Physical Activity Modulates Heat Shock Protein-72 Expression and Limits Oxidative Damage Accumulation in a Healthy Elderly Population Aged 60–90 Years

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Background. Reactive oxygen species production increases during aging, whereas protective mechanisms such as heat shock proteins (HSPs) or antioxidant capacity are depressed. Physical activity has been hypothesized to provide protection against oxidative damage during aging, but results remain controversial. This study aimed to investigate the effect of different levels of physical activity during aging on Hsp72 expression and systemic oxidative stress at rest and in response to maximal exercise.

Methods. Plasma antioxidant capacity (Trolox equivalent antioxidant capacity, TEAC), thiobarbituric acid-reactive species (TBARS), advanced oxidized proteins products (AOPP), and Hsp72 expression in leukocytes were measured before and after maximal exercise testing in 32 elderly persons (aged 73.2 years), who were assigned to two different groups depending on their level of physical activity during the past 12 months (OLow = moderate to low level; OHigh = higher level).

Results. The OHigh group showed higher aerobic fitness and TEAC (both representing 120% of OLow values) as well as lower oxidative damage (50% of OLow values) and Hsp72 expression. Exercise led to a lower increase in oxidative damage in the OHigh group. Aerobic fitness was positively correlated with TEAC and negatively with lipid peroxidation (TBARS). Hsp72 expression was negatively correlated with TEAC but positively correlated with TBARS levels.

Conclusions. The key finding of this study is that, in people aged 60 to 90 years, long-term high level of physical activity preserved antioxidant capacity and limited oxidative damage accumulation. It also downregulated Hsp72 expression, an adaptation potentially resulting from lower levels of oxidative damage.

The “free radical theory of aging” proposes that the accumulation of oxidative damage observed in elderly people (1,2) is a key component of the aging process (3). Although an increased reactive oxygen species (ROS) production is a strong stimulus for antioxidant enzymes (4), insufficient adaptation of this protective system in response to increased production of ROS during aging leads to accumulation of oxidative damage (1,5). Heat shock proteins (HSP), by preventing the accumulation of oxidized proteins, represent an alternative protective system (6). However, the induction of those proteins is also altered during aging. Indeed, we and others have recently shown a lower Hsp72 expression in leukocytes from elderly compared to young adults (7,8). Aging is then characterized by lower Hsp72 expression despite oxidant/antioxidant imbalance, both leading to the accumulation of oxidative damage that could be involved in most pathologies associated with aging or in the aging process itself (3,6).

Among the different strategies developed to counteract those deleterious alterations during aging, physical exercise has been suggested to prevent or decrease oxidative damage accumulation. In young adults, several weeks of endurance training increased the activity of antioxidant enzymes and Hsp72 expression in muscle, and reduced the levels of oxidative damage (9,10). However, in old people, results regarding the effect of physical exercise on oxidant/antioxidant balance or HSP expression still remain controversial. Small or no modification of the oxidant/antioxidant system or Hsp72 expression were reported after 6–16 weeks of aerobic (11,12) or resistance training (13–15) in elderly people. Slightly more pronounced adaptations were observed after 6 months of resistance training (16). Taken altogether, these results suggest that exercise-induced changes in oxidant/antioxidant balance or HSP expression could be sensitive to the duration of the exercise program and that long-term physical training may be necessary to generate significant adaptations.

We hypothesized that elderly people spontaneously committed to long-term (at least 1 year) regular physical activity, including sporting activity, should present significant adaptations in terms of oxidant/antioxidant status and HSP expression. We tested this hypothesis by investigating Hsp72 expression and oxidant/antioxidant status in selected elderly people characterized by different levels of physical activity.

