Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test

(glucocorticoids/ eicosanoids /depletion of mediators/ phospholipase A2/mast cell amines)


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ABSTRACT Human recombinant lipocortin 1 has been tested for anti-inflammatory activity in a conventional model of acute inflammation. Microgram amounts of the protein, locally administered, inhibited edema of the rat paw when induced by subplantar injections of carrageenin: the ED50 was 10-20 µg per paw, and inhibition (maximum of 60-70%) was not dependent upon an intact adrenal cortex. Doses of lipocortin that produced ~50% inhibition in the carrageenin test were inactive against edema elicited by bradykinin, serotonin, platelet-activating factor-acether, or dextran, whereas edema caused by Naja mocambique venom phospholipase A2 was strongly inhibited by lipocortin. The protein inhibited edema when rats were pretreated with agents that depleted mast-cell amines, kininogen, or polymorphonuclear leukocytes prior to initiation of the carrageenin edema but had no inhibitory action when rats were pretreated with the dual cyclooxygenase/ lipoygenase inhibitor BW 755C. These results demonstrate that human recombinant lipocortin has potent local anti-inflammatory activity, probably through selectively interfering with eicosanoid generation. Lipocortin is relatively ineffective against edema caused by mast-cell degranulation or kinins, except when degranulation is caused by phospholipase A2.

The lipocortins (1) are a family of proteins that can inhibit phospholipase A2 activity in in vitro systems. Partially purified preparations of these proteins inhibit the release of eicosanoids (2) and lyso-platelet-activating factor (3) from cells and inhibit carrageenin-induced pleurisy (4) and paw edema in the rat (2), but lack of a highly purified protein has curtailed more definitive experimental work.

Recently, one form of lipocortin, designated lipocortin 1, has been sequenced, and the human gene has been cloned (5). We have reported that this recombinant protein blocks the release of eicosanoids from the guinea pig perfused lung (6) and other (7) preparations, and we now demonstrate that microgram doses of the protein, locally administered, inhibit the eicosanoid- but not the amine-dependent component of carrageenin edema in the rat paw assay. Lipocortin retains activity in this test when animals are depleted of amines or of kininogen but does not retain activity when eicosanoid synthesis is blocked. Lipocortin is also inactive as an inhibitor of edema caused by agents that degranulate mast cells or by the products of mast-cell degranulation. Only when the latter is induced by injection of phospholipase A2 is lipocortin effective.

Our results show that lipocortin has strong anti-inflammatory properties and that these properties are related to the ability of the protein to decrease eicosanoid formation at the inflammation site. Our results also suggest that lipocortin can neutralize the effect of extracellular phospholipases.

MATERIALS AND METHODS

Lipocortin 1. Recombinant lipocortin 1 was produced in Escherichia coli and chemically purified (>99%) by procedures analogous to those described (5, 8) using P150 gel exclusion chromatography as the final purification step. Lipocortin was dissolved in 25 mM Tris-HCl buffer, pH 7.7, with 0.1-5 mM EDTA and bovine serum albumin at 0.1 mg/ml (Pierce, endotoxin poor). The endotoxin level in the final preparation (as estimated by the limulus assay) was such that rats received 1 ng of E. coli endotoxin per 10 µg of injected lipocortin.

Lipocortin was aliquoted in solutions of high concentration, frozen, and when thawed, used immediately. Two batches of lipocortin, designated batch A and batch B, were used in this study. The two batches varied in potency; batch A was about two to three times as potent as batch B, probably because of heterogeneity in the N terminus, the folding, or the oxidation state of the different preparations.

Carrageenin Paw Edema. Adult male Wistar rats (150-200 g) were used throughout this study. A 2% (wt/vol) solution of lambd a carrageenin (Sigma) in saline (0.9% NaCl) was prepared freshly for each experiment. Before injection, a stock solution of lipocortin (1 mg/ml) was thawed and diluted in saline; the EDTA was then titrated with calcium chloride. A subplantar injection of 0.1 ml of carrageenin was followed immediately by lipocortin (or vehicle or other solution) injected in a vol of 0.1 ml (at the same injection site when possible) into the right hind paw of lightly anesthetized rats (ether). The final concentration of carrageenin in the injection was therefore 1% in a vol of 0.2 ml.

Paw volumes were measured hourly for up to 5 hr using a hydroplethysmograph (Socrif, Varese, Italy), and the increase in volume caused by the irritant was estimated after subtracting the basal volume of the paw before injection.

