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The Janus-faced effects of hypoxia on astrocyte function

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Abstract

Astrocytes are increasingly recognized for their impact on neuronal function and viability in health and disease. Hypoxia has Janus-faced influences on astrocytes and their ability to support neuronal viability. For example, hypoxia induces astrocyte-dependent protection of neurons following hypoxia preconditioning. Yet, hypoxia induces processes in astrocytes that augment neuronal death in other situations, such as the coincidence of hypoxia with inflammatory signaling. A complex array of gene expression is induced by hypoxia within astrocytes and neurons through multiple transcription factors and intracellular molecular pathways. The hypoxia inducible factors (HIF) are transcription factors that are likely instrumental in orchestrating adaptive and pathological functions of astrocytes. As such, the HIFs are postulated to mediate both adaptive and pathological functions during hypoxia/ischemia. Identifying the conditions under which hypoxia induces signaling in astrocytes that alters autonomous or neuronal survival will undoubtedly have important implications regarding the development of new strategies for stroke treatment.

Keywords

astrocyte; hypoxia; HIF; ischemia; preconditioning

Introduction

Neurons are the principal cell type examined when unraveling the molecular processes induced by hypoxia and ischemia that influence cell survival during stroke. While this approach has merit, astrocytes and hypoxia-induced molecular processes within astrocytes also undoubtedly affect survival of brain parenchyma during hypoxia and ischemia. The conditions under which hypoxia-induced processes in astrocytes are protective or damaging to themselves or neurons are only beginning to be delineated. Numerous molecular pathways may be induced by hypoxia in astrocytes and contribute to these divergent phenotypes. These hypoxia-induced molecular pathways include the hypoxia inducible factors (HIF-1 α and HIF-2 α), NF- κ b, p53, cAMP, and c-jun, all of which may influence cell survival (JinMaoSimon and Greenberg 2001; Laderoute and others 2002; MichielsMinetMottet and Raes 2002; SchmidZhou and Brune 2004; Semenza 2000). Of these hypoxia-induced molecular processes, the HIFs have garnered particular interest because their abundance is markedly increased during hypoxia and they stimulate the expression of an abundance of targets that modify cell viability. These targets include those that enhance glycolysis, angiogenesis, alter mitochondrial function, and induce expression of pro-apoptotic bcl-2 family members (Bruick 2000; KimTchernyshyovSemenza and Dang

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2006; PapandreouCairnsFontanaLim and Denko 2006; Semenza 2000). This update will examine the hypoxia-induced signaling cascades in astrocytes and their influence on neuronal death, especially as they pertain to HIF dependent signaling.

Astrocytes and adenosine: a hypoxia-induced protective role of astrocytes

Astrocytes have a central role in controlling the extracellular concentration of adenosine, a molecule with known neuroprotective properties (HeurteauxLauritzenWidmann and Lazdunski 1995; Lin and others 2008; Wardas 2002). Hypoxia alters the abundance of multiple proteins that collaborate to enhance adenosine concentrations in the extracellular space (Fig. 1). Hypoxia enhances connexin 43 (Cx43) protein abundance in astrocytes, which can form both GAP junctions and hemichannels, the latter of which release ATP (Kang and others 2008; Lin and others 2008). Several enzymes are involved in the conversion of extracellular ATP to adenosine. The ecto-nucleoside triphosphate diphosphohydrolases (NTPDase), which include CD39 (NTPDase-1) and CD39L (NTPDase-2), are plasma membrane proteins which hydrolyze extracellular tri- and diphosphate nucleosides (ATP or ADP) to monophosphate nucleosides (AMP) (RobsonSevigny and Zimmermann 2006). AMP is subsequently converted to adenosine through the actions of the ecto 5'-nucleotidases (CD73). While the hypoxic regulation of CD39L has not been reported, both CD39 and CD73 are induced by hypoxia. In intestinal epithelial cells, CD73 is induced in hypoxia by HIF-1 (Synnestvedt and others 2002). Interestingly, hypoxia also reduces the abundance of the equilibrative nucleoside transporter 1 (ENT-1) in endothelial cells through a HIF dependent mechanism (Eltzschig and others 2005). Since ENT-1 transports adenosine from the extracellular space into the cell, reduced ENT-1 abundance is expected to increase extracellular adenosine concentration. In terms of the expression of these molecules in astrocytes, CD39L is the most prominent NTPDase isoform expressed by astrocytes (Wink and others 2006). CD73 and is expressed by astrocytes following injury (Nedeljkovic and others 2006). While ENT-1 transcript is present in cultured astrocytes its relative contribution compared to ENT-2 is likely modest (NagasawaKawasakiTanakaNagai and Fujimoto 2007). Regardless, this information suggests that hypoxia enhances extracellular adenosine abundance by enhancing Cx43, CD39, and CD73 and may further enhance extracellular adenosine by reducing ENT-1 expression (Fig. 1). Moreover, some of these hypoxia-induced changes are likely mediated by the HIFs.

