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研究課題名（和文）Studies on the association of lipid hydroperoxides and Alzheimer's disease by LC/MS

研究課題名（英文）Studies on the association of lipid hydroperoxides and Alzheimer's disease by LC/MS

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研究成果の概要（和文）：本研究では、酸化ストレスを与える神経細胞（ADモデル神経細胞）を対象に、未酸化脂質や過酸化脂質をLC/MSで同定し、未酸化・過酸化脂質の定性・定量評価とADモデル細胞の酸化ストレスの関係を調べた。酸化剤であるH2O2濃度を増やすことで酸化ストレスを上昇させ、細胞の形態が変化し、細胞内の活性酸素種が増加した。それと伴い、機能性脂質（プラズマローゲンとカルジオリピン等）の量が有意に減したが、一方、過酸化脂質が蓄積され、有意な増加を示した。これらの変動は脂質代謝の障害を引き起こし、酸化ストレスによる細胞損傷を示唆した。ADモデル細胞に脂質代謝の障害を起こし、過酸化脂質と酸化ストレス依存性を示唆した。

研究成果の学術的意義や社会的意義
ADは世界中で深刻な社会問題となり、ADのメカニズムに関する研究は人類の健康増進に貢献できる。本研究では、LC/MSによる確立されたリピドミクス分析方法を用いて、ADモデル神経細胞を対象に、病態による神経細胞の変化と酸化ストレスにおける過酸化脂質の量の変動の関連を研究した。酸化ストレスに受けた神経細胞内では未酸化脂質や過酸化脂質の網羅的に定性と定量をすることで、ADに関連する脂質代謝異常として分子レベルの病理メカニズムの解明が期待される。また、脂質の定性・定量評価を用い、ADに罹患する潜在的なリスクの予測や診断、及び薬物治療効果の評価に有用な指標になると考えられる。

研究成果の概要（英文）：Alzheimer's disease (AD) is a serious threat worldwide, in which oxidative stress is thought to play an important role in the pathological development. In this study, the AD cell model by using PC12 neuronal cells was established, the intracellular intact lipids and oxidized lipids were detected by LC/MS, and the contents of the molecular species were determined. Besides, cell morphology, viability, and reactive oxygen species levels were evaluated. As a result, lipid hydroperoxide molecular species accumulated with the increased H2O2 concentration, suggesting the oxidative damage in the molecular level. Moreover, the amounts of functional lipids including plasmalogens and cardiolipins were decreased along with the increased H2O2 concentration. This study showed that the lipid metabolism disorders occurred in AD model neurons, which suggested a close association between lipid hydroperoxides and AD.

研究分野：Analytical chemistry

キーワード：oxidative stress　oxidized lipids　lipid hydroperoxides　lipidomics　plasmalogen　antioxidant　LC/MS　PC12 neuronal cells

１．研究開始当初の背景

Alzheimer's disease (AD) is a progressive neurological disorder characterized by an accumulation of extracellular amyloid-β, formation of intracellular neurofibrillary tangles, neuroinflammation, brain atrophy, and a gradual progression of memory loss. There are 46.8 million people worldwide living with AD or other dementia in 2015, and this number will double every 20 years. Besides, AD is associated with an estimated health-care cost of US$172 billion per year. Currently, AD has become a terrible health problem, bringing medical trouble and financial burden to the whole society.

For pathological mechanism, there has been increasing evidences suggest that free radical-mediated oxidation of biological substrates is a key feature of AD pathogenesis. These reactive oxygen species (ROS), such as superoxide, peroxide, and hydroxyl radical, can be generated due to exogenous factors, such as radiation or drug exposure. It is widely known that oxidative stress is involved in neuronal cell death, which is one of the major causes of neurodegenerative diseases, such as AD and Parkinson's disease. In terms of AD, cytochrome c oxidase in nerve cell is specifically attacked. Consequently, electron transport, ATP production, oxygen consumption, and mitochondrial membrane potential all become impaired.

