Specific inhibition of leukotriene B₄ (LTB₄)-induced neutrophil emigration by 20-hydroxy LTB₄: implications for the regulation of inflammatory responses

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- 1 The interaction between leukotriene B₄ (LTB₄) and its metabolite, 20-hydroxy LTB₄ in the control of neutrophil emigration was examined in guinea-pig skin.
- 2 Leukotriene B₄ (10–300 ng) elicited a dose-dependent increase in neutrophil infiltration (as measured by myeloperoxidase activity) 4 h after injection into guinea-pig skin. In contrast, 20-hydroxy LTB₄ (30–1000 ng) displayed only weak inflammatory activity in this assay.
- 3 Although 20-hydroxy LTB₄ had low agonist activity, this metabolite caused a potent dose-dependent inhibition of responses to LTB₄ (100 ng), when administered systemically (ED₅₀ = 1.3 µg kg⁻¹, s.c.) without significantly affecting neutrophil infiltration in response to C5a (2 µg). Systemic administration of 20-carboxy LTB₄ (10 µg) did not affect neutrophil accumulation in response to LTB₄ or C5a. In addition, neither 15(S)-hydroxy 5(S)-HPETE (10 µg) nor lipoxin A₄ (10 µg) inhibited responses to LTB₄.
- 4 Addition of 20-hydroxy LTB₄ (10⁻¹¹–10⁻⁸ M) to human blood prior to isolation of the neutrophils led to concentration-dependent decrease in the number of LTB₄ receptors and decreased chemotactic responsiveness to LTB₄ without affecting responses to C5a. Incubation of blood with 20-carboxy LTB₄ (10⁻⁸ M) did not reduce LTB₄ receptor number or chemotactic responsiveness to LTB₄.
- 5 These data indicate that although 20-hydroxy LTB₄ is a weak agonist at LTB₄ receptors, it can desensitize neutrophils to the effects of LTB₄ via down-regulation of the high affinity receptor and thus provides evidence for a mechanism whereby inflammatory responses may be regulated.

Keywords: Leukotriene B₄; neutrophils; inflammation; 20-hydroxy LTB₄

Introduction

Leukocyte adhesion and emigration are cellular hallmarks of acute inflammation which can be elicited by a variety of neutrophil chemotactic factors. One such factor is leukotriene B₄ (LTB₄), an arachidonic acid metabolite of the 5-lipoxygenase pathway (Borgeat & Samuelsson, 1979). *In vitro*, LTB₄ causes neutrophil adhesion to endothelial cells (Gimbrone *et al.*, 1984; Hoover *et al.*, 1984) and stimulates neutrophil chemotaxis (Ford-Hutchinson *et al.*, 1980). *In vivo*, application of LTB₄ stimulates neutrophil adhesion in post-capillary venules (Dahlen *et al.*, 1981), emigration of neutrophils into tissues (Bray *et al.*, 1981; Higgs *et al.*, 1981; Movat *et al.*, 1984) and increased vascular permeability (Wedmore & Williams, 1981; Bjork *et al.*, 1982). LTB₄ is produced by PMN and other cell-types and has been detected at sites of experimentally-induced inflammation (Simmons *et al.*, 1983; Ford-Hutchinson *et al.*, 1984) and in several inflammatory disease states including psoriasis (Brain *et al.*, 1984), gout (Rae *et al.*, 1982), rheumatoid arthritis (Davidson *et al.*, 1983) and inflammatory bowel disease (Sharon & Stenson, 1984). The reduction in neutrophil infiltration by agents which inhibit the production of or antagonize the action of LTB₄ suggest that this mediator can play a central role in the recruitment of leukocytes to sites of inflammation (Salmon *et al.*, 1983; Foster *et al.*, 1990; Fretland *et al.*, 1990; Carter *et al.*, 1991).

