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Differential Effect of Dexamethasone on the Regulation of Phospholipase A_2 and Prostanoid Synthesis in Undifferentiated and Phorbolester-Differentiated U937 Cells

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The human undifferentiated histiocytic cell-line U937 can be induced to differentiate by incubation with 12-0-tetradecanoylphorbol-13-acetate (TPA) into macrophage-like cells. Dexamethasone reduced the prostaglandin production in TPA-differentiated U937 cells dose dependently, whereas undifferentiated U937 cells were dexamethasone insensitive. Concomitantly phospholipase A_2, the enzyme liberating the prostaglandin precursor arachidonic acid, was inhibited by dexamethasone in TPA-differentiated but not in undifferentiated U937 cells. The activity of lysophosphatide acyltransferase, the key enzyme of fatty acid reacylation into phospholipids, remained unchanged both in undifferentiated and TPA-differentiated U937 cells. The data suggest that responsiveness to glucocorticoid-dependent regulation of prostanoid synthesis is acquired by cells of the monocyte-macrophage lineage late in differentiation.

Key words: U937 cell line, differentiation, prostaglandins

Glucocorticoids are well-known inhibitors of prostaglandin and leukotriene synthesis in inflammatory cells such as macrophages [1]. Inhibition is receptor mediated [2] and dependent on protein synthesis [3]. The rate of eicosanoid synthesis is regulated by the availability of free arachidonic acid which can be metabolized into prostanoids and leukotrienes via the cyclooxygenase and lipoxygenase pathways. The release of the precursor fatty acid is primarily dependent on the enzyme phospholipase A_2 which cleaves arachidonic acid from membrane phospholipids [4]. Accordingly, lipocortin has been proposed as the glucocorticoid-induced protein responsible for the inhibition of eicosanoid synthesis possibly as a result of its antiphospholipase A_2 activity [5].

Recently, several questions have been raised concerning the validity of the lipocortin model. Davidson and Dennis were able to demonstrate that the phospholipase A_2 was not directly inhibited by lipocortin but by nonspecific substrate interactions of lipocortin

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with phospholipids [6]. This observation has been confirmed by other groups [7,8]. Furthermore, it rapidly became obvious that lipocortin belongs to a large family of cytoskeletal proteins present in every cell [9].

The inhibition of the eicosanoid synthesis by glucocorticoids, however, appears to be restricted to a few cell types. Thus, glucocorticoids, when administrated in vivo in healthy men [10] or rabbits [11], do not suppress the overall synthesis of prostaglandins in contrast to cyclooxygenase inhibitors [12].

In order to get more detailed information about the molecular mechanism of the inhibition of eicosanoid synthesis by dexamethasone, we used the undifferentiated human macrophage-like cell line U937, first described by Sundström and Nilsson [13]. U937 cells, which normally grow in suspension, can be differentiated by addition of 5×10^{-9} M 12-O-tetradecanoylphorbol-13-acetate (TPA) for 3 days. During the differentiation process the cells cease to divide and undergo a number of marked morphological changes [14].

Here we report that undifferentiated U937 cells are dexamethasone insensitive with respect to prostaglandin secretion, whereas a reduction of 50% was observed in TPA-differentiated cells. These results correlated with the inhibition of phospholipase A_2 in TPA-differentiated U937 cells but not in undifferentiated U937 cells.

MATERIALS AND METHODS

Materials

Cell culture media and supplements were obtained from Gibco (Karlsruhe, FRG); fetal calf serum was from Biochrom (Berlin, FRG). Dexamethasone phosphate was supplied by Merck (Darmstadt, FRG). Arachidonic acid was from NuCheck Prep. Inc. (Elysian, MN). Silica gel 60 was purchased from Fluca AG (9470 Buchs, CH). 5,5-Dithio-bis-(2-nitrobenzoic acid) (DTNB), 1-acyl-glycerophosphatidylcholine and 12-0-tetradecanoylphorbol-13-acetate were from Sigma (Deisenhofen, FRG). For radioimmunoassay, ^{3}H-labelled prostaglandins were obtained from NEN (Dreieich, FRG), goat antirabbit-γ-globulin from Boehringer-Calbiochem (Frankfurt, FRG) and prostaglandins from Upjohn (Kalamazoo, MI). NEN (Boston, MA) supplied arachidonoyl-1-^{14}C phosphatidylcholine.

