Exercise training modulates heat shock protein response in diabetic rats

Mustafa Atalay,1 Niku K. J. Oksala,1,2 David E. Laaksonen,1 Savita Khanna,1,3 Chitose Nakao,1 Jani Lappalainen,1 Sashwati Roy,3 Osmo Hänninen, and Chandan K. Sen1,3

1Department of Physiology, University of Kuopio, FIN-70211 Kuopio; and 2Departments of Pathology and Surgery, Tampere University Medical School, FIN-33521 Tampere, Finland; and 3Laboratory of Molecular Medicine, Department of Surgery, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, Ohio 43210

DIABETES IS ASSOCIATED WITH impairment of endogenous tissue defense mechanisms and vulnerability of tissues to various types of stress. A major component of the endogenous defense is the heat shock protein (HSP) family, which may protect against tissue damage by facilitating the refolding of denatured proteins and maintenance of structural integrity and by acting as molecular chaperones (3). A substantial role for HSPs in diabetes and oxidative stress has emerged (15, 23, 39). It has recently been observed that skeletal muscle glucose-regulated protein (GRP) 78 and HSP90 are elevated in Type 2 diabetes mellitus (15). In contrast, streptozotocin-induced diabetes (SID) was associated with impaired HSP72 synthesis in rats (40).

Uncontrolled oxidative stress, a state in which the increased production of reactive oxygen species overwhelms endogenous antioxidant protection (17, 36), may result in damage of lipids, proteins, and genome (36). However, at lower concentrations, reactive oxygen species also serve as messenger molecules and regulate cellular adaptations. There is a general consensus that oxidative stress may have an important role in the pathophysiology of diabetes and its complications (1, 7, 9, 21). Thus the management of oxidative stress represents a key therapeutic approach to treat diabetes and its complications (1, 24, 36). The cellular redox state is known to modulate the expression of stress proteins (31).

Habitual physical exercise is a powerful tool in preventive medicine, especially for the diabetic patient. Despite possible risks of acute exercise in relation to oxidative stress in diabetic patients (1, 24), our group has previously observed that regular moderate exercise and fitness may protect diabetic men against oxidative stress (24).

It has been well demonstrated that HSP has a major role in tissue protection and repair against a number of insults and pathological conditions (33, 39). Endurance training upregulates HSP expression (14, 28, 32, 33) and may provide an extra protection against oxidative stress (32, 37). The information regarding the protective effect of HSPs in tissue protection in diabetes is, however, limited. Exercise training serves as an excellent physiological model for studying mechanisms of HSP induction and enhancing tissue protection. The effect of chronic exercise on HSP expression in experimental diabetes has not been previously examined. In the present study, we tested the hypothesis that endurance training enhances tissue protection and decreases oxidative stress and tissue damage in diabetic animals through the induction of heat shock responses.

MATERIALS AND METHODS

Animals. The experimental protocol was approved by the Ethics Committee for the laboratory animal research of University of Kuopio, Finland. Animal care and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Male outbred Wistar rats (National Laboratory Animal Center, Kuopio, Finland) were maintained at 22 ± 2°C with 12:12-h dark-light cycles and had free access to standard rat chow and water. One-half of the rats were randomly assigned to the diabetic group, which was induced by the injection of streptozotocin, as described below. The other one-half of the rats were kept as a control group. Rats with sustained diabetes (glucosuria of at least 20 mmol/l 2 wk after streptozotocin injection) and the nondiabetic control rats were further randomly divided into untrained and trained groups (n = 8).

Address for reprint requests and other correspondence: C. K. Sen, Laboratory of Molecular Medicine, 512 Dorothy M. Davis Heart & Lung Research Institute, 473 W. 12th Ave., Columbus, OH 43210 (E-mail: sen-1@medctr.osu.edu).

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Preparation of diabetic rats. Diabetes was induced by a single intraperitoneal injection of streptozotocin at a dose of 60 mg/kg (prepared in 0.1 M citrate buffer, pH 4.5) to male 12-wk-old Wistar rats (1). The state of diabetes was confirmed by glucosuria by using glucose test strips (BM-Test-5L, Boehringer Mannheim) after 1 wk of streptozotocin injection. A dipstick urine test was repeated once a week during the study. Blood glucose levels were also measured at the end of the study in truncal blood collected immediately after decapitation by using a commercial kit (Gluco-quant Glucose/HK, Boehringer Mannheim) based on a hexokinase/glucose-6-phosphate dehydrogenase enzymatic method, as previously reported (13).

