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研究課題名（和文）内因性ピリドキサミン-アルデヒド付加体の探索：酸化ストレスマーカーとしての評価

研究課題名（英文）Lipid hydroperoxide-derived modifications to pyridoxamine: a novel biomarker of oxidative stress

研究代表者

李　宣和（Lee, Seon Hwa）

東北大学・薬学研究科・助教

研究者番号：６０５１９７７６

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研究成果の概要（和文）：ピリドキサミン（PM）は、化学ストレス、例えば糖尿病による糖化ストレスを低減する。そこで本研究では、酸化・脂質化ストレスに対するPMの効果を、脂質過酸化とタンパク質修飾の観点で精査した。まず、過酸化脂質由来の反応性アルデヒド(4-oxo-2(E)-nonenal, ONEなど)が、PMによりピロール環誘導体として捕捉される事を明らかにした。次いでヒト血清アルブミンとリノール酸過酸化物との反応、更に酸化ストレス条件下の細胞実験で、PMの修飾阻害効果を確認した。今回、PMの脂質過酸化由来のストレス抑制効果に加え、PM-ONE付加体の酸化ストレスマーカーとしての可能性も示した。

研究成果の学術的意義や社会的意義
今回、過酸化脂質由来の求電子性アルデヒド化合物とPMの反応機構、反応生成物、および修飾阻害効果を精査した。これらの知見からPMは、糖尿病のみならず、酸化・脂質化の関与する様々な慢性疾患（高脂血症、老化など）さらには生活習慣（喫煙、飲酒など）によるタンパク質ダメージの抑制効果も期待される。同様にPMは、化学発癌や薬物毒性などの原因となる求電子性代謝物の捕捉を通して、タンパク質・DNA修飾を抑制する効果も期待される。

研究成果の概要（英文）：Pyridoxamine (PM) is a drug candidate for various chronic diseases because it can inhibit advanced glycation end product formation. PM might therefore prevent protein damage from lipid hydroperoxide-derived aldehydes 4-oxo-2(E)-nonenal (ONE) and 4-hydroxy-2(E)-nonenal HNE). Upon reaction with PM, ONE and HNE yielded an identical product containing a pyrrole ring, which structure was characterized by LC-MS and NMR. When human serum albumin (HSA) was reacted with a linoleic acid hydroperoxide, ONE modified more residues than did HNE. Upon treatment with PM, the formation of ONE-modified HSA peptides, but not of HNE-modified peptides, was reduced. Concomitantly, PM-ONE adducts increased in a dose-dependent manner. The inhibition effect of PM was confirmed in the cell system subjected to oxidative stress. Therefore, PM can inhibit lipid hydroperoxide-derived damage to proteins by trapping ONE preferentially, and the resulting PM-ONE adducts can be used as a dosimeter for ONE production.

研究分野：Clinical analytical chemistry, Chemical toxicology

キーワード：Oxidative stress, Lipid peroxidation, Pyridoxamine, Mass spectrometry, Protein modification

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９、ＣＫ－１９（共通）

１．研究開始当初の背景

(1) Oxidative stress has been associated with a number of inflammatory and age-related degenerative diseases. Increased production of reactive oxygen species (ROS) and up-regulation of lipoxygenases or cyclooxygenases result in the formation of lipid hydroperoxides, which undergo decomposition to reactive aldehydes, such as 4-oxo-2(*E*)-nonenal (ONE) and 4-hydroxy-2(*E*)-nonenal (HNE). These reactive aldehydes covalently modify cellular macromolecules, leading to mutation and apoptosis. ONE-derived DNA modifications have been characterized and detected in mammalian tissue DNA. Reaction of the histidine and lysine residues with ONE resulted in the formation of a novel cyclic structure within bovine histone H4. We have also identified the major ONE- and HNE-derived modifications to angiotensin (Ang) II, which has been implicated in various cardiovascular diseases. The major modifications identified are as follows: ONE-derived, pyruvamide-Ang II (Ang P, N-terminal α-ketoamide), [N-ONE]-Ang II (4-ketoamide), [Arg^2(ONE–H_2O)]-Ang II; HNE-derived, [His^6(HNE)]-Ang II (Michael addition product). Biological activities of Ang II are significantly altered when its N-terminus undergoes oxidative modification.

