Influence of the presence of OB-Re on leptin radioimmunoassay

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Abstract

Leptin, a hormone derived from adipose tissue, regulates energy homeostasis and body weight. In the mouse, serum leptin levels, when measured by radioimmunoassay (RIA), increase by a factor of more than 50 times during pregnancy, compared with those in the non-pregnant state. It is well known that mouse placenta produces the secretory isoform of the leptin receptor, OB-Re. In order to investigate the issue of whether serum leptin levels are actually increased during pregnancy or whether the increased OB-Re concentration plays a role in this phenomenon, serum leptin levels were determined by the immunoprecipitation of leptin using anti-leptin antibody, and were found to be increased only by about ten times during pregnancy. To investigate the influence of OB-Re on leptin measurement by the RIA procedure, serum leptin levels were measured by the RIA after the addition of OB-Re to the serum. The apparent values of leptin levels increased in parallel with the amount of OB-Re added to the serum. Leptin levels, as determined by the RIA, might therefore provide artificially high values when serum levels of the secretory form of OB-R are high, in cases, for example, such as the last period of pregnancy in mice.


Introduction

Leptin (Zhang et al. 1994), a 16 kDa protein which is encoded by the obese gene, is mainly secreted from adipose tissues and regulates body weight by reducing food intake and increasing energy expenditure. Several alternate spliced isoforms (a–e, as well as others) of the leptin receptor (OB–R) have been cloned, and all of these, except for the OB-Re (secretory form), contain a single transmembrane domain (Chen et al. 1996, Ghilardi et al. 1996, Iida et al. 1996a,b). Plasma levels of leptin vary in proportion to adiposity in lean and obese rodents as well as in humans (Considine et al. 1995). The human placenta also produces leptin, the serum concentration of which increases about twofold during pregnancy (Hassink et al. 1997, Masuzaki et al. 1997). This is not the case for the rat, however, where the placenta does not produce significant amounts of leptin and adipose tissues per se increase leptin production during pregnancy (Kawai et al. 1997). In the mouse, the placenta secretes the OB-Re and, as a result, serum leptin levels, which are measured by radioimmunoassay (RIA), increase significantly during pregnancy (Gavrilova et al. 1997, Tomimatsu et al. 1997, Yamaguchi et al. 1998). In the case of a family with early-onset morbid obesity, as the result of a defect in the human OB–R gene (Clément et al. 1998), all of the isoforms of OB-R are produced as the secretory form in affected persons who have a homozygous mutation, and who show high levels of serum leptin. Other family members with this heterozygous mutation also show high levels of serum leptin considering their adiposity. These findings suggest that OB-Re increases the half-life period of leptin in vivo. The purpose of the present study was to examine the effect of the OB-Re isoform on the detection of leptin levels by RIA.

Materials and Methods

Animals

All work was performed in accordance with the Guidelines for Animal Experimentation, issued by the Japanese Association for Laboratory Animal Science in 1987. Non-pregnant ICR mice at 9 weeks of age and time-pregnant ICR mice were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). The day on which a vaginal plug was observed was designated as day 0. Blood samples were collected from the abdominal aorta of the mice, which were anesthetized by an intraperitoneal injection of pentobarbital. The blood samples were centrifuged and the supernatant, which was stored at ~ 80 °C until assayed, was used as the serum sample.

Partial purification of rat OB-Re

The CHO cell clone, which stably expresses rat OB-Re (Yamaguchi et al. 1998), was incubated with serum-free
medium S-Clone SF-02 (Sanko Junyaku Co. Ltd, Tokyo, Japan). The conditioned media was dialyzed against buffer A (25 mM HEPES (pH 7-4), 100 mM NaCl), followed by two passes through a wheat germ lectin Sepharose 6 MB column (Amersham Pharmacia Biotech Ltd, Amersham, Bucks, UK). The column was washed with buffer A and then eluted by 0·3 M N-acetyl-d-glucosamine in buffer A. The elute was concentrated and washed with buffer A with a Centricon Plus-20 (100 kDa molecular weight cut off) (Millipore Corp., Bedford, MA, USA), and used as the OB-Re for the purposes of this experiment (Matsuda et al. 1999). After further purification by ion-exchange chromatography using Fractogel EMD DEAE (S) (Merck Eurolab GmbH, Darmstadt, Germany), the N-terminal amino acid sequence of the OB-Re was determined to be LNLAYPTSPW (>95%). The N-terminal amino acid of OB-Re is situated one amino acid residue upstream of the predicted signal cleavage site (Iida et al. 1996a).

