Phosphatidylinositol 3-Kinase–Dependent Activation of Akt, an Essential Signal for Hyperthermia-Induced Heat-Shock Protein 72, Is Attenuated in Streptozotocin-Induced Diabetic Heart

Tetsuji Shinohara,[1] Naohiko Takahashi,[1] Tatsuhiko Ooie,[2] Masahide Hara,[1] Sakuji Shigematsu,[1] Mikiko Nakagawa,[2] Hidetoshi Yonemochi,[2] Tetsunori Saikawa,[2] and Hironobu Yoshimatsu[1]

We tested the hypothesis that phosphatidylinositol 3-kinase (PI 3-kinase)-dependent activation of Akt is essential for the expression of cardiac heat-shock protein 72 (HSP72) and that this pathway is impaired in the streptozotocin (STZ)-induced diabetic heart. STZ-induced male diabetic rats were treated with insulin (STZ-insulin group, $n = 26$) or vehicle (STZ-vehicle group, $n = 61$) for 3 weeks. Whole-body hyperthermia (43°C for 20 min) was applied, and the heart was isolated 24 h later. Compared with control heart, hyperthermia-induced HSP72 expression and phosphorylation of Akt were attenuated in the STZ-vehicle heart. Pretreatment with wortmannin attenuated hyperthermia-induced HSP72 expression and phosphorylation of Akt. In isolated perfused heart experiments, the hyperthermia-treated STZ-vehicle heart showed poor left ventricular functional recovery during reperfusion after no-flow global ischemia compared with hyperthermia-treated control heart. Insulin treatment restored HSP72 expression and reperfusion-induced functional recovery. In cultured neonatal rat cardiomyocytes, hyperthermia-induced HSP72 expression was enhanced by insulin, together with tolerance against hypoxia-reoxygenation injury. Wortmannin and LY294002 inhibited hyperthermia-induced HSP72 expression and phosphorylation of Akt. Our results indicate that activation of Akt, in a PI 3-kinase–dependent manner, is essential for hyperthermia-induced HSP72 expression in association with cardioprotection, suggesting impairment of this signaling pathway in the STZ-induced diabetic heart, probably due to insulin deficiency. *Diabetes* 55: 1307–1315, 2006

From the [1]Department of Internal Medicine 1, Faculty of Medicine, Oita University, Oita, Japan; and the [2]Department of Laboratory Medicine, Faculty of Medicine, Oita University, Oita, Japan.

Address correspondence and reprint requests to Naohiko Takahashi, MD, PhD, Department of Internal Medicine 1, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama, Oita 879-5593, Japan. E-mail: takanao@med.oita-u.ac.jp.

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CPP, coronary perfusion pressure; DMEM, Dulbecco's modified Eagle's medium; GSK, glycogen synthase kinase; HSE, heat-shock element; HSF1, heat-shock factor 1; HSP72, heat-shock protein 72; LDH, lactate dehydrogenase; LV, left ventricle; LVDP, LV developed pressure; PI 3-kinase, phosphatidylinositol 3-kinase; released CK, creatine kinase content; siRNA, small interfering RNA; STZ, streptozotocin.

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Studies from our laboratory and those of other investigators have shown that heat-shock protein 72 (HSP72), induced by hyperthermia, protects the heart against ischemia-reperfusion injury (1–3). Streptozotocin (STZ)-induced diabetic rats have been widely used as a model of insulin-deficient diabetes and are known to exhibit various cardiac abnormalities (4). However, the effects of STZ-induced diabetes on HSP72 expression in the heart remain inconclusive. Joyeux et al. (5) reported that, although hyperthermia induced comparable expression levels of HSP72 in normal and diabetic hearts, the size of the infarction induced by coronary artery occlusion followed by reperfusion was larger in the diabetic than in the normal heart. Based on these observations, Joyeux et al. (5) concluded that the myocardial protective effect of hyperthermia did not extend to STZ-induced diabetic rats and seemed to be unrelated to the HSP72 level. Recently, however, Qi et al. (6) reported that preconditioning with a κ-opioid receptor agonist, U50,488H, increased cardiac HSP72 expression and reduced the infarct size induced by coronary artery occlusion in the normal heart 24 h after preconditioning but not in the STZ-induced diabetic rat heart. They concluded that lack of a U50,488H cardioprotective effect could, at least in part, be due to impaired synthesis of HSP72 in the STZ-induced diabetic heart (6).

Activation of Akt promotes survival of some cell types, including cardiomyocytes (7). Hyperthermia is known to cause the activation of Akt via either a phosphatidylinositol 3-kinase (PI 3-kinase)–dependent or –independent pathway (8–11). However, little is known about the involvement of Akt in hyperthermia-induced HSP72 expression in the heart. Because insulin activates Akt in a PI 3-kinase–dependent manner (12,13), we hypothesized that *1*) whole-body hyperthermia could cause PI 3-kinase–dependent activation of Akt in the normal heart, resulting in HSP72 overexpression in association with protection against ischemia-reperfusion injury, and *2*) insulin-deficient STZ-induced diabetic rats have impaired PI 3-kinase–dependent activation of Akt, which results in the loss of hyperthermia-induced myocardial HSP72 expression and cardioprotection.

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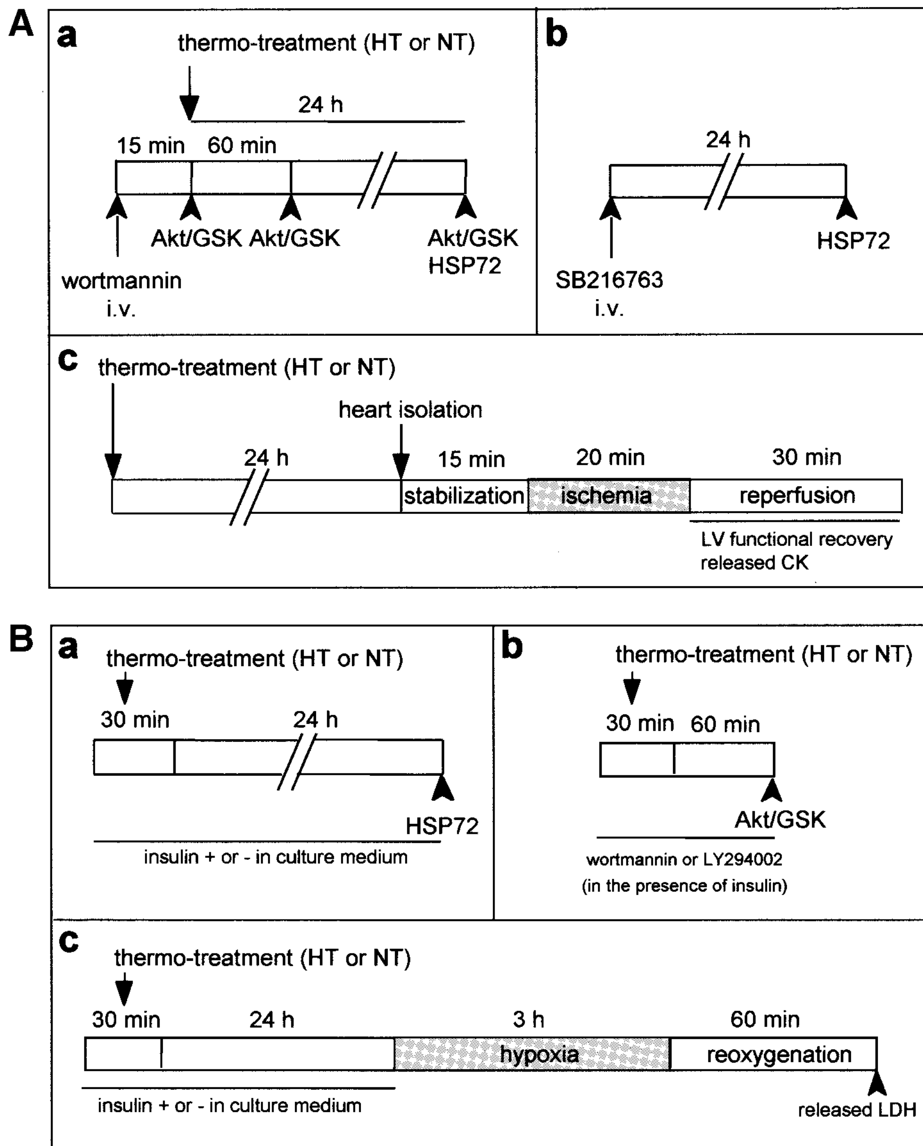

