Downregulation of the constitutively expressed Hsc70 in diabetic myocardium is mediated by insulin deficiency

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Abstract

The 70 kDa heat shock protein family plays important cardiac protective roles against myocardial injuries. Reduced myocardial protection is a common feature of diabetic myocardium. This study was carried out to define the changes in the 70 kDa heat shock protein family in the myocardium in the of streptozotocin-diabetes rats, and to explore the mechanisms through which diabetes alters the abundance of Hsp70/Hsc70 in cardiac muscle. In the diabetic myocardium, the abundance of Hsc70 was significantly reduced. The abundance of Hsp70 was low in cardiac muscle and was not induced in the diabetic myocardium. Unlike Hsp60, Hsp70 and Hsc70 did not augment insulin-like growth factor-I receptor signaling in cardiac muscle cells. In cultured cardiomyocytes, insulin directly increased the abundance of Hsc70, whereas insulin could not modulate Hsp70. Treating diabetic rats with insulin restored myocardial Hsc70 level, but phlorizin treatment failed to restore myocardial Hsc70. These in vivo and in vitro studies showed that downregulation of Hsc70 in diabetic myocardium was secondary to insulin deficiency. Thus, insulin played a major role in maintaining adequate expression of Hsc70 in cardiac muscle.


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Introduction

Heat shock proteins play cardiac protective roles during myocardial injuries. The expression of various heat shock proteins typically increases upon myocardial stress (Latchman 2001, Lepore et al. 2001, Delogu et al. 2002, Cornelussen et al. 2003). For example, the expression of Hsp70 is dramatically induced after myocardial ischemic injuries (Trost et al. 1998, Lepore et al. 2001). The 70 kDa family of heat shock proteins is involved in cellular protection during stress in various tissues (Erbs et al. 2004). Two isoforms of 70 kDa heat shock proteins, Hsp70 and Hsc70, exist in mammalian tissues (Garrido et al. 2003, Erbs et al. 2004, Giffard & Yenari 2004). Previous reports in the literature indicate that Hsc70 is constitutively expressed and only mildly induced during stress situations, while Hsp70 is highly inducible upon stress stimuli (Erbs et al. 2004, Giffard & Yenari 2004). Both Hsp70 and Hsc70 are capable of protecting cardiac muscle cells against injuries (Chong et al. 1998, Trost et al. 1998). Induction of Hsp70 is part of the defence mechanisms and may contribute to enhancement of myocardial protection during ischemic injury, as overexpressing Hsp70 in myocardium is associated with lesser ischemic myocardial damages (Mestril et al. 1996, Jayakumar et al. 2001). Despite the fact that the cardiac protective effects of 70 kDa heat shock proteins have been well documented (Mestril et al. 1996, Jayakumar et al. 2001, Latchman 2001), little is known about the comparative expression of Hsp70 and Hsc70 in the diabetic state.

Diabetic myocardium is associated with reduced myocardial protection, and myocardial injuries are exacerbated in diabetic patients (Shan et al. 2003). Our recent study has shown reduced Hsp60 in diabetic myocardium (Shan et al. 2003). The goals of this study were to define the changes in Hsp70 and Hsc70 in diabetic myocardium and to study how diabetes alters the abundance of Hsp70/Hsc70 in cardiac muscle. The results showed that the abundance of Hsc70 was significantly reduced in diabetic myocardium because of insulin deficiency. The abundance of Hsp70 is quite low in myocardium in vivo and was not induced by diabetes. In cultured cardiomyocytes, insulin increased the expression of Hsc70, whereas insulin had no effect on Hsp70. These findings provide new insight into how diabetes modulates 70 kDa heat shock protein family.

Materials and Methods

Materials

Mouse anti-Hsp70 and anti-Hsc70 monoclonal antibodies were purchased from StressGen Biotechnologies Corp.
made with samples run on the same gel and exposed on the same film. For final analysis, the intensity of signals was normalized to the control sample on the same gel.