Preparation of anti-OB-R antiserum and anti-leptin IgG Sepharose

A portion of the extracellular domain (from amino acid no. 205 to no. 344) (Iida et al. 1996a) of the rat OB-R was produced in E. coli using QIA expressionist® (Qiagen GmbH, Hilden, Germany) in forms of NH2-terminal predicted signal cleavage site (Iida et al. 1996). The resulting protein was used as the antigen for immunizing Hartley guinea pigs (obtained from Japan SLC, Inc.) and anti-OB-R antiserum was obtained as described previously (Yamaguchi et al. 1998).

Anti-leptin antiserum was obtained by immunizing a rabbit with recombinant mouse met-leptin. In order to prepare anti-leptin IgG Sepharose, IgG, which was purified from the antiserum using protein A Sepharose (Amersham Pharmacia Biotech Ltd), was coupled with Sepharose using HiTrap NHS-activated Sepharose (Amersham Pharmacia Biotech Ltd) according to the manufacturer’s protocol.

Western blot analysis

Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (2–15% gradient gel for OB-Re or 15% gel for leptin) (Laemmli 1970) and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech Ltd). The membranes were blocked by incubation for 1 h at room temperature in blocking buffer (20 mM Tris–HCl (pH 7·5), 150 mM NaCl, 5% skim milk, 0·1% Tween 20) and then incubated overnight at 4 °C in the same buffer which contained anti–OB-R antiserum (guinea pig) or anti-leptin antiserum (rabbit). The membranes were washed five times for 10 min each in a washing buffer (20 mM Tris–HCl (pH 7·5), 150 mM NaCl, 0·1% Tween 20) and then incubated for 1 h at room temperature in blocking buffer which contained the peroxidase-conjugated F(ab’)2 fragment of donkey anti IgG (H+L) from either a guinea pig or a rabbit (Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA), followed by five washes of 10 min each with the washing buffer. The bound antibody was visualized using the ECL chemiluminescent Western blotting detection system (Amersham Pharmacia Biotech Ltd) and exposed to X-ray film (Fuji Photo Film Co. Ltd, Tokyo, Japan). The intensities of the bands on the X-ray films were determined by scanning the X-ray film with a laser densitometer UltroScan XL (Amersham Pharmacia Biotech Ltd).

Deglycosylation of OB-Re

The OB-Re, which was produced by CHO cells, or 0·05 µl serum from a pregnant mouse at day 17 was denatured by boiling in a denaturation buffer for 3 min, followed by treatment with or without glycopeptidaseF (Takara Shuzo Co. Ltd, Otsu, Shiga, Japan) in a buffer which contained 0·1 M Tris–HCl (pH 8·6), 0·1% SDS, 21 mM 2-mercaptoethanol and 1% Nonidet P-40 for 16 h at 37 °C. After incubation, samples were subjected to Western blot analysis using anti-OB-R antiserum.

Detection of OB-Re-like protein using leptin Sepharose

Leptin Sepharose was prepared by coupling recombinant rat leptin (Murakami & Shima 1995, Mizuno et al. 1998) with Sepharose using HiTrap NHS-activated Sepharose. Each 10 µl serum from a non-pregnant mouse, a pregnant mouse at day 13 or day 17 or serum from a non-pregnant mouse to which had been added 0·5 µg OB-Re produced from CHO cells, was mixed with 300 µl IP buffer (20 mM Tris–HCl (pH 7·4), 0·5% Triton X-100, 0·3 M NaCl). This mixture was then mixed with leptin Sepharose for 16 h at 4 °C with or without 50 µg mouse leptin (Alpha Diagnostic International, San Antonio, TX, USA). After centrifugation at 8000 g at 4 °C, the leptin Sepharose was washed three times with IP buffer followed by one washing with a buffer containing 20 mM Tris–HCl (pH 7·4). After centrifugation, materials which were bound to the leptin Sepharose were eluted by the addition of sample buffer and the resulting eluted materials were subjected to Western blot analysis using anti-OB-R antiserum.

Detection of leptin in mouse serum by immunoprecipitation using anti-leptin IgG Sepharose

A 100 µl sample of serum obtained from four mice of each group (non-pregnant mice, pregnant mice at day 13 or day...
were mixed together. The total 400 µl serum sample from each group or several concentrations of mouse leptin (Alpha Diagnostic International) was mixed with 400 µl IP buffer for 1 h at 4 °C. The resulting mixture was then mixed with anti-leptin IgG Sepharose for 16 h at 4 °C. After centrifugation at 8000 g at 4 °C, the anti-leptin IgG Sepharose was washed three times with the IP buffer, followed by one wash with a buffer containing 20 mM Tris–HCl (pH 7·4). After centrifugation, the materials which were bound to the anti-leptin IgG Sepharose were eluted by the sample buffer and subjected to Western blot analysis using anti-leptin antiserum.