Neutrophils also have the unique capacity to metabolize LTB₄ via ω -oxidation to 20-hydroxy LTB₄ and further to 20-carboxy LTB₄ (Hansson *et al.*, 1981). 20-carboxy LTB₄ is essentially biologically inactive while 20-hydroxy LTB₄ is reported to bind to the LTB₄ receptor and possess limited biological activity (Ford-Hutchinson *et al.*, 1983; Clancy *et al.*, 1984). Nanomolar concentrations of these ω -oxidation products have been detected in patients with conditions such

as purulent peritonitis and acute respiratory distress syndrome (Kikawa *et al.*, 1986; Seeger *et al.*, 1991), suggesting that this may be an important pathway of LTB₄ inactivation at sites of inflammation *in vivo*.

In the present study we have compared the inflammatory activities of 20-hydroxy LTB₄ with those of LTB₄ after injection into guinea-pig skin. We have also investigated the possibility that 20-hydroxy LTB₄ may desensitize responses to LTB₄ *in vivo*.

Methods

Induction of inflammatory responses in guinea-pig skin

Male Hartley guinea-pigs (400–450 g) were anaesthetized in an atmosphere of Metofane (methoxyflurane) and their backs shaved. Leukotriene B₄, 20-hydroxy LTB₄, 20-carboxy LTB₄ (all from Biomol, Plymouth Meeting, PA, U.S.A.) and human recombinant C5a (Franke *et al.*, 1988) were dissolved in saline containing 0.25% bovine serum albumin (BSA). Duplicate intradermal injections of saline or mediators were given in a volume of 0.1 ml in a randomized fashion such that there were a maximum of 6 sites per guinea-pig. Four hours after injection animals were killed, skin removed and sites punched out with a 13 mm gasket punch. 20-Hydroxy LTB₄ or 20-carboxy LTB₄ were either co-injected with LTB₄ or C5a or given subcutaneously in the nape of the neck.

Extraction and assay of myeloperoxidase activity in skin samples

Skin sites were homogenized and assayed for myeloperoxidase by a modification of the method described by Lundberg & Arfors (1983). Skin samples were homogenized in phosphate-buffered saline (pH 7.2), containing 0.5% hexadecyltri-

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methyammonium bromide with a Polytron homogenizer (Brinkman, New York, U.S.A.). After homogenization, and 2 freeze/thaw cycles, the homogenates were centrifuged at 2,500 *g* for 30 min. Myeloperoxidase activity was then assayed in the supernatant by incubating 50 μ l of diluted sample with 150 μ l potassium phosphate buffer (pH 6.0) containing O-dianisidine (0.2 mg ml⁻¹) and hydrogen peroxide (0.001%) at 37°C for 15 min. The reaction was terminated by the addition of 100 μ l 0.4 M glycine (pH 10) and the absorbance read at 450 nm using a 96-well plate reader (Molecular Devices, Menlo Park, CA, U.S.A.). Known numbers of guinea-pig neutrophils (harvested from the peritoneal cavity after injection of casein) were included in each assay as a standard curve and the data expressed as numbers of neutrophils per skin site.

In vitro desensitization (chemotaxis and [³H]-LTB₄ binding) experiments using neutrophils isolated from whole blood

Aliquots (10 ml) of heparinized human blood were incubated with log dilutions of 20-hydroxy LTB₄ (10⁻¹¹–10⁻⁸ M final concentrations) for 5 min at 37°C. Neutrophils were then isolated as described below and the capacity to respond in the chemotaxis assay to either LTB₄ (5 nM) or C5a (5 nM) was evaluated (see below). In parallel, the ability of neutrophils to bind specifically [³H]-LTB₄ was evaluated according to a method adapted from Lin *et al.* (1984). Briefly, neutrophils at a density of 2.5 × 10⁶ cells per ml were incubated with 0.25–0.75 nM [³H]-LTB₄ (195 Ci mmol⁻¹, DuPont/NEN, Boston, MA, U.S.A.) in Hanks buffered saline (containing Ca²⁺ and Mg²⁺, 10 mM HEPES pH 7.25, 0.04% sodium bicarbonate) in the absence and presence of 1 μ M unlabelled LTB₄ at 4°C for 30 min. The assay was performed in triplicate in microtiter plates (Costar, Cambridge, MA, U.S.A.) with a total volume of 200 μ l and bound ligand was separated from free ligand using the betaplate apparatus (Pharmacia LKB, Piscataway, NJ, U.S.A.). The wash buffer was the same buffer as used for the incubation. Specific binding represents the value obtained when non-specific binding is subtracted from total binding.

