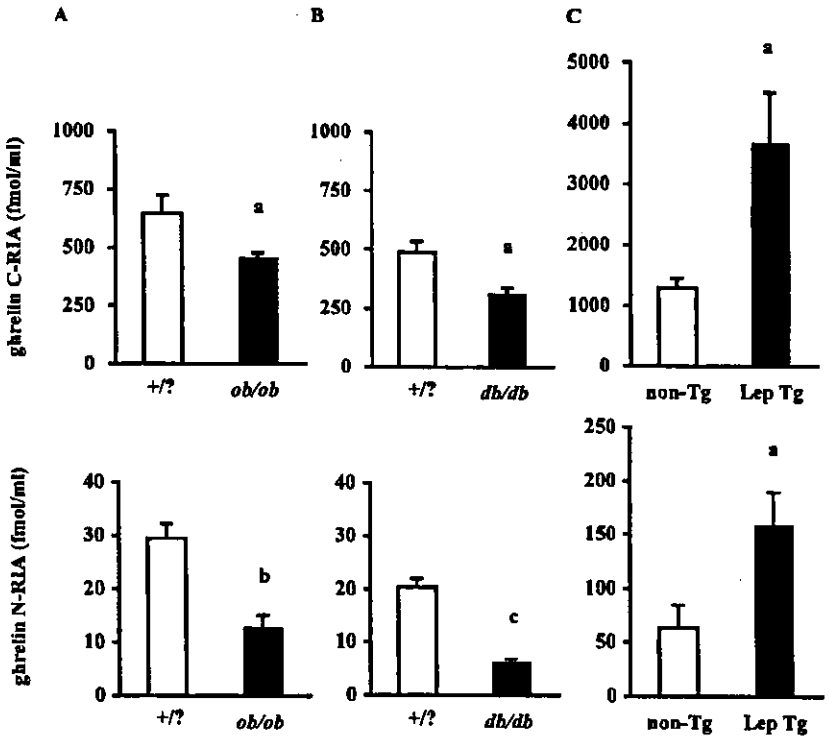

FIG. 1. Plasma ghrelin levels measured by C-RIA (*upper panel*) and N-RIA (*lower panel*) in *ob/ob* and *db/db* mice and Lep Tg mice. A, Plasma ghrelin levels in *ob/ob* and control (+/?) mice (n = 6/group). B, Plasma ghrelin levels in *db/db* and control (+/?) mice (n = 6/group). C, Plasma ghrelin levels in Lep Tg and control (nontransgenic, non-Tg) mice (n = 6/group). a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.0001$ (*vs.* control mice).

TABLE 2. Body weights and plasma ghrelin levels by C-RIA and N-RIA in leptin-injected *ob/ob* mice

	BW (g)	Plasma ghrelin (fmol/ml)	
		C-RIA	N-RIA
Leptin-injected	53.3 ± 3.0[a]	439.0 ± 24.4[a]	10.2 ± 2.9[a]
Saline-injected	56.5 ± 3.7	466.2 ± 24.6	12.8 ± 3.7

Values are given as the mean ± SEM. BW, Body weight.
[a] Not significant *vs.* saline-injected mice.

was increased by 12% in *ad libitum*-fed mice, but was decreased by 30% in food-restricted mice. Plasma ghrelin levels were elevated in food-restricted mice compared with *ad libitum*-fed mice. They were 7.6- and 11.2-fold higher by C-RIA and N-RIA, respectively, than those in *ad libitum*-fed mice ($P < 0.001$ for both).

Plasma ghrelin levels in Zucker fatty rats

The Zucker fatty and control rats were studied to examine changes in plasma ghrelin levels in obese animals in detail. Plasma ghrelin levels by C-RIA after 12-h fasting in fatty and control rats were 294.9 ± 25.4 and 485.5 ± 41.4 fmol/ml, respectively ($P < 0.001$). Twenty-four-hour fasting produced clearer results. Plasma ghrelin levels by C-RIA and N-RIA after 24-h fasting in the control rats were 1.7- and 2.6-fold higher than those in fatty rats, respectively (Table 4). There was also significant difference in blood glucose levels between fatty and control rats after 24-h fasting (Table 4). The molecular forms of plasma ghrelin in 24-h fasted control rats were examined by RP-HPLC coupled with C-RIA and N-RIA (Fig. 2, A and B). The recoveries of the immunoreactivities after RP-HPLC were more than 90% by C-RIA and 95% by N-RIA, respectively. One major peak eluted at the position of des-acyl ghrelin-(1–28), and another major peak eluted at the position of acylated rat ghrelin-(1–28) by RP-HPLC coupled with C-RIA (Fig. 2A, a and b, respectively). The molar ratio of des-acyl ghrelin to acylated ghrelin was 4.3:1. One major peak eluted at the position of acylated rat ghrelin-(1–28) by RP-HPLC coupled with N-RIA (Fig. 2B, b).

Effect of insulin-induced hypoglycemia on plasma ghrelin levels in fasted Zucker fatty rats

Plasma ghrelin levels after insulin injection in 24-h fasted Zucker fatty rats were examined to elucidate the role of blood glucose levels in the reduced plasma ghrelin levels in these animals. The blood glucose level declined to 48 ± 6 mg/dl 90 min after insulin injection, and hypoglycemia lasted until the end of the study (Fig. 3A). There was no significant change in blood glucose levels in saline-injected rats. Figure 3B shows the time course of plasma ghrelin levels in both groups. Plasma ghrelin levels by C-RIA 120 and 240 min after insulin injection were 197 and 228% of the initial values, respectively. The changes were significant ($P < 0.001$ *vs.* saline injection for both). Plasma ghrelin levels by N-RIA 120 and 240 min after insulin injection were 263% and 278% of the initial values, respectively. The changes were also significant ($P < 0.005$ *vs.* saline injection for both).

Effect of glucose injection on plasma ghrelin levels

The effect of glucose injection on plasma ghrelin levels was studied in 24-h fasted Sprague Dawley rats. Plasma ghrelin levels by C-RIA and N-RIA after 24-h fasting were elevated to 149% and 289% of the values before fasting, respectively

TABLE 3. Body weight changes and plasma ghrelin levels by C-RIA and N-RIA in food-restricted and *ad libitum*-fed mice

	Initial BW (g)	BW changes (g)	Plasma ghrelin (fmol/ml)	
			C-RIA	N-RIA
Food-restricted	22.1 ± 0.4[a]	−6.5 ± 0.2[b]	5318.9 ± 373.5[b]	340.8 ± 23.8[b]
Ad libitum-fed	23.4 ± 0.4	2.7 ± 0.3	703.2 ± 107.3	30.5 ± 11.5

Values are given as the mean ± SEM. BW, Body weight.
[a] Not significant *vs. ad libitum*-fed mice.
[b] $P < 0.001$ *vs. ad libitum*-fed mice.

TABLE 4. Blood glucose and plasma ghrelin levels by C-RIA and N-RIA after 24-h fasting in 15-wk-old Zucker fatty (*fa/fa*) and the control (+/?) rats

	Blood glucose (mg/dl)	Plasma ghrelin (fmol/ml)	
		C-RIA	N-RIA
fa/fa	97.0 ± 4.8[a]	303.9 ± 24.9[a]	38.9 ± 4.3[a]
+/?	69.8 ± 2.0	521.1 ± 41.4	102.1 ± 5.2

Values are given as the mean ± SEM.
[a] $P < 0.001$ *vs.* control rats (+/?).

