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Glucocorticoid – and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes *in vivo*

[1]Susan H. Peers, [2]Frazer Smillie, Amber J. Elderfield & [1,3]Roderick J. Flower

Pharmacology Group, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, Avon BA2 7AY

1 We have studied the occurrence, distribution and disposition of lipocortins (annexins) 1, 2 and 5 in mixed peritoneal leucocytes obtained from rats in which glucocorticoid levels were altered by adrenalectomy, administration of the glucocorticoid antagonist, RU486, or by injection of dexamethasone or hydrocortisone, as well as from rats in which the peritoneal cells were elicited by inflammatory stimuli.

2 In cells obtained from untreated rats with an intact adrenal cortex, lipocortins 1, 2 and 5 were readily detectable: the majority of each of the proteins was apparently located intracellularly with much smaller amounts in the membrane. Lipocortin 1 and to a lesser extent lipocortin 5 were also seen in a Ca^{2+}-dependent association with the external plasma membrane. Following administration of RU486 ($2 \times 20\ mg\ kg^{-1}$) the amounts of lipocortin 1 and 2 in cells were greatly reduced. Conversely, injection of hydrocortisone ($1\ mg\ kg^{-1}$) or dexamethasone ($0.08\ mg\ kg^{-1}$) caused an increase in the amount of lipocortin 1 and 2 in peritoneal cells within 30 min. Lipocortin 5 was unchanged by any manipulation of glucocorticoid levels.

3 Lipocortins 1 and 2 were elevated in both intracellular and membrane-associated fractions of macrophages elicited by intraperitoneal injection in inflammogens. This phenomenon also occurred in adrenalectomized animals.

4 Our data indicate that glucocorticoids control the synthesis of some members of the lipocortin family in rat mixed peritoneal cells but also suggest the existence of a separate system for controlling the generation of this protein. The significance of these observations is considered in relation to the mechanism of glucocorticoid hormone action on eicosanoid production.

Keywords: Lipocortin 1; annexins; glucocorticoids; peritoneal leucocytes; RU486

Introduction

The glucocorticoids can exert a rapid and powerful suppression of eicosanoids and platelet activating factor (PAF) synthesis by cells and in many systems a considerable body of evidence exists to suggest that this inhibitory effect is caused by the glucocorticoid-stimulated synthesis or release of a second messenger protein originally termed 'macrocortin', 'lipomodulin' or 'renocortin' (see Flower, 1988). It was surmised that these proteins were either identical or at least very similar and the name 'lipocortin' was coined to replace the earlier terminology (Di Rosa *et al.*, 1984). Biological evidence supported the concept that these proteins, or highly purified preparations containing them, duplicated certain effects of the glucocorticoids, in particular inhibition of eicosanoid synthesis and (some) anti-inflammatory effects (Blackwell *et al.*, 1982). It was also observed, again by use of biological and enzyme-assay techniques, that the glucocorticoids caused the 'induction' by cells of lipocortin and that this effect could be blocked by inhibitors of protein or RNA synthesis (Blackwell *et al.*, 1982). In rat peritoneal macrophages, however, the situation was complex, with glucocorticoids apparently influencing the 'release' as well as the synthesis of the protein (Carnuccio *et al.*, 1981).

Ion-exchange chromatography revealed that 'lipocortin' obtained from rat peritoneal lavage fluid was actually a mixture of at least four proteins with apparently similar molecular weights and biological activity (Blackwell *et al.*, 1982). There were also reports of larger proteins having similar properties (Coote *et al.*, 1983). Recently several of these have been purified, sequenced and cloned revealing a family of 6 proteins with substantial homologies (Wallner *et al.*, 1986; Pepinsky *et al.*, 1988). A surprising finding was that the calpactins, a family of proteins thought to be involved with the maintenance of the cytoskeleton or with exocytosis, were in some cases identical to the lipocortins, as were members of the 'chromobindin' family. Recognition of this fact has opened many new avenues of thought concerning the role of these proteins in cellular physiology. It has been suggested recently that this family should be renamed 'annexins' (Crumpton & Dedman, 1990).

