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Bioanalytical Applications

Advanced glycation end products of beta$_2$-microglobulin in uremic patients as determined by high resolution mass spectrometry☆

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ABSTRACT

By using a high resolution top-down and bottom-up approach we identified and characterized the AGEs of beta$_2$-microglobulin (β_2-m) formed by incubating the protein in the presence of glucose and of the main reactive carbonyl species. Glucose induced glycation on the N-terminal residue, while glyoxal (GO) and methylglyoxal (MGO) covalently reacted with Arg3. Carboxymethyl (CM-R) and imidazolinone (R-GO) derivatives were identified in the case of GO and carboxyethyl arginine (CE-R) and methyl-imidazolinone (R-MGO) for MGO. Interestingly, α,β-unsaturated aldehydes [4-hydroxy-2-nonenal (HNE); 4-oxo-2-nonenal (ONE); acrolein (ACR)] did not induce any covalent modifications up to 100 μM. The different reactivity of β_2-m towards the different RCS was then rationalized by molecular modeling studies. The MS method was then applied to fully characterize the AGEs of β_2-m isolated from the urine of uremic subjects. CM-R, CE-R and R-MGO were easily identified on Arg3 and their relative abundance in respect to the native protein determined by a semi-quantitative approach. Overall, the AGEs content of urinary β_2-m ranged from 0.2 to 1% in uremic subjects. The results here reported offer novel insights and technical achievements for a potential biological role of AGEs-β_2-m in pathological conditions.

1. Introduction

Advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) represent a quite heterogeneous and complex class of covalently modified proteins that are formed by different oxidative-based mechanisms and can occur either exogenously or endogenously [1]. Two main pathways are involved in AGEs formation, the first is based on the reaction of reducing sugars with protein primary amino groups (the amino terminus and the ε-amino group of Lys), followed by metal ion catalyzed rearrangements. The second involves the reaction of reactive carbonyl species (RCS) such as glyoxal (GO), methylglyoxal (MG) with nucleophilic protein sites, and in particular Arg, Lys and Cys. The RCS acting as AGEs precursors are generated by either sugar or AGEs decomposition/autoxidation, a series of reactions which are catalyzed by metal ions. ALEs are generated by only one pathway, based on the covalent adduction of lipid derived RCS (HNE, GO, MDA) with Arg, Lys, His and Cys nucleophilic residues [1].

AGEs and ALEs play a pathogenetic role in the onset and progression of different oxidative-based diseases, including diabetes [2–4], chronic renal failure [5,6], cardiovascular diseases [7,8] and neurological disorders [9]. Currently, great analytical effort is spent

Abbreviations: β_2-m, Beta$_2$-microglobulin; C-β_2-m, commercial β_2-m; nC-β_2-m, non-commercial β_2-m; RCS, reactive carbonyl species; AGEs, advanced glycoxidation end products; ALEs, advanced lipoxidation end products; AGEs-β_2-m, AGE-modified beta$_2$-microglobulin; GO, glyoxal; MGO, methylglyoxal; HNE, 4-Hydroxy-2-nonenal; ONE, 4-oxo-2-nonenal; ACR, acrolein; CM-R, carboxymethyl arginine; CE-R, carboxyethyl arginine; R-GO-I, arginine-glyoxal imidazolinone; R-MGO-I, arginine-methylglyoxal imidazolinone.

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on the identification, characterization and quantitative or semi-quantitative analysis of AGEs and ALEs, in both *in vitro* and *ex vivo* conditions. Such information is of great value, not only to better understand the pathogenetic role of AGEs/ALEs, but also to identify the oxidation pathways involved in protein modification as well as novel biomarkers and drug targets [10,11]. An elevated risk of tumor has been related to oxidative stress and increased levels of AGEs and ALEs have been observed in chronic diseases, such as cancer [12].

While it is quite easy to generate and characterize AGEs and ALEs by incubating the target protein with glucose or RCS in *in vitro* conditions (usually using supra physio-pathological concentrations of the reactants), the identification and characterization of such covalent adducts in *ex vivo* samples is much more challenging, not only because of the presence of the matrix, but also due to the low amounts of AGEs and ALEs in respect to the native proteins. In general, the most widely applied technique to detect AGEs/ALEs in *ex vivo* samples is based on proteomic approach consisting of Western blot analysis to identify AGEs/ALEs and MS to identify the protein bearing the oxidative modification. By using such an approach, human serum albumin (HSA) was identified as the main circulating protein undergoing a complex pattern of oxidative modifications in different physio-pathological conditions and forming several AGEs and ALEs which have been identified *in vitro* as well as *ex vivo* [13–16]. Besides HSA, other AGEs and ALEs of circulating or urinary proteins have so far been identified and among these β_2-m might represent a protein target of interest.

