15d-Prostaglandin J2 Protects Brain From Ischemia-Reperfusion Injury


Objective—Brain expresses abundant lipocalin-type prostaglandin (PG) D2 (PGD2) synthase but the role of PGD2 and its metabolite, 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) in brain protection is unclear. The aim of this study is to assess the effect of 15d-PGJ2 on neuroprotection.

Methods and Results—Adenoviral transfer of cyclooxygenase-1 (Adv-COX-1) was used to amplify the production of 15d-PGJ2 in ischemic cortex in a rat focal infarction model. Cortical 15d-PGJ2 in Adv-COX-1–treated rats was increased by 3-fold over control, which was correlated with reduced infarct volume and activated caspase 3, and increased peroxisome proliferator activated receptor-γ (PPARγ) and heme oxygenase-1 (HO-1). Intraventricular infusion of 15d-PGJ2 resulted in reduction of infarct volume, which was abrogated by a PPARγ inhibitor. Rosiglitazone infusion had a similar effect. 15d-PGJ2 and rosiglitazone at low concentrations suppressed H2O2-induced rat or human neuronal apoptosis and necrosis and induced PPARγ and HO-1 expression. The anti-apoptotic effect was abrogated by PPARγ inhibition.

Conclusion—15d-PGJ2 suppressed ischemic brain infarction and neuronal apoptosis and necrosis in a PPARγ dependent manner. 15d-PGJ2 may play a role in controlling acute brain damage induced by ischemia-reperfusion. (Arterioscler Thromb Vasc Biol. 2006;26:481-487.)

Key Words: COX-1 ■ 15d-PGJ2 ■ PPARγ ■ apoptosis ■ stroke

Postaglandin (PG) H synthase-1 (also known as cyclooxygenase-1 [COX-1]) is constitutively expressed in almost all mammalian cells.1 It is a bifunctional enzyme with a cyclooxygenase activity that converts arachidonic acid to PG G2 (PGG2) and a peroxidase activity that converts PGG2 to PGH2.2 PGH2 is converted to diverse prostanoids by specific enzymes. COX-1 plays an important role in maintaining physiological homeostasis and protecting brain tissues from ischemia-reperfusion (I/R) injury. COX-1 deleted mice are highly susceptible to ischemic brain infarction,3 whereas COX-1 overexpression protects brain from I/R damage, which is abrogated by a selective COX-1 inhibitor.4 COX-1 overexpression in ischemic brain augments the production of PGF2α, PGE2, and PGD2, and suppresses leukotriene B4 (LTB4) and LTC4. As LTB4 and LTC4 have been shown to be detrimental to brain tissue, whereas PG12 is protective,5-7 COX-1 overexpression tilts the eicosanoid balance toward tissue protection. PGD2 is elevated in COX-1 overexpressed brain tissues but its role in brain I/R injury is unclear. Brain is enriched in lipocalin-type PGD synthase (L-PGDS), which catalyzes the formation of abundant PGD2.8 The role of PGD2 in I/R brain injury is unclear. As 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2), a nonenzymatic product of PGD2, was shown to possess anti-inflammatory properties through activation of peroxisome proliferator activated receptor-γ (PPARγ),9-13 PGD2 has been implicated in tissue protection. However, it has recently been argued that the tissue 15d-PGJ2 level is too low to elicit an anti-inflammatory action in vivo, especially in vascular tissues.14 In view of abundant expression of L-PGDS and PGD2 in brain, we postulated that 15d-PGJ2 contributes to cerebral protection. Our experimental findings show a considerable amount of 15d-PGJ2 in ischemia brain, which was enhanced by adenoviral COX-1 gene transfer. 15d-PGJ2 and rosiglitazone reduced brain infarct volume, inhibited brain and neuronal apoptosis, suppressed NF-κB activation, and upregulated heme oxygenase-1 (HO-1) in a PPARγ-dependent manner.

