Weight increase and overweight are associated with DNA oxidative damage in skeletal muscle ☆

María-Pía de la Maza*, Daniela Olivares, Sandra Hirsch, Walter Sierralta, Vivien Gattás, Gladys Barrera, Daniel Bunout, Laura Leiva, Mireya Fernández*

Institute of Nutrition and Food Technology (INTA), University of Chile, Macul 5540, P.O. Box 138-11, Santiago, Chile

KEYWORDS
Weight maintenance;
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Summary
Background and aims: Weight maintenance within normal standards is recommended for prevention of conditions associated with oxidative injury. To compare oxidative damage in a post mitotic tissue, between adults differing in long-term energy balance.
Methods: During hernia surgery, a sample of skeletal muscle was obtained in 17 non-obese adults. Subjects were divided into two groups according to their self-reported weight change: weight maintainers (WM) reported <4 kg increase, and weight gainers (WG) reported >5 kg increment. Muscle immunohistochemistry for 8-hydroxy-deoxyguanosine (8OHdG), 4-Hydroxy-2-nonenal (4HNE), and TNF-α, as markers of oxidative injury and inflammation, were performed. As known positive controls for oxidative injury, we included 10 elderly subjects (66–101 yr). Anthropometric measures and blood samples for clinical laboratory and serum cytokines (TNF-α and IL-6) were obtained.
Results: 8OHdG was higher in WG compared with WM (149.1 ± 16.2 versus 117.8 ± 29.5, $P = 0.03$), and was associated with anthropometric indicators of fat accumulation. 4HNE was similar in WG compared with WM (10.9 ± 7.6 versus 9.8 ± 6.3) but noticeably higher in elderly subjects (21.5 ± 15.3, $P = 0.059$). TNF-α protein in WG was higher compared with WM (114.0 ± 41.7 versus 70.1 ± 23.3, $P = 0.025$), and was associated with weight increase.

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*Corresponding authors. Tel.: +56 2 9781495; fax: +56 2 2214030.
E-mail address: mpmaza@inta.cl (M.P. de la Maza).

Conclusions: Moderate self-reported weight increase, and body fat accumulation, suggesting long-term positive energy balance is associated with muscle DNA oxidative injury and inflammation.

Introduction

The most accepted hypothesis of aging proposes that physiological age-related changes are a consequence of oxidative damage to cellular macromolecules due to free radical attack originated in mitochondria.[1] Intracellular concentrations of oxidized macromolecules such as lipids, proteins and DNA, increase as a function of age in different animal species, including human beings.[2] This would signal the occurrence of an imbalance between free radical production during cell metabolism and the cellular antioxidant defense and repair systems.[3] Most evidences supporting this hypothesis of aging derive from animal studies, which confirm an inverse relationship between longevity and oxidative stress.[4] One of the most constant scientific findings is the prolongation of life span in animal models, through caloric restriction (CR).[5,6] The underlying mechanisms are still not completely understood, however the prevention of oxidative injury is the most widely accepted hypothesis.[7]

The age-related decline in muscle mass and performance has been attributed to oxidative damage of mitochondrial genome and enzymes (e.g. aconitase), which cause a deficiency in the activity of respiratory chain complexes.[8] In human beings, higher concentrations of protein carbonyls have been detected in post mitotic tissues such as brain, muscle and eye lens.[9] An age-dependent increase of human muscle oxidative damage to DNA, lipids and proteins was demonstrated by measuring 8-hydroxy-2-deoxyguanosine (8OHdG), malondialdehyde (MDA) and protein carbonyl groups, respectively, in skeletal muscle samples obtained during orthopedic surgery.[10] Nevertheless, whether these products of oxidative stress are the best biomarkers of ageing, is still a matter of debate.[11]

Epidemiological data support the anti-ageing effects of leanness,[12,13] however long-term follow-up studies are lacking.[14] On the other hand, some evidences indicate that obesity, the phenotypic expression of a positive energy balance, induces oxidative stress,[15–17] and a pro-inflammatory condition associated with elevation of adipose-derived cytokines and peptides.[18,19] In fact obese subjects die young, mostly from cardiovascular and metabolic complications associated with fat accumulation. An acceleration of ageing or of age-related cellular changes has not been directly demonstrated in overweight or obese subjects.

