

Effects of Diet and Time of the Day on Serum and CSF Leptin Levels in Osborne-Mendel and S5B/Pl Rats

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Abstract

ISHIHARA, YURI, CHRISTY L. WHITE, HARUAKI KAGEYAMA, ASAKO KAGEYAMA, DAVID A. YORK, AND GEORGE A. BRAY. Effects of diet and time of the day on serum and CSF leptin levels in Osborne-Mendel and S5B/Pl Rats. *Obes Res.* 2004;12:1067–1076.

Objective: To characterize the effects of dietary fat on the diurnal variation in serum and cerebrospinal fluid (CSF) leptin levels in Osborne-Mendel (OM) and S5B/Pl rats and quantitate the dose response to lower doses of leptin administered into the third cerebral ventricle.

Research Methods and Procedures: Rats were fitted with implanted vascular ports or third ventricular cannulas and fed either laboratory chow or one of two semipurified high-fat or low-fat diets. Leptin and insulin were measured by immunoassay.

Results: Serum leptin and insulin levels were positively correlated and had similar patterns of diurnal change. CSF leptin and insulin also had diurnal rhythms, with a peak at 7:00 AM, but the diurnal oscillations of leptin and insulin were significantly lower in the S5B/Pl rats than the OM rats. Thus, the ratio of CSF to serum leptin was significantly higher in the S5B/Pl rats than in the OM rats. Dietary fat had no effect on these diurnal patterns. There was a right shift in the dose response to leptin in the OM rats compared with the S5B/Pl rats. S5B/Pl rats treated with leptin had higher signal transduction and translation (STAT-3) mRNA levels compared with pair-fed or saline injected S5B/Pl rats. Hypothalamic suppressors of cytokine signaling mRNA levels

were not statistically different between the groups.

Discussion: The higher CSF-to-serum leptin ratio in the S5B/Pl rats, the enhanced suppression of food intake and body weight with leptin injections, and the higher STAT-3 activity in these animals suggest that S5B/Pl rats are more sensitive to leptin than OM rats.

Key words: blood-brain barrier, high-fat diet, leptin resistance, diurnal rhythm

Introduction

Leptin is a 16-kDa protein, produced mainly in adipose tissue, that plays an important role in regulating feeding behavior. Leptin is structurally related to cytokines, and its receptor is a member of the cytokine superfamily. The absence of leptin produces massive obesity in animals and humans, and treatment with leptin reduces food intake and body weight. Because circulating leptin levels are highly correlated with body fat, the high levels of leptin in the face of obesity suggest resistance to the action of leptin.

Several hypotheses have been proposed to explain leptin resistance. One of them is impaired leptin transport across the blood-brain barrier (BBB).¹ Decreased cerebrospinal fluid (CSF)-to-serum ratio of leptin has been reported in obese humans (1,2), obese CD-1 mice (3), high-fat (HF) diet-induced obese rats, genetically obese *fa/fa* rats (4), and Koletsky rats, which have a point mutation on the short form leptin receptor and have less transport across the BBB compared with their littermates (5). Another potential mechanism of leptin resistance is a defect in the receptor or in the postreceptor signaling pathways. Leptin activates

Received for review June 20, 2003.

Accepted in final form May 18, 2004.

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¹ Nonstandard abbreviations: BBB, blood-brain barrier; CSF, cerebrospinal fluid; HF, high fat; JAK-STAT, janus kinase signal transduction and translation; SOCS-3, suppressors of cytokine signaling; OM, Osborne-Mendel; LF, low fat; ICV, intracerebroventricular; STAT-3, Signal transduction and translation; RBC, red blood cell; RT-PCR, reverse transcription-polymerase chain reaction; CYC, cyclophilin.

signal transduction through the janus kinase signal transduction and translation (JAK-STAT) pathway through the long form of the leptin receptor. The suppressors of cytokine signaling (SOCS-3) block leptin-induced signal transduction and provide another candidate to explain leptin resistance at the postreceptor level. Indeed, adrenalectomy, which prevents obesity, activates the JAK-STAT signaling pathway and down-regulates the SOCS-3 pathway (6).

Osborne-Mendel (OM) rats are prone to obesity when eating an HF diet, but S5B/P1 rats resist dietary obesity when eating the same diet. Several studies have characterized the differences in feeding behavior and physiology between these two strains. OM rats have higher serum leptin and insulin levels than S5B/P1 rats, and OM rats are proportionally fatter. After a single high dose of leptin administered into the cerebral ventricles, OM rats and S5B/P1 rats have a similar acute decrease in food intake (7). The concentration of ketones in the serum and brain is higher in the resistant S5B/P1 rats (8), whereas the sympathetic nervous system is more responsive in the S5B/P1 rats (9). The suppression of food intake is greater and lasts longer in S5B/P1 rats than in OM rats after infusion of linoleate acid into the duodenum (10). The slow rectifying potassium channel in the taste bud shows a greater change in conductance in the S5B/P1 rat than in the OM rat in response to polyunsaturated fatty acids (11,12). These data imply that the response to fatty acids or a metabolite of fatty acids may be one of the major metabolic signals that differentiate the OM rat from the S5B/P1 rat.

In this study, we tested two hypotheses that might help explain leptin resistance in OM rats. The first hypothesis is that there might be a higher ratio of CSF to serum leptin, indicating increased leptin transport. To test this hypothesis, we have directly measured the diurnal variation of serum leptin, corticosterone, and insulin in both chow-fed OM and S5B/P1 rats. In addition, we measured both CSF and serum leptin in OM and S5B/P1 rats eating either low-fat (LF) or HF diets to assess the effect of dietary fat and strain on leptin concentration in the CSF. Finally we examined the effect of intracerebroventricular (ICV) injections of lower doses of leptin on food intake of OM and S5B/P1 rats to test their sensitivity to submaximal levels.

The second hypothesis is that the activity of signal transduction mediated by leptin through the JAK-STAT pathway in the hypothalamus might be different in OM and S5B/P1 rats and that this difference might contribute to leptin resistance in OM rats. To test this hypothesis, we injected different doses of leptin ICV on consecutive days and monitored food intake and body weight gain. We also measured hypothalamic SOCS-3 and signal transduction and translation (STAT-3) mRNA levels from rats given a single 1.0- μ g dose of leptin ICV.

