Journal of Ethnopharmacology 145 (2013) 162–167

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Aged garlic extract restores nitric oxide bioavailability in cultured human endothelial cells even under conditions of homocysteine elevation

Norbert Weiss [a,b,*], Louisa Papatheodorou [a], Naoaki Morihara [a,1], Robert Hilge [a], Nagatoshi Ide [a,2]

[a] Center for Vascular Medicine, Section Angiology, University of Munich Medical Center, City Campus, Munich, Germany
[b] Center for Vascular Medicine and Department of Medicine, Division of Angiology, University Hospital Carl Gustav Carus at the Technical University Dresden, TU Dresden, Germany

ARTICLE INFO

Article history:
Received 7 August 2012
Received in revised form
5 October 2012
Accepted 24 October 2012
Available online 2 November 2012

Keywords:
Aged garlic extract
Nitric oxide
Homocysteine
Tetrahydrobiopterin
Antioxidant
Endothelium

ABSTRACT

Ethnopharmacological relevance: Supplementation with aged garlic extract (AGE) has been shown to restore impaired endothelium-dependent vasodilator response in subjects with acutely elevated plasma homocysteine (Hcy) levels after an oral methionine load and in patients with chronic coronary artery disease. Moreover, AGE has been shown to inhibit the progression of coronary calcifications in patients with coronary artery disease. The molecular mechanisms, by which AGE preserves endothelial function is unknown. Our objective was to explore whether AGE preserves endothelial nitric oxide (NO) output even under conditions of elevated Hcy levels by preventing oxidative inactivation of the NO synthase cofactor tetrahydrobiopterin.

Material and methods: Endothelial (EA.hy 926) cells were incubated with hypoxanthine, aminopterin, thymidine and methionine (HAT/MET) to increase cellular Hcy levels, and with and without AGE. Agonist stimulated NO output was measured using the fluorescent probe DAF-2, and cellular thiol levels (Hcy, cysteine, reduced and oxidized glutathione) and cellular tetrahydrobiopterin levels were measured by high performance liquid chromatography.

Results: HAT/MET incubation resulted in significantly increased cellular Hcy levels, unaffected by coincubation with AGE. Elevated Hcy went along with significantly decreased NO output (to $34.4 \pm 4.4\%$ of control) and levels of tetrahydrobiopterin (from 4.67 ± 2.17 to 2.17 ± 0.97 pmol/mg). Incubation with AGE (5 mg/mL) in HAT/MET-treated cells prevented the declines in NO output and tetrahydrobiopterin levels. AGE increased cellular levels of cysteine and total glutathione, and prevented glutathione and tetrahydrobiopterin oxidation induced by elevated Hcy.

Conclusion: Incubation with AGE preserved normal NO output from endothelial cells even under conditions of elevated Hcy levels by increasing cellular thiol antioxidant and prevention of tetrahydrobiopterin oxidation. This suggests that AGE might be useful in the prevention of endothelial dysfunction.

1. Introduction

Elevated plasma homocysteine (Hcy) levels are associated with an increased risk of cardiovascular diseases (Bautista et al., 2002). Lowering plasma Hcy levels in patients with severe hyperhomocysteinemia has been shown to substantially reduce cardiovascular event rates (Yap et al., 2001). Whether or not reduction plasma Hcy levels in patients with mildly or moderately Hcy levels translates to a reduced risk for cardiovascular disease is still under debate. Intervention studies for lowering Hcy levels by B-vitamins has been shown to reduce the incidence of stroke (Wang et al., 2007) but not of coronary events and total cardiovascular mortality (Bonaa et al., 2006). In addition, not all hyperhomocysteinemic patients are responsive to B-vitamin supplementation in respect to Hcy lowering. Especially patients with chronic renal failure, who have a dramatically increased risk of cardiovascular disease partly mediated by increased plasma Hcy levels, do not sufficiently respond to folic acid and vitamin B12 supplementation (Dierkes et al., 2001; Austen et al., 2003). Therefore, alternative treatment strategies are needed.

