Vinegar Intake Enhances Flow-Mediated Vasodilatation via Upregulation of Endothelial Nitric Oxide Synthase Activity

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This study examined the effect of acetate on endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (HUVECs) by immunoblotting assay and the ability of acetic acid to upregulate flow-mediated vasodilatation in humans. In HUVECs, acetate induced a biphasic increase in the phosphorylated form of eNOS. The amount of phosphorylated eNOS was significantly increased by exposure to 200 μmol/l acetate for 20 min (early phase) and for 4 h (late phase). The inhibitors of cAMP-dependent protein kinase (PKA) and AMP-activated protein kinase (AMPK) blocked acetate-induced eNOS phosphorylation in the early and the late phase respectively. Furthermore, in postmenopausal women, maximum forearm blood flow (FBF) in response to shear stress increased in the vinegar (acetic acid) administered group compared to the placebo group. These results suggest that acetic acid-induced eNOS phosphorylation contributes to upregulation of flow-mediated vasodilatation in humans.

Key words: acetic acid; endothelial function; endothelial nitric oxide synthase (eNOS) phosphorylation; AMP-activated protein kinase (AMPK); PKA

Vinegar is commonly used as a seasoning. It has a very long history, and its existence was recorded in ancient Babylon (5000 BC).1 It has also been used traditionally as a folk medicine and is believed to have several health benefits. In animal studies, it has been found that vinegar has such effects as stimulation of Ca absorption,2 enhancement of recovery from fatigue,3 found that vinegar has such effects as stimulation of Ca absorption,2 enhancement of recovery from fatigue,3 increased in the vinegar (acetic acid) administered group compared to the placebo group. These results suggest that acetic acid-induced eNOS phosphorylation contributes to upregulation of flow-mediated vasodilatation in humans.

Hypertension, hypercholesterolemia, and postprandial hyperglycemia are major cardiovascular risk factors, all of which culminate in endothelial dysfunction and cause arteriosclerosis.10,11 Most of all, lowered production of endothelium-derived nitric oxide (NO) is the major feature of endothelial dysfunction, and endothelial NO synthase (eNOS) is responsible for NO production in blood vessels. Indeed, impairment of the eNOS/NO pathway is considered to lead to various cardiovascular disorders via endothelial dysfunction.12 Activation of eNOS is mostly dependent on phosphorylation at Ser-1177;13 phosphorylation at this site is catalyzed by several protein kinases, including cAMP-dependent protein kinase (PKA), protein kinase B (Akt), and AMP-activated protein kinase (AMPK).14 An infusion of acetate has been shown to increase portal blood flow via vasodilatation in rats.15 Additionally, Daugirdas and Nawab (1987) reported that acetate had a vasorelaxant effect in vascular smooth muscle.16 We, on the other hand, found that acetate induced AMPK phosphorylation both in mouse liver and rat hepatocytes.17 Thus these reports suggest that acetate contributes to enhancement of flow-mediated vasodilatation through AMPK activation.

To evaluate the effect of acetate in the endothelium, initially we examined to determine whether acetate would activate eNOS via AMPK activation or via other pathways in human umbilical vein endothelial cells (HUVECs). Furthermore, we conducted a pilot clinical study using plethysmography to assess the effect of intake of vinegar on flow-induced vasodilatation in humans.

Materials and Methods

Cell culture experiments.
Cell culture. HUVECs were isolated as described by Jaffe et al.18 and cultured in an endothelial growth medium, HuMedia-EG2 (KE-2150S, Kurabo, Osaka, Japan). The cells were cultured in collagen type I-coated 6-well plates (356400, BD, Tokyo).

Protein preparation. Nearly confluent HUVECs were starved for 4 h in HuMedia-EB2 (serum free medium: KE-2350S, Kurabo),

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Abbreviations: eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; FBF, forearm blood flow; AMPK, AMP-activated protein kinase; NO, nitric oxide
supplemented with 1% penicillin-streptomycin liquid (15070-063, Gibco, San Francisco, CA) and 0.1% bovine serum albumin (BSA) (A6003-25 g, Sigma-Aldrich, St. Louis, MO) to prevent serum factors from influencing the results. After starvation, the cells were placed in HuMedia-EB2, supplemented with 0.1% BSA and sodium acetate (final concentration: 0 for the control group, 100, 200 μM/L) and then incubated for 5 min to 4 h. After stimulation with acetate, the medium was replaced with 150 μL of RIPA buffer and the cells were scraped into microtubes. The scraped cells were sonicated twice for 5 s at 4°C and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatants were collected and the protein concentration determined by BCA protein assay.

