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The local anti-inflammatory action of dexamethasone in the rat carrageenin oedema model is reversed by an antiserum to lipocortin 1

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1 A local pre-injection of 1 μg dexamethasone sodium phosphate strongly inhibited (>60% inhibition at 3 h; $P<0.001$ at all time points) the development of carrageenin-induced paw oedema in the rat induced by a subplantar injection of 0.1 ml, 2% carrageenin.

2 Coinjection of a polyclonal rabbit antiserum raised against human 1-188 recombinant lipocortin 1, which also recognised the rat protein, reversed the inhibitory action of dexamethasone ($P<0.05$ at 4 h and 5 h). At the highest volume used (40 μl) control antisera were without any effect.

3 These data further support the concept that lipocortin 1 is involved in the anti-inflammatory mechanism of action of the glucocorticoids.

Keywords: Lipocortin 1; dexamethasone; carrageenin oedema

Introduction

Lipocortin 1 (also called annexin 1) is a member of a super family of proteins (Pepinsky *et al.*, 1988) characterized by the presence of multiple copies of a 17 amino acid motif thought to be responsible for the ability of these proteins to bind negatively charged phospholipids and calcium (Saris *et al.*, 1986).

Lipocortin 1 is induced by glucocorticoids in several systems both *in vivo* (Fuller & Verity, 1989; Ambrose & Hunninghake, 1990; Goulding *et al.*, 1990a) and *in vitro* (Errasfa *et al.*, 1985; Piltch *et al.*, 1989; Browning *et al.*, 1990; Solito *et al.*, 1991). Administration of purified human recombinant lipocortin 1 produces anti-inflammatory and anti-pyretic effects in rats and rabbits (Cirino *et al.*, 1989; Carey *et al.*, 1990; Davidson *et al.*, 1991), eicosanoid suppressive actions in cells *in vitro* (Cirino *et al.*, 1987; Cirino & Flower, 1987) and ameliorates ischaemic damage (infarct size and cerebral oedema) produced in rat brain by occlusion of the middle cerebral artery (Relton *et al.*, 1991).

In some systems neutralising antibodies to lipocortin 1 have been shown to reverse the actions of glucocorticoids. For example, Violette *et al.* (1990) were able to abrogate the effect of dexamethasone on squamous carcinoma cell differentiation by addition of the neutralising anti-lipocortin 1 monoclonal antibody 1A. Likewise, Carey *et al.* (1990) observed that the inhibitory action of dexamethasone on interleukin 1β-induced thermogenesis in the rat was reversed following an intracerebroventricular injection of a polyclonal rabbit antibody raised against 1-188 lipocortin 1. Relton and her colleagues (1991) also observed that this antibody exacerbated the ischaemic damage to the rat brain, pointing to a protective effect of endogenous lipocortin 1.

The rat hind paw carrageenin oedema model is a popular acute model of inflammation which is very sensitive to the inhibitory action of both local and systemic steroids as well as to lipocortin 1 (Cirino *et al.*, 1989). Here we show that the local inhibitory action of dexamethasone is abrogated by the anti-lipocortin 1 1-188 antiserum but not by sera drawn randomly from a panel of control rabbits. The data support the concept that exogenous steroids exert part of their anti-inflammatory effect through the induction of extracellular lipocortin 1.

Methods

Male Wistar rats (150–200 g) were purchased from Olac (U.K.) and retained in the animal unit for one week before use. They were transferred to the procedure room at least 24 h before the start of the experiment and accustomed to the experimental protocol. Hind paw oedema was induced by the injection of 0.1 ml carregeenin (2% w/v in sterile saline) and the paw volume measured hourly by plethysmometry for 5 h as previously described (Cirino *et al.*, 1989) using a hydroplethysmograph (model 7150, Ugo Basile, Camerio, Italy). Dexamethasone (1 μg: Decadron; MSD, Hoddesdon, UK) and/or antisera was injected locally in a volume of 0.05 ml, 10–15 min before the carrageenin injection. Oedema was expressed as volume (ml) increase above original volume of the paw as measured immediately before the first injection.

