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CHARACTERIZATION OF THE TRANSPORT SYSTEM OF PROSTAGLANDIN A_2 IN L-1210 MURINE LEUKEMIA CELLS

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Abstract—Prostaglandin (PG) A_2 has been shown to be actively incorporated into mammalian cells and transferred to the cell nucleus. To characterize the properties of the PGA_2 transfer system, we examined the status of PGA_2 in L-1210 cells with modified cellular glutathione (GSH) levels. PGA_2 in the cytosol fraction of the cells existed in its free-form, the GSH conjugate-form and a macromolecule associate-form (protein bound-form). When the GSH level was lowered under culture conditions, the amount of free-form increased while that of the protein bound-form was unchanged. When PGA_2-loaded cells were incubated in a salt solution, free- and conjugate-forms were emitted from the cells. A concomitant decrease and increase of protein bound PGA_2 in cytosol and nuclei, respectively, were observed. Subsequent studies with isolated cellular fractions revealed that PGA_2 bound to cytosolic protein was transported into the nuclear interior in a temperature-dependent manner. The binding of PGA_2 to the protein and subsequent transport to the nuclei were inhibited by PGJ_2 and 4-hydroxy-cyclopentenone, but not by PGB_2, PGD_2, PGE_2, $PGF_{2\alpha}$, arachidonic acid or oleic acid. *N*-Ethylmaleimide (NEM) and *p*-chloromercuribenzoate (PCMB) strongly interfered with these transfer processes, suggesting that sulfhydryl components are involved in the transport of PGA_2. Subfractionation of cytosol by gel chromatography proved the presence of two proteins (100–150 kDa and 25–35 kDa) that are capable of transporting PGA_2 to cell nuclei. Though 40–45 kDa proteins, which correspond to GSH *S*-transferases, bound to PGA_2, they lacked the nuclear transport activities.

Cyclopentenone prostaglandins (PGs†) such as PGA_2 and J_2 inhibit the growth of human and animal tumor cell lines [1, 2], and manifest many other biological activities, which include inhibition of virus replication [3, 4], stimulation of osteogenesis [5] and activation of cellular glutathione (GSH) biosynthesis via induction of γ-glutamylcysteine synthetase [6, 7]. According to Narumiya *et al.* [8, 9], these PGs are actively incorporated into mammalian cells and accumulate in cell nuclei. This uptake and accumulation in nuclei are closely correlated with the extent of cell growth inhibition [10].

Lines of evidence suggest that the accumulation of PGA_2 and J_2 into cell nuclei is the first key step in the elicitation of their biological activities, and the mechanism of PGA_2 nuclear transport has become a subject of much interest. Recently, Khan and Sorof [11] reported that PGA_2 and J_2 can bind non-covalently to purified rat liver fatty acid binding protein (FABP) with high affinity, and speculated that FABP could serve as an intracellular carrier of these PGs in the liver. However, the hypothesis remains to be tested, particularly for other tissues, and its role in the nuclear transport of PGs has not been fully elucidated.

We have shown previously that uptake of PGA_2 by L-1210 cells is suppressed markedly when cellular GSH is depleted by buthionine sulfoximine (BSO). The amount of PGA_2 in cytosol is also reduced by the depletion of GSH, but the accumulation of PG in the nuclei is not affected [12].

In the present study, we examined the intracellular behavior of PGA_2 using GSH-depleted and -enriched L-1210 cells, and characterized three PG transfer systems. We also demonstrated that specific cytosolic proteins are involved in the translocation of PGA_2 to the cell nucleus.

MATERIALS AND METHODS

Materials. [5,6,8,11,14,15(N)-^{3}H]PGE_2 (200 Ci/mmol) was obtained from Amersham International Plc (Buckinghamshire, U.K.). PGA_2, B_2, D_2, E_2, $F_{2\alpha}$, J_2, and arachidonic acid were purchased from Funakoshi Yakuhin (Tokyo, Japan). ATP, ADP, AMP, cAMP, GTP, *N*-acetylcysteine (*N*-AcCys), BSO, oleic acid, retinoic acid, *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), GSH and rat liver GSH *S*-transferase (GST) were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-Chloro-2,4-dinitrobenzene (CDNB) was from Tokyo Kasei (Tokyo, Japan). Dried powder of phosphate-buffered saline (PBS) and RPMI-1640 medium were from Nissui Seiyaku (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from M. A.

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† Abbreviations: PGs, prostaglandins; GSH, glutathione; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; FABP, fatty acid binding protein; BSO, buthionine sulfoximine; FBS, fetal bovine serum; CDNB, 1-chloro-2,4-dinitrobenzene; PBS, phosphate-buffered saline; GST, GSH *S*-transferase; TCA, trichloroacetic acid; and *N*-AcCys, *N*-acetylcysteine.

Bioproducts (Walkersville, MD). [^{3}H]PGA_2 was prepared from [^{3}H]PGE_2 by treatment with 0.1 N HCl at 37° for 6 hr, and purified by HPLC as described below. 4-Hydroxy-2-cyclopentenone (4-OH-cyclopentenone) was prepared as described previously [7]. All other chemicals used were of reagent grade. Sephadex G-150 (Superfine) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and Centricon-10 microconcentrator from the AMICON Division, W. R. Grace & Co. (Danvers, MA, U.S.A.).

Cell culture and pretreatment with BSO and N-AcCys. L-1210 murine leukemia cells were cultured with RPMI-1640, containing 10% FBS and penicillin (200 U/mL), in humidified air with 5% CO_2 at 37°. The cells (5×10^5 cells/mL, 20 mL of suspension) were cultured for 72 hr and collected by centrifugation at 300 *g* for 10 min. The cells obtained were resuspended in 20 mL (3.0 to 3.5 $\times 10^6$ cells/mL) of fresh medium containing 2 mM *N*-AcCys, a precursor of cysteine, or 1 mM BSO, an inhibitor of GSH biosynthesis, and then further cultured for 12 hr at 37°.

