Aged Garlic Extract Maintains Cardiovascular Homeostasis in Mice and Rats

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ABSTRACT  Nitric oxide (NO) plays an important role in controlling the physiological functions of the cardiovascular system. However, toxic peroxynitrite is produced by the reaction of NO with superoxide. We investigated the effect of aged garlic extract (AGE) on NO production, and on oxidative stress induced by peroxynitrite. A single dose of AGE temporarily increased NO production by 30–40% between 15 and 60 min after administration to mice. The time course of the fluctuation in NO levels in the AGE-treated group clearly differed from that in a group treated with an inducible NO synthase (iNOS) inducer. A selective constitutive NOS (cNOS) inhibitor overcame the effect of AGE. These results indicate that AGE increases NO production by activating cNOS, but not iNOS. In another experiment, the addition of AGE to a rat erythrocyte suspension reduced the rate of peroxynitrite-induced hemolysis in a concentration-dependent manner, suggesting that AGE protects erythrocytes from membrane damage induced by peroxynitrite. Because an increase in NO derived from cNOS and protection against peroxynitrite are important factors in the prevention of cardiovascular disease, our data strongly suggest that AGE could be useful in preventing cardiovascular diseases associated with oxidative stress or dysfunctions of NO production. J. Nutr. 136: 777S–781S, 2006.

KEY WORDS: • garlic • nitric oxide • peroxynitrite • cardiovascular diseases

Since ancient times, people of many different cultures have considered garlic (Allium sativum) to be a valuable healing agent. Garlic has been used as a cure for various ailments, including heart disease, cancer, and infection (1). However, chronic administration of raw garlic causes diverse toxic effects, such as anemia, weight loss, and growth reduction (2). Aged garlic extract (AGE), extracted for >10 mo, is less irritating and does not induce the toxic changes mentioned above (2,3). Furthermore, a large number of pharmacological studies found that AGE and its components possess antioxidative (4,5), antiaging (6), immunomodulatory (7), cardiovascular (8–11), and hepatoprotective (12,13) properties.

Nitric oxide (NO) is synthesized from L-arginine by NO synthases (NOS) in many of the cells of the cardiovascular system, including endothelial cells, macrophages, smooth muscle cells, platelets, and fibroblasts (14). Three kinds of NOS, i.e., neuronal NOS, inducible NOS (iNOS), and endothelial NOS, were reported to be responsible for NO biosynthesis in these cells (15). The small quantity of NO produced by constitutive NOS (cNOS; neuronal NOS and endothelial NOS) is an important cellular messenger with a major role in controlling physiological functions in the cardiovascular system (16,17). However, when excess NO is produced through upregulation of iNOS, toxic peroxynitrite is produced by a reaction with superoxide (18,19). Peroxynitrite is a potent oxidant that was shown to oxidize lipids and LDL and to promote platelet aggregation, thus aggravating the atherogenic process (20). Thus, NO possesses the character of a double-edged sword.

In this study, we examined the effect of AGE on NO production, and on oxidative stress induced by peroxynitrite.
MATERIALS AND METHODS

Aged garlic extract. AGE was prepared as follows. Cloves of garlic (Allium Sativum L.) were rinsed with purified water, sliced, and soaked in a water-ethanol mixture, which was then naturally extracted/aged for >10 mo at room temperature. The AGE we used contained ~256 g/L solid material and 6.3 g/L arginine, as reported previously.

Chemicals. We purchased (+)-1,2-Ethyl-2-(1-E)-hydroxymethyl-5-nitro-3-hexanenamide (NOR3), N6-monomethyl-l-arginine acetate (l-NMMA), and peroxynitrite from Dojindo Laboratories. Arginine, zinc sulfate, phosphoric acid, sulfanilamide, N-1-naphthylethylenediamine, sodium nitrite, potassium nitrate, sodium pyruvate, sodium chloride, disodium hydrogenphosphate and sodium dihydrogenphosphate were obtained from Wako Pure Chemical Industries. Diphénylhexemion chloride (DPI) was purchased from Research Biochemical International. Lipopolysaccharide (LPS; W.E. coli 055:B5) was obtained from DIFCO Laboratories. FAD, NADPH, nitrate reductase (EC 1.6.6.2), and lactate dehydrogenase (EC 1.1.1.27) were purchased from Boehringer Mannheim. LPS, L-NMMA, and DPI were dissolved in sterile saline. NOR3 was suspended in sterile saline. Other reagents were dissolved in distilled water.

