Expression of the Long and Short Leptin Receptor Isoforms in Peripheral Blood Mononuclear Cells: Implications for Leptin's Actions

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Leptin, the adipocyte-derived hormone, is secreted into the blood and regulates body weight via its receptors in the hypothalamus. Leptin receptors are also present in many peripheral tissues implicating leptin in the regulation of other body functions, including reproduction, liver and enteric metabolism, hematopoiesis, and immunity. Four splice variants of the leptin receptor have been identified in humans: the long isoform that has full intracellular signaling capacity and 3 shorter isoforms that differ in the length of their cytoplasmic tail. Here, we report the quantification by reverse transcriptase-polymerase chain reaction (RT-PCR) of the relative expression levels of the 2 major leptin receptor splice variants, the long (OB-R_L) and the shortest membrane bound variant (OB-R_S) in mononuclear cells from peripheral blood of 15 healthy human subjects (9 women and 6 men), with a body mass index (BMI) that ranged from 19.7 to 41.6. Both OB-R_L and OB-R_S were coexpressed in all mRNAs tested. However, the expression of the short form (OB-R_S), was on average 8-fold higher than the expression of the long form (OB-R_L) (120.8 ± 12.9 *v* 14.6 ± 3.0 relative intensity units, $P < .001$). The predominance of the short splice variant over the long one was apparent in all samples and ranged from 4- to 27-fold. There was no significant difference in the expression of either isoform between men and women. However, the relative expression of both OB-R_S and OB-R_L isoforms was significantly lower in the overweight (BMI > 26), compared with the lean subjects (BMI < 25) (78.8 ± 9.1 and 6.2 ± 1.1 *v* 148.8 ± 14.4 and 18.9 ± 4.0 relative intensity units, respectively, $P < .03$) and was inversely correlated with the BMI and plasma leptin levels ($P < .01$). In conclusion, the expression of OB-R_S and OB-R_L leptin receptor isoforms appears to be reduced in human peripheral blood mononuclear cells from obese individuals, with OB-R_S remaining the predominant leptin receptor isoform. This might have implications for the bioavailability and/or action of circulating leptin not only on these cells, but also on other target tissues.

THE LEPTIN RECEPTOR is a transmembrane protein having similarity with the class I family of cytokine receptors[1] (eg, leukemia inhibitory factor [LIF] receptor, granulocyte colony-stimulating factor [G-CSF] receptor). Leptin receptors have been identified in several tissues outside the appetite regulatory centers of the brain, suggesting that leptin also has important actions on peripheral tissues. Thus, leptin can act as a proliferative factor and affect hematopoiesis and reproduction,[2,3] promote angiogenesis,[4] suppress lipid synthesis in vitro[5] and in vivo,[6] influence lipid availability by acting on gastrointestinal cells,[7] act directly on T cells reversing immunosuppression,[8] and inhibit insulin transcription and supress basal and glucose-stimulated insulin secretion.[9-11]

There exist several isoforms of the leptin receptor generated by mRNA alternative splicing, which vary in the length of their cytoplasmic tail. In humans, 4 such variants have been identified to be expressed in a variety of tissues, including the brain, liver, pancreas, placenta, lung, skeletal muscle, small intestine, heart, and hematopoietic cells. The longest isoform is predicted to have a long intracellular domain of 305 amino acids, which contains the conserved sequence motifs necessary for the binding of the Janus activated kinases (JAK) and the STAT family of signal transducing factors, required for full leptin signaling function.[12] The shorter isoforms share the same extracellular domain with the long receptor isoform, but they have truncated cytoplasmic domains ranging from 36 to 98 amino acids in length. As a result, they lack the STAT binding element, thus having reduced signal transduction capabilities.[13-15] However, due to the existence of a functional JAK binding motif in their carboxyl terminal tail, these receptor isoforms can still transduce part of the leptin signaling action, which may actually be divergent to those of the long isoform.[13,16]

Interestingly, all leptin receptor splice variants are coexpressed in various tissues, but their levels of expression may differ between them.[17,18] The longest isoform appears to be the most abundant in the hypothalamus, while the shorter isoforms seem to predominate in peripheral tissues.[19,20] The wide spread expression of the shorter membrane bound isoforms in the periphery has led to the hypothesis that, by binding leptin, they could modulate in vivo the signaling activities of the long leptin receptor isoform.

