basal HIF-1α stabilization (as a result of media change), prolonged glucose deprivation did not further increase HIF-1α accumulation. Analysis of astrocytes incubated in glucose-free medium under normoxic conditions for extended periods (up to 48 h) showed no additional increase in HIF-1α accumulation, and glucose replenishment also had no effect (data not shown). Thus, although glucose withdrawal stabilizes low basal HIF-1α levels in astrocytes, additional oxygen deprivation is required to further induce protein accumulation.

Severe Insult Stimulates Rapid and Increased Secretion of VEGF from Astrocytes

Although glucose and oxygen deprivation increased cytoplasmic VEGF concentrations compared with normoxic controls at all time points (Fig. 3C), significantly more VEGF accumulated in anoxic samples. VEGF protein levels were significantly enhanced already by the 4-h time point during hypoxia, but, in comparison, anoxic samples already exhibited a threefold increase. Accumulation of secreted VEGF in media also increased in a similar time-dependent manner with threefold higher levels in anoxic compared with hypoxic glucose-deprived cells at each time point (Fig. 3D). Thus, VEGF production and secretion is significantly enhanced in oxygen and glucose-deprived astrocytes.
HIF-1 Only Partially Controls VEGF Induction in Astrocytes During Injury

To assess the contribution of HIF-1 to VEGF signaling by astrocytes during insult we performed HIF-1α knockdown studies. Using siRNA targeted inhibition, we could reliably abolish HIF-1α mRNA expression (Fig. 4A). A scrambled siRNA probe had no effect on HIF-1α stabilization. This knockdown translated to almost complete abrogation of protein stabilization during all insult conditions (Fig. 4B). However, despite near-complete Hif-1α knockdown, only 40–50% reduction of VEGF expression in astrocytes was observed in all samples regardless of the severity of insult. A graph of cytoplasmic VEGF levels, as measured by ELISA after 6 h, is shown (Fig. 4C). Thus, this data implies that although HIF-1 signaling does play a significant role, HIF-1-independent mechanisms also contribute to regulation of astrocytic VEGF.

VEGF Inhibition Reduces Proliferation and Cell Survival of Astrocytes

Exogenous application of VEGF has been shown to increase proliferation of astrocytes and surrounding cells in vivo. Since astrocyte VEGF expression is enhanced during injury we assessed whether endogenous VEGF modulates astrocyte proliferation and survival in vitro. Using the VEGF inhibitor SU1498, we show that blockade of VEGF signaling significantly reduces thymidine incorporation and therefore proliferation of astrocytes by up to 50% already after 6 h of exposure (Fig. 5, A and B). No differences were observed between hypoxic and anoxic cells, but the effect of SU1498 seemed more pronounced in the absence of glucose (Fig. 5B), presumably due to increased stress levels of the cells. Notably, inhibition had no effect on proliferation in the absence of insult, indicating that VEGF has no endogenous mitogenic effect in the absence of injury. Blocking of VEGF also led to increased cell death compared with untreated cells after 6 h of exposure to different severities of insult (Fig. 5C). Overall a gradual increase in cell death was observed with increased severity of insult as previously noted. Cell death levels after treatment were stable under normoxic conditions in the presence of glucose and increased only marginally in the absence of glucose. However, inhibitor treatment during more severe injury significantly augmented cell death by 40–50%. Thus, VEGF is an important astrocyte survival factor during injury.

DISCUSSION

The ability of cells to cope with reduced oxygen levels is critical to their survival, but hypoxic tolerance varies greatly among different cell types. During hypoxia or ischemic insult loss of astrocytes causes microvascular damage and exacerbates disturbance of brain homeostasis (48). Since astrocytes
are postulated to play an important role in supporting BBB function under various pathological circumstances, we investigated astrocytic responses to different degrees of injury. In particular, we focused on the temporal induction of HIF-1α and its target gene VEGF, a growth factor shown not only to be cytoprotective but also responsible for increased vascular permeability during injury (38, 51). We show that astrocytes, in contrast to most other cell types, require severe oxygen deprivation to prevent HIF-1α degradation and can survive severe ischemic conditions for up to 6 h. Although HIF-1 is not the sole regulator of astrocyte VEGF expression, endogenous VEGF secretion during injury is important for increased astrocyte survival and proliferation.

