

Intramuscular Heat Shock Protein 72 and Heme Oxygenase-1 mRNA Are Reduced in Patients With Type 2 Diabetes

Evidence That Insulin Resistance Is Associated With a Disturbed Antioxidant Defense Mechanism

Clinton R. Bruce,¹ Andrew L. Carey,² John A. Hawley,¹ and Mark A. Febbraio²

To examine whether genes associated with cellular defense against oxidative stress are associated with insulin sensitivity, patients with type 2 diabetes ($n = 7$) and age-matched ($n = 5$) and young ($n = 9$) control subjects underwent a euglycemic-hyperinsulinemic clamp for 120 min. Muscle samples were obtained before and after the clamp and analyzed for heat shock protein (HSP)72 and heme oxygenase (HO)-1 mRNA, intramuscular triglyceride content, and the maximal activities of β -hydroxyacyl-CoA dehydrogenase (β -HAD) and citrate synthase (CS). Basal expression of both HSP72 and HO-1 mRNA were lower ($P < 0.05$) by 33 and 55%, respectively, when comparing diabetic patients with age-matched and young control subjects, with no differences between the latter groups. Both basal HSP72 ($r = 0.75$, $P < 0.001$) and HO-1 ($r = 0.50$, $P < 0.05$) mRNA expression correlated with the glucose infusion rate during the clamp. Significant correlations were also observed between HSP72 mRNA and both β -HAD ($r = 0.61$, $P < 0.01$) and CS ($r = 0.65$, $P < 0.01$). HSP72 mRNA was induced ($P < 0.05$) by the clamp in all groups. Although HO-1 mRNA was unaffected by the clamp in both the young and age-matched control subjects, it was increased ($P < 0.05$) \sim 70-fold in the diabetic patients after the clamp. These data demonstrate that genes involved in providing cellular protection against oxidative stress are defective in patients with type 2 diabetes and correlate with insulin-stimulated glucose disposal and markers of muscle oxidative capacity. The data provide new evidence that the pathogenesis of type 2 diabetes involves perturbations to the

antioxidant defense mechanism within skeletal muscle. *Diabetes* 52:2338–2345, 2003

The pathogenesis of type 2 diabetes has not been fully elucidated; however, there is growing evidence linking this disease with oxidative stress (1–3). Oxidative stress, resulting from increased production or decreased removal of reactive oxygen species (ROS), plays a key role in the pathogenesis of late diabetes complications (2) and insulin-stimulated glucose uptake (4). To combat protein-related homeostatic disruption, such as oxidative stress, cells respond by synthesizing a family of highly conserved proteins termed heat shock proteins (HSPs). In skeletal muscle, the most widely studied family is the 70-kDa family, which contains the constitutive HSP73 and inducible HSP72 forms. HSP72 has been found to protect skeletal muscle against contraction-induced ROS formation (5). However, the 30-kDa family, also known as the heme oxygenases (HOs; HO-1 being the inducible form and HO-2 the constitutive form) is also expressed in skeletal muscle and plays an important role in the cellular defense against oxidative stress and the negative effects of proinflammatory cytokines (6). Recently, Kurucz et al. (7) observed a decreased expression of HSP72 mRNA in patients with type 2 diabetes, with this reduction being correlated with some markers of insulin resistance. This study (7) provided preliminary evidence that HSP72 is related to insulin resistance, but their results with respect to insulin sensitivity during a hyperinsulinemic clamp were not conclusive. Nonetheless, the authors hypothesized mechanisms by which stress proteins may be implicated in insulin resistance, and they suggested that further research in this area is warranted. Furthermore, in a preliminary study that compared $>5,000$ genes in skeletal muscle samples from patients with insulin resistance and healthy subjects, HSP72 was 1 of only 17 genes that were markedly lower in the patient population (8).

Little is known regarding the functional significance of HO-1 in skeletal muscle, although HO-1 provides protection against proinflammatory cytokines, which are known to be elevated in the skeletal muscle of patients with type 2 diabetes (9).

From the ¹Exercise Metabolism Group, School of Medical Sciences, RMIT University, Bundoora, Victoria, Australia; and ²Skeletal Muscle Research Laboratory, School of Medical Sciences, RMIT University, Bundoora, Victoria, Australia.

Address correspondence and reprint requests to Mark A. Febbraio, PhD, Associate Professor Research, Skeletal Muscle Research Laboratory, School of Medical Sciences, RMIT University, P.O. Box 71, Bundoora 3083, Victoria, Australia. E-mail: mark.febrario@rmit.edu.au.

Received for publication 24 March 2003 and accepted in revised form 6 June 2003.

CS, citrate synthase; C_{75} , critical threshold; DEXA, dual-energy X-ray absorptiometry; FFA, free fatty acid; FFM, fat-free mass; GDR, glucose disposal rate; β -HAD, β -hydroxyacyl-CoA dehydrogenase; HO, heme oxygenase; HSP, heat shock protein; IMTG, intramuscular triglyceride; JNK, c-Jun NH₂-terminal kinase; MPO, myeloperoxidase; NOS, nitric oxide synthase; PPAR α , peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TNF, tumor necrosis factor.

© 2003 by the American Diabetes Association.

TABLE 1

Subject characteristics and measures during whole-body insulin-stimulated glucose uptake in young healthy subjects, age matched healthy subjects, and patients with type 2 diabetes

	Young healthy subjects	Age-matched healthy subjects	Diabetic subject
Age (years)	23 ± 1	50 ± 2*	42 ± 3*
Body mass (kg)	79.2 ± 2.4	84.1 ± 3.3	93.6 ± 10.2
BMI	24.0 ± 1.0	27.4 ± 1.2	30.1 ± 2.2*
Percent body fat	12.3 ± 3	25.5 ± 4.8*	31.0 ± 2.9*
HbA _{1c} (%)	4.5 ± 0.1†	5.0 ± 4.8†	8.1 ± 0.7
Fasting blood glucose (mmol/l)	4.2 ± 0.1†	4.4 ± 0.1†	8.6 ± 1.8
Fasting plasma insulin (pmol/l)	40.5 ± 4.7†	60.5 ± 5.6	109.7 ± 24.5
Fasting plasma FFA (mmol/l)	0.7 ± 0.1†	0.8 ± 0.1†	1.2 ± 0.1
Clamp			
GDR (mg · kg FFM ⁻¹ · min ⁻¹)	16.6 ± 1.6†	11.0 ± 1.5*†	4.5 ± 1.9*
Blood glucose (mmol/l)	4.9 ± 0.1	5.1 ± 0.1	5.0 ± 0.1
Plasma insulin (pmol/l)	429 ± 35	545 ± 36	522 ± 35

Data are means ± SE. *Different compared with young healthy subjects ($P < 0.05$); †different compared with diabetic subjects ($P < 0.05$).

