Biochemical Pharmacology, Vol. 43, No. 5, pp. 1053–1060, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

FORMATION OF A PROSTAGLANDIN A_2-GLUTATHIONE CONJUGATE IN L1210 MOUSE LEUKEMIA CELLS

JUDY PARKER* and HELMUT ANKEL
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226, U.S.A.

(*Received* 2 *July* 1991; *accepted* 31 *October* 1991)

Abstract—Prostaglandins containing a cyclopentenone moiety are potent antiviral and antigrowth compounds. Some evidence indicates that these prostaglandins are conjugated to glutathione by cells. However, the metabolism of one group, the prostaglandins of the A type, is unclear due to conflicting reports. We studied the uptake and metabolism of prostaglandin A_2 (PGA_2) in mouse L1210 leukemia and L929 fibroblast cell lines in which this prostaglandin has antiviral and antigrowth effects. Both cell types took up the PGA_2 and then metabolized it to a more polar compound. Inside L1210 cells, PGA_2 was initially conjugated to glutathione and then reduced at the 9-keto position to form 9-OH-PGA_2-GSH. The 9-OH-PGA_2-GSH was then secreted from the cells and apparently degraded to form the CysGly and Cys derivatives. Intracellular glutathione was decreased markedly by the addition of the PGA_2 in L1210 and L929 cells. This result confirms that conjugation of PGA_2 to glutathione occurs in both cell types. Formation of the 9-OH-PGA_2-GSH and other glutathione-related conjugates was prevented when glutathione was depleted by growth in buthionine sulfoximine. The glutathione-depleted cells were insensitive to the cytotoxicity of the PGA_2, suggesting that one of the glutathione-related conjugates may be involved in the cytotoxicity of PGA_2. These results end the controversy over the metabolism of PGA_2 and suggest mechanisms for its antiviral and antigrowth actions.

Prostaglandins (PGs†) containing a cyclopentenone moiety (PGAs, PGJs and the related clavulones and punaglandins) are inhibitors of viral replication and cell growth at concentrations below the point at which they become cytotoxic. Thus, these compounds have potential as therapeutic agents and some may have a physiological antiviral and/or antitumor function. Prostaglandins of the A and J series have been found to inhibit the replication of Sendai [1], vesicular stomatitis [2], vaccinia [3], herpes simplex II [4], and encephalomyelocarditis [5] viruses. This group of viruses includes enveloped viruses with both single-stranded (−)RNA genomes and double-stranded DNA genomes, as well as a single-stranded (+)RNA virus lacking an envelope. In addition, the growth of several tumor cell lines is inhibited by prostaglandins, clavulones and punaglandins. The cell lines showing this response include L1210 leukemia [6] and B16 melanoma [7–9] from mouse, and HL60 promyelocytic leukemia [10], K562 chronic myelogenous leukemia [11], and melanomas [12] of human origin. Thus, it is clear that both the antiviral and the antigrowth effects of these prostaglandins are quite broad in scope. It is thought that the cyclopentenone moiety is required for both antiviral [4] and antigrowth [10, 13] actions of the prostaglandins. Yet, the mechanism(s) of the antiviral and antigrowth actions remains unknown.

* Corresponding author: Dr. Judy Parker, Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. Tel. (414) 257-8482; FAX (414) 257-2008.

† Abbreviations: PG, prostaglandin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MTT, 3(4,5-dimethyl-thiazoyl-2-yl)2,5-diphenyltetrazolium bromide; and PBS, phosphate-buffered saline.

To understand the biology and pharmacology of cyclopentenone prostaglandins, it is important to determine the metabolism of these compounds. Recently, it was reported that the cyclopentenone prostaglandin Δ^{12}-PGJ_2 is conjugated to glutathione in Chinese hamster ovary and rat hepatoma cells [14]. After conjugation, further metabolism led to reduction at the 11-keto and Δ^{12-13} moieties. This was the first evidence that cells other than red blood cells [15–18] and liver [19] also metabolize a cyclopentenone prostaglandin by conjugation to glutathione. The antigrowth effect of PGA_1 has been reported to be reversible while that of $\Delta^{12}PGJ_2$ is irreversible [20, 21]. Thus, it is unclear if metabolism of PGAs is different from that of $^{12}PGJ_2$. Furthermore, there are conflicting reports of the metabolism of PGA_1. Free PGA_1 was reported to accumulate inside L1210 leukemia cells and to bind to nuclear proteins [20, 22]. However, in red blood cells no free PGA_1 could be observed and a glutathione conjugate was formed inside the cells which was in part further reduced at the 9-keto position [16–18, 23]. We studied the metabolism of PGA_2 in L1210 and L929 cells in order to resolve these conficting reports of PGA metabolism.

METHODS

Chemicals. Roswell Memorial Park Institute (RPMI) medium 1640, minimal essential medium with Hanks' salts (MEM), horse serum, and fetal bovine serum were obtained from the Grand Island Biological Co. Ethyl acetate, benzene, acetonitrile, acetic acid, hydrochloric acid and C_{18} Sep Paks were from Fisher Scientific. Streptomycin sulfate was from Calbiochem, Triton X-100 from BioRad, and Biosafe II scintillation fluid from Research Products

International. Silica gel 60 thin-layer chromatography plates were from Merck (No. 5721). Tissue culture flasks, 96-well plates, and 12-well plates were from CoStar. Penicillin G, PGA_2, PGE_2, *N*-[2-hydroxyethyl]piperazine-*N'* -[2-ethanesulfonic acid] (HEPES), glutathione, cysteine, buthionine sulfoximine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 5-sulfosalicylic acid, and 3(4,5-dimethyl-thiazoyl-2-yl)2,5-diphenyltetrazolium bromide (MTT) were from the Sigma Chemical Co. PGA_2 was also purchased from Cayman Chemicals. The dipeptide CysGly was purchased from BaChem.

