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A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids *in vivo* in mice

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1 The injection of a suspension of a polyacrylamide gel (bio gel) into the dorsal subcutaneous area of mice induced an inflammatory reaction and the migration of neutrophils towards the inflamed site.

2 The intravenous administration of anti-inflammatory drugs (dexamethasone, indomethacin and lysine-acetylsalicylate) to polyacrylamide gel-treated mice inhibited the accumulation of neutrophils in the inflamed site.

3 A similar administration of a 36 K mouse lipocortin, induced a strong dose-dependent inhibition of neutrophil accumulation in the inflamed site.

4 Dexamethasone and lipocortin inhibited the production of eicosanoids, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) in the inflamed site of polyacrylamide gel-treated mice.

5 Lipocortin impaired both phospholipase A_2 (PLA_2) activity and chemotaxis of isolated inflammatory neutrophils.

6 The present studies show an *in vivo* anti-inflammatory effect of lipocortin similar to that of glucocorticosteroids. In agreement with recent data on the extracellular effects of various lipocortins, these results might implicate lipocortin(s) in the anti-inflammatory effects of glucocorticosteroids.

Introduction

Glucocorticosteroids are widely used as potent anti-inflammatory drugs (Lewis & Piper, 1981; Townley & Suliaman, 1987). The mechanism of action of glucocorticosteroids as anti-inflammatory agents has received a tentative explanation over the last ten years. These steroids induce the synthesis of phospholipase A_2 (PLA_2) inhibitory proteins now referred to as lipocortins (Hirata *et al.*, 1980; Blackwell *et al.*, 1980; Cloix *et al.*, 1983; Errasfa *et al.*, 1985). Lipocortins have been shown to inhibit soluble phospholipase A_2 *in vitro* and eicosanoid synthesis in whole cells. These effects could be responsible for part of the anti-inflammatory effects of glucocorticosteroids. To support this hypothesis, it has been shown that the anti-inflammatory effects of glucocorticosteroids involve gene expression and protein synthesis (Tsurufuji *et al.*, 1979) and are correlated with inhibition of eicosanoid production (Parente *et al.*, 1984). Recently, the full cDNA sequence of lipocortin I was obtained (Wallner *et al.*, 1986). It showed that this protein belonged to a family of at least six homologous intracellular proteins (Pepinsky *et al.*, 1988) and had no leader peptide to permit its secretion. Although lipocortins are intracellular proteins, extracellular lipocortin(s) have also been found in the supernatant of glucocorticosteroid-treated cells (Hirata *et al.*, 1980; Blackwell *et al.*, 1980; Cloix *et al.*, 1983; Errasfa *et al.*, 1985), suggesting that these proteins may be secreted and may interact with other cells. Indeed, the recombinant lipocortin I was shown to inhibit, both thromboxane A_2 release from guinea-pig activated lungs (Cirino *et al.*, 1987) and prostacyclin production in human endothelial cells (Cirino &

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Flower, 1987). Moreover, we could demonstrate that lipocortins were able to inhibit cellular phospholipase A_2 from mouse thymocytes (Rothhut *et al.*, 1987; Errasfa *et al.*, 1989) and guinea-pig alveolar macrophages (Errasfa *et al.*, 1988). In parallel, we have shown that a 36 K mouse lipocortin was able to impair the production of leukotriene B_4 (LTB_4) and Paf (platelet-activating factor) in rat inflammatory neutrophils (Fradin *et al.*, 1988). These cellular effects of lipocortins have not been explained yet in terms of their molecular mechanism, although their inhibitory effect on PLA_2 *in vitro* has been attributed to their ability to bind to acidic phospholipids (Davidson *et al.*, 1987; Aarsman *et al.*, 1987). Moreover, the ability of lipocortins to reproduce the anti-inflammatory effect of glucocorticosteroids *in vivo* needs to be studied with highly purified lipocortins.

In this study, we present results which indicate that lipocortins share the anti-inflammatory effects of glucocorticosteroids *in vivo*. Our data might implicate lipocortin(s) in the anti-inflammatory effects of glucocorticosteroids.

