

# Impaired Cathepsin L Gene Expression in Skeletal Muscle Is Associated With Type 2 Diabetes

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To identify abnormally expressed genes associated with muscle insulin resistance or type 2 diabetes, we screened the mRNA populations using cDNA differential display combined with relative RT-PCR analysis from muscle biopsies of diabetes-prone C57BL/6J and diabetes-resistant NMRI mice fed with a high-fat or normal diet for 3 or 15 months. Six abnormally expressed genes were isolated from the mice after a 3-month fat feeding; one of them was cathepsin L. No significant difference in mRNA levels of these genes was observed between fat- and normal-diet conditions in either strains. However, cathepsin L mRNA levels in muscle were higher in normal diet-fed C57BL/6J mice compared with normal diet-fed NMRI mice at 3 months ( $0.72 \pm 0.04$  vs.  $0.51 \pm 0.04$  relative units,  $P < 0.01$ ,  $n = 8-10$ ) and at 15 months ( $0.41 \pm 0.05$  vs.  $0.27 \pm 0.04$  relative units,  $P = 0.01$ ,  $n = 9-10$ ). Further, cathepsin L mRNA levels in muscle correlated inversely with plasma glucose in both strains regardless of diets at 3 ( $r = -0.49$ ,  $P < 0.01$ ,  $n = 31$ ) and 15 ( $r = -0.42$ ,  $P = 0.007$ ,  $n = 39$ ) months. To study whether cathepsin L plays a role in human diabetes, we measured cathepsin L mRNA levels in muscle biopsies taken before and after an insulin clamp from 12 monozygotic twin pairs discordant for type 2 diabetes and from 12 control subjects. Basal cathepsin L mRNA levels were not significantly different between the study groups. Insulin infusion increased cathepsin L mRNA levels in control subjects from  $1.03 \pm 0.30$  to  $1.90 \pm 0.32$  relative units ( $P = 0.03$ ). Postclamp cathepsin L mRNA levels were lower in diabetic twins but similar in nondiabetic twins compared with control subjects ( $0.66 \pm 0.22$ ,  $1.16 \pm 0.18$  vs.  $1.38 \pm 0.21$  relative units,  $P < 0.02$ , NS, respectively). Further, postclamp cathepsin L mRNA levels were correlated with insulin-mediated glucose uptake ( $r = 0.37$ ,  $P = 0.03$ ), particularly, with glucose oxidation ( $r = 0.37$ ,  $P = 0.03$ ), and fasting glucose concentrations ( $r = -0.45$ ,  $P < 0.01$ ) across all three study groups. In conclusion, muscle cathepsin L gene expression is increased in diabetes-prone mice and related to glucose tolerance. In humans, insulin-stimulated cathepsin L expression in skeletal muscle is impaired in diabetic but not in nondiabetic monozygotic twins, suggesting that the changes may be secondary to

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Insulin resistance can be induced by high-fat intake in animals and humans (1–4) and is seen at the levels of whole-body and skeletal muscle (4,5). The degree of fat-induced insulin resistance varies according to different genetic backgrounds in humans (6) and in experimental animals (7–10). The C57BL/6J mouse is genetically prone to develop insulin resistance and diabetes. When challenged with a high-fat diet, the C57BL/6J mouse shows elevated plasma insulin and glucose levels compared with a control mouse strain (8,10). Therefore, strain-specific and fat-responsive genes could contribute to insulin resistance and glucose intolerance in C57BL/6J mice (11). In an effort to identify such genes, we performed cDNA differential display (12) on skeletal muscle from diabetes-prone C57BL/6J and diabetes-resistant NMRI mice fed with a high-fat or normal diet. Among six putative candidate clones with abnormal expression in C57BL/6J compared with NMRI mice, we identified the cathepsin L gene. To test whether cathepsin L plays a role in human type 2 diabetes, we examined cathepsin L gene expression in muscle biopsies taken before and after an insulin clamp from 12 monozygotic twin pairs discordant for type 2 diabetes and from 12 control subjects (13,14). The study of monozygotic twins discordant for diabetes allowed us to study whether the observed differences were inherited (similar in diabetic and nondiabetic twins) or acquired (different between twin pairs).

## RESEARCH DESIGN AND METHODS

**Animals and diets.** Female mice of the C57BL/6J and NMRI strains were obtained at age 4 weeks from Bomholtgaard Breeding and Research Center (Ry, Denmark). Eight to 10 mice from each strain received either a high-fat diet (Research Diets, New Brunswick, NJ) or an ordinary rodent diet (Research Diets) for 3 and 15 months. Animals were kept four to five per cage in a temperature-controlled ( $22 \pm 1^\circ\text{C}$ ) room with a 12-h light-dark cycle. The mice had free access to food and water throughout the study periods. On a caloric base, the high-fat diet consisted of 16.4% protein, 25.6% carbohydrates, and 58.0% fat (total 23.4 kJ/g), and the normal diet consisted of 25.8% protein, 62.8% carbohydrates, and 11.4% fat (total 12.6 kJ/g). At the end of each study period, blood samples were taken at 11:00 A.M. from the intraorbital retrobulbar plexus during the nonfasting state for the measurement of plasma insulin and glucose. Mice were killed during anesthesia with an intraperitoneal injection of midazolam (0.14 mg/mouse, Dormicum; Hoffman LaRoche, Basel, Switzerland) and a combination of fentanyl (0.9 mg/mouse) and fentanyl (0.02 mg/mouse, Hypnorm; Janssen, Beerse, Belgium). Gastrocnemius muscles were taken immediately into liquid nitrogen and stored at  $-80^\circ\text{C}$  until analyzed. The study was approved by the Animal Ethics Committee at Lund University.

