15-Deoxy-Δ^{12,14}-prostaglandin J₂ activates PI3K-Akt signaling in human breast cancer cells through covalent modification of the tumor suppressor PTEN at cysteine 136

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Abstract
15-Deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂), one of the terminal products of cyclooxygenase-2-catalyzed arachidonic acid metabolism, has been shown to stimulate breast cancer cell proliferation and migration through Akt activation, but the underlying mechanisms remain poorly understood. In the present study, we investigated the effects of 15d-PGJ₂ on the activity of PTEN, the inhibitor of the phosphoinositide 3-kinase (PI3K)-Akt axis, in human breast cancer (MCF-7) cells. Since the α,β-unsaturated carbonyl moiety in the cyclopentenone ring of 15d-PGJ₂ is electrophilic, we hypothesized that 15d-PGJ₂-induced Akt phosphorylation might result from the covalent modification and subsequent inactivation of PTEN that has several critical cysteine residues. When treated to MCF-7 cells, 15d-PGJ₂ bound to PTEN, and this was abolished in the presence of the thiol-reducing agent dithiothreitol. A mass spectrometric analysis by using recombinant and endogenous PTEN protein revealed that the cysteine 136 residue (Cys136) of PTEN is covalently modified upon treatment with 15d-PGJ₂. Notably, the ability of 15d-PGJ₂ to covalently bind to PTEN as well as to induce Akt phosphorylation was abolished in the cells expressing a mutant form of PTEN in which Cys136 was replaced by serine (C136S-PTEN). The present study demonstrates for the first time that electrophilic 15d-PGJ₂ directly binds to cysteine 136 of PTEN and provides new insight into PTEN loss in cancer progression associated with chronic inflammation. These observations suggest that 15d-PGJ₂ can undergo nucleophilic addition to PTEN, presumably at Cys136, thereby inactivating this tumor suppressor protein with concomitant Akt activation.

1. Introduction
Breast cancer is one of the leading causes of cancer-related deaths in women [1, 2]. As in the case of the majority of other malignancies, inflammation is associated with pathogenesis of breast cancer. Cyclooxygenase-2 (COX-2), a key enzyme involved in inflammatory response, is frequently overexpressed in various cancer types including breast cancer [3-6]. Abnormally elevated COX-2 expression or activity causes stimulation of cancer cell proliferation, resistance to cancer cell apoptosis, and enhancement of angiogenesis and invasiveness which account for its oncogenic function [7]. COX-2 catalyzes the conversion of arachidonic acid to a series of prostaglandins (PGs), some of which have been reported to play roles in carcinogenesis [4, 5, 8]. Of note, aberrant induction of COX-2 in breast cancer is correlated with an increased production of 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) [8]. 15d-PGJ₂ has been known to have oncogenic characteristics [9]. 15d-PGJ₂ contains a chemically reactive α,β-unsaturated carbonyl moiety in its...
cyclopentenone ring. Because of its electrophilic nature, 15d-PGJ2 can form a covalent adduct with free thiols of cysteine residues present in various proteins via the Michael addition reaction. Proteins which are known to be covalently modified by 15d-PGJ2 include nuclear factor-kappa B (NF-kB) [10], activator protein-1 (AP-1) [11], H-Ras [12], the tumor suppressor protein p53 [13], eukaryotic initiation factor 4A [14], Rac1 [15], c-Jun [16] and Hu antigen R [17].

Our previous study demonstrated that 15d-PGJ2 induced COX-2 expression in human breast cancer (MCF-7) cells via the Akt-AP-1 signaling pathway [8]. Akt activation by 15d-PGJ2 was considered to be responsible for pro-inflammatory and plausible tumor-promoting effects of this cyclopentenone PG [8]. However, the molecular mechanism underlying 15d-PGJ2-induced Akt activation remains to be clarified. The tumor suppressor, phosphatase and tensin homolog (PTEN) has been demonstrated the intracellular production of ROS by 15d-PGJ2. As might be also potential targets for Michael addition by electrophilic reagents; these catalytic cysteine residues might be also potential targets for Michael addition by electrophilic oxidation or nitrosylation. These catalytic cysteine residues could serve as critical residues for redox-sensitive signaling.

2. Materials and methods

2.1. Materials

15d-PGJ2 was obtained from Cayman Chemical Co. (Ann Arbor, MI). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), diethiothreitol (DTT), L-ascorbic acid (vitamin C) and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). Antibodies against Akt, phospho-Akt, PTEN, and hemagglutinin (HA) tag were products of Cell Signaling Technology (Danvers, MA). Antibody against streptavidin-horseradish peroxidase conjugate was purchased from Sigma-Aldrich. Secondary antibodies were purchased from Thermo Scientific (Waltham, MA).

2.2. Cell culture

Human breast cancer MCF-7, MDA-MB-231 and SKBr-3 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in their recommended media supplemented with 10% fetal bovine serum and 100 ng/ml penicillin/streptomycin/fungizone mixture at 37 °C in a humidified atmosphere of 5% CO2 / 95% air.

2.3. Wound healing assay

MCF-7 cells (3 × 105 cells/ml) were plated into the ibidi culture insert on 6 well dishes. After 24-h incubation for appropriate cell attachment, the culture-insert was gently removed by using sterile tweezers. Cells were treated with 10 μM of 15d-PGJ2 alone or together with LY294002 for another 24 h. Cell migration was observed under the microscope.

