Biphasic Effects of 15-Deoxy-Δ^{12,14}-Prostaglandin J\textsubscript{2} on Glutathione Induction and Apoptosis in Human Endothelial Cells


Abstract—The lipid products derived from the cyclooxygenase pathway can have diverse and often contrasting effects on vascular cell function. Cyclopentenone prostaglandins (cyPGs), such as 15-deoxy-Δ^{12,14}-prostaglandin-J\textsubscript{2} (15d-PGJ\textsubscript{2}), a peroxisome proliferator–activated receptor-γ (PPARγ) agonist, have been reported to cause endothelial cell apoptosis, yet in other cell types, cyPGs induce cytoprotective mediators, such as heat shock proteins, heme oxygenase-1, and glutathione (GSH). Herein, we show in human endothelial cells that low micromolar concentrations of 15d-PGJ\textsubscript{2} enhance GSH-dependent cytoprotection through the upregulation of glutamate-cysteine ligase, the rate-limiting enzyme of GSH synthesis, as well as GSH reductase. The effect of 15d-PGJ\textsubscript{2} on GSH synthesis is attributable to the cyPG structure but is independent of PPAR, inasmuch as the other cyPGs, but not PPARγ or PPARα agonists, are able to increase GSH. The increase in cellular GSH is accompanied by abrogation of the proapoptotic effects of 4-hydroxyxenonenal, a product of lipid peroxidation present in atherosclerotic lesions. However, higher concentrations of 15d-PGJ\textsubscript{2} (10 μmol/L) cause endothelial cell apoptosis, which is further enhanced by depletion of cellular GSH by buthionine sulfoximine. We propose that the GSH-dependent cytoprotective pathways induced by 15d-PGJ\textsubscript{2} contribute to its antiatherogenic effects and that these pathways are distinct from those leading to apoptosis. (Arterioscler Thromb Vasc Biol. 2001;21:1846-1851.)

Key Words: cyclopentenone prostaglandins ■ glutathione ■ glutamate-cysteine ligase ■ endothelium ■ apoptosis

Prostaglandins of the J series (PGJs) are cyclopentenones synthesized from arachidonic acid via enzymatic conversion by cyclooxygenase and prostaglandin D\textsubscript{2} (PGD\textsubscript{2}) synthase, followed by nonenzymatic dehydration of PGD\textsubscript{2} to PGJ\textsubscript{2}, Δ\textsuperscript{12}-PGJ\textsubscript{2}, and 15-deoxy-Δ\textsuperscript{12,14}-PGJ\textsubscript{2} (15d-PGJ\textsubscript{2}). These compounds have generated considerable interest in vascular biology after the discovery of their ability to activate one of the ligand-activated nuclear receptors, peroxisome proliferator–activated receptor-γ (PPARγ). Through a mechanism in which 15d-PGJ\textsubscript{2} acts as a PPARγ agonist, it has been shown to inhibit production of proinflammatory cytokines in monocytes and the binding of these cells to the endothelium. However, cyclopentenone prostaglandins (cyPGs) have also PPAR-independent anti-inflammatory effects, including inhibition of nuclear factor-κB activation by inhibiting IκB kinase. This is important in atherogenesis, because some of the early events in lesion formation, such as monocyte recruitment and adherence, are mediated by nuclear factor-κB–dependent upregulation of monocyte chemoattractant protein-1, as well as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. Moreover, cyPGs induce cytoprotective mediators, such as heat shock proteins, heme oxygenase-1, and glutathione (GSH), further strengthening the notion that cyPGs are potentially antiatherogenic. However, cyPGs have also been shown to be cytostatic or cytotoxic to a number of cell types, including endothelial cells, vascular smooth muscle cells, and monocytes. Apoptosis caused by 15d-PGJ\textsubscript{2} in endothelial cells has been reported to be PPARγ dependent, whereas in a human neuroblastoma cell line, it has been proposed that the production of reactive oxygen species and secondary lipid peroxidation products leads to cell death.

The diverse effects of the cyPGs on vascular cells remain uncertain and potentially conflicting. Although inhibition of vascular smooth muscle cell growth can be antiatherogenic, endothelial cell apoptosis, perturbation of the endothelial cell layer integrity, and subsequent leakage of LDL and other serum-derived factors into the subendothelial space would promote lesion formation.

