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Hypoxia and hypoxia-inducible factor modulated gene expression in brain: involvement in neuroprotection and cell death

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Abstract Hypoxia, due to impaired cerebral blood flow, has hazardous effects on brain structure and function. Therefore, mechanisms should exist to meet the needs for hypoxic adaptation via regulation of gene expression. Signaling between the O_2 sensor and the regulator(s) of transcription is only partially characterized and requires regulatory transcription factors. Among these regulatory proteins, hypoxia-inducible factor–1 (HIF-1) appears to have a key role. HIF-1 modulates gene activity in response to low O_2 tensions in the developing and in the adult brain. Moderate hypoxia may elicit autoprotective mechanisms or hypoxia-induced regulators can contribute to mechanisms leading to cell death. Moreover, reactivation of embryonic gene expression may occur after injury-induced hypoxia. Thus, analyses of embryonic and pathogenic models should help to understand how hypoxia-mediated proliferative / cell death processes are involved in brain development and in the pathogenesis of acute or chronic neurodegenerative brain diseases.

Key words Hypoxia · Hypoxia-inducible factor · Cell death · Apoptosis · Brain development

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Abbreviations

ARNT – arylhydrocarbon receptor nuclear translocator
AP-1 – activator protein 1
ATF-1/CREB – activating transcription factor/cAMP responsive element binding protein
bHLH – basic-helix-loop-helix
C/EBPβ/NF-IL-6 – CAAT enhancer binding protein beta
CNS – central nervous system
EGR-1 – early growth response protein-1
EPAS1 – endothelial PAS domain protein 1
EPO – erythropoietin
EPOR – erythropoietin receptor
HIF-1 – hypoxia-inducible factor
HLF – HIF-1α-like factor
HRE – hypoxia response element
HRF – HIF-related factor
MAP – microtubule associated protein
MOP1 – member of the PAS superfamily 1
NFkB – nuclear factor kappa B
PAS – Per-ARNT-Sim
ROS – reactive oxygen species
Stat – signal transducer and activator of transcription
VEGF – vascular endothelial growth factor

Introduction

The maintenance of the structural and functional integrity of the brain is dependent on glucose and an uninterrupted oxidative metabolism. Anaerobic glycolysis is not able to provide sufficient energy to meet the energy equivalents required by the brain. Thus, hypoxia or reduced or interrupted cerebral blood flow have hazardous effects on brain structure and function. Therefore, the brain should be prepared to react fast and adaequately to reduced O_2 tensions. A powerful autoregulation is in place which increases cerebral blood flow to counteract hypoxia in the brain (Siejsö, 1978). However, pure hypoxia is a rather unique situation.

Under clinical conditions severe hypoxia in the brain mainly occurs as global ischemia in severe hypotension and cardiac arrest or as focal brain ischemia due to vascular occlusion. Global brain ischemia can be induced by the combined occlusion of extracranial arteries or by cardiac arrest (Ginsberg and Busto, 1989). The highly vulnerable CA1 neurons in the hippocampus die already after periods of 5 min of global brain ischemia (Schmidt-Kastner and Freund, 1991); neurons in the striatum are affected after about 10 min of ischemia, and cortical neurons after about 15–20 min, when normothermic and normoglycemic conditions are maintained (Pulsinelli et al., 1982). Under these conditions, neurons are quite selectively damaged, whereas astrocytes and vascular cells survive.

Focal brain ischemia develops in the case of a major arterial occlusion by an acute thrombus formation or embolism, resulting in stroke. Several animal models of stroke are in use which employ mechanical or photochemical occlusion of the middle cerebral artery (Ginsberg and Busto, 1989). Since there is continued collateral flow in the focal occlusion model, the level of brain ischemia is less severe and longer periods of ischemia are needed to cause damage. After induction of focal brain ischemia the neuropathological analysis then shows infarction in the core of the lesion. Moreover, a region of moderate flow reduction exists in the peri-infarct area in which neurophysiological functions are abolished, whereas the overall metabolism and structure is maintained; this region is typically described as the penumbra zone (Sharp et al., 2000).

