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Mass spectrometric characterizations of protein carbonylation: Comparison between three different conditions, oxidation by Cu^{II}/ascorbic acid, adduction of methyl glyoxal, and adduction of 4-hydroxy-2(*E*)-nonenal

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ABSTRACT

Oxidative stress can cause various protein modifications, mainly carbonylation. There are three major chemical ways to introduce hydrazine reactive carbonyls: (i) direct oxidation by reactive oxygen species (ROS), (ii) adduction of sugars or sugar-related aldehydes, and (iii) adduction of lipid-related aldehydes. Here, we performed a comparative study of three representative conditions: Cu^{II}/ascorbic acid (AA) (ROS), methylglyoxal (sugar-related), and 4-hydroxy-2(*E*)-nonenal (HNE) (lipid-related). Insulin β chain and Girard's reagent P were used as the model protein and hydrazine reagent, respectively. LC/MS analyses of reactions with HNE or Cu^{II}/AA-treated linoleic acid revealed that protein carbonylation under oxidative stress is the most efficient in the presence of polyunsaturated fatty acids.

1. Introduction

Increased production of reactive oxygen species (ROS) causes oxidative stress, which is implicated in the degenerative diseases of aging such as cancer, cardiovascular diseases, and brain dysfunctions [1]. Therefore, various protein modifications under oxidative stress have been studied in relation to aging and disease [2–4]. Several kinds of ROS-mediated reactions result in direct protein modifications [5,6], such as oxidative cleavage at the carboxyl side of proline (Pro) [7], oxidation of histidine (His) [8,9], sulfoxidation of methionine (Met) [10], sulfenylation and sulfinylation of cysteine (Cys) [11], carbonylation of lysine (Lys), arginine (Arg), and Pro [12,13], and so on. Reactions resulted from glycoxidation and lipoxidation processes have been studied as indirect modifications [14]. In addition, epimerization of N-terminal amino acids by lipoxidation products have been recently reported by the authors' group [15]. Among these oxidative stress-mediated protein modifications, carbonylation has been widely studied for biomarkers [16] because specific derivatization strategies for their detection have been established [17–19]. For example, hydrazine reagents that form hydrazone derivatives with carbonyl groups, such as 2,4-dinitrophenylhydrazine (DNPH), are the most frequently used reagents. Furthermore, in combination with anti-DNPH antibody, western blotting (WB) [20], 2D-Gel fingerprint imaging [21], and enzyme-linked immunosorbent assay [22] can be applied. Furthermore, biotinylated [23,24] and charged [25] hydrazine reagents can facilitate clean-up from biological samples and enable sensitive analysis by mass spectrometry (MS), respectively. Therefore, many studies (PubMed search on July 29, 2014; keywords: carbonylation; protein; oxidative stress; 2309 hits) [26] have been performed to evaluate oxidative stress *in vivo* and *in vitro*.

Abbreviations: AA, ascorbic acid; AcOH, acetic acid; ARA, arachidonic acid; DNPH, 2,4-dinitrophenylhydrazine; EIC, extracted ion chromatogram; ESI, electrospray ionization; FA, formic acid; GRP, Girard's regent P; HNE, 4-hydroxy-2(*E*)-nonenal; ONE, 4-oxo-2(*E*)-nonenal; Ins β, insulin chain β; LA, linoleic acid; LC, liquid chromatography; MG, methylglyoxal; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PB, phosphate buffer; ROS, reactive oxygen species; TIC, total ion chromatogram; V8, endoproteinase Glu-C; WB, western blotting.

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There are three major chemical ways to introduce carbonyl groups into proteins [17]: (i) oxidation of amino acid residues by ROS, (ii) adduction of molecules derived from sugars, and (iii) adduction of molecules derived from lipids. In ROS-mediated direct carbonylation, side chains of Lys, Arg, and Pro have been reported to transform carbonyl compounds, such as α-aminoadipic and γ-glutamic semialdehydes [12,13]. Aldehydes produced through the Maillard reaction, such as methylglyoxal (MG) and 3-deoxyglucosone, have been reported to react mainly with Lys and Arg to form advanced glycation end-products [27,28]. Aldehydes from lipid hydroperoxide, such as 4-hydroxy-2(*E*)-nonenal (HNE) and 4-oxo-2(*E*)-nonenal (ONE), have been reported to react mainly with His, Lys, and Arg to form lipoxidation products [29,30]. Furthermore, lysyl oxidase has been recognized as an extracellular enzyme that catalyzes formation of α-aminoadipic semialdehyde from lysine residues in collagen and elastin as an enzymatic protein carbonylation [31]. Yet to the best of our knowledge, there are no reports confirming the major carbonylation route, because most studies focus on one carbonylation source for the sake of simplicity, despite the fact that proteins are known to be oxidized in at least 35 different ways [17].

Here, we performed a comparative study of the formation of hydrazine reactive carbonylated proteins under each representative condition: Cu^{II}/ascorbic acid (AA) (as ROS), MG (as sugar-related), or HNE (as lipid-related). Insulin β chain (Ins β, Fig. 1a) was used as a model protein because it contains all the target amino acids of ROS (Lys, Arg, and Pro) and nucleophilic amino acids that can react with aldehydes (Lys, Arg, and His). Furthermore, Ins β in combination with endoproteinase Glu-C (V8, cleaves at the carboxyl side of glutamic acid) is ideal because V8 can avoid miscleavages resulting from modifications and can digest to give three peptides of appropriate lengths (Fig. 1b–d). Girard's reagent P (GRP, Fig. 1g) was used as a hydrazine reagent, because the quaternary amine structure is known to facilitate clean-up using a strong cation exchange column [25] and enhances ionization to enable sensitive detection by MS [25,32].

