Biochemical and Biophysical Research Communications 393 (2010) 631–636

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The immunological and chemical detection of N-(hexanoyl)phosphatidylethanolamine and N-(hexanoyl)phosphatidylserine in an oxidative model induced by carbon tetrachloride

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ARTICLE INFO

Article history:
Received 23 January 2010
Available online 12 February 2010

Keywords:
Lipid peroxidation
Aminophospholipid
Amide-type adduct
Oxidative modification
Monoclonal antibody

ABSTRACT

Lipid peroxidation products have a high reactivity against the primary amino groups of biomolecules such as aminophospholipids, proteins, and DNA. Until now, many papers have reported about the modification of biomolecules derived from lipid peroxides. Our group has also reported that aminophospholipids, such as phosphatidylethanolamine (PE), can be modified by lipid peroxidation including 13-hydroperoxyoctadecadienoic acid (13-HPODE). The aim of this study was to examine the oxidative stress *in vivo* by detecting the formation of N-(hexanoyl)phosphatidylethanolamine (HEPE) and N-(hexanoyl)phosphatidylserine (HEPS), a novel hexanoyl adduct, using a liquid chromatography/tandem mass spectrometry (LC/MS/MS) and a monoclonal antibody.

Consequently, we observed that the formation of HEPE and HEPS occurred in the red blood cell (RBC) ghosts modified by 13-HPODE and the oxidative stress model induced by carbon tetrachloride (CCl_4) using LC/MS/MS monitoring hexanoyl ethanolamine (HEEA), a head group of HEPE, and hexanoyl serine (HESE) as a part of HEPS. Furthermore, we obtained a novel type of monoclonal antibody against HEPE. This antibody could recognize HEPE in the liver of rats with oxidative stress *in vivo*.

Introduction

Several lines of evidence indicate that oxidative stress caused by reactive oxygen species (ROS) can modify proteins, DNAs, and lipids both *in vitro* and *in vivo* during the aging process [1,2]. Lipid peroxidation products, one of the secondary oxidative stress biomolecules formed following ROS, can lead to such oxidative modifications with proteins, DNAs, and lipids [3–5]. During lipid peroxidation, various lipid hydroperoxides, such as 9- and/or 13-HPODE as the intermediates, malondialdehyde (MDA), and 4-hydroxy-2-nonenal (4-HNE) are generated and lead to the formation of Schiff bases or Michael-type adductions [6–9].

Our group reported that amide-type adducts can be generated as one part of the major modification products by lipid peroxides [3,10]. Furthermore, N^{ε}-(hexanoyl)lysine (HEL), one of these adducts derived from ω-6 fatty acid hydroperoxides, can possibly be used as a marker to evaluate oxidative stress *in vivo* using LC/MS/MS [11]. A recent study suggested that N^{ε}-(propanoyl)lysine derived from an oxidized ω-3 fatty acid can also be an useful marker for oxidative damage caused by ω-3 fatty acid oxidation *in vivo* [12].

On the other hand, phospholipids are important components of biological membranes and are essential for the structural integrity and function of cells. Accordingly, the formation of oxidatively damaged phospholipids may also cause pathophysiological effects. Several recent reports indicated that the head groups of aminophospholipids are sensitive toward oxidation and that phosphatidylethanolamine (PE) is one of the main targets of the lipid peroxidation products undergoing the same reaction mechanism as proteins [5,13–16].

However, little is known about the modification reaction of lipid hydroperoxides with aminophospholipids, such as PE and phosphatidylserine (PS) *in vivo*, as compared to the detailed analyses of protein modification [3,11]. To characterize the modification mechanism of PE head groups and the serine moiety of PS by lipid peroxidation products, we investigated the formation of HEPE and HEPS as one of the plausible PS modification products using LC/MS/MS method [5]. Moreover, we succeeded in the preparation of the monoclonal antibody against HEPE in order to examine in detail the formation of HEPE using rats treated with CCl_4 as the oxidative stress model.

