Advanced Glycation End-Product Accumulation and Associated Protein Modification in Type II Skeletal Muscle With Aging

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One mechanism that may influence the quality of skeletal muscle proteins, and explain the age-related decline in contractility, is protein damage. Advanced glycation end-products (AGE) in vivo are useful biomarkers of damage. In this study, comparison of extensor digitorum longus (EDL) muscles from young (8 months), old (33 months), and very old (36 months) Fischer 344 Brown Norway F1 (F344BNF1) hybrid rats shows that muscles from the very old rats have a significantly higher percentage of myofibers that immunolabel intracellularly for AGE-antibody 6D12 compared to the younger age group. The AGE-modified proteins, determined in the semimembranosus muscles from young (9 months) and old (27 months) F344 rats, identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry include creatine kinase, carbonic anhydrase III, β-enolase, actin, and voltage-dependent anion-selective channel 1. Moreover, there is a significant increase in AGE modification of β-enolase with age. These results identify a common subset of proteins that contain AGE and suggest that metabolic proteins are targets for glycation with aging.

Skeletal muscle function diminishes with age. For instance, muscles from hind limbs of aged rodents produce less force than those muscles from younger rats (1–4). The reduced force production is, in part, due to age-related muscle atrophy, but recent studies demonstrate that the reduction in contractility is a result, at least in part, of defects in the skeletal muscle contractile proteins (1,5,6). These findings suggest that the quality of the skeletal muscle proteins is reduced in older animals.

One mechanism that may influence the quality of skeletal muscle proteins, and explain the age-related decline in contractility, is protein damage (e.g., posttranslational modification) (6). The formation of advanced glycation end-products (AGEs) in proteins occurs in vivo, via the Maillard reaction (7,8). AGE accumulation occurs, or is facilitated, under oxidative stress (9–11). In general, the accumulation of AGEs is tissue specific, alters the structural properties of proteins, and reduces their susceptibility to breakdown (10,12,13). Although there are many forms of AGEs, Nε-(carboxymethyl)lysine (CML) and Nε-(carboxyethyl)lysine (CEL) are major AGEs in vivo and are useful biomarkers of oxidative stress and damage (9). For instance, AGE accumulates in aging human skin collagen, lens crystallins, and arterial walls (14–16). It is elevated in skin collagen from diabetic persons, and levels correlate with the severity of complications (17). These findings suggest that glycoxidation reactions and oxidative stress may be important mechanisms in the pathogenesis of diabetic complications and possible age-related degenerative processes. In other words, AGE may be involved in the development of age-related deterioration of skeletal muscle function with age.

Studies investigating the effect of glycation on structure and function of skeletal muscle are limited (18). Myosin structure and actin–myosin interaction appear to be altered by exposing myosin to glucose in vitro. Two studies demonstrate that skeletal muscle function is altered in the presence of oxidative stress (18,19). Thus, age-related changes in muscle contractility performance may be related to posttranslational modifications, such as accumulation of AGEs, in key muscle proteins. However, to date, investigations focused on in vivo AGE accumulation in skeletal muscle from aged animals are not available.

In the present study, we examined AGE accumulation in muscles with type II skeletal muscle fibers and identified protein targets of AGE to test the hypothesis that age-related posttranslational modifications are underlying mechanisms for the decline in muscle function with aging.

**Materials and Methods**

Animal Protocol

For the determination of AGE accumulation in myofibers from the extensor digitorum longus (EDL) muscles, 17 male Fischer 344 Brown Norway F1 hybrid rats, 8 months old (n = 6), 33 months old (n = 6), and 36 months old (n = 5), representing 100%, 50%, and 30% survival rate for this rat strain, were used (National Institute on Aging [NIA] Strain Survival Information). For the identification of proteins that are targets of AGE, the semimembranosus muscles from eight male Fischer 344 rats, 9 months old (n = 4) and 27 months old (n = 4), representing 100% and 18% survival rate...
for this rat strain, were used (NIA Strain Survival Information). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Each animal was deeply anesthetized with intraperitoneal sodium pentobarbital (Fisher Scientific, Fair Lawn, NJ) 50–60 mg/kg. The EDL muscles were removed, placed on corks in embedding medium, and flash frozen in isopentane over liquid nitrogen. These specimens were stored at −80°C until cross-sections were cut. The semimembranosus muscles were removed, flash frozen in isopentane over liquid nitrogen, and stored at −80°C until processed. The EDL and semimembranosus muscles were selected for study because of their predominantly fast fiber type compositions (20).

