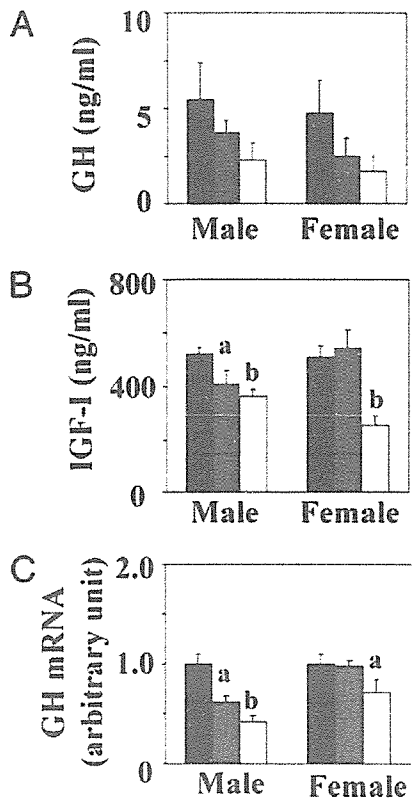

FIG. 4. Serum GH, IGF-I, and pituitary GH mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice (n = 8/group). A, Serum GH levels. B, Serum IGF-I levels. C, Pituitary GH mRNA levels. a, $P < 0.05$; b, $P < 0.01$ (*vs.* control mice).

5. No significant difference was noted in the levels between control and transgenic mice.

Hematoxylin eosin and immunohistochemical staining for GH of the pituitary

Hematoxylin eosin staining is shown in Fig. 5, A and B. The pituitary morphology of Tg 10–1 mice was not different from that of the control mice. Immunohistochemical staining for GH is shown in Fig. 5, C and D. The distribution of GH-immunoreactive cells in the pituitary of Tg mice was similar to that of control mice.

Effects of GHRH and ghrelin on GH release

Control and Tg 10–1 mice were used. Serum GH levels after GHRH administration in male and female Tg 10–1 mice were similar to those of control mice throughout the course of the experiment (Fig. 6A). There was no significant difference in serum GH level at each time point between both male and female Tg-10 and control mice. Serum GH levels 10 min after ghrelin administration in male Tg10–1 and control mice were 63.1 ± 6.8 and 72.6 ± 12.0 ng/ml, respectively (Fig. 6B, *left panel*). The difference was not significant. Serum GH levels 20 min after ghrelin administration in male Tg10–1 and control mice were 30.2 ± 6.7 and 61.2 ± 15.5 ng/ml, respectively, and levels after 30 min were 11.8 ± 1.4 and 21.9 ± 4.1 ng/ml, respectively (Fig. 6B, *left panel*). Both differences were significant ($P < 0.01$). Serum GH levels 10 min after ghrelin administration in female Tg10–1 and control mice were 8.7 ± 3.7 and 52.8 ± 8.2 ng/ml, respectively, and those after 20 min were 29.8 ± 6.3 and 78.5 ± 14.3 ng/ml, respectively (Fig. 6B, *right panel*). Both differences were significant ($P < 0.01$). Serum GH levels 30 min after ghrelin administration in female Tg10–1 and control mice were 22.8 ± 6.3 and 22.3 ± 8.8 ng/ml, respectively (Fig. 6B, *right panel*). The difference was not significant.

TABLE 5. Plasma ACTH, serum TSH, LH, and FSH levels of 8-wk-old control and transgenic mice (n = 8/group)

	Control	Tg 9-2	Tg 10-1
ACTH (pg/ml)	135 ± 35	144 ± 32	123 ± 44
TSH (ng/ml)	3.31 ± 0.06	3.37 ± 0.11	3.49 ± 0.13
LH (ng/ml)	31.5 ± 2.1	30.8 ± 1.7	27.7 ± 2.1
FSH (ng/ml)	268.4 ± 21.8	221.1 ± 43.9	253.5 ± 24.6

Values are given as the mean ± SEM.

Expression of GHS-R in the pituitary

GHS-R mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 1.56, and 3.46 AU, respectively (Fig. 7). The difference between control and Tg 10–1 mice was significant ($P < 0.01$).

Expression of hypothalamic neuropeptides that regulate GH secretion

GHRH mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 0.88, and, 0.80 AU, respectively (Fig. 8A). The differences between control and Tg 9–2 mice and control and Tg 10–1 mice were not significant. Somatostatin mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 1.08, and 0.97 AU, respectively (Fig. 8B). The differences between control and Tg 9–2 mice and control and Tg 10–1 mice were not significant.

Effects of continuous infusion of des-acyl ghrelin on GH-IGF-I axis and body weights

Male and female C57BL/6 mice were used. Serum GH levels after 10 d treatment with saline and des-acyl ghrelin in male mice were 5.8 ± 1.1 and 7.5 ± 2.0 ng/ml, respectively. The difference was not significant. Those with saline and des-acyl ghrelin in female mice were 9.2 ± 2.2 and 9.5 ± 1.8 ng/ml, respectively. The difference was not significant either. Serum IGF-I levels after 10 d treatment with saline and des-acyl ghrelin in male mice were 769.3 ± 16.6 and 768.7 ± 21.6 ng/ml, respectively. The difference was not significant. Those with saline and des-acyl ghrelin in female mice were 766.2 ± 13.4 and 719.4 ± 49.1 ng/ml, respectively. The difference was not significant either. Body weights and lengths in des-acyl ghrelin-injected mice were not significantly different from those in saline-injected mice in either males or females (data not shown).

Discussion

We have generated transgenic mouse lines that overexpress preproghrelin mRNA in a wide variety of tissues. The wide tissue distribution of preproghrelin mRNA in trans-

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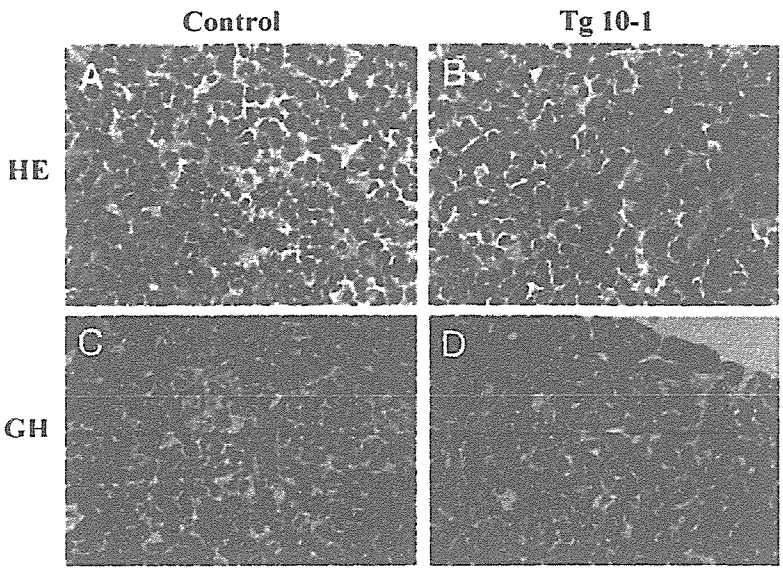

FIG. 5. Morphology of the pituitary and the localization of GH-immunoreactive cells in the pituitary of 8-wk-old male control (A and C) and Tg 10–1 (B and D) mice. A and B, Hematoxylin eosin (HE) staining. C and D, The localization of total and GH-immunoreactive cells in the pituitary. Original magnification, ×40. The immunoreactive cells are stained *brown* by the avidin-biotin complex methods.

genic mice was consistent with previous reports on transgenic mice using the CAG promoter (33, 34). Preproghrelin mRNA expression was increased, especially in Tg 10–1 mice, and its amount in the stomach reached 52-fold of that in control mice. Consistent with the elevated mRNA expression, peptide levels of total ghrelin (des-acyl plus acylated ghrelin) in various tissues were also elevated in transgenic mice. Plasma total ghrelin levels in transgenic mice showed marked results. Those in transgenic mice showed 10- and 44-fold of those in control mice. We originally intended to generate mice overexpressing biologically active ghrelin. Unexpectedly, acylated ghrelin levels were not changed in all tissues examined and plasma of transgenic mice, compared with those of control mice, indicating that transgenic mice overexpress only des-acyl ghrelin. The expression of acylated ghrelin has been reported in a small number of tissues, such as the stomach (X/A cells), duodenum, hypothalamus, and pancreatic α-cells (1, 31, 39, 40). These reports and our present data suggest that only a limited number of cell lineages may able to process proghrelin or acylate ghrelin. The underlying mechanism by which ghrelin is acylated is unknown to date. Further study is needed to clarify the mechanism of the acylation.

The acylation of ghrelin is assumed to be essential for its actions, and des-acyl ghrelin, which lacks the modification, is devoid of endocrine actions, based on previous studies (1, 41). However, recent studies indicated that des-acyl ghrelin may have some actions. Des-acyl ghrelin as well as acylated ghrelin causes a significant inhibition of cell proliferation in human breast carcinoma cell lines (29) and inhibits cell death in cardiomyocytes and endothelial cells through ERK1/2 and phosphatidylinositol 3-kinase/AKT (30). In addition, one study (42) reported that acylated and des-acyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of known GHS-Rs. Moreover, another study (28) indicated that des-acyl ghrelin may offset the action of acylated ghrelin on insulin secretion. Ghrelin has been shown to induce a reduction in serum insulin levels. In the study, coadministration of acylated plus des-acyl ghrelin did not re-

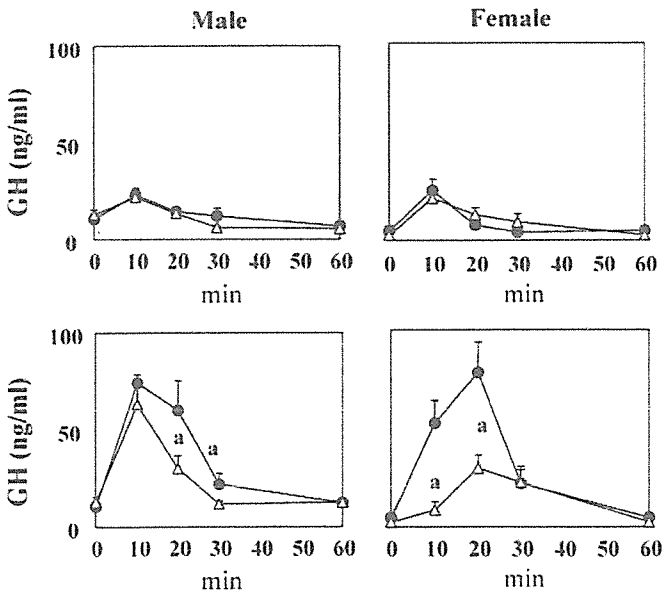

FIG. 6. The responses of GH to GHRH and ghrelin in 8-wk-old control (*closed circles*) and Tg 10–1 (*open triangles*) mice. A, Time course of serum GH levels after iv injection of 60 μg/kg GHRH (n = 8/each point). B, Time course of serum GH levels after iv injection of 40 μg/kg ghrelin (n = 8/each point). a, $P < 0.01$ (*vs.* control mice).

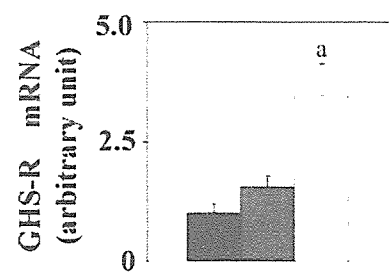

FIG. 7. Pituitary GHS-R mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis (n = 8/group). a, $P < 0.01$ (*vs.* control mice).