Methods

Lipocortin preparation

A 36 K lipocortin was purified either from mouse thymuses or lungs. Protein purification was performed as described previously (Errasfa *et al.*, 1988; Errasfa & Russo-Marie, 1988a). Briefly, thymuses or lungs were removed from mice, then homogenized and sonicated. A first centrifugation allowed the elimination of a crude material. From the resulting supernatant, proteins were extracted by calcium precipitation-EGTA solubilization, then purified on a Mono Q column HR 5/5 using fast protein liquid chromatography (Pharmacia). Biochemical and immunological analysis showed that the two 36 K proteins purified either from mouse lungs or thymuses were identical. Therefore, they are referred to as 36 K lipocortin, this lipocortin is identical to a 32 K lipocortin from human peripheral blood mononuclear cells (Errasfa & Russo-Marie, 1988a). Human recombinant lipocortin I was a gift from Dr Jeff Browning (Biogen, Cambridge, MA, U.S.A.).

Inflammatory model

The experimental inflammatory model has been described in detail previously (Errasfa & Russo-Marie, 1988b). Briefly, mice were anaesthetized with ether, and 2 ml of a 150 mg ml^{-1} polyacrylamide gel suspension were injected into the dorsal subcutaneous tissue of mice. After a 4–7 h period, mice were killed and the subcutaneous area washed with phosphate buffered saline (PBS) in order to collect the gel and the migrated inflammatory cells (polymorphonuclear leucocytes (PMNs) > 90% of total cells). The gel was decanted, then the cells present in the supernatant (Sn1) were counted using a Coulter counter ZBI. Sn1 was then centrifuged at 300 g at 4°C. Prostaglandin E_2 (PGE_2) and LTB_4 were measured in the resulting supernatant (Sn2) by a specific radioimmunoassay according to the method of Salmon *et al.* (1982).

Drug treatment of mice

(i) Mice received intravenously 200–300 μl of either dexamethasone (0.15 and 1.5 mg kg^{-1}), indomethacin (0.5 and 5 mg kg^{-1}) or lysine-acetylsalicylate (50 and 150 mg kg^{-1}). Control mice received ethanol at an appropriate dilution for matching dexamethasone- and indomethacin-treated mice, and saline for matching lysine-acetylsalicylate-treated mice. Two hours after dexamethasone or 1 hour after acetylsalicylate or indomethacin administration, mice received the polyacrylamide gel suspension.

(ii) Two hundred to three hundred μl of the protein (P) or its vehicle (V) (saline containing the same concentration of the buffer mixture used to purify the protein) were injected intravenously into mice. After a 0.5–1 h period, mice received the polyacrylamide gel suspension subcutaneously.

After administration of the anti-inflammatory drugs or the protein, and injecting the gel, mice were killed after a predetermined period (4–7 h). The number of migrated cells was counted and PGE_2 and LTB_4 measured as described above.

Neutrophil phospholipase A_2 activity estimation

Mice were killed 6 h after receiving the polyacrylamide gel. Cells were obtained as described above. They were washed with PBS and labelled with [^{3}H]-arachidonic acid (AA) (5 μCi per 10^8 cells per 20 ml of MEM-HEPES medium) for 30–40 min at 37°C. Cells were then washed with PBS containing 0.5% fatty acid free-bovine serum albumin (FAF-BSA) to eliminate unbound [^{3}H]-AA from the incubation medium. Labelled PMNs (25,000–30,000 c.p.m. per 3–4 × 10^6 cells in 0.45 ml) were resuspended in MEM-HEPES medium, pH 7.4 and preincubated 20 min at 37°C with 5 μg of mouse 36 K lipocortin. Control cells received the vehicle of the protein. The reaction was started by the addition of 0.45 ml of the