Tissue Staining and Analysis

Muscle cross-sections, 10 μm thick, were cut on a cryostat (Leica Microsystems, Nussloch, Germany) from the midbelly of the EDL muscle. Sections were placed on gelatinized slides and immunolabeled for AGES CML and CEL (34). The primary antibody used was mouse monoclonal immunoglobulin G (IgG) antibody 6D12 (Transgenic Inc., Kumamoto, Japan). Based on the information from the manufacturer, 6D12 does not recognize the early products, Schiff base, and Amadori products. It, however, shows positive reaction to AGE samples obtained from proteins, lysine derivatives, or monoamino-carboxylic acids. This result indicates the immunospecificity of 6D12 to a common structure among AGE structures. The epitope of 6D12 is CML protein adduct. This antibody also recognizes CEL. Dilution of the primary antibody was 0.83 μg/mL, or 1:300 (vol/vol) basis. Negative controls were included in which the primary antibody incubation step was omitted.

Next, myofibers were evaluated for the presence or absence of AGE immunolabeling (21). Muscle cross-sections were digitally imaged through a microscope at ×200 using a software imaging program (Diagnostic Instruments, Sterling Heights, MI). The goal was to determine, as closely as possible, the total number of AGE-positive and AGE-negative myofibers in each cross-section. Therefore, the number of images obtained per muscle cross-section was the greatest number that could fit side-by-side on a muscle cross-section. Two to six images were obtained per muscle cross-section, with the number of images depending on the size of the muscle itself. A mean of 797 fibers were counted per muscle. The number of AGE-positive and AGE-negative myofibers was determined from these images, and the percentage of AGE-positive fibers was calculated per muscle.

The evaluations for the presence of AGES were performed by four professional students who demonstrated good inter-rater reliability (95% agreement). Initial evaluations for AGE immunolabeling were blinded. However, over time, the evaluations in practicality became quasi-blinded due to the large differences in AGE immunolabeling between muscles from young (100% survival rate), old (50% survival rate), and very old (30% survival rate) F344BNF1 hybrid rats (100% and 18% survival rates, respectively) were fractionated into cytosolic proteins (22). Briefly, muscle samples were cooled in liquid nitrogen, ground using a mortar and pestle, exposed to Subfractionation Buffer 1 (20 mM imidazole, 0.25 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4), and immediately homogenized with a glass homogenizer (Kontes Duall, Vineland, NJ). Following a 15-minute centrifugation at 12,000 g at 4°C, the supernatant was collected, the remaining pellet was rehomogenized with Subfractionation Buffer 1, and centrifugation was repeated. The supernatants were combined to form a cytosolic protein preparation. The remaining pellet was homogenized in Subfractionation Buffer 2 (1 mM Tris(2-carboxyethyl)phosphine HCl [TCEP], 0.5% trifluoroacetic acid [TFA]) and centrifuged for 15 minutes at 12,000 g at 4°C. The supernatant was collected in a fresh tube, and the pellet was rehomogenized in Subfractionation Buffer 2 followed by centrifugation. These supernatants were combined, forming a myofibrillar protein preparation. Finally, a membrane protein preparation was prepared by homogenizing the remaining pellet in 3.5% sodium dodecyl sulfate (SDS). Protein concentrations were determined using the bichinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL). Bovine serum albumin was used as a protein standard.

