Dietary Supplementation with Aged Garlic Extract Inhibits ADP-Induced Platelet Aggregation in Humans

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ABSTRACT Garlic has been widely reported to protect against cardiovascular disease by reducing serum cholesterol concentrations and blood pressure and by inhibiting platelet aggregation. However, most of these studies have been performed in hypercholesterolemic subjects or in animal models. We performed a 13-wk study in normolipidemic subjects who ingested 5 mL of aged garlic extract (AGE, Kyolic) per day. Blood was drawn from these subjects at the beginning and end of the study. Aggregation of platelet-rich plasma was induced by ADP; full lipid profiles and liver function tests were determined on serum, and plasma concentrations of eicosanoids were also measured. Dietary supplementation with AGE significantly inhibited both the total percentage and initial rate of platelet aggregation at concentrations of ADP up to 10 μmol/L. The K₅₀ for ADP-induced aggregation were approximately doubled after supplementation with AGE, whereas the maximum rate of aggregation was unaffected. No significant changes in plasma thromboxane B₂ and 6-ketoprostaglandin F₁α concentrations or serum lipid profiles were observed. We conclude that AGE, when taken as a dietary supplement by normolipidemic subjects, may be beneficial in protecting against cardiovascular disease as a result of inhibiting platelet aggregation. J. Nutr. 130: 2662–2665, 2000.

KEY WORDS: • platelet aggregation • humans • garlic • ADP • eicosanoids

Garlic (Allium sativum) has been used for many centuries, as both a flavoring and a folk medicine. At present, the potential therapeutic and health-promoting effects of garlic are attracting considerable interest (Agarwal 1996). For example, garlic has been shown to be antitumorigenic (Kyo et al. 1998, Milner et al. 1990). Garlic and some of its constituents have also been shown to be protective against acetaminophen (Wang et al. 1996) and bromobenzene toxicities (Wang et al. 1999).

By far the most widely studied and reported health-promoting effect of garlic is cardioprotection. Cardiovascular disease is multifactorial, and garlic appears to exert its beneficial effects at several different sites in the pathogenesis of the disease. Several reports have claimed that garlic lowers plasma cholesterol concentrations, particularly those of LDL (Neil et al. 1996, Steiner et al. 1996). More recently, an aged garlic extract was shown to inhibit directly the formation of atherosclerotic plaques in de-endothelialized carotid arteries in rabbits fed a diet supplemented with 1% cholesterol (Efendy et al. 1997). The oxidative modification of LDL is now recognized as an important process in the development of atherosclerosis, and garlic has been shown to inhibit Cu²⁺-induced oxidation of LDL in vitro (Ike et al. 1997) and to protect cultured vascular endothelial cells from injury induced by oxidized LDL (Ike and Lau 1997). In addition, Munday et al. (1999) reported that ingestion of an aged garlic extract (AGE), but not raw garlic, inhibits the oxidation of subsequently isolated LDL.

Finally, garlic has also been shown to have antithrombotic effects in that it inhibits platelet aggregation in experimental animals (DeBoer and Folts 1989) and humans (Bordila et al. 1996, Lawson et al. 1992, Legnani et al. 1993, Steiner and Lin 1998) at high risk of cardiovascular disease. Indeed, the only study we are aware of that has focused on healthy subjects is that of Legnani et al. (1993) who showed that acute (6 h) and chronic (14 d) administration of dried garlic powder (Kwai, Lichtenberg Pharma GmbH, Berlin, Germany) inhibited ADP- and collagen-induced platelet aggregation. In this paper, we report the effects of an AGE (Kyolic) taken by healthy subjects as a dietary supplement for 13 wk on ADP-induced platelet aggregation and on the plasma concentrations of lipids and eicosanoids. In addition, the results of liver function tests before and after dietary supplementation with AGE are reported.

SUBJECTS AND METHODS

Aged garlic extract. Aged garlic extract (AGE, Kyolic), kindly provided by Wakunaga of America (Mission Viejo, CA), is formulated by soaking sliced raw garlic (Allium sativum) in 15–20% aqueous ethanol for up to 20 mo at room temperature. The extract is then filtered and concentrated under reduced pressure at low temperature. The content of water-soluble compounds is relatively high, whereas that of oil-soluble compounds is low. The AGE used in this trial contained 305 g/L extracted solids; S-allyl cysteine, the most abundant water-soluble organosulfur compound in AGE, was present at 1.47 g/L.

