Decreased Expression of Heat Shock Protein 72 In Skeletal Muscle of Patients With Type 2 Diabetes Correlates With Insulin Resistance

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Oxidative stress has been ascribed a role in the pathogenesis of diabetes and its complications, and stress proteins have been shown to protect organisms in vitro and in vivo against oxidative stress. To study the putative role of one of the most abundant cytoprotective stress proteins, inducible cytoplasmic 72-kDa-mass heat shock protein (Hsp-72), in the pathogenesis of diabetes, we measured its mRNA concentration in muscle biopsies from six type 2 diabetic patients and six healthy control subjects (protocol 1) as well as in 12 twin pairs discordant for type 2 diabetes and 12 control subjects undergoing a euglycemic-hyperinsulinemic clamp in combination with indirect calorimetry (protocol 2). The amount of Hsp-72 mRNA in muscle was significantly lower in type 2 diabetic patients than in healthy control subjects (in protocol 1: 5.2 ± 2.2 vs. 53 ± 32 million copies of Hsp-72 mRNA/μg total RNA, $n = 6$, $P = 0.0039$; in protocol 2: 3.2 ± 3.3 vs. 43 ± 31 million copies of Hsp-72 mRNA/μg total RNA, $n = 12$, $P = 0.0001$). Hsp-72 mRNA levels were also markedly reduced in the nondiabetic co-twins compared with healthy control subjects (5.8 ± 5.0 vs. 43 ± 31, $n = 12$, $P = 0.0001$), but they were also statistically significantly different from their diabetic co-twins when the difference between the pairs was compared ($P = 0.0280$). Heat shock protein mRNA content in muscle of examined patients correlated with the rate of glucose uptake and other measures of insulin-stimulated carbohydrate and lipid metabolism. In conclusion, the finding of decreased levels of Hsp-72 mRNA in skeletal muscle of patients with type 2 diabetes and its relationship with insulin resistance raises the question of whether heat shock proteins are involved in the pathogenesis of skeletal muscle insulin resistance in type 2 diabetes. *Diabetes* 51:1102–1109, 2002

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FFM, fat-free mass; Hsc-73, constitutively expressed cytoplasmic heat shock protein with 73-kDa molecular mass; HSP-70, heat shock protein family consisting of several stress proteins (Hsp-72, Hsc-73, mHsp-75, and Grp-78/BIP) with molecular mass ~70 kDa; Hsp-72, inducible cytoplasmic heat shock protein with 72-kDa molecular mass; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; PPAR, peroxisome proliferator–activated receptor; sRNA, synthetic RNA.

The pathogenesis of type 2 diabetes is still uncertain (1,2) but some evidence is accumulating showing that hyperglycemia is associated with the production of reactive oxidative intermediates, and with a disturbed antioxidant defense mechanism (3,4). In type 2 diabetes, insulin sensitivity is inversely correlated with the levels of plasma free radicals (5), and recently Rudich et al. (6) described a potential direct link between oxidative stress and insulin-stimulated glucose uptake. The authors found that in 3T3-L1 adipocytes, prolonged oxidative stress caused a 60% decrease in insulin-stimulated GLUT4 translocation to the plasma membrane by affecting steps distal to the activation of insulin receptor substrate-1 and phosphatidylinositol 3-kinase (7). Treating the cells with the antioxidant lipoic acid reversed these changes (8). The ability of stress proteins to protect cells against oxidative stress has been verified in several laboratories (9,10), and data pointing to the possible involvement of stress proteins in the diabetic state also came from laboratory experiments. Isolated islets from the diabetes-prone BB rat are vulnerable to the toxic effect of oxygen radicals or nitric oxide after heat shock because the BB rat has low levels of heat shock proteins in the islets (11). Transgenic mice carrying the calmodulin minigene develop severe insulin-dependent diabetes, and in parallel with this alteration, they lose their ability to react to cold exposure with a stress response. It is, however, not known whether this represents a primary defect or develops secondary to the diabetic state (12).

Heat shock proteins represent an evolutionary conserved family of polypeptides that protect other cellular proteins from damage by binding to them when they are denatured or inappropriately folded. They also participate in the assembly and disassembly of macromolecular complexes (13,14). The family of ~70-kDa heat shock proteins (HSP-70) represents one of the most abundant stress proteins in the human body. In the mammalian cytoplasm, two very similar 70-kDa heat shock proteins are present (15): constitutively expressed cytoplasmic 73-kDa-mass heat shock protein (Hsc-73) and inducible cytoplasmic 72-kDa-mass heat shock protein (Hsp-72), which plays a

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TABLE 1
Clinical characteristics of study subjects

Parameters	Protocol 1		Protocol 2		
	Type 2 diabetic subjects	Control subjects	Type 2 diabetic co-twins	Nondiabetic co-twins	Control subjects
n (F/M)	6 (1/5)	6 (0/6)	12 (5/7)	12 (5/7)	12 (6/6)
Age (years)	51 ± 4	48 ± 9	64 ± 3	64 ± 3	61 ± 2
BMI (kg/m^2)	25.1 ± 2.7	27.1 ± 4.2	30.1 ± 1.3	27.5 ± 1.3	26.0 ± 1.0
Fasting plasma glucose (mmol/l)	8.9 ± 0.8*	5.7 ± 0.2	11.4 ± 1.1*	6.0 ± 0.2‡	5.4 ± 0.2
Fasting plasma insulin (μU/ml)	ND	ND	12.7 ± 1.6*	7.3 ± 2.2	6.9 ± 0.9
2-h glucose (mmol/l)	ND	ND	19.5 ± 1.7*	8.1 ± 1.6‡	5.7 ± 0.3
Glucose uptake (mg · kg FFM^{-1} · min^{-1})	2.8 ± 1.2†	6.8 ± 2.2	5.2 ± 0.7*	8.5 ± 0.8‡	11.3 ± 0.9
Glucose storage (mg · kg FFM^{-1} · min^{-1})	ND	ND	2.8 ± 0.6*	4.8 ± 0.6‡	7.6 ± 0.9
Glucose oxidation (mg · kg FFM^{-1} · min^{-1})	ND	ND	2.4 ± 0.2*	3.7 ± 0.2	3.8 ± 0.2
Lipid oxidation (mg · kg FFM^{-1} · min^{-1})	ND	ND	1.23 ± 0.13*	0.76 ± 0.18	0.65 ± 0.07

Data are means ± SD. ND, not determined in the study. *$P < 0.01$ compared with control subjects †$P < 0.02$ compared with control subjects; ‡$P < 0.05$ compared with control subjects

significant cell maintenance and cytoprotective role under both physiological and stress conditions.