One-Dimensional Gel Electrophoresis

Prior to Western immunoblotting to screen for AGE modifications, membrane protein homogenates were electrophoretically separated by SDS–polyacrylamide gel electrophoresis (PAGE) using a Mighty Small SE 250 (Pharmacia Hoeffer, San Francisco, CA) system and a 10% resolving gel with a 3% stacking gel (5). Protein loads for preparations from skeletal muscle were 10 μg per lane, which gave an immune reaction within the linear range for the antibody. To increase the resolution of the proteins, the use of a large format gel (16 cm × 18 cm) for SDS–PAGE (Pharmacia Hoeffer) was necessary. For each sample, two gels were run in parallel (5). One gel was silver stained using the mass spectrometry (MS)-compatible Silver Stain Plus Kit (Bio-Rad, Hercules, CA). The alternate gel was used for Western immunoblotting. Images were captured using a GS800 Imaging System (Bio-Rad).

Two-Dimensional Gel Electrophoresis

First-dimension isoelectric focusing (IEF) was performed with the Protean IEF Cell (Bio-Rad) using 11 cm, pH 7–11 immobilized pH linear gradient (IPG) strips. IPG strips were rehydrated with 90 μg of cytosolic protein preparation at 50 V for 14 hours. Samples were focused at 250 V for 15 minutes, with a linear increase to 8000 V for 2.5 hours, and a final focusing step at 8000 V to reach a total of 35,000 V hours. For the second dimension, IPG strips were equilibrated for 10 minutes in Buffer A (6 M urea, 2% SDS, 375 mM Tris–HCl [pH 8.8], 20% glycerol, and 130 mM dithiothreitol [DTT]), followed by 10 minutes in Buffer A with 135 mM iodoacetamide substituted for DTT. The equilibrated strips were embedded in 0.5% (wt/vol) agarose on the top of 13% acrylamide gels. Second-dimension SDS–PAGE was performed using the Pharmacia Hoefer SE 600 system. For each sample, two gels were run in parallel. One
gel was silver stained using the MS-compatible Silver Stain Plus Kit (Bio-Rad). The alternate gel was used for Western immunoblotting. Images were captured using a GS800 Imaging system (Bio-Rad).

**Western Immunoblotting**

Following resolution of proteins by one- or two-dimensional gel electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a semidry transfer system (Bio-Rad). Membranes were incubated with mouse monoclonal IgG antibody 6D12 (1:2500) primary antibody. Biotinylated goat anti-mouse alkaline phosphatase-conjugated secondary antibody (1:3000), streptavidin (1:3000), along with biotinylated alkaline phosphatase (1:3000), and the substrate bromo-4-chloro-3-indoyl phosphate–nitroblue tetrazolium (BCIP-NBT) were used to visualize the immunoreaction. Membranes were imaged using the GS800 imaging system (Bio-Rad) and quantified by densitometric analysis using Sigma Scan Pro (SYSTAT Software, Inc., San Jose, CA). Subsequently, the relative amount of immunoreaction was determined, which is the density of the immunoreaction normalized to the density of the same protein from the silver-stained gel.

**Selection and Preparation of Proteins for MS**

To align protein bands or spots exhibiting an immunoreaction with bands or spots on silver-stained gels, images of Western immunoblots and their corresponding gels were printed on transparencies and overlaid on a light box. Selected bands were excised and proteins digested in-gel with trypsin overnight at 37°C as described (5). Prior to protease digestion, the cysteine residues were reduced with DTT and alkylated using iodoacetamide. Peptides were extracted from gels by repeated swelling and shrinking of the gel using 25 mM ammonium hydrogen carbonate and acetonitrile 1:1 (vol/vol), followed by 5% formic acid and acetonitrile 1:1 (vol/vol) (5). Extracted peptides were evaporated to near dryness in a Speed Vac and stored at −80°C prior to mass spectral analysis.