Furthermore, age-related muscle wasting (sarcopenia) has been attributed to increased expression of TNF-α,[20] a cytokine also related to obesity and centripetal fat distribution.[21] Actually in elders, the accretion of fat has been ascribed to the loss of muscle mass which decreases metabolic rate.[22,23] However, under certain circumstances, overnutrition can cause steatosis, lipotoxicity, and lipoapoptosis. The most affected tissues are β cells, myocardium and skeletal muscle.[24] Both aging and the metabolic syndrome could represent these lipotoxic conditions.[25,26] Nevertheless the relationship between adipose tissue growth and the expression of this cytokine in skeletal muscle has been studied mostly in obese or old patients, but has not been evaluated in young healthy subjects, as a measure of ''physiologic age''.

Therefore, the present study aimed to verify if healthy middle-aged non-obese weight gainers (WG) show signs of higher oxidative damage compared with weight maintainers (WM), and thus leaner subjects. With this purpose we compared oxidative injury and TNF-α protein expression in skeletal muscle, between subjects reporting maintenance of body weight during most of their adult life, and those reporting progressive weight increase and body fat accumulation, with a maximal body mass index (BMI) of 30 kg/m^2.

Subjects and methods

Among adult male subjects with abdominal or inguinal hernias, scheduled for surgery during the next few days, we selected those who accepted to participate in this study by signing a written informed consent. Exclusion criteria were age below 30 yr, cigarette smoking ($\geqslant$1 cigarette/day), vegetarianism or repeated dieting with weight fluctuations, diabetes mellitus, immune diseases, acute infectious conditions, BMI $>$ 30, ingestion of vitamin supplements, treatment with

corticosterone, hypolipemic drugs or propranolol. A sample of 10 healthy elderly subjects (age > 65 yr) was included as well-known positive controls for muscle oxidative damage.[10] Younger adults were divided into two groups according to their self-reported history of weight change; WM reported no change in body weight, or a minor raise (less than 4 kg over the last 10 yr) and WG stated a body weight increase > 5 kg during the last 10 yr, without exceeding 30 kg/m^2 BMI.

Every subject underwent a complete medical evaluation including clinical history, dietary recall (24 h and food frequency[27]), physical activity questionnaire,[28] physical examination, anthropometric evaluation (including weight, height, abdominal circumference and skinfold thickness at four sites to calculate percent body fat according the Durnin and Womersley[29]). Immediately before the surgical procedure a fasting blood sample was obtained for the following determinations: glucose, insulin, thyroid-stimulating hormone (TSH), lipoproteins (total cholesterol, HDL cholesterol and triglycerides), creatinine, uric acid, hepatic profile, C Reactive Protein (CRP), leptin, adiponectin, serum cytokines (TNF-α and IL-6) and TNF-α soluble receptor 1 (TNF-αSR1).

During the operation, a small sample (1 cm^3) of skeletal muscle (internal abdominal oblique) was obtained, a fraction was placed in Boin's solution, then included in paraffin for immunohistochemical detection of TNF-α and 8-OH-dG, by light microscopy. Another fraction was fixed in paraformaldehyde for immunogold detection of 4-Hydroxy-2-nonenal (4HNE) adducts by electron microscopy (EM).

This protocol was approved by INTA's ethics committee and by the equivalent councils of the three institutions in which the surgical procedures were carried out, according to the Helsinski declaration.

Laboratory procedures

Determination of serum glucose, lipoproteins, hepatic profile, uric acid and creatinine were performed using standard automated systems, using Roche kits. Serum insulin was measured by a DPC kit (normal range $<20\,\mu$U/dl), leptin and adiponectin by Linco RIA kits, and TSH by electroquimioluminiscence using a Roche kit (normal range = 0.27–4.2 mU/L). CRP was measured by immunoturbidometric analysis (normal range = 0–5 mg/l) through a Roche kit. Serum TNF-α, IL-6 and TNF-αSR1 concentrations were measured using high sensitivity ELISA kits (Quantikine R&D kits).

For immunohistochemical studies, after deparaffination with xylene and ethanol, the sections were treated with H_2O_2 in ethanol for 20 min.