(Fig. 4, *upper* and *lower panels*). Plasma ghrelin levels by C-RIA after 0 (saline injection), 2.0, and 5.0 g/kg glucose injections were 98%, 84%, and 78% of the values after 24-h fasting (Fig. 4, *upper panel*). The differences between 0 and 2.0 g/kg, and 0 and 5.0 g/kg were significant ($P < 0.05$ for both). Plasma ghrelin levels by N-RIA were reduced in a clearer dose-dependent manner. They were 87%, 69%, and 30% of the values after 24-h fasting (Fig. 4, *lower panel*). The differences between 0 and 2.0 g/kg, 0 and 5.0 g/kg, and 2.0 and 5.0 g/kg were significant ($P < 0.05$, $P < 0.0001$, and $P < 0.0001$, respectively).

Effect of severity of obesity on the secretory regulation of ghrelin

Younger and older Zucker fatty rats than 15-wk-old rats, which were used in the studies described above, were used. Eight- and 30-wk-old Zucker fatty and their control rats were studied. Body weights of 8-wk-old fatty and control rats were 282.5 ± 5.1 and 213.8 ± 1.6 g, respectively, and those of 30-wk-old fatty and control rats were 732.5 ± 22.5 and 408.7 ± 8.8 g, respectively. That is, 8-wk-old fatty rats were only 1.3-fold heavier than the control rats, whereas 30-wk-old fatty rats were 1.8-fold heavier than the control rats. Figure 5A shows the effect of 24- and 48-h fasting followed by 6-h refeeding in 8-wk-old rats. Plasma ghrelin levels before fasting determined by C-RIA in fatty and control rats were 295.4 ± 22.5 and 335.8 ± 36.2 fmol/ml, respectively. Plasma ghrelin levels after 24-h fasting in fatty and control rats were 96% and 187% of the values before fasting, and those after 48-h fasting were 150% and 165% of the control levels, respectively. The differences in plasma ghrelin levels between fatty and control rats were not significant before fasting, but were significant after 24- and 48-h fasting ($P < 0.001$ and $P < 0.05$, respectively). Plasma ghrelin levels by N-RIA showed similar results. Those before fasting in fatty and control rats were 45.1 ± 4.4 and 48.1 ± 9.4 fmol/ml, respectively. Plasma ghrelin levels after 24-h fasting in fatty and control rats were 95% and 222% of the values before fasting, and those after 48-h fasting were 147% and 180% of the prefasting values, respectively. The differences between fatty and control rats were not significant before fasting, but were significant after 24- and 48-h fasting ($P < 0.001$ and $P < 0.05$, respectively). Plasma ghrelin levels after 6-h refeeding in the control rats by C-RIA and N-RIA were reduced to 45% and 32% of the values after 48-h fasting, respectively. Plasma ghrelin levels after 6-h refeeding in fatty rats, however, were not reduced. They were 106% and 97% of the values after 48-h fasting by C-RIA and N-RIA, respectively. Figure 5B shows the effect of 24- and 48-h fasting followed by 6-h refeeding in 30-wk-old rats. Plasma ghrelin levels in older control rats were increased in the same manner as in younger ones. Plasma ghrelin levels after 24-h fasting by C-RIA and N-RIA were 200% and 269% of the values before fasting, respectively. On the contrary, plasma ghrelin levels after 24-h fasting in older fatty rats were not changed similarly to those in younger fatty rats. They remained, however, low even after 48-h fasting. They were 108.4% by C-RIA and 73.0% by N-RIA of the values before fasting. Moreover, plasma ghrelin levels after 6-h refeeding were clearly higher than those after 48-h fasting. They were 145% by C-RIA and 261% by N-RIA of the values after 48-h fasting.

Plasma ghrelin levels in obese human subjects

Plasma ghrelin levels were examined in human nondiabetic obese subjects. Figure 6, A and B, shows the mean of the plasma ghrelin levels and the correlation between BMIs and plasma ghrelin levels in obese and sex- and age-matched control subjects, respectively. Plasma ghrelin levels in obese and control subjects were 82.1 ± 5.4 and 145.2 ± 16.0 fmol/ml, respectively (Fig. 6A). The difference was significant ($P < 0.005$). There was a significant negative correlation between BMIs and plasma ghrelin levels (Fig. 6B; $r = -0.51$; $P < 0.0001$).

Discussion

In the present study we reported for the first time the secretory regulation of plasma ghrelin in obese animals distinguishing the active form of ghrelin from total ghrelin. We used two kinds of RIAs for the purpose, namely C-RIA for the carboxyl terminal and N-RIA for the amino terminal of ghrelin. The levels measured by N-RIA represent those of active form of ghrelin, as *n*-octanoyl modification, which is essential for the biological activity of ghrelin (7, 37, 38), is located on Ser^3 of ghrelin. On the other hand, the levels measured by C-RIA represent those of total ghrelin, including its inactive form (7).

Plasma ghrelin levels by C-RIA were reduced in genetically obese *ob/ob* and *db/db* mice compared with those in their control mice. Plasma ghrelin levels by N-RIA showed results similar to those by C-RIA, and they were approxi-

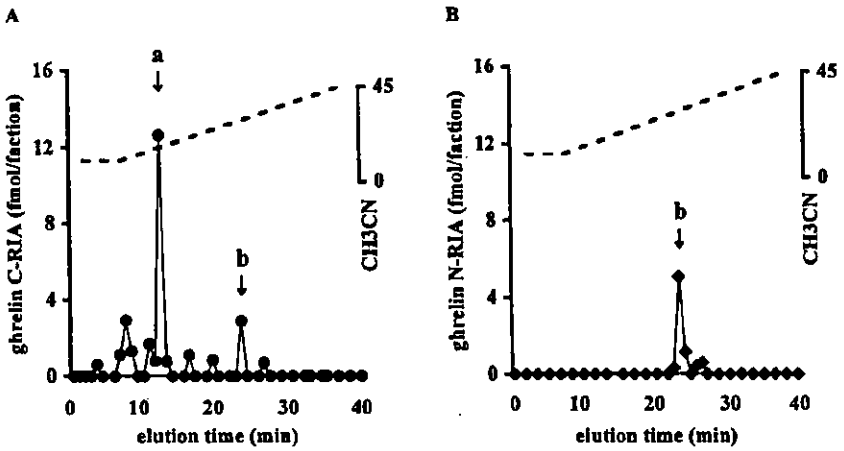

FIG. 2. Representative RP-HPLC coupled with C-RIA and N-RIA of plasma ghrelin in 24-h fasted Zucker control (+/?) rats. A, RP-HPLC coupled with C-RIA. B, RP-HPLC coupled with N-RIA. The *arrows* indicate the elution position of des-acyl ghrelin (a) and acylated full-length ghrelin (b).