Astrocytes and adenosine have a clear role in mediating the protective effects of hypoxic preconditioning (HPC) (Lin and others 2008). Hypoxic preconditioning is the observation that a sublethal exposure of mice to hypoxia 48–72 hrs prior to subsequent stroke reduces stroke volume. A similar phenomenon is induced in cultured neurons or astrocytes. In a recent study, which utilized astrogloma cell lines, preconditioning was ineffective at protecting astrocytes in the absence of Cx43 (Kang and others 2008). Cx43 forms hemichannels release ATP. Similarly, loss of Cx43 function *in vivo* eliminated HPC-mediated protection in mice (Lin and others 2008). Given that Cx43 is expressed only in astrocytes, this finding clearly implicates astrocytes as an important mediator of HPC derived neuronal protection. It has long been appreciated that adenosine has direct protective effects on neurons (Wardas 2002). Yet, since adenosine signaling also protects cultured astrogloma cells, astrocytes also are likely to mediate cell autonomous protection by releasing ATP, which is subsequently converted to adenosine. Similarly, other studies implicate adenosine and CD39/CD73 in ischemic preconditioning in the heart and kidney (Grenz and others 2007; Kohler and others 2007), suggesting a ubiquitous role of these pathways in mediating the protection provided by hypoxia preconditioning.

Astrocytes participate in hypoxia-induced neuroprotection mediated by erythropoietin

Astrocytes are a main source of erythropoietin (EPO), a protein with neuroprotectant properties (Marti and others 1997; Masuda and others 1994; Prass and others 2003; Ruscher and others

2002). Traditionally, HIF-1 was described to regulate EPO expression in hypoxia (Semenza and others 1997), while more recent reports suggest HIF-2 as the major inducer of EPO in hypoxia (ChavezBaranovaLin and Pichiule 2006; Gruber and others 2007). EPO demonstrates its ability to provide neuroprotection as it reduces neuronal death from oxygen glucose deprivation (OGD) (Ruscher and others 2002), glutamate toxicity (MorishitaMasudaNagaoYasuda and Sasaki 1997), and nitric oxide induced death (Sakanaka and others 1998). Similarly, EPO in conditioned media taken from hypoxic astrocyte cultures is protective to neurons exposed to OGD (ChavezBaranovaLin and Pichiule 2006). Not only does EPO protect neurons but also enhances viability of astrocytes exposed to nitric oxide or experiencing oxidative stress (DiazAssarafMiller and Schipper 2005; Liu and others 2006) (Fig. 2). Similar to the *in vitro* experiments, protective effects have been described for EPO *in vivo* during stroke (Bernaudin and others 1999; Prass and others 2003; Sakanaka and others 1998; Siren and others 2001). Moreover, intra-theal EPO receptor protein administration, which sequesters EPO and inhibits its activity, precludes HPC in rat stroke models (Prass and others 2003). Taken together, this evidence suggests that astrocyte-derived EPO is a crucial mediator of astrocyte and neuronal viability following HPC and during acute stroke.

Astrocyte HIF-1 and HIF-2, mediators of hypoxic preconditioning?