Methods

Culture of U937 cell line. U937 cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 50 μg/ml penicilline, 50 U/ml streptomycin, and 5% heat-inactivated fetal calf serum. To induce differentiation a final concentration of 5×10^{-9} M 12-0-tetradecanoylphorbol-13-acetate (TPA) was added to 5×10^5 cells/ml, and the cells cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 3 days the cells were washed twice with RPMI 1640.

Synthesis of eicosanoids. U937 cells and TPA-differentiated U937 cells were incubated overnight in the presence or absence of dexamethasone at concentrations indicated in RPMI 1640 medium containing 1% human-AB-serum in flat-bottomed microtiter plates (96 wells) or in 24-well plates (Nunc, Wiesbaden, FRG) at a cell density of 5×10^5 cells/ml. Arachidonic acid at different concentrations was added to the cells. After the times indicated, the supernatant was removed, and aliquots were used for determination of prostanoid production. The concentration of prostaglandin E_2 (PGE_2), thromboxane B_2 (TxB_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was measured by

radioimmunoassay (RIA) using a modified double-antibody method as described previously [15]. The antibodies recognized their respective prostaglandin with high specificity. The cross-reactivity against other prostaglandins and arachidonic acid at the concentrations used was less than 1%. The detection limit was about 20 pg/ml.

Cell preparation for enzyme assays. TPA-differentiated cells were harvested with a rubber policeman from culture dishes and nonadherent undifferentiated cells were centrifuged at 600 × *g* for 10 min. The pellet was resuspended in phosphate-buffered saline and sonicated 3 times for 10 sec at 50 W (Labsonic 1510, Braun). After low-speed centrifugation for 10 min at 600g to remove nuclear material and cell debris the supernatant was ultracentrifuged for 60 min at 100,000*g* (65 Ti Rotor, Beckmann) and the resulting pellet collected for phospholipase A_2 and lysophosphatide acyltransferase assays. The protein concentration of the particulate fraction was measured by a microtiter Bradford assay [16] using BSA as standard protein.

Determination of phospholipase A_2 activity. Phospholipase A_2 was determined essentially as described by Flesch and Ferber [17], using phosphatidylcholine (α-1-palmitoyl-2-arachidonyl [arachidonoyl-1-^{14}C]) as substrate. After evaporation under N_2 the lipids were briefly sonicated in distilled water containing 5 mg/ml bovine serum albumin (fatty acid free). The assay mixture contained 100 mM Tris/HCl, pH 8.5, 1 mM $CaCl_2$/1 mM $MgCl_2$, 50 μl of the 100,000*g* pellet, and 1 μM labelled lipid (25,000 cpm) as substrate in a total volume of 250 μl. The control mixtures contained a final concentration of 1 mM EDTA to remove Ca^{2+} and Mg^{2+} which are essential for total phospholipase A_2 activity. The mixture was incubated at 37°C in 5 ml glass tubes with shaking for 30 min. The reaction was stopped by adding 375 μl of ice-cold 2-propanol/1 N HCl (1/0.086, v/v). After addition of 700 μl heptane, the solution was vigorously shaken and warmed to room temperature. When a two-phase system was established 500 μl of the top heptane phase was transferred into Eppendorf tubes containing 200 μl heptane, and a small amount of silica-gel. After vigorous mixing and centrifugation at 13,000*g* for 5 min the activity of 500 μl of the supernatant was determined in a liquid scintillation counter.

Determination of lysophosphatide acyltransferase activity. Lysophosphatide acyltranseferase activity was determined as previously described [18]. Briefly, the method is based on the reaction of liberated coenzyme A with the dye 5,5-dithio-bis-(2-nitrobenzoic acid). The 100,000*g* pellet was resuspended and diluted in 20 mM Hepes/140 mM KCl buffer, pH 7.0, at concentrations between 20 μg and 80 μg protein/tube. After a pre-run with protein, 10 nmol arachidonoyl-CoA and DTNB were added to estimate hydrolase activities. The assay was started by addition of 25 nmol 1-palmitoyl-glycerophosphorylcholine to a final total volume of 500 μl. The absorption was measured in a Gilford spectrophotometer (Corning Lab. Sciences Company) at 412 nm at 37°C. The LAT activity was calculated after subtraction of the background hydrolase activity from the initial velocity.

