No effects of lifelong creatine supplementation on sarcopenia in senescence-accelerated mice (SAMP8)

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Submitted 28 January 2005; accepted in final form 16 February 2005

THE MARKED DECLINE in skeletal muscle mass and strength accompanying advancing age (sarcopenia) is an important public health burden. Presently, research aims to develop effective strategies to prevent age-related muscle atrophy. Hormone replacement therapy and exercise prescription have proven effective but face important limitations (7). Oral creatine supplementation can improve muscle adaptations during training (25) and promote muscle rehabilitation after leg immobilization (11), yet its long-term effectiveness in preventing sarcopenia is unknown. Short-lived additive effects of oral creatine supplementation and resistance training have been reported in older men (1, 5). However, a recent study from our laboratory failed to report beneficial effects of creatine supplementation (5 g/day) combined with exercise training over a 6-mo period in 55- to 75-yr-old men (4). In this respect, two experimental issues are of importance. First, it has recently been shown that, in healthy persons, after an initial rise during the first weeks of creatine supplementation (9, 12), muscle creatine content (and supposedly with it the related ergogenic or therapeutic effects) gradually returns to baseline levels during prolonged creatine supplementation (2). Possibly, long-term beneficial effects of creatine supplementation are only to be expected in pathological conditions that are characterized by a primary deficit in creatine metabolism leading to chronically decreased tissue creatine content such as in inborn errors of creatine synthesis and in gyrate atrophy (13, 20, 21). Second, despite the fact that a 6-mo to 1-yr intervention appears long according to experimental standards, such period represents only a minor fraction of the time window over which sarcopenia develops in older individuals. The current study aims to overcome the above-mentioned experimental drawbacks by investigating the effects of creatine supplementation during nearly the entire life span of mice.

Even in rodents, studying the effect of lifelong lifestyle or nutritional changes on the natural aging process takes approximately two years of follow-up. The different strains of senescence-accelerated mice (SAM) provide potentially interesting models to specifically study the aging process of various organs in an accelerated way (23). We have demonstrated that mice of the SAMP8 strain develop typical features of skeletal muscle senescence at a relatively young age (60 wk) compared with normal mice (100–120 wk; W. Derave, B. O. Eijnde, and P. Hespel, unpublished observation). In SAMP8 mice, muscle mass and tetanic force are reduced by up to 25% in 60-wk-old vs. 25-wk-old mice. Importantly, muscle total creatine and phosphocreatine content declines throughout lifetime by 35% in face of constant ATP and glycogen content (W. Derave, B. O. Eijnde, and P. Hespel, unpublished observation). We have previously shown that oral creatine supplementation (2–5% of food intake) can antagonize creatine depletion in soleus muscles of a transgenic mouse model of amyotrophic lateral sclerosis (3) and increase creatine content in rat muscles, most prominently slow oxidative muscles (19). Thus, with increasing age, SAMP8 mice exhibit a creatine-reduced/-deficient state in skeletal muscles, making them an adequate model for studying the effects of creatine replacement therapy in sarcopenia as well as studying the possible causal role of energy/creatinine depletion in age-related muscle degeneration. Multiple theories exist about the etiology of aging in general and skeletal muscle senescence in particular (28). An increasing number of studies, however, seem to indicate that mitochondrial metabolism plays an important role. The accumulation of mutations in mitochondrial DNA and the resulting mitochondrial dysfunction and energy deficiency are believed to causally relate to sarcopenia (18, 24). Tissues with high and fluctuating energy demand, such as neurons and skeletal muscle, are especially vulnerable to deficient mitochondrial function. In these tissues, the creatine kinase/phosphocreatine system plays an important role as an energy buffer and as a
transporter of energy from mitochondria (27). Creatine supplementation can enhance the function of the creatine kinase/phosphocreatine system (10). Thus creatine supplementation may be an effective intervention to maintain energy homeostasis in neuronal and muscular tissue by compensating for mitochondrial dysfunction and/or improving the function of the residual mitochondria. Indeed, creatine supplementation has proven protective in experimental models of several neurodegenerative disorders and mitochondrial cytopathies (29), yet its long-term potential to protect skeletal muscle from senescence-associated deterioration remains insufficiently explored.

Therefore, in the present study, SAMP8 mice were assigned to control or creatine-supplemented diet (2% of food intake) at the age of 10 wk until 60 wk, which is near the average survival date. Muscle mass, in vitro contractile properties, energy metabolite content, and fiber-type distribution and size were evaluated at the age of 10, 25, and 60 wk. It is hypothesized that creatine supplementation, by antagonizing the age-related decrease in muscle creatine content, can prevent loss in skeletal muscle mass and function.