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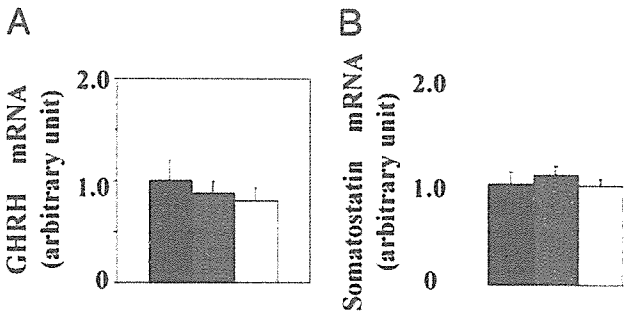

FIG. 8. Hypothalamic GHRH and somatostatin mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis. A, GHRH mRNA levels (n = 8/group). B, Somatostatin mRNA levels (n = 8/group).

sult in any changes in serum insulin levels in humans, suggesting that ghrelin action on insulin is modulated by des-acyl ghrelin.

The present study indicates that transgenic mice overexpressing des-acyl ghrelin show small phenotype. Longitudinal growth was the most reduced in female Tg 10–1 mice (20% reduction from control mice). The phenotype was not associated with changes in BMIs. These mice did not show decreased food intake or decreased body fat mass. In addition, they showed normal nutritional condition, based on their biochemical parameters, including blood glucose, serum total protein, and total cholesterol levels. These data indicate that the small phenotype of transgenic mice is not attributed to poor nutritional condition.

Serum IGF-I levels were significantly reduced in male and female transgenic mice, compared with control mice. Female Tg 10–1 mice had no less than 50% reduction in serum IGF-I levels, compared with control mice. Although the differences in serum GH levels between control and transgenic mice were not statistically significant, probably because of the pulsatile character of GH secretion, the levels tended to be reduced in transgenic mice, compared with control mice, and the mean GH level of Tg10–1 mice was only 50% of that of control mice. It should be emphasized that Tg 10–1 mice showed lower serum GH levels than Tg 9–2 mice. Body weights and lengths of the former were more reduced than the latter. It should be also noted that the former showed higher des-acyl ghrelin expression than the latter. Reduced pituitary GH mRNA levels in transgenic mice support the observation. The GH-IGF-I axis-specific alteration in transgenic mice was also indicated by the measurement of other anterior pituitary hormones than GH. Plasma ACTH, serum TSH, LH, and FSH levels were not altered.

The size and morphology of the pituitary including the somatotrope populations of transgenic mice were similar to those of control mice. These data indicated that there is no apparent change, suggesting developmental problems in the pituitary of transgenic mice.

Responses of GH to GHRH and ghrelin in transgenic mice exhibited intriguing results. Transgenic mice showed normal response of GH to GHRH. Alternatively, if we consider that the basal GH levels are lower in transgenic mice, the similar maximal response might indicate that they are hyperresponsive to GHRH. It is not likely that an insufficient dose of GHRH induced submaximal response of GH in both control and transgenic mice, judging from previous reports (43). On the other hand, the responses of GH to ghrelin were reduced in transgenic mice. It is noteworthy that the reduction was much greater in female transgenic mice than in male mice, if we take their serum IGF-I levels into account. Taken together our results and these reports indicate that overexpression of des-acyl ghrelin in our mice may result in reduction of GH response to endogenous ghrelin, and it may result in the reduced serum IGF-I levels in transgenic mice.

The reduced GH response to ghrelin in transgenic mice could be due to down-regulated the GHS-R. However, the pituitary GHS-R mRNA levels in the transgenic mice were rather elevated. It is not likely that overexpressed des-acyl ghrelin acts as a blocking agent to the GHS-R because ^{125}I-labeled acylated ghrelin bound to the GHS-R cannot be displaced by des-acyl ghrelin (20). Overexpressed des-acyl ghrelin may have some effects on endogenous GH secretion, modifying the action of endogenous ghrelin in transgenic mice via, for instance, another receptor or modulation of the signal transduction pathway after the GHS-R.

Previous reports indicated that the hypothalamus plays a critical role in the stimulatory effect of ghrelin on GH secretion as well as the pituitary (21, 22, 23). Because GH secretion is regulated chiefly by two hypothalamic hormones, GHRH and somatostatin, the expression of these hormones could be altered in transgenic mice. We could not find any significant difference in either GHRH or somatostatin mRNA levels between control and transgenic mice. These data might suggest that overexpressed des-acyl ghrelin acts on not only the pituitary but also the hypothalamus in the transgenic mice, judging from the fact that hypothalamus GHRH mRNA were not elevated, and somatostatin mRNA levels were not decreased despite the decreased serum GH levels.

We could not show, unfortunately, that continuous ip infusion of des-acyl ghrelin has some effect on serum GH and IGF-I levels or body weights. It should be noted, however, that plasma des-acyl ghrelin levels in transgenic mice reached 10- and 50-fold of those in control mice. Administration of a higher dose of des-acyl ghrelin, or longer administration, might result in alteration in the GH-IGF-I axis. On the other hand, the phenotype of transgenic mice might reflect direct effects of ubiquitous expression of des-acyl ghrelin. It should also be noted that high levels of des-acyl ghrelin were detected in a various tissues, especially in the pituitary, as well as in plasma of transgenic mice. The des-acyl ghrelin immunoreactive pituitary cells might play an important role in the mechanism for the altered GH-IGF-I axis in a paracrine or autocrine manner. It should be pointed out that preproghrelin mRNA is reported to be expressed in the normal pituitary (44), as we showed in the present study, suggesting its physiological role in GH secretion. The phenotype of transgenic mice may reflect the role. Further study is needed for this issue.

The mechanism underlying the sexual dimorphism in the responses of GH to ghrelin in transgenic mice is not fully understood. It might be due to the gender difference in the secretory regulation of GH. Female mice have been reported to be different from male mice in that they have noncyclical

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and rather low somatostatin output and that GHRH plays a dominant role in it (45). There might be a GHRH-dependent mechanism for the reduced response in transgenic mice. Indeed, one recent report (26) indicated that transgenic rats expressing an antisense GHS-R mRNA in the hypothalamic arcuate nucleus show marked gender difference in GH secretion. Although there was no significant difference in pulse frequency and baseline levels of GH between male control and transgenic rats, female transgenic rats showed lower baseline levels and fewer pulses of GH than female control rats (26).

The 94-amino acid proghrelin is cleaved to yield ghrelin. One previous study (46) demonstrated that C-terminal proghrelin peptides are present in the human circulation. Transgenic mice in the present study would also overexpress these peptides. We have not excluded the possibility that the phenotype of transgenic mice might be due to the effects of these peptides.

In conclusion, the present study demonstrates that transgenic mice overexpressing des-acyl ghrelin show small phenotype and altered GH-IGF-I axis. These observations may indicate a role of des-acyl ghrelin in the regulation of GH secretion.

Acknowledgments

The authors gratefully acknowledge the excellent technical support of Chieko Ishimoto and Hitomi Hiratani.

Received May 17, 2004. Accepted October 1, 2004.

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This work was supported by research grants from the Japanese Ministry of Education, Science, and Culture and the Japanese Ministry of Health, Labor, and Welfare.

References

1. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
2. **Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K, Nakazato M** 2000 Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. Biochem Biophys Res Commun 275:477–480
3. **Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K** 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 86:4753–4758
4. **Lu S, Guan JL, Wang QP, Uehara K, Yamada S, Goto N, Date Y, Nakazato M, Kojima M, Kangawa K, Shioda S** 2001 Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. Neurosci Lett 321: 157–160
5. **Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL** 2003 The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–661
6. **Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS** 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714–1719
7. **Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M** 2001 Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120:337–345
8. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S** 2001 A role for ghrelin in the central regulation of feeding. Nature 409:194–198
9. **Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K** 2002 Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. Endocrinology 143:3341–3350
10. **Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML** 2001 Circulating ghrelin levels are decreased in human obesity. Diabetes 50: 707–709
11. **Hanada T, Toshinai K, Kajimura N, Nara-Ashizawa N, Tsukada T, Hayashi Y, Osuye K, Kangawa K, Matsukura S, Nakazato M** 2003 Anti-cachectic effect of ghrelin in nude mice bearing human melanoma cells. Biochem Biophys Res Commun 301:275–279
12. **Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K** 2001 Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227–232
13. **Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal IJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR** 2001 Ghrelin causes hyperphagia and obesity in rats. Diabetes 50:2540–2547
14. **Dieguez C, Casanueva FF** 2000 Ghrelin: a step forward in the understanding of somatotroph cell function and growth regulation. Eur J Endocrinol 142: 413–417
15. **Seoane LM, Tovar S, Baldelli R, Arvat E, Ghigo E, Casanueva FF, Dieguez C** 2000 Ghrelin elicits a marked stimulatory effect on GH secretion in freely moving rats. Eur J Endocrinol 143:R7–R9
16. **Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K** 2000 Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 85:4908–4911
17. **Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD** 1997 Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 48:23–29
18. **Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M** 2002 The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab 87:2988
19. **Willesen MG, Kristensen P, Romer J** 1999 Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology 70:306–316
20. **Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R** 2001 Binding of ^{125}I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest 24:RC7–RC9
21. **Torsello A, Grilli R, Luoni M, Guidi M, Ghigo MC, Wehrenberg WB, Deghenghi R, Muller EE, Locatelli V** 1996 Mechanism of action of hexarelin. I. Growth hormone-releasing activity in the rat. Eur J Endocrinol 135:481–488
22. **Maheshwari HG, Rahim A, Shalet SM, Baumann G** 1999 Selective lack of growth hormone (GH) response to the GH-releasing peptide hexarelin in patients with GH-releasing hormone receptor deficiency. J Clin Endocrinol Metab 84:956–959
23. **Hataya Y, Akamizu T, Takaya K, Kanamoto N, Ariyasu H, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K, Nakao K** 2001 A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. J Clin Endocrinol Metab 86:4552–4555
24. **Okimura Y, Ukai K, Hosoda H, Murata M, Iguchi G, Iida K, Kaji H, Kojima M, Kangawa K, Chihara K** 2003 The role of circulating ghrelin in growth hormone (GH) secretion in freely moving male rats. Life Sci 72:2517–2524
25. **Sun Y, Ahmed S, Smith RG** 2003 Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 23:7973–7981
26. **Shuto Y, Shibasaki T, Otagiri A, Kuriyama H, Ohata H, Tamura H, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I** 2002 Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. J Clin Invest 109:1429–1436
27. **Hosoda H, Kojima M, Matsuo H, Kangawa K** 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 279:909–913
28. **Broglio F, Prodam F, Benso A, Gottero C, Destefanis S, Gauna C, van der Lely AJ, Ghigo E,** The peripheral but not the neuro-endocrine response to acylated ghrelin is modulated by non-acylated ghrelin in humans. Program of the 85th Annual Meeting of The Endocrine Society, Philadelphia, PA, 2003, p 264 (Abstract P1-553)
29. **Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, Deghenghi R, Reissmann T, Ghigo E, Muccioli G** 2001 Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. J Clin Endocrinol Metab 86:1738–1745
30. **Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A** 2002 Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 159:1029–1037
31. **Iwakura H, Hosoda K, Doi R, Komoto I, Nishimura H, Son C, Fujikura J,**

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Tomita T, Takaya K, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Kangawa K, Imamura M, Nakao K 2002 Ghrelin expression in islet cell tumors: augmented expression of ghrelin in a case of glucagonoma with multiple endocrine neoplasm type I. J Clin Endocrinol Metab 87:4885–4888

