An Endogenous Electrophile that Modulates the Regulatory Mechanism of Protein Turnover: Inhibitory Effects of 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 on Proteasome†

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ABSTRACT: Prostaglandin D_2 (PGD_2), a major cyclooxygenase product in a variety of tissues and cells, readily undergoes dehydration to yield electrophilic PGs, such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2). We have previously shown that 15d-PGJ_2 potently induces apoptosis of SH-SY5Y human neuroblastoma cells *via* accumulation of the tumor suppressor gene product p53. In the study presented here, we investigated the molecular mechanisms involved in the 15d-PGJ_2-induced accumulation of p53. It was observed that 15d-PGJ_2 potently induced p53 protein expression but scarcely induced p53 gene expression. In addition, exposure of the cells to 15d-PGJ_2 resulted in an accumulation of ubiquitinated proteins and in a significant inhibition of proteasome activities, suggesting that 15d-PGJ_2 acted on the ubiquitin−proteasome pathway, a regulatory mechanism of p53 turnover. The effects of 15d-PGJ_2 on the protein turnover were attributed to its electrophilic feature, based on the observations that (i) the reduction of the double bond in the cyclopentenone ring of 15d-PGJ_2 virtually abolished the effects on protein turnover, (ii) overexpression of an endogenous redox regulator, thioredoxin 1, significantly retarded the inhibition of proteasome activities and accumulations of p53 and ubiquitinated proteins induced by 15d-PGJ_2, and (iii) treatment of SH-SY5Y cells with biotinylated 15d-PGJ_2 indeed resulted in the formation of a 15d-PGJ_2−proteasome conjugate. These data suggest that the modulation of proteasome activity may be involved in the mechanism responsible for the accumulation of p53 and subsequent induction of apoptotic cell death induced by 15d-PGJ_2.

The prostaglandins (PGs)[1] are a family of structurally related molecules that are produced by cells in response to a variety of extrinsic stimuli and that regulate cellular growth, differentiation, and homeostasis (*1*, *2*). PGs are derived from fatty acids, primarily arachidonate, which are released from membrane phospholipids by the action of phospholipases. Arachidonate is first converted to an unstable endoperoxide intermediate by cyclooxygenases and then is subsequently converted to one of several related products, including PGD_2, PGE_2, $PGF_2\alpha$, prostacyclin (PGI_2), and thromboxane A_2, through the action of specific PG synthetases. PGD_2 is a major cyclooxygenase-catalyzed reaction product in a variety of tissues and cells and has significant effects on a number of biological processes, including platelet aggregation, the relaxation of vascular and nonvascular smooth muscles, and nerve cell functions (*3*). It has been shown that PGD_2 readily undergoes dehydration *in vivo* and *in vitro* to yield biologically active PGs of the J_2 series, such as PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (Figure 1) (*4*). Members of the J_2 series of the 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 (*5*).

Many types of mammalian cells undergo apoptosis during normal development or in response to a variety of stimuli, including DNA damage, growth factor deprivation, and abnormal expression of oncogenes or tumor suppressor genes (*6*−*8*). Apoptosis induced by these various agents appears to be mediated by a common set of downstream elements that act as regulators and effectors of apoptotic cell death.

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[1] Abbreviations: PGs, prostaglandins; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; PGD_2, prostaglandin D_2; COX-2, cyclooxygenase-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; s-LLVY-MCA, succinyl-leucine-leucine-valine-tyrosine-MCA; Boc-LSTR-MCA, butoxycarbonyl-leucine-serine-threonine-arginine-MCA; Z-LLE-βNA, benzyloxycarbonyl-leucine-leucine-glutamate-βNA; ALS, amyotrophic lateral sclerosis; Trx, thioredoxin 1; MAPK, mitogen-activated protein kinase.

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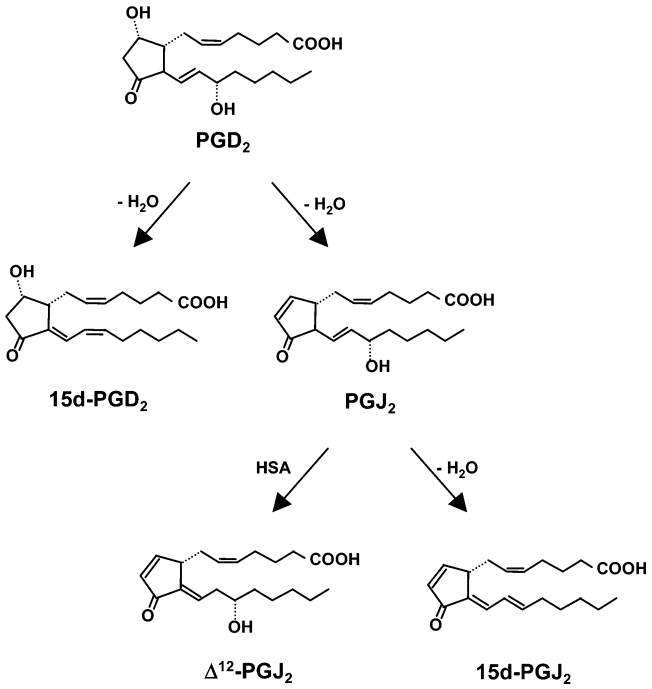

FIGURE 1: PGD_2 metabolic pathway.

In many cases, apoptosis requires the p53 tumor suppressor protein (*9*). Overexpression of p53 not only is induced by several cell death stimuli but also is itself sufficient to induce apoptosis in gene transfer assays (*10*). We have recently shown that 15d-PGJ_2 induces apoptosis of SH-SY5Y human neuroblastoma cells *via* accumulation of p53. Moreover, the Fas/Fas ligand (FasL) pathway has been identified as the downstream signaling mechanism in p53-mediated apoptosis (*11*).

In the study presented here, we investigated the molecular mechanism involved in the p53-mediated neuronal cell death induced by 15d-PGJ_2 and found that the accumulation of p53 could, at least in part, be attributed to disruption of the proteasome-dependent regulatory mechanism of protein turnover. Proteasome is a large multisubunit protease complex that selectively degrades intracellular proteins (*12*−*14*). Most of the proteins removed by these proteases are tagged for destruction by ubiquitination. Proteasome has a role to play in controlling cellular processes, such as metabolism and the cell cycle, through signal-mediated proteolysis of key enzymes and regulatory proteins. It also operates in the stress response by removing abnormal proteins and in the immune response by generating antigenic peptides. Thus, our findings suggest that 15d-PGJ_2 could be an endogenous modulator of the proteasome-dependent regulation of cellular functions.