RESULTS

U937 cells growing autonomously in suspension with a smooth and round surface can be induced to differentiate by the phorbol ester TPA. Besides cessation of proliferation, morphological changes such as adherence to the substrate and cell cluster became obvious (Fig. 1a,b). Dexamethasone did not affect either cell morphology or proliferation

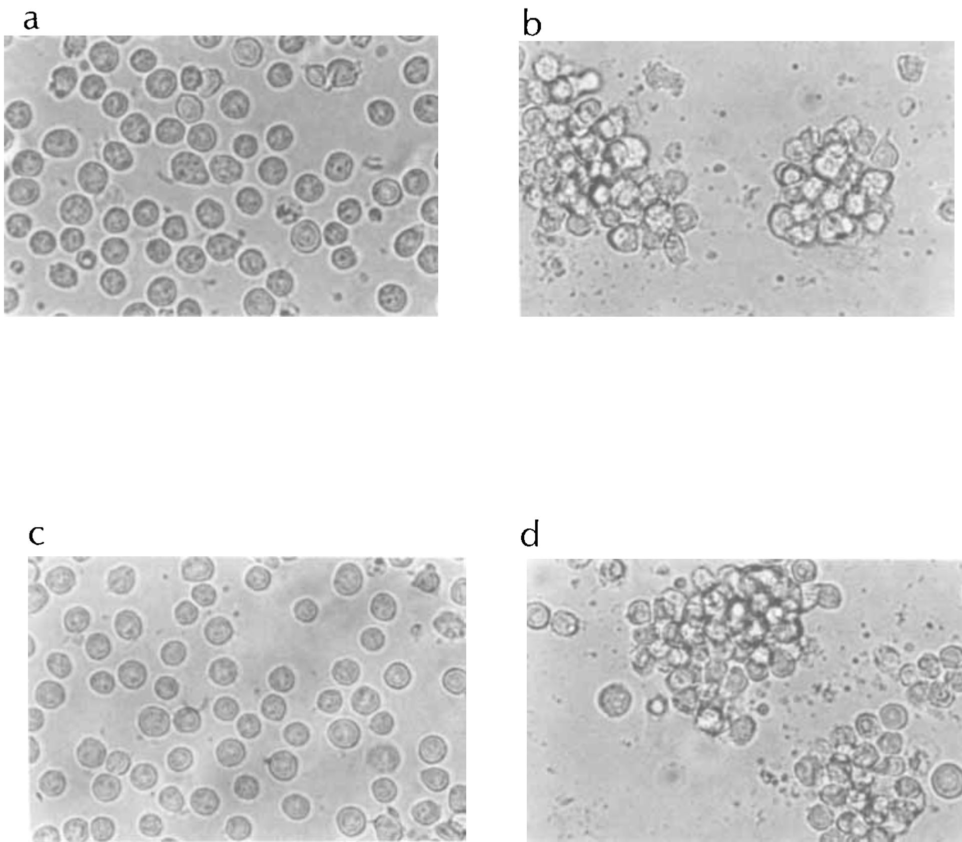

Fig. 1. Light microscopy of the U937 cell line. **a:** Undifferentiated U937 cells. **b:** TPA-differentiated U937 cells. **c:** Undifferentiated U937 cells incubated with 1×10^{-6} M dexamethasone for 24 hrs. **d:** TPA-differentiated U937 cells incubated with 1×10^{-6} M dexamethasone for 24 hours. a–d: ×400.

of undifferentiated or TPA-differentiated U937 cells and therefore cannot be considered to induce a dedifferentiation of these cells (Fig. 1b,d).

The TPA-differentiated U937 cells were capable of releasing significant amounts of prostaglandins. Figure 2 shows a time course of prostaglandin E_2 (PGE_2) secretion. The cells released PGE_2 spontaneously at a rate of approximately 1 ng/ml, which was markedly increased by the prostanoid precursor arachidonic acid in a concentration-dependent manner. After incubation overnight with 1×10^{-6} M dexamethasone both unstimulated and arachidonic-acid-stimulated PGE_2 secretion was reduced by approximately 50%. Similarly, dexamethasone reduced the synthesis of other prostaglandins. The effect of dexamethasone on the production of thromboxane B_2 (TxB_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is shown in Figures 3 and 4, respectively.

