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Inhibition of trophoblast invasiveness *in vitro* by immunoneutralization of leptin in the bat, *Myotis lucifugus* (Chiroptera)

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Abstract

In addition to effects on metabolism and appetite, leptin is a reproductive hormone produced and secreted by the placenta of many, but not all mammalian species. In mice, in which the placenta does not secrete leptin, exogenously added leptin stimulates invasiveness of early (but not late)-gestation trophoblast cells. We report a similar phenomenon occurs in *Myotis lucifugus* (little brown myotis), a species in which the placenta synthesizes and secretes leptin. Immunoneutralization of endogenously secreted leptin from cultured *M. lucifugus* trophoblast cells inhibited the ability of these cells to invade a matrigel matrix. The effect was not due to an inhibitory effect of the antibody on cell proliferation, nor was it a non-specific effect of antibody administration. Cell invasion was significantly reduced in untreated cells obtained from late-gestation placentas, and the antibody had no effect at that time. This occurred despite continued expression throughout gestation of the long (OBRb) and short (OBRa) isoforms of leptin receptor mRNA. This study suggests that an important function of leptin during pregnancy is an effect on trophoblast cell invasiveness, at a time when the placenta is becoming established. That this occurs in two phylogenetically unrelated and distant species, regardless of whether the placenta is a source of secreted leptin, suggests that this is a highly conserved reproductive action of leptin.

Keywords: Leptin; Trophoblast invasion; Leptin receptor; ObRb; ObRa; Little brown myotis

1. Introduction

The hormone leptin regulates energy homeostasis through its actions on metabolic rate and appetite. In addition, leptin is an important reproductive hormone, without which mammals are infertile (Chehab et al., 1996). One reproductive tissue that acts as a target for leptin is the placenta. This hypothesis is supported by the observation that leptin receptors are expressed in trophoblast cells in several species (Cervero et al., 2004; Henson et al., 1999; Kawai et al., 1999; Senaris et al., 1997; Thomas et al., 2001; Zhao et al., 2004), and that leptin influences secretion by trophoblast cells of human chorionic gonadotropin (Chardonnens et al., 1999; Islami et al., 2003a), vascular-endothelial growth factor (Islami et al., 2003b), and progesterone (Cameo et al., 2003). In addition to its actions on placental hormone secretion, leptin may be necessary for the development of the placenta. In *ob*/*ob* mice, which lack a functional leptin protein, pregnancy cannot be established without leptin treatment through the time of embryo implantation and initial placental development (Malik et al., 2001). The role of leptin during this period may be to increase trophoblast invasion of the uterus, as we have found that leptin stimulates invasion in primary cultures of mouse trophoblast cells from early pregnancy (Schulz and Widmaier, 2004). The ability of leptin to stimulate trophoblast invasion was dependent on the activity of matrix metalloproteinases, enzymes required for trophoblast cells to digest connective tissue and penetrate the maternal endometrium (Bischof et al., 1995; Schulz and Widmaier, 2004). Leptin has also been shown to promote the expres-

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sion of matrix metalloproteinase-2 (MMP-2), and the activity of MMP-9 in cultured human cytotrophoblast cells (Castellucci et al., 2000).

During pregnancy, circulating levels of leptin become elevated in all mammalian species studied thus far. However, the source of leptin during pregnancy varies among species. In humans and other primates, the placenta produces leptin (Henson et al., 1998; Henson et al., 1999; Masuzaki et al., 1997). Although one group has found leptin mRNA in mouse placenta (Hoggard et al., 1997; Hoggard et al., 2000), others including us have failed to find leptin message there (Gavrilova et al., 1997; Malik et al., 2005; Tomimatsu et al., 1997; Zhao et al., 2003), and leptin is not secreted by cultured mouse placental explants (Kronfeld-Schor et al., 2000). Instead, increased synthesis and secretion of leptin by maternal adipose tissue seems to account for elevated plasma leptin concentrations in the pregnant mouse (Kronfeld-Schor et al., 2000; Tomimatsu et al., 1997).

In addition to conventional animal models, we have studied leptin biology in the seasonally breeding, insectivorous bat *Myotis lucifugus* (little brown myotis). Part of the rationale for studying these animals is that the little brown myotis is a model for studying the energetics of pregnancy. Pregnant little brown myotis expend large amounts of energy flying, maintain low body fat and yet give birth to offspring approximately 25% the mother's mass at parturition (Kurta and Kunz, 1987). Moreover, as in primates, the placenta of the little brown myotis synthesizes leptin mRNA and secretes leptin *in vitro* (Kronfeld-Schor et al., 2000; Zhao et al., 2003). Thus, the little brown myotis is a model for studying the actions of leptin in placentas that produce leptin, including the human placenta.

