Comparison of Eicosanoids Production between Rat Polymorphonuclear Leukocytes and Macrophages: Detection by High-Performance Liquid Chromatography with Precolumn Fluorescence Labeling

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ABSTRACT—We developed a procedure for serial measurement of fluorescent derivatives of eicosanoids in biological samples by HPLC. The 9-anthryldiazomethane (ADAM)-derivatized sample was first fractionated through SEP-PAK silica into fraction 1 (eluate of chloroform:toluene) and fraction 2 (eluate of acetonitrile:methanol). Both fractions were loaded separately onto an ODS column, and eluted with a step-gradient of 85% and 95% acetonitrile for Fr-1 (HETE’s and arachidonic acid) and with 70% acetonitrile for Fr-2 (PG’s and LTB₄). The method was applied to the arachidonate products of rat peritoneal leukocytes which were stimulated with A23187. The polymorphonuclear leukocytes (PMNL), which were collected after stimulation with casein, released mainly LTB₄, 5-HETE, 6-K-PGF₁α, but little arachidonic acid. In contrast to PMNL, rat macrophages, which were collected after peritoneal injection of soluble starch and bacto peptone, released 5-HETE, arachidonic acid, and 6-K-PGF₁α, but no LTB₄. These differences might be partly caused by the differential rates of uptake or turnover of arachidonic acid into their membrane phospholipids.

When rat pleurisy was induced by carrageenin (1) or zymosan (2), we observed that PMNL appeared at the inflammatory site initially, followed by migration of monocytes/macrophages to the site. To determine the roles of leukocytes in the inflammatory reaction, the active products of these leukocytes, including the arachidonate metabolites, should be assessed. However, these arachidonate metabolites have a marked variety of biological activities including some conflicting actions. For example, it is well-known that PGI₂ inhibits platelet aggregation (3), and TXA₂ induces platelet aggregation (4). Therefore it is necessary to analyze all the arachidonate metabolites and arachidonic acid (AA) in biological samples. Previously we developed a method for the serial measurement of prostaglandins (PG’s) and leukotriene (LT) B₄ by high-performance liquid chromatography (HPLC) using a fluorescent derivatizing agent, 9-anthryldiazomethane (ADAM) (5–8). By this method, PG’s and LTB₄ released from casein-induced rat peritoneal leukocytes were assayed (8). In the present study, we devised a procedure for measuring hydroxyeicosatetraenoic acids (HETE’s) and AA together with the previously reported ADAM derivatives of PG’s and applied it to biological samples from rat peritoneal leukocytes.
MATERIALS AND METHODS

Agents

The following were obtained from commercial sources: [1-14C]-Arachidonic acid ([14C]-AA, 2.06 GBq/mmol, Amersham); 5(S)-hydroxyeicosatetraenoic acid (5(s)-HETE, Cayman Chemical Co.); arachidonic acid (AA, Nu chek Prep, Inc., Elysian, MN, U.S.A.); casein and starch soluble (Wako Pure Chemical Industries, Ltd., Osaka, Japan); bacto peptone (Difco Lab. Co., Detroit, MI, U.S.A.); EDTA-2Na (Dojindo Lab., Ltd., Kumamoto, Japan); bovine serum albumin (BSA, essential fatty acid-free) and trypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.); A23187 (Calbiochem. Co., La Jolla, CA, U.S.A.); and Percoll (Pharmacia LKB Biotechnology AB, Uppsala). 6-K-PGF1α, thromboxane (TX) B2, PGE2, and PGD2 were kindly supplied by Ono Pharmaceutical Co., Ltd., Osaka, Japan, and leukotriene (LT) B4 (Paesel Co., Germany), by Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan. 15(S)-Hydroxyeicosatetraenoic acid (15(s)-HETE) and 12(S)-hydroxyeicosatetraenoic acid (12(s)-HETE) (Cayman Chemical Co.) were gifts from Asahi Chemical Industry Co., Ltd., Tokyo, Japan. ADAM (9-anthryldiazomethane, Funakoshi Pharmaceutical Co., Tokyo) and 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861, Takeda Chemical Industries, Ltd., Osaka) were kind gifts.

