Hyperleptinemia of Pregnancy Associated with the Appearance of a Circulating Form of the Leptin Receptor

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Leptin is a hormone produced in adipose cells that regulates energy expenditure, food intake, and adiposity. In mice, we observed that circulating leptin levels increase 20–40-fold during pregnancy. Pregnant ob/ob females had no detectable serum leptin, demonstrating that the heterozygous conceptus was not the source of the leptin. However, leptin mRNA and protein levels in maternal adipose tissue were not elevated. The circulating leptin was in a high molecular weight complex, suggesting that the rise in leptin was due to expression of a binding protein. Indeed, quantitative assays of serum leptin binding capacity revealed a 40-fold increase, coincident with the rise in serum leptin. Leptin binding activity reached a capacity of 207 ± 15 nmol/liter of serum at day 18 of gestation, and half-maximal binding was observed with ~3 nM leptin. The binding protein was purified and partially sequenced, revealing sequence identity to the extracellular domain of the leptin receptor. We found that the placenta produces large amounts of the OB-Re isoform of leptin receptor mRNA, which encodes a soluble binding protein. Thus, the extreme hyperleptinemia of late pregnancy is attributable to binding of the leptin by a secreted form of the leptin receptor made by the placenta.

Leptin, a hormone produced in adipose cells, is important in the regulation of metabolic efficiency, energy expenditure, food intake, and adiposity (1–3). It serves as a signal reporting the degree of adiposity: circulating leptin levels correlate best with the amount of body fat (4, 5). Mice lacking a functional leptin (formerly ob or obese) gene become massively obese and develop diabetes mellitus due to overeating and decreased metabolic expenditure (6). These mice are also hypogonadal and hypercorticosteronemic, presumably on a hypothalamic basis. Leptin treatment of ob/ob (lep<sup>−/−</sup>/lep<sup>−/−</sup>) mice reverses all of these abnormalities, and in normal mice it causes decreased food intake, increased energy expenditure, weight loss, and precocious sexual maturity (7–11). Although much studied for its role in sexual maturity (7–11). Although much studied for its role in

Metabolism during pregnancy is quite different from metabolism in the nongravid state. It is altered to provide rapid growth of the fetus and placenta and to prepare the mother for nursing. Some species, including humans and mice, accumulate maternal fat during gestation, and then use it during lactation (13, 14). The importance of leptin in the regulation of adiposity and energy metabolism led us to investigate the physiology of leptin during gestation in the mouse. In addition, the observation that leptin mRNA is made by the human placenta (15, 16) suggested a role for leptin during pregnancy. To our surprise, we observed that, although mice have a profound hyperleptinemia in the third trimester of gestation, the murine placenta does not make leptin. There is, however, a massive increase in a circulating leptin-binding protein during pregnancy in mice.

MATERIALS AND METHODS

Mice—Mice (7–9 weeks old) were obtained from Charles River Laboratories (CD-1), Jackson Laboratories (C57BL/6J and C57BL/6J lep<sup>−/−</sup>/lep<sup>−/−</sup>) and laboratory stock (FVB/N) and maintained on a 12-h light/dark cycle and a standard pellet diet (NIH-07). Pregnancy in ob/ob Mice (17, 18)—Female C57BL/6J ob/ob mice were treated with either 2 or 5 µg of recombinant murine leptin (Peprotech or R & D Systems)/g of body weight/day via intraperitoneal injection, responding with ~1 g/day weight loss. After 7 days of treatment (about the time of apparent vaginal maturation), C57BL/6J males were introduced. Copulatory plugs were detected 5–18 days later. Eleven of the 12 mice became pregnant, with one spontaneous abortion at day 16. Leptin treatment was discontinued at various times during gestation. Of the five mice in which leptin was stopped on day 1, one showed no signs of pregnancy, and four carried to term. (Two of the four had been treated with progesterone (Sigma; 1 mg in 0.1 ml of sesame oil subcutaneously per day) (17)). The four mice not treated with leptin during gestation showed no signs of labor. At sacrifice on day 20, one day longer than the usual C57BL/6J gestation, the fetuses appeared hypoactive.

