Rapid Increase in Circulating Leptin in Ventromedial Hypothalamus-Lesioned Rats

Role of Hyperinsulinemia and Implication for Upregulation Mechanism

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The mechanisms of marked increase in plasma leptin soon after ventromedial hypothalamus (VMH) lesions were investigated. Although rats did not gain body weight or parametrial fat-pad mass 24 h after the operation, the acute VMH-lesioned rats exhibited substantial five- and fourfold increases in plasma leptin levels compared with sham-operated control rats in fed (22.6 ± 3.2 vs. 5.8 ± 1.2 ng/ml) and fasted (8.8 ± 2.0 vs. 2.3 ± 0.3 ng/ml) states, respectively. Plasma insulin concentration was doubled in VMH-lesioned rats compared with sham-operated controls in both fed and fasting states. Northern blot analysis revealed that mRNA of *ob* gene was not increased in parametrial fat pad of animals 24 h after the creation of VMH lesions. However, leptin content in the fat pad was significantly increased in VMH-lesioned rats compared with sham-operated controls (32.2 ± 4.7 vs. 17.4 ± 2.3 ng/g wet tissue). The leptin content in parametrial fat pad was highly correlated with plasma leptin concentrations ($r = 0.898$, $P < 0.001$). To define the effect of hyperinsulinemia on their hyperleptinemia, a small dose of streptozotocin (STZ) (25 mg/kg body wt) was intravenously administered into rats 5 days before the creation of VMH lesions. Plasma insulin levels were not increased after VMH lesions in STZ-pretreated rats. Plasma leptin levels were halved in the absence of hyperinsulinemia, but still remained twofold higher than those in their sham-operated counterparts (9.9 ± 1.3 vs. 4.8 ± 0.7 ng/ml). These results indicate that the destruction of VMH rapidly promotes leptin production before obesity develops through an enhanced translational process in which hyperinsulinemia occurring after VMH lesioning plays an important role. The present study also suggests that there are other mechanisms that rapidly upregulate leptin production in adipocytes in VMH-lesioned rats in which the target organ of this hormone has been destroyed. *Diabetes* 48:2034–2038, 1999

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SSC, sodium chloride–sodium citrate; STZ, streptozotocin; VMH, ventromedial hypothalamus.

Leptin, the peptide encoded by the obesity gene, is secreted by adipose cells and plays an important role in regulating food intake, energy expenditure, and adiposity (1,2). The weight-reduction action of leptin is thought to be mediated primarily by signal transduction through the leptin receptor in the hypothalamus (2). Bilateral lesions of the ventromedial nuclei in the hypothalamus (VMH) can produce obesity in animals, and lesioned rats have been widely used as a representative animal model of obesity (3,4). VMH-lesioned rats have a significant hyperphagia in spite of substantially increased leptin levels (5,6), indicating that a key target for the biological actions of leptin is destroyed by the creation of VMH lesions (7). It is generally accepted that plasma leptin concentrations well reflect the amount of adipose tissue in the whole body (2,8,9) and that an increase in adiposity is the primary cause of hyperleptinemia in obesity, including VMH lesion–induced obese animals (5).

In preliminary studies, we unexpectedly found that plasma leptin levels in rats were remarkably elevated only 24 h after the creation of VMH lesions before obesity developed. This observation led us to speculate that there is a yet unidentified mechanism that increases plasma leptin levels in VMH-lesioned rats, independent of adiposity. A number of clinical and experimental studies have shown that insulin directly increases leptin production (10,11). On the other hand, it has been reported that insulin levels are substantially elevated in VMH-lesioned rats (3,4). Therefore, the hyperinsulinemia following the creation of VMH lesions might be involved in a mechanism for stimulating leptin production. In this study, we tried to explore the mechanism that rapidly increases plasma leptin concentration where the target organ of leptin is destroyed in VMH-lesioned rats.

