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Original Contribution

Ex vivo detection of histone H1 modified with advanced glycation end products

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ABSTRACT

A number of oxidative stress agents cause DNA and protein damage, which may compromise genomic integrity. Whereas oxidant-induced DNA damage has been extensively studied, much less is known concerning the occurrence and fate of nuclear protein damage, particularly of proteins involved in the regulation and maintenance of chromatin structure. Protein damage may be caused by the formation of reactive carbonyl species such as glyoxal, which forms after lipid peroxide degradation. It may also result from degradation of early protein glycation adducts and from methylglyoxal, formed in the process of glycolytic intermediate degradation. Major adducts indicative of protein damage include the advanced glycation end product (AGE) carboxymethyllysine (CML) and argpyrimidine protein adducts. Thus, the formation of CML and argpyrimidine protein adducts represents potential biomarkers for nuclear protein damage deriving from a variety of sources. The purpose of this study was to identify and quantify AGE adducts formed in vivo in a nuclear protein, specifically histone H1, using CML and argpyrimidine as biomarkers. Histone H1 was isolated from calf thymus collected immediately after slaughter under conditions designed to minimize AGE formation before isolation. Using antibodies directed against oxidative protein adducts, we identified CML, argpyrimidine, and protein crosslinks present in the freshly isolated histone H1. Detailed mass spectroscopy analysis of histone H1 revealed the presence of two specific lysine residues modified by CML adducts. Our results strongly suggest that glycation of important nuclear protein targets such as histone H1 occurs in vivo and that these oxidative changes may alter chromatin structure, ultimately contributing to chronic changes associated with aging and diseases such as diabetes.

Genomic integrity requires that nuclear protein damage be minimized and, if it occurs, repaired. Alternatively, damaged proteins must be degraded and replaced to maintain homeostatic regulation of gene expression. There are a variety of systems serving to repair DNA and histone damage [1,2]. Oxidatively modified [2] and glycoxidated [1] histones are removed by nuclear proteasomes, thereby preserving genomic integrity. Nonenzymatic modifications of nuclear proteins may affect gene expression and contribute to the pathophysiology of diseases such as ethanol-induced liver toxicity [3] and diabetes [4], as well as contributing to aging. Proteins may be nonenzymatically glycated when reducing sugars react with amino groups of lysine residues, forming reactive α-oxoaldehydes such as glyoxal [5,6] and methylglyoxal [6]. These glycation products are up to 20,000 times more reactive than glucose. Protein glycation leads to the formation of stable advanced glycation end products (AGE) such as carboxymethyllysine (CML) [7,8] and argpyrimidine [8]. CML is the most abundant AGE biomarker formed by nonenzymatic glycation of proteins. Thus, CML is a widely used reporter for glycation-induced protein modification. However, it should be noted that CML protein adducts can also be formed by reactions other than glycation, for example, by lipid peroxidation via the glyoxal intermediate and by deoxyribose DNA degradation [9]. Other glycation biomarkers have recently been characterized, such as argpyrimidine [8], which is formed by the reaction of methylglyoxal (formed by nonenzymatic degradation of glycolytic intermediates such glyceraldehyde 3-phosphate [10]) with guanidino groups of protein arginine residues. The enzyme glyoxalase utilizes glutathione to provide a cellular defense against protein glycation [11] by neutralizing glyoxal and methylglyoxal to nonreactive products, thereby protecting intracellular proteins from nonenzymatic glycation.

The study of protein glycation in biomedical systems initially focused on the effects of hyperglycemia on extra- and intracellular proteins such as collagen [7], laminin, and hemoglobin [5]. Sugars other than glucose are also important physiological sources for inducing protein glycation [12]. In particular, pentoses were shown to be more reactive toward protein amino groups than hexoses, because pentoses dissociate more readily to the open carbonyl form than do hexoses [13]. Because the formation of high intracellular concentrations of reactive reducing sugars such as the pentose ADP-ribose is

Abbreviations: CML, N^{ε}-carboxymethyllysine; AGE, advanced glycation end product; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PARG, poly(adenosine diphosphoribose) glycohydrolase; PARP, poly(adenosine diphosphoribose) polymerase; PCA, perchloric acid; TCA, trichloroacetic acid.

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induced under conditions of oxidative stress resulting in DNA damage, we turned our attention to study nuclear proteins as targets for glycation.

When poly(ADP-ribose) polymerase (PARP) is activated after oxidative stress-induced DNA damage [14], polymers of ADP-ribose are formed and covalently react with nucleosomal histones as part of the DNA repair response. This process of introducing a high number of negative charges on histone molecules causes the separation of DNA from the nucleosomes and allows the DNA repair proteins access to initiate DNA repair [15]. On the other hand, histone-associated ADP-ribose polymers are quickly cleaved by the activity of poly(ADP-ribose) glycohydrolase (PARG), thereby restoring normal DNA and nucleosome interaction. High concentrations of ADP-ribose are generated in the local microenvironment of histones, which contain high amounts of arginine and lysine residues [16]. Treating cells with DNA-damaging alkylating compounds results in activation of PARP and ADP-ribose polymer turnover, which involves PARG activity. The high levels of ADP-ribose in the microenvironment of the chromatin resulted in the formation of histone H1-associated ADP-ribose monomers [17]. Chemical analyses of these monomers were consistent with glycation of histone H1. To search for evidence of nuclear protein glycation occurring in vivo, we looked for the presence of three biomarkers of protein AGE product formation in histone H1 from calf thymus carefully collected and analyzed immediately after slaughter, namely CML, argpyrimidine protein adducts, and protein crosslinks.

