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STIMULATION OF 5-LIPOXYGENASE ACTIVITY IN POLYMORPHONUCLEAR LEUKOCYTES OF RATS BY CASEINATE TREATMENT

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Abstract—In the present study, the lipoxygenase activity in polymorphonuclear (PMN) leukocytes of rats was characterized, and the change in lipoxygenase activity in peritoneal and peripheral PMN leukocytes challenged by caseinate was investigated. Peritoneal PMN leukocytes produced 5-HETE and LTB_4 as the major 5-lipoxygenase metabolites from arachidonic acid. The 5-lipoxygenase activity was calcium dependent. Caseinate treatment in rats significantly stimulated the 5-lipoxygenase activity in peripheral and peritoneal PMN leukocytes. Although it also stimulated the cyclooxygenase metabolic pathway, the effect was not as evident as that on the 5-lipoxygenase pathway. Since LTB_4 is a potent chemotactic factor for PMN leukocyte migration, the present results might explain the mechanism on the PMN leukocyte infiltration caused by caseinate treatment.

Metabolism of arachidonic acid leads to the formation of a group of biologically active compounds; prostaglandins and thromboxanes are formed by the cyclooxygenase pathway and hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs) by the lipoxygenase pathways. Borgeat and Samuelsson [1] have reported that rabbit polymorphonuclear (PMN) leukocytes biosynthesize 5-HETE and LTB_4 [5*S*, 12*R*-dihydroxy-6,14-*cis*-8,10-*trans* eicosatetraenoic acid] via the 5-lipoxygenase pathway upon the stimulation of calcium ionophore A23187. Using carrageenin-soaked polyester sponges in rats as an animal model, Simmons *et al.* [2] have observed that the concentration of LTB_4 during the early phase of the inflammatory response (4–8 hr) is sufficient to induce leukocyte aggregation, and chemotaxis and degranulation of PMN leukocytes *in vitro*. Therefore, LTB_4 may mediate, at least in part, the influx of PMN leukocytes and contribute to other events which characterize the inflammatory response. The authors suggested that PMN leukocytes are the major source of LTB_4 in the inflammatory locus.

Caseinate is a mixture of related phosphoproteins present in milk and cheese. It is present to the extent of 3% in bovine milk and is one of the most nutritive milk proteins since it contains all of the common amino acids. Intraperitoneal injection of 12% caseinate in saline induces the infiltration of PMN leukocytes into the peritoneal cavity. This is a routine method for collecting PMN leukocytes. In the present paper, we studied the effect of caseinate treatment on 5-lipoxygenase activity in rat PMN leukocytes.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Arachidonic acid (54.5 mCi/mmol), 12-L-[5,6,8,9,11,12,14,15-^{3}H(N)]-hydroxy-5,8,10,14-eicosatetraenoic acid (225 Ci/mmol), 15-L-[5,6,8,9,11,12,14,15-^{3}H(N)]-hydroxy-5,8,11,13-eicosatetraenoic acid (183 Ci/mmol), 5-D-[5,6,8,9,11,12,14,15-^{3}H(N)]-hydroxy-6,8,11,14-eicosatetraenoic acid (150 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [5,6,8,9,11,12,14,15-^{3}H(N)]Leukotriene B_4 (176 Ci/mmol) was purchased from Amersham International plc (Bucks, U.K.). Casein sodium salt, histopaque, calcium ionophore A23187 and indomethacin were obtained from the Sigma Chemical Co. (St. Louis, MO). RPMI medium was from GIBCO Laboratories (Grand Island, NY). Standards of prostaglandin E_2, thromboxane B_2, 5-HETE and LTB_4 were provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Thin-layer chromatographic plates of silica gel 60, 0.25 mm in thickness, were purchased from E. Merck (Darmstadt, F.R.G.). All reagents not specified above were of analytic grade.