RESEARCH DESIGN AND METHODS

Female Sprague-Dawley rats (Japan SCL, Hamamatsu, Japan) were kept in individual cages on a rotating 12-h light-dark cycle with free access to both standard rat diet (Oriental Food, Tokyo) and water. Operations for VMH lesions were carried out at 2:00–3:00 P.M. The animals were anesthetized by inhalation of Isoflurane (Forane; Dainabot, Osaka, Japan), and electrolytic bilateral VMH lesions were produced by the method previously described (12). Briefly, the stereotaxic coordinates for electrode placement were determined with reference to the interaural line and bregma. The top of the upper incisor bar was 3.0 mm above the interaural line. The coordinates for VMH lesioning were 2.8 mm posterior to the bregma, 0.6 mm lateral to the midsagittal line, and 0.5 mm dorsal from the base of the skull, according to the atlas of Paxinos and Watson (13). An anodal current

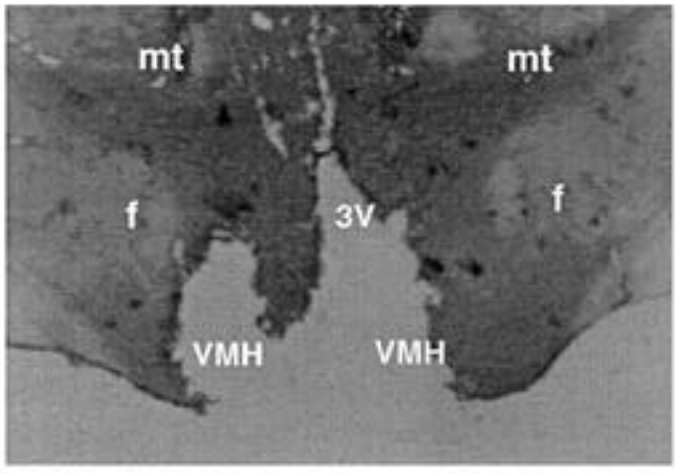

FIG. 1. A typical picture of a VMH lesion in rats. f, fornix; mt, mammillothalamic tract; 3V, third ventricle.

of 2 mA was passed for 20 s through a stainless-steel needle insulated with epoxylite except for 0.5 mm at the top. Control animals received sham VMH lesions (no current passed through the electrode). At the end of the experiments, the area of the hypothalamic lesions in all animals was determined histologically by cresyl-violet staining. A typical picture of VMH lesions is shown in Fig. 1. VMH-lesioned and sham-operated rats were fed normal rat diet ad libitum (fed state) or deprived of food (fasted state) after the operations. On the following day, blood samples were taken from the abdominal aorta under pentobarbital anesthesia (50 mg/kg body wt i.p.) at 2:00–3:00 P.M. (just 24 h after the operation). The parametrial fat pads were removed, weighed, immediately frozen in liquid nitrogen, and stored at –80°C until use. In streptozotocin (STZ)-pretreated rats, a small dose of STZ (25 mg/kg body wt) was dissolved in citrate buffer and administered into rats via the tail vein 5 days before the VMH operation. These rats showed normal insulin levels at baseline, but no increased insulin response to intravenous glucose administration (1 g/kg body wt) (data not shown). These STZ-pretreated rats underwent VMH lesions or sham operation, a blood sample was taken, and parametrial fat pads were removed 24 h after the operations in a fed state.

Northern blot analysis for abundance of *ob* mRNA. Total RNA was prepared from the tissue of each rat using Trizol reagent (Gibco BRL, Tokyo). The concentration of RNA was determined spectrophotometrically at 260 nm. RNA was loaded at 15 µg per lane on a 1% agarose/formaldehyde gel and transferred onto a nylon membrane (Hybond N+; Amersham, Arlington Heights, IL). Membranes were hybridized with [α-32P]dCTP-labeled cDNA probe. The probe was labeled with [α-32P]dCTP using the Megaprime DNA labeling system (Amersham). Prehybridization and hybridization were carried out overnight at 42°C. Membrane was washed twice in a solution of 2 × sodium chloride–sodium citrate (SSC) and 0.2% sodium pyrophosphate with 1% SDS at 65°C for 30 min, and then washed in 0.2 × SSC with 0.1% SDS for 15 min at ambient temperature. The blot was exposed to X-ray film (Fuji Photo Film, Tokyo) at –80°C using an intensifying screen. The signals were quantified with FUJIX Bio-Imaging Analyzer BAS2000 (Fuji). Equivalent loading was verified by the density of the methylene blue–stained rRNA bands after transfer.

