Geranylgeranylacetone, Heat Shock Protein 90/AMP-Activated Protein Kinase/Endothelial Nitric Oxide Synthase/Nitric Oxide Pathway, and Endothelial Function in Humans

Noritaka Fujimura, Daisuke Jitsuiki, Tatsuya Maruhashi, Shinsuke Mikami, Yumiko Iwamoto, Masato Kajikawa, Kazuaki Chayama, Yasuki Kihara, Kensuke Noma, Chikara Goto, Yukihito Higashi

Objective—Geranylgeranylacetone (GGA) induces expression of heat shock protein 90 (Hsp90), an adaptor molecule for assembly of endothelial nitric oxide synthase (eNOS) phosphorylation complex. The purpose of this study was to determine whether GGA enhances Hsp90 expression and augments endothelium-dependent vasodilation via upregulation of eNOS in humans.

Methods and Results—We evaluated the effects of GGA on human umbilical vein endothelial cells (HUVECs) and on forearm blood flow (FBF) responses to acetylcholine and sodium nitroprusside in 40 healthy young men. Hsp90, eNOS, AMP-activated protein kinase (AMPK), and Akt expression in HUVECs and peripheral blood mononuclear cells was detected by Western blot analysis. GGA increased Hsp90 expression and phosphorylation of eNOS and AMPK but not Akt in HUVECs and increased Hsp90 expression in peripheral blood mononuclear cells. Oral administration of GGA (600 mg) augmented the FBF response to acetylcholine. Infusion of N\textsuperscript{G}-monomethyl-L-arginine, an NO synthase inhibitor, completely abolished GGA-induced augmentation of the FBF response to acetylcholine. GGA also augmented the acetylcholine-stimulated NO release in smokers.

Conclusion—These findings suggest that GGA-induced activation of Hsp90/AMPK significantly increased NO-mediated vasodilation in healthy subjects, as well as in smokers. The use of GGA may be a new therapeutic approach for improving endothelial dysfunction. (Arterioscler Thromb Vasc Biol, 2012;32:00-00.)

Key Words: endothelial function ■ nitric oxide synthase ■ AMPK ■ geranylgeranylacetone ■ heat shock protein 90

Heat shock proteins (Hsps) are present in most cells, including endothelial cells, and play an important role in normal cellular homeostasis and protection of cells from damage in response to stress stimuli. The intracellular concentrations of Hsps can be increased by 2 to 3 times with stress stimuli, such as raised temperature, oxidative stress, hypoxia, inflammation, and ischemia-reperfusion injury.

Several investigators have focused on the interaction of endothelial nitric oxide synthase (eNOS) with Hsp90. Hsp90 is abundant in all eukaryotic cells, accounting for 1% to 2% of cytosolic proteins, and is localized to the cytoplasm, with small amounts found in the nucleus and cytoskeleton. Hsp90 is a molecular chaperone that can interact with signaling molecules, including Src family members, Raf, MEK, and G protein subunits. The Hsp90/Akt/AMP-activated protein kinase (AMPK)/eNOS pathway is an integral part of NO release. Hsp90 upregulates eNOS activity in endothelial cells by forming an eNOS-Hsp90 heterocomplex in response to not only stress stimuli but also stimulation of endothelial cells with vascular endothelial growth factor, estrogen, and statins, leading to eNOS-derived NO production. Geranylgeranylacetone (GGA) is widely used as an antiulcer agent. Recently, GGA has been shown to enhance the expression of Hsp90 in the gastric mucosa and rat liver.

Cardiovascular diseases are associated with endothelial dysfunction. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, resulting in cardiovascular complications. Therefore, from a clinical perspective, it is important to select an appropriate intervention that is effective in improving endothelial dysfunction in cardiovascular diseases. Pharmacological interventions that induce the expression of Hsp90 have some possibility to protect endo-

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Peripheral blood mononuclear cells were collected from the interface at 1200 rpm for 10 minutes. The samples were then centrifuged at 2050 rpm for 20 minutes, which were then layered into Ficol-Paque (Lymphoprep, Nycomed). The samples were then centrifuged at a concentration of 3 mmol/L in absolute ethanol. GFA-free ethanol solution was used as a control vehicle. These stock solutions were stored at 4°C in brown glass vials filled with nitrogen gas until use.

