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Inhibition of ionophore-stimulated leukotriene B_4 production in human leucocytes by monohydroxy fatty acids

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1 Leukotriene B_4 (LTB_4) release by calcium ionophore-stimulated human leucocytes was measured by use of selective solvent partition of reaction mixtures and an agarose microdroplet chemokinesis assay, and the inhibitory effects of four monohydroxy fatty acids were determined.

2 15-Hydroxy-eicosatetraenoic acid (15-HETE) was the most effective inhibitor of LTB_4 production with an approximate IC_{50} value of 6 μM and 99% inhibition at 50 μM, whereas 13-hydroxy-octadecadienoic acid (13-HODD) and 12-HETE were weaker inhibitors with approximate IC_{50} values of 32 μM and 23 μM, and 59% and 68% inhibition at 50 μM, respectively.

3 We suggest that 13-HODD and 12-HETE, which are present in large amounts in the lesions of the skin disease psoriasis, may act as endogenous modulators of 5-lipoxygenase activity in skin.

Introduction

The arachidonate 5-lipoxygenase metabolite, leukotriene B_4 (5S, 12R-dihydroxy-6, 14-*cis*-8,10-*trans*-eicosatetraenoic acid, LTB_4) is a potent neutrophil chemoattractant both *in vitro* (Ford-Hutchinson *et al.*, 1980; Goetzl & Pickett, 1980; Palmer *et al.*, 1980; Bray *et al.*, 1981) and in human skin *in vivo* (Camp *et al.*, 1984) and has been implicated in the pathogenesis of various inflammatory diseases. Biologically active amounts of LTB_4-like material have been recovered from the lesions of the common inflammatory and proliferative skin disease, psoriasis, and it has therefore been proposed that LTB_4 may play a role in the pathogenesis of the intraepidermal neutrophil infiltrate that characterizes this disease (Brain *et al.*, 1984 a,b; Camp *et al.*, 1984). Several monohydroxy fatty acids have also been identified in psoriatic skin lesions, the most abundant being the linoleic acid metabolite 13-hydroxy-9,11-octadecadienoic acid (13-HODD). This compound as well as 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and 9-hydroxy-10,12-octadecadienoic acid (9-HODD), are present in scale obtained by abrasion of psoriatic lesions in microgram amounts (Camp *et al.*, 1983). Unlike 12-HETE, neither 13-HODD nor 9-HODD were found to stimulate leucocyte movement in an agarose microdroplet chemokinesis assay (Camp *et al.*, 1983) but their other possible biological properties have not been studied.

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It has been reported that the arachidonate metabolite 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) is a potent inhibitor of the formation of 5-lipoxygenase products in leucocyte suspensions (Vanderhoek *et al.*, 1980). Because of the abundance of 13-HODD in psoriasis and the fact that its ω-configuration is identical to that of 15-HETE, we have investigated its ability to inhibit the formation of LTB_4 by 5-lipoxygenase in human neutrophils, and have compared its potency with that of 15-HETE, 9-HODD and 12-HETE.

Methods

Synthesis of monohydroxy fatty acids

15-HETE and 13-HODD were prepared by incubating arachidonic acid and linoleic acid, respectively, with soybean lipoxidase according to one of the methods of Baldwin *et al.* (1979). Briefly, fatty acid (100 mg) and enzyme (15 mg) were incubated at room temperature in 0.05 M sodium borate buffer, pH 9.0, containing a minimal quantity of aqueous ammonia (sp. gr. 0.88), in a total volume of 206 ml. After 30 min, 60 mg sodium borohydride dissolved in 2 ml borate buffer was added. After a further 30 min the reaction was stopped by acidification to pH 3.5 with 4 M HCl. The monohydroxy fatty acids were extracted into ethyl acetate, and following evaporation the residue