The lipocortin antiserum was raised in rabbit to human recombinant lipocortin 1 residues 1-188 which had been expressed in *E. coli* as previously described (Carey *et al.*, 1990).

SDS-PAGE and immunoblotting was performed by conventional techniques and lipocortin 1 was visualised using a highly specific antisera (Ab842) which showed no cross reactivity with other members of the lipocortin family (J. Browning, personal communication). Both full length human recombinant lipocortin 1 and Ab 842 were kindly supplied by Dr J. Browning, Biogen Research Inc., Cambridge, U.S.A.

Statistics

Student's t test (unpaired) was used for comparisons of individual data points when comparing control with dexamethasone-treated rats and when comparing antiserum and treated rats with vehicle-treated controls.

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Results

In a series of preliminary experiments we established a dose-response curve to locally injected dexamethasone in the rat paw. Injection of 0.1 μg dexamethasone was without effect whereas injection of 10 μg per paw produced a near maximal (>85%) inhibition. For the purposes of this experiment a maximal effect is not desirable and therefore we reduced the dose to 1 μg per paw which produced significant ($P<0.001$) inhibition at all time points > 1 h with an approximate inhibition of 65% (at the time of peak oedema) or 60% (area under the curve).

Figure 1 shows the effect of coinjection of 10, 20 and 40 μl of the rabbit polyclonal antiserum which recognises 1–188 lipocortin 1. The antiserum reverses the effect of the dexamethasone although the effect does not show a graded response to different doses. In practice 40 μl produced the maximum inhibition possible whilst 5 μl (data not shown) was without effect.

To determine whether this was a function of normal rabbit serum we tested a panel of 5 other batches of non-immune rabbit serum drawn at random from our colony. All were without effect at the highest dose used (40 μl). Figure 2 shows a matched experiment in which 40 μl of a control antiserum was contrasted with 40 μl of immune antiserum. Inhibition in the presence of the control antiserum was not significantly different from the dexamethasone alone, whilst 40 μl of immune antiserum almost completely abrogated the action of dexamethasone.

From Figures 1 and 2 it can be seen that both control and anti-lipocortin 1 antiserum appeared to enhance the oedematous response during the first hour. To test whether this was an effect of mast cell degranulation caused by serum alone, we performed an experiment in which rats were treated with 10 μg methysergide simultaneously with 20–40 μl serum injection into the paw. The treatment reduced substantially the oedema produced by serum injection at 30 min from 0.44 ± 0.03 ml ($n = 5$) to 0.14 ± 0.03 ml ($n = 5$), when 40 μl of serum was injected and from 0.30 ± 0.03 ml ($n = 5$) to 0.14 ± 0.05 ml ($n = 5$) when 20 μl was injected.

To ensure that the rabbit polyclonal anti-human recombinant lipocortin 1 1–188 antiserum recognised rat lipocortin, we performed a simple test in which we compared the ability of this antiserum and another highly specific antiserum (Ab842) to recognise rat lipocortin extracted from lavage macrophages and used Western blotting analysis. Both antibodies recognised an identical pattern of products in the rat macrophage extracts with a major band at 36 kDa (data not shown) corresponding to the native species of lipocortin 1.

The ability of the rabbit polyclonal antiserum to reduce the activity of lipocortin 1 could be caused by proteolytic activity in the serum. To eliminate this possibility we conducted an experiment in which immune and non-immune serum was incubated together with samples of authentic human lipocortin 1 for 1 h at 37°C after which the samples were extracted and subjected to Western blotting analysis. Figure 3 shows the results of this experiment from which it can be seen that a small amount of metabolism occurred in all samples tested, but that there was no difference between the immune and control sera in this respect.

