15-deoxy-Δ^{12,14}-prostaglandin J₂ up-regulates death receptor 5 gene expression in HCT116 cells: involvement of reactive oxygen species and C/EBP homologous transcription factor gene transcription

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Abstract

Although 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15dPGJ₂) was reported to up-regulate death receptor 5 (DR5) protein expression and sensitize TRAIL-induced cytotoxicity, its action mechanism remains unclear. Using HCT116 colon cancer cells, we found that sensitization of TRAIL-induced cytotoxicity by 15dPGJ₂ resulted from up-regulation of DR5 via gene transcription but was not associated with PPAR-γ activation. Moreover, 15dPGJ₂ induced GRP78, XBP1, and C/EBP homologous transcription factor (CHOP) expression in HCT116 cells, confirming that 15dPGJ₂ is an endoplasmic reticulum stress inducer. Knockdown of the CHOP gene by siRNA attenuated DR5 up-regulation and the sensitized cytotoxicity in colon cancer HCT116 and SW480. With deletion plasmids of DR5 promoters, we found that the CHOP-binding site was involved in activating the DR5 gene by 15dPGJ₂. A mechanistic study showed the contributions of reactive oxygen species (ROS) and intracellular calcium in CHOP and DR5 gene up-regulation. 15dPGJ₂ was also found to induce DR5 in two prostate cancer cell lines, LNCaP and PC3. Although in LNCaP DR5 up-regulation was accompanied by CHOP expression by 15dPGJ₂, no significant increase in CHOP expression or DR5 promoter activity was observed in PC3 cells. This inability to induce CHOP was not due to the p53-null in PC3 cells, as similar extents of increase in CHOP protein were found due to 15dPGJ₂ in both wild-type and p53-null HCT116 cells. In summary, the effect of up-regulation of DR5 by 15dPGJ₂ in colon cancer cells is independent of PPAR-γ and p53 but relies on CHOP induction through gene transcription involving ROS and calcium. [Mol Cancer Ther 2008;7(10):3429–40]

Introduction

Tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand (TRAIL), a member of the TNF ligand superfamily, can trigger apoptotic death events and is one of the major mediators of antitumor immunity (1, 2). Four distinct receptors can bind and interact with TRAIL. Through interaction on death receptors (DR4 and DR5), TRAIL recruits intracellular adaptor molecules that engage proximal caspase-8, which is activated by proteolysis and in turn activates the effector caspase-3, either by direct processing or indirectly through a mitochondrial apoptotic pathway. TRAIL also interacts with two decoy receptors, DcR1 and DcR2. The functional lack of a cytoplasmic death domain means that both receptors compete with DR4 and DR5 for binding to TRAIL (1, 3). TRAIL is able to induce apoptosis in various tumor cell types while having only negligible effects on normal cells (2). This advantage has made TRAIL a potential target in cancer therapy (4). Monoclonal antibodies that engage TRAIL death receptors have potent antitumor activity and are currently undergoing clinical trials (5).

An increasing number of publications have reported the predominance of TRAIL resistance in primary human tumor cells due to loss of either the targeted death receptors or distal signaling cascades (6). Sensitization of cancer cells by treating them with subtoxic concentrations of chemotherapeutic drugs and irradiation has been shown to restore TRAIL sensitivity in tumor cells (7, 8). Up-regulation of DR5 by different mechanisms, such as reactive oxygen species (ROS), c-Jun NH₂-terminal kinase, and C/EBP homologous transcription factor (CHOP), has also been reported (9–11).

Several studies have shown the close relationship between endoplasmic reticulum (ER) stress and DR5 expression. ER stress is induced when unfolded/misfolded proteins accumulate in the ER lumen, and calcium is depleted from the lumen (12). To cope with this stress, multiple intracellular signaling pathways are first induced to maintain ER homeostasis. However, under conditions of severe ER stress, the cell may eventually activate programmed cell death pathways. It is therefore reasonable...
to assume that manipulation of ER stress might provide new anticancer targets (13). Various ER stress inducers, such as MG132 (14), tunicamycin (TM; ref. 6), thapsigargin (TG; ref. 15), and 15-deoxy-A12,14-prostaglandin J2 (15dPGJ2; ref. 16), have consistently shown an ability to induce DR5 expression on cell surfaces. Although the molecular mechanisms for DR5 protein expression by ER stress inducers might vary with stimuli and cell types, the ER stress-inducible transcription factor, CHOP, has provided a link between ER stress and DR5.

