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Dexamethasone induces the expression of the mRNA of lipocortin 1 and 2 and the release of lipocortin 1 and 5 in differentiated, but not undifferentiated U-937 cells

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The effect of dexamethasone on mRNA and protein synthesis of lipocortins (LCT) 1, 2 and 5 has been investigated in U-937 cells A constitutive expression of both mRNAs and proteins was detected in undifferentiated U-937 cells This constitutive level was increased time- and dose-dependently by incubation with phorbol myristate acetate (PMA) In U-937 cells differentiated by 24 h incubation with 6 ng/ml PMA, dexamethasone (DEX) (1 μM for 16 h) caused an increased synthesis of the mRNA level of LCT-1 and 2, but not of LCT-5, over the level induced by PMA DEX had no effect in undifferentiated cells Moreover, DEX stimulated the extracellular release of LCT-1 and 5, but not of LCT-2, and inhibited the release of PGE_2 and TXB_2 only in the differentiated U-937 cells These results suggest that the responsiveness of these cells to glucocorticoids is dependent on the phase of cell differentiation The selective release of lipocortins by differentiated U-937 cells may explain, at least in part, the inhibition by DEX of the prostanoid release

Lipocortin expression, Cell differentiation, Dexamethasone, Eicosanoid inhibition

1. INTRODUCTION

Lipocortins have been defined as proteins induced by glucocorticoids through a receptor-dependent mechanism which are able to inhibit phospholipase A_2 enzyme activity [1]. This inhibition results in. (i) a decreased release of pro-inflammatory lipid metabolites like prostaglandins, leukotrienes and platelet-activating factor, (ii) an anti-inflammatory action which mimicks the effect of glucocorticoids (reviewed by Flower [2]) Lipocortins belong to a large family of calcium- and phospholipid-binding proteins which have been differently named. annexins, calpactins, chromobindins, anchorins, calcimedins, endonexins, calelectrins, inhibitor of blood coagulation, proteins I–III (for review see [3,4]) At the present, eight lipocortins have been identified and sequenced They share a common four-fold repeat structure [5] and are characterized by a 17-amino acid consensus sequence which may be implicated in the binding to biomembranes [6].

Lipocortins are a highly controversial topic Inhibition of PLA_2 activity, reduction of eicosanoid release and anti-inflammatory effect have been reported for LCT-1 [7-10], LCT-2 [11] and LCT-5 [12]. On the other hand, the inhibitory action on PLA_2 has been ascribed to substrate depletion rather than to a direct interaction with the enzyme [13–15] and negative data on the anti-inflammatory effect of LCT-1 have been reported [16] Conflicting results have also been published on the glucocorticoid property of inducing the synthesis of lipocortins Lipocortin induction by glucocorticoids has been observed in macrophages [17], renomedullary cells [18], neutrophils [19,20], thymocytes [21], fibroblasts [22,23], squamous carcinoma cells [24]. However, in different experiments it has been shown that DEX did not induce LCT-1 mRNA in fibroblasts, lymphocytes, alveolar macrophages and HeLa cells [25], as well as in U-937 cells [26] Moreover, several authors have reported inhibitory effects of glucocorticoids on eicosanoid release independent of lipocortin induction [27–29] This discrepancy has been ascribed to the complexity of steroid regulation of protein synthesis and the requirement for either additional factors or for a defined stage of cell differentiation has been recently suggested [30] It is also noteworthy that LCT-1 and -2 have been shown to be identical to the 35 kDa substrate for EGF-receptor/kinase and $pp60^{v\text{-}src}$ respectively [31-33] This observation raised the question of lipocortin involvement in the regulation of cell growth and differentiation. Subsequent work has demonstrated that differentiation of U-937 cells [26], HL-60 cells [34], human skin fibroblasts [35] was associated with increased expression of lipocortins

In this paper we have tested the hypothesis that the induction of the proteins by glucocorticoids is depend-

Abbreviations LCT, lipocortins, PLA_2, phospholipase A_2, PMA, phorbol 12-myristate 13-acetate, DEX, dexamethasone

