Biochemical Pharmacology, Vol. 40, No. 6, pp. 1307–1316, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

GLUCOCORTICOID-INDUCED REDUCTION OF PROSTANOID SYNTHESIS IN TPA-DIFFERENTIATED U937 CELLS IS MAINLY DUE TO A REDUCED CYCLOOXYGENASE ACTIVITY

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(*Received* 27 *December* 1989; *accepted* 21 *April* 1990)

Abstract—The molecular mechanism of the glucocorticoid-induced inhibition of prostanoid synthesis was investigated in human monoblastoid U937 tumor cells and phorbol ester (TPA)-differentiated U937 cells. Prostanoid synthesis was inhibited in TPA-differentiated U937 cells by glucocorticoids such as dexamethasone and prednisolone, whereas aldosterone and progesterone showed no inhibitory effect. None of these methods had any influence on prostanoid secretion of undifferentiated U937 cells. Receptor binding studies revealed the presence of glucocorticoid receptors in both undifferentiated and TPA-differentiated U937 cells ($K_D \approx 5 \times 10^{-9}$ M), however, the number of receptors per cell was increased 10-fold in TPA-differentiated U937 cells. Expression of lipocortin I and II as measured by Western blot analysis was not affected by dexamethasone. In TPA-differentiated cells, dexamethasone decreased the activities of two enzymes essential for prostanoid synthesis, cyclooxygenase and phospholipase A_2, by 60–70% and 30%, respectively. Cells pretreated with the translation inhibitor cycloheximide and dexamethasone showed similar cyclooxygenase and phospholipase A_2 activities as cells treated with cycloheximide alone. Western blot analysis demonstrated that the significantly decreased cyclooxygenase activity correlated with an inhibited protein synthesis. In this human macrophage-like model glucocorticoids thus interfere at least at two levels with prostanoid synthesis by inhibiting the activities of phospholipase A_2 as well as cyclooxygenase.

Glucocorticoids are effective drugs used for the treatment of chronic inflammatory diseases. The mode of action of glucocorticoids is very complex as almost all known pro-inflammatory mediators are modulated in their synthesis, release or degradation [1]. Though the molecular mechanism of glucocorticoid action is well defined on the receptor level, little is known about the specific proteins which are induced or repressed to mediate the observed effects. This is especially true for the influence on prostanoid metabolism. In most cells, glucocorticoids induce a reduction of prostanoid synthesis, however, there are other cells, such as renal interstitial cells [2] or amnion cells [3], where glucocorticoids increase prostanoid synthesis. The molecular mechanism of the glucocorticoid-induced inhibition of prostanoid synthesis appeared to correlate with an induction of lipocortins. These proteins were defined by their ability to inhibit phospholipase A_2 and consequently reduced the availability of the precursor of prostanoids, arachidonic acid [reviewed in Ref. 4]. This concept, however, does not seem to hold true in most systems. The induction of lipocortins by glucocorticoids was not confirmed in most cases [e.g. 5, 6]. Furthermore, these proteins seem to interact with the phospholipid substrate rather than specifically inhibit phospholipase A_2 [7, 8]. With respect to the multiple members of the lipocortin family as well as the variable effects of glucocorticoids in different cell types, these findings do not exclude an involvement of lipocortins in the mediation of glucucorticoid effects, however, lipocortins do not seem to have a central function.

Recently attention has focused on the effect of glucocorticoids on enzymes other than phospholipase A_2, namely cyclooxygenase. In mouse bone marrow-derived macrophages we could show a decreased enzymatic activity of the cyclooxygenase/PGE isomerase after dexamethasone treatment [9]. Similar results were obtained on the mRNA level in cultured rat vascular cells by Bailey *et al.* [10] and on the protein level of human skin fibroblasts by Raz *et al.* [11]. Thus there seems to be a more general mechanism of inhibition on the cyclooxygenase level induced by glucocorticoids, the mechanism of which has not yet been characterized.

In a previous communication we could show that the human monoblastoid cell line U937 is a useful model for the investigation of the mechanism of glucocorticoid action [12]. These cells can be differentiated along the monocyte–macrophage lineage by treatment with the phorbol ester TPA. TPA-induced differentiation resulted in cessation of proliferation in parallel with marked morphological and functional changes [13]. Only the differentiated cells

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responded to dexamethasone with regard to an inhibition of prostanoid secretion while prostanoid synthesis of the undifferentiated tumor cells was unaffected by glucocorticoids [12].

It was the aim of the present study to investigate the effects of glucocorticoids on the synthesis of prostanoids in this system in more detail. We could show that glucocorticoids interfere specifically at least with two enzymatic steps in the prostanoid synthesis, the release of arachidonic acid by phospholipase A_2 and the conversion of arachidonic acid by cyclooxygenase. Both effects were detectable only in the differentiated cells, which may in part be due to an increase in glucocorticoid receptor number, whereas lipocortins did not seem to be involved in these cells.

MATERIALS AND METHODS

Culture for the U937 cell line. U937 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured and differentiated with TPA (5×10^{-9} M) as described previously [13].

Prostanoid generation. Undifferentiated and TPA-differentiated U937 cells were incubated overnight in RPMI 1640 medium containing 1% heat inactivated human AB-serum without or with dexamethasone, prednisolone (Merck, Darmstadt, F.R.G.), Aldosterone (Ciba, Wehr, F.R.G.) or progesterone (The Sigma Chemical Co., Deisenhofen, F.R.G.) in flat bottom microtiter plates (96 wells) (Nunc, Wiesbaden, F.R.G.) at a cell density of 5×10^5 cells/mL. To induce prostanoid secretion both undifferentiated and TPA-differentiated U937 cells were stimulated with 2×10^{-5} M arachidonic acid (NuCheck Prep. Inc., Elysion, NN, U.S.A.). After 24 h, supernatants were removed and PGE_2,* TxB_2 and $PGF_{2\alpha}$ determined by a modified double sandwich radioimunoassay as described previously [14]. Cross-reactivity of the specific antibodies to other prostanoids was less than 0.1%. At the concentrations used, neither arachidonic acid nor the steroids or any other reagent used interfered with the radioimmunoassay.

Determination of glucocorticoid receptors. Glucocorticoid receptors of undifferentiated and TPA-differentiated U937 cells were determined as outlined in [15]. Briefly, undifferentiated and TPA-differentiated U937 cells were cultured in plastic dishes (Greiner, Nürtingen, F.R.G.) at a cell density of 5×10^5 cells/mL in RPMI medium containing 5% fetal calf serum. The cells were incubated with 5×10^{-9} M [^{3}H]dexamethasone (specific activity 95 Ci/mmol) (Amersham, Braunschweig, F.R.G.) and increasing concentrations of unlabelled dexamethasone for 30 min at 37°. Afterwards the cells were washed three times with culture medium and the free tritiated dexamethasone of each washing step determined by liquid scintillation counting. After the final washing step the pellets of undifferentiated U937 cells were resuspended in a hypotonic solution of 1.5×10^{-3} M $MgCl_2$. The same solution was used for harvesting the TPA-differentiated U937 cells with a rubber policeman. The amount of bound tritiated dexamethasone was determined by liquid scintillation counting. Binding sites per cell and the affinity constant were calculated by Scatchard analysis [16].