Cloning of *ob* cDNA fragment using reverse transcriptase–polymerase chain reaction. Total RNA (0.8 µg) from Sprague-Dawley rat white adipose tissue was reverse-transcripted by random priming with Moloney murine leukemia virus reverse transcriptase (Gibco BRL), and then the first-strand cDNA was used for polymerase chain reaction using specific oligonucleotide primers (5′-GTCCAAGAAGAAGAAGACCCCA-3′ and 5′-GGAGCCAAGGTTTCTTCCCT-3′, Genbank accession no. D45862). The resulting 592-bp fragment was cloned into TA-cloning vector, pGEM-T Easy (Promega, Madison, WI). The nucleotide sequence of the *ob* insert in pGEM was confirmed by double-strand sequencing.

Measurement of leptin content in parametrial fat pads. Leptin content in white adipose tissues was measured by the method of Bado et al. (14). Briefly, frozen parametrial fat pads (1.0 g) were completely homogenized in Krebs-Ringer phosphate buffer (HEPES-KRP, 5 ml) consisting of 50 mmol/l HEPES, 100 mmol/l NaCl, 5 mmol/l KCl, and 1 mmol/l each of $MgCl_2$, NaH_2PO and $CaCl_2$ (pH 7.4) using a homogenizer (PT-MR 3100; Polytron, Littau, Switzerland). The homogenates were centrifuged at 4,000*g* for 3 min to remove the lipid phase. The resulting infranatant (3.0 ml) was centrifuged at 15,000*g* for 60 min at 4°C and then the resulting supernatant (1.0 ml) was used for leptin and protein assays.

Other measurements. Plasma insulin concentrations were determined by a radioimmunoassay kit (Cat#RI-13K; Linco, St. Charles, MO) standardized against rat insulin. Plasma leptin concentrations were determined by a radioimmunoassay kit (Cat#RL-83K; Linco) for specifically determining rat leptin. Protein in adipose tissue was determined by the Lowry method (15).

Analysis. Data are expressed as means ± SE. Statistical significance was assessed by Student's *t* test (for two groups) or one-way analysis of variance (for more than two groups), and $P < 0.05$ was accepted as a significant difference.

RESULTS

Table 1 shows body weight before (initial) and 24 h after (final) the operation, body weight gain over 24 h, parametrial fat-pad weight, food intake for 24 h, and plasma glucose levels 24 h after the operation. Initial body weight was comparable between VMH-lesioned and sham-operated rats in the fed state. Although the food intake of animals with bilateral VMH lesions was increased twofold over that of sham-operated rats, the lesioned animals showed no body weight gain. Final body weight and parametrial fat-pad weight were comparable between VMH-lesioned and sham-operated rats in the fed state. Under the 24-h fasted state, the body weight of both VMH-lesioned and sham-operated rats significantly declined. There were no significant differences in final body weight or parametrial fat-pad mass between these two groups.

Plasma leptin and insulin levels in VMH-lesioned and sham-operated rats are depicted in Fig. 2. Plasma insulin was increased by twofold and leptin by fourfold in the lesioned rats, compared with control animals, in the fed state (3.2 ± 0.5 vs. 1.5 ± 0.4 ng/ml for insulin and 22.6 ± 3.2 vs. 5.8 ± 1.2 ng/ml for leptin, respectively). Plasma insulin levels in both control

TABLE 1
Body weight, parametrial fat-pad weight, food intake, and plasma glucose levels