2.4. Plasmids

The pSG5L-HA tagged PTEN expressing (wild-type), PTEN with C124S mutation (mutant type), and PTEN mock plasmids were purchased from Addgene (Cambridge, MA). Site-directed mutagenesis was performed by PCR assembly using pSG5L-HA-PTEN plasmid as a template. The following oligonucleotide and their complementary sequences were used to design the mutation in PTEN-Cys71 → Ser71, 5'- CAAGATATACAAATCTTAGTGCTGAAA-GACA-3', PTEN-Cys136 → Ser136 (C136S): 5'-CTCGTGTATGATTAACTGATATATC-3. The PCR fragments were annealed and 18 cycles of PCR were performed. The PCR fragments were then sub-cloned into the pSG5L expressing vector. The presence of site-directed mutations was confirmed by complete sequencing of the PTEN gene.

2.5. siRNA transient transfection

MDA-MB-231 cells were transfected with COX-2-siRNA or scrambled siRNA using the transfection reagent Lipofectamine RNAiMAX for 48 h according to manufacturer's instructions. The sequence of human COX-2-siRNA was 5'-CACCAGA-GUAAUACCUU-3' (forward) and 5'-AAGGCUUAACUCCUGUGG-3' (reverse) which was purchased from Bioneer Cooperation (Daejeon, Korea). Transfected cells were then treated with 15d-PGJ2 for 3 h and then harvested for the next experiments.

2.6. Western blot analysis

MCF-7 cells (2 × 105 cells/ml) were plated in a 60-mm dish and treated with 15d-PGJ2 under specified conditions. After rinse with phosphate buffered saline (PBS), the cells were exposed to the lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin and protease inhibitors] on ice for 30 min. After centrifugation at 13,000 g for 15 min, supernatant was separated and stored at −70 °C until use. The protein concentration was determined by using the BCA protein assay kit (Thermo Scientific, Waltham, MA). Protein samples were electrophoresed in a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane at 100 mA for 16 h. Blots were incubated in fresh blocking buffer (tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% nonfat dry milk, pH 7.4) for 1 h followed by incubation with appropriate primary antibodies in TBS with 3% bovine serum albumin (BSA). After washing with TBST three times, blots were incubated with horseradish peroxidase-conjugated
secondary antibody in TBST with 3% nonfat dry milk for 1 h at room temperature. Blots were washed again three times in TBST buffer, and transferred proteins were detected with an enhanced chemiluminescence detection kit (Abclone, Seoul, Korea).

2.7. Identification of reduced and oxidized forms of PTEN by immunoblot analysis

MCF-7 cells (2 × 10^5 cells/ml) were plated in a 100-mm dish and treated with 15d-PGJ2 under specified conditions. After rinse with PBS containing 10 mM N-ethylmaleimide (NEM), the cells were exposed to the lysis buffer [10 mM NEM, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na3EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na2VO4, 1 µg/ml leupeptin and protease inhibitors] on ice for 1 h. After centrifugation at 13,000g for 15 min, protein samples were subjected to SDS-PAGE, followed by Western blot analysis.

2.8. Measurement of ROS

To monitor the accumulation of intracellular ROS, the fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA) was used. Following treatment, cells were rinsed with Hank’s Balanced Salt Solution (HBSS) and were treated with 10 µM of DCF-DA. After 30 min incubation at 37°C, cells were examined under the fluorescence microscope.

2.9. Anchorage-independent growth assay

A total of 2 × 10^4 cells were seeded on a six-well plate in RPMI medium containing 10% fetal bovine serum and 0.33% agarose on top of a layer of medium containing 0.5% agarose and overlaid with medium containing 10% fetal bovine serum, which was changed every 3–4 days. Colonies were counted under a light microscope approximately 4 weeks after seeding. For each construct, three independent experiments were performed and at least 10 microscopic images per experiment were used for measurement of colony sizes under the microscope. In all experiments, a cutoff diameter of at least 100 µm was used to distinguish colonies from background debris. All diagrams report the mean number of colonies ± standard deviation (SD).

2.10. Docking simulation

Flexible docking of 15d-PGJ2 onto PTEN was performed by use of the SYBYL version 7.0 software program (SYBYL molecular modeling software, Tripos, Inc, version 7.0, St Louis, MO) and the Red Hat Linux operating system, version 7.0. The chemical structures of compounds were prepared in MOL2 format by use of the sketcher module in SYBYL, and partial atomic charges were calculated by the Gasteiger–Huckel method and assigned to the ligand atoms. To optimize the ligand structures, the conjugate gradient energy minimization of the ligand was run until the value converged to a maximum derivative of 0.001 kcal/mol Å. The x-ray crystal structure of complex between PTEN and tartrate (Protein Data Bank entry = 1DSR) was used as a target for the docking of flexible ligand by FlexX algorithm in SYBYL. All crystallographic water molecules were removed from target structure except for one that was involved in the hydrogen bond network inside the binding pocket. The active site was defined as all the amino acid residues enclosed within 6.5 Å radius sphere centered by the bound tartrate.

2.11. Molecular docking

The crystal structure of PTEN was obtained from RCSB Protein Data Bank (PDB code 5BZZ). The structure of PTEN was minimized using the Protein Preparation Wizard by applying an OPLS_2005 force field [34]. The model structure of 15d-PGJ2 was built by using Maestro build panel and minimized by using the Macromodel of Maestro in the Schrödinger suite program. Glide covalent docking was used to predict the covalently bound conformations of 15d-PGJ2. Docking space was defined as a 50 Å cubic box centered on Cys136, which was even set as a reactive residue. Michael addition by sulfur atom of Cys136 was provided in the default reaction types in Glide covalent docking. No docking constraint to reference position was applied even though a distance constrain between the reactive atoms was applied by the glide covalent docking protocol. Docking mode was set to “thorough” with a minimization radius of 3 Å from the ligand.