Endurance training of rats. Fourteen-week-old rats were trained on a treadmill in both training groups for 8 wk for 5 days/wk, as previously described (13). After 1 wk of familiarization of the rats to the treadmill, training began in control animals from 1.08 km/h, 30 min/day, with gradual increases in training speed and duration such that rats reached 1.08 km/h for 1.5 h/day by the end of the first week and 1.5 h/day for 1.5 h/day at week 4 and continued 5 days/wk for a total of 8 wk. In diabetic animals, the same training duration was used, but the intensity was slightly lower because of lower body weight and lower endurance capacity of the diabetic rats. Training started in diabetic animals from 1.08 km/h for 1.5 h/day by the end of the first week and reached 1.45 km/h for 1.5 h/day by week 4 and continued at this intensity for 4 more weeks. The rats in both groups tolerated the training and were able to increase the running distance and intensity, according to the training protocol, throughout the study.

Sample collection and preparation. After the 8-wk period of endurance exercise training, all of the rats were killed at rest by decapitation 72 h after the last training session. After decapitation, heart, liver, and red gastrocnemius (RG) and vastus lateralis (VL) muscles were quickly excised, freed from adipose and connective tissue, rinsed in ice-cold saline, and blotted on filter paper. Tissue samples were further cut into small pieces and placed in liquid nitrogen and stored at −70°C for later homogenization and biochemical assays (22). RG muscle was examined to represent the response of mainly oxidative muscle fibers, and superficial white portion of VL was studied as glycolytic muscle fibers.

Frozen tissues were ground in liquid nitrogen with a mortar and sonicated in a buffer containing 25% glycerol, 0.42 mol/l NaCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l ethylenediaminetetraacetic acid, 20 mmol/l N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 5 μmol/l dithiothreitol, and 5 μmol/l phenylmethylsulphoxide at +4°C. Unless otherwise stated, all chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) and were of analytic grade or the highest grade available. Protein extracts (20 μg protein/lane), together with molecular weight markers, were electrophoresed on SDS-PAGE (SDS/8 or 10% PAGE) and transferred to a nitrocellulose membrane (Protran, Schleicher and Schuell, Dassel, Germany). Equal

**P < 0.05, ***P < 0.01. Difference due to diabetes: #P < 0.05. Difference due to training: ++P < 0.01. ++‡‡P < 0.001. Interaction between diabetes and training: ‡‡‡P < 0.01.

Training

Diabetes

Heart, Liver, RG, VL muscles in response to SID and Tr.

Fig. 2. A: effect of SID and Tr on the expression of heat shock factor-1 (HSF-1) in heart, liver, and RG and VL muscles. Groups and bars are as in Fig. 1. Values are means ± SE. Difference due to SID: *P < 0.05. Difference due to endurance training: ††P < 0.05. **P < 0.01. ***P < 0.001. Interaction between diabetes and training: ++‡‡‡P < 0.01. B: Western blot images demonstrating tissue HSF-1 expression in heart, liver, and RG and VL muscles in response to SID and 8-wk endurance training.
transfer was checked and quantified by reversible protein staining of the nitrocellulose membrane with Ponceau S reversible membrane staining.

**Analysis of stress proteins by Western blot.** Western blot procedures were followed, as previously reported (11, 29). After blocking with a 5% (wt/vol) fat-free milk solution at 37°C for 1 h, membranes were treated overnight at 4°C with monoclonal antibodies (StressGen, Victoria, BC), recognizing the inducible form of HSP72, heme oxygenase 1 (HO-1), HSP90, and GRP75. A polyclonal primary antibody [heat shock factor (HSF)-1; Alexis, San Diego, CA] was used for the detection of HSF-1. Membranes were washed for 3 × 10 min with Tris-buffered saline containing 0.1% Tween 20. As secondary antibodies, horseradish peroxidase-conjugated anti-mouse (Santa-Cruz, Santa Cruz, CA), anti-rat (Zymed, San Francisco, CA), and anti-rabbit immunoglobulins (Santa-Cruz) were used, respectively. After 6 × 10 min of washing with Tris-buffered saline containing 0.1% Tween 20, immunoblots were visualized by using Renaissance Western blot chemiluminescence reagent (NEN, Life Sciences Products, Boston, MA) and quantified by using an image-analysis software (Scion Image, Frederick, MD).