32. **Mori K, Yoshimoto A, Takaya K, Hosoda K, Ariyasu H, Yahata K, Mukoyama M, Sugawara A, Hosoda H, Kojima M, Kangawa K, Nakao K** 2000 Kidney produces a novel acylated peptide, ghrelin. FEBS Lett 486:213–216
33. **Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M** 1995 Green fluorescent protein as a marker in transgenic mice. Dev Growth Differ 37:455–459
34. **Niwa H, Yamamura K, Miyazaki J** 1991 Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199
35. **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
36. **Bahary N, Leibel RL, Joseph L, Friedman JM** 1990 Molecular mapping of the mouse db mutation. Proc Natl Acad Sci USA 87:8642–8646
37. **Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S** 1995 Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1:1155–1161
38. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
39. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M** 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141: 4255–4261
40. **Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S** 2002 Ghrelin is present in pancreatic α-cells of humans and rats and stimulates insulin secretion. Diabetes 51:124–129
41. **Broglio F, Benso A, Gottero C, Prodam F, Gauna C, Filtri L, Arvat E, van der Lely AJ, Deghenghi R, Ghigo E** 2003 Non-acylated ghrelin does not possess the pituitaric and pancreatic endocrine activity of acylated ghrelin in humans. J Endocrinol Invest 26:192–196
42. **Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, Wells T** 2004 Ghrelin and des-octanoyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor. Endocrinology 145:234–242
43. **Clark RG, Robinson IC** 1985 Effects of a fragment of human growth hormone-releasing factor in normal and 'little' mice. J Endocrinol 106:1–5
44. **Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Oikawa S** 2001 Regulation of the ghrelin gene: growth hormone-releasing hormone upregulates ghrelin mRNA in the pituitary. Endocrinology 142:4154–4157
45. **Robinson IC** 1991 The growth hormone secretory pattern: a response to neuroendocrine signals. Acta Paediatr Scand Suppl 372:70–78;discussion 79–80
46. **Pemberton C, Wimalasena P, Yandle T, Soule S, Richards M** 2003 C-terminal pro-ghrelin peptides are present in the human circulation. Biochem Biophys Res Commun 310:567–573

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Plasma ghrelin levels in healthy elderly volunteers: the levels of acylated ghrelin in elderly females correlate positively with serum IGF-I levels and bowel movement frequency and negatively with systolic blood pressure

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Abstract

Aging is associated with a decrease in growth hormone (GH) secretion, appetite and energy intake. As ghrelin stimulates both GH secretion and appetite, reductions in ghrelin levels may be involved in the reductions in GH secretion and appetite observed in the elderly. However, only preliminary studies have been performed on the role of ghrelin in elderly subjects. In this study, we sought to clarify the physiologic implications of the age-related alterations in ghrelin secretion by determining plasma ghrelin levels and other clinical parameters in healthy elderly subjects. Subjects were ≥65 years old, corresponding to the SENIEUR protocol, had not had a resection of the upper gastrointestinal tract and had not been treated with hormones. One hundred and five volunteers (49 men and 56 women) were admitted to this study (73·4 ± 6·3 years old). Plasma levels of acylated ghrelin in elderly female subjects positively correlated with serum IGF-I levels and bowel movement frequency and negatively with systolic blood pressure. In elderly men, desacyl ghrelin levels correlated only weakly with bowel movement frequency. These findings suggest that the plasma levels of the acylated form of ghrelin may influence the age-related alterations in GH/IGF-I regulation, blood pressure and bowel motility. These observational associations warrant further experimental studies to clarify the physiologic significance of these effects.

Journal of Endocrinology (2006) **188,** 333 344

Introduction

Aging is associated with progressive decreases in growth hormone (GH) secretion, appetite and energy intake (Wurtman *et al.* 1988, Corpas *et al.* 1993, Morley 1997, Muller *et al.* 1999). This reduced GH secretion is termed 'somatopause' and may be a cause of age-related metabolic and physiologic changes, including reduced lean body mass and expansion of adipose mass. Altered blood lipid profiles also favor the development of vascular diseases that may increase overall mortality. The age-related reduction in energy intake has been termed 'the anorexia of aging' and predisposes to the development of undernutrition (Morley 1997). Common in older people, undernutrition has been implicated in the development and progression of chronic diseases commonly affecting the elderly, as well as in increasing mortality (Wurtman *et al.* 1988).

The mechanisms underlying the reduced GH secretion in aged animals and humans are complex (Muller *et al.* 1999,

DOI: 10.1677/joe.1.06442
0022 0795/06/0188 333 © 2006 Society for Endocrinology *Printed in Great Britain*
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Chapman 2000). Age-related changes appear to involve the function of hypothalamic peptides specifically regulating GH secretion, and GH-releasing hormone (GHRH) and somatostatin (SS), appear to play a major role in this event. Experimental evidence indicates that within the rat hypothalamus, GHRH synthesis is impaired with increased age; relative hyperfunction of the SS-ergic system is also found in this animal. The physiologic causes of the anorexia of aging are largely unknown and probably multifactorial (Morley 1997). Possible mechanisms include a reduction in the central and/or peripheral feeding drives and increased activity of central and/or peripheral satiety signals (Martinez *et al.* 1993, Morley 1997, de Jong *et al.* 1999).

Ghrelin, a 28-amino-acid peptide, exhibits a variety of actions, including vasorelaxation (Nagaya *et al.* 2001, Shimizu *et al.* 2003) and stimulation of GH secretion (Takaya *et al.* 2000, Arvat *et al.* 2001, Hataya *et al.* 2001), appetite (Korbonits *et al.* 2004, van der Lely *et al.* 2004) and gastrointestinal motility (Masuda *et al.* 2000, Trudel *et al.* 2002, Fujino *et al.* 2003). A portion of ghrelin possesses a unique fatty acid modification, *n*-octanoylation, at Ser 3 (Kojima *et al.* 1999). Of the two circulating forms of ghrelin, acylated and unacylated (desacyl), the acylated form is thought to be essential for ghrelin biologic activity. Recently, however, desacyl ghrelin was reported to influence both cell proliferation and adipogenesis (Cassoni *et al.* 2001, Bedendi *et al.* 2003, Broglio *et al.* 2004, Thompson *et al.* 2004), prompting us to hypothesize that alterations in ghrelin may be involved in the reduction of GH secretion and appetite in elderly subjects. Preliminary studies using small numbers of elderly subjects demonstrated that the mean plasma concentrations of total ghrelin in normal weight geriatric subjects were lower than those present in younger, normal-weight subjects (Rigamonti *et al.* 2002, Sturm *et al.* 2003). In addition, GH response to ghrelin administration in elderly subjects is lower than that seen in young subjects (Broglio *et al.* 2003). While ghrelin mRNA levels in the stomach gradually decrease with increasing age in rats, serum levels of total ghrelin did not exhibit obvious age-related variation (Liu *et al.* 2002). In contrast, studies in rat indicated that both stomach ghrelin secretion and ghrelin-induced GH secretion increased in aged rats in comparison to younger rats (Englander *et al.* 2004). Total ghrelin secretion also increases with aging in monkeys (Angeloni *et al.* 2004). Although the disparity between humans and other animal models may be due to species differences, the number of human subjects was rather small. In addition, no adjustment of plasma ghrelin levels by other parameters was attempted, leaving the results of these human studies in question.

In this study, we determined the plasma concentrations of the two ghrelin forms, acylated and desacyl ghrelin, and their relationship to various anthropometric, hormonal and metabolic parameters in 105 elderly volunteers. Using these measurements and appropriate analyses, we sought to clarify the age-related alteration in ghrelin secretion and the associated physiologic implications in elderly subjects.

Materials and Methods

Subjects

One hundred and thirty-seven (62 male and 75 female) elderly volunteers were registered for our study. Thirty-two that did not satisfy the criteria for this study were excluded. Finally, 105 (49 male and 56 female) volunteers aged 65–94 years were subjected to analysis. All of the subjects were Japanese, and were recruited from the outpatient clinics of Kyoto University Hospital ($n=66$ (male, $n=36$; female, $n=30$)) and Kyoto Preventive Medical Center ($n=39$ (male, $n=13$; female, $n=26$)). The inclusion criteria were as follows: 1. ≥ 65 years of age; 2. correspondence with the SENIEUR protocol (Ligthart *et al.* 1984); 3. provision of written consent to participate in this study. Patients with either past history of upper gastrointestinal tract resection or present use of either hormones or steroids were excluded. The SENIEUR protocol provides strict admission criteria for human immunogerontologic studies. This protocol used clinical information (infection, inflammation, malignancy and other conditions, including acute myocardial infarction, treated cardiac insufficiency, hypertension of arteriosclerotic or diabetic origin, dementia, pregnancy, malnutrition, alcoholism and drug abuse), laboratory data (erythrocyte sedimentation rate, hemoglobin levels, mean corpuscular volume, leukocyte count with differentiation, immunoelectrophoresis, urinalysis and serum concentrations of urea, alkaline phosphatase, glucose, ASAT, ALAT and protein) and pharmacologic interference (prescribed medication for the treatment of the disorders defined above, anti-inflammatory drugs, hormones and analgesics) (Ligthart *et al.* 1984). This study included two exclusion criteria (no past resection of the upper gastrointestinal tract and no current treatment with hormones or steroids) to optimize endocrinologic and metabolic examination of stomach-derived hormones. The subjects who met all criteria were recognized as healthy subjects. Younger subjects, in whom plasma ghrelin levels were used for comparison with those in elderly subjects, were described previously (Akamizu *et al.* 2005). They were 16 male and 20 female Japanese volunteers 21–61 years of age. None of the subjects suffered from any known medical conditions or were currently taking medication. The period of the study was from March to September 2004. The study protocol was approved by the ethics committees on human research of Kyoto University Graduate School of Medicine and Kyoto Preventive Medical Center. Written, informed consent was obtained from all subjects prior to enrollment.

Laboratory analyses and biomedical factors

Blood samples for hormone and glucose analyses were drawn from a forearm vein in the morning after overnight fast. Plasma samples were prepared as previously described (Kojima *et al.* 1999, Akamizu *et al.* 2005). Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Kyoto, Japan: 500 kallikrein inactivator U/ml), were centrifuged at 4 °C. We added 1 N mol/l HCl (10% volume of plasma volume) to the separated plasma immediately. Plasma levels of acylated and unacylated ghrelin were measured with two commercially available ELISA kits, the Active Ghrelin ELISA and Desacyl-Ghrelin ELISA respectively, according to the manufacturer's protocol (Mitsubishi Kagaku Iatron, Tokyo, Japan) (Akamizu *et al.* 2005). The minimal detection limits for acylated and desacyl ghrelin in this assay system were 2·5 and 12·5 fmol/ml respectively. The intra- and interassay coefficients of variation were 6·5% and 9·8% for acylated ghrelin and 3·7% and 8·1% for desacyl ghrelin respectively. Ghrelin measurements of samples from the older and young subjects were performed with the same kits, but not in the same assay. Plasma glucose was measured by the glucose oxidase method. Serum GH, insulin-like growth factor (IGF)-I and insulin concentrations were measured by immunoradiometric assay (IRMA), while serum leptin levels were measured by RIA (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Insulin resistance was calculated according to the homeostasis model of assessment of insulin resistance (HOMA-IR), calculated as insulin (μU/ml) × blood glucose (mmol/l)/22·5 (Haffner *et al.* 1997).

The questionnaire presented to all subjects collected information about their sleeping time duration, bowel movements, smoking habits, alcohol consumption, use of medication, past medical history and physical activity. The question about bowel frequency was, 'How often do you usually defecate – once a day, more than once a day or once per 2 or 3 days?'

Statistical analysis

Data are expressed as the mean ± S.D. We used Student's *t*-test to compare the means of the variables measured in both groups. The relationships between ghrelin concentrations and the variables studied were assessed by multiple regression analysis. The variables examined in the multiple regression models were site of recruitment, gender, age or age group (elderly and younger group), body-mass index (BMI), sleeping duration and blood levels of GH, IGF-I, insulin, glucose and leptin. The associations between ghrelin concentrations and blood pressure or bowel movement were assessed by multiple regression analysis after adjustment by the potential confounding factors according to gender. As the ghrelin distribution was slightly skewed, natural logarithms of ghrelin were used for the regression analysis. To identify the subsets of parameters that are statistically significantly related to each hormone level, we performed multiple regression analysis with a backward-elimination procedure after adjustment for the potential effect of site. All statistical analyses were performed by SAS, Version 8·02 (SAS Institute, Cary, NC, USA). *P* values less than 0·05 were considered to be statistically significant.