	Sham	VMH	Sham + fast	VMH + fast	Sham + STZ	VMH + STZ
n	5	5	3	5	3	3
Initial body weight (g)	284 ± 9	277 ± 4	280 ± 14	283 ± 5	270 ± 5	279 ± 3
Final body weight (g)	280 ± 10	275 ± 4	264 ± 14	264 ± 5	261 ± 7	275 ± 5
Body weight gain (g/day)	–3.8 ± 2.2	–2.2 ± 3.4	–16.3 ± 1.2	–19.8 ± 1.4	–9 ± 2.6	–4.3 ± 6.6
Fat-pad weight (g)	4.8 ± 0.6	5.1 ± 0.6	4.4 ± 0.6	5.0 ± 0.4	4.5 ± 0.7	3.8 ± 0.3
Food intake (g/day)	11.5 ± 0.9	28.8 ± 1.8*	0	0	11.4 ± 0.7	24.7 ± 2.1*
Glucose (mmol/l)	8.08 ± 0.38	7.52 ± 0.30	6.57 ± 0.76	5.48 ± 0.62	10.27 ± 1.67	9.30 ± 1.41

Data are means ± SE. Body weight was measured before (initial) and 24 h after (final) the creation of bilateral VMH lesions, food intake for 24 h after the lesions, and plasma glucose levels at 24 h post-operation. Fast, fasting state for 24 h after the operation; Sham, sham-operated control rats; STZ, STZ (25 mg/kg body wt)-injected rats 5 days before the VMH operation; VMH, VMH-lesioned rats. *$P < 0.05$ vs. sham-operated rats.

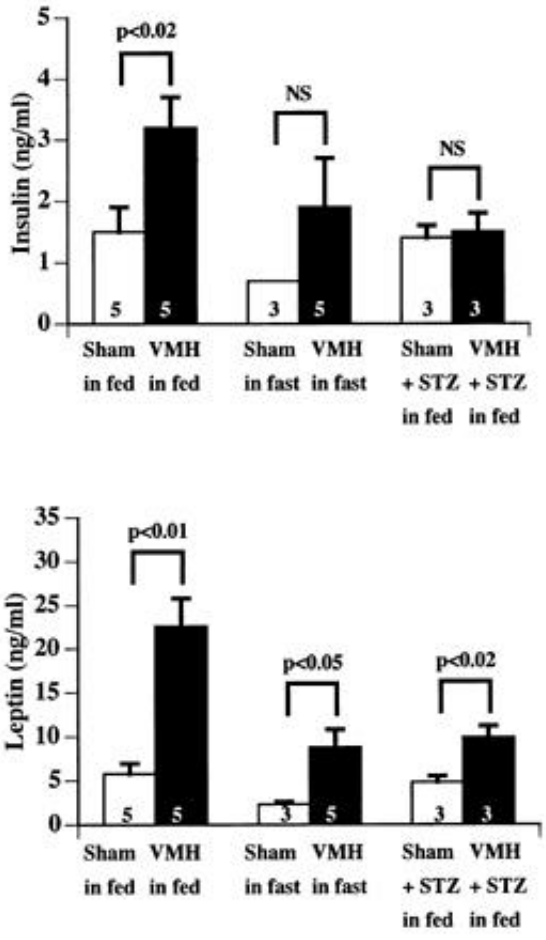

FIG. 2. Changes in plasma immunoreactive insulin and leptin concentrations in sham-operated and VMH-lesioned rats.

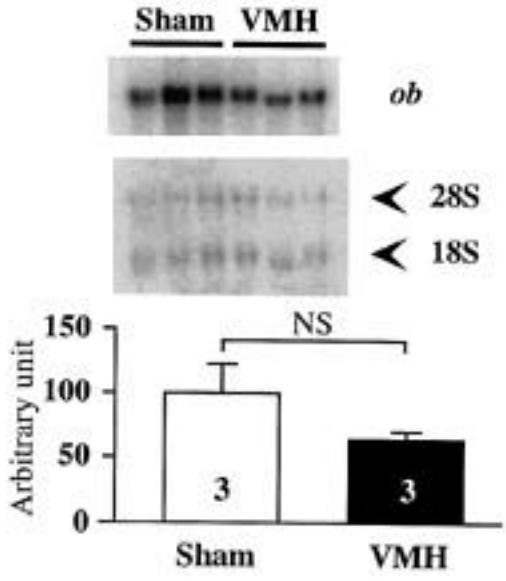

FIG. 3. Northern blot analysis for abundance of *ob* mRNA in parametrial fat pads of sham-operated and VMH-lesioned rats and quantification of the relative percent change of the lesioned rats. The lower blot shows the 18S and 28S ribosomal RNA bands as visualized by methylene blue staining.

