Weight Increase Is Associated with Skeletal Muscle Immunostaining for Advanced Glycation End Products, Receptor for Advanced Glycation End Products, and Oxidation Injury

Maria Pia de la Maza,1 Jaime Uribarri,2 Daniela Olivares,1 Sandra Hirsch,1 Laura Leiva,1 Gladys Barrera,1 and Daniel Bunout1

Abstract

Background: Tissue accumulation of advanced glycation end products (AGEs) is associated with ageing, both in diabetics and nondiabetic subjects.

Aim: The purpose of this study was to assess immunostaining for AGEs, specifically carboxymethyl-lysine (CML) and receptor for AGEs (RAGE), in muscle tissue of healthy male subjects differing in age and weight stability.

Methodology: Muscle tissue was obtained during hernia surgery in middle-aged men reporting weight maintenance (WM, n = 10) or weight gain (WG, n = 7), and also in 4 elderly men. Tissue immunostaining for CML and RAGE was performed.

Results: Intensity of CML and RAGE staining were highly correlated (r = 0.84) and also significantly associated with weight change and age. Muscle AGEs accretion was statistically associated with muscle expression of oxidative injury (8-hydroxy-deoxyguanosine and 4-hydroxy-2-nonenal) and inflammatory markers (tumor necrosis factor-α).

Discussion: The increase of skeletal muscle AGEs/RAGE and markers of inflammation and oxidative injury in association with weight gain and old age suggest a pathogenic role of AGEs in weight gain and in sarcopenia of aging.

Background

It has been extensively demonstrated that calorie restriction (CR) is able to modify longevity in animal models, from yeast to mammals.1–5 Experiments in nonhuman primates as well as epidemiological data and short-term CR interventions in human beings support most of the changes observed in CR rodents, although whether prolongation of life span is possible in primates is still not known.4

Long-term energy balance is necessarily reflected in body weight and composition changes over time. Therefore, we proposed that self-reported changes in body weight could be a potential indicator of caloric balance: subjects reporting minor to zero change in body weight during several years could resemble energy-restricted primates according to the protocol of the University of Maryland. In contrast, people reporting an increase in weight during the same period necessarily have had positive energy balances, similar to ad libitum-fed primates, which tend to exhibit a progressive increase in weight. In a previous study, we demonstrated that subjects who maintained their weight accumulated less 8-hydroxy-deoxyguanosine (8OHdG) and tumor necrosis factor-α (TNF-α) in skeletal muscles compared with the weight gainers, whose muscles were more similar to those of elderly men. These variables correlated with body fat, although all the studied men had body mass index (BMI) ≤30 kg/m2.6

Advanced glycation or glycoxidation end products (AGEs) are heterogeneous moieties that originate endogenously as a result of a hyperglycemic environment, such as in diabetes mellitus, or by oxidative reactions catalyzed by radical species. They can also derive from dietary sources or from smoking.7 Whatever their sources, AGEs accumulate mostly in extra- and intracellular long-lived proteins such as collagen, with cross-linking of protein fibers. Several studies

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have demonstrated an association between tissue levels of AGE (assessed by antibodies, mass spectrometry, or fluorescence) and ageing, both in diabetics and nondiabetic subjects.\(^8\)\(^9\)\(^10\) Many of the effects of AGEs are mediated by receptors such as receptor for AGEs (RAGE), whose interaction with AGE-modified proteins initiates cellular signals that activate nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) and other pathways, resulting in transcription of proinflammatory factors and increased oxidative stress.\(^11\) These deleterious effects are counteracted by AGER1, another AGE receptor, that also enhances AGE breakdown.\(^12\) Interestingly, in rodents, the low AGE content in the diet seems to a major determinant of the beneficial effects of CR on metabolic and inflammatory signs, as well as cardiac function, aging, muscle and bone function, as well as median life-span extension.\(^13\) Conversely, the addition of AGEs to a CR diet suppresses its life-extending effects and other benefits.\(^14\)

