Aging, sex differences, and oxidative stress in human respiratory and limb muscles

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Abstract

Oxidative stress is involved in the sarcopenia of aging muscles. On the grounds that ventilatory muscles are permanently active, and their activity may even increase with aging, we hypothesized that the levels of oxidative stress would probably be increased in the external intercostals of elderly healthy individuals. We conducted a case–control study in which reactive carbonyl groups, malondialdehyde–protein adducts, 3-nitrotyrosine immunoreactivity, Mn-superoxide dismutase (Mn-SOD), and catalase were detected using immunoblotting in external intercostals and quadriceps (open muscle biopsies) obtained from 12 healthy elderly and 12 young individuals of both sexes. In elderly subjects, reactive carbonyls, malondialdehyde–protein adducts, 3-nitrotyrosine, Mn-SOD, and catalase were significantly greater in the external intercostals than in the young controls. A post hoc analysis, in which men and women from both groups were analyzed separately, revealed that the external intercostals of elderly women, but not those of elderly men, showed significantly increased levels of reactive carbonyls, malondialdehyde–protein adducts, 3-nitrotyrosine, and Mn-SOD compared to those of control females. This study suggests that differences in muscle activity might explain the differential pattern of oxidative stress observed in human respiratory and limb muscles with aging as well as the likely existence of a sex-related regulation of this phenomenon in these muscles.

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Keywords: Aging; Respiratory and limb muscles; Sex-related differences; Protein oxidation; Protein tyrosine nitration; Antioxidant enzymes; Free radicals

Aging has been defined as a decline in performance and fitness with advancing age. Even in healthy individuals muscles become weaker and less powerful as age progresses, impairing their ability to perform essential physical activities of daily life, while increasing prevalence for both falls and morbidity. Indeed, the deleterious effects of aging are best observed in postmitotic tissues such as skeletal muscles and neurons, in which damaged or lost cells cannot be replaced by the mitosis of intact ones. The overall loss of muscle mass, quality, and strength is generally known as sarcopenia, which includes among others type II fiber atrophy, decreased mitochondrial volume and enzyme content, mitochondrial DNA mutations, and reduced muscle respiratory rate [1–4].

Although the etiology of sarcopenia of aging muscles is still under investigation, several factors have already been implicated, such as the loss of growth hormone and a reduction in both estrogen and androgen production [5], impaired glucose and/or fatty acid metabolism, nitrogen imbalance, decreased muscle protein synthesis, reduced physical activity, and oxidative stress [6–8]. In fact, aging has also been attributed to a greater accumulation of deleterious effects on tissues from reactive oxygen species (ROS) compared to those normally neutralized by intracellular antioxidant defenses [9,10]. It is nowadays commonly accepted that oxidative stress is primarily involved in the etiology of aging, especially in those tissues with high levels of oxygen metabolism such as skeletal muscles [6–8]. For instance, the diaphragm of senescent rodents was shown to increase its oxidative capacity [11], as well as its content of antioxidant enzymes such as superoxide dismutases...
age-dependent loss in sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase reported in aging rodent diaphragms\cite{11,12}. Furthermore, an age-dependent loss in sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase isoform 2a (SERCA2a) activity was also shown as a result of 3-nitrotyrosine accumulation in aging rat limb muscles\cite{13–15}. On the other hand, other studies have focused their attention on the assessment of the implications of oxidative stress in human muscles. In this regard, increased levels of oxidized glutathione, lipid peroxidation, both Mn-SOD and catalase activity, protein carbonylation, and DNA oxidation have been shown in the quadriceps muscles of elderly patients undergoing orthopedic surgery\cite{16–19}. Moreover, Mn-SOD activity was also shown to be higher in the rectus abdominis of elderly patients undergoing abdominal surgery\cite{20}, whereas catalase and glutathione transferase activities were shown to be lower in satellite cells from human quadriceps\cite{21}.

