Single Oral Dose of Geranylgeranylacetone Induces Heat-Shock Protein 72 and Renders Protection Against Ischemia/Reperfusion Injury in Rat Heart

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Background—Induction of heat-shock proteins (HSPs) results in cardioprotection against ischemic insult. Geranylgeranylacetone (GGA), known as an antiulcer agent, reportedly induces HSP72 in the gastric mucosa and small intestine of rats. The present study tested the hypothesis that oral GGA would induce HSP72 in the heart and thus render cardioprotection against ischemia/reperfusion injury in rats.

Methods and Results—Cardiac expression of HSPs was quantitatively evaluated in rats by Western blot analysis. Ten minutes of whole-body hyperthermia induced HSP72 expression in the rat hearts. A single oral dose of GGA (200 mg/kg) also induced expression of HSP72, which peaked at 24 hours after administration. Therefore, isolated perfused heart experiments using a Langendorff apparatus were performed 24 hours after administration of 200 mg/kg GGA (GGA group) or vehicle (control group). After a 5-minute stabilization period, no-flow global ischemia was given for 20, 40, or 60 minutes, followed by 30 minutes of reperfusion. During reperfusion, the functional recovery was greater and the released creatine kinase was less in the GGA group than in the control group. Electron microscopy findings revealed that the ischemia/reperfusion-induced damage of myocardial cells was prevented in GGA-treated myocytes.

Conclusions—The results suggest that oral GGA is cardioprotective against ischemic insult through its induction of HSP72. (Circulation. 2001;104:1837-1843.)

Key Words: ischemia ■ proteins ■ reperfusion ■ signal transduction

The heat-shock proteins (HSPs) are an important family of endogenous protective proteins that increase in response to a wide variety of stresses, such as heat shock, hypoxia, hydrogen peroxide, inflammation, and ischemia. Among the various sizes of HSPs, HSP72 is reportedly involved predominantly in cardioprotection; several studies have demonstrated that whole-body hyperthermia 24 hours before the onset of myocardial ischemia is protective against ischemia/reperfusion injury and is associated with proportional induction of HSP72 expression. Furthermore, recent studies revealed that both the myogenic cells and hearts of transgenic mice overexpressing HSP72 protein are resistant to ischemic injury.

Therefore, pharmacological interventions that induce the expression of cardiac HSPs have been focused intensively on a therapeutic approach, but several agents that increase the level of cardiac HSPs are toxic or have harmful side effects.

Geranylgeranylacetone (GGA), an acyclic polyisoprenoid, is an antiulcer drug developed in Japan that reportedly induced overexpression of the HSP70 family (HSP72 and HSP73) in the gastric mucosa and small intestine of rats when given orally. We therefore hypothesized that oral administration of GGA might induce HSP72 expression in the heart as well and provide protection against ischemia/reperfusion injury.

Materials
Monoclonal IgG cross-reactive to inducible HSP72, HSP60, and HSP27 antibodies was purchased from Stressgen Biotechnologies Corp. Primary antibodies of inducible nitric oxide synthase (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) were purchased from Transduction Laboratories. The primary antibody of thioredoxin was kindly provided by Professor J. Yodoi (Kyoto University, Japan). Horseradish peroxidase–linked F(ab')2 fragment from sheep anti-mouse immunoglobulin and reagents for Western blot detection by enhanced chemiluminescence (ECL) were pur-
chased from Amersham Pharmacia. Bradford protein assay kits were purchased from Bio-Rad Laboratories. GGA was provided by Eisai Co Ltd.

Animals
Male Sprague-Dawley rats (220 to 250 g) were used. To assess the effect of whole-body hyperthermia, rats were anesthetized with pentobarbital (20 mg/kg IP) and placed, with their heads on a pillow to avoid aspiration of water, for 10 minutes in a bath in which the water temperature was maintained at 43°C (heat-stress group). Rectal temperature was monitored throughout the whole-body hyperthermia. GGA, as an emulsion with 5 g% gum arabic and 0.008% tocopherol, was given orally at a dose of 200 mg/kg (GGA group). Rats in the control group were given the same dose of vehicle (control group). To examine the time-dependent expression of HSP72, rats were killed by deep anesthesia with pentobarbital (50 mg/kg IV) 6, 12, 24, and 48 hours after administration of GGA or vehicle and heat stress. The heart was rapidly removed and frozen in liquid nitrogen. The dose-dependent expression of HSP72 was evaluated by use of different doses of GGA (0, 50, 100, 200, and 400 mg/kg).