Strong induction of CHOP is regarded as a marker of ER stress (17, 18). Expression of CHOP is mainly regulated at the transcriptional level. The human CHOP promoter contains two ER-stress element motifs and two amino acid response element motifs. On unfolded protein response activation, the ER intramembrane transducers, PRK-like ER kinase and activating transcription factor (ATF) 6, are activated. Through phosphorylation of eukaryotic translation initiation factor 2α, PRK-like ER kinase induces translation of ATF4. Both ATF4 and ATF6 can target ER-stress element sites and transactivate the CHOP gene (17). Although studies have suggested that PRK-like ER kinase/eukaryotic translation initiation factor 2α/ATF4 signaling is more important than ATF6 in inducing CHOP, maximal induction of CHOP in the presence of both ATF6 is required (19). Evidence indicates that activation of a CHOP-binding site on the DR5 promoter region accounts for DR5 protein induction by 15dPGJ2 in human HCT116 colon cancer cells.

15dPGJ2 is not only an endogenous agonist of PPAR-γ (21) but also an ER stress inducer (22). Its effective induction of cell apoptosis resulting from ROS suggests its potential therapeutic value in anticancer therapy (23, 24). In contrast to other ER stressors, the mechanisms of the increased DR5 protein expression by 15dPGJ2 remain elusive. In human PC3 prostate cancer cells, 15dPGJ2 was shown to up-regulate the DR5 protein through mRNA stabilization rather than gene transcription (16). Paradoxically, 15dPGJ2 is capable of inducing CHOP gene in HeLa cells via activation of C/EBP and ER-stress element sites in the CHOP promoter (22). These unique and distinct results compared with other ER stress inducers sparked our interest in further investigating the molecular mechanisms underlying DR5 protein induction by 15dPGJ2 in human HCT116 colon cancer cells.

Materials and Methods

Reagents
RPMI 1640, fetal bovine serum, and gentamicin were obtained from Life Technologies. Polyclonal antibodies against GRP78, CHOP, XBP1, and horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal DR4 and DR5 antibodies were purchased from Abcam. The DcR1 antibody was purchased from BD PharMingen Technical. The DcR2 antibody was purchased from Stressgen. The DcR2 antibody was purchased from BD PharMingen Technical. The DcR1 antibody was purchased from BD PharMingen Technical. The DcR2 antibody was purchased from Stressgen. The DcR1 antibody was purchased from BD PharMingen Technical. The DcR2 antibody was purchased from Stressgen. The DcR1 antibody was purchased from BD PharMingen Technical. The DcR2 antibody was purchased from Stressgen. The DcR1 antibody was purchased from BD PharMingen Technical. The DcR2 antibody was purchased from Stressgen. The DcR1 antibody was purchased from BD PharMingen Technical. The DcR2 antibody was purchased from Stressgen.

15dPGJ2, BADGE, GW1929, rosiglitazone, ciglitazone, TM, TG, and 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) were purchased from Calbiochem. SP600125 was obtained from Tocris Cookson. GW9662, N-acetyl-l-cysteine (NAC), and brefeldin A (BFA) were from Sigma. DR5 promoter plasmids were provided by Prof. T. Sakai (Graduate School of Medical Science, Kyoto University). All materials for SDS-PAGE were obtained from Bio-Rad Laboratories. Soluble recombinant human TRAIL was purchased from PeproTech, and the DR5 agonist antibody was purchased from R&D Systems. 5-(6)-Chloromethyl-2,7′-dichlorodihydrofluorescein diacetate and Fluo-3/AM were from Molecular Probes.

Cell Culture
Human colon adenocarcinoma (HCT116, HT29, and SW480) and prostate cancer (PC3 and LNCaP) cell lines were obtained from the American Type Culture Collection. The culture medium used throughout these experiments was RPMI, containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Measurement of Cell Viability by the 3-(4,5-Dimethylthiazol-2-yl)2,5-Diphenyltetrazolium Bromide Assay
Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay as we described previously (25). The net absorbance (A350-630) indicates the enzymatic activity of mitochondria and implies cell viability.

Determination of Surface DR5 Protein by Flow Cytometry
After drug treatment, cells were harvested, washed twice with fluorescence-activated cell sorting washing buffer (1% FCS and 0.1% NaN3 in PBS), and incubated with the DR5 antibody at 4°C for 30 min. After washing with the fluorescence-activated cell sorting washing buffer three times, the fluorescence of cells was analyzed using a FACScan flow cytometer (BD Biosciences).

Caspase-3 Activity Assay
Colorimetric Assay Kit (BioVision) was used to evaluate caspase-3 activity in cell lysates. The absorbance at 405 nm was measured with a spectrophotometer. Comparison of the absorbance from apoptotic samples with an uninduced control represented the multiple of increase in caspase activity.

Immunoblotting Analysis
After stimulation, cell lysates were harvested as described previously (25), and equal amounts of the soluble protein, as determined by the Bradford protein assay, were denatured, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Proteins were then detected by specific antibodies followed by chemiluminescence detection (Amersham Biosciences).