Research Methods and Procedures

Animals

All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Pennington Biomedical Research Center. Age-matched 13-week-old male OM and S5B/P1 rats were selected from the breeding colony maintained in the animal facility of the Pennington Biomedical Research Center. They were housed in individual hanging wire-bottom cages supplied with an automated watering system in a room with a 12-hour light:dark cycle (7:00 AM to 7:00 PM) maintained at $22 \pm 2^\circ\text{C}$. All rats consumed a commercial diet (Rodent Chow 5001; Purina Mills, St. Louis, MO) ad libitum until the beginning of the experiments.

Experiment 1

After 1 week of adaptation to the cages while eating Purina Laboratory Chow, all rats were implanted with blood sampling ports and allowed to recover for a 7-day period. The rats were divided into two groups, and blood was sampled from these ports at either 6:00 AM, 12:00 PM, and 6:00 PM or 9:00 AM, 3:00 PM, and 9:00 PM.

Experiment 2

Rats from each strain were divided into two weight-matched groups. One-half of the rats of each strain were given a semipurified HF diet (56% energy as fat), and the other one-half were given a semipurified LF diet (10% energy as fat). The composition of these diets has been described previously (7). A single food jar was secured to each cage. Food intake was measured during the light and dark cycle for 48 hours, beginning on the 14th day after rats were placed on either an LF or HF diet. One-half of the rats in each diet group were anesthetized with a ketamine cocktail [1.10 mg/kg of mixture of xylazine (5 mg/mL), acepromazine (1.6 mg/mL), and ketamine HCl (80 mg/mL)]. Forty-eight hours after being started on the diet, paired CSF and serum samples were collected between 8:00 AM and 11:00 AM. The remainder of the rats were anesthetized after eating the special diets for 14 days, and the same procedures were repeated.

Experiment 3

Eight-week-old rats were adapted to a modified 12-hour light cycle (lights off 11:00 AM) for 4 weeks before the experiments. After 3 days of adaptation to the light cycle, rats were provided an HF diet. After 1 week of adaptation, a third ventricular cannula was implanted in each rat (R & D Systems, Minneapolis, MN). Each strain of rats was divided into two subgroups, with one subgroup receiving saline (2 μ L) ICV and the other subgroup receiving leptin (1 μ g/2 μ L) ICV. The daily schedule was as follows: at 7:00 AM, food jars were removed, and food intake and body weight were measured; rats received ICV injections be-

tween 9:30 AM and 10:30 AM; food was returned to the cages immediately before the lights went off at 11:00 AM. Food intake was measured 2, 4, 6, 12, and 24 hours after injection. Rats received five consecutive daily ICV injections and were allowed 14 days to recover while body weight and food intake were measured. They were then tested with different doses (0.01, 0.03, 0.1, 0.3, and 0.1 μg for S5B/P1 rats and 0.1, 1.0, 3, and 10 μg for OM rats) using the same schedule.

Experiment 4

Age-matched 8-week-old OM and S5B/P1 rats were adapted to the experimental room and modified light cycle (see Experiment 3) for 4 weeks. Rats were adapted to a pelleted HF diet (D12330; Research Diet, New Brunswick, NJ) that was fed ad libitum for 4 weeks. One-half of the vehicle-injected rats were pair-fed to the leptin-treated rats. For pair-feeding, two-thirds of the average food intake of the previous day for the leptin-treated rats was provided at 11:00 AM, and the remaining one-third amount of the food was given at 8:00 PM. Two weeks before the experiment started, they were fitted with a third ventricular cannula. Rats received either 2 μL of sterile physiological saline or 1.0 μg of leptin per rat dissolved in saline vehicle. Rats pair-fed to leptin-injected rats received 2 μL of saline. The ICV injections were repeated on 4 consecutive days between 7:00 AM and 9:00 AM. Rats were killed between 9:00 AM and 11:00 AM on the fourth day, 2 hours after receiving their ICV injections. After decapitation, trunk blood was collected for measurement of serum hormones. The hypothalamus was dissected from each rat, snap-frozen in liquid nitrogen, and stored at -80°C until used for total RNA analysis.

Surgical Procedures

Implantation of Vascular Ports. Rats were fasted overnight and anesthetized with ketamine cocktail injected subcutaneously. An incision was made lateral to the midline of the dorsal surface of the body, and a subcutaneous pocket was created to receive the vascular port (ROP-3S, 3.0Fr; Rat-O-port: Access Technologies, Skokie, IL). The port was filled with 0.15 mL of 0.9% saline solution. An incision was made from 0.5 cm below the jaw and 0.5 cm right of the midline of the neck, and the subcutaneous tissue was explored by blunt dissection to identify the right external jugular vein. A 1-cm length of vein was isolated from the surrounding tissue and a 4-0 silk ligature was placed on the distal side of the vein. The silastic catheter was tunneled subcutaneously from the incision on the back to the neck incision. A small incision was made on the vein, and the tip of the catheter was inserted caudally and advanced 3 cm into the vein. The catheter was secured to the vein using 4-0 silk. The back flow of blood was used as sign of patency and was evaluated both before and after the catheter was secure.

The incisions in the neck and back were closed with 3-0 Vicryl. The port was filled with 0.15 mL of heparinized saline (200 units/mL).

Third Cerebroventricular Cannulas. Rats were anesthetized by subcutaneous injection with 0.11 mL (for S5B/P1 rats) or 0.08 mL (for OM rats) per 100 grams body weight of a ketamine cocktail (80 mg/mL ketamine, 1.6 mg/mL acepromazine, and 5 mg/mL xylazine). Rats were placed in a stereotaxic apparatus, with the incisor bar set at -3.3mm below the interaural line. The tip of the vertically mounted, 14-mm-long, 25-gauge stainless steel cannula was lowered to the following coordinates: 2.5 mm posterior to bregma, 0.0 mm lateral to midsagittal line, and 8.5 mm ventral to the dura mater according to the atlas of Paxinos and Watson (13). The guide cannulas were secured in place using dental acrylic and three stainless steel anchor screws in the skull. Each guide was fitted with a 31-gauge stylet to prevent occlusion and contamination. Verification of the cannula placement was performed 5–7 days after surgery. Water consumption was monitored in non-water-deprived rats in response to ICV angiotensin II (20 ng/2 μL ; Sigma) delivered by 31-gauge injectors that projected 0.5 mm beyond the tip of the guide cannula. Rats that did not drink water within 2 minutes were excluded from the study.