**Human subjects.** A total of 12 Caucasian monozygotic twin pairs discordant for type 2 diabetes and 12 healthy subjects without family history of diabetes

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MAP4, microtubule-associated protein 4; mtCOX1, mitochondrial H-chain-encoded cytochrome C oxidase subunit 1; mtND2, mitochondrial H-chain-encoded NADH dehydrogenase subunit 2; mtND4, mitochondrial H-chain-encoded NADH dehydrogenase subunit 4.

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TABLE 1

Body weight, plasma glucose, and insulin in C57BL/6J and NMRI mice fed with a high-fat or normal diet for 3 or 15 months

	3 months		15 months	
	Fat diet	Normal diet	Fat diet	Normal diet
<b>C57BL/6J mice</b>				
Body weight (g)	27.2 ± 0.9*	21.3 ± 0.5	44.3 ± 1.6*	26.5 ± 0.8
Plasma glucose (mmol/l)	9.1 ± 0.6	7.8 ± 0.5	5.3 ± 0.3*	3.8 ± 0.2
Plasma insulin (pmol/l)	250.1 ± 37.4	211.2 ± 45.0	463.5 ± 29.0*	266.5 ± 46.7
<b>NMRI mice</b>				
Body weight (g)	40.2 ± 2.8	37.2 ± 1.3	48.0 ± 3.8	38.3 ± 1.7
Plasma glucose (mmol/l)	9.9 ± 0.2	9.5 ± 0.3	5.3 ± 0.2	5.5 ± 0.2
Plasma insulin (pmol/l)	401.4 ± 83.2†	170.8 ± 32.3	705.3 ± 144.7†	275.3 ± 64.4

Data are means ± SE (n = 8–10). \*P < 0.01, †P < 0.05 vs. normal diet–fed mice at the same time point.

participated in the study. Type 2 diabetes had been diagnosed after the age of 40 years based on a standardized 75-g oral glucose tolerance test (15). The control subjects were matched to the nondiabetic twins for age, sex, and BMI. Monozygosity of the twins was confirmed by genetic markers (14). Insulin sensitivity was measured by a 3-h euglycemic-hyperinsulinemic clamp (13,14). Plasma glucose in the diabetic patients was lowered to the basal level comparable with that of the control subjects by prior infusion of insulin over a period of 50 min 3–4 h before initiation of the insulin clamp (0 min) (13,14). Muscle biopsies of vastus lateralis were obtained in the basal state (0 min) and at the end of the clamp (+180 min), frozen immediately in liquid nitrogen, and stored at –80°C until analyzed. Informed consent was obtained from all subjects. The protocol was approved by the regional ethics committee, and the study was conducted according to the principles of the Declaration of Helsinki.

**cDNA differential display.** cDNA differential display was conducted using muscle biopsies from six individual mice fed a high-fat or normal diet for 3 months (two high-fat–fed C57BL/6J mice, two high-fat–fed NMRI mice, one normal diet–fed C57BL/6J mouse, and one normal diet–fed NMRI mouse). Total RNA was isolated from the muscle biopsies by the acid guanidinium thiocyanate method (16) and subjected to DNase I (Promega, Madison, WI) treatment according to the manufacturer’s instruction to avoid genomic DNA contamination. The differential display was performed as previously described (17). The selection of cDNA bands was directed to those present in a strain-specific or fat-responsive manner. Thus, those bands present or absent in high-fat–fed C57BL/6J mice compared with normal diet–fed C57BL/6J mice were recovered. The recovery of the corresponding cDNAs was achieved by elution of the DNA bands from the sequencing gel and PCR reamplification (17). The recovered cDNAs were subcloned using a T-Cloning kit (MBI Fermentas, Vilnius, Lithuania), sequenced using the Sequenase version 2.0 kit (USB, Cleveland, OH), and compared with sequences in the GenBank (www.ncbi.nlm.nih.gov).

**RT-PCR quantitation of gene expression**

**Gene expression in mice.** RNA expression was examined using a modified “primer-dropping” RT-PCR method (18). Total RNA was isolated from the muscle biopsies by the acid guanidinium thiocyanate method (16) and subjected to DNase I (Promega) treatment according to the manufacturer’s instruction to avoid genomic DNA contamination. The treated total RNA (400 ng) was then reverse-transcribed in a 40-μl reaction with a 5 μmol/l oligo(dT)<sub>18</sub> primer in the presence of 200 units of Superscript II Reverse Transcriptase (Life Technologies, Glasgow, Scotland) and 25 μmol/l dNTP for 60 min at 37°C. After heat inactivation of the RT at 95°C for 5 min, total cDNA was subjected to PCR coamplification of target genes together with cyclophilin as a reference gene. Cyclophilin was used as a reference gene because it is unaffected by insulin and the diabetic state (19). Validation of this method by RNase protection assay has been described in a previous study (17). The target gene primer pairs are (from 5’ to 3’) as follows: for the unknown clone, GAAACGGGTGTGTTTACGCTG, CCCACGGAAGTATATATCAC; for mitochondrial H-chain–encoded cytochrome C oxidase subunit 1 (mtCOX1), TTG GAACTGACTTTGCCAC; TTTGTTTTAGGTTGCGGTCTG; for cathepsin L, GTTGGCGTTTTAGCGCATCG, CCCATGGTCGAGGTTCTTTC; for mitochondrial H-chain–encoded NADH dehydrogenase subunit 4 (mtND4), TGAAGCTCCAATGCTGGGTC, CCATTTGAAGTCCCTCGGGCC; for mtND2, CTCCTGGCCATCGTACTCAAC, GAGTAGAGTGAGGGATCGGTTG; for microtubule-associated protein 4 (MAP4) homologue, GCTGGGCAGATTGCGCTC TC, TACGCTCAGTCACTACTACCC; and for mouse cyclophilin, TGGTCAAC CCCACCGTGTTC, TAGATGGACCTGCCGCAAGT. For cathepsin L measurement, the PCR (20 μl) contains 2 μl of RT reaction, 1× PCR buffer, 200 μmol/l dNTP, 5% dimethylsulfoxide, 5% formamide, 0.5 units Taq polymerase, 0.40 μmol/l cathepsin L primers, and 0.20 μmol/l cyclophilin primers. The PCR was

