Comparative analysis of expression and secretion of placental leptin in mammals

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Zhao, Jing, Thomas H. Kunz, Nancy Tumba, Laura Clamon Schulz, Chris Li, Monica Reeves, and Eric P. Widmaier. Comparative analysis of expression and secretion of placental leptin in mammals. Am J Physiol Regul Integr Comp Physiol 285: R438–R446, 2003. First published April 17, 2003; 10.1152/ajpregu.00776.2002.—Increased plasma level of leptin appears to be a ubiquitous feature of pregnant mammals. The mechanisms by which leptin levels are increased may be species specific, however, with some species upregulating adipose leptin production and others expressing leptin in the placenta. Placental leptin expression was examined in representative species of the two most abundant mammalian orders, Rodentia and Chiroptera, and in cultured human choriocarcinoma (BeWo) cells. Leptin mRNA was expressed in BeWo cells and in placentas of Myotis lucifugus (little brown bat), Eptesicus fuscus (big brown bat), and Rattus norvegicus (laboratory rat), but not in the common laboratory mouse Mus musculus. cAMP stimulated secretion of leptin from BeWo cells and also stimulated leptin mRNA expression in the cells. In addition to adipose and placental tissue, leptin transcript in M. lucifugus was detectable in heart, spleen, and liver, but not in lung, brain, and kidney. Hepatic expression was also observed in E. fuscus, but not in mice or rats, and did not appear to result from hepatic fat deposition. Leptin cDNA was cloned and sequenced from M. lucifugus placenta and shared up to 95% homology with other mammalian leptin cDNAs. It is concluded that 1) placental leptin expression and secretion are species-specific traits, 2) placental leptin production represents one of three major mechanisms for achieving high circulating maternal leptin levels during pregnancy, the others being upregulation of adipose leptin production and production of circulating leptin-binding proteins, and 3) hepatic leptin expression in pregnant insectivorous bats may be an adaptation resulting from the presence of extremely low amounts of subcutaneous fat during pregnancy and lactation in these species.

bats; adipose tissue; energy homeostasis; reproduction; placenta

LEPTIN IS AN ANOREXIGENIC hormone produced by adipose tissue and is one component of a complex regulatory pathway that acts to maintain energy homeostasis in mammals (40, 53). Treatment of infertile ob/ob mice with recombinant leptin not only leads to a loss of food intake and body weight but also restores fertility, sug-

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In New England, this species is active from April through September and hibernates for the rest of the year. Mating occurs in autumn, and sperm is stored in the female’s reproductive tract during hibernation (5). Ovulation and fertilization occur on arousal from hibernation in spring (25), and gestation lasts ~60 days, with one offspring produced each year (34). During pregnancy, greatly increased leptin levels are observed in maternal blood (29, 32, 50). Parturition begins around mid-June and is highly synchronized within a given colony. Litter size accounts for ~25% of adult body mass (31), and pups reach 84% of adult size by the end of lactation (30). Thus, during pregnancy and lactation, little brown bats face extraordinary energy demands, and leptin would be expected to be tightly controlled during these periods.

We have used *Mus musculus* and *M. lucifugus* as model species to 1) determine whether the rodent and bat placentas are capable of synthesizing leptin mRNA transcript, 2) determine whether placental leptin expression and secretion are under regulatory control, and 3) establish the gestational profile and tissue distribution of leptin mRNA in *M. lucifugus*. In part on the basis of our earlier observations (28, 29), we hypothesized that the placenta of *M. lucifugus*, but not *M. musculus*, would express leptin transcript, that leptin transcript in the placenta would be expressed throughout gestation, and that leptin expression would be primarily restricted to adipose and placental tissue.