Subjects
Forty healthy young men (mean age, 21.9±2.0 years) were selected as subjects so as to eliminate the possibility of alteration in endothelial function caused by factors such as hypertension, heart failure, atherosclerosis, hypercholesterolemia, diabetes mellitus, smoking, and aging. This was a double-blind, randomized, placebo and active drug study. The 40 subjects were randomly assigned to receive GGA (n = 20) or placebo (n = 20). To confirm the effectiveness of GGA as a therapeutic approach for endothelial dysfunction, we evaluated the effect of GGA on endothelial function in male smokers without cardiovascular risk factors using a protocol identical to the study protocol in healthy subjects. The 24 subjects were randomly assigned to receive GGA (n = 12; mean age, 28.4±3.6 years) or placebo (n = 12; mean age, 29.1±3.9 years). The study protocol was approved by the Ethics Committee of Hiroshima University Graduate School of Biomedical Sciences. Informed consent for participation in the study was obtained from all subjects.

Isolation of Peripheral Blood Mononuclear Cells
After a 30-minute rest period, 600 mg of GGA or placebo was orally administered to each subject. In a preliminary study, we confirmed that circulating levels of GGA after oral administration of GGA (600 mg) were almost equal to 10 μmol/L of GGA in the experimental HUVEC protocol. Peripheral blood samples were obtained from the forearm deep vein before and at 3, 6, 9, 12, and 24 hours after oral administration of GGA or placebo. Peripheral blood samples (7 mL each) were placed in ethylenediaminetetraacetic acid tubes. An equal volume of phosphate-buffered saline was added to the obtained samples, which were then layered into Ficol-Paque (Lymphoprep, Nycomed). The samples were then centrifuged at 2050 rpm for 20 minutes at 20°C to remove erythrocytes and mononuclear cells. Peripheral blood mononuclear cells were collected from the interface and then washed 2 times with phosphate-buffered saline (centrifugation at 1200 rpm for 10 minutes).

Western Blot Analysis
HUVECs and peripheral blood mononuclear cells were lysed on ice in the following lysis buffer: 1% NP-40, 25 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 137 mmol/L NaCl, 10% glycerol, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin. Lysates were scraped off the dish and centrifuged at 15000 rpm for 5 minutes at 4°C, and protein concentrations of the supernatants were determined using the DC protein assay (Bio-Rad). SDS-PAGE and Western blotting were performed using standard procedures. The antibodies used in Western blotting include Hsp90 antibody (SPA-830, StressGen), Hsp70 antibody (SPA-810, StressGen), eNOS antibody (610296, BD Transduction Laboratories), phospho-eNOS (Serine 1177) antibody (612393, BD Transduction Laboratories), Akt antibody (No. 9272, Cell Signaling Technology) and phospho-Akt antibody (No. 9271, Cell Signaling Technology), AMPK antibody (No. 2532, Cell Signaling Technology) and phospho-AMPK antibody (No. 2535, Cell Signaling Technology), polyclonal ERK1/2 antibody (No. 4659, Cell Signaling Technology), polyclonal anti-phospho-specific ERK1/2 antibody (No. 9106, Cell Signaling Technology), p38 mitogen-activated protein kinase antibody (No. 9212, Cell Signaling Technology), phospho-p38 mitogen-activated protein kinase (Thr180/Tyr182) antibody (No. 9211, Cell Signaling Technology), anti-JNK antibody (No. 9252, Cell Signaling Technology), and phospho-JNK antibody (No. 9251, Cell Signaling Technology). Compounds C was purchased from Sigma-Aldrich (P5499). Immunodetection was accomplished using an anti-sheep or anti-mouse secondary antibody (1:5000 dilution) and an enhanced chemiluminescence kit (Amersham).