Adenoviral vectors

The construction of recombinant adenoviruses expressing Hsp70, Hsc70, and the control adenovirus Ad-SR was described previously (Mestril et al. 1996). The rat Hsp70 or Hsc70 gene was inserted into the E1 region of the Ad5 vector. Hsp70 or Hsc70 was cloned into the multiple cloning site of the adenoviral shuttle plasmid pACCMVpLPASR. The plasmid was co-transfected with pJM17 in 293 cells to generate Ad-Hsp70 and Ad-Hsc70. The viruses were replicated in 293 cells, purified by Virakit from Virapau (Carlsbad, CA, USA) and the viral titers were determined by plaque assay in 293 cells. Cardiomyocytes were plated in 100 mm Petri dishes in high-glucose Dulbecco’s modified Eagle medium containing 10% Fetal bovine serum and 1% penicillin/streptomycin. When indicated, the cells were infected with adenoviruses of Ad-SR, Ad-Hsp70, or Ad-Hsc70 and incubated for 36–48 h at 37 °C, 5% CO2.

Statistical analysis

The data were expressed as mean ± s.e. based on data derived from three to six independent experiments. The intensity of bands from Western blots were scanned with densitometry and digitally analyzed. Statistical significance was tested by Student’s t-test or ANOVA with post hoc analysis when appropriate. A P-value below 0.05 was considered statistically significant.

Results

Changes in Hsp70 and Hsc70 in diabetic myocardium

The first series of experiments were to characterize the changes in the 70 kDa heat shock protein family, Hsc70 and Hsp70, in diabetic myocardium. Sprague–Dawley rats were injected with STZ or vehicle (plasma glucose 101 ±8 vs 366 ±34 mg/dl, P<0.001) and diabetic myocardium was harvested at the indicated time intervals. The abundance of myocardial Hsp70 and Hsc70 was analyzed with Western blots as shown in Fig. 1. Interestingly, the expression of Hsc70 was significantly reduced in myocardium after the onset of diabetes. Although the expression of inducible Hsp70 can be detected in cultured cardiomyocytes, the expression of Hsp70 is low in adult myocardium and there was no visible difference between the control and diabetic myocardium. These results indicate that there was a time-dependent downregulation of Hsc70 in diabetic myocardium, but there was no clear upregulation of Hsp70 in diabetic myocardium.

Hsp70 and Hsc70 did not modulate IGF-I receptor signaling

In the diabetic myocardium, there was downregulation of IGF-I receptor as we had expected (Fig. 1). Recent studies in
our laboratory have shown that Hsp60 modulated the abundance of myocardial IGF-I receptor and thus augmented IGF-I receptor signaling in cardiac muscle cells (Shan et al. 2003). Therefore, the next series of experiments were to investigate whether 70 kDa heat shock protein can modulate IGF-I receptor signaling in primary cardiomyocytes. To this end, the cardiomyocytes were infected with adenoviral vector carrying Hsp70 (Fig. 2). Compared with the cells infected

**Figure 1** Changes in 70 kDa heat shock proteins in diabetic myocardium. (A) The abundance of Hsc70 in streptozotocin-induced diabetic rat (STZ-DM) myocardium. Myocardium was harvested from control and diabetic rats and myocardial proteins were immunoblotted for Hsc70. Immunoblotting with α-actinin served as controls. (B) Immunoblotting of Hsp70 in control and STZ-DM myocardium. No visible Hsp70 proteins can be detected in normal or diabetic myocardium. Immunoblot of Hsc70 in cultured neonatal cardiomyocytes served as positive control. IGF-I receptor protein (IGF-IR) was visibly detected in the myocardium. (C) Time-course effects of diabetes on myocardial Hsc70 and IGF-I receptor. Each bar represents the mean ± S.E. summarized from multiple experiments (n=6–8 animals in each group). The abundance of heat shock protein was normalized to the abundance of α-actinin in each sample. *P<0.05 vs the controls.

**Figure 2** Overexpression of Hsp70 did not alter IGF-I receptor signaling in cardiomyocytes. Cardiomyocytes were infected with Ad-Hsp70 (70) or control virus Ad-SR (SR) (A), serum-deprived overnight, and then treated with IGF-I (10⁻⁸ M) for 1, 2, or 5 min. (B) Phosphorylation of IGF-I receptor (p-IGF-IR), Akt (p-Akt), and Erk (p-Erk1/2). IGF-I receptor phosphorylation was studied by sequential immunoprecipitation (anti IGF-I receptor antibodies and protein A/G plus) and immunoblotting (anti-phosphotyrosine antibodies). The abundance of IGF-IR was determined by immunoblotting with antibodies against IGF-IR β-subunits. Phosphorylation of Akt and Erk was determined by immunoblotting with phosphor-specific antibodies. (C & D) Positive controls were carried out in cardiomyocytes transduced with Ad-Hsp60 (60), which augmented IGF-I receptor signaling. Cont, control.
with control virus, expression of 70 kDa heat shock protein increased significantly in the cells transduced with Ad-Hsp70 (Fig. 2A). Overexpression of Hsp70 protein did not alter basal IGF-I receptor phosphorylation, Akt, or Erk. Upon IGF-I stimulation, receptor phosphorylation, Akt activation, and Erk activation were identical in the cells infected with Ad-SR and Ad-Hsp70 (Fig. 2B). In contrast, overexpression of Hsp60 lead to increased IGF-I receptor phosphorylation (Fig. 2C and D) as we previously reported (Shan et al. 2003). We also studied the effect of Hsc70 overexpression on IGF-I receptor signaling in cardiomyocytes. As shown in Fig. 3, IGF-I activation of IGF-I receptor, Erk, and Akt was not modulated by overexpression of Hsc70. These data suggested that, unlike 60 kDa heat shock protein, 70 kDa heat shock proteins do not modulate IGF-I receptor signaling in cardiac muscle.

