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Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids

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Abstract Accumulation of carbonyl derivatives of proteins (protein carbonyl) is taken as a biomarker of oxidative protein damage in aging and in various diseases. We detected protein carbonyls in situ in human diabetic arteriosclerotic tissues and characterized the formation of protein carbonyls. Protein carbonyls were identified in the thickened intima of arterial walls and co-localized with protein adducts formed by carbonyl amine chemistry between protein and carbonyl compounds derived from autoxidation of carbohydrates, lipids, and ascorbate, i.e. advanced glycation end products or glycoxidation products, such as carboxymethyllysine (CML) and pentosidine, and lipoxidation products, such as malondialdehyde (MDA) and 4-hydroxy-nonenal (HNE). In vitro incubation of proteins with ascorbic acid accelerated the production of protein carbonyls as well as CML and pentosidine, and incubation with arachidonate accelerated the production of protein carbonyls as well as CML, MDA, and HNE. By contrast, incubation of proteins with glucose resulted in the production of CML and pentosidine, but not protein carbonyls. Schiff base inhibitors, (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide and aminoguanidine, inhibited the production of protein carbonyls after incubation with ascorbate and arachidonate. The present study suggests that ascorbate and polyunsaturated fatty acids, but not glucose, represent potential sources of protein carbonyls, and that both the glycoxidation and lipoxidation reactions contribute to protein carbonyl formation in aging and various diseases.

Key words: Protein carbonyl; Advanced glycation end product; Glycoxidation; Lipoxidation; Aging; Atherosclerosis

1. Introduction

Accumulation of protein carbonyls is implicated in aging [1,2]. Relationships have been found between the content of protein carbonyls and cell age in human erythrocytes and in cultured fibroblasts [3], and between the content of protein carbonyls and subject age in human brain and in lens tissues [4,5]. Also in various diseases, rises in protein carbonyls have been demonstrated [4–7].

Several lines of evidence have demonstrated that protein carbonyls are generated in vivo primarily by the metal-catalyzed oxidation systems [1,2]. Other mechanisms of protein carbonyl formation are not fully understood. Furthermore, the histological distribution of protein carbonyls in diseases has been determined only in the brain tissues of patients with Alzheimer's disease [8] and remains to be established in other diseases.

Under oxidative stress, carbohydrates and lipids as well as proteins are the major targets of reactive oxygen species. Proteins are modified indirectly with reactive carbonyl compounds derived from the autoxidation of carbohydrates and lipids. Autoxidation of carbohydrates and ascorbate yields carbonyl compounds, e.g. glyoxal, arabinose, methylglyoxal, glycolaldehyde, and dehydroascorbate [9–12]. These carbonyls are reactive with protein amino groups and initiate the Maillard reaction which forms Schiff base and eventually advanced glycation end products (AGEs) or glycoxidation products, such as N^{ε}-carboxymethyllysine (CML) and pentosidine. Lipid peroxidation of polyunsaturated fatty acids, such as arachidonate, yields other carbonyl compounds: some are identical to those formed from carbohydrates [13], such as glyoxal and methylglyoxal, and others are characteristic of lipids, such as malondialdehyde (MDA) and 4-hydroxy-nonenal (HNE) [14]. The latter carbonyl compounds produce lipoxidation products, MDA-lysine and HNE-protein adducts [15,16]. It is of interest that the accumulation of glycoxidation and lipoxidation products in the serum and tissue proteins has been implicated in diseases similar to those with an increase in protein carbonyls [17–26].

We therefore hypothesized that both glycoxidation and lipoxidation reactions contribute to the formation of protein carbonyls. To test this, we detected protein carbonyls as well as glycoxidation and lipoxidation products in human diabetic arteriosclerotic tissues. Furthermore, we characterized the in vitro formation of protein carbonyls as well as glycoxidation and lipoxidation products.

2. Materials and methods

2.1. Human tissue specimens

Arterial tissue sections were obtained at autopsy from seven patients with non-insulin-dependent diabetic mellitus with poor glycemic control (hemoglobin A_{1C}, $>8\%$) (age 42–59). These specimens were fixed in 10% formalin and embedded in paraffin.

