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Eicosapentaenoic acid modulates neutrophil leukotriene B_4 receptor expression in cystic fibrosis

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SUMMARY

In patients with cystic fibrosis (CF), high intrapulmonary concentrations of the neutrophil chemotaxin leukotriene B_4 (LTB_4) are associated with specific reduction of LTB_4-induced chemotaxis of circulating neutrophils. The chemotactic abnormality is partially corrected by dietary supplementation with eicosapentaenoic acid (EPA). LTB_4-induced neutrophil chemotaxis is mediated by specific, high-affinity, cell surface LTB_4 receptors. The hypotheses that neutrophil LTB_4 receptors are down-regulated in CF, and that EPA normalizes receptor expression, were tested by measuring the number (R_{max}) and affinity (K_d) of LTB_4 receptors on neutrophils from eight CF patients before and after EPA (6 weeks of 2·7 g/day), and from nine normal individuals. High-affinity receptor R_{max} was depressed in CF patients ($0·6 \pm 0·2 \times 10^4$/cell (mean ± s.d.) *versus* $1·8 \pm 0·7 \times 10^4$/cell in normals), but corrected to normal ($2·0 \pm 1·9 \times 10^4$/cell) after EPA. High-affinity receptor K_d was depressed in CF patients ($0·4 \pm 0·3$ nM *versus* $1·4 \pm 0·5$ nM in normals), and also corrected to normal with EPA ($1·4 \pm 1·2$ nM). Low-affinity receptors were depressed, but did not change significantly with EPA. These results indicate that neutrophil responses in chronic inflammatory lung disease can be influenced directly by LTB_4 receptor modulation, and that this effect of EPA predominates over alterations in neutrophil signal transduction in situations of chronic exposure to LTB_4.

Keywords eicosapentaenoic acid neutrophils leukotriene B_4 receptors cystic fibrosis

INTRODUCTION

Leukotriene B_4 (5(S), 12(R)-dihydroxyeicosa-6, 14-*cis*, 8,10-*trans*-tetraenoic acid (LTB_4)) is a potent activator of neutrophils [1,2], and is produced by human macrophages and neutrophils in response to a variety of stimuli, including *Pseudomonas aeruginosa* [3]. The airways of patients with cystic fibrosis (CF), which are chronically colonized with *Ps. aeruginosa*, contain excessive numbers of neutrophils and significant amounts of LTB_4 [4–6]. LTB_4-induced chemotaxis of neutrophils from patients with CF is reduced compared with that of cells from normal subjects, whereas the response to formyl methionyl–leucyl–phenylalanine (fMLP) and casein are normal [7]. These observations suggest that LTB_4 produced in the airways reaches the pulmonary circulation in concentrations sufficient to affect the function of circulating neutrophils.

LTB_4 induces neutrophil chemotaxis and activation through specific surface receptors and G-protein-mediated signal transduction pathways [8,9]. Several points in these pathways are susceptible to modulation by the ω-3 unsaturated fatty acid eicosapentaenoic acid (EPA), though the mechanisms of such modulation are incompletely characterized. In normal subjects EPA inhibits both neutrophil production of, and responses to, LTB_4 [10–19]. The reduction in LTB_4-induced chemotaxis is due predominantly to inhibition of post-receptor signal transduction mechanisms, since it has been shown that phosphatidylinositol-specific phospholipase C activity is reduced, while the density and affinity of neutrophil LTB_4 receptors remains unchanged [19]. In contrast to the observations in normal subjects, LTB_4 chemotaxis of neutrophils from patients with CF is low, and increases in response to moderate doses (2·7 g/day for 6 weeks) of oral EPA [20]. It has been shown *in vitro* that exposure to LTB_4 causes down-regulation of high-affinity LTB_4 receptors on the surface of neutrophils and a parallel loss in chemotactic response to LTB_4 [21]. A similar effect may occur *in vivo* in patients with CF, since it has been demonstrated that those colonized with *Ps. aeruginosa* have high sputum concentrations of LTB_4 and demonstrate a selective reduction of LTB_4-induced neutrophil chemotaxis [20]. Dietary supplementation with EPA may reduce the amount of LTB_4 produced within the airway, allowing up-regulation of suppressed receptors on circulating neutrophils. The present study tests the hypothesis that LTB_4 receptors on

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circulating neutrophils are down-regulated in patients with CF, and that dietary supplementation with EPA increases LTB_4-induced neutrophil chemotaxis by normalization of LTB_4 receptor expression.