Undifferentiated U937 cells did not produce prostaglandins spontaneously and secreted only small amounts of prostaglandins when incubated with arachidonic acid. The stimulation of PGE_2 production was time and concentration dependent (Fig. 5). In contrast to TPA-differentiated U937 cells, pretreatment of undifferentiated U937 cells with dexamethasone did not affect prostaglandin synthesis.

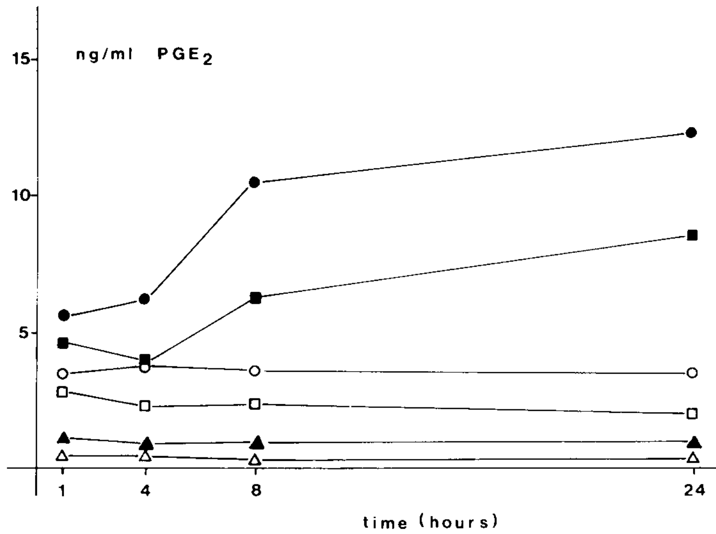

Fig. 2. Inhibition of PGE_2 secretion in dexamethasone-treated TPA-differentiated U937 cells. TPA-differentiated U937 cells were cultured in the presence (open symbols) or absence (filled symbols) of dexamethasone 1×10^{-6} M for 20 h. The cells were incubated with medium (▲, △) or with arachidonic acid 1×10^{-5} M (■, □) and 2×10^{-5} M (●, ○) for the times indicated. Data shown are means from a typical experiment with duplicate biological incubations and quadruplicate radioimmunoassay samples.

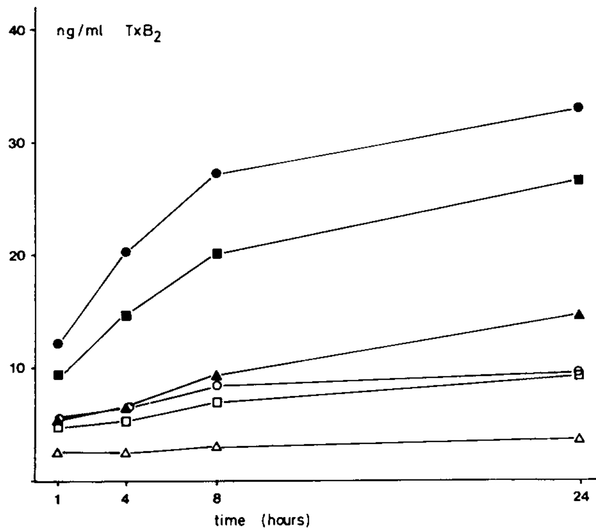

Fig. 3. Inhibition of TxB_2 secretion in dexamethasone-treated TPA-differentiated U937 cells. Legend as in Figure 1.

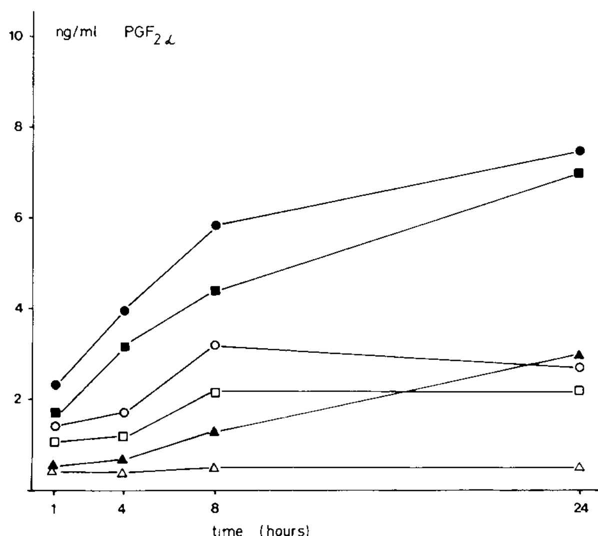

Fig. 4. Inhibition of $PGF_{2\alpha}$ secretion in dexamethasone-treated TPA-differentiated U937 cells. Legend as in Figure 1.