Preparation of peritoneal leukocytes

Peritoneal leukocytes were collected from male Sprague-Dawley (SD) rats (300–500 g, Japan SLC Co., Hamamatsu) at 16 hr after the intraperitoneal injection of 1% casein solution (30 ml/rat) in Ca2+-Mg2+-free Krebs-Ringer bicarbonate solution, as previously reported (8). Then the polymorphonuclear leukocytes (PMNL) were separated from the collected leukocytes by the Percoll gradient centrifugation method. Thirty percent (v/v) and 80% (v/v) isotonic Percoll solutions were carefully introduced into a polypropylene tube, and the cell suspension was gently set on the top of the upper layer. After centrifugation (400 × g, 30 min, 15°C), the upper layer (mainly monocytes) and the lower layer (mainly PMNL) were collected separately, and then each was washed with Ca2+-Mg2+-free HBSS. When examined under the light microscope after Giemsa staining, the upper fraction contained PMNL (>98.5%), and the other contained monocytes (>92%).

Peritoneal macrophages were collected from male SD rats, injected intraperitoneally with 5% soluble starch and bacto peptone (5 ml/100 g body weight). Four days after the injection, the rats were killed by cutting the carotid artery under diethyl ether anesthesia, and then the peritoneal cells were harvested (9). The cells were suspended in RPMI 1640 medium (Gibco Lab. Co., NY, U.S.A.) supplemented with 10% fetal bovine serum (Filtron Co., Australia), penicillin G potassium (105 U/l) and streptomycin sulfate (0.1 g/l) (Meiji Seika, Tokyo, Japan). The cells containing macrophages were seeded at 1 × 107 cells per 60-mm Corning tissue culture dish (Corning, #25010 DISH) and incubated for 2 hr at 37°C in an atmosphere of 5% carbon dioxide in air. After the incubation, the dishes were washed three times with the Hanks balanced salt solution (HBSS) to wash out non-adherent cells. The number of adherent cells finally remaining was 7 × 105/dish.

Incubation

The Ca-ionophore A23187 was dissolved in ethanol (4 mM) and diluted with HBSS before use. The peritoneal leukocytes were preincubated at 37°C for 5 min in HBSS and then stimulated with A23187 (12.5 μM). The supernatant of the cell suspension was collected, and its pH was adjusted to 3.0 with 1 N HCl. The macrophages in HBSS containing 0.1% BSA were incubated at 37°C in presence of A23187 (12.5 μM).

Arachidonate metabolites extraction and derivatization

The above supernatants were passed through a SEP-PAK C18 column (Waters Associates,
Milford, MA, U.S.A.), and the methanol eluate was then evaporated (6, 7). The residues were mixed with the same volume of 0.2% (w/v) ADAM ethylacetate solution (50 μl) and left in the dark overnight at room temperature (5–8). The reaction mixture was applied to a cartridge of SEP-PAK Silica (Waters Associates, Milford, MA, U.S.A.) that had already been washed with chloroform : toluene (1:1) and was then eluted with 5 ml of chloroform : toluene (1:1). This fraction, Fr-1, contains ADAM derivatives of HETE’s and AA. Then the column was eluted with 5 ml of acetonitrile : methanol (4:1) to yield Fr-2, containing ADAM-derivatives of PG’s, TXB2, and LTB4 as previously described (8). The recovery of this step was >90%. These fractions were evaporated and dissolved in methanol : ethylacetate (1:1).

High performance liquid chromatography

In addition to the equipments for HPLC described previously (6), a GP-A40 gradient programmer (Japan Spectroscopic Co., Ltd.) and a CC-12 Integrator (System Instrument Co., Ltd.) were used. Derivatized samples and authentic standards were injected into the HPLC apparatus equipped with an ODS-column (YMC A303 ODS, 4.6 × 250 mm, 5 μm; YMC Co. Ltd., Kyoto, Japan). As mobile phases for Fr-1 (HETE’s and AA), two stepwise solvent systems, CH3CN/H2O/H3PO4 (85/15/0.1) and CH3CN/H2O/H3PO4 (95/5/0.1), were used at 30°C at the flow rate of 1.0 ml/min. The mobile phase for Fr-2 (PG’s, TXB2, and LTB4) was the solvent CH3CN/H2O/H3PO4 (70/30/0.1) at 30°C at the flow rate of 1.0 ml/min. The fluorescence intensity of the eluate was monitored by a fluorescent detector at 412 nm with excitation at 365 nm. The chromatogram was analyzed by a CC-12 Integrator.

