Human muscle aging: ROS-mediated alterations in rectus abdominis and vastus lateralis muscles

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Abstract
Aging is related to the accumulation of reactive oxygen species (ROS)-mediated oxidative damage. Considering the heterogeneity of age-related changes and the involvement of muscles in different functions, we compared the aging process in different functional muscles. We studied age-related changes in rectus abdominis (RA) and vastus lateralis (VL) in subjects of different age (18–48- and 66–90-year-old). We analysed fiber distribution, antioxidant enzymatic systems: Mn and CuZn superoxide dismutase (MnSOD, CuZnSOD), glutathione peroxidase (GSHPx), catalase (CAT), as well as oxidative damage markers: lipoperoxide levels (LPO), carbonylated proteins (CP), reduced and oxidized glutathione (GSH, GSSG) content and the GSH/GSSG ratio. In the muscles analysed, type I fiber increases during aging with a consequent decrease in type II distribution.

In the elderly group RA MnSOD showed higher activity than VL. Furthermore, in RA MnSOD was higher in the elder group than in the younger group. CuZnSOD, as well as GSHPx and CAT activities remained unchanged. LPO levels in VL increase with age; moreover, in the elderly group VL showed higher value than RA. CP, GSH and GSSG remained unchanged, while GSH/GSSG decreases in RA during aging.

In conclusion, a relationship between aging and ROS seems to exist, but oxidative processes could evolve in different ways in muscles with different functions.

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Keywords: Rectus abdominis; Vastus lateralis; Aging; Oxidative stress; Antioxidant enzymes; Lipid peroxidation; Carbonylated protein content; Glutathione levels

1. Introduction
According to the mitochondrial theory of aging, it is widely accepted that aging is related to the accumulation of reactive oxygen species (ROS)-mediated oxidative damage and to a functional decline in mitochondria. Moreover, transgenic approaches with genes such as superoxide dismutase and catalase, involved in detoxifying superoxide and hydrogen peroxide in mitochondria and cytosol, and pharmacological approaches using mimetics of these antioxidant enzymes have shown that the endogenous production of ROS by normal physiological processes is a major limit to lifespan (Melov et al., 2000). Skeletal muscles are unique in their requirement and ability to undertake very rapid changes in energy supply and oxygen flux during contraction and it has been suggested that this makes the tissue particularly prone to ROS-mediated damage as a result of an increase in electron flux and corresponding leakage from the mitochondrial respiratory chain. In fact, the aging process is mediated by a vicious cycle of events, ultimately leading to cellular senescence. Central to this vicious cycle is an increase in oxidative stress, mediated by an increased production of ROS, and/or a reduced antioxidant capacity. The electron transport chain (ETC) is thought to be the greatest producer of ROS in skeletal muscle and it was demonstrated that mitochondrial production of ROS is maintained at high level inside the mitochondrial matrix (Lee andWie, 1997). The proximity of mitochondrial DNA to the primary source of ROS could incur oxidative insults and lead to mitochondrial DNA damage, resulting in defective ETC proteins, reduced ETC activity and enhanced production of ROS. The aging process also results in a decrease in the physiological capacity of neuromuscular systems. One effect of
this is the gradual loss of the muscle functional capacity that occurs during aging (Hyatt et al., 1990).

The reduced functionality typical of aged subjects, often associated with pain and disability, has been attributed to a number of changes taking place in the aging process, such as the gradual decrease in cross-sectional area, fiber denervation and fiber number loss, mainly of type II fibers (Coggan et al., 1990; Jakobsson et al., 1990; Lexell, 1995) while type I fibers increase with aging (Gollnick et al., 1972; Larsson and Karlsson, 1978, Lexell et al., 1988).