The concentration dependency of the dexamethasone effect was tested in the range of 1×10^{-8} to 1×10^{-6} M for PGE_2. Whereas none of the concentrations of dexamethasone used had any influence on the PGE_2 secretion of undifferentiated U937 cells, dexamethasone induced a concentration-dependent decrease of the PGE_2 production of TPA-differentiated U937 cells (Fig. 6).

To gain further insight into the mechanism of this phenomenon, the enzymes involved in regulating the availability of the prostaglandin precursor arachidonic acid were examined. Lysophosphatide acyltransferase, which reacylates fatty acid into

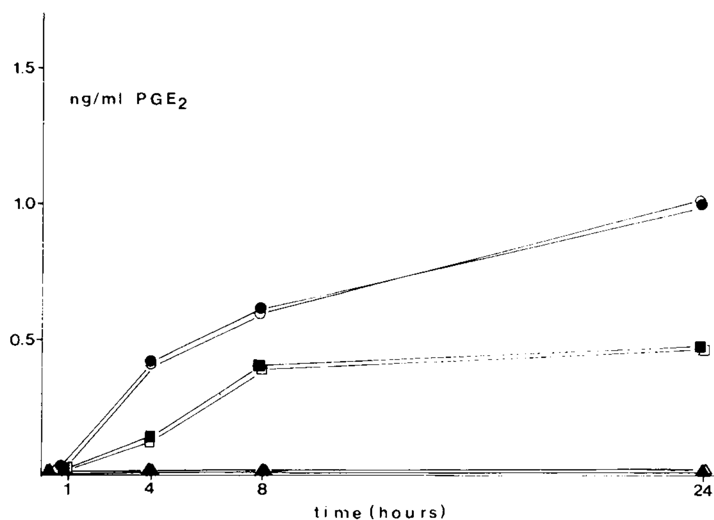

Fig. 5. Effect of dexamethasone on PGE_2 secretion in undifferentiated U937 cells. Legend as in Figure 1.

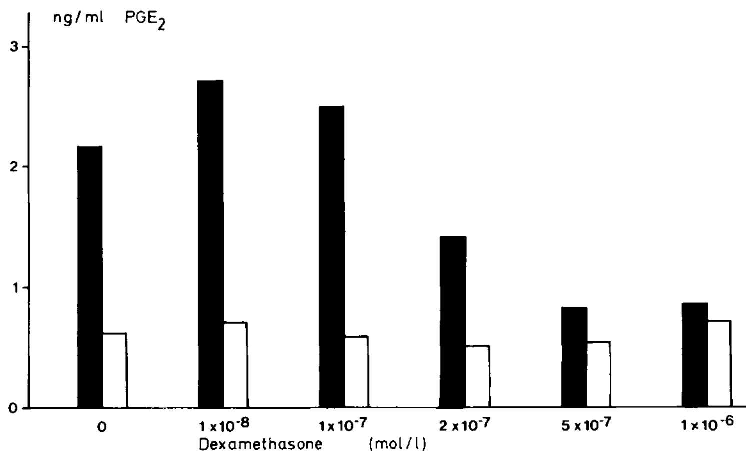

Fig. 6. Concentration-dependent effect of dexamethasone on the arachidonic-acid-induced PGE_2 secretion from undifferentiated and TPA-differentiated U937 cells. After preincubation in the presence or absence of dexamethasone at the concentrations indicated, the undifferentiated (open bars) and TPA-differentiated (filled bars) were stimulated with arachidonic acid 1×10^{-5} M for 24 h.

phospholipids, had the same enzyme activity of about 10 nmol/mg protein × min in both undifferentiated and differentiated U937 cells (Table I). Pretreatment of each cell type with dexamethasone had no significant effect on the lysophposphatide acyltransferase activity.

