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Action of corticosteroids in regulation of prostaglandin biosynthesis in cultured fibroblasts

(cyclo-oxygenase/prostacyclin/anti-inflammation/virus transformation/lipoxygenase)

K. A. CHANDRABOSE, E. G. LAPETINA, C. J. SCHMITGES, M. I. SIEGEL, AND P. CUATRECASAS

Department of Molecular Biology, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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ABSTRACT Various vasoactive agents (e.g., thrombin and bradykinin) and serum stimulate arachidonate production and thus prostaglandin biosynthesis in cultured fibroblasts. Treatment of 3T3 cells with the anti-inflammatory steroid, dexamethasone, inhibits this stimulation but has no inhibitory effect on the basal activity of phospholipase (or on prostaglandin content) in resting, confluent fibroblasts. In intact cells, the proportion of released arachidonic acid converted into prostaglandins is increased by steroid treatment; in quiescent, dense cells and in serum-treated cells, the total incorporation into prostaglandins is increased. Furthermore, the cyclo-oxygenase activity of homogenates from steroid-treated cells is increased very substantially. Thus, although steroids may affect phospholipase (EC 3.1.1.4) activities it is possible that these effects may be secondary to a more important stimulatory effect on cyclo-oxygenase activity which leads to selective alterations in prostaglandin biosynthesis. The steroid-induced increase in cyclo-oxygenase activity is not observed in a transformed variant of the same cell line. Fatty acid lipoxygenase (EC 1.13.11.12) activity exists in the particulate rather than the cytosolic fraction of 3T3 cells.

Prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$), which are produced by various fibroblast lines in culture (1, 2), have been implicated frequently in inflammatory reactions (3). Although anti-inflammatory steroids have been reported to inhibit prostaglandin synthesis (4), the mechanism of action of steroids has not been clearly established. High concentrations of anti-inflammatory steroids have been found to inhibit *in vitro* preparations of prostaglandin synthetase (5), while other authors observed no inhibition at moderate concentrations (6, 7). Chang *et al.* (8) reported that steroids prevent the release of prostaglandins rather than affect their biosynthesis. While Gryglewski *et al.* (9) suggested that steroids reduce the availability of substrate for prostaglandin synthesis, Hong and Levine (10), working with transformed mouse fibroblasts, observed that anti-inflammatory steroids could inhibit phospholipase activity in treated cells and postulated that this inhibition may be fundamental to the anti-inflammatory action of steroid hormones.

We have confirmed the inhibitory action of corticosteroids on the stimulated activity of phospholipases (EC 3.1.1.4) in Swiss 3T3 mouse fibroblasts, although the basal enzyme activity is apparently not affected. It is not clear whether this inhibition is a secondary, compensatory response of the intact cell since steroids rapidly induce an increase in fatty acid cyclo-oxygenase activity, the first step in the multienzyme complex known as prostaglandin synthetase.

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MATERIALS AND METHODS

3T3 (Swiss) fibroblasts and their transformed derivative (SV40, 3T3) were purchased from Microbiological Associates and were cultured in Dulbecco's modified Eagle's minimal essential medium with 10% fetal calf serum, penicillin, and streptomycin. [*1*-^{14}C]Arachidonate (specific activity 61 mCi/mmol) was purchased from Amersham. Cells, seeded at an initial density of 2×10^6 cells per 13-cm petri dish, were grown to confluency, (fed on the third day) and allowed to remain in medium without further change. Experiments were conducted only on completely quiescent cells. Cells were labeled for 15 hr with 0.1 μCi of [*1*-^{14}C]arachidonate per ml (prepared by sonicating the labeled substrate in the resting medium). After this labeling period, steroid dissolved in alcohol was added to a final concentration of 5 μM. Control cultures received only alcohol (1 μl/ml). At various times, the medium was removed, the cells were washed twice with minimal essential medium, and the medium was replaced with minimal essential medium. The cultures were further incubated with the specified additions for the indicated time, after which the medium was removed and lyophilized. Prostaglandins were extracted from the dried medium with ethylacetate, concentrated, back-extracted with ethyl acetate from phosphate buffer (pH 5), and concentrated. Aliquots were chromatographed as described below. Silica gel in radioactive areas was scraped from plates and radioactivity was measured by scintillation techniques.

