Intracerebroventricular Administration of Insulin and Glucose Inhibits the Anorectic Action of Leptin in Rats

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Obese individuals with glucose intolerance present with high serum levels of glucose, insulin, and leptin. These substances are potent inhibitors of feeding in the brain. Obese subjects still present with over-feeding despite elevation of the above factors. To elucidate the mechanism of this paradox, the effects of insulin and glucose on the anorectic action of leptin in the hypothalamus were examined. Adult male Sprague-Dawley rats (weighing 285–320 g) were pretreated with intracerebroventricular injection of insulin, glucose, or saline, followed by leptin (7.5 μg) or phosphate-buffered saline (PBS) injection into the third cerebral ventricle (icv). The cumulative food intakes were measured 24 hr after leptin icv. The tyrosine phosphorylation of signal transducer and activator transcription factor 3 (STAT3) in the hypothalamus was determined by Western blotting. In rats pretreated with saline and stimulated with leptin (saline/LEPTIN group), food intake diminished to about 50% of that of the saline/PBS group ($P < 0.005$). Food intake in the insulin/LEPTIN group was significantly higher compared with the saline/LEPTIN group ($P < 0.005$) and reached the level seen in the saline/PBS group. Similar data were obtained in glucose pretreatment experiments. Insulin and glucose icv resulted in reduction of leptin-induced STAT3 tyrosine phosphorylation compared with saline. Infusion of insulin and glucose icv did not alter peripheral blood glucose levels in all groups. High insulin or glucose levels in the brain could result in leptin resistance as manifested by food intake, which is probably due to the attenuation of STAT3 phosphorylation downstream the leptin receptor. Exp Biol Med 228:1156–1161, 2003

Key words: insulin; glucose; leptin; hyperphagia

Common features of obese people and animals with glucose intolerance are hyperinsulinemia, hyperglycemia, and hyperleptinemia (1–3). It has been reported that insulin leads to a decrease in food intake and an increase in sympathetic activity mediated by the hypothalamus as well as leptin (4, 5). A deficiency of insulin or insulin signal in the brain causes hyperphagia and body weight gain in rodents (6, 7). Glucose also has a satiety effect mediated by the hypothalamus (8, 9). Leptin secreted from adipocytes strongly reduces food intake via its hypothalamic action in animals (10, 11). These potent inhibitory effectors have been shown to be at a higher level in obese people, yet these individuals paradoxically present with over-feeding.

Dunbar and Lu (12) demonstrated that long-term insulin administration into the brain attenuated the leptin-induced increase in sympathetic activity in rats (12). However, there are no studies on the effect of insulin on the anorectic action of leptin. In this regard, previous studies showed that the Janus kinase/signal transducer and transcription activator (JAK/STAT) is a major signaling pathway in leptin signal transduction in the hypothalamus (13, 14). Furthermore, in an *in vitro* study, insulin was reported to interfere with leptin receptor signaling at the level of Janus kinase-2 (JAK-2), indicating that hyperinsulinemia contributes to the pathogenesis of leptin resistance (15). Wang *et al.* (16) demonstrated that voluntary hyperphagia induced leptin resistance coupled with insulin resistance in a few days in rats. However, it remains unclear whether the rise in insulin or glucose level in the hypothalamus is the real stimulus that modulates the leptin signaling pathway and consequently its anorectic action *in vivo*.

To elucidate the interactions between insulin, glucose, and leptin in the brain (i.e., the mechanism of the aforementioned paradoxical phenomenon), we assessed the separate effects of intracerebroventricular administration of insulin and glucose on the anorectic action of leptin in the hypothalamus in normal rats. In addition, we used Western

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blotting to analyze tyrosine phosphorylation of STAT3 in the hypothalamus, which is downstream of the leptin signal transduction. We also investigated the role of high levels of insulin and/or glucose in the hypothalamus as primary pathogenic factors of obesity.

