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Determination of 5-hydroperoxyeicosatetraenoic acid produced in rat basophilic leukemia cell line RBL-2H3 by high-performance liquid chromatography with chemiluminescence detection

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Abstract

A simple and sensitive method, applicable to quantification of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) produced in cells has been developed using high-performance liquid chromatography on a silica gel column with chemiluminescence detection. 5-HPETE was clearly separated from other positional isomers of HPETEs and hydroxyeicosatetraenoic acids with hexane–isopropanol–acetic acid (97:3:0.01, v/v) as the mobile phase. The lower limit of detection was about 100 pg. 5-HPETE produced in 10^7 cells of RBL-2H3 cells stimulated with A23187 was determined as 480±30 pg. In the present study, 5-HPETE, which occurs naturally, was detected and quantitated for the first time in intact cells. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Oxidative stress is defined as an increased hydroperoxide metabolism and an increased steady-state concentration of any of the reactive oxygen species [1]. Oxidative stress is thought to play a major role in the pathogenesis of a wide range of human diseases, however, intracellular changes in the redox state have been reported to regulate various cellular functions differentially in physiological conditions [2].

5-Lipoxygenase, the primary regulator of leukotriene synthesis, catalyses a two-step reaction from arachidonic acid to the unstable epoxide leukotriene A_4 (LTA_4) via 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) [3,4]. This reaction in intact cells requires the presence of 5-lipoxygenase activating protein (FLAP) [5], that increases the efficiency of 5-lipoxygenase conversion of 5-HPETE to LTA_4 [6,7]. 5-HPETE is also converted to 5-hydroxy-eicosatetraenoic acid (5-HETE) as a result of reduction by glutathione peroxidase [8]. Therefore, 5-lipoxygenase is an enzyme that participates in hydroperoxide metabolism. Actually, 5-lipoxygenase activity depended on intracellular concentration of lipid hydroperoxides that were primarily suppressed by phospholipid hydroperoxide glutathione peroxidase (PHGPx) [9].

On the other hand, 5-HPETE is one of lipid

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hydroperoxides which are reduced by PHGPx, in addition to being the critical intermediate in the biosynthesis of leukotrienes. 5-HPETE has been reported to have several biological functions, for example, as a direct activator of 5-lipoxygenase [10], as an inhibitor of neuronal Na^+, K^+-ATPase [11] and as a possible inducer of apoptosis [12]. Recently, Rao et al. [13] demonstrated that other hydroperoxide metabolites of arachidonic acid, 12-HPETE and 15-HPETE, induced the expression of c-Fos and c-Jun proteins and increased the activating protein 1 (AP-1) in vascular smooth muscle cells. Therefore, the separation and quantitation of cellular HPETEs is important in order to understand their metabolic dynamics and biological roles for cellular functions.

The detection of HPETE has commonly exploited the ultra-violet absorption of the conjugated diene (235 nm). However, this method lacks sufficient sensitivity and specificity for the quantitation of trace amounts of HPETEs in cells. Various methods have been developed to measured lipid peroxides in biological samples where the concentration can be expected to very low. These techniques included iodine liberation [14], chemiluminescence detection [15,16], ultra-violet absorption with an in-line diode-array detector [17,18], coulometeric detection [19], fluorescence labeling [20,21], and peroxidase [22] or cyclooxygenase activity [23]. However, any study with these techniques had not been directed to measure the amounts of cellular HPETE, which occurs naturally.

In the present study, we attempted to apply a chemiluminescence–HPLC system to the detection of cellular HPETE. This method has already been applied for the specific detection and quantitation of phospholipid hydroperoxides [24]. After some modifications of the method, a fair amount of 5-HPETE produced in cells has been successfully indicated. The present report also demonstrates the accumulation of 5-HPETE in A23187-stimulated RBL-2H3 cells using the present methods.

2. Experimental

2.1. Reagents

Arachidonic acid, 5-HETE, 12-HETE, 15-HETE, 5(S)-HPETE, 12(S)-HPETE, and 15(S)-HPETE were purchased from Funakoshi (Tokyo, Japan). A23187, cytochrome *c*, and sodium borohydride ($NaBH_4$) were obtained from Sigma (St. Louis, USA). Luminol and HPLC-grade solvent were from Wako (Osaka, Japan). All other chemicals were of the highest grade available.