EXPERIMENTAL PROCEDURES

Materials. 15d-PGJ_2 and 9,10-dihydro-15d-PGJ_2 were obtained from the Cayman Chemical Co. (Ann Arbor, MI). The anti-p53 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-ubiquitin polyclonal antibody was obtained from Biomega Co. (Foster City, CA). 26S proteasome was purified from rat liver as described previously (*15*). Succinyl-leucine-leucine-valine-tyrosine-MCA (s-LLVY-MCA) for the chymotrypsin activity and butoxycarbonyl-leucine-serine-threonine-arginine-MCA (Boc-LSTR-MCA) for the trypsin activity of proteasome and the proteasome inhibitor, MG132, were obtained from the Peptide Institute, Inc. (Osaka, Japan). Benzyloxycarbonyl-leucine-leucine-glutamate-βNA (Z-LLE-βNA), a substrate of the peptidyl-glutamyl peptide hydrolase activity of proteasome, was obtained from Sigma. Anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal and anti-20S proteasome β-subunit polyclonal antibodies were obtained from Chemicon (Temecula, CA) and Calbiochem (La Jolla, CA), respectively. Horseradish peroxidase-linked anti-goat and anti-mouse IgG immunoglobulins and enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham. The protein concentration was measured using the BCA protein assay reagent obtained from Pierce.

Cell Culture and Cell Viability. Human SH-SY5Y neuroblastoma cells (a kind gift of W. Maruyama of the National Institute of Longevity Sciences) were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% Nakashibetsu precolostrum newborn calf serum. Cells were seeded in plates coated with polylysine and cultured at 37 °C. Cell viability was quantified with the 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 an MTT solution (5 mg/mL) for 4 h. The cells were then lysed with 0.04 N HCl in 2-propanol, and cell viability was determined 24 h later by the level of formazan production from diphenyltetrazolium salt using a multiplate reader at 570 nm (630 reference filter).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated with the isogen reagent (Nippon Gene, Tokyo, Japan) and spectrophotometrically quantified. The RT reaction was performed with 10 μg of total RNA and an oligo(dT) primer using the First-Standard cDNA synthesis kit (Life Technology, Inc., Rockville, MD). The reaction mixture was then subjected to a brief incubation at 65 °C to inactivate the enzyme. PCRs were carried out in a final volume of 25 μL consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 200 μM amounts of each deoxyribonucleoside triphosphate, 0.5 μL of the RT first-standard cDNA products, each forward and reverse primer (1 μM each), and 1.25 units of r*Taq* DNA polymerase (Toyobo Co., Osaka, Japan). The reaction mixtures were heated at 94 °C for 3 min and then immediately cycled 35 times through a 1 min denaturing step at 94 °C, a 1.5 min annealing step at 54 °C, and a 1 min extension step at 72 °C. The following primers were used: p53, 5′-CAGCCAAGTCTGTGACTTGCACGTAC-3′ (forward) and 5′-CTATGTCGAAAAGTGTTTCTGTCATC-3′ (reverse).

Proteasome Activity. The peptidase activity of proteasome was measured using three peptidase activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase) with fluorogenic peptides as substrates according to the method of previous reports (*16*, *17*). Cells incubated with 15d-PGJ_2 were washed with phosphate-buffered saline twice and homogenized in 0.8% digitonin (pH 7.8) containing 2 mM EDTA for 10 min at 37 °C. Afterward, the lysates were centrifuged at 5000 rpm for 5 min, and the supernatants were collected as whole cell extracts. The activities of proteasome were measured as follows. Whole cell extracts were incubated with 200 μM fluorogenic peptide substrates for 30 min

at 37 °C in 100 μL of assay buffer [100 mM Tris-HCl (pH 8.0) containing 1 mM DTT]. After incubation, the reaction mixture was added to 2 mL of 100 mM Tris-HCl (pH 9.0) and 100 μL of 10% SDS followed by a measurement of the fluorescence of the cleavage products using a model F-2000 fluorescence spectrophotometer (Hitachi). The fluorescent cleavage products of s-LLVY-MCA and Boc-LSTR-MCA were detected at an emission wavelength of 460 nm (excitation wavelength of 380 nm), and the fluorescent group of Z-LLE-βNA was detected at 410 nm (excitation at 335 nm).

Preparation of Biotinylated 15d-PGJ$_2$. The carboxyl group of 15d-PGJ$_2$ was modified by amidation with EZ-link 5-(biotinamido)pentylamine by a modification of a previously described procedure (*18*). Briefly, 1 mg of 15d-PGJ$_2$ (isomeric mixture containing 90−95% of the *trans*,*cis*-12,14 isomer) was made to react with 1 mg of (5-biotinamido)-pentylamine in the presence of the condensing agent 1-ethyl-3-(dimethylaminopropyl)carbodiimide. The reaction was carried out overnight at room temperature in acetonitrile. Biotinylated 15d-PGJ$_2$ was then purified with a reverse-phase HPLC system equipped with a Develosil ODS-HG-5 column (4.6 mm × 250 mm) using a linear gradient from 10% aqueous acetonitrile to 100% acetonitrile for 30 min. The elution profiles were monitored by the absorbance at 310 nm. The biotinylated 15d-PGJ$_2$ was dried under argon and dissolved in DMSO for further use.

Labeling of Proteasome with Biotinylated 15d-PGJ$_2$ in SH-SY5Y Cells. SH-SY5Y cells were incubated with 10 μM biotinylated 15d-PGJ$_2$ for 2 h. Cells were washed twice with phosphate-buffered saline, harvested, and lysed in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% SDS, 1% Triton, and protease inhibitors. Cell lysates containing 200 μg of protein were incubated by batch with 50 μL of NeutrAvidin-Plus beads for 24 h at 4 °C with constant shaking. Beads were washed three times with lysis buffer by centrifugation at 6000 rpm for 1 min. Proteins were eluted by boiling the beads in Laemmli sample buffer for 5 min and analyzed by SDS−polyacrylamide gel electrophoresis followed by immunodetection with the anti-proteasome antibody.

