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NEUTROPHILS BIOSYNTHESIZE LEUKOTOXIN, 9, 10-EPOXY-12-OCTADECENOATE

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Summary: An epoxy derivative of linoleate, 9, 10-epoxy-12-octadecenoate, was demonstrated to be biosynthesized by neutrophils from various sources such as canine and human blood, and guinea-pig peritonea. It was nominated as leukotoxin from its 'toxic' activity onto mitochondrial respiration. From the reaction mixture of leukocytes with linoleate, an isomer of leukotoxin, 12, 13-epoxy-9-octadecenoate, and a 'non-toxic' hydroxy derivative of linoleate, 9-hydroxy-12-octadecenoate, were detected. Such a cascade reaction of linoleate by leukocytes was discussed. Biosynthesis of leukotoxin by neutrophils was substantially enhanced by the presence of calcium ion and calcium-ionophore, A_{23187}. Neutrophils contained leukotoxin, *ca.* 7 f moles/cell, which was extractable by 60% ethanol, but little of the isomer.

In the previous papers, we reported the existence of leukotoxin (LX), 9, 10-epoxy-12-octadecenoate, and its isomer (LX'), 12, 13-epoxy-9-octadecenoate, in lung lavages of rat after exposure to hyperoxia and in the reaction mixture of the lung lavage leukocytes with linoleate(1) as well as its existence in human burned skin (2). 'Toxic' effect of leukotoxin on mitochondrial respiration and on smooth muscle contraction was noted (1). Recently, we have been informed from Kato et al. (3) that they found exactly the same linoleate epoxides exist in the rice plant as self defensive substances against rice blast disease caused by a fungus, *Pyricularia orgzae*, of which characteristic is antimycin-sensitive. These facts indicate that LX is a common substance among animal and plant.

Abbreviations: HPLC, high performance liquid chromatography; GC-MS, gas-chromatography/mass spectrometry; NMR, nuclear magnetic resonance; RCI, respiratory control index; St III O_2, the rate of oxygen consumption in State III respiration; PLase, phospholipase; Percoll, polyvinylpyrrolidone-coated silica gel.

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In this paper, we will discribe the biosynthesis of this interesting substance by neutrophils from various animal and human sources in relation to biosynthesis conditions.

Materials and Methods

Materials: Modified Hank's balanced salt solution without calcium and magnesium (HBSS) was purchased from Sigma Chem. Co., and Percoll from Pharmacia. Linoleic acid of reagent grade was purchased from Wako Pure Chem. Co., and lipoxygenase from Sigma Chem. Co..

Preparation of neutrophils: Neutrophils were obtained from human or canine blood or from guinea-pig peritonea. Heparinized blood sample (200 ml) was mixed with its 1/5 volume of 6% dextran in physiological saline, and was settled for 30 min at room temperature for sedimentation of red blood cells. Leukocyte rich upper layer was mixed with its equal volume of HBSS, then centrifuged for 6 min at 400 x g. The resulting pellet containing leukocytes was subjected to hypotonic treatment to remove contaminating red blood cells (4). Neutrophils were separated from lymphocytes by density gradient centrifugation on Percoll (5). The neutrophils were washed twice with HBSS. The guinea-pigs (300 g body weight) were injected intraperitoneally with 30 ml of sterile isotonic saline containing 2% casein and the peritoneal exudate was collected 16 h later in siliconized flask, followed by centrifugation at 400 x g for 5 min. When necessary, the cell pellet was briefly exposed to hypotonic saline (0.45%) to lyse contaminating erythrocytes. Neutrophils were separated from mononuclear cells using Percoll as described above. The viability of the cells was measured by the trypan blue exclusion test (6).

Cell Count: Cells were counted by using a standard hemocytometer. The number of leukocytes was determined from these totals and from Wright-Giemsa-stained differential counts of cytocentrifuged preparations.

Biosynthesis of Leukotoxin: Linoleic acid was neutralized with 1 M Tris, then dissolved in 10% ethanol to be 10 mM, (substrate solution). 0.1 ml to 0.2 ml of the substrate solution and 1.9 ml to 1.8 ml of leukocyte suspension (1 to 5 x 10^7 cells per ml of HBSS) were mixed and incubated for 10 min at 30°C.