Cellular GSH content rose approximately 180% by the treatment with *N*-AcCys, while it decreased to 20% with BSO. After the pretreatment, the cells were collected and washed twice with PBS, and then suspended in PBS at the indicated cell densities. Cellular GSH content was determined by the fluorometric method as described previously [6].

Cellular uptake of PGA_2. PGA_2 uptake was examined by incubating 200 μL of cell suspension (1×10^7 cells) in PBS for 10 min with 10 μM [^{3}H]-PGA_2 (0.1 μCi) at 37°. The uptake was stopped by adding 5 mL of cold PBS to the suspension, and cells were collected by centrifugation at 300 *g* for 5 min at 4°. After washing twice with PBS, the cells were lysed by homogenization in 200 μL of Buffer A (see below). Next, a small portion of the lysates was taken into an isotope counting vial to measure the total uptake of PGA_2. The rest of the cell lysate was centrifuged at 15,000 *g* for 20 min at 4° and the supernatant was transferred to a counting vial. The pellet was suspended in 200 μL of Buffer A and transferred to a counting vial. The radioactivities in the supernatant and the pellet were regarded as PGA_2 accumulated in cytosol and nuclei, respectively, as previously described [9, 10].

Efflux of PGA_2 from cells. The cells suspended in 200 μL PBS were incubated with 10 μM [^{3}H]PGA_2 for 15 min at 20°. Then, the PGA_2-loaded cells were collected by centrifugation, washed, and resuspended in the original volume of PBS. After incubation for 5, 10 or 20 min at 37°, the cells were collected and lysed in Buffer A; then the radioactivities in subcellular fractions were determined.

Buffers. Buffer A: 100 mM Tris–Cl, pH 7.4, containing 5 mM $MgCl_2$, 2 mM $CaCl_2$ and 1 mM $NaHCO_3$. Buffer B: 250 mM sucrose in Buffer A.

Subcellular distribution of PGA_2. The supernatant and the pellet suspended in Buffer A were treated with 50 μL of 50% trichloroacetic acid (TCA), followed by centrifugation at 15,000 *g* for 10 min. The TCA-soluble fraction was collected and subjected to HPLC analysis as described below. The TCA-insoluble fraction was dissolved in 300 μL of 0.1 N NaOH and transferred to a counting vial, and the radioactivity was determined. Previous subcellular distribution studies [9, 10] have revealed that the cyclopentenone PGs incorporated were found only in the cytosol and nucleus, and centrifugation at 15,000 *g* allowed quantitative recovery of nuclear bound PGs (see Diagram A).

HPLC analysis. HPLC was performed on a Cosmosil $5C_{18}$-AR column (4.6 × 150 mm) using H_2O–acetonitrile (3:2), acidified with phosphoric acid to pH 3.5, as a mobile phase at a flow rate of 1.0 mL/min, and monitored at 230 nm [13]. Radiochromatography was conducted using a Beckman HPLC System Gold equipped with a Beckman 166 UV detector and a 171 radioisotope detector.

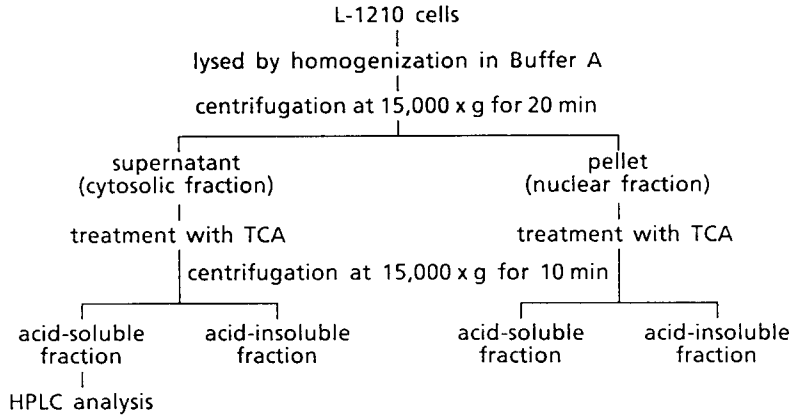
Identification of GSH-conjugate of PGA_2. [^{3}H]-PGA_2 (100 μM, 0.2 μCi) was incubated in 100 μL PBS for 10 min at 37° with either 5 mM GSH alone or 5 mM GSH plus 5 U of rat liver GST. The reaction was terminated by the addition of 30 μL of 50% TCA, and then centrifuged at 15,000 *g* for 10 min at 4°. The TCA-soluble supernatant was subjected to HPLC analysis described above.

Isolation of cytosol and nucleus. L-1210 cells (3.0 to 3.5 $\times 10^6$ cells/mL, 80 mL of the cell suspension) were washed twice with PBS and lysed in 1 mL of Buffer A. The lysate was then centrifuged at 15,000 *g* for 20 min at 4°, and the supernatant (cytosolic fraction) was dialyzed against Buffer A for 24 hr at 4°. To prepare the nuclear fraction, the 15,000 *g* pellet was suspended in 200 μL of Buffer A and layered on 1 mL of Buffer A containing 2.2 M sucrose, and then centrifuged at 15,000 *g* for 20 min at 4° [14]. The pellet (nuclear fraction) was washed once with Buffer A and resuspended with Buffer B.

