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Prostaglandin D₂ toxicity in primary neurons is mediated through its bioactive cyclopentenone metabolites

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Abstract

Prostaglandin D₂ (PGD₂) is the most abundant prostaglandin in brain but its effect on neuronal cell death is complex and not completely understood. PGD₂ may modulate neuronal cell death via activation of DP receptors or its metabolism to the cyclopentenone prostaglandins (CyPGs) PGJ₂, Δ¹²-PGJ₂ and 15-deoxy-Δ^{12,14}-PGJ₂, inducing cell death independently of prostaglandin receptors. This study aims to elucidate the effect of PGD₂ on neuronal cell death and its underlying mechanisms. PGD₂ dose-dependently induced cell death in rat primary neuron-enriched cultures in concentrations of 10 μM, and this effect was not reversed by treatment with either DP1 or DP2 receptor antagonists. Antioxidants *N*-acetylcysteine (NAC) and glutathione which contain sulfhydryl groups that can bind to CyPGs, but not ascorbate or tocopherol, attenuated PGD₂-induced cell death. Conversion of PGD₂ to CyPGs was detected in neuronal culture medium; treatment with these CyPG metabolites alone exhibited effects similar to those of PGD₂, including apoptotic neuronal cell death and accumulation of ubiquitinated proteins. Disruption of lipocalin-type prostaglandin D synthase (L-PGDS) protected neurons against hypoxia. These results support the hypothesis that PGD₂ elicits its cytotoxic effects through its bioactive CyPG metabolites rather than DP receptor activation in primary neuronal culture.

Keywords

Cell death; Cyclopentenone prostaglandins; DP receptors; Primary neuron; Prostaglandin D₂; Ubiquitinated protein

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Conflict of interest

The authors declare no conflict of interest. The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

1. Introduction

PGD₂ is the most abundant prostaglandin in brain and has been shown to be involved in the regulation of body temperature, the sleep–wake cycle, blood flow, neurotransmission and pain responses (Abdel-Halim et al., 1980; Chiu and Richardson, 1985). PGD₂ has been shown to have both protective and toxic effects in various models of CNS and neuronal injury, thus its role in modulating neuronal injury in disease states remains controversial (Li et al., 2008; Liang et al., 2005; Taniguchi et al., 2007; Wu et al., 2007; Xiang et al., 2007). PGD₂ may protect neurons from glutamate toxicity or ischemia–reperfusion injury through the activation of DP1 receptors in neuronal cells (Liang et al., 2005). PGD₂ may also produce vasodilatation by its effect on endothelial cells (Taniguchi et al., 2007). PGD₂ is also synthesized by activated microglia and astrocytes and modulates the inflammatory response in these cells (Kanda et al., 2013; Mohri et al., 2006). The mRNA for prostaglandin D synthase (PGDS) is found in leptomeninges, choroid plexus, and oligodendrocytes (Urade and Hayaishi, 2000; Urade et al., 1993). PGD₂ may also produce toxicity through its cyclopentenone prostaglandin (CyPG) metabolites via disruption of the ubiquitin proteasome pathway, resulting in accumulation of ubiquitinated proteins (Ub-proteins) and protein aggregates within neurons (Li et al., 2003, 2004; Liu et al., 2011; Wang et al., 2006; Wang and Figueiredo-Pereira, 2005). Chronic administration of a CyPG into the striatum produces pathological changes in neurons that closely approximate those found in Parkinson's disease (Pierre et al., 2009). Additionally, PGD₂, and its bioactive metabolite 15-deoxy $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂), have also been reported to induce apoptosis in various cell lines (Chambers et al., 2007; Chen et al., 2005; Kondo et al., 2002; Xiang et al., 2007; Zhu et al., 2010).

PGD₂ may produce its effects through both receptor-dependent and -independent mechanisms. PGD₂ binds to two cell membrane G protein-coupled receptors known as DP1 and DP2/CRTH2 (chemo-attractant homologous receptor expressed on TH2 cells) (Matsuoka and Narumiya, 2007). The DP1 and DP2 receptors have divergent effects on cAMP production, and are coupled to Gs and Gi proteins, respectively (Crider et al., 1999). Activation of the DP1 receptor decreases intracellular calcium and thus may have a neuroprotective effect, whereas DP2 receptor activation increases intracellular calcium, and may therefore exacerbate injury and cell death after ischemic injuries (Liang et al., 2005; Taniguchi et al., 2007). The effect of PGD₂ is dependent upon expression of DP1 and DP2 in various cell types and pathological conditions, thus PGD₂ and its receptors may have divergent effects upon cell death depending on the toxic stimulus and the cellular systems involved (Wu et al., 2007).

PGD₂ exerts its effects on a variety of cell types in brain. The present study aims to determine the direct effect of PGD₂ upon cell death in neurons using primary neuron enriched cultures. High levels of COX-2 expression are induced selectively within neurons in pathological conditions including ischemia, epilepsy and AD which may result in high levels of PGD₂ production within the neuron (Nakayama et al., 1998). These diseases are associated with selective neuronal death (Auer and Siesjo, 1988). The current studies were designed to ascertain whether PGD₂'s effects upon neurons are receptor-mediated or are mediated through the actions of its bioactive CyPG metabolites. Whether PGD₂'s toxic effect upon neurons can be abolished by treatment with DP receptor antagonists was tested. The conversion of PGD₂ to the J₂ series CyPGs in neuronal culture medium was quantified, and whether these CyPG metabolites could produce similar toxicity in primary neurons in the absence of PGD₂ was tested. Furthermore, whether PGD₂ toxicity is characterized by the same effects upon the ubiquitin proteasome pathway (UPP) as CyPGs was determined. The effect of blocking PGD₂ synthesis after hypoxia in neurons by lipocalin-type PGDS gene dysruption upon cell death and UPP function was evaluated.

