# Glutathione *S*-Transferases (GSTs) Inhibit Transcriptional Activation by the Peroxisomal Proliferator-Activated Receptor $\gamma$ (PPAR$\gamma$) Ligand, 15-Deoxy-$\Delta^{12,14}$Prostaglandin $J_2$ (15-d-$PGJ_2$)[†]

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ABSTRACT: 15-Deoxy-$\Delta^{12,14}$prostaglandin $J_2$ (15-d-$PGJ_2$), a terminal metabolite of the J-series cyclopentenone prostaglandins, influences a variety of cellular processes including gene expression, differentiation, growth, and apoptosis. As a ligand of peroxisomal proliferator-activated receptor $\gamma$ (PPAR$\gamma$), 15-d-$PGJ_2$ can transactivate PPAR$\gamma$-responsive promoters. Previously, we showed that multidrug resistance proteins MRP1 and MRP3 attenuate cytotoxic and transactivating activities of 15-d-$PGJ_2$ in MCF7 breast cancer cells. Attenuation was glutathione-dependent and was associated with formation of the glutathione conjugate of 15-d-$PGJ_2$, 15-d-$PGJ_2$−SG, and its active efflux by MRP. Here we have investigated whether the glutathione *S*-transferases (GST) can influence biological activities of 15-d-$PGJ_2$. MCF7 cells were stably transduced with human cytosolic GST isozymes M1a, A1, or P1a. These GSTs had no effect on 15-d-$PGJ_2$ cytotoxicity when expressed either alone or in combination with MRP1. However, expression of any of the three GSTs significantly inhibited 15-d-$PGJ_2$-dependent transactivation of a PPAR$\gamma$-responsive reporter gene. The degree of inhibition correlated with the level of GST expressed. Under physiologic conditions, the nonenzymatic rate of 15-d-$PGJ_2$ conjugation with glutathione was significant. Of the three GST isozymes, only GSTM1a-1a further stimulated the rate of 15-d-$PGJ_2$−SG formation. Moreover, GSTM1a-1a rate enhancement was only a transient burst that was complete within 15 s. Hence, catalysis plays little, if any, role in GST inhibition of 15-d-$PGJ_2$-dependent transactivation. In contrast, inhibition of transactivation was associated with strong GST/15-d-$PGJ_2$ interactions. Potent inhibition by 15-d-$PGJ_2$ and 15-d-$PGJ_2$−SG of GST activity was observed with $K_i$ in the 0.15−2.0 $\mu$M range for the three GST isozymes, results suggesting avid associations between GST and 15-d-$PGJ_2$ or 15-d-$PGJ_2$−SG. Electrospray ionization mass spectrometry (ESI/MS) studies revealed no stable adducts of GST and 15-d-$PGJ_2$ indicating that GST/15-d-$PGJ_2$ interactions are primarily noncovalent. These results are consistent with a mechanism of GST-mediated inhibition of transactivation in which GST binds 15-d-$PGJ_2$ and 15-d-$PGJ_2$−SG thereby sequestering the ligands in the cytosol away from their nuclear target, PPAR$\gamma$.

The cyclopentenone prostaglandins are arachidonic acid metabolites that influence a variety of cellular processes including induction of apoptosis and inhibition of cell growth, induction of adipocyte differentiation, and inhibition of inflammatory signaling pathways (*1*−*7*). 15-Deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15-d-$PGJ_2$),[1] a terminal product of prostaglandin $D_2$ metabolism, is among the most potent cyclopentenone prostaglandins. Its biological effects are mediated by both peroxisomal proliferator-activated receptor $\gamma$ (PPAR$\gamma$)-independent and PPAR$\gamma$-dependent mechanisms (*5*, *6*, *8*−*12*). Many PPAR$\gamma$-independent effects are believed to involve the formation of 15-d-$PGJ_2$/protein adducts (*11*−*14*) via reactions between protein thiols and the $\alpha,\beta$-unsaturated ketone on 15-d-$PGJ_2$ (*2*, *15*−*17*). Formation of these adducts inactivates or modifies the function of the target protein resulting in the observed biological effects (*11*−*14*).

15-d-$PGJ_2$ is also a ligand for the nuclear receptor PPAR$\gamma$. Activation of PPAR$\gamma$ by binding of this or other ligands facilitates PPAR$\gamma$/retinoid X receptor heterodimer formation, recruitment of transcriptional coeffectors, and binding of the PPAR$\gamma$/retinoid X receptor complex to its PPAR responsive elements (*1*, *6*, *8*, *18*−*20*). Consequently, ligand binding to PPAR$\gamma$ is associated with the altered expression of a variety of genes including those involved in adipocyte differentiation, cell proliferation, and lipid and glucose homeostasis (*8*, *19*, *21*). PPAR$\gamma$ is upregulated in cancer cells including those from colon and breast, and it has been suggested that the ligands of PPAR$\gamma$ may be useful in the treatment of some cancers (*3*−*5*, *22*−*24*).

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[1] Abbreviations: ESI, electrospray ionization; 15-d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$prostaglandin $J_2$; 15-d-$PGJ_2$−SG, glutathione conjugate of 15-d-$PGJ_2$; GST, glutathione *S*-transferase: HPLC, high-performance liquid chromatography; $IC_{50}$, drug concentration at which cell proliferation is inhibited 50% of control; MRP, multidrug resistance protein or multidrug resistance-associated protein; MS, mass spectrometry; PPAR and PPAR$\gamma$, peroxisome proliferator-activated receptor and peroxisome proliferator-activated receptor $\gamma$; PPRE, peroxisome proliferator-activated receptor responsive element.