2.2. Antibodies

Anti-pentosidine rabbit IgG [21], anti-HNE rabbit IgG [27], and anti-MDA mouse monoclonal IgG (kindly provided from Dr. Joseph L. Witztum) [28,29] were used for immunohistochemistry. Anti-AGE rabbit IgG [26], the major epitope structure of which was recently

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Abbreviations: AGE, advanced glycation end product; BSA, bovine serum albumin; CML, N^{ε}-(carboxymethyl)lysine; HNE, 4-hydroxy-nonenal; MDA, malondialdehyde; OPB-9195, (±)-2-isopropylidene-hydrazono-4-oxo-thiazolidin-5-ylacetanilide

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identified as CML [30], was also used. These antibodies were highly specific as demonstrated by minimal cross-reactivity assessed by competition with albumin modified with related epitopes [20].

2.3. Immunohistochemistry

Tissue sections (5 μm thickness) were mounted on a slide coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO), deparaffinized, and rehydrated in distilled water. For in situ detection of protein carbonyls, tissue sections were incubated with 0.5 mg/ml pronase (Dako, Glostrup, Denmark) for 5 min at room temperature and, after washing, reacted with or without 2,4-dinitrophenylhydrazine for 5 min at room temperature to derivatize protein carbonyl groups to 2,4-dinitrophenylhydrazone (DNP-hydrazone), followed by further incubation with 1:50 diluted rabbit anti-DNP antibody (Oncor, Gaithersburg, MD) for 2 h. The sections were washed and incubated with 1:100 diluted goat anti-rabbit IgG conjugated with peroxidase (Dako) for 2 h, followed by the detection with 3,3′-diaminobenzidine solution containing 0.003% H_2O_2. To confirm the specificity of immunostaining, competition experiment was performed by preincubation of tissue sections with 10 mM sodium borohydride for 30 min before 2,4-dinitrophenylhydrazine reaction. Non-immune rabbit IgG was used as a negative control.

For detection of glycoxidation and lipoxidation products, tissue sections were incubated with either anti-AGE (CML) rabbit IgG, anti-pentosidine rabbit IgG, anti-MDA mouse monoclonal IgG, or anti-HNE rabbit IgG overnight in humid chambers at room temperature, followed by incubation with goat anti-rabbit or -mouse IgG conjugated with peroxidase and the detection with 3,3′-diaminobenzidine and H_2O_2, as described above. The specificity of immunostaining was confirmed by competition experiments by incubating anti-AGE, anti-pentosidine, anti-HNE, or anti-MDA antibody for 4 h at 37°C with an excess of CML-, pentosidine-, MDA-, or HNE-BSA, respectively [20]. Non-immune rabbit or mouse IgG was used as a negative control.

2.4. In vitro incubation experiments

Fatty acid-free BSA (10 mg) was incubated at 37°C under air with either 100 mM glucose, 10 mM ascorbic acid, or 10 mM arachidonate (Sigma) in 5.0 ml of 0.1 M sodium phosphate buffer (pH 7.4), in the absence or presence of several concentrations of OPB-9195 [31] or aminoguanidine (Tokyo Chemical Industry, Tokyo, Japan) [32]. The experiment was repeated twice.

The protein carbonyl content was measured by the method as previously reported [16]. CML in the hydrolysates was measured as its *N*-trifluoroacetate methyl ester by selected ion monitoring gas chromatography/mass spectrometry (GC/MS), as described previously [33]. The CML standard [34] was a gift from Dr. John W. Baynes. Pentosidine was measured in hydrolysates of the incubation mixtures by reversed-phase high performance chromatographic assay using post-column fluorescence detection (Ex = 328 nm, Em = 378 nm) [21,35]. The preparation of pentosidine standard has been described previously [35]. MDA was measured using a colorimetric assay kit (Bioxyteckh LPO-586: Oxis International, Portland, OR). HNE was determined by an ELISA as described previously [36].

2.5. Statistical analysis

Data are expressed as means ± S.D. IC_{50} was calculated using regression analysis.

3. Results

3.1. Co-localization of protein carbonyls with glycoxidation and lipoxidation products in diabetic arteriosclerosis

Diabetic arteriosclerotic lesions were examined for protein carbonyls. Protein carbonyls were identified in diffusely thickened intima (neointima) of arterial walls (Fig. 1A). No protein carbonyls was detected in control tissue sections processed