Binding of Biotinylated 15d-PGJ$_2$ to Purified Proteasome. The proteasome solution (40 μg/mL) was coated overnight at 4 °C on the wells of the microtiter plate. Following the wells had been washed with PBS containing 0.05% Tween 20 (PBS/Tween), 100 μL of the biotinylated 15d-PGJ$_2$ solution was added to the wells. After incubation for 2 h at 37 °C, followed by washing with PBS/Tween, each well was filled with 200 μL of Block Ace solution (40 mg/mL) for 1 h at 37 °C. The peroxidase-conjugated NeutrAvidin solution was added and the mixture incubated for 1 h at 37 °C. After washing had been carried out, 100 μL of 0.05 M citrate buffer (pH 5.5) containing 0.4 mg/mL *o*-phenylenediamine and 0.003% H_2O_2 was added and the mixture incubated for several minutes at room temperature. The reaction was terminated by adding 50 μL of 2 M sulfuric acid, and the absorbance at 490 nm was read on a micro-ELISA plate reader.

Stable Transfection with Thioredoxin 1 (Trx) in SH-SY5Y Cells. SH-SY5Y cells were transfected with the pcTrx1 vector or with the vector alone using GenePORTER transfection reagent (Gene Therapy Systems, Inc.). In these experiments, 1×10^6 cells were incubated with the DNA-GenePORTER mixture (2 μg of DNA/10 μL of GenePORTER) in 1 mL of serum-free Opti-MEM (Gibco) at 37 °C. After incubation for 6 h, 1 mL of complete medium was added, and the cells were cultured for 18 h. Thereafter, stable transfectants were isolated by selection on 500 μg/mL G418 for ~3 weeks. Single clones of stably transfected cells were isolated by limiting dilution. Several G418-resistant stable clones were maintained in medium containing 500 μg/mL G418.

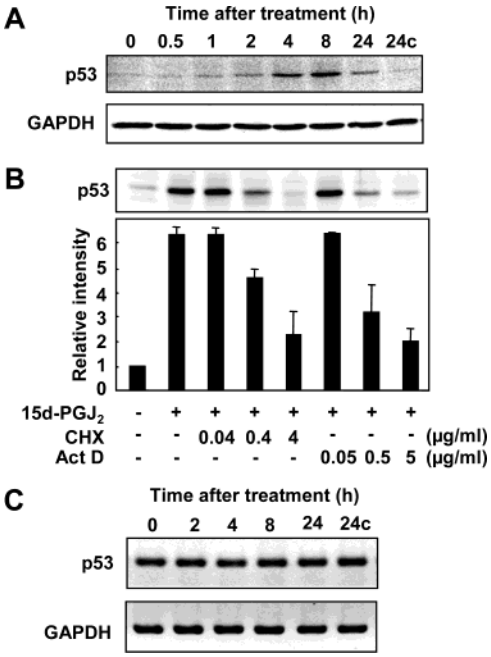

FIGURE 2: Induction of p53 in SH-SY5Y cells treated with 15d-PGJ$_2$. (A) Time-dependent induction of the p53 protein in SH-SY5Y cells treated with 10 μM 15d-PGJ$_2$. Lane 24c shows the level of p53 protein in the cells treated with vehicle (DMSO) alone for 24 h. (B) Effect of actinomycin D (Act D) or cycloheximide (CHX) on 15d-PGJ$_2$-induced accumulation of the p53 protein. The results that are shown are means ± the standard error of the mean of three independent experiments. (C) Time-dependent changes in p53 gene expression in SH-SY5Y cells treated with 10 μM 15d-PGJ$_2$ for different time intervals. Lane 24c shows the level of p53 mRNA in the cells treated with vehicle (DMSO) alone for 24 h.

RESULTS

15d-PGJ$_2$-Induced Accumulation of p53. Exposure of cells to p53-activating signals can lead within a relatively short time to a marked elevation in the level of the p53 protein. To some extent, this can be achieved by enhanced translation of the p53 mRNA, probably involving relief of a translational repression mechanism operating through the 3′-untranslated region of this mRNA (*19*). To establish the molecular mechanism involved in the p53-mediated neuronal cell death induced by 15d-PGJ$_2$, we investigated the regulatory mechanism of p53 protein accumulation. As shown in Figure 2A, the time course of p53 protein induction in SH-SY5Y cells treated with 15d-PGJ$_2$ (10 μM) revealed a maximal accumulation at 8 h followed by a gradual decline to 24 h. The 15d-PGJ$_2$-induced accumulation of the p53 protein was found to require both RNA and protein synthesis, because actinomycin D and cycloheximide, which block RNA and protein synthesis, respectively, inhibited the accumulation of the p53 protein in a dose-dependent manner (Figure 2B). Meanwhile, there was no apparent increase in the p53 mRNA

levels in response to the 15d-PGJ_2 treatment (Figure 2C). It should also be noted that the 15d-PGJ_2-induced accumulation of the p53 protein was also observed in other neuronal cell lines, such as neuronal-like pheochromocytoma PC12 cells and murine neuroblastoma Neuro 2A cells (data not shown). These data suggest that the 15-PGJ_2-induced accumulation of p53 may represent a general mechanism underlying neuronal cell death induced by the cyclopentenone PGs of the J_2 series.

15d-PGJ_2-Induced Modulation of Protein Turnover. The observations that the 15d-PGJ_2-induced accumulation of the p53 protein required both RNA and protein synthesis (Figure 2B) and that 15d-PGJ_2 scarcely induced gene expression of p53 (Figure 2C) suggested that 15d-PGJ_2 might affect the regulatory mechansim of p53 protein turnover. It is generally accepted that p53 protein turnover is regulated by multiple mechanisms, including regulation of p53 protein degradation and covalent modifications of p53, particularly protein phosphorylation (*20*). p53 becomes phosphorylated on multiple sites in response to various types of stress through the mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 (*21*). A suggested potential outcome of such phosphorylation is the stabilization of p53 through inhibition of p53 ubiquitination and degradation. We indeed observed that 15d-PGJ_2 significantly induced the phosphorylation (activation) of all three MAPKs; however, the 15d-PGJ_2-induced p53 accumulation was not suppressed by their specific inhibitors (data not shown). These data suggest that the MAPK pathways may not be involved in the 15d-PGJ_2-induced accumulation of p53 in SH-SY5Y cells.