Blood Sampling from Vascular Ports

After recovering from surgery for 7 days, blood was collected from the vascular port while rats had access to standard laboratory chow. A volume of 0.30 mL of fluid in the port was discarded, and 1.0 mL of blood was collected. Heparinized saline (0.15 mL, with 200 U/mL) was injected into the port to provide a heparin lock. Special care was taken not to stress the animals; familiarization with the procedures and frequent gentle handling were used. Serum samples were stored at -70°C until used for hormone assays.

Collection of CSF and Paired Serum Sample

Rats were anesthetized with the ketamine cocktail and placed in the stereotaxic apparatus. The skin was shaved over the posterior aspect of the head. The head was flexed acutely so that the external occipital protuberance in the neck region became prominent. A 27-gauge needle was inserted through the atlanto-occipital membrane into the cisterna magna. Approximately 0.2 mL of CSF was collected. The samples were centrifuged, and samples with $>10\ \mu\text{L}$ of red blood cell (RBC) contamination were discarded. The chest cage was opened, a 21-gauge needle was inserted transcardially, and blood samples were collected and stored at -70°C until used for hormone assay.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Five hypothalami from each group were selected. Total RNA was isolated from individual whole hypothalami using

TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer's protocol. Total RNA was quantified by spectrophotometer and confirmed by ethidium bromide staining of the 18S and 28S ribosomal RNA under ultraviolet light after electrophoresis on a 1% agarose/formaldehyde gel.

STAT-3 and SOCS-3 mRNA levels were determined by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) using cyclophilin (CYC) mRNA as an internal standard, as previously described (6). Taqman probe and primer were designed using Primer Express Software (Applied Biosystems, Branchburg, NJ), following the Applied Biosystems Guidelines. The primers for SOCS-3 were 5'-TGGCTACCCTCCAGCATCTT-3' and 5'-AGCTGGTCACTTTCTCATAGGA-3'; the Taqman probe was 5'-6-FAM-TCCAGGTGACCGTTGACAGTCTTCCG-BHQ-1-3'. Primers for STAT-3 were 5'-TCCAGTCAGC-CAGCTCTTCA-3' and 5'-AGCTGGCAGGGCTCTT-GTC-3'; the Taqman probe was 5'-6-FAM-CGGTCAGT-GTCTTCTGCACGTACTCCATT-BHQ-1-3'. Primers for CYC were 5'-CCCACCGTGTCTTTCGACAT-3' and 5'-TGCAAACAGCTCGAAGCAGA-3'; the Taqman probe was 5'-VIC-CAAGGGCTCGCCATCAGCCG-TAMRA-3'. Real time RT-PCR was performed in a 40- μ L final reaction volume using the Taqman 1000 reaction PCR core reagents kit (Applied Biosystems). The reaction mixture contained 1 \times Taqman buffer A, 5.5 mM magnesium chloride, 300 μ M deoxy-adenosine 5'-triphosphate (dATP), deoxy-cytosine 5'-triphosphate (dCTP), deoxy-guanosine 5'-triphosphate (dGTP) and 600 μ M deoxy-uridine 5'-triphosphate, 0.15 units AmpliTaq Gold DNA polymerase, 0.0615 units Multiscribe RT primers, forward (300 nM STAT-3, 300 nM SOCS-3, and 100 nM CYC) and reverse primers forward (300 nM STAT-3, 300 nM SOCS-3, and 200 nM CYC) and 1 ng of total RNA for STAT-3 and SOCS-3, and 1 ng of total RNA for CYC. Real time RT-PCR was performed on an ABI PRIZM 7700 sequence detector (Applied Biosystems) with the following conditions: 48 °C for 30 minutes and 95 °C for 10 minutes for one cycle, followed by 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. For quantitative analysis, standard total RNA from an intact rat hypothalamus was used to create each standard curve with threshold cycle vs. amount of RNA to ensure that the cycle used was on the linear portion of the reaction.

Radioimmunoassays

Commercial radioimmunoassays against rat/mouse leptin were used to assay leptin and a specific insulin for serum insulin (Linco Research, St. Charles, MO) and corticosterone (ICN, Costa Mesa, CA). CSF leptin and paired serum leptin in Experiment 2 were measured using a mouse/rat-leptin RIA kit (Alpco Diagnostics, Windham, NH).

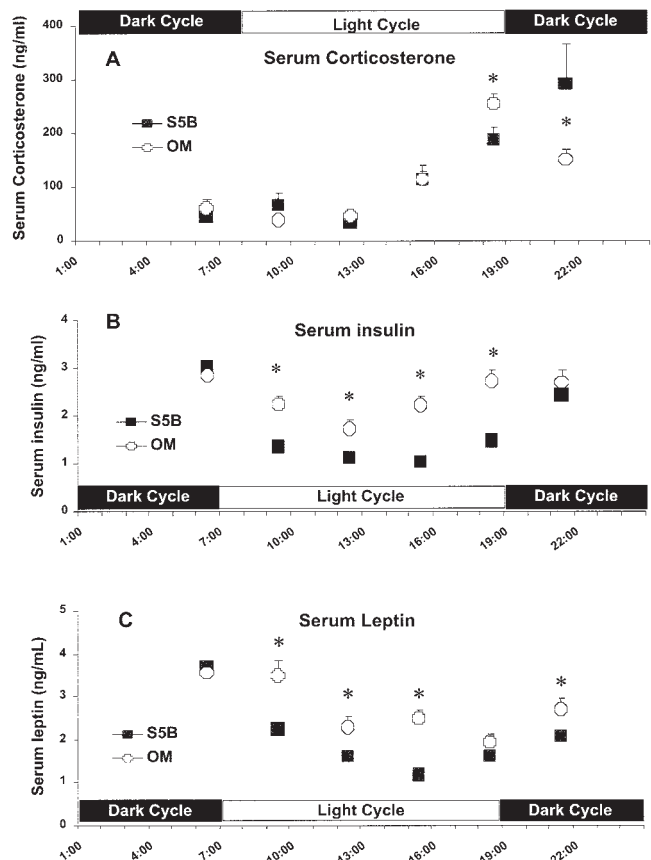


Figure 1: Diurnal variation of serum levels of corticosterone, insulin, and leptin was measured over a 15-hour period. (A) Serum corticosterone (ng/mL). (B) Serum insulin (ng/mL). (C) Serum leptin (ng/mL). Each point represents the means \pm SE for each strain at each time-point. * $p < 0.05$ compared between strains at each point.

