Cyclopentenone Prostaglandins as Potential Inducers of Intracellular Oxidative Stress*

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In the present study, we find that cyclopentenone prostaglandins (PGs) of the J2 series, naturally occurring derivatives of PGD2, are potential inducers of intracellular oxidative stress that mediates cell degeneration. Based on an extensive screening of diverse chemical agents on induction of intracellular production of reactive oxygen species (ROS), we found that the cyclopentenone PGs, such as PGA2, PGJ2, Δ12-PGJ2, and 15-deoxy-Δ12,14-PGJ2, showed the most potent pro-oxidant effect on SH-SY5Y human neuroblastoma cells. As the intracellular events that mediate the PG cytotoxicity, we observed (i) the cellular redox alteration represented by depletion of antioxidant defenses, such as glutathione and glutathione peroxidase; (ii) a transient decrease in the mitochondrial membrane potential (Δψ); (iii) the production of protein-bound lipid peroxidation products, such as acrolein and 4-hydroxy-2-nonenal; and (iv) the accumulation of ubiquitinated proteins. These events correlated well with the reduction in cell viability. In addition, the thiol compound, N-acetylcysteine, could significantly inhibit the PG-induced ROS production, thereby preventing cytotoxicity, suggesting that the redox alteration is closely related to the pro-oxidant effect of cyclopentenone PGs. More strikingly, the lipid peroxidation end products, acrolein and 4-hydroxy-2-nonenal, detected in the PG-treated cells potently induced the ROS production, which was accompanied by the accumulation of ubiquitinated proteins and cell death, suggesting that the membrane lipid peroxidation products may represent one of the causative factors that potentiate the cytotoxic effect of cyclopentenone PGs by accelerating intracellular oxidative stress. These data suggest that the intracellular oxidative stress, represented by ROS production/lipid peroxidation and redox alteration, may underlie the well-documented biological effects, such as antiproliferative and antitumor activities, of cyclopentenone PGs.

Oxidative stress is increasingly seen as a major upstream component in the signaling cascade involved in many of the cellular functions such as cell proliferation, inflammatory responses, stimulating adhesion molecule, and chemoattractant production (1). It has been suggested that some level of oxidative stress may be required in response to cytotoxic agents and converted into the redox regulatory system as a downstream signaling pathway (2). However, excess oxidative stress may be toxic, exerting cytostatic effects, causing membrane damage, and activating pathways of cell death (apoptosis and/or necrosis). Reactive oxygen species (ROS)1 generated during oxidative stress may be responsible for these effects due to their ability to damage cellular components, such as membrane lipids. Lipid peroxidation mediated by a free radical chain reaction mechanism yields lipid hydroperoxides as primary products, and subsequent decomposition of the lipid hydroperoxides generates a large number of reactive aldehydes, such as ketoaldehydes, 2-alkenals, and 4-hydroxy-2-alkenals (3). There is increasing evidence that these aldehydes are causally involved in most of the pathophysiological effects associated with oxidative stress in cells and tissues.

The prostaglandins (PGs) are a family of structurally related molecules that are produced by cells in response to a variety of extrinsic stimuli and regulate cellular growth, differentiation, and homeostasis (4, 5). PGs are derived from fatty acids, primarily arachidonate, that are released from membrane phospholipids by the action of phospholipases. Arachidonate is first converted to an unstable endoperoxide intermediate by cylooxygenases and subsequently converted to one of several related products, including PGG2, PGE2, PGF2α, prostacyclin (PGL2), and thromboxane A2, through the action of specific PG synthetases. PGG2 is a major cylooxygenase product in a variety of tissues and cells and has marked effects on a number of biological processes, including platelet aggregation, relaxation of vascular and nonvascular smooth muscles, and nerve cell functions (6). PGG2 readily undergoes dehydration in vivo and in vitro to yield additional biologically active PGs of the J2 series (Fig. 1A) (7–9). Members of J2 series of PGs, characterized by the presence of a reactive α,β-unsaturated ketone in the cyclopentenone ring (cyclopentenone PGs), have their own unique spectrum of biological effects, including antitumor activity, the inhibition of cell cycle progression, the suppression of viral replication, the induction of heat shock protein expression, and the stimulation of osteogenesis (10).

