with atorvastatin did not alter BAP, but decreased urinary NTx in 16 elderly patients (3 men and 13 women) with hypercholesterolemia, very similar to our results. Moreover, an *in vitro* study has recently shown antiresorptive effects of statins as well as bone-formative effects [3]. More recently, Staal *et al.* [5] have shown that statins *in vitro* inhibit bone resorption but do not increase bone formation, consistent with our results. Based on all these observations above, it is likely that clinical use of atorvastatin may have beneficial effects on bone metabolism mostly by reducing bone resorption rather than by stimulating bone formation.

Although our study found potentially beneficial effects of atorvastatin on bone metabolism, whether statins can really increase bone mass to reduce the risk of fractures has not reached consensus yet [2]. Since it has been suggested that the bone turnover markers are good predictors for the subsequent change of BMD, and their improvement independently contributes to reduction of fracture risk [23], the significant improvement in bone turnover by atorvastatin in our study is expected not only to augment their bone mass but also to reduce the future risk of fractures. However, the magnitude of the antiresorptive effect of statins, as measured by changes of bone turnover markers in our study, seems to be far less pronounced than that of bisphosphonates [2, 24], probably due to their lower affinity to bone. Further studies are therefore needed to resolve whether this beneficial but relatively weak effect of atorvastatin on bone metabolism found in our study is of clinical relevance.

Since the present study has some limitations, our results should be interpreted cautiously. First, it did not include large numbers of patients. Second, we had no control subjects to compare and be more certain of the changes found in our study. Thus, the improvement of bone metabolism in our study may be explained partly by dietary and exercise advice given since the baseline. In addition, another possibilityis is that hypercholesterolemia itself might have been associated with higher bone turnover, and that reduced bone resorption found in our study might have only reflected the hypercholesterolemia improved by atorvastatin. Indeed, Koshiyama *et al.* [25] have recently reported the possibility that hypercholesterolemia may be the main cause of abnormal bone metabolism in type 2 diabetes mellitus [26]. However, ΔNTx did not correlate with ΔTC, ΔTG, ΔLDL or ΔHDL in our study. Third, whether our short-term results will similarly be found in a long-term study remains unclear. Lastly, our subjects were recruited from patients with hypercholesterolemia, but not those diagnosed as osteoporosis. As Rosenson *et al.* [15] stated in their report, the antiresorptive effect of atorvastatin found in our patients with hypercholesterolemia might be more pronounced in osteoporotic subjects, as in the case with bisphosphonates [27]. Some beneficial effects of atorvastatin on bone metabolism might have been partially obscured in the present study.

In summary, in a 3-month prospective study, we could not find that atorvastatin increased BAP in the patients with hypercholesterolemia, contrary to our expectations from previous *in vitro* studies in the literature. On the other hand, we found that atorvastatin significantly reduced NTx in these patients. These findings indicate that atorvastatin may exert beneficial effects on bone metabolism in patients with hypercholesterolemia mostly by reducing bone resorption rather than by stimulating bone formation. Further studies with more patients and longer duration are warranted to evaluate its effects, if any, on the prevention of osteoporosis and subsequent fractures.

Reference

1. Miwa S, Watada H, Omura C, Takayanagi N, Nishiyama K, Tanaka Y, Onuma T, Kawamori R (2005) Antioxidative effect of fluvastatin in hyperlipidemic type 2 diabetic patients. *Endocr J* 52: 259–264.
2. Bauer DC (2003) HMG CoA reductase inhibitors and the skeletal: A comprehensive review. *Osteoporos Int* 14: 273–282.
3. Mundy G, Garrett R, Harris S, *et al.* (1999) Stimulation of bone formation in vitro and in rodents by statins. *Science* 286: 1946–1949.
4. Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S (2000) Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. *Biochem Biophys Res Commun* 271: 688–692.
5. Staal A, Frith JC, French MH, Swartz J, Gungor T, Harrity TW, Tamasi J, Rogers MJ, Feyen JH (2003) The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. *J Bone Miner Res* 18: 88–96.

6. Massy ZA, Keane WF, Kasiske BL (1996) Inhibition of the mevalonate pathway: benefits beyond cholesterol reduction? *Lancet* 347: 102–103.
7. Rejnmark L, Buus NH, Vestergaard P, Andreasen F, Larsen ML, Mosekilde L (2002) Statins decrease bone turnover in postmenopausal women: A cross-sectional study. *Eur J Clin Invest* 32: 581–589.
8. Kajinami K, Takekoshi N, Matsui S, Kanemitsu S, Okubo S, Kanayama S, Yamashita N, Sato R (2003) Effect of pretreatment vitamin D levels on in vivo effects of atorvastatin on bone metabolism in patients with heterozygous familial hypercholesterolemia. *Am J Cardiol* 92: 1113–1116.
9. Kuzuya M, Suzuki Y, Asai T, Koike T, Kanda S, Nakamura A, Satake S, Umegaki H, Iguchi A (2003) Atorvastatin, 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor, reduces bone resorption in the elderly. *J Am Geriatr Soc* 51: 1677–1678.
10. Berthold HK, Unverdorben S, Zittermann A, Degenhardt R, Baumeister B, Unverdorben M, Krone W, Vetter H, Gouni-Berthold I (2004) Age-dependent effects of atorvastatin on biochemical bone turnover markers: a randomized controlled trial in postmenopausal women. *Osteoporos Int* 15: 459–467.
11. Braatvedt GD, Bagg W, Gamble G, Davidson J, Reid IR (2004) The effect of atorvastatin on markers of bone turnover in patients with type 2 diabetes. *Bone* 35: 766–770.
12. Bjarnason NH, Riis BJ, Christiansen C (2001) The effect of fluvastatin on parameters of bone remodeling. *Osteoporos Int* 12: 380–384.
13. Stein EA, Farnier M, Waldstreicher J, Mercuri M; Simvastatin/Atorvastatin Study Group (2001) Effects of statins on biomarkers of bone metabolism: a randomised trial. *Nutr Metab Cardiovasc Dis* 11: 84–87.
14. Rejnmark L, Buus NH, Vestergaard P, Heickendorff L, Andreasen F, Larsen ML, Mosekilde L (2004) Effects of simvastatin on bone turnover and BMD: a 1-year randomized controlled trial in postmenopausal osteopenic women. *J Bone Miner Res* 19: 737–744.
15. Rosenson RS, Tangney CC, Langman CB, Parker TS, Levine DM, Gordon BR (2005) Short-term reduction in bone markers with high-dose simvastatin. *Osteoporos Int* 16: 1272–1276.
16. Watanabe S, Fukumoto S, Takeuchi Y, Fujita H, Nakano T, Fujita T (2001) Effects of 1-year treatment with fluvastatin or pravastatin on bone. *Am J Med* 110: 584–587.
17. Rejnmark L, Olsen ML, Johnsen SP, Vestergaard P, Sorensen HT, Mosekilde L (2004) Hip fracture risk in statin users — a population-based Danish case-control study. *Osteoporos Int* 15: 452–458.
18. Wada Y, Nakamura Y, Koshiyama H (2000) Lack of positive correlation between statin use and bone mineral density in Japanese subjects with type 2 diabetes. *Arch Intern Med* 160: 2865–2870.
19. Sirola J, Honkanen R, Kroger H, Jurvelin JS, Maenpaa P, Saarikoski S (2002) Relation of statin use and bone loss: A prospective population-based cohort study in early postmenopausal women. *Osteoporos Int* 13: 537–541.
20. Maeda T, Kawane T, Horiuchi N (2003) Statins augment vascular endothelial growth factor expression in osteoblastic cells via inhibition of protein prenylation. *Endocrinology* 144: 681–692.
21. Riggs BL, Melton LJ 3rd (1986) Involutional osteoporosis. *N Engl J Med* 314: 1676–1686.
22. Oxlund H, Andreassen TT (2004) Simvastatin treatment partially prevents ovariectomy-induced bone loss while increasing cortical bone formation. *Bone* 34: 609–618.
23. Garnero P, Dargent-Molina P, Hans D, Schott AM, Breart G, Meunier PJ, Delmas PD (1998) Do markers of bone resorption add to bone mineral density and ultrasonographic heel measurement for the prediction of hip fracture in elderly women? The EPIDOS prospective study. *Osteoporos Int* 8: 563–569.
24. Kress BC, Mizrahi IA, Armour KW, Marcus R, Emkey RD, Santora AC 2nd (1999) Use of bone alkaline phosphatase to monitor alendronate therapy in individual postmenopausal osteoporotic women. *Clin Chem* 45: 1009–1017.
25. Koshiyama H, Wada Y, Nakamura Y (2001) Hypercholesterolemia as a possible risk factor for osteopenia in type 2 diabetes mellitus. *Arch Intern Med* 161: 1678.
26. Majima T, Komatsu Y, Yamada T, Koike Y, Shigemoto M, Takagi C, Hatanaka I, Nakao K (2005) Decreased bone mineral density at the distal radius, but not at the lumbar spine or the femoral neck, in Japanese type 2 diabetic patients. *Osteoporos Int* 16: 907–913.
27. McClung MR, Geusens P, Miller PD, Zippel H, Bensen WG, Roux C, Adami S, Fogelman I, Diamond T, Eastell R, Meunier PJ, Reginster JY; Hip Intervention Program Study Group (2001) Effect of risedronate on the risk of hip fracture in elderly women. Hip Intervention Program Study Group. *N Engl J Med* 344: 333–340.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 353 (2007) 67–73
BBRC
www.elsevier.com/locate/ybbrc

NRSF regulates the developmental and hypertrophic changes of HCN4 transcription in rat cardiac myocytes

Shinobu Kuratomi [a], Akiko Kuratomi [a], Koichiro Kuwahara [b], Takahiro M. Ishii [c], Kazuwa Nakao [b], Yoshihiko Saito [d], Makoto Takano [a,*]

[a] *Department of Physiology, Jichi Medical University, Shimotsuke, Tochigi, Japan*
[b] *Department of Medicine and Clinical Sciences, Graduate School of Medicine, Kyoto University, Kyoto, Japan*
[c] *Department of Physiology, Graduate School of Medicine, Kyoto University, Kyoto, Japan*
[d] *First Department of Internal Medicine, Nara Medical University, Nara, Japan*

Received 13 November 2006
Available online 4 December 2006

Abstract

The HCN4 channel shows differential expression patterns during the embryonic development and hypertrophy of hearts. Briefly, HCN4 expression is maximally activated in embryonic hearts and quickly diminishes after birth. However, it is reactivated during cardiac hypertrophy. The sequence analysis of HCN4 gene revealed the presence of a conserved NRSE motif, which is known to bind the transcriptional factor neuron-restrictive silencing factor (NRSF). A promoter analysis of HCN4 with rat cardiac myocytes identified the region inducing a basal transcriptional activity. This region drove a high activity in embryonic myocytes, but not in neonatal myocytes treated with hypertrophic agents. After confirming that NRSF protein binds to the NRSE, HCN4 promoter activities modified by NRSE were evaluated. With wild-type NRSE, the promoter activity correlated well with the developmental and hypertrophic changes of HCN4 expression, whereas mutant NRSE constructs failed. We conclude that the NRSE–NRSF system was implicated in HCN4 expression in cardiac myocytes.

Keywords: HCN4 channel; NRSE; NRSF; Transcription; Cardiac myocyte; Development; Hypertrophy

Hyperpolarization-activated, cyclic nucleotide-gated cation channels (HCN channels) are expressed in a variety of cardiac cells and neurons. These channels generate an inward current termed I_f, which plays a crucial role in the autonomous rhythmic activity of excitable cells [1]. To date, four mammalian HCN isoforms (HCN1–4) have been identified, three of which (HCN1, HCN2, and HCN4) are present in the heart with varying expression levels in different cardiac regions [2,3]. Among these isoforms, HCN4 is best known as the predominant isoform in the pacemaker region located in the sinoatrial (SA) node [2,4]. The HCN4 channel gene is also a member of embryonic cardiac genes. The HCN4 channel is widely expressed in the heart in the early developmental stage of an embryo, but its expression diminishes with embryonic differentiation [5]. In most forms of cardiac hypertrophy, there is an increase in the expression of embryonic genes, including ion channels. The upregulation of HCN4 expression in the heart is considered to underlie arrhythmogenesis during cardiac hypertrophy and heart failure [6,7]. Therefore, the transcriptional regulation of the HCN4 appears to be important for understanding the mechanisms of differentiation of the electrophysiological properties of cardiac myocytes and the mechanisms underlying the electrical remodeling of diseased hearts [8].

The neuron-restrictive silencer element (NRSE [9]) has been identified as a negative regulatory element that silences neuronal gene expression in nonneuronal cells [10]. The repression is induced through the binding of the zinc finger

* Corresponding author. Fax: +81 285 40 6294.
E-mail address: takanom@jichi.ac.jp (M. Takano).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.119

transcriptional factor neuron-restrictive silencer factor (NRSF [11]). Using transgenic mice expressing dominant negative NRSF in their hearts, we previously reported that NRSF plays a critical role in the structural and functional alterations that occur during heart failure [12]. These transgenic mice exhibit increased vulnerability to arrhythmias and the upregulation of HCN4 channel expression in their hearts.

