European Journal of Pharmacology 633 (2010) 24–32

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and Analgesia

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

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ARTICLE INFO

Article history:
Received 16 September 2009
Received in revised form 18 January 2010
Accepted 2 February 2010
Available online 11 February 2010

Keywords:
Tolerance
Neuroprotection
Oxidative stress
HIF-1 (hypoxia-inducible factor-1) target gene
Prolyl hydroxylase
Desferrioxamine

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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doi:10.1016/j.ejphar.2010.02.008

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

as negative and positive controls, respectively, to indicate the position of the specific protein band of HIF-1α (120 kDa) on the Western blots (data not shown). The MCID image analysis software (InterFocus Imaging, Linton, Cambridge, UK) was used to quantify the area and density of protein bands.

To determine the protein levels of HIF-1α, Epo and VEGF after drug treatments, commercially available ELISA kits were used with samples of whole cell lysates or culture media. The assays were performed in accordance with the manufacturer's instructions.

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Dulbecco's Modified Eagle's Medium, fetal bovine serum, fungizone, penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Culture flasks and multiwell dishes were obtained from Nunc (Roskilde, Denmark). YC-1 was obtained from Cayman Chemical (Ann Arbor, MI, USA). DC Protein Assay Kit and nitrocellulose membrane were obtained from Bio-rad Laboratories (Hercules, CA, USA). COS-7 nuclear extracts lysate and rabbit polyclonal anti-HIF-1α antibody were obtained from Novus Biologicals (Littleton, CO, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Millipore (Billerica, MA, USA). Complete protease inhibitor cocktail and lumilight chemiluminescence substrate were obtained from Roche (Basel, Switzerland). Hyperfilm ECL was obtained from GE Healthcare (Amersham, Buckinghamshire, UK). ELISA kits for total HIF-1α, Epo and VEGF were obtained from R&D Systems (Minneapolis, MN, USA).

2.6. Statistical analysis

All experimental results are presented as mean ± S.E.M. of the average values of triplicates from at least 3 independent experiments. Differences between treatments were analyzed by repeated measures of one- or two-way ANOVA as appropriate with the Bonferroni *post-hoc* test, using Graphpad Prism version 5.0 (Graphpad Software, USA). *P*-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Effects of preconditioning with HIF-1 inducers against oxidative insult in astrocytes

Initial experiments were performed to determine the cytotoxicity profiles of, DFO, DHB, EDHB, and H_2O_2 in the primary astroglial cultures (Fig. 1A). EDHB and DFO all produced a significant decrease in cell viability at higher concentrations ($P<0.05$), although under these conditions DHB did not reduce cell viability. Non-toxic concentrations of the HIF-1 inducers (DFO, 0.1–1 mM; DHB, 0.5–1.5 mM; EDHB, 0.3–0.8 mM) were selected as preconditioning treatments, while the concentration of 1 mM H_2O_2 treatment for 24 h ($IC_{50} = 820 \pm 47$ μM; data not shown) was chosen as a model of oxidative injury as it consistently produced a significant decrease in the astrocyte viability by 50–75% of vehicle control (Fig. 1B, C, D).

To determine whether the preconditioning of astrocytes with the HIF-1 inducers could protect against oxidative injury, cultures were treated with the above-mentioned concentrations of the drugs for 4 or 24 h, and subsequently exposed to 1 mM H_2O_2 for 24 h on *div* 22. Preconditioning with the sub-toxic concentrations of DFO, DHB or EDHB significantly protected the astrocytes from H_2O_2-induced injury ($P<0.05$; Fig. 1B, C, D). The preconditioning effects of DFO were concentration- and time-dependent where protection induced by 1 mM DFO was greater than that found with 100 μM DFO, and 100 μM pretreatment with DFO for 24 h was more effective than for 4 h. The effects of EDHB appeared to be more potent than DHB, where concentrations of EDHB lower than those of DHB were sufficient to render maximal protection, perhaps due to the enhanced cell membrane-permeability of EDHB by ethyl modification of the chemical structure (Sasaki et al., 1987). DHB, but not EDHB, produced a concentration-dependent improvement in cell viability ($P<0.05$; Fig 1C, D). In addition to the MTT viability assay, morphological observations and cell counting after Hoescht staining were performed to confirm the changes in cell survival. Exposure to 1 mM H_2O_2 for 24 h significantly reduced the number of viable cells by approximately 50% and increased the number of cells with apoptotic-like nuclear condensation (Fig. 2B). Pretreatments with DFO (Fig 2E, G) and EDHB (Fig. 2F, H) reversed these effects caused by H_2O_2 injury by returning the number of live cells to essentially control levels. Data obtained from MTT

