Cardiac Muscle Protein Catabolism in Diabetes Mellitus: Activation of the Ubiquitin-Proteasome System by Insulin Deficiency

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Protein degradation is increased by both insulin deficiency and insulin resistance in humans and animal models. In skeletal muscle, this insulin-dependent increase in protein degradation involves activation of both caspase-3 and the ubiquitin-proteasome system. The influence of abnormal insulin signaling on protein metabolism in cardiac muscle is not well understood; therefore, we measured protein degradation in cardiac muscle of mice with streptozotocin-induced diabetes. Insulin deficiency increased both total muscle proteolysis (measured as tyrosine release in muscle slices or extracts) and the degradation of the myofibrillar protein actin (measured as the appearance of a 14-kDa actin fragment). Expression of ubiquitin mRNA and chymotrypsin-like activity in the proteasome were increased, indicating activation of the ubiquitin-proteasome system in diabetic mouse heart. We also evaluated possible signaling pathways that might regulate cardiac muscle proteolysis. Insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation, and Akt phosphorylation were decreased. Insulin replacement prevented the decrease in IRS-1/Akt phosphorylation, the increase in proteolysis, and attenuated the increase in ubiquitin mRNA. We conclude that insulinopenia accelerates proteolysis in cardiac muscle by reducing IRS-1/Akt signaling, which leads to activation of the ubiquitin-proteasome proteolytic pathway. (Endocrinology 149: 5384–5390, 2008)

Diabetes mellitus is associated with a marked increase in the risk of cardiac disease, including a cardiomyopathy that is characterized by impaired ventricular function, even though there is no atherosclerotic coronary heart disease or hypertension (1). The mechanisms underlying this type of heart disease in diabetes are complex. There could be involvement of hyperlipidemia, altered endothelial function, and/or decreased sympathetic neuronal function (2, 3). A major component of diabetic cardiomyopathy is cardiac remodeling, which involves increased turnover of cardiac muscle proteins. Destruction of cardiac muscle proteins also could affect contractile function by decreasing the content of myofibrillar protein. It is unclear how diabetes changes the regulation of protein metabolism in the heart.

Insulin is an important regulator of protein turnover in both skeletal and cardiac muscle. It is well established that insulin deficiency or insulin resistance accelerates the degradation of skeletal muscle protein (4, 5). The mechanisms underlying this response involve suppressed insulin receptor substrate-1 (IRS-1)/Akt signaling, which causes activation of caspase-3 and stimulation of the ubiquitin-proteasome system (5–7). Evidence also suggests that an intact insulin signaling system plays an important role in regulating cardiac size. In mice with a selective knockout of the insulin receptor in cardiomyocytes, the hearts were reduced in size by 20–30% (8). In cardiac muscle, insulin stimulates protein synthesis (9) and exerts an antiapoptotic effect on cardiomyocytes (10). Insulin may also increase cardiac contractility (9).

The importance of the ubiquitin-proteasome system in regulating protein degradation in skeletal muscle in diabetes is well documented. However, little information is known about the regulation of the ubiquitin-proteasome system in the heart (11, 12). For example, Kedar et al. (13) reported that the ubiquitin-proteasome system is critical for the degradation of key structural and functional proteins in the heart. This report suggested that the ubiquitin-proteasome system could regulate cardiac function in both normal and stressed situations by changing protein metabolism. In the present study, we evaluated the influence of insulin deficiency on muscle protein metabolism in the heart using a mouse model of acute type 1 diabetes.

In this study, cardiac muscle protein degradation and insulin signaling were examined by several different methods to determine the impact of insulin deficiency on cardiac muscle protein turnover. We investigated four major proteolytic processes: 1) the ATP-dependent, ubiquitin-proteasome system; 2) the lysosomal proteolytic pathway; 3) the calcium-activated proteolytic pathway; and 4) caspase-3-mediated proteolysis. Our results provide evidence that the IRS-1/Akt regulates cardiac muscle mass by modulating the activity of the ubiquitin-proteasome system and caspase-3-mediated proteolysis.

