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Mitochondria are targets for geranylgeranylacetone-induced cardioprotection against ischemia-reperfusion in the rat heart

Tetsuji Shinohara,¹ Naohiko Takahashi,¹ Hiroaki Kohno,¹ Kunitoshi Yamanaka,¹ Tatsuhiko Ooie,² Osamu Wakisaka,¹ Yukichi Murozono,¹ Yayoi Taniguchi,² Yasuko Torigoe,² Masahide Hara,¹ Tatsuo Shimada,³ Tetsunori Saikawa,² and Hironobu Yoshimatsu¹

¹Department of Internal Medicine 1, ²Department of Cardiovascular Science, Faculty of Medicine, and ³Department of Health Science, School of Nursing, Oita University, Oita, Japan

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Shinohara T, Takahashi N, Kohno H, Yamanaka K, Ooie T, Wakisaka O, Murozono Y, Taniguchi Y, Torigoe Y, Hara M, Shimada T, Saikawa T, Yoshimatsu H. Mitochondria are targets for geranylgeranylacetone-induced cardioprotection against ischemia-reperfusion in the rat heart. *Am J Physiol Heart Circ Physiol* 293: H1892–H1899, 2007. First published June 22, 2007; doi:10.1152/ajpheart.00493.2007.—It has been shown that orally administered geranylgeranylacetone (GGA), an anti-ulcer drug, induces expression of heat shock protein 72 (HSP72) and provides protection against ischemia-reperfusion in rat hearts. The underlying protective mechanisms, however, remain unknown. Mitochondria have been shown to be a selective target for heat stress-induced cardioprotection. Therefore, we hypothesized that preservation of mitochondrial function, owing to an opening of a putative channel in the inner mitochondrial membrane, the mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel, could be involved in GGA- or heat stress-induced cardioprotection against ischemia-reperfusion. Rats were treated with oral GGA or vehicle. Twenty-four hours later, each heart was isolated and perfused with a Langendorff apparatus. GGA-treated hearts showed better functional recovery, and less creatine kinase was released during a 30-min reperfusion period, after 20 min of no-flow ischemia. Concomitant perfusion with 5-hydroxydecanoate (5-HD, 100 μ M) or glibenclamide (10 μ M) abolished the GGA-induced cardioprotective effect. GGA also showed preserved mitochondrial respiratory function, isolated at the end of the reperfusion period, which was abolished with 5-HD treatment. GGA prevented destruction of the mitochondrial structure by ischemia-reperfusion, as shown by electron microscopy. In cultured cardiomyocytes, GGA induced HSP72 expression and resulted in less damage to cells, including less apoptosis in response to hypoxia-reoxygenation. Treatment with 5-HD abolished the GGA-induced cardioprotective effects but did not affect HSP72 expression. Our results indicate that preserved mitochondrial respiratory function, owing to GGA-induced HSP72 expression, may, at least in part, have a role in cardioprotection against ischemia-reperfusion. These processes may involve opening of the mitoK_{ATP} channel.

heat shock protein 72; mitochondrial ATP-sensitive potassium channel; 5-hydroxydecanoate; glibenclamide

CARDIOMYOCYTES POSSESS ABUNDANT mitochondria, which are key organelles involved in ischemia-reperfusion injury (14, 19). Damage to mitochondria causes reduced respiratory function and ATP synthesis, resulting in irreversible cardiomyocyte damage (14, 19). Our laboratory has previously reported that geranylgeranylacetone (GGA), an anti-ulcer agent, induces expression of heat shock protein 72 (HSP72) and provides

cardioprotection against ischemia-reperfusion injury in rat hearts (16, 25). Nevertheless, the precise mechanisms for GGA-induced protection remain unknown. Since mitochondria have been shown to be selective targets for heat stress (HS)-induced cardioprotection (20), we hypothesized that the preservation of mitochondrial function could be involved in GGA- or HS-induced cardioprotection against ischemia-reperfusion.

The mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel is a putative channel in the inner mitochondrial membrane (17). Although this channel has not yet been cloned, its pharmacological activation by nicorandil provides cardioprotection against ischemic insult or oxidative stress and its blockade by 5-hydroxydecanoate (5-HD) or glibenclamide prevents this protection (7, 24). It has also been reported that opening of the mitoK_{ATP} channel has an essential role in ischemic preconditioning-induced protection of mitochondria (6).

In the present study, using rat heart and rat neonatal cultured cardiomyocytes, we investigated the mechanism by which HSP72 expression induces cardioprotection. In experiments using isolated perfused hearts, we investigated the effects of either 5-HD or glibenclamide on left ventricular (LV) functional recovery and creatine kinase (CK) release during a 30-min reperfusion following 20 min of no-flow global ischemia. At the end of the reperfusion period, the mitochondria were isolated for evaluation of respiratory function and their ultrastructure. In experiments using cultured cardiomyocytes, the effect of 5-HD on GGA-induced cardioprotective effects was investigated using small-interfering RNA (siRNA) that targets HSP72 because GGA-induced cardioprotective effects may involve HSP72 expression.

MATERIALS AND METHODS

All experimental procedures were approved by and performed in accordance with the guidelines for the care and use of laboratory animals of the Physiological Society of Oita University, Japan.