Measurement of Forearm Blood Flow
Forearm blood flow (FFB) was measured with a mercury-filled Silastic strain-gauge plethysmography (EC-5R, DE Hokanson Inc) as previously described.17

Procedures
The study began at 8:30 AM. Subjects fasted the previous night for at least 12 hours. They were kept in a supine position in a quiet, dark, air-conditioned room (constant temperature of 22°C to 25°C) throughout the study. After subjects had maintained the supine position for 30 minutes, basal FBF was measured. Then, FBF responses to acetylcholine (Daiichi Pharmaceutical), an endothelium-dependent vasodilator, were measured. Acetylcholine infusion was administered at doses of 3.75, 7.5, and 15 μg/minute for 5 minutes, and sodium nitroprusside infusion was administered at doses of 0.75, 1.5 and 3.0 μg/minute for 5 minutes. These studies were carried out in a randomized fashion. Each study proceeded after FBF had returned to baseline. The same procedure was repeated 9 hours after oral administration of GGA (600 mg) or placebo.

To examine the effect of GGA on release of NO, we measured FBF in the presence of the NO synthase inhibitor L-NMMA (8-monomethyl-l-arginine (L-NMMA) (Clinalfa) in all subjects. The responses of forearm vasculature to acetylcholine after intraarterial infusion of L-NMMA (8 μmol/minute for 5 minutes) were evaluated.

Statistical Analysis
Results are presented as the means±SD. Values of P<0.05 were considered to indicate statistical significance. The Mann-Whitney U test was used to evaluate differences between before and after GGA administration. Comparisons between the 2 groups with respect to changes in parameters were performed with adjusted means on an ANCOVA, with baseline data used as covariates. Comparisons of dose-response curves of parameters during infusion of the drug were analyzed by ANOVA for repeated measures with Bonferroni correction.

Results
Effect of GGA on Expression of Hsp90 and Hsp70 in HUVECs
After GGA administration, Hsp90 and Hsp70 protein expression significantly increased at a GGA dose of 3 μmol/L, and the maximum effect was achieved at 10 μmol/L (Figure 1A and 1C). Hsp90 and Hsp70 protein expression levels in HUVECs were significantly increased at 60 minutes after administration of GGA at a concentration of 10 μmol/L, peaked at 90 minutes, and then returned to baseline levels within 240 minutes (Figure 1B and 1D).

Effect of GGA on Phosphorylation and Expression of eNOS in HUVECs
After GGA administration, eNOS phosphorylation significantly increased at a GGA dose of 3 μmol/L, and the maximum effect
was achieved at 10 μmol/L (Figure 2A). eNOS phosphorylation in HUVECs was significantly altered at 60 minutes after administration of GGA at a concentration of 10 μmol/L, peaked at 90 minutes, and then returned to baseline levels within 240 minutes (Figure 2B). The eNOS protein expression in HUVECs was not altered over a period of 90 minutes by GGA at any concentration and was not altered up to 240 minutes by GGA at 10 μmol/L (Figure 2A and 2B).

Effect of GGA on Phosphorylation and Expression of AMPK, Akt, and Mitogen-Activated Protein Kinases in HUVECs

After GGA administration, AMPK phosphorylation significantly increased at a GGA dose of 1 μmol/L, and the maximum effect was achieved at 10 μmol/L (Figure 2C). AMPK phosphorylation in HUVECs was significantly altered at 60 minutes after administration of GGA at a concentration of 10 μmol/L, peaked at 90 minutes, and then returned to baseline levels within 240 minutes (Figure 2D). AMPK protein expression in HUVECs was not altered over a period of 90 minutes by GGA at any concentration and was not altered up to 240 minutes by GGA at 10 μmol/L (Figure 2C and 2D). Akt protein expression and Akt phosphorylation in HUVECs were not altered over a period of 90 minutes by GGA at any concentration and were not altered up to 240 minutes by GGA at 10 μmol/L (Figure 2E and 2F). The expression of ERK1/2 protein, JNK protein, and p38 protein and the phosphorylation of ERK1/2, JNK, and p38 in HUVECs were not altered over a period of 90 minutes by GGA at any concentration and were not altered up to 240 minutes by GGA at any concentration and were not altered up to 240 minutes by GGA.
GGA at 10 μmol/L (Supplemental Figure I, available online at http://atvb.ahajournals.org).