In this study, we identified the presence and quantified the relative expression levels of 2 major leptin receptor splice variants, the long leptin receptor (OB-R_L) and the shortest membrane bound leptin receptor (OB-R_S), in peripheral mononuclear cells from lean and overweight individuals. This might have important implications for leptin's actions, as mononuclear cells via their leptin receptors could serve as a buffer/reservoir system for the clearance of circulating leptin.

MATERIALS AND METHODS

Patients

The study was approved by the hospital ethical committee. Peripheral blood (20 mL) was obtained from 15 healthy individuals (9 women and 6 men) after an overnight fast. Each subject gave their informed consent. All subjects had no history of endocrine abnormality and were under no medication. Their weight had been stable over the previous 6 months, and they were not on any specific diet.

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RNA Preparation

Mononuclear cells were isolated from blood samples using a Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient. Total RNA was isolated from the mononuclear cells using the TriPure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany) (20 to 25 μgr of RNA per 20 mL). The integrity of RNA samples was determined on agarose gels (1.2%) and spectrophotometrically using the absorption ratio at 260/280 nm. The RNA quantification was performed by measuring the absorption at 260 nm.

For the removal of any residual DNA contamination, RNA samples were preincubated with DNase I (Promega, Madison, WI), at 37°C for 25 minutes and then inactivated at 65°C for 5 minutes before the reverse transcription-polymerase chain reaction (RT-PCR) method.

RT-PCR

The RT-PCR method was performed using 2 different approaches for all samples in a Perkin-Elmer 9600 thermocycler (Perkin Elmer, Norwalk, CT). The first approach was the application of the RT-PCR reaction in 1-step using the Titan One tube RT-PCR system (Roche Molecular Biochemicals) according to the manufacturer's instructions, while the second approach was a 2-step RT-PCR reaction, a reverse transcription reaction step, followed by a PCR reaction. The results from both RT-PCR reactions were analyzed together.

For the first approach, 1.5 μg of total RNA was used, and the reaction was performed in a final volume of 25 μL, using 20 pmol of each leptin receptor primer or 5 pmol for each of the β-actin primers. Briefly, after the reverse transcription reaction, which took place at 49°C for 40 minutes, there followed 10 PCR cycles at 94°C for 30 seconds, 53°C (for the OB-R_S transcript) or 58°C (for the OB-R_L transcript) for 30 seconds, 68°C for 55 seconds. These were followed by 11 additional PCR cycles with the same denaturing and annealing steps, increasing in each 1 of them the elongation step by 5 seconds. Finally, the reaction was completed with 14 PCR cycles with the previous denaturing and annealing steps and an elongation step at 68°C for 115 seconds. Equal amounts from total RNA were used for either the leptin receptor or the β-actin RT-PCR reactions.

For the second approach, cDNA synthesis was performed using approximately 4 μg of total RNA, random hexanucleotides (200 pmol, Biolabs, Hertfordshire, UK), Moloney murine leukemia virus (M-MLV) reverse transcriptase (400 U, Promega), RNasin inhibitor (20 U, Promega), and deoxyribonucleoside triphosphate (dNTP) (0.4 mmol/L) at 37°C for 1½ hours. This was followed by the addition of 400 U M-MLV to the reaction, and the incubation was continued for another 1 and a half hours. The reverse transcription reaction was terminated with the addition of 3 U RNase H (GibcoBRL, Life Technologies, Paisley, UK) for 30 minutes at 37°C. The PCR reaction was subsequently performed in a final volume of 25 μL, using an aliquot (1/10) from the reverse transcription reaction and 20 pmol of each leptin receptor primer or 5 pmol of each β-actin primer, with the same denaturing and annealing steps as before, but an elongation step at 72°C for 90 seconds for a total of 35 cycles. Equal amounts from total RNA were used for either the β-actin or the leptin receptor PCR reactions.

Primer Design

Two different sets of specific primers were used to amplify the different leptin receptor splice variants (Fig 1). One pair (OB-R/2s and OB-R/Lr) was chosen to identify and amplify a 279-bp PCR product from the OB-R_S splice variant (huB219.3 or 6.4 short splice variant; GenBank acc. no. U66495).[2] The second pair (OB-R/1s and OB-R/Sr, Fig 1) was chosen to identify and amplify a 470-bp PCR product from the OB-R_L splice variant (huOB-R; GenBank acc. no. U43168).[1] The reverse primers (OB-R/Sr and OB-R/Lr) were chosen to be specific for the OB-R_S and OB-R_L splice variants, respectively (Fig 1).