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ent on the stage of cell differentiation The effect of DEX on mRNA and protein level of LCT-1, -2 and -5 has been investigated in U-937 cells which can be induced to differentiate from an immature monocytic phenotype into macrophage-like cells by incubation with phorbol esters [36,37]

2. MATERIALS AND METHODS

2 1 *Materials*

U-937 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Gibco, Paisley, UK) containing 2 mM L-glutamine (Serva Feinbiochimica Co , Heidelberg, Germany) together with 50 μg/ml gentamicin (Sigma Chemical, USA) and 10% foetal calf serum (FCS) (Hyclone, Logan, UK) in 5% CO_2 at 37°C in a controlled humidified incubator Phorbol 12-myristate 13-acetate, dexamethasone-21-phosphate, phenylmethylsulfonyl fluoride were from Sigma TXB_2 and PGE_2 RIA kit with minimal cross-reactivity (0 06 and 3 7, respectively) with PGE_1 were from NEN-E I DuPont de Nemours and Co , USA Nonidet P-40 was from Pharmacia-LKB (Biotechnology, Uppsala, Sweden) Oligo(dT) cellulose, random oligonucleotide primers, deoxynucleotide triphosphates, dideoxynucleotide triphosphates, RNase H, T_4 DNA polymerase, Klenow fragment of DNA polymerase I, and T_4 polynucleotide kinase were from Promega (Madison, WI, USA) Restriction endonucleases, *Eco*RI methylase, T_4 DNA ligase, and *Eco*RI linkers were from New England Biolabs (Beverly, MA, USA) Nitrocellulose and Nytran filters were from Schleicher&Schuell (Dassel, Germany) Ponceau S colorant was from Serva Feinbiochimica Acrylamide-bis, Triton X-100 and 4-chloro-1-naphtol were obtained from Bio-Rad (Hercules, CA, USA) Peroxidase conjugated IgG, fraction goat anti-rabbit Ig heavy and light chains specific were from Cappel (Westchester, PA, USA)

2 2 *Differentiation of U-937 cells*

U-937 cells were plated at 5×10^5 cells/ml in a medium containing 5% FCS and different concentrations of PMA (0 6, 6 60 ng/ml corresponding to 1, 10 and 100 nM, respectively) for different times of incubation (4 and 24 h) After the incubation the cells were washed with a buffer solution containing 1 mM PMSF then lysed with a phosphate buffer solution containing 0 1% NP-40 plus 1 mM PMSF and incubated on ice for 10 min After centrifugation the supernatants were boiled in SDS buffer and electrophoresed on 10% SDS PAGE according to Laemmli [38] On the basis of the results obtained (see below), in subsequent experiments the U-937 cells were incubated with or without 6 ng/ml PMA for 24 h Then DEX (1 μM) was added to both undifferentiated and differentiated cells and incubation carried out for a further 2 or 16 h in the presence of both PMA and DEX In some experiments at the end of incubations an aliquot of the medium was removed for prostanoid radioimmunoassay and the cells lysed as described above Cell viability was always >95% as measured by the Trypan blue exclusion test as well as the release into the supernatant of lactic dehydrogenase assayed according to Bergmeyer [39]

2 3 *Oligonucleotide synthesis*

Oligonucleotides were synthesized using an Applied Biosystem Instrument 380B DNA synthesizer and purified by preparative denaturing PAGE followed by a desalting G50 column When necessary oligonucleotides were labelled with T_4 polynucleotide kinase and (γ-^{32}P]ATP