It is also not known whether insulin stimulates the expression of these genes and/or the potential mechanisms by which genes associated with cellular defense against oxidative stress may be affecting insulin sensitivity. It has been hypothesized that both GLUT4 and the peroxisome proliferator-activated receptors (PPARs) interact with HSP72, and that this interaction may be responsible, in part, for the role of HSP72 expression in insulin resistance (7). The aim of the present study was, therefore, to investigate the role of selected genes associated with cellular defense against oxidative stress in the etiology of insulin resistance in human skeletal muscle. We performed a euglycemic-hyperinsulinemic clamp in patients with type 2 diabetes and control subjects, and we related this measure to HSP72 and HO-1 mRNA in skeletal muscle samples obtained before and after the clamp. In addition, we measured the mRNA content of GLUT4, PPAR- α , and PPAR- γ , as well as the maximal activity levels of the oxidative enzymes β -hydroxyacyl-CoA dehydrogenase (β -HAD) and citrate synthase (CS). We hypothesized that both HSP72 and HO-1 mRNA would be lower in patients with type 2 diabetes compared with healthy control subjects, and that their expression would correlate with an impaired glucose infusion rate during the clamp. We further hypothesized that the expression of these genes would be related to intramuscular oxidative capacity, but that GLUT4, PPAR- α , and PPAR- γ gene expression would be unimpaired in the skeletal muscle of patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. A total of 21 volunteers (7 patients with type 2 diabetes, 5 healthy but sedentary age-matched control subjects, and 9 young control subjects) participated in this investigation, which was approved by the human ethics committee of RMIT University. The physical characteristics of all study participants are presented in Table 1. Normal glucose tolerance was confirmed in young and age-matched control subjects by an oral glucose tolerance test (75 g glucose). The subjects in the diabetic group had a mean time of 4 ± 1 years since diagnosis of disease (range 6 months to 10 years) and were treated with diet ($n = 2$) or oral hypoglycemic agents ($n = 1$ sulfonylureas, $n = 1$ metformin, $n = 3$ a combination of sulfonylureas and metformin). Other than type 2 diabetes, these subjects had no other medical problems. All subjects were nonsmokers, and, apart from subjects in the diabetic group, none were taking medications known to alter carbohydrate metabolism. All participants had maintained a constant body mass during the 6 months preceding the experiment, and each was instructed to abstain from any form

of vigorous physical activity for 36 h before the hyperinsulinemic-euglycemic clamp experiment and to maintain their habitual diet. The subjects reported to the laboratory for this experiment after a 12- to 14-h overnight fast, and in the case of the type 2 diabetic patients, before the administration of any antidiabetic medication.

Experimental procedures. On their first visit to the laboratory, whole-body dual-energy X-ray absorptiometry (DEXA) (Lunar DPX; Lunar Radiation, Madison WI) was used to measure whole-body fat mass and fat-free mass (FFM). Before each scan, the DEXA was calibrated with known phantoms. On a subsequent visit to the laboratory, the euglycemic-hyperinsulinemic clamp was performed. An antecubital vein was cannulated for infusion of glucose and insulin, and a hand vein was cannulated retrogradely and was heated for sampling of arterialized blood. After collection of baseline blood samples, a percutaneous biopsy of the vastus lateralis muscle was obtained. After resting for 5 min, a primed (9 mU/kg)-continuous infusion of insulin (Actrapid; Novo Nordisk, Baulkham Hills, New South Wales, Australia) was commenced at a rate of 40 mU · m⁻² · min⁻¹, and blood glucose concentration was measured at 5-min intervals throughout the clamp. A variable-rate infusion of 20% glucose was used to maintain euglycemia (5 mmol/l) for the duration of the clamp (120 min). The blood glucose concentration in the diabetic group was allowed to decrease during the insulin infusion to 5 mmol/l and then maintained at this concentration for the remainder of the clamp. Samples (2 ml) for subsequent determination of plasma insulin concentration were obtained every 10 min, centrifuged, and stored at -80°C. Whole-body glucose uptake was calculated from the glucose infusion rate (mg · kg FFM⁻¹ · min⁻¹) required to maintain a blood glucose concentration of 5 mmol/l. A second muscle biopsy was obtained 120 min after the initiation of the insulin infusion. To prevent a decrease in plasma potassium concentration during the clamp, 30 mmol KCl (Slow-K; Novartis, Castle Hill, New South Wales, Australia) was administered orally.

Blood biochemistry. Blood glucose concentration was measured using an automated analyzer (2300 Stat Plus Glucose and L-Lactate analyzer; Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin concentration was determined by radioimmunoassay (Phadeseph, Insulin RIA; Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), HbA_{1c} was determined by specific ion-exchange chromatography (Sigma Diagnostics, Castle Hill, New South Wales, Australia), and plasma free fatty acid (FFA) concentration was measured using an enzymatic colorimetric method (NEFA C test kit; Wako, Richmond, VA). To provide an indication of ROS formation, plasma collected from diabetic and age-matched control subjects were analyzed for the enzyme myeloperoxidase (MPO), which catalyzes the formation of hypochlorous acid, a powerful oxidant derived from chloride ions and hydrogen peroxide (10). This analysis was performed using an enzyme-linked immunoassay (Calbiochem, San Diego, CA). Because of technical difficulties, we were unable to perform this analysis on plasma from young control subjects.