Materials. Monoclonal immunoglobulin G (IgG) cross-reactive to inducible HSP72 antibody was obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Horseradish peroxidase-linked F(ab')₂ fragment from sheep anti-mouse immunoglobulin and reagents for Western blot assay and enhanced chemiluminescence were purchased from Amersham (Piscataway, NJ). Bradford protein assay kits were purchased from Bio-Rad (Richmond, CA). 5-HD and glibenclamide were purchased from ICN Biomedicals (Costa Mesa, CA) and Wako (Osaka, Japan), respectively. GGA was provided by Eisai (Tokyo, Japan).

Address for reprint requests and other correspondence: T. Shinohara, Dept. of Internal Medicine 1, Faculty of Medicine, Oita Univ., 1-1 Idaigaoka, Hasama, Oita 879-5593, Japan (e-mail: shinohar@med.oita-u.ac.jp).

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Table 1. Hemodynamic parameters at baseline

	<i>n</i>	LVDP, mmHg	HR, beats/min	CPP, mmHg	dP/dt _{max} , mmHg/s	dP/dt _{min} , mmHg/s
CNT	8	104 ± 6	346 ± 18	81 ± 2	3,170 ± 172	-2,768 ± 83
GGA	9	96 ± 13	325 ± 9	80 ± 3	3,270 ± 114	-2,801 ± 112
HS	9	96 ± 6	331 ± 16	84 ± 3	3,161 ± 97	-2,762 ± 51
CNT-5-HD	8	102 ± 13	324 ± 17	84 ± 4	3,108 ± 145	-2,808 ± 152
GGA-5-HD	8	104 ± 6	324 ± 11	81 ± 4	3,189 ± 120	-2,924 ± 156
HS-5-HD	8	99 ± 8	342 ± 22	82 ± 3	3,050 ± 125	-2,781 ± 170
CNT	7	103 ± 5	341 ± 17	79 ± 4	3,088 ± 82	-2,807 ± 160
GGA	7	101 ± 4	321 ± 15	79 ± 2	3,287 ± 156	-2,844 ± 112
HS	7	100 ± 5	338 ± 15	80 ± 6	3,269 ± 101	-2,725 ± 128
CNT-Glib	7	101 ± 5	321 ± 11	81 ± 5	3,167 ± 72	-2,840 ± 126
GGA-Glib	7	101 ± 7	341 ± 18	83 ± 5	3,085 ± 164	-2,772 ± 155
HS-Glib	7	103 ± 5	327 ± 26	81 ± 5	2,987 ± 173	-2,745 ± 170

Values are means ± SE; *n*, number of hearts. LVDP, left ventricular developed pressure; HR, heart rate; CPP, coronary perfusion pressure; dP/dt_{max} and dP/dt_{min}, peak positive and negative first derivatives of LV pressure, respectively; CNT, control; GGA, geranylgeranylacetone; HS, heat shock; 5-HD, 5-hydroxydecanoate; Glib, glibenclamide.

was evaluated after 4 h with treatment of 10⁻⁵ M GGA. To evaluate the effect of 5-HD on GGA treatment, the cultured myocytes were treated with 10⁻⁵ M GGA or vehicle for 4 h in the culture medium, and 5-HD (500 μM) was added 10 min before hypoxia was induced. Some cardiomyocytes were lysed for Western blot analysis. To evaluate the tolerance to hypoxia-reoxygenation, the remaining cardiomyocytes were incubated in serum-free DMEM without glucose under a hypoxic gas mixture (95% N₂-5% CO₂) at 37°C for 3 h,

followed by reoxygenation under a normoxic gas mixture for 1 h. The supernatant was carefully collected for lactate dehydrogenase (LDH) determination using an LDH assay kit (Eiken Chemical, Tokyo, Japan). For the apoptosis assay, cells were fixed after reoxygenation in 4% paraformaldehyde solution in 0.1 M NaH₂PO₄ and permeabilized with 0.5% Triton X-100 (Sigma, St. Louis, MO). Apoptotic cells were detected with the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method using an

