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Characterizing Ischaemic Tolerance in Rat Pheochromocytoma (PC12) Cells and Primary Rat Neurons

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Abstract—Preconditioning tissue with sublethal ischaemia or hypoxia can confer tolerance (protection) against subsequent ischaemic challenge. *In vitro* ischaemic preconditioning (IPC) is typically achieved through oxygen glucose deprivation (OGD), whereas hypoxic preconditioning (HPC) involves oxygen deprivation (OD) alone. Here, we report the effects of preconditioning of OGD, OD or glucose deprivation (GD) in ischaemic tolerance models with PC12 cells and primary rat neurons. PC12 cells preconditioned (4 h) with GD or OGD, but not OD, prior to reperfusion (24 h) then ischaemic challenge (OGD 6 h), showed greater mitochondrial activity, reduced cytotoxicity and decreased apoptosis, compared to sham preconditioned PC12 cells. Furthermore, 4 h preconditioning with reduced glucose (0.565 g/L, reduced from 4.5 g/L) conferred protective effects, but not for higher concentrations (1.125 or 2.25 g/L). Preconditioning (4 h) with OGD, but not OD or GD, induced stabilization of hypoxia inducible factor 1α (HIF1α) and upregulation of HIF1 downstream genes (*Vegf, Glut1, Pfkfb3 and Ldha*). In primary rat neurons, only OGD preconditioning (4 h) conferred neuroprotection. OGD preconditioning (4 h) induced stabilization of HIF1α and upregulation of HIF1 downstream genes (*Vegf, Phd2 and Bnip3*). In conclusion, OGD preconditioning (4 h) followed by 24 h reperfusion induced ischaemic tolerance (against OGD, 6 h) in both PC12 cells and primary rat neurons. The OGD preconditioning protection is associated with HIF1α stabilization and upregulation of HIF1 downstream gene expression. GD preconditioning (4 h) leads to protection in PC12 cells, but not in neurons. This GD preconditioning-induced protection was not associated with HIF1α stabilization. © **2020 Published by Elsevier Ltd on behalf of IBRO.**

Key words: neuroprotection, preconditioning, ischaemia, hypoxia, HIF-1, glucose, OGD, GD.

INTRODUCTION

Over the years, neuroprotective therapies for ischaemic stroke have attracted many potential studies and have shown enticing promises in pre-clinical trials; however, ultimately, all candidates taken to clinical trial have failed (Patel and McMullen, 2017). Many drugs have been used to target single molecules to alter the ischaemic cascade, however, an alternate therapy called ischaemic preconditioning (IPC) has shown promising results in neuroprotection (Stevens et al., 2014; Wang et al., 2015). IPC is a phenomenon whereby brief nonlethal ischaemic challenge can upregulate protective mechanisms that build tolerance against critical ischaemia (Meng et al., 2015). Applying a stimulus typically associated with injury, but at the intensity/duration below the threshold for damage, can activate and amplify endogenous protective mechanisms, which then confer protection against subsequent insults (Stevens et al., 2014; Meng et al., 2015). Although the molecular processes of ischaemic tolerance are not fully understood, IPC is recognized to induce adaptive processes associated with vascular remodelling, erythropoiesis and angiogenesis, etc (Koch et al., 2014). A number of studies have shown auspicious IPC effects on neuroprotection both *in vitro* (Prasad et al., 2011; Hillion et al., 2005) and *in vivo* (Dave et al., 2001; Papadakis et al., 2013). One therapy related to *in vivo* studies is called remote ischaemic preconditioning (RIPC), the process of inducing protective effects to distant sensitive organs with temporary ischemia-and-reperfusion cycles to non-vital tissues. RIPC has shown positive results *in vivo* and mainly deals with cardiovascular as well as cerebral processes (Zhou et al., 2018; Liang et al., 2019).

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Abbreviations: GD, glucose deprivation; HPC, hypoxic preconditioning; IPC, ischaemic preconditioning; Nx, normoxia; OD, oxygen deprivation; OGD, oxygen glucose deprivation; PC12, Pheochromocytoma.

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Whilst IPC has shown intriguing results, another therapy, hypoxic preconditioning (HPC) has similar applications (Li et al., 2017). *In vitro*, ischaemic preconditioning (IPC) is typically achieved through oxygen glucose deprivation (OGD), whereas hypoxic preconditioning (HPC) involves oxygen deprivation alone (OD). Various *in vivo* studies have reported that HPC followed by recovery (for 1 or 2 days) protects against focal and global ischaemia in adult and neonatal brain (Gidday et al., 1994; Miller et al., 2001; Sharp et al., 2004). Both IPC- and HPC-induced tolerance require RNA translation and *de novo* protein synthesis. Matsuyama et al. (2000) suggested that a period of reperfusion following IPC/HPC is essential to mediate *de novo* protein synthesis and neuroprotection. IPC/HPC correlates with increased expression of hypoxia inducible factor1 (HIF1), which is the master regulator of cell responses to low oxygen (Sharp et al., 2004; Bradley et al., 2012; Karuppagounder and Ratan, 2012). HIF1 is comprised of heterodimeric subunits: HIF1α, which is oxygen responsive, and HIF1β which is a stable subunit. In the presence of oxygen, HIF1α is hydroxylated, ubiquitinated and degraded (Chen et al., 2012; Strowitzki et al., 2019). However, in the absence of oxygen, HIF1α is stabilized, dimerizing with HIF1β. HIF1 translocates to the nucleus resulting in upregulation of many genes including vascular endothelial growth factor (*Vegf*), erythropoietin (*Epo*), glucose transporters and glycolytic enzymes (Kaelin and Ratcliffe, 2008).

In this study, we investigated effects of OGD, glucose deprivation (GD) and OD preconditioning in an *in vitro* ischaemia model with the PC12 cell line and primary rat neurons. Additionally, we studied the effect of OGD, GD and OD on HIF1α and its downstream genes.

EXPERIMENTAL PROCEDURES

Materials

Rat adrenal pheochromocytoma (PC12) cells, Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L), Dulbecco's phosphate buffered saline (PBS), fetal bovine serum (FBS), heat-inactivated horse serum (HS), poly-D-lysine (50×), trypsin (50×), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Trypan Blue, protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), Tween-20, Tris, glycine, sodium-dodecyl sulphate (SDS), dithiothreitol (DTT), Triton X-100, paraformaldehyde (PFA), bovine serum albumin (BSA), goat anti-mouse IgG-FITC (cat# F0257) antibody were from Sigma-Aldrich (St Louis, MO, USA). Glucose-free Dulbecco's modified Eagle's medium, neurobasal medium, glucose-free neurobasal medium, 10,000 units penicillin and 10 mg streptomycin/mL, TrypLE (synthetic trypsin), Glutamax supplement, sodium pyruvate (100 mM), Hank's balanced salt solution (HBSS), L-glutamine (200 mM), B27 supplement (50×, serum free), Pierce BCA protein assay kit and Pierce ECL Western immunoblotting substrates were from ThermoFisher Scientific (Loughborough, UK). Laemmli buffer (4×), 4–15% Mini-PROTEAN TGX Precast polyacrylamide gel, skimmed milk, Precision Plus Protein Dual Color Standard were from Bio-Rad (Hertfordshire, UK). Amersham™ Protran® Premium nitrocellulose blotting membranes were from VWR (Leicestershire, UK), RIPA (radio-immuno precipitation assay) buffer (10×) were from New England Biolabs Ltd (Hertfordshire, UK), Mouse anti HIF1α monoclonal antibody (cat# NB100-105) was from Novus Biologics (Abington, UK), rabbit polyclonal anti-β-actin antibody (cat# ab119716) was from Abcam (Cambridge, UK), rabbit polyclonal anti-β-III-tubulin antibody (clone Tuj1, cat# 801213) was from Biolegend (CA, USA), goat polyclonal anti-mouse IgG horseradish peroxidise (HRP) affinity (cat# P0447), anti-rabbit IgG HRP affinity (cat# P0448) were from Dako, Agilent (Santa Clara, CA, USA). Vectashield mounting medium with DAPI was obtained from Vector Laboratories (Burlingame, CA, USA). The Tetro cDNA synthesis kit and SensiFAST™ SYBR Hi-ROX kits were from Bioline Reagents Ltd (London, UK). The RNeasy plus Mini Kit was from Qiagen (Manchester, UK). The non-radioactive cytotoxicity assay kits were from Promega (Southampton, UK). The Guava cell dispersal reagent, Guava nexin kit, Guava instrument cleaning fluid, Guava Easycheck kit were from Merck Millipore (Burlingon, MA, USA). Plastic materials for cell cultures including pipettes, T25 cell culture vessel, 96-, 24- and 12-well plates, were from Greiner Bio-One (Gloucestershire, UK).

Cell culture

PC12 cells. PC12 cells were cultured in 'complete' medium [high-glucose DMEM (containing 4.5 g/L glucose, L-glutamine and sodium bicarbonate, without pyruvate) supplemented with 5% FBS, 5% HS and 1% penicillin–streptomycin] as described previously (Singh et al., 2020).

Primary rat cortical neuronal culture. Rat embryos (embryonic day 17–18: E17–18) were removed from a pregnant Sprague-Dawley rat, which was humanely killed under Schedule 1 according to the Animals Scientific Procedures Act of 1986 (United Kingdom) with approval by the local ethics committee. A total of 38 embryos obtained from 3 different pregnant rats were used in this study. The embryonic brains were dissected and cortical neurons were isolated as described previously (Singh et al., 2020). Neurons were plated onto poly-D-lysine (5 mg/mL; 0.15×10^6 cells per cm^2) pre-coated plates and placed in a standard incubator with a humidified atmosphere containing 5% CO_2 at 37 °C. 'Complete' medium for these cultures was high glucose (4.5 g/L) neurobasal medium containing 2% B27 serum-free supplements, 2 mM L-glutamine and 1% penicillin and streptomycin.