Clearly, it is important to examine the cytotoxic and cytoprotective aspects of these compounds to gain a perspective on the biological actions of cyPGs in the vasculature.

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the present study, human endothelial cells were exposed to a range of concentrations of 15d-PGJ$_2$ commonly used in the literature. In the present study, we show that low micromolar concentrations of 15d-PGJ$_2$ cause a robust increase in cellular GSH through transcriptional upregulation of glutamate-cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis,$^{16}$ via a mechanism independent of PPARγ, whereas higher concentrations (10 μmol/L) cause endothelial cell apoptosis. Moreover, we show that low concentrations of 15d-PGJ$_2$ confer resistance against cell death caused by 4-hydroxynonenal (HNE), a lipid peroxidation product found in atherosclerotic lesions.

**Methods**

**Materials**

Prostaglandins and arachidonic acid were purchased from Cayman Chemicals. These were stored in 10 mmol/L aliquots in ethanol at −80°C and diluted immediately before use. Ciglitazone and Wy-14,643 were from BioMol, and HNE was from Calbiochem. The other reagents were purchased from Sigma Chemical Co.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Endothelial Cell Service Center (Dr Francois Booyse, Department of Medicine, University of Alabama at Birmingham) and cultivated in plastic ware coated with 0.05% gelatin/10 g/mL fibronectin (Biomedical Technologies) in endothelial cell growth medium (Clonetics) supplemented with 10% FBS. The experiments were performed in the same media with 2% FBS. Cells were used at passages 3 to 7 at confluence.

**Measurement of GSH, GSH Peroxidase, GSH Reductase, and GST Activities**

Total GSH (GSH+glutathione disulfide [GSSG]),$^{17}$ glutathione peroxidase (GPx),$^{18}$ and glutathione reductase (GR)$^{19}$ activities were measured as previously described. The GR activity in samples was determined as percentage of control. B, Cells were treated with increasing concentrations of 15d-PGJ$_2$ for 16 hours, and total GSH was assessed as nanomoles per milligram protein (prot). C, Cells were exposed to 5 μmol/L 15d-PGJ$_2$ for 0 to 24 hours before measurement of total GSH. D, Cells were exposed to 2.5 μmol/L (solid squares) or 10 μmol/L (open circles) 15d-PGJ$_2$ for 0 to 24 hours before measurement of GSH/GSSG ratio by high-performance liquid chromatography. Values are mean±SEM (n=3). *(P<0.05, **P<0.01, and ***P<0.001 vs control.

**Statistical Analysis**

Results of the experimental studies are reported as mean±SEM. Differences were analyzed by Student t test. A difference between groups of *P<0.05 was considered significant.

**Results**

**Effect of Prostaglandins, Arachidonic Acid, PPARα, and PPARγ Activators on GSH**

Total cellular GSH was increased in HUVECs on exposure to prostaglandin A$_1$ (PGA$_1$), PGD$_2$, PGJ$_2$, and 15d-PGJ$_2$ (Figure 1A). Of these compounds, PGA$_1$, PGJ$_2$, and 15d-PGJ$_2$ have a cyclopentenone structure, whereas PGD$_2$ converts nonenzymatically to PGJs.$^1$ Prostaglandin E$_2$ or arachidonic acid were not able to induce GSH, implying that the cyclopentenone structure is important for GSH induction. Because PGJs were the most potent inducers of GSH, we then examined the concentration and time-dependent induction of GSH by 15d-PGJ$_2$. Incubation of HUVECs with 0.5 to 5 μmol/L 15d-PGJ$_2$ for 16 hours caused an increase in GSH, which was significant with 0.5 μmol/L 15d-PGJ$_2$ (Figure 1B). However, with 10 μmol/L 15d-PGJ$_2$, or PGJ$_2$, there was no difference in GSH versus control (Figure 1A and 1B). The increase in total GSH was significant after 8 hours of incubation with 5 μmol/L 15d-PGJ$_2$ and occurred without a decrease in total GSH at the early time points (Figure 1C). The GSH/GSSG ratio (39±3 at 0 hours, n=3) did not change during incubation with a low concentration (2.5 μmol/L) of
induction at 6 hours was already significant with 0.5 μmol/L 15d-PGJ2 (Figure 2B). Although the induction of either GCL subunit mRNA was smaller with 10 μmol/L 15d-PGJ2 than with 2.5 μmol/L or 5 μmol/L 15d-PGJ2, GAPDH expression was decreased as well, and the ratio of GCL mRNA to GAPDH did not change. This indicates that the decline in GCL mRNA expression was due to the cytotoxic effect and general decrease in transcription rather than specific down-regulation of GCL with high concentrations of 15d-PGJ2. To determine whether the increase in GCL mRNA is attributable to a transcriptional induction, the cells were incubated with 15d-PGJ2 in the presence and absence of the transcriptional inhibitor actinomycin D (Figure 2C). Actinomycin D abolished the induction of GCL, implying that the induction of GCL subunits is transcriptional.