The objective of the present study was to characterize the transcriptional regulation through the NRSE–NRSF system in HCN4 expression during the development and hypertrophy of cardiac myocytes. Here, we identified a functional NRSE regulatory DNA element located within the first intron of the HCN4 gene and demonstrated that the NRSE–NRSF system regulated the HCN4 gene promoter during cardiac myocyte development and hypertrophy.

Materials and methods

Rapid amplification of cDNA ends (RACE). RNA ligase-mediated 5′-RACE was performed using the GeneRacer kit (Invitrogen) by using the additional reverse primers; HCN4-specific primer (5′-CAT GGC ACC GAA CTG GCG CTG CAT GAA G-3′) and HCN4-specific nested primer (5′-GCC GCG CCT CCC TCC ACT TTG ATA-3′). The amplified products were sequenced to determine the transcription start site.

Construction of a promoter reporter plasmid. Various fragments of the promoter of the mouse HCN4 gene were isolated from a mouse genomic bacterial artificial chromosome (BAC) and cloned into the luciferase reporter plasmid pGL3-Basic (Promega). The intronic fragments containing mouse HCN4 NRSE at its center were prepared by performing PCR. All constructs were verified by DNA sequencing.

Cell preparation and culture. Rat cardiac myocytes were cultured as previously described [13]. In neonatal myocyte assays, the cardiac ventricles were excised from 2- to 4-day-old Wistar rats. For embryonic myocytes, embryonic rat whole hearts were isolated from anesthetized pregnant rats (postcoitum day 12.5). After enzymatic digestion of their tissues, a cardiac myocyte fraction was prepared by Percoll gradient centrifugation (GE Healthcare). After 24 h of cell plating, the cells were cultured in serum-free media. In hypertrophy-inducing assays, the media were replaced by media containing hypertrophy-inducing agents (50 μM phenylephrine (PE) or 50 nM endothelin (ET)-1) 24 h after the first media change.

Transient transfection and luciferase reporter assay. Cardiac myocytes grown in 24-well plates were transfected by using Lipofectamine (Invitrogen). The cells were cotransfected with 0.6 μg of the luciferase reporter construct and 0.2 μg of the pRL-TK vector (Promega) expressing *Renilla* luciferase. Following a 72-h culture, the cell lysate was harvested, and the luciferase activity was measured using Promega's Dual-Luciferase Reporter Assay System. All luciferase activities were normalized with the *Renilla* luciferase activities.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from cultured neonatal rat cardiac myocytes by using NE-PER kit (Pierce). The radiolabeled probe was prepared using 22-bp oligonucleotides containing the intronic NRSE of HCN4 gene. The binding reaction was performed in a 20-μl final volume of a reaction buffer containing 20 mM Hepes (pH 7.6), 150 mM KCl, 2.5 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and 10 μg poly(dI–dC) per ml. The nuclear extract (5 μg of protein) was added to the reaction buffer and incubated with 10 fmol of a probe for 30 min on ice. In the competition experiments, 10- or 100-fold molar excess competitors were coincubated. For EMSA-antibody assays, the nuclear extracts were preincubated with 1 μg of antibodies. The samples were separated by electrophoresis on 5% polyacrylamide gels in 0.25× TBE buffer.

Chromatin immunoprecipitation (ChIP). Chromatin from neonatal rat primary cardiac myocytes was prepared using the ChIP assay kit (Upstate). The purified chromatin was immunoprecipitated using NRSF antibody (Upstate). The immunoprecipitated product was analyzed by PCR using the following primer pairs: NRSE ChIP primers 5′-AGA GGG TGG TAT ACA CTG GAG AAG-3′ (forward) and 5′-ACT ACA CTG GGA AGA TGA GAG GAT-3′ (reverse) and control ChIP primers 5′-AAT GGG ACT CCT CTT ACT CAT TTC T-3′ (forward) and 5′-AAA GTC CCT GAT GAC ACA CTA GTT C-3′ (reverse).

RT-PCR and quantitative real-time RT-PCR analysis. Conventional RT-PCR was performed using Platinum PCR SuperMix (Invitrogen). To avoid the amplification of genomic DNA, each pair of primers was designed to reside in the exons separated by introns. Quantitative real-time RT-PCR was conducted for HCN4 and GAPDH by using predesigned TaqMan Gene Expression Assays (Applied Biosystems). The reaction was performed on ABI Prism 7700 System (Applied Biosystems). The mRNA levels of HCN4 were normalized to the endogenous GAPDH.

Western blotting analysis. Nuclear proteins were prepared from ~10^7 cultured neonatal rat cardiac myocytes by using an NE-PER kit (Pierce). The proteins were separated on 6% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and incubated with a NRSF antibody (1:500; Upstate). Signals were detected using ECL system (GE Healthcare).

Statistical analysis. Data were expressed as means ± SE values. Statistical analysis was performed using Student's *t*-test. The *P* values less than 0.05 were interpreted to represent statistically significant differences.

Results

Identification of the mouse HCN4 promoter region and the NRSE within the intron region

To determine whether NRSF participates in the transcriptional regulation of HCN4, we searched for an NRSE-like sequence in the mouse HCN4 genomic sequence. The computer search revealed that an NRSE-like sequence was located ~2 kb upstream of exon 2 of the HCN4 gene (Fig. 1, upper panel). As shown in the inset of Fig. 1, the NRSE-like sequence identified within the intron of the HCN4 gene is highly conserved among several species and is homologous to the consensus NRSE, suggesting that it might mediate an important regulatory function in the HCN4 transcription. We next attempted to identify the promoter region. The mouse HCN4 gene consists of eight exons, and the translational initiation site (ATG) is located within exon 1. The transcription start site (+1) in the heart was determined by a 5′-RACE reaction performed with several primers anchored in exon 1 to amplify the total RNA isolated from mouse hearts. Cloning and sequencing of the 5′-RACE product revealed the start site located 401 bp upstream from the ATG. Several restriction sites used in the subsequent promoter truncation assays are shown in the promoter region (Fig. 1, lower panel).

Functional analysis of the HCN4 promoter region

We next evaluated the transcriptional activity of the fragment comprising bp −3075 to +400 of the region of the HCN4 gene in cardiac myocytes. A series of HCN4

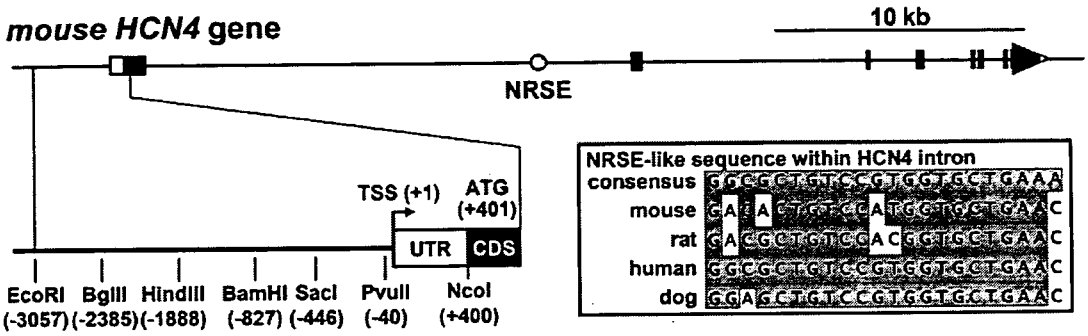

Fig. 1. Genomic structure of the mouse HCN4 gene and identification of the conserved NRSE. The boxes represent exons, including the coding sequence (CDS) and the untranslated region (UTR). The arrows indicate the transcription start site (+1). The inset shows the consensus sequence of NRSE and the conserved region of the intronic NRSE-like sequences in HCN4 among four species. The shadowed areas indicate the sequence that is homologous to the consensus NRSE.

promoter deletion luciferase reporter constructs were generated and transiently transfected into the primary cultures of neonatal rat cardiac myocytes (Fig. 2A). Serial truncations from −3057 to −446 bp regions resulted in an increase in transcriptional activity, suggesting the presence of potential negative regulatory elements in this region. Further deletion of the promoter region led to a decrease in reporter activity. Therefore, we concluded that the −446/+400 promoter region was responsible for the basal transcriptional activity of HCN4, and this fragment was used as the basic HCN4 promoter in the following experiments.

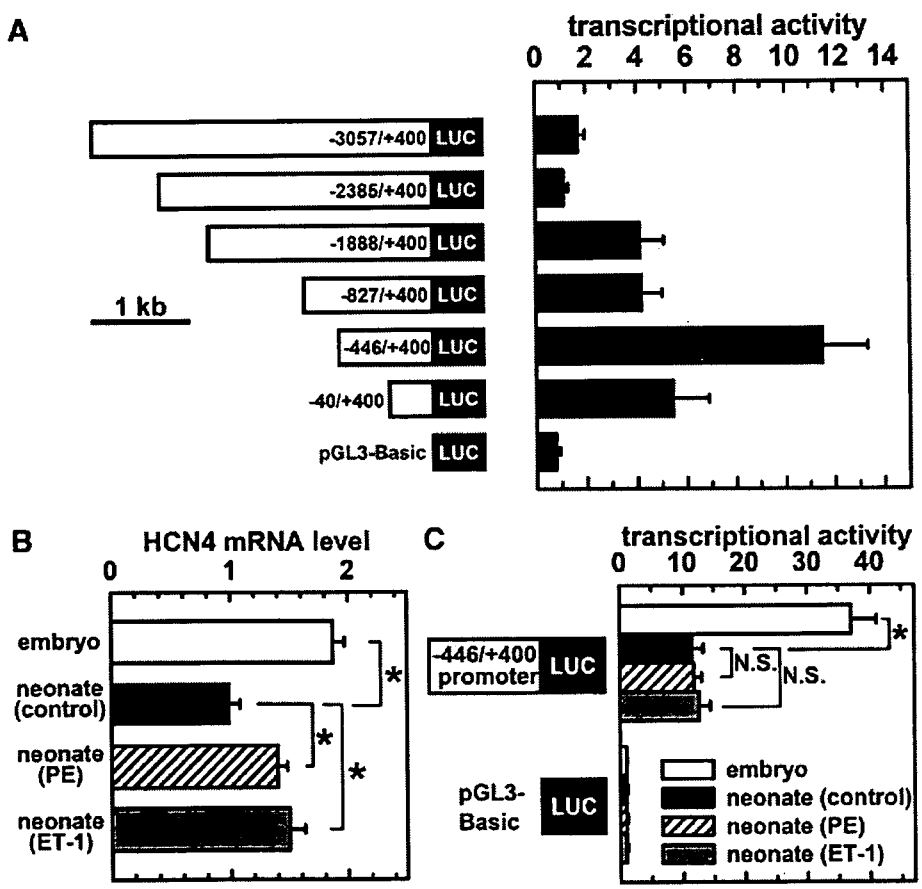

Fig. 2. Transcriptional activity of the mouse HCN4 promoter region. (A) Various 5′ truncated promoter reporter constructs were transfected into the neonatal rat primary cardiac myocytes. (B) Quantification of mRNA with real-time PCR using the primary cultures of embryonic, neonatal control, neonatal PE-treated, and neonatal ET-1-treated cardiac myocytes. The mRNA levels are expressed as normalized values relative to the neonatal control myocytes. (C) Transcriptional activity of the −446/+400 fragment in the embryonic, neonatal control, neonatal PE-treated, and neonatal ET-1-treated cardiac myocytes. All data are expressed as means ± SE values obtained from at least three separate assays carried out in quadruplicate. $^{*}P < 0.05$.

The HCN4 expression in cardiac myocytes is maximally activated in the early embryonic stage and diminishes in the course of embryonic development. However, HCN4 expression is reactivated during cardiac myocyte hypertrophy [6,7]. To assess whether the −446/+400 promoter activity quantitatively mimics the above pattern of HCN4 expression, we prepared several types of primary cultures of cardiac myocytes. We first compared the mRNA level of HCN4 in embryonic and neonatal myocytes. As shown in Fig. 2B, the mRNA level of HCN4 was higher in embryonic myocytes. When neonatal cardiac myocytes were stimulated with PE and ET-1 (commonly used hypertrophy-inducing agents) the HCN4 mRNA was also upregulated.

We then performed reporter assays with the −446/+400 promoter construct in these myocytes. In this assay, a pGL3-Basic vector was used as control. As shown in Fig. 2C, embryonic myocytes expressed ~3-fold higher activity compared to neonatal control myocytes, whereas no significant increases in the reporter activity were detected in neonatal myocytes stimulated with PE and ET-1. These results appear to indicate that −446/+400 promoter activity results in the high expression level of HCN4 in embryonic myocytes, but is not involved in HCN4 expression induced by PE and ET-1. The HCN4 gene appeared to contain other regulatory elements associated with the changes in HCN4 expression, and the intronic NRSE may be a putative important regulatory element.

