[2001] studied four heterozygous missense mutations in exon 6 of *TRPS1* in eight individuals with TRPS from seven different families. They included patients reported by Kajii [1994; also described by Sugio and Kajii, 1984], Nagai et al. [1994], and Vilain et al. [1999], and those from the Netherlands, Germany, Belgium, and Turkey. In addition, they found a heterozygous 2681T → A missense mutation in a 12-year-old boy with a phenotype compatible with TRPS III. His mother, however, had the same heterozygous mutation and a TRPS I phenotype. The G → A conversion at nt 2723 in the present woman has been found in a boy from the Netherlands (ID 5874) and in a girl from Germany (ID 7795). Nucleotide 2723 was also affected in a family reported by Nagai et al. [1994]. It seems that nt 2723 is a mutation hot spot for TRPS III.

In conclusion, we identified a missense mutation of *TRPS1* in a new Japanese family of TRPS III. Data of this study support and reinforce the recent finding that TRPS III results from missense mutations in exon 6 of *TRPS1* [Lüdecke et al., 2001].

ACKNOWLEDGMENTS

We thank Dr. Hermann-Josef Lüdecke for critical reading of the manuscript and Drs. Toshiro Nagai and Tomonobu Hasegawa for valuable discussion.

REFERENCES

Itin PH, Bohn S, Mathys D, Guggenheim R, Richard G. 1996. Trichorhinophalangeal syndrome type III. Dermatology 193:349–352.

Kajii T, Gonzalez IF, Matsuura S. 1994. Tricho-rhino-phalangeal syndrome type III. Am J Med Genet 49:349–350.

Lüdecke H-J, Schaper J, Meinecke P, Momeni P, Groß S, von Holtum D, Hirche H, Abramowicz MJ, Albrecht B, Apacik C, Christen H-J, Claussen U, Devriendt K, Fastnacht E, Forderer A, Friedrich U, Goodship THJ, Greiwe M, Hamm H, Hennekam RCM, Hinkel GK, Hoeltzenbein M, Kayserili H, Majewski F, Mathieu M, McLeod R, Midro AT, Moog U, Nagai T, Niikawa N, Ørstavik KH, Plöchl E, Seitz C, Schmidtke J, Tranebjærg L, Tsukahara M, Wittwer B, Zabel B, Gillessen-Kaesbach G, Horsthemke B. 2001. Genotypic and phenotypic spectrum in the tricho-rhino-phalangeal syndromes types I and III. Am J Hum Genet 68:81–91.

Momeni P, Glockner G, Schmidt O, von Holtum D, Albrecht B, Gillessen-Kasabach G, Hennekam R, Meinecke P, Zabel B, Rosenthal A, Horsthemke B, Lüdecke HJ. 2000. Mutations in a new gene, encoding a zinc-finger protein, cause tricho-rhino-phalangeal syndrome type I. Nat Genet 24:71–74.

Nagai T, Nishimura G, Kasai H, Hasegawa T, Kato R, Ohashi H, Fukushima Y. 1994. Another family with tricho-rhino-phalangeal syndrome type III (Sugio-Kajii syndrome). Am J Med Genet 49:278–280.

Niikawa N, Kamei T. 1986. The Sugio-Kajii syndrome, proposed tricho-rhino-phalangeal syndrome type III. Am J Med Genet 24:759–760.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning. New York: Cold Spring Harbor Laboratory Press. p 9.14.

Sugio Y, Kajii T. 1984. Ruvalcava syndrome: autosomal dominant inheritance. Am J Med Genet 19:741–753.

Vilain C, Sznajer Y, Rypens F, Desir D, Abramowicz M. 1999. Sporadic case of trichorhinophalangeal syndrome type III in a European patient. Am J Med Genet 85:495–497.

ELSEVIER

Journal of Diabetes and Its Complications 16 (2002) 119–122

JOURNAL OF Diabetes AND ITS Complications

Pathophysiogical role of leptin in lifestyle-related diseases Studies with transgenic skinny mice overexpressing leptin

Yoshihiro Ogawa*, Hiroaki Masuzaki, Ken Ebihara, Mitsuyo Shintani, Megumi Aizawa-Abe, Fumiko Miyanaga, Kazuwa Nakao

Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Received 25 June 2001; accepted 2 July 2001

Abstract

Leptin is a major adipocyte-derived hormone that is involved in the regulation of food intake and energy expenditure. Plasma leptin concentrations are elevated in obese subjects, suggesting its pathophysiological role in obesity-related lifestyle-related diseases. We have recently succeeded in the generation of transgenic skinny mice overexpressing leptin. They exhibit increased glucose metabolism and insulin sensitivity accompanied by a significant increase in insulin signaling for glucose utilization in the skeletal muscle and liver. They also show blood pressure elevation through the sympathetic activation. Introduction of the lethal yellow agouti (A^y) allele into transgenic skinny mice results in late-onset obesity and diabetes with blood pressure elevation similar to those found in nontransgenic agouti mice (A^y/+ mice). After caloric restriction, blood pressure elevation is reversed but insulin resistance still remains in A^y/+ mice in parallel with a reduction of plasma leptin concentrations. By contrast, blood pressure elevation is sustained but insulin resistance is reversed in transgenic mice overexpressing leptin with the A^y allele (Tg/+:A^y/+ mice), which remain hyperleptinemic. Collectively, our data suggest the pathophysiologic and therapeutic implication of leptin in obesity-related insulin resistance and hypertension.

Keywords: Leptin; Transgenic skinny mice; Diabetes; Hypertension

1. Introduction

Obesity is defined as increased mass of adipose tissue and confers a higher risk of cardiovascular and metabolic lifestyle-related diseases such as diabetes, hypertension, and hyperlipidemia (Spiegelman & Flier, 1996). The adipose tissue has been regarded as a triglyceride storage organ, but recent advances in molecular and cell biology have revealed that the adipose tissue is involved in the regulation of a variety of homeostatic processes as an important endocrine organ that secretes many biologically active substances such as tumor necrosis factor-α, free fatty acids, and so forth (Spiegelman & Flier, 1996).

Leptin is such an adipocyte-derived hormone that brings about decreased food intake and increased energy expenditure (Fig. 1), thereby representing one of the defense mechanisms against the development of overweight or obesity (Friedman & Halaas, 1996). Accordingly, the potential usefulness of leptin for treatment of obesity and obesity-related diseases has attracted the interest of many clinical investigators. Numerous studies have demonstrated that plasma leptin concentrations are elevated in obese subjects in proportion to the degree of adiposity (Considine et al., 1996), suggesting the state of impaired leptin action or leptin resistance. On the other hand, it is speculated, although paradoxically, that hyperleptinemia may play a role in the pathogenesis of obesity and obesity-related complications. In an attempt to assess the role of chronic hyperleptinemia in vivo, we have recently created transgenic skinny mice with elevated plasma leptin concentrations comparable to those found in markedly obese subjects (Ogawa et al., 1999). In this article, we discuss the possible pathophysiological role of leptin learned from transgenic skinny mice.

2. Transgenic skinny mice overexpressing leptin

We constructed a fusion gene comprising the human serum amyloid component promoter and mouse leptin

* Corresponding author. Tel.: +81-75-751-3173; fax: +81-75-771-9452.
E-mail address: ogawa@kuhp-kyoto-u.ac.jp (Y. Ogawa).

1056-8727/02/$ – see front matter
PII: S1056-8727(01)00204-5

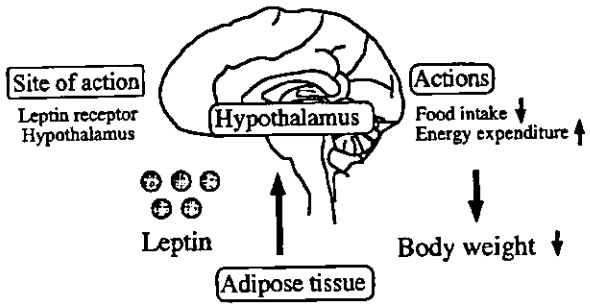

Fig. 1. Leptin as an adipocyte-derived circulating satiety factor that regulates food intake and energy expenditure.

cDNA coding sequences so that the hormone expression might be targeted to the liver (Ogawa et al., 1999). Plasma leptin concentrations are elevated in transgenic mice according to the copy numbers of the transgene incorporated, and have reached up to approximately 50 ng/ml in transgenic mice with 30 copies of the transgene. Food intake and body weight of transgenic mice are approximately 70% of those of nontransgenic littermates. They are apparently devoid of white and brown adipose tissue (Fig. 2). We, therefore, call these animals transgenic "skinny" mice. The animals have proved to be the useful model system to investigate the functional role of chronic hyperleptinemia in vivo (Aizawa-Abe et al., 2000; Masuzaki et al., 1999; Matsuoka et al., 2001; Ogawa et al., 1999; Yura et al., 2000).

3. Glucose metabolism in transgenic skinny mice

Evidence has accumulated indicating that leptin is involved in the regulation of glucose metabolism, thus suggesting the pathophysiological role of leptin in insulin resistance and diabetes. Transgenic skinny mice exhibit normoglycemia despite hypoinsulinemia relative to nontransgenic littermates (Ogawa et al., 1999). Glucose and insulin tolerance tests have revealed increased glucose metabolism and insulin sensitivity accompanied by increased insulin signaling in the skeletal muscle and liver (Fig. 2). These findings suggest that leptin might be an antidiabetic factor. Transgenic skinny mice, when treated with storeptozotocin (STZ), develop insulin-deficient diabetes similar to that in nontransgenic littermates. In response to a low dose of insulin that does not normalize diabetes in STZ-treated nontransgenic littermates, transgenic skinny mice with STZ-induced diabetes show a remarkable improvement of glucose metabolism. These observations suggest the potential usefulness of coadministration of leptin and insulin for treatment of insulin-deficient diabetes. Recently, we have crossed transgenic skinny mice and a mouse model of lipoatrophic diabetes (A-ZIP/F-1 mice) (Moitra et al., 1998) and demonstrated that chronic hyperleptinemia can rescue insulin resistance and diabetes in lipoatrophic diabetes on a long-term basis. Collectively, our data suggest the usefulness of leptin for treatment of diabetes of various causes.

4. Blood pressure elevation in transgenic skinny mice

Leptin is known to increase energy expenditure through the activation of sympathetic nervous system. Transgenic skinny mice show increased urinary catecholamine excretion relative to nontransgenic littermates, suggesting chronic sympathetic activation (Aizawa-Abe et al., 2000). They exhibit 10–15 mm Hg blood pressure elevation relative to nontransgenic littermates (Fig. 2). Blood pressure elevation is normalized in transgenic skinny mice treated with α-adrenoceptor, β-adrenoceptor, and sympathetic ganglionic blockers that do not affect blood pressure in nontransgenic littermates. These findings suggest that chronic hyperleptinemia can increase blood pressure through the sympathetic activation (Aizawa-Abe et al., 2000). It has been proposed that insulin resistance or hyperinsulinemia secondary to it may play a role in the pathogenesis of obesity-related hypertension (Landberg & Krieger, 1989). Our data suggest that chronic hyperleptinemia can cause blood pressure elevation through sympathetic activation without insulin resistance and hyperinsulinemia. Thus, leptin may be involved in the pathogenesis of obesity-related hypertension via insulin-independent mechanisms.

5. Pathophysiological implication of leptin in caloric restriction

Weight reduction is one of the most powerful strategies for treatment of obesity and obesity-related complications such as obesity-related insulin-resistant diabetes and hypertension. However, molecular mechanisms responsible for it are poorly understood. Plasma leptin concentrations are reduced during caloric restriction or body weight loss,

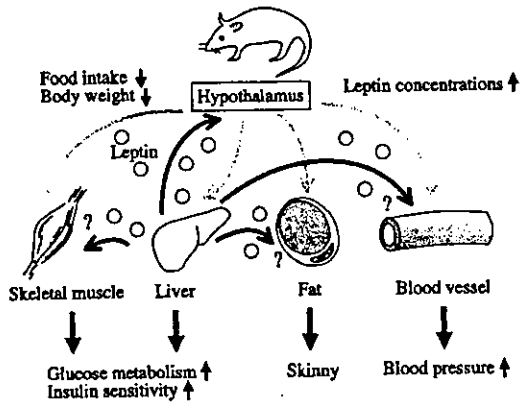

Fig. 2. Cardiovascular and metabolic phenotypes of transgenic skinny mice overexpressing leptin.

suggesting that leptin deficiency may play a role in the metabolic response to caloric restriction (Ahima et al., 1996). As a first step toward understanding the therapeutic implication of leptin in obesity-related insulin-resistant diabetes, we have crossed transgenic skinny mice (Tg/+) and KKA^y (A^y/+) mice, a well-established model of late-onset obesity and Type 2 diabetes (Masuzaki et al., 1999). The double transgenic mice (Tg/+:A^y/+ mice) are of normal weight with increased insulin sensitivity at younger ages. At older ages, however, they are phenotypically indistinguishable from A^y/+ mice; they both develop obesity, insulin-resistant diabetes, and blood pressure elevation similar to those in A^y/+ mice (Masuzaki et al., 1999). During caloric restriction, A^y/+ mice show hypoleptinemia in parallel with body weight reduction. However, in response to caloric restriction, Tg/+:A^y/+ mice remain to be hyperleptinemic despite body weight reduction, because of constitutive expression of the transgene in the liver. These observations suggest that Tg/+:A^y/+ mice are the unique experimental model to investigate the role of hypoleptinemia during caloric restriction (Masuzaki et al., 1999).