Statistics

The diurnal variation data were analyzed by repeated-measures ANOVA, with time being a repeated factor and autoregressive covariance structure. The data from the second experiment were analyzed by ANOVA (with strain, diet, and time as the main factors and all interactions).

Results

Experiment 1

The diurnal variation of corticosterone, insulin, and leptin was measured over a 15-hour period (Figure 1). Serum leptin levels (Figure 1C) of OM rats were significantly higher than those of S5B/P1 rats [$F(1,57) = 12.08$, $p = 0.0010$] at all time-points, except at 6:00 AM and 6:00 PM. There was a significant effect of the time of day on serum leptin [$F(5,91) = 79.72$, $p < 0.0001$]. There were also significant effects of the time of day on the serum leptin level for each strain of rats [OM: $F(5,91) = 23.65$, $p <$

0.0001, S5B/P1: $F(5,91) = 68.1, p < 0.0001$). The highest serum leptin levels in both strains were observed at 6:00 AM (OM, 3.55 ± 0.31 ng/mL; S5B/P1, 3.67 ± 0.16 ng/mL), but there was no statistically significant difference between the two strains at this time [$F(1,91) = 0.42, p = 0.5181$]. There were, however, significant differences in serum leptin levels between strains at other times except 6:00 PM {9:00 AM: [$F(1,91) = 13.09, p = 0.0005$], 12:00 PM [$F(1,91) = 6.21, p = 0.0145$], 3 PM: [$F(1,91) = 15.90, p = 0.0001$], and 9:00 PM [$F(1,91) = 4.50, p = 0.0366$]}. The lowest leptin level was observed at 6:00 PM in OM (1.94 ± 0.21 ng/mL) and 3:00 PM in S5B/P1 rats (1.15 ± 0.11 ng/mL).

OM rats had higher serum insulin levels than S5B/P1 rats [$F(1,57) = 16.19, p = 0.0002$]. There was a significant effect of the time of day on the concentration of endogenous insulin in serum [$F(5,94) = 21.80, p = 0.0001$]. There was also a significant effect of the time of day on the serum insulin level for each strain of rats [OM: $F(5,94) = 5.52, p = 0.0002$; S5B/P1: $F(5,94) = 21.11, p < 0.0001$]. The highest serum insulin levels were observed at 6:00 AM (OM: 2.86 ± 0.23 ng/mL; S5B/P1: 3.01 ± 0.17 ng/mL), and there were no statistically significant differences between the two strains at 6:00 AM [$F(1,94) = 0.31, p = 0.5804$]. There were significant differences in serum insulin levels between strains at 9:00 AM [$F(1,94) = 7.13, p = 0.0089$], 12:00 PM [$F(1,94) = 4.24, p = 0.0421$], 3:00 PM [$F(1,94) = 13.67, p = 0.0004$], and 7:00 PM [$F(1,94) = 13.76, p = 0.0004$], but not at 9:00 PM [$F(1,94) = 0.69, p = 0.4085$]. The lowest insulin level was observed at 12:00 PM in OM (1.72 ± 0.20 ng/mL) and 3:00 PM in S5B/P1 rats (1.03 ± 0.15 ng/mL).

There was a significant effect of the time of day on the concentration of endogenous serum corticosterone [Figure 1A; $F(5,94) = 52.37, p < 0.0001$; OM: $F(5,94) = 24.41, p < 0.0001$; S5B/P1: $F(5,94) = 33.30, p < 0.0001$]. OM and S5B/P1 rats both showed the highest corticosterone level at 9:00 PM (OM, 254.11 ± 18.56 ng/mL; S5B/P1, 291.61 ± 75.29 ng/mL). There was no significant effect of strain alone as a factor in serum corticosterone level [$F(1,57) = 0.80, p = 0.3747$]. However, for the strain \times time interaction, both 6:00 PM [$F(1,94) = 8.95, p = 0.0023$] and 9:00 PM [$F(1,94) = 18.44, p < 0.0001$] were significant.

Experiment 2

Body weights of OM rats were significantly greater than those of S5B/P1 rats of the same age. Over the 15-day study period, the OM rats eating the LF and HF diet showed a significant increase in body weight. Food intake was measured in animals eating both diets. Neither the strain of rats [$F(1,51) = 0.01, p = 0.9097$] nor the diet they ate [$F(1,51) = 0.9784, p = 0.9784$] affected food intake during the light or dark phase of the diurnal cycle (data not shown).