Depletion Experiments. In depletion experiments we followed the protocols as published by Di Rosa et al. (9, 10). In some experiments all depleting agents were used concurrently, and the dosing regimes were combined.

To deplete mast-cell amines, rats received six doses of compound 48/80 at 0.6 mg/kg i.p. at 12 hourly intervals followed by two doses of the same agent at 1.2 mg/kg, after which edema was initiated in the usual way by injection of carrageenin.

For the depletion of kininogen, rats were lightly anesthetized and injected repeatedly i.v. with cellulose sulfate at 1 mg/kg (Sigma) at 10-min intervals. After the third dose, rats were removed from the anesthesia chamber and assayed for edema not less than 3 hr later.

To be depleted of polymorphonuclear leukocytes, rats were given methotrexate at 2.5 mg/kg each day for 3 days and assessed on the fourth day for carrageenin-caused paw edema.

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Phospholipase A$_2$ Edema. In these experiments edema was induced in the rat paw by subplantar injection of a phospholipase A$_2$ purified from *Naja mocambique mocambique* (EC 3.1.1.4) (Sigma). In initial experiments a dose–response relationship between edema and amount of venom enzyme was established by injecting the hind paw with 5–30 $\mu$g in a vol of 0.1 ml. The 10– and 30-$\mu$g doses produced the same degrees of edema ($\approx 0.75$ ml at 30 min), differing only in time taken for the edema to subside. The 5-$\mu$g dose was $\approx 80\%$ of maximal, declined more rapidly, and was used for the remainder of our studies. The inflammatory properties of the venom phospholipase were greatly increased by calcium addition, and we routinely dissolved the venom in a solution containing 1 mM CaCl$_2$ before use.

Before injection, the venom was incubated for 15 min at 37$^\circ$C with either lipocortin or vehicle. Aliquots containing 5 $\mu$g of venom in 0.1 ml were injected into the paw, and the increase in volume was determined, as described above.

**Induction of Edema by Other Agents.** When injected into the rat hind paw, serotonin in doses of 0.2–20 $\mu$g produced a dose-related edema. We chose a dose of 10 $\mu$g as standard, which was premixed with lipocortin or vehicle before injection. Likewise, bradykinin produced dose-related edema in doses of 10–50 $\mu$g and compound 48/80 produced a dose-related response in doses of 0.2–10 $\mu$g. In the former case a standard dose of 25 $\mu$g was chosen, and in the latter case the dose was 5 $\mu$g. Lipocortin was premixed with these agents before injection.

Edema produced by all of these agents had a more rapid onset and shorter duration than that of carrageenin, and the swelling of the paw was measured at 30-min intervals for up to 3 hr.

**Adrenalectomized Rats.** Surgically adrenalectomized rats (Wistar) were obtained from Interfauna (Huntingdon, U.K.) and were maintained on saline instead of water. The animals were used for carrageenin induced paw-edema studies as described above not >14 days after adrenalectomy. Sham-operated animals, obtained from the same supplier at the same time, served as controls in these experiments.

**Statistics.** Student’s unpaired $t$ test was used to assess the significance of differences seen between groups of animals.

**RESULTS**

**Effect of Lipocortin on Carrageenin Edema.** To test the ability of lipocortin to inhibit carrageenin-induced edema, the protein was injected at doses of 2–100 $\mu$g (0.05–2.27 nmol) simultaneously with carrageenin. In 100 of 109 rats administered lipocortin, edema was inhibited. Most nonresponders were those that received the lowest protein dose.

Fig. 1 shows a series of matched experiments in which various (5–50 $\mu$g) doses of lipocortin (batch A) were given. The inhibition seen at the time of maximal edema (3 hr) was linearly related to the logarithm of the dose, and the ED$_{50}$ fell in the range of 10–20 $\mu$g (0.27–0.34 nmol). Not shown on Fig. 1 is the effect of injections of 2 or 100 $\mu$g of lipocortin: Doses of 2 $\mu$g or less were inactive, and injection of 100 $\mu$g produced no greater inhibition than that of 50 $\mu$g. Thus, the maximal inhibition in this test is 60–70% at the 3-hr time point. The ED$_{50}$ for batch B was $\approx 30$ $\mu$g (0.71 nmol).

