Aged Garlic Extract Ameliorates Physical Fatigue

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Aged garlic extract (AGE) has recently received attention as a potent anti-fatigue agent. The principal aim of this study was to elucidate the mechanism responsible for the ameliorating effect of AGE on physical fatigue in rats caused by repeated endurance exercise on a mechanical treadmill apparatus. Rats were subjected to endurance exercise 5 times per week for 4 weeks. AGE at a dosage of 2.86 g/kg was administrated to rats 30 min before every exercise. Succinate dehydrogenase (SDH) activity in the gastrocnemius and soleus muscles and superoxide dismutase (SOD) activity, nitric oxide (NO) metabolites, and lactic acid concentration in plasma were evaluated as biomarkers of physical fatigue. SDH activity was increased 2–4-fold by repeated endurance exercise in comparison with unexercised (intact) rats, and AGE further up-regulated this activity by 40%. SOD activity was increased 5-fold, whereas AGE maintained it at a level equivalent to that in intact rats. Levels of NO metabolites were slightly decreased, whereas AGE enhanced them 2-fold. Lactic acid concentration was not changed in any of the groups. These results indicate that AGE may facilitate the turnover of aerobic glucose metabolism, attenuate oxidative stress, and promote oxygen supply based on vasodilation, suggesting that AGE ameliorates the various impairments associated with physical fatigue.

Key words aged garlic extract; fatigue; rat; succinate dehydrogenase; nitric oxide; superoxide dismutase

Although recent advances in the medical field have been remarkable, lifestyle-related diseases such as hypertension, diabetes, heart disease, and cancer continue to increase, and are the major causes of death. As a result, the public have become much more health-conscious, and an increased proportion of people now exercise routinely to prevent lifestyle-related diseases and enhance their physical strength. Moderate exercise is useful for preventing illness and mental stress, but excessive exercise itself can be a form of stress and cause fatigue or various types of damage to the body.

In ancient Egypt and Rome, garlic (Allium sativum L.) was given to laborers and soldiers, probably to mitigate fatigue or to prompt recovery from physical exhaustion.1,2 Thereafter, garlic was used as a folk medicine to treat hypertension, heart disease, inflammation, diabetes and cancer.3 Recently, research has focused on the preventive and curative effects of garlic on cardiovascular disease and cancer.4–6 However, it is not practical to use raw garlic because it has various side effects such as anemia, weight loss, growth reduction, and decrease of caecal microflora and serum protein.5,6 Aged garlic extract (AGE), which is extracted for more than 10 months, is less irritating and toxic.5,7 A large number of pharmacological studies have demonstrated that AGE possesses anti-oxidative,8–10 anti-stress,11 immunomodulating12,13 cardiovascular14,15 and hepatoprotective16 properties. Moreover, AGE has been shown to be superior to raw garlic in terms of its anti-oxidation properties, and amelioration of physiological and psychological stress.10,11 These reports suggest that AGE may prevent various types of injury that can result from exercise, or enhance physical strength.

Therefore, we examined the effect of AGE in rats subjected to repeated endurance exercise on a treadmill. Here we report that AGE facilitates skeletal muscle oxidative enzyme activity and promotes oxygen supply based on NO production, suggesting that AGE contributes to enhancement of physical strength and endurance.

MATERIALS AND METHODS

Aged Garlic Extract AGE was prepared by rinsing garlic (Allium sativum L.) cloves with purified water, slicing them, and then soaking them in a water–ethanol mixture, which was then naturally extracted/aged for more than 10 months at room temperature. The AGE used in this study contained 28.6% (w/v) solid material. S-Allyl-l-cysteine (SAC) was also present as 0.1% of the total solids, calculated on a dry weight basis.