To test the hypothesis that heat shock proteins may be involved in the pathogenesis of skeletal muscle insulin resistance in type 2 diabetes, we measured the amount of Hsp-72 mRNA in muscle biopsies from patients with type 2 diabetes and healthy control subjects using a competitive RT-PCR method. We also measured Hsp-72 mRNA levels in muscle biopsies from monozygotic twins discordant for type 2 diabetes and control subjects without any family history of diabetes, and we related the mRNA concentrations to measures of insulin-stimulated glucose and lipid metabolism. Importantly, studying monozygotic twins discordant for diabetes allows some estimation of the relative contributions of primary (genetic) versus secondary (nongenetic) etiological factors to a given phenotype or metabolic defect. Thus, any defect present in the diabetic subjects but not in their genetically identical nondiabetic co-twins may have a nongenetic origin, whereas defects present in both the diabetic subjects and their identical nondiabetic co-twins (as compared with control subjects without family history of diabetes) may have a genetic origin.

RESEARCH DESIGN AND METHODS

The clinical characteristics of the subjects participating in the two protocols are shown in Table 1. In protocol 1, six type 2 diabetic and six nondiabetic control subjects participated in a 3-h euglycemic-hyperinsulinemic (blood glucose 5 mmol/l, insulin 70 mU/l) clamp. A needle biopsy from the vastus lateralis muscle was performed before the clamp. In protocol 2, 12 monozygotic twin pairs discordant for type 2 diabetes and 12 healthy age- and sex-matched control subjects with normal glucose tolerance (NGT) and without a family history of diabetes and hypertension participated. In this protocol, the clamp was combined with indirect calorimetry (see below) to allow measurements of substrate oxidation as previously explained (16). Monozygosity of the twins was confirmed by genetic markers (17).

Study protocols. All studies were started in the morning after a 10-h overnight fast. A polyethylene catheter was inserted into an antecubital vein for infusion of test substances. Another polyethylene catheter was inserted into a contralateral wrist vein for blood sampling. This hand was placed and maintained in a heated plexiglas box to obtain arterialized venous blood. Plasma glucose was normalized in the diabetic patients before each study by a prior intravenous insulin infusion given in order to overcome the known compensatory effect of hyperglycemia per se, as previously explained (16,17). The insulin infusion was stopped when the plasma glucose concentration had declined to a near-normal level, and a further period of 30 min elapsed while the plasma glucose concentration continued to decline to normoglycemia (i.e., plasma glucose concentration ~5 mmol/l). This time point marked the initiation of each experiment (time −120 min), when the tritiated glucose tracer bolus as described below was given. Except for the prior insulin infusion, the control subjects were studied in the fasting euglycemic state using a similar protocol with a primed continuous glucose tracer infusion from −120 min. Baseline measurements were performed during a predefined baseline "steady-state" period from −30 to 0 min, when tracer equilibrium was achieved. Insulin (Actrapid; Novo-Nordisk, Bagsvaerd, Denmark) was infused from 0 to +180 min at a constant rate of 40 mU · m^{-2} · min^{-1} in both the type 2 diabetic twins and the nondiabetic twins and control subjects. Plasma glucose concentration was maintained constant at euglycemia, using a variable glucose infusion. Plasma glucose concentration was monitored in arterialized blood every 5–10 min using an automated glucose oxidase method (Glucose Analyser 2, Beckman Instruments, Fullerton, CA). Measurements of insulin-stimulated glucose turnover rates were performed from +150 to +180 min during clamps (i.e., predefined insulin stimulated steady-state periods). The rate of nonoxidative glucose metabolism was calculated as the difference between total glucose disposal and glucose oxidation as measured by indirect calorimetry. Plasma concentrations of glucose and insulin were measured every 10 min during both baseline and insulin-stimulated steady-state periods. The glucose clamp studies were combined with a primed (22 μCi) continuous (0.22 μCi/min) infusion of [3-^{3}H]glucose (New England Nuclear, Boston, MA) to obtain valid measurements of peripheral glucose turnover rates as previously explained (17). Glucose turnover rates were expressed as milligrams per kilogram of fat-free mass (FFM) per minute and are presented throughout the paper as the mean values calculated over the two 30-min steady-state periods. Total body fat content (and thus FFM) was measured using the bioimpedance method (18). Needle biopsies were obtained in the basal state (time 0 min) from the vastus lateralis muscle. The biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analyzed. Informed consent was obtained from all subjects. The protocols were approved by the local ethics committees, and the study was conducted according to the principles of the Declaration of Helsinki.

Quantitative competitive RT-PCR. Total RNA was prepared from the muscle biopsies by the method of Chomczynski and Sacchi (19). RNA was quantitated by measuring absorbance at 260 nm in a spectrophotometer, and its quality and integrity were estimated from the 260:280-nm absorbance ratios. RNA samples were stored at −80°C. Oligonucleotides were designed by using the sequence of human Hsp-72 cDNA, published by Milner and Campbell (20), coding for the 72-kDa–molecular weight inducible heat shock protein (GenBank accession no. [GBAN] M59830 or M34269). The sequences chosen as primers were 100% conserved between the rat, mouse, and human cDNAs (21) and GenBank accession nos. M59830, L16764, and M35021. The four oligonucleotides (listed below) were synthesized by DNA Technology (Aarhus, Denmark). The sequence of the oligonucleotides were as follows: primer 1: AGA TCA TCG CCA ACG ACC AG (565–584, sense); primer 2: GTC AGC ACC ATG GAC GAG ATC TCC TCA GTG CTT CAT GTC CGA CTG (854–835 + 754–731, antisense); primer 3: AAT TTA ATA CGA CTC ACT ATA GGG AAG ATC ATC GCC AAC GAC CAG (T7 promoter sequence [22] plus 565–584, sense); primer 4: CAC CAT GGA CGA GAT CTC CT (854–835, antisense). Reverse transcription was performed using M-MLV RTase (Life Technologies, Rockville, MD), according to the vendor's recommendation. If the cDNA

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mixtures were not used immediately for the PCR, they were stored at −20°C. PCR was performed with standard mixtures using a Perkin-Elmer DNA Thermal Cycler. The amplification cycle profiles were 95°C (denaturing) for 1 min, 58°C (annealing) for 1 min, and 72°C (synthesis) for 30 s. The cycle number varied between 30 and 55, according to the actual requirement of the experiments.