Methods

Participants

Independently living elderly people were recruited from local associations that offer different activities for elderly people. Thirty-two 60- to 90-year-old elderly people (73.2 ± 7.4 years, men = 16, women = 16) were assigned to...
two different groups according to their level of physical activity. The level of physical activity was evaluated using the Modified Baecke Questionnaire for Older Adults (17). Sixteen participants (men = 8, women = 8) who were highly physically active (as defined by the following: score of physical activity = 20.7 ± 7.4 and who have been regularly engaged in sporting activities [at least 4 h/wk] during the past 5 years) were included in the old highly active group (OHigh). The participants included in the OHigh group performed at least two of the following sporting activities: bush walking/hiking, brisk walking, weight training, cycling, jogging, tennis, gymnastics, swimming. On average, they exercised 10 h/wk at least 9 mo/y. The other 16 participants (men = 8, women = 8) who had only a medium to low level of physical activity (score = 13.6 ± 3.9) and who were not engaged in any sporting activity constituted the old low active group (OLow). The two groups were paired for age, height, and weight, and all participants were under a Mediterranean-style diet (18). A complete medical history and physical examination including resting 12-lead electrocardiogram (ECG) and pulmonary function test were performed by a neurologist. Exclusion criteria were abnormal ECG, cardiovascular or metabolic disease, pulmonary disease, cancer and/or autoimmune disease, joint disease, neurological disease or cognitive impairment, and other pathologies that contraindicate maximal incremental testing (19). None of the participants took any medication or vitamin/mineral supplements, or had a body mass index > 27. The study design and purpose were fully explained to the participants, who all provided written consent to participate. The protocol was approved by the local ethics committee.

Study Design

During their first visit, the participants underwent a treadmill (LE-200 CE; Jaeger, Hoechberg, Germany) familiarization to determine their preferred walking speed (PWS) (20). At least 48 hours later, they went back to the laboratory in the morning, 2 hours after a standardized breakfast (same composition for every participant), to perform a maximal incremental test (GXT) on the treadmill at their PWS as previously described (20). All the participants were asked not to perform any type of physical activity at least 24 hours before the second visit to the laboratory. The GXT began with 2 sets of a 5-minute warm-up at PWS = 0% grade, then the workload was increased using a grade increase from 1%/min to 2%/min until the criteria for maximality were reached as previously described (21). During the test, oxygen consumption ($VO_2$), CO2 output, and ventilation were analyzed by breath using an online system (Oxycon Pro; Jaeger), and cardiac activity was monitored continuously using a 12-lead ECG. The ventilatory threshold ($T_{vent}$) was systematically determined by two different experts using two methods (19).

Before and after the performance of the GXT, blood samples were drawn from an antecubital vein. Fresh blood was used for immediate analysis by flow cytometry, and plasma or serum aliquots were frozen and kept at −80°C until analyses.

Analytical Methods

Plasma vitamin C and E concentrations were measured using high performance liquid chromatography (22,23). Plasma concentrations of cholesterol and triglycerides were measured using a routine enzymatic method (Coulter CPA; Coultruns France SA, Margency, France). Plasma antioxidant capacity was measured using the Trolox equivalent antioxidant capacity (TEAC) by colorimetry (NX2331; Randox Laboratories, Mauguito, France). Advanced oxidation protein products (AOPP), markers of protein oxidation, were measured by spectrophotometry (24). Plasma thiobarbituric acid–reactive species (TBARS), a marker of lipid peroxidation, were measured by spectrophotometry (25). Total and differential leukocyte counts were determined by using an automated hematology analyzer (Pentra 120 Retic; ABX Diagnostics, Montpellier, France).

Flow Cytometry

The expression of Hsp72 in leukocytes and the percentage of apoptotic and necrotic leukocytes were measured by flow cytometry on an EPICS-XL-MLC flow cytometer (Beckman-Coulter, Villepinte, France) as previously described (8). Briefly, 100 µL of EDTA-treated whole blood was added to 2 mL of a lysis solution containing ammonium chloride and was incubated for 10 minutes on ice in the dark. After centrifugation, lysed erythrocytes were removed, the overlay washed with phosphate-buffered saline (PBS), and cell concentration adjusted with PBS to 10^6 cells/mL.