For the 8OHdG assay, samples were treated with 10 μg/ml Proteinase K in phosphate-buffered saline (PBS) (0.05 M phosphate, 0.15 M NaCl, pH 7.4) with 1% bovine serum albumin (BSA-50, Rockland, USA) for 40 min at 37 °C, and incubated in 5% skim milk in PBS for 2 h. The sections were next incubated with monoclonal anti-8-OH-dG antibody (N45.1 Gentaur MOG-020P) (1:75) for 2 h at room temperature and overnight at 4 °C, and then sequentially exposed to biotin labeled horse anti-mouse IgG and ABC complex (Vectastain ABC elite kit, Vector Laboratories, USA). The sites of peroxidase binding were demonstrated with diamonobenzidine (DAB Vector Laboratories, USA, catalog no. SK-4100). Negative controls were immunostained as above, but with preimmune serum instead of anti-8OH-dG antibody.

For TNF-α immunohistochemistry, samples were treated with citrate-buffered saline (CBS) (10 mM citrate, 0.15 M NaCl, pH 6), at room temperature for 2 min and microwaved three times for 5 min. The sections were kept at room temperature for 20 min and incubated with 0.1 M glycine in Tris-buffered saline (TBS) (0.05 M Tris, 0.12 M NaCl, pH 7.4) for 15 min, blocked for 20 min with 1% preimmune serum goat in TBS, at room temperature. Sections were next incubated with anti-human TNF-α antibody (Rockland catalogue no. 209-401-306) (1:50) for 1 h at room temperature and overnight at 4 °C. After washing with 1% BSA in TBS samples were incubated with anti-rabbit IgG for 1 h at room temperature. The sites of peroxidase binding were demonstrated with diamonobenzidine (Rockland, USA catalogue no. DAB-50). Negative controls were immunostained as above, but with preimmune serum instead of anti-TNF-α antibody.

Sections were counterstained with hematoxylin for microscopic examination and then photographed. Two tissue sections without fat infiltration were studied in each subject, choosing five fields of each section for analysis. For 8OHdG, signals were quantified through Image-J 1.32, and data were expressed as particle number/area fraction. TNF-α staining was quantified through Matlab 6.5 R 13, 2002. Two photographs of each sample were selected, then four areas (200 $\times$ 200 pixels) of each photograph were analyzed by the software, according to Matkowskyj et al.[30] The numeric value obtained indicates the ratio between the immunostained sample and a negative control.

For 4HNE detection, after fixation in the cold, samples were washed with phosphate buffer, dehydrated in ethanol and infiltrated with LR Gold resin, then transferred into gelatine capsules filled with 0.8% (w/v) benzoyl peroxide in LR Gold. The blocks were then sectioned with a diamond knife and ultrathin sections were mounted on pioloform-coated gold grids and immediately incubated. The grids were transferred to droplets of 1% BSA in PBS and blocked for 30 min, then incubated for 14 h at 4 °C with antibody against HNE-adducts in 1% BSA/PBS. The attachment to the antibody was detected with goat anti-rabbit-Au10 nm. After 15 min post-fixation with 1% (v/v) glutaraldehyde in PBS, the grids will be thoroughly washed with water, contrasted with uranyl acetate and lead citrate, dried and examined under the EM. Results are expressed as number of gold particles/100 μm^2.

Statistical analysis

Statistical analysis was performed in the Statistica for Windows package. Data are expressed as mean $\pm$ SD. For parametric variables differences between groups were compared through Student's *t*-test, and for nonparametric data, Mann–Whitney *U*-test. Correlations between variables were analyzed by Pearson's or Spearman's correlation coefficients, respectively. Frequencies between groups were compared by the Fisher exact test.

Results

Twenty seven male volunteers were studied, 10 elders (ages 66–101 yr) and 17 younger adults (ages 34–47 yr). They were all healthy except for the hernias, hypertension (six cases, mostly among elders) and hyperlipidemia (seven cases). According to inclusion criteria, all were non-smokers and not dieting. We restricted the sample to healthy male subjects aged 33–48 yr, with a narrow BMI range (20–30 kg/m^2). We excluded women because they usually have wide variations in weight due to pregnancies and lactation. We also excluded subjects with BMI $> 30\ kg/m^2$ to avoid interference with obesity-related co-morbidities such as glucose intolerance, diabetes, sleep apnea, atherosclerosis, etc. Volunteers and their spouses were interrogated carefully about recent weight changes, which were nonsignificant. Table 1 shows clinical and laboratory data, comparing subjects according to age groups.