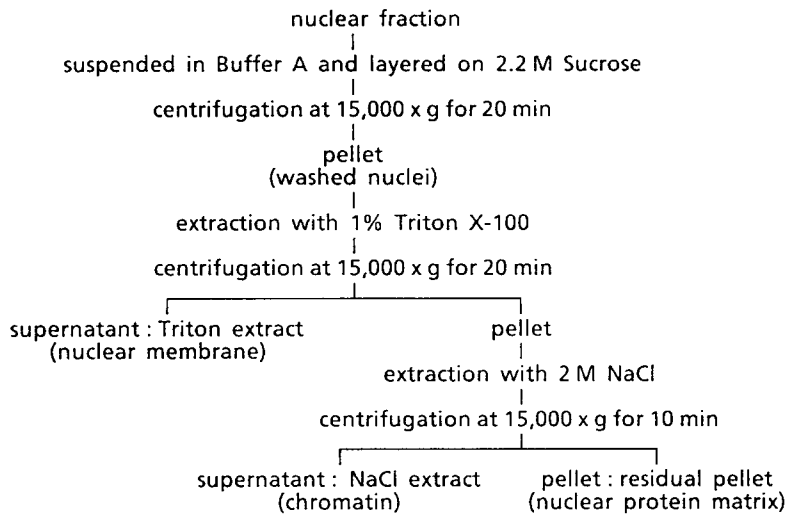
Nuclear transport of PGA_2 in a cell-free system. Cytosolic fraction (containing approx. 300–400 μg of soluble proteins) was incubated in 100 μL of Buffer B with 3 μM [^{3}H]PGA_2 (300 pmol, 0.1 μCi) for 10 min at 37°. Then, 30 μL of nuclear fraction (2–5 $\times 10^6$) was added to the reaction mixture, and the mixture was further incubated at 4, 20 or 37°. At the indicated times, the mixture was centrifuged at 15,000 *g* for 1 min at 4° to separate the cytosolic and nuclear fractions. The supernatant (cytosolic fraction) was isolated and mixed with 40 μL of 50% TCA, followed by centrifugation at 15,000 *g* for 10 min at 4°. The TCA-soluble fraction was collected and subjected to HPLC analysis. The TCA-insoluble precipitate was dissolved in 300 μL of 0.1 N NaOH to determine the radioactivity. The 15,000 *g* precipitate (nuclear fraction) was washed and suspended with 300 μL of Buffer A, and then transferred to a counting vial to determine the amount of PGA_2 transported to the cell nuclei. The PGA_2 transporting activity of the 15,000 *g* supernatant was found to be comparable to that of 105,000 *g* supernatant on the basis of protein concentration.

Intranuclear distribution of PGA_2. After incubating the cell nuclei with cytosolic fraction and [^{3}H]PGA_2, the nuclear fraction was collected and washed with Buffer A, and then suspended with 50 μL of Buffer A. A portion of the suspension was taken to measure the total amount of PGA_2 transferred to the nuclei,

A. Subcellular fractionation and subsequent TCA treatment of L-1210 cells



B. Subfractionation of nuclear fraction



and then the rest was mixed with 500 μL of Buffer A containing 1% Triton X-100. The mixture was maintained at 4° for 10 min, and then centrifuged at 15,000 *g* for 20 min at 4° to isolate the supernatant (Triton extract, corresponds to nuclear membrane). The pellet was suspended in 50 μL of Buffer A, mixed with 500 μL of Buffer A containing 2 M NaCl, and then kept at 4° for 10 min. The mixture was centrifuged at 15,000 *g* for 10 min at 4° to separate the supernatant (NaCl extract, chromatin fraction). The residual pellet (nuclear matrix) was suspended in 300 μL of Buffer A. The radioactivities in these three fractions were measured (see Diagram B).

Gel filtration. Dialyzed supernatant fraction of L-1210 cell extracts was centrifuged at 105,000 *g* for 60 min at 4°, and the isolated supernatant was concentrated with a Centricon-10 microconcentrator. The concentrate (containing approx. 35 mg protein) was incubated with 3 μM [^{3}H]PGA_2 (1.5 nmol, 1 μCi) in 500 μL of Buffer A for 10 min at 37° and then applied to a Sephadex G-150 column (1.5 × 20 cm) equilibrated with 50 mM KH_2PO_4, pH 7.0. Elution was performed with 50 mM KH_2PO_4, pH 7.0, at 4°, and the UV absorbance of the eluate (2 mL/tube) at 280 nm was measured. After counting the radioactivity with a portion of the eluate, the eluate was concentrated and its nuclear transfer activity was determined as follows. The concentrate (containing 300 μg protein) was incubated with nuclear fraction (3 × 10^6 nuclei) in 130 μL of Buffer B for 20 min at 37°. Then the mixture was centrifuged at 15,000 *g* for 1 min at 4°, and PGA_2-derived

Table 1. Effect of cellular GSH content on intracellular distribution of PGA_2 in L-1210 cells

Subcellular fraction	PGA_2 (pmol/10^6 cells)		
	Control	*N*-AcCys	BSO
Total	140.0 ± 2.9	148.5 ± 4.9	104.1 ± 5.3
15,000 *g* Sup. (cytosol)	70.9 ± 3.2	73.9 ± 4.7	39.5 ± 2.3
Acid-soluble fraction	59.7 ± 4.3	64.3 ± 4.8	30.9 ± 1.8
Acid-insoluble fraction	11.6 ± 2.1	10.1 ± 2.3	7.9 ± 2.7
15,000 *g* Pellet (nuclei)	68.3 ± 3.2	72.5 ± 3.1	66.3 ± 4.4
Acid-soluble fraction	15.5 ± 1.7	17.3 ± 3.1	14.9 ± 1.3
Acid-insoluble fraction	53.4 ± 4.9	56.3 ± 2.8	50.0 ± 4.7

L-1210 cells (7×10^7) were cultured in the presence of 2 mM *N*-AcCys or 1 mM BSO, or their absence (control) at 37° for 12 hr. After the treatment, the cells (1×10^7) were incubated with 10 μM [^{3}H]PGA_2 (0.2 μCi) in 200 μL of PBS at 37° for 10 min. The cells were washed and lysed in 200 μL of Buffer A followed by centrifugation at 15,000 *g* for 20 min at 4°. The supernatant (cytosol) and pellet (nuclei) were separated and treated with TCA; then the radioactivity in each fraction was determined. Values are means ± SD (N = 3).

radioactivity in the nuclear fraction was determined as described above.