Measurement of human neutrophil chemotaxis *in vitro*

Neutrophils were isolated from anticoagulated (heparin, Squibb-Monsam, Inc., Cherry Hill, N.J., U.S.A., 30 units ml⁻¹ final concentration) human blood obtained from normal donors according to the method of Ferrante & Thong (1978). Isolated neutrophils were resuspended (2.5 × 10⁶ cells ml⁻¹) in HBSS (supplemented with 10 mM

Figure 1 Increase in neutrophil infiltration (as measured by myeloperoxidase activity) after injection of leukotriene B₄ (LTB₄, open columns bars) or 20-hydroxy LTB₄ (solid columns) into the skin of separate groups of guinea-pigs. Results are expressed as the mean $\pm$ s.e.mean of data derived from 5 animals.

Figure 2 Inhibition of leukotriene B₄ (LTB₄, 100 ng)-induced neutrophil accumulation in guinea-pig skin by systemic (subcutaneous) administration of 20-hydroxy LTB₄. Each point represents the mean $\pm$ s.e.mean of data from 5–10 animals.

HEPES) containing 0.7 mM Mg²⁺ and 1.6 mM Ca²⁺ and recrystallized bovine serum albumin (BSA; 2 mg ml⁻¹ Sigma) and adjusted to pH 7.2. The chemotaxis assay was performed in a 48 well chamber apparatus (Neuroprobe, Cabin John, MD, U.S.A.) with cellulose nitrate filters (pore size, 3.0 μ m) as described (Harvath *et al.*, 1987). The total number of cells (observed at $\times$ 400 magnification) migrating from 20 μ m from beneath the surface monolayer to the leading front (usually $\sim$ 100–120 μ m per 45 min at optimal chemotactic factor concentration) in response to various concentrations of LTB₄, 20-hydroxy LTB₄ or 20-carboxy LTB₄ were summed with the aid of an Optimax image analyzer (Optimax, Hollis, N.H., U.S.A.) and provided an index of the chemotactic response. Each experimental condition was performed in duplicate and three to five fields were assessed for cell migration. Results are expressed as the percentage maximum response where 100 per cent was equal to the peak response seen in the presence of the most efficacious concentration of LTB₄. The number of cells migrating spontaneously (i.e. negative controls) was subtracted from all measurements prior to data transformation.

Results

LTB₄ (10–300 ng) caused a dose-dependent increase in neutrophil infiltration 4 h after injection into guinea-pig skin (Figure 1). In contrast, 20-hydroxy LTB₄ was much less active, producing inflammatory responses of similar magnitude to the saline controls. 20-carboxy LTB₄ also showed very little inflammatory activity when injected *in vivo*. In the experiments shown in Figure 1, the LTB₄ and 20-hydroxy LTB₄ were injected in separate groups of animals. Initially, LTB₄ and 20-hydroxy LTB₄ were injected at different sites in the same animals and we observed unexpectedly low control responses to LTB₄. These results prompted us to determine whether systemic injection of 20-hydroxy LTB₄ (injected subcutaneously at a distant site from the LTB₄ injections) could suppress the responses to LTB₄.

The results shown in Figure 2 indicate that 20-hydroxy LTB₄ inhibited neutrophil responses to LTB₄ (100 ng) *in vivo* at extremely low doses (ED₅₀ = 1.3 μ g kg⁻¹, s.c.). However, 20-hydroxy LTB₄ did not significantly affect neutrophil emigration in response to C5a (2 μ g) in animals where responses to LTB₄ were inhibited (Figure 3). This dose of C5a was submaximal and chosen because it gave a similar magnitude of response as 100 ng LTB₄. The inhibitory property of 20-hydroxy LTB₄ was not shared with 20-carboxy LTB₄ which did not affect responses to LTB₄ or C5a (Figure 4).

In addition, neither 15(S)-hydroxy 5(S)-HPETE nor lipoxin

Figure 3 Effect of systemic administration of 20-hydroxy leukotriene B₄ (20-OH-LTB₄) (3 µg) on accumulation of neutrophils in guinea-pig skin in response to LTB₄ (100 ng, open columns) or C5a (2 µg, solid columns) in the same animals. Each point represents the mean ± s.e.mean of data from 5 animals per group. **P* < 0.01 vs. control.

