ANTIFLAMMIN-2 (AF2) is a nonapeptide corresponding to the amino acid residues 246–254 of lipocortin-1 showing anti-inflammatory activity both in vitro and in vivo. The effect of AF2 on the thromboxane B₂ (TXB₂) and histamine release from isolated and perfused guinea-pig lungs has been studied. AF-2 (10–100 nM) inhibited leukotriene C₄ (LTC₄) (3 ng) and antigen-induced (ovalbumin, 1 mg) TXB₂ release in normal and sensitized lungs, respectively. In contrast AF-2 (100 nM) did not modify TXB₂ release induced by histamine (5 μg) or bradykinin (5 μg) in normal lungs. Antigen-induced histamine release was not affected by 100 nM AF-2 infusion. When tested in chopped lung fragments AF-2 (0.1–25 μM) did not modify the release of histamine and TXB₂ induced by antigen (ovalbumin, 10 μg ml⁻¹) or calcium ionophore A 23187 (1 μM). Our results show that the inhibitory effect of AF-2 on TXB₂ release is selective and depends on the stimulus applied. In this respect AF-2 mimics, at least in part, the actions of both glucocorticoids and lipocortin-1.

**Key words**: Antiflammins, Guinea-pig, Lung, Thromboxane B₂

**Introduction**

Uteroglobin¹ and lipocortin-¹² belong to a family of structurally related proteins referred to as annexins or lipocortins which are calcium-binding proteins.³–⁵ Lipocortins are glucocorticoid-inducible proteins which, by inhibiting phospholipase A₂ (PLA₂), are believed to be involved in the suppression of prostaglandin and leukotriene synthesis associated with some aspects of the anti-inflammatory activity of corticosteroids.⁶ Recently nonapeptide fragments of uteroglobin and lipocortin-1, named antiflammins,⁷ have been shown to inhibit PLA₂ in vitro and to possess anti-inflammatory activity in vivo.⁸–⁹ One of these nonapeptides, antiflammin-2 (AF-2, HDMNKVLDL) corresponds to the amino acid residues 246–254 of lipocortin-1.⁷ However, conflicting results have been reported concerning the biological activity of AF-2. The peptide has been shown to inhibit carrageenan-induced rat paw oedema,⁹ porcine pancreatic PLA₂⁷ and human polymorphonuclear leucocyte PLA₂ activity⁸ as well as PLA₁ activity in extracts of human psoriatic epidermis.¹⁰ In contrast, other authors have reported that AF-2 is unable to inhibit porcine PLA₂, eicosanoid-release by different cell types, and carrageenan-induced rat paw oedema.¹¹–¹³

In the light of these conflicting results we decided to evaluate the biological activity of AF-2 in the isolated perfused guinea-pig lung model where human recombinant lipocortin-1 has been shown to be very active in inhibiting LTC₄-induced TXB₂ release.¹⁴ We have also tested the effect of the peptide on histamine release in the same experimental model.

**Materials and Methods**

**Animals**: Male guinea-pigs (Dunkin Hartley, 300–400 g) were used. In some experiments animals were sensitized by subcutaneous and intraperitoneal injections of equal doses (100 mg) of ovalbumin¹⁵ and the lungs were then removed 3 weeks later.

**Mediator release from perfused lungs**: Lungs from normal or sensitized guinea-pigs were cannulated through the pulmonary artery, excised and suspended in a chamber where they were immediately perfused with oxygenated Krebs bicarbonate solution at 37°C using aWatson and Marlow 503 S peristaltic pump. The rate of perfusion was constant at 5 ml min⁻¹. AF-2 was infused at 0.1 ml min⁻¹ 30 min before stimulating TXB₂ or histamine release and throughout the experiment. Fractions of 5 ml, corresponding to a 1 min collection time, were collected and stored at −80°C for assay. Stimuli such as LTC₄ (3 ng), ovalbumin (1 mg), histamine (5 μg) or bradykinin (5 μg) were applied as a bolus injection (0.1 ml).

**Mediator release from chopped lungs**: Lungs from normal or sensitized male guinea-pigs were cannulated and perfused with Krebs bicarbonate as described above. Lungs were then removed, cut into small
pieces with a sharp blade and washed extensively. Triplicate samples (0.6 g wet weight) were incubated in Krebs bicarbonate solution at 37°C for 10 min in the presence of AF-2 (0.1–25 μM) and subsequently challenged with ovalbumin (10 μg/ml–1 sensitized lungs) or calcium ionophore A23187 (1 μM, normal lungs). The incubation was stopped 30 min later by transferring the samples to an ice bath. Incubation media were then centrifuged (50 × g) and aliquots for TXB2 and histamine determination immediately frozen and kept at −80°C until analysis. Lung fragments were resuspended in Krebs bicarbonate solution, boiled for 10 min and filtered. The filtrate was centrifuged (50 × g) and aliquots for histamine assay were kept at −80°C until analysis.

In some experiments lungs were cannulated in situ through the pulmonary artery and perfused with AF-2 for 30 min. The lungs were then rapidly removed, cut into small pieces and incubated and processed as described above.

Radioimmunoassay of thromboxane B2: Thromboxane B2 was measured by radioimmunoassay without prior extraction or purification as previously described16 and expressed as ng min⁻¹ in perfusion experiments.

Fluorimetric analysis of histamine: Histamine release was measured fluorimetrically17 and corrected for spontaneous release occurring in the absence of the inducer and expressed as μg min⁻¹ in the perfusion experiments.

Since it has been reported that a reduction of the biological activity of AF-2 is caused by the oxidation of the methionine residue6 some experiments were performed in the presence of dithiothreitol (10:1). However, we could not detect any differences in AF-2 activity.