Materials and Methods

Experimental Protocol. Adult male Sprague-Dawley rats weighing 285 to 320 g obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were housed at 22°C in a 12:12-hr light:dark cycle environment (light on at 0700, off at 1900), and provided with standard laboratory diet (Oriental Yeast, Tokyo) and tap water *ad libitum*. The experimental protocol was approved by the Animal Care and Use Committee at our institution.

Under pentobarbital anesthesia (40 mg/kg, intraperitoneal administration), a 22-gauge stainless steel guide cannula was stereotaxically implanted into the third cerebral ventricle (6 mm anterior to the interaural line). A stainless steel wire stylet (29 gauge) was inserted into the guide cannula to prevent leakage of the cerebrospinal fluid (CSF). These items were fixed to the skull with dental cement 7 days before the experiment.

Experiment 1. Insulin (3 mU diluted in saline, designated as insulin/LEPTIN group) (Humalin R, Eli Lilly, Japan) or same volume of saline (saline/LEPTIN 1 group) was applied to the third cerebral ventricle via the cannula (icv). Thirty min after the administration of insulin or saline, mouse recombinant leptin (7.5 μg dissolved in 5 μl of phosphate-buffered saline [PBS], purchased from R&D Systems Inc., Minneapolis, MN) or PBS (saline/PBS 1, insulin/PBS group) was infused intracerebroventricularly.

Experiment 2. Glucose (500 μg dissolved in saline, glucose/LEPTIN group) or the same volume of saline (saline/LEPTIN 2 group) was administered intracerebroventricularly. Thirty min later, leptin or PBS (saline/PBS 2, glucose/PBS groups) was infused as in Experiment 1.

Measurement of Food Intake. To measure the cumulative food intake of each group, food was weighed 2, 12, and 24 hr after intracerebroventricular infusion of leptin or PBS at 1900, the beginning of the dark phase. Ten to twelve rats were used in each group.

Measurement of Glucose Levels in Blood and Cerebrospinal Fluid. Blood samples were taken from the tail vein, and the CSF samples were taken from the cisterna magna. Glucose levels were measured immediately in these 2 samples by the glucose oxidase method.

Western Blotting. To examine whether leptin signal transduction could be altered by insulin or glucose, we analyzed the tyrosine phosphorylation of STAT3 in protein extracts from leptin-stimulated hypothalamus. Four rats in each group were decapitated 30 min after icv infusion of leptin or PBS; the whole hypothalamus was dissected out and immediately frozen in liquid nitrogen until assay. The hypothalamus was homogenized in lysis buffer containing (in mmol/l) 10 NaH_2PO_4, 1.0 EDTA, 250 sucrose, 150 KCl, and 1.0 phenylmethylsulfonyl fluoride, and then centrifuged at 15,000 rpm for 30 min. Protein concentrations of the supernatants were estimated according to the method of Lowry *et al.* (17). A 40-mg protein of the supernatant was mixed with SDS sample buffer and boiled for 5 min before loading onto SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and blocked by incubation for 1 hr at room temperature with 5% non-fat dry milk in Tris-buffered saline/0.1% Tween 20 (TBST). The membrane was incubated overnight with anti-phospho-STAT3 antibody (Cell Signaling Technology, USA) as the primary antibody, and then reacted with anti-rabbit IgG antibody (Amersham Pharmacia Biotech, UK) for 90 min after washing with TBST. Protein bands were visualized by enhanced chemiluminescence reaction, then reblotted with anti-STAT3 antibody (Cell Signaling Technology, USA) to determine hypothalamic STAT3 protein.