To meet the needs for hypoxic adaptation, an O_2 sensing system has to control the cellular functions via regulation of gene expression. This system should consist of the sensor protein from which the O_2 signal is transmitted to a regulatory transcription factor which then modulates gene activity. The signaling cascade between the O_2 sensor and the regulatory transcription factor(s) is only partially characterized and may require reactive oxygen species (ROS) and susceptible sites in the regulatory proteins (Kietzmann et al., 2000). Several transcription factors, such as activator protein 1 (AP-1), early growth response protein-1 (EGR-1) (Yan et al., 1999), nuclear factor kB (NFkB) (Koong et al., 1994), CAAT enhancer binding protein beta (C/EBPβ/NF-IL-6) (Yan et al., 1997) and the hypoxia-inducible factor (HIF-1) were found to be involved in the modulation of gene expression by O_2. Among these regulatory proteins the hypoxia-inducible factors (HIFs) have been shown to be of special interest.

Hypoxia-inducible factor–1 (HIF-1) modulates gene activity in response to low O_2 tensions in the adult brain

Hypoxia-inducible factors (HIF) are heterodimeric transcription factors consisting of an α- and a β-subunit, both belonging to the basic-helix-loop-helix (bHLH)-PAS protein superfamily. HIF-1α was first cloned from the human hepatoma cell line Hep3B (Wang et al., 1995) and is a member of the PAS superfamily 1 (MOP1) (Hogenesch et al., 1997). Meanwhile, the mouse and rat isoforms have also been cloned (Wenger et al., 1996; Kietzmann et al., 2001). Additionally, from human, mouse and rat two other α-subunits were cloned, HIF-2α (Hogenesch et al., 1997; Kietzmann et al., 2001) and HIF-3α (Gu et al., 1998; Kietzmann et al., 2001). HIF-2α (Wenger and Gassmann, 1997) known as endothelial PAS domain protein 1 (EPAS1) (Tian et al., 1997), HIF-1α-like factor (HLF) (Ema et al., 1997) or HIF-related factor (HRF) (Flamme et al., 1997) is the member of the PAS superfamily 2 (MOP2) (Hogenesch et al., 1997). HIF-3α is also referred to as MOP7 (Hogenesch et al., 1997). The HIF-β subunit is identical to a member of the arylhydrocarbon receptor nuclear translocator, ARNT family: either ARNT1, ARNT2 or ARNT3 (Wang et al., 1995; Semenza, 1999).

The level of the HIF-1α protein is regulated mainly by protein stabilization under hypoxic (Huang et al., 1996; Jiang et al., 1997) and protein degradation under normoxic conditions via a ubiquitination mechanism which is mediated by the von Hippel-Lindau tumor suppressor protein (Maxwell et al., 1999; Cockman et al., 2000; Tanimoto et al., 2000). Hypoxia-dependent upregulation of HIF-1α mRNA levels do not seem to play a major role (Wenger and Gassmann, 1997). Among the known HIF molecules, HIF-1 is the best studied so far.

Under low O_2 tensions, HIF-1 has been shown to enhance the expression of genes encoding erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glycolytic enzymes like phosphofructokinase or enolase (Semenza et al., 1994; Semenza et al., 1996). The transcriptional activation was achieved by the binding of HIF-1 to the hypoxia response element (HRE) located either in the 5' or the 3' region of the genes (Beck et al., 1991; Bunn and Poyton, 1996).

Constitutive and hypoxia-dependent expression of HIF-1α in neurons

It was reported that *in vivo* the HIF-1α and HIF-1β (ARNT1) mRNA was expressed in all human, rat, and mouse organs assayed, including the brain (Wiener et al. 1996). Since the brain is extremely sensitive to hypoxia and ischemia (Siesjö, 1978) the regulation of HIF-1 expression should be highly relevant for the brain. Indirect evidence for a neuroprotective role of the hypoxia-regulatory mechanisms and HIF-1 derives from the known neuroprotective effects provided by its two target gene products, EPO (Sakanaga et al., 1998) and VEGF (Jin et al., 2000). The question arises which cell elements in the brain express HIF-1α under baseline conditions, because these are the cells that have the full potential to react to ischemia or hypoxia with an increase of HIF-1.