**Matrix-Assisted Laser Desorption Ionization–Time of Flight MS**

In general, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) was performed to obtain peptide mass fingerprints (QSTAR XL quadrupole-TOF mass spectrometer; Applied Biosystems Inc. [ABI], Foster City, CA) (5). Prior to MALDI-TOF MS analysis, a portion of the peptide mixture was desalted using Millipore C18 ZipTips (Millipore, Danvers, MA) using the protocol of the manufacturer. Extracted peptides were crystallized using α-cyano-4-hydroxycinnamic acid diluted 1:1 (vol/vol) with a 50:50 acetonitrile/Nanopure water mixture and 0.1% trifluoroacetic acid. Full-scan mass spectra of the peptide mixtures from 500 to 3500 mass-to-charge ratio (m/z) and tandem mass spectral data of select ions were collected using a QSTAR XL quadrupole-TOF (ABI) mass spectrometer. The TOF region acceleration voltage was 4 kV, and the injection pulse repetition rate was 6.0 kHz. Laser pulses were generated with a nitrogen laser at 337 nm, ~9 μJ of laser energy using a laser repetition rate of 20 Hz. External calibration was performed using human angiotensin II (monoisotopic [MH+] of m/z 1046.5417; Sigma), and adrenocorticotropic hormone fragment 18–19 (monoisotopic [MH+] of m/z 2465.1989; Sigma). The ABI Bayesian Peptide Reconstruct Tool, which is very robust as multiple peaks in an isotope series are used during the Bayesian calculation, was used to generate a peak list for sample peptides. For the confirmation of the protein identity, tandem MS (MS/MS) was performed (QSTAR XL) using the rule that every 1000 m/z is 50 collision energy units and every 100 m/z above 1000 m/z is 5 more collision energy units.

Measured peptide m/z values were used to search the National Center for Biotechnology Information (NCBI) for protein identifications and database accession numbers using Mascot (www.matrixscience.com) or BioAnalyst (ABI) software. All searches were performed with a mass tolerance between 50 and 100 ppm. Positive identification required a minimum of three peptide matches and a probability score that indicates high concordance between the masses of experimentally derived peptides with theoretical masses of peptides from the matched protein, and a positive identification from at least one product ion spectrum.

**Statistical Analysis**

Descriptive statistical analysis included means and standard errors of the means. Differences between groups were determined by performing a Kruskal–Wallis one-way analysis of variance (ANOVA) on Ranks followed by Kruskal–Wallis Multiple-Comparison z Value Test.

**Table 1. Myofiber Percent AGE-Positive Immunolabeling in EDL Muscles From F344BNF1 Hybrid Rats**

<table>
<thead>
<tr>
<th>Age of Rats</th>
<th>% AGE Positive</th>
</tr>
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<tbody>
<tr>
<td>8 mo</td>
<td>1.38 ± 0.34</td>
</tr>
<tr>
<td>33 mo</td>
<td>5.92 ± 1.8</td>
</tr>
<tr>
<td>36 mo</td>
<td>22.64 ± 5.2*</td>
</tr>
</tbody>
</table>

Notes: Values indicate mean ± standard error of the mean.

*Significantly different from young group. Kruskal-Wallis one-way analysis of variance on Ranks: p < .001, followed by Kruskal-Wallis Multiple-Comparison z Value Test.

AGE = advanced glycation end-product determined by 6D12 antibody; EDL = extensor digitorum longus muscle; F344BNF1 = Fischer 344 Brown Norway F1; 8 months, 33 months, and 36 months represent 100%, 50%, and 30% survival rates for the F344BNF1 hybrid rat strain.

**RESULTS**

**AGE-Positive Myofibers**

The percentage of myofibers showing immunolabeling for AGE (6D12) was significantly greater in the very old rats than in the young rats, a nearly 10-fold difference, p < .001 (Table 1). The pattern of the AGE immunolabeling has two characteristic appearances at the individual myofiber level. One pattern is intracellular punctuate labeling (Figure 1C). The other pattern is labeling at the myofiber periphery.
In some instances, both patterns are present in the same cell (Figure 1D). With the techniques used in this study, it was not possible to determine whether the peripheral staining was on membrane proteins or on other proteins near the sarcolemma. Immunolabeling appeared in other tissues, such as connective tissue, within the skeletal muscle (Figure 1D).