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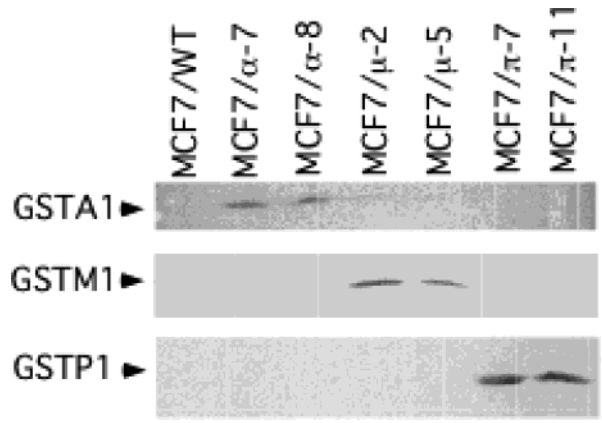


FIGURE 1: Western blot analysis of parental MCF7 cells transduced with GST. Fifty micrograms of protein from the whole cell lysates of parental cells (MCF7/WT) and cells transduced with expression vectors encoding GSTA1 (MCF7/α-7, MCF7/α-8), GSTM1a (MCF7/$\mu$-2, MCF7/$\mu$-5), and GSTP1a (MCF7/$\pi$-7, MCF7/$\pi$-11) were separated by electrophoresis, transferred, and examined using antibodies specific for α class GST (GSTA1), $\mu$ class GST (GSTM1), and $\pi$ class GST (GSTP1) as shown.

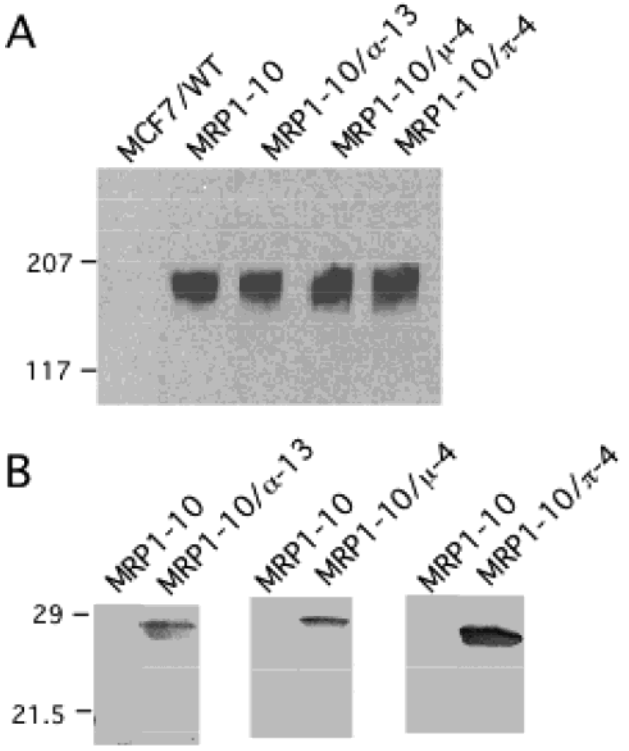


FIGURE 2: Western blot analysis of MRP1-expressing cells transduced with GST. Shown are Western transfers analyzed for MRP1 (A) or GST (B) as described in Experimental Procedures. The positions of molecular weight markers (kDa) are shown to the left. Each lane was loaded with 30 $\mu$g of protein derived from membrane (A) or whole cell lysate (B) preparations. Protein derived from parental (MCF7/WT), parental MRP1-expressing (MCF7/MRP1-10), and GST-transduced MRP1-expressing (MRP1-10/α-13, MRP1-10/$\mu$-4, and MRP1-10/$\pi$-4) cell lines are labeled.

Previous studies from our laboratory showed that 15-d-$PGJ_2$ cytoxicity and activation of PPAR$\gamma$-dependent transcription were attenuated by expression of the glutathione conjugate efflux transporters, MRP1 (ABCC1) and MRP3 (ABCC3) (*16*). This attenuation was glutathione-dependent and was associated with the formation of the glutathione conjugate of 15-d-$PGJ_2$, 15-d-$PGJ_2$−SG, and its MRP-dependent efflux. While 15-d-$PGJ_2$−SG formation occurs nonenzymatically, here we have examined whether the human cytosolic GST can influence the cellular activity of exogenously administered 15-d-$PGJ_2$. Using MCF7 breast cancer cells stably transduced with the human GST isozymes, M1a-1a, A1-1, and P1a-1a, we demonstrate that all three inhibit 15-d-$PGJ_2$ activation of PPAR$\gamma$-dependent reporter gene transcription. Kinetic analyses using purified recombinant GST indicate that the inhibitory effects of GST do not involve catalysis of 15-d-$PGJ_2$−SG formation but rather are attributable to avid noncovalent associations between GST and 15-d-$PGJ_2$ or its glutathione conjugate.

Table 1: GST Activities in Parental (MCF7/WT) MCF7 Cells and MCF7 Cells Transduced with GST (MCF7/$\mu$, α, and $\pi$ Clones) or MRP1 and GST (MRP1-10/$\mu$, α, and $\pi$ Clones)

| cell line | GST activity (nmol/(min·mg)) |
|---|---|
| MCF7/WT | 5 |
| MCF7/$\mu$-2 | 280 |
| MCF7/$\mu$-5 | 440 |
| MCF7/$\pi$-7 | 246 |
| MCF7/$\pi$-11 | 432 |
| MCF7/α-7 | 288 |
| MCF7/α-8 | 459 |
| MRP1-10/$\mu$-4 | 239 |
| MRP1-10/$\pi$-4 | 325 |
| MRP1-10/α-13 | 262 |

## EXPERIMENTAL PROCEDURES

*Cell Lines and Culture*. All cell lines were derived from parental MCF7 cells (MCF7/WT) and were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 10 $\mu$g/mL ciprofloxacin at 37 °C, 5% $CO_2$. Transduction and characterization of MCF7 cells stably expressing transgenic MRP1 (MCF7/MRP1-10 cells) have been described previously (*16*, *25*). For stable expression of transgenic GST, cDNA encoding the human GST isozymes M1a (*26*), A1 (*27*), and P1a (*28*) were inserted into pLHCX (Clontech) and introduced by transduction into MCF7/WT and MCF7/1-10 cells as described (*25*, *29*). GST and MRP1 expression were assessed by Western blot of cytosolic and membrane protein preparations, respectively, and by Northern blot (*30*, *31*). GST levels were quantified by enzyme activity toward 1-chloro-2,4-dinitrobenzene (*32*).

