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INDUCTION OF LIPOCORTIN 1 BY TOPICAL STEROID IN RAT SKIN

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Abstract—Western blotting and densitometric analysis of extracts obtained from EDTA extraction of skin segments showed greater extracellular Lipocortin 1 (LC1) in skin sites from steroid-treated animals compared to that seen in matched vehicle treated animals. Extracellular LC1 was maximal 3 hr after steroid, less was found in skin after 6 hr and levels had returned to basal at 18 hr. Pre-treatment of rats with the glucocorticoid receptor antagonist RU38486 (20 mg/kg) prevented the steroid induction of extracellular LC1 at both the 3 and 6 hr time-points. Systemic treatment of rats with betamethasone sodium phosphate (0.1–1 mg/kg) showed that the induction of LC1 on the cell surface was both time- and dose-dependent. Oedema in rat skin caused by 5-hydroxytryptamine (5-HT), platelet activating factor (PAF) and zymosan activated serum (ZAS) was assessed using ^{125}I-labelled human serum albumin. Following a 3 hr topical treatment with betamethasone-17-valerate the inflammatory activities of all of the tested stimuli were significantly attenuated demonstrating that at this time-point the topical steroid was biologically active. Topical steroid treatment of the skin resulted in a translocation of LC1 to the cell surface, which was maximal after a 3 hr period and was also temporally associated with the anti-inflammatory effect of these agents.

Key words: glucocorticoid; Lipocortin 1; RU38486; skin oedema

It is believed that the anti-inflammatory effects of the glucocorticoids are brought about in the same manner as the physiological effects of other steroid hormones, i.e. by the control of gene expression and ultimately the synthesis or suppression of regulatory proteins. Although the anti-inflammatory effects of these substances cannot be attributed to a single mechanism of action of the glucocorticoids, much attention has been focused upon one of the possible mediators of these effects, the glucocorticoid-inducible 37 kDa protein LC1‡ (see [1]).

LC1 is a member of a family of proteins possessing a highly conserved repeated 70 amino acid motif which confers calcium and phospholipid binding properties. LC1 inhibits the production of various prostanoids—mediators associated with the inflammatory response. It has been demonstrated that release of Thromboxane A_2 from perfused lungs *in vitro* [2] may be suppressed by LC1. Human recombinant LC1 inhibits carrageenin-induced oedema formation in the rat paw [3], a response known to be partially dependent upon the activity of newly synthesized prostanoids. The inhibitory action of LC1 has been, in part, attributed to an effect upon the activity of the enzyme phospholipase A_2 and therefore inhibition of the conversion of membrane phospholipids to arachidonic acid.

More recently however it has been found that LC1 has an effect upon the cellular component of certain inflammatory responses [4, 5]. Interestingly it has also been demonstrated that anti-serum raised against a fragment of LC1 was able to reverse the anti-inflammatory effects of dexamethasone in this model. This effect of LC1 upon inflammatory cells has been reiterated in other models. One particular study showed that the neutrophil accumulation stimulated following interleukin-1 administration into a mouse air pouch was attenuated following administration of both LC1 and glucocorticoids [5]. Probably more importantly it has been demonstrated that the inhibitory effect of the steroid dexamethasone in this model is prevented by prior treatment of animals with a neutralizing antibody to LC1, and thereby implicating LC1 in the anti-inflammatory effects observed here. Together these results indicate that at least some of the anti-inflammatory effects of the steroids may be due to the production of LC1 and its resultant effect.

Basal levels of LC1 have been identified in normal rat, porcine and human skin [6, 7]. At present there are no studies in the literature to suggest that the *topical* application of steroid results in an induction of LC1 synthesis or that this may be related to the anti-inflammatory actions of the topical steroids. Although it has been suggested that LC1 may in fact have a role to play in the pathogenesis of skin disease, such as psoriasis [7]. This group found that the localization of LC1 in diseased and normal skin differed and suggested that LC1 was translocated from the cytoplasm to the plasma membrane as psoriatic lesions were formed. In light of the above, we have investigated whether topical application of

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‡ Abbreviations: LC1, Lipocortin 1; 5-HT, 5-hydroxytryptamine; PAF, platelet activating factor; ZAS, zymosan activated serum; PMSF, phenylmethyl-sulphonyl fluoride; PGE_1, prostaglandin E_1.

steroid was related to any changes in both total content and localization of LC1 in the rat skin.