**MATERIALS AND METHODS**

**Collection of animals and tissues.** Little brown bats (*M. lucifugus*) were collected from maternity colonies in eastern Massachusetts from late May to late June over four seasons (1999–2002). Bats were captured with a harp trap from 2100 to 2300 as they returned from foraging and transferred to the laboratory in simulated roosts without food, as previously described (33). In addition, several big brown bats (*Eptesicus fuscus*) were hand captured from a maternity colony in southern New Hampshire. At ~10–12 h after capture, bats were weighed and then killed by decapitation. Embryos were removed and weighed to estimate the stage of gestation on the basis of a regression equation of embryo mass (EM) vs. gestational stage (GS) in *M. lucifugus* as follows: GS = \(-9.6789 \times EM^2 + 44.381 \times EM - 53.535\). This equation was derived by regressing mean embryo mass against date of collection. In some cases, gestational stages were arbitrarily divided into three trimesters to facilitate statistical comparisons across gestation: early pregnancy (from day 60 to day 40 from parturition), middle pregnancy (from day 40 to day 20 from parturition), and late pregnancy (from day 20 to day 0 from parturition). Immediately after death, placenta, liver, kidney, heart, brain, spleen, lung, and subcutaneous fat were dissected and frozen in liquid nitrogen for mRNA analyses.

Timed-pregnant Swiss-Webster mice and Sprague-Dawley rats (Harlan) were individually housed in the Boston University Laboratory Animal Care Facility (lights on between 0700 and 1900) with water and commercial laboratory chow available ad libitum. Animals were killed between 0800 and 1000 by exposure to CO2. Adipose tissue, liver, and placenta were immediately dissected and frozen in liquid nitrogen for RNA analyses. All procedures used in this study were approved by the Boston University Institutional Animal Care and Use Committee.

**In vitro incubation.** Placentas from bats at 23–48 days before parturition (as determined by the equation in *Collection of animals and tissues*) were weighed and dissected in half, and each half was separately minced with a razor into ~1-mm3 cubes. Placental fragments were washed with several changes of 1,000 volume equivalents of cold, serum-free Krebs buffer containing 4% (wt/vol) BSA and 0.1% (wt/vol) glucose to remove residual extracellular fluid and blood cells. Tissue fragments (1/2 placenta per tube) were incubated for 4 h in glass test tubes with 1 ml of serum-free Krebs buffer as described above, supplemented with the protease inhibitors leupeptin and aprotinin (25 μg/ml each) and 5 mM dibutyryl cAMP, 1–2 μM corticosterone [a naturally occurring steroid in *M. lucifugus* (48)], 10 nM–10 μM dexamethasone, or vehicle (dibutyric acid or ethanol). Each placental half served as its own control in a given experiment. Incubations were at 37°C in a humidified O2–CO2 chamber with gentle shaking. Culture media were collected every hour and replaced with 1 ml of fresh media. Collected media were centrifuged, and the supernates were dehydrated in a Centrivap concentrator (LabConco) and frozen for future hormone assays. Blank samples (no tissue) were processed similarly. Placental fragments were collected at the conclusion of an experiment and processed for RT-PCR analysis of leptin transcript (see below).

Leptin concentrations in culture media were determined by RIA using a human leptin kit (Linco Research) that we previously validated for *M. lucifugus* plasma leptin (29, 32, 50). Dehydrated media were first reconstituted to 10% original volume, which was empirically determined to result in leptin measurements that fell on the linear portion of the human leptin standard curve. Immunoreactive leptin was at or below the limit of detection in blank samples of reconstituted culture medium.

*Human choriocarcinoma (BeWo) cells were obtained from American Type Culture Collection and grown in RPMI 1640 culture medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were cultured in the presence of vehicle (butyric acid) or 1 mM dibutyryl cAMP for 6 days. Media were collected and replaced daily. A portion of the collected media from each culture dish was dried in a Centrivap concentrator and reconstituted to 10% of original volume for RIA of leptin using the human leptin RIA kit (Linco Research). Cells were harvested at the end of the 6th day in culture, and total RNA was extracted with TRIzol reagent. RT-PCR analysis of leptin transcripts was performed as described below.