Despite this progress in humans, it has never been established whether the deleterious effects of accumulating ROS are specifically confined to the limb muscles of elderly humans or whether these effects could also be observed in other muscles such as the respiratory muscles, which are essential to life and must remain active throughout the life span of the individual. In addition, both lung and respiratory muscle function and structure have been shown to be altered even in healthy elderly humans, leading to a significant loss in chest wall compliance\cite{22}. Although this is unlikely to be clinically relevant under healthy conditions, it may clearly have a significant impact on morbidity and mortality under stress conditions.

On this basis, we hypothesized that the levels of oxidative stress would probably be increased in the intercostal muscles of physically active, healthy elderly humans. To test this hypothesis, first we aimed at determining the levels of protein carbonylation and nitration in both the external intercostal and the quadriceps muscles of a healthy elderly population as opposed to healthy young individuals. Second, we also investigated whether oxidative stress levels might differ between elderly males and females in both respiratory and limb muscles. Third, we explored the levels of several antioxidant mechanisms in those muscles.

Materials and methods

Subjects

Twelve healthy elderly volunteer individuals (6 male and 6 female, 68 years) and 12 healthy young volunteer control subjects (identical gender distribution, 25 years) were recruited from the general population on an outpatient basis. This sample size was calculated on the basis of previous studies from our group\cite{23,24}. The cut-off age for the elderly individuals of 65 years and over was chosen specifically according to data previously published\cite{16}. All individuals were Caucasian. Exclusion criteria included current or past smoking habit, chronic respiratory failure, bronchial asthma, coronary disease, severe undernourishment (body mass index <20 kg/m\textsuperscript{2}), chronic metabolic diseases, suspected paraneoplastic or myopathic syndrome, and/or treatment with drugs known to alter muscle structure and/or function. This is a case–control study designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines for research on human beings. The Ethics Committee on Human Investigation at Hospital del Mar-IMIM approved all experiments. Informed written consent was obtained from all individuals.

Clinical, nutritional, and functional assessment

Clinical evaluation included medical history, complete physical examination, thorax radiology, and electrocardiogram. Nutritional evaluation included body mass index and analytical parameters. Forced spirometry was performed and static lung volumes were determined in all subjects using standard procedures, and reference values by Roca et al.\cite{25,26} were used. Inspiratory muscle strength was assessed through determination of maximal inspiratory pressure at the mouth (Sibelmed-163; Sibel, Barcelona, Spain) during an occluded maneuver from residual volume. Reference values by Wilson et al.\cite{27} were chosen.

Muscle biopsies

Muscle samples were obtained from the external intercostal and quadriceps (vastus lateralis) as the control muscle by open biopsy after procedures published elsewhere\cite{23,28–30} and were immediately frozen in liquid nitrogen and stored at −80°C for further analysis. All subjects were prevented from doing any potentially exhausting physical exercise 10 to 14 days before coming to the hospital to undergo the surgical procedures.

Biological muscle studies

Total carbonyl groups

Total levels of those highly reactive carbonyl groups in the protein side chains were detected by reaction (derivatization) with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of 2,4-dinitrophenylhydrazone (DNP)\cite{31}. The DNP-derivatized proteins were subsequently separated by electrophoresis and further subjected to immunoblotting with selective antibodies against the DNP moieties.

Aldehyde–protein adducts

The lipid peroxidation product malondialdehyde (MDA) can cause further cellular damage by binding to and modifying proteins, which leads to the formation of aldehyde–protein adducts. The characteristic feature of these adductions is the introduction of carbonyl groups into the modified proteins. MDA reacts with lysine residues to form Schiff base adducts that can also be detected in tissues using a selective antibody\cite{32}.

Immunoblotting

The effects of oxidants on muscle proteins and lipids were evaluated according to methodologies published elsewhere.
Frozen muscle samples from both external intercostals and quadriceps muscles (n = 12/group) were homogenized in a buffer containing Hepes 50 mM, NaCl 150 mM, NaF 100 mM, Na pyrophosphate 10 mM, EDTA 5 mM, Triton X-100 0.5%, leupeptin 2 μg/mL, PMSF 100 μg/mL, aprotonin 2 μg/mL, and pepstatin A 10 μg/mL. Samples were then centrifuged at 1000g for 30 min. The pellet was discarded and the supernatant was designated as a crude homogenate. Total muscle protein level in each sample was spectrophotometrically determined with the Bradford technique using different runs of triplicates in each case and bovine serum albumin (BSA) as the standard (Bio-Rad protein reagent; Bio-Rad, Inc., Hercules, CA, USA). The final protein concentration in each sample was calculated from at least two Bradford measurements that were almost identical. Equal amounts of total protein from crude muscle homogenates were always loaded (20 μg per sample/lane) onto the gels, as well as identical sample volumes/lanes.