Quantitative Reverse Transcription-PCR
Total RNA extraction, cDNA synthesis, and quantitative PCR were conducted as we described previously (26).
Primer sequences were as follows: CHOP forward primer (5'-TGGCCTTTTCTCTGCCGACACT-3'); CHOP reverse primer (5'-TGTGACCTTGCTGCTTGTTCTG-3'); DR5 forward primer (5'-CATCCATGGAAATGACCTTCTTC-3'); DR5 reverse primer (5'-GTGCAAGGACTTAACTCCAGT-3'); β-actin forward primer (5'-CGGGACCTGACTGAC-3'); and β-actin reverse primer (5'-AGGAAGGCTGGAAGAGTCG-3').

Small Interfering RNA for CHOP

The small interfering RNA (siRNA) for CHOP (accession no. NM_004083) and siCONTROL Nontargeting siRNA were synthesized by Dharmacon Research. Cells were transfected with siRNAs for 48 h using Dharmafect following the manufacturer’s instructions.

DR5 Promoter Activity Assay

Reporter genes of the DR5 promoter were prepared using endotoxin-free plasmid preparation kits (Qiagen). Cells were seeded into 24-well plates overnight before transfection with 0.1 μg plasmid using LipofectAMINE 2000 reagent (Invitrogen). Twenty-four hours later, cells were treated with 15dPGJ2 for another 24 h, and luciferase activity was determined as we described previously (25).

Measurement of Intracellular ROS and [Ca2+]i

For ROS measurement, cells were loaded with 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (1 μmol/L) for 30 min. Afterwards, cells were washed twice with PBS and then subjected to flow analysis (Becton Dickinson). For the [Ca2+]i measurement, cells were loaded with the calcium-sensitive fluorescein, Fluo-3/AM (3 μmol/L), for 30 min. After washing with PBS, different concentrations of 15dPGJ2 were added for 2 h, and the fluorescence intensity was determined with excitation and emission wavelengths at 488 and 530 nm, respectively, in a flow cytometer at room temperature. Acquisition and analysis were done with FACScan.

Statistical Evaluation

Values are expressed as mean ± SE of at least three experiments, which were done in duplicate. ANOVA was used to assess the statistical significance of the differences, and P < 0.05 was considered statistically significant.

Results

15dPGJ2 Sensitizes TRAIL-Induced Cell Death in HCT116 Colon Cancer Cells through DR5 Induction

As reported previously in other cell types (23, 24), 15dPGJ2 alone can cause cell death in HCT116 cells. In an attempt to investigate if 15dPGJ2 can sensitize TRAIL-induced cytotoxicity and if such an event results from expression changes in TRAIL death receptors rather than intracellular apoptotic pathways, we first needed to design experiments to minimize the cytotoxic interference from the action of 15dPGJ2 itself. To this end, we pretreated HCT116 colon cancer cells with 15dPGJ2 for 12 h and then washed out the drug before adding TRAIL and incubating for 24 h. Under this washout manipulation, we found that 10 μmol/L 15dPGJ2 caused no cytotoxicity, and only 20% cell toxicity was observed when the concentration was increased to 30 μmol/L. However, when challenging 10 and 30 μmol/L 15dPGJ2-pretreated cells with TRAIL, we found the weak cytotoxicity induced by TRAIL was enhanced (Fig. 1A, left).

To analyze whether 15dPGJ2 sensitization of TRAIL toxicity gave rise to activation of caspase, a critical mediator of death receptor-induced apoptosis, we used colorimetric caspase assay kits to determine caspase activity. We found that 15dPGJ2 (10 and 30 μmol/L) potentiated TRAIL to induce caspase-3 activity, but 15dPGJ2-primed cells by themselves showed no apparent induction of caspase activation (Fig. 1A, right). To further confirm that such sensitization resulted from up-regulation of death receptors, we tested the effect of the DR5 agonistic antibody. As shown in Fig. 1B, pretreatment with 10 or 30 μmol/L 15dPGJ2 still sensitized cell death in response to the DR5 agonistic antibody (0.5-2 μg/mL).

To explore the sensitization of 15dPGJ2 on TRAIL-mediated cell apoptosis, we analyzed the expression levels of TRAIL receptors both before and after 15dPGJ2 treatment. Immunoblotting data indicated that after treatment with 30 μmol/L 15dPGJ2 for 16 h, only protein levels of DR5, but not those of DR4, DcR1, and DcR2, were increased (Fig. 1C). The increased DR5 protein level induced by 15dPGJ2 displayed time and dose dependency. Moreover, the increased surface protein level of DR5 was evidenced by flow cytometry (Fig. 1D).

15dPGJ2-Induced DR5 Up-regulation Is PPAR-γ Independent

15dPGJ2 is an endogenous PPAR-γ agonist, so we wondered if DR5 induction is mediated by PPAR-γ. To address this point, we examined several PPAR-γ agonists and PPAR-γ antagonists. Despite the increase in DR5 protein by 15dPGJ2, three different PPAR-γ agonists, GW1929, rosiglitazone, and ciglitazone (each at 10 and 30 μmol/L) did not change DR5 protein expression with 16 h of incubation in HCT116 cells (Fig. 2A). In the antagonist experiment, we found that pretreating cells for 1 h with neither the PPAR-γ partial antagonist BADGE (30 μmol/L) nor the specific PPAR-γ antagonist, GW9662 (30 μmol/L), affected DR5 protein induction by 15dPGJ2 (Fig. 2B). Moreover, we found that the DR5 protein induced by 15dPGJ2 was not altered by overexpression of PPAR-γ (Fig. 2C). These results suggested that 15dPGJ2 induces DR5 up-regulation through PPAR-γ-independent pathways.