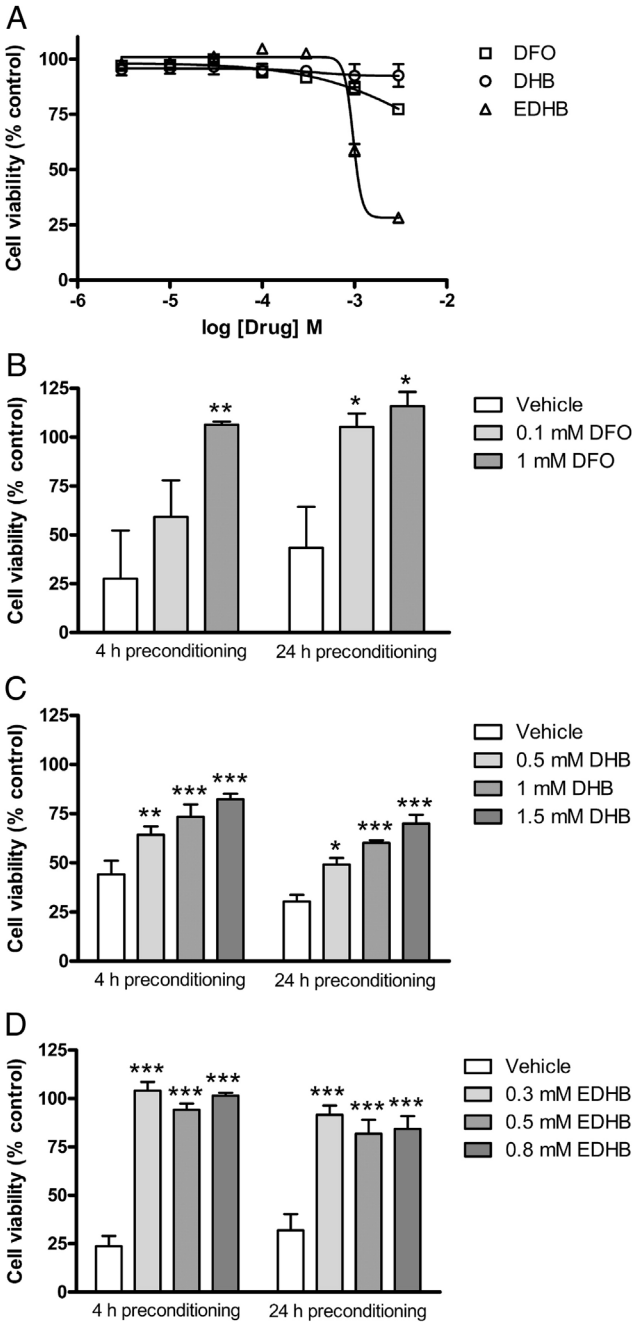

Fig. 1. Cytotoxicity profile of HIF-1 inducers and preconditioning against H_2O_2. Astrocytes were treated with 3 μM–3 mM of DFO, DHB and EDHB for 24 h to determine the non-toxic concentrations of these drugs (A). For the preconditioning experiments, astrocytes were pretreated with vehicle or sub-toxic concentrations of DFO (B), DHB (C) or EDHB (D) for 4 or 24 h. Cell viability of the astrocyte cultures was determined after a subsequent 24 h exposure to 1 mM H_2O_2. MTT assay was used to assess the viability of astrocytes after treatments. Data are expressed as mean ± S.E.M. from 3–4 independent experiments performed in triplicate. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to vehicle pretreatment by two-way ANOVA, Bonferroni *post-hoc* test.