Cytotoxicity was determined using the sulforhodamine B microtiter plate method (*16*, *33*). Stock solutions of 15-d-$PGJ_2$ (Cayman Chemical, Ann Arbor, MI) were stored at −20 °C in degassed 95% ethanol. The $IC_{50}$ values of 15-d-$PGJ_2$ for the cell lines were determined by interpolation of cell survival curves. Cytotoxicities were expressed as relative resistance ($IC_{50}$ control cell line ÷ $IC_{50}$ test cell line).

*Recombinant GST*. Bacterial expression vectors encoding human GST M1a, A1, and P1a were generated by polymerase chain reaction amplification of their respective cDNAs using the following oligonucleotide pairs: GSTM1a, 5′-TTTCTCGAGTAACAGGAGGAATTAACCATGCCCATGATACTGGGGTA-3′ and 5′-TTTGAATTCCTACTTGTTGCCCCAGACA-3′; GSTA1, 5′-TTTCTCGAGTAACAGGAGGAATTAACCATGGCAGAGAAGCCCAA-3′ and 5′-TTTGAATTCTTAAAACCTGAAAATCTTCCTT-3′; GSTP1a, 5′-TTTCTCGAGTAACAGGAGGAATTAACCATGCCGCCCTACACCGTGGTCTAT-3′ and 5′-TTTGAAT-

Table 2: Expression of GST Has No Effect on 15-d-PGJ$_2$ Cytotoxicity in MCF7 Cells

| cell line | GST | relative resistance[a] |
|---|---|---|
| | −MRP1 | |
| MCF7/WT | (−GST) | 1.0 |
| MCF7/$\mu$-2 | (+GSTM1a-1a) | 1.2 |
| MCF7/$\alpha$-7 | (+GSTA1-1) | 1.0 |
| MCF7/$\pi$-7 | (+GSTP1a-1a) | 1.0 |
| | +MRP1 | |
| MCF7/MRP1-10 | (−GST) | 2.0 |
| MRP1-10/$\mu$-4 | (+GSTM1a-1a) | 2.1 |
| MRP1-10/$\alpha$-13 | (+GSTA1-1) | 2.0 |
| MRP1-10/$\pi$-4 | (+GSTP1a-1a) | 2.1 |

[a] Relative resistance = $IC_{50}$/($IC_{50}$ of MCF7/WT cells). The $IC_{50}$ of MCF7/WT cells was 10 $\mu$M.

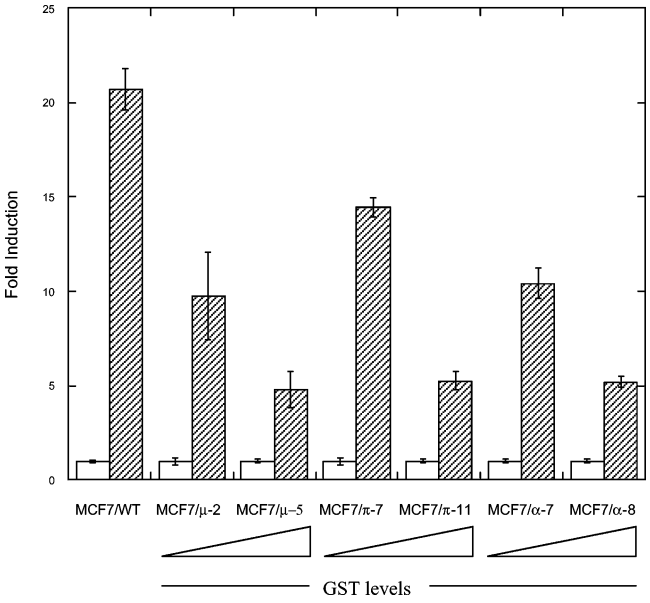


FIGURE 3: Expression of GST inhibits 15-d-PGJ$_2$ induction of PPRE reporter gene expression. The effect of GST on 15-d-PGJ$_2$ activation of PPAR$\gamma$-dependent reporter gene transcription was assessed by transient transfection of parental (MCF7/WT) or GST-transduced (GSTM1a, MCF7/$\mu$-2, MCF7/$\mu$-5; GSTP1a, MCF7/$\pi$-7, MCF7/$\pi$-11; GSTA1, MCF7/$\alpha$-7, MCF7/$\alpha$-8) cells as described in Experimental Procedures. Fold induction was defined as reporter gene expression in 15-d-PGJ$_2$-treated (20 $\mu$M) cells divided by reporter gene expression in the same cell line treated with vehicle only. Both vehicle control (open bars) and 15-d-PGJ$_2$-treated (shaded bars) transfections were done in triplicate with bars representing the mean values ± 1 sd. Triangles represent increasing levels of GST expression for each pair of isozymes expressed. The level of GST in the higher expressing member of each pair (MCF7/$\mu$-5, MCF7/$\pi$-11, MCF7/$\alpha$-8) was ~1.5 to 2-fold higher than the level in the lower expressing member (MCF7/$\mu$-2, MCF7/$\pi$-7, MCF7/$\alpha$-7) (see Table 1).

TCTCACTGTTTCCCGTTGCCATT-3′. Amplifications were accomplished with the Expand High Fidelity PCR system (Roche, Indianapolis, IN). The resulting amplified DNA fragments, containing a ribosomal binding sequence eight nucleotides upstream from the start codon, were inserted into the *Xho* I/*Eco*R I sites of pOXO4 (*34*) downstream of the T7 promoter. Competent BL21 Star bacteria (Invitrogen, Carlsbad, CA) were transformed with the GST expression vectors. Bacteria were cultured and recombinant GST expression was induced with isopropyl-$\beta$-D-thiogalactopyranoside as described (*34*). GSTs were isolated by glutathione−agarose affinity chromatography (*35*). SDS polyacrylamide gel electrophoresis analysis revealed that recombinant protein preparations were free of contaminating proteins (Coomassie staining). Protein concentrations were estimated (*36*) and specific activities determined (*32*). The specific activities of purified proteins were as follows: GSTM1a-1a, 166 $\mu$mol/(min·mg); GSTA1-1, 80 $\mu$mol/(min·mg), and GSTP1a-1a, 134 $\mu$mol/(min·mg).