(2) Pyridoxamine (PM) is one of vitamin B6 vitamer and functions as a coenzyme in enzymatic transaminations *in vivo*. PM is also a promising pharmacological agent for the treatment of diabetic complications and other chronic conditions. This is based on its multiple inhibitory effects, including (i) inhibition of advanced glycation end products formation, (ii) inhibition of advanced lipoxidation end products formation, and (iii) trapping of ROS. Recently, it was reported that PM can react with ONE much more rapidly than lysine and suggested its use for therapeutically scavenging ONE. However, ONE-derived modifications to PM and potential roles of PM-ONE adducts as biomarkers of oxidative stress have not been thoroughly investigated.

２．研究の目的

(1) To characterize lipid hydroperoxide-derived modifications to PM
(2) To determine the major PM adducts from the reaction with reactive aldehydes in the presence of human serum albumin (HSA) and the cells that treated with reactive aldehydes
(3) To determine lipid hydroperoxide-derived modifications to HSA and cellular proteins in the absence and presence of PM to confirm the inhibitory effect of PM
(4) To develop enrichment procedure and MS-based quantitative analytical methodology for the major PM-reactive aldehyde adducts selected as biomarker candidates
(5) To monitor selected PM-reactive aldehyde adducts in cell systems that subjected to various oxidative stress to evaluate them as novel biomarkers of oxidative stress

３．研究の方法

(1) PM was reacted with lipid hydroperoxide-derived reactive aldehydes ONE and HNE. The modifications on PM were characterized by LC/ESI-MS, MS/MS and NMR analyses.
(2) PM was reacted with ONE and HNE in the presence of HSA and the major stable PM adducts were determined. Lipid hydroperoxide-derived modifications to HSA were also monitored in the absence and presence of PM to confirm the inhibitory effect of PM.
(3) As for the selected PM-ONE/HNE adducts, enrichment and LC/ESI-selected reaction monitoring (SRM)/MS methodologies were developed.
(4) In rat L6 skeletal muscle cells, lipid peroxidation and the formation of lipid hydroperoxide-derived reactive aldehydes were induced by $CuSO_4$ and ascorbic acid (AscA). The selected PM-ONE/HNE adducts were monitored using the method developed above. Specific PM-ONE/HNE adducts that exhibit dose-dependent increase in levels against the extent of oxidative stress were recognized as novel biomarkers of oxidative stress.

４．研究成果

(1) Characterization of modifications to PM derived from ONE and HNE

- LC/ESI-MS analysis of the reaction between PM and ONE at 37 °C for 5 days revealed the presence of nine products, which include PM + ONE – H_2O (PO1 and PO2), 2PM + 2ONE – $2H_2O$ (PO3 and PO4), PM + 2ONE (PO5 and PO6), PM + ONE – $2H_2O$ + 2H (PO7), PM + ONE – $2H_2O$ (PO8), and PM + 2ONE – H_2O (PO9).
- LC/ESI-MS analysis of the reaction between PM and HNE at 37 °C for 5 days revealed the presence of two products PM + HNE (PH1) and PM + HNE – $2H_2O$ (PH2). Time course experiments showed that PH1 increases quickly (0–24 h) and then decreased (1–7 days) while PH2 increased to its maximum level at 5 days, suggesting dehydration of PH1 to PH2.

- Structures of the major products (PO1, PO7, and PO8) were tentatively identified by MS/MS analysis.
- LC/ESI-MS and MS/MS analyses indicated that PO7 and PH2 have identical LC and MS properties (Figure 1A).
- NMR analysis of the isolated PH2 (PO7) characterized a novel PM adduct containing pyrrole ring that can be derived from both ONE and HNE (Figure 1B).