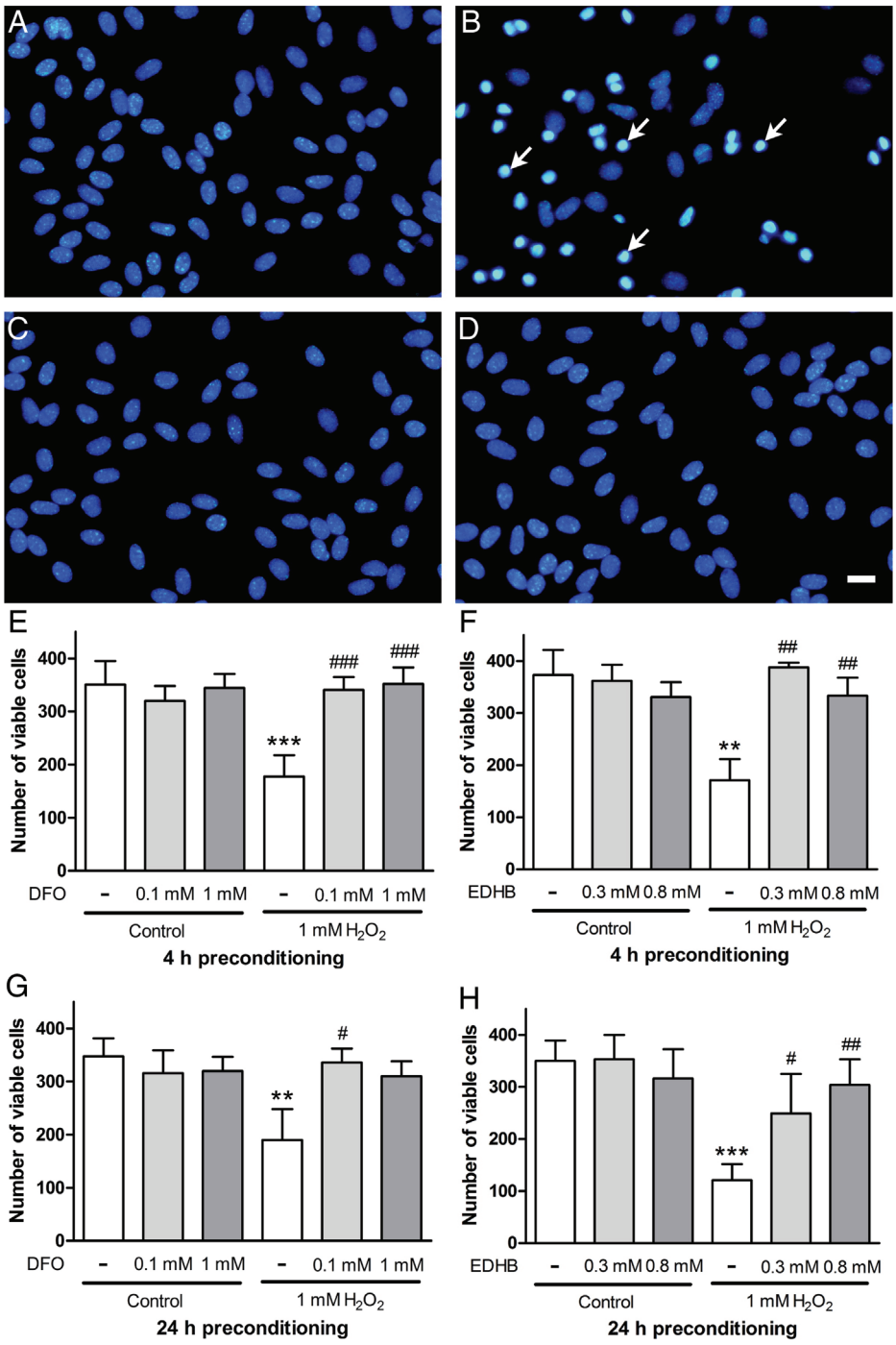

Fig. 2. DFO and EDHB preconditioning prevented loss of viable cells and reduced apoptotic-like nuclear condensation. Representative Hoechst staining photos of untreated astrocytes (A) showing normal morphology of dispersed chromatin in the nuclei. Astrocytes were preconditioned for 24 h with vehicle (B), 1 mM DFO (C) or 0.8 mM EDHB (D) and then treated with 1 mM H_2O_2 for 24 h. Nuclear condensation is indicated by the arrows. Scale bar represents 20 μm. Astrocytes were pretreated for 4 (E, F) or 24 (G, H) hours with DFO (E, G) or EDHB (F, H) and subsequently injured by 1 mM H_2O_2 for 24 h. Cells were fixed with 4% PFA and stained with Hoechst 33342. 4 random visual fields each from 2 wells per treatment conditions were counted. Numbers of viable cells are presented as mean ± S.E.M. per photo from 3–4 independent experiments. $^{**}P<0.01$ and $^{***}P<0.001$ compared to uninjured control; $\#P<0.05$, $\#\#P<0.01$ and $\#\#\#P<0.001$ compared to vehicle pretreatment by two-way ANOVA, Bonferroni *post-hoc* test.

assays (Fig. 1B, D) and cell counting (Fig. 2E–H) showed similar results, confirming MTT measurements as a reliable index of cell viability.

Since preconditioning with DFO and EDHB produced the most consistent and robust cytoprotective effects against H_2O_2-induced oxidative injury, we further explored the time required to effect protection. Cultures were pretreated for 0.5, 1, 2, 4, 8, 16 or 24 h with DFO and EDHB prior to a 1 mM H_2O_2 insult for 24 h (Fig. 3A, B). All preconditioning treatments produced significant improvements in cell viability ($P<0.05$). Surprisingly, the protection was induced rapidly by both compounds where preconditioning with DFO achieved complete protection within 4–8 h and pretreatment with EDHB fully protected the cells at 0.5 h.

3.2. Elevation in nuclear HIF-1α expression, intracellular and released VEGF by DFO and EDHB treatments

To assess whether treatments with DFO and EDHB alone could stabilize HIF-1α protein, Western immunoblotting was employed to

