Transactivation of the PPAR-Responsive Enhancer Module in Chemopreventive Glutathione S-Transferase Gene by the Peroxisome Proliferator-Activated Receptor-γ and Retinoid X Receptor Heterodimer

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ABSTRACT

Cancer chemopreventive agents transcriptionally induce glutathione S-transferase (GST), which can protect cells from chemical-induced carcinogenesis. Activation of either NF-E2-related factor-2 (Nrf2) or the C/EBPα/hemer binding protein-β (C/EBPβ) contributes to GST induction. Peroxisome proliferator-activated receptor-γ (PPARγ) and the retinoic acid X receptor (RXR) play roles in regulating cell differentiation and chemoprevention. This study examined GSTA2 gene induction by the PPARγ activator and 9-cis-retinoic acid (RA), a RXR ligand, and investigated the molecular basis of PPAR-RXR-mediated GSTA2 induction in the H4IIE hepatocytes. Either 15-deoxy-δ(12, 14)-prostaglandin J2 (PGJ2) or RA induced GSTA2 with Nrf2 and C/EBPβ activation. Compared with PGJ2 or RA alone, PGJ2 + RA enhanced GSTA2 induction, with increases in Nrf2 and C/EBPβ activation. PGJ2 + RA increased the luciferase reporter gene activity in the cells transfected with the −1.65-kb flanking region of the GSTA2 gene. Thiazolidinedione PPARγ agonists, troglitazone, rosiglitazone, and pioglitazone, in combination with RA, potentiated GSTA2 induction, confirming that the activation of the PPARγ and RXR heterodimer contributed to GSTA2 expression. Deletion of the antioxidant response element- or C/EBP-binding sites or the overexpression of dominant-negative mutant of C/EBP abolished the reporter gene expression. PGJ2 + RA increased the binding of the PPARγ − RXR heterodimer to the putative PPAR-response elements (PPREs) in the GSTA2 promoter. Specific mutations of these multiple PPRE sites resulted in the complete loss of its responsiveness to PGJ2 + RA, which suggests that these binding sites function as a PPRE-responsive enhancer module (PPREM). Transactivation of PPRE by the PPARγ − RXR heterodimer was verified by the effective GSTA2 induction in the cells treated with PGJ2 + RA after transfecting them with the plasmids encoding PPARγ and RXRα. In conclusion, the PPARγ − RXR heterodimer promotes GSTA2 induction by activating PPRE in the GSTA2 gene, as well as inducing Nrf2 and C/EBPβ activation.

INTRODUCTION

Glutathione S-transferases (GSTs) are responsible for the cellular metabolism as well as detoxification of several xenobiotics and carcinogenic compounds primarily in the liver and, to a much lesser extent, in the extrahepatic organs. Glutathione conjugates formed in the liver can either be excreted intact in bile, or they can be converted to mercapturic acids in the kidney and excreted in the urine (1). The loss of GST protection can increase the susceptibility of preneoplastic lesions in humans (20–23). The activated PPARγ forms a heterodimer with retinoid X receptor (RXR)α and binds to specific PPAR response elements in the promoter region of their target genes (24). Therefore, PPARγ agonists affect cell survival, growth, and differentiation by binding to the peroxisomal proliferator-response element (PPRE).

Hepatic stellate cells in the liver contain 40–70% of the body stores of retinoids. Retinoic acids, which are natural metabolites of circulating vitamin A, are essential for maintaining the normal pathway of epithelial tissues differentiation (25). Natural and synthetic retinoids are also effective in preventing a variety of cancers in animals and in reversing preneoplastic lesions in humans (26–29). A large-scale study of the cancer chemopreventive role of retinoic acids in human shows that retinoids exhibit a high degree of specificity in cancer chemoprevention (30). The major retinoids responsible for the transcriptional regulation of many processes in the development and differentiation are all-trans and 9-cis-retinoic acids (RAs), which affect the important biological processes via their interaction with the nuclear receptors (25). In particular, RA was identified as an activating ligand that is relatively selective for RXRα, which must heterodimerize with a permissive partner (24, 31, 32). RXRα is essential...
for the high-affinity DNA binding of PPAR and serves as an auxiliary DNA-binding factor.

From these observations, it is possible that PPARγ and RXRα agonists activate the expression of the liver-specific genes at the transcriptional level. The liver-specific target genes may include GSTs because there is an extremely high (i.e., ~10 mM) concentration of glutathione in the liver. The conjugation of xenobiotics with glutathione, which are catalyzed by all classes of GSTs, is fundamentally different from their conjugation with other amino acids, because the substrates of glutathione conjugation include an enormous range of electrophilic xenobiotics or xenobiotics, which are biotransformed to electrophiles (33). The GSTA2 gene is a representative member of the class α GSTs that contains a xenobiotic-response element, putative phenobarbital-responsive element, glucocorticoid-responsive element, and an ARE. However, no functionally active PPRE or PPRE-responsive enhancer module (PPREM) has yet been identified in the GSTA2 gene. In the present study, we initially found the putative multiple PPREs in the promoter region of the GSTA2 gene. We determined whether the PPARγ and RXR activators induce GSTA2 via activating PPARγ – RXR heterodimer binding to the putative PPREs in H4IIE hepatocytes and, if so, whether the putative PPREs function as a module where there needs to be multiple nuclear binding sites in close proximity. We used 15-deoxy-Da-prostaglandin J2 (PGJ2) and thiazolidinedione PPARγ ligands as the PPARγ agonists. It was found that the combined treatment of the PPARγ agonist and RA both activated and induced Nrf2 and C/EBPβ, which are the essential transcription factors for GSTA2 expression. More importantly, the PPAR-binding site cluster in the GSTA2 gene was identified as a functionally active PPREM.