Measurement of eNOS and AMPK activity. Activities of eNOS and AMPK were assessed by the ratio of the phosphorylated to the total activity of each protein. As the importance of eNOS phosphorylation at Ser-117713) and AMPK phosphorylation at Thr-17219) for their enzymatic activity have been demonstrated, we measured their phosphorylation levels as an indicator of enzymatic activities. Quantitation of phosphorylated and total eNOS or AMPK activity levels was carried out by immunoblotting using the following specific antibodies:12,20) anti-phospho-eNOS (Ser-1177) (#9571S, Cell Signaling, Danvers, MA); anti-eNOS (#9572, Cell Signaling); anti-phospho-AMPK (Thr-172) (#2535, Cell Signaling); and anti-AMPK (#2532, Cell Signaling).

Immunoblotting assays were performed as follows: The prepared protein (10 μg) was size-fractionated by SDS-PAGE (414855, Cosmo Bio, Tokyo) and then transferred electrophoretically to a PVDF membrane (RPN303F, GE Healthcare, Little Chalfont, England). The membrane was blocked by 1 h incubation in TBS buffer (1× TBS, 0.1% Tween 20) containing 5% BSA (A3059-50 g, Sigma-Aldrich) at room temperature, then immunoblotted with the primary antibody described above for 1 h at room temperature, followed by horseradish peroxidase-conjugated secondary antibody (65-6120, Zymed, San Francisco, CA) for 45 min at room temperature after washing (10 min × 3) with the same buffer. Immunoreactive bands were detected and quantified using an ECL Western blotting detection kit (RPN2209, GE Healthcare) and a lumino-image analyzer, LAS-1000plus (FUJI FILM, Tokyo). The results were presented as percentages of the control group.

Inhibitor assay. HUVECs were pre-treated with an inhibitor or an antagonist for 30 min in HuMedia-EB2 before acetate stimulation, and the cells were then incubated with acetate at 5 min or 4 h. The concentrations for the inhibitor and antagonist were as follows: Compound C (AMPK inhibitor) (171260, Merck, Tokyo) 40 μM/L; H89 (PKA inhibitor) (EI196-0025, Biomol, Plymouth Meeting, PA) 20 μM/L; S-Cyclopentyl-1, 3-dipropylxan-thine (DPCPX, adenosine receptor A1 antagonist) (C101-25MG, Sigma-Aldrich) 100 μM/L; 8-(3-Chlorostyryl) caffeine (CSC, adenosine receptor A2a antagonist) (C197-25MG, Sigma-Aldrich) 10 μM/L; and MRS1754 (adenosine receptor A2b antagonist) (M6316-5MG, Sigma-Aldrich) 200 μM/L. Protein preparation and immunoblotting were carried out in the manner described above.

Clinical study. Subjects’ characteristics. Twelve postmenopausal females who were healthy but excessively sensitive to cold were enrolled in this clinical study, because their flow-mediated vasodilatation was thought to be blunted by an imbalance of female hormones. Postmenopausal women have vasomotor symptoms such as hot flashes/flashes, cold hands and feet (‘cold flushes’ or ‘cold syndrome’), night sweats, palpitations, vertigo, headaches, or lower back pain, most likely due to a disruption in pathways modulated by female hormones. Estrogen, by inducing the activity of eNOS, increases NO production and has a favorable effect on arterial vasomotority.21-25) It is well demonstrated that postmenopausal status is associated with significantly reduced arterial NO activity26) and thus leads to an increase in local vascular resistance and to impairment of endothelium-dependent flow-mediated vasodilatation.27) Factors which contribute to excessive sensitivity to cold26) may thus lead to an instability of excessive sensitivity to cold. Vasomotor instability of excessive sensitivity to cold was determined by a 10-question interview,27) which contained typical complaints of those suffering from unusual coldness.27) The study subjects were women who had been without a menstrual cycle for the past 12 months consecutively26) and answered yes to more than seven questions in the interview. Subjects were excluded on the basis of the following criteria: food allergy, use of lipid-lowering drugs, smoking, blood donation (200 ml or more within month, or 400 ml or more within 3 months), or who were deemed unsuitable on the basis of a physician’s diagnosis of serious cardiovascular, endocrine, metabolic, respiratory, liver, or renal disorders requiring treatment. The ethics committee of Soiken Clinic approved the study protocol. In accordance with the Declaration of Helsinki, all subjects were adequately informed on this study by physicians, and written informed consent for participation was obtained from all subjects.