Wild type β_2-m is indeed responsible for dialysis related amyloidosis (DRA), an unavoidable and severe complication that occurs in chronically hemodialyzed patients. In DRA, mature fibrils consisting of β_2-m are deposited preferentially in the osteoarticular tissues and then, over the years, lead to bone and joint destruction [17]. Some studies suggest that AGEs of β_2-m (AGEs-β_2-m) contribute to the development of the inflammatory response through the activation of RAGE receptors, monocyte chemotaxis and the inhibition of collagen synthesis by human fibroblasts [18–22]. Several analytical strategies have been reported in order to identify and characterize AGEs-β_2-m in both plasma, urine and amyloid fibrils but no definitive structural assignment nor quantitative or semi-quantitative data have so far been provided. The identity of AGEs-β_2-m has mainly been carried out by using immunological techniques such as immunohistochemistry, Western blot and ELISA. The first AGE structure was identified in β_2-m purified from long-term hemodialysis patients as well by incubating, *in vitro*, β_2-m with glucose and the structure of the covalently modified β_2-m was assigned to glycated derivatives [23]. The primary glycated site was identified as the α-amino group of the amino terminal isoleucine which was then confirmed by MALDI-MS together with other minor glycated sites [24]. Pentosidine derivative was then identified by HPLC assay and competitive ELISA in amyloid-fibril β_2-m [25]. AGEs products formed by the reaction of β_2-m with RCS such as glyoxal and 3-deoxyglucosone, namely carboxymethyl lysine and imidazolinone were then identified by immunohistochemistry and immunological techniques [26–29]. Immunohistochemical studies also detected ALEs derivatives of β_2-m in long lived amyloid deposits, suggesting that RCS derived lipid peroxidation products such as MDA and HNE are also involved in the covalent modification of β_2-m [30]. Most of the results so far reported on AGEs-β_2-m are based on immunological approaches and are mainly carried out on β_2-m isolated by fibril deposits. Unequivocal structural confirmation by MS has been provided only for the glycated β_2-m and there is little information on the AGEs and ALEs of circulating and urinary β_2-m.

High resolution MS coupled to nano-LC is the most suitable analytical technique for studying protein covalent modifications in terms of sensitivity and of wealth of structural information. Several applications have so far been reported for the analysis of AGEs and ALEs in both *in vitro* and *ex vivo* conditions [10,31]. In this study an Orbitrap™ MS analyzer coupled to a nano-LC system was used to fully characterize AGEs and ALEs of β_2-m generated *in vitro*, by incubating β_2-m with RCS in physiopathological conditions. The approach was then applied to the identification and characterization of AGEs-β_2-m isolated from the urine of uremic patients. Moreover, a semi-quantitative analysis in order to determine the relative abundance of AGEs-β_2-m in respect to the native isoform has also been carried out.

2. Experimental

2.1. Reagents and materials

Ammonium bicarbonate (NH_4HCO_3), formic acid, ammonium hydroxide, methylglyoxal (MGO), glyoxal (GO), acrolein (ACR) and LC-MS grade solvents (Chromasolv®) were purchased from Sigma–Aldrich (Milan, Italy). LC-grade H_2O (18 MΩ cm) was prepared with a Milli-Q H_2O purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade. Sequence grade chymotrypsin was obtained from Roche Diagnostics S.p.A. (Monza, Italy).

4-Hydroxy-2-nonenal diethylacetal (HNE-DEA) and 4-oxo-2-nonenal diethylacetal (ONE-DEA) were synthesized according to the literature [32]. HNE and ONE were prepared by hydrolysis of HNE-DEA and ONE-DEA, respectively (1 h at room temperature in 1 mM HCl) and their concentration determined by UV spectroscopy (λ_{max} 224 nm; 13,750 M^{-1} cm^{-1}).

2.2. Beta$_2$-microglobulin

Recombinant wild type beta$_2$-microglobulin (rβ_2-m) was expressed as an inclusion body in *Escherichia coli* by following the procedure previously described [33]. Commercial β_2-m (C-β_2-m) from pooled urines of uremic subjects (patients with chronic renal tubular proteinuria) was from Sigma-Aldrich (Milan, Italy). Non-commercial β_2-m (nC-β_2-m) was isolated from the residual urine of a patient affected by DRA who underwent chronic hemodialysis (8 years) due to glomerulonephritis. Both samples were purified as reported by Berggard et al. [34]. Briefly, the procedure used for nC-β_2-m was as follows: twenty-four-hour urine was collected, dialyzed (membrane cut-off 6000) against water and finally lyophilized. Sample (total amount of 100 mg) was chromatographed on Sephacryl S300 column (2.5 × 100 cm) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with Tris HCl (pH 7.4; 20 mM) containing 150 mM NaCl. β_2-m was isolated by gel filtration, dialyzed against 5 mM NaH_2PO_4 pH 8 and finally purified by ion-exchange chromatography with a mono Q column 16/10HR (GE Healthcare Bio-Sciences AB). The protein concentration was determined spectrophotometrically at λ = 280 nm and considering the A0.1% of 1.7.

2.3. Sample preparation

2.3.1. In vitro studies: incubation of recombinant β_2-m with RCS and glucose

rβ_2-m (10 μM final concentration) was incubated for 24 h at 37 °C in NH_4HCO_3 buffer (50 mM, pH 7.8), in the absence or presence of ACR, HNE, ONE, GO or MGO at 1:1 or 1:10 protein:aldehyde molar ratios. Glycosylation was conducted by incubating rβ_2-m (10 μM final concentration) for 48 h at 37 °C in NH_4HCO_3 buffer (50 mM, pH 7.8), in the absence or presence of glucose at 5 mM, 15 mM and 50 mM final concentration. Intact protein analysis (see below) was performed at different incubation times (0, 2, 4, 6, 8 h);