Statistical Analysis. Data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using two-tailed Student's *t*-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Effects of Pretreatments on Food Intake. *Experiment 1: pretreatment with insulin icv.* Figure 1 shows the effects of insulin infusion into the brain on feeding behavior. The cumulative food intakes in the saline/

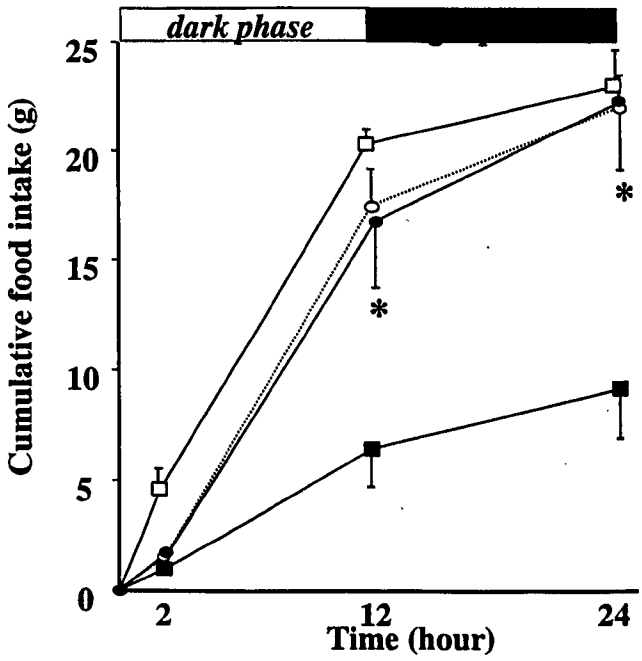

Figure 1. Acute effects of insulin icv on food intake in rats treated with central leptin infusion. Lines show the mean cumulative food intakes for 24 hr in the saline/PBS 1 group (saline icv + PBS icv, open squares), the saline/LEPTIN 1 group (saline icv+ leptin icv, solid squares), the insulin/LEPTIN group (insulin icv + leptin icv, solid circles), and the insulin/PBS group (insulin icv + PBS icv, open circles). The cumulative food intake in the insulin/LEPTIN group was significantly higher than that of the saline/LEPTIN group. *$P < 0.01$, compared with the saline/LEPTIN 1 group.

LEPTIN 1 group (saline icv + leptin icv) were significantly reduced compared with the saline/PBS 1 group (saline icv + PBS icv) at 2, 12, and 24 hrs (2 hrs: 1.0 ± 0.4 vs 4.7 ± 0.9 g, $P < 0.05$, 12 hrs: 6.5 ± 1.7 vs 20.3 ± 0.7 g, $P < 0.01$, 24 hrs: 9.3 ± 2.3 vs 23.0 ± 1.7 g, $P < 0.005$, each). In contrast with the saline/LEPTIN 1 group, those of the insulin/LEPTIN group (insulin icv + leptin icv) were significantly increased to the level seen in the saline/PBS 1 group (12 hrs: 16.8 ± 3.0 g, 24 hrs: 22.3 ± 3.2 g, $P < 0.01$ versus saline/LEPTIN group 1, respectively). Insulin icv alone (insulin/ PBS group) resulted in mild inhibition of feeding, but the difference in the cumulative food intake between the saline/PBS 1 and insulin/PBS groups was not significant. The level of peripheral blood glucose was not affected by insulin icv (Fig. 2).

Experiment 2: pretreatment with glucose icv. Figure 3 shows the effects of glucose icv on feeding behavior. Similar to Experiment 1, the cumulative food intakes of saline/LEPTIN 2 group were significantly lower compared with the saline / PBS 2 group. In comparison with the saline/ LEPTIN 2 group, those of the glucose/LEPTIN group (glucose icv + leptin icv) were restored close to the level observed in the saline/PBS 2 group (12 hrs: 16.1 ± 0.8 g, $P < 0.01$, 24 hrs: 18.9 ± 1.1 g, $P < 0.05$ versus saline/ LEPTIN 2 group, respectively). The inhibitory effect of glucose icv alone (the glucose/PBS group) on food intake was similar to that of insulin/PBS group. Administration of glucose icv increased the CSF glucose level by 20 mg/dl, but did not change that of peripheral blood (Fig. 4).

The above results indicated that the inhibitory effect of leptin on feeding was canceled by high levels of insulin and glucose in the hypothalamus.