**Northern blot analyses.** Poly(A) RNA was extracted using the Oligoex Direct mRNA kit (Qiagen); 10 μg of poly(A) RNA were run for 3 h on formaldehyde-1% agarose gels at 40 mM and then transferred to nylon membranes using a vacuum blotter (model 785, Bio-Rad) at 5 mmHg for 4 h. After transfer, membranes were baked at 80°C for 30 min to fix the mRNA. Membranes were prehybridized at 45°C with Ambion ULTRAhyb solution for 1 h and hybridized overnight at 45°C with 32P-labeled probe (106–107 cpm/ml). Blots were washed twice with 2× saline-sodium citrate (300 mM sodium chloride and 30 mM sodium citrate) plus 0.1% (wt/vol) SDS at 45°C for 5 min and twice with 0.1× saline-sodium citrate plus 0.1% SDS at 45°C for 15 min. Hybridized membranes were exposed overnight to X-ray film with intensifying screen at ~80°C. The oligonucleotide probe was 38 nt long (5’-GACT-GGCTGGTGATGAAAATGTCTATGGTCTGCTGACAAT-3’) and complementary in sequence to a highly conserved region
among different mammalian leptin cDNAs. The 5′ end was radiolabeled with [γ-32P]ATP using the KinaseMax 5′-end-labeling kit (Ambion).

RT-PCR and sequencing. The following custom-made primers were used for RT-PCR and sequencing. Forward primer 1 (5′-GRTCTYTTGCTTTGYYCTCTAC-3′) was a degenerate primer corresponding to a highly conserved region of human, mouse, rat, dog, and cat leptin cDNA and extended into the 5′-flanking region. Forward primer 2 (5′-ARRGTCCTCAGATGCACACAAAAAC-3′) was a degenerate primer; its origin was within the coding region of leptin cDNA. Both forward primers were used in separate reactions with the same reverse direction primer (5′-GCCACCCCTCBBGTGGAGTAG-3′) and a reverse transcription kit (Promega) according to the manufacturer’s protocol. PCRs were performed in a thermal cycler (model PTC-100, MJ Research) with the following conditions: 94°C for 1 min (step 1), 94°C for 40 s, 55°C for 40 s, and 72°C for 2 min for a total of 38 cycles (step 2), and 72°C for 2 min (step 3). These conditions were determined to be optimal for assessing the presence or absence of leptin transcript, which is not abundantly expressed in most tissues. PCR products were electrophoresed on 1.5% low-melt agarose gels in the presence of ethidium bromide and visualized under ultraviolet light. Products corresponding to the expected size that would include the intervening intron. Conditions for semiquantitative PCR using β-actin tran- script were the same as those described above except for the number of amplification cycles. It was determined that 25 amplification cycles resulted in actin transcript signal within the exponential increase and before the plateau in reaction product and that 33 cycles resulted in exponential increase of leptin reaction product (not shown). The primer pair for β-actin was purchased from Promega; a fragment of 285 bp was expected. PCR products were electrophoresed on 1% agarose gels in the presence of ethidium bromide and visualized with a digital imaging system from Molecular Analysis (Bio-Rad). RNA transcript expression was semiquantitatively assessed using the ratio of leptin to β-actin. Product intensities were analyzed using the NIH Image software.

Miscellaneous. Cortisol in the plasma of M. lucifugus was determined using a commercial RIA (ICN), which we previously validated for rat plasma (48, 49). Immunoblots for detection of glucocorticoid receptor were performed using rabbit anti-human glucocorticoid receptor antiserum (Santa Cruz Biotechnologies) at a dilution of 1:100. Several hypo-

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ANOVAs or t-tests were used to test for significant differences among treatment groups using PRISM software (GraphPad); significant differences were considered to exist at \( P < 0.05 \).

RESULTS

Expression of leptin transcript in placenta. To investigate expression of leptin in bats, we first designed a 38-mer oligonucleotide probe that was 100% homologous among human, mouse, and rat leptin cDNA and resided within the highly conserved binding domain of leptin. Northern blot analysis revealed a 4.5-kb band in mouse adipose tissue, consistent with other reports (53), and a single, smaller transcript of ~2.3 kb in bat adipose tissue (data not shown).

Placental mRNA from early-, middle-, and late-gestation M. lucifugus was hybridized with the probe described above. The blots revealed a single placental transcript of ~2.3 kb (Fig. 1A) that was identical in size to the transcript in M. lucifugus adipose tissue. After the data were normalized to β-actin expression, there was no significant change in leptin mRNA expression per unit mass of placenta from early to late gestation (Fig. 1B). Thus leptin transcript was expressed in M. lucifugus placenta in high amounts throughout gestation.