Experiment 1: Effect of AGE on NO production

Male ddY mice (5 wk old) were purchased from Japan SLC and housed, 4–6/plastic cage, under a 12-h light:dark cycle for 1 wk before use in the experiment. They had free access to a commercial diet (CE-2, Clea) and water. They were killed by bleeding after collection of blood under anesthesia. The in vivo experiments were approved by the Wakunaga Pharmaceutical Company Institutional Animal Care and Use Committee.

AGE (2.86 g/kg body weight, orally), NOR3 (10 mg/kg, orally), or l-NMMA (40 mg/kg, i.p.) was administered to the mice. Blood samples were collected 15 min after administration in the AGE and NOR3 groups and 1 h after administration in the l-NMMA group.

Separately, AGE (2.86 g/kg, orally) or LPS (30 mg/kg, i.p.) was administered to the mice, and blood samples were collected to measure changes in NO metabolites over time.

To clarify the mechanisms involved in the fluctuation of NO metabolites after AGE administration, mice were given AGE (2.86 g/kg, orally) or a dose of arginine equivalent to the arginine content of the AGE (63 mg/kg, orally). DPI (1 mg/kg, i.p.) was administered 2 h before the experiment, based on its duration of effect. Blood samples were collected to measure changes in NO metabolites over time.

Blood samples were taken from the right ventricle of the anesthetized mice with a heparinized syringe. The erythrocytes were separated from the plasma and buffy coat by centrifugation at 1000 × g for 10 min at 4°C, then washed 3 times with 10 volumes of PBS. The washed erythrocytes were suspended in PBS, and the hemocrit was measured using a Partec Counter PC-608 (ERMA). The hemocrits of the erythrocyte suspensions used in the experiments were adjusted to 10% using PBS. The erythrocyte suspensions were preincubated with various concentrations of AGE (1.4–5.7 g/L) at 37°C for 5 min. Peroxynitrite (300 μmol/L) was then added to each erythrocyte suspension to induce peroxidation, followed by further incubation for 30 min at 37°C. Each erythrocyte suspension was then separated by centrifugation at 1000 × g for 5 min at 4°C and the absorbance of the supernatant was measured at 540 nm. By comparing these values with the absorbance of distilled water (considered equivalent to 100% hemolysis), the percentage hemolysis obtained with peroxynitrite was calculated.

Statistical analysis. The data are expressed as means ± SEM. Significant differences between means were determined using one-way ANOVA followed by Duncan’s or Scheffe’s multiple-comparison test. All statistical analyses were performed using STATISTICA™ (StatSoft Japan).

RESULTS

Experiment 1: Effect of AGE on NO production

Fluctuations in NO production were investigated after administration of AGE, NOR3, and l-NMMA. Both AGE and NOR3 (a NO donor) increased NO levels in the plasma compared with the control (P < 0.05 and P < 0.01, respectively). On the other hand, the NOS inhibitor l-NMMA reduced NO levels in the plasma compared with the control (P < 0.01, Table 1).

We also examined the effect of AGE on NO production over time. AGE increased NO levels in the plasma at 15 (by 44%, P < 0.01), 30 (by 37%, P < 0.05), and 60 (by 44%, P < 0.01) min after treatment compared with the basal value, indicating a temporary stimulation of NO production (Fig. 1).

We further investigated the effect of LPS (a typical iNOS inducer) on NO production. All mice survived for up to 360 min after treatment with LPS. LPS gradually increased NO levels in the plasma from 120 min after treatment onwards, producing a significant 5.2-fold increase at 360 min compared with the basal value (P < 0.01, Fig. 2).

