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Preconditioning protects against oxidative injury involving hypoxia-inducible factor-1 and vascular endothelial growth factor in cultured astrocytes

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ABSTRACT

Tolerance to brain injury involves hypoxia-inducible factor-1 (HIF-1) and its target genes as the key pathway mediating a cascade of events including cell survival, energetics, and angiogenesis. In this study, we established the treatment paradigms for an *in vitro* model of tolerance to oxidative injury in primary astrocytic cultures and further examined the roles for the HIF-1 signalling cascade. Isolated murine astrocytes were preconditioned with sub-toxic concentrations of HIF-1 inducers and subsequently exposed to a H_2O_2 insult, where changes in cell viability and protein expression were determined. Preconditioning with non-damaging concentrations of desferrioxamine (DFO) and ethyl-3,4-dihydroxybenzoate (EDHB) significantly improved cellular viability after H_2O_2 injury treatment. Time course studies revealed that DFO and EDHB treatments alone induced sequential activation of HIF-1 signal transduction where nuclear HIF-1α protein accumulation was detected as early as 2 h, followed by downstream upregulation of intracellular and released VEGF from 4 h and 8 h onwards, respectively. The protective effects of DFO and EDHB preconditioning against H_2O_2 injury were abolished by co-treatment with cycloheximide, an inhibitor of protein synthesis. Importantly, when the anti-HIF-1 compound, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) was used, the cytoprotection and VEGF accumulation produced by DFO and EDHB preconditioning were diminished. These results indicate the essential role of the HIF-1 pathway in our model of tolerance against oxidative injury in cultured astrocytes, and suggest roles for astrocytic HIF-1 expression and VEGF release which may influence the function of surrounding cells and vasculature during oxidative stress-related brain diseases.

1. Introduction

Preconditioning with mild, non-damaging stress can induce tolerance against a subsequent severe insult (Dirnagl et al., 2003; Gidday, 2006). Recent studies have shown that hypoxia-inducible factor-1 (HIF-1) is a key transcription factor inducing adaptive responses during hypoxic or ischaemic conditions in the brain, in both scenarios of preconditioning and injury (Semenza, 2001). HIF-1 is composed of two protein subunits, HIF-1α (120 kDa) and HIF-1β (91–94 kDa) (Wang and Semenza, 1995). Since HIF-1β is constitutively expressed, the overall expression of HIF-1 is dependent on the breakdown of HIF-1α which is primarily regulated by enzymes known as HIF prolyl-hydroxylase domain-containing proteins (PHDs) (Bruick and McKnight, 2001). Under normoxic conditions, in the presence of di-oxygen, a ferrous ion and 2-oxoglutarate, PHDs catalyze the hydroxylation of specific proline residues (Pro-402 and Pro-564) in the oxygen-dependent degradation domain of HIF-1α. The hydroxylated form of HIF-1α is targeted by von Hippel–Lindau protein to form a complex which is ultimately subject to ubiquitination and proteasomal degradation (Yu et al., 2001).

During hypoxia, or by the means of pharmacological manipulation where PHD activity is inhibited, HIF-1α is stabilized enabling it to dimerize with HIF-1β. HIF-1 heterodimers can then bind to the DNA consensus sequence of the hypoxia-response element, and initiate transcription of target genes such as vascular endothelial growth factor (VEGF) and erythropoietin (Epo) (Sharp and Bernaudin, 2004). Previous studies have shown that compounds which reduce PHD activity and increase levels of HIF-1 can produce neuroprotection of hippocampal and cortical neurons (Hamrick et al., 2005; Siddiq et al., 2005; Zaman et al., 1999), as well as various ischaemic injury models in neonatal and adult rat brains *in vivo* (Bergeron et al., 1999; Jones et al., 2008; Mu et al., 2005; Siddiq et al., 2005). Overall, HIF-1 is involved in key events that are likely to contribute to neuroprotection, including neurogenesis, angiogenesis, glycolysis and cell proliferation/survival (Sharp and Bernaudin, 2004).

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In physiological and pathological states, astrocytes can influence the survival of neighbouring neurons by various mechanisms (Trendelenburg and Dirnagl, 2005) and enhancing astroglial viability may serve as an alternative neuroprotective strategy by preserving neuronal–astrocytic interactions. In particular, astrocytes are important in preventing oxidative stress by rapid removal of hydrogen peroxide (H_2O_2) which may react with iron to form cell-damaging hydroxyl radicals via the Fenton reaction (Dringen et al., 2005). Recent studies have shown that preconditioning with PHD inhibitors significantly reduced cell death after cytotoxic insults in C6 glioma cells (Yang et al., 2004; Yang et al., 2005) indicating the existence of HIF-1 signalling in astroglial cells and its possible involvement in the development of tolerance to brain injury. In the present study, we examined whether preconditioning with a number of compounds, that are known to inhibit PHD activity, can afford cytoprotection against H_2O_2-induced oxidative injury in primary cultures of forebrain astroglia through the activation of HIF-1. We found that preconditioning with desferrioxamine (DFO) and ethyl 3,4-dihydroxybenzoate (EDHB) conferred protection involving sequential induction of HIF-1α accumulation and VEGF upregulation, and that these effects were attenuated by the selective HIF-1 inhibitor YC-1.

