

# Uncoupling Protein 3 Is Reduced in Skeletal Muscle of NIDDM Patients

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Two recently described proteins in the mitochondrial uncoupling protein (UCP) family, UCP-2 and UCP-3, have been linked to phenotypes of obesity and NIDDM. We determined the mRNA levels of UCP-2 and UCP-3 in skeletal muscle of NIDDM patients and of healthy control subjects. No difference in the mRNA levels or in the protein expression of UCP-2 was observed between the two groups. In contrast, mRNA levels of UCP-3 were significantly reduced in skeletal muscle of NIDDM patients compared with control subjects. In the NIDDM patients, a positive correlation between UCP-3 expression and whole-body insulin-mediated glucose utilization rate was also noted. These results suggest that UCP-3 regulation may be altered in states of insulin resistance. *Diabetes* 47:1528–1531, 1998

Resting skeletal muscle metabolism is a significant determinant of whole-body energy expenditure (1,2). Uncoupling proteins (UCPs) function to uncouple respiration from oxidative phosphorylation and ATP synthesis, converting fuel into heat (3). A substantial part of the basal metabolic rate derives from leaking of protons across the inner mitochondrial membrane. Three UCPs have been described to date. UCP-1 is primarily expressed in brown adipose tissue (3,4). Transgenic mice in which brown fat is ablated develop obesity and insulin resistance (5). Conversely, UCP-1 overexpression in white adipose tissue in genetically obese *A<sup>y</sup>* mice prevents the development of obesity (6). However, because there is little brown fat present in adult humans, UCP-1 is unlikely to play a major role in controlling human energy metabolism.

In humans, skeletal muscle is an important site of thermogenesis, although the molecular mechanisms controlling this process remain unclear (7). The recently identified proteins, UCP-2 and UCP-3, may be candidates for the proton leak observed in skeletal muscle (8–10). UCP-2 and UCP-3 show 59 and 57% amino acid identity to UCP-1, respectively.

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PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; UCP, uncoupling protein.

In adult humans, UCP-2 is expressed in a large number of tissues, including white adipose tissue and skeletal muscle (8,11), whereas UCP-3 appears to be restricted to skeletal muscle (9,10).

Aberrant UCP function could underlie metabolic defects seen in both obesity and NIDDM. We hypothesized that expression levels of UCP-2 and/or UCP-3 may be decreased in skeletal muscle from insulin-resistant NIDDM patients and thus may potentially be involved in the pathogenesis of peripheral insulin resistance. Competitive reverse transcriptase-polymerase chain reaction (RT-PCR) was used to assess UCP-2 and UCP-3 mRNA levels in skeletal muscle obtained from nine NIDDM patients and eight healthy control subjects. UCP-2 protein expression in protein lysates from skeletal muscle was also determined.

## RESEARCH DESIGN AND METHODS

**Subject characteristics.** The study protocol was reviewed and approved by the institutional ethics committee of the Karolinska Institute, and informed consent was received from all subjects before their participation. The clinical characteristics of the subjects are presented in Table 1. The diabetic group consisted of nine male NIDDM patients whose mean duration of disease was 6.5 years and who ranged from those newly diagnosed to those with a duration of disease of 15 years. Patients were treated with insulin ( $n = 1$ ), a combination of sulfonylureas and insulin ( $n = 1$ ), sulfonylureas ( $n = 4$ ), or diet ( $n = 3$ ). The control group consisted of eight healthy male subjects. None of the study participants smoked or took any other medication. The subjects were instructed to abstain from any form of strenuous physical activity for 48 h before the experiment. On the day of the test, the subjects reported to the laboratory after an overnight fast, and in the case of the NIDDM patients, before the administration of any antidiabetic medication.

**Muscle biopsy.** Muscle biopsy specimens were obtained, as described previously (12), under local anesthesia from the vastus lateralis portion of the quadriceps femoris muscle and were immediately placed in liquid nitrogen.