The effect on serum leptin of HF or LF diets for 3 and 15 days is shown in Figure 2A. There were significant effects

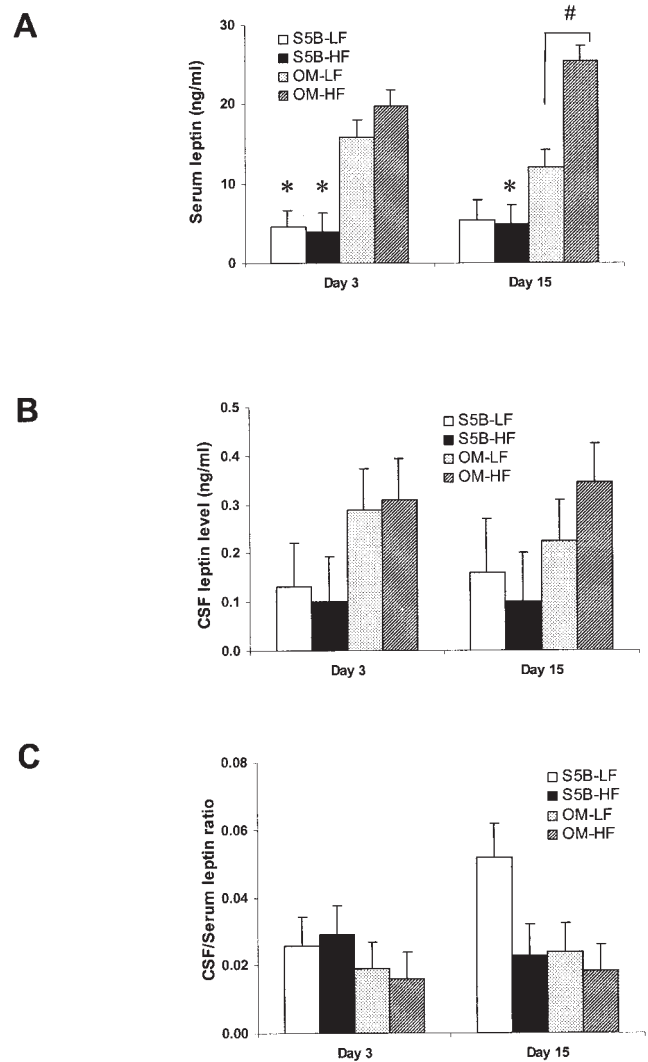


Figure 2: (A) Effect of an HF and LF diet at days 3 and 15 on serum leptin levels. The bars represent the mean \pm SE. Blood was collected by cardiac puncture after the collection of CSF. * $p < 0.05$ compared with respective strain for each diet. # $p < 0.05$ between diets and between strains. (B) Effect of an HF and LF diet at days 3 and 15 on CSF leptin levels. The bars represent the mean \pm SE. CSF (100–200 μ L) was collected from cisterna magna. The CSF samples were centrifuged to check for RBC contamination. Samples with $>10 \mu$ L of pellet of RBCs were discarded. There was a significant difference between the two strains ($p = 0.0122$) but not between diets or time. (C) Effect of diet on ratios of CSF/serum leptin. The bars represent the mean \pm SE. There was a significant effects of strains ($p = 0.0388$) but not of diet ($p = 0.1651$) or time ($p = 0.2780$).

of strain [$F(1,44) = 73.49, p < 0.0001$], diet [$F(1,44) = 6.80, p = 0.0124$], and strain \times diet [$F(1,44) = 8.43, p = 0.0057$], but no effect of day [$F(1,44) = 0.32, p = 0.5749$]. OM rats had significantly higher serum leptin levels at both day 3 [$F(1,44) = 6.210859, p < 0.0001$] and day 15

[$F(1,44) = 5.924086, p < 0.0001$]. No diet effects were evident at day 3, but on day 15, OM rats eating the HF diet had higher leptin levels compared with OM rats eating the LF diet [$F(1,44) = 4.422481, p = 0.0015$].

The effects on CSF leptin levels of the HF and LF diet at days 3 and 15 are shown in Figure 2B. The ANOVA showed that OM rats had significantly higher CSF leptin levels compared with the S5B/P1 rats [$F(1,42) = 6.86, p = 0.0122$]. However, there were no statistical differences in relation to diet [$F(1,42) = 0.04, p = 0.8504$] or days of treatment [$F(1,42) = 0.00, p = 0.9988$].

The effect of the HF or LF diet on the ratios of CSF/serum leptin at days 3 and 15 is shown in Figure 2C. There was a significant effect of strain [$F(1,40) = 4.57, p = 0.0388$], but neither diet [$F(1,40) = 2.00, p = 0.1651$] nor time [$F(1,40) = 1.21, p = 0.2780$] showed any significant effects on the CSF/serum leptin ratio.

Experiment 3

The body weights of OM rats were significantly greater than those of the S5B/P1 rats. The effects of varying doses of leptin on food intake and body weight of OM and S5B/P1 rats are shown in Figure 3 for the 0.3- μg dose and as a dose-response curve in Figure 4. After treatment with the 0.3- $\mu\text{g}/\text{kg}$ dose of leptin, there was a clear divergence in the responses of the OM and S5B/P1 rats (Figure 3). There were day [$F(341,11) = 28.24, p < 0.0001$], strain [$F(30,1) = 13.92, p < 0.0008$], treatment \times day [$F(341,11) = 4.14, p < 0.0001$], strain \times day [$F(341,11) = 6.14, p < 0.0001$], and treatment \times strain \times day [$F(341,11) = 3.12, p < 0.0005$] effects on body weight at the doses of 0.3 μg leptin. Body weights and food intake of S5B/P1 rats were both decreased by leptin. In contrast, this dose of leptin did not have a significant effect on body weight of OM rats and reduced food intake only after 4 and 5 days of treatment (Figure 3).

Figure 4 shows the dose response of food intake and body weight of OM and S5B/P1 rats to leptin. The figure was constructed using the food intake and body weight data for the fifth and last day of treatment at each dose (e.g., Figure 3). The absolute value of the difference between vehicle-treated and leptin-treated animals was plotted. There is a clear dose response of food intake and body weight. The data for the S5B/P1 rats are shifted to the left of those for the OM rats, indicating enhanced sensitivity of response to leptin in the S5B/P1 rats. The statistical analysis of each of the other doses is described below, but the data are not shown because they are similar to the data in Figure 3.