To assess the pathophysiological and therapeutic implications of leptin, we examined cardiovascular and metabolic responses of Tg/+:A^y/+ mice to caloric restriction (Fig. 3). In response to caloric restriction, both A^y/+ and Tg/+:A^y/+ mice lose weight with the normalization of plasma glucose concentrations (Aizawa-Abe et al., 2000; Masuzaki et al., 1999). However, insulin resistance remains in Tg/+:A^y/+ mice in parallel with a decrease in plasma leptin concentrations. By contrast, insulin resistance is reversed in Tg/+ and Tg/+:A^y/+ mice after caloric restriction (Masuzaki et al., 1999). These observations suggest that weight reduction in combination with leptin supplementation is more efficacious than weight reduction alone for treatment of obesity-related insulin resistance and diabetes.

We also examined blood pressure change in A^y/+ and Tg/+:A^y/+ mice during caloric restriction. Blood pressure elevation is reversed in A^y/+ mice after caloric restriction (Fig. 3). By contrast, Tg/+:A^y/+ mice are still hypertensive after caloric restriction (Aizawa-Abe et al., 2000). It is likely that leptin deficiency may reverse the sympathetic activation, thus contributing to blood pressure reduction after caloric restriction.

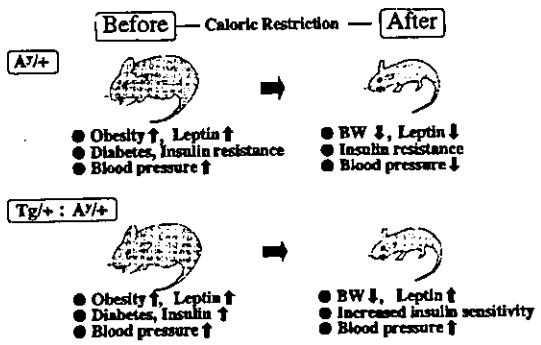

Fig. 3. Cardiovascular and metabolic responses of A^y/+ and Tg/+:A^y/+ mice to caloric restriction.

6. Conclusion

Leptin is a major adipocyte-derived hormone with multiple regulatory potentials and may be involved in the pathogenesis of lifestyle-related diseases. With its pleiotropic effect, leptin should be relevant from both pathophysiologic and therapeutic viewpoints. Our transgenic skinny mice overexpressing leptin have provided new insight into the pathophysiology of obesity-associated lifestyle-related diseases.

Acknowledgments

We thank Ms. Isa for secretarial assistance and Ms. M. Nagamoto for technical assistance. This work is supported in part by research grants from the Japanese Ministry of Education, Science, Sports, and Culture and the Japanese Ministry of Health and Welfare.

References

Ahima, R. S., Prabakanan, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., & Flier, J. S. (1996). Role of leptin in the neuroendocrine response to fasting. *Nature, 382*, 250–252.

Aizawa-Abe, M., Ogawa, Y., Masuzaki, H., Ebihara, K., Satoh, N., Iwai, H., Matsuoka, N., Hayashi, T., Hosoda, K., Inoue, G., Yoshimasa, Y., & Nakao, K. (2000). Pathophysiological role of leptin in obesity-related hypertension. *Journal of Clinical Investigation, 105*, 1243–1252.

Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., Mckee, L. J., Buer, T. L., & Caro, J. F. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine, 334*, 292–295.

Friedman, J. M., & Halaas, J. L. (1996). Leptin and the regulation of body weight in mammals. *Nature, 395*, 763–770.

Landberg, L., & Krieger, D. R. (1989). Obesity, metabolism, and the sympathetic nervous system. *American Journal of Hypertension, 2*, 125S–132S.

Masuzaki, H., Ogawa, Y., Aizawa-Abe, M., Hosoda, K., Suga, J., Ebihara, K., Satoh, N., Iwai, H., Inoue, G., Nishimura, H., Yoshimas, Y., & Nakao, K. (1999). Glucose metabolism and insulin sensitivity in transgenic mice overexpressing leptin with lethal yellow agouti mutation: usefulness of leptin for treatment of obesity-associated diabetes. *Diabetes, 48*, 1615–1622.

Matsuoka, N., Ogawa, Y., Masuzaki, H., Ebihara, K., Aizawa-Abe, M., Satoh, N., Ishikawa, E., Fujisawa, Y., Kosaki, A., Yamada, K., Kuzuya, H., & Nakao, K. (2001). Decreased triglyceride-rich lipoproteins in transgenic skinny mice overexpressing leptin. *American Journal of Physiology, 280*, E334–E339.

Moitra, J., Mason, M. M., Olive, M., Krylov, D., Gavrilova, O., Macus-Samuels, B., Feigenbaum, L., Lee, E., Aoyma, T., Eckhaus, M., Reitman, M. L., & Vinson, C. (1998). Life without white fat: a transgenic mouse. *Genes & Development, 12*, 3168–3181.

Ogawa, Y., Masuzaki, H., Hosoda, K., Aizawa-Abe, M., Suga, J., Suda, M., Ebihara, K., Iwai, H., Matsuoka, N., Satoh, N., Odaka, H., Kasuga, H.,

Fujisawa, Y., Inoue, G., Nishimura, H., Yoshimasa, Y., & Nakao, K. (1999). Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. *Diabetes*, *48*, 1822–1829.

Spiegelman, B. M., & Flier, J. S. (1996). Adipogenesis and obesity: rounding out the big problem. *Cell*, *87*, 377–389.

Yura, S., Ogawa, Y., Sagawa, N., Masuzaki, H., Itoh, H., Ebihara, K., Aizawa-Abe, M., Fujii, S., & Nakao, K. (2000). Accelerated puberty and late-onset hypothalamic hypogonadism in female transgenic skinny mice overexpressing leptin. *Journal of Clinical Investigation*, *105*, 749–755.

Clin Exp Immunol 2002; 128:21–26

Leptin inhibits stress-induced apoptosis of T lymphocytes

Y. FUJITA*, M. MURAKAMI*, Y. OGAWA*, H. MASUZAKI*, M. TANAKA*, S. OZAKI*, K. NAKAO* & T. MIMORI†
*Departments of *Medicine and Clinical Science and †Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan*

(Accepted for publication 20 December 2001)

SUMMARY

Leptin, which is secreted by adipocytes, the placenta and the stomach, not only controls appetite through leptin receptors in the hypothalamus but also regulates cell-mediated immunity. In this study we have demonstrated that continuous injection of leptin prevents the reduction in lymphocyte numbers normally observed in fasted and steroid-injected mice. Consistent with leptin-induced protection, we observed up-regulation of the bcl-xL gene as a result of signal transduction via leptin receptors on lymphocytes. We suggest that leptin might contribute to the recovery of immune suppression in malnourished mice by inhibiting lymphocyte apoptosis.

Keywords apoptosis bcl-xL corticosteroid leptin

INTRODUCTION

Nutritional status and immune function are closely related [1–3]. Food deprivation leads to impaired immune responses and an increase in the incidence of infectious disease, although the mechanism by which this occurs has yet to be elucidated. Adipose tissue preserves energy homeostasis through the storage of triglycerides. However, it has been found recently that a number of cytokine-like molecules, such as leptin [4], tumour necrosis factor-α (TNF-α) [5] and plasminogen activator inhibitor-1 (PAI-1) [6] are secreted from adipocytes, suggesting that adipose tissue may also play a role in the regulation of the immune and haematopoietic systems.

Leptin is secreted specifically by adipocytes [4], and serum leptin levels are proportional to body mass index. However, the placenta [7] and stomach [8] provide additional sources of leptin. Leptin decreases food intake, increases energy expenditure and reduces body weight via leptin receptors within the ventromedial hypothalamus [9], where leptin functions to inhibit the production of neuropeptide Y which stimulates food intake [10]. The murine leptin and leptin-receptor mutants *ob/ob* and *db/db*, respectively, serve as animal models of obesity, and develop marked obesity and diabetes due to deficiencies in leptin signalling [11]. In contrast, leptin transgenic mice with elevated plasma leptin concentrations lack brown or white adipose tissue, show reduced food intake, and are markedly lean in comparison with non-transgenic littermates [12].

Correspondence: Yoshimasa Fujita, Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606–8507, Japan.
E-mail: s75@kuhp.kyoto-u.ac.jp

The leptin receptor is expressed in peripheral tissues such as the kidney, lung and adrenal gland [13,14], and several *in vitro* studies have demonstrated that leptin acts directly on the leptin receptor [15,16]. There are at least five splice variants of the leptin receptor Ob-Ra–Ob-Re, and one of these five variants, Ob-Rb, possesses a long intracellular domain demonstrating homology with gp130, a subunit of the IL-6 family of cytokine receptors [17]. On the other hand, Ob-Ra, one of the shortest forms of the leptin receptor, lacks the STAT3 activation domain and is not considered essential for signal transduction [18].

Recent studies have revealed that Ob-Rb is expressed in fetal liver haematopoietic precursor cells, bone marrow and peripheral T cells [14,19]. In adult human bone marrow, both CD34 positive and negative cells express leptin receptor. These findings suggest the possibility that leptin not only regulates body weight, but also modulates the immune system. Indeed, leptin increases the proliferation of haematopoietic stem cell populations at the multilineage progenitor level [18], enhances alloproliferative mixed-lymphocyte reactions, and reverses cellular immune function in fasted mice [20]. In addition, leptin might act as a growth factor for both myeloid leukaemic cells [21] and lung cancer cells [22]. In addition, human white blood cell counts are correlated with body mass index and serum leptin levels [23]. Moreover, diminished cell-mediated immunity and decreased lymphocyte counts have been reported in *ob/ob* and *db/db* mice [24,25].

We demonstrate here that leptin receptor messenger RNA is expressed in lymphoid tissue, and that leptin both restores the decrease in lymphocyte numbers normally observed in fasted mice and prevents apoptosis of lymphocytes in steroid-injected mice. Consistent with the observed anti-apoptotic effect of leptin, we observed up-regulation of the bcl-xL gene by leptin. We suggest that leptin may contribute to recovery of immune

suppression in malnourished mice by inhibiting lymphocyte apoptosis.

MATERIALS AND METHODS

Mice and reagents

Female C57BL/6 mice (6 weeks old) and C57BL/Ks db/db (6 weeks old) were purchased from SLC (Kyoto, Japan) and CLEA (Tokyo, Japan), respectively. Leptin transgenic mice were bred in our laboratory [12]. Recombinant mouse leptin was purchased from R&D Systems (Minneapolis, USA). Hydrocortisone phosphate was purchased from Banyu Pharmaceutical Co. (Tokyo, Japan). All monoclonal antibodies (MoAbs), including hamster anti-CD3 MoAb (2C11), antimouse bcl-2 MoAb and rat antimouse bcl-x MoAb, were purchased from Becton-Dickinson (Franklin Lakes, USA). N-acetylsphingosine (C2-ceramide) was purchased from Sigma Aldrich Japan (Tokyo, Japan).

RT-PCR

Total RNA was extracted from cells using TRIzol® reagent (GIBCO/BRL, Rockville, USA). Five micrograms of total RNA was reverse-transcribed and PCR was performed using specific Ob-Ra and Ob-Rb primers [13].

Fasting and steroid injection experiments

Mice were kept without food for 60 h, with repeated intraperitoneal injection of 0, 1 or 10 μg/g initial mouse body weight of leptin in a solution of phosphate buffered saline (PBS) every 12 h. In a second experiment, 200 μg/g mouse body weight of hydrocortisone phosphate was administered intraperitoneally; 10 μg/g body weight of leptin was injected 2 h before and 4 h after administration of hydrocortisone phosphate. Mice were sacrificed 24 h after hydrocortisone injection.

Cell culture

1×10^5 cells/ml of T cell hybridoma A3·4C6 [26] were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml amphothericin B, 2 mM L-glutamine and 0·05 mM 2-mercaptoethanol, with or without recombinant mouse leptin, under conditions of 37°C and 5% CO_2 for 24 h. In the steroid-induced apoptosis experiment, cells were cultured with 10^{-6} M hydrocortisone for 24 h. In the ceramide-induced apoptosis experiment, cells were cultured with 10 μg/ml C2-ceramide for 18 h. In another experiment, cells were cultured for 5 h on 96-well plates (Costar 3590), coated with anti-CD3 antibody (10 g/ml) overnight.

Assay for DNA fragmentation

Agarose gel electrophoresis was carried out as previously described [27]. DNA was extracted from 1×10^6 cells/sample and suspended in 20 μL of lysis buffer (10 mM EDTA, 50 mM Tris HCl (pH 8·0), 0·5% sodium-N-lauroylsarcosinate) and 1 μL of 10 mg/ml RNaseA was added. Samples were then incubated at 50°C for 30 min, 1 μL of 10 mg/ml Proteinase K was added and incubated for 30 min at 50°C. Then, samples were analysed by agarose gel electrophoresis.

Cell staining

For cell surface analysis, 1×10^5 thymocytes were stained with FITC-conjugated anti-CD4 MoAb and PE-conjugated anti-CD8 MoAb and analysed by FACSCalibur® (Becton Dickinson), as described previously [27]. For detection of bcl-2 and bcl-xL gene products, cells were washed and fixed in PBS containing 4% paraformaldehyde for 20 min. Cells were then washed and suspended in staining buffer (1% BSA in PBS with 0·1% sodium azide) with 0·1% saponin, followed by incubation with hamster antimouse-bcl-2 MoAb or rat antimouse bcl-xL MoAb for 30 min on ice. After washing, cells were stained with FITC-conjugated antihamster IgG or FITC-conjugated antirat IgG, respectively, for 30 min on ice. After washing, cells were analysed by FACSCalibur®. In order to detect apoptosis, cells were stained with 50 μg/ml of propidium iodide after fixation with 70% ethanol for 4 h at 4°C and treatment with 100 μg/ml of RNaseA. After washing, apoptotic cells were determined as the proportion of hypodiploidal cells.