MATERIALS AND METHODS

Materials. [α-32P]dCTP (3000 mCi/mmol) and [γ-32P]ATP (3000 mCi/mmol) were purchased from New England Nuclear (Arlington Heights, IL). The random prime-labeling kit was supplied by Promega (Madison, WI). PGJ2 and tretinoin were purchased from Biomol Research Labs (St. Louis, MO). Pioglitazone and rosiglitazone were kindly supplied from Dong-A Pharmaceutical Co. (Shingal, Korea). RA, DTT, and 3-[N-morpholino]propanesulfonic acid were provided from Sigma Chemical (St. Louis, MO). Anti-PPARγ antibody and anti-RXRα antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The plasmid pGTB-1.65 construct containing the promoter region of the GSTA2 gene was generated according to procedures published previously (37). Briefly, total RNA was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose. The filter was then incubated with hybridization buffer containing 50% deionized formamide, 5× Denhardt’s solution, and 0.1% BSA (Pentex Fraction V), 0.1% SDS, 200 μg/ml sonicated salmon sperm DNA, and 5× saline-sodium phosphate-EDTA [1× saline-sodium phosphate-EDTA = 0.15 M NaCl, 10 mM Na2HPO4, and 1 mM NaEDTA (pH 7.4)] for 24 h at 4°C without probe. Hybridization was performed at 42°C for 18 h with a heat-denatured cDNA probe for GSTA2 that was random prime-labeled with [α-32P]dCTP. Filters were washed twice in 2× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate) and 0.1% SDS for 10 min at room temperature and twice in 0.1× SSC and 0.1% SDS for 10 min at room temperature. Filters were then washed once in a solution containing 0.1× SSC and 0.1% SDS for 1 h at 60°C. After quantitation of GSTA2 mRNA levels via scanning densitometry, the membranes were stripped and rehybridized with a 32P-labeled cDNA probe for 18S rRNA to control for RNA loading onto the membranes. Four separate experiments were performed with different RNA samples.

Immunoblot Analysis. SDS-PAGE and immunoblot analysis were performed according to procedures published previously (37). Briefly, proteins were separated by 7.5 or 12% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The replicate SDS-PAGE gels were stained with Coomassie Blue for verification of equal loading of proteins before immunoblotting. The nitrocellulose paper was incubated with an anti-GSTα antibody (Droto R&D, Detroit, MI), followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Specificity of the antibodies to

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Preparation of a cDNA Probe for GSTA2. A cDNA probe for the GSTA2 gene was amplified by reverse transcription-PCR using selective primers (37) and cloned in the pGEM-T vector (Promega).

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to methods published previously (14). Briefly, H4IIE cells in dishes were washed twice with ice-cold PBS and then scraped from the dishes with PBS and transferred to microtubes. Cells were then centrifuged at 2,500 × g for 3 min and allowed to swell after the addition of 100 μl of hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.5% NP40, 1 mM EDTA, and 0.5 mM phenylmethysulfonylfluoride. The lysates were incubated for 10 min on ice and then centrifuged at 7,200 × g for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM DTT, and 1 mM phenylmethysulfonylfluoride and then incubated for 30 min on ice. The samples were then centrifuged at 15,800 × g for 10 min to obtain supernatants containing nuclear fractions. Nuclear fractions were stored at −70°C until use.

Preparation of Cytosolic Fractions. H4IIE cells were washed twice with sterile PBS, scraped from their dishes, and sonicated to disrupt the membranes. Cytosolic fractions were prepared by differential centrifugation at 15,000 × g for 15 min and stored at −70°C until use. Protein content was determined by the Bradford assay (Bio-Rad protein assay kit; Bio-Rad, Hercules, CA).

Northern Blot Analysis. Total RNA was isolated from H4IIE cells using the single-step method of thiocyanate-phenol-chloroform RNA extraction, and northern blot analysis was carried out according to procedures described previously (37). Briefly, total RNA was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose paper. The northern blot paper was baked in a vacuum oven at 80°C for 2 h. The filter was then incubated with hybridization buffer containing 50% deionized formamide, 5× Denhardt’s solution, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% BSA (Pentex Fraction V). 0.1% SDS, 200 μg/ml sonicated salmon sperm DNA, and 5× saline-sodium phosphate-EDTA [1× saline-sodium phosphate-EDTA = 0.15 M NaCl, 10 mM Na2HPO4, and 1 mM NaEDTA (pH 7.4)] for 42°C for 1 h without probe. Hybridization was performed at 42°C for 18 h with a heat-denatured cDNA probe for GSTA2 that was random prime-labeled with [α-32P]dCTP. Filters were washed twice in 2× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate) and 0.1% SDS for 10 min at room temperature and twice in 0.1× SSC and 0.1% SDS for 10 min at room temperature. Filters were then washed once in a solution containing 0.1× SSC and 0.1% SDS for 1 h at 60°C. After quantitation of GSTA2 mRNA levels via scanning densitometry, the membranes were stripped and rehybridized with a 32P-labeled cDNA probe for 18S rRNA to control for RNA loading onto the membranes. Four separate experiments were performed with different RNA samples.
The GST subunit has been confirmed previously (8, 37). Immunoreactive protein was visualized through incubation with an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom; Ref. 8). Equal loading of proteins was verified by actin immunoblotting with a goat antiactin antibody (Santa Cruz Biotechnology). Three or four separate experiments were performed with different cytosolic samples. Changes in the levels of GSTA2 protein in the cells treated with PPARγ agonist + RA relative to that in untreated control were determined via scanning densitometry. Similarly, PPARγ, Nrf2, and C/EBPβ were immunologically detected with their respective antibodies (Santa Cruz Biotechnology). At least three separate experiments were performed with different subcellular fractions (lysates) to confirm changes in the protein levels.

Scanning Densitometry. Scanning densitometry of the Northern and immunoblots was performed with Image Scan & Analysis System (AlphaInnotech Corp., San Leandro, CA). The area of each lane was integrated using the software AlphaEase version 5.5, followed by background subtraction.