Lipid peroxidation is one of the oxidative damages in cell membranes, lipoproteins, and other lipid-containing structures. The accumulation of lipid hydroperoxides (L-OOH), which serve as the lipid oxidation products, will lead the whole body under the state of oxidative stress. L-OOH, such as the hydroperoxides of phosphatidylcholine (PC), phosphatidylethanolamine (PE), triglyceride (TG) has been known not only being toxic mediators but also exerting diverse biological effects. Therefore, there has been increasing studies on L-OOH in the recent years. However, there is few reports on L-OOH in AD patients, and there is no L-OOH analysis in nerve cells of AD-model.

２．研究の目的

The aims of this study were: to investigate the lipid hydroperoxide molecular species in AD-model nerve cells by LC/MS, and to uncover the association between their changes and oxidative stress in AD.

３．研究の方法

In this study, the LC/MS-based L-OOH omics will be initially combined with other index, including nerve cell damage degree (by cell viability), oxidation degree (by ROS level), mitochondrial function (by cardiolipin level), and nerve cell function evaluation (by plasmalogen level), to comprehensively refine the molecular characteristics in AD-model neuronal cells.

(1) AD-modeling via oxidative stress

① Cell culturing: The PC12 cells, as the commonly used neuronal cells, were purchased from JRCB cell bank and were cultured in RPMI 1640 medium. Then, the cells were differentiated by nerve growth factor.

② AD Modeling: According to the literature, the antioxidant H_2O_2 was used to induce oxidative stress for the differentiated neuronal cells. The dosage gradient of H_2O_2 was ranged from 200 to 2000 μM.

(2) Bioassay of AD-model nerve cells

① Cell viability: The CCK-8 kit was used to test the H_2O_2-induced cell damage.

② Intracellular ROS level: The cells were loaded with DCFH-DA to obtain the images by using the fluorescence microscope, and the fluorescent intensity was measured.

③ Plasmalogen profiling: The plasmalogens were extracted by Folch's method, and the plasmalogen profile was acquired by the established LC/MS method.

(3) Lipidomic analysis for L-OOH

① LC/MS conditions: The HPLC conditions such as column, mobile phase, and elution gradient were optimized for ensuring the usability for the all the L-OOH species separation. The MS scan was performed under both positive- and negative-modes for different L-OOH classes.

② Intact lipids and L-OOH analysis: The total lipids in cells were extracted by Folch's method, and analyzed by LC/MS under the optimized conditions. The identification of intact lipids and lipid hydroperoxides was conducted on the basis of high-resolution MS and MS/MS characteristics, as well as HPLC behavior with the

comparison of the in-house database.

④ Comparison and statistics: The intensity of each lipid species calibrated by internal standards were calculated for amount comparation and further statistical analysis.

4．研究成果

(1) AD model established by H_2O_2 inducement of PC12 cells

Firstly, the PC12 cells were cultured and differentiated, and then treated with H_2O_2 with a series of concentrations. The morphology was evaluated, as the representative photos showed in Figure 1A. The normally differentiated cells showed long and abundant axon, while in the modeling groups, the axon number and axon length decreased along with the dose of H_2O_2. The cell viability showed unchanged with low concentration of H_2O_2 (until 1000 μM), while for the higher dose groups the cell viability gradually decreased (Figure 1B). In parallel, the intracellular ROS levels were significantly higher than control in 1600, 1800, and 2000 μM groups, indicating that oxidative stress in the differentiated neuronal cells was induced by H_2O_2 (Figure 1C). These results suggested that the oxidative stress-induced AD model was established for the following studies.