Two different sets of experiments were conducted. For the purpose of comparison, external intercostal muscle samples from both elderly and young individuals (24) were always run together in the same gel, whereas quadriceps muscle samples were run in a different set of experiments, but with an identical approach being applied. In the different Western blot analyses, the same samples were always run together and kept in the same order. Proteins were then separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with nonfat milk, and incubated overnight with selective antibodies. The following antibodies were used to detect the different antigens and phenomena: anti-DNP moiety antibody (rabbit anti-DNP antibody, from the Oxyblot kit; Chemicon International, Inc., Temecula, CA, USA; dilution 1/150), anti-MDA antibody (Academy Bio-Medical Co., Inc., Houston, TX, USA; dilution 1/4000), anti-3-nitrotyrosine antibody (Cayman Chemical, Inc., Ann Arbor, MI, USA; dilution 1/1000), anti-Mn-SOD antibody (StressGen, Victoria, BC, Canada; dilution 1/5000), and anti-catalase antibody (Calbiochem, San Diego, CA, USA; dilution 1/2000). Tissue homogenates obtained from rat brain mitochondria and rat erythrocytes were used as positive controls for the enzymes Mn-SOD and catalase, respectively. Specific proteins from all samples were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. For each of the antigens, samples from the different groups were always detected in the same film under identical exposure times. The specificity of carbonyl groups was confirmed by avoiding the derivatization process [31] in some of the samples and by omission of the primary antibody and incubation of the membranes only with secondary antibody (goat anti-rabbit IgG, HRP-conjugated, from the Oxyblot kit; Chemicon International Inc.; dilution 1/300). Controls for specific binding of MDA–protein adducts were performed by omitting the primary antibody and incubating the membranes only with secondary antibody (peroxidase-conjugated AffiniPure rabbit anti-goat IgG (H+L); Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA; dilution 1/6000). Also, native BSA (Roche GmbH, Mannheim, Germany) was loaded onto the gels as a second negative control for MDA specificity. Finally, the following controls were also performed to confirm the specificity of 3-nitrotyrosine immunoreactivity: omission of the primary antibody and incubation of the membranes only with secondary antibody (HRP-conjugated goat anti-mouse immunoglobulin-specific polyclonal antibody; BD Biosciences Pharmingen, San José, CA, USA; dilution 1/4000), chemical reduction of nitrotyrosine to aminotyrosine with 100 mM dithionite (Sigma–Aldrich GmbH, Steinheim, Germany), and native BSA loading onto some gel lanes [34,35]. Blots were scanned with an imaging densitometer, and optical densities (OD) of specific proteins were quantified with Diversity Database 2.1.1 (Bio-Rad, Philadelphia, PA, USA). Values of total protein carbonylation, total MDA–protein adducts, and total protein tyrosine nitration in a given sample were calculated by addition of OD of individual protein bands in each case. Final optical densities obtained in each specific group of subjects corresponded to the mean values of the different samples (lanes) of each of the antigens studied. To validate equal protein loading among various lanes, PVDF membranes were stripped and reprobed with a mouse anti-α-subunit Na,K-ATPase antibody (Johns Hopkins University, Baltimore, MD, USA) in all cases. Optical densities in each histogram were expressed as the ratio of the optical densities of the specific antigen to those of α-subunit Na,K-ATPase.

Statistical analysis

Data are presented as means ± SD. Mann–Whitney nonparametric tests were used for unpaired comparisons between elderly and young individuals. In addition, analysis of variance was used to compare optical densities between elderly and young individuals of both sexes. Relationships between various parameters were studied by calculating the Spearman’s correlation coefficient. A p value of 0.05 or less was considered significant.