Cell lines and cell culture. Mouse L1210 leukemia cells (ATCC CCL219) were routinely grown in RPMI-1640 supplemented with 10% horse serum in glass bottles or in plastic flasks. Cells were subcultured three times per week and seeded at 0.2×10^6/mL. Mouse L929 fibroblast cells (ATCC CCL1) were grown in MEM supplemented with 10% fetal bovine serum and subcultured by standard techniques three times per week. All culture medium included penicillin and streptomycin.

Preparation of [^{3}H]prostaglandin A_2 and high performance liquid chromatography of prostaglandins. [5,6,8,11,12,14,15-^{3}H(N)]Prostaglandin E_2 (200 Ci/mmol), obtained from New England Nuclear was mixed with 0.5 mL of 0.01 N HCl (pH 2.05). The tube was filled with N_2, sealed, and heated at 70° for 15–18 hr. The sample was then extracted three times with 1 mL of ethyl acetate and the combined organic phase washed with 1 mL of H_2O. The organic phase was evaporated by a stream of N_2 and the residue was taken up in the high performance liquid chromatography (HPLC) column buffer (see below), The sample was next injected onto a Beckman Ultrasphere C_{18} (2 × 150 mm) column and isocratically eluted at 0.2 mL/min in 40% acetonitrile, 0.16% benzene, 0.24% acetic acid. The fractions containing [5,6,8,12,14,15-^{3}H(N)]-PGA_2 were pooled, extracted three times with ethyl acetate, and dried under a stream of N_2; the residue was dissolved in 100% ethanol. HPLC of ethyl acetate extracts from experiments with cells was as described above except that a Beckman Ultrasphere C_{18} column (4.6 × 150 mm) was used at a flow rate of 1 mL/min.

Uptake and metabolism of prostaglandin A_2. L1210 cells (2×10^6) were mixed with 10 μM PGA_2 or PGE_2 (containing 15,000–100,000 dpm of corresponding [^{3}H]prostaglandin) in 0.2 mL of RPMI-1640, 25 mM HEPES, (pH 7.4). Samples were heated at 37° in a water bath for the indicated times. Then 0.62 mL of cold phosphate-buffered saline (PBS) was added to the tubes and the cells were pelleted at 500 *g* for 3 min. The supernatant was transferred to a fresh tube, and the cells were washed with 0.3 mL PBS and then pelleted again. The cells were resuspended in 0.92 mL PBS, 0.2 mL RPMI-1640, 25 mM HEPES, and the cell suspension and the combined supernatants were acidified to pH 3.8 with 0.1 N HCl. Each sample was extracted three times with 1 mL of ethyl acetate, and aliquots of the residual aqueous phase and the combined ethyl acetate extracts were counted to determine the distribution of the PGA_2 or PGE_2. The volume of the L1210 cells was estimated using the equation $V = 4/3\pi r^3$, since these are spherical cells with an approximate diameter of 10 μm. Experiments with L929 cells followed a similar procedure except that the medium was MEM plus 25 mM HEPES and the cells were scraped off the plates.

Analysis of aqueous phase radioactivity. Aqueous phase samples were immediately loaded onto individual C_{18} Sep Pak columns and the columns washed in order with 10 mL of 50 mM ammonium acetate (pH 3), 10 mL H_2O, 10 mL heptane, and 10 mL ethanol (100%). The radioactivity in the ethanol eluate was concentrated by evaporation under a stream of N_2 and then injected into a Beckman Ultrasphere C_{18} (4.6 × 150 mm) HPLC column. The column was eluted with 25% acetonitrile in 50 mM ammonium acetate (pH 3.4) for 30 min. Then a linear gradient to 35% acetonitrile in 50 mM ammonium acetate was applied, followed by further elution with the latter mobile phase. The flow rate was 1 mL/min, and fractions of 1 mL were collected, mixed with scintillation fluid and counted for ^{3}H. The 9-OH-PGA_2-GSH was prepared essentially as described for $\Delta^{12}PGJ_2$-GSH [19] except that PGA_2 replaced the $\Delta^{12}PGJ_2$ and after 1 hr at 37° 12.5 mM sodium borohydride was added for an additional hour. Thin-layer chromatography was on silica gel 60 plates in *n*-butanol:acetic acid:H_2O (40:10:5) in which synthetic PGA_2-GSH (unreduced) had an $R_f = 0.28$.

In vitro *formation of PGA_2 polar metabolites.* Approximately 10^8 L929 cells were washed in phosphate-buffered saline and homogenized by 70 strokes of a Duall homogenizer in 2 mL of 1 mM EDTA, 0.25 M sucrose, 100 mM sodium phosphate (pH 7.0). The homogenate was centrifuged at 9000 *g* for 10 min at 4°. Reaction mixtures were 0.3 mL and contained 1 mM EDTA, 100 mM sodium phosphate (pH 7.0), 83 mM sucrose, plus 100 μM PGA_2 containing 13,500 dpm of [^{3}H]PGA_2. Glutathione (250 μM) and 0.1 mL of homogenate supernatant were present in some tubes as indicated. Tubes were shaken at 22° and at various times 50 μL aliquots were removed and mixed with 1 mL of 1% formic acid to lower the pH to 2.0. The samples were extracted three times with ethyl acetate and 0.5 mL of the residual aqueous phase was counted for ^{3}H by scintillation counting.