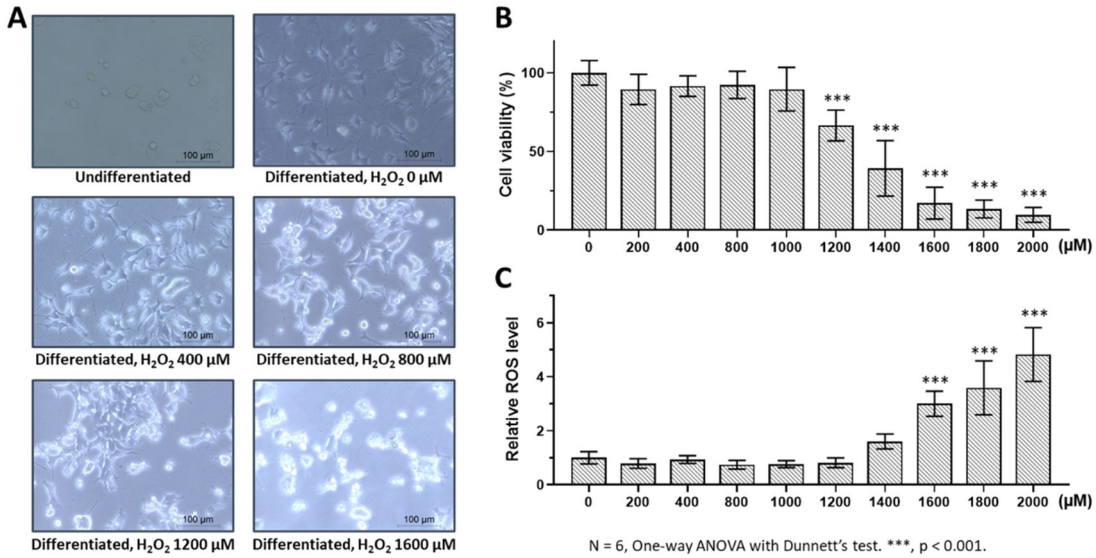

Figure 1. (A) Morphology of undifferentiated and differentiated PC12 cells of control, and with different concentration of H_2O_2; (B) The viability of for the cells treated with different concentration of H_2O_2; (C) The intracellular ROS levels for the cells treated with different concentration of H_2O_2.

(2) Identification of L-OOH by LC/MS/MS

The oxidized lipids, specifically the lipid hydroperoxides, were detected by LC/MS/MS, including the hydroperoxides for TG, for PC, and for PE. The identification was based on their HRMS signals, the retention behavior on HPLC, and the tandem MS fragmentations. The representative identification, taking PCOOH34:2 (16:0/18:2-OOH) as an example, is shown in Figure 2.

(3) Comparison of L-OOH levels among control and AD-model cells

The oxidation of lipids is widely accepted to play a crucial role in multiple pathological processes. Free radical oxidation of unsaturated fatty chains produces a variety of oxidized structures, such as L-OOH, as the key intermediates of oxidative reactions generally induced by ROS. L-OOH has been known not only being toxic mediators but also exerting diverse biological effects. Therefore, in this study, hydroperoxide species of TG (TGOOH), of PC (PCOOH), and of PE (PEOOH) were semi-quantitated individually, and the total level of each L-OOH class was compared between control and AD-model groups. TGOOH showed the greatest accumulation under oxidative stress, being with significance in 1400, 1600, and 1800 μM groups, and accounting for more than 20 folds of control in 1800 μM group (Figure 3A). While for PCOOH and PEOOH, the

accumulation showed the similar trends. These results were in accordance with the decrease of cell viability and the increase of the ROS level, suggesting the association of L-OOH and oxidative stress-related AD in PC12 cells.