Cell death was assayed after PGD₂ or CyPG incubation with rat primary neurons or primary neurons from lipocalin-type PGD synthase (L-PGDS) knock-out mice. PGD₂-induced neuronal toxicity was also evaluated in primary neurons after treatment with selective DP1 or DP2 receptor antagonists and compared to that of treatment with the sulfhydryl-containing antioxidants *N*-acetylcysteine (NAC) and glutathione (GSH), which can adduct and inactivate CyPGs, as well as with ascorbate and tocopherol, antioxidants lacking sulfhydryl groups that do not adduct CyPGs.

2. Materials and methods

Animal studies were performed with the approval of the University of Pittsburgh Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed in a temperature and humidity controlled environment with 12 h light cycles and free access to food and water.

2.1. Reagents and antibodies

Tocopherol, NAC, GSH, ascorbate, PGD₂, PGJ₂, 15d-PGJ₂, BW 245C (DP1 receptor agonist), 15(R)-15-methyl PGD₂ (DP2 receptor agonist), DP1 and DP2 receptor antagonists BW A868C and BAY-u3405 were purchased from Cayman Chemical (Ann Arbor, MI). Anti-PARP and anti-caspase-3 antibodies were from Cell Signaling (Danvers, MA). Anti-poly ubiquitinated protein antibody (FK2) was obtained from Enzo Life Sciences (Farmingdale, NY) and Streptavidin-HRP was from Pierce Biotechnology (Rockford, IL). Anti-GAPDH antibody was from Ambion (Austin, TX), and anti- β -actin antibody and all other chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

2.2. Rat primary neuronal culture

Cortical primary neuronal cultures were prepared from E17 fetal rats (Sprague-Dawley, Charles River, Wilmington, MA) as previously described (Li et al., 2008) and used for experiments after 9 DIV. Cells were grown in serum-free Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 and GlutaMAX (Invitrogen).

2.3. Cell treatment

Rat primary neurons were treated with 15d-PGJ₂, PGD₂, PGJ₂ or vehicle (methyl acetate) at 9 DIV. For dose response curves, cells were incubated with 0.6–50 μ M 15d-PGJ₂ or PGJ₂, 0.03–50 μ M PGD₂ or vehicle (methyl acetate) for 48 h. To determine the effect of PGD₂ receptor antagonists on cell death, cells were incubated with the DP1 receptor antagonist BW A868C (0.1 and 1 μ M) or the DP2 receptor antagonist BAY-u3405 (1 and 10 μ M) for 2 h then treated with 20 μ M PGD₂ or 20 μ M 15d-PGJ₂. Cell death was measured 48 h later. In antioxidant studies, cells were incubated with 1 mM NAC; 1 mM ascorbate; 6 mM GSH; or 2 mM tocopherol for 2 h, followed by treatment with 50 μ M PGD₂ or 30 μ M 15d-PGJ₂. Cell death was measured 48 h after PGD₂ or 15d-PGJ₂ treatment. To determine the effect of PGD₂ receptor agonists on poly-ubiquitinated protein levels in L-PGDS knock-out mouse primary neurons after hypoxia and normoxia, cells were incubated with the DP1 receptor antagonist BW 245C (200 nM) or the DP2 receptor agonist 15(R) 15-methyl PGD₂ (200 nM) for 2 h prior to hypoxia. Cells were harvested 48 h later ($n = 4$ per group) and immunoblotted using anti-poly Ub antibody. Protein densitometric measurements were normalized to their respective vehicle controls.

2.4. Cell death measurements

Cell death was assessed by measuring lactate dehydrogenase (LDH) release into medium (LDH assay, Pointe Scientific, Canton, MI) and indirectly using the MTT-based In Vitro Toxicology Assay Kit (Sigma, St. Louis, MO). For hypoxia studies, cells were treated with 1 μ M dizocilpine (MK 801, as 100% live control), or 20 μ M staurosporin (SP, as 100% dead control). Cell death was also measured by manually counting the percentage of neurons that stained with propidium iodide (PI) 48 h after hypoxia. Cells were stained with propidium iodide (PI, 0.1 μ g/ml) and Hoechst (100 ng/ml, Sigma) and counted from 12 fields with data expressed as percentage PI-stained cells normalized to the vehicle-treated group.

2.5. Western blot

Western blots were performed as previously described (Liu et al., 2011). For Ub-protein detection, cell lysates were resolved on a 4–20% linear gradient polyacrylamide gel (BioRad, Hercules, CA) and immunoblotted with anti-poly-ubiquitinated conjugates antibody (1:1000). For PARP and caspase-3 detection, cell lysates were resolved on 10% or 12% SDS–PAGE and immunoblotted with anti-PARP (1:1000) and anti-caspase-3 (1:1000) antibodies at 4 °C overnight. Blots were washed and the appropriate secondary antibodies applied. Protein signal was visualized with ECL reagents (Pierce). Blots were then stripped and re-probed using anti- β -actin antibody (1:5000) or anti-GAPDH (1:8000) as a loading control.