FIG. 1. Experimental protocol. *A*: Experiments using whole rat heart. Experiments were performed 4 weeks after STZ or vehicle injection. *B*: Experiments using cultured neonatal rat cardiomyocytes. GSK, GSK-3β. i.v., intravenous injection.

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RESEARCH DESIGN AND METHODS

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

Antibody to mouse HSP72 was purchased from Stressgen (San Diego, CA). Antibodies to rabbit Akt and phospho-Akt and glycogen synthase kinase (GSK)-3β and phospho–GSK-3β were purchased from Cell Signaling (Beverly, MA). Horseradish peroxidase–tagged secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham (Piscataway, NJ). Bradford protein assay kits were purchased from Bio-Rad (Richmond, CA). LY294002 and SB216763 were purchased from Promega (Madison, WI) and Wako (Osaka, Japan), respectively. Wortmannin and other chemical agents were purchased from Sigma (St. Louis, MO).

Housed Male Sprague-Dawley rats (200–250 g) were illuminated daily from 0700 to 1900 with temperature maintained at 21 ± 1°C. All animals were allowed free access to tap water and standard pellet rat chow (Clea Japan, Tokyo, Japan). Diabetes was induced by a single injection of STZ (60 mg/kg) dissolved in sterile sodium citrate buffer solution (0.1 mol/l citric acid and 0.2 mol/l sodium phosphate, pH 4.5) into the tail vein (14). Age-matched control rats ($n = 61$) were injected with an equivalent volume of citrate buffer solution (control group). One week after the STZ injection, rats with plasma glucose concentration >400 mg/dl were defined as diabetic. At that time point, the STZ-induced diabetic rats were randomly divided into two groups: those treated with insulin (STZ-insulin group, $n = 26$) and those treated with saline (STZ-vehicle group, $n = 61$). Insulin treatment was carried out by subcutaneous injection of 0.025 units/g Novolin U (Novo Nordisk, Bagsværd, Denmark) at 1900 every day for 3 weeks. Rats of the STZ-vehicle group and control group were treated with vehicle injection at the same time for the same period. Plasma glucose concentration was measured using a commercial test kit (GR-101; Terumo, Tokyo, Japan). Serum insulin concentration was quantitated using an insulin radioimmunoassay kit. Plasma concentrations of total cholesterol, triglyceride, and free fatty acid were colorimetrically measured using a spectrophotometer. Four rats from either the STZ-vehicle group or the control group were housed 12 weeks after vehicle or STZ injection.

Western blot analysis. Western blotting was performed as described previously (2,3). Briefly, rats were heparinized (500 IU/kg i.p.) and anesthetized with pentobarbital (50 mg/kg i.p.). Each heart was rapidly removed and frozen in liquid nitrogen. The tissues were homogenized with lysis buffer (50 mmol/l Tris-HCl at pH 7.4, 10% glycerol, 2 mmol/l EDTA, 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 50 mmol/l glycerophosphate, 2 mmol/l Na_3VO_4, 20 mmol/l NaF, 1 mmol/l phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Nonidet P-40). Samples were centrifuged, and the protein concentration was measured by the Bradford method (2,3). An equal amount of total protein in each fraction was electrophoresed on 8.5% SDS-PAGE and transferred electrophoretically onto a polyvinylidine fluoride membrane. After blocking with 0.5% nonfat milk, the membranes were incubated with antibodies, followed by an incubation with secondary antibodies. The proteins were detected by enhanced chemiluminescence after exposure to Hyperfilm. The amount of protein on the immunoblots was quantified using National Institutes of Health (Bethesda, MD) image analysis software.

PI 3-kinase–dependent phosphorylation of Akt and GSK-3β and HSP72 expression. As shown in Fig. 1*A*, *a*, 4 weeks after STZ or vehicle injection, each rat was anesthetized with pentobarbital (20 mg/kg i.p.) and immersed in a water bath at 43°C (hyperthermia) or 37°C (normothermia) for 20 min.

TABLE 1
Basic characteristics of the three groups of rats

Parameter	Control rats: vehicle treated	STZ-induced diabetic rats: Vehicle treated	STZ-induced diabetic rats: Insulin treated
n	8	8	8
Body weight (g)	417 ± 7	326 ± 11*	365 ± 6*†
Heart weight (g)	1.02 ± 0.02	0.89 ± 0.02*	0.91 ± 0.02*
Heart weight–to–body weight ratio (mg:g)	2.45 ± 0.06	2.76 ± 0.11‡	2.50 ± 0.09§
Blood glucose (mg/dl)	202 ± 7	535 ± 29*	207 ± 32†
Serum insulin (ng/ml)	4.20 ± 0.49	0.91 ± 0.14*	2.52 ± 0.35*†
Total cholesterol (mg/dl)	53.1 ± 2.8	57.6 ± 2.6	60.5 ± 4.9
Triglycerides (mg/dl)	103.4 ± 10.5	47.1 ± 4.0*	52.6 ± 6.9*
Free fatty acid (mg/dl)	1,297 ± 178	625 ± 65*	650 ± 101*

Data are means ± SE. *$P < 0.01$ vs. control group; †$P < 0.01$ vs. STZ-vehicle group; ‡$P < 0.05$ vs. control group; §$P < 0.05$ vs. STZ-vehicle group.

Rectal temperature was monitored to confirm changes in body temperature. Wortmannin (15 μg/kg), a PI 3-kinase inhibitor, or vehicle was injected into the tail vein 15 min before each thermo-treatment. Phosphorylation of Akt and GSK-3β was evaluated immediately before and 1 and 24 h after hyperthermia treatment ($n = 5$ for each). HSP72 expression was evaluated 24 h after each thermo-treatment ($n = 5$). In five rats of the control and STZ-vehicle group, SB216763, a GSK-3β inhibitor, was injected into the tail vein and hearts isolated 24 h later (Fig. 1*A*, *b*).