2 4 *Isolation of cDNA probes for human lipocortins*

A LCT-2 specific oligonucleotide probe, complementary to nucleotides 30 to 70 of the human LCT-2 cDNA [33] was synthesized as previously described [40] Briefly poly(A)$^+$) RNA from U-937 cells was prepared by two rounds of chromatography on oligo(dT) cellulose A cDNA library was constructed in the λgt11 vector, using a Boehringer-Mannheim cDNA synthesis kit and a Stratagene in vitro packaging kit, following the manufacturer's instructions Plaque hybridization, according to Maniatis et al [41], was performed at 65°C in 4× SSC A 950 bp *Eco*RI fragment from nucleotide 6 to the *Eco*RI site present in the human LCT-2 cDNA sequence was subcloned in Bluescript SK and completely sequenced with the dideoxy chain termination method [42] This fragment was used for hybridization on Northern blots Human LCT-1 and -5 cDNAs were obtained with the polynucleotide chain reaction method [43] using the λDNA obtained from the cDNA library described above For LCT-1, oligonucleotide CGCGCCATGGCAATGGTATCAGAATTCCTC (corresponding to amino acids 1–8) and oligonucleotide GCGCGAGCTCTTAGTTTCCTGGACAAAGAGCCAC (corresponding to amino acids 340–346) [44] were used as 5′ and 3′ primer, respectively For LCT 5, oligonucleotide CGCGCCATGGACAGGTTCTCAGAGGCA (corresponding to amino acids 1–7) and oligonucleotide GCGCGAGCTCTTAGTCATCTTCTCCACAGAGCAG (corresponding to amino acids 314–320) [5] were used as 5′ and 3′ primer, respectively Amplified DNAs were subcloned in the *Nco*I and *Sac*I restriction sites of the pGEM5Zf(+) vector (Promega) and sequenced with the dideoxy chain termination method [42] The *Nco*I–*Sac*I fragment was used for hybridization experiments

2 5 *Northern blot analysis*

Total RNA was obtained from 10–50 million cells, according to the method of Chomczynski and Sacchi [45] 5 μg of total RNA were size fractionated on denaturing 1 2% agarose gel and transferred to Gene Screen Plus membranes (NEN), as described by Maniatis et al [41] ^{32}P-labelled cDNA probes were obtained using the random primed DNA labelling kit from Boehringer, following the manufacturer's instructions and with an average specific activity of 500 million cpm per μg of DNA Hybridization was carried out in 5× Denhart's solution 4×SET and 0,1% SDS solution and washes in 2× SET and 0 1% SDS, at 65°C (all solution prepared according to Maniatis et al [41] Hybridization signals were normalized to an internal standard (β-actin) Autoradiography was carried out at -40°C, using intensifying screen and Kodak XAR film and autoradiographies were evaluated with a LKB Ultrascan XL Laser Densitometer

2 6 *Western blot analysis*

Intracellular and extracellular proteins were separated in 10% polyacrylamide gels according to Laemmli [38] and electroblotted to nitrocellulose membranes Immunodetection was performed using specific anti-lipocortin polyclonal antibodies (1 1000 dilution) (a kind gift from Dr J L Browning, Biogen, Cambridge, MA) and goat anti-rabbit immunoglobulin conjugated to peroxidase with 4-chloro-1-napthol as substrate

3. RESULTS

3.1 *Effect of differentiation of U-937 cells on lipocortin mRNA and protein synthesis*

Several (n=4) Northern and Western blotting experiments were performed on U-937 cells incubated for 4 and 24 h either with or without PMA (0 6–60 ng/ml). In Fig 1 a typical Northern blot analysis together with the densitometric scanning is shown. Undifferentiated cells showed a constitutive expression of the mRNAs of LCT-1, -2 and -5, with the mRNA of LCT-2 being more abundant than the others The LCT-1 mRNA synthesis was increased by approximately 1 5-fold by PMA high concentrations (6–60 ng/ml) after 24 h incubation, whereas the stimulation after 4 h was less pronounced (Fig 1a) After 4 h incubation the maximal stimulation (approx 3-fold) of the LCT-2 mRNA level was observed in the presence of 60 ng/ml PMA, whereas after

24 h the maximal increase (approx 4-fold) was reached at 6 ng/ml PMA (Fig. 1b). Similarly to LCT-2, the maximal stimulation (approx 4-fold) of the synthesis of the mRNA of LCT-5 was observed at 60 ng/ml PMA after 4 h and at 6 ng/ml PMA after 24 h (Fig. 1c).