was purified by Lipidex 5000 gel partition chromatography as previously described (Camp *et al.*, 1983). The monohydroxy fatty acid peak was identified by ultraviolet (u.v.) absorbance monitoring of column effluent, and was further purified by straight phase high performance liquid chromatography (h.p.l.c.) by use of a 25 cm × 8 mm i.d. Spherisorb S5W semipreparative column eluted with hexane/propan-2-ol/methanol/acetic acid (975/22/26/1, by vol) at 2.7 ml min^{-1}. The final concentration of 15-HETE and 13-HODD was determined by u.v. absorbance spectrophotometry. Racemic 12-HETE and 9-HODD were prepared by photo-oxidation of arachidonic and linoleic acids, respectively, followed by sodium borohydride reduction and chromatographic purification, as previously described (Camp *et al.*, 1983). The hydroxy fatty acids were stored at −20°C in methanol.

Assay for 5-lipoxygenase in human leucocyte suspensions

Mixed leucocytes (approximately 75% neutrophils) were prepared by dextran sedimentation of fresh human venous blood, as previously described (Camp *et al.*, 1983), and were suspended in Eagle's minimal essential medium buffered to pH 7.4 with 30 mM HEPES buffer (buffered MEM). In preliminary experiments, 10^6 leucocytes were incubated in a shaking water bath at 37°C with 2 μM ionophore A23187 for different time periods (1–10 min), or with a range of ionophore A23187 concentrations (0–5 μM) for 4 min, in a total vol of 0.1 ml. Reactions were stopped by addition of 0.5 ml 0.1 M sodium acetate buffer, pH 3.5, and the LTB_4 in the mixture was extracted by the following solvent partition system which removes contaminating monohydroxy fatty acids from the final residue. The acidified incubation mixture was partitioned twice with 0.8 ml ethyl acetate and following centrifugation the pooled organic phases were evaporated. The residue was resuspended in 0.5 ml 0.1 M sodium phosphate buffer, pH 8.4, and partitioned twice with 0.8 ml 1-chlorobutane. In this system monohydroxy fatty acids and less polar material are preferentially soluble in the organic phase, whereas LTB_4 and more polar material remain in the aqueous phase (Barr *et al.*, 1984). The aqueous phase was then acidified with 30 μl glacial acetic acid and extracted twice with 0.8 ml ethyl acetate. The pooled ethyl acetate was evaporated, vacuum desiccated and stored at −20°C in 50 μl methanol. Prior to bioassay, the methanol was evaporated *in vacuo* and the residues redissolved in 0.3 ml buffered MEM. These solutions were assayed in duplicate against standard LTB_4 after 100 fold dilution, by an agarose microdroplet chemokinesis method (Smith & Walker, 1980). Results are expressed as LTB_4 equivalents released per 10^6 cells.

In order to show that LTB_4 was the only chemokinetically active material present in the final residues assayed by the agarose microdroplet method, 10^6 leucocytes were incubated with 2 μM ionophore A23187 for 4 min in a total vol of 0.1 ml. The reaction mixture was extracted as described above, and the final ethyl acetate residue subjected to straight phase h.p.l.c. by use of a 25 cm × 4.9 mm i.d. Nucleosil 50–5 μm analytical column eluted with hexane/propan-2-ol/methanol/acetic acid (85:10:5:0.1, by vol) at 1 ml min^{-1}. One min fractions were collected and evaporated, and the residues assayed for chemokinetic activity.

Preliminary experiments had also shown that high concentrations of 15-HETE, 13-HODD, 12-HETE and 9-HODD (100 μM each) inhibited LTB_4-induced leucocyte movement in the agarose microdroplet chemokinesis assay (data not shown). Therefore 15-HETE, which had similar partition characteristics to the other monohydroxy fatty acids tested, was subjected to the solvent partition systems described above, and the final ethyl acetate residue tested for its effects on LTB_4-induced leucocyte movement in the agarose microdroplet assay.