Northern blotting

Total RNA was extracted from A3·4C6, spleen and thymus cells using TRIzol® reagent. Samples of 15 μg of RNA were applied to each lane. After electrophoresis, RNA was transferred to a nylon membrane and hybridized with a ^{32}P-labelled mouse bcl-xL specific cDNA probe. Autoradiography was performed for 24 h and analysed using BAS 2000® (Fuji Photo Film, Tokyo, Japan).

RESULTS

To clarify the involvement of leptin in the immune response, we first confirmed the expression of leptin receptor messenger RNA in lymphoid tissue. Both short and long isoforms of the leptin receptor, Ob-Ra and Ob-Rb, were detected in the spleen, thymus and bone marrow by RT-PCR (Fig. 1).

Next, we investigated the effect of leptin on the number of lymphocytes detected in fasted mice. After 60 h of fasting, decreased numbers of thymocytes and splenocytes were detected (Table 1). In the thymus, the $CD4^+CD8^+$ T cell population decreased dramatically from 85% (control) to 25% in fasted mice (Fig. 2a). On the other hand, the proportion of CD4 and CD8 single-positive T cells did not differ among the spleens of fasted and fed control mice (data not shown). Repeated injection of leptin reduced the observed decrease in lymphocyte numbers within the thymus of fasted mice, particularly that of the $CD4^+CD8^+$ T cell population (Table 1, Fig. 2a). The protective effect of leptin was observed to be dose-dependent (Table 1, Fig. 2a). However, one area that remained unclear was whether leptin exerted a direct effect on lymphocytes, or whether it exerted its effect through an indirect mechanism, for example, by counteracting the endocrinological disturbances associated with fasting.

Fasting is widely known to cause the stress-induced release of several hormones *in vivo* [28]. In particular, a steroid hormone derived from the adrenal glands is suspected to play an important role in fasting-induced lymphopenia because lymphocyte numbers do not decrease in adrenectomized mice, even during fasting [29]. To investigate the effect of leptin on the steroid-induced cell death of lymphocytes, hydrocortisone was intraperitoneally injected (200 μg/g body weight) into mice, with or without leptin (10 μg/g body weight). As noted during fasting, steroid injection decreased the number of lymphocytes, particularly $CD4^+CD8^+$T lymphocytes, within the thymus. Injection of leptin inhibited the steroid-induced decrease of thymocytes and splenocyte numbers (Table 1) and reversed the observed decline

in $CD4^+CD8^+$ thymocyte numbers (Fig. 2b). This result is consistent with the observed effect of leptin in fasted mice. Moreover, DNA fragmentation of thymocytes in steroid-injected mice was prevented by leptin administration (Fig. 3). In contrast, when leptin-receptor-defective *db/db* mice were treated with hydrocortisone, the proportion of $CD4^+CD8^+$ T cells within the thymus decreased, as was observed for normal mice, but the change was not reversed by injection of leptin (Fig. 2c). This result indicates that the protective effect of leptin against the decline in lymphocyte numbers is related specifically to the binding of leptin to its receptor. When combined, these results strongly suggest that leptin can reverse the decline in T cell numbers due to fasting by preventing steroid-induced apoptosis of lymphocytes by binding to its receptor.

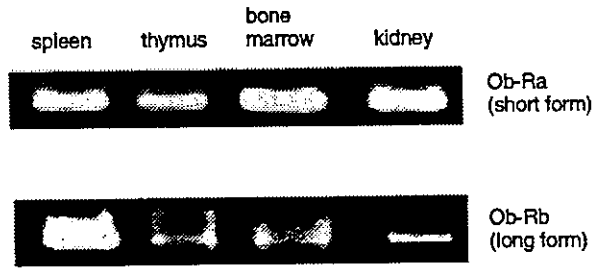

Fig. 1. The leptin receptor is expressed in lymphocytes. Short and long isoforms of the leptin receptor, Ob-Ra and Ob-Rb, were detected by RT-PCR in lymphocytes of the spleen, thymus and bone marrow. Kidney served as a positive control for expression of the leptin receptor.

Table 1. Leptin recovers the decrease in lymphocytes observed in starved and steroid injected mice

	Thymus		Spleen
Treatment	Thymocytes	Double positive thymocytes	Splenocytes ($\times 10^6$)
Untreated	177.0 ± 20.8	154.0 ± 25.4	125.0 ± 4.80
Starved	18.2 ± 11.5	5.12 ± 5.29	15.7 ± 6.15
Starved and leptin treated (1 μg/g)	25.8 ± 10.1	10.1 ± 6.20	28.1 ± 10.3
Starved and leptin treated (10 μg/g)	45.2 ± 3.79	27.7 ± 4.22	50.8 ± 8.29
Untreated	101.0 ± 6.42	83.0 ± 5.26	159.0 ± 66.1
Hydrocortisone treated	12.1 ± 5.25	2.39 ± 3.00	65.0 ± 16.3
Hydrocortisone and leptin (10 mg/g) treated	24.1 ± 5.30	9.87 ± 5.21	102.0 ± 13.1

The mice were treated as described in the Materials and methods section. Data are represented as the mean ± s.d., $n = 3$ per group.

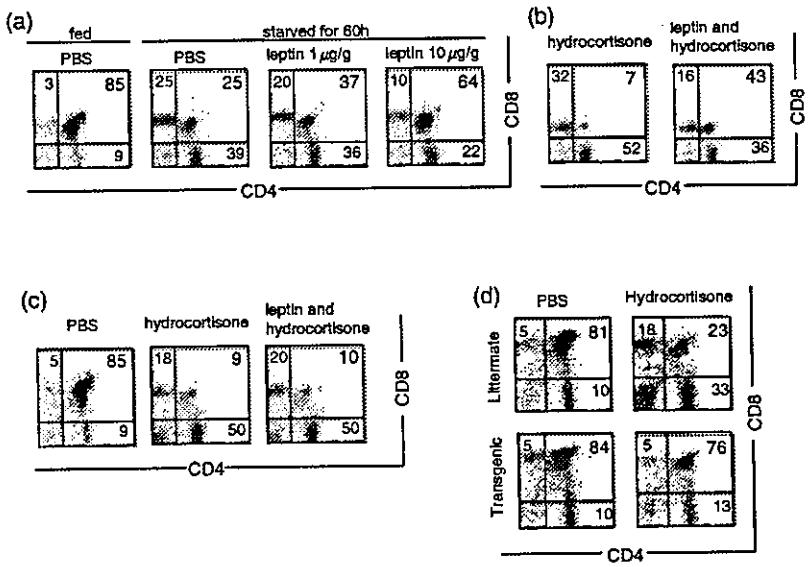

Fig. 2. (a) Leptin reverses the decrease in $CD4^-CD8^+$ thymocytes normally observed in fasted mice. (b) Leptin protects against steroid-induced apoptosis of $CD4^+CD8^+$ thymocytes. (c) Leptin cannot rescue thymocytes of *db/db* mice from steroid-induced apoptosis. (d) Thymocytes of leptin transgenic mice are resistant to steroid-induced apoptosis. This figure represents one of three separate experiments in which similar results were obtained. The percentage of fluorescence-positive cells that were detected is indicated in the corresponding squares.

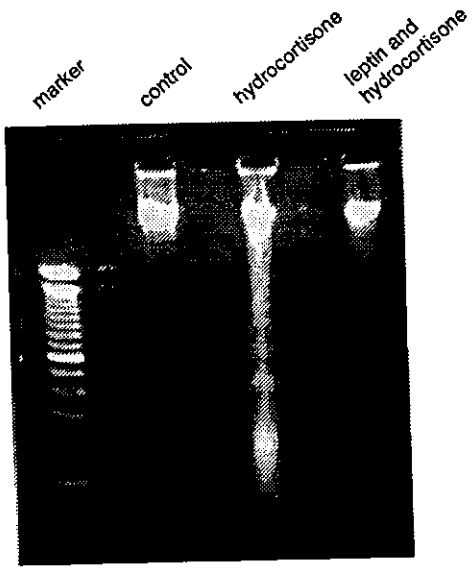

Fig. 3. Leptin prevents DNA fragmentation in thymic T cells induced by steroid administration.

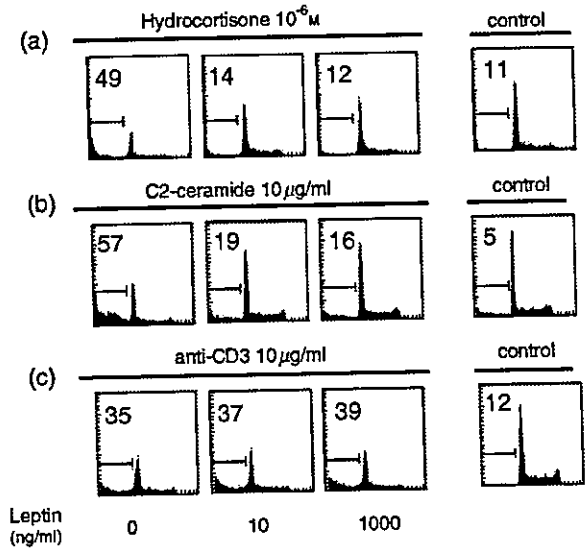

Fig. 4. Leptin prevents apoptosis induced by steroid (a) and ceramide (b), but does not prevent that induced by anti-CD3 MoAb (c). The T cell hybridoma, A3·4C6, was preincubated with or without leptin and apoptosis was induced. The proportion of apoptotic cells was analysed by FACSCalibur® and is shown in figures.

Next, we used leptin transgenic mice to confirm the anti-apoptotic effect of leptin on thymocytes. Plasma leptin concentrations in leptin transgenic mice are approximately 12-fold higher than those of non-transgenic littermates [12]. In the present study, no differences in the surface markers of lymphocytes in the spleen, thymus and bone marrow were observed between leptin transgenic mice and non-transgenic littermates (data not shown). However, when hydrocortisone was injected into leptin transgenic mice and non-transgenic littermates, lower levels of $CD4^+CD8^+$ thymocytes were not found in the leptin transgenic mice compared to non-transgenic littermates (Fig. 2d), suggesting that the thymocytes of leptin transgenic mice are protected from steroid-induced apoptosis by maintaining elevated plasma leptin levels.

To clarify the mechanism by which leptin prevents steroid-induced apoptosis, we performed *in vitro* experiments using a murine T cell hybridoma, A3·4C6 [26]. This hybridoma has been shown to be specific for sperm whale myoglobin and to express the leptin receptor (data not shown). After incubation with leptin for 24 h, followed by an additional 24 h with hydrocortisone, apoptotic cells were examined by flow cytometric analysis. Leptin decreased the proportion of apoptotic cells in a dose-dependent manner (Fig. 4a). In contrast, the anti-CD3 antibody-induced apoptotic death of T cells was not prevented by leptin (Fig. 4c), suggesting that leptin is involved in steroid-induced apoptosis but not in activation-induced cell death.

Recent studies have revealed that ceramide is likely to be one of the several second messengers involved in steroid-induced apoptosis [30]. To investigate whether leptin is effective in preventing ceramide-induced cell death, apoptosis of A3·4C6 induced by N-acetylsphingosine (C2-ceramide) was examined. Leptin decreased the number of apoptotic cells induced by ceramide in a dose-dependent manner (Fig. 4b), as it did following steroid-induced apoptosis (Fig. 4a). These results suggest the possibility that leptin prevents steroid-induced apoptosis downstream of ceramide.

As Ob-Rb displays homology with gp130, a common signal transduction molecule, leptin is thought to activate STAT3 in the same way as does gp130 receptor signalling [16]. Recent studies have revealed that STAT3 binds to the promoter region of bcl-xL, an antiapoptotic molecule of the bcl-2 family, and thereby enhances transcription of the protein [31]. We therefore investigated the possibility that the anti-apoptotic activity of leptin can be attributed to increased expression of bcl-xL following activation of STAT3. We performed flow cytometric analysis and Northern blotting to confirm bcl-xL expression at both the protein and messenger RNA level. When the T cell hybridoma A3·4C6, expressing Ob-Rb, was incubated in the presence of leptin for 24 h, expression of cytoplasmic bcl-xL protein was significantly increased in a dose-dependent manner (Fig. 5a). Moreover, bcl-xL messenger RNA in A3·4C6 was enhanced by leptin in a dose-dependent manner (Fig. 5b). Consistent with the results of *in vivo* experiments, bcl-xL messenger RNA was enhanced within the spleen and thymus of leptin-injected mice (Fig. 5c).

DISCUSSION

The mechanism by which malnutrition leads to immunodeficiency is not fully understood. However, leptin has been reported to be the key link between fasting and immunodeficiency [20], and to inhibit thymocyte apoptosis both *in vivo* and *in vitro* [32]. As fasting increases the production of steroid hormones, it may induce apoptosis of T cells. The present study offers compelling evidence that leptin inhibits the decline in lymphocyte numbers that normally accompanies fasting, in addition to T cell apoptosis following steroid injection. The evidence further suggests that leptin protects lymphocytes through up-regulation of bcl-xL via leptin receptors on lymphocytes. Only one-third of T cell hybridoma cells expressed bcl-xL after leptin stimulation and the observed increase in expression of the bcl-xL gene was not as dra-

matic as that observed following leptin stimulation of fasted mice (Fig. 5). These data suggest that bcl-xL alone is not responsible for the observed inhibitory effect of leptin. Fasting increases the level of steroid hormone within the serum and decreases the amount of circulating leptin [28], both of which can accelerate apoptosis of lymphocytes and impair the immune response.