Results

Characteristics of the study subjects

Table 1 indicates the main characteristics of both young and elderly individuals. Elderly individuals were significantly older and showed a significant reduction in the ratio of forced expiratory volume in 1 s to forced vital capacity as well as in the ratio of residual volume to total lung capacity. No significant correlations were found between physiological and biological variables.

Reactive carbonyl groups

Total protein carbonylation

As shown in Fig. 1A, anti-DNP antibody detected several protein bands with apparent masses ranging from 118 to 25 kDa in both the intercostal and the quadriceps muscles (left and right, respectively) of elderly and young subjects. Total protein carbonylation levels were significantly greater only in the intercostal muscles of the elderly individuals compared to the
Fig. 1. (A) Representative examples of carbonylated proteins in the external intercostal muscles (left) and vastus lateralis (right) of both elderly and young control subjects. Remaining unmodified in the quadriceps (Fig. 1B). In a post hoc analysis in which males and females were analyzed separately, the levels of total protein carbonylation in the elderly women compared to those of the young control females (Fig. 1C). No carbonyl groups were detected when proteins were not derivatized or when the primary antibody was omitted (data not shown).

MDA–protein adducts

Fig. 2A illustrates various MDA–protein adducts with apparent masses ranging from 71 to 26 kDa in both the intercostal and the quadriceps muscles (left and right, respectively) of elderly and young individuals. Total levels of MDA–protein adducts were significantly higher in both muscles in the elderly group of subjects compared to those of the young control individuals (Fig. 2B). In the post hoc analysis, total MDA–protein adducts were significantly greater in the external intercostals of the senescent women compared to those of the young control females, whereas in the quadriceps muscle, MDA–protein adducts were significantly greater in the elderly males compared to the young control men (Fig. 2C). No MDA–protein adducts were detected when the primary antibody was omitted or when native BSA was loaded (data not shown).

Protein tyrosine nitration

Several tyrosine-nitrated protein bands with apparent masses ranging from 69 to 16 kDa were detected in both the external intercostals and the quadriceps muscles (left and right, respectively) of elderly and young controls (Fig. 3A). Total muscle 3-nitrotyrosine levels were significantly greater in both the external intercostals and the quadriceps of the elderly subjects compared to those detected in the young control group (Fig. 3B). In the post hoc analysis, only in the elderly women compared to the young females was total protein tyrosine nitration significantly greater in both muscles (Fig. 3C). No tyrosine-nitrated proteins were detected when the primary antibody was omitted, or samples were reduced with dithionite, or when BSA was loaded (data not shown).

Antioxidant enzymes

Mn-SOD

As shown in Fig. 4A, Mn-SOD was detected in both the external intercostals and the quadriceps (left and right, respectively) of all subjects. Muscle protein content of this enzyme was significantly higher only in the intercostals of the elderly subjects compared to those of the young controls (Fig. 4B). Levels of this enzyme showed, however, a tendency to be greater in the quadriceps muscles of the elderly group compared to the young controls (Fig. 4B). In the post hoc analysis, muscle Mn-SOD protein was significantly greater in the external intercostals of the elderly females, compared to the young control women (Fig. 4C). In the quadriceps muscles, Mn-SOD content showed only a strong tendency to be higher, which did not reach statistical significance, in the elderly females compared to the young control women (Fig. 4C).

Catalase

Fig. 5A illustrates the presence of the enzyme catalase in both external intercostals and quadriceps (left and right, respectively) of all individuals. Muscle catalase protein content

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Table 1
Main characteristics and functional variables of the study subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Young</th>
<th>Elderly</th>
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<tr>
<td>All</td>
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<tr>
<td>Age, years</td>
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<td>68 ± 5***</td>
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<td>Weight, kg</td>
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<td>70 ± 7.7</td>
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<td>BMI, kg/m²</td>
<td>23.4 ± 3.4</td>
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<tr>
<td>FEV₁, % pred</td>
<td>97 ± 11</td>
<td>93 ± 12</td>
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<tr>
<td>FEV₁/FVC</td>
<td>83 ± 8</td>
<td>74 ± 4**</td>
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<tr>
<td>RV/TLC</td>
<td>24 ± 5</td>
<td>44 ± 5***</td>
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<tr>
<td>MIP, % pred</td>
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<td>110 ± 27</td>
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Males