NRSF represses the HCN4 promoter activity via the NRSE of the intron

To determine whether *cis*-regulatory functions are encoded in the intronic NRSE-like sequence of the HCN4 gene, we inserted the ~3-kb intron fragment containing the NRSE sequence at its center into the −446/+400 HCN4 promoter construct and performed reporter assays by using the neonatal rat cardiac myocytes (Fig. 3A). Compared to the transcriptional activity of the −446/+400 HCN4 promoter, that of the wild-type NRSE construct was attenuated to less than 18%. This supported the hypothesis that the inserted fragment encoded repression activity, and this activity was possibly mediated by the NRSE-like sequence. To confirm this, we prepared the construct of mutated NRSE [9,13]. When the mutant NRSE construct was transfected, the repression by the wild-type NRSE almost disappeared. These findings indicated that the intact NRSE sequence was necessary for the repression activity.

To investigate whether the nuclei of the cardiac myocytes bind to the NRSE of the HCN4 gene and to establish whether this binding activity is conferred by NRSF protein, we conducted an EMSA. A radiolabeled 22-bp oligonucleotide containing the HCN4 NRSE sequence was used as a probe. As shown by the arrow in Fig. 3B, a prominent slow-migrating DNA–protein complex was visualized with nuclear extracts. This complex was also formed with the consensus NRSE probe (data not shown). We next performed a competition experiments by using an unlabeled oligonucleotide containing wild-type or mutated NRSE. The signal for the prominent complex was abolished by the addition of wild-type NRSE oligonucleotide, whereas the mutated NRSE oligonucleotide was much less effective. This demonstrates that the major band was NRSE sequence-specific. We further examined whether the NRSE sequence-specific complex is related to the NRSF protein. The EMSA was performed with an NRSF antibody (Fig. 3C). The major complex was specifically eliminated by the NRSF antibody, but not by nonspecific antibodies. The interaction between the NRSF protein and the NRSE of HCN4 was also confirmed by the ChIP assay (Fig. 3D). The DNA immunoprecipitated with the NRSF antibody was amplified by the NRSE ChIP primers specifically recognizing the intronic NRSE region of HCN4, but the sample with the control IgG was not. Furthermore, coimmunoprecipitation was not observed with control ChIP primers, which recognized the first intron region of HCN4 but was located away from NRSE. Altogether, these findings indicated that NRSF directly binds to the NRSE of the HCN4 intron and represses HCN4 promoter activity in cardiac myocytes.

The NRSE–NRSF system is functionally important for cardiac HCN4 transcriptional regulation

Having confirmed that the NRSE–NRSF regulatory system is involved in the transcription of HCN4, we next investigated the functional role of NRSE–NRSF in the HCN4 expression in developmental and hypertrophic changes of cardiac myocytes. Because the −446/+400 promoter construct failed to mimic the HCN4 expression pattern (Fig. 2B and C), we conducted the reporter assays using the NRSE constructs with the primary cultures of embryonic, neonatal control, neonatal PE-treated, and neonatal ET-1-treated cardiac myocytes. To evaluate the NRSE–NRSF-specific transcriptional function, the reporter gene activities were normalized to the −446/+400 promoter activity expressed in the respective cells, and the activity in neonatal control myocytes was assigned a value of one (Fig. 4A). The embryonic myocytes expressed ~3.5-fold higher promoter activity compared to neonatal control cells, whereas the myocytes stimulated with PE and ET-1 displayed ~2-fold increases in the reporter activity. These data appeared to correlate closely with the HCN4 mRNA expression demonstrated in Fig. 2B. In contrast, mutant NRSE construct demonstrated no significant differences. These notable results indicate that the NRSE–NRSF system mediates an important regulatory function in the HCN4 expression of the embryonic myocytes and in the HCN4 upregulation induced by PE and ET-1.

Finally, we investigated whether NRSF could regulate the developmental change in HCN4 expression. We performed an RT-PCR to examine the mRNA levels of HCN4 and NRSF by using the total RNA isolated from rat hearts in the embryonic and adult stages. As shown

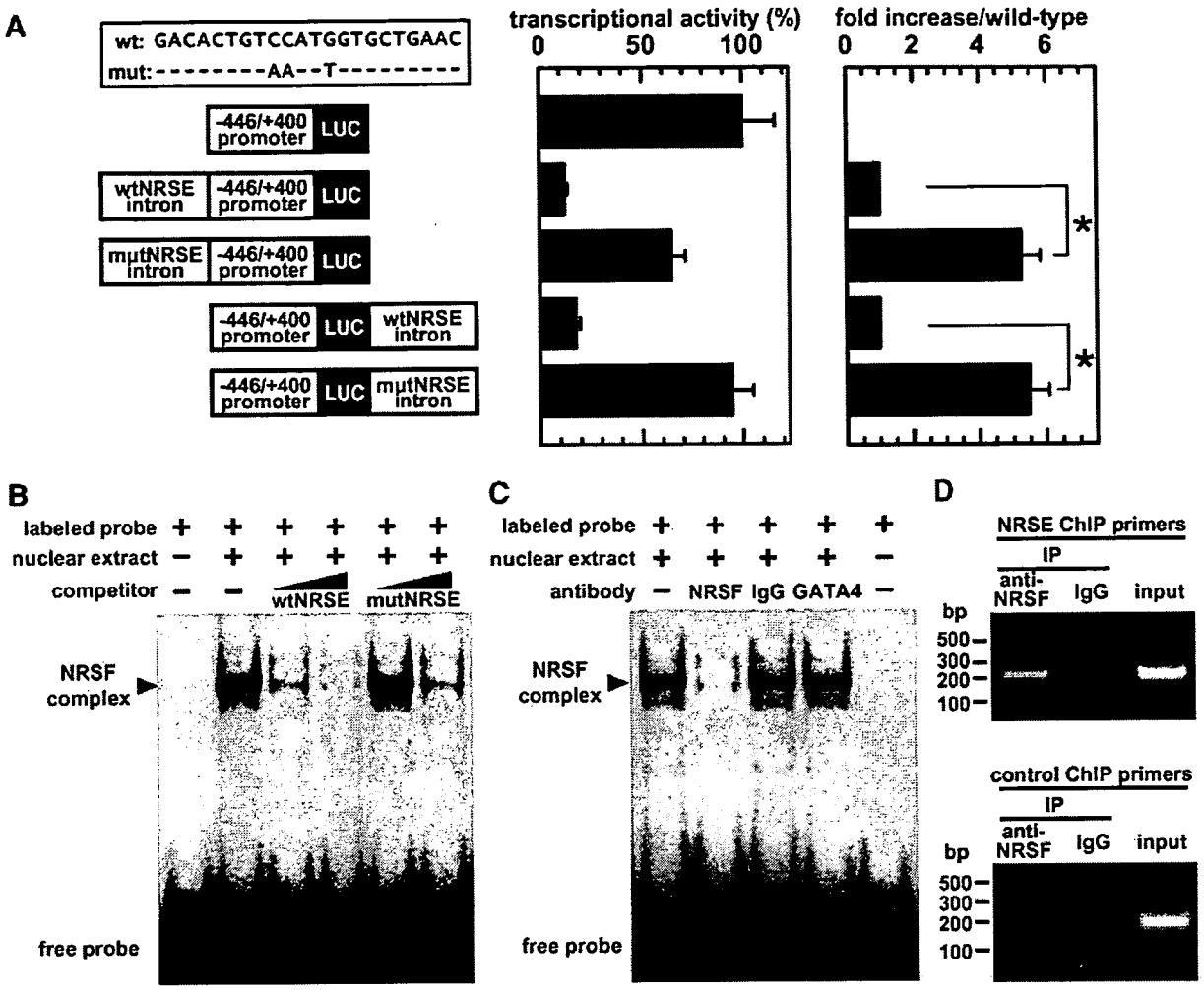

Fig. 3. Transcriptional activity of the intronic NRSE and NRSF binding to the HCN4 NRSE. (A) The inset shows the wild-type and mutated HCN4 NRSE. The constructs illustrated in the left were used in reporter assays with the control culture of neonatal rat cardiac myocytes. The bar graph in the right side demonstrates the fold increases by mutagenesis. The results are expressed as means ± SE $^*P < 0.05$. (B) EMSA with labeled HCN4 NRSE probe performed using the nuclear extracts from the neonatal cardiac myocytes. The binding activity of NRSF complex was competed by adding unlabeled wild-type NRSE oligonucleotide (wtNRSE) but not mutated NRSE (mutNRSE). (C) EMSA was performed using a NRSF antibody and nonspecific antibodies (normal IgG, GATA4 antibody). (D) The ChIP assay was performed by using a NRSF antibody and control IgG. The immunoprecipitates were analyzed using PCR with two sets of primers—NRSE ChIP and control ChIP primers. The 230-bp (upper panel) and 200-bp (lower panel) products correspond to the NRSE and nonNRSE regions, respectively.

in Fig. 4B, the expression of HCN4 mRNA decreases with development; conversely, the mRNA of NRSF increases. This inverse correlation between HCN4 and NRSF is consistent with our findings that NRSF negatively regulates HCN4 expression, implying that the increase in NRSF might repress HCN4 expression during the embryonic development of hearts. In contrast, our previous study has demonstrated that the mRNA level of NRSF did not decrease with hypertrophic change in cardiac myocytes [13]. Therefore, we assessed another mechanism of the downregulation of NRSE repression activity during cardiac myocyte hypertrophy. To examine whether the NRSE-specific DNA binding activity of nuclei was modulated with hypertrophic agents, EMSA was performed using the neonatal cardiac myocytes treated with PE and ET-1 for up to 24 h. As shown in Fig. 4C, the treatment with hypertrophic agents decreased the DNA binding activity in a time-dependent manner up to 3 h, and its activity was recovered at 8 h after stimulation. Western blot performed in parallel with corresponding nuclear extracts detected no clear differences in the NRSF protein levels (Fig. 4D). Therefore, the stimulus-induced change detected by EMSA appeared to correlate with the modulation of NRSF binding activity, rather than with the decrease in the NRSF protein levels. These results suggest that the upregulation of HCN4 expression induced by PE and ET-1 could be mediated by the downregulation of the NRSF bindings to the NRSE. In summary, we concluded that the NRSE–NRSF system was involved in the expression of HCN4 in the cardiac myocytes, although its mech-

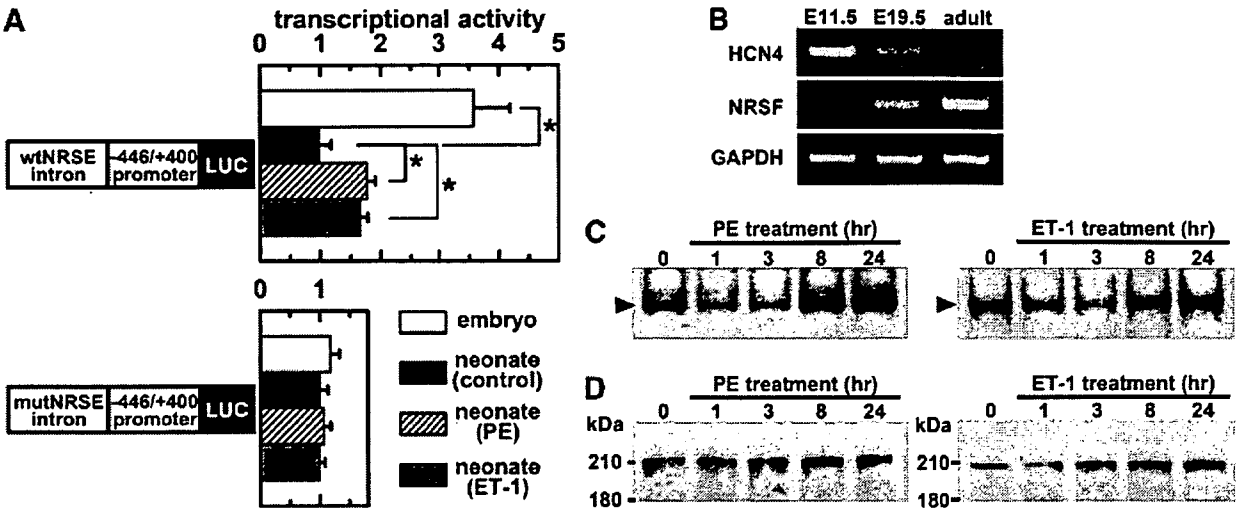

Fig. 4. (A) Transcriptional responses of the NRSE. Each experiment was performed at least three times in quadruplicate. The results are expressed as means ± SE $^*P < 0.05$. (B) RT-PCR analysis for HCN4, NRSF, and GAPDH. The RNA isolated form rat hearts in the embryonic (embryonic day 11.5, 19.5) and adult stages were used. (C) The modulation of NRSF DNA binding activity was analyzed by EMSA. The arrow denotes the NRSF–DNA complexes. (D) NRSF protein was analyzed by Western blotting.

anism may be different in the development and hypertrophy of cardiac myocytes.

Discussion

In the present study, we have focused on the transcriptional mechanisms that control the expression of the HCN4 in the embryonic development and hypertrophy of cardiac myocytes. Although previous studies have demonstrated changes in current density and mRNA levels during cardiac development, hypertrophy, and heart failure [6,7], this is the first analysis demonstrating that these related to the regulation of the transcriptional activity of the HCN4 gene.

