Insulin Deficiency Downregulated Heat Shock Protein 60 and IGF-1 Receptor Signaling in Diabetic Myocardium

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Heat shock protein (Hsp)60 and IGF-1 receptor signaling protect cardiac muscle against injury. The abundance of cardiac IGF-1 receptor can be upregulated by Hsp60, but how diabetes modulates cardiac muscle Hsp60 has not yet been defined. We investigated the changes of Hsp60 and IGF-1 receptor signaling in the diabetic myocardium and studied how diabetes modulates Hsp60 and IGF-1 receptor in diabetic myocardium. In the streptozotocin (STZ)-induced diabetic rat, downregulation of Hsp60 and IGF-1 receptor occurred 4 days after induction of diabetes. IGF-1 activation of IGF-1 receptor, Mek, and Akt were reduced accordingly in the diabetic myocardium. The independent effect of insulin and hyperglycemia on Hsp60 was investigated in primary cardiomyocytes. Incubating cardiomyocytes with insulin was associated with dose-dependent increase of Hsp60 protein. In contrast, the abundance of Hsp60 was not affected by high concentration of glucose in these cells. To further determine the independent effects of hyperglycemia and insulin deficiency on the changes of myocardial Hsp60 and IGF-1 receptor, we used phlorizin to normalize blood glucose in diabetic rats. In the phlorizin-treated diabetic rats, myocardial Hsp60 was lower than that of the normal controls. In contrast, insulin treatment normalized myocardial Hsp60 in the diabetic rats. Because phlorizin does not alter insulin secretion, Hsp60 expression was modulated by insulin and not by hyperglycemia. Similar changes of Hsp60 and IGF-1 receptor were observed in the skeletal muscle of STZ-induced diabetic rats. These findings suggest that insulin deficiency is a novel mechanism that leads to downregulation of Hsp60 in diabetic muscle tissues. The development of diabetic cardiomyopathy might have involved downregulation of Hsp60 and subsequent reduction of IGF-1 receptor signaling. *Diabetes* 54: 175–181, 2005

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Hsp, heat shock protein; STZ, streptozotocin.

Abnormalities of cardiovascular systems are common in diabetic patients, and the consequences of cardiac disease in diabetes are devastating (1). In addition to coronary artery disease and hypertension, diabetic patients often have evidence of a cardiomyopathy that may occur without apparent microvascular or macrovascular diseases (1–3). Similar myocardial abnormalities have also been found in animal models of diabetes (2). Although a number of biochemical and physiological changes had been described in diabetic myocardium, the mechanisms underlying the development of diabetic cardiomyopathy are not fully understood. Our recent study showed that the abundance of IGF-1 receptor is reduced in diabetic myocardium (4). Because IGF-1 has cardiac-protective action (5), reduced IGF-1 receptor can potentially lead to decreased myocardial protection and may play a fundamental role during the development of diabetic cardiomyopathy. We also discovered that heat shock protein (Hsp)60 increased the abundance of IGF-1 receptor in cardiac muscle cells and that reduced myocardial Hsp60 expression is involved in the downregulation of myocardial IGF-1 receptor in the diabetic state (4).

Hsps are chaperone molecules that may modulate intracellular signaling. Cardiac protective actions of Hsp have been described in the literature (6–8). Recent data from our and other laboratories have shown antiapoptotic effects of Hsp60 in cardiac muscle (9). Although these findings suggest that reduced Hsp60 and IGF-1 is a new paradigm contributing to the development of diabetic cardiomyopathy, the mechanism through which diabetes downregulates cardiac Hsp60 protein is not known. It is also not clear whether decreased myocardial IGF-1 receptor can lead to decreased IGF-1 signaling in vivo. This study was carried out to determine how diabetes alters myocardial Hsp60 and IGF-1 receptor signaling and to dissect the independent effects of hyperglycemia and insulin deficiency on Hsp60 protein in cardiac muscle.

RESEARCH DESIGN AND METHODS

Mouse anti-Hsp60 monoclonal antibody was purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Other antibodies were from Santa Cruz Biolabs (Santa Cruz, CA). Protein A/G PLUS-Agarose beads were from Santa Cruz Biolabs. Long R^3 IGF-1 is from GroPep (Adelaide, Australia), which is an IGF-1 analog that substitutes an Arg for the Glu at position 3 and a 13–amino acid extension peptide at the NH_2-terminus. Other

TABLE 1
Characteristics of experimental animals

	Body weight (g)	Blood glucose (mg/dl)
Control	266.7 ± 17.2	100.7 ± 8.1
Diabetes day 4	243.7 ± 15.6*	365.8 ± 34.4*
Diabetes day 7	239.0 ± 15.9*	395.8 ± 34.9*
Diabetes day 14	232.2 ± 13.4*	491.6 ± 38.2*
Diabetes day 21	222.8 ± 45.3*	572.8 ± 68.5*

Data are means ± SE. Body weight and plasma glucose were measured at 4, 7, 14, and 21 days after injection of STZ or vehicle (control). *$P < 0.05$ vs. controls.

chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ).