HPLC Analysis of Fatty Acids and Leukotoxin: It was followed as reported previously (1). Briefly: The reaction of leukocytes with linoleate was stopped by addition of ethanol to its final concentration of 15 to 60% and acidified to be pH < 3.0 by adding 1N HCl. The mixture was centrifuged and the ethanol concentration of the supernatant was adjusted to be 15% by adding distilled water. The mixture was passed through a Sep-Pak C_{18} cartridge equilibrated with 15% acidic ethanol. After washing the cartridge with 15% acidic ethanol and 35% acidic acetonitrile, fatty acids and LX were eluted from 75% acidic acetonitrile. They were extracted by diethyl ether and dissolved in aliquot of ethanol. The samples were injected into Develosil-ODS columns (5 μm particles, 0.46 x 15 cm and 0.46 x 25 cm, Nomura Chem. Co., Seto) mounted in a Shimadzu HPLC apparatus. Solvent system used was a mixture of acetonitrile:water:phosphoric acid (73:27:0.1). The flow rate (1.2 ml/min to 1.5 ml/min) and the column oven (CTO-6A) temperature (30° to 40°C) were controlled by a micro processor (SCL-6A). Fatty acids and LX were detected by their absorbance at 192 nm using an available-wavelength detector (SPD-6A) and their elution patterns were traced by a recorder (C-R3A). For a large scale preparation, a Develosil-ODS column (1.0 x 25 cm, Nomura Chem. Co., Seto) was used. A Gilson's fraction collector, model 201 with model 201-202 controller, was used for the collection of each fraction. Aliquots of each fraction were extracted by diethyl ether.

Determination of Chemical Structure: Samples of fatty acids derivatives were methylated on a phenylated methylsilicon column by phenyl-trimethylammonium hydroxide and analyzed by a Shimadzu GC-MS apparatus, GCMS-9020DF, with a data processor GCMS PAC-1100.

Measurement of Mitochondrial Respiration: Rat liver mitochondria were prepared as reported previously (7). Oxygen consumption by mitochondria was recorded at 20°C using a Beckman's 39550 oxygen electrode. RCI and St III O_2 were caluculated from traces of mitochondrial respiration.

Results

Biosynthesis of LX by Neutrophils: Fig. 1 A and B show HPLC patterns of the extracts from the reaction mixture of the human blood neutrophils with and

