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Reversal by aminoguanidine of the age-related increase in glycoxidation and lipoxidation in the cardiovascular system of Fischer 344 rats

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Abstract

Non-enzymatic glycoxydation and lipoxidation of proteins continues to stimulate great interest in gerontology as both markers and promoters of aging. The first aim of the study was to determine the age-related changes in levels of N^{ε}-(carboxymethyl)lysine (CML) and 4-hydroxy-2-nonenal (HNE) present on proteins of the cardiovascular system of Fischer 344 rats and identify the particular polypeptides being modified. The second objective was to evaluate whether pharmacological administration of aminoguanidine (1 g/L in the drinking water) could reverse protein glycoxidation and lipoxidation. CML content in serum, aorta, and heart proteins from 28-month-old rats was double of that found in 4-month-old animals. AG administration to old rats for 3 months from the age of 25 months lowered CML content by 15 ($P = .2275$), 44 ($P < .0001$), and 28% ($P = .0072$) in serum, aorta, and heart, respectively. Serum albumin, transferrin and immunoglobulins were most prominently adducted by both CML and HNE. While the extent of albumin and transferrin modification was comparable between age groups, CML and HNE bound to immunoglobulins increased in the sera of old rats as a result of the accumulation of immunoglobulin heavy and light chains. AG treatment prevented immunoglobulin accumulation in serum, suggesting a beneficial action on renal filtration. Lipoxidation of heart mitochondrial proteins was prevalent over glycoxidation, either as CML or pentosidine. Although AG prevented HNE-induced inactivation of the α-ketoglutarate dehydrogenase complex in vitro, it had no effect in rat hearts, suggesting AG could not reach the mitochondrial matrix.

Keywords: N^{ε}-(Carboxymethyl)lysine; 4-Hydroxy-2-nonenal; Amyloid; Heart; Mitochondria; Aging

1. Introduction

Chronic oxidative stress and defective glycemic control associated with aging alter the structure and compromise the function of proteins. While specific enzymes can remove certain types of modification(s), like methionine sulfoxide [1], advanced glycation (AGE) and lipoxidation end-products (ALE) modify proteins permanently. Some proportion of this damage cannot be removed by proteolysis and thus accumulates, thereby promoting further degeneration, in part exacerbated by receptors of AGE (RAGE) present on the surface of various cell types [2–4]. Because protein modifications can lead to structural disorganization, loss of activity, free radical generation, and insoluble aggregates, their formation is thought to play a central role in the decline of physiological functions seen in normal and pathological aging.

AGE and ALE are formed by carbonyl/amine or carbonyl/thiol chemistry between protein residues (Lys, His,

Abbreviations: CML, N^{ε}-(carboxymethyl)lysine; HNE, 4-hydroxy-2-nonenal; AG, aminoguanidine; AGE, advanced glycation end-products; ALE, advanced lipoxidation end-products; KGDC, α-ketoglutarate dehydrogenase complex; iNOS, inducible nitric oxidase synthase; NO, nitric oxide

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Cys) and reactive carbonyl compounds. Despite being continuously generated in the body by the oxidative metabolism of carbohydrates, amino acids and/or lipids, carbonyl compounds will only form irreversible adducts with certain proteins at specific residues [5,6]. N^{ε}-(Carboxymethyl)lysine (CML), the predominant AGE found in vivo [7], is formed by oxidative cleavage of the Amadori product fructoselysine or through the glycation of protein by glyoxal, which itself results from the autooxidation of carbohydrates, glycated peptides, amino acids, or lipids. ALE derive from the irreversible non-enzymatic modification of proteins by lipid-derived aldehydes, among which 4-hydroxy-2-nonenal (HNE) is one of the most reactive and abundant end products generated by the controlled oxidation of ω-6 polyunsaturated fatty acids [8]. Reactions between reactive carbonyls and protein side chain residues occur continuously in the body throughout life and increase exponentially in pathological conditions such as diabetes.

In theory, tissues with a slow rate of regeneration, such as the myocardium and the vascular endothelium, are especially prone to accumulating modified proteins over time. Also, elevated oxidative stress and increased DNA damage have been associated with the aging myocardium. Metabolically heart mitochondria are particularly active and constantly produce oxidants, even more so in aging animals [9,10]. Mitochondrial membranes are rich in polyunsaturated fatty acids and thus generate HNE prominently. In addition to oxidative damage, proteins of the aging heart may be under an increased risk for glycation due to heightened use of glucose for ATP synthesis [11]. Yet, there is little evidence on the extent and nature of oxidative post-translational modifications of proteins in the cardiovascular system during normal aging.

It is thought that pharmacological interventions that prevent damage from forming or remove existing damage would have the potential to maintain tissue integrity, thus greatly improving the health and vigor of the elderly. Aminoguanidine (AG) is a nucleophilic hydrazine that inhibits AGE and, possibly, ALE formation in vitro by trapping carbonyl compounds [12,13]. Numerous studies have shown the beneficial effects of AG in ameliorating or preventing complications caused by experimental diabetes [14–17]. In animal studies, AG was able to either prevent or retard age-related glomerular sclerosis [18], arterial stiffening [19], atherogenesis [20], and cardiac hypertrophy [18,19]. Hence, the potential usefulness of AG could be extended to normal aging, which is considered by many to share pathophysiological attributes with diabetes, such as insulin resistance and dyslipidemia.

The goal of the present study was to determine the extent of CML and HNE contained in proteins of the cardiovascular system of old Fischer 344 rats, and identify the most prominent proteins being modified. A subsequent goal was to evaluate whether the formation of these modifications could be prevented or substantially decreased by a 3 months administration of AG.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma unless specified otherwise in the text.

2.2. Animals and diets

Male rats (Fischer 344, 25-month-old) from the National Institute on Aging were housed individually in cages in a controlled environment (ambient temperature 22 ± 2 °C, 12:12-h light–dark cycle) and randomly assigned to one of the two dietary treatments for 3 months. A first group (AG treatment) was fed AIN-93M diet (Dyets Inc.) and given aminoguanidine HCl in the drinking water (AG, 1 g/L distilled water). A second group (control group, Ctl) was fed AIN-93M diet and given distilled water. All animals had free access to food and water until sacrifice. Food and water intake was recorded every other day. Body weight was measured weekly. In addition to old rats, 4-month-old F344 rats (young group, Y) were used as controls where appropriate. They were fed AIN-93M diet and water ad libitum for 2 weeks.

2.3. Tissue sampling

Rats were anesthetized with diethyl ether and blood samples obtained by an axial cut through the Pectoralis major, which severed the axillary artery. In addition, a midlateral incision was made in the chest and the heart and thoracic aorta removed. While heart mitochondria were immediately isolated, aortas were frozen in liquid nitrogen and stored at −80 °C until further processing. Blood was allowed to clot for 20 min at room temperature in the dark, and serum was obtained by centrifugation at 1000 × *g* for 10 min at 4 °C and stored at −80 °C. Protein content was determined using the bicinchoninic acid method (Pierce) and BSA as a standard.

