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The dual role of prostaglandin E_2 in excitotoxicity and preconditioning-induced neuroprotection

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Abstract

Cyclooxygenase-2 is harmful in models of cerebral ischemia yet plays a protective role in preconditioning-induced ischemic tolerance in the heart. This study examined the mechanisms underlying cyclooxygenase-2-mediated neurotoxicity and preconditioning-induced neuroprotection in an in vitro model of cerebral ischemia. Inhibition of cyclooxygenase-2 protects cortical neuronal cultures from death induced by oxygen–glucose deprivation and reduces oxygen–glucose deprivation-induced increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$). In the present study, we determined if prostaglandin E_2 (PGE_2) is responsible for this cyclooxygenase-2-mediated effect. Rat cortical cultures expressed mRNA for the prostanoid EP_1–EP_4 receptors. PGE_2 reversed the attenuation in $[Ca^{2+}]_i$ and the protection offered by cyclooxygenase-2 inhibition during oxygen–glucose deprivation. These effects likely occur via activation of the prostanoid EP_1 receptor since blocking this receptor during oxygen–glucose deprivation reduced $[Ca^{2+}]_i$ and neurotoxicity. Next, we considered if the moderate activation of this pathway, by preconditioning cultures with sub-lethal oxygen–glucose deprivation, influenced the development of tolerance to an otherwise lethal oxygen–glucose deprivation insult, 48 h later. Inhibition of cyclooxygenase-2 during oxygen–glucose deprivation–preconditioning abolished preconditioning-induced protection. Furthermore, cultures were rendered tolerant to oxygen–glucose deprivation by the transient exposure to exogenous PGE_2 24 h prior to the insult, indicating that this product of the cyclooxygenase-2 pathway is sufficient to induce ischemic tolerance. This study shows that cyclooxygenase-2 and PGE_2 are involved in both oxygen–glucose deprivation-induced neurotoxicity and preconditioning-induced neuroprotection. While neurotoxic in the context of lethal oxygen–glucose deprivation, the moderate activation of this signalling pathway confers ischemic tolerance.

Keywords: Cyclooxygenase-2; Prostaglandin E_2; Oxygen–glucose deprivation; Preconditioning; Ca^{2+}; Primary cortical cultures

1. Introduction

Cyclooxygenase-2 is a rate-limiting enzyme that catalyzes the biosynthesis of prostaglandins from arachidonic acid. This enzyme is constitutively present in the brain, particularly in the cortex and hippocampus (Breder et al., 1995; Kaufmann et al., 1996). The expression of cyclooxygenase-2 is regulated by physiological synaptic activity (Yamagata et al., 1993) and is strongly induced by pathological events associated with the excessive activation of *N*-methyl-D-aspartate (NMDA) receptors, such as cerebral ischemia in rodents (for review see Kaufmann et al., 1997), as well as ischemic stroke in humans (Sairanen et al., 1998; Iadecola et al., 1999). A causative role for cyclooxygenase-2 in ischemia is supported by the findings that neuronal overexpression of cyclooxygenase-2 in transgenic mice aggravates neuronal damage following transient focal ischemia (Dore et al., 2003), while cyclooxygenase-2-deficient mice are less susceptible to both focal and global ischemic brain injury (Iadecola et al., 2001; Sasaki et al., 2004). The protection afforded in the early stages of ischemia may be attributed to attenuated excitotoxicity

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since cyclooxygenase-2-null mice are resistant to neuronal injury produced by NMDA microinjection into the neocortex (Iadecola et al., 2001; Manabe et al., 2004). Moreover, NMDA exposure induces cyclooxygenase-2 expression in cultured cortical neurons and pharmacological inhibition of this enzyme protects against NMDA toxicity (Hewett et al., 2000; Carlson, 2003).

The mechanisms by which cyclooxygenase-2 is neurotoxic are poorly defined but may be due to an increase in the production of prostanoids like prostaglandin E_2 (PGE_2). In vitro, PGE_2 increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of astrocytes and stimulates glutamate release (Bezzi et al., 1998). This, in turn, leads to the activation of neuronal ionotropic glutamate receptors and results in $[Ca^{2+}]_i$ elevations (Bezzi et al., 1998; Sanzgiri et al., 1999). PGE_2-induced increases in neuronal $[Ca^{2+}]_i$ could potentially be neurotoxic in situations of excessive PGE_2 production. In rat cortical and hippocampal cultures, PGE_2 (5–25 μM) induces apoptosis (Takadera et al., 2002, 2004). On the other hand, PGE_2 (0.001–10 μM) is reported to protect cultured neurons from death induced by NMDA and glutamate (Cazevieille et al., 1994; Akaike et al., 1994; McCullough et al., 2004). The pro-death and pro-survival effects of PGE_2 likely occur via the activation of different G protein-coupled PGE_2 receptors, four of which have been identified (EP_1–EP_4). In general, the prostanoid EP_2 and EP_4 receptors stimulate adenylate cyclase, the EP_3 receptors inhibit adenylate cyclase and EP_1 receptors enhance $[Ca^{2+}]_i$ by influencing internal Ca^{2+} mobilization and influx of extracellular Ca^{2+} (Negishi et al., 1995). Activation of the prostanoid EP_2 receptor is normally associated with neuroprotection. The prostanoid EP_2 receptor agonist, butaprost, offers protection against glutamate and NMDA exposure in neuronal cultures (Akaike et al., 1994; McCullough et al., 2004). In contrast, the prostanoid EP_1/EP_3 receptor agonist, 17-phenyl trinor prostaglandin E_2 (17-pt-PGE_2), blocks the protection against NMDA exposure in cortical cultures that is normally afforded by cyclooxygenase-2 inhibition (Carlson, 2003). This suggests that the prostanoid EP_1 and/or EP_3 receptor mediates cyclooxygenase-2-dependent neurotoxicity.

We have previously shown that mimicking ischemia in vitro by depriving cortical cultures of oxygen and glucose leads to a rapid increase in cyclooxygenase-2 protein expression and that inhibition of cyclooxygenase-2 using the selective inhibitor, 1-[(4-methylsulfonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl)pyrazole (SC-58125), dose-dependently protects cultures against this insult. The protective effect of SC-58125 is accompanied by a decrease in both oxygen–glucose deprivation-induced glutamate release and elevations of $[Ca^{2+}]_i$ (Gendron et al., 2004). Given the key role for NMDA receptor activation in oxygen–glucose deprivation toxicity (Goldberg and Choi, 1993) and the involvement of the prostanoid EP_1/EP_3 receptor activation in NMDA toxicity (Carlson, 2003), we hypothesized that a reduction in PGE_2 production and prostanoid EP_1 receptor activation may explain why cyclooxygenase-2 inhibition during oxygen–glucose deprivation results in decreased $[Ca^{2+}]_i$ and neuroprotection.