Stimulation of leptin secretion from M. lucifugus placenta. Once it was established that leptin transcript was expressed in bat placenta, we examined control of leptin secretion from placental fragments in vitro. Preliminary experiments confirmed our published report (29) that leptin is secreted from placentas of M. lucifugus and also demonstrated that the secretion was increased by cAMP (not shown). In addition, cAMP significantly stimulated secretion of leptin from cultured human choriocarcinoma cells (Fig. 2A) and also significantly increased leptin mRNA in the cells (Fig. 2B).

Glucocorticoids potently stimulate leptin synthesis and secretion from adipose tissue cells (28, 39) and are important metabolic hormones during pregnancy. The hypothesis that glucocorticoids may stimulate placental leptin production was tested using placental fragments from M. lucifugus incubated with natural (corticosterone) or synthetic (dexamethasone) steroids for up to 8 h. Because the gestational profile of plasma glucocorticoids in any chiropteran species was unknown, however, we first determined cortisol (the major glucocorticoid in M. lucifugus) levels in plasma of pregnant bats. Plasma cortisol was ~50–350 ng/ml (~0.14–1 μM) in pregnant bats over the course of gestation (\( n = 41 \), data not shown) compared with ~15–170 ng/ml in postlactational M. lucifugus (48). Using a range of concentrations (10 nM–10 μM) spanning and exceeding those measured in pregnant M. lucifugus, however, we could not detect any effect of natural or synthetic glucocorticoids on leptin secretion.
or mRNA production in placental fragments from *M. lucifugus* (not shown). Failure to observe effects of glucocorticoids was not due to the absence of placental glucocorticoid receptors, because immunoblots revealed the presence of receptors in *M. lucifugus* placenta (Fig. 3).

**Leptin expression profile in *M. lucifugus***. Tissue distribution of leptin mRNA was examined in *M. lucifugus* using RT-PCR analysis of total RNA from adipose tissue, placenta, heart, liver, kidney, spleen, brain, and lung from late-gestation females. Leptin transcript was detected in adipose and placental tissue, with barely detectable levels in heart, liver, and spleen and below-detectable levels in lung, kidney, and brain (Fig. 4, A and B). These results were confirmed in a replicate analysis (not shown) with tissue from different animals, except the amount of expression (relative to that in adipose and placental tissue) was greater in heart and spleen than shown in Fig. 4A.

**Fig. 1.** A: representative Northern blot of leptin mRNA expression in placentas of *Myotis lucifugus* during the course of gestation. Top: a single 2.3-kb band. Bottom: expression of β-actin mRNA on the same blot shown as a loading control. Ten micrograms of semipurified mRNA were loaded in each lane. Positions of 18S and 28S rRNA are shown for comparison. B: summary of Northern blot analyses of leptin expression in placentas of *M. lucifugus* at different stages of gestation after normalization to β-actin mRNA using the ImageJ program. There was no significant correlation between days of gestation and leptin expression.

**Fig. 2.** A: secretion of leptin from cultured human choriocarcinoma (BeWo) cells over 6 days in the presence or absence of 1 mM dibutyryl cAMP. Values are means ± SE of 5–6 experiments. B: effect of dibutyryl cAMP on leptin mRNA expression from these cultured BeWo cells shown in a representative RT-PCR experiment [inset: leptin (top) and actin (bottom)] and as a summary of 4 replicate experiments (bar graph). Values are means ± SE. *P < 0.03 vs. control.
Because leptin mRNA is expressed in avian liver (46), but not in any known adult mammal, further studies were performed to verify the presence of leptin mRNA in *M. lucifugus* liver as seen in Fig. 4A. RT-PCR analysis of hepatic tissue from several individual little brown bats and a related species, *E. fuscus*, revealed expression of a single PCR product that corresponded to the predicted size of the partial leptin transcript (Fig. 4, C and D). No expression of leptin transcript was present in mouse or rat liver (Fig. 4, E and F). There was, however, expression of leptin transcript in rat placenta, but no detectable expression in mouse placenta (Fig. 4E). Northern blot analysis of hepatic mRNA from *M. lucifugus* detected a single transcript in liver that corresponded in size to that expressed in placenta (Fig. 5). The amount of leptin transcript (assessed by Northern blotting) in the liver of this individual animal collected in 2000 was greater than that identified in replicate animals collected in 2002 (assessed by RT-PCR; Fig. 4); this may have been due to individual or seasonal variability. No leptin expression was detected by Northern blotting in mouse liver or placenta (Fig. 5), confirming the RT-PCR results.