Astrocytes are less susceptible to injury than other brain cells, such as neurons. This is due, at least in part, to compensation for a lack of oxygen by switching to glycolytic metabolism (25) that allows the constant ATP levels required to ensure cell viability during stress (15). In most experimental injury paradigms, in vitro astrocytes crucially maintained their ATP levels at or above 50%. Remarkably, cell survival was only significantly compromised during severe insult, when oxygen and glucose deprivation was performed in parallel, and

![A](image1.png)  
**Fig. 4.** HIF-1α knockdown in astrocytes does not completely abrogate VEGF expression. RNA analysis shows HIF-1α mRNA levels are abolished by HIF-1α siRNA transfection but not a scrambled control probe under normoxic (Nx) and hypoxic (Hx) conditions (A). As a result, protein levels are also clearly abrogated under all injury conditions (B). Actin and Sp1 are used as input controls. Despite near-complete HIF-1α knockdown, short interfering RNAs (siRNAs) treatment does not fully abrogate VEGF expression in astrocytes during injury. A representative VEGF ELISA result shows a maximum of 50% inhibition was achieved under all conditions (C) in the presence or absence of glucose (+G and −G respectively, **P < 0.005; n = 3).**

![B](image2.png)  

![C](image3.png)  
**Fig. 5.** Inhibition of VEGF reduces astrocyte proliferation. Treatment of astrocytes for 6 h with a VEGF inhibitor (SU1498) resulted in significant reduction in astrocyte proliferation in the presence (A) and absence of glucose (B). Note that no significant changes were observed under normoxic conditions, despite increased concentration of the inhibitor. A 50% decrease was observed, however, at a concentration of 10 μM during severe insult (*P < 0.05; n = 3/4). Cell viability, assessed by trypan blue staining, was decreased following SU1498 treatment and oxygen glucose deprivation for 6 h (C). No significant difference was observed in normoxic incubated cells; however, increased severity of insult caused a concomitant rise in the number of dead cells.
cells were unable to recover from a 70% drop in ATP concentrations. A strong correlation seemed to exist between astrocyte proliferation and cell death during severe insult despite reduced ATP content.

Studies by Jelluma et al. (18) showed that astrocytes proliferate during sole glucose withdrawal, and our data confirm this and provide good evidence that injury severity is a strong proliferative stimulus. Both hypoxia and ischemia also activate and promote proliferation of resident astrocytes in vivo (33, 50), a process often referred to as astrogliosis or glial scarring. Suggested to be a reparative process to restore homeostasis through isolation of the damaged region, a glial scar may also detrimentally interfere with subsequent neural repair or axonal regeneration, and cause release of microglial-associated inflammatory factors as well as alter cellular interactions at the BBB. Our data suggest that preventing astrocytic proliferation, especially within the first 6 h of ischemic injury, may be beneficial to reduce scarring.

Although HIF-1 is the master regulator of hypoxic responses, stabilization of the HIF-1 heterodimeric complex (and thus a full stress response) in astrocytes required lower oxygen levels compared with most studied cells. This further underlines the resistance of these cells to reduced oxygen concentrations, although whether delayed HIF expression is a general characteristic of hypoxic-tolerant cells is an interesting question that deserves further investigation. It is, however, intriguing that forced expression of HIF-1 in a hippocampal cell line potentiated oxidative glutamate toxicity (3). The requirement of severe oxygen deprivation to stabilize HIF-1α may be additionally explained by the essential role of astrocytes in BBB maintenance. It is fitting that astrocytes are not sensitive to small changes in oxygen levels as this could have disastrous consequences on BBB function and neuronal homeostasis.

Notably, HIF-1 not being induced by hypoxia does not mean astrocytes are insensitive to changes in oxygen tension, indeed it has been shown that despite mild hypoxia not affecting astrocyte survival, substantial differences in metabolism still occur that contribute to their adaptation, such as regulation of astrocyte survival, substantial differences in metabolism still occur that contribute to their adaptation, such as regulation of astrocyte survival, substantial differences in metabolism still occur that contribute to their adaptation, such as regulation of astrocyte survival, substantial differences in metabolism still occur that contribute to their adaptation.