PATIENTS AND METHODS

Subjects, study design and analysis

A normal range for LTB_4 receptor density and affinity on neutrophils isolated from peripheral venous blood was established in nine healthy individuals. Subjects were recruited from laboratory staff and students who were non-smokers, taking no medication, and who had no evidence of inflammatory disease at the time of sampling. The median age of these subjects was 28 years (range 20–36 years), and five were male.

Nine patients with CF were recruited. All had a chronic productive cough, and *Ps. aeruginosa* cultured repeatedly from sputum samples for more than 12 months. None considered themselves to be functionally impaired, and none had been admitted to hospital for treatment within the previous month. All had Shwachman scores of over 75% at enrolment. All were taking pancreatic enzyme supplements, and none was receiving oral or inhaled corticosteroids. The median age of the group was 18 years (range 14–22 years), and seven were male.

Patients with CF had venous blood taken for estimation of neutrophil LTB_4 receptor status before dietary supplementation with 2·7 g/day of EPA in the form of fish oil capsules (Hi-MEGA; Roussel-Uclaf, Pennant Hills, Australia) for 6 weeks. A second venous blood sample was taken within 48 h of completing a total intake of 113 g of EPA. Compliance and tolerance were assessed by capsule-count and weekly review. One patient with CF withdrew from the study after 1 week, unable to tolerate the taste and belching associated with the fish oil. Each subject gave written informed consent for the study, as did the parents of subjects under 18 years of age. The study protocol was reviewed and approved by the Western Sydney Health Service Research and Ethics Committees.

Neutrophil LTB_4 receptor status of subjects with CF, before and after EPA, was compared with that of normal subjects by the Mann–Whitney *U*-test (unpaired samples). The significance of the change in neutrophil LTB_4 receptor status of subjects with CF before and after EPA was assessed by the Wilcoxon rank sum test (paired samples) for each of the eight subjects who completed 6 weeks of supplementation.

Neutrophil isolation

Neutrophils were isolated from 40 ml of venous blood sample by a modification of the method of Böyum [7,22], using 3·5% dextran sedimentation of erythrocytes, Ficoll (Ficol–Paque; Pharmacia, North Ryde, Australia) gradient separation of neutrophils from mononuclear cells, and hypotonic lysis of remaining erythrocytes. Separation was performed in calcium- and magnesium-free media at room temperature, and was complete within 2 h. Trypan blue dye exclusion demonstrated 98% cell viability.

LTB_4 receptor assay

Immediately after isolation, the density and affinity of LTB_4 receptors on the surface of intact neutrophils was assessed by a modification of the method of Goldman & Goetzl [21]. Neutrophils were resuspended at a concentration of $6{\cdot}7 \times 10^6$ cells/ml in Hanks' buffered salt solution (HBSS; ICN Biomedicals, Seven Hills, Australia) with 0·1% ovalbumin (OVA; Grade V, Sigma Aldrich, Castle Hill, Australia) and 10 mM HEPES (Calbiochem-Novabiochem, Alexandria, Australia), buffered to pH 7·3 with sodium hydroxide (HBSS/OVA/HEPES) at 4°C. Cell suspension (300 μl) was placed into 1·5 ml polypropylene centrifuge tubes suspended in an ice-water bath, each containing 40 pg ^{3}H-LTB_4 (Amersham, North Ryde, Australia) in 100 μl HBSS/OVA/HEPES and a range of concentrations of LTB_4 in 100 μl HBSS/OVA/HEPES. The amount of unlabelled LTB_4 in each tube ranged from 6·4 pg to 100 ng: concentrations were prepared by serial five-fold dilution of freshly resuspended stock LTB_4 in HBSS/OVA/HEPES. Binding of labelled LTB_4 to neutrophils was measured in triplicate at each concentration of competing unlabelled ligand. Cells were incubated in a shaking ice-water bath for 30 min, after which each suspension was layered over a 300-μl phthalate oil cushion (*n*-butyl phthalate (ICN Biomedicals, Seven Hills, Australia): dinonyl phthalate (Sigma Aldrich), 7:2, v/v) at 4°C, and the cells pelleted by centrifugation at 8000 *g* for 30 s in a Beckman Microfuge II with a cooled fixed-angle rotor. Supernatant HBSS/OVA/HEPES and phthalate oil were removed and the tip of each tube, containing the cell pellet, excised into scintillation vials. Five millilitres of scintillant (Ultima Gold; Canberra Packard, Hunters Hill, Australia) were added to each vial and the batch allowed to equilibrate at room temperature overnight before β-counting. The amount of ^{3}H-LTB_4 specifically bound to 2×10^6 cells at each concentration of competing ligand was entered as an individual point in the software package MacLigand v4.7 (Dr Peter J. Munson, Unit on Biostatistical Methodology, National Institutes of Health, Bethesda, MD). The program used recursive curve fitting of a two-site model to derive high- and low-affinity LTB_4 receptor density (R_{max}) and dissociation constants (K_d) for cells from each individual. This technique yielded reproducible results; the inter- and intra-individual variance in high-affinity receptor density and dissociation constants of two normal subjects, each tested on three occasions at least 1 month apart, was less than 10%.