Light-microscopic examination of cryostat sections of *M. lucifugus* liver revealed a lobular appearance, with cords of hepatocytes radiating out from a central vein typical of mammalian livers. No evidence for the presence of adipose-storing cells, which might have accounted for the presence of hepatic leptin, was observed when 16-μm sections were stained with Sudan black (not shown).

Because RT-PCR analysis was performed with degenerate primers based on the mouse leptin cDNA sequence, the results were verified by cloning and sequencing leptin cDNA from *M. lucifugus* placenta and using the sequence to generate a bat-specific oligonucleotide probe. A composite *M. lucifugus* leptin cDNA sequence was generated by directly sequencing several RT-PCR products and several different subclones. This sequence (GenBank accession no. AY055474) represented ~80% of the predicted coding region and was 87% homologous to human and 84% homologous at the nucleotide level to rat and mouse leptin cDNAs. On the basis of this sequence, a homologous oligonucleotide probe corresponding to the probe used in Figs. 1 and 5 (differing by 2 nt) was generated for use in Northern blots. As with the degenerate probes, a single transcript of 2.3 kb was observed on Northern blots of adipose and placental tissue (data not shown).
leptin transcript is species specific, even within the same order of mammals. We chose to examine rodents and bats, because members of these orders collectively comprise ~70% of all mammalian species (51) and are not closely related taxonomically (44). Thus patterns of leptin expression or regulation that are similar among rodents and bats may reveal ubiquitous regulatory mechanisms. Human choriocarcinoma cells and placentas of little brown bats, big brown bats, and rats, but not mice, expressed leptin transcript. Absence of expression in the mouse placenta [and our earlier report that mouse placenta does not secrete leptin in vitro (28)] supports previous observations (16, 47) but is in conflict with observations reported by Hoggard et al. (23), which may possibly be explained by the use of different strains of mice between studies (23, 28). On the basis of these results and those reported for baboons and humans (20), we conclude that leptin expression appears to be a common feature of placental physiology in mammals, but it is nonetheless not a ubiquitous feature. Thus it appears that generalities about placental leptin expression must be tempered at this time, because all related species within a single family may not express and secrete leptin from the placenta.

The placentas of certain species, such as humans (20), baboons (20), and little brown bats (unpublished observations), express the active form of the leptin receptor, OB-Rb. Together with the ability of the placenta to produce and secrete leptin, this suggests that the placenta may be a target organ for leptin and that placental leptin may have autocrine or paracrine activities. Indeed, leptin has been suggested to regulate placental growth and angiogenesis (3), function as a local immunomodulator (22), and possibly play a role in implantation by modulating expression of extracellular matrix-degrading enzymes (3).

The size of the leptin mRNA transcript was smaller in M. lucifugus than in the mouse (and other species (27, 37)). The reason for this difference is unknown. However, leptin mRNA contains a long 3’-untranslated region (53), which may be important for regulating message half-life, as has been reported for other transcripts with such regions (e.g., estradiol receptor transcript (42)). It is not possible to say at this time, however, whether the shortened 3’-untranslated region of the bat leptin mRNA plays a role in stability of the transcript.

We previously reported that leptin accumulates in media of placental fragments from M. lucifugus incubated in vitro in the absence of any stimulus (29). We report here that the secretion of leptin from M. lu-
Glucocorticoids potently stimulate leptin production and secretion from adipose cells (28, 39), and mammalian placentas express glucocorticoid receptors in trophoblast cells (12). Thus we tested the hypothesis that glucocorticoids would stimulate placental leptin expression and secretion but were unable to demonstrate any effect of natural or synthetic glucocorticoids on M. lucifugus placentas. This was true, despite the presence of immunoreactive glucocorticoid receptor and the use of steroid concentrations consistent with and higher than those found in plasma of pregnant M. lucifugus. Thus the results obtained in M. lucifugus are in contrast with those of Sudgen et al. (45), who demonstrated that glucocorticoids stimulated leptin secretion in vivo from rat placenta, and Coya et al. (9), who found that glucocorticoids upregulated expression and secretion of leptin in human placental cells.