2.4. Cytokine array

Serum cytokines were measured using the RayBio™ Rat Cytokine Array I (RayBiotech, Inc.), with 0.2 ml serum and by following the manufacturer's instructions. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies; antibody binding was visualized using an ECL™ Western Blotting System (Amersham Pharmacia Biotech Inc.). Signal intensity was quantified using an AlphaImager™ imaging system (Alpha Innotech Corporation).

2.5. Isolation of heart mitochondria

Heart mitochondria were isolated as previously described [21]. Hearts were minced in cold 0.3 M mannitol

containing 5 mM Mops, 5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.1% BSA (fatty acid-free, fraction V, Calbiochem-Novabiochem Corporation), pH 7.4, and subjected to collagenase treatment (Type IV, Sigma) at 4 °C for 40 min. Digestion was stopped with EGTA and the buffer was decanted. The heart digest was homogenized, using a Potter-Elvehjem homogenizer, in 8 volumes of cold 0.3 M mannitol, 5 mM Mops, 5 mM KH_2PO_4, 1 mM EGTA, 0.1 mM *n*-tosyl-L-phenylalanine chloromethyl ketone, aprotinin (8 Trypsin Inhibitor Unit/l), 0.5 μM leupeptin, 0.5 μM pepstatin A, and 0.1% BSA (fatty acid-free), pH 7.4. The homogenate was then centrifuged at 1500 × *g* for 10 min at 4 °C. The pellet, representing the non-mitochondrial fraction, was mixed in a minimum volume of buffer and stored under N_2 at −20 °C until further processing. The supernatant was further centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting pellet was washed twice by centrifugation at 10,000 × *g* for 10 min at 4 °C and resuspended to a final concentration of 15 mg protein/ml. This crude preparation is enriched in subsarcolemmal mitochondria, but also contains interfibrillary mitochondria. Protein content was determined using the bicinchoninic acid method (Pierce) and BSA as the standard.

2.6. Isolation of proteins contained in heart non-mitochondrial fraction and aorta

The lipids contained in the cardiac non-mitochondrial fraction were extracted with methanol: chloroform (2:1), according to Bligh and Dyer [22], so that the methanol: chloroform:water ratio was 2:1:0.8. After gently mixing for 2 h at room temperature under N_2, chloroform and water were added so that the methanol:chloroform:water ratio became 1:1:0.9. After mixing, the samples were centrifuged at 900 × *g* for 25 min, and the lipid layer decanted. Proteins contained in the aqueous phase were precipitated with an equal volume of 20% [w/v] trichloroacetic acid, centrifuged at 29,000 × *g* for 30 min at 4 °C, washed with 5% trichloroacetic acid, centrifuged at 29,000 × *g* for 30 min at 4 °C, and dried using a SPD111V Speed Vac® concentrator (Thermo Electron Corporation). Proteins were hydrolyzed with degassed 6 N HCl under N_2 at 110 °C for 18 h, neutralyzed with 5 N NaOH and 0.5 M phosphate buffer (pH 7.4), filtered using 0.2-μm Acrodisc® LC13 PVDF syringe filters (Gelman Sciences), and stored at −80 °C.

Thoracic aortas were rinsed in ice-cold PBS, finely minced, and homogenized in PBS using a Potter-Elvehjem homogenizer. Extracts were delipidized and the resulting proteins precipitated and hydrolyzed as mentioned above for the heart samples.

To assess protein content, free amino acids present in heart and aorta hydrolyzates were measured by fluorometrically monitoring the formation of *o*-phthaldehyde-primary amine derivatives (excitation 360 nm, emission 460 nm) using a 96-well plate reader (CytoFluor® 4000, Applied Biosystems). The reaction mixture consisted of 80 μl hydrolyzate and 160 μl 0.1% [w/v] *o*-phthaldehyde (Fluka), 0.5% [v/v] β-mercaptoethanol in 0.4 M boric acid, pH 10.4. L-Leucine (Fluka) was used as the standard and the data are expressed as Leu equivalent.

2.7. N^{ε}-(carboxymethyl)lysine (CML) content in serum, aorta, and heart determined by ELISA

CML content in heart and aorta hydrolyzates and serum was determined by competitive indirect ELISA, using a protocol adapted from Makita et al. [23]. Ninety-six-well plates (Maxisorp, Nunc) were coated with 0.15 ml CML–BSA (4 μg/ml in PBS) for 2 h at room temperature, washed three times with 0.2 ml PBS and blocked with 0.15 ml PBS containing 0.1% BSA and 0.05% [v/v] Tween® 20 for 1 h at room temperature. After washing the plates, 50 μl of competitive antigen (sample or CML–BSA standard) was added, followed by 100 μl PBS containing 0.1% BSA, 0.05% [v/v] Tween® 20 and 8 ng mouse anti-CML monoclonal antibody (clone 6D12, Wako Chemicals USA Inc.). Plates were incubated for 3 h at room temperature with constant agitation, then washed three times with 0.2 ml PBS, and developed with alkaline phosphatase-linked anti-mouse antibody (Pierce, diluted 1:2500) utilizing *p*-nitrophenyl phosphate as a colorimetric substrate. Plates were read at 405 nm using a SpectraMax plate reader (Molecular Dynamics). CML levels were computed as B/B_0 ratio, where B is the competitor OD_{405} minus background OD_{405} (in absence of primary antibody), and B_0 is the total OD_{405} (in the absence of competitor) minus background OD_{405}. CML–BSA standard was prepared under sterile conditions by incubating BSA (10 mg/ml, fatty acid-free, globulin-free, Sigma) with 0.5 M D-glucose in 0.2 M $NaPO_4$ (pH 7.4) containing 1 mM EDTA and 1 mM Na azide at 37 °C in the dark for 60 days to yield an OD of 12 mM^{-1} albumin at 350 nm.

2.8. Profile of CML-modified proteins in serum and heart mitochondria determined by Western blotting

Sera or crude mitochondria were mixed with sample buffer (125 mM Tris–HCl, pH 6.8, 4% [w/v] SDS, 20% [v/v] glycerol, 2% [v/v] β-mercaptoethanol, 0.02% [w/v] bromophenol blue), then heat-denatured (95 °C, 5 min). The proteins (serum, 10 μg; mitochondria, 70 μg) were subjected to reducing SDS–PAGE, followed by electroblotting onto nitrocellulose membranes and incubated with mouse anti-CML monoclonal antibody (clone 6D12, Wako Chemicals USA Inc., dilution 0.16 μg/ml) in blocking buffer (20 mM Trizma® base, 150 mM NaCl, 0.1% [v/v] Tween® 20, 5% [w/v] non-fat dry milk, 0.5% [w/v] BSA, pH 7.5). Membranes were rinsed three times for 10 min and incubated with horseradish peroxidase-conjugated secondary antibodies. Antibody binding was visualized using an ECL™ Western Blotting System (Amersham Pharmacia Biotech Inc.). Specificity of the

primary antibody was evaluated by pre-incubation with a specific antigen, 0.5 mg CML–BSA, prior to Western blotting.

