Dexamethasone-induced translocation of lipocortin (annexin) 1 to the cell membrane of U-937 cells

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Lipocortin (annexin) 1 is a putative mediator of the inflammatory effects of glucocorticoids. By flow cytometric analysis (FACS) we have studied the effect of dexamethasone on the cellular localization of lipocortin 1. U-937 cells were incubated with or without 10 nM phorbol 12-myristate 13-acetate (PMA) to induce cell differentiation. Then 1 μ M dexamethasone was added and incubation carried out for increasing times (1–24 h). Dexamethasone caused a time-dependent biphasic translocation of lipocortin 1 from the intracellular compartment to the cell membrane with maximal membrane expression at 4 and 24 h. In differentiated U-937 cells the steroid-induced membrane accumulation of lipocortin 1 was significantly higher than that of undifferentiated cells. The accumulation of the protein in the cell membrane may precede its release which is stimulated by dexamethasone in differentiated U-937 cells. Since extracellular lipocortin 1 has anti-inflammatory properties the modulation of the translocation/secretion process of the protein by glucocorticoids may be part of their mechanism of action.

Keywords: Lipocortin 1; dexamethasone; flow cytometry; membrane translocation

Introduction Lipocortin (annexin) 1 is a member of a family of proteins endowed with calcium and phospholipid binding properties which has been proposed as a putative mediator of the anti-inflammatory action of glucocorticoids. This hypothesis is based on experimental evidence showing that steroid drugs are able to induce the synthesis and/or release of the protein (Goulding *et al.*, 1990) and that extracellular lipocortin 1 shares many of the anti-inflammatory effects of glucocorticoids (Becherucci *et al.*, 1993). The extracellular release of lipocortin 1 has been questioned by experimental evidence showing that it is an intracellular protein lacking in hydrophobic signal peptide (Frey *et al.*, 1991). On the other hand Christmas *et al.* (1991) have observed that lipocortin 1 is secreted by the human prostate gland through a novel secretory mechanism of high sorting efficiency.

We have previously shown that dexamethasone induces the expression of the mRNA of lipocortin 1 and the secretion of the protein in differentiated, but not undifferentiated U-937 cells (Solito *et al.*, 1991). These cells have been used in the present paper to investigate by flow cytometric analysis (FACS) the effect of dexamethasone on the cellular localization of lipocortin 1.

Methods *Intracellular and cell membrane immunofluorescence of U-937 cells* U-937 cells plated at $5 \times 10^5 \text{ ml}^{-1}$ were incubated for 24 h either with or without 10 nM PMA. Then, in the appropriate experiments, 1 μ M dexamethasone was added and cells further incubated for increasing times (1–24 h). To assess intracellular expression, cells were fixed and permeabilized according to Dent *et al.* (1989). Immunofluorescence was performed as described in the Beckton Dickinson Monoclonal Handbook. Briefly, cells resuspended in phosphate-buffered saline (PBS) containing 1% BSA were incubated with human IgG (1 mg ml^{-1}) for 20 min at 4°C to prevent non-specific binding. After washing, the cells were incubated either with the anti-CD14 antibody (1:10 dilution) or with the anti-lipocortin 1 antibody (1:100 dilution) for 20 min at 4°C. After washing the FITC anti-rabbit Ig antibody (5 $\mu\text{g ml}^{-1}$) was added and cells further incubated for 20 min at 4°C. Before FACS cell viability was assessed by the propidium iodide method, as described by Shapiro (1988). Only viable cells have been used for the analysis.

Flow cytometric analysis (FACS) The quantitative FACS was performed on a FACSTAR flow cytometer (Beckton Dickinson) equipped with an Argon ion laser beam operating at 488 nm using 200 mW of power to excite the FITC and the propidium iodide. Log fluorescence histograms (256-channel) were obtained from approx. 10000 viable cells for each sample. With the Consort 30 Data Analysis System from Beckton Dickinson, mean channel number fluorescence was used to assess differences in fluorescence intensity.

Materials Human monocytic U-937 cells were obtained from the American Type Culture Collection and cultured as previously described (Solito *et al.*, 1991). Dexamethasone-21-phosphate, phorbol 12-myristate 13-acetate (PMA), propidium iodide, were purchased from the Sigma Chemical Company. Fluorescein isothiocyanate (FITC)-labelled antibodies anti-human and anti-rabbit Ig were from Boehringer Mannheim Biochemica, Germany. FITC-labelled antibody against CD14 was from Beckton-Dickinson, Mountain View, CA, U.S.A. Human IgG were from Cappel, West Chester, PA, U.S.A. Anti-human lipocortin 1 rabbit polyclonal antibody (no. 842) was kindly supplied by Dr J.L. Browning, Biogen, Cambridge, MA, U.S.A. This antibody recognized a human recombinant lipocortin 1 endowed with anti-inflammatory activity (Becherucci *et al.*, 1993).

Results U-937 cells incubated for 24 h with 10 nM PMA showed a dramatic increase in the membrane positivity ($59.1 \pm 14.6\%$ of positive cells) to CD14, a specific macrophage antigen, compared to control cells ($0.3 \pm 0.1\%$, $n = 6$, $P < 0.01$). This result demonstrates that 24 h treatment with 10 nM PMA induced immature monocytic U-937 cells to differentiate into macrophage-like cells confirming previous observations (Hoff *et al.*, 1992).