Cell preparation for the enzyme assays. Undifferentiated and TPA-differentiated U937 cells at a density of 5×10^5 cells/mL were incubated overnight in culture dishes without or with dexamethasone, prednisolone, aldosterone or progesterone at the concentrations indicated. Adherent TPA-differentiated U937 cells were washed twice with phosphate-buffered saline (PBS) and harvested in 0.1 M *N*-tris(hydroxymethyl)-methylglycine buffer (Tricine) (Serva, Heidelberg, F.R.G.), pH 8.5, with a rubber policeman. Non-adherent undifferentiated U937 cells were centrifuged at 600 *g* for 10 min and washed twice in PBS. The pellets of both undifferentiated and TPA-differentiated U937 cells were resuspended in 0.1 M Tricine, pH 8.5, and homogenized by sonication three times for 10 sec at 50 W (Labsonic 1510, Braun, Melsungen, F.R.G.) on ice. After centrifugation for 30 min at 100,000 *g* the resulting pellet was resuspended in Tricine, pH 8.5. Protein concentration of the crude membrane fraction was measured by a micro Bradford protein assay [17] using bovine serum albumin as standard.

In experiments using the translation inhibitor cycloheximide the cells were incubated for 12 hr without or with 10 μg/mL (3.5×10^{-5} M) cycloheximide. Afterwards culture medium without or with 1×10^{-6} M dexamethasone was added for a further incubation period of 12 hr. Cell viability of the cycloheximide treated U937 cells was greater than 85%

Phospholipase A_2 assay. Determination of phospholipase A_2 was performed as originally described by Flesch and Ferber [18] with minor modifications [9] using phosphatidylcholine L-*a*-1-palmitoyl-2-arachidonyl (arachidonyl-1-(^{14}C)) (NEN, Boston, MA, U.S.A.) as substrate. The phospholipid was suspended by sonication in twice distilled water containing bovine serum albumin (essentially fatty acid-free). The final concentrations in the assay were: 1 μM lipid corresponding to about 25,000 cpm, 5 mg/mL albumin, 0.05 M Tris–HCl, pH 8.5, 0.01 M $CaCl_2$ and 10 to 20 μg protein in a total volume of 250 μL. Control assays were performed with 0.01 M EDTA instead of $CaCl_2$. After incubation for 30 min at 37° the liberated fatty acid was extracted by heptane extraction as described in [9]. The enzyme activity was calculated from the percentage of liberated fatty acid.

Cyclooxygenase/PGE_2-isomerase assay. The enzyme assay was performed as described recently [19] and based on the radioimmunologic determination of PGE_2 synthesized from arachidonic acid. In controls 2.5×10^{-4} M diclofenac (Sigma), an inhibitor of cyclooxygenase [20] reduced the content of immunoreactive PGE_2 to less than 10%. The assay mixture containing 100 μg protein of the crude membrane fraction, 8×10^{-6} M hemoglobin, 3.2×10^{-4} M tryptophan (Sigma), and 3.75×10^{-3} M reduced glutathione (Boehringer, Mannheim, F.R.G.) in 0.05 M Hepes/0.14 M KCl pH 8.0, was

* Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PGE_2, prostaglandin E_2; $PFG_{2\alpha}$, prostaglandin $F_{2\alpha}$; TxB_2, thromboxane B_2.

preincubated for 10 min at 37°. The reaction was started by the addition of 1×10^{-5} M arachidonic acid. The total volume consisted of 200 μL. After 15 min the assay was stopped by freezing in liquid nitrogen. Incubation time and arachidonic acid concentration have been shown to be optimal for measuring cyclooxygenase activity in this system [19].

Western blot analysis. Expression of lipocortin I and II and cyclooxygenase was determined by Western blot analysis in undifferentiated and TPA-differentiated U937 cells treated without or with 1×10^{-6} M dexamethasone for 24 hr. The cells were harvested in PBS as described above. After sonication nuclei and cellular debris were removed by centrifugation for 10 min at 600 *g*.

The cell homogenate was used for lipocortin Western blots. Since cyclooxygenase is known to appear as a membrane bound enzyme crude membranes were prepared by centrifugation of the cell homogenate for 30 min at 100,000 *g*. Equal amounts of protein were denatured according to Laemmli [21] and resolved by one-dimensional SDS–PAGE using a 10% polyacrylamide gel. Proteins were electrophoretically transferred (4°, 1 hr, 200 V) from polyacrylamide gel to Immobilon PVDF transfer membrane (Millipore, Bedford, U.S.A.) in a transfer buffer (0.06 M glycine, 7.8×10^{-3} M Tris and 20% methanol, v/v, pH 8.3–8.5). Nonspecific protein binding sites of the transfer membrane were blocked by incubation overnight in a blocking buffer (5% low fat dry milk, 0.1 M Tris, 0.9% NaCl and 0.02% NaN_3, w/v, pH 7.4).

The polyclonal rabbit anti-lipocortin I and II antibodies, kindly provided by Dr R. B. Pepinsky (Biogen Corp., Boston, MA, U.S.A.), were diluted in the blocking buffer as outlined in [22]. To visualize the immunopositive proteins iodinated [^{125}I]goat anti-rabbit IgG (1×10^6 cpm/mL) was used. Before iodination [23, 24] goat anti-rabbit antibodies (Calbiochem, Frankfurt, F.R.G.) were purified by FPLC (Pharmacia LKB, Freiburg, F.R.G.) using a protein A Sepharose column. After washing 8–10 times with 100 mM Tris, pH 8.0, the blot was dried and exposed to an X-ray film (Kodak X-OMAT, Rochester, NY, U.S.A.) for autoradiography at −70°.

For cyclooxygenase Western blots the polyclonal rabbit antibody was diluted 1:50 in the blocking buffer [25]. Immunopositive proteins were detected by incubation with biotinylated goat anti-rabbit antibody (Amersham, Braunschweig, F.R.G.) and visualized by streptavidin peroxidase complex [26]. Western blot analysis of cyclooxygenase revealed the staining of two bands, the second of which has previously been described as a degradation product of the cyclooxygenase polypeptide [25]. The density of the cyclooxygenase band was determined by laser densitometric scanning (Pharmacia LKB, Freiburg, F.R.G.).

The molecular weight markers used were α-lactalbumin (14 kD), soy bean trypsin inhibitor (20 kD), carbonic anhydrase (30 kD), ovalbumin (43 kD), bovine serum albumin (67 kD) and phosphorylase b (94 kD) (Pharmacia).