**Euglycemic-hyperinsulinemic clamp.** Insulin-mediated glucose utilization was determined using the euglycemic-hyperinsulinemic clamp procedure, which has been described in detail previously (13,14). This procedure and the muscle biopsy were performed on separate occasions.

**RNA extraction and cDNA synthesis.** Muscle biopsy specimens (25–35 mg) were removed from liquid nitrogen and were homogenized, using a Polytron mixer, in 1 ml guanidium thiocyanate-phenol solution (Sigma Tri-Reagent; Sigma, St. Louis, MO), and total RNA was extracted according to the manufacturer's instructions. The integrity of the extracted RNA was verified by gel electrophoresis, and 2  $\mu$ g of RNA was used as template for subsequent cDNA synthesis in the reverse transcription reaction, using Promega Reverse Transcription System (Promega, Madison, WI) in a 20- $\mu$ l reaction volume according to the manufacturer's instructions. After synthesis was complete, cDNA was diluted tenfold and stored in aliquots at  $-20^{\circ}\text{C}$ .

**Quantification of mRNA.** UCP-2 and UCP-3 mRNA was quantified by reverse transcription followed by competitive RT-PCR, during which known amounts of standard DNA was coamplified with the target cDNA in the same tube. The standard was designed to use the same polymerase chain reaction (PCR) primers as the target but yield a PCR product differing sufficiently in size to allow separation and quantitation of the two amplicons by gel electrophoresis. A multispecific standard with target sequences for UCP-2, UCP-3, and  $\beta$ -globin (Fig. 1) was generated using overlap extension and amplification by PCR (15). The standard was

TABLE 1  
Subject characteristics

	NIDDM	Control
<i>n</i>	9	8
Age (years)	57 ± 2	55 ± 2
BMI (kg/m <sup>2</sup> )	27.3 ± 1.2	26.7 ± 0.8
Glucose utilization (μmol · kg <sup>-1</sup> · min <sup>-1</sup> )	27.35 ± 4.5*	37.02 ± 3.4
Fasting glucose (mmol/l)	9.1 ± 0.8†	5.3 ± 0.4
Fasting insulin (pmol/l)	97 ± 20‡	36 ± 8
HbA <sub>1c</sub> (%)	6.9 ± 0.4†	4.6 ± 0.1
Duration of diabetes (years)	6.7 ± 2.1	—

Data are means ± SE. \**P* < 0.03; †*P* < 0.001; ‡*P* < 0.02. *P* values express significant differences from control subjects.

purified in large quantity and stored as a concentrated stock at -80°C. Working solutions at defined concentrations were prepared by serial dilution, and several aliquots of each dilution were stored at -20°C.

**Competitive PCR.** Each cDNA was coamplified in the presence of known amounts of the multispecific competitor in a PCR reaction with specific primers for UCP-2, UCP-3, or β-μglobin to establish an equivalence point for each of the three genes. To reduce errors, the same working dilution of competitor was used for the analysis of all three genes, and each determination was carried out on two separately synthesized batches of cDNA from each muscle biopsy. The results were then normalized and presented by reference to the mRNA levels of the constitutively expressed β-μglobin gene (15). The absence of contamination by genomic DNA was checked by omitting reverse transcriptase in the reactions. Furthermore, the PCR primers were designed to hybridize in separate exons, thus yielding a product of a different size when hybridizing to genomic DNA. The primer sequences and the resulting product sizes are given in Table 2. The UCP-3 primer pair recognizes sequences shared by both the long (UCP-3<sub>L</sub>) and short (UCP-3<sub>S</sub>) forms of UCP-3 transcripts (16).