Cell treatment conditions. For both PC12 and neuronal cultures, media was refreshed (50%) after two days *in vitro* (DIV2), then every 2–3 days, until treatment, when 100% changes were performed. For preconditioning (PC) experiments, 100% media changes were made on a staggered basis (6 h, then 4 h, then

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2 h), such that all treatments were changed to reperfusion simultaneously (Fig. 1). Reperfusion was for 24 h (100% change; 'complete' medium at 21% O_2, 5% CO_2 at 37 °C) which was then followed by an OGD insult (100% medium change). For untreated control (medium changes matched 6 h sham-PC; no insult applied), sham-PC (full glucose and oxygen until insult), and OD, the cells were treated with 'complete' medium. For GD and OGD, the cells were treated with 'glucose-free' medium (identical supplements to 'complete' media, but without glucose; for PC12 cells high glucose DMEM was replaced with glucose-free DMEM; for primary rat neurons high glucose neurobasal medium was replaced with glucose-free neurobasal). For normoxia (Nx) and GD, the cells were incubated in 21% O_2, 5% CO_2 at 37 °C in a humidified incubator, whereas for OD and OGD, the cells were incubated 0.3% O_2, 5% CO_2, 94% N_2 at 37 °C in purpose-built INVIVO$_2$ 400 humidified hypoxia workstation (Ruskinn Technologies, Bridgend, UK). For OD and OGD treatments, media in filter-capped flasks was placed within the INVIVO$_2$ chamber for 24 h prior to use, to deplete oxygen. In studies involving various glucose concentration in PC12 cells, high glucose DMEM (4.5 g/L glucose) was diluted with glucose-free DMEM (0 g/L glucose) to achieve the desired glucose concentration, i.e. 0.5625, 1.125, or 2.25 g/L. All concentrations were then tested as preconditioning (4 h) in normoxia (Nx-PC) and hypoxia (Hyp-PC) followed by 24 h reperfusion and OGD insult (6 h). For untreated control, the cells were treated with high-glucose DMEM (4.5 g/L glucose), matched treatment conditions; with no insult applied. In these experiments, treatment with 4.5 g/L in normoxia (matching other experiments) was considered sham-PC.

Assessment of cell viability

MTT assays. Cell mitochondrial activity was evaluated using the standard colorimetric assay as described previously (Singh et al., 2020). The activity of mitochondria in control cells (complete media in normoxic conditions) was assigned as 100%, while treatment samples were normalised against the control group value.

Lactate dehydrogenase (LDH) release assay. LDH assay was conducted according to manufacturer's protocol and was described previously (Singh et al., 2020). The data were expressed as the mean percent of LDH release from the positive control (deliberately induced cell death by addition of cell lysis buffer).

Trypan blue exclusion assay. Trypan blue exclusion was used to determine viable cells present in PC12 cell suspensions as described previously (Singh et al., 2020). Cell viability was expressed as percentage of viable cells in the total number of cells.

Flow cytometry analysis. Apoptosis in PC12 cells was detected with a Guava Nexin Kit containing Annexin V and 7-AAD double stain according to manufacturer's protocol and was described previously (Singh et al., 2020). A total of 10,000 events in the gate were acquired for each sample and three samples were acquired per condition. The data were expressed as % of cells in each quadrant. Cells in the lower left quadrant represented viable cells (Annexin V and 7-AAD negative cells); the cells in lower right quadrant represent early apoptotic cells (Annexin V positive and 7-AAD negative cells); cells in upper right column represent necrotic/late apoptotic cells (Annexin V and 7-AAD positive cells). Data were analysed using Guava analysis software (Merck Millipore, MA, USA).

Immunofluorescence. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min, and then were permeabilised using 0.1% Triton X-100 in PBS for 15 min and blocked by incubating with 5% BSA in PBS-T (PBS, 0.1% Triton X-100) for 1 h at room temperature. This was followed by overnight incubation at 4 °C with primary antibody (Tuj1, rabbit anti-β-III-tubulin, 1:500 in 1% BSA in PBS-T). Following three PBS washes, cells were incubated in secondary antibody (goat anti-mouse IgG-FITC, 1:200 in 1% BSA in PBS-T) for 3 h at room temperature. Coverslips were then washed with PBS and mounted onto slides with Vectashield mounting medium with nuclear stain: DAPI (4′,6-diamidino-2-phenylindole). Images were taken by Hamamatsu (C4742-95) digital camera attached with Nikon Eclipse 80i fluorescence microscope, and were double merged (consisting of FITC Tuj1$^+$ and DAPI$^+$ channels) with NIS-Element BR 3.22.14 software (Nikon, Tokyo, Japan).

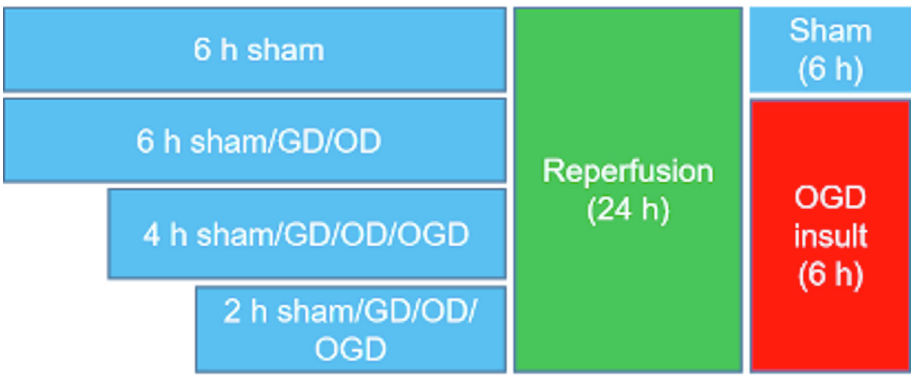
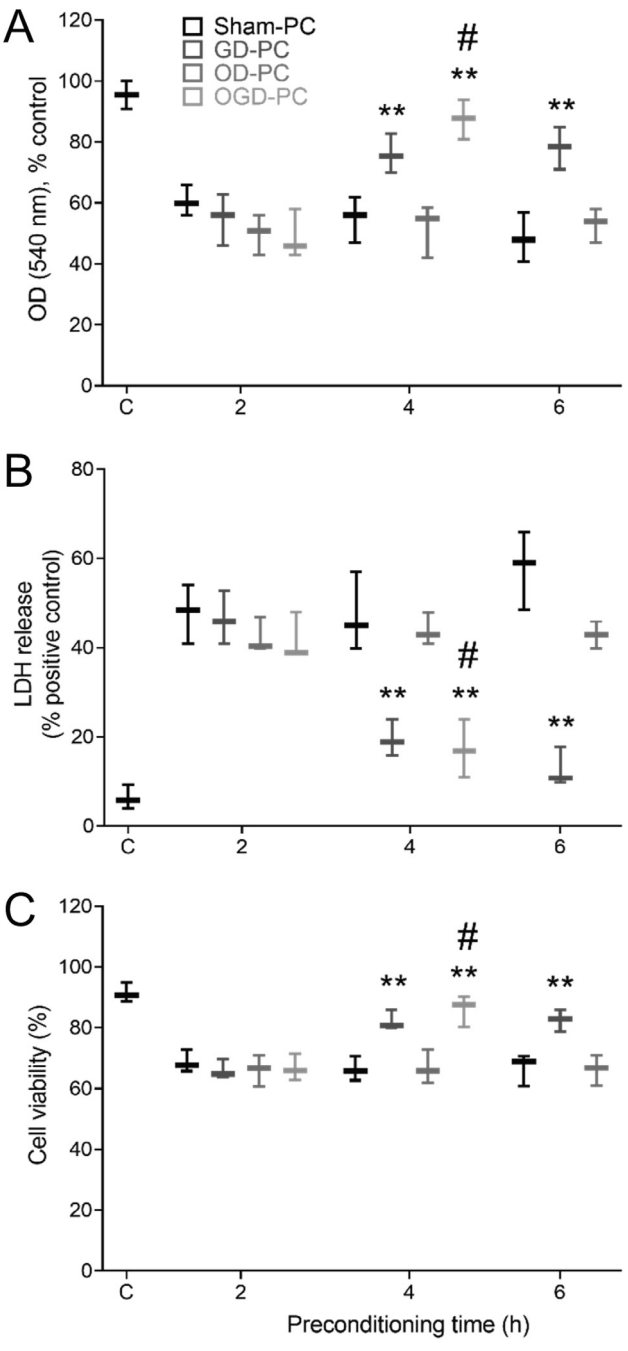


Fig. 1. Timeline of glucose-deprivation (GD), oxygen deprivation (OD) or oxygen and glucose deprivation (OGD) preconditioning. PC12 cells and primary rat neurons were subjected to GD, OD, OGD or sham treatments (2, 4 or 6 h) followed by reperfusion for 24 h ('complete' medium at 21% O_2, 5% CO_2 at 37 °C) which was then followed by an OGD insult (6 h). Untreated controls were maintained in complete medium.

Protein extraction and immunoblotting

Proteins were extracted from cultures and processed as described previously (Singh et al., 2020). Twenty to 40 μg protein was denatured for 5 min in 4× Laemmli buffer at 95 °C. Samples were electrophoresed and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk powder in 1× PBS-T for 1 h then incubated overnight at 4 °C with a mouse anti HIF1α monoclonal antibody (1:500) in 1% milk powder of PBS-T buffer. After the overnight incubation, membranes were washed in 1× PBS-T three times for 5 min each and were incubated for 1 h in the goat polyclonal anti-mouse IgG antibody conjugated with HRP (1:1000) in 1% milk powder of 1× PBS-T. After being washed three times with 1× PBS-T, the membranes were developed by Pierce ECL Western immunoblotting substrates, and imaged with ChemiDoc MP Imaging system (Biorad, California, USA). Thereafter, the membranes were treated with mild stripping buffer and re-probed with a rabbit polyclonal anti-β-actin antibody (1:1000) and a subsequent goat polyclonal anti-rabbit IgG antibody conjugated with HRP (1:1000), and the imaged as above. The protein levels were quantified by densitometric analysis using Image J (NIH, USA). Values were normalized to β-actin, for the same sample. All treatment conditions were normalised to untreated control. Untreated controls in all the replicates were normalised to a single untreated control immunoblot and an average across all the untreated control samples was obtained. Raw immunoblots were included in Supplementary Methods (Figs. S2, S3).



Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted from cells using the RNeasy plus Mini Kit and was converted into cDNA by the Tetro cDNA synthesis kit in accordance with the manufacturer's protocol, as described previously (Singh et al., 2020). Amplification of 100 ng cDNA template per reaction was performed by using the SensiFAST SYBR Hi-ROX kit in a Techne Prime Pro 48 Real-time qPCR machine (ThermoFisher Scientific, Loughborough, UK) as described previously (Singh et al., 2020). The primers [including glucose transporter 1 (*Glut1*), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (*Bnip3*), prolyl hydroxylase 2 (*Phd2*), 6-phosphofructo-2-kinase/fructose-2,6-bipho sphatase 1 (*Pfkfb1*), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*Pfkfb3*), lactate dehydrogenase A (*Ldha*)] were as follows:

Actin, 5′-TGCCCTAGACTTCGAGCAAGA-3′ (forward) and 5′-CATGGATGCCACAGGATTCCATAC-3′ (reverse); *Glut1,* 5′-GGTGTGCAGCAGCCTGTGTA-3′ (forward) and 5′-GACGAAC AGCGACACCACAGT-3′ (reverse); *Hif1α,* 5′-TCAAGTCAGCAACGTGGAAG-3′

Fig. 2. PC12 cells response to glucose-deprivation (GD), oxygen deprivation (OD) or oxygen glucose deprivation (OGD) preconditioning (PC). Cells were exposed to sham-PC, GD-PC, OD-PC or OGD-PC (2, 4 or 6 h) followed by 24 h reperfusion and 6 h OGD insult. **(A)** MTT assay. Compared to untreated controls, all conditions showed reduced mitochondrial activity ($p \leq 0.01$). All were similar to sham-PC, except for significantly lower reduction (less toxicity) for GD (4, 6 h)-PC and OGD (4 h)-PC, versus sham-PC (same timepoint; $^{**}p \leq 0.01$), and for OGD (4 h)-PC versus GD-PC (same timepoint; $^{\#}p \leq 0.05$). **(B)** LDH assay. Compared to untreated control, all conditions showed greater LDH release ($p \leq 0.01$). For most conditions, LDH release was similar to sham-PC, except for significantly lower LDH release (less cell death) for GD (4, 6 h)-PC and OGD (4 h)-PC, versus sham-PC (same timepoint; $^{**}p \leq 0.01$), and for OGD (4 h)-PC versus GD-PC (same timepoint; $^{\#}p \leq 0.05$). **(C)** Trypan blue assay. Compared to untreated control, all conditions showed reduced cell viability ($p \leq 0.01$). For most conditions, cell viability was similar to sham-PC (~73%), except for significantly greater viability (less cell death) for GD (4, 6 h)-PC and OGD (4 h)-PC, versus sham-PC (same timepoint; $^{**}p \leq 0.01$), and for OGD (4 h)-PC versus GD-PC (same timepoint; $^{\#}p \leq 0.05$).

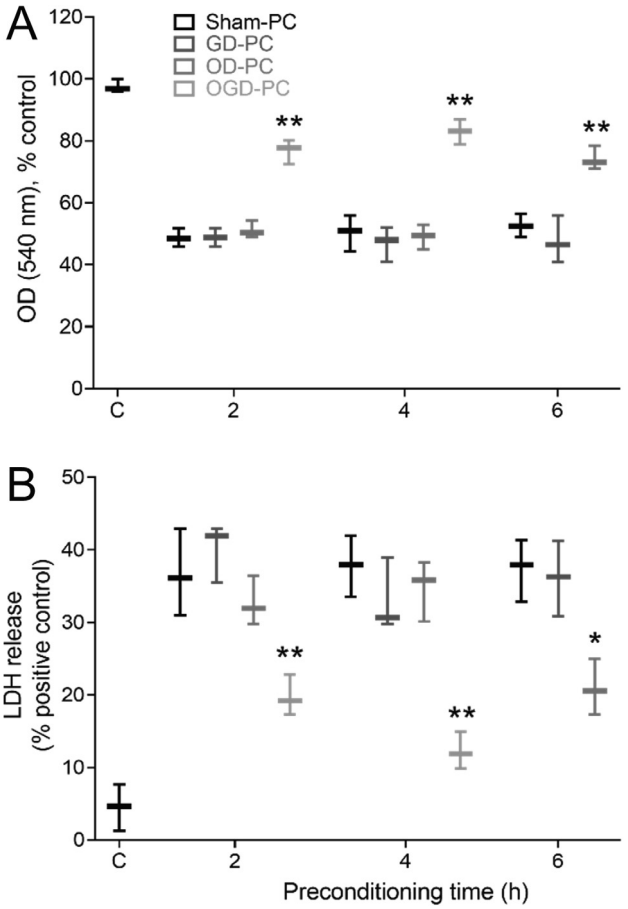


Fig. 3. Primary neuronal culture response to glucose-deprivation (GD), oxygen deprivation (OD) or oxygen glucose deprivation (OGD) preconditioning (PC). Cells were exposed to sham-PC, GD-PC, OD-PC or OGD-PC (2, 4 and 6 h) followed by 24 h reperfusion and 6 h OGD insult. **(A)** MTT assay. Compared to untreated controls, all conditions showed reduced mitochondrial activity ($p \leq 0.01$). All were similar to sham-PC, except for significantly lower reduction (less toxicity) for OGD (2, 4 h)-PC ($^{**}p \leq 0.01$) and OD (6 h)-PC ($^{*}p \leq 0.05$), versus sham (same timepoint). **(B)** LDH assay. Compared to untreated control, all conditions showed greater LDH release ($p \leq 0.01$). For most conditions, LDH release was similar to sham-PC, except for significantly lower release (less cell death) for OGD (2, 4 h)-PC and OD (6 h)-PC, versus sham-PC (same timepoint; $^{**}p \leq 0.01$; $^{\#}p \leq 0.05$).

(forward) and 5′-TATCGAGGCTGTGTCGACTG-3′ (reverse); *Vegf,* 5′-TTACTGC TGTACCTCCAC-3′ (forward) and 5′-ACAGGACGGCTTGAAGATA-3′ (reverse); *Phd2,* 5′-TGCATACGCCACAAGGTACG-3′ (forward) and 5′-GTAGGTGA CGCGGGTACTGC-3′ (reverse); *Bnip3,* 5′-TTTAAACACCCGAAGCGCACAG-3′ (forward) and 5′-GTTGTCAGACGCCTTCCAATG TAGA-3′ (reverse); *Pfkfb1,* 5′-AACCGCAACATGACCT TCCT-3′ (forward) and 5′-CAACACAGAGGCCCAG CTT A-3′ (reverse); *Pfkfb3,* 5′-CTGTCCAG CAGAGGC AAGAA-3′ (forward) and 5′-CGCGGTCTGGATGG TACTTT-3′ (reverse); *Ldha,* 5′-AAGGTTATGGCTCC CTTGGC-3′ (forward) and 5′-TAGTGACGTG TGACA GTGCC-3′ (reverse)

Actin was used as an internal control to normalise the relative levels of mRNA. Quantification of mRNA expression was performed using the comparative delta Ct method.

Data analysis

For studies in PC12 cells, biological replicates were performed using cells being derived from separate flasks (different streams of cultured cells), whereas in studies performed with primary neurons, biological replicates were performed on cells derived from different rats. For each biological replicate, three technical replicates were performed. In experiments employing 96 well plates, at least 8 well replicates were performed in each plate. In this study, the dataset obtained for each treatment condition was independent of other conditions (i.e. independent sampling; one rat, one number). The data obtained from each of the biological replicates was averaged. The data was represented as mean + standard deviation (S.D.) in text. In the box and whisker plots, the boxes depict the median and the 25th and 75th quartiles and the whisker depict the 5th and 95th percentile. The data were tested for normality using Anderson-Darling normality test. For normally distributed data, one-way or two-way ANOVA with Tukey's post hoc analysis was performed. For data that were not normally distributed, the non-parametric Kruskal–Wallis test was used. For all data analysis, PRISM version 8 (Graph Software Inc, CA, USA) for Windows version 10 was used. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Initial experiments were performed to determine the time points at which OGD induced toxicity in both PC12 cells and primary cortical rat neurons, to inform choices for ischaemic challenge (insult; in which cell death should be evident), and for preconditioning (in which cell death should be minimal or absent). Three different cell viability assays were utilised. LDH and trypan blue exclusion assays detected permanent loss of cell membrane integrity, indicative of cell death, and therefore culture viability. The MTT assay assessed changes in mitochondrial respiration. Differences versus control may indicate altered cellular metabolism, reduced proliferation, or cell death. Diminished mitochondrial activity due to ischaemia can potentially be reversed, upon timely reperfusion (Bopp and Lettieri, 2008).

A 6 h exposure to OGD induced toxicity in both PC12 cells and primary cortical rat neurons. In PC12 cells, 6 h OGD caused significant reductions in mitochondrial activity (MTT, OD; 73.6 ± 3.4% versus 100 ± 4.8% in untreated control, *Two-way ANOVA:* $F_{(3,80)} = 191.9$, $p \leq 0.01$) and cell viability (trypan blue exclusion assay; 76.1 ± 3.2% versus 93.9 ± 3.4% in untreated control, *Two-way ANOVA:* $F_{(3,80)} = 192.6$, $p \leq 0.01$). There was also significant increase in LDH release (24.3 ± 2.4% versus 6.4 ± 0.2% in untreated control, *Two-way ANOVA:* $F_{(3,80)} = 154.2$, $p \leq 0.01$). Similarly, 6 h OGD was found cytotoxic in primary rat neuron cultures, demonstrated by reduced mitochondrial activity (54.7

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± 5.1% versus 100% in untreated control, *Two-way ANOVA:* $F_{(4,30)} = 49.1$, $p \leq 0.01$) and increased LDH release (34.2 ± 6.9% versus 4.2 ± 1.8% in untreated control, *Two-way ANOVA:* $F_{(4,30)} = 62.6$, $p \leq 0.01$).