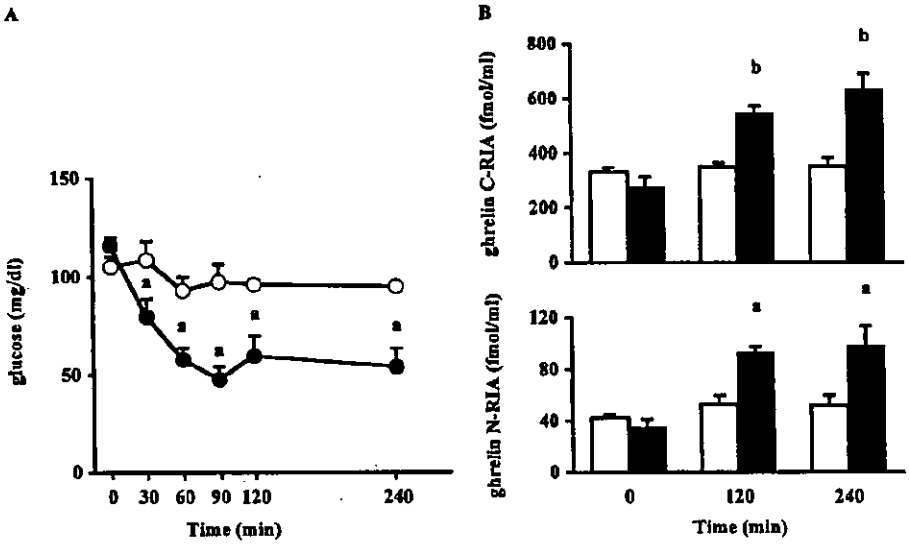

FIG. 3. The effect of 8.0 U/kg NPH insulin injection in 24-h fasted Zucker fatty (*fa/fa*) rats (n = 8/group). A, Changes in blood glucose levels after sc injection of saline (○) and insulin (●). B, Changes in plasma ghrelin levels measured by C-RIA (*upper panel*) and N-RIA (*lower panel*) after sc injection of saline (□) and insulin (■). a, $P < 0.005$; b, $P < 0.001$ (*vs.* injection of saline).

mately 2–5% of the latter. We thus demonstrated that plasma levels of both total ghrelin and the active form of ghrelin are reduced in obese animals. These results are compatible with previous reports on obese Caucasian subjects (35) and rats fed a high fat diet (42), in which total plasma ghrelin levels are shown to be reduced, and with a report on reduced ghrelin mRNA levels in *db/db* mice (34). Plasma ghrelin levels in Lep Tg mice, which are characterized by disappearance of lipid from adipose tissue (40), showed inverse results. They were highly elevated in these skinny mice and reached 2.9-fold by C-RIA and 2.5-fold by N-RIA of those in the control mice, whereas there was a discrepancy in stomach and plasma ghrelin levels in Lep Tg mice. Stomach ghrelin levels may represent only the storage of ghrelin in the stomach and may not reflect its secretion into the bloodstream. To confirm the results in Lep Tg mice in another animal model with low body weight, food-restricted mice were studied. They were fed with 70% of the average food intake of the control mice, mimicking the feeding states in Lep Tg mice, which consume approximately 70% of the food of non-Tg mice (40). We observed that plasma ghrelin levels were also highly elevated in food-restricted mice. These results of plasma ghrelin levels in Lep Tg mice and food-restricted mice are compatible with previous reports by us and others on patients with anorexia nervosa, whose plasma ghrelin levels are highly elevated (17, 36). We also observed that plasma ghrelin levels are dramatically reduced when Lep Tg mice gain weight by feeding a high fat diet (Ebihara, K., and Y. Ogawa, unpublished data). Taken together, these data indicate that plasma levels of total ghrelin and the active form of ghrelin reflect chronic feeding states. Although we observed higher plasma ghrelin levels in non-Tg mice compared with the control mice in the group of *ob/ob* and *db/db* mice, this may be accounted for by gender difference in plasma ghrelin levels. Female animals tend to show higher plasma ghrelin levels than male animals (Ariyasu, H., unpublished data).

The mechanism for the reduced plasma ghrelin levels in obese animals and humans is unknown. It is conceivable that the deficiency in leptin action could result in the reduced plasma ghrelin levels in the obese animals used in this study, as leptin is lacking in *ob/ob* mice (43), and leptin receptor is lacking in *db/db* mice and Zucker fatty rats (44–46). Previous studies, however, showed the opposite results. The charac-

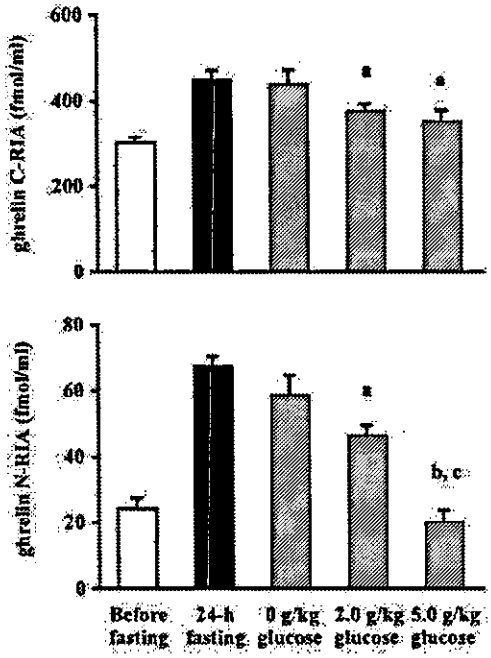

FIG. 4. Plasma ghrelin levels measured by C-RIA (*upper panel*) and N-RIA (*lower panel*) after ip injection of 0 (saline injection), 2.0, and 5.0 g/kg glucose in 24-h fasted Sprague Dawley rats (n = 8/group). a, $P < 0.05$; b, $P < 0.001$ (*vs.* injection of 0 g/kg glucose). c, $P < 0.001$ (*vs.* injection of 2.0 g/kg glucose).

teristic negative correlation between plasma ghrelin levels and BMIs (17, 35) instead leads to the idea that ghrelin secretion may be negatively regulated by leptin, because plasma leptin levels positively correlate with BMIs (47). In addition, the present study showed that leptin replacement in *ob/ob* mice did not result in restored plasma ghrelin levels. It is not likely that the amount of injected leptin used in this study was not enough, because it can elevate plasma leptin levels to the extent of those in Lep Tg mice for a few hours (40, 48). Taken together, the lack of leptin action does not seem to result in the reduced plasma ghrelin levels in these obese animals. Further study, however, is needed to elucidate the role of leptin in plasma ghrelin levels in obese subjects.

Plasma ghrelin levels in genetically obese rats were also examined. Zucker fatty (*fa/fa*) rats showed lower plasma ghrelin levels than the control rats. The results of RP-HPLC coupled with C-RIA and N-RIA for plasma ghrelin were compatible with those for ghrelin in the stomach (7, 18, 34). These data indicate that acylated full-length ghrelin and desacyl ghrelin are the two major forms of this hormone in rat plasma and confirm the validity of C-RIA and N-RIA. In fasted conditions, Zucker fatty rats showed higher blood glucose levels than the control rats, compatible with a previous report (49). The difference between fatty and control rats in glucose levels led us to the hypothesis that the higher glucose levels may be involved in the reduced plasma ghrelin levels in the fasted fatty rats, because sugar intake, but not stomach expansion, decreases circulating ghrelin levels in rodents (31). The effect of short-term changes in blood glucose levels on plasma ghrelin levels in Zucker fatty rats was examined. NPH insulin injection created prolonged hypoglycemia in these animals, and the nadir blood glucose values were comparable to blood glucose levels in 24-h fasted control rats. The hypoglycemia-stimulated ghrelin secretion and plasma levels of both total ghrelin and the active form of ghrelin 120 and 240 min after insulin injection reached 200–280% of the initial values, respectively. The values exceeded those in 24-h fasted control rats. It should be noted that hypoglycemia induced by rapid insulin had much less effect on plasma ghrelin levels (Ariyasu, H., unpublished data), suggesting slow secretory regulation of ghrelin by hypoglycemia. Then the effect of glucose injection on plasma ghrelin levels was examined in fasted Sprague Dawley rats. Plasma levels of both total ghrelin and the active form of ghrelin were reduced by glucose injection in a dose-dependent manner. These data suggest that reduced blood glucose results in elevated plasma ghrelin levels in fasted rats of normal weight and that high blood glucose levels may be involved in the reduced plasma ghrelin levels in obese animals. These data also indicate that short-term stimulation of ghrelin secretion, *i.e.* hypoglycemia, restores the reduced plasma ghrelin levels in obese animals, suggesting the exquisite secretory regulation of ghrelin in both chronic and acute phases of energy homeostasis.