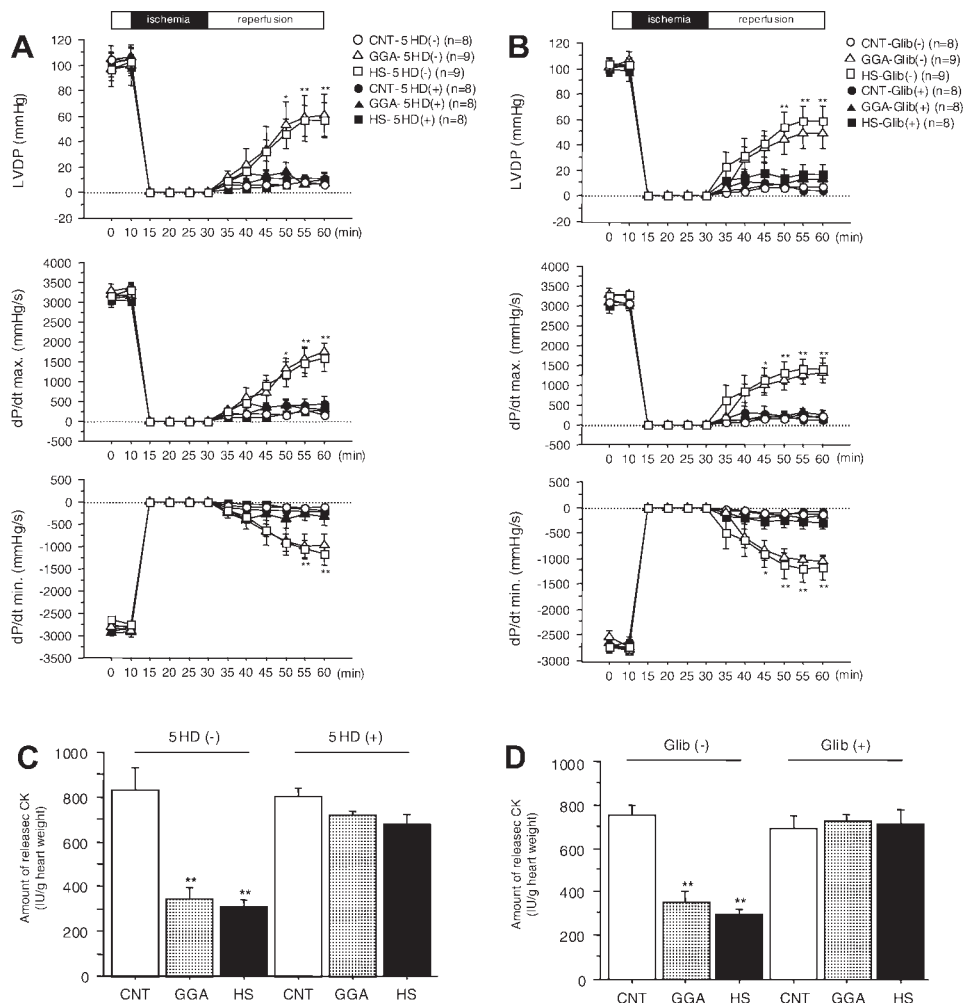


Fig. 2. A and B: effects of 5-HD (A) and Glib (B) on serial changes of LVDP and peak positive and negative first derivatives of LV pressure (dP/dt_{max} and dP/dt_{min}, respectively) during the experimental period. Twenty minutes of no-flow global ischemia was followed by 30 min of reperfusion. Data are means ± SE; *n* = 8 or 9 hearts/group. **P* < 0.05 and ***P* < 0.01 vs. corresponding 5-HD- or Glib-perfused groups. C and D: effects of perfusion with 5-HD (C) or Glib (D) on GGA- or HS-induced reduction in amount of CK released during the 30-min reperfusion period. Amount of released CK is expressed relative to ventricular weight. Data are means ± SE. ***P* < 0.01 vs. control group.

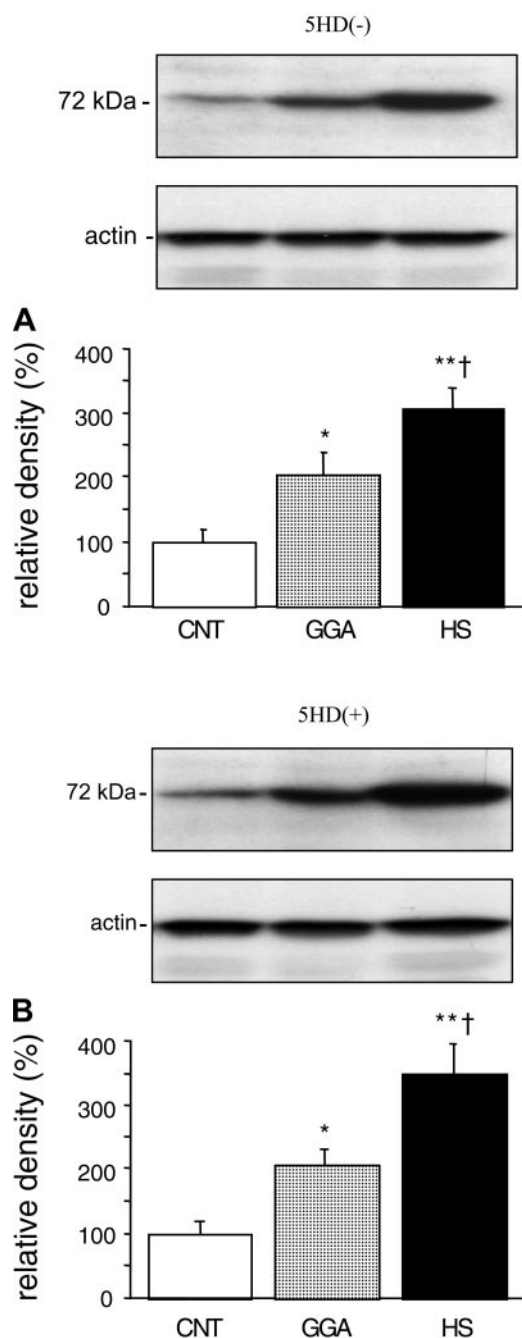


Fig. 3. Effects of 5-HD perfusion on GGA- or HS-induced HSP72 expression. *A*: HSP72 expression without 5-HD perfusion. *B*: HSP72 expression with 5-HD perfusion. Hearts were isolated at the end of the reperfusion period and prepared for Western blot analysis. Representative immunoblots and relative band densities of cardiac HSP72 are shown. Data are means \pm SE; $n = 7$ hearts/group. * $P < 0.05$ and ** $P < 0.01$ vs. control group. † $P < 0.05$ vs. GGA group.