On the other hand, because the rapid demise of p53 is known to be largely achieved through the proteasome-dependent protein degradation pathway (*20*), it was hypothesized that 15d-PGJ_2 might act on this mechanism. Indeed, the effects of 15d-PGJ_2 on SH-SY5Y cells were fully reproduced by treatment of the cells with a specific proteasome inhibitor, MG132, demonstrating significant induction of p53 accumulation and cytotoxicity (Figure 3A,B). Although MG132 is a reversible inhibitor of proteasome, we observed significant inhibition of proteasome activity by MG132 (Figure 3A, bottom panel), and the level of proteasome inhibition observed in the cells seemed to be correlated with the extent of accumulation of p53. Moreover, treatment of cells with 15d-PGJ_2 or MG132 resulted in the enhanced accumulation of ubiquitinated proteins (Figure 3C). It was also observed that 15d-PGJ_2 partially inhibited the proteasome activities, such as chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase activities (Figure 3D). These observations suggest that the modulation of proteasome may be involved in the mechanism responsible for the accumulation of p53 and ubiquitinated proteins in the cells exposed to 15d-PGJ_2.

The Electrophilic Center of 15d-PGJ_2 Represents the Active Site for Modulation of Protein Turnover. It was anticipated that the α,β-unsaturated carbonyl group in the cyclopentenone ring of 15d-PGJ_2 might be a prerequisite for the inhibition of p53 turnover. To prove this hypothesis, SH-SY5Y cells were exposed to 15d-PGJ_2 and its analogue 9,10-dihyro-15-deoxy-$\Delta^{12,14}$-PGJ_2 (9,10-dihydro-15d-PGJ_2) (Figure 4A), and changes in the protein turnover, monitored by the accumulation of p53 and ubiquitinated proteins, were

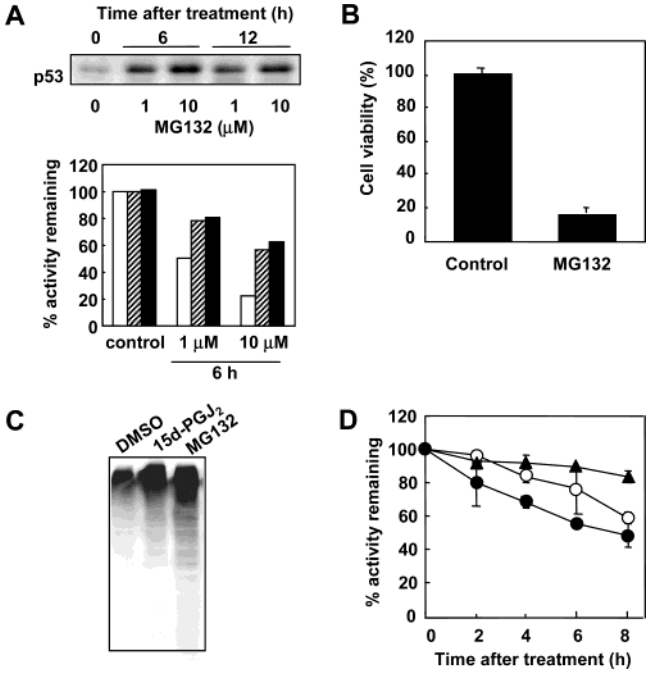

FIGURE 3: Involvement of the proteasome pathway in the 15d-PGJ_2-induced accumulation of p53 and ubiquitinated proteins. (A) Effect of a specific proteasome inhibitor MG132 on p53 accumulation (top panel) and proteasome activity (bottom panel) in SH-SY5Y cells. The cells were exposed to 1 or 10 μM MG132: (empty bars) chymotrypsin-like activity, (hatched bars) trypsin-like activity, and (filled bars) peptidylglutamyl peptide hydrolase activity. (B) Cytotoxicity of MG132. The cells were exposed to 10 μM MG132 for 24 h. The results that are shown are means $\pm$ the standard error of the mean of three independent experiments. (C) Enhanced accumulation of ubiquitinated proteins in the cells exposed to 15d-PGJ_2 or MG132. The cells were exposed to 10 μM 15d-PGJ_2 or 10 μM MG132 for 24 h. (D) Effect of 15d-PGJ_2 on proteasome activities in SH-SY5Y cells. The proteasome activities were measured using the fluoropeptides, s-LLVY-MCA for the chymotrypsin-like activity (▲), Boc-LSTR-MCA for the trypsin-like activity (●), and Z-LLE-βNA for the peptidylglutamyl peptide hydrolase activity (○), as proteolytic substrates. In the absence of 15d-PGJ_2, we could not see any changes in the proteasome activities during the incubation periods. The results that are shown are means $\pm$ the standard error of the mean of three independent experiments.

examined. As shown in panels B and C of Figure 4, 15d-PGJ_2 and MG132 significantly inhibited the accumulation of p53 and ubiquitinated proteins, which was well correlated with the level of proteasome inhibition, whereas 9,10-dihydro-15d-PGJ_2 had no significant effects on the turnover of these proteins. Thus, the reduction of the double bond in the cyclopentenone ring of 15d-PGJ_2 virtually abolished the effect of 15d-PGJ_2, indicating that these biological activities of 15d-PGJ_2 can be attributed to its electrophilic center.

Effect of Trx Overexpression on 15d-PGJ_2-Induced Modulation of Protein Turnover. The data thus far suggested that cellular redox status might play a role in the 15d-PGJ_2-induced p53 accumulation. On the basis of the previous findings that Trx, a key molecule in the maintenance of cellular redox balance, is known to play critical roles in protecting cells against oxidative stress and in mediating signal transduction (22), we investigated the role of this redox regulator in 15d-PGJ_2-induced accumulation of p53 and ubiquitinated proteins. To this end, we established Trx-overexpressing derivatives of SH-SY5Y cells (*23*). For the experiments described below, results were compared between