Plasma ghrelin levels were further examined using younger and older Zucker fatty rats. Moreover, they were examined in various feeding states in these studies. The time course of plasma ghrelin levels by fasting followed by refeeding in 8-wk-old rats showed intriguing results. Plasma ghrelin levels showed no significant difference by C-RIA or N-RIA between fatty and control rats when they were freely fed. They showed marked elevation after 24-h fasting in the control rats, and the values reached 1.9-fold of those before fasting and remained at almost the same levels after 48-h fasting. On the contrary, plasma ghrelin levels did not show any change in fatty rats after 24-h fasting. They were elevated after 48-h fasting, but did not reach the levels in control rats. The delayed secretory regulation of ghrelin by fasting in obese animals raised the idea that short-term secretory regulation of ghrelin is modified by an excess energy deposit. The older fatty rats showed clearer results and confirmed this idea. Eight-week-old fatty rats weighed only 1.3 times as much as the control rats, whereas 30-wk-old fatty rats weighted 1.8 times as much as the control rats. Although older control rats showed almost the same pattern of plasma ghrelin levels as the younger ones, older fatty rats showed a more delayed pattern after fasting than younger fatty rats. The larger energy deposit in older fatty rats than younger ones may explain these augmented results. Refeeding experiments after 48-h fasting also showed intriguing results. Plasma ghrelin levels were reduced to basal values after refeeding in the control rats, but not in fatty rats. Plasma ghrelin levels were unchanged in 8-wk-old fatty rats and were even elevated in 30-wk-old fatty rats. Obese animals appear to be less sensitive to negative stimulation for the secretory regulation of ghrelin, and plasma ghrelin levels may be still elevated due to the preceding fasting. These results are in keeping with our observation of the feeding behavior of the refed rats in this study if we consider that ghrelin is a potent stimulator for food intake (22–30). The

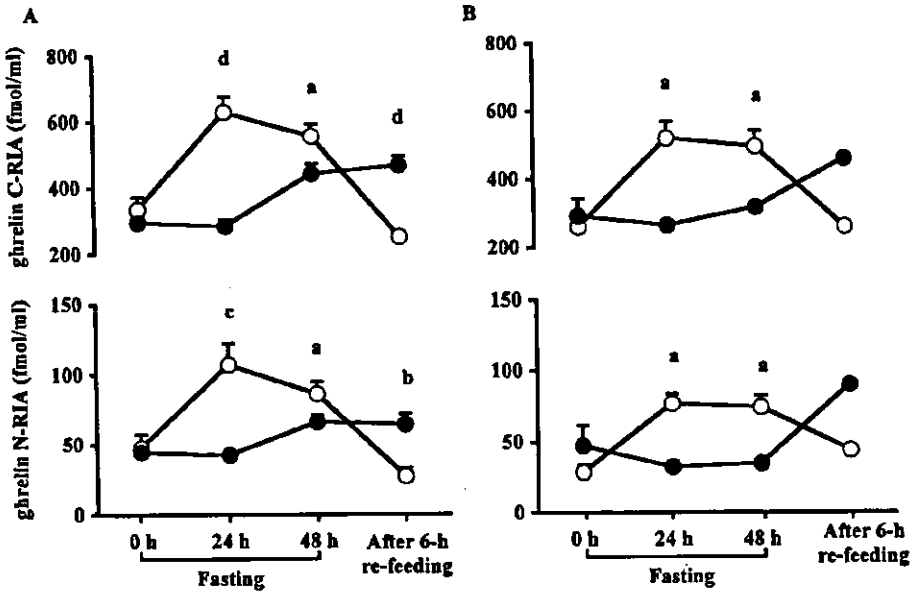

FIG. 5. The effect of 24- and 48-h fasting followed by 6-h refeeding in older and younger Zucker fatty (*fa/fa*; ●) and control (+/?; ○) rats (n = 8/group). A, Changes in plasma ghrelin levels measured by C-RIA (*upper panel*) and N-RIA (*lower panel*) in 8-wk-old rats. B, Changes in plasma ghrelin levels measured by C-RIA (*upper panel*) and N-RIA (*lower panel*) in 30-wk-old rats. a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.001$; d, $P < 0.0001$ (*vs.* control rats).

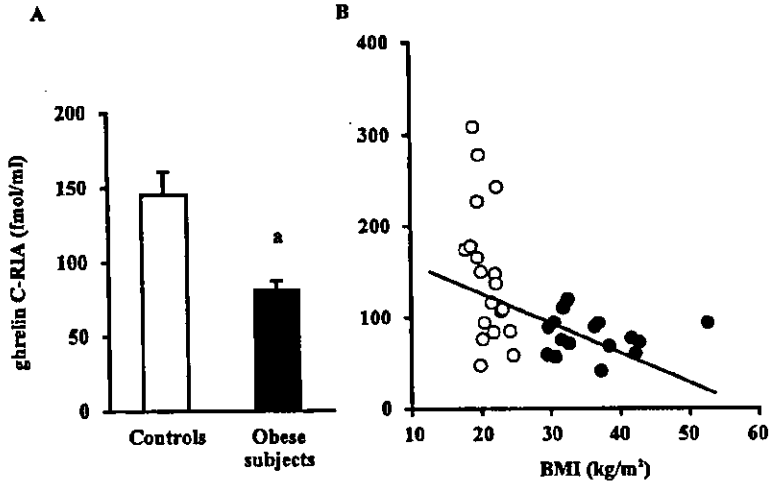

FIG. 6. Plasma ghrelin levels in 17 obese Japanese subjects (9 men and 8 women; BMI, >25.0 kg/m^2) and 20 sex- and age-matched control subjects. A, Mean plasma ghrelin levels measured by C-RIA in obese and control subjects. a, $P < 0.0005$. B, Correlation between BMIs and plasma ghrelin levels in obese (●) and control (○) subjects. $r = -0.51$; $P < 0.0001$.

control rats almost stopped eating after 6-h refeeding with satisfaction, whereas fatty rats were still eating even after that.

Plasma ghrelin levels in obese human subjects in the present study confirmed the results in the animals mentioned above. We clearly demonstrated a negative correlation between BMIs and plasma ghrelin levels in obese Japanese subjects, expanding a previous study showing reduced plasma ghrelin levels in obese Caucasians (35). These data are also compatible with studies by us and others of patients with anorexia nervosa (17, 36). These patients show high plasma ghrelin levels, and their BMIs have a negative correlation with these levels.

We could not detect marked discrepancy between ghrelin levels measured by C-RIA and N-RIA in the present study. Measuring the acylated form of this hormone, however, may be of advantage if we consider that ghrelin is biologically active only in the acylated form (1). There may be physiological or pathological conditions under which plasma levels of total ghrelin and the active form of ghrelin are discrepant. Measuring the active form of ghrelin may be of importance in such conditions. Further study is needed for this issue.