apoptosis kit (Medical Biological Lab, Nagoya, Japan). Slides were treated with a mixture of terminal deoxynucleotidyl transferase, FITC-dUTP, and TdT buffer II at 37°C for 1 h. After being washed with PBS, the cells were mounted with mounting medium of 4',6-diamidino-2-phenylindole.

siRNA transfection. The siRNAs targeting HSP72 were obtained from Ambion (Austin, TX) and transfected into cells at a concentration of 10 nM with siPORT NeoFX Transfection (Ambion) (23). The

control cells were transfected with negative control siRNA. Twenty-four hours after transfection, cells were incubated with GGA or vehicle for 4 h. HSP72 expression was evaluated, and hypoxia-reoxygenation was performed.

Statistical analysis. Data are expressed as means \pm SE. Serial changes in LVDP, LVEDP, CPP, heart rate, and dP/dt were analyzed by two-way ANOVA and by the Bonferroni/Dunn test, unless otherwise specified. The hemodynamic parameters at baseline, the ratio of released CK to ventricular weight, the LDH content in the cultured medium, and the percentage of apoptotic cells were compared among groups by one-way ANOVA followed by the Bonferroni/Dunn test. The relative intensity of each protein and RCI were compared using the Mann-Whitney *U*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Isolated perfused heart experiments. As shown in Table 1, LVDP, heart rate, CPP, and dP/dt did not differ among the 12 groups at baseline. Figure 2, *A* and *B*, shows the serial changes in LVDP and dP/dt during the experimental period. The recovery of LVDP and dP/dt with reperfusion was greater in the GGA and HS groups compared with the control group ($P < 0.05$ for each by ANOVA). Perfusion with 5-HD abolished the improved functional recovery observed in the GGA- and HS-treated hearts (Fig. 2*A*). Heart rate and CPP did not differ significantly among the 12 groups at any period (data not shown). Perfusion with glibenclamide had the same effect as 5-HD (Fig. 2*B*). The amount of CK released during the 30-min

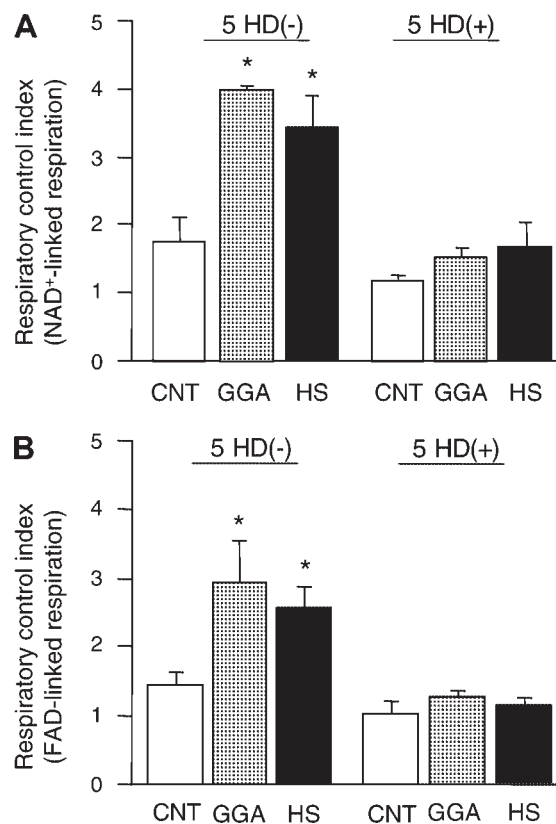


Fig. 4. Respiratory control index (RCI). RCI was calculated as the ratio of the state 3 to state 4 mitochondrial oxygen consumption rates. Mitochondria were isolated at the end of the reperfusion period. NAD⁺- (A) and FAD-linked respiration (B) is shown. Data are means \pm SE; $n = 7$ hearts/group. * $P < 0.05$ vs. control group.

reperfusion was lower in the GGA and HS groups than in the control group ($P < 0.01$) (Fig. 2, C and D). Perfusion with 5-HD abolished the reduction in CK release observed in the GGA and HS groups (Fig. 2C). Perfusion with glibenclamide had the same effect as 5-HD (Fig. 2D).

GGA-induced cardiac HSP72 expression. Figure 3, A and B, shows the cardiac HSP72 expression level in the hearts at the end of reperfusion. Irrespective of perfusion with 5-HD, the hearts treated with GGA or HS showed increased HSP72 expression levels compared with control hearts ($P < 0.05$ and $P < 0.01$, respectively). The HSP72 expression level in HS-treated hearts was greater than that in GGA-treated hearts ($P < 0.05$).

Mitochondrial respiration. Figure 4, A and B, shows the respiratory function of mitochondria at the end of the reperfusion period. The RCIs for malate (complex I substrate)- and succinate (complex II substrate)-energized mitochondria were increased in the GGA and HS groups compared with the control group ($P < 0.05$ for each). Concomitant perfusion with 5-HD abolished the improved respiration function of mitochondria in GGA- and HS-treated hearts.