Chemicals Hydroxylamine, hypoxanthine, hydroxylamine O-sulfonic acid, potassium cyanide (KCN), ethylenediamine-N,N,N,N′,N′,N′-tetraacetic acid disodium salt dihydrate (EDTA-2Na), potassium dihydrogen phosphate, sodium tetraborate, sulfuric acid, N,N,N′-triethyl-N′-naphthylethylenediamine, acetic acid, zinc sulfate, phosphoric acid, sulfanilamide, sodium nitrite, potassium nitrate, sodium pyruvate, sodium succinate, sodium cyanide, aluminum chloride and calcium chloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). Determiner LA was purchased from Kyowa Medex Co., Ltd. (Tokyo, Japan). Xanthine oxidase (bovine milk, XOD) and cytochrome c (rat heart) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). FAD, NADPH, nitrate reductase (Aspergillus species) and lactate dehydrogenase (rabbit muscle) were purchased from Boehringer Mannheim (Mannheim, Germany).

Animals and Treatments Male Wistar rats (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed, 2–3 per plastic cage, under a 12-h light/dark cycle for one week before use in the experiment. They were allowed free access to a commercial diet (CE-2, Clea, Tokyo, Japan) and water. The in vivo experiments were approved by the Institutional Animal Care and Use Committee at Wakunaga Pharmaceutical Co. Ltd. A total of 21 rats were divided into three groups (7 per group): intact (unexercised), control and AGE groups. The intact group was not subjected to exercise, while everyday exercise on a mechanical treadmill apparatus. Rats were subjected to endurance exercise 5 times per week for 4 weeks. AGE at a dosage of 2.86 g/kg was administrated to rats 30 min before every exercise. Succinate dehydrogenase (SDH) activity in the gastrocnemius and soleus muscles and superoxide dismutase (SOD) activity, nitric oxide (NO) metabolites, and lactic acid concentration in plasma were evaluated as biomarkers of physical fatigue. SDH activity was increased 2–4-fold by repeated endurance exercise in comparison with unexercised (intact) rats, and AGE further up-regulated this activity by 40%. SOD activity was increased 5-fold, whereas AGE maintained it at a level equivalent to that in intact rats. Levels of NO metabolites were slightly decreased, whereas AGE enhanced them 2-fold. Lactic acid concentration was not changed in any of the groups. These results indicate that AGE may facilitate the turnover of aerobic glucose metabolism, attenuate oxidative stress, and promote oxygen supply based on vasodilation, suggesting that AGE ameliorates the various impairments associated with physical fatigue.
cise or oral AGE administration, and the control and AGE groups were subjected to treadmill exercise for 4 weeks (5 days per week). The exercise-loaded rats were made to run on a mechanical treadmill apparatus (model KN-73, Natsume Seisakusho Co., Ltd., Tokyo, Japan) consisting of a wide, endless belt riding on metal rollers. An acrylic box, partitioned into ten individual compartments measuring 10×57 cm, was suspended to cover the belt, providing a limited running area for each rat. Motivation was provided by an electric shock nozzle plate at the rear of each compartment. The belt was set at an angle of 5 degrees, running at a constant speed of 20 m/min. AGE was administered orally at a dose of 2.86 g/kg, 30 min before the exercise, for 4 weeks. The control group was treated similarly, but was administrated distilled water orally instead of AGE. Blood, gastrocnemius and soleus samples were collected immediately after the last exercise loading. Blood was taken from the abdominal aorta using a heparinized syringe with the rat anesthetized. Plasma was obtained after centrifugation of the heparinized blood and used for measurement of lactic acid, superoxide dismutase (SOD) activity and nitric oxide (NO) metabolites. Gastrocnemius and soleus samples were homogenized for 2 min with 5 volumes of 0.3 M phosphate buffer (pH 7.4). Sodium succinate was added to yield a final concentration of 17 mM, and mixed for 2 min. Then, sodium cyanide, cytochrome c, aluminum chloride and calcium chloride were added to final concentrations of 10 mM, 17 mM, 0.4 mM and 0.4 mM, respectively. This mixture was measured spectrophotometrically with a commercial available kit (Kyowa Medex).