The topical application of steroid cream, betamethasone-17-valerate, results in an inhibition of oedema formation in response to several putative mediators of inflammation [8]. This group has now investigated whether the anti-inflammatory effects of topically applied steroid occurs in parallel with an induction of LC1 in the rat skin. The exact location of the biologically active pool of LC1 is unclear. There have been suggestions that LC1, which is EDTA-extractable, found on cell membrane surfaces after steroid treatment may be the most important pool of LC1 with respect to its anti-inflammatory properties [9]. In light of these suggestions the LC1 content of skin washes, containing EDTA for extraction, have been measured, as well as assessment of total content in whole skin homogenates following steroid treatment of rat skin, using the western blotting technique and densitometric analysis.

MATERIALS AND METHODS

Materials. PAF, ZAS, 5-HT, PGE_1, Evans blue, betamethasone-17-valerate, anti-sheep IgG peroxidase conjugate, diaminobenzadine, EDTA and PMSF were all purchased from Sigma Chemical Co. (Poole, U.K.) The rainbow coloured molecular weight markers were purchased from Amersham Laboratories (Amersham, U.K.) and ^{125}I-labelled human serum albumin from Amersham International PLC (U.K.). Acetone was obtained from BDH Chemical Co. (U.K.). Hypnorm (fentanyl/fluanisone) was obtained from Janssen Pharmaceuticals Ltd (U.K.) and Hypnovel (midazolam hydrochloride) from Roche Products Ltd (U.K.). Betamethasone sodium phosphate was purchased from Glaxo Group Ltd (U.K.). The glucocorticoid antagonist RU486 (mifepristone) was a generous gift of Dr R. Deraedt of Roussel-Uclaf, Romainville, France. The sheep LC1 polyclonal antibody was supplied by Dr Jamie Croxtall.

PAF and PGE_1 were made up in ethanol and 5-HT in sterile distilled water and all stored at −20°. Evans blue (1%) was made up in 0.9% NaCl (saline) and stored at room temperature. ZAS was prepared as described by Williams and Jose [13] and stored at −20°. All dilutions were made in sterile saline on the day of use. A suspension of RU38486 was made in sterile water on the day of use.

Steroid and steroid antagonist pre-treatment. Male Wistar rats (250–300 g) were anaesthetized using an anaesthetic cocktail comprised of hypnorm™: sterile water: hypnovel™ in a ratio of 1:2:1 at a dose of 4 mL/kg. Rats were shaved and a depilatory (Louis Marcel) cream applied to remove all of the remaining hair. A minimum of 1 hr was allowed to elapse prior to application of the steroid. After this time betamethasone-17-valerate (4 mg/mL, 500 μL) dissolved in acetone was pipetted evenly over a marked area (11 × 10 cm) on the back of the rat. The dose of steroid used in this study was comparable to amounts used for assessment in the vasoconstriction assay, the assay technique for topical steroids [10]. Vehicle and untreated controls were also carried out. The topical applications were applied to the rats 1–18 hr prior to experimental use.

Systemic steroid treatment involved administration of betamethasone sodium phosphate (0.1–1 mg/kg) either 1 or 3 hr prior to removal of skin samples.

In some experiments the steroid antagonist RU38486 (20 mg/kg) was given in suspension orally to conscious rats 20 and 2 hr prior to the experiment.

LC1 extraction. For total lipocortin measurements in rat skin, each skin biopsy was homogenized using an Ultraturrax homogenizer in PBS (2.8 mL) containing EDTA (10 mM) and PMSF (1 mM). Each sample was then centrifuged and the supernatant removed. From each rat three skin sites were taken and the homogenates pooled to represent one sample.

