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Inhibition by gossypol of tumor promoter-induced arachidonic acid metabolism in rat peritoneal macrophages

Kazuo Ohuchi [a], Masako Watanabe [a], Noriyasu Hirasawa [a], Susumu Tsurufuji [a], Tsuneo Ozeki [b] and Hirota Fujiki [c]

[a] *Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba Aramaki, Sendai, Miyagi,* [b] *The 3rd Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu and* [c] *National Cancer Center Research Institute, Tokyo (Japan)*

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Rat peritoneal macrophages were prelabeled with [^{3}H]arachidonic acid. The release of radioactivity into the medium was increased by treatment with TPA-type tumor promoters, such as TPA, teleocidin and aplysiatoxin, and the non-TPA-type tumor promoter, thapsigargin. Gossypol, at concentrations of 3 and 10 μM, inhibited the release of radioactivity stimulated by both types of tumor promoter, although the mechanism of stimulation of arachidonic acid metabolism is different in the two types of tumor promoter. Stimulation of prostaglandin E_2 production by these tumor promoters was also inhibited by treatment with gossypol. Calcium ionophore A23187-stimulated release of radioactivity and prostaglandin E_2 production were also inhibited by gossypol treatment. The mechanism of inhibition by gossypol of prostaglandin E_2 production is discussed.

Introduction

In a two-stage carcinogenesis experiment on mouse skin, several tumor promoters were present which have been classified into 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-type tumor promoters [1–4], such as TPA, teleocidin and aplysiatoxin, and non-TPA-type tumor promoters such as palytoxin [5] and thapsigargin [6]. Recently, we have shown [7,8] that arachidonic acid metabolism in macrophages is stimulated by treatment with these tumor promoters at very low doses, irrespective of their type, and we hypothesized that the tumor promoters are associated with the ability to stimulate arachidonic acid metabolism. In order to assign a significant role of arachidonate metabolites in tumor promotion, it is useful to apply a specific inhibitors of arachidonic acid metabolism consecutively during one stage of promotion. Gossypol, a polyphenolic compound from cotton plant, was found to inhibit calcium ionophore A23187-stimulated release of leukotrienes in RBL-1 cells as determined by radioimmunoassay [9], and to inhibit 5- and 12-lipoxygenases of RBL-1 cells [10], suggesting that gossypol is a useful specific inhibitor of arachidonate 5- and 12-lipoxygenases. However, during a series of experiments carried out to identify inhibitors of tumor promoter-stimulated

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; HPLC, high-performance liquid chromatography.

Correspondence: K. Ohuchi, Department of Biochemistry, Faculty of Pharmaceutical Science, Tohoku University, Aoba Aramaki, Sendai, Miyagi 980, Japan.

arachidonic acid metabolism we found that gossypol inhibits the release of radioactivity from [^{3}H]arachidonic acid-labeled macrophages and inhibits synthesis of prostaglandin E_2, a cyclooxygenase product. In this paper, we will show that gossypol has an inhibitory effect on arachidonic acid release probably by inhibiting phospholipases, and as a consequence to inhibits synthesis synthesis of a cyclooxygenase product, prostaglandin E_2.

Materials and Methods

Preparation of rat peritoneal macrophages. A solution containing soluble starch (Wako Pure Chemical, Tokyo, Japan) and bacto peptone (Difco Laboratories, Detroit, MI, U.S.A.) (5% each) was injected into male rats (the Sprague-Dawley strain, specific pathogen free) of 300–350 g, (Charles River Japan Inc., Kanagawa, Japan) intraperitoneally at a dose of 5 ml per 100 g body weight. 4 days after the injection, the rats were killed by cutting the carotid artery under diethyl ether anesthesia and peritoneal cells were harvested [7].

Macrophage culture. The cells were suspended in Eagle's minimum essential medium (Nissui Inc., Tokyo, Japan) and supplemented with 10% (v/v) calf serum (Grand Island Biochemical Co., Grand Island, NY, U.S.A.), penicillin G potassium (36 mg/l) and streptomycin sulfate (100 mg/l) (Meiji Seika Co., Tokyo, Japan). The macrophages were seeded at $6 \cdot 10^6$ cells per 60 mm Falcon tissue culture dish (Division of Becton, Dickinson and Co., Cokeysville, MD, U.S.A.) for 2 h at 37° C. After the incubation, the dishes were washed three times with the medium to wash out non-adherent cells [7].