Gel Shift Assay. A double-stranded DNA probe containing the GSTA2 gene ARE end-labeled with [γ-32P]ATP, and T4 polynucleotide kinase was used for gel shift analysis. The sequence of the ARE-containing oligonucleotide was 5'-GATCATGGCATGGCATTGCACTAGGTGACAAAGCA-3'. Similarly, C/EBP gel shift analysis was carried out with the radiolabeled oligonucleotide, 5'-TGGAGATTGCCAATCTGCA-3', which contained the C/EBP consensus sequence. The consensus PPRE sequence was 5'-CCAAGGTCAAAGGT-

Fig. 1. Expression of peroxisome proliferator-activated receptor (PPARγ) in H4IIE cells and other types of cells. Total cell lysates were prepared from H4IIE cells, primary cultured hepatocytes, hepatic stellate cells, 3T3-L1 preadipocytes, and 3T3-L1 adipocytes, as described in “Materials and Methods.” Immunoblot analyses were conducted with the lysate preparations. Each lane contained 10, 20, or 30 μg of lysate proteins. Closed and open arrowheads, PPARγ1 and PPARγ2, respectively. Results were confirmed by repeated experiments.

Fig. 2. Induction of GSTA2 by PGJ2. A, immunoblot analyses of the GSTA2 protein. Immunoblot analyses show the levels of GSTA2 protein in H4IIE cells treated with 100-1000 nM PGJ2 for 24 h (left) or 300 nM PGJ2 for 1–24 h. Each lane was loaded with 10 μg of cytosolic proteins. Immunoreactive protein was visualized through incubation with horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit. Equal loading of proteins was verified by probing the replicate blots for actin. Changes in GSTA2 protein levels relative to control were assessed by scanning densitometry. B, Northern blot analyses of GSTA2 mRNA. Northern blot analysis was performed with total RNA fractions (30 μg each) prepared from the cells incubated with 100-1000 nM PGJ2 for 24 h (left) or in the cells treated with 300 nM PGJ2 for 3–24 h (right). The equal loading of RNA in each lane was confirmed by rehybridization of the stripped membrane with a 32P-labeled probe for 18S rRNA. Changes in the GSTA2 mRNA expression relative to control were assessed by scanning densitometry and normalized by RNA loading. C, immunoblot analyses of NF-E2-related factor (Nrf2) or CAAT/enhancer binding protein (C/EBPβ) and gel shift assays of Nrf2 or C/EBPβ DNA binding. Nuclear fractions were prepared from H4IIE cells treated with 300 nM PGJ2 for 1–12 h. Gel shift assays were conducted with nuclear extracts prepared from H4IIE cells exposed to PGJ2 (300 nM) for 1–12 h and 5 μg of radiolabeled antioxidant response element (ARE) or C/EBP-binding oligonucleotide. Arrowheads, the DNA bound with proteins. Results were confirmed by three separate experiments, and a representative immunoblot is shown. Data represent the mean ± SD with three separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman-Keuls test (significant as compared with control, *P < 0.05; **P < 0.01; control level = 1).
CATGTT-3'. To study the binding of nuclear hormone receptors to the DNA-binding sites, double-stranded PPRE oligonucleotides derived from PPRE1, 5'-CCATCGGGTGAATGCATCTGT-3'; PPRE2, 5'-TTGGCGAGAAGGATCAGTA-3'; and PPRE3, 5'-AACAGGACAAGATGATGA-3' present in the promoter region of the GSTA2 gene were used as probes. The reaction mixture contained 4 μl of 5 × binding buffer [containing 50 mM Tris-Cl (pH 7.5), 20% glycerol, 5 mM MgCl2, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, and 0.25 mg/ml poly dI-dC], 10 μg of nuclear extract, and sterile water up to a total volume of 20 μl. The reaction mixture was preincubated without probe at room temperature for 10 min. The probe (1 μl containing 10^6 cpm) was then added, and DNA-binding reactions were carried out for 30 min at room temperature.

In some analyses, specificity of binding was determined by competition experiments, which were carried out by adding a 10-fold molar excess of an unlabeled ARE, C/EBP, or PPRE oligonucleotide to the reaction mixture before the labeled probe was added. SP-1 oligonucleotide (5'-ATTCGATCGGGGCGGGGGCACGC-3') was used as a negative control for competition experiments. In other analyses, known as immuno-inhibition assays, antibodies directed to Nrf2, C/EBPα, C/EBPβ, PPARγ, and RXRα (2 μg each) were added to the reaction mixture 20 min after the labeled probe was added, and the reaction was then continued for 1 h at 25°C. Samples were separated on 4% polyacrylamide gels at 100 V. The gels were fixed with 40% methanol/10% acetic acid, dried, and subject to autoradiography.

**Luciferase Reporter Gene Analysis.** We used the dual-luciferase reporter assay system (Promega). Briefly, H4IIE cells (7 × 10^4 cells/well) were plated in six-well plates overnight, serum starved for 12 h, and transiently transfected with each GSTA2 promoter-luciferase construct and pRL-SV plasmid (a plasmid that encodes for Renilla luciferase and is used to normalize transfection efficacy) in the presence of LipofectAMINE Plus Reagent (Life Technologies, Inc, Gaithersburg, MD) for 3 h. Transfected cells were incubated in DMEM containing 1% FCS (Life Technologies) for 3 h and exposed to PGI2 + RA (50 nm each) in medium containing 10% FCS for 18 h at 37°C. Firefly and Renilla luciferase activities in cell lysates were measured using a Luminoskan luminometer (Thermo Labsystems, Helsinki, Finland). The activity of firefly luciferase was measured by adding Luciferase Assay Reagent II (Promega) according to the manufacturer’s instruction, and, after quenching the reaction, the Renilla luciferase reaction was initiated by adding Stop & Glo reagent (Promega). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase.
induced GSTA2 in H4IIE cells. PGJ2, at concentrations of 300 or 1000 nM, increased the GSTA2 protein levels (Fig. 2, left panel).

