An acyclic polyisoprenoid derivative, geranylgeranylacetone protects against visceral adiposity and insulin resistance in high-fat-fed mice

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Adachi H, Kondo T, Ogawa R, Sasaki K, Morino-Koga S, Sakakida M, Kawashima J, Motoshima H, Furukawa N, Tsuruzoe K, Miyamura N, Kii H, Araki E. An acyclic polyisoprenoid derivative, geranylgeranylacetone protects against visceral adiposity and insulin resistance in high-fat-fed mice. Am J Physiol Endocrinol Metab 299:E764–E771, 2010. First published August 17, 2010; doi:10.1152/ajpendo.00075.2010.—Induction of heat shock protein (HSP)72 improves insulin resistance and obesity in diabetic animal models. Geranylgeranylacetone (GGA), known as an antiulcer drug, induces HSP72 and protects organs against several cellular stresses. This study investigated whether GGA administration would induce HSP72 in liver and render physiological protection against high-fat feeding in mice. A single and 4-wk oral administration of 200 mg/kg GGA was performed in high-fat diet (HFD)-fed mice. Metabolic parameters, cytokines, and gene expressions related to insulin signaling were evaluated. A single administration of GGA induced HSP72 in liver of normal chow-fed and HFD-fed mice. Insulin resistance after HFD was slightly ameliorated. Four weeks of GGA administration also increased HSP72 in liver and significantly improved insulin resistance and glucose homeostasis upon glucose challenge. Activation of c-jun NH2-terminal kinase (JNK) was attenuated, and insulin signaling was improved in the liver of HFD mice. Visceral adiposity was decreased in GGA-treated mice, accompanied by reduced leptin and increased adiponectin levels. GGA can be a novel therapeutic approach to treat metabolic syndrome as well as type 2 diabetes by improving insulin signaling and reducing adiposity. These beneficial effects of GGA could be mediated through HSP72 induction and JNK inactivation in the liver.

Insulin stimulates a signaling network composed of a number of molecules, initiating the activation of insulin receptor tyrosine kinase and phosphorylation of the insulin receptor substrate (IRS) proteins (e.g., IRS-1 and IRS-2) (49). In recent years, it has become apparent that obesity is also linked to oxidative stress and endoplasmic reticulum (ER) stress (12, 19, 40, 41). These stress-signaling pathways are activated in various tissues in several metabolic disorders such as hyperglycemia, insulin resistance, and chronic inflammation, leading to the activation of c-Jun NH2-terminal kinase (JNK) pathway. JNK is now known to be involved in the progression of insulin resistance (19, 23, 31) and phosphorylates IRS-1 on Ser307, rendering it a poor substrate for the activated insulin receptor (20). The suppression of the JNK pathway in obese diabetic mice markedly improves insulin resistance and ameliorates glucose tolerance (24).

Heat shock protein (HSP)72 is a major stress-inducible molecular chaperone that plays a key role in maintaining correct protein folding, assembly, and appropriate protein transport (32). HSP72 can modulate stress-activated JNK signaling by direct inhibition (13, 42) and/or dephosphorylation (53) of JNK. Some researchers (5, 29) have reported previously that the mRNA expression of HSP72 was reduced in patients with type 2 diabetes and was inversely correlated with their insulin sensitivity. Our group and others have reported recently that induction of HSP72 by various means is capable of decreasing the amount of visceral adiposity and improving insulin resistance in mice models of type 2 diabetes partly through the inhibition of JNK activity (6, 14, 33, 34). From the clinical point of view, there is considerable interest in the discovery of adiposity-reducing and insulin-sensitizing agents through the increase of HSP72 levels in vivo.