**Western blot analysis.** Muscle biopsy specimens were removed from liquid nitrogen and homogenized in ice-cold homogenizing buffer (50 mmol/l HEPES [pH 7.6], 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l Na<sub>2</sub>VO<sub>4</sub>, 10 mmol/l NaF, 30 mmol/l Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10% [vol/vol] glycerol, 1 mmol/l benzamidine, 1 mmol/l dithiothreitol, 10 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride), and lysates cleared by centrifugation at 15,000*g* for 10 min (4°C). Protein was determined using a commercial kit (Bio-Rad, Richmond, CA). Aliquots (30 μg) of the supernatant were solubilized in Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis (10% resolving gel), and transferred to nitrocellulose membranes. Immunodetection of UCP-2 protein was performed using a polyclonal UCP-2 antibody. The antibody was raised against a peptide based on the NH<sub>2</sub>-terminal of UCP-2. After incubation with secondary antibody, the nitrocellulose membrane was placed in a phosphorimager cassette for 24 h, after which the UCP-2 protein was visualized by phosphorimaging and quantified by densitometry.

**Statistical analysis.** Data are presented as means ± SE. Statistical differences were determined using Student's unpaired *t* test. Significance of correlations was determined by simple regression analysis.

## RESULTS

Age and BMI were similar between the two groups (Table 1). Glycemic control, as evaluated by HbA<sub>1c</sub>, was moderate (6.9 ± 0.4%). The normal range for HbA<sub>1c</sub> in our laboratory is <5.2%. The mean peripheral glucose utilization rate achieved

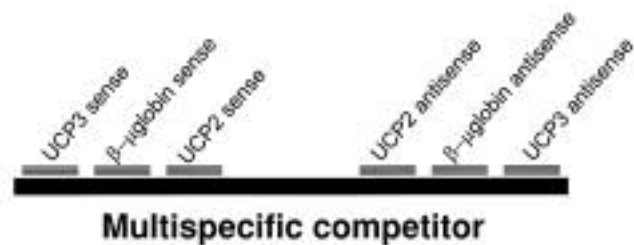


FIG. 1. Schematic outline of the organization of the multispecific competitor. The sequence of primers and the resulting product sizes are given in Table 2.

during steady-state hyperinsulinemia was reduced by 26% in the NIDDM patient group compared with the control group (Table 1). Mean steady-state serum insulin and plasma glucose levels during the last 100 min of the clamp were similar between the two groups (547 ± 67 vs. 516 ± 53 pmol/l for insulin and 5.9 ± 0.2 vs. 5.2 ± 0.1 mmol/l for glucose in NIDDM patients vs. control subjects, respectively; NS).

In human skeletal muscle, UCP-3 was more abundant than UCP-2. A significant degree of variability in the mRNA level of UCP-3 was observed in the skeletal muscle of healthy individuals (Fig. 2). However, mRNA levels of UCP-3 were reduced in the skeletal muscle of NIDDM patients compared with control subjects (41% reduction, *P* < 0.005) (Fig. 2). The skeletal muscle of NIDDM patients did not differ from that of control subjects in that both had a similar abundance of UCP-2 mRNA (Fig. 3A) and protein expression (Fig. 3B).

No correlation was observed between BMI and UCP-3 expression levels in skeletal muscle. In all subjects, a weak negative correlation between BMI and UCP-2 expression in skeletal muscle was observed (*r* = 0.59, *P* < 0.05; data not shown). Interestingly, we observed a positive correlation between UCP-3 expression level in skeletal muscle and insulin sensitivity as determined by the glucose utilization rate during the hyperinsulinemic-euglycemic clamp in the NIDDM patients (*r* = 0.66, *P* < 0.01) (Fig. 2, inset). No such relationship was evident in healthy control subjects.

## DISCUSSION

Both UCP-2 and UCP-3 map to regions of human chromosome 11 and mouse chromosome 7, loci that are associated with hyperinsulinemia and obesity (8). Thus, alterations in the regulation of UCP-2 and UCP-3 could potentially occur in both obesity and NIDDM. The coding region of the UCP-2 gene has been examined in two recent studies of large cohorts of obese subjects, and in neither study have any disease-associated polymorphisms or mutations been reported (17,18). The UCPS appear to be under complex regulation. Thiazolidinedione treatment in human brown preadipocytes