Statistical analysis. Statistical analysis was performed using the means ± SD of independent experiments. To compare the values obtained from cells with varying absolute activities, the mean of each set of control cells (duplicate to quadruplicate incubations) was taken as 100% and the mean of the respective cells incubated with dexamethasone calculated as a percentage of the control. Percentage data are given as means ± SD of N different cell cultures. The statistical significance of the inhibition values so obtained was calculated using the nonpaired two-tailed Student's *t*-test. A P value <0.05 was considered to indicate a statistically significant difference between two sets of data.

RESULTS

Differential effect of glucocorticoids on prostanoid secretion in undifferentiated and TPA-differentiated U937 cells

Undifferentiated U937 cells were capable of secreting PGE_2 when incubated with 20 μM arachidonic acid for 24 hr. Neither dexamethasone, prednisolone, aldosterone nor progesterone had any influence on PGE_2 release in the range of 1×10^{-9} M to 1×10^{-6} M (Fig. 1a). During TPA-induced differentiation PGE_2 production of TPA-differentiated U937 cells was enhanced 100-fold after addition of exogenous arachidonic acid. By preincubation of TPA-differentiated U937 cells with the glucocorticoids dexamethasone and prednisolone both spontaneous (data not shown) and arachidonic acid stimulated PGE_2 secretion were markedly decreased, whereas aldosterone and progesterone did not alter PGE_2 production except at the highest concentration tested (1×10^{-5} M) (Fig. 1b). In all experiments dexamethasone was more potent than prednisolone in reducing PGE_2 release. TPA-differentiated U937 cells also secreted significant amounts of TxB_2 and $PGF_{2\alpha}$. At a concentration of 1×10^{-6} M dexamethasone the synthesis of all these cyclooxygenase products was decreased by more than 85% (85 ± 5%, 89 ± 3% and 87 ± 4% for PGE_2, TxB_2 and $PGF_{2\alpha}$, respectively ($N = 6$, $P < 0.001$)) (Fig. 2).

Glucocorticoid receptor studies of undifferentiated and TPA-differentiated U937 cells

Glucocorticoid receptors were detectable in both undifferentiated and TPA-differentiated U937 cells (Fig. 3). The affinity constant (K_D) was $\approx 5 \times 10^{-9}$ M and remained unchanged during TPA-induced differentiation. The number of binding sites per cell, however, increased from about 2700 in undifferentiated U937 cells up to about 26,000 in TPA-differentiated U937 cells.

Effect of dexamethasone on phospholipase A_2 activity

Phospholipase A_2 activity was detectable in membranes of both undifferentiated and TPA-differentiated U937 cells. The enzyme activity in membranes of undifferentiated U937 cells was not altered by preincubation of the cells with 1×10^{-6} M dexamethasone overnight whereas dexamethasone decreased phospholipase A_2 activity in membranes of TPA-differentiated U937 cells by 31 ± 5% ($N = 8$, $P < 0.001$). Treatment of TPA-differentiated U937 cells with 10 μg/mL (3.5×10^{-5} M) cycloheximide decreased phospholipase A_2 activity by 50% which

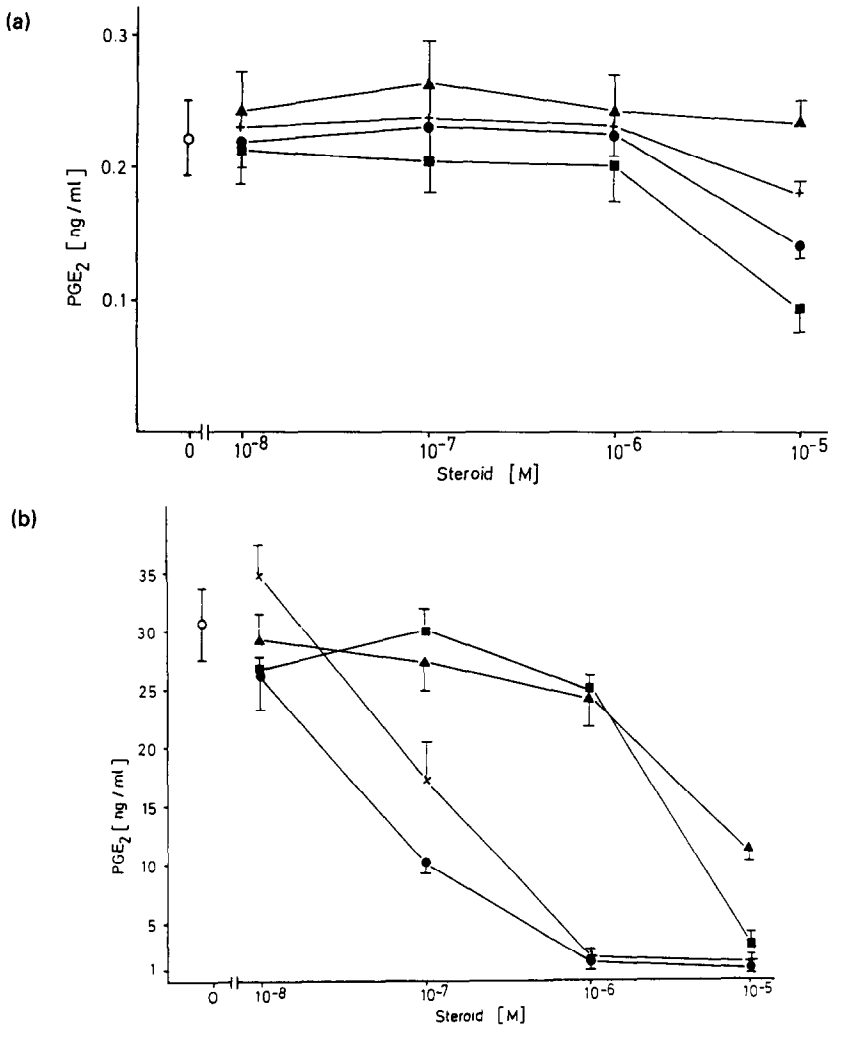

Fig. 1. Effect of steroids on prostaglandin E_2 secretion of undifferentiated and TPA-differentiated U937 cells. Undifferentiated (a) and TPA-differentiated U937 cells (b) were incubated overnight without (○) or with dexamethasone (●), prednisolone (+), aldosterone (■) and progesterone (▲) at the concentrations indicated. Arachidonic acid (final concentration 2×10^{-5} M) was then added to the cultures. After 4 hr supernatants were removed and the concentration of PGE_2 determined by RIA. Data are given in ng/mL. The arachidonic acid control data are the means ± SD of six separate wells. The data for steroid-treated cells are the means of two separate wells assayed in duplicate.

was identical when cycloheximide and dexamethasone were added together (Table 1). Aldosterone and progesterone had no influence on phospholipase A_2 activity of TPA-differentiated U937 cells (data not shown).

Lipocortin I and II Western blot analysis

The expression of lipocortin I and II was augmented during TPA-induced differentiation. Dexamethasone failed to further increase the amount of lipocortin I and II in either undifferentiated and TPA-differentiated U937 cells (Fig. 4).