1 Supported by Wakunaga of America Company Limited.
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0022-3166/00 $3.00 © 2000 American Society for Nutritional Sciences.
Subjects. - Apparently healthy subjects (n = 23; 12 men, 11 women, age range 22–45 y) who were not taking medication for any known disease completed the study which had the prior approval of the Ethics Committee of Liverpool John Moores University. Subjects consumed 5 mL of AGE (taken in a small volume of fruit juice) daily for 13 wk between 0700 and 0900 h; otherwise, subjects followed their usual diet and lifestyle (including alcohol intake). Three subjects were smokers. All subjects refrained from taking aspirin or other drugs known to affect hemostasis and/or platelet aggregation for 2 wk before and during the study. Blood samples (34 mL) were taken after an overnight (12-h) fast immediately before ingestion of AGE and under similar conditions after 13 wk of ingestion of AGE (i.e., 24 h after the last dose). The major portion of the sample (27 mL) was added to 38 g/L trisodium citrate in the ratio 9:1 (v/v; blood/anticoagulant) for platelet aggregation studies, and 4 mL was allowed to clot at room temperature for preparation of serum (1500 g for 20 min) for eicosanoid assays. Serum and plasma samples for biochemical analyses were stored at −70°C for up to 3 mo before analysis.

Platelet aggregation. - Platelet aggregation was determined within 2 h of blood being drawn. Platelet-rich plasma (PRP) was prepared by centrifugation of blood at 1500 g for 8 min, whereas platelet-poor plasma (PPP) was prepared by centrifuging PRP at 1500 g for a further 20 min. Platelets in PRP were counted using a Neubauer hemocytometer (Merck Eurolab, Lutterworth, UK) and, if necessary, the cell count was adjusted to 2.5 × 10^5 cells/mL by dilution with PPP. Aggregation was performed in a PAP-4 Platelet Aggregation Profiler (Bio/Data, Horsham, PA), which was first calibrated using PRP (0% aggregation) and PPP (100% aggregation). Aggregation was carried out at 37°C in 0.2 mL of PRP in microwell tubes and was initiated by adding ADP (stock 200 μmol/L) to final concentrations of up to 10 μmol/L. The aggregation curves were recorded and analyzed using the associated Bio/Data software for total percentage aggregation and the maximal initial rate of aggregation (i.e., % aggregation/min). At low concentrations of ADP, aggregation is biphasic and its rate was derived from the primary wave of aggregation. To calculate K_M and R_max values for aggregation from plots of rate of aggregation (R) vs. ADP concentration (A), the three classical transformations used for enzyme kinetic data were applied, namely, Lineweaver-Burk (1/R vs. 1/A), Eadie-Hofstee (R vs. 1/A) and Hanes-Woolf (A/R vs. A) plots (Garrett and Grisham 1995). K_M and R_max values were obtained from regression analysis of each plot.

Biochemical analyses. - Plasma 6-keto-prostaglandin F_1α (6-keto-PGF_1α) and thromboxane B_2 (TXB_2) were quantified using Amprep C2 columns (100 mg) and enzymeimmunoassay kits supplied by Amersham International (Amersham, Bucks., UK). To extract the eicosanoids from plasma, columns were conditioned by rinsing with 2 mL of methanol followed by 2 mL of distilled water. Plasma (1 mL) was acidified to pH 3 with 1 mol/L HCl and applied to the column. The column was washed with 5 mL of each of distilled water, 10% (v/v) ethanol and petroleum ether before the eicosanoids were eluted with 5 mL of methyl formate. The methyl formate eluate was evaporated to dryness under N_2 and the residue redissolved in 250 μL of assay buffer (0.1 mol/L phosphate buffer, pH 7.5, containing 9 g/L saline, 1 g/L bovine serum albumin and preservative). Aliquots (50 μL) of the redissolved extract were assayed for 6-keto-PGF_1α and TXB_2 by a horse radish peroxidase-linked enzymeimmunoassay method according to the manufacturer’s instructions.