Materials and methods

Chemicals. Lipid A was obtained from Funakoshi. Pecoll was purchased from GE Healthcare. Ethanol amine, lipoxygenase from soy-

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doi:10.1016/j.bbrc.2010.02.043

bean, phospholipase D (PLD), zirconyl chloride hydrate, and bovine serum albumin (BSA) were obtained from Sigma. The second antibodies labelled alexa fluor were purchased from Invitrogen. 13-HPODE was enzymatically synthesized from linoleic acid by soybean lipoxygenase as previously described [3,17]. All other chemicals were obtained from Wako Pure Chemicals.

Synthesis of HEEA, HESE, HEEA-[d11], and HESE-[d11]. HEEA and HEEA-[*d11*] as an internal standard of HEEA were synthesized following the previously reported method [5]. The synthesized HEEA and HEEA-[*d11*] were confirmed by liquid chromatography/mass spectrometry (LC/MS) at *m*/*z* 159 and 170, respectively. Part of the HEEA was used for the preparation of HEEA-BSA via the carbodiimide method for use as an antigen for screening of the monoclonal antibody against HEPE. In the case of HESE, 0.1 g of L-serine was dissolved in 1.6 ml of 50% ethanol and then reacted with 96 μl of hexanoic anhydride. The reaction mixture was purified by reverse HPLC. HESE-[*d11*] as an internal standard of HESE was synthesized as well as HEEA-[*d11*]. HESE and HESE-[*d11*] were also confirmed by LC/MS at *m*/*z* 203 and 214.

Sample preparation and analyses of HEEA and HESE with LC/MS/MS. Sample preparation and LC/MS/MS analysis of HEEA and HEEA-[*d11*] basically carried out according to the procedures of previous reported paper [5]. Part of procedures was partially modified for further purification of HEEA and HESE. We added the next procedures after PLD hydrolysis. After residues were extracted with 1 ml methanol [5], 100 μl of 200 mM zirconyl solution and 150 μl of NaH_2PO_4 solution were added to methanol solutions to purify by the zirconyl reaction [18]. After incubation at room temperature for 15 min, the samples were centrifuged at 9000 rpm at 4 °C for 15 min. The upper layers were evaporated. The obtained samples were resuspended in 100 μl of ethanol, and 10 μl of each sample was then applied twice to LC/MS/MS. For the quantification, HEEA-[*d11*] and HESE-[*d11*] were added to samples at a concentration of 7.8 nM as an internal standard after PLD hydrolysis. Liquid chromatography was done using Develosil ODS-HG-5 ($2\psi \times 75$ mm) as the column. Solvent A was 0.1% formic acid and solvent B was CH_3CN. The gradient program for HEPS and HEPS-*d11* was as follows: initial, A 100%; 15 min, A 70%; 20 min, A 0%; 25 min, A 0%; 26 min, A 100%; 34 min, A 100%. The level of HEEA and HESE was corrected by protein measured by BCA assay reagent kit (Thermo).

Modification of RBC ghost membrane with 13-HPODE. The RBC ghosts were prepared by the same method as previously described [19]. The obtained RBC ghost membrane was mixed with 13-HPODE (0, 1, 2, 5, 10 mM: final concentration) and incubated at 37 °C for 3 days (72 h). After incubation, the reaction was stopped by adding 250 mM of ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA) solution and 30 mM of butylhydroxytoluene (BHT) solution. The detection of HEEA and HESE were previously described.

Fractionation of RBC using percoll method by density. One hundred milliliter of rabbit serum was mixed with 100 ml of isotonic solution (10 mM phosphate buffer containing 152 mM NaCl (pH7.4)) and then centrifuged at 3500 rpm and 4 °C for 20 min to remove the upper layer. This process was repeated three times. The obtained RBC was fractionated by percoll method [20]. Briefly, 1 ml of 80% percoll in 2.5 M sucrose was added to 15 ml tube, then filled with 150 μl of RBC. Moreover, 1 ml of 70% percoll in 2.5 M sucrose and 1 ml of 60% percoll in 2.5 M sucrose were separately added. This solution was centrifuged at 13,000 rpm and 4 °C for 35 min. After adding 5 volumes of isotonic solution, the percoll of the obtained fractions mixed with isotonic solution was removed by centrifugation (1300 rpm for 10 min, 4 °C). This process was repeated twice. After removal of the percoll, the HEEA and HESE concentrations in the RBC fraction were measured by the LC/MS/MS method.