For intracellular Hsp72 expression, leukocytes were permeabilized using a Fix and Perm kit according to the manufacturer’s instructions (An-der-Grub, Vienna, Austria). Then, 10^6 cells were incubated for 20 minutes on ice in the dark with a solution containing 1 µg of either monoclonal antibody specific to Hsp72 (SPA-810; immunoglobulin G1 IgG1), clone C92F3A-5; StressGen Biotechnologies, Victoria, Canada) or of the isotype-matched monoclonal antibody (MG101; IgG1; Caltag Laboratories, Burlingame, CA). Cells were then washed and incubated 15 minutes on ice in the dark with the secondary fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse F(ab’)_2-IgG (SAB-102; StressGen Biotechnologies). Leukocytes were analyzed for Hsp72 expression, and differentiation between lymphocyte, monocyte, and granulocyte populations was performed according to difference in granularity and size. Data were presented as the percentage of Hsp72-positive cells and mean fluorescence intensity (MFI) in positive cells and were corrected for background fluorescence from the negative control.

The percentage of apoptotic and necrotic leukocytes was measured using the Annexin V-FITC kit for flow cytometry (ADK-700; StressGen Biotechnologies) according to the manufacturer’s instructions. Cells positive for only Annexin V were considered apoptotic, and cells positive for both Annexin V and propidium iodide were considered necrotic.

Statistical Analysis

The significant probability level was set at $p < .05$, and all values are given as mean ± standard deviation (SD). Differences between the two groups for anthropometric or aerobic parameters and plasma antioxidants were tested using one-way analysis of variance (ANOVA). The effect of the GXT on oxidative damage markers, leukocyte counts, and Hsp72 expression was tested using ANOVA for
RESULTS

Anthropometric and Aerobic Fitness Values

No significant differences were observed for anthropometric data (Table 1). The OHigh group showed significantly higher VO2max and VO2 TiE values (120% of OLow group values, Table 1).

Leukocyte Counts

Significantly lower lymphocyte, monocyte, and granulocyte counts were observed in the OHigh group, at rest and postexercise, but no difference was observed concerning the percentage of apoptotic or necrotic leukocytes (Table 2). Granulocyte count significantly increased postexercise in both groups (Table 2).

Antioxidant Capacity

The OHigh group had significantly higher TEAC and vitamin E concentration (Table 3), and interestingly, TEAC was positively correlated with VO2max and VO2 TiE (respectively: r = 0.52, p = .002; r = 0.63, p < .001; n = 32, Figure 1a for VO2 TiE).

Lipid and Protein Oxidation Markers

The OHigh group had significantly lower resting and postexercise AOPP and TBARS values. The performance of GXT induced an increase in TBARS in the two groups, although AOPP increased in only the OLow group (Figure 2). A significantly negative correlation was observed between resting TBARS and VO2 TiE (r = −0.56, p = .001; Figure 1b).

Hsp72 Expression

The OHigh group showed half the percentage of lymphocytes expressing Hsp72 observed in the OLow group and a > 50% lower MFI in monocytes and granulocytes at rest and postexercise when compared to the OLow group (Figure 3). This difference was further supported by a negative correlation between MFI in monocytes and granulocytes and VO2 TiE (respectively: r = −0.52, p = .002; r = −0.55, p = .001; n = 32, Figure 1c for granulocytes). Hsp72 expression in leukocytes was also positively correlated with oxidative damage (TBARS with: MFI in monocytes r = 0.38, p = .03; MFI in granulocytes r = 0.45, p = .01, Figure 1d) and negatively correlated with antioxidant capacity (TEAC with MFI in monocytes r = −0.48, p = .005; MFI in granulocytes r = −0.52, p = .002). No change in Hsp72 expression could be observed in either group in response to GXT.

DISCUSSION

The key finding of this study was that, at rest and following exercise, highly physically active 60- to 90-year-old elderly people showed: (i) lower Hsp72 expression, (ii) higher antioxidant capacity, and (iii) lower levels of oxidative damage. This result was further supported by the fact that aerobic fitness was positively correlated with antioxidant capacity and negatively with Hsp72 expression or oxidative damage. Such adaptations, so far, have been reported only in young and highly trained persons. A disturbance of the oxidant/antioxidant system during aging both at rest and in response to acute exercise is well documented, and physical activity has been suggested to prevent this alteration, thus contributing to slowdown the aging process. During acute exercise, the increased production of ROS acts as a biological signal to stimulate