2.2. Extraction of arachidonic acid metabolites from A23187-stimulated RBL-2H3 cells

Rat basophilic leukemia (RBL-2H3) cells were maintained in RPMI-1640 medium [25] (Gibco BRL, Grand Island, New York, USA) with 5% fetal calf serum. Harvested cells (10^7 cells) were preincubated for 5 min in 1 ml of the reaction mixture (phosphate-buffered saline, pH 7.4, 10 μg/ml or 50 μ*M* of arachidonic acid and 1 m*M* of $CaCl_2$). Stimulation of RBL-2H3 cells was carried out by the addition of 10 μ*M* calcium ionophore A23187. After 5 min of stimulation, 3 ml of ethyl acetate was added to the mixture. The pH of the mixture was adjusted to 3.5 with 0.1 *M* HCl and then the ethyl acetate phase was collected and evaporated off. After the evaporation, samples were stored at −80°C prior to analysis.

Extracted samples were dissolved in methanol with 5 mg of $NaBH_4$. After 1 h of incubation at room temperature, reduced products of HPETEs were re-extracted with ethyl acetate as described above.

2.3. Separation and quantitation of 5-HPETE, 5-HETE and LTB_4

HPLC was carried out with a two-pump system (CCPS; Tosoh, Tokyo, Japan) for delivery of the solvent used as the mobile phase and the solution of chemiluminescence reagents, respectively. A LiChrosorb Si-60 silica gel column (240×4 mm; Merck, Darmstadt, Germany) was used. Standard solutions (0.1–100 ng of 5-HPETE) or lipid extracts were dissolved in 100 μl of ethanol. Fifty μl of this solution (corresponding to 5×10^6 of RBL-2H3 cells) was subjected to HPLC. The mobile phase consisted of hexane–isopropanol–acetic acid (97:3:0.01, v/v). The flow rate was 4 ml/min. The eluate was monitored at 235 nm with the LC-10AS UV spectrophotometric detector (Shimadzu, Kyoto, Japan). At the post-column junction after the UV detector, the

chemiluminescence reagent was fed into the eluate at a flow rate of 0.8 ml/min. The chemiluminescence reagent, contained 20 μg/ml of cytochrome *c* and 20 μg/ml of luminol in 50 m*M* of borate buffer, pH 10.8. Isomers of HPETEs were detected by the photon emission of luminol at 430 nm with the 825-CL chemiluminescence detector (Japan Spectroscopic, Tokyo, Japan). 5-HETE and LTB_4 were calculated from their absorption at 235 nm and at 280 nm, respectively.

3. Results and discussion

3.1. Separation and quantitation of 5-HPETE on normal-phase HPLC

A typical chromatogram of synthetic isomers of HPETEs and HETEs is shown in Fig. 1. Separation was accomplished on a normal-phase HPLC, which had become a standard method for analysis of hydroxy fatty acids isomers. 5-HPETE was successfully separated from 12-HPETE and 15-HPETE by using an isocratic mobile phase of hexane–isopropanol–acetic acid (97:3:0.01, v/v). However, separation between 12-HPETE and 15-HPETE was insufficient under the present conditions (Fig. 1A). These hydroperoxides of fatty acids were clearly separated from their corresponding reduced forms, HETEs (Fig. 1B).

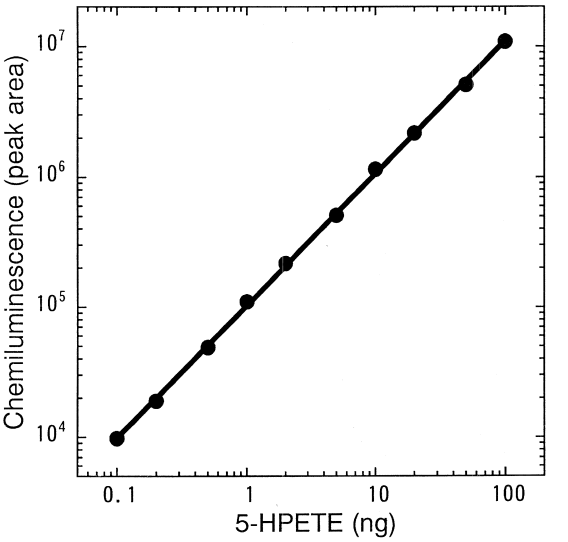

Fig. 2. Calibration line of 5-HPETE in the chemiluminescence–HPLC.