Table 2 depicts clinical and laboratory features of subjects, divided according to their change in body weight for the period of 10 yr. As expected, anthropometric measurements and leptin concentrations were higher among WG compared to WM. CRP was also higher in WG. Weight increase correlated significantly with BMI ($r = 0.66$), waist and hip circumferences ($r = 0.83$ and 0.81, respectively) and body fat% ($r = 0.7$). Three out of 9 WM had body fat >25%, compared with seven out of

Table 1 Clinical and laboratory data in relation to age.

	Adults ($n = 17$)	Elders ($n = 10$)	*P*
Age (yr)	42 $\pm$ 4	74 $\pm$ 10	< **0.001**
Sistolic pressure (mmHg)	128 $\pm$ 13	144 $\pm$ 24	**0.03**
Diastolic pressure (mmHg)	81 $\pm$ 8	79 $\pm$ 13	0.63
Body mass index (kg/m^2)	26 $\pm$ 3	25 $\pm$ 4	0.59
Body fat (%)	25.6 $\pm$ 5	25.4 $\pm$ 4	0.89
Hemoglobin (g/dl)	15.1 $\pm$ 0.6	14.5 $\pm$ 1.7	0.23
C Reactive Protein (mg/dl)	0.12 $\pm$ 0.04	0.41 $\pm$ 0.3	**0.0003**
Total cholesterol (mg/dl)	205 $\pm$ 43	206 $\pm$ 35	0.99
HDL cholesterol (mg/dl)	47 $\pm$ 8	45 $\pm$ 9	0.60
Tryglycerides (mg/dl)	155 $\pm$ 138	141 $\pm$ 68	0.75
Creatinine (g/dl)	0.95 $\pm$ 0.1	1,1 $\pm$ 0,3	0.07
TSH (μU/ml)	2.3 $\pm$ 1	3.6 $\pm$ 3	0.16
Glucose (mg/dl)	95.2 $\pm$ 8.4	98 $\pm$ 10	0.50
Insulin (μU/ml)	6 $\pm$ 3	5 $\pm$ 4	0.78
HOMA	1.3 $\pm$ 0.8	1.2 $\pm$ 1.0	0.75
Leptin (ng/l)	5.9 $\pm$ 2.7	6.2 $\pm$ 4.0	0.82
TNF-α (pg/ml)	0.8 $\pm$ 1.0	1.1 $\pm$ 1.2	0.38
IL-6 (pg/ml)	2.2 $\pm$ 1.7	3.6 $\pm$ 3.3	0.17
TNF-α S. receptor (pg/ml)	701 $\pm$ 242	909 $\pm$ 308	0.06

Data expressed as means $\pm$ SD.
Detection limits for TNF-α, IL-6 and TNF-α SR1 are 0.5–32, 0.156–10.0 and 78–5000 pg/dl, respectively.

Table 2 Clinical and laboratory data in relation to body weight change.

	Weight maintainers ($n = 9$)	Weight gainers ($n = 8$)	P
Age (yr)	42±4	42±4	0.97
BMI (kg/m^2)	24.7±2.7	27.2±1.7	**0.03**
Weight change (kg)	0.4±2.6	7.2±2.3	**<0.001**
Body fat (%)	23.4±5	28.0±3	**0.04**
Waist (cm)	86.6±6.9	96.6±2.8	**0.002**
Waist/hip	0.91±0.04	0.95±0.03	0.12
SAP (mmHg)	132±13	123±11	0.18
DAP (mmHg)	81±9	81±7	0.86
Energy intake (kcal/d)	1978±399	2098±569	0.62
Total cholesterol (mg/dl)	200±43	211±45	0.61
HDL cholesterol (mg/dl)	52±7	42±6	**0.005**
Tryglycerides (mg/dl)	155±163	156 ±116	0.98
Creatinine (g/dl)	0.97±0.1	0.92±0.1	0.38
C Reactive Protein (mg/dl)	0.10±0	0.14±0.1	**0.045**
TSH (μU/ml)	2.3±1	2.3±1	0.97
Glucose (mg/dl)	95±6.9	96±10.5	0.88
Insulin (μU/ml)	5.9±3.2	5.7±3.0	0.93
HOMA	1.38±0.8	1.24±0.9	0.73
Leptin (ng/l)	4.5±2.1	7.5±2.5	**0.018**
Adiponectin (μg/dl)	13.3±4.1	11.9±3.5	0.47
TNF-α (pg/ml)	1.03±1.0	0.47±0.9	0.24
IL-6 (pg/ml)	2.7±2.3	1.7±0.7	0.24
TNF-α S. receptor (pg/ml)	647.8±226	760.3±259	0.35