In contrast to a previous report that found an association between obesity, immune suppression and the presence of infectious disease [33] and cancer [34], our results suggest that obesity might confer resistance to infection. This paradoxical observation can be explained by considering the possibility that the leptin signal is less transducible in obese patients.

Ceramide is generated by hydrolysis of sphingomyelin followed by activation of acidic sphingomyelinase. It functions as an intracellular second messenger, mediating the sphingomyelin signalling pathway. Recently, ceramide has been examined as a common intermediator of several apoptotic stimuli, including steroid administration [30]. However, it is not involved in Fas-induced apoptosis [35,36]. The apoptotic effect of ceramide is due to its induction of cytochrome c release from mitochondria [37]. The bcl-2 gene family can inhibit ceramide-induced apoptosis [38,39].

The present study confirms the findings of reduced numbers of lymphocytes in fasting, and illustrates that leptin prevents the decline in lymphocytes during fasting, in addition to preventing apoptosis of T cells following steroid injection [32]. This may be explained partly by the ability of leptin to up-regulate bcl-xL through the leptin receptors on lymphocytes. However, leptin might not be able to rescue a T cell hybridoma from activation-induced cell death through CD3 stimulation, which is known to involve Fas and the Fas ligand [40–42]. The different effects of leptin on steroid-induced apoptosis and activation-induced cell death might be explained by the bcl-2 related gene products, which prevent apoptosis downstream of ceramide.

Leptin was found originally to modulate body weight but, more recently, it has become recognized as an immune regulator. Administration of leptin to fasted mice has been observed to reverse impairment of T cell function [20]. Although the present study illustrates that leptin inhibits the stress-induced apoptosis of T cells, other effects with regard to immunity remain unclear.

As shown in the present study, lymphocytes can be preserved during fasting by administration of exogenous leptin. This suggests that leptin might be used therapeutically to treat immunodeficiency caused by severe malnutrition and cachexia in cancer and AIDS patients. Such applications may be assessed by further investigation using mouse models of AIDS [43]. On the other hand, it is possible that the anti-apoptotic effects of leptin might result in autoreactive T cells and lead to the development of autoimmune disease. The possibility that leptin might lead to autoimmunity is currently under investigation using our leptin transgenic mice.

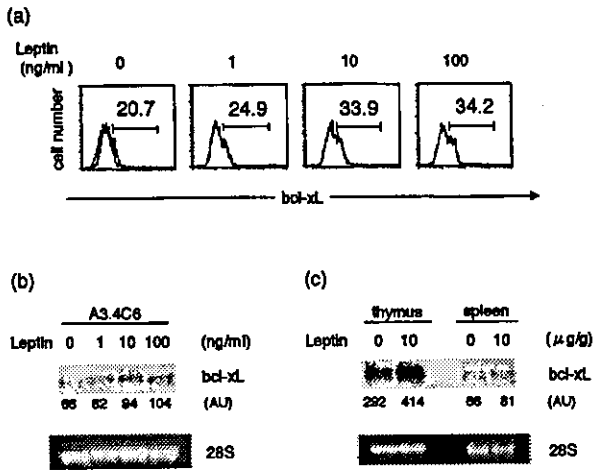

Fig. 5. (a) Leptin induced bcl-xL protein in the T cell hybridoma. The T cell hybridoma, A3·4C6 was incubated in the presence or absence of leptin for 24 h. Expression of the bcl-xL gene was detected with cell-staining using antibcl-xL MoAb and a fluorescent conjugated second antibody. The dotted line indicates cell staining using the fluorescent conjugated antibody without antibcl-xL MoAb. (b) Expression of bcl-xL messenger RNA is enhanced by leptin. A3·4C6 were incubated with graded concentrations of leptin for 24 h and RNA was extracted as described in the materials and methods section. (c) C57/BL6 mice were injected with either PBS or leptin (10 μg/g) and RNA was extracted from the thymus and spleen 24 h later. Northern blotting was performed as described in the materials and methods section. The 28S ribosomal RNA bands were stained with ethidium bromide and are shown in the lower panels of this figure. The relative band intensities of bcl-xL are indicated by assignment of arbitrary units (AU).

ACKNOWLEDGEMENTS

We thank K. Ohmura for his excellent technical assistance. This work was supported by grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Chandra RK. Nutrition and immune system: an introduction. Am J Clin Nutr 1997; 66:460S–3S.
2 Scrimshaw NS, SanGiovanni JP. Synergism of nutrition, infection and immunity: an overview. Am J Clin Nutr 1997; 66:464S–77S.
3 Calson J, Ainley CC, Wolstencroft RA, Norton KRW, Thompson RPH. Cell-mediated immunity in anorexia nervosa. Clin Exp Immunol 1986; 64:370–5.
4 Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse *obese* gene and its human homologue. Nature 1994; 372:425–32.
5 Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993; 259:87–91.
6 Shimomura I, Funahashi T, Takahashi M *et al.* Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. Nature Med 1996; 2:800–3.
7 Masuzaki H, Ogawa Y, Sagawa N *et al.* Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. Nature Med 1997; 3:1029–33.
8 Bado A, Levasseur S, Attoub S *et al.* The stomach is a source of leptin. Nature 1998; 394:790–3.
9 Sato N, Ogawa Y, Katsuura G *et al.* Pathophysiological significance of the obese gene product, leptin, in ventromedial hypothalamus (VMH) – lesioned rats: evidence for loss of its satiety effect in VMH-lesioned rats. Endocrinol 1997; 138:947–54.
10 Stephens TW, Basinski M, Bristow PK *et al.* The role of neuropeptide Y in the antiobesity action of the *obese* gene product. Nature 1995; 377:530–2.
11 Coleman DL. Obese and diabetes: two mutant genes causing diabetes–obesity syndromes in mice. Diabetologia 1978; 14:141–8.
12 Ogawa Y, Masuzaki H, Hosoda K *et al.* Increased glucose metabolism

and insulin sensitivity in transgenic skinny mice overexpressing leptin. Diabetes 1999; **48**:1822–9.

13 Hoggard N, Mercer JG, Rayner DV, Moar K, Trayhurn P, Williams LM. Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and *in situ* hybridization. Biochem Biophys Res Commun 1997; **232**:383–7.

14 Cioffi JA, Shafer AW, Zupancic TJ *et al.* Novel B219/OB receptor isoforms. Possible roles of leptin in hematopoiesis and reproduction. Nature Med 1996; **2**:585–9.

15 Sivitz WI, Walsh SA, Morgan DA, Thomas MJ, Haynes WG. Effects of leptin on insulin sensitivity in normal rats. Endocrinology 1997; **138**:3395–401.

16 Bai Y, Zhang S, Kim KS, Lee JK, Kim KH. *Obese* gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones. J Biol Chem 1996; **271**:13939–42.

17 Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M, Friedman JM. Leptin activation of Stat3 in the hypothalamus of wild-type and *ob/ob* mice but not *db/db* mice. Nature Genet 1996; **14**:95–7.

18 Yamashita T, Murakami T, Otani S, Kuwajima M, Shima K. Leptin receptor signal transduction. OBRa and OBRb of *fa* type. Biochem Biophys Res Commun 1998; **246**:752–9.

19 Bennett BD, Solar GP, Yuan JQ, Mathias J, Thomas GR, Matthews W. A role for leptin and its cognate receptor in hematopoiesis. Current Biol 1996; **6**:1170–80.

20 Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 1998; **394**:897–901.

21 Konopleva M, Mikhail A, Estrov Z *et al.* Expression and function of leptin receptor isoforms in myeloid leukemia and myelodysplastic syndromes: proliferative and anti-apoptotic activities. Blood 1999; **93**:1668–76.

22 Tsuchiya T, Shimizu H, Horie T, Mori M. Expression of leptin receptor in lung: leptin as a growth factor. Eur J Pharmacol 1999; **365**:273–9.

23 Wilson CA, Bekele G, Nicolson M, Ravussin E, Pratley RE. Relationship of the white blood cell count to body fat: role of leptin. Br J Haematol 1997; **99**:447–51.

24 Chandra RK. Cell-mediated immunity in genetically obese (C57BL/6J ob/ob) mice. Am J Clin Nutr 1980; **33**:13–6.

25 Mandel MA, Mahmoud AAF. Impairment of cell-mediated immunity in mutation diabetic mice (db/db). J Immunol 1978; **120**: 1375–7.

26 Ozaki S, Durum SK, Muegge K, York-Jolley J, Berzofsky JA. Production of T-T hybrids from T cell clones: direct comparison between cloned T cells and T hybridoma cells derived from them. J Immunol 1988; **141**:71–8.

27 Murakami M, Tsubata T, Okamoto M *et al.* Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. Nature 1992; **357**:77–80.

28 Ahima RS, Prabakaran D, Mantzoros C *et al.* Role of leptin in the neuroendocrine response to fasting. Nature 1996; **382**:250–2.

29 Wing EJ, Magee DM, Barczynski LK. Acute starvation in mice reduces the number of T cells and suppresses the development of T-cell-mediated immunity. Immunology 1988; **63**:677–82.

30 Cifone MG, Migliorati G, Parroni R *et al.* Dexamethasone-induced thymocyte apoptosis. Apoptotic signal involves the sequential activation of phosphoinositide-specific phospholipase C, acidic sphingomyelinase, and caspases. Blood 1999; **93**:2282–96.

31 Catlett-Falcone R, Landowski TH, Oshiro MM *et al.* Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999; **10**:105–15.

32 Howard JK, Lord GM, Matarese G *et al.* Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in *ob/ob* mice. J Clin Invest 1999; **104**:1051–9.

33 He GW, Ryan WH, Acuff TE *et al.* Risk factors for operative mortality and sternal wound infection in bilateral internal mammary artery grafting. J Thorac Cardiovas Surg 1994; **107**:196–202.

34 Garfinkel L. Overweight and cancer. Ann Intern Med 1985; **103**:1034–6.

35 Watts JD, Gu M, Polverino AJ, Patterson SD, Aebersold R. Fas-induced apoptosis of T cells occurs independently of ceramide generation. Proc Natl Acad Sci USA 1997; **94**:7292–6.

36 Hsu SC, Wu CC, Luh TY, Chou CK, Han SH, Lai MZ. Apoptotic signal of Fas is not mediated by ceramide. Blood 1998; **91**:2658–63.

37 Ghafourifar P, Klein SD, Schucht O *et al.* Ceramide induces cytochrome c release from isolated mitochondria. J Biol Chem 1999; **274**:6080–4.

38 Yang J, Liu X, Bhalla K *et al.* Prevention of apoptosis by bcl-2: release of cytochrome c from mitochondria blocked. Science 1997; **275**: 1129–32.

39 Kluck RM, Wetzel EB, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for bcl-2 regulation of apoptosis. Science 1997; **275**:1132–6.

40 Dhein J, Walczak H, Baumler C, Debatin KM, Krammer PH. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature 1995; **373**:438–41.

41 Brunner T, Mogil RJ, LaFace D *et al.* Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature 1995; **373**:441–4.

42 Ju ST, Panka DJ, Cui H *et al.* Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 1995: **373**:444–8.

43 Cohen J. Building a small-animal model for AIDS, block by block. Science 2001; **293**:1034–6.

ORIGINAL ARTICLE

Plasma leptin levels and cardiac sympathetic function in patients with obstructive sleep apnoea-hypopnoea syndrome

K Shimizu, K Chin, T Nakamura, H Masuzaki, Y Ogawa, R Hosokawa, A Niimi, N Hattori, R Nohara, S Sasayama, K Nakao, M Mishima, T Nakamura, M Ohi

Thorax 2002;**57**:429–434

See end of article for authors' affiliations

Correspondence to: Dr K Chin, Department of Physical Therapeutics, Kyoto University Hospital of Medicine, 54 Shogoin Kawahara-cho, Sakyo-Ku, 606–8507 Kyoto, Japan; chink@kuhp.kyoto-u.ac.jp

Revised version received 29 August 2001
Accepted for publication 6 November 2001

Background: The control of body weight and cardiac sympathetic function in patients with obstructive sleep apnoea-hypopnoea syndrome (OSAHS) are important because both factors have significant effects on the mortality of these patients. It has recently been reported that OSAHS has a significant effect on the secretion of leptin, a hormone involved in the control of body weight and sympathetic nerve activity. In addition to the circadian rhythm of leptin secretion, the effects of one night of treatment with nasal continuous positive airway pressure (nCPAP) and the mechanism of the effects of nCPAP on nocturnal leptin secretion in patients with OSAHS has not yet been elucidated.
Methods: Blood samples were obtained at 21.00 hours, 00.00 hours, 03.00 hours, and 06.30 hours from 21 subjects with OSAHS (mean apnoea and hypopnoea index 52.4/h), with and without nCPAP treatment. Iodine-123 (I^{123})-meta-iodobenzylguanidine (MIBG) imaging was used to evaluate myocardial sympathetic function before nCPAP treatment.
Results: Plasma leptin reached a peak level at 00:00 hours ($p<0.01$) in patients with OSAHS, both with and without nCPAP treatment. The first night of nCPAP treatment significantly decreased the plasma leptin levels at 03.00 hours (without nCPAP: mean (SE) 21.6 (4.7) ng/ml; with nCPAP: 19.3 (4.1) ng/ml, $p<0.02$) and at 06.30 hours (without nCPAP: 17.6 (3.8) ng/ml; with nCPAP: 15.2 (3.2) ng/ml, $p<0.01$). The magnitude of the decrease in leptin levels after nCPAP treatment was significantly correlated with cardiac sympathetic function measured before nCPAP treatment ($p<0.03$).
Conclusions: Patients with OSAHS undergo nocturnal increases in leptin levels in spite of interruption of sleep due to apnoea and hypopnoea, a trend seen in normal subjects. Plasma leptin levels in patients with OSAHS decreased significantly after the first night of nCPAP treatment. Enhanced cardiac sympathetic function in these patients may contribute to the leptin levels before nCPAP treatment and vice versa.