Earlier timepoints (2, 4 h OGD) did not induce obvious toxicity (data not shown), although for primary neuronal cultures, 4 h OGD showed reduced MTT activity (MTT: 75.5 ± 7.9% versus 100 ± 8.2% in untreated control, *Two-way ANOVA*: $F_{(4,30)} = 49.1$, $p \leq 0.05$). In this same primary neuronal culture experiment (4 h OGD), LDH release was not significantly elevated: 7.4 ± 1.2% versus 4.2 ± 1.8% in untreated control, $p > 0.05$, indicating that neuronal viability was not reduced.

Therefore, 6 h OGD was chosen as the condition for ischaemic insult, while 2 and 4 h OGD, being sublethal, were used as preconditioning treatments on both PC12 cells and primary rat neurons, followed by reperfusion and the ischaemic insult (Fig. 1): GD-PC (2, 4, 6 h), OD-PC (2, 4, 6 h), and OGD-PC (2, 4 h).

In PC12 cultures, for all preconditioning treatments (including sham-PC), 6 h OGD resulted in reduced mitochondrial activity, increased LDH release, and reduced cell viability, versus untreated controls (Fig. 2). However, some protection against these insults was conferred by OGD-PC (4 h) and GD-PC (4, 6 h), compared to sham-PC at the same timepoint, for all three assays. OGD-PC (4 h) conferred greater protection than GD-PC (4 h), evidenced by reduced toxicity across all three assays. Results for all other preconditioning treatments were comparable to time-matched sham-PC, for all three assays. Results for sham-PC did not vary across timepoints.

In primary neuronal cultures, for all preconditioning treatments (including sham-PC), 6 h OGD resulted in reduced mitochondrial activity and increased LDH release, versus untreated controls (Fig. 3). However, some protection against these insults was conferred by OGD-PC (2, 4 h), OD-PC (6 h) compared to sham-PC at the same timepoint. Results for all other preconditioning treatments were comparable to time-matched sham-PC, for all three assays. Results for sham-PC did not vary across timepoints.

Primary rat neuronal cultures were examined by fluorescence microscopy (Fig. 4). Untreated cultures

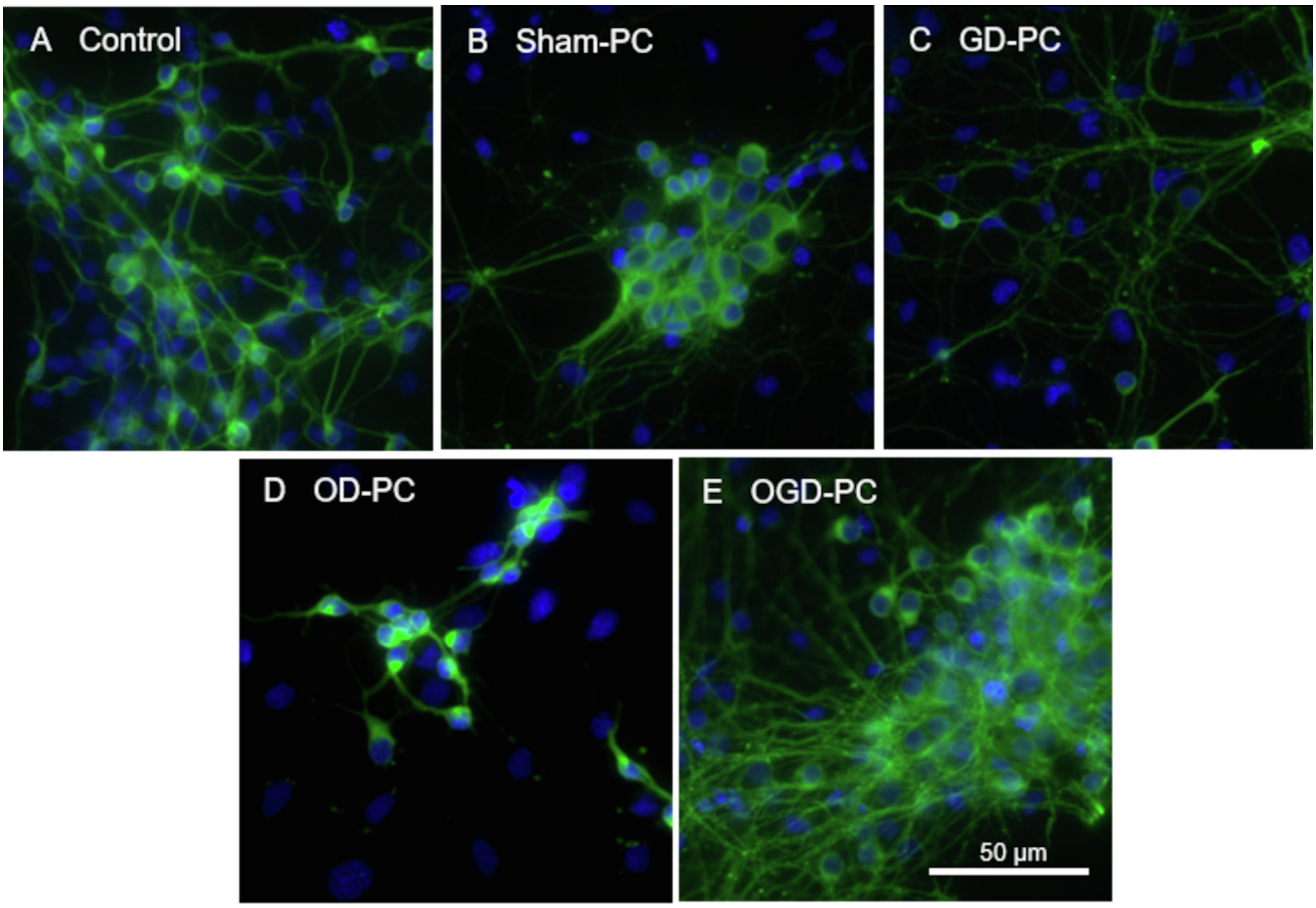

Fig. 4. Representative double merged micrographs of primary cortical neurons following glucose-deprivation (GD), oxygen deprivation (OD) or oxygen glucose deprivation (OGD) preconditioning (PC). Cells were exposed to sham-PC, GD-PC, OD-PC or OGD-PC (4 h) followed by 24 h reperfusion and 6 h OGD insult, except untreated control (full glucose and normoxia throughout). In all conditions, the majority of nuclei ($DAPI^+$, blue) were associated with $Tuj1^+$ staining (green; indicating neuronal identity). Fluorescence micrographs show **(A)** untreated control cultures, **(B)** sham-PC, **(C)** GD-PC, **(D)** OD-PC and **(E)** OGD-PC cultures. $Tuj1^+$ cells in **(B)**, **(C)** and **(D)** exhibited shorter and fewer neurites, and reduced cell densities in comparison to control. $Tuj1^+$ cells in **(E)** had longer and more numerous neurites, and higher cell densities, compared to **(B)**, **(C)** and **(D)**, with morphologies resembling **(A)**, consistent with the protective effects reported here for OGD-PC.

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were composed primarily of neurons (65.3 ± 9.2% Tuj1$^+$) with numerous elongated neurites (Fig. 4A). In all preconditioned cultures, post-OGD insult, cell density was reduced (sham-PC: 41 ± 14 nuclei per micrograph, GD-PC: 28 ± 12, OD-PC: 36 ± 9, OGD-PC: 97 ± 11, all $p \leq 0.01$ versus untreated: 148 ± 21; *one-way ANOVA:* $F_{(4,30)} = 37.1$). The same analysis showed that cell count for OGD-PC was greater than that for sham-PC, OD-PC and GD-PC, suggesting protective effects. The relationships revealed in these data were consistent with LDH assays (Fig. 3B). For sham, OD-PC and GD-PC, neurites were typically shorter and fewer, in comparison to untreated control (Fig. 4B-D). For OGD-PC, neurites were fewer in number and shorter than untreated controls, but more numerous and longer than in equivalent sham-PC cultures (Fig. 4E).

As 4 h GD-PC was cytoprotective in PC12 cells, we performed further studies to determine the role of glucose by preconditioning PC12 cells with varying glucose concentrations (4 h, with normoxia or hypoxia). For all preconditioning treatments, subsequent insults resulted in reduced mitochondrial activity, increased LDH release, and reduced cell viability, versus untreated controls, with the sole exception of 0 g/L hypoxia in the trypan blue assay (Fig. 5). For mitochondrial activity, some protection against these insults was conferred by glucose reduction (0.565 g/L) or complete deprivation (0 g/L), in both normoxic and hypoxic conditions (Fig. 5A). Complete GD (0 g/L) offered greater protection against mitochondrial toxicity in hypoxia (versus normoxia, 0 g/L). Reduced glucose concentrations exhibited a dose-dependent protective effect in the LDH assay. In both normoxic and hypoxic conditions, all concentrations showed lower LDH release (less cell death) versus complete medium (4.5 g/L), with 0.565 and 0 g/L showing significant reductions in LDH release versus 2.25 g/L (for same oxygen conditions; Fig. 5B). No differences were noted between normoxic and hypoxic conditions. In terms of viability assessed by trypan blue assay, some protection was evident in the absence of glucose (0 g/L), for both normoxia (83.7 ± 4.4%) and hypoxia (89.2 ± 5.1%) , versus 4.5 g/L normoxia (70.1 ± 4.2%) (Fig. 5C). All other glucose concentrations were indistinguishable from full glucose (4.5 g/L), with no differences evident between normoxia and hypoxia.

