Lipocortin-1 Is an Endogenous Inhibitor of Ischemic Damage in the Rat Brain

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Summary

Lipocortin-1 (annexin-1) is an endogenous peptide with antiinflammatory properties. We have previously demonstrated lipocortin immunoreactivity in certain glial cells and neurons in the rat brain (Strijbos, P. J. L. M., F. J. H. Tilders, F. Carey, R. Forder, and N. J. Rothwell. 1990. Brain Res. In press.), and have shown that an NH_2-terminal fragment (1–188) of lipocortin-1 inhibits the central and peripheral actions of cytokines on fever and thermogenesis in the rat in vivo (Carey, F., R. Forder, M. D. Edge, A. R. Greene, M. A. Horan, P. J. L. M. Strijbos, and N. J. Rothwell. 1990. Am. J. Physiol. 259:R266; and Strijbos, P. J. L. M., J. L. Browning, M. Ward, R. Forder, F. Carey, M. A. Horan, and N. J. Rothwell. 1991. Br. J. Pharmacol. In press.). We now report that intracerebroventricular administration of lipocortin-1 fragment causes marked inhibition of infarct size (60%) and cerebral edema (46%) measured 2 h after cerebral ischemia (middle cerebral artery occlusion) in the rat in vivo. The lipocortin-1 fragment was effective when administered 10 min after induction of ischemia. Ischemia caused increased expression of lipocortin-1 around the area of infarction as demonstrated by immunocytochemistry. Intracerebroventricular injection of neutralizing antilipocortin-1 fragment antiserum increased the size of infarct (53%) and the development of edema (29%). These findings indicate that lipocortin-1 is an endogenous inhibitor of cerebral ischemia with considerable therapeutic potential.

Lipocortins are a family of calcium binding proteins with diverse actions, including antiinflammatory properties, possibly acting by inhibition of arachidonic acid production and subsequent reduction in eicosanoid synthesis (see reference 1). However, there is now some doubt over their mechanisms of action (2), and several have been demonstrated; e.g., phospholipid binding, calcium binding, and receptor phosphorylation (see references 3 and 4). Nevertheless, potent antiinflammatory effects of lipocortin-1 have been demonstrated on carrageenin-induced paw edema in the rat in vivo (5). Lipocortins have been proposed as mediators of glucocorticoid actions, and their synthesis can be induced by endogenous or synthetic steroids (see reference 1), although in some tissues lipocortin induction appears to be independent of glucocorticoid status (6).

Several reports have indicated that lipocortin-1 is present in the brain (7–9), and we have described its cellular distribution in the rat brain in some detail (10). A high density of lipocortin-1-like immunoreactivity is present in both glial and neuronal cells throughout the brain, with the latter most evident in the dentate gyrus, CA1, CA2, and CA3 neurones of the hippocampus. Central administration of an active recom-

binant fragment (1–188 amino acids) of lipocortin-1 causes dose-dependent inhibition of the actions of cytokines on fever and metabolism in the conscious rat (11). Inhibition of the actions of endogenous lipocortin-1 within the brain, by cerebral injections of neutralizing antibody raised to the lipocortin-1 fragment, significantly reverses the inhibitory effects of glucocorticoids on cytokine action (11). These observations indicate that both endogenous and exogenous lipocortin-1 have important biological actions within the central nervous system.

Interruption of blood supply to the brain (cerebral ischemia) results in damage to neurones within the central nervous system, ultimately leading to cell death. The mechanisms of this neurodegenerative process are not fully understood, but apparently involve increased extracellular calcium concentrations, release of excitatory amino acids, increased synthesis of arachidonic acid and eicosanoids, and formation of edema (12). Cytokines have also been strongly implicated in the neurodegenerative responses to brain injury (13), although their involvement in cerebral ischemia has not yet been investigated. Modification of these processes provide possible targets for therapeutic intervention, which may be of benefit in the treat-

ment of stroke or brain injury. In view of the actions of lipocortin-1 already described, it is likely that this peptide could inhibit several of the events that ultimately lead to neuronal death after ischemia.

The objective of the present study was to investigate the effects of exogenous and endogenous lipocortin-1 on cell death and edema formation after cerebral ischemia in the rat. The results indicate that lipocortin-1 is a potent, endogenous inhibitor of brain damage which may be of considerable therapeutic benefit.