*PPAR$\gamma$-Dependent Transactivation Assays*. Cells were transiently transfected with the PPAR$\gamma$-responsive reporter gene, PPREx3-TK-LUC, as described previously (*16*). Twenty-four hours later, transfected cells were treated with 20 $\mu$M 15-d-PGJ$_2$ or vehicle for 1 h. Luciferase reporter gene activity was determined 24 h after 15-d-PGJ$_2$ or vehicle treatment (*16*).

*ESI/MS Analysis of GST*. Recombinant GSTM1a-1a, A1-1, and P1a-1a (100 $\mu$g/mL) were incubated with 15-d-PGJ$_2$ or vehicle control for 3 h at 37 °C in 0.1 M potassium phosphate, pH 7.5. When the eicosanoid was included, the molar ratio of 15-d-PGJ$_2$ to GST monomer was 5:1. Incubations were washed with 10 volumes of $dH_2O$ and concentrated to 50 $\mu$L using an Apollo 7 mL high-performance centrifugal concentrator (Orbital Biosciences, Topsfield, MA). Acetonitrile and formic acid were added to final concentrations of 50% and 0.1% (v/v), respectively. Samples were stored at −20 °C and were analyzed on a Micromass Quattro II mass spectrometer (Waters, Milford, MA) equipped with a *z*-spray and a triple quadrupole analyzer. Spectra (*m*/*z* 600−1800) were obtained in positive ion mode with the following instrument settings: capillary voltage, 3.5 kV; cone voltage, 36−117 V; source temperature, 80 °C; and ion energy, 1.5 V. Protein masses were determined from raw ion profiles using the maximum entropy calculation program (Masslynx software, Waters, Milford, MA).

*Enzyme Kinetics*. The rates of 15-d-PGJ$_2$ conjugation were determined at 25 °C in 0.1 M potassium phosphate, 140 mM KCl, and 2 mM glutathione with or without GST (GSTM1a-1a, 0.26 $\mu$g/mL; GSTA1-1, 0.27 $\mu$g/mL; or GSTP1a-1a, 0.07 $\mu$g/mL). Reactions were initiated by the addition of 20 or 500 $\mu$M 15-d-PGJ$_2$ and terminated at 15, 30, 45, and 60 s by the addition of perchloric acid to 5% (v/v). Reaction mixtures were separated by analytical HPLC (*16*). Elutions were monitored spectrophotometrically at 272 nm, the isosbestic point for 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG ($\epsilon_{272}$ = 7300 $cm^{-1}M^{-1}$). 15-d-PGJ$_2$−SG was quantified by integrating areas under the elution peaks (~34 min). Actual concentrations were calculated by comparisons with the elution profiles of 15-d-PGJ$_2$ standards (elution at ~72 min).

Inhibition of GST catalysis by 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG was assessed using 1-chloro-2,4-dinitrobenzene as the variable substrate as described (*35*). Purified 15-d-PGJ$_2$−SG (*16*) or 15-d-PGJ$_2$ was preincubated at 25 °C with 0.1 M potassium phosphate (pH 6.5) with or without GST (GSTM1a-1a, 0.26 $\mu$g/mL; GSTA1-1, 0.27 $\mu$g/mL; or GSTP1a-1a, 0.07 $\mu$g/mL) for 5 min. Reactions were initiated by the addition of 2 mM glutathione and 0.25−2 mM 1-chloro-2,4-dinitrobenzene.

## RESULTS

*Cell Lines*. Parental (MCF7/WT) and MRP1-expressing (MCF7/MRP1-10) cells were stably transduced with expres-