Western Immunoblotting
Western blotting was performed as previously described. The frozen heart preparations were homogenized with SDS sample buffer, centrifuged, and boiled. The total protein concentration of myocardium was quantified by the Bradford method. The preparations were diluted in dissociation buffer. An equal amount of total protein in each fraction was conducted on 8.5% SDS-PAGE and transferred electrophoretically to a polyvinylidine difluoride membrane. After transferral and blocking with 0.5% nonfat milk, the membrane was incubated with antibodies. The proteins were detected by enhanced chemiluminescence with exposure to Hyperfilm (Amersham Pharmacia). The amount of protein on the immunoblots was quantified by use of National Institutes of Health image software.

Isolated Perfused Heart Experiments
Twenty-four hours after administration of 200 mg/kg of GGA (GGA group) or vehicle (control group), the rats were heparinized (500 IU/kg IP) and anesthetized with pentobarbital (50 mg/kg IP). Each heart was isolated and perfused retrogradely by the Langendorff method with Krebs-Henseleit buffer (mmol/L): NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, glucose 11.0; pH 7.4) equilibrated with a 95% O2/5% CO2 gas mixture at 36.5°C at a rate of 10 minutes of constant-pressure perfusion, the perfusion flow was determined for each heart, which was then perfused at a constant pressure of 75 mm Hg. A water-filled latex balloon was inserted through the mitral orifice into the left ventricle (LV), and the LV end-diastolic pressure was adjusted to 0 to 5 mm Hg. During the initial 10 minutes of constant-pressure perfusion, the perfusion flow rate was determined for each heart, which was then perfused at a determined perfusion rate with a microtub pump while the heart was covered with water-jacketed glassware and the relative humidity was maintained at ≈90%. Normothermic no-flow global ischemia was initiated for 20, 40, or 60 minutes, followed by reperfusion for 30 minutes. The coronary effluent during the 30 minutes of reperfusion was collected for measurement of creatine kinase activity (released CK).

LV pressure was monitored with a pressure transducer to obtain the peak positive and negative first derivatives of LV pressure (dP/dtmax and dP/dtmin). LV developed pressure (LVDP) was defined as the difference between the LV systolic and diastolic pressures. Coronary perfusion pressure (CPP) was defined as the intrathoracic pressure measured at the level of aortic cannulation. LV pressure, CPP, and ECG were continuously recorded on a polygraph recorder (WS-681G, Nihon Kohden) and stored on a PCM data recorder (RD-111T, TEAC) for later analysis.

Electron Microscopy Findings
After 30 minutes of reperfusion after 20 minutes of no-flow ischemia, the LV papillary muscles were immediately removed from both GGA-treated and control hearts and cut into small blocks, which were then fixed in cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde and paraformaldehyde at 4°C for 2 hours and postfixed in cacodylate-buffered (pH 7.4) 2% osmium tetroxide/0.5% potassium ferrocyanide at 4°C for 2 hours. The tissue blocks were dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed under a transmission electron microscope (JOEL-100CX).

Statistical Analysis
Data are expressed as mean±SEM. Serial changes in LVDP, CPP, and dP/dt were analyzed by 2-way ANOVA followed by the Bonferroni-Dunn test, unless otherwise specified. The ratio of released CK to ventricular weight was analyzed with an unpaired t test. The relative intensity of each protein was compared by Mann-Whitney’s U test. A value of P<0.05 was considered significant.

Results
Cardiac Expression of HSP72 and Other Protective Proteins
HSP72 in the GGA group was observed from 6 hours and peaked at 24 hours after GGA administration, but the expression level at 48 hours after administration decreased compared with that at 12 and 24 hours (Figure 1). No time-dependent change in the expression of HSP72 was observed in the control group.

The dose-dependent effects of GGA on the expression of HSP72 were also assessed 24 hours after administration (Figure 2). HSP72 expression increased in a dose-dependent manner, peaking at 200 mg/kg, but increasing the dose up to 400 mg/kg did not result in a further increase in expression.