Materials and Methods

Cerebral Ischemia. Cerebral ischemia was induced by permanent unilateral occlusion of the middle cerebral artery (MCA)[1] in anesthetized rats. Young, adult (200–250 g), male Sprague Dawley rats (Charles River Breeding Laboratories, Kent, UK) were stereotaxically implanted with guide cannulae in the third ventricle of the brain under pentobarbitone anesthesia at least 4 d before the experiment. MCA occlusion (14) was performed under halothane (2% in oxygen, nitrous oxide) anesthesia. The left MCA was exposed through a small sub-temporal craniectomy, and was occluded by electrocoagulation using bipolar diathermy at the point where it crossed the olfactory tract, and at the level of the inferior cerebral vein, a cut was then made between the points of coagulation. The wound was sutured and recovery of consciousness in all animals was achieved within 10 min of surgery.

Assessment of Infarct Volume. Rats were killed either 2 or 24 h after MCA occlusion. Brains were removed and 500-μm coronal sections cut, immediately placed individually into tetrazolium dye staining medium, and incubated for 30 min at room temperature. The positively stained area on each slice was stereotaxically sketched by eye and infarct area for each section assessed using Magiscan 2 image analysis.

Cerebral Edema. Tissue density of hemispheric brain sections was assessed by percoll gradient (adapted from reference 15). Animals were killed 2 h after MCA occlusion and their brains rapidly removed. 1-mm sections were cut from each hemisphere at the level of bregma, and the equilibriation depth of each slice in the percoll gradient was measured after 2 min. The tissue water content of each sample was calculated from the equilibrium depth that was directly proportional to the specific gravity of the tissue.

Distribution of Lipocortin-1 in the Brain. Lipocortin-1 immunoreactivity was determined using an antiserum raised in rabbits to a recombinant lipocortin-1 fragment (1–188) (11). We have demonstrated by Western blot analysis and gelatin models that this antiserum binds to endogenous rat lipocortin-1 (1; and Carey et al., unpublished data). Rats were killed, the brains rapidly removed and immersion fixed (4% paraformaldehyde in 0.1 M phosphate buffer), frozen, and 10-μm frontal sections cut. Sections were washed with TBS at room temperature and incubated overnight at room temperature with the primary polyclonal lipocortin-1 fragment antiserum (1:100, with TBS containing 0.3% Triton X-100). Immunoreactivity was visualized using FITC-labeled secondary antiserum incubated for 1 h at room temperature. Control sections were incubated with lipocortin-1 fragment antiserum (1:100) preabsorbed with an excess of lipocortin-1 fragment (10^{-6} M, 22 μg/ml) and showed no lipocortin-1 immunoreactivity.

Experimental Protocol. 10 min after MCA occlusion, rats were injected intracerebrally with either lipocortin-1 fragment (human

recombinant lipocortin-1, NH_2-terminal 1–188 amino acids) that had been expressed in *Escherichia coli* (2) at a dose of 1.2 μg in 3 μl PBS or sterile vehicle (0.9% saline) alone (controls) via a previously implanted indwelling guide cannula.

In separate experiments, animals were injected intracerebroventricularly with either antiserum to the lipocortin-1 fragment or nonimmune serum (3 μl) using the same protocol as above. Animals were killed either 2 or 24 h after surgery for assessment of infarct volume, after 2 h for cerebral edema, or after 7 d for lipocortin distribution.

Statistical Analysis. Values are presented as means ± SEM. Statistical differences were assessed by Student's t test for unmatched data using two-tailed probabilities throughout.