Measurement of Lactic Acid  Plasma lactic acid concentration was determined spectrophotometrically with a commercially available kit (Koyo Medex).

Determination of SDH Activity  SDH activity was determined according to the method of Cooperstein et al.  Briefly, frozen gastrocnemius and soleus samples were homogenized for 2 min with 5 volumes of 0.3 M phosphate buffer (pH 7.4). Sodium succinate was added to yield a final concentration of 17 mM, and mixed for 2 min. Then, sodium cyanide, cytochrome c, aluminum chloride and calcium chloride were added to final concentrations of 1 mM, 17 mM, 0.4 mM and 0.4 mM, respectively. This mixture was measured within a few minutes at 550 nm. SDH activity was calculated from the ferricytochrome c concentration and protein content.

Determination of SOD Activity  SOD activity was determined using the method of Oyanagui.  Briefly, the plasma (0.1 ml), 0.2 ml of reagent A (aqueous solution, pH 7.0, containing a final concentration of 0.2 mM hydroxylamine plus 0.2 mM hypoxanthine), or hypophosphatized powder containing a final concentration of 0.2 mg/ml=1.77 mM hydroxylamine O-sulfonic acid plus 0.2 mM hypoxanthine upon addition of water), and water or KCN (1 mM final concentration, 0.5 ml) were mixed. The reaction was started by adding 0.2 ml of reagent B (containing final concentrations of 1.25 mM/ml XOD, 0.1 mM EDTA-2Na, 20.8 mM KH2PO4 and 15.6 mM Na2B4O7). This mixture (1.0 ml) was incubated for 30 min at 37 °C without shaking and then treated with 2.0 ml of reagent C (coloring reagent containing a final concentration of 300 μg/ml sulfamic acid, 5 μg/ml N-1-naphthylenediamine, and 16.7% acetic acid). The final mixture was allowed to stand for 20 min at room temperature and optical absorption was measured at 550 nm.

Quantification of NO Metabolites  Nitrite (NO2⁻) and nitrate (NO3⁻) were used as an index of NO production.  These NO metabolites were determined using the method of Schmidt et al.  Briefly, plasma was diluted 4-fold with distilled water. NADPH, FAD and nitrate reductase were added to yield final concentrations of 50 μM, 5 μM and 200 U/l, respectively. This mixture were incubated for 20 min at 37 °C, and then lactate dehydrogenase and sodium pyruvate were added to give final concentrations of 10 mg/l and 10 mM respectively. This mixture were further incubated for 5 min at 37 °C to oxidize NADPH, and then nitrite was measured using the Griess reaction. The resulting samples were deproteinized by adding a 5% volume of zinc sulfate to give a final concentration of 93 mM. After centrifugation at 1000×g for 15 min at room temperature, the supernatant was mixed with the same quantity of Griess reagent (5.8 mM sulfanilamide, 25 g/l phosphoric acid and 5.3 mM N-1-naphthylenediamine). After 10 min of color development at room temperature, the absorbance was measured at a wavelength of 540 nm. Each plasma sample was assayed in duplicate. Control values were obtained by treating mixture as described, but using 25 g/l phosphoric acid instead of complete Griess reagent. Calibration curves were obtained using sodium nitrite and potassium nitrate in distilled water. Values obtained by this procedure represent the sum of nitrite and nitrate in the samples.

Protein Assay  Protein contents were determined by the method of Lowry et al., with bovine serum albumin as a standard.

Statistical Analysis  The data are expressed as mean±standard error of the mean (S.E.M.). Data were analyzed using Student’s t-test. Differences at p<0.05 were considered to be significant.

RESULTS

Body weight changes were not influenced by either exercise or AGE treatment (data not shown).