Effects of pretreatments on leptin-induced STAT3 phosphorylation. Recently, we reported the results of time course studies of leptin-induced phosphorylation of STAT3 in the rat hypothalamus (18). Lysates derived from cerebroventricularly stimulated hypothalamus were subjected to anti-phospho-STAT3 immunoblotting. Phosphorylation of STAT3 appeared 15 min after injection of 7.5 μg leptin, and the maximal induction of STAT3 phosphorylation was noted at 30 min after the injection (18).

Experiment 1: pretreatment with insulin icv. We investigated the effects of insulin on leptin-induced STAT3 phosphorylation in the rat hypothalamus 30 min after leptin icv. Insulin or saline was administered followed by leptin or

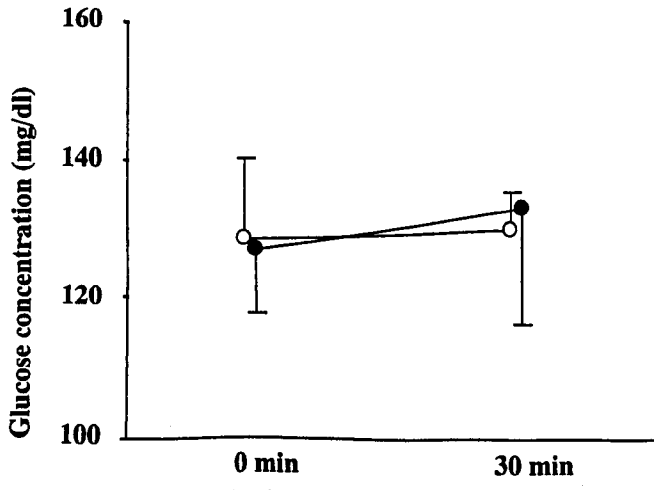

Figure 2. Peripheral blood glucose concentration after insulin icv. The concentration of blood glucose at 30 min after such infusion was not different from the control in both the insulin / LEPTIN group (○) and the saline/LEPTIN group (●).

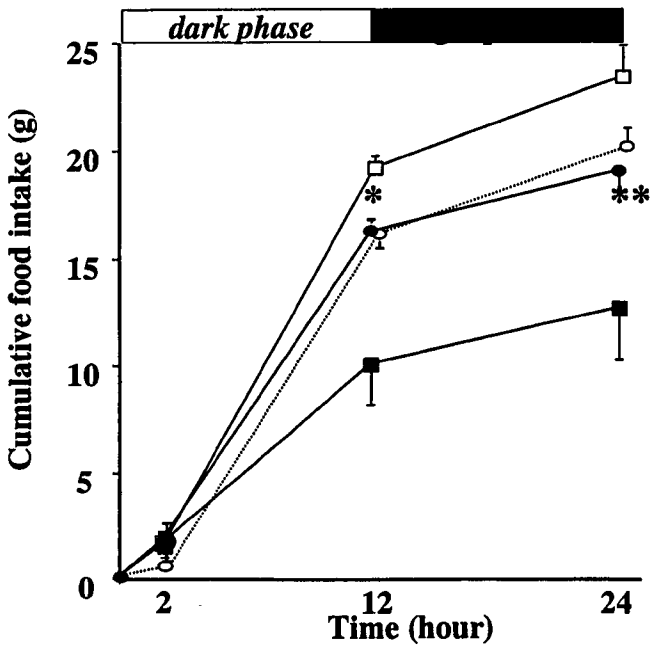

Figure 3. Acute effects of glucose icv on food intake in rats treated with central leptin infusion. Lines represent the mean cumulative food intakes for 24 hr in the saline/PBS 2 group (saline icv + PBS icv, open squares), the saline/LEPTIN 2 group (saline icv + leptin icv, solid squares), the glucose/LEPTIN group (glucose icv + leptin icv, solid circles), and the glucose/PBS group (glucose icv + PBS icv, open circles). The cumulative food intake in the glucose/LEPTIN group was significantly higher than that of the saline/LEPTIN 2 group. $*P < 0.005$, $**P < 0.05$ compared with the saline/LEPTIN 2 group.