Gel mobility shift assay. As previously described (15), we used the double-stranded heat-shock element (HSE) oligonucleotide (5′-CTAGAAGCT TCTAGAAGCTTCTAG-3′) labeled with ^{32}P filling in the 5′ overhangs with Klenow polymerase. Because heat-shock factor 1 (HSF1) is normally present in the cell in an inactive form, we were able to use whole-cell lysates. After the samples were incubated with ^{32}P-labeled HSE for 30 min at room temperature, they were loaded onto a 4.5% nondenaturing acrylamide gel and electrophoresed. For cold competition experiment, the sample was incubated with a 200-fold molar excess of unlabeled HSE for 15 min before the addition of labeled HSE. The gel was dried under vacuum and analyzed with an image analyzer (BIO-image BAS 2000; Fuji Film, Tokyo, Japan).

Isolated perfused heart experiments. As shown in Fig. 1*A*, *c*, 24 h after whole-body hyperthermia ($n = 8$ for each group) or normothermia ($n = 8$ for each group), each rat was heparinized and anesthetized. Subsequently, the heart was isolated and perfused retrogradely with Krebs-Henseleit buffer (pH 7.4; 118 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 25.0 mmol/l Na_2HCO_3, and 11.0 mmol/l glucose) equilibrated with a 95% O_2-5% CO_2 gas mixture at 36.5°C at a constant pressure of 75 mmHg. A water-filled latex balloon was inserted through the mitral valve orifice into the left ventricle (LV), and the LV end-diastolic pressure was adjusted to 0–5 mmHg. No-flow global ischemia was initiated for 20 min, followed by reperfusion for 30 min. The coronary effluent during the 30-min reperfusion period was collected for measurement of creatine kinase content (released CK). LV pressure was monitored using a pressure transducer to obtain the peak positive and negative first derivatives of LV pressure (dP/dt_{max} and dP/dt_{min}). LV developed pressure (LVDP) was defined as the difference between the LV systolic and diastolic pressure. LV pressure, coronary perfusion pressure (CPP), and electrocardiogram were continuously recorded on a polygraph recorder (WS-681G; Nihon Kohden, Tokyo, Japan) and stored on a PCM data recorder (RD-111T; TEAC, Tokyo, Japan) for later analysis.

In vitro experiments using neonatal rat cardiomyocytes. Neonatal cardiomyocytes were prepared from 3-day-old Wistar rats (3). Briefly, cardiomyocytes were placed onto 35-mm culture dishes at a density of 5×10^6 per dish and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C under an atmosphere of 5% CO_2 and 95% room air. On day 3, the medium was switched to serum-free DMEM supplemented with 5 μg/ml transferrin, and the cells were incubated for 24 h. The cultured myocytes were incubated at 42°C (hyperthermia) or 37°C (normothermia) for 30 min in the presence or absence of insulin (1 μg/ml) in the culture medium. HSP72 expression was evaluated 24 h after hyperthermia or normothermia (Fig. 1*B*, *a*). Phosphorylation of Akt and GSK-3β in the presence of insulin was evaluated 1 h after each thermo-treatment (Fig. 1*B*, *b*). In some dishes, 40 nmol/l wortmannin or 20 μmol/l LY294002 was added to the medium. To evaluate tolerance against hypoxia-reoxygenation, hyperthermia or normothermia was applied to cells, which were then incubated for 24 h in the presence or absence of insulin. The cardiomyocytes were incubated in serum- and insulin-free DMEM without glucose under a hypoxic gas mixture (95% N_2 and 5% CO_2) at 37°C for 3 h. The cells were reoxygenated for 1 h with a normoxic gas mixture, and the supernatant was collected for determination of lactate dehydrogenase (LDH) using an LDH assay kit (Eiken Chemical, Tokyo, Japan) (Fig. 1*B*).

Small interfering RNA transfection. The small interfering RNAs (siRNAs) targeting HSP72 were purchased from Ambion (Austin, TX) and transfected to cells at a concentration of 10 nmol/l with siPORT NeoFX Transfection (Ambion). The control cells were transfected with negative control siRNA. Then, 24 h after transfection, cells were incubated at hyperthermia (42°C) or normothermia (37°C) for 30 min in the presence or absence of insulin in the culture medium. HSP72 expression was evaluated 24 h after hyperthermia or normothermia.

Statistical analysis. Data are expressed as means ± SE. Serial changes in LVDP, dP/dt, CPP, and heart rate were analyzed by two-way ANOVA. Comparison of physiological and serum parameters, the relative intensity of each protein, the ratio of released CK to LV weight, and LDH content in the cultured medium were analyzed using one-way ANOVA followed by the Bonferroni-Dunn test. A P value <0.05 was considered statistically significant.

RESULTS

Whole-heart experiments. Table 1 summarizes the basic characteristics of the three experimental groups used in the isolated perfused heart experiments (normothermia-treated rats, $n = 8$ for each group). Body and heart weights were lower in the STZ-vehicle than in the control group ($P < 0.01$ for each). However, the ratio of heart to body weight was higher in the STZ-vehicle than in the control group ($P < 0.05$). Insulin treatment restored the reduction in body weight ($P < 0.01$) and the increased ratio of heart to body weight ($P < 0.05$). Plasma glucose concentrations were higher and serum insulin concentrations lower in the STZ-vehicle than the control group ($P < 0.01$) but could be restored by insulin treatment ($P < 0.01$ for each). Although plasma total cholesterol concentration was not significantly different among the three groups, serum triglyceride and free fatty acid concentrations were lower in the STZ-vehicle than the control group ($P < 0.01$ for each). Insulin treatment did not significantly restore the low triglyceride and free fatty acid concentrations.

Figure 2 shows PI 3-kinase–dependent HSP72 protein expression. As shown in Fig. 2*A*, when compared with corresponding normothermia treatment groups, hyperthermia resulted in a 2.9-fold increase in HSP72 expression in the control heart but only a 1.7-fold increase in the STZ-vehicle heart ($P < 0.01$ for each). Thus, HSP72 expression was greater in the control heart than in the STZ-vehicle heart ($P < 0.01$). Insulin treatment (STZ-insulin heart) restored HSP72 expression ($P < 0.01$). Pretreatment with wortmannin attenuated hyperthermia-induced HSP72 expression in the control heart ($P < 0.01$) but not in the STZ-vehicle heart (Fig. 2*B*). Reduced HSP72 expression in response to hyperthermia was also evident