Control of body weight and cardiac sympathetic function are both clinically important in patients with obstructive sleep apnoea-hypopnoea syndrome (OSAHS) because they have significant effects on the morbidity and mortality of such patients. Leptin is a circulating hormone that is expressed abundantly throughout the body—specifically in adipose tissue,[1–3] although it is also secreted from other tissues including human placenta and stomach.[4 5] Leptin induces a complex response that affects control of body weight and energy expenditure.[1] It has recently been reported that circulating plasma leptin levels are raised in men with newly diagnosed untreated OSAHS.[6–8] Earlier studies of nasal continuous positive airway pressure (nCPAP) in the treatment of patients with OSAHS suggested that OSAHS has a significant impact on serum leptin levels, with significantly decreased levels following nCPAP treatment of 3–4 days, 1 month, and 6 months.[6 7] However, these studies did not report the first night effects of nCPAP on serum leptin levels. Patients with OSAHS have significantly higher muscle sympathetic nerve activity (MSNA) than controls,[10] but significantly decreased MSNA and blood pressure during sleep have been reported after the first night of nCPAP treatment.[10] It is therefore possible that changes in serum leptin levels occur after only one night of nCPAP. In addition to the effects of one night of treatment with nCPAP, it is unknown whether a nocturnal increase in serum leptin levels (as occurs in both lean and obese subjects) also occurs in patients with OSAHS.[11] We have therefore studied the nocturnal variation in plasma leptin levels and the first night effects of nCPAP treatment in patients with OSAHS.

It is also important to understand the mechanism which drives these changes in leptin levels because leptin has been reported to be associated with an increased risk of myocardial infarction, haemorrhagic stroke, and abnormal fibrinolysis in men and postmenopausal women.[12–14] Although a relationship between leptin levels and MSNA in patients with OSAHS has not been found,[9] MSNA levels do have a significant positive correlation with leptin concentrations in healthy non-diabetic men.[15] We have therefore studied the relationship between the changes in plasma leptin levels and cardiac sympathetic nerve function.[16]

METHODS

Subjects

Twenty one men of mean (SE) age 45.0 (2.4) years and body mass index (BMI) 28.9 (0.8) kg/m^2 with OSAHS (apnoea-hypopnoea index (AHI) 52.4 (3.4) episodes/h) underwent nCPAP treatment. Polysomnography was performed[16 17] before nCPAP was started and again on the first night of nCPAP treatment. There was a 1 week interval between the two polysomnographic examinations. OSAHS was established on the basis of clinical and polysomnographic criteria.[16 17] In addition to clinical symptoms, an AHI of >20 events/hour was used as a selection criterion because these patients were good candidates for nCPAP treatment. Six of the patients had hypertension (five of whom were prescribed antihypertensive drugs), four had diabetes (one of whom was prescribed a glucosidase inhibitor while the others received only diet therapy),

Table 1 Characteristics of study patients

Subject no	Age (years)	BMI (kg/m²)	Pao_2 (kPa)	$Paco_2$ (kPa)	%VC (%)	FEV_1/FVC (%)
1	45	31.1	11.6	5.0	97.0	81.9
2	40	29.9	11.7	5.3	98.9	90.7
3	64	33.0	10.2	6.0	99.1	79.1
4	28	34.9	9.8	5.7	95.0	83.1
5	49	24.6	11.9	5.5	89.0	79.2
6	60	29.5	10.9	5.8	83.8	82.9
7	50	27.8	9.6	6.1	96.0	59.7
8	65	28.4	10.5	5.7	114.0	69.1
9	35	25.1	13.1	5.9	109.6	80.6
10	44	29.9	12.2	5.1	133.7	85.1
11	37	31.9	11.1	5.7	105.0	77.9
12	56	24.7	13.9	5.5	135.9	71.1
13	35	34.0	11.1	5.7	94.4	87.0
14	63	26.0	10.8	4.8	141.9	68.3
15	43	30.7	9.2	5.2	121.2	83.5
16	31	21.9	10.6	5.3	94.9	81.2
17	33	26.0	11.4	5.5	106.2	75.1
18	44	28.5	11.4	5.9	121.0	81.8
19	52	33.6	10.8	5.0	116.9	74.6
20	41	30.0	10.0	5.8	103.7	74.2
21	47	25.0	11.0	5.9	104.0	86.7
Mean	45	28.9	11.1	5.5	107.7	78.7
SE	2.4	0.8	0.2	0.1	3.4	1.6

BMI=body mass index; Pao_2=arterial oxygen tension; $Paco_2$=arterial carbon dioxide tension; %VC=vital capacity (% predicted); FEV_1/FVC=ratio of forced expiratory volume in 1 second to forced vital capacity.

and two patients had both hypertension and diabetes (one patient received an antihypertensive drug and an oral hypoglycaemic agent; the other was on antihypertensive drugs only). No patient was given subcutaneous insulin. These treatment regimens had been adhered to several months before nCPAP therapy was started. Control leptin samples were obtained at night (n=21) and in the morning (n=5) from the patients.

The study was approved by the Institutional Committee for the Protection of Human Subjects and all patients gave informed consent before the study.

Polysomnography

Polysomnography was started at 21.00 hours and ended at 06.30 hours. Surface electrodes were applied using standard techniques to obtain an electroencephalogram, an electromyogram of the chin, an electrocardiogram, and an electrooculogram. Sleep was defined according to the criteria of Rechtschaffen and Kales.[18] Ventilation was monitored by inductive plethysmography (Respitrace; Ambulatory Monitoring, Ardsley, NY, USA). Airflow was monitored by thermistors (Nihon Kohden, Tokyo, Japan) placed at the nose and mouth, while arterial oxygen saturation (Sao_2) was monitored continuously with a pulse oximeter (Pulsox-7; Minolta, Osaka, Japan). A polygraph (Polygraph System RM-6000; Nihon Kohden, Tokyo, Japan) was run continuously at 10 mm/s to record all of the above physiological data simultaneously throughout the course of the experiment. All parameters were stored in a data recorder (A-621; Sony Precision Technology, Tokyo, Japan) for subsequent analysis. Before blood sampling at 21.00 hours and 06.30 hours, blood pressure was measured three times and the mean value was calculated. The nCPAP pressure was titrated manually throughout one night. Apnoea was defined as the cessation of airflow at the nose and mouth lasting for more than 10 seconds. Hypopnoea was defined as a decrease of 50% or more in thoracoabdominal motion associated with a fall in the baseline oxygen saturation of 4% or more. All AHI values were calculated to express the number of episodes of apnoea and hypopnoea per hour of total sleep time.

Blood sampling and analysis

Patients ingested a light hospital meal in the evening. From 20.00 hours to 06.30 hours on the following day the patients did not eat or drink anything. Before polysomnography an 18-gauge catheter was inserted into the right antecubital vein. The catheter was connected to tubing, extended sufficiently so as not to disturb sleep, and locked by heparinised saline. Blood samples were withdrawn for analysing leptin, glucose, and immunoreactive insulin (IRI) at 21.00 hours, 00.00 hours, 03.00 hours, and 06.30 hours. In five of the 21 patients blood samples were taken at two separate times 06.30 hours before nCPAP treatment was initiated. Blood samples were immediately centrifuged at 4°C for 10 minutes and stored at −80°C for later analysis.

MIBG imaging

Iodine-123(^{123}I)-meta-iodobenzylguanidine (MIBG) imaging has been used to evaluate myocardial sympathetic function in various cardiac diseases.[19 20] The patients underwent ^{123}I-MIBG cardiac imaging after polysomnography without nCPAP treatment. Each subject received 30 mg potassium iodine on the day before the study began, continuing until the day after the study ended in order to block tracer uptake by the thyroid gland. Using a gamma camera equipped with a low energy, parallel hole, general purpose collimator, planar images were obtained at rest in the anterior view over a 3 minute interval at 15 minutes (early image) and 3 hours (delayed image) after an injection of 111 MBq MIBG. The regions of interest within the heart were set manually on these planar images. The mean heart counts from the early image and from the delayed image were calculated. On the planar images a region in the upper mediastinum was used to calculate the mean mediastinal counts. The heart to mediastinum count ratios were calculated as an index of the myocardial uptake of MIBG. The heart to mediastinum count ratios from the early image (H/M early) and from the delayed image (H/M delay) were used as indices of cardiac sympathetic function.[19 20]

Assays

The plasma leptin level was measured by radioimmunoassay (RIA) with intra-assay and interassay coefficients of variation of 5.3% (n=10) and 5.9% (n=10), respectively.[21] Serum levels of IRI were measured by RIA (Insulin-RIA-beads II, Dainabot, Tokyo, Japan) and plasma glucose was determined by an enzyme method (Glu-L 2; Wako Chemical, Osaka, Japan).

Table 2 Effects of nCPAP on obstructive sleep apnoea syndrome

	nCPAP(−)	nCPAP(+)	p value*
AHI (/h)	52.4 (3.4)	4.2 (1.1)	<0.0001
REM time (%)	7.5 (1.2)	13.8 (2.0)	0.0051
Non-REM time (%)	92.5 (1.2)	86.2 (2.0)	0.0051
SWS time (%)	0.5 (0.4)	4.0 (1.1)	0.0004
TST (min)	470.0 (14.3)	453.9 (23.0)	0.6637
Sao_2 <90% (% time)	30.1 (4.3)	0.6 (0.3)	<0.0001
Sao_2 <85% (% time)	17.2 (3.7)	0.2 (0.1)	<0.0001
Morning SBP (mm Hg)	123.9 (4.3)	113.1 (3.0)	0.0087
Morning DBP (mm Hg)	85.6 (2.9)	76.6 (2.2)	0.0029

nCPAP=nasal continuous positive airway pressure; nCPAP(−)=without nCPAP treatment; nCPAP(+)=first night of nCPAP treatment; AHI=apnoea hypopnoea index; REM=rapid eye movement; SWS=slow wave sleep; TST=total sleep time; Sao_2=arterial oxygen saturation; SBP=systolic blood pressure; DBP=diastolic blood pressure.
*Wilcoxon signed rank test. Values are mean (SE).

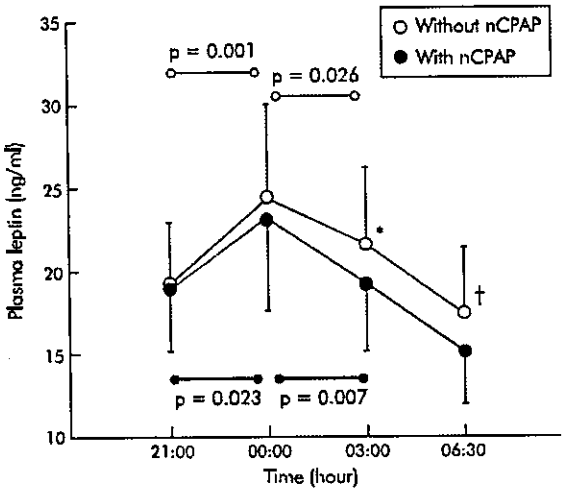

Figure 1 Nocturnal profiles of plasma leptin levels. Plasma leptin levels at 03.00 hours (*p<0.02) and 06.30 hours (†p<0.01) decreased significantly with nCPAP treatment. A peak level was seen at 00.00 hours which decreased significantly on both nights of the study. Plasma leptin levels at 00.00 hours with and without nCPAP treatment were significantly higher than the immediate values at 21.00 hours and 03.00 hours. Statistical analysis was performed by the Friedman test and Wilcoxon signed rank test and intragroup analysis was performed using the Wilcoxon signed rank test.

