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Efficacy of HSP72 induction in rat liver by orally administered geranylgeranylacetone

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Abstract It is well known that heat-shock proteins (HSPs) have a cytoprotective function as "molecular chaperones" when cells are exposed to several stress conditions. Geranylgeranylacetone (GGA) is an antiulcer drug that was developed in Japan and it has recently been reported to induce HSP72 in rat gastric mucosa. In this experiment, we investigated the induction of HSP72 in rat liver in response to oral administration of GGA and assessed its ability to induce tolerance to warm ischemic injury by this approach. We prepared donor rats by orally administering GGA to them and compared HSP72 expression in graft liver, survival rates, and serum TNF-α concentrations after liver transplantation with the findings in controls. The survival rates were significantly increased when the livers were obtained from donor rats given GGA. Western blotting revealed expression of HSP72 in graft livers given GGA, and the serum TNF-α levels were significantly suppressed in the rats given GGA. Oral administration of GGA induced HSP72 in graft livers, and they were better able to tolerate warm ischemic injury. Oral administration of GGA appears to provide a promising new strategy for preventing ischemia-reperfusion injury.

Key words Geranylgeranylacetone · Heat-shock protein 72 · Liver transplantation · Warm ischemic injury · Ischemia reperfusion injury

Introduction

Many studies have shown the importance of heat-shock proteins (HSPs) to cell survival under stress conditions [1, 2, 4, 9]. HSPs are highly conserved proteins in all organisms [14] and are rapidly synthesized by cells in response to a variety of stresses [3, 5, 8, 11]. Their cytoprotective functions are thought to be mediated by their function as "molecular chaperones" [2].

In liver transplantation, and liver surgery in general, warm ischemia reperfusion injury causes functional and structural damage to liver cells. Because it is important to prevent such injury, many strategies have been reported [7, 12, 16]. While HSPs are well known to play a beneficial role in preventing ischemia reperfusion injury [13, 15, 19, 21], a simpler and more practical strategy has been desired from a clinical standpoint.

Geranylgeranylacetone (GGA), an acyclic polyisoprenoid, is an antiulcer drug developed in Japan and has long been used clinically without serious adverse reactions. Recently, GGA has been demonstrated to induce HSP72 in rat gastric mucosa and to prevent ulcer formation [15]. If HSP72 could be induced in liver by oral administration of GGA, it would provide a useful and practical strategy for preventing warm ischemia reperfusion injury.

In this study, we investigated the induction of HSP72 in rat liver in response to oral administration of GGA and assessed the ability to induce tolerance to warm ischemic injury by this approach. We also determined the serum TNF-α levels of rats following ischemia and

reperfusion to investigate whether GGA can suppress cytokine production.

Materials and methods

Animals

Male, inbred Brown Norway rats weighing 180–250 g were obtained from Seac Yoshitomi (Fukuoka, Japan) and housed in wire-mesh cages in a room maintained at 24 °C on a 12-h light-dark cycle. The animals were allowed free access to standard laboratory chow and water. The recipients were fasted overnight before the operation. The rats were housed under pathogen-free conditions in the Cooperative Research Center according to the guidelines set by the National Institutes of Health (NIH publication No. 86–23, revised 1985).

Preparation of donor rats

Donor rats were divided into three groups: group I ($n = 5$), no treatment, control group; group II ($n = 7$), vehicle as emulsion with 5% gum arabic containing 0.008% α-tocopherol administered orally for 4 weeks; and group III ($n = 8$), GGA, including 0.008% α-tocopherol, 200 mg/kg per day as emulsion with 5% gum arabic administered orally for 4 weeks. The GGA was kindly provided by Eisai (Tokyo, Japan).

Orthotopic liver transplantation

The method of hepatectomy and orthotopic liver transplantation was based on the cuff technique described by Kamada and Calne [10]. Before hepatectomy, the donor liver was perfused in situ with 5 ml chilled saline via the portal vein. Following cuff application, 3 ml warm (37 °C) saline was used to reperfuse the graft via the cuff, and the liver graft was stored in saline for 45 min at 37 °C. At the end of the warm ischemia, the liver was flushed out with 3 ml of cold (4 °C) saline and transplanted orthotopically. The effluent was collected after the warm ischemia, and the concentration of glutamic pyruvic transaminase (GPT) was measured. After transplantation, the rats were intravenously infused with 1 ml Ringer's lactate solution. The 7-day survival rates were compared, and autopsy was performed on all rats that died before 7 days to determine the cause of death. Blood samples were obtained from some rats 6 h after transplantation to measure serum TNF-α.

