Multidrug Resistance-Associated Protein 1 Mediates 15-Deoxy-Δ^{12,14}-prostaglandin J₂-Induced Expression of Glutamate Cysteine Ligase Expression via Nrf2 Signaling in Human Breast Cancer Cells

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ABSTRACT: 15-Deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) is a representative J-series cyclopentenone prostaglandin bearing an electrophilic α,β-unsaturated carbonyl group. In the present study, treatment of human breast cancer MCF-7 cells with 15d-PGJ₂ caused the up-regulation of the glutamate cysteine ligase catalytic (GCLC) subunit, the rate-limiting enzyme in glutathione (GSH) synthesis. 15d-PGJ₂ treatment caused nuclear translocation and transactivation of Nrf2, a redox-sensitive transcription factor responsible for induced expression of antioxidant and other cytoprotective genes. siRNA knockdown of Nrf2 abrogated 15d-PGJ₂-induced GCLC expression. Following 15d-PGJ₂ treatment, the intracellular GSH level was initially diminished but eventually enhanced even above the basal level. The reactive oxygen species (ROS) scavenger N-acetylcysteine (NAC) abolished the 15d-PGJ₂-induced Nrf2 activation and GCLC expression. Pharmacologic inhibition or siRNA knockdown of Akt, the target of phosphoinositide 3-kinase (PI3-K), attenuated 15d-PGJ₂-induced Nrf2 activation and GCLC expression, and NAC treatment inhibited phosphorylation of Akt, and subsequently Nrf2 activation and GCLC upregulation. 9,10-Dihydro-15-PGJ₂, a nonelectrophilic analogue of 15d-PGJ₂ that lacks the ability to form a conjugate with GSH, failed to induce activation of Akt and Nrf2 as well as ROS generation. These findings, taken all together, suggest that intracellular accumulation of ROS formed as a consequence of initial depletion of GSH can activate Akt, which in turn induces Nrf2 activation and subsequently the expression of GCLC, leading to the restoration of GSH. Interestingly, the extracellular GSH level was increased, concomitantly with the depletion of the intracellular GSH following 15d-PGJ₂ treatment. However, 15d-PGJ₂ was unable to influence both intra- and extra-cellular GSH levels when multidrug resistance-associated protein 1 (MRP1), the efflux pump for GSH conjugates, was blocked by its antagonist, MK571. Moreover, 15d-PGJ₂-induced GCLC expression was attenuated by the MKS71 and also by siRNA knockdown of MRP1, suggesting that MRP1 contributes to 15d-PGJ₂-mediated up-regulation of GCLC by pumping out the 15d-PGJ₂-GSH conjugate. It is speculated that 15d-PGJ₂, once effluxed through MRP, liberates from the GSH conjugate, and the free 15d-PGJ₂ re-enters the cell and forms the GSH conjugate again. In conclusion, MRP1 mediates Nrf2-dependent up-regulation of GCLC in 15d-PGJ₂-treated MCF-7 cells, possibly via a putative recycling loop of 15d-PGJ₂-GSH conjugation.

INTRODUCTION

15-Deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂), a representative J-series cyclopentenone prostaglandin, is one of the major terminal products of the cyclooxygenase-2 (COX-2)-dependent arachidonic acid cascade.¹ Its synthesis and release can be stimulated during the inflammatory process and under tumorigenic conditions.²,³ 15d-PGJ₂ acts as an endogenous ligand for peroxisome proliferator-activated receptor γ (PPARγ) and has been considered as a bifunctional regulator of cell proliferation and death.⁴,⁵ This cyclopentenone prostaglandin exerts an oncogenic function by directly interacting with cellular proteins, which does not necessarily depend on PPARγ.⁶–⁸ 15d-PGJ₂ has been shown to induce activation of nuclear factor-erythroid 2 p45 (NF-E2)-related factor (Nrf2), a master regulator of the basal and inducible expression of diverse cytoprotective proteins.⁹,¹⁰ Nrf2 is normally sequestered in the cytoplasm by Keap1 and undergoes ubiquitination and proteasome-dependent degradation.¹¹ Nrf2 activation is initiated by direct oxidation or modification of Keap1 cysteine thiol and/or phosphorylation of Nrf2.¹²,¹³ Subsequently, Nrf2 is liberated from its cytoplasmic repressor Keap1 and translocated into the nucleus.¹⁴

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Glutamate cysteine ligase (GCL), a representative antioxidant enzyme, is a heterodimer composed of the catalytic (GCLC) and modulatory (GCLM) subunits. Human GCLC promoter contains the antioxidant response element (ARE), which is the cis-acting consensus sequence responsible for Nrf2-dependent regulation of gene expression. Up-regulation of GCLC leads to increased biosynthesis of GSH, the most abundant intracellular nonprotein thiol that participates in cellular defense by scavenging reactive oxygen species (ROS) and electrophiles.