A calibration graph was prepared by using authentic HPETEs (Fig. 2). A linear relationship between peak areas and amounts of HPETE was obtained in the range from 0.1–100 ng. The limit of detection by chemiluminescence is much lower than that achieved

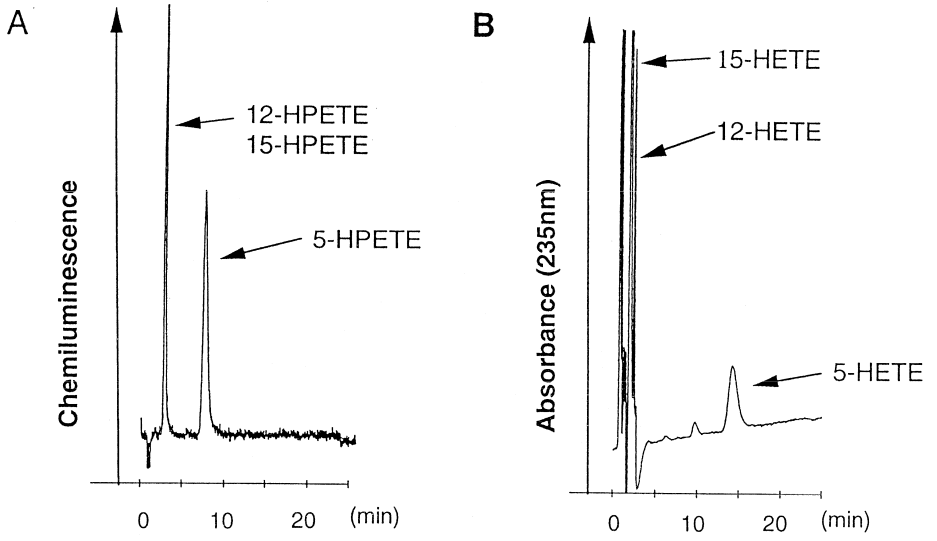

Fig. 1. Separation of isomers of HPETE and HETE by HPLC on silica gel column. (A) Isomers of HPETE (5 ng) were separated by HPLC and monitored with a chemiluminescence detector. (B) Isomers of HETE (100 ng), separated by HPLC, were monitored at 235 nm.

by monitoring the absorbance of the conjugated diene at 235 nm (more than 10 ng), a method that has commonly been used for the detection of HPETEs and HETEs. Each value, determined from five experiments, had a difference to the mean value of 3%.

High sensitivity for the detection of HPETE is required to minimize effects of a background signal. To obtain a stable baseline on a chemiluminescence–HPLC, it was essential to optimize the proportion of solvents in the mobile phase and the mixing ratio of the chemiluminescence reagent to the eluent. Although, being a common solvent for HPLC, methanol did not improved the stabilization of the baseline on a chemiluminescence–HPLC. Yamamoto et al. [15] selected a mixture of acetonitrile and butanol as the mobile phase for separation of lipid hydroperoxides included phospholipids, cholesterol, fatty acids and triacylglycerol. However, no stable baseline was obtained in the present HPLC system. As a consequence of testing various solvent systems for the mobile phase, hexane and isopropanol had been chosen for basal mobile phase solvents. It was said that isopropanol was not suitable for a chemiluminescence–HPLC because of its susceptibility to oxidation, however, the quenching effect of isopropanol was limited and did not affect the quantitation of 5-HPETE in the present study, as shown in Fig. 1. Trace amounts of acetic acid added to the mobile phase can eliminate the tailing of each peak. To eliminate noise, it is necessary that the concentration of the chemiluminescence reagent is not in excess of 20% in the mixed solution. When the proportion of hexane in the mobile phase is increased, the ratio of the chemiluminescence reagent to the mobile phase should be decreased. After all the optimization, the present conditions for HPLC improved the separation and the sensitivity of 5-HPETE.

