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# Interactions of Prostaglandin $A_2$ with the Glutathione-Mediated Biotransformation System

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**ABSTRACT.** The cyclopentenone prostaglandin $A_2$ ($PGA_2$) is known to inhibit cell proliferation, and metabolism of this compound thus might be important in controlling its ultimate function. The glutathione-related metabolism of $PGA_2$ was therefore investigated both with purified glutathione *S*-transferase P1-1 (GSTP1-1) and with IGR-39 human melanoma cells. Firstly, the irreversible inhibition of human GSTP1-1 and its mutants C47S, C101S, and C47S/C101S was studied. $PGA_2$ appeared to inhibit GSTP1-1 mainly by binding to the cysteine 47 moiety of the enzyme. This binding was reversed by a molar excess of GSH, indicating that retro-Michael cleavage occurs. Secondly, after exposing IGR-39 human melanoma cells to $PGA_2$, both diastereoisomers of the $PGA_2$–glutathione conjugate are excreted into the medium, although with a clear excess of the *S*-form, due to its preferential formation by the GSTP1-1 present in the cells. Thirdly, the effect of $PGA_2$ on intracellular GST activity was determined by quantification of the excreted glutathione conjugate *S*-(2,4-dinitrophenyl)glutathione (DNPSG) after exposure to 1-chloro-2,4-dinitrobenzene. DNPSG excretion was inhibited after incubation with 10 or 20 μM $PGA_2$ for 1 or 4 hr, as a result of glutathione depletion, reversible GST inhibition, and covalent modification of intracellular GST. Furthermore, $PGA_2$ also inhibited transport of DNPSG by the multidrug resistance-associated protein, an effect that was reversible and competitive. In conclusion, $PGA_2$ modulates all three aspects of the glutathione-mediated biotransformation system, i.e. GSH levels, GSTP1-1 activity, and transport of GSH conjugates. A role for GSTP1-1 as a specific transport protein inside the cell is indicated. BIOCHEM PHARMACOL **57**;12:1383–1390, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** glutathione *S*-transferases; prostaglandin $A_2$; cells; metabolism; inhibition

Prostaglandin $A_2$, a prostaglandin containing an α,β-unsaturated ketone moiety, is an inhibitor of cell proliferation of animal and human cell lines [1–5] by arresting the cell cycle in the $G_1$ phase [4, 6]. Therefore, $PGA_2$§ has been suggested as a potential chemotherapeutic agent [1, 2, 5].

Cyclopentenone prostaglandins are transported into cells by a specific carrier system and subsequently transferred to the nucleus [7], where they accumulate [8]. Accumulation in the nucleus results in inhibition of DNA synthesis [1], induction of apoptosis [9], induction of synthesis of heat shock proteins [10, 11], and regulation of GSH levels by induction of γ-glutamylcysteine synthetase [12]. These prostaglandins are further reported to possess antiviral [13] and antitumor [14] activity. Although the molecular mechanisms are not totally clear, it has recently been shown that cyclopentenone prostaglandins inhibit nuclear factor kappa B transcription and at the same time activate heat shock transcription factor [15].

The α,β-unsaturated carbonyl moiety of prostaglandin $A_2$ seems to be required for the antiproliferative effect [3] and for the reaction with cellular proteins [16–18]. Metabolism of $PGA_2$ can thus be very important in the eventual effect of this compound. It was shown as early as 1975 that prostaglandins of the A class conjugate with GSH both chemically and when catalysed by GST [19]. Recently, Bogaards *et al.* [20] demonstrated that the various isoenzymes of GST can stereoselectively catalyse the GSH conjugation of prostaglandin $A_2$ and $J_2$, and that this stereoselective formation of the *R*- or the *S*-form of the conjugate is isoenzyme-dependent. Furthermore, when L1210 mouse leukemia cells were exposed to $PGA_2$, it was demonstrated that prostaglandin $A_2$-SG was formed and excreted into the medium [21]. On the other hand, GSH appears to play an important role in the uptake and metabolism of $PGA_2$ [8, 17], but GSH levels do not seem to be critical for the growth inhibitory effect [8].

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§ *Abbreviations:* BSO, D,L-buthionine-*S*,*R*-sulfoximine; CDNB, 1-chloro-2,4-dinitro benzene; DNPSG, *S*-(2,4-dinitro phenyl)glutathione; GST, glutathione *S*-transferase; MRP, multidrug resistance-associated protein; PG, prostaglandin; $PGA_2$-SG, prostaglandin $A_2$–glutathione conjugate; and HBSS, Hanks' balanced salt solution.

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Another component, which thus is probably a contributing factor in the metabolism of $PGA_2$, is GST activity. As previously shown, α,β-unsaturated carbonyl compounds inhibit GSTP1-1 both reversibly and by covalent modification of the cysteine residues [22, 23]. This particular isoenzyme is interesting, because it has been associated with drug resistance against alkylating anticancer agents [24].