The studies were extended to verify the effects of PGJ2 in most tissue types (15). We determined whether PGJ2 weakly increased the Nrf2 level in the nuclear fraction (Fig. 2, right panel). Gel shift analysis of protein binding to the C/EBP-binding site was performed with the nuclear extracts using a radiolabeled C/EBP-binding oligonucleotide. PGJ2 minimally changed C/EBP DNA binding, which varied during the 6–12-h time period (Fig. 2, right panel).

**GSTA2 Induction by RA.** Next, we determined whether RA could induce GSTA2. Treatment of the H4IIE cells with RA (10–300 nM), a ligand of RXR, for 24 h increased the GSTA2 protein level in a concentration-dependent manner (Fig. 3A). RA at concentrations of 100 or 300 nM induced GSTA2 2–3-fold with the maximum induction being observed at 300 nM. A time course study showed that RA gradually induced the GSTA2 protein level from 6 h with the maximum increase being noted at 24 h. Northern blot analyses confirmed that the level of GSTA2 mRNA increased after exposure of cells to 300 nM RA (Fig. 3B). The GSTA2 mRNA level peaked at 12 h.

This study next examined whether or not RA activated Nrf2 and C/EBPβ. RA at 300 nM weakly induced the nuclear translocation of Nrf2 and C/EBPβ (Fig. 3C), as evidenced by the subcellular fractionation and immunoblot analysis. Gel shift analyses confirmed that RA at 300 nM weakly increased Nrf2 and C/EBPβ binding to their respective consensus DNA oligonucleotides (Fig. 3C). Immuno-inhibition experiments using specific antibodies directed against Nrf2, PPARγ, and RXRα indicated that an increase in the Nrf2-ARE DNA-binding activity by RA was dependent on Nrf2 (Fig. 3C).

Correspondingly, the shift in the C/EBP DNA-binding complex was the result of C/EBPβ.

**Synergistic Induction of GSTA2 by PGJ2 + RA.** PPARγ heterodimerizes with the RXR for activation, and the PPARγ–RXR heterodimers are widely expressed in the major organs, including the liver (15). We determined whether PGJ2 + RA enhanced GSTA2 induction. GSTA2 was induced 2-fold by 100 nM RA but not 100 nM PGJ2. In contrast to the weak induction of GSTA2 by each agent alone, PGJ2 + RA (100 nM each) synergistically increased the GSTA2 protein level (Fig. 4A). A preliminary study reported that PGJ2 + RA induced GSTA2 to the greatest extent at the 1:1 molar ratio. To determine the dose-response effect, the GSTA2 level was further assessed in the cells treated with PGJ2 + RA each at equal concentrations (i.e., 15, 50, or 100 nM) for 24 h (Fig. 4B). The dose-response study revealed that GSTA2 induction was efficaciously increased by the PGJ2 + RA treatment (i.e., 3–4-fold increases at concentrations of 15 or 50 nM). Therefore, 50 nM PGJ2 and RA each were selected for the subsequent experiments.

**Synergistic Induction of GSTA2 by PPAR Agonists in Combination with RA.** The studies were extended to verify the effects of the thiazolidinedione PPARγ agonists in combination with RA on the GSTA2 expression. Immunoblot analysis revealed that troglitazone (1–10 μM) + RA (50 nM) synergistically induced GSTA2, whereas either troglitazone (1–10 μM) or RA alone only weakly increased the GSTA2 protein level (Fig. 5A). Rosiglitazone at 1–10 μM moderately induced GSTA2, which was also enhanced by a combination treatment with RA. Similarly, pioglitazone + RA markedly increased the
GSTA2 expression level when compared with the individual agent alone (Fig. 5A). These results strongly support the conclusion that the activation of the PPARγ and RXR heterodimer leads to the induction of GSTA2.

Additional experiments were performed in the primary cultured hepatocytes to confirm that the activation of the PPARγ and RXR heterodimer induces GSTA2 in normal cells. Because the enhancement effect for GSTA2 expression by the PPARγ agonist was saturated at 10 μM in H4IIE cells, 1 or 3 μM the PPARγ agonist were chosen for this experiment. Troglitazone, rosiglitazone, or pioglitazone in combination with RA induced GSTA2 to a greater extent when compared with the PPARγ agonist alone at the concentrations examined (Fig. 5B). Hence, the PPARγ and RXR heterodimer enhance GSTA2 expression in hepatocytes.

Activation of Nrf2 and C/EBPβ by PGJ2 + RA. To confirm that PGJ2 + RA (50 nM each) activates Nrf2, immunoblot and gel shift assays were performed with the nuclear extracts of the cells treated with PGJ2 + RA. PGJ2 + RA increased the nuclear Nrf2 levels and its binding to a radiolabeled ARE consensus oligonucleotide at 6–12 h. Binding of the protein to the ARE was competed with an excess amount of unlabeled ARE (Fig. 6A). Immunoblot and gel shift analyses showed that the PGJ2 + RA treatment increased the C/EBPβ levels in the nuclear fractions and C/EBPβ binding to the C/EBP binding oligonucleotide compared with the control (Fig. 6B). Competition experiments using excess quantities of unlabeled C/EBP or SP-1 oligonucleotides confirmed the specificity of C/EBP DNA binding. Supershift experiments indicated that the C/EBP-binding complex comprised of C/EBPβ (Fig. 6B). The band intensity of C/EBP DNA-binding complex was reduced by the anti-C/EBPβ antibody but not the anti-PPARγ or -RXRα antibodies. Immunocytochemistry confirmed that Nrf2 and C/EBPβ, which were located mainly in the cytoplasm of control cells, had perinuclear and nuclear localization at 6–12 h in the cells treated with PGJ2 + RA, suggesting that these transcription factors translocate into the nucleus (data not shown).