RESULTS

Scatchard plots of the data from both normal and CF patients demonstrated both high- and low-affinity binding sites (Fig. 1), allowing the derivation of high- and low-affinity receptor R_{max} and K_d in all individuals tested.

Neutrophils isolated from nine normal individuals had a mean ($\pm$ s.d.) of $1{\cdot}8 \times 10^4$ ($\pm 0{\cdot}7 \times 10^4$) high-affinity LTB_4 receptors/cell, with a mean K_d of $1{\cdot}4 \pm 0{\cdot}5$ nM. Low-affinity receptors were more variable, with a mean R_{max} of 200×10^4 ($\pm 260 \times 10^4$) receptors/cell, and a mean K_d of 1744 ± 1656 nM. While these values are somewhat higher than those initially described by Goldman & Goetzl [21], they are consistent with later estimates in normal individuals [9,23,24].

The neutrophils of patients with CF, before EPA supplementation, had $0{\cdot}6 \times 10^4$ ($\pm 0{\cdot}2 \times 10^4$) high-affinity LTB_4 receptors/cell, significantly fewer than those on normal neutrophils ($P = 0{\cdot}0004$, by Mann–Whitney *U*-test) (Fig. 2). The mean K_d of high-affinity receptors from CF neutrophils was $0{\cdot}4 \pm 0{\cdot}3$ nM, significantly lower than those of normal subjects ($P = 0{\cdot}0014$, by Mann–Whitney *U*-test) (Fig. 2). Low-affinity receptors from CF patients were fewer (mean R_{max} of 68×10^4 ($\pm 63 \times 10^4$) LTB_4 receptors/cell) and had a lower mean K_d,

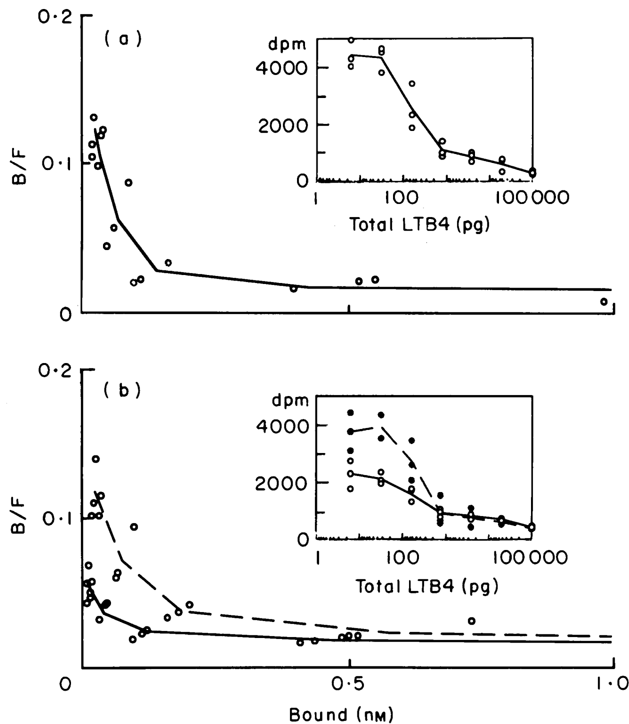

Fig. 1. Scatchard plots of displacement of ^{3}H-leukotriene B_4 (LTB_4) from LTB_4 receptors on the surface of intact neutrophils. Representative plots from a healthy control (a) and from a patient with cystic fibrosis before (solid line) and after (dashed line) supplementation with eicosapentaenoic acid (EPA) (b) are shown. Each point represents the proportion of bound to free (B/F) LTB_4 at a particular concentration of bound LTB_4. The curves are lines of best fit for each set of points, derived using the program MacLigand v4.7. A two-site model provides the best fit for each plot, allowing derivation of the high- and low-affinity receptor density (from *x*-axis intercepts of each segment) and dissociation constant (from slopes of each segment of line of best fit). Insets show the binding curves from which the Scatchard plots were derived.