Results

Plasma ghrelin concentrations in elderly subjects

We examined the anthropometric, hormonal and metabolic parameters of elderly volunteers (Table 1). The levels of acylated ghrelin in plasma were not significantly different between male and female subjects, while plasma levels of desacyl ghrelin in female subjects were significantly higher than those observed in male subjects (male, 53·3 ± 41·5 fmol/ml; female, 72·0 ± 46·1 fmol/ml; $P=0·031$). In comparison to our previous study of younger volunteers (mean age = 33·5 ± 9·0, $n=36$) (Akamizu *et al.* 2005), plasma levels of acylated ghrelin in elderly female subjects were significantly reduced from the levels in younger female subjects (11·9 ± 9·8 vs 19·9 ± 9·8 fmol/ml; $P=0·004$) (Fig. 1A and B). We did not observe any significant differences in the plasma levels of acylated ghrelin in men (9·3 ± 11·6 vs 10·9 ± 6·1 fmol/ml) or desacyl ghrelin levels in both sexes (male, 53·3 ± 41·5 vs 49·1 ± 23·5 fmol/ml; female, 72·0 ± 46·1 vs 79·8 ± 53·9 fmol/ml) between the elder and younger subjects. The ratios of acylated to desacyl ghrelin (A/D ratio) in elderly female subjects were significantly lower than those in younger female subjects (16·3 ± 8·2 vs 26·8 ± 7·8; $P=0·001$). Men did not exhibit any significant differences between the age groups (18·0 ± 11·0 vs 22·6 ± 8·8; $P=0·101$) (Fig. 1C). Age, BMI, insulin, leptin and HOMA-IR levels, however, also differed significantly between the sexes. In addition, the volunteers were recruited from two independent sites. Although nearly all of the parameters, including ghrelin levels, did not differ significantly between the sites, the A/D ratios in both sexes and diastolic blood pressure (BP) values in men were significantly different (Table 1). To account for these differences, recruitment site, gender and age group were included as independent variables in the multiple regression analyses.

Correlations of ghrelin concentrations with various parameters in elderly subjects

In contrast to the results in Student's *t*-test, plasma levels of acylated ghrelin in women were not correlated with age group in the multiple regression analyses ($P=0·914$) (Table 2), suggesting that those in elderly female subjects

Table 1 Characteristcs of elderly subjects and their plasma ghrelin concentrations

		Male				Female				
	All ($n=105$)	All ($n=49$)	KPMC ($n=13$)	KUH ($n=36$)	*P* value*	All ($n=56$)	KPMC ($n=26$)	KUH ($n=30$)	*P* value*	*P* value**
Parameters										
Age (years)	73·4 ± 6·3	75·0 ± 7·0	72·8 ± 4·7	75·8 ± 7·5	0·119	72·0 ± 5·4	69·4 ± 4·7	74·2 ± 5·1	**0·001**	**0·017**
Height (cm)	155·8 ± 8·4	162·1 ± 6·5	163·1 ± 5·1	161·8 ± 6·9	0·488	150·3 ± 5·5	151·3 ± 4·2	149·3 ± 6·4	0·162	**0·001**
Body weight (kg)	55·5 ± 8·5	58·3 ± 9·0	60·4 ± 4·8	57·5 ± 10·0	0·182	53·1 ± 7·2	54·3 ± 7·2	52·1 ± 7·3	0·261	**0·002**
BMI (kg/m^2)	22·9 ± 2·8	22·1 ± 2·6	22·8 ± 1·9	21·9 ± 2·7	0·205	23·5 ± 2·8	23·7 ± 2·9	23·4 ± 2·8	0·671	**0·008**
Acylated ghrelin (fmol/ml)	10·7 ± 10·7	9·3 ± 11·6	6·4 ± 2·8	10·4 ± 13·3	0·099	11·9 ± 9·8	9·8 ± 8·3	13·8 ± 10·8	0·129	0·213
Desacyl ghrelin (fmol/ml)	63·2 ± 44·8	53·3 ± 41·5	65·1 ± 33·6	49·0 ± 43·7	0·183	72·0 ± 46·1	77·3 ± 51·3	67·3 ± 41·4	0·431	**0·031**
A/D ratio (%)	17·1 ± 9·6	18·0 ± 11·0	11·7 ± 6·8	20·3 ± 11·5	**0·003**	16·3 ± 8·2	12·5 ± 5·6	19·6 ± 8·6	**0·001**	0·379
GH (ng/ml)	2·5 ± 3·7	2·5 ± 4·7	3·9 ± 6·7	2·0 ± 3·7	0·349	2·5 ± 2·6	2·8 ± 2·8	2·2 ± 2·4	0·395	0·929
IGF-I (ng/ml)	125·5 ± 36·8	128·5 ± 41·1	134·9 ± 20·8	126·2 ± 46·3	0·370	122·8 ± 32·7	121·7 ± 28·5	123·8 ± 36·5	0·813	0·440
Insulin (μU/ml)	6·1 ± 4·0	5·0 ± 3·1	4·3 ± 1·2	5·2 ± 3·6	0·222	7·2 ± 4·4	6·6 ± 3·2	7·7 ± 5·3	0·319	**0·004**
Glucose (mg/dl)	94·1 ± 9·0	95·0 ± 9·2	92·5 ± 6·4	96·0 ± 9·9	0·156	93·3 ± 8·9	92·4 ± 9·9	94·1 ± 8·0	0·492	0·323
Leptin (ng/ml)	7·9 ± 6·2	4·4 ± 2·3	4·5 ± 1·3	4·3 ± 2·5	0·825	11·0 ± 6·9	10·2 ± 5·6	11·6 ± 8·0	0·440	**0·001**
HOMA-IR	1·4 ± 1·0	1·2 ± 0·8	1·0 ± 0·3	1·2 ± 0·9	0·136	1·7 ± 1·1	1·5 ± 0·7	1·8 ± 1·4	0·223	**0·007**
Systolic BP (mmHg)	140·2 ± 19·6	136·2 ± 19·5	145·8 ± 20·3	132·7 ± 18·3	0·055	143·7 ± 19·1	142·6 ± 20·2	144·6 ± 18·4	0·699	0·050
Diastolic BP (mmHg)	82·4 ± 10·4	81·0 ± 11·0	87·5 ± 10·0	78·7 ± 10·5	**0·014**	83·6 ± 9·8	85·7 ± 11·4	81·7 ± 7·9	0·141	0·215
Sleeping duration (h)***	6·7 ± 1·0	6·8 ± 1·3	6·7 ± 1·1	6·9 ± 1·3	0·594	6·6 ± 0·8	6·8 ± 0·7	6·5 ± 0·8	0·172	0·437

KPMC, Kyoto Preventive Medical Center; KUH, Kyoto University Hospital.
*KPMC vs KUH; **male vs female; ***information of one male subject in KUH is unknown.
Bold values: $P<0·05$.
Système International (SI) units for GH, micrograms per liter (conversion factor, 1·0); for IGF-I to nanomoles per liter (0·131); for glucose, millimoles per liter (0·05551); for insulin, picomoles per liter (6·945); for leptin, nanomoles per liter (0·08).

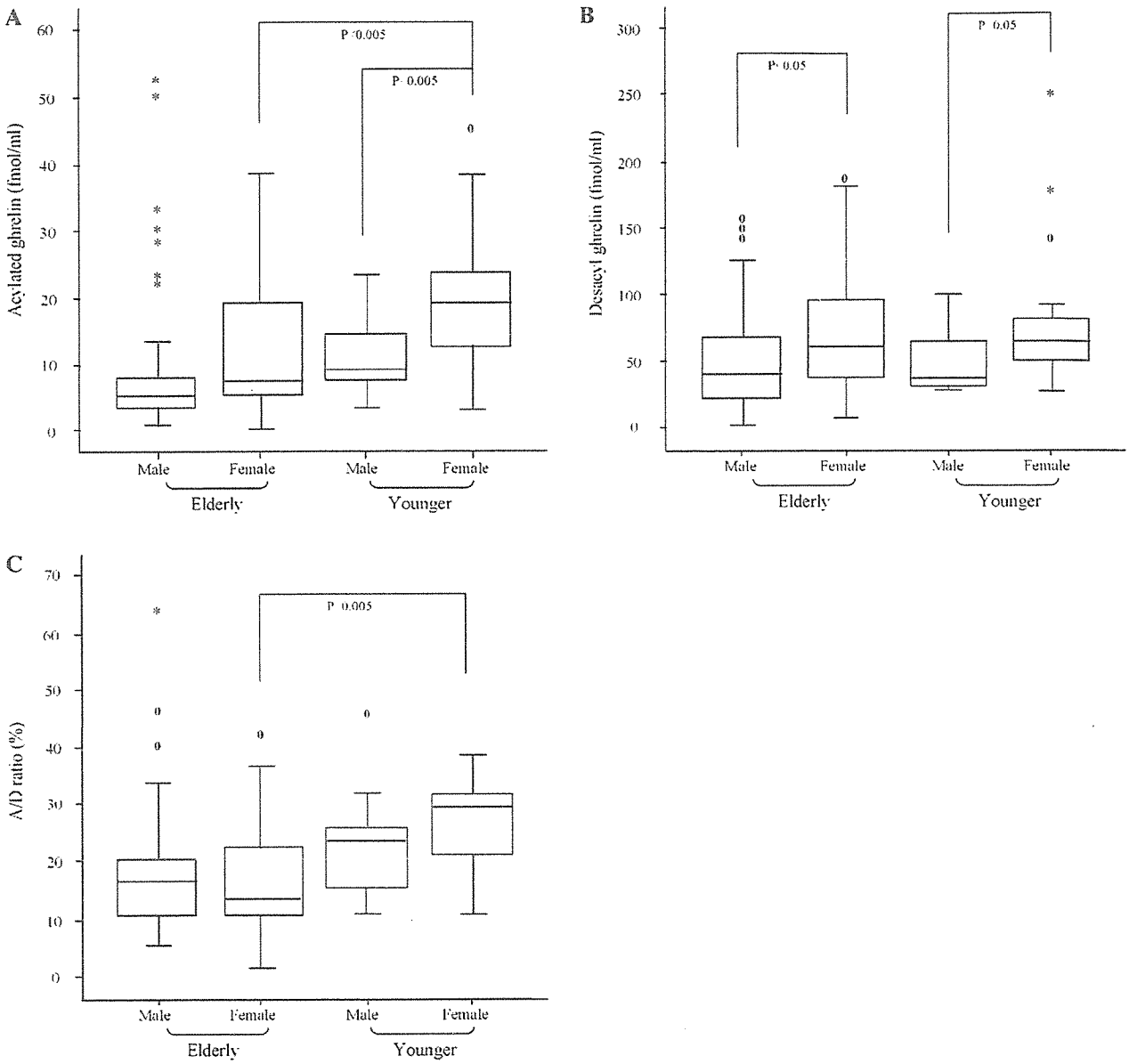

Figure 1 Comparison of plasma acylated ghrelin levels (A), desacyl ghrelin levels (B) and (C) A/D ratios between elderly and younger subjects. The values for subjects younger than 65 years are derived from our previous studies (Akamizu *et al.* 2005). Results are shown as box and whiskers plots. The upper hinge of the box represents the 75th and the lower the 25th percentile. The median is shown as a line across the box. The whiskers above and below the boxes represent the largest and smallest observed scores that are less than 1·5 box lengths from the box. Values farther away are potential outliers. If zero (0) appears, the value is between 1·5 and 3 interquartile ranges from the top or bottom edge of the box. If an asterisk (*) appears, the value is more extreme.