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<tbody>
<tr>
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</tr>
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<td>Weight, kg</td>
<td>69 ± 13</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
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<tr>
<td>FEV₁, % pred</td>
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</tr>
<tr>
<td>FEV₁/FVC</td>
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<tr>
<td>RV/TLC</td>
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<td>MIP, % pred</td>
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Females

<table>
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<tr>
<td>Weight, kg</td>
<td>61 ± 12</td>
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<tr>
<td>BMI, kg/m²</td>
<td>23.9 ± 2.5</td>
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<tr>
<td>FEV₁, % pred</td>
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<tr>
<td>FEV₁/FVC</td>
<td>83 ± 7</td>
</tr>
<tr>
<td>RV/TLC</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>MIP, % pred</td>
<td>111 ± 19</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. Abbreviations used: BMI, body mass index; FEV₁, forced expiratory volume in 1 s; pred, predicted; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; MIP, maximal inspiratory pressure. Statistical significance of the results is between young and elderly individuals.

*  p ≤ 0.05.
** p ≤ 0.01.
*** p ≤ 0.001.
was significantly higher in both muscles in the elderly group of subjects compared to the young controls (Fig. 5B). In the post hoc analysis, the levels of catalase showed a only tendency to be higher in both intercostals and quadriceps in the elderly men compared to the young control males (Fig. 5C).

**Discussion**

The main findings in this study are that in skeletal muscles of healthy elderly individuals compared to muscles of a healthy young population: (1) total carbonyl group formation, MDA–
protein adducts, and protein tyrosine nitration were significantly greater in the aging external intercostals, whereas only MDA–protein adducts and 3-nitrotyrosine immunoreactivity were significantly higher in the senescent quadriceps; (2) whereas both Mn-SOD and catalase proteins were significantly higher in the aging external intercostals, only catalase, and not Mn-SOD, was greater in the senescent quadriceps; and (3) a post hoc analysis specifically revealed that elderly women showed
significantly greater levels of both total protein carbonylation and nitrilation, as well as Mn-SOD content, in their intercostal muscles, whereas elderly men showed an increase only in MDA–protein adducts in their quadriceps and a strong tendency toward higher catalase levels in their respiratory and limb muscles.

It has been proposed that accumulation of the deleterious effects of ROS throughout the entire life of the individual could be largely responsible for aging [6,8,9]. Indeed, the detrimental effects of aging are best seen in postmitotic cells such as neurons and muscle fibers. ROS-mediated protein damage was said to be involved in the aging process as a result of studies showing that exposure of enzymes from young animals to ROS led to changes in their activity similar to those observed in aging tissues [36,37]. In the light of both the current literature [6–12,16–21] and the results encountered in our study, it is possible to conclude that accumulation of ROS is likely to be involved in the etiology of aging-induced reduced muscle performance. It is also noteworthy that on the basis of both the reported decline in mitochondrial function with aging and the fact that mitochondria are the main oxygen radical source in muscle fibers, the reactive species superoxide anion is likely to be the major player in this process. For instance, it has been well established that in aging, mitochondrial function is characterized by increased electron leakage from the respiratory chain, slowed turnover, and reduced efficiency [38–42]. Also, as the half-life of superoxide anion is very short and it does not cross membranes, its oxidative effects will probably occur within the mitochondria, thus further impairing mitochondrial function.