Expression of leptin synthesis in bat placenta did not change throughout gestation when normalized to β-ac- tin expression. This agrees with our previously reported results which demonstrated that the rate at which leptin accumulated in the media of minced placental fragments was similar throughout gestation when normalized to leptin secreted per gram of placenta (29). Thus leptin appears to be constitutively expressed in the placenta throughout the course of gestation in M. lucifugus. This suggests that as gestation advances, increased placental leptin secretion is due to the increase in placental mass, rather than upregulation of leptin per gram of tissue (or per cell). However, other studies have demonstrated that placental leptin expression increases as gestation advances in humans and rats (10, 15) and decreases from early to late gestation in baboons (21), indicating that not only the expression of placental leptin, but also its temporal pattern of expression during pregnancy, is species specific.

In addition to adipose and placental tissue, leptin is also expressed in pituitary (26), stomach (8), ovary (43), skeletal muscle (8), mammary gland (8), brain (8), endometrium (18), avian liver (46), and several fetal tissues, such as liver, heart, lung, and hair follicle (22). The present study revealed that leptin transcript was expressed in variable amounts in several tissues in bats, with the strongest and most consistent expression in adipose and placental tissue. Although we did not have sufficient material to further characterize leptin expression in most tissues, we report here that the liver of adult little brown bats and big brown bats, but not rats or mice, expresses leptin transcript. The expression level on Northern blots was greater than that observed using RT-PCR. Although we cannot explain this difference, it may reflect the fact that tissue samples for each analysis were obtained from different sampling seasons, and individual animals from one season may have been exposed to different environmental and metabolic challenges at the time of capture, resulting in variable degrees of hepatic leptin expression. Notwithstanding this individual variability, this is the first report of a healthy adult mammal expressing leptin in the liver. Fetal mice (24) and fetal sheep (6, 13), but not their adult counterparts, have been shown to express hepatic leptin. In both cases, this occurs at a developmental time when adipose stores are minimal or undetectable (1). Similarly, hepatic leptin in chickens is most closely correlated with metabolism during the posthatching period (2), when subcutaneous adipose tissue is minimal. During pregnancy, M. lucifugus and E. fuscus contain very little detectable adipose stores, which may reflect their high metabolic rate and energy use at that time. Thus it is possible that hepatic expression of leptin is a widespread phenomenon in vertebrates that compensates for reduced or absent subcutaneous fat stores. It would be interesting to determine whether adult animals that do not normally express leptin in liver do so during periods of prolonged starvation and depletion of body fat. Likewise, during the prehibernatory season, M. lucifugus gains considerable amounts of adipose tissue, and it would be interesting to determine whether hepatic leptin expression decreases at that time.

Leptin has been cloned or partially sequenced in several species, including human, rat and mouse (53), and big brown bats (11). The homology of the nucleotide sequence of M. lucifugus leptin cDNA to reported sequences for other eutherian mammals was 79–95%. Leptin was strongly expressed in adipose and placental tissue of the little brown bat and moderately in liver, heart, and spleen. It is not known whether leptin expression in placenta, liver, heart, and spleen was due to the presence of a small amount of adipocytes in these organs. However, placental leptin expression has been clearly documented within syncytiotrophoblast in human and baboon placenta (20), and we could detect no lipid-staining cells in the liver of M. lucifugus. In chickens, liver expression of leptin occurs in hepatocytes (41), but this is unknown for fetal mammals or for adult M. lucifugus.

In summary, we demonstrate that expression of leptin and its secretion from the placenta are species specific, under the acute regulation of cAMP but not...
glucocorticoids, and that livers of little brown bats and a related species, big brown bats, express leptin mRNA. The latter may represent a common feature of animals, in which stores of adipose tissue are restricted because of the life stage or natural history of the species. Although increased maternal leptin appears to be ubiquitous in pregnant mammals, generalities about the mechanisms that result in increased plasma leptin during pregnancy are not possible.

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