For determination of extracellular LC1 three skin segments, from each rat, were washed individually with PBS (0.5 mL) containing EDTA (2 mM) and PMSF (1 mM) and were pooled to represent one sample.

The protein content of each sample was determined using the Bradford protein assay technique [11] using a Coomassie blue dye and the light absorbance measured using a programmable ELISA plate reader (Anthos reader 2001, Anthos Labtec) at a wavelength of 570 nm. Estimation of protein content in each sample was made by comparison to a standard curve to albumin. To assess the LC1 content each sample was subjected to western blotting analysis.

Measurement of LC1 utilizing western blotting analysis. Samples were loaded, in volumes of not more than 20 μL on to slab gels for SDS–PAGE [12]. Following separation the proteins were electrotransferred onto nitrocellulose sheets (0.5 μm; Immobilon PVDF transfer membrane, Millipore). After incubation with a milk (5% w/v) blocking solution the sheets were incubated with a specific sheep antibody for LC1 (1:5000) overnight, followed by anti-sheep IgG (peroxidase-linked) (1:2000). The blots were developed using diaminobenzadine (0.05% w/v). The molecular weights of the immunoreactive bands were determined by comparison to the migration of molecular weight standards ranging from 14.4 to 200 kDa.

Densitometric analysis on each western blot (using a VIDS V densitometer, Meggitt Controls, Ai Cambridge Ltd) was carried out to allow quantification of the bands.

Oedema measurement. Male Wistar rats (250–300 g), were pre-treated (3 hr) with betamethasone-17-valerate, anaesthetized and 2.0 μCi/kg of ^{125}I-labelled human serum albumin in Evans blue solution (1%) was injected intravenously. Following this, eight different agents were injected intradermally into the rat skin in a volume of 0.1 mL, each agent in duplicate. These comprised of 5-HT (2.5×10^{-8} mol/site), PAF (5×10^{-8} mol/site) and ZAS (50% per site). After a period of 30 min blood was collected by intracardiac puncture and the animal killed with anaesthetic overdose. The skin was removed and each skin site punched out using a 17 mm diameter hole punch. Both skin sites and plasma samples, prepared by centrifugation of collected blood at 795 *g* for 5 min, were radioactivity assessed using a Beckman gamma counter.

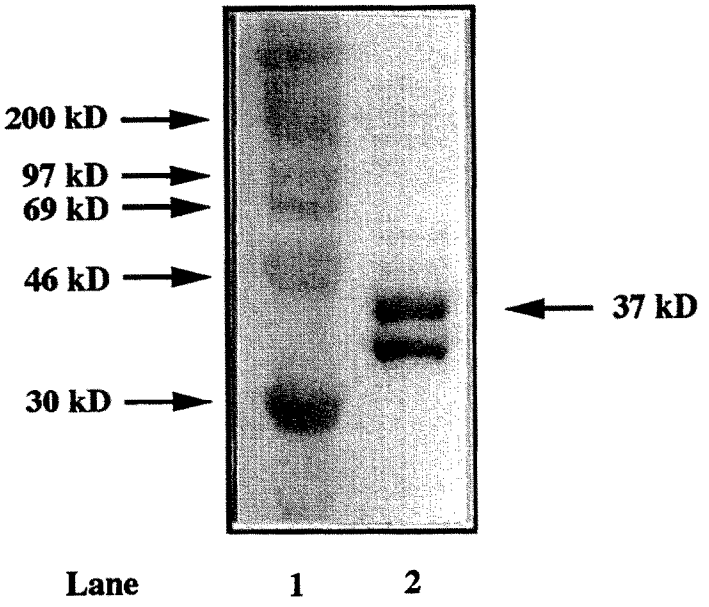

Fig. 1. Measurement of the molecular weight of LC1 by western blotting analysis using specific antibody raised against LC1 upon homogenized rat skin extract. Lane 1 shows the molecular weight marker and lane 2 the bands picked up by the specific antibody in a skin homogenate sample.