Furthermore, Evers *et al.* [25] recently demonstrated that the glutathione conjugates of prostaglandin $A_2$ are transported by MRP.

These recent findings prompted us to further investigate the glutathione-related metabolism of prostaglandin $A_2$ with purified enzymes as well as in the IGR-39 human melanoma cell line.

## MATERIALS AND METHODS

### *Materials*

GSH and NADH were obtained from Boehringer, Mannheim. Prostaglandins $A_2$ and $J_2$ were purchased from Sigma. CDNB was obtained from Aldrich Chemie. HPLC-grade trifluoroacetic acid was obtained from Baker. HPLC-grade methanol was from Labscan. DNPSG was synthesised analogously to Sokolovsky *et al.* [26].

[$^3$H]Glycine was purchased from Amersham. GSTP1-1 was purified as previously described [27]. The three mutants of GSTP1-1, C47S, C101S, and C47S/C101S, were a generous gift from Dr. M. LoBello (Dept. of Biology, University of Rome 'Tor Vergata,' Rome, Italy) [28]. Specific activities were 40, 50, 87, and 72 units/mg for GSTP1-1, C47S, C101S, and C47S/C101S, respectively.

### *Inhibition Studies with Purified GSTP1-1 and Its Mutants C47S, C101S, and C47S/C101S*

Incubations were performed to determine time-dependent covalent inhibition of glutathione *S*-transferase P1-1 and its mutants C47S, C101S, and the double mutant C47S/C101S, as previously described [23]. Incubation mixtures (250 μL) containing 0.2 M potassium phosphate (pH 7.4), supplemented with 0.2 mM EDTA, 0.5 μM enzyme, and 10, 25, or 250 μM prostaglandin $A_2$ were incubated at 25°. Methyl acetate was used as a solvent. At various time points during a 4-hr incubation period, GST activity was measured according to Habig *et al.* [29] adapted for a Thermomax microplate reader (Molecular Devices Corp.), as earlier described [22]. Because inactivation of GSTP1-1 by other α,β-unsaturated carbonyl derivatives was reversed by incubating the modified enzyme with an excess of GSH (retro-Michael reaction) [23, 30], the reversibility of the binding of $PGA_2$ to GSTP1-1 was investigated as previously described [23].

### *Cellular Exposure and Cytotoxicity Assay*

Human melanoma cancer cells (IGR-39) were provided by the Dr. Daniel den Hoed kliniek (Rotterdam, The Netherlands). IGR-39 cells were cultured in RPMI 1640 medium (GIBCO, Life Technologies), supplemented with 10% fetal bovine serum, 50 mg/L gentamicin, at 37° in a humid atmosphere containing 5% $CO_2$. To exclude cytotoxic effects during the assay for GSH conjugation, only concentrations of prostaglandin $A_2$ giving at least 90% viable cells were used in the experiments. Cytotoxic effects were determined by lactate dehydrogenase-leakage assay [31]. Approximately $50 \times 10^4$ cells/mL were plated onto a 24-well tissue cluster Costar, and cultured as described above for a maximum period of 24 hr. Cells were exposed for 1.5 or 4 hr in quadruplicate in Hanks' balanced salt solution (HBSS without phenol red and $NaHCO_3$, obtained from GIBCO), supplemented with $NaHCO_3$ to a final concentration of 0.35 g/L. Concentrations used were 2, 5, 10, and 20 μM $PGA_2$, dissolved in methyl acetate (final concentration in the medium always ≤0.5%). Control incubations containing only the solvent were included. No cytotoxicity was detected with the concentrations used.

### *Cellular $PGA_2$-SG Formation*

The formation and transport of the prostaglandin $A_2$–glutathione conjugates (*R*- and *S*-forms of $PGA_2$-SG) was studied by loading IGR-39 human melanoma cells, containing primarily GSTP1-1 and probably a minor amount of GSTM1a-1a [22], with [$^3$H]glycine, which is subsequently incorporated into intracellular GSH.

In brief, cells were plated onto 25-cm$^2$ flasks at a density of $50 \times 10^4$ cells/mL and incubated at 37° for a maximum of 24 hr in 5 mL medium. The cells were loaded for 3 hr with 45 μCi [$^3$H]glycine (14.8 Ci/mmol) in supplemented medium. After 3 hr, the medium was removed and the cells were washed three times with HBSS (w/o phenol red). Thereafter, cells were exposed to 10 or 20 μM $PGA_2$ in HBSS. At time intervals of 1 hr, 1 mL of HBSS was sampled, immediately frozen on dry ice, and stored at −20° until further analysis of the amount of excreted $^3$H-labeled glutathione conjugates. After 4 hr, cells were washed three times with HBSS and were harvested in cold PBS. Cells were frozen and stored at −20° until further analysis. The effect of GSH depletion on the formation of the two $PGA_2$-SG diastereoisomers was studied by incubating the cells overnight with 50 μM BSO, and subsequent exposures to [$^3$H]glycine and $PGA_2$ were conducted in the presence of 50 μM BSO as well.