Silica gel plates (Brinkmann) were developed by ascending chromatography using the top layer of ethylacetate/2,2,4-trimethylpentane/acetic acid/water (90:50:20:100, vol/vol). Recovery was monitored by the addition of tritiated PGE_2 and arachidonate to the incubations immediately before they were stopped.

The cyclo-oxygenase assays were conducted on cells disrupted by hand homogenization in a tight-fitting glass homogenizer. Crude homogenate was assayed for 2 min in 0.1 M phosphate buffer (pH 8.5), containing 0.2–50 μM [*1*-^{14}C]arachidonate (2000–200,000 cpm per assay) and 0.6 mM epinephrine bitartrate in a total volume of 100 μl. Reactions were terminated by the addition of 1 ml of chloroform/methanol (2:1, vol/vol). The solvent was partitioned against 0.2 ml of 1% formic acid and the chloroform layer was evaporated, chromatographed, and quantified as described above.

Prostaglandins were characterized by the following criteria: (*a*) labeled products cochromatographed with authentic prostaglandins in two different chromatographic systems; (*b*) cells labeled with palmitate or stearate and identically processed did

Abbreviations: PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; 6-keto-$PGF_{1\alpha}$, prostaglandin-6-keto-$F_{1\alpha}$.

Table 1. Production of metabolites from endogenous arachidonate under basal and stimulated states

Addition	Metabolite, cpm × 10^{-3}						
	6-keto-$PGF_{1\alpha}$	PGE_2	PGD_2	PGA_2	HETE	Arachidonate	Total
Serum	2.46	9.40	2.45	4.33	4.80	84.07	107
Serum + steroid	9.26	17.45	2.61	4.61	2.00	55.13	91
Thrombin	13.24	34.46	4.07	7.41	7.91	113.3	180
Thrombin + steroid	10.51	26.22	2.22	2.18	4.88	64.4	110
Bradykinin	32.19	68.54	15.74	8.18	18.73	276.3	419
Bradykinin + steroid	19.82	35.79	4.81	7.06	9.47	92.40	169
MEM	3.59	7.96	1.11	1.97	2.45	36.0	53
MEM + steroid	7.73	14.69	1.69	3.60	2.84	39.14	69

Arachidonate metabolites were analyzed. The duration of treatment with stimuli were 20 min with thrombin (1 unit/ml) or bradykinin (1 μg/ml), 30 min with serum (10%, vol/vol), and 40 min with minimal essential medium (MEM). The last column is the total amount of arachidonate and its metabolites found in the culture medium per 10^7 cells. Cells were pretreated with 5 μM dexamethasone for 6 hr and it was present during subsequent incubations. Prostaglandins not tabulated contained less than 100 cpm. HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

not show radioactive products cochromatographing with the prostaglandins; (*c*) preincubation of cells with 10 μM indomethacin for 15 min reduced the labeling in these products to 5% of the control values; (*d*) the products found from *in vivo* experiments were produced *in vitro* from [*1*-^{14}C]arachidonate; and (*e*) the labeled products from these cells cochromatographed with arachidonate metabolites from human platelet lysates, a well-characterized prostaglandin-synthesizing system. The two other common products of arachidonate metabolism are 12-hydroxy-5,8,10-heptadecatrienoic acid and 12-hydroxy-5,8,10,14-eicosatetraenoic acid. Both were detected in the cells and cochromatographed with hydroxyheptadecatrienoic and hydroxyeicosatetraenoic acids produced by platelet lysates.

These cells also make another prostaglandin product, prostaglandin-6-keto-$F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). The labeled compound cochromatographed with authentic 6-keto-$PGF_{1\alpha}$ in two different solvent systems. Moreover, its biosynthesis was selectively inhibited by 15-hydroperoxytetraenoic acid in intact cells and its formation in *in vitro* assays was selectively inhibited by reduced glutathione (11). Since the immediate precursor of 6-keto-$PGF_{1\alpha}$ is prostacyclin (11), we conclude that prostacyclin is an arachidonate metabolite in these cells.