Measurement of radioactivity released from [^{3}H]arachidonic acid-labeled macrophages. The adherent cells were further incubated in 4 ml of the medium containing 1 μCi of [^{3}H]arachidonic acid (61 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.) in order to label the cellular lipids for 20 h at 37° C [7]. To remove free [^{3}H]arachidonic acid, the cells were washed five times with 2 ml of the medium without calf serum. The cells were then incubated in 4 ml of the medium containing each tumor promoter and various concentrations of gossypol and supplemented with 0.1% (w/v) fatty acid-free bovine albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) instead of calf serum. Each tumor promoter and gossypol-acetic acid was dissolved in ethanol and an aliquot of the solution was added to the medium. The control medium contained the same amount of the vehicle. The final concentration of ethanol was adjusted to 0.1%. TPA and gossypol-acetic acid were purchased from Sigma. Teleocidin was isolated from *Streptomyces mediocidicus* [2], and aplysiatoxin was isolated from marine blue-green alga, *Lyngbya majuscula* [11]. Thapsigargin [6,8,12,13] (6-(acetoxy)-2,3,3a,4,5,6,6a,7,8,9b-decahydro-3,3a-dihydroxy-3,6,9-trimethyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-2-oxo-4-(1-oxobutoxy)-azuleno[4,5-*b*]furan-7-yl octanoate) was a gift from Dr. S.B. Christensen, Royal Danish School of Pharmacy, Denmark. A23187 was purchased from Hoechst Japan Ltd., Tokyo, Japan. At the appropriate times of incubation, 100 μl of the medium was withdrawn and counted for the released radioactivity.

HPLC analysis. After 20 min incubation of [^{3}H]arachidonic acid-labeled macrophages in the medium containing 1.5 μM A23187 and various concentrations of gossypol, the medium was withdrawn and centrifuged at 1600 · g for 5 min at 4° C. Then the supernatant fraction was applied to an octadecylsilyl silica cartridge (Sep-Pak C18 cartridge, Waters Associates, Milford, MA, U.S.A.) which was conditioned by washing with 10 ml of methanol and followed by 10 ml of H_2O before use. After application of the supernatant fraction, the cartridge was washed with 10 ml of H_2O followed by 10 ml of 15% methanol and the lipophylic components were finally eluted with 10 ml of 100% methanol. The methanol fraction was evaporated to dryness under reduced pressure and the residue was dissolved in a minimum volume of 50% ethanol in 0.01 M phosphate buffer (pH 7.4) and subjected to high-performance liquid chromatography (HPLC) on a reverse-phase column (NOVA PAK C18, Waters Associates). Each sample was run on a linear gradient program [8,14,15] from 100% solvent A (93.4% 0.01 M phosphate buffer (pH 7.4), 6% methanol and 0.6% *t*-amyl alcohol) to 100% solvent B (99.4% methanol and 0.6% *t*-amyl alcohol) for 100 min at a flow rate of 1 ml/min and 1-ml fractions were collected. The

radioactivity in each fraction was counted and expressed as counts per min per dish.

Radioimmunoassay of prostaglandin E_2. The prostaglandin E_2 level in the medium was radioimmunoassayed after incubating the non-labeled macrophages ($6 \cdot 10^6$ cells) for 40 min in 4 ml of the medium containing each tumor promoter and various concentrations of gossypol supplemented with 0.1% (w/v) fatty acid-free bovine albumin (Sigma) instead of calf serum. Prostaglandin E_2 anti-serum was purchased from Seragen, Inc. (Boston, MA, U.S.A.).