2.12. Transient transfection

MCF-7 cells were seeded at a density of 1 × 10^5 cells/ml in 60-mm dish and grown to 90% confluence in complete growth media. Transient transfections with pSGSL-HA-PTEN derivatives were performed using the Lipofectamine 2000 transfection reagents according to the instructions supplied by the manufacturer (Invitrogen, Carlsbad, CA). After 12 to 24 h transfection, cells were treated with 15d-PGJ2 for additional 12 h and lysed for Western blot analysis.

2.13. Immunoprecipitation analysis

MCF-7 cells (2 × 10^5 cells/ml) were plated in a 100-mm dish and treated with biotinylated-15d-PGJ2 under specified conditions. After rinse with PBS, the cells were exposed to the lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na3EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na2VO4, 1 µg/ml leupeptin and protease inhibitors] on ice for 30 min. After centrifugation at 13,000g for 15 min, supernatant was separated and stored at −70°C until use. The protein concentration was determined by using the BCA protein assay kit. Protein lysates (500 µg) were subjected to immunoprecipitation by shaking with a primary antibody against PTEN or HA (Cell Signaling Technology, Beverly, MA) at 4°C for 12 h followed by addition of protein G agarose bead suspension (25% slurry, 40 µl) from each sample was collected after 70°C for 2 min, immunoprecipitated beads were collected by discarding the supernatant and rinsed with cell lysis buffer. After final wash, immunoprecipitate was resuspended in 50 µl of 2 × SDS electrophoresis sample buffer and boiled for 5 min. Supernatant (40 µl) from each sample was collected after centrifugation and loaded onto 8% SDS polyacrylamide gel. Proteins were then transferred from the gel to a PVDF membrane and immunoblotted with HRP-conjugated streptavidin antibody.

2.14. Mass spectrometric analysis

Sample preparation. 15d-PGJ2 (30 µM) or vehicle DMSO was added to recombinant PTEN for 1 h at room temperature, and PTEN peptides were subjected to trypsinization. For analysis of endogenous PTEN, MCF-7 cells were transiently transfected with pSGSL-HA-WT-PTEN and treated with 10 µM of 15d-PGJ2 for 6 h. Cell lysates were prepared by lysis buffer for 30 min in ice and trypsinized. The mass spectral analysis was performed by Diatech Korea Co. (Seoul, Korea).

Enzymatic in-gel digestion. 15d-PGJ2 treated or vehicle treated PTEN samples were separated by NuPAGE® 4–12% Bis-Tris Gel
In-vitro gelation and gel digestion were performed using respective horseradish peroxidase (HRP) conjugated secondary antibody (rabbit). The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine tetrahydrochloride. Finally, counterstaining was performed using Mayer’s hematoxylin.

2.17. Statistical analysis

When necessary, data were expressed as mean ± SD, and statistical analysis for single comparison was performed using a one-way ANOVA. At least three independent experiments were averaged. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.005\), compared to groups.

3. Results

3.1. 15d-PGJ \(_2\) induces the activation of Akt in breast cancer cells

To explore the effect of 15d-PGJ \(_2\) on activation of the oncoprotein Akt in breast cancer cells, we utilized three different subtypes of breast cancer cell lines [estrogen receptor (ER)-positive, MCF-7; ER-negative, MDA-MB-231; Her-2 enriched: SKBR-3], all of which harbor the wild-type PTEN gene [35]. At first, we intended to determine whether the effect of 15d-PGJ \(_2\) on Akt activation is a general phenomenon in breast cancer cells which contain functional PTEN gene. However, while the level of phosphorylated Akt was constitutively elevated in SKBR-3 cells, a luminal B type breast cancer cells exhibiting the elevated level of Her-2 protein, both MCF-7 and MDA-MB-231 cells showed a relatively low level of Akt phosphorylation (Supplementary Fig. S1A). Because of a highly elevated level of constitutively phosphorylated Akt in SKBR-3 cells due to Her-2 overexpression [36], further increase in Akt phosphorylation upon 15d-PGJ \(_2\) treatment was not achievable (Supplementary Fig. S1B). For this reason, we used the other two cell lines, MCF-7 and MDA-MB-231, for further experiments. When MCF-7 and MDA-MB-231 cells were treated with 15d-PGJ \(_2\) (10 \(\mu\)M), there was a time-dependent increase in phosphorylation of Akt, whereas the protein levels of Akt and its upstream regulator PTEN barely changed (Fig. 1A and 1B). The increased phosphorylation of Akt was also dependent on the concentration of 15d-PGJ \(_2\) in MCF-7 cells (Supplementary Fig. S1C). Again, the expression of PTEN as well as Akt was not changed. beside breast cancer cells, incubation of human colon cancer HCT-116 cells, harboring wild-type PTEN gene [37], with 15d-PGJ \(_2\) also induced Akt phosphorylation (Supplementary Fig. S2A). This suggests that 15d-PGJ \(_2\)-promoted Akt activation can be manifested in other cancer cells which express wild-type PTEN protein. Additionally, the stimulatory effect of 15d-PGJ \(_2\)-induced Akt phosphorylation on migration of MCF-7 cells was confirmed by the wound healing assay. 15d-PGJ \(_2\)-induced cell migration was suppressed when the PI3K inhibitor (LY294002) was co-treated with 15d-PGJ \(_2\) (Fig. 1C).

COX-2, which is a rate-limiting enzyme in the arachidonic acid cascade, was upregulated at 48 h in MCF-7 cells (Fig. 1A) and at 3 h in MDA-MB-231 cells (Fig. 1B) following 15d-PGJ \(_2\) (10 \(\mu\)M) treatment. Upregulation of COX-2 by 15d-PGJ \(_2\) may augment its formation as a terminal product. To identify whether 15d-PGJ \(_2\)-induced Akt phosphorylation is associated with COX-2 expression, MDA-MB-231 cells were transfected with COX-2 siRNA. Even though COX-2 gene was silenced, 15d-PGJ \(_2\)-induced Akt phosphorylation was not abrogated (Fig. 1D). Likewise, pharmacologic inhibition of COX-2 with celecoxib failed to affect the 15d-PGJ \(_2\)-induced Akt phosphorylation in MDA-MB-231 cells (Fig. 1E) and also in HCT-116 cells (Supplementary Fig. S2B). These results indicate that 15d-PGJ \(_2\)-induced Akt phosphorylation is not associated with COX-2 upregulation.

