Biochemical and Biophysical Research Communications 458 (2015) 920–927

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Metabolism and cytotoxic effects of phosphatidylcholine hydroperoxide in human hepatoma HepG2 cells

Yuuri Suzuki [a], Kiyotaka Nakagawa [a, *], Shunji Kato [a], Naoto Tatewaki [b], Shunsuke Mizuochi [a], Junya Ito [a], Takahiro Eitsuka [b], Hiroshi Nishida [b], Teruo Miyazawa [a, c]

[a] Food and Biodynamic Chemistry Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan
[b] Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata 956-8603, Japan
[c] Food Biotechnology Innovation Project NICHe, Tohoku University, Sendai 980-8579, Japan

ARTICLE INFO

Article history:
Received 10 February 2015
Available online 20 February 2015

Keywords:
Phosphatidylcholine hydroperoxide
Metabolism
Cytotoxic effects
Unusual apoptosis
Human hepatoma HepG2 cells
Atherosclerosis

ABSTRACT

In this study, we investigated cellular uptake and metabolism of phosphatidylcholine hydroperoxide (PCOOH) in human hepatoma HepG2 cells by high performance liquid chromatography–tandem mass spectrometry, and then evaluated whether PCOOH or its metabolites cause pathophysiological effects such as cytotoxicity and apoptosis. Although we found that most PCOOH was reduced to PC hydroxide in HepG2 cells, the remaining PCOOH caused cytotoxic effects that may be mediated through an unusual apoptosis pathway. These results will enhance our fundamental understanding of how PCOOH, which is present in oxidized low density lipoproteins, is involved in the development of atherosclerosis.

1. Introduction

Oxidized low density lipoprotein (Ox-LDL) is now recognized as a causative factor in atherosclerosis [1]. Many studies have shown cytotoxic and apoptotic effects of Ox-LDL on monocytes/macrophages, smooth muscle cells and endothelial cells [2], implying that Ox-LDL plays a role in atherosclerosis progression and plaque instability.

Ox-LDL contains predominantly oxidized phosphatidylcholine (PC), which is hypothesized to play a role in the promotion of atherosclerosis [3]. During the course of LDL oxidation, PC is primarily oxidized to PC hydroperoxide (PCOOH), while further modification of PCOOH yields various secondary products such as PC hydroxide (PCOH) and PC with a truncated *sn*-2 acyl group (truncated PC) [3,4]. Among these oxidatively modified PCs, PCOOH was thought to be the most causative agent in terms of atherosclerosis [3,4], but which oxidized PCs show cytotoxic, apoptotic and atherogenic effects remain unclear.

The mechanisms of cellular uptake and metabolism of PCOOH are also unknown. To the best of our knowledge, only one study, by Bao and Williamson, has examined PCOOH metabolism [5]. They incubated human hepatoma cell line (HepG2) with PCOOH, and found that PCOOH was mainly converted to PCOH in the cells. However, there are no subsequent reports concerning PCOOH metabolism in the literature.

Based on these earlier findings, in this study we quantitatively evaluated cellular uptake and metabolism of PCOOH in liver cells and other cultured cells by high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS), and then performed cell culture studies in order to discriminate whether PCOOH or its metabolites (PCOH, truncated PC, etc.) are principally related to pathophysiological processes such as cytotoxicity and apoptosis induction.

2. Materials and methods

2.1. Reagents

1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0–18:2 PC) was purchased from Avanti Polar Lipids (Alabaster, AL). PCOOH was enzymatically synthesized from PC using soybean lipoxygenase-1

Abbreviations: Toc, α-tocopherol; JNK, c-Jun-NH_2-terminal kinase; DAPI, 4′, 6-diamino-2-phenylindole; ESI, electro-spray ionization; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; GPx, glutathione peroxidase; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cells; BSO, L-buthionine-sulfoximine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; Ox-LDL, oxidized low density lipoprotein; PAzPC, 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine; PONPC, 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosohocholine; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PCOH, phosphatidylcholine hydroxide; PHGPx, phospholipid hydroperoxide glutathione peroxidase; SM, sphingomyelin.

* Corresponding author. Fax: +81 22 717 8905.
E-mail address: nkgw@biochem.tohoku.ac.jp (K. Nakagawa).

http://dx.doi.org/10.1016/j.bbrc.2015.02.063
0006-291X/

and chromatographically purified [6]. The predominant *sn*-2 residue of the purified PCOOH was 13-hydroperoxyoctadecadienoic acid. PCOH was prepared by reducing PCOOH with $NaBH_4$. Sphingomyelin (SM) was obtained from Nagara Science (Gifu, Japan). L-Buthioninesulfoximine (BSO) was from Sigma (Tokyo, Japan). All other reagents were of analytical grade.

2.2. Cells

The human hepatocellular carcinoma cell line HepG2 was obtained from the RIKEN cell bank (Tsukuba, Japan). The monocytic cell line THP-1 was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). The cells were cultured in RPMI-1640 medium (Sigma) containing 0.3 g/l L-glutamine and 2.0 g/l sodium bicarbonate supplemented with 10% fetal bovine serum (FBS) (Dainippon Sumitomo Pharmaceutical, Osaka, Japan), 100 kU/l penicillin (Gibco BRL, Rockville, MD) and 100 mg/l streptomycin (Gibco). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Preparation of test medium

Test samples (PCOOH, PCOH, or PC) were dissolved in methanol at a concentration of 13 mM. The stock solution was diluted with test medium (RPMI 1640 medium containing 0.3% FBS) to achieve the desired final concentration (e.g., 0–50 μM PCOOH). The final concentration of methanol in the test medium was less than 0.4% (v/v), which did not affect cell viability. Other test samples (BSO and α-tocopherol (Toc)) were dissolved in water or ethanol followed by preparation of test medium. Medium with solvent (methanol) alone was similarly prepared and used as the control medium.