Materials: The composition of Krebs bicarbonate solution (mM) was the following: NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.17, CaCl₂·6H₂O 0.25 and glucose 8.4. Other agents used were: calcium ionophore A23187 (Calbiochem), ovalbumin grade II, histamine hydrochloride, bradykinin, TXB₂, LTC₄ (Sigma); phtaldialdeyd (Fluka), radio-labelled TXB₂ (Amersham). AF-2 was a generous gift from Dr P. Doyle (Wellcome Foundation, Beckenham, UK). Antibody anti-TXB₂ was kindly supplied by Dr J. Salmon (Wellcome, UK).

Results

Mediator release from perfused lungs: The basal release of TXB₂ from isolated perfused normal guinea-pig lungs was 6.35 ± 1.8 ng min⁻¹; n = 12. The basal release was not affected by 10 nM AF-2 (7.2 ± 1.1 ng min⁻¹; n = 4), 30 nM AF-2 (6.5 + 1.7 ng min⁻¹; n = 4) and 100 nM AF-2 (7.35 ± 0.8 ng min⁻¹; n = 4).

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

FIG. 1. Time course of TXB₂ release from isolated and perfused normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

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Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.

Normal guinea-pig lungs challenged with a bolus injection of LTC₄ (3 ng). Control (○). AF-2 was infused at 10 (▲), 30 (△) and 100 nM (▲). Each point represents the mean ± SE (vertical bars) of four lungs.
Inhibition of thromboxane B_2

**FIG. 2.** Time course of TXB_2(A) and histamine (B) release from isolated and perfused sensitized guinea-pig lungs challenged with a bolus injection of antigen (ovalbumin 1 mg). Control (O); infusion of AF-2 100 nM (●). Each point represents the mean ± SE (vertical bars) of six lungs.

Mediator release from chopped lungs: In another set of experiments we evaluated the effect of AF-2 infusion on the release of TXB_2 and histamine from chopped guinea-pig lungs. The mediators were released from normal lungs by 1 µM calcium ionophore A23187 and by antigen (10 µg ml⁻¹ ovalbumin) from sensitized lungs. None of the AF-2 concentrations tested (0.1–25 µM) was able to significantly modify TXB_2 and histamine released induced by these agents. Similar results were obtained when lungs were perfused in situ with AF-2 at the above concentrations and fragments subsequently challenged with the same stimuli (data not shown).

**Discussion**

These data show the ability of AF-2 to inhibit the TXB_2 release induced by LTC_4 or antigen in normal and sensitized guinea-pig lungs respectively. However, when other stimuli, such as histamine and bradykinin were used, no inhibition of TXB_2 release was observed.

Human recombinant lipocortin-1 ( annexin-1) when infused through normal guinea-pig lung preparation inhibited LTC_4-induced TXB_2 release. Glucocorticoids have been shown to inhibit TXB_2 release from guinea-pig isolated perfused lungs challenged by different stimuli such as leukotrienes, histamine or antigen while they did not prevent TXB_2 generation induced by bradykinin.

For these reasons we studied the effect of the nonapeptide, referred to as antiflammin-2, corresponding to lipocortin-1 sequence 246–254, on TXB_2 generation from guinea-pig lungs challenged with different stimuli in order to compare its biological activity to the effect of glucocorticoids and lipocortin-1.

Our results suggest that the inhibitory activity of AF-2 on TXB_2 release is dependent on the challenge used and mimics, at least in part, the action of both glucocorticoids and lipocortin-1. In fact AF-2, like glucocorticoids, significantly inhibits TXB_2 release induced by LTC_4 and it is unable to block the releasing action of bradykinin in normal lungs. In this respect it is interesting to observe that dexamethasone has an inhibitory activity on the bradykinin-induced eicosanoid release from the inflamed lungs while it is ineffective in normal lungs. The reason for the lack of activity of both glucocorticoids and AF-2 in normal lungs is not clear.

It has been suggested that separated PLA₂ pools may exist or that a phospholipase C linked pathway could be involved. The main difference between AF-2 and dexamethasone was observed when the lung was stimulated with histamine. In fact AF-2 was unable to block the histamine-induced TXB_2 release in normal lungs, which has been reported to be inhibited by glucocorticoids. We found that AF-2 was unable to inhibit the antigen-induced histamine release from sensitized lungs. This observation is consistent with previous results reported by us suggesting that another glucocorticoid-induced protein (vasocortin) different from lipocortin is responsible for the glucocorticoid inhibition of histamine release.

The release for the conflicting results reported by different authors on the biological activity of AF-2 is not clear. In fact, the mode of action of lipocortins and lipocortin-derived peptides as PLA₂ inhibitors it is not yet understood. Thus until now it has not been clearly demonstrated if the PLA₂ inhibition depends on a direct interaction between the enzyme and the inhibitor or it is due to the binding of the inhibitor to the substrate. Another aspect which could affect the biological activity of the peptide might be its oxidation possibly occurring during the experimental procedures. In fact it has been suggested that AF-2 might be inactivated by spontaneous oxidation of its methionine residue in the position 3 and consequently it might be effective only in the presence.
of a reducing agent. In this respect we did not observe any significant difference in the inhibition of TXB$_2$ release by AF-2 in the presence or absence of diithiothreitol.

Furthermore the inhibitory action of AF-2 on TXB$_2$ generation seems to be dependent not only on the stimulus but also on the experimental model used. Indeed, the peptide was completely ineffective when assayed on TXB$_2$ release from chopped lungs.

Corticosteroids are very powerful drugs in asthma therapy but their mechanism is not yet clearly understood and their clinical use mostly empirical. Antiflammin-2 could be used as a useful tool in exploring the mechanism through which glucocorticosteroids could affect the airway regulatory mechanisms.

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