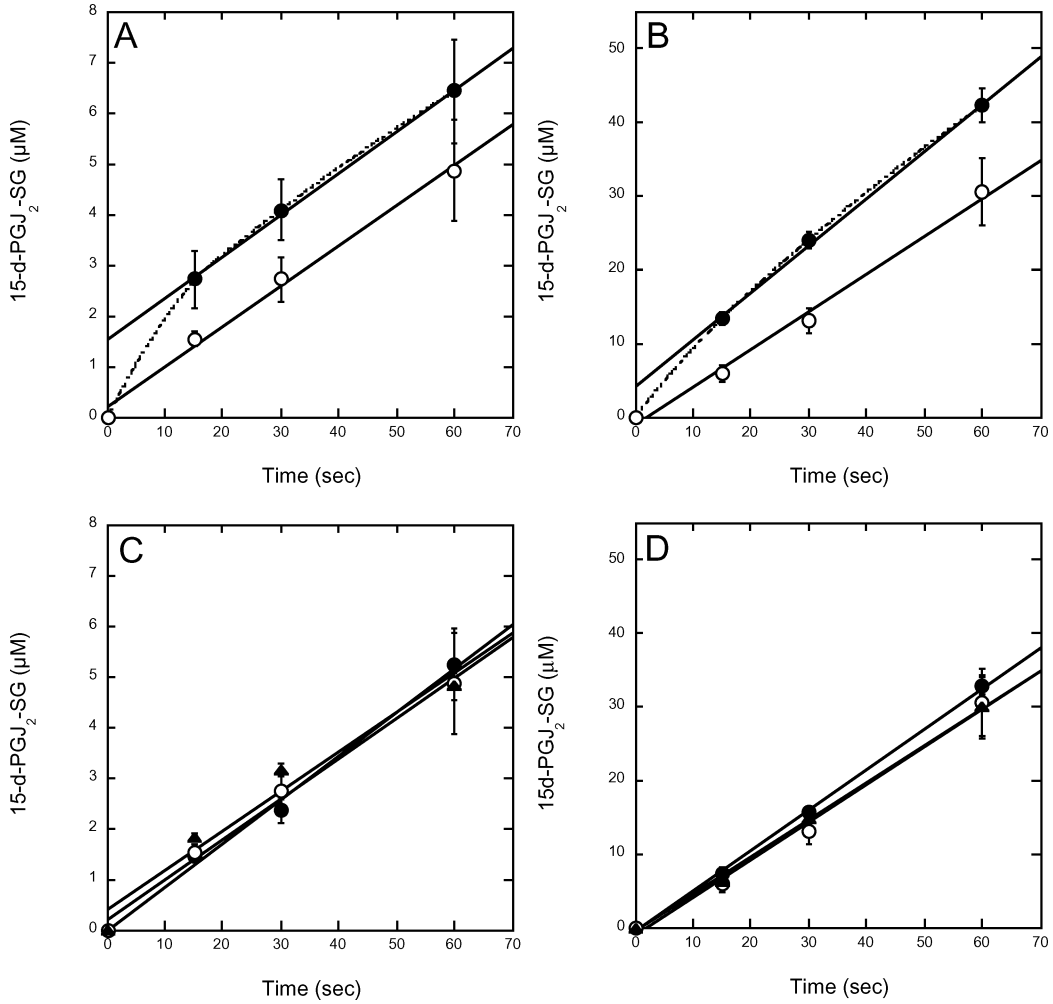


FIGURE 4: Role of GST in the rates of 15-d-$PGJ_2$−SG formation. The rates of glutathione conjugation with 15-d-$PGJ_2$ were determined using 20 $\mu$M (A and C) or 500 $\mu$M (B and D) 15-d-$PGJ_2$ and 2 mM glutathione at 25 °C, pH 6.5, as described in Experimental Procedures. Formation of 15-d-$PGJ_2$−SG is shown in the absence of GST (○, A−D) or in the presence of GSTM1a-1a (●, A and B), GSTA1-1 (●, C and D), or GSTP1a-1a (▲, C and D). Points represent the mean values of triplicate determinations ± 1 sd.

sion vectors encoding GSTM1a-1a, -A1-1, and -P1a-1a. Western blot analysis verified that the correct isoforms are expressed in the parental derivatives transduced with GSTA1 (MCF7/α-7 and -8), GSTM1a (MCF7/$\mu$-2 and -5), and GSTP1a (MCF7/$\pi$-7 and -11) (Figure 1) and in the MRP1-derivatives transduced with GSTA1 (MRP1-10/α-13), GSTM1a (MRP1-10/$\mu$-4), and GSTP1a (MRP1-10/$\pi$-4) (Figure 2B). Substantial levels of GST are expressed as evidenced by quantitative enzyme assay (Table 1). Transduced GST had no effect on MRP1 expression (Figure 2A).

*Effect of GST Isozymes on 15-d-$PGJ_2$ Cytotoxicity and PPARγ-Dependent Transcriptional Activation*. Attenuation of 15-d-$PGJ_2$ cytotoxicity and transactivation by MRP1 are associated with formation of the glutathione conjugate, 15-d-$PGJ_2$−SG, and its efflux (*16*). Hence, we determined whether GST could further influence 15-d-$PGJ_2$ activities. As shown in Table 2, expression of the three isozymes had no effect on 15-d-$PGJ_2$ cytotoxicity in MRP1-minus cell lines (compare MCF7/WT versus MCF7/$\mu$-2, MCF7/α-7, and MCF7/$\pi$-7), nor were the GSTs able to augment the 2-fold resistance to 15-d-$PGJ_2$ cytotoxicity conferred by MRP1 (compare MCF7/MRP1-10 versus MRP1-10/$\mu$-4, MRP1-10/α-13, and MRP1-10/$\pi$-4). In contrast, expression of any of the three GST isozymes significantly inhibited 15-d-$PGJ_2$ activation of PPAR-dependent transcription (Figure 3). Moreover, the degree of inhibition was correlated with the level of GST expressed.

*GST Catalysis of 15-d-$PGJ_2$−SG Formation*. To ascertain the mechanism of GST-mediated inhibition of 15-d-$PGJ_2$ transactivation, we examined the abilities of the three isozymes to increase the rate of 15-d-$PGJ_2$ conjugation with glutathione. Under the conditions used (2 mM glutathione, pH 6.5, 25 °C), the nonenzymatic rate of 15-d-$PGJ_2$−SG formation was substantial—4.8 $\mu$M/min at 20 $\mu$M 15-d-$PGJ_2$ (Figure 4A,C, open circles) and 31 $\mu$M/min at 500 $\mu$M 15-d-$PGJ_2$ (Figure 4B,D, open circles). Addition of GSTM1a-1a resulted in a transient increase in the rates of 15-d-$PGJ_2$−SG formation (Figure 4A,B, closed circles). However by 15 s, the rates of GSTM1a-1a-associated conjugate formation were nearly indistinguishable from the nonenzymatic rates. Hence, with increasing reaction times, the contribution of GSTM1a-1a to overall 15-d-$PGJ_2$−SG formation is minimal. Addition of GSTA1-1 or GSTP1a-1a had no effect on the rates of 15-d-$PGJ_2$−SG formation even at early reaction times (Figure 4C,D, closed circles and triangles, respectively). These results indicate that inhibition by GST of 15-d-$PGJ_2$ reporter gene transactivation cannot be attributed to catalysis of 15-d-$PGJ_2$−SG formation.