Methods

Stroke Model

The rat focal cerebral infarction model has been described previously.15 In brief, male Long-Evans rats were anesthetized, right middle
cerebral artery (MCA) was ligated reversibly with a 10-0 suture, and both common carotid arteries were occluded with aneurysm clips. At the indicated time point, the aneurysm clips and the suture were removed and blood flow in all 3 arteries was restored. The animals were kept in an air-ventilated incubator at 24.0±0.5°C for 24 hours and then euthanized under anesthesia. Brains were quickly removed and the ischemic or contralateral cerebral cortex was isolated and frozen. Infarct volume was measured by incubating coronally dissected brain slices with 2.3,5-triphényltiazolium chloride as previously described. All procedures were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals and approved by the Academia Sinica Animal Studies Committee.

**Cell Culture and H$_2$O$_2$-Mediated Oxidative Stress**

Rat primary cortical neuron cultures were prepared from 14- to 15-day-old fetus according to procedures previously described. All the experiments were performed on cultured neurons after 12 to 14 days in vitro. More than 90% of cells stained positive for microtubal associated protein-2. Human BE(2)-C neuroblastoma cells (American Type Culture Collection) were grown to 70% confluence in a humidified 5% CO$_2$ atmosphere. H$_2$O$_2$, 15d-PGJ$_2$ (Cayman), bisphenol A diglyceryl ether (BADGE) (Fluka), rosiglitazone (Cayman), zVADfmk, and zDEVDfmk (Biovision) were added to serum-deprived BE(2)-C cells either alone or in various combinations for 12 hours. Extent of cytotoxicity was assessed by lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays according to manufacturer’s instructions (Roche).

**Preparation of Replication-Defective Recombinant Adenoviral Vectors**

Viral vectors were prepared as previously described. We constructed the replication-defective recombinant adenoviral (rAd) vector a human phosphoglycerate kinase (PGK) promoter to drive COX-1 (Adv-COX-1), green fluorescent protein (Adv-GFP), or PGK alone to serve as the control (Adv-PGK).

**Intracerebral Ventricular Infusion of Adenoviral Constructs and 15d-PGJ$_2$**

The procedure was performed as previously described. Briefly, anesthetized rats were placed in a stereotaxic apparatus; 10 plaque-forming units (pfu) or 10 L of 15d-PGJ$_2$ (1 to 50 pg) were infused into the right lateral ventricle at a rate of 5 µL/min at the following coordinates: Anterior, 2.5 mm caudal to bregma; Right, 2.8 mm lateral to midline; and Ventral, 3.0 mm ventral to dural surface. Periodic confirmation of proper placement of the needle was performed with infusion of fast green. To delineate the distribution of the transgene expression, we infused Adv-GFP into the right lateral ventricle for 72 hours and GFP was visualized under microscopy. GFP was detected in the lining of ependymal cells and cells surrounding the right ventricle in all 8 coronal brain slices but not in the left ventricle (Figure I, available online at http://atvb.ahajournals.org).

**Measurements of Brain Tissue PGD$_2$ and 15d-PGJ$_2$**

Brain tissues for PGD$_2$ and 15d-PGJ$_2$ analysis were prepared as previously described. Briefly, the ischemic cortex was homogenized in 1 mL ice-cold buffer and centrifuged at 55 000g for 1 hour. Eicosanoids were extracted with a Sep-Pak C18 cartridge and analyzed by enzyme immunoassays using reagents from Cayman for PGD$_2$, and R&D Systems for 15d-PGJ$_2$.

**Western Blot Analysis**

Analysis of proteins in the cortex and BE(2)-C cells by Western blotting was performed as described previously, using antibodies for COX-1 (Cayman, 1:1000), COX-2 (Cayman, 1:1000), PPARγ (Santa Cruz, 1:500), HO-1 (ABR, 1:2000), GAPDH (BD Pharmingen, 1:10000), active caspase-3 (Cell signaling, 1:500), Poly (ADP-ribose) polymerase (PARP) (Cell signaling, 1:1000), and 1kB-α (Santa Cruz, 1:1000). Protein bands were visualized by an enhanced chemiluminescence system (Pierce).