Since many of the molecular processes that contribute to HPC, including EPO, may be induced by the HIFs, it begs the question as to the dependence of HIF signaling in astrocytes for preconditioning mediated cytoprotection. In other organ systems, HIF-1 appears crucial for “early” HPC mediated protection. For example, haplotype insufficiency of HIF-1 completely eliminates the protective effects of ischemic preconditioning in the heart when the preconditioning stimulus was applied just a few minutes before the ischemic insult (Cai and others 2008). This early HPC likely occurs through unique mechanisms compared to the delayed HPC that is maximal at 72 hours after hypoxia exposure and is typically examined for protection against stroke. In fact, loss of HIF-1 function in neurons *in vivo* does not alter the protection mediated by delayed HPC against focal stroke (Baranova and others 2007). Thus, neuronal HIF-1 function is dispensable for delayed HPC-mediated protection. With regards to astrocytes, conditioned media collected from hypoxic astrocyte cultures loses much of its protection if HIF-2 function is eliminated in astrocytes, while loss of HIF-1 had a minimal effect (ChavezBaranovaLin and Pichiule 2006). It was demonstrated that this loss of protection was the result of loss of HIF-2 mediated induction of EPO expression in astrocytes (Fig. 2). It is yet to be determined if astrocyte-specific function of HIF-1 or HIF-2 will be required for HPC mediated protection against stroke *in vivo*.

Hypoxia, astrocytes, and VEGF: a double edged sword

Many studies have shown that astrocytes secrete VEGF (Lee and others 1999; Sinor and others 1998), the expression of which is induced by hypoxia. During hypoxia and following stroke VEGF expression is induced through HIF-1 (Baranova and others 2007; RempéLelliVangeisonJohnson and Federoff 2007). VEGF abundance is also enhanced by the increased half life of VEGF transcript mediated by the binding of HuR protein to its 3' UTR during hypoxia (LevyChungFurneaux and Levy 1998). VEGF mediates angiogenesis, has direct neuroprotective effects to neurons in culture (JinMao and Greenberg 2000), and is important for inhibiting neuronal death in the days following stroke (Zhao and others 2006). VEGF has been ascribed an important role in preconditioning. A neutralizing antibody against VEGF prevented the HPC observed *in vitro* when cerebellar granular neurons are exposed to a subsequent lethal dose of hypoxia (Wick and others 2002). In rats, preconditioning was mirrored by an increase in VEGF levels in astrocytes (Bernaudin and others 2002). Yet, it should be noted that astrocytes are not the sole source of VEGF in the brain. In fact, both microglia and neurons contribute to VEGF abundance following hypoxia (Baranova and others

2007; PlateBeckDannerAllegrini and Wiessner 1999). Therefore, the coordinated expression of VEGF by these multiple cell types likely contributes to its neuroprotective effect.

While VEGF is neuroprotective under multiple hypoxia/ischemia paradigms, administration of VEGF during acute stroke increases stroke volume by increasing blood brain barrier permeability and edema (Zhang and others 2000) (Fig. 2). In contrast, when administered two days after stroke onset, VEGF enhances angiogenesis and recovery from stroke. An elegant study demonstrated that matrix metalloproteinase-9 (MMP-9) has pathological and adaptive functions, which are dependent on its temporal expression and actions upon VEGF (Zhao and others 2006). MMPs cleave extracellular matrix proteins, which are important in modulating cellular interactions during tissue remodeling in disease and trauma. It is well described that MMP-9 increases blood brain barrier permeability and increases ischemia-induced damage during acute stroke (CunninghamWetzel and Rosenberg 2005; Jian Liu and Rosenberg 2005). For example, MMP-9 knockout mice have less severe strokes and inhibitors of the MMP-9 reduce stroke volume (Asahi and others 2000). In stark contrast, several days post-ischemia, MMP-9, which is highly expressed by astrocytes, serves a neuro-protective role by cleaving and activating VEGF (Zhao and others 2006). These temporal distinct and divergent actions of VEGF and MMPs demonstrate the important concept that molecules released by astrocytes may change from a pathological to adaptive phenotype depending on the temporal profile of expression following ischemia.

