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Glucocorticoid-induced phospholipase A_2-inhibitory proteins mediate glucocorticoid teratogenicity *in vitro*

(glucocorticoid action/cleft palate/prostaglandins/arachidonic acid)

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ABSTRACT **Dexamethasone induces the synthesis of a phospholipase A_2-inhibitory protein (PLIP) of molecular weight ≈55,000 from calf thymus and PLIPs of molecular weights 55,000, 40,000, 28,000, and 15,000 from A/J mouse thymus and from 12-day embryonic B10.A mouse palates. Sufficient quantities of calf thymus PLIP and of the 15,000 molecular weight mouse thymus and palate PLIPs were prepared and tested as inhibitors of programmed cell death in the medial-edge epithelium of single mouse embryonic palatal shelves in culture. All of the proteins tested prevent the loss of the medial-edge epithelium and, thus, produce the teratogenic effects of glucocorticoids in the palatal culture model. This teratogenic action of both PLIP and glucocorticoids is reversed by arachidonic acid, the precursor of prostaglandins and thromboxanes, suggesting that PLIP mediates the effects of glucocorticoids by inhibiting phospholipase A_2.**

Although glucocorticoids are known to produce cleft palate in various mammals (1–5), little is known about the precise mechanism by which they produce their teratogenic action. The degree of cleft palate produced by glucocorticoids is in direct proportion to their anti-inflammatory activity. The steroids act as a consequence of their binding to cytoplasmic receptor proteins, followed by the translocation of the ligand–receptor complex into the nucleus, which in turn affects transcription of various RNA species (6–8). It has been demonstrated recently that glucocorticoids induce their anti-inflammatory action by producing a protein that inhibits phospholipase A_2, the enzyme that produces arachidonic acid from phospholipids (9–12). Thus, the availability of arachidonic acid, the substrate for prostaglandin and thromboxane biosynthesis, is reduced after exposure to glucocorticoids. The synthesis of this protein is mediated by the glucocorticoid receptor and is blocked by inhibitors of RNA and protein synthesis (12).

The clefting action of glucocorticoids involves (*i*) a delay of the elevation of the embryonic palatal shelves from a vertical position to a horizontal position *in vivo* (13–16) and (*ii*) inhibition of palatal shelf medial-edge lysosomal enzyme activity and epithelial breakdown both *in vivo* (15, 16) and in single embryonic palatal shelves *in vitro* (15, 16). Glucocorticoid-induced palatal teratogenicity can be corrected significantly by arachidonic acid in the rat (17) and mouse (18, 19) *in vivo* and in the mouse *in vitro* (18, 19). The corrective effect of arachidonic acid can be prevented by indomethacin, an inhibitor of cyclooxygenase, *in vivo* and *in vitro* (17–19). These results indicate that glucocorticoids inhibit the release of arachidonic acid and prevent the subsequent production of prostaglandins or thromboxanes, or both, in the induction of cleft palate, thereby paralleling their anti-inflammatory action. This led us to postulate that the teratogenic action of glucocorticoids may be mediated also by the production of phospholipase A_2-inhibitory proteins (PLIPs). In this paper, we report that the teratogenic action of glucocorticoids *in vitro* is produced by a dexamethasone-induced calf thymus PLIP and that this teratogenic action of PLIP, like that of glucocorticoid (17–19), is reversed by arachidonic acid.