In the adult rat brain, HIF-1α and HIF-1β mRNA ex-

pression was found in all regions, but mainly in neurons (Bergeron et al. 1999). In newborn rats cerebral cortex and hippocampus were the major sites of HIF-1 mRNA expression (Bergeron et al., 2000). Western blots showed a constitutive expression of HIF-1α in the brain, whereas the results of immunohistochemical studies varied with the antibody and histological preparation used. Accordingly, low levels of HIF-1α immunoreactivity were found in neurons of the hippocampus and cortex of rats (Jin et al., 2000), whereas in hypoxic brains neurons, astrocytes, ependymal cells, or endothelial cells showed strong HIF-1α immunoreactivity (Chavez et al. 2000). HIF-2α was initially thought to be an endothelial cell specific regulator which is also found in the brain (Plate, 1999; Marti et al., 2000). Meanwhile it was found that HIF-2α was expressed also by other cell types such as hepatocytes (Kietzmann et al., 2001). The dimerization partners for HIF-1α appeared to be expressed with a slightly different cellular distribution; whereas HIF-1β/ARNT1 was found in normoxic cerebral neurons and glial cells, ARNT2 was detected only in neurons (Drutel et al., 1996, 1999, 2000). Additionally, two splice variants of HIF-1α in brain were identified, one of which dimerizes with ARNT2 even more avidly than with HIF-1β/ARNT1. Thus, at least two HIF-heterodimers appear to exist in neurons: the normal HIF-1α/ARNT1 dimer and the dimer consisting of the spliced HIF-1aa and ARNT2. In contrast to the HIF-1α/ARNT1 complex, HIF-1aa/ARNT2 did not recognize the HIF-1-binding site of the hypoxia-induced erythropoietin (Epo) gene (Drutel et al., 2000), suggesting that it controls the transcription of a distinct set of genes.

The combined evidence from in situ hybridization studies and immunohistochemistry renders it likely that neurons express HIF-1α; this has been confirmed in our recent studies (Fig. 1).

Global hypoxia/ischemia enhances HIF-1 gene expression

Numerous studies have addressed changes of gene and protein expression in animal models of global and focal brain ischemia (Koistinaho and Hökfelt, 1995; Sharp et al., 2000). In specific, HIF-1 has been studied in animal models of hypoxia, global brain ischemia, focal ischemia and neonatal hypoxia/ischemia.

In hypoxia, an increase of HIF-1 mRNA was found in the brain, kidney, and lung when rats or mice were exposed to reduced ambient O_2 concentrations for 30 to 60 min (Wiener et al., 1996). After 15 min of global brain ischemia in rats, an increase of HIF-1α protein levels was found between 8 and 24 h of recirculation in the hippocampus using Western blots, and corresponding immunohistochemistry showed scattered neurons in the hippocampus and more dense staining in the cortex (Jin et al., 2000 b); VEGF levels were increased between 4 and 72 h with a similar anatomical pattern.

Regulation through HIF-1α and HIF-2α contributes to angiogenesis in general by controlling important growth factors (Carmeliet, 2000), which also applies to the brain (Plate, 1999; Marti et al., 2000). Angiogenesis is known to occur after chronic hypobaric hypoxia in the adult brain (Mironov et al., 1994; Harik et al., 1995; Patt et al., 1997; Boero et al., 1999), and increases of VEGF have been reported (Kuo et al., 1999). However, HIF-1α was found to rapidly accumulate during the onset of hypoxia (10 % O_2); it remained at high levels for only 14 days and then decreased by 21 days despite the continuous low arterial oxygen tension (Chavez et al., 2000). When 21-day-hypoxia adapted rats were then exposed to a more severe hypoxic challenge using 8 % oxygen, HIF-1α accumulated again. Thus, HIF-1 appears to have a role in vascular remodeling and metabolic changes in chronic hypoxia.

