様式Ｃ－１９

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研究課題名（和文）過酸化脂質由来の化学修飾アンジオテンシンの解析：心血管系疾患への新規アプローチ
研究課題名（英文）Lipid hydroperoxide-derived modifications to angiotensin peptides: a novel approach for cardiovascular disease
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研究成果の概要（和文）：アンジオテンシン(Ang)II と過酸化脂質由来の親電子性分解物（4-oxo-2(*E*)-nonenal (ONE)及び 4-hydroxy-2(*E*)-nonenal (HNE)）の反応において、Ang II 上の主な修飾位置が、N-末端α-アミノ基、Asp^1、Arg^2および His^6 である事を見出した。ONE 由来の新規修飾 Ang II として、N-末端アスパラギン酸の脱炭酸及び脱アミノ化による生成物（pyruvamide-Ang II）を同定した。次いで、得られた修飾 Ang II の生物活性を精査するため、非放射性 Ang II type 1 (AT_1)レセプターアッセイ法及びアミノペプチダーゼ(AP) A 酵素アッセイ法を、感度・選択性・スループットに優れ質量分析(MS)を活用して開発した。これらの結果より、Ang II の N-末端上の酸化修飾が、AT_1 レセプター及び APA との相互作用を阻害する事を確認し、心血管系の機能調節に影響を与える可能性を示唆した。

研究成果の概要（英文）：In the reactions of angiotensin (Ang) II with lipid peroxidation-derived bifunctional electrophiles, 4-oxo-2(*E*)-nonenal (ONE) or 4-hydroxy-2(*E*)-nonenal (HNE), the major modifications have been identified at the N-terminus, Asp^1, Arg^2, and His^6 of Ang II. The identities of ONE- and HNE-modified Ang IIs were confirmed by mass spectrometry (MS) before and after the reaction with sodium borohydride. A novel ONE-derive pyruvamide-Ang II was formed via oxidative decarboxylation of N-terminal aspartic acid. The unexpected formations of Arg- and His-modifications were confirmed using model reactions with N^{α}-*tert*-butoxycarbonyl-amino acids. To investigate the biological effects of modified Ang IIs, MS-based label-free Ang II type 1 (AT_1) receptor binding assay and aminopeptidase (AP) A enzyme assay have been developed. Using these methodologies, we have confirmed that the oxidative modifications on the N-terminus of Ang II disrupt interactions with AT_1 receptor and APA, which could affect the regulation of cardiovascular function.

交付決定額

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１．研究開始当初の背景

(1) Oxidative stress has been implicated in the degenerative diseases of aging such as cancer, cardiovascular disease and brain dysfunction. Increased production of reactive oxygen species (ROS) during oxidative stress results in the formation of lipid hydroperoxides, which undergo decomposition to the α,β-unsaturated aldehyde genotoxins such as 4-oxo-2(*E*)-nonenal (ONE), 4-hydroxy-2(*E*)-nonenal (HNE). There is increasing evidence that lipid hydroperoxide-derived modifications to glutathione, DNA bases and proteins are involved in cellular cytotoxicity, mutations and altered gene regulations.

(2) Angiotensin (Ang) II and its metabolites (Ang III, Ang IV and Ang A) have been implicated in various cardiovascular diseases, such as hypertension, atherosclerosis and heart failure. Oxidative stress has been considered as a central mechanism of these diseases based on the involvement of ROS in numerous signaling pathways of Ang peptides. Another major consequence of ROS-derived damage to cardiovascular system is lipid peroxidation with production of the genotoxic aldehydes. However, little attention has been given to the potential for the lipid hydroperoxide-derived modifications to Ang peptides that could modulate their biological functions.

２．研究の目的

(1) To characterize lipid hydroperoxide-derived modifications to Ang peptides.

(2) To develop the mass spectrometry (MS)-based analytical methodology to profile Ang and modified Ang peptides in biological systems.

(3) To test biological activities of modified Ang peptides.

(4) To investigate alternative molecular mechanisms of cardiovascular diseases.

(5) To provide biomarkers for oxidative damage and therapeutic targets for patients with cardiovascular diseases.

３．研究の方法

(1) Ang II was allowed to react with lipid hydroperoxide-derived bifunctional electrophiles such as ONE or HNE. Reactions were monitored by liquid chromatography (LC)/UV and/or LC-electrospray (ESI)/MS in different reaction times and concentrations.

(2) The modifications on Ang II were characterized by LC-ESI/MS and/or matrix assisted laser desorption ionization-time of flight (MALDI-TOF)/MS.