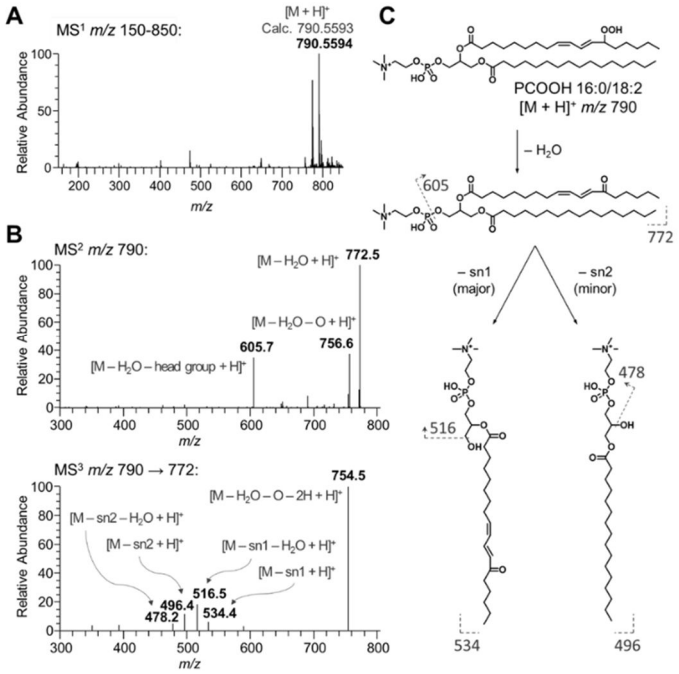

Figure 2. Identification of fatty acyl composition in the lipid hydroperoxide PCOOH 16:0/18:2. (A) The high-resolution MS^1 spectrum showed an identical signal at *m/z* 790.5594 as $[M+H]^+$; (B) In MS^2 spectrum, the signals of *m/z* 772 and *m/z* 756 indicated the existence of the hydroperoxyl group, while *m/z* 605 was assigned as the $[M-H_2O$-headgroup$+H]^+$; (C) The MS^3 spectrum not only gave the signal of hydroperoxyl group cleavage as $[M-H_2O-O-2H+H]^+$) at *m/z* 754, but also showed two pairs of signals, *m/z* 534, 516, and *m/z* 496, 478, which were assigned as $[M-FA16:0+H]^+$ and $[M-FA18:2OOH+H]^+$, respectively.

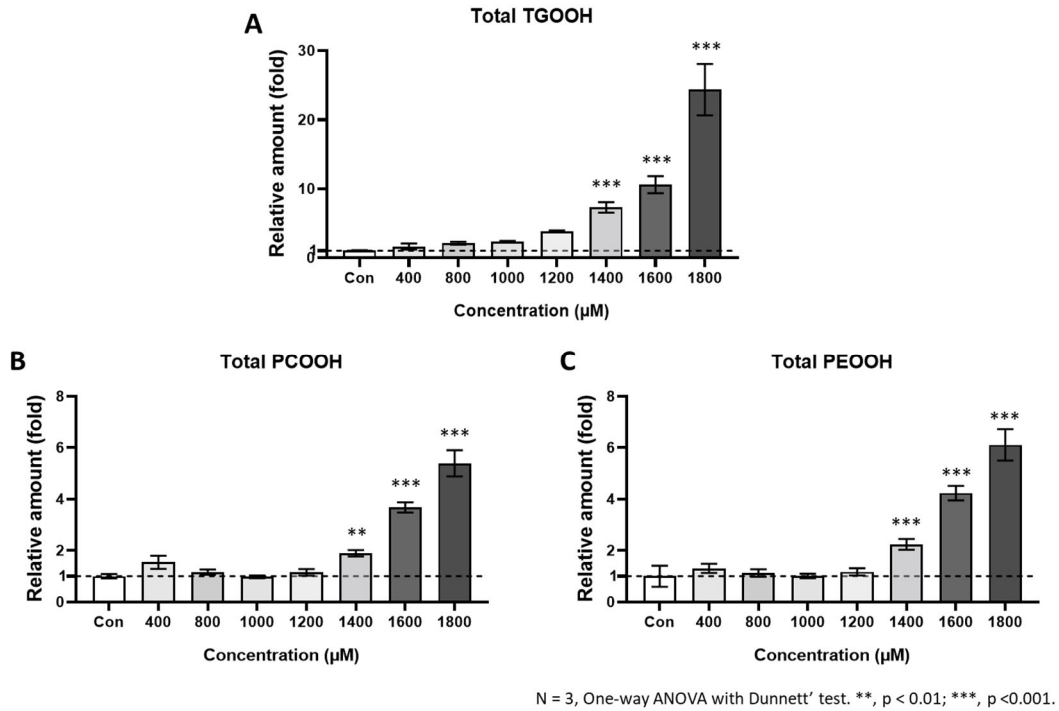

Figure 3. Levels of lipid hydroperoxides in differentiated PC12 cells. (A) Total triglyceride hydroperoxides (TGOOH); (B) Total phosphatidylcholine hydroperoxides (PCOOH); (C) Total phosphatidylethanolamine hydroperoxides (PEOOH).