The intracellular GSH level is an important determinant of redox status and is hence subjected to precise regulation. Multi-drug resistance-associated protein 1 (MRP1), an ATP-binding cassette (ABC) transporter family member, is one of the factors involved in GSH homeostasis. This plasma membrane protein facilitates the efflux of diverse electrophiles out of the cell in the form of GSH conjugates, resulting in the consumption of intracellular GSH. It has been shown that the GSH conjugate of 15d-PGJ₂ is a substrate for MRP1 as well.

In the present study, we found that treatment of human breast cancer (MCF-7) cells with 15d-PGJ₂ caused transient GSH depletion but consequently enhanced the intracellular GSH content via Nrf2-dependent GCLC up-regulation. This prompted us to investigate whether the altered GSH homeostasis accounts for Nrf2 activation and subsequent GCLC upregulation in 15d-PGJ₂-treated MCF-7 cells. Here, we report that MRP1 takes part in the recycling of 15d-PGJ₂ through efflux of the 15d-PGJ₂-GSH conjugate in MCF-7 cells.

### EXPERIMENTAL PROCEDURES

**Materials.** 15d-PGJ₂, 9,10-dihydro-15d-PGJ₂ (H₂-15d-PGJ₂), and the biotinylated products of both compounds were obtained from Cayman Chemical Co. (Ann Arbor, MI). N-Acetyl-L-cysteine (NAC), cycloheximide, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], metaphosphoric acid (MPA), and an antibody against GCL was kindly provided by Dr. Terrance J. Kavanagh (University of Wisconsin-Madison). Antibodies against Nrf2 and histone H1 were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). Antibodies against GCLM antibody was a product of Thermo Scientific (Fremont, CA). The GCLC antibody was kindly provided by Dr.授權 J. Kavanagh (University of Washington). The GSTP antibody was obtained from Assay Designs (Ann Arbor, MI). Antibodies against Nrf2 and histone H1 were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). An monoclonal antibody against MRP1 was manufactured by Abcam (Cambridge, UK). Antibodies for Akt and phospho-Akt were products of Cell Signaling Technology (Beverly, MA). Secondary antibodies and anti-Lamin B were obtained from Zymed Laboratories Inc. (Carlsbad, CA). Dichlorofluorescein diacetate (DCF-DA) and propidum iodide (PI) were manufactured by Molecular Probes Inc. (Carlsbad, CA). Small interfering RNA (siRNA) for the Akt gene and control vector PKD were purchased from Upstate (Lake Placid, NY). siRNA for the negative control, Nrf2, and MRP1 were custom prepared at Invitrogen (Carlsbad, CA). Control plasmid pEF, Ti-Luc, ARE-Luc, and GC-MT-Luc were kindly provided by Dr. Jeffery A. Johnson (University of Wisconsin—Madison).

**Cell Culture and Measurement of Viability.** MCF-7 cells were maintained in RPMI 1640 with 10% fetal bovine serum and 100 ng/mL antibiotics at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The cells were plated at an appropriate density according to each experimental scale. Cell viability was determined by the MTT assay as described previously.

**Reverse Transcriptase—Polymerase Chain Reaction (RT-PCR).** Total RNA was isolated from MCF-7 cells using TRIzol (Invitrogen) following the manufacturer’s instructions. RT-PCR was performed according to standard procedures. PCR conditions for GCLC, GCLM, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), the house keeping gene, were as follows: for GCLC and GCLM, 35 cycles for 95 °C for 1 min, 51 °C for 2 min, and 72 °C for 1 min; for GAPDH, 26 cycles for 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 2 min. The primer pairs were as follows (forward and reverse, respectively): GCLC, S'-GAGGCTATGTCAGACATTGTTGC-3' and S'-GTGATCTCC-TCTGACCGAGGTCGGTG-3'; GCLM, S'-GTCCAGCGCAGGCG-AGGAGCTTCGAT-3' and S'-GATCAGTTGTAGCACAAGCGGT-ATG-3'; GAPDH, S'-AAGGTCGAGATCAGGATT-3' and S'- GAGCGTGGGTGTCCTCTCT-3'. Amplification products were resolved by 1.0–1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. All primers were purchased from Bionics (Seoul, Korea).