Serum TNF-α concentration after transplantation

Blood samples were obtained 6 h after transplantation, and the serum was stored at –80 °C for later determination of the TNF-α concentration with a Factor-Test-XTM mouse TNF-α ELISA kit (Genzyme, Cambridge, Mass., USA).

Western blotting analysis

Western blotting analysis was used to detect HSP72 in the tissues as previously reported [20]. Frozen tissue specimens were homogenized in lysis buffer and then clarified by centrifugation at 15000 *g* for 15 min at 4 °C. The protein content of the supernatants was determined by means of the Bradford protein assay (BioRad Laboratories, Richmond, Calif., USA). Thirty micrograms of protein were then resolved by electrophoresis on an 8% polyacrylamide gel with 0.1% sodium dodecyl sulfate, and the gel-resolved proteins were transferred to nitrocellulose transfer membranes (Schleicher and Schuell, Keene, N.H., USA). The blots were incubated with primary antibody specific for the inducible form of HSP70 (monoclonal SPA-810; Stress Gen Biotechnologies, Victoria, Canada). The filters were then incubated with anti-mouse immunoglobulin horseradish peroxidase-linked second antibody (NA 931; Amersham International, Aylesbury, UK) and reacted with the enhanced chemiluminescence detection reagent (Amersham International). The light emitted by the enhanced chemiluminescence method was recorded on X-ray film (Fuji medical X-ray film; Fujifilm, Tokyo, Japan).

Table 1 Graft survivals. Group I, no treatment, control; group II, oral vehicle for 4 weeks; group III, oral geranylgeranylacetone for 4 weeks (200 mg/kg per day as emulsion with 5% gum arabic, including 0.008% α-tocopherol)

Group	Survival rates	
Group I	0%	0/5
Group II	0%	0/7
Group III	87.5%	7/8

Statistical analysis

Statistical analysis was performed by using the unpaired *t*-test. A probability value < 0.05 was considered statistically significant. Values are expressed as mean ± SD.

Results

Survival of liver-transplanted rats

Table 1 shows the survival of the liver-transplanted rats. All rats in groups I and II died of primary non-functioning grafts within 2 days after transplantation (survival rates: 0/5 and 0/7, respectively), whereas the 1-week survival rate of the transplanted rats in group III was 87.5% (7/8).

Enzyme levels in the effluent

The GPT content of the effluent from livers after 45 min of exposure to warm ischemia is shown Table 2. The GPT concentrations were significantly elevated in the livers of the group II rats compared with the group III rats (1759.3 ± 822.1 vs 919.2 ± 558.6 IU/l; $P < 0.02$). The increased GPT level in the effluent of the group II rats was thought to be due to plasma membrane injury during the warm ischemia.

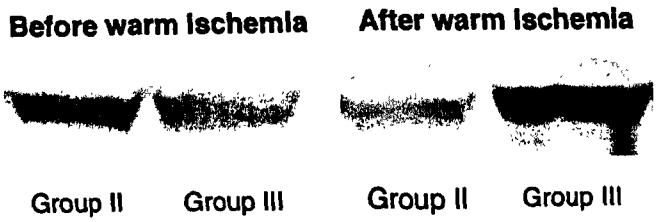

Fig. 1 Heat-shock protein 72 (HSP72) expression in liver detected by western blotting. The expression of HSP72 in livers from the rats administered garanylgeranylacetone for 4 weeks is strongly increased after 45 min warm ischemia compared with livers from vehicle-group rats

Table 2 Enzyme levels in the effluent from warm ischemic rat livers. After 45 min of warm ischemia, rat livers were flushed with 5 ml cold saline and the concentrations of glutamic pyruvic transaminase *(GPT)* were measured. Results are expressed as a mean ± SD

Group	GPT
Group II	1759.3 ± 822.1 IU/l
Group III	919.2 ± 558.6 IU/l
P value	0.02

Table 3 TNF-α level in the serum 6 h after reperfusion. Results are expressed as a mean ± SD

Group	TNF-α
Group II	230.3 ± 15.4 pg/ml
Group III	102.5 ± 74.5 pg/ml
P value	0.05

TNF-α assay

The serum TNF-α level 6 h after reperfusion are shown Table 3. The TNF-α concentrations were significantly elevated in the group II rats compared with the group III rats (230.3 ± 15.4 vs 102.5 ± 74.5 pg/ml; $P < 0.05$).