**Independent effects of insulin on cardiac Hsc70 and Hsp70 in vitro**

In order to investigate the independent effect of insulin and hyperglycemia on 70 kDa heat shock protein, cardiomyocytes were incubated with increasing concentrations of insulin or D-glucose and the cells were harvested for immunoblotting with specific antibodies. Insulin increased the abundance of Hsc70 in neonatal cardiomyocytes. However, Hsp70 proteins could not be induced by insulin (Fig. 4A and B). Hyperglycemia increased the expression of Hsp70, but not the expression of Hsc70 (Fig. 4C and D). The effect of D-glucose on Hsp70 expression was most significant at extremely high concentrations. D-Mannitol also induced a dose-dependent increase in Hsp70 (Fig. 4E and F), suggesting higher osmolality could induce Hsp70 in cardiomyocytes. D-Mannitol hyperosmolality did not alter the expression of Hsc70 in cardiomyocytes (Fig. 4F).

**The effect of insulin and phlorizin treatment on myocardial Hsc70 in vivo**

To dissect further the independent effect of insulin deficiency and hyperglycemia in vivo on myocardial 70 kDa heat shock proteins, we treated the diabetic animals with either insulin or phlorizin to normalize blood glucose. Phlorizin inhibits sodium–glucose co-transporter in renal tubule, promotes glucosuria, and normalizes blood glucose levels in diabetic animals (Laybutt et al. 2002). Body weight was restored and blood glucose was normalized in insulin treated rats, while in the phlorizin-treated animals only blood glucose was restored (Table 1). The abundance of myocardial Hsc70 was normalized after insulin therapy in diabetic rats, but phlorizin treatment failed to restore Hsc70 content in diabetic myocardium (Fig. 5). These data provide in vivo evidence that insulin deficiency is the key factor leading to down-regulation of myocardial Hsc70 in diabetic myocardium. Hyperglycemia does not appear to be an important factor contributing to the regulation of myocardial Hsc70 in vivo in the ranges of hyperglycemia seen in these diabetic rats. Furthermore, these data indicate that it is possible to normalize Hsc70 level in diabetic myocardium.

**Changes in Hsp70 and Hsc70 in kidney, adipose tissue, and skeletal muscle**

In order to determine whether diabetes altered 70 kDa heat shock protein in other tissues, various tissues were isolated from diabetic and control rats, homogenated, and immunoblotted with specific antibodies (Fig. 6). In the kidney, Hsc70 was increased in the diabetic rats. Insulin treatment normalized kidney Hsc70 levels; phlorizin treatment also corrected kidney Hsc70 levels. In the adipose tissue, Hsc70 is mildly increased in the diabetic rats, insulin treatment normalized Hsc70 levels and phlorizin therapy partially corrected this defect. Skeletal muscle Hsc70 was
Figure 4 Independent effect of insulin and hyperglycemia on 70 kDa heat shock proteins in cardiomyocytes. (A) The effect of insulin on Hsp70 and Hsc70. After overnight serum deprivation, cardiomyocytes were incubated with various concentrations of insulin or vehicles for 24 h. Cell lysates were harvested and analyzed with immunoblots. (B) The effect of insulin on Hsp70 and Hsc70. Data represent results summarized from multiple experiments. The abundance of heat shock protein was normalized to the abundance of α-actinin in each sample. (C) The effect of D-glucose on Hsp70 and Hsc70. Cardiomyocytes were incubated with various concentrations of D-glucose in growth medium for 24 h. Cell lysates were harvested and analyzed with immunoblots. Primary cardiomyocytes could not be grown well at <180 mg/dl of glucose, thus glucose concentration started at 200 mg/dl. (D) Effect of hyperglycemia on Hsp70 and Hsc70 in vitro. Bar graph represents densitometry analysis from multiple experiments. The abundance of heat shock protein was normalized to the abundance of α-actinin in each sample. (E) The effect of D-mannitol on Hsp70 and Hsc70. Cardiomyocytes were incubated with various concentrations of D-mannitol for 24 h. Cardiomyocytes cannot be grown without glucose, therefore, 200 mg/dl D-glucose was present in the growth medium. (F) Effect of osmolality on Hsp70 and Hsc70. Bar graph represents densitometry analysis from multiple experiments. The abundance of heat shock protein was normalized to the abundance of α-actinin in each sample.