Preconditioning is the phenomenon by which a non-lethal stressor induces tolerance against a subsequent lethal insult (for review see Kirino, 2002). Cyclooxygenase-2 activity is required for ischemic preconditioning in the heart (for review see Bolli, 2000), yet little is known of the cycloxogyenase-2 requirement for tolerance induction in the brain. In our laboratory, cerebral ischemic preconditioning is modeled by subjecting cortical neuronal cultures to sub-lethal oxygen–glucose deprivation which induces delayed tolerance to a subsequent prolonged exposure to oxygen–glucose deprivation. We have previously shown that tolerance to oxygen–glucose deprivation can be generated by moderately activating signaling events that have the potential to be neurotoxic when induced by a more prolonged or intense insult (Tauskela et al., 2003). Since oxygen–glucose deprivation-induced cyclooxygenase-2 activation is neurotoxic, we hypothesized that the moderate activation of this enzyme by preconditioning is required to induce tolerance to oxygen–glucose deprivation in neurons.

To assess the involvement of PGE_2 in oxygen–glucose deprivation-induced neurotoxicity and in preconditioning-induced neuroprotection, the present study used rat cortical neuronal cultures to determine whether: 1) PGE_2 and activation of the prostanoid EP_1 receptor are involved in cyclooxygenase-2-mediated neuronal death induced by long duration oxygen–glucose deprivation; 2) cyclooxygenase-2 and PGE_2 are involved in preconditioning-induced ischemic tolerance.

2. Materials and methods

2.1. Materials

Tissue culture dishes and plates were purchased from either Du Pont-Life Technologies (Burlington, ON, Canada) or VWR Canlab (Mississauga, ON, Canada). Minimal essential medium (with Earle's salt and L-glutamine) was purchased from Wisent Canadian Laboratories (St-Bruno, QC, Canada), horse serum from Hyclone Laboratories (Logan, UT, USA) and fetal bovine serum from Gemini Bio-Products (Woodland, CA, USA). Uridine, 5-fluoro-2′deoxyuridine, poly-L-lysine, propidium iodide and the prostanoid EP_1 receptor antagonist, 8-Chloro-dibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid 2-acetylhydrazide (SC-19220), were purchased from Sigma (St. Louis, MO, USA). TriReagent® was purchased from Molecular Research Center (Cincinnati, OH, USA). Superscript™ II Reverse Transcriptase, 5X First Strand Buffer, dithiothreitol (DTT), Oligo(dT) and agarose were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). The Perkin-Elmer reagents, $MgCl_2$, 10X PCR II Buffer and Amplitaq® DNA Polymerase were purchased from Applied Biosystems (Foster City, CA, USA). Primers were ordered from Sigma Genosys (Oakville, ON, Canada). Fluo-4 AM was bought from Molecular Probes (Eugene, OR, USA). SC-

58125 and PGE_2 were purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Preparation of fetal neuronal cortical cultures

All procedures using animals were approved by the Institute for Biological Sciences Animal Care Committee. Primary cortical cultures were prepared as previously described (Black et al., 1995) using embryonic day 18 fetuses from timed-pregnant Sprague–Dawley rats purchased from Charles River Canada (St. Constant, QC, Canada). Briefly, the cortical region of the fetal brain was dissected and cells were dissociated and resuspended in plating medium consisting of minimal essential medium containing 2 mM glutamine and supplemented to 25 mM glucose, 10% fetal bovine serum and 10% horse serum. Cells were plated at a density of $1.7–1.85 \times 10^6$ cells/ml in 12-well tissue culture plates coated with poly-L-lysine. Cultures were treated with mitotic inhibitors (15 μg/ml 5-fluoro-2′-deoxyuridine and 35 μg/ml uridine) on the fourth day in vitro to minimize glial growth. At 7 days in vitro, one-half of the medium was replaced with medium identical to plating medium, except that it did not contain fetal bovine serum. Experiments were performed on mixed neuronal-glial cell cultures at 15–19 days in vitro.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

To study mRNA expression of the prostanoid receptors ($EP_1–EP_4$), total RNA was extracted from rat cortical cultures as well as from liver and lung tissue from an adult rat using TriReagent®, as per the manufacturer's instructions. RNA (2 μg) was converted into cDNA in a 10 μl reaction consisting of 0.1 μM Oligo(dT), 0.01 M DTT, 1 mM dNTPs and 20 U Superscript™ II Reverse Transcriptase in 1X First Strand Buffer. The reaction mixture was incubated at 42 °C for 60 min, 80 °C for 5 min, followed by cooling at 4 °C before being diluted with RNase-free water to a final volume of 100 μl. One microliter of the reverse transcriptase reaction was used for PCR amplification in a final volume of 25 μl consisting of 1.5 mM $MgCl_2$, 300 μM dNTPs, 0.04 U/μl Amplitaq® DNA Polymerase and 100 nM of left and right primers in 1X PCR II Buffer. PCR cycling conditions were as follows: 94 °C, 5 min (1 cycle); 94 °C, 1 min; 59 °C, 30 s; 72 °C, 1 min (35 cycles); 72 °C, 7 min. Primer sequences and product sizes are given in Table 1. PCR products were electrophoresed on 1.5% agarose gels and bands were visualized by staining with ethidium bromide. Gels were photographed using Northern Eclipse imaging software (EMPIX Imaging Inc., Mississauga, ON, Canada).

Table 1
Primer sequences and expected product sizes for RT-PCR analysis of the prostanoid $EP_1–EP_4$ receptors

Gene	Left and right primers (5′→3′)	GenBank accession #	Product size (bp)
EP_1	CGGTGGTGTGAGCCTTTAAT CCCTTTCAGATCCCACTTCA	NM_013100	353
EP_2	GTACTGTCCTGGGACGTGGT TTCCTTTCGGGAAGAGGTTT	NM_031088	340
EP_3	GACGGCTATCCAGCTTATGG	X83855 for EP3alpha	380
	CTCAGGGAAGCAGGTATTGC	X80133 for EP3beta	291
EP_4	ATGAGCATTGAGCGCTACCT ATGTAAGAGAAGGCGGCGTA	NM_032076	224