2.9. Profile of 4-hydroxynonenal (HNE)-modified proteins in serum and heart mitochondria determined by Western blotting

The extent of HNE-modified proteins in serum and heart mitochondria was determined by Western blotting as described above for CML-proteins. Blots were incubated with mouse monoclonal antibody to HNE-modified keyhole limpet hemocyanin (Oxis International, Inc.) in blocking buffer. Specificity of the primary antibody was evaluated by pre-incubation with its specific antigen, 0.5 mg HNE–BSA, prior to Western blotting. HNE–BSA was produced by incubating BSA (10 mg/ml, fatty acid-free, globulin-free, Sigma) with 1 mM HNE (Oxis International, Inc.) and 1 mM EDTA in PBS overnight, followed by dialysis against PBS.

2.10. Mass spectrometry

The identity of serum polypeptides modified by CML and HNE in old rats was determined using nanoscale liquid chromatography coupled to electrospray ionization tandem mass spectrometry (nano-LC ESI MS/MS). Serum proteins were separated by 10% SDS–PAGE under reducing conditions and stained with Coomassie Blue R-250. The bands were removed from the gel, destained in 10% methanol/10% acetic acid, washed with 100 mM NH_4HCO_3, dehydrated with acetonitrile, and dried under vacuum before digestion with modified porcine trypsin (Promega Corporation) in 50 mM ammonium bicarbonate, 5 mM calcium chloride, overnight at 37 °C.

Nano-LC ESI MS/MS experiments were performed on an API-US QTOF mass spectrometer (Micromass UK Ltd.) equipped with the CapLC system (Waters Corporation). The stream select module was configured with an OPTI-PAK Symmetry300 C_{18} trap column (Waters) connected in series with a nanoscale analytical column. The latter was packed with 4-μm C_{12} Jupiter Proteo particles (Phenomenex) according to the pressurized bomb method described previously [24] using a fused-silica column with an integral frit, ''PicoFrit'' (360 μm o.d. × 75 μm i.d. × 40 cm, 15-μm tip) from New Objective (New Objective, Inc.). The inlet end of the analytical column was connected to a ZU1XC metallic union (Valco Instruments Co. Inc.), where the electrospray voltage was applied.

Peptide samples (5 μl) were injected onto the trap column at 10 μl/min, desalted and back-flushed to the analytical column at 0.5 μl/min using gradient elution. The gradient consisted of 5–50% solvent B in 35 min, followed by 50% B for 15 min and 50–90% B in 5 min (solvent A = 5% acetonitrile, 0.1% formic acid; solvent B = 95% acetonitrile, 0.1% formic acid). QTOF parameters were set as follows: the electrospray potential was set to 3.5 kV (applied to the Valco union), the cone voltage set to 35 V, the extraction cone set to 2 V, the source temperature set to 100 °C. The MS survey scan was *m/z* 400–1600 with a scan time of 1 s and the collision energy was set to 5 eV. For operation in the MS/MS-mode, the scan time was increased to 2 s and the isolation width was set to include the full isotopic distribution of each precursor (∼4-Da mass window). Doubly, triply and quadruply protonated peptide ions selected by the data dependant software were subjected to collision-induced dissociation (CID) using appropriate collision energies for adequate fragmentation. Upon the completion of a run, the MS/MS spectra were searched against the non-redundant NCBI protein database using MASCOT software (Matrix Science Inc.).

2.11. α-Ketoglutarate dehydrogenase complex (KGDC) activity

KGDC was used as a reporter enzyme of HNE-mediated protein inactivation [25]. The effect of AG on HNE-mediated inactivation of KGDC was assessed by incubating AG (0–2 mM) with HNE (0.3 mM) in 50 mM Tris–HCl (pH 7.6) for 10 min at 30 °C under agitation, followed by the addition of purified porcine heart KGDC (5 mU, Sigma), further incubation for 30 min, and measurement of KGDC activity. Under these conditions HNE did not interfere with Tris-buffer. KGDC activity was determined spectrophotometrically using a SpectraMax 96-well plate reader (Molecular Dynamics) by monitoring the reduction of NAD^+ at 340 nm for 10 min at 30 °C. Assay mixture was composed of 2 mM NAD^+, 0.6 mM thiamine pyrophosphate, 0.24 mM CoASH, 0.1 mM dithiothreitol, 1 mM $MgCl_2$, 20 μM $CaCl_2$, 25 mM oxamate (a lactate dehydrogenase inhibitor) and 5 mM α-ketoglutarate in 50 mM Tris–HCl (pH 7.6). The same assay was used to determine heart KGDC activity in young, old untreated, and old AG-treated rats using 15 μg mitochondrial proteins solubilized with *n*-octyl β-D-glucopyranoside (0.15% [w/v] in 50 mM Tris–HCl, pH 7.6).

2.12. Statistical analysis

Data were analyzed by unpaired two-tailed Student's *t*-test or single-factor ANOVA followed by Student–Newman–Keuls multiple comparison test using the Statview package (SAS Institute Inc.). Proportions were arcsine transformed prior to analysis. All statistical tests were performed to the 95% level of confidence.

3. Results

3.1. Weight gain, food, water and AG intake

Twenty five-month-old male F344 rats free of obvious cardiovascular disease were provided with AG in the

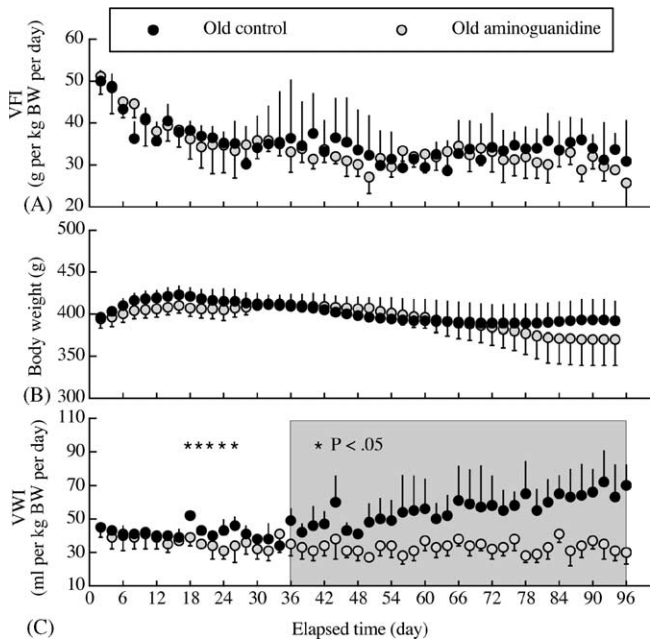

Fig. 1. Effect of AG administration to male F344 rats from ages 25 to 28 months on voluntary food intake (VFI) (A), body weight (B), and voluntary water intake (VWI) (C). Each time point represents the mean of 5 animals ± S.D. $^{*}P < .05$, Student *t*-test.

