The Hypothalamic Leptin Receptor in Humans

Identification of Incidental Sequence Polymorphisms and Absence of the *db/db* Mouse and *fa/fa* Rat Mutations

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Leptin-receptor gene expression in hypothalamic tissue from lean and obese humans was examined. The full-length leptin receptor, that is believed to transmit the leptin signal, is expressed in human hypothalamus. There was no difference in the amount of leptin-receptor mRNA in seven lean (BMI 23.3 ± 0.9 kg/m^2) and eight obese (BMI 36.9 ± 1.5) subjects as determined by reverse transcription–polymerase chain reaction. A sequence polymorphism (A→G) was detected at position 668 of the leptin receptor cDNA. This second base substitution changed a glutamine to an arginine at position 223 of the leptin receptor protein. Of 15 subjects analyzed, 11 were heterozygous for this base change and 3 were homozygous. The occurance of the polymorphic allele(s) did not correlate with BMI in the population studied. The mutation responsible for the defect in the leptin receptor in *db/db* mice was not detected in any obese human, nor was the *fa/fa* rat mutation. These results provide evidence that the leptin resistance observed in obese humans is not due to a defect in the leptin receptor.

A point mutation in the hypothalamic leptin receptor in *db/db* mice has recently been identified (1,2). This mutation results in alternative splicing of the receptor coding region and the addition of a 106-bp insert containing a premature termination signal. This defect in the leptin receptor is hypothesized to lead to the obese phenotype in *db/db* mice. Indeed, unlike *ob/ob*, diet-induced obese, and normal mice (3–5), *db/db* mice do not respond to the administration of exogenous leptin (6).

We have previously demonstrated by radioimmunoassay that obese humans have fourfold more serum leptin than do lean individuals (7). Elevated leptin in obese humans has also been observed by Western blot analysis (8). Furthermore, we did not detect any defects in the adipose tissue mRNA for leptin in >100 subjects (9). Taken together, these observations provide strong evidence that defects in the function of the *OB* gene and the adipocyte are not the cause of obesity in humans. Because the appropriate response to an elevation in leptin is a reduction in food intake and an increase in energy expenditure ultimately resulting in weight loss, it appears that obese humans are resistant to endogenous leptin.

The present study provides evidence that defects in the leptin receptor itself are not the cause of leptin resistance in obese humans.

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CSGE, conformation-sensitive gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcriptase.

RESEARCH DESIGN AND METHODS

Tissue. Human brains were obtained shortly after autopsy from the Washington, DC, Medical Examiner's Office. Tissue from seven lean black males (BMI 23.3 ± 0.9 kg/m^2, range 19.2–26.3; age 46 ± 7.1 years) and eight obese black males (BMI 36.9 ± 1.5, range 30.3–43; age 45.1 ± 4.9 years) was obtained. Death was not from neurological causes. Gross and microscopic pathological examinations of the brains were normal in all cases. All had toxicology screens negative for alcohol or illicit drugs. The hypothalamus was blocked coronally anterior to the mamillary bodies and was rapidly frozen in isopentane and dry ice and stored at −70°C until use.

Reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was obtained by guanidinium thiocyanate–phenol–chloroform extraction (10). First-strand cDNA was synthesized from 1 μg of total RNA using random hexamers (GeneAmp RNA PCR kit, Perkin-Elmer/Cetus, Branchburg, NJ). For the amplification reaction, six sets of primers based on the published sequence of the human leptin receptor (11) were synthesized: −78 to −59, 710–731; 588–607, 1367–1388; 1248–1267, 2015–2034; 1881–1902, 2668–2687; 2551–2573, 3346–3363; and 3147–3167, 3554–3573. Each primer pair defined a product that encompassed ~800 bp of the leptin receptor. PCR reactions (12) were performed in a Perkin-Elmer/Cetus model 9600 thermocycler using 10 pmol of each primer and the entire product of the reverse transcription reaction. Thermocycling conditions were as follows: 30-s denaturation at 94°C, 30-s annealing at 55°C (except for set 3, with which a 60°C annealing temperature was used), and 60-s extension at 72°C for 35 cycles. An additional 2-min extension step at 72°C was added after the 35 cycles.