2.4. Oxygen–glucose deprivation

Oxygen–glucose deprivation was performed as previously described (Tauskela et al., 1999). To test the effect of PGE_2 and SC-58125 on oxygen–glucose deprivation-induced death, cultures were washed twice in a glucose-free balanced salt solution (BSS) composed of (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 20 HEPES and 0.03 glycine, pH 7.4. Cultures were pre-incubated with PGE_2 (10 μM) for 60 min at 37 °C in glucose-free BSS. The buffer was then removed and replaced with glucose-free BSS ± PGE_2 (10 μM) ± SC-58125 (25 μM). Cells were exposed to an anaerobic environment (95% N_2/5% CO_2, O_2 partial pressure of 10–15 Torr) by placing cultures in a 37 °C incubator housed in an anaerobic glove-box (Forma Scientific, Marjetta, OH, USA). Cellular morphology was monitored periodically during the course of oxygen–glucose deprivation using a microscope placed in the glove-box. Once cells began to swell (60–75 min), oxygen–glucose deprivation was terminated by returning the cultures to their stored medium ± PGE_2 (10 μM) and placing them in a standard incubator maintained at 37 °C. Control cultures were treated the same as those exposed to oxygen–glucose deprivation, except that BSS + 3 mM glucose was used throughout the experiment and cultures were kept in the standard incubator. The final concentration of DMSO (0.2%), the vehicle used to dissolve PGE_2 and SC-58125, was constant for all treated conditions. To test the effect of blocking the prostanoid EP_1 receptor during oxygen–glucose deprivation, cultures were pre-incubated with the prostanoid EP_1 receptor antagonist, SC-19220 (1–100 μM), for 60 min, exposed to oxygen–glucose deprivation in the presence of SC-19220 and returned to SC-19220-containing medium.

2.5. Assessment of cell injury

Neuronal death was assessed 24 h following treatments as previously described (Tauskela et al., 2001). Cultures were exposed to 0.03 μg/μl propidium iodide in BSS + glucose for 45 min at room temperature. The fluorescence intensity (Ex = 530 ± 20 nm; Em = 645 ± 40 nm) from 4 locations within each well was measured on a platereader (Cytofluor 2350, Millipore, Bedford, MA, USA). The percentage of propidium iodide uptake was determined by subtracting the fluorescence measured in untreated sister cultures, then normalizing values to the fluorescence representing 100% neuronal death, which was obtained by exposing sister cultures to 10 μM NMDA for 24 h. Thus, the percentage of propidium iodide uptake is equivalent to the percentage of dead cells above control levels. Death in untreated control wells was <5%.

2.6. $[Ca^{2+}]_i$ assay

The $[Ca^{2+}]_i$ was determined using platereader measurements of fluorescence intensity from cells loaded with the Ca^{2+}-sensitive, high-affinity fluorescent dye, Fluo-4 AM ($K_d \cong 345$ nM; Ex = 485 ± 20 nm; Em = 530 ± 20 nm) (Tauskela et al., 2003). To prepare the dye, 15 μl DMSO, 15 μl of 20% pluronic acid and 400 μl of BSS + glucose were sequentially added to 50 μg Fluo-4 AM, vortexing for 5 min between each step. The dye solution was then added to 10.1 ml BSS + glucose and vortexed again for 5 min. Cells

were bathed in 400 μl/well of the dye solution for 60 min at 37 °C, washed with glucose-free BSS and allowed to equilibrate for 30 min at room temperature. The buffer was replaced with glucose-free BSS ± drug and the initial fluorescence intensity was immediately measured from 4 locations within each well. After a 60 min pre-incubation period, the buffer was removed and replaced with fresh glucose-free BSS ± drug and cultures were exposed to oxygen–glucose deprivation. Following oxygen–glucose deprivation, plates were washed once and fluorescence intensities were measured. The fold-change in fluorescence was calculated by dividing the final reading of each well by the initial reading and then normalizing to values of drug-free control cultures. Control cultures were treated the same as those exposed to oxygen–glucose deprivation, except that BSS + glucose was used throughout the experiment and cultures were kept in the standard incubator during the oxygen–glucose deprivation period.

2.7. Oxygen–glucose deprivation–preconditioning

Cultures were preconditioned by exposure to a sub-lethal duration of oxygen–glucose deprivation (50–60 min) in the absence or presence of SC-58125 on day 1, allowed to recover for 48 h and then subjected to a prolonged exposure to oxygen–glucose deprivation (60–75 min) on day 3 (Fig. 1). To reduce inter-plate variability, all cultures exposed to oxygen–glucose deprivation on day 3 were tested in the same 12-well plate (Plate A). Sister cultures (Plate B) were preconditioned in the presence or absence of SC-58125 but not subjected to oxygen–glucose deprivation on day 3, to ensure that the preconditioning stimulus and SC-58125 were non-lethal. Briefly, on day 1, cultures to be preconditioned were pre-incubated for 60 min in glucose-free BSS ± 50 μM SC-58125. Non-preconditioned cultures were pre-incubated in glucose-containing BSS ± 50 μM SC-58125. Following the pre-incubation period, both Plate A and Plate B were placed in the anaerobic chamber for 50–60 min for preconditioning of glucose-free wells. In the presence of glucose, anoxia is not sufficiently stressful to precondition or to kill neurons. After being removed from the anaerobic chamber, cultures were washed twice in order to remove the SC-58125 and returned to drug-free media. On day 3, preconditioned and non-preconditioned cells in Plate A were subjected to a prolonged oxygen–glucose deprivation insult by placing them in the anaerobic chamber for 60–75 min in glucose-free BSS. Plate B was also placed in the anaerobic chamber but cells were bathed in BSS + glucose. Following oxygen–glucose deprivation, cultures were returned to conditioned media and cell death was assessed 24 h later.

2.8. PGE_2-preconditioning

To precondition cultures with PGE_2, cells were exposed to three consecutive 60 min applications of PGE_2 (10 μM) in BSS + glucose on day 1. Non-preconditioned cultures were exposed to only BSS + glucose. Following the third application, cultures were washed and returned to their stored media. The following day, cultures were subjected to oxygen–glucose deprivation and neuronal death was assessed on day 3. To determine if higher concentrations of PGE_2 are lethal, cultures were exposed to three consecutive 60 min applications of PGE_2 (25, 50 and 100 μM) in BSS + glucose on day 1 and neuronal death was assessed on day 3.

2.9. Statistical analysis

Three to six wells were investigated per condition from a minimum of 3 different platings. Data is presented as the mean ± S.E.M. Statistical comparisons were made by Student's *t*-test or analysis of variance (ANOVA). When significant differences were observed, Dunnett's test was employed for multiple comparisons. Statistical significance was inferred at $P < 0.05$.