SDH Activity in Skeletal Muscle  SDH activity in skeletal muscle was measured as an index of tricarboxylic acid cycle turnover. As shown in Table 1, SDH activity in gastrocnemius muscle of control group was found to be a significant increase of 2.4-fold in comparison with the intact group (p<0.01). AGE significantly up-regulated this increase compared with the control group (p<0.05). On the other hand, SDH activity in soleus muscle of control group was found to be a significant increase of 4.6-fold in comparison with the intact group (p<0.01). AGE up-regulated this increase compared with the control group, but its effect was found to be not strong in contrast to gastrocnemius muscle (0.05<p<0.1).

Table 1. Effect of AGE on SDH Activity in Skeletal Muscle

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Exercise</th>
<th>M. gastrocnemius (nmol/min/mg protein)</th>
<th>M. soleus (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>7</td>
<td>-</td>
<td>1.98±0.29</td>
<td>1.96±0.23</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>+</td>
<td>4.70±0.61**</td>
<td>9.18±1.68**</td>
</tr>
<tr>
<td>AGE</td>
<td>7</td>
<td>+</td>
<td>6.60±0.60*</td>
<td>13.34±2.16</td>
</tr>
</tbody>
</table>

Endurance exercise was loaded to rats with treadmill 5 times per week for 4 weeks. AGE was administered orally with 2.86 g/kg for 4 weeks on 30 min before loading of exercise. SDH activity was determined using gastrocnemius and soleus removed from lower limb after loading of last exercise. Each value represents mean±S.E.M. **(vs. intact, p<0.01), * (vs. control, p<0.05) significant difference by Student’s t-test.
Lactic Acid, SOD Activity and NO Metabolites in Plasma

Lactic acid, SOD activity and NO metabolites were measured as an index of anaerobic glucose metabolism, relationship between exercise and free radicals, and vasodilation, respectively. Lactic acid was not increased by treadmill exercise. AGE did not also affect lactic acid levels (Fig. 1). SOD activity in the control group was significantly increased 5.1-fold compared with the intact group (Fig. 2). However, AGE significantly down-regulated, as same level as intact group. Lactic acid was not influenced in comparison with the intact group. Therefore, mild condition (20 m/min) of moderate exercise for 4 weeks was set in contrast to the method of Ushijima et al., and the function of AGE was focused on prevention of oxidative stress, and promotion of aerobic glucose metabolism turnover and oxygen supply based on vasorelaxation. In particular, we examined SOD activity and NO metabolites in plasma, as a close relationship between exercise tolerance and oxygen has been observed. Furthermore, we evaluated SDH activity in skeletal muscle as an index of tricarboxylic acid cycle turnover and lactic acid in plasma as an index of anaerobic glucose metabolism.

ATP is an essential energy source during exercise, and is necessary for effective supply of oxygen. Although muscle can produce 30—32 molecules of ATP via the tricarboxylic acid cycle in the presence of an adequate oxygen supply, it can produce 2 molecules of ATP through anaerobic glucose metabolism when the supply of oxygen is insufficient. Moreover, it has been reported that endurance training increases the activities of enzymes involved in both aerobic glucose metabolism and the tricarboxylic acid cycle.24—26) In this study, we investigated lactic acid and SDH activity as an index of anaerobic and aerobic glucose metabolism, respectively. SDH activity in the gastrocnemius, which is a fast muscle related to instantaneous force, and the soleus, which is a slow muscle related to endurance, was significantly increased in the control group as compared with the intact group. Lactic acid was not influenced in comparison with the intact group. Therefore, this type of training may favor aerobic glucose metabolism over anaerobic glucose metabolism. On the other hand, AGE elevated SDH activity in skeletal muscle compared with the control group, indicating that AGE enhances the aerobic glucose metabolic ability of skeletal muscle during exercise.