**AGE-Positive Proteins**

Protein identities were obtained using MALDI-TOF MS and are outlined in Table 2. Peptide fingerprints from full scans were used to obtain an initial identification. MS/MS sequencing of one or more peptides confirmed the protein’s identity. Figure 2 shows a representative MALDI-TOF mass spectrum of peptides generated from an in-gel trypsin digest.

**Table 2. MALDI-TOF MS Identification of AGE Immunoreactive Bands**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number*</th>
<th>Mass (d)</th>
<th>Score</th>
<th>% Coverage</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage-dependent anion-selective channel 1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>gi[6755963]</td>
<td>30853</td>
<td>112</td>
<td>54</td>
<td>11</td>
</tr>
<tr>
<td>α-enolase&lt;sup&gt;1&lt;/sup&gt;</td>
<td>gi[6978811]</td>
<td>47279</td>
<td>88</td>
<td>37</td>
<td>11</td>
</tr>
<tr>
<td>Creatine kinase&lt;sup&gt;1&lt;/sup&gt;</td>
<td>gi[6978661]</td>
<td>43224</td>
<td>177</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>Actin&lt;sup&gt;1&lt;/sup&gt;</td>
<td>gi[71620]</td>
<td>42372</td>
<td>109</td>
<td>32</td>
<td>10</td>
</tr>
<tr>
<td>Carbonic anhydrase 3&lt;sup&gt;1&lt;/sup&gt;</td>
<td>gi[31377484]</td>
<td>29703</td>
<td>74</td>
<td>46</td>
<td>6</td>
</tr>
</tbody>
</table>

*Accession numbers were taken from National Center for Biotechnology Information (NCBI)nr database.

<sup>1</sup>MOWSE score was taken from Mascot (www.matrixscience.com); score > 54 indicates a significant match.

<sup>2</sup>Protein was confirmed with peptide sequencing by tandem MS (MS/MS).

MALDI-TOF MS = matrix-assisted laser desorption ionization–time of flight mass spectrometry; AGE = advanced glycation end-product.
of a protein band that was identified as actin. Ten peptides representing 32% of the primary sequence for actin were identified from the peptide mass fingerprint (Figure 2A and Table 2). In this example, one peptide was sequenced by MS/MS analysis, thus providing unambiguous identification of the protein. The MS/MS spectrum for peptide 1790 m/z is shown in Figure 2B. The fragmentation of this peptide produced a product ion spectrum containing 7 of 16 b ions and 12 of 16 y ions that matched the amino acid sequence for residues 241–256 of actin.

**DISCUSSION**

In this study, comparison of EDL muscle from young, old, and very old F344BNF1 hybrid rats shows that the muscle from the very old rats has a significantly higher percentage of myofibers that immunolabel intracellularly for 6D12 (an AGE antibody) compared to the muscles from the younger group. The AGE-modified proteins include creatine kinase, carbonic anhydrase III, β-enolase, actin, and voltage-dependent anion-selective channel 1 (VDAC1), with β-enolase showing an accumulation of AGE with age.

**AGE-Positive Fibers**

This immunohistochemical investigation reveals that the extent of the immunoreactivity for the anti-AGE monoclonal antibody, 6D12, increases in myofibers with aging. The biosynthetic pathways of AGE in vivo have not yet been elucidated. However, several studies report that AGE-modified proteins are generated through different metabolic pathways (e.g., peroxidation of polyunsaturated fatty acids, glycoxidation), with oxidation playing an important role in CML formation (10). The extent depends on the tissue and disease state. For example, muscle shows the least glycation of several tissues, with the basal level of glycation in muscle protein of 0.2 mmol/mol lysine (23). However, the level of glycation in muscle protein exhibits a 3-fold increase in muscles from diabetic rats compared with nondiabetic rats (21).

According to the glycation hypothesis and oxidative stress theory of aging, accumulation of AGEs of the Maillard reaction alters the structural properties of proteins and reduces their susceptibility to degradation (12,13). Decreased susceptibility of AGE-proteins to degradation by the proteasome, the function of which is also compromised during aging, might contribute to the buildup of modified proteins (24–26).