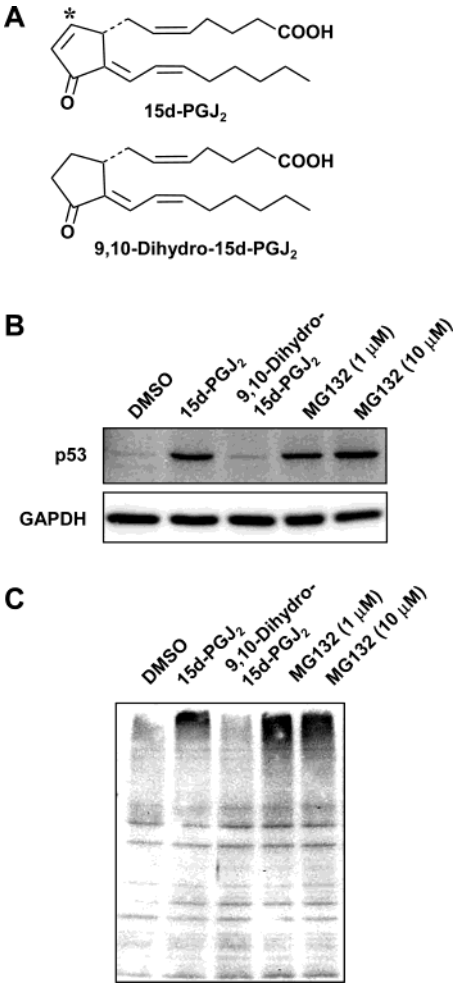

FIGURE 4: Electrophilic center of 15d-PGJ_2 which is involved in the accumulation of p53 and ubiquitinated proteins. (A) Chemical structures of 15d-PGJ_2 (top) and 9,10-dihydro-15d-PGJ_2 (bottom). The asterisk denotes the electrophilic carbon (position 9). (B) Effect of 15d-PGJ_2, 9,10-dihydro-15d-PGJ_2, and MG132 on p53 accumulation and proteasome activity in SH-SY5Y cells. The proteasome activity was measured using the fluoropeptides, s-LLVY-MCA, for the chymotrypsin-like activity as the proteolytic substrate. (C) Effect of 15d-PGJ_2, 9,10-dihydro-15d-PGJ_2, and MG132 on accumulation of ubiquitinated proteins in SH-SY5Y cells. In panels B and C, the cells were exposed to 15d-PGJ_2 (10 μM), 9,10-dihydro-15d-PGJ_2 (10 μM), or MG132 (1 or 10 μM) for 8 h.

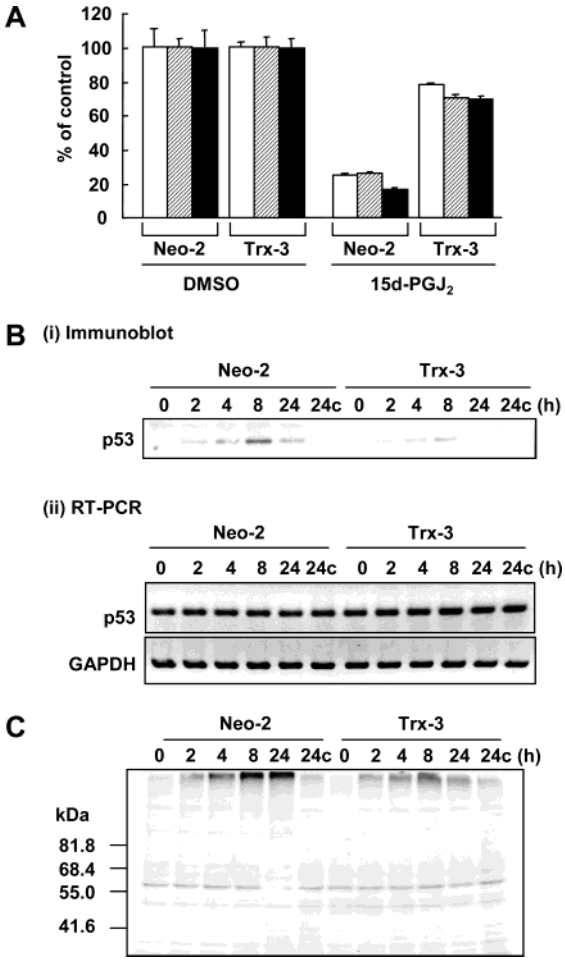

FIGURE 5: Effect of overexpression of Trx on 15d-PGJ_2-induced reduction of proteasome activities and accumulation of p53 and ubiquitinated proteins. Both control (Neo-2) and Trx-overexpressed (Trx-3) cells were incubated with 10 μM 15d-PGJ_2. (A) Effect of overexpression of Trx on 15d-PGJ_2-induced reduction of proteasome activities. The proteasome activities were measured using the fluoropeptides, s-LLVY-MCA for the chymotrypsin-like activity (empty bars), Boc-LSTR-MCA for the trypsin-like activity (hatched bars), and Z-LLE-βNA for the peptidylglutamyl peptide hydrolase activity (filled bars), as proteolytic substrates. The results that are shown are means $\pm$ the standard error of the mean of three independent experiments. (B) Effect of overexpression of Trx on 15d-PGJ_2-induced accumulation of p53. (C) Effect of overexpression of Trx on 15d-PGJ_2-induced accumulation of ubiquitin−protein conjugates.

empty-vector transfected control cells (Neo-2) and Trx-transfected cells (Trx-3), to assess the effectiveness of expressed Trx in protecting against 15d-PGJ_2-induced modulation of protein turnover. As shown in Figure 5A, the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase activities of proteasome in the Neo-2 cells exposed to 10 μM 15d-PGJ_2 decreased to 26, 28, and 18% of the control values of untreated cells, respectively, after incubation for 24 h, whereas the inhibition of proteasome activities was significantly abrogated by the overexpression of Trx. In addition, the protection against 15d-PGJ_2-induced reduction of proteasome activities was associated with the retardation of the accumulation of detrimental proteins, such as p53 and ubiquitinated proteins. As shown in Figure 5B, while there were no significant changes in the p53 mRNA levels in both Neo-2 and Trx-3, it was observed that the level of p53 protein in the Neo-2 cells exposed to 15d-PGJ_2 (10 μM) for 8 h was ~5-fold greater than that of vehicle-treated cells and that the p53 protein accumulation in the Trx-overexpressed cells (Trx-3 cells) exposed to 15d-PGJ_2 was inhibited to ~50% of the vector control. Moreover, we also observed that the 15d-PGJ_2-induced accumulation of ubiquitinated proteins was significantly suppressed by the Trx overexpression (Figure 5C). These data suggest that the 15d-PGJ_2-induced modulation of protein turnover is closely associated with the cellular redox status.