When any body changes occur, it should be considered whether neuromuscular responses are the natural outcome of aging or simply the reflection of an age-related decline in physical activity. In a review of muscle fibre type transitions Pette and Staron (1997) suggest that age-related changes may be muscle specific. Regarding a possible heterogeneity of age-related changes in muscle and the involvement of different muscles in different functions, it seems appropriate to compare the aging process in separate functional muscles (Dutta and Hadley, 1995). In fact, an important question is whether the aging process affects all human muscles equally. The aim of the present study was therefore to compare ROS-mediated alterations during aging in two types of human muscles: rectus abdominis (RA) and vastus lateralis (VL). We chose these muscles for their different embryological origin (RA: pubic crest and pubic symphysis; VL: upper intertrochanteric line, base of greater trochanter, lateral line as aspera, lateral supracondylyar ridge and lateral intermuscular septum), different nerve supplies (RA: anterior primary rami (T7-12); VL: posterior division of femoral nerve (L3,4)) and different functions (RA: flexes the trunk, aids forced expiration and raises intra-abdominal pressure; VL: extends the knee). RA is a typical posture muscle, while VL is a typical movement muscle.

2. Experimental procedures

Chemicals—unless otherwise specified, all chemicals and reagents used were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were of analytical grade or the highest grade available.

Subjects and muscle biopsy—the study was approved by the Ethics Committee of the University of Chieti and conformed to the standards set by the Declaration of Helsinki (last modified in 2000). After subjects had been fully informed of the aim of the experiments and of the risks involved in the biopic procedure, written informed consent was obtained.

Forty-one subjects were enrolled in the study and divided into two groups according to the age of donors: 18–48 (RA: 8 = 8, 3 women and 5 men; VL: n = 5, 3 women and 2 men) and 66–90-year-old (RA: n = 12, 8 women and 4 men; VL: n = 16, 11 women and 5 men).

Subjects taking part in the study had undergone surgery for various reasons (simple bone lesions, lipomas, varicose veins, crural and internal hernias, ovariectomies for cysts, hysterecctomy, cholecystectomy) under general anaesthesia. The biopsies were quickly immersed in liquid nitrogen and stored in its vapour to avoid disidratation until their analysis.

Hospitalised patients were subjected to routine haematological and urinary tests.

Neuromuscular or other chronic diseases, which are known to lead to changes in muscle, and diseases that considerably impair motor activity, were excluded.

Fiber identification—in order to distinguish type I and II fibers we used a histochemical analysis of myosin-ATPase.

To obtain a representative picture of the histochemical profile of the muscle studied, the central portion of the biopsy was chosen for examination; serial transverse sections of the muscles were cut at 10 μm using microtome at −20 °C. The distribution of type I and II fibers was determined from sections stained for myosin-ATPase activity at pH 4.37 and 10.3 (Brooke and Kaiser, 1970). The stained sections were magnified (×120) with a microscope and photographed; an area with 100–200 fibers was classified and counted.

Preparation of tissue samples—the muscle biopsies were divided into four samples. The first sample was diluted 20% (w/v) in buffer Tris–HCl 20 mM, pH 7.4 and homogenized twice: first with a Polytron and the second time with a Potter Braun homogenizer. The homogenate obtained was used for lipids peroxidation levels (LPO) determination.

The second sample was homogenized as previously described, in 0.32 M sucrose and 1 mM ethylenediaminetetra-acetate (EDTA) buffer. The homogenate was centrifuged at 800×g for 15 min and the supernatant obtained was centrifuged at 5000×g for 10 min at 4 °C (Beckman J-21C Centrifuge). The supernatant was centrifuged at 14000×g for 20 min to obtain the mitochondrial pellet that was resuspended in sucrose–EDTA medium and utilized to evaluate superoxide dismutase Mn-dependent activity (MnSOD). The cytosol obtained after mitochondrial fraction was used to determine superoxide dismutase CuZn-dependent (CuZnSOD), catalase (CAT) and glutathione peroxidase (GSHPx).

The third sample was used to assay protein carbonyl content; the muscle sample was homogenized in 5 mM phosphate buffer (pH 7.5) with protease inhibitors (leupeptin 0.5 μg/ml, aprotinin 0.5 μg/ml, pepstatin 0.7 μg/ml) and 0.1% Triton X-100. The homogenate was centrifuged at 700×g for 20 min and biochemical analysis was performed on the resulting supernatant.