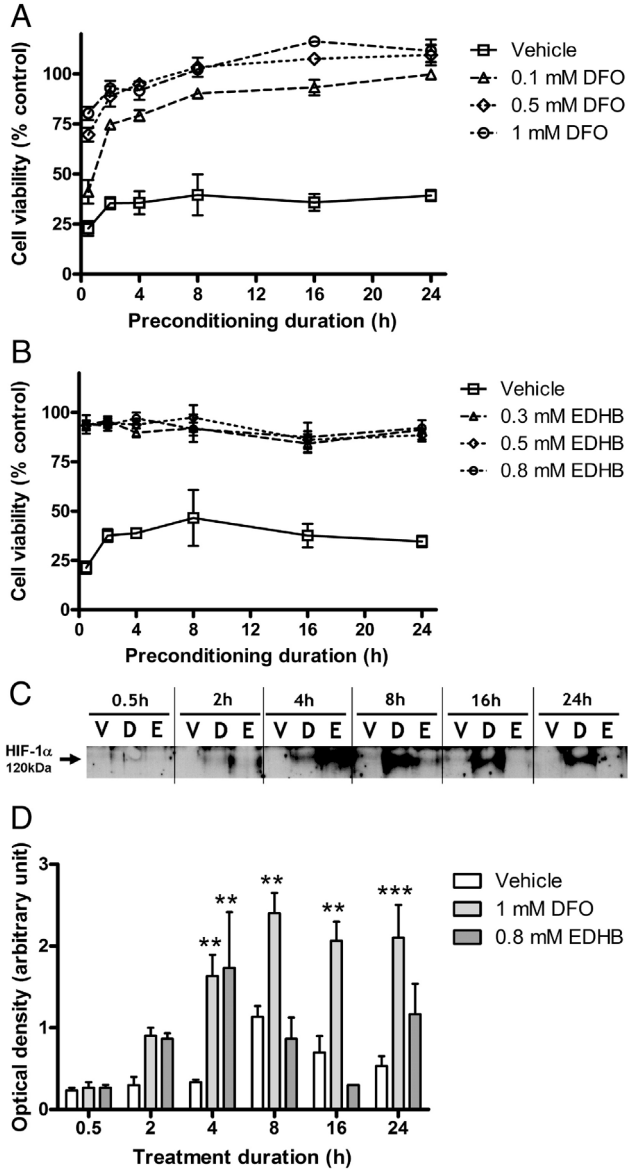

Fig. 3. Time course of DFO and EDHB cytoprotective effects and nuclear HIF-1α expression. Astrocytes were pretreated with vehicle, 1 mM DFO (A) or 0.8 mM EDHB (B) for up to 24 h period and exposed to 1 mM H_2O_2 insult for 24 h. Cell viability of the astrocytic cultures was assessed by MTT assay. Data are expressed as mean ± S.E.M. from 3–4 independent experiments performed in triplicate. All concentrations of DFO and EDHB at all time points are significantly different to the vehicle pretreatment ($P<0.05$). Nuclear extracts were obtained from astrocytes treated with vehicle, 1 mM DFO or 0.8 mM EDHB for various periods over 24 h. Western blotting analysis was performed to determine the nuclear expression of HIF-1α. A representative Western blot (C) and the densitometric measurements from 3 independent experiments (D) are shown. $^{**}P<0.01$ and $^{***}P<0.001$ compared to vehicle pretreatment by two-way ANOVA, Bonferroni *post-hoc* test.

detect the levels of HIF-1α in the nuclear extracts of DFO- and EDHB-treated astrocytes. An accumulation of HIF-1α was found 2 h after treatment with DFO or EDHB. DFO-stimulated expression was sustained at maximum levels from 8 to 24 h, whereas EDHB-induced a more transient expression of HIF-1α which peaked at 4 h (Fig. 3C, D), and the time courses of action of the changes in HIF-1α expression were significantly different ($P<0.05$).

VEGF is one of the HIF-1 downstream targets induced by hypoxia and hypoxia-mimetic compounds in astrocytes (Ijichi et al., 1995; Sinor et al., 1998), so we measured the levels of intracellular and released VEGF by ELISA. Intracellular VEGF expression was up-regulated by both 1 mM DFO and 0.8 mM EDHB treatments from 4 h onwards, whereas an increasing amount of VEGF was released into the culture media from 8 h (Fig. 4A, B). In addition, VEGF levels were measured in culture media after pretreatment with DFO or EDHB for 4 or 24 h and subsequent 24 h H_2O_2 insult (Fig. 4C, D). DFO- and EDHB-stimulated release of VEGF was