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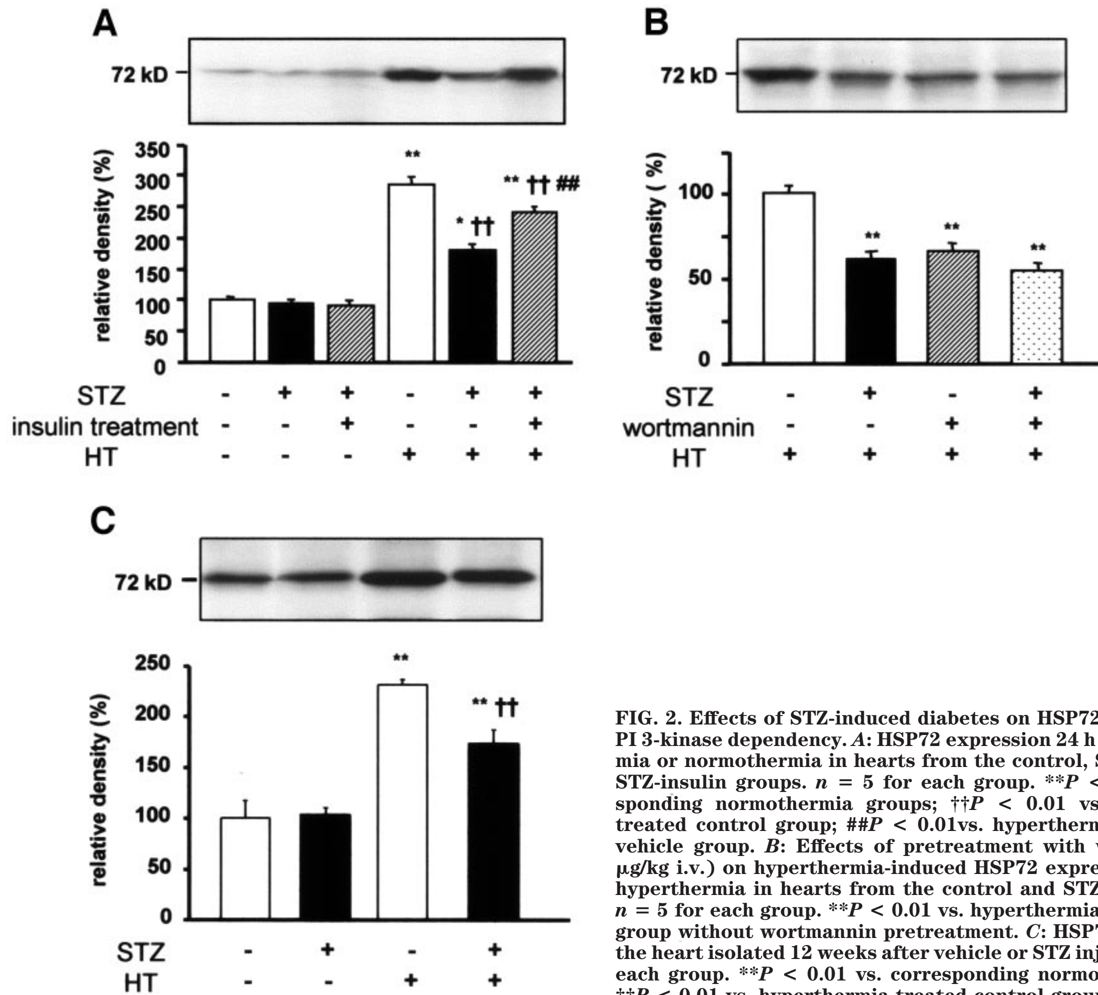

FIG. 2. Effects of STZ-induced diabetes on HSP72 expression and PI 3-kinase dependency. *A*: HSP72 expression 24 h after hyperthermia or normothermia in hearts from the control, STZ-vehicle, and STZ-insulin groups. *n* = 5 for each group. ***P* < 0.01 vs. corresponding normothermia groups; ††*P* < 0.01 vs. hyperthermia-treated control group; ##*P* < 0.01vs. hyperthermia-treated STZ-vehicle group. *B*: Effects of pretreatment with wortmannin (15 μg/kg i.v.) on hyperthermia-induced HSP72 expression 24 h after hyperthermia in hearts from the control and STZ-vehicle groups. *n* = 5 for each group. ***P* < 0.01 vs. hyperthermia-treated control group without wortmannin pretreatment. *C*: HSP72 expression in the heart isolated 12 weeks after vehicle or STZ injection. *n* = 5 for each group. ***P* < 0.01 vs. corresponding normothermia groups; ††*P* < 0.01 vs. hyperthermia-treated control group.

in the STZ-induced diabetic rat heart at 12 weeks after STZ injection ($P < 0.01$; Fig. 2*C*).

Figure 3*A* and *B* depict hyperthermia-induced phosphorylation of Akt in the control and STZ-vehicle hearts. Two representative bands for phospho-Akt and total Akt and the relative density of phospho-Akt at three time points (immediately before and 1 and 24 h after hyperthermia, Fig. 1*A*, *a*) are shown. A robust increase in phospho-Akt in the control heart was observed 1 h after hyperthermia treatment, which was inhibited by pretreatment with wortmannin ($P < 0.01$). Such an increase in Akt phosphorylation was attenuated in the STZ-vehicle heart ($P < 0.01$), resulting in no significant difference from the wortmannin-pretreated control heart. Akt phosphorylation in either group disappeared 24 h after hyperthermia treatment. Figure 3*C* and *D* depicts phosphorylation of GSK-3β. Two representative bands for phospho–GSK-3β and total GSK-3β and the relative density of phospho–GSK-3β are shown. A remarkable increase in phospho–GSK-3β in the control heart was observed 1 h after hyperthermia treatment, which was inhibited by pretreatment with wortmannin ($P < 0.01$). Such an increase in GSK-3β phosphorylation was attenuated in the STZ-vehicle heart ($P < 0.01$), resulting in no significant difference from the wortmannin-pretreated control heart. GSK-3β phosphorylation in either group disappeared 24 h after hyperthermia treatment. As illustrated in Fig. 3*E*, even in the absence of hyperthermia, SB216763 induced HSP72 expression in control and STZ-vehicle hearts 24 h after injection.

Serial changes in LVDP and dP/dt of hearts isolated from normothermia-treated rats (Fig. 4*A*) and hyperthermia-treated rats (Fig. 4*B*) in the control, STZ-vehicle and STZ-insulin groups during the reperfusion period are shown in Fig. 4. Neither of these two parameters nor heart rate or CPP showed a significant difference among the three experimental groups at the baseline period (data not shown). In the normothermia-treated heart, reperfusion-induced LV functional recovery was poor in all three groups. In contrast, the hyperthermia-treated control heart showed a better LV functional recovery compared with the normothermia-treated control heart ($P < 0.05$ by ANOVA). However, such an improved functional recovery was not observed in the hyperthermia-treated STZ-vehicle heart. In contrast, the hyperthermia-treated STZ-insulin heart showed a better LV functional recovery than the STZ-vehicle heart ($P < 0.05$ by ANOVA). With respect to released CK, in the control group, it was reduced in the hyperthermia-treated hearts compared with the normothermia-treated hearts (154 ± 26 vs. 371 ± 37 IU/g, $P < 0.01$). However, in the STZ-vehicle group, its reduction by hyperthermia was not significant (299 ± 35 vs. 378 ± 58 IU/g). In contrast, in the STZ-insulin group, released CK was lower in hyperthermia- compared with normothermia-treated hearts (184 ± 33 vs. 332 ± 35 IU/g, $P < 0.05$).