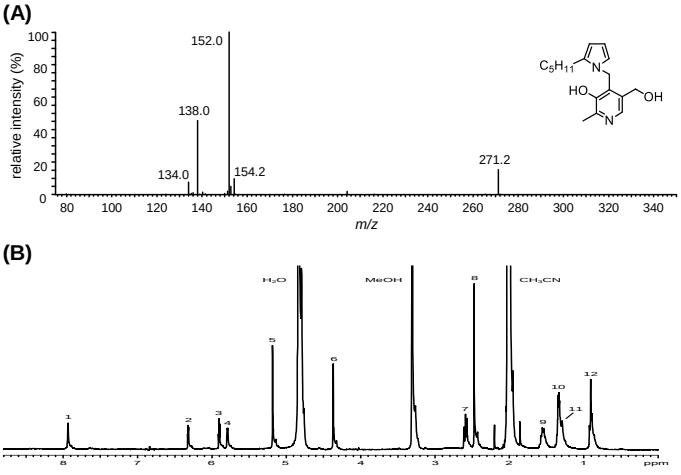

Figure 1. Analysis of PH2 (PO7) by (A) MS/MS and (B) NMR.

(2) Investigation of inhibition effect of PM on ONE/HNE-derived modifications to HSA

- In the incubation of HSA with ONE or HNE, Lys residues provided the most favorable modification sites for both aldehydes, and the number of HNE-modified sites was higher than that of ONE modified sites. When HSA was allowed to react with a linoleic acid hydroperoxide (13-HPODE) in the presence of AscA, ONE modified more residues (10 Lys, 3 His, 2 Arg) than did HNE (8 His, 2 Lys), indicating the relative reactivity of aldehydes towards amino acid residues.
- Upon treatment with increasing concentrations of PM, the levels of all PM-ONE/HNE adducts increased dose-dependently. The MS peak of PO1 was the most intense, followed by PO7/PH2 and PO8. The normalized MS peak intensities of the ONE-modified peptides decreased in a PM dose-dependent manner. In particular, the levels of peptides modified at K162, K190, and K525 showed a clear dose-dependent decrease. In contrast, no dose dependent change in concentration was observed for the selected HNE-modified peptides. Our results demonstrate that PM can inhibit lipid hydroperoxide-derived damage to proteins by trapping ONE preferentially.

(3) Investigation on the inhibition effect of PM on cellular protein damage

- SRM conditions such as mass transition, collision energy (eV), and *S*-lens RF amplitude (V), were optimized for PM adducts as follows: PH1, *m/z* 325.1→152.1, 24, 107; PO1/PO2, *m/z* 305.2→152.1, 25, 110; PO7/PH2, *m/z* 289.2→152.1, 23, 110; PO8, *m/z* 287.2→138.1, 14, 90.
- After the pretreatment of PM (0±100 μM), the cell were incubated with Ang II in the presence of AscA and $CuSO_4$ to induce lipid peroxidation followed by decomposition of lipid hydroperoxides to reactive aldehydes.
- LC/ESI-SRM/MS analysis revealed the presence of PO1 and PO2 even without PM treatment, indicating the formation of PM in the cell. By the addition of PM, levels of PO1 and PO2 were increased in a dose-dependent manner (Figure 2).

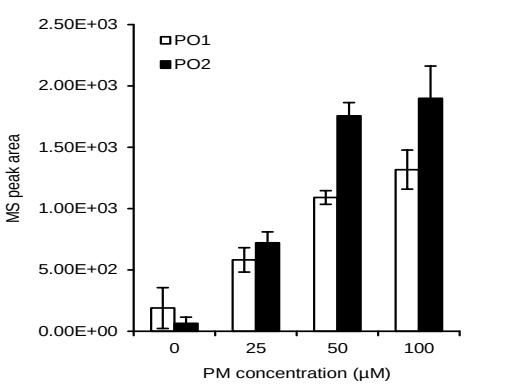

Figure 2. Changes in levels of PO1 and PO2 extracted from cell culture medium after incubation with Ang II, AscA and $CuSO_4$.

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〔図書〕（計　　件）

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http://www.pharm.tohoku.ac.jp/~bunseki/bunseki-e.html
researchmap
https://researchmap.jp/read0140803
ORCID iD
https://orcid.org/0000-0003-1095-5516

６．研究組織

(1)研究分担者

研究分担者氏名：大江　知行

ローマ字氏名：(OE, Tomoyuki)

所属研究機関名：東北大学

部局名：大学院薬学研究科

職名：教授

研究者番号（8 桁）：10203712

(2)研究協力者
研究協力者氏名：
ローマ字氏名：

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