METHODS

**Animals.** Male SAMP8/Ta (senescence-accelerated mice prone) were obtained from breeding pairs, kindly donated by Dr. M. Hosokawa (Council of SAM Research, Kyoto, Japan). The animals were reared and maintained under conventional breeding conditions with food and water ad libitum and were kept on a 12:12-h light-dark cycle. The experimental protocol was approved by the Ethical Commission for Animal Research of K.U. Leuven. A total of 78 mice were included in the study, divided over five experimental groups, and 12 mice died before investigation (Table 1). Mice were investigated at 10, 25, or 60 wk of age. From the age of 10 wk, mice received either creatine-free rodent pellets (Kliba, Cossenay-Gare, Switzerland) or similar pellets that were enriched with 2% creatine monohydrate (Creapure; Degussa, Trostberg, Germany). Daily food intake was monitored in a subsample of mice.

**Muscle contractile properties.** At the time of investigation (10, 25, or 60 wk), mice of all five experimental groups were anesthetized with an intraperitoneal infusion of pentobarbital sodium (Nembutal, 50 mg/kg body wt). After careful dissection of the soleus, extensor digitorum longus (EDL) and tibialis anterior muscles from both legs, mice were killed by cervical dislocation. The soleus and EDL muscles were quickly frozen in liquid nitrogen and stored at −80°C for later biochemical assays. Soleus and EDL muscles were electrically stimulated with capacitor discharges between platinum electrodes.

Table 1. Initial and final number of mice included in each group and the percentage surviving

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Treatment</th>
<th>Initial No.</th>
<th>Final No.</th>
<th>Survival, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Control</td>
<td>12</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>Control</td>
<td>13</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>Creatine</td>
<td>11</td>
<td>11</td>
<td>100</td>
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</table>

**Muscle metabolites.** Muscle metabolites were measured on the soleus, EDL, and tibialis muscles that were not subjected to the contraction protocol. Total muscle water content was measured by weighing the samples before and after 48 h of freeze-drying. The concentration of metabolites was measured on freeze-dried samples (soleus, EDL, and tibialis anterior) and calculated as micromole per gram dry weight. Tissue glycogen content was determined on pieces of ±1 mg dry sample. Glycogen was hydrolyzed in 1 M HCl at 100°C for 2 h, and glucose residues were determined enzymatically by a hexokinase method on a fluorometer. The other half of soleus and EDL muscles, ATP, phosphocreatine, and free creatine were extracted by perchloric acid precipitation (3 N perchloric acid) on ice, neutralization with 2 N KOH, and analyzed enzymatically with standard fluorometric assays (15).

**Immunohistochemistry.** Muscle fiber type determination was performed by means of immunofluorescent staining with specific anti-type I and anti-type IIA antibodies and digital morphometric analysis. Soleus muscle was chosen because the most pronounced atrophy was observed in this muscle. Transverse sections (10 μm) were cut from the midbelly area of soleus muscles with a Leica CM1850 cryostat (Leica, Nussloch, Germany) at −20°C and mounted on glass slides, with each slide containing two serial sections of soleus muscles from 60-wk-old control and creatine-treated groups. Sections were brought to room temperature and fixed in PBS containing 4% paraformaldehyde and 0.1% Triton X-100 for 10 min and rinsed for 2 min in wash buffer (0.5% BSA in PBS). Next, sections were incubated for 30 min in 10 mM NH4Cl, rinsed for 2 min in wash buffer, and incubated in PBS containing 1% BSA for 30 min. Sections were incubated overnight at 4°C in wash buffer containing two primary monoclonal antibodies directed against fast myosin type IIA (1:20 dilution; N2.261 supernatant; Developmental Studies Hybridoma Bank) and slow myosin type I (1:20 dilution; A4.840 supernatant; Developmental Studies Hybridoma Bank). Thereafter, slides were washed 3 × 5 min in wash buffer and 60 min at room temperature in wash buffer containing the secondary antibodies Alexa Fluor 350 anti-mouse IgG1 (Molecular Probes, Leiden, The Netherlands) and FITC anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL), each in a 1:500 dilution. Cover slips were mounted with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). Two to three digital images were taken from each section with a Nikon Eclipse E1000 microscope (Nikon, Boehrnhedorp, Germany) at a ×20 magnification with appropriate bandpass filters for Alexa Fluor350 and FITC. Morphometric analysis of double-labeled immunofluorescent images was performed with Lucia G Software (LIM, Prague, Czech Republic). On average, 292 fibers were outlined per section and identified as type I fibers (green staining), hybrid fibers coexpressing type I and type IIA (green and blue staining), type IIA fibers (blue staining), and type IIX/B fibers (no staining). Fiber type...
distribution as percentage number and percentage area and the average fiber size per type were calculated for each of the fiber categories.