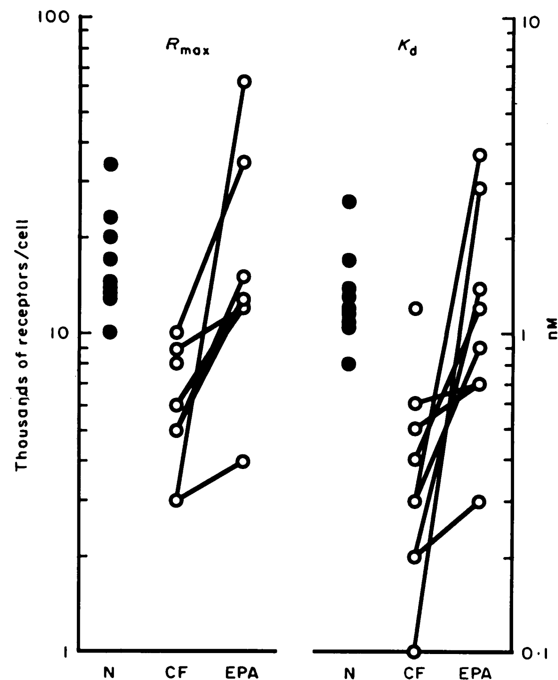

Fig. 2. High-affinity leukotriene B_4 (LTB_4) receptor density (R_{max}) and dissociation content (K_d) on human neutrophils. Both are significantly lower, in neutrophils from patients with cystic fibrosis (CF, ○) than in those of normal individuals (N, ●). Patients with cystic fibrosis, given 2·7 g/day of eicosapentaenoic acid for 6 weeks (EPA, ○), increased both R_{max} and K_d to levels not significantly different from those of normal individuals.

266 ± 207 nM, than those in normal subjects ($P = 0{\cdot}045$ and $P = 0{\cdot}009$, respectively) (Fig. 3).

The number and K_d of high-affinity receptors increased in eight CF patients who completed the course of EPA supplementation—all but one to at least the range seen in normal individuals, and one to values greater than those of normal subjects (Fig. 2). The increase in high-affinity receptor number and K_d in CF patients after EPA supplementation was significant, with P values for both of 0·012 by Wilcoxon signed rank test. R_{max} and K_d of high-affinity receptors from CF patients after EPA were not significantly different from those of the normal controls ($P = 0{\cdot}497$ and $P = 0{\cdot}562$, respectively, by Mann–Whitney U-test).

The number and K_d of low-affinity receptors increased in some CF patients who completed the course of EPA supplementation (Fig. 3), but the difference in low-affinity receptor number and K_d in CF patients after EPA supplementation was not significant, with P values of 0·327 and 0·401, respectively, by Wilcoxon signed rank test. Low-affinity receptor number and K_d in CF patients after EPA were also not significantly different from those of normal controls ($P = 0{\cdot}564$ and $P = 0{\cdot}054$, respectively, by Mann–Whitney U-test).

DISCUSSION

These findings support the hypothesis that LTB_4 receptors are reversibly down-regulated in CF. Compared with normal controls, CF patients have fewer high-affinity LTB_4 receptors on the surface of their circulating neutrophils, a finding consistent with *in vivo* densensitization by prior exposure to LTB_4. This abnormality is completely corrected by doses of EPA which substantially correct a corresponding abnormality of neutrophil chemotactic response to LTB_4 in CF [20]. Sperling *et al.* noted a similar reversal of depressed neutrophil chemotactic response to LTB_4 in patients with rheumatoid arthritis given equivalent doses of EPA, and first proposed the mechanism of *in vivo* densensitization in a disease with chronic local overproduction of LTB_4 [25]. Receptor down-regulation could be due to occupation of available receptors, internalization of occupied receptors, or a shift of receptors from high-affinity to low-affinity binding states. Published data are consistent with a change in the disposition of the receptors within the neutrophil plasma membrane, rather than internalization, irreversible binding or denaturation [26]. Studies *in vitro* have shown recovery of high-affinity receptors within 1 h of LTB_4-induced down-regulation [27], and there is no evidence of a granular cytoplasmic store of LTB_4 which could be recycled to the plasma membrane [28].