Twenty-four hours after administration of vehicle or GGA, the expression of HSP27 was not significantly different between the control and GGA groups (Figure 3A). The expression of HSP60 was significantly decreased in the GGA group compared with the control group (Figure 3B). iNOS or nNOS (data not shown) could not be detected in either group (Figure 4A). Although eNOS was detected in both groups, the level of expression was not significantly different (Figure 4A). There was no significant difference in expression of thioredoxin between the groups (Figure 4B).

Isolated Perfused Heart Experiments
Figure 5 shows the serial changes in LVDP and dP/dt during the reperfusion periods. At baseline, none of these parameters differed between the GGA and the control groups (Table). During no-flow global ischemia, LVDP and dP/dt quickly decreased to zero. On reperfusion, both LVDP and dP/dt increased and dP/dtmax decreased. The recovery of LVDP and dP/dt was greater in the GGA group than in the control group when 20 or 40 minutes of ischemia was applied. When 60 minutes of ischemia was applied, the functional recovery of both groups was very poor, resulting in an insignificant difference between the groups. Despite various ischemic periods, neither CPP nor heart rate differed significantly between the groups throughout the experimental period (data not shown).

Figure 6 shows the amount of released CK during the 30-minute reperfusion period. When 20 or 40 minutes of ischemia was applied, the released CK was lower in the GGA
no significant difference was observed, however, when 60 minutes of ischemia was applied.

**Electron Microscopy Findings**

In control rats (Figure 7a and 7b), most of myocardial cells had atrophic nuclei, swollen mitochondria, and irregularly arranged myofibrils. In particular, the cristae of the subsarcolemmal mitochondria were destroyed and the intracellular spaces were enlarged. In contrast, the ultrastructure of the myocardial cells of GGA-treated rats (Figure 7c and 7d) was preserved, although some cells were slightly damaged. The nuclei were generally oval, having densely packed chromatin disposed around the periphery of the nucleus. Myofibrils were arranged regularly, and Z bands were clearly identified. Most of the sarcolemmal, interfibrillar, and perinuclear mitochondria possessed well-developed cristae, although there were a few swollen mitochondria. The T tubules were slightly expanded.

**Discussion**

The major findings in the present study are that oral GGA induced HSP72 expression in the rat heart and that hearts treated with GGA were protected against ischemia/reperfusion injury. During reperfusion, the functional recovery was greater and released CK was less in the GGA group than in the control group when 20 or 40 minutes of ischemia was applied. The protective effects disappeared, however, when 60 minutes of ischemia was applied. Thus, the GGA-induced cardioprotection observed with shorter duration of ischemia may be attenuated with more prolonged duration of ischemia.

In general, members of the HSP70 family bind transiently to nascent proteins and act as intracellular chaperones, helping to stabilize these proteins until they achieve their final conformation.1,16 Diverse stresses, heavy metals, amino acid analogues, inflammation, and oxidative/ischemic stress induce the expression of HSP genes. In stress conditions, the HSP70 family may stabilize denatured proteins within the cell, possibly facilitating their removal or repair, leading to protection and/or restoration of cell function during recovery from the stress.16

Several studies have demonstrated that whole-body hyperthermia 24 hours before cardiac ischemia is protective against ischemia/reperfusion injury and is associated with induction of HSP72,2–4 suggesting a significant role of this protein in cardioprotection. Heat stress, however, increases some other cytoprotective proteins, such as catalase,17 superoxide dismutase,18 or other members of the HSP family.19 In addition,
heat stress preserves mitochondrial activity, increases levels of high-energy phosphates, and attenuates the calcium paradox after ischemia/reperfusion. Therefore, it has been questioned whether increased HSP72 levels per se provide protection against ischemia-related insult. This problem has been resolved by recently developed molecular technology; it has been found that both the myogenic cells and hearts of transgenic mice overexpressing HSP72 protein are resistant to ischemic injury. Thus, it is currently thought that its action as a molecular chaperone, with various other biological

**Figure 3.** Expression of HSP27 and HSP60 after oral GGA (G) or vehicle (CNT, C) by Western blot. Hearts were isolated 24 hours after administration of GGA (200 mg/kg) or vehicle. A, Representative bands of HSP27 (top) and its quantification (bottom). B, Representative bands of HSP60 (top) and its quantification (bottom). Density of proteins in GGA group is expressed relative to that in control group. Data are mean±SEM. *P<0.05 GGA group vs CNT group.