Streptozotocin (STZ)-induced diabetes was obtained by injecting STZ (80 mg/kg body wt, i.p.) into Sprague-Dawley rats. Blood glucose levels were monitored by tail-vein sampling. The diabetic rats were harvested at different intervals after the onset of diabetes (random glucose >200 mg/dl). When indicated, the diabetic rats were treated with ultralente insulin (3–9 units, s.c., b.i.d.) or phlorizin (500 $mg \cdot kg^{-1} \cdot d^{-1}$) to normalize blood glucose. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Primary cultures of neonatal cardiomyocytes were prepared from Sprague-Dawley rats according to a protocol that we described previously (10). Cardiomyocytes were plated in 100-mm petri dishes and incubated at 37°C, 5% CO_2. When indicated, after overnight serum deprivation, cardiomyocytes were incubated with increasing concentrations of insulin (0–1.0 mU/ml), D-glucose (200–1000 mg/dl), or D-mannitol (0–800 mg/dl). Primary cardiomyocytes could not be grown at <180 mg/dl glucose; thus, D-glucose concentration in our study was at least 200 mg/dl.

Western blot. The tissues and cells were lysed with lysis buffer (137 mmol/l NaCl, 20 mmol/l Tris-HCl [pH 7.5], 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 2 mmol/l EDTA [pH 8.0], 3 μg/ml aprotinin, 3 μg/ml leupeptin, 2 mmol/l phenylmethylsulfonyl fluoride, 20 mmol/l NaF, 10 mmol/l NaPP, and 2 mmol/l Na_3VO_4). Equal amounts of proteins from each sample were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane and incubated with a blocking buffer (3% BSA in 20 mmol/l Tris-HCl [pH 7.5], 137 mmol/l NaCl, and 0.1% Tween 20) for 1 h at room temperature. The membranes were incubated sequentially with primary antibodies overnight at 4°C, washed two times (20 mmol/l Tris-HCl [pH 7.5], 137 mmol/l NaCl, and 0.1% Tween 20), incubated with horseradish peroxidase–conjugated secondary antibodies (1:7500 to 1:13,000 dilution) for 1 h at room temperature, washed three times, and then detected with ECL (Amersham Biosciences, Arlington Heights, IL).

Immunoprecipitation. The cells were lysed, and the lysates (1000 μg of protein in 1 ml) were preabsorbed with 10 μl of protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 30 min on a rocking platform and spun for 5 min at 10,000 rpm for 20 s, and the supernatant was incubated with specific primary antibody at 4°C for 6 h. After incubation with 20 μl of protein A/G agarose beads for 1.5 h at 4°C, the immunocomplexes were collected by centrifugation and washed three times with ice-cold washing buffer (137 mmol/l NaCl, 20 mmol/l Tris-HCl [pH 7.5], 1% Triton X-100, 2 mmol/l EDTA [pH 8.0], 2 mmol/l PMSF, and 2 mmol/l Na_3VO_4). The final products were briefly boiled and resolved with SDS-PAGE and immunoblotted with specific antibodies as indicated.

Statistical analysis. The data were expressed as mean ± SE on the basis of data derived from three to six independent experiments. The intensity of bands from Western and Northern blots was scanned with densitometry and digitally analyzed. Statistical significance was tested by Student's t test or ANOVA with post hoc analysis when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Changes of Hsp60 and IGF-1 receptor proteins in diabetic myocardium. IGF-1 receptor signaling enhances myocardial protection (11), and Hsp60 may modulate myocardial survival through its antiapoptotic actions and through modulation of IGF-1 receptor signaling (4,9). To investigate whether the occurrence of diabetes led to perturbation of Hsp60 and IGF-1 receptor, we induced diabetes by STZ in Sprague-Dawley rats (Table 1). Time-course study showed significant reduction of cardiac Hsp60 and IGF-1 receptor after induction of diabetes (Fig. 1). There