2.6. L-PGDS knock-out mouse breeding and genotyping

Lipocalin-type prostaglandin D₂ synthase (L-PGDS) knock-out mice were obtained from Dr. Myungsoo Joo at Vanderbilt University School of Medicine and bred 5 generations to obtain heterozygous L-PGDS (+/–) and homozygous L-PGDS (–/–) null mice. PCR was performed using DNA extracted from tail snips using QuickExtract DNA Extraction Solution (Epicentre Biotechnologies, Madison, WI) with the following cycling parameters: 94 °C, for 45 s; 54 °C, for 30 s; 72 °C for 90 s, 30 cycles. Primers were as follows: L-PGDS null primers – (5'-ATCGCCTTCTATCGCCTTCTTGACGAGT-3') and (5'-TCTTGAGAGTGCACAGAGCAAAGGAGTC-3'); wild-type primers – (5'-CGGGAGAAGAAAGCTGTATTGTAT-3') and (5'-GCTGTAGGTGTAGTGTCCAGGAG-3'). Reaction products were separated on a 1.8% agarose gel containing ethidium bromide and photographed under UV light.

2.7. Cortical primary neuron-enriched culture from L-PGDS knock-out mouse

Primary neuronal culture was prepared as previously described (Li et al., 2008). Briefly, L-PGDS heterozygous and null mouse fetal cortical brain tissue was dissected out, freed of meninges and trypsinized for 30 min. in 0.25% trypsin in Neurobasal Medium (Life Technologies). Cells from like genotypes were pooled before plating, ensuring a more even distribution of cells between plates. Cells were used for studies after 9 DIV.

2.8. Hypoxia/normoxia

Hypoxia was performed in primary neurons from L-PGDS knock-out mice in deoxygenated medium using a hypoxic glove box (Coy Laboratories, Grass Lake, MI) flushed with 92% argon, 5% CO₂ and 3% H₂ for 2–3 h as described previously resulting in ~40–50% cell death after 24 h reperfusion. Staurosporin (20 μ M, a 100% cell death internal standard) and 1 μ M MK801 (a 100% cell survival internal standard) treatments were included in each cell death assay experiment.

2.9. Proteasome activity

20S proteasome activity was measured using a Chemicon Proteasome Activity Assay kit following manufacturer's instructions (Millipore, Billerica, MA) (Liu et al., 2011). Briefly, primary neurons from L-PGDS knock-out mice underwent normoxia or hypoxia treatment. Cells were harvested 24 h later in cell lysis buffer containing: 50 mM Hepes (pH 7.4), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 and 2 mM ATP. Protein concentration was measured using the bicinchoninic acid method (Pierce). Cell lysate (50 μ g) was incubated with labeled 20S proteasome substrate LLVY-AMC for 2 h at 37 °C. Free AMC fluorophore was read using a BioTek Flx800 multi-detection micro-plate reader (Winooski, Vermont) with λ_{ex} 360 nm and λ_{em} 460 nm. Relative Fluorescence Units (RFU) for each sample were recorded as the index of proteasome activity.

2.10. Detection of PGD₂ conversion to its metabolites in primary neuron culture medium

Primary neurons were prepared as described above and treated with freshly prepared 50 μ M PGD₂ made from a stock solution (10 ng/ml, in methyl acetate). Samples of PGD₂ stock, vehicle (methyl acetate) and medium from cell culture plates 30 min after PGD₂ treatment, and at 24 h were assayed for PGD₂, 15d-PGD₂ and J₂ series CyPG concentrations by ultraperformance liquid chromatography (UPLC)–MS/MS, as previously described with modifications (Liu et al., 2013; Miller et al., 2009). Samples were loaded onto Oasis HLB solid phase extraction cartridges (Waters, Oasis, Milford, MA) and washed with 1 ml volumes of 5% methanol before elution with 100% methanol. Extracts were dried under nitrogen and reconstituted in 125 μ L of 80:20 methanol: de-ionized water. Analytes were separated via reverse phase UPLC with an Acquity UPLC BEH C18 1.7 μ M, 2.1 mm $\times$ 100 mm column. Mass spectrometric (MS) analysis was performed via a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (ThermoFisher Scientific). Quantization by selected reaction monitoring (SRM) analysis on PGD₂, Δ^{12} -PGJ₂, 15d-PGJ₂, and 15d-PGD₂ was performed by monitoring their m/z transitions, 351 $\rightarrow$ 271, 333 $\rightarrow$ 271, 315 $\rightarrow$ 271, and 333 $\rightarrow$ 271, respectively. Parameters were optimized to obtain the highest [M–H][–] ion abundance for each analyte. Data were acquired using Xcalibur Software version 2.0.6. Because Δ^{12} -PGJ₂ and PGJ₂ are not independently resolved using this method of UPLC MS/MS detection, the data presented here are shown as the sum of both PGJ₂ and Δ^{12} -PGJ₂ (PGJ₂/ Δ^{12} -PGJ₂).

2.11. Cell treatment with CyPG mixture

The CyPG mixture (MIX) contains 16.8 μ M PGJ₂, 3.2 μ M Δ^{12} -PGJ₂ and 1.6 μ M 15d-PGJ₂. Cells were treated with 50 μ M PGD₂, CyPG MIX or vehicle (methyl acetate) for 48 h. Cell death was measured (n = 12 per group) and immunoblotting (n = 3 per group) was performed as described above. Results are normalized to vehicle-treated groups.