and VMH-lesioned rats in the fasted state were half of those in control and VMH-lesioned rats in the fed state. Fasted VMH-lesioned rats still had higher insulin levels than did fasted controls (1.9 ± 0.8 vs. 0.7 ± 0.0 ng/ml), although this did not attain a statistical significance, probably because of the small number of animals. Plasma leptin levels in both lesioned and sham-operated rats in the fasted state were half of those in the fed state; however, the levels in fasted animals were still fourfold greater in VMH-lesioned rats than in sham-operated rats (8.8 ± 2.0 vs. 2.3 ± 0.3 ng/ml). Pretreatment of STZ did not affect body weight gain or food intake before the VMH operation; thus, initial body weight was comparable among groups (Table 1). Body weight did not rise after the operations in either group of rats, but rather decreased, and the parametrial fad-pad weight was comparable between STZ-pretreated VMH-lesioned rats and their sham-operated counterparts (Table 1). The STZ pretreatment did not affect food intake in either lesioned or sham-operated rats after the operations, but slightly raised plasma glucose levels in both groups (Table 1). Plasma insulin levels in VMH-lesioned rats pretreated with STZ were not increased, but were similar to those in sham-operated rats pretreated with STZ (Fig. 2). STZ pretreatment halved plasma leptin levels in the lesioned rats (22.6 ± 3.2 to 9.9 ± 1.3 ng/ml); however, the levels in these rats were still twofold higher than those in their sham-operated counterparts (4.8 ± 0.7 ng/ml). There was a substantial positive correlation between plasma leptin and insulin levels in all rats ($r = 0.66$, $P < 0.0005$).

Northern blot analysis of *ob* gene expression in parametrial white adipose tissue is depicted in Fig. 3. Abundance of *ob* gene in the parametrial fat tissue was similar between VMH-lesioned and sham-operated rats 24 h after the creation of VMH lesions in the fed state. Although VMH-lesioned rats did not exhibit *ob* gene overexpression, they had twofold higher leptin content in parametrial adipose tissue expressed as either leptin content per gram of fat tissue or per milligram of protein (Fig. 4*A* and *B*). There was a very close correlation between plasma leptin concentration and leptin content in the adipose tissue (Fig. 4*C* and *D*).

DISCUSSION

We demonstrated in this study that plasma leptin concentration was increased four- to fivefold only 24 h after the creation of VMH lesions in rats. Satoh et al. (16) have already demonstrated that plasma leptin levels were 2.1-fold increased in VMH-lesioned rats 1 day after the lesions; however, they did not observe an increase in *ob* gene expression, nor did they measure leptin content in adipose tissues. Our VMH-lesioned rats had much higher plasma leptin levels (22 ng/ml) than those in Satoh's study (8 ng/ml). Nonetheless, we also failed to observe an increased abundance of *ob* gene expression in the VMH-lesioned rats. There are two steps in protein synthesis: transcription and translation. The former refers to gene expression; thus, the transcription process was not enhanced in VMH-lesioned rats. To explore mechanisms of the hyperleptinemia, we attempted to measure leptin content in adipose tissue, and found that the tissue content of leptin was remarkably increased in the VMH-lesioned rats and that there was a close correlation between leptin content and circulating leptin. The results suggest that the translational process of leptin synthesis is exclusively enhanced in the acute VMH-lesioned rats.