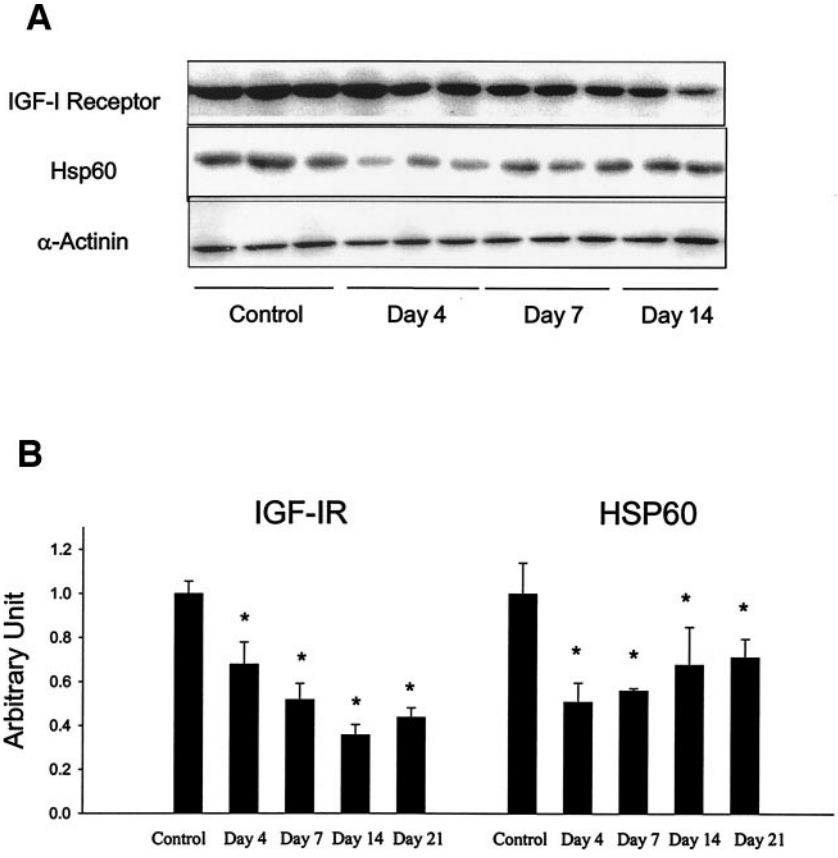

FIG. 1. Changes of Hsp60 and IGF-1 receptor in diabetic myocardium. *A*: The abundance of Hsp60 and IGF-1R in STZ-induced diabetic rat myocardium. Myocardium was harvested from control and diabetic rats, and myocardial proteins were immunoblotted for Hsp60 and IGF-1R. *B*: Time-course effects of diabetes on myocardial Hsp60 and IGF-1R. Each bar represents mean ± SE summarized from multiple animals. *$P < 0.05$ vs. controls.