Electron microscopic findings. In mitochondria isolated from the control group, most of the inner and outer membranes were disrupted, displaying disrupted cristae formation and a swollen matrix (Fig. 5). These alterations were reduced, however, in mitochondria isolated from the GGA and HS groups, although some mitochondria were substantially damaged.

GGA-induced HSP72 expression in cultured rat neonatal cardiomyocytes. Figure 6, A and B, shows the effects of concentration- and time-dependent GGA induction of HSP72

in cultured cardiomyocytes. When assessed 4 h after GGA application, a GGA concentration of 10^{-5} M induced the greatest HSP72 expression level (Fig. 6A). HSP72 expression reached a maximum level 4 h after application of 10^{-5} M GGA (Fig. 6B).

The effect of 5-HD against GGA-induced HSP72 expression in cultured rat neonatal cardiomyocytes. Figure 6C shows representative immunoblots and relative band densities of HSP72 in cultured rat neonatal cardiomyocytes. Irrespective of the application of 5-HD, cardiomyocytes treated with GGA showed increased HSP72 expression compared with that in control cardiomyocytes ($P < 0.01$).

The effects of GGA and 5-HD treatment on hypoxia-reoxygenation injury in cultured rat neonatal cardiomyocytes. Figure 7, A and B, shows representative photomicrographs of TUNEL staining and the percentages of apoptotic cells in cultured cardiomyocytes after hypoxia-reoxygenation. GGA treatment reduced the number of TUNEL-positive cells ($18.2 \pm 2.0\%$ vs. $7.4 \pm 1.1\%$, $P < 0.01$). However, concomitant application of 5-HD abolished the protective effects of GGA ($16.2 \pm 1.7\%$ vs. $7.4 \pm 1.1\%$, $P < 0.01$). GGA treatment reduced the level of LDH release in response to hypoxia-reoxygenation compared with vehicle treatment (0.016 ± 0.005 vs. 0.050 ± 0.004 IU/ml, $P < 0.01$) (Fig. 7C). However, a concomitant application of 5-HD abolished the protective effects of GGA (0.042 ± 0.006 vs. 0.016 ± 0.005 IU/ml, $P < 0.01$).

The effect of HSP72-siRNA on HSP72 expression and hypoxia-reoxygenation injury. As shown in Fig. 8A, the HSP72-targeted siRNA effectively suppressed the GGA-induced

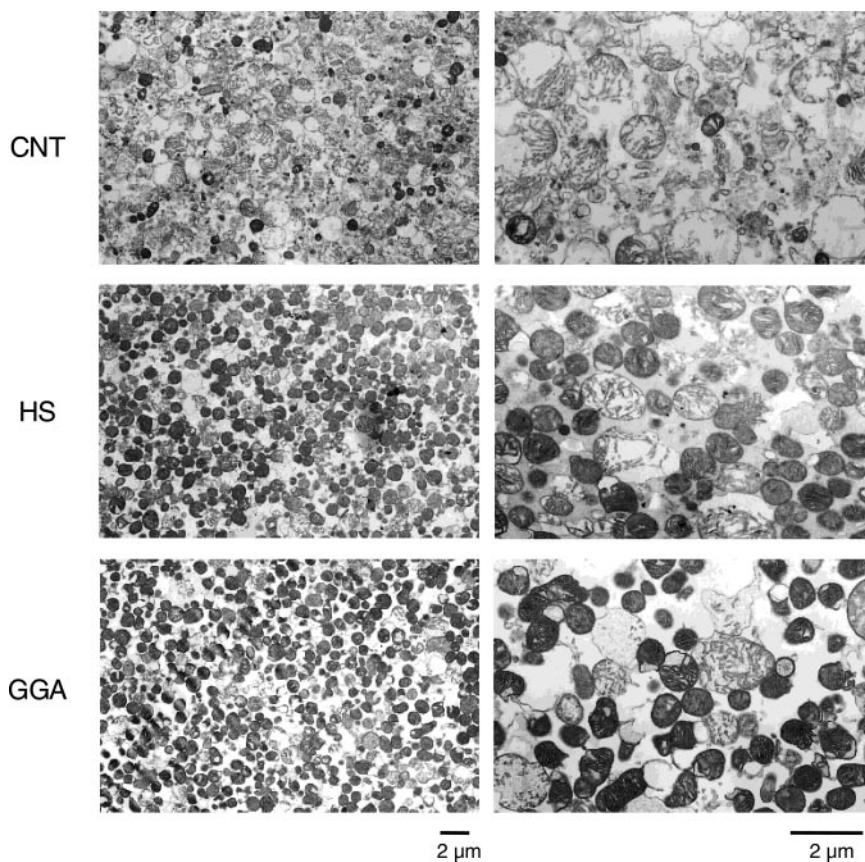


Fig. 5. Electron microscope results. In isolated mitochondria from the control group, most of the inner and outer membranes were disrupted, displaying destroyed cristae formation and a swollen matrix. These alterations were reduced, however, in isolated mitochondria from the GGA or HS group, although some mitochondria were still markedly damaged.