The HCN gene family encodes proteins responsible for the ionic conductance termed I_f. Among the ion currents expressed by the heart, I_f is known to possess unique properties [1]. I_f is activated by membrane hyperpolarization, not by depolarization, and is carried by both Na^+ and K^+. Early electrophysiological studies suggested that I_f plays an important role in the ionic conductance during cardiac pacemaker depolarization, thus determining the heart rate and rhythm generated by the SA node cells [14]. Embryonic cardiac myocytes also show spontaneous rhythmic activity, and it has been proposed that I_f plays a pivotal role in this electrical function [5,15]. Therefore, the HCN4 gene regulatory mechanism appears to be important for understanding the mechanism behind cardiac cells acquiring spontaneous activity. The present study demonstrated the functional evidences that NRSF was associated with the expression of HCN4 in embryonic hearts. Additionally, our preliminary results demonstrate that the mRNA levels of HCN4 and NRSF display an inverse expression pattern between the SA node and the ventricle of adult rat hearts (unpublished data). This finding may suggest that cardiac cells without automatic rhythmic activities used the NRSF silencing system to repress HCN4 expression.

Heart failure patients experience a number of changes in the electrical function of the heart that lead to potentially lethal cardiac arrhythmias. Arrhythmias associated with cardiac hypertrophy and heart failure are likely to involve multiple pathophysiological mechanisms. Action potential prolongation, increase in intracellular Na^+, and altered Ca^{2+} handling are consistently reported in failing hearts [16]. These changes are possibly due, at least in part, to the decreases in the number of K^+ channels and the reexpressions of fetal-type ion channels, including HCN4. Nevertheless, their gene regulatory mechanisms remain poorly understood. In this regard, our present study has provided a new insight that the NRSE–NRSF gene regulatory system might participate in cardiac electrical remodeling. The elucidation of the transcriptional mechanisms of ion channels and transporters may provide a clue to prevent the electrical remodeling in diseased hearts.

Acknowledgments

We thank Prof. Kawakami and Prof. Endo (Jichi Medical University) for valuable discussions and Ms. Y. Ohori for excellent technical assistance. This work was supported by Grants-in-Aid from Takeda Science Foundation, the Vehicle Racing Commemorative Foundation, and the Japan Medical Association.

References

[1] R.B. Robinson, S.A. Siegelbaum, Hyperpolarization-activated cation currents: from molecules to physiological function, Annu. Rev. Physiol. 65 (2003) 453–480.

[2] W. Shi, R. Wymore, H. Yu, J. Wu, R.T. Wymore, Z. Pan, R.B. Robinson, J.E. Dixon, D. McKinnon, I.S. Cohen, Distribution and prevalence of hyperpolarization-activated cation channel (HCN) mRNA expression in cardiac tissues, Circ. Res. 85 (1999) e1–e6.

[3] C. Marionneau, B. Couette, J. Liu, H. Li, M.E. Mangoni, J. Nargeot, M. Lei, D. Escande, S. Demolombe, Specific pattern of ionic channel gene expression associated with pacemaker activity in the mouse heart, J. Physiol. 562 (2005) 223–234.

[4] T.M. Ishii, M. Takano, L.H. Xie, A. Noma, H. Ohmori, Molecular characterization of the hyperpolarization-activated cation channel in rabbit heart sinoatrial node, J. Biol. Chem. 274 (1999) 12835–12839.

[5] K. Yasui, W. Liu, T. Opthof, K. Kada, J.K. Lee, K. Kamiya, I. Kodama, $I(f)$ current and spontaneous activity in mouse embryonic ventricular myocytes, Circ. Res. 88 (2001) 536–542.

[6] M. Fernandez-Velasco, N. Goren, G. Benito, J. Blanco-Rivero, L. Bosca, C. Delgado, Regional distribution of hyperpolarization-activated current (I_f) and hyperpolarization-activated cyclic nucleotide-gated channel mRNA expression in ventricular cells from control and hypertrophied rat hearts, J. Physiol. 553 (2003) 395–405.

[7] U.C. Hoppe, E. Jansen, M. Sudkamp, D.J. Beuckelmann, Hyperpolarization-activated inward current in ventricular myocytes from normal and failing human hearts, Circulation 97 (1998) 55–65.

[8] B. Rosati, D. McKinnon, Regulation of ion channel expression, Circ. Res. 94 (2004) 874–883.

[9] N. Mori, C. Schoenherr, D.J. Vandenbergh, D.J. Anderson, A common silencer element in the SCG10 and type II Na^+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells, Neuron 9 (1992) 45–54.

[10] C.J. Schoenherr, A.J. Paquette, D.J. Anderson, Identification of potential target genes for the neuron-restrictive silencer factor, Proc. Natl. Acad. Sci. USA 93 (1996) 9881–9886.

[11] C.J. Schoenherr, D.J. Anderson, The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes, Science 267 (1995) 1360–1363.

[12] K. Kuwahara, Y. Saito, M. Takano, Y. Arai, S. Yasuno, Y. Nakagawa, N. Takahashi, Y. Adachi, G. Takemura, M. Horie, Y. Miyamoto, T. Morisaki, S. Kuratomi, A. Noma, H. Fujiwara, Y. Yoshimasa, H. Kinoshita, R. Kawakami, I. Kishimoto, M. Nakanishi, S. Usami, M. Harada, K. Nakao, NRSF regulates the fetal cardiac gene program and maintains normal cardiac structure and function, EMBO J. 22 (2003) 6310–6321.

[13] K. Kuwahara, Y. Saito, E. Ogawa, N. Takahashi, Y. Nakagawa, Y. Naruse, M. Harada, I. Hamanaka, T. Izumi, Y. Miyamoto, I. Kishimoto, R. Kawakami, M. Nakanishi, N. Mori, K. Nakao, The neuron-restrictive silencer element-neuron-restrictive silencer factor system regulates basal and endothelin 1-inducible atrial natriuretic peptide gene expression in ventricular myocytes, Mol. Cell. Biol. 21 (2001) 2085–2097.

[14] K. Yanagihara, H. Irisawa, Inward current activated during hyperpolarization in the rabbit sinoatrial node cell, Pflugers Arch. 385 (1980) 11–19.

[15] J. Stieber, S. Herrmann, S. Feil, J. Loster, R. Feil, M. Biel, F. Hofmann, A. Ludwig, The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart, Proc. Natl. Acad. Sci. USA 100 (2003) 15235–15240.

[16] G.F. Tomaselli, D.P. Zipes, What causes sudden death in heart failure? Circ. Res. 95 (2004) 754–763.

0021-972X/07/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 92(2):532–541

doi: 10.1210/jc.2006-1546

Efficacy and Safety of Leptin-Replacement Therapy and Possible Mechanisms of Leptin Actions in Patients with Generalized Lipodystrophy

Ken Ebihara, Toru Kusakabe, Masakazu Hirata, Hiroaki Masuzaki, Fumiko Miyanaga, Nozomi Kobayashi, Tomohiro Tanaka, Hideki Chusho, Takashi Miyazawa, Tatsuya Hayashi, Kiminori Hosoda, Yoshihiro Ogawa, Alex M. DePaoli, Masanori Fukushima, and Kazuwa Nakao

Department of Medicine and Clinical Science (K.E., T.K., M.H., H.M., F.M., N.K., T.T., H.C., T.M., T.H., K.H., Y.O., K.N.), Kyoto University Graduate School of Medicine, and Department of Clinical Trial Management (M.F.), Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan; and Amgen Inc. (A.M.D.), Thousand Oaks, California 91319

Background: Lack of leptin is implicated in insulin resistance and other metabolic abnormalities in generalized lipodystrophy; however, the efficacy, safety, and underlying mechanisms of leptin-replacement therapy in patients with generalized lipodystrophy remain unclear.

Methods: Seven Japanese patients with generalized lipodystrophy, two acquired and five congenital type, were treated with the physiological replacement dose of recombinant leptin during an initial 4-month hospitalization followed by outpatient follow-up for up to 36 months.

Results: The leptin-replacement therapy with the twice-daily injection dramatically improved fasting glucose (mean ± SE, 172 ± 20 to 120 ± 12 mg/dl, $P < 0.05$) and triglyceride levels (mean ± SE, 700 ± 272 to 260 ± 98 mg/dl, $P < 0.05$) within 1 wk. The leptin-replacement therapy reduced insulin resistance evaluated by euglycemic clamp method and augmented insulin secretion at glucose tolerance test with different responses between acquired and congenital types. Improvement of the fatty liver was also observed. The efficacy and safety of the once-daily injection were comparable to those of the twice-daily injection. The leptin-replacement therapy ameliorated macro- and microalbuminuria and showed no deterioration of neuropathy and retinopathy of these patients. The leptin-replacement therapy is beneficial to diabetic complications and lipodystrophic ones. Two patients developed antileptin antibodies but not neutralizing antibodies. The therapy was well tolerated, and its effects were maintained for up to 36 months without any notable adverse effects such as hypoglycemia, high blood pressure, or reduction of bone mineral density.

Conclusions: The present study demonstrates the efficacy and safety of the long-term leptin-replacement therapy and possible mechanisms of leptin actions in patients with generalized lipodystrophy. (*J Clin Endocrinol Metab* 92: 532–541, 2007)

LEPTIN PLAYS A MAJOR role in the regulation of energy homeostasis (1). The plasma leptin concentration increases in proportion to the degree of adiposity (2–6). Besides the antiobesity actions, leptin has a wide range of actions including antidiabetic actions (6–8).

Generalized lipodystrophy is a heterogeneous group of diseases characterized by a profound deficiency of adipose tissue (9) and is commonly associated with severe insulin-resistant diabetes, hypertriglyceridemia, and fatty liver (10, 11). In lipoatrophic patients, these metabolic abnormalities develop as a consequence of decreased mass of the adipose tissue (12–14), and consequently, plasma leptin concentrations are markedly reduced (15). We and others demonstrated that the leptin administration or transgenic overexpression of leptin reverses the metabolic abnormalities in different mouse models of lipodystrophy, indicating that the metabolic abnormalities in lipoatrophic patients are caused mainly by a shortage of leptin (16, 17). Recently, the 4-month leptin-replacement therapy with twice-daily injection protocol was reported to improve glucose and lipid metabolism in nine female patients with lipodystrophy in the United States (18).

In the present study, we evaluated the efficacy and safety of long-term leptin-replacement therapy on seven Japanese patients with generalized lipodystrophy.

First Published Online November 21, 2006

Abbreviations: AGL, Acquired generalized lipodystrophy; CGL, congenital generalized lipodystrophy; CT, computed tomography; HbA1c, glycosylated hemoglobin; L/S, liver to spleen; r-metHuLeptin, recombinant methionyl human leptin.

Subjects and Methods

Subjects

Eligible criteria were according to the study protocol of the National Institutes of Health (18). We evaluated seven patients with generalized lipodystrophy including two patients with acquired generalized lipodystrophy (AGL) and five patients with congenital generalized lipodystrophy (CGL). Patients with CGL were further analyzed for mutations in either *seipin* (19) or 1-acylglycerol-3-phosphate *O*-acyltransferase2 (*AGPAT2*) genes (20). Table 1 summarizes the baseline clinical characteristics of seven patients treated in the present study.

