Thioredoxin as a Molecular Target of Cyclopentenone Prostaglandins

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Prostaglandin (PG) D2, a major cyclooxygenase product in a variety of tissues and cells, readily undergoes dehydrogenation to yield the biactive cyclopentenone-type PGs of the J series, such as 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2). We have shown previously that 15d-PGJ2 is a potent electrophile that causes intracellular oxidative stress and redox alteration in human neuroblastoma SH-SY5Y cells. In the present study, based on the observation that the electrophilic center of 15d-PGJ2 was involved in the pro-oxidant effect, we investigated the role of thioredoxin 1 (Trx), an endogenous redox regulator, against 15d-PGJ2-induced oxidative cell injury. It was observed that the 15d-PGJ2-induced oxidative stress was significantly suppressed by the Trx overexpression. In addition, the treatment of SH-SY5Y cells with biotinylated 15d-PGJ2 resulted in the formation of a 15d-PGJ2-Trx adduct, indicating that 15d-PGJ2 directly modified the endogenous Trx in the cells. To further examine the mechanism of the 15d-PGJ2 modification of Trx, human recombinant Trx treated with 15d-PGJ2 was analyzed by mass spectrometry. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of the 15d-PGJ2-treated human recombinant Trx demonstrated the addition of one molecule of 15d-PGJ2 per protein molecule. Moreover, the electrospray ionization-liquid chromatography/mass spectrometry/mass spectrometry analysis identified two cysteine residues, Cys-35 and Cys-69, as the targets of 15d-PGJ2. These residues may represent the direct sensors of the electrophilic PGs that induce the intracellular redox alteration and neuronal cell death.

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 (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 cyclooxygenase and subsequently converted into one of several related products, including PGD2, PGE2, PGF2α, prosta-cyclooxygenase (COX), thromboxane A2, through the action of specific PG synthetases. Among them, PGD2 is a major cyclooxygenase 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 (3). It has been shown that PGD2 readily undergoes dehydrogenation in vivo and in vitro to yield biologically active PGs of the J2 series, such as PGJ2, Δ12-PGJ2, and 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) (4–7). Members of the J2 series of the PGs, unlike other classes of eicosanoids, characterized by the presence of an electrophilic α,β-unsaturated carbonyl group in the cyclopentenone ring, have their own unique spectrum of biological effects, including inhibition of macrophage-derived cytokine production (8, 9) and IκB kinase (10, 11), induction of synovioyte and endothelial cell apoptosis (12), induction of glutathione S-transferase gene expression (13) and intracellular oxidative stress (14), and potentiation of apoptosis in neuronal cells (15). Moreover, recent studies have shown that 15d-PGJ2 directly inhibits the NF-κB-dependent gene expression through coherent modification of critical cysteine residues in IκB kinase (10) and the DNA-binding domains of NF-κB subunits (11, 16).

Thioredoxin 1 (Trx) is a small and ubiquitously expressed protein originally identified in Escherichia coli and is evolutionarily conserved from prokaryotes to higher eukaryotes (17–19). Human thioredoxin was cloned as an adult T cell leukemia-derived factor or interleukin-1-like factor (20, 21). Trx is an essential cofactor electron donor for ribonucleotide reductase but also has many other cellular functions, including regulation of transcription factors and apoptosis, and can act exogously as a redox active growth factor (18, 22). In addition, Trx is known to play important roles in the redox regulation of signal transduction and in cytoprotection against oxidative stress (23, 24). The catalytic activity of Trx resides in its active site where the two redox-active cysteine residues (Cys-32 and Cys-35) undergo reversible oxidation/reduction. In addition to the conserved cysteine residues in the active site, three additional...
Thioredoxin as a Molecular Target of 15d-PGJ₂