Statistics. Results were analyzed with a factorial ANOVA with age (25 and 60 wk) and treatment (control and creatine) as independent variables and with post hoc Tukey’s tests in case of significant interaction. Data from 10-wk-old mice are only shown as indicative baseline values, but were not included in statistical analyses. Statistics were performed using Statistica software (Statsoft, Tulsa OK). Data are presented as means ± SE, and significance level was set at \( P \leq 0.05 \).

RESULTS

Survival, body weight, and food intake. The number of mice surviving at age 60 wk was 67% in the control group (C) vs. 76% in the creatine group (CR). However, statistical analysis of the cumulative proportion surviving did not reveal a difference between the groups (Table 1). Body weight was 27.5 ± 0.7 g at week 10 and increased to 36.1 ± 1.6 g at week 25 and 36.7 ± 2.2 g at week 60 for C and CR, respectively (not significant [NS] for age and treatment). Between week 10 and week 60, C and CR on average consumed 6.1 ± 0.2 g and 6.0 ± 0.3 g food•mouse\(^{-1}\)•day\(^{-1}\), respectively (NS).

Muscle metabolites. As shown in Table 2, ATP content was not significantly influenced by age or treatment in either soleus or EDL muscles. Phosphocreatine and total creatine content, however, declined from 25 to 60 wk in both muscle types (\( P < 0.05 \)). Creatine supplementation elevated neither muscle phosphocreatine nor total creatine stores. In soleus, there was a trend for a main effect of creatine treatment on ATP (\( P = 0.07 \)) and total creatine (\( P = 0.11 \)) content. Glycogen content in tibialis anterior decreased with age (\( P < 0.05 \)). This decrease was more pronounced in CR than in C (Table 2).

Muscle mass. The time course of soleus, EDL, and tibialis anterior muscle mass is summarized in Table 3. Muscle mass decreased in all three muscle types from 25 to 60 wk of age (\( P < 0.05 \)). The relative decrease was more pronounced in soleus (20–25%) than in EDL and tibialis anterior (10%). Creatine treatment did not prevent age-associated muscle mass loss, but instead even tended to reduce muscle mass of mice aged 25 and 60 wk (\( P = 0.07 \) for soleus and \( P = 0.09 \) for EDL).

Muscle contractile properties. Maximal tetanic force (Table 4) during electrically evoked in vitro contractions was lower in soleus and EDL of 60-wk-old mice compared with 25-wk-old mice, irrespective of the treatment received. When corrected for cross-sectional area, maximal tetanic tension was not affected by either age or treatment. Table 5 summarizes contraction and relaxation time and peak duration of single-twitch contractions. In soleus, contraction time, peak duration, and relaxation time were prolonged at older age, whereas in EDL only contraction time was increased (\( P < 0.05 \)). Creatine supplementation did not affect these contractile properties in any muscle type at either age.

Fiber type distribution and size. Soleus muscles were predominantly composed of equal proportions of type I and type II fibers.
AIA fibers, with minor fractions of hybrid type-I/IIA fibers and type-IIIX fibers (Fig. 1A). Compared with 25 wk, in soleus muscles at 60 wk of age the proportion of type I fibers had increased vs. a decrease in type IIX fibers \( (P < 0.05) \). This fast-to-slow transition was not influenced by creatine supplementation (Fig. 1A). The average cross-sectional area of type IIX fibers decreased with advancing age, whereas the size of the other fiber types remained constant from week 25 to 60 (Fig. 1B). Again, no effect of creatine supplementation was observed.

**DISCUSSION**

Given the wide popularity of creatine as a dietary supplement in athletic populations, as well as its growing use as an adjuvant therapy in some specific neuromuscular disorders (10, 29), short-term effects of creatine supplementation on skeletal muscle have been investigated extensively in the last 15 years. However, the effects of chronic creatine supplementation remain to be elucidated. The present study hypothesized that oral creatine supplementation could prevent the age-related decrease in muscle creatine content in SAMP8 mice. Previous studies have shown that, in rodents, oxidative muscles are more responsive than glycolytic muscles in terms of increasing their creatine content in response to loading (19). In the present study, total creatine content of the oxidative soleus but not the glycolytic EDL increased by 20% in creatine-treated compared with control mice at 25 wk of age (after 15 wk of supplementation), which is comparable with effects usually observed in rodents and humans (10, 19), although the difference did not reach statistical significance. In the subsequent 35 wk, however, creatine supplementation could not prevent the decline in creatine content of either soleus or EDL muscles. This is surprising, since one would expect that effects of chronic creatine supplementation are most prominent in a state of lowered tissue creatine content (2). This finding may indicate that the low levels of creatine and phosphocreatine in aged mouse muscles are homeostatically regulated and cannot be influenced despite continuous exogenous supply. Consequently, low muscle creatine content in older age does not appear to be a problem of impaired endogenous creatine synthesis or decreased systemic availability.