Pretreatment with compound C (20 μmol/L), a specific inhibitor of AMPK, for 30 minutes inhibited the GGA-induced AMPK phosphorylation, Hsp90 expression, and eNOS phosphorylation (Figure 3) but had no effect on the expression of AMPK and eNOS.

Baseline Clinical Characteristics
The baseline clinical characteristics before and at 9 hours after oral administration of GGA or placebo in the 40 healthy subjects the 24 smokers are summarized in Supplemental Tables I and II, respectively. All parameters, including the glucose, insulin, and lipid profiles and the systemic and forearm hemodynamics, were similar before and after administration of GGA or placebo in the healthy subjects and smokers.

Figure 2. Geranylgeranylacetone (GGA) stimulated endothelial nitric oxide synthase (eNOS) phosphorylation in human umbilical vein endothelial cells (HUVECs). eNOS phosphorylation by GGA was dependent on AMP-activated protein kinase (AMPK) phosphorylation and was independent of Akt phosphorylation in HUVECs. HUVECs were stimulated with GGA at the indicated concentrations for 90 minutes (A, C, and E) and with 10 μmol/L GGA for the indicated times (B, D, and F) (n=5 each). *P<0.0001 vs GGA of 0 μmol/L or for 0 minutes.

Figure 3. Pretreatment with compound C (20 μmol/L) for 30 minutes inhibited geranylgeranylacetone (GGA)-induced AMP-activated protein kinase (AMPK) phosphorylation, heat shock protein 90 (Hsp90) expression, and eNOS phosphorylation but had no effect on expression of AMPK and eNOS in human umbilical vein endothelial cells (HUVECs).

Effect of GGA on Expression of Hsp90 and Hsp70 in Peripheral Blood Mononuclear Cells
Oral administration of GGA significantly enhanced the synthesis of Hsp90 and Hsp70 in peripheral blood mononuclear cells of the healthy subjects (Figure 4A and 4C) and smokers.
The induction became apparent from 6 hours to 12 hours after administration of GGA and returned to the baseline level within 24 hours. Placebo did not alter the synthesis of Hsp90 and Hsp70 in the healthy subjects and smokers.

**Effect of GGA on Vascular Function**

Intraarterial infusion of acetylcholine and sodium nitroprusside significantly increased FBF in a dose-dependent manner in the healthy subjects and smokers. The FBF responses to acetylcholine and sodium nitroprusside were similar in the 2 groups of healthy subjects and smokers. Oral administration of geranylgeranylacetone (GGA, n=20), but not the placebo (n=20), augmented the forearm blood flow (FBF) response to acetylcholine (A and C) in the healthy subjects. Sodium nitroprusside-stimulated vasodilation (B and D) was similar before and after GGA (n=20) or placebo (n=20) administration in the healthy subjects. Oral administration of geranylgeranylacetone (GGA, n=12), but not the placebo (n=12), augmented the FBF response to acetylcholine (E and G) in all subjects. Sodium nitroprusside-stimulated vasodilation (F and H) was similar before and after GGA (n=12) or placebo (n=12) administration in smokers.
Intraarterial infusion of L-NMMA reduced baseline FBF from 4.3 ± 1.3 to 2.8 ± 0.9 mL/minute per 100 mL tissue after GGA administration in the healthy subjects (P < 0.001) and baseline FBF from 4.1 ± 1.3 to 2.6 ± 0.8 mL/minute per 100 mL tissue after GGA administration in smokers (P < 0.001) and abolished GGA-induced augmentation of FBF response to acetylcholine in the healthy subjects (Figure 6A) and smokers (Figure 6B). No significant change was observed in arterial blood pressure or heart rate after intraarterial infusion of either acetylcholine or sodium nitroprusside in any of the subjects.