Measurement of gene expression. A portion of muscle (~30 mg) was extracted for total RNA using a modification of an acid guanidium thiocyanate-phenol chloroform extraction method described elsewhere (11). To visualize the integrity of the total RNA, 0.5 μ g was fractionated on a 1% denaturing agarose gel. The total RNA was subsequently quantified two to three more times before 1 ng of each total RNA sample was reverse-transcribed in a 10- μ l reaction containing 1 × TaqMan reverse transcriptase buffer, 5.5 mmol/l MgCl₂, 500 mmol/l each 2'-deoxynucleoside and 5'-triphos-

phate, 2.5 mmol/l random hexamers, 0.4 units/ml RNase inhibitor, 1.25 units/ml Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA) and made up to volume with H₂O (0.05% diethylpyrocarbonate treated). Control samples were also analyzed, where all of the above reagents are added to RNA samples except the Multiscribe reverse transcriptase. The reverse transcription reactions were performed using a GeneAmp PCR system 2400 (PerkinElmer, Wellesley, MA) with conditions at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Then, 2 ml 0.5 mol/l EDTA (pH 8.0) was added to each sample, and they were stored at -20°C until further analysis. Real-time PCR was used to quantitate human HSP72, HO-1, PPAR-γ, PPAR-α, and GLUT4 expression from the cDNA samples. Human probe and primers were designed (Primer Express version 1.0; Applied Biosystems) from the human gene sequence accessed from Gen-Bank/EMBL. To limit the possibility of nonspecific amplification of each gene, we designed the nucleic acid sequences to span an exon-exon junction when possible in order to limit the possibility of genomic DNA contamination. The probe and primer sequences are presented in Table 2.

Each TaqMan probe was labeled with the fluorescent tags FAM (6-carboxyfluorescein) at the 5' end and TAMRA (6-carboxy-tetramethylrhodamine) at the 3' end. We also amplified ribosomal 18S mRNA as our reference gene, since this gene is known to be constitutively expressed. The TaqMan probes and primers for this gene were supplied in a control reagent kit (Applied Biosystems). We quantitated gene expression using a multiplex comparative critical threshold (C_T) method (iCycler iQ; Bio-Rad, Hercules, CA). A C_T value reflects the cycle number at which the DNA amplification is first detected. This method detected our reference gene (human 18S) and gene of interest in a single tube, where the primers for 18S were limited to ensure that adequate amounts of reagents were present for the amplification of both genes. It was possible to detect 18S in the same tube as our genes of interest because the reporter dyes attached to the TaqMan probes fluoresce at different emission wavelength maxima. In preliminary experiments, we determined the relative efficiency of amplification of 18S versus the genes of interest. These experiments revealed approximate equal efficiencies of 18S and genes of interest amplifications over different starting template concentrations. We also performed experiments to demonstrate that multiplex versus nonmultiplex experiments had no effect on C_T values, and that primer-limited multiplex 18S versus non-primer-limited nonmultiplex 18S reactions also had no effect on C_T values. Finally, we have determined the linear dynamic range for starting template concentrations.

PCRs were carried out in 25-μl reactions of TaqMan universal PCR master mix (1 ×), 50 nmol/l TaqMan 18S probe, 20 nmol/l 18S forward primer, 80 nmol/l 18S reverse primer, and probes and primers at specific concentrations ranging from 50 to 150 nmol/l (probes) and 50 to 900 nmol/l (primers) for each gene of interest. The specific concentrations for each gene were optimized in preliminary experiments. Based on preliminary experiments, 5 ng of cDNA was used for HSP72, PPAR-γ, PPAR-α, and GLUT4, and 20 ng of cDNA was used for HO-1. This resulted in similar C_T thresholds for all genes (see RESULTS section). cDNA and control preparations not containing reverse transcriptase were amplified using the following conditions: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min for HSP72, GLUT4, and PPAR-α. Conditions for HO-1 and PPAR-γ were identical but were run for 50 cycles because of the low basal expression of these genes. This was determined from preliminary experiments. Furthermore, in preliminary experiments, polymerized products were run on a gel to visualize a single band. Of note, in previous experiments where we have followed these procedures, we have compared our gene expression data obtained from real-time PCR methodology with that obtained using a Northern blot, and we have demonstrated comparable results (12).

For each sample, a ΔC_T value was obtained by subtracting 18S C_T values from the C_T of the gene of interest. Therefore, a higher ΔC_T value indicates a lower relative expression. To determine the basal mRNA expression when comparing groups, we used the young control subjects' preclamp value as the control. Hence, a ΔC_T value was obtained for each of the young control subjects and averaged. This value was subtracted from the ΔC_T value for each subject preclamp to derive a Δ - ΔC_T value. The expression of each gene was then evaluated by 2^{-Δ - ΔC_T}. Because the ΔC_T value for each subject in young control subjects was 0, the 2^{-Δ - ΔC_T} for the young control subjects group preclamp became 1. To determine the effect of insulin on the mRNA expression in each group, a ΔC_T value was obtained by subtracting 18S C_T values from each gene of interest, using the preclamp value as the control. Preclamp values for each subject were subtracted from the postclamp samples for each subject to derive a Δ - ΔC_T value. The expression was then evaluated by 2^{-Δ - ΔC_T}, with all preclamp values for each subject being 1.