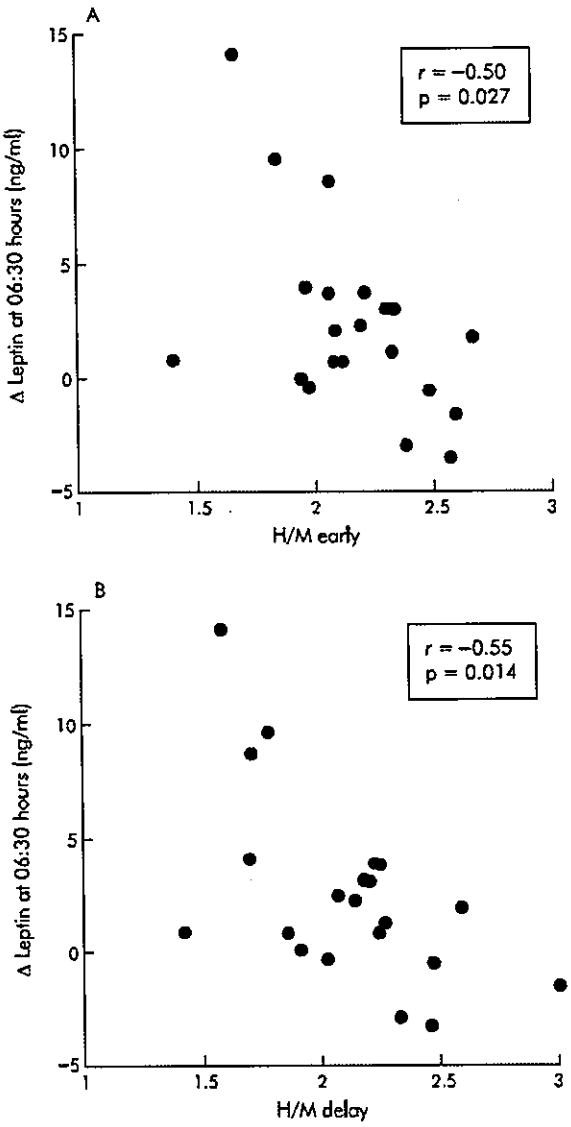

Figure 2 Correlation between (A) early heart to mediastinal count ratios, an index of myocardial uptake of MIBG (H/M early), and the magnitude of changes in plasma leptin levels (Δleptin) at 06.30 hours before and after nCPAP treatment ($r = -0.50$, $p=0.027$); and (B) correlation between delayed heart to mediastinal count ratios (H/M delay) and Δleptin at 06.30 hours before and after nCPAP treatment ($r = -0.55$, $p=0.014$).

Data analysis

The data are presented as mean (SE) values. A non-parametric method was used for data analysis. Changes in plasma leptin levels during sleep were compared using the Friedman test. If a significant difference was found, differences between the peak values and the immediate values (before and after peak values) were compared by the Wilcoxon signed rank test, as were paired data of plasma leptin, glucose, and serum IRI concentrations at each sampling time before and after nCPAP treatment. The plasma leptin levels measured at two separate times in five of the subjects at 06.30 hours were also compared using the Wilcoxon signed rank test. Spearman rank correlation coefficients were calculated to analyse the correlation between variables. These calculations were performed using StatView software for Windows (Version 4.5; Abacus Concepts, Berkeley, CA). A p value of <0.05 was considered significant.

RESULTS

Anthropometric data, arterial blood gas tensions, and spirometric data are presented in table 1. The effects of nCPAP treatment on OSAHS are shown in table 2; sleep apnoea, nocturnal oxygen saturation, blood pressure, and sleep architecture all showed improvements.

Nocturnal profile of plasma leptin levels

On each night the control leptin levels at 21.00 hours (before sleep) were the same (before nCPAP, 24.5 (5.6) ng/ml; just before an application of nCPAP, 23.2 (5.6) ng/ml, p=0.39). Plasma leptin levels measured at 06.30 hours before nCPAP treatment at two separate times in five patients were not significantly different from one another (first sample: 12.0 (3.6) ng/ml; second sample: 12.0 (4.1) ng/ml, p=0.85). The nocturnal profile of plasma leptin levels at 21.00 hours, 00.00 hours, 03.00 hours, and 06.30 hours in patients with OSAHS with and without nCPAP treatment are shown in fig 1. The trend towards a nocturnal increase was seen both with and

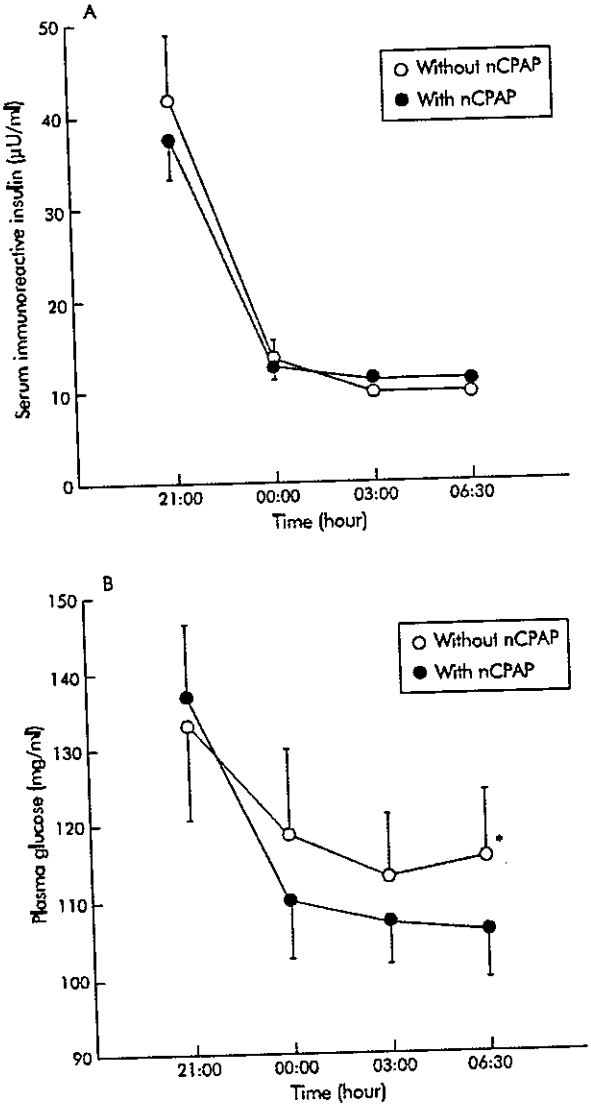

Figure 3 Nocturnal profiles of (A) serum immunoreactive insulin levels and (B) plasma glucose levels. No significant difference was seen in serum immunoreactive insulin levels with and without nCPAP treatment at each sampling time; plasma glucose levels were significantly decreased with nCPAP treatment at 06.30 hours (*p=0.027).

without nCPAP treatment. On both nights plasma leptin levels peaked at 00.00 hours ($p<0.05$). On the first night of nCPAP treatment plasma leptin levels decreased significantly at 03.00 hours (without nCPAP, 21.6 (4.7) ng/ml; with nCPAP, 19.3 (4.1) ng/ml, p=0.016) and at 06.30 hours (without nCPAP, 17.6 (3.8) ng/ml; with nCPAP, 15.2 (3.2) ng/ml, p=0.009). The magnitude of changes in leptin levels with and without nCPAP treatment did not correlate significantly with the magnitude of changes in any parameters during polysomnography including AHI and blood pressure (table 2).

MIBG scintigram

H/M early and H/M delay did not correlate significantly with serum leptin levels at any point before or after nCPAP treatment. The results of MIBG scintigraphy before nCPAP treatment and the magnitude of the difference in plasma leptin levels before and after nCPAP treatment (Δleptin) showed a correlation only at 06.30 hours (H/M early: $r = -0.50$, p=0.027; H/M delay: $r = -0.55$, p=0.014; fig 2).

Nocturnal profile of glucose and insulin levels

Nocturnal plasma glucose and serum IRI levels were measured in 19 of the 21 patients (fig 3). At 21.00 hours, about 3 hours after the evening hospital meal, plasma glucose and serum IRI showed no significant differences with or without nCPAP treatment, but a significant difference was seen in the plasma glucose level at 06.30 hours between those treated with nCPAP and those not treated ($p<0.05$, Wilcoxon signed rank test).

DISCUSSION

We have shown that the nocturnal rise in the plasma leptin concentration is seen in patients with OSAHS whether or not they receive nCPAP treatment. After the first night of treatment with nCPAP the plasma leptin concentration decreased significantly at 03.00 hours and at 06.30 hours, although the control levels at 21.00 hours (before sleep) were the same. We have also shown that H/M early and H/M delay (indices of myocardial MIBG uptake showing cardiac sympathetic function and integrity of the heart) were significantly correlated with the magnitude of the decrease in the plasma leptin level at 06.30 hours compared with the level before nCPAP treatment—that is, plasma leptin levels in OSAHS patients who had exacerbated cardiac sympathetic function before nCPAP treatment decreased in proportion to the magnitude of their exacerbated cardiac sympathetic function activity before starting nCPAP treatment.

We have previously reported that plasma leptin levels decreased in the morning after treatment with nCPAP for 3–4 days, 1 month, and 6 months.[9] It has recently been reported that serum leptin levels are significantly increased in patients with OSAHS compared with age and weight matched controls,[6–8] and these levels were found to decrease significantly after 6 months of treatment with nCPAP.[8 9] However, the precise timing of the fall in leptin levels after the introduction of nCPAP treatment has remained uncertain. In this study we have shown that plasma leptin levels begin to decrease during the first night of treatment with nCPAP. Although the AHI on the first night of treatment was small (table 2), it is possible that plasma leptin levels might decrease significantly with nCPAP treatment before 03.00 hours if nCPAP titration was more perfect because the data during the first night of treatment—especially in the early part of the polysomnographic study—are often imperfect.

The nocturnal increase in plasma leptin levels was seen in patients with OSAHS both with and without nCPAP treatment—a trend seen in both lean and obese subjects.[11] It has been reported that there is a correlation between the diurnal rhythm of leptin and meal times.[12] However, the meal times of the patients before and after nCPAP were the same in our study, so our patients showed nocturnal increases in plasma leptin levels despite sleep fragmentation due to AHI, although the leptin levels at 03.00 hours and 06.30 hours decreased significantly after one day of nCPAP treatment.

From the results of the cardiac MIBG scintigram and plasma leptin levels before and after nCPAP treatment, the decrease in plasma leptin levels seen after one day of treatment might be explained by the effect of nCPAP on sympathetic nerve activation. This, together with the effects on blood pressure found in this study, is consistent with another report suggesting that nCPAP decreases MSNA and blood pressure during sleep.[10] Previous reports have also shown that increased cardiac sympathetic function is improved after one month of nCPAP treatment.[16] In addition, Snitker *et al*[15] reported that MSNA levels had a significant positive correlation with leptin concentrations in healthy non-diabetic men. The decrease in sympathetic nerve activity due to nCPAP

treatment might therefore also reduce serum leptin levels. On the other hand, several reports have shown that leptin administration increases sympathetic nerve activity,[23 24] and it has recently been reported that leptin may play a role in the pathogenesis of some forms of obesity related hypertension.[25] Decreased leptin levels in patients with OSAHS following treatment with nCPAP might therefore improve sympathetic nerve activity including blood pressure (table 2). Further studies are needed to determine whether the decrease in serum leptin levels improves the sympathetic nerve activity or whether improved sympathetic nerve activity causes serum leptin levels to decrease. Although nCPAP treatment significantly improves AHI, REM time %, and the severity of hypoxaemia, as indicated by the percentage of time with Sao_2 <85% or 90% and blood pressure (table 2), these measurements were not correlated with the decrease in the serum leptin levels. Further research is therefore also required to determine the factors that significantly impact on serum leptin levels.

The plasma glucose level was found to be reduced at 06.30 hours after one night of nCPAP treatment, but IRI levels did not change. From these results we speculate that insulin activity for glucose also improved after the first night of nCPAP treatment. It has been reported that exogenously administered leptin improves insulin activity[26 27]; in our study the insulin activity for glucose improved in spite of decreased leptin levels. Phillips *et al*[9] recently reported that OSAHS may be accompanied by further resistance to the metabolic effects of leptin, greater than the resistance evident in obesity alone. Further improvements in leptin resistance to the metabolism of insulin and glucose after nCPAP treatment might therefore enhance the action of insulin on glucose more than the decrease in leptin levels.

The primary limitation of our study was that blood samples were obtained only four times while the serum leptin level cycles every 7 minutes.[28] However, the nocturnal profile of the leptin levels before and after nCPAP treatment was the same as in previously reported studies,[11 28] although the levels at 03.00 hours and 06.30 hours decreased significantly after nCPAP treatment. The serum leptin levels therefore appear to be accurate despite the longer sampling intervals.

A second limitation of this study is that there was no statistically significant correlation between MIBG uptake before treatment and any of the eight measurements of plasma leptin before and after nCPAP treatment, and Δleptin was significantly correlated with the early and delayed MIBG uptake at 06.30 hours only. Confidence in the significance of Δleptin at 06.30 hours may therefore be reduced by the amount of statistical testing performed, and the relationship between leptin levels and cardiac sympathetic function should be studied further, including long term effects of nCPAP treatment on leptin levels and MIBG scintigraphy.

Ideally, we would have liked to have done complementary studies of leptin and glucose measurements in weight matched subjects without OSAHS during a single night of sham nCPAP. However, it is difficult to find weight matched subjects without OSAHS in Japan because East Asian subjects are more likely to develop OSAHS at a lower BMI than Western subjects.[29] The control leptin levels corresponding to the two measurements at 21.00 hours and the two measurements at 6.30 hours were the same before nCPAP treatment, but following nCPAP treatment the serum leptin levels decreased significantly at 03.00 hours and 06.30 hours. Treatment with nCPAP for one night therefore significantly lowered the leptin levels, although no weight matched control subjects were studied.

A further limitation of this study is that no women were included. It has been reported that the circulating concentration of leptin is significantly higher in both premenopausal and postmenopausal women than in men, even when the levels were corrected for differences in BMI or percentage body fat (premenopausal women > postmenopausal women > men).[30] The changes in leptin levels with and without nCPAP treatment would therefore have been different if women had been included in the study. Although the overall results would have been similar, further studies are needed to apply the results of this study to women.

This study has shown that decreased serum leptin levels in the morning may be a marker of improved cardiac sympathetic nerve function. This finding has clinical relevance in light of the negative associations of increased leptin levels—namely, an increased risk of myocardial infarction,[12] haemorrhagic stroke,[13] and abnormal fibrinolysis in men and postmenopausal women.[14] It has also been reported that hypercoagulable states are improved by nCPAP treatment.[17] Although leptin levels change according to body weight[1 2] and with meal times,[22] continuous measurement of leptin levels before and after the initiation of nCPAP treatment, in addition to the measurement of body weight, may prove helpful in evaluating the prognostic factors and efficacy of treatment in patients with OSAHS.