Data and statistical analysis. The densitometric data where required were subjected to parametric analysis using the Student's *t*-test for paired data. In some cases the densitometric values obtained from samples of animals treated with the steroid or its vehicle are expressed as a ratio of that achieved in untreated controls.

In the case of the oedema measurements the responses in the steroid treated animals were compared to those in the vehicle treated animals. Statistical differences between the two groups were determined with the use of the Students' *t*-test for unpaired data.

In both cases values were considered to be significantly different when $P < 0.05$.

RESULTS

LC1 measurement

LC1 was found to be present in homogenates of rat skin. The antiserum utilized for these experiments was found to detect not only intact LC1 (37–38 kDa) but also a 35 kDa metabolite (see Fig. 1). In agreement with Fava *et al.* [6], in whole skin homogenates, basal expression of LC1 in untreated animals with bands equivalent to native LC1 and a 35 kDa protein, being expressed following western blotting of whole skin homogenized extracts were found. On visual assessment treatment with the topical steroid demonstrated only very subtle differences in the total cellular LC1 content compared to control rats in agreement with densitometric analysis, as shown in Table 1; however, these differences did not reach statistical significance. There was also a slight increase above control of the cellular LC1 content in vehicle treated rats and in fact this was very similar to the increases noted with the steroid treatment (Table 1). This was most probably simply due to the fact that an external insult would result in a rise in LC1 content. The

Table 1. Densitometric measurement values of western blots measuring LC1 in rat skin samples

Pre-treatment (hr)	Total LC1		EDTA-extractable LC1	
	Steroid	Vehicle	Steroid	Vehicle
3	0.76 ± 0.13	1.09 ± 0.25	2.49 ± 0.75*	1.06 ± 0.27
6	1.24 ± 0.16	1.27 ± 0.08	1.73 ± 0.51	1.16 ± 0.06
18	1.43 ± 0.21	1.44 ± 0.18	1.16 ± 0.17	0.88 ± 0.08

Each value represents the mean ±SE of $N = 4$ in the case of the measurements for the homogenates and $N = 5$ in the case of the EDTA-extractable LC1 values. Each densitometric value is expressed as a ratio of that measurement given for the corresponding matched control, i.e. the response to the control was equal to 1. Statistical significance is indicated by * for $P < 0.05$.