Increases in the Nrf2 and C/EBPβ Expression Levels by PGJ2 + RA. Nrf2 and C/EBPβ expression was next examined to determine whether it was affected by PGJ2 + RA. Immunoblot analysis revealed that PGJ2 + RA notably increased the Nrf2 levels in the total cell lysates at 6–12 h (Fig. 7A). C/EBPβ expression also began to increase 3 h after the PGJ2 + RA treatment. C/EBPβ was induced 2–3-fold after 6–12 h in the cells exposed to PGJ2 + RA (Fig. 7B). This indicates that PGJ2 + RA increased the Nrf2 and C/EBPβ expression level, suggesting that induction of Nrf2 and C/EBPβ might also contribute to Nrf2- and C/EBPβ-mediated GSTA2 induction.
Analysis of ARE and C/EBP-Response Element in the GSTA2 Promoter. The functional role of ARE and C/EBP-response element in transactivation of the GSTA2 gene by PGJ2 + RA was next examined. The reporter gene assays were performed using the H4IIE cells transfected with pGL-1651, which contained the luciferase structural gene downstream of the −1.65-kb GSTA2 promoter region (Fig. 8A).

Exposure of the pGL-1651-transfected cells to PGJ2 + RA resulted in a 6-fold increase in the luciferase activity (Fig. 8B). Either PGJ2 or RA alone (50 nM each) was minimally active. To precisely define the role of the ARE sequence for the induction of GSTA2 by PGJ2 + RA, this study prepared pGL-ΔARE, which is a chimeric gene construct with the ARE deleted (Fig. 8A). The relative luciferase-inducible activity by PGJ2 + RA was markedly decreased in the H4IIE cells transfected with pGL-ΔARE, compared with that in the cells transfected with pGL-1651 (Fig. 8B). C/EBPβ was next examined to determine whether it plays a role in GSTA2 induction by PGJ2 + RA. The luciferase inducibility was completely abolished in the cells transfected with pGL-ΔC/EBP (Fig. 8B), which is a chimeric gene construct where the C/EBP response element was deleted (Fig. 8A). To further confirm the functional role of C/EBPβ in GSTA2 transactivation, the constitutively active dominant-negative mutant of C/EBP (AC/EBP) was expressed in combination with the pGL-1651 luciferase reporter in H4IIE cells. The expression of AC/EBP substantially inhibited the ability of PGJ2 + RA to stimulate reporter gene expression from the pGL-1651 plasmid (Fig. 8B, inset). The ability of PGJ2 + RA to stimulate reporter gene expression from pGL-1651 was not inhibited by transfecting the cells with pCMV500, a control plasmid. These results provide evidence that the ARE and C/EBP-response elements are both necessary for GSTA2 induction by PGJ2 + RA.

Binding of the PPARγ and RXR Heterodimer to the PPREs. Studies have shown that PPARγ heterodimerizes with RXR (probably RXRα), which is activated by RA (38, 39). Searching the GSTA2
promoter region with the PPRE consensus sequence, AGGTACAGG- 

GTCAG, resulted in three putative response elements (i.e., PPRE1, 

PPRE2, and PPRE3), to which the activating PPARγ – RXR hetero-
dimer might bind. The putative PPRE1 sequence present at −792 

bp from the transcription start site contained a reverse DR1 site, 

whereas the other DR1 sites named PPRE2 and PPRE3 were present 
at −746 and −549 bp, respectively (Fig. 9A).

As part of the efforts to examine how PGJ2 + RA enhances the 

GSTA2 gene induction, the activities of protein binding to the 

PPREs were determined. Gel shift analyses revealed that PGJ2 + RA 

increased the band intensities of protein binding to either the PPRE1 or 

PPRE2 oligonucleotide at 3–12 h (Fig. 9, B and C). Similarly, 

PGJ2 + RA treatment resulted in increases in protein binding to the 

PPRE3 at 3–6 h (Fig. 9D). The protein–PPRE3-binding complex was 
immunodepleted with either an anti-PPARγ antibody or anti-RXRα 

antibody (Fig. 9E). Competition experiments using excess amounts 
of unlabeled SP-1 or the PPRE3 oligonucleotide confirmed the 
specificity of protein DNA binding (Fig. 9E). The specificity of 

PPARγ – RXR heterodimer binding to the other PPREs was also 

confirmed by immunodepletion and competition analyses with the 

unlabeled oligonucleotides (data not shown).

Functional Analysis of the PPREs in the GSTA2 Promoter. 

When the PPRE1 sequence located between the nucleotides –792 and 

−781 bp in pGL-1651 was mutated (Fig. 10A; i.e., pGL-mPPRE1), the 

inducible luciferase activity by PGJ2 + RA was completely reduced to 

that of the control (Fig. 10B). For comparison, this study determined 

the effect of 3-methylcholanthrene, an AhR-xenobiotic- 

response element-mediating GSTA2 inducer, on the expression of 

pGL-mPPRE1 promoter reporter gene. Exposure of the H4IIE cells 

transfected with pGL-mPPRE1 to 3-methylcholanthrene increased the 

luciferase activity 8-fold compared with the untreated control (Fig. 

10B). The increase in luciferase activity was comparable with that in 
pGL-1651-transfected cells treated with 3-methylcholanthrene. These 

results indicated that the PPRE1 in the GSTA2 gene is selectively 

activated by PGJ2 + RA. The role of the PPRE2 or PPRE3 site in 

responsiveness was also examined using mutation analyses (Fig. 

10A). Mutation of each of the PPRE sites almost completely abolished 

the inducible expression of the pGL-1651 luciferase chimeric gene by 

PGJ2 + RA (Fig. 10C). Either pGL-mPPRE2 or pGL-mPPRE3 also 

responded to 3-methylcholanthrene. These results demonstrate that 

PGJ2 + RA directly activates the PPRE enhancer activities in the 

GSTA2 gene and that the multiple PPRE-binding sites are essential for 

the full ligand responsiveness.