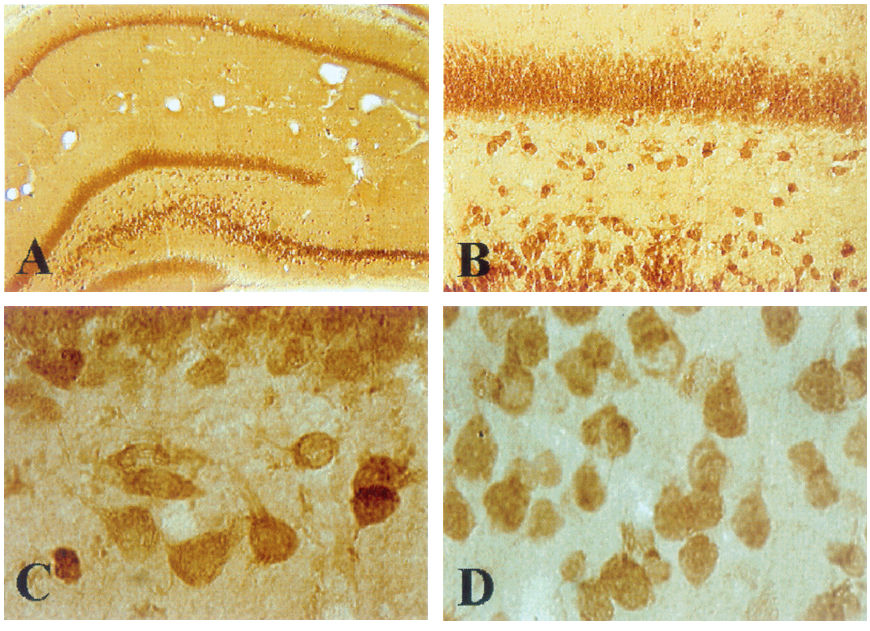

Fig. 1 Neuronal expression of HIF-1α. Rat brain sections (50 µm) were prepared on the vibratome and immunohistochemistry was performed with an antibody against rat HIF-1α as described (Kietzmann et al. 2001). Strong HIF-1α-like immunoreactivity is found in neurons throughout the forebrain. *A* Hippocampus, x 25; *B* Dentate gyrus and hilar region, x100; *C* Hilar neurons, x400; *D* Cortical neurons, x400.

Focal brain ischemia enhances HIF-1 gene expression

An extensive study of the HIF-1 system in focal brain ischemia after permanent occlusion of the middle cerebral artery in rats was reported by Bergeron et al. (1999). Using in situ hybridization, the mRNAs encoding HIF-1α, glucose transporter-1 and several glycolytic enzymes were found up-regulated in the penumbra, beginning 7.5 h after the onset of ischemia, and further increasing at 19 and 24 h. Western blot analysis showed an increase of both HIF-1α and HIF-1β levels in the penumbra at 20 h after the onset of ischemia, and similar increases of both subunits were found in experiments with hypoxia in the same study (Bergeron et al., 1999). Marti et al. (2000) induced permanent focal ischemia in the mouse and studied late stages of the infarction process with in situ hybridization; HIF-1α mRNA was found in a small perifocal rim whereas HIF-2α mRNA was enhanced in the cells of the vessel walls 3 d after ischemia. The mRNA of the HIF-1 target genes VEGF and of both VEGF receptors were also upregulated in the peri-focal region (Marti et al., 2000). Thus, upregulation of HIF-1α mRNA levels occurred in addition to presumed increases at the protein level, although mRNA changes occurred late after the onset of ischemia as compared to typical stress genes (Sharp et al., 2000). Since HIF-1α is not immediately expressed at high levels in the truly ischemic neurons, a cumulative effect of moderate ischemia or hypoxia seems to be required to activate HIF-1 gene expression.

Studies of human brain tissues with acute infarction are not available. Nevertheless, it is possible that an increase in the level of HIF-1α might also occur in stroke. In fact, in patients undergoing coronary bypass surgery after myocardial infarction, gene expression studies in heart biopsy specimens revealed a dramatic upregulation of the HIF-1α mRNA and VEGF mRNA (Lee et al., 2000).

Moderate and severe hypoxia may elicit divergent cellular responses: involvement of HIF-1

Moderate hypoxia elicits an autoprotective mechanism

Moderate and brief insults to the brain can lead to an autoprotective metabolic and molecular regulation which is called "preconditioning" (Chen and Simon, 1997). For example, short brain ischemia can "precondition" the brain for a period of severe ischemia which then elicits less damage. In the neonate it was found that hypoxic preconditioning with 8 % O_2 for 3 hours protected the newborn rat brain against subsequent hypoxic-ischemic injury, and this initial hypoxic exposure markedly increased HIF-1α and HIF-1β expression (Bergeron et al., 2000). This preconditioning effect could be also achieved by the application of two chemical inducers of HIF-1, cobalt chloride ($CoCl_2$) or desferrioxamine (DFX) in very young rats *in vivo*. HIF-1α and HIF-1β protein levels were markedly increased after intraperitoneal injection of $CoCl_2$ (60 mg/kg), and moderately increased levels were found after intraperitoneal injection of DFX (200 mg/kg) 1 to 3 hours after the injections. Preconditioning with $CoCl_2$ or DFX 24 hours before hypoxia-ischemia resulted in 75 and 56 % brain protection, respectively (Bergeron et al., 2000).

