Lipid Hydroperoxide-mediated Protein Modification: Mechanistic Study and Screening Strategy by Mass Spectrometry

(過酸化脂質由来のタンパク質修飾に関する研究:
質量分析法によるメカニズム解析とスクリーニング法の開発)

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東北大学大学院　薬学研究科　生命薬科学専攻
臨床分析化学分野

髙橋　亮

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[Background & Objective]

Increased production of reactive oxygen species (ROS) during oxidative stress has been associated with a number of age-related degenerative diseases, cancer, cardiovascular diseases, and neurological disorders. ROS exhibit facile reactivity with DNA bases, proteins, and polyunsaturated fatty acids (PUFAs). Linoleic acid (LA), one of representative PUFAs, can be converted to hydroperoxides (13-hydroperoxy-9,11 (*Z*,*E*)-octadecadienoic acid, 13-HPODE), which undergo homolytic decomposition to bifunctional electrophiles such as 4-oxo-2(*E*)-nonenal (ONE). These reactive aldehydes are involved in protein dysfunctions and altered gene regulations through the modification of amino acid residues and crosslinking of proteins. Thus, there have been intensive efforts to ascertain the structural nature of lipid hydroperoxide-mediated protein modifications, followed by detecting them in biological samples. However, most previous studies have focused on either the structural characterization of modifications on a model amino acid or the analysis of protein modification by a single electrophile because of the difficulties in dealing with complex samples. Therefore, it is required to conduct a comprehensive study of the protein modifications that result from various peroxidation products of PUFAs. In my proposal, following approaches (Figure 1) have been applied to find novel biomarkers for lipid peroxidation and improve understanding of the relationship between lipid hydroperoxide-mediated protein modifications and degenerative diseases: i) A bioactive peptide, angiotensin (Ang) II (DRVYIHPF), was employed to characterize its modification by multiple electrophiles [1, 2]. ii) Based on the results, the proteomic methodology was developed for the screening of lipid hydroperoxide-mediated protein modification using an isotope data dependent scan (IDDS) and stable isotope labeling by fatty acid in

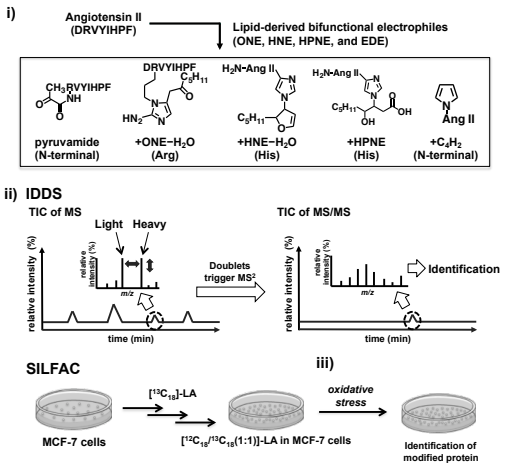

Figure 1. Outline of the current study. i) Identification of lipid-derived modifications to Ang II, ii) Development of IDDS and SILFAC, and iii) Screening of modified protein in the cell system.

cell culture (SILFAC). iii) The developed methodology was then applied to screen out modified proteins in MCF-7 cells subjected to conditions of oxidative stress.

[Methods]