The amount of hypothalamic leptin-receptor gene expression was determined using methods previously described (13). In brief, first-strand cDNA was generated as described above. The reaction was then split equally into two separate tubes for the PCR reaction. The first tube was used to amplify the ~800 bp of the receptor cDNA enclosed by primer set 5. The second tube was used to generate a 440-bp fragment of the β-actin cDNA using primers from Clontech (Palo Alto, CA). The leptin-receptor amplification reaction was linear from cycles 30 to 45 for both the lean (r = 0.99) and obese (r = 0.99) subjects. The data in Fig. 1 were obtained after 35 cycles of amplification for the receptor and 25 cycles for actin. No product was obtained with this primer set in the absence of the RT.

Conformation-sensitive gel electrophoresis (CSGE) (14) was performed with modifications as described by Considine et al. (9). cDNA samples with potential sequence variations were purified using the QIAquick Spin Kit (Quiagen, Chatsworth, CA) and sequenced using automated protocols for fluorescent detection of dideoxy termination

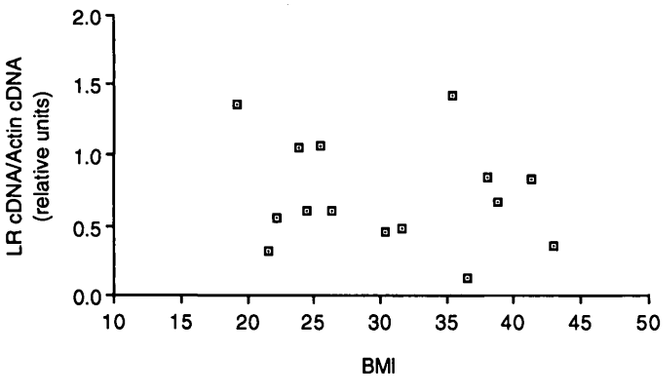

FIG. 1. Lack of correlation of leptin-receptor gene expression with BMI. The relative amount of leptin-receptor mRNA in human hypothalamus was determined by RT-PCR.

products on a model 377 DNA sequencing system (Applied Biosystems, Foster City, CA).

RESULTS

Expression of hypothalamic leptin receptor in lean and obese individuals. Leptin-receptor gene expression in lean versus obese individuals was examined by measuring the incorporation of radiolabeled downstream primer into an ~800-bp PCR product. There was no difference in the amount of leptin-receptor mRNA between the lean and obese groups, nor was there a correlation between leptin-receptor gene expression and BMI (Fig. 1). No difference in the size of the PCR product from lean and obese individuals was observed, ruling out the presence of alternative splicing in obese humans, as observed in *db/db* mice.

Analysis for sequence variations in the leptin receptor. Utilizing the six primer pairs described above, the complete cDNA sequence of one lean hypothalamic leptin receptor was generated. Sequence analysis was in agreement with the human sequence previously determined (11). The remaining 14 subjects were screened for potential sequence variations in the leptin-receptor cDNA product by CSGE. A single homoduplex band (Fig. 2*A*) was observed for most subjects in most regions of the leptin receptor, suggesting that the leptin receptor transcripts were derived from a single species of mRNA. However, several heteroduplex species were observed in the region defined by primer set 2 (588–607, 1367–1388) (Fig. 2*B*). Sequencing of a PCR product from this region identified a single-base substitution (A→G) at position 668 of the leptin-receptor cDNA. This substitution, in the second base of the codon at position 223 of the leptin receptor protein, changed a glutamine to an arginine. The base substitution generated a restriction site for *Hpa* II in the cDNA, which enabled confirmation of the substitution (Fig. 2*C*). This polymorphism was very common in the study population; it was detected in all but one subject. Three subjects appeared to be homozygous for the polymorphism.

Sequence variations were detected in two other areas of the leptin receptor. Four subjects were heterozygous for an A→G substitution at position 326. This second base change did not change the amino acid. Three subjects exhibited a G→A substitution at position 3057. This third base change did not change the amino acid; affected subjects were heterozygous for the variation. No relationship between any of the sequence variations and the lean or obese phenotype could be established.