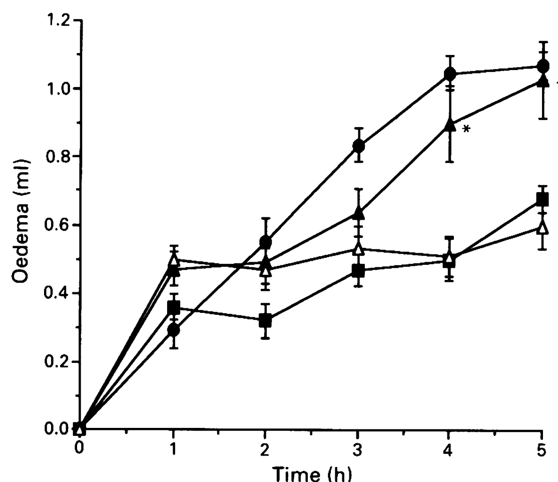

Figure 2 Dexamethasone inhibition of carrageenin-induced rat paw oedema: reversal by anti-lipocortin 1 antibody and lack of effect of control antiserum. Dexamethasone (1 μg) (■) or saline vehicle (●) was pre-injected with 40 μl anti-lipocortin 1 antiserum (▲) or non-immune rabbit serum (Δ) 15 min before carrageenin, $n = 4$. *Significantly different from dexamethasone alone ($P<0.05$).

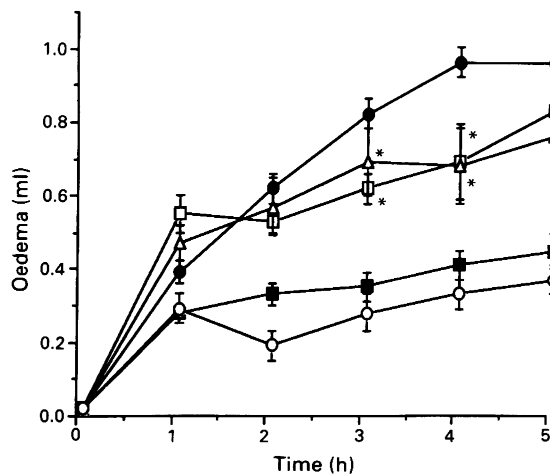

Figure 1 Inhibition of carrageein-induced rat paw oedema by local dexamethasone and reversal by anti-lipocortin 1 antisera. Dexamethasone (1 μg) (■, $n = 26$) or saline vehicle (●, $n = 27$) was pre-injected with 10 μl (O, $n = 9$), 20 μl (Δ, $n = 10$) or 40 μl (□, $n = 10$) of antibody or vehicle 15 min before carrageenin, and the paw volume recorded for 3 h. Vertical bars indicate s.e.mean, *significantly different from dexamethasone alone ($P<0.05$).

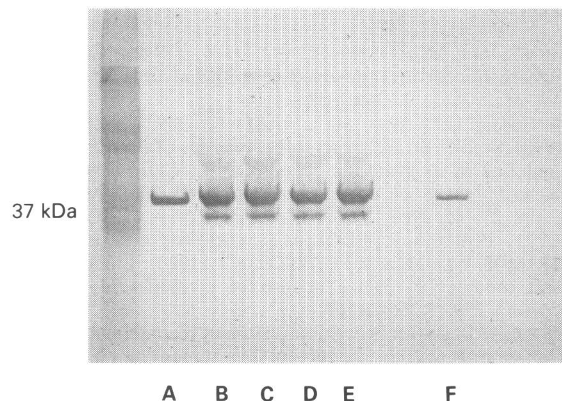

Figure 3 A Western blot shows the degree of proteolytic degradation of lipocortin 1 by the antisera used in this experiment.

Extreme left hand lane molecular weight markers. Lanes (A) = lipocortin 1 + buffer only; (B) = lipocortin 1 + anti lipocortin antiserum (C–E) = non immune control sera. (F) = lipocortin 1 standard, not incubated.

Discussion

The classical approach to the investigation of the function of endogenous compounds is the use of antagonists or antibodies. In the absence of antagonists to the protein lipocortin 1, one is compelled to rely upon experiments with immunological reagents which recognise the purified protein. Data described in this paper using this approach strongly suggest that the acute local anti-inflammatory action of dexamethasone is at least partially dependent upon the generation of lipocortin 1.