Experimental Procedures

Materials—15d-PGJ₂ and 9,10-dihydro-15d-PGJ₂ were obtained from Cayman Chemicals (Ann Arbor, MI). Horseradish peroxidase-linked anti-mouse IgG immunoglobulins and enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Biosciences. The antibodies against NSF and ubiquitin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Biomedica Co. (Foster City, CA), respectively. The protein concentration was measured using the BCA protein assay reagent obtained from Pierce. UltraLink Immobilized NeutrAvidin Plus and 1-ethyl-3-(dimethylaminopropyl)carbodiimide were obtained from Pierce. The sequence grade modified trypsin was purchased from Promega. Human recombinant Trx (hTrx) was produced by a previously described method (26) and kindly provided by Ajinomoto Co. Inc. (Kawasaki, Japan). 2,7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was from Molecular Probes, Inc. (Eugene, OR). Cell Culture—SH-SY5Y cells were grown in Cosmedium-001 (CosmoBio, Tokyo, Japan) containing 5% Nakanishietha precolostrum newborn calf serum, 100 μg/ml penicillin, and 100 units/ml streptomycin. The cells were seeded in plates coated with poly-lysine and cultured at 37 °C. Cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (14). Flow Cytometry Analysis of Intracellular ROS Production—DCFH-DA was used to measure the ROS (27, 28). Cells were incubated with 10 μM 2,7'-dichlorodihydrofluorescein diacetate (dissolved in Me₂SO) 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, scraped from the plate, and resuspended at 1 × 10⁶ cells/ml in PBS containing 10 mM EDTA. The fluorescence was measured using a flow cytometer (Epics XL, Beckman Coulter). Preparation of Biotinylated 15d-PGJ₂—The carboxyl group of 15d-PGJ₂ was modified by amiation with EZ-Link 5-biotin-maleimido-penta-tylamine by a modification of a previously described procedure (16). Biotinylated 15d-PGJ₂ was purified through a reverse-phase HPLC eluted with a linear gradient of acetonitrile/water/acidic acid. The modified PG was then dried under argon and dissolved in Me₂SO for further use.

Immunohistochemical Detection of Biotinylated 15d-PGJ₂-Binding Proteins—Cells were fixed overnight in PBS containing 2% paraformaldehyde and 0.2% picric acid at 4 °C. The 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 2% bovine serum albumin and the primary anti-biotin antibody. The cells were then incubated for 1 h in the presence of fluorescein isothiocyanate—labeled goat anti-mouse IgG (Amersham Biosciences), rinsed with PBS containing 0.3% Triton X-100, and covered with anti-fade solution. Images of the cellular immunofluorescence were acquired using a confocal laser scanning microscope (Fluoroview; Olympus Optical Co., Ltd., Tokyo, Japan) with a ×40 objective (488-nm excitation and 518-nm emission).

Stable Transfection with Trx in SH-SY5Y Cells—SH-SY5Y cells were transfected with pCMV-1 vector or with the vector alone using GenePORTER™ transfection reagent (Gene Therapy Systems, Inc.). In these experiments, 1 × 10⁶ cells were incubated with DNA-GenePORTER™ mixture of 1 μg of DNA/10 μl of GenePORTER™ of serum-free Opti-MEM (Invitrogen) at 37 °C. After 6 h of incubation, 1 ml of complete medium was added, and cells were cultured for 18 h. Thereafter, stable transfectants were isolated by selection on 500 μg/ml G418 for ~3 weeks. Single clones of the stably transfected cells were isolated by limiting dilution. Several G418-resistant stable clones were maintained in medium containing 500 μg/ml G418.