For the preparation of mini-genes coding for different segments of HSP-70 cDNA, 1 μg of total cellular RNA (isolated from the heart of a heat-treated SPRD rat) was reverse-transcribed using primer 2 or 4. cDNAs from the two reactions were then amplified using primers 1 and 4, resulting in the production of a 210-bp-long (80 bp was deleted by primer 2 during reverse transcription) or a 290-bp-long product. The fragments were isolated from agarose gel and were used as templates for the subsequent rounds of PCR experiments. In these experiments, using primers 3 and 4, the PCR products were tagged 5-prime to the DNA constructs with the promoter of the T7 bacteriophage RNA polymerase. The pieces containing the T7 promoter were isolated from agarose gels and were stored at −20°C in known concentrations. Synthetic RNA (sRNA) standards were prepared by using the constructed mini-genes and reagents necessary for in vitro transcription (SP6/T7 Transcription Kit; Boehringer-Mannheim, Mannheim, Germany), as recommended by the manufacturer. Aliquots containing 1 μg sRNA in 10 μl diethyl pyrocarbonate–water were prepared and were stored at −80°C. The quantitative RT-PCR was performed as follows: 200 ng of total cellular RNA from each sample was mixed together with at least four different concentrations (10-fold and/or 3.16-fold serial dilutions) of the deleted-length sRNA template and was subjected to the RT-PCR procedure described above. PCR products were separated on agarose gels, stained with ethidium bromide (example is shown in Fig. 1*A*), and photographs were scanned by a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA). Ratios of the 210-bp (competitor) and 290-bp (target) bands were determined and were plotted against the known concentrations of the competitor on a double-log scale. Target RNA concentrations were determined from the *x*-axis intercept at the point where the ratio of the two products was equal (Fig. 1*B*). Measurements were repeated with each sample, and if the SD between the two measurements was <30%, the average results were taken as final. If the deviation was larger, the experiment was repeated again, until the range (SD <30%) was reached or until the correlation coefficient calculated from all the combined data was >0.9.

Specificity of the reactions. The inducible Hsp-72 and the constitutively expressed cognate Hsc-73 show significant homology at both nucleic acid and protein levels (20). Therefore, we determined the specificity of the assay to detect Hsp-72 in different biological samples. The nucleotide sequence of the PCR products made from RNA isolated from a nondiabetic subject and a patient with type 2 diabetes was determined with standard dideoxy-sequencing (ABI equipment; Institute of Biochemistry, BRC, Szeged, Hungary). In both subjects, the sequencing reaction showed the presence of only one PCR template, which had 98.4% sequence identity with the human Hsp-72 cDNA (GenBank accession no. M59830).

Accuracy was checked by determining the changes in Hsp-72 mRNA concentrations after heat shock in cultured NIH-3T3 cells. These cells express low basal levels of Hsp-72 but respond to heat stress robustly (23). After heat shock (43°C for 30 min), total RNA was isolated from the cells at different time intervals and Hsp-72 mRNA level was determined by the competitive RT-PCR assay. At 30 min after the heat shock, a significant elevation (80- to 120-fold) of Hsp-72 mRNA level was seen, which lasted for ~60 min. At 4 h after the heat shock, the Hsp-72 mRNA had approached the baseline. In two independent experiments, highly comparable kinetics were obtained. Very similar kinetic data were reported by Deguchi et al. (24) by measuring the transcription of the hsp-70 gene with the nuclear run-on method.

Reproducibility of the method. Intra- and interassay variation of the competitive RT-PCR method was determined using known concentrations of target sRNA. Intra-assay variation was determined in three independent experiments, using three different concentrations of the target, and was found to be 6.6 ± 3.5, 9.7 ± 7.2, and 13.8 ± 12.1%, respectively. The interassay variation was 34.4% and was determined from five separate experiments, using new reagents each time.

Statistical methods. Nonparametric statistical methods were used: Mann-Whitney *U* test for group comparison and Wilcoxon's signed-rank test for paired data. Correlation analysis was done with the Spearman test; rho (*R*) correlation coefficients and probability values were calculated. $P < 0.05$ was considered statistically significant. All statistical calculations were performed with StatView software (SAS Institute, Cary, NC).

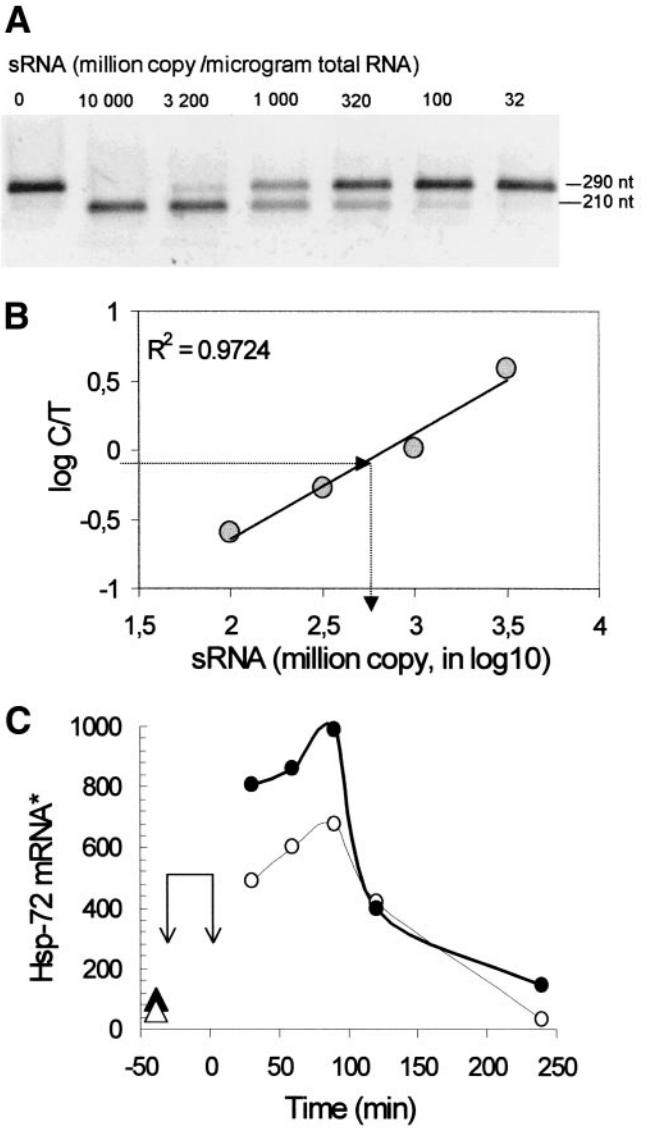

FIG. 1. Quantitation of Hsp-72 mRNA in human muscle biopsies and heat-shocked NIH-3T3 cells, using competitive RT-PCR. *A*: Equal amounts of total RNA isolated from human muscles was added to titrated quantities (as indicated) of sRNA (competitor). RT-PCR of this sRNA yielded a product that was 80 nucleotides (nt) shorter (210 nt) than the amplification output generated from the native human Hsp-72 mRNA (290 nt, target), and that could therefore be distinguishable on the gel. *B*: The ratios of the amplification products from the competitor and target (C/T) were plotted against the number of copies of competitor added, and the plot was used to determine the equivalence point (indicated by arrows) between sRNA and target RNA. *C*: Kinetics of Hsp-72 expression after heat treatment of mammalian cells. NIH-3T3 cells were heat-treated at 43°C for 30 min and then incubated at 37°C. Total RNA was isolated at the indicated time points after heat treatment, and the amount of Hsp-72–specific message was determined with the competitive quantitative method. Results from two independent experiments (open or filled circles) are shown. Arrows indicate the beginning and the end of heat treatment. Basal Hsp-72 levels are shown by the two triangles. *10^6 copy/μg total RNA.