In experiments to determine the effects of monohydroxy fatty acids on the production of LTB_4 by ionophore A23187-stimulated leucocyte suspensions, 10^6 leucocytes were pre-incubated for 4 min at 37°C in 95 μl buffered MEM with different concentrations of 15-HETE, 13-HODD, 12-HETE and 9-HODD. After 4 min, ionophore A23187, dissolved in 5 μl buffered MEM, was added to give a final concentration of 2 μM. After a further 4 min, the reaction was stopped and the mixture extracted as described above.

Cell viability was assessed by trypan blue dye exclusion before and after incubation of leucocytes (10^6 cells per 0.1 ml) with 50 μM 15-HETE for 4 min at 37°C.

Materials

LTB_4 was a gift from Dr J. Rokach, Merck Frosst, Pointe Claire-Dorval, Canada. Arachidonic acid, linoleic acid and sodium borohydride were obtained from Sigma, Poole, Dorset. Soybean lipoxidase (Fluka) was supplied by Fluorochem, Glossop, Derbyshire. Calcium ionophore A23187 (free acid) was from Calbiochem and was supplied by Cambridge Bioscience, Hardwick, Cambridgeshire. The sources of materials used in the agarose microdroplet chemokinesis assay were as previously described (Camp *et al.*, 1983) except for Indubiose A37 which was obtained from Uniscience, London. Organic solvents (h.p.l.c. grade) were obtained from Fisons, Loughborough, Leicestershire and all other reagents were from B.D.H., Poole, Dorset.

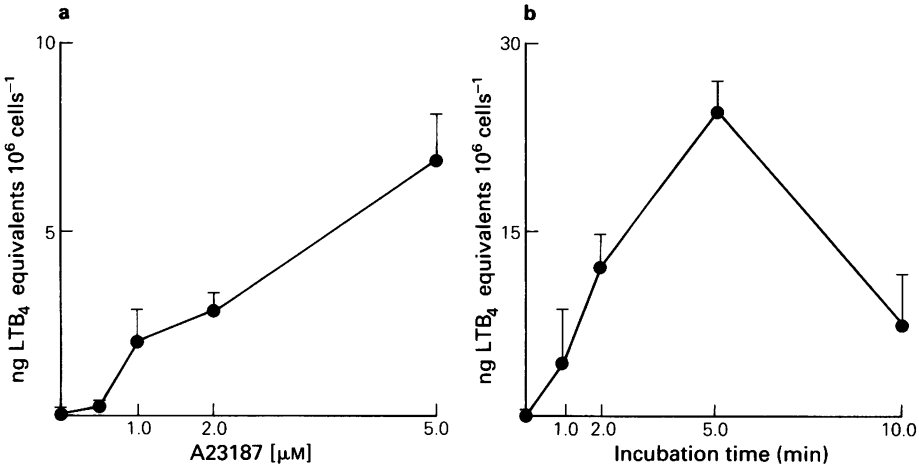

Figure 1 Effect of calcium ionophore A23187 concentration (a) and incubation time (b) on the generation of leukotriene B_4 (LTB_4)-like activity by mixed human leucocytes. Each 0.1 ml reaction mixture contained 10^6 leucocytes and was incubated for 4 min, $n = 4$ (a) or with 2 μM A23187, $n = 6$ (b). Points show mean values and bars show s.e.m.

Results

Figure 1 shows the effects of ionophore A23187 concentration and incubation time on the production of LTB_4-like material by suspensions of mixed human leucocytes. A near linear rate of LTB_4 production was evident at an ionophore concentration of 2 μM and incubation time of 4 min (Figure 1b). These conditions were adopted in subsequent experiments. When 11 aliquots of leucocytes prepared from the same fresh blood sample were incubated with 2 μM calcium ionophore A23187 for 4 min and the LTB_4 equivalents determined by the agarose microdroplet method as described (using leucocytes from a single donor for the assay), an intra-assay coefficient of variation of 15% was obtained. However, when 24 aliquots of leucocytes, each from different fresh blood samples, were incubated, extracted and assayed (with leucocytes from different blood samples for each agarose microdroplet assay), the LTB_4 equivalents produced per 10^6 cells varied from 1.0–25.5 ng, giving an inter-assay coefficient of variation of 86%.