MATERIALS AND METHODS

Purification and Assay of PLIP. The PLIP was produced from calf thymuses by using a modification of the procedure of Hirata *et al.* (12). Calf thymus tissue slices (100 g) were incubated in 500 ml of RPMI 1640 medium in the presence of 50 μM dexamethasone, penicillin (100 units/ml), and streptomycin (100 mg/ml) for 16–18 hr, and PLIP was partially purified by the following three steps. Preliminary separation of PLIP was performed by adsorbing the medium from incubation on a DEAE-cellulose column and eluting the inhibitory protein from the column with 0.25 M Tris·HCl buffer. The PLIP fraction was dialyzed against deionized water overnight, and the pH was adjusted to 7.6 with NaOH. This protein fraction was purified further on a second DEAE-cellulose column with a linear gradient of Tris·HCl buffer (0.1–0.3 M). Finally, the PLIP fraction was purified by Sephadex G-100 gel filtration and was electrofocused as described (20) to determine the pI value of this protein. This partially purified PLIP fraction was dialyzed and lyophilized and was used in the single-shelf culture system as described by Goldman *et al.* (16) to test its ability to inhibit programmed cell death in the medial-edge epithelium of embryonic palatal shelves.

PLIP was assayed by its ability to inhibit snake venom phospholipase A_2 in the procedure described by Hirata *et al.* (12) with some modifications (see Table 1 for a definition of 1 unit of PLIP). Phospholipase A_2 activity was measured *in vitro* with and without PLIP. The reaction mixture contained, in a total volume of 1 ml, 50 mM Tris buffer (pH 8.0), 0.1 unit of snake venom phospholipase A_2, and 20,000 dpm of 1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylcholine (59.3 mCi/mmol; 1 Ci = 37 GBq) and was incubated at 37°C for 15 min. The reaction was stopped by adding 6 ml of hexane to the incubation tubes, and each tube was mixed for 10 sec. [^{14}C]Arachidonic acid released by phospholipase A_2 action was extracted into the hexane layer. Three milliliters of the hexane layer was assayed for radioactivity to measure the phospholipase A_2 activity.

Embryonic Single-Shelf Culture. This procedure as described before (15, 16) permits normal disruption of the medial-edge epithelium in cultured shelves. In brief, single pala-

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Abbreviation: PLIP, phospholipase A_2-inhibitory protein.