Table 1 Characteristics of experimental animals. Diabetes was induced by streptozotocin injection (STZ-DM). Insulin or phlorizin were injected into subsets of diabetic rats to correct hyperglycemia

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
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<tbody>
<tr>
<td>Control</td>
<td>262.7 ± 13.1</td>
<td>101.8 ± 6.6</td>
</tr>
<tr>
<td>STZ-DM</td>
<td>227.8 ± 11.6*</td>
<td>396.2 ± 35.6*</td>
</tr>
<tr>
<td>Insulin-treated STZ-DM</td>
<td>262.8 ± 26.7†</td>
<td>119.7 ± 11.8†</td>
</tr>
<tr>
<td>Phlorizin-treated STZ-DM</td>
<td>236.7 ± 13.0*</td>
<td>135.2 ± 28.3†</td>
</tr>
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Data represent means ± s.e. *P < 0.05, vs controls. †P < 0.05, vs STZ-DM.
downregulated in diabetic rats, similar to the changes in cardiac muscle. Insulin therapy completely restored Hsc70 levels in skeletal muscle, while phlorizin therapy partially increased Hsc70 levels in skeletal muscle. These experiments suggest that downregulation of Hsc70 in skeletal muscle involved both insulin deficiency and hyperglycemia. In various diabetic tissues, the changes in Hsp60 generally paralleled the changes in IGF-I receptor (Arispe et al. 2002). The changes in Hsc70 did not necessarily parallel the changes in IGF-I receptor in each tissue investigated, which provide additional support to our in vitro observation that 70 kDa heat shock proteins did not modulate IGF-I receptor protein (Figs 2 and 3).

Discussion

The expression of constitutive Hsc70 was reduced in diabetic myocardium, and the inducible Hsp70 was not induced in diabetic myocardium. Since these two heat shock proteins may protect cardiac muscle against injuries, these findings may have functional implications during the development of diabetic cardiomyopathy. Our data also indicate that insulin is a key factor in maintaining adequate constitutive expression of Hsc70 in cardiac muscle. Insulin can directly induce the expression of Hsc70 in cardiac muscle, which may contribute to the cardiac protective action of insulin.

The 70 kDa family of heat shock proteins has two isoforms in mammalian cells. Hsc70 is a constitutively expressed 73 kDa protein, and Hsp70 is a 72 kDa protein that is inducible by stress (Garrido et al. 2003, Giffard & Yenari 2004). These two isoforms share a high degree of sequence homology; both are composed of a 44 kDa ATPase domain, a 18 kDa peptide-binding domain, and a 10 kDa C-terminal domain (Giffard & Yenari 2004). Extensive evidence suggests both isoforms are involved in assisting protein folding, transporting protein across the membrane, regulating stress response, cooperating with other chaperone systems, and aiding cell survival. Despite the remarkable similarities in their structure and function, delicate differences exist between these two isoforms (Garrido et al. 2003, Giffard & Yenari 2004). The subcellular localization is not entirely identical. Hsp70 has been found to bind the 40S ribosomal subunit, whereas

Figure 5  The effects of insulin and phlorizin on cardiac Hsc70 and IGF-I receptor in streptozotocin-induced diabetic rat. When indicated, diabetic rats were treated with insulin or phlorizin for 6 days after the induction of diabetes. (A) Representative photo of myocardial Hsc70 and IGF-IR immunoblot. (B) Densitometry analysis of myocardial Hsc70 and IGF-IR. Bar graph represents densitometry analysis from multiple experiments (n=6–8 animals in each group). The abundance of heat shock protein was normalized to the abundance of α-actinin in each sample. Data represent means±s.e. *P<0-05 vs controls; †P<0-05 vs untreated diabetes.
Hsc70 can interact with nascent polypeptides in ribosomes (Beck & De Maio 1994, Arispe et al. 2002). The functional significance of these dissimilarities is not yet known. A recent study by Atalay et al. (2004) showed that the levels of the inducible Hsp70 (Hsp72) were downregulated in the myocardium of STZ-induced diabetic rats. However, in our study, we did not detect a significant change in Hsp70/72 in myocardium. The abundance of Hsc70 was not investigated in the study by Atalay et al. (2004).