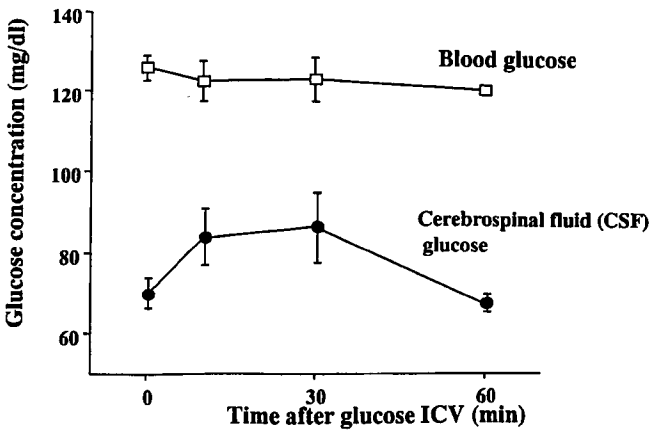

Figure 4. Glucose concentration in peripheral blood (open squares) and cerebrospinal fluid (CSF: solid circles) after glucose icv. Glucose concentration in CSF sample taken from the cisterna magna was increased by 15% (the peripheral blood glucose level did not vary) after infusion of glucose at 500 μg/rat into the third cerebral ventricle.

PBS icv. The resultant lysates of the hypothalamus were subjected to immunoblotting with anti-phospho-STAT3 or anti-STAT3 antibodies. STAT3 phosphorylation of samples from the insulin/LEPTIN group was markedly reduced compared with that of the saline/PBS 1 group. Reblotting the membranes with anti-STAT3 antibodies showed that insulin administration did not alter the amounts of hypothalamic STAT3 proteins (Fig. 5A). As shown in Figure 5B, densitometric analysis of the phosphorylated STAT3 bands revealed that insulin administration decreased leptin-induced STAT3 phosphorylation by 60% compared with that of the saline/LEPTIN 1 group.

Experiment 2: pretreatment with glucose icv. Similar to Experiment 1, we investigated the effects of glucose icv on hypothalamic STAT3 phosphorylation. STAT3 phosphorylation was reduced by 50% in samples from the glucose/LEPTIN group, compared with that of the saline/ LEPTIN 2 group. Glucose administration did not affect the amounts of hypothalamic STAT3 proteins (Fig. 6A and B).

Discussion

In what is called a "satiation period", obesity, an important risk factor of glucose intolerance, is increasing in the world (19). High levels of anorexigenic hormones or nutrients (i.e., hyperinsulinemia, hyperleptinemia, and hyperglycemia) are commonly seen in obese people; however, these anorectic factors fail to suppress feeding. Odeleye *et al.* (20) reported that fasting hyperinsulinemia was a predictor of increased body weight gain and was a possible risk factor for the development of obesity in Pima Indian children. Though many investigators have reported the actions of leptin, a potent regulator of food intake and body weight homeostasis, the interactions between insulin, glucose, and leptin in the brain remains poorly defined. This study was performed on the hypothesis that high concentrations of insulin or glucose in the brain act to suppress the anorectic action of leptin, based on the above paradoxical clinical findings. High plasma insulin or glucose concentration produces significant elevation of insulin or glucose level in the CSF (21, 22). Changes in peripheral insulin or glucose concentration alter plasma levels of various hormones that are involved in the central regulation of feeding. To exclude the influence of other factors, we administered insulin and glucose into the third cerebral ventricle and established a hyperinsulinemic or hyperglycemic milieu, which was limited to the rat brain. In this study, we demonstrated that a rise in insulin or glucose in the hypothalamus decreased leptin-induced STAT3 tyrosine phosphorylation in this area of the brain and inhibited subsequent anorectic action of leptin in rats.