Results

MCA occlusion resulted in reproducible and extensive damage to the striatum and cortex (assessed from tetrazolium staining of brain slices), measured 2 h after surgery. No
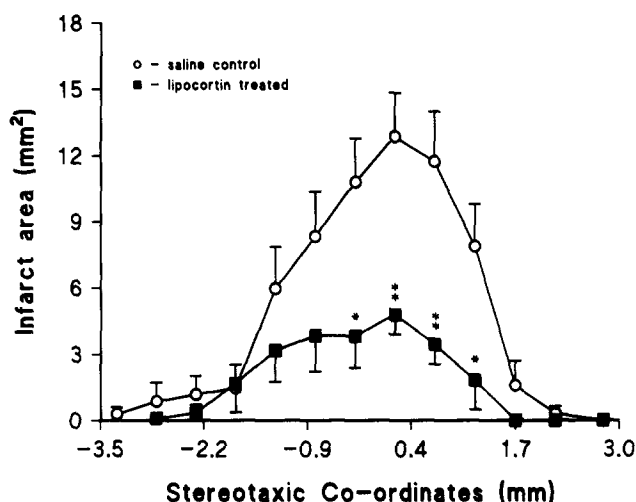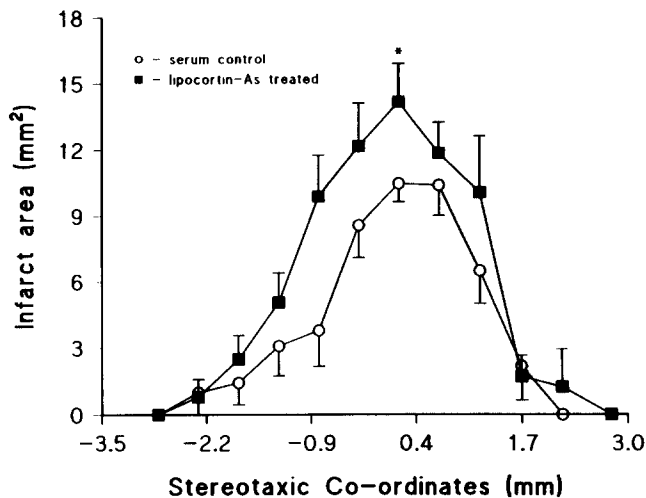

Figure 1. Effect of lipocortin-1 fragment on infarct size. Animals were injected intracerebroventricularly with either saline (3 μl/rat, n = 10) (*circles*) or lipocortin-1 fragment (1.2 μg in 3 μl/rat, n = 10) (*squares*) 10 min after MCA occlusion and killed 2 h later to assess infarct area (mm^2). Mean ± SEM, * = $p < 0.05$, ** = $p < 0.01$.
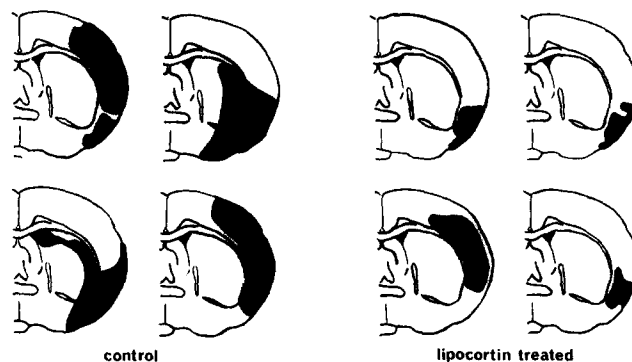
Figure 2. Effect of lipocortin-1 fragment on infarct size. Examples taken of coronal sections taken just anterior to bregma from brains of animals in the saline-treated (n = 4) and lipocortin-1 fragment–treated (n = 4) groups 2 h after MCA occlusion (see Fig. 1).

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[1] *Abbreviation used in this paper:* MCA, middle cerebral artery.

Figure 3. Effect of lipocortin-1 fragment antiserum on infarct size. Animals were injected intracerebroventricularly with either nonimmune serum (3 μl/rat, n = 10) (circles) or lipocortin-1 fragment antiserum (3 μl/rat, n = 10) (squares) 10 min after MCA occlusion and killed 2 h later to assess infarct area (mm^3). Mean $\pm$ SEM, * = p < 0.05.

damage was observed in any sham-operated animals (data not shown). A single intracerebroventricular injection of lipo-cortin-1 fragment (1.2 μg/rat), 10 min after the induction of cerebral ischemia, caused marked inhibition of this damage assessed 2 h after surgery (Figs. 1 and 2). The total volume of the infarct was reduced by 60% (control, 32.03 $\pm$ 4.3 vs. lipocortin, 13.32 $\pm$ 3.6 mm^3, n = 10, p < 0.05). A very similar pattern of infarction, although more extensive, was observed when damage was assessed 24 h after MCA occlu-

sion. Lipocortin-1 fragment, administered 10, 30, and 60 min after ischemia, caused reductions in infarct volume assessed 24 h later of 53%, 39%, and 27%, respectively (10 min control; 80.53 $\pm$ 13.2 vs. lipocortin; 37.9 $\pm$ 6.1 mm^3, n = 10, p < 0.05), (30 min control; 94.79 $\pm$ 10.7 vs. lipocortin; 58.28 $\pm$ 9.6, n = 10, p < 0.05), (60 min control 66.82 $\pm$ 10.3 vs. lipocortin; 48.77 $\pm$ 12.2, n = 10).