The $PGA_2$-SG conjugates were separated and quantitated by HPLC analysis as previously described [20]. In short, 300 μL medium was injected on a Zorbax reversed phase $C_{18}$ column (250 × 4.6 mm), eluted with a flow rate of 1 mL/min, with 50 mM aqueous ammonium acetate (pH 3.4)/acetonitrile (75:25, v/v) isocratically for 30 min, followed by a linear gradient from 25 to 50% acetonitrile in 30 min. Radioactivity was detected using on-line radiochemical detection (Canberra Packard A500). A 0.5-mL liquid flow cell was used. As scintillation cocktail, Flo Scint A (Packard Instruments) was used with a flow rate of 2 mL/min. The conjugates were identified by comparison of the retention times with those of the synthesized reference compounds.

### *GST Inhibition Studies in IGR-39 Human Melanoma Cells*

The inhibition of GST activity towards the substrate CDNB in IGR-39 human melanoma cells was determined as previously described [22]. Briefly, cells were plated onto 6-well tissue clusters, and exposed for 1 hr to 10 or 20 μM $PGA_2$ in HBSS (w/o phenol red). Control incubations containing only the solvent were included. After 1- or 4-hr exposure, CDNB was added in HBSS to a final concentration of 10 μM and mixed; then, 0.2 mL aliquots were taken at four time points (1, 5, 10, and 20 min) and mixed with 5 μL 0.04M *N*-acetyl-L-cysteine. Samples were immediately frozen at −20° until further analysis. After 20 min, cells were harvested in PBS and immediately frozen. The medium samples and the intracellular contents were analyzed on HPLC to determine DNPSG formation. Intracellular GSH concentration [32], GST activity [29], and protein content [33] were determined as previously described [22].

The effect of GSH depletion on the inhibition of GST activity in IGR-39 human melanoma cells was investigated by incubating cells overnight with 50 μM BSO, after which the exposure to $PGA_2$ was performed, also in the presence of 50 μM BSO.

### *Statistical Methods*

Levels of significance were tested by one-way ANOVA ($P < 0.05$), and comparisons between groups were made using Tukey's test ($P < 0.05$).

## RESULTS

### *Inhibition Studies with Purified GSTP1-1*

Time-dependent inhibition of GSTP1-1 and the mutants C47S, C101S, and C47S/C101S by prostaglandin $A_2$ was studied. In Fig. 1A, the percentage of remaining GST activity after incubating 10, 25, or 250 μM of the prostaglandin $A_2$ with GSTP1-1 is presented. Remaining activity after 3 hr was 74%, 56%, and 34%, respectively. Incubating these concentrations with the C47S mutant still resulted in a decrease in GST activity, albeit less evident than with the parent enzyme and only at the highest concentration used (250 μM). Remaining activities were 90%, 85%, and 80%. When the C101S mutant was used in the incubations, the rate of inactivation was somewhat faster, but the remaining activity after 3 hr was similar to that in incubations with GSTP1-1. When both cysteine residues were mutated into a serine, no inhibition at all could be observed. A typical representation of the inhibition of the enzyme activity of GSTP1-1 and the various mutants after exposure to 250 μM $PGA_2$ is presented in Fig. 1B.

In order to confirm if $PGA_2$ reacts with cysteine residues of GSTP1-1, the modified GSTP1-1 was incubated with 2.5 mM GSH to distinguish whether a retro-Michael cleavage occurred. The catalytic activity of GSTP1-1 towards