RESULTS

Clinical and metabolic characteristics. In the type 2 diabetic patients in protocol 1, insulin-stimulated glucose uptake rate was ~60% lower than in the nondiabetic control subjects ($P = 0.0163$) (Table 1). In protocol 2, the fasting plasma glucose, insulin, and 2-h glucose concentrations were slightly higher in the nondiabetic co-twins than in the control subjects; seven of them had impaired glucose tolerance (IGT) and were slightly insulin resistant. The diabetic twins had 55% lower rates of insulin-stimulated glucose uptake ($P = 0.0002$), 63% lower rates of

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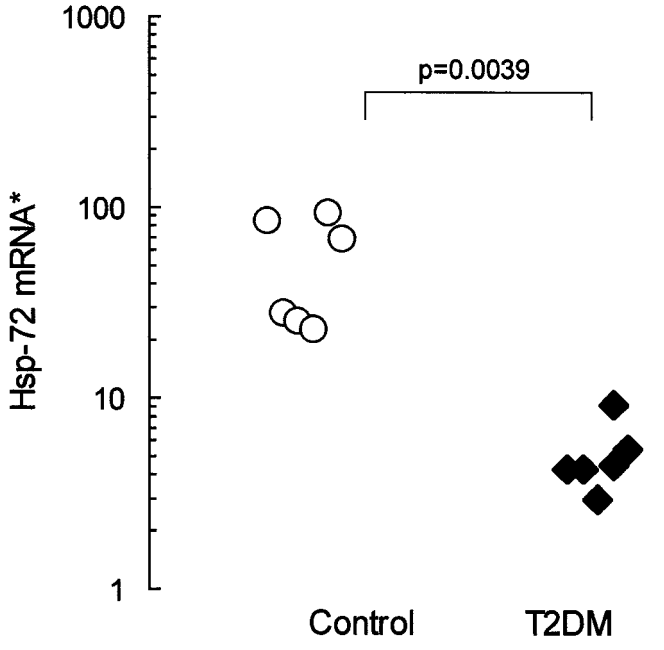

FIG. 2. Hsp-72 mRNA concentration in muscle tissues of control and type 2 diabetic patients. Amount of Hsp-72 messenger RNA was determined in muscle biopsies of six nondiabetic (control) and six type 2 diabetic patients (T2DM) using the competitive RT-PCR as described in RESEARCH DESIGN AND METHODS. Individual data are expressed, using $\log_{10}$ scale, as copies of Hsp-72 mRNA in 1 μg total RNA. The difference between the two groups is statistically significant (Mann-Whitney *U* test, $P = 0.039$). *10^6 copy/μg total RNA.

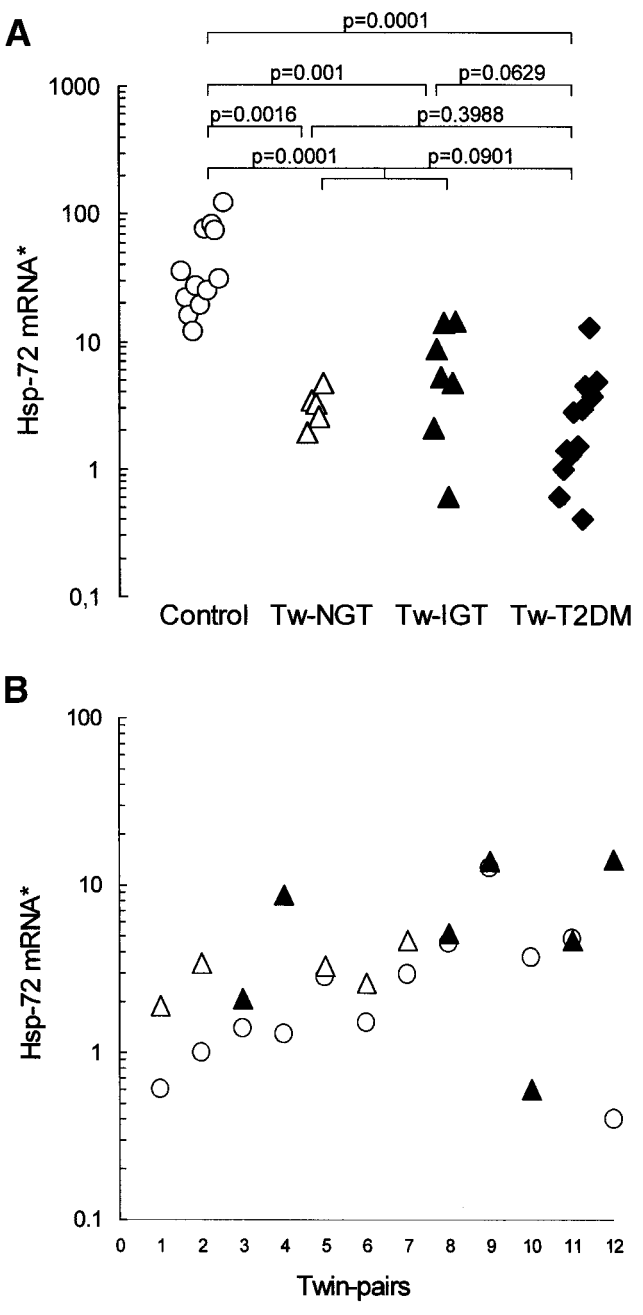

FIG. 3. Hsp-72 mRNA levels in muscle biopsies of control subjects and identical monozygotic twins discordant for type 2 diabetes. The amount of Hsp-72 messenger RNA was determined using the competitive RT-PCR as described in RESEARCH DESIGN AND METHODS. *A*: Individual data are shown, using $\log_{10}$ scale, as copies of Hsp-72 mRNA in 1 μg total RNA. Statistical differences between the different groups were calculated with the nonparametric Mann-Whitney *U* test. *B*: Comparison of the expression of Hsp-72 mRNA between nondiabetic and diabetic individuals in twin-pairs. ○, twins with type 2 diabetes; ▲, twins with IGT; △, twins with NGT. Statistical differences (Wilcoxon-paired test) between nondiabetic and diabetic twins: $P = 0.0431$ for type 2 diabetes vs. NGT; $P = 0.1755$ (NS) for type 2 diabetes vs. IGT; $P = 0.028$for type 2 diabetes vs. NGT+IGT. *10^6 copy/μg total RNA. Tw, twin; T2DM, type 2 diabetes.

glucose storage ($P = 0.0005$), and 37% lower rates of glucose oxidation ($P = 0.0003$) compared with the control subjects. The nondiabetic monozygotic co-twins also had 25% lower rates of insulin-stimulated glucose uptake (8.5 ± 0.8 vs. 11.3 ± 0.9 mg · kg FFM^{-1} · min^{-1}, $P = 0.0404$), which was mainly caused by a 37% decrease in the rate of glucose storage (4.8 ± 0.6 vs. 7.6 ± 0.9 mg · kg FFM^{-1} · min^{-1}, $P = 0.0243$) compared with the control subjects. Lipid oxidation rates during insulin infusion were elevated in the type 2 diabetic twins compared with the other study groups, but they were not statistically different in nondiabetic twins and control subjects (Table 1).