Data expressed as means±SD.
Detection limits for TNF-α, IL-6 and TNF-α SR1 are 0.5–32, 0.156–10.0 and 78–5000 pg/dl, respectively.

eight WG ($P = 0.036$). Differences in calculated energy intake and physical activity level (expressed as metabolic equivalents or METs), did not reach statistical significance when comparing WG to WM.

Tissue samples were adequate for immunohistochemistry, except for one (WG), that was removed for statistical analysis regarding TNF-α and 4HNE. Representative images for 8OHdG and TNF-α staining in the different study groups are shown in Figs. 1 and 2(I) respectively. 8OHdG was significantly higher in WG (149.1±16.2) compared with WM (117.8±29.5), $P = 0.03$, and the latter did not differ from values obtained in elderly subjects (156.7±46.2). Moreover, 8OHdG was associated with weight ($r = 0.5$, $P = 0.045$), BMI ($r = 0.5$, $P = 0.047$), weight change ($r = 0.5$, $P = 0.043$), body fat ($r = 0.65$, $P = 0.007$), waist circumference ($r = 0.66$, $P = 0.005$), hip circumference ($r = 0.55$, $P = 0.025$), waist/hip ratio ($r = 0.52$, $P = 0.038$) and leptin concentration ($r = 0.51$ $P = 0.044$), but not to present energy intake. No associations were observed between 8OHdG immunostaining and serum metabolic or inflammation parameters. Respecting muscle TNF-α protein, WG exhibited higher staining than WM (114.0±41.7 versus 70.1±23.3 units/pixel, respectively, $P = 0.025$), and similar to elders (102.2±24.5 units/pixel) (Fig. 2(II)). Muscle TNF-α correlated positively with weight change ($r = 0.63$, $P = 0.012$), dietary fat intake, waist and hip circumferences ($r = 0.52$, 0.57 and 0.60, respectively, $P < 0.04$), and leptin concentration ($r = 0.59$, $P = 0.017$), but not significantly with BMI and body fat.

Immunostaining images and analysis of 4HNE adducts are shown in Fig. 3(I) and (II). Mean values for WM, WG and elderly subjects were 9.8±6.2, 10.9±7.6 and 21.5±15.3 particles/100 μm^2 ($P = 0.059$). No correlations were found between 4HNE immunostaining and anthropometric metabolic variables.

When data from young adults were re-analyzed according to BMI (> or <26 kg/g^2) instead of weight change, 8OHdG was significantly higher in overweight compared with normal weight subjects (142.8±25.3 versus 113.7±25.5 versus $P = 0.04$), however differences in TNF-α and 4HNE did not reach statistical significance (102.6±35.9 versus 67.1±36.1 and 11.1±6.8 versus 8.7±7.7, $P > 0.05$, respectively).

Discussion

In this study we demonstrate, for the first time in humans, that WM (and thus, on average leaner