Uptake of [14C]-AA into rat PMNL and macrophages

Rat PMNL (1 × 10⁷ cells) were incubated with [14C]-AA (222 kBq/ml) for 10, 20, and 40 min. Rat macrophages (5 × 10⁵ cells, in a 24-well culture plate, Corning, #25820) were incubated with [14C]-AA (780 kBq/ml) for 0.25, 1, and 2 hr. These cells were washed 2 times with HBSS buffer, and macrophages were incubated with 0.25% trypsin and 0.05% EDTA in phosphate-buffered saline (PBS) for 30 min to detach them. Radioactivity incorporated into these cells was counted with a liquid scintillation counter (Beckman).

Statistical analysis

Results were analyzed for statistical significance by Student’s t-test for paired observations.

RESULTS

Development of assay method for serial measurement of arachidonate metabolites

Figure 1 (A and B) show typical HPLC chromatograms of ADAM-derivatives of authentic arachidonate metabolites. All arachidonate metabolites were derivatized with ADAM and separated into two fractions by SEP-PAK silica. The two fractions (Fig. 1A, Fr-1 and Fig. 1B, Fr-2) were separately loaded onto an ODS column. The elution condition for Fr-1 gave good separation of 15-HETE, 12-HETE, 5-HETE, and AA. In order to confirm if the estimated LTB4 and 5-HETE were the products of lipoxygenase action, we examined the effect of a specific 5-lipoxygenase inhibitor, AA861 (10). AA861 (0.1 μM and 1.0 μM) significantly reduced the amount of 5-lipoxygenase products such as LTB4 and 5-HETE in a dose-dependent manner when PMNL were
stimulated with A23187 as shown in Table 1. However, AA861 (0.1 μM and 1.0 μM) dose-dependently increased the AA level in the incubation medium.

Fig. 1. Typical chromatograms of ADAM derivatives of authentic arachidonate metabolites. A (Fr-1) and B (Fr-2). Authentic arachidonate metabolites and AA were derivatized with ADAM and then fractionated through SEP-PAK silica into Fr-1 and Fr-2. The two fractions were injected separately into an HPLC apparatus equipped with an ODS-column (YMC A303 ODS, 4.6 × 250 mm, 5 μm, YMC Co., Ltd., Kyoto). As mobile phases for Fr-1 (HETE's and AA), two stepwise solvent systems, i.e., CH3CN/H2O/H3PO4 (85/15/0.1) and CH3CN/H2O/H3PO4 (95/5/0.1), were used at 30°C at the flow rate of 1.0 ml/min (A). The mobile phase for Fr-2 (PG's, TXB2, and LTB4) was the solvent of CH3CN/H2O/H3PO4 (70/30/0.1) at 30°C at the flow rate of 1.0 ml/min (B). Fluorescence intensity of the eluate was monitored at 412 nm with excitation at 365 nm. ADAM derivatives of the following amounts of authentic AA metabolites were injected: 10 ng of HETE’s and AA, 5 ng of PG’s and TXB2, and 10 ng of LTB4.

Table 1. Effect of AA861 on the release of 5-lipoxygenase metabolites and arachidonic acid from rat peritoneal leukocytes when stimulated with A23187

| Treatment            | LTB4    | 5-HETE   | AA       |
|----------------------|---------|----------|----------|
| A23187 alone         | 211.8 ± 11.6 | 360.2 ± 10.9 | 19.5 ± 2.6 |
| A23187 + AA861 (0.1 μM) | 87.6 ± 5.8*  | 197.5 ± 10.9* | 22.9 ± 1.3 |
| A23187 + AA861 (1.0 μM) | < 1      | 27.3 ± 2.6*  | 46.9 ± 2.1* |
| Control              | < 1      | < 1       | < 1      |

Rat peritoneal leukocytes (4 × 10⁷ cells) were stimulated with A23187 (12.5 μM) for 10 min, and 5-lipoxygenase metabolites and arachidonic acid (AA) in the incubation medium were assayed by the ADAM-method as described in the text. Values are means ± S.E. from 4 samples. * indicates that the value is statistically different from that of A23187 alone at P < 0.05. < 1: below the detection limit.
Serial measurement of arachidonate metabolite production in rat PMNL