drinking water (1 g/L, AG group) or without AG (Ctl group) for 3 months. AG did not significantly affect mean body weight (AG rats: 370 ± 31 g versus Ctl rats: 392 ± 23 g) or food intake compared to control untreated rats (Fig. 1). Water intake was significantly ($P < .05$) elevated in control rats between day 18 and 26, and from day 36 until the end of the feeding trial compared to AG-treated rats. Mean water intake increased from 45 to 70 ml kg^{-1} BW day^{-1} in control rats during the 3-month-trial, while in AG-treated rats water intake averaged 42 ml kg^{-1} BW day^{-1} the first week and remained constant at 35 ± 3.9 ml kg^{-1} BW day^{-1} throughout the trial (Fig. 1). Since AG was added to the drinking water at 1 g/L, AG intake paralleled water intake and on average amounted to 35 ± 3.9 mg AG kg^{-1} BW day^{-1}.

3.2. Serum cytokines

Given that AG inhibits inducible nitric oxide synthase (iNOS) and nitric oxide is viewed as a pro-inflammatory agent, we sought to determine whether the 3 months administration of AG would repress cytokine production. As shown in Fig. 2 there was no significant difference in the serum cytokine profile of old untreated versus old AG-treated rats. Regardless of age or treatment, tissue inhibitor of metalloproteinase-1 (TIMP-1) and monocyte chemoattractant protein-1 (MCP-1) were the most abundant cytokines (Fig. 2B). MCP-1 and LPS-induced CXC chemokine (LIX) were elevated in old rats compared to young animals.

3.3. Validation of the ELISA used to determine CML

A competitive indirect ELISA was developed using commercial monoclonal antibodies (clone 6D12) and synthesized CML–BSA as the adsorbed or competitive antigen. Monoclonal antibodies did not react with native BSA when used as a plate coating (Fig. 3A) or as a competitive antigen (Fig. 3B). In contrast, the monoclonal antibodies bound efficiently to CML–BSA synthesized either from D-glucose (Fig. 3A and B) or glucose-6-phosphate (data not shown). The standard competition curve defined a dynamic range of 15–300 pmol CML–BSA equivalent per well (Fig. 3C). Monoclonal antibody specificity for CML was demonstrated by competitive Western blotting of serum proteins, where pre-incubation of the antibodies with CML–BSA abolished the signal (Fig. 3D).

3.4. Effect of AG on CML content in serum, aorta, and heart proteins

As determined by ELISA, CML content was significantly elevated in the serum ($P = .005$), aorta ($P < .0001$), and heart ($P = .0002$) proteins of old untreated rats compared to their young counterparts (Fig. 4). When given to old rats for 3 months AG lowered CML content by an average of 15, 44 and 28% in serum ($P = .2275$), aorta ($P < .0001$), and heart ($P = .0072$), respectively.

3.5. Effect of AG on CML- and HNE-modified proteins in serum

Owing to the age-associated increase in total CML in serum proteins, we sought to identify the polypeptides being modified. The profile of serum proteins differed between age groups. In old rats we observed an intensification of three bands on the Coomassie Blue stain (Fig. 5A). These polypeptides also corresponded to those being modified by CML and HNE (Fig. 5B and C). The specificity of these observations was reinforced by competition tests carried out with the antibodies used (Fig. 5D and E). Among the polypeptides that accumulate in the serum of old rats, we have identified components of immunoglobulins (both heavy and short chains, Table 1). In all cases, the administration of AG ameliorated the accumulation of these proteins in the sera of old rats and/or the extent of their formation.

3.6. Effect of AG on CML- and HNE-modified proteins in heart mitochondria

Since aging is associated with glycemic dysregulation and increases in lipid-derived aldehydes, we sought to determine the extent of CML- and HNE-modified proteins in subcellular compartments. We chose to focus the analysis on heart mitochondria, which actively take up and oxidize carbohydrates and lipids for the production of

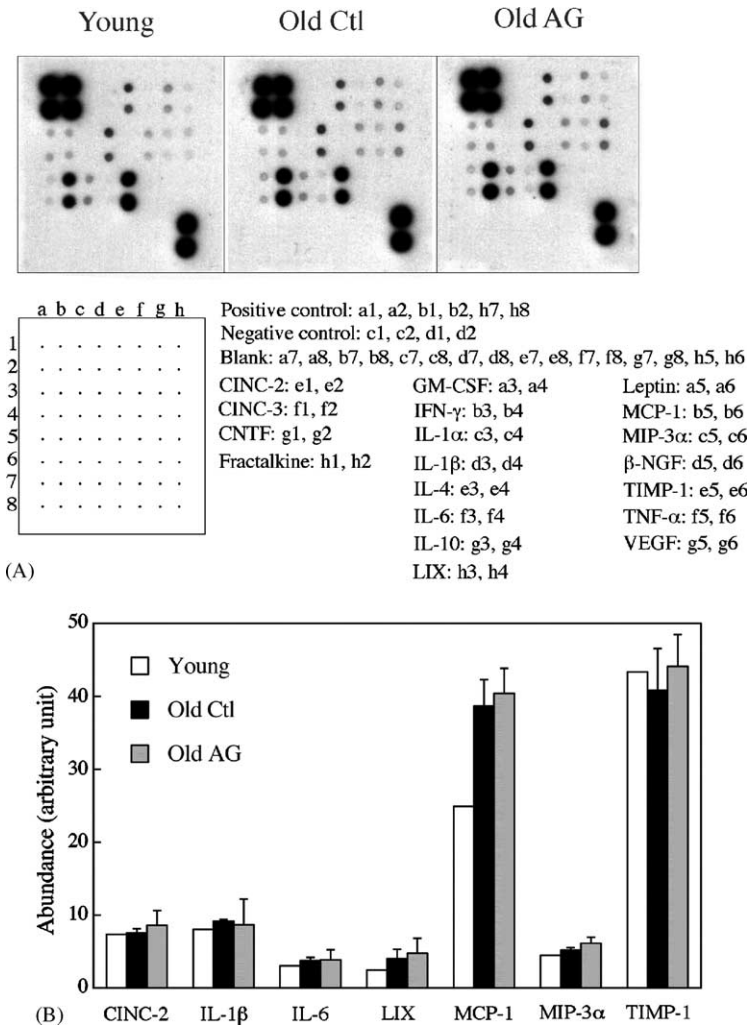

Fig. 2. Expression of serum cytokines, chemokines, growth factors and leptin in 4-month-old (Young, $n = 2$), untreated 28-month-old (Old Ctl, $n = 3$), and AG-treated 28-month-old (Old AG, $n = 3$) rats (A), and arbitrary quantification of the predominant cytokines (mean ± S.D.) (B). Cytokine-induced neutrophil chemoattractant-2 and -3 (CINC-2 and -3), ciliary neurotropic factor (CNTF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), interleukines (IL), LPS-induced CXC chemokine (LIX), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-3α (MIP-3α), nerve growth factor-beta (β-NGF), tissue inhibitor of metalloproteinase-1 (TIMP-1), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF).