The phenomenon that the iron chelator DFX activated HIF-1 and mediated a preconditioning response indicates that HIF-1 has additional roles under stress situations different from hypoxic regulation. Thus, the iron chelators DFX and mimosine permitted protection from oxidative stress-induced apoptosis mediated by glutathione depletion in cortical neuronal cultures (Zaman et al., 1999). This was associated with enhanced DNA binding of HIF-1 as well as an increased expression of glycolytic enzymes and EPO in cortical cultures and the H19-7 hippocampal neuronal cell line. Likewise, cobalt chloride, which also activated HIF-1 and ATF-1/CREB in cortical cultures, prevented oxidative stress-induced death in these cells (Zaman et al., 1999).

On the other hand neurons were preconditioned by a non-lethal interval (60 min) of oxygen/glucose deprivation in rat primary cortical neuron cultures (Ruscher et al. 1998). Again, oxygen/glucose deprivation induced a rapid and transient increase in HIF-1 DNA binding activity. However, a reduced induction of HIF-1 binding activity was observed when the preconditioning occurred 48 h prior to the insult (Ruscher et al., 1998).

Thus, the pharmacological intervention with drugs stimulating HIF-1 expression or enhancing the HIF-1 protein amounts could become a neuroprotection method in anticipated ischemia, e. g., in elective cardiac or cerebro-vascular surgery.

Hypoxia-induced regulators contribute to mechanisms leading to cell death

HIF-1 has multiple facets (Semenza 1999, 2000). While most studies suggest an overall positive function for HIF-1 under chronic hypoxic conditions, there is a proposal in the current literature that acute HIF-1 upregulation may also serve as a signal of severe hypoxic cell damage (Semenza, 1999). This insight was initially derived from studies on tumors and the important tumor-suppressor p53 which controls apoptosis. First, hypoxia is a strong inducer of p53 in tumors; in this way hypoxia in rapidly growing tumors with poor vascularization provided a physiological pressure for the expansion of variant tumor cells with p53 mutations inactivating its apoptotic function (Graeber et al., 1996). Second, HIF-1α stabilized p53 and thereby contributed to an apoptotic response (An et al., 1998). Third, p53 inhibited HIF-stimulated transcription (Blagosklonny et al., 1998). Finally, embryonic stem cells from mice showed reduced proliferation and increased apoptosis when exposed to hypoxia and hypoglycemia, whereas HIF-1α "knock-