**HSF-1 binding activity.** A gel mobility shift assay was performed, as previously described (19). The protein extracts were prepared similarly as for the Western blot and mixed with isotope-labeled probes corresponding to the two overlapping heat shock elements. Protein-DNA complexes were resolved on a nondenaturing polyacrylamide gel. Gels were dried, and the radioactivity was detected by autoradiography.

**Analysis of protein carbonyls by Western blot.** The tissue extracts were prepared, as described above. The protein carbonyls were derivatized with 2,4-dinitrophenyl hydrazine immediately before the electrophoresis, as previously described (11). Protein extracts (20 μg protein/lane) were electrophoresed on 10% SDS-PAGE, and Western blot procedures were followed, as described above. As primary antibody, rat monoclonal antibody to 2,4-dinitrophenol (Zymed Laboratories, San Francisco, CA) was applied at 1:1,000 dilution overnight at 4°C. The membranes were incubated with a secondary antibody: horseradish peroxidase-conjugated mouse anti-rat antibody (Zymed Laboratories) was used at 1:10,000 dilution for 1 h at room temperature.

**Analysis of citrate synthase activity.** Citrate synthase (CS) activity was measured, as previously described (35). Total protein concentration was measured by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

**Histology.** Frozen tissue specimens were immersed in phosphate-buffered formalin overnight at +5°C. Routine paraffin embedding and tissue processing were performed. Sections of 3-μm thickness were mounted on glass slides and stained with hematoxylin-eosin. The slides were covered with cover slides and DPX mounting medium. The digital images were acquired at ×20 magnification by using a microscope with a charge-coupled device camera.
Statistical analyses. Two-way ANOVA was used to test the effect of diabetes and endurance training. The equality of variances was checked with Levene’s test. Statistical significance was defined as \( P < 0.05 \). All data are expressed as means ± SE.

RESULTS

Effects of experimental diabetes. The efficiency of streptozotocin treatment was evident by glucosuria (> +++) measured by using glucose test strips and blood glucose levels. As our laboratory previously reported from the same study, blood glucose levels at rest in diabetic, untrained rats were remarkably higher compared with those of the corresponding nondiabetic rats (7.00 ± 1.00 vs. 19.17 ± 3.80 mmol/l) (12).

SID decreased overall HSP72 levels in heart, liver, and VL muscle \(( P < 0.01, 0.05, and 0.05, \) respectively; Fig. 1). The effect of SID was most evident or largely restricted to the trained group \(( P \) for the interaction between presence of diabetes and training status <0.05 for heart, liver, and VL). Myocardial HSP72 levels were 60% lower in SID rats at rest compared with nondiabetic rats (Fig. 1). HSF-1 protein content was significantly lower in heart and RG muscle of SID animals \(( P < 0.05)\) and tended to decrease in liver tissue \(( P < 0.06; \) Fig. 2). Gel mobility shift image of HSF-1 binding activity in heart, liver, and RG and VL muscles in response to SID and 8-wk endurance training is presented in Fig. 3. SID increased the expression of HO-1 in liver and RG and VL muscles \(( P < 0.001, 0.05, and 0.05, \) respectively; Fig. 4). HSF90 expression was higher in heart but lower in liver of the diabetic rats compared with nondiabetic rats \(( P < 0.001 and 0.05, \) respectively; Fig. 5). GRP levels were, however, higher in liver of the SID rats \(( P < 0.01; \) Fig. 6). SID increased the oxidative stress marker protein carbonyl concentration in all of the tissues examined \(( P < 0.01 for VL, and for the rest of the tissues \( P < 0.05; \) Fig. 7). This effect was, however, more prominent and largely restricted to the untrained rats.

SID induced accumulation of lymphocytes in the heart tissue (Fig. 8) of untrained animals. No obvious histological changes were observed in the liver in SID or endurance-trained animals (figure not shown). SID also brought about an apparent increase in the volume of interstitial space and interstitial swelling in VL muscle (Fig. 9). SID induced similar morphological changes in RG muscle too (figure not shown).