The present study is the first to provide evidence of the presence of ROS-mediated protein modifications in one of the main respiratory muscles, the external intercostal, in active senescent humans with no comorbidity. It should be emphasized that in this study, the three indirect indices employed to analyze muscle oxidative stress levels, protein carbonylation as measured by the DNPH assay, protein tyrosine nitrilation, and MDA–protein adducts, were clearly increased in this respiratory muscle in the elderly humans compared to muscles from the young controls. It is also worth mentioning that all three oxidative stress indices as well as both Mn-SOD and catalase content were markedly greater in the aging intercostals, whereas this was not exactly the case for the senescent quadriceps of the same individuals. The fact that external intercostals must be permanently active and that certain significant pulmonary function impairment was shown in our healthy elderly individuals might help account for these findings. Actually, respiratory muscle function may be increased in elderly individuals as a result of modifications in respiratory mechanics [43–46]. In line with this, it has been shown that the mechanics of the respiratory system undergo structural and functional changes with aging, which somehow mimic those occurring in pulmonary emphysema, but at a much lower intensity [43–46]. In fact, as lung compliance increases because of reduced lung elasticity, chest wall compliance steadily declines with advancing age [22,45]. Hence, it is plausible that the maintenance of an adequate ventilation in humans is achieved at the expense of an increased activity of the respiratory muscles, even in healthy aging.

In aging quadriceps, however, catalase and both MDA–protein adducts and protein tyrosine nitrilation, but not carbonyl groups from the DNPH assay or Mn-SOD content, were significantly increased in the elderly subjects compared to young controls. Given the fact that the widely used DNPH assay indiscriminately detects total protein carbonyl groups formed by diverse species such as hydroxyl and alklyperoxyl radicals, MDA, and hydroxynonenal [47], and that lipid peroxidation is a common phenomenon of oxidative stress [48,49], we decided specifically to detect whether MDA–protein adducts were increased in aging muscles. Indeed, MDA–protein adducts were found to be greater in both the intercostals and the quadriceps of the elderly human subjects compared with the young controls, suggesting that peroxidation of polyunsaturated membrane lipids is likely to be a continuing event during the aging process, at least in muscles. In line with this, other studies [16–19] have already shown that lipid peroxidation, oxidized glutathione, protein oxidation as measured by the DNPH assay, and DNA oxidation were increased in the vastus lateralis of hospitalized patients undergoing orthopedic surgery (hip replacement, bone lesions) at ages above 65 years [16–20]. As to the activity of Mn-SOD enzyme, this was reported to be increased in senescent quadriceps in some studies [16,17], whereas no significant variations in its activity were reported in a most recent study [20]. Mn-SOD activity was actually increased in the rectus abdominis of patients of the study in question, who underwent abdominal surgery for various reasons [20]. In all of these studies, catalase activity remained unchanged in the senescent muscles analyzed [16,17,20]. In our study, however, catalase was significantly increased in both muscles of the elderly individuals, whereas Mn-SOD content showed only a tendency to be greater in the quadriceps and was significantly increased only in the external intercostals of the same subjects. The facts that hospitalized patients undergoing a surgical procedure (with certain previous immobilization) were...
assessed in the former studies, whereas physically active healthy elderly subjects from the general population were used in ours, and that protein content, and not enzyme activity, was explored in our study might help account for these discrepancies.

Clearly, the content of the mitochondrial isoform Mn-SOD is increased in senescent human muscles probably to counteract the deleterious effects (compensatory mechanism) produced by enhanced levels of superoxide anion in those muscles. Also, in our study this is more evident in the

Fig. 3. (A) Representative examples of protein expression of Mn-SOD in both the external intercostal muscles (left) and the vastus lateralis (right) of both elderly and young control subjects. Monoclonal anti-α-subunit Na,K-ATPase antibody was used to control for equal loading across various lanes. Corresponding positive control (+C, rat brain mitochondria) is indicated. (B) Optical densities in the histograms are expressed as the ratio of the optical density of Mn-SOD to that of α-Na,K-ATPase (mean values ± SD). Mn-SOD protein content was significantly higher (***p < 0.001) in the external intercostals (left) of the elderly subjects compared to those of the young controls. In the quadriceps, however, Mn-SOD levels showed only a tendency to be greater, which did not reach statistical significance (p = 0.1), in the elderly compared to the young controls (right). (C) Optical densities in the histograms are expressed as the ratio of the optical density of Mn-SOD to that of α-Na,K-ATPase (mean values ± SD). In a post hoc analysis, Mn-SOD levels were significantly increased (**p < 0.01) in the external intercostals (left) of the elderly women compared to those in the young control females. In the vastus lateralis (right), Mn-SOD levels showed only a tendency to be greater, which did not reach statistical significance (p = 0.1), in the elderly women compared to the young control females.

assessed in the former studies, whereas physically active healthy elderly subjects from the general population were used in ours, and that protein content, and not enzyme activity, was explored in our study might help account for these discrepancies.

