Human Neutrophil Chemotaxis in Response to Diepoxides of Linolenic Acid

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Diepoxides of linolenic acid were found to be chemoattractants. The concentration that produces 50% of maximal chemotaxis was 4.5 10^{-7} mol/l for human neutrophils when investigated in a chemotaxis test based on the method of spectrophotometric determination of myeloperoxidase activity with buffers containing bovine serum albumin. Leukotriene B_4 was used as positive control. The concentration of leukotriene B_4 that produces 50% of maximal chemotaxis was 1.8 nmol/l. No chemotactic activity was observed when monoepoxides of linoleic or linolenic acid, diepoxides of linoleic acid or triepoxides of linolenic acid were used. Mono-, di- and triepoxides of polyunsaturated fatty acids were synthesized with meta-chloroperbenzoic acid, separated by TLC and HPLC and identified by GC/MS.

Introduction

The directional movement of cells towards a gradient of specific chemoattractants is called chemotaxis. Chemotaxis can be observed in many biological processes, e.g. embryogenesis, fertilization, wound healing, inflammatory reactions and the spread of tumor cells (Snyderman and Goetzl, 1981). Various assays have been established to investigate the chemotactic activity of cells including the Boyden chamber assay (Boyden 1962), the agarose diffusion test (Nelson *et al.,* 1978) and the determination of the activity of specific enzymes like myeloperoxidase (Stelmaszynski and Zgliczynski, 1974) neutrophil granulocytes (PMNs) are among the best investigated cells and are known to respond to many chemotaxins. Besides formyl-peptides like formyl-methionyl-leucyl-phenylalanine (FMLP), some of the most potent chemotaxins are oxidized polyunsaturated fatty acids (PUFA) such as leukotriene B_4 (LTB_4) (Palmblad *et al.,* 1981), 12-hydroxyeicosatetraenoic acid (12-HETE) (Turner *et al.,* 1975) and 8,15-dihydroxyeicosatetraenoic acid (8,15-diHETE) (Kirsch *et al.,* 1988). Other lipid peroxidation products, e.g. 4-hydroxynonenal and other 4-hydroxy-aldehyds which have been shown to accumulate during inflammatory reactions (Dubouloz and Dumas, 1954; Bragt *et al.,* 1979), also attract PMNs (Curzio *et al.,* 1985). Epoxides of PUFAs are produced during heart infarction (Dudda and Spiteller, 1995). In addition, infiltrated PMNs have been observed in the infarcted area. As the PMN infiltration could be, in part, due to the formation of epoxides it seems to be possible, that these metabolites are involved in the pathophysiological process of this disease. Therefore, we were interested to investigate the chemotactic activity of diepoxides of linolenic acid.

Abbreviations: BSA, bovine serum albumin; DEL, HPLC fraction of diepoxides of linoleic acid; DELEN, HPLC fraction of diepoxides of linolenic acid; ED_{50}, concentration that produces 50% of maximal chemotaxis; FMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hank's balanced salt solution; HPLC, high pressure liquid chromatography; HETE, hydroxyeicosatetraenoic acid; HTAB, hexadecyltrimethylammonium bromide; LPO, lipid peroxidation; LTB_4, leukotriene B_4; mcPBA, *meta*-chloroperbenzoic acid; MEL, HPLC fraction of monoepoxides of linoleic acid; MELEN, HPLC fraction of monoepoxides of linolenic acid; MPO, myeloperoxidase; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide; MW, molecular weight; NAPP, sodium phosphate buffer; PMN, neutrophil granulocytes; PUFA, polyunsaturated fatty acid; PVP, polymer of 1-vinyl-2-pyrrolidinone; THF, tetrahydrofurane; TMB, tetramethylbenzidine.

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Materials and Methods

GC/MS

GC/MS measurements were carried out using a fused silica DB-1 capillary column (length: 30 m; inner diameter: 0.32 mm; carrier gas: H_2 2 ml/min; temp. prog.: 80°C-280°C at 3°C/min). The GC was coupled to a Finnigan MAT 312 double focussing mass spectrometer operated under EI conditions at 70 eV.

TLC

TLC separation of epoxides was achieved using 20 cm x 20 cm coated plates with a 0.75 mm layer of silica gel $60F_{254}$. A 4:1 mixture of cyclohexane and ethylacetate was used as mobile phase. Samples up to 50 mg were separated on one plate.