Arachidonate metabolites produced from rat PMNL stimulated by A23187 were measured by the above method: Typical chromatograms are shown in Fig. 2A (Fr-1) and Fig. 2B (Fr-2). The PMNL released larger amounts of 5-HETE, 6-K-PGF1α, TXB2, PGE2, PGD2, and LTB4 than the amount of AA into the medium, when the cells were stimulated with A23187 (12.5 μM). Time courses of the released products of the PMNL are shown in Fig. 3. PMNL rapidly released 5-HETE, whose release was maximum at 5 min after the

![Fig. 2. Typical chromatograms of ADAM derivatives of arachidonate metabolites produced by rat polymorphonuclear leukocytes stimulated with A23187. A (Fr-1) and B (Fr-2): Rat PMNL (1 X 10^7 cells) which were obtained by Percoll density gradient centrifugation were stimulated with A23187 (12.5 μM) for 5 min, and arachidonate metabolites in the medium were extracted and derivatized with ADAM and then assayed as described in the Methods. HPLC was performed in the same way as Fig. 1.](image)

![Fig. 3. Time-course curves of arachidonate metabolites released from rat PMNL. A and B: Time-course curves of arachidonate metabolites and AA in the medium released from PMNL (1 X 10^7 cells) when stimulated with A23187 (12.5 μM). Panel A shows time-course curves of 5-HETE (△) and AA (▲) in Fr-1; Panel B shows those of PG's (6-K-PGF1α, ●; PGE2, △; PGD2, ▲), TXB2 (○) and LTB4 (□) in Fr-2. The value at 0 time was plotted as 0, since the medium showed a value less than the detection limit of 1 ng at 0 time. Values are the means from 3 samples; vertical bars indicate S.E. of the mean. All values are statistically different from those of non-stimulated cells at P < 0.05.](image)
addition of A23187. However, the AA release was relatively slow and gradually increased up to 40 min incubation with A23187. Fifteen-HETE and 12-HETE were not released by these cells. The time course of LTB₄ was similar to that of 5-HETE. However, the releases of PGE₂ and PGD₂ increased at slower rates than that of LTB₄. The release of 6-K-PGF₁₀ showed a slow rate until the initial incubation time of 10 min, and then the rate of the release increased markedly.

**Arachidonate metabolites production by rat macrophages**

Soluble starch and Bacto peptone induced rat peritoneal macrophages secreted many kinds of arachidonate metabolites into the incubation medium, when stimulated with A23187 (12.5 µM). Figure 4 shows typical chromatograms of arachidonate metabolites and AA produced from the macrophages stimulated with A23187 (12.5 µM) for 1 hr. As shown in the figure, the macrophages released AA, 5-HETE, and cyclooxygenase products: 6-K-PGF₁₀, TXB₂, PGE₂ and PGD₂. The time courses of products from the macrophages when they were stimulated with A23187 are shown in Fig. 5. 5-HETE and AA were slowly released from the macrophages when stimulated with A23187.

**Uptake of [¹⁴C]-arachidonic acid into the rat PMNL and macrophages**

Figures 6 and 7 show the time courses of [¹⁴C]-AA uptake into rat PMNL and macrophages. The PMNL showed rapid uptake of [¹⁴C]-AA and reached almost a plateau after 10–40 min. In contrast, the macrophages showed a continuous increase in the uptake of labeled AA, and its uptake was still increasing at 2 hr.

![Fig. 4. Typical chromatograms of ADAM derivatives of arachidonate metabolites produced by rat peritoneal macrophages stimulated with A23187. A (Fr-1) and B (Fr-2): Rat peritoneal macrophages (1 X 10⁷ cells), which were obtained by injection of soluble starch and bacto peptone, were stimulated with A23187 (12.5 µM) for 1 hr. Arachidonate metabolites in the supernatants were extracted and derivatized with ADAM and assayed. The Fr-1 chromatogram shows ADAM derivatives of 5-HETE and AA, and the Fr-2 chromatogram shows those of the cyclooxygenase products.](image-url)
Fig. 5. Time-course curves of arachidonate metabolites released from rat peritoneal macrophages. Time course curves of 5-HETE, AA, 6-K-PGF1α, and TXB2 in the medium when rat macrophages (1 × 10⁷ cells) were seeded and stimulated with A23187 (12.5 μM) as described in the Methods section. The value at 0 time was plotted as 0, since the medium showed a value less than the detection limit of 1 ng at 0 time. Values are the means from 3 samples; vertical bars indicate S.E. of the mean. All values of A23187-stimulated cells are statistically different from those of the control at P < 0.05. ▲: A23187, ○: control.

