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Developmental Changes in *ob* Gene Expression and Circulating Leptin Peptide Concentrations

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We examined the developmental changes in murine white and brown adipose tissue leptin and circulating immunoreactive total leptin concentrations. The ~4.4 kb leptin mRNA levels were higher at 2 and 7d postnatal ages, but declined to adult levels by the 14d stage and remained so until 160d. Paralleling the mRNA concentrations, leptin peptide levels also were higher at 2d, 7d, and 14d, declining to adult values by the 21d weaning stage. No difference in mRNA levels was observed between brown-enriched and white adipose tissue. No sexual dimorphism was observed in the leptin mRNA or peptide levels between 14 and 160d; however, at 2 and 7d, while no sex related differences were observed in the peptide levels, adipose mRNA concentrations were mildly higher in males than in the females. We conclude that leptin mRNA and peptide levels are higher during consumption of a high fat milk diet. High levels of leptin with increasing food intake and body weight gain signify hypothalamic leptin receptor resistance during the immediate postnatal period. © 1997 Academic Press

Body weight is dependent upon food intake and energy expenditure. The white adipose tissue serves as a storage depot for excess substrate (1), while brown adipose tissue releases energy as heat (2,3). Presence of excessive white adipose tissue defines obesity. Transgenic mice with ablation of brown adipose tissue are obese and hyperphagic suggesting a link between food intake and energy expenditure (4). Leptin, the circulating translated product of the *ob* gene which is synthesized in both white and brown adipose tissue serves as a marker of obesity and reflects the physiological fed state in an animal (5,6,7). Leptin, has been reported to cross the adult blood-brain barrier and interact with leptin receptors in the central nervous system (8-10), thereby signalling the brain regarding the body's fat energy stores (11,12). In the hypothalamus, leptin is known to suppress the synthesis of the orexigenic hypothalamic neuropeptide Y (10,12), thereby causing a diminution in food intake and ultimately weight loss (13,14). This mechanism of leptin action serves as a feed back pathway by which satiety is accomplished after ingestion.

Neuropeptide Y is expressed in fetal and postnatal brain and is known to enhance food intake during the rodent's suckling phase of development (15). In addition, leptin is expressed by rat adipose tissue as early as the first postnatal day (6). However, whether satiety is accomplished by leptin during this phase of development is unknown. Prior to determining its functional role it is essential to delineate the level of expression of leptin mRNA along with circulating leptin concentrations during the suckling phase when a high-fat milk diet is ingested in comparison to the high carbohydrate ingesting postweaned and adult stages of development. To accomplish this goal we undertook the present study and noted that leptin mRNA levels in both the white and brown enriched adipose tissue are higher during the early suckling phase. This developmental pattern is reflected by higher circulating leptin peptide concentrations.

MATERIALS AND METHODS

Animals. Balb-C mice were purchased from Jackson Laboratories and housed under 12 hour light and dark cycles with free access to standard rat chow. The care and use of animals followed the guidelines established by the National Institutes of Health and were approved by the Magee-Womens Research Institutional Committee for Animal Care and Use.

Sample collection. On varying postnatal ages (from 2 to 160 days) the mice received intraperitoneal phenobarbital and were euthanized. Blood was obtained from the left ventricle, the samples centrifuged at 4°C, the plasma removed, aliquoted, and stored at −20°C until further use. Fat tissue from the interscapular region which was visibly brownish-pink in color was removed and labeled brown adipose tissue. Subcutaneous fat found around the abdomen which was visibly yellow in color was labeled white adipose tissue. The tissues were quick frozen in liquid nitrogen and stored at −70°C until further use.

Northern blot analysis. Poly (A+) enriched RNA was extracted from the brown and white fat using the miniribosep mRNA extrac-

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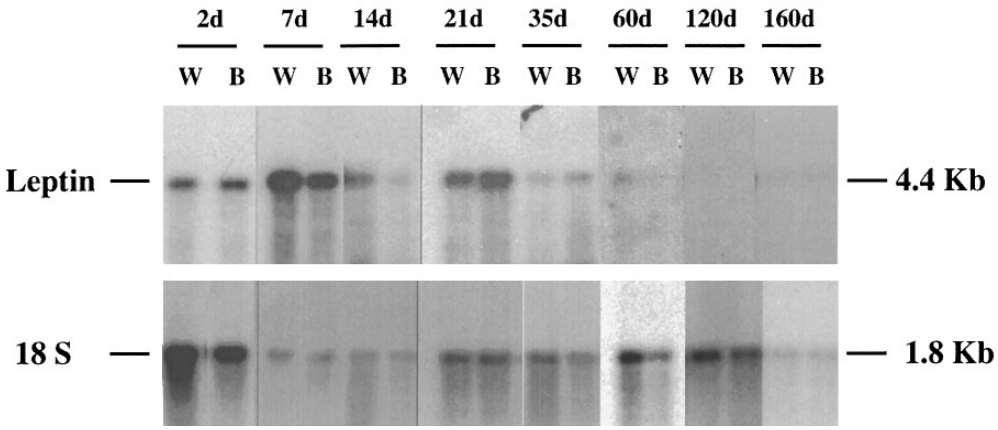