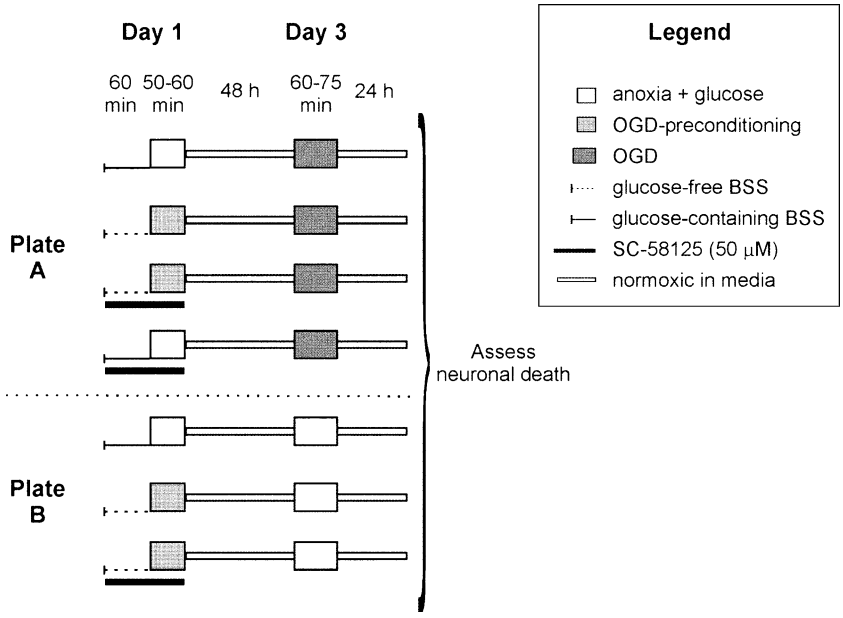

Fig. 1. Protocol for assessing the effect of cyclooxygenase-2 inhibition during oxygen–glucose deprivation–preconditioning on tolerance induction against otherwise lethal oxygen–glucose deprivation. See the Materials and methods section for a complete description of the protocol. OGD = oxygen–glucose deprivation, BSS = balanced salt solution.

3. Results

3.1. Mixed neuronal–glial cortical cultures express mRNA for the EP_1–EP_4 prostanoid receptors

Studies examining the presence of EP_1–EP_4 prostanoid receptor mRNA expression in neuronal cortical cultures have yielded different results depending on the species from which they were prepared (rat versus mouse) and the presence of astrocytes in the cultures (Takadera et al., 2002; Carlson, 2003). Therefore, before assessing whether PGE_2 is responsible for the detrimental effects of cyclooxygenase-2 in oxygen–glucose deprivation, the expression of PGE_2 receptors in our mixed rat neuronal/glial cortical cultures was examined by RT-PCR analysis. Rat cortical cultures expressed mRNA for prostanoid receptors EP_1, EP_2, EP_3 and EP_4 (Fig. 2). In regard to the prostanoid EP_3 receptor, the prominent amplification product in both liver and cortical samples was 380 bp, corresponding to the EP_3-alpha splice variant; the band at 291 bp corresponds to the EP_3-beta splice variant (Table 1). A third band (approximately 475 bp) was also amplified using cDNA from cortical cultures but not from liver. To our knowledge, none of the rat mRNA sequences for the prostanoid EP_3 receptor currently in the Genbank database would result in a product of this size with the primers used to amplify the EP_3 receptor. However, a BLAST search of nucleotide databases using the prostanoid EP_3 receptor primers did not target any matching genes other than those coding for the EP_3 receptor. This product may thus represent a tissue-specific splice variant not previously identified.

3.2. PGE_2 suppresses the protection against oxygen–glucose deprivation offered by cyclooxygenase-2 inhibition

We have shown that cyclooxygenase-2 inhibition with SC-58125 significantly protects both mouse and rat mixed cortical cultures from oxygen–glucose deprivation-induced neuronal death (Gendron et al., 2004). If cyclooxygenase-2-mediated death occurs via increased PGE_2 synthesis, then the exogenous addition of PGE_2 should reverse the protective effect of cyclooxygenase-2 inhibition. To test this, neuronal death was compared between cultures exposed to oxygen–glucose deprivation (60–75 min) in the presence of SC-58125 (25 μM) alone and cultures exposed to both SC-58125 and PGE_2 (10 μM). SC-58125-treated cultures were exposed to the inhibitor only during oxygen–glucose deprivation. Cultures treated with PGE_2 during oxygen–glucose deprivation were also pre-incubated with PGE_2 (10 μM) for 60 min prior to oxygen–glucose deprivation and returned to PGE_2 (10 μM)-containing media following oxygen–glucose deprivation. This concentration of PGE_2 induces increases in $[Ca^{2+}]_i$ in astrocytes and neurons (Bezzi et al., 1998). Neither SC-58125 nor PGE_2 were toxic under control conditions (data not shown). The addition of SC-58125 during oxygen–glucose deprivation resulted in significant protection, as assessed 24 h post-insult (Fig. 3A). PGE_2 partially, yet significantly, reversed the protection afforded by SC-58125 (Fig. 3A). In the absence of SC-58125, PGE_2 had no significant effect on oxygen–glucose deprivation toxicity. Since oxygen–glucose deprivation resulted in a high degree of neuronal death, any aggravation of death caused by PGE_2 may have been masked. Therefore, to determine if PGE_2 aggravates oxygen–glucose deprivation-induced death, a shorter duration of oxygen–glucose deprivation (60–65 min) causing a less severe insult was employed. Lower concentrations of PGE_2 (0.1 and 1.0 μM) were also tested on the basis of reported neuroprotection (Cazevieille et al., 1994; Akaike et al., 1994; Yagami et al., 2003; McCullough et al., 2004). PGE_2 did not significantly effect oxygen–glucose deprivation-induced death at any of the concentrations tested (Fig. 3B).

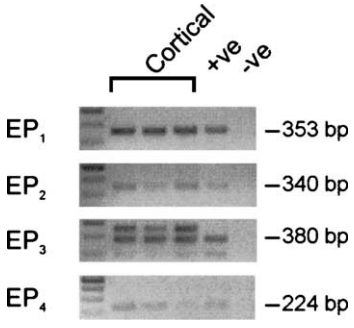

Fig. 2. Mixed neuronal/glial cortical cultures express prostanoid EP_1–EP_4 receptor mRNA. Total RNA was isolated from 3 different platings of rat cortical cultures for RT-PCR analysis using primers specific for prostanoid EP_1, EP_2, EP_3 or EP_4 receptor mRNA. RNA extracted from lung of an adult rat was used as a positive control (+ve) for the prostanoid EP_1, EP_2 and EP_4 receptors while RNA from liver served as a control for the prostanoid EP_3 receptor. As a negative control (−ve), cDNA was omitted from the PCR reaction. The RT-PCR products were electrophoretically fractioned on a 1.5% agarose gel stained with ethidium bromide and visualized by UV illumination.