In the present study, as part of an effort to identify endogenous inducer of intracellular oxidative stress and to elucidate the molecular mechanism underlying the oxidative stress-mediated cell degeneration, we examined the oxidized fatty acid

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1The abbreviations used are: ROS, reactive oxygen species; PGs, prostaglandins; 15d-PGJ2, 15-deoxy-Δ12,14-PGJ2; PGD2, prostaglandin D2; HNE, 4-hydroxy-2-nonenal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; DiOC6(3), 3,3′-dihexyloxycarbocyanine iodide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PBS, phosphate-buffered saline; mAb, monoclonal antibody; NAC, N-acetylcysteine; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; Δψ, mitochondrial membrane potential.
metabolites for their ability to induce intracellular ROS production in a human neuroblastoma SH-SY5Y cell and found that the J2 series of the PGs represent the most potent inducers. In addition, the intracellular ROS production was accompanied by the alteration of cellular redox status and the production of lipid peroxidation-derived highly cytotoxic aldehydes, such as acrolein and 4-hydroxy-2-nonenal (HNE), which could also induced the intracellular ROS production. These data suggest that intracellular oxidative stress constitutes a pivotal step in the pathway of cellular dysfunction induced by the PGs.

EXPERIMENTAL PROCEDURES

Materials—PGs and several other lipid peroxidation products were purchased from the Cayman Chemical Co. (Ann Arbor, MI). Horseradish peroxidase-linked anti-goat and anti-mouse IgG immunoglobulins and enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. The protein concentration was measured using the BCA protein assay reagent obtained from Pierce. 3,3′-Diethyloxacarbocyanine iodide (DiOC6(3)), carbonyl cyanide m-chlorophenylhydrazone (CCCP), and N-acetylcysteine (NAC) were from Sigma. 2,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was from Molecular Probes Inc. (Eugene, OR).

Cell Culture—SH-SY5Y cells were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% Nakashibetsu precoolerum newborn calf serum, 100 μg/ml penicillin, and 100 units/ml streptomycin. Cells were seeded in plates coated with polylysine and cultured at 37 °C.

Cell Viability—Cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells incubated with PGs or other chemicals were treated with 10 μl of MTT solution (5 mg/ml) for 4 h. The cells were then lysed with 0.04 N HCl in isopropyl alcohol, and the absorbance was read at 570 nm.

Flow Cytometry Analysis of ROS and Mitochondrial Membrane Potential (△Ψm)—DCFH-DA was employed to measure ROS (11, 12). Cells were incubated with 10 μM 2,7′-dichlorodihydrofluorescein diacetate (dissolved in dimethyl sulfoxide) for 30 min at 37 °C and then treated with different agents for an additional 30 min at 37 °C. After chilling on ice, the cells were washed with ice-cold PBS, resuspended from the plate, and re-suspended at 1 × 10^6 cells/ml in PBS containing 10 mM EDTA. For the detection of △Ψm, 40 nM DiOC6(3) (13) in the absence or presence of 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) was added and incubated for 15 min at 37 °C. The fluorescence was measured using a flow cytometer (Epics XL, Beckman Coulter).

Glutathione Assay—Measurement of GSH in the cells was performed fluorometrically according to the method of Sissin and Hilf (14). In brief, the cells incubated with HNE or NAC were washed twice with PBS (pH 7.0) and extracted with the 25% (w/v) metaphosphoric acid solution containing 5 mM EDTA. After ultracentrifugation (105,000 × g, 30 min), 1.8 ml of 0.1M phosphate solution (pH 8.0) containing 5 mM EDTA and 100 μl of the o-phenaldehyde solution (1 mg/ml) were added to the resulting supernatant (100 μl), and then the fluorescence intensity at 420 nm was then determined with activation at 350 nm.