Lipocortin 1 (annexin 1 or calpactin II) is a 37 kDa member of this family and when prepared in its correctly folded form the recombinant human protein has eicosanoid-inhibitory and anti-inflammatory properties strongly reminiscent of the original biological extracts (Cirino & Flower, 1987a; Cirino *et al.*, 1987; 1989). Although biological evidence suggested that (at least) some members of the lipocortin family were 'induced' or 'released' by glucocorticoids, the question of whether lipocortin 1 is regulated in this way has proved to be more complex than expected. Some workers have observed increases in lipocortin 1 or its mRNA following exposure of cultured cells to steroids (Wallner *et al.*, 1986; Fuller & Verity, 1989; Piltch *et al.*, 1989) whereas other groups have not (Hullin *et al.*, 1989; Bienkowski *et al.*, 1989). It has been suggested that the concurrent presence of growth factors is required for steroid induction (Phillips *et al.*, 1989) or that cells must be in a differentiated state before lipocortin 1 induction can occur (Solito *et al.*, 1991).

[1] Present address: Department Biochemical Pharmacology, William Harvey Research Institute, St Bartholomew's Hospital Medical College, Charterhouse Square, London, EC1M 6BQ. [2]Department of Biochemistry, School of Biological Sciences, University of Bath. [3]Author for correspondence.

The situation *in vivo* or in cells *ex vivo* also seems complex: Northup *et al.*, (1988) were unable to detect an increase in lipocortin 1 protein in mouse macrophages following glucocorticoid treatment but Mitchell *et al.* (1988) detected an increase in lipocortin 1 in human amnion cells following dexamethasone treatment. In man, Goulding *et al.* (1990) reported an increase in monocyte lipocortin 1 following intravenous injection of hydrocortisone and Browning *et al.* (1990) described *de novo* synthesis of lipocortin 1 in primary cultures of peripheral monocytes maintained in the presence of glucocorticoids. Glucocorticoids also increase the lipocortin concentration of bronchoalveolar lavage fluid in man (Ambrose & Hunninghake, 1990). Recently (Vishwanath *et al.*, 1992) adrenalectomy has been shown to reduce the levels of lipocortin 1 mRNA and protein in several tissues in the rat.

Using Western blotting techniques we have now investigated the effects of glucocorticoids upon lipocortin 1 synthesis in rat peritoneal leucocytes, one of the systems in which lipocortin 'induction' was first described. Some of these results have appeared in abstract form (Smillie *et al.*, 1989).

Methods

General note on cell collection

Care was taken in all these experiments to minimize artefacts attributable to the fluctuating endogenous steroid background of the rats. All cell collections were made at the same time of day (early p.m., to avoid the corticosterone surge) from rats which were acclimatized and accustomed to the regular animal house routine for at least 2 weeks. Positive action was taken to minimize disturbances to the colony which might arise while removing rats from stock cages.

Collection of cells

Glucocorticoids or saline were administered subcutaneously in a volume of 0.1 ml; saline or paraffin oil (1 ml) was injected intraperitoneally. RU486, prepared as an aqueous suspension, was administered by mouth (1 ml).

Following appropriate treatment, rats were killed by exposure to CO_2 gas and 15–20 ml of either ice-cold Krebs solution or saline (as indicated) containing heparin (5 u ml^{-1}) was injected intraperitoneally and the cavity and its contents lightly massaged. The cell-rich fluid ($10-15 \times 10^6$cells/rat; >90% macrophages) was removed and immediately placed on ice. The pooled cell number was assessed with a haemocytometer and the numbers adjusted to 50×10^6 cells in each separate sample before collection of the cells by centrifugation (1000 *g*, 15 min, 4°C). The cell-free supernatant was removed (and in some cases retained, see below) and lipocortins were then extracted as described below. If not extracted immediately the pellet was snap-frozen by immersion of the tube in dry ice and stored at −20°C.

Extraction of lipocortin

Two points of importance were established in preliminary experiments. Firstly, lipocortins tend to precipitate out of Ca^{2+}-containing solutions particularly when these are stored for any length of time. To guard against such losses of the protein, EDTA was routinely incorporated with all extraction buffers. Secondly, in the case of lipocortins 1 and 5, a proportion of the total cellular protein can, under some conditions, become externalised onto the external surface of the cell. This can be removed following suspension of the cells in a Ca^{2+}-free solution and centrifugation.

Our initial investigation revealed three distinct 'pools' of lipocortins.

(a) *Cell surface extracellular lipocortins ('E')* which were recovered by washing cells in saline and transferring the cell-free fluid after centrifugation directly into tubes containing a molar excess of EDTA. These samples were vortex-mixed, snap-frozen and used in the analytical procedures without further manipulation.

(b) *Intracellular lipocortins ('P')*. Cell pellets were resuspended in ice-cold Tris buffer pH 7.4 (50 mM) containing 10 mM EDTA. The cells were lysed by freeze-thawing, the samples were centrifuged (2 min, 13,000 r.p.m.) and the supernatant decanted and retained for analytical work.