run for 29 cycles (94°C, 30 s; 55°C, 30 s; 72°C, 30 s) and followed by a final extension at 72°C for 10 min. Similar PCRs were conducted for measurement of mRNA levels of mtCOX1, mtND4, mtND2, MAP4 homologue, and the unknown genes. The PCR conditions were optimized according to the primer-dropping method (18) for each individual gene. PCR products were separated on a 2% agarose gel containing ethidium bromide, photographed with UPP-110HA printing paper (Sony, Tokyo), and quantitated using a Personal Densitometer SI scanner together with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The mRNA levels were expressed relative to that of cyclophilin.

**Cathepsin L gene expression in humans.** Total RNA extraction from the muscle biopsies, DNase I treatment, and reverse transcription were conducted the same as described above. After heat inactivation of the RT at 95°C for 5 min, total cDNA was subjected to PCR coamplification of cathepsin L together with cyclophilin as a reference gene. The primer pair for cathepsin L are (from 5’ to 3’) GTCAGTGTGGTCTTGTTGG and AAGGACTCATGACCTGCATC; the primer pairs for human cyclophilin are GTCTCCTTTGAGCTGTTGC and CTGGGAACCAITTTGTGTTGG. To study the effect of insulin on cathepsin L gene expression, we measured cathepsin L mRNA levels in skeletal muscle biopsies from 12 control subjects before and after a euglycemic clamp using the following conditions. The PCR (20 μl) contains 2 μl of RT reaction, 1× PCR buffer, 200 μmol/l dNTP, 5% dimethylsulfoxide, 5% formamide, 0.5 units Taq polymerase, 0.25 μmol/l cathepsin L primers, and 0.1 μmol/l cyclophilin primers. The PCR was run for 34 cycles (94°C, 30 s; 55°C, 30 s; 72°C, 30 s) and followed by a final extension at 72°C for 10 min. The PCR conditions were optimized according to the primer-dropping method (18). PCR products were analyzed as described above. To examine whether altered gene expression of cathepsin L is associated with type 2 diabetes, we measured basal and postclamp mRNA concentrations of this gene in all three groups. We used the similar conditions as described above for measuring basal cathepsin L concentrations, and we used different conditions for measurement of post-clamp cathepsin L concentrations. These procedures were used because some postclamp samples had cathepsin L concentrations too high to measure when using the conditions described above. To avoid substrate competition and allow a direct comparison of them between the groups, we changed the conditions for postclamp cathepsin L concentration measurements. We also analyzed cathepsin L gene expression using real-time RT-PCR in basal and postclamp samples from 5 subjects and in postclamp samples from 11 nondiabetic subjects.

**Analytical measurements.** Plasma glucose was determined with an automated glucose oxidase method (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA). Plasma insulin in mouse and human samples was measured using a double-antibody radioimmunoassay (Linco, St. Charles, MO, and Pharmacia Diagnostics, Uppsala, Sweden, respectively).

**Statistical analysis.** Data are expressed as means ± SE. Statistical analysis was performed using an NCSS 6.0.21 statistical package (NCSS Statistical Software, Kaysville, UT). The significance of difference within or between groups was tested by Mann-Whitney rank tests. The relationship among variables was analyzed by Spearman correlations.

**RESULTS**

**Effect of fat feeding on insulin and glucose levels and body weight in C57BL/6J and NMRI mice.** Plasma insulin levels in the high-fat–fed C57BL/6J mice did not differ at 3 months but were higher at 15 (P = 0.005) months compared with the normal diet–fed C57BL/6J mice (Table 1). Plasma insulin levels in the high-fat–fed NMRI

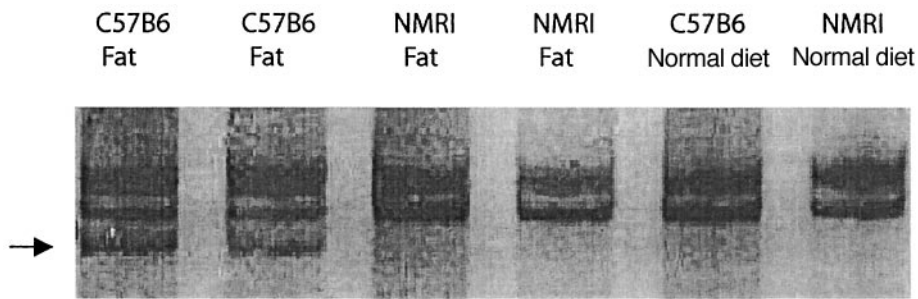


FIG. 1. An autoradiograph of cDNA differential display of a representative candidate cDNA band (arrow) with overexpression in high-fat-fed C57BL/6J mice at 3 months. Total RNA was isolated from gastrocnemius biopsies, and the differential display was performed as described in the text.