Assays—Leptin was measured by RIA<sup>1</sup> (Linco), with interassay coefficients of variation of 16% and 5% for the low and high standards, respectively. Typically, 50 µl of nonpregnant mouse serum or 2 µl of day 18 serum were assayed. The binding protein interfered with the RIA beginning at day 15 of pregnancy as shown by assay of serum from pregnant ob/ob mice, which had no detectable leptin. Western Lep-tin was measured by Western blotting after immunoprecipitation (19). Fat pad protein extracts were prepared as described by Barr et al. (19).

Isolation and Analysis of RNA—RNA was extracted using RNA STAT-60 (Tel-Test), and poly(A) RNA was isolated using a Mini-Oligo(dT) Cellulose Spin Column kit (5 Prime → 3 Prime, Boulder, CO). Northern blots (Maximum Strength Nytran Plus; Schleicher & Schuell) were hybridized using Rapid-hyb (Amersham Corp.) according to the manufacturer’s instructions and exposed to film or quantitated with a PhosphorImager (Molecular Dynamics). Probes were a murine leptin coding region fragment (1285; base pairs 50–566 in GenBank<sup>™</sup> accession number U18812), a 1.9-kilobase pair fragment of chicken β-actin (20), a human leptin receptor extracellular region (base pairs 166–1395 in GenBank<sup>™</sup> number U43168), and an 81-base pair region (GTATG-TGTACCTGACTTTTCCATGGATTAGTACGACACTGACTGCCAATTCGATATAAAATCATTATATGAACACCTGCTGCAAGTTATCTGATAATAAAATCATTTAATGACACTGACTGCCAATTCGATATAAAATCATTATATGAACACCTGCTGCTGCAAGTTATCTGATAATAAAATCATTTAATGACACTGACTGCCAATTCGATATAAAATCATTATATGAACACCTGCTGCTGCAAGTTATCTGATAATAAAATCATTTAATGACACTGACTGCCAATTCGATATAAAATCATTATATGAACACCTGCTGCTGCAAGTTATCTGATAATAAAATCATTTAATGACACTGACTCCCTGA<sup>†</sup> from expressed sequence tag AA028211 (Genome Systems) containing all of the OB-Re isoform-specific exon of the leptin receptor (21).

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<sup>1</sup>The abbreviations used are: RIA, radioimmune assay; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
Leptin-binding Protein

Leptin in pregnancy. A, maternal serum leptin levels in pregnant CD-1 mice. Leptin was measured by RIA (○) and Western blot (●). The line is the mean of the RIA data. The day 4 and 9 RIA data include five points each. Two points (day 17 Western blot of 543 ng/ml and day 18 RIA of 640 ng/ml) are off scale. B, Western blot analysis of serum leptin. Serum from CD-1 mice (nonpregnant (Nonpreg), day 4 (d4), day 9 (d9), day 14 (d14), and day 18 (d18)) and nonpregnant db/db and ob/ob mice was immunoprecipitated, electrophoresed, and immunoblotted with antileptin antibody. Either 50 or 500 μl of serum was used, as indicated. The db/db, d14, and d18 sera were from individual animals, and the other samples were each pooled from two mice. C, RNA from parametrial fat (8 μg) of individual CD-1 mice was probed for leptin and reprobed for β-actin. Quantitation (mean ± S.E., n = 4) of the leptin:actin mRNA ratios is shown as a percentage of the nonpregnant mean. The day of gestation is indicated; np indicates nonpregnant.

Figure 1

Hyperleptinemia Is Not Due to Production by the Conceptus. We next asked if the high maternal leptin was due to synthesis by the conceptus. A significant fetal contribution seemed unlikely because of the low leptin levels in day 18 fetal blood and amniotic fluid (above). Since leptin is expressed by the placenta in humans (15, 16), we expected that this would also be true in mice. However, we were unable to detect leptin RNA in placenta or embryonic membranes, even with a Northern blot containing 30 μg of poly(A) placental RNA (Fig. 2A). We estimate that the level of leptin RNA in placenta is less than V1000 the amount in fat. Moreover, no leptin-specific signal

1C) and protein levels (data not shown) did not increase during pregnancy. Although we cannot rule out either increased flux resulting in more leptin secretion or leptin production from other fat depots, the simplest explanation is that the high leptin levels in pregnancy are not caused by increased leptin synthesis by maternal fat.
was detected in these tissues by in situ hybridization.  