(c) *Membrane-bound lipocortins ('M')*. The broken cell debris from the previous step was re-extracted with ice cold detergent-containing buffers (Tris pH 7.4 (50 mM), EDTA (10 mM), 2% NP40). The pellets were resuspended and allowed to stand for 30–60 min at 4°C centrifugation for 5 min at 13,000 r.p.m. The supernatant was retained.

An aim of this study was to compare the amounts of lipocortins in different compartments of the cell and to detect any changes in location that might occur following steroid treatment. For this reason the three different 'fractions' obtained as described above were always diluted to the same final volume (200 µl) so that a direct comparison of relative abundances could be made on Western blots.

Gel electrophoresis and immunoblotting of lipocortin

Samples (representing the extracts of 0.5×10^6 cells) were boiled for 5 min in buffer containing 2% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol and then applied to a 1.5 mm 10% polyacrylamide minigel. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by conventional methods (Laemmli, 1970), the samples were transferred electrophoretically onto nitrocellulose and lipocortins detected by use of antibodies raised in rabbits against human recombinant lipocortin 1 (Ab 842), human native lipocortin 2 (Ab 774) and rat lipocortin 5 (Ab 890) respectively. No cross reactivity between these three species was observed with the particular batches of antisera used in these experiments (personal communication from Dr J. Browning, Biogen Research Inc). Bands were visualized by the deposition of peroxide-oxidized diaminobenzidine following incubation with peroxidase-linked anti-rabbit antibody. Data are shown as Western blots from representative experiments ($n > 3$).

Materials

Dexamethasone sodium phosphate (Oradexon-Organon) was obtained from Organon (Cambridge, UK) and heparin (25,000 u ml^{-1}) from Evans (Greenford, UK). Bovine serum albumin (fatty acid-free), hydrocortisone sodium phosphate, buffer salts and 3, 3 diaminobenzidine tetrahydrochloride dihydrate (DAB) were supplied by Sigma (Poole, UK). Sodium dodecyl sulphate, acrylamide and bis-acrylamide were purchased from BDH (Dagenham, UK) and 2-mercaptoethanol from Fisons (Loughborough, UK).

Surgically adrenalectomized Wistar rats were obtained from Interfauna UK Ltd or Olac and maintained in the animal house for 7–14 days with saline drinking water prior to use. Sham-operated control animals were purchased with each batch for purposes of comparison.

The glucocorticoid antagonist RU486 (mifepristone) was a generous gift of Dr R. Deraedt of Roussel-Uclaf, Romainville, France. Authentic human recombinant lipocortin 1 and all the antisera used here (all of which were highly specific rabbit polyclonal preparations) were the generous gift of Dr J. Browning of Biogen Research Inc (Cambridge MA, U.S.A.).

Results

Recovery of lipocortins from cells

Our first observation demonstrated that the mass of lipocortin 1 recovered from the cells of control rats (i.e. rats receiving no exogenous steroid) was extremely variable between batches of rats and that different cell handling techniques produced different profiles of lipocortin and its major metabolites. The variations observed between batches of rats could, at least in part, be attributed to different hormonal backgrounds, although it is clear that there are other factors which are also important.

Figure 1 shows immuno blots of the EDTA-extractable (see below) lipocortin 1 obtained from an identical number of cells from different batches of rats picked to illustrate this point. When extracted and processed under identical conditions the amount of lipocortin 1 and the range of species present in the samples ranges from 'undetectable' (lane A, B) to 'easily detectable' (lanes, C,D,E,). Lanes F and G are from cells taken from a matched 'sham-operated' group (lane F) and an adrenalectomized (lane G) group. It is clear that endogenous corticosteroids profoundly alter the amount found in resting cells in the peritoneal cavity. However the effect of adrenalectomy observed so clearly in Figure 1 was by no means an invariable finding and we encountered instances where the amount present in cells from adrenalectomized rats was high, strongly suggesting that in addition to the steroid background other humoral factors such as acute phase proteins may also be important (see below).