There are several examples that support the idea that ghrelin has actions involved in energy homeostasis as well as GH release (21). GHSs have been demonstrated to have an orexigenic action, and we and others recently showed that central administration of ghrelin induces food intake in rodents (22–30). Ghrelin injection induces the expression of Fos protein in the hypothalamic arcuate nucleus and then stimulates the expression of NPY and AGRP (27–29). A role of NPY and AGRP as mediators of feeding effect of ghrelin is suggested by studies in which antagonists of either NPY or AGRP were shown to attenuate the orexigenic potency of ghrelin (27–29). Continuous administration of ghrelin induces food intake even in humans (50). Meanwhile, peripheral daily administration of ghrelin or ipamorelin, one of the

GHSs, induces adiposity independent of food intake or GH secretion in rodents (31, 32). These data suggest that ghrelin may act in various ways, thereby increasing energy deposit. An alteration in the secretory regulation of ghrelin in obese subjects may play an important role in these actions of ghrelin.

In conclusion, the present study demonstrates that the short-term regulation of plasma levels of both total ghrelin and the active form of ghrelin is delayed in obese animals and that insulin-induced hypoglycemia restores the delayed regulation, suggesting that blood glucose levels are involved in the delayed regulation in obese animals. These observations are in keeping with the hypothesis that ghrelin is involved in acute and chronic energy homeostasis.

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Role of Leptin in Pregnancy—A Review

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Leptin is an adipocyte-derived hormone that decreases food intake and body weight via its receptor in the hypothalamus. In rodents, it also modulates glucose metabolism by increasing insulin sensitivity. We previously reported that leptin is produced by human placental trophoblasts. We also revealed that leptin gene expression in the placenta was augmented in severe pre-eclampsia, and suggested that placental hypoxia may play a role in this augmentation. Maternal plasma leptin levels correlated well with mean blood pressure, but not with body mass index. Plasma leptin levels in pre-eclamptic women with IUGR were higher than those without IUGR ($P<0.05$).

We further examined the effects of hyperleptinemia on the course of pregnancy by using transgenic mice (Tg) overexpressing leptin. In pregnant Tg mice, food intake was significantly less than non-Tg, and the fetal body weights were reduced to approximately 70 per cent of those of non-Tg.

Resistin is a novel adipocyte–derived hormone that decreases insulin sensitivity and increases plasma glucose concentration, thus contributing the development of obesity-related type II diabetes mellitus. We recently found that resistin gene is expressed in the human placenta as well as adipose tissue. In this review, possible roles of placental leptin and resistin are discussed.
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INTRODUCTION

Dramatic changes in energy metabolism are well recognized in pregnant women. Increased food intake, decreased insulin sensitivity and hyperlipidemia are major features during pregnancy (Abrams and Pickett, 1999). These changes are beneficial in providing energy to the fetus and preparing the mother for nursing. It is proposed that maternal adaptation to the pregnant state is mainly due to placental hormones, such as prolactin, placental lactogen and steroid hormones (Abrams and Pickett, 1999). However, the mechanism is not fully understood.

Recently, leptin was introduced as an adipocyte-derived messenger of energy metabolism (Zhang et al., 1994). Subsequently, we revealed that leptin is produced in the human placenta and is secreted into both maternal and fetal circulation (Masuzaki et al., 1997). Since leptin receptor is abundantly expressed in various maternal tissues, placenta and fetal tissues, physiological and pathophysiological roles in pregnancy are expected (Holness et al., 1999; Ahima and Flier, 2000; Henson and Castracane, 2000; Ashworth et al., 2000; Reitman et al., 2001). However, the role of leptin in pregnancy has not fully elucidated to date, since production and metabolism of leptin during pregnancy are significantly different among species.

In this review, we first introduce leptin as a novel messenger of energy metabolism. Secondly, we present our data on leptin production in the human placenta and discuss possible roles of leptin in pregnancy. Finally, we introduce our recent findings on the production of resistin in the human placenta. Resistin is the newly discovered adipocyte-derived peptide hormone that regulates insulin resistance and development of type II diabetes mellitus (Steppan et al., 2001).

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1. LEPTIN AS A MESSENGER OF ENERGY METABOLISM

Leptin is a peptide hormone that consists of 146 amino acids, which is expressed abundantly and specifically in the adipose tissue (Zhang et al., 1994). It decreases food intake via its cognate receptor (Ob-R) in the hypothalamus (Campfield et al., 1995; Halaas et al., 1995). In addition, leptin activates sympathetic nervous system and increases energy expenditure (Pelleymounter et al., 1995; Masuzaki et al., 1999; Ogawa et al., 1999). Thus, leptin decreases body weight and adiposity as a novel messenger of energy metabolism.

Following the discovery of genes for leptin (Zhang et al., 1994) and its receptor (Tartaglia et al., 1995), a number of animal studies and in vitro studies revealed various functions

of leptin (Ahima and Flier, 2000). In addition to the satiety action and activation of sympathetic nervous system, leptin acts also as a peripherally produced metabolic signal to the neuro-endocrine and reproductive systems, and plays a crucial role in reproduction. In leptin deficient *ob/ob* mice, reproductive function is impaired, but is recovered by leptin supplementation (Chehab et al., 1996). On the other hand, in leptin over-expressing transgenic mice, the onset of puberty is accelerated (Yura et al., 2000). Moreover, leptin affects various peripheral functions, such as glucose and lipid metabolism (Masuzaki et al., 1999; Ogawa et al., 1999), insulin sensitivity (Kim et al., 2000; Ebihara et al., 2001), hematopoiesis (Cioffi et al., 1996; Mikhail et al., 1997), angiogenesis (Sierra-Honigmann et al., 1998), blood pressure (Shek et al., 1998; Aizawa-Abe et al., 2000), progesterone secretion in the ovary (Karlsson et al., 1997), etc.

2. LEPTIN AS A PLACENTA-DERIVED HORMONE

Recently, we found that plasma leptin levels in pregnant women are significantly higher than those in non-pregnant women (Masuzaki et al., 1997). To elucidate the site of leptin production during pregnancy, we measured leptin levels in maternal and cord plasma.

Irrespective to BMI, plasma leptin levels in pregnant women were significantly higher than in non-pregnant women. When the plasma leptin levels were measured consecutively during 40 normal pregnancies and puerperium, plasma leptin levels in the first trimester were twice as high as the non-pregnant levels. In the second and third trimester, plasma leptin levels further increased to approximately 35 ng/ml, and returned to normal non-pregnant level within 24 h, suggesting that the major source of leptin in maternal plasma is the placenta (Masuzaki et al., 1997).

To confirm leptin production by the placenta, we examined leptin gene expression in human pregnant uteri. Northern blot analysis identified in the placental chorionic tissue, a single leptin mRNA species of the same size (~4.5 kb) as in mature adipocytes. Expression of the leptin gene was found abundantly in the first trimester chorionic villi, and slightly in the third trimester chorion laeve, and amnion. Immunohistochemically, both syncytiotrophoblasts and cytotrophoblasts were stained positively for leptin (Masuzaki et al., 1997).

Plasma leptin levels in umbilical arteries and umbilical veins were significantly lower than those in paired maternal plasma. Moreover, leptin levels in umbilical artery were significantly lower than those in paired umbilical vein (Yura et al., 1998a).

All these results indicate that leptin is synthesized in the human placenta, and is secreted to both maternal and fetal circulations.