Hypoxia and inflammation co-conspire to induce glutamate release by astrocytes

A critical role performed by astrocytes in the brain is their ability to uptake extracellular glutamate. This property serves to limit excitotoxicity and it recycles glutamate to glutamine, which can then be shunted back to neurons (BakSchousboe and Waagepetersen 2006). In astrocytes, hypoxia causes selective down-regulation of the glutamate transporters EAAT-1 (aka GLAST) and EAAT-2 (aka GLT-1) (Fig. 3), which are expressed primarily on astrocytes (Dallas and others 2007). This hypoxia-mediated reduction in glutamate transporter is mediated by NF κ B, not HIF. Not surprisingly, loss of astrocyte glutamate transporters increases ischemic injury in rats both *in vivo* and *in vitro* (Rothstein et al., 1996). Thus, this process likely contributes to excitotoxicity during stroke. Furthermore, like hypoxia, TNF α reduces EAAT2 expression (BoycottWilkinsonBoylePearson and Peers 2008). Since inflammatory processes are prominent in the days following ischemia, this effect of TNF α on EAAT2 could also contribute to excitotoxicity and neuronal death in the post-ischemic period.

IL-1 β and hypoxia co-conspire to induce astrocytes to release glutamate, which compromises neuronal viability during hypoxia and inflammation (FogalLiLobnerMcCullough and Hewett 2007). Multiple past studies, using blocking antibodies, antagonist, viral vectors, and genetic means, demonstrated that IL-1 β mediates damage in hypoxia and ischemia both *in vivo* and *in vitro* (FogalLiLobnerMcCullough and Hewett 2007; Hara and others 1997; Ohtaki and others 2003; YangZhaoDavidson and Betz 1997). A recent investigation demonstrated that IL-1 β likely mediates its pathological effect through signaling of the IL-1R1 receptor on astrocytes (FogalLiLobnerMcCullough and Hewett 2007). By exposure to a combination of hypoxia and IL-1 β , the authors selectively induce neuronal death in neurons in co-cultures of neurons and astrocytes. Interestingly, astrocytic expression of IL-1R1 was necessary for neuronal injury induced by hypoxia and IL-1 β , and loss of IL-1R1 function on neurons was without effect (FogalLiLobnerMcCullough and Hewett 2007). This hypoxia and IL-1 β dependent neuronal death was mediated by increased transport of cysteine from the extracellular space to the intracellular compartment, which was accompanied by a release of glutamate into the media by astrocytes through the actions of system X_c (Fig. 3). This enhanced extracellular glutamate resulted in neuronal death. Given that ubiquitous loss of IL-1R1 receptor function reduced stroke volume in mice, these results likely have relevance to cell death *in vivo*. HIF-1 α is

implicated in release of IL-1 β by astrocytes (Zhang and others 2006) and NF- κ b induces IL-1 β in several cell types. Thus, it would be of interest to understand if HIF-1 and NF- κ b contribute to this process. Beyond IL-1 β , it is also well appreciated that during inflammation other cytokines/chemokines, such as TNF- α and stromal derived factor-1, also cooperate to induce release of glutamate from astrocytes leading to neurotoxicity (Bezzi and others 2001). While, to date, this process has only been described with neuroinflammation, both TNF α and stromal derived factor-1 are expressed following stroke (Barone and others 1997; Hill and others 2004) and in theory could mediate similar excitotoxic effects following ischemia.

Astrocyte HIF-1 as a mediator of neuronal death in hypoxia

While astrocytes are quite resistant to cell death when challenged with hypoxia alone, hypoxia induces neuronal death in astrocyte/neuron co-cultures. Since HIF-1 can induce the expression of neuroprotective targets in hypoxia, we were interested in examining the effect of HIF-1 function in astrocytes on hypoxia-induced neuronal death in co-cultures. Surprisingly, loss of HIF-1 function selectively in astrocytes was markedly protective against hypoxia-induced neuronal death (VangeisonCarrFederoff and Rempe 2008), suggesting a pathological effect of astrocytic HIF-1 on neuronal viability. In contrast, loss of HIF-1 in neurons modestly reduced the ability of neurons to withstand a hypoxic challenge. Thus, hypoxic-signaling through HIF-1 has cell-type specific and divergent effects on neuronal viability.