HSP72 expression

Western blotting analysis revealed HSP72 expression in the livers (Fig. 1). Expression of HSP72 in the livers of the rats administered GGA for 4 weeks was strongly increased after 45 min of warm ischemia compared with the livers of the vehicle group, whereas after administration of GGA for 4 weeks without warm ischemia, expression of HSP 72 in the liver was as weak as in the livers of the vehicle rats.

Discussion

Our study clearly showed a significantly higher survival rate at 1 week in the liver-grafted rats given GGA long term than the liver-transplanted rats not given GGA. Expression of HSP72 in the liver of the rats given GGA for 4 weeks was strongly induced after 45 min of warm ischemia compared with the livers from the vehicle group. However, expression of HSP72 in the liver after administration of GGA for 4 weeks without warm ischemia was just as weak as in the livers of vehicle rats. This suggests that GGA rapidly induced HSP72 in the liver under stress, in comparison with control. In addition, TNF-α production was more suppressed in the transplanted rats administered GGA long term than in the vehicle group. These results suggest that the warm ischemic injury and subsequent reperfusion injury are mitigated as a result of induction of HSP72 by GGA. More specifically, the HSP72 induced by GGA not only increased the survival rates of livers transplanted after warm ischemia, but protected the hepatocytes from warm ischemic injury, as indicated by the leakage of liver enzyme into the effluent after warm ischemia, and it also inhibited the activation of non-parenchymal cells (especially Kupffer cells).

Many studies have suggested that HSP synthesis is induced when cells are exposed to sublethal stress, such as hyperthermia [8], chemical agents [5], an immune response [11], or ischemia [3]. HSPs are thought to support the transportation, folding, and rearrangement of other proteins as "molecular chaperones" [2]. In particular, members of the HSP70 family play a major role in the folding, unfolding, and translocation of polypeptides as well as in the assembly and disassembly of oligomeric protein complexes. Under stress conditions, disassembly of oligomeric complexes and unfolding of polypeptides occurs, and HSPs prevent or reverse these events. HSPs may accelerate the removal of denatured proteins [11] and in this way HSPs play one of the most critical roles in induced tolerance to stress conditions.

We have previously shown that the expression of the mRNA of GRP78, a member of the HSP70 family induced by fasting, was correlated with the outcome of rat liver transplantation after warm ischemia [18]. However complete donor fasting is far from the actual situation in clinical practice, and a simpler method needs to be considered. GGA is an antiulcer drug developed in Japan and has been used clinically to treat gastritis or gastric ulcer patients since 1984 without severe adverse reactions. GGA was recently reported to induce HSP72 in rat gastric mucosa and inhibit ethanol-induced ulcer formation [6], and we expected to be able to apply this newly discovered function of GGA to liver tissue. Accordingly, we investigated the ability of orally administered GGA to induce HSP72 in the liver. The results showed surprising graft survivals in the rats given

GGA. We think that this simple method of inducing HSP72 in the liver may serve as a new strategy for preventing ischemic reperfusion injury.

It has been suggested that the stress response in mammalian cells occurs via transcriptional induction of heat-shock genes mediated by activation of a preexisting pool of heat-shock factor that binds to heat-shock element [17], and Hirakawa reported that GGA may especially induce transcriptional activation of heat-shock genes in rat gastric mucosal cells [6]. The mechanism of HSP72 induction by GGA in rat liver is probably the same as in rat gastric mucosa, and our study showed that serum TNF-α levels 6 h after reperfusion were more suppressed in transplanted rats administered GGA long term than in the vehicle group. Although we do not know how GGA suppresses TNF-α production, we think that the HSP72 induced by GGA affects non-parenchymal cells (especially Kupffer cells), and these aspects of GGA function should be investigated further.

In summary, oral administration of GGA effectively induced HSP72 in graft liver, and the livers were better able to tolerate warm ischemic injury when obtained from the rats administered GGA. Oral administration of GGA is considered to be a promising new practical strategy for preventing ischemia reperfusion injury, not only for liver transplantation but for liver surgery in general.

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