Intramuscular triglycerides, CS, and β-HAD activity. Muscle (~30 mg) was freeze-dried under vacuum for 24 h. The sample (~10 mg) was viewed under a microscope (6.3×) at room temperature for dissection and removal of

TABLE 2
Primers and probe sequences for real-time PCR

Gene	Forward primer	Reverse primer	TaqMan probe
HSP72	5' CCAAGCAGACGCAGATCTTTC 3'	5' GCCCTCGTACACCTGGATCA 3'	5' CCTACTCCGACAACCAACCCGGG 3'
HO-1	5' TTCTCCGATGGGTCTTACACT 3'	5' TCACATGGCATAAAGCCCTACA 3'	5' CAACTGTGCGCACCA 3'
PPAR-γ	5' AAACITCAAGAGTACCAAGTGCAA 3'	5' GGCATTATTAGAGCTGAGTCTTCTC 3'	5' CAAAGTGGAGCCCTGCATCTCCACCTTATT 3'
PPAR-α	5' AGCTTTGGCTTTACGGAAATACCA 3'	5' CCACAGGATAAAGTCAACCGAGGA 3'	5' TCACGGACAGCCTTTCAACAGCTTC 3'
GLUT4	5' CCTGCCAGAAAAGAGTCTGAAGC 3'	5' ATCCTTCAGCTCAGCCAGCA 3'	5' CAGAAAACATGGGCCAGCCTGTCA 3'

all traces of adipose tissue, connective tissue, and blood contaminants. This procedure yielded ~7–8 mg of dry-weight dissected muscle, from which a direct measure of intramuscular triglyceride (IMTG) content was determined, as previously described (13). The coefficient of variation for this measurement was ~8%. Muscle (5–10 mg) was homogenized in 1:50 dilution (wt/vol) of a 175-mmol/l potassium buffer solution, and CS activity was assayed spectrophotometrically at 25°C. β -HAD activity was assayed spectrophotometrically at 25°C, measuring the disappearance of NADH using the same homogenate as for CS (14).

Statistics. Data are presented as the means \pm SE. Differences between experimental groups were determined using a one-way ANOVA for all measures except the effect of insulin on mRNA expression. For this measure, a two-way (group \times clamp) ANOVA was used. Significant differences were located using a Newman-Kuels post hoc test. To examine the relationship between gene expression and markers of insulin sensitivity, data from all groups were combined and examined with Pearson product correlations. Statistical significance was accepted at $P < 0.05$.

RESULTS

Subject characteristics and whole-body insulin-stimulated glucose uptake. BMI and percent body fat were lower ($P < 0.05$) in young control subjects compared with the diabetic group, whereas percent body fat was lower ($P < 0.05$) in young control subjects compared with both age-matched and diabetic subjects. Fasting blood glucose, plasma insulin, FFA, and HbA_{1c} concentrations were higher ($P < 0.05$) in the diabetic group compared with age-matched and young control subjects. The glucose disposal rate (GDR) was higher ($P < 0.05$) in young control subjects compared with age-matched control subjects and the diabetic group. In addition, GDR was higher ($P < 0.05$) in age-matched control subjects compared with the diabetic group (Table 1). Neither blood glucose nor plasma insulin were different when comparing groups during the clamp (Table 1).

Basal mRNA expression. Both HSP72 and HO-1 were reduced ($P < 0.05$) in the diabetic group by 33 and 55%, respectively, compared with young control subjects. No differences were observed in basal gene expression when comparing age-matched control subjects with young control subjects for these genes (Fig. 1). No differences were observed in basal mRNA expression of GLUT4 (1.32 ± 0.21 - and 0.69 ± 0.13 -fold change for age-matched control subjects and the diabetic group relative to young control subjects = 1), PPAR- α (1.66 ± 0.57 - and 1.10 ± 0.59 -fold change for age-matched control subjects and the diabetic group relative to young control subjects = 1), and PPAR- γ (0.90 ± 0.38 - and 1.07 ± 0.19 -fold change for age-matched control subjects and the diabetic group relative to young control subjects = 1). The average C_T value (means \pm SD) for the five genes for young control subjects ranged from 27.1 ± 0.8 (HSP72) to 31.2 ± 2.1 (PPAR- γ).

Insulin-stimulated mRNA expression. There was a main effect ($P < 0.05$) of the clamp on HSP72 mRNA expression, demonstrating insulin-stimulated HSP72 mRNA expression in all groups, but there were no differences when comparing groups. In contrast, HO-1 mRNA expression was not affected by the clamp in young or age-matched control subjects, but it was increased ($P < 0.05$) approximately 70-fold from preclamp levels in the diabetic group. Insulin did not affect the mRNA expression of GLUT4, PPAR- α , or PPAR- γ in any group (Table 3).

IMTG content and muscle oxidative capacity. IMTG content was ~150% higher ($P < 0.05$) in the diabetic group compared with age-matched and young control subjects.

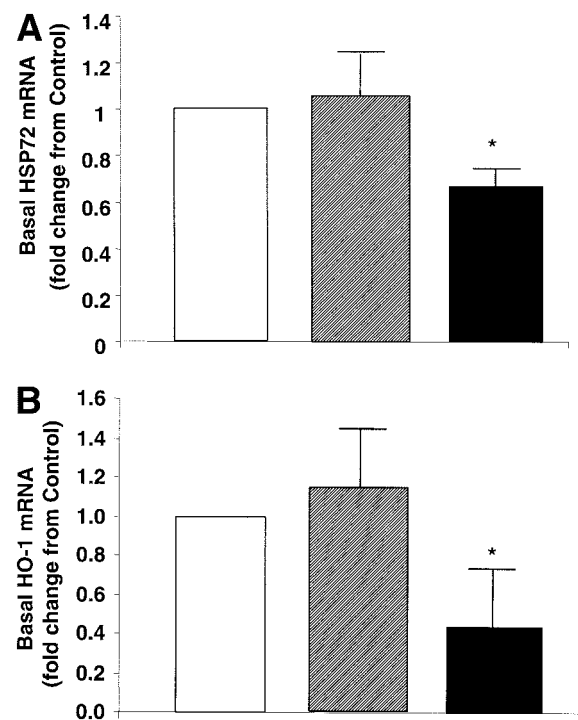


FIG. 1. Basal HSP72 (A) and HO-1 (B) mRNA expression in biopsy samples obtained from the vastus lateralis in young healthy subjects (□), age-matched healthy control subjects (▨), and patients with type 2 diabetes (■). Values are means \pm SE. *Difference ($P < 0.05$) from other groups.