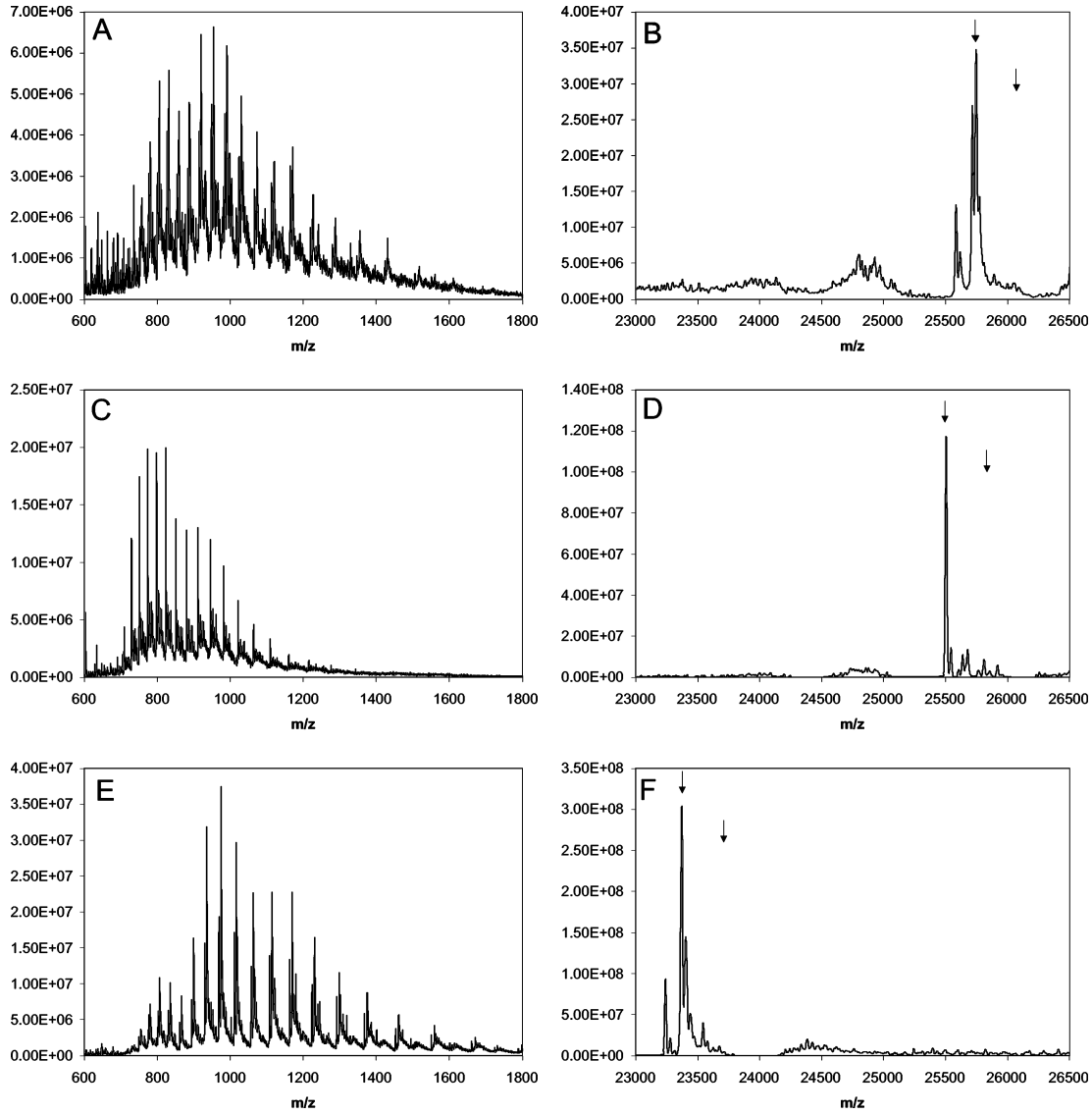


FIGURE 5: Electrospray ionization mass spectrometry analyis of GST treated with 15-d-PGJ$_2$. Recombinant GSTs were incubated 3 h with a 5-fold molar excess of 15-d-PGJ$_2$ followed by ESI/MS analysis as described in Experimental Procedures. Shown are the positive ion mode electrospray mass spectra (A, C, and E) and the true mass scale maximum entropy transformed spectra (B, D, and F) for GSTM1a-1a (A and B), GSTA1-1 (C and D), and GSTP1a-1a (E and F). The left upper arrows in each transformation represent the masses of the native, recombinant GST monomers (M1a, 25 748; A1, 25 505; and P1a-1a, 23 372), while the right lower arrows represent predicted masses of 15-d-PGJ$_2$ monoadducts of the corresponding GSTs.

*Noncovalent Interactions between GST Isozymes and 15-d-PGJ$_2$ or 15-d-PGJ$_2$−SG*. Next, we determined whether GST might inhibit transactivation by binding the ligand or its glutathione conjugate in noncatalytic interactions. Purified GSTs were incubated with a 5-fold molar excess of 15-d-PGJ$_2$ for 3 h at 37 °C in phosphate buffer (pH 7.5) and analyzed by ESI/MS to determine whether the putative GST/15-d-PGJ$_2$ interactions were covalent. Analyses of these spectra for each isozyme revealed a single dominant species corresponding to the mass of the unmodified GST (Figure 5B,D,F). These spectra were indistinguishable from those obtained in the absence of 15-d-PGJ$_2$ (not shown). We conclude that little, if any, stable 15-d-PGJ$_2$ adduct is formed with GST under the conditions used.

Enzyme kinetic analysis was used to evaluate potential noncovalent interactions between 15-d-PGJ$_2$ or its conjugate with GST. Using 1-chloro-2,4-dinitrobenzene as the variable substrate, we measured the effect of 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG on GST activity. As shown in Figure 6, both compounds were potent, concentration-dependent inhibitors of all three isozymes with $K_i$ constants in the 0.15−2.0 $\mu$M range (Table 3), results suggesting that 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG form relatively avid noncovalent associations with the cytosolic GSTM1a-1a, A1-1, and P1a-1a.