CHOP Mediates DR5 Gene Up-regulation by 15dPGJ2 in Various Colon Cancer Cell Lines

Because CHOP is an inducible ER stress marker involved in DR5 gene transcription, and 15dPGJ2 was found to be an ER stress inducer, we wondered if the induced ER stress response, particularly CHOP expression, accounts for 15dPGJ2-induced DR5 up-regulation. In this context, we found that 15dPGJ2 was capable of up-regulating GRP78, CHOP, and XBP1 (Fig. 3A and B). These effects exhibited concentration and time dependence. To clarify whether the induction effect on CHOP resulted from gene expression, mRNA levels were measured by real-time PCR. As shown...
Figure 1. 15dPGJ2 sensitizes TRAIL-induced and DR5 antibody-induced apoptosis and increases DR5 protein expression in HCT116 cells. Cells were pretreated with 15dPGJ2 at different concentrations for 12 h. After washout of 15dPGJ2, cells were treated with vehicle, TRAIL (A), control IgG, or a DR5 agonistic antibody (B) for 24 h at the indicated concentrations. Cellular viability was assessed by the MTT assay (A and B), and caspase-3 activity was determined from cell lysates (A). C, cells were treated with 15dPGJ2 with the indicated concentrations and for the specified periods. Cell lysates were used for determining the protein expressions of DR5, DR4, DcR1, and DcR2. β-Actin level was used as an internal control. A representative study is shown, and three additional experiments yielded similar results. D, after treatment with 30 μmol/L 15dPGJ2 for 16 h, the surface DR5 protein level was determined by flow cytometry. Mean ± SE from three independent experiments. *, P < 0.05, significant potentiation of TRAIL-induced or DR5 agonist antibody-induced effects and an increase in DR5 protein expression by 15dPGJ2.
in Fig. 3C, 15dPGJ2 (10 and 30 μmol/L) treatment for 3, 6, or 9 h significantly induced CHOP mRNA expression in a time-dependent manner, indicating that CHOP is induced by 15dPGJ2 at the transcriptional level.

To verify if CHOP plays an important role in DR5 up-regulation by 15dPGJ2, we transfected CHOP siRNA and at 48 h stimulated HCT116 cells with 30 μmol/L 15dPGJ2 for 16 h. Accompanying the CHOP reduction, the induction of DR5 protein by 15dPGJ2 was efficiently reduced, and 15dPGJ2-mediated sensitization of TRAIL toxicity was abrogated (Fig. 4A). Moreover, 15dPGJ2-stimulated increases in CHOP and DR5 mRNA were inhibited by CHOP siRNA treatment (Fig. 4B). These results suggest that CHOP is involved in the action of induction of DR5 gene expression and TRAIL sensitization by 15dPGJ2.

Next, we carried out a luciferase assay using reporter plasmids containing various deletion sizes of the DR5 promoter to identify the 15dPGJ2-responsive element in the DR5 promoter. First, we found that 15dPGJ2 increased the promoter activity of DR5 (Fig. 4C). Similar activation extents were detected in the 5′-deleted plasmids, pDR5/-605, pDR5/-318, and pDR5/-289. However, activation of pDR5/-252 by 15dPGJ2 was markedly diminished. As the CHOP binding site is located at -289/-252, these results confirm the essential role of CHOP in 15dPGJ2-induced DR5 gene transcription.

To understand if such CHOP-dependent DR5 induction by 15dPGJ2 also occurred in other human colon cancer cell lines, we tested SW480 and HT29 cells and found that 15dPGJ2 was still able to up-regulate CHOP and DR5 (Fig. 4D, left). Moreover, other ER stressors, including TG, TM, and BFA, possessed similar effects as 15dPGJ2. Among these stimuli, we found the effects of TM exhibited its peak effects at 16 h and then gradually declined, whereas other stimuli induced responses were sustained at 16 to 24 h (data not shown). Our results also confirmed that the DR5 induction by 15dPGJ2 in SW480 and HT29 cells was mediated by CHOP: in the presence of CHOP siRNA, this DR5 induction effect was diminished. Extending this observation to cell function, we found that pretreatment with 15dPGJ2 enhanced cell susceptibility to TRAIL by HCT116 and SW480 cells. In contrast, TRAIL (50 ng/mL) had no significant toxicity within 24 h of incubation in HT29 cells receiving 15dPGJ2 pretreatment (Fig. 4D, right).