No differences were observed in IMTG content when comparing young control subjects with age-matched control subjects (Fig. 2). CS (12.6 ± 1.5 vs. 5.4 ± 0.7 vs. $3.6 \pm 0.5 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ for young control, age-matched control, and diabetic subjects, respectively) and β -HAD (9.4 ± 0.9 vs. 5.8 ± 1.2 vs. $4.9 \pm 0.7 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ for young control, age-matched control, and diabetic subjects, respectively) maximal activity was higher ($P < 0.05$) in young control subjects compared with age-matched control and diabetic subjects. Although there was a tendency for the maximal activity of these enzymes to be reduced in the diabetic group compared with age-matched control subjects, the results were not significantly different.

Plasma MPO concentration. Plasma MPO content was higher ($P < 0.05$) in the diabetic group compared with age-matched control subjects. However, the time \times treatment interaction for MPO was not significantly different ($P > 0.05$) when comparing the diabetic group with age-matched control subjects (Fig. 3).

Relationship between HSP72 and HO-1 mRNA expression and markers of insulin sensitivity, oxidative capacity, and intramuscular lipid content. Because both HSP72 and HO-1 mRNA were reduced in the diabetic group, we performed correlations to determine whether this reduction was associated with metabolic factors that are important for insulin resistance. Taking note that a higher ΔC_T is indicative of lower mRNA expression, HSP72 mRNA was inversely correlated with GDR ($r = -0.75$, $P < 0.001$), β -HAD ($r = -0.61$, $P < 0.01$), and CS ($r = -0.65$, $P < 0.01$). Although not statistically significant, there was a tendency for HSP72 mRNA to be correlated with IMTG content ($r = 0.46$, $P < 0.06$) (Fig. 4).

TABLE 3

Effect of insulin stimulation on mRNA expression of HSP72, HO-1, PPAR- α , PPAR- γ , and GLUT4 in biopsy samples obtained from the vastus lateralis in young healthy subjects, age-matched healthy subjects, and patients with type 2 diabetes

Gene	Young healthy subjects	Age-matched healthy subjects	Diabetic subjects
HSP72*	3.20 \pm 1.58	4.00 \pm 1.95	4.72 \pm 2.19
HO-1	1.15 \pm 0.28	0.94 \pm 0.49	67.35 \pm 39.18†
PPAR- γ	1.53 \pm 0.94	1.18 \pm 0.19	0.92 \pm 0.19
PPAR- α	1.11 \pm 0.19	0.88 \pm 0.39	1.71 \pm 0.69
GLUT4	1.30 \pm 0.48	0.86 \pm 0.22	1.20 \pm 0.27

Values are means \pm SE and expressed as the value obtained from the sample collected post-clamp relative to the value obtained from the sample obtained before the clamp. *Significant main effect ($P < 0.05$) of the hyperinsulinemic-euglycemic clamp; †indicates significant effect ($P < 0.05$) of the hyperinsulinemic-euglycemic clamp in diabetic patients.

Likewise, HO-1 mRNA was significantly inversely correlated with GDR ($r = -0.50, P < 0.05$), and there was a tendency for HO-1 mRNA to be inversely correlated with CS ($r = -0.46, P < 0.06$) (Fig. 5)

DISCUSSION

The results from this study provide novel evidence that genes associated with antioxidant defense mechanisms are markedly reduced in patients with type 2 diabetes, and that the reduction in the basal expression of these genes is associated with impaired insulin sensitivity. We have demonstrated a strong relationship between the basal expression of HSP72 and HO-1 and the GDR determined during a 2-h hyperinsulinemic-euglycemic clamp, and we have shown that the reduction in muscle oxidative capacity and accumulation of intramuscular lipid is also associated with the expression of this gene. Hence, our data suggest that skeletal muscle insulin resistance in patients with type 2 diabetes is, in part, caused by a dysfunction in antioxidant defense mechanisms, and our data support the recently proposed hypothesis that decreased stress protein expression may be a primary factor leading to the development of type 2 diabetes (3).

HSP72 and insulin resistance. Our observation of a reduction in the basal expression of HSP72 mRNA in the skeletal muscle of patients with type 2 diabetes supports previous investigations (7,8). Our data are novel with respect to the expression and induction of HSP72 in these patients and the possible mechanisms by which HSP72 could be exerting its effect. First, in a previous study (7), the relationship between HSP72 mRNA expression and insulin-stimulated glucose uptake during a hyperinsuline-

mic-euglycemic clamp in patients with type 2 diabetes and age-matched control subjects tended to be different but was not significant ($r = 0.525, P = 0.081$). The authors were therefore unable to conclude with conviction that this relationship was marked. Our data extend their findings by clearly demonstrating that the relationship is indeed marked. Even in young and age-matched control subjects who were not classed as insulin resistant, the relationship was nonetheless apparent (Fig. 4). This may have been related to the higher BMI of age-matched compared with young control subjects (Table 2). We further demonstrated a significant correlation between the expression of this gene and muscle oxidative capacity, as well as a moderate relationship between IMTG accumulation and HSP72 mRNA (Fig. 4).

In a previous study (7), the authors speculated on the mechanism by which HSP72 expression may be affecting insulin sensitivity. They suggested that GLUT4 may interact with HSP72 because both are known to interact with F-actin. In addition, these authors suggested that the PPARs may also play a role because HSP72 and the PPARs form a complex *in vivo* in the rat. In the present study, we found that the expression of these genes was not defective in the diabetic group relative to young and age-matched control subjects. In addition, we found no reduction in GLUT4 total protein (data not shown). Because we did not measure the trafficking of GLUT4 to the plasma membrane during the clamp or the total protein or ligand binding efficiency of the PPARs, our data cannot categorically rule out the possibility that these proteins may interact with HSP72. However, the results argue against such an hypothesis.

The fact that HSP72 mRNA was markedly associated

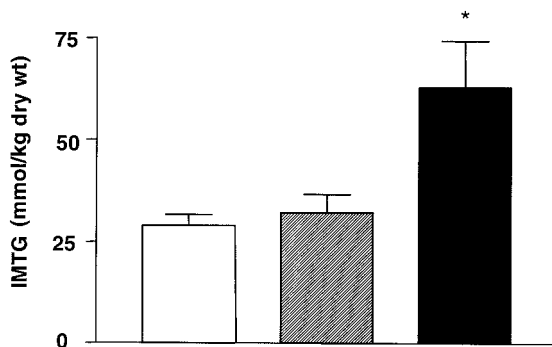


FIG. 2. IMTG in biopsy samples obtained from the vastus lateralis in young healthy subjects (□), age-matched healthy control subjects (▨), and patients with type 2 diabetes (■). Values are means \pm SE. *Difference ($P < 0.05$) from other groups.