Results

When [^{3}H]arachidonic acid-labeled macrophages were incubated in the medium containing TPA (10 ng/ml), a release of radioactivity into the medium was increased with incubation time (Fig. 1). In the presence of gossypol, TPA-stimulated release of radioactivity was inhibited in a dose-dependent manner (Fig. 1). Gossypol at 1 μM showed no inhibitory effect. Significant inhibition was observed at 3 and 10 μM. At these concentrations, gossypol inhibited the release of radioactivity untill 60 min of incubation. Gossypol at 30 μM inhibited the release of radioactivity more effectively than at 10 μM (data not shown). When

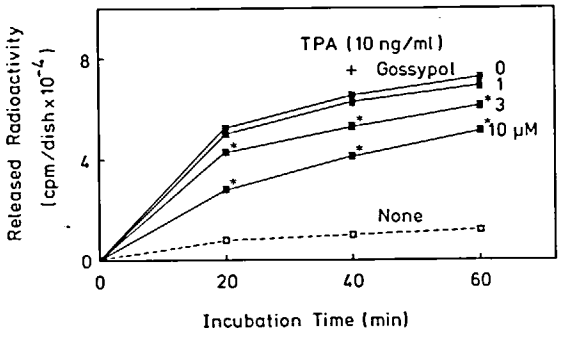

Fig. 1. Effects of various concentrations of gossypol on TPA-induced radioactivity release from [^{3}H]arachidonic acid-labeled macrophages. [^{3}H]Arachidonic acid-labeled macrophages ($6 \cdot 10^6$ cells) were incubated at 37°C in 4 ml of medium supplemented with 0.1% (w/v) fatty acid-free bovine albumin containing 10 ng/ml TPA and the indicated concentrations of gossypol. The values are the means ± S.E. from four dishes. The results were confirmed by two additional experiments. Statistical significance: * $P < 0.001$ vs. corresponding control. o-----o expresses spontaneous release of radioactivity.

TABLE I

EFFECTS OF GOSSYPOL ON TPA-INDUCED PROSTAGLANDIN E_2 PRODUCTION

$6 \cdot 10^6$ macrophages were incubated at 37°C for 40 min in 4 ml of the medium supplemented with 0.1% (w/v) fatty acid-free bovine albumin containing 10 ng/ml of TPA and the indicated concentrations of gossypol. Prostaglandin E_2 levels in the medium were radioimmunoassayed. Values are the means ± S.E. from four dishes. Similar results were obtained by a separate experiment.

Treatment	Prostaglandin E_2 (ng/ml)
None	4.9 ± 0.2
TPA (10 ng/ml)	30.5 ± 0.4
TPA (10 ng/ml) + gossypol (1 μM)	30.7 ± 1.0
TPA (10 ng/ml) + gossypol (3 μM)	23.6 ± 0.6 *
TPA (10 ng/ml) + gossypol (10 μM)	20.5 ± 0.2 *

Statistical significance: TPA 10 (ng/ml) vs. none, $P < 0.001$; * $P < 0.001$ vs. TPA 10 (ng/ml).

non-labeled macrophages were incubated in the medium containing TPA (10 ng/m), prostaglandin E_2 production was increased prominently when measured at 40 min of incubation (Table I). Gossypol at 1 μM did not inhibit TPA-stimulated prostaglandin E_2 production, but significant inhibition was observed at 3 and 10 μM, as was also observed for inhibition of radioactivity release (Fig. 1).

Table II summarizes the effect of gossypol at concentrations of 3 and 10 μM on the release of radioactivity and prostaglandin E_2 production stimulated by the TPA-type tumor promoters, teleocidin and aplysiatoxin, each at 10 ng/ml. Again, gossypol at 3 and 10 μM inhibited both the release of radioactivity and prostaglandin E_2 production dose dependently.

Thapsigargin, a non-TPA-type tumor promoter, at 10 ng/ml also stimulated the release of radioactivity and prostaglandin E_2 production as shown in Table III. However, at 40 min incubation, the increase of the release of radioactivity and prostaglandin E_2 production by thapsigargin treatment was not as prominent as that by treatment with TPA-type tumor promoters (Tables I and II). Both the increased release of radioactivity and the increased prostaglandin E_2 production caused by thapsigargin treatment were inhibited significantly by gossypol at concentrations of 3 and 10 μM. At

TABLE II

EFFECTS OF GOSSYPOL ON TELEOCIDIN- AND APLYSIATOXIN-INDUCED RELEASE OF RADIOACTIVITY AND PROSTAGLANDIN E_2 PRODUCTION

[^{3}H]Arachidonic acid-labeled macrophages ($6 \cdot 10^6$ cells) were incubated at 37 °C for 40 min in 4 ml of the medium supplemented with 0.1% (w/v) fatty acid-free bovine albumin containing teleocidin (10 ng/ml) or aplysiatoxin (10 ng/ml) and the indicated concentrations of gossypol. For measurements of the prostaglandin E_2 level, non-labeled macrophages ($6 \cdot 10^6$ cells) were incubated at 37 °C for 40 min. The results were confirmed by a separate experiment. Values are the means ± S.E. from four dishes.