3.3. PGE_2 induces elevations in $[Ca^{2+}]_i$

We have reported that the protective effect of cyclooxygenase-2 inhibition during oxygen–glucose deprivation is accompanied by a decrease in $[Ca^{2+}]_i$ (Gendron et al., 2004). Since PGE_2 induces $[Ca^{2+}]_i$ increases in astrocytes and neurons (Bezzi et al., 1998), we investigated whether PGE_2 reverses the protection afforded by SC-58125 by increasing $[Ca^{2+}]_i$. Cultures loaded with the fluorescent Ca^{2+}-indicator, Fluo-4 AM, were exposed to PGE_2 (10 μM)±SC-58125 (25 μM) before (60 min) and during oxygen–glucose deprivation. The fold-change in fluorescence was calculated by dividing the fluorescence intensity measured following oxygen–glucose deprivation by the initial reading and then normalizing to values of drug-free control cultures. Under normoxic/normoglycemic conditions, SC-58125 had no effect on $[Ca^{2+}]_i$ whereas PGE_2 significantly increased $[Ca^{2+}]_i$ (Fig. 4). During oxygen–glucose deprivation, SC-58125 (25 μM) attenuated oxygen–glucose deprivation-induced rises in $[Ca^{2+}]_i$, though not as effectively as previously observed using 50 μM SC-58125 (Gendron et al., 2004). A lower dose of SC-58125 was employed in the present study to minimize the final concentration of vehicle (DMSO) which was kept constant in all treated samples. PGE_2 significantly increased $[Ca^{2+}]_i$ during oxygen–glucose deprivation, thereby reversing the SC-58125-induced attenuation of $[Ca^{2+}]_i$ (Fig. 4).

3.4. Inhibition of the prostanoid EP_1 receptor protects cortical cultures from oxygen–glucose deprivation

Since activation of the prostanoid EP_1 receptor increases Ca^{2+} influx and intracellular Ca^{2+} mobilization (Negishi et al., 1995), we next considered whether activation of this receptor is involved in death induced by oxygen–glucose deprivation. Cultures were pre-incubated (60 min) with the prostanoid EP_1 receptor antagonist,

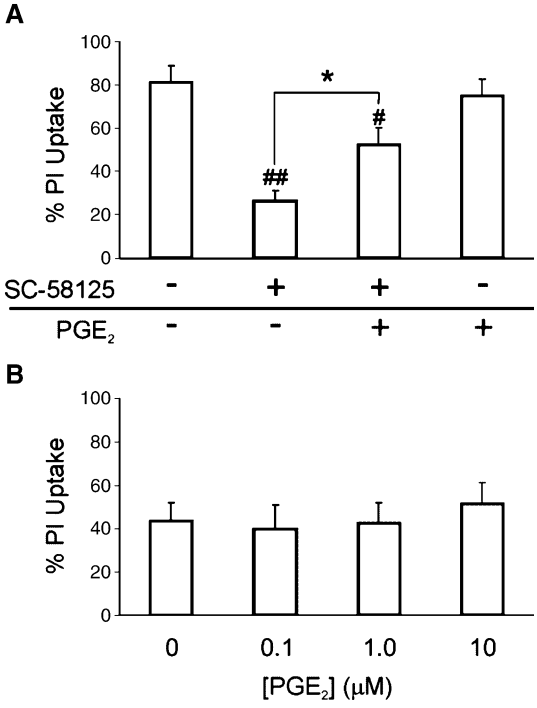

Fig. 3. PGE_2 suppresses the protection against oxygen–glucose deprivation offered by cyclooxygenase-2 inhibition but has no effect on oxygen–glucose deprivation toxicity in the absence of SC-58125. Rat cortical cultures were subjected to oxygen–glucose deprivation in the presence or absence of SC-58125 (25 μM) and/or PGE_2 (0.1–10 μM). Cultures treated with PGE_2 were pre-incubated with PGE_2 for 60 min, exposed to oxygen–glucose deprivation in the presence of PGE_2 and returned to PGE_2-containing media. Neuronal death was assessed 24 h after oxygen–glucose deprivation by propidium iodide (PI) uptake. A. Neuronal death was compared between cultures exposed to oxygen–glucose deprivation (60–75 min) in the presence of SC-58125 (25 μM) alone and cultures exposed to both SC-58125 (25 μM) and PGE_2 (10 μM). B. The effect of PGE_2 (0.1–10 μM) on oxygen–glucose deprivation-induced death was tested using a duration of oxygen–glucose deprivation (60–65 min) that caused a less severe insult. Data represents the mean ± S.E.M. obtained from experiments performed using 4 different platings of cells in A and 5 different platings in B. $^{\#, \#\#}$Significant ($^{\#}P<0.05$ and $^{\#\#}P<0.001$) difference compared to oxygen–glucose deprivation performed in the absence of drugs, as determined by one-way ANOVA. *Significantly ($P<0.05$) different as determined by Student's *t*-test.

SC-19220 (1, 10 or 100 μM), exposed to oxygen–glucose deprivation in the presence of SC-19220 and returned to SC-19220-containing media. Blocking the prostanoid EP_1 receptor resulted in significant protection against oxygen–glucose deprivation in a dose-dependant manner (Fig. 5A). To determine if blocking the prostanoid EP_1 receptor would attenuate oxygen–glucose deprivation-induced increases of $[Ca^{2+}]_i$, cultures loaded with Fluo-4 AM were exposed to oxygen–glucose deprivation in the presence or absence of 10 μM SC-19220. SC-19220 significantly reduced oxygen–glucose deprivation-induced increases in $[Ca^{2+}]_i$ (Fig. 5B).

3.5. Cyclooxygenase-2 is involved in tolerance induction by oxygen–glucose deprivation–preconditioning

If rat cortical cultures are preconditioned by exposure to sub-lethal oxygen–glucose deprivation, they are protected from a subsequent otherwise lethal oxygen–glucose deprivation insult 24–72 h later (Tauskela et al., 2001). While the signalling events initiated by preconditioning are not fully understood, tolerance can be induced by the moderate activation of signalling pathways that have the potential to be lethal when excessively induced by a more prolonged or intense insult (Tauskela et al., 2003). Given that the activation of cyclooxygenase-2 during oxygen–glucose deprivation is lethal (Gendron et al., 2004), we hypothesized that the moderate activation of cyclooxygenase-2 during preconditioning is required for tolerance induction. To test this, rat cortical cultures were preconditioned by sub-lethal oxygen–glucose deprivation in the presence or absence of SC-58125 (Fig. 1). Since SC-58125 has