At the initial dose of 1.0 μg of leptin ICV, there were significant effects of treatment [$F(37,1) = 11.48, p = 0.0017$], day [$F(37,1) = 51.52, p < 0.0001$], and strain \times day effect [$F(570,15) = 3.97, p < 0.0001$] on body weight. The body weights of both OM and S5B/P1 rats were re-

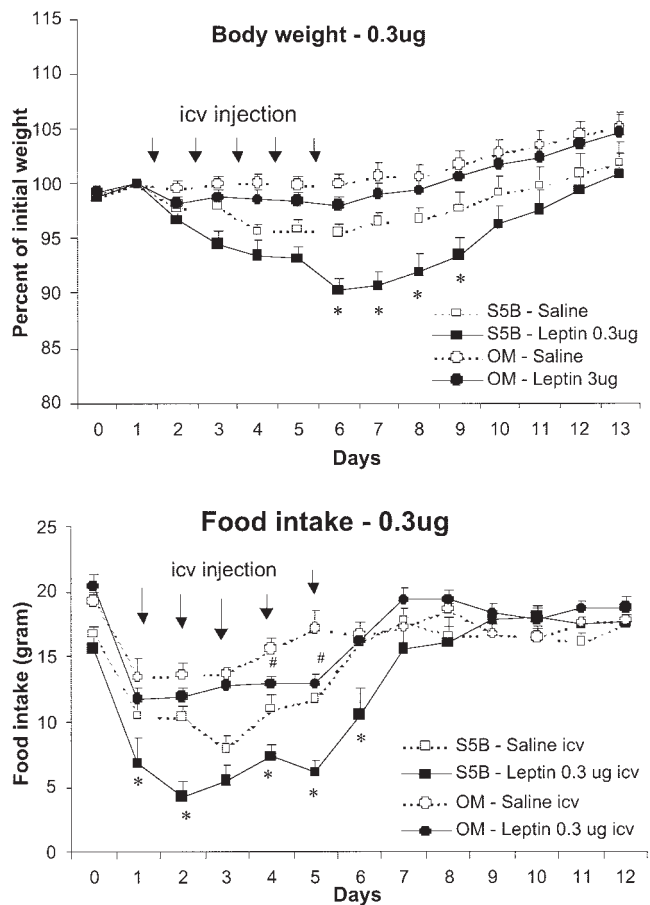


Figure 3: Effect of repeat ICV injections of 0.3 μg leptin on body weight and food intake of S5B/P1 and OM rats. Each point is the mean \pm SE for 5 to 7 animals/group. There is a larger decrease in food intake and body weight in the S5B/P1 rats than in the OM rats.

duced by leptin treatment (data shown only in dose response). There were day [$F(38,15) = 59.04, p < 0.0001$], strain [$F(38,1) = 29.38, p < 0.0001$], treatment \times day [$F(38,15) = 7.35, p < 0.0001$], and strain \times day [$F(38,15) = 3.75, p = 0.0005$] effects on food intake. Leptin-treated S5B/P1 rats had lower food intake than saline-treated S5B/P1 rats on days 1 to 5, but higher food intake on days 10 and 11 after cessation of the leptin treatment. Similar effects were observed in leptin-treated OM rats (data shown only in dose response).

The difference in sensitivity to ICV leptin between OM and S5B/P1 rats was most evidenced after the 0.1- μg dose of leptin (data shown only in dose response). There were day [$F(239,10) = 11.37, p < 0.0001$], strain [$F(24,1) = 8.90, p < 0.0065$], treatment \times day [$F(239,10) = 2.33, p < 0.0123$], strain \times day [$F(239,10) = 4.76, p < 0.0001$], and treatment \times strain \times day [$F(239,10) = 1.98, p < 0.0364$] effects on body weight. Leptin reduced the body weight of S5B/P1 rats but had no effect on OM rats. Similarly, there

Dose Response to Leptin

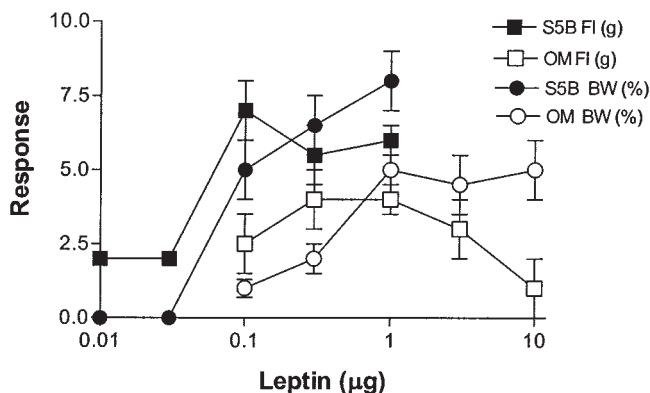


Figure 4: Dose response to leptin in OM and S5B/P1 rats. Each point is the difference between the absolute value of the vehicle-treated and the leptin-treated animals after the fifth injection of leptin.

were day [$F(238,10) = 10.55, p < 0.0001$], treatment \times day [$F(238,10) = 1.96, p = 0.0387$], strain \times day [$F(238,10) = 3.94, p < 0.0001$], and treatment \times strain \times day [$F(238,10) = 2.40, p < 0.0098$] effects on food intake. Leptin significantly reduced food intake of S5B/P1 rats but had no effect on OM rats (data shown only in dose response). Higher doses of leptin (3 and 10 $\mu\text{g}/\text{d}$) did, however, have significant effects on body weight and food intake in OM rats (data shown only in dose response), and the effects on body weight were prolonged after cessation of treatment with leptin. There was only a day effect for 0.01 and 0.03 μg ICV leptin-treated S5B/P1 rats on body weight [$F(180,9) = 13.02, p < 0.0001$] and food intake [$F(180,9) = 15.33, p < 0.0001$; data shown only in dose response].

Experiment 4

Food intake of the rats in Experiment 4 is shown in Figure 5. The S5B/P1 rats treated with leptin ICV and pair-fed to leptin-treated rats lost body weight compared with saline-treated rats on the third and fourth days. Compared with the day 1 weight, saline-treated rats lost 7.1% of their body weight, leptin-treated rats lost 12.1%, and the pair-fed rats lost 12.2%. For OM rats, the percentage of body weight loss compared with the first day of the experiment was 3.1% for saline-treated, 5.0% for leptin-treated, and 5.8% for pair-fed rats.