A third set of primers (β-act1 and β-act2) that recognized and amplified a PCR product of 587 bp in the human β-actin mRNA (GenBank acc. no. AB004047) were also used. All primers were chosen to lie in different exons and to flank intron sequences. All primer sequences are shown below:

OB-R/1s: CCCATTGAGAAGTACCAGTTCAGTC
OB-R/Sr: CATGATTAGACTTCAAAGAATGTCCG
OB-R/2s: CAAGAATTGTTCCTGGGCACAAGGAC
OB-R/Lr: CCTGGGCCTCTATCTCCCATGAGC
β-act1: CCAAGGCCAACCGCGAGAAGATGAC
β-act2: AGGGTACATGGTGGTGCCACCAGAC.

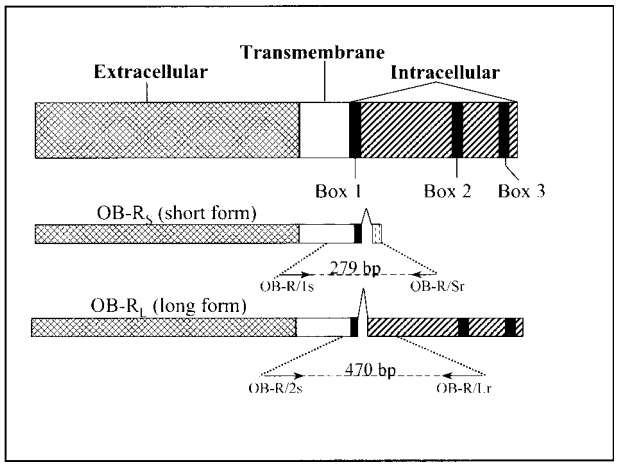

Fig 1. Diagrammatic representation of the full-length leptin receptor protein. The different protein domains are schematically represented with various hatchings. Box 1, 2, and 3 (black boxes) represent the conserved binding motifs necessary for JAK2 (box 1 and 2) and STAT3 (box 3) activation, respectively. The alternatively spliced variants for the OB-R_S and the OB-R_L are shown below with the different pairs of primers specific for each variant (OB-R/1s and OB-R/Sr, OB-R/2s and OB-R/Lr) used for the RT-PCR. The length of each specific PCR product is also indicated.

Image Analysis Documentation System

In each RNA sample, RT-PCR reactions were performed in triplicate. All PCR products were run on 1.2% to 1.5% agarose gels and were visualized with ethidium bromide staining. Product lengths were estimated using standard molecular weight markers (pUC18-HinfI). Data were collected and analyzed by an ImageMaster VDS Analyser System (Amersham Pharmacia Biotech). The mean integrated optical density of each PCR band was determined by the program according to a density calibration step tablet after subtraction of the background. Measurements for each PCR band were normalized using the corresponding integrated optical density of the β-actin mRNA as standard. Expression of OB-R_S and OB-R_L isoforms were referred to as the mean of the all independent RT-PCR experiments in relative integrated optical density units.

Immunoassay

Leptin levels were measured in the serum from all individuals using a human leptin enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Oxon, UK). Serum samples were diluted 50-fold before the assay. The sensitivity of the assay was less than 7.8 pg/mL. The intraassay coefficient of variation was less than 3.3%.

Statistical Analysis

Statistical analysis was performed using the SPSS for Windows 1997 version 8.0 software (SPSS, Chicago, IL). Data are expressed as means

± SEM. Comparisons between the mean values and correlations were performed with nonparametric statistics (Mann-Whitney test and Spearman's correlation coefficient, respectively). Upper level of statistical significance was chosen to be equal to 5%.

RESULTS

Table 1 shows the clinical characteristics of all individuals studied.