Covalent Binding of 15d-PGJ_2 to Cellular Proteasome. Because the protein levels of the proteasome subunit (19S subunit S1 and 20S β-subunit) were nearly unchanged by treatment of SH-SY5Y cells with 15d-PGJ_2 (data not shown), it was presumed that the partial loss of proteasome activity (Figure 3D) might be ascribed to the direct interaction of 15d-PGJ_2 with proteasome. To assess if the inhibition of proteasome activity elicited by 15d-PGJ_2 in SH-SY5Y cells could be due to the covalent modification of proteasome,

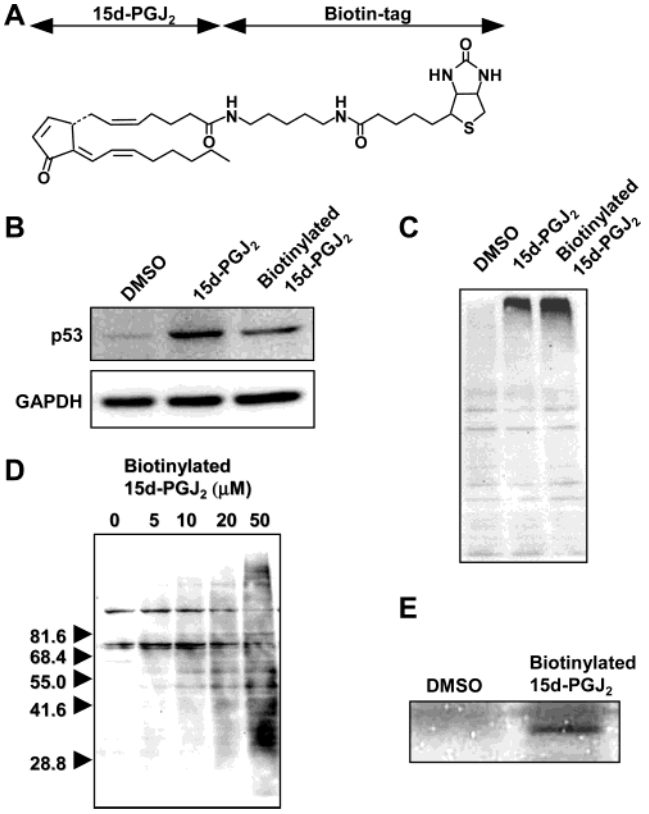

FIGURE 6: Modification of proteasome by a biotinylated 15d-PGJ_2 derivative in SH-SY5Y cells. (A) Chemical structure of biotinylated 15d-PGJ_2. (B) Effect of 15d-PGJ_2 and its biotinylated derivative on p53 accumulation in SH-SY5Y cells. (C) Effect of 15d-PGJ_2 and its biotinylated derivative on accumulation of ubiquitinated proteins in SH-SY5Y cells. In panels B and C, the cells were exposed to 10 μM 15d-PGJ_2 or 10 μM biotinylated 15d-PGJ_2 for 8 h. (D) Biotinylated 15d-PGJ_2 labeling of cellular proteins in SH-SY5Y cells. SH-SY5Y cells were treated with biotinylated 15d-PGJ_2 for 2 h. Biotinylated 15d-PGJ_2 bound to cellular proteins was detected by immunoblot experiments using HRP-bound NeutrAvidin. (E) Biotinylated 15d-PGJ_2 labeling of proteasome in SH-SY5Y cells. SH-SY5Y cells were treated with 10 μM biotinylated 15d-PGJ_2 for 2 h, and cell lysates were incubated by batch with NeutrAvidin beads. After the mixture had been extensively washed, proteins bound to the resin through biotinylated PG were eluted by boiling in Laemmli sample buffer, and proteasome present in the eluate was detected by immunoblot analysis using an anti-proteasome β-subunit antibody.

we prepared a biotinylated 15d-PGJ_2 analogue (Figure 6A) and studied its interaction with proteasome in intact cells. As shown in panels B and C of Figure 6, the biotinylated 15d-PGJ_2 derivative retained the activities of native 15d-PGJ_2. In addition, it appeared that numerous proteins were modified by the biotinylated PG derivative (Figure 6D). To demonstrate the modification of proteasome by 15d-PGJ_2 in intact cells, SH-SY5Y cells were treated with biotinylated 15d-PGJ_2, and cell lysates were incubated by batch with NeutrAvidin beads. After being extensively washed, proteins bound to the resin through biotinylated PG were eluted by being boiled in Laemmli sample buffer, and a 20S proteasome β-subunit present in the eluate was detected by immunoblot analysis (Figure 6E). The result clearly demonstrated that 15d-PGJ_2 covalently bound to proteasome in the cells.

Covalent Binding of 15d-PGJ_2 to Purified 26S Proteasome. To further assess the covalent binding of proteasome to 15d-PGJ_2, the protein (proteasome) coated on an immunoplate was incubated with the biotinylated 15d-PGJ_2, and 15d-PGJ_2 conjugates generated in the coating protein were then detected with an ELISA using the peroxidase-conjugated NeutrAvidin. As shown in Figure 7A, the incubation of proteasome with biotinylated 15d-PGJ_2 led to the dose-dependent formation of 15d-PGJ_2−protein conjugates, whereas BSA used as a control scarcely reacted with 15d-PGJ_2. Direct interaction of proteasome with 15d-PGJ_2 was also demonstrated by Western blot analysis (data not shown). Moreover, exposure of the purified 26S proteasome to 15d-PGJ_2 resulted in a dose-dependent reduction of proteasome activities: the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase activities of proteasome decreased to 25, 84, and 50% of the control values of untreated proteasome, respectively, after treatment for 8 h (Figure 7B). Thus, it appears that proteasome possesses a high affinity for 15d-PGJ_2. These observations suggest that proteasome may be a potential target of 15d-PGJ_2 and that the 15d-PGJ_2-induced accumulation of p53 and apoptosis are, at least in part, mediated by direct interaction of proteasome with 15d-PGJ_2.

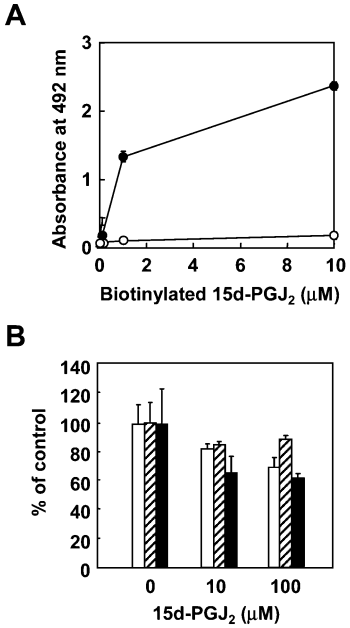

FIGURE 7: Binding of 15d-PGJ_2 to purified 26S proteasome. (A) Binding of 15d-PGJ_2 to proteasome *in vitro*. Proteins coated on the immunoplate were incubated with biotinylated 15d-PGJ_2, and immunoreactivity of the biotinylated 15d-PGJ_2 bound to the proteins with peroxidase-conjugated NeutrAvidin was examined with an ELISA: (●) proteasome and (○) BSA. The results that are shown are means ± the standard error of the mean of three independent experiments. (B) Effect of 15d-PGJ_2 on proteasome activities in purified 26S proteasome *in vitro*. The purified 26S proteasome (0.2 mg/mL) was incubated with 15d-PGJ_2 (0−1 mM) for 1 h at 37 °C, and the proteasome activities were measured: (empty bars) chymotrypsin-like activity, (hatched bars) trypsin-like activity, and (filled bars) peptidylglutamyl peptide hydrolase activity. The results that are shown are means ± the standard error of the mean of three independent experiments.