Materials and Methods

Animals

Mice (C57BL/6) were purchased from The Jackson Laboratory (Bar Harbor, ME). The experiments were approved by the Institutional Animal Care and Use Committee of Emory University. Mice were housed for 1 week before experiments. Animals were fasted overnight, and blood was collected for glucose and insulin measurements. Animals were then treated with streptozotocin or saline, as indicated, and blood glucose was measured weekly or biweekly. Animals were sacrificed at the end of the experiment, and tissues were harvested for analysis.

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Abbreviations: amc, 7-Amino-4-methylcoumarin; DTT, dithiothreitol; E-64, trans-epoxysuccinyl-l-leucylamido-4-guanidino butane; FOXO, forkhead O transcription factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRS-1, insulin receptor substrate-1; STZ, streptozotocin.

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Physiological parameters

<table>
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<tr>
<th>Parameter</th>
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<th>Diabetes + Ins (n = 6)</th>
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<tr>
<td>Blood glucose (mg/dl)</td>
<td>133.8 ± 2.7</td>
<td>423.3 ± 11.6b</td>
<td>169.7 ± 8.6</td>
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<td>Plasma insulin (ng/ml)</td>
<td>1.12 ± 0.03</td>
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<td>Body weight (g)</td>
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<tr>
<td>Heart weight (mg)</td>
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<td>84.5 ± 6b</td>
<td>100 ± 3</td>
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All data are presented as the mean ± SE. Ins, Insulin.

a P < 0.05 vs. control.
b P < 0.01 vs. control.
c P < 0.05 vs. control.
d p = 0.05 days after STZ treatment.
e p < 0.01 vs. control.
f p < 0.05 vs. control.

Cardiac muscle protein degradation

Protein degradation in cardiac muscle was evaluated by three different assays. Total protein degradation was measured in left ventricular slices, whereas breakdown of soluble cardiac muscle protein and myofibrillar proteins were determined in flash-frozen muscles. As described for isolated skeletal muscle (14), we measured the rate of tyrosine release from slices of the left ventricle (~15 mg/slice). The slices were fixed at resting size (15) on a plastic support and then incubated in Krebs-Ringer bicarbonate buffer (135.5 mM NaCl; 4.7 mM KCl; 24.8 mM NaHCO3; 1 mM MgSO4·7H2O; 2.5 mM CaCl2; 10 mM glucose) containing 0.5 mM cycloheximide to block tyrosine reutilization. After a 30-min preincubation, the muscle was transferred to a flask containing fresh media and incubated at 37 °C for 2 h. All incubation flasks were gassed with 95%/O2/5%/CO2 for 3 min at the beginning of the preincubation and experimental periods. Degradation of proteins was measured by assaying the free tyrosine in the trichloroacetic acid soluble supernatant using a fluorometric technique (16). The influence of diabetes on the different proteolytic pathways was assessed by adding proteolytic inhibitors to the media and measuring tyrosine release (17, 18). The ubiquitin-proteasome pathway was specifically inhibited by adding 20 μM epoxomicin (Peptides International Inc., Louisville, KY). Epoxomicin is a cell-permeable, potent, selective, and irreversible inhibitor of proteasome function. It does not inhibit nonproteasomal proteases, including calpain and lysosomal cathepsins, at concentrations less than 50 μM (19).

In some experiments, both lysosomal and calcium-activated proteases were blocked. This was accomplished by adding 10 mM metylimine, 200 μM valine, 170 μM leucine, 100 μM isoleucine, and 1 μM/ml insulin to the medium to inhibit lysosomal function, whereas calcium-activated proteases were blocked by deleting calcium from the Krebs-Ringer bicarbonate buffer and adding 50 μM trans-epoxysuccinyl-l-leucylamido-4-guanidino butane (E-64) to the media. E-64 is a potent inhibitor of both the calcium pathway (calpains) and the lysosomal proteases cathepsin B, D, H, and L. At 50 μM, E-64 does not inhibit the proteasome (20).