Table 1 shows the results of intracellular and cell membrane expression of lipocortin 1. U-937 cells incubated for 24 h with 10 nM PMA (i.e. differentiated cells) showed an approximately 30 fold increase of intracellular lipocortin 1 expression (60.3 ± 0.4 mean fluorescence) as compared with control (i.e. undifferentiated) cells (2.4 ± 0.1). On the other hand, the extent of lipocortin 1 expression on the membrane was very similar in both differentiated (36.4 ± 2.4) and undifferentiated cells (38.0 ± 3.1). Treatment with dexamethasone of differentiated cells caused a decrease of the intracellular expression of the protein (6.9 ± 0.1 at 2 h) and a

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Table 1 Intracellular and cell membrane expression of lipocortin 1 in U-937 cells

Dexamethasone incubation-times (h)	Intracellular		Cell membrane	
	Control cells	PMA-treated cells (mean fluorescence)	Control cells	PMA-treated cells
0	2.4 ± 0.1	60.3 ± 0.4**	38.0 ± 3.1	36.4 ± 2.4
1	2.3 ± 0.1	8.4 ± 0.2**	19.2 ± 2.0	45.8 ± 1.6**
2	5.8 ± 0.3	6.9 ± 0.1	53.9 ± 2.4	233.2 ± 12.4**
4	25.1 ± 0.5	25.6 ± 0.5	184.0 ± 4.2	290.6 ± 6.5**
8	5.0 ± 0.1	13.1 ± 0.1**	3.7 ± 0.1	2.6 ± 0.1
16	16.9 ± 0.4	21.8 ± 0.6*	41.6 ± 0.9	80.6 ± 1.3**
24	15.1 ± 0.3	10.3 ± 0.3*	47.6 ± 1.1	193.7 ± 8.2**

U-937 cells were incubated with or without 10 nM phorbol 12-myristate 13-acetate (PMA) for 24 h. Then 1 µM dexamethasone was added and incubation carried out for different times. At the end of incubations, intracellular and cell membrane expression of lipocortin 1 was determined by FACS as described in Methods. Data are means ± s.e.mean of the channel number fluorescence measured in the scans of 6 different cell preparations ($n = 6$). The mean non-specific fluorescence measured with the FITC-labelled anti-human Ig antibody (intracellular: 11.5 ± 0.8 , $n = 6$; cell membrane: 5.9 ± 0.2 , $n = 20$) has been subtracted from the table values. * $P < 0.005$, ** $P < 0.001$ vs corresponding control cell values (Student's two-tailed t test).

concurrent dramatic increase of membrane protein accumulation (233.2 ± 12.4 at 2 h) which peaked at 4 h (290.6 ± 6.5). At 8 h, membrane expression fell to negligible amounts and again increased at 16 and especially at 24 h (193.7 ± 8.2). In control cells the glucocorticoid also induced a biphasic membrane translocation of the protein which again peaked at 4 h (184.0 ± 4.2) and 24 h (47.6 ± 1.1). These values were, however, significantly lower than those of PMA-treated cells. In separate experiments after 24 h PMA incubation, cells were further incubated for increasing times (1–24 h) in absence of dexamethasone. In these cells both intracellular and cell membrane positivity of lipocortin 1 did not significantly change with time as compared with the values observed after 24 h PMA (not shown).

Discussion These results demonstrate that dexamethasone induces a biphasic translocation of lipocortin 1 from the intracellular compartment to the cell membrane of U-937 cells. The steroid effect is probably specific inasmuch as glucocorticoids do not interfere with the differentiation process induced by PMA in these cells (Hoff *et al.*, 1992). We have previously observed the release of lipocortin 1 from differentiated U-937 cells stimulated with dexamethasone (Solito *et al.*, 1991). Since the steroid-induced membrane accumulation is significantly higher in differentiated U-937 cells, the translocation of large amounts of lipocortin 1 to the membrane may precede the release of the protein in the extracellular milieu. Extracellular lipocortin 1 has anti-inflammatory properties (Becherucci *et al.*, 1993); it is then conceivable that the modulation of the translocation/

secretion of the protein by glucocorticoids represents part of their mechanisms of action.

The time-course of steroid-induced membrane accumulation of lipocortin 1 in differentiated U-937 cells shows that the expression peaks at 4 h, decreases to very low amounts at 8 h and increases again at 16 and 24 h. This pattern is somewhat specular to what occurs intracellularly where the initial high level of the protein decreases at 1–2 h and increases again at later times. The biphasic accumulation of lipocortin 1 on cell membrane induced by dexamethasone may appear surprising; however, a very similar time-course has been reported previously for the release of 'macroscortin' (an earlier name for lipocortin 1) induced by hydrocortisone in rat macrophages (Blackwell *et al.*, 1981; Carnuccio *et al.*, 1981). These authors have shown that macroscortin is stored preformed in macrophages and that hydrocortisone induces a biphasic release of the protein. On the basis of the previous and the present experimental evidence we propose that glucocorticoids initially stimulate the translocation to the membrane and release of the preformed lipocortin 1 with subsequent depletion of intracellular stores. This process is then followed by a new wave of synthesis which allows for new protein to be translocated and released. Further work is necessary to ascertain whether the resynthesis phase is a steroid-stimulated event or whether there is an internal feedback mechanism which initiates protein synthesis when intracellular concentrations fall below a certain level.

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