The molecular weight of authentic recombinant human lipocortin 1 by SDS-PAGE is 37–38 kDa, and rat protein has approximately the same mass (Pepinsky *et al*., 1986). The polyclonal antiserum used here detected intact (37 kDa) lipocortin 1 as well as metabolites of calculated molecular weights of 33.3 and 25 kDa. A high mol. wt. metabolite sometimes seen (e.g. in Figure 3) is probably the 45 kDa asymmetrically-clipped homodimer or adduct referred to by Pepinsky *et al*. (1989). A lipocortin 2 metabolite was also routinely detected. The reasons for variations in the amount of recovered intact lipocortins and metabolites are not clear. Collection of cells or extraction of lipocortin in buffers containing soybean trypsin inhibitor ($1\ mg\ ml^{-1}$), trasylol ($100\ u\ ml^{-1}$) or phenylmethylsulphonyl fluoride (PMSF) (200 mM) did not affect the distribution of intact protein and metabolites (data not shown). Other high molecular weight immunoreative species were frequently observed with all of our antisera.

Figure 1 Variable content of lipocortin 1 in rat 'resting' mixed peritoneal leucocytes. The immunoblot shows the range (and molecular weight species) of lipocortin 1 recovered by EDTA extraction in 0.5×10^6 lysed cells from several control (lanes A–E), sham-operated (F) and adrenalectomized (G) rats. Lane H is authentic recombinant human lipocortin.

Location of cellular lipocortins

Table 1 summarises the overall disposition of lipocortins in the mixed peritoneal cells as observed during these experiments. Lipocortin 1 is readily detectable in the intracellular lysate of cells from steroid-treated rats and in some samples from control rats also and may be detected extracellularly attached to the membrane when peritoneal lavage is performed in Ca^{2+}-containing medium. However under routine conditions little was found in the membrane (Figure 2). However, if this latter pool is concentrated 4–5 fold small amounts of the protein may be detected. Lipocortin 2 is less abundant intracellularly, could not be detected extracellularly and was not easily detectable in the membrane under normal conditions. Lipocortin 5 is present predominantly in the cell lysate, but could also be detected in smaller amounts in the cell surface fractions.

Effect of glucocorticoids in vivo

Injection of dexamethasone ($0.08\ mg\ kg^{-1}$) causes a time-dependent increase in the intracellular content of the lipocortins 1 and 2 (Figure 3) in peritoneal leucocytes. By microdensitometry, the intensities of the lipocortin 1 bands (relative to the control) at 15, 30, 60 and 120 min following dexamethasone injection were 0.48, 3.69, 4.16 and 6.52 respectively. The corresponding figures for the lipocortin 2

Table 1 The disposition of lipocortins 1, 2, and 5 (L1, L2 and L5) in rat mixed peritoneal leucocytes following saline or dexamethasone treatment

	Relative abundances of lipocortins in cells from					
	Saline-treated rats			*Dexamethasone treated rats*		
Fraction	L1	L2	L5	L1	L2	L5
Cell surface (E)	(+)	UD	(+)	+	UD	+
Intracellular (I)	+/++	+	++	+++/++++	+++	++
Membrane bound (M)	(+)	(+)	UD	(+)	(+)	UD

The scores were assessed by visual examination of the Western blots generated during these experiments (data collected from >15 individual experiments). Rats received either saline vehicle or dexamethasone $0.08\ mg\ kg^{-1}$ and cells were collected, processed and extracted as described in the text.
UD: Protein undetectable area following × 4 concentration.
(+): Protein undetectable in the normal dilution range but just detectable following × 4 concentration.
\+ Protein present in small amounts but visually detectable.
++ Protein present in moderate amounts and easily detectable.
+++ Protein present in large (or very large ++++) amounts and strongly visible.

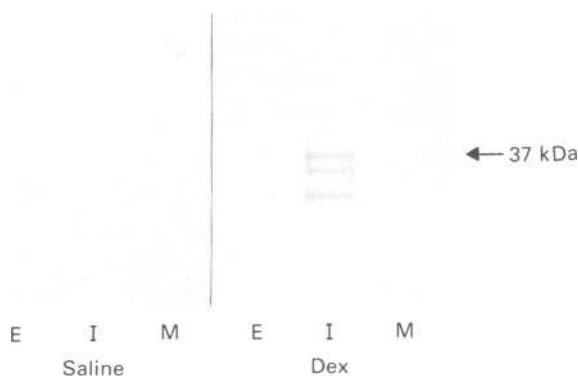

Figure 2 Disposition of lipocortin 1 in rat leucocytes. Recovery of lipocortin 1 from the cell surface (external-'E'), the intracellular (internal-'I') or cell membrane ('M') fractions of rat mixed peritoneal leucocytes obtained in a matched experiment from rats treated with saline or dexamethasone (Dex) for 1 h. Each lane represents the extract obtained from 0.5×10^6 cells.