mice were significantly higher at 3 ( $P = 0.01$ ) and 15 ( $P = 0.01$ ) months compared with the normal diet-fed NMRI mice (Table 1). Plasma glucose levels in the high-fat-fed C57BL/6J mice were similar at 3 months, but significantly higher after 15 months ( $P = 0.001$ ) compared with the normal diet-fed C57BL/6J mice (Table 1). No significant deterioration of glucose levels was seen in the high-fat-fed NMRI mice compared with the normal diet-fed NMRI mice at 3 and 15 months (Table 1). Body weight of the high-fat-fed C57BL/6J mice was 28% higher at 3 months ( $P < 0.001$ ) and 69% higher at 15 months ( $P < 0.001$ ) compared with the normal diet-fed C57BL/6J mice (Table 1). However, body weights of the high-fat- and normal diet-fed NMRI mice were similar at 3 months and only slightly higher at 15 ( $P = 0.06$ ) months in the high-fat-fed C57BL/6J mice compared with the normal diet-fed NMRI mice (Table 1).

**Mouse gene expression.** Using cDNA differential display, we cloned six cDNA candidates. Five of them were present only in the high-fat-fed C57BL/6J panel and one only in the high-fat-fed NMRI panel. A representative candidate band is shown in Fig. 1. Sequencing analysis revealed that the five cDNA clones from the high-fat-fed C57BL/6J panel matched the genes of cathepsin L, mtND4, mtND2, mtCOX1 with 100% sequence identity, and the gene of MAP4 with >80% sequence identity. The cDNA clone from the high-fat-fed NMRI panel was unknown. Of note, mtND2 mRNA levels were correlated with those of mtND4 ( $r = 0.41$ ,  $P = 0.01$ ,  $n = 33$ ) and with mtCOX1 ( $r = 0.48$ ,  $P < 0.01$ ,  $n = 33$ ) in all mouse groups at 3 months. Further, cathepsin L mRNA levels were also correlated with mRNA levels of mtCOX1 ( $r = 0.37$ ,  $P = 0.03$ ,  $n = 33$ ) and mtND2 ( $r = 0.51$ ,  $P < 0.01$ ,  $n = 33$ ) across all mouse groups after 3 months feeding. Regarding the five genes isolated from the high-fat-fed C57BL/6J mice, mRNA levels of these genes were not significantly different between the high-fat- and normal diet-fed conditions after 3 months (Table 2). Neither was the mRNA level of the unknown clone, which was isolated from the high-fat-fed NMRI mice, different between high-fat- and normal diet-fed conditions after 3 months (Table 2). However, the

mRNA levels of mtND2, cathepsin L, and the unknown clone were significantly higher in the normal diet-fed C57BL/6J mice than in the normal diet-fed NMRI mice at 3 months (Table 2). There was an inverse correlation between cathepsin L gene expression and plasma glucose levels among all study groups after 3 months of feeding ( $r = -0.49$ ,  $P < 0.01$ ,  $n = 31$ ) (Fig. 2A), with the strongest correlation observed in the normal diet-fed NMRI mice ( $r = -0.69$ ,  $P = 0.05$ ,  $n = 8$ ). No correlation was observed between mRNA of other clones and glucose tolerance (data not shown).

After 15 months, we observed strain-specific but not diet-specific differences in muscle mRNA levels of cathepsin L and mtND2, i.e., the mRNA levels of cathepsin L ( $0.41 \pm 0.05$  vs.  $0.27 \pm 0.04$  relative units,  $P < 0.01$ ,  $n = 9-10$ ) and mtND2 ( $0.97 \pm 0.03$  vs.  $0.67 \pm 0.03$  relative units,  $P < 0.01$ ,  $n = 9-10$ ) were higher in normal diet-fed C57BL/6J mice than in normal diet-fed NMRI mice. A similar inverse correlation between cathepsin L mRNA and plasma glucose levels as observed at 3 months was observed in all study groups at 15 months ( $r = -0.42$ ,  $P = 0.02$ ,  $n = 39$ ) (Fig. 2B). The strongest correlation was observed in the normal diet-fed C57BL/6J mice ( $r = -0.72$ ,  $P = 0.03$ ,  $n = 9$ ).

**Clinical characteristics of human subjects.** Fasting plasma glucose levels were higher in the diabetic and nondiabetic twins than in the control subjects (Table 3). Plasma insulin concentrations in the basal state and during the clamp were similar in the diabetic and nondiabetic twins and the control subjects (Table 3). Plasma glucose concentrations in the basal state were higher in the diabetic twins than in the nondiabetic twins ( $7.0 \pm 0.2$  vs.  $5.5 \pm 0.2$  mmol/l,  $P < 0.001$ ) and control subjects ( $7.0 \pm 0.2$  vs.  $5.4 \pm 0.2$  mmol/l,  $P < 0.001$ ). The diabetic twins had a 55% decrease in the rate of insulin-stimulated glucose uptake ( $P < 0.01$ ), 63% decrease in glucose storage ( $P < 0.01$ ), and 37% decrease in glucose oxidation ( $P < 0.01$ ) compared with the control subjects (Table 3). The nondiabetic monozygotic co-twins also had a 25% decrease in insulin-stimulated glucose uptake ( $8.5 \pm 0.8$  vs.  $11.4 \pm 0.9$