To explore potential pathological mechanisms of HIF-1 we explored the influence of iNOS on neuronal death. Pathological roles of NO include oxidative damage through peroxynitrate production, energy failure by inhibiting several enzymes in the mitochondrial respiratory chain and ATP production (for review see (Iadecola 1997)). Furthermore, NO has been implicated in DNA damage, PARP activation, and induction of apoptosis. In astrocyte cultures, loss of HIF-1 function markedly reduced iNOS transcript abundance (VangeisonCarrFederoff and Rempe 2008) confirming that NO may be one mechanism by which astrocyte HIF-1 is toxic to neurons. In fact, inhibiting iNOS function with both non-specific and specific inhibitors protects neurons in co-culture with astrocytes from hypoxia-induced cell death (VangeisonCarrFederoff and Rempe 2008). Yet, compared to loss of HIF-1 function in astrocytes, inhibition of iNOS function was not nearly as effective at reducing hypoxia-induced neuronal death. This observation suggests that astrocytic HIF-1 induces other pathological processes in astrocytes leading to neuronal death independent of iNOS, which are yet to be defined.

The interplay between HIF-1 and NF- κ b, and its relevance to astrocytes

While both HIF-1 and NF- κ b are induced by hypoxia and inflammation, a recent *in vivo* study demonstrates a direct dependence of hypoxia-mediated induction of HIF-1 abundance on NF- κ b function (Rius and others 2008). That is, removal of NF- κ b function largely attenuated hypoxia-induced HIF-1 protein abundance in multiple tissues *in vivo*, including the brain. HIF-1 α and VEGF protein abundance in the brain, which was induced by placing mice into hypoxia, was significantly diminished with loss of NF- κ b function. Interestingly, in these conditional transgenic mice, loss of NF- κ b function was almost exclusively observed in astrocytes, not neurons. Thus, NF- κ b function was required for maintenance of the HIF-1 response in astrocytes, which had a significant effect on overall HIF-1 levels in brain and VEGF abundance (Rius and others 2008). Thus, HIF-1 abundance in astrocytes is prominent *in vivo* and the transcription of HIF-1 α is dependent on NF- κ b function. This interaction may not only be one-sided since in some cell types HIF-1 can induce NF- κ b function (Scortegagna and others 2008). This interplay between HIF-1 and NF- κ b in astrocytes is of interest as it suggests a complex interaction of these transcription factors in determining cell viability during hypoxia/ischemia. For example, similar to HIF-1, NF- κ b also induces iNOS (KleinertPautzLinker and Schwarz 2004), which could work in concert with HIF-1 to induce neuronal death (see above).

Similarly, both HIF-1 and NF- κ B are implicated in inducing IL-1 β (MalekBorowiczJargiello and Czuczwar 2007; Zhang and others 2006) which could lead to astrocytic release of glutamate during hypoxia/inflammation (see above and Fig. 3). Moreover, cyclooxygenase, a NF- κ B target, alters astrocyte viability with oxygen glucose deprivation (LeeSongGiffard and Chan 2006). Finally, NF- κ B induces MMPs, which are important determinants of stroke volume (Fig. 4). Given the prominent role of NF- κ B during inflammation, the complex interactions of HIF-1 and NF- κ B in astrocytes may be particularly important in the subacute phase of stroke when inflammatory processes are prominent.

Does neuroprotection equal glioprotection?