were not significantly different from those in younger female subjects. In addition, plasma levels of desacyl ghrelin in elderly subjects were not associated with gender (P=0·175). Although age and blood levels of GH and insulin were correlated with plasma levels of acylated and/or deacyl ghrelin in younger subjects, no parameter correlated with them in elderly subjects or elderly males. In elderly females, however, acylated ghrelin levels positively correlated with IGF-I (P=0·010). As this positive correlation between ghrelin and IGF-I levels was surprising, we examined the interaction between BMI and IGF-I by dividing female subjects into two groups based on the median value of BMI, 23·3. Acylated ghrelin levels correlated significantly with IGF-I levels in the group with lower (<23·3) (P=0·014), but not higher (>23·3) (P=0·090), BMI values (Fig. 2). We did not observe any

Table 2 Mulitiple regression analysis between plasma ghrelin concentrations and various parameters in healthy elderly and younger subjects

	All						**Elderly**									**Younger*** (All) ($n=36$)		
	Male ($n=65$)			Female ($n=76$)			All ($n=105$)			Male ($n=49$)			Female ($n=56$)					
	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)
Acylated ghrelin																		
Site	–	–	–	–	–	–	0·172	0·433	0·7	−0·076	0·831	0·1	0·540	0·087	6·2	–	–	–
Gender	–	–	–	–	–	–	0·366	0·138	2·3	–	–	–	–	–	–	0·196	0·439	2·2
Age group	−0·184	0·661	0·3	0·047	0·914	<0·1	–	–	–	–	–	–	–	–	–	–	–	–
Age	–	–	–	–	–	–	0·001	0·957	<0·1	0·005	0·873	<0·1	−0·012	0·721	0·3	−0·044	**0·002**	31·0
BMI	−0·058	0·333	1·6	0·064	0·281	1·7	−0·026	0·621	0·3	−0·070	0·426	1·7	0·149	0·065	7·2	0·067	0·136	8·1
GH	−0·003	0·906	<0·1	0·046	0·080	4·4	0·015	0·608	0·3	−0·003	0·938	<0·1	0·094	0·123	5·1	0·036	**0·026**	17·1
IGF-I	0·003	0·204	2·8	0·006	**0·011**	9·2	0·006	0·113	2·7	0·003	0·588	0·8	0·015	**0·010**	13·6	−0·001	0·523	1·5
Insulin	−0·032	0·374	1·4	−0·008	0·811	<0·1	−0·015	0·632	0·2	−0·024	0·657	0·5	−0·008	0·847	<0·1	−0·067	**0·015**	20·2
Glucose	−0·001	0·913	<0·1	0·008	0·559	0·5	0·000	0·988	<0·1	0·000	0·993	<0·1	0·008	0·619	0·5	−0·028	0·086	10·6
Leptin	0·015	0·796	0·1	−0·010	0·717	0·2	0·020	0·470	0·6	−0·009	0·920	<0·1	−0·052	0·176	3·9	−0·002	0·904	<0·1
Sleeping time	–	–	–	–	–	–	−0·008	0·938	<0·1	−0·030	0·813	0·1	0·157	0·369	1·8	–	–	–
Desacyl ghrelin																		
Site	–	–	–	–	–	–	−0·317	0·102	2·8	−0·566	0·124	6·1	−0·044	0·858	<0·1	–	–	–
Gender	–	–	–	–	–	–	0·293	0·175	2·0	–	–	–	–	–	–	0·012	0·959	<0·1
Age group	0·076	0·858	<0·1	0·428	0·214	2·3	–	–	–	–	–	–	–	–	–	–	–	–
Age	–	–	–	–	–	–	−0·007	0·670	0·2	−0·004	0·881	<0·1	−0·015	0·550	0·8	−0·014	0·231	5·3
BMI	−0·011	0·848	<0·1	0·022	0·646	0·3	−0·040	0·387	0·8	−0·046	0·604	0·7	0·047	0·457	1·2	0·034	0·409	2·5
GH	0·022	0·447	1·0	0·037	0·075	4·6	0·021	0·407	0·7	0·012	0·756	0·3	0·060	0·213	3·4	0·027	0·063	12·3
IGF-I	0·003	0·264	2·2	0·004	**0·024**	7·3	0·002	0·460	0·6	0·000	0·994	<0·1	0·008	0·094	6·0	0·001	0·467	2·0
Insulin	−0·038	0·297	1·9	−0·023	0·395	1·1	−0·009	0·745	0·1	−0·018	0·740	0·3	−0·007	0·826	0·1	−0·064	**0·013**	20·9
Glucose	−0·010	0·475	0·9	0·010	0·340	1·3	0·006	0·573	0·3	−0·002	0·937	<0·1	0·016	0·212	3·4	−0·024	0·105	9·4
Leptin	0·018	0·766	0·2	0·003	0·903	<0·1	0·025	0·308	1·1	0·020	0·819	0·1	−0·014	0·634	0·5	0·005	0·772	0·3
Sleeping time	–	–	–	–	–	–	−0·036	0·667	0·2	−0·004	0·974	<0·1	−0·002	0·989	<0·1	–	–	–

Bold values: $P<0·05$.
β: regression coefficient.
r^2 (%): squared partial correlation coefficient.
*The number of younger subjects is too small to be stratified by gender for multiple regression analysis.

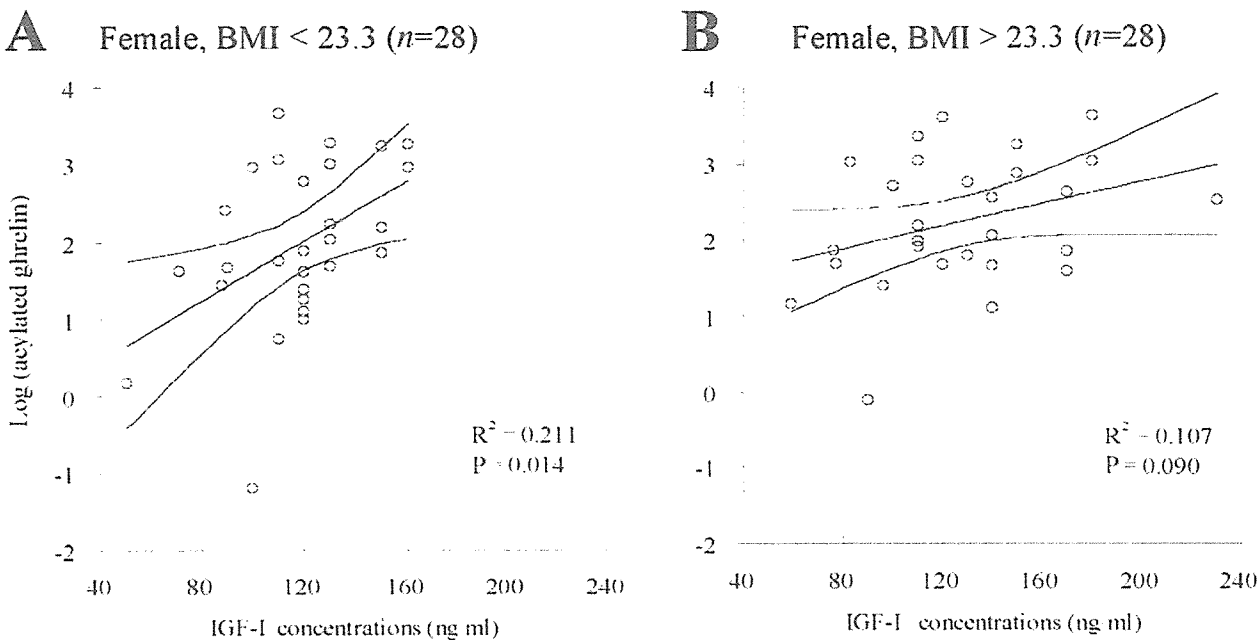

Figure 2 Linear regression analysis of the relationship between IGF-I and plasma acylated ghrelin levels in elderly female subjects of differing BMI levels. (A) Subjects with lower BMI (<23·3, $n=28$); (B) those with higher BMI (>23·3, $n=28$).

significant correlations between either acylated ghrelin and GH levels or GH and IGF-I levels in the group with a lower BMI. Although statistically not significant, plasma levels of acylated and desacyl ghrelin in elderly female subjects tended to be positively associated with BMI, while those in elderly men tended to be negatively associated (Table 2 and Fig. 3).

In the multivariate model, acylated ghrelin levels in women, but not in men, correlated with systolic BP levels, independently of site, age, BMI, sleeping duration,

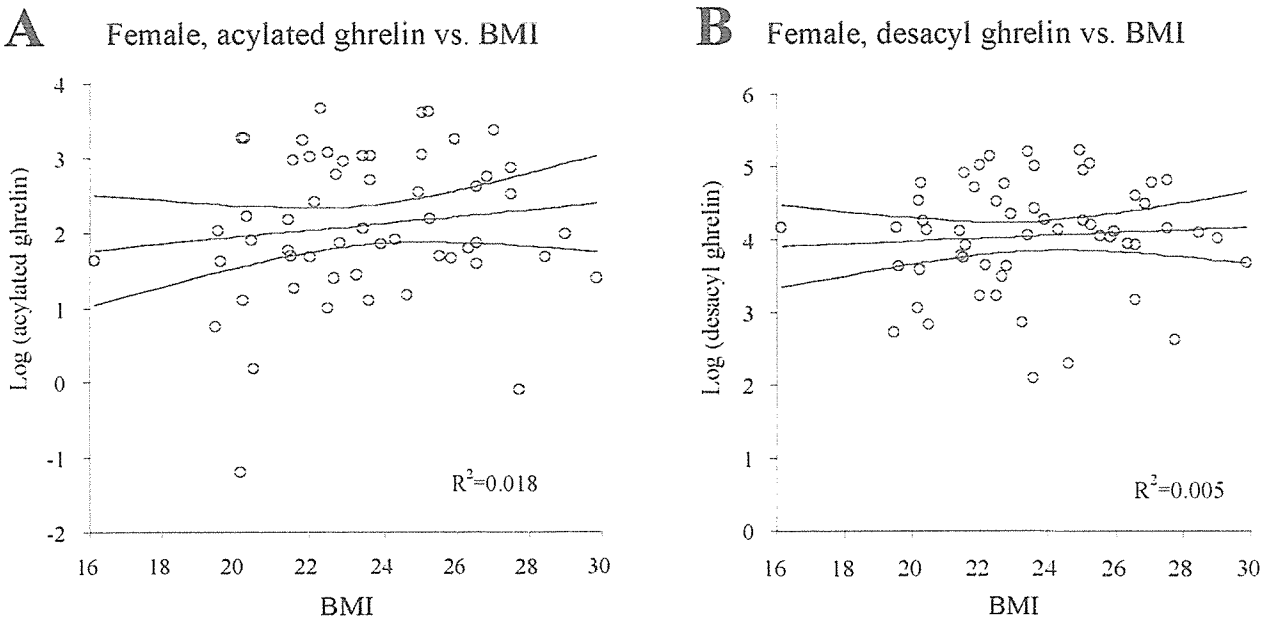

Figure 3 Linear regression analysis of the relationship between BMI and plasma levels of acylated (A) or desacyl (B) ghrelin in elderly female subjects.