In our previous study, leptin stimulated trophoblast invasion in cells from early, but not late pregnancy in the mouse. In both the mouse and myotis, plasma leptin concentrations and total leptin receptor levels (as evidenced by northern blotting) increase from early to late pregnancy (Zhao et al., 2004). However, there are multiple isoforms of the leptin receptor generated by alternative splicing. The longest isoform, ObRb, is the only one capable of signaling through the JAK/STAT pathway, and of optimal signaling through the MAPK pathway (reviewed in Schulz and Widmaier, 2006). A shorter isoform, ObRa, is also capable of weak signaling through the MAPK and IRS-1 pathways (Bjorbaek et al., 1997). In the rat, placental expression of ObRa, but not ObRb mRNA, increases from mid-(d16) to late (d22) pregnancy (Smith and Waddell, 2002). In the baboon (*Papio* sp.), neither isoform changes significantly with pregnancy stage (Green et al., 2000). Here we test the hypothesis that leptin secreted by trophoblast cells promotes invasiveness *in vitro* in *M. lucifugus* placenta from the first trimester of pregnancy. In addition, we report the sequence of the ObRa and ObRb isoforms from the little brown myotis, and examine expression of ObRb mRNA in the placenta throughout gestation. Because *M. lucifugus* and *M. musculus* are evolutionarily distant from each other, and because they are members of the two most specious mammalian orders, common functions of leptin on placental physiology would suggest that the effects of leptin are conserved and widespread among mammals.

2. Materials and methods

Reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

2.1. Animals

All animal procedures were approved by the Boston University IACUC and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Pregnant *M. lucifugus* weighing up to 12.7 g were captured at a maternity roost in Massachusetts from May to June 2003–2005. Bats were captured using a harp trap as they emerged from the roost at dusk and were transferred to the laboratory in simulated wooden roosts (Kunz and Kurta, 1988), where they remained overnight. Myotis were sacrificed the following morning by decapitation in order to obtain placentas. 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) as previously described (Zhao et al., 2003). Gestation length is approximately 60 days in *M. lucifugus* (Wimsatt, 1945).

2.2. Trophoblast cell isolation

Trophoblast cells were cultured by using a previously described protocol for trophoblast cells from mice (Thordarson et al., 1987), with specified modifications (Kronfeld-Schor et al., 2000). Briefly, placentas were separated from the underlying endometrium using dissecting forceps in a sterile dish containing wash medium (Medium 199, 20 mM Hepes, 10 mM $NaHCO_3$, penicillin/streptomycin). The placentas were then incubated in dissociation medium (wash medium containing 1 mg/ml collagenase, 20 μg/ml DNase) for 1 h at 37 °C, with periodic trituration to separate cells. Cells were washed to remove dissociation medium, then filtered to remove undigested tissue. The cells were separated on an isotonic 40% Percoll gradient. The trophoblast cell layer was collected and plated in NCTC-135 medium, with 20 mM Hepes, $NaHCO_3$, 1.65 mM cysteine, 10% fetal calf serum, and penicillin/streptomycin.

2.3. Matrigel invasion assays

For each experiment, isolated trophoblast cells from 3 to 10 bats from the same trimester of pregnancy (either first or third) were pooled and divided into matrigel invasion chambers, at a density of $0.5–1 \times 10^5$ cells per insert in a 24-well plate (BD Biosciences, Bedford, MA). The cells were cultured overnight in NCTC-135 containing 10% FCS at 37 °C, in an air/5% CO_2 atmosphere. Medium was replaced the following morning with 1 mL serum-free NCTC-135 (controls) or one of the following additions. To block leptin that was secreted into the medium by the cultured cells, an antibody to human leptin (Diagnostic Systems Laboratories R01725) was added to the medium at a 1:200 final dilution. To determine the specificity of the anti-leptin antibody, cells were treated with an irrelevant antibody which was generated against chicken immunoglobulin, at a 1:200 final dilution. Duplicate wells were used for each treatment. After 24-h incubation, cells remaining above the insert membrane were removed by gentle scraping with a swab. Cells that had invaded through the matrigel to the bottom of the insert were fixed in 1.5% paraformaldehyde, 0.1% Triton X-100 for one hour. After being washed in 0.1 M PBS, the cells were stained with hematoxylin and eosin. The insert was then washed in 70% ethanol and briefly air dried, then mounted on a glass slide with a coverslip by using glycerol mounting medium. The slides were coded blindly to prevent counting bias, and the number of invaded cells on each membrane counted under a light microscope. The number of invaded cells for each experimental sample ($n = 1$) represents the average of duplicate wells.