Level of Hsp-72 mRNA in muscle biopsies. In protocol 1, despite the substantial individual deviations within the groups (from 3 to 10 × 10^6 copy/μg total RNA in the diabetic subjects and from 23 to 92 × 10^6 copy/μg total RNA in the control subjects), there was an ~10-fold, statistically significant ($P = 0.0039$) difference between the two groups (Fig. 2). In protocol 2, the amount of Hsp-72 mRNA was also compared between monozygotic twins discordant for type 2 diabetes and healthy control subjects. The control subjects with no family history of diabetes showed the highest level of Hsp-72 mRNA, which was significantly different from the values in the diabetic ($P = 0.0001$) and nondiabetic twins ($P = 0.0001$) (Fig. 3*A*). No significant difference was found in Hsp-72 mRNA between the diabetic and nondiabetic groups of co-twins ($P = 0.0901$) (Fig. 3*A*); however, if the difference between the twin-pairs were analyzed with matched rank test, a statistically significant difference was found between the diabetic and nondiabetic (NGT or IGT) twin-pairs ($P = 0.028$) and also between diabetic twins and their co-twins with NGT ($P = 0.0431$). Twins with IGT did not show statistically significant alteration compared with their diabetic co-twins ($P = 0.1755$) (Fig. 3*B*).

Correlation of Hsp-72 mRNA levels with measures of carbohydrate and lipid metabolism. When the Hsp-72 mRNA concentration was correlated with the rate of glucose uptake, as measured in patients participating in

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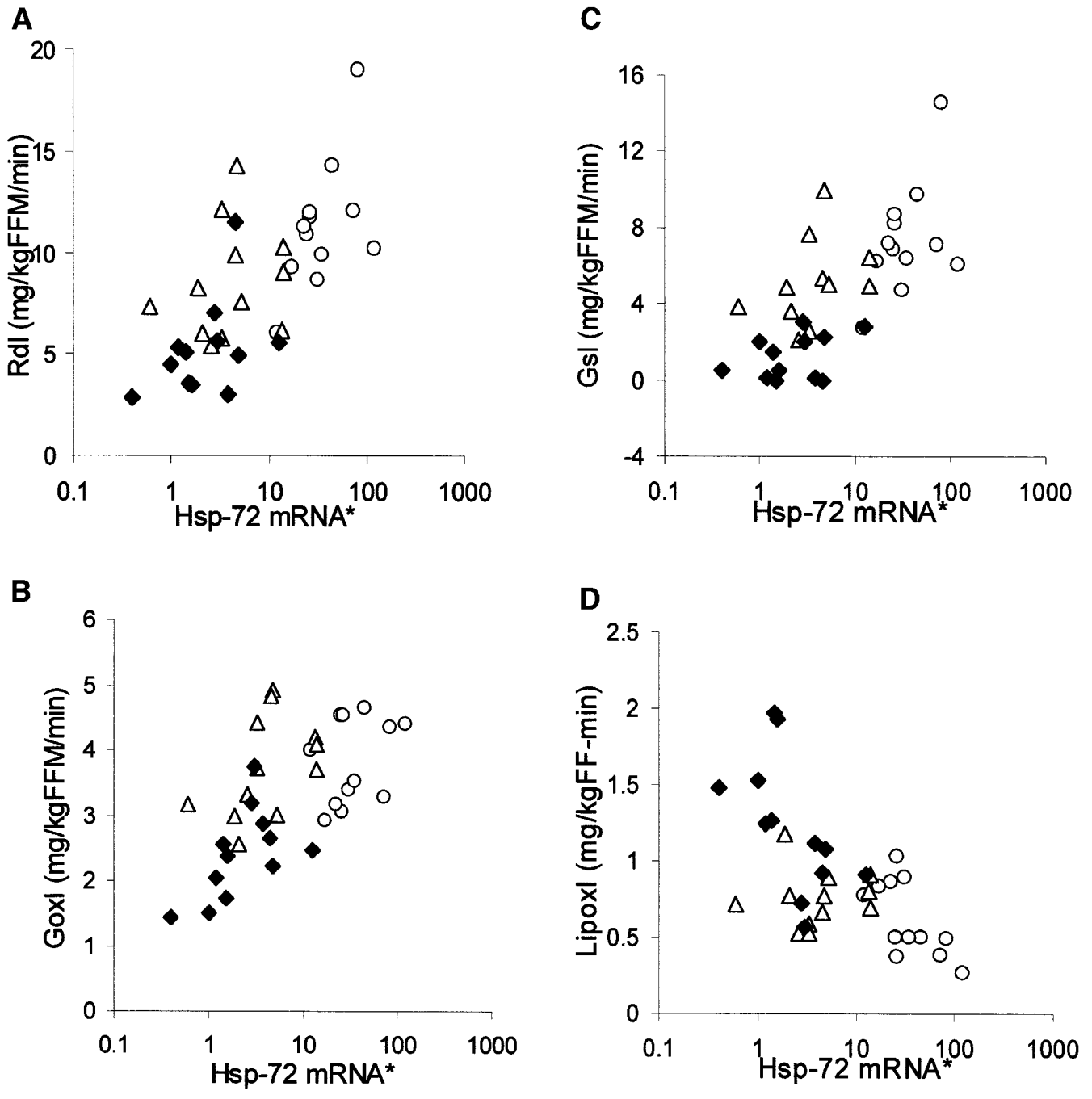

FIG. 4. Correlation between muscle Hsp-72 mRNA expression and rates of glucose and lipid turnover, as determined in protocol 2 (n = 36). All of the values are expressed as milligrams per kilogram FFM per minute. ○, control subjects; △, nondiabetic twins; ♦, twins with type 2 diabetes. *A*: Insulin-stimulated total glucose uptake (RdI): control subjects: R = 0.531, P = 0.078 NS; nondiabetic subjects: R = 0.581, P = 0.0053; twins: R = 0.519, P = 0.0128; twins with type 2 diabetes: R = 0.441, NS. *B*: Insulin-stimulated glucose oxidation (GoxI): controls: R = 0.364, NS; nondiabetic subjects: R = 0.253, NS; twins: R = 0.564, P = 0.0068; twins with type 2 diabetes: R = 0.594, P = 0.0487. *C*: Net glycogen synthesis during insulin infusion (GsI): controls: R = 0.322, NS; nondiabetic subjects: R = 0.322, NS; twins: R = 0.514, P = 0.0137; twins with type 2 diabetes: R = 0.487, NS. *D*: lipid oxidation during insulin infusion (LipoxI): control subjects: R = −0.634, P = 0.0355; nondiabetic subjects: R = −0.425, P = 0.0416; twins: R = −0.389, P = 0.0623 (NS); twins with type 2 diabetes: R = 0.643, P = 0.0329 for. *10^6 copy/μg total RNA.