Recent in vitro studies confirmed that most of the leptin produced by the placenta is secreted into the maternal circulation, but that a considerable proportion of leptin is also released into fetal circulation (Linnemann et al., 2000; Hoggard et al., 2001; Lepercq et al, 2001). Linnemann et al. (2000) and Lepercq et al. (2001) reported that a relatively low proportion (1.6 per cent and 5 per cent, respectively) of placental leptin is secreted into the fetal circulation. Lepercq et al. (2001) also measured leptin mRNA expression in the fetal adipose tissue by RT-PCR and suggested that umbilical leptin levels can be taken as a marker of fat tissue in human fetuses. In contrast, Hoggard et al. (2001) reported that a higher proportion (13.6 per cent) of leptin is released into fetal circulation. Thus, the origin and physiological significance of leptin in the fetal circulation are the interesting aim of the future investigation.

3. HYPERLEPTINEMIA IN COMPLICATED PREGNANCY

To explore the pathophysiological significance of leptin in complicated pregnancy, we measured the plasma leptin level and placental leptin mRNA expression in pregnant women with pre-eclampsia.

Pre-eclampsia is a hypertensive disorder, which develops in late pregnancy and is usually associated with fetal growth retardation due to placental dysfunction. To explore the pathophysiological significance of leptin in pre-eclampsia, we measured the plasma leptin level and placental leptin mRNA expression in pregnant women with pre-eclampsia.

Pre-eclampsia was divided into two subgroups, mild and severe pre-eclampsia, according to the definition by The American College of Obstetricians and Gynecologists (ACOG). No significant differences in plasma leptin levels were observed between the mild pre-eclampsia group and its gestational age-matched control group. By contrast, plasma leptin levels in the severe pre-eclampsia group were approximately threefold higher than those in its gestational age-matched control group. Plasma leptin levels in the severe pre-eclampsia group were also significantly higher than those in the mild pre-eclampsia group (Mise et al., 1998).

Leptin mRNA expression was markedly augmented in the placental tissue form pre-eclamptic women as compared to gestational age-matched normal pregnant women. The leptin mRNA level in a severe pre-eclampsia was markedly higher than those in mild pre-eclampsia. In this study, placental leptin mRNA levels were roughly parallel to plasma leptin levels in all the pre-eclamptic women examined (Mise et al., 1998).

We next compared the plasma leptin levels with maternal and fetal clinical features of pre-eclampsia. Plasma leptin levels showed a positive correlation with the mean arterial blood pressure in pregnant women with pre-eclampsia, but not with body mass index (BMI). Plasma leptin levels showed a negative correlation with DSD or delta SD of neonatal body weight, the degree of fetal growth restriction.

On the other hand, plasma leptin levels in pre-eclamptic women who delivered small-for-date (SFD) newborns were significantly higher than those in women who delivered

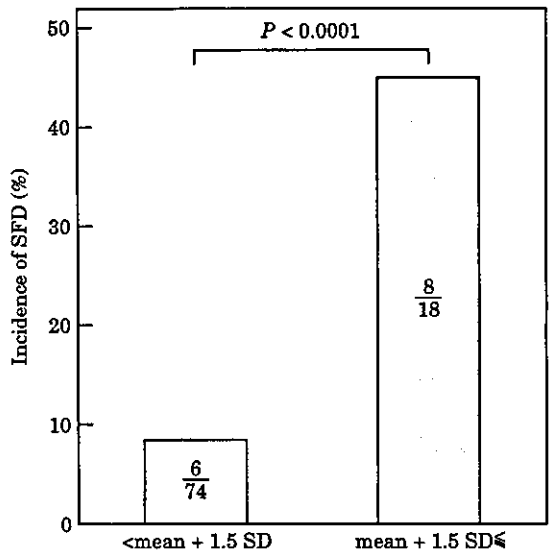

Figure 1. Incidence of SFD in pregnant women with plasma leptin levels greater than mean + 1.5 s.d. The incidence of small-for-date (SFD) infant (44.4 per cent) in pregnant women with plasma leptin levels larger than mean + 1.5 s.d. was significantly ($P<0.0001$) higher than that in women with plasma leptin levels less than mean + 1.5 s.d. (8.1 per cent). Statistical analysis was performed with both Mann–Whitney U test and analysis of variance (ANOVA) followed by Fisher's protected least significance difference test.

appropriate-for-date (AFD) newborns. Moreover, the incidence of SFD in pregnant women with plasma leptin levels higher than mean + 1.5 standard deviation was significantly higher than that in women with normal leptin levels (Figure 1). These findings suggest a close correlation between fetal growth restriction and the elevated plasma leptin levels.

We previously demonstrated that leptin synthesis and secretion are increased during the course of forskolin-induced cellular differentiation from cytotrophoblasts to syncytiotrophoblasts in human choriocarcinoma cell line, BeWo cells, and suggested that BeWo cells are the useful in vitro model system with which to access the regulation of leptin production in placental trophoblasts (Masuzaki et al., 1997). To elucidate the mechanisms for augmented placental production of leptin in pre-eclampsia, we examined the effects of hypoxia on leptin secretion using BeWo cells. Treatment of BeWo cells with 20 μM forskolin induced dose- and time-dependent increases in leptin and hCG secretions. Hypoxic stimulation was performed by exposing cells to 5 per cent oxygen condition. Up to 48 h, no significant differences in leptin levels in the culture media were observed between BeWo cells cultured under hypoxic conditions with 5 per cent oxygen and those cultured under standard conditions with 20 per cent oxygen. In BeWo cells cultured for 72 h under 5 per cent oxygen, leptin secretion was increased approximately threefold relative to those cultured under 20 per cent oxygen. By contrast, hCG levels in the culture media from BeWo cells cultured under 5 per cent oxygen were decreased significantly as compared to those cultured under 20 per cent oxygen (Mise et al., 1998).

Figure 2 shows a hypothetical relationship between the augmented leptin secretion from placenta and fetal growth restriction in severe pre-eclampsia. In severe pre-eclampsia with hypertension, maternal utero-placental blood flow is impaired. The impaired placental circulation causes chronic disturbance of nutrient supply and finally results in fetal growth restriction. On the other hand, placental hypoperfusion also produces local hypoxia, which consequently augments leptin gene expression in the placenta. It is plausible that the elevated leptin levels in the maternal circulation may aggravate hypertension, since leptin activates sympathetic nervous system and stimulates catecholamine secretion (Shek et al., 1998; Aizawa-Abe et al., 2000). Thus, maternal plasma leptin levels possibly reflect the feto-placental milieu in pregnancy complicated with fetal growth restriction.

Augmented placental leptin mRNA expression is also reported in pregnancies complicated with diabetes mellitus, especially in pregnant women treated with insulin (Lepercq et al., 1998; Lea et al., 2000). Leptin concentration in both maternal plasma and placental tissue are increased in these patients. A physiological role of insulin in the regulation of leptin production in the human placenta has been proposed (Lepercq et al., 1998). On the other hand, plasma leptin levels in the pregnant women complicated with mild gestational diabetes mellitus were lower as compared to women with normal glucose tolerance after adjusting for body mass index and fasting insulin levels (Festa et al., 1999). Leptin gene expression in adipocytes is reported to be stimulated by insulin (MacDougald et al., 1995; Rentsch and Chiesi, 1996).