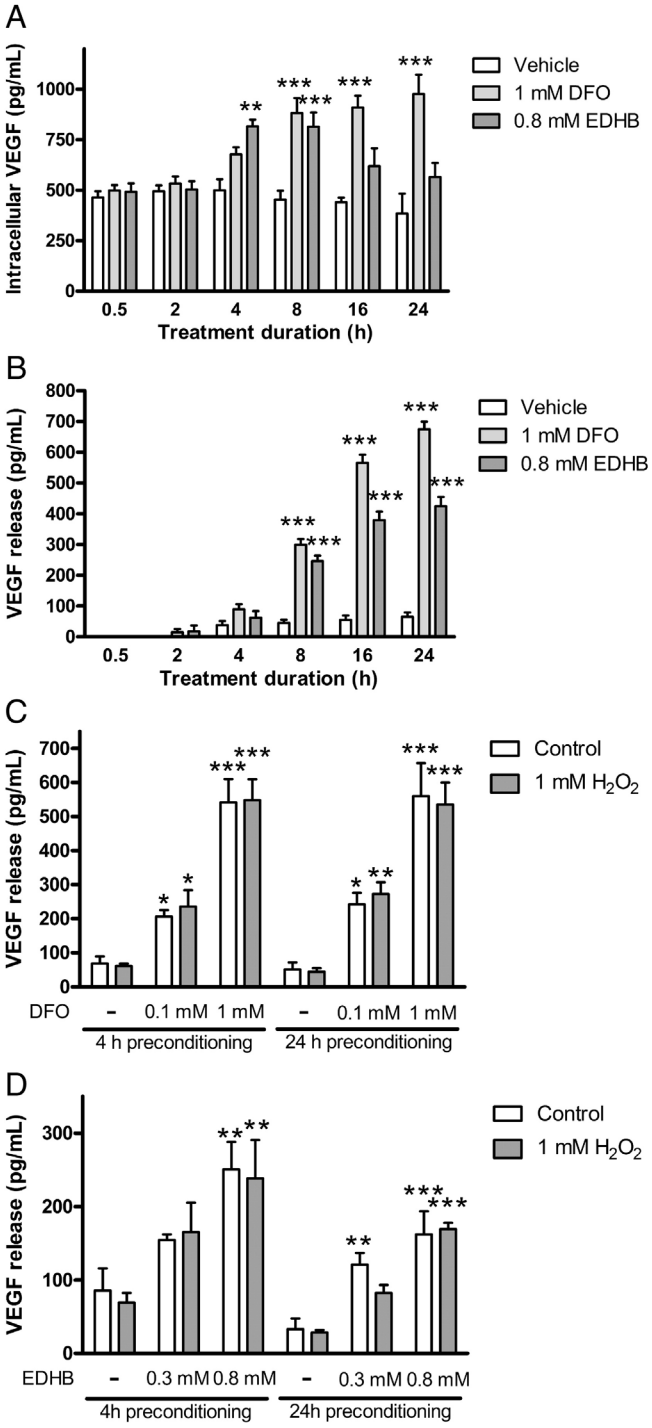

Fig. 4. Effects of DFO and EDHB treatments on intracellular and released VEGF levels. Astrocytes were treated with vehicle, 1 mM DFO or 0.8 mM EDHB for up to 24 h. At the end of the treatments, culture media and whole cell lysates were collected to measure the intracellular (A) and released (B) VEGF levels by ELISA. VEGF levels were also determined in culture media after 4 or 24 h pretreatments with DFO (C) or EDHB (D) and subsequent exposure to 24 h 1 mM H_2O_2. Data are expressed as mean ± S.E.M. from 3–4 independent experiments performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to vehicle treatment by two-way ANOVA, Bonferroni *post-hoc* test.

still evident after H_2O_2-induced injury and H_2O_2 did not affect the amount of VEGF compared to control. Different temporal profiles were observed between the treatments ($P<0.05$; Fig. 3D, 4A, B) where the actions of DFO were more sustained over time, while protein expression induced by EDHB was short-lived. Overall, treatments with DFO and EDHB stimulated HIF-1α accumulation, VEGF production and VEGF release in a cascade-related manner.

3.3. Mechanistic insights into the effects of PHD inhibitors on HIF-1α and VEGF expression

Induction of HIF-1 accumulation and transcriptional activity can be blocked by the protein synthesis inhibitor cycloheximide (CHX), implicating a requirement of *de novo* protein synthesis for HIF-1 signalling (Semenza et al., 1994). Hence, we tested if CHX (100 μM) could reduce the cytoprotection afforded by DFO or EDHB preconditioning. Co-incubation of astrocytic cultures with 100 μM CHX failed to reverse the protection of astrocytes by 1 mM DFO and 0.8 mM EDHB for 24 h (data not shown). Because the protection produced by 1 mM DFO and 0.8 mM EDHB was so robust at the higher concentrations, we decided to test the effects of lower concentrations of the preconditioning treatments in further mechanistic studies. The protective effects of preconditioning with lower concentrations of DFO (30 μM) and EDHB (10 and 30 μM) for 8 h were partially prevented by the presence of CHX (Fig. 5A, B).

Next we sought to define the involvement of HIF-1 signal transduction in our tolerance model by suppressing its downstream effects. YC-1 was first identified as a soluble guanylyl cyclase (sGC) activator (Ko et al., 1994), but more recently has been shown to inhibit HIF-1α expression via sGC/cGMP-independent mechanisms (Chun et al., 2001). YC-1 (100 μM) treatment alone produced a minor effect (<20%) on mitochondrial activity of the astrocytes as indicated by the data from MTT assays, although morphological observations and cell counting of the YC-1-treated astrocytes demonstrated no significant effects on viability (data not shown). Improvements in cell viability induced by pretreatment with DFO or EDHB for 8 h prior to H_2O_2 insults were significantly attenuated by co-incubation with YC-1 (Fig. 5C, D). To confirm that the reversal effect of YC-1 on cytoprotection was due to inhibition of the HIF-1 pathway, levels of HIF-1α and intracellular and released VEGF were determined. There were no significant changes in HIF-1α protein levels detected in whole cell lysates using the HIF-1α ELISA at the 8 h time point using the lower concentrations of DFO and EDHB (Fig. 6A). The upregulation of intracellular and released VEGF in DFO-treated astrocytes was diminished by the effects of YC-1 (Fig. 6B, C). EDHB significantly increased VEGF release to a smaller extent than DFO under this treatment paradigm, but YC-1 co-treatment maintained the amount of released VEGF at the basal level (Fig. 6C). These results suggest the likely involvement of HIF-1 induction and downstream effects in our model of tolerance against oxidative injury in astroglia.