VEGF, a well-described HIF-1α target gene, is strongly induced during development but is constitutively expressed at nearly undetectable levels in adulthood under normoxic conditions (2, 5, 28). VEGF is also upregulated during pathological processes in the brain (28, 31, 32) and in vitro (8, 14, 17, 39). Indeed hypoxic exposure moderately increased cytoplasmic accumulation and basal release of VEGF in our astrocyte cultures in a time-dependent manner. In vivo this response seems to be advantageous since enhanced VEGF release probably protects neighboring cells through paracrine mechanisms (8, 47). Importantly, in our model increased severity of insult progressively augmented VEGF release, with glucose-free astrocyte-conditioned media containing dramatically elevated concentrations compared with glucose-containing conditioned media, in agreement with other studies of hypoglycaemic VEGF induction in glioblastoma and monocyes (37, 40).

Although moderate upregulation of VEGF probably enhances survival of surrounding cells, a rapid and massive increase in concentrations would have an overall negative effect on surrounding vasculature causing increased permeability and vascular remodelling and resulting in BBB disruption (38, 51). Indeed the temporal upregulation of VEGF in our model reflects the profile of BBB leakage in vivo, with VEGF induced as early as 1–3 h after focal cerebral ischemia onset and peaking at 24–48 h (9, 32). Thus, taken together this data implies that astrocytes have the capacity to secrete significant amounts of VEGF during ischemic injury that can induce BBB leakage. Thus, preventing the action of high VEGF concentrations may not only attenuate significant proliferation of astrocytes but also improve BBB integrity.

Surprisingly, our data advocates that insult-induced VEGF expression in astrocytes does not fully depend on HIF-1. Notably, hypoxic elevation of VEGF occurred in the absence of detectable HIF-1α stabilization, suggesting that a HIF-1-independent regulatory mechanism also exists during mild oxygen deprivation. Knockdown of HIF-1α by siRNA further supported this finding, reducing VEGF expression by only 50%. Although an obvious alternative candidate is HIF-2α, during our study constitutive HIF-2α expression levels were unaffected by hypoxia (data not shown) in agreement with others (29). Thus, how VEGF is hypoxically induced in the absence of HIF-1 remains intriguing and deserves further investigation. Crucially, very recent evidence by Arany et al. (4) shows that the transcriptional coactivator PGC-1α powerfully regulates VEGF expression independent of HIF signaling during nutrient and oxygen insufficiency in a number of cells. Such a mechanism may also be plausible for astrocytes. Exogenous application of VEGF promotes astrocyte proliferation and can act as a paracrine cytoprotective factor in vivo (24, 26, 47). However, the possible impact of endogenous VEGF secretion on astrocyte responses to injury per se have not been well documented. Importantly, we show that endogenously produced VEGF plays an important role in astrocyte fate during injury. Blockade of VEGF signaling pathways significantly decreased proliferation but also increased astrocyte cytotoxicity. Thus, endogenous VEGF secretion plays a significant autocrine role in proliferation and survival after injury via receptor-mediated effects. The finding that abrogation of autocrine VEGF signaling modulates astrocyte mitogenicity and degeneration provides an impetus for examining the role of VEGF in astrogial scarring after central nervous system...
injury. Since our data illustrate that varying levels of VEGF have differential outcome on astrocytes, fine-tuning protein concentrations could have beneficial effects on both injury progression and recovery. Complete inhibition of VEGF, a survival factor for many cells, would probably be detrimental, as it is involved in many physiological processes such as wound repair (21). However, it seems concentrations that reduce or delay astrocyte proliferation, but support cytoprotective function, could still be identified. Hopefully, the positive knockon effect of this would be reduced permeability of the BBB vasculature. More studies are needed to assess the feasibility of such possibilities.

Perspectives and Significance

We show that cultured astrocytes require severe oxygen deprivation for HIF-1α induction to occur. During severe insult, VEGF secretion correlates with HIF-1α induction, although evidence also suggests additional regulation via HIF-1-independent mechanisms during mild injury. Importantly, endogenous VEGF plays a significant autocrine role in promoting both survival and proliferation of astrocytes during injury. Since impairment or alteration of astrocyte function results in microvascular damage and accelerates neuronal death/hyperactivation, and VEGF has an important role in BBB leakage and central nervous system edema formation, and VEGF has an important role in BBB leakage and central nervous system edema formation, respectively.

ACKNOWLEDGMENTS

The authors thank Dr. Martijn Moransard for critical reading of the manuscript.

GRANTS

This study was supported by Swiss National Science Foundation, Stiftung 3R, and EUROXY (EU) grants (to M. Gassmann and O. O. Ogunsola).

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