There are several reports on the analysis of promoter region of leptin gene in mouse and human adipose tissue (He et al., 1995; Hwang et al., 1996; De la Brousse et al., 1996; Miller et al., 1996). These reports demonstrated that C/EBPα is involved in the adipocyte-specific transcription of the mouse and human leptin genes. Miller et al. (1996) reported that only 217 bp of 5′ sequence are required for basal adipose tissue-specific expression of the leptin gene as well as enhanced expression by C/EBPα. On the other hand, Ebihara et al. reported that DNA sequences between −1885 and −1830 of human leptin 5′-franking sequences are involved in the trophoblast-specific transcription of human leptin gene. By electrophoresis mobility shift assay, this sequence is not blocked by oligonucleotide encompassing the consensus C/EBP binding site (Ebihara et al., 1997). Moreover, leptin gene expression in trophoblast was enhanced by activation of protein kinase C (Yura et al., 1998b), while leptin gene expression in adipose tissue was inhibited by protein kinase C (Pineiro et al., 1998). These findings together suggest that regulation of leptin gene expression in trophoblast is different from that in adipose tissue. Elucidation of the precise mechanism for augmentation of leptin gene expression in the placenta of diabetic pregnancy requires further investigation.

Leptin gene expression is also augmented in hydatidiform mole (Sagawa et al., 1997). Augmented leptin expression in the placenta may stimulate the proliferation of trophoblast, since trophoblast expresses functional leptin receptor (Henson et al., 1998; Bodner et al., 1999).

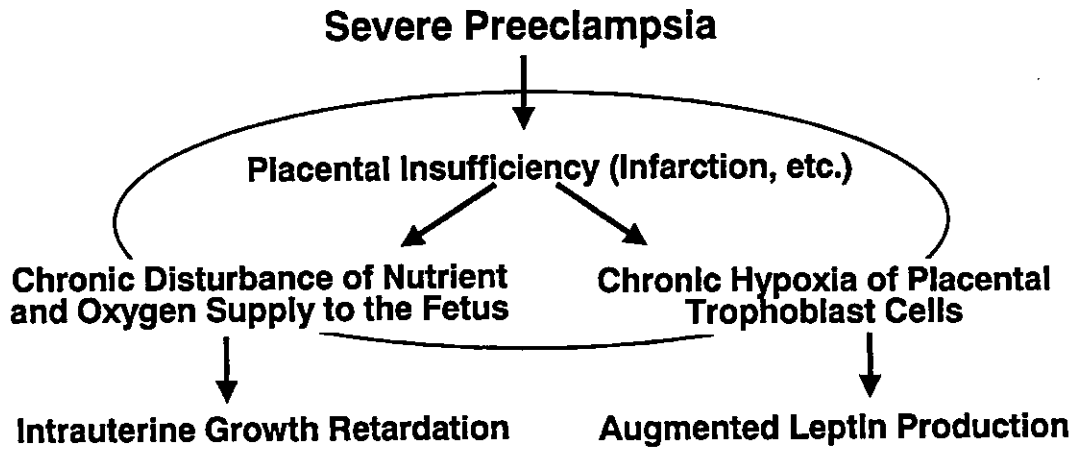

Figure 2. Relationship between augmented leptin secretion and fetal growth suppression in severe pre-eclampsia (a hypothesis). Placental insufficiency causes both augmentation of placental leptin production and fetal growth suppression. Hyperleptinemia may have association with fetal growth retardation as is discussed in the experiment with leptin over-expressing transgenic mice.

4. EXPERIMENT WITH LEPTIN OVER-EXPRESSING TRANSGENIC MICE

We further examined the effects of hyperleptinemia on the course of pregnancy by using leptin over-expressing transgenic (Tg) mice. In mice, leptin gene is not expressed in the placenta. Therefore, we generated transgenic mice over-expressing leptin under the control of the liver-specific human serum amyloid P component (SAP) promoter. The human SAP promoter is highly specific to the liver and is active only after birth (Masuzaki et al., 1999; Ogawa et al., 1999). Pregnant Tg mice were obtained by mating normal male non-Tg mice to female Tg mice (10–12 weeks of age).

Plasma leptin levels in the non-pregnant Tg mice at 12 weeks of age were 10 times higher than those in control non-Tg mice. Daily urinary norepinephrine excretion in Tg mice was significantly higher than that in non-Tg mice, indicating the activation of sympathetic nervous system by heperleptinemia (Aizawa-Abe et al., 2000).

In mice, placenta secretes soluble leptin receptor into maternal circulation, which binds to leptin and inhibits leptin clearance from plasma (Gavrilova et al., 1997). Thus, in non-Tg mice, plasma leptin levels increased 20-folds during pregnancy. On the other hand, plasma leptin levels in leptin over-expressing Tg mice before pregnancy (81.4 ± 15.4 ng/mL, mean ± s.d., $n=4$) were 10-times higher than those in non-Tg control mice, and interestingly, they were further elevated to 611 ± 199 ng/mL ($n=4$) in late pregnancy.

The elevated plasma leptin levels did not suppress the amount of food intake in non-Tg mice throughout the pregnancy, suggesting the so-called leptin-resistance in pregnant mice. Leptin resistance in rodent and obese human is proposed to be related with decreased cerebrospinal fluid/serum leptin ratio, which is regulated by the transport of leptin across the blood-brain barrier (Caro et al., 1996; Kastin & Pan, 2000). However, food intake was significantly suppressed in leptin over-expressing Tg mice compared to non-Tg mice, suggesting the possibility that even in the pregnant state, leptin can be functional when its level is high enough (Sagawa et al., 2001).

Blood pressure in the non-pregnant Tg mice was significantly higher than that in control non-Tg mice (Aizawa-Abe et al., 2000). The systolic blood pressure of Tg mice at 18th day of pregnancy (113 ± 5.0 mmHg, $n=4$) showed higher tendency than that of non-Tg mice (102.6 ± 2.6 mmHg, $n=4$), although the difference was not statistically significant. The litter size of leptin over-expressing Tg mice at 19th day of pregnancy (7.5 ± 1.29; mean ± s.d., $n=4$) was similar to that of non-Tg control mice (7.25 ± 1.26, $n=4$). Therefore, we calculated the mean fetal body weight of each litter. When we used these values for statistical analysis, the mean fetal body weights of leptin over-expressing Tg mice (0.95 ± 0.03 g, $n=4$) were significantly ($P<0.001$) less than those of non-Tg control mice (1.22 ± 0.01, $n=4$) (Sagawa et al., 2001).

These results suggest that hyperleptinemia may affect the fetal growth by modulating maternal food intake and vascular tone. Another major function of leptin is regulation of glucose metabolism and insulin sensitivity (Masuzaki et al., 1999; Ogawa et al., 1999; Kim et al., 2000). In an experimental animal with mutation of leptin receptor, leptin administration prevents spontaneous gestational diabetes (Yamashita et al., 2001). Although these data are based on rodent experiment, it is possible that hyperleptinemia may also affect fetal growth through this pathway, since maternal plasma glucose level is one of the essential nutrients for fetal growth. Further study of the regulation of maternal glucose metabolism by leptin is necessary to confirm the role of placental leptin in fetal growth in humans.

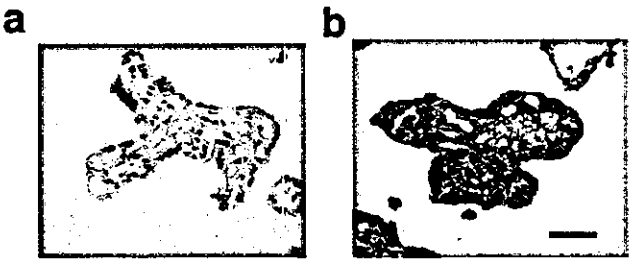

Figure 3. In situ hybridization analysis of resistin gene expression in term placenta. Placental tissue was collected from a pregnant woman at elective caesarean section at 38 weeks gestation. a: sense probe, b: anti-sense probe. Horizontal bar indicates 100 μm.