Synthesis of N-(hexanoyl) 1,2-dipalmitoyl-sn-glycero-3-phophatidylethanolamine (HEDPPE). HEDPPE was prepared by the reaction of hexanoic anhydride and DPPE (5/2, w/w) with 1 ml of $CHCl_3$ and incubated at 37 °C for 24 h. The product was separated and purified using a silica G60 thin-layer chromatography (TLC) plate and developed with $CHCl_3/CH_3OH/NH_4OH$ (80/20/2, v/v/v).

Preparation of monoclonal antibody against HEPE. The liposome containing HEDPPE was used as an antigen (HEPE antigen). DPPE/HE-DPPE/cholesterol/L-α-phosphatidyl-DL-glycerol dimyristoyl (DMPG) were mixed at the ratio of 4/3/7/1 (w/w/w/w). Furthermore, 30 μl of lipid A was added and dissolved in one drop of chloroform. This mixed solution was evaporated by a rotary evaporator and prepared as a thin mixture layer in a glass flask. A 0.3 M glucose solution was added to the flask and mixed by sonication. After confirming formation of the emulsion, the solution was transferred to a serum tube and then placed in liquid nitrogen, and incubated for 30 min. This solution was then incubated at room temperature. This procedure was repeated three times. The obtained solution containing small uilamellar vesicles (antigen containing SUV) was estimated by phospholipids test Wako C (Wako) and quantified the concentration of the phospholipids. We prepared the HEPE antigen by sonicating the antigen containing SUV for 30 s when the Balb/c mice (female, 6 weeks at first immunization) were first immunized with 100 μl of the HEPE antigen (0.5 mg/ml). After increasing the immunoreactivity of the antibody derived from the serum of mice, the spleen cells were fused with murine myeloma cells by a standard polyethylene glycol method. For the screening and cloning of the hybridoma, aliquots of the hybridoma supernatants were tested for reactivity against HEEA-BSA by an enzyme-linked immunosorbent assay (ELISA). The ELISA and competitive ELISA followed the previously reported method [12].

Animal experiment. Six-week old Wistar male rats were obtained from Chubu Kagaku Shizai Co., Ltd., (Nagoya, Japan). The obtained rats were separated into two groups. One group was the control to intraperitoneally inject the corn oil. The other group was the pharmacological induced oxidative stress group by two intraperitoneal injections of CCl_4 (1 mg/kg) (24 h and 4 h before sacrificed). The blood was obtained from the heart before rats were perfused with 0.9% normal saline solution (NSS), then the livers were extracted and immediately stored at −80 °C until preparation of the homogenate solution. They were homogenized in 10 times its volume of phosphate-buffered saline (PBS) containing 5% EDTA and 5% BHT for the evaluation of HEEA and HESE using the LC/MS/MS method. One of the livers derived from the control rats or CCl_4 injected rats was fixed by the compound (Sakura Finetechnical Co., Ltd., Tokyo) under liquid nitrogen after being perfused with 0.9% NSS. The cryostat sections were prepared at 10 μm for the immunohistochemical analysis using the fluorescent labeled antibody. The protein contents of the homogenates were determined using a BCA protein assay. The thiobarbituric acid reactive substances (TBARS) were measured as previously described [21].

Immunofluorescence stain using HEPE antibody on liver section. The cryostat sections were washed with PBS and fixed by 3.7% paraformaldehyde solution for 20 min. The fixed sections were treated with PBS containing 5% Triton X-100 for 10 min at room temperature. Next, a blocking step was performed using PBS containing 1% BSA and 1.35% rabbit normal serum for 30 min. The anti-HEPE monoclonal antibody and anti-HEL rabbit polyclonal antibody [3] were applied to cryostat sections and incubated at 4 °C overnight. After incubation, the 2nd antibody (anti-mouse antibody labeled Alexa Fluor 488 and anti-rabbit antibody labeled Alexa Fluor 568) was applied to the section and incubated at room temperature for 1 h. The sections were washed by PBS for 5 min at each step and this wash step was performed 3 times. After the last wash step of the second antibody labeled fluorescence, the sections