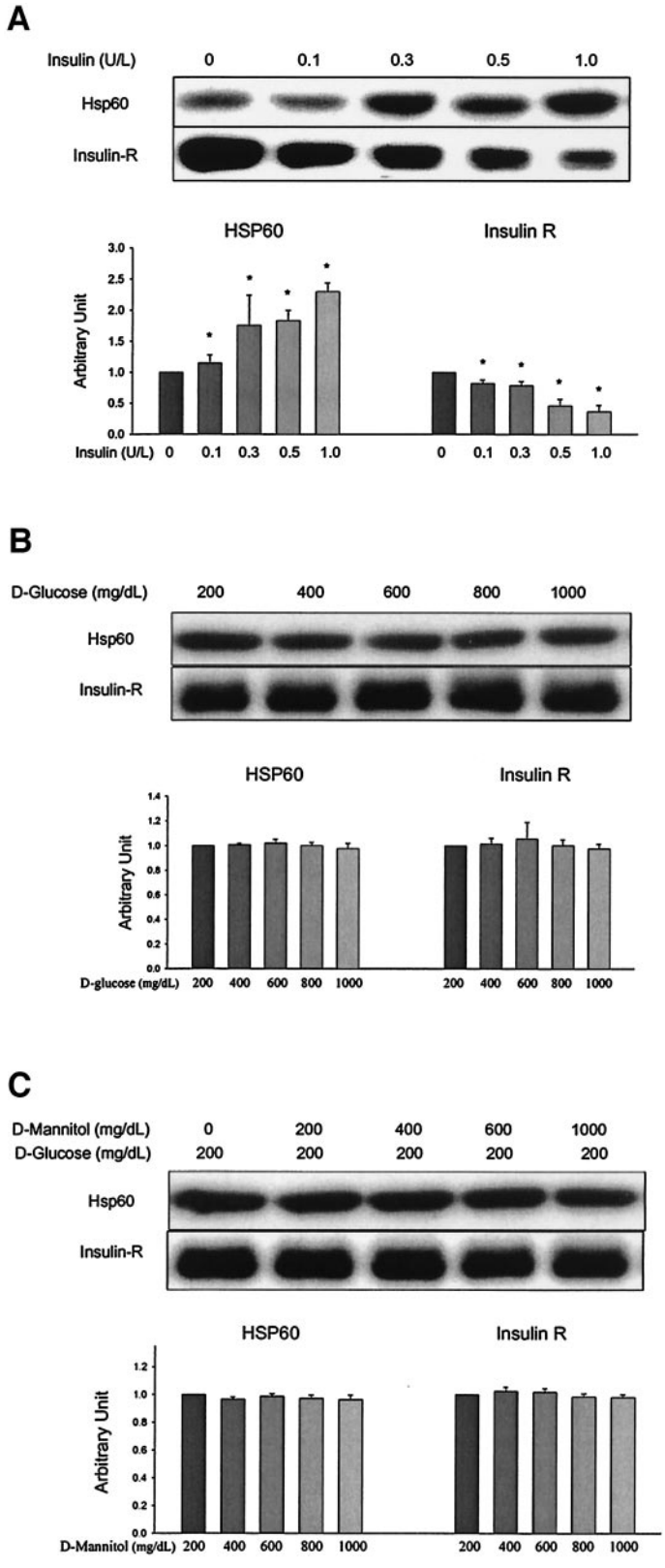

FIG. 2. The independent effects of insulin on Hsp60 in cardiomyocytes. Cardiomyocytes was incubated with various concentrations of insulin, D-glucose, or D-mannitol. The abundance of Hsp60 protein was analyzed with immunoblotting. Primary cardiomyocytes could not be grown well at <180 mg/dl glucose; thus, glucose concentration started at 200 mg/dl. *A*: Insulin upregulated Hsp60. *B*: The changes of Hsp60 in response to increasing concentrations of D-glucose. *C*: The changes of Hsp60 and insulin receptor in response to increasing concentrations of D-mannitol. Bar graph represents densitometry analysis from multiple experiments.

TABLE 2
Characteristics of experimental animals

	Body weight (g)	Blood glucose (mg/dl)
Control	262.7 ± 13.1	101.8 ± 6.6
STZ-induced diabetic rats	227.8 ± 11.6*	396.2 ± 35.6*
Insulin-treated STZ-induced diabetic rats	262.8 ± 26.7*	119.7 ± 11.8†
Phlorizin-treated STZ-induced diabetic rats	236.7 ± 13.0†	135.2 ± 28.3†

Data are means ± SE. When indicated, the diabetic rats were treated with insulin or phlorizin for 6 days. *$P < 0.05$ vs. controls; †$P < 0.05$ vs. STZ-induced diabetic rats.

was a sudden reduction of cardiac Hsp60 after the onset of diabetes and a mild increase between day 4 and day 21 (Fig. 1). Progressive reduction of cardiac IGF-1 receptor lagged behind downregulation of Hsp60 and reached a nadir at approximately day 14, which supports our recent in vitro study that Hsp60 can modulate IGF-1 receptor in cardiac muscle cells (4). These findings showed persistent reduction of cardiac Hsp60 and IGF-1 receptor in diabetic myocardium.

Independent effects of glucose and insulin on Hsp60 in neonatal cardiomyocytes. The following experiments are designed to determine how diabetes downregulates Hsp60 and IGF-1 receptor in cardiac muscle cells. Hyperglycemia and inadequate insulin action (insulin deficiency/resistance) are potential candidates that mediate the effects of diabetes on their expressions. To define the effects of hyperglycemia on Hsp60 in cardiomyocytes, we incubated neonatal cardiomyocytes with insulin for 8 h, and the abundance of Hsp60 was determined with immunoblots. As shown in Fig. 2*A*, there was a dose-dependent increase of Hsp60 protein in the cardiomyocytes that were incubated with insulin. In contrast, the abundance of Hsp60 was not affected by high concentration of glucose (Fig. 2*B*) and mannitol (Fig. 2*C*) in the cardiomyocytes. These results indicated that the abundance of Hsp60 protein could be upregulated by insulin but not by hyperglycemia or hyperosmolarity.

In vivo effects of insulin and phlorizin on the abundance of Hsp60 and IGF-1 receptor in diabetic myocardium. To determine further the independent effects of hyperglycemia and insulin deficiency on the changes of myocardial Hsps in vivo, we used insulin and phlorizin to normalize blood glucose in STZ-induced diabetic rats. Phlorizin inhibits Na-glucose cotransporter in renal tubule, which can promote glucosuria and normalize blood glucose levels in diabetic animals (12). As shown in Table 2, insulin-treated rats restored body weight and normalized blood glucose, whereas phlorizin-treated animals restored only blood glucose level. In the phlorizin-treated diabetic rats, myocardial Hsp60 and IGF-1 receptor remained lower than those of controls (Fig. 3), which supports our hypothesis that cardiac Hsp60 was not regulated by blood glucose. In contrast, insulin treatment normalized the abundance of cardiac Hsp60 and IGF-1 receptor in STZ-induced diabetic rats. These data provide in vivo evidence that cardiac Hsp60 and IGF-1 receptor were regulated by insulin but not by hyperglycemia.