5. PLACENTAL RESISTIN AS A REGULATOR OF MATERNAL ENERGY METABOLISM

Resistin is a newly identified adipocyte-derived hormone that decreases insulin sensitivity and increases plasma glucose concentration, thus contributing to the development of type II diabetes mellitus (Steppan et al., 2001). Therefore, we examined the involvement of resistin in maternal glucose metabolism during pregnancy.

Resistin is proposed to link obesity to insulin resistance (Flier, 2001). Adipocyte secretes various substances that modulate insulin sensitivity, such as free fatty acid, TNF-α, and leptin. Resistin was recently cloned by Steppan et al. (2001) and expression in the adipose tissue was decreased by the treatment with thiazolidinedione, an anti-diabetic drug. Thus, resistin is proposed to be a major factor that induces insulin resistance and hyperglycemia in obese individuals.

In mice experiment, pretreatment with recombinant resistin significantly increased plasma glucose levels after glucose injection and blunted the suppression of plasma glucose levels by insulin injection (Steppan et al., 2001). Thus, resistin interferes insulin action and increases plasma glucose levels in vivo. Therefore, we examined resistin gene expression in the human placenta.

Northern blot analysis revealed resistin mRNA expression in term placenta as well as in the amniotic membrane (Sagawa et al., unpublished results). Resistin mRNA expression was also detected in a trophoblastic cell line (BeWo cells) and a very faint band was detected in decidua vera tissue. In situ hybridization revealed positive staining for resistin mRNA in placental villi, mainly in syncitiotrophoblast, while no staining was observed with a sense RNA probe (Figure 3).

We next examined changes in resistin gene expression during human pregnancy. Resistin gene expression in term placental tissue was significantly greater than that in chorionic villous tissue in the first trimester ($P<0.01$) (Sagawa et al., 2001). By contrast, the resistin gene was expressed to a lesser degree in adipose tissue than in term placental tissue (Sagawa et al., unpublished results). Moreover, resistin gene expression in adipose tissue of pregnant women at term did not differ from that of non-pregnant women.

Maternal insulin sensitivity is regulated by various factors including placenta-derived hormones. Several placenta-derived hormones, such as prolactin, human placental lactogen and steroid hormones, are considered to decrease insulin sensitivity. Placental production of these hormones is increased in accordance with the increasing size of the placenta. On the other hand, we have reported that the human placenta produces leptin (Masuzaki et al., 1997), a well known adipoctye-derived hormone, which increases insulin sensitivity and corrects the hyperglycaemia in leptin deficient *ob/ob* mice (Pelleymounter et al., 1995). Moreover, in the present study, we present evidence that resistin is a new member of placental hormone in humans. As resistin is supposed to induce insulin resistance and increase the plasma glucose level (Flier, 2001; Steppan et al., 2001), it is possible that placental resistin may contribute to the regulation of maternal glucose metabolism in concert with various placental hormones, including leptin.

CONCLUSION

Figure 4 schematically illustrates the hypothesis of physiological functions of placental leptin and resistin.

The leptin receptor is expressed in various tissues, such as hypothalamus, muscle, liver, adipose tissue, etc. (Tartaglia et al., 1995; Hoggard et al., 1997; Karlsson et al., 1998). In addition to adipose tissue-derived leptin, placenta-derived leptin may act on the hypothalamus, and regulate maternal energy expenditure and neuroendocrine functions (Chehab et al., 1996; Yura et al., 2000). On the other hand, placental leptin may also act on maternal peripheral tissues, such as muscle, liver, or pancreas, and regulate glucose metabolism and insulin sensitivity (Masuzaki et al., 1999; Ogawa et al., 1999; Ebihara et al., 2001). In addition, placental leptin is transferred to the fetus (Linnemann et al., 2000; Hoggard et al., 2001; Lepercq et al., 2001) and may regulate fetal development and growth (Hoggard et al., 1997).

This study presents evidence that resistin is expressed in the human placenta, and the expression in this tissue is higher than that in adipose tissue. Thus, it is plausible that placenta-derived resistin may have physiological significance in the regulation of maternal glucose metabolism by decreasing insulin sensitivity during human pregnancy.

In summary, the present study provides the evidence for leptin as a novel placenta-derived hormone in humans, and suggests the physiologic and pathophysiological significance of leptin in fetal growth in normal and complicated pregnancy. Moreover, we have demonstrated for the first time that the resistin gene is expressed in human placental tissue, leading to a novel view of resistin as a placenta-derived regulator of glucose metabolism during pregnancy. However, the regulatory mechanism of resistin gene expression in the human placenta has not yet elucidated. Moreover, interaction between leptin and resistin actions has not been studied. Further investigation on the effects of both leptin and resistin on the maternal glucose metabolism and insulin sensitivity may provide better understanding of the placental role in the maternal energy metabolism and fetal growth.

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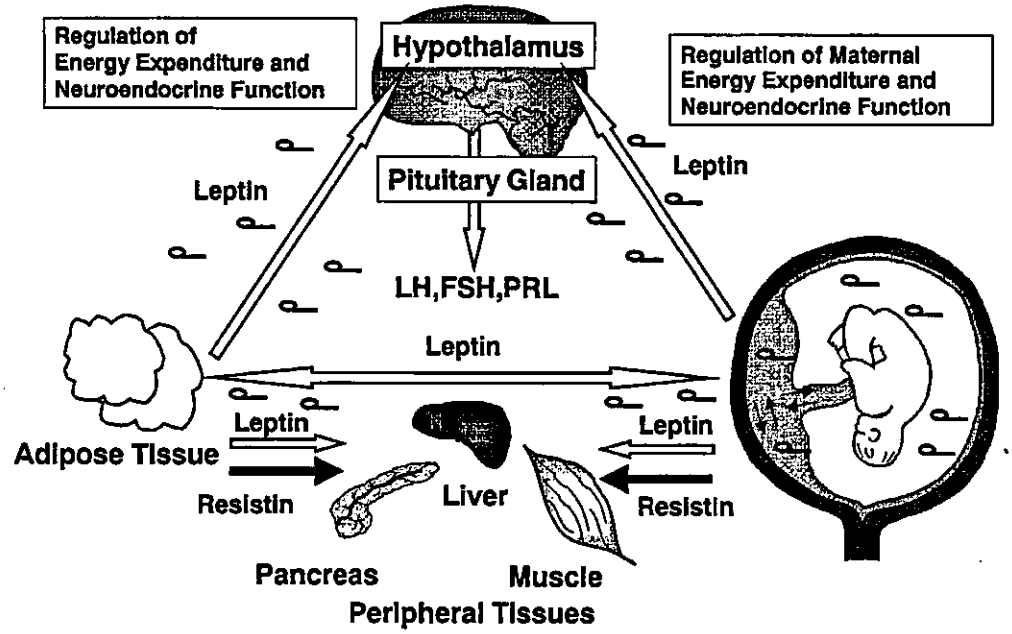

Figure 4. Physiological functions of placental leptin and resistin. Placenta-derived leptin may act on hypothalamus, and regulate the maternal energy expenditure and neuroendocrine functions, since leptin receptor is expressed in the hypothalamus. Placenta-derived leptin may also affect on the glucose metabolism in the liver, pancreas and muscle, since these organs express functional leptin receptors. In addition, placenta secretes leptin into fetal circulation, although the role of leptin in the fetal growth and development has not been proven. It is possible that placenta-derived resistin may modulate maternal glucose metabolism since resistin administration decreases insulin sensitivity in mice.

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