Effects of endurance training. Eight weeks of endurance training upregulated oxidative metabolism in skeletal muscle. This was evident with increased CS activity in RG and VL muscles \(( P < 0.001 and 0.05, \) respectively; Table 1). Although there was no significant interaction between diabetes and training, the training effect on CS activity seemed to be more pronounced in RG muscle of diabetic animals compared with the nondiabetic rats (74 vs. 30% induction). Overall, CS activities in RG and VL muscles were, however, lower in SID rats, and this effect was more evident in untrained SID rats \(( P < 0.05 and 0.001, \) respectively; Table 1). Endurance training significantly increased the expression of HSP72 in heart, liver, RG, and VL muscles of both diabetic and nondiabetic rats (two-way ANOVA: \( P < 0.01, 0.001, 0.01, \) and 0.001, respectively; Fig. 1). However, for HSP levels, we observed a significant interaction between diabetes and training in heart, liver, and VL muscle \(( P < 0.05)\), indicating a lower training effect on HSP induction in these tissues of diabetic rats compared with nondiabetic rats. Endurance training induced HSF-1 protein content in all of the tissues examined \(( P < 0.01, 0.001, 0.05, \) and 0.05, respectively; Fig. 2). HSF-1 binding activity measured in mobility shift assay was clearly apparent after endurance training only in nondiabetic control animals; there was, however, no statistical interaction between diabetes and training (Fig. 2). HSF-1 binding activity measured in mobility shift assay was clearly apparent after endurance training only in nondiabetic control animals (Fig. 3). Endurance training did not affect other HSP expression, except GRP75 induction in RG muscle of both groups \(( P < 0.05)\).

Despite the trend of increase in nondiabetic control groups, endurance training did not affect protein carbonyl levels significantly in any of the tissues examined.

Eight weeks of endurance training did not have any apparent effect on the histology of the tissues in nondiabetic rats, except for the triglyceride deposition in VL muscle of both normal and SID rats (Fig. 9). However, endurance training normalized SID-induced lymphocyte accumulation in heart tissue (Fig. 8). SID-induced tissue swelling in VL muscle was not prevented by endurance training (Fig. 9). Similar morphological changes were also observed in RG muscle (figure not shown).
DISCUSSION

In the present study, we present the first evidence for impaired overall HSP72 expression in heart, liver, and VL muscle of SID rats. Endurance training upregulated HSP72 levels in all of the tissues examined. However, this induction was severalfold lower in diabetic animals than in control rats. We observed that endurance training induced HSF-1 activation in control rats but not in SID rats. This finding may be one of the mechanisms behind the impaired HSP response in SID. In addition, histological examinations revealed that tissues of SID animals were more vulnerable to inflammation at rest as well as after chronic exercise. Regular training, however, may attenuate the inflammation that accompanies SID in heart tissue.

Induction of diabetes by streptozotocin brought about a variety of tissue-specific changes in HSP levels. Diabetes decreased overall levels of HSP72 in heart, liver, and VL muscle, although decreased HSP72 levels were detected only in heart and liver of the untrained animals. Limited information is available on the effects of diabetes on skeletal muscle and heart HSP expression. Joyeux et al. (18) showed that heat shock preconditioning did not provide any cardioprotection in diabetic rats, despite a clear protective effect in control animals. However, there was no significant difference in the HSP expression between diabetic and control rats (18). On the other hand, in another study, induction of HSP70 expression by heat was lower in liver and adrenal gland of SID rats, which could be interpreted as impaired cytoprotective capacity (40). Decreased tissue regeneration and lower HSP72 expression have been previously reported in SID animals (5).
Table 1. Effect of streptozotocin-induced diabetes and 8-wk endurance training on citrate synthase activity in heart, red gastrocnemius muscle, and vastus lateralis muscle

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Con-Tr</th>
<th>SID</th>
<th>SID-Tr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.165±0.90</td>
<td>1.239±0.108</td>
<td>1.017±0.20</td>
<td>1.140±0.42</td>
</tr>
<tr>
<td>RG</td>
<td>328±44</td>
<td>426±16*</td>
<td>233±15*</td>
<td>406±35*$| $</td>
</tr>
<tr>
<td>VL</td>
<td>125±10</td>
<td>149±18†</td>
<td>92±10†</td>
<td>110±9$‡$</td>
</tr>
</tbody>
</table>

Values are means ± SE. Groups are as follows: Con, nondiabetic control sedentary rats; Con-Tr, Con rats after 8-wk endurance training (Tr); SID, streptozotocin-induced diabetic (SID) sedentary rats; SID-Tr, SID rats after Tr. RG, red gastrocnemius; VL, vastus lateralis. Differences due to SID: *P < 0.05, †P < 0.01. Differences due to Tr: $P < 0.05, §P < 0.001.