RESULTS

The effect of steroid treatment on arachidonate metabolism was investigated in dense, quiescent cultures of 3T3 cells in the presence and absence of various stimuli. Since analysis of cell-monolayer lipids showed no detectable prostaglandin products, as also reported by others (1), the total radioactivity in arachidonate products in the medium is a reasonable estimate of the total phospholipase activity. In most cases, steroid pretreatment reduced the total amount of arachidonate and its metabolites (Table 1). However, in nonstimulated cells steroid pretreatment failed to inhibit phospholipase activity (Table 1).

Despite this apparent reduction of total phospholipase activity, as reflected by the lowering of total arachidonate metabolites secreted into the medium, the proportion of released arachidonate that was converted into prostaglandin products was consistently higher in steroid-treated than in control cells (Table 1). This was particularly striking in serum-stimulated cells where, despite a small reduction of total arachidonate metabolites, the absolute amounts of 6-keto-$PGF_{1\alpha}$ and PGE_2, the major prostaglandin products in 3T3 cells, were increased by 4- and 2-fold, respectively. Therefore, in this case the observed reduction in free arachidonate resulting from steroid treatment can be explained entirely by an increase in prostaglandin synthesis. The hypothesis that this apparent increase in prostaglandin production is due to an effect of steroid treatment on cyclo-oxygenase activity rather than on changes in arachidonic acid concentration within the cells is further supported by the observation that in unstimulated cells there was an absolute increase in amounts of prostaglandin products formed in steroid-treated cells. Even when thrombin and bradykinin were used as stimulating agents for phospholipase, and where the decreased total amount of arachidonate metabolites may reflect an overall reduction of phospholipase activity, moderate but consistently enhanced percentages of prostaglandin synthetase products were observed.

Since these results suggested two opposing biochemical effects of dexamethasone at the level of the intact cell, *in vitro* assays of cyclo-oxygenase activity were used to dissociate such effects of steroids from changes in phospholipase activity. The cyclo-oxygenase activity had a broad pH optimum between 7.8 and 9.5, and the formation of all compounds was linear for at least 2 min. While the amounts of PGE_2 and $PGF_{2\alpha}$ increased (but nonlinearly) up to 15 min, there was no increase in the

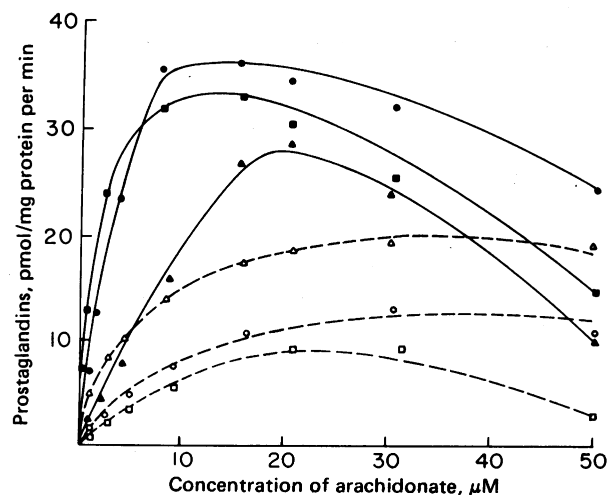

FIG. 1. Effect of dexamethasone treatment of cultured fibroblasts on the rate of conversion of arachidonate to prostaglandins (i.e., cyclo-oxygenase activity) by cell homogenates. Protein concentration (12) was 1 mg/ml in control and 0.6 mg/ml in steroid-treated cell homogenates. The cyclo-oxygenase assay reactions were terminated after 1 min. Cells were preincubated for 6 hr with 5 μM dexamethasone. Filled symbols represent activities from steroid-treated cells, open symbols from control cells. △ and ▲, PGE_2; □ and ■, 6-keto-$PGF_{1\alpha}$; ○ and ●, $PGF_{2\alpha}$. Addition of steroid to the assay mixture had no effect on specific activity of control homogenate.

Table 2. Subcellular location of fatty acid cyclo-oxygenase and lipoxygenase in 3T3 cells

	Control				Steroid			
Enzyme source	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	HETE	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	HETE
Homogenate	10.6	8.2	11.1	15.6	38.2	43.3	31.6	28.9
800 × *g* pellet	2.8	1.6	3.5	2.4	3.5	2.8	3.7	4.2
100,000 × *g* pellet	18.2	14.5	21.3	28.7	31.3	38.7	26.1	26.5
Cytosol	<1	<1	<1	<1	<3	<3	<3	<3

The pellets were resuspended in a volume of buffer equal to the original homogenate before cyclo-oxygenase assay (see Fig. 1). Equal aliquots of homogenate, pellets, and cytosol were assayed. The data are expressed as pmol of product formed/min per mg of protein. HETE, hydroxyeicosatetraenoic acid.

amount of 6-keto-$PGF_{1\alpha}$ after 2 min. The activities were linear with protein concentrations up to 1.4 mg/ml.