Fig. 4. (A) Representative examples of protein expression of Mn-SOD in both the external intercostal muscles (left) and the vastus lateralis (right) of both elderly and young control subjects. Monoclonal anti-α-subunit Na,K-ATPase antibody was used to control for equal loading across various lanes. Corresponding positive control (+C, rat brain mitochondria) is indicated. (B) Optical densities in the histograms are expressed as the ratio of the optical density of Mn-SOD to that of α-Na,K-ATPase (mean values ± SD). Mn-SOD protein content was significantly higher (***p < 0.001) in the external intercostals (left) of the elderly subjects compared to those of the young controls. In the quadriceps, however, Mn-SOD levels showed only a tendency to be greater, which did not reach statistical significance (p = 0.1), in the elderly compared to the young controls (right). (C) Optical densities in the histograms are expressed as the ratio of the optical density of Mn-SOD to that of α-Na,K-ATPase (mean values ± SD). In a post hoc analysis, Mn-SOD levels were significantly increased (**p < 0.01) in the external intercostals (left) of the elderly women compared to those in the young control females. In the vastus lateralis (right), Mn-SOD levels showed only a tendency to be greater, which did not reach statistical significance (p = 0.1), in the elderly women compared to the young control females.
external intercostal muscle, where its increased activity due to modifications of the respiratory mechanics with age [22,43–46] led to greater levels of oxidative stress than those observed in the quadriceps muscles of the same individuals. Furthermore, this finding also reinforces the concept that superoxide anion is probably the main oxygen species involved in aging-induced reduced muscle performance. Finally, catalase content was increased in both the respiratory and the limb muscles of the elderly humans in the present study, indicating that the hydrogen peroxide formed by the action of Mn-SOD will be scavenged by catalase, a ubiquitous enzyme that catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen.

Our study is also the first to provide evidence of increased levels of tyrosine protein nitration in both respiratory and limb muscles of healthy elderly humans as opposed to muscles from young control individuals. Viner et al. [13,15] already demonstrated that, in senescent rat muscles, 3-nitrotyrosine selectively accumulated on the SERCA2a, probably through peroxynitrite formation, even in the presence of an excess of SERCA1 isoform [14]. Collectively, it can be concluded that in vivo nitration of senescent muscles might be mediated by induced activity of nitric oxide synthases (NOS) such as the constitutive neuronal NOS, whose levels were shown to be increased in aging muscles [50]. It has been clearly established that nitric oxide reacts extremely fast with superoxide anion (the
reaction is six times faster than the scavenging of superoxide by superoxide dismutases) to form peroxynitrite [51], further leading to protein tyrosine nitration in tissues. Future studies to explore the nature and function of the different tyrosine-modified proteins in human senescent muscles are clearly required.

Another remarkable finding in our study is that total carbonyl groups as measured by the DNP assay, MDA–protein adducts, protein tyrosine nitration, and Mn-SOD levels of the external intercostals on the one hand, and 3-nitrotyrosine immunoreactivity of the quadriceps on the other, were significantly modified only in the elderly postmenopausal women and not in the elderly men. This might imply the presence of a sex-related mechanism in the redox regulation of these aging muscles. Indeed, there is evidence that estrogens have strong protective antioxidant properties against oxidative stress in muscles and other tissues [52–55]. This suggests that muscles from young females, as opposed to males of a similar age, are strongly protected against ROS-mediated deleterious effects. Actually, the fact that in the external intercostals of the young males as opposed to the young females, protein carbonylation was significantly greater and that MDA–protein adducts showed a tendency to be higher, strongly supports this hypothesis. In fact, although levels of these three indices of oxidative stress were indeed increased in the intercostals of the elderly males, they did not reach statistical significance compared to those of the young males, because levels in the muscles of the latter were already relatively higher as opposed to those of the young females. Furthermore, probably as a protective compensatory mechanism, the content of Mn-SOD was also increased in the intercostal muscles and showed a tendency to be higher in the quadriceps of the postmenopausal females compared to that observed in the young controls. Clearly, future studies are required to fully elucidate the nature of the sex-related differences regarding oxidative stress development in human senescent muscles.