(4) Depletion of cardiolipins in AD-model cells

Cardiolipins (CL) exist as crucial functional phospholipid in mitochondria, which play a key role in energy use and lipid metabolism. In this study, CL species were semi-quantitated, and the total CL level was compared between control and AD-model groups (Figure 4A). As the result, the total CL decreased with the inducement of H_2O_2 in a dose-dependent manner, which was significant from 1200 μM. The depletion of CL as the

functional lipid under H_2O_2-induced oxidative stress indicated that in the current AD model, the cells were with the damaged mitochondrial function, and thus, could not function the β-oxidation normally, which resulted in the lipid metabolism disorders.

(5) variation of plasmalogen profile in AD-model cells

Plasmalogens are a kind of phospholipid with beneficial health functions, of which the functions include mediating dynamics of the cell membrane, being involved in signal transduction, and contributing to endogenous antioxidant activity. Plasmalogen insufficiency is associated with various diseases and pathologic processes, including Alzheimer's disease, respiratory disease, cell membrane alterations, fatty alcohol accumulation, and other lipid metabolic disorders. Therefore, in this study, the plasmalogen profile was also assessed (Figure 4B). It is noted that only the ethanolamine plasmalogen (PlsEtn) species were detected, but not choline plasmalogen, suggesting the cell-specificity of plasmalogen characteristics. The Total PlsEtn, accounted as the sum of all the molecular species, revealed a slight decrease along with the inducement of H_2O_2,of which the 1600 and 1800 μM groups showed statistical significance. Moreover, the less unsaturated degree of the PlsEtn species, the more loss under the oxidative stress in the AD-model cells (Figure 4C), which suggested the dysregulation of plasmalogen profile and the possible dysfunction of these functional phospholipids. And, the dose-dependent manner of the loss for certain PlsEtn species was consisted with the loss of CL and the accumulation of L-OOH.