Table 3 Relationship between plasma ghrelin concentrations and blood pressure in healthy elderly subjects

	Acylated ghrelin		Desacyl ghrelin		A/D ratio	
	β	*P**	β	*P**	β	*P**
Male						
Systolic blood pressure	−0·003	0·789	0·009	0·467	−0·012	0·050
Diastolic blood pressure	0·012	0·616	−0·016	0·525	0·028	0·027
Female						
Systolic blood pressure	−0·022	**0·039**	−0·016	0·074	−0·006	0·291
Diastolic blood pressure	−0·007	0·719	−0·001	0·969	−0·007	0·565

*Adjusted by recruitment site, age, BMI, sleeping duration, blood pressure (mutually) and blood levels of GH, IGF-I, insulin, glucose and leptin.
Bold values: $P<0·05$.

diastolic BP levels and blood levels of GH, IGF-I, insulin, glucose and leptin ($P=0·039$) (Table 3). Finally, acylated ghrelin levels and A/D ratio in women, but not in men, also correlated significantly with frequencies of bowel movement ($P=0·014$ and $P=0·008$ respectively) (Table 4). In men, desacyl ghrelin levels correlated with this parameter ($P=0·037$). There were no significant correlations between ghrelin levels and smoking habits in any subject groups. Gender difference was not an independent determinant of plasma ghrelin levels; β and P values for sex were $\beta=0·366$ and $P=0·138$ (acylated), and $\beta=0·293$ and $P=0·175$ (desacyl) respectively.

Other hormone levels

The correlations between other hormone levels and physiologic parameters in healthy elderly subjects are summarized in Table 5. Significantly, in both sexes, serum GH and IGF-I levels correlated negatively with BMI and age respectively, while serum leptin levels correlated positively with BMI. Plasma glucose levels positively correlated with both serum IGF-I levels and age. Serum IGF-I levels in females correlated positively with plasma concentrations of acylated ghrelin and negatively with serum GH levels, while serum leptin levels in men were significantly associated with age.

Discussion

Although two studies have demonstrated that mean plasma concentrations of total ghrelin in elderly, normal-weight subjects were 36% (Rigamonti *et al.* 2002) and 20% (Sturm *et al.* 2003) lower than those seen in younger, normal-weight subjects, these studies used a small number of subjects. In addition, only total ghrelin levels were examined, and no attempt was made to investigate gender differences. In this study, we demonstrated that the

Table 4 Relationship between plasma ghrelin concentrations and bowel movement in healthy elderly subjects

		Acylated ghrelin		Desacyl ghrelin		A/D ratiio	
	Number	Mean	S.D.	Mean	S.D.	Mean	S.D.
Bowel movement (male)							
≥2/day	15	10·8	10·1	62·9	42·6	15·4	6·0
1/day	29	9·4	13·1	52·7	41·4	17·8	10·3
<1/day	5	4·0	3·1	27·7	34·2	27·0	21·5
P value* (≥1/day vs <1/day)		0·144		**0·037**		0·249	
Bowel movement (female)							
≥2/day	11	15·0	9·8	91·1	43·0	15·6	4·8
1/day	38	12·3	10·2	71·3	47·8	17·3	9·0
<1/day	7	5·5	4·4	45·2	28·6	11·8	6·1
P value* (≥1/day vs <1/day)		**0·014**		0·188		**0·008**	

*Adjusted by recruitment site, age, BMI, sleeping duration and blood levels of GH, IGF-1, insulin, glucose and leptin.
Bold values: $P<0·05$.

Table 5 Mulitiple regression analysis between other hormone levels and various parameters in healthy elderly subjects*

	Male			Female		
	Parameters	β	*P*	Parameters	β	*P*
Hormones						
GH						
	BMI	−0·971	0·001	BMI	−0·411	0·001
	Sleeping time	1·347	0·007			
	Leptin	0·610	0·048			
IGF-I						
	Age	−3·262	0·001	Leptin	3·177	0·001
	Glucose	1·540	0·003	Age	−2·317	0·002
	Leptin	4·466	0·028	BMI	−5·987	0·004
				Acylated ghrelin	0·900	0·012
				GH	−3·739	0·013
Insulin						
	BMI	0·602	0·001	Leptin	0·438	0·001
Glucose						
	IGF-I	0·098	0·009	IGF-I	0·128	0·002
	Age	0·528	0·012	Age	0·552	0·040
	BMI	1·006	0·048			
Leptin						
	BMI	0·513	0·001	BMI	1·410	0·001
	Age	0·106	0·017	Insulin	0·506	0·001
				IGF-I	0·044	0·010

β: regression coefficient.
*Multiple regression analysis with backward-elimination procedure was performed after adjustment for the effect of recruitment site; candidate independent parameters were age, BMI, sleeping duration and blood levels of GH, IGF-I, insulin, glucose and leptin.

plasma levels of acylated ghrelin in elderly male and female subjects were respectively 20% and 40% lower than those seen in younger subjects. In contrast, plasma concentrations of desacyl ghelin in elderly subjects of both sexes did not differ from those observed in younger subjects in both sexes. As a result, the A/D ratios in elderly female subjects were significantly lower than those in younger female subjects. In addition, plasma acylated ghrelin levels did not show significant gender difference, while plasma desacyl ghrelin levels in elderly female subjects were significantly higher than those in elderly male subjects, although gender difference was not an independent determinant for them. The reductions in acylated ghrelin levels observed in elderly female subjects may be partially related to a higher BMI than that seen in younger women (23·5 ± 2·9 vs 20·3 ± 1·9; $P<0·001$), as plasma levels of acylated ghrelin in all females were not correlated with age group in the multiple regression analyses. Other modifying factors, especially menopause, should be considered as possibly affecting plasma acylated ghrelin levels in women. In support of this hypothesis, Kellokoski *et al.* (2005) recently reported that estrogen replacement therapy increases plasma levels of acylated ghrelin. Further studies will be necessary to delineate the mechanisms by which estrogen affects the production and/or secretion of acylated ghrelin.

ELISAs used for the measurement of plasma ghrelin levels in this study were two-site sandwich assays with two monoclonal antibodies. One monoclonal antibody recognizes the octanoyl-modified (Active Ghrelin kit) and the other the nonmodified N-terminal portion of ghrelin (Desacyl-Ghrelin kit) (Akamizu *et al.* 2005). The ratio of acylated to (acylated plus desacyl) ghrelin (A/(A+D) ratio) determined by ELISAs was lower than that of acylated to total ghrelin previously determined by RIA, which measures total ghrelin with an antiserum against the C-terminal region of ghrelin. This finding suggests that a fragmented form of ghrelin lacking the N-terminal region may naturally exist in human plasma or may be artificially produced during the RIA procedure. If so, then approximately 40–60% of the total ghrelin measured by RIA is probably fragmented. As a fragmented form of ghrelin is not measured in these two assays, its existence and physiologic implications should be considered and investigated in the future. A limitation of the study was that the measurements of ghrelin concentrations were not undertaken by inclusion of samples from both young and elderly subjects in the same assays. This increases the risk that interassay variation or drift may have reduced our ability to compare concentrations between the two age groups. To mitigate this risk, we used the same kind of assay kit.

A negative correlation between BMI and plasma levels of total or acylated ghrelin was reported by many investigators, including us (Ariyasu *et al.* 2001, Tschop *et al.* 2001, Akamizu *et al.* 2005). On the contrary, plasma levels of both acylated and desacyl ghrelin in elderly female subjects tended to be associated positively with BMI, while those in elderly men tended to be negatively associated (Table 2 and Fig. 3). Particularly, the relationship between plasma acylated ghrelin levels and BMI should be noted, although statistically not significant ($\beta=0{\cdot}149$, $P=0{\cdot}065$). These findings suggest that the regulation of ghrelin secretion and/or production in elderly female subjects is altered in comparison to that seen in younger subjects. This altered regulation might be related to the anorexia and undernutrition associated with aging. For example, plasma ghrelin levels may not rise sufficiently when elderly subjects lose weight, resulting in poor appetite and a state of negative energy balance.

In women, acylated ghrelin concentrations correlated positively with IGF-I independently of recruitment site, age, BMI, sleeping duration or blood levels of GH, IGF-I, insulin, glucose and leptin. While a negative correlation between ghrelin and IGF-I levels was reported in children and adolescents (Bellone *et al.* 2002, Whatmore *et al.* 2003), such a correlation has not been observed in adult subjects (Dall *et al.* 2002, Malik *et al.* 2004). Recently, Poykkyo *et al.* (2005) reported a negative correlation between plasma ghrelin and IGF-I in adult subjects with obesity, insulin resistance and type 2 diabetes. The association was particularly strong in both men and subjects in the higher BMI tertiles (maximum: 29·2 or less). In women, the correlation disappeared in the lowest BMI tertile (minimum: 26·5 or more). In agreement with this report, we did not observe a significant correlation between plasma ghrelin and IGF-I levels in the higher BMI population (>23·3). Acylated ghrelin levels, however, correlated positively with IGF-I levels with lower BMI values (<23·3). The positive correlation of ghrelin and IGF-I observed in elderly subjects implicates the dysregulation of ghrelin secretion and/or production during aging, suggesting that the negative feedback regulation of IGF-I may be lost. The IGF-I levels observed in the lower BMI group, 117·4 ± 25·1 ng/ml, may be too low to inhibit ghrelin secretion. In this group, the positive correlation suggests that ghrelin regulates IGF-I production by affecting GH secretion. Although we could not identify significant correlations between either acylated ghrelin and GH levels or GH and IGF-I levels, such associations between plasma ghrelin levels and serum GH levels have been observed in previous studies (Yoshimoto *et al.* 2002, Akamizu *et al.* 2005). Thus, the regulation of ghrelin/GH/IGF-I axis in elderly women with low IGF-I levels may be different from that seen in the younger subjects with normal IGF-I levels.

Acylated ghrelin levels in elderly women correlated negatively with systolic BP. The inverse relationship between total ghrelin levels and BP has previously been reported in pregnant women (Makino *et al.* 2002) and patients with hypertension (Poykko *et al.* 2003). Ghrelin, which exerts vasorelaxant or vasodilatory effects *in vitro* (Okumura *et al.* 2002, Shimizu *et al.* 2003), decreases BP (Nagaya *et al.* 2001). Our study also demonstrated the novel correlation of ghrelin levels with frequency of bowel movement in elderly subjects. It should be noted that the smaller number of men than women might have resulted in the borderline correlation between male acylated ghrelin level and the frequency of bowel movement. Ghrelin administration in humans stimulates peristalsis (Takaya *et al.* 2000, Nagaya *et al.* 2001, Akamizu *et al.* 2004) and enhances gastric and intestinal motilities in rats (Masuda *et al.* 2000, Trudel *et al.* 2002, Fujino *et al.* 2003). These findings suggest that ghrelin might play a role in the regulation of bowel motility.

In this study, we confirmed that both blood IGF-I and glucose levels were significantly correlated with age (Davidson 1979, Corpas *et al.* 1993, Muller *et al.* 1999). Serum leptin levels, adjusted for various parameters including BMI, exhibited a significant positive association with age in men (Table 5), but a nonsignificant negative association with age in women (data not shown). This finding corresponds to a report by Baumgartner *et al.* (1999) suggesting that the differences among men and the changes with age in serum leptin levels are associated with differing circulating levels of testosterone. Although plasma glucose levels correlate positively with serum IGF-I levels, few investigators, as far as we know, have reported this positive correlation. As several regulatory factors affect both blood glucose and IGF-I levels, further investigations will be necessary to clarify the mechanisms underlying this correlation. Finally, we confirmed previously observed correlations between BMI and serum GH or leptin levels in elderly subjects (Baumgartner *et al.* 1999, Iranmanesh *et al.* 1991, Chapman 2004).

In summary, we measured plasma levels of acylated and desacyl ghrelin in healthy elderly subjects. The levels of acylated ghrelin in women correlated positively with IGF-I levels, suggesting that the negative feedback mechanism does not function properly in nonobese elderly subjects. These results suggest, however, that ghrelin may regulate IGF-I levels through control of GH. Acylated ghrelin concentrations in women correlated with both systolic BP and the frequency of bowel movements. These findings strongly suggest that, in elderly women, acylated ghrelin may play a role in the regulation of the GH/IGF-I axis, BP and bowel movements. The obvious next step is to explore and confirm these physiologic effects of ghrelin experimentally. In addition, analysis of 24-h acylated and desacyl ghrelin secretion is extremely important to determine the physiologic control of ghrelin secretion during the lifespan. Finally, understanding the relationship between plasma ghrelin levels and these clinical parameters in the elderly may provide therapeutic opportunities to target ghrelin in disorders related to aging.