Carey *et al.* (1990) were able successfully to demonstrate that the same antiserum as was used in this study, reversed the action of dexamethasone as an anti-pyretic agent in the rat. Lipocortin 1 itself was active as an antipyretic in this model. Lipocortin 1 was also protective in the ischaemic brain damage model of Relton *et al.* (1991) and central administration of the same antiserum exacerbated the damage caused by ischaemia, suggesting a protective role for endogenous lipocortin 1.

Other antisera to lipocortin 1 have also been shown to reduce or block the action of glucocorticoids. Violette *et al.* (1990) demonstrated that hydrocortisone caused the terminal differentiation of a human squamous cell carcinoma line and that this effect was neutralised by the co-presence of a monoclonal anti-lipocortin antibody but not by a non-neutralising anti-lipocortin 1 monoclonal (i.e. an antibody which binds the protein but does not prevent its biological effect). A similar phenomenon was observed by Croxtall & Flower (1992) in which dexamethasone-induced inhibition of A549 cell proliferation was blocked in the presence of the monoclonal anti-lipocortin 1 antibody 1A.

Before the advent of purified recombinant human lipocortin 1 and the appropriate antisera, some highly suggestive experiments which pointed in the same direction were performed with the monoclonal antibody RM23 (Flower *et al.*, 1984) raised against the 'anti-phospholipase' activity generated by steroid treatment of the rat macrophage. This factor was almost certainly a member of the lipocortin family and RM23 inhibited the reduction in macrophage prostaglandin synthesis by hydrocortisone and reversed the local anti-inflammatory effect of dexamethasone in the rat carrageenin-induced pleurisy model.

Although highly suggestive these results could not be taken as definitive since the actual nature of the protein target recognised by the antibody was not completely characterized. We have ensured in these experiments that the rabbit anti-human 1–88 lipocortin 1 antibody does in fact recognise the rat protein. The protein sequence of rat and human lipocortin 1 is almost 90% homologous (Kovacic *et al.*, 1991) so such a cross-reactivity is probably to be anticipated.

The idea that externally applied antisera can reverse the action of hormones such as the glucocorticoids implies strongly that the lipocortin 1 target is extracellular. This indeed is congruent with other evidence. For example the external application of lipocortin to cells or its intravenous administration to animals (Cirino *et al.*, 1989; Carey *et al.*, 1990; Davidson *et al.*, 1991) produces a prompt and dramatic effect on eicosanoid generation, inflammation or fever. Furthermore, the existence of a protein binding site with a high affinity for lipocortin has been detected on the surface of some human cells with the obvious implication that this is a receptor or a target enzyme (Goulding *et al.*, 1990b).

The injection of serum itself, in doses of 20–40 μl, into the carrageenin-inflamed paw caused a non-significant but repoducible increase in the oedema responses at the 1 h time point. This seemed to be caused by mast cell degranulation as a small oedema produced by injection of the serum alone was blocked substantially with methysergide.

As lipocortin 1 produces many effects by acting on the outside of cells it is susceptible to the action of proteases and indeed treatment of cells with proteases has been reported to abrogate the actions of steroids in some systems (Hirata *et al.*, 1980). To exclude the fact that our rabbit serum contained proteolytic activity which might destroy lipocortin, we compared the ability of control and immune serum to degrade authentic human lipocortin 1. There was no apparent difference in the ability of the sera to degrade this protein over a long period of incubation and indeed only a fraction of the protein was degraded at all. This argues against the possibility of non-specific proteolytic mechanisms.

The glucocorticoids can alter the expression of almost 1% of the mammalian genome and it is therefore only to be expected that they have pleiotropic actions on the inflammatory response. Nevertheless it should be possible to identify key effectors which mediate particular aspects of their action. We believe lipocortin 1 to be one of these effector proteins and consider that the evidence presented in this paper further strengthens this concept by demonstrating the specific reversing activity of the antisera against local administered dexamethasone.

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