The calcium-dependent phospholipase A_2 could easily be detected in the U937 cell line (Table I). When phospholipase A_2 activity was compared in the membranes of undifferentiated and TPA-differentiated U937 cells, the enzyme activity was increased approximately twofold during differentiation. Pretreatment overnight with 1×10^{-6} M dexamethasone resulted in unchanged or only slightly higher phospholipase A_2 activity in membranes of undifferentiated U937 cells (+4.0 ± 6.7%) (n = 3). The phospholipase A_2 activity in membranes of TPA-differentiated U937 cells was decreased by 37.8% ± 10.7% (n = 3). Dexamethasone directly added to the membranes did not alter phospholipase A_2 activity. The inhibition of phospholipase A_2 by dexamethasone in membranes of TPA-differentiated U937 cells was reversed by preincubation with cycloheximide (Fig. 7).

TABLE I. Phospholipase A_2 and Lysophosphatide Acyltransferase Activity in Membranes of Dexamethasone-Treated Undifferentiated and TPA-Differentiated U937 Cells*

	Experiment 1		Experiment 2		Experiment 3	
	LAT	PLA_2	LAT	PLA_2	LAT	PLA_2
Undifferentiated U937	7.5	3.85	12.1	6.4	9.3	2.8
Dex/undifferentiated U937	8.6	3.93	10.7	6.3	10.7	3.12
TPA-differentiated U937	9.1	8.3	10.7	11.6	9.4	5.1
Dex/TPA-differentiated U937	8.9	4.3	11.1	7.3	10.5	3.7

*Crude membrane fractions were prepared from cells that had been incubated for 20 h with or without dexamethasone 1×10^{-6} M. Enzyme activities were determined as described in Materials and Methods and are given for lysophosphatide acyltransferase (LAT) in nmol/mg protein × min and for phospholipase A_2 (PLA_2) in pmol/mg protein × min, respectively.

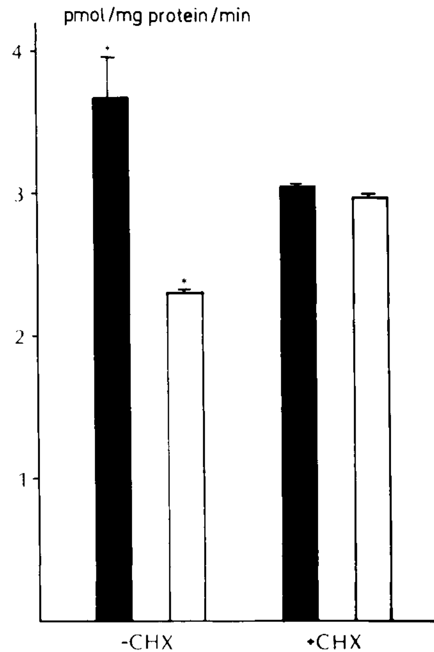

Fig. 7. Effect of cycloheximide on the inhibition of phospholipase A_2 activity by dexamethasone in membranes of TPA-differentiated U937 cells. After preincubation with and without 10 μg/ml cycloheximide (CHX) for 12 h, TPA-differentiated U937 cells were cultured with (open bars) or without (filled bars) 1×10^{-6} M dexamethasone for 12 h. Crude membrane fraction was prepared and phospholipase A_2 activity measured as described in Materials and Methods. Data are means ± SD of two separate experiments with duplicate determinations (*$P \leq 0.001$ as evaluated by Student's t-test).

DISCUSSION

The human histiocytic cell line U937 was capable of synthesizing prostaglandins when exogenous arachidonic acid was supplied, confirming similar observations made by Cobb and co-workers [19]. After differentiation with TPA into macrophage-like cells, prostaglandin synthesis was enhanced by a factor between 20 and 30.

Dexamethasone markedly inhibited prostaglandin synthesis in TPA-differentiated U937 cells, the synthesis of all prostaglandins measured being reduced to the same extent. The inhibition was dependent on the dexamethasone concentration, and on the time of preincubation. In contrast to these findings dexamethasone over a wide concentration range proved to be completely ineffective in reducing prostaglandin production in undifferentiated U937 cells.