RESULTS

Subcellular distribution of PGA_2 in L-1210 cells. Prior to the distribution study, L-1210 cells were treated with *N*-AcCys or BSO to modify the cellular GSH levels as described in Materials and Methods. When 10 μM [^{3}H]PGA_2 and L-1210 cells were incubated for 10 min at 37°, about 50% of the radioactivity incorporated into the cells was recovered in the cytosol of control and *N*-AcCys-treated cells, and approximately 35% in the cytosol of BSO-treated cells; the amounts of PGA_2 transferred to nucleic were virtually the same for all cells (Table 1).

Determination of PGA_2 GSH-conjugate and PGA_2 in cytosol. Following incubation of L-1210 cells with [^{3}H]PGA_2 and fractionation of the cellular components, the cytosol was treated with TCA. The protein-bound PGA_2, which was precipitated by TCA, became extractable with ethyl acetate by treating with NaOH and was identified by HPLC. The TCA-nonprecipitable fraction was directly subjected to HPLC analysis providing two radioactive peaks; the major peak (peak 1) was eluted at 2.5 min and a minor peak (peak 2) was eluted at 12.5 min. Peak 1 and peak 2 correspond to PGA_2 GSH-conjugate and free PGA_2, respectively [12]. The area ratios of peak 1/peak 2 obtained from control, *N*-AcCys- and BSO-treated cells were 20.4, 19.5 and 1.4, respectively, and the total radioactivity of peak 2 in BSO-treated cells was about five times greater than that of the control and *N*-AcCys-treated cells. Thus, PGA_2 in cytosol existed in three different forms: free-form, conjugate-form and protein bound-form.

Efflux and nuclear translocation of cytosolic PGA_2. To characterize the status of PGA_2 effluxed from the cells and that remaining in the cells, [^{3}H]PGA_2 and L-1210 cells were incubated at 20° for 15 min, washed with buffer, and then incubated at 37°. The net uptake of PGA_2 is greater at 20° than at 37°, and most of the PG remains in the cytosol, whereas the nuclear transfer system is activated at or close to 37° [9]. As shown in Fig. 1, two typical changes were observed in both control and BSO-treated cells. First, the amount of GSH-conjugate within the cells decreased, while it increased in the medium. Free-form PGA_2 in the cytosol and nuclei decreased during the initial 5 min of incubation. Meanwhile, the free-form became detectable in the medium but began to decrease 5 min later, suggesting that reuptake of PGA_2 occurred. Second, the amount of PGA_2 in the TCA-insoluble fraction of cytosol (protein-bound PGA_2) decreased time dependently to half of the original amount after 20 min. Concomitantly, the PG in the TCA-insoluble fraction of the nuclei, obtained from control and BSO-treated cells, increased from 25.5 to 43.0 and from 30.0 to 45.9 pmol/10^6 cells, respectively. These results suggest that the GSH-conjugate and the free-form of PGA_2 were exported from the cells, while the protein bound-form was transferred from cytosol to nuclei.

Transport of PGA_2 from cytosol to nuclei in a cell-free system. The study was extended to a cell-free system to examine whether PGA_2 bound to cytosolic proteins can be transferred to nuclei. The cytosolic fraction isolated from L-1210 cells was dialyzed for 24 hr at 4°, thus reducing the GSH in the cytosol to less than 5% of the initial amount. When the dialyzed cytosol was incubated with 3 μM [^{3}H]PGA_2 (300 pmol) for 10 min at 37°, and then treated with TCA, about 75% of the radioactivity was recovered in the TCA-insoluble fraction (protein-bound PGA_2), and the rest was recovered in the TCA-soluble fraction (free-form) (Fig. 2). When the nuclear fraction was added to this incubation mixture, the protein-bound PGA_2 decreased time dependently during subsequent incubation and reached a plateau at 20 min, though the amount of PGA_2 in the cytosolic TCA-soluble fraction did not change during