NO produced during exercise induced vasodilation accompanied by an increase of blood flow, suggesting that exercise is effective for preventing hypertension. It has been reported that moderate chronic exercise attenuates the elevation of blood pressure in young spontaneously hypertensive rats, and that the concentration of total plasma nitrite is significantly higher in exercised rats than in controls. They concluded that the increased NO formation was responsible for the low blood pressure in the exercised rats. However, in our studies, NO metabolites in the plasma of exercised rats were similarly to those in the intact group. We assume that this exercise may
increase blood pressure. On the other hand, AGE increased NO metabolites compared with the control group. In other words, we consider that AGE inhibited the elevation of blood pressure accompanying chronic exercise. The small quantity of NO produced by constitutive NOS (eNOS) plays an important role as a cellular messenger controlling physiological functions in the cardiovascular system.\(^3^8,3^9\) However, when excess NO is produced through up-regulation of inducible NOS (iNOS), toxic peroxynitrite is produced through a reaction with superoxide.\(^3^0,3^1\) We have reported that AGE increases NO production by activating cNOS, but not iNOS.\(^3^4\) From our previous study, AGE is seemed to enhance NO production by cNOS and dilates blood vessels, thus increasing the supply of oxygen to skeletal muscle.

A large number of pharmacological studies have investigated the relationship between exercise and reactive oxygen species. In general, exercise changes blood flow distribution and reduces the supply of oxygen. Visceral tissue causes a decrease in blood flow and induces an ischemic state.\(^3^2—3^4\) Accordingly, a hypoxic state produces active oxygen derived from the xanthine oxidase system.\(^3^5\) SOD catalyzes the conversion of superoxide radicals to oxygen and hydrogen peroxide, and appears to protect cells against reactive free radicals produced under oxidative conditions. In this study, we examined the anti-oxidative effect of AGE on free radicals produced during exercise loading. The exercise increased SOD activity and facilitated the ability to scavenge free radicals. Superoxide radicals have been reported to induce increase of SOD.\(^3^6\) Our results also suggested that the elevation of SOD activity may be responsible for the increase of superoxide radicals after exercise tolerance. The exercise under severe condition may favor anaerobic glucose metabolites over aerobic glucose metabolites, resulting in increase of lactic acid in plasma, and/or may induce liver damage. However, the exercise under mild condition used in our study did not cause liver damage in macroscopic observation and did not increase lactic acid levels in plasma, suggesting that our condition was not seemed to induce liver damage. On the other hand, AGE significantly inhibited the increase of SOD activity thus AGE may be related to the anti-oxidative effects of AGE. AGE had a protective effect against ischemia and did not induce liver damage in macroscopic observation and our condition was not seemed to induce liver damage. On the other hand, AGE significantly inhibited the increase of SOD activity and reduced the supply of oxygen. Visceral tissue causes a decrease in blood flow and induces an ischemic state.\(^3^2—3^4\) Accordingly, a hypoxic state produces active oxygen derived from the xanthine oxidase system.\(^3^5\) SOD catalyzes the conversion of superoxide radicals to oxygen and hydrogen peroxide, and appears to protect cells against reactive free radicals produced under oxidative conditions. In this study, we examined the anti-oxidative effect of AGE on free radicals produced during exercise loading. The exercise increased SOD activity and facilitated the ability to scavenge free radicals. Superoxide radicals have been reported to induce increase of SOD.\(^3^6\) Our results also suggested that the elevation of SOD activity may be responsible for the increase of superoxide radicals after exercise tolerance. The exercise under severe condition may favor anaerobic glucose metabolites over aerobic glucose metabolites, resulting in increase of lactic acid in plasma, and/or may induce liver damage. However, the exercise under mild condition used in our study did not cause liver damage in macroscopic observation and did not increase lactic acid levels in plasma, suggesting that our condition was not seemed to induce liver damage. On the other hand, AGE significantly inhibited the increase of SOD activity thus AGE may be related to the anti-oxidative effects of AGE. AGE had a protective effect against ischemia and did not induce liver damage.

In conclusion, the present findings indicate that AGE facilitates skeletal muscle oxidative enzyme activity and promotes oxygen supply through NO production, suggesting that AGE contributes to enhancement of physical strength and endurance.

REFERENCES