Assay of PGA_2 cytotoxicity. L929 cells (5×10^4/well) were seeded into a 96-well plate in MEM, 10% fetal bovine serum with and without 50 μM buthionine sulfoximine and incubated for 17 hr at 37°. The medium was then removed and plates were washed three times with phosphate-buffered saline. Then 0.1 mL of MEM plus 25 mM HEPES was added with and without 100 μM PGA_2 in triplicate. Buthionine sulfoximine (50 μM) was added to those wells which had been grown previously with buthionine sulfoximine and the cells were incubated for 6 hr at 37°. Then 20 μL/well of MTT (5 mg/mL in phosphate-buffered saline) was added on top of the medium for 1 hr at 37°. The medium was removed, 100 μL of 0.04 N HCl in isopropanol was added, and the dissolved, reduced MTT was determined by absorbance at 570 nm in a Biotek reader.

Effect of PGA_2 on cellular glutathione. L1210

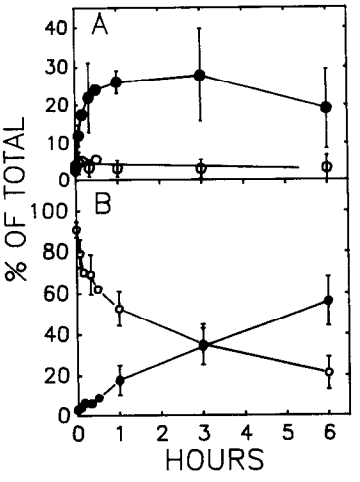

Fig. 1. Uptake and metabolism of PGA_2 in L1210 cells. L1210 cells (2×10^6) were incubated at 37° in 0.2 mL of RPMI 1640, 25 mM HEPES (pH 7.4) with 10 μM PGA_2 (containing 15,000–100,000 dpm of [^{3}H]PGA_2). At various times the cells were separated from the medium and each sample was extracted with ethyl acetate at pH 4 as in Methods. Radioactivity in the samples is presented as percent of total for each time point. Values are means ± SD of five experiments except at 10 and 30 min where the values are the average of two experiments only. Key: (A) cells: (○—○) ethyl acetate phase, and (●—●) aqueous phase; (B) medium: (○—○) ethyl acetate phase, and (●—●) aqueous phase.

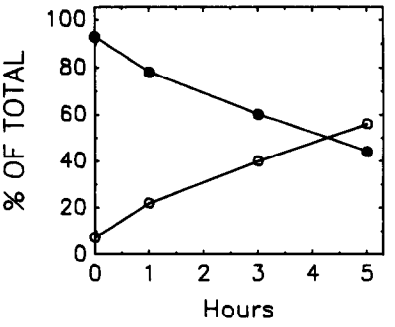

Fig. 2. Formation of polar metabolites of PGA_2 in L929 cells. L929 cells (approx. 10^6/well) in a 12-well plate were exposed to 50 μM PGA_2 (containing 18,000 dpm of [^{3}H]-PGA_2) in 0.3 mL of MEM, 25 mM HEPES pH 7.4. At each time shown, the cells and medium were extracted together at pH 4 with ethyl acetate as in Methods. Radioactivity partitioning into each phase is shown as percent of total. Each point is the average of two duplicate wells. Key: (○—○) aqueous phase, and (●—●) organic phase.

cells (0.5×10^6/mL) were mixed with various concentrations of PGA_2 or PGE_2 in RPMI-1640, 25 mM HEPES. Three experiments had a total of 2 mL cells/sample and one experiment had 10 mL/sample. After 1 hr at 37° the cells were pelleted at 500 *g* for 3 min, resuspended in 1 mL of PBS, and transferred to an Eppendorf tube. The cells were pelleted again and then dissolved in 30 μL of 1% Triton X-100. Each sample then received 38 μL of 10% 5-sulfosalicylic acid and 50 μL H_2O. Precipitated protein was removed by centrifugation at 10,000 *g* for 10 min. Aliquots of the supernatant were then assayed for glutathione.

We measured total glutathione (> 95% GSH) in the supernatants using the DTNB/glutathione reductase procedure first described by Owens and Belcher [24]. We followed the modification of Anderson [25], however; total sample volume was 0.215 mL and we read the absorbance of the samples every minute at 405 nm in a Biotek 96-well plate reader.

RESULTS AND DISCUSSION

We found that the L1210 cells took up PGA_2 and immediately metabolized it to a more polar compound(s) in experiments where radioactive PGA_2 was used to trace the total PGA_2 (Fig. 1). Formation of the polar compound(s) was detected by the fact that it partitioned into aqueous solution rather than ethyl acetate at pH 3–4, whereas PGAs were extracted into ethyl acetate under these conditions. The polar metabolite(s) appeared in the medium after it was observed in the cells. The L929 cells also metabolized PGA_2 to a polar compound (Fig. 2). PGA_2 (as organic soluble radioactivity) was present in the cells at about 1 μM. Under identical conditions PGE_2 remained 98% or more in the medium after 6 hr, although some breakdown of the PGE_2 into more polar compounds did occur (not shown). Clearly, PGE_2 does not become cell associated in contrast to the accumulation of a PGA_2 metabolite inside the cells. This suggests that the cyclopentenone moiety (which appears when PGE_2 is dehydrated to PGA_2) is required for formation of the polar metabolites which are found in the cells.

High performance liquid chromatography (HPLC) was used to identify the metabolites of PGA_2 in both the medium and the cells. The radioactive material which could be extracted into ethyl acetate from the medium was analyzed by HPLC. This material was all PGA_2 initially and although the PGA_2 decreased over time (as the cells took it up) no nonpolar metabolites that extract into ethyl acetate appeared (not shown). The radioactivity which extracts into the aqueous phase was found both in cells and in the medium. The predominant polar metabolite of PGA_2 from inside the cells eluted at 18 min with minor peaks at 12, 16 and 22 min (Fig. 3A). In the samples from the medium, the major peak eluted at 17 min with minor peaks at 11, 20, 22 and 24 min (Fig. 3B). Synthetic PGA_2-GSH reduced at the 9-keto position (9-OH-PGA_2-GSH) eluted as a major peak at 18 min and a minor peak at 16 min (not shown). As we used sodium borohydride to prepare the 9-OH-PGA_2-GSH standard, two stereoisomers could result, explaining the presence of two peaks in the standard. Unreduced PGA_2-GSH eluted at 21 min and PGA_2 eluted at 75 min under these conditions. The predominant polar metabolite in both the cells and the medium thus appeared to be 9-OH-PGA_2-GSH. Only one stereoisomer of 9-OH-