## DISCUSSION

Previous work from our laboratory showed that expression of MRP1 and MRP3 reduces 15-d-PGJ$_2$ cytotoxicity and PPRE-dependent activation of transcription (*16*). These MRP effects are associated with formation of the 15-d-PGJ$_2$ glutathione conjugate, 15-d-PGJ$_2$−SG, and its efflux by MRP. Accordingly, in the present work, we examined whether cytosolic GSTs, which catalyze glutathione conjugation, can reduce cellular activities of 15-d-PGJ$_2$ or augment MRP-mediated attenuation of 15-d-PGJ$_2$ activities. Using transgenic cell lines expressing GSTM1a-1a, -A1-1, or -P1a-1a alone or in combination with MRP1, we show that these isozymes of GST have no effect on 15-d-PGJ$_2$ cytotoxicity

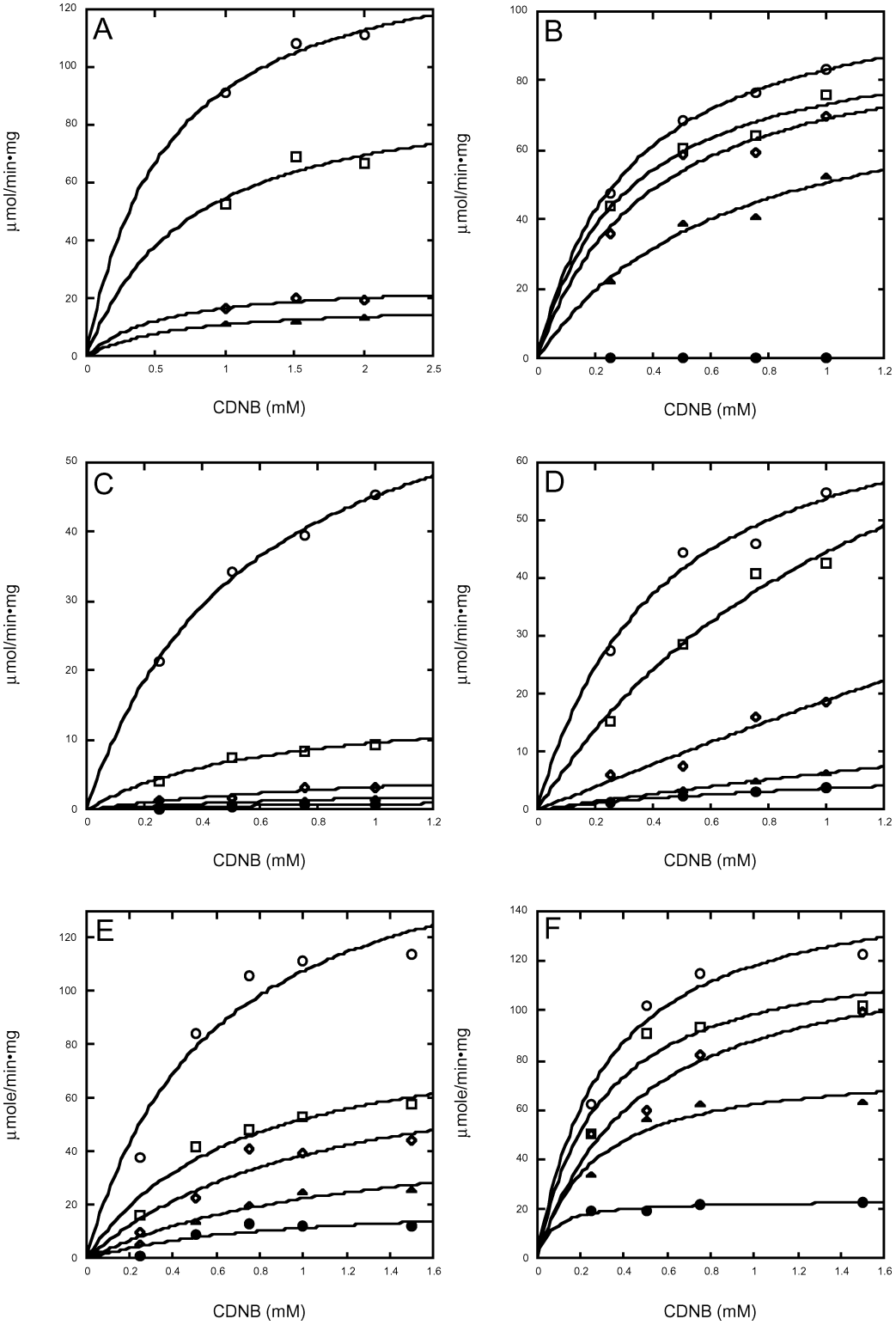


FIGURE 6: 15-d-$PGJ_2$ and its glutathione conjugate, 15-d-$PGJ_2$−SG, are potent inhibitors of GST activity. The effect of 15-d-$PGJ_2$ (A, C, and E) and 15-d-$PGJ_2$−SG (B, D, and F) on GST (M1a-1a, A and B; A1-1, C and D; P1a-1a, E and F) catalysis of 1-chloro-2,4-dintrobenzene conjugation with glutathione were examined. The amount of inhibitor (15-d-$PGJ_2$ or 15-d-$PGJ_2$−SG) added is shown in order of increasing concentration as follows: ○, no inhibitor < □ < ◇ < ▲ < ●. Concentrations of 15-d-$PGJ_2$ were (A) 0, 0.1, 0.5, and 1.0 $\mu$M; (C) 0, 1.0, 5.0, 10, and 20 $\mu$M; and (E) 0, 0.5, 1.0, 5.0, and 10 $\mu$M. Concentrations of 15-d-$PGJ_2$−SG were (B) 0, 0.05, 0.1, 0.3, and 0.6 $\mu$M; (D) 0, 0.5, 1.0, 3.0, and 6.0 $\mu$M; and (F) 0, 0.5, 1.0, 2.0, and 10 $\mu$M.

with or without MRP1 (Table 2). In contrast, all three isozymes confer significant inhibition of 15-d-$PGJ_2$-mediated, PPAR$\gamma$-dependent reporter gene transactivation. The extent of inhibition for each isozyme was correlated with the level of that isozyme expressed (Figure 3).