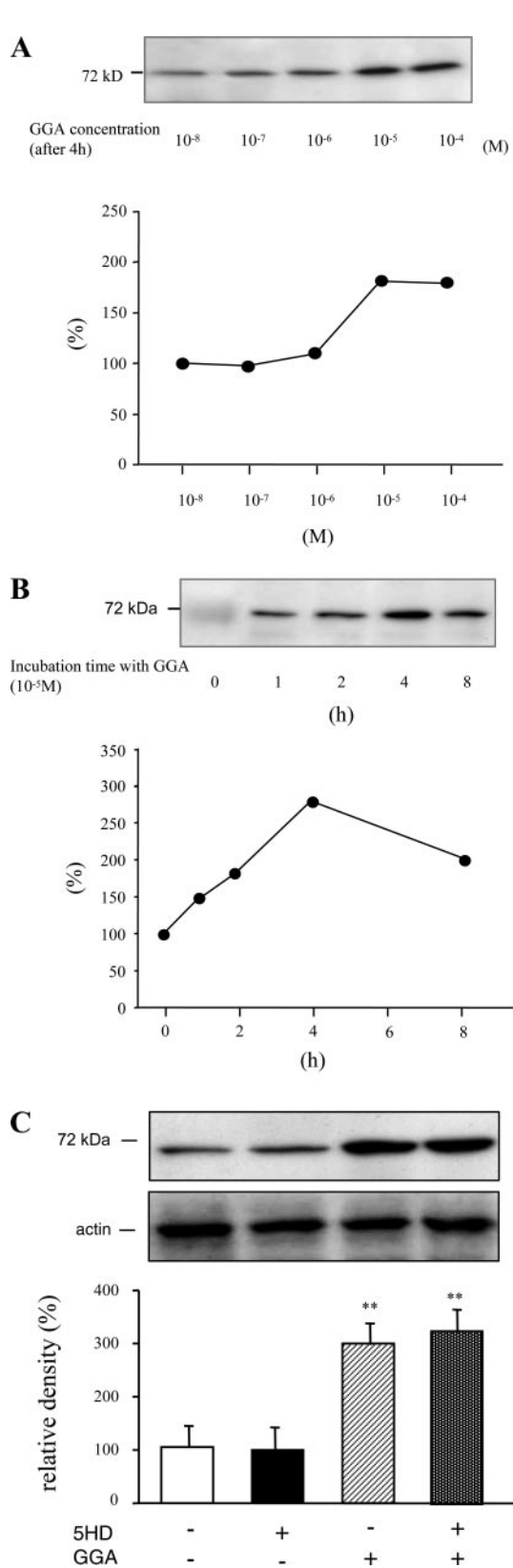


Fig. 6. *A* and *B*: representative immunoblots and relative band densities of GGA-induced HSP72 expression in cultured cardiomyocytes according to GGA dose and incubation time. *C*: effect of 5-HD on GGA-induced HSP72 expression in cultured rat neonatal cardiomyocytes. Data are means \pm SE; $n = 5$ independent cultures/group. $**P < 0.01$ vs. vehicle group.