Analysis of serum was carried out using a fully automated Monarch 2000 Chemistry System (Instrumentation Laboratory, Warrington, UK). All test kits and quality control sera were purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Total cholesterol and triglyceride concentrations were determined by the CHOD-PAP and GPO-PAP methods, respectively. HDL was measured as the cholesterol remaining in the supernatant after precipitation of apolipoprotein B–containing lipoproteins with heparin/manganese chloride (HDL precipitant, Boehringer Mannheim). LDL cholesterol was calculated using the Friedewald equation (Friedewald et al. 1972).

Serum alanine aminotransferase (ALT) and aspartate aminotransferase were assayed at 37°C by NAD^+–linked reactions. Alkaline phosphatase and y-glutamyltransferase were measured at 37°C by kinetic colorimetric methods utilizing the production of p-nitrophenol and 5-amino-2-nitrobenzozene, respectively.

Statistical analysis. - The significance of differences in various parameters before and after AGE supplementation was assessed by Student’s paired t test using the Minitab (State College, PA) statistical package.

RESULTS

The extent of platelet aggregation in response to ADP was reduced after dietary supplementation with 5 mL of AGE/d for 13 wk (Fig. 1A). This was most dramatic at low concentrations of ADP (P < 0.05; 0.5, 1 and 2 μmol/L) when the extent of aggregation was submaximal. At ADP concentrations ≥ 4 μmol/L, total percentage aggregation was maximal at 70–80% and was not significantly affected by ingestion of AGE.

The rate of ADP-induced platelet aggregation was similarly reduced after dietary supplementation with AGE (Fig. 1B). This was significant at all concentrations of ADP up to 10 μmol/L. All three transformations of these data showed that ingestion of AGE had little effect on the R_max for ADP-induced aggregation, whereas the K_M was approximately doubled (P < 0.007 for the Eadie-Hofstee and Hanes-Woolf transformations, Table 1).

Dietary supplementation with 5 mL of AGE/d for 13 wk had little effect on serum lipid concentrations in healthy humans. It is noteworthy that serum triglycerides were decreased by 13% and total and LDL cholesterol were decreased by 3% each; however, these differences were not significant.

FIGURE 1 ADP-induced platelet aggregation in humans before and after ingestion of aged garlic extract (AGE) for 13 wk. Platelet aggregation was initiated by the addition of various concentrations of ADP to platelet-rich plasma (PRP). Aggregation curves were analyzed for: (A) the total percentage aggregation, and, (B) the initial rate of aggregation (%/min). Values are means ± sem, n = 23. *Significantly different (P < 0.05) before and after ingestion of AGE.
in Figure 1 calculated from three different linear transformations of the data shown first 2 mo of AGE administration with concentrations decrease-al. 1987). Our study lasted 3 mo and, indeed, it has been after long-term (6 mo) dietary administration of AGE (Lau et al. 1987) been reported that a hypocholesterolemic effect occurs only decrease in serum cholesterol concentrations when the sub-jects under investigation were normolipidemic. It has also been reported that relatively high concentrations of water-soluble compounds, and low concentrations of oil-soluble compounds, this study, we used a liquid aged garlic extract (AGE, Kyolic) extracts, dried extracts and oil macerates. The chemical com-pounds and low concentrations of oil-soluble compounds, and is standardized to S-allylcysteine, its major organosulfur constituent. In contrast, the major organosulfur compound in the garlic powder (Kwai) used by Legnani et al. (1993) was allicin.

Supplementation with AGE had no significant effects on serum total, LDL or HDL cholesterol and triglyceride concentrations (Table 2). This is in contrast to some previously reported studies (Agarwal 1996, Lau et al. 1987, Steiner et al. 1996, Warshafsky et al. 1993) and could be due to the fact that the subjects who took part in our study were essentially normo-lipidemic. Using the European Atherosclerosis Society guidelines, 11 of 23 of our subjects had serum cholesterol concentrations < 5.2 mmol/L (200 mg/dL), whereas the remaining 12 had concentrations of 5.2–7.8 mmol/L, none exceeded 7.8 mmol/L (300 mg/dL). Lau et al. (1987) also did not observe a decrease in serum cholesterol concentrations when the sub-jects under investigation were normolipidemic. It has also been reported that a hypocholesterolemic effect occurs only after long-term (6 mo) dietary administration of AGE (Lau et al. 1987). Our study lasted 3 mo and, indeed, it has been reported that an initial rise in serum cholesterol is seen in the first 2 mo of AGE administration with concentrations decreasing by mo 4 (Lau et al. 1987).