In Fig 2 the corresponding Western blot analysis on the intracellular proteins is shown. Constitutive expression of the intracellular proteins was observed in the undifferentiated U-937 cells. The synthesis of LCT-1 was stimulated by PMA with a peak at 60 and 6 ng/ml after 4 and 24 h, respectively (Fig 2a) After 4 h incubation the maximal increase of LCT-2 was also observed at 60 ng/ml PMA, whereas after 24 h the maximum was reached at 0.6 ng/ml (Fig 2b) The synthesis of LCT-5 was stimulated to a similar extent after 4 h by the three concentrations of PMA, whereas after 24 h the maximal increase was reached at 60 ng/ml PMA (Fig 2c) During these experiments no release of the proteins in the supernatants was observed (not shown) These results confirm and extend to LCT-5 previous observations [26,34,35] that lipocortins are constitutively expressed proteins and that the process of cell differentiation is accompanied by an increased level of both mRNA and protein synthesis On the other hand, the differentiation process did not cause the release of any of the proteins in the extracellular medium

3 2 *Effect of dexamethasone on undifferentiated and differentiated U-937 cells*

On the basis of the results of the previous experiments we decided to investigate the effect of dexamethasone on the expression of lipocortins in U-937 cells either undifferentiated or differentiated by incubation with 6 ng/ml PMA for 24 h In a first series of experiments (n=4) the cells incubated with or without PMA were further incubated for 2 h in the presence of 1 μM DEX A typical Northern blot analysis with the densitometric scanning from these experiments is shown in Fig 3 Consistent with the previous results, PMA (6 ng/ml for 24 h) was able to increase several-fold the synthesis of lipocortin mRNA The increase of lipocortin mRNA by PMA was a constant observation in all Northern blot analyses performed, although the extent of the stimulation varied between experiments The messenger level was not modified by the treatment with the glucocorticoid (1 μM for 2 h) in either undifferentiated or differentiated U-937 cells The corresponding Western blot analyses on intracellular proteins (not shown) confirmed the stimulation by PMA of the protein synthesis which was not significantly altered by DEX Also under these conditions lipocortins were not released into the medium (not shown).

In a second series of experiments (n=6), the cells were incubated with or without 6 ng/ml PMA for 24 h, then 1 μM DEX was added and incubation carried out for 16 h. A typical Northern blot analysis with the densitometric scanning from these experiments is shown in Fig