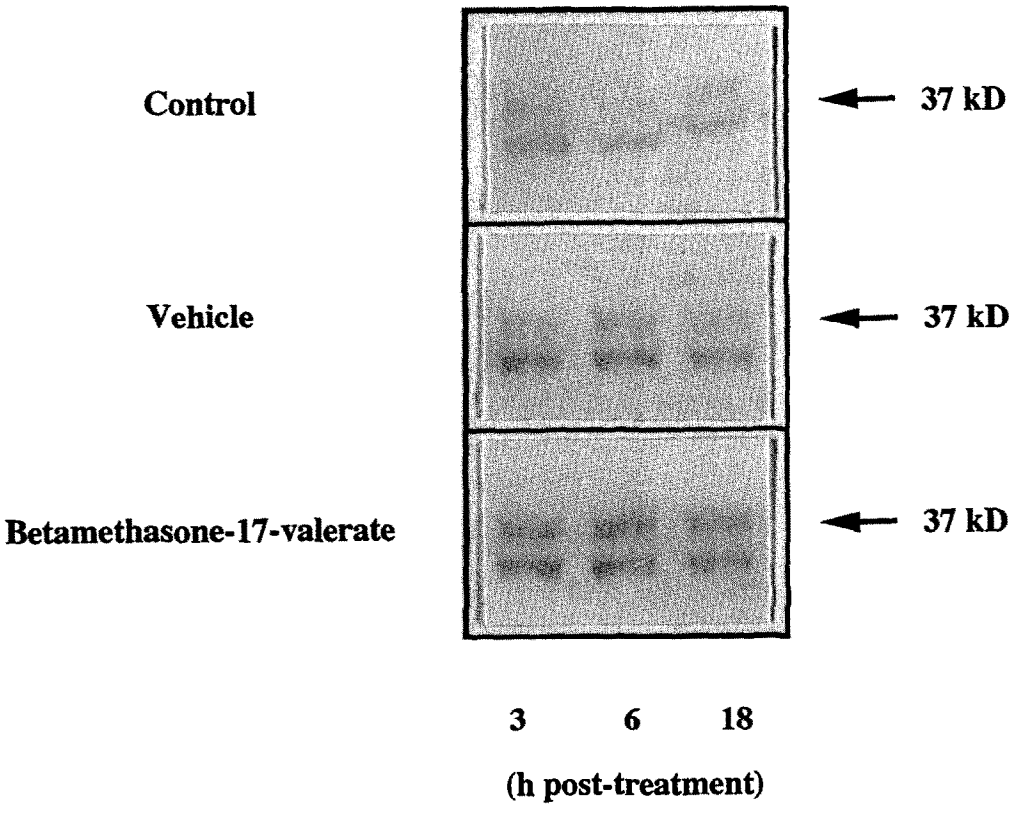

Fig. 2. The induction of EDTA-extractable LC1 in skin sites obtained from rats treated topically with the steroid betamethasone-17-valerate (2 mg dissolved in 500 μL of acetone evenly pipetted over the skin surface) over a time period of 3–18 hr after treatment. Skin sites were washed with buffer containing both EDTA (2 mM) and PMSF (1 μM) and these were then subjected to western blotting analysis. LC1 was identified by using a specific sheep antibody for LC1. Each sample represents the EDTA-washes obtained from three skin sites from each animal. Controls represent untreated animals.

procedure of removing the hair and applying the steroid or vehicle itself appeared sufficient to produce such a change.

Betamethasone-17-valerate treatment resulted in an increase in an extracellular EDTA-extractable pool of LC1 above that obtained in vehicle treated or control untreated skin samples. Western blotting of samples from the control untreated animals showed that little LC1 exists in this particular pool in comparison to steroid treated skin (e.g. see Fig. 2). This was further emphasized with densitometric analysis, shown in Table 1. The ratios of the densitometric readings for treated compared to untreated skin shows that at 3 hr the amount of LC1 was at a maximum and decreased over time with there being less present after 6 and 18 hr, the 3 hr time-point was found to be statistically different to that achieved in controls ($P < 0.01$, $N = 5$) and significantly different from vehicle-treated skin ($P < 0.05$). A single experiment to determine if the maximum LC1 recovery occurred earlier than 3 hr showed this not to be the case, confirming the 3 hr time-point as the maximum. As with whole-skin homogenates, the vehicle did have some effect upon cell surface LC1 above that achieved in control untreated animals, with slight, but insignificant, increases above untreated control occurring at 6 and 18 hr post-treatment.

Figure 3 shows a typical western blot demonstrating that pre-treatment of animals with the steroid receptor antagonist resulted in a decrease of extracellular LC1 content in skin treated with steroid and RU38486 in comparison to skin taken from animals treated with the steroid only. This treatment appeared to block the LC1 translocation at both the 3 and 6 hr time-points ($N = 4$). Densitometric analysis of bands from antagonist treated animals gave values of 0.47, 0.32, 1.5 and 0.93 when expressed as a ratio of the readings obtained from the same western blot for betamethasone treatment after 3 hr. Analysis at the 6 hr time-point, expressed in the same manner as that for the 3 hr time-point, gave values for the antagonist treated animals of 0.62, 0.63, 0.98 and 0.55. Statistically significant differences were achieved only at the 6 hr time-point, giving $P < 0.05$.