out" embryonic cells showed no apoptotic response (Carmeliet et al., 1998); thus, HIF-1α regulation appears to be associated with cell death. Accordingly, Halterman and Federoff (1999) suggested that coordinated expression of HIF-1α and p53 contributed to ischemia-induced neuronal cell death. Cortical neuron cultures exposed to oxygen-glucose deprivation and under blockade of glutamate receptors (to prevent excitotoxic cell death) showed a delayed apoptotic cell death; this type of neuronal death could be attenuated by viral gene transfer of a dominant negative form of HIF-1α which depended on the presence of p53 (Halterman et al., 1999). Thus, different levels of hypoxia elicit different responses, with moderate levels of HIF-1 faciliting energy supply and high levels of HIF-1 interacting with the apoptotic machinery through p53, eventually contributing to cell death (Semenza 1999). This concept of HIF-1 participation in cell death does not seem to be speculative since the transcription of the gene encoding Nip3, a proapoptotic member of the Bcl-2 family, was strongly induced in response to hypoxia (Bruick, 2000). Furthermore, it was found that the Nip3 promoter contains a functional HIF-1-responsive element (HRE) and is potently activated by both hypoxia and forced expression of HIF-1α. Thus, Nip3 may play a dedicated role in the pathological progression of hypoxia-mediated apoptosis after ischemic injury and possibly in necrotic events (Bruick, 2000). Accordingly, studies about the role of Nip3 in the brain have been initiated (Schmidt-Kastner and Bruick, in prep.). A second hint for a role of HIFs in cell death comes from Drutel et al. (1999, 2000). The authors showed that antisense knock-down of HIF-1α, HIF-1β/ARNT1 and ARNT2 reduced proliferation of PC12 cells, and reduction of ARNT2 increased apoptosis in PC12 cells. In view of these novel studies linking HIF-1 to cell death, the upregulation of HIF-1α in the hippocampus after global brain ischemia (Jin et al. 2000 b) needs to be interpreted with caution. A role for HIF-1α in delayed neuronal death *in vivo* should be considered if gene expression is upregulated in the vulnerable CA1 neurons in a delayed fashion even if this does not lead to increased protein levels ("attempted regulation"). In fact, a fine-grained analysis of HIF-1α mRNA expression in vulnerable and resistant neurons of the hippocampus is underway in the laboratory of the authors, and a delayed upregulation of HIF-1α mRNA was found in the vulnerable CA1 neurons (Schmidt-Kastner et al., 2001); at the same time, CA1 cells expressed pro-apoptotic genes such as bax and caspase-3 (unpublished data). Another potentially harmful aspect of HIF-1 upregulation is the increase of LDH (Bergeron et al., 1999; Semenza, 1999) which could be associated with increased lactate production that, in turn, may have negative consequences after brain ischemia (Siejsö, 1978). Thus, careful experimental studies of the HIF-1 system in acute brain ischemia are required before existing or novel stimulators of HIF-1α production can be used to protect the brain in conditions of hypoxia/ischemia.

Involvement of hypoxia-inducible factor-1 (HIF-1) in the modulation of gene activity in response to low O_2 tensions in the developing brain

The morphogenesis of the CNS requires coordinated actions between the genetic program and the embryonic microenvironment. It was proposed that "physiological hypoxia" (Chan-Ling and Stone, 1993), among other factors, regulates the expression of genes products of which are involved in the shaping of the embryonic body. Indeed, areas of transient hypoxia exist in the rat embryo as demonstrated by application of the nitroimidazole derivative EF5 which serves as a marker for hypoxic cells and tissues (Chen et al., 1999). Especially, the hindbrain, otic vesicles and the first branchial arches were found to display hypoxia (Chen et al., 1999). It seems likely that "physiological hypoxia" contributes, at least, to the regulation of two major developmental events: vascularization of the CNS *anlage* and cell death (Chen et al., 1999). Cell death may then contribute to the closure of the neural tube (Weil et al., 1997).

Two hypoxia-induced and HIF-1 regulated genes appeared to participate in the vascularization process of the brain. Whereas ample evidence exists that VEGF and its receptors are involved in angiogenesis (Millauer et al., 1993, Flamme et al., 1997, Aitkenhead et al., 1998, Ogunshola et al., 2000), neuronal guidance, neurogenesis, and neuroprotection (Yang and Cepko 1996, Marti and Risau 1999, Marti et al., 2000), comparably little is known about the roles played by EPO and its receptors (EPOR) during neurodevelopment. Since the EPO and the EPOR gene are expressed in various tissues of the postimplantation mouse (Yasuda et al., 1993), in the human retina (Juul et al. 1998) and in other parts of the developing CNS (Li et al., 1996; Juul et al., 1999; Liu et al., 1997; Dame et al., 2000), it was suggested that EPO is involved in the proliferation and differentiation of astrocytes, neurons and endothelial cells. In addition, the supposed proliferative potential of the EPO/EPOR system appears to be supported by its known antiapoptotic effects mediated by the binding of Stat5 to the promotor region of the bcl-xL gene as demonstrated for erythroid precursor cells (Silva et al., 1999).