HPLC

HPLC was performed on a Spherisorb ODSII 5μm column (250 x 8 mm) with a solvent flow rate of 2 ml/min using UV detection at 212 nm. The compounds were separated using gradient elution with water/acetonitrile.

Trimethylsilylation

0.3 mg of the substrate was dissolved in 10 μl THF (purified and dried on molecular sieve) and 20 μl MSTFA were added. The mixture was allowed to stand at room temperature for 12 h. 1 μl of the mixture was subjected to GC/MS.

Synthesis of epoxides of PUFA

300 mg of linoleic acid or linolenic acid were dissolved in 20 ml CH_2Cl_2 and added to 300 mg mcPBA dissolved in 10 ml saturated $NaHCO_3$ solution. After 2 h at 0°C the reaction products were extracted with CH_2Cl_2, the organic phase was washed with saturated $NaHCO_3$ solution and dried over Na_2SO_4. After evaporation of the solvent the residue was subjected to TLC. Monoepoxides were obtained by scraping off the silica gel from r_f=0.40–0.50, diepoxides of linoleic acid and linolenic acid were found in the zone between r_f=0.17–0.25, triepoxides of linolenic acid were detected in the zone between r_f=0.1–0.17. The TLC fractions were eluted with ethylacetate and subjected to HPLC after evaporation of the solvent. Single peaks were collected. An aliquot was trimethylsilylated and the compounds identified by GC/MS.

Isolation of neutrophils from human blood

Blood was taken from a healthy white 59 years old male.

Preparation of polymorphonuclear neutrophilic granulocytes (PMNs)

PMNs of a healthy 59 years old white man were isolated from heparinized (100 int. units/ml Liquemin®) venous blood. Erythrocytes were removed by sedimentation in 6% (w/v) dextran (MW about 70.000) in physiological saline (0.9% NaCl; w/v) for 45 min at 37°C and subsequent centrifugation of the leukocyte-rich supernatant on Ficoll-Histopaque® (density: 1.077 g/cm) for 20 min at 550×g, 4°C. Remaining erythrocytes in the pellet were removed by hypotonic lysis in 0.2% (w/v) sodium chloride. Isotonic conditions were immediately restored by 1:2 dilution with 1.6% (w/v) sodium chloride. The leukocytes were washed by twofold centrifugation for 10 min at 400×g, 4°C and resuspension in physiological saline. The supernatant was discarded and the isolated PMNs were resuspended in Hank's balanced salt solution without phenol red, pH 7.4, supplemented with 0.1% (w/v) purified BSA. Viability of the cells was estimated by staining with trypan blue and exceeded 95%.

Chemotaxis assays

The chemotaxis assay was performed with modified Boyden chambers and PVP-coated polycarbonate filters (pore size: 3μm). The upper compartment contained $2x10^6$ human PMNs in 0.2 ml HBSS supplemented with BSA. The lower compartment was filled with 0.5 ml buffer alone or with the chemoattractant in various concentrations. The chambers were incubated for 60 min at 37°C in a humidified atmosphere containing 5% CO_2 in air. The number of PMNs migrated into the lower compartment was estimated spectrophotometrically by measuring the activity of the enzyme myeloperoxidase (MPO) in a calibrated assay.

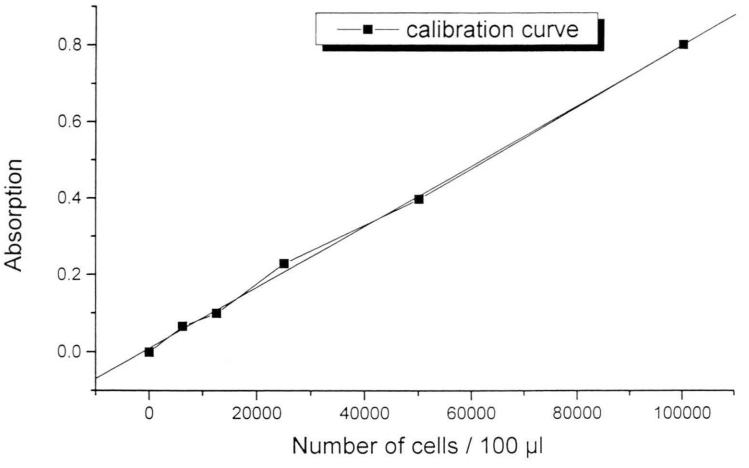

Fig. 1. Calibration curve of 655 nm absorption in relation to the number of neutrophil granulocytes diluted in hexadecyltrimethylammonium bromide buffer.