Study design

The study protocol was approved by the ethical committee of Kyoto University Graduate School of Medicine (approval number 331). Informed written consent was obtained from all subjects and their families. Recombinant methionyl human leptin (r-metHuLeptin) was provided by Amgen, Inc. (Thousand Oaks, CA). For the first year, r-metHuLeptin

TABLE 1. Characteristics of the patients at baseline

Patient no.	1	2	3	4	5	6	7	Mean ± SE
Age (yr)	11	29	19	16	15	23	33	21 ± 3
Sex	F	M	M	F	F	F	F	
Type of lipodystrophy	AGL	CGL[a]	CGL[b]	AGL	CGL[b]	CGL[b]	CGL[a]	
Body weight (kg)	31.0	32.0	52.8	34.6	43.5	52.0	53.9	42.8 ± 3.9
BMI (kg/m^2)	16.0	14.2	19.7	13.9	17.9	21.2	20.3	17.6 ± 1.1
Body fat (%)[c]	5.0	4.7	5.0	5.8	5.4	5.7	10.0	5.9 ± 0.7
Age of DM onset (yr)	9	11	10	15	6	5	14	10 ± 1
Duration of DM (yr)	2	18	9	1	9	18	19	11 ± 3
Leptin (ng/ml)	0.92	0.82	1.23	1.15	0.90	1.23	1.40	1.09 ± 0.08
Fasting glucose (mg/dl)	208	142	105	138	247	221	130	172 ± 21
HbA1c (%)	10.0	10.3	8.8	7.9	7.7	10.2	10.2	9.3 ± 0.4
Triglyceride (mg/dl)	1941	69	1031	1246	89	254	232	695 ± 273
Total cholesterol (mg/dl)	250	183	194	298	185	285	233	233 ± 18
L/S ratio	0.78	1.23	0.95	0.35	1.12	0.88	0.73	0.86 ± 0.11
Urinary albumin (mg/d)	48.4	6.9	31.0	778	20.3	359	11.3	179.3 ± 110.5
Blood pressure (mm Hg)	114/48	120/82	126/54	92/52	108/56	104/64	108/70	110 ± 4/61 ± 5
Bone mineral density (g/cm^2)[c]	0.94	0.88	1.32	0.90	1.06	1.37	1.33	1.11 ± 0.08
Diet therapy (kcal/d)	1500	1500	1800	1500	1500	1600	1500	
Antidiabetic therapy	Pioglitazone (15 mg/d)	Glibenclamide (2.5 mg/d), voglibosie (0.6 mg/d)	Insulin (60 IU/d)	Pioglitazone (30 mg/d)	None	Pioglitazone (45 mg/d)	Insulin (20 U/d)	
Lipid-lowering therapy	None	None	None	Pravastatin (40 mg/d), bezafibrate (400 mg/d)	None	None	None	

BMI, Body mass index; DM, diabetes mellitus; L/S ratio, ratio of liver to spleen for CT attenuation values.
[a] CGL due to neither *seipin* nor *AGPAT2* mutation.
[b] CGL due to *seipin* mutation.
[c] Body fat and bone mineral density were measured by dual energy x-ray absorptiometry.

was administered as twice-daily sc injection (18). The physiological replacement dose was estimated to be 0.02 mg/kg·d for men, 0.03 mg/kg·d for girls under 18 yr of age, and 0.04 mg/kg·d for women on the basis of information provided by Amgen. Patient 1 was treated with 100% of the replacement dose for the entire period. Patient 2 was treated with 100% for the first and second month and 200% thereafter. Patients 3–7 were treated with 50% for the first month, 100% for the second month, and 200% thereafter. All patients were evaluated as inpatients for the first 4 months. After discharge, patients attended local clinics for every leptin injection, and all of the leptin injections were done by medical doctors because self-injection of r-metHuLeptin, which was not approved as a drug, was not permitted in Japan. Each patient had been prescribed a diet of fixed calories indicated in Table 1 beginning at least 2 months before the initiation of leptin-replacement therapy, and this was not altered throughout the therapy. The dose of antidiabetic and lipid-lowering drugs was tapered or the treatment discontinued as needed. After 12 months of twice-daily leptin treatment, we reduced the dosing frequency to once daily without change of total daily dose. At present, total duration of leptin-replacement therapy was 36 months for patient 1 and 2, 24 months for patient 3, 18 months for patient 4, 8 months for patient 5, and 2 months for patients 6 and 7.

Biochemical analysis

Plasma leptin levels were determined by the immunoassay (Linco, St. Charles, MO). Plasma glucose, serum triglycerides, total cholesterol, alanine aminotransferase, aspartate aminotransferase, and serum and urine creatinine levels were determined according to standard methods with the use of automated equipment. Glycosylated hemoglobin (HbA1c) levels were measured by ion-exchange HPLC. Serum insulin levels were determined by immunoassays (Shibayagi Co., Ltd., Gunma, Japan). Urine albumin excretion was assayed with a human albumin ELISA kit (Sanko Junyaku Co., Ltd., Tokyo, Japan). Antibodies to leptin in serum was tested with the use of a solid-phase RIA, and the potential neutralizing effects of antibodies on leptin bioactivity were assessed in an *in vitro* bioassay developed by Amgen (Thousand Oaks, CA) (21).

Procedures

Body fat and whole-body bone mineral density were determined by dual-energy x-ray absorptiometry (QDR-2000; Hologic Inc., Bedford, MA). The oral glucose tolerance test (75 g) was performed after an overnight fast. In patients under insulin therapy, insulin injection was stopped from the previous night. The Σ values of plasma glucose (PG) levels and serum insulin (IRI) levels were calculated by the sum of the values at 0, 30, 60, 90, 120, and 180 min after administration. Insulin action on glucose uptake in peripheral tissues was evaluated using the hyperinsulinemic-euglycemic glucose clamp technique (22). Fatty liver was diagnosed by both ultrasound and computed tomography (CT) imaging. Liver volume was calculated with the use of CT imaging. Lipid contents of liver and skeletal muscle were determined by magnetic resonance imaging performed on a 1.5-T system (Magnetom Symphony; Siemens Medical System, Erlangen, Germany). The signal intensity of the same region on both the in-phase image (I_{in}) and the out-of-phase image (I_{out}) was measured. The fat index (FI) was defined by the following formulae: $FI = (I_{in} - I_{out}) / I_{in}$. Tissue lipid content was calculated using FI as previously reported (23).

Statistical analysis

Data were expressed as the mean ± SE. Comparison between baseline data and data obtained at various times was assessed by ANOVA and completed by Fisher's probable least-significant difference test, as required.

Results

Baseline characteristics

Three of five CGL patients were homozygous for the same nonsense mutation (R275X) of the *seipin* gene as we previously reported (Table 1) (24). The remaining CGL patients had neither *seipin* nor *AGPAT2* gene mutation (24, 25). All the patients had markedly decreased body fat, hypoleptinemia, and uncontrolled diabetes with high fasting glucose levels and HbA1c levels, despite the diet and exercise therapy and the use of oral antidiabetic drugs or insulin. Their age of onset and duration of diabetes are also summarized in Table 1. Three of seven patients had marked fasting hypertriglyceridemia at the level above 1000 mg/dl. The mean ± SE of the total cholesterol level was 233 ± 18 mg/dl. Five patients were diagnosed to have fatty liver, and their ratios of liver to spleen (L/S ratio) for CT attenuation values were under 0.95. Four of seven patients had elevated urine albumin excretion (>30 mg/d), and two of them had macroalbuminuria (>300 mg/d). All the patients showed normal blood pressure (mean ± SE, 110 ± 4/61 ± 5 mm Hg) and bone mineral density (mean ± SE, 1.11 ± 0.08 g/cm^2).

High compliance of leptin-replacement therapy

All of the leptin injections were done by medical doctors. For the initial 4 months, all the patients received 100% of scheduled leptin injections as inpatients. After discharge, patients attended local clinics for every leptin injection and received over 98% as outpatients thereafter.

Achievement of physiological replacement of leptin

At any dose, peak plasma levels occurred 2 h after the leptin injection. The peak plasma leptin levels at the doses of 50, 100, and 200% under the protocol of twice-daily injections were 4.05 ± 0.19, 9.80 ± 1.70, 18.95 ± 1.58 (mean ± SE) ng/ml, respectively. The peak plasma leptin level of the 400% dose under the protocol of once-daily injections was 34.48 ± 2.11 (mean ± SE) ng/ml. Thus, the elevations of plasma leptin level were dose dependent, and physiological replacement was achieved as expected.

Rapid effects on glucose and triglyceride levels

The fasting plasma glucose levels decreased day by day in all the patients, and a significant reduction was achieved within 7 d (mean ± SE, 172 ± 20 mg/dl at baseline *vs.* 120 ± 12 mg/dl after 7 d, $P < 0.05$) (Table 2). By 4 months, all the patients, except patient 6, were able to discontinue all of the antidiabetic drugs (Table 1). Patient 6 could reduce the dose of the antidiabetic drug by 2 months.

The fasting triglyceride levels also decreased day by day in all the patients, and a significant reduction was achieved within 7 d (mean ± SE, 700 ± 272 mg/dl at baseline *vs.* 260 ± 98 mg/dl after 7 d, $P < 0.05$) (Table 2). Lipid-lowering drugs of patient 4 were able to be discontinued by 4 months.

Glucose tolerance tests

As shown in Fig. 1A, the mean plasma glucose levels in response to the oral 75-g glucose load were dramatically improved already at 1 month and were maintained at 2 and 4 months in all patients. The insulin levels were distinctly low before the treatment in both AGL and CGL patients (Fig. 1, B and C). The changes after the initiation of the leptin-replacement therapy of serum insulin levels showed a marked contrast between AGL and CGL patients. Glucose-induced insulin secretion was dramatically improved already at 1

TABLE 2. Changes of fasting plasma glucose and serum triglyceride levels for first month of the leptin-replacement therapy

Patient no.	Fasting plasma glucose (mg/dl)					Fasting serum triglyceride (mg/dl)				
	Baseline	1 d	3 d	7 d	30 d	Baseline	7 d	14 d	21 d	28 d
1	208	177	160	108	76	1941	653	210	204	218
2	142	115	119	102	94	69	51	49	72	50
3	105	100	89	105	108	1031	122	108	97	115
4	138	125	126	96	108	1246	589	320	425	496
5	247	227	204	182	151	254	399	134	151	194
6	221	185	169	141	130	89	63	54	131	74
7	141	135	133	105	125	269	54	85	95	99
Mean ± SE	172 ± 20	152 ± 17	143 ± 14	120 ± 12[a]	113 ± 9[b]	700 ± 272	260 ± 98[a]	110 ± 38[b]	168 ± 46[b]	178 ± 58[b]

[a] $P < 0.05$ compared to baseline.
[b] $P < 0.01$ compared to baseline.

month in AGL patients (Fig. 1B), whereas no apparent improvement in insulin secretion was observed even after 4 months of the therapy in CGL patients (Fig. 1C). To evaluate the ability of insulin secretion, we calculated the values of ΣIRI/ΣPG in a 75-g oral glucose tolerance test. The values of ΣIRI/ΣPG were substantially increased at 1 month in two AGL patients, and additional increases were observed at 2 and 4 months, whereas those in five CGL patients remained unchanged even after 4 months of the therapy (Fig. 1D).

Hyperinsulinemic-euglycemic clamp study

The glucose infusion rates during the hyperinsulinemic-euglycemic clamp study were distinctly low at baseline in all the patients (mean ± SE, 2.5 ± 0.3 mg/kg·min; range, 1.60–3.6 mg/kg·min). The increase of glucose infusion rate was observed but not statistically significant at 1 month on the treatment (mean ± SE, 3.7 ± 0.3 mg/kg·min, $P = 0.062$ *vs.* at baseline). A significant increase was achieved at 2 months (mean ± SE, 4.4 ± 0.4 mg/kg·min, $P < 0.01$ *vs.* at baseline) and an additional increase was observed at 4 months (mean ± SE, 5.6 ± 1.0 mg/kg·min, $P < 0.001$ *vs.* at baseline). By contrast to insulin secretion, no apparent difference between AGL and CGL patients was observed on the changes of insulin sensitivity.

In patient 4, the hyperinsulinemic-euglycemic clamp study was performed at 10 d. A substantial increase of glucose infusion rate was detected already at 10 d (2.52 mg/kg·min at baseline and 4.63 mg/kg·min at 10 d) and again at 1 month (4.59 mg/kg·min), which was comparable to that at 4 months (5.06 mg/kg·min).

Effects on fatty liver

Five of seven patients were diagnosed to have apparent fatty liver. The L/S ratios of CT attenuation value in five of seven patients were 0.74 ± 0.10 (mean ± SE) (Table 1). The L/S ratio of CT attenuation value in these patients improved from 0.74 ± 0.10 (mean ± SE) to 1.09 ± 0.06 (mean ± SE) by 2 months and further improved thereafter. Consistent with this, in these patients, the alanine aminotransferase level decreased from 80.5 ± 24.2 to 32.3 ± 4.6 U/liter (mean ± SE), and the ASL level decreased from 42.3 ± 11.1 to 21.5 ± 4.3 U/liter (mean ± SE) by 2 months, and these values were also further improved thereafter. The liver volume also decreased in all patients who had fatty liver at baseline (mean ± SE,

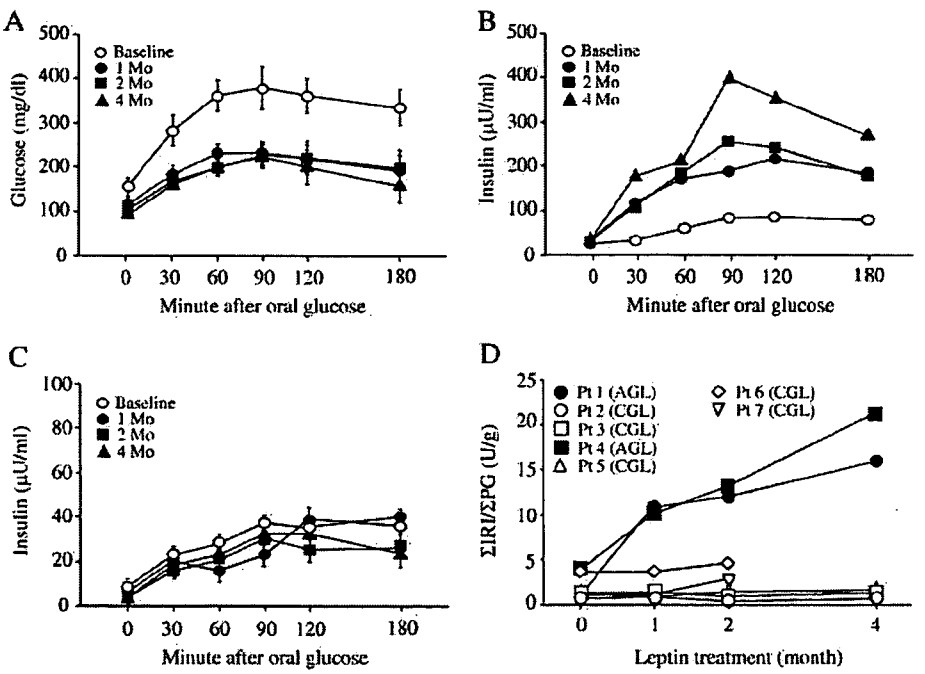

FIG. 1. Mean ± SE plasma glucose levels in all seven patients (A), mean serum insulin levels in two AGL patients (B), mean ± SE serum insulin levels in five CGL patients (C), and ΣIRI/ΣPG of each patient (D) after an oral 75-g glucose-tolerance test before and after 1, 2, and 4 months of the leptin-replacement therapy. ΣIRI, Sum of the plasma insulin levels before and 30, 60, 90, 120, and 180 min after an oral 75-g glucose load; ΣPG, sum of the plasma insulin levels before and 30, 60, 90, 120, and 180 min after an oral 75-g glucose load; ΣIRI/ΣPG, value of ΣIRI divided by ΣPG.