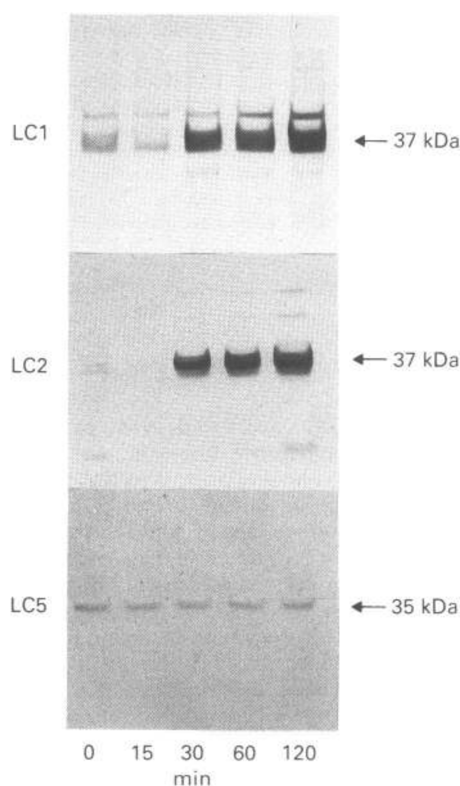

Figure 3 Time course of glucocorticoid induction of lipocortins 1 and 2. Lipocortins 1, 2 and 5 in EDTA extracts of 0.5×10^6 peritoneal mixed cells obtained from adrenalectomized rats receiving dexamethasone (0.8 mg kg^{-1}, s.c.) at different times following steroid treatment. Western blots were prepared in triplicate and probed with antisera to lipocortin 1, 2 and 5. Lipocortin 1 appears as a doublet: the lower band represents the 37 kDa species but the upper band may be an asymmetrically clipped adduct such as that described by Pepinsky *et al.* (1989).

bands were 1.3, 28.9, 39.5 and 43.2. The amount of immunoreactive lipocortin 5 was unchanged at any time.

Early experiments indicated that lipocortin 1 was attached to the exterior of peritoneal cells by a Ca^{2+}-dependent mechanism (Cirino & Flower, 1987b), and indeed lipocortin-like biological activity was discovered in the peritoneal lavage fluid of steroid-treated rats (Blackwell *et al.*, 1982). Figure 4 shows that immunoreactive lipocortin 1 is present in increased amounts in both peritoneal cells (37 kDa band 3.3 relative to control) and in concentrated ($\times$ 10) Ca^{2+}-free lavage fluid (37 kDa band 5.8 relative to control) following dexamethasone injection.

The effect of glucocorticoid receptor antagonists

Pretreatment of rats with the steroid receptor antagonist, RU486, not only prevents dexamethasone-induced increases in peritoneal leucocyte intracellular lipocortins 1 and 2, but also reduces the amount of these proteins in cells from rats which had not received exogenous steroid (Figure 5). Microdensitometry measurements of the bands gave the following relative intensities: lipocortin 1–control (1.0), RU486 (0), dexamethasone (1.67) and hydrocortisone (2.32):lipocortin 2–control (1.0), RU486 (0), dexamethasone (1.98) and hydrocortisone (2.74). Lipocortin 5 was unaffected by RU486. These findings suggest that endogenous steroids are important in regulating the cellular content of lipocortins 1 and 2, as suggested by previous findings of reduced lipocortin activity from adrenalectomized rats (Blackwell *et al.*, 1982). To achieve maximum effect, rats were pretreated with RU486 at 18 h and 2 h before cell harvesting; shorter pretreatment reduced, but did not completely eliminate the effects of exogenous steroids (data not shown).

Hydrocortisone also induces lipocortin 1 and 2 in peritoneal cells, but a higher dose (1 mg kg^{-1}; 1 h pretreatment) was required, consistent with its lower anti-inflammatory potency compared to dexamethasone (Figure 5).

Steroid-independent regulation of rat peritoneal cell lipocortins

Our experiments indicated variability in the basal amounts of lipocortins in cells from control animals despite our attempts to minimize stress to the colony (see Figure 1). In addition, although cells from adrenalectomized rats generally contained less lipocortin 1, some batches of animals acquired very high amounts following surgery, and clearly this could not be caused by endogenous steroids. These observations suggested an additional non-glucocorticoid control mechanism for regulation of lipocortin synthesis.