IGF-1 receptor signaling in the diabetic myocardium. We previously reported that downregulation of Hsp60

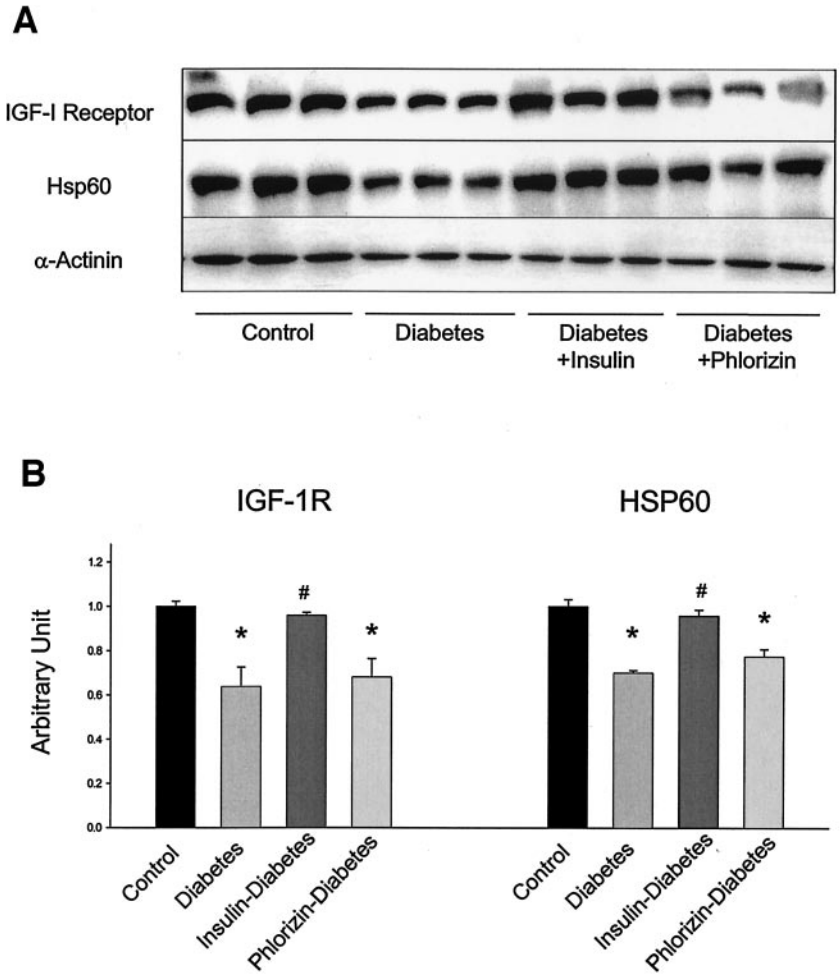

FIG. 3. The effects of insulin and phlorizin on cardiac Hsp60 and IGF-1 receptor in STZ-induced diabetic rats. When indicated, diabetic rats were treated with insulin or phlorizin for 6 days after the induction of diabetes. *A*: A representative photo of myocardial Hsp60 and IGF-1R immunoblot. *B*: Densitometry analysis of myocardial Hsp60 and IGF-1R. Bar graph represents densitometry analysis from multiple experiments. Data represent mean ± SE; **P* < 0.05 vs. controls; #*P* < 0.05 vs. untreated diabetes.

decreased IGF-1 receptor abundance and hence attenuated IGF-1 receptor signaling in neonatal cardiomyocytes. The next series of experiments were to study whether downregulation of IGF-1 receptor in diabetic myocardium attenuated IGF-1 receptor signaling in vivo. After overnight fasting, control and diabetic rats received an injection of Long R^3 IGF-1 (100 μg/kg body wt) via inferior vena cava. Long R^3 IGF-1 is an IGF-1 analog that has low affinity for IGF binding proteins. Tyrosine phosphorylation of IGF-1 receptor was studied by immunoprecipitation with anti–IGF-1 receptor and then immunoblotting with anti-phosphotyrosine antibodies (Fig. 4*A*). Compared with the controls, autophosphorylation of IGF-1 receptor was decreased ~50% ($P < 0.0001$) in the myocardium of STZ-induced diabetic rats. Immunoblotting with anti–IGF-1 receptor β subunit antibodies showed that the abundance of IGF-1 receptor was decreased in the diabetic myocardium as we had anticipated ($P = 0.0045$). Although insulin activated insulin receptor phosphorylation in vivo as expected, the insulin receptors were not activated by Long R^3 IGF-1 in the control and diabetic myocardium. Therefore, Long R^3 IGF-1 at this dosage did not cross-activate insulin receptor signaling in the heart. The abundance of myocardial insulin receptor was upregulated in this model ($P = 0.0012$) as previously reported (13). To define signaling changes downstream from IGF-1 receptor, we studied phosphorylation of Akt and Mek. As shown in Fig. 4*B* and *C*, IGF-1 receptor–activated Akt and Mek were accordingly decreased in the myocardium of STZ-induced diabetic rats (Akt, ↓47.2 ± 4.1%, $P = 0.0014$; MEK, ↓56.5 ± 8.4%, $P = 0.0013$). Compared with IGF-1 receptor, the abundance of Akt and Mek was not significantly reduced in diabetic myocardium (Fig. 4*C*). Thus, reduced activation of Akt and Mek was due to decreased activation of IGF-1 receptor.