Several types of controls were examined with batch A of the protein. Boiled lipocortin (100$^\circ$C for 15 min) at a dose of 10 $\mu$g was without effect at any time (one experiment with five rats per group, 5.0 $\pm$ 5.1% inhibition; control inhibition, 40.0 $\pm$ 6.1%). “Sham” lipocortin, a preparation extracted from *E. coli* lacking the lipocortin plasmid but containing a similar qualitative and quantitative mix of endotoxin and *E. coli* material was also tested at a “dose” corresponding to the amount of *E. coli* material contained in 10 $\mu$g of lipocortin (one experiment with five rats per group). No inhibition was seen; in fact, a substantial potentiation (24.4 $\pm$ 13.7% at the 3-hr time point) was seen relative to the saline-injected control. Finally, to exclude the possibility that lipocortin preparations contained a factor that stimulated the adrenal cortex—thereby reflexly suppressing the inflammatory response—we tested the 10-$\mu$g dose in adrenalectomized rats (one experiment with five rats per group). An inhibitory effect indistinguishable from that observed in normal rats was seen with a 40.0 $\pm$ 8.9% inhibition at 3 hr.

In one further experiment (four rats per group) we tested the ability of lipocortin to inhibit carrageenin edema once edema was established. For this test, we injected a 10-$\mu$g dose of lipocortin 1 hr and 2 hr after carrageenin. We noted that a reduction in the edema relative to controls in both treated groups occurred at subsequent time points, which achieved statistical ($P < 0.05$) significance within 1 hr of dosing and which had reached 77.8 $\pm$ 11.1% and 74.0 $\pm$ 10.6% inhibition values (relative to vehicle-treated controls), respectively, 2 hr after giving the protein.

**Depletion of Mediators.** Carrageenin edema depends upon several factors, including the release of mast-cell amines, the generation at the site of inflammation of eicosanoids, the migration of polymorphonuclear leukocytes, and the actions of kinins. Because lipocortin generally produced a maximal inhibition of 60–70%, it was evident that, unlike the glucocorticoids themselves, lipocortin could not inhibit all components of the inflammatory response.

To determine which mediators were sensitive to lipocortin, we conducted a series of depletion experiments in which a fixed dose of lipocortin (batch B, 20 $\mu$g) was tested in rats pretreated with depleting agents. Table 1 summarizes the results of these experiments and also shows the mean inhibition by lipocortin of edema in control animals or those treated with depleting drugs. Treatment of rats with agents that depleted mast-cell amines, kininogen, or eicosanoids reduced the overall edema response, indicating that one or
Table 1. Summary of data from depletion experiments

<table>
<thead>
<tr>
<th>Depleting agent</th>
<th>Maximal edema, ml (n)</th>
<th>Lipocortin inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Lipocortin (20 μg)</td>
</tr>
<tr>
<td>None</td>
<td>0.79 ± 0.05 (11)</td>
<td>0.49 ± 0.05 (11)</td>
</tr>
<tr>
<td>i. Compound 48/80</td>
<td>0.58 ± 0.06 (5)</td>
<td>0.39 ± 0.04 (5)</td>
</tr>
<tr>
<td>ii. Cellulose sulfate</td>
<td>0.38 ± 0.04 (6)</td>
<td>0.14 ± 0.05 (6)</td>
</tr>
<tr>
<td>iii. Methotrexate</td>
<td>0.74 ± 0.06 (5)</td>
<td>0.53 ± 0.04 (4)</td>
</tr>
<tr>
<td>i + ii + iii</td>
<td>0.34 ± 0.06 (4)</td>
<td>0.08 ± 0.1 (3)</td>
</tr>
<tr>
<td>BW 755C</td>
<td>0.31 ± 0.07 (5)</td>
<td>0.37 ± 0.06 (5)</td>
</tr>
</tbody>
</table>

Batch B was used in these experiments. Maximal edema values are given as x ± SEM.
*Derived from mean values in control and lipocortin edema columns.
†Derived planimetrically from the complete inflammation curve over time.
§P < 0.05 relative to control value.
¶P < 0.01 relative to control value.

more inflamogenetic components had been removed. Treatment with methotrexate had little effect. Lipocortin reduced the residual edema further after rats were treated with cellulose sulfate to remove kininogen or compound 48/80 to remove mast-cell amines, although the anti-inflammatory properties of lipocortin seemed reduced when rats had been pretreated with only methotrexate. When rats received all three depleting agents, lipocortin was strikingly effective in reducing edema. In these rats there was a small peripheral edema in all paws, which itself was lipocortin sensitive, and treatment with the latter reduced the basal-paw volume as well as the carrageen-induced edema. Lipocortin could not further reduce the edema of rats that had been "depleted" by prior treatment with a maximal dose (50 mg/kg) of the dual cyclooxygenase/lipoxygenase inhibitor BW 755C.