To assay the degradation of cardiac muscle soluble protein, we used a modification of the proteasomal proteolysis assay reported by Goldberg and colleagues (21). The heart was removed, and, after removal of slices for metabolism tests, two thirds of the remaining heart was freeze clamped in liquid nitrogen. Muscle extracts were prepared by pulverizing cardiac muscle (~50 mg tissue) in liquid nitrogen and homogenizing the powder in ice-cold harvest buffer (5 mM Tris-HCl (pH 8.8), 1% glycerol, 1 mM EDTA, 1 mM EGTA, freshly constituted 1 mM β-Me, and 50 μM EP-475) (22). The homogenates were centrifuged at 30,000 × g for 30 min, then the soluble fractions were used to measure protein degradation, and the pellets were used to determine actin cleavage (22). For the protein degradation determination, muscle extracts were dia-

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**Figure 1.** Protein degradation is elevated in the cardiac muscle of STZ-treated diabetic mice. A, Protein degradation was measured as tyrosine (tyr) release from isolated skeletal and cardiac muscle from control (Ctrl), diabetic (STZ), or insulin-treated diabetic (STZ+Ins) mice. The protein degradation rates are depicted in the bar graph and represent the means ± SE (n = 9 per group). §, P < 0.05 vs. skeletal muscle control; *, P < 0.05 vs. cardiac muscle control. B, Protein degradation was measured as tyrosine release from isolated cardiac muscle from control or diabetic (STZ) mice. The entire bar (white and black) represents total protein degradation. Black bars represent the remaining degradation activity after inhibition of the lysosomal plus calcium-activated proteolytic pathways (left) or proteasome proteolytic pathway (right). Therefore, the white portions of the bars only indicate the activities of the degradation pathways specified below the bars. Data are reported as the means ± SE (n = 6 per group). *, P < 0.05 vs. control.
Signaling Technologies); and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000 dilution; Chemicon International, Inc., Temecula, CA). The IRS-1 tyrosine phosphorylation was measured in muscle extracts by immunoprecipitation with the IRS-1 antibody, followed by Western blot analysis using the PY-20 (antiphosphotyrosine) antibody (1: 2000 dilution, BD Biosciences, San Jose, CA).

Northern blots

Hearts were removed and immediately freeze clamped in liquid nitrogen. Total RNA from the heart was isolated using TriReagent (Molecular Research Center, Inc., Cincinnati, OH), and used in Northern blots for ubiquitin and GAPDH as described (24). Autoradiographic band intensities were quantified by densitometry (Bio-Rad Gel Doc system) using the corresponding GAPDH to correct for variations in RNA loading and transfer.

Proteasome activity

To measure the chymotrypsin-like peptidase activity of the proteasome, frozen pulverized cardiac muscle was homogenized in a protease and fluorescence was measured in a fluorometer (Shimadzu Scientific Instruments) using a 380-nm excitation wavelength and a 460-nm emission wavelength. Free amc (Chemicon International) was used to prepare a standard curve, and all readings were calculated relative to the fluorescence intensity of the standard curve. The difference between the substrate cleavage activity levels in the presence and absence of epoxomicin was used to calculate the contribution of proteasome.

Caspase-3 activity

Cardiac muscle was pulverized under liquid nitrogen and homogenized in lysis buffer [100 mM HEPES, 10% sucrose, 0.1% Nonidet P-40, and antiprotease cocktail (1 tablet/10 ml Roche) (pH 7.4)]. The activity of caspase-3 was measured using a fluorogenic substrate (Ac-DEVD-amc), with or without a caspase-3 inhibitor (Ac-DEVD-CHO) in the caspase assay buffer (CaspACE Assay System; Promega Corp., Madison, WI). Incubations were performed at 37 C for 1 h, and fluorescence was measured in a fluorometer (Shimadzu Scientific Instruments) with a 380-nm excitation wavelength and a 460-nm emission wavelength. The difference between the substrate cleavage activity levels in the presence and absence of caspase-3 inhibitor was used to calculate caspase-3 activity (14).