Biotinylated 15d-PGJ₂ Labeling of Trx in SH-SY5Y Cells—SH-SY5Y cells at 50% confluence were incubated with 50 μM biotinylated 15d-PGJ₂ for 1 h. The cells were washed with PBS, harvested, and lysed in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS, 1% Triton, plus protease inhibitors. Cell lysates containing 200 μg of protein were immunoprecipitated with 50 μg of NeutrAvidin-Plus beads at 4 °C with constant shaking. The beads were rinsed three times with lysis buffer by centrifugation at 6,000 rpm for 1 min. The proteins were eluted by boiling the beads in Laemmli sample buffer for 5 min and analyzed by SDS-PAGE followed by immunodetection with anti-Trx monoclonal antibody. In addition, the cell lysates were incubated with 3 μg of anti-Trx antibody overnight at 4 °C. The mixture was then centrifuged (2,000 rpm, 5 min), rinsed three times with lysis buffer, and then boiled with the Laemmli sample buffer, and the biotinylated proteins were then subjected to immunoblot and detection with HRP-conjugated NeutrAvidin and ECL.

Enzyme-linked Immunosorbent Assay (ELISA)—To coat the wells of the microtiter plate, 100 μl/well hrTrx (0.1 mg/ml) in PBS was used and then incubated overnight at 4 °C. Following washing with TBS/Tween, 100 μl of the biotinylated 15d-PGJ₂ solution was added to the wells. After incubation for 2 h at 37 °C followed by washing with TBS/Tween, each well was filled with 200 μl of Block Ace solution (4 mg/ml) for 1 h at 37 °C. The HRP-conjugated NeutrAvidin was then added to the wells at 100 μl/well for 1 h at 37 °C. After washing, 100 μl of 0.05 M citrate buffer, pH 5.0, containing 0.4 mg/ml o-phenylenediamine and 0.003% H₂O₂ was added and incubated for several minutes at room temperature in the dark. The reaction was terminated by the addition of 2 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader.

Titration of Trx Sulphydryls with DTNB—The reactivity of the sulphydryl groups in hrTrx was determined by titration with DTNB by the method of Ellman (30), as modified by Riddles et al. (31). A 0.1 mg/ml hrTrx sample was treated with 0.1 mM 15d-PGJ₂, and after dialysis against PBS, the protein was desulfurated with 0.1 M dithiothreitol containing 13 mM EDTA and 133 mM Tris, pH 8, and reacted with DTNB (1 mM) at room temperature for 5 min. The reaction was monitored as an increase in absorbance at 412 nm. The concentration of sulphydryl groups was calculated using a standard curve with N-acetylcycteine.

Mass-assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)—The native and 15d-PGJ₂-modified hrTrx were mixed with a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (Sigma) containing 75% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room tempera-
ture and pressure. The trypsin-digested hrTrx were mixed with a saturated solution of α-cyano-4-hydroxy-cinnamic acid (Sigma) containing 50% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room temperature and pressure. The analyses were performed using an Autoflex matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker, Bremen, Germany) with a nitrogen laser (337 nm). All analyses were in the positive ion mode, and the instrument was calibrated immediately prior to each series of studies.

Electrospray Ionization-Liquid Chromatography/Mass Spectrometry (ESI-LCMS/MS) Analysis—The ESI-LCMS (and ESI-LCMS/MS) analyses were performed on an LCQ ion trap mass system (ThermoQuest) equipped with an electrospray ion source. The electrospray system employed a 5-kV spray voltage and a capillary temperature of 260°C.

Peptide Mapping—The native and 15d-PGJ2-modified hrTrx (1 mg/ml) were digested with modified trypsin in 0.25 ml of 50 mM Tris-HCl buffer, pH 8.8, at 37°C for 24 h using an enzyme:substrate ratio of 1:100 (w/w). Peptide samples were analyzed by a reversed-phase HPLC, a system that consisted of a nanospace SI-1 HPLC system (SHISEIDO Co., Ltd., Tokyo, Japan) with a FP-1520 fluorescence detector (Jasco Co., Tokyo, Japan), using a Capcell Pak C18 UG120 column (2.0 × 250-mm inner diameter; SHISEIDO Co., Ltd., Tokyo, Japan). These samples were eluted with a linear gradient of water containing 0.1% formic acid (Solvent A) and acetonitrile containing 0.08% formic acid (Solvent B) (time = 0–3 min, 10% B; 3–45 min, 10–40% B; 45–50 min, 40–50% B; 50–52 min, 50–80% B). The flow rate was 0.2 ml/min, and the column temperature was controlled at 40°C. The chromatograms were recorded at 215 nm.