**Statistical Analysis**

Analysis of variance (ANOVA) was used to compare the temporal expression of proteins, infarct volumes, and eicosanoid levels. The level of significance for differences between groups was further analyzed with post-hoc Fisher’s protected t tests by GB-STAT 5.0.4 (Dynamic Microsystem, Inc, Silver Springs, Md). P<0.05 was considered significant.

**Results**

**Adenoviral COX-1 Gene Transfer Increased 15d-PGJ$_2$ in Ischemic Brain**

Adv-COX-1 infusion 72 hours before a 50-minute MCA occlusion resulted in increased COX-1 proteins, PGD$_2$, and 15d-PGJ$_2$ levels accompanied by a reduction in infarct volume (Figure 1a). The extent of 15d-PGJ$_2$ increase (∼3-fold) correlated with that of COX-1 increase (∼3-fold) (Figure 1b). COX-2 was markedly suppressed whereas PPARγ and HO-1 were elevated by Adv-COX-1 treatment (Figure 1b). EMSA analysis reveals increased p50/p65 NF-κB DNA binding in ischemic cortex as previously reported, which was abrogated by Adv-COX-1 (Figure II, available online at http://atvb.ahajournals.org).

**15d-PGJ$_2$ and Rosiglitazone Reduced Infarct Volume**

To confirm the protective effect of 15d-PGJ$_2$, we infused 15d-PGJ$_2$ 24 hours before a 50-minute MCA occlusion. 15d-PGJ$_2$ at 1 pg reduced the infarct volume by >50% (Figure III, available online at http://atvb.ahajournals.org). However, when 15d-PGJ$_2$ was infused immediately after the 50 minutes of ischemia, it failed to reduce infarct volume significantly even when the dose was increased to 50 pg (Figure III).
reduced the infarct size in right cortex when the MCA occlusion was shortened to 30 minutes (Figure IV, available online at http://atvb.ahajournals.org) in a concentration-dependent manner (Figure 2a). It remained effective when administered 2 hours after ischemia but was no longer effective at 3 hours after ischemia (Figure 2a). To determine whether the anti-infarct action of 15d-PGJ2 is mediated via PPARγ, we infused GW9662, a selective inhibitor of PPARγ. GW9662 completely abrogated the protective effect of 15d-PGJ2 (Figure 2b). Infusion of rosiglitazone (50 ng) immediately after the 30-minute ischemia reduced the infarct volume by >80% and remained effective when infused 2 hours, but not 3 hours, after ischemia. 15d-PGJ2 and rosiglitazone reduced IkBα degradation to a similar extent (Figure V, available online at http://atvb.ahajournals.org). These results suggest that 15d-PGJ2 reduced infarct volume via a PPARγ-dependent pathway.

**PPARγ Ligands Upregulated PPARγ Protein Levels**

The PPARγ protein level in ischemic brain was higher than control (Figure 3a), which was concentration-dependently enhanced by 15d-PGJ2 (Figure 3a), and rosiglitazone (data not shown). 15d-PGJ2 upregulated PPARγ in normal brains in a time-dependent manner, with a 3-fold increase in the protein level 12 hours after infusion (Figure 3b).

**Adv-COX-1, 15d-PGJ2, and Rosiglitazone Inhibited Brain Tissue Caspase 3 Activation**

To determine whether the tissue protective effects are mediated by blocking apoptosis, we measured activated caspase-3 in ischemic cortex treated with Adv-COX-1 or PPARγ ligands. Activated caspase-3 was increased in ischemic cortex, which was abrogated by Ad-COX-1, 15d-PGJ2 (Figure 4a and 4b), or rosiglitazone infusion (data not shown). 15d-PGJ2 at 50 pg or rosiglitazone at 50 ng reduced activated caspase-3 to the basal level and Adv-COX-1 suppressed caspase 3 activation to a similar extent.