**Western Blot Analysis.** MCF-7 cells were exposed to the cell lysis buffer (Cell Signaling Technology) containing the protease inhibitors for 30 min on ice followed by centrifugation at 13,000g for 15 min. The protein concentration of the supernatant was measured by using the bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL). Proteins were separated by running through 7–12% of sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membrane (Gelman Laboratory, Ann Arbor, MI). The blots were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS)-T buffer [PBS containing 0.1% Tween-20] for 1 h at room temperature followed by incubation with a 1:1000 dilution of primary antibodies for 2 h or overnight. Equal lane loading was ensured by using antactin. The blots were rinsed three times with PBS-T for 10 min each. Washed blots were exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h and washed again three times with PBS-T. Then, the transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Transient Transfection.** MCF-7 cells were seeded at a density of 1 × 10⁵ cells/mL in a 60-mm dish and grown to 50% confluence in complete media. Then plasmids were transfected into the cells with Fugene 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany). In the case of siRNAs, reverse transfection was performed using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. MCF-7 cells were seeded at a density of 1 × 10⁵ cells/mL in a 100-mm dish with a preincubated mixture of siRNA and Lipofectamine RNAiMAX. After 72 h of transfection, cells were treated with 15d-PGJ₂ for an additional 24 h, then lysed with cell lysis buffer. The target sequences for siRNAs were as follows: Nrf2, S'-AAU AGU AGU AGC UGG AAA AAC ATT TT-3' (forward) and S'-GUU UUU CCA GCC CAU ACU CUU TT-3' (reverse); MRP1, S'-AAG CAG AGC UGA AUG AGU AGG G-3' (forward) and S'-CCC UCU UAC UCU UAC AGC UCG UCU U-3'.

**ARE Luciferase Assay.** MCF-7 cells were transfected with Ti-Luc, ARE-Luc, GC-MT-Luc using Fugene 6 reagents. After 72 h, the medium was changed, and the cells were treated with 15d-PGJ₂ for an additional 24 h, then washed with PBS, lysed with reporter lysis buffer (Promega, Madison, WI), and centrifuged at 13,000g for 1 min at 4 °C. The supernatant was stored at −70 °C for the luciferase assay. Twenty microliters of cell lysate was mixed with the same amount of the luciferase assay reagent (Promega) at room temperature, and the luciferase reporter activity was measured by a luminometer (Auto Lumat LB983, EG and B Berthold, Bad widbad, Germany). The ARE-Luc vector contains a human NQO1 ARE consensus sequence that is identical to the GCLC ARE core sequence.

**Immunocytochemistry for Nrf2.** MCF-7 cells grown on 4-well chamber slides were treated with 15d-PGJ₂ and/or other compounds. Cells were rapidly rinsed with PBS and fixed with 5% acetic acid in
methanol for 30 min at −20 °C. After washing with PBS, the cells were blocked by PBS-T containing 5% bovine serum albumin (Pierce) for 2 h at room temperature. Anti-Nrf2 antibody was diluted 1:100 in blocking buffer and incubated overnight at 4 °C. Afterward, cells were washed with PBS and labeled with FITC-conjugated secondary rabbit antibody (Zymed) diluted 1:1000 for 1 h at room temperature. Stained cells were visualized and photographed under a confocal microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

Preparation of Nuclear Proteins. Confluent cells in 100-mm dishes were treated with 15d-PGJ2. Cells were gently washed with ice-cold PBS, scraped in 1 mL of PBS, and centrifuged at 3,000g for 5 min at 4 °C. Pellets were suspended in 100 μL of hypotonic buffer A for 15 min on ice, and 2 μL of 10% Nonidet P-40 solution was added for 5 min. The mixture was then centrifuged for 10 min at 12,000g. The pellets were suspended in 50 μL of buffer C for 1 h on ice and centrifuged for 10 min at 12,000g. The supernatant containing nuclear proteins was collected and stored at −70 °C after quantification. The composition of buffers was previously described.24

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed to determine the ARE binding activity using a DNA–protein binding detection kit (GIBCO) according to the method described elsewhere.24

Binding of Biotinylated 15d-PGJ2 to GSH in Intact Cells. The 15d-PGJ2–GSH conjugate was detected using the biotin–streptavidin system according to the previous protocol.2 Antibody against GSH for immunoprecipitation was purchased from Virogen (Waterstown, MA).

Measurement of the Intracellular ROS Accumulation. To monitor the intracellular accumulation of ROS, the fluorescent probe DCF-DA was utilized. Following the treatment of cells with 15d-PGJ2 or H2O2, 15d-PGJ2, cells were rinsed with PBS and exposed to 10 μM DCF-DA. After a 15-min incubation at 37 °C, cells were examined under a confocal microscope equipped with an argon laser (488 nm, 200 mW).