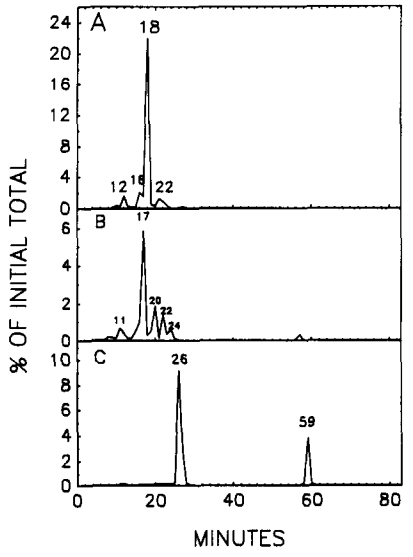

Fig. 3. HPLC analysis of PGA_2 metabolites extracted into aqueous phase. L1210 cells were incubated with PGA_2 as in Fig. 1 except that 20 μM PGA_2 containing 200,000 dpm of [^{3}H]PGA_2 was used. Glutathione was depleted in one sample by growing the cells in 1 mM buthionine sulfoximine 24 hr before and during the PGA_2 exposure. Radioactivity extracted into the aqueous phase was desalted and analyzed on an Ultrasphere C_{18} column as described in Methods. Elution was begun with 25% acetonitrile in 50 mM ammonium acetate (pH 3.4) for 30 min; then a linear gradient over 10 min to 35% acetonitrile in the ammonium acetate was applied, followed by elution with the latter. Fractions (1 mL) were collected and ^{3}H was determined by scintillation counting. Results are shown as percent of initial total radioactivity at the start of the experiment. (A) Control cell-associated material. (B) Control medium. (C) Medium from glutathione-depleted cells.

Table 1. Amino acid analysis

	Ratio of amino acid to glutamic acid		
	Glutamic acid	Glycine	Cysteic acid
Synthetic PGA_2-GSH	1.0	0.88	0.18
Major cell product	1.0	1.15	0.24

Synthetic PGA_2-GSH and the aqueous phase of cell samples were purified by C_{18} Sep Pak and then thin-layer chromatography as described in Methods. The samples were eluted with 50:50 ethanol:H_2O from the section of the plate comigrating with the PGA_2-GSH standard. Samples were oxidized with performic acid (to convert cysteine to cysteic acid) and hydrolyzed in 6 N HCl.

PGA_2-GSH was found as would be expected to result from enzymatic reduction. A small amount of unreduced PGA_2-GSH was also detected in the cell samples (22 min).

It is likely that the peaks at 20 and 24 min were breakdown products of the 9-OH-PGA_2-GSH which were generated by the action of γ-glutamyl transpeptidase and peptidases after the major cellular metabolite was secreted. These enzymes do, in fact, degrade other GSH conjugates [26]. We have detected such products (PGA_2-Cys and PGA_2-CysGly) in the medium by thin-layer chromatography which does not separate the reduced and unreduced compounds. Atsmon *et al.* [14] have also observed this degradation of $\Delta^{12}PGJ_2$-GSH in Chinese hamster ovary and rat hepatoma cells. Another report also found PGA_1 and 9-OH-PGA_1 conjugated to cysteine and CysGly after 72 hr in media from B104 neuroblastoma and C6 glioma cells [27].

We examined metabolism of PGA_2 in L1210 cells that were depleted of intracellular glutathione to < 2% of the control level by growth in the presence of buthionine sulfoximine. Only 5% of the total radioactivity became cell associated after 3 hr in the glutathione-depleted cells compared to 34% cell-associated radioactivity in control cells. The polar metabolites from the glutathione-depleted cells had the following distribution (as percent of total): 59 min, 1%; 12 min, 0.3%; PGA_2-GSH (reduced and unreduced), 0.5%; other, 1% (not shown). The GSH-depleted cells also contained about 2% organic soluble material which was presumably PGA_2. Thus, these cells contained approximately 2% of the amount of PGA_2-GSH found in the control cells. This is in good agreement with the depletion of GSH to about 2% of the control cell amount and it is clear that loss of glutathione blocked production of the PGA_2-GSH.

Polar metabolites were also found in the medium of the glutathione-depleted cells. When analyzed by HPLC there were peaks at 26 and 59 min, but none of the peaks seen in medium from control cells were present (Fig. 3C). The peak at 59 min sometimes appeared when PGA_2 and glutathione were combined in the test tube and therefore was probably a result of nonenzymatic breakdown of the PGA_2 and did not contain glutathione. The peak at 26 min comigrated with an unreduced PGA_2-Cys standard. This unreduced PGA_2-Cys probably resulted from direct conjugation of cysteine to PGA_2 since it cannot be a breakdown product of the 9-OH-PGA_2-GSH.