The availability of the prostanoid precursor arachidonic acid is regulated by phospholipase A_2 and lysophosphatide acyltransferase activity; therefore we examined these two enzymes involved in the deacylation-reacylation cycle. Lysophosphatide acyltransferase activity was unaffected by dexamethasone in the membranes of both undifferentiated and TPA-differentiated U937 cells. In contrast phospholipase A_2 was significantly reduced in membranes of dexamethasone-treated TPA-differentiated U937 cells. Inhibition of prostanoid synthesis by glucocorticoids as a receptor-mediated process has been demonstrated to be dependent on protein synthesis [3]. Cycloheximide, an inhibitor of protein synthesis, reversed the reduction of membrane-associated phospholi-

pase A_2 activity by dexamethasone in TPA-differentiated U937 cells. Together with the lack of a direct effect of dexamethasone on phospholipase A_2, the data suggest a specific, receptor-mediated process of dexamethasone in TPA-differentiated U937 cells. As shown for the prostaglandin production, treatment of undifferentiated U937 cells with dexamethasone had no effect on either phospholipase A_2 or lysophosphatide acyltransferase activity.

Recently, Duval and co-workers have reported that dexamethasone at concentrations of 1×10^{-6} M even stimulated arachidonic acid release in undifferentiated U937 cells [20]. Our results with respect to phospholipase A_2 activity and prostaglandin secretion do not show this effect clearly. However, no inhibitory effect of dexamethasone could be observed, which confirms the findings of Duval et al. in undifferentiated U937 cells.

These results suggest that the capacity to respond to glucocorticoids such as dexamethasone is acquired late in the differentiation into macrophage-like cells. Dexamethasone-induced de-differentiation of TPA-differentiated U937 cells, resulting in a reduced phospholipase A_2 activity and prostaglandin secretion, is unlikely since dexamethasone had no influence on either morphology or proliferation in TPA-differentiated U937 cells. The failure of glucocorticoids to suppress prostanoid synthesis and phospholipase A_2 activity in undifferentiated U937 cells could be explained by a lack of specific receptors in these cells. However, Duval et al. have demonstrated the presence of glucocorticoid receptors in these cells [20], and our own preliminary experiments confirm this observation. Taken together these observations suggest differences at the postreceptor level responsible for the different effects of glucocorticoids on prostanoid synthesis.

Glucocorticoids affected prostanoid synthesis in unstimulated TPA-differentiated U937 cells and those challenged with the precursor fatty acid arachidonic acid. Spontaneous prostanoid synthesis depends on cleavage of arachidonic acid from complex lipids. Accordingly, phospholipase A_2 activity in membranes of TPA-differentiated U937 cells was reduced by dexamethasone. Although other lipases and esterases (besides phospholipase A_2) which liberate arachidonic acid as an initial step for prostaglandin synthesis cannot be excluded, inhibition of phospholipase A_2 by dexamethasone might contribute to reduction of prostaglandin secretion. Besides incorporation and liberation, exogenously added arachidonic acid might also in part be metabolised directly into prostaglandins. Since it is only in TPA-differentiated U937 cells that dexamethasone treatment decreases arachidonic-acid-induced prostaglandin synthesis, regulation might also occur at a level distal of arachidonic acid availability. Experiments are being done at present in order to test this possibility.

In recent years lipocortins, a family of proteins with the ability to inhibit phospholipase A_2 activity, have been described by several groups. Induction of lipocortins has been proposed as the mechanism underlying glucocorticoid dependent inhibition of eicosanoid synthesis [21–23]. Human lipocortin, however, has been cloned from the undifferentiated U937 cell line, demonstrating unequivocally its presence in these cells [24]. Accordingly, using Western blot analysis, Huang and co-workers detected lipocortin I and II in undifferentiated U937 cells [25]. Thus the glucocorticoid unresponsiveness of the cells cannot be ascribed to a lack of these proteins, although structure differences or differences in their inducibility may be relevant.

In conclusion, undifferentiated and TPA-differentiated U937 cells present an interesting model for the examination of the molecular mechanism of glucocorticoids, as the cells, although genetically identical, show markedly different sensitivity towards dexamethasone with respect to membrane-associated phospholipase A_2 activity and prostaglandin secretion.

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