In vitro experiments using neonatal rat cardiomyocytes. In the presence of insulin, hyperthermia treatment resulted in a 4.8-fold increase in HSP72 content in cardiomyocytes compared with normothermia-treated cells ($P <$

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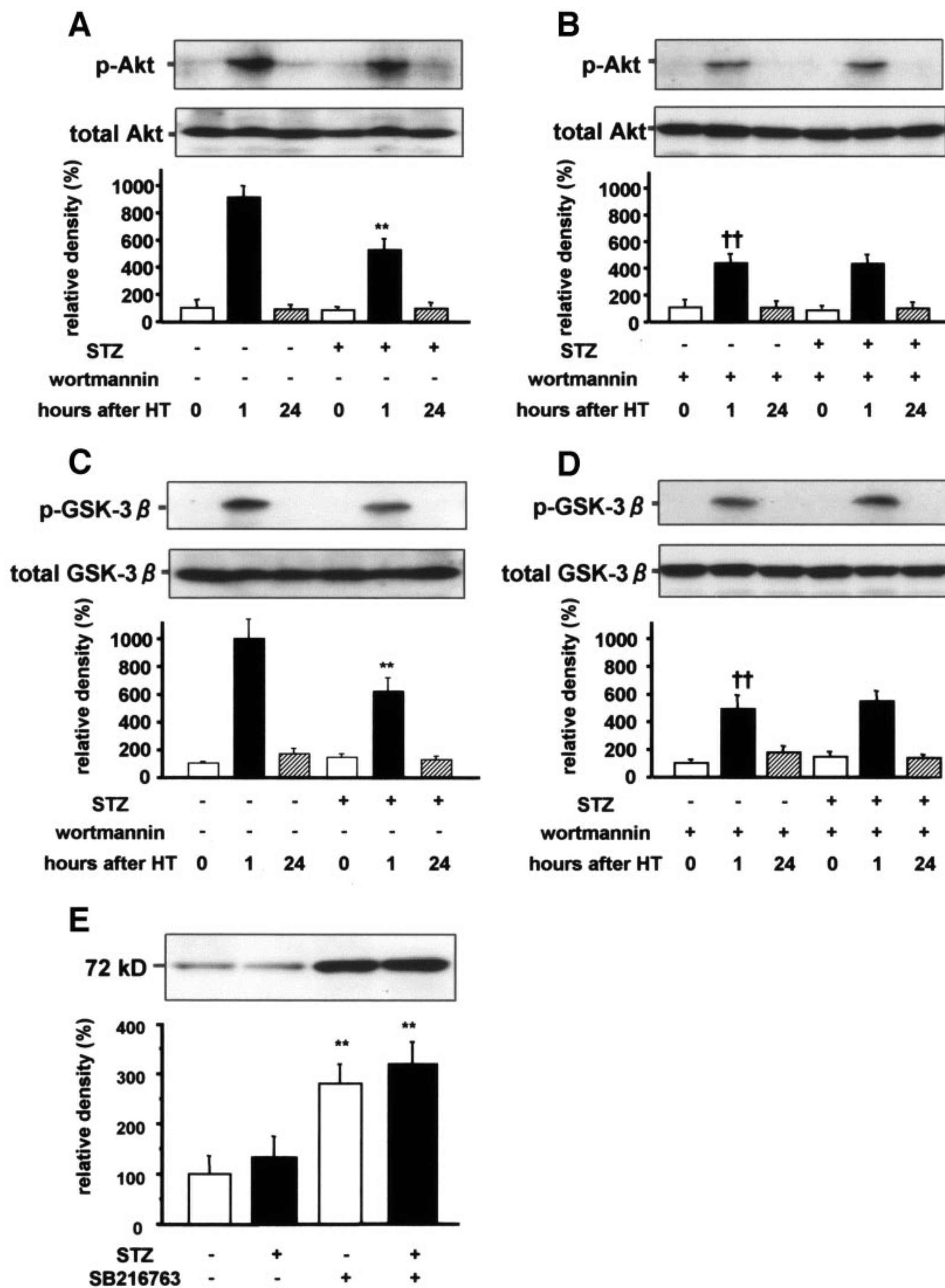

FIG. 3. Effects of STZ-induced diabetes on phosphorylation of Akt and GSK-3β and PI 3-kinase dependency and effects of GSK-3β inhibition on HSP72 expression. *A* and *B*: Two representative bands for phospho–Ser^{473}-Akt and total Akt and the relative density of phospho–Ser^{473}-Akt at three measured points, i.e., immediately before and 1 and 24 h after hyperthermia, in hearts from the control and STZ-vehicle groups. *A*: Phosphorylation of Akt without wortmannin pretreatment. *B*: Phosphorylation of Akt with wortmannin pretreatment. *n* = 5 for each group. ***P* < 0.01 vs. corresponding control group; ††*P* < 0.01 vs. corresponding wortmannin-untreated group. *C* and *D*: Two representative bands for phospho–Ser^{9}-GSK-3β and total GSK-3β and the relative density of phospho–Ser^{9}-GSK-3β at three measured points, i.e., immediately before and 1 and 24 h after hyperthermia, in hearts from the control and STZ-vehicle groups. *C*: Phosphorylation of GSK-3β without wortmannin pretreatment. *D*: Phosphorylation of GSK-3β with wortmannin pretreatment. *n* = 5 for each group. ***P* < 0.01 vs. corresponding control group; ††*P* < 0.01 vs. corresponding wortmannin-untreated group. *E*: HSP72 expression 24 h after intravenous injection of SB216763 (15 mg/kg) in hearts from the control and STZ-vehicle groups. *n* = 5 for each group. ***P* < 0.01 vs. corresponding SB216763-untreated groups.

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0.01; Fig. 5*A*). In the absence of insulin, hyperthermia treatment resulted in a 2.7-fold increase in the HSP72 content compared with normothermia-treated cells ($P < 0.05$; Fig. 5*A*). Thus, hyperthermia-induced HSP72 expression was greater in cardiomyocytes cultured with insulin than in those without insulin ($P < 0.01$). Figure 5*B* shows the effects of 40 nmol/l wortmannin or 20 μmol/l LY294002 on insulin-induced augmentation of HSP72 expression. Hyperthermia-induced HSP72 overexpression was attenuated by the addition of wortmannin or LY294002 ($P < 0.01$ for each). Figure 5*C* and *D* demonstrates the phosphorylation of Akt and GSK-3β, respectively, when assessed 1 h after hyperthermia treatment in the presence of insulin. Phospho-Akt increased 3.2-fold in hyperthermia-treated cells compared with normothermia-treated cells ($P < 0.01$; Fig. 5*C*). The increase was inhibited by 40 nmol/l wortmannin ($P < 0.01$) and 20 μmol/l LY294002 ($P < 0.05$). Similarly, phospho–GSK-3β increased 2.6-fold in hyperthermia-treated cells compared with normothermia-treated cells ($P < 0.01$; Fig. 5*D*). The increase was inhibited by 40 nmol/l wortmannin and 20 μmol/l LY294002 ($P < 0.01$ for each). As shown in Fig. 5*E*, the gel mobility shift assay revealed hyperthermia-induced HSF1-HSE DNA binding in cells cultured in the presence of insulin. However, the binding was very weak in cells cultured in the absence of insulin. In the presence of insulin, the level of LDH in the culture medium after hypoxia-reoxygenation was reduced in hyperthermia-treated cells compared with