Eventually, sarcopenia of old age results in muscle weakness that may predispose elderly individuals to a higher risk of falls and loss of autonomy as well as to increased morbidity. Indeed, Powers et al. [56] already demonstrated that diaphragm force generation was reduced with aging in rats. In another study, maximum transdiaphragmatic pressure was considerably lower in elderly individuals compared to that in the young controls [57], possibly contributing to further respiratory muscle failure of senescent patients with severe lung disease. In line with this, it has recently been demonstrated that aging has additive effects on protein oxidation and diaphragm force reserve impairment of senescent animals mechanically ventilated [58]. This is of vital importance because the difficulty of weaning elderly patients from mechanical ventilation represents a major clinical defeat in intensive care units [59,60]. Moreover, aging was shown to be an independent predictor of weaning complications of patients after cardiac surgery [61]. More recently, aging was also shown to be an important incidence predictor of the development of the highly prevalent chronic obstructive pulmonary disease (COPD) [62], the prevalence of which has also been shown to progressively increase in the female sex. In conclusion, alterations of the respiratory mechanics described in aging are unlikely to be clinically relevant under healthy conditions; however, they may clearly have a significant impact on morbidity and mortality under stress conditions. This is important, because elderly humans have also been shown to be more prone to suffer from respiratory illnesses or to take medication that might also influence ROS production in muscles [45].

Study critique

The main limitation of our study has to do with the relatively small sample size used in contrast to previous reports [16–20], in which no clear inclusion criteria were provided to recruit the subjects. Actually, from an ethical point of view, the utilization of a population size larger than that required for the obtaining of significant results is not usually recommended. Despite the relatively small number of subjects studied, we found consistent significant results in terms of protein expression of the different indices analyzed. Furthermore, in our study, extremely restrictive inclusion and exclusion criteria were employed in order to carefully select healthy elderly and young subjects from the general population. In addition, the volunteer subjects used in the study were physically active and had not been hospitalized for any reason in the months previous to study entry, as opposed to former studies, in which subjects underwent orthopedic surgery for different bone lesions, implying a certain degree of immobilization both before and during surgery [16–20]. This is important, because immobilization might have influenced oxidative stress levels in those muscles, as has been shown in other studies [63–65], despite the fact that biopsies from the young controls were also obtained under identical conditions.

In the current study, another remarkable difference from previous reports has been the assessment of the aging effects on human respiratory muscles and not only on the limb muscles. Because this has involved the use of relatively “invasive” procedures, especially that of the external intercostals, we were compelled to use a relatively reduced number of subjects in this study, who were sampled on an outpatient basis in order to ensure their maximum comfort. Open thoracotomy is the gold standard technique to obtain biopsies from the diaphragm, the main inspiratory muscle. However, this surgical practice can be applied to subjects only in the presence of serious illnesses such as lung neoplasms. Therefore, normal healthy individuals cannot be exposed to such an invasive procedure. Furthermore, under certain conditions, the external intercostals or other rib cage muscles have been shown to be progressively recruited [66–68], making them an excellent target for study. Finally, clear accessibility of the external intercostals by means of an open biopsy on an outpatient basis as well as the fact that this model enabled us to carefully select the subjects, excluding any comorbid condition, clearly justified the design of the current study.

Conclusions

Our study is the first to provide evidence of increased ROS-mediated effects on muscle proteins in the external intercostals of healthy elderly humans, which may have additional effects
on other conditions such as mechanical ventilation and COPD. Furthermore, it also suggests the likely existence of a sex-related regulation of the oxidative stress levels in limb muscles and especially in the respiratory muscles. These findings might eventually offer a target for therapeutic intervention in the aging process.

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