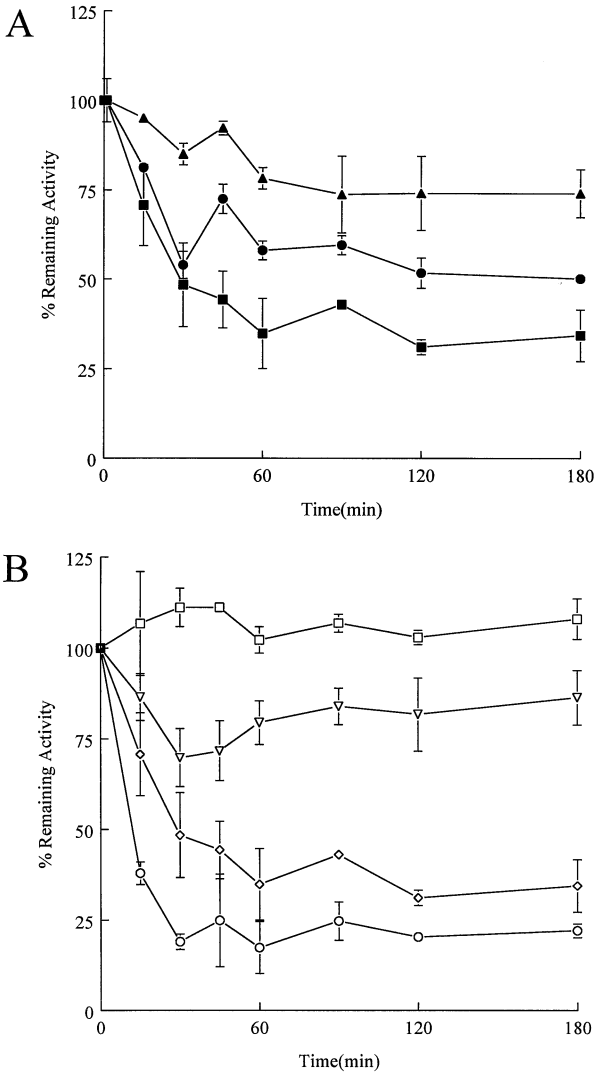


**FIG. 1. (A) Inhibition of GSTP1-1 presented as % of control by various concentrations of prostaglandin $A_2$. Symbols used are: ▲, 10 μM; ●, 25 μM; and ■, 250 μM. All points are mean values ± SE calculated from six determinations. (B) Inhibition of GSTP1-1 and its mutants by 250 μM prostaglandin $A_2$. The activity of GSTP1-1 (◇), the C47S mutant (▽), the C101S mutant (○), or the double mutant C47S/C101S (□) was monitored for 3 hr of incubation with 250 μM $PGA_2$. All points are mean values ± SE calculated from six determinations.**

CDNB was restored to almost 100% after 24 hr for both 25 and 250 μM $PGA_2$ (data not shown).

### *Cellular $PGA_2$-SG Formation*

After IGR-39 human melanoma cells were loaded with [$^3$H]glycine, exposure to 10 or 20 μM prostaglandin $A_2$ resulted in excretion of both diastereoisomers of the $PGA_2$-SG conjugates for at least 4 hr. In Fig. 2A, a typical chromatogram of medium of human IGR-39 melanoma cells exposed to 20 μM $PGA_2$ for 4 hr is depicted, showing that the two diastereoisomers of $PGA_2$-SG are the main metabolites present in the medium. Figure 2B shows the amount of excreted $PGA_2$-SG diastereoisomers in the

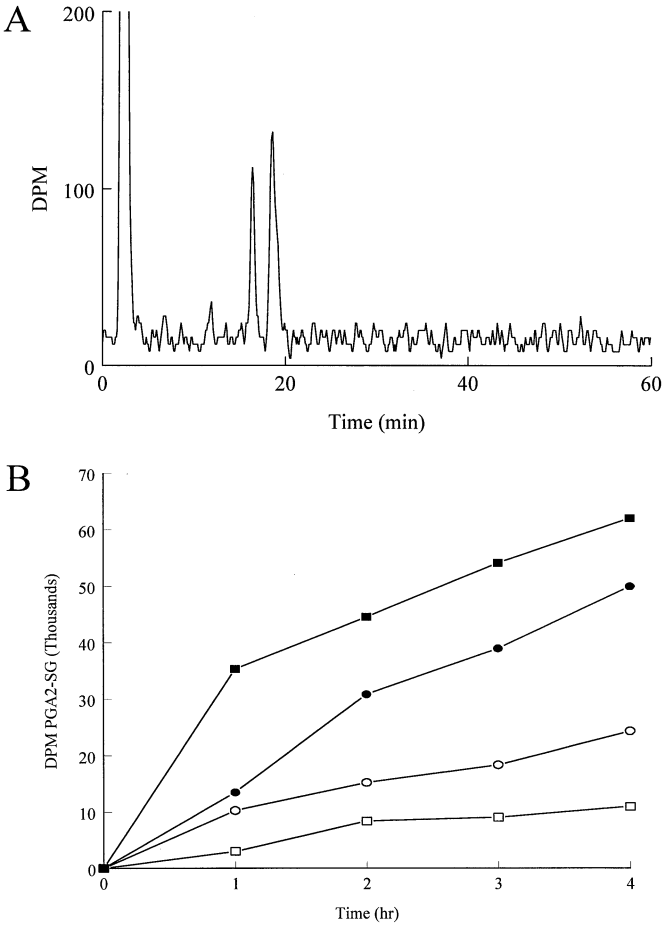


**FIG. 2. IGR-39 human melanoma cells were loaded with [$^3$H]glycine after which 10 or 20 μM PGA was added and the amount of prostaglandin $A_2$-glutathione conjugate was measured in the medium. (A) A typical HPLC chromatogram of medium of human IGR-39 melanoma cells exposed to 20 μM $PGA_2$ for 4 hr. Peaks are: t = 2.5 min: [$^3$H]glycine; t = 16.5 min: *R*-$PGA_2$-SG; and peak = 18.7 min: *S*-$PGA_2$-SG. (B) Excretion of the *S* and *R* forms of $PGA_2$-SG, presented as amount of dpm present in the medium for 4 hr. One out of two experiments is shown. Symbols used are: ■, *S*-$PGA_2$-SG after exposure to 10 μM; □, *R*-$PGA_2$-SG after exposure to 10 μM; ●, *S*-$PGA_2$-SG after exposure to 20 μM; ○, *R*-$PGA_2$-SG after exposure to 20 μM.**