FIG. 1. Representative Northern blots demonstrating the ~4.4 kb leptin mRNA (top panel) and the 1.8 kb 18S rRNA (bottom panel) in white (W) and brown-enriched (B) adipose tissue obtained from different ages (2d to 160d) are shown. Due to age-dependent differences in leptin mRNA amounts, differing autoradiographic exposure times are depicted to allow demonstration of leptin mRNA bands optimally at all ages.

tion kits (Collaborative research, Bedford, MA). RNA was quantitated spectrophotometrically and the purity of the samples was assessed as a ratio of 1.8-2.0 at 260/280 nM wavelength. Five micrograms of poly (A+) enriched RNA was loaded on 1.2% agarose-2.2 M formaldehyde slab gels and run at 22V for 16 hours. The gels were stained with ethidium bromide and visualized under ultraviolet light to confirm the integrity of RNA samples. The RNA from the gels were transferred to Nytran membranes (Micron Separations, Inc., Westboro, MA) and crosslinked by UV light in a stratalinker at 1200 $\times$ 100 microjoules for 45 s (Stratalinker 1800, Stratagene, La Jolla, CA). The filters were prehybridized for 2 hours at 42°C in 50% formamide, 0.1% polyvinyl pyrollidone, 0.1% bovine serum albumin, 0.1% Ficoll, 75 mM sodium chloride, 50 mM sodium monobasic phosphate, 1.25 mM EDTA, 0.2% SDS, and 200 μg of denatured salmon sperm DNA. The filters were subjected to hybridization with 1×10^6 cpms/ml of ^{32}P-randomly primed 550 bp rat leptin cDNA (16) for 24 hours. The filters were washed at room temperature in 2 $\times$ SSC (1 $\times$ SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and 0.1% SDS. The final wash was performed in 0.1% SSC and 0.1% SDS for 30 minutes at 45°C. The filters were subsequently exposed to X-ray film at −70°C for differing lengths of time until optimal resolution was achieved (17). The same filters were stripped and reprobed under the same conditions with a ^{32}P-randomly primed 1.8 kb rat 18S ribosomal RNA cDNA (18) to correct for the interlane loading variability. The leptin mRNA levels were quantitated by densitometry and the values were expressed as a ratio to the 18S rRNA optical density.

Radioimmunoassay. Leptin peptide concentrations in ng/ml were assessed by the double antibody radioimmunoassay (19) using an anti-murine leptin antibody and murine leptin standards (Linco Co., St. Louis, MO).

Data analysis. All results are expressed as a mean±SEM. Developmental changes were analyzed by the one way analysis of variance and intergroup differences validated by the Newman Kuel's test at a $p<0.05$.

RESULTS

Figure 1 demonstrates representative Northern blots depicting the ~4.4 kb leptin mRNA and the corresponding 1.8 kb 18S rRNA in white and brown adipose tissue obtained at different postnatal ages. Figure 2 shows the densitometric quantitation of leptin mRNA standardized to the 18S mRNA. While no difference between the white and brown adipose tissue leptin mRNA levels are noted at a given age, a sexual dimorphism with slightly though not significantly higher male versus female leptin mRNA concentrations are observed at 2 and 7d, this difference disappearing between 21d and 160d. Overall pooled leptin mRNA concentrations from white and brown-enriched adipose tissues obtained from male and female mice are higher (2 to 5-fold) at 2 and 7 days of age, declining at 14d to achieve adult (120-130d) values. A second slight increase in leptin mRNA is observed at 35d to 60d around the time of puberty and sexual maturation (Figure 3). Radioimmunoassayable mouse leptin peptide concentrations mimick this age-dependent decline in leptin

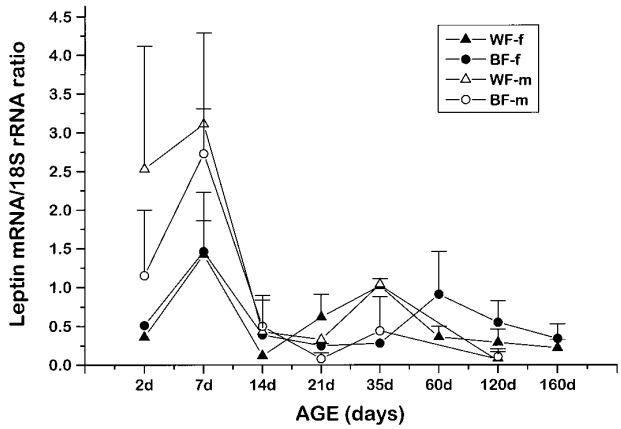

FIG. 2. Leptin mRNA to 18S rRNA ratios are expressed in white (WF) and brown-enriched (BF) fat, in male (m) versus female (f) animals at ages between 2d and 160d.