Effect of PPARγ1 – RXRα Overexpression on the GSTA2 

Transactivation. This study examined the effects of PPARγ1 and 

RXRα overexpression with or without ligands on the Nrf2, C/EBPβ, 

and GSTA2 gene expression. The H4IIE cells were transfected with 

the plasmids encoding PPARγ1 and RXRα, which were then exposed 

to PGJ2 + RA. Either PPARγ1 or RXRα alone elicited a modest 

increase in the Nrf2 expression level. The concomitant expression 
of PPARγ1 and RXRα to the larger extent enhanced the level of Nrf2 

level in the total cell lysates (Fig. 11A, top panel). Furthermore, 

PGJ2 + RA potentiated Nrf2 induction in the cells transfected with 

the PPARγ1 and RXRα plasmids. Similarly, PPARγ1 and RXRα 

overexpression caused an increase in the C/EBPβ level in the total 

cell lysates. C/EBPβ was further induced by PGJ2 + RA in the cells 

transfected with the plasmids (Fig. 11A, top panel).

Additional experiments were performed using PPARγ selective 

agonists with or without RA to confirm that the ligand activation of 

PPARγ and RXR led to Nrf2 or C/EBPβ, pioglitazone, or rosiglitazone alone (3 μM) failed to induce Nrf2 in the cell lysates. 

As expected, an increased Nrf2 expression level was noted in the cells 
treated with pioglitazone + RA or rosiglitazone + RA (Fig. 11A, 

bottom panel). However, troglitazone (3 μM) with or without RA 

induced Nrf2, which suggested that troglitazone might elicit oxidative 

stress. Pioglitazone, rosiglitazone, or troglitazone in conjunction with 

RA induced C/EBPβ to the greater extent, compared with their 

respective treatment alone (Fig. 11A, bottom panel).

The extents of protein binding to the PPRE-binding sites in the 

GSTA2 gene were next determined. PPARγ1 and RXRα overexpres-

sion in the H4IIE cells resulted in an increase in the band intensity of 

protein binding to PPRE3, which was enhanced by the PGJ2 + RA 
treatment (Fig. 11B). The addition of either the anti-PPARγ or -RXRα 

antibodies decreased the intensity of the shifted band, indicating that 

the binding proteins include PPARγ and RXRα.

To examine the effect of the PPARγ and RXR heterodimer on 

transactivation of the GSTA2 gene, functional reporter gene analysis 

was performed on the cells transfected with pGL-1651. The functional 

role of the PPARγ and RXR heterodimer in responsiveness was 

verified by an increase in the luciferase inducibility (Fig. 11C). The 
luciferase activity in the cells transfected with the plasmids encoding
PPARγ and RXRs was comparable with that of the control cells treated with PGJ2 + RA. The luciferase reporter gene expression level was further increased after exposing the cells transfected with the PPARγ and RXRα plasmids to PGJ2 + RA (Fig. 11C). The results demonstrate that the PPARγ and RXR heterodimer, which interacts with the multiple PPRE-binding sites, makes crucial contribution to the inducible expression of the GSTA2 gene.

**DISCUSSION**

The nuclear receptors are ligand-dependent transcription factors that regulate the gene networks involved in controlling cell growth, morphogenesis, differentiation, and homeostasis. Activation of the nuclear receptor homodimers and heterodimers in response to the ligands requires binding of the nuclear receptors to the response elements that contain two core recognition sequences (i.e., half-sites). PPARs, including PPARα, PPARβ/δ, and PPARγ, constitute a subfamily of the nuclear receptor superfamily activated by a variety of natural and chemical ligands. The PPAR genes are involved in regulating the lipid metabolism (40–42). The RXRs, as members of the nuclear receptor superfamily, are the common heterodimeric partner for many receptors, including PPAR. The RXRs are modular proteins with a highly conserved central DNA-binding domain and less conserved ligand-binding domain (43). RXR serves as a permissive partner of PPARs. Although the effects of the PPAR subfamily on the lipid metabolism have been extensively studied, a proper examination of the role of PPARs in the transcriptional regulation of the Phase II detoxification genes has not been reported.

There are reports showing that PPARγ1 is expressed in the adipocytes and hepatic stellate cells with PPARγ2 being less frequently expressed (15, 16, 44). In the current study, it was shown that PPARγ1 was sufficiently expressed in the H4IE and primary-cultured hepatocytes. It was revealed that either PPARγ or RXR agonist results in the induction of GSTA2 with increases in the mRNA and that treatment of the cells with the PPARγ and RXR agonists synergistically induces GSTA2. This was also observed in the cells treated with nanomolar concentrations of PGJ2 and RA. PGJ2 at nanomolar concentrations served as a ligand of PPARγ. Enhanced GSTA2 induction by PGJ2 + RA, compared with that by each agent alone, demonstrates that PPARγ and RXR heterodimer activation may contribute to the enzyme induction. This was further supported by the observation that the PPARγ ligand, troglitazone, rosiglitazone, or pioglitazone, in combination with RA, induced GSTA2 to a much greater extent. A discrepancy in the effective concentration range between PGJ2 (50 nM) and thiazolidinedione PPARγ agonist (1–10 μM) for GSTA2 induction may have resulted from the difference in their equilibrium dissociation rate constants for PPARγ. This is re-
activities in the cells transfected with the PPRE mutant plasmation on the induction of luciferase activity by PGJ2 mutant constructs of pGL-1651.