Animals. Male Wistar rats aged 3 months were provided by the Animal Center of National Cheng Kung University Medical Center, Tainan, Taiwan. All animals were maintained on a commercial laboratory feed under a constant light–dark cycle (8.00 a.m. to 8.00 p.m.), and water was provided *ad lib.*

Preparation of peritoneal PMN leukocytes. Peritoneal PMN leukocytes were collected according to the method of stimulation with caseinate [3]. Rats were treated with intraperitoneal injection of 6 ml of 12% caseinate dissolved in saline. After 16–24 hr, the PMN leukocytes were harvested by washing the peritoneal cavity with 40 ml of saline. Cells were transferred into a plastic tube containing 10 U/ml of heparin. Red blood cells were removed by the hypotonic lysis with 24 ml of water for 20 sec. The tonicity was restored by the addition of 8 ml of 3.6% saline. Cells were then washed twice in saline and suspended in RPMI medium. Then the number of PMN leukocytes was determined by staining the cells with toluidine blue or Giemsa and counting in a hemacytometer.

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Preparation of peripheral PMN leukocytes. Peripheral PMN leukocytes were purified according to the previously reported methods [4, 5]. The rat whole blood was obtained directly from the abdominal aorta in the presence of 3.8% solution of sodium citrate (9 : 1, v/v) and pooled. The blood was mixed with one-fifth volume of 6% dextran solution in a 50 ml screw-cap round-bottom tube. The mixture was allowed to settle for 1 hr at room temperature. Red blood cells were settled with dextran. The white blood cell-rich plasma was collected and centrifuged at 400 *g* for 10 min. The cell pellet was resuspended in 6 ml of RPMI medium and layered over 3 ml of histopaque followed by centrifugation at 400 *g* for 30 min. The fractions with PMN leukocytes were collected and pooled. The residual red blood cells were removed by hypotonic saline lysis. The PMN leukocyte fraction was treated with 5 ml of 0.2% saline solution. After vortexing for 20 sec, 5 ml of 1.6% saline were added followed by centrifugation at 400 *g* for 10 min. This hypotonic treatment was repeated once and PMN leukocytes were then suspended in RPMI medium.

Assay of lipoxygenase activity in cell-free system. The PMN leukocytes were suspended in the assay buffer (25 mM Tris–HCl containing 14 μM indomethacin and 1 mM EDTA, pH 7.4) and disrupted by sonication with a model W-375 sonicator (Heat Systems-Ultrasonics, Inc.). The homogenates were centrifuged at 10,000 *g* for 20 min and the lipoxygenase activity in the supernatant was assayed. A mixture of 0.5 ml of cell supernatant prepared from 1×10^7 cells, and 0.2μCi of [1-^{14}C]arachidonic acid and 1 mM $CaCl_2$ was incubated at 37° for 20 min. The reaction was stopped by acidification with 1 N HCl. Products were extracted with ethyl acetate and evaporated with nitrogen gas. Residues were dissolved in a small aliquot of absolute ethanol and applied to thin-layer chromatographic plates. The plates were developed in a solvent system of petroleum ether : diethyl ether : acetic acid (50 : 50 : 1, v/v/v). The radioactive products were detected by a Berthold TLC scanner and autoradiography. Zones corresponding to the radioactive products were scraped into a scintillation vial for the determination of radioactivity by LKB Rackbeta liquid scintillation counter.

Arachidonic acid metabolism by intact PMN leukocytes. PMN leukocytes were suspended in Tris–HCl buffer (50 mM, pH 7.5) with NaCl (100 mM). Incubation mixture contained 2.5×10^6 cells, 0.2 μCi of [1-^{14}C]arachidonic acid, 1 μM calcium ionophore A23187 and 2 mM $CaCl_2$ in a final volume of 1 ml, unless stated otherwise. The mixture was incubated at 37° for 20 min. Extraction, separation and quantitation of arachidonate products were performed by the same methods as described above except when the products were separated by thin-layer chromatography in the organic phase of a solvent system of ethyl acetate : 2,2,4-trimethylpentane : acetic acid : water (11 : 5 : 2 : 10, v/v/v/v).