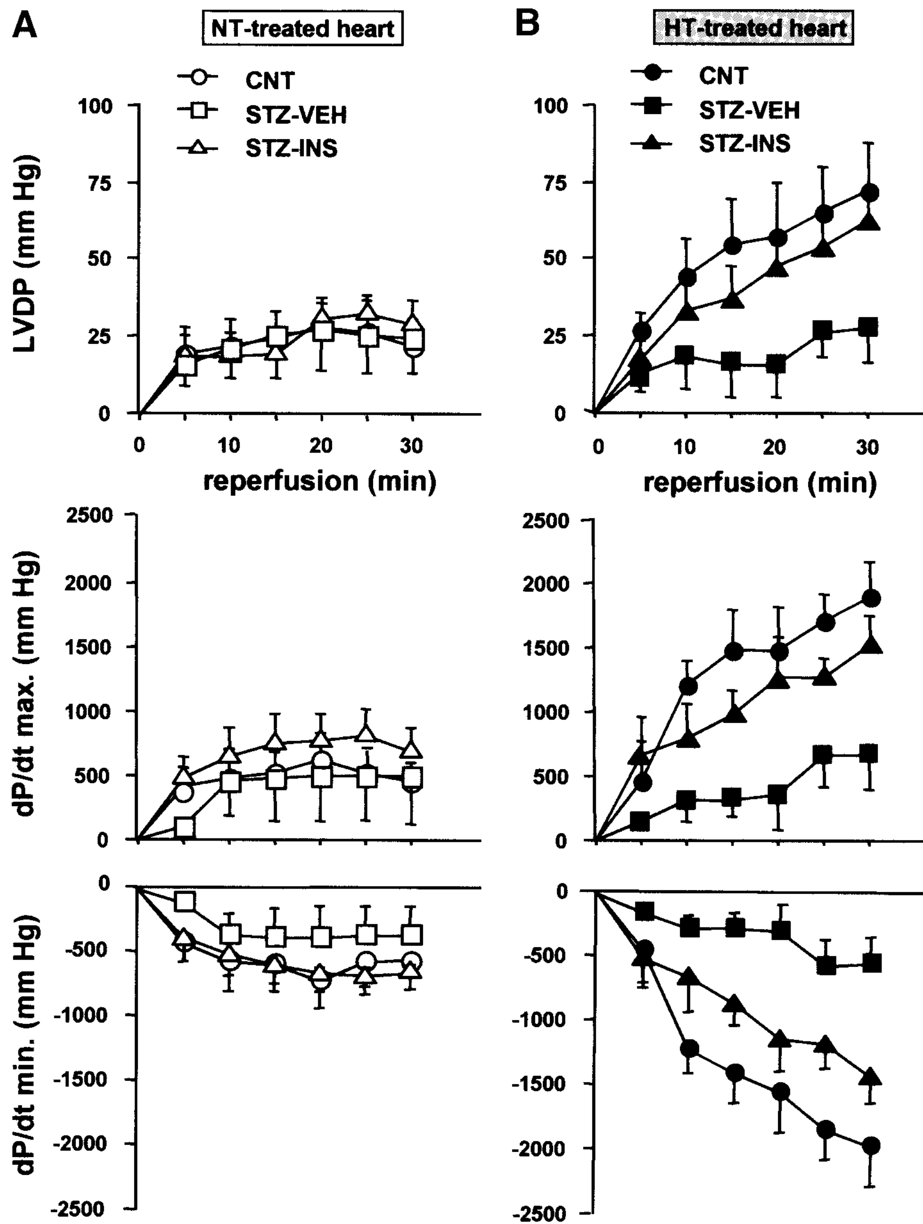

FIG. 4. Serial changes in LVDP, dP/dt_{max}, and dP/dt_{min} during the reperfusion period. The 30-min reperfusion followed 20 min of no-flow global ischemia. *A*: Normothermia-treated heart. *B*: Hyperthermia-treated heart. $n = 8$ for each group.

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normothermia-treated cells (0.012 ± 0.003 vs. 0.038 ± 0.009 IU/ml, $P < 0.05$). However, in the absence of insulin, the reduction by hyperthermia was not significant (0.032 ± 0.006 vs. 0.044 ± 0.009 IU/ml).

The effects of HSP72 siRNA are shown in Fig. 6*A*. Hyperthermia-induced HSP72 expression was reduced in cells incubated with HSP72 siRNA when compared with cells incubated with control siRNA, irrespective of the presence or absence of insulin ($P < 0.01$ for each). Regarding the levels of LDH after hypoxia-reoxygenation (Fig. 6*B*), hyperthermia reduced the LDH release from control siRNA-treated cells ($P < 0.05$). However, LDH release from cells incubated with HSP72 siRNA was greater than that in control siRNA-treated cells, irrespective of the presence or absence of insulin ($P < 0.01$ for each).

DISCUSSION

The main findings of the study are as follows (1). Whole-body hyperthermia induced phosphorylation of Akt and GSK-3β and HSP72 expression in a PI 3-kinase–dependent manner in the heart, which was attenuated in STZ-induced diabetic rats (2). Inhibition of GSK-3β with an intravenous injection of SB216763 induced HSP72 overexpression per se, irrespective of the presence of diabetes (3). Attenuated hyperthermia-induced HSP72 expression in the STZ-induced diabetic heart was restored by insulin treatment in association with improved reperfusion-induced LV functional recovery (4). In cultured neonatal rat cardiomyocytes, hyperthermia induced phosphorylation of Akt and GSK-3β and HSP72 expression in a PI 3-kinase–dependent manner (5). Insulin enhanced PI 3-kinase–dependent hyperthermia-induced HSP72 expression in association with tolerance against hypoxia-reoxygenation injury. These findings suggest that PI 3-kinase–dependent Akt phosphorylation (activation) and subsequent GSK-3β phosphorylation (inhibition) form an essential signal for hyperthermia-induced HSP72 expression in the heart, and that this signaling pathway is impaired in the STZ-induced diabetic heart. Because hyperthermia upregulates various