TABLE 2

Gene expression in C57BL/6J and NMRI mice fed with a high-fat or normal diet for 3 months

	NMRI/normal diet	NMRI/fat	C57B6/normal diet	C57B6/fat
mtCCIX1	$0.47 \pm 0.04$	$0.51 \pm 0.06$	$0.53 \pm 0.08$	$0.74 \pm 0.05$
Cathepsin L	$0.51 \pm 0.04$	$0.61 \pm 0.03$	$0.72 \pm 0.04^*$	$0.71 \pm 0.02$
mtND4	$0.59 \pm 0.09$	$0.76 \pm 0.04$	$0.73 \pm 0.06$	$0.90 \pm 0.09$
mtND2	$0.39 \pm 0.08$	$0.66 \pm 0.05^\dagger$	$0.71 \pm 0.07^*$	$0.80 \pm 0.07$
MAP4 homologue	$0.55 \pm 0.03$	$0.62 \pm 0.04$	$0.59 \pm 0.04$	$0.64 \pm 0.04$
Unknown	$0.80 \pm 0.03$	$0.83 \pm 0.02$	$0.92 \pm 0.02^*$	$0.94 \pm 0.02$

Data are means  $\pm$  SE in relative units ( $n = 8-10$ ). \* $P < 0.01$ ;  $\dagger P < 0.03$  vs. NMRI/normal diet.

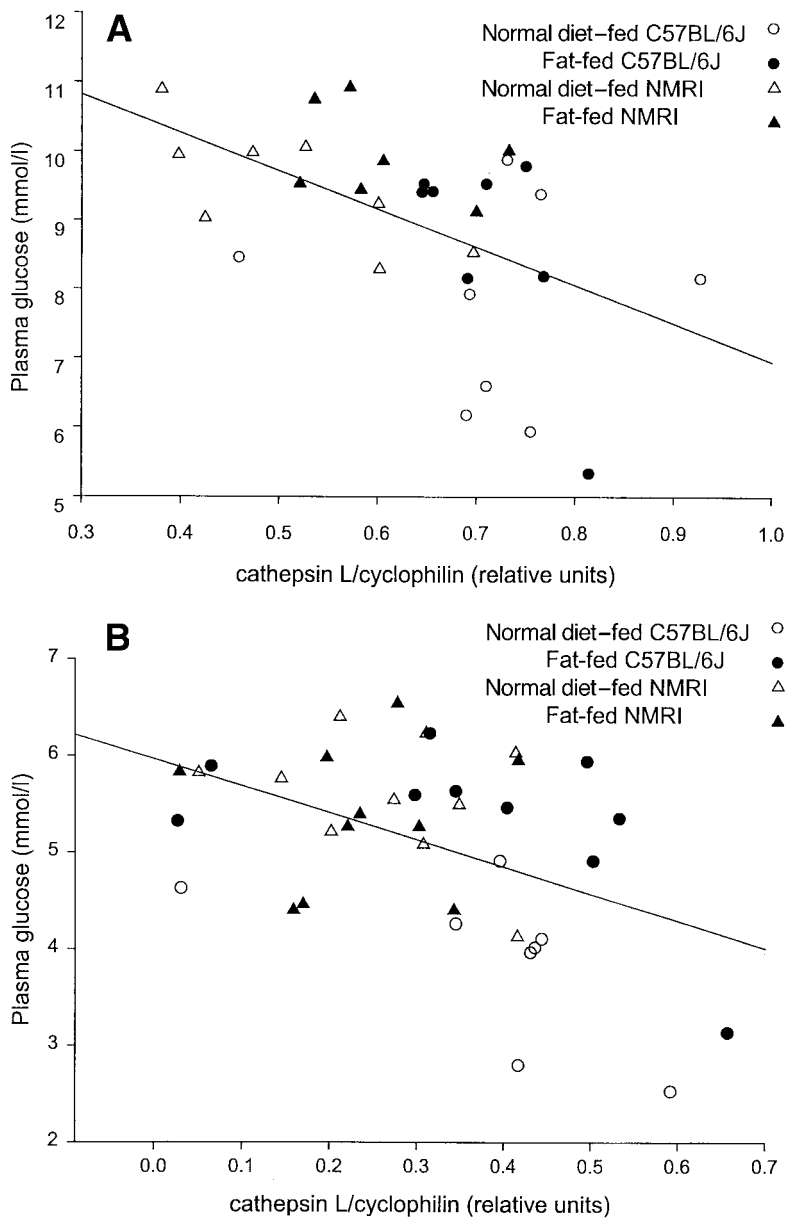


FIG. 2. The inverse correlations between mouse cathepsin L gene expression and plasma glucose levels among all study groups at 3 months (A) ( $r = -0.49, P < 0.01, n = 31$ ) and 15 months (B) ( $r = -0.42, P = 0.02, n = 39$ ). Cathepsin L mRNA was analyzed using a relative quantitative RT-PCR method and expressed relative to cyclophilin.