Previously, we demonstrated that positive caloric balance is associated with increased oxidative stress in muscle.\(^6\) Because oxidative stress is closely associated with tissue levels of AGEs and RAGE,\(^15\) we wanted to test this association in muscle tissue. Therefore, we performed immunostaining for AGEs, specifically carboxymethyl-lysine (CML), and RAGE in muscle tissue obtained from adult males who were either weight maintainers or gainers and compared them to muscle tissue in elderly men.\(^6\) Our results show that AGEs accumulate and expression of RAGE is markedly increased in muscle tissue as weight and age increase.

### Subjects and Methods

We selected adult male subjects scheduled for surgery of inguinal hernia, who agreed to participate in this study by signing a written informed consent approved by our local ethics committee. According to age and self-report of weight change, we divided the sample in three study groups: (1) Weight maintainers (WM), who reported no change in body weight, or a minor increase (less than 4 kg) over the last 10 years and aged 30–50 years; (2) weight gainers (WG), who stated a body weight increase >5 kg during the last 10 years with ages between 30 and 50 years, and a BMI \(\leq\)30 kg/m\(^2\); and (3) healthy elderly (E) subjects, aged >65 years. This report is based on a subsample of subjects in a previously published study on muscle tissue oxidative damage,\(^6\) thus exclusion criteria, clinical, and laboratory evaluations are similar, but the sample size is smaller. Anthropometric evaluation included weight, height, abdominal circumference, and skinfold thickness at four sites to calculate percent body fat according the Durnin and Womersley.\(^16\)

During the operation, a small sample (1 cm\(^3\)) of skeletal muscle (internal abdominal oblique) was obtained, and a

### Table 1. Demographic and Anthropometric Data of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>WM (n = 10)</th>
<th>WG (n = 7)</th>
<th>E (n = 4)</th>
<th>ANOVA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41 ± 4</td>
<td>42 ± 5</td>
<td>67 ± 2(^{2a})</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71 ± 8</td>
<td>83 ± 8(^{b})</td>
<td>64 ± 8</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24.8 ± 3</td>
<td>27.4 ± 2</td>
<td>23.8 ± 3</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>Weight change (kg)</td>
<td>1.1 ± 2.2</td>
<td>6.4 ± 0.8</td>
<td>23.4 ± 2</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>24.3 ± 6</td>
<td>27.4 ± 2</td>
<td>23.4 ± 2</td>
<td>0.288</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>92 ± 8</td>
<td>99 ± 7</td>
<td>91 ± 4</td>
<td>0.116</td>
<td></td>
</tr>
<tr>
<td>Right handgrip strength (kg)</td>
<td>44 ± 8</td>
<td>43 ± 6</td>
<td>31 ± 1(^{a})</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>Left handgrip strength (kg)</td>
<td>40 ± 8</td>
<td>43 ± 6</td>
<td>31 ± 48(^{b})</td>
<td>0.040</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Statistically significant if \(p < 0.05\).

\(^{a}\)Significantly different from WM and WG.

\(^{b}\)Significantly different from WM.

WM, Weight maintainers; WG, weight gainers; E, elderly; ANOVA, analysis of variance; BMI, body mass index.