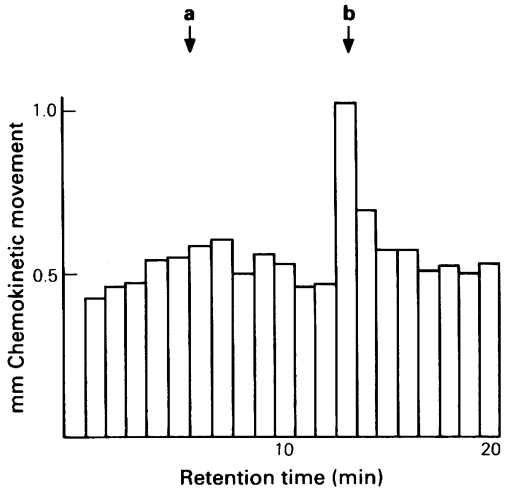

Figure 2 Chemokinetic activity in straight phase h.p.l.c. fractions after selective solvent extraction of 4 min incubations containing 10^6 leucocytes and 2 μM A23187 in 0.1 ml. Each h.p.l.c. fraction was evaporated, redissolved in 0.3 ml buffered MEM and assayed in duplicate after 100 fold dilution. Activity is expressed as the distance moved by leucocytes. The elution times of standard 12-HETE (a) and LTB_4 (b) are shown.

After straight phase h.p.l.c. of extracts of reaction mixtures, assay of evaporated fractions showed a single peak of chemokinetic activity occurring at the same retention time as standard LTB_4. Similar results were obtained in three experiments, one of which is illustrated in Figure 2.

In the preliminary experiments in which 15-HETE (100 μM in 0.1 ml buffered MEM) was subjected to extraction using the described solvent partition system, the final ethyl acetate residue had no effect on LTB_4-induced leucocyte movement in the agarose microdroplet assay (data not shown). The results obtained from experiments in which monohydroxy fatty acids were incubated with ionophore A23187 stimulated leucocytes are shown in Figure 3. In view of interassay variation, each result is expressed as a percentage of the LTB_4 equivalents produced by 10^6

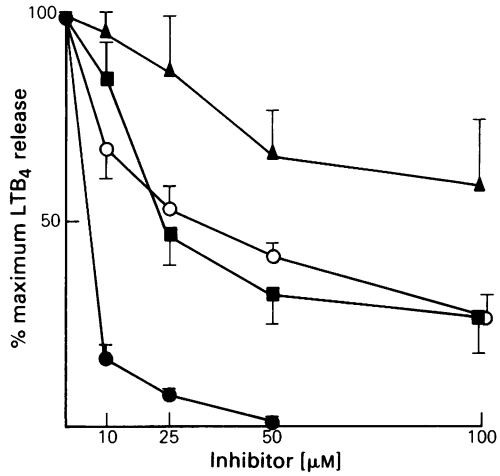

Figure 3 Inhibitory effect of 15-HETE (●, $n = 8$)., 13-HODD (○, $n = 6$), 12-HETE (■, $n = 6$) and 9-HODD (▲, $n = 4$) on leukotriene B_4 (LTB_4) production by ionophore A23187-stimulated mixed human leucocytes. Each point represents the mean of n experiments and bars show s.e.mean. Each result is expressed as a percentage of the LTB_4 equivalents produced by 10^6 cells in the absence of hydroxy fatty acid. Approximate IC_{50} values in μM for 15-HETE, 13-HODD, 12-HETE and 9-HODD are 6, 32, 23 and > 100 respectively, and the percentage inhibition at 50 μM was 99, 59, 68 and 34 respectively.

cells in the absence of hydroxy fatty acid. Approximate IC_{50} values for each hydroxy fatty acid were obtained from the curves shown in Figure 3, and are given in the legend which also shows the percentage reduction of LTB_4 production in the presence of 50 μM concentrations of each hydroxy fatty acid. Trypan blue dye exclusion studies showed more than 90% viability after incubation of leucocytes with 50 μM 15-HETE for 4 min (n = 5).