In addition to the cell viability assays, apoptosis was assessed (flow cytometry; Annexin-V/7-AAD) in the

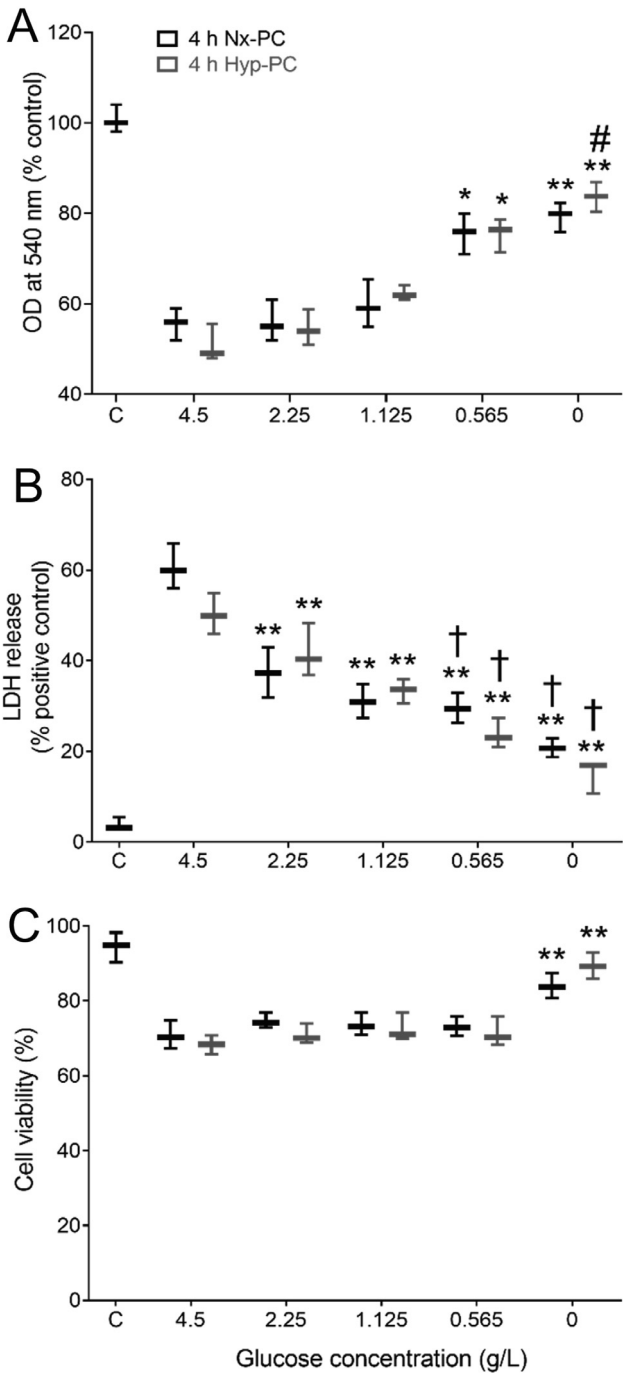


Fig. 5. Reduced-glucose preconditioning (PC) of PC12 cells conferred protective effects against ischaemic insult. PC12 cells were preconditioned in normoxia (Nx-PC; 21% O_2) or hypoxia (Hyp-PC; 0.3% O_2) for 4 h with varying glucose concentrations (0, 0.565, 1.125, 2.25, 4.5 g/L), followed by 24 h reperfusion and 6 h OGD insult, except untreated control, C (full glucose and normoxia throughout). **(A)** MTT assay. Compared to untreated controls, all conditions showed reduced mitochondrial activity ($p \leq 0.05$). All similar to sham (4.5 g/L Nx-PC), except for significantly lower reduction (less toxicity) for the lowest glucose concentrations (0 and 0.565 g/L), with or without hypoxia (versus 4.5 g/L Nx-PC, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$). Complete glucose deprivation (0 g/L) with hypoxia precondition showed less toxicity than GD in normoxia ($^{\#}p \leq 0.05$ versus Nx-PC, 0 g/L). **(B)** LDH assay. Compared to untreated controls, all conditions showed greater LDH release ($p \leq 0.05$). In comparison to sham (4.5 g/L Nx-PC), all reductions of glucose concentration (0–2.25 g/L), with or without hypoxia, were associated with significantly lower LDH release (less cell death; $^{**}p \leq 0.01$ versus 4.5 g/L Nx-PC). Both 0.565 and 0 g/L also showed significant reductions in LDH release versus 2.25 g/L (for same oxygen conditions; $^{\dagger}p \leq 0.05$). No differences were noted between normoxic and hypoxic conditions. **(C)** Trypan blue assay. Compared to untreated controls, all conditions showed reduced cell viability ($p \leq 0.01$) except 0 g/L Hyp-PC ($p > 0.05$). For all reduced-glucose concentrations, cell viability was similar to sham (4.5 g/L Nx-PC; ~60–70%). Viability was greater without glucose in both normoxic and hypoxic conditions ($^{**}p \leq 0.01$ versus 4.5 g/L Nx-PC).

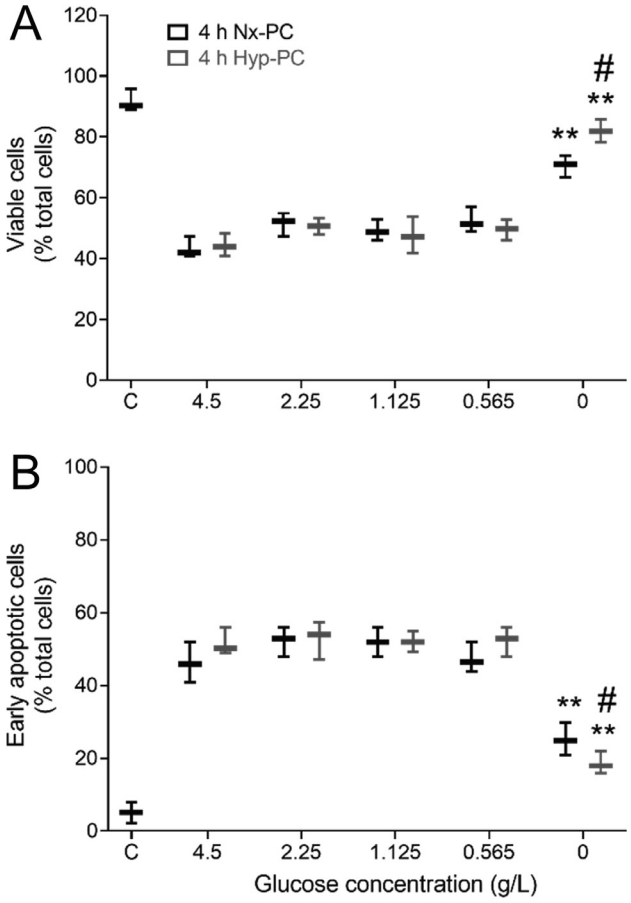


Fig. 6. Flow cytometric analysis of the effect of varying glucose concentration on preconditioning-induced tolerance to ischaemic insult. PC12 cells were preconditioned in normoxia (Nx-PC; 21% O_2) or hypoxia (Hyp-PC; 0.3% O_2) for 4 h with varying glucose concentrations followed by 24 h reperfusion and 6 h OGD insult, except untreated control, C (full glucose and normoxia throughout). **(A)** Bar chart representing viable (7-AAD$^-$) cells as percentage of all cells. Compared to untreated control, all conditions showed reduced cell viability ($p \leq 0.01$), except 0 g/L Hyp-PC ($p > 0.05$). For all reduced (non-zero) glucose concentrations, cell viability was similar to sham (4.5 g/L Nx-PC; ~45–55%). Viability was greater without glucose in both normoxic and hypoxic conditions ($^{**}p \leq 0.01$). Preconditioing with complete glucose deprivation (0 g/L) in hypoxia had more viable cells compared to Nx-PC, 0 g/L ($^{\#}p \leq 0.05$). **(B)** Bar chart representing early apoptotic (Annexin-V$^+$/7-AAD$^-$) cells as percentage of all viable (7-AAD$^-$) cells. Compared to untreated controls, all conditions showed greater expression of marker for early apoptosis ($p \leq 0.05$). For all reduced (non-zero) glucose concentrations, percentage early apoptotic cells was similar to sham (4.5 g/L Nx-PC; ~45–55%). A lower percentage of early apoptotic cells was seen in both normoxic and hypoxic conditions without glucose ($^{**}p \leq 0.01$). Preconditioing with complete glucose deprivation (0 g/L) in hypoxia induced a lower percentage of early apoptotic cells compared to Nx-PC, 0 g/L ($^{\#}p \leq 0.05$).

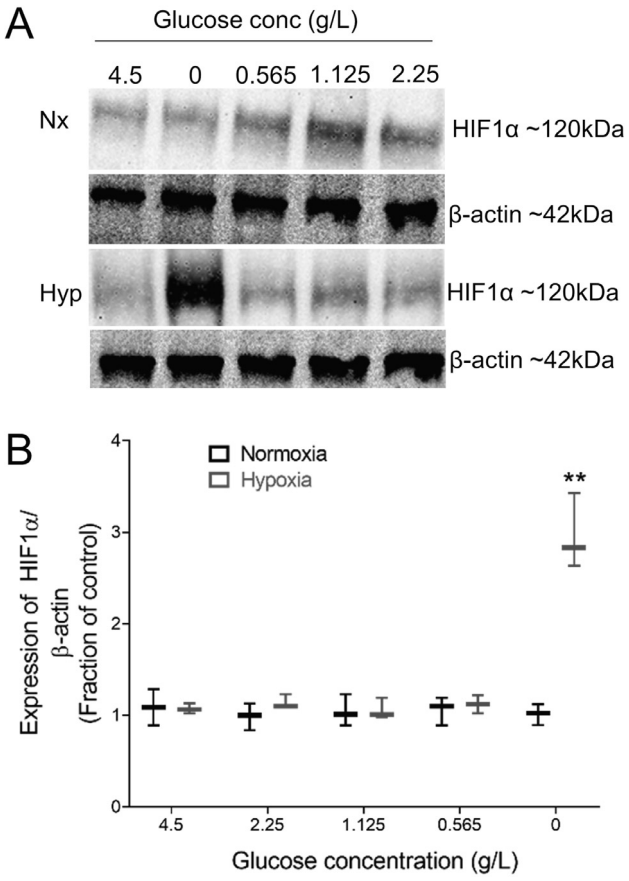


Fig. 7. HIF1α levels in PC12 cells cultured in normoxia and hypoxia with varying glucose concentration. Immunoblot analysis of HIF1α levels in PC12 cells treated in normoxia (Nx; 21% O_2) and hypoxia (Hyp; 0.3% O_2) with varying glucose concentrations of 0, 0.565, 1.125, 2.25, 4.5 g/L for 4 h **(A)** Representative HIF1α immunoblots were shown with corresponding β-actin; **(B)** Bar chart representing normalised HIF1α expression. Compared to sham (4.5 g/L, Nx), all conditions showed no significant increase in HIF1α expression except complete glucose deprivation (0 g/L) with hypoxia ($^{**}p \leq 0.01$ versus Nx-PC, 4.5 g/L).