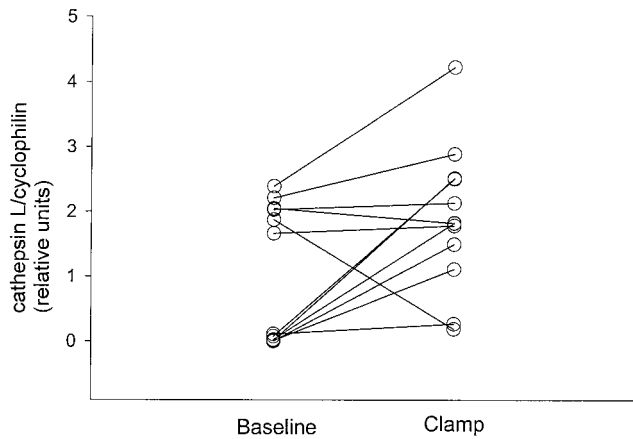
$\text{mg} \cdot \text{kg fat-free mass}^{-1} \cdot \text{min}^{-1}, P < 0.03$ ), which was particularly due to a 37% decrease in the rate of glucose storage ( $4.8 \pm 0.6$  vs.  $7.6 \pm 0.9 \text{ mg} \cdot \text{kg fat-free mass}^{-1} \cdot \text{min}^{-1}, P < 0.02$ ) compared with the control subjects (Table 3).

**Cathepsin L gene expression in humans.** Insulin infusion increased cathepsin L gene expression in control subjects from  $1.03 \pm 0.30$  to  $1.90 \pm 0.32$  relative units ( $P = 0.03$ ) (Fig. 3). Basal cathepsin L expression tended to be lower in the diabetic and nondiabetic twins compared with

TABLE 3  
Clinical characteristics of human subjects

	Diabetic twins	Nondiabetic co-twins	Control subjects
n (F/M)	12 (5/7)	12 (5/7)	12 (5/7)
Age (years)	$64 \pm 3$	$64 \pm 3$	$61 \pm 2$
BMI ( $\text{kg}/\text{m}^2$ )	$30.1 \pm 1.3$	$27.5 \pm 1.3$	$26.0 \pm 1.0$
Fasting plasma glucose (mmol/l)	$11.4 \pm 1.1^*$	$6.0 \pm 0.2^\dagger$	$5.4 \pm 0.2$
Glucose uptake ( $\text{mg} \cdot \text{kg FFM}^{-1} \cdot \text{min}^{-1}$ )	$5.2 \pm 0.7^*$	$8.5 \pm 0.8^\dagger$	$11.4 \pm 0.9$
Glucose storage ( $\text{mg} \cdot \text{kg FFM}^{-1} \cdot \text{min}^{-1}$ )	$2.8 \pm 0.6^*$	$4.8 \pm 0.6^\dagger$	$7.6 \pm 0.9$
Glucose oxidation ( $\text{mg} \cdot \text{kg FFM}^{-1} \cdot \text{min}^{-1}$ )	$2.4 \pm 0.2^*$	$3.7 \pm 0.2$	$3.8 \pm 0.2$
Insulin baseline (pmol/l)	$69.6 \pm 16.5$	$50.9 \pm 6.4$	$45.9 \pm 5.0$
Insulin clamp (pmol/l)	$528.1 \pm 51.6$	$497.9 \pm 40.9$	$559.6 \pm 32.3$

Data are means  $\pm$  SE. \* $P < 0.01$ ;  $^\dagger P < 0.05$  vs. control subjects. FFM, fat-free mass.

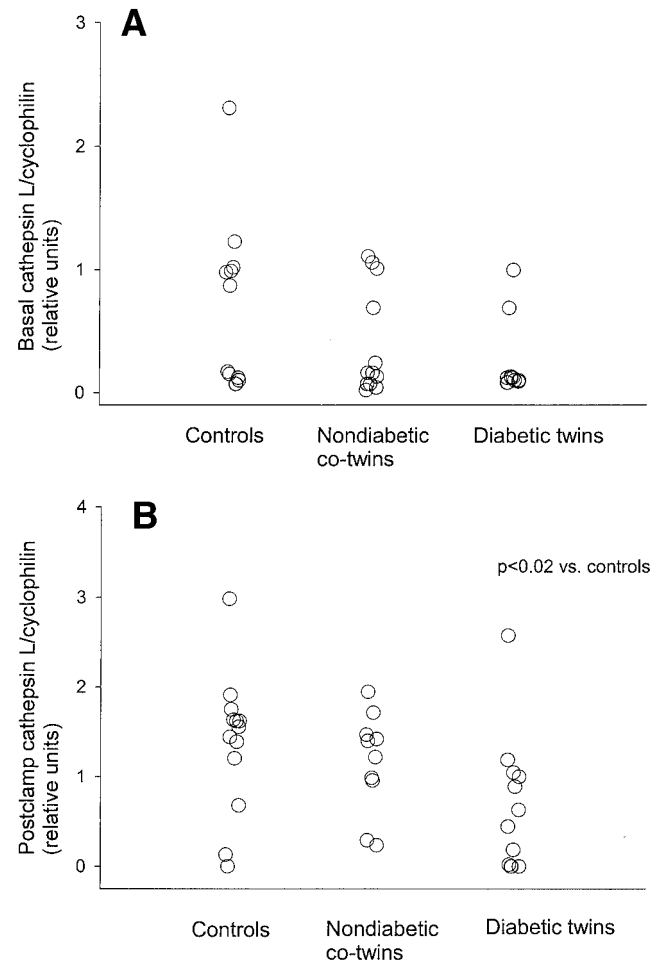


**FIG. 3.** The stimulatory effect of insulin on muscle cathepsin L gene expression ( $P = 0.03$ ) in 12 control subjects. Total RNA was isolated from vastus lateralis biopsies taken before and after an insulin clamp as described in the text. Cathepsin L mRNA was analyzed using a relative quantitative RT-PCR method and expressed relative to cyclophilin.