Glutathione Peroxidase Assay—GSH peroxidase activity was determined according to the method of Lawrence and Burk (15). One unit was defined as the amount of enzyme required to oxidize 0.5 μmol of NADPH (corresponding to 1 μmol of reduced GSH) per min.

Measurements of Acrolein and HNE Levels—The levels of acrolein and HNE were measured, as their protein-bound forms, by competitive enzyme-linked immunosorbent assays (ELISA), using anti-protein-bound HNE (mAbHNEJ2) (16) and anti-protein-bound acrolein (mAb5F6) (17) monoclonal antibodies, as previously reported (18).

Immunoblot Analysis—For detection of the ubiquitinated proteins, whole cell lysates from SH-SY5Y cells treated with 15d-PGJ2 were incubated with SDS sample buffer for 5 min at 100 °C. The samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One gel was used for staining with Coomassie Brilliant Blue; the other gel was transblotted onto a nitrocellulose membrane, stained with anti-ubiquitin polyclonal antibody (Biomeda Co., Foster City, CA). This procedure was followed by the addition of horseradish peroxidase conjugated to rabbit anti-mouse IgG and ECL reagents. The bands were visualized by Cool Saver AE-6955 (ATTO, Tokyo, Japan).

Immunocytochemistry—For immunocytochemistry, cells were fixed overnight in PBS containing 2% paraformaldehyde and 0.2% picric acid at 4 °C. Membranes were permeabilized by exposing the fixed cells to PBS containing 0.3% Triton X-100. The cells were then sequentially incubated in PBS solutions containing blocking serum (5% normal goat serum) and immunostained with anti-protein-bound acrolein monoclonal antibody (mAb5F6) (17) or polyclonal antibodies that specifically recognize protein-bound HNE (19). The cells were then incubated for 1 h in the presence of fluorescein isothiocyanate-labeled goat anti-rabbit and Cy5-labeled goat anti-mouse, rinsed with PBS containing 0.3% Triton X-100, and covered with anti-fade solution. Images of cellular immunofluorescence were acquired using a confocal laser microscope (Bio-Rad) with a 40× objective (488-nm excitation and 518-nm emission).

Statistical Analysis—The paired Student’s t test was used to compare the significance of the differences between data.

RESULTS

Cyclopentenone PGs as Potential Inducers of Intracellular ROS Production—To identify endogenous inducer of intracellular oxidative stress, we screened a large number of lipophilic chemicals, including oxidized fatty acid metabolites, on induction of intracellular ROS production and found that some of the PG derivatives showed potent pro-oxidant effects on human neuroblastoma SH-SY5Y cells. As shown in Fig. 1, the intracellular ROS production in SH-SY5Y cells was significantly induced by PGA2 and by the PGD2 metabolites, such as PGJ2,
D\textsuperscript{2}-PGJ\textsubscript{2}, and 15d-PGJ\textsubscript{2}. Among the J\textsubscript{2} series of PGs, the intracellular ROS production was most potently induced by 15d-PGJ\textsubscript{2}, which was followed by its precursors, PGJ\textsubscript{2} and D\textsuperscript{2}-PGJ\textsubscript{2}. The level of ROS in the cells exposed to 15d-PGJ\textsubscript{2} (10 \(\mu\)M) was ∼10-fold higher than that of the control. Other oxidized fatty acids, including (9R)-hydroxy-(10E,12Z)-octadecadienoic acid, (+)-13-hydroxy-(9Z,11E)-octadecadienoic acid, (9S)-hydroperoxy-(10E,12Z)-octadecadienoic acid, (13S)-hydroperoxy-(9Z,11E)-octadecadienoic acid, 9-oxo-(10E,12Z)-octadecadienoic acid, 13-oxo-(9Z,11E)-octadecadienoic acid, (+)-13-hydroxy-(9Z,11E)-octadecadienoic acid cholesteryl ester, and (+)-9(10)-epoxy-(12Z)-octadecenoic acid, had no significant effects on the ROS production (data not shown).