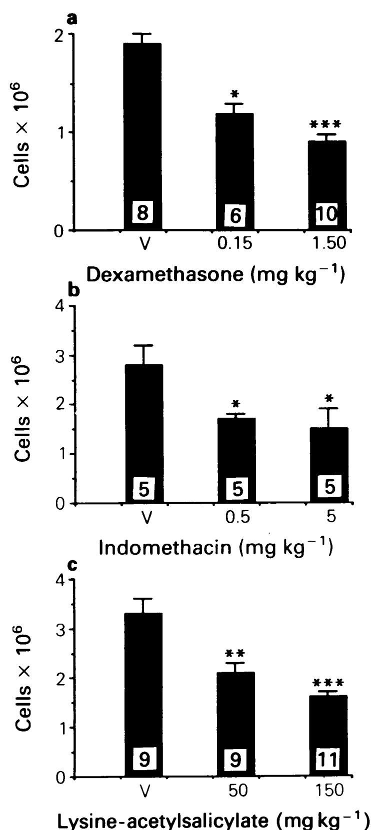

Figure 1 Inhibition of cell migration induced by anti-inflammatory agents in polyacrylamide gel-induced inflammation in mice. Mice received, intravenously, (a) dexamethasone, (b) indomethacin or (c) lysine-acetylsalicylate. Then, they received 2 ml of a 150 mg ml^{-1} gel suspension subcutaneously. Five hours later, the animals were killed and the number of the migrated PMNs counted as described in Methods. The number of animals is indicated inside each column. Each column represents the mean number of cells per animal. Vertical bars show s.e.mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

cell suspension to 50 μl of the calcium ionophore A23187 or the chemotactic peptide N-formyl-methionyl-leucyl-phenylalamine (fMLP) at a known concentration. Unstimulated cells received the solvent (DMSO) at the appropriate concentration (0.01%). The reaction was terminated by the addition of 500 μl of 10 mM EGTA containing 0.5% FAF-BSA, pH 7.4. The samples were centrifuged for 5 min at 10,000 g, and aliquots of the supernatants were counted by liquid scintillation spectrometry to determine the released radioactivity. The cellular PLA_2 activity of PMNs was estimated as the release of [^{3}H]-AA from the cells.

Measurement of neutrophil chemotaxis

The chemotaxis assay was performed as described by Harvath *et al.* (1980). PMNs were obtained from mice that had received the suspension of polyacrylamide gel 6 h previously. The cells were washed with PBS pH 7.4 and adjusted to 10^6 cells ml^{-1} in the following buffer (mM): NaCl 145, KCl 5, $MgSO_4$ 1, HEPES 10, glucose 10, $CaCl_2$ 1, pH 7.4. The cells (0.45 ml) were incubated with increased concentrations of the prôtein or with its vehicle (50 μl) at 37°C for 20–30 min. Then 50 μl of the cell suspension was subjected to the chemotaxis assay using 1 μM FMLP for 40 min at 37°C in 5% CO_2 in a humidified atmosphere. A 48-well micro chemotaxis assembly (Neuro Probe, Inc) was used fitted with 25 × 80 mm polycarbonate membranes of 3 μm pore size (Nucleopore). The migrating cells which traversed and adhered to the membrane were stained with Diff-Quik staining reagents. The cells were counted by use of a light microscope. The result of a single well is expressed as a mean of counts from ten separate fields observed at 40 ×. The final result of each assay is expressed as the mean ± s.e.mean of several replicate wells.

Measurement of leukocytes and platelets in mouse whole blood

Blood samples (50 μl) were collected from the retroorbital vein of mice. Total leukocyte and platelet counting was performed with a Coulter counter ZBI.

Chemicals

The following products were purchased from Sigma; dexamethasone, calcium ionophore A23187, FMLP, BSA-FAF, EGTA and indomethacin. Lysine-acetylsalicylate (Aspegic) was from Egic Joullié, France. Polyacrylamide gel (Bio Gel, P4, 200–400 mesh) was purchased from Biorad (Paris, France). DMSO was from Merck (Paris, France). HEPES and MEM were purchased from Eurobio (Paris, France). Prostaglandin E_2 (PGE_2) was a gift from J. Pike (The Upjohn company, Kalamazoo, Michigan, U.S.A.). [^{125}I]-PGE_2 and antibodies against PGE_2 were from Pasteur Institute (Paris, France). [^{3}H]-LTB_4 and [^{3}H]-arachidonic acid were purchased from Amersham (Paris, France) and the kit for LTB_4 radioimmunoassay from Wellcome (Paris, France).