Methods and materials

Materials

Histone H1 protein, potassium phosphate buffer, pH 7.4, bovine serum albumin, sodium azide, HCl, NaOH, sodium dodecyl sulfate, SP Sephadex C25, Dowex 2X8–100 ion-exchange resin, formic acid, ammonium formate, *N,N,N′,N′*-tetramethylethylenediamine, and acrylamide/bisacrylamide were purchased from Sigma–Aldrich. Reverse-phase HPLC utilized a C18 μBondapak column and Xterra-C18 column purchased from Waters (Milford, MA, USA).

Histone extraction from calf thymus

A modification of a previous method was used [18]. The whole preparation was carried out at 4 °C unless stated otherwise in the text. Calf thymus was obtained immediately after slaughter. It was transported to the laboratory on an ice pack within a period of 30 min. Membranes and connective tissue were removed as rapidly as possible to minimize proteolysis of histone H1, keeping the tissue at 4 °C throughout the isolation process as described below. Two hundred grams of thymus was minced with scissors into 2-cm chunks and 50-g batches were homogenized for 2 min with 700 ml of 0.14 M NaCl, adjusted to pH 5 with 2 M HCl, cooling the blender jar throughout the procedure. The pH of the homogenate was adjusted to pH 5 with 1 N HCl and then the homogenate was centrifuged for 30 min at 1500 *g*. The supernatant was discarded and one-half the original volume was added to each tube. Tubes were shaken to dislodge the pellet; the suspension was transferred to the blender, mixed for 1 min, and centrifuged at 1500 *g* for 20 min. This process was repeated three to five more times until the supernatant solution was very clear.

H1 extraction

The chromatin-containing pellet was suspended in 800 ml of 5% perchloric acid (PCA) by blending for 2 min. The solution was centrifuged at 1500 *g* for 30 min followed by repeating the process using 400 ml of PCA. The combined supernatants were clarified by filtration through a fine-sintered glass funnel and trichloroacetic acid (TCA; 100%, w/v) was then added to the solution drop-wise with continuous mixing to give a final TCA concentration of 18%. The precipitate was collected by centrifuging in glass tubes at 1500 *g* and 20 ml of water was used to dissolve the precipitate. The solution was clarified by filtering it through a small funnel with cotton to eliminate any insoluble material present. After extensive dialysis against water, the sample was lyophilized. If very pure material was required, 100 mg of the histone H1-rich precipitate was dissolved in 5 ml of water and applied to a 100×2.5-cm column of P60 equilibrated with 0.01 N HCl. Histone H1 was eluted with 5 ml of 0.01 N HCl and the fractions (OD_{235}) were pooled and then dialyzed against water. Columns were run at room temperature.

Storage

Lyophilized histone was stored at room temperature. Histone solutions were made in deionized redistilled water and were stored frozen at −20 °C until used.

CML isolation and quantification

To determine the CML content of a protein fraction, the dry pellet was hydrolyzed with 6 M HCl at 110 °C for 12 h after sodium borohydride reduction [7]. Next, the hydrolysate was subjected to chromatography on a 1-ml cation-exchange column (sulfonic acid, SP-Sephadex C25) followed by a 1-ml anion-exchange column (ammonium, Dowex X8-100) with some modification as previously described [1]. The sample was dissolved in 10 ml of 200 mM formic acid and applied to a previously equilibrated 1-ml SP–Sephadex resin with 100 mM formic acid. The sample was washed two times each with 200, 100, 50, and 10 mM formic acid solutions and eluted with 10 ml of 25 mM ammonium formate (pH 10.5). This solution was applied to a 1-ml Dowex X-100 cation resin column previously equilibrated with 2 M potassium hydroxide. The column was washed twice with 10 ml of 25 mM ammonium formate, pH 10.5, and twice with 10 ml of 12.5 mM ammonium formate, pH 9.5. Then 10 ml of water was added to the column and the sample was eluted with 5 ml of 100 mM HCl and 5 ml of 25 mM HCl. Ten milliliters of 25 mM HCl was added to the eluted solutions and the aliquots were then pooled into a 50-ml tube. The collected samples were lyophilized and dissolved in 10 ml of water. An aliquot of [^{14}C]CML was used to monitor recovery, which averaged 70%. HPLC (Agilent) was used to analyze CML and lysine content. The HPLC conditions for CML analysis included a C18 reverse-phase Xterra column (3.0×250 mm, particle size 5 μm), a running solution of 50 mM NaCl containing 1% sodium azide and 10% methanol, and a flow rate of 1 ml/min. The column was heated to 37 °C and the sample was monitored with an Agilent fluorescence detector at $340_{ex}/450_{em}$ nm. Standard amino acid analysis was used to determine lysine content after OPA derivatization using a C18 μBondapak column (3.9×300 mm, particle size 10 μm, pore size 125 Å). The flow rate was 2 ml/min and sample elution was monitored in the same manner as described for CML.

Immunoblotting of CML, argpyrimidine, and histone H1

Isolated histone H1 was subjected to 12% (w/v) SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Proteins were stained with Ponceau red to control the efficiency of the blotting and the appropriate loading of the lanes. The membrane was blocked with 5% albumin in a solution of Tris-buffered saline (TBS) with Tween 20, pH 7.4, at room temperature for 1 h. The mouse CML monoclonal antibody supplied by R&D Systems was used at 1:1500 dilution and incubated for 1 day. The secondary antibody was a goat anti-mouse conjugated with peroxidase from Jackson ImmunoResearch Laboratories and prepared for use at 1:1500 dilution, held at 4 °C, and discarded after 1 day. The argpyrimidine monoclonal antibody supplied by NOF Corp. was