In a previous paper, Wallner *et al.* (1986) noted that 'elicited' macrophages contained large amounts of lipocortin 1 compared to 'resident' peritoneal cells, suggesting that cell

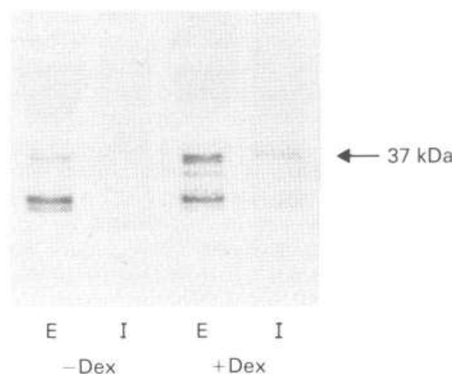

Figure 4 Dexamethasone-induced increase in lipocortin 1 in peritoneal lavage fluid. Mixed peritoneal cells were collected by saline lavage (20 ml) from rats receiving vehicle (– Dex) or dexamethasone 0.8 mg kg^{-1} s.c. 1 h beforehand (+ Dex). The cells were extracted by EDTA-lysis and the saline lavage fluid was concentrated $\times$ 10 prior to Western blotting. 'E' = external (lavage) lipocortin 1; 'I' = internal lipocortin 1. The samples represent the extracts from 0.5×10^6 cells or 1% of the total lavage fluid.

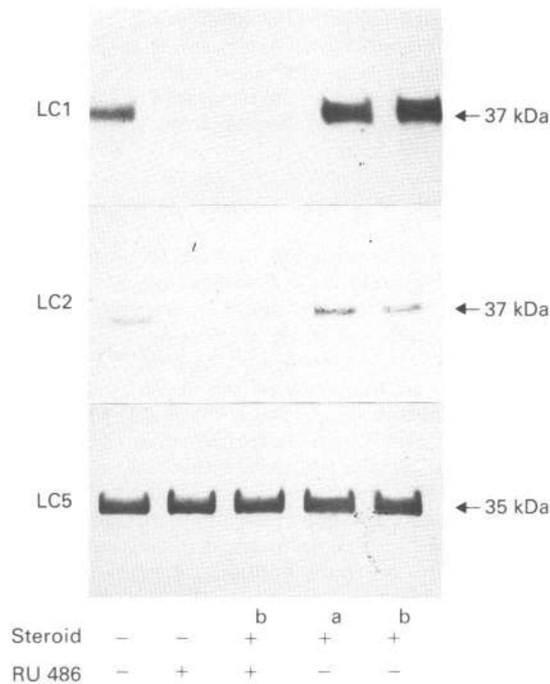

Figure 5 The effect of pretreatment with the glucocorticoid antagonist, RU486, on the content of lipocortins 1, 2 and 5 in rat mixed peritoneal cells. EDTA extracts of cells (0.5×10^6) were obtained from rats receiving saline, RU486 ($20\ mg\ kg^{-1} \times 2$) or steroid (a = hydrocortisone, $1\ mg\ kg^{-1}$; b = dexamethasone, $0.08\ mg\ kg^{-1}$ 1 h before collection) either alone or in combination. Western blots were prepared in triplicate and probed with antisera to lipocortins 1, 2 and 5.

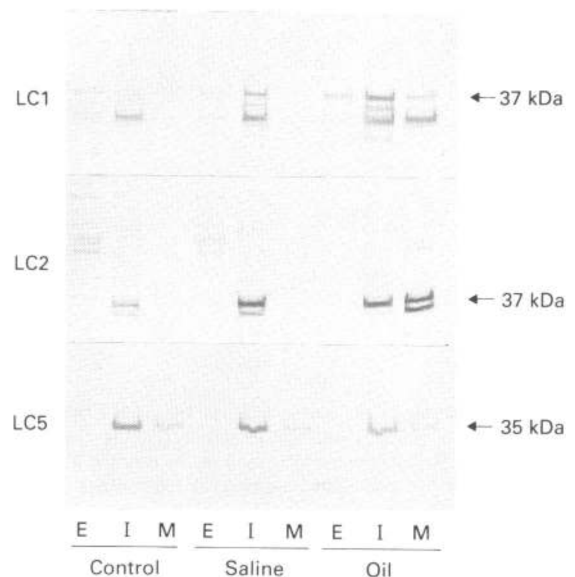

Figure 6 The effects of inflammogens upon lipocortin 1, 2 and 5 in rat leucocytes. The immunoblot shows increased synthesis and differential distribution of lipocortins 1, 2 and 5 caused by the i.p. injection into adrenalectomized rats of 1 ml saline or 1 ml paraffin oil. Control adrenalectomized rats were untreated. Cells were collected from rats 24 h after injection by peritoneal lavage in Ca^{2+}-containing fluid and counted. Equivalent numbers of cells (0.5×10^6) from each group of rats were used to prepare the 'extracellular' (E), 'intracellular' (I) and 'membrane soluble' (M) fractions. Western blots were prepared in triplicate and probed with antisera to lipocortins 1, 2 and 5.