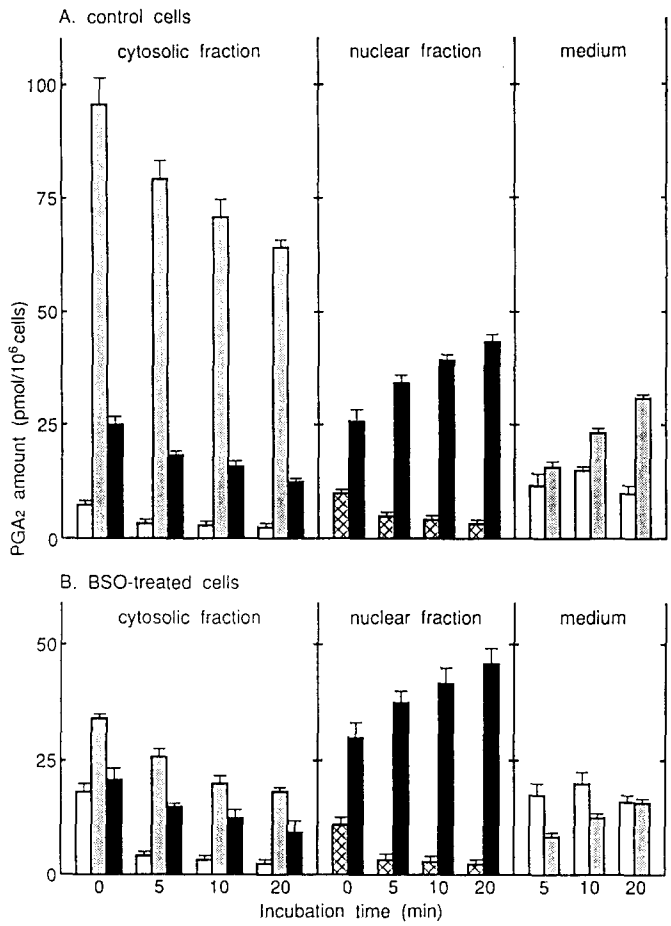


Fig. 1. Effect of GSH depletion on efflux and subcellular distribution of PGA_2. L-1210 cells (6×10^7) were treated with or without 1 mM BSO at 37° for 12 hr. After the treatment, a portion of the cells (1×10^7) was incubated with 10 μM [^{3}H]PGA_2 (0.2 μCi) in 200 μL of PBS at 20° for 15 min. The cells were then washed and resuspended in 200 μL of PBS. After incubation of the washed cells for 0, 5, 10 and 20 min at 37°, the suspensions were centrifuged briefly to separate the cells and the medium. Subcellular fractionation of the cells and HPLC analysis were carried out as described under Materials and Methods. Upper and lower panels show the results obtained from control and BSO-treated cells. Key: (□) PGA_2 in free-form, (▨) GSH-conjugate, (■) bound-form (acid-insoluble fraction), and (⊠) PGA_2 in acid-soluble supernatant of the nuclear fraction. Values are means ± SD of three experiments.

the incubation period. The radioactivity in the nuclei was increased, and more than 95% of the radioactivity in the nuclei was recovered in TCA-insoluble precipitates. On the other hand, when the nuclear fraction was incubated in the absence of cytosol, [^{3}H]PGA_2 bound to the nuclei was reduced to less than half of that of the incubation with cytosol. Similar profiles of PGA_2-derived radioactivity with the cytosolic and nuclear fractions isolated from BSO-treated cells were observed (data not shown).

Inhibition of pGA_2 nuclear transport. PGJ_2 and several 4-substituted cyclopentenone derivatives inhibit nuclear accumulation of PGA_2 [7]. We therefore examined the effects of related compounds on the binding of 3 μM [^{3}H]PGA_2 to cytosolic proteins and its subsequent nuclear translocation (Table 2). PGJ_2 significantly inhibited the binding as well as the nuclear transfer of PGA_2. 4-OH-Cyclopentenone inhibited the protein binding. PGB_2, PGD_2, PGE_2, $PGF_{2\alpha}$, arachidonic acid, oleic acid, retinoic acid and nucleotides (ATP, ADP, AMP, cAMP, GTP) did not affect either binding to cytosolic proteins or nuclear transfer under the same conditions employed (data not shown).

Intranuclear distribution of PGA_2. The cell nuclei, radiolabeled by the transfer system of PGA_2 as described above, was first treated with 1% Triton X-100 to isolate nuclear membranes and then with 2 M NaCl to isolate chromatin. The resulting residues correspond to the nuclear matrix [15]. The [^{3}H]-PGA_2-derived radioactivity was recovered mainly in the nuclear matrix, which was approximately 80% of the total nuclear radioactivity. However, when an excess amount of PGJ_2 had been present during the initial incubation of cytosol and [^{3}H]PGA_2, the subnuclear distribution profile was changed markedly: the proportion of Triton extract was increased, while the radioactivities in the NaCl

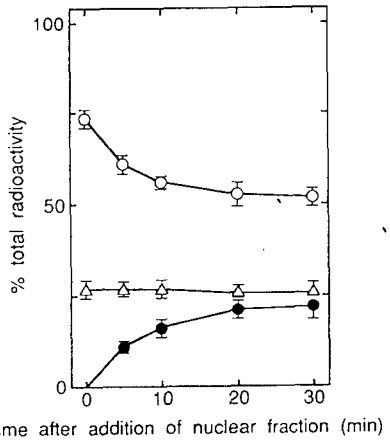


Fig. 2. Transfer of cytosolic protein-bound PGA_2 to nuclei. Dialyzed cytosolic fraction containing 300 μg protein was incubated with 3 μM [^{3}H]PGA_2 (0.1 μCi) in 100 μL of Buffer B for 10 min at 37°. After the incubation (at 0 min), 30 μL of the nuclear fraction (2×10^6 nuclei) were added to the mixture; then incubation was continued at 37° for 5, 10, 20 or 30 min, followed by centrifugation at 5000 g for 1 min at 4° to separate the cytosolic and the nuclear fractions. The cytosolic fraction was treated with TCA, and centrifuged at 15,000 g for 10 min at 4°. The nuclear fraction was washed once with Buffer A and then suspended in 300 μL of Buffer A. The radioactivities in the acid-soluble (Δ) and acid-insoluble precipitate (○) of the cytosolic fraction and the nuclear fraction (●) are shown. Values are means ± SD of triplicate samples.

extract and residual pellet were reduced markedly (Table 3). The results suggest that PJG_2 competed with the PGA_2 transport to the nuclear interior.

Effect of temperature on nuclear transport. Previous work showed that the nuclear transport of the PG

Table 3. Effect of PGJ_2 on nuclear translocation and subnuclear distribution of PGA_2

Subnuclear fraction	PGA_2 (pmol/10^6 nuclei)	
	Control	PGJ_2
Total	16.5 ± 0.3	6.8 ± 0.1
Triton extract	2.2 ± 0.1	3.1 ± 0.1
NaCl extract	0.5 ± 0.1	0.3 ± 0.1
Residual pellet	13.4 ± 0.2	3.4 ± 0.2

Cytosolic and nuclear fractions were prepared as described under Materials and Methods.