Systemic treatment of animals with betamethasone sodium phosphate (0.1–1 mg/kg) resulted in both a dose- and time-dependent increase in the extracellular LC1 found in the skin (for typical responses

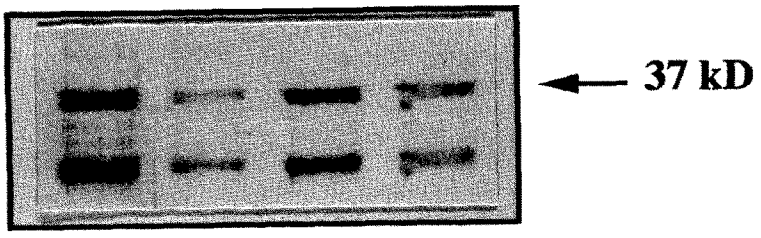

Fig. 3. Typical inhibition of steroid-induced EDTA-extractable LC1 induction using the steroid receptor antagonist RU38486 (20 mg/kg 20 and 2 hr prior to steroid treatment) in rat skin, treated topically with the steroid betamethasone-17-valerate. Skin sites were washed with buffer containing EDTA (2 mM) and PMSF (1 μM) and these were then subjected to western blotting analysis. LC1 was identified using a specific sheep antibody for LC1. Each sample represents the EDTA-washes obtained from three skin sites from each rat.

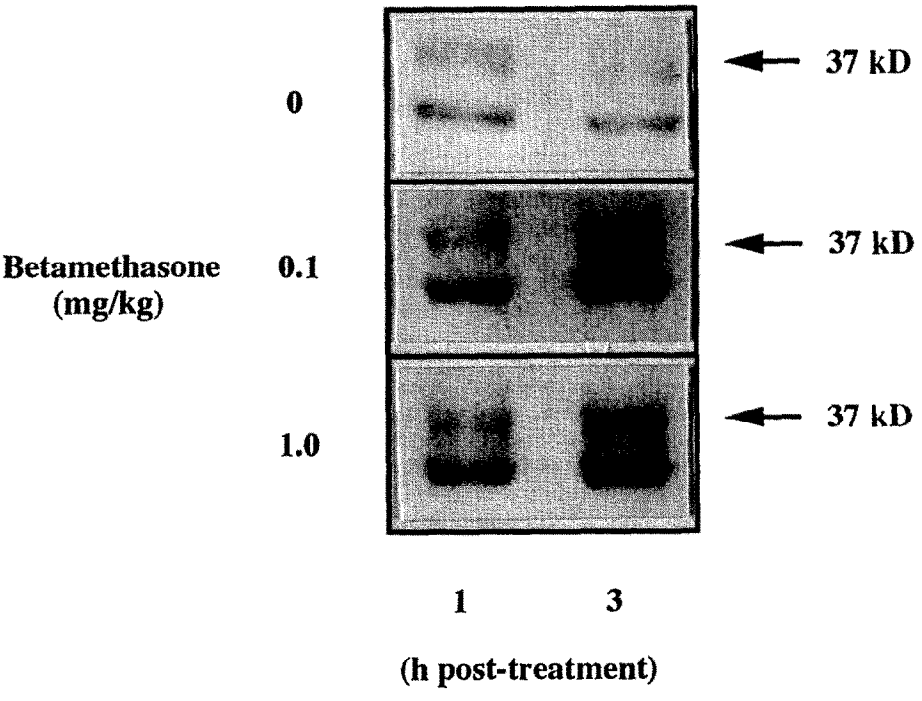

Fig. 4. A typical western blot showing the effect of systemic betamethasone sodium phosphate (0.1–1 mg/kg) given with a 1 and 3 hr pre-treatment upon EDTA-extractable LC1 in rat skin. Skin sites were washed with buffer containing EDTA (2 mM) and PMSF (1 μM) and these were then subjected to western blotting analysis. LC1 was identified using a specific sheep antibody for LC1. Each sample represents the EDTA-washes obtained from three skin sites from each rat. Controls represent saline treated animals.

see Fig. 4). Mean ratio values of 1.14 ± 0.3, 1.51 ± 0.46, 1.65 ± 0.14 and 1.91 ± 0.2 were obtained for treatments of 0.1 mg/kg at the 1 and 3 hr time-points, and 1 mg/kg at the 1 and 3 hr time-points, respectively ($N = 4$ in each case). Significant differences were achieved with the higher dose of 1 mg/kg giving $P < 0.05$ and 0.01 after 1 and 3 hr, respectively.