Acknowledgements

We thank Drs Hidenori Arai, Hisanori Horiuchi, Makoto Tanaka, Yutaka Nagano and Hiroshi Dekigai, Department of Geriatric Medicine, Kyoto University Hospital, for recruiting elderly subjects; Ms Hiratani, Ishimoto, and Takehisa for excellent technical assistance; and Ms Keiko Amemiya and Mizuho Hirata for excellent secretarial assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This study was supported by funds from the Ministry of Education, Science, Culture, Sports and Technology of Japan, and the Ministry of Health, Labor, and Welfare of Japan, and Research Grants by Chiyoda Foundation, Foundation for Growth Science, and Yamanouchi Foundation for Research on Metabolic Disorders.

References

Akamizu T, Takaya K, Irako T, Hosoda H, Teramukai S, Matsuyama A, Tada H, Miura K, Shimizu A, Fukushima M *et al.* 2004 Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. *European Journal of Endocrinology* **150** 447–455.

Akamizu T, Shinomiya T, Irako T, Fukunaga M, Nakai Y, Nakai Y & Kangawa K 2005 Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. *Journal of Clinical Endocrinology and Metabolism* **90** 6–9.

Angeloni SV, Glynn N, Ambrosini G, Garant MJ, Higley JD, Suomi S & Hansen BC 2004 Characterization of the rhesus monkey ghrelin gene and factors influencing ghrelin gene expression and fasting plasma levels. *Endocrinology* **145** 2197–2205.

Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S *et al.* 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *Journal of Clinical Endocrinology and Metabolism* **86** 4753–4758.

Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF *et al.* 2001 Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. *Journal of Clinical Endocrinology and Metabolism* **86** 1169–1174.

Baumgartner RN, Waters DL, Morley JE, Patrick P, Montoya GD & Garry PJ 1999 Age-related changes in sex hormones affect the sex difference in serum leptin independently of changes in body fat. *Metabolism* **48** 378–384.

Bedendi I, Alloatti G, Marcantoni A, Malan D, Catapano F, Ghe C, Deghenghi R, Ghigo E & Muccioli G 2003 Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. *European Journal of Pharmacology* **476** 87–95.

Bellone S, Rapa A, Vivenza D, Castellino N, Petri A, Bellone J, Me E, Broglio F, Prodam F, Ghigo E *et al.* 2002 Circulating ghrelin levels as function of gender, pubertal status and adiposity in childhood. *Journal of Endocrinological Investigation* **25** RC13–RC15.

Broglio F, Benso A, Castiglioni C, Gottero C, Prodam F, Destefanis S, Gauna C, van der Lely AJ, Deghenghi R, Bo M *et al.* 2003 The endocrine response to ghrelin as a function of gender in humans in young and elderly subjects. *Journal of Clinical Endocrinology and Metabolism* **88** 1537–1542.

Broglio F, Gottero C, Prodam F, Gauna C, Muccioli G, Papotti M, Abribat T, Van Der Lely AJ & Ghigo E 2004 Non-acylated ghrelin counteracts the metabolic but not the neuroendocrine response to acylated ghrelin in humans. *Journal of Clinical Endocrinology and Metabolism* **89** 3062–3065.

Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, Deghenghi R, Reissmann T, Ghigo E & Muccioli G 2001 Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. *Journal of Clinical Endocrinology and Metabolism* **86** 1738–1745.

Chapman IM 2000 Hypothalamic growth hormone-IGF-1 axis. In *Endocrinology of Aging*, pp 23–44. Eds JE Morley & L van den Berg. Totowa, NJ, USA: Humana Press.

Chapman IM 2004 Endocrinology of anorexia of ageing. *Best Practice and Research. Clinical Endocrinology and Metabolism* **18** 437–452.

Corpas E, Harman SM & Blackman MR 1993 Human growth hormone and human aging. *Endocrinology Review* **14** 20–39.

Dall R, Kanaley J, Hansen TK, Moller N, Christiansen JS, Hosoda H, Kangawa K & Jorgensen JO 2002 Plasma ghrelin levels during exercise in healthy subjects and in growth hormone-deficient patients. *European Journal of Endocrinology* **147** 65–70.

Davidson MB 1979 The effect of aging on carbohydrate metabolism: a review of the English literature and a practical approach to the diagnosis of diabetes mellitus in the elderly. *Metabolism* **28** 688–705.

de Jong N, Mulder I, de Graaf C & van Staveren WA 1999 Impaired sensory functioning in elders: the relation with its potential determinants and nutritional intake. *Journals of Gerontology. Series A, Biological Sciences and Medical Sciences* **54** B324–331.

Englander EW, Gomez GA & Greeley GH Jr 2004 Alterations in stomach ghrelin production and in ghrelin-induced growth hormone secretion in the aged rat. *Mechanisms of Ageing and Development* **125** 871–875.

Fujino K, Inui A, Asakawa A, Kihara N, Fujimura M & Fujimiya M 2003 Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *Journal of Physiology* **550** 227–240.

Haffner SM, Miettinen H & Stern MP 1997 The homeostasis model in the San Antonio Heart Study. *Diabetes Care* **20** 1087–1092.

Hataya Y, Akamizu T, Takaya K, Kanamoto N, Ariyasu H, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K *et al.* 2001 A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. *Journal of Clinical Endocrinology and Metabolism* **86** 4552.

Iranmanesh A, Lizarralde G & Veldhuis JD 1991 Age and relative adiposity are specific negative determinants of the frequency and amplitude of growth hormone (GH) secretory bursts and the half-life of endogenous GH in healthy men. *Journal of Clinical Endocrinology and Metabolism* **73** 1081–1088.

Kellokoski E, Poykko SM, Karjalainen AH, Ukkola O, Heikkinen J, Kesaniemi YA & Horkko S 2005 Estrogen replacement therapy increases plasma ghrelin levels. *Journal of Clinical Endocrinology and Metabolism* **90** 2954–2963.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402** 656–660.

Korbonits M, Goldstone AP, Gueorguiev M & Grossman AB 2004 Ghrelin – a hormone with multiple functions. *Front Neuroendocrinology* **25** 27–68.

Ligthart GJ, Corberand JX, Fournier C, Galanaud P, Hijmans W, Kennes B, Muller-Hermelink HK & Steinmann GG 1984 Admission criteria for immunogerontological studies in man: the SENIEUR protocol. *Mechanisms of Ageing and Development* **28** 47–55.

Liu YL, Yakar S, Otero-Corchon V, Low MJ & Liu JL 2002 Ghrelin gene expression is age-dependent and influenced by gender and the level of circulating IGF-I. *Molecular and Cellular Endocrinology* **189** 97–103.

Makino Y, Hosoda H, Shibata K, Makino I, Kojima M, Kangawa K & Kawarabayashi T 2002 Alteration of plasma ghrelin levels associated with the blood pressure in pregnancy. *Hypertension* **39** 781–784.

Malik IA, English PJ, Ghatei MA, Bloom SR, MacFarlane IA & Wilding JP 2004 The relationship of ghrelin to biochemical and anthropometric markers of adult growth hormone deficiency. *Clinical Endocrinology (Oxf)* **60** 137–141.

Martinez M, Hernanz A, Gomez-Cerezo J, Pena JM, Vazquez JJ & Arnalich F 1993 Alterations in plasma and cerebrospinal fluid levels of neuropeptides in idiopathic senile anorexia. *Regulatory Peptides* **49** 109–117.

Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M & Kangawa K 2000 Ghrelin stimulates gastric acid secretion and motility in rats. *Biochemical and Biophysical Research Communications* **276** 905–908.

Morley JE 1997 Anorexia of aging: physiologic and pathologic. *American Journal of Clinical Nutrition* **66** 760–773.

Muller EE, Locatelli V & Cocchi D 1999 Neuroendocrine control of growth hormone secretion. *Physiology Review* **79** 511–607.

Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, Hayashi Y & Kangawa K 2001 Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **280** R1483–R1487.

Okumura H, Nagaya N, Enomoto M, Nakagawa E, Oya H & Kangawa K 2002 Vasodilatory effect of ghrelin, an endogenous peptide from the stomach. *Journal of Cardiovascular Pharmacology* **39** 779–783.

Poykko SM, Kellokoski E, Horkko S, Kauma H, Kesaniemi YA & Ukkola O 2003 Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. *Diabetes* **52** 2546–2553.

Poykko SM, Ukkola O, Kauma H, Kellokoski E, Horkko S & Kesaniemi YA 2005 The negative association between plasma ghrelin and IGF-I is modified by obesity, insulin resistance and type 2 diabetes. *Diabetologia* **48** 309–316.

Rigamonti AE, Pincelli AI, Corra B, Viarengo R, Bonomo SM, Galimberti D, Scacchi M, Scarpini E, Cavagnini F & Muller EE 2002 Plasma ghrelin concentrations in elderly subjects: comparison with anorexic and obese patients. *Journal of Endocrinology* **175** R1–5.

Shimizu Y, Nagaya N, Teranishi Y, Imazu M, Yamamoto H, Shokawa T, Kangawa K, Kohno N & Yoshizumi M 2003 Ghrelin improves endothelial dysfunction through growth hormone-independent mechanisms in rats. *Biochemical and Biophysical Research Communications* **310** 830–835.

Sturm K, MacIntosh CG, Parker BA, Wishart J, Horowitz M & Chapman IM 2003 Appetite, food intake, and plasma concentrations of cholecystokinin, ghrelin, and other gastrointestinal hormones in undernourished older women and well-nourished young and older women. *Journal of Clinical Endocrinology and Metabolism* **88** 3747–3755.

Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A *et al.* 2000 Ghrelin strongly stimulates growth hormone release in humans. *Journal of Clinical Endocrinology and Metabolism* **85** 4908–4911.

Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC & Wells T 2004 Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* **145** 234–242.

Trudel L, Tomasetto C, Rio MC, Bouin M, Plourde V, Eberling P & Poitras P 2002 Ghrelin/motilin-related peptide is a potent prokinetic to reverse gastric postoperative ileus in rat. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **282** G948–G952.

Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E & Heiman ML 2001 Circulating ghrelin levels are decreased in human obesity. *Diabetes* **50** 707–709.

van der Lely AJ, Tschop M, Heiman ML & Ghigo E 2004 Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocrinology Reviews* **25** 426–457.

Whatmore AJ, Hall CM, Jones J, Westwood M & Clayton PE 2003 Ghrelin concentrations in healthy children and adolescents. *Clinical Endocrinology (Oxf)* **59** 649–654.

Wurtman JJ, Lieberman H, Tsay R, Nader T & Chew B 1988 Caloric and nutrient intakes of elderly and young subjects measured under identical conditions. *Journal of Gerontology* **43** B174–B180.

Yoshimoto A, Mori K, Sugawara A, Mukoyama M, Yahata K, Suganami T, Takaya K, Hosoda H, Kojima M, Kangawa K *et al.* 2002 Plasma ghrelin and desacyl ghrelin concentrations in renal failure. *Journal of the American Society of Nephrology* **13** 2748–2752.