To clarify the mechanism by which AGE increases NO production, we investigated the effects of arginine (a NOS substrate) on NO production levels in mouse plasma1,2

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>NO metabolites (μmol/L)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>31.2 ± 2.8</td>
<td>-</td>
</tr>
<tr>
<td>AGE</td>
<td>5</td>
<td>41.2 ± 2.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NOR 3</td>
<td>6</td>
<td>69.0 ± 2.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>l-NMMA</td>
<td>5</td>
<td>13.8 ± 2.1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Experimental values were compared with those for the control group using Duncan’s multiple comparison test after one-way ANOVA [F(3, 19) = 63.890, P < 0.001].

2 Blood was collected from 15 min after administration in the AGE and NOR 3 groups, and 1 h after administration in the l-NMMA group.
and DPI (a selective cNOS inhibitor) on NO production (Figs. 3 and 4). Although AGE contains some arginine, a dose of arginine equivalent to the arginine content of the AGE did not increase NO levels in the plasma compared with the basal value (Fig. 4). However, DPI significantly decreased NO levels in the plasma at 60 and 120 min after treatment compared with the basal value (P < 0.05, Fig. 3). DPI administered before AGE completely eliminated the effect of the AGE (Fig. 4).

**Experiment 2: Effect of AGE on peroxynitrite-induced oxidative stress**

The addition of peroxynitrite to rat erythrocyte suspensions produced 4 times more hemolysis than in control erythrocyte suspensions without peroxynitrite (Table 2). AGE (1.4–5.7 g/L) significantly inhibited this increase in hemolysis in a dose-dependent manner (P < 0.05 or P < 0.01). However, AGE (1.4–5.7 g/L) did not affect the degree of hemolysis that occurred in the absence of peroxynitrite.

**DISCUSSION**

NO has biological functions in areas as diverse as blood pressure modulation, vasodilation, neurotransmission, and inhibition of platelet adhesion and aggregation (22). However, peroxynitrite is a potent oxidant that causes tissue injury, neurodegenerative disease, inflammation, and atherosclerosis (23–27). In particular, peroxynitrite-induced cardiovascular disease is related to an effect on erythrocytes because these are in constant contact with the endothelial cells of the blood vessels, where peroxynitrite is formed (28–30). We consider that both an increase in NO derived from cNOS and protection against peroxynitrite are likely to be important factors in the prevention of cardiovascular disease.

We investigated NO production in the body by measuring stable NO metabolites in the blood. We confirmed that levels of NO metabolites change in the presence of a NO donor or NOS inhibitor. Both AGE and an NO donor significantly increased NO levels (Table 1). AGE rapidly increased NO production by ~30–40% after administration, but NO production had returned to the basal level at 120 min after administration (Fig. 1). This rapid response may be explained by the fact that S-allylcysteine, one of the major compounds in AGE, is rapidly and easily absorbed from the gastrointestinal tract (within 15 min of administration of AGE) in animals (31).

To clarify the mechanism by which AGE increases NO production, we investigated the effect of DPI (a selective cNOS inhibitor) on NO production. DPI significantly decreased the NO level in mouse plasma at 60 and 120 min after treatment compared with the basal value (Fig. 3). We determined the time of administration of DPI based on the absorption of AGE and the activity profile of DPI. NO production was inhibited when DPI was administered 120 min before AGE, in contrast to the increase seen when AGE was administered alone (Fig. 4). Because our AGE contained 6.3 g/L arginine (a substrate of...
NOS), we investigated the effect of an equivalent dose of arginine. However, treatment with arginine alone did not increase NO production (Fig. 4). These results suggest that the enhancement of NO production that occurred after administration of AGE does not depend on the arginine content of the AGE, and could therefore be caused by an increase in cNOS activity. LPS (an iNOS inducer) began to increase NO production at 120 min after administration. Levels had increased by 5.2-fold at 360 min and continued to rise for up to 720 min after administration (Fig. 2). Nevertheless the pattern of NO production in response to AGE was obviously different from that seen with LPS (Figs 1 and 2). Thus, the results obtained after administering LPS further substantiated the theory that NO production after the administration of AGE might result from cNOS activity. Although cNOS normally exists as an inactive homodimer, its expression and activation depend on the intracellular Ca\(^{2+}\) concentration. We therefore hypothesized that AGE may activate cNOS by accelerating Ca\(^{2+}\) influx.