Statistical analysis

Results are presented as mean ± sem. Densitometric data for blots are expressed as a percentage of the control mean density after normaliza-
tion to loading controls. To identify significant differences between two groups, comparisons were made using the Student’s t test. Differences with $P$ values less than 0.05 were considered significant.

**Results**

**Protein degradation is increased in cardiac muscle of diabetic mice**

We studied nine pairs of mice [diabetic (STZ-treated) mice and pair-fed controls]. In addition, insulin was administered to six STZ-treated mice (Table 1). Heart weights of diabetic, control, and STZ plus insulin mice were 84.5 ± 6, 101 ± 4, and 100 ± 3 mg, respectively ($P < 0.01$; diabetes vs. control or STZ plus insulin group). The body weights of the diabetic mice (18.2 ± 1.8 g) were lower than either control (22.5 ± 2.2 g; $P < 0.05$) or STZ plus insulin (21.7 ± 3.5 g; $P < 0.05$) mice. The heart weight to body weight ratios were not statistically different in any group.

We measured total protein degradation rates in slices of the left ventricle and compared them with the rates measured in skeletal muscle. In control animals, protein degradation in cardiac muscle was 2-fold higher than in skeletal muscle ($P < 0.01$), indicating that cardiac muscle has a higher protein turnover rate compared with skeletal muscle (6). Compared with hearts of control mice, the rate of protein degradation was increased 31% in hearts of STZ-treated mice (Fig. 1A). Insulin replacement attenuated the increase in cardiac muscle protein degradation. When calcium-activated and lysosomal proteolysis was inhibited in cardiac tissue, there was a 30% decrease in the protein degradation in both control and diabetic mouse cardiac tissue. However, even with the calcium-activated and lysosomal proteolysis pathways inhibited, hearts from diabetic mice still exhibited increased protein degradation relative to the control mice. Thus, the increased protein degradation appears to result from stimulation of the ubiquitin proteasome system (Fig. 1B). To confirm this, epoxomicin, a specific proteasome inhibitor, was included in the incubation media. Under this condition, degradation due to the proteasome was 42% greater in the diabetic mouse heart than in the control mouse heart (Fig. 1B). To evaluate whether similar events occur in the right ventricle, we also measured protein degradation in this tissue. We found no significant difference in the rates of protein degradation between the right and left ventricles in either diabetic or control mice (data not shown). Thus, the accelerated protein degradation in cardiac muscle of diabetic mice is due to enhanced activity of a proteasome-dependent proteolytic system.

To evaluate this finding in more detail, we measured the degradation of soluble proteins in the heart muscle. Consistent with results from the measurement of total protein degradation in the cardiac muscle slice, degradation of soluble proteins was 1.8-fold higher in cardiac muscle when compared with skeletal muscle (Fig. 2). Evidence that the difference involved the ubiquitin-proteasome pathway was obtained by comparing protein degradation in cardiac extracts with or without addition of ATP. ATP is essential for the proteolytic activity of the ubiquitin proteasome system (21). In the absence of ATP, there was no significant difference between protein degradation rate in control vs. diabetic mice. In the presence of an ATP generation system, there was a greater increase in protein degradation in cardiac muscles from diabetic mice compared with those from control mice (Fig. 2). This indicated that the increased protein degradation in diabetic mouse heart is ATP dependent.

To provide additional evidence for activation of the ubiquitin proteasome system, we analyzed the level of ubiquitin mRNA in cardiac muscle of diabetic and control mice. There was a marked increase in ubiquitin mRNA in muscle of diabetic mice when compared with nondiabetic normal control mice. Insulin administration partially blocked the increase in ubiquitin mRNA (Fig. 3). We also found that there was a 2-fold increase in the chymotrypsin-like activity in the proteasome in heart muscle from diabetic mice compared with control mice. Again, insulin treatment partially blunted this response (Fig. 4). Together, these results suggest that diabetes activates the ubiquitin-proteasome system to degrade cardiac muscle protein.