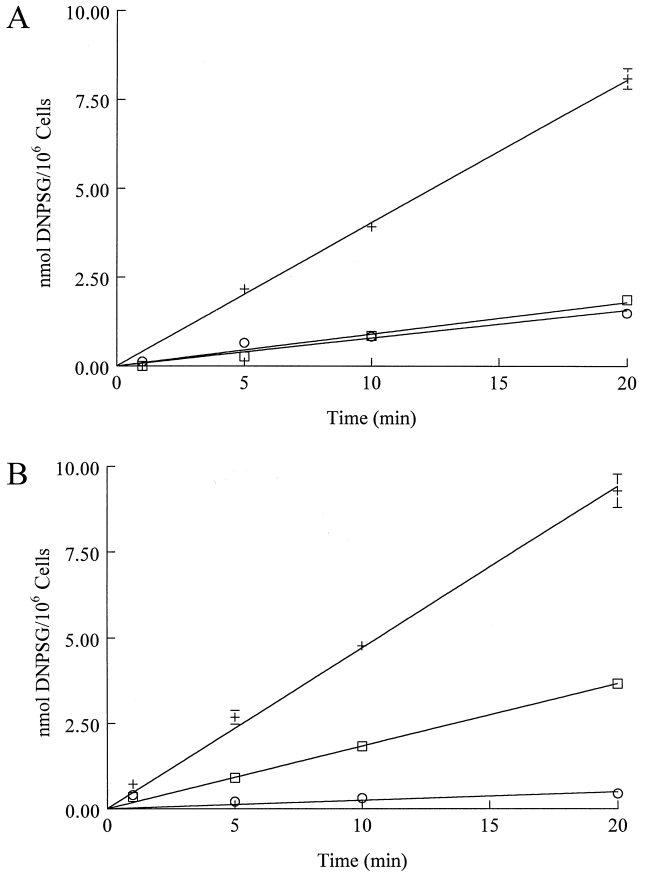


**FIG. 3. The DNPSG excretion of IGR-39 human melanoma cells after exposure to prostaglandin $A_2$. Cells were exposed to 10 or 20 μM $PGA_2$ for 1 (A) and 4 hr (B) respectively, and subsequently to 10 μM CDNB for 20 min. GSH conjugation was determined by the excretion of DNPSG into the medium. Incubations were performed in triplicate. Values are presented as nmol per 10$^6$ cells. Symbols used are: +, control; □, 10 μM; and ○, 20 μM. Results from one out of two experiments are shown.**

medium presented in dpm. Exposure to $PGA_2$ clearly resulted in a higher amount of the *S*-form in medium during these 4 hr. The *S*:*R* ratios after 4 hr were 5.7 and 2.0 for exposure to 10 and 20 μM $PGA_2$ respectively, but the total amount of excreted $PGA_2$-SG was similar for the two concentrations. Intracellularly, no conjugates could be detected. Overnight exposure of the cells to 50 μM BSO before incubation with $PGA_2$ resulted in no detectable $PGA_2$-SG conjugate in the medium.

### *GST Inhibition in IGR-39 Human Melanoma Cells*

The model substrate for GST activity, CDNB, was used to determine the effect of $PGA_2$ on GST activity in IGR-39 human melanoma cells. To determine the effect of prostaglandin $A_2$ on cellular GST activity, cells were exposed to 10 or 20 μM $PGA_2$ for 1 or 4 hr.

The time-dependent DNPSG excretion and the effect of 1- and 4-hr exposure to $PGA_2$ are presented in Fig. 3, A and B, respectively. As the excretion of DNPSG was in general linear over a time-span of 20 min, linear regression ($y = ax$) was used to describe the time-dependent DNPSG excretion. The correlation coefficient ($r^2$) for the regression was in general greater than 0.95. A 1-hr exposure to 10 or 20 μM $PGA_2$ thus resulted in a decrease in DNPSG excretion into the medium. However, the effect at 20 μM was almost equal to that at 10 μM. Exposure to the same concentrations for 4 hr resulted in a dose-dependent decrease in DNPSG excretion.

As expected, overnight preincubation with 50 μM BSO resulted in no detectable DNPSG excretion into the medium after either 1- or 4-hr exposure to 10 or 20 μM $PGA_2$.