RESULTS

Pro-oxidant and Cytotoxic Effects of 15d-PGJ2 Can Be Attributed to Its Electrophilic Center—It has been suggested that the α,β-unsaturated carbonyl group in the cyclopentenone ring of 15d-PGJ2 is a prerequisite for the induction of intracellular oxidative stress and cytotoxicity (14). To prove this hypothesis, human neuroblastosma SH-SY5Y cells were exposed to 15d-PGJ2 and its analog, 9,10-dihydro-15d-PGJ2 (Fig. 1A), and induction of ROS production and cytotoxicity were examined. As shown in Fig. 1B, the intracellular ROS production in SH-SY5Y cells was induced by 15d-PGJ2 in a dose-dependent manner. The level of ROS in the cells exposed to 15d-PGJ2 (50 μM) was ~30-fold higher than that of the control. In contrast to this potent pro-oxidant effect of 15d-PGJ2, 9,10-dihydro-15d-PGJ2 had no significant effects on the ROS production in a manner similar to the induction of the ROS production, 15d-PGJ2 (20 μM) resulted in a rapid decrease in the MTT reduction levels to 10% of the basal levels after 24 h of exposure, whereas the MTT reduction levels were maintained at about basal level in the cells exposed to 9,10-dihydro-15d-PGJ2 (Fig. 1C). Thus, the reduction of the double bond in the cyclopentenone ring of 15d-PGJ2 virtually abolished the pro-oxidant and cytotoxic effects of 15d-PGJ2, indicating that these biological activities can be attributed to the electrophilic center of 15d-PGJ2. Although proliferative effects of cyclopentenone-type PGs have been described in several cell types when used at nanomolar or low micromolar concentrations (32–34), we did not see such effect in human neuroblastosma SH-SY5Y cells (Fig. 1C).

Effect of Trx Overexpression on 15d-PGJ2-induced Oxidative Stress—The fact that cyclopentenone PGs are susceptible to nucleophilic addition reactions with thiols suggests that the action of the cyclopentenone PGs is closely related to the direct reaction with glutathione and/or other thiol compounds. However, i-buthionine-S,R-sulfoximine, a specific inhibitor of glutathione biosynthesis, itself, did not so effectively induce intracellular ROS production and cell death, indicating that the effects of the cyclopentenone PGs may not merely result from glutathione depletion alone (14). This and the finding that the electrophilic center of 15d-PGJ2 is involved in the pro-oxidant effect (Fig. 1) suggested that other cellular redox molecules might play crucial roles in protection against 15d-PGJ2-induced oxidative cell injury. Based on the previous findings that Trx, a key molecule in the maintenance of cellular redox balance, plays critical roles in protecting against oxidative stress and mediating signal transduction (35), we investigated the role of this redox regulator on the 15d-PGJ2-induced ROS production. To this end, we established the Trx-overexpressing derivatives of SH-SY5Y cells by stable transfection with Trx cDNA. The Trx expression vector (pTrx-1) was introduced into the SH-SY5Y cells with the selectable pcDNA, and four clones with resistance to G418 were selected. As a control, SH-SY5Y cells transfected with a control vector were similarly selected and the G418-resistant clones were isolated. As shown in Fig. 2A, the Trx-transfected clones Trx-1 and Trx-3 demonstrated a readily detectable expression of the Trx protein by immunoblot analysis with the anti-Trx monoclonal antibody, whereas the
Covalent binding of 15d-PGJ2 to endogenous Trx in SH-SY5Y cells. To determine covalent binding of 15d-PGJ2 to Trx, we also examined cell lysates subjected to control cells within 24 h of exposure, whereas the Trx-overexpressed cells exposed to 15d-PGJ2 was inhibited about 40% of the vector control. Next, we examined the effect of Trx on the 15d-PGJ2-induced cytotoxicity. The transfected SH-SY5Y cells were examined by MTT assay for sensitivity to cytotoxicity induced by continuous exposure (24 h) to 15d-PGJ2. As shown in Fig. 2C, 15d-PGJ2 (5 or 10 μM) resulted in a decrease in the MTT reduction levels to 10% of the basal levels on vector control cells within 24 h of exposure, whereas the Trx-overexpressing cells were more resistant to 15d-PGJ2-induced cell death than the control cells. We also examined the effect of Trx on the accumulation of ubiquitinated proteins and p53, both of which have been suggested to be involved in the 15d-PGJ2-induced cell death (14, 15), and found that Trx could have significantly inhibited the accumulation of these proteins (data not shown). These data strongly suggest that Trx may be involved, at least in part, in the protection against the 15d-PGJ2 cytotoxicity.