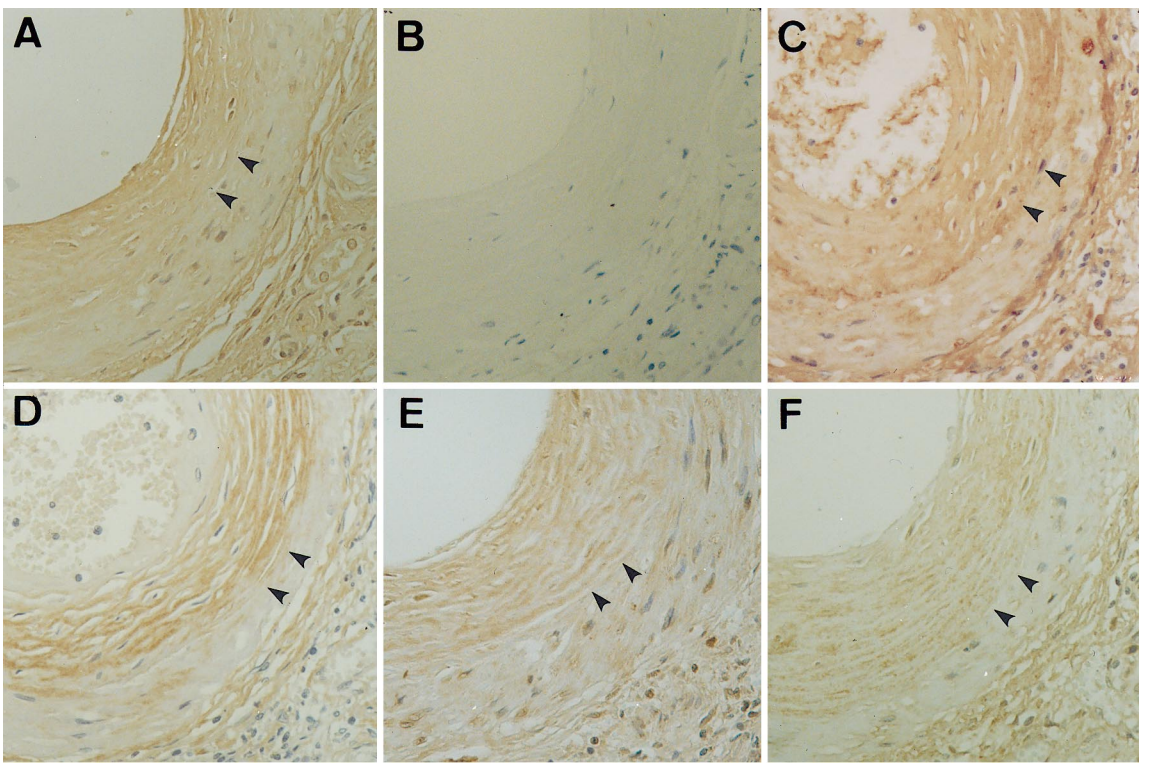

Fig. 1. Immunohistochemical detection of protein carbonyls as well as glycoxidation and lipoxidation products in diabetic arteriosclerosis. Arteriosclerotic tissue sections from a 54-year-old non-insulin-dependent diabetic male were immunostained for protein carbonyls (A and B), CML (C), pentosidine (D), MDA (E), and HNE (F). Nuclei were counterstained with Meyer's hematoxylin. Protein carbonyls are present in the diffusely thickened intima of arterial walls (indicated by arrows in A), but are undetectable in control tissue section processed usnder the same conditions except for the absence of the 2,4-dinitrophenylhydrazine reaction (B). Protein carbonyls co-localize with the glycoxidation and lipoxidation products. A–F, ×200.

under the same conditions except for the absence of the 2,4-dinitrophenylhydrazine reaction (Fig. 1B) or in tissue sections preincubated with sodium borohydrate before 2,4-dinitrophenylhydrazine reaction (data not shown). The distribution of protein carbonyls coincided with those of two glycoxidation products, CML (Fig. 1C) and pentosidine (Fig. 1D), and two lipoxidation products, MDA (Fig. 1E) and HNE (Fig. 1F). Specificity of immunostainings was demonstrated by the absence of immunoreaction with non-immune mouse or rabbit IgG or in the presence of an excess of competitors (data not shown).