Measurement of the Intracellular GSH Level. The GSH content was measured using the GSH-400 kit (Oxis International, Portland, OR) or the HPLC separation/fluorometric detection method of Neuschwander-Tetri and Roll. The latter method was previously described.23 The GSH-400 kit is based on a colorimetric reaction using 4-chloro-1-methyl-7-trifluoromethyl-quinolinum methylsulfate. Cells were rinsed with PBS, suspended in 200 μL of 5% MPA solution, and incubated on ice for 15 min. Then cells were collected using a scraper and centrifuged at 13,000g for 15 min at 4 °C. After centrifugation, 25 μL of R1 solution (solution of chromogenic reagent in HCl) was added to 450 μL of mixture of the supernatant and S3 buffer. Following the addition of 25 μL of R2 solution (30% of NaOH), the mixtures were incubated at room temperature for 10 min. The absorbance of the mixtures was read at 400 nm. The protein concentration was determined using the BCA kit.

Measurement of the Extracellular GSH Level. The extracellular GSH level was determined using the GSH-400 kit as well. Following the treatment of 15d-PGJ2, the cell culture medium without phenol red was collected and centrifuged at 3,000g for 5 min at 4 °C. Then, 200 μL of 5% MPA was added to the supernatant for protein precipitation and centrifuged at 13,000g for 10 min at 4 °C. After centrifugation, sequential addition of R1 and R2 and detection of the absorbance were performed as described for measurement of the intracellular GSH level.

Statistical Analysis. When necessary, data were expressed as the means ± SD, and statistical analysis for single comparisons was performed using Student’s t test. The criterion for statistical significance was P < 0.05.

RESULTS

15d-PGJ2 Induces GCLC Expression in MCF-7 Cells. MCF-7 cells were treated with 10 μM 15d-PGJ2, and expression levels of both GCLC and GCLM were measured at various time points. 15d-PGJ2 treatment resulted in the induction of GCLC expression at both mRNA (Figure 1A) and protein (Figure 1C) levels, whereas GCLM expression barely changed (Figure 1A and C). The expression of GCLC and its mRNA transcript was dependent on the concentration of 15d-PGJ2 (Figure 1B and D). 15d-PGJ2-mediated GCLC up-regulation was significantly blocked by cycloheximide, an inhibitor of protein biosynthesis in eukaryotes (Figure 1E), suggesting that GCLC induction by 15d-PGJ2 is achieved via the de novo protein synthesis.

Nrf2 Regulates 15d-PGJ2-Induced GCLC Expression in MCF-7 Cells. As the human GCLC promoter contains a consensus ARE sequence, we investigated whether Nrf2 could regulate 15d-PGJ2-induced GCLC expression.24 As illustrated in Figure 2A, the nuclear accumulation of Nrf2 was evident from 6 h following 15d-PGJ2 treatment, which was further verified by immunocytochemical analysis (Figure 3B). This led to enhanced Nrf2-ARE binding as determined by EMSA (Figure 2B). The luciferase reporter gene assay revealed that the Nrf2 transcriptional activity was elevated by 15d-PGJ2 treatment, which was reduced by a mutation of GC-box (GC-MT Luc), a critical ARE segment for Nrf2 transactivation (Figure 2C).25 To further confirm that Nrf2 is essential for 15d-PGJ2-induced GCLC expression, we adopted the siRNA strategy. We observed that siRNA knockdown of Nrf2 abrogated 15d-PGJ2-induced GCLC expression (Figure 2D).

15d-PGJ2-Induced Nrf2 Activation, Up-Regulation of GCLC Expression, and Increased GSH Synthesis in MCF-7 Cells Are Mediated via PI3K-Akt Signaling. 15d-PGJ2 treated
The luciferase activities were measured after transiently transfected with the ARE luciferase construct (Ti-Luc, ARE-quence as described in Experimental Procedures. (C) MCF-7 cells were radiolabeled oligonucleotide probe harboring an ARE consensus sequence. (D) MCF-7 cells were transfected with scrambled siRNA or Nrf2-ARE DNA binding activity was assessed by EMSA out of the 15d-PGJ2-GSH conjugate could facilitate the subsequent interaction between 15d-PGJ2 and GSH, likely due to the chemical equilibrium shifted in favor of conjugation between two entities. The 15d-PGJ2-GSH conjugate, once pumped out of the cell, may then undergo dissociation, and the resulting free lipophilic 15d-PGJ2 may re-enter the cell. To test this hypothesis, the MRP1 blocker (MK571) and MRP1-siRNA were utilized. In the presence of MK571, 15d-PGJ2 was unable to induce both GCLC expression (Figure 5A) and a subsequent GSH upregulation through activation of PI3K-Akt signaling. 15d-PGJ2-Induced Up-Regulation of GCLC Is Potentially Mediated by MRP1 in MCF-7 cells. MRP1, also known as the GSH-S-conjugate (GS-X) pump, is an ABC transporter possibly capable of effluxing the 15d-PGJ2-GSH conjugate out of the cell.25 Thus, we hypothesized that MRP1-mediated pumping out of the 15d-PGJ2-GSH conjugate could facilitate the subsequent interaction between 15d-PGJ2 and GSH, likely due to the chemical equilibrium shifted in favor of conjugation between two entities. The 15d-PGJ2-GSH conjugate, once pumped out of the cells, may then undergo dissociation, and the resulting free lipophilic 15d-PGJ2 may re-enter the cell. To test this hypothesis, the MRP1 blocker (MK571) and MRP1-siRNA were utilized. In the presence of MK571, 15d-PGJ2 was unable to induce both GCLC expression (Figure 5A) and a subsequent GSH enhancement (Figure 5D) in MCF-7 cells. This is probably due to insufficient accumulation of ROS responsible for Akt activation as a consequence of the blockage of MRP1-mediated efflux of the 15d-PGJ2-GSH conjugate (Figure 5B). Similarly, the siRNA knockdown of MRP1 attenuated 15d-PGJ2-induced GCLC upregulation (Figure 5C), lending further support to the assumption that MRP1 would contribute to 15d-PGJ2-induced ROS generation and downstream events. Moreover, 15d-PGJ2 transiently elevated the extracellular GSH level at 1 h after 15d-PGJ2 treatment (Figure 5E), in parallel with the decrease in the intracellular GSH level (Figure 4A). In the presence of MK571, however, 15d-PGJ2 failed to cause both transient depletion of the intracellular GSH (Figure 5F) and elevation of the extracellular GSH (Figure 5G), supporting the possibility of MRP1-mediated efflux and a subsequent dissociation of the 15d-PGJ2-GSH conjugate in the extracellular part.