'activation' might increase lipocortin 1 levels in some way; alternatively a second signal resulting from the inflammatory reaction might induce lipocortin synthesis. Intraperitoneal injection of inflammogens, such as zymosan or paraffin oil, results in a sterile inflammatory response featuring infiltration initially of neutrophils and subsequently of monocytic cells. Twenty-four hours following the injection, the elicited cells are predominantly monocyte/macrophages (>90%) although these cells differ phenotypically from resting, resident macrophages.

In cells elicited by injection of 1 ml paraffin oil, or to a lesser extent 1 ml sterile saline, lipocortin 1 and 2 levels are greatly increased compared to resident cells. The distribution of lipocortins was different, with lipocortin 1 being readily, and lipocortin 2 faintly, detectable in the membrane pool from these elicited cells. Lipocortin 5 levels and distribution were unaltered. These changes could not be due to an increase in endogenous corticosteroids following injection of inflammogen, since essentially similar observations were made in adrenalectomised rats (Figure 6). A similar 'induction' of lipocortins was seen in cells elicited by i.p. injection of zymosan (10 mg) or glycogen (10 mg, data not shown) indicating that this was not a feature only of oil-elicited cells, or an artefact of extraction following phagocytosis of oil droplets.

Discussion

The data presented in this paper clearly demonstrate that glucocorticoids can promote an increase in the mass of immunoreactive lipocortins 1 and 2 in extracts from the mixed peritoneal leucocytes of rats. This effect is rapid, is not unique to dexamethasone and is dependent upon glucocorticoid receptor occupation and therefore presumably upon gene modulation.

The doses of the glucocorticoids used in this study are moderate anti-inflammatory doses in the rat and the experiments in which the glucocorticoid antagonist, RU486, was used imply that lipocortin 1 and 2 are sensitive to changes in endogenous levels of glucocorticoids. The fact that cells from adrenalectomized animals generally contained less lipocortin 1 than cells from control animals would also tend to confirm this. No change in the mass of immunoreactive lipocortin 5 was seen with changing endocrine status of the animal or glucocorticoid treatment implying that unlike the closely related species, lipocortins 1 and 2, the lipocortin 5 gene is not glucocorticoid-sensitive.

We have not measured the amount of cellular mRNA for these lipocortins so we cannot pinpoint precisely the mechanism of 'induction': the most obvious possibility is direct enhancement of gene transcription but there could be other explanations. For example, glucocorticoids could promote the translation of a pre-existing pool of lipocortin mRNA or could stimulate movement of the protein from one protein compartment of the cell into the EDTA-extractable phase, perhaps by causing changes in phosphorylation state of the protein, although the reverse redistribution has been proposed (Sheets *et al.*, 1987). We could not detect a substantial pool of any lipocortin elsewhere in the cell using conventional extraction techniques, implying that the most likely explanation for the increase is *de novo* synthesis following gene activation or translation of a pre-existing pool of mRNA. The results obtained here support the biological and enzyme assay data obtained in earlier studies (Blackwell *et al.*, 1982) and are in line with a recent publication demonstrating a reduction in lipocortin mRNA in the rat following adrenalectomy (Vishwanath *et al.*, 1992).

Kovacic *et al.* (1991) have cloned and sequenced the rat

lipocortin 1 gene and compared it with the human gene. A consensus nucleotide sequence for glucocorticoid receptor binding is located in both human and rat genes in intron 1. This finding provides a notional explanation at least for the effect of glucocorticoids on the expression of this protein. Furthermore, the human gene contains a motif characteristic of response elements regulated by substances such as interferons, mitogenic agents and serum factors. Once again, this could explain the ability of cells to respond to substances other than glucocorticoid hormones. A similar observation was made by Browning and his colleagues (1990) who pointed out that the human lipocortin 1 gene also contains an AP1 site consistent with the inducibility of the gene in some cells by phorbol esters (cf. Beinkowski *et al.*, 1989). The lipocortin 1 promoter region also revealed a homology with regulatory elements in the haptoglobin promoter region (Oliviero & Cortese, 1989), a gene which is induced by a combination of IL-6 and steroids, as well as another resembling an acute phase recognition element (APRE-2β), which might prove to be important in gene regulation during chronic inflammatory episodes. Although the synthesis of many acute phase proteins are driven by cytokines and steroids in combination it is also possible to achieve an acute phase response in the absence of glucocorticoids: this could be triggered by surgery (such as adrenalectomy or sham adrenalectomy) or by intercurrent infections.