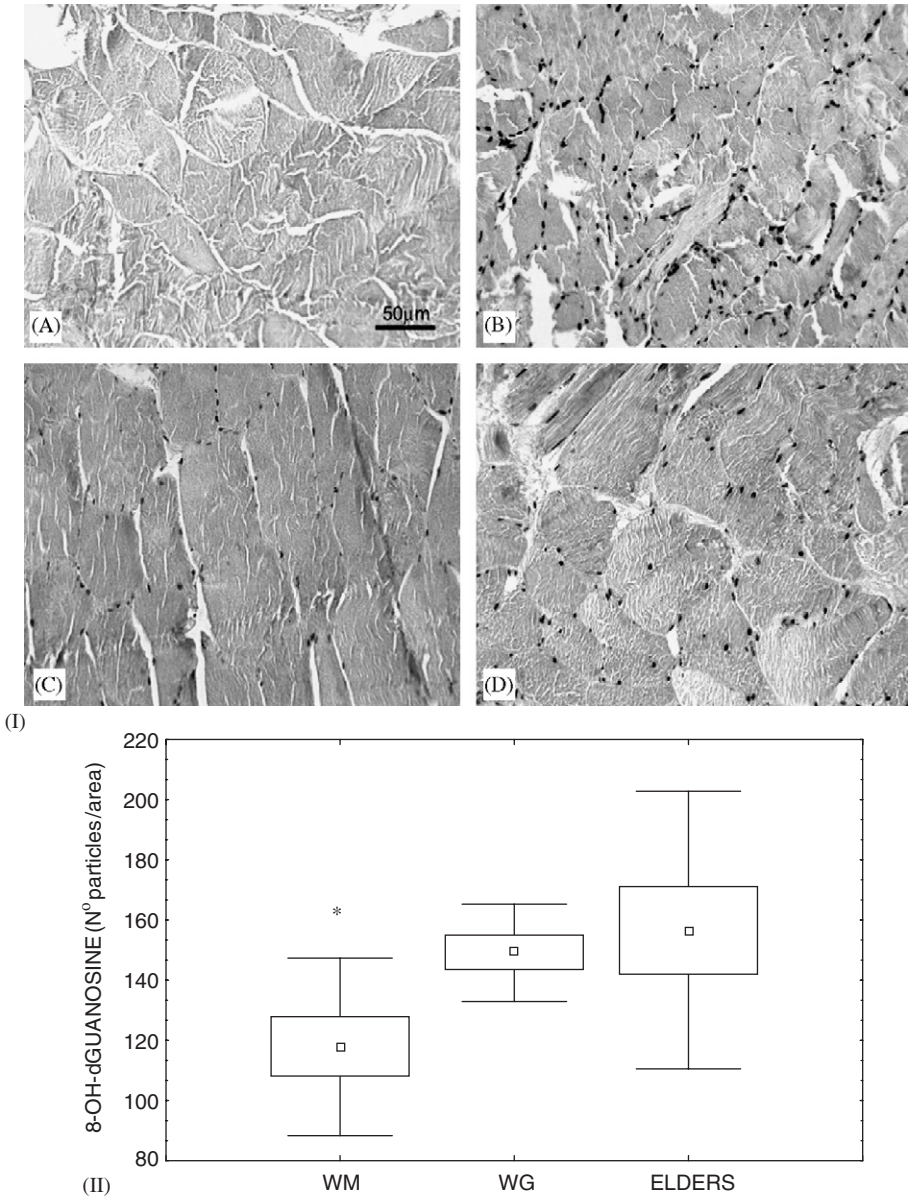

Figure 1 (I) Immunostaining for 8OhdG: (A) negative control, (B) muscle from subject >65 yr (positive control), (C) muscle from weight maintainer, and (D) muscle from weight gainer. (II) Comparison of 8OHdG immunostaining, analyzed through Image-J. WM = weight maintainers. WG = weight gainers. *$P<0.05$ compared with WG.

subjects) exhibit less DNA oxidative stress and muscle injury, evidenced by lower 8OHdG and TNF-α. In this sense, WG (usually overweight) behave as elderly subjects. Similar results are observed if groups are divided according to BMI ($<$ or >26 kg/m^2). The lower DNA oxidative injury of WM reported here, suggests that this model of weight maintenance could be useful for the study of ageing in humans. However it requires a non-mitotic tissue, such as skeletal muscle. In fact in our previous study, with a comparable sample, we did not detect any difference in 8OHdG concentrations in DNA obtained from blood mononuclear cells.[31] The other oxidation marker analyzed (4HNE) increased only in elderly subjects, suggesting that its enrichment requires a longer period of time, unlike mitochondrial DNA which has been reported as more susceptible to oxidative injury.[2] However, the technique employed does not allow sub-cellular compartamentalization analysis to assess if 8OHdG origin is mitochondrial or genomic.