**Figure 4.** Expression of NOS and thioredoxin after oral GGA (G) or vehicle (CNT, C) by Western blot. Hearts were isolated 24 hours after administration of GGA (200 mg/kg) or vehicle. A, Representative bands of eNOS (top) and iNOS (middle) and quantification of eNOS (bottom). B, Representative bands of thioredoxin (top) and its quantification (bottom). Density of proteins in GGA group is expressed relative to that in control group. Data are mean±SEM.
functions, is essential for the cardioprotective effects of HSP72.

In the present study, GGA, which is widely used in the clinical setting as an antiulcer drug, induced HSP72 expression in the heart. The expression of small HSP (HSP27) and chaperonin (HSP60), both known to provide protection,1 was not induced by GGA. GGA has been reported to induce other protective proteins in several tissues, 21,22 such as nNOS in cultured guinea pig gastric pit cells 21 and thioredoxin in cultured hepatocytes, 22 which may act as an antioxidant. 23 In the present study, however, a single oral dose of GGA (200 mg/kg) did not induce expression of NOS (including iNOS, nNOS, and eNOS) or thioredoxin. On the basis of these findings, the cardioprotective effects by GGA observed in the present study are suggested to be caused predominantly by expression of HSP72.

HSP72 synthesis is controlled by a specific family of transcription factors, the heat-shock factors (HSFs). 24,25 HSF-1 is present in the cytoplasm as an inactive form of monomer, which is activated when exposed to heat stress. 1 It has been reported that exposure of cells to a heat-shock temperature of 42°C results in transient activation of HSF-1; its DNA-binding activity increases rapidly, during which time the intracellular levels of HSP72 increase.24

The mechanisms by which cardiac HSP72 is induced by GGA, as observed in the present study, remain to be clarified. In cultured guinea pig gastric mucosal cells, Hirakawa et al12 demonstrated that GGA caused rapid activation of HSF-1 and HSP72 messenger RNA, resulting in subsequent protein expression of HSP72 in association with protection against ethanol-induced exfoliation and damage. Similar mechanisms may underlie the induction of cardiac HSP72 expression by GGA.

Although several signal transductions have been postulated to be involved in the cardioprotective effects induced by HSP72,1 recent studies indicate the significance of c-Jun N-terminal kinase (JNK). 26,27 After ischemia/reperfusion, cardiomyocytes die via apoptosis as well as necrosis, and the antiapoptotic effect of HSP72 in heat-stressed cells is reportedly related to suppression of the stress kinase JNK.26 A more recent study demonstrated that HSP72 downregulates JNK by accelerating its dephosphorylation, which reduces the susceptibility of myogenic cells to simulated ischemia/reperfusion.27 Future investigation of the involvement of downregulation of JNK in the cardioprotective mechanisms induced by GGA is required.

Heat stress, resulting in expression of HSPs, is a potential intervention for the acquisition of cardioprotection, but clinical application of this therapeutic concept may be more easily achieved by oral administration of drugs that can safely induce HSP expression. GGA is a nontoxic antiulcer agent that may be capable of reducing clinical ischemia/reperfusion injury.

**Limitations of the Study**

First, GGA induces broad classes of the protective proteins, and the present study estimated the expression of only...
HSP72, HSP27, HSP60, NOS, and thioredoxin. For example, involvement of the other protective proteins, including various families of HSPs, such as αB-crystallin, HSP10, and HSP90, in GGA-induced cardioprotection remains to be estimated. Second, a recent study using confocal immunofluorescence microscopy demonstrated that an elevation of temperature induced expression of HSP72 in blood vessels but not in cardiomyocytes. The present study used whole-heart preparations to determine the expression of HSP72 by Western blot, and it remains to be studied whether induction of HSP72 by GGA may occur in cardiomyocytes or vascular cells. Finally, we estimated functional recovery during reperfusion using isolated perfused heart experiments. The results need to be confirmed by in vivo experiments in the future.

Conclusions
This study demonstrates for the first time that oral GGA induced HSP72 expression, which is associated with acquisition of resistance to ischemic insults, in rat hearts. GGA is a nontoxic agent, and the beneficial effects of clinical use of GGA in ischemic heart disease should be further assessed.

References


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