In the developmental period of the tree shrew *Tupaia belangeri* which spans from the closure of the anterior neuropore to the onset of the vascularization of the neuroepithelium, five phases of a straightforward, spatially and temporally defined apoptotic process have been characterized. This process occurs in the forebrain and eyes and beside *Tupaia*, most probably, also in other tetrapods (Knabe and Kuhn 1998, Knabe et al., 2000). Three dimensional reconstructions revealed an oriented "band"-like pattern of dead neuroepithelial cells. Interestingly, this band extends from the dorsal to the ventral midline of the forebrain and bifurcates at the position of the later optic chiasm. Most probably these cell death events are involved in late bilateralization processes of the entire forebrain (Knabe and Kuhn 1998,

Knabe et al., 2000). The maximally extended band of cell death occured immediately prior to the onset of the vascularization of the forebrain. Therefore, the cell death events are likely to be regulated by physiologically occurring hypoxia possibly involving HIF-1.

This would then be consistent with the finding that HIF-1 plays an important role in the development of the brain, its vasculature and in the regulation of the genes encoding metabolic enzymes during embryonic development (Semenza, 1999). Analysis of the development of knockout homozygous *Hif1a-/-* embryos that express no HIF-1α protein revealed that development is arrested by E9.0 and that the mice die by E10.5 (Iyer et al., 1998, Ryan et al., 1998). The reason for this demise was impaired vascular development and regression of blood vessels in the cephalic region (Iyer et al., 1998 a).

Associated with the disturbed vascular development, massive cell death was observed within the cephalic mesenchyme. Malformations became apparent in *Hif1a-/-* embryos between E8.5 and E9.5; exactly at the time when heterozygote *Hif1a*±/± embryos first express HIF-1α protein (Iyer et al., 1998 a).

HIF-1 responses may also play an important role in the development of the retina because relative hypoxia seems to be the driving force for the growing vasculature in the retina (Chan-Ling and Stone, 1993; Stone et al., 1995). When *hyper*oxia is used as a treatment of lung dysfunction of premature infants, it suppresses the HIF-mediated and VEGF-driven angiogenesis leading to improper vascular development in the eye. Upon return to normoxic conditions the improper vascularization can lead to poor oxygenation of the retina and thus hypoxia results in blindness (oxygen induced retinopathy). Ozaki et al. (1999) studied the retinal development of mice which were exposed to *hyper*oxia at P0 or at P7 for five days and then returned to normoxia. In normal mice, a strong increase in HIF-1α paralleled the postnatal development of vessels, whereas in mice exposed to *hyper*oxia, HIF-1α was immediately down-regulated. After return to normoxia HIF-1α increased strongly, indicating the development of hypoxia due to incomplete vascularization during hyperoxia. Studies on hyperoxia in the brain at different stages of development and HIF-1 remain to be performed.

Hypoxia and ischemia also play an important role in the perinatal period. In a study on very young postnatal rats, combined ischemia/hypoxia was found to increase HIF-1α mRNA and protein expression, and the expression was shifted in a way that neuronal levels of HIF-1α declined and expression in vascular cells increased (Bergeron et al., 2000). Thus, the HIF-1α system can be activated in very young rats.

However, evidence was collected that prolonged moderate hypoxia is needed rather than acute severe hypoxia to obtain an accumulation of HIF-1α. Accordingly, in a model of severe perinatal asphyxia no upregulation of HIF-1α in the brains was found; by contrast, exposure of the dams to prolonged moderate hypoxia led to an upregulation of HIF-1α in the brains of the pups (Chiappe-Gutierrez et al., 1998). These studies confirm that acute severe hypoxia is not a good stimulus for the induction of HIF-1α.

Theoretically, subtle dysfunction of HIF-mediated regulation could be associated with developmental disturbances of the brain; in fact, subtle developmental problems have been associated with the brain dysfunction seen in schizophrenia (Bogerts, 1999).

Hypoxia-inducible factor–1 (HIF-1) modulates gene activity in response to low O_2 tensions in the brain during aging