**Upstream Signaling Pathways of 15dPGJ2-Induced CHOP and DR5 Up-regulation**

After identifying CHOP induction for DR5 gene expression, we tried to clarify the upstream signaling cascades underlying the action of 15dPGJ2 in DR5 protein and gene transcription. Previous studies showed the regulatory roles of mitogen-activated protein kinases (9, 27), calcium (28), and ROS (10, 29) in DR5 gene regulation. To understand the roles of these signaling mediators, we pretreated cells with the antioxidant NAC (5 mmol/L), a thiol antioxidant that is known to function as both a redox buffer and an ROS scavenger, the MEK inhibitor U0126 (10 μmol/L), the p38 mitogen-activated protein kinase inhibitor SB203580 (10 μmol/L), the c-Jun NH2-terminal kinase inhibitor SP600125 (10 μmol/L), and the calcium chelator BAPTA/AM (30 μmol/L), for 1 h, followed by stimulation with 30 μmol/L 15dPGJ2 for 16 h. As shown in Fig. 5A, NAC abolished DR5 and CHOP protein expressions caused by...
BAPTA/AM also inhibited such responses, whereas the three mitogen-activated protein kinase inhibitors had minimal effects. We further attempted to determine if these effects were evident in transcription levels, so we pretreated these inhibitors for 1 h and then stimulated them with 15dPGJ2 for 9 h. As shown in Fig. 5B, NAC almost completely abolished DR5 and CHOP mRNA expressions. BAPTA/AM inhibited both mRNA levels by about 30% to 50%, whereas mitogen-activated protein kinase inhibitors had no effects.

### 15dPGJ2 Increases Intracellular ROS and Calcium

Our results suggest the involvement of ROS and calcium in the 15dPGJ2-induced up-regulation of CHOP and DR5 gene transcription. Indeed, previous studies reported that 15dPGJ2 can generate ROS in cancer cells (23, 30), and ROS can induce CHOP production (31, 32). To confirm this suggestion, we determined intracellular levels of ROS and calcium. Flow cytometric studies revealed that 50 μmol/L 15dPGJ2 time-dependently induced increases in ROS (Fig. 5C). Likewise, 15dPGJ2 (3-30 μmol/L) dose-dependently elevated intracellular calcium levels after 1 h of treatment (Fig. 5D). When we pretreated cells with 5 mmol/L NAC and then stimulated them with 15dPGJ2 for 1 h, we found that the calcium increase induced by 15dPGJ2 was significantly abolished.

### 15dPGJ2 Has Cell Type Specificity in CHOP Induction, Which Is Independent of p53, ROS, and Calcium

In contrast to our findings in colon cancer (HCT116, SW480, and HT29) cells that CHOP plays a critical role in DR5 gene transcription, a previous study showed that 15dPGJ2 induces DR5 protein expression in prostate cancer cell line PC3 through mRNA stability rather than gene transcription (16). Therefore, we were interested in confirming this finding and to understand if CHOP induced by 15dPGJ2 and other ER stressors has cell type specificity. To this end, we tested two prostate cancer cell lines, LNCaP and PC3. We found in both prostate cancer cells TG, TM, and BFA simultaneously induced CHOP and DR5. 15dPGJ2 had similar effects in LNCaP cells, but in PC3 cells it only up-regulated DR5 (Fig. 6A, left). The noneffectiveness of 15dPGJ2 in inducing CHOP in PC3 cells was evident at 48 h of incubation (data not shown).

After observing that 15dPGJ2 could not increase CHOP in PC3 cells, we wondered if p53 plays a role in ER stress-mediated CHOP production. This assumption was based on the facts that, different from LNCaP cells, PC3 cells are p53-null, and the p53 binding site is positioned at the -1,847 site of the human CHOP promoter (33). To address this issue, we did experiments in wild-type and p53-null HCT116 cells and treated cells with different ER stressors. Our results showed that CHOP was induced to similar extents by TG, BFA, and 15dPGJ2 (Fig. 6B, left). These results exclude the involvement of p53 in CHOP gene transcription by ER stressors.