In a separate experiment, intracerebroventricular injection of antiserum to lipocortin-1 fragment, significantly increased (53%) the extent of neuronal damage (control; 22.58 $\pm$ 2.4 vs. lipocortin-As; 34.6 $\pm$ 4.1 mm^3, n = 10, p < 0.05) assessed 2 h after the induction of ischemia (Fig. 3).

Cerebral edema was assessed from tissue density, and expressed as the difference between the infarcted (left) and the undamaged (right) hemisphere sections. Tissue density measurements for undamaged right hemisphere sections were almost identical to values for both left and right hemisphere samples from unoperated control animals (data not shown). In animals subjected to MCA occlusion and injected with either saline or nonimmune serum, significant edema was apparent within 2 h. Injection of the lipocortin-1 fragment (1.2 μg/rat) caused significant (46%) inhibition of edema without affecting the uninjured tissue (Fig. 4 a) (control; 1.81 $\pm$ 0.25 $\times$ 10^{-3} vs. lipocortin; 0.98 $\pm$ 0.18 $\times$ 10^{-3} gH$_2$O/ml, n = 10, p < 0.05).

In contrast, administration of lipocortin antiserum (3 μl/rat) enhanced the extent of edema in the infarcted hemisphere by 29% (Fig. 4 b) again, without affecting the uninjured tissue (control; 1.68 $\pm$ 0.12 $\times$ 10^{-3} vs. lipocortin-As; 2.15 $\pm$ 0.32 $\times$ 10^{-3} gH$_2$O/ml, n = 6).

