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Studies on the induction of lipocortin-1 by glucocorticoids

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Summary. - Part of the anti-inflammatory efficacy of glucocorticoids has been ascribed to the inhibition of eicosanoid formation which is brought about by lipocortins (LC, phospholipase A_2 inhibitory proteins) whose synthesis is induced by corticosteroids. We have investigated the effect of glucocorticoids on eicosanoid formation and lipocortin-1 induction in a human cell line *in vitro* and in patients with inflammatory lung disease *in vivo*. Human promyelocitic U-937 cell line was differentiated by 24 h incubation with phorbol myristate acetate (PMA, 6 ng/ml), then dexamethasone (1μM) was added for further 16 h. In these conditions the steroid treatment caused both the release of lipocortin-1 in the cellular supernatant and the inhibition of eicosanoid release. These results suggest that the responsiveness of these cells to steroids is dependent on the phase of cell activation-differentiation. The selective release of the lipocortin-1 may explain the inhibition of eicosanoid formation. Patients with inflammatory lung disease underwent glucocorticoid treatment at clinically effective doses. LC-1 expression was significantly stimulated in alveolar macrophages, but not in blood-derived lympho-monocytes. These results suggest that also *in vivo* cell responsiveness to glucocorticoids is acquired during cell differentiation from blood monocyte to tissue macrophage.

Key words: glucocorticoids, lipocortins, cell differentiation.

Riassunto *(Studi sulla induzione della lipocortina 1 da parte dei glucocorticoidi).* - I glucocorticoidi svolgono, in parte, la loro azione antiinfiammatoria bloccando il rilascio di eicosanoidi. Tale inibizione è mediata dalle lipocortine (LC, proteine inibenti l'attività fosfolipasica A_2), la cui sintesi viene indotta dai farmaci steroidei. Nel presente lavoro è stato studiato l'effetto dei glucocorticoidi sia sulla formazione di eicosanoidi che sull'induzione della lipocortina 1, in due differenti sistemi: a) *in vitro* in una linea cellulare umana; b) *in vivo* in pazienti affetti da malattie polmonari a patogenesi infiammatoria. La linea cellulare promielocita umana U-937 è stata differenziata dal trattamento per 24 h con forbolo miristato acetato (PMA 6 ng/ml), quindi sottoposta ad ulteriore trattamento con desametasone (1μM) per successive 16 h. In tali condizioni il trattamento steroideo ha indotto sia il rilascio nel sopranatante di coltura della lipocortina 1 sia l'inibizione del rilascio di eicosanoidi. Questi risultati suggeriscono che la capacità delle cellule in coltura di rispondere all'azione steroidea dipende dal grado di differenziamento. Il conseguente rilascio della lipocortina 1 può spiegare l'inibizione della formazione di eicosanoidi. Un gruppo di pazienti affetti da malattie polmonari a base infiammatoria è stato sottoposto a trattamento con dosi farmacologiche di metilprednisolone. Dopo il trattamento farmacologico è stato osservato che i macrofagi alveolari, a differenza delle cellule mononucleate del sangue, presentavano un aumento significativo della concentrazione di lipocortina 1 ed una inibizione del rilascio di eicosanoidi. Questi risultati suggeriscono che anche *in vivo* la capacità di rispondere agli steroidi è acquisita durante il processo da monocita ematico a macrofago tissutale.

Parole chiave: glucocorticoidi, lipocortine, differenziamento cellulare.

Introduction

The anti-inflammatory actions of the glucocorticoids have been attributed to the induction of a group of phospholipase A_2 (PLA_2) inhibitory-proteins, collectively called lipocortins (LC) [1-3]. LCs are thought to reduce the release by membrane phospholipids of free arachidonic acid by inhibiting PLA_2 activity, although other mechanisms of action have been proposed [4].