Oral administration of GGA also significantly increased the synthesis of Hsp90 in peripheral blood mononuclear cells in the healthy subjects and smokers. GGA augmented acetylcholine-induced vasodilation in forearm circulation in healthy subjects and smokers through an increase in NO production, whereas the vasodilator responses to sodium nitroprusside did not alter after GGA administration. These findings suggest that GGA has a beneficial effect on endothelial cell function but not on smooth muscle cell function and that GGA augments endothelial function through activation of the Hsp90/AMPK/eNOS/NO pathway in humans.

In endothelial cells, eNOS exists in multiprotein complexes such as caveolins 1 and 3, Akt, AMPK, Hsp90, G protein-coupled receptors, and mitogen-activated protein kinase family members. Hsp90 is essential for regulating eNOS activity. It has been shown that Hsp90 does not influence the eNOS/caveolin interaction but enhances the ability of calmodulin to displace caveolin from eNOS. The Hsp90 inhibitor geldanamycin inhibits NO production in response to various stimuli, indicating that eNOS activity depends on interaction with Hsp90. In addition, overexpression of Hsp90 increases the amount of Hsp90 that is bound to eNOS and directly induces the activation of eNOS. Under the condition of coexpression of eNOS and Hsp90, Hsp90 increased NOS activity in a dose-dependent manner. In the present study, GGA increased the synthesis of Hsp90 both in HUVECs and peripheral blood mononuclear cells and increased eNOS phosphorylation in HUVECs. These findings suggest that GGA induces activation of eNOS, especially increased eNOS phosphorylation, but not eNOS protein expression through enhancement of Hsp90 protein expression. Hsp90 is responsible for the stability and activity of a wide variety of signaling kinases and proteins. Hsp90 can act as an allosteric modulator of eNOS by inducing a conformational change and as a scaffold for the recruitment of molecules, including kinases and phosphatases, that regulate eNOS activity. Although it is not clear what kind of molecule is enhanced by GGA in endothelial cells, it is thought that some signaling molecules may contribute to GGA-induced Hsp90 expression.

GGA enhanced the phosphorylation of AMPK in HUVECs. In addition, the AMPK inhibitor compound C inhibited the GGA-induced Hsp 90 expression and phosphorylation of AMPK and eNOS. Under pathophysiological conditions, in the present study, we confirmed that GGA also activated AMPK in endothelial cells. Growing evidence indicates that AMPK can phosphorylate eNOS on serine 1177. A possible explanation of the GGA-induced augmentation of endothelial function is as follows: calcium-calmodulin, together with Hsp90, displaces eNOS from

Discussion

In the present study, we demonstrated that GGA, which is not only an antulcer agent but also a specific Hsp inducer, significantly increased Hsp90 and the phosphorylation of eNOS and AMPK but not eNOS and AMPK protein expression, Akt protein expression, and Akt phosphorylation, in HUVECs. Compound C, an AMPK inhibitor, inhibited the GGA-induced Hsp 90 expression and phosphorylation of AMPK and eNOS.
ampak/Hsp90 and Endothelial Function

Caveolin-1; the interaction of Hsp90 with AMPK and eNOS allows Hsp90 to serve as a docking site for phosphorylation of eNOS serine 1177 by AMPK; Hsp90 stabilizes the binding of calmodulin and eNOS; electron flux is caused from the reduction of the peroxide domain of eNOS; and NO is released from 1-arginine in endothelial cells.