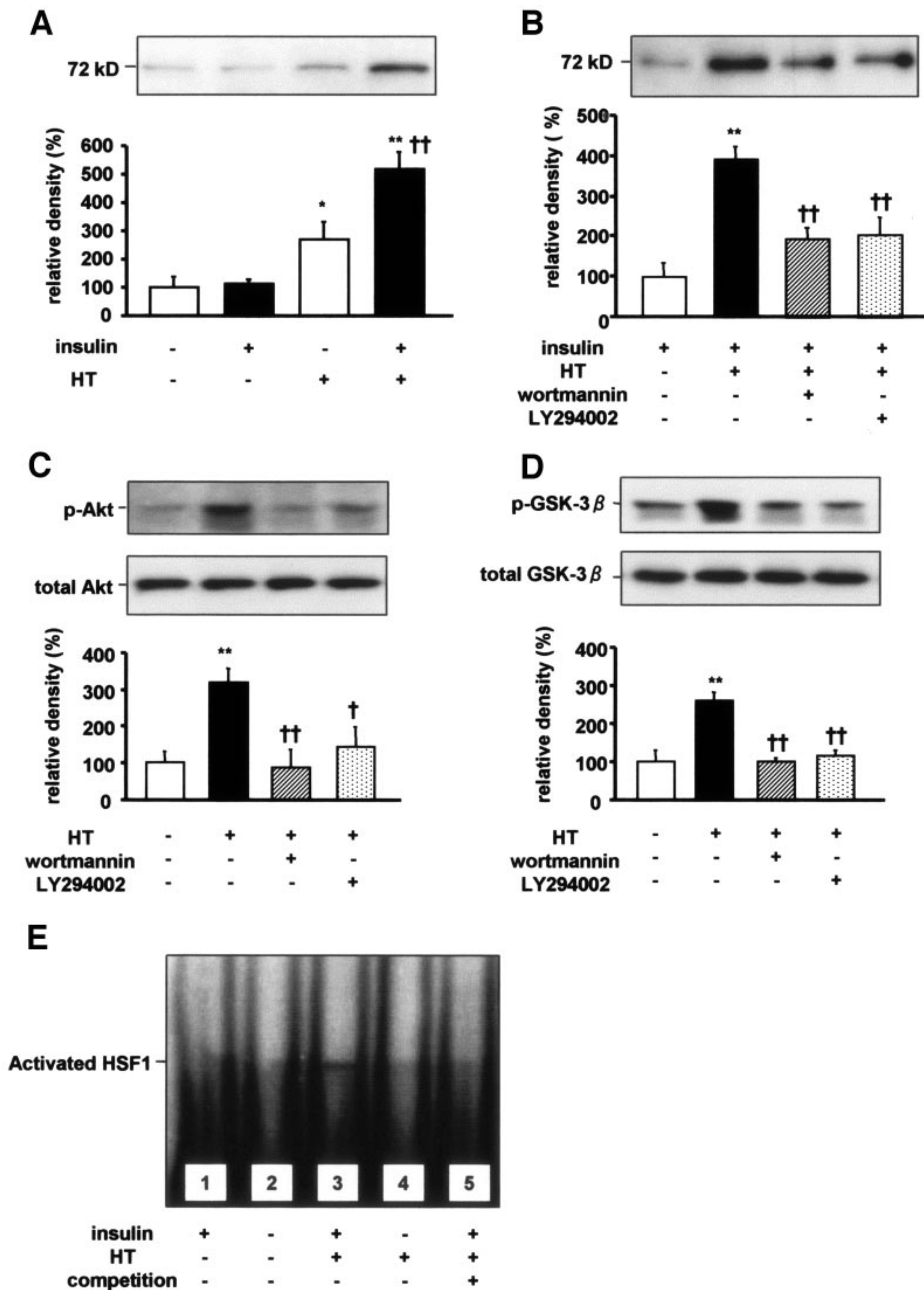

FIG. 5. Effects of insulin on HSP72 expression and phosphorylation of Akt and GSK-3β, PI 3-kinase dependency, and insulin-induced HSF1 activation in cultured neonatal rat cardiomyocytes. *A*: HSP72 expression in cells cultured in medium 24 h after hyperthermia or normothermia in the absence or presence of 1 μg/ml insulin. Four independent cultures were evaluated. $*P < 0.05$ and $**P < 0.01$ vs. corresponding normothermia-treated cells; ††$P < 0.01$ vs. hyperthermia-treated cells cultured in the absence of insulin. *B*: Effects of 40 nmol/l wortmannin or 20 μmol/l LY294002 on hyperthermia-induced HSP72 expression 24 h after hyperthermia in cells cultured in the presence of 1 μg/ml insulin. Four independent cultures were evaluated. $**P < 0.01$ vs. normothermia-treated cells without wortmannin or LY294002; ††$P < 0.01$ vs. hyperthermia-treated cells without wortmannin or LY294002. *C*: Phosphorylation of Akt. *D*: Phosphorylation of GSK-3β. Two representative bands for phospho–Ser^{473}-Akt and total Akt and the relative density of phospho–Ser^{473}-Akt (*C*) and for phospho–Ser^{9}-GSK-3β and total GSK and the relative density of phospho–Ser^{9}-GSK-3β (*D*), 1 h after treatment with normothermia or hyperthermia, are shown. Four independent cultures were evaluated. $**P < 0.01$ vs. normothermia-treated cells without wortmannin or LY294002; †$P < 0.05$ and ††$P < 0.01$ vs. hyperthermia-treated cells without wortmannin or LY294002. *E*: Gel mobility shift assay showing activation of HSF1 1 h after hyperthermia application in the presence of insulin (*lane 3*).

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cytoprotective proteins, it should be possible to estimate whether increased HSP72 levels are directly related to the experimentally observed protective effects. In the present study, cardiomyocytes transfected with HSP72 siRNA showed a blunted increase in HSP72, in accordance with the loss of tolerance against hypoxia-reoxygenation injury (Fig. 6), indicating the direct contribution of hyperthermia-induced HSP72 expression to protection against ischemic insults.

This is the first report demonstrating that Akt activation is involved in hyperthermia-induced HSP72 expression in the heart. Using cultured human neuroblastoma cells, Bijur et al. (11) reported that hyperthermia rapidly activated Akt in a PI 3-kinase–dependent manner and inhibited GSK-3β, resulting in HSP72 overexpression. In contrast, in COS-7 and NIH 3T3 cells, hyperthermia-induced activation of Akt was unaffected by treatment with wortmannin (8). On the other hand, in human embryonic kidney 293 cells, Shaw et al. (9) reported that hyperthermia-induced activation of Akt was dependent on PI 3-kinase. In the present study, whole-body hyperthermia-induced activation of myocardial Akt was abrogated by wortmannin. Furthermore, in cultured cardiomyocytes, hyperthermia-induced Akt activation was also abrogated by wortmannin or LY294002. These observations suggest that in the heart, hyperthermia-induced activation of Akt is convincingly PI 3-kinase–dependent. GSK-3β is a ubiquitously expressed protein-serine/threonine kinase, and its