### Table 2. Laboratory Data of the Three Study Groups

<table>
<thead>
<tr>
<th>Serum levels (units)</th>
<th>WM (n = 10)</th>
<th>WG (n = 7)</th>
<th>E (n = 4)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>95.8 ± 8</td>
<td>82.2 ± 33</td>
<td>92.7 ± 9</td>
<td>0.3972</td>
</tr>
<tr>
<td>Insulin (uU/mL)</td>
<td>8.1 ± 4</td>
<td>6.2 ± 3</td>
<td>3.6 ± 2</td>
<td>0.1134</td>
</tr>
<tr>
<td>Leptin (ng/L)</td>
<td>5.8 ± 4</td>
<td>6.8 ± 2</td>
<td>4.3 ± 2</td>
<td>0.3909</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.13 ± 0.11</td>
<td>0.14 ± 0.1</td>
<td>0.28 ± 0.2</td>
<td>0.1043</td>
</tr>
<tr>
<td>Adiponectin (ug/mL)</td>
<td>11.8 ± 4</td>
<td>12.5 ± 4</td>
<td>13.8 ± 4</td>
<td>0.7553</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>210 ± 38</td>
<td>201 ± 36</td>
<td>206 ± 28</td>
<td>0.8755</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>50 ± 10</td>
<td>41 ± 6</td>
<td>44 ± 6</td>
<td>0.1091</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15 ± 0.6</td>
<td>15 ± 0.1</td>
<td>14 ± 1.3</td>
<td>0.2828</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>5.4 ± 1</td>
<td>5.4 ± 1</td>
<td>5.3 ± 1</td>
<td>0.9860</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Statistically significant if \(p < 0.05\).

WM, Weight maintainers; WG, weight gainers; E, elderly; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol.
### Table 3. Immunostaining for CML and RAGE in the Three Study Groups

<table>
<thead>
<tr>
<th></th>
<th>WM (n = 10)</th>
<th>WG (n = 7)</th>
<th>E (n = 4)</th>
<th>ANOVA p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML (Units/pixel)</td>
<td>35.0 ± 7.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.6 ± 5.7</td>
<td>67.7 ± 9.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RAGE (Units/pixel)</td>
<td>35.4 ± 7.4</td>
<td>62.5 ± 8.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.8 ± 3.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4HNE (number of particles/μm²)</td>
<td>5.5 ± 5</td>
<td>9.1 ± 6</td>
<td>19.3 ± 13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.025</td>
</tr>
<tr>
<td>80HdG (number of particles/area)</td>
<td>132.1 ± 36</td>
<td>150.6 ± 19</td>
<td>169.5 ± 52</td>
<td>0.199</td>
</tr>
<tr>
<td>TNF-α (Units/pixel)</td>
<td>62.9 ± 29</td>
<td>118.6 ± 42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>106.4 ± 28</td>
<td>0.0096</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Statistically significant if p < 0.05.

<sup>a</sup>Significantly different from WG and E.

<sup>b</sup>Significantly different from WM.

<sup>c</sup>Significantly different from WM.

WM, Weight maintainers; WG, weight gainers; E, elderly; CML, carboxymethyl-lysine; RAGE, receptors for advanced glycation end products; 4HNE, 4-hydroxy-2-nonenal; 8OHdG, 8-hydroxy-deoxyguanosine; TNF-α, tumor necrosis factor-α.

**FIG. 1.** Representative immune staining for carboxymethyl-lysine (CML) in the three study groups. Matlab analysis of color density demonstrated lower intensity in muscle tissue from weight maintainers compared with weight gainers, which are closer to elderly subjects.
fraction was placed in Bouin’s solution and then placed in paraffin for immunohistochemical detection of CML and RAGE by light microscopy. After deparaffinization with xylene and ethanol, the sections were treated with H2O2 in ethanol for 20 min and then blocked with diluted normal blocking serum for 20 min at room temperature. Next, these 4-mm sections were incubated with anti-CML (1:50) (CMS-10 monoclonal antibody; Abcam, Inc, Cambridge, MA) or RAGE (1:1000) (PA1-075; Affinity Purified Bioreagents, Golden, CO) antibodies for 30 min at room temperature and at 4°C overnight, and then sequentially exposed to biotin-labeled horse anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG and ABC complex (Vectastain ABC elite kit, Vector Laboratories, USA). The sites of peroxidase binding were demonstrated with diaminobenzidine (DAB, catalogue no. SK-4100; Vector Laboratories, USA). Negative controls were immunostained as above, but with preimmune serum instead of specific antibodies. Sections were counterstained with hematoxylin for examination by microscopy and then photographed. Signals were quantified through Matlab 6.5 (R 13, 2002). Calculations are based on two tissue sections per subject, choosing five fields of each section for analysis. Two photographs and four areas of each sample (200 × 200 pixels) were selected to be analyzed by the software according to Matkowskyj et al.17 The numeric value obtained indicates the ratio between the immunostained sample and a negative control.