3.2. *In vitro formation of protein carbonyls as well as glycoxidation and lipoxidation products*

To characterize the formation of protein carbonyls on proteins, BSA was incubated under air with glucose, arachidonate, or ascorbic acid, all of which are autoxidized and yield

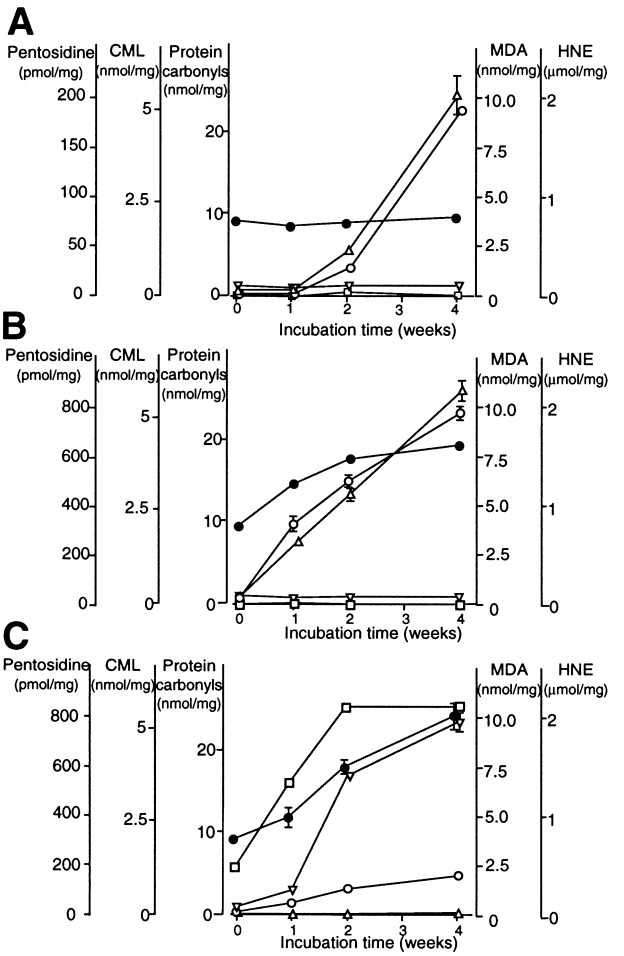

Fig. 2. In vitro formation of protein carbonyls as well as glycoxidation and lipoxidation products. BSA (10 mg) was incubated at 37°C with either 100 mM glucose (A), 10 mM ascorbic acid (B), or 10 mM arachidonate (C) in 0.1 M phosphate buffer (pH 7.4), and the yields of protein carbonyls, CML, pentosidine, MDA, and HNE in the incubation mixtures were determined at intervals. The protein carbonyl content in the control BSA incubated for 4 weeks without precursors is 8.26 nmol/mg albumin and the levels of CML, pentosidine, MDA, and HNE in the control BSA incubated for 4 weeks without precursors are below the detection limits. ●, protein carbonyls; ○, CML; △, pentosidine; □, MDA; ▽, HNE. Representative data from two experiments are shown. Data are expressed as means ± S.D.

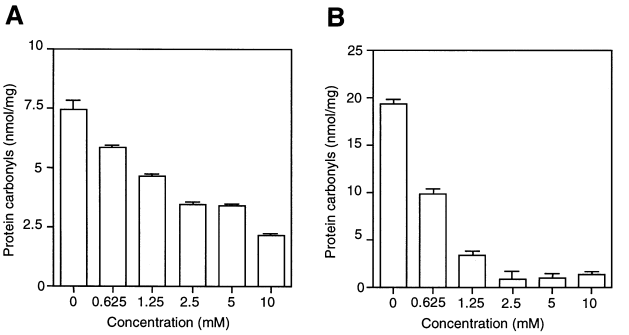

Fig. 3. Inhibitory effect of OPB-9195 in vitro on the formation of protein carbonyls in BSA. BSA (10 mg) was incubated under air at 37°C for 4 weeks with either 10 mM ascorbate (A) or arachidonate (B) in 0.1 M phosphate buffer (pH 7.4) in the presence of several concentrations of OPB-9195. The yields of protein carbonyls in the incubation mixtures were then determined. Representative data from two experiments are shown. Data are expressed as means ± S.D.

carbonyl compounds capable of reacting with proteins. Incubation of BSA with glucose (Fig. 2A) and ascorbate (Fig. 2B) yielded a time-dependent increase of glycoxidation products, CML and pentosidine, but not of lipoxidation products, MDA and HNE. Incubation of BSA with arachidonate yielded an increase of CML, MDA, and HNE, but not of pentosidine (Fig. 2C). It is noted that incubation with ascorbate as well as arachidonate yielded protein carbonyls (closed circles in Fig. 2). By contrast, protein carbonyls were not detectable after a 4 week incubation with glucose.