Changes of Hsp60 and IGF-1 receptor in skeletal muscles and kidney. In the skeletal muscles, the changes of Hsp60 and IGF-1 receptor were similar to that of cardiac muscle (Fig. 5*A*). Hsp60 and IGF-1 receptor concomitantly reduced in the diabetic skeletal muscle, and insulin treatment restored Hsp60 and IGF-1 receptor to normal. However, phlorizin treatment was unable to restore Hsp60 and IGF-1 receptor in the skeletal muscle. Although these experiments suggest that insulin regulates Hsp60–IGF-1 in a mechanism similar to cardiac muscle, regulation of Hsp60 and IGF-1 in kidney was different. In the kidney, both Hsp60 and IGF-1 receptor were increased significantly (Fig. 5*B*), which is in agreement with our previous observation in the kidney isolated from ZDF rats (4). When these diabetic rats were treated with insulin for 6 days, the levels of Hsp60 and IGF-1 receptor returned to normal. Phlorizin treatment failed to normalize Hsp60 and IGF-1 receptor in the kidney, which suggested that kidney Hsp60 was not regulated by hyperglycemia (Fig. 5).

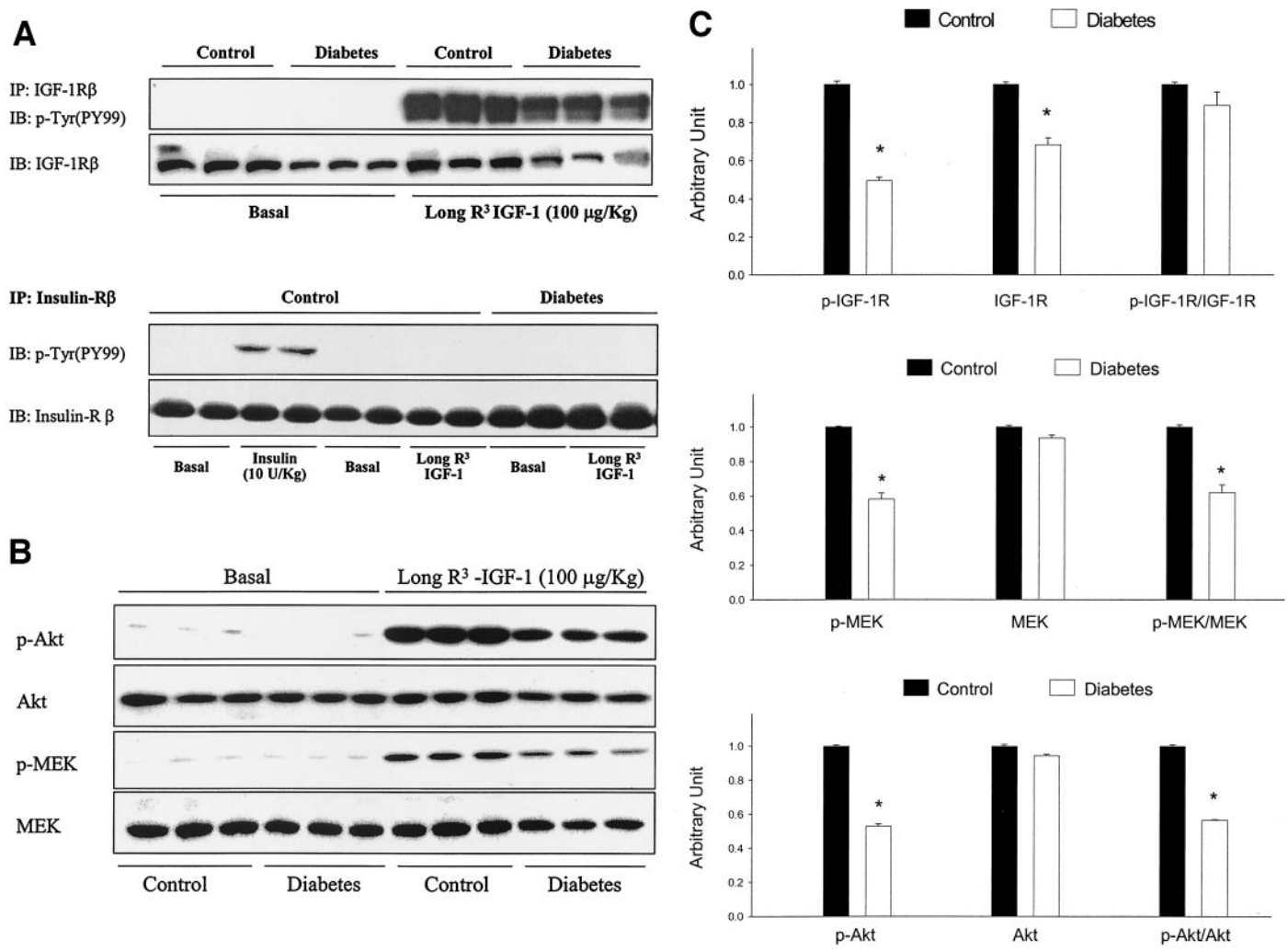

FIG. 4. Attenuated myocardial IGF-1 receptor signaling in STZ-induced diabetic rats. After overnight fasting, control and diabetic rats received an injection of Long R^3 IGF-1 (100 μg/kg body wt) for 2 (IGF-1R phosphorylation) to 5 (Akt/Mek activation) min. Myocardial proteins were extracted and immunoprecipitated (IP) with anti–IGF-1R or anti-insulin receptor antibodies and then immunoblotted (IB) with specific antibodies. *A*: Activation of IGF-1 receptor phosphorylation. *B*: Phosphorylation of Akt and Mek. *C*: Comparison of protein phosphorylation versus protein content. Bar graph represents densitometry analysis from multiple experiments. *$P < 0.05$ vs. control myocardium.

DISCUSSION