Immunohistochemical examination of brain tissue, 7 d after the induction of cerebral ischemia, revealed a marked increase
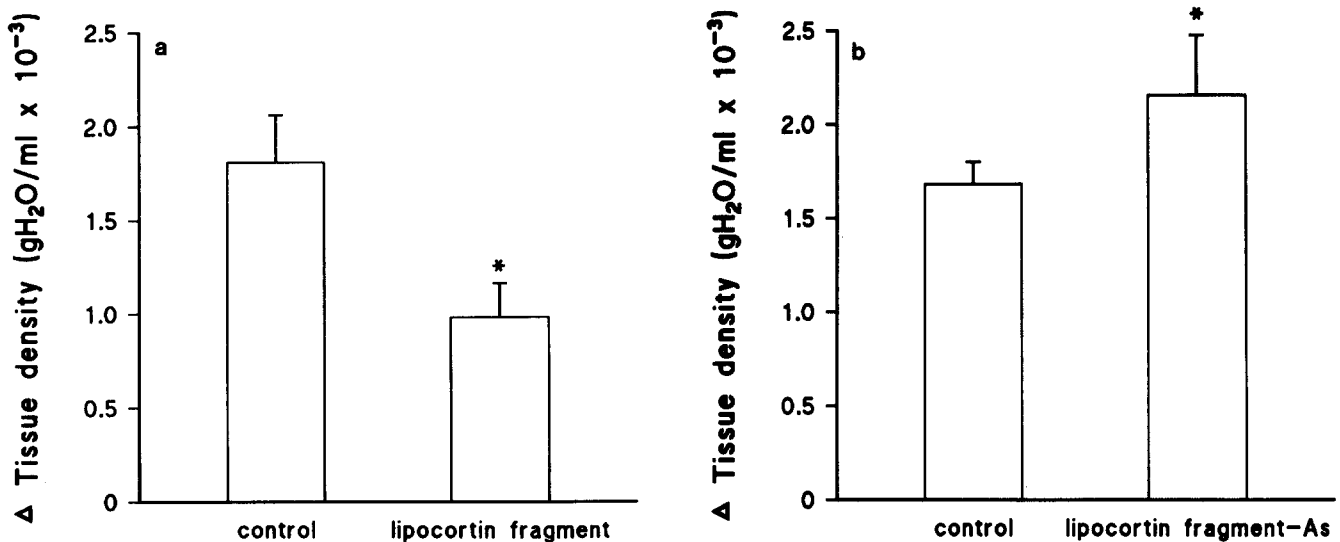
Figure 4. (a) Effect of lipocortin-1 fragment on cerebral edema. Animals were injected intracerebroventricularly with either saline (3 μl/rat, n = 10) or lipocortin-1 fragment (1.2 μg in 3 μl/rat, n = 10) 10 min after MCA occlusion. Results are expressed as the difference in tissue density (gH$_2$O/ml $\times$ 10^{-3}) between left (damaged) and right (undamaged) hemisphere samples 2 h after MCA occlusion. Mean $\pm$ SEM, * = p < 0.05. (b) Effect of lipocortin-1 fragment antiserum on cerebral edema. Animals were injected intracerebroventricularly with either nonimmune serum (3 μl/rat, n = 6) or lipocortin-1 fragment antiserum (3 μl/rat, n = 6) 10 min after MCA occlusion. 2 h later, animals were killed and tissue density assessed. Mean $\pm$ SEM, * = p < 0.05.
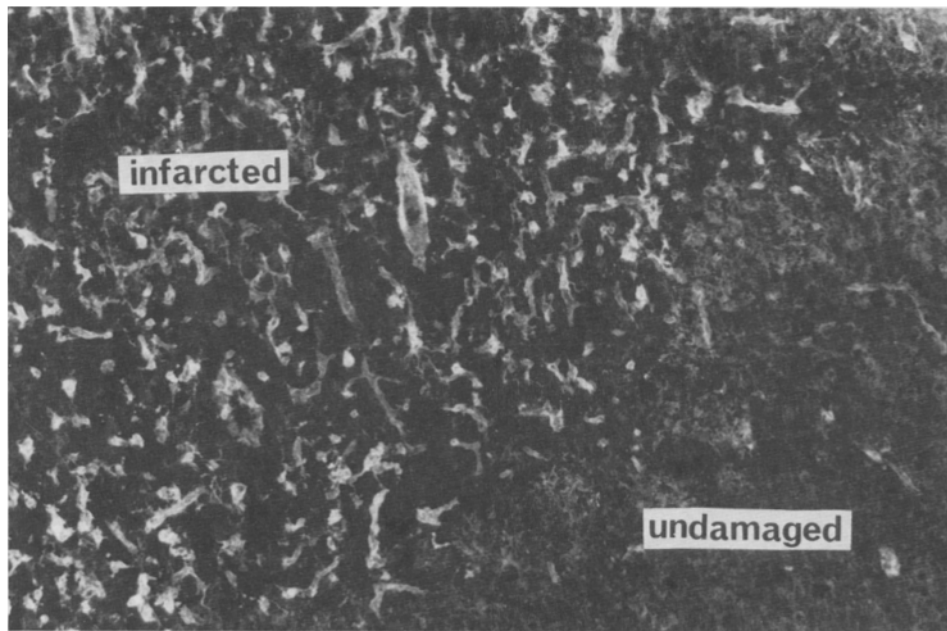
Figure 5. Immunohistochemical staining of lipocortin-1 in infarcted tissue 7 d after MCA occlusion. Light staining indicates the presence of immunoreactive lipocortin-1 in the area of infarction.

in lipocortin-1 immunoreactivity in infarcted areas of cortex compared to undamaged tissue (Fig. 5). No changes in lipocortin-1 distribution were observed in the brains of sham-operated animals.

Discussion

The results of this study demonstrate that a recombinant fragment of lipocortin-1 is a potent inhibitor of neuronal damage and edema after cerebral ischemia in the rat, and further suggest that endogenous lipocortin-1 may be induced during ischemia and act to limit subsequent damage.