In the present study, SID increased liver and skeletal muscle HO-1 levels, consistent with the results of Cosso et al. (8). HO-1 mRNA levels were increased by 1.8-fold in the liver of spontaneously diabetic rats compared with nondiabetics (8). HO-1 (HSP32) induction has been recognized as a sensitive marker of oxidative stress, and its overexpression protects against oxidative damage in several cell types (3, 26). HO-1 induction in response to SIDs agrees with increased oxidative protein damage and increased oxidative stress in SID rats.

In contrast to the levels in the heart, we observed a lower HSP90 expression in liver tissue of SID rats compared with the control animals. Previously, HSP90 has been shown to be associated with steroid hormone receptor maturation, which may have further implications in the pathogenesis of diabetes (38). We observed increased levels of hepatic GRP75 in the diabetic animals. In an earlier study, Parfett et al. (30) showed that mRNA of GRP78, a cytosolic form of GRP75, was induced in liver tissue of the nonobese diabetic mice. However, in our study, SID did not induce GRP expression in the other tissues examined.

Diabetes increased levels of protein carbonyls significantly in all of the tissues examined. This effect was, however, evident only in untrained animals. Our group and others have shown that diabetes is associated with increased oxidative stress, and elevated protein carbonyl levels have been reported both in Type 1 and Type 2 diabetes, as well as in experimental diabetes (2, 10, 12, 13, 24). Consistent with the protein oxidation in diabetic animals, we observed significant tissue inflammation, which was evident by tissue swelling in skeletal muscle and lymphocyte infiltration in heart.

The endurance-training protocol used in the present study was effective, as attested by the 74% increase in CS activity in skeletal muscle of the trained SID rats. Eight weeks of training also increased triglyceride deposition in skeletal muscle, which represents the increased capacity of fat oxidation, a well-defined, early adaptation of the skeletal muscle to endurance training (16, 34).

Endurance training increased the levels of HSP72 in the heart, liver, and skeletal muscle of both SID and nondiabetic control rats. A large number of papers investigating the effects of training on HSP levels in heart and skeletal muscle of the nondiabetic rats have been published. Most of the studies agree that endurance training induces HSP72 expression in heart and skeletal muscle of rats and humans (14, 20, 27, 28, 32, 33, 37). In contrast, we observed markedly lower HSP72 induction by endurance training in diabetic animals than in control rats, indicating impaired HSP72 induction in diabetic animals. These findings are in agreement with the previous reports showing that, in SID rats, heat-induced HSP72 expression was impaired (40).

Oxidative stress activates the heat shock response (6), and an increased HSP response after endurance training has been shown to decrease lipid peroxidation in the myocardium of nondiabetic rats (32). However, in this study, we did not observe any tight correlation between oxidative stress and HSP response in most of the tissues examined. Therefore, upregulation of HSP72 by endurance training seems to have been induced by factors other than exercise-induced oxidative stress.

We detected a lack of HSF-1 activation measured by mobility shift assay and impaired induction of HSP-1 expression in endurance-trained SID rats. In contrast, there was an increased HSF-1 activation, expression, and trimerization in nondiabetic animals than in SID rats. HSF-1 is the major heat shock transcription factor that binds to the heat shock responsive element in the promoter of the heat shock genes and regulates the rapid induction of HSP synthesis in response to various environmental stresses (19, 29). Increased expression of HSF-1 enhanced cytoprotective capacity and correlated with constitutive HSF-1 DNA binding activity in murine fibroblasts (25). The overexpression of HSF-1 in that model, however, did not result in overproduction of HSP after heat shock (25). A potential mechanism by which SID might deteriorate HSF-1 activation is via upregulation of glycogen synthase kinase-3 (GSK-3). GSK-3 was initially described as a key enzyme that regulates glycogen metabolism, but is currently known to be involved in a diverse array of cell functions, including suppression of HSF-1 activity (4). Because GSK-3 activity was not measured in the present study, this assumption is speculative.

In the histological examination, we detected interstitial swelling in the skeletal muscle and lymphocyte inflammation in the heart of SID animals. The SID-induced inflammatory changes in the heart were restored with endurance training, in parallel with increased HSP levels.

In conclusion, our results demonstrate increased oxidative stress and an overall impaired HSP response in SID rats. HSP72 induction and HSF-1 transcriptional activity were attenuated in diabetic rats compared with nondiabetic control rats, although endurance training increased HSP72 expression in SID rats. Our results suggest that exercise training may have applications in offsetting compromised HSP-mediated tissue defenses in diabetes.

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HEAT SHOCK RESPONSE AND EXERCISE IN DIABETES

REFERENCES