**Correlation between ROS Production and Cytotoxicity**—To examine the correlation between ROS production and cytotoxicity in SH-SY5Y cells exposed to PGs, we examined the cytotoxic effects of PGs by MTT assay. As shown in Fig. 2A, among the PGs tested, the J series of PGs resulted in a rapid decrease in MTT reduction levels to <40% of basal levels within 24 h of exposure. A significant cytotoxicity was also observed when the cells were treated with PGE\subscript{2} and PGD\subscript{2} at the concentration of 25 \(\mu\)M (data not shown). In contrast to these PGs, the MTT reduction levels were maintained at 80–90% of basal levels in SH-SY5Y cells exposed to other PGs, such as 13,14-dihydro-15-keto-PGA\subscript{2}, PGA\subscript{2}, PGE\subscript{2}, and 15-keto-PGE\subscript{2}. As shown in Fig. 2B, 15-PGJ\subscript{2} induced cell death in time- and dose-dependent manners. Even 5 \(\mu\)M 15d-PGJ\subscript{2} did cause a 40% decrease in the MTT reduction level after 24 h of incubation, indicating that 15d-PGJ\subscript{2}, the terminal metabolite of PGD\subscript{2}, may represent the most potent cytotoxic metabolite. Similar results were obtained from other cell viability assays, such as crystal violet and trypan blue exclusion assays (data not shown). As shown in Fig. 2C, the PG-induced ROS production was well correlated with the cytotoxicity. The correlation between ROS production and cytotoxicity was also suggested by the observation that \(\alpha\)-tocopherol, a lipophilic antioxidant, significantly inhibited the PG cytotoxicity (Fig. 2D).

**Redox Alteration Induced by Cyclopentenone PGs**—A potential pathway that might mediate these effects of cyclopentenone PGs involves alteration of cellular redox status. To examine whether cyclopentenone PGs could influence the redox status, we measured the intracellular GSH levels and GSH peroxidase activity. As shown in Fig. 3A, the GSH levels were partially diminished by treatment with 15d-PGJ\textsubscript{2}. It was also observed that the 15d-PGJ\textsubscript{2} treatment of the cells resulted in a significant decrease in the GSH peroxidase activity (Fig. 3B). To confirm whether the PG-induced redox alteration plays a role in mediating the ROS production and cytotoxicity, SH-SY5Y cells were pretreated with the thiol compound NAC prior to the exposure to 15d-PGJ\textsubscript{2}, and then the ROS production and cell viability were examined. As shown in Fig. 3, C and D, the NAC pretreatment resulted in an increased survival as well as in the inhibition of ROS production in the cells. These data suggest that redox alteration may be closely related to the action of cyclopentenone PGs.

**Mitochondria as the Source of ROS**—It is believed that mitochondrial oxidative phosphorylation is the major endogenous source of the ROS and is involved in a wide variety of disorders (20). Therefore, it was anticipated that the ROS detected in the cells exposed to PGs may originate from the mitochondria, one of the major ROS-producing organella. In this context, we measured the alteration of the mitochondrial membrane potential (\(\Delta \Phi\)), which is a component of the overall proton motive force that drives the ATP production in the mitochondria. As
shown in Fig. 4A, 15d-PGJ₂ induced a significant decrease in mitochondrial Δψ, suggesting that the PG acted on the process of oxidative phosphorylation. We then examined the effect of CCCP treatment on the 15d-PGJ₂-induced ROS production. CCCP has a dissociable proton and acts by carrying protons across the inner mitochondrial membrane, resulting in depletion of mitochondrial electrochemical gradient (Δψ) by dissipating the proton gradient. As shown in Fig. 4B, CCCP alone did not cause ROS production, whereas the pretreatment of CCCP led to a dose-dependent inhibition of intracellular ROS production induced by 15d-PGJ₂. These results suggest that mitochondrial electron transport chain is involved in the 15d-PGJ₂-induced ROS production.