3.2. COX-2 regulates growth and migration of breast cancer cells

To determine whether COX-2 regulates growth and migration of breast cancer cells, we first performed soft agar colony formation assay. The colony formation inhibition upon COX-2 knockdown was not achieved (Fig. 2A). In vivo, the growth inhibition of breast cancer cells by the COX-2 inhibitor was not observed (Fig. 2B). To decipher the opposite role of COX-2, we hypothesized that COX-2 plays a stimulatory role in tumor growth; in other words, in vivo COX-2 knockdown may not be an effective strategy to inhibit tumor growth. To support this assumption, we conducted the knockdown efficiency of COX-2 expression in vivo (Fig. 2C). COX-2 silencing significantly inhibited the migration of breast cancer cells in vitro (Fig. 2D). These results suggest that COX-2 can be a potential therapeutic target in breast cancer.

3.3. COX-2 regulates the PI3K/Akt signaling in breast cancer cells

To determine whether COX-2 regulates PI3K/Akt signaling in breast cancer cells, we first performed the knockdown efficiency of COX-2 expression in vivo and also in vitro (Fig. 3A and 3B). COX-2 knockdown significantly inhibited the migration of breast cancer cells in vitro (Fig. 3C). These results suggest that COX-2 can be a potential therapeutic target in breast cancer.

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3.2. ROS does not appear to play a role in 15d-PGJ2-induced Akt phosphorylation

15d-PGJ2 and other cyclopentenone PGs have been shown to induce the production of ROS capable of inducing Akt phosphorylation [8]. Therefore, we initially examined whether ROS could be involved in 15d-PGJ2-induced Akt phosphorylation. Akt activation through PI3K-mediated phosphorylation is counteracted by PTEN. Human PTEN protein contains seven cysteine residues, of which cysteine 71 and cysteine 124 are known to be responsible for the regulation of its phosphatase activity [38]. Hydrogen peroxide (H2O2) has been shown to inactivate PTEN by inducing formation of
a disulfide bond between these two spatially adjacent cysteine residues [29]. To determine whether 15d-PGJ2-generated ROS could oxidize PTEN or not, MCF-7 cells were treated with 15d-PGJ2 for indicated periods of time. We utilized MCF-7 cells for this study because these cells have been known to exhibit relatively low intracellular ROS content compared with MDA-MB-231 cells [39]. Electrophoresis under non-reducing conditions showed that oxidized PTEN migrated faster as a discrete isoform, which occurred only in cells treated with H2O2, but not in cells treated with 15d-PGJ2 (Fig. 2A). To clarify whether 15d-PGJ2-produced ROS mediates Akt phosphorylation, MCF-7 cells were pre-incubated with ROS scavengers, trolox which is a water-soluble vitamin E analog and vitamin C. Though both antioxidants efficiently attenuated the 15d-PGJ2-induced intracellular ROS accumulation (Fig. 2B), neither trolox nor vitamin C inhibited 15d-PGJ2-induced Akt phosphorylation (Fig. 2C and 2D). Another antioxidant NAC, which also acts as a thiol reducing agent, abrogated 15d-PGJ2-promoted Akt activation (Supplementary Fig. S1D). All together, these results suggest that oxidative inactivation of PTEN through disulfide formation is unlikely to account for 15d-PGJ2-induced

Fig. 2. Non-involvement of ROS-induced oxidation of PTEN in its inactivation by 15d-PGJ2 and effects of ROS scavengers on 15d-PGJ2-induced Akt phosphorylation in MCF-7 cells. (A) Redox status of PTEN was measured by SDS-PAGE under non-reducing condition. MCF7 cells were treated with 10 μM of 15d-PGJ2 for indicated time points. H2O2 (1 mM) was used as a reference to oxidize PTEN, and DTT (1 mM) was used to keep PTEN in a reduced state. Two discrete forms of PTEN were identified as separate bands. Reduced PTEN migrated slower than the oxidized form due to difference in the molecular mass. PTEN control represents immunoblot of PTEN under reducing condition. (B) Cells were treated with 10 μM of 15d-PGJ2 for 12 h in the presence or absence of trolox (1 mM) or vitamin C (1 mM). Images of cellular fluorescence were acquired by using a fluorescence microscope. (C) Cells were exposed to 15d-PGJ2 (10 μM) in the absence or presence of trolox (0.1, 0.5 or 1 mM) for 12 h. (D) Cells were treated with 15d-PGJ2 in the absence or presence of vitamin C (0.1, 0.5 or 1 mM) for 12 h. The activation of Akt through phosphorylation was determined by Western blot analysis.
enhancement of Akt activation in MCF-7 cells.