Figure 2. Role of Nrf2 activation in 15d-PGJ2-induced GCLC expression. (A) 15d-PGJ2-induced nuclear translocation of Nrf2 was examined by Western blot analysis. The nuclear fraction was prepared from MCF-7 cells treated with 10 μM of 15d-PGJ2 for the indicated time periods. Lamin B was used to ensure that equal amounts of nuclear proteins were loaded. (B) The Nrf2-ARE DNA binding activity was assessed by EMSA at the indicated time intervals after treatment with 15d-PGJ2, using a radiolabeled oligonucleotide probe harboring an ARE consensus sequence as described in Experimental Procedures. (C) MCF-7 cells were transiently transfected with the ARE luciferase construct (Ti-Luc, ARE-Luc, and GC-MT-Luc). The luciferase activities were measured after treatment with 15d-PGJ2 (10 μM) for 12 h. The Ti-Luc plasmid was used as the mock vector. Luciferase activities were normalized by cotransfection with β-gal. Values represent the means ± SD of three samples. (D) MCF-7 cells were transfected with scrambled siRNA or Nrf2 siRNA for 72 h, followed by treatment with 15d-PGJ2 for an additional 24 h. The expression level of GCLC was examined by Western blot analysis.

with MCF-7 cells increased the phosphorylation of Akt in a time-dependent fashion (Figure 3A). LY294002, a PI3K inhibitor, abrogated 15d-PGJ2-induced nuclear translocation of Nrf2 as assessed by the immunocytochemical assay (Figure 3B). Likewise, the transient transfection with Akt-siRNA suppressed the accumulation of Nrf2 in the nucleus provoked by 15d-PGJ2 (Figure 3C), corroborating the fact that Nrf2 activation by 15d-PGJ2 is regulated via the PI3K-Akt pathway. Treatment with LY294002 also abolished the 15d-PGJ2-induced GCLC expression (Figure 3D) and subsequent GSH biosynthesis (Figure 3E). Likewise, Akt knockdown with siRNA blocked the up-regulation of GCLC induced by 15d-PGJ2 (Figure 3F).

15d-PGJ2 Reduces Intracellular GSH Levels due to the Formation of the GSH Conjugate in MCF-7 Cells, Leading to ROS Generation. GSH biosynthesis is predominantly catalyzed by GCL.26 We therefore measured the intracellular GSH contents at various time intervals after treatment with 15d-PGJ2. Notably, the intracellular GSH level in 15d-PGJ2-treated MCF-7 cells was initially reduced but eventually enhanced even above the basal level (Figure 4A). Elevation of the intracellular GSH level is likely to be triggered as a feedback response to the initial reduction of GSH, which appears to be attributed to GSH conjugation with 15d-PGJ2. 15d-PGJ2 has been reported to form a GSH adduct, both enzymatically and nonenzymatically.22,27 In the present study, we were also able to demonstrate that 15d-PGJ2 directly bound to GSH at 1 h by using a biotin-streptavidin system (Figure 4B). Moreover, 15d-PGJ2 induced the expression of glutathione S-transferase π (GSTP) responsible for GSH conjugation with electrophiles at 1 h (Figure 4C), which might catalyze 15d-PGJ2-GSH conjugation.28