1.88 ± 0.12 l at baseline to 1.50 ± 0.10 l at the end of the second month).

In patient 4, measurements of tissue lipid content were performed using magnetic resonance imaging before and after 3 and 10 d and 1, 2, and 4 months of the leptin-replacement therapy. At baseline, lipid content in her liver was clearly increased (29.0%), whereas that in her skeletal muscle was not increased (4.3%). After the leptin-replacement therapy, a distinct change of lipid content in the liver was not detected at 3 and 10 d (31.5 and 28.4%, respectively), but a substantial and gradual decrease was detected at 1 month and again at 2 and 4 months (23.5, 17.5, and 9.6%, respectively). On the other hand, in the skeletal muscle, no distinct change of lipid content was detected even at 4 months (4.2%).

Metabolic controls after discharge for 8 months

After the initial 4 months of hospitalization, the patients were continuously followed as outpatients on the protocol of twice-daily injection. Their fasting glucose levels (Fig. 2A), HbA1c levels (Fig. 2B), glucose infusion rates during the hyperinsulinemic-euglycemic clamp study (Fig. 2C), triglyceride levels (Fig. 2D), total cholesterol levels (Fig. 2E), and liver volumes (Fig. 2F) at 8 and 12 months were almost unchanged when compared with those at 4 months, the end of the hospitalization.

Once-daily leptin injection

After 12 months of twice-daily leptin injection, the treatment protocol was altered to once-daily dosing without change of total daily dose in patient 1–4. The alteration of leptin injection protocol did not affect the plasma glucose levels before breakfast, lunch, and dinner in four patients (Fig. 3, A–C). Consistent with these results, HbA1c (Fig. 2B) levels and results of the 75-g oral glucose tolerance test (data not shown) in these patients did not change after the protocol alteration. Likewise, glucose infusion rates during the hyperinsulinemic-euglycemic clamp study, triglyceride levels, total cholesterol levels, and liver volumes were unchanged after the alteration of the treatment protocol (Fig. 2, C–F).

Long-term effects

The duration of leptin-replacement therapy was 36 months for patients 1 and 2, 24 months for patient 3, and 18 months for patient 4. The fasting plasma glucose levels and HbA1c levels were well controlled throughout the therapy period (Fig. 2, A and B). The improved glucose infusion rates during the hyperinsulinemic-euglycemic clamp study, decreased triglyceride and total cholesterol levels, and liver volumes after 4 months of leptin-replacement therapy as inpatients

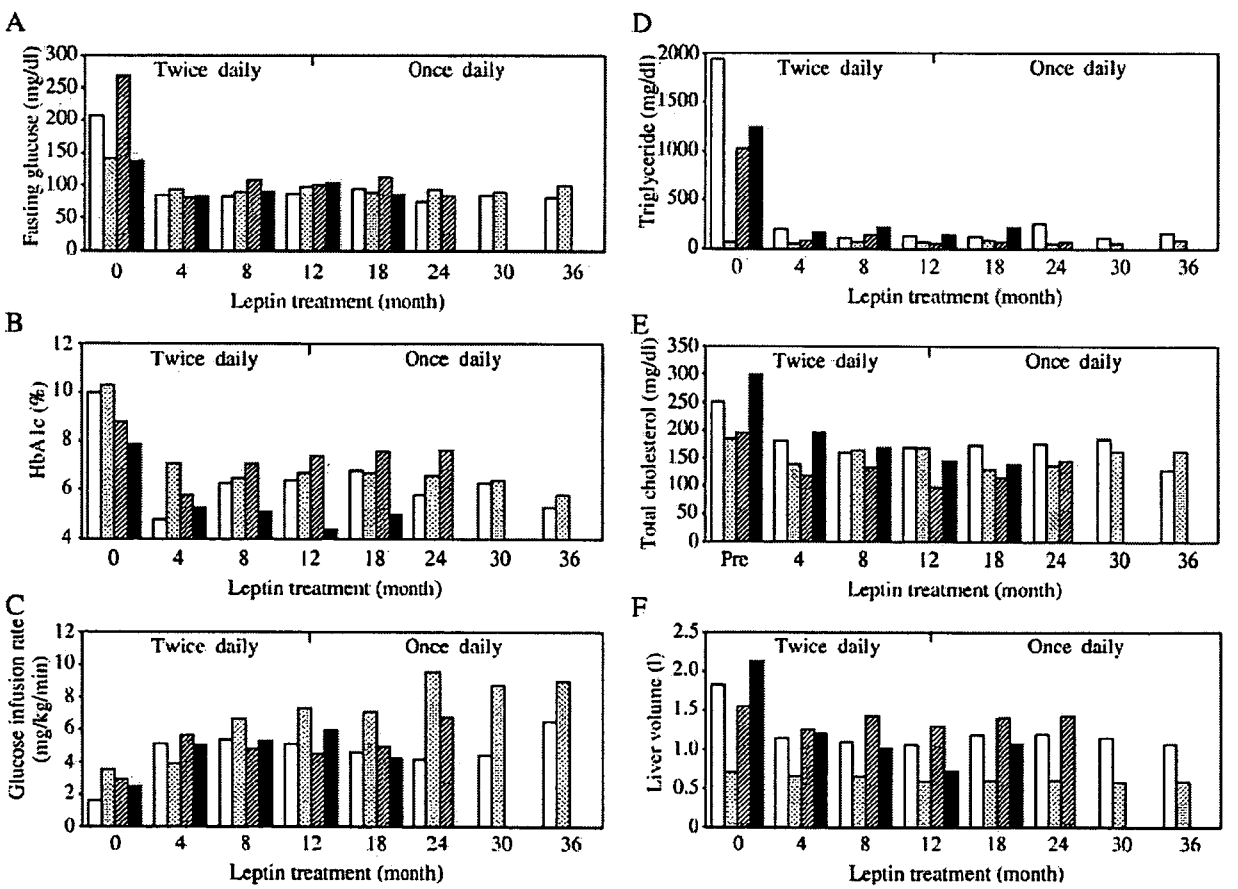

FIG. 2. Fasting plasma glucose levels (A), HbA1c levels (B), glucose infusion rates during the hyperinsulinemic euglycemic clamp study (C), triglyceride levels (D), total cholesterol levels (E), and liver volumes (F) before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), and patient 4 (*black bars*).

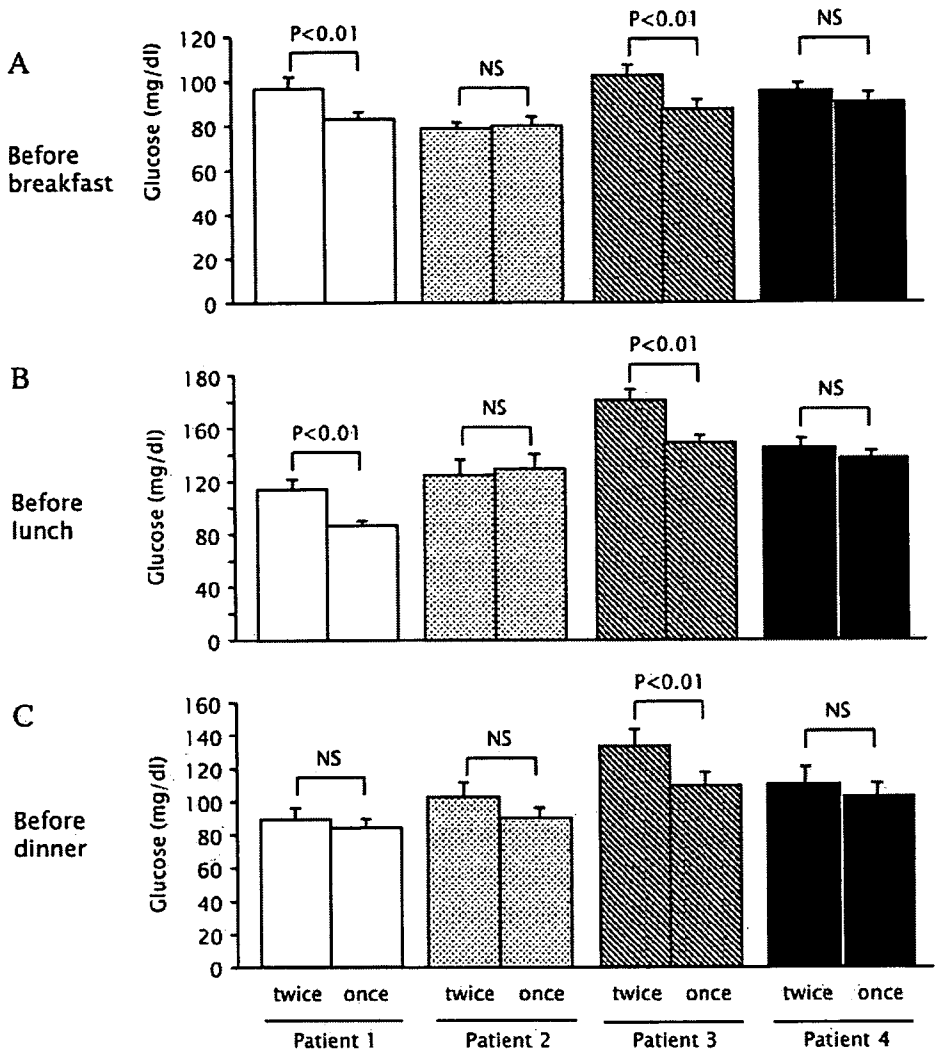

FIG. 3. Comparison of the mean (± SE) plasma glucose levels during the 12th month under the protocol of twice-daily leptin injection and during the 13th month under the protocol of once-daily leptin injection before breakfast (A), lunch (B), and dinner (C) in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), and patient 4 (*black bars*). NS, No significance difference ($P > 0.05$) between groups.

were well controlled throughout the therapy period (Fig. 2, C–F).

Antileptin antibodies

Patients 2 and 3, both CGL patients, showed elevations of basal plasma leptin levels, 75.0 and 42.4 ng/ml at the end of the 12th month, respectively. We detected antileptin antibodies in both patients. Antibodies from these patients did not neutralize the action of leptin at all in a bioassay.

Diabetes and other complications

All seven patients had normal renal functions at baseline; however, two patients had microalbuminuria (>30 mg/d), and two patients had macroalbuminuria (>300 mg/d) (Table 1). In addition, five of seven patients had elevated creatinine clearance (mean ± SE, 206.5 ± 22.0 ml/min·1.73 m^2) at the level above 125 ml/min·1.73 m^2. After the initiation of leptin-replacement therapy, urine albumin excretion of patients 1 and 3 with microalbuminuria began to decrease gradually within 1 month and was normalized within 2 months (Fig. 4A). Macroalbuminuria of patients 4 and 6 was also regressed to microalbuminuria within 3 and 1 month, respectively (Fig. 4B). In parallel, the creatinine clearance of the five patients with glomerular hyperfiltration significantly decreased to 129.5 ± 24.5 ml/min·1.73 m^2 (mean ± SE) for the 4-month leptin-replacement therapy ($P < 0.05$). These beneficial effects of leptin on urine albumin excretion and glomerular hyperfiltration were stable for up to 36 months.

Six of seven patients showed no diabetic retinopathy, but patient 7 had a nonproliferative retinopathy at baseline. No deterioration of her retinopathy was observed during the therapy. Six of seven patients had no diabetic neuropathy at baseline, although patient 6 showed neurogenic bladder. During the therapy period, her neurogenic bladder did not worsen, and no patients developed diabetic retinopathy or diabetic neuropathy.

Five of seven patients had moderate to severe acanthosis nigricans at baseline, but the acanthosis nigricans was improved in five patients after the leptin-replacement therapy.