Accumulation of Protein-bound Aldehydes and Ubiquitinated Proteins in SH-SY5Y Cells Exposed to Cyclopentenone PGs—In addition to the ROS production, we found that the PG cytotoxicity was accompanied by the production of lipid peroxidation-derived highly cytotoxic aldehydes, such as acrolein and HNE (Fig. 5A), in the cells. The levels of acrolein and HNE were measured, as the protein-bound forms, by competitive ELISA and immunocytochemical assays. As shown in the Fig. 5B, 15d-PGJ₂ enhanced the productions of the protein-bound acrolein and HNE in a time-dependent manner. Maximum 3- and 8-fold increases in the production of acrolein and HNE, respectively, was observed, and the production of both modified proteins persisted for at least 24 h. Noncytotoxic PGs, such as PGA₂, PGB₂, and PGE₂, did not produce the protein-bound aldehydes (Fig. 5, C and D). Immunocytochemical analyses showed that exposure of the cells to 15d-PGJ₂ resulted in the appearance of acrolein and HNE reactivity in essentially all cells (Fig. 6). Since these aldehydes are known to be the most reactive electrophiles, it is likely that the cytotoxic effect of 15d-PGJ₂ was potentiated by these aldehydes.

On the other hand, it was anticipated that the PG-induced oxidative stress leading to the formation of oxidatively modified proteins provokes the misfolding of proteins, which may then be targeted for degradation by the ubiquitin-dependent proteolytic pathway (21). To examine whether the ubiquitin pathway is activated by the PG-induced oxidative stress, ubiquitin-protein conjugates generated in the cells were analyzed by an immunoblot analysis. As shown in Fig. 7A, the J series of PGs most significantly induced the generation of ubiquitin-protein conjugates with high molecular weights (>100 kDa); other PGs, including PGA₂, 15-keto-PGA₂, PGB₂, PGD₂, PGE₂, and 15-keto-PGE₂, were less effective or ineffective. Upon incubation with 15d-PGJ₂, the ubiquitinated proteins were detected from 30 min to 24 h and returned to the level of the control at 48 h (Fig. 7B). These data suggest that the PG-induced oxidative stress may lead to the increased ubiquitination of aberrant proteins, including oxidatively modified proteins.

Intracellular ROS Production and Cell Death Induced by Reactive Aldehydes—More strikingly, we found that the lipid peroxidation-derived reactive aldehydes could be a second source of ROS in the cells. As shown in Fig. 8A, when SH-SY5Y
cells were treated with a variety of reactive aldehydes, both acrolein and HNE caused a significant increase in the ROS levels. Interestingly, the \( \alpha,\beta \)-unsaturated aldehydes, such as crotonaldehyde and 2-nonenal, possessing an analogous functionality to acrolein and HNE, were all inactive. We also found that both acrolein and HNE showed the most potent cytotoxicity (Fig. 8B). In addition, generation of ubiquitinated protein was observed in the cells exposed to the aldehyde (HNE) (Fig. 8C). These results are consistent with the observation (Fig. 2) that the ROS production in the cells exposed to PGs was closely associated with the cytotoxicity. These data suggest that the reactive aldehydes, such as acrolein and HNE, may potentiate the effect of 15d-PGJ\(_2\) by accelerating the ROS production and redox alteration in the cells (Fig. 9).