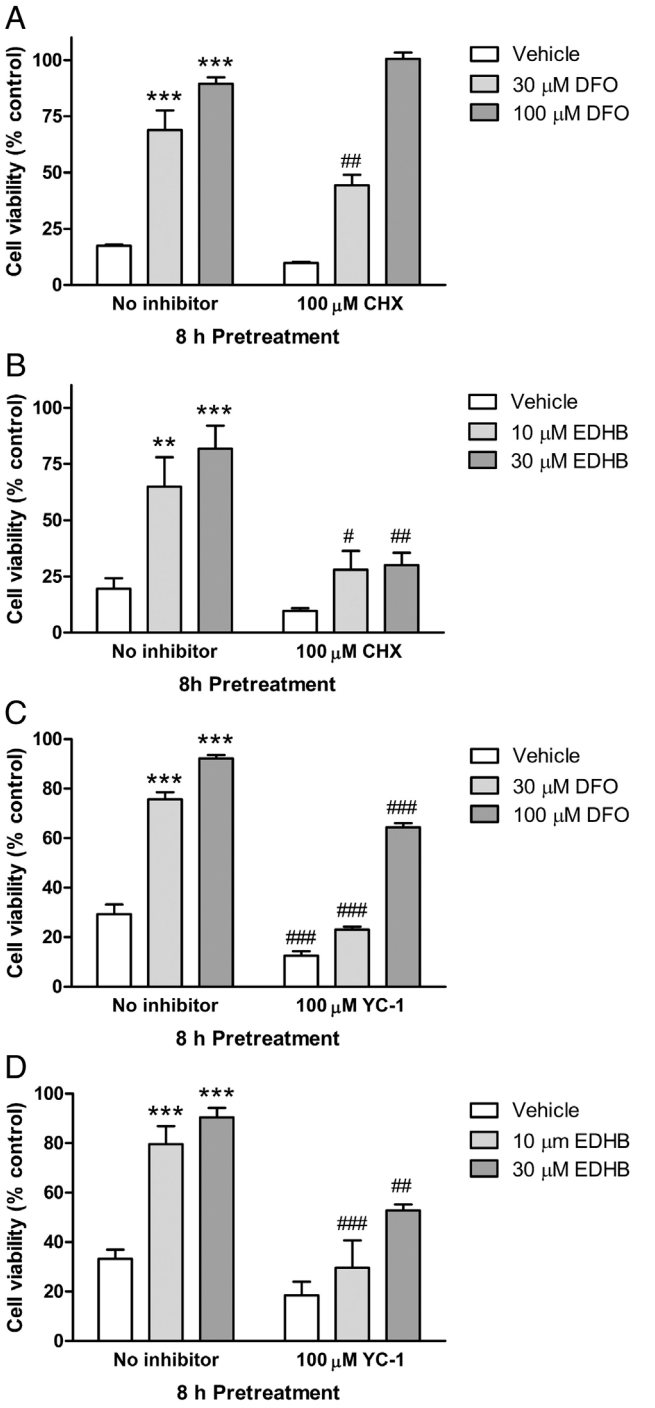

Fig. 5. Effects of CHX and YC-1 on DFO- and EDHB-induced cytoprotection against H_2O_2 injury. Astrocytes were pretreated with vehicle, DFO (A, C) and EDHB (B, D) in the absence or presence of 100 μM CHX (A, B) or 100 μM YC-1 (C, D) for 8 h, and subsequently exposed to 1 mM H_2O_2 insult for 24 h. Cell viability of the astrocytic cultures was assessed by MTT assay. Data are expressed as mean ± S.E.M. from 3 independent experiments performed in triplicate. $^{**}P<0.01$ and $^{***}P<0.001$ compared to vehicle pretreatment; $\#P<0.05$, $\#\#P<0.01$ and $\#\#\#P<0.001$ compared to corresponding pretreatment without CHX or YC-1 co-incubation by two-way ANOVA, Bonferroni *post-hoc* test.

4. Discussion

Preconditioning with hypoxia and hypoxia-mimetics induces tolerance to injury in various *in vitro* and *in vivo* models and is believed to involve changes in expression of HIF-1 and its target gene (Semenza, 2001). Recent studies examining conditional knockout of HIF-1α in different types of cells have highlighted the complex roles of HIF-1 signalling in brain injury and repair, and in particular, the need to elucidate the importance of cell-specific regulation and possible complex contributions of this system to injury or repair processes (Chavez et al., 2006; Helton et al., 2005; Vangeison et al., 2008). In the present study, we established an *in vitro* model of tolerance to oxidative injury in primary astrocytic cultures using a number of PHD inhibitors which have previously been shown to induce HIF-1 expression (Chan et al., 2002; Wright et al., 2003). We observed that DFO, DHB and EDHB were able to protect the astrocytes against H_2O_2-induced injury. Indeed, DFO and EDHB produced robust and consistent protective actions, and when we explored the mechanisms involved, HIF-1α accumulation was found to be induced by the preconditioning treatments with downstream upregulation of intracellular and released VEGF (Fig. 7). Overall, our findings reveal the existence of an astrocytic HIF-1-mediated