<table>
<thead>
<tr>
<th>Leukocyte Counts</th>
<th>OHigh</th>
<th>Postexercise</th>
<th>OLow</th>
<th>Postexercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes, 10^9/L</td>
<td>1.39 ± 0.20</td>
<td>1.38 ± 0.25</td>
<td>1.79 ± 0.40*</td>
<td>1.99 ± 0.57*</td>
</tr>
<tr>
<td>Monocytes, 10^9/L</td>
<td>0.32 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td>0.43 ± 0.13*</td>
<td>0.48 ± 0.18*</td>
</tr>
<tr>
<td>Granulocytes, 10^9/L</td>
<td>2.83 ± 0.48</td>
<td>3.32 ± 0.76*</td>
<td>4.50 ± 2.00*</td>
<td>4.77 ± 2.21*</td>
</tr>
<tr>
<td>Apoptotic leukocytes, %</td>
<td>4.8 ± 2.1</td>
<td>5.5 ± 1.6</td>
<td>5.0 ± 2.1</td>
<td>3.8 ± 2.3</td>
</tr>
<tr>
<td>Necrotic leukocytes, %</td>
<td>2.0 ± 0.9</td>
<td>2.6 ± 0.4</td>
<td>2.5 ± 1.0</td>
<td>2.3 ± 1.2</td>
</tr>
</tbody>
</table>

Notes: Values are means ± standard deviation.
*Different from OHigh (p < .05).
1Different from resting values (p < .05).
OHigh = old highly active group; OLow = old low active group.

Table 1. Anthropometric and Aerobic Fitness Values

<table>
<thead>
<tr>
<th>Anthropometric and Aerobic Fitness Values</th>
<th>OHigh</th>
<th>OLow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>71.9 ± 5.0</td>
<td>74.6 ± 6.4</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>64.1 ± 8.2</td>
<td>69.4 ± 10.6</td>
</tr>
<tr>
<td>Height, cm</td>
<td>164 ± 10</td>
<td>166 ± 8</td>
</tr>
<tr>
<td>VO2max (mL/kg/min)</td>
<td>32.4 ± 3.2</td>
<td>27.4 ± 3.7*</td>
</tr>
<tr>
<td>VO2 TiE (mL/kg/min)</td>
<td>26.3 ± 2.5</td>
<td>21.8 ± 2.3*</td>
</tr>
</tbody>
</table>

Notes: Values are means ± standard deviation.
*Different from OHigh (p < .05).
the activity of antioxidant enzymes or the expression of HSP. This improvement of the protective systems can in turn limit the accumulation of oxidative damage (4). In young people, the chronic stimulation of the protective systems might improve the oxidant/antioxidant balance and decrease the resting expression of Hsp72 (26). However, little and contradictory information is available concerning the adaptations of elderly people to physical activity.

Table 3. Plasma Antioxidant Concentrations

<table>
<thead>
<tr>
<th>Plasma Antioxidants</th>
<th>OHigh</th>
<th>OLow</th>
<th>OHigh</th>
<th>OLow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Postexercise</td>
<td>Rest</td>
<td>Postexercise</td>
</tr>
<tr>
<td>TEAC, mmol/L</td>
<td>1.27 ± 0.07</td>
<td>1.26 ± 0.08</td>
<td>1.10 ± 0.11*</td>
<td>1.10 ± 0.12*</td>
</tr>
<tr>
<td>Vitamin C, μmol/L</td>
<td>53.6 ± 37.5</td>
<td>56.5 ± 37.5</td>
<td>55.3 ± 16.7</td>
<td>51.4 ± 22.0</td>
</tr>
<tr>
<td>Vitamin E, μmol/L/(triglycerides + cholesterol)</td>
<td>5.4 ± 0.4</td>
<td>5.7 ± 0.8</td>
<td>3.9 ± 0.4*</td>
<td>4.1 ± 0.6*</td>
</tr>
</tbody>
</table>

Notes: Values are means ± standard deviation. *Different from OHigh (p < .05).

TEAC = Trolox equivalent antioxidant capacity; OHigh = old highly active group; OLow = old low active group.