We next measured degradation of the nonsoluble, myofibrillar proteins by assessing actomyosin-actin cleavage (5, 22). We found an increased level of the characteristic 14-kDa actin fragment that results from caspase-3-mediated proteolysis in the heart of diabetic mice (Fig. 5). Treating the diabetic mice with insulin significantly attenuated the increase in actin cleavage.

The activation of caspase-3 is an initial step that stimulates protein degradation by the ubiquitin proteasome system in skeletal muscle of diabetic mice. There was evidence for the increased proteolytic activity of caspase-3 because the level of the characteristic 14-kDa actin cleavage fragment was elevated in heart muscle of diabetic mice. To confirm this finding, we measured caspase-3 activity in cardiac muscles of pair-fed control, diabetic, and diabetic mice treated with insulin. The activity was increased 33% in diabetic heart vs. control heart, and insulin administration reversed this response (Fig. 6).

We also evaluated the effect of insulinopenia on protein synthesis in isolated heart muscle slices. Protein synthesis was measured as the incorporation of [U-14C] phenylalanine
FIG. 5. Actin cleavage is increased in cardiac muscle of diabetic mice. Actin cleavage was measured in control (Ctrl), diabetic (STZ), or insulin-treated diabetic (STZ+Ins) mice. Western blot analysis (left) of heart muscle protein shows both intact actin (42 kDa) and the 14-kDa actin fragment cleavage product (indicated by arrows). GAPDH (bottom panel) was used as a loading control. The bar graph (right) shows the mean value of the 14-kDa fragment band intensity, expressed as a percentage of untreated control value after normalization by the density of the GAPDH bands. Insulin-deficient mice (middle bar) had substantially higher levels of the actin fragment compared with control mice or insulin-treated diabetic mice. Data are reported as the means ± SE (n = 6 per group). *, P < 0.01 vs. control.

(7). We found no significant differences in the rates of protein synthesis measured in heart slices from diabetic mice, control mice, and diabetic mice treated with insulin (data not shown).

Finally, we examined whether signaling through the IRS-1/Akt pathway in the heart was altered by diabetes because signaling defects in this system have been linked to increased proteolysis in skeletal muscle (5). Diabetes caused a significant decrease in IRS-1 tyrosine phosphorylation and in the phosphorylation of Akt (Fig. 7). Insulin therapy improved both abnormalities. The forkhead O transcription factor (FOXO) is a substrate of Akt and activates the ubiquitin E3 ligase when it is dephosphorylated. We measured phosphorylation of FOXO and found that phosphorylation of threonine 32 was decreased in hearts of diabetic mice. Insulin partially restored FOXO phosphorylation to the level measured in control animals (Fig. 8).

Discussion

Diabetes mellitus is an independent risk factor for the development of heart failure. This occurs from the observation that diabetes is associated with contractile dysfunction of the heart (26). Our results suggest that the decreased cardiac contractile activity in diabetes is due to the loss of cardiac muscle protein. Protein turnover is an important physiological process in the heart, considering that the entire complement of proteins in the heart is replaced on average every 30 d (27). Indeed, we found that diabetes caused a decrease in cardiac wet weight and that this was due to an increased degradation rate of cardiac muscle proteins. By measuring cardiac wet weight over dry weight, we were able to assess protein turnover with the same tissue sample.

Consistent with our results of decreased heart weights, others have reported that the heart atrophies in STZ-induced, type 1 diabetic animals. Singh et al. (28) and Liu et al. (29) reported a 25 and 20% decrease, respectively, in the heart weights in STZ mice compared with values from control mice. In our study the reduced heart weight in diabetes was due to an increased rate of degradation of total heart protein (Fig. 1A and Table 1). Interestingly, the rate of protein degradation was 1.8- to 2-fold higher in cardiac muscle than skeletal muscle, regardless of whether the animal was diabetic or a normal control (Figs. 1A and 2). Others have reported that individual cardiac muscle proteins turn over at rates 1.5- to 3-fold higher than skeletal muscle (29, 30).