the control subjects ( $0.22 \pm 0.09$ ,  $0.40 \pm 0.13$  vs.  $0.67 \pm 0.20$  relative units, respectively; NS) (Fig. 4A). Postclamp cathepsin L mRNA levels were lower in diabetic twins but similar in nondiabetic twins compared with control subjects ( $0.66 \pm 0.22$ ,  $1.16 \pm 0.18$  vs.  $1.38 \pm 0.21$  relative units,  $P < 0.02$ , NS, respectively) (Fig. 4B). The postclamp cathepsin L mRNA level correlated with the fasting blood glucose level ( $r = -0.45$ ,  $P < 0.01$ ,  $n = 35$ ), the rate of insulin-mediated glucose uptake ( $r = 0.37$ ,  $P = 0.03$ ,  $n = 35$ ), and the rate of glucose oxidation ( $r = 0.37$ ,  $P = 0.03$ ,  $n = 35$ ) across the three study groups. Similar correlations were also observed in the nondiabetic twins between the postclamp cathepsin L mRNA level and insulin-mediated glucose uptake ( $r = 0.70$ ,  $P = 0.02$ ,  $n = 10$ ) (Fig. 5) or insulin-mediated glucose oxidation ( $r = 0.88$ ,  $P < 0.01$ ,  $n = 10$ ). There was no significant correlation between the postclamp cathepsin L mRNA level and insulin-mediated glucose storage. In addition, we also analyzed cathepsin L gene expression using real-time RT-PCR in basal and postclamp samples from 5 subjects and in postclamp samples from 11 nondiabetic subjects. In keeping with the results from “primer-dropping” RT-PCR, we observed a stimulatory effect of insulin on cathepsin L gene expression (basal  $2.7 \pm 0.35$  relative units, postclamp  $3.8 \pm 0.7$  relative units,  $P = 0.06$ ,  $n = 5$ ). Postclamp cathepsin L gene expression measured with real-time PCR correlated with insulin-mediated glucose uptake ( $r = 0.64$ ,  $P = 0.03$ ,  $n = 11$ ).

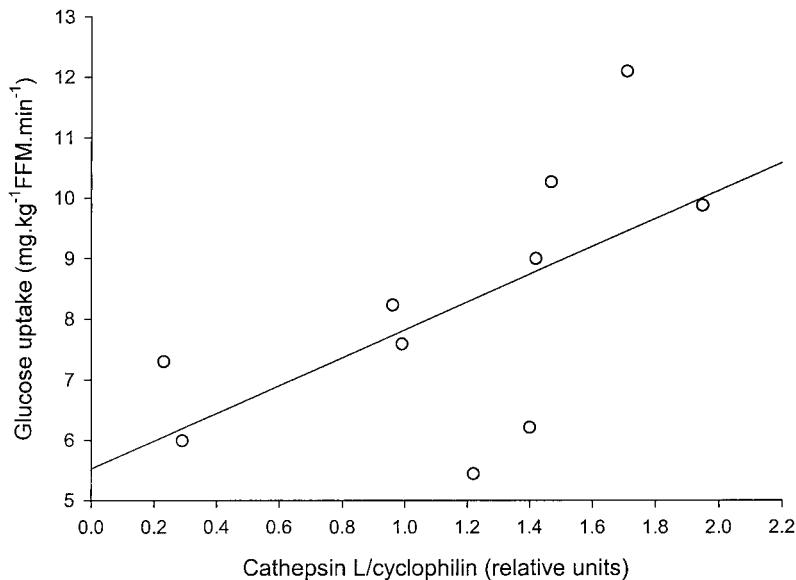
## DISCUSSION

The high-fat diet induced insulin resistance in both C57BL/6J and NMRI mice as judged from the baseline hyperinsulinemia, whereas only C57BL/6J mice showed elevated glucose levels in response to the high-fat diet. Using cDNA differential display, we isolated six differential candidate cDNA clones. Five of them were present in the cDNA panel of C57BL/6J mice fed with a high-fat diet, and one clone was present in the cDNA panel of NMRI mice fed with a high-fat diet. The five cDNA clones from the high-fat-fed C57BL/6J panel represented genes encoding



**FIG. 4.** Basal (A) and postclamp (B) cathepsin L mRNA expression in diabetic twins, nondiabetic twins, and control subjects. Basal cathepsin L mRNA expression tended to be lower in the diabetic (0.22 relative units, 95% CI 0.04–0.42,  $n = 12$ ) and nondiabetic twins (0.40 relative units, 0.12–0.67,  $n = 12$ ) than in the control subjects (0.67 relative units, 0.24–1.1,  $n = 12$ ). Postclamp cathepsin L expression was decreased in the diabetic twins (0.66 relative units, 0.18–1.1,  $n = 12$ ,  $P < 0.02$ ) but not in nondiabetic twins (1.16 relative units, 0.76–1.6,  $n = 10$ , NS) compared with control subjects (1.38 relative units, 0.91–1.8,  $n = 12$ ). Total RNA was isolated from vastus lateralis biopsies taken before and after an insulin clamp as described in the text. Cathepsin L mRNA was analyzed using a relative quantitative RT-PCR method and expressed relative to cyclophilin. Postclamp cathepsin L mRNA levels were measured under different conditions from those in Fig. 3; thus, data were not directly comparable to the data in Fig. 3.