Next, we studied the protein expression GCL of by Western blotting. 15d-PGJ2 caused a 2-fold induction of GCLM in 16 hours, whereas GCLC protein levels were not significantly changed at this time point (Figure 2D).

**Effect of 15d-PGJ2 on Other GSH-Metabolizing Enzymes**

To study the effects of 15d-PGJ2 on other GSH-metabolizing enzymes, we exposed the cells to 0.5 to 10 μmol/L 15d-PGJ2 for 16 hours and measured GPx, GST, and GR activities. Neither GPx nor GST was significantly changed by 15d-PGJ2 (results not shown). However, GR showed a concentration-dependent induction with 0.5 to 5 μmol/L 15d-PGJ2. The induction was significant at 0.5 μmol/L 15d-PGJ2 (0.23±0.01 U/mg protein versus control 0.16±0.01 U/mg protein, P<0.01; n=3) and was maximal at 2.5 to 5 μmol/L 15d-PGJ2 (0.32±0.01 U/mg protein), whereas 10 μmol/L 15d-PGJ2 decreased GR by ∼25% (0.12±0.003 U/mg protein, P<0.05 versus control; n=3). Because synthesis of GSH is also dependent on the reuse of exported GSSG or GSH conjugates via γ-glutamyl transpeptidase (γ-GT),24 we studied the effect of the specific inhibitor of γ-GT, acivicin, on the induction of GSH by 15d-PGJ2. In the presence of 5 μmol/L acivicin, the percent increase in GSH on exposure to 15d-PGJ2 (5 μmol/L) for 16 hours was significantly smaller (174±17% versus 263±17% 15d-PGJ2 only, P<0.01; n=3), implying that γ-GT is important for the increase in intracellular GSH.

**Effect of 15d-PGJ2 on Endothelial Cell Viability**

Incubation of endothelial cells with 10 μmol/L 15d-PGJ2 or PGJ2 caused significant cytotoxic changes in endothelial cell morphology in the initial experiments. To assess cell death caused by 15d-PGJ2, endothelial cells were treated with increasing concentrations of 15d-PGJ2 with and without 250 μmol/L buthionine sulfoximine (BSO), a specific inhibitor of GCL, 24 hours before and during 16 hours of treatment with 15d-PGJ2. With this treatment regimen, GSH was decreased by 90%. Incubation with 10 μmol/L 15d-PGJ2 caused a significant (P<0.001) increase in the apoptotic (annexin V–FITC positive) cell population compared with untreated controls (Figure 3A). Although 5 μmol/L 15d-PGJ2 by itself caused no cytotoxicity, it did so in combination with BSO, and BSO also caused a significant exacerbation of the cytotoxicity of 10 μmol/L 15d-PGJ2 (Figure 3A).

The possibility that lower nontoxic concentrations of 15d-PGJ2 that cause GSH induction could confer resistance
This lipid peroxidation product has been shown to induce apoptosis in a number of cell types, including HUVECs,25,26 and it is present in human atherosclerotic lesions. 27 Incubation of cells with 2.5 μmol/L 15d-PGJ2 for 24 hours before exposure to 20 μmol/L HNE for 16 hours. C, HUVECs were incubated with indicated concentrations of 15d-PGJ2 and BSO (10 μmol/L) for 24 hours before exposure to 20 μmol/L HNE for 16 hours. Values are mean±SEM (n=3). ***P<0.001 BSO treated vs untreated controls (A). **P<0.01 and *P<0.05 vs HNE treatment without 15d-PGJ2 pretreatment (B); *P<0.05 and **P<0.01 vs respective controls (C).