Covalent Binding of 15d-PGJ2 to Human Recombinant Trx—Because Trx contains reactive sulphydryl groups, it can be hypothesized that Trx will react with 15d-PGJ2. Apart from low molecular weight 15d-PGJ2-glutathione adduct, the 15d-PGJ2-thiol conjugates are associated with high molecular weight proteins. The list of 15d-PGJ2-modified proteins so far includes IκB kinase (10), Trx reductase (36), and keap1.2 Accordingly, we studied the direct interaction between 15d-PGJ2 and endogenous Trx in SH-SY5Y cells. We investigated whether 15d-PGJ2 reacts with Trx or other proteins, we prepared a biotinylated 15d-PGJ2 (Fig. 3A), which retains the α,β-unsaturated ketone substituent and the electrophilic β-carbon of 15d-PGJ2. As shown in Fig. 3B, incorporation of the biotinylated 15d-PGJ2 into the cells was observed in SH-SY5Y cells by immunocytochemical detection, suggesting the utility of the biotinylated 15d-PGJ2. We then attempted to detect the 15d-PGJ2-Trx adduct in the cells exposed to the biotinylated 15d-PGJ2. To this end, the SH-SY5Y cells were treated with 50 μM biotinylated 15d-PGJ2 for 1 h, and the cell lysate was incubated with NeutrAvidin beads. After washing with lysis buffer, proteins bound to the resin through biotinylated 15d-PGJ2 were eluted with SDS-PAGE sample buffer, and Trx was detected by immunoblot analysis with the anti-Trx monoclonal antibody (Fig. 3C). Alternatively, cell lysates were subjected to immunoprecipitation with an anti-Trx monoclonal antibody, and the presence of biotinylated 15d-PGJ2-modified proteins was detected by immunoblot analysis with HRP-conjugated NeutrAvidin (Fig. 3C). Thus, it appeared that 15d-PGJ2 reacted to an appreciable extent with endogenous Trx, itself, in intact SH-SY5Y cells.

MALDI-TOF MS Analysis of 15d-PGJ2-treated hrTrx—To further elucidate the mechanism for modification of Trx by 15d-PGJ2, we attempted to identify the modification sites of the protein by monitoring the formation of ubiquitinylated 15d-PGJ2 Michael adducts by mass spectrometric methods. As shown in Fig. 5, the MALDI-TOF MS analysis of the native hrTrx revealed a peak of m/z 11,785. When hrTrx was incubated with 0.1 mM 15d-PGJ2 in 50 mM sodium phosphate buffer, pH 7.4, for 2 h at 37°C, some unmodified hrTrx subunits were observed, as was the peak (m/z 12,099) corresponding to the addition of one molecule of 15d-PGJ2 per protein. Further incubations resulted in the appearance of peaks corresponding to the addition of one to two molecules of 15d-PGJ2 (data not shown).
LC/MS analysis of the tryptic peptides from the native hrTrx provided identification of the peptides accounting for 92% of the protein sequence (see Fig. 6A and Table I). Relative to the calculated mass of the unmodified peptide, which showed an increased mass of +316 Da, the singly charged N-terminal product ions (b8 and b11) and its H2O loss ions (b8 and b11) were detected in the MS/MS analysis of the tryptic peptides from the 15d-PGJ2-treated hrTrx (see Fig. 6B and Table I).