Next, we wanted to understand how 15dPGJ2 regulates cell susceptibility to TRAIL in prostate cancers and if p53 plays a critical role in this event. Results of cell viability shown in Fig. 6A (right) indicate that LNCaP was less sensitive to TRAIL-induced cytotoxicity than were PC3 cells. More intriguingly, the TRAIL toxicity in neither prostate cancer cell line was affected by the presence of 15dPGJ2. In p53-null HCT116 cells, TRAIL toxicity was reduced compared with that in wild-type HCT116 cells, but 15dPGJ2 was still capable of enhancing TRAIL toxicity regardless of the presence or absence of p53 (Fig. 6B, right).
Figure 4. CHOP mediates 15dPGJ2-induced DR5 gene transcription and contributes to TRAIL-induced cytotoxicity in colon cancer cells. CHOP siRNA or siCONTROL was transfected into HCT116 cells. After 48 h of transfection, cells were treated with 30 μmol/L 15dPGJ2 or DMSO (vehicle) for 16 h (A) or 9 h (B). Protein (A) and mRNA (B) levels of CHOP, DR5, and β-actin were determined. In some siRNA manipulated cells, 15dPGJ2 (30 μmol/L) was added for 12 h followed by washing out and then treatment with TRAIL (50 ng/mL) for 24 h. Cell viability was determined by the MTT assay. C, DR5 promoter activity was determined by transient transfection of cells with reporter plasmids containing various sizes of the DR5 promoter and luciferase gene followed by stimulation with 15dPGJ2. D, various colon cancer cells with or without siRNA treatment were stimulated with TG (1 μmol/L), TM (10 μg/mL), BFA (1 μg/mL), or 15dPGJ2 (30 μmol/L) for 24 h. Protein levels of DR5, CHOP, and β-actin were determined. After treating cells with 15dPGJ2 (30 μmol/L) for 12 h followed by washing out and then treatment with TRAIL (50 ng/mL) for 24 h, cell viability was determined by the MTT assay. Mean ± SE of three independent experiments. *, P < 0.05, significant reduction in 15dPGJ2-induced up-regulation of CHOP mRNA, DR5 mRNA, DR5 promoter activity, and sensitization of cell death in response to TRAIL.
Figure 5. ROS and intracellular calcium are involved in CHOP induction caused by 15dPGJ2. A, HCT116 cells were pretreated with NAC (5 mmol/L), U0126 (10 μmol/L), SB203580 (SB; 10 μmol/L), SP600125 (SP; 10 μmol/L), or BAPTA/AM (30 μmol/L) for 1 h and then stimulated with 15dPGJ2 (30 μmol/L) for 16 h. Protein levels of CHOP and DR5 were measured in cell lysates by an immunoblot analysis. Protein levels were quantified by densitometry, normalized to the β-actin level, and expressed as percentages of the control group without agent treatment. B, with similar treatment, except for 9 h of incubation with 15dPGJ2 (30 μmol/L), CHOP and DR5 mRNA expression levels were determined by quantitative PCR and expressed as percentages of the control. C, to determine the intracellular content of ROS, HCT116 cells were stimulated with 15dPGJ2 (30 μmol/L) for 3, 6, or 9 h and further loaded with 5-(6)-chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate for 30 min; the fluorescence was measured by flow cytometry. D, to determine the content of intracellular calcium, HCT116 cells were loaded with calcium-sensitive fluorescein Fluo-3 (3 μmol/L, 30 min). HCT116 cells were stimulated with 15dPGJ2 (3, 10, and 30 μmol/L) for 3 h. In some experiments, HCT116 cells were pretreated with NAC (5 mmol/L) for 1 h followed by stimulation with 15dPGJ2 (30 μmol/L). After 3 h of incubation, fluorescence indicating the intracellular calcium level was measured by flow cytometry. Mean ± SE of at least three separate experiments. *; P < 0.05; significant reduction in 15dPGJ2-induced protein or mRNA levels of CHOP and DR5 and induction of ROS and [Ca2+]i.
Figure 6. Cell-specific effects of 15dPGJ2 on DR5 up-regulation and TRAIL toxicity: p53 is not involved in 15dPGJ2-induced CHOP expression. LNCaP, PC3 (A), and HCT116 (wild-type and p53-/-; B) cells were treated with 30 μmol/L 15dPGJ2, 1 μmol/L TG, 10 μg/mL TM, or 1 μg/mL BFA for 24 h (A) or the time indicated (B). Protein levels were determined by immunoblotting. Cell viability in LNCaP, PC3, and HCT116 (wild-type and p53-/-) cells, which were pretreated with 15dPGJ2 (30 μmol/L) for 12 h followed by washing out and then treated with TRAIL at the concentrations indicated for 24 h, was determined by the MTT assay. C, a DR5 promoter activity assay as described in Fig. 4C was conducted in 30 μmol/L 15dPGJ2-treated wild-type HCT116 and PC3 cells. D, intracellular ROS and calcium were measured as described in Fig. 5C and D in HCT116 and PC3 cells after treatment with 15dPGJ2 (30 μmol/L). *, P < 0.05, significant sensitization of cell death in response to TRAIL (B) and reduction of 15dPGJ2-induced DR5 promoter activity in HCT116 cells (C). #, P < 0.05, less susceptibility of p53-null HCT116 cells to TRAIL.
To confirm the unique effect that 15dPGJ2 cannot up-regulate DR5 gene transcription in PC3 cells (16), we did a promoter activity analysis. Results of the reporter assay of the DR5 promoter showed the ability of 15dPGJ2 to increase promoter activity analysis. Results of the reporter assay of regulating DR5 gene transcription in PC3 cells (16), we did a fluorescence analysis ruled out this possibility, as marked production of both signaling mediators was detected in 15dPGJ2-treated PC3 cells as observed in HCT116 cells (Fig. 6D).