We postulated that early depletion of the intracellular GSH level, as a result of conjugation with 15d-PGJ2, provokes ROS generation, which is reciprocally linked to the cellular GSH contents. We observed that ROS accumulation occurred concomitantly when the cellular GSH level dropped following 15d-PGJ2 treatment (Figure 4D). To ensure that 15d-PGJ2-induced ROS formation could mediate cellular stress response, thereby upregulating GCLC, MCF-7 cells were exposed to the antioxidant NAC. NAC cotreatment abolished the expression of GCLC as well as the phosphorylation of Akt induced by 15d-PGJ2 (Figure 4E), indicative of the ROS involvement in 15d-PGJ2-induced GCLC upregulation through activation of PI3K-Akt signaling.
Cyclopentenone Moiety of 15d-PGJ2 Is Essential for Its Up-Regulation of GCLC Expression in MCF-7 Cells. The cyclopentenone moiety of 15d-PGJ2 has been proposed as an important structural feature for some of the effects exerted by this electrophilic prostaglandin. As depicted in Figure 6A, H2-15d-PGJ2, a hydrogenated analogue of 15d-PGJ2 lacking the electrophilic α,β-unsaturated carbonyl group, was unable to undergo conjugation with GSH (Figure 6B). In line with this observation, H2-15d-PGJ2 failed to induce ROS accumulation (Figure 6C) and provoked neither GCLC upregulation (Figure 6D) nor GSH elevation (Figure 6E). Furthermore, phosphorylation of Akt (Figure 6D) and nuclear translocation of Nrf2 (Figure 6F) were not evident in cells treated with H2-15d-PGJ2. These results clearly indicate that the electrophilic carbon 9 (marked with an asterisk in Figure 6A) located in the cyclopentenone ring is essential for the formation of the 15d-PGJ2-GSH conjugate and activation of Akt and Nrf2 signaling.

**DISCUSSION**

GSH, the most predominant intracellular nonprotein thiol, is an essential component of cellular defense against oxidants and electrophilic toxicants. The balance between GSH and its disulfide form (GSSG) determines cellular redox status. The formation of GSH adducts with various electrophiles occurs nonenzymatically or is catalyzed by GSTs. Conjugation with GSH is generally regarded as a detoxification process, but it may also modulate signal transduction indirectly through alteration of the cellular redox status.

According to the “hard and soft acids and bases” theory, GSH is regarded as a soft nucleophile that preferentially reacts with soft electrophiles, such as Michael addition acceptors with the α,β-unsaturated carbonyl group. 15d-PGJ2 has been reported to conjugate with GSH at the carbon 9 in MCF-7 and HepG2 cells, due to the electrophilic α,β-unsaturated carbonyl moiety.
localized in the cyclopentenone ring.\textsuperscript{22,30} In the present study, we found that 15d-PGJ\textsubscript{2}-induced transient reduction of the intracellular GSH content coincided with 15d-PGJ\textsubscript{2}-GSH conjugation in MCF-7 cells. We speculate that the formation of a GSH conjugate of 15d-PGJ\textsubscript{2} may result in initial depletion of the intracellular GSH level, which provokes the up-regulation of GCLC responsible for the \textit{de novo} synthesis of GSH as a feedback response. Diethylmaleate, capable of depleting GSH through conjugation, showed an intracellular GSH profile similar to that attained with 15d-PGJ\textsubscript{2} treatment and also induced GCLC expression in MCF-7 cells (Song, N.-Y. and Surh, Y.-J., unpublished observation). Consistent with our observations, Liu et al. reported that 4-hydroxynonenal, an electrophilic end-product of lipid peroxidation, caused an initial depletion of GSH through conjugation, followed by a marked increase in GCL-catalyzed GSH biosynthesis.\textsuperscript{31} Thus, the formation of the 15d-PGJ\textsubscript{2}-GSH conjugate appears to contribute to 15d-PGJ\textsubscript{2}-induced upregulation of GCLC in MCF-7 cells by altering the intracellular GSH pool. In contrast to 15d-PGJ\textsubscript{2}, \(H_2\)-15d-PGJ\textsubscript{2}, a non-electrophilic analogue of 15d-PGJ\textsubscript{2}, was incompetent in conjugating with GSH and inducing GCLC expression.