While this discussion and research to date has principally examined the impact of hypoxia on astrocyte function and how it alters neuronal viability, astrocyte viability is an absolute requirement for neuronal survival. Thus, it is also pertinent to consider how hypoxia-induced signaling in astrocytes alters cell-autonomous viability. Certainly, some of the hypoxia-induced molecular changes that are induced during hypoxia, such as adenosine and EPO not only protect neurons but are also reported to protect astrocytes or astroglial cell lines from toxic stress (DiazAssarafMiller and Schipper 2005; Lin and others 2008; Liu and others 2006). The protective effects of these molecules on both neurons and astrocytes likely diminish stroke volume. While more speculative, the activation of System X_c in astrocytes, by the combination of hypoxia and IL-1 β , may also enhance astrocyte viability. As discussed above, hypoxia and IL-1 β enhance the uptake of cysteine into astrocytes (FogalLiLobnerMcCullough and Hewett 2007). Since cysteine is converted to glutathione, an important anti-oxidant, the uptake of cysteine may diminish astrocyte cell death in ischemia. In fact, glutathione is protective to astrocytes in ischemic and toxic paradigms (Cho and others 2003; Gabryel and Malecki 2006; GabryelToborek and Malecki 2005; KimJoe and Han 2003). Yet, this presumably adaptive process in astrocytes has the unintended toxic effect on neurons by inducing the release of glutamate from astrocytes (see above). Thus, when identifying hypoxia-induced processes to target for intervention to reduce stroke damage, studying the effects of these targets on both neuronal and astrocyte viability will be essential to maximize the beneficial effects of any intervention.

Summary: during stroke, will the “good” or “bad” effects of hypoxia in astrocytes predominate?

Of course it is overly simplistic to delineate a hypoxia-induced molecular pathway within astrocytes as “good” or “bad”. Clearly, these pathways have varied actions that depend on multiple factors. Mild hypoxia induces preconditioning and neuroprotection when delivered days prior to ischemia, demonstrating its adaptive function under these temporal conditions. In contrast, the presence of inflammatory cytokines during mild hypoxia may compromise neuronal viability to an otherwise non-injurious hypoxic stimulus by altering astrocyte function (FogalLiLobnerMcCullough and Hewett 2007). Similarly, signaling between astrocytes and microglia may induces neuronal cell death during inflammation (Bezzi and others 2001), which is prominent following stroke. Certainly the timing of expression of hypoxia-induced signaling in astrocytes is important for determining its effect on stroke volume. For example, VEGF and MMPs augment stroke-related damage in the acute stroke period, but transform their function to an adaptive role in the days following ischemia. Finally, beyond the effects of hypoxia-signaling in astrocytes on neuronal viability, it is also pertinent to consider how these changes will alter astrocyte viability. By better defining these varied effects of hypoxia signaling in astrocytes we should enhance our understanding of how to reduce damage accompanying stroke.

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Figure 1. Hypoxia and HIF-1 alter the levels of multiple proteins that control abundance of extracellular adenosine

Hypoxia enhances the conversion of ATP, ADP, and AMP to adenosine through the actions of CD39L, CD39 and CD73. Hypoxia also diminishes expression of ENT-1, which diminishes uptake of adenosine from the extracellular space. (In this and remaining figures, the yellow cell is an astrocyte whereas the blue cells depict a neuron.)

Figure 2. The HIFs induce EPO and VEGF, which has adaptive and pathological effects
 EPO, which is a neuroprotectant and glioprotectant, is primarily induced by hypoxia in astrocytes through a HIF-2 dependent mechanism. VEGF is induced by hypoxia primarily through HIF-1. VEGF induces edema in the acute phase of stroke, but has protective effects when expressed several days following stroke onset. (BBB=blood brain barrier)

Figure 3. Hypoxia and inflammation may increase extracellular glutamate concentration

Hypoxia decreases expression of some glutamate transporters, which would be predicted to increase the extracellular glutamate concentration. A combination of mild hypoxia and IL-1 β induces cysteine uptake and glutamate release in astrocytes through the action of System X_c. Cysteine can be converted to glutathione, an antioxidant.

Figure 4. Pathways by which hypoxia may act upon astrocytes to alter neuronal or astrocytic viability

Loss of HIF-1 function in astrocytes is protective to neurons, which is partially mediated by iNOS. NF-κb maintains HIF-1 levels in astrocytes and may have other effects such as induction of cyclooxygenase (COX) or MMP, which alter astrocyte and neuronal viability, respectively.