Finally, the fourth sample used for the determination of glutathione levels was weighed, homogenized in 5% sulfosalicylic acid (SSA) 10% w/v, and centrifuged at 9500×g for 5 min, and the GSH and GSSG contents of the supernatants were measured. All the procedures described were performed on ice.

Biochemical determination—mitochondrial and cytosolic SOD activities were measured by monitoring at 550 nm the rate of cytochrome c reduction by superoxide radicals produced by a xanthine/xanthine oxidase system. In particular, in order to avoid the contamination by cytosolic SOD, we determined MnSOD activity in the presence of 1 mM KCN (Flöhè and Otting, 1984).

GSHPx activity was detected spectrophotometrically by measuring at 340 nm NADPH oxidation in the presence of
GSH and hydrogen peroxide (H$_2$O$_2$) (Flohe and Gunzler, 1984).

Catalase activity was evaluated by measuring at 240 nm H$_2$O$_2$ decay (Aebi, 1984).

Malondialdehyde and 4-hydroxy-2-nonenal were evaluated colorimetrically at 586 nm according to Erdelmeier and co-workers method (1998).

Protein carbonyl content was detected using the 2,4-dinitrophenyl hydrazine (DNPH) procedure (Levine et al., 1990).

The difference between the DNPH-treated and the HCl-treated (blank) samples was determined at 366 nm and the results were expressed as nmol carbonyl groups/mg of protein.

Sample protein concentration was evaluated by using bovine albumin as a standard (Lowry et al., 1951). All the biochemical determination described were performed in duplicate.

Statistical analysis—statistical analysis was carried out with the one-way (ANOVA) analysis of variance and Fisher’s test for multiple comparison. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Fiber distribution

RA and VL showed a significant increase in type I fibers % during aging (RA: 66–90 vs 18–48, $P<0.02$; VL: 66–90 vs 18–48, $P<0.01$) and a consequent decrease in type II fibers % evident both in the RA and VL of elderly subjects as opposed to their younger counterpart (RA: 66–90 vs 18–48, $P<0.01$; VL: 66–90 vs 18–48, $P<0.01$) (Table 1).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Type I fiber %</th>
<th>Type II fiber %</th>
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<tr>
<td>Rectus abdominis</td>
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<tr>
<td>18–48-year-old</td>
<td>43.587 ± 3.263</td>
<td>57.662 ± 3.754</td>
</tr>
<tr>
<td>66–90-year-old</td>
<td>59.142 ± 3.837*</td>
<td>40.858 ± 3.837**</td>
</tr>
<tr>
<td>Vastus lateralis</td>
<td>39.300 ± 9.733</td>
<td>60.700 ± 9.733</td>
</tr>
<tr>
<td>18–48-year-old</td>
<td>57.800 ± 3.936***</td>
<td>42.263 ± 3.937†</td>
</tr>
<tr>
<td>66–90-year-old</td>
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</table>

Values are expressed as mean ± SEM. *$P<0.02$ fiber I % in rectus abdominis of subjects aged 66–90 vs fiber I % in rectus abdominis of subjects aged 18–48. **$P<0.01$ fiber II % in rectus abdominis of subjects aged 66–90 vs fiber II % in rectus abdominis of subjects aged 18–48. ***$P<0.01$ fiber I % in vastus lateralis of subjects aged 66–90 vs fiber I % in vastus lateralis of subjects aged 18–48. †$P<0.01$ fiber II % in vastus lateralis of subjects aged 66–90 vs fiber II % in vastus lateralis of subjects aged 18–48.

3.2. MnSOD and CuZnSOD activities

MnSOD increased in RA in the aged group as opposed to the younger one (66–90 vs 18–48, $P<0.006$). Furthermore, in the elderly group the increase in MnSOD was more evident in RA than in VL (RA 66–90 vs VL 66–90, $P<0.01$) (Fig. 1a).

On the other hand, in RA and VL CuZnSOD activity was not dissimilar, considering both age and the different muscles (Fig. 1b).