Treatment	Released radioactivity (cpm/dish $\cdot 10^{-4}$)	Prostaglandin E_2 (ng/ml)
None	0.97 ± 0.04	3.5 ± 0.2
Teleocidin (10 ng/ml)	6.35 ± 0.12	32.3 ± 0.5
Teleocidin (10 ng/ml) + gossypol (3 μM)	5.42 ± 0.06 *	23.2 ± 0.4 *
Teleocidin (10 ng/ml) + gossypol (10 μM)	4.51 ± 0.13 : *	18.1 ± 0.3 *
Aplysiatoxin (10 ng/ml)	7.01 ± 0.09	34.2 ± 0.2
Aplysiatoxin (10 ng/ml) + gossypol (3 μM)	5.86 ± 0.07 *	25.2 ± 0.4 *
Aplysiatoxin (10 ng/ml) + gossypol (10 μM)	4.95 ± 0.11 *	19.0 ± 0.4 *

Statistical significance: none vs. teleocidin (10 ng/ml) or aplysiatoxin (10 ng/ml), $P < 0.001$; * $P < 0.001$ compared to the corresponding control.

10 μM gossypol, thapsigargin-stimulated release of radioactivity and prostaglandin E_2 production were lowered to the basal level (Table III).

When the prelabeled macrophages were incubated in the presence of 1.5 μM A23187, the release of radioactivity into the medium 20 min after the incubation was significantly increased (Table IV). The A23187-stimulated release of radioactivity was also inhibited by gossypol at 3 and 10 μM in a dose-dependent manner (Table IV). Radiochromatograms after HPLC resolution of [^{3}H]arachidonic acid and its metabolites released into the medium 20 min after the incubation are shown in Fig. 2. On stimulation with A23187, the macrophages released several arachidonic acid metabolites, the retention times of which resembled those of 6-ketoprostaglandin $F_{1\alpha}$, prostaglandin E_2, leukotriene B_4 and 12-hydroxy-

TABLE III

EFFECTS OF GOSSYPOL ON THAPSIGARGIN-INDUCED RELEASE OF RADIOACTIVITY AND PROSTAGLANDIN E_2 PRODUCTION

[^{3}H]Arachidonic acid-labeled macrophages ($6 \cdot 10^6$ cells) were incubated at 37 °C for 40 min in 4 ml of the medium supplemented with 0.1% (w/v) fatty acid-free bovine albumin containing thapsigargin (10 ng/ml) and the indicated concentrations of gossypol. For measurements of the prostaglandin E_2 level, non-labeled macrophages ($6 \cdot 10^6$ cells) were incubated at 37 °C for 40 min. The results were confirmed by a separate experiment. Values are the means ± S.E. from four dishes.

Treatment	Released radioactivity (cpm/dish $\cdot 10^{-4}$)	Prostaglandin E_2 (ng/ml)
None	0.91 ± 0.05	3.2 ± 0.1
Thapsigargin (10 ng/ml)	1.41 ± 0.03	4.9 ± 0.1
Thapsigargin (10 ng/ml) + gossypol (1 μM)	1.38 ± 0.04	4.6 ± 0.2
Thapsigargin (10 ng/ml) + gossypol (3 μM)	1.09 ± 0.02 *	3.8 ± 0.1 *
Thapsigargin (10 ng/ml) + gossypol (10 μM)	0.88 ± 0.04 *	2.8 ± 0.1 *

Statistical significance: none vs. thapsigargin 10 (ng/ml), $P < 0.001$; * $P < 0.001$ compared to thapsigargin (10 ng/ml).

eicosatetraenoic acid. Some other radioactive peaks were also found (Fig. 2, upper panel). On treatment with 10 μM gossypol, the peak heights of

TABLE IV

EFFECTS OF GOSSYPOL ON A23187-INDUCED RELEASE OF RADIOACTIVITY FROM PRE-LABELED MACROPHAGES

[^{3}H]Arachidonic acid-labeled macrophages ($6 \cdot 10^6$ cells) were incubated at 37 °C for 20 min in 4 ml of the medium supplemented with 0.1% (w/v) fatty acid-free bovine albumin containing 1.5 μM of A23187 and the indicated concentrations of gossypol. Values are the means ± S.E. from four dishes. Similar results were obtained from two additional experiments.