Surprisingly, inhibition of transactivation was not dependent upon GST catalysis of 15-d-$PGJ_2$−SG formation. Indeed, only GSTM1a-1a stimulated the rates of conjugate formation, and this increase was transient, probably due to rapid product inhibition, such that the net effect of GST on total 15-d-

Table 3: Kinetic Constants for 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG Inhibition of GST

| | $K_i$ ($\mu$M)[a] | |
|---|---|---|
| | 15-d-PGJ$_2$ | 15-d-PGJ$_2$−SG |
| GSTM1a-1a | 0.15 | 0.15 |
| GSTA1-1 | 0.18 | 0.58 |
| GSTP1a-1a | 1.9 | 2.0 |

[a] $K_i$ values were determined from secondary plots ($1/V^{app}$ or $K_m^{app}/V^{app}$ versus inhibitor concentration ($I$)) derived from data presented in Figure 6. The lowest value ($-I$ intercept) of the two plots was reported as the $K_i$ for each GST/inhibitor pair.

PGJ$_2$−SG was minimal (Figure 4). Mass spectrometry and kinetic analyses were used to determine whether GST might inhibit 15-d-PGJ$_2$ transactivation via noncatalytic binding of the ligand. ESI/MS suggested that 15-d-PGJ$_2$ does not form stable, covalent adducts with GST under the conditions examined (Figure 5). However, both 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG were potent inhibitors of the activities of all three GST toward their common substrate, 1-chloro-2,4-dinitrobenzene (Figure 6, Table 3). The $K_i$'s (Table 3) were in the 0.15−2.0 $\mu$M range indicating avid noncovalent interactions between 15-d-PGJ$_2$ or its conjugate, 15-d-PGJ$_2$−SG, and GSTM1a-1a, -A1-1, or -P1a-1a.

This result is consistent with a mechanism of GST inhibition of PPAR$\gamma$-dependent transactivation in which the ligands, 15-d-PGJ$_2$ and its glutathione conjugate, bind cytosolic GST and are thereby sequestered away from their nuclear target, PPAR$\gamma$. Inhibition of transcription via binding of these lipophilic, amphipathic ligands to GST represents a potentially new biological consequence of a long recognized property of GST—namely, the ability of GST to bind a variety of nonsubstrate ligands (*37*−*39*). The plausibility of this interpretation is addressed by examining the affinities of GST for the ligands and the levels of GST expressed in the cells. Kinetic analyses suggest that affinities of GST for 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG are in the low micro- to high nanomolar range (Table 3). Based upon the cellular GST activities (Table 1) and the specific activities of purified proteins (Experimental Procedures), the level of GST monomer expressed in transgenic cell lines is estimated between 10 and 34 $\mu$M. While we do not know the actual concentrations of 15-d-PGJ$_2$ or 15-d-PGJ$_2$−SG achieved in treated cells, these affinities and levels appear suited to accommodate binding of a significant proportion of free 15-d-PGJ$_2$ and 15-d-PGJ$_2$−SG present in the cytosol of cells treated with 20 $\mu$M 15-d-PGJ$_2$.

The significance of 15-d-PGJ$_2$−SG binding to GST deserves some discussion. While this conjugate has lost its reactive Michael acceptor site, it may retain significant biological activity. First, 15-d-PGJ$_2$−SG, if unbound, may represent a reservoir of 15-d-PGJ$_2$ that can form by retro-Michael addition. Because the purified conjugate appears quite stable (C. Paumi, unpublished), this reaction is likely a relatively minor source of intracellular 15-d-PGJ$_2$. Alternatively, and we believe more significantly, 15-d-PGJ$_2$−SG may, like its parent compound 15-d-PGJ$_2$, be an activating ligand of PPAR$\gamma$. Indeed, the ligand-binding pocket of PPAR$\gamma$ is quite large and accepts a variety of ligands (*40*). Moreover, data from our laboratory has shown that 15-d-PGJ$_2$−SG associates with the purified ligand binding domain of PPAR$\gamma$ with a $K_d \approx 0.5$ $\mu$M (unpublished observations), representing an affinity comparable to that estimated for the parent molecule, 15-d-PGJ$_2$ (*1*, *41*). Hence, binding and cytosolic sequestration of 15-d-PGJ$_2$−SG may effect quantitatively important inhibition of overall PPAR$\gamma$-dependent transactivation by 15-d-PGJ$_2$/15-d-PGJ$_2$−SG.

In these experiments, GST is selective for the inhibition of PPRE-dependent transactivation but has no measurable effect on cytotoxicity. The reasons for this selectivity are uncertain. One potential contributing explanation is that transactivation may be a more sensitive measure than cytotoxicity for discerning differences in 15-d-PGJ$_2$ effects on the various cell lines. Alternatively, the selectivity of GST for transactivation may be due to differences in the pathways of 15-d-PGJ$_2$ cytotoxicity versus PPAR$\gamma$ activation. It has been suggested that 15-d-PGJ$_2$ cytotoxicity has a large PPAR$\gamma$-independent component (*42*), a process that may involve covalent adduct formation between 15-d-PGJ$_2$ and crucial thiols of proteins associated with cell growth, signaling, survival, and apoptosis (*11*−*14*, *43*). Formation of the glutathione conjugate eliminates the reactive, adduct-forming Michael acceptor of 15-d-PGJ$_2$. 15-d-PGJ$_2$−SG is thus less reactive than the parent compound and therefore unlikely to form cytotoxic covalent adducts. Hence, the PPAR$\gamma$-independent component of cytotoxic processes competes with glutathione conjugation of 15-d-PGJ$_2$, conjugation that, for this particular cyclopentenone prostaglandin, is essentially unaffected by GSTM1a-1a, -A1-1, or -P1a-1a. However as a ligand of PPAR$\gamma$, 15-d-PGJ$_2$−SG may retain transcriptional activity. According to this view, cytosolic sequestration of the conjugate by GST would be expected to have a relatively greater influence on transactivation than on the PPAR$\gamma$-independent component of cytotoxicity. Finally, sequestration by GST may have a more general role in the modulation of activities associated with other eicosanoids including those that can also form glutathione conjugates.

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