Given the inability of creatine supplementation to correct the lowered creatine content in aging muscle, it seems unlikely that other effects on muscular function and morphology are to be expected, since elevated intramyocellular creatine availability is thought to be the primary effect of oral creatine supplementation that drives other biochemical and functional adaptations (6). Indeed, in the present results, the deterioration of muscle morphology and contractile parameters with advancing age could not be alleviated by creatine treatment. As measures of atrophy, muscle mass and fiber size decreased with age, which could not be prevented by creatine treatment. Instead, muscle mass decline rather tended to be enhanced by creatine supplementation in soleus \( (P = 0.07) \) and EDL \( (P = 0.09) \). Atrophy of type IIX fibers also occurred without protective effects from creatine supplementation. Maximal tetanic force, which is greatly determined by the physiological cross-sectional area, confirmed the above-mentioned changes with age and the lack of changes with creatine treatment on muscle atrophy. Together, these data seem to support the conclusion that creatine...
supplementation is not an effective long-term strategy to prevent senescence-associated sarcopenia. Such contention is compatible with our earlier study in humans that showed one year of creatine supplementation not to beneficially impact muscular functional capacity in older males enrolled in an exercise training program (4). In contrast to the latter study, where intervention covered only a fraction of the total atrophy period and where subjects did not exhibit lowered creatine stores, the present study had the additional advantage that creatine could be supplemented during nearly the entire life span in an animal model that displays lowered creatine stores at older age (~35%).

The aging process in muscular tissue includes not only atrophy, as evidenced by decreased muscle mass, strength, and fiber size but also a marked slowing of muscle contraction. In the SAMP8 model of muscle senescence, isometric twitch contractions exhibit significantly slower contraction and relaxation. In addition, fiber type distribution follows the expected fast-to-slow transition. Both effects, however, appeared to be irrespective of treatment received. Thus, as for atrophy, creatine supplementation seems unable to alter the age-related slowing of muscle contractility in SAMP8 mice.

Spiked by some remarkable studies published by one research group in the late nineties (14, 16), suggesting a role for creatine in the treatment of neuromuscular degenerative diseases, we and others have initiated studies in an attempt to reproduce and expand these observations. Presently, however, a number of negative findings have contradicted the usefulness of long-term creatine treatment in amyotrophic lateral sclerosis (3, 8, 17), Huntington’s disease (22, 26), and sarcopenia (4). Therefore, the current study confirms the emerging idea that long-term creatine supplementation is of little value in the prevention or treatment of neuromuscular degeneration. Even though some studies have reported mild positive effects of short-term creatine supplementation in patients with neuromuscular disorders, evidence for beneficial effects of long-term treatment are entirely lacking (17). However, long-term creatine supplementation can be effective in diseases that are characterized by a primary creatine deficiency, such as in patients genetically lacking arginine-glycine amidinotransferase or guanidinoacetate methyltransferase enzymes (13, 21).

To our knowledge, the present study is the first to explore the impact of creatine supplementation during nearly the entire life span of animals. Although a detailed pharmacotoxic profile was not the aim of this study, it is interesting to note that lifelong creatine supplementation in mice had neither beneficial nor detrimental effects on survival and body weight evolution.

In conclusion, oral creatine supplementation (2% of food intake) from 10 to 60 wk in SAMP8 mice cannot prevent the age-associated reduction in creatine content in skeletal muscle. Likewise, the deterioration in morphological and contractile muscle properties with advancing age is not influenced by lifelong creatine supplementation.

GRANTS

This study is supported by grants from Onderzoeksraad K.U. Leuven (Grant no. OT99/38) and from the Flemish Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO-Vlaanderen Grant no. G.0255.01 and 1.5172.02N). B. O. Eijnde obtained a research fellowship from the Onderzoek K.U. Leuven, and W. Derave is a recipient of a postdoctoral fellowship from the Flemish Fonds voor Wetenschappelijk Onderzoek Vlaanderen. The mice pellets were kindly provided by Degussa (Trossberg, Germany). The monoclonal MHC antibodies, developed by Helen M. Blau, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA).

REFERENCES