Antioxidant treatment strategies are a promising alternative for high-risk patients, especially patients with chronic renal failure. Supplementation with lipophilic antioxidants like vitamin E, which primarily protects cell membranes from lipid peroxidation, has not been shown to be effective in decreasing cardiovascular risk in high-risk populations (Yusuf et al., 2000; Lonn et al., 2002). On the

* Corresponding author at: Center for Vascular Medicine and Department of Medicine – Division of Angiology, University Hospital Carl Gustav Carus at the Technical University Dresden, Fetscherstrasse 74, D-01307 Dresden, Germany. Tel.: +49 351 458 4243; fax: +49 351 458 4359.
E-mail address: norbert.weiss@uniklinikum-dresden.de (N. Weiss).
[1] Present address: Wakunaga Pharmaceuticals Co., Ltd. Hiroshima, Japan.
[2] Present address: Wakunaga of America Co., Ltd., Mission Viejo, CA, USA.

0378-8741/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2012.10.045

other hand, a small pilot clinical study in patients with end-stage renal disease has documented a significant reduction in cardiovascular events by supplementation with cysteine donors, which increase cellular total thiol and glutathione levels, and therefore may act as intracellular antioxidants (Tepel et al., 2003).

Garlic (*Allium sativum* L) and its constituents have been used in traditional medicine for centuries. Among the various garlic preparations available, aged garlic extract (AGE) is uniquely produced by an up to 20-month natural aging process. During this aging process, the odorous, harsh and irritating compounds in raw garlic are converted naturally into stable and safer organosulfur compounds. AGE contains primarily water-soluble organosulfur compounds, such as *S*-allylcysteine and *S*-allylmercaptocysteine, which may act as cellular donors of thiol containing reducing equivalents, comparable to *N*-acetylcysteine, and water-soluble nonsulfur compounds and Maillard reaction products (Amagase et al., 2001; Ryu and Rozen, 2003).

Garlic and AGE show many pharmacological effects, including prevention of cardiovascular diseases (Efendy et al., 1997; Rahman and Billington, 2000; Campbell et al., 2001; Budoff et al., 2004,2009; Williams et al., 2005; Weiss et al., 2006), enhancement of immune function (Kyo et al., 2001; Ichikawa et al., 2006) and antioxidant properties (Ide and Lau, 1999;Lau, 2001; Dillon et al., 2002). Cardiovascular effects of AGE are thought to be at least partly due to its antioxidant and thiol modifying properties (Ide and Lau, 2001; Lau, 2001; Mousa and Mousa, 2005). More recently, AGE has been reported to improve endothelial function and retard the progression of coronary calcifications in patients with coronary artery disease, who are already treated with statins (Budoff et al., 2004, 2009).

Endothelial dysfunction caused by decreased bioavailability of NO is a key event in vascular pathobiology associated with elevated Hcy levels (Weiss, 2005). This is at least partly due to a decrease in cellular tetrahydrobiopterin levels owing to increased oxidative inactivation under conditions of elevated Hcy levels (Topal et al., 2004). We have previously shown that a cysteine donor increases cellular levels of total thiols and glutathione and preserves normal endothelial function even under conditions of elevated Hcy levels in a mouse model of mild hyperhomocysteinemia (Weiss et al., 2002). In addition we have shown that AGE supplementation restored impaired endothelium-dependent vasodilator response in subjects with acutely elevated plasma Hcy levels after an oral methionine load (Weiss et al., 2006).

In the present in-vitro study we thought to elucidate whether supplementation of AGE in cultured endothelial cells preserves NO output in cells exposed to elevated Hcy levels and explored possible mechanisms behind it.

2. Material and methods

Chemicals used were obtained from Sigma-Aldrich, Taufkirchen, Germany, unless otherwise stated.