Inhibition of cyclooxygenase activity by glucocorticoids

Cyclooxygenase activity was enhanced more than 10-fold during differentiation with TPA. Dexamethasone did not alter cyclooxygenase activity in undifferentiated U937 cells. In membranes of TPA-differentiated U937 cells, however, cyclooxygenase activity was inhibited by 60 ± 5% (N = 10) after incubation over night with 1×10^{-6} M dexamethasone (Fig. 5). Beside PGE_2 small amounts of TxB_2 and $PGF_{2\alpha}$ were detected in the *in vitro* cyclooxygenase assay. The effects of dexamethasone on TxB_2 and $PGF_{2\alpha}$ paralleled those of PGE_2. The extent of dexamethasone-induced inhibition of cyclooxygenase activity was similar to the decrease achieved by treatment of the cells with 10 μg/mL (3.5×10^{-5} M) cycloheximide (Fig. 6). Cycloheximide in combination with dexamethasone did not further influence the activity of the enzyme. The inhibitory effect was specific for glucocorticoids since dexamethasone and prednisolone decreased cyclo-

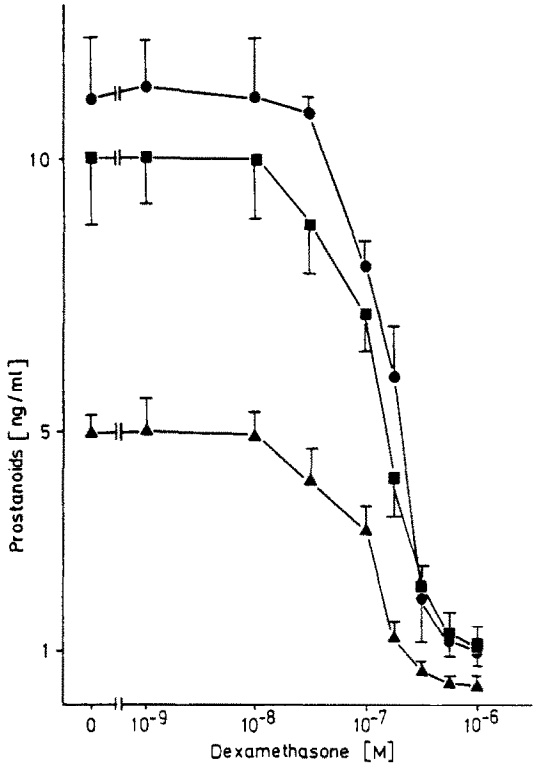

Fig. 2. Dose-dependent inhibition of PGE_2, TxB_2 and $PGF_{2\alpha}$ secretion in TPA-differentiated U937 cells by dexamethasone. TPA-differentiated U937 cells were cultured overnight without or with dexamethasone at concentrations indicated. Prostanoid secretion was stimulated for 24 hr by 2×10^{-5} M arachidonic acid. Concentrations of PGE_2 (●), TxB_2 (■) and $PGF_{2\alpha}$ (▲) were determined by RIA as described in Materials and Methods. Data are the means ± range of two wells assayed in duplicate and given in ng/mL.

Table 1. Effect of cycloheximide and dexamethasone on phospholipase A_2 activity in membranes of TPA-differentiated U937 cells

	Control	Dexamethasone
Without cycloheximide	100%	69 ± 4%
With cycloheximide	48 ± 4%	43 ± 4%

After treatment for 12 hr without or with 10 μg/mL (3.5×10^{-5} M) cycloheximide TPA-differentiated U937 cells were incubated for 12 hr without or with 1×10^{-6} M dexamethasone. Crude membranes were prepared as described in Materials and Methods. Data are means ± SD of two separate experiments with duplicate determinations and given as a percentage of the control. Control phospholipase A_2 activity was 4.15 pmol/mg protein/min.

oxygenase activity whereas aldosterone and progesterone showed no significant effect (Fig. 7).

Dexamethasone reduced the enzyme activity in a dose dependent manner to 81.2 ± 2.8%, 54.9 ± 9.6% and 36.1 ± 13.9% compared to control cells by 10^{-8} M, 10^{-7} M and 10^{-6} M dexamethasone, respectively (N = 3, $P < 0.005$) (Fig. 8).

Cyclooxygenase Western blot analysis

TPA-differentiated U937 cells incubated for 24 hr without or with 1×10^{-6} M dexamethasone were examined for their content of cyclooxygenase. Western blot analysis and subsequent laser densitometry revealed a 50% reduced expression of the cyclooxygenase protein following treatment with dexamethasone (Fig. 9).

DISCUSSION

Glucocorticoids are potent anti-inflammatory

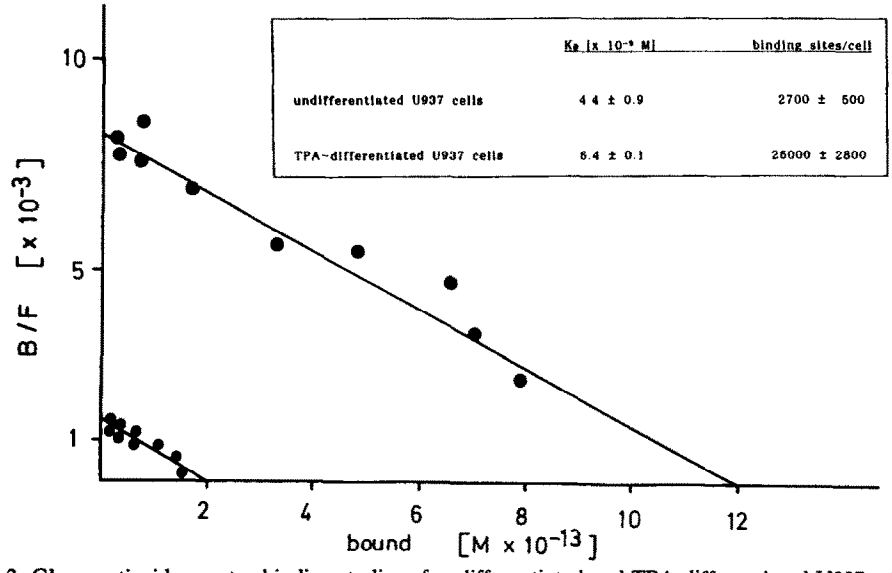

Fig. 3. Glucocorticoid receptor binding studies of undifferentiated and TPA-differentiated U937 cells. Undifferentiated and TPA-differentiated U937 cells were incubated with 5×10^{-9} M tritiated dexamethasone and increasing concentrations of unlabelled dexamethasone. After 30 min the cells were washed three times. Radioactivity was measured by liquid scintillation counting. Binding sites per cell and receptor affinity was calculated by Scatchard analysis. The figure represents data from a single experiment. Data in the table are the means ± range of two separate experiments.