**DISCUSSION**

PGD\(_2\) is known to be sequentially metabolized to PGJ\(_2\), \( \Delta_{12}\)-PGJ\(_2\), and 15d-PGJ\(_2\) (Fig. 1A) (7–9). A comparison of the PG biosynthetic pathway with the pro-oxidant profile reveals that induction of intracellular oxidative stress is mediated mainly by the metabolites of PGD\(_2\), the most active of which is the terminal metabolite 15d-PGJ\(_2\) (Fig. 1B). The induction potency was 15d-PGJ\(_2\) > \( \Delta_{12}\)-PGJ\(_2\) > PGJ\(_2\) > PGD\(_2\), indicating a gain in biological potency as the catabolism of PGD\(_2\) proceeds. Since PGJ\(_2\) is easily converted to \( \Delta_{12}\)-PGJ\(_2\), the activity of PGJ\(_2\) may be mediated by \( \Delta_{12}\)-PGJ\(_2\). If this is the case, the dienone structure of \( \Delta_{12}\)-PGJ\(_2\) and 15d-PGJ\(_2\) may be critical for the ROS production. As far as we know, this is the first report that demonstrated the intracellular ROS production by PGs.

A characteristic of cyclopentenone PGs is that they contain \( \alpha,\beta \)-unsaturated ketones, which are very susceptible to nucleophilic addition reactions with thiols, and are essential for the actions of the PGs (22, 23). It has been shown that PGA\(_1\) forms a monoconjugate with thiols, whereas \( \Delta_{12}\)-PGJ\(_2\) forms a bisconjugate with two thiols (24, 25). It has also been shown that the binding of PGA\(_1\) to synthetic polymer-supported thiols as a model of thiol-containing proteins is reversible but that of \( \Delta_{12}\)-PGJ\(_2\) is irreversible (26). Several previous observations have also indicated that PGD\(_2\) and its J-ring metabolites might exert effects through interactions with intracellular proteins as follows: (i) Narumiya et al. (27) have shown that radiolabeled \( \Delta_{12}\)-PGJ\(_2\) is actively incorporated into cells and transferred to the nucleus, where it is associated with proteins; and (ii) some PGs, including PGD\(_2\), PGJ\(_2\), and \( \Delta_{12}\)-PGJ\(_2\), have been shown to bind with high affinity to liver fatty acid-binding protein and intracellular protein involved in the uptake, intracellular transport, and metabolism of free fatty acids and their acyl-CoA esters (28).

Although the PGs, such as PGA\(_1\) and PGE\(_1\), have been reported to cause rapid degenerative changes in differentiated murine neuroblastoma cells in culture (29, 30), the molecular mechanism underlying the PG-induced cell degeneration had not been analyzed. Based on the observations that (i) 15d-PGJ\(_2\) partially reduced intracellular GSH levels (Fig. 3A), (ii) the 15d-PGJ\(_2\) treatment of the cells resulted in a significant decrease in the GSH peroxidase activity (Fig. 3B), and (iii) the

**FIG. 4.** Mitochondria as the source of intracellular ROS. A, time-dependent alterations of \( \Delta\phi \) induced by 15d-PGJ\(_2\). The cells were incubated with 40 nM DiOC\(_6\)(3) in the absence or presence of 15d-PGJ\(_2\) for 15 min at 37 \(^\circ\)C. B, effect of CCCP pretreatment on the 15d-PGJ\(_2\)-induced ROS production. The cells incubated with 10 \( \mu \)M DCFH-DA for 30 min were pretreated with CCCP (0–100 \( \mu \)M) for 30 min and then treated with 10 \( \mu \)M 15d-PGJ\(_2\) for 30 min.