Figure 1. a, Correlation between oxygen consumption at the ventilatory threshold (VO₂ T_EQ) and plasma antioxidant capacity (Trolox equivalent antioxidant capacity, TEAC): r = 0.63, p < .001; n = 32. b, Correlation between VO₂ T_EQ and thiobarbituric acid–reactive species (TBARS): r = −0.56, p = .001; n = 32. c, Correlation between VO₂ T_EQ and mean fluorescence intensity (MFI) in heat shock protein 72 (Hsp72)-positive granulocytes: r = −0.55, p = .001; n = 32. d, Correlation between TBARS and MFI in granulocytes: r = 0.45, p = .01; n = 32.
particularly during long-term involvement in physical activity. Small or no modifications of the oxidant/antioxidant system were reported after 12–16 weeks of aerobic (11,12) or resistance training (15) in elderly people. However, 6 months of resistance training slightly reduced the oxidative damage in 60- to 83-year-olds (16), so it could be hypothesized that adaptations in response to training occur at a slower rate in elderly people. A decreased cell-signaling capacity, as illustrated by the impaired gene expression for antioxidant enzymes observed in skeletal muscles during aging (4), could contribute to the attenuated adaptations to training of antioxidant enzymes or HSP. However, our study showed significant positive adaptation in the OHigh group, and oxidant/antioxidant markers levels in this group were similar to those previously reported by our group in active individuals 50 years younger (8). This finding suggests that a large improvement in oxidant/antioxidant balance is still possible in old age with a long-term training program.

The performance of GXT resulted in a significant increase in lipid peroxidation markers in both of our groups, as previously observed in young and old persons (8), but with significantly lower levels in the OHigh group. This lower oxidative stress in the OHigh group could be explained by the higher plasma antioxidant capacity observed in those same individuals. Interestingly, the OHigh group also had lower monocyte and granulocyte counts, both leukocyte subsets being involved in phagocytic activity and thus ROS production (27). Thus, lower phagocytic cell counts in the OHigh group could have contributed to limiting oxidative damage. Although muscular adaptations were beyond the scope of our study, it should be noted that regular physical activity is also known to decrease ROS production by mitochondria, a major ROS source during exercise (28).

To our knowledge, this study is the first one to provide evidence of a down-regulation of Hsp72 in elderly people involved in long-term physical activity, including sporting activities, an adaptation previously reported only in young adults (26). The limited oxidative damage accumulation observed in our OHigh group could represent a potential mechanism for this adaptation. Indeed, the activation of the heat shock factor1 (HSF1), the transcription of the hsp70 gene, and the induction of Hsp72 are known to be highly redox sensitive (29). These observations suggest that decreasing ROS production or increasing antioxidant capacity may limit Hsp72 induction. In our study, by lowering oxidative damage, physical activity could have thus reduced the activation of HSF1 and the induction of Hsp72; this result could explain the down-regulation of Hsp72 in our highly active elderly participants. The role of ROS in the induction of Hsp72 was indirectly supported by the correlations between oxidant/antioxidant markers and Hsp72 expression, where low levels of antioxidants or high levels of oxidative damage were associated with high expression of Hsp72. The capacity of the OLow group to still synthesize Hsp72 represents an important adaptation that could contribute to limiting oxidative damage accumulation and preventing cell dysfunction, alterations often described during aging or under pathological conditions (30). Even if those individuals were not physically active, they were still independently living and free of any disease.

The present study, as a cross-sectional study, does not provide definitive evidence on the effect of physical activity on Hsp72 expression or oxidant/antioxidant balance during aging, but certainly adds important knowledge to the topic. Taking into account the limited specificity of TBARS as a marker of lipid peroxidation, we have also measured plasma levels of AOPP, a protein oxidation marker. These two markers are consistent with each other, have been validated, and are widely used in clinical practice as markers of oxidative damage (8,31).

**Conclusion**

The main finding of this experiment was the down-regulation of Hsp72 as well as the protective effect against oxidative damage accumulation in relatively higher levels of physical activity during aging. Highly active and healthy elderly people aged 60–90 years were characterized by a better oxidant/antioxidant balance when compared to age-matched, less active people. A significant down-regulation of the protective Hsp72 was also observed in the highly active participants, probably resulting from reduced oxidative damage. These results are of high relevance considering the pivotal role of oxidative stress and Hsp72 expression in cardiovascular risks and “unsuccessful aging” (32,33). This study provides evidence for the promotion of long-term physical activity during aging to preserve aerobic
capacity and to improve HSP expression and oxidant/antioxidant status.

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