Inhibition of PLA_2 activity will ultimately result in a decreased formation of arachidonic acid derived mediators of inflammation such as prostaglandins and leukotrienes [5, 6]. Human lipocortin-1 has been the first of these molecules to be sequenced and cloned [3, 7], and the presence of a unique N-terminal sequence and 4 repetitive calcium and phospholipid binding regions have been revealed [8]. This type of molecular architecture is thought to be a common feature of other calcium and phospholipid proteins called annexins [9]. The repetitive binding sequence of LC-1 may be responsible for the properties of this molecule, although it has been reported that the presence of all 4 binding regions is not a requirement for the inhibition of PLA_2 activity *in vitro* [8]. LCs have been detected in a variety of cell types, including monocytes, neutrophils and macrophages [10-12].

Intracellularly, lipocortins are found not only in a soluble cytosolic portion but also associated with elements of the cytoskeleton which they bind to in a calcium-dependent manner (calpactin) [13]. Associated with lipids, especially negatively charged phospholipids of the plasma membrane, these proteins could function as a link between membrane and cytoskeleton and could play a role in cytodinamic processes. They are also capable of fusing vescicles either to each other or to membranes and are, therefore, presumed to be involved in exocytotic processes [14]. A link between the glucocorticoid-mediated anti-inflammatory effect and the lipocortins was recognized at the beginning of the 80's. Their effects correlate with both an increase in total mRNA levels and the *de novo* protein synthesis which is in agreement with the delayed onset of action of the steroids. It has, therefore, been assumed that this effect is mediated by newly synthesized proteins.

In this paper we have investigated the role of glucocorticoids on the induction of lipocortin-1 in: i) U-937 cells, a human histiocytic cell line; ii) in peripheral blood lympho-monocytes and alveolar macrophages obtained from patients with inflammatory lung disease, treated with clinically effective doses of glucocorticoids.

Effect of dexamethasone on lipocortin-1 expression in U-937 cells

U-937 cells were incubated with of without 6 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h, then 1 μM dexamethasone (DEX) was added and the incubation was carried out for 16 h. After the incubation the cells were lysed with a phosphate buffer solution containing 0.1% Nonidet P-40 plus 1 mM phenylmethylsulphonyl fluoride (PMSF), and the supernatants were precipitated. Both samples were separated in 10% polyacrylamide gels according to Laemmli [15] and electroblotted to nitrocellulose membranes. Immunodetection was performed using a specific anti-lipocortin-1 polyclonal antibody and goat anti-rabbit immunoglobulin conjugated to peroxidase with 4-cloro-1-napthol as substrate. The corresponding Western blot analysis is shown in Fig. 1.

The synthesis of the intracellular protein was stimulated by PMA. The intracellular lipocortin synthesis was not modified by DEX treatment in both undifferentiated and differentiated U-937 cells. On the other hand, the release in the cell supernatant of lipocortin-1 was observed only in differentiated U-937 cells further incubated with 1 μM DEX for 16 h. This release could not be due to cell death since cell viability assessed by trypan blue exclusion and lactic dehydrogenase release was always > 95%. Results in Table 1 show that differentiated U-937 cells released approx. 10-fold more TXB_2 than undifferentiated cells. Dexamethasone treatment inhibited TXB_2 release only in differentiated U-937 cells. Similar results have been obtained with PGE_2 (not shown). The observation of the release of lipocortin-1 from differentiated U-937 cells is intriguing since lipocortins are intracellular proteins lacking hydrophobic signal sequences. It is of interest that earlier investigations reporting the steroid-induced release [16, 17] of lipocortins have been recently confirmed in humans following *in vivo* administration of glucocorticoids [18, 19]. Moreover, the selective release of lipocortins 1 and 5, but not of lipocortin-4 in the human prostate fluid has been recently reported by Christmas *et al.* [20]. It has been recently proposed that lipocortins 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 pathway through the endoplasmic reticulum and Golgi apparatus [21, 22]. 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

Fig. 1. - Western blotting analysis of lipocortin-1. U-937 cells ($5 \cdot 10^5$ cells/ml) were incubated with or without PMA (6 ng/ml). After 24 h 1 μM dexamethasone (DEX) was added where indicated. The analysis is representative of 6 experiments with similar results.