Myeloperoxidase activity

MPO activity was determined after lysis of migrated PMNs with buffer containing 0.5% (w/v) hexadecyltrimethylammonium bromide (HTAB) and 10 mmol/l morpholinopropane sulfonic acid (MOPS), pH 7.0. Oxidation of 0.0065% (w/v) tetramethylbenzidine (TMB) was initiated by incubation with 0.5 mmol/l H_2O_2 in sodium acetate-citrate buffer (0.1 mol/l), pH 6.0, for 30 min at room temperature in the presence or absence of a sample containing MPO in a final volume of about 265 µl. The reaction was terminated by addition of 1 N acetic acid and the MPO catalysed H_2O_2-dependent oxidation of TMB was measured spectrophotometrically at 655 nm in a microplate reader. The enzyme activity was calibrated by estimating the MPO activity of defined numbers of disintegrated non-migrated cells.

Results

The number of migrated cells was calculated using a calibration curve of the 655 nm absorption in relation to the number of PMNs (see Fig. 1).

According to GC/MS analysis only diepoxides of linolenic acid were found in the HPLC fraction. These diepoxides of linolenic acid demonstrated chemotactic activity towards human neutrophils with an ED_{50} value of 4.5 10^{-7} mol/l in our experiments (see Fig. 2).

LTB_4 used as positive control exhibited an ED_{50} value of 1.8 nmol/l which is about two orders of magnitude lower than the ED_{50} of the zone con-

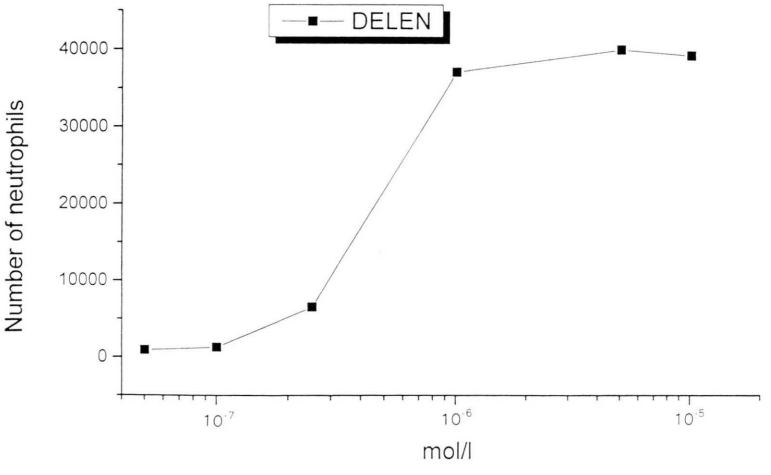

Fig. 2. Chemotaxis of neutrophil granulocytes in relation to the concentration of diepoxylinolenic acid (DELEN) (incubation time: 60 min); number of cells calculated from the calibration curve (see Fig. 1).

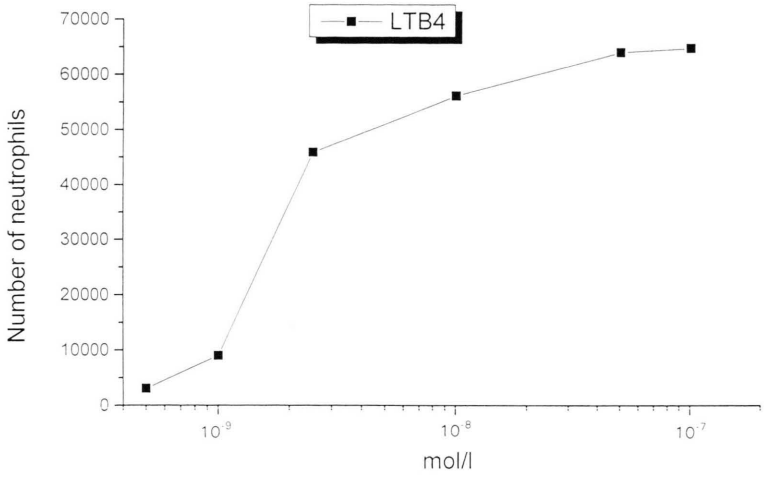

Fig. 3. Chemotaxis of neutrophil granulocytes in relation to the concentration of Leukotriene B_4 used as positive control (incubation time: 60 min); number of cells calculated from the calibration curve (see Fig. 1).

taining the diepoxides of linolenic acid (see Fig. 3). At the concentration of maximal activity the diepoxides of linolenic acid attracted about two times less neutrophils with regard to their total number than LTB_4 at its maximal concentration. Data are given in Table I.