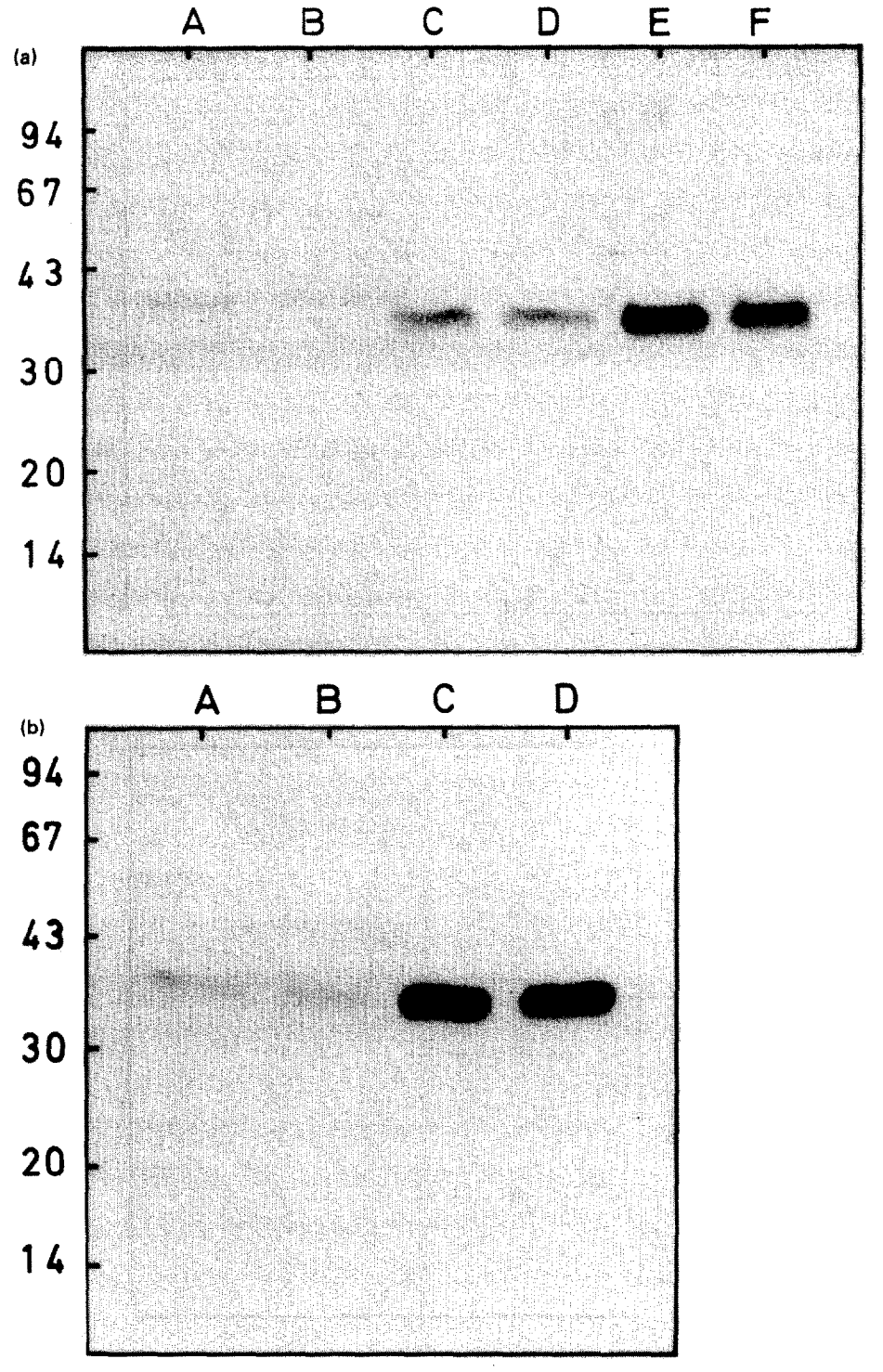

Fig. 4. Effect of dexamethasone on lipocortin I and II expression in undifferentiated and TPA-differentiated U937 cells. Undifferentiated and TPA-differentiated U937 cells were incubated for 24 hr without or with 1×19^{-6} M dexamethasone. Proteins of the cells prepared as described in methods were fractioned on a 10% SDS–polyacrylamide gel and processed for Western blot as described in Materials and Methods. The incubation times for the antibodies were similar for lipocortin I and II. The blots were exposed for autoradiography for 2 and 5 days for lipocortin II and II, respectively. (a) Lipocortin I, A: recombinant human lipocortin I (10 ng); B: recombinant human lipocortin I (1 ng); C: undifferentiated U937 cells/control; D: undifferentiated U937 cells/dexamethasone-treated; E: TPA-differentiated U937 cells/control; F: TPA-differentiated U937 cells/dexamethasone-treated. (b) Lipocortin II, A: undifferentiated U937 cells/control; B: undifferentiated U937 cells/dexamethasone-treated; C: TPA-differentiated U937 cells/control; D: TPA-differentiated U937 cells/dexamethasone-treated.

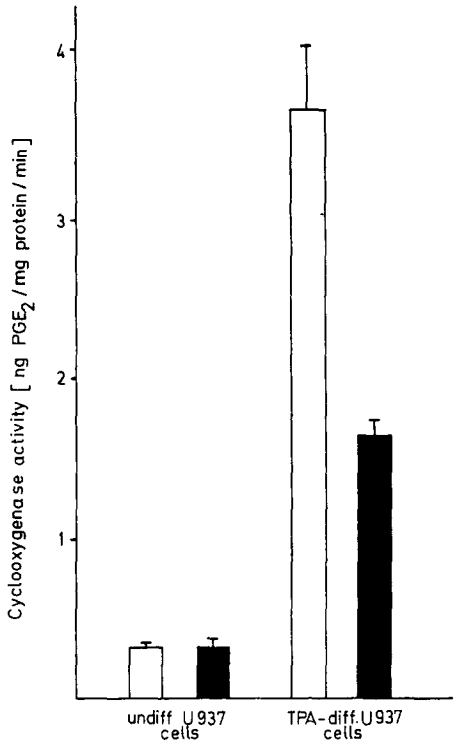

Fig. 5. Differential effect of dexamethasone on cyclooxygenase activity of undifferentiated and TPA-differentiated U937 cells. Undifferentiated and TPA-differentiated U937 cells were incubated overnight without (open bars) or with 1×10^{-6} M dexamethasone (filled bars). Crude membrane fractions were prepared as described in Materials and Methods. Equal amounts of protein were incubated with arachidonic acid and the content of PGE_2 formed determined by RIA. Data are the means ± SD of three separate experiments each performed as quadruplicate and expressed as ng PGE_2/mg protein/min. The inhibition of the TPA-treated cells by dexamethasone was significant with $P < 0.001$.