Four of five female patients who were of reproductive age had hypogonadotropic amenorrhea at baseline as previously reported (26, 27) but resumed and sustained normal menses

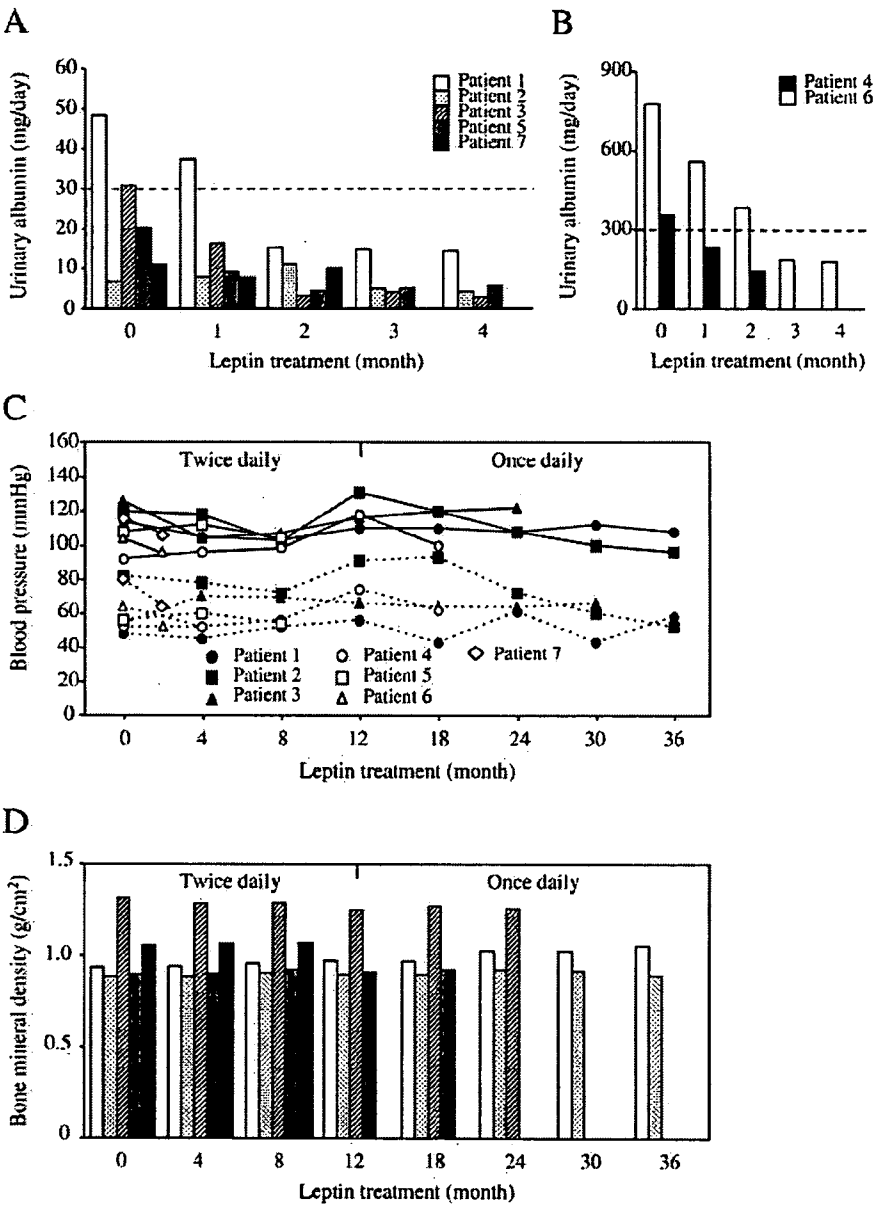

FIG. 4. A and B, Daily excretion levels of urinary albumin before and after 1, 2, 3, and 4 months of the leptin-replacement therapy in patients 1, 2, 3, 5, and 7 and in patients 4 and 6. C, Blood pressure before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in each patient. *Solid lines* indicate systolic blood pressure, and *broken lines* indicate diastolic blood pressure. D, Whole-body bone mineral density before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), patient 4 (*gray bars*), and patient 5 (*black bars*).

after the initiation of the leptin therapy. In an 11-yr-old girl, the menarche was observed after 12 months of the leptin therapy.

All the patients indicated an improvement in feeling of satisfaction after a meal within 1 or 2 d after the initiation of leptin therapy. This effect was sustained throughout the leptin therapy. For the first 4 months, a tendency of body weight reduction was observed in all the patients, but this change was not significant (mean ± SE, 40.9 ± 3.5 to 38.1 ± 3.1 kg, $P = 0.55$). After the first 4 months, the body weight was almost unchanged throughout the leptin therapy.

Adverse effects

We carefully observed blood pressure in the patients. At baseline, no patients showed hypertension (Table 1), and no distinct elevation of blood pressure was observed at any time throughout the therapy period (Fig. 4C).

No patients showed abnormal bone mineral density (Table 1). Whole-body bone mineral densities of the patients were unchanged for up to 36 months (Fig. 4D).

In all the patients, no other adverse effects of the leptin-replacement therapy including skin reactions at injection sites were detected for up to 36 months.

Discussion

In the present study, all of the leptin injections were done by medical doctors, because self-injection of leptin, which is not approved as a drug, is not permitted in Japan. In addition, all the patients were evaluated as inpatients during the initial 4 months of the leptin-replacement therapy. After leaving the hospital, the patients attended local clinics every day for every leptin injection. This allowed close supervision of leptin-replacement therapy, and the patients' lifestyles including diet and exercise were maintained constant. This

condition could minimize the influences of compliance of leptin injection and changes of diet and exercise. Although we could not include a randomized, placebo-treated control group in the present study because of the rarity and clinical diversity of generalized lipodystrophy, it is highly likely that the improved metabolic control is due to the leptin therapy rather than to an improvement in general compliance associated with participation in the study.

In previous reports, improvements of glucose and triglyceride levels, glucose tolerance, and insulin sensitivity were reported at 1 month (18, 25, 28). The present study clearly shows that significant reductions of fasting glucose levels are achieved within 7 d after the initiation of the leptin-replacement therapy, and substantial reductions of the triglyceride levels are also gained within 7 d (Table 2). These rapid and powerful effects of leptin-replacement therapy were further confirmed with the glucose tolerance test and hyperinsulinemic-euglycemic glucose clamp study performed after 7 or 10 d in patient 4. These rapid effects on glucose and lipid metabolism in the present study are comparable to the rapid effects of leptin administration in two different mouse models of generalized lipodystrophy (16, 17).

After 12 months of the twice-daily leptin treatment, we tried to alter the leptin injection protocol to a once-daily injection without change of total daily dose. This protocol alteration did not affect the controls of glucose and lipid metabolism, and these controls were maintained for up to 24 months (Figs. 2 and 3). These observations demonstrate that a once-daily leptin injection is sufficient to control glucose and lipid metabolism in patients with generalized lipodystrophy.

In the present study, we detected antileptin antibodies in two of four tested patients. Both of them were CGL patients, whereas we did not detect antileptin antibodies in AGL patients. This observation raises the possibility that antileptin antibodies more easily develop in CGL patients than AGL patients. Antibodies from both of our CGL patients did not neutralize the leptin action *in vitro* bioassay. In at least one child with congenital leptin deficiency, the transient appearance of neutralizing antibodies against leptin was reported (21). It is possible to speculate that neutralizing antibodies against leptin more easily develop in patients with congenital leptin deficiency than CGL patients, who have a little leptin levels.

The leptin-replacement therapy substantially ameliorated or did not worsen diabetic complications. Amelioration of proteinuria in the present study is consistent with our and other's previous reports that leptin-replacement therapy significantly alleviates the glomerular injury and proteinuria of lipoatrophic diabetes in mice and humans (29, 30). Although we could not perform renal biopsies, it is highly likely that proteinuria observed in our patients is due to diabetic nephropathy because their proteinuria and hyperfiltration were evidently improved in parallel with the metabolic improvement. These findings indicate that leptin is useful to treat, at least, a certain type of diabetic nephropathy.

The leptin-replacement therapy did not induce elevation of blood pressure in any patients throughout the therapy period (Fig. 4C). We previously demonstrated that a high plasma leptin level that is 10 times of that in normal controls elevates blood pressure through the activation of the sympathetic nervous system in mice (31). It is highly likely that the leptin-replacement therapy at the physiological replacement dose does not affect blood pressure.

Bone mineral density of the patients was within normal range at baseline and was unchanged during the therapy period for up to 36 months (Fig. 4D), consistent with the study reported previously (32). We also previously demonstrated that leptin is a powerful inhibitor of bone formation in mice (33). Although the present study indicates that the leptin-replacement therapy at the physiological replacement dose does not affect bone mineral density in humans, careful follow-up is necessary for young patients.

The effect of leptin on β-cell function remains unclear. Leptin treatment decreased serum insulin levels in mouse models of lipodystrophy (16, 17) and human lipoatrophic patients in the United States (18). These decreases of insulin levels were explained by the reduction of glucose levels rather than the suppressive effect of leptin. Indeed, insulin levels peaked earlier in lipoatrophic patients in the United States, although their overall amounts of insulin secreted in response to the glucose load were less after the leptin therapy than at baseline (28). On the other hand, we here demonstrate that leptin-replacement therapy dramatically improves insulin secretion in Japanese AGL patients. Because glucose-lowering therapy often leads to the restoration of β-cell function in patients with diabetes, this effect can be explained at least in part by the cancellation of glucotoxicity (34). The different responses of insulin secretion to leptin-replacement therapy between AGL and CGL patients could be accounted for by the different duration of diabetes. The impaired insulin responses to the glucose load in CGL patients suggests that their β-cell functions were already exhausted before leptin-replacement therapy. Although whether leptin has an additional effect on β-cell is unknown, we here demonstrate that leptin-replacement therapy is beneficial to the treatment of impaired β-cell function.

The mechanisms through which leptin exerts its insulin-sensitizing actions are unclear at present. Fat accumulation in the insulin target organs, which causes so-called lipotoxicity, is considered to be one of the mechanisms for insulin resistance in patients with lipodystrophy (35). Because in patient 4 with AGL, the improvement of insulin sensitivity was observed before a substantial decrease of tissue lipid content in the liver and muscle, additional studies are necessary to clarify the relationship between insulin resistance and the tissue lipid content in humans.

Based on the effect of the leptin-replacement therapy, it is highly likely that leptin deficiency is the main cause of the metabolic abnormalities associated with lipodystrophy. However, the adipose tissue is recognized as the largest endocrine organ. Therefore, it is possible to speculate that these hormones other than leptin may be involved to some degree in the pathogenesis of lipoatrophic diabetes.

Using leptin-overexpressing transgenic skinny mice (8), we previously reported that leptin treatment is useful for treatment of not only lipoatrophic diabetes mice (17) but also other diabetic mice models (36, 37). These observations along with dramatic effects and safety of the leptin therapy in the

present study indicate possible application of the leptin therapy to diabetes and its complications.

In summary, under strict control of lifestyle and an extremely high compliance of leptin injection, we demonstrate that the leptin-replacement therapy improves both insulin sensitivity and insulin secretion dramatically and rapidly improves glucose and lipid metabolism in patients with generalized lipodystrophy, and its effects are maintained for up to 36 months without any adverse effects. In addition, the leptin-replacement therapy is beneficial to diabetic complications and lipodystrophic ones. The once-daily leptin injection is sufficient to control glucose and lipid metabolism for a long time. It is concluded that leptin-replacement therapy is an effective and safe treatment for long-term improvement of glucose and lipid metabolism and complications in generalized lipodystrophy.

Acknowledgments

We thank Tomoyuki Akashi, Ikuyo Ogawa, and Yasushi Yokokawa (Hamanomachi General Hospital, Fukuoka, Japan); Koichi Kitamura (Kitamura Clinic, Kyoto, Japan); Masanori Adachi and Katsuhiko Tachibana (Kanagawa Children's Medical Center, Kanagawa, Japan); Katsuya Aizu and Hiroshi Mochizuki (Saitama Children's Medical Center, Saitama, Japan); Mari Satoh (Toho University Omori Medical Center, Tokyo, Japan); and Yoichiro Oda (Chigasaki Municipal Hospital, Kanagawa, Japan) for referring the patients originally. We also thank the patients and their families for participating in this study.

Received July 17, 2006. Accepted November 15, 2006.

Address all correspondence and requests for reprints to: Ken Ebihara or Kazuwa Nakao, Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: Kebihara@kuhp.kyoto-u.ac.jp or Nakao@kuhp.kyoto-u.ac.jp.

This work was supported by grants from the Japanese Ministry of Education, Science, Sports, and Culture; the Japanese Ministry of Health, Welfare, and Labor; the Japan Medical Association; Japan Research Foundation for Clinical Pharmacology; the Fujisawa Foundation; the Takeda Science Foundation; and The Study Grant for Japan Insulin Study Group.

Disclosure Statement: K.E., T.K., M.H., H.M., F.M., N.K., T.T., H.C., T.M., T.H., K.H., Y.O., M.F., and K.N. have nothing to disclose. A.M.D. is employed by Amgen Inc.