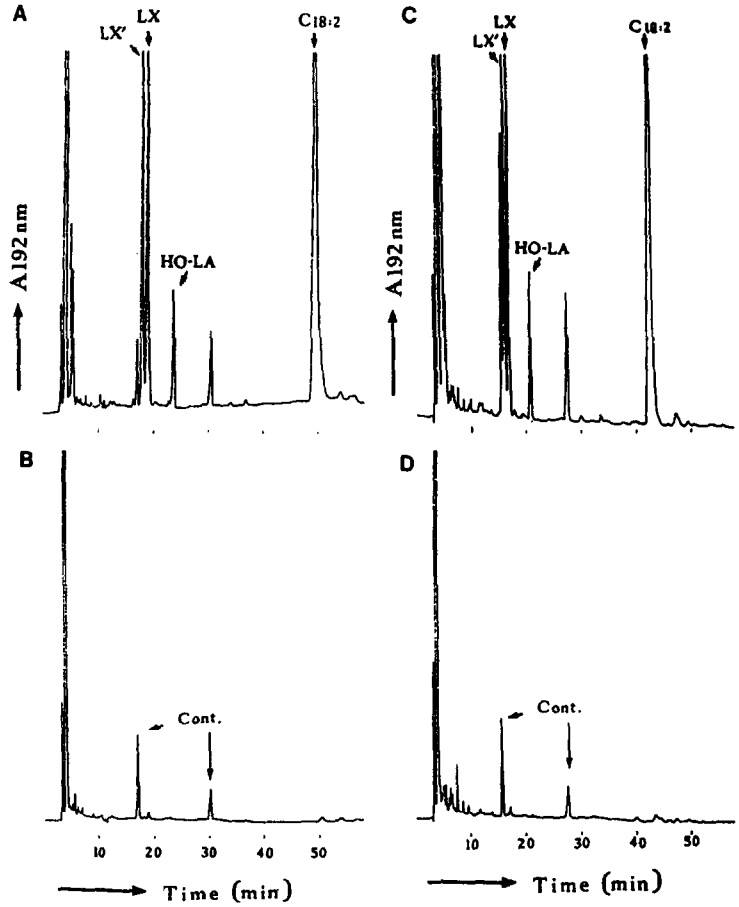

Fig. 1. HPLC analyses of fatty acids and LX extracted from the linoleate-neutrophils reaction mixture. Fatty acids and LX were extracted by 15% ethanol from the reaction mixture of human blood neutrophils (1.6×10^7 cells/ml) with (A) or without (B) linoleate (0.75 mM), or from that of guinea-pig peritoneal neutrophils (1.6×10^7 cells/ml) with (C) or without (D) linoleate (0.75 mM). After a partial purification by Sep-Pak C_{18} cartridge, aliquot of the sample (equivalent to one-fiftyth of the reaction mixture) was injected to HPLC mounted with Develosil-ODS columns (0.46 x 15 cm and 0.46 x 25 cm). Column temperature was set at 30°C (A and B) or 40°C (C and D). Solvent was acetonitrile:water:phosphoric acid (73:27:0.1). The flow rate was 1.2 ml/min until 30 min, then 1.5 ml/min until the end of analysis. Elution of fatty acids was monitored by their absorbance at 192 nm. Each peak was subjected to GC-MC analysis and identified as indicated in the figure. LX,LX' or HO-LA stands for leukotoxin,its isomer or 9-hydroxy-linoleate as described in the text.

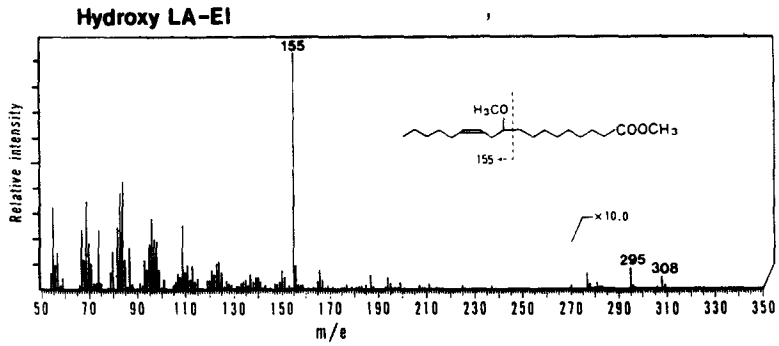

Fig. 2. Analysis of 9-hydroxy-linoleate by GC-MC measurements. Hydroxy-linoleate fractionated as shown in Fig. 1, were methylated on a phenylated methylsilicon column by phenyl-trimethylammonium hydroxide methanol solution, then subjected to GC-MS measurements. Its chemical ionization mass spectrum is illustrated.

without linoleate, and Fig. 1 C and D those of the guinea-pig peritoneal neutrophils. Those of the canine blood neutrophils showed quite similar patterns (data not shown). No significant difference in the patterns was induced by the difference of the oven temperature, 30°C and 40°C (Fig. 1. A *vs.* C). LX, 9, 10-epoxy-12-octadecenoate, and its isomer LX', 12, 13-epoxy-9-octadecenoate, are detected as a couple of sharp peak. Their GC-MS patterns and NMR data and their 'toxic' activity onto mitochondrial respiration were the same as reported in the previous paper (1). Another major peak in HPLC pattern designated HO-LA in Fig. 1 was identified as a hydroxy-linoleate, 9-hydroxy-octadecenoate, from its GC-MS pattern, as shown in Fig. 2. The hydroxy-linoleate showed no 'toxic' activity onto rat liver mitochondrial function such as RCI or St III O_2.

Preliminary experiment demonstrated that a preparation of sonicated neutrophils produced LX from hydroperoxy-linoleate which was prepared from linoleate by lipoxygenase in a test tube (data not shown).