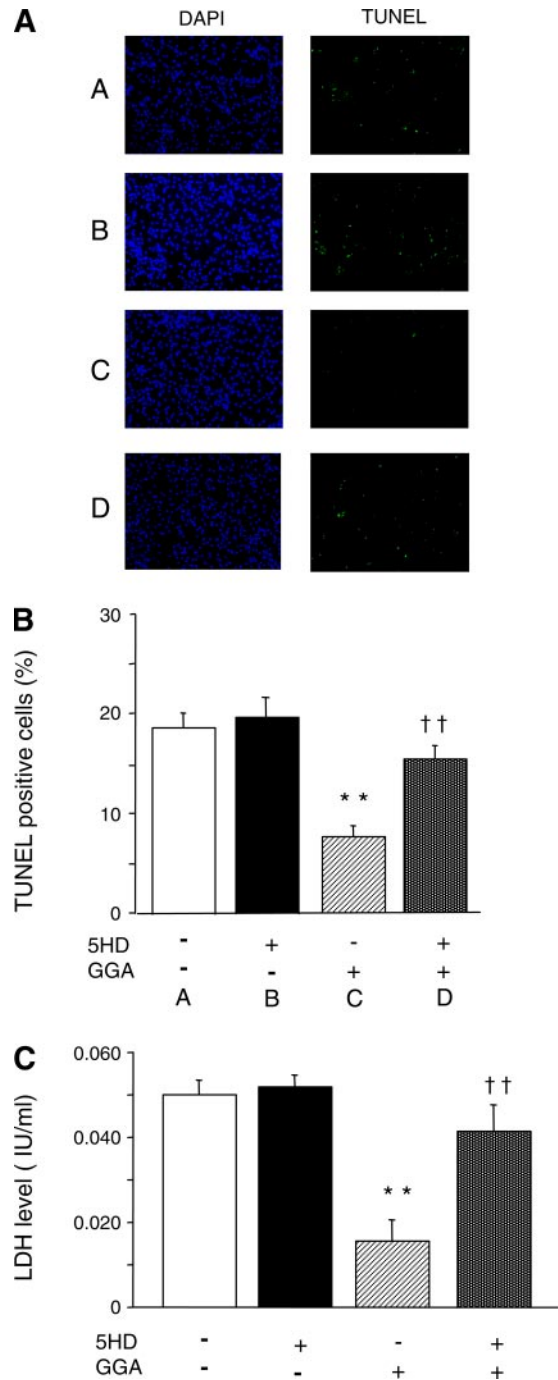


Fig. 7. Effects of GGA and 5-HD treatment on hypoxia-reoxygenation injury in cultured rat neonatal cardiomyocytes. *A*: representative photomicrographs of TUNEL staining in cultured cardiomyocytes after hypoxia-reoxygenation. Nuclei with green staining indicate TUNEL-positive cells. *B*: quantitative results of TUNEL staining for different groups. *C*: amount of LDH released in response to hypoxia-reoxygenation. DAPI, 4',6-diamidino-2-phenylindole. Five independent cultures were evaluated. Data are means \pm SE; $**P < 0.01$ vs. vehicle group; $\dagger\dagger P < 0.01$ vs. GGA-treated cells in the absence of 5-HD.

HSP72 expression in the presence or absence of 5-HD. In GGA-treated cardiomyocytes, the LDH release in response to hypoxia-reoxygenation was increased with 5-HD treatment (0.012 ± 0.002 vs. 0.030 ± 0.004 IU/ml, $P < 0.01$) (Fig. 8*B*). In the absence of 5-HD, treatment with HSP72-targeted siRNA

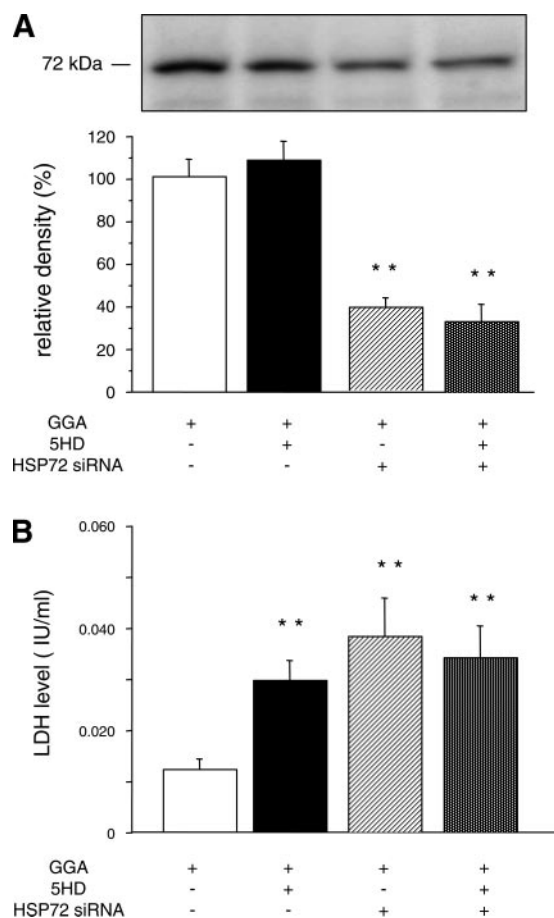


Fig. 8. Effect of HSP72-specific small-interfering (siRNA) on HSP72 expression and hypoxia-reoxygenation injury in cultured cardiomyocytes. *A*: representative immunoblots and relative band densities of HSP72. *B*: amount of LDH release in response to hypoxia-reoxygenation. Data are means \pm SE; $n = 5$ independent cultures/group. ** $P < 0.01$ vs. GGA-treated cells in the absence of 5-HD and HSP72-specific siRNA.

inhibited the GGA-induced protective effects on LDH release (0.012 ± 0.002 vs. 0.038 ± 0.008 IU/ml, $P < 0.01$). Concomitant treatment with 5-HD did not affect the amounts of LDH released (0.030 ± 0.004 vs. 0.034 ± 0.006 IU/ml, not significant).

DISCUSSION

In the present study, we demonstrated that rat hearts treated with GGA or HS were protected from ischemia-reperfusion injury. This was shown by better LV function recovery and reduced CK release during reperfusion. Preservation of mitochondrial respiratory function and structure was also demonstrated, when assessed at the end of reperfusion. These observations indicate that upregulation of HSP72 with GGA or HS protects the mitochondria from ischemia-reperfusion injury, resulting in improved preservation of cardiac function.

Regarding the role of mitochondria as a target for the protective effects of HS, Polla et al. (20), using cultured cells, reported that treatment with HS prevented hydrogen peroxide-induced changes in mitochondrial membrane potential and cristae formation and that the protective effects of HS were well correlated with the expression of HSP72. Jayakumar et al. (15) reported that rat hearts transfected with the HSP72 gene