Figure 4 Effect of systemic administration of 20-carboxy leukotriene B₄ (20-COOH LTB₄) on accumulation of neutrophils in guinea-pig skin in response to LTB₄ (100 ng, open columns) or C5a (2 µg, solid columns). Results are expressed as the mean ± s.e.mean of data from 5 animals per group.

Figure 5 Inhibition of leukotriene B₄ (LTB₄)-induced inflammatory responses by 20-hydroxy LTB₄ (10 µg) at various times after dosing. Results are expressed as the mean ± s.e.mean of data from 5 animals per group.

Figure 6 Inhibition of leukotriene B₄ (LTB₄)-mediated neutrophil chemotaxis (a) and specific [³H]-LTB₄ binding to isolated neutrophils (b) pre-exposed to 20-hydroxy LTB₄ in whole blood. Log dilutions of 20-hydroxy LTB₄ were added to whole human blood and incubated at 37°C for 5 min. Neutrophils were then isolated as described in the methods section and assayed for their ability to respond in the chemotaxis assay to LTB₄ (5 nM) (●) or C5a (5 nM) (■) (a) and to bind specifically [³H]-LTB₄ (b). The values shown represent the mean ± s.e.mean for 3 independent experiments.

A₄ inhibited responses to LTB₄ when administered under the same conditions as 20-hydroxy LTB₄ at doses up to 10 µg (for 15(S)-hydroxy 5(S)-HPETE % control response = 88.3 ± 17.3, *n* = 5; for lipoxin A₄, % control response = 102.7 ± 10.6, *n* = 5). Figure 5 shows that the effect of 20-hydroxy LTB₄ was longlasting (responses still maximally inhibited at 2 h after dosing), but reversible (responses were not significantly reduced at 4 h after dosing). When 20-hydroxy LTB₄ was coadministered locally with LTB₄ no inhibition was observed at doses which were devoid of systemic activity.

In vitro, pretreatment of human neutrophils in whole blood with 20-hydroxy LTB₄ led to a concentration-dependent and specific inhibition of the chemotactic response to LTB₄ (IC₅₀ = 0.15 ± 0.07 nM) and loss of high affinity receptors for [³H]-LTB₄ (IC₅₀ = 4.0 ± 1.8 nM) (Figure 6). No inhibition of either chemotaxis (% control response = 101 ± 3.6) or receptor expression (% control response = 93.0 ± 7.6) was noted in response to 20-carboxy LTB₄ (10⁻⁸ M). Moreover, pre-exposure of neutrophils to 20-hydroxy LTB₄ in blood did not inhibit chemotaxis in response to the complement fragment, C5a (Figure 6).

Discussion

There is increasing evidence that LTB₄ plays a central role in leukocyte recruitment and the ensuing tissue damage that occurs in inflammatory diseases. Leukotriene B₄ has been detected at sites of inflammation, both in laboratory animals

(Simmons *et al.*, 1983; Ford-Hutchinson *et al.*, 1984) and in human diseases (Rae *et al.*, 1982; Davidson *et al.*, 1983; Brain *et al.*, 1984; Sharon & Stenson, 1984) and has been shown to possess potent leukocyte chemotactic properties *in vitro* and *in vivo*. Treatments which lower LTB₄ pharmacologically (Foster *et al.*, 1990; Carter *et al.*, 1991; Salmon *et al.*, 1983) or by dietary means (Lefkowitz, 1988) have been demonstrated to diminish leukocyte recruitment *in vivo*. In addition, agents which antagonize LTB₄ at its receptor have also displayed anti-inflammatory activity (Fretland *et al.*, 1990; 1991).

Further evidence to support a role for LTB₄ in inflammation can be inferred from the finding that an effective mechanism exists for its inactivation at sites of inflammation. Thus, LTB₄ can be metabolized by neutrophils and possibly other cell types via ω -oxidation to products which have much reduced biological activity (Hansson *et al.*, 1981; Ford-Hutchinson *et al.*, 1983; O'Flaherty *et al.*, 1986). Furthermore, these products (20-hydroxy LTB₄ and 20-carboxy LTB₄) have been detected in inflammatory exudates (Kikawa *et al.*, 1986; Seeger *et al.*, 1991), suggesting that ω -oxidation is an important pathway of LTB₄ inactivation *in vivo*.