Discussion

In initial experiments, a near linear rate of production of LTB_4-like activity was obtained when 0.1 ml reaction mixtures containing 10^6 leucocytes were incubated with 2 μM calcium ionophore A23187 for 4 min. The lower levels of LTB_4 measured after 10 min incubations (Figure 1b) are likely to be the result of a combination of binding of LTB_4 to the cells as well as metabolism of LTB_4 by the cells to the chemokinetically less active ω-oxidation products, 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (Bray, 1983). In other initial experiments, 100 μM concentrations of the four monohydroxy fatty acids to be tested were found to inhibit LTB_4-induced leucocyte movement in the agarose microdroplet chemokinesis assay. However, it was shown that the solvent extraction method used prior to the chemokinesis assay removed the inhibitory monohydroxy fatty acids, such that they had no effect on the final assay.

Under the above optimal conditions, the four monohydroxy fatty acids were tested for their capacity to inhibit the formation of LTB_4 by ionophore stimulated leucocytes. The results (Figure 3) show that 15-HETE is a more potent inhibitor than 13-HODD and 12-HETE, as indicated by its lower IC_{50} value and the almost complete inhibition of LTB_4 formation at a 15-HETE concentration of 50 μM. The IC_{50} value of 6 μM obtained for 15-HETE is thus very similar to the value of 6.2 μM reported by Vanderhoek *et al.* (1980). The potency of 9-HODD was much less, such that an IC_{50} value could not be obtained.

The work of Wilkinson *et al.*, (1985) suggests that at least part of the inhibitory effect of 15-HETE on LTB_4 formation via 5-lipoxygenase is through competition of 15-HETE with arachidonic acid for the substrate binding site on 5-lipoxygenase, with the resulting metabolism of 15-HETE to 5,15-dihydroxy-eicosatetraenoic acid. We propose that the inhibitory effect of 12-HETE on LTB_4 production by ionophore-stimulated leucocytes may be, at least in part, through such a mechanism, whereby 12-HETE competes with arachidonic acid for substrate binding sites on 5-lipoxygenase and is itself metabolised to 5S,12S-dihydroxy-6,10-*trans*-8,14-*cis*-eicosa-tetraenoic acid. The latter compound has been reported to be a product of arachidonic acid metabolism by separate 5-lipoxygenase and 12-lipoxygenase enzymes, acting independently (Borgeat *et al.*, 1981), and would be expected to be formed by the action of 5-lipoxygenase on 12-HETE. However, 9-HODD and 13-HODD, which lack a double bond in the 5 position, are thereby not susceptible to metabolism by 5-lipoxygenase. Their inhibitory effect on LTB_4 production may therefore be through true 5-lipoxygenase inhibition, and not through substrate competition. Inhibition of LTB_4 production by stimulated leucocytes does not appear to be a non-specific effect of different monohydroxy fatty acids, in view of the weak inhibitory capacity of 9-HODD relative to the other compounds tested (Figure 3).

Quantification of 13-HODD and 12-HETE in extracts of the skin lesions of psoriasis has shown levels of approximately 85 μmol and 40 μmol per kg surface scale respectively (Camp *et al.*, 1983). It is therefore possible that these hydroxy fatty acids might be capable of acting as endogenous modulators of 5-lipoxygenase activity in human skin.

This work was presented to the Society for Investigative Dermatology as a poster (Fincham & Camp, 1984).

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