3.3. 15d-PGJ₂–induced Akt phosphorylation is mediated through S-alkylation of PTEN

15d-PGJ₂ has an electrophilic α,β-unsaturated carbonyl moiety within the cyclopentenone ring, so it can covalently modify cysteine residues in cellular proteins. We speculated that 15d-PGJ₂ could carbonylate and inactivate PTEN. In order to determine the direct interaction of 15d-PGJ₂ with PTEN, we utilized biotin-tagged 15d-PGJ₂. Treatment of MCF-7 cells with biotinylated 15d-PGJ₂ resulted in formation of a PTEN adduct in a time-dependent fashion (Fig. 3A). The binding of 15d-PGJ₂ to PTEN was also observed in MDA-MB-231 (Fig. 3B) and HCT-116 cells (Supplementary Fig. S2C). To test the possibility that 15d-PGJ₂-induced Akt activation was attributable to thiol modification of PTEN, MCF-7 cells were pre-incubated with the sulfhydryl reducing agent DTT that is expected to keep the cysteine thiol residues of PTEN in a reduced state. As illustrated in Fig. 3C, the binding of biotinylated 15d-PGJ₂ to PTEN was abrogated in the presence of DTT. In addition, DTT pretreatment abolished the ability of 15d-PGJ₂ to activate Akt through phosphorylation in MCF-7 cells (Fig. 3D) and HCT-116 cells (Supplementary Fig. S2D).

To verify whether the electrophilic cyclopentenone ring of 15d-PGJ₂ plays a critical role in the activation of Akt through PTEN thiol

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**Fig. 3. Possible involvement of PTEN thiol modification in the 15d-PGJ₂–induced Akt phosphorylation in breast cancer cells.** (A) MCF-7 cells were incubated with biotinylated 15d-PGJ₂ (30 μM) for indicated time periods. (B) MDA-MB-231 cells were treated with biotin-conjugated 15d-PGJ₂ (20 μM) for 1 and 3 h. (C) MCF-7 cells were exposed to biotin-conjugated 15d-PGJ₂ (10 μM) with or without DTT (1 mM) treatment for 6 h. Cell lysates were subjected to immunoprecipitation with the PTEN antibody followed by immunoblot analysis with HRP-conjugated streptavidin. (D) MCF-7 cells were incubated with 15d-PGJ₂ (10 μM) with or without DTT (0.2 or 1 mM) for 12 h. The levels of phosphorylated Akt were assessed by Western blot analysis.
modification, MCF-7 cells were incubated with 15d-PGJ$_2$ or its non-electrophilic analog 9,10-dihydro-15d-PGJ$_2$. Unlike 15d-PGJ$_2$, 9,10-dihydro-15d-PGJ$_2$ lacking the $\alpha,\beta$-unsaturated carbonyl moiety in the cyclopentenone ring failed to interact with PTEN (Fig. 4A) and to induce the phosphorylation of Akt (Fig. 4B). In addition to the covalent binding, other types of interactions, such as hydrogen bonding, may also contribute to the 15d-PGJ$_2$-induced PTEN inactivation. The binding energies of the x-ray complex structures were estimated by FlexX. FlexX is a computer program for predicting the geometry of the protein-ligand complex and estimates the binding affinity. The dock pose with the least binding energy has the highest affinity and hence is the best docked conformation. As shown in Fig. 4C, the docking energy of 15d-PGJ$_2$ with PTEN was calculated to be $-24.32$ kcal/mol whereas that of 9,10-dihydro-15d-PGJ$_2$ with PTEN was $-20.16$ kcal/mol, suggesting the interaction between 15d-PGJ$_2$ and PTEN is more stable than the interaction between 9,10-dihydro-15d-PGJ$_2$ and PTEN.

**Fig. 4. Requirement of the cyclopentenone ring of 15d-PGJ$_2$ for its covalent interaction with PTEN and subsequent activation of Akt in MCF-7 cells.** (A) MCF-7 cells were incubated with 30 $\mu$M each of biotinylated 15d-PGJ$_2$ or biotinylated 9,10-dihydro-PGJ$_2$. The interaction between PTEN and 15d-PGJ$_2$ was assessed by immunoblot analysis, and the incorporation of biotinylated 15d-PGJ$_2$ into PTEN immunoprecipitates was detected with HRP-streptavidin. (B) MCF-7 cells were treated with 15d-PGJ$_2$ or 9,10-dihydro-PGJ$_2$ (10 $\mu$M each) for 12 h. The expression levels of phosphorylated and total Akt were measured by Western blot analysis. (C) Flexible docking analysis comparing the relative affinity of 15d-PGJ$_2$ and 9,10-dihydro-PGJ$_2$ for PTEN was performed by using the SYBYL version 7.0 software program and the Red Hat Linux operating system, version 7.0.
3.4. Site-directed mutation of PTEN cysteine 136 negates its covalent modification and subsequent inactivation by 15d-PGJ2.

The catalytically active site of human PTEN contains several cysteine residues, such as cysteine 71 and 124 that are prone to oxidation. In addition, mutation of the cysteine 136 residue of PTEN has been implicated in Cowden’s disease which is a risk factor of breast cancer [40]. To elucidate which of the aforementioned cysteine residues of PTEN could be a target of 15d-PGJ2, we performed site-directed mutagenesis by replacing distinct cysteine residues with serine. As a result, three different PTEN mutant constructs (Cys71S, Cys124S, and Cys136S) were generated (Fig. 5A). MCF-7 cells were transiently transfected with either wild-type or each of aforementioned mutant PTEN vectors. Notably, MCF-7 cells harboring PTEN mutation at cysteine 136 (Cys136S-PTEN) exhibited the markedly reduced level of phosphorylated Akt upon 15d-PGJ2 treatment, compared with cells expressing the other PTEN cysteine mutant constructs (Fig. 5B). Furthermore, 15d-PGJ2-induced MCF-7 cell migration was abolished by transfection with C136S-PTEN plasmid (Fig. 5C). Cys136 mutation in PTEN to serine also significantly reduced the binding of biotinylated 15d-PGJ2 to endogenous PTEN in MCF-7 (Fig. 5D) and MDA-MB-231 (Fig. 5E) cells. These results suggest that the cysteine 136 residue of PTEN may represent a critical target for covalent modification by 15d-PGJ2 in intact cells.