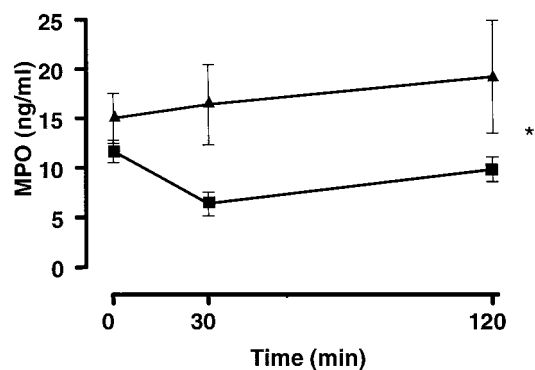


FIG. 3. Plasma MPO content in plasma collected before and during a hyperinsulinemic-euglycemic clamp in age-matched control subjects (▨) and the diabetic group (▲). *Main group effect ($P < 0.05$).

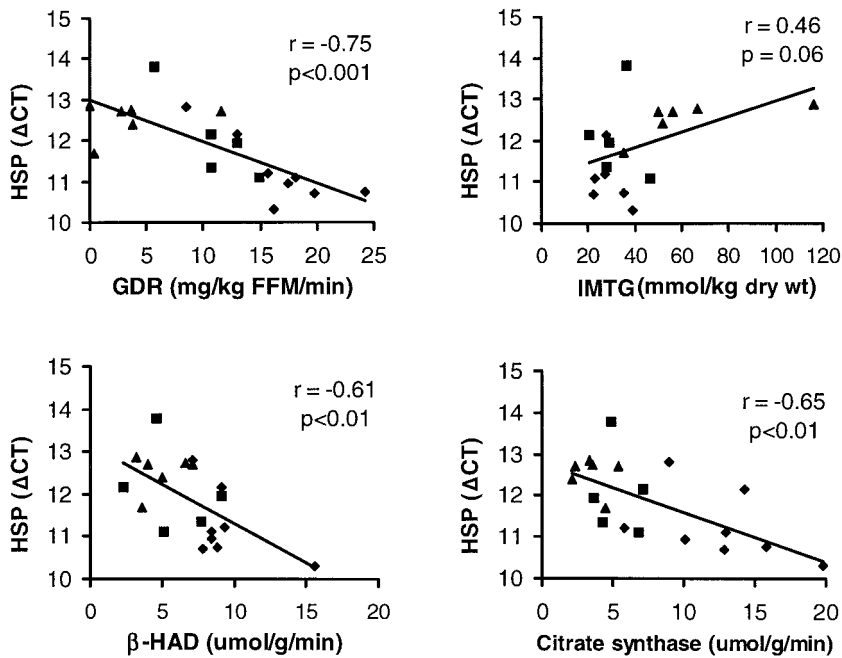


FIG. 4. Relationship between HSP mRNA expression and GDR, IMTG content, β-HAD activity, and CS activity. A decrease of 1 in the C_T value denotes a halving in copy number. ♦, young control subjects; ■, age-matched control subjects; ▲, the diabetic group.

with muscle oxidative capacity and moderately associated with IMTG accumulation raises two hypotheses. First, it is well known that HSP72 plays a pivotal role in mitochondrial biogenesis (15). Cytosolic HSP72 is a major precursor protein that interacts with the trans-outer mitochondrial membrane complex to import proteins into the mitochondria. Given the relationship between type 2 diabetes and impaired muscle oxidative capacity (16,17), along with the relationship in the present study, we propose that impaired HSP72 expression in patients with type 2 diabetes may play a role in impaired oxidative capacity. In addition, our observation that HSP72 was moderately associated with accumulation of IMTG raises an alternative hypothesis. It has recently been demonstrated that c-Jun NH₂-terminal kinase (JNK) activity is abnormally elevated in obesity (18). Furthermore, an absence of JNK results in significantly improved insulin sensitivity and enhanced insulin receptor signaling capacity in two different models of mouse obesity (18). Of note, HSP72 inhibits JNK via inhibition of its dephosphorylation (19,20). Because an accumulation of IMTG is associated with insulin resistance (21), one role of intramuscular HSP72 may be to downregulate JNK, a mechanism that would be impaired in patients with type 2 diabetes.

A major limitation to the present study is that like previous investigations (7,8), we did not obtain sufficient muscle tissue to examine HSP72 protein content. However, we have previously demonstrated a remarkable similarity between HSP72 mRNA and protein expression

in contracting human skeletal muscle (22), although this response is sometimes variable in subjects undergoing exercise (23). Nonetheless, our previous data (22), together with the evidence of a strong relationship between HSP72 gene transcription and protein translation (24), suggest that protein levels were also reduced in the muscles of the patients with type 2 diabetes.