Ang II was allowed to react with ONE, 4-hydroxy-2(*E*)-nonenal (HNE), 4,5-epoxy-2(*E*)-decenal (EDE) or 4-hydroperoxy-2(*E*)-nonenal (HPNE). Each reaction mixture was analyzed by liquid chromatography (LC)-ultraviolet (UV), matrix-assisted laser desorption ionization (MALDI)/time-of-flight (TOF)-mass spectrometry (MS), and LC/electrospray ionization (ESI)-MS to identify modified Ang II. MALDI/postsource decay TOF-MS and tandem mass spectrometry (MS/MS) were also performed to confirm each modification before and after sodium borohydride reduction. For further investigation of the mechanisms and structures of each modification, prolonged incubation and model reactions using N^{α}-*tert*-butoxycarbonyl-amino acids were performed. 13-HPODE and [$^{13}C_{18}$]-13-HPODE were prepared using soybean lipoxidase from LA and [$^{13}C_{18}$]-LA, respectively. The mixture of 13-HPODE and [$^{13}C_{18}$]-13-HPODE was purified using normal phase LC system, and their combined concentration was determined by UV spectroscopy. A mixture (1:1) of 13-HPODE and [$^{13}C_{18}$]-13-HPODE was then allowed to react with Ang II or human serum albumin (HSA) in the presence of L-ascorbic acid (AscA). The major modifications derived from LA peroxidation were determined and the settings of IDDS were optimized by analyzing the reaction mixtures. MCF-7 cells were cultured with [$^{13}C_{18}$]-LA-albumin and the conditions were optimized for the cells to produce a 1:1 ratio of LA and [$^{13}C_{18}$]-LA by monitoring LA-containing major phospholipids (PLs) in the cells. Total lipid extraction was performed by Bligh-Dyer method and the resulting lipids were purified on Oasis HLB cartridge columns. The sample was analyzed by LC/ESI-selected reaction monitoring (SRM)/MS in the negative ion mode. Fragment ions arising from *sn*-1 and *sn*-2 positions were used for Q3 ions. The SILFAC cells were treated with calcium ionophore A23187 and AscA at 37 °C for 24 h. The cells were lysed in CelLytic M buffer containing protease and phosphatase inhibitor cocktail and the proteins were extracted. After acetone purification, the pellets were dissolved in 8 M urea. The solution was reduced and alkylated with dithiothreitol and iodoacetamide, respectively. Trypsin was added in a ratio of 1:50 (w/w) after 10–fold dilution with 50 mM ammonium bicarbonate, followed by incubation overnight at 37 °C. A portion of the sample (1 μL) was analyzed by LC/ESI-MS/MS or LC/ESI-IDDS/MS. Data were analyzed using Proteome Discoverer 1.3. Peak list was searched by Mascot 2.3.01 against

UniProtKB/Swiss-Prot (2014_11, 547085 sequences).

[Results]

The most abundant ONE-derived modification to Ang II was pyruvamide-Ang II (Ang P) that formed *via* oxidative decarboxylation of N-terminal Asp. It was followed by [Arg^2(ONE–H_2O)]-Ang II and a 4-ketoamide form of [*N*-ONE]-Ang II. Michael addition product of [His^6(HNE)]-Ang II and dehydrated Michael addition product of [His^6(HNE–H_2O)]-Ang II were major HNE-derived modifications. The specific conformation of Ang II induced dehydration of the Michael addition product. EDE preferentially modified the N-terminus and His^6 of Ang II. In addition to the N-substituted pyrrole of [*N*-C_4H_2]-Ang II and Michael addition products of [His^6(EDE)]-Ang II, their hydrated forms were detected as major products. Substantial amounts of [*N*-(EDE–H_2O)]-Ang II isomers were also formed. The main HPNE-derived products were [His^6(HPNE)]-Ang II and [*N*-(HPNE–H_2O)]-Ang II. However, ONE, HNE, and malondialdehyde-derived modifications were dominant, because HPNE is a precursor of these aldehydes. These modified Ang IIs were detected as predominant products in the reaction between Ang II and 13-HPODE mixture. The characteristic isotope pattern (^{12}C:^{13}C=1:1) observed in MS spectra and the mass difference of the $[M + H]^+$ doublet also aided the identification of [*N*-(9,12-dioxo-10(*E*)-dodecenoic acid)]-Ang II and [His^6(9-hydroxy-12-oxo-10(*E*)-dodecenoic acid)]-Ang II as major LA-derived modifications. IDDS was optimized to trigger when the doublet with corresponding mass difference (*e.g.* 4.5 Da for double charged ions of ONE, HNE, and HPNE-adducts) and a ratio of 1:1 were detected in the survey scan. The optimal match tolerance for the intensity of the doublet was determined to be 0.15. The exclusion mass width was then adjusted to avoid triggering a data dependent scan of interfering peaks. When the value was set at ± 5 ppm, interfering peaks were excluded effectively. Using the current settings, it was possible to screen all the major 13-HPODE-derived modifications to Ang II and HSA. As for HSA, IDDS (ΔDa=4.514 and 3.009) revealed 9 (ONE × 2 and HPNE × 7) and 24 (ONE × 1, HNE × 2, and HPNE × 21) modifications, respectively. After the optimization of SRM conditions for the analysis of PLs, time course experiment was performed to determine the optimal time for incorporation of [$^{13}C_{18}$]-LA into MCF-7 cells. The metabolic incorporation was observed after 6 h of incubation and reached its maximum level in 72 h. SILFAC was achieved by three times of [$^{13}C_{18}$]-LA-albumin treatment (1.4 μM) in 120 h, followed by one subculture. Finally, global screening using SILFAC and LC/ESI-IDDS/MS identified the presence of 133 modified peptides. Among them,