At the end of the total experimental exposure (1 or 4 hr

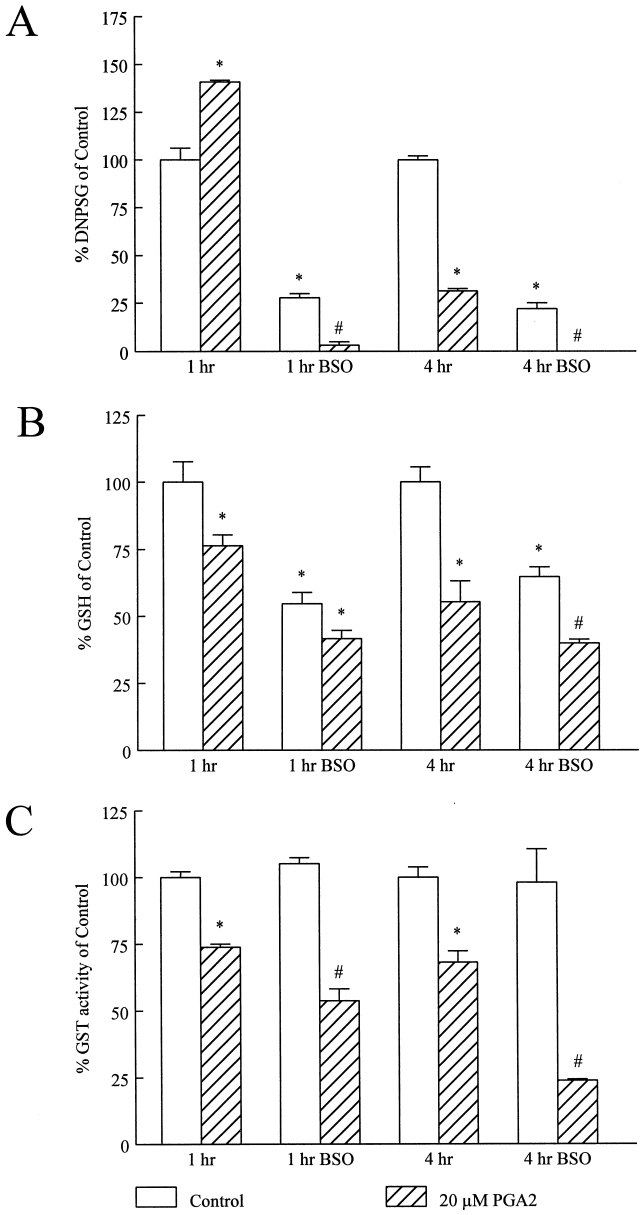


**FIG. 4. The effect of 20 μM prostaglandin $A_2$ on intracellular DNPSG (A), GSH levels (B), and GST activity (C) in IGR-39 human melanoma cells. Cells were incubated overnight with 50 μM BSO or in medium and subsequently exposed to 20 μM $PGA_2$ for 1 or 4 hr (with 50 μM BSO or in medium), after which CDNB was added for 20 min. The remaining intracellular DNPSG, GSH levels, and GST activity were determined and expressed as % of control incubations. Incubations were performed in triplicate. *, significantly different from control incubations ($P < 0.05$); #, significantly different both from control incubations and from control incubations with 50 μM BSO ($P < 0.05$).**

plus 20 minutes), intracellular concentration of DNPSG was measured in the supernatant. As is shown in Fig. 4A, intracellular DNPSG after a 1-hr exposure to prostaglandin $A_2$ was significantly higher compared to control values. After a 4-hr of exposure to 10 or 20 μM $PGA_2$, intracellular DNPSG was decreased dose dependently to 52% and 20%, respectively.

Overnight preincubation of the IGR-39 human melanoma cells with 50 μM BSO resulted in a reduction of intracellular DNPSG to 30% for a 1-hr control exposure and to about 20% for a 4-hr control exposure. After subsequent exposure to $PGA_2$, almost no intracellular DNPSG could be detected.

In Fig. 4, B and C, the remaining intracellular GSH levels and GST activity after the total exposure period in the supernatant are presented as the percentage of values obtained from control exposures. After 1-hr exposure to 10 or 20 μM $PGA_2$, GSH levels were reduced to around 80%, as was the GST activity. After 4 hr of $PGA_2$ exposure, GSH levels were halved for both concentrations, and GST activity decreased to 68% remaining activity after exposure to 20 μM. No significant decrease in GST activity was detected after 4-hr exposure to 10 μM $PGA_2$.

When cells were incubated overnight with 50 μM BSO, GSH levels dropped to about 50%, but the GST activity was not influenced. Subsequent exposure to 10 or 20 μM $PGA_2$ for 1 hr did not further deplete GSH levels, while after 4 hr GSH levels were slightly decreased to 41% of control levels. Overnight preincubation with 50 μM BSO reduced GST activity to 74% and 54% of control values after 1 hr, and to 43% and 23% after exposure for 4 hr to 10 or 20 μM $PGA_2$, respectively.