It has been previously demonstrated that insulin activates HSP72 gene expression in a human hepatoma cell line (25). We have shown that HSP72 mRNA is induced by muscle contraction (11,22,23) and recombinant human interleukin-6 infusion (26). To our knowledge, however, no previous studies have examined the effect of a hyperinsulinemic-euglycemic clamp on HSP72 mRNA expression in human skeletal muscle. Our data demonstrate that HSP72 mRNA is induced to a small extent by this intervention, but that this induction is not defective in patients with type 2 diabetes (Table 3). It is not surprising that the clamp induced HSP72 mRNA in skeletal muscle because insulin activates signal transducer and activator of transcription (STAT) proteins (27), which are present in skeletal muscle (28) and activate HSP72. However, it was somewhat surprising that the fold induction of HSP72 by the clamp was uniform across groups, given the lower basal level in the diabetic group. We have no evidence for whether this small induction would lead to the translation of HSP72 protein in this time frame, and we therefore have difficulty interpreting these data. In addition, we cannot determine whether the increased expression was caused

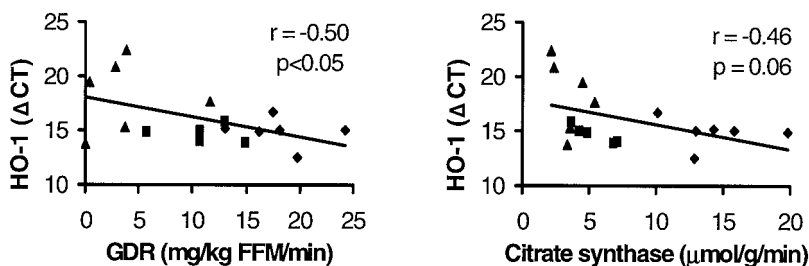


FIG. 5. Relationship between HO-1 mRNA expression and GDR and CS activity. A decrease of 1 in the C_T value denotes a halving in copy number. ♦, young control subjects; ■, age-matched control subjects; ▲, the diabetic group.

by the insulin or the enhanced glucose uptake associated with the clamp, although one would expect that the response was due to the former because, in a preliminary study, we have shown that increasing plasma glucose content reduces both circulating HSP72 protein and intramuscular HSP72 mRNA (29). Nonetheless, our findings may have implications for those presented by Kurucz et al. (7) because in that study a bolus of insulin was provided to the patients with type 2 diabetes before the muscle biopsy. This may have, in fact, reduced the difference in HSP72 mRNA when comparing the patient with the control group.

We cannot conclude from our data that the decrease in basal HSP72 mRNA mediates insulin resistance because we only present associations. However, our data, coupled with those presented previously in humans (7,8), as well as earlier studies in diabetic rodents showing that pharmacological increases in HSP72 expression improves insulin sensitivity (30), provide some evidence that HSP72 is directly involved in the pathogenesis of insulin resistance.

HO-1 and insulin resistance. Comparatively little is known about the functional significance of HO-1 in skeletal muscle. To our knowledge, this is the first study to measure the expression of this gene in the muscles of patients with type 2 diabetes. Like HSP72, HO-1 expression was markedly reduced in the diabetic group (Fig. 1) and correlated with GDR (Fig. 5). However, it must be noted that the range in expression for HO-1 when comparing individuals was much greater compared with HSP72. Given that a decrease of 1 in the C_T value corresponds to a halving of the copy number of the gene, the range in HSP72 was 10–14 (Fig. 4), whereas the range for HO-1 was 12–22 (Fig. 5), with the highest C_T values in the patients in the diabetic group. Hence, our data show that patients with type 2 diabetes have markedly reduced HO-1 gene expression even relative to HSP72. It has been suggested that HO-1 provides protection against the effects of proinflammatory cytokines (6), and a polymorphism of the promoter region of the human HO-1 gene is associated with the susceptibility to pathogenesis associated with type 2 diabetes (31).

It is well known that the proinflammatory cytokine tumor necrosis factor (TNF)- α is implicated in the etiology of insulin resistance and type 2 diabetes, primarily by reducing tyrosine phosphorylation of insulin receptor substrate-1 (rev. in 32). Although the relationship between HO-1 and TNF- α has not been investigated in skeletal muscle, HO-1 inhibits the deleterious effects of TNF- α in cultured fibroblasts (33). It is possible that there is a relationship between HO-1 and TNF- α in the muscles of patients with type 2 diabetes, and this warrants investigation. Interestingly, HO-1 is also induced in rat skeletal myoblasts by the nitric oxide (NO) donor sodium nitroprusside (34), and in neuronal NO synthase (NOS) protein, expression is reduced in the skeletal muscle of patients with type 2 diabetes (35). It is possible, therefore, that HO-1 mRNA expression is reduced in the skeletal muscle of patients with type 2 diabetes because of the reduced NOS expression. Of note, although HO-1 was not induced by insulin in young or age-matched control subjects, there was a \sim 70-fold increase in insulin-induced gene expression in the diabetic group. Although this observation shows that insulin has an abnormal effect on HO-1 gene

expression with type 2 diabetes, we are unable to shed light on this finding. We can only speculate that due to the aberrant insulin signaling cascade in patients with type 2 diabetes, insulin may have resulted in intramuscular stress or ROS production. Of note, MPO content was higher in the diabetic group compared with age-matched control subjects (Fig. 3). Stored in primary granules of neutrophils, MPO catalyzes the formation of hypochlorous acid, a powerful ROS (10). Although we were not able to measure MPO in young control subjects, our data in the diabetic group and age-matched control subjects provide further evidence that the pathogenesis of type 2 diabetes involves oxidant production.

In summary, we have provided evidence that the expression of genes associated with providing cellular defense against oxidative stress are markedly reduced in the skeletal muscles of patients with type 2 diabetes. Furthermore, the expression of these genes is associated with whole-body insulin sensitivity and muscle oxidative capacity. Our data cannot demonstrate any causal relationship between markers of oxidative stress and insulin resistance, and it must be noted that glucose disposal during the clamp was lower in age-matched control subjects compared with young control subjects, yet the expression of our measured genes was similar when comparing these groups. This demonstrates that the factors associated with insulin sensitivity are complex and multifactorial. Nonetheless, our data provide compelling evidence of a strong association between antioxidant defense and insulin sensitivity, therefore suggesting that reduced stress protein expression in skeletal muscle may be a primary factor in the etiology of type 2 diabetes. This hypothesis requires investigation, since stress proteins can be pharmacologically induced, suggesting a potential novel therapeutic target for muscle insulin resistance.

ACKNOWLEDGMENTS

This study was supported by the Australian Research Council (DP0209570-MAF) and EFFEM Foods Pty. Australia (to J.A.H.).

We acknowledge the assistance of Drs. Adamandia D. Kriketos and Gregory J. Cooney for measuring the IMTGs. We also thank Sally Clark, Kate Greenway, Mitchell Anderson, and Dr. David Newman for technical assistance.