In vitro assays of the cyclo-oxygenase of steroid-treated 3T3 cells demonstrated substantially greater activity than those of control cells (Fig. 1). Although $PGF_{2\alpha}$ was only a trace metabolite *in vivo* (legend of Table 1), it was a substantial product *in vitro*. In homogenates of control cells, regardless of the arachidonate concentration, the prostaglandin synthetase produced more PGE_2 than $PGF_{2\alpha}$ and even less 6-keto-$PGF_{1\alpha}$. However, in the steroid-treated homogenates, the product distribution varied with the substrate concentration. The steroid-treated enzyme preparation was much more susceptible to substrate inhibition than the control synthetase. Nevertheless, the most important observation is the fact that the specific activity of the enzyme from steroid pretreated cells increased from 50 to 400%, depending upon the specific prostaglandin measured. Despite these large effects (i.e., increases) in maximal velocities, the concentrations of arachidonate necessary for half-maximal activity occur over a relatively narrow range, between 1–9 and 3–5 μM for the enzyme from control and steroid-treated cells, respectively.

The steroid-induced increase in cyclo-oxygenase activity was relatively more sensitive to the duration of incubation with steroid than to the steroid concentration. Thus, 1–10 μM dexamethasone caused similar increases in activity when the incubation time is the same. Furthermore, the increase in activity after cells are incubated with steroid for 2 hr was less than 50% of that observed after 4 hr. Incubations of 4–6 hr with dexamethasone appeared to be optimal, and a minimum of 2 hr was necessary for demonstrable changes in activity.

Cell fractionation experiments indicated that fatty acid cyclo-oxygenase appeared to be localized predominantly in the 100,000 x *g* pellet, in agreement with previously published reports (Table 2) (7). However, lipoxygenase activity, which until recently was generally believed to be cytosolic (13, 14), was found almost exclusively in the particulate fraction of the 3T3 cell and therefore fractionates with cyclo-oxygenase. In steroid-treated cells, instead of the specific activity of the cyclo-oxygenase increasing during fractionation of crude homogenates to particulate fractions by centrifugation at 100,000 x *g*, as observed in the control cells, the specific activity of the steroid-treated cells actually decreased. Preliminary recombination experiments demonstrated moderate increases in the specific activity when cytosolic fractions from steroid-treated cells were added back to the 100,000 × *g* pellets from either steroid-treated or control cells. However, these increases still did not reach the cyclo-oxygenase activities achieved in the crude homogenates of steroid-treated cells. In addition, in these experiments the effect of added cytosol was more apparent at high rather than low concentrations of arachidonate.

Table 3. Cyclo-oxygenase and lipoxygenase activities of growing and quiescent 3T3 cells

	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	HHT	HETE
Growing cells	15.7	12.6	17.8	9.2	22.8
Dense, quiescent cells	16.1	13.1	16.7	8.6	23.0

Growing cells were homogenized and assayed (see Fig. 1) after one complete cycle of division. Quiescent cells were used 1 week after they were seeded. Similarly seeded controls were monitored for 3 days for cessation of growth by measuring cell number. The data represent pmol/min per mg of protein. HHT, hydroxyheptadecatrienoic acid; HETE, hydroxyeicosatetraenoic acid.

Previous studies (15) have shown that steroids, especially dexamethasone, can stimulate cell growth in 3T3 cells. In the present experiments, overnight incubations of cells with dexamethasone in the absence of added mitogen increased the number of cells in a resting population. If growing cells in fact have higher cyclo-oxygenase activity compared to dense, quiescent cells, then the activity of this enzyme may be related to growth initiation in an important way. However, the cyclo-oxygenase activities of growing and dense, quiescent cells were not appreciably different (Table 3), suggesting that the effect of steroid on cyclo-oxygenase activity in 3T3 cells may not be growth related; similar results have been observed with β-methasone and with a different clone of 3T3 cells.*

In contrast to normal cells, virally (simian virus 40) transformed 3T3 cells did not exhibit the steroid-induced increase in cyclo-oxygenase activity when measured in either homogenates or 100,000 × *g* pellets (Table 4). This is consistent with the reported refractoriness of transformed cells to glucocorticoids in studies of stimulation of growth initiation by steroids (14).