To confirm that the homoduplexes detected did not occur as a result of a recessive mutation(s) in the transcripts derived from both alleles of the leptin-receptor gene, subject PCR products were heteroduplexed with the PCR products derived from the lean individual who was screened by direct sequencing. CSGE analyses of the PCR products did not detect any additional heteroduplex species in the 14 subjects screened in this manner.

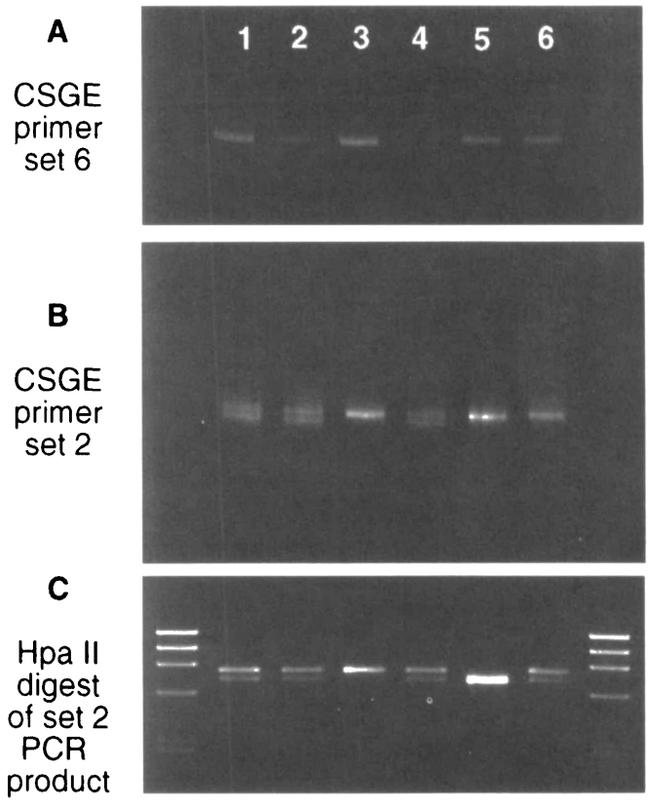

FIG. 2. Detection of sequence variation in the leptin receptor. Four obese (1,2,4,5) and two lean (3,6) subjects representative of the study population are illustrated. No heteroduplex species were detected in the PCR product generated by primer pair 6 (*A*). Heteroduplex species were detected in the PCR product from primer set 2 (*B*) indicating the presence of sequence variation. *Hpa* II restriction digest confirms the presence of the A→G substitution (*C*). Subject 5 is homozygous for the A→G substitution, subject 3 lacks the base change, and the remaining subjects are heterozygous. The size markers in panel C are ϕx174 DNA-*Hae* III digest.

DISCUSSION

The cDNA sequences of the leptin receptor and several truncated variants have recently been deduced (1,2), and based on this information, we have begun a preliminary examination into the possibility that a defective hypothalamic leptin receptor is the cause of leptin resistance in humans. To this end, using RT-PCR, we have confirmed the reported sequence of the human leptin receptor and have demonstrated that it is present in the hypothalamus. We did not detect the insertion of an additional 106-bp nucleotide sequence in the PCR product derived from the obese subjects. This observation rules out the possibility that the *db/db* mouse leptin-receptor defect is present in obese humans. Using the RT-PCR technique previously demonstrated to detect a twofold elevation in *OB* gene expression in obese humans (13), we found no difference in the amount of hypothalamic leptin-receptor mRNA in our study population. Although only 15 subjects were analyzed, this finding sug-

gests that human leptin resistance is not due to a lack of hypothalamic leptin receptor.