We will not know precisely what influence other than glucocorticoids are definitely associated with an induction of lipocortin 1 until the molecular biology of the promoter site is more carefully worked out. From the data presented in this paper however it is clear that there is a prominent non-glucocorticoid driven pathway for lipocortin 1 induction in the rate peritoneal macrophage.

Of interest is the discovery that lipocortin 1 is externalized by cells. In solutions containing physiological calcium concentrations this material seems predominantly associated with the cell surface by a Ca^{2+}-dependent mechanism and can be easily detached in low Ca^{2+} solutions. This phenomenon almost certainly explains the observation of Blackwell *et al.* (1982) that the 'polypeptide I' component of 'macrocortin' in crude peritoneal lavage fluid (now known to be lipocortin 1) was elevated by pretreatment of rats by steroids. In these experiments, the cells from treated rats were maintained for a short while in a Ca^{2+}-free environment thus promoting 'release' of lipocortin into the medium. Lipocortin 1 does not possess a signal sequence and the method by which it is externalized is not clear although this phenomenon has been observed by others (Christmas *et al.*, 1991) and lipocortin 5, which also lacks a formal signal sequence, has also been located pericellularly by other groups (Pfaffle *et al.*, 1988). This pool of lipocortin is significant as Carnuccio *et al.* (1981) and Cirino & Flower (1987b) reported that it is the external membrane-associated lipocortin 1 that produces its biological effects. Furthermore, the models in which lipocortin 1 has been shown to have biological activity are those in which the protein has been applied externally (Cirino & Flower, 1987a; Cirino *et al.*, 1987; 1989; Davidson *et al.*, 1991).

The presence of increased intracellular and membrane lipocortins in activated/elicited cells, even from adrenalectomized animals, may explain some of the variability in lipocortin levels seen in control animals, and almost certainly explains its presence in cells from some batches of adrenalectomized and sham-operated animals. A sub-clinical infection following the operation, or even the surgical trauma itself, could lead to cell infiltration, and hence to high background levels in resident cells.

'Membrane-bound' lipocortins have been noted in human placental membranes (Sheets *et al.*, 1987) and in porcine heart (Pula *et al.*, 1990) and A431 cells (Ando *et al.*, 1991). In the first two examples, lipocortin 1, and 5 and 6 respectively could be extracted by non-ionic detergents but not by EGTA, and hence resemble the membrane-bound pools observed in elicited cells. In the case of placental lipocortin 1, this pool may be modified by phosphorylation. The situation in A431 cells appears to be different, in that here, lipocortin 1 forms a covalent polymer following the action of tissue transglutaminase and cannot be extracted from membranes by Triton X-100, although plasmin treatment is effective. The identity of the cellular membrane containing the 'membrane bound' detergent-extractable lipocortin 1 has not been confirmed, although in placenta, the plasma membrane seems the most likely candidate. It may be that the 'membrane-bound' pool in the elicited cells represents 'nascent' lipocortin 1.

Although many workers have used cell lines to investigate lipocortin 1 synthesis we have always worked with primary cells because we believe these to be more relevant to the study of inflammation *in vivo* and indeed, the situation in such cells seems different from many cultured cell lines. The experiments reported in this study indicate that the lipocortin system is very sensitive to glucocorticoid control and responds very quickly to changes in steroid levels. Elsewhere we have reported an elevation of lipocortin 1 in peripheral blood monocytes taken from human volunteers receiving intravenous hydrocortisone (Goulding *et al.*, 1990). In addition, Browning *et al.* (1990) have reported that human peripheral monocytes cultured in [^{35}S]-methionine labelled media synthesized labelled lipocortin 1 in response to dexamethasone. It seems that primary cells respond well to glucocorticoids both *in vivo* and *in vitro* but that this capacity may be lost or in some way impaired following long term culture. The increase in lipocortin 1 and 2 associated with elicited cells implies glucocorticoid-independent mechanism for induction associated with activation or transformation.

Lipocortin 1 has anti-inflammatory properites (Cirino *et al.*, 1989; Errasfa & Russo-Marie, 1989) and the observation that its synthesis is regulated by endogenous and exogenous corticosteroids is therefore of great significance. Since lipocortin 2 is also regulated by steroids we propose that this molecule should also be considered as a potential mediator of steroid hormone action.

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