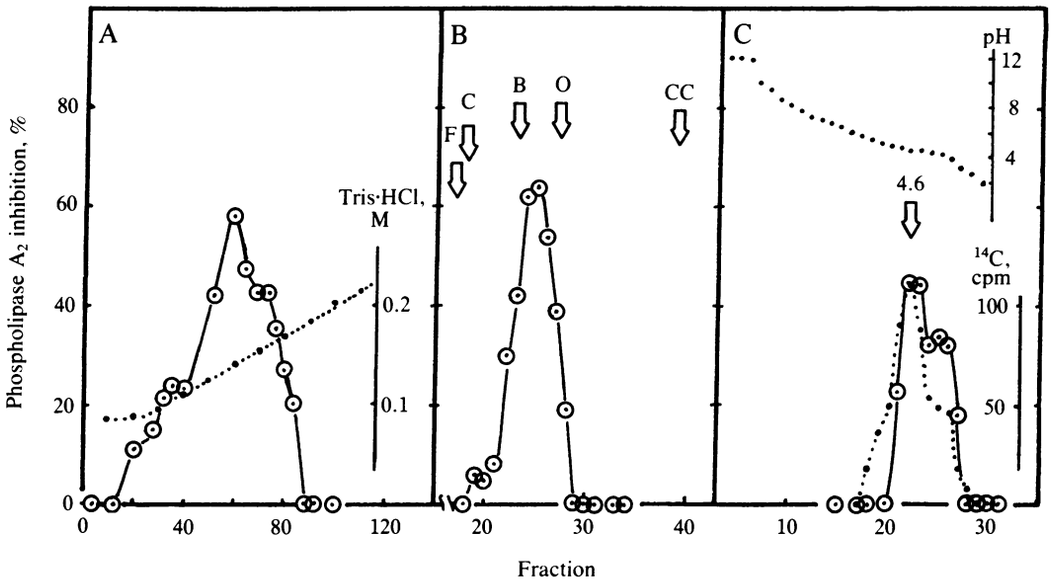

FIG. 1. Partial purification of PLIP. (*A*) DEAE-cellulose column chromatography under a linear gradient system. The concentration of Tris·HCl (•···•) was determined by conductivity, and the phospholipase A_2-inhibitory activity (—) was determined by assaying *Naja Naja* snake venom phospholipase A_2 in the presence of a 20-μl aliquot of each fraction as described. (*B*) Gel filtration of PLIP. The PLIP fraction from the DEAE-cellulose column was dialyzed against deionized water and was lyophilized. The dried material was dissolved in 2 ml of 0.01 M Tris·HCl buffer (pH 8.0) and was gel-filtered through a Sephadex G-100 column (2 × 30 cm) with the same buffer. Locations of standard proteins are as follows: F, ferritin; C, catalase (V_o); B, bovine serum albumin (M_r, 67,000); O, ovalbumin (M_r, 43,000); and CC, cytochrome *c* (M_r, 12,300). Each fraction contained 2.0 ml of filtrate. (*C*) pI value of PLIP. [^{14}C]Leucine-labeled PLIP was prepared by incubating calf thymus with [^{14}C]leucine (51.63 mCi/mmol; 0.5 mCi/200 ml of incubation mixture) and was partially purified by the method mentioned above. The partially purified protein was electrofocused as described (20). Each fraction contained 0.5 ml. Radioactivity (•···•) was determined in a 100-μl aliquot of each fraction, and phospholipase A_2-inhibitory activity (—) was determined by using a 20-μl aliquot of each fraction.

tal shelves from 13.5-day embryos were cultured on Millipore filters over medium consisting of 10% fetal bovine serum plus penicillin (100 units per ml) and streptomycin (100 μg per ml) in minimal essential medium (ME medium). PLIP was solubilized in ME medium, sterilized by Millipore filtration, and included in the culture medium as a 2% addition. Arachidonic acid was diluted with ethanol and included as a 0.5% addition. All additions to the culture medium were made at the expense of appropriate volumes of ME medium. The cultures were incubated at 37–38°C in humidified 5% CO_2/95% air for 48–60 hr. The shelves were fixed in a 4% formaldehyde/1% $CaCl_2$ solution, dehydrated, and embedded in glycol methacrylate. Sections (2.0 μm) were prepared by using glass knives and were stained with Lee's methylene blue-basic fuchsin. Transverse sections were cut through the middle third of both control and test shelves (15, 16). The medial edge of each shelf was routinely scored as epithelial-free or epithelial-bound by at least two experienced observers working independently.

RESULTS

Separation of PLIP. The results show that after the second DEAE column purification of the calf thymus material, a protein with phospholipase A_2-inhibitory activity appeared at 0.15 M salt concentration (Fig. 1*A*). The molecular weight of the PLIP by Sephadex G-100 analysis was calculated to be ≈55,000 (Fig. 1*B*). The pI value of this protein fraction as determined by isoelectrofocusing is about 4.6 (Fig. 1*C*). In one experiment, incubation with [^{14}C]leucine in the medium gave an electrofocusing pattern with the radioactivity and phospholipase A_2-inhibitory activity of this fraction appearing at the same position (Fig. 1*C*), indicating that a PLIP was synthesized during the incubation period from the amino acid source of the medium.

Effect of PLIP on the Embryonic Single-Shelf Epithelial Breakdown: Calf Thymus PLIP. This protein fraction, when tested in cultures of single palatal shelves from CD-1 and B10.A mouse embryos, produced a dose-dependent inhibition of embryonic palatal epithelial breakdown in both glucocorticoid-susceptible strains (Table 1). At a concentration of 34 milliunits/ml of culture medium, PLIP permitted breakdown of the medial-edge epithelium in only 12% of the CD-1 shelves and 25% of the B10.A shelves. This degree of inhibi-