high-energy phosphates. Mitochondrial proteins were isolated from the hearts of young, untreated old, and AG-treated old rats, separated by SDS–PAGE under reducing conditions and subjected to Western blotting as described above. Blots for CML-proteins showed a great deal of non-specific binding (Fig. 6A). Only one band, localized between 60 and 65 kDa, may contain CML. The intensity of the signal was not affected by the age of the animal or the administration of AG.

Among the proteins modified by HNE in the heart, a polypeptide identified previously as dihydrolipoamide succinyltransferase (the E2 component of α-ketoglutarate dehydrogenase complex, E2-KGDC) was increasingly adducted in old rats [25]. AG administration did not appear to lower this biomarker of aging (Fig. 6B), whereas in vitro, AG showed the potential to prevent the inhibition of KGDC induced by HNE (Fig. 6C). In this assay, AG and HNE were first incubated together for 10 min, at which point purified KGDC was added, further incubated for 30 min and the enzyme activity measured. Under these conditions AG restored KGDC activity in a concentration-dependent manner by a mechanism thought to involve the scavenging of HNE. However, this protective effect of AG was not seen in vivo. KGDC activity, which increases in old F344 rat hearts as a compensatory mechanism to maintain the bioenergetics of the myocardium, remained unchanged in AG-treated old rats compared to untreated old rats (Fig. 6D).

4. Discussion

Non-enzymatic glycoxidation and lipoxidation of proteins continues to stimulate great interest in geriatric research because modifications of native proteins are recognized as markers as well as contributors of the aging

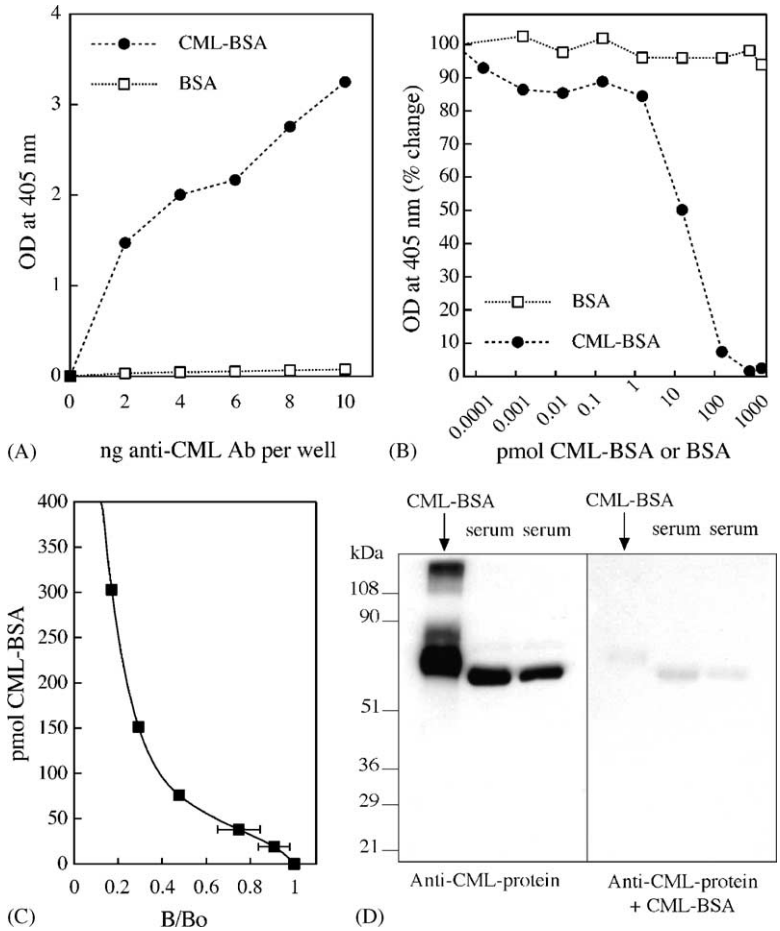

Fig. 3. Specificity and standard curve of competitive indirect ELISA for CML. Binding efficiency of anti-CML monoclonal antibodies (clone 6D12) to synthesized CML–BSA or native BSA used to coat the plate wells (0.6 μg protein/well) (A). Displacement curves of anti-CML antibodies (10 ng Ab/well coated with 0.6 μg CML–BSA/well) by 1.5×10^{-4} to 1.5×10^{3} pmol CML–BSA or native BSA used as a competitive antigen (B). Typical standard curve of the competitive indirect ELISA (mean of three independent curves ± S.D.) using 0.6 μg CML–BSA to coat each well, 15×10^{-3} to 300 pmol CML–BSA/well as competitive antigen, and 8 ng anti-CML Ab/well (C). Competitive Western blot showing the specificity of anti-CML monoclonal antibodies (clone 6D12) for synthetic CML–BSA and rat serum CML-modified proteins (D).

process. While glycation and subsequent glycoxidation occur over a period of weeks, thereby affecting long-lived proteins such as collagen, elastin, myelin, and crystallin, conditions such as diabetes and aging where oxidants and reactive carbonyls are elevated will promote adduction of shorter-lived proteins and thus impair specific functions otherwise unaffected. Yet, little is known about the range of proteins affected or the level of glycoxidation

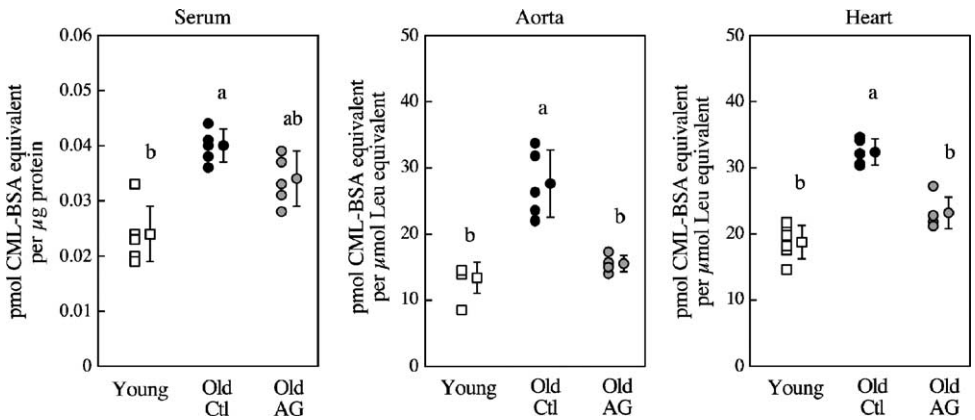

Fig. 4. Contents of CML-modified proteins in the serum (A), aorta (B), and heart of 4-month-old (Young), untreated 28-month-old (Old Ctl), and AG-treated 28-month-old (Old AG) rats. Individual observations and mean ± S.D. for 5–6 animals are shown. Values not sharing a common letter are significantly different ($P < .05$, single-factor ANOVA).