DISCUSSION

It has been shown that 15d-PGJ_2 potently induces apoptosis of SH-SY5Y human neuroblastoma cells *via* accumulation of p53 (*11*). Herein, we investigated the molecular mechanism involved in the p53-mediated neuronal cell death induced by 15d-PGJ_2 and found that the accumulation of p53 could be attributed to the disruption of the proteasome-

dependent regulatory mechanism of protein turnover. Moreover, we provided evidence that the PG inhibited the proteasome pathway *via* a mechanism in which 15d-PGJ_2 directly interacted with proteasome. These findings suggest that, under inflammatory conditions, the enhanced production of PGs and their metabolites, including 15d-PGJ_2, may cause disruption of the proteasome pathway function, which could further result in the buildup of pro-apoptotic and detrimental proteins, such as p53 and ubiquitinated proteins. This may, at least in part, be the mechanism underlying the 15d-PGJ_2-induced apoptosis of SH-SY5Y cells.

PGs are physiologically present in body fluids in picomolar to nanomolar concentrations (*24*); however, the rate of arachidonate metabolism is highly increased under several pathological conditions, including hyperthermia, infection, and inflammation (*25*), and local PG concentrations in the micromolar range have been detected at the sites of acute inflammation (*26*). Upregulation of PG biosynthesis is suggested to be involved in the pathophysiological processes relevant to inflammatory responses. On the basis of the findings that (i) PGD_2, a precursor of cyclopentenone PGs, was one of the most abundantly produced PGs in several tissues and (ii) PGD_2 could be converted readily to the J_2 series of PGs in the presence of plasma *in vitro* (*27*), it was anticipated that the levels of PGD_2 derivatives might reach functionally significant levels in inflammation and its related disorders. Indeed, despite the rapid metabolism and potential for sequestration of these compounds by binding proteins, several previous studies have successfully determined the *in vivo* levels of the J_2 series of PGs. (i) Hirata *et al.* (*28*) have detected significant quantities (~150 ng/24 h in human males) of Δ^{12}-PGJ_2 in human and monkey urine and larger amounts of other, uncharacterized Δ^{12}-PGJ_2 immunoreactive compounds in human urine. (ii) Gilroy *et al*. (*29*) have observed an elevated level of cyclopentenone PG synthesis in the late phases of inflammation. On the other hand, Shibata *et al*. (*4*) have recently established an immunochemical approach for the detection of 15d-PGJ_2. The authors have raised a murine monoclonal antibody 11G2, which clearly distinguished 15d-PGJ_2 from other PGs. Characterization of the antibody revealed that the monoclonal antibody was directed almost exclusively against free 15d-PGJ_2. The development of specific antibodies against 15d-PGJ_2 has made it possible for us to obtain evidence that points to the occurrence of this PG *in vitro* and *in vivo* being highly likely. We indeed demonstrated that the extracellular concentrations of 15d-PGJ_2 in RAW264.7 macrophages exposed to lipopolysaccharide *in vitro* could reach the low micromolar range (*4*). This and the observations that (i) 15d-PGJ_2 *in vivo* was mainly detected in the cytoplasm of most of the foamy macrophages in human atherosclerotic plaques (*4*) and (ii) an enhanced intracellular accumulation of 15d-PGJ_2 was observed in the lipopolysaccharide-stimulated RAW264.7 macrophages (*4*) suggest that, under defined pathological processes, steady-state concentrations of 15d-PGJ_2 may be far above what is generally believed. Kondo *et al*. (11) have demonstrated for the first time that 15d-PGJ_2 accumulates in the spinal cord of sporadic amyotrophic lateral sclerosis (ALS) patients, mainly occurring in the motor neurons of the anterior horn. This finding suggested that COX-2 upregulation, through its pivotal role in inflammation, followed by the enhanced intracellular production of 15d-PGJ_2 might be involved in the pathogenesis of inflammation-related disorders, including neurodegenerative diseases. Indeed, the involvement of PGs in neurodegenerative disorders has been suggested by clinical and epidemiological studies. For example, nonsteroidal anti-inflammatory drugs, which inhibit PG synthesis, reduce the rate of deterioration of the cognitive behavior in Alzheimer's disease patients (*30*, *31*). In addition, rheumatoid arthritis patients who are on high doses of anti-inflammatory drugs have a reduced incidence of this disease (*32*, *33*). Recent observations have also suggested a role for inflammatory-related events in the progression and propagation of the neurodegenerative process in ALS. (i) COX-2 is upregulated in the anterior horn of the spinal cord samples from ALS patients (*34*); (ii) inhibition of COX-2 protects motor neurons in an organotypic model of ALS (*35*).

In our previous study, as part of an effort to identify the 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 metabolites for their ability to induce intracellular ROS production in SH-SY5Y cells *in vitro* and found that the J_2 series of PGs represent the most potent inducers (*36*). This and the observations that intracellular ROS production was accompanied by the alteration of the cellular redox status and the production of lipid peroxidation-derived highly cytotoxic aldehydes, such as acrolein and 4-hydroxy-2-nonenal, which could also induce intracellular ROS production, suggest that intracellular oxidative stress constitutes a pivotal step in the pathway of cellular dysfunction induced by the PGs. We have also shown that 15d-PGJ_2 induces apoptosis of SH-SY5Y human neuroblastoma cells *via* accumulation of p53 (*11*). It is generally accepted that the accumulation of active p53 in response to stress occurs mainly through post-translational mechanisms (*20*). Pivotal is the increase in the protein half-life of p53. p53 is usually a very labile protein, turning over with a half-life sometimes as short as a few minutes. In response to DNA damage and other types of stress, p53 is markedly stabilized. It has been suggested that a rapid increase in the p53 concentration without a need for *de novo* transcription is particularly advantageous in cells with severely damaged genomes. Meanwhile, an elevation in the level of the p53 protein can also be achieved by improved translation of the p53 mRNA, involving relief of a translational repression mechanism operating through the 3′-untranslated region of this mRNA (*19*). In the study presented here, we found that 15d-PGJ_2 affected the regulatory mechansim of p53 protein turnover. To test whether the electrophilic center of 15d-PGJ_2 is involved in the modulation of protein turnover, we examined the effect of 9,10-dihydro-15d-PGJ_2, an analogue of 15d-PGJ_2, on the accumulation of p53 and ubiquitinated proteins and found that the reduction of the double bond in the cyclopentenone ring of 15d-PGJ_2 virtually abolished the effects of 15d-PGJ_2 on protein turnover (Figure 4). Thus, the action of 15d-PGJ_2 appeared to operate *via* mechanisms that depend on the reactivity of its electrophilic α,β-unsaturated ketones. This observation, in turn, suggested that cellular redox molecules might play crucial roles in the regulation of the biological functions of cyclopentenone PGs. We therefore investigated the role of Trx, a key molecule in the maintenance of the cellular redox balance, in the 15d-