Oedema

Pre-treatment (3 hr) with topically applied betamethasone-17-valerate significantly ($P < 0.05$ for both 5-HT in the absence and presence of PGE_1 and PAF alone and $P < 0.01$ for PAF and ZAS in the presence of PGE_1 and ZAS alone, $N = 6$ in each case) inhibited the oedema formation to all of the stimuli tested (Fig. 4). The range of the inhibitory effect being from 35–48% inhibition of the control response. Saline alone produced a small response, 13.0 ± 2.5 μL of plasma ($N = 5$) in vehicle treated animals, which was significantly ($P < 0.05$) attenuated to 6.7 ± 1.1 μL in steroid treated animals ($N = 5$).

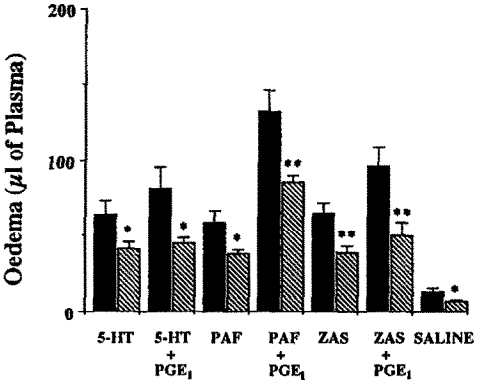

Fig. 5. The inhibitory effects of topically applied betamethasone-17-valerate (2 mg dissolved in 500 μL of acetone evenly pipetted over the skin surface, with a 3 hr pre-treatment) upon oedema formation induced by 5-HT (2.5×10^{-8} mol/site), PAF (5×10^{-8} mol/site) and ZAS (50% per site) given alone or in the presence of PGE_1 (3×10^{-10} mol/site). The open columns represent the responses in vehicle control treated animals and the filled bars the steroid treated animals. Responses are expressed as means (with SE mean shown by the vertical bars) of $N = 5$ rats. Significant inhibition of oedema formation was evaluated using the unpaired Student's *t*-test (two-tailed) and are indicated by * for $P < 0.05$ and ** for $P < 0.01$.

DISCUSSION

The mechanism of action of the topical steroids has remained unclear since their introduction for the treatment of skin disease in the early 1960s. Certain anti-inflammatory effects of the steroids have been attributed, at least in part, to the activity of a steroid-inducible protein called LC1. This study was carried out in an attempt to identify if an induction of LC1 occurred following topical steroid treatment in parallel with anti-inflammatory activity.

In agreement with previous findings LC1 was found to be present in the skin of control untreated animals and this suggests that endogenously produced glucocorticoids, or some other factor, at normal physiological levels result in the induction of lipocortin in the skin. In fact, Vishwanath *et al.* [14] demonstrated that adrenalectomy of rats resulted in a down-regulation of the messenger ribonucleic acid for LC1 and a decrease in the levels of the protein, demonstrating quite clearly that endogenous glucocorticoids are involved in the maintenance of normal physiological levels of LC1. Betamethasone-17-valerate treatment resulted in subtle changes in LC1 content in the homogenates of whole skin. However, the vehicle alone also appeared to increase the LC1 content above control. This was most likely due to the fact that the physical procedure of applying the vehicle itself resulted in a slight induction of this protein. The induction of intracellular LC1 has been found to occur even in adrenalectomized rats following the i.p injection of irritant [15]. No apparent differences between vehicle control and steroid-treated skin were seen in the total LC1 content of the homogenates at either 3 or 6 hr post-treatment with the steroid. These results suggest that this particular steroid treatment did not result in a net increase in LC1 formation in the skin.