protocol 1, a tendency (r = 0.525) of higher heat shock protein mRNA level coinciding with stronger capacity to take up glucose could be observed, but this tendency was not statistically significant (P = 0.0814, data not shown). Correlation between Hsp-72 mRNA levels and different parameters of glucose and lipid metabolism were examined within different subgroups in protocol 2 (summarized in Fig. 4 and Table 2). In the subgroup of nondiabetic subjects (pooled control subjects and nondiabetic cotwins), strong positive correlations with the insulin-stimulated rate of glucose uptake (r = 0.581, P = 0.0053) (Fig. 4*A*) and glucose storage (r = 0.559, P = 0.0073) (Fig. 4*C*), and inverse correlations with the rate of lipid oxidation (r = −0.425; P = 0.0416; Fig. 4*d*) and the 2-h glucose level (r = −0.716, P = 0.0006) (Table 2), were found. In the subgroup of control subjects without family background

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TABLE 2
Correlation between skeletal muscle Hsp-72 mRNA and plasma fasting glucose or insulin levels, and 2-h glucose levels during oral glucose tolerance testing in different subgroups of patients from protocol 2

Parameters	Control subjects without family background of diabetes	Nondiabetic subjects	Twins (all)	Diabetic subjects
n	12	24	24	12
Fasting glucose	−0.106 (NS)	−0.397 (NS)	−0.391 (NS)	−0.455 (NS)
Fasting insulin	−0.091 (NS)	−0.197 (NS)	−0.129 (NS)	−0.210 (NS)
2-h glucose	−0.744 (0.0236)*	−0.716 (0.0006)*	−0.327 (NS)	−0.263 (NS)

Data are *R* (*P*). *Statistically significant correlations.

of diabetes ($n = 12$), the Hsp-72 mRNA levels showed negative correlations with the 2-h glucose levels ($r = -0.744$, $P = 0.0236$) (Table 2) and with the rate of lipid oxidation ($r = -0.634$, $P = 0.0142$) (Fig. 4*D*). The pooled data of twins showed that Hsp-72 mRNA level in this subgroup correlates positively with glucose uptake ($r = 0.519$, $P = 0.0128$) (Fig. 4*A*), glucose oxidation ($r = 0.564$, $P = 0.0068$) (Fig. 4*B*), and glucose storage ($r = 0.514$, $P = 0.0137$) (Fig. 4*C*). In the subgroup analysis of diabetic twins, there was positive correlation ($r = 0.594$, $P = 0.0487$) (Fig. 4*B*) between the amount of Hsp-72 mRNA and the rate of insulin-stimulated glucose oxidation, and there was negative correlation between Hsp-72 mRNA levels and oxidation of lipids ($r = -0.643$, $P = 0.0329$) (Fig. 4*D*). No statistically significant correlations were found between the stress protein transcript levels and either fasting plasma glucose or insulin concentrations in any of the subgroups (Table 2).

DISCUSSION

We provide the first evidence that there is decreased expression of the Hsp-72 mRNA in skeletal muscle from patients with type 2 diabetes, and that the level of Hsp-72 mRNA correlates with the rate of insulin-stimulated glucose and lipid turnover and glucose tolerance. In both of our protocols, we found an order of magnitude of difference between Hsp-72 mRNA levels measured in type 2 diabetic patients compared with control subjects without a family history of diabetes. Because we determined the amount of specific mRNA from the muscle biopsies, we have no proof as to whether the decreased Hsp-72 mRNA levels are also reflected by a decreased amount of protein. Measurement of expression at the level of transcript does not provide information about post-transcriptional control of gene expression, and, indeed, recent experimental evidence suggests that in general there is no obvious correlation between mRNA and protein expression levels (25,26). However, in the experiments cited above, the translation efficiency of different transcripts were compared where control mechanisms determining nuclear export, transcript stability, translational regulation, and protein degradation were acting on different templates, obviously with different outcomes. Comparing the expression of the same gene under different metabolic circumstances at both the transcriptional and the translational levels might result in a different conclusion. Saghizadeh et al. (27) studied the expression of tumor necrosis factor-α message in muscle biopsies of normal and diabetic subjects and found that the cytokine mRNA is upregulated in the diabetic tissue. The same upregulation of mRNA was found in cultured muscle biopsies of diabetic patients, with a concomitant increase in the production of the cytokine as well (27). The magnitude of the difference between Hsp-72 mRNA levels of the healthy and diabetic groups (10-fold) in our experiments, and the fact that stress protein expression in general is strongly regulated at the level of transcription (28,29), support the idea that in this case as well the differences in the levels of transcript were translated into differences in the amounts of protein.

Hsp-72 mRNA levels in skeletal muscle of the patients correlated with several parameters of carbohydrate and lipid metabolism. Already in protocol 1, with a limited amount of data, the rate of insulin-stimulated glucose uptake showed a tendency to increase with elevated heat shock protein mRNA expression. Analysis of the data of different subgroups of patients from protocol 2 showed that depending on the subgroup and on the number of patients in the subgroups, Hsp-72 mRNA level correlated strongly with different insulin-stimulated metabolic changes, such as the increase in the uptake, oxidation, and storage of glucose and the decrease in the oxidation of lipids. In addition, in the subgroup of pooled nondiabetic subjects and control subjects without family background of diabetes, the amount of the stress protein transcript showed strong correlation with the 2-h glucose levels during the oral glucose tolerance test. Although we cannot conclude from the data that the decreased Hsp-72 mRNA levels are the cause or consequence of the deranged metabolic state in type 2 diabetes, there is some support for a causal relationship. Because Hsp-72 mRNA expression did not show any correlation with either fasting plasma glucose or insulin level in any of the subgroups, it confirms our hypothesis that the altered stress protein mRNA level is not simply the consequence of the chronically elevated plasma glucose or insulin. The possible regulatory effect of glucose on the expression of the heat shock protein mRNA can also be ruled out by the fact that during our protocols, diabetic patients, at 1 h before the time of biopsy, were made normoglycemic. We cannot exclude the possibility that the acute administration of insulin (9.4 ± 2.1 IU added intravenously during a period of 52 ± 8 min) used for adjusting euglycemia in diabetic subjects affected Hsp-72 mRNA levels. However, this bolus was not given to nondiabetic co-twins, who possessed similarly low levels of the heat shock protein mRNA, and acute insulin treatment has been shown to increase the expression of the inducible 70-kDa-mass heat shock proteins in hepatocytes in vitro (30). This insulin-stimulated increase is opposed by our finding. Equally important is the fact that there is much less correlation between

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muscle Hsp-72 mRNA content and the basal rates of glucose and lipid metabolisms. Using the same subgroups, Hsp-72 mRNA showed correlation with basal-rate glucose oxidation or storage only in the group of twins or in the diabetic group, respectively (data not shown). These data strongly support the possible association between the stress protein and the action of insulin.