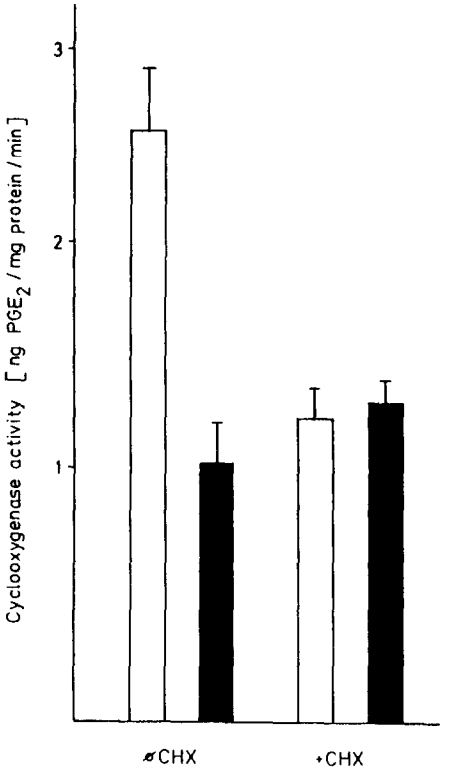

Fig. 6. Effect of cycloheximide and dexamethasone on cyclooxygenase activity of TPA-differentiated U937 cells. After incubation without or with cycloheximide (CHX, 10 μg/mL (3.5×10^{-5} M)) for 12 hr TPA-differentiated U937 cells were treated for 12 hr without (open bars) or with 1×10^{-6} M dexamethasone (filled bars). Crude membrane fractions were prepared as described in Materials and Methods. Equal amounts of protein were incubated with arachidonic acid and the content of PGE_2 formed determined by RIA. Data are the means ± SD of three separate experiments each performed as quadruplicate and expressed as ng PGE_2/mg protein/min. Inhibition was significant with $P < 0.005$.

drugs. Their beneficial effect is probably due to their ability to suppress a variety of mediators of inflammation including cytokines and prostanoids [1]. A decade ago Danon *et al.* demonstrated inhibition of prostanoid synthesis by glucocorticoids, a process requiring protein synthesis [26]. However, the molecular mechanism of glucocorticoid-induced inhibition of prostanoid secretion still remains a matter of debate.

We have used the U937 cell line as a model for investigating the mechanism of glucocorticoid action. At concentrations of 10^{-6} M and below, none of the steroids used had any effect on the prostanoid secretion of undifferentiated U937 tumor cells. During TPA-induced differentiation along the monocyte/macrophage lineage the cells acquired glucocorticoid sensitivity. Dexamethasone and prednisolone inhibited the PGE_2 release while aldosterone and progesterone had no significant effect on PGE_2 production in TPA-differentiated U937 cells, indicating the specificity of the observed effects. The inhibitory effect observed with all steroids at a concentration of 10^{-5} M in both types of cells was considered to be non specific and due to membrane perturbing effects of the extremely high concentration of sterane-like molecules. In contrast to our results Bienkowski *et al.* did not observe a reduction of arachidonic acid-induced TxB_2 secretion by dexamethasone [27]. This apparent discrepancy might be due to the short preincubation time with dexamethasone before the addition of arachidonic acid. In our experiments a preincubation with dexamethasone of at least 5 hr was required to achieve a significant decrease in prostanoid synthesis.

Receptor binding studies revealed the presence of glucocorticoid receptors in both TPA-differentiated U937 cells and undifferentiated cells, confirming the results of Duval *et al.* [28]. The affinity constant (K_D) remained unchanged during differentiation while the number of binding sites per cell was induced 10-fold in TPA-differentiated U937 cells. This is in agreement with the findings of Isacke *et al.* [5], who showed that dexamethasone enhanced the level of metallothionin mRNA in both undifferentiated and TPA-differentiated U937 cells, but that the response

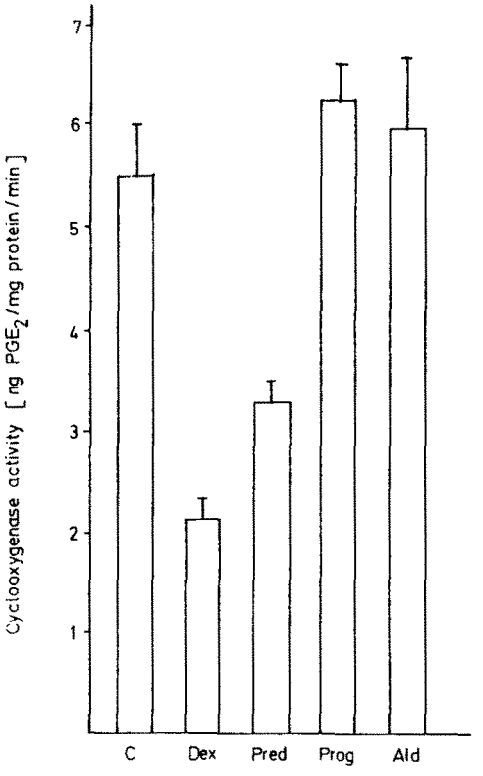

Fig. 7. Effect of different steroids on cyclooxygenase activity of TPA-differentiated U937 cells. TPA-differentiated U937 cells were incubated overnight with culture medium (C), dexamethasone (Dex), prednisolone (Pred), aldosterone (Ald) or progesterone (Prog) at a concentration of 1×10^{-6} M. Crude membrane fractions were prepared as described in Materials and Methods. Equal amounts of protein were incubated with arachidonic acid and the content of PGE_2 formed determined by RIA. Data are the means ± SD of quadruplicate determinations expressed as ng PGE_2/mg protein/min. A typical experiment out of three similar ones is shown.

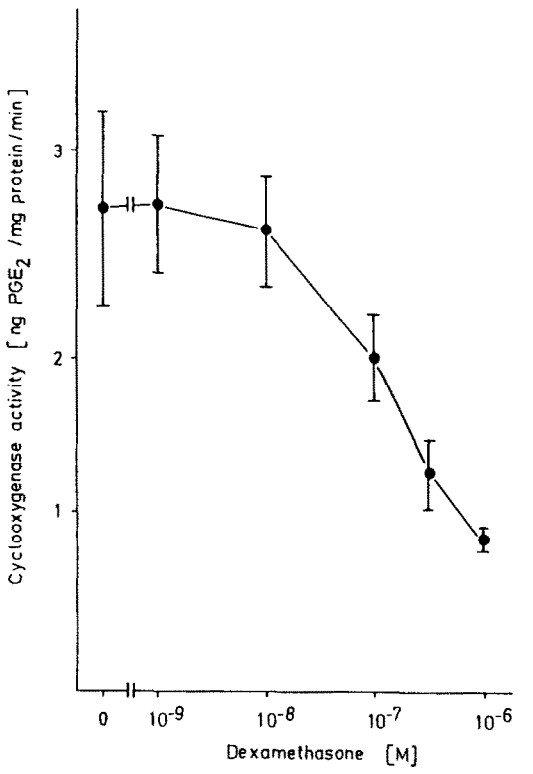

Fig. 8. Dose dependency of dexamethasone-induced inhibition of cyclooxygenase activity of TPA-differentiated U937 cells. TPA-differentiated U937 cells were incubated overnight without or with dexamethasone at the concentrations indicated. Crude membrane fractions were prepared as described in Materials and Methods. Equal amounts of protein were incubated with arachidonic acid and the content of PGE_2 formed determined by RIA. Data are the means ± range of two separate experiments each performed in quadruplicate and expressed as ng PGE_2/mg protein/min.

was 10-fold higher in TPA-differentiated U937 cells. There are, however, examples in the literature, where the number of receptors would not correspond with the glucocorticoid effect, e.g in certain lymphocytes [29]. Thus the oumber of glucocorticoid receptors might not be a sufficient explanation, and alterations at the post-receptor level have to be considered for the differential effect of glucocorticoids on prostanoid synthesis in undifferentiated and TPA-differentiated U937 cells.