To identify the 15d-PGJ2 modification site, the 15d-PGJ2-modified peptides, **Tp-1** and **Tp-2**, were further analyzed by ESI-LC/MS/MS without additional chromatography. The MS/MS spectrum of the [M + H]+ at m/z 1,940.7 from the 15d-PGJ2-modified peptide (**Tp-1**: LVVVDFAATWCQPCK) is shown in Fig. 7. In the MS/MS analysis, the singly charged N-terminal product ions (b6–18, b9–18, b10–18, and b12–18) were observed. The C-terminal fragment ions (y4, y6, y7, y8, y9, y10, y11, y12, and y13), N-terminal product ion (b14), and the H2O loss fragment ions (y3–18) were observed to increase 316 Da, suggesting that the 15d-PGJ2 modification site is in the sequence on Cys-35 but not Cys-32. The MS/MS spectrum of the [M + 2H]+2 at m/z 1,518.9 from the 15d-PGJ2-modified peptide (**Tp-2**: YSNVFLESADVDDCQDVASECEVK) is shown in Fig. 8. In the MS/MS analysis, the singly charged N-terminal product ions (b7, b8, b9, b11, b12, b13, b15, b16, and b17) and its H2O loss fragment ions (b6–18, b7–18, b8–18, b9–18, b11–18, b14–18, b15–18, b16–18, and b17–18) and doubly charged N-terminal product ions (b202+ and b212+) were observed. The singly charged C-terminal product ions (y4, y5, y6, y7, y8, y9, y11, y12, y13, y14, and y15) and doubly charged product ions (y182+, y192+, and b232+) were observed to increase 316 Da. These data suggest that the 15d-PGJ2 modification is associated with Cys-69 on the peptide Tp-2.

**DISCUSSION**

PGs are physiologically present in body fluids in picomolar to nanomolar concentrations (37); however, the arachidonate me-
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Fig. 5. MALDI-TOF MS analysis of 15d-PGJ₂-treated hrTrx. hrTrx (0.1 mg/ml) was incubated with 0.1 mM 15d-PGJ₂ in PBS for 30 min at 37 °C.

The production of oxidative cell damage by Trx might be achieved through a direct electrophile scavenger function. To prove this hypothesis, using a biotinylated 15d-PGJ₂, we explored the incorporation of 15d-PGJ₂ into cellular proteins (Fig. 3B) and cellular Trx (Fig. 3C). Our results show that biotinylated 15d-PGJ₂ modifies Trx when added to intact cells. These results raise the possibility that Trx plays an important role in the protection against the pro-oxidant effects of the electrophilic PG, at least at the pharmacological doses employed in most biochemical studies. Several previous observations have indicated that PGD₂ and its J-ring metabolites might exert effects through interactions with intracellular proteins. Narumiya et al. (56) have shown that radiolabeled Δ₁₂ PGJ₂ is actively incorporated into cells and transferred to the nucleus, where it is associated with proteins. Some PGs, including PGD₂, PGJ₂, and Δ₁₂-PGJ₂, have been shown to bind with high affinity to the 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. 15d-PGJ₂ has been shown to directly inhibit NF-κB activation either by blocking IκB kinase activity through covalent modifications of critical cysteine residues in IκB kinase β or by interacting with cysteine residues in the DNA-binding domain of the NF-κB subunit p65 (10, 58). Moreover, the NF-κB p50 subunit was also identified to be a target for covalent modification by 15d-PGJ₂ leading to the inhibition of DNA binding (16). Moos et al. (36) have also reported the covalent modification and inhibition of Trx reductase by cyclopentenone PGs. More recently, Olivia et al. (59) have shown that 15d-PGJ₂ induces H-Ras activation mediated by direct interaction of 15d-PGJ₂ to the cysteine residue (Cys-184) of H-Ras. This and our observations suggest cyclopentenone PGs could induce redox alteration due, at least in part, to the inhibition of the Trx-dependent regulatory systems.