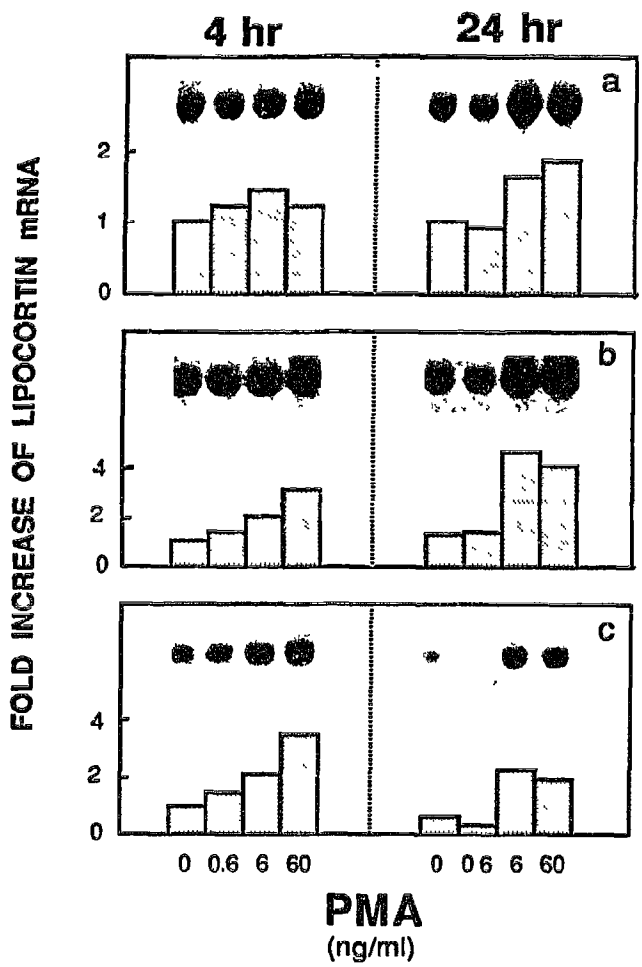

Fig 1 Effect of PMA-induced differentiation on lipocortin mRNA level U-937 cells (5×10^5 cells/ml) were cultured in RPMI 1640 with 5% FCS and incubated either for 4 and 24 h with or without PMA at the indicated concentrations At the end of incubations Northern blot analyses were performed using 5 μg total mRNA as described under Experimental Procedures Exposure time was 48 h at −40°C with an intensifying screen The densitometric scanning below the corresponding hybridization signal shows the normalized quantification The analysis is representative of 4 experiments with similar results (a) LCT-1, (b) LCT-2, (c) LCT-5

4 Again the level of lipocortin mRNAs was increased several-fold by PMA treatment (note the difference in the densitometric scale compared to Fig. 3). In undifferentiated U-937 cells the amount of the lipocortin mRNAs was not modified by DEX treatment. In contrast, in differentiated U-937 cells further incubated for 16 h with 1 μM DEX the level of mRNA of LCT-1 and 2 rose approx. by 5- and 2 5-fold, respectively. The LCT-5 messenger was not substantially altered by the glucocorticoid treatment. The corresponding Western blot analysis is shown in Fig 5 The synthesis of intracellular lipocortins was stimulated by PMA. Again the intracellular protein synthesis was not modified by DEX treatment in both undifferentiated and differentiated U-937 cells On the other hand, the release in

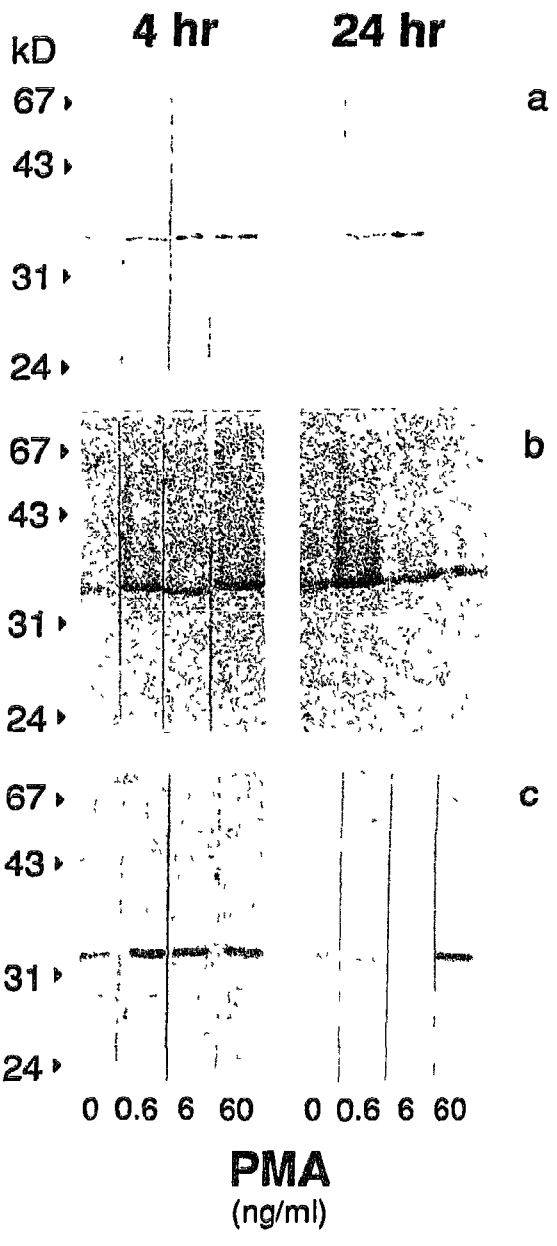

Fig 2 Effect of PMA-induced differentiation on lipocortin synthesis Western blot analysis of intracellular proteins from the same experiment shown in Fig 1 Immunodetection was performed using specific anti-lipocortin polyclonal antibodies (1/1000 dilution) as described under Experimental Procedures The analysis is representative of 4 experiments with similar results (a) LCT-1 (b) LCT-2, (c) LCT-5

the cell supernatant of LCT-1 and -5, but not LCT-2, was observed in differentiated U-937 cells further incubated with 1 μM DEX for 16 h. This release could not be due to cell death, as suggested by Isacke et al. [26], since cell viability assessed by trypan blue exclusion and lactic dehydrogenase release was always >95%. Moreover, in the case of cell lysis, all three proteins should have been detected in the cell supernatant.