3.5. 15d-PGJ2 binds to the cysteine 136 residue of both recombinant human PTEN and an endogenous form of PTEN present in human breast cancer cells.

As an initial approach towards determining whether Cys136 of PTEN is directly modified by 15d-PGJ2, vehicle-treated recombinant PTEN and 15d-PGJ2-modified recombinant PTEN were subjected to trypsin digestion followed by mass spectrometric analysis. The nano-LC-ESI-MS/MS of 15d-PGJ2-modified PTEN showed 315.22 kDa difference at Cys136 compared to control (Fig. 6A). This change likely corresponds to modification of Cys136 by 15d-PGJ2 in purified recombinant human PTEN protein incubated with this cyclopentenone PG. The direct interaction between Cys136 of PTEN and 15d-PGJ2 was also evident in 15d-PGJ2-treated MCF-7 cells which were transiently transfected with wild-type PTEN (Fig. 6B). Furthermore, molecular docking analysis predicted the covalent interaction between the cyclopentenone moiety of 15d-PGJ2 and Cys136 of PTEN. As shown in Fig. 6C, Cys136 is found to be located at close proximity to the electrophilic carbon in the ω,β-unsaturated carbonyl group of 15d-PGJ2. The covalent interaction between 15d-PGJ2 and PTEN was stabilized through hydrogen bond interaction with valine 166 backbone of PTEN. Additionally, the eicosatetraenoic acid substituent of 15d-PGJ2 makes a possible salt bridge with lysine 147 of PTEN. These findings suggest that 15d-PGJ2 is able to directly bind to the cysteine 136 residue of PTEN, which in turn suppresses PTEN activity.

3.6. 15d-PGJ2 increases colony formation and tumor growth in a xenograft mouse model.

To clarify whether 15d-PGJ2-mediated thiol modification at Cys136 of PTEN contributes to carcinogenesis, we examined the induction of anchorage-independent growth of MCF-7 cells harboring WT-PTEN or C136S-PTEN. 15d-PGJ2 was added to MCF-7 cells 6 h before initiating the anchorage independent assay. After 3 weeks, C136S-PTEN-transfected MCF-7 cells exposed to 15d-PGJ2 exhibited significantly reduced growth on soft agar compared with 15d-PGJ2 treated cells harboring WT-PTEN (Fig. 7A).

To evaluate pro-tumorigenic effect of 15d-PGJ2 in vivo, MDA-MB-231 cells were injected into the Balb/c nude mice subcutaneously. Considering that administration of 15d-PGJ2 at a high dose (5 mg/kg) or repeatedly even at low dose caused cytotoxic activity [41, 42], mice were subjected to a subcutaneous administration near the tumor area of vehicle (control) and 15d-PGJ2 (2 mg/kg) twice a week for 3 weeks. Tumor growth was gradually increased in mice receiving 15d-PGJ2 on the day 23 and later thereafter (Fig. 7B and Fig. 7C). Elevated expression of phosphorylated Akt was observed in the tumor tissue of 15d-PGJ2 treated mice (Fig. 7D, Fig. 7E; and Supplementary Fig. S3A). Furthermore, increased cellularity was observed in the tumor xenograft in 15d-PGJ2-treated mice as assessed by hematoxylin and eosin (H&E) staining (Supplementary Fig. S3B).

4. Discussion

Chronic inflammation is one of the representative risk factors for cancer development and progression [3]. Abnormally increased COX-2 expression has been frequently observed in breast carcinogenesis [43, 44]. Thus, overexpression of COX-2 is commonly associated with poor prognosis and also linked to metastasis and angiogenesis in breast cancer [43, 45]. Mice genetically engineered to overexpress COX-2 in mammary glands developed malignancies, while COX-2 knockout mice were less susceptible to mammary carcinogenesis [46]. Furthermore, immunohistochemical analysis in tissue array specimens of 1576 invasive breast cancer revealed elevated expression of COX-2 in 37.4% of the tumors, which correlated with poor distant disease-free survival [45].

It has been reported that combination of COX-2 inhibitors with anti-cancer drugs may reduce their side effects [47, 48]. A clinical trial of pre-operative short-term celecoxib treatment showed transcriptional alterations supporting anti-tumor activity and suppression of Ki-67 protein in primary breast cancer tissues suggesting that COX-2 inhibition in combination with other anticancer drugs could provide clinical benefit in breast cancer therapy [49]. In addition, a case-control study has revealed that the regular use of aspirin and other nonsteroidal anti-inflammatory drugs is inversely related to risk of breast cancer [50].

15d-PGJ2 is the representative cyclopentenone PG which is one of the terminal products in the COX-2-mediated arachidonic acid metabolism [51]. Under physiologic conditions, PGs are present in body fluid in picomolar to nanomolar concentration [52]. However, the rate of arachidonic acid metabolism is highly increased under inflammatory conditions [53]. 15d-PGJ2 has been detected at low micromolar concentrations at the site of inflammation, specifically 25 pg/ml in the supernatant of activated macrophage, which would be equivalent to 0.8 μM [54]. For this reason, interaction between 15d-PGJ2 at a micromolar concentration and signaling molecules is facilitated when COX-2 is constitutively overexpressed. In addition, it has been reported that even though micromolar concentrations of 15d-PGJ2 were added to cells, only picomolar amounts were detected in cells [55]. Other studies, using radioactively labelled 15d-PGJ2, showed that only a small proportion (less than 5%) of this reactive lipid molecule is associated with cell proteins when added to fibroblasts in culture [56]. Likewise, we found that the efficacy of 15d-PGJ2 internalization is approximately 1–4% depending on treatment concentration at first time (Supplementary Fig. S4).