via intracoronary infusion showed protection of mitochondrial respiratory function after ischemia-reperfusion, which was associated with improved preservation of ventricular function. They suggested that one of the mechanisms for HSP72-mediated cardioprotection may be the opening of the mitoK_{ATP} channel, although no evidence was shown (15). The important role of the mitoK_{ATP} channel in myocardial ischemic tolerance after HS treatment has since been reported in animal studies, both in vitro and in vivo (1, 12, 18). In isolated perfused rat hearts, preperfusion with either 10 μ M glibenclamide or 100 μ M 5-HD abolished the reduction in regional ischemia-induced infarct size produced by whole body HS treatment 24 h before ischemia (12). With the use of an in vivo rabbit model of acute myocardial infarction, the reduction in infarct size with HS treatment 24 h before coronary occlusion was abolished with intravenous 0.3 mg/kg glibenclamide or 5 mg/kg 5-HD administered 10 min before coronary occlusion (18). These studies, however, did not evaluate mitochondrial respiratory function in response to ischemia-reperfusion (1, 12, 18). In our perfused rat heart experiment, concomitant perfusion with 100 μ M 5-HD or 10 μ M glibenclamide abolished the GGA- or HS-induced improvement in LV functional recovery during reperfusion. Furthermore, GGA- or HS-treated hearts showed preserved mitochondrial respiratory function, with preservation of mitochondrial ultrastructure, when assessed at the end of the reperfusion period. The preservation of mitochondrial respiratory function with GGA or HS was abolished by treatment with 100 μ M 5-HD. The pharmacological selectivity of these widely used mitoK_{ATP}-channel blockers, at the conventional inhibitory concentrations (1, 12, 18) used in the present study, may indicate the important role of the mitoK_{ATP} channel in GGA- or HS-induced preservation of mitochondrial respiratory function, leading to cardioprotection against ischemia-reperfusion.

The role of the mitoK_{ATP} channel in modulating cardiac mitochondrial function has been investigated previously (6, 13). Using isolated mitochondria from rat ventricles, Holmuhamedov et al. (13) demonstrated that the pharmacological opening of mitoK_{ATP} channel induced rapid membrane depolarization, a decrease in the rate of ATP synthesis, and a resultant compensatory increase in the rate of mitochondrial respiration. In addition to energy production, mitochondria can store Ca²⁺, a function that is critical for the maintenance of cellular Ca²⁺ homeostasis (5). The mitoK_{ATP} channel opening was also shown to prevent cellular Ca²⁺ overload by accumulating and retaining Ca²⁺ in the matrix (13). These mechanisms may underlie the cardioprotection induced by GGA or HS, as demonstrated in the present study. Further studies are needed to show how enhanced expression of HSP72, induced by GGA or HS, modulates the activity of the mitoK_{ATP} channel. As shown in Fig. 3, at the end of the reperfusion period, HSP72 was abundantly expressed in GGA- or HS-treated heart, even in those concomitantly perfused with 5-HD. Consistent with this finding, the study of Hoag et al. (12) reported that the enhanced expression of HSP72 in HS-treated rabbit heart was not reduced by preperfusion with glibenclamide or 5-HD. Taken together, these results indicate that HSP72 degradation does not underlie the ability of 5-HD to abolish cardioprotection.

Until now, we have attributed the opening of the mitoK_{ATP} channel, and the resultant preservation of mitochondrial func-

tion, to HSP72-induced cardioprotection by GGA or HS. However, another mechanism may be involved. Following ischemia-reperfusion, cardiomyocytes die, by apoptosis as well as necrosis, and the anti-apoptotic effect of HSP72 in HS-treated cells has been reported to be related to suppression of the stress kinase c-Jun NH₂-terminal kinase (JNK) (8). A more recent study demonstrated that HSP72 downregulates JNK by accelerating its dephosphorylation, which reduces the susceptibility of myogenic cells to simulated ischemia-reperfusion (9). However, HSP72 essentially acts as a molecular chaperone (3). HSP72 supports the transport of newly synthesized mitochondrial proteins, including respiratory chain enzymes, into mitochondria. The well-preserved enzyme activity of the mitochondrial respiratory chain may be due to the enhanced RCI after ischemia-reperfusion observed in mitochondria isolated from GGA- or HS-treated hearts.

Although we confirmed the protective effects of HSP72 expression in cultured cardiomyocytes using siRNA that targets HSP72, the effects of HSP72 induction could not be isolated in experiments *in vivo*. In addition to the HSP family, GGA is known to induce expression of many classes of protective proteins (11, 21). Therefore, the protective effects of GGA observed in experiments *in vivo* could not be solely explained by the effects of HSP72. In this regard, effective elimination of HSP72 expression by HSP72-specific siRNA was observed *in vitro*, together with the elimination of protection against hypoxia-reoxygenation. These results strongly support the validity of our hypothesis that GGA-induced HSP72 expression provides cardioprotection.

There are several limitations in the present study. First, we evaluated the effects of HSP72-siRNA only in experiments that investigated hypoxia-reoxygenation injury, *i.e.*, LDH release. Future studies will be required to investigate the effects of HSP72-siRNA on mitoK_{ATP} channel effectors as RCI. Second, although 5-HD is generally regarded as a specific inhibitor of mitoK_{ATP} channel, it has been clarified that 5-HD has multiple actions independent of mitoK_{ATP} channel (10). Thus alternative mechanisms should be taken into consideration as interpreting our results using 5-HD.

In conclusion, our observations indicate that the preserved mitochondrial respiratory function with GGA-induced HSP72 expression may, at least in part, have a role in cardioprotection against ischemia-reperfusion. An opening of the mitoK_{ATP} channel may be involved in these processes.

GRANTS

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