Treatment	Released radioactivity (cpm/dish $\cdot 10^{-4}$)
None	0.41 ± 0.02
A23187 (1.5 μM)	6.01 ± 0.09
A23187 (1.5 μM) + gossypol (1 μM)	5.78 ± 0.10
A23187 (1.5 μM) + gossypol (3 μM)	4.10 ± 0.11 *
A23187 (1.5 μM) + gossypol (10 μM)	2.50 ± 0.06 *

Statistical significance: A23187 (1.5 μM) vs. none, $P < 0.001$; * $P < 0.001$ vs. A23187 (1.5 μM).

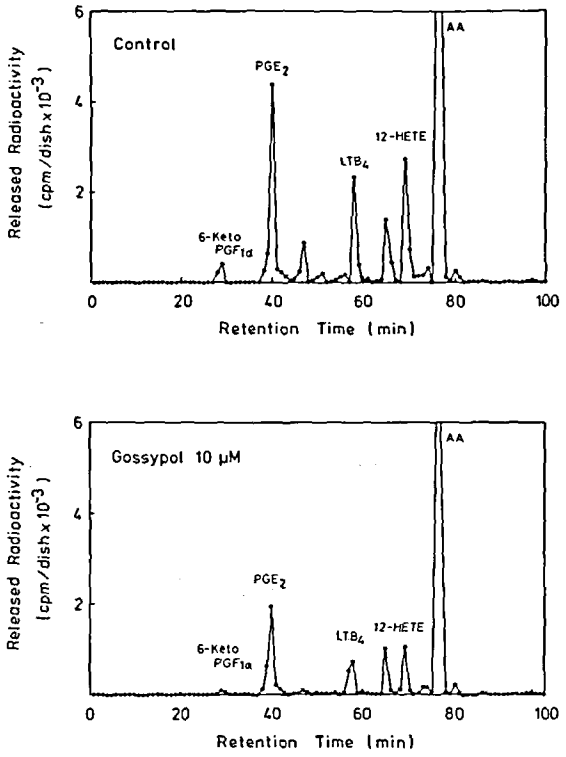

Fig. 2. HPLC analysis of [^{3}H]arachidonic acid metabolites released into the medium by treatment with A23187 and the effects of gossypol. [^{3}H]Arachidonic acid-labeled macrophages ($6 \cdot 10^6$ cells) were incubated at 37 °C in 4 ml of the medium supplemented with 0.1% (w/v) fatty acid-free bovine albumin containing 1.5 μM of A23187 with (lower panel) or without (upper panel) gossypol (10 μM). The retention times of authentic [^{3}H]arachidonic acid and some of its metabolites were as follows: arachidonic acid (AA), 78 min; 12-hydroxyeicosatetraenoic acid (12-HETE), 70 min; leukotriene B_4 (LTB_4), 58 min; prostaglandin E_2 (PGE_2), 40 min; and 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), 29 min as indicated in the figure.

these arachidonic acid metabolites were decreased (Fig. 2, lower panel).