Dietary supplementation with AGE had little effect on liver function as judged by the serum activity of four enzymes (Table 2). However, it is noteworthy that AGE caused a significant decrease in the serum activity of ALT. This cy-tosolic enzyme is released into blood from damaged hepatocytes. Thus, this observation suggests that AGE is hepatoprotective and is in agreement with both in vitro (Wang et al. 1999) and in vivo (Nakagawa et al. 1988) studies in animals that have demonstrated that AGE protects against known hepatotoxins such as carbon tetrachloride and bromobenzene.

Possibly the most important result of our clinical trial was that after 3 mo of dietary supplementation, AGE inhibited both the total percentage and initial rate of ADP-induced platelet aggregation (Fig. 1). Steiner and Lin (1998) con ducted a similar study in which 7.2 g of dried AGE (equivalent to ~25 mL of liquid AGE) was given as a daily supplement to hypercholesterolemic men for 10 mo. In that study, inhibition of platelet aggregation induced by epinephrine and collagen was seen, but not of aggregation induced by ADP; inhibition of platelet adhesion to fibrinogen was also seen. Steiner and Lin (1998) reported the median effective concentration (EC50) of ADP for platelet aggregation to be ~1.5 μmol/L, which is similar to the KM values reported in Table 2. Although the EC50 for ADP was unaffected by the supplementation regimen used by Steiner and Lin (1998), the KM for ADP was approxi-mately doubled by our supplementation regimen (Table 1), indicating a decrease in the affinity of the platelet ADP receptor for its ligand.

PGL3, the major arachidonic acid metabolite formed by the vascular endothelial cells, is a potent vasodilator and inhibitor of platelet aggregation. In contrast, the major arachidonic acid metabolite formed by platelets is TXA2, which is a potent vasoconstrictor and stimulator of aggregation. It is the balance between these eicosanoids that is important in regulating hemostasis and platelet aggregation. These eicosanoids are extremely short-lived in plasma and are invariably measured as their stable metabolites, 6-keto-PGF1α and TXB2. Supplemen-tation with AGE produced apparent decreases in plasma 6-keto-PGF1α and TXB2 concentrations of 18 and 32% respec-tively (Table 2). However, these decreases were not significant and, perhaps more importantly, AGE had little or no effect on the balance between these eicosanoids. This implies that di-

### TABLE 1

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<th>Kinetic parameters for ADP-induced platelet aggregation in humans before and after ingestion of aged garlic extract (AGE) for 13 wk1,2</th>
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<td>Lineweaver-Burk</td>
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1 KM values for ADP and maximal rates of aggregation (Pmax) were calculated from three different linear transformations of the data shown in Figure 1(b). 2 Values are means ± SEM, n = 23. * Significantly different (P < 0.05) before and after ingestion of AGE.

HDL cholesterol was unchanged (Table 2). Similarly, little change was seen with serum enzymes, although serum ALT was significantly decreased by nearly 20% after ingestion of AGE (Table 2). Plasma concentrations of TXB2 and 6-keto-PGF1α tended to be decreased (32 and 18%, respectively) after ingestion of AGE; however, these decreases were not signifi-cant (Table 2).

### DISCUSSION

A major problem in interpreting studies that investigate the health-promoting effects of garlic supplementation is that many different garlic preparations (and dosing regimens) have been used. The main garlic preparations available commer-cially are pressed garlic juice, garlic oil, dry powder, liquid extracts, dried extracts and oil macerates. The chemical com-position of these preparations varies widely (Lawson 1996). In this study, we used a liquid aged garlic extract (AGE, Kyolic) that contains relatively high concentrations of water-soluble compounds and low concentrations of oil-soluble compounds, and is standardized to S-allylcysteine, its major organosulfur constituent. In contrast, the major organosulfur compound in the garlic powder (Kwai) used by Legnani et al. (1993) was allicin.

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### TABLE 2

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<th>Serum lipids and enzymes and plasma eicosanoids in humans before and after ingestion of aged garlic extract (AGE) for 13 wk1</th>
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<td>Serum lipids, mmol/L</td>
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<td>Aspartate aminotransferase</td>
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<td>Plasma eicosanoids, ng/L</td>
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<td>6-Keto prostaglandin F1α</td>
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<td>Thromboxane B2</td>
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1 Values are means ± SEM, n = 23. * Significantly different (P < 0.05) before and after ingestion of AGE.
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