TABLE 2  
Primer sequences and resulting product sizes

Name	Sense primer	Antisense primer	Size of PCR product	
			Gene	Competitor
β-μglobin	5' CCA GCA GAG AAT GGA AAG TC 3'	5' GAT GCT GCT TAC ATG TCT CG 3'	269	309
UCP-2	5' GCT CGT AAT GCC ATT GTC A 3'	5' ACA GTG GCC AGC GCT ACT GTA 3'	220	269
UCP-3	5' ATA CAG CGG GAC TAT GGA C 3'	5' GAA GTA CTG GCC TGG AGG TG 3'	288	400

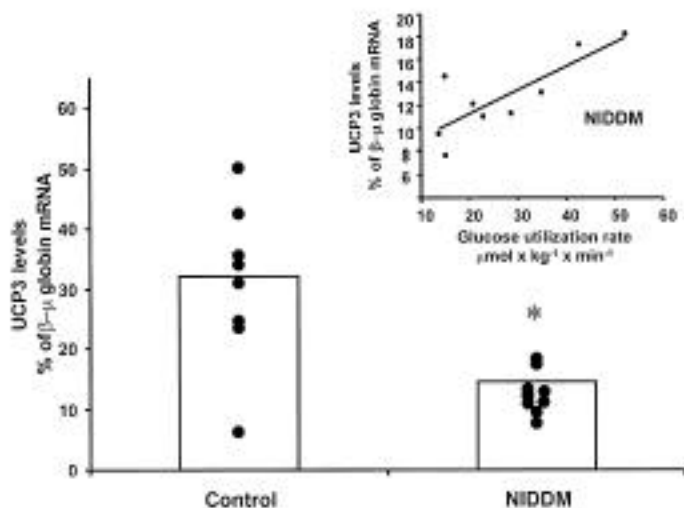


FIG. 2. Individual mRNA levels of UCP-3 in skeletal muscle from NIDDM patients and from control subjects. Results are reported as a percentage of  $\beta$ - $\mu$ globulin mRNA. The superimposed bars show means  $\pm$  SE ( $31.04 \pm 4.70$  and  $12.74 \pm 1.15$  for control vs. NIDDM subjects, respectively). \* $P < 0.005$  versus control values. Inset shows correlation between UCP-3 mRNA levels and insulin-mediated glucose utilization observed in NIDDM patients ( $P < 0.01$ ).

results in an increase in UCP-1 expression (19). UCP-2 has also been shown to be upregulated by thiazolidinediones in skeletal muscle and adipocyte cell lines (20), and by leptin (21). Hypothyroidism in rats is associated with decreased UCP-3 levels in skeletal muscle, while hyperthyroidism is associated with increased expression of UCP-3 (22). Recently, Millet et al. (23) showed that 5-day calorie restriction leads to upregulation of both UCP-2 and UCP-3 in skeletal muscle, and of UCP-2 in adipose tissue, in both lean and obese human subjects. Interestingly, the induction of gene expression in response to food restriction did not differ between the two groups. Furthermore, no significant difference was noted in UCP-2 and UCP-3 expression levels between the lean and obese subjects studied (23). Similarly, Surwit et al. (24) found that resistance to high-fat feeding-induced obesity in mice is associated with induction of UCP-2 expression in white adipose tissue and UCP-1 expression in brown adipose tissue, but not with changes in UCP-3 expression in muscle. However, a similar high-fat feeding challenge in rats resulted in a twofold increase in UCP-3 expression in skeletal muscle and a 1.6-fold increase in UCP-2 expression in adipose tissue, indicating possible species differences (25).

Here we report that mRNA levels of UCP-3 are reduced in skeletal muscle from NIDDM subjects compared with healthy control subjects. Furthermore, in NIDDM subjects, UCP-3 expression is positively correlated with whole-body insulin-mediated glucose utilization. These results suggest that UCP-3 regulation may be altered in states of insulin resistance. Further studies are needed to investigate whether this defect contributes to the pathogenesis of NIDDM.