Identification of the lipoxygenase products. Residues containing [1-^{14}C]arachidonic acid and its metabolites after ethyl acetate extraction were dissolved in 20 μl of absolute ethanol, and mixed with the same volume of standard [^{3}H]LTB_4, [^{3}H]15-HETE, [^{3}H]12-HETE and [^{3}H]5-HETE, respectively. Metabolites of [1-^{14}C]arachidonic and tritiated standards were separated by reverse phase of high performance liquid chromatography (HPLC). A column (4× 250 mm) of LiChrosorb RP-18 (10 μm) was used and 52% acetonitrile in 17 mM phosphate buffer (pH 3) was used as the mobile phase. The flow rate was set at 1 ml/min and 0.4 ml portions were collected. Radioactivity of ^{3}H and ^{14}C in each fraction was then counted.

RESULTS

Metabolism of arachidonic acid in PMN leukocytes

In cell-free assays, [1-^{14}C]arachidonic acid was incubated with supernatant of cell homogenate of peritoneal PMN leukocytes in the presence of 14 μM indomethacin. Two major metabolites were formed on thin-layer chromatogram. They co-migrated with standard 5-HETE and LTB_4, respectively. When [^{14}C]-labeled samples were chromatographed with tritiated standards by high performance liquid chromatography, one product was eluted with standard [^{3}H]5-HETE and the other with [^{3}H]LTB_4. Their retention time was 30 and 8 min, respectively. Formation of 5-HETE and LTB_4 fraction constituted the total 5-lipoxygenase activity in cells. The increase in the 5-lipoxygenase activity was linear in fashion up to 1.4×10^7 cells used as enzyme source. The enzyme activity was calcium dependent as reported by Jakschik and Lee [6]. Presence of 1 mM $CaCl_2$ induced the maximal effect. Therefore, all subsequent assays were carried out in the presence of 1 mM $CaCl_2$ with the cell homogenate prepared from 1×10^7 cells.

In intact cell assays, peritoneal PMN leukocytes in the presence of 1 μM calcium ionophore A23187 and 2 mM $CaCl_2$ transformed the exogenous [1-^{14}C]arachidonic acid via the cyclooxygenase and the lipoxygenase pathways. LTB_4, 5-HETE, PGE_2 and TXB_2 were formed. Production of these products was linear in fashion up to 2.5×10^6 cells used in incubation. Therefore, all subsequent assays were carried out using 2.5×10^6 cells in the reaction mixture.

Arachidonate metabolism in PMN leukocytes treated with caseinate

Change in arachidonic acid metabolism in PMN leukocytes stimulated by caseinate was then studied. Arachidonic acid metabolism by peripheral and peritoneal PMN leukocytes stimulated by caseinate was compared with that by peripheral PMN leukocytes of control animals. Both cell-free and intact cell assays were performed. In cell-free assays, 5-lipoxygenase activity in PMN leukocytes was higher induced by caseinate treatment (Table 1). Formation of 5-HETE by peripheral and peritoneal PMN leukocytes induced by caseinate treatment was 3.4-fold and 3.6-fold higher than that of control peripheral PMN leukocytes, respectively. Formation of LTB_4 by peripheral and peritoneal PMN leukocytes treated with caseinate was 2.4-fold and 2.6-fold higher than that of control peripheral PMN leukocytes, respectively.

In intact-cell assays, the peripheral and peritoneal PMN leukocytes of rats treated with caseinate also

Table 1. Effect of caseinate treatment on 5-lipoxygenase activity in cell-free homogenates

Groups	LTB_4 (cpm)	5-HETE (cpm)
Peripheral (control)	1966 ± 179 (100%)	2730 ± 378 (100%)
Peripheral (caseinate)	4743 ± 497** (241%)	9398 ± 1500** (344%)
Peritoneal (caseinate)	5162 ± 621** (262%)	9726 ± 1052** (356%)