Statistical analysis

The results are expressed as mean ± s.e.mean. Means were compared by use of Student's *t* test. In

our experimental conditions, differences were considered to be significant when P values were less than 0.05.

Results

Effect of anti-inflammatory drugs on cell migration

The injection of a suspension of polyacrylamide gel into the dorsal subcutaneous tissue of mice induced an inflammatory reaction, the latter was characterized by the accumulation of PMNs, and the production of inflammatory mediators such as PGE_2 and LTB_4 in the inflamed site of mice. The intravenous administration of all anti-inflammatory drugs (dexamethasone, indomethacin, lysine-acetylsalicylate) reduced the migration of PMNs toward the polyacrylamide gel (Figure 1).

Effect of two lipocortins (LCI and 36 K) on cell migration

Intravenous injection of the 36 K lipocortin in mice reduced dose-dependently the migration of PMNs to the subcutaneous tissue of mice (Figure 2a). A comparable inhibitory effect on cell accumulation was observed when mice received an intravenous injection of human recombinant lipocortin I (15 μg per animal): the number of migrated cells was $(10.1 \pm 1.9) \times 10^6$ in vehicle-treated mice versus $(4.1 \pm 0.5) \times 10^6$ in lipocortin I-treated mice (mean $\pm$ s.e.mean, $n = 6$, $P < 0.01$).

Intravenous administration of boiled 36 K lipocortin (10 μg per animal) to the animals had no effect on the inflammatory reaction: $(9.1 \pm 2.5) \times 10^6$ cells in vehicle-treated mice versus $(8.1 \pm 2.0) \times 10^6$ cells in boiled lipocortin-treated mice ($n = 4$). Administration of the 36 K lipocortin to the animals did not induce any change in their whole blood platelet and leukocyte counts. Platelets and leukocytes were $(6.7 \pm 1.6) \times 10^8$ and $(1.7 \pm 0.3) \times 10^6$ cells ml^{-1} respectively in 36 K lipocortin-treated mice versus $(6.4 \pm 1.2) \times 10^8$ and $(1.5 \pm 0.4) \times 10^6$ cells ml^{-1} respectively in vehicle-treated mice ($n = 10$). Moreover, the percentage of circulating blood neutrophils and mononuclear cells was not changed (data not shown).