RT-PCR reactions were performed in all RNAs using the leptin receptor-specific primers and the β-actin primers. PCR specific bands of the expected lengths for the OB-R_S (279 bp) and the OB-R_L (470 bp) leptin receptor splice variants and for β-actin (587 bp) mRNA were detected in all samples. Figure 2A shows a representative agarose gel of typical RT-PCR reactions. It is clear that the OB-R_S product incorporates several-fold higher levels of optical density than the OB-R_L product. This was evident in all individuals tested. The integrated optical density of each OB-R_S or OB-R_L PCR band, measured in arbitrary units, was quantified and normalized in all samples against the integrated optical density of the β-actin PCR product. The average level of expression for the OB-R_S isoform from all samples was determined to be approximately 8-fold higher than the expression of the long form (OB-R_L) (120.8 ± 12.9 *v* 14.6 ± 3.0 relative intensity units, $P < .001$) (Fig 2B), but varied significantly between samples from 4- to 27-fold. Similar differences between the relative expression levels of the OB-R_S and OB-R_L transcripts were observed in men and women.

It is noteworthy that there was no significant difference in the expression of either OB-R_S or OB-R_L transcripts between men and women (94.3 ± 18.9 and 12.9 ± 7.4 *v* 138.4 ± 15.6 and 15.1 ± 2.8, respectively) (Fig 3A). However, the relative expression levels of the OB-R_S and OB-R_L isoforms were found to be significantly lower in the overweight/obese compared with the lean individuals (78.8 ± 9.1 and 6.2 ± 1.1 *v* 148.8 ± 14.4 and 18.9 ± 4.0 relative intensity units, respectively, $P < .03$) (Fig 3B). Moreover, there was an inverse relationship of the relative expression of either leptin receptor isoform with the body mass index (BMI) ($r = -.69$, $P < .01$ and $r = -.57$, $P < .03$, respectively) and the serum leptin levels ($r = -.65$, $P < .01$ and $r = -.70$, $P < .01$, respectively). Figure 4 shows the correlations of OB-R_S with BMI and leptin levels. Leptin levels were clearly higher in the overweight/obese versus lean subjects (26.1 ± 7.2 *v* 7.2 ± 1.7 ng/mL, $P < .01$) (Table 1).

DISCUSSION

This study has shown that both the long leptin receptor splice variant (OB-R_L) and the shortest membrane bound (OB-R_S) splice variant are expressed in blood mononuclear cells from healthy individuals with a consistent, albeit variable, predominance of the short one. This several-fold higher expression of the OB-R_S over the OB-R_L leptin receptor splice variant was observed in both men and women, whether lean or overweight. Overweight individuals, however, had lower relative expression levels of both leptin receptor mRNA transcripts compared with lean individuals. We cannot exclude the possibility that this might be due to a gender influence, given the predominance of female and male subjects in the lean and overweight groups, respectively. However, we believe this is an unlikely possibility, because there was no significant difference in the mRNA levels of either leptin receptor transcript between men and women. In addition, there was an overall significant inverse correlation of both leptin receptor transcripts with the BMI and the circulating leptin levels, suggesting that the degree of adiposity and/or the elevations in leptin levels, rather than gender, might influence the absolute or relative expression of OB-R_L and OB-R_S.

Assuming that the downregulation of leptin receptor transcripts reflects a similar reduction in leptin receptor numbers on the cell surface, this might contribute to the apparent leptin resistance of obese individuals. In support of this possibility, there is experimental evidence to suggest that the expression of leptin receptors is sensitive to both genetic and physiologic interventions that cause a change in circulating leptin.[21,22] Thus, *ob/ob* mice that lack leptin have elevated levels of total leptin receptor expression.[21] In addition, reduction of leptin mRNA expression induced by exposure to cold resulted in an increase of leptin receptor expression.[22] Moreover, the exogenous administration of leptin reduced, while prolonged fasting increased, the leptin receptor mRNAs levels in the mouse hypothalamus.[21,22] It is of note, however, that Kielar et al[18] found no difference in the expression levels of the short leptin receptor splice variant in biopsies of visceral fat from obese and lean