Assays
Serum leptin levels were measured with an RIA kit (Linco Research, Inc., St Louis, MO, USA) (Ma et al. 1996). In this analysis, the bound/free separation of the tracer is achieved by means of double-antibody methods. Briefly, leptin standards or mouse sera (100 µl) were mixed with 125I-labeled mouse leptin and incubated with anti-leptin antibody for 20 h at 4 °C. A precipitating reagent, which contained anti-IgG antibody was then added to each sample, followed by an additional 20 min of incubation at 4 °C. After next centrifuging the samples for 20 min at 2000 g and 4 °C, the supernatants were decanted and the radioactivity in the pellets was counted to determine the bound radioactivity. The log values of the leptin standards were then plotted vs (the bound counts (B)/zero standard bound counts (B0)) to generate a standard curve for the calculation of the unknowns.

Calculations and statistical analysis
Results are presented as mean ± s.d. values. The data presented in Fig. 6 were analyzed between groups by one-way analysis of variance (ANOVA) using the Statview computer software program (Abacus Concepts, Inc., Berkeley, CA, USA). When the ANOVA showed significant differences, post-hoc analyses were performed with Fisher’s test. Significance was accepted as P<0·05.

Results
OB-Re-like immunoreactivity in sera from pregnant mice
The serum leptin levels of the mice used in this study were determined by means of an RIA kit (Linco Research, Inc.). As has been previously reported (Gavrilova et al. 1997, Tomimatsu et al. 1997), the serum leptin levels of mice were increased after the mid-gestational stage. The serum leptin levels of non-pregnant mice (9 weeks of age) and pregnant mice at day 13 or day 17 were 4·1±2·0 (n=10), 90·4±28·9 (n=11) and 283·6±145·0 ng/ml (n=10) respectively. The levels of OB-Re in the sera of all mice were determined by Western blot analysis and representative data are shown in Fig. 1A. Bands approximately 140 kDa in size, which are consistent with the presence of OB-Re, were detected in the sera from pregnant mice at day 13 and day 17. The intensities of these bands in pregnant mice at day 17 were about six times as high as were found in pregnant mice at day 13, based on the use of laser densitometry. In addition to the

Figure 1 Detection of OB-Re-like immunoreactivity in sera by Western blot analysis using anti-OB-R antiserum. Each 0·2 µl serum from a non-pregnant mouse (NP) (A), a pregnant mouse at day 13 (D13) or day 17 (D17) and several concentrations of rat OB-Re produced by CHO cells (OB-Re) (A, B) were subjected to Western blot analysis using anti-OB-R antiserum. Arrowheads represent the OB-Re-like immunoreactivity in the sera from pregnant mice.
OB-Re-like bands, both larger and smaller bands were also clearly observed in sera from the non-pregnant mice. Two such bands were also faintly detected in the sera from pregnant mice at both day 13 and day 17. However, they do not appear to be OB-Re-related proteins, since no binding to leptin was observed (see below). The molecular weight, as determined by SDS-PAGE, of the OB-Re-like protein in the sera from pregnant mice at day 13 and day 17 was larger than the rat OB-Re protein produced by CHO cells. The intensities of the bands of the OB-Re-like protein in the sera from pregnant mice at day 13 and day 17 were equivalent to the intensities of the bands, which corresponded to the rat OB-Re protein which was electrophoresed in parallel, at levels of 3–8 µg/ml (day 13) and about 30 µg/ml (day 17) (Fig. 1B).

Leptin-binding activity of OB-Re-like protein

In order to clarify that the OB-Re-like protein is able to bind leptin, each sample of serum from non-pregnant mice and pregnant mice at day 13 or day 17 was precipitated with leptin Sepharose and subjected to Western blot analysis using anti-OB-R antiserum. The arrowhead represents the OB-Re-like immunoreactivity in the sera from pregnant mice. Each 10 µl serum from a pregnant mouse at day 17 (D17) (B) or serum from a non-pregnant mouse (NP) with or without 0.5 µg OB-Re produced by CHO cells in advance (C) was precipitated with leptin Sepharose with or without 50 µg mouse leptin and subjected to Western blot analysis using anti-OB-R antiserum. The rat OB-Re produced by CHO cells was electrophoresed in parallel with these samples for a size marker (OB-Re).