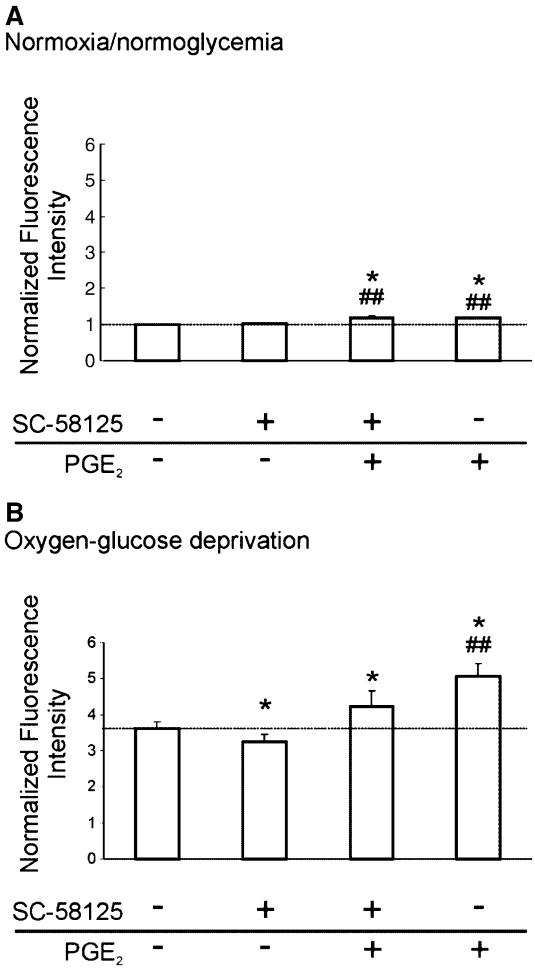

Fig. 4. PGE_2 induces elevations in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was examined in rat cortical cultures loaded with the fluorescent Ca^{2+}-sensitive dye, Fluo-4 Am. Cultures were pre-incubated with PGE_2 (10 μM) and/or SC-58125 (25 μM) in BSS containing glucose (A) or lacking glucose (B) for 60 min. Prior to oxygen–glucose deprivation, the buffer was removed and replaced with fresh buffer containing or lacking PGE_2 (10 μM) and/or SC-58125 (25 μM). Cultures were then placed in the standard incubator (A) or in the anaerobic chamber (B). The fold-change in fluorescence was calculated by dividing the final reading of each well by the initial reading and then normalizing to values of drug-free control cultures. Data represents the mean ± S.E.M. obtained from experiments done in duplicate using 3 different platings of cells. $^{\#\#}$Significant ($P<0.001$) difference compared to drug-free controls, as determined by one-way ANOVA. *Significantly ($P<0.05$) different compared to drug-free controls, as determined by Student's *t*-test.

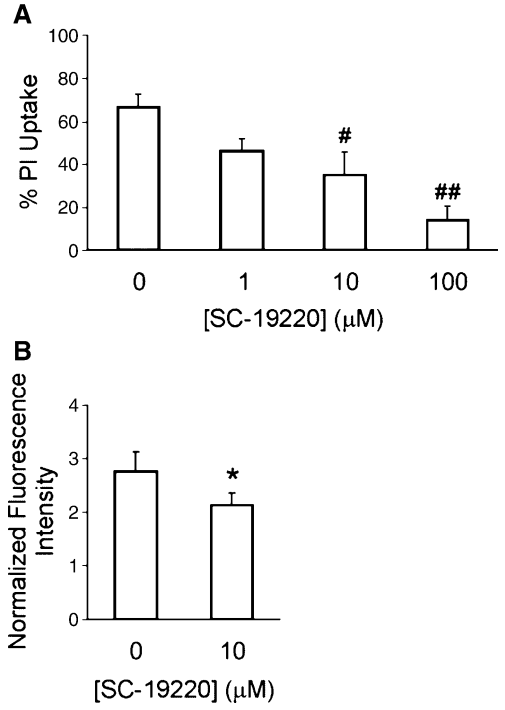

Fig. 5. The prostanoid EP_1 receptor antagonist, SC-19220, protects cultures from oxygen–glucose deprivation-induced death and attenuates oxygen–glucose deprivation-induced rises in $[Ca^{2+}]_i$. A. Rat cortical cultures were subjected to oxygen–glucose deprivation in the presence or absence of the prostanoid EP_1 receptor antagonist, SC-19220 (1–100 μM). Cultures were pre-incubated with the SC-19220 for 60 min, exposed to oxygen–glucose deprivation in the presence of SC-19220 and returned to SC-19220-containing media. Neuronal death was assessed 24 h after oxygen–glucose deprivation by propidium iodide (PI) uptake. B. $[Ca^{2+}]_i$ was examined in rat cortical cultures loaded with the fluorescent Ca^{2+}-sensitive dye, Fluo-4 AM. Cultures were pre-incubated with/without SC-19220 (10 μM) in glucose-free BSS for 60 min. Prior to oxygen–glucose deprivation, the buffer was removed and replaced with fresh buffer containing or lacking SC-19220 (10 μM) and cultures were placed in the anaerobic chamber. The fold-change in fluorescence was calculated by dividing the final reading of each well by the initial reading and then normalizing to values of drug-free control cultures. Data represents the mean±S.E.M. obtained from experiments performed using 4 different platings of cells in A and in B. $^{\#,\#\#}$Significant ($^{\#}P<0.05$ and $^{\#\#}P<0.001$) difference compared to oxygen–glucose deprivation performed in the absence of drugs, as determined by one-way ANOVA. *Significantly ($P<0.05$) different compared to oxygen–glucose deprivation performed in the absence of SC-19220, as determined by Student's *t*-test.

a long half-life (over 24 h; (Nakayama et al., 1998)), prolonged oxygen–glucose deprivation was performed 48 h after the preconditioning event. Oxygen–glucose deprivation–preconditioning, whether in the presence or absence of SC-58125, was non-lethal (data not shown). As expected, oxygen–glucose deprivation–preconditioning significantly protected cultures against the otherwise lethal oxygen–glucose deprivation insult (Fig. 6). Cultures that were exposed to SC-58125 during the preconditioning event, however, were not significantly protected. As a control, non-preconditioned cultures were treated with SC-58125 48 h prior to the prolonged oxygen–glucose deprivation. These cultures were not protected against oxygen–glucose deprivation, suggesting SC-58125 was fully eliminated from cultures by day 3.

3.6. Transient PGE_2-preconditioning induces tolerance to oxygen–glucose deprivation

Since cyclooxygenase-2 is required for tolerance induction, we investigated whether the transient exposure of cultures to the cyclooxygenase-2 product, PGE_2, was sufficient to induce tolerance to oxygen–glucose deprivation. To precondition rat cultures, cells were exposed to three consecutive 60 min PGE_2 (10 μM) applications in glucose-containing BSS, after which time cultures were washed and returned to their media. Non-preconditioned cultures were exposed to glucose-containing BSS in the absence of PGE_2. The following day, cultures were subjected to oxygen–glucose deprivation and neuronal death was assessed 24 h later. Cultures preconditioned with PGE_2 were significantly protected against oxygen–glucose deprivation-induced death compared to non-preconditioned controls (Fig. 7). PGE_2-preconditioning on its own was not lethal (Fig. 7). In some preconditioning models, optimal protection is achieved using the maximum non-lethal dose of a preconditioning stimulus. Therefore, higher concentrations of PGE_2 were tested to determine whether 10 μM PGE_2 falls near the threshold of lethality. Interestingly, three consecutive 60 min applications of PGE_2 at 25, 50 or 100 μM were not lethal (data not shown).