REFERENCES

1. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: a new perspective of an old paradigm (Review). *Diabetes* 48:1–9, 1999
2. Evans JL, Goldfine ID, Maddux BA, Grodsky GM: Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocrine Rev* 23:599–622, 2002
3. Hooper PL: Diabetes, nitric oxide, and heat shock proteins. *Diabetes Care* 26:951–952, 2003
4. 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
5. Molka MB, Zoppi CC, Alves AA, Silveira IR, Marangoni S, Periera-Da Silva I, Novello JC, Macedo DV: HSP72 as a complimentary protection against oxidative stress induced by exercise in the soleus muscle of rats. *Am J Physiol Regul Integr Comp Physiol* 279:R1539–R1545, 2000
6. Barreiro E, Comtois AS, Mohammed S, Lands LC, Hussain SN: Role of heme oxygenases in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress. *Am J Physiol Lung Cell Mol Physiol* 283:L476–L484, 2002
7. Kurucz I, Morva A, Vaag A, Eriksson KF, Huang X, Groop L, Koranyi L: Decreased expression of heat shock protein 72 in skeletal muscle of

- patients with type 2 diabetes correlates with insulin resistance. *Diabetes* 51:1102–1109, 2002
8. 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
 9. 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
 10. Carlin G, Djursater R: Peroxidation of phospholipids promoted by myeloperoxidase. *Free Radic Res Commun* 4:251–257, 1988
 11. Febbraio MA, Koukoulas I: HSP72 gene expression progressively increases in human skeletal muscle during prolonged, exhaustive exercise. *J Appl Physiol* 89:1055–1060, 1999
 12. Steensberg A, Febbraio MA, Osada T, Schjerling P, van Hall G, Saltin B, Pedersen BK: Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol* 537:633–639, 2001
 13. Kriketos AD, Furler SM, Gan SK, Poynten AM, Chisholm DJ, Campbell LV: Multiple indexes of lipid availability are independently related to whole body insulin action in healthy humans. *J Clin Endocrinol Metab* 88:793–798, 2003
 14. Lowry OH, Passonneau JV: *A Flexible System of Enzymatic Analysis*. New York, Academy Press, 1972
 15. Hood DA: Invited Review: Contractile activity-induced mitochondrial biogenesis in skeletal muscle. *J Appl Physiol* 90:1137–1157, 2001
 16. Simoneau JA, Veerkamp JH, Turcotte LP, Kelley DE: Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *FASEB J* 13:2051–2060, 1999
 17. He J, Watkins S, Kelley DE: Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* 50:817–823, 2001
 18. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333–336, 2002
 19. Dorion S, Landry J: Activation of the mitogen-activated protein kinase pathways by heat shock. *Cell Stress Chaperones* 7:200–206, 2002
 20. Meriin AB, Yaglom JA, Gabai VL, Zon L, Ganiatsas S, Mosser DD, Zon L, Sherman MY: Protein-damaging stresses activate c-Jun N-terminal kinase via inhibition of its dephosphorylation: a novel pathway controlled by HSP72. *Mol Cell Biol* 19:2547–2555, 1999
 21. Boden G, Lebed B, Schatz M, Homko C, Lemieux S: Effect of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects. *Diabetes* 50:1612–1617, 2001
 22. Febbraio MA, Steensberg A, Walsh R, Koukoulas I, van Hall G, Saltin B, Pedersen BK: Reduced glycogen availability is associated with an elevation in HSP72 in contracting human skeletal muscle. *J Physiol* 538:911–917, 2002
 23. Walsh RC, Koukoulas I, Garnham A, Moseley PL, Hargreaves M, Febbraio MA: Exercise increases serum Hsp72 in humans. *Cell Stress Chaperones* 6:386–393, 2001
 24. 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
 25. 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
 26. Febbraio MA, Steensberg A, Fischer CP, Keller C, Hiscock N, Pedersen BK: IL-6 activates HSP72 gene expression in human skeletal muscle. *Biochem Biophys Res Commun* 296:1264–1266, 2002
 27. Coffey PJ, van Puijnenbroek A, Burgering BM, Klop-de Jonge M, Koenderman L, Bos JL, Kruijer W: Insulin activates Stat3 independently of p21ras-ERK and PI-3K signal transduction. *Oncogene* 15:2529–2539, 1997
 28. Frost RA, Nystrom GJ, Lang CH: Regulation of IGF-I mRNA and signal transducers and activators of transcription-3 and -5 (Stat-3 and -5) by GH in C2C12 myoblasts. *Endocrinology* 143:492–503, 2002
 29. Febbraio MA, Steensberg A, Keller C, Nielsen HB, Krstrup P, Ott P, Secher NH, Pedersen BK: Glucose ingestion attenuates contracting muscle HSP72 mRNA and hepatosplanchnic HSP72 release during exercise in humans (Abstract). *Med Sci Sports Exerc* 35:S196, 2003
 30. Kurthy M, Mogyorosi T, Nagy K, Kukorelli T, Jednakovits A, Talosi L, Biro K: Effect of BRX-220 against peripheral neuropathy and insulin resistance in diabetic rat models. *Ann N Y Acad Sci* 967:482–489, 2002
 31. Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, Chang MJ, Wu TC, Chen LC, Ding YA, Pan WH, Jou YS, Chau LY: Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. *Hum Genet* 111:1–8, 2002
 32. Hotamisligil GS: The role of TNFalpha and TNF receptors in obesity and insulin resistance. *J Intern Med* 245:621–625, 1999
 33. Petrache I, Otterbein LE, Alam J, Wiegand GW, Choi AM: Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 278:L312–L319, 2000
 34. Vesely MJ, Sanders R, Green CJ, Motterlini R: Fibre type specificity of haem oxygenase-1 induction in rat skeletal muscle. *FEBS Lett* 458:257–260, 1999
 35. Bradley SJ, Stephens TJ, Canny BJ, Kingwell BA, McConell GK: Lower skeletal muscle N-NOS unchanged by exercise training in type 2 diabetics (Abstract). *Med Sci Sports Exerc* 34:S275, 2002