EDTA extraction of skin segments yielded measureable quantities of LC1, as shown in Fig. 2. This suggests that LC1 may be situated on cell surfaces, an observation noted in several studies [9, 16, 17], as well as being found intracellularly. The possible significance of this "pericellular" pool was indicated by Croxtall and Flower [9], when it was suggested that inhibition by LC1 antibody of the anti-inflammatory effects of steroids demonstrated in several studies could be best explained by the existence of a biologically active pericellular pool of LC1. In these studies extracellular LC1 appeared maximum after 3 hr and then decreased over the next 15 hr. Therefore, although there appeared to be minimal differences in the LC1 content in the homogenates of vehicle and steroid treated samples at the earlier time-points in comparison to untreated controls, it did appear that the steroid treatment was having an effect upon LC1 disposition.

Experiments with the steroid-receptor antagonist RU38486 demonstrated that the apparent translocation of LC1 was a receptor-mediated event since in antagonist-treated animals the amount of EDTA-extractable LC1 found after topical steroid was reduced in comparison to animals not treated with RU38486. This also implies that the effects of the betamethasone-17-valerate in this model are not due to a non-specific effect of the vehicle or the steroid itself but rather due to specific activation of steroid receptors.

From Fig. 4 it also appears that systemic steroid treatment will result in a similar increase of the EDTA-extractable pool of LC1 and this was found to be both dose- and time-dependent, a classical feature of specific steroid action. Thus, it appears that systemic as well as topical steroid treatment may result in an increase in the expression of cell surface LC1.

Since LC1 was maximum at 3 hr following topical steroid treatment it was checked to see if in fact this change was also accompanied by biological activity, i.e. an anti-inflammatory effect. Figure 5 shows that this treatment had a profound effect upon oedema formation in the skin in response to a selection of different mediators of inflammation. The stimuli used for these experiments were chosen to include as many features of an acute inflammatory response

encompassed by what is termed the "two-mediator hypothesis" [18, 19]. This states that oedema formation is dependent upon two factors, the magnitude of the venular permeability and the extent of arteriolar vasodilation. In this study both direct-acting stimuli, such as 5-HT and PAF, and neutrophil-dependent stimuli, such as ZAS, were used as agents which produced an increase in the venular permeability. These were given either in the absence or the presence of the vasodilator PGE_1. Thus, it would appear that the topical steroid treatment employed in this study inhibited the inflammatory actions of both neutrophil-dependent and -independent stimuli. This group has previously shown that the systemic treatments employed in this study produce anti-inflammatory effects in the skin [8], with the dose of betamethasone sodium phosphate with a 3 hr pre-treatment having the greatest inhibitory effect upon oedema formation and, as in this study, is accompanied with the greatest LC1 content.

Thus, both of the treatments which result in an increase in EDTA-extractable LC1 are accompanied by anti-inflammatory activity. The suggestion from these findings that LC1 may be anti-inflammatory is in accordance with other studies which have shown that native LC1 and peptide fragments possess powerful anti-inflammatory activity [3, 20]. The exact mechanism of action of LC1 as an anti-inflammatory agent in these studies may be manifold. It has been demonstrated in normal human skin that topical steroid treatment will result in a decrease in the epidermal phospholipase A_2 (PLA_2) activity [21], thereby resulting in a decrease in the generation of certain pro-inflammatory mediators, such as prostaglandins and PAF, associated with the PLA_2-lipid mediators cascade. Although exactly how LC1 interacts with PLA_2 to produce this effect is unclear, i.e. whether a direct effect upon the enzyme itself or due to an interaction with the phospholipid substrate [22]. Also, it is certain that LC1 has direct effects upon the neutrophil-dependent component of specific inflammatory responses. It is possible that if LC1 is responsible for the anti-inflammatory action of the steroids in this study that its mode of action could be either of the aforementioned, however this is a matter which warrants further investigation.

In conclusion, it can be said that although topical betamethasone-17-valerate had little discernable effect upon total LC1 content in the skin it did appear to alter the localization of this protein. This is the first report of changes in LC1 induced by application of topical steroid. The fact that the induction of LC1 is also associated with anti-inflammatory activity suggests that these two factors may be related, however this requires further investigation for a definitive answer to be elucidated.

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