Figure 3. Cytotoxic vs cytoprotective effects of 15d-PGJ2 in HUVECs. A, HUVECs were exposed to 5 and 10 μmol/L 15d-PGJ2 for 16 hours in the absence (open bars) and presence (solid bars) of GCL inhibitor BSO (250 μmol/L) 24 hours before (control) and during treatment with 15d-PGJ2. The cells were collected and labeled with annexin V-FITC, and percentage of apoptotic (annexin V–FITC positive) cells was assessed. B, HUVECs were incubated without (open bars) and with (solid bars) 2.5 μmol/L 15d-PGJ2, for 24 hours. The cells were then washed with HBSS and incubated further with indicated concentrations of HNE for 16 hours. C, HUVECs were incubated with indicated concentrations of 15d-PGJ2 and BSO (10 μmol/L) for 24 hours before exposure to 20 μmol/L HNE for 16 hours. Values are mean±SEM (n=3). ***P<0.001 BSO treated vs untreated controls (A). **P<0.01 vs HNE treatment without 15d-PGJ2 pretreatment (B); *P<0.05 and **P<0.01 vs respective controls (C).

against lipid peroxidation products associated with atherogenesis was also examined. To address this, HUVECs were first treated with 15d-PGJ2 for 24 hours, after which the cells were washed with HBSS and incubated further with HNE. This lipid peroxidation product has been shown to induce apoptosis in a number of cell types, including HUVECs,25,26 and it is present in human atherosclerotic lesions.27 Incubation with 15 μmol/L HNE caused a statistically significant (P<0.05 versus untreated control) increase in the apoptotic cell population. Incubation of cells with 2.5 μmol/L 15d-PGJ2 for 24 hours before HNE treatment attenuated cytotoxicity, and this effect was most striking with the highest concentration (20 μmol/L) of HNE (Figure 3B). The lowest concentration of 15d-PGJ2 that was protective was 0.5 μmol/L (Figure 3C). To assess the role of GSH on cytoprotection against HNE, the cells were treated with 1 μmol/L 15d-PGJ2 in the presence of 10 μmol/L BSO, which caused an ≈90% reduction of GSH versus 15d-PGJ2 alone. BSO decreased the cytoprotective effect of 15d-PGJ2 treatment (Figure 3C). BSO alone did not enhance the already substantial cytotoxicity of this concentration of HNE (Figure 3C).

Discussion
In the present study, we demonstrate that 15d-PGJ2 has dual effects on endothelial cell survival and apoptosis. Low concentrations are cytoprotective via a mechanism that involves a major contribution from an increase in intracellular GSH, whereas higher concentrations are cytotoxic.

The concentrations of 15d-PGJ2 found at local sites of inflammation are not clear, because estimations are complicated by the reactivity of the α,β-unsaturated carbonyl group characteristic to cyPGs, which renders them susceptible to conjugation.28 Thus, the low concentrations assessed in some pathological settings do not necessarily represent the concentrations to which the cells are exposed at sites of inflammation.29,30 PGD2 synthesis has been shown to be upregulated in synthetic phenotype of smooth muscle cells in the atherosclerotic intima.31 It is plausible that in a pathophysiological setting, a wide range of concentrations of the PGJs can occur with a corresponding spectrum of biological responses. It should be noted, however, that although PGD2 readily undergoes hydration into PGJs in the presence of albumin in vitro, the extent by which this occurs in vivo is still under debate because the studies have been focused on finding free PGJs rather than PGGJ-protein adducts.28

In the present study, we show that 0.5 to 5 μmol/L 15d-PGJ2 causes an increase in cellular GSH, which is preceded by transcriptional upregulation of GCLM and GCLC mRNA (Figure 2A through 2C). However, changes in GCLC mRNA were not reflected in increased protein (Figure 2D). Nevertheless, a 2-fold upregulation of GCLM protein was observed. Although GCLC has all the catalytic activity and is feedback-inhibited by GSH, the light subunit has an important regulatory role, by decreasing the Kᵢ for GSH, thereby increasing the catalytic activity.22,33 Therefore, in increase in GCLM protein without changes in catalytic subunit expression is sufficient to increase GSH synthesis.