2. Materials and methods

2.1. Reagents

Ins β (FVNQHLC$^{\#}$GSH LVEALYLVC$^{\#}$G ERGFFYTPKA, C$^{\#}$ = oxidized-Cys (—SO_3H); bovine) (Fig. 1a), AA, MG (Fig. 1e), linoleic acid (LA), and V8 were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). HNE (Fig. 1f) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). GRP [1-(hydrazinocarbonyl-methyl) pyridinium chloride] (Fig. 1g) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Formic acid (FA), acetic acid (AcOH), and copper(II) sulphate pentahydrate ($CuSO_4{\cdot}5H_2O$) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), disodium hydrogenphosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), and ammonium bicarbonate (NH_4HCO_3) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Acetonitrile (HPLC grade) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Amicon Ultra-0.5 centrifugal filter devices (0.5 mL, cutoff 3 kDa) were purchased from EMD Millipore Co. (Bedford, MA, USA). Ultrapure water was obtained from a Milli-Q Integral 10 (EMD Millipore, Billerica, MA, USA) equipped with a 0.22 μm membrane cartridge. Nitrogen (N_2) and helium (He) gases for MS were purchased from Taiyo Nippon Sanso Co. (Tokyo, Japan).

2.2. LC/ESI-MS

Liquid chromatography (LC) was carried out using an Agilent 1100 system (Agilent Technologies Inc., Santa Clara, CA, USA) consisting of a G1312A pump, a G1367A autosampler, a G1314A photodiode array detector, and a G1316A column oven. A Jupiter 4u Proteo 90A (150 × 2.1 mm i.d., 4 μm, 90 Å; Phenomenex, Torrance, CA, USA) column was used at 35 °C with solvent A (0.1% FA in water, v/v) and solvent B (0.1% FA in acetonitrile, v/v). The linear gradient used was as follows: 1% B at 0 min, 1% B at 5 min, 50% B at 40 min, 100% B at 41 min, 100% B at 51 min, 1% B at 52 min, and 1% B at 70 min with a flow rate of 0.2 mL/min.

Electrospray ionization (ESI)-MS and tandem mass spectrometry (MS/MS) was carried out using an LCQ-Deca (Thermo Fisher Scientific Inc., Waltham, MA, USA), which is a 3D-ion trap mass spectrometer equipped with an ESI interface. The optimal operating conditions were as follows: mode, positive; source voltage, 4.5 kV; heated capillary temperature, 300 °C; sheath and auxiliary gas (N_2) pressures, 85 and 15 (arbitrary units), respectively. Full scanning analyses were performed in the *m/z* range of 400–2000. MS/MS analyses were performed using data-dependent scanning mode and the parameters were as follows: collision gas, He; precursor, top 3 ions; default charge state, 2; default isolation width, 2.00; normalized collision energy, 30.0; activation Q, 0.250; activation time, 30.00 ms; minimum signal required, 100,000; and minimum MS^n signal required, 5000. Data analyses were performed using Xcalibur™ version 2.0 SR2 (Thermo Fisher Scientific Inc.).

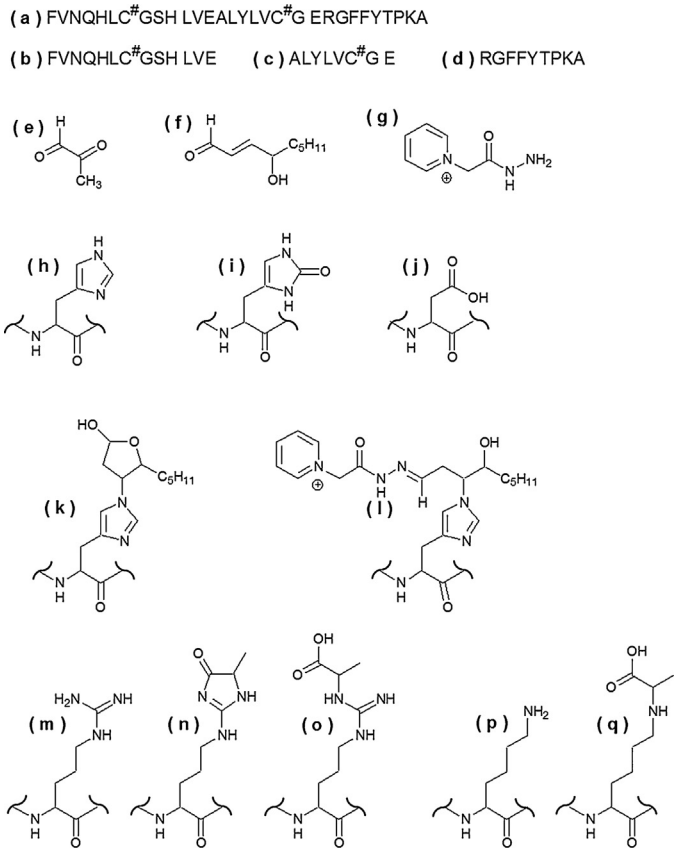

Fig. 1. Structures of compounds appearing in the manuscript. (a), Ins β; (b), peptide 1; (c), peptide 2; (d), peptide 3; (e), methylglyoxal (MG); (f), 4-hydroxy-2(*E*)-nonenal (HNE); (g), Girard's reagent P (GRP); (h), histidine (His); (i), 2-oxo-histidine (2-oxo-His); (j), asparatic acid (Asp); (k), HNE-His adduct; (l), HNE-His adduct derivatized with GRP; (m), arginine (Arg); (n), N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (MG-H); (o), N^{ω}-(carboxyethyl)-Arg; (p), lysine (Lys); and (q), N^{ω}-(carboxyethyl)-Lys. C$^{\#}$ in (a)–(c) indicates oxidized cysteine (—SO_3H).