Diabetic cardiomyopathy is associated with a number of microscopic changes, such as capillary basement membrane thickening, proliferation of small arterioles, degeneration and loss of cardiomyocytes, accumulation of ground matrix, and focal fibrosis of myocardium (7,14). Despite these anatomical and functional changes having been recognized for some time, the molecular mechanisms underlying these changes in diabetic myocardium are not fully understood. Numerous biochemical abnormalities have been described in diabetic myocardium, including reprogramming of contractile protein expression to fetal pattern (13), increased oxidative stress (15), decreased sarcoplasmic reticulum calcium ATPase (16), increased troponin I phosphorylation (17), increased apoptosis of cardiac muscle cells (18), increased expression and activation of protein kinase C isoforms (19), alterations in glucose and fatty acid metabolism (14), and abnormal expression of growth factors and their receptors (13,19).

Although these changes might have contributed to the development of cardiac dysfunction, most are not specific to diabetic cardiomyopathy because similar biochemical abnormalities were found in other forms of cardiomyopathy. However, few changes seem to be specific for diabetes cardiomyopathy. One of these changes is the abundance of IGF-1 receptor and Hsp60. The abundance of IGF-1 receptor in myocardium is reduced in animal models of diabetes, as shown in the present study. This is different from ischemic cardiomyopathy and hypertrophic cardiomyopathy, which are associated with increased IGF-1 receptor expression and increased Hsp60 expression in myocardium (20–22), probably representing a myocardial self-defense mechanism (8). Because IGF-1 has cardiac protective action, reduced IGF-1 receptor and its signaling can potentially lead to decreased myocardial protection during myocardial ischemia and thus may play a fundamental role during the development of diabetic cardiomyopathy. We recently discovered that Hsp60 inhibits degradation of IGF-1 receptor, which in turn increases the abundance of IGF-I receptor and augments the antiapoptotic actions of IGF-1 in cardiac muscle cells (4). The time-course study (Fig. 1) supports the concept that decreased cardiac expression of Hsp60 contributed to down-regulation of IGF-1 receptor in diabetic myocardium.