PC12 cells for these conditions (representative dotplots in Fig. S1). For all preconditioning treatments, subsequent 6 h OGD resulted in reduced cell viability (AV$^-$/7-AAD$^-$, Fig. 6A) and increased apoptosis (AV$^+$/7-AAD$^-$, Fig. 6B), versus untreated control, with the sole exception of 0 g/L hypoxia. Some protection against these insults was conferred by complete GD, in both normoxic (GD-PC) and hypoxic (OGD-PC) conditions. Complete GD (0 g/L) offered greater protection against apoptosis in hypoxia (OGD-PC; versus normoxia, 0 g/L: GD-PC). All other glucose concentrations were indistinguishable from full glucose (4.5 g/L), with no differences between normoxia and hypoxia.

Of all the preconditioning treatments (OGD, OD, GD) studied, 4 h OGD-induced protection was the most consistent across PC12 cells and primary neurons. Therefore, these OGD (4 h) was used to assess effects on expression of HIF1α and various downstream (HIF1-dependent) genes. In both PC12 and primary neuronal cultures, HIF1α protein levels were upregulated in response to complete GD (0 g/L) in hypoxia (OGD), but not other conditions tested (Figs. 7 and 8). For PC12 cells, testing included varied levels of GD. The extent of upregulation was similar for both cell types (~2.6–3.5-fold).

In PC12 cells, *Vegf*, *Glut1, Pfkfb3* and *Ldha* were significantly upregulated in cells exposed to complete GD (0 g/L) in hypoxia (OGD) compared to 4.5 g/L normoxia (untreated control), but not in other treatment

conditions. All treatment conditions had no significant effect on *Hif1α*, *Bnip3 and Pfkfb1* expression (Fig. 9). In primary neurons, *Vegf*, *Phd2* and *Bnip3* were significantly upregulated in cells exposed to OGD compared to normoxia (untreated control), but not in OD and GD treatment conditions. All treatment conditions had no significant effect on *Hif1α*, *Glut1 and Pfkfb1* expression (Fig. 10).

DISCUSSION

One of the most promising avenues of research in stroke therapeutics is the concept of HPC and IPC, in which brief periods of brain hypoxia or ischaemia have been shown to reduce the damaging effects of subsequent severe ischaemic insults (Stevens et al., 2014; Wang et al., 2015). Both IPC and HPC have shown promise by targeting various pathways. Studies have commonly pointed out that reprogramming of normal transcriptional responses to low-level ischaemia induce neuroprotective responses, thereby limiting the impact of a subsequent injurious ischaemic event (Stevens et al., 2014; Wang et al., 2015; Meller and Simon, 2015). Experiments employing various preconditioning doses (strength and duration of stimulus) have demonstrated hormetic responses (Calabrese et al., 2020). Hormesis is an adaptive response of cells induced by moderate stress, which may involve transcription factors such as Nrf-2 and NF-κB (Calabrese and Agathokleous, 2019). In this study, the effects of preconditioning with OGD, GD and OD were studied in both PC12 cells and primary rat neurons, and assessed in terms of protection against subsequent OGD insult. Additionally, the stabilisation of HIF1α and its downstream genes upregulation, which has been commonly reported to play a crucial role in HPC induced tolerance (Bradley et al., 2012; Karuppagounder and Ratan, 2012), were also studied.

Our study was initially performed on PC12 cells, as a study (Hillion et al., 2005) has previously found IPC protected PC12 cells against a subsequent lethal OGD insult. PC12 cells are popular because of their versatility for pharmacological manipulation, their ease of culture and the large quantity of experimental data available in the literature. PC12 cells are a widely used and accepted model for neurochemical and neurobiological studies (Hillion et al., 2005). Pilot experiments revealed the maximal nonlethal stress tolerated by PC12 cells to be 4 h OGD. Exposure to 6 h OGD induced obvious toxicity and was therefore chosen as the duration for ischaemic insult. Various preconditioning treatments (GD, OD or OGD) followed by reperfusion were tested for protection against 6 h OGD insult. For PC12 cells, preconditioning with GD or OGD for 4 h was found to offer some protective effects. Shorter periods (2 h) of GD or OGD preconditioning were not protective. In our initial studies, we did not find 4 h of OD preconditioning protective, therefore we hypothesized that glucose concentration might play an essential role in IPC induced tolerance. There is limited literature on GD preconditioning for neuroprotection, but one study found that exposing the heart to transient GD confers a preconditioning-like protection against a subsequent ischaemia/reperfusion injury *in vivo* (Awan et al., 2000).

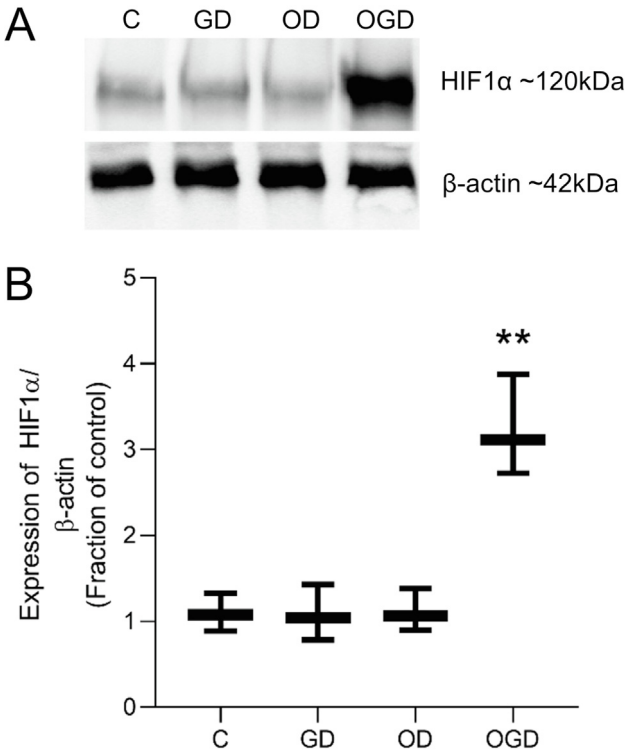
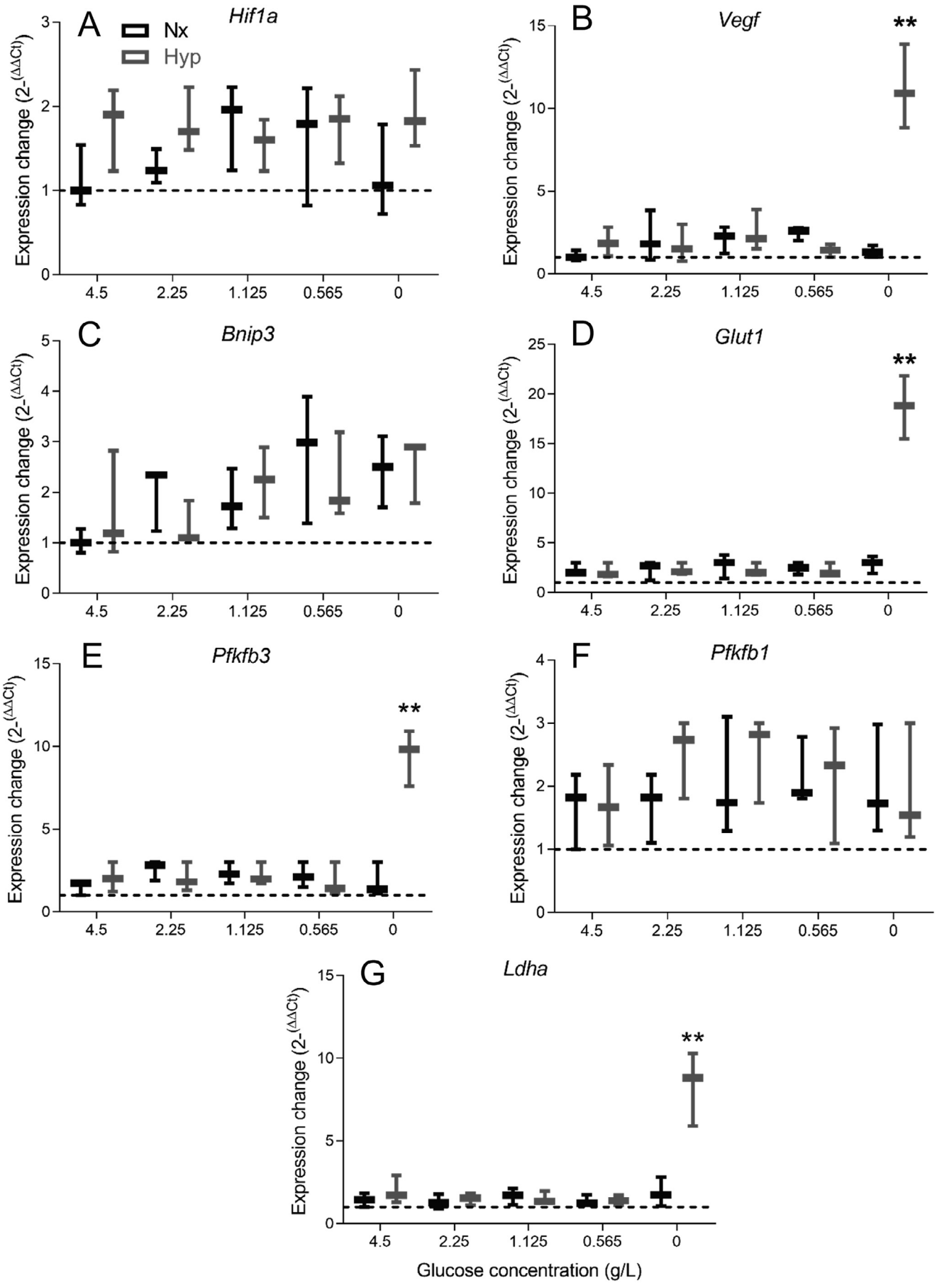