In the present investigation, as well as in a previous survey,[31] we found that self-reported weight change is associated with body fat accumulation, namely abdominal, in healthy non-obese middle-aged men. This is the necessary consequence of a positive energy balance, although cross sectional dietary recalls may not recognize it, as

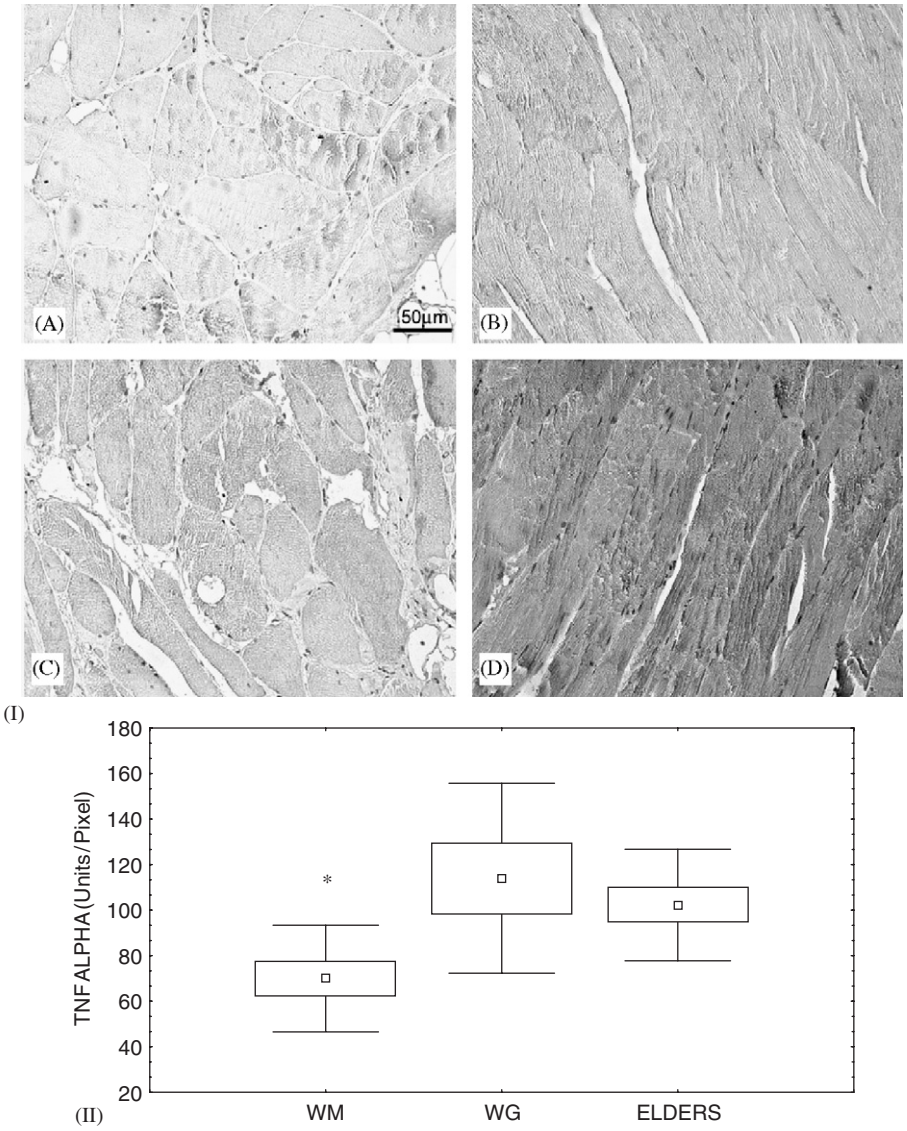

Figure 2 (I) Immunostaining for TNF-α: (A) negative control; (B) muscle from subject >65 yr (positive control); (C) muscle from weight maintainer; and (D) muscle from weight gainer. (II) Comparison of TNF-α protein, analyzed through Matlab 6.5. WM = weight maintainers. WG = weight gainers. *$P<0.05$ compared with WG.

has been shown by the doubly labelled water technique.[32] Overweight individuals are more responsive to food-derived hedonic stimulus, but this intermittent overeating can be overlooked by food surveys.[33] As representative of the Chilean population, our volunteers were mostly sedentary, thus differences in body composition are not the result of variations in energy expenditure but, most probably, the result of small but continuing positive energy balances, which we were not able to detect through short-term dietary and physical activity recalls.