Table 1. PLIP activity and reversal

Treatment	Mouse strains* CD-1		B10.A	
Control	65/65	(100)	28/28	(100)
Cortisol (0.1 μg/ml)†	2/10	(20)‡	1/6	(17)‡
PLIP (milliunits/ml)				
85.0	0/3	(0)‡	—	—
34.0	2/17	(12)‡	1/4	(25)‡
17.0	3/6	(50)‡	2/5	(40)‡
8.5	2/5	(40)‡	2/3	(67)‡
0.85	2/7	(29)‡	2/4	(50)‡
0.085	2/4	(50)‡	2/4	(50)‡
0.0085	3/5	(60)‡	2/2	(100)
PLIP (34 milliunits/ml)				
With arachidonic acid (1 μg/ml)	5/8	(63)§	—	—

Inhibition by dexamethasone-induced PLIP in calf thymus of medial-edge epithelial breakdown in cultured single palatal shelves from CD-1 and B10.A mouse embryos, and reversal of PLIP inhibition by arachidonic acid in CD-1 shelves.

*Ratios represent the number of shelves with epithelial breakdown per total number of cultured shelves, and numbers in parentheses indicate the percentage of cultured shelves with epithelial breakdown.

†One unit of PLIP inhibits 1 unit of *Naja Naja* snake venom phospholipase A_2 activity. One unit of the enzyme hydrolyzes 1 μmol of L-α-phosphatidylcholine to L-α-lysophosphatidylcholine and a fatty acid per min at pH 8.9 at 25°C.

‡Statistically significant ($P < 0.001$) compared to controls.

§Statistically significant ($P < 0.001$) compared to PLIP (34 microunits/ml) without added arachidonic acid.

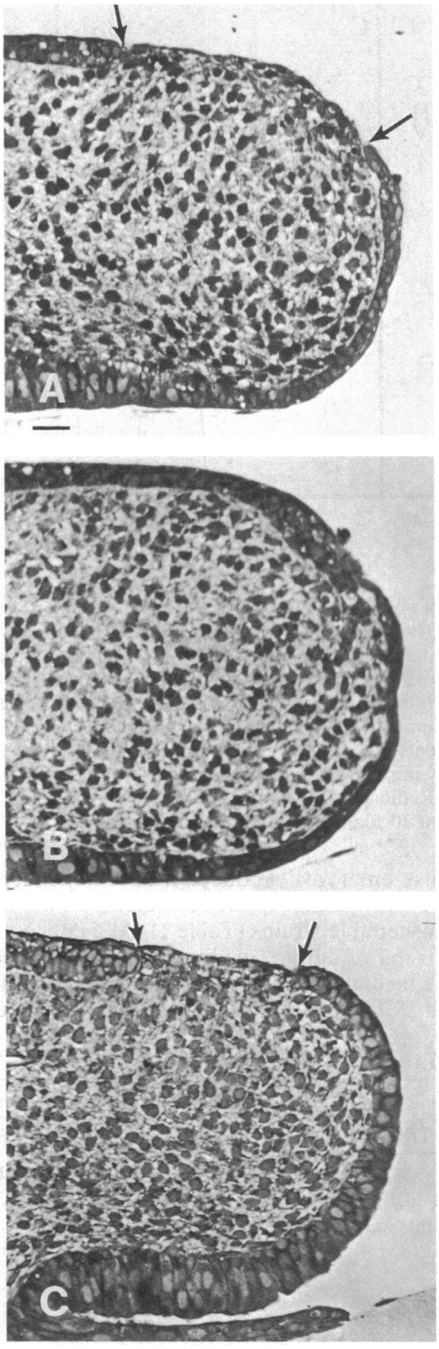

FIG. 2. Transverse sections of the medial edge of single palatal shelves cultured for 48–60 hr. In *A*, *B*, and *C*, the upper surface is oral and the lower surface is nasal, and the magnifications are the same. (*A*) Control with an epithelial-free medial edge (between arrows). (*B*) Shelf treated with PLIP (34 milliunits/ml), which inhibits the breakdown of the medial-edge epithelium. (*C*) Shelf in which the PLIP inhibition is reversed by arachidonic acid (1 μg/ml), resulting in the loss of epithelium at the medial edge (between arrows). As *A* and *C* illustrate, the palatal medial edge is not always maintained precisely in its normal anatomical position in single shelves in culture. (Bar in *A* = 50 μm.)