Test materials. Three test samples and a placebo were used, prepared by Mizkan Group Corporation (Aichi, Japan). The three test samples contained rice vinegar (test sample A), black vinegar made from unpolished rice (unpolished rice vinegar, test sample B), and black vinegar made from forbidden rice, which is a kind of ancient rice (forbidden rice vinegar, test sample C). Each sample contained 15% vinegar, and all the vinegars contained 4-5% w/v acetic acid; there were no differences in composition (calorie, 15 kcal; lipid, 0 g; protein, lower than 1 g; carbohydrate, 3 g) among the three vinegar and placebo drinks. The placebo contained freeze-dried rice vinegar powder made from 15 ml rice vinegar and lactic acid solution, and the total volume was adjusted to 100 ml. Acetic acid was removed from the rice vinegar during the freeze-dry process. Lactic acid was added to adjust the sensory attributes of placebo to as close as possible to those of the test samples. Subjects ingested 100 ml of one of the test samples or the placebo per day.

Effect of intake of vinegar on endothelial function. We conducted a double-blind crossover study. The clinical study protocol was summarized in Fig. 1. Twelve subjects were enrolled, and were randomly divided into four groups. Two of the 12 subjects dropped out of the study for personal reasons. Therefore a total of 10 subjects (54.30 ± 5.02 years old, mean ± SD) were enrolled. The subjects took one of the test samples or the placebo at breakfast every day for 3 d. On the fourth day, when forearm blood flow (FBF) was to be measured, the subjects took the test sample or the placebo under the direction of the study director before FBF was measured (details described below). The washout period between interventions was 10 d (×2.5 the intake period, based on the clinical study of Hirota et al., 200727)). Each subject was asked to avoid making any changes in lifestyle, dietary habits, alcohol consumption, or amount of exercise during the study. Furthermore, ingestion of diets and drinks containing significant amounts of vinegar and excessive exercise were forbidden.

FBF measurement using strain-gauge plethysmograph. All measurements were made under the same conditions. The subjects entered a air-conditioned room (21 ± 2°C). After 30 min, they ingested one of the samples or the placebo, and stayed in the room for more than 105 min. They were subsequently placed in a supine position, and three cuffs and a strain-gauge were attached to the forearm opposite to subject handness. The subjects had rested for 15 min in the supine position, measurement of FBF was started using a strain-gauge plethysmograph (EC-6; D.E. Hokanson, Bellevue, WA). Arterial FBF was initially measured as basal blood flow. Then a cuff over the forearm was inflated to a pressure of 200 mmHg and the forearm was compressed for 5 min. Subsequently, cuff occlusion was released and arterial FBF was measured every 15 s for a further 5 min.

Analytical methods. Flow-induced dilatation of the forearm arteries was evaluated as the increase in the rate of maximum FBF after release of forearm cuff occlusion as compared to the baseline FBF.

Statistical analysis. The results of cell culture experiments and the clinical study were expressed as mean values ± SE. In comparison with the control group in the cell culture experiments and with the placebo group in the clinical study, differences among groups were analyzed by Dunnett’s multiple comparison test. A p value of less than 0.05 was accepted as statistically significant. Data were processed using the software package SPSS ver.11.5 (SPSS Japan, Tokyo).

Results. Exposure of HUVEC to acetate for 5 min to 4 h induced an increase in eNOS phosphorylation at Ser-1177 (Fig. 2A, B), but there was no effect on total eNOS expression (Fig. 2B). As shown in Fig. 2A, when cells...
were incubated with 100 or 200 μmol/l of acetate for 5–20 min (short-term stimulation) and 2–4 h (long-term stimulation), the amount of phosphorylated eNOS significantly increased in a dose-dependent manner in each case (Fig. 2A, p < 0.05).