2.4. Cellular uptake and metabolism of PCOOH

HepG2 cells (1.0×10^6) were pre-incubated with 10% FBS/RPMI-1640 in 10 cm dishes. Twenty-four hours later, the medium was replaced with test medium containing PCOOH. Then, the cells were incubated for 4 h. Similarly, THP-1 cells were treated with test medium. After treatment, the number of cells was evaluated in an OneCell counter (Bio Medical Science, Tokyo, Japan) with an electronic microscope. Cells and medium were then subjected to LC–MS/MS analysis.

2.5. LC–MS/MS analysis

Cells (1.0×10^7) were suspended in 650 μl water, whereas medium (300 μl) was diluted with 300 μl water. The sample (600 μl cell suspension or diluted medium) was then mixed with 2.4 ml chloroform–methanol (2:1, v/v; containing 1.0 mg SM). The mixture was partitioned by centrifugation at 1000 × *g* for 20 min at −20 °C into two phases: chloroform layer (lower organic phase) and methanol–water layer (upper aqueous phase). The lower chloroform layer (lipid fraction) was collected. The remaining aqueous layer was re-extracted with Folch theoretical lower phase [7], and subjected to centrifugation 1000× *g* for 20 min at −20 °C. Lipid fractions were combined and evaporated under nitrogen gas. The dried extract was re-dissolved in 240 μl chloroform–isopropanol (2:1, v/v), and a portion (40 μl) was loaded onto an aminopropyl Sep-Pak cartridge (100 mg) (Waters, Tokyo, Japan) equilibrated with chloroform–isopropanol (2:1, v/v). The cartridge was rinsed with 750 μl chloroform–isopropanol (2:1, v/v), and the oxidized PCs were then eluted with 1.5 ml methanol. The eluent was evaporated, and the residue was dissolved in 200 μl methanol. A final aliquot of 10 μl was injected for LC–MS/MS analysis.

For LC–MS/MS, an ODS column (Atlantis T3 column, 3.5 μm, 2.1 × 100 mm; Waters) was used at 40 °C. The mobile phase consisted of two components: A, water containing 0.1 mM sodium acetate and B, methanol containing 0.1 mM sodium acetate. The gradient profile was as follows: 0–4 min, 70–90% B linear; 4–10 min, 90% B; 10–17 min, 90–100% B linear; 17–30 min, 100% B; 30–30.1 min, 100–70% B linear; 30.1–35.0 min 70% B. The flow rate was 0.2 ml/min. Oxidized PCs were analyzed using a 4000 QTRAP LC–MS/MS System (AB SCIEX, Tokyo, Japan). MS/MS parameters were optimized with synthesized PCOOH and PCOH standards under electro-spray ionization (ESI) (positive). Oxidized PCs in cells and medium were determined using the multiple reaction monitoring (MRM) mode as follows: PCOOH, *m*/*z* 812.4 > 541.4; PCOH, *m*/*z* 796.5 > 613.4 [8]. Extraction efficiencies of PCOOH and PCOH standards spiked with cell suspension (or medium) were over 90%. Truncated PCs were analyzed using MRM transitions from the literature [9]: 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosohocholine (PONPC; *m*/*z* 740.6 > 634.5) and 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (PAzPC; *m*/*z* 664.6 > 201.0).

2.6. Cell cytotoxic assay

HepG2 cells (1.0×10^4) were pre-incubated with 10% FBS/RPMI-1640 in 96-well plates. Twenty-four hours later, the medium was replaced with test medium containing 50 μM PCOOH, PCOH or native non-oxidized PC for 24 h. Then, the cells were observed under a microscope, and the number of viable cells was evaluated using WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, 10 μl WST-1 reagent was added to the medium, and incubated at 37 °C for 2 h. Absorbance (450/655 nm) of the medium was measured with a microplate reader. In addition to these experiments, test medium containing either 50 μM PCOOH + 100 μM BSO (glutathione (GSH) synthesis inhibitor) or 50 μM PCOOH + 100 μM BSO + 50 μM Toc was prepared, and cell cytotoxicity assays were performed. Cellular GSH levels were evaluated by using GSH-Glo™ Glutathione Assay (Promega, Madison, WI).

2.7. Mitochondrial membrane potential

HepG2 cells (1.0×10^4) were seeded onto Poly-D-lysine-coated coverslips set in a 24-well plate and cultured for 24 h. Cells were treated with test medium for 4–24 h. After treatment, the medium was replaced with fresh medium containing 200 nM MitoTracker Red (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and incubated for 30 min at 37 °C. After washing with cold phosphate-buffered saline, the cells were fixed with 2% paraformaldehyde for 15 min. The cells were then stained with 4′,6-diamino-2-phenylindole (DAPI) solution (5 μg/ml) for 15 min. Intracellular localization of the probes was determined by a fluorescent microscope.

2.8. Western blot analysis

After a 24 h pre-incubation of HepG2 cells (1.0×10^6) with 10% FBS/RPMI-1640 in a 10 cm dish, the cells were treated with the test medium for 4–24 h. Cells were sonicated in ice-cold CelLytic™ M (Sigma) including 1.0% protease inhibitor cocktail. After the homogenates were centrifuged at 15,000× *g* for 15 min at 4 °C, the supernatant was collected, and the protein concentration was measured. Cellular proteins (10 μg/well) were separated by SDS-PAGE (8–14% gel). After blocking with skim milk, the membranes were incubated with primary antibodies for p53, phospho-p38 (*Thr180*/*Tyr182*), phospho-c-Jun-NH_2-terminal kinase (JNK)