References

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432
2. Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS 1995 Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1:1311–1314
3. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM 1995 Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. Nat Med 1:1155–1161
4. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF 1996 Serum immunoreactive-leptin concentrations in normal-weight and obese human. N Engl J Med 334:292–295
5. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI 1995 Identification and expression cloning of a leptin receptor, OB-R. Cell 83:1263–1271
6. Spiegelman BM, Flier JS 1996 Adipogenesis and obesity: rounding out the big picture. Cell 87:377–389
7. Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ 1997 Acute stimulation of glucose metabolism in mice by leptin treatment. Nature 389: 374–377
8. Ogawa Y, Masuzaki H, Hosoda K, Aizawa-Abe M, Suga J, Suda M, Ebihara K, Iwai H, Matsuoka N, Satoh N, Odaka H, Kasuga H, Fujisawa Y, Inoue G, Nishimura H, Yoshimasa Y, Nakao K 1999 Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. Diabetes 48:1822–1829
9. Reitman ML, Arioglu E, Gavrilova O, Taylor SI 2000 Lipodystrophy revisited. Trends Endocrinol Metab 11:410–416
10. Lawrence RD 1946 Lipodystrophy and hepatomegaly with diabetes, lipaemia, and other metabolic disturbances: a case throwing new light on the action of insulin. Lancet 1:724–731, 773–775
11. Rossini AA, Self J, Aoki TT, Goldman RF, Newmark SR, Meguid MM, Soeldner JS, Cahill GF 1977 Metabolic and endocrine studies in a case of lipoatrophic diabetes. Metabolism 26:637–650
12. Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS 1998 Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. Genes Dev 12:3182–3194
13. Moitra J, Mason MM, Olive M, Krylov D, Gavrilova O, Marcus-Samuels B, Feigenbaum L, Lee E, Aoyama T, Eckhaus M, Reitman ML, Vinson C 1998 Life without white fat: a transgenic mouse. Genes Dev 12:3168–3181
14. Gavrilova O, Marcus-Samuels B, Graham D, Kim JK, Shulman GI, Castle AL, Vinson C, Eckhaus M, Reitman ML 2000 Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. J Clin Invest 105:271–278
15. Andreelli F, Hanaire-Broutin H, Laville M, Tauber JP, Riou JP, Thivolet C 2000 Normal reproductive function in leptin-deficient patients with lipodystrophy. J Clin Endocrinol Metab 85:715–719
16. Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL 1999 Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. Nature 401:73–76
17. Ebihara K, Ogawa Y, Masuzaki H, Shintani M, Miyanaga F, Aizawa-Abe M, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Gavrilova O, Reitman ML, Nakao K 2001 Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. Diabetes 50:1440–1448
18. Oral EA, Simha V, Ruiz E, Andewelt A, Premkumar A, Snell P, Wagner AJ, DePaoli AM, Reitman ML, Taylor SI, Gorden P, Garg A 2002 Leptin-replacement therapy for lipodystrophy. N Engl J Med 346:570–578
19. Magre J, Delepine M, Khallouf E, Gedde-Dahl Jr T, Van Maldergem L, Sobel E, Papp J, Meier M, Megarbane A, Bachy A, Verloes A, d'Abronzo FH, Seemanova E, Assan R, Baudic N, Bourut C, Czernichow P, Huet F, Grigorescu F, de Kerdanet M, Lacombe D, Labrune P, Lanza M, Loret H, Matsuda F, Navarro J, Nivelon-Chevalier A, Polak M, Robert JJ, Tric P, Tubiana-Rufi N, Vigouroux C, Weissenbach J, Savasta S, Maassen JA, Trygstad O, Bogalho P, Freitas P, Medina JL, Bonnicci F, Joffe BI, Loyson G, Panz VR, Raal FJ, O'Rahilly S, Stephenson T, Kahn CR, Lathrop M, Capeau J; BSCL Working Group 2001 Identification of the gene altered in Berardinelli-Seip congenital lipodystrophy on chromosome 11q13. Nat Genet 28:365–370
20. Agarwal AK, Arioglu E, De Almeida S, Akkoc N, Taylor SI, Bowcock AM, Barnes RI, Garg A 2002 AGPAT2 is mutated in congenital generalized lipodystrophy linked to chromosome 9q34. Nat Genet 31:21–23
21. Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM, O'Rahilly S 2002 Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. J Clin Invest 110:1093–1103
22. Greenfield MS, Doberne L, Kraemer F, Tobey T, Reaven G 1981 Assessment of insulin resistance with the insulin suppression test and the euglycemic clamp. Diabetes 30:387–392
23. Kawamitsu H, Kaji Y, Ohara T, Sugimura K 2003 Feasibility of quantitative intrahepatic lipid imaging applied to the magnetic resonance dual gradient echo sequence. Magn Reson Med Sci 2:47–50
24. Ebihara K, Kusakabe T, Masuzaki H, Kobayashi N, Tanaka T, Chusho H, Miyanaga F, Miyazawa T, Hayashi T, Hosoda K, Ogawa Y, Nakao K 2004 Gene and phenotype analysis of congenital generalized lipodystrophy in Japan: a novel homozygous nonsense mutation in *seipin* gene. J Clin Endocrinol Metab 89:2360–2364
25. Ebihara K, Masuzaki H, Nakao K 2004 Long-term leptin-replacement therapy for lipoatrophic diabetes. N Engl J Med 351:615–616
26. Oral EA, Ruiz E, Andewelt A, Sebring N, Wagner AJ, DePaoli AM, Gorden P 2002 Effect of leptin replacement therapy on pituitary hormone regulation in patients with severe lipodystrophy. J Clin Endocrinol Metab 87:3110–3117
27. Musso C, Cochran E, Javor E, Young J, DePaoli AM, Gorden P 2005 The long-term effect of recombinant methionyl human leptin therapy on hyperandrogenism and menstrual function in female and pituitary function in male and female hypoleptinemic lipodystrophic patients. Metabolism 54:255–263
28. Javor ED, Cochran EK, Mussa C, Young J, DePaoli AM, Gorden P 2005 Long-term efficacy of leptin replacement therapy in patients with generalized lipodystrophy. Diabetes 54:1994–2002
29. Suganami T, Mukoyama M, Mori K, Yokoi H, Koshikawa M, Sawai K, Hidaka S, Ebihara K, Tanaka T, Sugawara A, Kawachi H, Vinson C, Ogawa Y, Nakao K 2004 Prevention and reversal of renal injury by leptin in a new mouse model of diabetic nephropathy. FASEB J 19:127–129
30. Javor ED, Moran SA, Young JR, Cochran EK, DePaoli AM, Oral EA, Turman MA, Blackett PR, Savage DB, O'Rahilly S, Balow JE, Gorden P 2004 Proteinuric nephropathy in acquired and congenital generalized lipodystrophy:

baseline characteristics and course during recombinant leptin therapy. J Clin Endocrinol Metab 89:3199–3207
31. Maron SA, Patten N, Young JR, Cochran E, Sebring N, Reynolds J, Premkumar A, DePaoli AM, Skarulis MC, Oral EA, Gorden P 2004 Changes in body composition in patients with severe lipodystrophy after leptin replacement therapy. Metabolism 53:513–519
32. Aizawa-Abe M, Ogawa Y, Masuzaki H, Ebihara K, Satoh N, Iwai H, Matsuoka N, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Nakao K 2000 Pathophysiological role of leptin in obesity-related hypertension. J Clin Invest 105:1243–1252
33. Elefteriou F, Takeda S, Ebihara K, Magre J, Patano N, Kim CA, Ogawa Y, Liu X, Ware SM, Craigen WJ, Robert JJ, Vinson C, Nakao K, Capeau J, Karsenty G 2004 Plasma leptin level is a regulator of bone mass. Proc Natl Acad Sci USA 101:3258–3263
34. Brownlee M 2003 A radical explanation for glucose-induced β-cell dysfunction. J Clin Invest 112:1788–1790
35. Petersen KF, Oral EA, Dufour S, Befroy D, Ariyan C, Yu C, Cline GW, DePaoli AM, Taylor SI, Gorden P, Shulman GI 2002 Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. J Clin Invest 109:1345–1350
36. Masuzaki H, Ogawa Y, Aizawa-Abe M, Hosoda K, Suga J, Ebihara K, Satoh N, Iwai H, Inoue G, Nishimura H, Yoshimasa Y, Nakao K 1999 Glucose metabolism and insulin sensitivity in transgenic mice overexpressing leptin with lethal yellow agouti mutation: usefulness of leptin for the treatment of obesity-associated diabetes. Diabetes 48:1615–1622
37. Miyanaga F, Ogawa Y, Ebihara K, Hidaka S, Tanaka T, Hayashi S, Masuzaki H, Nakao K 2003 Leptin as an adjunct of insulin therapy in insulin-deficient diabetes. Diabetologia 46:1329–1337

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

Cell Metabolism
Short Article

Central Melanocortin Signaling Restores Skeletal Muscle AMP-Activated Protein Kinase Phosphorylation in Mice Fed a High-Fat Diet

Tomohiro Tanaka,[1] Hiroaki Masuzaki,[1,*] Shintaro Yasue,[1] Ken Ebihara,[1] Tetsuya Shiuchi,[2] Takako Ishii,[1] Naoki Arai,[1] Masakazu Hirata,[1] Hiroshi Yamamoto,[3] Tatsuya Hayashi,[4] Kiminori Hosoda,[1] Yasuhiko Minokoshi,[2] and Kazuwa Nakao[1]
[1] Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan
[2] Department of Developmental Physiology, National Institute for Physiological Sciences, Okazaki 444-8585, Japan
[3] Department of Surgery, Shiga University of Medical Science, Otsu 520-2192, Japan
[4] Department of Human Coexistence, Kyoto University Graduate School of Human and Environmental Studies, Kyoto 606-8501, Japan
*Correspondence: hiroaki@kuhp.kyoto-u.ac.jp
DOI 10.1016/j.cmet.2007.04.004

SUMMARY

Little is known about the role of the central melanocortin system in the control of fuel metabolism in peripheral tissues. Skeletal muscle AMP-activated protein kinase (AMPK) is activated by leptin and serves as a master regulator of fatty acid β-oxidation. To elucidate an unidentified role of the central melanocortin system in muscle AMPK regulation, we treated conscious, unrestrained mice intracerebroventricularly with the melanocortin agonist MT-II or the antagonist SHU9119. MT-II augmented phosphorylation of AMPK and its target acetyl-CoA carboxylase (ACC) independent of caloric intake. Conversely, AMPK/ACC phosphorylation by leptin was abrogated by the coadministration of SHU9119 or in KKA^{y} mice, which centrally express endogenous melanocortin antagonist. Importantly, high-fat-diet-induced attenuation of AMPK/ACC phosphorylation in leptin-overexpressing transgenic mice was not reversed by central leptin but was markedly restored by MT-II. Our data provide evidence for the critical role of the central melanocortin system in the leptin-skeletal muscle AMPK axis and highlight the system as a therapeutic target in leptin resistance.

INTRODUCTION

Leptin augments fatty acid β-oxidation in skeletal muscle and enhances whole-body insulin sensitivity, thereby serving as a promising therapeutic candidate for the treatment of insulin resistance and dyslipidemia (Shimabukuro et al., 1997; Minokoshi et al., 2002). In agreement with this notion, we and others have demonstrated the clinical efficacy of leptin in the treatment of diabetes, dyslipidemia, and steatosis in patients with lipodystrophy (Oral et al., 2002; Ebihara et al., 2007). The clinical application of leptin has been hampered, however, by the fact that leptin does not fully exert its metabolic effect in prevalent forms of human obesity (Maffei et al., 1995) and in diet-induced obese rodents (El-Haschimi et al., 2000).

Using transgenic skinny mice overexpressing leptin in liver (LepTg mice), we recently demonstrated that enhanced lipid metabolism and insulin sensitivity in LepTg mice are attenuated on a high-fat diet (HFD) (HFD-LepTg) despite persistent hyperleptinemia, compared with HFD-fed nontransgenic (HFD-non-Tg) littermates (Tanaka et al., 2005). Even with pronounced hyperleptinemia, skeletal muscle AMPK activity is attenuated in HFD-LepTg mice to the level of HFD-non-Tg mice (Tanaka et al., 2005). Noteworthy is the fact that switching HFD back to a standard diet (STD) leads to a significant recovery of muscle AMPK activity in LepTg mice before they regain their skinny phenotype (Tanaka et al., 2005), suggesting the reversible nature of the dietary lipid-induced leptin resistance.

AMPK is activated by decreased energy stores and orchestrates energy-sparing reactions in a cell-specific manner (Hardie et al., 2006). In skeletal muscle cells, AMPK activation stimulates glucose uptake, glycolysis, fatty acid β-oxidation, and mitochondrial biogenesis (Hardie et al., 2006) and critically mediates leptin-induced fatty acid β-oxidation (Minokoshi et al., 2002). Our previous work demonstrated that skeletal muscle AMPK activity closely parallels insulin sensitivity and inversely correlates with energy efficiency in LepTg mice under STD or HFD feeding (Tanaka et al., 2005), indicating that AMPK activity should be a novel biochemical marker of leptin sensitivity in vivo.

The central melanocortin system consists of endogenous melanocortin agonists and receptors. Endogenous agonist is synthesized as pro-opiomelanocortin (POMC) preprohormone and is proteolytically cleaved to produce melanocyte-stimulating hormones (MSHs). In the brain,