Received 21 July 2005
Accepted 27 October 2005
Made available online as an Accepted Preprint
18 November 2005

Diabetologia (2006) 49: 1264–1273
DOI 10.1007/s00125-006-0226-3

ARTICLE

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Ghrelin prevents development of diabetes at adult age in streptozotocin-treated newborn rats

Received: 21 November 2005 / Accepted: 17 January 2006 / Published online: 29 March 2006
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Abstract *Aims/hypothesis:* Ghrelin, a stomach-derived hormone, functions in multiple biological processes, including glucose metabolism and cellular differentiation and proliferation. In this study, we examined whether early treatment with ghrelin can regenerate beta cells of the pancreas in an animal model of diabetes mellitus, the n0-STZ model, in which neonatal rats are injected with streptozotocin (STZ) at birth. *Methods:* Following administration of ghrelin to n0-STZ rats from postnatal days 2 to 8, we examined beta cell mass, mRNA expression levels of insulin and of pancreatic and duodenal homeobox 1 (*Pdx1*) gene, and pancreatic morphology on days 21 and 70. In addition, we investigated the effects of ghrelin on beta cell replication. *Results:* By day 21, ghrelin treatment increased pancreatic expression of insulin and *Pdx1* mRNA in n0-STZ rats. The number of replicating cells was also significantly increased in the ghrelin-treated n0-STZ model. At day 70, n0-STZ rats exhibited hyperglycaemia, despite slight increases in plasma insulin levels. Ghrelin treatment resulted in the improvement of plasma glucose levels, which were associated with normal plasma insulin levels. Pancreatic insulin mRNA and protein levels were significantly increased in ghrelin-treated n0-STZ model animals. *Conclusions/interpretation:* These findings suggest that ghrelin promotes regeneration of beta cells in STZ-treated newborn rats. Thus, early administration of ghrelin may help prevent the development of diabetes in disease-prone subjects after beta cell destruction.

Keywords Diabetes mellitus · Ghrelin · Insulin · Pancreatic beta cell · Pdx1 · Streptozotocin

Abbreviations BrdU: 5-bromodeoxyuridine · FBG: fasting blood glucose · GHS-R: growth hormone secretagogue receptor · Pdx1: pancreatic and duodenal homeobox 1 · STZ: streptozotocin

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Introduction

Ghrelin was originally isolated from rat stomach as a growth-hormone-releasing acylated peptide [1]. This peptide hormone stimulates food intake and growth hormone secretion [2, 3], but also plays a regulatory role in glucose metabolism and insulin secretion. Ghrelin may have direct biological action within the pancreas, potentially functioning during pancreatic development. Although these results remain controversial, in vivo peripheral administration of ghrelin modulated plasma insulin and glucose levels [4–7]. The growth hormone secretagogue receptor (GHS-R), for which ghrelin can serve as a ligand, is expressed in the pancreas [8, 9]. Ghrelin gene expression is markedly higher in the fetal pancreas than in the stomach [10], suggesting ghrelin might be involved in the development, proliferation or differentiation of the pancreas. Ghrelin has been reported to possess mitogenic and anti-apoptotic actions in adipocyte cells; treatment of pre-adipocytes with ghrelin induced cellular proliferation and differentiation into mature adipocytes [11, 12].

Diabetes mellitus is a common and serious disease. Of the abundant experimental trials designed to explore novel therapies for the disease, one attempted to increase insulin secretion from the pancreas by promoting the proliferation or regeneration of beta cells [13]. In this study, we examined the effects of ghrelin on the development of hyperglycaemia and the regeneration of beta cells in streptozotocin (STZ)-treated newborn rats, which show rapid remission but gradual development of diabetes in adult animals. This model has been traditionally used to study pancreatic regeneration [14–16]. We investigated the changes in the islets of Langerhans morphology and pancreatic insulin mRNA and protein levels following ghrelin administration in this animal model. Further, we studied the resulting alterations in the expression levels of pancreatic and duodenal homeobox 1 (Pdx1), one of the major pancreatic transcriptional factors thought to play a key role in the development and regeneration of the pancreas [17–19].

Materials and methods

Animals

All animal experimental procedures were approved by the Kyoto University Graduate School of Medicine committee on animal research.

Female and male Sprague–Dawley rats, purchased from Charles River (Yokohama, Japan), were allowed free access to tap water and a standard pellet rat diet (352 kcal/100 g, CE-2; Clea, Tokyo, Japan). Females were caged with a male for one night; pregnancy was detected by abdominal palpation 14 days later. Natural birth occurred 22 days after mating.

We examined four experimental groups: (1) a Control group, in which newborn rats received a single i.p. injection of citrate buffer; (2) a Ghrelin group, which received twice daily s.c. injections of ghrelin (100 μg/kg body weight) for 7 days (from day 2 to 8) after birth; (3) an n0-STZ group, which received a single i.p. injection of streptozotocin; and (4) an n0-STZ/Ghrelin group, which received a single i.p. injection of streptozotocin followed by injections of ghrelin as in the Ghrelin group. Dams were randomly assigned to four groups and pups from the same litter were assigned to the same group. The numbers of dams in each of the four groups were five (Control), five (Ghrelin), seven (n0-STZ) and seven (n0-STZ/Ghrelin).

At birth, newborns received a single i.p. injection of 100 mg/kg body weight of STZ (Sigma, Steinheim, Germany), freshly dissolved in citrate buffer (0.05 mmol/l, pH 4.5). Pups were left with their mothers. All neonates were tested on day 2 for glycosuria with Multistix SG (Bayer Medical, Leverkusen, Germany). Only those animals that were glycosuric (3+ value with the Multistix SG test) at day 2 after birth were included in the n0-STZ model group. Ghrelin treatment was started after glycosuria was confirmed.

Animals were killed at 21 or 70 days after birth by bleeding following anaesthesia with an i.p. injection of sodium pentobarbital (50 mg/kg). Blood samples were collected from the inferior vena cava, centrifuged immediately at 20,000 ×g for 2 min at 4°C, and stored at −80°C until assayed.

Pancreas removal and treatment

After excision, pancreases were removed and weighed. To measure insulin content, pancreases (35–50 mg) were homogenised and centrifuged in 5 ml acid-ethanol (0.15 mol/l HCl in 75% [vol/vol] ethanol) at 1,000 g for 20 min; the supernatants were stored at −80°C. For immunohistochemistry, additional pancreases were fixed in 4% paraformaldehyde fixative for 24 h and embedded in paraffin.

Immunohistochemistry

Insulin and Pdx1 were detected immunohistochemically on 3-μm-thick tissue sections using an indirect peroxidase-labelling technique. Sections were incubated for 1 h at room temperature or overnight at 4°C with primary antibodies (guinea-pig anti-porcine insulin [Dako, Glostrup, Denmark], or rabbit anti-mouse/rat IDX-1 [Chemicon, Temecula, USA]). Insulin- and Pdx1-positive staining was visualised using a 3,3′-diaminobenzidine tetrahydrochloride kit (DakoCytomation) and fluorescence, respectively. Quantitative evaluations of insulin- and Pdx1-positive areas were performed using a computer-assisted image analysis procedure using an Olympus BX 51 microscope connected via a DP 12 digital camera and Mac SCOPE Ver 2.6 software (Mitani, Fukui, Japan). The insulin- and Pdx1-positive area, as well as the area of the total pancreatic section, was evaluated on ten non-consecutive sections of the pancreas of five animals per group at each point. For semi-quantitative analysis, more than six fields were observed per section. The relative volume of insulin- and Pdx1-positive cells was determined by a stereological morphometric method, calculating the ratio between the areas occupied by immunoreactive cells and that occupied by total pancreatic cells.

Beta cell replication

Pancreatic sections that had not already been used for morphometric studies were used to examine beta cell replication. Sections were double-stained for phospho-histone H3 (Ser10) and insulin. Sections were incubated with a 1/50 dilution of anti-phospho-histone H3 (Ser10) antibody (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. Sections were then incubated with guinea-pig anti-insulin antibody for 1 h at room temperature. After six washes with PBS, the sections were incubated with the secondary antibody conjugated with fluorescence (Alexa fluor 488 and 546; Molecular Probes,

Eugene, OR, USA) in blocking solution for 30 min at room temperature. After rinsing with PBS, sections were mounted with Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and examined with confocal laser scanning microscopy (Leica Microsystems, Mannheim, Germany). A minimum of 1,000 beta cell nuclei were counted per section.

RNA extraction and RT-PCR methods

Total RNA was extracted from rat pancreases as previously described [20]. To synthesise the first-strand cDNA, the extracted products were used as templates in reactions containing RT (Invitrogen, Carlsbad, CA, USA). RT-PCR analysis was performed as previously described [20]. The first-strand cDNA was used in subsequent PCR analyses, in combination with the following oligonucleotide primers: insulin-1: 5′ TAGACCATCAGCAAGCAGGTC, 3′ CAC ACCAGGTACAGAGCCT; *Pdx1*: 5′ AGGAGGTGCAT ACGCAGCAG, 3′ GAGGCCGGGAGATGTATTTGTT.

The identity of the PCR products was confirmed by agarose gel electrophoresis.

Real-time PCR

We mixed 1 μl of the amplified cDNA with 25 μl 2×PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 23 μl sterile distilled water and 0.5 μl 10 pmol/μl sense and antisense primers specific for either insulin or *Pdx1*. Forty cycles of PCR amplification were carried out using the Thermal Cycler System (ABI PRISM 7700; Applied Biosystems) for 15 s at 95°C followed by 60 s at 60°C. The concentration of each mRNA product was quantified using calibration curves expressing the fluorescence intensity against the amount of standardised PCR product. All expression data were normalised to the amount of 18S ribosomal RNA (Applied Biosystems) amplified from the same individual sample.

Analytical techniques

Plasma and blood glucose levels were determined using a glucose analyser (Antisense 2; Sankyo, Tokyo, Japan). Insulin was extracted from pancreases as described [21]. Insulin concentrations were measured using a Lebis insulin ELISA kit (Shibayagi, Gunma, Japan).

Statistical analysis

Values are expressed as means±SEM. Differences between each group of rats were evaluated using a Mann–Whitney test or a Bonferroni-corrected ANOVA as appropriate. A value of $p<0.05$ was considered statistically significant.

Results

Effects of ghrelin treatment from day 2 to day 8 after birth: studies on day 21

The characteristics of the 21-day-old rats are summarised in Table 1. After screening n0-STZ and n0-STZ/Ghrelin groups for glucosuria (3+ or more) on day 2 after birth, animals were kept for an additional 19 days. While significant changes in body weight and fasting blood glucose (FBG) levels were observed between the Control and Ghrelin groups, no changes in these parameters and insulin levels were observed between the n0-STZ and n0-STZ/Ghrelin groups (Table 1). There were no significant differences in body weight, FBG levels and insulin levels between the Control and n0-STZ groups or between the Ghrelin and n0-STZ/Ghrelin groups, although a significant change in FBG levels was observed between the Control and n0-STZ/Ghrelin groups.

Insulin mRNA expression levels within the pancreas were markedly reduced in the n0-STZ group in comparison with Control animals. These levels, however, returned to levels similar to those of the Control group following ghrelin treatment (n0-STZ/Ghrelin group) (Fig. 1a). The pancreatic insulin mRNA expression level of the n0-STZ group was approximately one-third of those of the Control and n0-STZ/Ghrelin groups. In addition, insulin immunostaining of the pancreas was reduced to approximately one-twentieth compared with that of the Control group (Fig. 1b–f). These finding are surprising, because the n0-STZ group exhibited plasma insulin levels similar to those of the Control and n0-STZ/Ghrelin groups. Therefore, they suggested that neonatal beta cells, even if the number becomes small, are able to secrete insulin to keep the plasma level normal.

Table 1 Characteristics (means±SEM) of fasted 21-day-old rats

	Control	Ghrelin	n0-STZ	n0-STZ/Ghrelin
Body weight (g)	37.8±1.1 (20)	45.1±0.8 (8)*	37.6±1.2 (15)	36.3±1.3 (9)
FBG (mmol/l)	6.3±0.1 (17)	5.2±0.3 (8)**	5.9±0.2 (15)	5.6±0.1 (8)***
Plasma insulin (pmol/l)	55±6 (7)	41±5 (8)	55±13 (6)	60±2 (6)

Number of rats in parentheses
Statistical analysis (Mann–Whitney test) was carried out between all groups
*$p<0.005$, **$p<0.0005$, ***$p<0.05$ (vs Control)