As an independent test of the possibility that the conceptus produces the circulating leptin, we mated ob/ob females with wild type males. The ob/ob maternal tissues cannot make leptin, so any circulating hormone would be from the heterozygous conceptus’ paternal copy of the gene. Leptin treatment was used to induce fertility, and then treatment was discontinued (see “Materials and Methods”). When plasma from pregnant ob/ob mice was assayed for leptin by Western blotting, none was detected (Fig. 2B). Taken together, these data demonstrate that production of leptin by the conceptus does not contribute to the hyperleptinemia of pregnancy.

Leptin-binding Proteins in Pregnant Serum—The lack of increased leptin levels in maternal adipose tissue and the lack of leptin production by the conceptus suggested that the maternal hyperleptinemia might be caused by an increase in the half-life of the hormone. One factor that can affect hormone clearance is interaction with binding proteins. To test for leptin binding activities, we analyzed the distribution of leptin in serum using gel filtration (Fig. 3). As previously reported (22, 23), serum from nonpregnant mice contained bound and free leptin. In contrast, >95% of the leptin in serum from pregnant mice was present in a bound form. It is notable that in nonpregnant db/db mice, which have comparably elevated leptin levels, only 21% of the leptin was in a bound form (Fig. 3 and Ref. 23).

Next, we undertook a quantitative analysis of leptin binding. Serum was incubated with 125I-leptin and known amounts of cold leptin, and the amount of bound leptin was calculated from the gel filtration profile. Half-maximal binding was observed at ~3 nM leptin in both nonpregnant (Fig. 4A) and day 18 serum (data not shown). Binding capacity in nonpregnant serum was 5.3 ± 1.8 nmol/liter of serum (n = 4). Leptin binding capacity increased slightly to 9.6 ± 4.1 nmol/liter of serum (n = 4) at day 12 of gestation, and by day 18 it was 207 ± 15 nmol/liter (n = 3), a 40-fold increase (Fig. 4B). The profile of serum leptin binding capacity during pregnancy correlated with that of serum leptin, suggesting that hyperleptinemia in pregnant mice might be caused by an increase of leptin half-life due to interaction with binding proteins.

To identify the leptin-binding proteins, we analyzed complexes formed between 125I-leptin and serum proteins using nondenaturing gel electrophoresis. In serum from nonpregnant mice, three labeled bands were observed (bands A, D, and E), that were not affected by incubation with excess unlabeled leptin (Fig. 5, lanes 1–3). When 125I-leptin was incubated with serum from pregnant mice, two additional complex bands (bands B and C) were observed (lanes 4–6). Band C was competed by cold leptin, and its appearance during gestation correlated with the increase in binding capacity (lanes 7–10). Band C was also present in serum from pregnant (but not nonpregnant) ob/ob mice (lanes 11 and 12). Band D was a minor band with properties similar to those of band C. Band D had the mobility of leptin bound to albumin; leptin aggregates have a similar mobility (data not shown). Band E contained free leptin. These data suggest that band C contained leptin bound to a specific binding protein induced during gestation.

To characterize the binding proteins, we incubated serum with immobilized leptin, washed the column, and then eluted the bound proteins with either SDS or leptin. SDS-PAGE of the eluate from beads incubated with pregnant serum revealed a band at ~140 kDa that was not found upon elution of beads.

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2 C. Bondy and J. Zhou, personal communication.
3 The calculations assume 100% bioactivity of the recombinant leptin. The concentration at half-maximal binding and the binding capacity are upper limits and would be reduced in proportion to the reduction in leptin bioactivity.
incubated with nonpregnant serum (Fig. 6, lanes 4–6). Most of the other bands in the eluate were nonspecific (present in the wash, lane 3), not pregnancy-specific (lane 6), or present in the leptin used for elution (not shown; see also Fig. 7, lane 20). The 270–300-kDa band was not reproducible. The nonpregnant samples contained a ~176-kDa protein, which probably corresponds to a previously noted binding protein (23).

