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Original article

Aged garlic extract exerts endothelium-dependent vasorelaxant effect on rat aorta by increasing nitric oxide production

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ABSTRACT

Background: Clinical trials have shown that aged garlic extract (AGE) is effective in reducing blood pressure of hypertensive patients. However, the mechanisms involved remain to be elucidated.
Purpose: The aim of the present study was to investigate the vasorelaxant effect of AGE on the aorta and its mechanism of action in order to clarify the blood pressure-lowering action of AGE.
Methods: The vasorelaxant effect was evaluated in isolated rat aortic rings. After aortic rings were contracted by 3×10^{-6} M norepinephrine (NE) for 30 min, AGE and other test drugs were added to the aortic rings. All results were expressed as percentages of the maximal NE-induced contraction.
Results: AGE induced the concentration–dependent vasorelaxation of isolated rat aortic rings that had been precontracted with norepinephrine. The effect of AGE was severely impaired in aortic rings lacking endothelium. In addition, the effect of AGE was inhibited by a nitric oxide synthase (NOS) inhibitor and a nitric oxide (NO) scavenger. Moreover, AGE treatment of aorta significantly increased the NO production. When various constituents of AGE were tested, the vasorelaxation of aorta was observed only in the presence of L-arginine, a substrate of NOS.
Conclusion: AGE causes endothelium-dependent vasorelaxation of aorta via stimulation of NO production and that L-arginine in AGE serves as a key agent for NOS-mediated NO production.
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Introduction

The functional disorder of vascular smooth muscle and/or endothelium is closely related to atherosclerotic development (Sehgel et al., 2013; Sena et al., 2013; Uryga and Bennett 2016). Vascular endothelial cells play a pivotal role for the contraction and relaxation of vascular smooth muscle as a source of several vasoactive substances such as nitric oxide (NO), hydrogen sulfide (H_2S), and endothelin, so that the failure in endothelial function is involved in a variety of lifestyle-related diseases, including hypertension, diabetes, hyperlipidemia, and obesity (Wang et al., 2008). It has been reported that garlic induces the endothelium-dependent relaxation on rat aorta (Oztürk et al., 1994) and that some polysulfides derived from garlic promote the production of H_2S in isolated rat aorta (Benavides et al., 2007).

Aged garlic extract (AGE) is one of the garlic preparations produced through extraction for more than 10 months. While fresh garlic (*Allium sativum* L.) includes some irritating and unstable compounds such as allicin and ajoene, AGE is characterized by non-irritating and stable sulfur compounds such as *S*-allylcysteine (SAC), *S*-1-propenylcysteine (S1PC), and *S*-allylmercaptocysteine (SAMC) (Kodera et al., 2003; Matsutomo and Kodera, 2016). Recent studies have revealed that AGE has the ability to lower blood pressure in uncontrolled hypertensives (Ried et al., 2013), and suppresses elevation of systolic blood pressure with age in spontaneously hypertensive rats (SHR) (Harauma and Moriguchi, 2006), and increases the blood concentration of NO metabolites in mice (Morihara et al., 2002). These observations suggest the involvement of AGE in the production of vasoactive substances such as NO during the vasoconstrictive and vasorelaxant response of aorta.

The aim of the present study was to investigate the vasorelaxant effect of AGE on the aorta and its mechanism of action in order to clarify the blood pressure-lowering action of AGE.

Abbreviations: ACh, acetylcholine; AGE, aged garlic extract; COX, cyclooxygenase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; eNOS, endothelial nitric oxide synthase; GSAC, γ-glutamyl-S-allylcysteine; H_2S, hydrogen sulfide; L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PAG, DL-propargylglycine; S1PC, S-1-propenylcysteine; SAC, S-allylcysteine; SAMC, S-allylmercaptocysteine; SHR, spontaneously hypertensive rats; SMC, S-methylcysteine.

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Materials and methods

Chemicals

Norepinephrine hydrochloride (NE), acetylcholine chloride (ACh), and DL-propargylglycine (PAG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), indomethacin, L-arginine, L-cysteine hydrochloride monohydrate, and solvents for HPLC and LC-MS were obtained from Wako (Osaka, Japan). L-Methionine was from Ajinomoto-Takara Co. (Tokyo, Japan). L-Glutamic acid and chemicals for chemical synthesis and post-column HPLC detection reagents were from Tokyo Chemical Industry Co. (Tokyo, Japan). *S*-Methylcysteine (SMC), *S*-methylmercaptocysteine, SAC, S1PC, γ-glutamyl-*S*-allylcysteine (GSAC), SAMC, and γ-glutamyl-*S*-allylmercaptocysteine were synthesized and purified according to previous reports (Matsutomo and Kodera, 2016). Nα-(1-Deoxy-D-fructos-1-yl)-L-arginine was prepared according to the previous report (Ryu et al., 2001). These reagents were dissolved in below-mentioned Krebs buffer.