This study also demonstrated that AGE significantly suppresses peroxynitrite-induced hemolysis in a dose-dependent manner (Table 2). Kondo et al. (30) showed that the antioxidant actions of albumin, glutathione, and N-acetylcysteine suppress peroxynitrite-induced hemolysis. It is thought that the antioxidant activity of albumin in the vascular compartment results from the scavenging of reactive oxygen and nitrogen species that are generated by basal aerobic metabolism (32,33). The main constituent responsible for the antioxidant properties of albumin is the thiol group within the cysteine moiety (34). In addition, the thiol residue of albumin reacts preferentially with peroxynitrite leading to thiol oxidation (35). Because glutathione and N-acetylcysteine also contain thiol groups, we consider that their effects are likely to be similar to that of albumin. AGE contains not only sulfur-containing compounds derived from garlic, but also other compounds such as Maillard reaction products formed during its natural aging process. Compared with the common antioxidants mentioned above, garlic compounds do not possess a free thiol group, but contain an S-allyl moiety or an allyl sulfoxide moiety. These may be important for the prevention of peroxynitrite-induced hemolysis. Other potential mechanisms of action may involve scavenging of peroxynitrite or erythrocyte membrane stabilization (36,37).

NO has different effects in cardiovascular disease depending on whether it is produced by iNOS or sNOS. Although the small quantity produced via sNOS in atherosclerotic lesions has a protective effect, peroxynitrite arising from the larger quantities of NO produced by iNOS contributes to vascular injury (36,38). In this report, we demonstrated that AGE increases NO levels through stimulation of eNOS, but not iNOS and protects against peroxynitrite-induced damage. These findings suggest that AGE may be extremely useful in the prevention of cardiovascular disease. It was reported that fresh garlic powder increases cNOS activity, but a quantity of arginine equivalent to that found in fresh garlic powder does not affect cNOS activity (39,40). Garlic contains many other amino acids, and Das et al. (40) speculated that any of these might be responsible for the increase in NO activity in response to fresh garlic powder. Furthermore, wild garlic (Allium ursinum), another species of garlic, was found to block NO inhibitor–induced hypertension by antagonizing the inhibitory effect on NO production in vivo (41). Because cardiovascular disease is generally chronic in nature, long-term intake of garlic may contribute to its prevention. However, it is not practical to use raw garlic because this has a variety of unwanted side effects such as anemia, weight loss, and growth reduction (2). AGE, which is extracted over a period of 10 mo, is less irritating and less toxic, so that intake can be tolerated for extended periods of time without the side effects of raw garlic (2,3). For this reason AGE could be useful for the long-term prevention of cardiovascular disease.

In conclusion, the study indicates that AGE increases NO levels by stimulating cNOS, but not iNOS, and that this increase is not due to the arginine content of AGE. Moreover, AGE protects erythrocytes from peroxynitrite-induced membrane damage. These findings suggest that chronic intake of AGE could be useful for the prevention of cardiovascular diseases resulting from oxidative stress associated with high NO activity or dysfunctions of NO production.

### LITERATURE CITED


### TABLE 2

**Effect of AGE on hemolysis of rat erythrocytes induced by peroxynitrite**

<table>
<thead>
<tr>
<th>Group</th>
<th>Peroxynitrite (+)</th>
<th>Peroxynitrite (−)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.4 ± 0.3</td>
<td>15.3 ± 1.0</td>
<td>−</td>
</tr>
<tr>
<td>AGE 1.4 mg/mL</td>
<td>3.7 ± 0.3</td>
<td>11.5 ± 0.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AGE 2.9 mg/mL</td>
<td>3.0 ± 0.3</td>
<td>7.5 ± 1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AGE 5.7 mg/mL</td>
<td>4.3 ± 0.5</td>
<td>4.4 ± 0.4</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1. Values are means ± SEM, n = 5. Experimental values were compared with the control group using Scheffe’s multiple comparison test after one-way ANOVA [F (3, 16) = 32.649, P < 0.001].
2. Rat erythrocyte suspensions (standardized to a hematocrit of 10%) were incubated with AGE with or without 300 μm/L peroxynitrite in PBS at 37°C in air for 35 min. The extent of hemolysis was measured as described in Materials and Methods.