2.1. Aged garlic extract

AGE is manufactured as follows: garlic cloves (*Allium sativum* L.) grown under strictly controlled organic conditions without the use of chemical fertilizers, herbicides or pesticides are sliced and soaked in an aqueous ethanol solution and extracted/aged up to 20 months at room temperatures. Chromatographic experiments including a LC/MS method identified that AGE as used in this study contained *S*-allylcysteine as a major sulfur-containing compound in the range of 1.6–2.4 mg/g (dry weight) (Ryu and Rozen, 2003). Other constituents such as *S*-allylmercaptocysteine, fructosyl-arginine and tetrahydro-β-carboline derivates have also been identified (Ryu et al., 2001; Amagase, 2006; Ichikawa et al., 2006). *S*-Allylcysteine concentration is used to standardize the extract according to the US Pharmacopeia/Natural Formula (USP/NF) garlic fluid extract monograph (Anonymus, 2005). AGE was kindly provided as an aqueous solution by Wakunaga of America Co. Ltd. (Mission Viejo, CA, USA), and added to the cell culture medium to be adjusted at 5 mg/mL as the final concentration.

2.2. Cell culture

In-vitro experiments were performed using the human endothelial cell line EA.hy 926 (Edgell et al., 1983) derived from fusing human umbilical vein endothelial cells with the permanent human cell line A549. The cells were a generous gift from Dr. Cora Edgell, University of North Carolina, Chapel Hill, NC, USA. Passages 34 to 38 were used in this study. EA.hy 926 cells maintain an endothelial phenotype characterized by expression of endothelial cell (EC) markers and endothelial NO synthase, by expression of adhesion molecules and secretion of chemokines (Postea et al., 2006). Cells were maintained in DMEM containing 900 mg/L D-glucose, 10% heat-inactivated FBS, and antibiotics (100 units/mL penicillin G sodium and 100 μg/mL streptomycin sulfate) (Invitrogen, Karlsruhe, Germany). Culture plates were maintained in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Cells were subcultured after treatment with 0.05% trypsin and 0.53 mmol/L disodium EDTA (Invitrogen). Cells were seeded in culture flasks, and allowed to grow to confluence before experimental treatment. Viability of cells throughout the experiment was always $>95\%$ as determined by trypan blue exclusion.

2.3. Induction of elevated homocysteine levels

Cellular levels of Hcy were increased by methionine supplementation (2 mmol/L) and inhibition of folate metabolism using aminopterin (0.4 μmol/L). DNA synthesis was rescued by supplementation of hypoxanthine (100 μmol/L) and thymidine (16 μmol/L) for 48 h (L-methionine and HAT-media supplement).

2.4. Determination of nitric oxide output from endothelial cells

Cellular NO output was measured using 4,5-diaminofluoresceine (DAF-2, Cayman Chemical, distributed by IBL, Hamburg, Germany) as described previously (Weiss et al., 2001) with slight modifications (Leikert et al., 2001). Briefly, after treatment with the test substances as indicated, cells were washed twice, and incubated with DAF-2 (0.1 μmol/L) and L-arginine (100 μmol/L) in PBS. Cells were stimulated using calcium ionophore A23187 (10 μmol/L) and incubated in the dark for 60 min at 37 °C and 5% CO_2. Cells pretreated with L-NAME (2 mmol/L) served as negative control. After incubation, the cell supernatant was taken, centrifuged, and fluorescence of the supernatant was measured after exciting at 495 nm at an emission wave length of 515 nm using a L5 SS fluorescence spectrometer equipped with FL WinLab 4.0 software (Perkin Elmer, Waltham, MA, USA). The excitation and emission slit width was set at 10 nm, and the detector amplification at 850 V. Blanks (DAF-2 solution with L-arginine and calcium ionophore) were subtracted from measured values. Values obtained where within the linear range of the assay as shown by comparison with standard curves generated using the NO donor diethyl-NONOate (2 nmol/L to 1 μmol/L, Cayman Chemical). Equal loading of cell culture plates with endothelial cells was confirmed by measuring protein concentrations of cells detached from the plates at the end of the experiments. Measurements are expressed as arbitrary units according to fluorescence intensities of the readings.