The possible nature of this potential association is speculative at this point. Stress proteins represent a highly conserved family of polypeptides with significant involvement in the creation and functional preservation of the protein machinery in living cells (31). Evidence has accumulated showing that stress proteins are indispensable for the cells not only under stressful conditions but also under physiological circumstances (32,33). The inability of an essential protein to form its native structure under physiological conditions may be the basis for human diseases (34).

The insulin receptor requires the contribution of different chaperones to its folding, assembly, and transport (35). It has been shown in fetal hepatocytes subjected to heat stress that HSP-70s associate with the insulin receptor to prevent recycling and damage (36). Given the conservation of the signaling cascade starting from the insulin receptor and the complexity of the downstream events involved in the transduction of the signal, it can easily be imagined that signal accuracy is maintained by scaffolding proteins, which preserve specificity of the reaction and assist in stabilizing the otherwise weak interaction between components. The scaffolding and catalytic roles of heat shock proteins is known for the glucocorticoid receptor (rev. in 37), and recently even catalytic activity has been shown to depend on the interaction between the enzyme and heat shock proteins (38).

GLUT4 is responsible for most of the insulin-stimulated increase in glucose transport (39,40), and this transporter is another possible candidate for heat shock protein interference. The GLUT4 transporting vesicles contain and interact with several different proteins, some of which are likely to require constant chaperone inspection. Both GLUT4 (41) and Hsp-72 (42) are known to interact with F-actin, which plays an indispensable role in the relocalization of intracellular compartments. Although no heat shock protein associated with GLUT4 vesicles have been identified so far, several putative glucose transporter binding proteins have been recognized, and their molecular weights (28, 70, and 85 kDa) are very close to those of the most abundant stress proteins (43). Interestingly, physical exercise, known to increase insulin sensitivity in diabetic patients (44) by inducing translocation of the GLUT4 transporter to the plasma membrane (45), also elevates heat shock protein expression in skeletal muscle during workout (rev. in 46). Also relevant to the issue is the association that has been shown between the Hsp-72 protein and the peroxisome proliferator–activated receptors (PPARs) in that they form a complex in vivo in the rat. The possibility thus remains that Hsp-72 may play a role in the folding, subcellular localization, and/or signaling pathway of PPARs (47) as well.

We attempted to examine the origin of the defect in heat shock protein mRNA expression by studying monozygotic twins discordant for type 2 diabetes. This is a very special population, in which individuals possess some kind of type 2 diabetes genetic constitutions without having overt diabetes. This population is superior to first-degree relatives because relatives on average may only have a 50% chance of carrying the true type 2 diabetes genetic constitution, whereas identical twins are 100% predisposed to it. Defects in insulin action precede the overt disease, and in our study the nondiabetic co-twins were not entirely discordant for glucose tolerance and insulin sensitivity, i.e., the nondiabetic co-twins were significantly more insulin resistant and had higher glucose levels than the control subjects. Despite this, however, compared with control subjects, nondiabetic twins showed severely decreased expression of Hsp-72 mRNA, which was similar to that of frank diabetic twins, suggesting that the decrease in Hsp-72 mRNA in the nondiabetic co-twins was not the consequence of insulin resistance; rather, it might represent an inherited trait. On the other hand, when Hsp-72 mRNA content of twin-pairs with the same genetic background was compared, nondiabetic twins had higher message level than their diabetic pairs. Interestingly, when twins with IGT were compared with their co-twins, no notable deviations were found, whereas Hsp-72 mRNA levels in twins with NGT differed statistically significantly from those of their diabetic pairs. The results from the monozygotic twins discordant for type 2 diabetes are more consistent with a genetic as compared with a nongenetic origin of this defect; however, they are in harmony with the dogma that most forms of type 2 diabetes are polygenic, with superimposed environmental influences.

In conclusion, the finding of decreased levels of Hsp-72 mRNA in skeletal muscle of patients with type 2 diabetes and its relationship with insulin resistance raises the question of whether heat shock proteins are involved in the pathogenesis of skeletal muscle insulin resistance in type 2 diabetes. During the preparation of the revised version of this manuscript, preliminary data using microarrays to compare the expression profile between insulin-resistant and insulin-sensitive subjects reported decreased Hsp-70 expression in vastus lateralis muscle from the insulin-resistant individuals (48).

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REFERENCES

1. Beck-Nielsen H, Groop L: Metabolic and genetic characterization of prediabetic states: sequence of events leading to non-insulin-dependent diabetes mellitus. *J Clin Invest* 94:1714–1721, 1994
2. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: a new perspective on an old paradigm (Review). *Diabetes* 48:1–9, 1999
3. Parthiban A, Vijayalingam S, Shanmugasundaram KR, Mohan R: Oxidative stress and the development of diabetic complications—antioxidants and lipid peroxidation in erythrocytes and cell membrane. *Cell Biol Int* 19:987–993, 1995
4. Muchova J, Liptakova A, Orszaghova Z, Garaiova I Tison P, Carsky J, Durackova Z: Antioxidant systems in polymophonuclear leucocytes of type 2 diabetes mellitus. *Dibet Med* 16:74–78, 1999
5. Paolisso G, D'Amore A, Volpe C, Balbi V, Saccomanno F, Galzerano D, Gugliano D, Varricchio M, D'Onofrio F: Evidence for a relationship