**FIG. 5.** Detection of protein-bound reactive aldehydes in SH-SY5Y cells exposed to 15d-PGJ\(_2\). A, chemical structures of lipid peroxidation products, acrolein, and HNE. B, competitive ELISA analysis of protein-bound acrolein (open square) and protein-bound HNE (closed square) in the cells exposed to 10 \( \mu \)M 15d-PGJ\(_2\). C, competitive ELISA analysis of protein-bound HNE in the cells exposed to 10 \( \mu \)M PGs. D, competitive ELISA analysis of protein-bound acrolein in the cells exposed to 10 \( \mu \)M PGs. B, C, and D, the data represent means ± S.D. of triplicate determinations.
NAC pretreatment significantly inhibited both ROS production and cytotoxicity by 15d-PGJ2 (Fig. 3, C and D), intracellular redox status appeared to represent a critical parameter for the PG-induced ROS production and cytotoxicity. The fact that cyclopentenone PGs are susceptible to nucleophilic addition reactions with thiols (22, 23) suggests that the action of cyclopentenone PGs is closely related to direct reaction with GSH and/or other thiol compounds. However, L-buthionine-(SR)-sulfoximine, a specific inhibitor of GSH biosynthesis, itself did not so effectively induce intracellular ROS production and cell death, indicating that the effects of cyclopentenone PGs may not merely result from the GSH depletion alone and suggesting an involvement of other redox regulators. From this standpoint, it is noteworthy that the cyclopentenone PG dramatically induced a depletion of GSH peroxidase activity (Fig. 3). GSH peroxidase contains a selenocysteine residue, which is essential for peroxidase activity (31, 32). This selenocysteine residue resembles a cysteine residue in terms of chemical properties but has a higher reactivity (33, 34). ROS and electrophiles, such as cyclopentenone PGs and reactive aldehydes, are likely to react with the selenocysteine residue of GSH peroxidase via a Michael-type addition reaction, resulting in the depletion of GSH peroxidase activity. The redox alteration, represented by the depletion of the antioxidant defenses, may be closely associated with the induction of ROS production, leading to the acceleration of oxidative stress, and may be crucial for the PG-induced cell death (Fig. 9).

On the other hand, the findings that (i) the production of ROS and reactive aldehydes represents the early cellular event observed within 30 min, (ii) the PG-induced ROS production was well correlated with the cytotoxicity (Fig. 2C), and (iii) α-tocopherol, a lipophilic antioxidant, significantly inhibited the PG cytotoxicity (Fig. 2D) suggest that intracellular ROS production may be involved, at least in part, in the cell death induced by the cyclopentenone PGs (Fig. 9). Although the detailed mechanism for the PG-induced ROS production is currently unknown, the accumulating data suggest the involvement of mitochondria, one of the major ROS-producing organelles. It is believed that inhibition of electron transport chain components involved in oxidative phosphorylation is the major pathway for generation of ROS (35). The findings that cyclopentenone PGs could directly modify cellular proteins, such as IκB kinase β (36, 37) and human serum albumin (38), suggest that reversible or irreversible modification of intracellular proteins with cyclopentenone PGs is involved in the mitochondrial ROS production followed by cell death. Cyclopentenone PGs may therefore inhibit the process of oxidative phosphorylation by direct modification of electron transport chain components, leading to the accumulation of electrons in the early stages of electron transport chain, where they can be donated directly to molecular oxygen to give ROS. ROS may further inactivate the iron-sulfur (Fe-S) centers of electron transfer chain complexes I, II, and III, resulting in shutdown of mitochondrial energy production and dysfunction of mitochondrial oxidative phosphorylation (35). ROS could also react with the thiol groups of GSH localized at the mitochondrial membrane level (39) and contribute to the lower GSH level. A low level of GSH could favor the decrease in Δψ, which would subsequently activate the opening of permeability transition pores to finally induce the release of cell death-promoting fac-

FIG. 6. Immunocytochemical detection of protein-bound HNE and protein-bound acrolein in SH-SY5Y cells exposed to 15d-PGJ2. The cells were incubated with 10 μM 15d-PGJ2 at 37 °C. The digitized images were colorized and combined using Adobe Photoshop, 3.0. Fluorescein isothiocyanate fluorescence (protein-bound HNE, green) is shown in the left column of panels (a–d); Cy3 fluorescence (protein-bound acrolein, red) is shown in the center column of panels (e–h), and the corresponding combined (superimposed) images are shown in the right column of panels (i–l) (yellow represents colocalization).
titors, including cytochrome c, the apoptosis-inducing factor, and latent forms of specialized proteases called caspases (20). Indeed, the 15d-PGJ2-induced reduction in cell viability was morphologically characterized by rounded cells and condensed nuclei. In addition, we have observed that 15d-PGJ2-induced DNA fragmentation in SH-SY5Y cells. These observations suggest that cyclopentenone PGs may induce apoptotic cell death.2