We also found that the major metabolite from the cells comigrated with synthetic PGA_2-GSH in thin-layer chromatography (which cannot separate the reduced and unreduced versions of this compound [16]). An amino acid analysis of the predominant polar metabolite from the cells (isolated by thin-layer chromatography) found glutamic acid and glycine in equimolar amounts; however cysteine (as cysteic acid) was present in lower amounts (Table 1). Synthetic PGA_2-GSH analyzed under the same conditions gave nearly identical results. The low yield of cysteine was probably due to incomplete performic acid oxidation during sample preparation for amino acid analysis. If the PGA_2 molecule is covalently bound via the thiol of the Cys residue, then the thiol would be resistant to performic acid oxidation. Only the cysteine released from the PGA_2 during the performic acid treatment would be

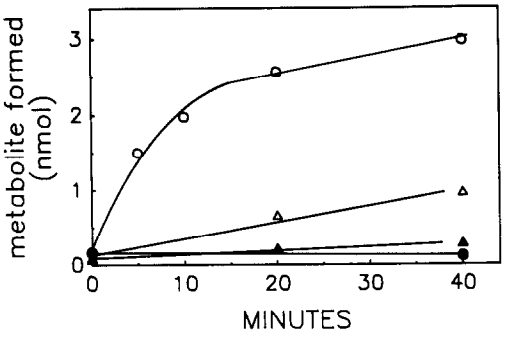

Fig. 4. Formation of polar metabolites of PGA_2 in L929 cell-free extracts. A cell-free extract prepared from L929 cells was mixed with 0.25 mM glutathione and 0.1 mM PGA_2 containing 13,500 dpm of [^{3}H]PGA_2 as described in Methods. At times shown aliquots were removed, acidified to pH 2, and extracted three times with ethyl acetate. The radioactivity in the aqueous phase was determined by scintillation counting and indicates the amount of polar metabolite(s) produced. Key: (○—○) extract + glutathione + PGA_2; (△—△) glutathione + PGA_2; (▲—▲) extract + PGA_2; and (●—●) PGA_2 alone.

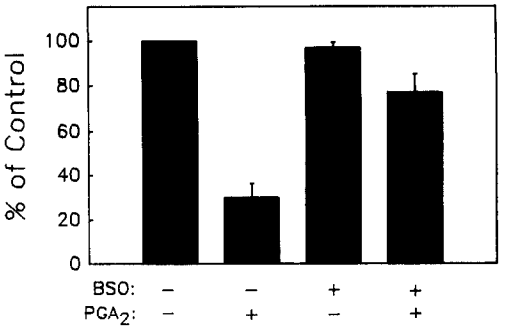

Fig. 5. Prevention of PGA_2 cytotoxicity by depletion of glutathione. L929 cells in 96-well plates were grown for 17 hr with and without 50 μM buthionine sulfoximine. The medium was removed and the monolayers were washed three times with phosphate-buffered saline. Then MEM, 25 mM HEPES with and without 100 μM PGA_2 was added and buthionine sulfoximine readded to the appropriate wells. After 6 hr at 37°, MTT was added and incubation continued for another hour. Then the amount of reduced MTT (indicating the cells with functional mitochondria) was determined by absorbance at 570 nm. Results (mean ± SD of four experiments) are the absorbance at 570 nm shown as percent of control (A_{570nm} = 0.39 ± 0.04) with no buthionine sulfoximine or PGA_2.

oxidized. This could explain the small amount of cysteic acid that was found.

The nucleophilic thiol group can react spontaneously with α,β-unsaturated carbonyl compounds such as the cyclopentenone moiety on PGA_2 provided the concentrations of each reactant are sufficient [28]. However, we found that extracts from L929 cells catalyzed the formation of the polar metabolite from PGA_2 (Fig. 4). Glutathione *S*-transferases can catalyze conjugation of PGA_1 with glutathione *in vitro* [29]. Thus it is probable that inside the cells this ubiquitous family of enzymes is involved in the formation of the PGA_2-GSH since the concentration of the PGA_2 is never very high inside the cell. Indeed, Atsmon *et al.* [14] also found that cell extracts catalyze the formation of $\Delta^{12}PGJ_2$-GSH. Cagen *et al.* [16] showed that the thiol is indeed covalently linked to C-11 of PGA_1 in the 9-OH-PGA_1-GSH produced by red blood cells. Our 9-OH-PGA_2-GSH standard had the GSH linked at the C-11 position, since it was produced by spontaneous reaction followed by reduction with sodium borohydride. It is therefore likely that the cell product we observed was also linked at C-11 to glutathione, since a different linkage would affect the HPLC retention time.

We investigated whether PGA_2 would become more toxic when glutathione was not present. Intracellular glutathione in L929 cells was depleted to < 2% of control levels by growth in the presence of buthionine sulfoximine. Surprisingly, loss of glutathione protected the L929 cells from the toxicity of PGA_2 (Fig. 5). The results of the MTT spectrophotometric assay were additionally supported by the fact that the cell monolayer was destroyed by 100 μM PGA_2 but this did not occur in cells depleted of glutathione by buthionine sulfoximine. Since the PGA_2-GSH conjugate and derivatives are not made in glutathione-depleted cells, these data suggest that perhaps one of these conjugates is responsible for the toxicity of PGA_2.

We wanted to know whether the conjugation of the PGA_2 to glutathione affected the content of glutathione in the L1210 and L929 cells. The formation of total polar metabolites was not saturated as the prostaglandin A_2 concentration was increased in L1210 cells (Fig. 6A). In parallel to the formation of polar metabolite, the glutathione content of the cells was reduced (Fig. 6B). The decrease in glutathione was specific for prostaglandin A_2 since ethanol (as used for the highest prostaglandin addition) and prostaglandin E_2 were unable to decrease the glutathione content. The content of glutathione in L929 cells was similarly reduced by PGA_2.*

Glutathione content is variable for different cell types and fluids but usually in the range of 0.1 to 10 mM. The L1210 cells as we grow them had approximately 1 nmol glutathione/10^6 cells or 1 mM. After a 1 hr exposure to 30 μM PGA_2, 2.5 nmol of polar metabolite was made by 2×10^6 cells (Fig. 6A). This value is close to the 1.8 nmol predicted to be lost in 1 hr from 2×10^6 cells (90% decrease at 25 μM PGA_2, Fig. 6B). The slight discrepancy may be due to the fact that glutathione controls its own synthesis. When intracellular glutathione is low, γ-glutamylcysteine synthetase is released from feedback inhibition by glutathione and more glutathione is consequently produced [30]. Thus, it is likely that additional glutathione is synthesized in the cells exposed to the prostaglandin and more metabolite can be made than would be predicted from the basal level of glutathione. It has been

* Parker J and Ankel H, manuscript in preparation.