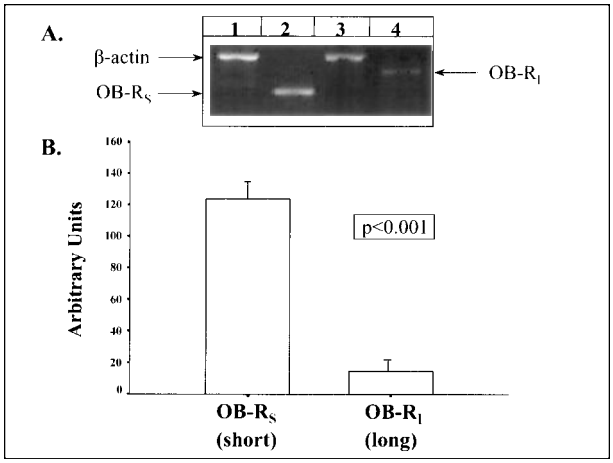

Fig 2. (A) Representative RT-PCR reactions showing the OB-R_S and the OB-R_L spliced variants in total RNA from the peripheral mononuclear cells of a healthy individual. Lanes 2 and 4 show the RT-PCR–specific bands for the OB-R_S and OB-R_L leptin receptor isoforms, respectively, and lanes 1 and 3 show the RT-PCR band for the β-actin mRNA from the same individual. (B) Quantification of the relative expression levels of the OB-R_S and the OB-R_L leptin receptor isoforms from all individuals studied. Bars represent the means ± SEM.

Table 1. Clinical Characteristics of the Individuals Studied

	Lean (BMI <25)	Overweight/ Obese (BMI >26)
No. (F:M)	9 (8:1)	6 (1:5)
Age (yr)	34 ± 2.6	37 ± 2.0
BMI	21.3 ± 0.3	33.5 ± 2.6*
Leptin (ng/mL)	7.2 ± 1.7	26.1 ± 7.2†

NOTE. Values are mean ± SEM.

*$P < .001$ *v* lean.

†$P < .01$ *v* lean.

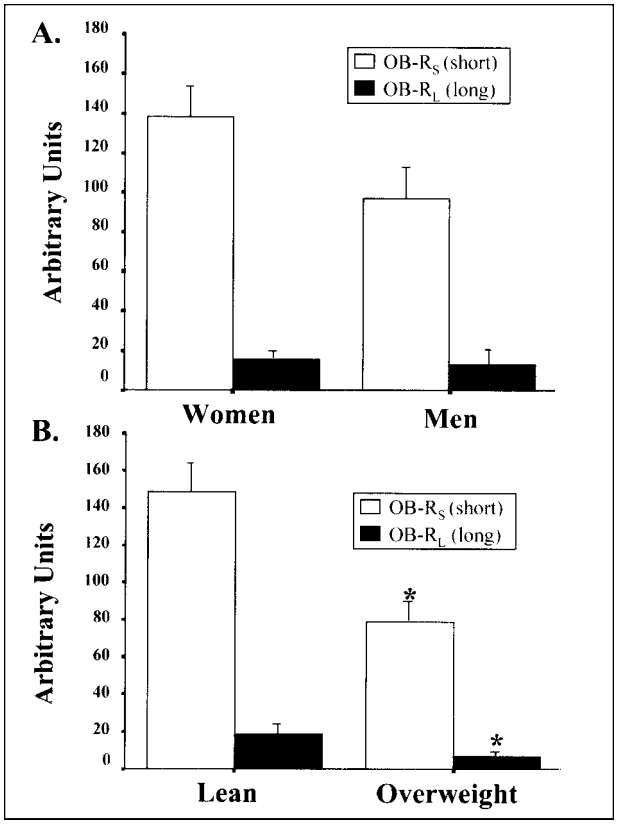

Fig 3. Comparison of the relative expression levels of the OB-R$_S$ and the OB-R$_L$ leptin receptor isoforms in peripheral mononuclear cells between women and men (A) and lean and overweight individuals (B). Bars are means ± SEM. *$P < .05$ *v* lean individuals.

patients. It might be that the smaller number of patients studied, combined with the large variability in the expression of the 2 isoforms, was responsible for their inability to detect significant differences. Alternatively, the regulation of the leptin receptor isoforms might differ between tissues.