To obtain further information about the mechanism of glucocorticoid action we examined two key enzymes involved in the regulation of prostanoid synthesis.

Phospholipase A_2 is involved in the control of prostanoid synthesis by regulating the availability of the precursor arachidonic acid [30]. The enzyme activity was reduced by glucocorticoids in membranes of TPA-differentiated U937 cells. In the presence of cycloheximide, which by itself strongly inhibited phospholipase A_2, no additional effect of dexamethasone was observed, suggesting the induction of a regulatory protein by glucocorticoids and excluding any direct steroid–protein interaction. In undifferentiated U937 cells, dexamethasone did not alter phospholipase A_2 activity.

Lipocortins which inhibit phospholipase A_2 *in vitro* have recently attracted much attention as potential mediators of the anti-inflammatory action of glucocorticoids [4]. However, the role of lipocortins in the regulation of phospholipase A_2 remains uncertain, since inhibition of phospholipase A_2 activity does not appear to be a result of lipocortin enzyme interactions [7, 8]. Western blot analysis showed that expression of lipocortin I and lipocortin II was not induced in either undifferentiated or TPA-differentiated U937 cells after incubation with 1×10^{-6} M dexamethasone. In accordance with our results Isacke *et al.* have shown recently that mRNA levels of lipocortin I and II were not altered by dexamethasone in U937 cells [5], confirming results previously obtained in murine peritoneal macrophages [6].

Cyclooxygenase occupies a central position in the

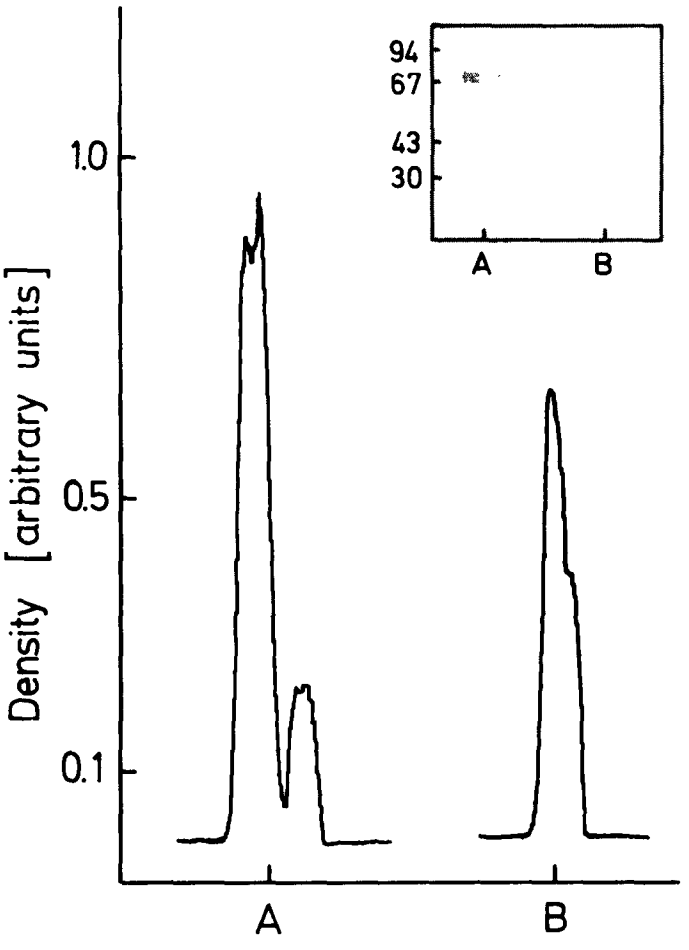

Fig. 9. Dexamethasone induced reduction of cyclooxygenase expression in TPA-differentiated U937 cells. TPA-differentiated U937 cells were treated for 24 hr without (A) or with 1×10^{-6} M dexamethasone (B). Equal amounts of crude membranes prepared as described in Materials and Methods were subjected to polyacrylamide gel electrophoresis and processed for Western blot. Relative intensities of the cyclooxygenase band were determined by laser densitometric scanning of the blot.

generation of prostanoids. Glucocorticoid treatment of TPA-differentiated U937 cells decreased the cyclooxygenase/PGE isomerase activity up to 70%. We thus confirmed our previously observed dexamethasone-induced reduction of cyclooxygenase/PGE isomerase activity in mouse bone marrow-derived macrophages [7]. The effect was specific for glucocorticoids as compared to other steroids. The *in vitro* assay of cyclooxygenase was optimized for the conversion of exogenously added arachidonic acid into PGE_2, but in addition to PGE_2 small amounts of TxB_2 and $PGE_{2\alpha}$ were also formed in the assay. The effect of glucocorticoids was the same on the formation of all prostanoids investigated, suggesting cyclooxygenase as the primary target of glucocorticoid action. However, an impairment of the distal enzymes of the prostanoid synthesis pathway, e.g. PGE_2 isomerase, TxB_2 synthase and $PGF_{2\alpha}$ synthase by glucocorticoids cannot be excluded completely. The effect on cyclooxygenase activity was confirmed by Western blot analysis, which showed a decreased amount of cyclooxygenase protein in TPA-differentiated U937 cells after glucocorticoid treatment. These results are in agreement with data of Raz *et al.* in human skin fibroblasts [11] and those of Bailey *et al.* in endothelial cells [10]. As in the case of the inhibition of phospholipase A_2, addition of cycloheximide to the cell cultures by itself strongly inhibited the cyclooxygenase activity and no additional effect on the dexamethasone-induced inhibition was observed. This is in accordance with the action of both substances on the transcriptional and/or translational level, the exact mechanism of which remains to be established in the case of glucocorticoids. However, the data presently available do not exclude the possibility of an additional induction of an inhibitory protein which might interact directly with the cyclooxygenase.

In summary, inhibition of both phospholipase A_2 and cyclooxygenase activity contributed to the observed reduction of prostanoid secretion by glucocorticoids. Reduced phospholipase A_2 activity might result in diminished prostanoid production due to inadequate precursor supply. However, inhibition of

cyclooxygenase activity was even more pronounced suggesting that inhibition of cyclooxygenase activity represents an important step in glucocorticoid-induced inhibition of prostanoid synthesis.