tion is generally comparable to that obtained with cortisol (0.1 μg/ml) in culture (Table 1). Fig. 2 illustrates the loss of the medial-edge epithelium in a CD-1 control (Fig. 2*A*) and retention of this epithelial population in a CD-1 shelf treated with PLIP at 34 milliunits/ml (Fig. 2*B*). Addition of arachidonic acid (1 μg/ml) to the culture medium containing PLIP (34 milliunits/ml) produced a highly significant ($P < 0.001$) reversal of the PLIP inhibition in CD-1 shelves (Table 1 and Fig. 2*C*).

PLIPs from Mouse Thymus and Embryonic Palate. We obtained four PLIPs from A/J mouse thymocytes and day 12 B10.A embryonic palates (incubated with dexamethasone under identical conditions as with calf thymus) with molecular weights of 55,000, 40,000, 28,000, and 15,000. Sufficient quantities of the 15,000 molecular weight PLIP from both thymus and palate were prepared and tested for inhibitory activity in the culture model. The 15,000 molecular weight thymus PLIP in concentrations ranging from 0.0051 to 0.05 milliunits/ml inhibited epithelial breakdown in 33–100% of the cultured shelves. Similarly, the 15,000 molecular weight palatal PLIP in concentrations from 2.5 to 250 milliunits/ml inhibited the epithelial breakdown in 40–100% of the cultured shelves. Thus, the PLIPs of different origin and different molecular weights produce the effects of glucocorticoids in the culture model.

DISCUSSION

Glucocorticoids exhibit a variety of biological effects, such as enzyme induction, anti-inflammatory action, and teratogenic action (cf. 12, 20–22). Both the teratogenic and anti-inflammatory actions appear to be mediated by PLIPs. Blackwell *et al.* (10) and Hirata *et al.* (12) isolated such proteins, which demonstrated a considerable similarity in their anti-inflammatory activity (including the inhibition of prostaglandin biosynthesis) to that of glucocorticoids. Here, we demonstrate the induction of PLIPs by glucocorticoids that are able to produce the *in vitro* teratogenic action of glucocorticoids in a palatal shelf culture model.

We have demonstrated that a PLIP from calf thymus has a molecular weight of ≈55,000, whereas mouse thymus and embryonic palate produce four different PLIPs of molecular weights 55,000, 40,000, 28,000, and 15,000. Blackwell *et al.* (10) showed that a PLIP induced by glucocorticoids in guinea pig lungs and rat leukocytes had a molecular weight of 15,000 and called it "macrocortin" (10). Hirata *et al.* (12) demonstrated that a PLIP induced by glucocorticoids in rabbit neutrophils had a molecular weight of 40,000 and called it "lipomodulin" (12). At present, it is not known whether these proteins are fragments from one single PLIP or are different proteins. At any rate, the present results demonstrate that PLIPs of different molecular weights and different species are capable of duplicating the effects of glucocorticoids in the shelf culture model.

PLIP also has been detected in calf thymuses incubated in the absence of dexamethasone (data not shown). However, the amount present is substantially less than that produced by dexamethasone-treated calf thymuses. Hirata *et al.* (12) also reported such an endogenous level of PLIP in the absence of glucocorticoids.

Thus, this paper demonstrates that glucocorticoids induce the production of PLIPs in the calf thymus, mouse thymus, and mouse embryonic palate that are able to mimic the *in vitro* teratogenic action of glucocorticoids—namely, the inhibition of epithelial breakdown in embryonic palatal shelves *in vitro* and the reversal of teratogenic action by arachidonic acid.

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