B, PPAR/GSTA2 by RA alone, RA potentiated the enzyme induction by contributes to GSTA2 induction. Despite the weak induction of mutated.
siently transfected with pGL-mPPRE1, in which the PPRE1 was luciferase reporter assays were performed in H4IIE cells transiently transfected with pGL-mPPRE1, in which the PPRE1 was mutated. The experimental value for luciferase activity represented the mean ± SD with four separate experiments (significant as compared with pGL-1651-transfected cells exposed to PGJ2 + RA, **, P < 0.01). 3-MC, 3-methylcholanthrene

The expression of the Phase II detoxifying enzyme is transcriptionally activated partly through the activation of Nrf2 via the ARE in the promoter regions of the target genes (2, 3). Nrf2 activation plays an important role in the induction of many Phase II detoxifying enzymes, and the lack of Nrf2/ARE activation increases the sensitivity to xenobiotics, including carcinogens (46–48). We reported previously that tert-butylhydroquinone, a representative prooxidant, activated Nrf2 for GSTA2 induction (9, 49). In the current study, it was shown that either PGJ2 or RA alone at relatively high concentrations elicited Nrf2 translocation and its DNA binding in the H4IIE cells, whereas PGJ2 or RA at nanomolar concentrations required each other for Nrf2 activation. In addition, PGJ2 + RA induced Nrf2. We found that the promoter region of the Nrf2 gene contains the putative PPRE site (GenBank no. U70474, mouse Nrf2 promoter), which potentially interacts with the activating PPARγ – RXR heterodimer. Therefore, both the presence of the binding sites for PPARγ – RXR in the promoter region of the Nrf2 gene and the efficacious GSTA2 induction by nanomolar concentrations of PGJ2 and RA indicate that the PPARγ and RXR heterodimer activates the Nrf2 gene induction, which contributes to the GSTA2 induction.

Previous studies showed that cancer chemopreventive agents, including oltipraz and flavonoids, activate C/EBPβ and stimulate C/EBPβ binding to the C/EBP-response element in the GSTA2 gene (13, 14). The essential role of the C/EBP-binding site in GSTA2 induction was further supported by an experiment using a dominant-negative mutant C/EBP (AC/EBP) in the reporter gene assay, which holds significant implication for the finding of C/EBPβ as an important transcription factor for GSTA2 induction. The NH2-terminal transactivation domains of C/EBPβ interact with CBP/p300 coactivator after binding to the C/EBP site, which is essential for C/EBPβ-mediated gene transactivation (50). In the present study, it was observed that a combined treatment of the cells with either PGJ2 and RA induced the nuclear translocation of C/EBPβ and activating C/EBPβ binding to the C/EBP response element. In addition, PGJ2 + RA increased the C/EBPβ expression level. The C/EBPβ gene contains PPRE sites (GenBank no. AY056052). It is likely that C/EBPβ induction via PPARγ and RXRα heterodimer binding to the PPREs in the promoter region of the C/EBPβ gene is also responsible for inducing GSTA2.

This study provides evidence that both the ARE and C/EBP-binding site have essential roles in the transactivation of the GSTA2 gene by PPARγ and RXR ligands, as evidenced by the binding site-deleted promoter–luciferase assay. The complete blockage of the PPARγ and RXRα-mediated induction of the GSTA2 gene as a result of a deletion mutation of either the ARE or C/EBP-binding site confirms the previous observation that the binding of Nrf2 and C/EBPβ to their response elements is simultaneously necessary for the full gene transactivation (Fig. 12A; Refs. 13 and 14). It is inferred that the formation of the transcriptional protein complexes on the two essential binding sites is required for the full responsiveness to chemical inducers.

The retinoic acid receptor and RXRα heterodimer activate tran-
Fig. 11. The functional role of peroxisome proliferator-activated receptor (PPAR)y and retinoid X receptor (RXR)x overexpression in gene transactivation. A, the effects of PPARy1 and RXRx heterodimers in the expression of NF-E2-related factor (NF2) and CCAAT/enhancer binding protein (C/EBP)y. The levels of NF2 and C/EBP were immunochemically measured in the cells transfected with the plasmids encoding PPARy1 and/or RXRx. Cells were transfected with an empty vector or the PPARy1 and/or RXRx plasmids (500 ng each) in the presence of LipofectAMINE for 3 h, exposed to the medium containing 1% FCS for 12 h, and then treated with prostaglandin J2 (PGJ2, 9 cis-retinoic acid (RA); 50 nM each) for an additional 12-h time period. pCMX and pCDNA3.1 were included as empty vectors to transfect cells with the same amount of plasmids (a total of 1 μg of DNA). In another set of experiments, H4IIE cells were transfected with PPARy-selective agonists (3 μM) with or without RA (50 nM) each for 12 h. The levels of NF2 and C/EBP were immunochemically determined in the cell lysates. Each lane contained 15 μg of proteins. Results were confirmed by repeated experiments. B, gel shift analysis of PPARy and RXRx binding to the putative peroxisome proliferator-activated receptor-response element (PPRE). Nuclear extracts were prepared from the H4IIE cells transfected with the PPARy1 and/or RXRx plasmids with or without PGJ2 + RA (50 nM each). Fifteen μg of nuclear extracts were incubated for 30 min with 5 ng of radiolabeled PPRE oligonucleotide and separated on 4% polyacrylamide gel. Arrowhead, the protein PPRE-binding complex. The nuclear extract was immunoabsorbed with an anti-PPARy antibody or an-RXRx antibody (2 μg). Excess of unlabelled PPRE oligonucleotide was added to the reaction mixture for a competition assay. Results were confirmed by repeated experiments. C, activation of the pGL-1651 gluthathione S-transferase A2 gene by PPARy1 and RXRx overexpression. H4IIE cells were transfected with the pGL-1651 luciferase reporter plasmid (1 μg) and plasmid of Renilla luciferase (5 ng) after transfection with the PPARy1 and/or RXRx plasmid (500 ng each) for 3 h and incubated for 12 h in the medium with 1% FCS. The cells were treated with PGJ2 + RA (50 nM each) for 18 h, and the cell extracts were assayed for firefly and Renilla luciferase activities. The fold inductions of the normalized luciferase activity were compared with that in control cells transfected with the pGL-1651 (1 μg) and empty plasmids (500 ng each of pCMX and pCDNA3.1). Data represented the mean ± SE with three separate experiments (significant as compared with the cells transfected with empty plasmids, **, P < 0.01; significant as compared with the cells transfected the PPARy1 and RXRx plasmids, ##, P < 0.01).