ACKNOWLEDGEMENT

The authors thank T Ueda for manuscript preparation. This work was supported in part by grants from Takei Ueda Memorial Fund; the Japanese Ministry of Education, Science, Sports, and Culture (grant No.13670598); and the Japanese Ministry of Health and Welfare.

Authors' affiliations
K Shimizu, T Nakamura, A Niimi, N Hattori, M Mishima, Department of Respiratory Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
K Chin, T Nakamura, Department of Physical Therapeutics, Kyoto University Hospital of Medicine, Kyoto, Japan
R Nohara, R Hosokawa, S Sasayama, Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine
H Masuzaki, Y Ogawa, K Nakao, Department of Clinical Science, Kyoto University Graduate School of Medicine
M Ohi, Department of Internal Medicine, Osaka Kaisei Hospital, Japan

REFERENCES

1 **Auwerx J,** Staels B. Leptin. *Lancet* 1998;**351**:737–42.
2 **Considine RV,** Sinha MK, Heiman ML, *et al.* Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996;**334**:292–5.
3 **Masuzaki H,** Ogawa Y, Isse N, *et al.* Human obese gene expression. Adipocyte-specific expression and regional differences in the adipose tissue. *Diabetes* 1995;**44**:855–8.
4 **Masuzaki H,** Ogawa Y, Sagawa N, *et al.* Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. *Nature Med* 1997;**100**:1029–33.
5 **Sobhani I,** Bado A, Vissuzaine C, *et al.* Leptin secretion and leptin receptor in the human stomach. *Gut* 2000;**47**:178–83.
6 **Ip MS,** Lam KS, Ho C, *et al.* Serum leptin and vascular risk factors in obstructive sleep apnea. *Chest* 2000;**118**:580–6.
7 **Vgontzas AN,** Papanicolaou DA, Bixler EO, *et al.* Sleep apnea and daytime sleepiness and fatigue: relationship to visceral obesity, insulin resistance, and hypercytokinemia. *J Clin Endocrinol Metab* 2000;**85**:1151–8.
8 **Phillips BG,** Kato M, Narkiewiczk K, *et al.* Increases in leptin levels, sympathetic drive, and weight gain in obstructive sleep apnea. *Am J Physiol Heart Circ Physiol* 2000;**279**:H234–7.
9 **Chin K,** Shimizu K, Nakamura T, *et al.* Changes in intra-abdominal visceral fat and serum leptin levels in patients with obstructive sleep apnea syndrome following nasal continuous positive airway pressure therapy. *Circulation* 1999;**100**:706–12.
10 **Somers VK,** Dyken ME, Clary MP, *et al.* Sympathetic neural mechanisms in obstructive sleep apnea. *J Clin Invest* 1995;**96**:1897–904.
11 **Sinha MK,** Ohannesian JP, Heiman ML, *et al.* Nocturnal rise of leptin in lean, obese, and non-insulin dependent diabetes mellitus subjects. *J Clin Invest* 1996;**97**:1344–7.
12 **Soderberg S,** Ahren B, Jansson JH, *et al.* Leptin is associated with increased risk of myocardial infarction. *J Intern Med* 1999;**246**:409–18.
13 **Soderberg S,** Ahren B, Stegmayr B, *et al.* Leptin is a risk marker for first-ever hemorrhagic stroke in a population-based cohort. *Stroke* 1999;**30**:328–37.
14 **Soderberg S,** Olsson T, Eliasson M, *et al.* Plasma leptin levels are associated with abnormal fibrinolysis in men and postmenopausal women. *J Intern Med* 1999;**245**:533–43.
15 **Snitker S,** Pratley RE, Nicolson M, *et al.* Relationship between muscle sympathetic nerve activity and plasma leptin concentration. *Obes Res* 1997;**5**:338–40.

16 **Otsuka N**, Ohi M, Chin K, *et al*. Assessment of cardiac sympathetic function with iodine-123-MIBG imaging in obstructive sleep apnea syndrome. *J Nucl Med* 1997;**38**:567–72.
17 **Chin K**, Ohi M, Kita H, *et al*. Effects of NCPAP therapy on fibrinogen levels in obstructive sleep apnea syndrome. *Am J Respir Crit Care Med* 1996;**153**:1972–6.
18 **Rechtschaffen A**, Kale A. *A manual of standardized terminology, techniques and scoring system for sleep stages of human subjects*. Washington, DC: National Institute of Health, 1968.
19 **Meriet P**, Benvenuti C, Moyse D, *et al*. Prognostic value of MIBG imaging in idiopathic dilated cardiomyopathy. *J Nucl Med* 1999;**40**:917–23.
20 **Lanza GA**, Giordano A, Pristipino C, *et al*. Abnormal cardiac adrenergic nerve function in patients with syndrome X detected by ^{123}I metaiodobenzylguanidine myocardial scintigraphy. *Circulation* 1997;**96**:821–6.
21 **Hosoda K**, Masuzaki H, Ogawa Y, *et al*. Development of radioimmunoassay for human leptin. *Biochem Biophys Res Commun* 1996;**221**:234–9.
22 **Schoeller DA**, Cella LK, Sinha MK, *et al*. Entrainment of the diurnal rhythm of plasma leptin to meal timing. *J Clin Invest* 1997;**100**:1882–7.
23 **Collins S**, Kuhn CM, Petro AE, *et al*. Role of leptin in fat regulation. *Nature* 1996;**380**:677.
24 **Haynes WG**, Morgan DA, Walsh SA, *et al*. Receptor mediated regional sympathetic nerve activation by leptin. *J Clin Invest* 1998;**100**:270–8.
25 **Aizawa-Abe M**, Ogawa Y, Masuzaki H, *et al*. Pathophysiology role of leptin in obesity-related hypertension. *J Clin Invest* 2000;**105**:1243–52.
26 **Barzilai NJ**, Wang J, Massilon D, *et al*. Leptin selectivity decreased visceral adiposity and enhanced insulin action. *J Clin Invest* 1997;**100**:3105–10.
27 **Shimomura I**, Hammer RE, Ikemoto S, *et al*. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 1999;**401**:73–6.
28 **Licinio J**, Mantzoros C, Negrao AB, *et al*. Human leptin levels are pulsatile and inversely related to pituitary-adrenal function. *Nature Med* 1997;**3**:575–9.
29 **Li KK**, Powell NB, Kushida C, *et al*. A comparison of Asian and white patients with obstructive sleep apnea syndrome. *Laryngoscope* 1999;**109**:1937–40.
30 **Rosenbaum M**, Nicolson M, Hirsch J, *et al*. Effects of gender, body composition, and menopause on plasma concentrations of leptin. *J Clin Endocrinol Metab* 1996;**81**:3424–7.

0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(9):3341–3350

doi: 10.1210/en.2002-220225

Delayed Short-Term Secretory Regulation of Ghrelin in Obese Animals: Evidenced by a Specific RIA for the Active Form of Ghrelin

HIROYUKI ARIYASU, KAZUHIKO TAKAYA, HIROSHI HOSODA, HIROSHI IWAKURA, KEN EBIHARA, KIYOSHI MORI, YOSHIHIRO OGAWA, KIMINORI HOSODA, TAKASHI AKAMIZU, MASAYASU KOJIMA, KENJI KANGAWA, AND KAZUWA NAKAO

Department of Medicine and Clinical Science (H.A., H.I., E.K., K.M., Y.O., K.H., K.N.), Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; Translational Research Center (K.T., T.A., K.K.), Kyoto University Hospital, Kyoto 606-8507, Japan; Division of Molecular Genetics, Institute of Life Science (M.K.), Kurume University, Kurume, Fukuoka 839-0861, Japan; and Department of Biochemistry (H.H., K.K.), National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

Ghrelin is an acylated peptide, whose lipid modification is essential for its biological activities. Previous studies demonstrated that it strongly stimulates GH release and has a potent orexigenic action. Meanwhile, there is enough evidence showing that feeding states influence plasma ghrelin levels. Fasting stimulates ghrelin secretion, and feeding reduces plasma ghrelin levels. In this study we examined the regulation of plasma ghrelin by fasting in genetically obese animals considering its molecular forms. Plasma levels of active form of ghrelin as well as those of total ghrelin were reduced in *ob/ob* and *db/db* mice compared with those in their control mice. Zucker fatty (*fa/fa*) rats also showed lower plasma ghrelin levels by fasting than the control rats. Insulin-induced hypoglycemia, however, stimulated ghrelin secretion in the fasted fatty rats. Moreover, glucose injection was revealed to reduce plasma ghrelin levels in rats. The effect of the severity of obesity on secretory regulation of ghrelin was also studied. Older fatty rats showed low plasma ghrelin levels even after 48-h fasting. These data suggest that the short-term secretory regulation of total ghrelin and the active form of ghrelin is delayed in obese animals and that blood glucose levels may be involved in the delayed regulation. (*Endocrinology* 143: 3341–3350, 2002)

THE CRUCIAL discovery of ghrelin has provided insight into the novel regulatory system of GH release (1). Artificial compounds named GH secretagogues (GHSs) release GH *in vitro* and *in vivo* through a specific receptor (2–6). Ghrelin was identified as an endogenous ligand for the receptor. Detailed structural analysis revealed that it is an acylated peptide of 28 amino acidic residues (1, 7, 8). It strongly stimulates GH release in a clear dose-dependent manner in animals and humans (1, 9–14) in concert with GH-releasing hormone (15). Previous studies revealed its unique tissue distribution, that is, the stomach is the major site of production of ghrelin, and ghrelin is also expressed in the hypothalamus (1, 16–20), suggesting its possible involvement in energy homeostasis as well as in GH release (21). Consistent with this hypothesis, GHSs and ghrelin stimulate food intake via hypothalamic neuropeptide Y and agouti-related protein (AGRP), when it is centrally administered in rats (22–30). Meanwhile, GHSs and ghrelin induce adiposity in animals independently of food intake or GH release (31, 32).

Plasma ghrelin levels are regulated by acute feeding states. We and others revealed that plasma ghrelin levels are elevated by fasting and reduced by feeding in animals and humans (17, 25, 28, 33). Oral glucose intake, but not stomach expansion, reduces plasma ghrelin levels in rats (31), and ghrelin mRNA in the gastric fundi is increased by insulin injection (34). A few previous studies have studied the relationship between chronic feeding states and plasma ghrelin levels. Plasma ghrelin levels are reduced in obese human subjects (35), and ghrelin mRNA expression in the stomach is reduced in genetically obese *db/db* mice (34). We and others recently reported that plasma ghrelin levels are markedly elevated in patients with anorexia nervosa (17, 36). These observations raise the idea that ghrelin may serve as an indicator of energy deposit such as leptin.

However, these studies are lacking in structural information of ghrelin in the altered plasma levels. Ghrelin is a unique hormone in that it is a 28-amino acid peptide that contains an *n*-octanoyl modification on Ser^3, and the lipid modification is essential for ghrelin-mediated stimulation of GH release. Des-acyl ghrelin, the des-*n*-octanoyl form of ghrelin, has almost no biological activities (7, 37, 38). Moreover, although fasting and feeding seem to be the major determinant factors of plasma ghrelin levels in subjects with normal body weight as mentioned above, little is known about the effect of short-term changes in energy balance on them in obese subjects.

The present study attempt to establish the difference between obese and lean subjects in the secretory regulation of ghrelin, considering its molecular forms. We examined

Abbreviations: AGRP, Agouti-related protein; BMI, body mass index; C-RIA, RIA for the carboxyl terminal; GHS, GH secretagogue; Lep Tg, leptin transgenic; NPH, neutral protamine Hagedorn; N-RIA, RIA for the amino terminal; RP-HPLC, reverse phase HPLC; TFA, trifluoroacetic acid.

plasma ghrelin levels in genetically obese *ob/ob* and *db/db* mice and Zucker fatty (*fa/fa*) rats using two kinds of RIAs that recognize total ghrelin and the active form of ghrelin separately. We also examined them in leptin transgenic (Lep Tg) mice. Lep Tg mice were recently generated transgenic mice on a C57BL/6J background overexpressing leptin under the control of the liver-specific human serum amyloid-P component promoter (39–41). The hyperleptinemia causes reduced food intake and disappearance of lipid from adipose tissue in these mice. Here we show that plasma levels of both total ghrelin and the active form of ghrelin after fasting are reduced in *ob/ob* and *db/db* mice and elevated in Lep Tg mice compared with those in their control mice. To study the secretory regulation of ghrelin further, we use Zucker fatty (*fa/fa*) rats and clearly demonstrate that insulin-induced hypoglycemia restores the reduced response of ghrelin secretion in them. In addition, we show that the secretory regulation of ghrelin by fasting is more reduced in older, *i.e.* more obese, fatty rats. The data in this study suggest that short-term secretory regulation of ghrelin reflects energy deposit and that blood glucose levels are involved in the altered regulation.

Materials and Methods

All procedures in animal experiments were approved by the Kyoto University Graduate School of Medicine committee on animal research. Blood samples were collected from the inferior vena cava of the mouse or the jugular vein of the rat under anesthesia with diethyl ether unless indicated. The human study was approved by the ethical committee on human research of Kyoto University Graduate School of Medicine, and all subjects gave their written informed consent.