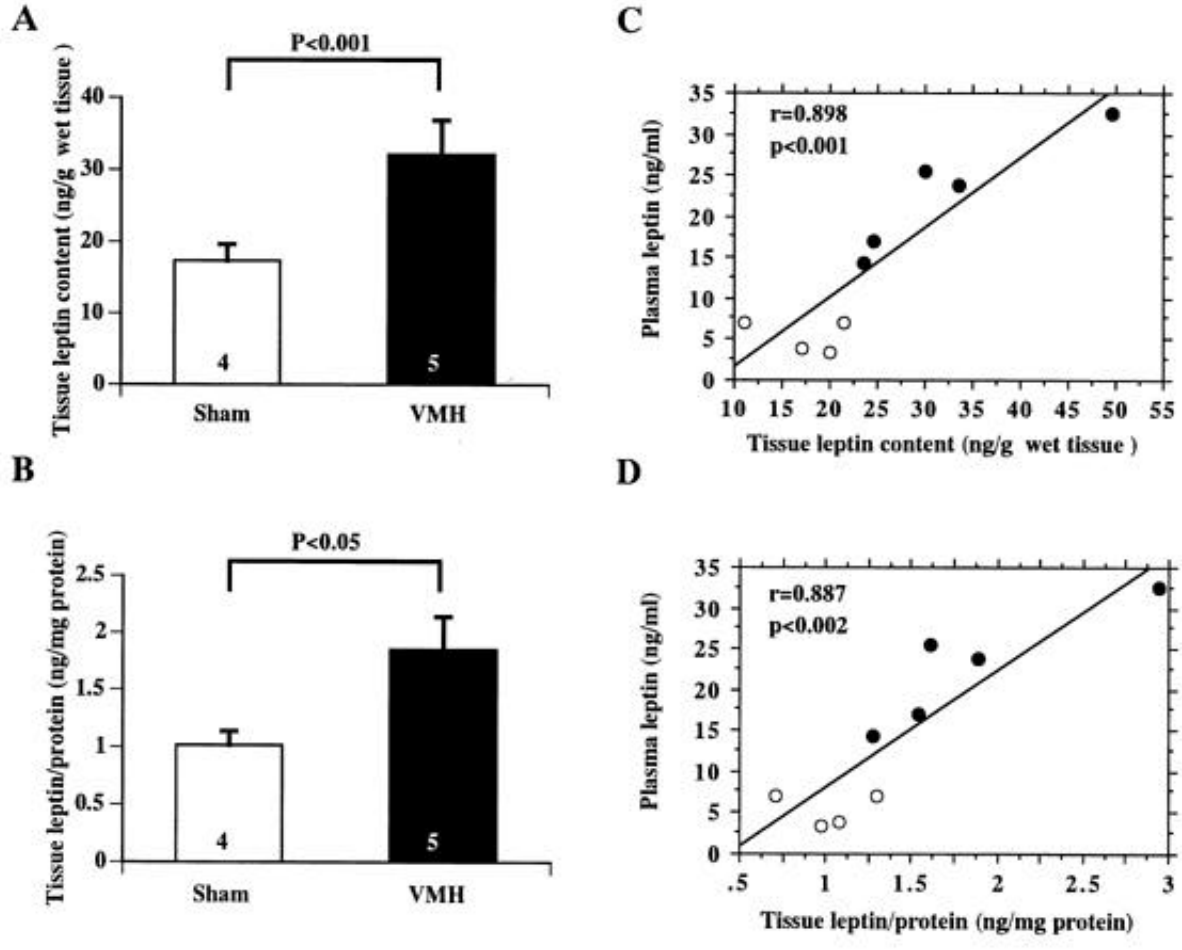

FIG. 4. Leptin contents in parametrial fat pads of sham-operated and VMH-lesioned rats. Leptin contents are expressed as nanograms per gram of wet tissue (*A*) or nanograms per milligram of protein (*B*). The correlations between plasma leptin concentration and leptin contents in parametrial fat pads are expressed by nanograms per gram of wet tissue (*C*) or nanograms per milligram of protein (*D*). ○, sham-operated rats; ●, VMH-lesioned rats.