(3) Further reactions such as sodium borohydride reduction or reaction with model amino acids were carried out for additional structural information.

(4) To investigate the biological effects of modified Ang IIs, MALDI-TOF/MS-based label-free Ang II type 1 (AT_1) receptor binding assay and LC-ESI/MS-based aminopeptidase (AP) A enzyme assay have been developed.

４．研究成果

(1) LC/UV analysis of the reaction between Ang II and ONE revealed the presence of four major products (O1-O4). When the reaction was monitored for 48 h, the formations of O1, O3 and O4 increased and O1 was the most abundant product throughout the incubation. However, O2 was not stable so that its formation was decreased gradually. The stable products were isolated and analyzed by MS. As a results, we have identified three major adducts, such as pyruvamide-Ang II (O1), the 4-ketoamide form of [N-ONE]-Ang II (O3), and the Arg-modified [Arg^2(ONE − H_2O)]-Ang II (O4).

(2) MALDI-TOF/MS analyses of O1 revealed an MH^+ at *m/z* 1001.5 corresponding to the loss of 45 Da from Ang II. MALDI-postsource decay-TOF/MS analysis revealed the modification occurred at N-terminal aspartic acid. Based on the MS data and comparison with authentic standard, O1 was characterized as pyruvamide-Ang II (Ang P). Thus, the initial reaction of ONE with N-terminal α-amino group of Ang II results in the formation of Schiff base intermediate, which undergoes decarboxylation followed by

hydrolysis to provide an α-keto amide (pyruvamide) moiety at the N-terminus of Ang II. Therefore, we have made a novel discovery that ONE mediates not only adduct formation but also oxidative decarboxylation of N-terminal aspartic acid.

(3) LC/UV analysis of the reaction between Ang II and HNE revealed the presence of eleven major products. Products H1-H5 have an identical MH^+ corresponding to the addition of HNE. The products H6-H10 have an identical MH^+, which corresponded to the addition of HNE and loss of H_2O. When the incubation was continued for 14 days, the formations of H1-H5 were gradually decreased whereas the formations of H6-H10 increased steadily reaching maximum levels after 14 days. H1-H5 were characterized as Michael addition products at His residue and they were shown to be dehydrated to form H6-H10.

(4) To confirm the formation of dehydrated Michael addition product, we carried out the model experiments with N^{α}-*tert*-butoxycarbonyl (*t*Boc)-His and HNE. In contrast to the Ang II reaction, the LC-ESI/MS analysis revealed an exclusive formation of *t*Boc-His-HNE, a Michael addition product, which was added two hydrogen atoms by the reaction with $NaBH_4$. However, it was not dehydrated even after 14 days of incubation. These results indicate that a dehydration of the Michael addition product in Ang II reaction could be induced by the special conformation of Ang II. Recent conformational studies on Ang II have proposed that a bioactive conformation is stabilized by hydrogen boding interactions between guanidinium (Arg^2), hydroxylate (Tyr^4), imidazole (His^6) and carboxylate (Phe^8). This means that the hydroxyl group on the hemiacetal ring formed by the reaction of His6 on Ang II with HNE can be easily protonated by C-terminal carboxyl group, which facilitates its dehydration reaction.

(5) We have developed a label-free AT_1 receptor binding assay using MALDI-TOF/MS, which allows the rapid, sensitive, and reproducible screening of a large number of samples. The affinity of competitors can be directly compared to that of Ang II by quantifying Ang II dissociated from the receptor-ligand complex using 50% methanol in water. MS sensitivity was maximized by choosing a suitable matrix and sample plate for Ang II. A stable-isotope-labeled internal standard was employed for accurate quantitation. When the assay was applied to the modified Ang IIs, it was revealed that the affinity of Ang P (K_i = 42 nM) was about 40 times lower than that of Ang II and the affinity of Ang C (cyclized form of Ang P, K_i = 2500 nM) was even lower than that of Ang P.

(6) Ang II can be converted to Ang III by APA-mediated cleavage of N-terminal aspartic acid. We have developed the LC-ESI/MS-based APA enzyme assay to investigate the metabolism of modified Ang IIs. When Ang II was incubated with APA in the presence of Ca, the formation of Ang III was observed in 20 min. However, Ang P and Ang C were not metabolized at all to Ang III even after a 24 h-incubation. Therefore, these results have confirmed that the oxidative modifications on the N-terminus of Ang II disrupt interactions with AT_1 receptor and APA, which could affect the regulation of cardiovascular function.

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〔その他〕
ホームページ等
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