A fixed dose of lipocortin (batch B, 20 μg) was ineffective when edema was produced by bradykinin, serotonin, dextran, or platelet-activating factor-acether instead of carrageenin (Table 2). Indomethacin and BW 755C in doses that completely suppress eicosanoid formation were also without effect in these tests, whereas invariably glucocorticoids inhibited markedly (see Table 2).

For compound 48/80, however, lipocortin produced a small (35.9 ± 5.1%, n = 11), but significant (P < 0.001), inhibition at the time of maximal edema, as did 15 μg of indomethacin (28.2 ± 5.0%, n = 10; P < 0.001). These data strongly suggest that eicosanoids were involved at the time of maximal edema as well as mast-cell amines. Again, dexamethasone inhibited the edema at all time points (see Fig. 2).

Edema Produced by Phospholipase A2. In 81 of 85 rats tested lipocortin, in doses of 2–20 μg (batch B) premixed with phospholipase A2, inhibited the formation of phospholipase edema. Maximal inhibition varied with the batch of enzyme, but inhibition never exceeded 85% and was always achieved with equimolar ratios of lipocortin to phospholipase. Increasing the ratio beyond 1:1 produced no further inhibition.

Fig. 3 represents a typical experiment with a single batch of venom enzyme; it shows the time course of edema produced by injecting 5 μg of the venom phospholipase and the dose-dependent inhibition produced by preincubating for 15 min with 5 and 10 μg of human recombinant lipocortin 1. The maximal inhibition achieved in this assay, as determined by inactivation of the venom enzyme with a 10-fold molar excess of p-bromophenacyl bromide before injection was 76.9 ± 4.9% (one experiment with 10 rats).

**DISCUSSION**

Our data demonstrate that human recombinant lipocortin 1 possesses anti-inflammatory properties in a well-characterized model of acute inflammation and supports an earlier contention regarding the biological activity of this protein (2, 4). In a series of parallel experiments (data not shown) we tested locally administered indomethacin as an inhibitor of carrageenin-induced paw edema and determined the ED50 to be 30 μg (84 nmol). As the ED50 for lipocortin is 50 nmol, lipocortin is >150-fold more active on a molar basis at the 3-hr time point than is indomethacin. In doses as high as 5 mg/kg orally (which completely suppress prostaglandin production) indomethacin produced a maximal inhibition of 62.8 ± 2.7% (n = 30) of carrageenin edema at the 3-hr time point—a maximum very similar to that of lipocortin.

Table 2. Summary of lipocortin effects in other inflammatory models

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Dextran (0.1 ml, 3%)</th>
<th>PAF-acether (1 μg)</th>
<th>Bradykinin (25 μg)</th>
<th>Serotonin (10 μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Edema, ml (n)</td>
<td>Inhibition, % (P)</td>
<td>Edema, ml (n)</td>
<td>Inhibition, % (P)</td>
</tr>
<tr>
<td>Saline</td>
<td>0.42 ± 0.06 (5)</td>
<td>0.33 ± 0.003 (13)</td>
<td>0.52 ± 0.02 (18)</td>
<td>0.55 ± 0.03 (20)</td>
</tr>
<tr>
<td>Lipocortin (20 μg)*</td>
<td>0.34 ± 0.05 (5)</td>
<td>0.28 ± 0.03 (10)</td>
<td>0.44 ± 0.05 (10)</td>
<td>0.54 ± 0.08 (9)</td>
</tr>
<tr>
<td>Dexamethasone (1 mg/kg)</td>
<td>NT</td>
<td>0.08 ± 0.02 (10)</td>
<td>0.37 ± 0.04 (8)</td>
<td>0.32 ± 0.05 (10)</td>
</tr>
<tr>
<td>Methysgeride (10 μg)*</td>
<td>NT</td>
<td>0.34 ± 0.03 (12)</td>
<td>NT</td>
<td>0.13 ± 0.09 (5)</td>
</tr>
<tr>
<td>Indomethacin (5 mg/kg)</td>
<td>NT</td>
<td>0.30 ± 0.03 (12)</td>
<td>0.45 ± 0.07 (8)</td>
<td>NT</td>
</tr>
<tr>
<td>BW 755C (50 mg/kg)</td>
<td>NT</td>
<td>0.30 ± 0.03 (5)</td>
<td>0.51 ± 0.04 (5)</td>
<td>0.54 ± 0.04 (10)</td>
</tr>
</tbody>
</table>