**15d-PGJ2 Suppressed Neuronal Apoptosis and Necrosis**

The in vivo data reveal that 15d-PGJ2 protected brain tissues from I/R-induced cell death. To ascertain its effect on protecting neuronal survival, we evaluated the effect of 15d-PGJ2 on H2O2-induced neuronal cell death in human BE(2)-C and rat primary cortical neuron culture. Because H2O2 has been reported to be a major mediator of I/R-induced neural cell apoptosis and necrosis,20,21 we measured H2O2-induced LDH release and MTT reduction and several apoptotic changes. H2O2 exerts a similar concentration-dependent increase in LDH release from cultured rat neurons (Figure VIa, available online at http://atvb.ahajournals.org) and BE(2)-C cells (Figure VIb) with a reciprocal MTT reduction. 15d-PGJ2 at 100 to 200 nM effectively suppressed H2O2-
induced LDH release from rat and human neurons (Figure 5a), which was abrogated by BADGE, a PPARγ inhibitor (Figure 5a). Paradoxically, 15d-PGJ2 at higher concentrations (>5 μmol/L) induced LDH release from BE(2)-C cells (Figure 5b). Similar to 15d-PGJ2, rosiglitazone at 0.5 μmol/L suppressed LDH release by >50%, which was abrogated by BADGE (Figure VIIa, available online at http://atvb.ahajournals.org), but paradoxically induced LDH release at higher concentrations (>10 μmol/L) (Figure VIIb). Caspase inhibitors blocked H2O2-induced LDH release and restored the inhibitory action of 15d-PGJ2 even in the presence of BADGE (Figure 5c).

To confirm that 15d-PGJ2 protects neurons from H2O2-induced apoptosis, we measured apoptotic cells by flow cytometry. 15d-PGJ2 inhibited H2O2-induced neuronal apoptosis by >50%, which was abrogated by BADGE (Figure 6a). Moreover, 15d-PGJ2 inhibited H2O2-induced caspase 3 activation and PARP cleavage in BE(2)-C cells, which were also abrogated by BADGE (Figure 6b).

15d-PGJ2 increased PPARγ protein levels by ~2-fold, which was not influenced by H2O2 treatment (Figure VIIIa, available online at http://atvb.ahajournals.org). Because HO-1 is an important mediator of cell survival, we determined whether HO-1 level is altered by H2O2 and 15d-PGJ2 treatment. 15d-PGJ2 increased HO-1 protein levels by ~10-fold (Figure VIIIb). H2O2 did not alter HO-1 level, nor did it interfere with the stimulatory action of 15d-PGJ2 (Figure VIIIb). By contrast, the HO-1 stimulatory action of 15d-PGJ2 was blocked by BADGE (Figure VIIIb).

Discussion

Results from this study indicate that a considerable quantity of 15d-PGJ2 is generated in I/R-injured cortex, which is augmented by COX-1 overexpression. Elevation of 15d-PGJ2 was correlated with a >50% reduction in infarct volume and a >80% reduction in activated caspase 3. 15d-PGJ2 has a potent effect on controlling the expansion of the infarct size as administration of 15d-PGJ2 intraventricularly at 1 pg was sufficient to induce ~50% reduction in infarct volume. These results suggest that the endogenously generated 15d-PGJ2 may be involved in suppressing I/R-induced infarct expan-
However, the causal relationship between the endogenous generation of 15d-PGJ\textsubscript{2} and reduction in infarct volume is not fully established in our study as we have not performed time-course experiments to demonstrate that 15d-PGJ\textsubscript{2} generation precedes the reduction in the infarct size. Work is in progress to characterize the relationship between endogenous 15d-PGJ\textsubscript{2}, infarct size, and biochemical markers.