3.3. GSHPx and CAT activities

GSHPx and CAT activities did not present changes (Table 2).

3.4. LPO levels

LPO levels, represented in Fig. 2, reported a significant increase during aging in VL (VL: 66–90 vs 18–48, $P<0.01$); in the elderly group this muscle also showed higher LPO levels than its RA counterpart (VL 66–90 vs RA 66–90, $P<0.05$).

Fig. 1. Aging and muscle differences in MnSOD (a) and CuZnSOD (b) activities in rectus abdominis and vastus lateralis in two experimental groups aged 18–48 and 66–90. Values are expressed as mean ± SEM. *$P<0.006$ rectus abdominis of 66–90- vs rectus abdominis of 18–48-year-old subjects. †$P<0.010$ rectus abdominis of 66–90- vs vastus lateralis of 66–90-year-old subjects.
3.5. CP content

As shown in the graph (Fig. 3), the protein carbonyl content did not differ in the two types of muscles of the two experimental groups.

3.6. GSH, GSSG levels and GSH/GSSG

Levels of GSH and GSSG did not change in the experimental groups. However, the GSH/GSSG ratio in RA decreased with age (RA: 66–90 vs 18–48 \( P \neq 0.01 \)) (Table 3).

4. Discussion

Aging is responsible for a number of physiological modifications in the human body that include a decrease in aerobic and functional capacities. Human skeletal muscle is a postmitotic tissue with a remarkable ability to change its mass in response to variations in the level of physical activity. Furthermore, muscle cells are characterised by a high mitochondrial activity capacity in response to variations in energy demands. The mitochondrial theory of aging focuses its attention on the central role of mitochondria as a source of ROS. Skeletal muscle cells also possess an elaborate antioxidative defence system capable of converting ROS into more stable species. The aging process has a marked impact on human skeletal muscle with a consequent impairment of the contractile function.

Reduction in muscle strength results in a greater tendency to fall, impaired mobility and disability in elderly adults (Schultz, 1995). This age-related loss of muscle strength can be attributed mainly to the loss of muscle mass, a phenomenon known as senile sarcopenia (Rogers and Evans, 1993). Our results on fiber type composition showed an age-related reduction in type II fiber % and a consequent increase in type I %. These data are in accordance with the studies comparing groups of young and old subjects, where a greater amount of type I fiber was found in the aged muscle (Larson et al., 1978; Larson, 1983; Jakobsson et al., 1988), but our results seem to indicate that the different use and function of RA and VL do not influence age-dependent changes in fiber composition.

Furthermore, the literature shows some studies describing the effects of aging on histomorphometric (Kirkeby and Garbarsch, 2000) or functional (Monemi et al., 1999; Sciote and Morris, 2000) and biochemical (Carmeli et al., 2002) changes in different human muscles. However, comparisons of biochemical changes during aging are limited to enzyme activities and energy reserve system (Muller-Hocker, 1990; Houmard et al., 1998; Pastoris et al., 2000), while no comparison on oxidative stress-related changes in different human muscles is made.

Age-dependent SOD activity changes in skeletal muscle are a matter of debate: some experimental studies show an age-dependent increase in SOD activity (Lammi-Keefe and Swan, 2000) or functional (Monemi et al., 1999; Sciote and Morris, 2000) and biochemical (Carmeli et al., 2002) changes in different human muscles. However, comparisons of biochemical changes during aging are limited to enzyme activities and energy reserve system (Muller-Hocker, 1990; Houmard et al., 1998; Pastoris et al., 2000), while no comparison on oxidative stress-related changes in different human muscles is made.

Age-dependent SOD activity changes in skeletal muscle are a matter of debate: some experimental studies show an age-dependent increase in SOD activity (Lammi-Keefe and Swan, 1984); other researchers report no changes in CuZnSOD activity (Gianni et al., 2004), while finding an increase in MnSOD activity in different rat tissues (myocardium, skeletal tissue and liver) (Ji et al., 1990; Judge et al., 2005).