There were significant effects of treatment on serum hormone levels [$F(76,2) = 27.62, p < 0.0001$] and of treatment \times strain on serum leptin levels [OM rats: $F(76,2) = 11.58, p < 0.0001$; S5B/P1 rats: $F(76,2) = 16.3, p < 0.0001$]. OM rats treated with leptin had higher leptin levels than pair-fed ($p < 0.0001$) or saline-treated OM

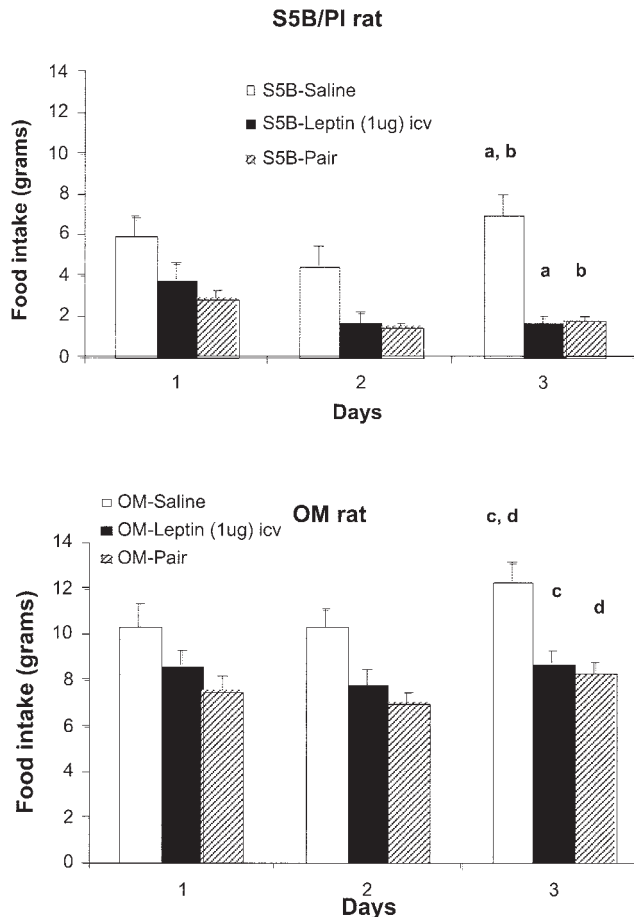


Figure 5: Comparison of the effect on food intake after consecutive ICV injections of leptin (1 μg) or saline compared to a group pair-fed to those receiving leptin. The data are presented as means \pm SE. Bars with the same letters are significantly different from one another ($p < 0.05$).

rats ($p = 0.002$). S5B/P1 rats treated with leptin also had higher leptin levels than pair-fed ($p < 0.0001$) and saline-treated S5B/P1 rats ($p < 0.0001$). In contrast to leptin, there were no significant effects of strain, treatment, or strain \times treatment interactions on plasma corticosterone. For insulin, there were significant effects of strain [$F(76,1) = 10.30, p = 0.002$] and treatment by strain [$F(76,1) = 0.952, p = 0.0028$]. OM rats treated with leptin had higher insulin levels than leptin-treated S5B/P1 rats ($p = 0.032$).

The levels of STAT-3 and SOCS-3 mRNA in relation to CYC mRNA in the hypothalamus of OM and S5B/P1 rats are shown in Figure 6. OM rats had higher STAT-3/CYC mRNA ratios than S5B/P1 rats, but these were not increased by leptin. In contrast, leptin increased the STAT-3/CYC mRNA ratio in the hypothalamus of S5B/P1 rats by $>70\%$. Pair-feeding had no effect in OM or S5B/P1 rats. The variance in assays of SOCS-3 mRNA was large, and no significant effects of strain or treatment were observed (Figure 6).

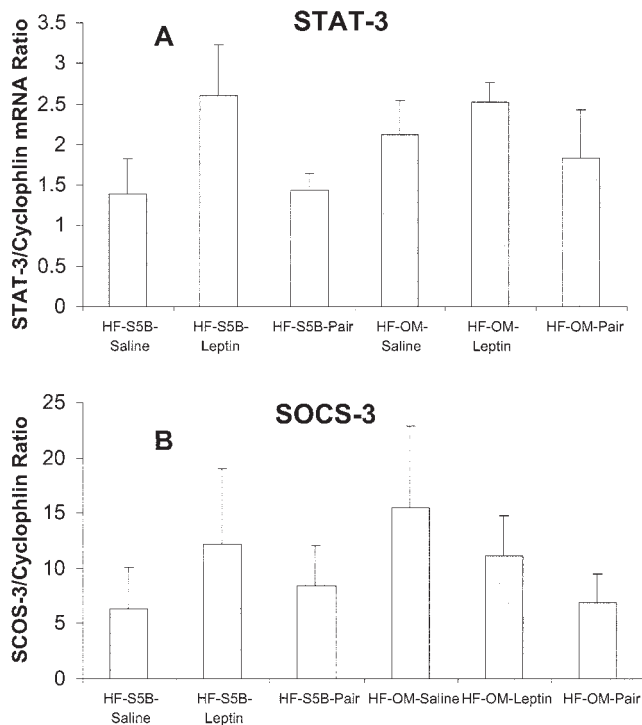


Figure 6: (A) Hypothalamic STAT-3 total RNA to cyclophilin RNA ratio. The data are presented as means \pm SE. (B) Hypothalamic SOCS-3 total RNA to cyclophilin RNA ratio. The data are presented as means \pm SE.

Discussion

These experiments, for the first time, directly measured both the CSF and serum leptin values in two strains of rats that differ in their susceptibility to obesity and have shown that the resistant (S5B/P1) rats had greater CSF/serum leptin levels than the rats susceptible to obesity (OM). The diurnal variation in serum leptin and insulin levels was also greater in the S5B/P1 rats than in the OM rats. Although the peak values of leptin and insulin were similar in the two strains of rats, the diurnal excursion of leptin and insulin in the OM rats was only one-half that of the S5B/P1 rats. Because the OM rats have more body fat than the S5B/P1 rats, this difference in excursion might be a reflection of their difference in fatness.