3.2. Quantitation of 5-HPETE in RBL-2H3 cells stimulated with A23187

The present method was applicable to quantitate intracellular 5-HPETE. The quantitation was carried out by the addition of arachidonic acid to RBL-2H3 cells prior to stimulation with A23187. Lipids extracted from cells were subjected to normal-phase HPLC and the accumulation of cellular 5-HPETE was observed (Fig. 3). The peaks of HPETE and HETE were monitored in terms of chemiluminescence (Fig. 3A–C) and absorbance at 235 nm (Fig. 3D–F). No detectable 5-HPETE was found in non-stimulated RBL-2H3 cells and there were no interfering peaks around the peaks of HPETE and HETE (Fig. 3A and D). An appreciable peak of 5-HPETE was detected in A23187-stimulated RBL-2H3 cells by chemiluminescence, but not by UV absorbance (Fig. 3B and E). Significant amounts of 5-HETE were also detected in activated RBL-2H3 cells by UV absorbance (Fig. 3E). 5-HPETE was identified on the basis of its retention time, as compared to that of authentic standard, and also by the determination of the products of the reduction of HPETE. The peak of 5-HPETE was completely eliminated by reduction with sodium $NaBH_4$ (Fig. 3C), and an increase in area of the peak that corresponded to 5-HETE was observed (Fig. 3F). A23187-stimulated RBL-2H3 cells (10^7 cells) produced 480 ± 30 pg of 5-HPETE and 385 ± 20 ng of 5-HETE within 5 minutes after the stimulation by A23187. Significant amounts of LTB_4 were also produced in A23187-stimulated RBL-2H3 cells, namely, 65 ± 13 ng/10^7 cells (data not shown). Conversion of the exogeneously added arachidonic acid (10 μg) into LTB_4, 5-HETE and 5-HPETE was about 5% under our conditions.

The present study has successfully indicated a fair amount of 5-HPETE produced in cells. Existence of 5-HPETE in cells was showed by a lot of studies with various methods, however, quantitation of that has not been proceeded in a direct way before. These results indicate that cellular 5-HPETE was rapidly converted into its metabolites, namely 5-HETE and LTA_4, and was kept to a fairly low level. Without adding arachidonic acid, RBL-2H3 cells were produced, 1.82 ng of LTC_4 and 0.25 ng of LTB_4 per 10^7 cells in the previous study [9]. However, the production of 5-HETE was less than a half of that of LTB_4 by estimation from conversion of [^{14}C]-arachidonic acid. Therefore, 5-HPETE produced excessively in cells was converted more effectively to 5-HETE than to LTA_4.

3.3. Effect of diethyl malate on 5-HPETE accumulation

The activity of 5-lipoxygenase is regulated by several factors, such as Ca^{2+}, ATP, and FLAP [26–