Although the *db/db* mouse mutation was not present in the cDNA of obese humans, other mutations might exist that could result in a defective leptin receptor. A single-base substitution that results in an amino acid change has recently been identified in the *fa/fa* rat (15). This amino acid substitution, hypothesized to affect dimerization of the receptor, may be the underlying cause of obesity in this rat model. Therefore, we screened the cDNA of the coding region of the human leptin receptor for sequence variations by CSGE. Sequence variations were detected in several regions of the leptin receptor. Most variations were single-base substitutions limited to three or four subjects and did not result in a change of amino acid. The *fa/fa* rat mutation was not detected in any of the subjects studied. However, a single-base substitution at position 668 of the cDNA was detected in 14 of the 15 individuals studied. This second base substitution changed a glutamine to an arginine in the leptin-receptor protein. It is not likely that this polymorphism results in leptin resistance for at least two reasons. First, the change was not limited to obese subjects but was detected in six out of seven lean individuals. Second, most subjects were heterozygous for the base change and their mRNA expressed both forms of leptin-receptor messages. Although this amino acid change must ultimately be modeled and the altered protein must be tested for its ability to transmit the leptin signal, it does not appear that this change in the leptin receptor is the cause of leptin resistance in humans. Note that the study population in which the polymorphism was identified is composed entirely of African-American men.

It is important to note that CSGE, like all PCR-based genome scanning techniques, may fail to detect ~10% of all single-base mismatches (14). In addition, these methods fail to detect insertions or deletions that interfere with amplification of the mutant allele. We have attempted to minimize this possibility by the generation of overlapping PCR products. However, such heterozygous mutations could result in the generation of mutant leptin receptors that might have dominant-negative effects. For these reasons, the present study cannot completely eliminate the possibility that functionally significant leptin-receptor mutations exist in the patient population studied.

If the hypothalamic leptin receptor is normal in obese humans, what is the defect that results in leptin resistance? Leptin receptors that are hypothesized to transport leptin into the cerebrospinal fluid have been identified in the choroid plexus (11). If access of leptin to the hypothalamus is through the choroid plexus, a defect in leptin transport in obese humans could result in leptin resistance. Alternatively, the resistance to leptin observed in obese humans may be due to a postreceptor defect in signal transduction or effector response.

In summary, the leptin receptor is expressed in the hypothalamus of humans. There was no difference in the amount of leptin receptor mRNA in lean or obese subjects, nor was a defect similar to that in the *db/db* mouse or *fa/fa* rat leptin receptor observed. Although further work is necessary to elucidate the exact mechanism of leptin-receptor signal transduction, it does not appear that defects in the leptin receptor itself are responsible for the leptin resistance characteristic of human obesity.

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REFERENCES

1. Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP: Evidence that the diabetes gene encodes the leptin receptor: Identification of a mutation in the leptin receptor gene in *db/db* mice. *Cell* 84:491–495, 1996
2. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM: Abnormal splicing of the leptin receptor in *diabetic* mice. *Nature* 379:632–635, 1996
3. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F: Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. *Science* 269:540–543, 1995
4. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM: Weight reducing effects of the plasma protein encoded by the *obese* gene. *Science* 269:543–546, 1995
5. Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, MacKellar W, Rosteck PR Jr, Schoner B, Smith D, Tinsley FC, Zhang XY, Heiman M: The role of neuropeptide Y in the antiobesity action of the *obese* gene product. *Nature* 377:530–532, 1995
6. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P: Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269:546–549, 1995
7. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 334:292–295, 1996
8. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM: Leptin levels in human and rodent: Measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. *Nature Med* 1:1155–1161, 1995
9. Considine RV, Considine EL, Williams CJ, Nyce MR, Zhang P, Opentanova I, Ohannesian JP, Kolaczynski JW, Bauer TL, Moore JH, Caro JF: Mutation screening and identification of a sequence variation in the human *ob* gene coding region. *Biochem Biophys Res Commun* 220:735–739, 1996
10. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extracts. *Anal Biochem* 162: 156–159, 1987
11. 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, Woolf EA, Selent-Munro C, Tepper RI: Identification and expression cloning of the leptin receptor, OB-R. *Cell* 83:1263–1271, 1995
12. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N: Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354, 1985
13. Considine RV, Considine EL, Williams CJ, Nyce MR, Magosin SA, Bauer TL, Rosato EL, Caro JF: Evidence against either a premature stop codon or the absence of obese gene mRNA in human obesity. *J Clin Invest* 95:2986–2988, 1995
14. Ganguly A, Rock MJ, Prockop DJ: Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* 90:10325–10329, 1993
15. Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CT, Hess JF: Leptin receptor missense mutation in the fatty Zucker rat. *Nature Genet* 13:18–19, 1996