Recent data highlight the contribution of adipose tissue and body weight to oxidative stress. Weindruch et al.[34] reported that lower body weight mediates some of the mortality-reducing effects of calorie restriction. Moreover, data from Barzilai et al.[35] suggest that most of the life-extending benefits of this can be attributed to reduced fat stores, probably through a decrease in concentration of fat-derived peptides, such as cytokines, complement factors and substrates. In rats, some age-related alterations, such as insulin resistance can be reverted through surgical removal of visceral fat.[36] Among the numerous substances produced by adipose tissue, adiponectin seems to be inversely associated with oxidative stress, while leptin has the opposite relationship.[37] In the present study, muscle TNF-α protein was associated with weight increase and abdominal fat. WM exhibited significantly lower muscle TNF-α, the same as calorie restricted animals,[38] sustaining the

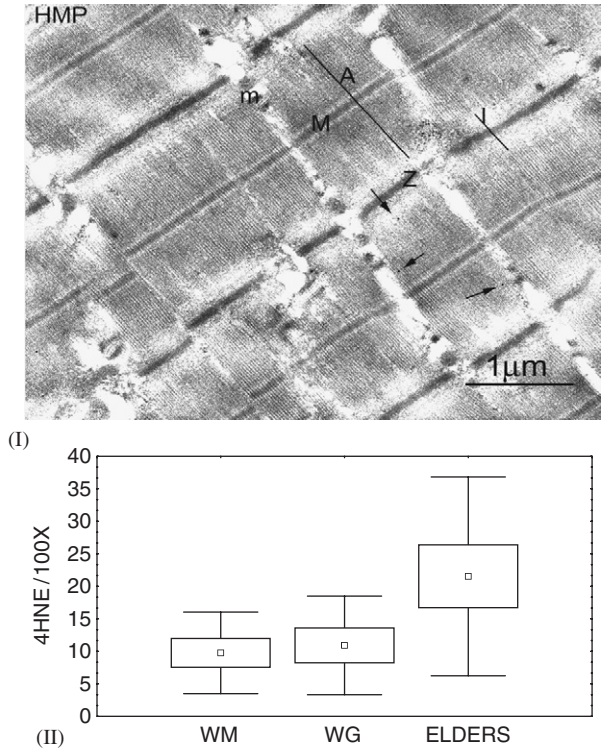

Figure 3 (I) Immunogold staining for 4HNE adducts in muscle from elderly subject with 19 gold particles/ 100 μm^2. (II) Comparison of 4HNE particles between study groups.

contribution of body fat to the aging process, and disorders such as cancer[39,40] and cardiovascular diseases.[41,42] The exact cellular origin of muscle TNF-α in our samples cannot be identified, however we excluded slides in which adipose tissue infiltration was evident on light microscopy, therefore our results are not comparable to increased TNF-α RNAm reported in muscles filled with intramyocytic triglycerides, in morbidly obese patients.[43] Furthermore, CD68 immunostaining (data not shown) was scarce, and close to capillary vessels, sites not incorporated in Matlab analyzes. Future studies must explain if tissue TNF-α increase is derived from the activation of the signaling cascade involving NFκB, as a consequence of oxidative stress, or from adipose-tissue signals, such as IL-6 and leptin. Whether the higher expression of TNF-α alters muscle protein turnover or induces apoptosis, as in old animals and humans,[34,44] remains to be studied in the future. In the present study, serum concentrations of cytokines TNF-α and IL-6 did not correlate with adiposity, suggesting paracrine signaling, however the exclusion of obese subjects must be borne in mind. Even so, the less specific inflammation marker CRP, although within normal ranges, was significantly higher in WG, and correlated positively with waist circumference.

In conclusion, we demonstrate that healthy nonobese middle-aged men with a self-reported weight increase averaging 7 kg over the last 10 yr, express more TNF-α and accumulate DNA oxidative injury in skeletal muscle, compared with subjects that maintain a stable body weight. Thus, even mild accretion of adipose tissue is associated with oxidative stress and inflammation in human muscle, resembling older people. Further studies must explore the mechanisms involved in these findings and their long-term consequences.

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