Next we investigated the effect of acetate on activity of AMPK, which catalyses eNOS phosphorylation, as described above. Exposure of HUVEC to acetate for 4 h induced an increase in AMPK phosphorylation at Thr-172 (Fig. 3A, B), but there was no effect on total AMPK expression (Fig. 3B). As shown in Fig. 3A, when cells were incubated with either 100 or 200 μmol/l acetate for 2–4 h, the levels of phosphorylated AMPK increased significantly (p < 0.05), but exposure to acetate for 5–60 min had no effects on AMPK phosphorylation. Furthermore, when cells were exposed to 100 or 200 μmol/l acetate with 40 μmol/l compound C (AMPK inhibitor) for 4 h, acetate-induced eNOS phosphorylation at Ser-1177 was suppressed (Fig. 3C), whereas it had little effect on short-term (20 min) acetate stimulation (Fig. 3D). Furthermore, H89 (a PKA inhibitor) suppressed eNOS phosphorylation induced by short-term (20 min) acetate exposure (Fig. 4A). On the other hand, adenosine receptor antagonists (A₁ antagonist, 100 μmol/l DPCPX; A₂A antagonist, 10 μmol/l CSC; and A₂B antagonist, 200 nmol/l MRS1754) did not prevent eNOS phosphorylation by short-term (20 min) acetate stimulation in HUVEC (Fig. 4B, C, D), although eNOS activation via PKA is reportedly evoked by adenosine receptor stimulation of the rat aorta.³⁰

To elucidate the efficacy of acetic acid on improving flow-mediated dilatation in human blood vessels, we performed a plethysmography test in postmenopausal women with excessive sensitivity to cold. This test revealed an increase in maximum (normalized) FBF in response to shear stress in all the vinegar intake groups (Fig. 5). The maximum FBF, which was measured immediately after the release of forearm occlusion (0 min), increased 13.1-fold from the basal blood flow in the rice vinegar intake group, 14.1-fold in the unpolished rice vinegar intake group, and 19.5-fold in the forbidden rice vinegar intake group (p < 0.05 in comparison with the placebo intake group), whereas it increased 8.0-fold in the placebo intake group (Fig. 5). In comparison with the placebo group, the increase rates of maximum FBF in the forbidden rice vinegar group in comparison to the placebo group (p < 0.05), but there were no statistical differences between the rice vinegar or unpolished rice vinegar and placebo groups (Fig. 5). The increase rates of maximum FBF in all groups dropped to almost the same level at 15 s after release of forearm occlusion. On the other hand, there were no significant differences in systolic (SBP) and diastolic (DBP) blood pressure before and after 4 d of intake in the vinegar and placebo groups (SBP/DBP (mean ± SD, n = 10): initial values, 121.4 ± 15.6/68.6 ± 6.4 mmHg; unpolished rice vinegar post-intake values, 121.0 ±
Discussion

In this study, we found that acetate activated eNOS in the endothelium, and further, that intake of vinegar, the main component of which is acetic acid, upregulated flow-mediated vasodilatation in postmenopausal women in our preliminary clinical study.

In HUVEC, acetate phosphorylated the Ser-1177 of eNOS in a biphasic manner at 5–20 min (the early phase eNOS phosphorylation) and 2–4 h (the late phase eNOS phosphorylation) (Fig. 2). Additionally, the extent of acetate-induced phosphorylation of eNOS in both phases increased in a dose-dependent manner in a range of 50–200 µmol/l of acetate. The early and late phases of phosphorylation peaked at 20 min and 4 h respectively. AMPK Thr-172 was phosphorylated at 2–4 h, but there were no significant differences at earlier time points up to 1 h (Fig. 3A). Furthermore, AMPK inhibitor compound C reduced the late but not the early phase of eNOS phosphorylation by acetate (Fig. 3C, D). These results indicate that the late eNOS phosphorylation by acetate was dependent on AMPK activation. When acetate is metabolized by acetyl-CoA synthase, AMP is simultaneously produced: acetate + CoA + ATP → acetyl-CoA + AMP + pyrophosphate. Confirming this reaction, it has been reported that in hepatocytes, acetate activates AMPK.17,31) The late phase effect in the endothelium in the present study was most likely an identical effect of acetate.