Figure 2  Leptin-binding activities of OB-Re-like immunoreactivity in mouse sera and rat OB-Re produced by CHO cells. (A) Each 10 µl serum from a non-pregnant mouse (NP) and a pregnant mouse at day 13 (D13) or day 17 (D17) was precipitated with leptin Sepharose and subjected to Western blot analysis using anti-OB-R antiserum. The arrowhead represents the OB-Re-like immunoreactivity in the sera from pregnant mice. Each 10 µl serum from a pregnant mouse at day 17 (D17) (B) or serum from a non-pregnant mouse (NP) with or without 0.5 µg OB-Re produced by CHO cells in advance (C) was precipitated with leptin Sepharose with or without 50 µg mouse leptin and subjected to Western blot analysis using anti-OB-R antiserum. The rat OB-Re produced by CHO cells was electrophoresed in parallel with these samples for a size marker (OB-Re).
The rat OB-Re produced by CHO cells and serum from a pregnant mouse at day 17 were treated with glycopeptidaseF, followed by a Western blot analysis (Fig. 3). Both the glycopeptidaseF-treated OB-Re-like protein and the rat OB-Re showed the same electrophoretic patterns. These results suggest that the OB-Re-like proteins in the serum from pregnant mice are, in fact, OB-Re molecules which are glycosylated to different degrees.

**Measurement of leptin concentration by immunoprecipitation using anti-leptin IgG Sepharose**

Each serum sample from non-pregnant mice, pregnant mice at day 13 or day 17 or several concentrations of mouse leptin were mixed with anti-leptin IgG Sepharose and the resulting precipitated materials with the anti-leptin IgG Sepharose were subjected to Western blot analysis using anti-leptin antiserum. Similar data were observed in three independent experiments and representative data are shown in Fig. 4A. Bands which are slightly larger than 14 kDa in size were detected in all mouse sera samples and appear to be mouse leptin. Judging by the characteristics of the leptin standards, which were run in parallel, leptin levels in the mouse sera were from 2.5 to 5 ng/ml (non-pregnant mice) and about 40 ng/ml (pregnant mice at day 13 and day 17).

In order to determine whether anti-leptin IgG Sepharose is able to immunoprecipitate the leptin–OB-Re complex, the materials which were precipitated with anti-leptin IgG Sepharose (Fig. 4A) were subjected to Western blot analysis using anti-OB-R antiserum (Fig. 4B). OB-Re-like bands of approximately 140 kDa in size were observed in sera from pregnant mice at day 13 and day 17. The anti-leptin IgG Sepharose was able to precipitate, not only free leptin, but also, at least in part, leptin which bound OB-Re (leptin–OB-Re complex).

**Figure 3** Deglycosylation of OB-Re from mouse serum and rat OB-Re produced by CHO cells. The rat OB-Re produced by CHO cells (OB-Re) or serum from a pregnant mouse at day 17 (D17) was treated with or without glycopeptidaseF, followed by Western blot analysis using anti-OB-R antiserum.

**Figure 4** Measurement of leptin concentration by immunoprecipitation. A total of 400 µl sera from each group of mice (non-pregnant mice (NP), pregnant mice at day 13 (D13) or day 17 (D17)) or several concentrations of mouse leptin were immunoprecipitated with anti-leptin IgG Sepharose, followed by Western blot analysis using anti-leptin antiserum (A) or anti-OB-R antiserum (B).
The levels of OB-Re which inhibit the immunoprecipitation of leptin by the anti-leptin IgG Sepharose were investigated. Mouse leptin and several concentrations of rat OB-Re produced by CHO cells were mixed, and subjected to immunoprecipitation with anti-leptin IgG Sepharose, followed by Western blot analysis using anti-leptin antiserum. As shown in Fig. 5, at levels up to 48 µg/ml OB-Re, immunoprecipitation was not inhibited by the presence of OB-Re. At a level of 192 µg/ml, however, a weak inhibition was detected.