It is an interesting question whether and how the constant availability of HIF-1 in neurons changes during aging. Frenkel-Denkberg et al. (1999) exposed young and old mice to hypoxia for 30 to 60 min. They found a severe impairment in the capacity of the older animals to form a HIF-1-HRE complex in brain samples and postulated that an attenuation in the capacity to form HIF-1-HRE complexes in senescent tissues may explain this decreased ability to respond adaequately to hypoxic stress. Similar results were obtained when rabbit vascular smooth muscle cells from young (6–8 month) and old (4–5 years) animals were analyzed; in the cells of the old animals the HIF-1α protein levels and HIF-1 DNA-binding were found to be reduced compared to the cells from the young animals. This was accompanied by a decrease of the hypoxia-induced VEGF promoter driven luciferase gene expression in the cells from the older animals (Rivard et al., 2000). Thus, changes in HIF-1α production and degradation could play a role in conditions such as aging and vascular dementia. Lower HIF-1 production in the aging brain could be associated with a less efficient compensation of small ischemic/hypoxic events and an increased incidence of mini-strokes. Dramatic changes of ubiquitin are seen in many neurodegenerative disorders (Sherman and Goldberg, 2001), and a cellular disturbance of the proteasome function could also affect HIF-1 levels.

Reactivation of embryonic gene expression after injury

Evidence exists that groups of genes and related cellular processes which help in sculpting the developing CNS are reactivated during pathogenic and/or repair processes in the adult. As mentioned, hypoxic microenvironments which are linked to apoptotic/neuroprotective and/or angiogenic events are of relevance both in the developing and in the diseased brain.

Since EPO has been suggested to have proliferative as well as anti-apoptotic effects in the developing CNS (Liu et al., 1997), this might also hold true for the brain after ischemic insults. For instance the proliferative potential of EPO has been observed in brain endothelial cells in which hypoxia-induced EPO production activated the

transition from the G0 to G1 phase (Yamaji et al., 1996). Furthermore, the hypoxia-induced apoptosis was decreased in cultured human embryonic NT2 cells and in cultured rat hippocampal neurons when recombinant EPO was added (Juul et al., 1998, Lewczuk et al., 2000). In mammals, the earliest extrinsic macrophages which most probably are precursors of microglial cells colonize the developing forebrain immediately prior to its vascularization and as shown in the tree shrew *Tupaia belangeri* in response to preexisting bands of apoptotic cell death (Knabe and Kuhn 1999, Knabe et al., 2000), i. e., under conditions assumed to be hypoxic. In the impaired brain microglial cells which also invade hypoxic regions have been proposed to be candidates contributing to HIF regulation (Marti et al., 2000).

In the adult brain the EPO/EPO-R system is expressed in subsets of neurons and astrocytes. After ischemic insults EPO/EPO-R expression has been localized also to microglia/macrophage-like cells, endothelial cells and reactive astrocytes at the border zones of the lesions (Bernaudin et al., 1999), whereas EPO/EPO-R are downregulated in areas containing insult-induced dead cells.

It is tempting to speculate that the expression of the EPO/EPO-R system in hypoxic zones after brain ischemia in the adult represents the reactivation of an embryonic gene expression pattern which is currently being investigated by the authors. Similar to the EPO/EPO-R system neurotrophins which protect neurons during development have been shown to exert neuroprotection in an injury model of neonatal hypoxia by blocking the activation of caspase 3 (Han et al., 2000). In addition, after ischemia-induced injury of the forebrain, "reactive" astrocytes express GABA (Lin et al., 1993) as do O-2A progenitor cells which are the precursor cells of type two astrocytes. Moreover, it has been shown that the mRNA of the immature splice variant of the microtubule associated protein MAP2, i. e., MAP2C, is reexpressed in the hippocampus after global brain ischemia (Saito et al., 1995). Under the conditions of focal brain ischemia reexpression of the intermediate filament nestin (Duggal et al., 1997) was noted in astrocytes suggesting an embryonic reversion of the mature cytoskeleton as a response of astrocytes to cerebral injury.

However, reactivation processes do not need to be coupled only to injuries directly mediated by ischemia. They occur also in macroglial cells after application of freezing lesions to the cerebral cortex which then results in the persistence or reappearance of radial glial cells in neonatal rats (Rosen et al., 1994). Moreover, an intermediate filament associated protein, IFAP-70/280 kDa, which is expressed in embryonic radial glial cells, is reexpressed in reactive astrocytes extending radially from stab induced lesions in the adult (Yang et al., 1997).

Thus, comparative analyses of well-defined embryonic and pathogenic models may improve the knowledge on how proliferative as well as cell death processes are involved in the proper and impaired development of the brain and also in the pathogenesis of acute or chronic neurodegenerative diseases after hypoxia-associated injuries.

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