Batch B was used throughout this experiment. Edema values are given as x ± SEM. NT, not tested in this laboratory; NS, not significant at the 5% level. PAF, platelet-activating factor.
*Local administration.
†Data taken from Peers (11).
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Fig. 2. Effect of lipocortin (batch B), indomethacin, and dexamethasone on the edema produced by compound 48/80 in the rat paw. The data are expressed as mean edema (ml) ± SEM, and values marked with an asterisk are statistically different from controls at a minimum significance level of $P < 0.05$. ○, Control edema ($n = 14$); ○, 40 μg of indomethacin given locally into the paw ($n = 10$); ○, 20 μg of lipocortin given locally ($n = 11$); and ●, dexamethasone at 1 mg/kg given 3 hr before the inflammogenic stimulus ($n = 10$).

In their classic studies, Di Rosa and his colleagues (9, 10) drew attention to the multifactorial nature of the carrageenin-induced edema of the rat paw and attempted to analyze the role of each mediator with the use of depleting agents. Our findings agree broadly with their results, in that they point to a strong involvement of mast-cell amines and kinins as well as prostanooids. Our results indicate that lipocortin retained activity when the overall edema was reduced by prior depletion of amines, kininogen, or polymorphonuclear cells—presumably because the eicosanoids were the major remaining proinflammatory mediators. Di Rosa et al. observed a similar phenomenon while using indomethacin. A puzzling feature of the depletion experiments is the apparent reduction by methotrexate of the lipocortin effect. This observation may mean either that migrating polymorphonuclear cells contribute a certain fraction of the eicosanoids responsible for the edema or that lipocortin exerts some of its actions by decreasing the migration of these cells.

Our general conclusion concerning the mechanism of action of locally administered lipocortin was further supported by our experiments in which the protein was tested against serotonin, bradykinin, dextran, or platelet-activating factor-acethyldopa. In no case did lipocortin produce a statistically significant influence on these types of inflammation in the fixed dose that we used in this study. Only in paw edema induced by injections of compound 48/80 was there any significant effect of lipocortin, and this effect occurred at a time point at which indomethacin also displayed some inhibitory effect. These results suggest that while the release of mast-cell amines is the most important component of compound 48/80-induced edema, eicosanoids exert a significant effect at least at one time point.

The failure of lipocortin to produce any anti-inflammatory effect when tested against many of the agents mentioned above indicates that lipocortin is selective in its action. Because glucocorticoids are effective against all forms of edema, obviously an additional mechanism must be invoked to explain how glucocorticoids bring about other types of anti-inflammatory action. Di Rosa’s group (9, 10) suggested that another induced protein mediates the ability of steroids to reduce dextran edema (12).

The only time when lipocortin was effective against an edema that depended upon release of mast-cell amines was in the instance of phospholipase A$_2$-induced inflammation. This type of edema is probably caused either by a direct lytic effect of the phospholipase or by products of its catalytic activity, because such an edema can be profoundly inhibited by pretreatment of the enzyme with the active-site inhibitor p-bromophenacyl bromide. The inhibition of phospholipase-induced edema may be biologically relevant, as lipocortin could directly neutralize the activity of extracellular phospholipases thought to be important in some forms of inflammation (13, 14).

Many proteins or protein extracts have been reported to possess anti-inflammatory activity, but with few exceptions (e.g., superoxide dismutase) these proteins have never been completely characterized or the activity has been associated with endotoxin or reflex stimulation of the adrenal cortex (15–18). Endotoxin itself has been reported to have anti-inflammatory effects, but the ED$_{50}$ in the rat paw edema test was 2–5 μg per paw—at least 10$^2$-fold greater than the amounts we administered (18).

That human recombinant lipocortin 1 is anti-inflammatory is particularly significant because the glucocorticoids induce the lipocortin gene (5). Other lines of evidence point indirectly to the importance of lipocortin as an important mediator of steroid action: inflammation and lipid-mediated release in adrenalectomized rats is greatly enhanced relative to sham-operated animals (19), suggesting that endogenous lipocortin exerts a continual inhibitory tone. Autoantibodies to lipocortin have also been reported (20, 21) in the plasma of patients with chronic inflammatory diseases, and the titer of these antibodies seems to correlate positively with the clinical phenomenon of “steroid-resistance” (21).

We believe that the synthesis and release of lipocortin may partially explain the marked anti-inflammatory actions of steroids and that after local administration of lipocortin, anti-inflammatory action is achieved by action on eicosanoid generation. Nor can we rule out additional mechanisms of action when the protein is given by routes other than the one described here.

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