scription in response to the retinoic acid receptor-specific ligands on the DR5 elements but constitutively repress transcription on the DR1 elements (51). The PPARy and PPARy – RXRx heterodimers have different DNA-binding specificities with preferential binding of the latter to DR1. The present finding of PPARy and RXRx heterodimer binding to the DR1 and functional role of the heterodimer in transactivating the GSTA2 gene excludes the possibility that the retinoic acid receptor-RXR heterodimer is involved in the transactivation of the GSTA2 gene. This is in parallel with the suppression of GSTP1 expression by RA in the presence of retinoic acid receptor as a result of the decreased transcription from its promoter (52). PGJ2 alone (at nanomolar concentrations) is a weak inducer of GSTA2, because the PPARy – RXRx heterodimer requires the binding of an RXR ligand. The role of the PPARy and RXRx heterodimer in GSTA2 gene transactivation was confirmed by the comparable induction of GSTA2 by the thiazolidinedione PPARy activator and RA. SRC-1 is a coactivator of PPARy (53). Binding of the ligand-activated PPARy – RXRx heterodimer to its DNA-binding sites stimulates the interaction between PPARy – RXRx and p160/SRC-1 (53).

The observation that the ligand-activated PPARy – RXRx heterodimer binds the regulatory PPREs independent of their activation and the induction of NF2 and C/EBP led us to examine the functional role of each of these PPRE-binding sites containing the DRs related to the hexamer AGGTCA in the GSTA2 gene. Specific mutations of these nuclear binding sites in the GSTA2 promoter, which are present as a three PPRE cluster, resulted in the complete loss of its responsiveness to PGJ2 + RA. We showed that all of the putative PPRE sites comprising DR1 are functionally active. Therefore, the binding of the activating PPARy – RXRx heterodimer to all of the PPRE sites is crucial for the inducible gene activation (Fig. 12B). Blockage of the ligand-dependent transcriptional response as a result of a mutation of the respective binding site suggests that the multiple putative PPREs function as a module in which the nuclear binding sites in close proximity to each other are essential for full ligand responsiveness. Therefore, it is highly likely that the PPAR-binding site cluster is the functionally active PPREM in the GSTA2 gene. Consistent with this, the essential role of the PPARy – RXRx heterodimer and PPRE-binding elements in activation, the GSTA2 gene was further supported by the reporter gene induction experiments. It was observed that transcription of the GSTA2 gene was further enhanced by exposing the cells transfected with the plasmids encoding PPARy and RXRx to their corresponding ligands. This study on the regulation of GSTA2 gene expression by the PPARy – RXRx heterodimer at the promoter containing DR1 elements brings additional insights into the transcriptional control mechanism of the Phase II enzyme involved in carcinogen detoxification.

NF2 and C/EBP play essential roles in gene transactivation. Now, we report that the activating PPARy and RXRx heterodimer functionally regulates PPREM activity via the proximal promoter of the GSTA2 gene and confers full responsiveness to the PPARy and RXRx.
activating Nrf2 and C/EBP direct gene transactivation, as well as the coordinate induction of the PPRE GSTA2 element (of the antioxidant- and C/EBP-response elements stimulates the expression of the GSTA2 gene, as well as by induction of activating C/EBPβ and Nrf2. The PPARγ – RXR heterodimer induces C/EBPβ and Nrf2. 

ligands in association with the activating Nrf2 and C/EBPβ (Fig. 12B). Both Nrf2 and C/EBPβ are up-regulated in a PPARγ- and RXR-dependent manner. However, the induction of Nrf2 and C/EBPβ resulted in little or no activation of the GSTA2 promoter in the cells transfected with a GSTA2 promoter–reporter construct containing the sequencially mutated PPAR response elements. These results suggest that the PPAR response module, which was activated by the ligand-bound PPARγ and RXR heterodimer, may be essential for the formation of the transactivation complexes comprising Nrf2 and C/EBPβ. The essential role of PPARγ binding to its target DNA element for enhancesome-mediated gene activation is further supported by the findings that activating transcription factors, such as PPARγ, Nrf2, and C/EBPβ, form complexes with coregulators (e.g., CBP/p300; Ref. 53) and that PPARγ binds to Nrf2 (54). An additional study revealed that PPARγ and RXRα agonists induced other forms of GST, including GSTA3 in H4IIE cells (data not shown). A search of the GenBank database showed that the putative PPRE containing DR1 element is located in the promoter region of the GSTA3 gene (GenBank no. AF067442). Therefore, the PPARγ – RXR heterodimer may serve as a common transcriptional factor complex for inducing the GSTs in the liver.

In conclusion: (a) PPARγ agonist + RA both activate and induce Nrf2 and C/EBPβ; (b) activating Nrf2 and C/EBPβ binding to the antioxidant- and C/EBPβ-response elements stimulates the expression of the GSTA2 gene in response to the PPARγ and RXR agonists; (c) the promoter region of the GSTA2 gene contains the multiple PPRE cluster, which interacts with the PPARγ – RXR heterodimer; and (d) the PPRE-binding sites present in the GSTA2 promoter might function as a PPREM for transactivation of the GSTA2 gene. The fact that PPARγ – RXR activation plays a key role in GST induction via direct gene transactivation, as well as the coordinate induction of the activating Nrf2 and C/EBPβ, provides implication that dietary components, including fatty acids and retinoids, strongly affect GST regulation, whose transcription confers cancer chemoprevention, as well as the detoxification of xenobiotics and toxicants.

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Transactivation of the PPAR-Responsive Enhancer Module in Chemopreventive Glutathione S-Transferase Gene by the Peroxisome Proliferator-Activated Receptor-γ and Retinoid X Receptor Heterodimer

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