15d-PGJ2 has been reported to act as a double-edged sword in cancer pathology [51, 57]. 15d-PGJ2 exhibits potent anti-proliferative properties in many types of malignant cells as evidenced by its inhibition of cancer cell growth or induction of apoptosis [41, 58]. 15d-PGJ2 induces apoptosis by modulating genes involved in cell cycle and cell survival in several types of cancer cells [59]. Besides its anti-inflammatory and anti-proliferative functions, 15d-PGJ2 exerts oncogenic effects. 15d-PGJ2 stimulates...
Fig. 5. Cys136 of PTEN as a putative target for covalent modification by 15d-PGJ2. (A) A schematic structure of PTEN with three critical cysteine residues (Cys71, Cys124 and Cys136) located in the catalytic domain. PTEN mutant plasmids were prepared by performing site-directed mutagenesis, in which each of above cysteine residues was replaced by serine. (B) Cells were transiently transfected with HA-MOCK, HA-WT-PTEN, HA-C71S-PTEN, HA-C124S-PTEN or HA-C136S-PTEN expressing vector for 24 h. Transfected cells were then incubated with 15d-PGJ2 (10 μM) for another 12 h to determine the activation of Akt. Whole cell lysates were prepared to detect phosphorylated Akt, total Akt and HA-tag by immunoblot analysis. HA-tag was used to ensure the equal expression of mutant vectors. (C) MCF-7 cells were transiently transfected with pSG5L-HA-WT-PTEN or -C136S-PTEN plasmid for 24 h followed by 15d-PGJ2 treatment (10 μM). Cell migration was visualized under a confocal microscope. (D) MCF-7 cells were transiently transfected with pSG5L-HA-WT-PTEN or -C136S-PTEN construct before treatment with biotinylated 15d-PGJ2 (30 μM) for 6 h. PTEN was immunoprecipitated with monoclonal HA antibody and analyzed by Western blot analysis, followed by detection with HRP-conjugated streptavidin. (E) Similarly, MDA-MB-231 cells were transiently transfected with pSG5L-HA-WT-PTEN or -C136S-PTEN construct before treatment with biotinylated 15d-PGJ2 (20 μM) for 1 h. PTEN was immunoprecipitated with monoclonal PTEN antibody followed by detection with HRP-conjugated streptavidin.
Fig. 6. Mass spectrometric analysis of PTEN modified by 15d-PGJ2. Human recombinant PTEN (A) and PTEN expressed in MCF-7 cells which were transiently transfected with wild-type PTEN (B) were analyzed by nano-LC-ESI-MS/MS. (A) After treatment of recombinant PTEN with 15d-PGJ2 (30 μM) for 1 h at room temperature, PTEN peptides were prepared by trypsin digestion. The tryptic peptides were loaded onto a fused silica microcapillary column. The column was directly connected to LTQ linear ion-trap mass spectrometer (Finnigan, CA) equipped with a nano-electrospray ion source. Conditions for the mass spectral analyses and other experimental details are described in Materials and Methods. (B) MCF-7 cells transiently transfected with pSG5L-HA-WT-PTEN were treated with 10 μM of 15d-PGJ2 at room temperature for 6 h followed by lysis for 30 min on ice. Cell lysates were trypsinized and subjected to mass spectrometry as described above. (C) Molecular docking analysis was performed by computer modeling. Glide covalent docking was used to predict the covalently bound conformations of 15d-PGJ2. Docking space was defined as a 50 Å cubic box centered on Cys136, which was even set as a reactive residue. Michael addition by sulfur atom of Cys136 was provided in the default reaction types in Glide covalent docking.
Fig. 7. Effects of 15d-PGJ2 on anchorage-independent growth of MCF-7 cells and human breast cancer xenograft tumor growth in vivo. (A) Before embedded to 0.33% agarose media, MCF-7 cells were transfected with WT-PTEN or C136S-PTEN plasmids. After 48 h-incubation, transfected cells were treated with 15d-PGJ2 for another 6 h and then grown in soft agar for 1 month. The number of colonies from three independent experiments is presented as means ± SD. Scale bar, 100 μm. Magnification, x40. (B) The representative photograph of MDA-MB-231 xenograft tumors. 15d-PGJ2 markedly increased the tumor size in the xenograft mice model. A total of 5 × 10^6 MDA-MB-231 cells were injected into dorsal flank of the Balb/c nude mice. Mice were treated with 15d-PGJ2 subcutaneously near the tumor sites twice a week for 3 weeks. (C) Tumor growth curves of the 15d-PGJ2 treated group and the control group. Data are reported as mean ± SD. (D) Western blot analysis of P-Akt, total Akt, and PTEN in xenograft tumor tissues. The quantification for the relative expression of P-Akt is shown in Supplementary Fig. S3A and presented means ± SD (n = 4). (E) Immunohistochemical analysis of P-Akt in mouse xenograft tumors. Scale bar, 100 μm. Magnification, x 100.
the proliferation of colorectal cancer cells [60], leukemia cells [61] and lung cancer cells [62] and has properties to induce angiogenesis [63, 64] and metastasis [64, 65]. In addition, it has been reported that 15d-PGJ2 contributes to mouse skin tumor formation [66]. Although aforementioned events induced by 15d-PGJ2 vary depending on tissue and cell types as well as concentrations and duration of exposure to it, many studies have shown that this cyclopentenone PG promotes cell proliferation at relatively low concentrations while it exhibits cytotoxic effects at a higher concentration [61, 62, 67, 68]. Our previous study demonstrated that 15d-PGJ2 induces COX-2 expression via Akt signaling [8]. However, the precise mechanism by which 15d-PGJ2 induces Akt activation was not elucidated. Our present study clearly unravels that 15d-PGJ2 suppresses the phosphatase activity of PTEN through direct interaction, thereby activating the PI3K-Akt signaling pathway.