Phosphorylation of eNOS at Ser-1177 is regulated by AMPK, PKA, Akt, or calmodulin kinase-II (CaMK-II).32) The present results indicated that AMPK activation due to acetate did not lead to phosphorylation of eNOS in the early phase, but rather in the late phase. Although neither Akt nor CaMK-II inhibitors reduced early phosphorylation due to acetate (data not shown), phosphorylation was abolished by H89, a PKA inhibitor (Fig. 4A). Therefore, it is possible that the early eNOS phosphorylation by acetate was due to PKA. In a previous report, an infusion of acetate resulted in vasodilatation via adenosine receptors A1 and A2.15) In addition, Ray and Marshall (2006) suggested that PKA activates eNOS via adenosine receptors A1 and A2A in the rat endothelium,30) but the present study indicates
that none of the A₁, A₂A, or A₂B antagonists affected eNOS phosphorylation due to acetate (Fig. 4B, C, D). In line with our results, it has been reported that acetate-induced vasorelaxation was associated with a rise in the cyclic AMP (cAMP) level. In that report, the rise in intracellular cAMP levels occurred immediately after incubation with acetate. Furthermore, it has been reported that cAMP activates PKA in the regulatory pathway of endothelial nitric oxide production in blood vessels. While we did not measure cAMP levels in this study, early eNOS phosphorylation by acetate may be independent of adenosine receptors, possibly via PKA, and dependent on yet other mechanisms involving a rise in the cAMP level.

The present results indicate that acetate affected eNOS activity in the endothelium. To investigate the effect of vinegar on endothelial function in humans, we conducted a pilot clinical study with postmenopausal women suffering unusual sensitivity. Postmenopausal status is considered to be a condition associated with a...
decline in flow-induced dilatation. In addition, Ushiroyama et al. (2005) reported reduced blood vessel extensibility in women who were excessively sensitive to cold. Hence we selected postmenopausal women with excessive sensitivity to cold as subjects who had reduced blood vessel extensibility in this pilot study. The results showed that the maximum FBF in all the vinegar intake groups increased more than 1.5 times in comparison to the placebo intake group, indicating that vinegar intake upregulated flow-responses in the postmenopausal women. After an intake of diet containing vinegar, the incremental serum acetate level was more than 50 mmol/l in humans. As described above, eNOS phosphorylation in HUVEC occurred at acetate concentrations of 50–200 mmol/l. Therefore, this effect of vinegar may at least in part be mediated by acetate-
induced eNOS activation. Previous clinical studies indicated that rice vinegar intake reduces high blood pressure in humans. Kajimoto et al. (2001) have demonstrated that blood pressure began to decrease only after 2 weeks of initial vinegar intake, and significant differences were observed only after 4 weeks. In this study, the vinegar intake periods were only 4 d. Possibly, that is not be enough for significant differences to develop. However, vinegar intake for 4 d ameliorated that is not be enough for significant differences to develop. Thus, vinegar intake for 4 d ameliorated flow-mediated vasodilatation in the present study. Thus, the anti-hypertensive effect of vinegar may be attributed to upregulation of flow-mediated vasodilatation. Notably, the maximum FBF in the forbidden rice vinegar intake group was highest among all groups and significantly higher than in the placebo group. Forbidden rice is reported to contain more anthocyanin than other rice. Xu et al. (2004) have demonstrated that anthocyanins induced E N O S phosphorylation. Thus the highest maximum FBF in the forbidden rice vinegar intake group might be attributed to an additional effect of anthocyanin or to a synergistic effect with acetate. 

Vinegar intake is reported to be beneficial for hypertension, hypercholesterolemia, and postprandial elevation of blood glucose. These symptoms are associated with endothelial dysfunction, which leads to cardiovascular disease through the development of arteriosclerosis. Endothelial dysfunction is caused by mal-regulation of eNOS enzymatic activity or inactivation of NO through oxidative stress rather than eNOS gene downregulation. The present study indicates that acetate upregulated eNOS activity by enhancement of phosphorylation of Ser-1177 in HUVEC, and that vinegar intake increased flow-mediated vasodilatation, as shown by plethysmography. There are various effective functional foods to alleviate the above mentioned symptoms, such as hypotensive peptides, hypocholesterolemic polyphenols, and fibers that control postprandial elevation of blood glucose. However, there have been no reports that these foods activate eNOS. As foods in general contain little acetate, taken together with these foods, vinegar may be an effective functional food in preventing cardiovascular diseases through improvement of endothelial function. Further study is necessary to confirm this effect.

The present investigation indicates that acetate induces eNOS phosphorylation in a dose-dependent, biphasic manner, most likely via PKA (the early phase) and AMPK (the late phase) in HUVECs (Fig. 6). Intake of vinegarmight enhance flow-mediated vasodilatation in humans through eNOS phosphorylation. 

References