Fig. 8. Effects of glucose-deprivation (GD), oxygen deprivation (OD) or oxygen and glucose deprivation (OGD) on HIF1α levels in primary rat neurons. Immunoblotting analysis of HIF1α level in primary rat neurons treated with in GD, OD and OGD in comparison to sham for 4 h. **(A)** Representative HIF1α immunoblots were shown with corresponding β-actin; **(B)** Bar chart representing normalised HIF1α expression. Compared to sham, all conditions showed no significant increase in HIF1α expression except OGD (** $p \leq 0.01$ *versus sham).*

We performed further studies by preconditioning PC12 cells with varying glucose concentrations, with and without hypoxia. Mitochondrial activity was found to be less dramatically impaired when cultures were preconditioned with the complete absence of glucose, and at the lowest reduction of glucose tested (0.565 g/L), with or without hypoxia. This suggests a protective effect of GD in terms of mitochondrial toxicity. Although both offered benefit, preconditioning with 0 g/L in hypoxia (OGD) offered greater protection against mitochondrial toxicity compared to 0 g/L in normoxia (GD). LDH assay results were slightly different, where both normoxia and hypoxia preconditioned cells with 0 to 2.25 g/L of glucose were found to significantly reduce LDH release. Similarly, significantly greater cell viability were found in cells preconditioned in normoxia and hypoxia in the absence of glucose. Flow cytometric analysis (Annexin V/7-AAD) showed significant reductions in apoptotic cell death in GD (35%) and most significantly OGD (20%) preconditioned cells in comparison to sham (50%) preconditioned cells. Interestingly, all the three assays and flow cytometry showed a protective effect in GD and more effectively in OGD preconditioned PC12 cells against a subsequent OGD insult. Longer term studies will be required to


A
Hif1a
Nx
Hyp
B
Vegf
**
C
Bnip3
D
Glut1
**
E
Pfkfb3
**
F
Pfkfb1
G
Ldha
**
Expression change (2-(ΔΔCt))
4.5
2.25
1.125
0.565
0
Glucose concentration (g/L)

determine whether preconditioning limited the maximum extent of these toxic effects, or simply delayed/slowed their onset, such that similar levels of toxicity may eventually occur, but at a later timepoint. If the latter, there may still be therapeutic value, as delayed progression of pathology could offer a longer window within which to deliver therapeutic interventions to rescue neural tissue.

Next, we validated these discoveries in a more representative model of neural tissue: primary rat neurons. Primary rat neurons have been widely used in both *in vitro* and *in vivo* models for the study of IPC. The outcomes of neurons in OGD depends on a number of factors, such as components of OGD medium, the duration and severity of OGD, neuron maturity and origin of neurons (Gao et al., 2015; Zhang et al., 2017; Liang et al., 2019). For example, Bhuiyan et al. (2011) found DIV10 rat cortical neurons were more resistant to OGD-reperfusion injury than DIV7 neurons. Koszegi et al. (2017) found that hippocampal CA1 neurons were more vulnerable to OGD induced cell death than cortical neurons. In our study, we used relatively mature (DIV10–14) neurons, and found that OGD (4 h) reduced mitochondrial activity but did not cause LDH release (indicative of cell death), suggesting non-lethal (reversible) toxicity. Prolonged OGD (6 h), however, induced substantial cell death, and was deemed cytotoxic. Similar results were reported in a study in which 6 h OGD (0% O_2; versus 0.3% O_2 here) resulted in significant neuronal death (LDH assay: 56.7% vs 19.5% in control) and apoptosis (TUNEL positive cells, out of total cells :16.5% vs 2% in control) (Zhang et al., 2017). Tian et al. (2017) also showed significant increase in LDH leakage (~3-fold versus control) and reduced cell viability (~50% of control) by 6 h OGD (1% O_2) in primary rat cortical neurons. However, Bhuiyan et al. (2011) showed that 4 h (but not 2 h) OGD (0% O_2), followed by 24 h reperfusion resulted in significant cytotoxicity (LDH assay: 60% vs 10% control). Another study also showed that 1 h of OGD (1% O_2) followed by 24 h of reperfusion resulted in a decrease in viability of approximately 20% indicating a moderate degree of cellular stress compared with 3 h OGD where the viability was decreased by more than 50% (Wappler et al., 2013). Both Bhuiyan et al. (2011) and Wappler et al. (2013) used Earle's balanced salt solution (EBSS) for OGD conditions in contrast to our and other studies that used glucose-free Neurobasal medium supplemented with B27. A recent study by Sünwoldt et al. (2017) found that B27 protected neurons from cell death during OGD in comparison to neurons incubated in EBSS.

Primary rat neuronal cultures were examined by fluorescence microscopy (4 h PC and untreated). Untreated cultures were composed primarily of neurons (65.3 ± 9.2% $Tuj1^+$) with numerous elongated neurites (Fig. 4A). In all preconditioned cultures, post-OGD insult, cell density was reduced (sham-PC: 41 ± 14 nuclei per micrograph, GD-PC: 28 ± 12, OD-PC: 36 ± 9, OGD-PC: 97 ± 11, all $p \leq 0.01$ versus untreated: 148 ± 21; *one-way ANOVA:* $F_{(4,30)} = 37.1$). The same analysis showed that cell count for OGD-PC was greater than that for sham-PC, OD-PC and GD-PC, suggesting protective effects. The relationships revealed in these data were consistent with LDH assays (Fig. 3B). For sham, OD-PC and GD-PC, neurites were typically shorter and fewer, in comparison to untreated control (Fig. 4B–D). For OGD-PC, neurites were fewer in number and shorter than untreated controls, but more numerous and longer than in equivalent sham-PC cultures (Fig. 4E).

Preconditioning with 2 and 4 h OGD conferred cytoprotection (greater mitochondrial activity, reduced LDH release, versus sham-PC) after 6 h OGD in primary neurons. Consistent with LDH assay results, fluorescence microscopy (Tuj1) also revealed greater cell density and healthier neurons in cultures preconditioned with 4 h OGD versus sham-PC (significant reduction in cell density). This is consistent with a number of studies using primary neurons *in vitro*. Tauskela et al. (2003) showed that OGD preconditioning (60–70 min) in rat cortical neurons significantly increased neuronal viability following 75–90 min OGD insult. Gao et al. (2015) showed that OGD preconditioning (15 min) in mouse hippocampal neurons enhanced neuron viability (77.3% versus 51.5% in control) after a subsequent OGD insult (55 min). Keasey et al. (2016) demonstrated that OGD preconditioning (30 min) in rat hippocampal neurons enhanced neuron viability (~75% versus 51.5% in control) after a subsequent OGD insult (90 min). Furthermore, Arthur et al. (2004) showed OD preconditioning (25 min) 24 h prior to OGD insult (40 min, 0% O_2 in EBSS) significantly reduced cortical neuronal death (22% versus 83% in control). Further, Bickler et al. (2015) found that HPC in hippocampal brain slices induced neuroprotective effects against OGD insult. Energy deprivation (both glucose- and amino acid-free media) protected rat cortical neuronal cells against OGD (180 min; viability was 80.1 ± 1.3% in the 9 h energy deprivation group versus 33.1 ± 0.5% in the untreated group), including the following observations: reduced mitochondrial membrane potential, decreased free radical formation, attenuated intracellular free calcium surge upon glutamate receptor stimulation, and elevated level of glutathione (Ga'spa et al., 2006). Our observation that IPC (OGD 2, 4 h) induced protection against subsequent insult was consistent with the studies listed above. Same duration (2, 4 h) of OD and GD pre-

Fig. 9. Gene expression in PC12 cells cultured in normoxia and hypoxia with varying glucose concentration. Hypoxic gene expression in PC12 cells treated with normoxia (Nx; 21% O_2) and hypoxia (Hyp; 0.3% O_2) with varying glucose concentrations of 0, 0.565, 1.125, 2.25, 4.5 g/L for 4 h. Compared to sham (4.5 g/L, Nx), all conditions showed no significant ($p > 0.05$) fold expression changes in *Hif1α*, *Bnip3* and *Pfkfb1*. Complete glucose deprivation (0 g/L) in hypoxia resulted in significant fold expression increases in *Vegf, Glut1, Pfkfb3,* and *Ldha* compared to sham ($^{**}p \leq 0.01$). Compared to sham (4.5 g/L Nx), no significant fold expression changes in *Vegf*, *Glut1*, *Pfkfb3* and *Ldha* expression were seen by all other conditions. For all the genes, the expression was normalized to β-actin house-keeping gene. The dot line represents the basal gene expression.