Table 1. - Effect of dexamethasone on TXB_2 release from U-937 cells

Sample	TXB_2
Differentiated cells	100 ± 0.32
Differentiated cells + dexam.	48.3 ± 1.9(*)
Undifferentiated cells	9.8 ± 0.1
Undifferentiated cells + dexam.	10.6 ± 0.1

U-937 cells ($5 \cdot 10^5$/ml) were incubated with or without PMA (6 ng/ml). After 24 h 1 μM dexamethasone was added where indicated. After a further 16 h the concentration of TXB_2 in the supernatant was measured by radioimmunoassay. Results are expressed as % change of TXB_2 release from differentiated U-937 cells (114.8 ± 0.01, n = 3).
(*) $p < 0.001$.

Table 2. - Effect of 6-methyl-prednisolone treatment on lipocortin-1 concentration in blood lympho-monocytes and alveolar macrophages

	Lipocortin-1 (pg/10^6 cells)	
	Before treatment	After treatment
Lympho-monocytes	6.8 (4.9 - 12.5)	6.1 (4.2 - 9.1)
Alveolar macrophages	8.8 (5.8 - 7264.3)	1001.1(*) (7.5 - 40248.0)

Lipocortin 1 concentrations in the cell extracts were measured by ELISA. Results are expressed as median (range).
(*) $p < 0.05$ *vs* before treatment value.

lipocortins. Moreover, the selective release of lipocortin-1 by differentiated U-937 cells may explain the inhibition by dexamethasone of TXB_2 and PGE_2 release.

Effect of glucocorticoids on cells from patients with inflammatory lung disease

Peripheral blood lympho-monocytes and alveolar macrophages from broncho-alveolar lavage were collected from 8 patients with inflammatory lung disease before and after treatment with 6-methyl-prednisolone (6-MP, 40 mg/day for a month). Subsequently, the release of eicosanoids by these cells was assessed by radioimmunoassay, and lipocortin-1 concentrations were measured using a sandwich enzyme-linked immuno-assorbent assay (ELISA). A monoclonal antibody of defined specificity to lipocortin-1 [23] was attached to the solid phase and a rabbit polyclonal anti-lipocortin antibody used to detect bound lipocortin-1 from samples. The protein content of cell lysates was assayed against a standard curve of recombinant human lipocortin-1 [3]. The detection limit of the assay was 100 pg/ml. The lipocortin-1 associated with cells was expressed in terms of pg of protein per 10^6 cells. Table 2 shows the cumulated data of lipocortin-1 ELISA in lympho-monocytes and alveolar macrophages from patients treated with the glucocorticoid. Very low amounts of the protein were detected in mononuclear cells with no significant difference before and after treatment. On the other hand, the administration of 6-MP induced a significant increase of lipocortin-1 concentration in alveolar macrophages, but not in peripheral blood cells. Similarly, the release of eicosanoids was found to be significantly inhibited by 6-MP treatment in alveolar macrophages, but not in blood lympho-monocytes (not shown). This study shows that a clinically effective glucocorticoid treatment in patients with inflammatory lung disease was able to both inhibit eicosanoid formation and induce synthesis of lipocortin-1 in alveolar macrophages. Neither inhibition of eicosanoid production nor induction of lipocortin-1 was demostrable in blood mononuclear cells from these patients. The different effect of glucocorticoids on arachidonic acid (AA) metabolism in blood leukocytes and alveolar cells confirms recent data in healthy volunteers by Sebaldt *et al.* [24]. The different effect of glucocorticoids on eicosanoid formation and lipocortin-1 synthesis in blood mononuclear cells and alveolar macrophages suggests that steroid sensitivity is acquired during maturation of the blood monocyte into tissue macrophages. It is of interest that lipocortin induction by glucocorticoids has been shown only in differentiated cells and organs like guinea-pig lungs, rat peritoneal macrophages [16, 25], rabbit neutrophils [17], rat renomedullary cells [26], human skin fibroblasts [27], rat thymocytes [28], human lung cells [18, 19], human squamous carcinoma cells [29]. In conclusion the state of differentiation of the cells both *in vitro* and *in vivo* appears to be critical for the cell responsiveness to glucocorticoids in terms of both eicosanoid inhibition and lipocortin-1 induction.

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