[^{3}H]PGA_2 (300 pmol, 0.1 μCi) was incubated with cytosol containing 400 μg of soluble proteins in 100 μL of Buffer B at 37° for 10 min in the presence of 30 nmol PGJ_2. Subsequently, 30 μL of nuclear fraction (4×10^6 nuclei) was added to the reaction mixture, and the mixture was further incubated for 20 min at 37°. The nuclear fraction was then collected by centrifugation and washed once with Buffer B. The extraction of PGA_2-derived radioactivity from the nuclei was performed as described in Materials and Methods. Values are means ± SD (N = 3).

incorporated into the cells is very slow at 4 and 20° but efficient at 37° [9]. To examine the effect of temperature on PGA_2 transport in a cell-free system, [^{3}H]PGA_2 and cytosol were incubated at 37° for 10 min, and then incubated with nuclear fraction for 20 min at 4, 20 or 37°. As shown in Table 4, radioactivities detected in the nuclei at 4 and 20° were about 40% of those found at 37°, while those in the residual pellet at 4 and 20° were 20% of those incubated at 37°. The radioactivities extractable with 1% Triton X-100 and 2 mM NaCl were essentially the same at all temperatures.

Involvement of protein thiol in nuclear transport of PGA_2. PGA_2, PGJ_2 and 4-OH-cyclopentenone

Table 2. Inhibition of PGA_2 transfer to cell nuclei by PGJ_2 and 4-hydroxy-2-cyclopentenone

Compound (300 μM)	PGA_2 (pmol)		
	Remained in cytosol		
	Acid-soluble	Acid-insoluble	Transferred to nuclei
Experiment 1			
None	59.4 ± 5.4	240.9 ± 4.2	
PJG_2	117.6 ± 4.2*	182.8 ± 4.3*	
4-OH-Cyclopentenone	107.4 ± 4.1*	195.3 ± 3.0	
Experiment 2			
None	57.8 ± 4.5	170.6 ± 3.8	65.7 ± 3.5
PGJ_2	111.4 ± 3.4*	164.7 ± 4.3	23.6 ± 1.1*
4-OH-Cyclopentenone	105.4 ± 3.7*	156.6 ± 1.9	40.5 ± 2.0

Assay mixtures containing 3 μM [^{3}H]PGA_2 (300 pmol, 0.1 μCi), 300 μM (30 nmol) of the various unlabeled compounds and cytosol fraction (400 μg proteins) in 100 μL of Buffer B were incubated at 37° for 10 min (Expt. 1). At the end of the incubation, 30 μL of nuclear fraction (2×10^6 nuclei) was added, and the mixture was further incubated at 37° for 20 min (Expt. 2). The amounts of PGA_2 in the acid-soluble and -insoluble fractions were determined as described in Materials and Methods. Values are means ± SD (N = 3).

* Significantly different from control, $P < 0.01$.

Table 4. Temperature-dependent transfer of PGA_2 into nuclei

Subnuclear fraction	PGA_2 (pmol/10^6 nuclei)		
	At 4°	At 20°	At 37°
Total	6.0 ± 0.1	6.3 ± 0.4	14.5 ± 0.2
Triton extract	3.5 ± 0.2	3.7 ± 0.1	3.4 ± 0.3
NaCl extract	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Residual pellet	1.9 ± 0.2	2.0 ± 0.1	10.4 ± 0.3

[^{3}H]PGA_2 (300 pmol, 0.1 μCi) was incubated with 100 μL of cytosolic fraction containing 300 μg of proteins at 37° for 10 min. Subsequently, 30 μL of nuclear fraction (3×10^6 nuclei) were added and the mixture was further incubated for 20 min at the indicated temperature. The nuclear fraction was separated by brief centrifugation, and then treated with Triton X-100 and NaCl as described in Materials and Methods. Values are means ± SD (N = 3).

are known to interact with various thiols. Therefore, we examined the possible involvement of protein thiols in protein binding and intracellular transport of PGA_2 (Table 5). The cytosol was incubated with 3 μM [^{3}H]PGA_2 in the presence of 1 mM NEM or PCMB, and the mixture was further incubated with the nuclear fraction. These two compounds inhibited binding of PGA_2 to cytosolic proteins and nuclear transport of PGA_2; the extents of inhibition were similar to that of PGJ_2.

The PGA_2-derived radioactivities in the NaCl extract and residual pellet were reduced significantly by NEM and PCMB, while the radioactivity extracted with 1% Triton X-100 was elevated about 2-fold. These results suggest that, although thiol functions are essential for the binding of PGA_2 with the protein involved in the nuclear transport, they also affect the transport process into the nuclear interior.

When the nuclear fraction was preincubated with 1 mM NEM or PCMB for 10 min at 37°, washed, and then used for the study of PG transport to the nucleus, a significant reduction of the radioactivities in the residual nuclear matrix was found (Expt. 2 in Table 5).

Molecular properties of protein(s) involved in the nuclear transport. The 105,000 *g* supernatant was incubated with 3 μM [^{3}H]PGA_2 for 10 min at 37° and fractionated by gel filtration. As shown in Fig. 3, the radioactivity was distributed into three peaks: the first (peak 1) was associated with proteins of high molecular mass (more than 100 kDa), the second (peak 2) with 40–50 kDa proteins, and the third one (peak 3) with the elution range of free PGA_2. On the other hand, the nuclear transport activity was dissociated into two peaks: the major one overlapped peak 1 while a small peak was found with proteins of low molecular mass (25–35 kDa). Peak 2 was devoid of the transport activity.