**AGE-Modified Proteins**

There is a preponderance of evidence that long-lived proteins have AGE; however, short-lived proteins also accumulate AGE when O2 is available (27). Cellular proteins are more highly glycated than extracellular proteins, which is consistent with the localization of the 6D12
antibody in our immunohistochemistry muscle cross-sections (28).

With age, Poggioli and colleagues (29) found a restricted set of proteins in human peripheral blood lymphocytes to be AGE-modified. Although the protein identities were not investigated, these proteins were not the most abundant proteins. In the present work, creatine kinase, β-enolase, carbonic anhydrase III, and voltage-dependent anion-selective channel 1 (VDAC1) are targets for AGE. In other words, AGE selectively targets several critical enzymes involved in energy production. Posttranslational modifications (oxidative modifications) of proteins are usually associated with inhibition of enzyme function as a result of conformational changes, oxidation of key cysteine residues, and cross-linking of proteins. Creatine kinase activity plays a critical role in energy metabolism in skeletal and cardiac muscles. Creatine kinase catalyzes the reversible transfer of a phosphoryl group from adenosine triphosphate (ATP) to creatine to produce adenosine diphosphate (ADP) and phosphocreatine. Many in vitro and in vivo experiments on cardiac myocytes show that creatine kinase activity is inhibited by reactive oxygen species (30,31). By comparison, carbonic anhydrase III is a zinc metallo-enzyme and plays an important role in maintaining muscle carbohydrate metabolism as well as the reversible hydration of carbon dioxide (32). This protein also has an age-associated increase in carbonyl content and a decrease in specific activity (33).

β-enolase is one of the most abundant proteins in cells (34) and catalyzes the conversion of 2-phosphoglycerate into phosphoenolpyruvate in the glycolysis pathway. Increasing evidence links β-enolase-dependent pathways to several pathologies and oxidative stress. For example, β-enolase has been identified as a target for oxidation in bacteria and in Alzheimer’s disease and as a nitrated protein in aging skeletal muscle (5,35). It is thought that β-enolase is a scavenger of AGE because lysines are available for modification at the exposed surface of the protein. This scavenging process may spare other proteins from AGE modification and consequent functional impairment. β-enolase is a good candidate for this role, as glycation of this protein has only a limited impact on cell physiology. Indeed, glycation leads to a decrease of β-enolase activity, but no changes were detected in glycolytic flux (34).

VDAC1 is located in the outer mitochondrial membrane. It plays an important role as a controlled passage for adenine nucleotides, Ca²⁺, and other metabolites in and out of mitochondria. Recently, it has been shown to be involved in cell-signaling events, such as mitochondria-mediated apoptosis (36). Modulation of VDAC1 occurs via several proapoptotic and anti-apoptotic cascades; however, modulation by reactive oxygen species is not known (37). Our detection of age-dependent glycation is consistent with recent reports demonstrating posttranslational modification of this protein in hearts of aging rats (38).

In the present work, actin is a target for AGE. Posttranslational modification of actin, such as nitration, has been found in aging skeletal muscle (5). It has been shown that Cys374 of actin monomer (G-actin) is particularly sensitive to the stress of oxidation by hydrogen peroxide and nitric oxide donors, leading to a reduction in actin polymerization (F-actin), which plays a major role in myofibril contraction (39,40). In vitro myofibrillar glycation (myosin, actin) is found to decrease adenosine triphosphatase (ATPase) activity (41).

Further identification of other proteins, assessment of the precise nature of the modification(s) and modification site(s), as well as the study of the impact of such modification(s) on the function of these proteins may provide insight on the effects of AGE modification in the aging process of skeletal muscle.

Summary

This study demonstrates that EDL myofibers in very old rats exhibit AGE accumulation in vivo, and that this AGE accumulation can occur on the myofiber periphery, intracellularly, or both. Creatine kinase, carbonic anhydrase, β-enolase, actin, and VDAC1 are proteins identified to be targets of AGE. The functional significance of these findings is unknown at this time.

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