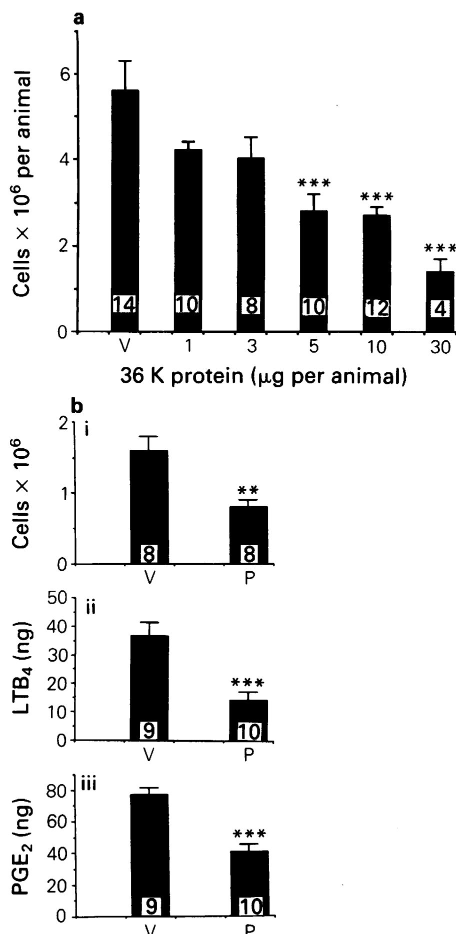

Figure 2 Effect of intravenous administration of the 36 K lipocortin on the migration of PMNs in inflamed mice. Mice received the protein 0.5–1 h before administration of 2 ml of a 150 mg ml^{-1} polyacrylamide gel suspension. (a) Inhibition of cell migration (expressed as cells per animal) by increasing doses of the 36 K lipocortin. Mice were killed 7 h after receiving the polyacrylamide gel suspension. (b) Inhibition of cell migration (i) and of production of leukotriene B_4 (LTB_4) (ii) and prostaglandin E_2 (PGE_2) (iii) by 10 μg of the 36 K lipocortin. Mice were killed 4 h after receiving the polyacrylamide gel suspension. The number of animals per group is indicated inside the columns. Protein-treated (P) mice were compared to vehicle-treated (V) mice. Values are expressed as means with vertical bars showing s.e.mean. ** $P < 0.01$ and *** $P < 0.001$.

Effect of dexamethasone and 36 K lipocortin on eicosanoid production

The inhibition of cell accumulation induced by the protein was accompanied by an inhibition of PGE_2 and LTB_4 production in the inflamed site (Figure 2b). When results were expressed as ng per 10^6 cells, the amounts of LTB_4 decreased significantly ($P < 0.001$) from 24.6 ± 1.5 (vehicle) to 13.9 ± 2.5

Table 1 Amounts of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) in the inflamed site of mice receiving either dexamethasone (0.15 and 1.50 mg kg^{-1}) or its vehicle

	Vehicle	*Dexamethasone* (mg kg^{-1}) *0.15*	*1.50*
LTB_4			
ng per exudate	67 ± 5	47 ± 5*	37 ± 7**
ng per 10^6 cells	36 ± 4	34 ± 2	28 ± 3
PGE_2			
ng per exudate	19 ± 2	nd	13 ± 2*
ng per 10^6 cells	12 ± 1	nd	16 ± 2

The experimental protocol is the same as described in Figure 1. The amounts of LTB_4 and PGE_2 are expressed as ng per exudate and as ng per 10^6 cells. Results are shown as mean ± s.e.mean; n = between 5 and 9 animals per group. ND: not done. * $P < 0.05$, ** $P < 0.01$.

(36 K protein), whereas the amounts of PGE_2 were not different (47.0 ± 4.6 and 52.0 ± 5.0) in vehicle and protein-treated mice, respectively.

Similarly, dexamethasone induced an inhibition of eicosanoid production (ng per mice) in the inflamed site of mice. However, when eicosanoids were expressed as ng per 10^6 cells, the amount of LTB_4 was decreased whereas levels of PGE_2 were not (Table 1).

Effect of 36 K lipocortin on [^3H]-arachidonic acid release from isolated neutrophils

As shown in Figure 3, when the 36 K lipocortin was incubated with inflammatory mouse PMNs, it inhibited [^{3}H]-arachidonic acid release from both A23187- and FMLP (0.1 μM)-stimulated PMNs. The boiled protein was inactive (data not shown).