Paraffin-fixed muscle sections of these subjects were used for immunohistochemical detection of 8OHdG and TNF-α.

FIG. 2. Representative immune staining of receptor for advanced glycation end products (RAGE) in the three study groups. Matlab analysis of color density demonstrated lower intensity in weight maintainers compared with weight gainers, which are closer to elderly subjects.
Likewise, paraformaldehyde-fixed samples were employed for immunogold detection of 4-hydroxy-2-nonenol (4HNE) adducts by electron microscopy (EM). Details of these procedures were described previously.\textsuperscript{6}

**Statistical Analysis**

Statistical analysis was performed in the Stata for Windows package, version 8.0. Data are expressed as mean ± SD. Differences between groups were compared using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis to evaluate between which groups the differences were significant. Correlations between variables were analyzed by Pearson correlation coefficients.

**Results**

The sample consisted of 10 WM (age 41 ± 4 years), 7 WG (age 42 ± 5 years), and 4 E (age 67 ± 2 years). Demographic, anthropometric and laboratory data of the three study groups are shown in Tables 1 and 2.

CML and RAGE immunostaining results were available for 7 WM and 7 WG, but RAGE staining was available in only 2 samples from elderly subjects. Values for CML and RAGE in skeletal muscle are depicted in Table 3. WM showed less staining for CML and RAGE compared with both WG and E. WG had similar CML staining compared with E. Representative photographs are depicted in Figs. 1 and 2.

CML immunostaining correlated significantly with weight change ($r = 0.7$), age ($r = 0.58$), right handgrip strength ($r = -0.54$), and high-density lipoprotein cholesterol (HDL-C) ($r = -0.5$), but not with weight, BMI, or body fat. Staining for RAGE also correlated significantly with weight change ($r = 0.7$) and age ($r = 0.6$), but not to any other anthropometric or laboratory variable measured.

Intensities of CML and RAGE staining were highly correlated ($r = 0.84, p = 0.0014$), and both were also positively associated with muscle TNF-α ($r = 0.55$ and 0.75, $p = 0.02$ and 0.0014, respectively). RAGE immunostaining was significantly associated with 8OHdG ($r = 0.55, p = 0.034$) and 4HNE ($r = 0.69, p = 0.007$). CML staining was not associated with these muscle oxidation adducts (Figs. 3–5).

**Discussion**

In the present study, we found more skeletal muscle immunostaining for AGEs (specifically CML) and its receptor (RAGE) in healthy middle-aged men who reported weight increase, without exceeding a BMI of 30 kg/m$^2$, compared to weight maintainers. The immunostaining of weight gainers was similar to that found in muscle from elderly men, although definite conclusions would require a bigger sample size of the latter. Immunostaining was highly correlated with weight change over the last 10 years, stressing its relevance to long-term positive energy balance.
FIG. 4. Correlation plots between 8-hydroxy-deoxyguanosine (8OHdG), carboxymethyl-lysine CML (A), and 8OHdG and receptor for advanced glycation end products (RAGE) (B).

FIG. 5. Correlation plots between 4-hydroxy-2-nonenal (4HNE) and carboxymethyl-lysine (CML) (A) and 4HNE and receptor for advanced glycation end products (RAGE) (B).
Both CML and RAGE immunostaining were highly associated with skeletal muscle TNF-α expression, a known mediator of inflammation and loss of muscle tissue in cachexia and ageing,\textsuperscript{18,19} both conditions that can be prevented by calorie restriction.\textsuperscript{20,21} RAGE, but not CML, immunostaining correlated positively with other well-known markers of DNA and protein oxidative (8OHdG and 4HNE) damage that accumulate with advanced age.