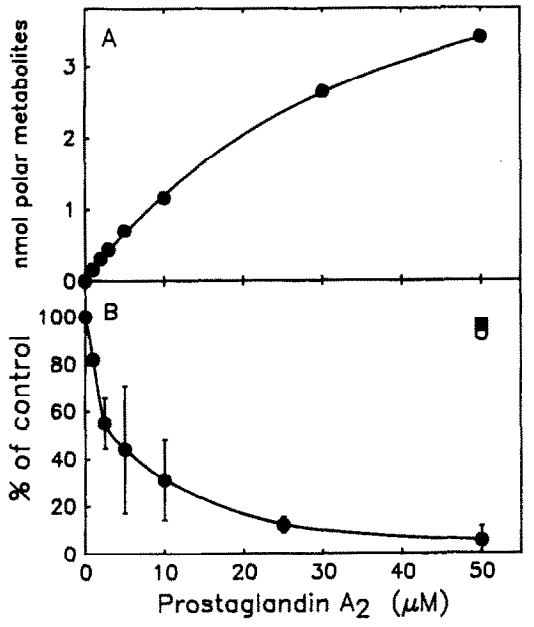

Fig. 6. Effect of PGA_2 concentration on PGA_2-GSH formation and glutathione content in L1210 cells. (A) L1210 cells were mixed with different concentrations of PGA_2 for 1 hr as in Fig. 2. Each sample contained 13,000 dpm of [^{3}H]PGA_2. The combined cells and medium were acidified and extracted with ethyl acetate to determine the total amount of polar radioactivity in each sample. Results are presented as nanomoles polar metabolites per sample. (B) L1210 cells were mixed with different concentrations of PGA_2 or PGE_2 in RPMI 1640, 25 mM HEPES (pH 7.4) for 1 hr. Then the glutathione content of the cells was determined as in Methods. Results presented as percent of control are the means ± SD of four experiments except that 1 μM was tested only once. Glutathione content of control cells was 350 ± 140 pmol/10^6 cells for the four experiments. Key: (●—●) PGA_2, (○) PGE_2, and (■) ethanol (0.125%).

reported that PGA_2 induces an increase in glutathione in the L1210 cells [31]. This increase was due to enhanced transcription of the γ-glutamylcysteine synthetase and was not observed until after 6 hr of PGA_2 treatment. The authors suggested it might be nuclear binding of PGA_2 that leads to enhanced transcription of this gene. We found that at times earlier than 6 hr PGA_2 led to a loss of glutathione in the same L1210 cells. It is possible that it is the loss of glutathione that stimulates transcription of the γ-glutamylcysteine synthetase gene.

We now understand how PGA_2 is metabolized in two cell lines that exhibit antiviral and antigrowth effects of the cyclopentenone prostaglandins. This knowledge has major implications for the mechanism(s) of the antiviral and and antigrowth actions of these prostaglandins. We found 1 μM intact PGA_2 and accumulation of 9-OH-PGA_2-GSH to nearly 200 μM inside the L1210 cells. Our results thus indicate that the metabolism of PGA_2 in L1210 and L929 cells is similar to that of the red blood cell [16, 18] and contradict a previous report of intact PGA_1 accumulating to 200 μM inside L1210 cells [22]. Thus, if intact PGA_2 is indeed the active compound for either of these effects, then it must act either at 1 μM inside the cells or from outside the cells. The alternative is that the reaction of cyclopentenone prostaglandins with glutathione is directly involved in the antiviral and/or antigrowth effects.

We have shown that neither the PGA_2-GSH and its derivatives nor the decrease in glutathione content caused by PGA_2 was responsible for the antiviral action of PGA_2.* Yet it is possible that one of the conjugates is responsible for the antigrowth effect of PGA_2 since the toxicity of PGA_2 at 6 hr was dependent upon the presence of glutathione. Nonetheless, it is not clear if the acute toxicity we measured at 6 hr is the same mechanism that accounts for the antigrowth effect of PGA_2. Gouin *et al.* [32] reported previously that a PGA_1-CysGly conjugate has antigrowth effects on B104 neuroblastoma and C6 glioma cells. However, others found that glutathione depletion enhances the antigrowth effect of $\Delta^{12}PGJ_2$ [14]. Thus, one report supports and one contradicts the hypothesis that one of the conjugates is responsible for the antigrowth effect. In the latter work diethyl maleate was combined with buthionine sulfoximine to deplete glutathione, and it is possible that the addition of the diethyl maleate had some non-specific effects on the cells.

Lastly, the depletion of intracellular glutathione by these prostaglandins may be a critical step in the antiviral or antigrowth action. It has already been shown that reduction of intracellular glutathione with buthionine sulfoximine blocks the progression of mitogen-stimulated peripheral blood mononuclear cells into S phase [33] and inhibits the growth of several other cell types [34–36]. It is interesting that the cyclopentenone prostaglandins also block progression through the cell cycle of HeLa cells [21, 37] and melanoma cells [38] in late G_1. The fact that depletion of glutathione enhances the antigrowth effect of $\Delta^{12}PGJ_2$ [14] could be interpreted as support for the hypothesis that loss of glutathione is the antigrowth mechanism (i.e. combination of two mechanisms to deplete glutathione). Yet, as discussed above, γ-glutamylcysteine synthetase is induced by long exposure to PGA_2 [31]. If this is a general effect of cyclopentenone prostaglandins, then cells should be able to eventually return glutathione to normal levels in the presence of the prostaglandin as long as substrates for glutathione synthesis are not exhausted. Thus, although we have some intriguing prospects, more analysis is needed before the antigrowth or antiviral mechanism of the cyclopentenone prostaglandins will be understood.