Insulin signal in the brain plays an important role in the regulation of food intake and energy expenditure (7). Sipols *et al.* (6) demonstrated that STZ-induced diabetes in rats was associated with hyperphagia and intracerebroventricular insulin infusion inhibited food intake and increased hypothalamic gene expression of neuropeptide Y (NPY), an orexigenic peptide. These findings suggest that existence of normal insulin level in the brain is indispensable for proper regulation of feeding behavior. On the other hand, Kellerer *et al.* (15) reported that insulin inhibited leptin receptor signaling at the level of JAK-2 in HEK293 cells, indicating that hyperinsulinemia contributes to the pathogenesis of leptin resistance. Furthermore, Peraldi *et al.* (23) reported that insulin induced a suppressor of cytokine signaling-3 (SOCS-3), which is an inhibitor of leptin signaling in the hypothalamus (24). Considered together with these early *in vitro* findings, the present results suggest that a high level of insulin in the hypothalamus is the underlying mechanism of leptin resistance. Thus, we suggest that any deviation from the physiological normal range of insulin (i.e., both lack of insulin signaling and high levels in the brain) can cause disorders of central feeding regulation.

Contrary to our findings, Carvalheira *et al.* (25) demonstrated that insulin increased the leptin-induced STAT3

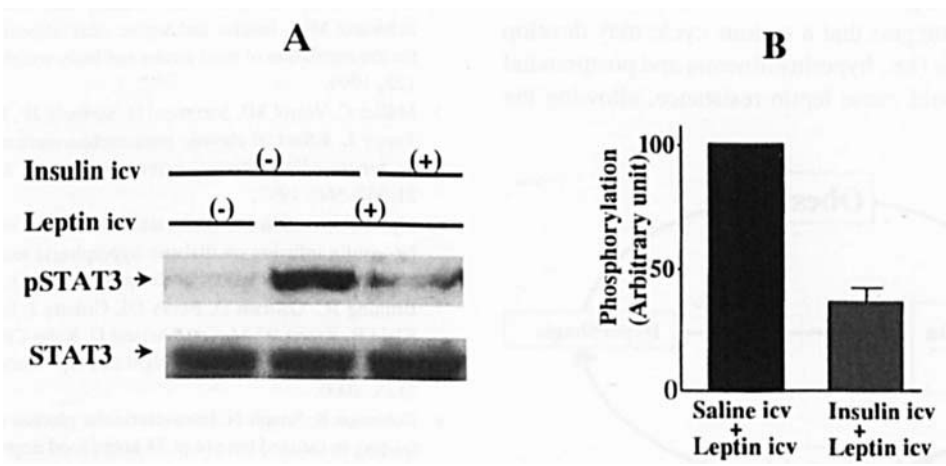

Figure 5. Effect of insulin icv on STAT3 phosphorylation in the rat hypothalamus. Leptin was injected into the third cerebral ventricle 30 min after insulin icv. Hypothalamic lysates were subjected to Western blotting using specific-anti-phospho-STAT3 and anti-STAT3 antibodies (A). The density of densitometrically analyzed bands of phosphorylated STAT3 was corrected relative to the intensity of STAT3 protein (B). The hypothalamic leptin-induced STAT3 phosphorylation in the insulin/LEPTIN group was 60% lower than that of the saline/LEPTIN 1 group. Data are mean ± SEM.