Current studies in the literature suggest that Hsp70 is the isoform that can be modulated in the mammalian myocardium. In the hypoxic human and rabbit hearts, expression of the inducible Hsp70 was significantly increased, whereas Hsc70 expression was not affected (Rafiee et al. 2003). Similarly, induction of myocardial Hsp70 was found in rats receiving coronary artery ligation (Tanonaka et al. 2001), but the level of myocardial Hsc70 was not increased. In various tissues including the heart, the 70 kDa heat shock protein family has a cell-protective function. Transgenic expression of Hsp70 reduced the size of myocardial infarction (Hutter et al. 1996). One of the key mechanisms through which Hsp70 and Hsc70 protect cells against injuries is through suppression of apoptosis signaling; overexpression of Hsp70 or Hsc70 leads to resistance against stress-induced caspase activation (Mosser et al. 2000, Giffard & Yenari 2004). In cardiac muscle cells, overexpression of Hsc70 attenuated oxidative injuries and enhanced cell survival (Chong et al. 1998). Hsp70 has been found to inhibit apoptosis induced by tumor necrosis factor (TNF), oxidative stress, ceramide, and ultraviolet (UV) radiation (Giffard & Yenari 2004). In addition to suppressing caspases, Hsp70 isoforms could antagonize apoptosis protease activating factor 1 and apoptosis inducing factor, modulate the Bcl-2 family, and inhibit dephosphorylation of c-jun-N-terminal kinase (Giffard & Yenari 2004).

We have recently reported that another heat shock protein, Hsp60, is capable of augmenting IGF-I receptor signaling in cardiac muscle cells (Shan et al. 2003). Unlike Hsp60, Hsp70 and Hsc70 did not modulate IGF-I receptor signaling as shown in this study. We also observed that Hsp60 was reduced in diabetic myocardium (Shan et al. 2003). These observations indicate a milieu of heat shock protein downregulation in diabetic myocardium. Insulin deficiency is a likely culprit behind cardiac Hsp downregulation. Very little is known regarding the regulation of the Hsp70 family in diabetes. Many questions remain; for example, which signaling pathways do insulin receptors use to induce Hsc70 expression? How many heat shock protein families are affected by diabetes and insulin? Does downregulation of
myocardial Hsc70 also occur in human diabetes? Does heat shock protein dysregulation ultimately lead to progression of diabetic cardiomyopathy? These questions are beyond the scope of this study but should be pursued in the future.

One caveat in our experimental models is the cell culture model used. Neonatal cardiomyocytes represent a reasonable in vitro model for cardiac muscle cells, but glucose metabolism and growth factor response can be different from adult cardiac muscle. Our in vivo and in vitro studies were in agreement regarding the effect of insulin on myocardial Hsc70 downregulation but there was a discrepancy between our in vitro and in vivo data regarding Hsp70 and hyperglycemia. In vitro studies showed that hyperglycemia increased Hsp70 in cultured cardiomyocytes; however, Hsp70 was not induced in diabetic myocardium. In cultured renal cells, hyperosmolality could induce Hsp70 expression (Cohen et al. 1991), but how osmolality regulates cardiac Hsp70 has not been investigated. It is not clear why Hsp70 was not induced in the diabetic myocardium. The complex in vivo biochemical changes in diabetic myocardium might have complicated the modulation of myocardial Hsp70.

Regulation of Hsp70/Hsc70 appears to be complex in different tissues. The effect of diabetes, hyperglycemia, and insulin varies in the four tissues we have studied (Fig. 6). Reduced expression of Hsp70 has been found in the wound bed of diabetic db/db mice (McMurtry et al. 1999), suggesting that delayed wound healing in diabetic animals may involve dysregulation of Hsp70. In the diabetes-prone BB rats, there was a lack of Hsp70 induction during beta cell destruction (Burkart et al. 2000). Specific mechanisms underlying differential regulation of Hsc70 in these tissues will require further research.

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