The covalent binding of 15d-PGJ₂ to Trx was examined upon incubation of hrTrx with biotinylated 15d-PGJ₂. The observations (Fig. 4) that the biotinylated 15d-PGJ₂ was significantly incorporated into hrTrx and that the exposure of

of 9,10-dihydro-15d-PGJ₂, an analog of 15d-PGJ₂, on the induction of ROS production and cell damage and found that the reduction of the double bond in the cyclopentenone ring of 15d-PGJ₂ virtually abolished the pro-oxidant and cytotoxic effects of 15d-PGJ₂ (Fig. 1). Thus, the pro-oxidant action of 15d-PGJ₂ appeared to operate via mechanisms that depend upon the reactivity of its electrophilic α,β-unsaturated ketones.

The reactive center of the cyclopentenone PGs has been proposed to account for some of their receptor-independent biological actions (10, 51). They can covalently react by means of the Michael addition reaction with nucleophiles, such as the free sulfhydryls of glutathione and cysteine residues in cellular proteins that play an important role in the control of the redox cell-signaling pathways (10, 51, 52). This, in turn, suggested that cellular redox molecules might play crucial roles in the regulation of the biological functions of cyclopentenone PGs. In the present study, we investigated the role of Trx on the 15d-PGJ₂-induced oxidative cell damage. Trx, a key molecule in the maintenance of the cellular redox balance, has been shown to play critical roles in protecting against oxidative stress and mediating signal transduction (53). Indeed, the overexpression of cytoplasmic Trx protected against oxidative stress-induced cell death (35, 54). Andoh et al. (55) have also shown that, using serum deprivation and 1-methyl-4-phenylpyridinium as models, Trx prevents the oxidative stress-induced apoptosis of SH-SY5Y cells. Consistent with these results, Trx conferred protection on SH-SY5Y cells against 15d-PGJ₂-induced cell damage (Fig. 2).

It was proposed that the protection of oxidative cell damage by Trx might be achieved through a direct electrophile scavenger function. To prove this hypothesis, using a biotinylated 15d-PGJ₂, we explored the incorporation of 15d-PGJ₂ into cellular proteins (Fig. 3B) and cellular Trx (Fig. 3C). Our results show that biotinylated 15d-PGJ₂ modifies Trx when added to intact cells. These results raise the possibility that Trx plays an important role in the protection against the pro-oxidant effects of the electrophilic PG, at least at the pharmacological doses employed in most biochemical studies. Several previous observations have indicated that PGD₂ and its J-ring metabolites might exert effects through interactions with intracellular proteins. Narumiya et al. (56) have shown that radiolabeled Δ₁₂ PGJ₂ is actively incorporated into cells and transferred to the nucleus, where it is associated with proteins. Some PGs, including PGD₂, PGJ₂, and Δ₁₂-PGJ₂, have been shown to bind with high affinity to the 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. 15d-PGJ₂ has been shown to directly inhibit NF-κB activation either by blocking IκB kinase activity through covalent modifications of critical cysteine residues in IκB kinase β or by interacting with cysteine residues in the DNA-binding domain of the NF-κB subunit p65 (10, 58). Moreover, the NF-κB p50 subunit was also identified to be a target for covalent modification by 15d-PGJ₂ leading to the inhibition of DNA binding (16). Moos et al. (36) have also reported the covalent modification and inhibition of Trx reductase by cyclopentenone PGs. More recently, Olivia et al. (59) have shown that 15d-PGJ₂ induces H-Ras activation mediated by direct interaction of 15d-PGJ₂ to the cysteine residue (Cys-184) of H-Ras. This and our observations suggest cyclopentenone PGs could induce redox alteration due, at least in part, to the inhibition of the Trx-dependent regulatory systems.