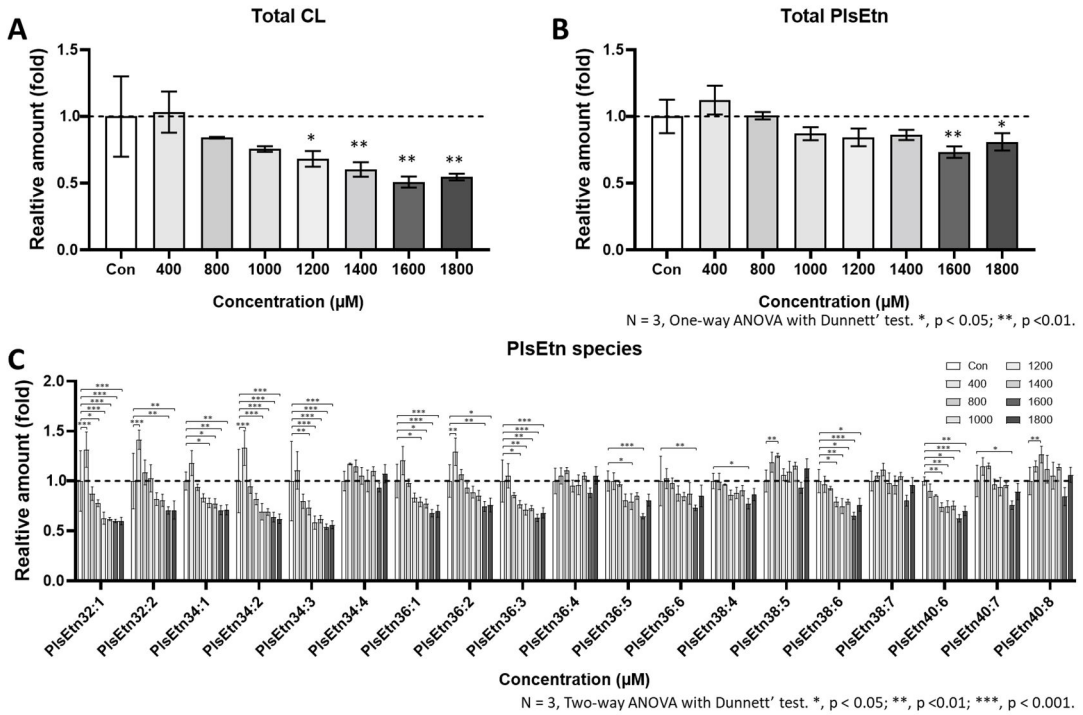

Figure 4. Variation of functional lipids in AD-model cells. (A) Depletion of cardiolipins; (B) Changes of ethanolamine plasmalogen; (C) Varied composition of molecular species ethanolamine plasmalogen.

In summary, in this study, the AD cell model by using PC12 neuronal cells was established, the intracellular intact lipids and oxidized lipids were detected by LC/MS, and the contents of the molecular species were determined. Besides, cell morphology, viability, and reactive oxygen species levels were evaluated. As a result, the lipid hydroperoxide molecular species accumulated with the increased H_2O_2 concentration, suggesting the oxidative damage in the molecular level. Moreover, the amounts of functional lipids including plasmalogens and cardiolipins were decreased along with the increased H_2O_2 concentration. This study showed that the lipid metabolism disorders occurred in AD model neurons, which suggested a close association between lipid hydroperoxides and AD.

５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　3件／うち国際共著　2件／うちオープンアクセス　1件）

１．著者名 Wu Yue、Chen Zhen、Chiba Hitoshi、Hui Shu-Ping	４．巻 322
２．論文標題 Plasmalogen fingerprint alteration and content reduction in beef during boiling, roasting, and frying	５．発行年 2020年
３．雑誌名 Food Chemistry	６．最初と最後の頁 126764～126764
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.foodchem.2020.126764	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Chen Zhen、Liang Qiangrong、Wu Yue、Gao Zijun、Kobayashi Satoru、Patel Joy、Li Cairong、Cai Fei、Zhang Youhua、Liang Chongsheng、Chiba Hitoshi、Hui Shu-Ping	４．巻 16
２．論文標題 Comprehensive lipidomic profiling in serum and multiple tissues from a mouse model of diabetes	５．発行年 2020年
３．雑誌名 Metabolomics	６．最初と最後の頁 115:1~115:13
掲載論文のDOI（デジタルオブジェクト識別子） 10.1007/s11306-020-01732-9	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Wu Yue、Chen Zhen、Jia Jiaping、Chiba Hitoshi、Hui Shu-Ping	４．巻 10
２．論文標題 Quantitative and Comparative Investigation of Plasmalogen Species in Daily Foodstuffs	５．発行年 2021年
３．雑誌名 Foods	６．最初と最後の頁 124:1～124:12
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/foods10010124	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Wu Yue、Chen Zhen、Darwish Wageh S.、Terada Koh、Chiba Hitoshi、Hui Shu-Ping	４．巻 67
２．論文標題 Choline and Ethanolamine Plasmalogens Prevent Lead-Induced Cytotoxicity and Lipid Oxidation in HepG2 Cells	５．発行年 2019年
３．雑誌名 Journal of Agricultural and Food Chemistry	６．最初と最後の頁 7716～7725
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acs.jafc.9b02485	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

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3．学会等名
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2020年

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2．発表標題
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3．学会等名
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4．発表年
2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関			
米国	New York Institute of Technology			