**Influence of OB-Re on mouse leptin RIA**

When leptin levels determined by the mouse leptin RIA procedure were compared with the levels determined by the immunoprecipitation of leptin, leptin levels of non-pregnant mice were similar. However, serum leptin levels of pregnant mice at day 17, as determined by RIA, were about seven times higher than the levels determined by the immunoprecipitation of leptin. Since serum OB-Re levels increase during pregnancy to the levels of about 30 µg/ml at day 17 of pregnancy, the apparent increases in leptin levels determined with the mouse leptin RIA might also exist when OB-Re is also present in the samples. Pooled sera from non-pregnant mice, which had been mixed with several concentrations of rat OB-Re protein, were subjected to the mouse leptin RIA procedure. As shown in Fig. 6, the apparent values of the serum leptin levels, as determined by the mouse leptin RIA, gradually increased in parallel with increasing concentrations of rat OB-Re protein, which is also present in the serum. Levels of rat OB-Re protein in excess of 6 µg/ml in the serum led to a significant apparent elevation in leptin levels, as determined by the RIA. A similar result was observed in a second independent experiment using another batch of rat OB-Re. Thus, the presence of OB-Re leads to an overestimation of serum leptin levels, as determined by the RIA procedure.

**Discussion**

Serum leptin levels of the pregnant mice in this report, which were measured by RIA, increased to a level of 90·4 (day 13) and 283·6 ng/ml (day 17 of pregnancy). Although leptin levels, which were measured by immunoprecipitation using anti-leptin IgG Sepharose, also increased, serum leptin levels of both groups of pregnant mice on day 13 and day 17 were only about 40 ng/ml. Meanwhile, levels of OB-Re in the pregnant mice sera were determined to be from 3 to 8 µg/ml (day 13) and approximately 30 µg/ml (day 17 of pregnancy) by Western blot analysis using our rat OB-Re protein as a standard. When OB-Re was added to serum from non-pregnant mice at a level comparable to those of the serum from pregnant mice at day 17, the apparent value of the serum leptin level determined by the mouse leptin RIA procedure increased to a level of about 1.4-fold that of the actual leptin level. In the leptin RIA kit, the antibody-bound leptin is separated from the free leptin by double-antibody methods. When the concentration of 125I-labeled leptin is C and that of unlabeled leptin in the sample is X, the unknown bound counts/maximum bound counts (B/B0) can be given by the following calculation: B/B0 = C/(X+C). If the unknown bound counts (B) is lowered by
competition at the level of leptin binding between, for example, OB-Re and anti-leptin antibody, the leptin concentration (X) in the sample could appear to be increased. At a level of OB-Re such as in the sera from pregnant mice at day 17, immunoprecipitation of leptin using the anti-leptin IgG Sepharose seems not to be affected by OB-Re. However, at higher levels of OB-Re, such as 192 ng/ml, the immunoprecipitation of leptin was clearly inhibited by the presence of OB-Re. This inhibition may be caused by competition at the level of leptin binding between OB-Re and anti-leptin IgG Sepharose. Considering the overestimation of 1.4-fold at day 17 of pregnancy, serum leptin levels of these mice, as determined by the RIA procedure (283.6 ng/ml), were much higher than those determined by immunoprecipitation (40 ng/ml). By, for example, being denatured during its purification step, the leptin-binding ability of the OB-Re used in this experiment might be lower than that of OB-Re which normally exists in pregnant mouse sera. In such cases, leptin levels at day 17 of pregnancy which were determined by RIA could, in fact, be overestimated by a factor which is considerably larger than the 1.4-fold and the leptin levels which were determined by immunoprecipitation (40 ng/ml) could, in fact, be underestimated. That is, the actual leptin levels at day 17 of pregnancy could be higher than the leptin level determined by immunoprecipitation (40 ng/ml) and lower than that by RIA (283.6 ng/ml). When sera from pregnant mice at day 17 were denatured with 1:25 M acetic acid and 5 M urea and were subjected to the leptin RIA after neutralization, leptin levels at day 17 of pregnancy, in fact, decreased to levels which were from 30 to 40% of the levels found in undenatured sera (data not shown). Therefore, a leptin level at day 17 of pregnancy of 100 ng/ml represents a reasonable figure. The OB-Re which exists in pregnant mouse sera was unable to bind to the leptin Sepharose after the denaturation and neutralization steps.

The serum leptin levels of pregnant mice at day 13 and day 17 were undoubtedly higher than that of non-pregnant mice because these values, which were determined by the immunoprecipitation of leptin using the anti-leptin IgG Sepharose, were about 40 ng/ml at both day 13 and day 17 of pregnancy. An elongation of the half-life period of leptin in vivo by OB-Re may be one of the factors in the observed increased serum leptin levels. However, when serum levels of the secretory form of OB-R are high, in cases, for example, such as the last period of pregnancy in mice, the leptin levels might not, in fact, be so high as determined by the leptin RIA.

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