Geranylgeranlyacetone (GGA), which is a safe and widely available antiulcer drug without serious adverse effects, has potential benefits for the treatment of cardiac (38, 46) and cerebral (35, 56) ischemia-reperfusion injury as well as neurodegenerative disorders (25). GGA exerts such cytoprotective effects through HSP72 induction in various tissues, including gastric mucosa, intestine, myocardium, retina, central nervous system, and liver (11, 16, 22, 38, 55). Although it has been recognized that GGA induces HSP72, it is still unknown whether GGA can ameliorate glucose metabolism in diabetic conditions or not.

In this study, we have investigated the metabolic effects of GGA on high-fat diet (HFD)-induced obese diabetic mice.

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MATERIALS AND METHODS

Materials. The following antibodies were used in this study. Mouse monoclonal anti-HSP70 (SPA-810; Stressgen Biotechnologies, Victoria, Canada), anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-JNK (Thr183/Tyr185), and JNK antibodies were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for actin, phospho-insulin receptor-β (IRβ; 1162/1163), and IRβ were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for IRS-1 and IRS-2 were from Upstate Biotechnology (Lake Placid, NY). GGA was generously given by Eisai (Tokyo, Japan).

Animals. The C57BL/6j mice (Kyudo, Kumamoto, Japan) were kept on a 12:12-h light-dark cycle and allowed free access to food and water. Only male mice were used in this study. The mice were maintained on food (standard chow or HFD) and water ad libitum. High-fat-fed group mice were fed a HFD for 4 wk (14% lard, 14% beef tallow, 25% casein, 20% sucrose, 15% cornstarch, 5% cellulose; the caloric intake from total fat content of the diet was 60%). All procedures were approved by the Animal Care and Use Committee of Kumamoto University (no. A19-039).

GGA administration. GGA induced HSP72 expression in a dose-dependent manner and peaked at 200 mg/kg. Increasing the dose (≥400 mg/kg) did not result in a further increase in HSP72 expression (39). Thus, we used the 200 mg/kg of GGA for induction of HSP72 in this study. GGA, as an emulsion with 5% gum arabic and 0.008% tocopherol, was given orally at a dose of 200 mg/kg (GGA group). Mice in the control group were given the same dose of vehicle (control group) under light anesthesia with diethyl ether.

RT-PCR. Total RNA was extracted from mice liver and brown adipose tissue (BAT) with TRizol (Invitrogen). The first-strand cDNA synthesis containing 1 μg of total RNA was primed with oligo(dT) (Takara, Tokyo, Japan). To quantify phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) transcripts, the LightCycler System (Roche Diagnostics, Meylan, France) was used (3). PCR products were performed using SYBR Green I master mix and specific primers for mouse PEPCK upstream primer (5'-GCCCTGATGGGATGAACTTT-GATGC-3') and downstream primer (5'-AAAGTTTCCAGCACCAG-GAT-3'), G-6-Pase upstream primer (5'-GAGGAAAAAGGAAAAGC-GACG-3') and downstream primer (5'-AAAGTTTCCAGCCACA-GAAT-3'), and uncoupling protein-1 (UCP1) upstream primer (5'-TTTTGAGGAAAGCACCCTAT-3') and downstream primer (5'-ACCCGGATGCTGAAAGAAGGC-3').

To detect β-actin mRNA, forward primer 5'-GTGGGCGCCTTAC-GACACCA-3' (nucleotide 25 to 45 of murine β-actin coding sequence) and reverse primer 5'-CCCTTTGTGATGTCACCGATATT-3' (nucleotide 541 to 564 of murine β-actin coding sequence) were used. Quantitative results of PEPCK, G-6-Pase, and UCP1 mRNA were normalized for the levels of β-actin mRNA. To assess the specificity of the amplified PCR products, a melting curve analysis was performed after the last cycle.