Schiff base inhibitors, OPB-9195 and aminoguanidine, inhibit both the glycoxidation and lipoxidation reactions, because their hydrazine nitrogen atoms are capable of trapping reactive carbonyl compounds, directly or via the free base upon hydrolysis, by the hydrazone formation (unpublished). Therefore, the involvement of glycoxidation and lipoxidation reactions in the formation of protein carbonyls was further evaluated using these Schiff base inhibitors. OPB-9195 inhibited in a dose-dependent manner the formation of protein carbonyls after incubation with ascorbate and arachidonate: half-maximal inhibition (IC_{50}) was observed at an OPB-9195 concentration of 2.13 mM for ascorbate-derived protein carbonyls (Fig. 3A) and of 0.64 mM for arachidonate-derived protein carbonyls (Fig. 3B). The corresponding figure for aminoguanidine was 5.87 mM for arachidonate-derived protein carbonyl formation. The lower efficacy of aminoguanidine is further illustrated by the fact that even 10 mM aminoguanidine achieved only 29.4% inhibition of ascorbate-derived protein carbonyl formation. The enhanced effectiveness of OPB-9195 might be due to the fact that the hydrazine nitrogen atom of aminoguanidine has decreased nucleophilicity because of the adjacent guanidinium cation (unpublished).

4. Discussion

We provide evidence suggesting that both the glycoxidation and lipoxidation reactions contribute to the increase in protein carbonyls. First, protein carbonyls were identified in colocalization with both the glycoxidation and lipoxidation products in the thickened intima of diabetic arterial walls. Second, the glycoxidation and lipoxidation reactions of BSA with ascorbic acid and arachidonate accelerated the production of protein carbonyls in addition to the glycoxidation and

lipoxidation products. Finally, inhibitors of the glycoxidation and lipoxidation reactions, OPB-9195 and aminoguanidine, inhibited the production of protein carbonyls after incubation with ascorbate and arachidonate.

That protein carbonyls as well as glycoxidation and lipoxidation products originate from the glycoxidation and lipoxidation reactions can account for the simultaneous accumulation of protein carbonyls, and glycoxidation and lipoxidation products in aging and various diseases, e.g. atherosclerosis, diabetes, uremia, rheumatoid arthritis, cataractogenesis, and neurologic disorders.

Oxygen free radicals produced by the metal catalyzed oxidation systems have been assumed as the primary mechanism for the formation of protein carbonyls in vivo [1,2]. Protein carbonyls are also generated by oxygen free radicals produced by ionizing radiation [37]. The present conclusion that both glycoxidation and lipoxidation reactions contribute to the increase in protein carbonyls, is in good agreement with the assumption by Stadtman [1] that glycation or Maillard reaction may contribute to the formation of protein carbonyls. However, glucose, which is the major source of glycation or Maillard reaction in vivo, turned out not to represent the source of protein carbonyls. Unexpectedly, ascorbate and polyunsaturated fatty acids represented potential sources of protein carbonyls. Of course, most careful consideration would be required to assess the formation of protein carbonyls in vivo, taking into account the concentration of each source and the duration of protein exposure to each source.

It remains unknown to what extent the glycoxidation and lipoxidation reactions contribute to the formation of protein carbonyls in vivo, as compared to the metal-catalyzed oxidation systems. At present, it is difficult to assess the contribution of each mechanism to the observed age-related increase and in various diseases. Further development of analytical procedures will be required to address this issue of interest.

Smith et al. [8] reported the histological distribution of protein carbonyls in the neuronal cytoplasm and nuclei of neurons and glia, and in neurofibrillary tangles in brain tissues of patients with Alzheimer's disease. In this regard, of particular interest are recent findings by us and others that glycoxidation products, CML and pentosidine, and lipoxidation products, MDA and HNE, were identified immunohistochemically in the neuronal cytoplasm and in neurofibrillary tangles in brain tissues of patients with Alzheimer's disease [24,25,29,30].

We demonstrated the distribution of protein carbonyls for the first time in vascular lesions, i.e. in diabetic arteriosclerosis. Our preliminary studies identified protein carbonyls also in the thickened intima of arterial walls of senile atherosclerosis and uremic arteriosclerosis, in co-localization with the glycoxidation and lipoxidation products (our unpublished observation). Therefore, the accumulation of protein carbonyls in vascular lesions might be a phenomenon common in most, not if all, types of vascular damage.

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