In the present study we have confirmed that 20-hydroxy LTB₄ and 20-carboxy LTB₄ have significantly reduced inflammatory activities compared to LTB₄. As 20-hydroxy LTB₄ has been shown to bind to LTB₄ receptors on neutrophils with 10–20 times less affinity than LTB₄ (Clancy *et al.*, 1984; O'Flaherty *et al.*, 1986; Jackson *et al.*, 1992), we also investigated the possibility that 20-hydroxy LTB₄ may antagonize the inflammatory effects of LTB₄ *in vivo*. Surprisingly, when 20-hydroxy LTB₄ was co-injected with LTB₄ in guinea-pig skin, the LTB₄ responses at distant control sites were also diminished. Consequently, we found that systemic (subcutaneous) administration of extremely low doses of 20-hydroxy LTB₄ can effectively block inflammatory responses to LTB₄. 20-Carboxy LTB₄ did not possess this property of 20-hydroxy LTB₄ and the effect was specific for LTB₄ since 20-hydroxy LTB₄ administration did not inhibit neutrophil infiltration in response to C5a. However, 20-hydroxy LTB₄ also caused a small (but nonsignificant) reduction in the myeloperoxidase signal in response to C5a which we believe reflects a LTB₄-dependent component to the eosinophil infiltration that occurs in response to C5a. As eosinophils contain myeloperoxidase-like activity the low numbers infiltrating the

skin sites may make a small contribution to the signal and would account for the slight reduction caused by 20-hydroxy LTB₄ in response to C5a. Studies are underway to investigate the LTB₄-dependent component of C5a-induced eosinophil infiltration using selective markers of eosinophil infiltration.

Doses of 20-hydroxy LTB₄ which were devoid of systemic activity did not inhibit responses to LTB₄ when co-injected locally. These data suggest that 20-hydroxy LTB₄ acted by desensitizing neutrophils to the action of LTB₄ in the circulation and did not inhibit responses to LTB₄ by local antagonism. This concept is supported by *in vitro* data where pre-exposure of neutrophils in whole blood to 20-hydroxy LTB₄ selectively inhibited neutrophil chemotaxis to LTB₄ and reduced the number of high affinity receptors for LTB₄ which have previously been shown to transduce the chemotactic response and can be eliminated by chemotactic deactivation of neutrophils to LTB₄ (Goldman & Goetzl, 1984). In fact *in vitro* it has already been shown that 20-hydroxy LTB₄ can desensitize neutrophil responses to LTB₄ and *vice versa* (O'Flaherty *et al.*, 1986). However, 20-hydroxy LTB₄ appears to be more potent as an inhibitor in the functional chemotaxis assay than in inhibiting receptor expression which may reflect a redistribution of receptors on the cell surface that are functionally uncoupled for chemotaxis but are still capable of binding ligand. A distribution of N-formyl peptide receptors into specialised membrane domains has previously been shown to occur upon ligand binding and may be relevant to the above finding with LTB₄ receptors (Jesaitis *et al.*, 1988). This selective desensitization of LTB₄-mediated function is similar to that which occurs in neutrophils from patients with cystic fibrosis (Lawrence & Sorrell, 1992).

These data indicate that although 20-hydroxy LTB₄ possesses only weak inflammatory activity it can cause specific desensitization to the inflammatory effects of LTB₄ and therefore may serve as a probe to investigate the role of LTB₄ in animal models of inflammatory disease. A similar proposal was recently put forward by Marleau *et al.* (1993) where infusion of LTB₄ itself was used to induce a state of desensitization in the rabbit. Furthermore, as 20-hydroxy LTB₄ has been detected at sites of inflammation in concentrations sufficient to produce these inhibitory effects, this process of desensitization may serve as a mechanism to regulate inflammatory processes and a reduced capacity to metabolize LTB₄ via ω -hydroxylation may predispose to chronic inflammatory disease.

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