The Binding Protein Is a Soluble Form of the Leptin Receptor Produced by the Placenta—To establish which protein(s) accounted for the pregnancy-specific binding activity, preparative scale isolation of leptin-binding proteins was performed. The eluted proteins were incubated with 125I-leptin, electrophoresed under nondenaturing conditions, and visualized by Coomassie staining (Fig. 7, lanes 1–7) and autoradiography (lanes 8–14). Of the four Coomassie-staining bands, only band C bound 125I-leptin in a cold leptin-inhibitable manner (lanes 5, 6, 12, 13; note that the intensity of band C in lane 12 is reduced due to leptin remaining from the elution). Band C from the affinity-purified fraction had the same mobility as the pregnancy-regulated band C from whole serum (lanes 10 and 12).

Next, the Coomassie-staining bands from the nondenaturing gel were eluted and separated by SDS-PAGE (lanes 15–21). It is clear that the affinity column eluate contains both the 140-

kDa binding protein and leptin (16, 32, and 64 kDa; lanes 16 and 20; on a reducing gel, only the 16-kDa form was present). Bands B and C are mostly the 140-kDa binding protein (whose size was unchanged under reducing conditions), with a minor ~60-kDa protein, which has the same mobility as albumin (lanes 17, 18, and 21). Band D is composed of leptin (lanes 19 and 20). These data show that the 140-kDa binding protein is the major protein eluted from the affinity column and is identical to band C of the affinity column.

Purified leptin binding protein was subjected to SDS-PAGE, the 140-kDa band was excised and digested with trypsin, and...
the resulting peptides were separated by HPLC. One of the peptides was sequenced, yielding a single, high confidence sequence, ISWDSQTMAPFPLQYQVK, which matches exactly amino acids 254–271 of the leptin receptor (24). Three other peaks were examined by mass spectrometry, one of which was a mixture and was not examined further. The other two peaks matched predicted leptin receptor tryptic peptides in size and absorbance properties (GPEFWR at positions 640–645, absorbing at 277 and 292 nm (observed $M_r$ = 792.4, predicted $M_r$ = 790.9) and EAAEIVSATSLLVDSVLQSYEVQVR at positions 281–307, not absorbing at 277 or 292 nm (observed $M_r$ = 2817.3, predicted $M_r$ = 2820.1)). Thus, the leptin-binding protein is a soluble form of the leptin receptor.

To identify the source of the binding protein, RNA from pregnant and nonpregnant mice was hybridized with a probe for the extracellular region of the leptin receptor. The 5.1-kilobase pair band encoding full-length leptin receptor was not clearly visible in blots using 20 μg of total RNA. However, a very abundant 2.8-kilobase pair band was present on the same blots in RNA from placenta and, at much lower levels, pregnant liver, but not in pregnant or nonpregnant brain, muscle, heart, lung, kidney, spleen, adipose, adrenal, ovary, or uterus (data not shown). The OB-Re mRNA splicing isoform of the leptin receptor is predicted to encode a soluble protein, lacking the transmembrane domain, but this mRNA was detectable only by polymerase chain reaction (21, 25). A complete sequence for the OB-Re isomorph-specific region was obtained (see “Materials and Methods”) and used to measure OB-Re mRNA levels during pregnancy (Fig. 8). It is clear that the 2.8-kilobase pair mRNA is the OB-Re isoform and that its levels increase dramatically in placenta at the end of pregnancy, while remaining low in other tissues, including liver. These data suggest that the increase in leptin-binding protein during pregnancy is due to increased levels of placental OB-Re isoform mRNA, encoding a soluble leptin-binding protein.