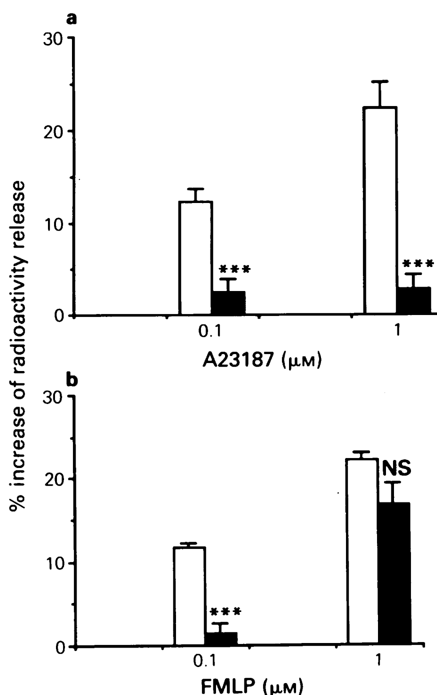

Figure 3 Inhibition of PMN phospholipase A_2 (PLA_2) activity by 5 μg 36 K lipocortin. PMNs were obtained from mice after they had received, 6 h before, 2 ml of a 150 mg ml^{-1} gel suspension. The cells were labelled with [^{3}H]-arachidonic acid, then incubated with the 36 K lipocortin for 20 min. Cell stimulation by (a) the calcium ionophore A 23187 or (b) N-formyl-methionyl-leucyl-phenylalanine (FMLP) and measurement of the released radioactivity are described in Methods. Results are expressed as % increase of radioactivity released from stimulated cells versus DMSO-treated cells. Vehicle-treated cells (open columns, $n = 3$) were compared to protein-treated cells (solid columns, $n = 6$). Results are expressed as mean with vertical bars showing s.e.mean. *** $P < 0.001$.

Effect of 36 K lipocortin on neutrophil chemotaxis

The effect of the 36 K lipocortin on PMN chemotaxis was investigated *in vitro*. As shown in Figure 4, 36 K lipocortin impaired, in a dose-dependent manner, PMN migration toward the chemotactic stimulant FMLP.

Discussion

Our findings show that purified lipocortin(s) can reproduce some of the anti-inflammatory effects of glucocorticosteroids in mice with an inflammatory lesion, since: (i) purified mouse 36 K lipocortin and recombinant lipocortin I decreased the accumulation of PMNs at the injured site; (ii) the mouse 36 K lipocortin decreased the amounts of LTB_4 and PGE_2 in the exudate of the injured animals; (iii) the mouse 36 K protein impaired, as glucocorticosteroids do (Hirata *et al.*, 1980), both cellular PLA_2 activation and chemotaxis of mouse PMNs *in vitro*. Taken together, these data suggest that lipocortins might be related to glucocorticosteroids in some way. Indeed, lipocortins were initially found as extracellular proteins after glucocorticosteroid treatment both in cell

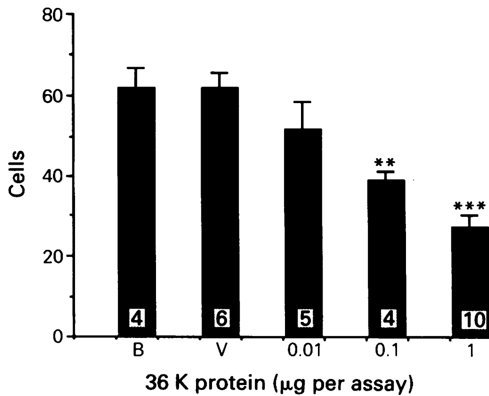

Figure 4 Inhibition of PMN chemotaxis by the 36 K lipocortin. PMNs were obtained from mice after they had received, 6 h before, 2 ml of a 150 mg ml^{-1} gel suspension. They were prepared as indicated in Methods, and incubated with increasing concentrations of the 36 K lipocortin. Cells (50 μl) were then subjected to the migration assay in the chemotaxis assembly. The number of cells which migrated toward the chemoattractant peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) were counted as described in Methods. The number of replicates is indicated inside the columns. B: buffer medium-treated cells, V: cells incubated with vehicle for the protein. Protein-treated cells were compared to vehicle-treated cells. ** $P < 0.01$, *** $P < 0.001$.

supernatants (Hirata *et al.*, 1980; Cloix *et al.*, 1983; Errasfa *et al.*, 1985) and rat peritoneal lavage fluid (Blackwell *et al.*, 1980). In addition, the first complete purification and partial sequencing of lipocortin I were performed on extracellular lipocortin (Pepinsky *et al.*, 1986), although the cDNA sequence of lipocortin I showed that this protein had no leader peptide to permit its secretion. Moreover, recently it has been found that recombinant lipocortin I inhibits both thromboxane release from guinea-pig isolated, perfused lungs (Cirino *et al.*, 1987) and prostacyclin from human endothelial cells (Cirino & Flower, 1987), and that other lipocortins are able to inhibit PLA_2 activity of mouse isolated thymocytes (Rothhut *et al.*, 1987; Errasfa *et al.*, 1989) and guinea-pig alveolar macrophages (Errasfa *et al.*, 1988). Taking these results into account, we hypothesized that if lipocortins were able to inhibit cellular PLA_2 activity when added exogenously, they may also be able to reproduce part of the anti-inflammatory effects of glucocorticosteroids when injected into the whole animal *in vivo*. Our results showing similar effects for lipocortins and glucocorticosteroids support such a hypothesis but do not give us any insight into the putative mechanism of action. We, therefore, tried to estimate how lipocortins might be anti-inflammatory.