To confirm this finding, the 105,000 *g* supernatant was first chromatographed on a Sephadex G-150 column and then the eluates were incubated with [^{3}H]PGA_2 and nuclei at 37°. In agreement with the

Table 5. Effect of SH-reagents on translocation of PGA_2 into nuclei

Subnuclear fraction	PGA_2 (pmol/10^6 nuclei)		
	Control	NEM	PCMB
Experiment 1			
Total	16.3 ± 1.2	6.3 ± 0.2*	6.4 ± 0.1*
Triton extract	2.3 ± 0.1	4.6 ± 0.1*	4.7 ± 0.1*
NaCl extract	0.5 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Residual pellet	13.8 ± 1.2	1.5 ± 0.1*	1.2 ± 0.1*
Experiment 2			
Total	14.4 ± 0.3	11.1 ± 0.1	12.1 ± 0.2
Triton extract	2.7 ± 0.2	4.7 ± 0.2*	4.0 ± 0.2*
NaCl extract	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Residual pellet	11.0 ± 0.3	5.7 ± 0.3*	7.6 ± 0.3*

Experiment 1: [^{3}H]PGA_2 (300 pmol, 0.1 μCi) was incubated with cytosolic fraction containing 400 μg of soluble proteins at 37° for 10 min in the presence of 1 mM NEM or PCMB; then 30 μL of nuclear fraction (4×10^6 nuclei) was added to the mixture. After additional incubation at 37° for 20 min, the nuclear fraction was separated, and treated with Triton X-100 and NaCl as described in Materials and Methods. Values are means ± SD (N = 3).

Experiment 2: Cytosolic fraction (400 μg of soluble proteins) was incubated with [^{3}H]PGA_2 (300 pmol, 0.1 μCi) for 10 min at 37°. Nuclear fraction (4×10^6 nuclei) suspended in 100 μL Buffer B was treated with 1 mM NEM or pCMB for 10 min at 37°. After the treatment, the nuclei were washed once with Buffer B and centrifuged. The washed nuclei were suspended in 30 μL of Buffer B, and then incubated for 20 min at 37° with 100 μL of cytosolic fraction preexposed to [^{3}H]-PGA_2. The nuclear fraction was separated by centrifugation and treated with Triton X-100 and NaCl. Values are means ± SD (N = 3).

* Significantly different from control, $P < 0.01$.

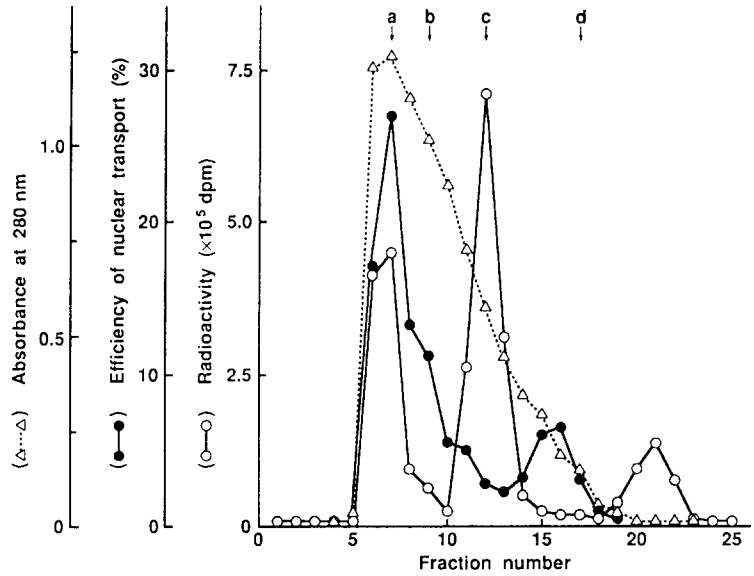


Fig. 3. Gel filtration profiles of [^{3}H]PGA_2-derived radioactivity and PGA_2 nuclear transfer activity. The 105,000 *g* supernatant, containing 35 mg protein, was incubated with 3 μM [^{3}H]PGA_2 in 500 μL of Buffer A for 10 min at 37°. After the incubation, the mixture was chromatographed on a Sephadex G-150 column, and the eluate was analyzed as described in Materials and Methods. The position of molecular markers is shown by arrows. Key: (a) aldolase (158 kDa); (b) bovine serum albumin (67 kDa); (c) ovalbumin (43 kDa); and (d) chymotrypsinogen (25 kDa).

prior finding, the nuclear transport activity was associated with peak 1 and 25–35 kDa protein. Peak 2, based on its molecular weight, was considered to be GST. When the 105,000 *g* supernatant was incubated with [^{3}H]PGA_2 in the presence of 1 mM CDNB, a substrate for GST, peak 2 disappeared and peak 3 became larger. Commercially available rat and porcine GST, though they bound to PGA_2, did not show nuclear transport activities.