2.12. Statistical analysis

Data are expressed as means $\pm$ SE and were analyzed using one-way ANOVA with Fisher LSD post hoc testing to calculate differences between groups. L-PGDS proteasome activity was analyzed using ANOVA with Bonferroni post hoc testing. Results were considered to be significant when P < 0.05.

3. Results

3.1. PGD₂ and its bioactive metabolites PGJ₂ and 15d-PGJ₂ induce primary neuronal cell death

To test the effect of PGD₂ treatment upon neuronal cell death, rat primary neuron-enriched cultures were treated with 0.03–50 μ M PGD₂ for 48 h and cell death was measured using the

LDH assay. As shown in Fig. 1A, PGD₂ induced primary neuronal cell death in a dose-dependent manner with 10 μM PGD₂ significantly increasing cell death.

PGD₂ readily undergoes nonenzymatic dehydration to yield several bioactive metabolites, the J₂ series CyPGs, which include PGJ₂, Δ¹²-PGJ₂ and 15d-PGJ₂ (Shibata et al., 2002). To determine the effects of these J₂ series CyPGs on neuronal cell death, primary neurons were incubated with PGJ₂ or 15d-PGJ₂ at 0.6–50 μM for 48 h. Similar to PGD₂, both PGJ₂ and 15d-PGJ₂ treatment resulted in dose-dependent neurotoxic effects. In comparison to PGD₂, which induces cytotoxic effects at 10 μM, PGJ₂ and 15d-PGJ₂-induced cell toxicity occurs at lower doses: 2.5 μM and 5 μM respectively (Fig. 1A).

As a secondary measure of cell death, PI and Hoechst staining was performed in primary neurons treated with vehicle, 50 μM PGD₂ or 30 μM 15d-PGJ₂ for 48 h. Consistent with the above cell death assay results, there was a significant increase in the percentage of PI-stained cells in PGD₂ and 15d-PGJ₂-treated cells as compared to vehicle controls (Fig. 1B), further providing evidence that PGD₂ and its bioactive metabolite 15d-PGJ₂ impart cytotoxic effects on neuronal cells.

To examine the role of apoptosis in PGD₂ and 15d-PGJ₂-treated primary neuronal cell death, primary neurons were incubated with 10 or 50 μM PGD₂, 10 or 25 μM 15d-PGJ₂, or vehicle for 24 h. Caspase-pathway activation was examined by detecting caspase-3 and PARP cleavage using immunoblots probed with anti-caspase-3 and anti-PARP antibodies. As shown in Fig. 1C, both PGD₂ and 15d-PGJ₂ treatment decreased the protein level of full length caspase-3, suggesting increased cleavage of caspase-3. Consistent with this result, cleaved PARP was detected in neurons treated with 50 μM PGD₂ or 25 μM 15d-PGJ₂ indicating that induction of apoptosis contributes to the cytotoxic effects of PGD₂ and 15d-PGJ₂ in primary neurons. Because prostaglandins have also been reported to induce toxicity via disruption of the ubiquitin proteasome system (UPS), resulting in intracellular accumulation of ubiquitinated proteins (Ub-proteins), poly-ubiquitinated-protein levels in cells treated with PGD₂, 15d-PGJ₂ or vehicle were then detected. Compared with vehicle-treated neurons, 24 h incubation with PGD₂ or 15d-PGJ₂ significantly induced Ub-protein accumulation, especially those of high molecular weight (Fig. 1D).

3.2. PGD₂ induces neuronal cell death and ubiquitinated protein accumulation through a DP receptor-independent mechanism in primary neurons

PGD₂ has been reported to elicit some of its downstream effects through the binding and activation of two G-protein coupled receptors: the DP1 and DP2 receptors. To investigate the role of DP receptors, the DP1 receptor antagonist BW A868C (0.1 and 1 μM) (Hatanaka et al., 2010) and the DP2 receptor antagonist BAY-u3405 (1 and 10 μM) (Zhu et al., 2010) were incubated with primary neurons in combination with 10 and 20 μM PGD₂. Neuronal cell death was measured 48 h after treatment. Inhibition of DP2 receptor activation with BAY-u3405 failed to attenuate PGD₂-induced neuronal cell death (Fig. 2A), while the DP1 receptor antagonist BW A868C exacerbated PGD₂-induced neurotoxicity as measured by the MTT assay, although this effect did not reach significance in the LDH assay (Fig. 2B). BAY-u3405 and BW A868C had no effect on cell death or viability alone. Both DP1 and DP2 receptor antagonists failed to attenuate PGD₂-induced neuronal cell death, suggesting that the neurotoxic effects of PGD₂ are mediated through a DP receptor-independent mechanism. In addition, DP1 and DP2 receptor antagonists failed to attenuate 15d-PGJ₂-induced neuronal cell death (Fig. S1).

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neuro.2013.08.001>.

To determine the effect of PGD₂ receptor antagonists on PGD₂ induced ubiquitinated protein accumulation and apoptosis, primary neurons were incubated with the DP1 receptor antagonist BW A868C (1 μ M) or the DP2 receptor antagonist BAY-u3405 (10 μ M) or vehicle (methyl acetate) combined with or without 20 μ M PGD₂ for 24 h. As shown in Fig. 2C, PGD₂ treatment resulted in the cleavage of PARP and caspase-3 as well as dramatic accumulation of ubiquitinated proteins in primary neurons; these effects were not altered by treatment with either DP1 or DP2 antagonists.