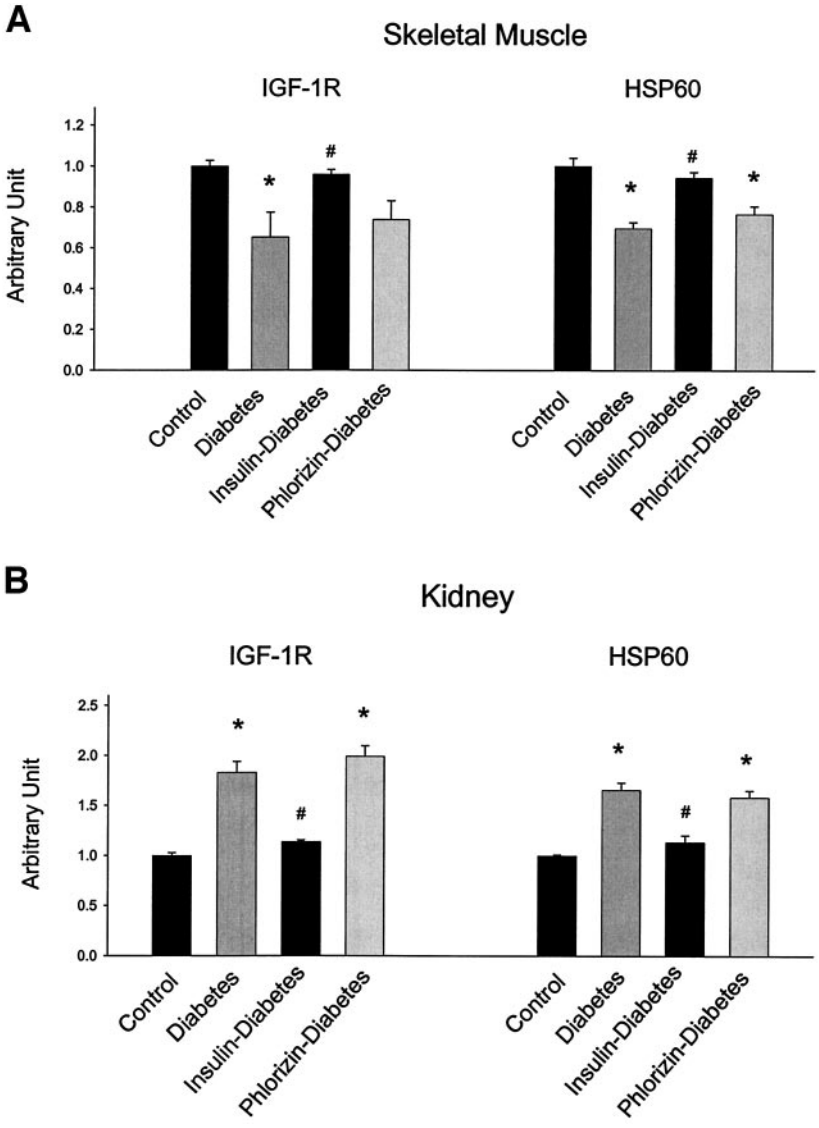

FIG. 5. The changes of Hsp60 and IGF-I receptor in the skeletal muscle and kidney of STZ-induced diabetic rats. *A*: The effects of insulin and phlorizin on skeletal muscle Hsp60 and IGF-1 receptor. *B*: The effects of insulin and phlorizin on kidney Hsp60 and IGF-1 receptor. Bar represents mean ± SE summarized from multiple animals. *$P < 0.05$ vs. controls.

Hsp60 may enhance myocardial protection through at least two different mechanisms. In addition to its effect on IGF-1 receptor signaling, Hsp60 modulates Bcl-2 protein family and increases cardiomyocytes resistance to apoptosis induction (9,23). Diabetic myocardium is different from ischemic cardiomyopathy regarding the expression of Hsp60. As discussed earlier, Hsp60 expression is increased during ischemic/reperfusion injury, probably representing a myocardial self-defense mechanism in response to ischemic/reperfusion injury. Despite Hsp60 playing an important role in myocardial protection, how Hsp60 is regulated in cardiac muscle has not been investigated in the past. This study showed that insulin could increase the expression of Hsp60 in cardiac muscle, and insulin deficiency led to reduced expression of Hsp60 in myocardium of STZ-induced diabetic rats. Our findings suggest that insulin deficiency or inadequate insulin action may be the underlying mechanism leading to downregulation of Hsp60 in cardiac muscle, which contributes to decreased IGF-1 receptor signaling and reduced myocardial protection.

The changes of Hsp60 and IGF-1 receptor are nearly identical in skeletal muscle and cardiac muscle, and both can be restored after insulin therapy. However, regulation of kidney Hsp60 and IGF-1 receptor is different from muscle tissues. We previously reported that kidney Hsp60 and IGF-1 receptor was upregulated in ZDF rats; thus, these changes are not model specific. Increased expression of IGF-1 in diabetic kidney has been observed, and increased IGF-1 signaling has been implicated in the development of diabetic nephropathy (24). Thus, the mechanisms through which diabetes modulates Hsp60 are not the same in all tissues. However, parallel changes of Hsp60 and IGF-1 receptors in different tissues continue to support our in vitro observation that Hsp60 can upregulate the abundance of IGF-1 receptor.

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