Preconditioning by transient exposure to PGE_2 may induce tolerance to oxygen–glucose deprivation via the activation of Ca^{2+}-dependent signalling, given that Ca^{2+} is a key mediator in tolerance induction (Tauskela and Morley, 2004). To determine whether activation of the prostanoid EP_1 receptor is involved in PGE_2-preconditioning, cultures were to be preconditioned in the presence of SC-19220. If activation of the prostanoid EP_1 receptor is required for tolerance induction, the addition of a prostanoid EP_1 receptor antagonist would abolish protection offered by PGE_2-preconditioning. However, exposing cultures to three consecutive 60 min applications of SC-19220 (1–100 μM) 24 or 48 h prior to

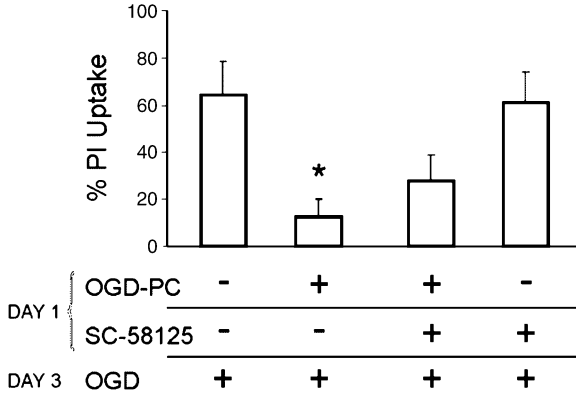

Fig. 6. Inhibition of cyclooxygenase-2 during oxygen–glucose deprivation–preconditioning attenuates tolerance induction. On day 1, cultures were preconditioned by exposure to a sub-lethal duration of oxygen–glucose deprivation (OGD-PC; 50–60 min) in the absence or presence of SC-58125 (50 μM), allowed to recover for 48 h and then subjected to a prolonged exposure to oxygen–glucose deprivation (OGD; 60–75 min) on day 3, as depicted in Fig. 1. Neuronal death was assessed 24 h following prolonged oxygen–glucose deprivation by propidium iodide (PI) uptake. Data represents the mean±S.E.M. obtained from experiments performed using 4 different platings of cells. *Significantly ($P<0.05$) different compared to oxygen–glucose deprivation performed in non-preconditioned cultures, as determined by one-way ANOVA.

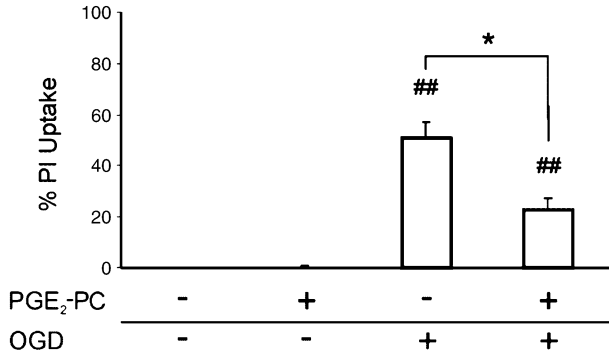

Fig. 7. Transient PGE_2-pre-conditioning induces tolerance to oxygen–glucose deprivation. PGE_2 was used as a preconditioning stimulus (PGE_2-PC). Rat cortical cultures were exposed to three consecutive 60 min applications of PGE_2 (10 μM) in BSS+glucose. Non-preconditioned cultures were exposed to BSS+glucose in the absence of PGE_2. Following the third application, cultures were washed and returned to their stored media. The following day, cultures were subjected to oxygen–glucose deprivation (OGD) and neuronal death was assessed 24 h later by propidium iodide (PI) uptake. Data represents the mean±S.E.M. obtained from experiments performed using 6 different platings of cells. ##Significant ($P<0.001$) difference compared to non-treated controls, as determined by one-way ANOVA. *Significantly ($P<0.05$) different, as determined by Student's *t*-test.

oxygen–glucose deprivation provided substantial protection, presumably due to the presence of residual SC-19220 (data not shown). Since SC-19220 could protect on its own, we could not determine if blocking the prostanoid EP_1 receptor during PGE_2-preconditioning would eliminate the induction of tolerance.

4. Discussion

While numerous studies provide evidence that cyclooxygenase-2 induction is detrimental in models of ischemia and excitotoxicity, the mechanisms by which cyclooxygenase-2 produces neurotoxic effects are poorly defined. As a cyclooxygenase-2 reaction product upregulated in models of ischemia and excitotoxicity, PGE_2 seems a probable mediator of cyclooxygenase-2-dependent injury (Gaudet et al., 1980; Stevens and Yaksh, 1988; Hewett et al., 2000; Carlson, 2003; McCullough et al., 2004). However, in vitro studies investigating the role of PGE_2 and its analogues have yielded conflicting results, showing that they either exert toxic (Takadera et al., 2002, 2004), protective (Cazevieille et al., 1994; Akaike et al., 1994; Carlson, 2003; McCullough et al., 2004) or no (McCullough et al., 2004) effect on neuronal survival.

We have previously shown that inhibition of cyclooxygenase-2 by SC-58125 dose-dependently protects rat cortical neuronal cultures against oxygen–glucose deprivation, an effect that is accompanied by a decrease in $[Ca^{2+}]_i$ (Gendron et al., 2004). Our current results suggest that PGE_2 is in part responsible for the neurotoxic effects of cyclooxygenase-2, since PGE_2 partially reverses the protection to oxygen–glucose deprivation that is normally afforded by cyclooxygenase-2 inhibition. Similar findings were reported by Carlson (2003) who found that PGE_2 and the prostanoid EP_1/EP_3 receptor agonist, 17-pt-PGE_2, reverse protection against NMDA exposure in mouse cortical cultures conferred by cyclooxygenase-2 inhibition (Carlson, 2003). Similarly, 17-pt-PGE_2 reverses the protective effect of cyclooxygenase-2 inhibition against NMDA-induced neocortical lesions in mice (Manabe et al., 2004). Taken together, these findings suggest that PGE_2 is involved in the NMDA-mediated component of oxygen–glucose deprivation toxicity via the activation of the prostanoid EP_1 and/or EP_3 receptor. This is supported by our findings that PGE_2 reversed the attenuation in $[Ca^{2+}]_i$ during oxygen–glucose deprivation that was offered by cyclooxygenase-2 inhibition. Indeed, PGE_2 elicited increases in $[Ca^{2+}]_i$ under both control conditions and during oxygen–glucose deprivation, though the effect was most pronounced during oxygen–glucose deprivation. This may be because, under normoxic/normoglycemic conditions, cells are better suited to counteract the elevation of $[Ca^{2+}]_i$ using energy-dependent processes that are compromised during oxygen–glucose deprivation (Arundine and Tymianski, 2003). The prostanoid EP_1 receptor is likely responsible for the increase in $[Ca^{2+}]_i$, since the prostanoid EP_1 receptor antagonist, SC-19220, reduced oxygen–glucose deprivation-induced elevations of $[Ca^{2+}]_i$ and offered significant neuroprotection against oxygen–glucose deprivation.