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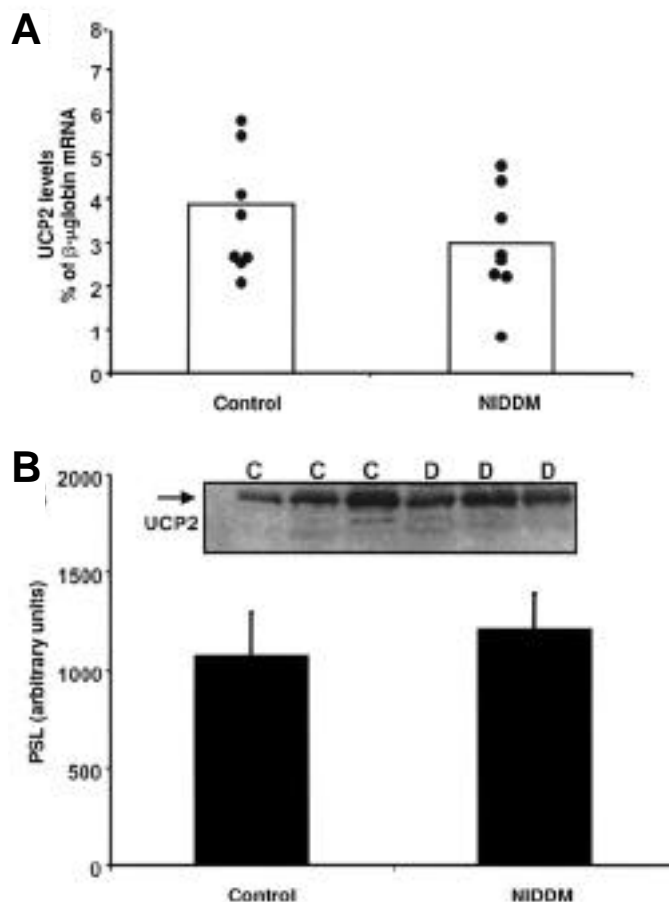


FIG. 3. **A:** Individual mRNA levels of UCP-2 in skeletal muscle from NIDDM patients and healthy control subjects. Results are reported as a percentage of  $\beta$ - $\mu$ globulin mRNA. The superimposed bars show means  $\pm$  SE ( $3.64 \pm 0.50$  and  $2.71 \pm 0.47$  for control vs. NIDDM subjects, respectively). **B:** UCP-2 protein expression in skeletal muscle from NIDDM and control subjects (means  $\pm$  SE), expressed in arbitrary phosphoimager units. Inset shows a representative autoradiogram. C, control; D, NIDDM; PSL, photostimulated luminescence.

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#### REFERENCES

- Zurlo F, Larson K, Bogardus C, Ravussin E: Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J Clin Invest* 86:1423-1427, 1990
- Zurlo F, Nemeth PM, Choksi RM, Sesodia S, Ravussin E: Whole-body energy metabolism and skeletal muscle biochemistry characteristics. *Metabolism* 43:481-486, 1994
- Jacobsson A, Stadler U, Glotzer MA, Kozak LP: Mitochondrial uncoupling protein from mouse brown fat: molecular cloning, genetic mapping, and mRNA expression. *J Biol Chem* 260:16250-16254, 1985
- Bouillaud F, Weissenbach J, Ricquier D: Complete cDNA-derived amino acid sequence of rat brown fat uncoupling protein. *J Biol Chem* 261:1487-1490, 1986
- Lowell BB, Susulic VS, Hamann A, Lawitts JA, Himms-Hagen J, Boyer BB, Kozaki LP, Flier JS: Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* 366:740-742, 1993
- Kopecky J, Clarke G, Enerbäck S, Spiegelman B, Kozak LP: Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. *J Clin Invest* 96:2914-2923, 1995
- Chagnon YC, Perusse L, Bouchard C: Familial aggregation of obesity, candidate genes and quantitative trait loci. *Curr Opin Lipidol* 8:205-211, 1997
- Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi MC, Bouil-