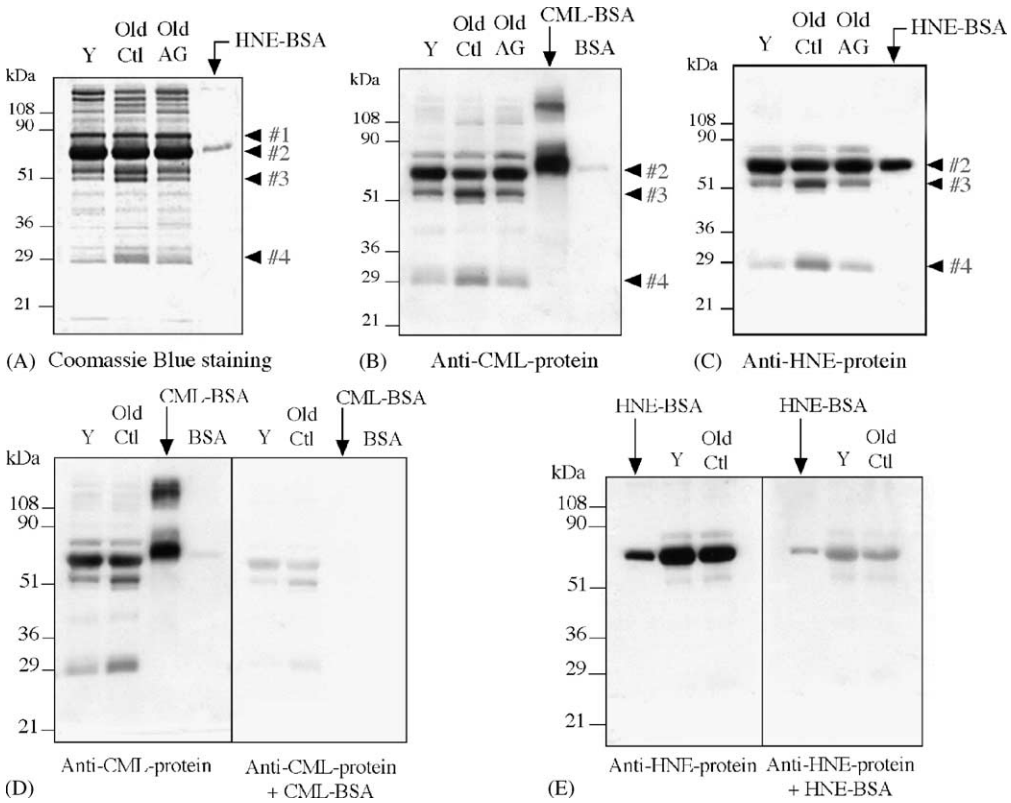

Fig. 5. Extent and nature of CML and HNE modifications in serum proteins of 4-month-old (Y), untreated 28-month-old (Old Ctl), and AG-treated 28-month-old (Old AG) rats. Total protein stain (A) showing the age-related increase in specific polypeptides #3 and #4, subsequently identified as immunoglobulin heavy and light chains by mass spectrometry (see Table 1). For reference polypeptide #1 is transferrin, and #2 is albumin. AG administration lowered their accumulation in serum. Immunoglobulin heavy and light chains that accumulate in the sera of untreated old rats also exhibit increased levels of CML (B) and HNE adducts (C). AG administration to old rats lowered the extent of CML and HNE modifications in serum. Blots are representative of four animals per group. Competitive Western blots showing the specificity of the antibodies used for rat serum CML (D) and HNE (E) modified proteins.

in subcellular compartments in relation to the blood stream and extracellular environment. Likewise the extent of protein lipoxidation is largely undocumented in animal models of aging. In contrast to glycation/glycoxidation, the process of lipoxidation may follow non-linear kinetics and depend upon specific factors, such as the lipid environment and turnover, the rate of oxidant production, and the status of antioxidant defense mechanisms. As a result lipoxidation may occurred sporadically at locations yet to be identified or unattainable to current drug therapies. The cardiovascular system represents an ideal subset of organs and tissues to examine the extent of glycoxidation and lipoxidation of native proteins because its constituents are slowly renewed, metabolically aerobic, and derive energy from glucose and fatty acids, which also are precursors of AGE and ALE.

To address some of these questions we developed a sensitive and specific ELISA that quantifies CML present in serum, aorta, and heart proteins obtained from young and old F344 rats, and used immunologic and mass spectrometric techniques to specifically identify the proteins modified by CML and HNE. Moreover, we evaluated whether AG, a scavenger of reactive carbonyl compounds and a drug used in the treatment of the complications of diabetes, was able to lower CML and HNE accumulation in these tissues and proteins when administered to old F344 rats from 25 to 28 months of age. Given the role played by CML- and HNE-induced protein inactivation and cross-linking in the chronic disorders associated with aging, it is reasonable to assume that preventing or lessening the formation of these protein adducts will help maintaining the optimum health of older adults.