Discussion

TRAIL is a recently identified anticancer agent, which induces apoptosis by interacting with two death-inducing receptors, DR4 and DR5. Both receptors engage the same downstream apoptotic mechanism and play crucial roles in synergistic cytotoxicity associated with TRAIL and other chemotherapeutic agents (34). In the search for new therapeutic strategies, we found that 15dPGJ2 enhanced TRAIL-induced apoptosis in some colon cancer (HCT116 and SW480) cell lines. In addition, our data confirmed the findings of Nakata et al. by showing that such a sensitization effect on cytotoxicity was partially mediated by DR5 induction (16). After DR5 induction by 15dPGJ2, cell death of HCT116, elicited by either TRAIL or the agonistic DR5 antibody, was dramatically enhanced, suggesting the effective function of these up-regulated death receptors. In this study, we further explored the crucial role of CHOP in mediating DR5 promoter activation.

As reported, 15dPGJ2 is an endogenous activator of PPAR-γ; however, PPAR-γ activation cannot account for all of the actions of 15dPGJ2 (35, 36). Herein, we produced some evidence to prove this suggestion. First, we found only 15dPGJ2 can stimulate DR5 expression in HCT116 cells, but other PPAR-γ agonists (GW1929, rosiglitazone, and ciglitazone) did not. Second, when treating cells with the PPAR-γ antagonists, BADGE and GW9662 (37, 38), 15dPGJ2-induced DR5 up-regulation was not altered. Third, 15dPGJ2-induced DR5 expression was not altered in HCT116 cells overexpressing PPAR-γ. These results highlight the need to elucidate the PPAR-γ-independent action mechanisms of 15dPGJ2. 15dPGJ2, an endogenous eicosanoid product and also an electrophilic mediator of cellular responses, is usually produced by activated macrophages and mast cells. At micromolar concentrations, it is thought to exert many biological activities through both PPAR-dependent and PPAR-independent mechanisms and is implicated in anti-inflammation and anti-tumor functions (39–41). However, its physiologic role is still a subject of discussion, because the levels of 15dPGJ2 are in the picomolar range in body fluids (42). Our current findings of enhancing TRAIL toxicity further suggest that 15dPGJ2 is a natural mediator for limiting tumor progression. Our results indicate that, like the potency for general biological actions of 15dPGJ2, micromolar levels are required for 15dPGJ2 to induce DR5 in cancer cells (e.g., 3-30 μmol/L in HCT116 cells). Determining whether such concentrations can be produced in the inflammatory microenvironment adjacent to cancer cells requires further investigation.

Our results in HCT116 cells confirmed earlier observations in PC3 cells that DR5 protein expression induced by 15dPGJ2 occurs in a PPAR-γ-dependent manner (16). However, studies dissecting molecular mechanisms gave distinct findings for the action of 15dPGJ2 in both cancer cell types. Our data with mRNA measurement, promoter activity assay, and siRNA approach suggest that DR5 induction by 15dPGJ2 in three colon cancer cell types occurs at the transcriptional level and is mediated by increasing CHOP gene transcription. These results are consistent with those reported in HeLa cells, where CHOP was increased by 15dPGJ2 (22). In contrast, in PC3 cells, 15dPGJ2-induced DR5 expression did not occur through an increase in DR5 mRNA transcription but through mRNA stabilization (16). To further clarify if cell types determine the distinct action mechanisms of 15dPGJ2, we tested the DR5 promoter activity and CHOP expression in PC3 cells. As a result, we found that 15dPGJ2 did not up-regulate CHOP protein or DR5 promoter activity in PC3 cells, unlike results seen in colon cancer cells, implying that the regulatory mechanisms for DR5 gene transcription are cell type specific. However, the unique effect in PC3 cells could not be applied to other ER stressors or other prostate cancer cell types. Our data showed that ER stressors like TG, TM, and BFA could still dramatically up-regulate CHOP and DR5 in PC3 cells. Moreover, in LNCaP prostate cancer cells, 15dPGJ2 had the ability to induce both CHOP and DR5. Therefore, we suggest that signaling pathways involved in the regulation of CHOP gene expression by 15dPGJ2 in PC3 cells might differ from those in other cell types. It is necessary to further compare if regulation details of the essential transcription factor(s) for CHOP promoter activation, such as ATF4, by 15dPGJ2 are cell type different.