Preparation of AGE

The specific procedure and specifications of preparation of AGE are listed in, and comply with the US Pharmacopeia/Natural Formula for garlic fluid extract monograph (United States Pharmacopeial Convention I, 2015). Briefly, AGE was prepared by rinsing cloves of garlic (*Allium sativum* L.) with purified water, and slicing them and soaking them in ethanol 20–50% (v/v), which was then extracted/aged for more than 10 months at room temperature. The AGE was concentrated to about 50% in vacuo. The concentrate was dried using a Pulvis GB22 spray dryer (Yamato, Japan) at an inlet temperature of 150 °C, an outlet temperature of 90 °C, an atomizing air pressure of 0.14 MPa, and a drying air flow of 0.45 m^3/min. The yield of dry extract used in this experiment was more than 20%, and we confirmed that it contains SAC not less than 0.05% as the marker compound. The powder obtained was stored at 4 °C before use.

Analysis of AGE

HPLC analysis was performed by post-column HPLC method using a NEXERA X2 system (Shimadzu, Japan) and LC-MS analysis was carried out on a system consisting of an Ultimate 3000 (Dionex/Thermo Fisher Science, USA) coupled to a Q-Exactive (Thermo Fisher Science, USA) according to previous report (Matsutomo and Kodera, 2016). The column utilized for separation was a Cadenza CD-C18 column (2.0 mm × 150 mm, 3 µm) (Imtakt Corporation, Japan).

Animals and preparation of isolated aortic rings

The experimental system to evaluate the response of rat aorta was performed following the method of Andriambeloson et al. (1997). Male Wistar rats (12 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Rats were anesthetized using isoflurane and killed by decapitation, and the descending thoracic aorta was carefully excised and placed in a petri dish filled with Krebs buffer containing (in mM): NaCl 118.5, KCl 4.74, $CaCl_2$ 2.5, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 24.9, and glucose 11.1. The aorta was cleaned of excess connective tissue and fat and cut into rings approximately 3 mm in length. In the group of endothelium-denuded aorta, the endothelium layer was removed by gently rubbing inside the lumen using a rough-surfaced needle. The aortic rings were attached to a force-displacement transducer FD Pick Up (Nihon Kohden, Japan) connected to a Polygraph System RM-6200 (Nihon Kohden) and recorded on a Thermal Array Recorder (Nihon Kohden) to measure their contractile force. The tissue bath was maintained at 37 °C and bubbled with a 95% O_2–5% CO_2 gas mixture. The aortic rings were allowed to equilibrate for 90 min under the initial tension of 2 g in 20 ml organ baths containing Krebs buffer, and then rinsed with Krebs buffer 4–5 times. The presence or absence of endothelium was confirmed by visual inspection under microscope as well as observing the relaxation response of aortic rings to 1×10^{-3} M ACh after 3×10^{-6} M NE addition. These animal studies were approved by the Animal Care and Use Committee of Wakunaga Pharmaceutical Co., Ltd. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication, 8th Edition, 2011).

Concentration-response curve of NE contraction

The following experiments were performed on the aortic rings after equilibration. NE was added to the tissue bath at progressively increasing cumulative concentrations ranging from 1×10^{-8} to 1×10^{-4} M at 2 min intervals.

Concentration-response curve of ACh and AGE relaxation

Aortic rings were contracted by a single submaximal concentration of 3×10^{-6} M NE. When the contractile response to NE was stable 30 min after NE addition, ACh (from 3×10^{-8} to 1×10^{-3} M) or AGE (from 0.001 to 1% w/v) was added to the aortic rings at progressively increasing cumulative concentrations at 2 min intervals. Each constituent of AGE was added respectively at the concentration equivalent to 0.5% (w/v) AGE and then the vasorelaxation was measured 2 min after addition of each constituent. All results are expressed as percentages of the maximal NE-induced contraction.

Investigation of mechanism of AGE action on relaxation

L-NAME (1×10^{-4} M), PAG (5×10^{-5} M), and indomethacin (5×10^{-6} M) were respectively added to the rings in an organ bath 5 times at 10 min intervals, and the rings were contracted by NE at 3×10^{-6} M 10 min after last addition of each inhibitor. cPTIO (1×10^{-4} M) and ODQ (1×10^{-6} M) were added 25 min after and 10 min before NE contraction, respectively. AGE was added to the tissue bath at progressively increasing cumulative concentrations ranging from 0.001 to 1% (w/v) in the presence of L-NAME and at a single concentration of 0.5% (w/v) in the presence of PAG, indomethacin, cPTIO, and ODQ 30 min after NE addition. In the experiments with PAG, indomethacin, cPTIO, and ODQ, the vasorelaxation was measured 2 min after addition of AGE. The above-mentioned experiments were performed on aortic rings that retained endothelium. All results are expressed as percentages of the maximal NE-induced contraction.

Measurement of NO metabolites

Part of Krebs buffer in the bath was collected 10 min after the addition of 0.5% (w/v) AGE, and NO metabolites level (the sum of NO_2^- and NO_3^- level) was analyzed using nitrate/nitrite colorimetric assay kit (Cayman chemical, USA).

Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM). The statistical significance of differences was determined

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