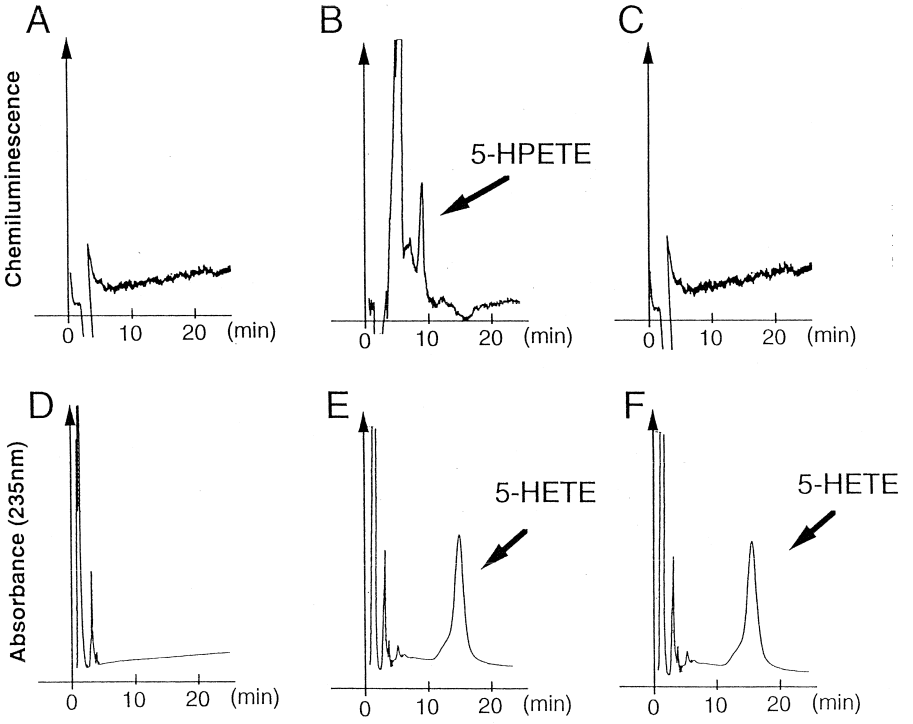

Fig. 3. Chromatogram showing 5-HPETE and 5-HETE in RBL-2H3 cells that had been stimulated with A23187. RBL-2H3 cells were stimulated with 10 μ*M* of A23187 for 5 min in the presence of 50 μ*M* of arachidonic acid. HPETE and HETE were monitored in terms of chemiluminescence (A, B, C) and absorbance at 235 nm (D, E, F), respectively. 5-HPETE and 5-HETE were detected in unstimulated RBL-2H3 cells (A, D) and in cells that had been stimulated with A23187 (B, E). 5-HPETE extracted from A23187-stimulated RBL-2H3 cells was reduced by treatment with $NaBH_4$ and the resultant material was analyzed by HPLC (C, F).

30]. There is evidence that fatty acid hydroperoxides might also activate 5-lipoxygenase [31,32]. A requirement for fatty acid hydroperoxides in the activation of lipoxygenase suggests that the activity of 5-lipoxygenase might be regulated by its own product, namely 5-HPETE [33]. Actually, 5-lipoxygenase activity depended on intracellular concentration of lipid hydroperoxides which were primarily suppressed by PHGPx [9]. To estimate the influence of glutathione peroxidases on the formation of 5-HPETE and LTB_4, the accumulation of 5-HPETE in RBL-2H3 cells was determined by the present method after treatment with diethyl malate (DEM) which was a inhibitor of glutathione peroxidases by the depletion of cellular glutathione (GSH), as a cofactor of glutathione peroxidases. Cellular GSH showed a remarkably decreased from 28.0±4.2 nmol/mg protein to 1.48±0.22 nmol/mg protein after the DEM treatment (data not shown). Detectable amounts of 5-HPETE and LTB_4 were not observed in A23187-stimulated RBL-2H3 cells, however considerable amounts of both lipoxygenase metabolites were produced by the addition of arachidonic acid in medium (Table 1). The production of 5-HPETE and LTB_4 was further increased by the depletion of GSH with DEM. The amounts of 5-HPETE and LTB_4 in DEM-treated cells were en-

Table 1
Effect of diethyl malate on the production of 5-HPETE and LTB_4. RBL-2H3 cells were preincubated for 2 h at 37°C with or without 1 m*M* diethyl malate (DEM) and/or 50 μ*M* of arachidonic acid (AA), and then stimulated with 10 μ*M* of A23187 for 5 min at 37°C. 5-HPETE and were quantitated as in the Experimental. Values are given as mean±SD of results from three independent experiments

	5-HPETE (ng)	LTB_4 (ng)
A23187 alone	n.d.	n.d.
A23187+AA	6.5±5.1	207.3±27.2
A23187+AA+DEM	32.1±11.2	535.0±85.9
A23187+DEM	n.d	144.6±51.7

hanced 5 and 2.5 times higher than those in non-treated cells, respectively. These results suggest that the increase in the production of LTB_4 by treatment with DEM would be due to the inhibition of glutathione peroxidases and the accumulation of 5-HPETE, which acts as the precusor of LTB_4 and also regulates the lipoxygenase activity.

4. Abbreviations

HPETE	hydroperoxyeicosatetraenoic acid
HETE	hydroxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
DCFH-DA	5,6-carbozy-2′,7′-dichlorofluorescein-diacetate
DEM	diethyl malate
FLAP	5-lipoxygenase-activating protein
PBS	phosphate-buffered saline
GSH	glutathione
PHGPx	phospholipid hydroperoxide glutathione peroxidase
$NaBH_4$	sodium borohydride

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