Western blotting for the signaling molecules. Mice were anesthetized with 100 mg/kg pentobarbital sodium. Following the loss of pedal and corneal reflexes, saline or 5.0 U of regular insulin was injected from inferior vena cava. The liver, mesenteric fat, pancreas, and quadriceps were dissected out 5 min after insulin injection and immediately frozen in liquid nitrogen. Western blotting was performed as described previously (47). Thirty micrograms of protein was resolved by SDS-polyacrylamide gel electrophoresis and blotted with the indicated antibodies. Proteins were detected with a Chemiluminescence Blotting Substrate kit (Roche Diagnostics, Mannheim, Germany). Immunoreactive bands were quantified using the National Institutes of Health Image analysis software.

Organ weight and morphological analysis. The liver, subcutaneous fat, epididymal fat, mesenteric fat, retroperitoneal fat, BAT, pancreas, and quadriceps were obtained after overnight fasting. Weight of each organ was measured and expressed as g/100 g body wt. Tissues were fixed in paraflin and cut into 10 sections (5 mm thickness) separated by 200 mm. Sections were stained with hematoxylin-eosin (HE) and visualized using BX-8000 microscope (Keyence, Osaka, Japan). The area of adipocytes in epididymal fat was measured and calculated using BX-II Dynamic Cells Count (Keyence, Osaka, Japan).

Blood analysis. Blood glucose was assayed with Glucose Test NEO (Sanwa Kagaku). Serum insulin, adipocytokines (adiponectin and leptin), and plasma lipid parameters (total cholesterol and triglyceride) were determined with LipoSEARCH (Skylight Biotech, Akita, Japan).

Glucose, pyruvate, and insulin tolerance tests. Glucose tolerance tests (GTT) and pyruvate tolerance test (PTT) were performed on fasted (15 h) mice. Mice received an intraperitoneal injection of glucose (2 g/kg body wt) or oral pyruvate load (2 g/kg body wt), and blood glucose levels were assayed immediately before and at 15, 30, 60, 90, and 120 min after administration. Insulin tolerance tests (ITT) were performed on fasted (6 h) mice. Mice received an injection of human regular insulin (0.75 units/kg body wt; Novo Nordisk, Bagsvaerd, Denmark) into the intraperitoneal space, and blood glucose levels were assayed immediately before and at 30, 60, 90, and 120 min (high-fat-fed mice) after injection.

Statistical analyses. Quantitative data are presented as means ± SD of at least three independent experiments. Statistical analysis was based on Student’s t-test for paired or unpaired data as appropriate. A P value <0.05 was used to determine statistical significance.

RESULTS

The effects of 200 mg/kg GGA single treatment in normal and HFD mice. To determine the effect of short period treatment, we performed single oral administration of 200 mg/kg GGA or PBS (control) on C57BL/6j male mice fed with normal diet. Six hours after administration, intraperitoneal (ip) GTT was performed. There was no difference in the blood glucose level between GGA and control groups (Fig. 1A). On the other hand, mice fed with HFD for 1 wk, the blood glucose levels at 90 and 120 min during ip GTT were significantly lower in GGA group compared with control group (Fig. 1B). There were no significant differences in area under the curve (AUC) of glucose between the control and GGA group (control vs. GGA: 548.8 ± 32.1 vs. 516.3 ± 24.7 mg·h−1·dl−1; P = 0.26).

To assess HSP72 induction by GGA, we examined by Western blotting analysis the HSP72 protein levels 6 h after single administration of 200 mg/kg GGA. GGA effectively increased HSP72 expression in HFD mice liver (Fig. 1C) and, to a lesser extent, in muscle (Fig. 1D) but not in mesenteric fat (Fig. 1E) or pancreatic tissue (Fig. 1F). Because induction of HSP72 attenuates JNK activation, phosphorylation of JNK was also examined in both tissues. In a reciprocal correlation with HSP72 induction, phospho-JNK was reduced in liver but not in other tissues examined (Figs. 1, C–F).