The relation between glyoxidation and ageing has been described previously\textsuperscript{8–10}; however, its relation with positive energy balances reflected by weight increase without reaching obesity has not been studied previously. This association does not seem to depend exclusively on body fat mass or distribution because no correlations were found with anthropometric parameters or with serum adiponectin or leptin. Because we did not measure muscle lipid deposition, we cannot exclude the possibility that the relation between AGEs and positive energy balance with slowly progressive weight increase can induce visceral deposition and oxidation of lipids, as postulated by Unger et al.\textsuperscript{22} However, immunostaining measurements were performed in muscle areas without fat accretion.

There are few studies regarding skeletal muscle accumulation of AGEs in humans. Biopsies performed in the vastus lateralis muscle of elderly and young subjects showed a 200% increase in the accumulation of pentosidine in elderly subjects who had lower functional capacity and strength compared with their young counterparts.\textsuperscript{23} Increased expression of RAGE or CML together with higher NF-κB DNA-binding activity and hydrogen production have been reported in muscle biopsies of patients with several muscular diseases.\textsuperscript{24,25} Similarly, progressive accumulation of AGE-modified proteins with aging and modification of critical enzymes in muscle energy production have been reported in experimental animals.\textsuperscript{26} The accumulation of AGEs in myocytes is considered a pathogenic mechanism for the myocardial damage observed in diabetes mellitus.\textsuperscript{27} Moreover, early glycated products such as glycated albumin are also capable of stimulating the production of reactive oxygen species (ROS) in cardiac myocytes.\textsuperscript{28}

Apart from their effect on stimulation of ROS formation, AGE-modified proteins are known to become resistant to degradation by the ubiquinone proteasome degradation machinery.\textsuperscript{29} This leads to accumulation of modified proteins in tissues, which has been considered as one of several potential mechanisms producing age-related functional decline of diverse organs.\textsuperscript{30} Therefore, AGEs can have an important pathogenic role in the muscular decline or sarcopenia of the elderly. Previously, we have shown the association between age-related accumulation of oxidation products such as 4HNE adducts and telomere attrition in skeletal muscle.\textsuperscript{31}

The correlation of AGE accumulation and RAGE expression with the expression of these inflammatory and oxidative markers in muscle further reinforces a potential role for pathogenic role of AGEs in the sarcopenia of aging.

Studies in healthy subjects have documented increasing levels of circulating AGEs with age as well as an association of circulating AGEs with markers of oxidative stress.\textsuperscript{15} These findings support an important effect of AGE-activated signal pathways on determining systemic oxidative stress. Any tissue/cellular effect of AGEs represents the final balance between a tendency to increase oxidative stress through stimulation of receptors such as AGER1.\textsuperscript{12,14} Future studies should also measure circulating levels of AGEs as well as tissue immunostaining of AGER1 to define muscle tissue AGE homeostasis in more detail.

A potential criticism of this study is the use of self-reported weight changes to classify subjects. We used self-reported weight change because it has previously been found to be associated with actual weight\textsuperscript{23} and with body fat mass. Another obvious limitation is the small sample size.

The current study cannot determine the mechanism of the association between excessive caloric intake, reflected by weight gain, and tissue accumulation of AGEs. One postulation would be that a higher caloric intake increases oxidative stress and AGEs, which in turn would lead to progressive tissue damage. Recent animal data, however, suggest the possibility that increased dietary AGE intake, unrelated to calories, might lead to both tissue AGE deposition and even weight gain.\textsuperscript{34}

In summary, we have found that muscle tissue from weight gainers and elderly subjects show marked increase of immunostaining for CML and RAGE in association with immunostaining of markers of oxidative stress and inflammation. This raises a potential role for AGEs in the pathogenesis of two important medical problems, weight gain in the young population and sarcopenia in the old population. Further research will be needed to define this role.

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Author Disclosure Statement

All authors declare that no competing financial interests exist.

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