Discussion

In our previous paper [7,8], we showed that the tumor promoters of mouse skin are associated with the ability to stimulate arachidonic acid metabolism, irrespective of their type, at very low doses. TPA-type tumor promoters, such as TPA, teleocidin and aplysiatoxin are shown to stimulate arachidonic acid metabolism through the activation of protein kinases, since an inhibitor of protein kinases, H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine) [16], inhibits both the release of radioactivity and prostaglandin E_2 production stimulated by the TPA-type tumor promoter [17]. On the other hand, H-7 failed to inhibit thapsigargin-stimulated arachidonic acid metabolism [17], suggesting that the mechanism of arachidonic acid metabolism stimulation by thapsigargin is different from that of the stimulation by the TPA-type tumor promoters. The TPA-type tumor promoters are reported to have a common receptor, presumably protein kinase C [18], for which thapsigargin has only a very poor affinity [6]. Furthermore, thapsigargin requires extracellular calcium for the stimulation of the arachidonic acid metabolism, while the TPA-type promoters do not [17]. This finding and other reports showing that thapsigargin is a potent Ca^{2+} mobilisator [19–21] also suggests that the mechanism is different in the two types of tumor promoter [17,22]. In this paper, we show that gossypol acts to inhibit the release of radioactivity from [^{3}H]arachidonic acid-labeled macrophages and prostaglandin E_2 production whether the stimulation of arachidonic acid metabolism is induced by the TPA-type tumor promoters (Fig. 1 and Table II) or by the non-TPA-type tumor promoter, thapsigargin (Table III). Thapsigargin treatment induced a weak stimulation of arachidonic acid metabolism when measured 40 min after the incubation (Table III). Thapsigargin requires much longer incubation period to express its full activity than TPA-type tumor promoters do [17]. When measured 6 h after treatment with thapsigargin (10 ng/ml), the prostaglandin E_2 level in the medium was significantly higher than that 6 h after treatment with TPA (10 ng/ml) [8]. Since the degree of stimulation by thapsigargin treatment was low at 40 min, gossypol at 10 μM suppressed the release of radioactivity and prostaglandin E_2 production to the basal level (Table III). As gossypol effectively inhibits the arachidonic acid metabolism stimulated by thapsigargin which requires extracellular calcium for the manifestation of its effect [17], gossypol was thought to suppress calcium ionophore-stimulated arachidonic acid metabolism. In fact, gossypol inhibited the release of radioactivity stimulated by A23187 (Table IV). After resolution

by HPLC, the radioactive peaks of [^{3}H]arachidonic acid metabolites were also found to be decreased by treatment with 10 μM gossypol (Fig. 2). A radiochromatogram obtained after treatment with 1 μM gossypol was similar to that of the control (Fig. not shown). We have not identified the arachidonic acid metabolites of which the retention times resemble leukotriene B_4 and 12-hydroxyeicosatetraenoic acid. It is possible that other lipoxygenase products such as 5-hydroxyeicosatetraenoic acid or 15-hydroxyeicosatetraenoic acid might comigrate with leukotriene B_4 or 12-hydroxyeicosatetraenoic acid, respectively. Hamasaki and Tai [10] reported that gossypol is a specific inhibitor of 5- and 12-lipoxygenses of RBL-1 cells, with ID_{50} values of 0.3 μM and 0.7 μM, respectively. However, in the present work, 1 μM gossypol showed no effect on the production of [^{3}H]arachidonic acid metabolites of which the retention times corresponded to those of leukotriene B_4 or 12-hydroxyeicosatetraenoic acid (Fig. not shown). At least 3 μM of gossypol was necessary to lower the radioactive peak heights of these metabolites. It should be noted that the production of cyclooxygenase products such as prostaglandin E_2 or 6-ketoprostaglandin $F_{1\alpha}$ were also suppressed in parallel with the decrease of the production of the lipoxygenase products (Fig. 2). Consequently we must conclude that gossypol, in cell culture systems, inhibits both the synthesis of cyclooxygenase products and lipoxygenase products through the inhibition of arachidonic acid release. Although we have not examined whether gossypol has a direct inhibitory effect on isolated phospholipases, the inhibition of the release of radioactivity from [^{3}H]arachidonic acid-labeled macrophages by gossypol treatment strongly suggests that gossypol does have an inhibitory effect on phospholipases at a cellular level. The inhibition of prostaglandin E_2 production by gossypol treatment might be caused through the inhibition of the release of arachidonic acid, since the inhibition rate of prostaglandin E_2 production resembled that of the release of radioactivity (Tables II and III). These results suggest that gossypol might be useful as an inhibitor of the phospholipases responsible for arachidonic acid metabolism at a cellular level. However, since gossypol is reported to be an inhibitor of several nucleotide-metabolizing enzymes, such as ATPase [23–25], lactate dehydrogenase [26,27] and adenylate cyclase [28], and to inhibit the production and/or release of endothelium-derived relaxing factor [29], the inhibition of phospholipases by gossypol might, not surprisingly be an effect of inhibition of a number of enzymes in the cells. Further investigation is necessary in order to clarify the effectiveness of gossypol on tumor promotion.

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