3.3. GSH and NAC, but not ascorbate or tocopherol, attenuate PGD₂ or 15d-PGJ₂-induced neuronal cell death

To determine whether the generation of reactive oxygen species (ROS) contributes to PGD₂ and 15d-PGJ₂-induced neuronal cell death, the antioxidants GSH, NAC, ascorbate or tocopherol were applied to primary neurons in combination with PGD₂ or 15d-PGJ₂. Cells were incubated with 50 μ M PGD₂ or 30 μ M 15d-PGJ₂ for 1 h prior to treatment with 1.0 mM NAC, 1.0 mM ascorbate, 6 mM GSH, 2 mM tocopherol or vehicle. Cell death and cell viability were measured 48 h later using LDH and MTT assays. As shown in Fig. 3, treatment with 1 mM NAC or 6 mM GSH significantly decreased cell death induced by PGD₂ and 15d-PGJ₂, whereas treatment with 1 mM ascorbate or 2 mM tocopherol failed to improve cell survival.

Ascorbate and tocopherol are electron donors that quench free radical production (Traber and Stevens, 2011). GSH is the most prevalent intracellular antioxidant, reacting directly with free radicals non-enzymatically and is the electron donor in the reduction of peroxides. In addition, GSH maintains the thiol redox potential in cells by preserving the reduced form of sulfhydryl groups in proteins (Dringen et al., 2000). NAC is a precursor molecule for GSH synthesis and a reducing agent for oxidized GSH. In addition to their ability to clear ROS, GSH and NAC are also cysteine-containing peptides capable of reducing disulfide bonds (Xiang et al., 2007). GSH covalently binds CyPGs, inactivating them (Brunoldi et al., 2007). The thiol-reducing agents GSH and NAC, protected primary neuronal cells against PGD₂ and 15d-PGJ₂-induced cell death, whereas antioxidants lacking thiol-reducing ability such as ascorbate or tocopherol, failed. These findings indicate that excessive ROS production may not be the critical mechanism responsible for PGD₂ or 15d-PGJ₂-induced primary neuronal cell death.

3.4. The bioactive metabolites of PGD₂, the J₂ series of CyPGs, alone mimic the cytotoxic effects of PGD₂ on primary neurons

PGD₂ is readily dehydrated to generate 15-deoxy $\Delta^{12,14}$ -PGD₂ (15d-PGD₂) and the bioactive J₂ series of CyPGs including 15d-PGJ₂, PGJ₂, and Δ^{12} -PGJ₂ in water (Shibata et al., 2002). To determine how PGD₂ is converted into CyPGs in culture media, we measured the concentrations of PGD₂ and its J₂ series CyPG metabolites in culture medium after addition of PGD₂ to the medium. We then tested whether the mixture of CyPGs produced by degradation of PGD₂ produced cell death in the absence of PGD₂.

A mass spectrometric (MS) method was developed to detect and quantify the metabolism of PGD₂ to its metabolites in neuronal culture medium. Freshly prepared neuronal culture medium containing 50 μ M PGD₂ was applied to cell culture dishes containing primary neurons to observe PGD₂ metabolism under normal culture conditions, and to empty culture dishes to observe auto-metabolism without neuronal influence. Dishes were incubated for 24 h at 37 °C, and 1 ml of medium was collected at the indicated time points for analysis. Concentrations of free PGD₂, 15d-PGD₂, 15d-PGJ₂, PGJ₂ and Δ^{12} -PGJ₂ in the medium were measured using UPLC-MS/MS. As shown in Table 1, the concentration of PGD₂ in culture medium in the presence of primary neurons decreased from 50 μ M to 47.71 ± 1.63

μM after 30 min incubation, and to $7.32 \pm 0.1 \mu\text{M}$ after 24 h. The concentration of free $\text{PGJ}_2/\Delta^{12}\text{-PGJ}_2$ increased from $1.69 \pm 0.24 \mu\text{M}$ at 30 min to $11.71 \pm 1.15 \mu\text{M}$ at 24 h. Because PGJ_2 and $\Delta^{12}\text{-PGJ}_2$ have the same m/z value, the two compounds were not separated in these experiments and are reported here as the sum of the two. The concentration of free 15d-PGJ_2 increased from $0.04 \pm 0.01 \mu\text{M}$ at 30 min to $1.53 \pm 0.04 \mu\text{M}$ at 24 h. Besides these J_2 series CyPGs, PGD_2 also generates other products including 15d-PGD_2 , whose concentration reached $7.89 \pm 0.19 \mu\text{M}$ at 24 h. Similar patterns of PGD_2 metabolism were observed in medium incubated in the absence of primary neurons: $[\text{PGD}_2]$ decreased to $7.12 \pm 0.14 \mu\text{M}$ after 24 h, whereas $[15\text{d-PGD}_2]$, $[15\text{d-PGJ}_2]$ and $[\text{PGJ}_2/\Delta^{12}\text{-PGJ}_2]$ increased to $8.43 \pm 0.53 \mu\text{M}$, $1.61 \pm 0.03 \mu\text{M}$ and $19.62 \mu\text{M}$, respectively. PGD_2 and its metabolites were below detection limits in vehicle-treated control medium.