It is difficult to predict if cyclopentenone prostaglandins have a physiological role as antiviral and antigrowth agents. PGA_2 has been observed in several tissues and fluids [39] but it has been argued that this is entirely due to artifact from the acid extraction used to isolate the prosta-glandins [40]. Indeed, acidic pH [41] as well as albumin [42–44] accelerates the dehydration of PGE to PGA and PGD to PGJ. It seems probable that PGA and PGJ do occur in certain tissues and fluids which are

* Parker J and Ankel H, manuscript in preparation.

naturally acidic (such as stomach, duodenum, skin, vagina, and urine) provided that the precursors PGE and PGD are present. It is not clear if enough cyclopentenone prostaglandins would be present in these tissues to have any natural protective effects. However the pharmacological potential of these compounds certainly is great.

Acknowledgements—We thank Dr. Liane Mende-Mueller of the Protein and Nucleic Acid Facility of the Cancer Center of the Medical College of Wisconsin for the amino acid analysis and Frank Laib for the mass spectroscopy. This work was supported by grants from the National Institutes of Health (AI 23450) and from the Cancer Center of the Medical College of Wisconsin.

REFERENCES

1. Santoro MG, Amici C, Elia G, Benedetto A and Garaci E, Inhibition of virus protein glycosylation as the mechanism of the antiviral action of prostaglandin A in Sendai virus-infected cells. *J Gen Virol* **70**: 789–800, 1989.
2. Santoro MG, Jaffe BM and Esteban M, Prostaglandin A inhibits the replication of vesicular stomatitis virus: effect on virus glycoprotein. *J Gen Virol* **64**: 2797–2801, 1983.
3. Benavente J, Esteban M, Jaffe BM and Santoro MG, Selective inhibition of viral gene expression as the mechanism of the antiviral action of PGA_1 in vaccinia virus-infected cells. *J Gen Virol* **65** 599–608, 1984.
4. Yamamoto N, Fukushima M, Tsurumi T, Maeno K and Nishiyama Y, Mechanism of inhibition of herpes simplex virus replication by Δ^7-prostaglandin A_1 and Δ^{12}-prostaglandin J_2. *Biochem Biophys Res Commun* **146**: 1425–1431, 1987.
5. Ankel H, Mittnacht S and Jacobsen H, Antiviral activity of prostaglandin A on encephalomyocarditis virus-infected cells: A unique effect unrelated to interferon. *J Gen Virol* **66**: 2355–2364, 1985.
6. Fukushima M, Kato T, Ota K, Arai Y, Narumiya S and Hayaishi O, 9-Deoxyl-Δ_9-prostaglandin D_2, a prostaglandin D_2 derivative with potent antineoplastic and weak smooth muscle-contracting activities. *Biochem Biophys Res Commun* **109**: 626–633, 1982.
7. Honn KV, Dunn JR II, Morgan JR, Bienkowski M and Marnett LJ, Inhibition of DNA synthesis in Harding–Passey melanoma cells by prostaglandins A_1 and A_2: Comparison with chemotherapeutic agents. *Biochem Biophys Res Commun* **87**: 795–801, 1979.
8. Honn KV, Romine M and Skoff A, Prostaglandin analogs as inhibitors of tumor cell DNA synthesis. *Proc Soc Exp Biol Med* **166**: 562–567, 1981.
9. Bregman MD, Sander D and Meyskens FL Jr, Prostaglandin A_1 and E_1 inhibit the plating efficiency and proliferation of murine melanoma cells (Cloudman S-91) in soft agar. *Biochem Biophys Res Commun* **104**: 1080–1086, 1982.
10. Honda A, Mori Y, Iguchi K and Yamada Y, Antiproliferative and cytotoxic effects of newly discovered halogenated coral prostanoids from the Japanese stolonifer *Clavularia viridis* on human myeloid leukemic cells in culture. *Mol Pharmacol* **32**: 530–535, 1987.
11. Santoro MG, Crisari A, Bandetto A and Amici C, Modulation of the growth of a human erythroleukemic cell line (K562) by prostaglandins: Antiproliferative action of prostaglandin A. *Cancer Res* **46**: 6073–6077, 1986.
12. Gregman MD and Meyskens FL Jr, Inhibition of human malignant melanoma colony-forming cells *in vitro* by prostaglandin A_1. *Cancer Res* **43**: 1642–1645. 1983.
13. Honn KV and Marnett LJ, Requirement of a reactive α,β-unsaturated carbonyl for inhibition of tumor growth and induction of differentiation by "A" series prostaglandins. *Biochem Biophys Res Commun* **129**: 34–40, 1985.
14. Atsmon J, Freeman ML, Meredith MJ, Sweetman BJ and Roberts LJ II, Conjugation of 9-deoxy-Δ^9,Δ^{12}(E)-prostaglandin D_2 with intracellular glutathione and enhancement of its antiproliferative activity by glutathione depletion. *Cancer Res* **50**: 1879–1885, 1990.
15. Bito L and Baroody RA, Impermeability of rabbit erythrocytes to prostaglandins. *Am J Physiol* **229**: 1580–1584, 1975.
16. Cagen LM, Fales HM and Pisano JJ, Formation of glutathione conjugates of prostaglandin A_1 in human red blood cells. *J Biol Chem* **251**: 6550–6554, 1976.
17. Smith JB, Silver MJ, Ingerman CM and Kocsis JJ, Uptake and inactivation of A-type prostaglandins by human red cells. *Prostaglandins* **9**: 135–145, 1975.
18. Heasley LE and Brunton LL, Prostaglandin A_1 metabolism and inhibition of cyclic AMP extrusion by avian erythrocytes. *J Biol Chem* **260**: 11514–11519, 1985.
19. Atsmon J, Sweetman BJ, Baertschi SW, Harris TM and Roberts LJ II, Formation of thiol conjugates of 9-deoxy-Δ^9,Δ^{12}(E)-prostaglandin D_2 and Δ^{12}(E)-prostaglandin D_2. *Biochemistry* **29**: 3760–3765, 1990.
20. Narumiya S, Ohno K, Fukushima M and Fujiwara M, Site and mechanism of growth inhibition by prostaglandins. III. Distribution and binding of prostaglandin A_2 and Δ^{12}-prostaglandin J_2 in nuclei. *J Pharmacol Exp Ther* **242**: 306–311, 1987.
21. Ohno K, Sakai T, Fukushima M, Narumiya S and Fujiwara M, Site and mechanism of growth inhibition by prostaglandins. IV. Effect of cyclopentenone prostaglandins on cell cycle progression of G_1-enriched HeLa S3 cells. *J Pharmacol Exp Ther* **245**: 294–298, 1988.
22. Narumiya S and Fukushima M, Site and mechanism of growth inhibition by prostaglandins. Active transport and intracellular accumulation of cyclopentenone prostaglandins, a reaction leading to growth inhibition. *J Pharmacol Exp Ther* **239**: 500–505, 1986.
23. Heasley LE, Watson MJ and Brunton LL, Putative inhibitor of cyclic AMP efflux: Chromatography, amino acid composition, and identification as a prostaglandin A_1-glutathione adduct. *J Biol Chem* **260**: 11520–11523, 1985.
24. Owens CWI and Belcher RV, A colorimetric micro-method for the determination of glutathione. *Biochem J* **94**: 705–711, 1965.
25. Anderson ME, Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* **113**: 548–555, 1985.
26. Meister A, Glutathione metabolism and its selective modification. *J Biol Chem* **263**: 17205–17208, 1988.
27. Vulliez-Le Normand B, Gouyette A, Heidet V, Nagel N and Dray F, Structural identification of prostaglandin A_1 biotransformation products from tumor cells. *Prostaglandins* **35**: 515–521, 1988.
28. Nicolet BH, The addition of mercaptans to certain double bonds. *J Am Chem Soc* **57**: 1098–1099, 1935.
29. Cagen LM, Pisano JJ, Ketley JN, Habig WH and Jakoby WB, The conjugation of prostaglandin A_1 and glutathione catalyzed by homogeneous glutathione *S*-transferases from human and rat liver. *Biochim Biophys Acta* **398**: 205–208, 1975.
30. Richman PG and Meister A, Regulation of γ-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. *J Biol Chem* **250**: 1422–1426, 1975.
31. Ohno K and Hirata M, Induction of γ-glutamylcysteine synthetase by prostaglandin A_2 in L-1210 cells. *Biochem Biophys Res Commun* **168**: 551–557, 1990.