In the present study, GGA did not alter Akt protein expression and Akt phosphorylation in HUVECs. It is known that the phosphatidylinositol 3-kinase/Akt pathway plays an important role in eNOS activation. However, interestingly, Brouet et al. have shown that Hsp90 stimulates eNOS activation independently of Akt phosphorylation on serine 1177. It is likely that GGA activates eNOS through enhancement of Hsp90 phosphorylation independently of Akt. Recently, Zhu et al. have shown that oral administration of GGA attenuates coronary endothelial dysfunction induced by ischemic reperfusion in the rat heart, at least in part through activation of the phosphatidylinositol 3-kinase/Akt pathway to improvement in endothelial function is unclear, differences in species, vessels, or pathophysiological conditions may influence the effect of GGA on the phosphatidylinositol 3-kinase/Akt pathway.

Stress stimuli, including heat shock, oxidative stress, hypoxia, inflammation, and heavy metals, induce the expression of Hsps in endothelial cells. However, the intracellular signaling cascade that ultimately leads to the activation of Hsp gene transcription and subsequent protein synthesis after stress exposure remains unclear. It is thought that several stress stimuli induce the expression of Hsps by activation of heat shock transcription factor. Indeed, Hirakawa et al. reported that GGA increases the Hsp70 mRNA level and directly activates heat shock transcription factor in cultured guinea pig gastric mucosal cells. Increased Hsp90 protein expression may result from the activation of heat shock transcription factor by GGA.

Jednakovitis et al. reported that preservation of endothelial function might be related to sustained levels of Hsp72 in spontaneously hypertensive rats. In addition, a protective effect of transfection of the Hsp70 gene to a donor heart on reperfusion injury has been shown. In the present study also, GGA significantly enhanced Hsp70 expression in HUVECs and peripheral blood mononuclear cells. Hsp70 family members, including Hsp70 and Hsp72, may contribute to improvement in endothelial function by GGA, although the precise mechanism involved in this process is unclear.

Study Limitation

We evaluated the synthesis of Hsp90 in peripheral blood mononuclear cells instead of human endothelial cells because obtaining endothelial cells from the artery is a great burden for subjects. We consider GGA to be a potent inducer of Hsp90 in human forearm endothelial cells, as well as HUVECs and peripheral blood mononuclear cells.

In the present study, we showed that GGA enhanced AMPK phosphorylation-related activation of eNOS associated with hsp90 in HUVECs. In addition, 1-NMMA abolished the GGA-induced augmentation of endothelium-dependent vasodilation in both healthy subjects and smokers. These findings suggest that GGA increases NO production from endothelial cells through activation of the AMPK/Hsp90/eNOS pathway. However, it is unclear whether GGA directly correlates with NO production.

Conclusions

GGA augments endothelium-dependent vasodilation, at least in part through activation of the Hsp90/AMPK/eNOS/NO pathway. Endothelial dysfunction is an initial step in arteriosclerosis, leading to cardiovascular and cerebrovascular outcomes. Therefore, from a clinical perspective, it is important to select an appropriate intervention that is effective in improving endothelial function in patients with cardiovascular diseases. Use of GGA may provide a new therapeutic approach for improving endothelial function in patients with cardiovascular diseases. Additional studies on the effects of GGA in patients with essential hypertension, heart failure, diabetes mellitus, and other cardiovascular diseases in which endothelium-dependent vasodilation is impaired are needed.

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Disclosures

None.

References


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Supplemental Figure I
Geranylgeranyacetone (GGA) did not alter the expression of ERK1/2 protein, JNK protein, and p38 protein or the phosphorylation of ERK1/2, JNK, and p38 in human umbilical vein endothelial cells (HUVEC). HUVEC were stimulated with GGA at indicated concentrations for 90 minutes (A, C and D) and with 10 µmol/L GGA for indicated times (B, D and F) (n=5, respectively).
**Supplemental Table I**

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Table I. Clinical characteristics of the control group and CCA group in healthy subjects.
### Supplemental Table II

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<td>1 ± 0.0</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
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<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

Table 2: Clinical characteristics of the control group and cGTA group in months.