The metabolic effects of 200 mg·kg−1·day−1 GGA treatment for 4 wk in HFD-fed mice. We next determined the long-term effects of 200 mg·kg−1·day−1 GGA treatment on HFD mice. High-fat feeding was started 1 wk before GGA administration. During the treatments, body weight, food intake, fasting blood glucose, and insulin were measured weekly. After the 4 wk of treatment, body weight in GGA group was significantly lower (Fig. 2A), whereas food intake was not different (Fig. 2B). Fasting blood glucose and insulin levels were significantly decreased by 28 and 80%, respectively (Fig. 2, C and D). Basal insulin resistance estimated by homeostasis model assessment of insulin resistance (HOMA-IR) was also significantly reduced (control vs. GGA: 1.65 ± 0.34 vs. 0.12 ± 0.06, P <
0.01). Upon glucose challenge, blood glucose levels at every time point were decreased significantly in GGA group (Fig. 2E). AUC of glucose on ip GTT was significantly decreased during 4 wk of GGA treatment compared with control (AUC in control vs. GGA: 519.4 ± 40.1 vs. 398.9 ± 33.6 mg·h·dl⁻¹, P = 0.00014; Fig. 2E).

An ip ITT also showed significant suppression of blood glucose levels at 90 and 120 min (Fig. 2F).

Adipose tissue weight reduction in long-term GGA treatment in high-fat-fed mice. Since long-term GGA treatment improved glucose homeostasis and insulin resistance, several cytokines whose expressions were modulated in the diabetic state were measured. Serum leptin level was reduced by ~70% (Fig. 3A), and adiponectin was increased by 30% (Fig. 3B). TNFα showed a trend of reduction but did not reach significance (P = 0.28; Fig. 3C). Because lower leptin and higher adiponectin suggest the reduction of adiposity in GGA-treated mice, organ weight of various tissues was measured. Muscle mass dissected from mixture of gastrocnemius and soleus muscle was increased in the GGA group (Fig. 3D). Subcutaneous and epididymal fat showed a tendency of decreased weight (P = 0.07 and 0.06, respectively), and both mesenteric and retroperitoneal fat weight were significantly decreased in the GGA group (Fig. 3D). UCP1 mRNA expression in BAT was significantly increased by ~35% in GGA group (Fig. 3E). HE staining of epididymal fat sections displayed smaller size cell distribution (Fig. 3F), and the fat area was significantly lower in GGA group (Fig. 3G).

HSP72 induction inhibited the phosphorylation of JNK and improved insulin signaling in GGA-treated liver. It was shown previously that GGA induced HSP72 in several tissues, including liver (11, 16, 22, 38, 55). Long-term administration of GGA increased HSP72 levels in liver, BAT, and pancreas but not in muscle and white adipose tissues (Fig. 4A). In the liver of GGA-treated mice, whereas basal JNK protein expression was comparable in two groups, phosphorylation of JNK was suppressed (Fig. 4B). In parallel with improved insulin resistance in vivo, insulin-stimulated phosphorylation of both IR subunit and Akt were increased in the liver of GGA-treated mice. Ser³⁰⁷ phosphorylation of IRS-1 was upregulated in the liver of control mice, whereas it was almost undetectable in GGA-treated mice (Fig. 4B). Because it is reported that induction of HSP72 in muscle is beneficial to treat diabetes, we carefully investigated HSP72 in muscle but failed to detect an increase of HSP72 even after long-term treatment with GGA (data not shown).

To investigate whether the GGA effect on glucose homeostasis improvement is explained by suppression of hepatic....
Gluconeogenesis, PTT was performed. Glucose excursion on pyruvate load was significantly attenuated at 60 min, and AUC on PTT was also decreased in the GGA group compared with controls (AUC in control vs. GGA: 556.5 ± 38.9 vs. 495.6 ± 35.6 mg·h·dl⁻¹, P = 0.04; Fig. 4C). Total RNA recovered from the liver of control and GGA-treated groups was prepared for quantitative RT-PCR. In the fasting state, mRNA levels of both PEPCK and G-6-Pase were significantly decreased by 69 and 81%, respectively, in GGA-treated group compared with control (Fig. 4D).