We evaluated the protein degradation process using several different techniques. We measured the release of tyrosine from isolated cardiac muscle slices and from soluble muscle proteins. Tyrosine release was used because muscle neither synthesizes nor degrades tyrosine, and its loss from the muscle reflects the net degradation of proteins (4). The release of tyrosine from incubated muscles has been used extensively with skeletal muscles isolated from small rodents, and the rate of protein degradation is linear with time (15). We studied small muscles to avoid potential limitations in the diffusion of oxygen, glucose, amino acids, etc. In addition, small muscles maintain levels of ATP and phosphocreatine, and show almost neutral protein balance during incubations for two or more hours (15). However, we rec-
recognize that results from incubation of cardiac muscle slices could differ because of the release of tyrosine from edges of the cardiac slice due to cut trauma. To minimize this possibility, the muscle slices were preincubated for 30 min before the experimental period was begun. This essentially washes out any tyrosine contributed by broken cells at the cutting interface. We confirmed our tissue slice data using an independent method of assessing protein degradation, namely by measuring the accumulation of the 14-kDa actin cleavage product from caspase-3 activity (22). An alternative approach would be to evaluate protein metabolism in isolated papillary muscles (31), but again, trauma could affect these measurements. In addition, it is possible that the rate of protein turnover in papillary muscles might be different from that in ventricular muscle. Surprisingly, we did not find significant differences in the measured rates of protein synthesis among diabetic hearts, control hearts, and the hearts of diabetic mice treated with insulin. This interesting result suggests that the large loss of cardiac mass in the diabetic animal is due predominantly to an increase in protein degradation.

By measuring protein degradation in the presence of different types of proteolytic inhibitors, we determined that the ubiquitin-proteasome system is the major proteolytic pathway degrading cardiac muscle in diabetic mice; inhibiting the proteasome blocked the protein degradation induced by insulin deficiency. Further evidence included the findings that: 1) ubiquitin mRNA was increased substantially in hearts of diabetic mice; and 2) the chymotrypsin-like activity in the proteasome was increased in hearts of diabetic mice. We also found that the lysosomal and calcium-activated proteolytic pathways comprised about 20–30% of the cardiac muscle degradation. This was the same in the diabetic heart as in the normal control heart. Consistent with our conclusions, others have reported that the ubiquitin-proteasome system is important for the degradation of key cardiac proteins such as myosin heavy chain (32) and cardiac troponin I (13). Liu et al. (29) have also reported that ubiquitin mRNA was increased in the hearts of diabetic rats.

The increase in protein degradation in cardiac muscle of diabetic animals is similar to the increases in skeletal muscle proteolysis in experimental animal and patients with sepsis (33), trauma (34), cancer (35), or uremia (36). Our data also show that activation of caspase-3 is associated with increased cardiac muscle protein degradation. Others have reported that caspase-3 is active in normal cardiac tissue and activated during ischemia-reoxygenation, suggesting a central role for this protease in regulating cardiac muscle protein metabolism (26). Our results indicate that the insulin signaling pathway, specifically
the IRS-1/Akt pathway, is a key regulatory system in both cardiac and skeletal muscle.

In conclusion, the mechanisms by which diabetes mellitus confers an increased risk of heart diseases are multifactorial (2, 3). Our results extend to the list of abnormalities by showing that diabetes activates proteolytic pathways in the heart that contribute to the loss of cardiac protein and, potentially, cardiac function. The latter is important because others have demonstrated that insulin deficiency depressed heart contractile function (28, 37). This involves the breakdown of the myofibrillar protein actin as a result of activation of the ubiquitin-proteasome system. These responses could contribute to diabetic cardiomyopathy.

Acknowledgments

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