Caseinate treatment and enzyme assay were described in Materials and Methods. Each value is the mean ± SEM from 7 individual animals. Values in parentheses are the percentages compared with their parallel controls.
** $P < 0.01$.

showed more active arachidonate metabolism than the peripheral PMN leukocytes of control rats (Table 2). In the metabolism via 5-lipoxygenase pathways, the peripheral and peritoneal PMN leukocytes of rats treated with caseinate produced about 3-fold more 5-HETE and LTB_4 than the peripheral PMN leukocytes of control rats. The peripheral and peritoneal PMN leukocytes of rats treated with caseinate also produced more PGE_2 and TXB_2, which are the metabolites via fatty acid cyclooxygenase pathways, than the peripheral PMN leukocytes of control rats.

DISCUSSION

Beetens *et al.* [7] reported that two Δ^6-trans-isomers of LTB_4 (5*S*,12*S*- and 5*S*,12*R*-dihydroxy-6,8,10,14-EEEZ-eicosatetraenoic acid) were formed together with LTB_4 when rat peritoneal PMN leukocytes were stimulated with calcium ionophore A23187. The proportion of the isomers are 67% for LTB_4 and 33% for its two *trans*-isomers. LTB_4 is the major product among the three isomers. In the present studies, formation of LTB_4 was confirmed by HPLC analysis with 52% acetonitrile in 17 mM phosphate buffer (pH 3) as the mobile phase. Although the product was eluted with standard [^{3}H]LTB_4, the possibility was still not excluded of the presence of two *trans*-isomers of LTB_4 in the fraction of LTB_4, which were all transformed from [1-^{14}C]arachidonic acid. As the standards of both 6-*trans*-isomers are not available, their formation cannot be further confirmed. Therefore, the 5-lipoxygenase activity observed in this study may represent the formation of two *trans*-isomers as well as that of 5-HETE and LTB_4.

Activation of PMN leukocytes by immunological and other stimuli (e.g. calcium ionophore A23187) releases the endogenous arachidonic acid which results in an increase in cellular biosynthesis of LTB_4 and 5-HETE [1]. The conversion of the exogenous arachidonate by cell homogenate and intact cells was directly determined in the present studies. This kind of experimental design excluded the involvement of the release of arachidonic acid in cells during the activation by caseinate. The results (Tables 1 and 2) indicate that the stimulatory effect of caseinate is due to the direct stimulation of cellular 5-lipoxygenase activity. However, its mechanisms remain to be determined. The cyclooxygenase pathway was also stimulated by caseinate (Table 2), but the effect was not as evident as that on the 5-lipoxygenase pathway.

In summary, the 5-lipoxygenase pathway in PMN leukocytes was stimulated by caseinate treatment. As soon as arachidonic acid is released from phospholipid of cell membrane, PMN leukocytes treated with caseinate would biosynthesize more LTB_4. Since LTB_4 is as potent on a molar basis as the formyl peptides or C_{5a} as a chemotactic factor for PMN leukocyte migration [8, 9], the present findings might provide the plausible explanation on the mechanism leading to the accumulation of PMN leukocytes in caseinate-treated locus.

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Table 2. Effect of caseinate treatment on arachidonate metabolism in intact PMN leukocytes

Groups	LTB_4 (cpm)	5-HETE (cpm)	PGE_2 (cpm)	TXB_2 (cpm)
Peripheral (control)	898 ± 76 (100%)	3831 ± 662 (100%)	458 ± 43 (100%)	287 ± 72 (100%)
Peripheral (caseinate)	3178 ± 436** (354%)	10638 ± 1509** (278%)	1155 ± 114** (252%)	520 ± 69** (181%)
Peritoneal (caseinate)	3230 ± 352** (360%)	12650 ± 1121** (330%)	1213 ± 98** (264%)	490 ± 51** (170%)

Caseinate treatment and enzyme assay were described in Materials and Methods. Each value is the mean ± SEM from 7 individual animals. Values in parentheses are the percentages compared with their parallel controls.
** $P < 0.01$.

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