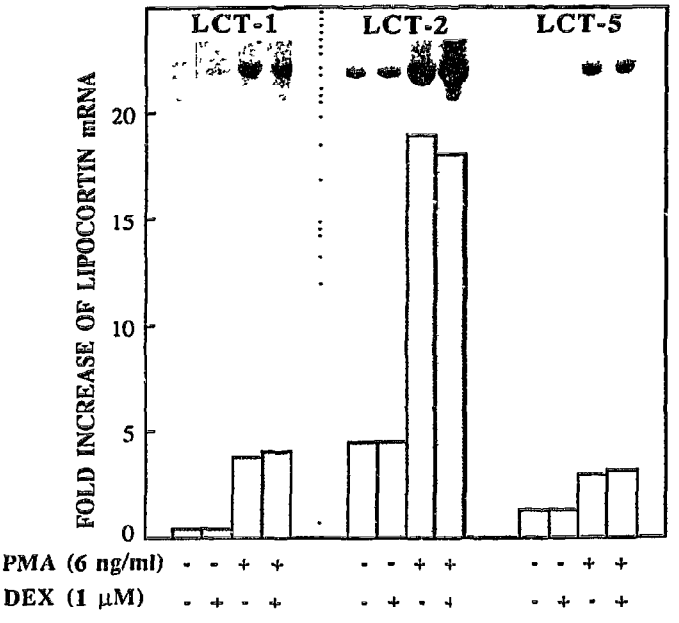

Fig 3 Effect of DEX (short incubation) on lipocortin mRNA level U-937 cells (5×10^5 cells/ml) were cultured in RPMI 1640 with 5% FCS and incubated either with or without 6 ng/ml PMA After 24 h, 1 μM DEX was added where indicated and incubation carried out for a further 2 h At the end of the incubations Northern blot analyses were performed using 5 μg total mRNA as described under Experimental Procedures Exposure time was 18 h at −40°C with an intensifying screen The densitometric scanning below the corresponding hybridization signal shows the normalized quantification The analysis is representative of 4 experiments with similar results

3 3 *Arachidonic acid metabolisms in undifferentiated and differentiated U-937 cells*

It has been shown that dexamethasone is able to inhibit eicosanoid synthesis only in differentiated U-937 cells [46] To investigate this the concentration of TXB_2 and PGE_2 was measured by radioimmunoassay in the supernatant of cells incubated under the following conditions (i) undifferentiated U-937 cells, (ii) undifferentiated U-937 cells incubated in the presence of 1 μM DEX for 16 h, (iii) U-937 cells differentiated by treatment with 6 ng/ml PMA for 40 h; (iv) U-937 cells incubated with 6 ng/ml PMA for 24 h and with both PMA and 1 μM DEX for a further 16 h. The results are reported in Table I Undifferentiated cells released low amounts of both TXB_2 and PGE_2 After PMA-induced differentiation the concentration in the supernatants of TXB_2 and PGE_2 rose dramatically by approx. 10- and 80-fold, respectively. DEX treatment had no effect on prostanoid release by undifferentiated cells, but significantly inhibited the release by differentiated U-937 cells.

4. DISCUSSION

The hypothesis that part of the anti-inflammatory action of glucocorticoids is mediated by lipocortins acting as 'second messengers' is based on two mainstays. the inducibility of lipocortins by glucocorticoids and the

capability of these proteins to interfere with PlA_2 activity.

In this paper we have addressed the issue of the regulation by glucocorticoids of the expression of LCT-1, -2, and -5 in U-937 cells, a human monocytic cell line, under a variety of experimental conditions The results show that in differentiated U-937 cells long incubations (16 h) with DEX caused (i) an increased synthesis of the mRNAs of LCT-1 and LCT-2 over the expression induced by the differentiation process; (ii) the selective extracellular release of LCT-1 and LCT-5.