Our findings further suggest that CHOP, although it cannot be applied to all cancer cell types, may still be an important mediator for DR5 gene transcription in response to ER stress and may be a good target for new anticancer agents that have cooperative potential with TRAIL. This aspect shows that ER stress inducers, such as MG132, TM, and TG, can up-regulate DR5 expression through CHOP (9, 14, 20). In this study, we provide functional evidence that CHOP-mediated DR5 induction might contribute to the increased cell susceptibility to TRAIL after 15dPGJ2 treatment. Using CHOP siRNA in HCT116 cells prevented 15dPGJ2 from inducing increased TRAIL toxicity. Such enhanced cytotoxicity was also observed in TRAIL-sensitive SW480 cells but not in TRAIL-resistant HT29 cells. In prostate cancer cells, TRAIL had higher sensitivity in PC3 than LNCaP cells. Regardless of the sensitivity, we found that 15dPGJ2 pretreatment did not affect TRAIL toxicity in
either prostate cancer cell line. Therefore, this suggests that the validity of increasing DR5 expression to enhance the TRAIL-induced antitumor effect is a cell-specific phenomenon. We suggest that, apart from the death receptor levels, other intracellular death pathways that cause cancer cells to be more resistant to TRAIL are also important gateways to achieve TRAIL sensitization. In this aspect, inhibition of antiapoptotic molecules such as cellular FLICE-like inhibitory protein, myeloid cell line-1, B-cell lymphoma-2, and inhibitors of apoptosis are effective tools to improve the ultimate clinical utility of TRAIL (43).

After identifying the cell-specific DR5 regulation by 15dPGJ2, we further elucidated the upstream molecules required for CHOP gene regulation. Because of the α,β-unsaturated carbonyl moiety present in its cyclopentenone ring structure, 15dPGJ2 is an electrophile, which covalently reacts via 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 controlling redox cell signaling pathways (44, 45). In this study, we found that ROS and calcium are two major mediators for 15dPGJ2-induced CHOP gene expression in HCT116 cells. Pharmacologic data suggest the existence of at least two upstream cascades for CHOP gene induction. One is ROS- and calcium-dependent pathway, whereas the other is ROS-dependent but calcium-independent pathway. This suggestion is based on two lines of evidence. Measurements by flow cytometry indicated that the calcium increase caused by 15dPGJ2 resulted from ROS production, because blockade of ROS by a scavenger abolished the calcium response. Moreover, all stimulation of mRNA and protein expressions of CHOP and DR5 was almost completely abrogated by NAC (a ROS scavenger) but only partial reduced by BAPTA (a calcium chelator). In this aspect, previous studies showed that ROS is a molecular mediator inducing CHOP (31, 32, 46) and DR5 (10, 11, 47) expression. In this study, we elucidated for the first time the cell-specific regulation of DR5 by 15dPGJ2. As mentioned earlier, insufficient induction of CHOP by 15dPGJ2 primarily explained the lack of activation of the DR5 promoter in PC3 cells. Despite such a distinction, we unexpectedly still detected similar responses of ROS and calcium increases in PC3 cells as found in HCT116 cells. These results suggest a differential mechanism, in addition to ROS and calcium, might be required to activate the CHOP gene in PC3 cells in a cooperative manner, and such regulation is specific to 15dPGJ2 but is not a general event for most ER stressors. Because a previous study showed the involvement of the C/EBP site and ER-stress element in activating the CHOP gene by 15dPGJ2 in HeLa cells (22), we were interested in further dissecting the linkage between second messengers (ROS and calcium) and transcription factors in activating the CHOP gene in HCT116 and PC3 cells.

Depending on the stimuli, a controversial requirement of p53 in CHOP gene regulation has been reported. Although p53-independent CHOP up-regulation was shown (48, 49), CHOP was shown to be a p53 target gene on encountering stress like hypoxia (33) and DNA damage (50). To further elucidate the exact case for 15dPGJ2, especially under a situation showing inconsistent CHOP induction in 15dPGJ2-treated HCT116 (p53-containing) and PC3 (p53-null) cells, we therefore compared the CHOP induction responses in wild-type and p53-null HCT116 cells. We clearly suggest that p53 is not required for CHOP regulation in response to 15dPGJ2, TG, and BFA. Other evidence comes from the CHOP induction results by ER stressors in SW480 and HT29 cells (both are p53 mutant colon cancer cell lines). Our current data further rule out the contributions of mitogen-activated protein kinases (ERK, p38, and c-Jun NTerminal kinase) in 15dPGJ2-elicted CHOP and DR5 expressions, although they possibly play roles under different conditions in CHOP expression and activation.

In conclusion, we herein depict the molecular mechanisms for the regulation of CHOP gene expression by 15dPGJ2. We show that 15dPGJ2 triggers CHOP gene expression in human colon cancer (HCT116, SW480, and HT29) and LNCaP prostate cancer cells through a PPARγ-independent mechanism. The increased CHOP induction is mediated by the activation of ROS and calcium, but not p53, and plays a critical role in activating the DR5 promoter. It thereby leads to up-regulation of the DR5 protein and sensitization of cancer cells to TRAIL-mediated apoptosis. These results strongly imply differential mechanisms regulating DR5 expression in various cancer cell types, which might modulate the effectiveness of drug combinations with TRAIL for preventing and/or treating cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
15-deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) up-regulates death receptor 5 gene expression in HCT116 cells: involvement of reactive oxygen species and C/EBP homologous transcription factor gene transcription

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