When compared to 4-month-old F344 rats, the serum, aorta and heart of 28-month-old rats exhibited significantly higher CML- and HNE-proteins. Serum albumin was the predominant protein adducted by CML and HNE in the circulation. As shown by antigen competition assays, the antibodies used to detected serum CML- and HNE-modified proteins were highly specific. The extent to which albumin was modified did not change between young and old animals, suggesting that all potential sites are already modified at the age of 4 months. In addition to albumin, proteins modified by CML and HNE included transferrin, and immunoglobulin heavy and light chains. All these polypeptides were concomitantly adducted by CML and HNE. The extent of CML and HNE modification of immunoglobulins was noticeably higher in old rats. Because serum immunoglobulin content increased in old rats concurrently with CML and HNE modifications, the rise of these adducts may result from an accumulation of

Table 1
Identification of serum proteins becoming increasingly modified by CML and HNE in old rats using amino acid sequence tags obtained from ESI-QTOF analyses[a]

Identified protein	NCBI number	Molecular mass (Da)[b]	Sequence coverage (%)	Peptide sequence	Position
Transferrin (liver regeneration-related protein)	33187764	76346	21	KGTDFQLNQLQGKK	122–135
				HTTIFEVLPQKADR	226–239
				NGDGKEDLIWEILK	274–287
				SKDFQLFGSPLGKDLLFK	298–315
				DLLFKDSAFGLLRVPPR	311–327
				LYLGHSYVTAIR	332–343
				GYYAVAVVK	446–454
				TAQWNIPMGLLFSR	478–491
				VSTVLTAQKDLFWK	616–629
				DLLFRDDTK	647–655
				LPEGTTYEEYLGAEYLQAVGNIR	660–682
Albumin	19705431	68674	19	FKDLGEQHFK	35–44
				LVQEVTDFAK	66–75
				RHPYFYAPELLYYAEK	169–184
				AFKAWAVAR	234–242
				MSQRFPNAEFAEITK	243–257
				NYAEAKDVFLGTFLYEYSRR	342–360
				RHPDYSVSLLLR	361–372
				YTQKAPQVSTPTLVEAAR	435–452
				KQTALAELVK	549–558
Immunoglobulin γ2a heavy chain	1220486	51558	15	APEWLGFIR	63–71
				TKDVLTITLTPK	267–278
				FSWFIDDVEVHTAQTHAPEK	295–314
				SVSELPIVHR	322–331
				VNSGAFPAPIEK	343–354
				GPQVYTMAPPK	365–375
Immunoglobulin κ light chain constant region	125142	11725	30	DGVLDSVTDQDSK	49–61
				DSTYSMSSTLSLTKVEYER	62–80
Immunoglobulin κ light chain variable region	7650276	10591	13	FSGSGSGTDFTLK	48–60

[a] MS/MS ion search was performed using the following parameters: peptide tolerance ± 0.3 Da, MS/MS tolerance ± 0.3 Da, monoisotopic mass, one missed cleavage.
[b] Value from the National Center for Biotechnology Information (NCBI) protein database.

moderately modified immunoglobulins rather than from an increase in adduct-to-polypeptide ratio. The accumulation of modified immunoglobulins indicated that old F344 rats showed signs of primary amyloidosis, a disease of the immune system where plasma cells produce excess amounts of antibodies, first accumulating in the circulation and progressing from the blood into almost all organs. If deposition takes place in the kidneys, renal function may become impaired and lead to decreased waste clearance. In an attempt to dilute accumulating wastes, old rats increase their water intake, as seen in this study and by others [26]. The administration of AG ameliorated this phenotype by lowering the absolute amount of immunoglobulins to levels found in young rats and by normalizing water consumption. Alternatively, AG may improve renal ultrastructure [27] and function directly, as previously reported [18,28] thus facilitating rapid clearance of AGE-peptides [29] and, secondarily, normalize blood chemistry and water intake.

While AG prevents CML- and HNE-protein formation in vivo, its use is also associated with the inhibition of the inducible form of nitric oxidase synthase (iNOS) [30–33], with possible repercussion on the immune response. The blockade of nitric oxide (NO) production, which induces cellular immune defenses, may be viewed as detrimental to the animal's health. Based on the overall condition of the animals herein studied, i.e. their locomotor activity, alertness, body weight, and food intake, AG-treated rats appeared to be in good health. The assessment of serum cytokines and examination of organs (lungs, heart, liver, and spleen) further rebutted the possibility that AG-treated rats were immunosuppressed. It is worth noting that rather than detrimental, iNOS inhibition by AG proved beneficial to cardiac contractile function in aged mice [34]. Old C57BL/6 mice display a time-dependent decrease in left-ventricle systolic and diastolic function that was linked to elevated cardiac iNOS levels and higher concentrations of NO_x and cGMP. AG administration (10 mg/kg BW, i.v. or infusion) acutely restored ventricular systolic and diastolic function by lowering myocardial cGMP, which otherwise interferes with Ca^{2+} and cAMP inotropic action. In view of our data, one may ask whether the inhibition of iNOS by AG and thus the lowering of localized NO concentrations contributed to the decrease in CML- and