It should be noted that PGE_2 only partially reversed the protection of SC-58125 against oxygen–glucose deprivation, suggesting additional mechanisms are responsible for cyclooxygenase-2-mediated death. For instance, other prostaglandins or reactive oxygen species, a by-product of prostaglandin synthesis, could contribute to cyclooxygenase-2 mediated neurotoxicity (Pepicelli et al., 2002). Moreover, there is evidence that cyclooxygenase-2 mediates excitotoxicity via the aberrant expression of cell cycle-related proteins (Mirjany et al., 2002).

Given that PGE_2 reverses the protection offered by SC-58125, one would expect PGE_2 to aggravate oxygen–glucose deprivation-toxicity when applied in the absence of a cyclooxygenase-2 inhibitor. However, even at a concentration (10 μM) able to reverse the protective effect of SC-58125, PGE_2 had no significant effect on oxygen–glucose deprivation-toxicity when applied on its own. Several possibilities may explain this observation. First, in the absence of SC-58125, cyclooxygenase-2 activity may result in the synthesis of prostanoids with antagonistic effects to PGE_2. Second, the endogenous synthesis of PGE_2 may be sufficient to saturate receptors such that the exogenous application of PGE_2 would have no additional effect. Finally, in the absence of SC-58125, PGE_2 may have little effect on oxygen–glucose deprivation-induced toxicity if the duration of oxygen–glucose deprivation is such that both PGE_2-treated and non-treated cultures reached the critical point of calcium overload. Yet, this last option is unlikely since PGE_2 did not

aggravate death induced by oxygen–glucose deprivation of shorter durations.

Previous studies using cortical neuronal cultures report that low doses (0.001–1 μM) of PGE_2 are neuroprotective against NMDA and glutamate exposure when applied either during or both during and after the insult (Cazevieille et al., 1994; Akaike et al., 1994). In contrast, neither 0.1 nor 1 μM PGE_2 offered protection against oxygen–glucose deprivation in the present study. This discrepancy may be due to differences in PGE_2 receptor expression and in the type of cultures used (i.e. mixed cultures versus neuron-enriched cultures). For example, McCullough et al. (2004) observed that PGE_2 (0.01–1 μM) is protective against excitotoxic stimuli in dissociated hippocampal neuronal cultures but not in hippocampal organotypic slices which preserve astrocytic/neuronal interactions. The combined activation of neuronal and astrocytic EP prostanoid receptors is believed to result in a net neutral effect on survival (McCullough et al., 2004). In contrast to studies showing that PGE_2 offers protection to excitotoxic insults, Takadera at al. (2002) report that PGE_2 (5–25 μM) induces apoptosis when present in cortical neuronal cultures for 48 h. In the present study, cultures exposed to 10 μM PGE_2 for 24 h demonstrated no signs of neuronal death.

Since cyclooxygenase-2 mediates myocardial ischemic preconditioning (Guo et al., 2000; Shinmura et al., 2000), the present study sought to determine whether this enzyme is also involved in tolerance induction in cortical neurons. In gerbils, cerebral ischemic preconditioning reduces cyclooxygenase-2 mRNA expression following an otherwise lethal ischemic event (Colangelo et al., 2004). While the study correlates a decrease in cyclooxygenase-2 expression with a better outcome post-ischemia, it does not address whether preconditioning-induced cyclooxygenase-2 activity is required for tolerance induction. Our finding that inhibition of cyclooxygenase-2 during oxygen–glucose deprivation–preconditioning attenuates the protection against prolonged oxygen–glucose deprivation that is normally afforded by preconditioning, indicates that cyclooxygenase-2 is required for tolerance induction. This is consistent with the facts that cyclooxygenase-2 is markedly induced by NMDA receptor activation in cortical neuronal cultures (Hewett et al., 2000; Carlson, 2003) and blocking NMDA receptor activation during oxygen–glucose deprivation–preconditioning abolishes tolerance (Grabb and Choi, 1999).

It is generally believed that signalling events that have the potential to be lethal make effective preconditioning inducers if they are titrated to just below the threshold of neuronal injury and, essentially, bring neurons to the "brink of death". For example, optimal protection against oxygen–glucose deprivation is achieved by preconditioning neurons with the maximum non-lethal exposure to oxygen–glucose deprivation or NMDA that can be tolerated (Tauskela et al., 2003; Raval et al., 2003). Therefore, it could be argued that the presence of a cyclooxygenase-2 inhibitor during oxygen–glucose deprivation–preconditioning would render the preconditioning event less stressful and thus result in a lesser degree of tolerance. However, the transient application of PGE_2 24 h prior to oxygen–glucose deprivation was sufficient to induce tolerance thereby underlining the importance of cyclooxygenase-2 and its product in preconditioning-induced tolerance. The protection afforded by PGE_2-preconditioning is not likely due to remaining PGE_2 having direct protective effects during oxygen–glucose deprivation since PGE_2 was not protective when applied during oxygen–glucose deprivation. Interestingly, unlike oxygen–glucose deprivation- or NMDA-preconditioning, PGE_2-preconditioning offered robust protection even though this preconditioning model does not appear to have the potential to be lethal. A concentration of PGE_2 one order of magnitude higher than the concentration used to induce tolerance was not lethal. This represents an unexpected departure from the "brink of death" requirement of other preconditioning stimuli and warrants further investigation. By falling so far from the threshold of damage, PGE_2-preconditioning may be more clinically relevant than traditional preconditioning stimuli.

This study shows that cyclooxygenase-2 and PGE_2 are involved in both oxygen–glucose deprivation-induced neurotoxicity and preconditioning-induced neuroprotection. While neurotoxic in the context of oxygen–glucose deprivation, the same abbreviated signalling events confer tolerance to oxygen–glucose deprivation. The outcome depends on the cellular context in which these signaling events are activated. Future studies should examine whether PGE_2 confers tolerance in vivo as it does in vitro. If this proves to be the case, the chronic inhibition of cyclooxygenase-2 could abolish the potential protective effect of endogenous PGE_2, thus rendering neurons more vulnerable to ischemia. Though the mechanisms by which PGE_2 induces ischemic tolerance remain to be studied, agonists of this prostanoid may prove to be beneficial to patients undergoing surgical procedures that put them at a greater risk for a cerebral infarct, as well as people more likely to suffer a stroke based on known risk factors such as hypertension, obesity and genetic predisposition. On the other hand, the acute inhibition of cyclooxygenase-2 during and/or following ischemia, when levels of this enzyme and PGE_2 are pathologically elevated, may prove to be an effective therapeutic strategy.

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