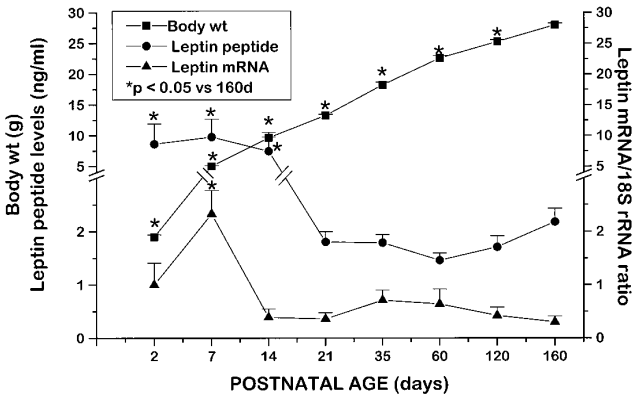

FIG. 3. Body weights (2d: n=6, 7d: n=9, 14d: n=6, 21d: n=10, 35d: n=15, 60d: n=8, 120d: n=10, 160d: n=12), leptin:18S rRNA ratios in all adipose tissue (white and brown-enriched) obtained from male and female mice (2d: n=6; 7d: n=9, 14d: n=6, 21d: n=6, 35d: n=6, 60d: n=8, 120d: n=10, 160d: n=12), and circulating immunoreactive leptin peptide levels from male and female mice at ages ranging from 2d to 160d (2d: n=4, 7d: n=5; 14d: n=4, 21d: n=10, 35d: n=15, 60d: n=5, 120d: n=9, 160d: n=4).

mRNA amounts (Figure 3). A four-fold increase in circulating peptide concentrations is observed at 2, 7, and 14d when compared to 21 to 160d. No sexual dimorphism in circulating leptin concentrations was observed at all the ages studied, therefore the results were pooled. Figure 3 also demonstrates the body weights of mice ranging from 2 to 160 days, with the 2, 7, and 14 days denoting the suckling phase, the 21d representing the weaning stage, and the 60d time point being when sexual maturation takes place. No differences in body weight were observed between age-matched male and female mice, thus the values were pooled.

DISCUSSION

In the present study we have observed the presence of leptin mRNA in both the interscapular and abdominal fat, with the former being more enriched in brown fat and the latter consisting mainly of white fat. The absence of a difference in leptin mRNA levels between these two types of fat may be true or merely reflect the presence of considerable amounts of white fat infiltrating the interscapular region more so in the adult than in the immediate postnatal stages. Further, unlike adult studies particularly in the human (20), no sexual dimorphism in leptin mRNA or peptide levels were noted, although a trend towards a male predominance with respect to leptin mRNA levels was observed during the early suckling phase.

The significantly higher expression of leptin associated with greater concentrations of circulating leptin peptide during the suckling phase may reflect the high fat milk diet leading towards building of fat stores along with energy expenditure necessary to generate endogenous heat (2,3). Alternatively the animals during the suckling phase continue to ingest milk thereby being satiated. This satiation may be secondary to the high circulating leptin concentrations. However, unlike the adult (21), despite high circulating leptin levels, the pups during the postnatal period continue to demonstrate no suppression in hypothalamic NPY levels (22), feed frequently and gain weight. This suggests that the hyperleptinemia is reflective of a hypothalamic leptin receptor insensitivity. Whether the long isoform of the leptin receptor capable of intracellular signalling develops (23-25) and is present in the neonatal hypothalamic nuclei in adequate numbers so as to mediate the biological action remains to be investigated.

We conclude that leptin mRNA levels in both the white adipose and brown enriched adipose tissue are higher during the early suckling phase. This increase is reflected in the increase in circulating leptin protein concentrations during the early suckling phase. Despite the increase in circulating leptin levels, these postnatal animals continue to suckle and gain body weight. The postnatal hyperleptinemia may signify a developmental leptin insensitivity of the hypothalamus due to inadequate development of the leptin receptor or its intracellular signalling pathway.

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