15d-PGJ\textsubscript{2} is highly effective in reducing infarct volume when administered before a 50-minute MCA occlusion but loses its activity when administered after occlusion. Its window of effectiveness extends to 2 hours, but not 3 hours, when administered after a shorter (30 minutes) and therefore a milder ischemic insult. These results suggest that 15d-PGJ\textsubscript{2} acts on early pathophysiological events after ischemic injury.

It has been proposed that acute cerebral injury immediately after transient focal ischemia is attributed to energy failure and excitotoxicity that results in neuronal necrosis.\textsuperscript{22} Acute ischemic injury also results in mitochondrial damage, which may lead to apoptosis. The extent of neuronal necrosis and apoptosis is influenced by the duration of MCA occlusion. A severe insult after a prolonged MCA occlusion causes predominantly neuronal necrosis, whereas a mild insult such as a 30-minute MCA occlusion incurs predominantly apoptosis.\textsuperscript{23,24} Our results show that 15d-PGJ\textsubscript{2} is capable of suppressing neuronal necrosis and apoptosis, thereby restricting the infarct development after a 50-minute or 30-minute MCA occlusion, albeit with a different window of effectiveness. Evidence supporting the action of 15d-PGJ\textsubscript{2} includes: (1)

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**Figure 5.** Control of H\textsubscript{2}O\textsubscript{2}-induced LDH release. a, BE(2)-C were treated with H\textsubscript{2}O\textsubscript{2} in the presence or absence of 15d-PGJ\textsubscript{2} and BADGE. b, BE(2)-C cells were treated with 15d-PGJ\textsubscript{2} at increasing concentrations. c, BE(2)-C cells were treated with H\textsubscript{2}O\textsubscript{2} and a combination of compounds as indicated. Each bar in a to c refers to mean±SD (n=3). *P<0.05. **P<0.01.

**Figure 6.** Inhibition of neuronal apoptosis. a, BE(2)-C cells treated with H\textsubscript{2}O\textsubscript{2}, 15d-PGJ\textsubscript{2}, and/or BADGE for 12 hours. Sub-G\textsubscript{0} apoptotic cells were analyzed by flow cytometry. Upper panel shows percentages of Sub-G\textsubscript{0} in representative flow profiles and the lower panel mean±SD of 3 experiments. b, BE(2)-C cells were treated with or without H\textsubscript{2}O\textsubscript{2}, 15d-PGJ\textsubscript{2}, and/or BADGE for 12 hours. Caspase 3 was determined as described in Methods. The upper panels show representative Western gels and the lower panels, mean±SD of densitometry of 3 experiments.
pretreatment of rat or human neurons with 15d-PGJ2 prevented H2O2-induced cytotoxicity as detected by LDH leakage and reduction in MTT staining; (2) 15d-PGJ2 prevented H2O2-induced neuronal apoptosis; and (3) administration of 15d-PGJ2 intraventricularly abrogated caspase 3 activation in the ischemic cortex. Because necrosis develops rapidly after a severe ischemic injury, 15d-PGJ2 is ineffective in controlling infarct size unless it is administered before I/R injury. By contrast, neuronal apoptosis induced by shorter ischemia takes place not as rapidly and therefore is responsive to 15d-PGJ2 inhibition even after tissue damage has occurred.

Several pieces of evidence support the requirement of PPARγ activation for the protective action of 15d-PGJ2. First, the effect of 15d-PGJ2 on reducing infarct volume in vivo was abrogated by a PPARγ inhibitor, GW9662. Second, the effect of 15d-PGJ2 on protecting neuronal cytotoxicity and apoptosis was abrogated by another PPARγ inhibitor, BADGE. Third, known PPARγ ligands such as rosiglitazone inhibited I/R-induced brain infarction in vivo and H2O2-induced neuronal apoptosis and cyto toxicity in vitro. Activation of PPARγ has been shown to upregulate HO-1 expression25,26 and suppress the expression of an array of genes by blocking the transcriptional activity of transactivators such as NF-κB.12,13 Our data confirm that 15d-PGJ2 upregulates HO-1 expression, inhibits NF-κB activation, and suppresses COX-2 expression in ischemic cortex and cultured neurons by a PPARγ-dependent pathway. HO-1 possesses tissue protective properties, whereas NF-κB mediated genes, such as COX-2,12,13 which aggravates tissue damage by producing pro-inflammatory prostanooids and reactive oxygen species. Upregulation of the protective HO-1 coupled with suppression of COX-2 and other NF-κB–dependent genes via PPARγ activation represents a major mechanism by which 15d-PGJ2 and rosiglitazone protect against I/R-induced neuronal necrosis and apoptosis and thereby limit the expansion of the infarct size.