PTEN is a phosphoinositide-3-phosphatase that converts PIP3 to PIP2 [69]. PTEN is frequently mutated or inactivated in many different types of cancer [18, 23]. PTEN undergoes post-translational modification via phosphorylation, acetylation and reversible oxidation of cysteine residues present in its catalytic domain [70]. Several studies have demonstrated that 15d-PGJ2 can stimulate production of intracellular ROS [71-74]. While molecular mechanisms underlying intracellular ROS generation by 15d-PGJ2 remain to be defined, one plausible mechanism might be the conjugation of 15d-PGJ2 with reduced glutathione (GSH), an essential component of the cellular antioxidant defense system [71, 74].

In the present study, we confirmed that 15d-PGJ2 treatment increased intracellular ROS accumulation in MCF-7 cells. However, 15d-PGJ2-induced phosphorylation of Akt was not reduced by ROS scavengers. While treatment with antioxidants, such as trolox and vitamin C, failed to inhibit 15d-PGJ2-induced Akt phosphorylation, co-treatment with NAC abrogated the Akt phosphorylation (Supplementary Fig. S1D). Since NAC functions not only as an antioxidant but also as a thiol modifying agent, it is speculated that the inhibitory effect of NAC on 15d-PGJ2-induced Akt phosphorylation is more likely to be due to its thiol reducing rather than ROS scavenging capability. It has been reported that PTEN can be inactivated through disulfide bond formation between two spatially adjacent cysteine residues, i.e., Cys124 and Cys71 in NIH3T3 and HeLa cells treated with H2O2 [75]. In the present study, we found that PTEN underwent oxidation in MCF-7 cells upon treatment with H2O2. However, such oxidation of PTEN was not detected in cells treated with 15d-PGJ2. This finding is consistent with the previous study demonstrating that oxidation of PTEN following arachidonic acid treatment is not identical to PTEN oxidation by hydrogen peroxide [24]. Therefore, it is conceivable that the levels of ROS formed in MCF7 cells challenged with 15d-PGJ2 may not exceed the threshold level required for oxidizing PTEN cysteine thiols.

Cysteine residues, the least abundant of amino acids, have been known to be major targets for redox modulation [76]. Because of its thiol group, cysteine seems to have unique roles in redox control [77]. In most cells, protein thiols are conserved in their reduced state, and GSH serves as a thiol-redox buffer maintaining cytosolic proteins in their reduced form [78]. 15d-PGJ2 represents a Michael reaction acceptor capable of reacting with the nucleophilic cysteine thiols. Although this reaction has been known to be reversible, numerous studies uncovered protein adducts formed by this electrophilic cyclopentenone PG [79, 80]. Since only a specific set of cysteine residues are available for covalent modification, this post-translational modification is likely to be selective. Even though...
Various cytosolic proteins bear reactive thiols, only about 10% forms protein adducts with 15d-PGJ2 and Δ12-PGJ2 [81]. 15d-PGJ2 was found to directly bind to GSH, consequently provoking cellular oxidative stress [74]. Besides GSH, several key signaling proteins undergo S-alkylation by 15d-PGJ2. These include NF-κB [10], AP-1 [11], H-Ras [12, 82], p53 [13], and HIF-1α [64]. Therefore, 15d-PGJ2 appears to have multiple target proteins whose functions/activities are regulated by covalent modification and subsequent alteration in their 3-dimensional structures.

PTEN has an amino-terminal catalytic domain (residues 7 to 185) and a carboxy-terminal C2 domain (residues 185 to 351). The N-terminal domain is responsible for phosphatase activity involved in substrate binding, whilst the C-terminal domain mediates membrane recruitment of signaling proteins [38]. The HCKAKGKR catalytic signature motif in PTEN constitutes the phosphate binding loop (called P loop, comprising residues 123 to 130) that is located at the bottom of active site pocket [83]. This highly conserved loop is important for substrate accommodation. In particular, Cys124 and Arg130 residues in this motif are known to be essential for catalytic activity of PTEN [38]. Moreover, Cys124 is also associated with redox-sensitive regulation of phosphatase activity of PTEN [29]. In this context, we initially speculated that 15d-PGJ2 may covalently modify Cys124 of PTEN, thereby inhibiting its catalytic activity. However, mass spectral data revealed that the actual binding occurs on Cys136. This observation was corroborated by spectral and molecular docking analyses. Moreover, mutation of Cys136 in PTEN markedly reduced its modification by biotinylated 15d-PGJ2 as well as 15d-PGJ2-induced Akt phosphorylation, indicative of Cys136 as a bona fide binding site of this cyclopentenone PG. In agreement with these results, 15d-PGJ2-induced colony formation in PTEN-WT-transfected MCF-7 cells was abolished in cells harboring C136S-PTEN.

In summary, treatment of human breast cancer cells with 15d-PGJ2 led to increased PI3K-mediated phosphorylation of Akt. The mechanism underlying Akt activation by 15d-PGJ2 is attributable to functional inactivation of PTEN through direct modification on the cysteine 136 residue located in the catalytic domain of this tumor suppressor (Fig. 8). Thus, the Akt activation during chronic inflammation-associated breast carcinogenesis may be attributable, at least in part, to 15d-PGJ2-mediated covalent modification and subsequent inactivation of PTEN, which may contribute to cancer cell proliferation, migration and survival. To the best of our knowledge, this is the first report identifying a specific amino acid of endogenous PTEN covalently modified, leading to the loss of its catalytic activity. Our findings provide a novel mechanism underlying activation of the PI3K-Akt axis through post-translational modification and consequent functional inactivation of PTEN.

Conflicts of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.03.016.