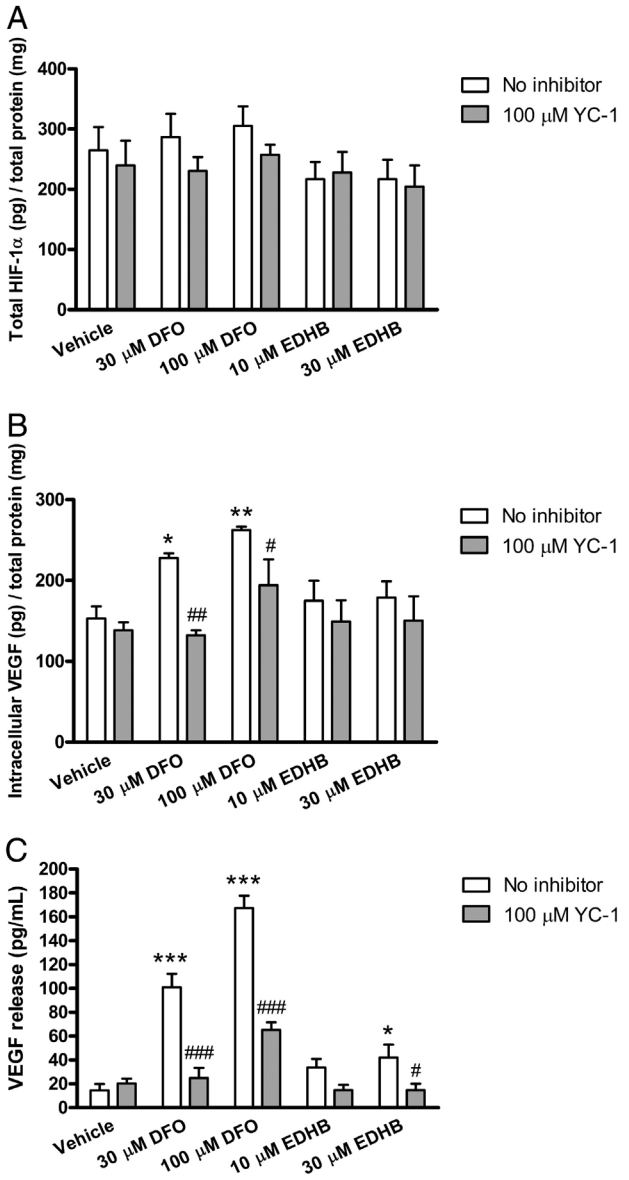

Fig. 6. Effect of YC-1 co-incubation on DFO- and EDHB-induced HIF-1α and VEGF expression. Astrocytes were treated with vehicle, DFO or EDHB for 8 h, with or without 100 μM YC-1 co-incubation. At the end of the treatments, media from each treatment condition was collected and cells were harvested in RIPA buffer. ELISAs were performed in whole cell lysates for total HIF-1α (A) and intracellular VEGF (B), and media samples for VEGF release (C). Data are expressed as mean ± S.E.M. from 3 independent experiments performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to vehicle pretreatment, $\#P<0.05$, $\#\#P<0.01$ and $\#\#\#P<0.001$ compared to corresponding pretreatment without YC-1 co-incubation by two-way ANOVA, Bonferroni *post-hoc* test.

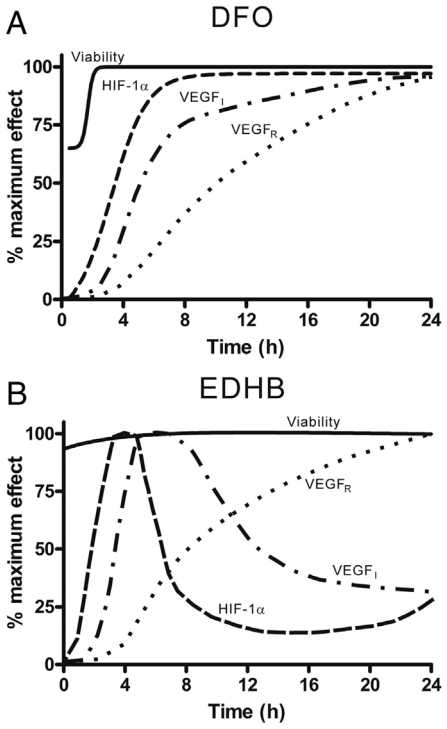

Fig. 7. Schematic representation of time courses of cellular events in DFO- and EDHB-treated astrocytes. The timing over 24 h of cellular events in astrocytes treated with DFO (A) or EDHB (B) observed in this study. Viability = increased cellular survival against oxidative injury after preconditioning, HIF-1α = HIF-1α accumulation, $VEGF_I$ = intracellular VEGF expression and $VEGF_R$ = VEGF release.

neuroprotective cascade with the potential to positively support the function of surrounding cells in the brain.

The mechanisms of action of all the PHD inhibitors employed in this study can be divided into 2 categories: (1) transition metals or iron chelators that remove the PHD-bound ferrous ion and (2) structural analogues of 2-oxoglutarate that competitively antagonize co-substrate binding (see Introduction). EDHB is an analogue of 2-oxoglutarate that also chelates iron with an affinity substantially lower than DFO, but is capable of causing iron deficiency *in vitro* (Wang et al., 2002). However, it is unclear whether the putative mechanisms of HIF-1 induction by these agents have differential effects on transactivation of selective HIF-1 target genes. This concept also reflects the possibility that other general mechanisms, independent of HIF-1, may be involved in the development of tolerance to injury by chemicals that can induce HIF-1 activation. Indeed, HIF-1 induction may also occur indirectly as a secondary event through stimulation of other signalling pathways that are not common to all HIF-1 inducers. For example, DFO, but not $CoCl_2$, can induce cyclooxygenase-2 (COX-2) expression and the COX-2 inhibitor NS-398 suppressed the DFO-induced expression of HIF-1α in colon cancer cell lines (Woo et al., 2006). Our studies demonstrated that preconditioning with DFO, DHB and EDHB improved the viability of astrocytes. Chavez et al. (2006) have reported that HIF-2 might be an important mediator of the adaptive response of astrocytes to hypoxia or hypoxia-mimetic agents by increasing the levels of Epo. Indeed, Epo is predominantly a target gene of HIF-2 (Rankin et al., 2007; Warnecke et al. 2004). We failed to observe any changes in Epo levels using ELISA (data not shown), indicating that the Epo/HIF-2 system appears not to be involved in the protection by PHD inhibitors under the present experimental conditions.

HIF-1 is the master transcription factor that mediates cellular adaptations in response to a low oxygen environment. Herein, we described the changes in expression of HIF-1α and one of the most characterized HIF-1 target genes VEGF, following DFO and EDHB treatments. Consistent with several other studies that showed an inducible VEGF expression in hypoxic astrocytes (Behzadian et al., 1998; Chavez et al., 2006; Sinor et al., 1998), we detected a prominent increase in the levels of HIF-1α and VEGF after DFO and EDHB treatments. We observed that HIF-1α accumulation began within 2 h of treatment with DFO or EDHB. While DFO-stimulated expression of HIF-1α was sustained, EDHB-induced expression was transient and peaked at 4 h (Fig. 7). Similar to the HIF-1α expression pattern, different temporal profiles were observed for VEGF production and release, where the DFO increased levels of VEGF for a sustained period of time, and the VEGF induced by EDHB was short-lived (Fig. 7). These data could be interpreted as reflecting an equilibrium between a synthetic pool and a releasable pool of VEGF, with "spillover" being a response to the strength of stimulus. Apart from its established role as a potent angiogenic mitogen, the functions of VEGF within the CNS have emerged as both neurotrophic

and neuroprotective, (Storkebaum et al., 2004). Given that VEGF is a multi-targeted factor that acts on many types of cells in the brain, its release from astroglia in response to injury could mediate cytoprotective mechanisms including preventing neuronal death, stimulating neurogenesis and modulation of the vasculature (Brockington et al., 2004; Rosenstein and Krum, 2004).