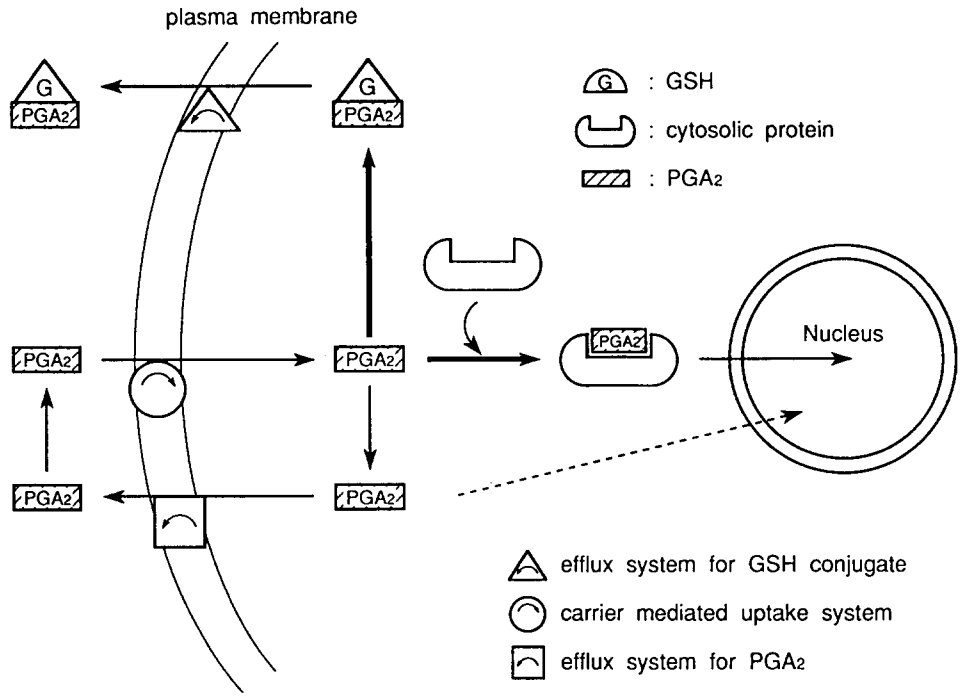
DISCUSSION

Previous findings suggest the presence of three different transfer systems in the transport of cyclopentenone PGs in L-1210 cells. One is the uptake system which introduces PGs into the cells via specific carrier. The second is the efflux system which transfers the PGs from the cells. The third is the nuclear transport system which transfers the PGs from the cytosol to the nuclei [8, 9]. Changes in the intracellular concentration and subcellular distribution of the PGs are considered to reflect the integrated effect of these three systems. GSH depletion causes reduction of both PGA_2 uptake and its distribution in cytosol, but does not affect accumulation of the PG in cell nuclei [12].

In this study, we found that the elimination of PGA_2 in cytosol was due to the efflux as its GSH-conjugate as well as its free-form. The conjugate was detectable in the extracellular space by HPLC analysis soon after incubation of the cells at 37°, indicating that considerable amounts of PGA_2 incorporated into the cells were conjugated with cellular GSH, as observed in CHO cells [16], and effluxed from the cells. On the other hand, PGA_2 bound to the proteins in cytosol decreased during incubation of the cells, and, concomitantly, the amount of PGA_2 in nuclei increased, indicating that the protein-bound PGA_2 was transferred to nuclei. The mechanism of PGA_2 transport is tentatively proposed in Scheme 1.

An ATP-dependent export mechanism of GSH-conjugate has been proposed based on experiments using plasma membrane inside-out vesicles prepared from rat heart, liver and human erythrocytes [17–19]. The same mechanism may be involved in the efflux of PGA_2 GSH-conjugate.

With regard to the intracellular dynamics of PGA_2, we assume that some specific cytosolic proteins are involved in the nuclear transport system of the PG. The validity of this assumption is supported by several findings. First, GSH depletion did not affect the accumulation of PGA_2 in the nuclei and the amounts of PGA_2 bound to protein in cytosol, although it significantly affects the amount of the free-form in cytosol [12]. Second, in a cell-free system, when PGA_2 was incubated with cytosolic proteins and then further incubated with cell nuclei, the PG bound to the protein was transferred to the cell nuclei, but the amount of free-form of the PG did not change during the second incubation. Third, when the cell nuclei were incubated with PGA_2 in the absence of cytosol fraction, the nuclear transport of the PG was small. Fourth, the nuclear transport of PGA_2 occurred in a temperature-dependent manner, and the binding of PGA_2 to the protein was specifically inhibited by PGJ_2 and 4-OH-cyclopentenone. PGJ_2 inhibited subsequent transport of PGA_2 into the nuclear interior. A similar inhibition profile and temperature sensitivity were observed in previous studies on subcellular distribution of PGA_2 [7, 9].



Scheme 1. Proposed pathway for translocation of PGA_2 in L-1210 cells.

In this study, when [^{3}H]PGA_2 was incubated with cytosolic proteins, a major proportion of the PG-derived radioactivity was recovered as TCA-insoluble precipitates. The interaction of PGA_2 and protein thiol may occur because PGA_2, J_2 and 4-OH-cyclopentenone have an α,β-unsaturated carbonyl group in their structure, which is very susceptible to reactive addition of thiol groups. PGJ_2 and 4-OH-cyclopentenone inhibited the binding of PGA_2 to the protein and, as a result, subsequent transport of the PG into cell nuclei. Furthermore, the binding of PGA_2 and its transport to nuclei were inhibited significantly by thiol-alkylating agents such as NEM and PCMB. As these two processes were not inhibited by a 100-fold excess concentration of arachidonic acid and oleic acid, typical ligands of FABP [20–22], we infer that the cytosolic protein responsible for the transport of PGA_2 into nucleus is not FABP [11]. Also, it is not likely that GST is the major protein responsible for the nuclear transport of the PG, because the 40–50 kDa protein in cytosol, which bound to PGA_2 lacked the transfer activity. Commercially available rat and porcine GST also did not show the transfer activities. Thus, our results strongly suggest the presence of transfer protein specific for cyclopentenone PGs in cytosol. As PGA_2 easily isomerizes to PGB_2 in body fluid and its existence has not been proven unequivocally, the protein may function physiologically as the carrier of PGJ_2 *in vivo*.

Our gel filtration study revealed that at least two cytosolic proteins are capable of transporting PG to cell nuclei: a protein with a high molecular mass (more than 100 kDa) and a smaller one (25–35 kDa). We speculate that the presence of these two components is related to the multiple biological functions of PGA_2 [2, 3, 5, 6]. Treatment of nuclei with NEM and PCMB markedly attenuated the efficiency of the PG transport, suggesting that sulfhydryl components are involved in the intra-nuclear dynamics of PGA_2. It may be hypothesized that PGA_2 is released from the cytosolic protein at the nuclear membrane or in the nuclear interior, and then the PG binds to the thiol groups of the target molecules. This needs to be verified by closer examination of the intracellular status of PGA_2.

Lines of evidence suggest that PGA_2 and J_2 cause induction and/or suppression of gene expression at the transcriptional levels, which is related to cell growth inhibition or stimulation of GSH biosynthesis [6, 7, 23–25]. Further studies on the status of PGA_2 in the cell nucleus should provide insight into the physiological significance of PG transport to the nucleus.

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