The increased expression of LCT-1 and -2 mRNAs is in apparent disagreement with recent results by Isacke et al. [26] and by Bienkowski et al [27] In U-937 cells treated with DEX these authors did not observe any increase of the messenger RNA coding for these two proteins over the level induced by PMA. It should be noted, however, that in the experiments by Isacke et al [26], the Northern blot analyses were performed after a maximum of 6 h incubation in the presence of both PMA and DEX, whereas in our experiments the cells were incubated for 24 h with PMA and for a further 16 h in the presence of both PMA and DEX It has been demonstrated that after 72 h incubation with PMA U-937 cells differentiate into an immature macrophage intermediate rather than into mature macrophage-like cells [37] It is therefore conceivable that in short incubations (6 h) with PMA, U-937 cells cannot achieve the critical differentiation state in which they become sensitive to the action of glucocorticoids. In agreement with this, it has been shown that the number of glucocorticoid receptors in differentiated U-937 cells is approx. 10-fold higher than in undifferentiated cells [47]. This critical differentiation state may account for cell responsiveness to corticosteroids explaining the diversity of reports on positive [21,44] and negative [25,27] data on the incubation of lipocortin mRNA expression

Table I

Effect of dexamethasone (DEX) on TXB_2 and PGE_2 release by U-937 cells

U-937 cells (5×10^5 cells/ml) cultured in RPMI 1640 with 5% FCS were incubated either with or without 6 ng/ml PMA After 24 h 1 μM DEX was added where indicated After a further 16 h an aliquot of the supernatants was collected for prostanoid radioimmunoassay

Sample	TXB_2 (ng/ml)	PGE_2 (pg/ml)
Undifferentiated cells	11 3±0 01	23 4±2 5
Undifferentiated cells + DEX	12 2±0 1	28 5±3 5
PMA-differentiated cells	114 8±0 01	1900 7±34 5
PMA-differentiated cells + DEX	55 4±2 2*	144 0±6 0*

Values are means ± SE of 3 experiments performed in triplicate (n=3)

*P<0 0005 vs PMA-differentiated cells (Student's t test for paired samples)

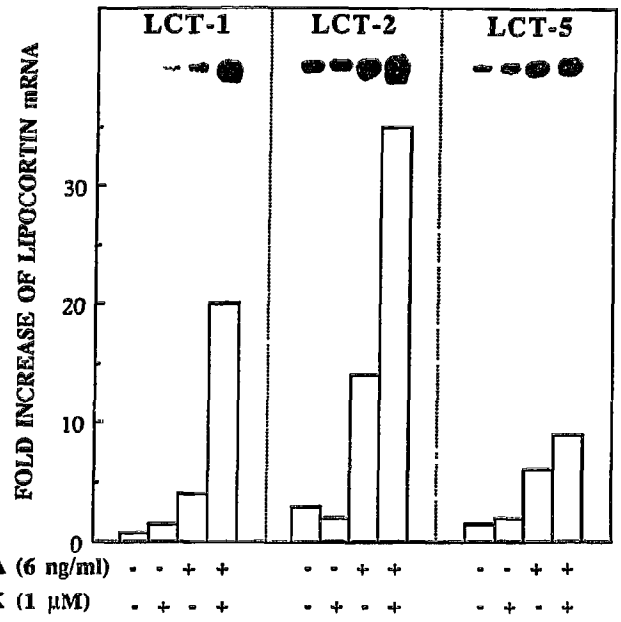

Fig 4 Effect of DEX (long incubation) on lipocortin mRNA level U-937 cells (5×10^5 cells/ml) were cultured in RPMI 1640 with 5% FCS and incubated either with or without 6 ng/ml PMA After 24 h 1 μM DEX was added where indicated and incubation carried out for a further 16 h At the end of the incubations Northern blot analyses were performed using 5 μg total mRNA as described under Experimental Procedures Exposure time was 18 h at −40°C with an intensifying screen The densitometric scanning below the corresponding hybridization signal shows the normalized quantification The analysis is representative of 6 experiments with similar results

by glucocorticoids This hypothesis is supported by the fact that lipocortin mRNA induction has been detected in rat macrophages [44] and thymocytes [21] after in vivo administration of DEX. In addition, long incubations (>12 h) of the differentiated cells in the presence of steroid drugs may be necessary to observe gene activation since incubation times up to 4 h were insufficient to stimulate mRNA expression [27].