PGJ_2-induced accumulation of p53 and ubiquitinated proteins. Consistent with the observation that Trx prevented oxidative stress-induced cell death (*37*−*39*), overexpression of Trx conferred protection on SH-SY5Y cells against loss of proteasome activities and accumulation of p53 and ubiquitinated proteins induced by 15d-PGJ_2 (Figure 5).

Proteasome is responsible for the majority of cellular proteolysis in eukaryotic cells and may contribute to controlling the intracellular levels of a variety of short-lived proteins (*40*−*42*). Substrates of proteasome include a number of cell regulatory molecules, such as cyclins, the Myc oncogene protein, and p53; the regulated degradation of these molecules has been linked to the control of cell proliferation and cell cycle progression (*40*−*42*). In the study presented here, we demonstrated that the increased intracellular level of p53 was associated with the partial reduction of proteasome activities (Figure 3D). Moreover, the proteasome inhibitor (MG132) similarly induced p53 accumulation and cell death in SH-SY5Y cells (Figure 3). These and the observations that (i) treatment of the cells with biotinylated 15d-PGJ_2 generated the 15d-PGJ_2−protein conjugates (Figure 6) and (ii) the exposure of the purified 26S proteasome to biotinylated 15d-PGJ_2 resulted in dose-dependent formation of 15d-PGJ_2−protein conjugates (Figure 7) suggested that the partial inactivation of proteasome activities might be ascribed to reversible or irreversible modification of proteasome with 15d-PGJ_2 in the same way as the known proteasome inhibitors, such as lactacystin (*43*), eponemycin (*44*), nitric oxide (*45*), and 4-hydroxy-2-nonenal (*46*, *47*), all of which irreversibly modify the 20S catalytic subunit of the 26S proteasome. On the other hand, there are also alternative explanations for the prostaglandin effects that must be considered in the future study. For example, (i) 15d-PGJ_2 may cause dissociation of a proteasome activator, such as 19S cap or PA28 regulator. (ii) 15d-PGJ_2 may induce phosphorylation or other events caused by lasting changes in the proteasome structure with inhibitory effects. (iii) As reported by Mullally *et al*. (*48*), 15d-PGJ_2 may inhibit the proteasome pathway via inhibition of the cellular ubiquitin isopeptidase activity.

It is believed that p53 protein turnover is regulated by multiple mechanisms, including regulation of p53 protein degradation and covalent modifications of p53, particularly protein phosphorylation. The observations that the 15d-PGJ_2-induced p53 accumulation was not suppressed by the MAPK specific inhibitors (T. Yamada, T. Shibata, and K. Uchida, unpublished observation) suggest that the MAPK pathways may not play a significant role in 15d-PGJ_2-induced p53 accumulation, although contributions of other protein kinases cannot be ruled out. As an alternative mechanism, it is also likely that 15d-PGJ_2-induced oxidative stress may be associated with the partial inhibition of proteasome activity. 15d-PGJ_2 has been shown to induce an intracellular oxidative stress, leading to the generation of lipid peroxidation products, including 4-hydroxy-2-nonenal (*36*). In addition, Bulteau *et al*. (*47*) have shown that 4-hydroxy-2-nonenal directly interacts with 20S proteasome α-like subunits iota, C3, and an isoform of XAPC7, leading to the partial inhibition of proteasome activity. 4-Hydroxy-2-nonenal and other reactive species may, therefore, contribute to the partial inhibition of proteasome activity in the cells exposed to 15d-PGJ_2. These and the observation that 15d-PGJ_2 directly modified numerous proteins in the cells (Figure 6D) suggest that the modification of proteasome by 15d-PGJ_2 may be the one event which contributes to proteasome inhibition and p53 accumulation.

Several previous observations have indicated that 15d-PGJ_2 might exert effects through interactions with intracellular proteins. It has been shown that 15d-PGJ_2 inhibits NF-κB by directly blunting IκB kinase and therefore blocks NF-κB-dependent antiapoptotic gene expression (*49*−*52*). 15d-PGJ_2 can also bind to and activate H-Ras (*53*), which may result in the activation of downstream signaling pathways associated with apoptosis. Moreover, we have recently identified thioredoxin 1, a regulatory molecule of apoptosis signaling kinase 1, as a target for covalent modification by 15d-PGJ_2 (*23*). Given these reactivities of cyclopentenone PGs toward redox active proteins, it is not unlikely that the cyclopentenone PG may adduct a number of cellular proteins and thus act on multiple pathways regulating apoptosis independently of a proteasome−p53 pathway. We are currently investigating the further upstream cell signaling induced by 15d-PGJ_2, which may provide new clues to the proapoptotic potential of the cyclopentenone PG.

We demonstrated here that one of the key targets in 15d-PGJ_2-induced activation of the p53-dependent apoptotic pathway is proteasome. The p53 accumulation induced by this PGD_2 metabolite occurs through covalent modification of the constituent proteins. Because proteasome regulates the majority of cellular proteolysis, the impairment of proteasome activity by 15d-PGJ_2 may have dramatic effects on cellular homeostasis. A similar role was recently proposed for 4-hydroxy-2-nonenal, a major lipid peroxidation product generated during oxidative stress (*46*, *47*). 4-Hydroxy-2-nonenal is structurally related to 15d-PGJ_2, because it contains an α,β-unsaturated aldehyde capable of reacting as a bifunctional electrophile. In this way, it may serve as an endogenous factor that is involved in neuronal cell degeneration associated with oxidative stress. Thus, identifying the role of proteasome inhibition in the neurodegenerative process is necessary for understanding how neuron death occurs in age-related neurodegenerative disorders.

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