Research on cerebral ischemia is dependent upon the use of surgical procedures in laboratory animals that are presumed to have some direct relevance to clinical conditions of ischemia. The protocol carried out in the present study, unilateral MCA occlusion in the rat, is widely recognized as one of the most reliable, reproducible, and relevant experimental models of focal ischemia currently practiced (see reference 16). Animals regain consciousness rapidly, with no overt behavioral defects, and the location and type of damage is comparable to that seen after ischemia in humans. A variety of methods have been used to assess the extent of neurological damage resulting from cerebral ischemia in experimental animals, some of the most common of these are blood flow or tissue density measurement, behavioral evaluation, and histological assessment. Tetrazolium chloride is a histochemical indicator of mitochondrial respiratory enzymes, and was used in the present study because of its ability to reliably delineate areas of brain infarction at a gross anatomical level using fresh, unfixed tissue samples (17). The percoll density gradient used in this study is an adaptation of that described by Marmarou et al. (15), and applies the specific gravity method to quantify the extent of cerebral edema. This type of gradient is uniform and linear, and offers advantages over more conven-

tional techniques involving weighing and drying since it is both less time consuming and more precise.

The recombinant fragment of lipocortin-1 has similar biological activity to the full molecule both in vitro and in vivo (18). However, in studies to directly compare the effects of the two molecules, we have demonstrated that the fragment apparently has greater activity when administered intracerebrally, probably due to improved availability to the brain, and has the additional advantage of greater stability (18). The dose of the fragment used in the present study was based on that which is maximally effective at inhibiting the central actions of cytokine-induced fever and hypermetabolism (11). However, since the site of action on ischemic brain tissue is probably some distance from the third ventricle, it is likely that higher doses, different sites of injection, or times of administration may be more effective. The actions of many anti-ischemic agents are improved considerably by administration before the insult, and in some cases this is the only means of obtaining significant effects (e.g., reference 19). We have observed potent effects on infarct size when lipocortin-1 fragment is injected 10 min after the induction of ischemia, and it is possible that further protection may be afforded by pretreatment of animals with this molecule. Furthermore, the results obtained from MCA-occluded animals 24 h after a single dose of lipocortin-1 fragment demonstrate that the protective effect of lipocortin does not appear to be a delay in the manifestation of the neurological consequences of cerebral ischemia, but rather a permanent inhibition of the development of neuronal damage. The chronic effects of lipocortin-1 fragment beyond 24 h after MCA occlusion have not yet been studied.

The antilipocortin-1 fragment antiserum used in this study is able to bind and neutralize both the fragment itself and the full recombinant molecule (11). Western blot analysis indicates that it is also able to bind to endogenous lipocortin

from the rat brain (Carey et al., unpublished data). It is therefore reasonable to assume that the potentiating effects of this antiserum on neuronal damage and cerebral edema after MCA occlusion is a result of the inhibition of endogenous lipocortin within the rat brain. The observed increase in lipocortin-like immunoreactivity in infarcted brain tissue (Fig. 5), although preliminary, suggests that some factor associated with ischemia stimulates the synthesis of the molecule, which may then serve as an endogenous inhibitor of neuronal damage. We have also observed an increase in lipocortin-like immunoreactivity around the site of mechanical brain injury in the rat (Strijbos et al., unpublished data).

Glucocorticoids are potent inducers of lipocortin-1 in several tissues and cell lines (4). However, the synthetic glucocorticoid dexamethasone phosphate apparently does not modify lipocortin-1 content of rat brain (10). These observations, together with the knowledge that glucocorticoids offer little or no protection against ischemic brain damage (20),

indicate that the actions of lipocortin described here are not related to glucocorticoid status or effects.

The mechanisms involved in the protective effect of lipocortin-1 on ischemic tissue are unknown. Inhibition of arachidonic acid release by lipocortin could decrease the synthesis of eicosanoids and inhibit the release and actions of the excitatory amino acid glutamate (21, 22), both of which may limit ischemic damage. Inhibition of the rise in intracellular calcium, which is central to the chain of ischemic damage, offers another possible site of action of lipocortin.

Research directed towards the treatment of stroke has, until now, focussed on the inhibition of the mediators of neuronal damage. The results presented here indicate that lipocortin-1 is an endogenous inhibitor of damage, and as such may offer a new avenue for therapeutic intervention. It is postulated that lipocortin may also be beneficial in myocardial ischemia and other forms of neurodegeneration.

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