Relevantly, it has been recently reported that in three different mouse cell lines the induction of the LCT-1 mRNA by DEX required between 8 and 24 h incubation [48]

The intracellular synthesis of lipocortins was not affected by either short or long exposure to DEX in both undifferentiated and differentiated U-937 cells confirming previous observations [26–28,47] The constitutive expression of these proteins may mask stimulatory effects by glucocorticoids on protein synthesis. Indeed, we have recently reported that macrophages from adrenalectomized rats have a very low level of constitutive lipocortins In these cells the synthesis of intracellular LCT-1, -2, and -5 was dramatically increased by in vivo administration of DEX to the animals [49].

The observation of the glucocorticoid-stimulated release of LCT-1 and LCT-5 from differentiated U-937 cells is intriguing since lipocortins are intracellular pro-

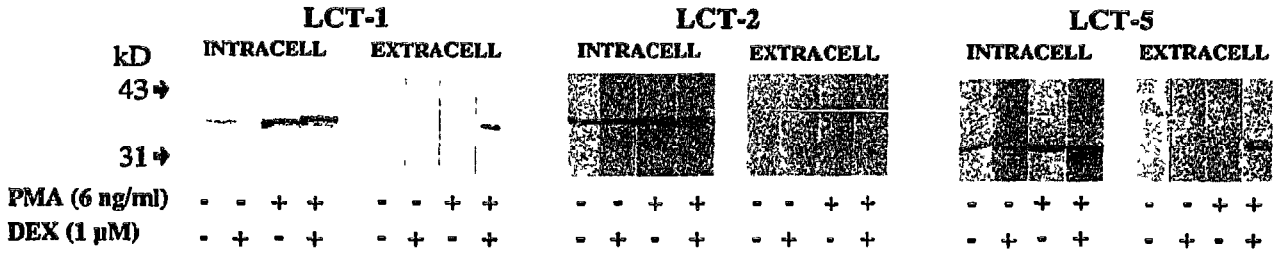

Fig 5 Effect of DEX (long incubation) on lipocortin synthesis and release Western blot analysis of intracellular and extracellular proteins from the same experiment shown in Fig 4 Immunodetection was performed using specific anti-lipocortin polyclonal antibodies (1/1000 dilution) as described under Experimental Procedures The analysis is representative of 6 experiments with similar results

teins lacking hydrophobic signal sequences It is of interest that earlier investigations reporting the steroid-induced release of lipocortins [17–19] have been recently confirmed in humans following in vivo administration of glucocorticoids [50,51] Moreover, the selective release of LCT-1 and -5 (annexins I and V), but not of LCT-4 (annexin IV), in the human prostate fluid has been recently reported by Christmas et al [52] It has been recently proposed that lipocortin(s) belong to a new class of secretory proteins which do not have hydrophobic sequences and are likely to be secreted through pathways which do not involve the classical secretion route through the endoplasmic reticulum and Golgi apparatus [53,54] On this basis it is tempting to suggest that in differentiated cells glucocorticoids are able to activate an as yet unknown process leading to selective release of lipocortins

This hypothesis is also supported by the results shown in Table I which demonstrate that DEX treatment was able to inhibit prostanoid release only in differentiated U-937 cells It has been shown that both PLA_2 and cyclooxygenase activities are significantly inhibited by DEX in diferentiated U-937 cells [47]. These observations are consistent with the concept that in differentiated cells glucocorticoids are able to stimulate the release of active species of lipocortins which in turn impair PLA_2 activity, as it has been recently suggested for human squamous carcinoma cells [24] It is then feasible that the inhibitory effect of glucocorticoids on arachidonic acid metabolism in differentiated U-937 cells is brought about by a combined inhibitory action on both PLA_2 and cyclooxygenase

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