Neither the HPLC fraction containing the monoepoxides of linolenic acid nor that of linoleic acid revealed any chemotactic activity. Even the HPLC fractions containing the diepoxides of linoleic acid and the triepoxides of linolenic acid did not attract human neutrophils.

Table I. Chemotaxis of PMNs in relation to the concentration of LTB_4 and diepoxylinolenic acid determined by 450 nm absorption (incubation time: 60 min); number of neutrophils calculated from the calibration curve (see Fig. 1).

LTB_4 [mol/l]	Absorption	Neutrophils (calculated)
0.5×10^{-9}	0.035 ± 0.002	3082
1.0×10^{-9}	0.082 ± 0.003	9018
2.5×10^{-9}	0.374 ± 0.018	45897
10.0×10^{-9}	0.455 ± 0.025	56127
50.0×10^{-9}	0.517 ± 0.011	63958
100.0×10^{-9}	0.523 ± 0.036	64716

DELEN [mol/l]	Absorption	Neutrophils (calculated)
0.5×10^{-7}	0.018 ± 0.000	935
1.0×10^{-7}	0.021 ± 0.003	1314
2.5×10^{-7}	0.062 ± 0.001	6492
10.0×10^{-7}	0.304 ± 0.012	37056
50.0×10^{-7}	0.327 ± 0.023	39961
100.0×10^{-7}	0.321 ± 0.021	39203

Discussion

It is well known that liver and other tissues contain cytochrome P450-linked epoxidases, which were shown to react with any double bond of arachidonic acid by epoxidation (Fitzpatrick and Murphy, 1989; Capdevila *et al.*, 1984). Hydroperoxides of PUFA produced by lipoxygenase in stressed tissue (Esterbauer *et al.*, 1992) also epoxidize double bonds of other PUFA. The level of PUFA epoxides increases in inflamed tissue consequently by activation of both epoxidases (Capdevila *et al.*, 1990) and lipoxygenases (Wolfe, 1982).

Thus, epoxides of linoleic acids were detected in burnt tissue (Ozawa *et al.*, 1989). They showed cytotoxic properties and were therefore called leukotoxins (Ozawa *et al.*, 1988). Recently, Dudda and Spiteller detected these compounds in heart tissue after myocardial infarction (Dudda and Spiteller, 1995). Epoxides of linoleic acid and linolenic acid were also detected in rice plants after attack of rice blast fungus (Kato *et al.*, 1983).

LTB_4, an epoxidation product of arachidonic acid produced during LPO, is one of the most potent chemotaxins. Therefore, we examined, if epoxides of other PUFA might have properties comparable to LTB_4. Indeed, we detected that the HPLC fraction containing several isomeric diepoxides of linolenic acid showed chemotactic ac-

tivity towards human neutrophils. Since we failed to separate the HPLC fraction in single isomeric compounds, we can not state exactly which one of the isomers acts as a chemotaxin or if it is a common property of all of them. The concentration necessary to induce a maximal response was higher than that of LTB_4 used as positive control. It might well be that the potency is underestimated, if only one of the isomers acts as chemotaxin.

The 9,10,12,13-diepoxides of linolenic acid resemble structurally LTB_4 with regard to the distance of the oxygen atoms and the anionic carboxylic group. These results support the assumption that amphipathic molecules are often effective chemotaxins (Hazelbauer, 1978).

Interestingly, neither the mono- and diepoxides of linoleic acid nor the mono- and triepoxides of linolenic acid revealed any chemotactic activity towards PMNs. This result indicates a specific receptor for epoxides. This assumption is in agreement with our findings that α-hydroxyaldehyds derived from plasmalogenepoxides produced in LPO do not reveal chemotactic activity (unpublished results) in contrast to 4-hydroxyaldehyds (Curzio *et al.*, 1985).

Our results demonstrate that not only hydroxylated derivatives of PUFA can act as chemotaxins, but also epoxidated PUFA. Since epoxides are formed during LPO following cell injury, they might play a role during necrotaxis, the directional movement of cells towards an injured cell (Bessis, 1974).

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