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hrTrx to the biotinylated 15d-PGJ₂ resulted in the loss of cysteine residues confirms that 15d-PGJ₂ is a chemically reactive species capable of alkylating nucleophilic sites in the protein Trx under physiological conditions. In this study, we have used a combination of MALDI-TOF MS and ESI-LC/MS (ESI-LC/MS/MS) to elucidate the mechanism for modification of Trx by 15d-PGJ₂. The MALDI-TOF MS analysis of 15d-PGJ₂-treated hrTrx showed that the reaction of Trx with 15d-PGJ₂ resulted in the formation of at least two adducts, the major of which is a monoadduct (Fig. 5). The 15d-PGJ₂-Trx adduct was further digested with trypsin, and the peptides (Tp-1 and Tp-2), containing the 15d-PGJ₂-cysteine adduct, were sequenced by ESI-LC/MS/MS. Among two catalytic site cysteine residues (Cys-32 and Cys-35) and three other cysteine residues (Cys-62, Cys-69, and Cys-73) in human Trx, the sequencing analysis showed the site of modification to be exclusively at Cys-35 and Cys-69 (Figs. 6–8). These data suggest that 15d-PGJ₂ may not be randomly incorporated into free SH groups but instead may alkylate cysteine groups in specific environments. The preference for alkylation of Cys-35 to Cys-32 was unexpected, because (i) the active site of Trx is known to have the cysteines on a protruding loop with Cys-32 more exposed than Cys-35 (60), and (ii) Cys-32 has a lower pKₐ than Cys-35 (61). Indeed, Erve et al. (62) showed that S-(2-chloroethyl)glutathione exclusively alkylates Cys-32 with no alkylation at Cys-35. Although the detailed mechanism for this preferential alkylation of Cys-35 by 15d-PGJ₂ remains unclear, the modification of one of the two active site cysteines may be directly associated with the abolishment of its redox regulatory functions, because Cys-35 is critical to releasing the reduced protein substrate followed by the formation of a disulfide linkage with Cys-32. On the other hand, the non-catalytic cysteine residues have also been reported to undergo covalent modification. Haendeler et al. (63) showed recently that Trx is S-nitrosylated on Cys-69, which is

![Figure 6](image-url)
located between an acidic and a basic amino acid in the proposed consensus motif for S-nitrosylation (64, 65). It has been suggested that S-nitrosylation on Cys-69 is involved in an anti-apoptotic mechanism of Trx that differs from the anti-apoptotic mechanisms mediated by the binding of proapoptotic proteins to Cys-32 and Cys-35 (63). Because this S-nitrosylation is required for scavenging ROS and for preserving the redox regulatory activity of Trx, the 15d-PGJ2 modification of Cys-69 may result in an increase in the formation of ROS. This may be associated with our previous finding (14) that 15d-PGJ2 and other cyclopentenone-type PGs can potently induce intracellular oxidative stress in SH-SY5Y human neuroblastoma cells.

In summary, our results identified the Trx as a molecular target for the covalent modification by 15d-PGJ2, providing a biochemical basis for the redox alteration by cyclopentenone PG. In addition, we identified the two target cysteine residues, Cys-35 and Cys-69, of the 15d-PGJ2 modification within Trx. The Trx modification by 15d-PGJ2 may be one of the mechanisms by which 15d-PGJ2 induces intracellular oxidative stress and neuronal cell death.

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