Human subjects also have a diurnal rhythm for leptin and insulin and a pulsatile relationship within defined peaks. The average circadian amplitude of leptin between zenith and nadir was 75.6% in lean human subjects, 51.7% in obese subjects, and 60.7% in obese subjects with type 2 diabetes (13), and there is also dampened pulsatility (14) in the obese. HF diets have also been reported to reduce the diurnal variation (15), but the animals studied in our experiments were eating chow diet. Alternatively, it is possible that the attenuation in the diurnal cycle of leptin in OM rats might contribute to their sensitivity to HF diets, on which they rapidly become obese.

The factors that regulate the diurnal rhythm of leptin have been studied in both humans and animals (16–18). In vitro, corticosteroids have been shown to up-regulate leptin mRNA levels in adipocytes (19,20). In humans, there is a strong negative correlation between plasma leptin levels and both cortisol levels and adrenocorticotropic hormone levels in humans (21), although administration of glucocorticoids increases leptin levels as it does in animals (22,23). Pathological elevation of corticosterone, as seen in patients with Cushing's syndrome, is also associated with elevated leptin levels but normal diurnal rhythm (24–27), but it is not clear whether this is the result of high glucocorticoid levels or increased visceral fat (28,29). However, in patients with adrenal insufficiency, modulation of corticosteroids within physiological levels does not alter leptin levels or its diurnal pattern (30). Furthermore, in patients with anorexia nervosa, the diurnal variation of leptin is absent, although the cortisol rhythm is preserved. These data, together with the observation that corticosterone levels were similar throughout the day in the OM and S5B/P1 rats, suggest that adrenal steroids are not responsible for the differences in maximal diurnal excursion of leptin.

Insulin also regulates leptin synthesis and secretion in vivo and in cell culture (20,31–34). Hyperinsulinemia in humans increases serum leptin levels dose-dependently, and during euglycemic clamp, there is a dose relationship between insulin and leptin, provided the clamp is of sufficient duration (35). The plasma leptin level can be shifted acutely by changing the timing of meals, whereas plasma cortisol is not affected by meal pattern (36). In physiological conditions, acute changes in either cortisol or insulin levels do not immediately change the leptin levels, but these hormones do seem to modulate leptin levels over time.

Several studies support the hypothesis that there is reduced access of leptin to the brain in obesity. The CSF-to-serum ratio of leptin may be decreased in obese humans, providing support for this idea (1,2). Peripheral infusion of leptin has not been shown to decrease body weight of New Zealand obese mice, a polygenic form of obesity, or in the yellow (A^y) mouse, which has a single mutation in the promoter of the *agouti* gene. Decreased leptin transport across the BBB has also been observed in obese CD-1 mice (3) and in Koletsky rats that have a point mutation on the short form of the leptin receptor (5). Likewise, rats with lesions in the paraventricular nucleus or in the posterior-dorsal amygdala have decreased leptin uptake across the BBB (37). Interestingly, normal transport of leptin is observed in *ob/ob* and *db/db* mutant mice (38). This study showed that the CSF-to-plasma leptin ratio was lower in the fatter OM rats, which is consistent with the idea of reduced blood-brain transport associated with increased body fat. Although the introduction of an HF diet is associated with a rapid loss of the response to peripheral leptin (39), we did not observe any diet-dependent changes in the CSF/serum

leptin ratio, suggesting that mechanisms other than the BBB must also be involved in HF diet-related leptin resistance. Furthermore, the transport of leptin into the brain seems to be independent of circulating leptin levels (40).

There are regional variations in uptake of leptin across the BBB (41,42). At lower concentrations (1 ng/mL), the transport across the BBB is fastest in the hypothalamus, whereas at higher (30 ng/mL) concentrations, the hippocampus, cerebellum, midbrain, pons-medulla, or occipital cortex has greater transport than the hypothalamus (42). This suggests that different regions of the brain may have different thresholds for access to leptin. Whether such differences between OM and S5B/P1 rats could account for the apparent differences in CSF-to-plasma leptin ratios remains to be explored.

Our previous studies have shown that OM and S5B/P1 rats have a similar response to high doses of leptin given ICV (7). However, in this study, we extended these observations to show that S5B/P1 rats were more responsive to lower doses of leptin, suggesting a rightward shift in the dose-response curve in OM rats. This suggests that mechanisms other than transport into the brain are involved in the resistance to leptin in OM rats. Leptin acts on receptors that belong to the cytokine superfamily. The activation of the JAK-STAT pathway is modulated through the long form (Rb) receptor that is widely distributed in the hypothalamus. This signaling pathway is inhibited by *SOCS-3*, a gene that is also induced by leptin. In the yellow obese (A^{vy}) mouse, a model of leptin-resistant obesity, *SOCS-3* mRNA levels are high, which would lead to more *SOCS-3* and inhibit the activation of leptin-signaling pathways.

We have previously shown that the prevention of obesity that follows adrenalectomy is associated with a constitutive activation of the JAK-STAT pathway and a down-regulation of *SOCS-3* signaling (6). We also have shown that the levels of the long form receptor protein (Rb) were reduced in the hypothalamus of OM rats, although mRNA levels were increased (43). The data in this manuscript extend these observations to show a functional impairment in the leptin signaling of OM rats, because the inducer of *STAT3* mRNA by leptin was attenuated. This impaired response may be a reflection of the reduced receptor protein level. In contrast, we were unable to show any differences in *SOCS-3* mRNA between OM and S5B/P1 rats or with leptin treatment. This may reflect the time of death of the animals or the fact that the whole hypothalamic RNA were sampled rather than just the arcuate nucleus-paraventricular nucleus areas and other circuits that regulate energy balance.

ICV injection is an experimental method of delivering agents to the brain and by-passing transport across the BBB. After intravenous injection of leptin, ICV levels reach their peak by 20 minutes (44). Our study indicated that 2 hours after ICV injection of leptin, serum leptin levels increased by ~9.2-fold in S5B/P1 rats and 3.7-fold in OM rats com-

pared with those in saline-treated control rats. This indicated that a large amount of the ICV leptin leaks into the serum compartment from the ventricles and that this leptin might initiate peripheral signaling. This observation suggests the need for caution in interpreting the response to centrally administered leptin.

Acknowledgments

This work was supported, in part, by NIDDK Grant 32,089. We thank Julia Volaufova and Silvia Morris for statistical analysis.

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