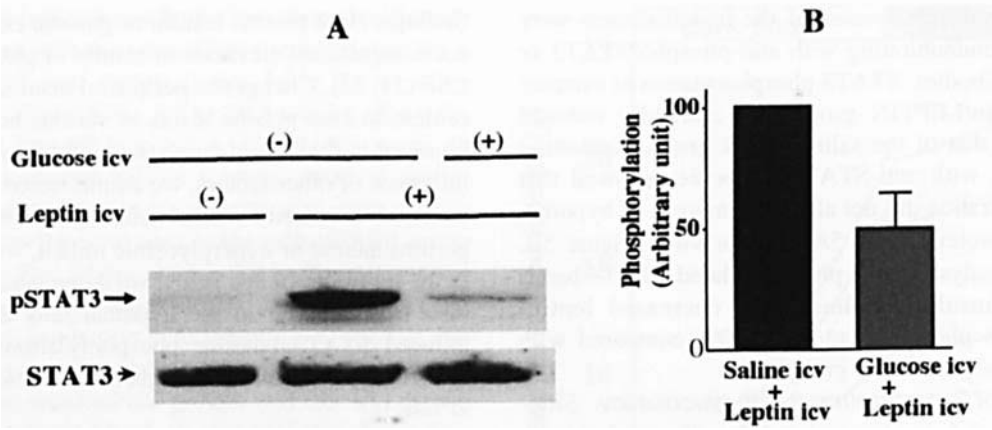

Figure 6. Effect of glucose icv on STAT3 phosphorylation in the rat hypothalamus. Leptin was injected into the third cerebral ventricle 30 min after glucose icv. Hypothalamic lysates were subjected to Western blotting using specific-anti-phospho-STAT3 and anti-STAT3 antibodies (A). Densitometric analysis of the phosphorylated STAT3 bands is shown (B). In the glucose/LEPTIN group, leptin-induced STAT3 phosphorylation was 50% lower than that of the saline/LEPTIN 2 group. Data are mean ± SEM.

phosphorylation in the rat hypothalamus. Different from our method, they simultaneously infused insulin and leptin into the cerebral ventricle. This methodological difference might explain the dissimilar results. In this regard, the authors did not examine the effect of co-administration on feeding behavior.

Our results showed that high glucose levels in the hypothalamus ameliorated the anorectic action of leptin and decreased leptin-induced STAT3 phosphorylation, similar to Experiment 1 with high insulin in the hypothalamus. Previous animal studies reported the satiety effect of glucose, which is mediated by the hypothalamus (8, 9). There is also evidence to suggest that glucose-responsive neurons in the ventromedial hypothalamus (VMH) are involved in this effect (9, 26). This hypothalamic area also contains abundant long-form leptin receptors (Ob-Rb) (27). Glucose-leptin interaction may be present in VMH, but our experiments did not identify the underlying mechanism(s) of glucose inhibition of leptin signal transduction. Further studies are needed to investigate such mechanism(s).

Our findings suggest that a vicious cycle may develop in obese individuals (i.e., hyperinsulinemia and postprandial hyperglycemia) could cause leptin resistance, allowing the induction of hyperphagia, which again increases insulin and glucose levels (Fig. 7). Thus hyperphagia *per se* may perpetuate hyperphagia. Alternatively, hyperinsulinemia and hyperglycemia may be risk factors for the development or the worsening of obesity.

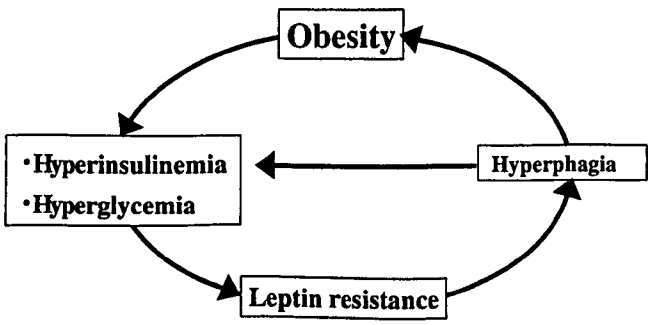

Figure 7. A vicious cycle of hyperphagia in obese individuals. Hyperinsulinemia and postprandial hyperglycemia could cause leptin resistance, allowing the induction of hyperphagia, which again increases insulin and glucose levels.

In conclusion, we have demonstrated in the present study that a rise in insulin and glucose in the rat brain could lead to leptin resistance as manifested by food intake. This effect is probably due to attenuation of STAT3 phosphorylation downstream the leptin receptor.

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