Thus, oxidative stress leads to a marked reduction in mitochondrial energy production, and an increase in oxidative stress could activate the mitochondrial permeability transition pore and initiate apoptosis.

It is also noticeable that the increased intracellular ROS production induced by cyclopentenone PGs was accompanied by the production of lipid peroxidation products, such as acrolein and HNE, in the cells (Figs. 5 and 6). Among all the α,β-unsaturated aldehydes, acrolein and HNE represent the strongest electrophiles and show the highest reactivity with nucleophiles, such as proteins and DNA (3). These aldehydes have been shown to be toxic to cultured cells (40–42) and have received considerable attention as endogenous cytotoxic agents that potentially could play a role in the pathogenesis of many degenerative diseases, including cardiovascular and neurodegenerative disorders (43, 44). The observations in the present study are in line with the accumulating body of literature supporting the role of lipid peroxidation at some point in the pathogenesis of these disorders. We also found that these reactive aldehydes further exerted an increased production of intracellular ROS (Fig. 8). The intracellular oxidative stress results in the production of reactive aldehydes, which may trigger the production of ROS and accelerate oxidative stress (Fig. 9). These sequential events may lead to the delayed onset of cell death. Due to the fact that cell death depends mainly on the extent of stress and involves multiple cellular processes, chronic exposure to ROS and reactive aldehydes may be required for the phenotypic expression of cell death.

It has recently been shown that cyclopentenone PG-like compounds, αJ2-iso prostanes, are formed in vivo as the products of isoprostane pathway (38, 45). As the reactive α,β-unsaturated carbonyl seems essential for the cyclopentenone PG-induced ROS production and cytotoxicity, it is anticipated that all of the cyclopentenone isoprostanes may exert similar effects. In addition, the observations that the cyclopentenone isoprostanes are detected in abundant quantities following induction of oxidative injury (38, 45) suggest that not only cyclopentenone PGs but also cyclopentenone isoprostanes may exert biological effects relevant to the pathobiology of oxidative stress.

PGs are physiologically present in body fluids in picomolar-to-nanomolar concentrations (46); however, arachidonic metabolism is highly increased in several pathological conditions, including hyperthermia, infection, and inflammation (47), and local PG concentrations in the micromolar range have been detected at sites of acute inflammation (48). In addition, elevated cyclopentenone PG synthesis has also been detected in the late phases of inflammation (49). Therefore, up-regulation of PG biosynthesis is suggested to be involved in the pathophysiological processes relevant to inflammatory responses. The findings that (i) PGD2, the precursor of cyclopentenone PGs, is one of the most abundantly produced PGs in several tissues, (ii) PGD2 can be converted readily to J2 PGs in the presence of plasma in vivo (8), and (iii) the cyclopentenone PGs, such as 15d-PGJ2, have been shown to be formed from PGD2 in vivo (9) strongly suggest that levels of PGD2 derivatives may reach functionally significant levels in inflammation and its related disorders.

In conclusion, these experiments demonstrate that the J2 series of the PGs represent the most potent inducers of intracellular oxidative stress and that the production of ROS in the
cells is closely associated with the excitotoxic effect of PGs. Although the present data were obtained from the in vitro experiments using a cell line, it is suggested that the intracellular oxidative stress may underlie the well-documented antiproliferative and antitumor effects of cyclopentenone PGs. The present study may therefore represent a first step in establishing a link between the intracellular oxidative stress and cell degeneration induced by the PGs.

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