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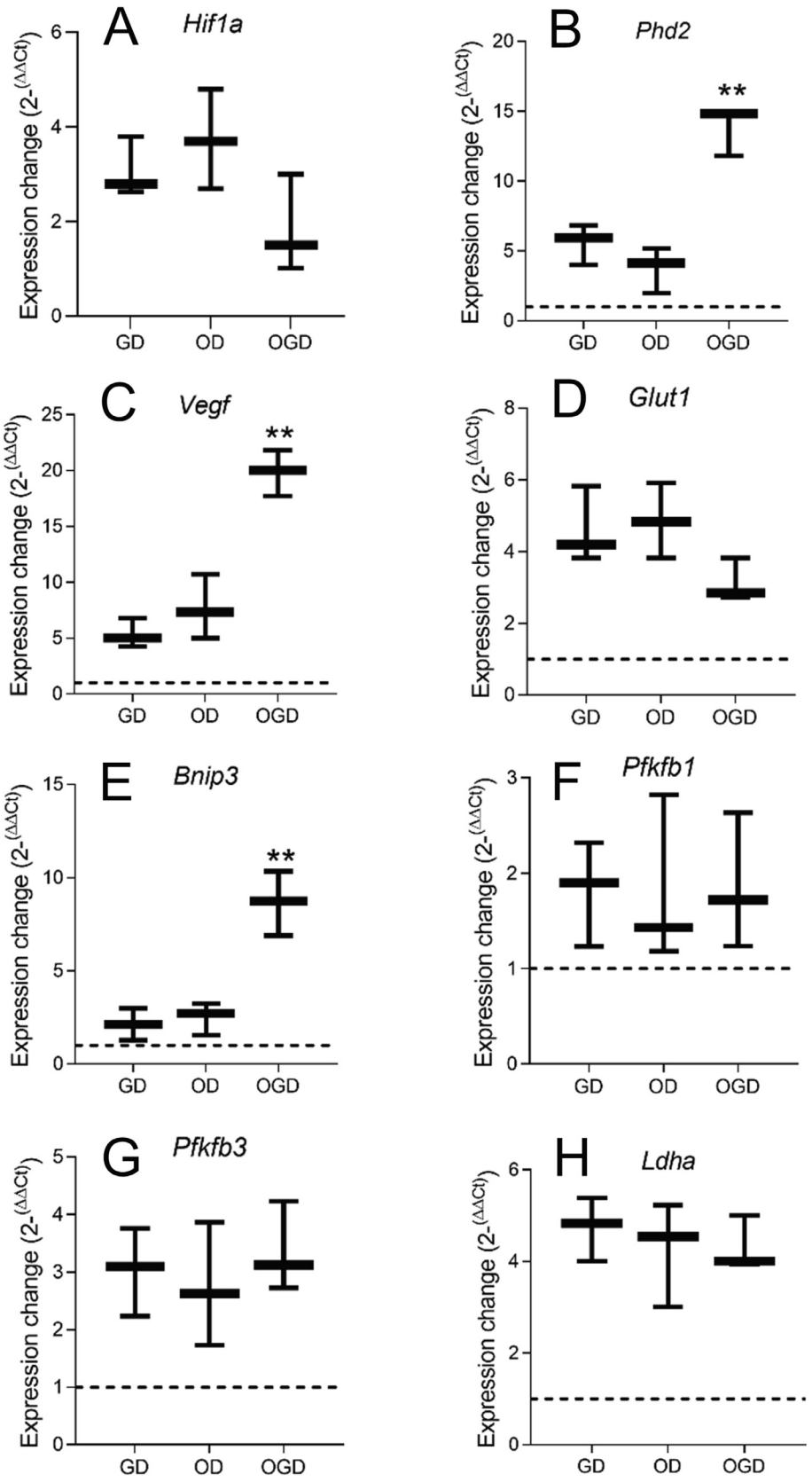

conditioning failed to protect neurons. However, preconditioning with a longer period (6 h) OD, but not GD induced ischaemic tolerance in neurons. As we aimed to compare the strength of OGD, OD and GD in this study, we used the same duration (4 h) on HIF1α and its downstream gene expression in primary neurons and PC12 cells. Further studies are required to explore the effect of longer duration of OD or GD on ischaemic tolerance, HIF1α and their downstream genes expression.

HIF1 is stabilized during hypoxia/ischaemia and upregulates hundreds of human genes that code for various adaptive cellular processes (Kaelin and Ratcliffe, 2008). As HIF1 plays an important role in promoting adaptive changes during hypoxia/ischaemia, we studied HIF1α protein expression in PC12 cells exposed to varying glucose concentrations in normoxia and hypoxia. We found that, HIF1α was upregulated in PC12 cells exposed to 4 h OGD, but not GD or OD. This is consistent with a study showing that OD (0.3%, 12 h) did not stabilise HIF1α in PC12 cells (Zhang et al., 2017). Similarly, HIF1α protein was upregulated in primary neurons exposed to 4 h OGD, but not GD or OD, which is consistent with a study showing significant HIF1α upregulation in rat cortical neurons at 6 h OGD (Zhang et al. 2017).

HIF1 protein upregulation is due to inhibition of the proteasomal degradation, while *Hif1α* gene expression was not significantly affected by hypoxia and ischaemia. Our results for PC12 cells and primary rat neurons are consistent with various studies that indicate that during hypoxia and ischaemia HIF1α protein expression is mainly regulated at the post-transcriptional level (Martín-Aragón et al., 2017; Singh et al., 2020). A number of HIF1 downstream genes such as *Glut1*, *Vegf*, *Pfkfb3* and *Ldha* were significantly upregulated in PC12 cells exposed to OGD only. *Vegf* is an important downstream target gene of HIF1

allowing for angiogenesis to increase blood flow during ischaemia (Ramakrishnan et al., 2014). During ischaemia, GLUT-1 (an integral membrane glycoprotein) upregulation is considered as an adaptive mechanism to increases glucose uptake, allowing cells to maintain or regain ATP levels by increasing flux through glycolytic pathways during ischaemia (Navale and Paranjape, 2016). Both *Pfkfb3* and *Ldha* are transcribed during ischaemia to promote anaerobic glycolysis to generate energy (Minchenko et al., 2003; Yamamoto et al., 2014). *Pfkfb3* is ubiquitously expressed in several proliferating cells and tissues, whereas *Pfkfb1* is restricted to muscle and liver cells (Minchenko et al., 2003). *Bnip3* upregulation was reported at time points starting at 12 h, with peak upregulation at 72 h (Liu et al., 2017). Similar to PC12 cells, *Vegf* was significantly upregulated by OGD in primary neurons. Additionally, primary neurons exhibited significant upregulation of *Phd2* and *Bnip3* in cells exposed to OGD, whilst there were no significant changes in *Hif1α*, *Glut1, Pfkfb1, Pfkfb3* and *Ldha* gene expression regardless of the treatment group. Hypoxia increases *Phd2* gene expression indicating the existence of a feedback loop potentially limiting HIF1α accumulation during hypoxia (Appelhoff et al., 2004). *Bnip3* expression has been noted for pro- and anti-apoptotic events and has been shown to contribute to delayed neuronal death via mitophagy (Shi et al., 2014). Unlike in PC12 cells, no significant expression of *Pfkfb3* and *Glut1* was found in primary neurons by OGD. Bolanos (2016) reported that PFKFB3 was abundantly expressed in astrocytes to promote anaerobic glycolysis but not typically found in neurons in the brain. *Actin* was used as an internal control due to its general expression across all eukaryotic cell types. *Actin* is the commonly used internal control in *in vitro* studies involving ischaemia/reperfusion studies (Lis et al., 2005).

In addition to the HIF signalling pathway, other molecules and signalling pathways are associated with IPC/HPC. During hypoxia, reactive oxygen species (ROS) formation increases, which has a significant detrimental impact on cell viability (Chen et al., 2018). Isoprostanes can be quantified as an indication of lipid and carbonyl protein oxidant-induced damage, indicative of oxidative stress (Montuschi et al., 2004). As ROS are mostly formed in the mitochondria (Chen et al., 2018), mitochondrial bioenergetics should be characterized more fully in future studies. Additionally, glutathione assays (GSH) and glutathione disulphide assays (GSSG) could be performed to assess the performance of cellular anti-oxidative defences in these conditions. Future studies should also include how heat shock-induced responses to preconditioning may be associated with any hormetic effects (Calabrese and Agathokleous, 2019).

It is worth noting that the PC12 cell line used in our study is a rat adrenal medulla cancer cell line. It has been observed that cancer cells rely on glucose-dependent glycolytic pathway and lactate production for energy needs, unlike normal cells that relied on oxidative phosphorylation (Potter et al., 2016). This is known as the "Warburg effect" and could be important for tumorigenesis. Some cancer genes, such as *Ras, cMys* and *P53* were involved in the regulating of Warburg effect (Dang and Semenza, 1999). The PC12 cells could be more sensitive to GD, and less sensitive to OD compared to other cells, e.g. neurons (Teng et al., 2006). The Warburg effect, therefore could play an important role in the GD preconditioning induced tolerance we observed in PC12 cells.

In conclusion, ischaemic tolerance is induced in both PC12 cells and primary rat neurons by OGD preconditioning, which is associated with HIF1α accumulation and upregulation of its downstream genes. Similar period of OD preconditioning does not result in cytoprotection in both types of cell, nor accumulate HIF1α. However, GD preconditioning protects PC12 cells, but not the primary neurons from OGD injuries. GD induced tolerance is not associated with HIF1α accumulation but could be due to Warburg effect. The role of the glycolytic pathway in PC12 cells during GD needs further investigation to understand the mechanism underlying the crucial role of GD in ischaemic tolerance.

AUTHOR CONTRIBUTIONS

This project was conceived by R.C. The experiments were designed by A.S. and R.C., and were performed by A.S, O.C., E.R. and K.A. Data were analysed and interpreted by A.S, S.J. and R.C. The manuscript was prepared by A.S. and O.C. and revised by S.J., L.Z., R. C. All authors have read and approved the final copy of the manuscript.

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Fig. 10. Gene expression in primary rat neurons exposed to oxygen deprivation (OD) or oxygen and glucose deprivation (OGD). Hypoxic gene expression in primary rat neurons treated with sham, GD, OD or OGD for 4 h. Compared to sham, all conditions showed no significant ($p > 0.05$) fold expression changes in *Hif1α*, *Glut1*, *Pfkfb1*, *Pfkfb3* and *Ldha*. OGD resulted in significant fold expression increases in *Phd2, Vegf* and *Bnip3* compared to sham ($^{**}p \leq 0.01$). No significant fold expression changes in *Phd2*, *Vegf* and *Bnip3* by GD or OD versus sham. For all the genes, the expression was normalized to β-actin house-keeping gene. The dot line represents the basal gene expression.

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DECLARATION OF CONFLICTING OF INTERESTS

None.

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APPENDIX A. SUPPLEMENTARY DATA

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