An important question may arise as to how 15d-PGJ\textsubscript{2}-GSH conjugation leads to an early reduction of the intracellular GSH level, which is in the millimolar range in most cells, whereas the concentration of 15d-PGJ\textsubscript{2} treated with MCF-7 cells in this study is much lower (10 \(\mu\)M). Such a large concentration difference prompted us to speculate the possible existence of a 15d-PGJ\textsubscript{2} recycling loop as schematically proposed in Scheme 1. The MRP1/GS-X pump, a member of the ABC transporter family, is a ubiquitously expressed efflux pump especially for GSH conjugates.\textsuperscript{21,32} It has been reported that the 15d-PGJ\textsubscript{2}-GSH conjugate is efficiently pumped out of the cell by MRP1.\textsuperscript{22} We therefore presumed that MRP1 could take part in 15d-PGJ\textsubscript{2} recycling, which accounts for the initial depletion of intracellular GSH and concurrent ROS production. In support of this supposition, the inhibition of MRP1 abrogated not only 15d-PGJ\textsubscript{2}-induced ROS accumulation but also GCLC expression. Once pumped out, the 15d-PGJ\textsubscript{2}-GSH conjugate may undergo dissociation to release free 15d-PGJ\textsubscript{2} in the extracellular compartment as GSH conjugation is a reversible process. The deconjugation of GSH adducts has been reported to occur spontaneously or enzymatically under the physiological conditions.\textsuperscript{22,27} Brunoldi et al.

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\caption{Transient reduction of intracellular GSH accumulation through conjugation with GSH and upregulation of GSTP and GCLC in 15d-PGJ\textsubscript{2}-treated MCF-7 cells. (A) The cells were treated with 15d-PGJ\textsubscript{2}, and the intracellular GSH level was determined at various time intervals by HPLC as described in Experimental Procedures. Values represent the means \(\pm\) SD of three samples. (B) MCF-7 cells were exposed to 10 \(\mu\)M biotinylated 15d-PGJ\textsubscript{2} for 1 h, GSH was immunoprecipitated with the GSH antibody and analyzed by Western blot, followed by detection with HRP-conjugated streptavidin. (C) The time-dependent alterations in the expression of GST after treatment with 15d-PGJ\textsubscript{2} were determined by Western blot analysis. (D) The intracellular ROS level in 15d-PGJ\textsubscript{2}-treated MCF-7 cells was assessed on the basis of the DCF-DA fluorescence. Cells were exposed to 10 \(\mu\)M 15d-PGJ\textsubscript{2} for the indicated time periods, followed by acquisition of the cellular fluorescence images using a confocal laser-scanning microscope. (E) MCF-7 cells were exposed to 15d-PGJ\textsubscript{2} with or without NAC (1, 5, and 10 mM) for 24 h, and the expression levels of GCLC and p-Akt were examined using corresponding antibodies.}
\end{figure}
showed the reversibility of the conjugation reaction between 15d-PGJ2 and GSH in HepG2 cells. We found that the extra-cellular GSH level was transiently increased following 15d-PGJ2 treatment, concomitantly with the depletion of the intracellular GSH content. In addition, 15d-PGJ2-induced fluctuation of the intra- and extracellular GSH levels was not observed when MRP1 was blocked by a pharmacologic antagonist. These data suggest a possibility that the 15d-PGJ2-GSH conjugate effluxed via MRP1 is broken down to liberate 15d-PGJ2, which is lipophilic and can be recycled after re-entering the cell, while hydrophilic GSH resides outside the cell.

It is well known that the cellular GSH content is inversely related to the intracellular ROS level. Excessive ROS generation predisposes cells to oxidative death and dysfunction of important proteins, promoting the pathogenesis of various human diseases as well as aging. Mild ROS challenge, however, can trigger cellular stress signal transduction cascades to initiate adaptive survival responses in a timely manner. ROS stimulate the mitogen-activated protein kinase and PI3K signal transduction pathways via the induction of Ca2+ fluxes and/or inhibition of certain protein phosphatases. We observed that the early decline in the intracellular GSH level provoked by 15d-PGJ2 was accompanied by accumulation of ROS, resulting in activation of the PI3K-Akt signaling. A plausible mechanism responsible for ROS-induced PI3K activation is a dysfunction of phosphatase and the tensin homologue deleted on chromosome 10 (PTEN), an antagonist for PI3K signaling. Kwon et al. demonstrated that ROS such as hydrogen peroxide can oxidize PTEN via the formation of an intramolecular disulfide bond between its adjacent cysteine residues. As a consequence of the reversible oxidation of PTEN, its negative regulation of the PI3K is shut down, allowing activation of downstream signals mediated by the PI3K-Akt pathway.