**DISCUSSION**

GGA, an acyclic polyisoprenoid, is an antiulcer drug developed in Japan (16) and is known to protect against organ and cell damages via its potent HSP-inducing function (25, 35–38, 46, 56) as well as reducing proinflammatory mediators (15, 28).

The induction of HSPs is regulated at the transcriptional level by interaction with a consensus cis-element (heat shock element) and a heat shock transcription factor 1 (HSF1) that specifically binds to heat shock element located in the upstream region of HSP genes (2). GGA causes rapid activation of HSF1, which is followed by increased expression of HSP72 mRNA in gastric mucosal cells (16). GGA enhances dephosphorylation and nuclear translocation of HSF1, HSF1-DNA binding, and HSP72 mRNA expression. It was shown previously that GGA suppressed the H₂O₂ and ethanol-induced activation of JNK, caspase-9, and caspase-3-like proteases, leading to significant inhibition of apoptosis in rat hepatocytes (21).

Recently, it was reported that BGP-15, which is a hydroxylamine derivative, increases HSP72 protein level in the skeletal muscles, prevents JNK phosphorylation, and ameliorates insulin resistance in ob/ob mice (6) and nondiabetic patients with
impaired glucose tolerance (30). BGP-15 is thought to activate HSP72 via modification of membrane microdomain-associated stress-sensing and -signaling mechanisms (50, 52) and by prolonging the binding of HSF1 to heat shock response element (27). Since GGA causes rapid activation of HSF1 itself, the mechanisms of induction of HSP72 and its operant organ may be different from those of BGP-15. But whether GGA could alleviate hyperglycemia and insulin resistance in diabetic mice is unclear.

Here, we demonstrated that a single oral administration of 200 mg/kg GGA slightly but significantly improved glucose tolerance in HFD obese mice, whereas no significant change was observed in normal diet-fed mice. These observations may indicate that HFD-induced obesity causes stress conditions, since GGA is reported to exert larger effects in stressed conditions (38, 55). Upregulation of HSP72 protein was observed mainly in the liver but not in muscle, adipose tissues, or pancreas of HFD mice after a single GGA administration. Upon long-term GGA treatment, HSP72 was also increased in liver, BAT, and pancreas but not in muscle or white adipose tissues.

HSP72 is postulated to ameliorate insulin resistance by direct inhibition of JNK (13, 42) and accelerating the rate of JNK dephosphorylation (53). Consistent with this, we observed that GGA decreased the phosphorylation of JNK in high-fat-fed mice. The involvement of JNK in the inhibitory serine phosphorylation of IRS-1 has been suggested to be responsible, in part, for the ability of JNK to downregulate insulin signaling (1). It has been shown previously that JNK1 mediates obesity-induced insulin resistance by inducing the serine phosphorylation of IRS-1 (20) and that JNK activation can be blocked by HSP72 (42), and it has also been reported that insulin signaling is enhanced in JNK-knockout mice (17).

Here, we propose a novel concept that GGA induces HSP72 and ameliorates insulin signaling mainly through the JNK inactivation in the liver. Previous reports describe that the target organ of insulin sensitization by HSP72 is the muscle and not the liver (6, 14). However, in this study, fasting blood glucose, fasting insulin, HOMA-IR, glucose excursion on PTT, and gluconeogenic enzyme expression (PEPCK and G-6-Pase) were all decreased dramatically after treatment of GGA. Since HOMA-IR is a simple estimating method of insulin sensitivity...
that is derived from fasting glucose and insulin levels, it is likely that it reflects primary hepatic insulin action but not muscular effects (18). Hepatic steatosis is also significantly and independently associated with HOMA-IR (44), suggesting that HOMA-IR represents mostly hepatic insulin resistance at the basal state. The results on PTT indicate that GGA suppresses gluconeogenesis in liver, which is also confirmed by PEPCK and G-6-Pase gene expression. In this context, amelioration of hepatic insulin resistance contributes mainly to improved whole body insulin sensitivity. Hepatic steatosis was also suppressed in the GGA group (data not shown), which further supports our hypothesis that the liver is a major target organ of GGA. Because we could not fully exclude the possibility of the contribution of muscle in metabolic improvements, muscle glucose uptake should be addressed in future studies.