Acknowledgements—The lipocortin I and II antibodies were kindly provided by Dr R. B. Pepinsky (Biogen Corp., Boston, MA, U.S.A.). The authors thank Dr W. Sierralta and Dr P. W. Jungblut (Max-Plank-Institut für Experimentelle Endokrinologie, Hannover, F.R.G.) for their helpful advice with the steroid receptor studies and Dr N. Topley for the critical reading of the manuscript. The technical assistance of Mrs A Ehlers is gratefully acknowledged. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 244/B5).

REFERENCES

1. Bowen DF and Fauci AS, Adrenal Corticosteroids. In. *Inflammation: Basic Principles and Clinical Correlates* (Eds. Gallin JI, Goldstein M and Snyderman R), pp. 877–893. Raven Press, Ltd, New York, 1988.
2. Erman A, Hassid A, Baer PG and Nasjletti A, Treatment with dexamethasone increases glomerular prostaglandin synthesis in rats. *J Pharmacol Exp Ther* **239**: 296–301, 1986.
3. Potestio FA, Zakar T and Olson DM, Glucocorticoids stimulate prostaglandin synthesis in human amnion cells by a receptor-mediated mechanism. *J Clin Endocrinol Metab* **67**: 1205–1210, 1988.
4. Flower RJ, Lipocortin and the mechanism of action of the glucocorticoids. *Br J Pharmacol* **94**: 987–1015, 1988.
5. Isacke CM, Lindberg RA and Hunter T, Synthesis of p36 and p35 is increased when U937 cells differentiate in culture but expression is not inducible by glucocorticoids. *Mol Cell Biol* **9**: 232–240, 1989.
6. Northup JK, Valentine-Braun KA, Johnson LK, Severson DL and Hollenberg MD, Evaluation of the anti-inflammatory and phospholipase-inhibitory activity of calpactin II/lipocortin. *J Clin Invest* **82**: 1347–1352, 1988.
7. Davidson FF, Dennis EA, Powell M and Glenney JR Jr, Inhibition of phospholipase A_2 by lipocortins and calpactins. *J Biol Chem* **262**: 1698–1705, 1987.
8. Aarsman AJ, Mynbeek G, van den Bosch H, Prieur B, Comera C, Jordan L and Russo-Marie F, Lipocortin inhibition of extracellular and intracellular phospholipases A_2 is substrate concentration dependent. *FEBS Lett* **219**: 176–180, 1987.
9. Goppelt-Struebe M, Wolter D and Resch K, Glucocorticoids inhibit prostaglandin synthesis not only at the level of phospholipase A_2 but also at the level of cyclooxygenase/PGE isomerase. *Br J Pharmacol* **98**: 1287–1295, 1989.
10. Bailey JM, Makheja AN, Pash J and Verma M, Corticoids suppress cyclooxygenase messenger RNA levels and prostanoid synthesis in cultured vascular cells. *Biochem Biophys Res Commun* **157**: 1159–1163, 1988.
11. Raz A, Wyche A and Needleman P, Temporal and pharmacological division of fibroblast cyclooxygenase expression into transcriptional and translational phases. *Proc Natl Acad Sci USA* **86**: 1657–1661, 1989.
12. Koehler L, Hass R, Goppelt-Struebe M, Kaever M and Resch K, Differential effect of dexamethasone on prostanoid synthesis in undifferentiated and TPA-differentiated U937 cells. *J Cell Biochem* **40**: 397–406, 1989.
13. Hass R, Bartels H, Topley H, Hadam M, Koehler L, Goppelt-Struebe M and Resch K, TPA-induced differentiation and adhesion of U937 cells: changes in ultrastructure, cytoskeletal organisation and expression of cell surface antigens. *Eur J Cell Biol* **48**: 282–293, 1989.
14. Kaever V, Goppelt-Struebe M and Resch K, Enhancement of eicosanoid synthesis in mouse peritoneal macrophages by the organic mercury compound thimerosal. *Prostaglandins* **35**: 885–902, 1988.
15. Leake RE and Habib F, Receptor analysis and characterization. In: *Steroid Hormones* (Eds. Green B and Leake RE), pp. 85–87 IRL Press at Oxford University Press, Oxford, 1987.
16. Scatchard G, The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
17. Redinbaugh HG and Campbell WH, Adaption of the dye-binding protein assay to microtiter plates. *Anal Biochem* **147**: 144–147, 1985.
18. Flesch I and Ferber E, Effect of cellular fatty acid composition on phospholipase A_2 activity of bone marrow-derived macrophages and their ability to induce lucigenin-dependent chemiluminescence. *Biochim Biophys Acta* **889**: 6–14, 1986.
19. Koehler L, Hass R, Wessel K, DeWitt DL, Kaever V, Resch K and Goppelt-Struebe M, Altered arachidonic acid metabolism during differentiation of the human monoblastoid cell line U937. *Biochim Biophys Acta* **1042**: 395–403, 1990.
20. Brune K, Rainsford KD, Wagner K and Peskar BA, Inhibition by antiinflammatory drugs of prostaglandin production in cultured macrophages. *Arch Pharmacol* **315**: 269–278, 1981.
21. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
22. Pepinsky RB, Tizard R, Mattaliano RJ, Sinclair LK, Miller GT, Browning JL, Chow EP, Burne C, Huang K-S, Pratt D. Wachter L, Hession C, Frey AZ and Wallner BP, Five distinct calcium and phospholipid binding proteins share homology with lipocortin I. *J Biol Chem* **263**: 10799–10811, 1988.
23. Hunter WM and Greenwood FC, Preparation of iodine-131 labelled human growth factor hormone of high specific activity. *Nature* **194**: 495–496, 1962.
24. Israel S, Hahn T, Holtmann H and Wallach D, Binding of human TNF-α to high-affinity cell surface receptors; effect of IFN. *Immunol Lett* **12**: 217–224, 1986.
25. Hedin L, Gaddy-Kurten D, Kurten R, DeWitt DL, Smith WL and Richards JS, Prostaglandin endoperoxide synthase in rat ovarian follicles: content, cellular distribution, and evidence for hormonal induction preceding ovulation. *Endocrinology* **121**: 722–731, 1987.
26. Danon A and Assoline G, Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. *Nature* **273**: 552–554, 1978.
27. Bienkowski MJ, Petro MA and Robinson LJ, Inhibition of thromboxane A synthesis in U937 cells by glucocorticoids. *J Biol Chem* **264**: 6536–6544, 1989.
28. Duval D, Lynde P, Hatzfeld A and Hatzfeld J, Dexamethasone-induced stimulation of arachidonic acid release by U937 cells grown in defined medium. *Biochim Biophys Acta* **887**: 204–213, 1986.
29. Ranelletti FO, Piantelli N, Iacobelli S, Musiani P, Longo P, Lauriola L and Marchetti P, Glucocorticoid receptors and *in vitro* corticosensitivity of peanut-positive and peanut-negative human thymocyte subpopulations. *J Immunol* **127**: 849–855, 1981.
30. Irvine RF, How is the level of free arachidonic acid controlled in mammalian cells. *Biochem J* **204**: 3–16, 1982.