After confirming the conversion of PGD_2 to its bioactive CyPG metabolites in culture medium, we examined whether treatment with a mixture of these CyPGs without PGD_2 would elicit deleterious effects similar to those of PGD_2 on neuronal cells. For this purpose, primary neurons were treated with either vehicle, $50 \mu\text{M}$ PGD_2 , or a CyPG mixture (CyPG MIX) containing PGJ_2 , $\Delta^{12}\text{-PGJ}_2$ and 15d-PGJ_2 at concentrations corresponding to those found in cell-free culture medium 24 h after $50 \mu\text{M}$ PGD_2 incubation. Previously performed experiments separating PGJ_2 and $\Delta^{12}\text{-PGJ}_2$ yielded a ratio of $83.6 \pm 2.5\%$ PGJ_2 to $16.4 \pm 4.3\%$ $\Delta^{12}\text{-PGJ}_2$, in post-ischemic brain (Liu et al., 2013); therefore, the CyPG mixture applied to neurons was comprised of $16.8 \mu\text{M}$ PGJ_2 , $3.2 \mu\text{M}$ $\Delta^{12}\text{-PGJ}_2$ and $1.6 \mu\text{M}$ 15d-PGJ_2 . Cell death and cell viability assays were performed 48 h later. Both CyPG MIX and $50 \mu\text{M}$ PGD_2 treatments significantly induced neuronal cell death and decreased cell viability to a similar extent compared with vehicle-treated control (Fig. 4A). Furthermore, 24 h incubation with CyPG MIX induced cleavage of Caspase-3 and PARP as well as augmented high molecular weight Ub-protein accumulation in primary neurons similar to that found after treatment with $50 \mu\text{M}$ PGD_2 (Fig. 4B). Thus, these results show that the CyPG metabolites of PGD_2 produce cell death and biochemical changes characteristic of PGD_2 -induced cell death in the absence of PGD_2 . These results support the hypothesis that PGD_2 's toxic effects in primary neurons are mediated through its CyPG metabolites.

L-PGDS is an enzyme responsible for PGD_2 biosynthesis in the brain. To further examine the role of PGD_2 generation in neuronal cell death and Ub-protein accumulation, primary cortical neurons from L-PGDS homozygous null ($-/-$) and heterozygous ($+/-$) mice were subjected to hypoxia and the effects of PGD_2 synthesis inhibition on hypoxia-induced cell death and Ub-protein accumulation were observed. As shown in Fig. S2, primary neurons from L-PGDS null mice were more resistant to hypoxia-induced cell death than neurons from heterozygous mice. In addition, Ub-protein levels were significantly lower in L-PGDS null cells 24 h after hypoxia as compared to heterozygous cells. This difference in Ub-protein accumulation was accompanied by an observed increase in proteasome activity in L-PGDS null neurons compared with L-PGDS heterozygous neurons post-hypoxia while the null and heterozygous cells exhibited similar proteasome activities under normoxic conditions. Furthermore, incubation with neither the DP1 receptor agonist BW 245C (BW) nor the DP2 receptor agonist 15(R) 15-methyl PGD_2 (15-M) had a significant effect on Ub-protein accumulation in primary neurons from L-PGDS null mice (Fig. S2D), further supporting that Ub-protein accumulation is DP1 and DP2 receptor-independent.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neuro.2013.08.001>.

4. Discussion

The major findings of this study are: (1) Treatment of primary neuron-enriched cultures with PGD₂ induces cell death, and blocking synthesis of PGD₂ by disruption of the L-PGDS gene protects neurons against hypoxic injury, consistent with a primarily toxic effect of PGD₂ in this model system. (2) The DP2 antagonist BAY-u3405 did not protect neurons from PGD₂-induced cell death or Ub-protein accumulation, suggesting that PGD₂'s toxic effects are not mediated by DP2 receptor activation. (3) PGD₂ was converted to CyPGs in culture medium, and these CyPGs alone produced neuronal cell death, accumulation of Ub-proteins and apoptosis in a nearly identical fashion to PGD₂. (4) Both PGD₂ and CyPG-induced cell death was blocked by treatment with NAC and GSH but not ascorbate or tocopherol.

PGD₂ and its metabolites may play an important role in mediating neural injury in a variety of disease states including ischemia, trauma and inflammatory disorders. There is an increase of free arachidonic acid due to increased activity of phospholipases (Katsura et al., 1993), and induction of expression of cyclooxygenase 2 in these disorders (Nakayama et al., 1998; Nogawa et al., 1997). These events result in the increased production of the intermediate prostaglandin metabolite PGH₂. PGD₂ is formed from PGH₂ by two distinct PGD synthases: the glutathione-dependent hematopoietic PGD synthase (H-PGDS) and the lipocalin-type PGD synthase (L-PGDS) (Lee et al., 2012; Urade and Hayaishi, 2000). The present results are consistent with previous studies that have shown that PGD₂ is directly toxic to neurons (Kondo et al., 2002; Li et al., 2008; Yagami et al., 2001). In these studies, PGD₂ treatment induced cell death in neuronal culture systems devoid of other cellular elements at concentrations in the 10 μ M range. CyPGs are more potent toxins than PGD₂ in primary neurons, with 15d-PGJ₂ producing toxicity at doses of 1–2.5 μ M in some studies (Liu et al., 2013; Yagami et al., 2003). The current study demonstrates that disruption of the L-PGDS gene protects neurons from hypoxia in vitro, and since PGD₂ is the major product of L-PGDS, these results are consistent with a toxic role for PGD₂ in neurons.