2. Materials and methods

2.1. Cell cultures and drug treatments

All experiments were performed in accordance with the ethical code of the National Health and Medical Research Council (Australia) and with the approval of Howard Florey Institute Animal Experimentation Ethics Committee. Swiss white mice were obtained from Animal Resources Centre (Perth, WA, Australia). Primary astrocyte cultures were prepared by a previously described method with some modifications (Moldrich et al., 2002). In brief, the forebrains of postnatal day 1–2 Swiss white mice were removed, digested and grown in a 75 cm^2 culture flask with astrocytic medium (AM) containing Dulbecco's Modified Eagle's Medium, 10% certified fetal bovine serum, 1% penicillin/streptomycin and 0.25% Fungizone™. At 10 days *in vitro* (*div*), cells were detached and subcultured into 24-well or 48-well culture dishes at the optimum density of 2×10^4 cells/cm^2. Shaking (15–18 h) during re-plating of cultures facilitated the removal of non-astrocyte population of cells that reside loosely on top of astrocytes, thereby improved the overall purity of the secondary cultures of astroglia (McCarthy and de Vellis, 1980). Cells were maintained in a humidified incubator (Themo Fisher Scientific, Waltham, MA, USA) supplied with 5% CO_2 at 37 °C and complete media changes were carried out every 3 days. Cultures established from these procedures in our laboratory contain >90% of cells positive for the specific marker for astrocytes, glial fibrillary acidic protein (Moldrich et al., 2002).

Drug treatments were conducted when the cultures became confluent at 22 *div*, astrocytes were preconditioned (for between 0.5 and 24 h) with sub-toxic concentrations of PHD inhibitors (DFO, 0.1–1 mM; 3,4-dihydroxybenzoate (DHB), 0.1–3 mM; EDHB, 0.3–0.8 mM) and then immediately exposed to hydrogen peroxide (H_2O_2; 0.1–1 mM) for a further 24 h. All drugs were prepared in AM with the exception that 10% (v/v) dimethyl sulfoxide (DMSO) was used to solubilize DHB, EDHB and 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1). The final concentration of DMSO did not exceed 0.15% which was found to have no effect on the viability of the astrocytes (data not shown).

2.2. Measurement of cellular viability

Cellular viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983). At the end of drug treatments, MTT (0.5 mg/ml) was incubated with the cells at 37 °C in 5% CO_2 for 30 min. After aspiration of the media, DMSO was added to each well to dissolve the formazan product. The absorbance was determined at wavelength 570 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Hoescht staining and cell counting

Cells were rinsed with phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature. After washes with PBS, 1 μg/ml of Hoechst 33342 in PBS was added to each well and incubated for 15 min. The cells were observed under fluorescent microscopy (Olympus IX71, Tokyo, Japan). For quantification of cell survival, 4 photomicrographs of random visual fields each from 2 wells per treatment condition were taken with a digital camera (Olympus C-5050, Tokyo, Japan) for blind counting on the numbers of viable cells with normal nuclear morphology displaying dispersed chromatin. Quantification of cells was facilitated by using *Image J* software from the National Institutes of Health (http://rsb.info.nih.gov/ij/). Photomicrographs were converted to 8-bit binary images. Automated counting of cells was performed with the analyze particles function by specifying appropriate size and circularity of objects. The overlapping nuclei were excluded from the automated counting by setting an upper threshold of particle size and were manually counted with the cell counter tool. The number of cells with condensed nuclei was also manually counted and subtracted from the total cell number to obtain the number of viable cells.

2.4. Whole cell lysate and nuclear extract preparation

For whole cell lysates, cells were rinsed with PBS and lysed by gentle shaking for 1 h in radioimmunoprecipitation assay buffer (RIPA: 100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). The cell suspensions were collected and frozen at −20 °C until use. For nuclear extracts, astrocytes were washed with PBS, harvested in homogenization buffer (15 mM Tris–HCl, pH 7.6, 1 mM dithiothreitol (DTT), 0.25 mM sucrose, 1 mM $MgCl_2$, 0.5 mM phenylmethanesulphonylfluoride, 2.5 mM EDTA), and centrifuged at 5000*g* for 10 min at 4 °C. After removal of the supernatant (cytosolic fraction), cell pellet was resuspended in 10 volume extraction buffer (10 mM Tris–HCl, pH 7.6, 0.5 mM DTT, 1.5 mM $MgCl_2$, 0.82 M NaCl, 25% glycerol, 0.5 mM EDTA) and sonicated for 20 s using a Microson ultrasonic cell disrupter (Heat Systems Ultrasonic, Farmingdale, NY, USA). Samples were gently mixed and centrifuged at 20,000*g* for 15 min. The supernatant (nuclear fraction) was collected and frozen at −20 °C until use. All buffers used above were kept ice-cold and supplemented with Complete Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail. Protein content was determined using a DC Protein Assay Kit.

2.5. Western blot analysis and ELISA

Equal amount of protein samples (50 μg) was electrophoresed on 6% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane. The membrane was washed with Tris-buffered saline (TBS; 50 mM Tris–HCl, 1.5% NaCl, pH 7.4) and incubated with blocking solution (3% w/v skim milk in TBS) for 2 h at room temperature. After blocking, the membrane was incubated overnight with primary antibody (rabbit polyclonal HIF-1α; 1:1000 dilution) at 4 °C. The membrane was washed with TBS containing 0.1% Tween-20 followed by incubation with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:2000 dilution) for 2 h at room temperature. Protein bands were visualized by Lumilight chemiluminescence substrate and exposure of the membrane to Hyperfilm ECL. Nuclear extracts of untreated and $CoCl_2$-treated COS-7 cells were used