## DISCUSSION

Prostaglandin $A_2$ is known to inhibit cell proliferation [1–5], and numerous studies have been done on the metabolic fate of this prostaglandin. Recent findings concerning the formation and transport of the diastereoisomeric glutathione conjugates shed further light on this metabolism [20, 25]. The present study aimed to obtain a more complete picture of the glutathione-related metabolism of $PGA_2$ by investigating interactions with purified enzymes as well as in a cellular system using the IGR-39 human melanoma cell line.

To determine whether $PGA_2$ can inhibit GSTP1-1 by covalent modification of the cysteine residues, $PGA_2$ was incubated with purified GSTP1-1 and three mutant enzymes missing one or both cysteine residues. It was demonstrated that $PGA_2$ inhibits GST activity by binding to the cysteine moieties of the enzyme. After mutation of the cysteine 47 residue of GSTP1-1, the enzyme could still be inhibited by $PGA_2$, but only at the highest concentration (250 μM) used and to a minor extent compared to native GSTP1-1. When only cysteine 101 was mutated, the extent of inactivation reached was similar to that of native GSTP1-1. When both cysteine 47 and 101 were mutated into a serine, no inhibition of GST activity could be detected. Again, these findings show that cysteine 47 is the principal target for modification by an α,β-unsaturated ketone, as we have previously shown for a number of other α,β-unsaturated carbonyl compounds [23, 34].

Whether $PGA_2$ acts as substrate or inhibitor of GST probably depends on the intracellular GSH concentration

[34]; thus, both processes were studied in IGR-39 human melanoma cells. First, the formation of $PGA_2$-SG conjugates was studied. In these cells, GSTP1-1 is the major GST expressed, but there is also minor amount of GST belonging to another subfamily present, probably GSTM1a-1a [22]. After these cells were loaded with [$^3$H]glycine and exposed to $PGA_2$, the $PGA_2$-SG conjugate excreted into the medium consisted for the most part of the *S*-diastereoisomer.

As Evers *et al.* [25] showed that transport of the two $PGA_2$-SG diastereoisomers by the MRP/GS-X pump is not stereoselective, it can be assumed that the higher amount of the *S*-form in the medium after exposure of IGR-39 human melanoma cells to $PGA_2$ is the result of the preferential formation of this form by the GST present in the cells. GSTP1-1 is stereoselective for the formation of the *S*-form of $PGA_2$-SG, whereas GSTM1a-1a showed no stereoselectivity [20], our results thus being in line with these findings. However, although the total amount of excreted $PGA_2$-SG was similar for both concentrations investigated, the *S*:*R* ratio was lower after exposure to 20 μM than after exposure to 10 μM. This might be the result of an increased contribution of the chemical reaction. Since GST activity was inhibited covalently from 80 to 68% after exposure to 20 μM $PGA_2$ for 1–4 hr, it is plausible that the chemical reaction becomes more significant. Another possibility is that, due to covalent interaction of $PGA_2$ with the cysteine 47 residue, the affinity of the enzyme for GSH decreases dramatically as well as changing the conformation of the active site. This results in a reduced ability to catalyze the reaction and possibly affects the stereoselectivity as well [35]. On the other hand, degradation of the *S*-$PGA_2$-SG might occur extracellularly or intracellularly, or the *S*-form might be the preferential conjugate for further intracellular metabolism or transport to the nucleus.

Parker and Ankel [21] ascribed for formation of only one 9-OH-$PGA_2$-SG diastereoisomer in L1210 mouse leukemia cells to enzymatic reduction of the other diastereoisomer. However, in view of the recent findings of Bogaards *et al.* [20] and the results of the present study, the stereoselective formation of one diastereoisomer by GST can also be an explanation. However, it should be emphasized that other metabolic pathways for $PGA_2$-SG might also play a role.

As the total formation of GSH conjugates did not increase after exposure to 20 μM compared to exposure to 10 μM, the uptake of $PGA_2$ might be a limiting factor. The uptake of $PGA_2$ is mediated by a specific carrier [7], and this carrier might have a $V_{max}$ which is already reached at 10 μM $PGA_2$. Furthermore, $PGA_2$ can be processed in different routes besides the glutathione-mediated biotransformation system, which might be dose-dependent.

The effect of $PGA_2$ on GST activity and on transmembrane transport was studied using a method that we had developed to determine GSH-related metabolism in intact cells by measuring DNPSG excretion [22]. In the present study, $PGA_2$ was demonstrated to inhibit DNPSG excretion after as little as 1-hr exposure to a concentration of 10 μM. However, no clear dose–response relationship in excretion could be detected, as we showed for most other α,β-unsaturated carbonyl compounds [22]. Intracellular DNPSG, which was measured in supernatant after the total exposure period, was increased upon 1-hr exposure to 10 or 20 μM $PGA_2$, and again no dose–response relationship could be detected. It appears that only 10% of the total amount of conjugate was excreted after exposure to $PGA_2$ compared to 40% in control incubations. This observation and the fact that total DNPSG formation was not decreased after 1-hr incubation with $PGA_2$ indicates that DNPSG transport out of the cell is inhibited.