32. Gouin E, Vulliez-Le Normand B, Gouyette A, Heidet V, Nagel N and Dray F, Tumor cell biotransformation products of prostaglandin A_1 with growth inhibitory activity. *Biochem Biophys Res Commun* **141**: 1254–1260, 1986.
33. Messina JP and Lawrence DA, Cell cycle progression of glutathione depleted human peripheral blood mononuclear cells is inhibited at S phase. *J Immunol* **143**: 1974–1981, 1989.
34. Atzori L, Dypbukt JM Sundqvist K, Cotgreave I, Edman CC, Moldeus P and Grafstrom RC, Growth-associated modifications of low-molecular-weight thiols and protein sulfhydryls in human bronchial fibroblasts. *J Cell Physiol* **143**: 165–171, 1990.
35. Djurhuus R, Svardal AM and Ueland PM, Growth state dependent increase of glutathione by homocysteine and other thiols, and homocysteine formation in glutathione depleted mouse cell lines. *Biochem Pharmacol* **39**: 421–429, 1990.
36. Kang YJ and Enger MD, Glutathione content and growth in A549 human lung carcinoma cells. *Exp Cell Res* **187**: 177–179, 1990.
37. Ohno K, Fukushima M, Fujiwara M and Narumiya S, Induction of 68,000-dalton heat shock proteins by cyclopentenone prostaglandins. *J Biol Chem* **263**: 19764–19770, 1988.
38. Bhuyan BK, Adams EG, Badiner GJ, Li LH and Barden K, Cell cycle effects of prostaglandins A_1, PGA_2, and PGD_2 in human and murine melanoma cells in culture. *Cancer Res* **46**: 1688–1693, 1986.
39. Attallah A, Payakkapan W, Lee J, Carr A and Brazelton E, PGA: Fact, not artifact. *Prostaglandins* **5**: 69–72, 1974.
40. Middleditch BS, PGA: Fact or artifact? *Prostaglandins* **9**: 409–410, 1975.
41. Stehle RG and Oesterling TO, Stability of prostaglandin E_1 and dinoprostone (prostaglandin E_2) under strongly acidic and basic conditions. *J Pharm Sci* **66**: 1590–1595, 1977.
42. Fitzpatrick FA and Wynalda MA, Albumin-lipid interactions: prostaglandin stability as a probe for characterizing binding sites on vertebrate albumins. *Biochemistry* **20**: 6129–6134, 1981
43. Kikawa Y, Narumiya S, Fukushima M, Wakatsuka H and Hayaishi O, 9-Deoxy-Δ^9, Δ^{12}-13,14-dihydroprostaglandin D_2, a metabolite of prostaglandin D_2 formed in human plasma. *Proc Natl Acad Sci USA* **81**: 1317–1321, 1984.
44. Polet H and Levine L, Metabolism of prostaglandins E, A, and C in serum. *J Biol Chem* **250**: 351–357, 1975.