In contrast to reports of EPA supplementation in subjects with inflammatory disease, several studies of the effects of 3 g/day of dietary EPA on the neutrophils of normal subjects have demonstrated a decreased chemotactic response to LTB_4 [12,15,29]. Georgilis & Klempner reported that *ex vivo* expo-

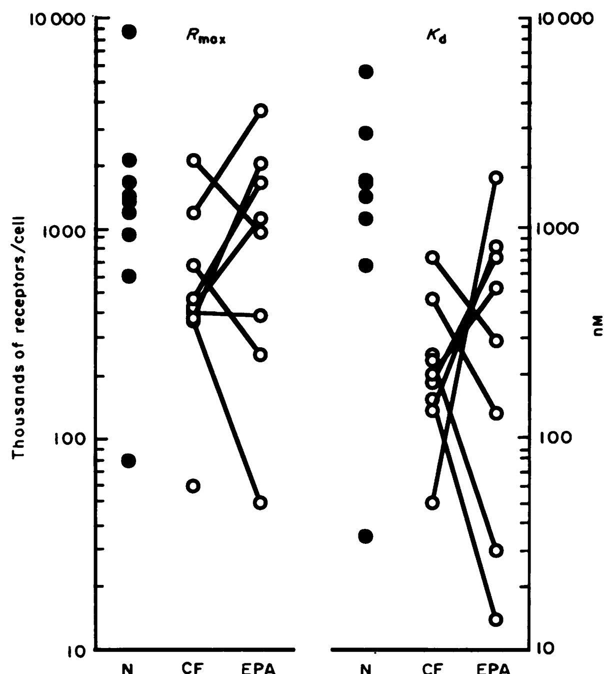

Fig. 3. Low-affinity leukotriene B_4 (LTB_4) receptor density (R_{max}) and dissociation content (K_d) on human neutrophils. Both are significantly lower, in neutrophils from patients with cystic fibrosis (CF, ○) than in those of normal individuals (N, ●). The change in low-affinity receptor numbers or affinity in patients with CF given 2·7 g/day of eicosapentaenoic acid for 6 weeks (EPA, ○) was not significant.

sure of neutrophils from normal subjects to EPA produced a decrease in LTB_4 receptor density, but used a method of analysis which did not distinguish between high- and low-affinity LTB_4 receptors [30]. Sperling *et al.* have recently reported that prolonged administration of high doses of dietary EPA (9 g/day) to normal subjects attenuates the chemotactic response, apparently through an effect on phosphatidylinositol-specific phospholipase C, with no change in the density or affinity of neutrophil LTB_4 receptors [19]. Such a post-receptor effect of dietary EPA may also be operating in patients with CF, since EPA fails to correct chemotaxis of CF neutrophils to normal range [20].

The direct demonstration of changes in high-affinity receptor status with EPA indicates that receptor down-regulation does occur in cystic fibrosis, presumably as a negative feedback response to chronic overproduction of LTB_4 in the lung. The effect of EPA is likely to be secondary to reduced production of LTB_4 in the airway of CF patients, with consequent decrease in exposure of circulating neutrophils to LTB_4 in the pulmonary circulation. This has not yet been proven *in vivo*, though in a limited study of patients with CF who received a 6-week course of EPA, we noted a trend towards reduction in sputum concentration of LTB_4 [20]. The mechanisms of receptor modulation by EPA in CF are not clear, though several reports suggest changes in LTB_4 receptor, or G-protein, disposition in the neutrophil membrane are possible [26,27,31]. Receptor expression may also be influenced by indirect effects of EPA on post-receptor regulatory mechanisms, such as protein kinase C and intracellular calcium [17,32,33]. The limitation of the *in vivo* effect of EPA to high-affinity receptors suggests that G-protein-mediated receptor affinity [27], rather than receptor synthesis, is being affected. Disease states where chronic excessive production of LTB_4 may alter receptor-mediated responses, such as CF and rheumatoid arthritis, provide an opportunity to explore both physiological and pathological feedback and control mechanisms of human neutrophil activation.

Since receptor effects are generally ligand-specific, the success of LTB_4 receptor modulation in CF supports a significant role for LTB_4 in the pathogenesis of CF lung disease. Given the relative ease and safety of the administration of moderate doses of dietary EPA, the observation that LTB_4-induced neutrophil response can be modulated at receptor level, *in vivo*, suggests further exploration of EPA as a therapeutic tool in CF is indicated.

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