for cathepsin L, mtND4, mtND2, mtCOX1, and a homologue of MAP4. The sequence identity of the cDNA clone from high-fat-fed NMRI panel was unknown. The genes of mtND2, mtND4, and mtCOX1 are all encoded by the mitochondrial H-chain. It has been demonstrated that transcription of genes on the mitochondrial H-chain is under coordinated regulation (17,20,21). The observed correlations for mRNA expression of mtND2, mtND4, and mtCOX1 in the present study are consistent with this notion. We did not see a significant effect of fat feeding on mRNA levels of these six isolated clones measured by RT-PCR (Table 3), as suggested by the cDNA differential display. This discrepancy could have resulted from extrinsic and intrinsic factors such as the experimental design, the band selection, and the low stringent conditions of the differential display, i.e., the short 10-bp primers and the low annealing temperature. We also have to keep in mind



**FIG. 5.** The correlation between postclamp cathepsin L gene expression and insulin-mediated glucose uptake in the nondiabetic twins ( $r = 0.70$ ,  $P = 0.02$ ,  $n = 10$ ). Cathepsin L mRNA was analyzed using a relative quantitative RT-PCR method and expressed relative to cyclophilin. FFM, fat-free mass.

that the differential display was performed on one to two individual samples; therefore, biologic variations can also account for the discrepancy. Despite this, we observed a similar overexpression of cathepsin L and mtND2 in the high-fat-fed C57BL/6J mice with both cDNA differential display and RT-PCR. Of these two genes, cathepsin L gene expression appeared to be associated with glucose intolerance, because an inverse correlation was seen between cathepsin L gene expression and plasma glucose across all groups of mice at 3 and 15 months.

Cathepsin L is a ubiquitously expressed lysosomal cysteine proteinase and is primarily responsible for intracellular protein degradation (22). It has been ascribed a key role in T-cell selection in the thymus (23) and in skin metabolism and hair formation (24). Increased cathepsin L gene expression has been found in wasting skeletal muscle of septic rats (25), whereas decreased cathepsin L gene expression and enzyme activity has been reported in mesangial cells cultured with glucose (26). Further, decreased cathepsin L enzyme activity has been reported in hypertrophied kidneys of streptozotocin-induced diabetic rats (27,28). In keeping with this, we observed an inverse correlation between muscle cathepsin L mRNA expression and plasma glucose levels in mice. Lower postclamp cathepsin L gene expression was also seen in muscle of type 2 diabetic patients, whereas basal cathepsin L gene expression was unchanged. Of note, the cathepsin L mRNA levels correlated with the rate of insulin-mediated glucose uptake, particularly with the rate of insulin-mediated glucose oxidation. This could imply that cathepsin L is in some way involved in glucose transport, which is often coregulated with oxidation. In fact, the ED<sub>50</sub> value (effective dose to produce 50% of the maximum effect) for stimulation of glucose oxidation by insulin is only half of that for stimulation of glucose storage by insulin (29). Interestingly, another cysteine proteinase, calpain-10, was recently identified by positional cloning as a susceptibility gene for type 2 diabetes (30). It is therefore interesting to note that the lower muscle calpain-10 mRNA level also was associated with the lower rate of

24-h carbohydrate oxidation in normal glucose-tolerant humans (31).

Insulin has been ascribed a stimulatory effect on expression of some genes involved in glucose metabolism and insulin action, such as p85 $\alpha$  of phosphatidylinositol 3-kinase, GLUT4, hexokinase II, glycogen synthase, and mitochondrial-encoded ND1 and COX1 in human muscle (17,32–34). In the present study, we demonstrate a stimulatory effect of insulin on cathepsin L gene expression in human muscle. The underlying mechanism for this effect is currently unknown. The observation of impaired cathepsin L gene expression after insulin stimulation in the diabetic twins compared with the control subjects would point at the presence of insulin resistance. This defect is most likely the consequence rather than the cause of the diabetic state, because the impaired postclamp cathepsin L gene expression was seen only in the diabetic but not in the nondiabetic monozygotic co-twin. In keeping with this, decreased cathepsin B and L activity in hypertrophied kidneys of streptozotocin-treated diabetic animals can be normalized by insulin treatment (27,28). Direct evidence is lacking for the effect of glucose on cathepsin L gene expression in muscle. However, in rat mesangial cells, it has been shown that 6-h glucose incubation increased cathepsin L mRNA and protein expression as well as enzyme activity, whereas glucose infusion for >12 h decreased cathepsin L mRNA and protein levels in this cell model (26). A negative effect of long-time glucose exposure (7 days) on cathepsin B and D mRNA expression has also been demonstrated in mesangial cells (35). These results suggest that glucose may acutely stimulate cathepsin L gene expression but, during chronic exposure to it, may downregulate cathepsin L gene expression. If this is also the case in vivo, it is possible that the observed decrease in muscle cathepsin L gene expression in this study reflects insulin resistance and hyperglycemia. Interestingly, the cathepsin L gene is located on chromosome 9q21-22 (36), which has shown suggestive linkage to type 2 diabetes in Finnish, Mexican-American, Pima Indian, and Han Chinese populations (37–41).

In summary, insulin upregulated cathepsin L gene ex-

pression in skeletal muscle, and this effect was impaired in diabetic twins. The study also suggests a relationship between cathepsin L and glucose metabolism. Because cathepsin L gene expression was normal in nondiabetic twins, changes in cathepsin L gene expression may develop as a consequence of the deranged glucose metabolism rather than represent an inherited trait.

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