Although we observed that 4 wk of GGA treatment induced HSP72 in the liver and pancreas, single treatment of GGA did not alter HSP72 levels in pancreas. Since JNK activation by hyperglycemia causes pancreatic β-cell apoptosis, induction of HSP72 in the pancreas may improve β-cell function through the inhibition of phosphorylation of JNK (23). Although we observed that the effects of GGA correlated with HSP72 induction, we could not completely prove that GGA improves glucose metabolism only through the HSP72-JNK pathway. It is necessary to assess the impact of the HSP72-JNK pathway using HSP72-knockout mice and/or HSF1-knockout mice (48) to determine whether the beneficial effects of GGA are exerted mainly through HSP72.

We show here that mice treated for 4 wk with GGA reduced body weight gain by high-fat feeding and improved glucose homeostasis and insulin sensitivity compared with high-fat-fed control mice. Long-term treatment of GGA showed more remarkable improvements than single administration in terms of glucose metabolism. Moreover, there was a dramatic decrease of fat accumulation in GGA-treated mice compared with controls. Serum leptin, which inversely correlates to the degree of adiposity that was decreased, and adiponectin, which has insulin-sensitizing effects since it enhances inhibition of hepatic glucose output as well as glucose uptake, were increased remarkably in GGA-treated group. We did not measure the other factors, such as rectal temperature, O₂ consumption, lipid metabolism, and lipid absorption. However, JNK1⁻/⁻ mice showed decreased adiposity by unknown mechanisms (17, 51), and HSP72 overexpression in skeletal muscle prevents high-fat...
feeding-induced increases in body weight and fat pad weight, which is associated with enhanced mitochondrial enzyme activity (6). In this context, the HSP72-JNK inhibitory pathway may be a major target to reduce intra-abdominal adiposity. Indeed, these beneficial effects of GGA are highly similar to those in HFD mice treated with hyperthermia plus mild electrical stimulation, which can effectively upregulate HSP72 as well (33). We also postulate another possible mechanism of reducing adiposity in GGA-treated mice. HSP72 induction increases peroxisome proliferator-activated receptor-γ coactivator-1α mRNA expression (our unpublished data), which could increase mitochondrial biogenesis. UCP1 mRNA up-regulation may be one of the effects of mitochondrial biogenesis. It is speculated that molecular chaperones such as HSP72 activate mitochondrial biogenesis (7). It is also known that mitochondrial dysfunction by several stresses can be recovered by activation of heat shock response (4, 43, 54). Hyperthermia also increases growth hormone (GH) expression (45). Adult GH deficiency results in increased adiposity and metabolic disturbances that can be reversed by GH supplementation. Therefore, activation of GH expression/release by HSP72 might contribute to improve the body compositions.

In terms of drug dosage, 200 mg·kg⁻¹·day⁻¹ GGA is rather high compared with a clinical dose in human. The dose chosen in this study was determined by the references in other reports that used 200 mg/kg GGA mainly for single administration (10, 11, 16, 21, 22, 25, 26, 38, 39). Lower dose optimization must be necessary to apply this treatment in a clinical setting.

In summary, we have shown that oral administration of GGA improved glucose metabolism and reduced adiposity in an animal model of type 2 diabetes. This effect of GGA could be explained mainly by induction of HSP72 in the liver. Targeted induction of HSP72 can be a potential therapeutic strategy for the treatment of diabetes and metabolic syndrome.

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