Fig. 6. Time-course curves of [14C]-arachidonic acid uptake into the rat PMNL. Rat PMNL (1 × 10⁷ cells) were incubated in the presence of [14C]-AA (222 kBq/ml) for 10, 20, and 40 min. Then the cells were washed with HBSS buffer 2 times, and radioactivity in the cells was counted with a liquid scintillation counter. Values are the means from 4 samples; vertical bars indicate S.E. of the mean.

Fig. 7. Time-course curves of [14C]-arachidonic acid uptake into the rat macrophages. Rat macrophages (5 × 10⁵ cells) were incubated in the presence of [14C]-AA (780 kBq/ml) for 0.25, 1, and 2 hr. Then the cells were washed with HBSS buffer 2 times and detached with 0.25% trypsin and 0.05% EDTA in PBS. Radioactivity in the cells was counted with a liquid scintillation counter. Values are the means from 4 samples; vertical bars indicate S.E. of the mean.
DISCUSSION

We have previously reported an isocratic ADAM-HPLC method for detection and quantification of PG's, TXB₂, PG metabolites, and LTB₄ (5–8). In the present study, an improved stepwise ADAM-HPLC method that could also detect HETE's was devised. HETE's which were eluted in the first eluate (Fr-1) from SEP-PAK had been discarded in the previous method as a wash. However, in our present method, HETE's and AA were detected in Fr-1. They showed good resolution, and a linear relationship was obtained for each of them. This means the derivatization with ADAM and the assay is quantitative. The present procedure has a merit over the previously reported assay using a radioisotope (11), for it is able to detect 8 different AA metabolites, including AA, in one particular sample which was applied twice on the HPLC apparatus. Moreover, the present method has the advantage that it can also evaluate endogenous AA, whereas other methods prelabelling cells with radioactive precursors have the disadvantage that the labeled precursors may be taken up only into a particular pool and their distribution might be different from the endogenous AA one.

We applied the present method to analyze the AA metabolites of rat leukocytes. Rat peritoneal leukocytes secreted 5-HETE, LTB₄ and AA. Furthermore, 5-HETE and LTB₄ were found to be lipoxygenase products by the inhibition with AA861, a 5-lipoxygenase inhibitor (10) (Table 1). The pattern of product release by A23187-stimulated PMNL differed in several aspects from the product release patterns of macrophages. The most distinct difference was the release of LTB₄: PMNL released mainly LTB₄, but macrophages released none. Macrophages released a larger amount of AA than the PMNL did (Fig. 5). The small amount of AA released from the PMNL might be partly due to the fast turnover of AA. This difference was further examined by the experiment of uptake of exogenous labeled arachidonic acid into these cells, as shown in Figs. 6 and 7. PMNL showed a plateau of incorporation of the labeled AA after approximately 10 min incubation, while macrophages continuously incorporated labeled AA up to 2 hr. In this experiment, during the initial 10–15 min, the rate of uptake of AA was higher in macrophages than PMNL, if incorporated AA was compared in terms of counts/min/cell. There are several papers on macrophages (12, 13) and PMNL (14, 15) that have reported results consistent with ours.

Some reports demonstrated that murine resident macrophages generate LTB₄ when stimulated with A23187 (16–18) or endotoxin (19), while the metabolites in macrophages are differentially altered by endotoxin tolerance (19) and Listeria immunization (20), suggesting that macrophages collected from an inflammatory site may have different natures from resident macrophages.

In conclusion, this HPLC method is simple and convenient for measuring arachidonate metabolites in the supernatant of peritoneal leukocyte cultures. The method can analyze all metabolites of AA in one particular sample at once.

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