We observed that 15d-PGJ2 and rosiglitazone exhibit a concentration-dependent paradoxical effect on cytotoxicity. 15d-PGJ2 induces LDH release at ≥5 μmol/L but protects neurons from necrosis and apoptosis at ≤1 μmol/L. This observation may explain the conflicting data reported in the literature. 15d-PGJ2 was reported to induce apoptosis of several cell types including neurons28–32 and protect cerebellar granular cells from apoptosis.33,34 A detailed review of those reports reveals that the paradoxical effects of 15d-PGJ2 may be caused by use of different 15d-PGJ2 concentrations. Studies using 15d-PGJ2 ≥10 μmol/L reported a pro-apoptotic effect, whereas those using concentrations ≤1 μmol/L were anti-apoptotic. The reason for this paradoxical action is unclear. Because rosiglitazone has a similar concentration-dependent paradoxical effect, it is tempting to speculate that PPARγ is involved. Further studies are needed to resolve this puzzle.

Our results reveal that PPARγ expression in rat brain tissues is increased by I/R and further enhanced by 15d-PGJ2 and rosiglitazone. These results are consistent with an autoregulation of PPARγ by its ligands. It is unclear how 15d-PGJ2 upregulates PPARγ. 15d-PGJ2 may enhance PPARγ at the transcriptional level or, alternatively, at the level of protein stability. Because PPARγ upregulation by 15d-PGJ2 in BE(2)-C cells was blocked by BADGE, it is reasonable to conclude that ligand-induced PPARγ upregulation requires PPARγ activation, which creates a positive feedback loop for tissue protection.

Thiazolidinediones (TZD) have been shown to protect brain from I/R injury35–37 and reduce myocardial infarction.38 Pioglitazone and troglitazone administered intraperitoneally reduced infarct volume and improve neurological function accompanied by suppression of COX-2 and IL-1β in the rat stroke model.35 Oral administration of pioglitazone for 4 days improved blood flow and reduced infarct volume in the rat model.36 Our results show that intraventricular administration of rosiglitazone 2 hours after ischemia was effective in controlling infarct size. However, the effect was abated when administered after 3 hours. Thus, TZD drugs that are now commonly used clinically for treating diabetes may be useful in preventing I/R-induced tissue injury in humans. Although TZD alone has a narrow therapeutic window, its combination with PGI2 analogs may prolong the therapeutic window and achieve a synergistic effect as PGI2 protects against tissue damage by different mechanisms.39,40

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Figure S-IV

(a) Western blot analysis showing the expression of IκB-α and GAPDH under different conditions: Sham, Ischemia, PGJ2 10pg, and PGJ2 50pg. The graph below the blots represents the fold of control for IκB-α expression.

(b) Western blot analysis for IκB-α and GAPDH, with Rosiglitazone treatment indicated for each condition (+ for treated, - for control).

(c) Western blot analysis for IκB-α and GAPDH, with 15d-PGJ2, BADGE, and H2O2 treatments. The graph below the blots represents the fold of control for IκB-α expression.
Figure S-V

[Graph showing MTT reduction and LDH release as a function of H$_2$O$_2$ concentration with different treatments indicated.]

- V, ·· H$_2$O$_2$
- H$_2$O$_2$+PGJ$_2$ (100nM)

MTT reduction (% of control)

LDH release (% of total killed)

H$_2$O$_2$ concentration (μM)