The results of the current and previously reported studies support the hypothesis that PGD₂ exerts its toxicity through mechanisms that are independent of the DP receptors. The present data and a previous study found that DP antagonists do not prevent cell death induced by PGD₂ (Syed et al., 2010; Wu et al., 2007; Zhu et al., 2010). The concentrations of PGD₂ needed to produce cell death in this study and others are approximately 1000 fold greater than concentrations needed to activate the DP2 receptor (Wright et al., 1999). PGD₂ toxicity induced cell death characterized by Ub-protein accumulation, as was found with 15d-PGJ₂ in this and previous studies (Li et al., 2004; Liu et al., 2011, 2013). DP1 and DP2 antagonists failed to attenuate PGD₂ induced Ub-protein accumulation or apoptosis (Fig. 2), while DP receptor agonists had no significant effect upon the accumulation of Ub-proteins in L-PGDS null cells (Fig. S2). Only small amounts of ³H-PGD₂ bind to the membrane fraction of brain homogenates, inconsistent with a high degree of PGD₂ binding to membrane bound DP1 and DP2 receptors (Yagami et al., 2003; Yamamoto et al., 2011).

DP1 receptor activation results in decreased intracellular Ca⁺⁺ and has been shown to have a protective effect against neuronal cell death due to a variety of insults (Ahmad et al., 2010; Liang et al., 2005; Saleem et al., 2007). Liang et al. found that PGD₂ protected hippocampal neuronal cultures and organotypic slice cultures from glutamate neurotoxicity (Liang et al., 2005). Saleem et al. found that DP1 receptor-specific agonists prevent cell death in neuronal culture due to excitotoxicity and that DP1 receptor null mice had larger infarctions after temporary middle cerebral artery occlusion, consistent with a protective role for DP1 receptor activation (Saleem et al., 2007). DP1 agonists reduced the extent of excitotoxic neuronal cell death produced by direct injection of *N*-methyl-D-aspartate into the cortex of young and aged mice (Ahmad et al., 2010). There is some evidence of a protective effect

mediated via activation of DP1 in this study. The DP1 receptor antagonist BW A868C significantly exacerbated PGD₂-induced cell death in one of two cell death assays. However, in this study, PGD₂ itself produces cell death rather than protects primary neurons; thus the potential protective effects of DP1 receptor activation by PGD₂ are overwhelmed by PGD₂'s direct toxic effects upon neurons in this in vitro model.

These and other results are consistent with the hypothesis that PGD₂ toxicity in this model is due to conversion of PGD₂ to its bioactive CyPG metabolites. Shibata et al. found that PGD₂ is non-enzymatically converted to the CyPGs PGJ₂ and 15d-PGJ₂ in water, and that this conversion is accelerated in the presence of albumin (Shibata et al., 2002). The current results indicate that PGD₂ added to culture medium is converted to PGJ₂/Δ¹²-PGJ₂ and 15d-PGJ₂. Neuronal cell death induced by PGD₂ is characterized by the accumulation of Ub-proteins (Li et al., 2003, 2004; Wang et al., 2006; Wang and Figueiredo-Pereira, 2005); this effect was mimicked by treatment with 15d-PGJ₂ or the CyPG mixture. CyPGs such as 15d-PGJ₂ readily react with sulfhydryl groups and thus bind to and are inactivated by GSH and NAC. GSH and NAC both ameliorated cell death induced by either 15d-PGJ₂ and PGD₂. The free radical scavengers, ascorbate and tocopherol, antioxidants that do not bind to CyPGs, had no effect. Taken together, these results suggest that PGD₂ toxicity is mediated by its CyPG metabolites.