104 peptides were identified only by IDDS.

[Discussion]

Many studies have suggested that the N-terminal Asp^1, Arg^2, and His^6 of Ang II play key roles in its biological activity and receptor binding. Therefore, Ang II modifications identified in this study could modulate its functions *in vivo*. Indeed, Ang P was shown to disrupt the interaction with the Ang II type I receptor, and it did not undergo further metabolism *via* aminopeptidase A, which converts Ang II to Ang III. The presence of ONE, HNE, and HPNE-derived modifications in the 13-HPODE mixture-treated HSA suggests that these modifications could occur *in vivo* and serve as biomarkers of lipid hydroperoxide-mediated damage.

To screen such modifications effectively, conditions for IDDS were optimized and SILFAC was developed by incubating MCF-7 cells with $[^{13}C_{18}]$-LA-albumin. It is important to control the cell culture to produce LA and $[^{13}C_{18}]$-LA in a constant ratio, such as 1:1 in the present study. Their hydroperoxides, 13-HPODE and $[^{13}C_{18}]$-13-HPODE (1:1) generate reactive aldehydes as a mixture of ^{12}C and ^{13}C isomers (1:1), which react with proteins in the cells and produce modified proteins as a mixture of ^{12}C and ^{13}C isomers (1:1). The isotope mass intensity ratio (1:1) and mass difference of the $[M + H]^+$ doublet are critical factors to trigger IDDS. Although the ratio of LA and $[^{13}C_{18}]$-LA was maintained to be 1:1 until the cell were subjected to oxidative stress, ratios of modified protein produced were found to be slightly altered. This alteration could be caused by the metabolic conversion of $[^{13}C_{18}]$-LA to $[^{12}C_{2,}\ ^{13}C_{18}]$-arachidonic acid (AA), and decomposition of endogenous AA to generate identical reactive aldehydes. The problem was overcome by modulating the setting of match tolerance for IDDS.

It was shown that the current screening strategy using SILFAC and IDDS can be applied to cell systems without complicated enrichment procedure. IDDS also help to reduce data analysis time by filtering only modified peptides for MS/MS. Therefore, this proteomic screening methodology can be a powerful tool for fast and comprehensive identification of lipid hydroperoxide-mediated protein modifications, which facilitates uncovering novel biomarkers for lipid peroxidation in various cell systems.

[List of publications]

[1] Lee, S. H., Takahashi, R., Goto, T., Oe, T. (2010) *Chem. Res. Toxicol.* **23**, 1771-1785.
[2] Takahashi, R., Goto, T., Oe, T., Lee, S. H. *Chem-Biol. Interact.* (submitted).