Because both leptin receptor isoforms (short and long) can bind leptin and mediate its internalization and degradation,[23] the apparently higher expression of OB-R$_S$ in circulating mononuclear cells could serve as a control mechanism of leptin's signaling action via OB-R$_L$, not only on these cells, but also on other target tissues, including the brain, bone marrow, immune system, gonadal axis, pancreas, and gastrointestinal tract. Interestingly, a relative abundance of the short membrane bound leptin receptor isoform has also been demonstrated in other peripheral tissues both in humans and mice. Thus, Gainsford et al[24] and Bennett et al[3] have detected a relative abundance of the short isoform over the long one in mouse and human hematopoietic cells from fetal and adult tissues. Using an RNase protection assay, Chilardi et al[14] were also able to demonstrate expression of both leptin receptor splice variants with predominance of the short isoform in most mouse tissues outside the brain, a finding also reported by Löllmann et al.[19] It might, therefore, be that the differential expression of the leptin receptor isoforms together with their putatively different signaling capabilities plays a role in the pleiotropic effects of leptin's actions. The soluble isoform, for example, can act as leptin binding protein to reduce serum-free leptin levels, whereas the membrane bound isoforms may act on different signaling pathways to activate other effector systems, leading eventually to different biological actions. Interestingly, in obese individuals, the majority of leptin circulates in the free presumably bioactive form, while in lean subjects, the majority is in the bound form, and this might be affected by the individual metabolic state.[25] It is not clear in our study whether the lower expression of the leptin receptor transcripts, and particularly, of the more abundant short splice variant in the obese individuals is adequate to influence the circulating free leptin molecules. It is more likely that the expected concomitant downregulation of the long fully functional leptin receptor transcript would contribute to the leptin resistance observed in obese individuals, rather that this represents a compensatory mechanism of increasing the availability of the free leptin form.

Accumulating evidence suggests that the leptin receptor mediates leptin signaling through activation of the JAK and the STAT members of transcription factors.[12,26] Thus, a box 1 motif (Fig 1) responsible for the activation/phosphorylation of the JAK2 and mitogen-activated protein kinase (MAPK) kinases is shared by both the long and the short leptin receptor isoforms in their cytoplasmic domains, and transient transfection assays have shown that both isoforms are capable of inducing leptin-dependent phosphorylation of these kinases.[16] Unlike the long receptor isoform, however, the short membrane bound isoform is unable to induce leptin-dependent activation of the STAT

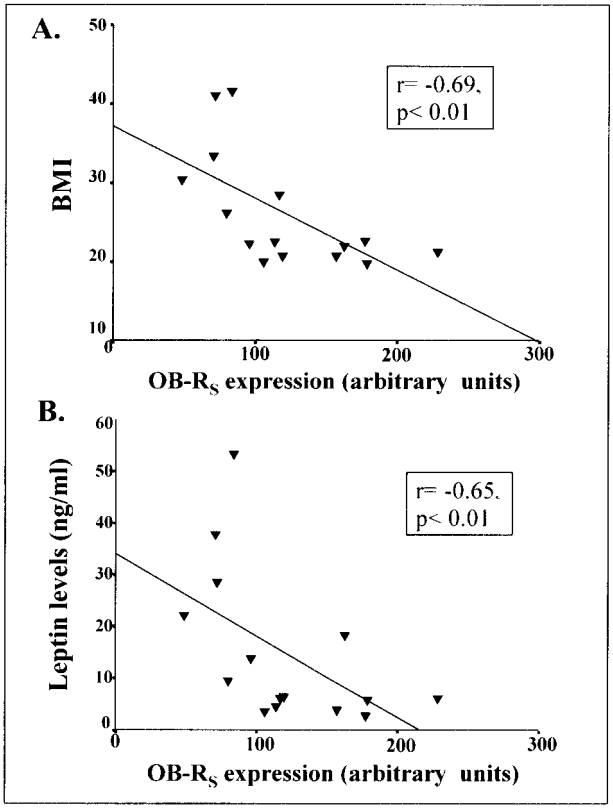

Fig 4. Correlation between the relative expression levels of the short leptin receptor isoform with BMI (A) and leptin levels (B) in the individuals studied.

pathway, because it lacks the STAT binding element in its truncated cytoplasmic domain (Fig 1).[13,15]

In conclusion, OB-R_S is the predominant leptin receptor transcript in human peripheral mononuclear cells with important implications for the bioavailability and/or actions of circulating leptin, not only on these cells, but also on other target tissues reached by leptin via the bloodstream. Furthermore, the overall lower expression levels of the leptin receptor splice variants in overweight compared with lean individuals might suggest that hyperleptinemia could regulate leptin receptor numbers, contributing to the obesity-related leptin resistance. Alternatively, low leptin receptor numbers might result in a compensatory increase of leptin levels and may contribute to the considerable variation in leptin levels at high percent body fat.

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