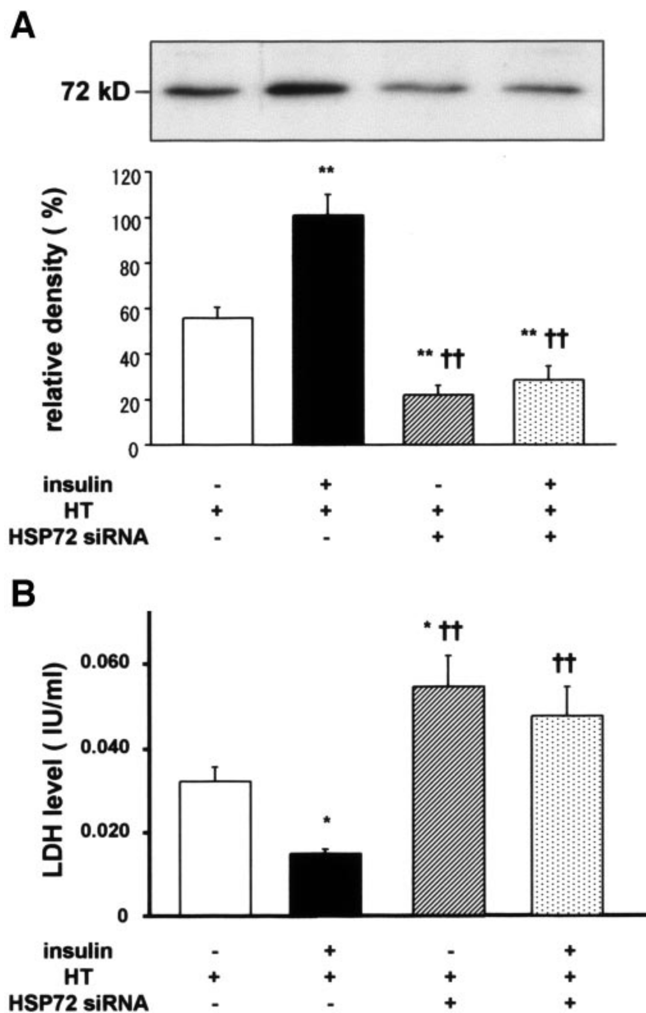

FIG. 6. Effects of siRNA directed against the HSP72 gene. *A*: HSP72 expression in cells cultured in medium 24 h after hyperthermia in the absence or presence of 1 μg/ml insulin. Four independent cultures were evaluated. ***P* < 0.01 vs. hyperthermia-treated and control siRNA-transfected cells cultured in the absence of insulin. ††*P* < 0.01 vs. hyperthermia-treated and control siRNA-transfected cells cultured in the presence of insulin. *B*: Levels of LDH in the culture medium after hypoxia-reoxygenation. Four independent cultures were evaluated. **P* < 0.05 vs. hyperthermia-treated and control siRNA-transfected cells cultured in the absence of insulin. ††*P* < 0.01 vs. hyperthermia-treated and control siRNA-transfected cells cultured in the presence of insulin.

activity is inhibited by Akt phosphorylation (16). The inhibitory effect of GSK-3β on HSF1 has been demonstrated previously (17,18). For instance, He et al. (17) showed that overexpression of GSK-3β in HeLa cells resulted in rapid deactivation of HSF1 during the recovery phase after hyperthermia treatment. In the present study, intravenous injection of SB216763, a GSK-3β inhibitor, induced a marked HSP72 overexpression without hyperthermia and irrespective of the presence of diabetes. With respect to PI 3-kinase dependency, hyperthermia-induced phosphorylation of GSK-3β was inhibited by wortmannin or LY294002. However, because the activity of GSK-3β is regulated by Akt, inhibition of hyperthermia-induced GSK-3β by wortmannin or LY294002 was probably due to the consequence of PI 3-kinase–dependent inhibition of Akt. Taken together, it can be concluded that activation of Akt in response to hyperthermia and subsequent inhibition of GSK-3β is an essential signaling pathway for hyperthermia-induced HSP72 overexpression in the heart.

In the present study, the STZ-induced diabetic heart showed attenuated hyperthermia-induced HSP72 expression in association with reduced Akt and GSK-3β phosphorylation. PI 3-kinase inhibition by wortmannin abolished phosphorylation of Akt and GSK-3β and HSP72 expression in the control heart but not in the STZ-induced diabetic heart, resulting in no difference between the two groups. These observations suggest that an impaired response to hyperthermia in activating Akt in a PI 3-kinase–dependent manner underlies the mechanism for depressed HSP72 expression in the STZ-induced diabetic heart. Regarding the mechanisms involved, it is noteworthy that insulin reportedly activates Akt in a PI 3-kinase–dependent manner in several cell types, including cardiomyocytes (12,13). In the present study, insulin treatment restored hyperthermia-induced HSP72 expression in the STZ-induced diabetic heart. In addition, in cultured cardiomyocytes, hyperthermia-induced HSP72 expression was enhanced by insulin in a PI 3-kinase–dependent manner. As shown in Fig. 5*E*, the gel mobility shift assay demonstrated that the blunted increase in HSP72 in the absence of insulin occurs at the transcriptional level. Taken together, insulin deficiency appears to play a key role in the depressed activation of PI 3-kinase–dependent Akt in response to hyperthermia in the STZ-induced diabetic heart.

Recent evidence has led to the idea that the cardioprotective effects of HSP72 are the result of its action as a molecular chaperone, in addition to the other biological functions of this protein (1,19–22). However, in the STZ-induced diabetic heart, the association between the level of HSP72 expression and its protective effects remains inconclusive (5,6). In disagreement with our observation, Joyeux et al. (5) reported that although HSP72 was abundantly induced by hyperthermia, its protective effects against ischemia-reperfusion were not observed in the STZ-induced diabetic heart. Nevertheless, our observation that depressed reperfusion-induced LV functional recovery in the STZ-induced diabetic heart was restored by insulin treatment in association with restored inducible levels of HSP72 expression suggests that impaired HSP72 synthesis could play some part in the loss of hyperthermia-induced cardioprotection. This hypothesis is supported by the observation that HSP72 overexpressing cardiomyocytes, cultured in the presence of insulin, showed protection against hypoxia-reoxygenation compared with cardiomyocytes expressing less HSP72, cultured in the absence of insulin.

There are several limitations in the study. First, we used several agents, including wortmannin, LY294002, and SB216763, at a single concentration. Although this is consistent with previous reports (11,23), the use of single doses of these chemicals did not demonstrate precise pharmacological mechanisms. Whereas we attributed PI 3-kinase–dependent activation of Akt to HSP72 overexpression, based on the effects of wortmannin and LY294002, other mechanisms might have been operating. In fact, neither wortmannin nor LY294002 could completely suppress Akt phosphorylation induced by hyperthermia (Figs. 3 and 5). Second, it is still controversial as to whether the STZ-induced diabetic heart is sensitive to or tolerant of ischemic insult (24,25). We reported previously that diabetic hearts, isolated 12 weeks after STZ injection, showed better reperfusion-induced LV functional recovery (26,27). In the present study, the hyperthermia-treated STZ-induced diabetic heart showed poor reperfusion-induced LV functional recovery compared

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with the hyperthermia-treated control heart. On this occasion, we used heart isolated 4 weeks after STZ injection. This relatively short duration of diabetes may explain the disparity in sensitivity to ischemia-reperfusion injury (24,25). As shown in Fig. 2*C*, HSP72 expression in hearts isolated 12 weeks after STZ injection was still attenuated. Thus, other protective pathways might be responsible for cardioprotection in the 12-week STZ diabetic heart. For instance, myocardial concentrations of creatine phosphate during reperfusion were greater in the 12-week STZ-induced diabetic rat heart (28). We also reported that translocation of the protein kinase C ϵ-isoform from the cytosol to particulate during ischemia plays a key role in cardioprotection against ischemia-reperfusion injury in the 12-week STZ-induced diabetic heart (26). Finally, insulin deficiency stimulates lipolysis in adipose tissue, increasing the delivery of free fatty acid from adipose tissue to liver and consequently, triglyceride production in liver. STZ-induced diabetic rats have shown increased serum triglyceride and free fatty acid levels (29,30). In disagreement, our STZ-induced diabetic rats showed lower serum concentrations of triglyceride and free fatty acid. This observation may be explained by the hypothesis that, in our model, lipolysis in adipose tissue occurred at an earlier period after STZ injection with subsequent depletion in adipose tissue. Under such conditions, insulin supplementation might have little effect on serum triglyceride and free fatty acid concentrations.

In conclusion, our results indicate that activation of Akt in a PI 3-kinase–dependent manner and subsequent inhibition of GSK-3β are essential for HSP72 expression in association with cardioprotection. The results also suggest that this signaling pathway is impaired in the STZ-induced diabetic heart, probably due to insulin deficiency.

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