DISCUSSION

Anti-inflammatory drugs have been shown to inhibit prostaglandin synthesis. Nonsteroidal drugs, such as aspirin and indomethacin, have been clearly established as inhibitors of fatty acid cyclo-oxygenase (16), the first enzyme in the metabolic conversion of arachidonic acid to prostaglandins. On the other hand, anti-inflammatory steroids have been thought to inhibit arachidonate metabolism at the level of membrane phospholipase activities, thus acting by limiting the availability of arachidonate for conversion to prostaglandins (10). Until now steroids were thought to have no effect on fatty acid cyclo-oxygenase.

This report confirms earlier observations (9, 10) that treatment of cells with anti-inflammatory steroids inhibits endogenous phospholipase activities. However, this inhibition is only evident when phospholipases are stimulated by exogenous factors. For example, when cells are pretreated with dexamethasone, the total phospholipase activity after stimulation with thrombin or bradykinin is severely inhibited (Table 1).

* A clone of 3T3 cells was kindly supplied by J. J. Collins, Department of Surgery, Duke Medical Center, Durham, NC 27710.

Table 4. Cyclo-oxygenase and lipoxygenase activities of 3T3 and SV3T3 cells incubated in the presence of dexamethasone

Enzyme source	Control			Steroid		
	6-keto-$PGF_{1\alpha}$	PGE_2	HETE	6-keto-$PGF_{1\alpha}$	PGE_2	HETE
3T3						
Homogenate	13.5	10.64	22.1	28.6	22.7	25.6
100,000 × *g* pellet	18.9	13.7	28.9	25.1	23.6	27.8
SV3T3						
Homogenate	14.6	15.8	26.8	13.7	14.8	22.5
100,000 × *g* pellet	17.5	18.6	27.5	14.2	15.9	25.3

Assays were conducted as described in Table 2. The 3T3 cells were from confluent, resting cultures. SV40, 3T3 cells were used at comparable cell densities. Cultures were treated with dexamethasone (5 μM) for 4 hr. Both cell types had identical schedules of medium changes. The data are expressed as pmol/min per mg of protein. HETE, hydroxyeicosatetraenoic acid.

Moderate inhibition is seen when serum is the stimulus, and unstimulated cells exhibit no inhibition.

Notably, steroid pretreatment of cultured cells leads to increased conversion of endogeneously released arachidonate to prostaglandins, especially in resting cells or those stimulated only with serum (Table 1). This increased metabolism of arachidonate is clearly the result of higher cyclo-oxygenase activities in cells treated with steroid, as demonstrated by studies on broken cell preparations (Fig. 1 and Table 2). This effect of steroids must be considered seriously in explanations of possible anti-inflammatory actions.

It is not known whether a decrease in phospholipase or an increase in cyclo-oxygenase activity is the earlier event in the action of anti-inflammatory steroids or whether one effect is simply the results of the other (e.g., by compensation). This is an important question, especially since we recently reported (17) that cyclic AMP inhibits thrombin-induced phospholipid breakdown in platelets. Prostacyclin, a potent stimulator of platelet adenylate cyclase, was found to be the most effective inhibitor of platelet phospholipase activity. The facts that the cyclo-oxygenase activity can be elevated by steroid treatment even under conditions where phospholipase activity is unaltered (Table 1 and Fig. 1), that there is increased production of prostacyclin in steroid-treated 3T3 cells (Table 1), and that *in vitro* cyclo-oxygenase assays (Fig. 1 and Table 2) reveal the formation of higher amounts of 6-keto-$PGF_{1\alpha}$ (the breakdown product of prostacyclin) could suggest that the effect of steroids on cyclo-oxygenase might occur early and that the resulting increase in prostacyclin production via cyclic AMP could account for the observed decrease in phospholipase activity. We do not yet know whether this temporal sequence of events actually occurs.

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