- laud F, Seldin MF, Surwit RS, Ricquier D, Warden CH: Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet* 15:269–272, 1997
9. Vidal-Puig A, Solanes G, Grujic D, Flier JS, Lowell BB: UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem Biophys Res Commun* 235:79–82, 1997
  10. Boss O, Samec S, Paoloni GA, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* 408:39–42, 1997
  11. Gimeno RE, Dembski M, Weng X, Deng N, Shyjan AW, Gimeno CJ, Iris F, Ellis SJ, Woolf EA, Tartaglia LA: Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. *Diabetes* 46:900–906, 1997
  12. Zierath JR, He L, Gumá A, Odegaard-Wahlström E, Klip A, Wallberg-Henriksson H: Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* 39:1180–1189, 1996
  13. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214–E223, 1979
  14. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H: Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes* 47:1281–1286, 1998
  15. Laville M, Aubeoef D, Khalfallah Y, Vega N, Riou JP, Vidal H: Acute regulation by insulin of phosphatidylinositol-3-kinase, Rad, Glut 4, and lipoprotein lipase mRNA levels in human muscle. *J Clin Invest* 98:43–49, 1996
  16. Solanes G, Vidal-Puig A, Grujic D, Flier JS, Lowell BB: The human uncoupling protein-3 gene: genomic structure, chromosomal localization, and genetic basis for short and long form transcripts. *J Biol Chem* 272:25433–25436, 1997
  17. Urhammer SA, Dalgaard LT, Sorensen TI, Moller AM, Andersen T, Tybjaerg-Hansen A, Hansen T, Clausen JO, Vestergaard H, Pedersen O: Mutational analysis of the coding region of the uncoupling protein 2 gene in obese NIDDM patients: impact of a common amino acid polymorphism on juvenile and maturity onset forms of obesity and insulin resistance. *Diabetologia* 40:1227–1230, 1997
  18. Otabe S, Clement K, Rich N, Warden C, Pecqueur C, Neverova M, Raimbault S, Guy-Grand B, Basdevant A, Ricquier D, Froguel P, Vasseur F: Mutation screening of the human UCP 2 gene in normoglycemic and NIDDM morbidly obese patients: lack of association between new UCP 2 polymorphisms and obesity in French Caucasians. *Diabetes* 47:840–842, 1998
  19. Digby JE, Montague CT, Sewter CP, Sanders L, Wilkison WO, O'Rahilly S, Prins JB: Thiazolidinedione exposure increases the expression of uncoupling protein 1 in cultured human preadipocytes. *Diabetes* 47:138–141, 1998
  20. Camirand A, Marie V, Rabelo R, Silva JE: Thiazolidinediones stimulate uncoupling protein-2 expression in cell lines representing white and brown adipose tissues and skeletal muscle. *Endocrinology* 139:428–431, 1998
  21. Zhou YT, Shimabukuro M, Koyama K, Lee Y, Wang MY, Trieu F, Newgard CB, Unger RH: Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation. *Proc Natl Acad Sci U S A* 94:6386–6390, 1997
  22. Gong DW, He Y, Karas M, Reitman M: Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta3-adrenergic agonists, and leptin. *J Biol Chem* 272:24129–24132, 1997
  23. Millet L, Vidal H, Andreelli F, Larrouy D, Riou JP, Ricquier D, Laville M, Langin D: Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. *J Clin Invest* 100:2665–2670, 1997
  24. Surwit RS, Wanf S, Petro AE, Sanchis D, Raimbault S, Ricquier D, Collins S: Diet-induced changes in uncoupling proteins in obesity-prone and obesity-resistant strains of mice. *Proc Natl Acad Sci U S A* 95:4061–4065, 1998
  25. Matsuda J, Hosoda K, Itoh H, Son C, Doi K, Tanaka T, Fukunaga Y, Inoue G, Nishimura H, Yoshimasa Y, Yamori Y, Nakao K: Cloning of rat uncoupling protein-3 and uncoupling protein-2 cDNAs: their gene expression in rats fed high-fat diet. *FEBS Lett* 418:200–204, 1997