Lipocortins inhibited both cell migration and eicosanoid production *in vivo* suggesting that at least two inflammatory parameters were altered: (i) cell migration i.e. chemotaxis and/or (ii) LTB_4 and PGE_2 production i.e. PLA_2 activity. In order to estimate more precisely whether *in vivo* PLA_2 activity was altered by lipocortin, we compared both the amounts of eicosanoids at the inflamed site and the ratio of these amounts correlated to the number of migrated PMNs. When results were expressed per cell, the amounts of LTB_4 decreased significantly, whereas the amount of PGE_2 were unchanged. Since LTB_4 is known to derive mainly from PMNs, these results suggested that PLA_2 activity of PMNs was impaired in lipocortin- and dexamethasone-treated mice, although the amounts of PGE_2 were not altered. This discrepancy between inhibition of PGE_2 and LTB_4 production may be due to the fact that PGE_2 originates from cells, located in the subcutaneous tissue, not taken into account when results were expressed per cell. To analyse further this effect, the same lipocortin used *in vivo* was assayed *in vitro* to check its ability to alter the cellular PLA_2 activity of neutrophils isolated from the inflammatory tissue. Indeed the 36 K lipocortin was able to inhibit the release of [^{3}H]-AA induced by both the calcium ionophore A23187 and 0.1 μM FMLP, indicating that on those neutrophils, lipocortin was able to inhibit PLA_2. The absence of effect for the higher dose of FMLP is not clear, it might be because at a high dose FMLP induces the release of AA through another activation pathway not involving PLA_2.

The second parameter likely to be altered by lipocortin was PMN chemotaxis. We therefore used the same approach to check whether lipocortin could alter the *in vitro* chemotaxis of isolated neutrophils. The 36 K lipocortin was able to inhibit the FMLP-induced chemotaxis in a dose-dependent manner. Therefore, the *in vitro* impairment of both PMN chemotaxis and PLA_2 activity by the 36 K lipocortin could occur *in vivo* and could participate in the *in vivo* inhibition of cell migration and eicosanoid production at the inflamed site in mice. Recently, we have shown (Fradin *et al.*, 1988) that the same 36 K lipocortin was able to impair the production of two chemoattractant agents; LTB_4 and Paf in rat PMNs. This effect could contribute to the anti-inflammatory effect induced by the 36 K lipocortin.

The ability of lipocortins to bind to acidic phospholipids *in vitro* in a calcium-dependent manner is known to be responsible for *in vitro* inhibition of both PLA_2 activity (Aarsman *et al.*, 1987; Davidson *et al.*, 1987) and blood coagulation (Funakoshi *et al.*,

1987; Chap *et al.*, 1988). Hence, we hypothesize that lipocortins bind to membrane phospholipids of activated cells within the vessel, inducing a modification of the membrane which leads to protection against a further PLA_2 attack of membrane phospholipids. PMNs would then become unable to migrate towards the inflamed site and to release PLA_2-derived mediators. The possibility that the inhibitory effect of lipocortins on PMN chemotaxis is related to PLA_2 inhibition is plausible but is not yet proven. Further investigations are required to explain the physiological presence of extracellular lipocortins and their *in vivo* effect. Nevertheless, the recently described phospholipase A_2 inhibitory effect by lipocortins in whole cells allows us to speculate that these proteins may play a role in the protection of cells against their activation by different stimuli.

Addendum

After completion of this manuscript, a paper was published (L. Miele *et al.*, 1988) describing the anti-inflammatory effect of lipocortin I and uteroglobin in the carrageenan-induced rat paw oedema.

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