After 4-hr exposure to $PGA_2$, the inhibition of DNPSG excretion showed a dose–response effect and total DNPSG formation was significantly decreased: a drop in intracellular as well as extracellular DNPSG concentration was observed. Although 4-hr exposure to $PGA_2$ evidently inhibits DNPSG formation, inhibition of transport still seems to play a role as well; after exposure to 20 μM $PGA_2$, only 12% of the total amount of conjugate formed was excreted, compared to 38% and 32% after exposure to control medium and 10 μM $PGA_2$, respectively. Recently, it has been demonstrated that transport of [$^{14}$C]ethacrynic acid–glutathione conjugate into microsomal vesicles of yeast cells transformed with *MRP1* cDNA dose dependently is inhibited by $PGA_1$ and $PGA_2$ in the presence of GSH. To investigate whether a glutathione conjugate was involved, both diastereoisomers of $PGA_2$-SG were synthesized and appeared to be good substrates for MRP and probably two other transporters as well [25]. Although it is not clear which transport pumps are present and functional in IGR-39 human melanoma cells, our results indicate that the maximum inhibition of the transport pumps is already reached at the lowest concentration used (10 μM). Assuming that the MRP/GS-X pump plays a role, exposure to this concentration probably results in an intracellular concentration above the $K_m$ value for this pump (ca. 1 μM) [25]. The percentages of DNPSG excretion of the total amount of DNPSG formed after 4-hr exposure indicate that the inhibition of DNPSG transport by $PGA_2$ is reversible, as after 10 μM exposure almost no inhibition is observed.

According to our previous findings [22], inhibition of DNPSG formation can be the result of the depletion of glutathione, reversible inhibition, and/or covalent modification of glutathione *S*-transferases. Therefore, both GSH concentration and GST activity were measured after the total exposure period in supernatant. GSH was depleted to 80% and 50% of control values after 1 and 4 hr, respectively. The loss of GSH notably differs from values obtained after exposure of L1210 cells to similar concentrations of $PGA_2$; in that study, however, and in addition to the use of a different cell line, a different assay was performed both for exposure and glutathione determination [21]. GST activity was reduced to 80% (10 μM) and 74% (20 μM) after 1-hr exposure and to 68% at 20 μM after 4 hr. Although depletion of GSH decreases spontaneous GSH conjugation, it is not very likely to affect GST activity, as the $K_m$ value

of GSH for GST is low [36, 37] compared to the normal intracellular GSH concentration [38]. GST activity is probably inhibited by $PGA_2$ both by reversible inhibition and covalent modification. $PGA_2$ then covalently modifies GSTP1-1, not only as purified enzyme, but in cells as well. We previously showed this to be true for ethacrynic acid and curcumin [22]. Further evidence for this phenomenon is obtained when IGR-39 human melanoma cells are exposed to $PGA_2$ after depletion of GSH to 50% of control values by overnight incubation with BSO. These conditions resulted in a decrease in GST activity to 50% after 1-hr exposure to 20 μM, and dropping to 23% after 4 hr. Apparently, GST is covalently modified more effectively when GSH levels are below a certain protecting level.

In L-1210 cells, GSH depletion to about 17% of control values resulted in a 50% reduction in $PGA_2$ uptake [8], causing a drop in cytosolic $PGA_2$ but not in nuclear amounts of $PGA_2$ [17]. Our results demonstrate that very small amounts of $PGA_2$ can already covalently inhibit GST activity, especially when GSH is depleted. This indicates that GSTP1-1 is at least one of the cellular proteins that binds $PGA_2$, as suggested by Ohno and co-workers [17]. This again suggests that GSTP1-1 might play a role in scavenging alkylating agents, especially when GSH concentrations are low, or conversely might serve as 'storing protein' for physiologically important compounds such as $PGA_2$.

The mechanisms that play a role in the inhibition of cell proliferation by cyclopentenone prostaglandins are still not clear. Binding to cellular proteins seems to be related to inhibition of cell proliferation [18] as is the induction of heat shock proteins [10, 11]. Furthermore, c-*myc* expression is inhibited by $PGA_2$, which results in an inhibition of cell proliferation [39]. $PGA_2$ has apoptosis-inducing properties, and this also might be a way in which the compound influences cell proliferation [9].

Clearly, $PGA_2$ is transported by a cytosolic protein to the nucleus where it is most likely bound or transferred to sulfhydryl-containing proteins and/or transcription factors, which can regulate cell proliferation and other cellular functions [17, 40]. This study indicates that GSTP1-1 is a potential candidate for a transport protein.

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