Our previous data indicated that in human muscle the activity of MnSOD increases significantly during aging.

### Table 2

<table>
<thead>
<tr>
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<th>GSHPx (mU/mg of proteins)</th>
<th>CAT (nmol/min/mg of proteins)</th>
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<tbody>
<tr>
<td>Rectus abdominis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–48-year-old</td>
<td>131.85 ± 16.14</td>
<td>60.45 ± 6.98</td>
</tr>
<tr>
<td>Rectus abdominis 66–90-year-old</td>
<td>151.21 ± 21.77</td>
<td>69.69 ± 12.96</td>
</tr>
<tr>
<td>Vastus lateralis</td>
<td></td>
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</tr>
<tr>
<td>18–48-year-old</td>
<td>137.40 ± 43.95</td>
<td>52.12 ± 12.87</td>
</tr>
<tr>
<td>Vastus lateralis 66–90-year-old</td>
<td>196.42 ± 33.18</td>
<td>54.17 ± 5.93</td>
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</table>

Values are expressed as mean±SEM.

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Fig. 2. Aging and muscle differences in LPO levels in rectus abdominis and vastus lateralis in two experimental groups aged 18–48 and 66–90. Values are expressed as mean±SEM. *\( P < 0.01 \) vastus lateralis of 66–90- vs vastus lateralis of 18–48-year-old subjects. \( ^{*}P < 0.05 \) vastus lateralis of 66–90- vs rectus abdominis of 66–90-year-old subjects.

Fig. 3. Aging and muscle differences in CP content in rectus abdominis and vastus lateralis in two experimental groups aged 18–48 and 66–90. Values are expressed as mean±SEM.
Values are expressed as mean±SEM. *P<0.01 rectus abdominis of subjects aged 66–90 vs rectus abdominis of subjects aged 18–48.

<table>
<thead>
<tr>
<th></th>
<th>Rectus abdominis 18–48-year-old</th>
<th>Rectus abdominis 66–90-year-old</th>
<th>Vastus lateralis 18–48-year-old</th>
<th>Vastus lateralis 66–90-year-old</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (nmol/g tissue)</td>
<td>861.34±102.06</td>
<td>761.58±152.10</td>
<td>600.83±127.24</td>
<td>790.73±164.73</td>
</tr>
<tr>
<td>GSSG (nmol/g tissue)</td>
<td>74.52±13.10</td>
<td>174.98±51.21</td>
<td>43.18±11.18</td>
<td>222.54±105.05</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>22.96±6.38</td>
<td>7.64±2.56*</td>
<td>23.07±2.34</td>
<td>18.33±4.62</td>
</tr>
</tbody>
</table>

(Pansarasa et al., 1999), particularly in females (Pansarasa et al., 1999). Furthermore, these data indicated that the activity of total SOD is lower in the old than in the young, suggesting a possible decrease in CuZnSOD with age. The total SOD comprises cytosolic CuZnSOD and mitochondrial MnSOD, together, these results suggest that aging is related to superoxide associated mitochondrial stress. Given the short half-life of superoxide, and its inability to freely cross membranes (Lynch and Fridovich, 1978), any stress from the mitochondrial ETC will occur only in mitochondria.

On the other hand, our results indicate that in RA and VL there is no change in CuZnSOD activity, also confirming that there may be a mitochondrial compartmentalization of oxidant stress in the aging of two muscles with completely different functions.

As regards the enzymes of glutathione system, there are few data on human skeletal muscles, since almost all of the studies performed were carried out on rat skeletal muscles. The majority of reports on cytosolic GSHPx activity point to an increase in its activity. On the other hand, mitochondrial GSHPx activity decreased slightly with age, but the pattern is quite complex (Ji et al., 1990). Moreover, in rat skeletal muscles CAT activity did not change in adult rats but increased in the old ones (Ji et al., 1990).

The results we obtained for GSHPx and CAT activities agree with the data reported by our lab (Pansarasa et al., 1999) and are consistent with those reported by Ji and co-workers (1992).