Previous studies have shown that *de novo* protein synthesis is required for HIF-1 signalling, where CHX inhibited HIF-1 production and its ability to induce transcription (Semenza et al., 1994). In the present study, we found that CHX could not affect the astrocyte protection induced by DFO (1 mM) or EDHB (0.8 mM) at the concentrations initially employed. Indeed, the protection using high concentrations of DFO and EDHB was so strong, presumably because the response produced was towards the maximal end of the concentration–response relationship, therefore we elected to use lower concentrations in all subsequent mechanistic studies. Using this strategy, the protective effects of preconditioning with lower concentrations of DFO and EDHB for 8 h were reduced by CHX treatment, indicating that new protein synthesis was required for the protection produced by DFO and EDHB.

To define the involvement of HIF-1 in the tolerance produced by the PHD inhibitors (DFO and EDHB) we used pharmacological inhibition of HIF-1. YC-1 was initially identified as a compound which could activate soluble sGC (Ko et al., 1994), and more recently has been shown to inhibit HIF-1α expression via mechanisms independent from sGC/cGMP (Chun et al., 2001). YC-1 suppressed the hypoxia-induced upregulation of VEGF and Epo, and is associated with a reduction in HIF-1α protein accumulation and DNA-binding activity in Hep3B cells (Chun et al., 2001). More recently, YC-1 was found to decrease HIF-1α stability by directly binding to a specific amino acid region (720–780) located within the inhibitory domain of HIF-1α (Kim et al., 2006). We were able to observe that the protection caused by preconditioning with DFO or EDHB was attenuated by YC-1. To confirm that the effect of YC-1 was due to blockade of HIF-1 signalling, we measured levels of HIF-1α and VEGF. While we did not observe any changes in HIF-1α protein expression across the different treatment conditions, we were able to determine that YC-1 could attenuate the increase in VEGF caused by both DFO and EDHB. Our results indicate that HIF-1 and its downstream effectors are important for tolerance produced by PHD inhibitors, DFO and EDHB, against oxidative stress in astrocyte cultures.

We applied H_2O_2 to the astroglial cultures to simulate the conditions of oxidative stress which is implicated in the pathogenesis of various neurological disorders. The concentration of H_2O_2 used as an oxidative insult in this study is somewhat higher than those used in previous studies, and may relate to differences in culture conditions including cell density, age of cultures, treatment period and the severity of injury (Prieto and Alonso, 1999; Rajapakse et al., 2003). Exposure to H_2O_2 (1 mM) for 24 h consistently produced in our culture system 50–75% reduction in astrocytic viability, which is associated with a mixture of predominantly apoptotic-like cell death, indicated by Annexin V and propidium iodide staining (data not shown; Diwakarla et al., 2009). The removal of exogenous H_2O_2 in cultured astrocytes is carried out by glutathione peroxidase and catalase with a high clearance rate such that the concentration of H_2O_2 is reduced by half within minutes (Dringen and Hamprecht, 1997). The iron chelator, DFO did not affect the rate of H_2O_2 clearance and pre-incubation with DFO, but not post-incubation, reduced cell death-related lactate dehydrogenase release from cultured astrocytes after exposure to H_2O_2 for 1 h (Liddell et al., 2004). Our results, offer an alternative explanation wherein the reduction in cellular toxicity requires activation of HIF-1 by DFO prior to the H_2O_2 insult. While astrocytes have a relatively higher capacity for the detoxification of peroxides than neurons (Dringen et al., 2005; Hirrlinger et al., 2002), it is possible that enhanced astrocyte survival can provide protective effects to the surrounding neurons during oxidative injury (Maragakis and Rothstein, 2006; Trendelenburg and Dirnagl, 2005). Indeed, several *in vitro* studies have indicated that the viability and functioning of neuronal cells after various toxic insults can be improved by co-culture with astroglia (Desagher et al., 1996; Drukarch et al., 1997; Lamigeon et al., 2001).

In conclusion, our findings indicate that pharmacological preconditioning treatments stimulating the activation of HIF-1 signalling can produce cytoprotective effects against oxidative damage in primary astrocytes. Due to the pivotal role of HIF-1 and its multi-targeted downstream effectors such as VEGF in the CNS, we speculate that the astroglial HIF-1 system may serve as a therapeutic target for neuroprotection and repair in oxidative stress-related neurological disorders.

Acknowledgements

The authors thank Dr. Chew Ling Lau (Howard Florey Institute) for the assistance in establishing astrocytic cultures and Western immunoblotting. This work was supported in part by Neurosciences Victoria (N.M.J.), Percy Baxter Charitable Trust and a Program Grant (#236085) from the National Health and Medical Research Council (Australia) of which P.M.B. is a Senior Principal Research Fellow. P.W.Y.C. was a recipient of a Research Scholarship from the University of Melbourne.

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