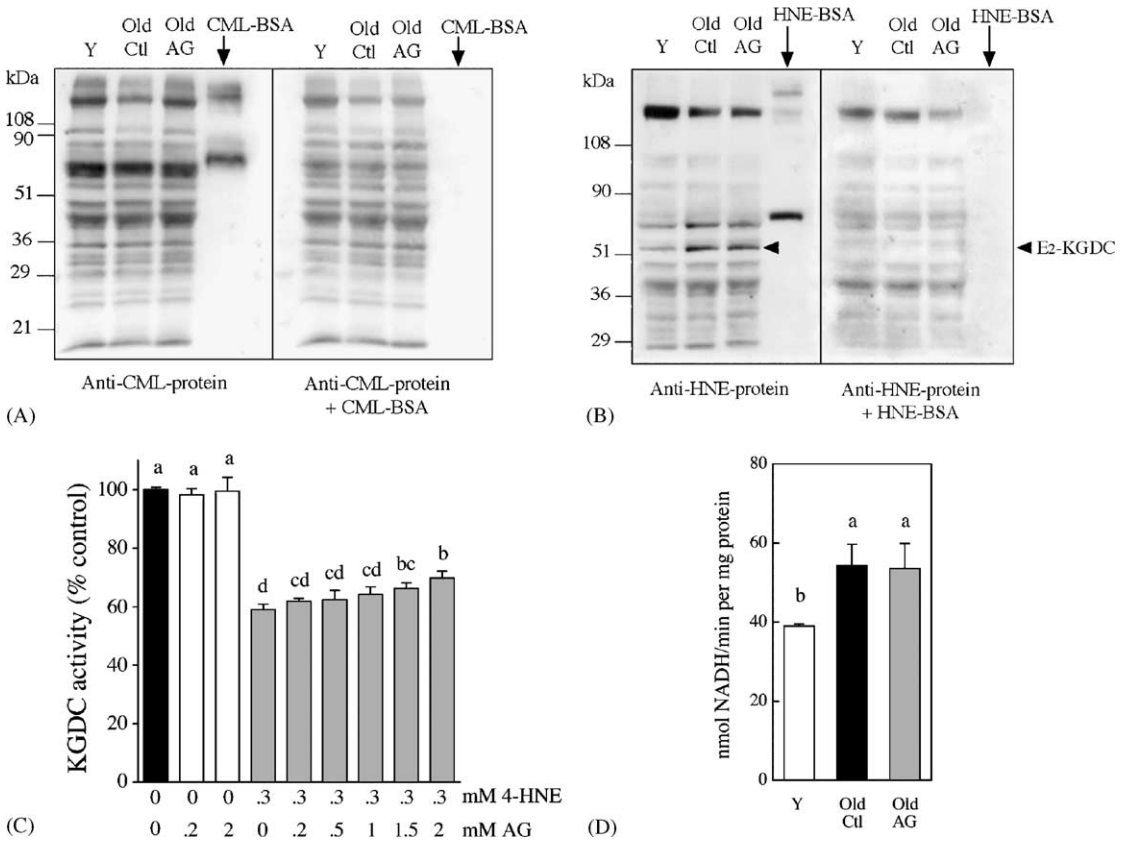

Fig. 6. Extent and nature of CML and HNE modifications in heart mitochondrial proteins of 4-month-old (Y), untreated 28-month-old (Old Ctl), and AG-treated 28-month-old (Old AG) rats. Immunoblots of mitochondrial proteins incubated with antibodies to CML-protein (A) and HNE-protein (B) in the presence or absence of competing antigen (0.5 mg CML–BSA or HNE–BSA, respectively). Of note, the degree to which HNE binds to E2-KGDC from old F344 rats is unchanged by AG administration. Blots are representative of three animals per group. AG prevents dose-dependently HNE-induced inactivation of reporter enzyme KGDC in vitro (C, see Section 2 for details). Data are shown as mean ± S.D. for five observations. Rat heart KGDC activity in experimental animals (D). Data are shown as mean ± S.D. for 5–6 animals. Values not sharing a common letter are significantly different ($P < .05$, single-factor ANOVA).

HNE-modified proteins in the different regions of the cardiovascular system. Regarding CML and more broadly AGE, indirect evidence suggested that NO did not take part in their formation. Indeed, the comparison of three iNOS inhibitors, AG, L-NAME, and methylguanidine, showed that only AG prevented renal AGE formation in diabetic rats [35]. However, there is good evidence to support the role of NO in the generation of lipid peroxidation products, including HNE, in biological systems challenged by oxidative stress [36,37]. Thus, the AG-associated lowering of serum HNE-proteins seen in the present study may, in part, result from the inhibition of iNOS.

As the major epitope in proteins modified with AGE, CML appears to be a useful biomarker of aging. The content of CML in proteins isolated from the thoracic region of the aorta and the non-mitochondrial fraction of the heart was substantially elevated with age. Thus, all cardiovascular tissues studied showed an age-related increase in CML. Our results are consistent with the predictions of the Maillard hypothesis, which identifies primarily glucose as the culprit, which cumulatively modifies proteins throughout the body, regardless of cell type. AG administration prevented this alteration, but with an efficacy that depended on tissue type. In fact, AG was most potent upon aorta and heart CML than in serum. This could reflect the compartmentalization and flux of CML, first originating in the circulation then migrating and diluting out into organs. Practically, the measurement of CML in the aorta and heart may be helpful for assessing the effectiveness of various strategies for intervention in the aging process.

Oxidative damage to proteins is thought to play a critical role in development of the aging heart phenotype. Because mitochondria produce a great deal of reactive oxygen species, mitochondrial proteins are among the primary targets of oxidative damage. In order to examine the extent of CML- and HNE-induced modifications in these organelles, heart proteins were separated into mitochondrial and non-mitochondrial proteins. The assessment of CML in mitochondrial proteins did not reveal any major differences between 4- and 28-month-old rats. An explanation may be that glucose, the principal precursor of CML, is excluded from mitochondria and thus does not form Schiff base adducts with mitochondrial proteins. Yet, while glucose is an important precursor of CML, other sources play a role, e.g. the degradation of lipids [38] and the

metabolism of amino acids, such as serine [39,40]. Nevertheless, CML content in mitochondrial proteins was negligible, which could also be explained by the rapid turn over of mitochondria (every 6–10 days) and their proteins. Likewise, pentosidine, a fluorescent AGE resulting from a cross-link between lysine and arginine residues, could not be detected by using Western blotting in mitochondria isolated from old rat hearts (data not shown).

While glycoxidation of heart mitochondrial proteins was modest in old F344 rats, modifications by HNE reached a detectable steady-state level. Confirming previous reports, aging of the heart was associated with an increase in mitochondrial HNE-proteins [25], in particular in HNE adducts to dihydrolipoamide succinyltransferase, i.e. the E2 enzyme component of the α-ketoglutarate dehydrogenase complex (E2-KGDC). As mitochondria are rich in polyunsaturated fatty acids (precursors of HNE) and prominent sites of oxidant production in the cell, short-lived proteins such as enzymes are modified by ALE. Based on the available evidence, the binding of HNE to mitochondrial proteins was limited to a few polypeptides, re-enforcing the view that the biochemical characteristics of a protein (the number and hindrance of potential target residues, the incidence of vicinal acidic or basic residues, the binding of phosphate [6,41]) rather than stochasticity determine its susceptibility to ALE modification.

AG is a nucleophilic hydrazine that inhibits AGE formation by trapping carbonyl compounds and also affects post-Amadori rearrangements [42]. Owing to its reactivity toward a broad range of carbonyl compounds, AG was previously shown to prevent the modification of proteins by ALE in vitro [12,13]. As one of the most readily formed lipid-derived aldehydes and commonly used model modifier of proteins in vitro, we studied HNE-induced inactivation of KGDC in the presence or absence of AG. In this system, AG provided some degree of protection by preventing KGDC inhibition in a dose-dependent manner, suggesting that AG is a scavenger of HNE. We then asked whether this interaction would benefit the aging animal and chose cardiac KGDC, a rate-determining enzyme of the citric acid cycle, as the target protein. The E2 component of KGDC is indeed increasingly adducted by HNE in the heart of 24- to 28-month-old F344 rats and, as a compensatory mechanism, KGDC becomes catalytically more efficient, with no change in protein content [25]. Although AG was able to counteract HNE-induced inactivation of KGDC in vitro, this effect remained modest and was not seen in the AG-treated rats. AG administration did not prevent the binding of HNE to E2-KGDC nor did it lower the age-associated compensatory increase in KGDC activity, suggesting that AG could not reach and remove the HNE generated in the mitochondrial matrix.

In summary, our results show that old F344 rats exhibit an elevated degree of glycoxidation and lipoxidation in serum, aorta, and heart proteins, leading to negative repercussion on kidney function. Modified proteins include serum albumin, transferrin, immunoglobulin, and mitochondrial dihydrolipoamide succinyltransferase. AG administration for 3 months significantly inhibited CML- and HNE-protein formation in aorta and heart, lowered serum content of CML-modified immunoglobulin, even though it was ineffective against lipoxidation of cardiac mitochondrial proteins in vivo. The use of AG as a pharmacological tool to prevent some of the negative outcomes associated with aging should be matter of further investigations.

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