Obese and "skinny" mice

Twenty-week-old male genetically obese *ob/ob* and *db/db* and their control (+/?) mice were purchased from Japan CLEA (Tokyo, Japan). Generation of Lep Tg skinny mice has been reported previously (40). Twelve-week-old female Lep Tg and nontransgenic control mice were used. The transgenic mice were used as heterozygotes. These animals were housed in a temperature-, humidity-, and light-controlled room (12-h light/12-h dark cycle, lights on at 0800 h) and allowed free access to water and standard rat chow (352 kcal/100 g, CE-2, Japan CLEA) before the study. The body weight was measured, and 400 μl blood were sampled at 1100 h after 12-h fasting. Plasma ghrelin was measured using two kinds of RIAs as described below. After blood samples were drawn, the mice were killed by chloroform, and the stomach was immediately removed and frozen in liquid nitrogen. The samples were stored at −80 C until use, and then they were prepared for ghrelin RIAs as described below. To rule out the direct effect of leptin deficiency on plasma ghrelin levels in *ob/ob* mice, they were injected with leptin. Twenty-week-old mice were purchased and housed as described above. They were ip injected with 1.0 mg/kg leptin (PeproTech EC, London, UK) or saline after 12-h fasting, and 400 μl blood were sampled 3 h after injection for the measurement of plasma ghrelin. The effect of food restriction on plasma ghrelin levels was also studied. Eight-week-old C57/BL6 mice were purchased and housed as described above. They were fed 70% of the average daily food intake of the control mice for 14 d, and 400 μl blood were sampled after overnight fasting for the measurement of plasma ghrelin.

Plasma ghrelin levels in Zucker fatty rats and effect of insulin-induced hypoglycemia

Fifteen-week-old genetically obese Zucker fatty (*fa/fa*) and the control (+/?) rats were purchased from Japan CLEA. These animals were housed in a temperature-, humidity-, and light-controlled room (12-h light/12-h dark cycle, lights on at 0800 h) and allowed free access to water and standard rat chow (352 kcal/100 g, CE-2, Japan CLEA) before the study. Their body weight was 542.5 ± 27.2 g (mean ± SD). They were fasted for 24 h for the measurement of blood glucose and plasma ghrelin. The blood samples of the control rats were also subjected to reverse phase HPLC (RP-HPLC) coupled with C-RIA for the carboxyl terminal and N-RIA for the amino terminal. Then fatty rats were sc injected with 8.0 U/kg human neutral protamine Hagedorn (NPH) insulin (Humulin N, Eli Lilly Japan, Kobe, Japan) to examine the effect of hypoglycemia on plasma ghrelin levels. About 25 μl blood were obtained 30, 60, 90, 120, and 240 min after insulin injection by making a small incision on the tail for the measurement of blood glucose. For the measurement of plasma ghrelin, 600 μl blood were sampled before and after 12- and 24-h fasting and 120 and 240 min after insulin injection. Blood glucose was measured using One Touch II (Life Scan, Milpitas, CA), and plasma ghrelin was measured by two kinds of RIAs.

Effect of glucose injection on plasma ghrelin levels in fasted rats

Eight-week-old male Sprague Dawley rats were purchased, housed, and fed as described above. Their body weight was 200.0 ± 5.1 g (mean ± SD). They were fasted for 24 h and then ip injected with 2.0 ml saline or glucose solutions containing 2.0 or 5.0 g/kg glucose. For the measurement of plasma ghrelin, 600 μl blood were sampled before fasting (at 1100 h), after 24-h fasting, and 90 min after injection. Plasma ghrelin was measured by two kinds of RIAs.

Effect of severity of obesity on plasma ghrelin levels

Younger and older Zucker fatty rats were studied to determine the effect of severity of obesity on plasma ghrelin levels by fasting. Eight- and 30-wk-old Zucker fatty and the control rats were purchased and housed as described above. The body weight was measured, and they were fasted for 48 h, except for free access to water. Then the animals were given free access to food (standard rat chow) and water for 6 h. For the measurement of plasma ghrelin, 600 μl blood were sampled before and after 24- and 48-h fasting (at 1100 h) and after 6-h refeeding. Plasma ghrelin was measured using two kinds of RIAs.

Plasma ghrelin levels in obese human subjects

Seventeen obese Japanese subjects with no apparent medical illness [body mass index (BMI), >25.0 kg/m^2] were recruited. They consisted of nine men and eight women. Their age and BMI were 53 ± 4 yr and 35.8 ± 1.5 kg/m^2 (mean ± SD), respectively. Twenty-one sex- and age-matched control subjects were also studied. Their age and BMI were 49 ± 5 yr and 21.1 ± 0.6 kg/m^2, respectively. Blood samples were drawn between 0800–1000 h after overnight fasting, and plasma ghrelin was measured by C-RIA as described below.

Preparation of stomach samples from mice

Stomach samples were prepared from mice as previously described (7, 18). Each sample was diced and boiled for 7 min in a 5-fold volume of water for the measurement of ghrelin. The solution was adjusted to 1.0 M acetic acid and 20 mM hydrogen chloride after boiling, and the tissue was homogenized. The supernatant was obtained after centrifugation at 10,000 rpm for 30 min.

Preparation of plasma samples

Plasma samples were prepared as previously described (1, 17). Blood samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan; 1000 kallikrein inactivator U/ml), and centrifuged at 4 C. For N-RIA, hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after separation of plasma.

Measurement of ghrelin

Measurement of mouse and rat ghrelin. Two kinds of polyclonal antibodies were raised against the amino terminal (Gly^1-Lys^{11}) and the carboxyl terminal (Gln^{15}-Arg^{28}) of rat ghrelin in rabbits as previously described

(7, 38). Mouse ghrelin has a completely identical structure as rat ghrelin (Iwakura, H., and K. Hosoda, manuscript submitted). One milliliter of the prepared plasma sample was diluted with an equal volume of 0.9% NaCl and loaded onto a Sep-Pak C_{18} cartridge (Waters Corp., Milford, MA) preequilibrated with 0.9% NaCl. For the prepared stomach samples, supernatant after the centrifugation was loaded onto a Sep-Pak C_{18} cartridge preequilibrated with 0.9% NaCl. The cartridge was washed with 3.0 ml 5% CH_3CN/0.1% trifluoroacetic acid (TFA) and eluted with 3.0 ml of 60% CH_3CN/0.1% TFA. The eluate was evaporated, lyophilized, and dissolved in RIA buffer [50 mM sodium phosphate buffer (pH 7.4), 0.5% BSA, 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA-2Na, and 0.05% NaN_3]. Two kinds of RIAs, C-RIA for the carboxyl terminal and N-RIA for the amino terminal of ghrelin, were carried out. Two tracer ligands were synthesized: [Tyr^0]rat ghrelin for antighrelin-(1–11) antiserum and [Tyr^{29}]rat ghrelin-(13–28) for antighrelin-(13–28). These ligands were radioiodinated by the lactoperoxidase methods. After radioiodination, monoiodinated ligands were purified by RP-HPLC on a μBondasphere C_{18} column (3.9 × 150 mm; Waters Corp., Milford, MA). The tracers were stored at −20 C in 0.1% BSA. Each RIA incubation mixture was composed of 100 μl standard ghrelin or unknown sample and 200 μl antiserum diluted with RIA buffer containing 0.5% normal rabbit serum. The antighrelin-(1–11) and antighrelin-(13–28) antisera were used at final dilutions of 1:6,000,000 and 1:20,000, respectively. After 12-h incubation, 100 μl ^{125}I-labeled tracers (15,000 cpm) were added. After an additional 36-h incubation, 100 μl antirabbit IgG goat serum were added. Free and bound tracers were separated after 24-h incubation by centrifugation at 3,000 rpm for 30 min. After aspiration of the supernatant, radioactivity in the pellet was counted with a γ-counter (ARC-600, Aloka, Tokyo, Japan). The minimal detectable quantities by C-RIA and N-RIA were 5.0 and 0.5 fmol/tube, respectively. The intraassay coefficients of variation of C-RIA and N-RIA were 6.0% and 3.0%, respectively, and the interassay coefficients of variation were 9.0% and 6.0%, respectively. The recoveries of ghrelin were more than 95% for both C-RIA and N-RIA.

Measurement of human ghrelin. Plasma ghrelin was measured as reported previously (1, 17). Briefly, polyclonal antibody against the carboxyl terminal of human ghrelin, which has an identical structure as rat ghrelin, was used. The RIA was performed similarly as described above.

Characterization of plasma ghrelin in Zucker control rats

Plasma ghrelin was characterized using RP-HPLC coupled with C-RIA and N-RIA as previously described (7, 18, 34). Plasma samples of 24-h fasted Zucker control rats were prepared and loaded on the Sep-Pak C_{18} cartridge as described above. The eluate was subjected to RP-HPLC on a μBondasphere C_{18} column. The RP-HPLC was performed using a linear gradient of CH_3CN from 10–60% in 0.1% TFA for 40 min. An aliquot of each fraction obtained by RP-HPLC was evaporated and lyophilized, and one fifth of each fraction was subjected to two kinds of RIAs for the measurement of ghrelin.

Data analysis

Results are expressed as the mean ± SE unless noticed. Comparisons between groups were performed with unpaired *t* test. The changes in body weight, blood glucose levels, and plasma ghrelin levels were compared by ANOVA using Fisher's test. Simple linear regression analysis was used to evaluate correlation between BMIs and plasma ghrelin levels. $P < 0.05$ was considered statistically significant.

Results

Stomach and plasma ghrelin levels in genetically obese ob/ob and db/db mice

Body weights and stomach ghrelin levels measured by C-RIA and N-RIA of *ob/ob* and *db/db* and their control mice are summarized in Table 1. The *ob/ob* and *db/db* mice weighed 90.0% and 79.0% more than their control mice, respectively ($P < 0.005$). Stomach ghrelin levels were significantly lower in these obese mice than in their control mice ($P < 0.005$ by C-RIA and $P < 0.05$ by N-RIA, for both). Figure 1, A and B, shows plasma ghrelin levels in *ob/ob*, *db/db*, and their control mice. Plasma ghrelin levels by C-RIA in *ob/ob* and control mice were 452.5 ± 25.4 and 646.5 ± 78.0 fmol/ml, respectively (Fig. 1A, *upper panel*). Those in *db/db* and the control mice were 313.2 ± 23.0 and 486.2 ± 49.7 fmol/ml, respectively (Fig. 1B, *upper panel*). The differences between obese and their control mice were significant ($P < 0.05$ for *ob/ob* and $P < 0.05$ for *db/db*). Plasma ghrelin levels by N-RIA in *ob/ob* and control mice were 12.7 ± 2.4 and 29.3 ± 2.8 fmol/ml, respectively (Fig. 1A, *lower panel*). Those in *db/db* and control mice were 6.1 ± 0.6 and 20.3 ± 1.6 fmol/ml, respectively (Fig. 1B, *lower panel*). The differences between obese and their control mice were also significant ($P < 0.005$ for *ob/ob* and $P < 0.0001$ for *db/db*).

TABLE 1. Body weights and stomach ghrelin levels measured by C-RIA and N-RIA in genetically obese *ob/ob* and *db/db* mice, Lep Tg mice, and their control mice (+/? for *ob/ob* and *db/db* mice and non-Tg for Lep Tg mice)

	BW (g)	Stomach ghrelin (fmol/mg)	
		C-RIA	N-RIA
ob/ob	60.2 ± 3.7[a]	30.9 ± 3.1[a]	8.3 ± 1.3[b]
+/?	31.7 ± 1.1	60.1 ± 2.5	14.6 ± 1.1
db/db	51.2 ± 4.8[a]	21.7 ± 3.2[a]	5.2 ± 0.6[b]
+/?	28.6 ± 2.7	38.3 ± 1.5	8.4 ± 0.6
Lep Tg	12.8 ± 1.0[a]	62.5 ± 4.4	9.9 ± 0.8[b]
non-Tg	17.1 ± 1.1	54.7 ± 1.9	13.1 ± 0.5

Values are given as the mean ± SEM. BW, Body weight.
[a] $P < 0.005$ *vs.* their control mice.
[b] $P < 0.05$ *vs.* their control mice.

Stomach and plasma ghrelin levels in Lep Tg mice

Lep Tg mice weighed 25.1% less than the control mice (Table 1; $P < 0.005$). No visible adipose tissue was found in sc, epididymal, mesenteric, or retroperitoneal fat depots from Lep Tg mice, as previously described (40). Stomach ghrelin levels in them are shown in Table 1. Lep Tg mice showed higher stomach ghrelin level by C-RIA than the control mice, although the difference was not significant. On the other hand, stomach ghrelin level by N-RIA was significantly lower in Lep Tg mice compared with control mice. Figure 1C shows plasma ghrelin levels in Lep Tg and control mice. Plasma ghrelin levels by C-RIA in Lep Tg and the control mice were 3680.5 ± 839.0 and 1283.3 ± 164.6 fmol/ml, respectively (Fig. 1C, *upper panel*). The difference was significant ($P < 0.05$). Plasma ghrelin levels by N-RIA in Lep Tg and the control mice were 158.1 ± 31.2 and 62.9 ± 21.2 fmol/ml, respectively (Fig. 1C, *lower panel*). The difference was also significant ($P < 0.05$).

Effect of leptin injection in ob/ob mice

Table 2 shows plasma ghrelin levels in leptin-injected *ob/ob* mice. Plasma ghrelin levels by C-RIA or N-RIA showed no significant difference between leptin- and saline-injected *ob/ob* mice.

Effect of food restriction on plasma ghrelin levels

Table 3 shows body weight changes and plasma ghrelin levels in food-restricted and *ad libitum*-fed mice. Body weight