DISCUSSION

We report a dramatic increase of circulating leptin levels during gestation with no detectable increase in leptin production. The serum levels of a leptin-binding protein derived from the leptin receptor increased with kinetics nearly identical to that of leptin. Since leptin is cleared by the kidney, at least partially by filtration (26), binding of the 16-kDa leptin to the binding protein (140 kDa if monomeric) should decrease its clearance. Thus, the most likely explanation for the hyperleptinemia is reduced renal clearance of bound leptin.

We demonstrated that the binding protein contains the extracellular region of the leptin receptor. Transmembrane leptin receptors bind leptin half-maximally at ~0.7 nM (24). The apparent affinity of leptin-binding protein is similar to this value (half-maximal binding at ~3 nM, an upper limit). More detailed comparisons of receptor and binding protein binding properties are needed. For example, it is not known if other ligands also bind or bind differentially or if other factors influence the leptin-binding protein interaction. The apparent binding capacity of 207 pmol/liter (also an upper limit) corresponds to 29 μg of binding protein/ml of serum.

Lee et al. (21) reported an mRNA splicing variant of the leptin receptor, Ob-Re, that is predicted to encode a secreted protein of 805 amino acids. The difference between this size and 140 kDa is at least partially due to glycosylation. While the simplest hypothesis is that the binding protein is the translation product of OB-Re mRNA, we cannot exclude the possi-

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4 O. Gavrilova, V. Barr, B. Marcus-Samuels, and M. Reitman, unpublished observations.

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**FIG. 7. Purification of leptin-binding protein.** Samples were incubated with $^{125}$I-leptin and then electrophoresed in a nondenaturing gel. Lanes 1–7 show a Coomassie stain, and lanes 8–14 show an autoradiograph, of the same gel. Lanes contain nonpregnant serum (5 μl; lanes 1 and 8 and lanes 2 and 9), pregnant serum (5 μl; lanes 3 and 10 and lanes 4 and 11), or affinity-purified leptin binding proteins (roughly equivalent to 100 μl of serum; lanes 5 and 12 and lanes 6 and 13). Unlabeled leptin competitor (10 μg) was included in lanes 2 and 9, lanes 4 and 11, and lanes 6 and 13. Bands B–D were eluted from a duplicate of lanes 5 and 12 (without $^{125}$I-leptin), subjected to SDS-PAGE, and stained with Coomassie Blue (lanes 16), the leptin used for elution off the affinity column (lane 20), and bovine serum albumin (lane 21).

**FIG. 8. Leptin receptor RNA isoform OB-Re is expressed in third trimester placenta.** Total RNA (20 μg) from placenta and from maternal kidney, liver, and uterus, as indicated, was hybridized with a probe specific for the OB-Re isoform of the leptin receptor. Samples were prepared on day 9, 14, or 18 of pregnancy or from nonpregnant female CD-1 mice. Placentas from two different mice are shown for each stage of gestation. The sizes, in kilobases, of RNA standards are indicated. Ethidium bromide staining (bottom) confirms comparable loading of the RNA samples.
bility that some of the circulating binding protein is produced either by proteolysis of the cell surface receptor or by translation of novel leptin receptor mRNAs that also encode truncated proteins.

What is the effect of leptin binding to binding protein? Binding proteins can act as a buffer and increase peptide half-life, or they can promote hormone clearance (e.g. corticotropin-releasing factor-binding protein (27)). It is possible for a hormone-binding protein complex to be a more potent agonist than free hormone (28). However, in most peptide hormone/binding protein systems (such as growth hormone (29), insulin-like growth factor-I (30), and multiple interleukins (31)), the bound form of the hormone is unable to bind and activate its receptor. In such cases, the binding protein acts as an inhibitor of hormone action. The similarities between leptin and growth hormone are particularly striking (32). Growth hormone is also a member of the cytokine receptor family, and its level in the circulation rises dramatically during pregnancy due to production of a soluble binding protein encoded by an alternatively spliced receptor RNA (22, 23). However, no

In summary, we demonstrate that in the third trimester of pregnancy, mice show a profound increase in leptin and leptin binding proteins are now being investigated, including its role in leptin physiology beyond pregnancy in the mouse.

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