PGD₂ and its CyPG metabolites are toxic at concentrations in the 10 μM range in in vitro studies, while PGD₂ and the highest concentrations of these CyPGs found in brain under pathological conditions are in the 100 nM range (Liu et al., 2011, 2013). However, this discrepancy does not exclude a role for PGD₂ or CyPGs in neuronal injury in vivo. The measured concentrations represent average brain concentration; local cellular and intracellular concentrations of PGD₂ and its CyPG metabolites may be much higher. COX-2 is expressed at much higher levels in circumscript populations of neurons such as hippocampus (Chen et al., 1995; Nakayama et al., 1998), potentially resulting in greatly elevated local PGD₂ concentrations near the site of production. The recent observation that PGF2α, a metabolite of arachidonic acid by COX-2, is immunocytochemically detected at dramatically higher levels in hippocampal neurons, supports this hypothesis (Takei et al., 2012). Furthermore, the biological effects of CyPGs may not be solely governed by their concentrations, but rather by cumulative covalent modification of protein targets (Higdon et al., 2012). Chronic exogenous administration of low concentrations CyPGs into striatum has been shown to produce changes in brain that closely resemble the neuropathological features of Parkinson's disease including accumulation of Ub-proteins within neurons (Pierre et al., 2009). Repeated administration of lower doses of 15d-PGJ₂ over 96 h produced neuronal cell death in vitro (Liu et al., 2013). These results and the results from the current study suggest that PGD₂ may have direct pathological effects upon neurons via its CyPG metabolites. It should be noted that the current study utilizes a model system that is devoid of vascular, glial and inflammatory cells that could potentially mediate the protective effects of PGD₂. Thus, additional studies are required to determine if PGD₂ and its CyPGs metabolites play an important role in brain pathology in disease states in in vivo models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Effects of PGD₂ and its CyPG metabolites on primary neuronal cell death and Ub-protein accumulation. (A) Rat primary neurons underwent treatment with 0.03–50 μM PGD₂, PGJ₂ or 15d-PGJ₂. Cell death was determined 48 h later by measuring LDH release into medium. Data are expressed as means ± SE and are normalized to Vehicle. $n = 6$ per group. $*P < 0.05$; $\&P < 0.001$. (B) Rat primary neurons were treated with PGD₂ (50 μM), 15d-PGJ₂ (30 μM) or Vehicle (Veh, methyl acetate) for 48 h then stained with propidium iodide (PI) and Hoechst. Cells were photographed (upper) and PI and Hoechst stained cells were counted. Cell death is expressed as %PI normalized to Vehicle (means ± SE). $n = 12$ per group. $\&P < 0.001$; NS: not significant. (C and D) Rat primary neurons were treated with vehicle (veh, methyl acetate), 10 or 50 μM PGD₂ (left panels), or 10 or 25 μM 15d-PGJ₂ (right panels). Cells were harvested 24 h later for Western blot analysis with β-actin as loading control. (C) Representative immunoblots of PARP and caspase-3 cleavage. $n = 3$ per group. Full: full length; clvd: cleaved. (D) (Middle) Immunoblots of ubiquitinated proteins as detected with anti-polyubiquitin antibody. Upper panel is quantitative results of densitometric

measurement of ubiquitinated protein band densities normalized to their respective vehicle-treated groups. ($n = 3-4$ per group). $*P < 0.05$ vs. Veh.

Fig. 2.

Effects of DP1 and DP2 receptor antagonists on PGD₂-induced cell death. (A and B) Rat primary neuronal cultures were treated with 10 or 20 μM PGD₂ and the DP1 receptor antagonist BW A868C (B) or the DP2 receptor antagonist BAY-u3405 (A), or vehicle (methyl acetate) for 48 h. Cell death was measured by the LDH assay and cell viability was measured by MTT assay. *n* = 6–12 wells per group. Data are means ± SE and are normalized to the vehicle-treated group. **P* < 0.05; ***P* < 0.01. (C) Representative immunoblots of poly-ubiquitinated proteins (Poly-Ub), PARP and caspase-3 cleavage in cell culture lysate 24 h after treatment with 1 μM BW A868C, 10 μM BAY-u3405 or vehicle (methyl acetate) with and without PGD₂ (20 μM). GAPDH served as a loading control, *n* = 4 per group.

Fig. 3.

Effect of antioxidants on PGD₂ and 15d-PGJ₂-induced neuronal cell death. Rat primary neurons were treated with vehicle (methyl acetate), PGD₂ (50 μM, *left panels*) or 15d-PGJ₂ (30 μM, *right panels*) and *N*-acetyl-L-cysteine (NAC), ascorbate (Asc), glutathione (GSH) or tocopherol (Toc) at the indicated concentrations for 48 h. Cell death was measured by the LDH assay and cell viability with the MTT assay. *n* = 6 per group. Data are means ± SE normalized to vehicle-treated groups. **P* < 0.05; &*P* < 0.01; ***P* < 0.001 vs. prostaglandin-only treated group.

Fig. 4.

Effects of PGD₂ metabolites on cell death. (A) Rat primary neurons were treated with PGD₂ (50 μ M) or a PGD₂ metabolite mixture (CyPG MIX: 16.8 μ M PGJ₂, 3.2 μ M Δ^{12} -PGJ₂, 1.6 μ M 15d-PGJ₂) for 48 h. Cell death was measured using the LDH assay and MTT cell viability assay. $n = 12$ per group. $\&P < 0.001$ vs Veh. (B) Representative immunoblots of cell lysates after 24 h treatment with Veh, PGD₂ or CyPG MIX for caspase-3 and PARP (full: full length; clvd: cleaved), and poly-ubiquitinated proteins. β -Actin was used as a loading control. right: graphical representation of densitometric measurement of poly-ubiquitinated proteins normalized to vehicle-treated group. $n = 3$ per group. $*P < 0.05$ vs Veh. (A and B) Data are expressed as means $\pm$ SE.

PGD₂ conversion to its metabolites.

Table 1

	Vehicle		Medium + PGD ₂ (with cells)		Medium + PGD ₂ (without cells)	
	0 h	24 h	30 min	24 h	24 h	24 h
PGD ₂	NF	NF	47.71 ± 1.63	7.32 ± 0.10	7.12 ± 0.14	
PGI ₂ /Δ ¹² -PGI ₂	NF	NF	1.69 ± 0.24	11.71 ± 1.15	19.62 ± 0.71	
15d-PGD ₂	NF	NF	0.84 ± 0.18	7.89 ± 0.19	8.43 ± 0.53	
15d-PGI ₂	NF	NF	0.04 ± 0.01	1.53 ± 0.04	1.61 ± 0.03	

Data are mean [CyPGs] ± SE (μM), n = 3 per group.

NF, not found.