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between oxidative stress and insulin action in non-insulin-dependent (type II) diabetic patients. *Metabolism* 43:1426–1429, 1994
6. Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N: Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes* 47:1562–1569, 1998
7. Tirosh A, Potashnik R, Bashan N, Rudich A: Oxidative stress disrupts insulin-induced cellular redistribution of insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. A putative cellular mechanism for impaired protein kinase B activation and GLUT4 translocation. *J Biol Chem* 274:10595–602, 1999
8. Rudich A, Tirosh A, Potashnik R, Khamaisi M, Bashan N: Lipoic acid protects against oxidative stress induced impairment in insulin stimulation of protein kinase B and glucose transport in 3T3-L1 adipocytes. *Diabetologia* 42:949–957, 1999
9. Marber MS, Mestril R, Chi S-H, Sayen MR, Yellon DM, Dillmann WH: Overexpression of the rat inducible 70 kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest* 95:1446–1456, 1995
10. Park Y-M, Han M-Y, Blackburn RV, Lee YJ: Overexpression of Hsp25 reduces the level of TNFa-induced oxidative DNA damage biomarker, 8-hydroxy-2′-deoxyguanosine, in L929 cells. *J Cell Physiol* 174:27–34, 1998
11. Bellmann K, Hui L, Radons J, Burkart V, Kolb H: Low stress response enhances vulnerability of islet cells in diabetes-prone BB rats. *Diabetes* 46:232–236, 1997
12. Matz JM, Lavoi KP, Epstein PN Blake MJ: Thermoregulatory and heat-shock protein response deficits in cold-exposed diabetic mice. *Am J Physiol* 270:R525–R532, 1996
13. Hartl FU: Molecular chaperones in cellular protein folding. *Nature* 381: 571–579, 1996
14. Benjamin IJ, McMillan DR: Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res* 83:117–132, 1998
15. Gething MJ, Sambrook J: Protein folding in the cell. *Nature* 355:33–45, 1992
16. Vaag A, Alford F, Beck Nielsen H: Intracellular glucose and fat metabolism in identical twins discordant for non-insulin-dependent diabetes mellitus (NIDDM): acquired versus genetic metabolic defects? *Diabet Med* 13:806–815, 1996
17. Vaag A, Henriksen JE, Madsbad S, Holm N, Beck-Nielsen H: Insulin secretion, insulin action, and hepatic glucose production in identical twins discordant for non-insulin-dependent diabetes mellitus. *J Clin Invest* 95:690–698, 1995
18. Lukaski HC, Johnson PE, Bolonchuck WW, Lykken GI: Assessment of fat-free mass using bioelectrical impedance measurements of the human body. *Am J Clin Nutr* 41:810–817, 1985
19. Chomczynski P, Sacchi C: Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987
20. Milner CM, Campbell RD: Structure and expression of the three MHC-linked Hsp70 genes. *Immunogenetics* 32:242–251, 1990
21. Gunter E, Walter L: Genetic aspects of the HSP-70 multigene family in vertebrates. *Experientia* 50:987–1001, 1994
22. Riedy MC, Timm EA, Stewart CC: Quantitative RT-PCR for measuring gene expression. *Biotechniques* 18:70–76, 1995
23. Kurucz I, Tombor B, Prechl J, Erdo F, Hegedus E, Nagy Z, Vitai M, Koranyi L, Laszlo L: Ultrastructural localization of Hsp-72 examined with a new polyclonal antibody raised against the truncated variable domain of the heat shock protein. *Cell Stress Chaperones* 4:139–152, 1999
24. Deguchi Y, Negoro S, Kishimoto S: Age-related changes of heat shock protein genetranscription in human peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 157:580–584, 1988
25. Anderson L, Seilhamer J: A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18:533–537, 1977
26. Gygy SP, Rochon Y, Franza BR, Aebersold R: Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19:1720–1730, 1999
27. Saghizadeh M, Ong JM, Garvey WT, Henry RR, Kern PA: The expression of TNF alpha by human muscle: relationship to insulin resistance. *J Clin Invest* 97:1111–1116, 1996
28. Morimoto RI, Kline MP, Bimston DN, Cotto JJ: The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays Biochem* 32:17–29, 1997
29. Wu C: Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol* 11:441–469, 1995
30. Ting L-P, Tu C-L, Chou C-K: Insulin-induced expression of human heat-shock protein gene hsp-70. *J Biol Chem* 264:3404–3408, 1989
31. Agashe VR, Hartl FU: Roles of molecular chaperones in cytoplasmic protein folding. *Semin Cell Dev Biol* 11:15–25, 2000
32. Fink AL: Chaperone-mediated protein folding. *Physiol Rev* 79:425–449, 1999
33. Beissinger M, Buchner J: How chaperones fold proteins. *Biol Chem* 379:245–259, 1998
34. Thomas PJ, Qu B-H, Pedersen PL: Defective protein folding as a basis of human disease. *TIBS* 20:456–459, 1995
35. Bass J, Chiu G, Argon Y, Steiner DF: Folding of insulin receptor monomers is facilitated by the molecular chaperones calnexin and calreticulin and impaired by rapid dimerization. *J Cell Biol* 141:637–646, 1998
36. Zachayus JL, Benatmane S, Plas C: Role of Hsp70 synthesis in the fate of the insulin-receptor complex after heat shock in cultured fetal hepatocytes. *J Cell Biochem* 61:216–229, 1996
37. Ylikomi T, Wurtz JM, Syvala H, Passinen S, Pekki A, Haverinen M, Blauer M, Tuohimaa P, Gronemeyer H: Reappraisal of the role of heat shock proteins as regulators of steroid receptor activity. *Crit Rev Biochem Mol Biol* 33:437–466, 1998
38. Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC: Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 392:821–824, 1998
39. Rea S, James DE: Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. *Diabetes* 46:1667–1677, 1997
40. Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ, Okada S: Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! *J Biol Chem* 274:2593–2596, 1999
41. Wang Q, Bilan PJ, Tsakiridis T, Hinek A, Klip A: Actin filaments participate in the relocalization of phosphatidyliositpl3-kinase to glucose transporter-containing compartments and in the stimulation of glucose uptake in 3T3–L1 adipocytes. *Biochem J* 331:917–928, 1998
42. Tardieux I, Baines I, Mossakowska M, Ward GE: Actin-binding proteins of invasive malaria parasites and the regulation of actin polymerization by a complex of 32/34-kDa proteins associated with heat shock protein 70kDa. *Mol Biochem Parasitol* 93:295–308, 1998
43. Jung CY: Proteins that interact with facilitative glucose transporters: implication for function. *Exp Physiol* 83:267–273, 1998
44. Albright A, Franz M, Hornsby G, Kriska A, Marrero D, Ullrich I, Verity LS: American College of Sports Medicine position stand: exercise and type 2 diabetes. *Med Sci Sports Exerc* 32:1345–1360, 2000
45. Wojtaszewski JF, Hansen BF, Gade, Kiens B, Markuns JF, Goodyear LJ, Richter EA: Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49:325–331, 2000
46. Fehrenbach E, Niess AM: Role of heat shock proteins in the exercise response. *Exerc Immunol Rev* 5:57–77, 1999
47. Huang Q, Alvares K, Chu R, Bradfield CA, Reddy JK: Association of peroxisome proliferator-activated receptor and Hsp72. *J Biol Chem* 269: 8493–8497, 1994
48. Patti ME, Butte A, Cusi K, Kohane I, Landaker EJ, Defronzo R, Mandarino LJ, Kahn CR: Analysis of differential gene expression in skeletal muscle from subjects with insulin resistance and type 2 diabetes (Abstract). *Diabetes* 50 (Suppl. 2):A247, 2001

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