Studies on lipid peroxidation, responsible for age-dependent reduction in membrane fluidity, have shown that lipid peroxidation (unstimulated and iron-stimulated) strongly decreases in the skeletal muscle of aged NMR-I mice (Salminen et al., 1988) whereas studies on human muscle LPO (Mecocci et al., 1999; Pansarasa et al., 1999; Fano et al., 2001) demonstrate that there is an age-dependent increase in MDA content. In previous works we pointed out that the age-related increase in LPO levels is influenced by sex (Pansarasa et al., 2000) and by fiber composition (Pansarasa et al., 2002). The data obtained in the present research confirm an increase in lipid peroxidation. However, we observed an increase in LPO only in VL.

Fiber I distribution is very similar in RA and VL, thus suggesting that the different use of these muscles is the cause of major lipid peroxidation.

Protein carbonyl content, a marker of protein oxidation, did not change during aging. However, a trend towards increasing amounts was observed among the youngest and the eldest groups for both muscles in accordance with Mecocci and co-workers (1999). Protein oxidation by hydroxyl radicals and reactive nitrogen species (RNS) were measured in several tissues of aging rats. Neither o-tyrosine nor 3-nitrotyrosine increased significantly with age in the tissues measured, suggesting that ROS and RNS do not accumulate with the advancing of age (Leeuwenburgh et al., 1998).

The difficulty in showing significant changes in CP in chronic physiological process such as aging could be linked to the fact that myofibrillar carbonyl content was shown to have significantly increased 3 h after acute oxidative stress, but was normalized within 6 h (Nagasawa et al., 1997). This rapid removal of oxidized myofibrillar proteins is unexpected in a tissue with a relatively slow protein turnover; thus, during aging, the efficient removal of oxidized proteins by proteolysis allows the maintenance of very slow changes in a protein oxidative marker.

The concentration and the ratio of GSH vs GSSG can undergo dynamic changes under various physiological and pathological conditions and are often documented as sensitive measures of tissue oxidative stress (Sies et al., 1980; Adam et al., 1983; Halliwell and Gutteridge, 1985; Denek and Fanburg, 1989). Some tissues, such as eye lens and liver, have the highest GSH concentration (~10 mM), whereas skeletal muscle as a whole has one of the lowest GSH amounts (~1 mM). However, skeletal muscle is highly heterogeneous in a variety of metabolic properties, such as oxidative potential, patterns of fuel utilizations and antioxidant enzyme activity (Laughlin et al., 1990). The muscle levels of GSH and GSSG, as well as GSH/GSSG, are therefore expected to reflect these metabolic characteristics. Moreover, muscle fibers with oxidative capacity (e.g. mitochondrial enzyme activity) also have much higher levels of GSH and total glutathione content than those with a lower oxidative potential (Ji et al., 1992).

On the basis of an increase in type I fibre in both muscles, our study pointed out that GSH and GSSG levels remained unchanged, thus confirming that during aging there are no alterations of membrane glutathione transport and that there is a fiber-specific adaptation of GSH system in skeletal muscle (Leeuwenburgh et al., 1994). The GSH/GSSG ratio decreases significantly only in the RA of the elderly group. Previously, Bejma and Ji (1999) showed that in old rats the GSH/GSSG ratio slightly increases in deep VL. These data are not in contrast with our results; in fact, in human VL the GSH/GSSG ratio did not change, showing that, independently of fiber type distribution, the function of these muscles could be more relevant to biochemical adaptation. It is actually difficult to explain why in RA the oxidative signs are different than in VL.

The increase in MnSOD is typical in the muscle, plasma or
erythrocytes of training subjects (Marzatico et al., 1997; Ortenblad et al., 1997; Brites et al., 1999), while RA is a typical tonic postural muscle; the increase in MnSOD could therefore rather represent an adaptation to age-dependent alterations of other antioxidant systems, such as the decrease in GSH/GSSG.

In conclusion, our data are the first evidence that oxidative stress in human muscles could evolve in different ways and that muscle function seems to play a specific role in aging-related changes in different muscles.

References