What caused the marked increase in plasma leptin soon after VMH lesions? There are reportedly many factors that stimulate leptin production (2), of which hyperphagia, increased body fat, and hyperinsulinemia may be potential candidates in VMH-lesioned rats. Hyperphagia and an increase in body fat, which reflect weight gain, were soon manifested in the VMH-lesioned rats; however, only 1 day of overfeeding seems to be insufficient to gain significant body weight. In fact, their body weights were probably decreased after operation in the present study because of stress or surgical invasion during the operations. Especially in the fasted state, body weight was decreased in VMH-lesioned rats. Despite the lack of body weight gain in both fasted and fed states and the lack of hyperphagia in the fasted state, plasma leptin concentrations were always higher in VMH-lesioned rats than in sham-operated controls. These results suggest that the destruction of the VMH can stimulate leptin production even in the absence of increased adiposity or hyperphagia.

A number of clinical and experimental studies have demonstrated that insulin stimulates the expression of the *ob* gene in adipocytes, thereby increasing plasma leptin levels (2,11,17). It has been recognized that hyperinsulinemia plays an obligatory role in developing obesity in VMH-lesioned rats (18). In this study, we observed that plasma insulin levels were significantly elevated in rats only 24 h after the creation of VMH lesions, when obesity had not yet developed. Therefore, it is reasonable to assume that hyperinsulinemia following VMH lesions stimulates leptin production. To confirm whether hyperinsulinemia is involved in the mechanism for hyperleptinemia associated with VMH-lesioned rats, we injected a small dose of STZ before VMH operation to suppress insulin response to the creation of the lesions, and we found that the lack of hyperinsulinemia resulted in significantly decreased plasma leptin levels in these animals. In preliminary experiments, we found that plasma insulin levels were not significantly altered 2, 6, and 12 h after the lesions but were remarkably elevated at 24 h (A.S., T.H., H.K., S.I., unpublished observations). Therefore, it is unlikely that insulin levels in STZ-pretreated VMH-lesioned rats had been elevated at some time during the preceding 24 h. We also observed a marked reduction in plasma leptin levels concomitant with decreased insulin levels in VMH-lesioned rats after 24 h of fasting as described above. Taken together, there is no doubt that hyperinsulinemia following VMH lesions plays an important role in the hyperleptinemia in VMH-lesioned rats. However, plasma leptin remained at higher lev-

els in the STZ-pretreated lesioned rats even in the absence of hyperinsulinemia, suggesting that there is another mechanism that rapidly stimulates leptin production in these animals, independent of hyperinsulinemia. It has been proposed that hyperinsulinemia following a VMH lesion was an important factor in hyperphagia (3). In the present study, STZ-pretreated VMH-lesioned rats were hyperphagic despite the lack of hyperinsulinemia. However, Inoue et al. (18) previously demonstrated that even without hyperinsulinemia, VMH-lesioned rats increased food intake by 20%, which may be attributed to derangement of the autonomic nervous system by a VMH lesion. It is well known that STZ-induced diabetic rats are hyperphagic. Therefore, both derangement of the autonomic nervous system and STZ-induced hyperglycemia might make VMH-STZ rats hyperphagic even in the absence of hyperinsulinemia.

Several studies have shown that leptin receptors are localized in the arcuate nucleus and the VMH at high density (19). However, it seems unlikely that the destruction of the VMH impairs clearance of circulating leptin. Leptin receptors distribute not only in the central nervous system, but also in various peripheral tissues (20,21). Kinetic studies have demonstrated that circulating plasma leptin is rapidly cleared by peripheral organs such as kidney (22). Vila et al. (23) recently reported that the clearance of injected radiolabeled leptin was almost identical between Zucker fatty rats and their lean counterparts, although the former had marked hyperleptinemia and a defect of the leptin receptor; this suggests that plasma leptin concentration is little affected by its clearance. Plasma leptin and leptin content in the adipose tissue were remarkably increased, with high correlation between plasma leptin and adipose leptin content in VMH-lesioned rats in this study. Therefore, at present, hyperleptinemia in VMH-lesioned rats would be attributable to increased production in adipose tissue and less to impaired clearance.

In conclusion, VMH lesions immediately stimulate the translational process of leptin synthesis in adipose tissue. Hyperinsulinemia plays an important role in this process, and there may be other factors that also promote it. Further studies are required to identify putative factors other than hyperinsulinemia that stimulate leptin production immediately after the destruction of the key target site of this hormone.

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