Thiol modification of Keap1 and phosphorylation of Nrf2 have been suggested as two principal mechanisms underlying

Figure 5. Possible involvement of MRP1 in 15d-PGJ2-induced up-regulation of GCLC in MCF-7 cells. (A) MCF-7 cells were treated with 15d-PGJ2 in the absence or presence of MK571 (1, 10, and 100 μM), an inhibitor of MRP1, and the expression of GCLC was analyzed by Western blot analysis. (B) MCF-7 cells were exposed to 10 μM 15d-PGJ2 with or without 100 μM MK571 for 3 h, and the intracellular ROS level was measured using DCF-DA as described in Experimental Procedures. (C) MCF-7 cells were transfected with MRP1-siRNA or negative control siRNA for 72 h, followed by the treatment with 15d-PGJ2 for an additional 24 h. Expression of GCLC was examined by Western blot analysis. (D) MCF-7 cells were exposed to 15d-PGJ2 in the absence or presence of MK571 as described for B, and the intracellular GSH levels were determined using the GSH-400 kit. (E and G) MCF-7 cells were treated with 15d-PGJ2 (10 μM) in the absence (E) or presence (G) of MK571 for the indicated time periods, and the culture medium was collected. The extracellular GSH contents were measured utilizing the GSH-400 kit as described in Experimental Procedures. (F) The intracellular GSH level was measured under the same condition as that described in G.
Nrf2 activation. It has been previously reported that thiol adduction of Keap1 by 15d-PGJ2 facilitates the release of Nrf2 from Keap1, while another study has revealed that 15d-PGJ2-induced thiol modification of Keap1 may not be sufficient to disrupt the Keap1-Nrf2 complex.38,39 Besides direct modification of Keap1 with 15d-PGJ2, the ROS accumulated as a consequence of the formation of the 15d-PGJ2-GSH conjugate appear to be involved in 15d-PGJ2-induced Nrf2 activation through inactivation of PTEN and subsequent activation of PI3K-Akt as addressed above.

The mechanism of Nrf2 activation via PI3K-Akt signaling is not explicitly characterized. One possibility is that activated Akt may directly phosphorylate Nrf2, resulting in its liberation from Keap1. Huang et al. have reported that protein kinase C phosphorlylates serine 40 present in the Neh2 domain of Nrf2, thereby enhancing the Nrf2-ARE transcriptional activity.40 Alternatively, Akt may phosphorylate and inactivate glycogen synthase kinase-3β, resulting in nuclear exclusion and degradation of Nrf2 through phosphorylation, resulting in Nrf2 activation.41,42

While GCL and its product GSH play pivotal roles in detoxification and elimination of electrophilic carcinogens, their elevated levels in tumor cells may confer resistance to chemotherapeutics.43,44 We also observed that 15d-PGJ2-induced up-regulation of GCLC and subsequent elevation of GSH rendered human breast cancer MCF-7 cells resistant to doxorubicin-induced cell death (Song, N.-Y. and Surh, Y.-J., unpublished observation). Moreover, we have previously demonstrated that 15d-PGJ2 induces Nrf2-dependent upregulation of MRP1 in MCF-7 cells, which is involved in chemoresistance.24 Hence, 15d-PGJ2, one of the terminal products of the COX-2 pathway, may confer resistance to various chemotherapeutics through Nrf2-mediated coordinated upregulation of GCLC and MRP1 in MCF-7 cells.

In conclusion, MRP1 plays a prominent role in Nrf2-driven GCLC expression in 15d-PGJ2-treated MCF-7 cells, possibly through pumping out the 15d-PGJ2-GSH conjugate and expediting the recycling of 15d-PGJ2 subsequently released from the conjugate. During the recycle process, the intracellular GSH level

Figure 6. Requirement of the cyclopentenone ring in the 15d-PGJ2-induced up-regulation of GCLC expression in MCF-7 cells. (A) The chemical structures of 15d-PGJ2 and its non-electrophilic analogue H2-15d-PGJ2. The asterisk represents the electrophilic carbon center. (B) MCF-7 cells were exposed to 10 μM biotinylated 15d-PGJ2 or the same concentration of biotinylated H2-15d-PGJ2 for 1 h. GSH was immunoprecipitated with the GSH antibody and analyzed by Western blot, followed by detection with HRP-conjugated streptavidin. (C) MCF-7 cells were treated with 10 μM each of 15d-PGJ2 or H2-15d-PGJ2 for 3 h, and the cellular ROS accumulation was determined using DCF-DA. (D, E, and F) MCF-7 cells were treated with 10 μM 15d-PGJ2 or the same concentration of H2-15d-PGJ2, and the expression of GCLC and p-Akt (D), the intracellular GSH level (E), and the nuclear translocation of Nrf2 (F) were assessed.
is transiently reduced followed by elevation of the extracellular GSH, which is attributed to repetitive formation and efflux of the 15d-PGJ$_2$-GSH conjugate. As a result, the redox-sensitive transcription factor Nrf2 is activated via ROS-dependent Akt phosphorylation, leading to sequential upregulation of GCLC and GSH. We thus suggest the transient GSH depletion due to conjugation between 15d-PGJ$_2$ and GSH followed by putative recycling as a novel mechanism responsible for the transactivation of Nrf2-mediated genes by 15d-PGJ$_2$ in addition to the thiol modification of Keap1 (Scheme 1).

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