Calcium as a mediator of LX biosynthesis: Table I summarizes the effect of calcium ion on the biosynthesis of LX by guinea-pig peritoneal neutrophils. Calcium and its ionophore significantly enhanced the LX biosynthesis. The optimum calcium concentration was 0.4 mM so far as tested. Higher concentration of calcium decreased the viability of leukocytes. It could be concluded that calcium ion is one of mediators of LX biosynthesis.

Table I. Biosynthesis of Leukotoxin by Neutrophils

Additions	Leukotoxin biosynthesized (f mole/cell)	Viability (%)
None^{+}	5.5	99.5
LA	6.4	93.7
LA + Ca^{++}*	13.6	87.9
LA + Ca^{++}* + A_{23187}**	19.0	83.9

$^{+}$ no incubation; * Ca^{++} = 0.4 mM; ** A_{23187} = 0.5 μM

Guinea-pig peritoneal neutrophils (2.5 x 10^7 cells/ml) was incubated with 0.5 mM linoleate and other additions at 30°C for 10 min. At the end of incubation, viability of the neutrophils was determined. Leukotoxin was extracted from the mixture by 60% ethanol, and analyzed as described in the text. $C_{10:0}$ was used as an internal standard.

Endogenous LX content in neutrophils: It was found that neutrophils contained a substantial amount of LX in the cell, but little LX'. Extraction of LX by various concentration of ethanol from guinea-pig peritoneal neutrophils was summarized in Table II. Increase in ethanol concentration up to 60% effectively released LX into medium from neutrophils (*ca.* 7 f moles/cell). Further increase in ethanol concentration over 60% did not affect the amount of LX released.

Table II. Extraction of Leukotoxin from Neutrophils with Various Concentration of Ethanol

EtOH (final conc.)	Leukotoxin released (f mole/cell)
15%	1.7
30%	5.4
60%	6.8

Ethanol was added to a suspension of guinea-pig peritoneal neutrophils (5.2 x 10^7 cell/ml) to its final concentration as indicated. The mixture was acidified to be pH<3.0 by adding 1N HCl, then centrifuged. Leukotoxin released from the neutrophils into the medium was measured by HPLC as described in the text.

Discussion

From HPLC analyses (Fig. 1), it is obvious that neutrophils obtained from both human blood and guinea-pig peritonea biosynthesizes LX about the same amount under the same conditions. Neutrophils from other animal sources such as canine blood (data not shown) and rat lung lavages (1) were found to biosynthesize LX in a similar rate. In Fig. 1, a peak other than LX and LX' was detected in the both samples. By GC-MS analyses, the peak was identified as a hydroxy-linoleate, 9-hydroxy-12, 13-octadecenoate (Fig. 2). 9-Hydroxy-linoleate might be converted from LX by cytosolic and/or microsomal epoxide hydrolases (8), as a consequence of linoleate cascade reaction. Predominant existence of LX, but little of LX', in the neutrophils *per se* (Table II) and that of 9-hydroxy-linoleate, but little of 12-hydroxy-linoleate in the reaction mixture of the neutrophils with linoleate (Fig. 1) suggest that LX is a biosynthesized product of the neutrophils and LX' is a by-product of LX during the extraction procedure.

By preliminary experiments, we found an antibiotic activity of LX against *Candida albicans* and *staphylococcus aureus*, besides of its 'toxic' activity to mitochondrial respiration and smooth muscle contraction. The existence of LX and LX' in rice plant as self defensive substances against a fungus reported by Kato *et al*. (3) and a potent biosynthesizing capability of the neutrophils from various animal sources reported previously (1) and here suggest that the gene to manusfect biosynthesis of LX, as a self defensive substance, originates from an early stage of evolution before the separation of animal and plant.

Predominant existence of $C_{18:2}$ (*ca*. 5 f moles/cell) in C-2 position of neutrophils phospholipids (over 60% in phosphatidylcholine and in phosphatidyl ethanolamine) reported by Yano et al. (9) is noted in relation to our present results. Under the pathologic conditions of human and animals, such as increase in Ca^{++} and PLase A_2 activity resulting release of unsaturated fatty acids from cell membranes, there could be abnormal over-production of LX by

neutrophils and subsequently disturbance of the functions such as cardiovascular shock due to the toxicity of LX.

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