A strong candidate for mediating GCL induction by 15d-PGJ2 is the electrophile responsive element (EpRE). It is present in both GCL genes and accounts for basal transcriptional activity, as well as β-naphthoflavone– and pyrroline dithiocarbamate–induced GCL expression.33,34 This element is activated on exposure to electrophiles through dissociation of transcription factor Nrf2 from its cytoplasmic docking protein Keap1 and the subsequent nuclear translocation and transactivation of EpRE.35,36 The actual mechanism of dissociation of Nrf2 from Keap1 is currently unknown, but it is thought to involve thiol modifications or phosphorylation of Keap1, Nrf2, or both.35–37 Thus, it is possible that 15d-PGJ2 binds to thiol groups, thereby activating Nrf2, or it may activate redox-sensitive kinase pathways and cause EpRE activation. Current studies in our laboratory are directed at elucidating the mechanism by which 15d-PGJ2 causes transcriptional induction of GCL and whether EpRE is involved.

15d-PGJ2 has been shown to inhibit GPxs in the human neuroblastoma cells14 and induce GST in hepatoma cell lines.38 These activities were not significantly altered in endothelial cells (results not shown). However, GR activity...
was increased in a concentration-dependent manner with low concentrations of 15d-PGJ2. The regulation of GR is poorly known, but recently, a functional EpRE element has been found in its promoter region (R.T. Mulcahy, unpublished data, 2001). In the endothelium, the upregulation of GR activity may provide an additional mechanism to increase the level of reduced GSH. In the present study, high concentrations of 15d-PGJ2 as well as another PPARγ activator, ciglitazone, caused endothelial cell death. These results are consistent with previous reports demonstrating a PPARγ-dependent increase in endothelial cell apoptosis.11 Recently, other, PPAR-independent, mechanisms of cytotoxicity of 15d-PGJ2 involving increased production of reactive oxygen species have been suggested.14 This would be consistent with the data showing that BSO enhanced the cytotoxicity of 15d-PGJ2 (Figure 3A). However, reactive oxygen species are not necessarily involved in the process, because GSH readily conjugates with 15d-PGJ2, thereby modulating its biological effects.28 Similarly, inhibition of the cytotoxicity of 15d-PGJ2 by thiol antioxidant N-acetylcysteine14 may be through conjugation, because 15d-PGJ2 reacts with low molecular weight thiols even in the absence of cells.28 Clearly, further studies are needed to define the mechanisms involved in endothelial cell apoptosis caused by 15d-PGJ2.

In the literature, up to 20 μmol/L 15d-PGJ2 has been used in human endothelial cells with appreciable cytotoxicity.29 It is difficult to compare the concentrations used in different studies, because 15d-PGJ2 is unstable, and the actual concentrations from commercial sources may vary.28 Another aspect that may explain some of the inconsistencies in the literature are different serum concentrations of the growth media, which have a major impact on the cytotoxicity of 15d-PGJ2 and thiazolidinediones.11,39 Oxidized derivatives of fatty acids are implicated in the pathogenesis of atherosclerosis. HNE, an aldehyde generated during oxidation of polyunsaturated fatty acids, is thought to be a major mediator of the adverse effects of lipid peroxidation.37 The proapoptotic effects of HNE are well documented in a number of cell types, including endothelial cells.25,26 In the present study, low concentrations of 15d-PGJ2 were protective against toxic effects of HNE. This effect was partially inhibited by BSO, suggesting that GSH contributes to the cytoprotective effects of 15d-PGJ2. Because cyPGs also induce other cytoprotective molecules such as heat shock proteins9 and heme oxygenase-1,10 these may play an additional role in cytoprotection.

Recent work by Taba et al29 has shown that shear stress induces lipocalin-type PGD2 synthesis and increases the release of PGD2 and 15d-PGJ2 to the medium. Furthermore, shear stress activates enzymes involved in liberation of arachidonic acid and cyclooxygenase-2.29,40–42 Laminar shear stress has well-documented antiapoptotic effects,43,44 which have been shown to be mediated partially through GSH.44 Recently, we have shown that shear stress can increase GSH synthesis45; therefore, it will be of interest to assess the role of increased production of 15d-PGJ2 on GSH induction and cytoprotection by laminar flow.

In summary, we have shown that low nontoxic levels of 15d-PGJ2 cause a potent induction of cellular GSH through transcriptional upregulation of the rate-limiting enzyme, GCL, as well as GR. Moreover, preconditioning of endothelial cells with low concentrations of 15d-PGJ2 confers resistance against HNE cytotoxicity. We propose that the modulation of GSH-dependent cytoprotection by PGJs contributes to their antiatherogenic effects.

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